
Current Research Trends in Biological Science
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# *Preface*

*This book covers all areas of biological research. The contributions by the authors include luminescent bacteria; MDR bacteria; enzyme; gamma hemolytic; Loligo sp.; Loligo edulis; H. nehereus; aflatoxin B1; groundnut paste; validation method; Andrographis paniculata; 5- fluorouracil; benzene; leukaemia; carcinogen; race-nonspecific resistance; durable resistance; QTL analysis; positional cloning; marker-assisted selection; pyramiding; rice; blast disease; soymilk; composition; characterize; seed coat color; Glycine max; black soybean; yeast; storage; viability; lectin; Quercus fusiformis; protein purification; HPLC; blood group specificity; pH stability; banana peels; brewery effluent; inorganic anions; removal efficiency; acute toxicity; Eleginops maclovinus; LC50-96h; ammonia; rice; drought stress; traditional varieties; coefficient of variabilities and heritability; cellulase; Penicillium sp.; lignocellulosic biomass; solid state fermentation; Metosartan; RNase A; desynapsis; netosis; sperm count; pre maturation; apoptosis; Dalbergia melanoxylon; tissue culture; callus induction; explants; murashige and skoog; dared; Ruta montana L.; fertility; sperm motility; sperm counts; histological alterations etc. This book contains various materials suitable for students, researchers and academicians in the field of biological research.*

***Chapter 1***
Print ISBN: 978-93-90206-33-9, eBook ISBN: 978-93-90206-34-6

# Bioluminescence of Symbiont Bacteria in Marine Life

**Delianis Pringgenies[1*], Kartika Dewi[1] and Pramita Apriliyani[1]**

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## ABSTRACT

Bioluminescence is a natural phenomenon found in living things that emit light without emitting heat. Bioluminescence can be produced in three ways, namely from special photocytes cells, glands in tissues, and luminescent bacteria that are symbiotic to the host. Bioluminescence in *Loligo sp* and *Harpadon nehereus* is known to originate from luminescent bacteria which are symbiotic in luminescent organs. However, information about symbiotic bacteria in other types of luminescence biota and its potential in Indonesian waters has not been widely explored. This study aims to determine the potential of luminescence symbiotic bacteria in *Loligo sp*. and *Loligo edulis* from Jepara waters and *Harpadon nehereus* from the Malacca Strait waters, Indonesia as an anti-bacterial agent and to prove differences in the luminescence proteins produced by symbiotic bacteria and their hosts. Sample collection is done by isolating the bacteria from luminescent organs which are then used as research samples. The study was conducted using a qualitative activity test on pathogenic bacteria and analysis of SDS-PAGE electrophoresis protein in bacteria and its host. The study found 2 isolates of luminescent symbiotic bacteria that were potential as antibacterial agents against Multi Drug resistant (MDR) pathogens of *Escherichia coli*, with inhibition zones of 32.59 mm and of *Enterobacter sp*.5 with inhibition zones of 28.44 mm. Luminescent symbiotic bacteria of *L. edulis* have the potential to be antibacterial agent against *Vibrio harveyi, E. coli, Staphylococcus aureus*, and *Bacillus sp*. Analysis of *H. nehereus* symbiotic bacteria showed gamma hemolytic in blood agar test. Protein analysis of SDS-PAGE electrophoresis showed that the enzymes found insymbionic bacteria were different from the enzymes in their host.

*Keywords: Luminescent bacteria; MDR bacteria; enzyme; gamma hemolytic; Loligo sp.; Loligo edulis; H. nehereus.*

## 1. INTRODUCTION

Bioluminescence is the most important source of light in deep sea ecosystems. Resulted from the oxidation of substrate catalyzed by enzymes, it enables living organism to radiate light [1]. The emission of light that occurs benefits the organism because it is useful for foraging, avoiding predators, identification or communication with others, and for camouflage [2].

Biota with bioluminescence activity comes mostly from the sea, with more than 700 luminescent genera and 80% of them are marine life [3]. Luminescent organs that are rich in nutrients are ideal places for symbiotic bacteria, which naturally creates competition among the luminescent bacterial community. In addition to the ability to create light, luminescence symbiotic bacteria evolve to provide protection for their host, which is itself an effort to survive in competition. In addition, there is also the use of luminescence bacterial isolate genes that are utilized in the field of ecology as agents of heavy metal biosensor in aquatic environments [4]. This indicates that bioluminescence is a promising focus in the fields of biotechnology and ecology. The source of the study can be obtained from bioluminescence organisms found in Indonesian waters, which holds potential as a source of isolates

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of luminescent bacteria. Indonesian waters are vast waters with a rich diversity of marine organisms, including bioluminescence organisms from Indonesia such as *Anomalops katoptron* from the Indonesian island of Banda [5] and *Loligo duvauceli* from Jepara [6]. Bioluminescence in *Loligo duvauceli* is known to originate from the symbiotic bacteria in the luminescent organs of this species. [6]. Luminescent bacteria have genes that produce the luciferase enzyme and long chains of aldehydes needed in bioluminescence chemical reactions [7]. This symbiosis is classified as a type of mutualistic symbiosis because luminescent organisms get a variety of benefits from the light produced, while the symbiont bacteria obtain a place that is rich in nutrients [8].

Based on the explanation, this research was conducted to determine the luminescence ability of squid from *Loligo sp.* and *Loligo edulis* from Jepara waters and on fish from *Harpadon nehereus* species from the Malacca Strait Waters, Indonesia. Specifically, this study will seek to prove that the ability of bioluminescence originates from symbiotic bacteria rather than innate host abilities, as well as to determine the further potential of the symbiotic luminescent bacteria. This study also aims to prove that the protein that emits light from the symbiotic bacteria is different from the protein that comes from their host.

## 2. METHODS

### 2.1 Sampling and Isolation of Luminescent Microbes on Squids

Five squids were collected by catching using floating net mesh from 1-2 m depth from the waters of Jepara. The samples were kept in a cool box for further preparation in the laboratory. Isolation of luminescent microbes was performed by pulverizing the light organ of the squid, after which a $10^{-5}$ dilution was made. The microbial suspension was then spread onto Zobell 2216E media composed of 2.5 g of peptone, 0.5 g of yeast extract, and 15 g of agar per liter. The microbial culture was then incubated for 2x24 hours in room temperature. The resulting microbial colonies were isolated. The colonies producing the most luminescence were purified to obtain purified culture.

Determination of luminescent symbiotic microbial isolates in squid light organs was based on the morphology of the luminescence bacterial colonies grown on Nutrient Agar (NA) media in petri dishes as well as observing the emission of light by bacteria in a dark room. The resulting isolates were purified to obtain purified isolates of luminescent microbes. The observation for the morphology of the luminescence bacterial colony includes color, shape, surface and the edge of the colony and biochemical identification.

### 2.2 Sampling and Isolation of Luminescent Microbes on the Bombay Duck (*Harpadon nehereus*)

Twenty Bombay duck fish samples, each approximately 23 cm in length, were collected from Tanjung Balai Karimun Island, Riau Islands, Indonesia. The collected samples were stored in a cool box prior to laboratory preparation. The samples were rinsed with sterilized saline water and were put in the open on a room temperature. After 8 hours of dark room observation, luminescent microbes occurred on the surface of the body. One gram of skin tissue from the samples were sampled for serial dilution. The resulting microbial suspension was then spread onto Zobell 2216E media composed of 2.5 g of peptone, 0.5 g of yeast extract, and 15 g of agar per liter. The microbial culture was then incubated for 2x24 hours in room temperature. Single purified colony with the most luminescence was inoculated into a new medium for molecular identification and hemolysis test.

### 2.3 Identification of Microbe

Characterization of microbes were performed through TCBS (Thiosulfate-citrate-bile salts-sucrose) isolation, Oxidase test, Indole test, Oxidative fermentative (Sucrose and Lactose) test, Motility test, $H_2S$ test and Voges-Proskauer test. Gram staining was also performed according to standard method. Melocular analysis was performed in accordance to the following stages.

## 2.4 Amplification of 16S Partial Ribosomes from Isolated Luminescent Microbes

Genomic DNA from luminous bacterial microbial isolate was used as a template for PCR amplification. Amplification of 16S ribosomal RNA (rRNA) was performed using 27f (5′-AGAGTTT-GATCMTGGCTCAG-3′) and 1492r (5′TACGGYTACCTTGTTACGACTT- 3′) primers. Amplification took place in a thermal cycler with 30 rounds followed by denaturation of DNA at 94ºC for 1 minute, at 55ºC for primary annealing for 1 minute, and extension at 72ºC for 1 minute. The final extension was carried out at 72ºC for 10 minutes. PCR products were analyzed using gel electrophoresis (1%) and the resulting images were analyzed using UVI Doc HD5 (UVITEC Cambridge).

The sequencing process was carried out at Genetika Science inc, Indonesia. The result of the sequencing was the nucleotide of the 16S rRNA gene region data, which was then processed and edited using BioEdit software, and was submitted to the NCBI database that stores information on nucleotide sequences.The phylogenetic tree was made using Blast tool software and designed by NCBI database search tool.

## 2.5 Antimicrobial Activities of Luminous Microbes from *Loligo sp*

This process is carried out by an overlay method [9]. Purified culture of bioluminescence microbe isolated from *Loligo sp* was used against MDR pathogenic microbe of the species *Klebsiella sp*., *Enterobacter sp*. 5, *Enterobacter sp*. 10, *CNS*, *Pseudomonas sp*., *and Escherichia coli*. Pathogenic microbe samples were obtained from Dr. Kariadi Central General Hospital, Semarang. MDR microbes were introduced in liquid Zobell 2216E plain media and then incubated for 24 hours at room temperature while being spun at 90 rpm. 1 mL of MDR microbial suspension was taken and put into 100 mL soft Zobell 2216E plain agar (1% of total volume), after which it was introduced to Zobell 2216E saline solid media which had been cultured with luminescent microbial isolates from the squid sample, then incubated at room temperature for 1- 2 days. The bacterial isolates which formed inhibition zones were sorted and were put into another iteration. Isolates which still showed viable result after iteration were taken and stored for further analysis. Quantitative tests using paper disks were then performed. Antimicrobial tests were carried out by streaking microbe isolate onto the paper disk with 20 µL concentration. The resulting inhibition zone was then measured with a caliper.

## 2.6 Antimicrobial Activities of Luminous Microbes from *Loligo edulis*

This process was performed using agar diffusion method according to Kirby-Bauer [10]. This test was performed both qualitatively and quantitatively using NA media. Purified culture of bioluminescence microbe isolated from *Loligo edulis* was used against vibriosis-causing pathogenic bacteria in animals, *V. harveyi* and *V. alginolyticus*, and pathogens which cause various types of infections in humans, *E. coli, S. aureus* and *Bacillus sp*. The qualitative test was performed first to determine whether the luminescent microbe isolate has the ability to inhibit the growth of the pathogenic microbes. Antimicrobial tests were carried out by streaking microbe isolate onto the paper disk with 20 µL concentration. The standard of microbial turbidity is equivalent to 0.5 McFarland. Pathogenic microbe sample was inoculated into NA media, after which paper disk was placed in the middle of the medium and was incubated for 4 x 24 hours at room temperature. The qualitative test result is a positive if transparent zone was formed around the paper disk. Otherwise, the test result is considered negative. Isolates showing positive result were put into quantitative testing.Quantitative test is performed to determine the diameter of inhibition zone. Quantitative test method is the same as the method used in qualitative tests. The formation of the barrier zone diameter was measured for each incubation period of 24 hours, 48 hours, 72 hours and 96 hours using a caliper.

## 2.7 Hemolytic Activity of Luminous Microbes Isolated from *Harpadon nehereus*

Blood agar is a differential media enriched with 5% human blood. This medium is used to determine the pathogenic properties of microbes. The test was performed by inoculating the luminous microbe isolated from *Harpadon nehereus* into blood agar, and incubated in room temperature form 48 hours. After the observation period expired, observation was made for changes in coloration which indicate the ability of the microbe to destroy blood cells. Hemolysis alpha occurs when the agar surrounding the colony changes color into green or brown hue. Beta hemolysis indicated by the appearance of

clear colors surrounding the colonies, whereas gamma hemolysis is indicated by no change in agar (Lesmana *et al.,* 2001) [11].

### 2.8 Protein Analysis by SDS-PAGE Electrophoresis

The analysis is performed to compare the protein obtained from luminescent bacteria (emitting light), luminescent bacteria (not emitting light), and luminescent organ sac of the subject. The electrophoresis method used was SDS-PAGES 12% with a buffer system, Laemli [12]. The protein standards used are LMW protein standards and Coomassie blue dye.

## 3. RESULTS

### 3.1 Luminescent Microbe from *Loligo* sp.

Isolation of symbiont microbe of light organs from *Loligo* sp. resulted in 40 microbial isolates. After light emission observation, 2 microbial isolates were found to produce the most luminescence. Isolation results of microbes in *Loligo*sp light organ are presented in Fig. 1.

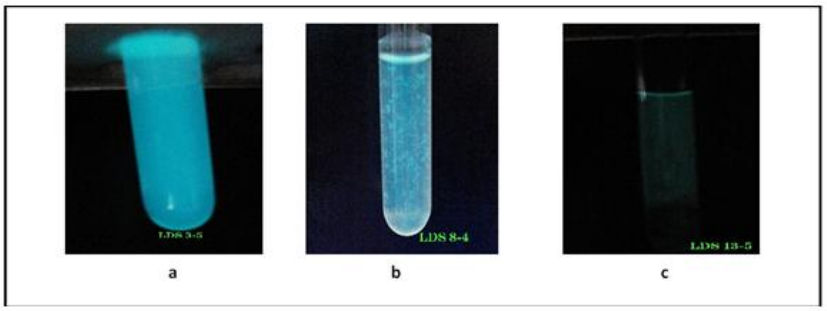



**Fig. 1. Levels of light emitted by microbial isolates; (a) high, (b) medium, (c) low**

### 3.2 Luminescent Microbe from *Loligo edulis*

Isolation of symbiont microbe of light organs from *Loligo edulis* resulted in 1 microbial isolate.Determination of luminescent symbiotic microbial isolates in the light organ of *Loligo edulis* was based on the morphology of the luminescence bacterial colonies grown on Nutrient Agar (NA) media in petri dishes as well as observing the emission of light by bacteria in a dark room. The light produced was bluish in hue (Fig. 2). The resulting isolates were purified to obtain purified isolates of luminescent microbes. The microbial colony was milk white in color, with circular shape, convex surface, and even margin.



**Fig. 2. Light emitted by the microbial isolate on NA media in a dark room**

### 3.3 Luminescent Microbe from the Bombay Duck (*Harpadon nehereus*)

Light emission from microbes obtained from the first serial dilution from the Bombay duck (*Harpadon nehereus*) (Fig. 3). The dilution result was introduced into agar media and the suspension was incubated for 48 hours. Based on the levels of light emitted, four colonies were selected to be purified. The purification resulted in one isolate which retained its capability in producing light (Fig. 4).

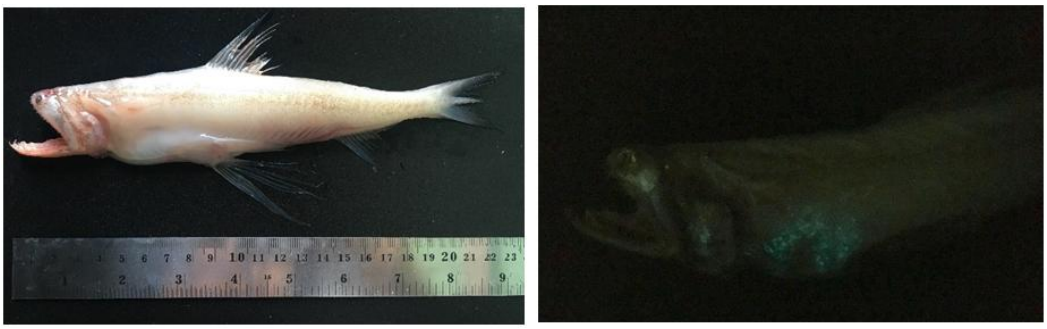


**Fig. 3. Host of luminescent microbe (a) fresh Bombay duck (b) light-emitting Bombay duck**

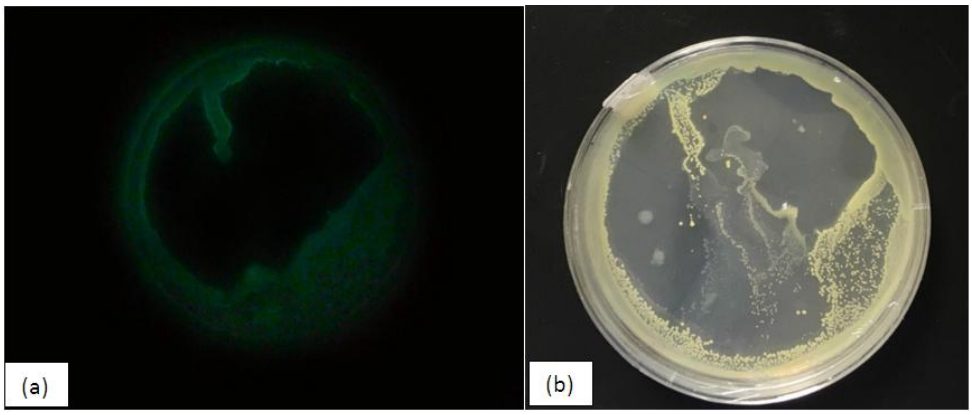



**Fig. 4. Microbial isolate obtained from the Bombay duck (a) in a dark room (b) in a room with lighting**

The luminescent microbe isolated from the Bombay duck was capable of emitting light in petri dish in the same way that it had done in its host (Fig. 4). Bioluminescence from the microbial isolate was obtained after 48 hours of incubation in room temperature. The light emission lasted for a period of 8 hours. The microbe was unstable in nature, which means that its bioluminescent property only occurred in the primary isolation. The microbe lost its ability to produce light after culture iteration from the isolate which had lost its bioluminescent property.

### 3.4 Antibacterial Activities of Luminous Microbes from *Loligo sp*

The sensitivity test of luminescent microbial isolate on the growth of pathogenic microbe showed that L2 bacterial isolate was bioactive or capable in inhibiting the growth of test microbes. This research uses *V. harveyi*, *V. alginolyticus, E. coli, S. aureus* and *Bacillus* sp. as test pathogens.

The qualitative test result of isolate L2 against the test pathogens (*V. harveyi*, *V. alginolyticus, E. coli, S. aureus* and *Bacillus* sp.) are presented in Table 1.

The qualitative test of L2 isolates resulted in positive (+) against *V. harveyi, E. coli, S. aureus* and *Bacillus sp*., whereas against *V. alginolyticus* the result was negative (-) (Table 1). This means that L2 isolates are able to inhibit the growth of *V. harveyi, E. coli, S. aureus* and *Bacillus sp*. but it is not that of *V. alginolyticus*.

The quantitative sensitivity test result of isolate L2 against the test pathogens (*V. harveyi*, *V. alginolyticus, E. coli, S. aureus* and *Bacillus* sp.) are presented in Table 2.

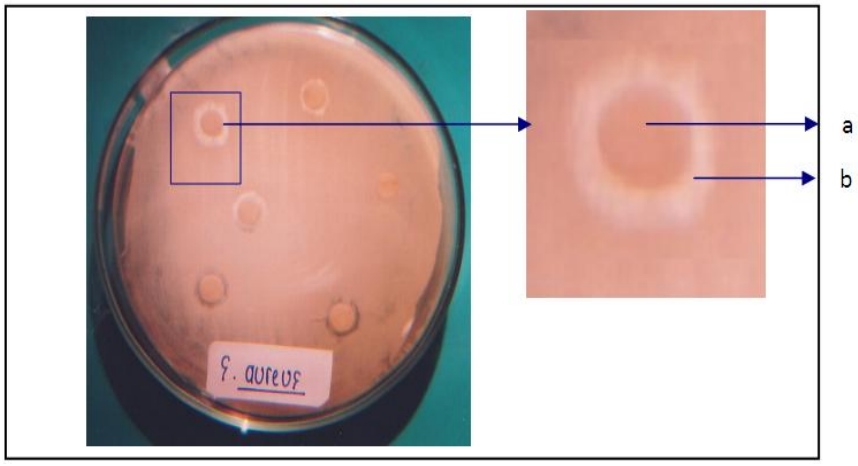



**Fig. 5. Formation of inhibition zone diameter (a- paper disk, b= inhibition zone**

**Table 1. Qualitative Test Results of Isolate L2 against Test Pathogens**

| Isolate Code | Test microbe | | | | |
|---|---|---|---|---|---|
| | ***V. harveyi*** | ***V. alginolyticus*** | ***E. coli*** | ***S. aureus*** | ***Bacillus* sp.** |
| L2 | + | - | + | + | + |

***Note:*** *+: Inhibition zone formed. -: Inhibition zone not formed*

**Table 2. Inhibition Zone of Luminescent Microbe in *L. edulis* against pathogenic microbe**

| Pathogenic Microbe | Incubation Period (h) | Inhibition zone diameter (in mm) |
|---|---|---|
| *V. harveyi* | 24 | 8.87 |
| | 48 | 8.79 |
| *E. coli* | 24 | 8.31 |
| | 48 | 8.21 |
| *S. aureus* | 24 | 9.02 |
| | 48 | 9.07 |
| *Bacillus* sp | 24 | 9.01 |
| | 48 | 8.83 |

## 3.5 Result of Protein Analysis by SDS-PAGE Electrophoresis

Observation of the position of the array of proteins produced by electrophoresis between luminescent bacteria (emitting light) and luminescent bacteria (not emitting light) did not indicate a significant difference. The only difference observed was the concentration of protein, color or protein concentration in the protein strain in the non-emitting luminescent bacteria, which was lower than the concentration in the protein strain of the light-emitting luminescent bacteria. The array of proteins which became the focus was around 80, 58, 50, 34 and 19 kDa molecular weights, which appeared on bothprotein strains of luminescent and non-luminescent bacteria Fig. 6.

Judging from its position, the array of typical protein strains of light-emitting bacteria and non light -emitting bacteria are not possessed by squid ink protein strains. This confirms the assumption that the protein contained in the sac is different to the protein produced by the symbiotic bacteria. Only protein strain of the sac possesses the array of protein groups around LMW 14, 15, 17, 21 and 32 kDa.

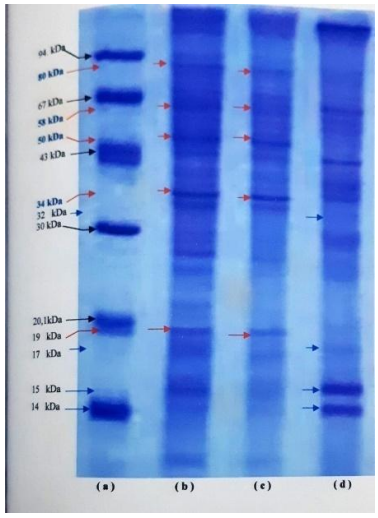



**Fig. 6. Result of Protein Analysis by SDS-PAGE Electrophoresis. (a) standard, (b) luminescentbacteria (Emitting light) (c) luminescent bacteria (not emitting light), (d) squid sac**

## 4. DISCUSSION

Bioluminescence in marine animals can be produced through a photophore which inherent special cells called photocytes, or through glands [2], or can be produced by light organs of fish that are symbiotic with luminescent bacteria, Luminescent microbes naturally have genes which allow them to produce luciferase enzymes and long aldehyde chains needed in bioluminescence chemical reactions [7]. Luciferase in an enzyme responsible in catalyzing luciferin substrate, which results in visible light thorough bioluminescence chemical reaction. The chemical reaction of bioluminesce is as follows:

$$FMNH_2 + RCHO + O_2 \longrightarrow FMN + H_2O + RCOOH + h\nu\ (490\ nm)$$

Luminescent bacteria isolated from *Loligo sp.* produce blue colored luminescence in petri dishes, and luminescent bacteria isolated from *Loligo edulis* have strong blue luminescence. On the other hand, luminescent bacteria from *H. nehereus* have weak green luminescence. The blue-green color has a wavelength range of approximately 490-509 nm.

Luciferase isolated from various types of bacteria shows the maximum spectral range of bioluminescence around 490 nm to 509 nm which ranges in the blue-green range [13]. 470 nm, which translates into the color blue, is the range of spectra for species for the genus *Photobacterium* [14]. Blue-green is a color that is often produced from bioluminescence chemical reactions because most marine organisms have the most sensitive visual pigments in the blue-green region [15]. In addition to that, blue is the color with the farthest-reaching wavelength in sea water (lmax ~ 475 nm) [14]. Green is the next most common color and is more commonly found in benthic and shallow coastal species, perhaps due to an increase in turbidity from particles in the water that spread blue light and support the transmission of longer wavelengths [15].

Two luminescent microbial isolates from *Loligo sp*. were identified as Uncultured bacterium clone 1P-1-G05 and Uncultured bacterium clone 3g10a, while luminescent microbial isolate from *Loligo edulis* was identified as *Photobacterium phosphoreum*. Luminescent bacteria from *H. nehereus* was identified as *Alteromonasmacleodii* strain NBRC 102226.Luminescent bacteria isolated from *Loligo sp*. or *Loligo edulis* showed strong and stable luminescence, meaning they can be recultured. On the other hand luminescent microbial isolates from H. nehereus displayed weak and unstable luminescence, meaning that they will lose their luminosity if they are cultured from darkened colonies. Luminescent microbes naturally possess a gene called the *Lux operon*, consisting of luxCDABEG. This *Lux operon* is responsible for the production of light in bacteria. The complex *Lux operon* system

is a microbial mechanism for quorum sensing [16]. Quorum sensing is a form of communication between microbial cells to form density and in this case produce light. From this very complex bioluminescence system, it is thought that several factors are the reason that several strains of luminescent bacteria carry lux genes when isolated from their host or natural habitat but these microbes do not express lux genes, case in point is *vibrio cholerae* [17], or when isolated from its host the luminescent microbe loses its natural phenotype making luminescence only occurs during the first isolation but becomes dark after being re-cultured [18].

Luminescent microbes from the sea, both those living in symbiosis with light organs or living free, which have so far been mostly identified as *Vibrio*, *Photobacterium* and *Alteromonas (shewanella)* [19]. Exploration of luminescent microbes from various waters and bioluminescence organisms continues with so many reported new species that add to the list of luminescent microbes. Most of these species are identified as the genus *Vibrio* or *Photobacterium*.Four luminescent microbes isolated from the coastal region of ChahBahar port, Sea of Oman, were identified as new strains and were reported to GeneBank. The four species mentioned are *Vibrio* sp. Persian 1, *Vibrio* sp. Persian 2, *Vibrio* sp. Persian 3, and *Vibrio* sp. Persian 4 [20]. Isolation of luminescent bacteria from squid taken from Malaysian waters was identified as *Photobacterium leiognathi,* whereas free living luminescent microbe from these waters was identified as *Vibrio sp.* [21].

Symbion microbes have the potential to produce secondary metabolites to protect host organisms, as a defense against pathogenic microorganismjas, and for the purposes of interspecies communication [20]. Some luminescent microbes are reported to have antimicrobial properties, although so far luminescent microbes have not been explored as new potential for sources of antimicrobial agents for sources of antimicrobial agents for emerging global threats of *multi drug resistence* microbes (Molina *et al.,*2016) [22].

Isolate from *Loligo sp.* was identified as *Uncultured bacterium* clone 1P-1-G05 with antimicrobial potential against *E. coli* MDR pathogenic microbe, which was capable of forming 32.59 mm inhibition zone, whereas the luminescent microbe identified as *Uncultured bacterium* clone 3g10a which displayed antimicrobial properties against *Enterobacter* sp.5 MDR pathogenic microbe and formed 28.44 mm of inhibition zone. The luminescent microbe *Photobacterium phosphoreum* isolated from *L. edulis* showed antibacterial properties against *V. harveyi, E. coli, S. aureus*, and *Bacillus sp*. Several luminescent microbes have also been reported to show antimicrobial properties; [23] reported 7 species of luminous microbes (*V. harveyi, V. campbellii, V. owensii, V. rotiferianus, V. alginolyticus, P. damselae* and *P. leiognathi*) showing antimicrobial activity against one pathogenic bacterium with inhibition zones ranging from 7 to 25 mm. [24] reported that luminescent bacteria were isolated from the mangrove ecosystem of Roach Park, Tuticorin, India, generally from the species *Vibrio sp*. and had antibacterial activity against human pathogenic microbes *Escherichia coli, Staphylococcus aureus*, and *Bacillus subtilis*. In addition, luminescent microbes isolated from *Lagocephalus spadiceus* (half-smooth golden puffer fish), *Leiognathus equulus* (ponyfish), *Scomber japonicus* (Pacific chub mackerel), and *Lutjanus argentimaculatus* (red snapper) were identified as *Vibrio sp*. (98%), *Vibrio anguillarium* strain X0906 (96%), *Vibrio* sp. (93%), and uncultured bacterium clone F2G (89%). The luminescent microbes also have the ability as antibacterial against MDR microbes *S. aureus* and *K. pneumonia* [22].

In general, the open surface area of marine organisms are rich in nutrients, giving rise to interest and competition among microbial communities [20,25]. This competition champions microbes that are able to produce metabolites that are beneficial for both the host and the microbe itself. It is known that luminescent microbes are symbiotic, and that they mostly live inside light-producing organs, such as those found in the light organs of *Loligo* sp. and *L. edulis*. The cum-squid light organ is only intended for certain types of luminescent microbes and is not a residence for non-luminescent microbes [7]. So that luminescent microbes in symbiosis with light organs no longer need to compete with other microbes that do not have luminous properties. However, this study found that luminescent microbes from *Loligo sp*. was able to inhibit the growth of MDR microbial pathogens and luminescent microbes L. written was able to inhibit the growth of various human pathogenic microbes [23] states that this ability is obtained because of the compounds produced by these microbes are beneficial for their survival through intra and interspecies chemical communication.

Microbial isolate from *H. nehereus* shows hemolysis gamma with no discoloration around the growing colony on the blood agar media. This shows that the microbes do not produce toxic substances capable of breaking down blood cells. Hemolysis is indicated by discoloration surrounding the colony on blood agar. This test is conducted to categorize bacteria in pathogenic and non-pathogenic groups by looking at the ability of bacteria to break down blood cells. Hemolysis alpha is the ability of bacteria to break down a portion of red blood cells, which occurs when the surrounding colonies turn green or brownish. Beta hemolysis is indicated by the appearance of clear colors surrounding the colonies, whereas gamma hemolysis is indicated by no change in agar [11]. In the future, the isolates of luminescent bacteria can be explored further for the potential they have given that these microbes do not produce blood cell breaking toxins.

Bioluminescence microbes have yet been explored intensively as new, potential antibiotic sources. So that later research can be further developed for such uses and to discover more properties of the antimicrobial compounds produced by luminescent microbes. Luminescent microbes have been known to have many benefits in the field of biotechnology, so further research to study bacterial self-defense mechanisms and bacterial evolution so that they can fight various pathogenic microbes is viable.

SDS-PAGE electrophoresis analysis of squid ink sacs and its symbiotic bacteria indicated that the protein found in bacteria was different from that of the sac. Meanwhile, observations that show that light emanates from bacteria means the source of the bioluminescence potential is the symbiotic bacteria. The facts found in this study indicate that the bioluminescence ability of *Loligo sp* and *H. nehereus* was the result of activity of their symbiotic bacteria, rather than innate abilities of both species.

## 5. CONCLUSION

Microbial isolation from light organ of *Loligo* sp. found two isolates emitting light with bluish hue. Isolate LDS 12-4 was identified as *Uncultured bacterium* clone 1P-1-G05 with antimicrobial potential against *E. coli* MDR pathogenic microbe, which was capable of forming 32.59 mm inhibition zone. Isolate LDS 18-5 was identified as *Uncultured bacterium* clone 3g10a which displayed antimicrobial properties against *Enterobacter* sp.5 MDR pathogenic microbe and formed 28.44 mm of inhibition zone. The analysis of luminescent microbe from *L. edulis* indicated the microbe was *Photobacterium phosphoreum* of which strong light emitted was bluish in hue. This species also showed strong antimicrobial properties against *V. harveyi, E. coli, S. aureus,* and *Bacillus* sp. The analysis of luminescent microbe from *H. nehereus*the Bombay duck was *Alteromonas macleodii* and this species was found to be capable of producing light with bright greenish hue. The microbe also displayed gamma hemolysis.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

## REFERENCES

1. Haddock SHD, Mark AM, James FC. Bioluminescence in the Sea. Annu. Rev. Mar. Sci. 2010; 2:443–93.
   DOI:10.1146/annurev-marine-120308-081028
2. Herring PJ. Luminescence in cephalopods andfish, Symp, Zool, Soc, London. 1977;38:127-159.
3. Shimomura O. Bioluminescence: Chemical Principles and Methods. Singapore (SG): World Scientific Publishing; 2006.
4. Charrier T, Durand MJ, Affi M, Jouanneau S, Gezekel H, Thouand G. Bacterial bioluminescent biosensor characterisation for on-line monitoring of heavy metals pollutions in waste water treatment plant effluents. Biosensors, Pier Andrea Serra, Intech Open; 2010.
   DOI: 10.5772/7210

5. Hellinger J, JaÈgers P, Donner M, Sutt F, Mark MD, Senen B, Tollrian R, Herlitze S. The flashlight fish *Aanomalops katoptron* uses bioluminescent light to detect prey in the dark. Plos One LoS One. 2017;12(2):1-18.
6. Pringgenies D, Jørgensen JM. Morphology of the Luminous Organ of Squid *Loligoduvaucelii*' Orbigny, 1839. Acta Zoologica. 1994;75(4):305-309.
7. Dunlap PV. Encyclopedia of Microbiology Bioluminescence, Microbial: Physiology. Elsevier Inc. University of Michigan, Ann Arbor; 2009.
8. Pringgenies D, Sastrodihardjo S, Nganro NR, Nyoman. Bacteria Symbiosis in Light Organ of the Squid *Loligo duvauceli* and cuttlefish *Sepia* sp Phuket Marine Biolical Center, Thailand. Spec. No. 2001;25(1):145-148.
9. Terkina IA, Parfenova VV, Ahn TS. Antagonistic activity of actinomycetes of Lake Baikal. Biochemistry and Microbiology. 2006;42:173-176 hlm.
10. Lay BW. AnalisisMikroba di Laboratorium. PT RajagrafindoPersada, Jakarta. 1994;168 hlm.
11. Lesmana M, Lebron CI, Taslim D, et al. *In vitro* antibiotic susceptibility of Neisseria gonorrhoeae in Jakarta, Indonesia. Antimicrob Agents Chemother. 2001;45(1):359-362. DOI:10.1128/AAC.45.1.359-362.2001
12. Laemli UK. Cleavage of structural proteins during the assembly of head of bacteriphage T4. Nature. 1970;227:680-685.
13. Lee FJ. A glucose-repressible gene encodes acetyl-CoA hydrolase from Saccharomyces cerevisiae. J Biol Chem. 1990;265(13):7413-8.
14. Daubner SC, Astorga AM, Leisman GB, Baldwin TO. Yellow light emission of *Vibrio fischeri*strain Y-1: purification and characterization of the energy-accepting yellow fluorescent protein. Proc Natl Acad Sci USA. 1987;84:8912–8916.
15. Herring PJ. The Spectral Characteristics of Luminous Marine Organisms. Proc. R. Soc. Lond. 1983;220:183-217.
16. Widder EA. Bioluminescence. In: "Adaptive Mechanisms in the Ecology of Vision." Edited by: S.N. Archer, M.B.A. Djamgoz, E. Loew, J.C. Partridge & S. Vallerga. Kluwer Academic Publishers, Dordrecht, the Netherlands. 1999;555–581.
17. Bassler BL. Cell-to-cell communication in bacteria. Journal Cell. 2002;109:421–424.
18. Nealson KH, Hastings JW. Bacterial bioluminescence: its control and ecological significance. Microbiol Rev. 1979;43:496–518.
19. Dunlap PV, Urbanczyk H. Luminous Bacteria in Prokaryotes. Springer-Verlag, Berlin. 2013;495-528.
20. Jabalameli F, Kalantar-Neyestanaki D, Asadollahi K, Taherikalani M, Emaneini M. Reply to: Molecular methods require for confirmation blaAIM (Adelaide imipenemase) producing Pseudomonas aeruginosa. Burns. 2014;40(7):1419-1420. DOI:10.1016/j.burns.2014.05.020
21. Yaser NA, Faiz MA, Foong A, Aslizah MZ, Iwana I. Isolation and identification of bioluminescent bacteria in squid and water of Malaysia. Int'l Journal of Advances in Agricultural & Environmental Engg. (IJAAEE). 2014;1(2):225-228.
22. Molina BSD, de Lima LR, Gomes LC, de Lima LS, Alcalde CR. Nutritive value of diets with inactive dry yeast for saanen goats in the peripartum period. Rev. Caatinga. 2016;29(1):200-205
23. Ramesh CH, Mohanraju R, Murthy KN, Karthick P, Narayana S. Impact of light, temperature, salinity, and glycerol on the intensity of luminescence and growth of marine bioluminescent bacteria *Vibrocampbellii* (strain SF1), Curr. Sci. 2014;106:511-513.
24. Arulmoorthy MP, Karunakaran K, Vignesh R, Vasudevan S, Srinivasan S. Identification and antimicrobial potential of bioluminescent bacteria isolated from the mangrove ecosystem of the Roach Park, Tuticorin, South east coast of India. BMR Microbiology. 2014;1:1-15.
25. Gram L, Jette M, Jesper BB. Antibacterial Activity of Marine Culturable Bacteria Collected from a Global Sampling of Ocean Surface Waters and Surface Swabs of Marine Organisms. Marine Biotechnology; 2009. DOI:10.1007/s10126-009-9233-y

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***Chapter 2***
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# Experimentation on the Validation of the Method for Determining Aflatoxin B1 in Groundnut Pastes Sold in the Markets of Abidjan (Côte d'Ivoire)

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## ABSTRACT

This study was carried out to validate the method of dosage of Aflatoxin B1 in the groundnut pastes for high performance liquid chromatography, according to the NFV03-110 Mai 2010 standard. The tests of the validation were performed out starting from ten samples. The linearity of calibration established between 0 and 3.0 µg/L was proven by determining the square of the coefficient of correlation of Pearson ($R^2$ = 0.9985). The limits of detection and quantification were respectively of 0.01 µg/kg and 0.02 µg/kg. The coefficients of variation of the tests of repeatability and reproducibility are respectively 3.4% and 3.79%, lower than 5%. The rate of recovery were estimated at 107.6%. The test of conformity did not show a significant difference (P > 0,05) between the average concentration in aflatoxine B1 (2.02 ± 1.44 µg/kg) and that of the certified value (2 µg/kg). The method of quantifying aflatoxin B1 in peanut paste by using HPLC can be regarded as acceptable for an analysis of routine.

*Keywords: Aflatoxin B1; groundnut paste; validation method.*

## 1. INTRODUCTION

The groundnut (*Arachis hypogaea*) is an oleaginous plant belonging to *the* genus *Arachis*, of the *Fabaceae* family which includes the majority of leguminous seeds and havinga high importance economic and nutritive [1-2]. It is a tropical plant originating from South America which was introduced in Africa, Asia and Europe [3]. The groundnut is the fifth culture among the most significant oilseeds in the world [4], the second edible source of oil [5], the third significant source of plant proteins and the twelfth vegetable production in the world [6].

In Africa, the sale of groundnuts is an income-generating activity for people in both rural and urban areas [7,8]. The groundnut is mainly transformed into oil, paste, flour and various derivatives which enter into the composition of foodstuffs. It is a good source of lipids, proteins, minerals, carbohydrates, fibres and vitamin E [9].

Despite its economic and nutritional potential, groundnut encounters enormous difficulties in harvesting and storage. According to [10], the groundnut is one of the oilseeds most contaminated by toxin-producing moulds most often during cultivation, harvesting and storage. Furthermore, according to [11], certain moulds such as the genera *Aspergillus*, *Penicillium* and *Fusarium* are able of producing toxins known as mycotoxins in groundnut seeds and its derivatives which can be very toxic for the consumer. However, according to [12], the complete elimination of the mycotoxins in the foodstuffs proves to be quasi-impossible because of their thermal stability.

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In sub-Saharan Africa and particularly in Côte.d'Ivoire, the groundnut paste is primarily produced in an artisanal way and marketed directly on the markets without any control. However, studies conducted in some countries, including Mali, Benin, Nigeria and Kenya, have revealed its contamination by mycotoxins [13-14]. Among these mycotoxins, aflatoxin B1 is the one characterized by the highest toxicity and most carcinogenic that can be found in many food products including the groundnut and its derivatives [15]. Health control requires that the level of aflatoxin B1 contamination of the groundnut dough be accurately determined for public decision-making. The first step in this process is therefore the validation of the method for the determination of aflatoxin B1 using high performance liquid chromatography as a principle.

## 2. MATERIALS AND METHODS

### 2.1 Material of Study

The material of study consists of ten samples of groundnut dough collected in various markets of the town of Abidjan having for laltitude: 5.3364 and longitude: -4.0266 with 5° 20′ 11″ North and 4° 1′ 36″ Western.

### 2.2 Reagents

The reagents used are all ~~of~~ analytical grade. These are standard solutions of aflatoxine B1 in particular, methanol grade HPLC (Fisher Bioblock Scientific, France), acétonitrile grade HPLC (Fisher Bioblock Scientific, France), acid acetic (Sigma Bioblock Scientific, France), dihydrogenated potassium phosphate (Fulka, France), sodium hydrogenocarbonate (Fulka, France).

### 2.3 Equipment

Aflatoxin B1 dosage was performed on a High Performance Liquid Chromatograph (Shimadzu, Kyoto, Japan) equipped with a fluorescence detector, a vacuum pump, an automatic injector, a Shim-pack CLCG-ODS C18 column (4 µm x 150 mm x 4.6 mm) and an operating software (Kroma 3000). The operating conditions are shown in Table 1.

**Table 1. Chromatographic conditions**

| Chromatograph | Operating conditions |
|---|---|
| Column | Shim-pack CLCG-ODS C18, 4 µm, 150 mm x 4,6 mm |
| Mobile phase | Methanol/acetonitrile (50:50 ; v/v) |
| Flow | 0,5 mL/min |
| Temperature | 40°C |
| Volume injected | 20 µl |
| Detecting | Fluorescence : λ excitation : 365 nm ; λ emission 435 nm |
| Duration of analysis | 15 minutes |

**Table 2. Validation of the method of determination of aflatoxine B1**

| Parameters | Results |
|---|---|
| Linearity (coefficient of correlation, $R^2$) | 0,9985 |
| Limit of détection (LOD) | 0,01 µg/kg |
| Limit of quantification (LOQ) | 0,02 µg/kg |
| Repetability (Coefficient of variation, CV) | 3,4% |
| Reproductibility (Coefficient of variation, CV) | 3,79% |
| Rate of recovery | 107,6% |
| Concentration of aflatoxine B1 obtained in the reference samples | 2,02±1,44[a] µg/kg |
| Certified value of aflatoxine B1 | 2[a] µg/kg |

*The values carrying the same letter on the same column do not present a significant difference (P<5 %) according to the test of Duncan*

## 2.4 Method of Validation

The validation of the method of analysis was carried out according to the method of the French Association of Standardization [16]. This procedure includes the study of the linearity of the calibration line, the determination of the limit of detection and the quantification, the calculation of the coefficient of variation for the tests of repeatability and reproducibility, and the calculation of the recovery rate for the exactitude of the tests.

### 2.4.1 Test of the linearity

Linearity of aflatoxin B1 was tested between 0 and 3.0 µg/L using 5 calibration points (0 µg/L, 0.2 µg/L, 0.6 µg/L, 1.3 µg/L and 3.0 µg/L). Five separate points were performed.

### 2.4.2 Limit of detection and limit of quantification

The limits of detection (LD) and quantification (LQ) were calculated from the white stamps and 10 distinct tests were analyzed by CLHP. LD = Average of the white + 3 times standard deviation (σ).

LQ = Average of the white + 10 times standard deviation (σ).

### 2.4.3 Tests of repeatability and reproducibility

To test repeatability, extracts from the same sample at a concentration of 0.5 µg/L were injected ten times on the same day into the chromatographic system. The coefficient ofvariation (CV) is obtained by making the standard deviation of the concentration results on the average of the same calculated concentrations.

For reproducibility, a sample with a concentration of 5 µg/L is injected each day in the chromatographic system during ten days (i.e. 10 injections). The coefficient of variation (CV) is calculated by making the standard deviation of the results of concentrations on the average of the same calculated concentrations.

### 2.4.4 Rate of recovery

Ten test specimens distinct from reference samples of groundnut paste were analyzed to appreciate the rate of recovery by the method for determination of aflatoxine B1. After extraction, purification and injection into the chromatographic system, the calculated concentrations made it possible to determine the rate of recovery by making the concentration read of the matrix injected on the theoretical concentration.

## 2.5 Method of Dosage of Aflatoxine B1

Aflatoxin B1 was determined according to [17] methods including the steps of extraction, purification on an immuno-affinity column and quantification using calibration after analysis by High Performance Liquid Chromatography.

A quantity of 25 g of groundnut paste was added to 100 mL of methanol/water solution (80:20, v/v) in a centrifuge pot. The mixture was homogenized using an agitator (Ultra Turax, France) for 3 minutes. The homogenate was centrifuged at 4000 rpm for 5 minutes and then filtered through Whatman N°4 paper in 25 mL Erlenmeyers. After filtration, a volume of 3 mL of the filtrate was added to 32 mL of phosphate saline buffer (PBS), for a total extraction volume of 35 mL. Thirty (30) mL of extract was deposited on an immunoaffinity column (Vicam, USA) at a flow rate of 1.5 mL/min. The column was then washed with 10 mL of PBS at a flow rate of 2 mL/min; the air in the column was removed by a vacuum pump during 30 seconds. The aflatoxins were eluted with 1.5 mL of acetonitrile solution in three 0.5 mL steps separated by a 1 minute pause between each step at a flow rate of 0.5 mL/min. The eluate was diluted with 1.5 mL of the PBS solution on the column at a flow rate of 0.5 mL/min.

The eluate was then collected in a 2 mL tube and dried under nitrogen at 40°C. The resulting residue was dissolved in 500 µL of methanol and then assayed by High Performance Liquid Chromatography.

### 2.6 Analyse Statistique

In case of significant differences between the parameters studied, an analysis of variance (ANOVA) was performed and the ranking of the means was done according to Duncan's test at the 5% α significance level. Differences are considered significant for values of $P<0.05$. The data were submitted in Excel and the results were presented in graph and table format.

## 3. RESULTS

The results of the validation are consigned in Table 2. The square of the coefficient of correlation of Pearson (R2) calculated to appreciate the linearity is 0.9985. The test of linearity showed a normal distribution on all the range of calibration from 0 to 3.0 µg/L. The limits of detection and quantification respectively 0.01 µg/kg and 0.02 µg/kg showed a high sensitivity of the technique used for the determination. The calculated coefficients of variation were 3.4% for the test of repeatability and 3.79% for the test of reproducibility. The rate of recovery obtained was 107.6%. The test of conformity did not show a significant difference with risk 0.05 between the average concentration obtained in the reference samples of groundnut paste (2.02 ± 1.44 µg/kg) and the certified value (2 µg/kg).

Fig. 1 presents the chromatogram of a reference sample containing aflatoxin B1 to a concentration of 5 µg/L and Fig. 2, the calibration curve.

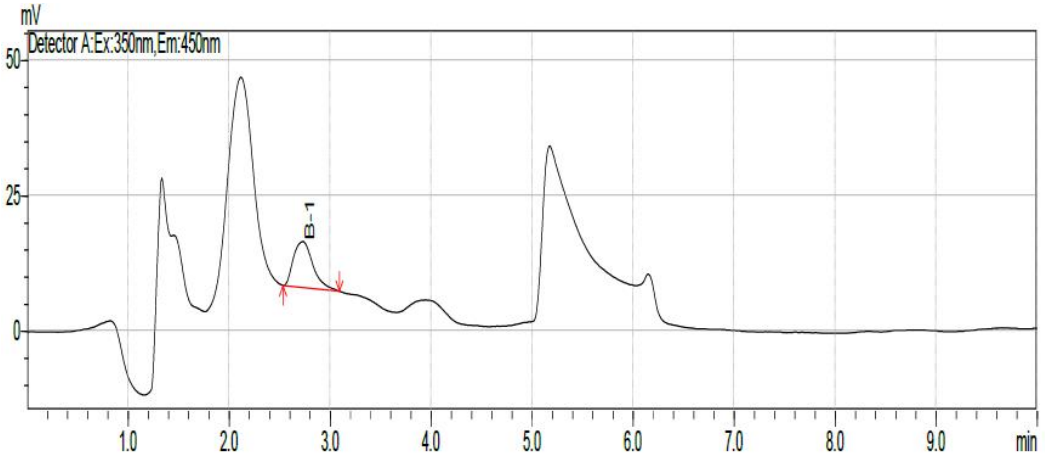



**Fig. 1. Chromatogram of a reference sample contaminated to 5 µg/L of aflatoxine B1**

## 4. DISCUSSION

The method used for this study was validated in terms of linearity, detection limit and limit of quantification, precision (repeatability and reproducibility tests) and recovery rate (extraction yield). These various parameters of validation are usually used for the validation of the method for the determination of aflatoxins by high-performance liquid chromatography [18] and [19], but also recommended by ICH (International Conferences Harmonization) and FDA (Food and drug administration) [20]. The test of the linearity highlights the normal distribution on all the range of calibration between 0 to 3.0 µg/L of aflatoxin B1. The linearity test shows the normal distribution over the entire calibration range from 0 to 3.0 µg/L aflatoxin B1. The correlation coefficient obtained for the regression line is greater than 0.990, thus it can be concluded that the method used is linear in the concentration ranges studied as reported by [21]. The results obtained for the detection and quantification limits are satisfactory.

The method is thus applicable and makes it possible to detect this substance with a sensitivity tested satisfactory. These results corroborate those of [22] who obtained detection and quantification limits of 0.01 µg/kg and 0.03 µg/kg respectively for the validation of the method for the determination of aflatoxin B1 in groundnut. The coefficients of variation calculated for the sample matrix of the repeatability and reproducibility tests are less than 5% and therefore acceptable. It can be said that the method is therefore applicable to the analyzed substance. The recovery rate obtained in this study is within the internationally recommended range (70-110%), therefore the method is applicable for this concentration range. The results obtained are comparable to those of other authors [22] and [23] who obtained 80-105% and 98.1-102.6% of the recovery rate for the validation of the method for the determination of aflatoxin B1 in groundnut, respectively. The dosed additions and determination of the certified concentrations of the reference samples also showed the accuracy of the assay results of the different samples because no significant difference was observed with the compliance test. Therefore, the results of the validation tests are consistent with the values indicating the acceptability of an analytical technique as indicated by the experts of the Joint FAO/IAEA Committee [24] and documented in the American Public Health Association (APHA) Collection of Standard Methods [25].

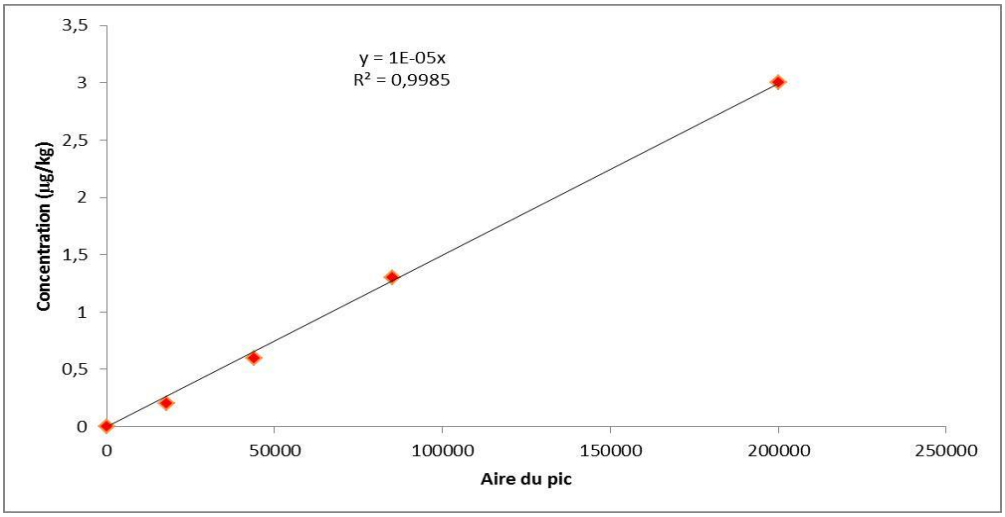



**Fig. 2. Calibration curve of aflatoxine B1**

## 5. CONCLUSION

The study carried out presented the results of the application of the high performance liquid chromatography method for the analysis of aflatoxin B1. The validation procedure confirmed that the proposed method for the determination of aflatoxin B1 in groundnut dough gave satisfactory results with internationally acceptable values for an analytical technique. Validation is essential for the risk assessment of mycotoxins in foodstuffs and particularly in peanut pastes sold in markets in order to prevent and ensure food safety for the Ivorian consumer and the quality of the product made available in local markets.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

## REFERENCES

1. Eke-Ejiofor J, Kiin-Kabari DB, Chukwu EC. Effect of processing method on the proximate, mineral and fungi properties of groundnut (*Arachis hypogea*). Seed Journal ofAgricultural and Biological Science. 2012;3:257-261.
2. Noba K, Ngom A, Guèye M, Bassène C, Kane M, Diop I, Ndoye F, Mbaye MS, Kane A, Ba AT. L'arachide au Sénégal: état des lieux, contrainteset perspectives pour la relance de la filière. Oilseeds Crpos and Lipids Journal Organisation. 2014;21(2):1-5.
   DOI: 10.1051/ocl/2013039
3. Ferguson ME, Jarvis A, Stalker HT, Williams DE, Guarino L, Valls JF, Pittman RN, Bramel PJ. Biogeography of wild Arachis (Leguminosae): distribution and environmental characterisation. Biodiversity and Conservation. 2005;14:1777-1798.
4. Sanginga N, Bergvinson D. Un plan d'action pour la transformation de l'agricultureafricaine. Oléagineuxet Niébé. 2015;27.
5. Knoden JL, Dufour L, Bindelle J. Fabrication de beurre de cacahuète. Collection Manuels et Techniques, Belgique. 2003;14.
6. FAOSTAT (Food and Agriculture Organization of the United Nations). Statistics Food. Production data; 2008.
   Available online at: http://faostat.fao.org.
7. FAO (Fond des Nations Unies pour l'Alimentationet de l'Agriculture). Etude sur la commercialisation des produitsmaraichersetcéréaliers et identification de nouveaux débouchés dans la région des savanes. 2007;95.
8. Diedhiou PM, Bandyopadhyay R, Atehnkeng J, Ojiambo PS. Aspergillus colonization and aflatoxin contamination of maize and sesame kernels in two agro-ecological zones in Senegal. Journal of Phytopathology. 2011;159(4):268-275.
   DOI: http://dx.doi.org/10.1111/j.1439-0434.2010.01761.x
9. Griel AE, Eissenstat B, Juturu V, Hsieh G, Kris-Etherton PM. Improved diet quality with peanut consumption. Journal of the American College of Nutrition. 2004;23:660-668.
10. Chapeland-Leclerc F, Papon N, Noël T, Villard J. Moisissures etrisquesalimentaires (mycotoxicoses). Revue Française des Laboratoires. 2005;373.
11. Wagacha JM, Mutegi CK, Christie ME, Karanja LW, Kimani J. Changes in Fungal Population and Aflatoxin Levels and Assessment of Major Aflatoxin Types in Stored Peanuts (*Arachishypogaea Linnaeus*). Journal of Food Research. 2013;2(5):10-23.
12. FAO (Fond des Nations Unies pour l'Alimentationet de l'Agriculture). Programmemixte FAO/OMS sur les normesalimentaires. Comité du codex sur les contaminants dans les aliments, septième session, Moscou (Russie). 2013;19.
13. Adjou SE, Yehouenou B, Sossou CM, Soumanou MM, Souza CA. Occurrence of mycotoxins and associated mycoflora in peanut cake product (kulikuli) marketed in Benin. African Journal of Biotechnology. 2012;11(78):14354-14360.
   DOI: http://dx.doi.org/10.5897/AJB12.324
14. Ndung'u JW, Makokha AO, Onyango CA, Mutegi CK, Wagacha JM, Christie ME, Wanjoya AK. Prevalence and potential for aflatoxin contamination in groundnuts and peanut butter from farmers and traders in Nairobi and Nyanza provinces of Kenya. Journal of Applied Biosciences. 2013;65:4922-4934.
15. Soleimany F, Jinap S, Abas F. Determination of mycotoxins in cereals by liquid chromatography tandem mass spectrometry. Food Chemistry. 2012;130:1055-1060.
16. AFNOR (Association Française de Normalisation). Analyse des produitsagricolesetalimentaires - protocole de caractérisationenvue de la validation d'uneméthoded'uneméthoded'analyse quantitative par construction du profild'exactitude. NF V03-110 Mai 2010. (Indice de classement : V03-110). Paris : Edition AFNOR. 2010;57.
17. AOAC (Association of Official Analytical Chemists). Aflatoxin M1 in liquid milk, immunoaffinity column by liquid chromatography. Official Methods of Analysis of AOAC International, 18th edition, AOAC, USA. Natural Toxins-chapter. 2005;49:45-47.

18. Alvarado-Hernández JR, Carvajal-Moreno M, Rojo-Callejas F, Ruiz-Velasco S. Aflatoxins in Natural Peanuts (*Arachis hypogaea L.)* of Mexico: Validation of the Biochemical Methods for Extraction and Quantification. Journal of Plant Biochemistry Physiology. 2016;4(2):1-11.
DOI:10.4172/2329- 9029.1000168
19. Gurav NP, Medhe S. Analysis of aflatoxins B1, B2, G1 and G2 in Peanuts: Validation Study. Analycal Chemistry. 201;17(2):1-9.
20. Adhikari R, Singh BH. A review on method development and validation using HPLC. International Journal for Pharmaceutical Research Scholars. 2016;5(3):167-172.
21. Dhib A. Validation d'uneMéthoded'Analyse des pesticides dans les tomatesfraîches par Chromatographieen Phase Gazeuseassociée à uncomptage par Scintillation Liquide. Diplômed'Ingénieur, Université de Tunis, Tunis. 2011;59.
22. Yazdanpanah H, Zarghi A, Shafaati AR, Foroutan SM, Aboul-Fathi F, Khoddam A, Nazari F, Shaki F. Analysis of Aflatoxin B1 in Iranian Foods Using HPLC and a Monolithic Column and Estimation of its Dietary Intake. Iranian Journal of Pharmaceutical Research. 2013;12 (supplement):83-89.
23. Baltac C, İlyasoğlu H, Yüksel F. Single-Laboratory Validation for the Determination of Aflatoxin B1, B2, G1, and G2 in Foods Based on Immunoaffinity Column and Liquid Chromatography with Postcolumn Derivatization and Fluorescence Detection. Food Analytical Methods. 2013;6(1):36-44.
24. FAO (Food and Agriculture Organization of the United Nations). Validation of analytical methods for food control. Report of a Joint FAO/AIEA Expert Consultation, FAO Food and Nutrition Paper N° 68, FAO, Rome, Italie. 1997;17.
25. APHA (American Public Health Association). Standard methods for the examination of water and wastewater. In American Public Health Association. Franson M. A. H., 27st edition, Washington DC. 2005;72.

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***Chapter 3***
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# Experimental Investigation on Tumour Suppressive and Organ Protective Effects of Aqueous *Andrographis paniculata* Leaves Extract on Benzene Induced Leukaemia Bearing Rats

**Akanni E. Olufemi[1*], O. R. Folarin[1] and C. Igbeneghu[1]**

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## ABSTRACT

**Aims:** To investigate and compare the protective and ameliorative role of aqueous leaf extract of *Andrographis paniculata* and standard drug 5-Fluorouracil on selected organs of male Wistar rats exposed to benzene carcinogen.
**Study Design:** Histological assessment of protective and ameliorative activity of the aqueous leaf extract of *Andrographis paniculata* and standard drug 5-Fluorouracil in some organs of experimental animals exposed to benzene carcinogen.
**Place and Duration of Study:** Department of Biomedical Sciences Ladoke Akintola University of Technology, College of Health Sciences, Osogbo. Nigeria between May and August 2012.
**Methodology:** Seventy-two (72) young male Wistar rats weighing 100-120 g were grouped into six (A-E), each group comprised of 6 rats in two replicates of three each (n=12).
Group A (control) received distilled water and normal saline (0.5 ml/kg each), group B received benzene chromasolv (0.2 ml at 1:10 dilution (water/2-propanol), group C received aqueous Andrographis paniculata (10 mg/kg bodyweight), group D was administered 5 -fluorouracil only (5 mg/kg body weight) for four weeks, group E was pretreated with Andrographis paniculata for 4 weeks prior to the administration of benzene chromasolv and group F was post treated with *Andrographis paniculata* for 4 weeks after pre-exposure to benzene chromasolv for 8 weeks. Leukaemia burden was assessed using haematological parameters such as Packed cell volume, Haemoglobin concentration, Red blood cells count and Total leukocyte count in the control and treatment groups.
**Results:** Results showed that leukemia was induced within 8 weeks as a significantly elevated WBC (leukocyte) counts and anaemia over the group not exposed to benzene which served as baseline were noted ($p < 0.05$).Organ histology showed varying lesions of the heart (mild to marked) coronary congestion, severe vacuolar degeneration and necrosis of the hepatocytes with cellular infiltration by mononuclear cells, diffuse tubular degeneration and necrosis with renal interstitial hemorrhage observed in the groups exposed to benzene carcinogen, 5-Fluorouracil and co-treatments of these two agents. However, hepato-renal and heart histio-architectures were intact in the extract pre-treatment and post-treatment groups.
**Conclusion:** These results suggest that *Andrographis paniculata* might be more effective than some other drugs currently in use as cancer suppressive chemotherapeutic agents and also may be a novel bioagent for the treatment of acute or chronic injuries induced on the liver, kidneys and heart by toxicants and carcinogens.

*Keywords: Andrographis paniculata; 5- fluorouracil; benzene; leukaemia; carcinogen.*

## 1. INTRODUCTION

Since time immemorial, plants have been used for maintaining health and curing diseases. It is a widely recognized fact that many pharmacologically active drugs are derived from natural resources

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such as medicinal plants [1,2]. Therefore, it is reasonable to search for novel drug molecules in herbs. With cancer being a widespread threat to humanity, plants play an important role in cancer prevention, as well as in therapy.

Medicinal plants provide new active chemopreventive molecules. In addition; treatment with plants can ease side-effects as well as provide support to the fears and anxieties of the sick. Some of the plants with reported anticancer properties are *Crocus sativus, Vitex, agnus-cactus, Withania somnifera, Curcuma longa, Scutellaria baicalensis, Gleditsia sinensis, Magnolia officinalis, Acanthopanax gracilistylus.*

*Andrographis paniculata Nees* popularly called king of the bitter is a medicinal plant belonging to the family Acanthaceae. As a perennial shrub *A. paniculata* is found mostly in evergreen, pine and deciduous forests and along road sides. It grows luxuriously in mild humid locations with tropical temperature and high rainfall [3]. It is a native to India and Sri Lanka and also found cultivated extensively in some parts of Europe, Southern Asia, China, Thailand and west Africa [4].

In folk medicine, *A. paniculata* is mostly used as heat repellant, poison-antidote and to prevent common cold and upper respiratory tract infection [5]. In Ayurvedic and Unani system of medicine (China), its leaf extringent “Kalmegh” is locally prepared cold and used as a common bitter tonic for treating chronic hepatitis and other ailments [6]. In modern research, *A. paniculata* is reported to exhibit wide range of biological activities including: antibacterial [7,8], antiviral [9,10,11], anti-inflammatory [12,13], anti-oxidant [14,15], immunomodulating/ immunostimulatory [16,17,18], Anti-diarrhoeal [19,20], Hepatoprotective and choleretic activity [21,22] and anticancer [23,24,25,26]. The plant also showed potential therapeutic action for curing hypertension and diabetes in human [27,28]. The enormous therapeutic use of *A. paniculata* for curing several human diseases has led to its usage as general antibiotics for farm animals. *A. paniculata* is extensively used in Taiwan to reduced mortality and cure digestive and respiratory tracts infection in broilers production and other live stocks [29].

The characteristic secondary metabolites found in this plant have enhanced its effectiveness as a potent medicinal plant for both human and animals [30,31]. The Chemical composition of *Andrographis paniculata* showed that it is a rich source of diterpenoids and flavonoids including other metabolites [32]. “Andrographolide” a diterpene lactone (SCHRI,1973), which is the major bioactive component isolated from *Andrographis paniculata,* has a colourless crystalline appearance with a bitter taste and is responsible for pharmacological activities observed from this plant [33-37]. The underlying molecular mechanisms have also been investigated and has been attributed to the nuclear transcription factor kappa B (NF-kB) which is the molecular target for the anti-inflammatory activity of *Andrographis paniculata* [38,39].

Benzene chromasolv is an environmental carcinogen, a haematoxin that is linked to increased incidence of cancer in humans and laboratory animals (Sigma-Aldrich Co. LLC.). Several types of neoplasm’s have been reported to be associated with benzene exposure in rats and mice after oral dosing or inhalation exposures for example it causes chromosome aberrations in humans and laboratory animals and such chromosomal rearrangements are relevant steps in the carcinogenic process [40].

Fluorouracil or 5-FU is a drug that is a pyrimidine analog which is used in the treatment of cancer [41]. The chemotherapy agent has been in use against cancer for about 40 years, it acts in several ways, but principally as a thymidylate synthase inhibitor. Interrupting the action of this enzyme blocks synthesis of the pyrimidine thymidine which is a nucleoside required for DNA replication so rapidly dividing cancerous cells undergo cell death [41].

Fluorouracil effects are felt system wide but fall most heavily upon rapidly dividing cells that make heavy use of their nucleotide synthesis machinery, such as cancer cells, but also other cells in parts of the body that are rapidly dividing, may also feel its effect after prolong usage. Adverse effects of this drug include myelosuppression, mucositis, dermatitis, diarrhea [42] and both acute central

nervous system (CNS) damage and progressively worsening delayed degeneration of the CNS in mice [43].

### 1.1 Aims of the Present Study

Despite the popular use and numerous therapeutic benefits of *Andrographis paniculata*, little is known about its anti cancer property especially on in vivo studies. This work aimed at investigating and comparing the possible protective and ameliorative roles of aqueous extract of the leaves and standard drug 5-fluorouracil which possess cancer chemopreventive activity on some organs (liver, heart and kidney) of wistar rats exposed to benzene carcinogen.

## 2. MATERIALS AND METHODS

### 2.1 Reagents

Benzene chromasolv (Sigma-Aldrich Co. LLC.), 5 Fluorouracil (5-FU), 10% buffered formalin, distilled water.

### 2.2 Plant Material

*Andrographis paniculata* leaves were harvested from Osogbo Local Government Area, Osun State and authenticated at Department of Botany and Phamacogonosis Obafemi Awolowo University, Ile Ife. The leaves were air dried at room temperature (24-27°C) for fifteen days; the air dried leaves were then milled into powder using electric blender (Phillips).

### 2.3 Preparation of Extract

Milled leaves weighing 150 grams was soaked in 1,500 mLs of distilled water for extraction and was kept at room temperature for 48 hours after which it was filtered through Whatman filter paper (No.1), concentrated using a rotary evaporator at low temperature (40-50°C). The extracts were preserved in airtight containers and kept at 4°C until further use.

### 2.4 Experimental Animals

Seventy-two young male Wistar rats weighing 100-120 g, purchased from the Experimental Animal house of Ladoke Akintola University of Technology, Ogbomoso Nigeria, were used for this study. These animals were kept in cages made with wood and galvanized wire mesh, at the College of Health Science Animal House, Mercyland campus in two replicates of six per group of 12 rats each. They were exposed to half daily cycle of light and dark. The diet and drinking water were provided *ad libitum*.

### 2.5 Experimental Design

Seventy-two (72) young male Wistar rats weighing 100-120 g were randomly allocated into six groups (A-E), each group comprised of 6 rats in two replicates of three each (n=12).

Group A: Received distilled water (0.5 ml/kg) + normal saline (0.5ml/kg) orally for four weeks.
Group B: was given benzene chromasolv (0.2 ml at 1:10 dilution (water/2-propanol) 50/50 v/v in water) 48 hourly for 4 times and allowed for 8 weeks prior to the administration of 5-Fluorouracil (5 mg/kg body wt) daily via tail vein for 4 weeks.
Group C: received *Andrographis paniculata* (10 mg/kg body wt) via gastric gavage daily using oral cannula for 4 weeks.
Group D: was administered 5-Fluorouracil only (5 mg/kg body wt) daily via tail vein for four weeks.
Group E: was pretreated with *Andrographis paniculata* (10 mg/kg body wt) for 4 weeks prior to the administration of benzene chromasolv (0.2ml at 1:10 dilution (water/2-propanol) 50/50 v/v in water) for 4 weeks.

Group F: was post treated with *Andrographis paniculata* (10 mg/kg body wt) for 4 weeks after pre-exposure to benzene chromasolv (0.2 ml at 1:10 dilution (water/2-propanol) for 8 weeks. The rats were sacrificed by cervical dislocation at the end of the treatment period.

## 2.6 Sample Collection

Each animal was weighed before sacrifice. The liver, kidney and heart were harvested and fixed in 10% buffered formalin in labeled bottles.

## 3. RESULTS AND DISCUSSION

Our previous study showed leukaemia induction in experimental rats exposed to benzene chromasolv within four weeks [44]. In this study leukaemia induction was evaluated using the estimated haematological parameters (blood counts) as in the Table 1. Results reflects a significant difference ($p<0.05$) between leukocyte counts of the rat group induced with leukaemia (marked leukocytosis) with anaemia (reduced haemoglobin concentration, red blood cells count and packed cell volume) when compared with rats that treated with water and saline. Therapeutic and chemo preventive properties of the extract are also evidenced by reversal of leukocytosis and anaemia in the various treated groups.

**Table 1. Mean±SD haematological parameters in the treatment groups and leukaemia control group**

| Treatment group | Leukocyte Count cmm | Red cells count $X10^9$/mm | Haemoglobin Concentration (g/dl) | Packed cells volume PCV (%) |
|---|---|---|---|---|
| A | 5.84 ±0.92 | 8.21±0.50 | 13.38±0.81 | 48.93 ±4.03 |
| B | 6.64 ±0.68 | 7.79±0.64 | 13.23±1.18 | 50.20±5.68 |
| C | 7.80 ±1.40 | 8.40±0.49 | 14.52±0.71 | 50.22±2.95 |
| D | 8.22±1.35 | 7.85±0.32 | 13.82±0.62 | 48.18±2.88 |
| E | 5.76±1.97 | 7.54±0.69 | 12.90±0.66 | 42.98±3.70 |
| F | 6.12±1.40 | 7.51±0.17 | 13.13±0.41 | 44.65±1.39 |
| G | 12.03±0.49* | 3.77±0.36* | 7.50±0.62* | 25.60±1.21* |

** Significant difference between the leukaemia control group and other treatment groups ($p<0.05$)*

A, Water and saline plus feed for 4 weeks; B, Leukaemia induction for 8 weeks prior to treatment with 5 FU; C, Aqueous *Andrographis paniculata* leaves extract with feed only; D, 5 FU only; E, Aqueous *Andrographis paniculata* leaves extract administration prior to leukaemia induction; F, Leukaemia induction followed by treatment with Aqueous *Andrographis paniculata* leaves extract, G, Leukaemia positive control.

## 3.1 Histopathology

The histio-architecture of the heart was retained in the control (Plate 1A), *Andrographis paniculata* (Plate 1C), *Andrographis paniculata* pre-treated + benzene chromasolv (Plate 1E) and benzene chromasolv pre-treated + *Andrographis paniculata* (Plate 1F) rats. However, mild to marked coronary congestion were observed in the heart of 5-Fluorouracil only (Plate 1D) and benzene chromasolv + 5-Fluorouracil treated rats (Plate 1B).

The kidneys of the control, *Andrographis paniculata*, *Andrographis paniculata* pre-treated + benzene chromasolv and benzene chromasolv pre-treated + *Andrographis paniculata* rats showed no visible lesion. The kidneys of benzene chromasolv + 5-Fluorouracil treated rats' demonstrated diffuse tubular necrosis and interstitial cellular infiltration. Similarly, there was marked tubular degeneration and necrosis coupled with some areas of hemorrhage into the interstitial in the kidney of 5-Fluorouracil treated- rats (Plate 2).

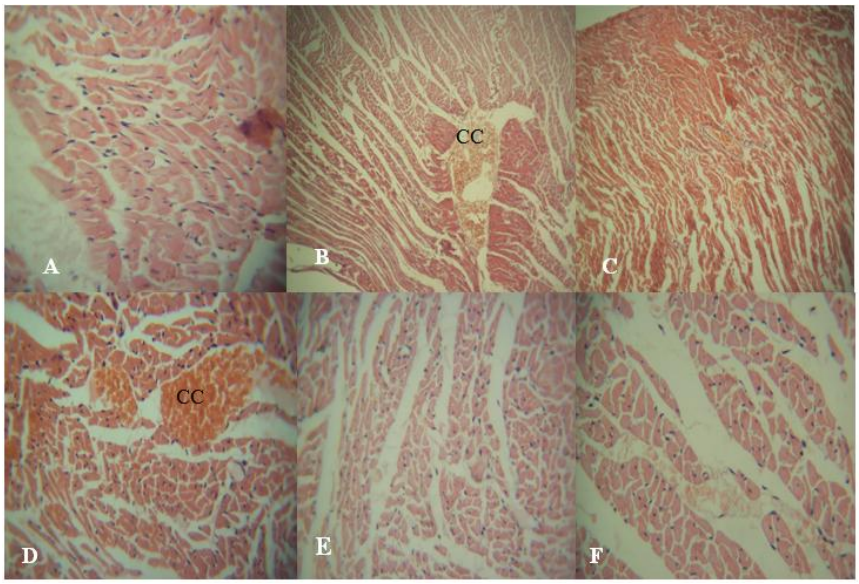



**Plate 1. Shows effect of aqueous extract of *Andographis paniculata* and 5-fluorouracil on the heart of male wistar rats exposed to benzene carcinogen**

*A. No visible lesions seen, B. There is mild coronary congestion (CC), C. no visible lesions D. there is marked coronary congestion (CC) in the heart, E. no visible lesions F. no visible lesions. Magnification: X400; Stain: H&E*

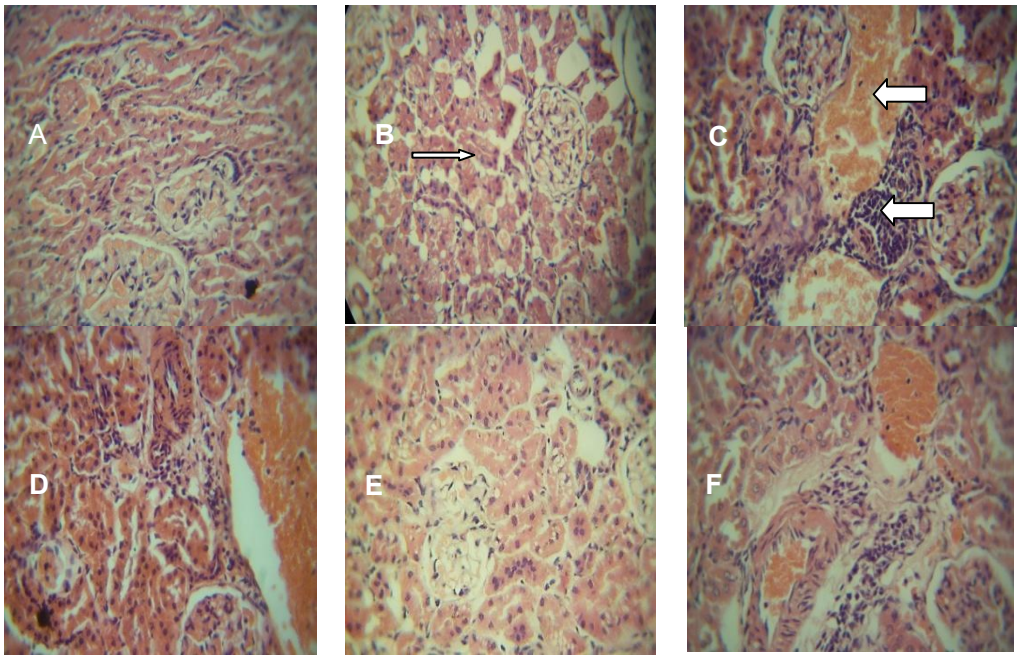



**Plate 2. Shows effect of aqueous extract of *Andographis paniculata* and 5-fluorouracil on the kidney of male wistar rats exposed to benzene carcinogen**

*A. No visible lesion B. Diffuse tubular necrosis (white arrow) and interstitial cellular infiltration in the kidney, C. mild congestion with perivascular cellular infiltration (white arrow), both closely associated with the glomeruli in the kidney, D. there is marked tubular degeneration and necrosis and there are also areas of haemorrhage into the interstitial in the kidney, E. no visible lesions F. no visible lesions. Magnification: X400; Stain: H&E*

The liver architecture of the control, *Andrographis paniculata*, *Andrographis paniculata* pre-treated + benzene chromasolv and benzene chromasolv pre-treated + *Andrographis paniculata* rats showed no visible lesion. On the contrary, the liver of benzene chromasolv + 5-Fluorouracil and 5-Fluorouracil only treated rats' showed varying degrees of severe vacuolar degeneration and necrosis with mild to moderate periportal cellular infiltration by mononuclear cells (Plate 3)

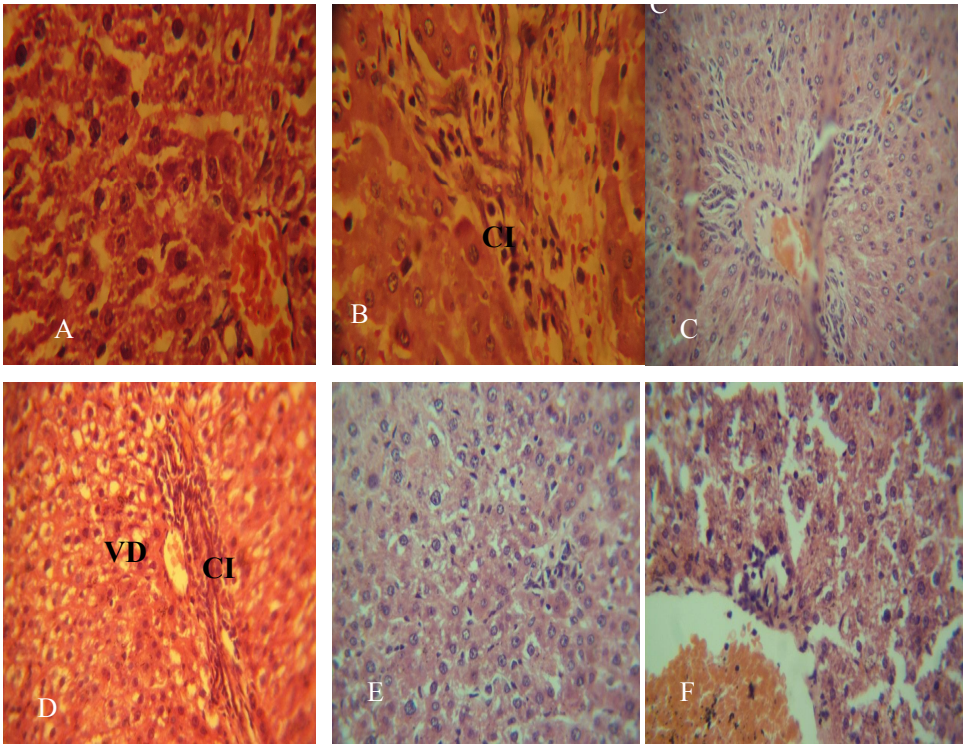



**Plate 3. Shows effect of aqueous extract of *Andographis paniculata* and 5-fluorouracil on the liver of male wistar rats exposed to benzene carcinogen**

*A. No visible lesions B. Severe vacuolar degeneration and necrosis with mild periportal cellular infiltration (CI) by mononuclear cells in the liver. C. No visible lesions D. mild to moderate portal cellular infiltration (CI), with mild diffuse and severe vacuolar degeneration (VD) of hepatocytes in the liver. E. no visible lesions seen lesions F. no visible lesions. Magnification: X400; Stain: H&E*

The various lesions of the heart (mild to marked coronary congestion), the liver (severe vacuolar degeneration and necrosis with mild to moderate periportal cellular infiltration by mononuclear cells) and the kidneys (diffuse tubular necrosis, tubular degeneration, renal interstitial hemorrhage and cellular infiltration) observed in this study are typical of histopathological lesions associated with 5-Fluorouracil and benzene chromasolv damage [40]. This has been attributed to DNA damaging ability of the agents [40]. In the same vein, the aggravated damage to the organs of rats co-treated with 5-Fluorouracil and benzene chromasolv is suggestive of the deleterious effects of Fluorouracil (anticancer drug) on normal cells after prolong usage.

The morphological appearance of the heart, kidneys and liver of the rats treated with Andrographis *paniculata* only was not disrupted in this study. This may indicate the safety of this herb as a natural and non-toxic traditional nutritional supplement for the management of liver, kidneys and cardiovascular disorders, as was previously reported [45,46,47].

Similarly, rats pre-treated with *Andrographis paniculata* and later exposed to benzene carcinogen or post-treated with *Andrographis paniculata* after exposure to benzene chromasolv pre-treatment had no distortion in their histio-architectures. This indicates that the plant extract could offer either protective or ameliorative properties accordingly. The possible mechanism underlying the hepato-

renal and cardio-protections of *A. paniculata* may be attributed to its antioxidant and free radical-scavenging properties [48].

This study also revealed that *Andrographis paniculata* could be more effective as a cancer suppressive chemotherapeutic agent than the standard drug 5-fluorouracil. Due to mild congestion with perivascular infiltration, both closely associated with renal glomeruli, of the group exposed to 5-fluorouracil only. It suggested that further research be carried out to determine the dosage and duration of usage of the drug.

## 4. CONCLUSION

In conclusion, the observed protective and ameliorative effects of *A. paniculata* could enhance the use of its aqueous leaf extract of as a novel bioagents for the treatment of acute or chronic injuries induced on the liver, kidneys and heart by toxicants and carcinogens.

## ETHICAL APPROVAL

Experimental protocol was approved by Institutional Ethics Committee of Ladoke Akintola University of Technology, College of Health Sciences, Osogbo, Osun - State, Nigeria. Animals were handled in accordance with international principles guiding the, use and handling of experimental animals (United States National Institutes for Health Publication, 1985).

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

## REFERENCES

1. Zong BH, Cao, Wang F. Anticancer polysaccharides from natural resources: A review of recent research, Carbohydrate Polymers. 2012;90(4):1395–1410.
2. Efferth T, Koch E. Complex interactions between Phytochemicals. The multi-target therapeutic concept of phytotherapy, Current Drug Targets. 2011;12(1):122–132.
3. DMPRD Division of Medical Plants Research and Development, Department of Medical Science, Ministry of Public Health) Handbook of Medicinal Plant for Primary Public Health. Text and Journal. 2009;53.
4. Akbar S. Andrographis paniculata: a review of pharmacological activities and clinical effects. Alternative Medicine Review. 2011;16(1):66-77.
5. Gabrielian ES, Shukarian AK, Goukasova GI, Chandanian GL, Panossian AG, Wikman G, Wagner H. A double blind, placebo-controlled study of *Andrographis paniculata* fixed combination Kan Jang in the treatment of acute upper respiratory tract infections including sinusitis. Phytomedicine. 2002;9(7):589-97.
6. Bensky D, Clavey S, Stoger E, Gamble A. Chinese herbal medicine. Materia medica. 2004;3: 437-40.
7. Dharmadasa RM, Samarasinghe K, Adhihetty P, Hettiarachchi PL. Comparative Pharmacognostic Evaluation of *Munronia Pinnata* (Wall.) Theob.(Meliaceae) and its substitute *Andrographis paniculata* (Burm. f.) Wall. Ex Nees (Acanthaceae). World Journal of Agricultural Research. 2013;1(5):77-81.
8. Sukesh K, Shafi Thompson T, Densingh J. Phytochemical investigation and antibacterial activity of *Gymnema sylvestre* and *Andrographis paniculata* from Western Ghats. Int. J. Phytomed. 2011;3:254-60.
9. Dua VK, Verma G, Dash AP. *In vitro* antiprotozoal activity of some xanthones isolated from the roots of *Andrographis paniculata*. Phytotherapy Research: An International Journal Devoted to

Pharmacological and Toxicological Evaluation of Natural Product Derivatives. 2009;23(1):126-8.

10. Manjusha GV, Rajathi K, Alphonse JK, Meera KS. Antioxidant potential and antimicrobial activity of *Andrographis paniculata* and *Tinospora cordifolia* against pathogenic organisms. J Pharm Res. 2011;4(2):452-5.
11. Radha R, Sermakkani M, Thangapandian V. Evaluation of phytochemical and antimicrobial activity of *Andrographis paniculata* nees (Acanthaceae) aerial parts. Int. J. of Pharm. Life Sci. 2011;2(2):562-7.
12. Sheeja K, Shihab PK, Kuttan G. Antioxidant and anti-inflammatory activities of the plant *Andrographis paniculata* Nees. Immunopharmacology and Immunotoxicology. 2006;28(1):129-40.
13. Radhika P, Rajendra YP, Sastry BS, Lakshmi KR. Anti-inflammatory activity of chloroform extract of *Andrographis paniculata* Nees Stem. Res J Biotechnol. 2009;4(2):35-8.
14. Premanath R, Devi NL. Antibacterial, antifungal and antioxidant activities of *Andrographis paniculata* Nees. Leaves. International Journal of Pharmaceutical Sciences and Research. 2011;2(8):2091.
15. Mishra US, Mishra A, Kumari R, Murthy PN, Naik BS. Antibacterial activity of ethanol extract of *Andrographis paniculata*. Indian Journal of Pharmaceutical Sciences. 2009;71(4):436.
16. Puri A, Saxena R, Saxena RP, Saxena KC, Srivastava V, Tandon JS. Immunostimulant agents from *Andrographis paniculata*. Journal of Natural Products. 1993;56(7):995-9.
17. Hidalgo MA, Romero A, Figueroa J, Cortés P, Concha II, Hancke JL, Burgos RA. Andrographolide interferes with binding of nuclear factor-κB to DNA in HL-60-derived neutrophilic cells. British Journal of Pharmacology. 2005;144(5):680-6.
18. Qin LH, Kong L, Shi GJ, Wang ZT, Ge BX. Andrographolide inhibits the production of TNF-α and interleukin-12 in lipopolysaccharide-stimulated macrophages: Role of mitogen-activated protein kinases. Biological and Pharmaceutical Bulletin. 2006;29(2):220-4.
19. Gupta S, Choudhry MA, Yadava JN, Srivastava V, Tandon JS. Antidiarrhoeal activity of diterpenes of *Andrographis paniculata* (Kal-Megh) against *Escherichia coli* enterotoxin *in vivo* models. International Journal of Crude Drug Research. 1990;28(4):273-83.
20. Sindermsuk J. The antibacterial activities of the pure extract compounds from *Andrographis paniculata* on predominate pathogenic enteric bacilli in Thailand. Bull Dept. Med. Serv. 1993; 18:394-400.
21. Trivedi NP, Rawal UM, Patel BP. Hepatoprotective effect of andrographolide against hexachlorocyclohexane-induced oxidative injury. Integrative Cancer Therapies. 2007;6(3):271-80.
22. Shukla B, Visen PK, Patnaik GK, Dhawan BN. Choleretic effect of andrographolide in rats and guinea pigs1. Planta Medica. 1992;58(02):146-9.
23. Butler MS, Robertson AA, Cooper MA. Natural product and natural product derived drugs in clinical trials. Natural Product Reports. 2014;31(11):1612-61.
24. Chun JY, Tummala R, Nadiminty N, Lou W, Liu C, Yang J, Evans CP, Zhou Q, Gao AC. Andrographolide, an herbal medicine, inhibits interleukin-6 expression and suppresses prostate cancer cell growth. Genes & cancer. 2010;1(8):868-76.
25. Lin HH, Tsai CW, Chou FP, Wang CJ, Hsuan SW, Wang CK, Chen JH. Andrographolide down-regulates hypoxia-inducible factor-1α in human non-small cell lung cancer A549 cells. Toxicology and Applied Pharmacology. 2011;250(3):336-45.
26. Mulukuri NS, Mondal NB, Prasad MR, Renuka S, Ramakrishna K. Research article isolation of diterpenoid lactones from the leaves of *Andrographis paniculata* and its anticancer activity. Int J Pharmacogn Phytochem. 2011;3:39-42.
27. Dandu AM, Inamdar NM. Evaluation of beneficial effects of antioxidant properties of aqueous leaf extract of Andrographis paniculata in STZ-induced diabetes. Pakistan Journal of Pharmaceutical Sciences. 2009;22(1):49-52.
28. Augustine AW, Narasimhan A, Vishwanathan M, Karundevi B. Evaluation of antidiabetic property of Andrographis paniculata powder in high fat and sucrose-induced type-2 diabetic adult male rat. Asian Pacific Journal of Tropical Disease. 2014;4:S140-7.
29. Kabeeruddin M. Kitabul Advia. Delhi, India: Aligarh Barqi Press; 1937;2:148

30. Sharma M, Sharma A, Tyagi S. Quantitative HPLC analysis of andrographolide in Andrographis paniculata at two different stages of life cycle of plant. Acta Chim. Pharm. Indica. 2012;2(1):1-7.
31. Sivananthan M, Elamaran M. Medicinal and pharmacological properties of Andrographis paniculata. Int J Biomol Biomed 2013b. 2013;3(2):1-2.
32. Pholphana N, Rangkadilok N, Thongest S, Ruchirawat S, Ruchirawat M, Satayavivad J. Determine and variation of three active diterpenoids in *Andrographis paniculata* (Burm.f.) Nees. Phytochem. Anal. 2004;15:365-371.
33. Kapil A, Koul IB, Banerjee SK, Gupta BD. Antihepatotoxic effects of major diterpenoid constituents of *Andrographis paniculata*. Biochemical Pharmacology. 1993;46:182–185.
34. Visen PK, Shukla B, Patnaik GK, Dhawan BN. Andrographolide protects rat hepatocytes against paracetamol-induced damage. Journal of Ethnopharmacology Cancer Therapy. 1993; 40:131–136.
35. Shen YC, Chen CF, Chiou WF. Suppression of rat neutrophil reactive oxygen species production and adhesion by the diterpenoid lactone andrographolide. Planta Medica. 2000;66:314–317.
36. Li J, Luo L, Wang X, Liao B, Li G. Inhibition of NF-kappaB expression and allergen-induced airway inflammation in a mouse allergic asthma model by andrographolide. Cellular and Molecular Immunology. 2009;6:381–385.
37. Abu-Ghefreh AA, Canatan H, Ezeamuzie CI. *In vitro* and *in vivo* anti-inflammatory effects of andrographolide. International Immunopharmacology. 2009;9:313–318.
38. Xia YF, Ye Q, Li YD, Wang J G, He XJ, Lin X. Andrographolide attenuates inflammation by inhibition of NF-kappa B activation through covalent modification of reduced cysteine 62 of p50. J Immunol. 2004;173:4207–17.
39. Hidalgo MA, Romero A, Figueroa J, Cortes P, Concha II, Hancke JL. Andrographolide interferes with binding of nuclear factor-kappaB to DNA in HL-60-derived neutrophilic cells. Br J Pharmacol. 2005;144:680–6.
40. Andrew W, Janet S. (Benzene Department of Human Services (USA); 1997.
41. Longley DB, Harkin DP, Johnston PG. 5-fluorouracil: mechanisms of action and clinical strategies. Nat. Rev. Cancer. 2003;3(5):330–8.
42. Chu E. Ode to 5-Fluorouracil. Clinical Colorectal Cancer. 2007;6(9):609.
43. Mark DN. Chemotherapy-induced Damage to the CNS as a Precursor Cell Disease by, University of Rochester; 2011.
44. Akanni EO, Alli OAT, Oloke JK. Anti-leukemic and immunomodulatory effects of fungal metabolites of *Pleurotus pulmonarius* and *Pleurotus ostreatus* on benzene-induced leukemia in Wister rats. Korean J Hematol. 2012;47(1):67–73.
45. Vetriselvan S, Rajamanikkam V, Subasini PD, Arun G. Comparative evaluation of hepatoprotective activity of *Andrographis paniculata* and Silymarin in ethanol induced hepatotoxicity in albino wistar rats. Der Pharmacia Lettre. 2010;2(6):52-59.
46. Singh P, Srivastava MM, Khemani LD. Renoprotective effects of *Andrographis paniculata* (Burm.f.) Nees in rats. Upsala Journal of Medical Sciences. 2009;114:136-139.
47. Sheeja K, Guruvayoorappan C, Kuttan G. Antiangiogenic activity of *Andrographis paniculata* extract and andrographolide. Int Immunopharmacol. 2007;7:211-21.
48. Ojha SK, Nandave M, Kumari S, Arya DS. Antioxidant activity of *Andrographis paniculata* in ischemic myocardium of rats. Global Journal of Pharmacology. 2009;3(3):154-157.

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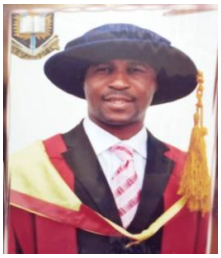
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# Mapping and Map-based Cloning of QTL Conferring Durable Resistance to Rice Blast towards Rice Breeding Strategy

**Kazutoshi Okuno[1,2*] and Shuichi Fukuoka[3]**

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## ABSTRACT

Rice blast caused by *Pyricularia oryzae* (syn. *Magnaporthe oryzae*) is a destructive disease of rice worldwide that poses a serious threat to rice production. Strengthening blast resistance is an important objective in rice breeding programs. Race-specific resistance genes (*R*-genes) confer complete resistance to blast, but new races of blast pathogen can overcome it. To avoid the genetic vulnerability of *R*-genes, race-nonspecific resistance in Japanese upland rice varieties has been focused on its durability. However, linkage drag between genes controlling this type of blast resistance and undesired traits has hindered its use. We genetically dissected race-nonspecific resistance to rice blast. Among detected QTLs, a single recessive resistance gene, *pi21*, was identified by map-based cloning. The use of race-nonspecific resistance genes like *pi21* has improved durable resistance in rice breeding programs.

*Keywords: Race-nonspecific resistance; durable resistance; QTL analysis; positional cloning; marker-assisted selection; pyramiding; rice; blast disease.*

## 1. INTRODUCTION

Rice blast caused by the fungus *Magnaporthe grisea* (*Pyricularia oryzae*) is a major biotic constraint in rice cropping regions worldwide. Between 10% and 30% of the annual rice harvest is lost because of infection by rice blast fungus [1]. Genetic improvement of resistance against rice blast is a significant and primary target in rice breeding programs.

Based on plant-pathogen interactions, the resistance to rice blast is categorized into two types, race-specific and race-nonspecific. Many race-specific resistance genes (*R*-genes) have been identified in a broad range of the world rice germplasm [2]. *R*-genes dramatically enhance blast resistance and result in stable rice production, but their extensive use poses a serious risk of the generation of new races of the blast pathogen and the quick breakdown of resistance (genetic vulnerability). In Japan, since the first breakdown of resistance occurred only 2 years after release of the resistant variety, 'Kusabue' with *Pik* introgressed from a Chinese variety in 1963, the resistance by several different *R*–genes has similarly broken down in the following years.

Similar trends were reported in Korea and Colombia [3]. Hence, rice breeders have paid their attention to the use of race-nonspecific resistance.

Japanese upland rice varieties are potential gene donors of race-nonspecific resistance.

Previous genetic studies indicated that the resistance of Japanese upland rice varieties was controlled by multiple genes or polygenes and two of them were possibly linked to the phenol reaction(*Ph*) locus

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on chromosome 4 or lax panicle (*lax*) locus on chromosome 11 [4,5]. However, the conventional genetic approaches could not determine the exact number of genes conferring the resistance and their chromosomal locations. Moreover, upland rice varieties have undesired characteristics, particularly poor grain and eating quality. Prior to the use of upland rice varieties to improve durable resistance to rice blast, the genetic dissection of the resistance is required.

This paper reviews mapping and map-based cloning of QTL conferring race-nonspecific resistance and its use for rice breeding programs to strengthen durable resistance to rice blast.

## 2. MAPPING OF QTLS CONFERRING RACE-NONSPECIFIC RESISTANCE TO RICE BLAST

QTLs for race-nonspecific resistance to rice blast were analyzed in progeny derived from the cross between Japanese upland and paddy rice varieties. In an 'Owarihatamochi' (resistant, upland) × 'Nipponbare' (moderately susceptible, paddy) cross, two resistance QTL alleles on chromosome 4 and one on chromosome 12 from 'Owarihatamochi' were identified [6,7]. Each QTL explained from 13.7% to 45.7% of the total phenotypic variation. One of QTL on chromosome 4 was inherited as a single recessive gene and was designated *pi21*.

Resistance QTL from other upland rice varieties were also detected in similar regions to those in 'Owarihatamochi' on chromosomes 4, 11 and 12 [8,9]. Resistance QTL alleles from other varieties were located mainly in a 30-Mb region of chromosome 4 and on chromosomes 1, 3, 6 and 11. Accumulated evidences highlight the target regions for improving race-nonspecific resistance to rice blast.

## 3. CHARACTERIZATION OF GENES FOR RACE-SPECIFIC RESISTANCE

More than 100 loci for resistance to blast have been identified and more than 30 of them have been cloned [10]. Race-specific *R* genes encode nucleotide-binding site (NBS) leucine-rich repeat (LRR) proteins that interact with pathogen effectors and trigger defense reactions according to the gene-for-gene model of recognition [11]. The exceptions are *Pid2* encoding a receptor-like kinase [12] and *Ptr* encoding anArmadillo repeat protein [13]. Recent studies in rice and *Arabidopsis* showed that different genes for NBS–LRR proteins, such as a pair of tightly linked NBS–LRR genes, cooperate in pathogen recognition and resistance. In contrast, the number of genes that have been cloned for race-nonspecific resistance is so limited.

## 4. MAP-BASED CLONING OF RACE-NONSPECIFIC RESISTANCE GENE, *PI21*

Linkage analysis and progeny testing narrowed down the *Pi21* locus to a small region carrying a single gene locus, *Os04g0401000* [14]. This gene encodes a protein with a putative heavy-metal-binding domain and a proline-rich region. Comparison of sequences between resistant and susceptible varieties identified 21- and 48-bp deletions in the resistant variety, suggesting that one of these deletions confers the resistance. Transgenic complementation testing confirmed that a loss-of-function mutation in the gene improves resistance to rice blast.

Information on the variation of QTL alleles allows the use of a wide range of germplasm. In the case of the *pi21* gene, Asian cultivated rice (*Oryza sativa* L.) has 12 haplotypes determined by insertion/deletion variations at three sites in the proline-rich region, which is presumed to be involved in protein–protein interactions in multicellular organisms. Each of the 12 haplotypes carries one of the two deletions or two smaller deletions compared with 'Owarihatamochi' haplotype, but it is difficult to predict the resistance/susceptibility phenotype from DNA sequences. Inoculation testing using a series of backcrossed lines carrying each of the *Pi21* haplotypes in the same genetic background indicated that only the line carrying the 'Owarihatamochi' haplotype showed improved resistance to blast. The results suggest that the two deletions in the resistance *pi21* allele are optimal to cause the loss of function, which increases resistance to blast.

## 5. MAP-BASED CLONING OF OTHER QTLS FOR RACE-NONSPECIFIC RESISTANCE

*Pi35* is a major resistance QTL on chromosome 1 which was found in a Japanese breeding line, 'Hokkai 188' and has maintained resistance under natural field conditions since 1961 [15]. Linkage analysis and complementation testing revealed that *Pi35* is allelic to *Pish*, a typical *R*-gene that encodes an NBS–LRR protein [16]. Six differences in the deduced amino acid sequences were identified between *Pi35* and *Pish*. One of the four residues in the LRR region is significantly associated with race-nonspecific resistance [17]. Three other residues in the LRR region and two residues in the NBS domain are also associated with the resistance, suggesting that a combination of multiple functional polymorphisms in the gene confers race-nonspecific resistance.

*Pi63* is a major resistance QTL in a 30-Mb region of chromosome 4 which were found in a Japanese upland rice variety, 'Kahei' [9]. Linkage analysis and complementation testing demonstrated that this gene encodes an NBS–LRR protein and is located within an *R*-gene cluster [18,19]). Interestingly, the resistance conferred by *Pi63* is isolate-specific. Such characteristic has not been detected in the genetic background of the donor variety because of the effect of the race-nonspecific *pi21* allele and alleles of other resistance QTLs. Increased expression of *Pi63* in transgenic lines led to moderate resistance against pathogen isolates that produce a highly susceptible phenotype in the NIL for *Pi63*.

*Panicle blast 1* (*Pb1*) on chromosome 11 is a gene derived from the *indica* variety 'Modan' [20]. Plants carrying this gene are susceptible to blast during young vegetative stages, but the resistance level increases as the plants grow and persists even after heading. Map-based cloning of *Pb1* revealed that it encodes an atypical NBS–LRR protein that has no P-loop and some motifs in the NBS domain are degenerated [21]. *Pb1* transcript level increased during the growth of *Pb1*-resistant varieties and effectively control panicle blast. *Pb1*-mediated resistance seems to be mediated by a signaling pathway distinct from that involving typical NBS–LRR proteins.

## 6. BREEDING OF DURABLE RESISTANCE TO RICE BLAST USING *PI21*

Most upland rice varieties in Japan are donors of *pi21* but their morphological and physiological characteristics are distinct from those of elite paddy rice varieties. Breeding efforts to introduce resistance alleles of major QTLs from upland rice started in the 1920s. But the breeding trials were unsuccessful because of the co-introduction of undesirable characteristics from the donors by tight linkage drag between resistance gene and genes controlling eating quality and other traits.

To remove donor chromosome segments around the *Pi21* locus and elsewhere in the genome, a line carrying the *pi21* allele was backcrossed with an elite paddy rice variety, 'Koshihikari' and the progeny carrying a single 1.8-Mb fragment around the *Pi21* locus from the donor was selected. Plants with recombination events within a 40-kb interval containing the *Pi21* locus were selected from approximately 6000 progenies. Eating quality of a progeny line carrying the 'Koshihikari' chromosomal segment from a point less than 2.4 kb downstream of the *Pi21* locus was equivalent to that of 'Koshihikari', and the line was highly resistant to blast. In contrast, a progeny line carrying the donor chromosomal sequence up to 37 kb downstream of the *Pi21* locus showed inferior eating quality. These results clearly show that the resistance *pi21* allele does not penalize agronomic traits and the cause of the association is tight linkage between genes conferring the resistance and undesirable traits.

The promising line with improved blast resistance and desirable grain characteristics was released as 'Tomohonami' ('Chubu 125') in 2009 [22]. 'Tomohonami' has been used as an excellent donor of *pi21* at more than 15 prefectural breeding stations and 6 research centers of the National Agriculture and Food Research Organization, Japan. More than 15 breeding lines carrying *pi21* have been developed. Recently, 'Fufufu', a line derived from 'Tomohonami' carrying *pi21* and *Apq1* for high-temperature tolerance during ripening, was released.

Slow induction of defense by *pi21* contributes to pathogen control without penalty on yield, as confirmedby field tests at several locations. The *pi21* alleles are effective against diverse fungus races, so the use of *pi21* might not be a strong driving force for changes in the structure of pathogen

populations. The durability of resistance conferred by *pi21* is required to be proved by prolonged resistance of varieties under natural field conditions. Monitoring of newly released varieties carrying *pi21* will provide further evidence to confirm or disprove the durability of resistance conferred by *pi21*.

## 7. GENE PYRAMIDING FOR SUSTAINABLE CONTROL OF RICE BLAST

Gene pyramiding (combining multiple resistance alleles in the one genetic background) is a way to enhance durable resistance in crop plants. If a single genotype confers durable resistance, this approach is desirable for breeders because breeding procedures though DNA marker-assisted selection are efficient and effective.

*R*-gene pyramids improve resistance to diverse pathogen isolates. A comprehensive survey of a series of gene pyramids detected interaction among genes. Among combinations between one of the *Pigm*, *Pi2*, *Pi9*, *Pi40* and *Piz* genes and one of the *Pi1*, *Pi33* and *Pi54* genes, *Pigm/Pi1*, *Pigm/Pi54* and *Pigm/Pi33*provided the best resistance at both seedling and heading stages [23]. These results highlight the importance of screening for favorable gene combinations to maximize the resistance.

Broadening the spectrum of resistance by pyramiding *R*-genes may prompt the counter-evolution of the pathogen; for example, the resistance of a variety with four *R*-genes was overcome one year after its release. The emergence of super-races that overcome the resistance of *R*-gene pyramids might increase over time when a single variety carrying an *R*-gene pyramid is cultivated in a large area. An epidemiological survey and simulation study on pathogen race dynamics suggest that replacement by varieties with different *R*-genes leads to drastic changes in the pathogen population structure that increase the risk of disease outbreak. Hence, *R*-gene pyramids, each resulting in a strong selection pressure against its pathogen, may not improve the durability of resistance. Further study of the effect of *R*-gene pyramids on pathogen population dynamics in the field is necessary to develop the guidelines for their use.

Combining multiple resistance QTL alleles is considered to additively enhance race-nonspecific resistance. However, breeders and researchers know that disease resistance sometimes interacts with genetic backgrounds and/or environmental factors. The data on resistance to blast over two decades support this idea in the context of race-specificity and temperature-dependent resistance and indicate the existence of genetic loci that modulate the resistance or its mode of action. To understand how resistance allele interacts with such factors, it is important to determine the appropriate number and combinations of resistance genes. However, knowledge of the impact of QTL pyramiding on the robustness of plant defense in rice is limited.

In the genetic background of the susceptible ‘Aichiasahi’, the average reduction of lesion area by *pi21* in eight field trials was 87% compared with the recurrent parent, whereas that by the minor QTL alleles was 39% by *Pi34*, 45% by *qBR4-2* and 22% by *qBR12-1* [24]. Although the effects of these minor QTL alleles were sometimes undetectable, their combinations dramatically reduced lesion area both in field tests and in glasshouse inoculation tests. The line with four resistance alleles had a lesion area of ≤1%, which was similar area to the donor and was only 6% of that in the line carrying *pi21* only, suggesting that the QTL pyramid conferred robust resistance. Similar results were obtained for a series of lines with one to four resistance QTL alleles, despite the presence of background noise from donors. A more important observation is that a QTL pyramid improves the stability of resistance; the coefficient of variation of lesion area across field tests in the line carrying four resistance QTL alleles was smaller than those in lines with only one or two. That study demonstrated the importance of minor QTL alleles for improving the stability of resistance, even if the effect of each of them is sensitive to the environment.

One of the concerns in the use of QTL pyramids is linkage drag. Most resistance QTL alleles have not been used in commercial varieties by conventional breeding programs, possibly because of linkage drag, as in the case of *pi21*. The use of breeding lines with remaining undesirable traits decreases the efficiency of breeding proportionally to the number of resistance QTL alleles. Therefore, the resistance QTL should be precisely located in the genome and the breeding program should start from linkage drag elimination. The fitness cost of resistance is another issue that should be evaluated in the future.

## 8. CONCLUSION

Progress in understanding the genetic control of race-nonspecific resistance in Japanese upland rice varieties has led to a breakthrough in rice breeding, and marker-assisted pyramiding of relevant genes guarantees germplasm enhancement of the resistance. Varieties carrying *pi21* will provide further evidence of the durability of resistance in large scale of cultivation areas in the future. Pyramiding other resistance QTL alleles into only *pi21* holding varieties is recommended to achieve robust disease control in disease-prone areas.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

## REFERENCES

1. Skamnioti P, Gurr SJ. Against the grain: safeguarding rice from rice blast disease. Trends in Biotechnology. 2009;27:141-150.
2. Sharma TR, Rai AK, Gupta SK, Vijayan J, Devanna BN, Ray S. Rice blast management through host-plant resistance: Retrospect and prospects. Agr Res. 2012;1:37-52.
3. Bonman JM. Durable resistance to rice blast disease - environmental-influences. Euphytica. 1992;63:115-123.
4. Higashi T, Saito S. Linkage groups of field resistance genes of upland rice variety “Sensho” to leaf blast caused by Pyricularia oryzae Cav. (in Japanease with Einglish summary). Japan J Breed. 1985;35:438-448.
5. Maruyama K, Kikuchi F, Yokoo M. Gene analysis of field resistance to rice blast (Pyricularia oryzae) in Rikuto Norin Mochi 4 and its use for breeding. (in Japanease with Einglish summary). Bull Natl Inst Agric Sci, Ser D. 1983;35:1-31.
6. Fukuoka S, Okuno K. QTL analysis and mapping of pi21, a recessive gene for field resistance to rice blast in Japanese upland rice. Theor Appl Genet. 2001;103:185-190.
7. Fukuoka S, Mizobuchi R, Saka N, Suprun I, Matsumoto T, Okuno K et al. A multiple gene complex on rice chromosome 4 is involved in durable resistance to rice blast. Theor Appl Genet. 2012;125:551-559.103:185-190.
8. Kato T, Endo I, Yano M, Sasaki T, Inoue M, Kudo S. Mapping of quantitative trait loci for field resistance to rice blast in upland rice, ‘Sensho’ (in Japanese). Breed Res. 2002;3:119-124.
9. Miyamoto M, Yano M, Hirasawa H. Mapping of quantitative trait loci conferring blast field resistance in the Japanese upland rice variety Kahei. Breed Sci. 2001;54:257-261.
10. Ashkani S, Rafii MY, Shabanimofrad M, Ghasemzadeh A, Ravanfar SA, Latif MA. Molecular progress on the mapping and cloning of functional genes for blast disease in rice (*Oryza sativa* L.): current status and future considerations. Crit Rev Biotechnol. 2016;36:353-367.
11. Cesari S, Thilliez G, Ribot C, Chalvon V, Michel C, Jauneau A, et al. The rice resistance protein pair RGA4/RGA5 recognizes the Magnaporthe oryzae effectors AVR-Pia and AVR1-CO39 by direct binding. Plant Cell. 2013;25:1463-1481.
12. Chen X, Shang J, Chen D, Lei C, Zou Y, Zhai W, et al. A B-lectin receptor kinase gene conferring rice blast resistance. Plant J. 2006;46:794-804.
13. Zhao HJ, Wang XY, Jia YL, Minkenberg B, Wheatley M, Fan JB, et al. The rice blast resistance gene Ptr encodes an atypical protein required for broad-spectrum disease resistance. Nat. Commun. 2018;9.
DOI: 10.1038/s41467-018-04369-4
14. Fukuoka S, Saka N, Koga H, Ono K, Shimizu T, Ebana K, et al. Loss of function of a proline-containing protein confers durable disease resistance in rice. Science. 2009;325:998-1001.
15. Nguyen TTT, Koizumi S, La TN, Zenbayashi KS, Ashizawa T, Yasuda N et al. Pi35(t), a new gene conferring partial resistance to leaf blast in the rice cultivar Hokkai 188. Theor Appl Genet. 2006;113:697-704.
16. Takahashi A, Hayashi N, Miyao A, Hirochika H. Unique features of the rice blast resistance Pish locus revealed by large scale retrotransposon-tagging. Bmc Plant Biology. 2010;10.
DOI: 10.1186/1471-2229-10-175

17. Fukuoka S, Yamamoto SI, Mizobuchi R, Yamanouchi U, Ono K, Kitazawa N, et al. Multiple functional polymorphisms in a single disease resistance gene in rice enhance durable resistance to blast. Sci Rep-UK. 2014;4.
DOI: 10.1038/srep04550
18. Xu X, Chen H, Fujimura T, Kawasaki S. Fine mapping of a strong QTL of field resistance against rice blast, Pikahei-1(t), from upland rice Kahei, utilizing a novel resistance evaluation system in the greenhouse. Theor Appl Genet. 2008;117:997-1008.
19. Xu X, Hayashi N, Wang CT, Fukuoka S, Kawasaki S, Takatsuji H, et al. Rice blast resistance gene Pikahei-1(t), a member of a resistance gene cluster on chromosome 4, encodes a nucleotide-binding site and leucine-rich repeat protein. Mol Breed. 2014;34:691-700.
20. Fujii K, Hayano-Saito Y, Saito K, Sugiura N, Hayashi N, Tsuji T, et al. Identification of a RFLP marker tightly linked to the panicle blast resistance gene, Pb1, in rice. Breed Sci. 2000;50:183-188.
21. Hayashi N, Inoue H, Kato T, Funao T, Shirota M, Shimizu T, et al. Durable panicle blast-resistance gene Pb1 encodes an atypical CC-NBS-LRR protein and was generated by acquiring a promoter through local genome duplication. Plant J. 2010;64:498-510.
22. Saka N, Fukuoka S, Terashima T, Kudo S, Shirota M, Ando I, et al. Breeding of a new rice variety " Chubu 125 " with high field resistance for blast and excellent eating quality. Res Bull Aichi Agric Res Ctr. 2010;42:171-183.
23. Wu YY, Xiao N, Chen Y, Yu I, Pan CH, Li YH et al. Comprehensive evaluation of resistance effects of pyramiding lines with different broad-spectrum resistance genes against Magnaporthe oryzae in rice (*Oryza sativa* L.). Rice. 2019;12:11.
DOI: 10.1186/s12284-019-0264-3
24. Fukuoka S, Saka N, Mizukami Y, Koga H, Yamanouchi U, Yoshioka Y et al. Gene pyramiding enhances durable blast disease resistance in rice. Sci Rep. 2015;5:7773.
DOI: 10.1038/srep07773.

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# Research on Microbiological and Biochemical Analysis of Soymilk Produced and Sold within Calabar Metropolis

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## ABSTRACT

This study was aimed at evaluating the microbiological and biochemical content of soymilk produced and sold locally within Calabar metropolis. Samples of soymilk were obtained from five areas in Calabar metropolis namely: Bogobiri, Watt market, Akim barracks, Army barracks and Marian market. Standard microbiological techniques including the pour plate method and relevant biochemical tests were used to isolate, characterize and identify the found microrganisms. The probable bacterial isolates were identified as: *Pseudomonas, Bacillus* and *Klebsiella species* while the fungi species were that of *Yeast* and *Aspergillus*. There was significant differences in bacterial and fungal count across the locations sampled ($P$<0.05). The mean bacterial counts were highest in samples from Army barracks 6.90±0.01x$10^8$ cfu/ml and lowest in Marian market 4.80±0.04x$10^8$ cfu/ml while the mean fungal count was highest in Army barracks 6.50±0.17x$10^8$ cfu/ml and lowest in Akim barracks 3.50±0.22x$10^8$ cfu/ml. Proximate composition analysis of the foods sampled revealed high carbohydrate content (41.32±0.04% - 53.70±0.04%), lipid content (1.50±0.03% - 5.00±0.04%) and fibre content (1.00±0.08% - 1.60±0.04%) while the range for other proximate composition indices such as ash (1.60±0.01% - 3.10±0.02%), moisture (31.80±0.04% - 36.50±0.15%), and protein content (10.68±0.30% - 19.08±0.30%) were relatively low. Elemental analysis of the soymilk samples showed relatively good amounts of calcium, zinc, iron and sodium which were within the permissible limits set by WHO guidelines for food standard. Lead, Mercury and Cadmium were not detected indicating that the source of water was portable and the soil or cultivation was devoid of harmful/toxic metals. Samples from Marian market however had the highest nutritional and mineral composition.

*Keywords: Microbiological; biochemical; soymilk; composition; characterize.*

## 1. INTRODUCTION

Soymilk is a healthy satisfying beverage obtained from the water extract of soybeans. It has a smooth creamy texture with an off-white emulsion-like suspension containing water soluble vitamins, proteins and carbohydrates [1]. Soymilk is a substrate used for the production of a number of fermented products such as fermented beverages, culture drinks, yogurt-like products, frozen desserts, cheese substitutes, tofu, sauces and other typical Asian products. Different edible microorganisms (bacteria, yeast and fungi) and their enzymes particularly glycosidases, amylases, proteases and lipases are used in the preparation of these fermented (cereal) foods to yield nontoxic products, with flavours, aromas and textures pleasant and attractive to the human consumer [2]. Soy-based foods are rich in high quality proteins, essential amino acids, minerals, vitamins (especially A and B) and vegetable oil etc. However, consumption of soymilk is hindered due to the presence of unpleasant off-flavours carried over from soybean as well as various oligosaccharides mainly raffinose and stachyose that may cause a gastrointestinal discomfort known as flatulence to consumers [3]. Microorganisms found in soymilk contribute to its spoilage. The spoilage rate is increased by storage temperature that favours the growth and proliferation of microorganisms liable to use soymilk as substrate. Generally, results show that the spoilage rate is lower at refrigeration temperature than room storage [4,5].

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The consumption of locally produced cereal foods and drinks are becoming very popular, with acceptability cutting across the various multi-ethnic groups and socioeconomic classes [6]. They have economic potentials especially now that emphasis is on development of local foods. The high cost and scarcity of milk supplies in developing countries has led to the development of alternative milk supplies from vegetable sources such as soymilk [2]. This is a vital substitute when solving malnutrition problems in developing countries like Nigeria [3]. Many women in Nigeria have learnt the skills of producing soymilk commercially as a strategy for poverty alleviation. These beverages are however prone to microbial spoilage if not properly processed, handled and stored. Bacteria such as *Lactobacillus lactis* have been found growing in soymilk [7]. This study was therefore, undertaken to evaluate the microbiological, proximate and mineral content of soymilk sold within Calabar metropolis in order to ascertain its safety level and quality.

## 2. MATERIALS AND METHODS

### 2.1 Collection and Preparation of Samples

Two liquid soymilk samples each were purchased from hawkers in five areas in Calabar metropolis namely: Watt market, Akim barracks, Army barracks, Bogobiri, and Marian market. The samples were properly labeled and transported to the microbiology and chemistry laboratory of the University of Calabar for microbiological, proximate and mineral content analysis respectively.

### 2.2 Microbiological Analysis

A tenfold serial dilution of the soymilk samples were carried out in triplicates using the pour plate method on Nutrient agar and MacConkey agar for total bacterial count while Sabouraud dextrose agar (SDA) was used for total fungal count [8].

#### 2.2.1 Determination of total bacterial count

The pour plate method of Frazier and Westhoff [9] was used. Nutrient agar was inoculated with serial dilutions of the samples and incubated at 37°C for 24-48 hours after which the colonies were counted.

#### 2.2.2 Determination of coliform count

MacConkey agar was inoculated with serial dilutions of the sample by the pour plate method and incubated at 37°C for 24-48 hrs. The different representative colonies from MacConkey agar plates were then picked and subjected to various biochemical test for confirmation such as oxidase test, sugar fermentation test, methyl red – voges prausker test, urease and Simon's's citrate agar test [8].

#### 2.2.3 Determination of fungi counts

Fungi counts were determined on Sabouraud dextrose agar plates (SDA). The SDA media was modified with streptomycin to inhibit bacterial contamination. One ml of the diluted samples were poured into an empty, sterile 9 ml Petri dish followed by pouring SDA into the prepared plates and incubated for 3-5 days at 37°C. Colonies were then counted and recorded after incubation period. SDA slants were prepared for further identification. Fungi were then identified by cultural and morphological characteristics (using a microscope) based on mycelium structure, branch conditions, conidiophore presence, shape etc [8].

#### 2.2.4 Purification, characterization and identification of isolates

Following enumeration, repeated sub culturing was carried out on nutrient agar plates for bacteria growth and SDA plates for fungi growth. Pure isolates were stocked in nutrient agar slants for bacteria and SDA slants for fungi and stored in the refrigerator at 4°C for further studies. Characterization and identification of bacteria isolates were carried out according to Medical microbiology laboratory procedures [10], and *Bergey's Manual of Determinative Bacteriology* [11], fungi isolates were identified according to [10,12,13].

## 2.3 Proximate Composition

Biochemical analysis of the soymilk samples was carried out for pH, ash, moisture, crude fibre, lipid, protein, carbohydrate, total titratable acidity and mineral content according to the method described by AOAC [14].

## 2.4 Statistical Analysis

Data were expressed as means ± standard deviation of triplicate determinations. Analysis was done by one-way analysis of variance (ANOVA) using the randomized complete block design and the least significant difference test to assess the difference between the blocks. A significant difference was considered at the level of 5% probability [15].

## 3. RESULTS

## 3.1 Microbiological Analysis

The bacterial species contained in the soymilk samples were *Pseudomonas, Bacillus* and *Klebsiella species* as presented while the fungal species isolated were species of *Yeast* and *Aspergillus* (Table 1).

The mean bacterial counts were highest in samples from Army market $6.90\pm0.01x10^{8}$ cfu/ml and lowest in Marian market samples $4.80^{d}\pm0.04x10^{8}$ cfu/ml while the mean fungal count was highest in Army market $6.50\pm0.17x10^{8}$ cfu/ml and lowest in Akim barracks $3.50\pm0.22x10^{8}$ cfu/ml (Table 2). There was significant difference in bacterial and fungal load across the various locations sampled at P>0.05 according to LSD test.

**Table 1. Microscopic and biochemical characteristics of bacterial isolates**

| Isolates | Gram's reaction | Morphology | Catalasee | Oxidase | Indole | Ornithine | Motility | Glucose | Sucrose | Lactose | Acid | Gas | Citrate | Urease | Methyl red | Voges Proskauer | Probable org. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $S_1$ | - | Rods | + | - | - | - | - | + | + | + | - | - | - | - | + | + | *Pseudomonas* |
| $S_2$ | - | Rods | + | - | + | - | - | + | + | + | + | - | - | - | + | + | *Pseudomonas* |
| $S_3$ | - | Rods | + | + | - | - | + | + | + | + | + | - | - | - | + | + | *Bacillus spp* |
| $S_4$ | - | Rods | - | - | - | - | - | + | + | + | + | - | + | + | - | + | *Klebsiella* |

*Legend: - represents a negative reaction; + represents a positive reaction*
*Samples $S_1$ – $S_4$ are codes for the isolates obtained from soymilk samples*

## 3.2 Proximate Composition of Cereal Foods and Drinks

The proximate composition of soymilk samples showed that they contained a high carbohydrate and protein content and a low ash and moisture (Table 3). The pH ranged from 5.45±0.03% in Akim barracks to 6.63±0.01% in Marian market. Ash content range was from 1.60±0.03% in Watt market to 3.10±0.02% in Marian market, moisture content ranged from 31.80±0.04% in Watt market to 1.60±0.15% in Marian market, crude fibre content ranged from 1.00±0.08% in Bogobiri and Army barracks to 1.60±0.04% in Watt market, fat content ranged from 1.50±0.03% in Akim barracks to 16.00±0.04% in Army barracks while the carbohydrate content ranged from 41.32±0.04% in Watt market to 53.70±0.04% in Army barracks. The total titratable acidity range was lowest in Marian market (0.35±0.24%) and highest in Watt and markets (0.75±0.05%).

## 3.3 Mineral Content

The result of the mineral composition of soymilk samples (Table 4) showed that: Army barracks samples had the highest level of Fe, Mn and Cu (51.62±0.02 mg/l, 0.62±0.04 mg/l and 0.42 mg/l respectively). Akim barracks samples had the highest level of Na (35.62±0.02 mg/l), Watt market samples had the highest amount of Zn (44.22±0.04a mg/l) while Marian market samples had the highest level of Ca (22.61±0.01 mg/l). Ca and Cu levels were lowest in Bogobiri (8.95±0.01 mg/l and 0.05±0.01 mg/l respectively), Mn and Zn were lowest in Akim barracks samples (0.18±0.03 mg/l and 17.88±0.04 mg/l respectively), while Watt market samples were also least in Fe and Na content (23.54±0.03 mg/l and 20.32±0.03 mg/l respectively).

**Table 2. Mean microbial count of soymilk samples**

| Parameters | Locations | | | | |
|---|---|---|---|---|---|
| | **Bogobiri** | **Watt market** | **Akim barracks** | **Army barracks** | **Marian market** |
| Bacterial count in $10^8$ cfu/ml | $6.44^{a}$±0.02 | $6.62^{ab}$±0.05 | $5.40^{bc}$±0.06 | $6.90^{cd}$±0.01 | $4.80^{d}$±0.04 |
| Fungal count in $10^8$ cfu/ml | $5.00^{a}$±0.32 | $6.30^{ab}$±0.36 | $3.50^{bc}$±0.22 | $6.50^{cd}$±0.17 | $4.80^{d}$±0.18 |

*Values are means of triplicate determinations ± standard deviation. LSD for bacteria = 11.53, LSD for fungi = 9.25. Means followed by similar superscripts within the same row are not significantly (P<0.05) different according to LSD test*

**Table 3. Proximate composition of soymilk drinks (in %) sold in selected locations within Calabar metropolis**

| Sample | Bogobiri | Watt market | Akim barracks | Army barracks | Marian market |
|---|---|---|---|---|---|
| pH | $5.83^{cd}$±0.05 | $6.45^{ab}$±0.05 | $5.45^{de}$±0.03 | $6.27^{bc}$±0.02 | $6.63^{a}$±0.01 |
| Ash content | $2.00^{ab}$±0.01 | $1.60^{bc}$±0.03 | $2.00^{ab}$±0.03 | $2.00^{ab}$±0.05 | $3.10^{a}$±0.02 |
| Moisture Content | $33.30^{a}$±0.15 | $31.80^{cd}$±0.04 | $33.30^{de}$±0.04 | $26.00^{bc}$±0.13 | $36.50^{ab}$±0.12 |
| Crude Fibre | $1.00^{cd}$±0.12 | $1.60^{a}$±0.04 | $1.20^{bc}$±0.07 | $1.00^{cd}$±0.08 | $1.40^{ab}$±0.21 |
| Fat Content | $3.50^{d}$±0.03 | $4.60^{a}$±0.04 | $1.50^{bc}$±0.06 | $5.00^{cd}$±0.05 | $2.30^{ab}$±0.22 |
| Protein Content | $14.00^{bc}$±0.25 | $19.08^{a}$±0.40 | $10.68^{de}$±0.30 | $12.30^{cd}$±0.30 | $14.51^{ab}$±0.30 |
| Total carbohydrate | $46.20^{bc}$±0.21 | $41.32^{e}$±0.04 | $51.32^{cd}$±0.02 | $53.70^{b}$±0.04 | $42.19^{a}$±0.07 |
| Titratable Acidity | $0.65^{bc}$±0.06 | $0.75^{a}$±0.05 | $0.75^{a}$±0.01 | $0.68^{ab}$±0.33 | $0.35^{cd}$±0.24 |

*Values are means of triplicate determinations ± standard deviation. LSD = 5.14. Means followed by similar superscripts within the same row are not significantly (P>0.05) different according to LSD test*

## 4. DISCUSSION

The bacterial count was very high compared to that reported in other investigations [16,17] *Klebsiella* isolated from this study is an important member of the coliform group. It is part of the normal flora of the intestine of human and vertebrates and can therefore cause gastroenteritis, diarrhea and urinary tract infections [18]. Its presence is not surprising since other authors had reported post processing contamination from producers, water, utensils and animal in the environment [19]. *Bacillus* spp. has also been implicated in food poisoning especially in cereals that have been cooked and stored at warm temperature [20]. Reports available indicate that the toxin produced by *Bacillus* spp. can cause *pneumonia* and bronchopneumonia [8]. Besides, *Bacillus cereus* is known to produce heat resistant spores that cannot be eliminated by boiling. *Pseudomonas* and *Klebsiella* species with frequencies of 15%, and 10% respectively have all been implicated in the spoilage of food and beverages [21]. *Aspergillus* spp. has been identified as a common spoilage organism of carbohydrate foods and storage micro-flora of many cereals [22]. Yeast has been implicated in the secondary role of converting starch to ethanol and $CO_2$ thereby releasing esters and phenolic flavours into the food. Therefore, the high microbial load obtained from soymilk sampled in this study may indicate high rate of laxity in hygiene especially during the processing and storage of these foods.

**Table 4. Mineral composition of soymilk drinks in mg/l sold locally in selected locations in Calabar metropolis**

| Locations | Ca | Fe | Mg | Mn | Zn | Pb | Cu | Hg | Cd | Na |
|---|---|---|---|---|---|---|---|---|---|---|
| Bogobiri | $8.95^{e}$±0.01 | $41.2^{a}$±0.01 | $21.1^{cd}$±0.01 | $0.37^{ef}$±0.02 | $30.2^{b}$±0.03 | ND | $0.05^{fg}$±0.01 | ND | ND | $22.61^{bc}$±0.01 |
| Watt Market | $19.66^{cd}$±0.02 | $23.54^{b}$±0.03 | $16.5^{de}$±0.01 | $0.42^{f}$±0.01 | $44.22^{a}$±0.04 | ND | $0.06^{fg}$±0.01 | ND | ND | $20.32^{bc}$±0.03 |
| Akim market | $22.11^{bc}$±0.01 | $27.88^{ab}$±0.02 | $14.23^{de}$±0.04 | $0.18^{f}$±0.03 | $17.88^{cd}$±0.04 | ND | $0.11^{fg}$±0.03 | ND | ND | $35.62^{a}$±0.02 |
| Army Barracks | $14.22^{de}$±0.01 | $51.62^{a}$±0.02 | $21.42^{cd}$±0.01 | $0.62^{f}$±0.04 | $33.12^{b}$±0.03 | ND | $0.41^{fg}$±0.02 | ND | ND | $27.44^{bc}$±0.01 |
| Marian market | $22.61^{d}$±0.01 | $34.73^{ab}$±$0.03^{ab}$ | $17.34^{de}$±0.01 | $0.56^{f}$±0.02 | $41.00^{a}$±0.01 | ND | $0.24^{fg}$±0.02 | ND | ND | $32.43^{bc}$±0.03 |

*Values are means of triplicate determinations ± standard deviation. LSD = 9.25. Means followed by similar superscripts within the same column are not significantly (P>0.05) different according to LSD test*

The pH range of the soymilk samples were not significantly different ($p>0.05$) and were comparable to pH values of (6.2%) reported by [4]. Low pH values are beneficial as they discourage the growth of pathogens which may cause gastrointestinal disorders. The moisture content range in this study was within the range reported by [16] of 79.40% - 91.00% and [17] 68.50% - 91.49%. Lower moisture values may be due to loss of water by evaporation. This implies that the hawked soymilk will have a longer shelf life as high moisture favours microbial growth and hence spoilage [23]. The fiber content of samples from Army barracks, Bogobiri and Watt market (Table 3) were considerably higher than 0.29% - 0.90% recorded by [16], 0.01% by [5] and 0.10% - 0.35% by [17]. Fiber helps in the maintenance of human health and has been known to reduce cholesterol levels in the body. Emphasis has been placed on the importance of keeping fibre intakes low in the nutrition of infants and pre-school children [24] since it leads to reduced digestibility, reduced vitamin and mineral availability, lowers calorie density [25], and has local effects on the intestinal mucosa of infants. The ash content in this study was higher than that reported by [17] of 0.80% - 1.00%. This may be due to the differences in particle size of the flour before sieving. The smaller the particle size of the flour, the more endosperms that will be exposed and as such more minerals will be extracted from soymilk. It further confirms its high mineral content as shown in Table 4. This agrees with the findings that plants that contain a large quantity of ash also provides an appreciable quantity of minerals required by the body [26]. The fat content of Watt market and Marian market (Table 3) were close to that obtained in high level (17.00%) and Wadata (18.00%) of Markurdi metropolis [17] and 19.89% reported by [27]. The protein content range of 10.68±0.30% - 19.08±0.40% in this study was quite higher than the values 6.78% - 7.76% obtained by [17] and 3.40% - 3.50% obtained by [16]. This may be attributed to the varying degree of heat treatments which may have resulted in the destruction or inactivation of some amino acids for example cysteine. Differences in nutrient content observed in this study as compared to reports by other researchers may be due to differences in cereal varieties used in production, differences in variety of bean seeds used and the mode or technique used in processing and preparing them.

Soymilk sampled for this study contained a high amount of calcium, iron, magnesium, sodium, and zinc, manganese and copper were present in small amounts while mercury, cadmium and lead were not detected. Recommended permissible limit of Zinc, copper, lead, and Cadmium are 99.0 mg/kg, 40.0 mg/kg, 0.30 mg/kg and 0.20 mg/kg respectively as set by [28]. The concentrations of zinc, copper, lead and cadmium found in soymilk samples were within these permissible limits set by FAO/WHO guidelines for food standard [28]. These toxic metals are carcinogenic affecting the kidney and liver and may be due to the water used for its preparation or from the soil used to plant the grains especially if phosphate fertilizer is used. Therefore, their absence shows that the drinks are not toxic but are safe for drinking. The various elements present in soymilk when analyzed statistically showed a high significant difference ($p<0.05$).

These mineral elements which are essential micro-nutrients required for the growth of bones and teeth, activation of enzymes, formation of red blood cells, melanin pigment formation, growth of bones and teeth, maintaining osmotic pressure, water balance and intracellular fluids in the body. Toxic metals like lead, mercury and cadmium were not identified in this work. Their carcinogenic effects often affect the kidney and liver. This may imply that these drinks were free from toxic metals and therefore not toxic to the body.

## 5. CONCLUSION

The result of this study carried out in Calabar metropolis revealed that soymilk samples sold in Army barracks were found to be highly contaminated with microorganisms some of which were of fecal origin. Samples from Akim Barracks were preferably lower in microbial content and as such less likely to undergo spoilage quickly. Samples from Marian market were observed to have the highest nutritional value and mineral composition. Most locally hawked soymilk in Calabar metropolis have been shown to contain little amounts of food spoilage organisms, coliforms, and fecal contaminants. Proximate composition of some these foods/ drinks revealed high carbohydrate, lipid and fiber content while other proximate composition indices such as ash content, moisture, and protein were relatively low. The Soymilk samples had good amounts of calcium, zinc, iron and sodium while lead, mercury

and cadmium were not detected indicating that the source of water was portable and the soil or cultivation was devoid of harmful/toxic metals.

It is therefore recommended that these foods should be properly processed with clean treated municipal water in order to avoid contamination with enteropathogenic bacteria. Also, health education training should be organized for the local people on the importance of cleanliness of their environment and the use of sterilized packaging materials for processing and packaging of these products, as well as the risk accrued to public health when these recommendations are not met by local producers.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

## REFERENCES

1. Iwe MO. The science and technology of soybeans. Rojoint Communication Services Ltd. Enugu, Nigeria. 2003;20-25.
2. Yuan S, Chang SK, Liu Z, Xu, B. Elimination of tripsin inhibitor activity and beany flavor in soymilk by conservative blanching and Ultra-High Temperature (UHT) processing. J. Agric. Food Chem. 2008;56(17):7957-7963.
3. Aderiye MO. Microbiology of soymilk production. M. Sc Thesis, University of Lagos. 1990;32.
4. Osuntogun B, Aboaba OO. Microbiological and physio-chemical evaluation of some non-alcoholic beverages. Park. J. Nutr. 2004;3(3):188-189.
5. Onourah CE, Adejare OA, Uhiare NS. Comparative physiochemical evaluation of soymilk and soya cake produced by three different methods. Nig. Food J. 2007;25: 28-38.
6. Barnett H, Hunter B. Illustrated genera of imperfect fungi. 3rd Ed. Burgess publishing company, Minneapolis, USA. 1972;73-75.
7. John OO, Arandhati KA. Medical Laboratory Science, Theory and Practical. Tata McGraw Hill Publishing Company Ltd., India. 1995;32-34.
8. A.O.A.C. Official methods of analysis 15th Ed. Association of official Analytical Chemist, Washington D.C. 1990;808,831-835,1113.
9. Ihekoronye AI, Ngoddy PO. Integrated food science and technology in the tropics. 2nd Ed. 1985;189.
10. Ikya JK, Gerah MO, Igyor MA. Analysis of chemical, microbiological and sensory qualities of soymilk sold in Gboko metropolis from Benue State. J. Agric. Vert. Sci. 2013;2:173–181.
11. Liamngee K, Terna TP, Bem AA, Orpin JB, Mzungu I, Obaje M, Anum T. Microbial analysis of soybean milk sold in Makurdi metropolis. J. Environ. Sci., Toxicol. Food Technol. 2013;3(3):97–104.
12. Pelcazar MI, Charre CS, Noel RK. Microbiology 5th edition, McGraw Hill Publishing Company, New Delhi. 1993;272.
13. Oranusi SU, Umoh VJ, Kwaga JKP. Hazard and critical control points of kunu-zaki, a non-alcoholic beverage in Northern Nigeria. Food Microbiol. 2003;20(1):127-132.
14. Wonang DL, Amienyo CA, Ekeleme OP, Dazol DG. Bacteriological assessment of “kunu” a local beverage sold in Jos, Plateau State. J. Environ. Sci. 2001;4(1): 5-7.
15. Wikipedia. Soyamilk; 2008.
Available:http://en.wikipedia.org/wiki
(Accessed 17th of May 2016)
16. Onweluzo JC, Nwakalor C. Development and evaluation of vegetable milk from *Treculia Africana* (decne) seeds. Pakistan Journal of Nutrition. 2009;8(3):233-238.
17. Cheesbrough M. District Laboratory Practice in Tropical Countries. Part 2. Cambridge University Press, Cambridge, United Kingdom. 2000;23-30
18. Frazier WC, Westhoff DC. Food microbiology 4th ed, McGraw Hill, New Delhi and New York. 2010;196-218.
19. Treagan L, Pulliam L. Medical microbiology laboratory procedures. In: Saunders, W. B. (Eds) Characterisation of bacteria, 9th Ed, Longman Press, Philadelphia. 1982;318.
20. Bergey DH, Holt JG. Bergey’s Manual of Determinative Bacteriology. 9th Ed. 1994;233-234.

21. Nester EW, Robert CE, Lidisfrom ME, Pesrau NN. Microbiology 3rd Ed, Saunders College Publishing, Japan. 2013;115-116.
22. Rhodes A, Fletcher DL. Principles of Industrial Microbiology, Pergaman Press, Oxford. 1966;119.
23. Battey AS, Duffy S, Schaffner DW. Modelling yeast spoilage in cold filled ready to drink beverages with *Saccharomyces cerevisiae*, *Zygosaccharomyces bacilli* and *Candida lipolytica.* Appl. Environ. Microbio. 2002; 68(4):1901-1906.
24. Eromesele IC, Eromesele CO. Studies on the chemical composition and physio-chemical properties of seeds of some used plants. Plant Food Human Nutr. 1993;43: 251-258.
25. Jansen GR. A consideration of allowable fibre levels in weaning foods. Food Nutr. Bull. 1980;2(4):38-46.
26. Rao-pu. Nutrient composition and biological evaluation of Mesta *(Hibiscus sadariffa*) seeds. Plant Food for Human Nutri. 1996;49:34-37.
27. Habeeb OH. Genotype and environmental interaction for yield in field soybeans. Philipi. J. Crop Sci. 1998;8:3-9.
28. Food Agricultural Organisation. Fermented cereals; A global perspective. (FAO Agricultural services Bulletin No. 138); 1999.
Available:http://www.fao.org/docrep/x2184E00.htm
(Accessed 15 March 2015)

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***Chapter 6***
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# Genetic Inheritance of Black Seed Coat in Soybean

**Valeria Carpentieri-Pipolo[1*]**

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## ABSTRACT

The standard phenotype of R gene is black hilum on black seed. The genetic control of several soybean (*Glycine max* L. Merr.) natural variants has not been studied. The genetic type T16 is the only occurrence with brown hilum on black seed coat. The aim of this chapter is to further a discussion the genetic control of seed black seed coat in soybean. The commercial cultivars Bragg, BR13 and BR6 present yellow seeds, their natural variants Bragg P and BR13P present black seed color and BR6M, LCV91-26 brown seed color. T16 was combined with these genotypes and it was found that the genetic control of the brown hilum trait in black seed coat of the T16 genotype was controlled by two loci segregating independently and controlling the expression of the color of the hilum and the seed coat color. The expression of the brown hilum trait in black seed coat is dependent on locus *T_*, which controls pubescence color; therefore it occurs only in genotypes with tawny brown pubescence (*T_*). Novel black soybean cultivars, breeding for specific characteristics, selected for direct use could, more suitable for human consumption will increase opportunities for exploring the global market of health and functional food industry and contribute as food source of both calorie and protein to combating hunger in undeveloped countries.

*Keywords: Seed coat color; Glycine max; black soybean.*

## 1. INTRODUCTION

Black soybeans containing black seed coats have been a widely used crop for many years due to their dietary health benefits and folk medicine. Consumption of soy foods has been recognized to lower the risk of aging-associated diseases, including cardiovascular disease and cancer, among others [1]. Pigmentation of seed coats is induced via the deposition of a number of flavonoids which derives from an anthocyanin biosynthesis and are secondary metabolites derived from flavonoid pathway such as proanthocyanidins and anthocyanins [1]. Because black soybeans are the only color reported to contain anthocyanins and only brown and black soybeans contain proanthocyanins, this may result in a differing level or type of bioactivity among soybeans with different seed coat colors, moreover these compounds also play a relevant role in plant pathogen defense and protection from UV light exposure [2]. The health-promoting traits of soybean seed are attracting great interest of the market, therefore breeders are working on development of novel soybean lines with a selected seed coat color to be used as bioactive ingredients in functional foods, targeting different healthproblems.

The visual appearance of the soybean seed has been altered as result of domestication during the transition of the wild *Glycine soja*to the current cultivated *Glycine max* [3]. The majority of commercial high yielding soybean cultivars that are grown and consumed throughout the world today exhibit yellow seed coats, whereas the majority of known accessions of the wild progenitor, *Glycine soja*possess black seed coats or, brown seed coats with a range of hilum colors (brown, black, imperfect black, buff, yellow). The genes controlling natural variation of seed coats pigmentation trait in soybean (*Glycine max* (L.) Merr.) are not completely understood. It remains poorly understood mainly because seed coat color of soybean was so far limited as markers to assess hybridity in breeding programs. The color of the seedcoat is important assayed visible marker to detect the

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presence of “off-types” in soybean seed lots of pure strains, is a mandatory descriptor for solicitation of plant variety protection and it has been used to identify mixtures of plantvarieties.

Several natural mutations associated seed coat color have been selected in soybean cultivars. These mutations may represent an important source of genes for the development of soybean food type cultivars in breeding programs.

The study of genetic control of black seed coat trait in T16, and the isolines LC 91-26, BraggP, BR13P and BR6 with brown seeds was investigated by Carpentieri-Pipolo et al. [4] the mainly results are discussed in the presentmanuscript.

## 2. GENETIC CONTROL OF THE PIGMENTS THATDETERMINE THE SEED COAT COLORS INSOYBEAN

Three independent loci (I, R, and T) mainly control the biosynthesis of the pigments that determine the seed coat colors. The *R* gene produces black hilum and black seed coat. In additional it is found that the locus *R* is also involved in the variegation phenotype affecting seed coat color. The *r-m* allele of this gene produces striped seed coat superimposed on an brown seed color, and the locus *R* and two additional independent loci *I* anthocyanin inhibitor and *T* tawny, control pigmentation and anthocyanin accumulation [5].

The *I* allele inhibits the production and accumulation of pigments over the entire seed coat, resulting in uniformly yellow-colored seeds, whereas the *i*allele leads to completely pigmented seeds by allowing the production and accumulation of pigments over the entire seed coat. The *I* locus controls the presence or absence and spatial distribution of flavonoid pigments and has four alleles (*I*, $i^j$, $i^k$, *i*). In the presence of *I* the total inhibition of pigmentation occurs; the double recessive allele $i^j$ allows full pigmentation of the seed; with $i^j$allele, pigmentation is restricted to the hilum; with the allele $i^k$ pigmentation in hilum and in part of the seed coat occurs. Plants carrying the *I* or $i^j$allele show complete inhibition of pigmentation in the seed coat or pigmentation only in the hilum, respectively, resulting in a yellow seed phenotype. However, there are four main seed coat colors (black, brown, yellow and green), and the inheritance of black and brown pigments is genetically independent from the green and yellow ones [6-9]. The remaining two alleles, $i^j$ and $i^k$, inhibit pigmentation except in the hilum and a saddle-shaped region, respectively. Yellow soybean cultivars carry the *I* allele for a light (no pigmented) hilum or the $i^j$allele for a dark (pigmented) hilum [6-9] as observed T16 and Bragg, that carry *i*and $i^j$, respectively. The dominance relationships among the four alleles are $I > i^j > i^k > i$. Inhibition of seed coat pigmentation by the *I* locus, at least the *I* and $i^j$alleles, is the result of RNA silencing of chalcone synthase genes, a key enzyme in a flavonoids biosynthesis.

The dominant allele *T* conferred brown (or tawny) and *t* conferred gray pubescence color. Further, *T* generally darkens hilum and/or seed coat color in combination with genotypes at the *I*, $W_1$, *R* and *O* loci [9,10]. The allelic series *T/t-r/t* produces the seed coat or hilum colors of black, red-buff and buff, respectively [9]. The dominant allele *T* was completely shown to suppress the development of abnormal pigmentation around the hilum region and to partially suppress the cracking of seed coat associated with chilling treatments. One recessive gene conditioning the phenotype spread hilum in the T48 genotype was found. The expression of the spread hilum is dependent of locus *T_*, which controls pubescence color; therefore it occurs only in genotypes with brown pubescence (*T_*), which characterizes pleiotropic effect of this locus on the trait spread hilum [3,10].

The inheritance black hilum/black seed coat was described by Kovinich et al. [6], Carpentieri-Pipolo et al. [4] Yang et al. [8] and Senda et al. [7]. More recently Gillman et al. [3] identified the specific gene and causative basis behind the phenomenon of brown hilum/seed coat coloration in soybean (*R* locus), and discovered an allelic series of four loss of function mutations affecting our *R* locus gene candidate ($R_2R_3MYB$ gene). The presence of each one of these mutations was perfectly correlated with the brown seed coat/hilum phenotype in a broadly distributed survey of soybean cultivars. These findings strongly suggest that loss of function for one particular seed coat-expressed $R_2R_3MYB$ gene is responsible for the brown seed coat/hilum phenotype in soybean.

The allelic sequence *R*, *r-m*, and *r* controls the pigment color and the presence of stripes in the seed coat. The *r-m* allele is dominant over *r* and recessive to *R*. The *r-m* allele exhibits instability in somatic and germinal cell layers and the revertant phenotypic forms are not stable with expression forms of the allele at high frequency [11]. The expression of this not typical phenotypic germinal instability was suggested as transposable elements residing in or near the *r-m* allele. Since the revertants of *r-m* alleles may be not be identical to the standard *R* and *r* forms they were designated by Chandlee and Vodkin [12] as designate *R** and *r** . There is one phenotypic difference between the standart*R* allele, and the *R** carryng brown hilum in black seed and *r* carryng r-m* (striped seed coat); thus the locus *R* is not stable and could produce all three forms *R**, *r-m* and *r* [11].

## 3. INHERITANCE OF BLACK SEEDS GENETIC TYPE T16, AND IN THE NATURAL VARIANTS BRAGGP, BR 13P, BR 6M, LC91-26

The inheritance of black seeds and brown hilum in genetic type T16, and in the natural variants BraggP, BR 13P, BR 6M, LC 91-26 were reported by Carpentieri-Pipolo et al. [4]. The main results of this study will be followeddiscussed.

The standard phenotype *R* gene is black hilum on black seed [3]. The genetic type T16 is the only occurrence with brown hilum on black seed coat in the *Glycine max* accession of USDA soybean germplasm.

The genetic type T236 is an *Glycine max* accession of USDA Soybean Germplasm Collections with white flower, gray pubescence and a usual red-buff (Rbf) seed coat. LC 91-26 is a T236 isoline, it is homozygous (*r-m*/*r-m*) (brown striped). The standard commercial soybean cultivars Bragg and BR13 present yellow seed coat with a black hilum and the standard BR6 present brown seed coat with a brown hilum. The isolines, natural variants of those cultivars are BraggP and BR13P with black hilum and black seed coat and BR6M with Brown hilum and brown seed coat respectively, the seed coat phenotypes of these soybean access are shown in the Fig. 1.

Table 1 summarize of genotypic of parental used accessing $F_1$ and $F_2$ generations resulting from crosses between soybeans genotypes T16 and Bragg, T16 and BraggP, T16 and BR 13P, T16 and BR 6M, T16 and LC 91-26, T16 × T236.

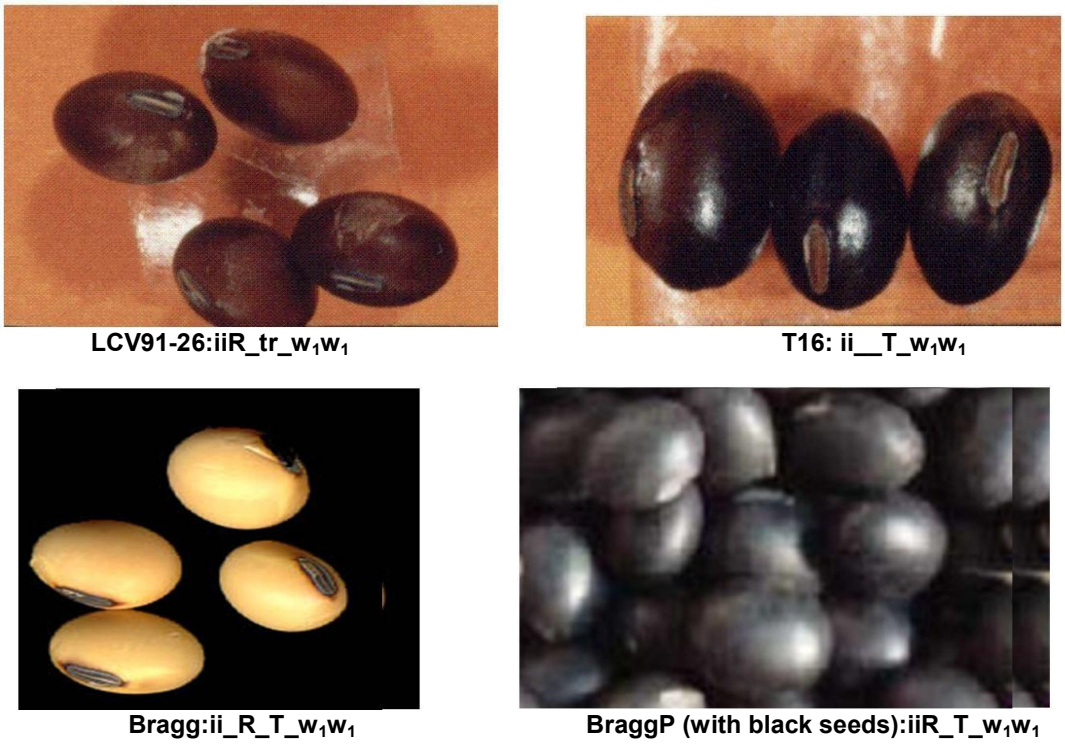
No difference between the original and reciprocal crosses was found, suggesting that there was no effect of maternal control on the characteristic hilum color. Hilum tissue is not maternally-derived, in contrast to the seed coat. Phenotypes of $F_1$seeds showed that the black hilum was dominant over the brown hilum, a previously expected result as described by Chandlee and Vodkin [11] in their study of the relation between black and brown pigment in soybean hilum and seed coat. Regarding the seed coat, the yellow color was dominant over the black one in the crossing between T16 and Bragg, and over tanned-red in the crossing between T16 and T236, as described by Seo et al. [12].

$F_2$ segregation from T16 and Bragg crossing indicated that the brown hilum pigmentation in black seed coat is controlled by a single allele *R* (Table 2). These results shown that the phenotype brown hilum pigmentation in black seed coat in the genetic type T146 (*R*) is controlled by a singleallele.

In the crosses between T16 and BraggP and T16 and BR 13P, the $F_2$ segregation presented correspondence to the 3:1 proportion (black hilum: brown hilum). This result was confirmed by the low value observed in the chi-square shows that the inheritance of seed color the hilum color were determined by a single gene in these crosses, and the black hilum dominates over brown hilum.

The dominance relation (3:1) was observed in the crosses between T16 and BR 6M and T16 and LCV 91-26 with dominance of brown striped seed coat color over brown seed coat. However, there was only segregation of seed coat color, and all the seeds showed brown hilum, confirming that all T16 and BR6 Brown genotypes and LCV91-26 (brown striped) have the same genetic constitution for hilum color (Table 2). Chandlee and Vodkin [11] have examined the inheritance of *r- m* allele through several generation and found that it exhibits unusual allele instability which changes in gene expression that can convert to the other expression form of the alleles at high frequencies. This behavior has not been seen in other unstable alleles in soybean and suggests that an insertion sequence or transposable elements reside in or near the *r-m*allele.

The $F_2$ population from T16 and Bragg demonstrated segregation in four different phenotype classes (yellow seed coat with black hilum, yellow seed coat with brown hilum, black seed coat with black hilum and black seed coat with brown hilum). $F_2$ showed concordance with the expected one of9:3:3:1 for two loci segregating independently and controlling the expression of the color of the hilum and the seed coat color (Table 2).



**LCV91-26:iiR_tr_$w_1w_1$** **T16: ii__T_$w_1w_1$**

**Bragg:ii_R_T_$w_1w_1$** **BraggP (with black seeds):iiR_T_$w_1w_1$**

**Fig. 1. Phenotype and genotypes of genetic types LCV 91-26 (red-buff seed coat, white flower and gray pubescence) LCV 91026 is natural variant of T236, T16 (black seed coat, brown hilum, white flower and tawny tawny brown pubescence), commercial cultivar Bragg (yellow seed coat, black hilum, white flower and tawny brown pubescence) and the natural variants BraggP (black seed coat, black hilum white flower and tawny brown pubescence), Bragg P is natural variant of Bragg**

Three SSR markers (Satt212, Satt417and Sat_090) linked to the *Lx* genes in the $F_{2:3}$ Segregation analysis of population from the cross between the soybean cultivars BR 36 X BRS 213, and BRS BRS155 X 213.

**Table 1. Description of phenotypic effects of four different genetic loci affecting seed coat and hilum colors in soybean**

| Genotype | Phenotypes | | | | Genotype |
|---|---|---|---|---|---|
| | **Seed coat** | **Pubescence** | **Flower** | **Hilum** | |
| T 16 | Black | Tawny | white | Brown | *iirrTTw*$_1$*w*$_1$ |
| Bragg | Yellow | Tawny | white | Black | *I*$^i$*I*$^i$*RRTTw*$_1$*w*$_1$ |
| Bragg P | Black | Tawny | white | Black | *iiRRTT w*$_1$*w*$_1$ |
| BR13P | Black | Tawny | white | Black | *iiRRTT w*$_1$*w*$_1$ |
| BR6M | Brown | Tawny | white | Brown | *iirrTTw*1*w*1 *w*$_1$*w*$_1$ |
| LC 91-26 | Black + striped | Tawny | white | Black | *ii r-m r-m TT w*$_1$*w*$_1$ |
| T 236 | Red-buff | gray | white | Brown | *iirrttoo w*$_1$*w*$_1$ |

*Source: Carpentieri-Pipolo et al. [4]*

**Table 2. Segregation analysis of $F_2$ population from the cross between the genetic typeT1 and the natural variants Bragg P, BR13 P, BR6 B, T 236 and LCV 91-26**

| | Number of observed $F_2$ individuals | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Seed coat | | | | | | | | | | |
| **Cross** | **Yellow** | **Black** | **Brown** | **Brown + striped** | | **Red buff** | **Buff** | | **Theoretical frequencies** | ***χ*2** | **P** |
| | Hilum | | | | | | | | | | |
| | Black | Brown | Black | Brown | Brown | Brown | Red buff | Buff | | | |
| T16 × Bragg P | | | 237 | 80 | | | | | 03:01 | 0.009 | 0.922 |
| T16 × BR13 P | | | 494 | 160 | | | | | 03:01 | 0.099 | 0.751 |
| T16 × BR6 M | | | | | 98 | 291 | | | 03:01 | 0.007 | 0.930 |
| T16 × LCV 91-26 | | | | | 87 | 276 | | | 03:01 | 0.206 | 0.649 |
| T16 × Bragg | 244 | 84 | 72 | 24 | | | | | 9:3:3:1 | 1.324 | 0.723 |
| T16 × T236 | | | 146 | 45 | | | 45 | 15 | 9:3:3:1 | 0.160 | 0.983 |

*$X^2$ Chi-square value ; P: probability.*
*Source: Carpentieri-Pipolo et al. [4]*

In the cross between T16 and T236, segregation of the $F_2$ population in four phenotype classes also occurred (black seed coat with black hilum, black seed coat with brown hilum, red-buff seed coat with buff hilum and red-buff seed coat with red-buff hilum) which agreed with the results reported by Seo et al. [12] from the crossing between T236 and Clark isoline L68-2077 (*r-m*). The obtained results indicating two loci segregating independently. The $F_2$ population have shown two phenotypes for pubescence color: tawny brown and gray pubescence. Plants with gray pubescence (*tt*) did not produce seeds with tawny brown hilum on black seed coat (*T_*), it only was observed in the plants with tawny brown pubescence, therefore the expression of the brown hilum trait on black seed coat is dependent on locus *T_*, which controls pubescence color; therefore it occurs only in genotypes with tawny brown pubescence (*T_*), these phenomenon characterize epistatic effect of the T locus on the hilum brown trait on seeds with black coat. The pubescence color allele, T, is involved in the dihydroxylation of the B ring in the formation of cyandin-3-glucoside that id necessary for function in flavonoids and anthocyanins biosynthesis.

## 4. CONCLUDING REMARKS AND FUTUREPERSPECTIVES

Our study concerning the analyses of the black seed coat/hilum various genotypes of black soybean. The genetic control of the brown hilum trait in black seed coat in the T16 genotype is controlled by a single recessive. There is no cytoplasmic effect in the expression of this trait and although belonging to the same allelic sequence, the genetic control of the brown hilum in black seed coat is different and independent from the one that determines black hilum in black seeds coats. Moreover the expression of the brown hilum trait on black seed coat is dependent on locus *T_*, which controls pubescence color, its occur only in genotypes with tawny brown pubescence (*T_*). The information of this chapter could have utility in breeding programs for developing novel black soybean varieties to directly use in daily food. The information also could help for development of molecular markers of traits which are linked with seed color to be used in marker-assisted breeding.

Novel black soybean cultivars, breeding for specific characteristics more suitable for human consumption with good acceptability will increase opportunities for exploring value-added processing products, to foster the global market of health and food industry. Moreover considering that soybean is the world's faster expanding crop with potential yield in temperate, subtropical and tropical zones, soybean varieties with black coat, selected for direct use could contribute as food source of both calorie and protein intake to combating hunger in undeveloped countries.

## COMPETING INTERESTS

Author has declared that no competing interests exist.

## REFERENCES

1. Jeng TL, Shih YJ, Wu MT, Sung JM. Comparisons of Ivonoids and anti- oxidative activities in seed coat, embryonic axis and cotyledon of black soybeans. Food Chemistry. 2010;123:1112-1116.
2. Zabala G, Vodkin L. Cloning of Pleiotropic T Locus in Soybean and Two Recessive Alelles That Dif-ferentially Affect Structure and Expression of Encoded Flavonoid 3' Hydroxylase, Genetics. 2003;163:295-309.
3. Gillman JD, Tetlow A, Lee JD, Shannon JG, Bilyeu K. Loss-of-function mutations affecting a specific glycine max R2R3 MYB Transcription Factor Result in Brown Hilum and Brown Seed Coats. BMC Plant Biology. 2011;11:155-167.
   Available:http://dx.doi.org/10.1186/1471- 2229-11-155
4. Carpentieri-Pípolo V, Almeida LA, Kiihl RAS. Inheritance of R locus expressing brown hilum on black seed coat in soybean. American Journal of Plant Sciences. 2015;6:1857- 1861.
   Available:http://dx.doi.org/10.4236/ajps.2015.611186
5. Bernard RL, Weiss MG. Qualitative Genetics, 117-149 in Soybean: Improvement, Production and Uses, In: Caldwell, B.E., Ed., 3rd Edition, American Society of Agronomy, Madison; ; 2004.
6. Kovinich N, Saleem A, Arnason JT, Miki B. Combined analysis of transcriptome and metabolite data reveals extensive differences between black and brown nearly-isogenic soybean (*Glycine max*) Seed Coats Enabling the Identification of Pigment Isogenes. BMC Genomics. 2011;12: 381-399.
   Available:http://dx.doi.org/10.1186/1471-2164-12-381
7. Senda M, Kurauchi T, Kasai A, Ohnishi S. Suppressive Mechanism of Seed Coat Pigmentation in Yellow Soybean. Breeding Science. 2012;61:523-530.
   Available:http://dx.doi.org/10.1270/jsbbs.61.523
8. Yang K, Jeong N, Moon JK, Lee YH, Lee SH, Kim HM, Hwang CH, Back K, Palmer RG, Jeong SC. Genetic Analysis of Genes Controlling Natural Variation of Seed Coat and Flower Colors in Soybean. Journal of Heredity. 2010;101:757-768.
   Available:http://dx.doi.org/10.1093/jhered/esq078
9. Guo Y, Qiu LJ. Allele-specific marker development and selection efficiencies for both flavonoid 3'-Hydroxylase and Flavonoid 3'5'-Hydroxylase Genes in Soybean Subgenus Soja. Theoretical and Applied Genetics. 2013;126:1445-1455.
   Available:http://dx.doi.org/10.1007/s00122-013- 2063-3
10. Carpentieri-Pípolo V, Souza A, Almeida LA, Kiihl RAS, Pípolo AE. Herança de hilo esparramado em sementes de soja. Pesquisa Agropecuária Brasileira. 2007;42:127-129.
    Available:http://dx.doi.org/10.1590/S0100-204X2007000100017
11. Chandlee JM, Vodkin LO. Unstable Expression of a Soybean Gene during Seed Coat Development. Theoretical and Applied Genetics. 1989;77:587-594.
    Available:http://dx.doi.org/10.1007/BF00274285
12. Seo YW, Specht JE, Graef GF, Graybosch RA. Inheritance of Red-Buff Seed Coat in Soybean. Crop Science. 1993;33:754-758.
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# Storage of Yeasts

**Nirmala Ravimannan[1*]**

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## ABSTRACT

Several methods have been used in the storage of yeasts. Traditional methods and low technology methods were done to compare the viability of yeasts during storage. The shelf life is limited during storage of yeasts on petri dishes or slants in the refrigerator. Sub culturing should be done after 3 months. The yeast cultures did not lose viability for six months when stored under distilled water. It is a very cheap method. Yeast cultures stored under mineral oil remained viable for more than a year.

*Keywords: Yeast; storage; viability.*

## 1. INTRODUCTION

There are several methods used in the storage of yeasts. It is important to preserve and maintain yeasts because they are used as starter cultures in the manufacture of bakery products and alcoholic beverages. So it is preferable to maintain a pure source of starter culture which is a valuable brewing ingredient. The basic principle of microbial preservation of starter cultures is to avoid any change in the physiological and genetic characteristics [1]. Yeasts also play a major role in the preparation of traditional fermented products in which starter culture is not added. Maintenance of stock cultures of yeast is essential in laboratories to use in various studies involving identification of strains and protein expression etc. Among several methods devised for maintaining yeast cultures, periodic subculture on agar slants or plates is the most common one. It is important to note that the method used should be such that the viability and the biochemical properties of the yeast culture are not lost. Previous studies done by [2], [3] and [4] showed the feasibility of storing fungi and some yeasts [5] in distilled water for a few months. This is the least expensive method. Covering fungal cultures on agar slants under mineral oil was studied by [5]. Though it is an old method it was found that the cultures were viable for many years. Liquid nitrogen storage is considered to be the optimum procedure for long term preservation of yeast samples in a viable state [7]. This method is very expensive. Therefore there is a need to find a long term storage method which is cheap.

## 2. ISOLATION AND IDENTIFICATION OF YEASTS FROM NATURAL SOURCES

### 2.1 Preparation of Isolates from Grains/ Fruits

Grapes are soaked in water for a few days. The isolate is prepared by taking the inoculums from the outer layer of the skin of grapes and the soaked water. These are plated on peptone yeast extract agar after preparing appropriate dilutions. Identification is usually based on morphological and biochemical properties using Lodder's manual [8]. Pure cultures are prepared by sub culturing on the same media.

### 2.2 Preparation of Isolates from Potato

Potato is boiled and sucrose is added to the mashed potato. This is allowed for a few days. Isolates are prepared by taking the inoculum from the surface of the mashed potato as well as from the water.

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These are plated on peptone yeast extract agar after preparing appropriate dilutions. Identification is based on morphological and biochemical properties using Lodder's manual [8]. Pure cultures are prepared by sub culturing on the same media.

## 3. DIFFERENT METHODS OF STORAGE

### 3.1 Periodic Subculture on Plates

Peptone yeast extract agar is prepared under sterilized conditions. The medium is poured (15 ml each) into sterile petri dishes. After setting, the inoculum of yeast is streaked on the surface of the medium. The plates are kept in an inverted position at 27°C for a few days and observed for the growth of yeast. These plates are kept in a sealed plastic bag and stored in a refrigerator. The cultures are checked monthly for three months for purity, viability and morphological and biochemical properties. Viability is tested by using pour plate method. Morphological and biochemical properties are tested by following the Lodder's manual [8]. Sub culturing is done at 3 months interval.

Shelf life is limited when storing yeasts on petri dishes in the refrigerator. It has to be sub cultured after 3 months.

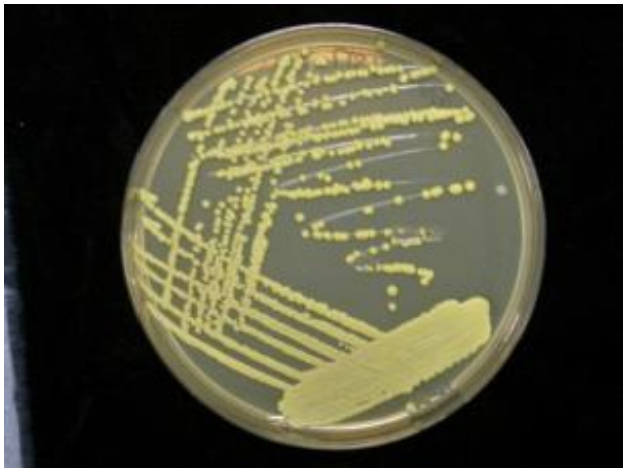


**Fig. 1. Yeast culture streaked on plate**



**Fig. 2. *Saccharomyces cerevisiae* on Peptone yeast extract medium**

### 3.2 Periodic Subculture on Agar Slopes

Peptone yeast extract agar (23 g/l) is dispensed in Mac Cartney bottles and after the usual sterilization procedure, is allowed to set at an inclined angle to form a slope. Duplicates are prepared for each yeast strain. The yeast cultures are inoculated following the standard procedures for yeasts [9] and stored at 4°C. The cultures are checked monthly for three months for purity, viability and morphological and biochemical properties. Viability is tested by using pour plate method. Morphological and biochemical properties are tested by following the Lodder's manual [8]. Sub culturing is usually done at 3 months interval.

### 3.3 Storage in Distilled Water

Mac Cartney bottles are sterilized after filling with distilled water. The discs are cut by using a cork borer from peptone yeast extract agar plates. A loopful of inocula are added onto the surface of the media discs and stored at 4°C after labeling. This can also be done by taking a small amount of yeast colony from a plate and transferring into the Mac Cartney bottle such that the suspension inside the tube become only slightly blurred and not milky. The cultures are checked monthly for three months for purity, viability and morphological and biochemical properties. Viability is tested by using pour plate method. Morphological and biochemical properties are tested by following the Lodder's manual [8]. The transferring of the discs is done at few months interval depending on the observations made on viability, purity etc.

From previous studies, the viability of the yeasts stored under distilled water tested after 6 months, showed that percentage of viable cells varied between 65-70% in all the subcultures.

Distilled water storage appears promising, fairly stable and more pleasant to handle though survival is for a shorter period when compared to mineral oil storage. This will significantly reduce the cost of yeast storage which is appropriate for a developing country like ours.

### 3.4 Storage under Mineral Oil

Peptone yeast extract agar (23 g/l) is dispensed in Mac Cartney bottles and after the usual sterilization procedure, is allowed to set at an inclined angle to form a slope. Duplicates are prepared for each yeast culture. Each of the culture was inoculated onto peptone yeast extract agar slant by using a sterile wire loop separately in zig-onto peptone yeast extract agar slant by using a sterile wire loop separately in zig-zag manner. The oil is sterilized by heating at 121˚C , 15 lb/inch$^2$ followed by drying at 170˚C for 1-2 hrs. The cultures are covered by mineral oil (liquid paraffin) up to 1 cm level. To prevent the contamination of oil from spores blown out from cultures while pouring oil into the cultures, small quantities of the oil in individual containers are autoclaved and added as single doses. These are stored at room temp. Retrieval is done by removing a small piece of yeast colony with a sterile loop, draining off the oil and streaking the inoculum into a plate or tube containing peptone yeast extract agar medium whenever required.

Cultures stored under mineral oil require transfer after a long period of inoculation due to reduced growth and metabolic activities. As it is covered by mineral oil there is reduced oxygen tension. The sterilized mineral oil does not allow oxygen or moisture to penetrate. Thus, sterilized condition is maintained. Some difficulties arise while sub culturing in a tropical country like ours. In the tropics, contamination of culture media during isolation, sub culturing etc is often more likely to occur than in temperate areas, partly because of the large amount of dust often present in the air and also due to air movement which may occur because of open windows. All the yeast cultures stored under mineral oil appeared in good condition after one year. Many of them showed a white to tan filamentous growth extending from the surface of the slant into the mineral oil. When observed under the microscope, this growth was found to be made up of some single cells. Upon subculture on peptone yeast extract agar medium, growth appeared within 24 hrs and almost all the subcultures showed abundant growth in 48

hrs. The microscopic examination of the yeast cultures and the biochemical tests agreed with the observation made before. Figure below shows how bottles of mineral oil storage are arranged in a storage cabinet.

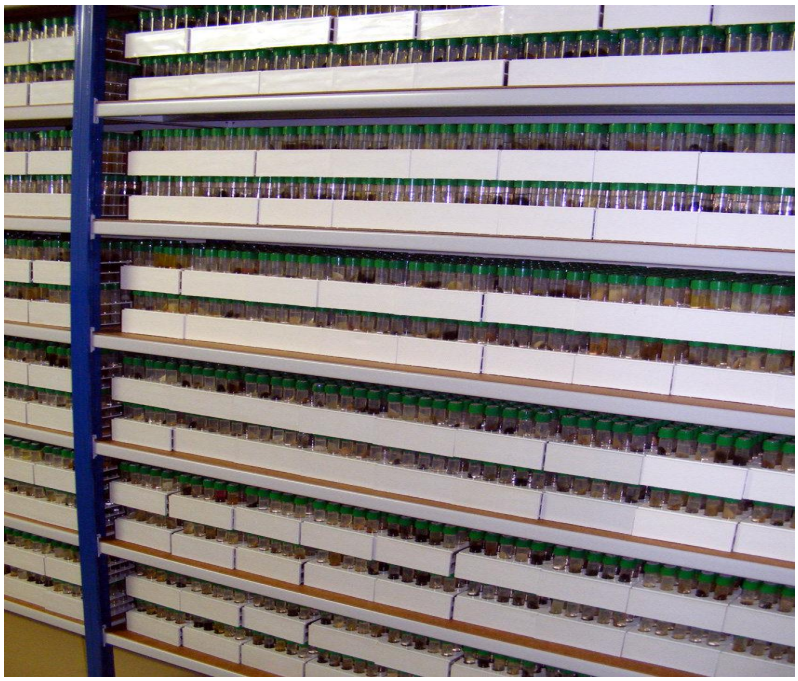


**Fig. 3. Mineral oil storage**

### 3.5 Preparation of Starter Cultures

Starter culture of yeast is prepared from agar plates under storage. Sterile wort is prepared (volumes 70 ml, 500 ml, 3.8 L). A single colony is taken by a sterile loop from the surface of the plate and immersed in the sterile wort and swirled well. The conical flasks are capped loosely and kept at 27°C for 48hrs. It is observed for the sedimentation of yeasts at the bottom of the conical flasks. This is used as the source for successive starters. The starter cultures can be dried and preserved in rice cakes or plant fibre strands for subsequent brewing process [10].

## 4. CONCLUSION

Few traditional methods of storage of yeasts were compared in this study. Distilled water storage is a cheap method in which the yeasts remained viable for six months. Though mineral oil storage provides a long term storage there is a chance of contamination during sub culturing. Therefore sub culturing should be done carefully for yeasts stored under mineral oil.

## COMPETING INTERESTS

Author has declared that no competing interests exist.

## REFERENCES

1. Cheong C, Wackerbauer K, Beckmann M, Jang KH, Kang SA. Effect of cultivation conditions on trehalose content and viability of brewing yeast following preservation via filter paper or lyophilization methods. Biotechnol Bioprocess Eng. 2008;13:690-696.

2. Smith, D. and Onion, A.H.S. (ed). A comparison of some preservation techniques for fungi. British Mycological Society; 1983.
3. Smith D, Onions AHS. (ed) The preservation and maintenance of living fungi. International Mycological Institute; 2006.
4. Ravimannan N. 'Comparison of different preservation techniques of fungal cultures ' Proceedings of the 26th Annual session of Institute of Biology; 2006.
5. Kali A, Charles MVP. A Cost-effective technique of yeast stock culture maintenance in laboratory.Int J Pharm Bio Sci. 2014;5(1):(B):65-69.
6. Onions AHS. Preservation of fungi. In: Methods in Microbiology. New York, Academic Press. 1971;4:113-151.
7. Kockova-Kratochvflova A, Kovacovska, R. A Contribution to the quantitative evaluation of yeast cultures stored in liquid nitrogen. Folia Microbiol. 1986;31:367-384.
8. Lodder J. The yeasts: Taxonomic study. Amsterdam, North Holland publishing Co; 1970.
9. Kirshop BE, Snell JJS. Maintenance of microorganisms: A manual of laboratory methods. Academic Press; 1984;207.
10. Nyanga LK, Nout MJR, Smid EJ, Boekhout T, Zwietering MH. Yeasts preservation: Alternatives for lyophilisation. World J Microbiol Biotechnol. 2012;28(11):3239-3244.

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She completed special degree in Botany in the University of Jaffna in 1988 and was successful in obtaining an MPhil degree in Food Science and Technology in the Post graduate Institute of Agriculture, Peradeniya in 1997. She has been attached to the Department of Botany, University of Jaffna as a senior lecturer. She is a member of the Ecotourism foundation. She is interested in forest ecology, biodiversity etc. Apart from Sri Lanka, she visited forests in Queensland, Malaysia and North India. She has published several papers in antioxidant activity in biochemistry, preservation of fungi and yeasts in food microbiology and few papers in conservation of mangroves, forests etc. She served as Head/Botany from 1998 to 2011 in the University of Jaffna, Sri Lanka.

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***Chapter 8***
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# Determination and Characterization of Lectin Activity from Texas Live Oak (*Quercus fusiformis*)

**Ruby A. Ynalvez[1*], Carmen Cruz-Mac Kinnon[1] and Marcus A. Ynalvez[2]**

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## ABSTRACT

Lectin is a protein of non-immunological origin. It is a protein with agglutination properties. They have biological properties that can be exploited for medicinal and therapeutic purposes. The objective of this study was to isolate and characterize lectin activity in Texas Live Oak (*Quercus fusiformis*). More specifically, the study aimed to determine the lectin's blood group specificity and pH stability, determine effects of seasonal variation, soil moisture and soil pH on lectin activity. The study also aimed to determine the presence of antifungal activity in *Q. fusiformis* extracts. Lectin activity was detected and compared via agglutination and protein assays. Protein partial purification was accomplished using diethylaminoethyl ion-exchange chromatography matrix. High Performance Liquid Chromatography (HPLC) was used to assess purity of the lectin. Results showed that *Q. fusiformis* extracts' lectin activities are stable at a pH range of 5.2 - 9.2 but with a significant decrease in activity above pH 9.2. Lectin activity was significantly higher when assayed against sheep red blood cells as compared to other blood groups tested. *Quercus fusiformis* extract is devoid of antifungal activity against *Aspergillus niger* and *Rhizopus stolonifer*. Seasonal variation, soil moisture, and soil pH do not significantly correlate with lectin activity. Results from HPLC showed presence of three peaks indicating a partial purification of the *Q. fusiformis* lectin.

*Keywords: Lectin; Quercus fusiformis; protein Purification; HPLC; blood group Specificity; pH stability.*

## 1. INTRODUCTION

Lectin is a protein of non-immunological origin and contains at least one non-catalytic carbohydrate-binding site. Lectins' ability to recognize and specifically bind reversibly to carbohydrates distinguishes them among other proteins [1,2]. Their capability of specifically recognizing and binding to sugars of erythrocytes *in vitro* merits the term agglutinin [2], the process is termed agglutination. Lectins' specificities such as binding to glycoprotein receptors on cellular membranes allow the study of the physiological means in cellular communication such as cell-to-cell recognition [3,4]. Thus, lectins play a pivotal role in cellular communication and protein-carbohydrate interactions [5,6,7]. Lectin's sugar-binding property readily distinguishes them as biotechnological tools to define carbohydrate structure and physiological dynamics [8-12]. This characteristic is exploited for biomedical research for wide applications [13-21]. Sources of lectins within an organism vary from organism to organism. Lectins also vary in terms of their function, structures, biological activity, concentration, as well as in organ and cellular location [7,22-23].

Isolation and purification of lectins may be done through a variety of protein purification methods [24-34]. Methods for purifying lectins vary due to lectin sources (*i.e.* plant or animal). Methods also will depend on lec-tins' structure, specificity, physiochemical properties and biological activity [35]. For example, in studying of *Apuleia leiocarpa* seed lectin (ApuISL) for antibacterial potential; the lectin was chromatographically isolated from a crude extract with 150 mM NaCl by using a chitin column. The resulting ApuISL adsorbed to the matrix was eluted using 1.0 M acetic acid [13]. In the isolation of

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*Callianara surinamensis* leaf pinnulae lectin, proteins were precipitated in ammonium sulphate and separated using a Sephadex G-75 chromatography [36]. Another example is lectin isolation and purification from black turtle bean (*Phaseolus vulgaris)* through the use of reverse micellar extraction achieved by dissolving sodium bis (2-ethylhexyl) sulfosuccinate in HPLC isooctane [37].

Texas Live Oak (*Quercus fusiformis*) is a thicket-forming shrub (or a large spreading tree). It is a drought-tolerant and cold-hardy plant compared with its look-a-like *Quercus virginiana.* The Texas Live Oak is native to Oklahoma and in north central, central, and southern Texas [38,39]. *Q. fusiformis* belongs to the family Fagaceae and may also be known as Escarpment Live Oak, Plateau Live Oak, Scrub Live Oak and West Texas Live Oak [38]. Several native plant species of Texas have yet to be examined as potential lectin sources, including Texas Live Oak. Previous study reported lectin to be present in the leaf, stem and fruit of *Q. fusiformis* with the highest lectin activity expressed in the leaves [23]. In this regard, the objective of this study was to isolate, partially purify and characterize leaf lectin activity from *Q. fusiformis*. This study focused on conventional methods of protein purification, which included centrifugation and column chromatographic techniques. High Performance Liquid Chromatography (HPLC) assessed the purity of the lectin. The lectin was characterized in terms of its animal blood group specificity, pH stability, seasonal variation's effect on lectin activity, and soil moisture and soil pH effect on lectin activity. The antifungal property of the *Q. fusiformis* extract was also determined. The research study was significant since it contributed to the inventory of lectins found in plants. It also established an efficient and effective protocol to isolate and partially purify lectin from *Q. fusiformis*.

## 2. METHODOLOGY

### 2.1 Sample Collection

Leaf samples were collected from Texas Live Oak trees found on the Texas A & M International University campus. Five trees were selected to sample and represent the Texas Live Oak tree population (N27˚34'24.3" W99˚26'00.3"; N27˚34'23.4" W99˚26'12.3"; N27˚34'22.3", W99˚26'17.3"; N27˚34'34.5" W99˚25'49.0"; N27˚34'23.3" W99˚25'59.1"). Leaf samples collected were mature sized leaves that ranged from 8 centimeter to 12 centimeter in size. Leaf samples were stored in assigned labeled Ziploc™ bags placed in a −40˚C freezer or samples were immediately homogenized.

### 2.2 Crude Extraction

Leaves were washed, ground and homogenized using a Waring® Laboratory variable-speed blender. Cold 0.01 M Tris-Cl buffer (0.15 M NaCl), pH 9.4 (1:8 w/v) was added and homogenized for one minute for each respective sample. Samples were stirred for one hour using a VWR® Dyla- Dual™ Hot Plate Stirrer at 1000 rpm in a cold room (4˚C) to optimize homogenization. Then, the homogenate was filtered using cheesecloth and centrifuged at 8000 rpm at 10˚C for 30 minutes using an Avanti® JE Centrifuge JA-20 Rotor. The crude extract was stored (at −40˚C) for later use or immediately used for agglutination assays.

### 2.3 Preparation of Red Blood Cells

Two-hundred microliter aliquot of blood samples including human (collected from Laredo Medical Center Laboratory), horse, rabbit and sheep (purchased from Biomérieux® company) were mixed with 10 mL 0.01 M, phosphate buffer saline (PBS), pH 7.2 (0.15 M NaCl) in a 15 mL tube. The blood suspension was centrifuged at room temperature using a Hamilton Bell® VanGuard V6500 Biohazard centrifuge with fixed speed of 3500 rpm for five minutes. At the end of centrifugation, the supernatant was discarded. A second wash or until the supernatant was clear to remove lysed red blood cells was done using 10 mL of 0.01 M, PBS. The pellet was dissolved with PBS to obtain a 2% blood suspension.

### 2.4 Agglutination Assays

The crude extract and purified fractions were assayed for the presence of lectin activities using Corningware™ 96-well microtiter U-plates, 0.01 M PBS and 2% blood suspension. The sample was

diluted by a serial two -fold dilution in PBS (50 µL) and incubated with a 2% suspension of RBCs (50 µL) at room temperature for an hour or until the negative control showed a red button formation. Agglutination activity was detected based on the RBCs appearance on the well; a positive result appears as a red-carpet layer, while negative results, appear as a red button in the bottom of the well.

## 2.5 Protein Content Determination

The protein content of crude extract samples was determined with the Bradford method [40] using a Quick-Start™ Bradford Protein Assay Kit.

## 2.6 Characterization of Lectin Activities

### 2.6.1 Blood group specificity

The blood group specificity was tested in four different blood groups; horse, human, rabbit and sheep. The animal blood was purchased as defibrinated blood from Biomérieux®. Human red blood cells were collected from the Laredo Medical Center Laboratory Department, as postpartum blood samples and screened negative for both HIV and blood-borne communicable diseases.

### 2.6.2 Effect of pH on *Q. fusiformis* Lectin Activity

Crude extracts of *Q. fusiformis* were studied for the effect of pH by using buffers of different pH levels: 5.2, acetate buffer; 6.2 and 7.2 phosphate buffer; 8.2 and 9.2, Tris-Cl buffer.

### 2.6.3 Effect of seasonal variation on *Q. fusiformis* lectin activity

Leaf samples were collected in three seasons (fall, winter, and summer) to determine if seasonal variation had an effect on *Q. fusiformis* lectin activity. Summer season consisted of hot temperatures with an average of 104˚F, with mostly clear skies, and long days. The fall season, had an average temperature of 93˚F, with partly cloudy skies and with the most rain due to hurricane season. The winter season consisted of an average temperature of 50˚F, with cloudy skies and short days. Two fall seasons and two winter seasons were observed while only one summer season was observed. Crude extracts of *Q. fusiformis* were prepared with 0.01 M phosphate buffer, 0.15 M NaCl, pH 7.2.

### 2.6.4 Effect of soil moisture on *Q. fusiformis* Lectin activity

The soil samples were collected by the Auger Method. Soil was sampled and subsampled by depth. Soil ex-amined was under the *Q. fusiformis* crown and collected from three *Q. fusiformis* locations. *Q. fusiformis* roots are at least 50 centimeters deep [38,39], thus depth focused on the 20 - 30 centimeter depth, which was the best estimation for soil analysis. Samples were stored in a cold room (4˚C) or used immediately. Soil moisture was determined by examining moisture loss of soil subsamples on aluminum weight trays. Samples were weighed using an analytical balance. A moist, 2.00 gram subsample was used for each triplicate subsample. Soils were oven-baked for 24 hours at 105˚C to dry and weighed once more to record dry soil mass.

### 2.6.5 Effect of Soil pH on *Q. fusiformis* Lectin activity

Soil samples collected for soil moisture determination were also sampled to determine soil pH. Soil samples were put in paper bags to air-dry for three to seven days, followed by sieving with a two-millimeter (mm) mesh screen #10. Five g of air-dry soil sample was placed into a 50 mL labeled centrifuge tube. A total of three replicates were performed. Ten mL of deionized water was added to the 50 mL centrifuge tube for a soil water ratio of 1:2. Solutions were shaken for one hour at 120 revolutions per minute (rpm); subsequently, solutions were centrifuged at 15,000-×g for 5 minutes. The resulting supernatant was used to record pH using the VWR® symphony pH meter.

### 2.6.6 Determination of the Antifungal Activity of *Q. fusiformis* Extracts

Two antifungal assays, an anti-*Aspergillus niger* and an anti-*Rhizopus stolonifer* assays were done. Potato Dextrose Agar (PDA) plates were marked with a circular circumference on the bottom of the

plate with a 60 millimeter (mm) perimeter. Fungal cultures were grown at 28˚C until they reached the drawn boundaries which took approximately 24 hours. After the mycelial colony had grown, sterile blank paper discs (6 mm in diameter, Grade AA) were saturated with 20 µL aliquot solutions of *Q. fusiformis* freeze-dried crude extracts that were diluted with sterile water. The concentrations, 125 µg/µl, 250 µg/µl and 500 µg/µl were tested. The discs were placed at the outer rim of the mycelial colony (which was within the new circumference of 60 mm away from the original circumference of the petri plate). The plates were incubated at 28˚C for 24 hours. After incubation, crescents or zones of inhibition were measured using a Vernier caliper. A negative control was used (sterile water) which allowed mycelial growth. On the other hand, a positive control (nystatin) was used to demonstrate the formation of crescents or zones of inhibition around the disc (Fig. 1).

## 2.7 Purification of *Q. fusiformis* Lectin

The lectin from *Q. fusiforms* crude extracts was partially purified by one step chromatography using an ion-exchange column chromatography with a Bio-Rad® BioLogic™ low-pressure chromatography system. All absorbance readings were at 280 nm. The flow rate was 1 ml/min and collections of eluate with 3 mL/fraction. Twen-ty-six mL of *Q. fusiformis* crude extract was applied to DEAE-cellulose column, which was equilibrated and washed with Buffer A, 0.01 M Trizma-Cl with 0.15 M NaCl, pH 9.2. After washing, Buffer B, 0.01 M Trizma-Cl with 0.5 M NaCl , pH 9.2, was run into the column until absorbance was below < 0.01, then elution Buffer C, 0.01 M Trizma-Cl with 1.0 M NaCl, pH 9.2, was applied into the column. A Bio-Rad® fraction collector #731 - 8304) was used to collect fractioned proteins. Fractioned proteins were pooled and dialyzed to remove the salts. Dialysis was performed with Thermo Scientific SnakeSkin® dialysis tubing. The dialysate used was deionized water. The dialyzed samples were then freeze-dried.

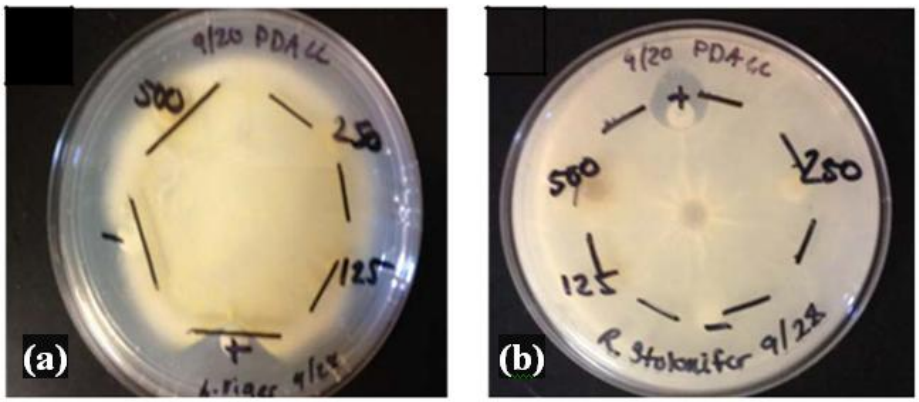



**Fig. 1. Antifungal activity assay against (a) *A. niger* and (b) *R. stolonifer.* The negative control was sterile water and the positive (+) control was the antifungal agent, nystatin showing crescent and zone of inhibition for *A. niger and R. stolonifer*, respectively**

High Performance Liquid Chromatography (HPLC) separation was performed on Prostar PS 220 system which has a UV detector and Rheodyne injector with 20 µl loop volume. Galaxie© Software was applied for data collecting and processing. Purity assessment of the dialyzed and freeze-dried sample (2 µL of freeze-dried sample diluted in 100 µL deionized $H_2O$) was analyzed by HPLC. A Phenomenex RP-C18 (250 × 4.60 mm) column was used as the stationary phase. A gradient of 100% water to 100% methanol was used as mobile phase. The mobile phase was pumped at 1 ml/min and the eluents were monitored at 215 nm and 280 nm. The injection vo-lumes of samples were 20 µl.

## 2.8 Statistical Analysis

Data for blood group specificity and pH stability were obtained from five replicates. An analysis of variance (ANOVA) associated with a 5 × 4 factorial experiment in randomized complete blocks design was performed using the Statistical Analysis System (SAS 9.3)® software. Mean comparisons were

performed by way of a Bonferroni test using a type I error rate of 0.05. Data analysis for seasonal variation was carried out using a one-way ANOVA. Bivariate correlations among seasonal variation, soil moisture and pH were estimated and tested using a Pearson correlation analysis with level of significance for a two-tailed test set at 0.01.

## 3. RESULTS AND DISCUSSION

Lectin activity from *Q. fusiformis* leaves was investigated, using agglutination assays. In the presence of lectin, sugars on the surface of red blood cells form an interaction with the lectin resulting in agglutination. This is evident by the formation of a carpet layer on the bottom of a microtiter plate wells. On the other hand, in the absence of lectin, a distinctive red button is formed on the bottom of the microtiter plate well. The reciprocal of dilution is calculated as titer value, which reflects lectin activity. The higher the titer value the higher is the lectin activity. Different factors *i.e.* blood group specificity; pH and seasonal variation are known to affect lectin activities [41-44]. In this study, how these factors affect *Q. fusiformis* lectin activity were investigated. Results of the study will aid in the characterization of *Q. fusiformis* lectin.

### 3.1 Blood Group Specificity Study of *Q. fusiformis* Lectin

The blood group specificity of *Q. fusiformis* lectin activity was investigated in four blood groups (horse, human, rabbit and sheep). The extracts of *Q. fusiformis* agglutinated to all blood groups tested, making *Q. fusiformis* a non-blood group specific lectin. Lectin specific activity (SA) was expressed as titer over milligrams of protein. For analysis, lectin specific activity was transformed to natural logarithm of specific activity (ln SA). ANOVA results reported a difference among the four blood groups tested, which was subjected to a Bonferroni test using a Type I error rate ($\alpha$ of 5%) for mean comparisons to determine significant differences (Table 1, Fig. 2). The Bonferroni test showed a significant difference in the ln specific activity values of *Q. fusiformis* lectin between sheep blood (ln SA = 8.30) and the three blood groups (horse, ln SA = 6.29), human (ln SA = 7.21) and rabbit (ln SA = 7.02). As shown in Fig. 2, ln specific activity using sheep blood showed a significantly higher lectin activity as compared to the other three blood groups. However, lectinln specific activities of *Q. fusiformis* using horse, human and rabbit blood groups were not significantly different from each other.

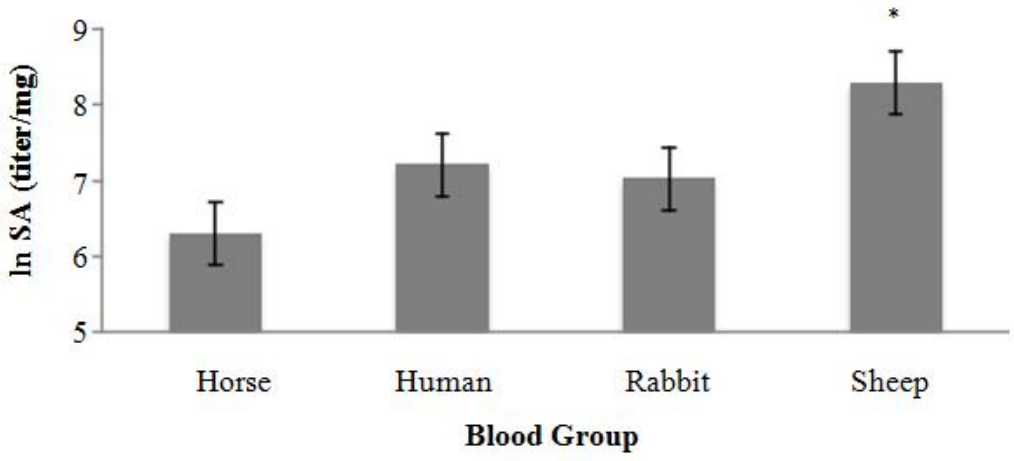



**Fig. 2. Blood group specificity of*Q. fusiformis*lectin based on ln SA of meancomparisons analyzed with a Bonferroni test using a type I error rate of 5%**
**Significant ($p<0.05$) compared to horse, human and rabbit blood groups*

Specificity is influenced by the limited number of contacts with carbohydrates and depth of the sugar binding sites [45]. In addition, any modification or substitution to a binding site can influence binding specificity [46]. The observed differences in *Q. fusiformis* lectin activity with different blood groups

may be due to differences in carbohydrate-lectin binding interactions which can be attributed to differences in carbohydrates presented on the cell surfaces of the different blood groups. (I will add text here to update from Molecules 2016 so add citation number)

**Table 1. Determinations of blood group specificity of *Q. fusiformis* crude extract lectin**

| Blood group | Titer[a] | Protein Content(mg/mL)[b] | HA[c] | SA (titer/mg)[d] | lnSA[e] |
|---|---|---|---|---|---|
| Horse | 6.08 | 0.743 | 304 | 959.59 | 6.29[f] |
| Human | 18.16 | 0.743 | 908 | 2094.11 | 7.21[f] |
| Rabbit | 15.2 | 0.743 | 760 | 2542.26 | 7.02[f] |
| Sheep | 49.44 | 0.743 | 2472 | 10,654.62 | 8.3[g] |

[a] *Titer is the reciprocal of the lowest dilution that was positive for lectin activity.*
[b] *Protein content was determined using Bradford assay.*
[c] *Hemagglutination Activity (HA), is titer multiplied with sample volume (50 uL).*
[d] *SA, Specific Activity is HA divided by the protein content.*
[e] *In Specific activity (SA), is the natural logarithm of specific activity. Values of ln SA are mean values from Bonferroni test using a type I error rate of 5%.*
[f] *Blood groups horse, human and rabbit show no significant difference from each other.*
[g] *Sheep blood group is significantly different from horse, human and rabbit*

The carbohydrates on the erythrocyte cellular surfaces are distinguishable among the four different blood groups [47]. There are reported monosaccharide determinants in the different blood groups, fucose in horse [48], galactose in human and [48-50] and mannose in rabbit erythrocytes [51]. Horse, human and rabbit red blood cells may contain carbohydrate components on the cellular surface binding sites that are relatively less recognized by the *Q. fusiformis* lectin binding site. On the other hand, the carbohydrates found on the cellular surface of sheep red blood cells may contain carbohydrate units in a structure and position more specific and with higher affinity for the binding of *Q. fusiformis* lectin, subsequently increasing sheep erythrocyte agglutination. Moreover, lectin characteristics such as, multivalency may determine cross-linking interaction in binding recognition. Spatial distribution of multivalency among lectin structures may produce a higher level of specificity [51,52].

*Quercus fusiformis* lectin activity is similar to other plant lectins that are also non-blood group specific. *Erythrina speciosa* lectin was characterized as a non-blood group specific. Its lectin activity was examined in the human blood ABO system and animal blood groups, rabbit, mouse, sheep and horse [53]. Likewise, the blood group specificity for leaf lectin in *Kalanochoe crenata* was characterized as a non-blood group specific lectin, agglutinating to the different types of human blood red cells of the ABO system [36]. *Artocarpus incisa* seeds were also examined in a wide range of blood groups including human ABO system and animal blood groups, cow, goat, rabbit, pig and sheep. *Artocarpus incisa* seed lectin resulted in non-blood group specificity in humans ABO system, while rabbit blood group activity was not different from human ABO and the other four blood groups were significantly different from human and rabbit agglutination [54]. Similarly, *Bryopsis plumosa* lectin from a green marine alga agglutinated sheep and horse erythrocytes [55]. The blood group specificity of *Q. fusiformis* lectin activity suggests *Q. fusiformis* lectin is non-blood group specific with higher specificity directed towards sheep erythrocytes. The present study is the first to report the non-blood group specificity of the *Q. fusiformis* lectin.

## 3.2 Effect of pH on *Q. fusiformis* Lectin Activity

Crude extracts of *Q. fusiformis* lectin were prepared and incubated in wide range of pH values with stirring for 24 hours at 4˚C. A Bonferroni test using a Type I error rate ($\alpha$ of 5%) was used to compare mean natural logarithm-transformed specific activity (ln SA) across pH levels (5.2, 6.2, 7.2, 8.2 and 9.2). Results indicated that *Q. fusiformis* lectin was stable at a pH range 5.2 to 8.2 while that for pH 9.2 was significantly lower than at pH 5.2 - 8.2 (Table 2, Fig. 3). Different pH conditions were found to have profound effects on the tertiary and quaternary structure of proteins and can perturb protein conformational stability [56]. Lectins have different optimum pH to maintain their stabilities [57].

Literature reported that pH stabilities of plant lectins vary. For example, *Morus rubra* [58] and *Ipomoea asarifolia* [59] leaf lectin crude extracts were stable at pH 7.5, while the Korean mistletoe lectin crude extract was stable at pH 8.0 [60]. On the other hand, the pH stability of *Kalanochoe crenata* leaf lectin was at pH values 2 to 12 [36]. *Kalanochoe crenata* leaf lectin activity was stable from pH 2 to 7.5, and the activity was lost at a pH higher than 9.2 [36]. The effect of pH on lectin activity was studied in the leaves of *Chorchorus olitours* also in a broad range of pH values from pH 2.0 to 10.5. *Chorchorus olitours* exhibited a high agglutination activity from pH 7.2 to 8.0 with a dramatic decrease of lectin activity below acidic conditions of pH 6.5 and above basic pH of 9.0 [47]. The pH stability of *Q. fusiformis* lectin activity at a wide range of pH is quite similar to other leaf lectins.

## 3.3 Effect of Seasonal Variation on *Q. fusiformis* Lectin Activity

Previous studies have reported lectin sources that display a seasonal variation in lectin activity [61]. For example, Elderberry (*Sambucus nigra*) and Black locust (*Robinia pseudoacacia*) bark lectins demonstrated a seasonal variation in content where lectin accumulation was higher during summer to winter compared to spring [43]. On the other hand, lectin activities in cultivated red alga *(Kappaphycus alvarezii*) varied with environmental characteristics such as heavy rain, solar radiation and low seawater temperatures [61]. It was hypothesized that lectin fluctuation found in both Elderberry and Black locust barks as contributed by seasonal variation resembles the behavior of plant storage proteins [43].

**Table 2. Effect of pH on *Q. fusiformis* crude extract lectin**

| pH | Titer[a] | Protein Content (ug/uL)[b] | HA[c] | SA(titer/mg)[d] | lnSA[e] |
|---|---|---|---|---|---|
| 5.2 | 18.6 | 0.532 | 930 | 2912.27 | 7.33[f] |
| 6.2 | 19.8 | 0.697 | 990 | 3053.03 | 7.25[f] |
| 7.2 | 36.1 | 0.856 | 1805 | 8147.53 | 7.73[f] |
| 8.2 | 24.5 | 0.78 | 1225 | 4944.42 | 7.32[f] |
| 9.2 | 12.5 | 0.847 | 607 | 1255.98 | 6.39[g] |

[a] *Titer is the reciprocal of the lowest dilution that was positive for lectin activity.*
[b] *Protein content was determined using Bradford assay.*
[c] *Hemagglutination Activity (HA), is titer multiplied with sample volume (50 µL).*
[d] *SA, Specific Activity is HA divided by the protein content.*
[e] *ln Specific activity (SA), is the natural logarithm of specific activity. Values of ln SA are mean values from Bonferroni test using a type I error rate of 5%.*
[f] *pH levels not significant difference from each other, lectin activity was stable*
[g] *pH level significantly different from 5.2 - 9.2*

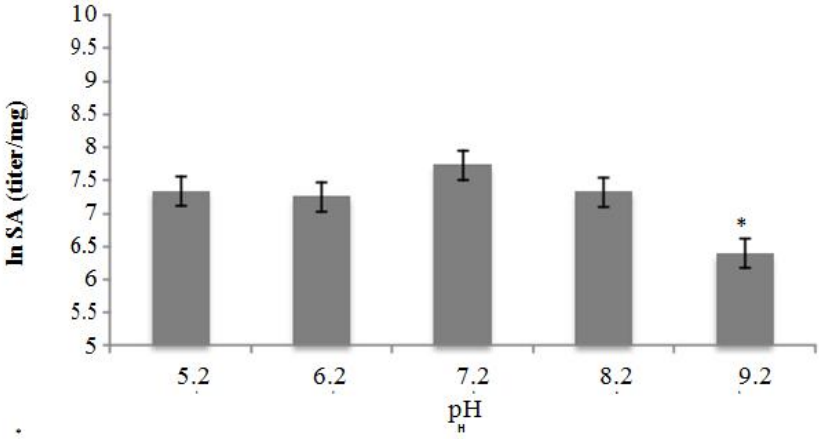



**Fig. 3. The effect of pH on *Q. fusiformis* lectin activity based on mean com parisons of ln SA analyzed with a Bonferroni test using a 5% type-I error rate**
*Significant, (p < 0.05) compared to pH range of 5.2 - 8.2*

Although, many plant lectins mimic the behavior of plant storage proteins, these lectins should not be classified as storage proteins [2]. Costa et al. [44] studied *Phthirusa pyrilfolia* leaf lectin with an activity higher during sunny weather. This was attributed to mistletoe's slower rate of photosynthesis promoting high lectin production.

In this study, *Q. fusiformis* leaf samples were collected during the fall, winter and summer seasons and lectin activities were compared. The mean ln SA (specific activity) was 7.6, 7.8 and 8.2 for fall, winter and summer, respectively (Table 3). Results of the correlation analysis determined that there was no significant correlation in mean ln SA for *Q. fusiformis* lectin during the different seasons, fall, winter and summer (Fig. 4). Compared to previous studies, this study has shown that *Q. fusiformis* lectin activity was not affected by seasonal variations.

## 3.4 Effect of Soil Moisture and Soil pH on *Q. fusiformis* Lectin Activity

Since the productivity of plants including biomolecule synthesis is affected by environmental factors, soil properties, moisture and pH were examined to determine if any of these factors affect lectin activity in *Q. fusiformis*. To date, the literature with regards to soil properties namely, moisture and pH affecting lectin activity are scarce. The lectin activity of common beans (*Phaseolus vulgaris*) was examined in three different soil types in a semi-arid region. The result of this study reported differences in lectin concentration and suggested environmental factors to contribute to lectin difference [62]. Results of the present study showed the average soil pH reading for 20-30 centimeter depth in fall was pH 8.36 and in winter was pH 8.31. On the other hand, the average moisture loss for the 20 - 30 centimeter depth in fall was 3% and for winter 5.6%.

The soil moisture and pH were subjected to a correlation analysis with level of significance for a two-tailed test at level 0.01 as shown in Table 4. Soil moisture and pH reflect the chemical status of soil conditions for the *Q. fusiformis*. Laredo soil is usually alkaline throughout [63]. *Since* the pH results of soil are basic, this may indicate that there may be some nutrients unavailable to *Q. fusiformis*, the production and expression of lectin may be affected. On the other hand, *Q. fusiformis* is a drought-tolerant tree and only requires water every three to four weeks. Analysis of the results for soil moisture and soil pH showed no significant correlation with ln SA for *Q. fusiformis* lectin.

**Table 3. Effect of seasonal variation of *Q. fusiformis* crude extract lectin**

| Seasons | Titer[a] | Protein Content[b] | HA[c] | SA (titer/mg)[d] | lnSA[e] |
|---|---|---|---|---|---|
| Fall | 5 | 0.15683 | 200 | 2540.06 | 7.6676 |
| Winter | 9 | 0.165167 | 450 | 2792.97 | 7.8715 |
| Summer | 4 | 0.112667 | 200 | 3930.9 | 8.2296 |

[a] *Titer is the reciprocal of the lowest dilution that was positive for lectin activity.*
[b] *Protein content was determined using Bradford assay.*
[c] *Hemagglutination Activity (HA), is titer multiplied with sample volume (50 uL).*
[d] *SA, Specific Activity is HA divided by the protein content.*
[e] *In Specific activity (SA), is the natural logarithm of specific activity. Values of ln SA are mean values from one-way ANOVA, t-test*

## 3.5 Determination of the Antifungal Activity of *Q. fusiformis* Extracts

There are two physiological proposed roles for plant lectins; the first is in plant defense mechanisms and the second, symbiotic nitrogen-fixing bacteria association [6,63,64]. Plant lectins have been investigated for their resistance to insects [65], bacteria [1] [28] [64] [66] [67] and fungi [1] [2] [13] [68] [69]. The study of *Q. fusiformis* lectin is relevant since new compounds and especially plant lectins with antifungal activity are of high interest due to rising fungal resistance to common fungicides. There are only a few plant lectins reported to have antifungal activities.

The antifungal activity of *Q. fusiformis* lectin was tested against *Aspergillus niger* and *Rhizopus stolonifer.* These two fungi are common pathogens in fruits and vegetables and cause rot disease [70]. Freeze-dried crude extracts of 125 µg/µl, 250 µg/µl and 500 µg/µl concentrations did not show any antifungal activity against *A. niger* and *R. stolonifer*. Similarly, the knife bean (*Canavalia gladiata*)

lectin, was devoid of antifungal activity when tested against three fungi *Botrytis cinerea*, *Myocospharella arachidcola* and *Fusarium oxysporum* [71]. In contrast, plant mistletoe (*Phthirusapyrfolia)* lectin was tested against eleven fungi (*Aspergillus niger*, *A. flavus*, *A. fumigatus*, *Rhizopus arrhizue*, *Paeciloyces variottie*, *Fusarium moniliforme*, *F. lateritium*, *Candida albicans*, *C. burneses*, *C. tropicalis*, *C. parapsilosis*, *Saccharomyces cerevisiae* and *Rhizoctnia solani*) and demonstrated antifungal activity against two fungi *F. lateritium and R. solani* [44]. Red kidney bean *(Phaseolus vulga-ris)*lectin reported by Ye et al. [72], *also* expressed antifungal activity but towards three fungal species, *Fusarium oxysporum*, *Rhizoctonia solani* and *Coprinus comatus*.

**Table 4. Pearson correlation analysis for the effect of soil moisture and soil pH on lectin activity of *Q. fusiformis***

| | | ln SA | SA | Soil moisture 20 - 30 cm | Soil pH 20 - 30 cm |
|---|---|---|---|---|---|
| ln SA | Correlation | 1 | 0.972 | 0.454** | 0.479** |
| | Sig. (2-tailed) | | 0.000 | 0.089 | 0.071 |
| | N | 15 | 15 | 15 | 15 |
| SA | Correlation | 0.972 | 1 | 0.432** | 0.442** |
| | Sig. (2-tailed) | 0.000 | | 0.108 | 0.099 |
| | N | 15 | 15 | 15 | 15 |
| soil moisture 20-30 cm | Correlation | 0.454** | 0.432** | 1 | 0.844 |
| | Sig. (2-tailed) | 0.089 | 0.108 | | 0.000 |
| | N | 15 | 15 | 15 | 15 |
| soil pH 20-30 cm | Correlation | 0.479** | 0.442** | 0.844 | 1 |
| | Sig. (2-tailed) | 0.071 | 0.099 | 0.000 | |
| | N | 15 | 15 | 15 | 15 |

**Correlation is significant at the level 0.01 (2-tailed)*

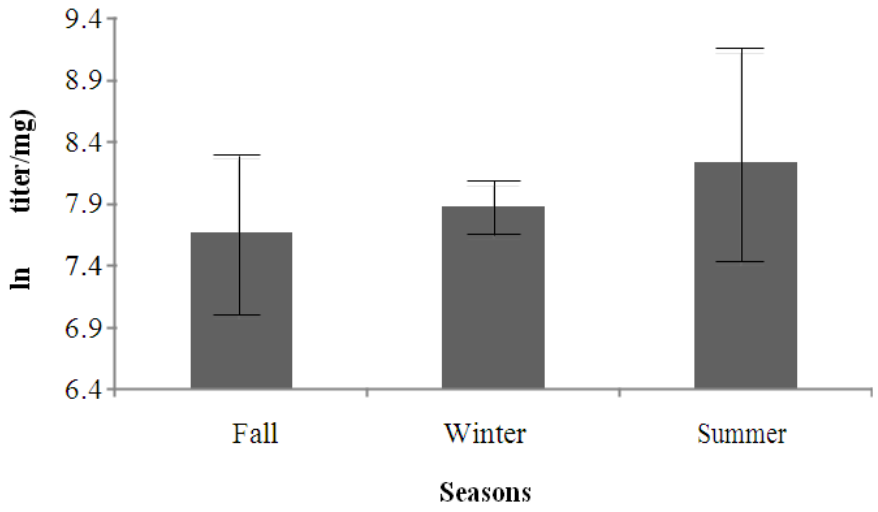



**Fig. 4. Seasonal variation results from correlation analysis of lectin mean ln SA**

## 3.6 Purification of *Q. fusiformis* Lectin

The crude extraction of lectin from *Q. fusiformis* was achieved by homogenization in Tris-Cl, pH 9.2 (0.15 M NaCl) buffer and stirred at 4˚C for one hour. Partial purification of *Q. fusiformis* crude extract was carried out by a one-step column chromatography technique using the ion-exchange chromatography column. The weak anionic DEAE-cellulose column (Pall Life Sciences AcroSep™, Product No. 20067-C001) consisted of a matrix with positive charges that will bind negatively charged proteins. Crude extracts of *Q. fusiformis* at pH 9.2 ran through the DEAE column chromatography

resulted in the binding of proteins. The chromatography profile showed three peaks, peaks are labeled with abbreviation letter D to indicate DEAE. As shown in Fig. 5, the *Q. fusiformis* crude extract resulted in the separation of proteins that corresponded to 3 peaks. The first peak (D1), the major peak, contained unbound proteins. The second peak (D2) and third peak (D3) corresponded to bound proteins that were eluted from the DEAE column using half stepwise salt concentration gradient 0.5 and 1.0M, respectively.

**Table 5. Lectin activity of the dialyzed and lyophilized fractions from D1, D2 and D3**

| Peak | Titer | HA |
|---|---|---|
| D1 | 144 | 7200 |
| D2 | 20 | 1000 |
| D3 | 10 | 500 |

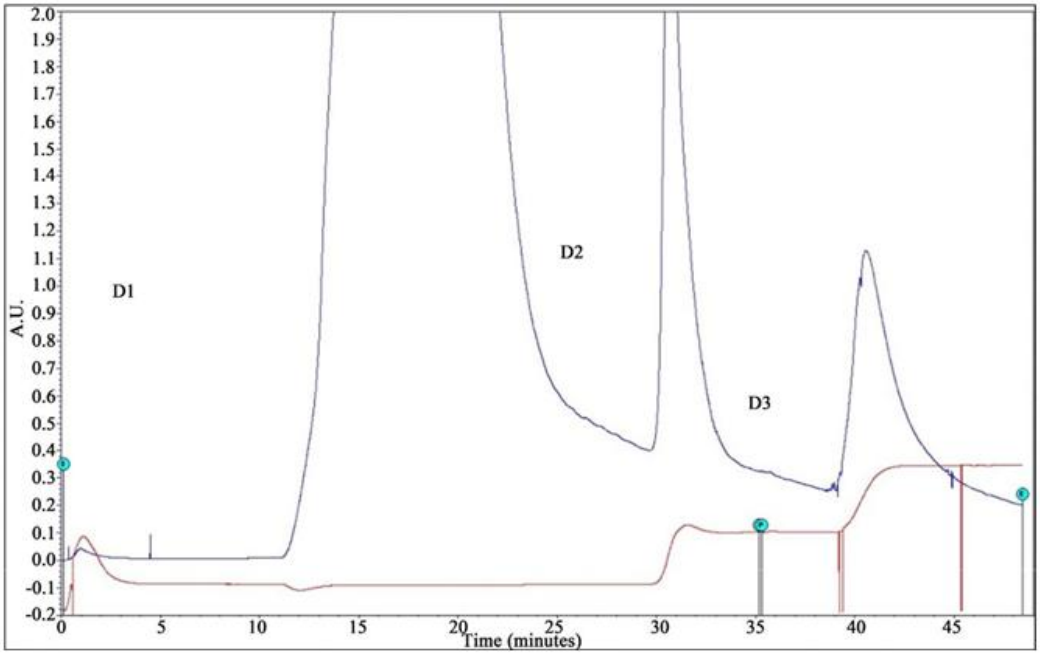



**Fig. 5. Purification of *Q. fusiformis* lectin by DEAE ion-exchange column chromatography**

*Quercus fusiformis* DEAE fractions, D1, D2 and D3 were tested for agglutination activities. These fractions did not exhibit any agglutinating activity. The absence of lectin activity in DEAE fractions may be for two reasons. First, fractions may not contain enough lectin concentration to have an activity detectable by the sensitivity of the agglutination assay due to dilution effect of column chromatography. Secondly, the high salt concentration used to elute proteins in peaks D2 and D3 may indicate an effect on conformational structure and stability of the lectin. Thus, fractions from each peak were pooled, dialyzed and lyophilized. Dialyzed samples were measured for pH with litmus paper and demonstrated a neutral pH.

Lectin activity is affected when pH conditions are modified for basic or acidic conditions [57]. This is true for *Q. fusiformis* lectin activity, and provides support for the effect of pH results when lectin activity decrease at pH level 9.2. The lyophilized samples were diluted with 200 µl of millipore water and subjected to agglutination assays to detect presence of lectin activity. Table 5 shows results of the agglutination assays. Results showed that all peaks contained lectin activity. The binding of *Q. fusiformis* lectin to the DEAE column could be attributed to the lectin amino acid composition. It can be assumed that *Q. fusiformis* lectin has a predominance of negatively charged aspartic acid and/or glutamic acid residues. These amino acids would give the lectin a net negative charge at the basic pH

due to the R-carboxylic groups ($COO^-$) present. Thus, the negatively charged *Q. fusiformis* lectins bind to the positive charged matrix of DEAE. For example, red marine alga (*Vidalia obtusilo-ba* C.) lectin was purified through two chromatographic steps, with its first step being an ion exchange DEAE-cellulose column. This lectin was reported to be rich in aspartic acid, glutamic acid and leucine [73].

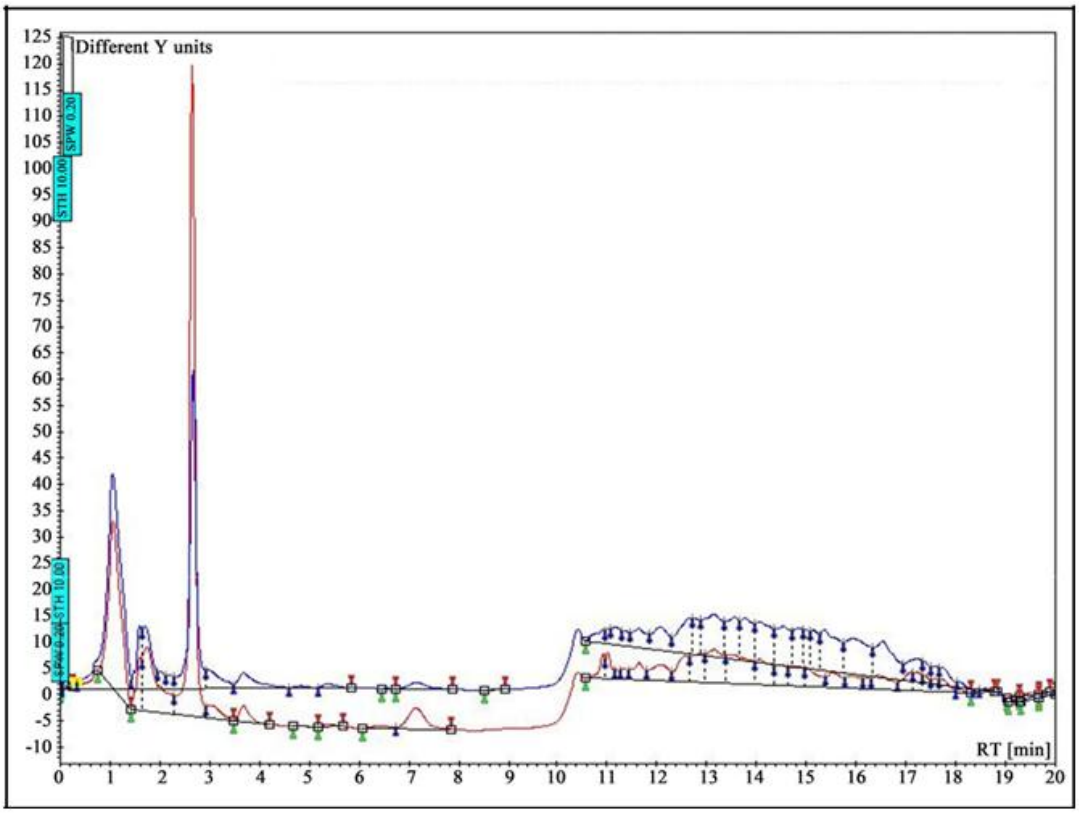



**Fig. 6. HPLC elution profile of peak 2, D2 from DEAE ion exchange column chromatography of *Q. fusiformis* extract showed three peaks**

## 3.7 Determination of *Q. fusiformis* Purity via HPLC

The first peak, D1 contained all unbound proteins including unbound lectins, thus D1 was not subjected to purity determination. Between D2 and D3, D2 was chosen for purity determination owing to the relatively higher pro-tein concentration as well as higher lectin activity observed in peak 2, D2. The purity of sample D2 was assessed using high performance liquid chromatography (HPLC). Based on the analytical assessment of D2, *Q. fusiformis* lectin is either only partially purified or D2 contains at least three isolectins since 3 peaks (retention times, 1.0, 1.5 and 2.5 min) were obtained after HPLC (Fig. 6). Isolectins are defined as closely related lectins difficult to separate since they have a similar amino acid composition.

## 4. CONCLUSIONS

*Quercus fusiformis*' lectin was non-blood group specific, with significantly higher affinity for sheep blood. Also, *Q. fusiformis* lectin was stable within a pH range of pH 5.2 to 8.2, with optimal activity at 7.2 and a significant decrease in activity at pH 9.2. The pH sensitivity of *Q. fusiformis* lectin indicates that the three-dimensional conformational structure and its binding sites are disrupted by ionic interactions occurring in different buffers and pH values. Seasonal variation was examined and no significant difference was found in lectin activity in *Q. fusiformis.* Environmental factors examined in

this study focused on soil properties, soil moisture and pH. These soil properties had no significant effect on lectin activity in *Q. fusiformis.* Lectin is hypothesized to play a role in plant defense and exhibit antifungal properties. Crude extracts from *Q. fusiformis* were examined for antifungal activity for potential inhibition and was devoid of fungal activity in the two fungi tested, *A. niger* and *R. stolonifer.*

The one step ion-exchange column chromatography technique with DEAE column resulted in two adsorbed peaks, with lectin activities. HPLC results indicated a partially purified lectin. Results of the present study pro-vide a characterization and the partial purification of *Q. fusiformis* lectin. Future efforts are needed that include upscale purification and further characterization of the lectin under study. The sugar inhibition test may reveal sugar specificity of *Q. fusiformis* lectin. Highly concentrated lectin and purified lectin may be examined for other biological activities including antifungal activities. In addition, determination of antifungal activities against other fungal species is recommended. The DEAE isolate can be further purified using a galactose affinity column owing to *Q. fusiformis* observed affinity to sheep's blood.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

## REFERENCES

1. Charungchitrak S, Petsom A, Sangvanich P, Karnchanatat A. Antifungal and Antibacterial Activities of Lectinfrom the Seeds of Archidendron jiringa Nielsen. Food Chemistry. 2011;126: 1025-1032.
   Available:http://dx.doi.org/10.1016/j.foodchem.2010.11.114
2. Peumans WJ, Van Damme EJM. Lectins as Plant Defense Proteins. Plant Physiology. 1995;109:347-352.
   Available:http://dx.doi.org/10.1104/pp.109.2.347
3. Jiang QL, Zhang S, Tian M, Zhang SY, Xie T, Chen DY, Chen YJ, He J, Liu J, Ouyang L, Jiang X. Plant Lectins, from Ancient Sugar-Binding Proteins to Emerging Anti-Cancer Drugs in Apoptosis and Autophagy. Cell Proliferation. 2015;48:17-28.
   Available:http://dx.doi.org/10.1111/cpr.12155
4. Ghazarin H, Idoni B, Oppenheimer SB. A Glycobiology Review: Carbohydrates, Lectins, and Implications in Cancer Therapeutics. Acta Histochemistry. 2016;113(3):236-247.
   Available:http://dx.doi.org/10.1016/j.acthis.200.02.004
5. Nagre NN, Chachadi VB, Eligar SM, Shubhada C, Pujari R, Shastry P, Swamy BM, Inamdar SR. Purification and Characterization of a Mitogenic Lectin from Cephalosporium, a Pathogenic Fungus Causing Mycotic Keratitis. Biochemistry Research International; 2010.
   Article ID: 854656
   Available:http://dx.doi.org/10.1155/2010/854656
6. Sharon N, Lis H. History of Lectins: From Hemagglutinins to Biological Recognition Molecules. Glyco-biology. 2004;14:53R-62R.
   Available:http://dx.doi.org/10.1093/glycob/cwh122
7. De Schutter K, Van Damme EJM. Protein-Carbohydrate Interactions as Part of Plant Defense and Animal Immunity. Molecules. 2015;20:9029-9053.
   Available:http://dx.doi.org/10.3390/molecules20059029
8. Christian B, Thomas JB, Melissa W, de Graaf AMA, van Delft FL, den Brok MH, Adema GJ. Targeting Aberrant Sialylation in Cancer Cells Using a Fluorinated Sialic Acid Analog Impairs

Adhesion, Migration, and in Vivo Tumor Growth. Molecular Cancer Therapeutics. 2013;12: 1935-1946.
Available:http://dx.doi.org/10.1158/1535-7163.MCT-13-0279

9. Lam SK, Ng TB. Lectins: Production and Practical Applications. Applied Microbiology Biotechnology. 2011;89:45-55.
Available:http://dx.doi.org/10.1007/s00253-010-2892-9
10. Oliveira C, Nicolau A, Teixeira JA, Domingues L. Cytotoxic effects of native and recombinant fru-talin, a plant galactose-binding lectin, on hela cervical cancer cells. Journal of Biomedicine and Biotechnology; 2011.
Article ID: 568932
Available:http://dx.doi.org/10.1155/2011/568932
11. Poiroux G, Pitié M, Culerrier R, Ségui B, Van Damme EJM, Peumans WJ, Bernadou J, Levade T, Rougé P, Barre A, Benoist H. Morniga G: A Plant Lectin as an Endocytic Ligand for Photosensitizer Molecule Targeting Toward Tumor-Associated T/Tn Antigens. Photochemistry and Photobiology. 2011;87:370-377.
Available:http://dx.doi.org/10.1111/j.1751-1097.2010.00858.x
12. Mahalingam A, Geonnotti AR, Balzarini J, Kiser PF. activity and safety of synthetic lectins based on benbenzoboroxole-functionalized polymers for inhibition of HIV entry. Molecular Pharmaceutics. 2011;8:2465-2475.
Available:http://dx.doi.org/10.1021/mp2002957
13. Procopio TF, Patriota LLdS, de Moura MC, de Silva PM, de Oleveira AP, Carvalho LVdN, Lima TdA, Soares T, da Silva TD, Coelho LCBB, Pitta MGdR, Rego MJB., de Figueiredo RCB, Paiva PMG, Napoleao TH. CasuL: A New Lectin Isolated from Calliandra surinamensis Leaf Pinnulae with Cytotoxicity to Cancer Cells, Antimicrobial Activity and Antibiofilm Effect. International Journal of Biological Macromolecules. 2017;98:419-429.
Available:http://dx.doi.org/10.1016/j.ijbiomac.2017.02.019
14. Dias RdO, Machado LdS, Migliolo L, Franco LF. Koharudin LM, Gronenborn AM. Insights into Animal and Plant Lectins wit Antimicrobial Activities. Molecules. 2015;20:519-541.
Available:http://dx.doi.org/10.3390/molecules20010519
15. Koharudin LM, Gronenborn AM. Antiviral Lectins as Potential HIV Microbicides. Current Opinion in Virology. 2014;7:95-100.
Available:http://dx.doi.org/10.1016/j.coviro.2014.05.006
16. Liu Z, Luo Y, Zhou TT, Zhang WZ. Could Plant Lectins Become Promising Anti-Tumor Drugs for Causing Autophagic Cell Death? Cell Proliferation. 2013;46:509-515.
17. Randy CF, Cheung H, Leung H, Pan WL, Ng TB. A Calcium Ion-Dependent Dimeric Bean Lectin with Antiproliferative Activity toward Human Breast Cancer MCF-7 Cells. Protein Journal. 2013;32:208-215.
Available:http://dx.doi.org/10.1007/s10930-013-9477-2
18. Li WW, Yu JY, Xu HL, Bao KK. Concanavalin A: A Potential Anti-Neoplastic Agent Targeting R. A; 2011.
19. Fang EF, Lin P, Wong JH, Tsao SW, Ng TB. A Lectin with Anti-HIV-1 Reverse Transcriptase, An-titumor And Nitric Oxide Inducing Activities From Seeds of Phaseolus vulgaris Cv. Extralong Autumn Purple Bean. Journal Agricultural Food Chemistry. 2010;58:2221-2229.
Available:http://dx.doi.org/10.1021/jf903964u
20. Liu B, Bian HJ, Bao JK. Plant Lectins: Potential Antineoplastic Drugs from Bench to Clinic. Cancer Letters. 2010;287:1-12.
Available:http://dx.doi.org/10.1016/j.canlet.2009.05.013
21. Swanson MD, Winter HC, Goldstein IJ, Markovitz DM. A Lectin Isolated from Bananas Is a Potent Inhibitor of HIV Replication. Journal of Biological Chemistry. 2010;285:8646-55.
Available:http://dx.doi.org/10.1074/jbc.M109.034926
22. Raja SB, Murali MR, Kumar NK, Devaraj SN. Isolation and Partial Characterization of a Novel Lec-tin from Aegle marmelos Fruit and Its Effect on Adherence and Invasion of Shigellae to HT29 Cells. Plos One. 2011;6:e16231-e16231.

Available:http://dx.doi.org/10.1371/journal.pone.0016231

23. Ynalvez RA, Fuentes LM, Sanchez CV. Comparison and Temperature Study of Lectin Activities in Texas Live Oak (Quercus fusiformis) Crude Extracts. Journal of Plant Science. 2011;6:124-134.
    Available:http://dx.doi.org/10.3923/jps.2011.124.134
24. Roy R, Murphy PV, Gabius H-J. Multivalent Carbohydrate-Lectin Interactions: How Synthetic Chemistry Enables Insights into Nanometric Recognition. Moelcules. 2016;21:629.
    Available:http://dx.doi.org/10.3390/molecules21050629
25. Xiao X, He H, Ding X, Yang Q, Liu X, Liu S, Rang J, Wang T, Zuo M, Xia L. Purification and Cloning of Lectin that Induce Cell Apoptosis from Allium chinense. Phytomedicine. 2015;22: 238-244.
    Available:http://dx.doi.org/10.1016/j.phymed.2014.12.004
26. Silvia MCC, Santana LA, Mentele R, Ferreiraa RS, de Miranda A, Silva-Luccac RA, Sampaioa MU, Cor-reiad MTS, Oliva MLV. Purification, Primary Structure and Potential Functions of a Novel Lectin from Bauhinia forficata Seeds. Process Biochemistry. 2012;47:1049-1059.
    Available:http://dx.doi.org/10.1016/j.procbio.2012.03.008
27. Bashir H, Khan T, Masood A, Hamid R. Isolation, Purification and Characterization of a Lectin from a Local Kashmiri Variety of Soybean (Glycine Max). Asian Journal Biochemistry. 2010;5: 145-153.
    Available:http://dx.doi.org/10.3923/ajb.2010.145.153
28. Gomes FS, Procópio TF, Lima TA, Napoleão TH, Coelho LCB, Paiva PMG. Isolation and Antimicrobial Activity of Lectin from Schinus terebinthifolius Leaves. Journal of Biotechnology. 2010;150:453.
    Available:http://dx.doi.org/10.1016/j.jbiotec.2010.09.661
29. Laija SN, Mahesh S, Smitha LS, Remani P. Isolation and Partial Characterization of Two Plant Lec-tins. Current Research Journal of Biological Sciences. 2010;2:232-237.
    Available:http://www.maxwellsci.com/print/crjbs/v2-232-237.pdf
30. Silva MC, Corrêa AD, Donizete dos Santos C, Marcos FCA, Patto de Abreu CM. Partial Purifica-tion of Leaf Lectin from Manihot esculenta and Screening Its Fungicidal Activity. Journal of Agricultural Biotechnol-ogy and Sustainable Development. 2010;2:136-141.
    Available:http://www.isabb.academicjournals.org/article/article1379332438_Silva%20et%20al.pdf
31. Lin P, Ye X, Ng T. Purification of Melibiose-Binding Lectins from Two Cultivars of Chinese Black Soybeans. Acta Biochimica et Biophysica Sinica. 2008;40:1029-1038.
    Available:http://dx.doi.org/10.1111/j.1745-7270.2008.00488.x
32. Chen HP, Xu LL. Isolation and characterization of a novel chitosan-binding protein from non-heading chinese cabbage leaves. Journal of Integrative Plant Biology. 2005;47:452-456.
    Available:http://dx.doi.org/10.1111/j.1744-7909.2005.00022.x
33. Kaur A, Singh J, Kamboj SS, Sexana AK, Pandita RM, Shamnugavel M. Isolation of an N-Acetyl-D-Glucosamine Specific Lectin from the Rhizomes of Arundo donax with Antiproliferative Activity. Phytochemistry. 2005;66:1933-1940.
    Available:http://dx.doi.org/10.1016/j.phytochem.2005.06.026
34. Almanza M, Vega N, Pérez G. Isolating and Characterizing a Lectin from Galactialindenii Seeds That Recognizes Blood Group H Determinants. Archives of Biochemistry and Biophysics. 2004;429:180-190.
    Available:http://dx.doi.org/10.1016/j.abb.2004.06.010
35. Kuku A, Odekanyin O, Adreniran K, Adewusi M, Olonade T. Purification of a Mannose/Glucose-Specific Lectin with Antifungal Activity from Pepper Seeds (*Capsicum annuum*). African Journal of Biochemistry Re-search. 2009;3,272-278.
    Available:http://www.researchgate.net/publication/228472330_Purification_of_a_mannoseglucose-specific_lectin_with_antifung
36. Carvalho AdS, Da Silva MV, Gomes FS, Paiva PMG, Malafaia C., Da Silva TD, Vaz AFdM, He S, da Silva AG, Arruda IRdS, Napoleao TH, Carneiro-da-Cunha MdG, Correia MTdS.

Purification, Characterization and Antibacterial Potential of a Leectin Isolated from Apuleia leiocarpa seeds. International Journal of Biological Macromolecules. 2015;75:402-408.
Available:http://dx.doi.org/10.1016/j.ijbiomac.2015.02.001

37. He S, Shi J, Walid E, Zhang H, Ma Y, Xue SJ. Reverse micellar extraction of lectin from black turtle bean (Phaseolus vulgaris): Optimisation of Extraction Conditions by Response Surface Methodology. Food Chemistry. 2015;166:93-100.
Available:http://dx.doi.org/10.1016/j.foodchem.2014.05.56
38. Simpson BJ. A Field Guide to Texas Trees. Lone Star Books, Houston; 1999.
39. Tull D, Miller GO. A Field Guide to Wildflowers, Trees and Shrubs of Texas. Lone Star Books, Houston; 1999.
40. Bradford MM. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utiliz-ing the Principle of Protein-Dye Binding. Analytical Biochemistry. 1976;72:248-254.
Available:http://dx.doi.org/10.1016/0003-2697(76)90527-3
41. Naeem A, Saleemuddin M, Hasan Khan R. Glycoprotein targeting and other applications of lectins in biotechnology. Current Protein and Peptide Science. 2007;8:261-271.
Available:http://dx.doi.org/10.2174/138920307780831811
42. Muramoto K, Kado R, Takei Y, Kamiya H. Seasonal Changes in the Multiple Lectin Compositions of the Acorn Barnacle Megabalanus rosa as Related to Ovarian Development. Comparative Biochemistry and Physiology. 1991;98:603-607.
43. Nsimba-Lubaki M, Peumans WJ. Seasonal Fluctuations of Lectins in Barks of Elderberry (Sambucus ni-gra) and Black Locust (*Robinia pseudoacacia*). Plant Physiology. 1986;80:747-751.
Available:http://dx.doi.org/10.1104/pp.80.3.747
44. Costa RMP, Vaz AFM, Oliva MLV, Coelho LCB, Correia MTS, Carneiro-da-Cunha MG. A New Mistletoe Phthirusa pyrifolia Leaf Lectin with Antimicrobial Properties. Process Biochemistry. 2010;45:526-533.
Available:http://dx.doi.org/10.1016/j.procbio.2009.11.013
45. Drickamer K. Multiplicity of Lectin-Carbohydrate Interactions. Nature Structural Biology. 1995;2: 437-439.
Available:http://dx.doi.org/10.1038/nsb0695-437
46. Drickamer K, Taylor E. Glycan Arrays for Functional Glycomics. Genome Biology. 2002;3, Reviews1034.1-Reviews1034.4.
Available:http://genomebiology.com/2002/3/12/reviews/1034
47. Khan MMH, Rahman ATM, Uddin MS, Khatun S, Pervin F, Absar N. Purification and Characterization of Lectins from Jute (*Chorchorus olitorius*) Leaves. Journal of the Chinese Chemical Society. 2008;55:1171-1177.
Available:http://dx.doi.org/10.1002/jccs.200800173
48. Wu AM, Lisowska E, Duk M, Yang Z. Lectins as Tools in Glycoconjugate Research. Glycoconjugate Journal. 2009;26:899-913.
Available:http://dx.doi.org/10.1007/s10719-008-9119-7
49. Kusui K, Takasaki S. Structural Study of N-Linked Sugar Chains of Sheep Erythrocyte Membrane Glyco-proteins. Glycoconjugate Journal. 1998;15:3-10.
Available:http://dx.doi.org/10.1023/A:1006967614009
50. Shibuya N, Goldstein IJ, Van Damme EJM, Peumans WJ. Binding properties of a mannose-specific lectin from the snowdrop (*Galanthus nivalis*) Bulb. Journal of Biological Chemistry. 1988;263:728-734.
Available:http://www.jbc.org/content/263/2/728
51. Loris R. Principles of Structures of Animal and Plant Lectins. Biochimica et Biophysica Acta. 2002;1572:198-208.
Available:http://dx.doi.org/10.1016/S0304-4165(02)00309-4

52. Sacchettini JC, Baum LG, Brewer CF. Multivalent Protein-Carbohydrate Interactions. A New Para-digm for Supermolecular Assembly and Signal Transduction. Biochemistry. 2001;40:3009-3015.
Available:http://dx.doi.org/10.1021/bi002544j
53. Konozy EHE, Mulay R, Faca V, Ward RJ, Greene LJ, Roque-Barriera MC, Sabharwal S, Bhide SV. Purification, Some Properties of a D-Galactose-Binding Leaf Lectin from Erythrina indica and Further Charac-terization of Seed Lectin. Biochimie. 2002;84:1035-1043.
Available:http://dx.doi.org/10.1016/S0300-9084(02)00003-2
54. Moreira RA, Castelo-Branco CC, Monteiro ACO, Tavares RO, Beltramini LM. Isolation and Partial Characterization of a Lectin from Artocarpus incisa L. Seeds. Phytochemistry. 1998;47, 1183-1188.
Available:http://dx.doi.org/10.1016/S0031-9422(97)00753-X
55. Han JW, Jung MG, Kim MJ, Yoon KS, Lee KP, Kim GH. Purification and characterization of a d-mannose specific lectin from the green Marine Alga, Bryopsis plumosa. Phycology Research. 2010;58:143-150.
Available:http://dx.doi.org/10.1111/j.1440-1835.2010.00572.x
56. Ugwu SO, Apte SP. The Effect of Buffers on Protein Conformational Stability. Pharmaceutical Technology. 2004;28:86-108.
57. Utarabhand P, Akkayanont P. Purification of a Lectin from Parkiajavanica Beans. Phytochemistry. 1995;38:281-285.
Available:http://dx.doi.org/10.1016/0031-9422(94)00550-D
58. Sureshkumar T, Priya S. Purification of a Lectin from M. rubra Leaves Using Immobilized Metal Ion Af-finity Chromatography and Its Characterization. Applied Biochemistry and Biotechnology. 2012;168:2257-2267.
Available:http://dx.doi.org/10.1007/s12010-012-9934-y
59. Salles HO, Vasconcelos IM, Santos LFL, Oliveira HD, Costa PP, Nascimento NRF, Santos CF, Sousa DF, Jorge ARC, Menezes DB, Monteiro HSA, Gondim DMF, Oliveira JTA. Towards a Better Understanding of Ipomoea asarifolia Toxicity: Evidence of the Involvement of a Leaf Lectin. Toxicon. 2011;58:502-508.
Available:http://dx.doi.org/10.1016/j.toxicon.2011.08.011
60. Wu J, Wang J, Rao P. Lunatin, a Novel Lectin with Anitfungal and Antiproliferative Bioactivities from Phaseolus billb. AInternational Journal of Biological Macromolecules. 2016;89:717-724.
Available:http://dx.doi.org/10.1016/j.ijbiomac.2016.04.092
61. Hung LD, Hori K, Nang HQ, Kha T, Hoa LT. Seasonal Changes in Growth Rate, Carrageenan Yield and Lectin Content in the Red Alga Kappaphycus alvarezii Cultivated in Camranh Bay, Vietnam. Journal of Applied Phycology. 2009;21:265-272.
Available:http://dx.doi.org/10.1007/s10811-008-9360-2
62. Gonzalez de Mejía E, Maldonado SHG, Gallegos JAA, Camacho RR, Rodriguez ER, Hernandez JLP, Chavira MMG, Castellanos JZ, Kelly JD. Effect of Cultivar and Growing Location on the Trypsin In-hibitors, Tannins, and Lectins of Common Beans (Phaseolus vulgaris L.) Grown in the Semiarid Highlands of Mexico. Journal of Agricultural Food Chemistry. 2003;51, 5962-5966.
Available:http://dx.doi.org/10.1021/jf030046m
63. United States Department of Agriculture. Natural Resources Conservation Service,. Texas Online Soil Survey Manuscript, Webb County; 1985.
64. Singh SS, Wang H, Chan YS, Pan W, Dan X, Yin CM, Akkouh O, Ng TB. Lectins from Edible Mushrooms. Molecules. 2015;20:446-469.
65. Vandenborre G, Smagghe G, Van Damme EJM. Plant Lectins as Defense Proteins against Phytophag-ous Insects. Phytochemistry. 2011;72:1538-1550.
Available:http://dx.doi.org/10.1016/j.phytochem.2011.02.024
66. Ratanapo S, Ngamjunyaporn W, Chulavatnatol M. Interaction of a Mulberry Leaf Lectin with a Phyto-pathogenic Bacterium, P. syringaepvmori. Plant Science. 2001;160:739-744.
Available:http://dx.doi.org/10.1016/S0168-9452(00)00454-4
67. Ayouba A, Causse H, Van Damme EJM, Peumans WJ, Bourne Y, Cambillau C, Rougé P. Interactions of Plant Lectins with the Components of the Bacterial Cell Wall Peptidoglycan. Biochemical Systematics and Ecology. 1994;22:153-159.
Available:http://dx.doi.org/10.1016/0305-1978(94)90005-1

68. Albuquerque LP, Santana GMS, Melo AMA, Coelho LCB, Silva MV, Paiva PMG. Deleterious Effects of Microgramma vaccinifolia Rhizome Lectin on Fusarium Species and Artemiasalina. Journal of Biotechnology. 2010;150:S453.
Available:http://dx.doi.org/10.1016/j.jbiotec.2010.09.662
69. Hossain MA, Maiti MK, Basu A, Sen S, Ghosh AK, Sen SK. Transgenic Expression of Onion Leaf Lectin Gene in Indian Mustard Offers Protection against Aphid Colonization. Crop Science. 2006;46:2022-2032.
Available:http://dx.doi.org/10.2135/cropsci2005.11.0418
70. Yildirim I, Turhan H, Özgen B. The Effects of Head Rot Disease (*Rhizopus stolonifer*) on Sunflower Genotypes at Two Different Growth Stages. Turkish Journal of Field Crops. 2010;15: 94-98.
Available:http://www.field-crops.org/assets/pdf/product513202a2be6f4.pdf
71. Wong JH, Ng TB. Isolation and Characterization of a Glucose/Mannose/Rhamnose-Specific Lectin from the Knife Bean Canavalia gladiata. Archives of Biochemistry and Biophysics. 2005;439:91-98.
Available:http://dx.doi.org/10.1016/j.abb.2005.05.004
72. Ye XY, Ng TB, Tsang PWK, Wang J. Isolation of a Homodimeric Lectin with Antifungal and Anti-viral Activities from Red Kidney Bean (*Phaseolus vulgaris*) Seeds. Journal of Protein Chemistry. 2001;20:367-375.
Available:http://dx.doi.org/10.1023/A:1012276619686
73. Melo FR, Benevides NMB, Pereira MG, Holanda MK, Mendes FNP, Oliveira SRM, Freitas ALP, Silva LMC. Purification and Partial Characterization of a Lectin from the Red Marine Alga Vidalia obtusiloba C. Agardh. Brazilian Journal of Botany. 2004;27:263-269.
Available:http://dx.doi.org/10.1590/S0100-84042004000200006

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She pursued a B.S. Biology degree at Texas A&M International University. After having graduated with a B.S. in Biology she continued on to earn her Master's Degree. As the recipient of TAMIU's Lamar Bruni Vergara Graduate Assistantship, she embraced the challenge of her thesis entitled "Partial Purification and Characterization of Texas Live Oak (*Quercus fusiformis*) Lectin". Throughout the process, she learned the importance of scientific research and literature, and gained invaluable skills applicable later in life. The result of her hard work was recognition in an article in the peer-reviewed journal *Advances in Bioscience and Biotechnology*. Afterwards, she began a career as a secondary science teacher in Laredo's United Independent School District. While there, she built rapport with her students and challenged them to be resourceful and to think outside the box. As a volunteer sponsor for the Environmental Club, she taught students about community service and the importance of stewardship of natural resources. After five dedicated years, she moved to the Laredo Independent School District where she was recognized for her efforts utilizing technology resources in the classroom. The honor received gave her the impetus to challenge herself professionally and change career from classroom teacher to Digital Learning Specialist.

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***Chapter 9***
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# Experiments on Removal of Inorganic Anions in Brewery Effluent Using Banana Peels

**J. N. Okereke[1*], O. I. Ogidi[2] and K. O. Obasi[3]**

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## ABSTRACT

Of the various water pollution problems, effluents from industries are major contributors. A research aimed at ascertaining the removal efficiency of anions (Cl, $SO_4$, $PO_4$, $NO_3$ ) and N in brewery industry, using ripe and unripe *Musa sapientum* (banana) peels at three different concentrations (1 g, 2 g and 4 g), was carried out. Basic laboratory protocols involving the use of Spectrophotometric methods were adopted to determine chemical properties of the effluent samples. The outcome of this work revealed that the concentrations of the inorganic anions of the treated brewery effluent sample were observed to decline compared to the untreated effluent sample. This research work shows that banana peels are effective precursors for the removal of inorganic anions from brewery effluent.

*Keywords: Banana peels; brewery effluent; inorganic anions; removal efficiency*.

## 1. INTRODUCTION

Presently, the world is populated by over seven billion people [1]. One of the most challenging problems of developing nations is ineffective management of vast volume of wastes generated from different industrial activities. More challenging is the unsafe disposal of effluents into the ambient environment. Effluents produced in various industries constitute potential hazards to our environment as they introduce various contaminants.

Of major contributors to various water pollution problems, are industrial effluents. Among these problems include eutrophication, which can catalyze the growth of algae, increased water purification cost, interference with the recreational value of water, health risks to humans and livestock, excessive loss of oxygen and undesirable changes in aquatic populations [2]. Water pollution as a result of phosphorus and nitrogen compounds has been a major source of eutrophication, thereby raising some concerns for environmentalists and environmental engineers. Also, several damages to the quality of receiving water, is due to effluent discharges from industrial and domestic wastewaters [3].

There is greater environmental awareness on the need to treat wastewater effluent before discharging into receiving water bodies in the recent years. This has led to the designing of standard scientific protocols for the removal of contaminants from wastewater [4]. Emphasis is placed on the treatment of industrial wastewater effluent since local and international authorities require that wastewaters from industries be treated and made to meet set standards before they are discharged into the water bodies. The need of safe and economical methods for the removal of inorganic ions and other contaminants in waters has developed interest towards the search for low cost treatment alternatives. There is therefore need for agro-based, in-expensive adsorbents to be explored and their feasibility to remove these contaminants, studied [5].

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Recently, a lot of natural products, usually referred to as wastes, such as plantain peels, peanut shells, corn cob, coconut shell, sugar cane bagasse and apple waste, have been reported to be used in the removal of inorganic ions [6,7]. However, [8] suggested other devices in purifying water in homes and industries.

Paucity of information exists on the removal efficiency of inorganic anions from brewery industry using ripe and unripe *Musa sapientum* (banana) peels that necessitated this investigation.

## 2. MATERIALS AND METHODS

### 2.1 Collection of Industrial Effluent Samples

Brewery effluent samples for this research were collected from the outlet of Pabod Breweries Ltd, Oginigba, Trans-Amadi, Port Harcourt using sterile 4-litre plastic container.

### 2.2 Preparation of Bio-Sorbent Precursor

Ripe and unripe bananas used were sourced from Swali Market in Yenagoa, Bayelsa State, Nigeria. The bananas were washed in tap water to remove sand and other debris and were peeled. The peels were sun-dried for 8days. They were washed again after sun-drying to remove the dirt adhered during the sun-drying process. Wetted biomass was air-dried to remove free water from the surface and oven-dried for 4 hours at 50°C. Separately, the crispy peels were pulverised into fine powder using a sterilized Milling Machine. The pulverised peels were sieved through a 0.25 mm sieve size and stored in air-tight bottle prior to the laboratory investigations at room temperature.

### 2.3 Experimental Procedure

Three separate concentrations of the pulverized peels were used. Respectively, 1 g, 2 g and 4 g, was mixed with 400 ml of the effluent per concentration. The sample placed in the conical flask apparatus were stirred at 120rpm speed using rotary shaker at room temperature to obtain homogenous mixture for 2 hours. When 2 hours of mixing has elapsed, the sample was allowed to settle for 10 hours and filtered using Whatman no.1 filter paper to remove the biosorbent precursors. This was followed by the analyses of the respective inorganic anions.

### 2.4 Determination of Inorganic Anions

Using the standard protocols described by [9], inorganic anion concentrations were determined.

#### 2.4.1 Chloride (Argentometric method)

Measured volume (50 ml) of the wastewater sample was put in conical flask and 3–4 drops of indicator potassium chromate solution and standard silver nitrate solution slowly added from the burette and the solution shaken well. At the end point, light yellow colour is sustained. The titration was repeated until a concordant volume was obtained.

#### 2.4.2 Phosphate (Ascorbic acid method)

Some quantity of effluent (50 ml) was measured into an acid-cleaned, dry 125 ml Erlenmeyer flask and 1 drop of phenolphthalein indicator added. When red color developed, 8 ml of 5N sulfuric acid was added until the color disappeared. The mixture was allowed at least 10 minutes (but not more than 30 minutes) for color development. Absorbance at 880 nm was measured using a reagent blank to zero the spectrophotometer. The reagent blank was made using 50 ml of distilled water carried through the digestion step and ascorbic acid procedure. The final concentration of phosphate was determined using the following equation: Mg/l P = mg/l from the curve x 50 ml divided by initial volume used (ml).

### 2.4.3 Nitrate–nitrogen (Colorimetric method)

Through a copper-coated Cadmium reduction column, effluent sample was passed. Recall that Nitrate in the sample was reduced to nitrite in a buffer solution. The nitrite was then determined by diazotizing with sulfanilamide and coupling with N-1- naphthylethylene diaminedihydrochloride to form a colorazo dye. The absorbance was measured at 540 nm which is linearly proportional to the concentration of 'nitrite + nitrate' in the sample. Nitrate concentrations were obtained by subtracting nitrite values, which have been separately determined without the cadmium reduction procedure, from the 'nitrite + nitrate' values.

### 2.4.4 Sulphate (Turbidometric method)

Into a clean and acid-washed beaker with 50 ml of buffer solution, deionised water (100 ml) was poured and then transferred into a clean, 125-ml Erlenmeyer flask containing a clean magnetic stirring bar. Measured quantity (10 ml) of deionised water, 6 ml of buffer reagent, and 10 ml of the standard solution were added into the flask. This was swirled gently to ensure mixing. About 0.1 g - 0.2 g of $BaCl_2$ was added to the flask which was immediately placed on the magnetic stirrer and stirred for 58 to 62 seconds. After a minute of stirring, the solution was allowed to stand undisturbed for about 2 minutes. The absorbance was measured by a spectrophotometer at the wavelength of 420 nm and sulphate concentration determined by comparison of the reading with a standard curve.

## 2.5 Efficiency Calculation

Removal efficiency of inorganic anions by banana peels was mathematically calculated. This was done by subtracting the initial concentration from the final concentration, multiplied by 100 and, divided by the initial concentration.

## 3. RESULTS AND DISCUSSION

## 3.1 Analysis of Inorganic Anions

Mean inorganic anions (Cl, $SO_4$, $PO_4$, $NO_3$ and N) concentrations of untreated brewery effluent samples were within the range of 5.34 mg/l – 547 mg/l with $NO_3$ recording highest; $PO_4$, lowest. After treatment, their concentrations decreased within the range of 516 mg/l – 3.34 mg/l, (Tables 1 & 2).

**Table 1. Mean results for inorganic anions of brewery effluent before and after treatment with ripe banana peels**

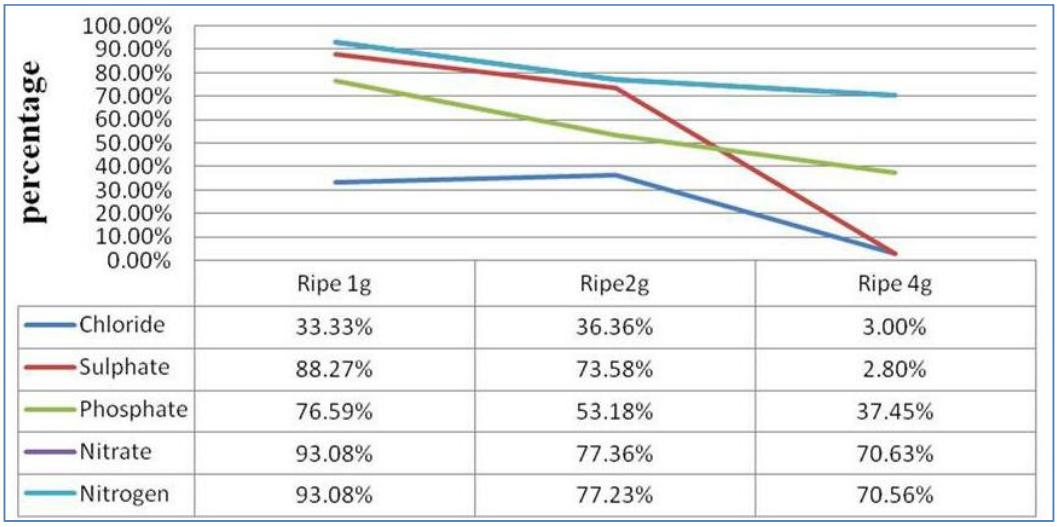
| Inorganic anions | Before treatment | After treatment | | |
|---|---|---|---|---|
| | | ripe (1 g) | ripe (2 g) | ripe (4 g) |
| Chloride (ppm) | 468.60 | 312.40 | 298.20 | 454.40 |
| Sulphate (ppm) | 348.70 | 40.10 | 92.10 | 338.80 |
| Phosphate (ppm) | 5.34 | 1.25 | 2.50 | 3.34 |
| Nitrate (ppm) | 547.00 | 37.80 | 123.80 | 160.60 |
| Nitrogen (ppm) | 123.00 | 8.50 | 28.00 | 36.20 |

**Table 2. Mean results for inorganic anions of brewery effluent before and after treatment with unripe banana peels**

| Inorganic anions | Before treatment | After treatment | | |
|---|---|---|---|---|
| | | unripe (1 g) | unripe (2 g) | unripe (4 g) |
| Chloride (ppm) | 468.60 | 397.60 | 369.20 | 440.20 |
| Sulphate (ppm) | 348.70 | 39.80 | 85.80 | 240.10 |
| Phosphate (ppm) | 5.34 | 2.23 | 2.90 | 2.50 |
| Nitrate (ppm) | 547.00 | 516.00 | 25.80 | 0.00 |
| Nitrogen (ppm) | 123.00 | 116.80 | 5.80 | 0.00 |

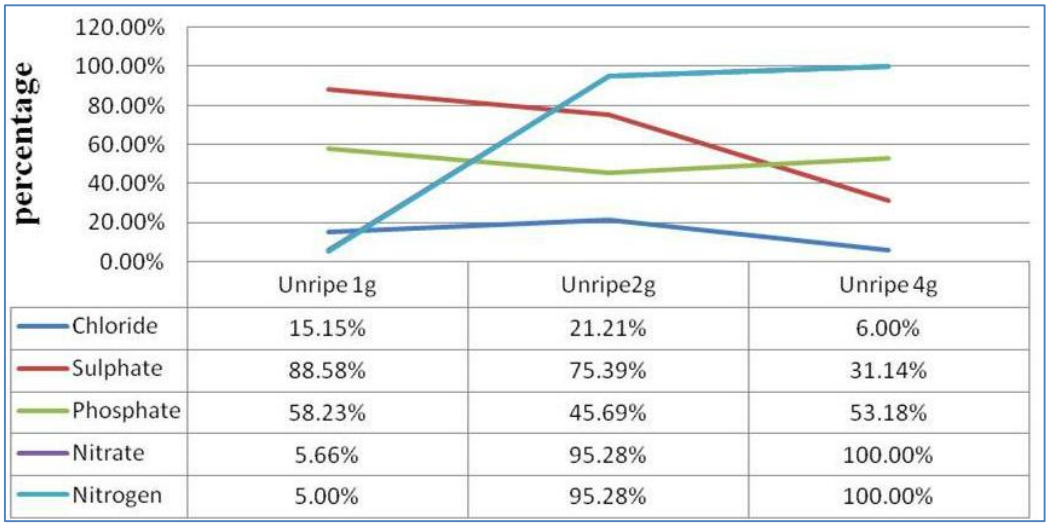
## 3.2 Efficiency Calculation

Efficiency of percentage removal of inorganic anions on the effluent samples was calculated. The result showed that nitrate and nitrogen had the highest percentage removal with both ripe and unripe banana peels on the effluent sample (Figs. 1&2).




| | Ripe 1g | Ripe2g | Ripe 4g |
|---|---|---|---|
| Chloride | 33.33% | 36.36% | 3.00% |
| Sulphate | 88.27% | 73.58% | 2.80% |
| Phosphate | 76.59% | 53.18% | 37.45% |
| Nitrate | 93.08% | 77.36% | 70.63% |
| Nitrogen | 93.08% | 77.23% | 70.56% |

**Fig. 1. Efficiency of percentage removal of inorganic anions in brewery effluent sample usig ripe banana peels**




| | Unripe 1g | Unripe2g | Unripe 4g |
|---|---|---|---|
| Chloride | 15.15% | 21.21% | 6.00% |
| Sulphate | 88.58% | 75.39% | 31.14% |
| Phosphate | 58.23% | 45.69% | 53.18% |
| Nitrate | 5.66% | 95.28% | 100.00% |
| Nitrogen | 5.00% | 95.28% | 100.00% |

**Fig. 2. Efficiency of percentage removal of inorganic anions in brewery effluent sample using unripe banana peels**

## 4. DISCUSSION

To avoid its negative effects on health and environment, Chloride concentration must be finely tuned to the minimal inhibitory concentration (0.7 ppm) [10]. However, untreated and treated brewery effluent samples had Chloride levels below (NEMA) standard. The results obtained are synonymous with the findings of other researchers like [8].

Sulphate anion plays a very vital role in hardness of water. It may undergo transformation to sulphur or hydrogen sulphide depending largely upon theredox potential of water. Hydrogen sulphide in water results from the decomposition of organic matter or bacterial reduction of sulphate under anaerobic condition [11]. In the present investigation, the mean concentration of sulphate ranged from 39.8 to 348.7 mg/l and average value was found to be 70 mg/L, which is about 25 times higher than EPA standards. This results obtained may be as a result of the assertion made above.

On the other hand, Phosphate can be present as dissolved or particulate matter. It is an essential plant nutrient and is often the most limiting nutrient to plant growth in fresh water. They enter waterways from human and animal wastes, phosphate rich bedrock, wastes from laundry cleaning and industrial processes, and fertilizer runoff [12]. If too much phosphate is present, algae and water weeds grow wildly, choke the water way and use up large amount of oxygen resulting into death of aquatic organisms due to dissolved oxygen depletion. According to [13], it is not possible to find a high phosphate reading if the algae are already blooming, as the phosphates will already be in the algae but not in water. However, phosphate is an essential nutrients to plants life, but when found in excess quantities, stimulates excessive plant growth such as algae bloom [14]. Although, both untreated and treated effluent samples are below the [15] permissible limits (10 mg/l).

The discharge of nitrate presents several water quality concerns. Nitrate contributes to aging (eutrophication) of water bodies, which eventually leads to depletion of dissolved oxygen levels. In cases of direct or indirect potable reuse, nitrate is a potential health hazard. Nitrates in drinking water are associated with blue baby syndrome, and the EPA has set treatment standards [16]. Unpolluted water usually contain only minute amount of nitrate [17]. Elevated levels in nitrate have been reported to exhibit delayed reactions to light and sound stimuli and can cause methaemogloobinemia [18,19]. Some of the results both untreated and treated brewery effluent sample are above EPA standard which implies that the conditions stated above, can result if untreated effluents from breweries are discharged into water bodies.

Some of the mean results both untreated and treated of the effluent samples are above [15] permissible limits. The high value of Nitrogen in the effluent sample is probably due to the use of food preservatives containing nitrogenous compound and presence of organic pollutants rich in nitrogen content in the effluents from the industry. Nitrogen is an essential plant element and is often the limiting nutrient in marine waters. The major routes of entry of nitrogen into bodies of water are municipal and industrial wastewater; private sewage disposal systems, decaying plant debris and discharge from car exhausts [20]. Effluents from brewery industry are generally rich in protein content [21]. Nitrogen may cause aquatic biological productivity to increase, resulting in low dissolved oxygen and eutrophication of lakes, rivers, estuaries, and marine waters [13].

## 5. CONCLUSION

From this work, banana peels (ripe and unripe) are effective precursors in the removal of inorganic anions from brewery effluent. It is worthy of note that the cost implication of using conventional methods of inorganic anions removal from brewery effluent is high compared to using low cost agro waste (banana peels) which is economical, cost effective and eco-friendly. We recommend that banana peels be used for inorganic anions removal from industrial effluents.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

## REFERENCES

1. Cable Network News (CNN) 13th February, Comment on the World Population Growth; 2011.
2. Akpor O, Muchie M. Environmental and Public Health Implications of Wastewaters Quality. African Journal of Biotechnology. 2011;10(13):2379-2387.

3. Surchi K. Agricultural wastes as low cost adsorbents for Pb removal: Kinetics Equilibrium and Thermodynamics. International Journal of Chemistry. 2011;3(3):103-112.
4. Zahra N. Lead removal from water by low cost adsorbents: A review. Pakistan Journal of Analytical and Environmental Chemistry. 2012;13(1):1-8.
5. Uttam S, Rajesh K. A review: Treatment of wastewater with low cost adsorbent. VSRD International Journal of Technology and Non-Technical Research. 2013;4:3.
6. Namasivayam C, Sangeetha D. Recovery of nitrate from wastewater by $ZnCl_2$ activated carbon from coconut coir pith, an agricultural solid waste. Indian Journal of Chemical Technology. 2005;12:513-521.
7. Castro R, Castano L, Ferreira G, Pedro M, Padilha P, Margarida J, Saeki M, Zara L, Marco, A., Martines A, Castro G. Banana peel applied to the solid phase extraction of Copper and Lead from river water: Pre - concentration of metal ions with a fruit waste. Industrial and Engineering Chemistry Research. 2011;50:3446-3451.
8. Okereke JN, Nnoli MC, Okereke CBN, Ahumibe NC. Efficiency of Water-purifying Devices used in Homes and Industries, Journal of Research in Ecology. 2013;2(2):108–114.
9. APHA. Standard Methods for the Examination of water and wastewater. 18th Edition. American Public Health Association, Washington, DC. 1998;45-60.
10. Virto R, Sanz D, Ivarez I, Condon S, Raso J. Relationship between inactivation kkinetics of a Listeria monocytogenes suspension by chlorine and its chlorine demand, J. of Applied Microbiol. 2004;97:1281-1288.
11. Metcalf, Eddy. WasteWater Engineering Treatment; Disposal and Reuse, Tata McGraw-Hill Publishing Company Limited, New Delhi; 1999.
12. Mosley L, Sarabject S, Aalbersborg B. Water Quality Monitoring in Pacific Island Counties. Handbook forwater quality. Suva-Fiji Island. The University of South Africa Pacific; 2004.
13. Perry RH, Green DW, Maloney JO. Perry's Chemical Engineers Handbook. 7$^{th}$ Ed. New York. McGraw-Hill; 2007.
14. Igbinosa E, Oko A. Impact of discharge wastewater effluents on the physiscochemical qualities of a receiving watershed in a typical rural community. Int. J. Environ. Sci. Tech. 2009;6(2):175-182.
15. NEMA. The Uganda National Environment (Standards for Discharge of effluent into water or land) Regulations, Kampala, Uganda; 1999.
16. Powlson D, Addiscott T, Benjamin N, Cassman K, De-Kok T, Van- Grinsven H, L'Hirondel L, Avery A, Van- Kessel C. When does nitrate become a risk for humans? Journal of Environmental Quality. 2008;37(2):291-295.
17. Jaji M, Bamgbose O, Arowolo T, Odukoya O. Water quality assessment of Ogun River, south west Nigeria. Environ. Monit. Assess. 2007;133:447–482.
18. Fatoki S, Ogumfowokan A. Pollution assessment in the Keiskamma River and in the impoundment downstream. Water SA. 2008;29(3):183-187.
19. Robillard P, Sharpe W, Wistock B. Nitrates in Drinking Water. Pennsylvania State kUniversity. Agric Biology Eng; 2003.
20. USEPA. Wetland Trends in Michigan since 1800: A preliminary Assessment; 1986.
21. Meertens H, Ndege L, Enserink H. Dynamics in farming systems: Changes in time and space in Sukumaland, Tanzania. Royal Tropical Institute/Amsterdam. 1995;48-60.

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***Chapter 10***
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# Experiment for the Determination of Acute Toxicity of Ammoniain Juvenile Patagonian Blenny (*Eleginops maclovinus*)

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## ABSTRACT

Deionized ammonium ($NH_3$) acute toxicity (LC50-96h) in Patagonian blenny juveniles (*Eleginops maclovinus*) was assessed. Concentrations of deionized ammonium in salt water were prepared by using 24.09 ± 2.1 g ammonium chloride ($NH_4Cl$). Fish were exposed in triplicates to different ammonium concentrations: 0.05; 0.094; 0.175; 0.325 and 0.605 mg $NH_3$ $L^{-1}$. Additionally, a control group was included. Experimental fish were kept at a photoperiod of 16:8h. Average temperatures were 16.24 ± 1.40°C. Oxygen concentration was 7.16 ± 0.40 mg $L^{-1}$. Water pH was 7.89 ± 0.2. LC50-96 h, was estimated by using Probit statistical method (95% intervals) using EPA software (1993). Juveniles of *E. maclovinus* showed a LC50-96h of 0.413 mg $NH_3$ $L^{-1}$ value, different from most marine species. This study presents the first record of ammonia toxicity in marine species of Chile.

*Keywords: Acute toxicity; Eleginops maclovinus; LC50-96h; ammonia.*

## 1. INTRODUCTION

Altough the salmon and trouts are the main exportation products from Chilean aquaculture, the urgent aquaculture diversification of Chilean fish farming is necessary. Currently, the Chilean aquaculture production has a 98% FOB in salmon farming, and only the 3.2% includes other species. The "Patagonian blenny" (*Eleginops maclovinus*, Georges Cuvier, 1830) is an endemic species with high potential for aquaculture production [1,2,3].

The *E. maclovinus* belongs to Perciformes order and it is distributed in Pacific Ocean from Valparaiso (33° S) to Tierra del Fuego island (54° S) and in the Atlantic Ocean until Buenos Aires province (39° S) [4], and was reported around Falkland Islands [5,6]. It is a prothandric hermaphrodite with sexual reversion in function of its corporal size and age, under intraspecific competence [7]. It is an omnivorous species and its diet varies according to geography, substrate and season [8].

An intensive production system requires adequate environmental conditions that promote growth and development of all organisms and in this context it is necessary for each species such as dissolved oxygen, carbon dioxide and total ammonium (TAN) that determine the success of the fish farming activities [9,10,11,12]. The total ammonium is the total sum of ammonium ($NH_4$+) and ammoniac

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($NH_3$), the latter is considered a product of body protein and nucleic acid biochemical degradation [13,14,15,16]. The ammoniac tolerance in freshwater fishes varied between 0.068 and 2.00 mg $NH_3$ $L^{-1}$ and between 0.09 and 3.35 mg $NH_3$ $L^{-1}$ in marine fishes [16]. In teleost fishes the nitrogen is normally eliminated through gills and urine [17] Yu). The pH is affected by the presence of ammonium ion or ammoniac [18].

Ammonium is a toxic product of fish excretion ($NH_4$<1.5 mg $L^{-1}$), derived from the ammonia in its urine (free ammonia), which in small concentrations is even more toxic ($NH_3$<0.0125 mg $L^{-1}$). A chemical reaction of free ammonia ($NH_3$-N) and ionized ammonia ($NH_3^+$-N) occurs in water, depending on the pH of the water, temperature, and ionic strength. If the pH decreases H + increases and $NH_4$+ is formed, if it increases, $NH_4$ + will release H + and $NH_3$ will form. Above pH 9 ammonia will form, which is more toxic to fish [19].

The previous turbot studies (*Scophthalmus maximus*) showed that this species is less tolerant to normoxia condition (80-85% oxygen saturation), in comparison to tolerance to under hiperoxia condition (110-115% oxygen saturation) [20]. These studies agree with low oxygen environmental level might enhance the ammoniac toxic effects [17]. An evidence of this factor is the depolarization that is produced in gills under ammoniac presence, simultaneously an excessive ATP consumption by the glutamate receptor activation (NMDA). As a result of intoxication, the individuals have convulsions and dead [21,18]. Other ammoniac effects are alterations in cerebellum tissue [22,23]; increment of respiratory and cardiopulmonary function [24], swimming behavior [25] and loss of fish reproductive capacity [26].

The studies of ammonia have been carried out with marine and freshwater fishes. The toxic effects are notoriously different for both groups. Data was obtained from determination of lethal media concentration ($LC_{50}$) that corresponds to the level of 50% of the population; this is the main procedure [27].

The fish sensibility to ammoniac depends on the species, for example, the $LC_{50}$ for *S. maximus* juveniles is from 1.7 to 2.7 mg $L^{-1}$ of ammoniac, and is different compared to 0.2 to 0.7 mg $L^{-1}$ in salmonid juveniles [28]. There is not available information about ammoniac tolerance for “Patagonian blenny” *E. maclovinus*. The aim of the present study is to determine the first ecotoxicological parameters, specifically the acute ammoniac toxicity $LC_{50\text{-}96h}$ in *E. maclovinus* under farming conditions.

## 2. MATERIALS AND METHODS

“Patagonian blenny” *E. maclovinus* juveniles were obtained from Quillaipe marine station of Fundacion Chile (41°31’S; 72°44’W). Fish were acclimated during 14 days period prior the experiment. During this period the fishes were fed ad libitum with salmon pellet, this feeding was suspended 48 hours before the experiment. The procedure used was suggested by Franson (2002). For the $LC_{50\text{-}96h}$ determination fish specimens of an average weight 24.09 ± 2.1 g, were selected and separated in triplicates in a control group, and five experimental groups with five individuals for each experimental unit, and these were exposed to starvation during 48 hours. The acute toxicity assay was carried out in a close recirculating system with 0.015 $m^3$ tanks. Each 12 hour the temperature, pH and oxygen values were measured. The photoperiod used was of 18 hours light: 6 hours dark. Concentrations of deionized ammonium in salt water were prepared by using 24.09 ± 2.1 g ammonium chloride ($NH_4Cl$). Fish were exposed in triplicates to different ammonia concentrations: 0.05; 0.094; 0.175; 0.325 and 0.605 mg $NH_3$ $L^{-1}$. Additionally, a control group was included. The ammoniac and ammonium chloride concentrations were specified in Table 1. Mortality and swimming fish were recorded twice daily and classified as a normal (N), lethargic swimming (L) and dead (†), when no movement in their gills showing no reaction to mechanical stimuli [29]. Ammonia was monitored at 0; 24; 48; 72 and 96 h in according to [30]. To determine LC50-96h, Probit statistical method was used with a range of 95% by the program EPA.

## 3. RESULTS

During the experimental period (96 hr) the temperature varied between 16.24 ± 1.4°C, the dissolved oxygen concentration in 7.16 ± 0.40 mg $L^{-1}$, pH between 7.89 ± 0.20, salinity 33ppm. The utilized fish individuals in the experiment did not show abnormal behaviour and mortality during acclimatation. There was no mortality in T1 group (fish reared in 0.05 mg $L^{-1}$ ammoniac concentration). However at 48 hr after the experiment beginning, fish showed lethargic swim (Table 2); 72 hr after the experimental period, the fish from T1 group showed lethargic swim (Table 2). The group exposed to high ammoniac concentration (0.605 mg $L^{-1}$) did not show mortality during the experimental period (Table 2). 48 hours after the beginning of experiment, the fish showed lethargic swim (Table 2). 48 hours after the beginning of experiment, both groups showed intoxication effects. The first group T1 exposed to low ammoniac concentration (0.050 mg$NH_3$ $L^{-1}$) was not mortality, whereas the T2 (0.094 mg$NH_3$ $L^{-1}$) group had 80% survival, T3 (0.175 mg$NH_3$ $L^{-1}$) had 32% survival, T4 (0.325 mg$NH_3$ L-$^{1}$) had 12% survival, and finally T5 (0.605 mg$NH_3$ $L^{-1}$) had 100% mortality after 96 hours (Table 3). Direct correlation between survival time and $NH_3$ was observed and it showed a low survival under high ammoniac concentration. The $LC_{50\text{-}96h}$ ammoniac calculation, for *E. maclovinus* juveniles was of 0.413 mg$NH_3$ $L^{-1}$ (the limit at 95% was between 0.233 to 0.611 mg$NH_3$ $L^{-1}$), that indicated that the same species has low tolerance to ammoniac concentration in comparison to other marine species.

**Table 1. Concentration ammonium deionized ($NH_3$) and ammonium chloride**

| Treatments | $NH_3$ (mg$L^{-1}$) | $NH_4CL$ (g$L^{-1}$) |
|---|---|---|
| T 1 | 0.050 | 0.500 |
| T 2 | 0.094 | 0.931 |
| T 3 | 0.175 | 1.732 |
| T 4 | 0.325 | 3.224 |
| T 5 | 0.605 | 6.000 |

**Table 2. Swim behaviour of fish individuals during a 96 h period**

| mg$NH_3$ $L^{-1}$ | Treatment | Time | | | | |
|---|---|---|---|---|---|---|
| | | 0 | 24 | 48 | 72 | 96 |
| Control | Control | N | N | N | N | N |
| 0.050 | T1 | N | N | N | L | L |
| 0.094 | T2 | N | N | N | L | L |
| 0.175 | T3 | N | N | N | L | L |
| 0.325 | T4 | N | N | L | L | L |
| 0.605 | T5 | N | L | † | † | † |

*Normal (N), 100% lethargic (L) y 100% mortality (†)*

**Table 3. Survival of *E. maclovinus* juveniles in five deionized ammonium concentration (mg$NH_3L^{-1}$) during 96 h**

| mg$NH_3$ $L^{-1}$ | Treatment | n | Survival (%) |
|---|---|---|---|
| Control | Control | 5 | 100 ± 0.0 |
| 0.050 | T1 | 5 | 100 ± 0.0 |
| 0.094 | T2 | 5 | 80 ± 8.0 |
| 0.175 | T3 | 5 | 32 ± 7.0 |
| 0.325 | T4 | 5 | 12 ± 5.0 |
| 0.605 | T5 | 5 | 0.0 ± 0.0 |

## 4. DISCUSSION

The $LC_{50\text{-}96h}$ for *E. maclovinus* obtained of 0.413 mg$NH_3$ $L^{-1}$ (limited at 95% between 0.233 to 0.611 mg$NH_3$ $L^{-1}$) is comparable with the results obtained for *Menidiaberyllina* that has a $LC_{50}$of 0.61 mg$NH_3L^{-1}$. The tolerancelevel for other marine species are of 2.29 mg$NH_3$ $L^{-1}$ for *Cyprinodon*

*variegatus* and 3.35 for *Gateropus aculeatus* [31]. On the basis of these results it can be considered that *E. maclovinus* has tolerance ranks similar to inland water fishes, such as *Oncorhynchusmykiss* in example it observed a tolerance level of0.291 mg$NH_3$ $L^{-1}$ [32], *Salmotrutta* was of 0.592 mg$NH_3L^{-1}$, *Prosopium williamsoni* was of 0.358 mg$NH_3$ $L^{-1}$, and *Ictalurus punctatis* that was of 0.50 mg$NH_3L^{-1}$ [33].

The ammoniac is considered as the most toxic fraction of TAN that is defined by the sum of ammonium ion and ammoniac [34,35]. The ammoniac effect is related to temperature, salinity, oxygen and pH [36,37], on this basis, the temperature observed in the present study was, within the optimal tolerance level [18], and whereas at salinity level at high NaCl concentration the ammoniac toxic effect is lower [18,37,38]. Also, in increment condition of sodium and calcium in oxygen saturated water the ammoniac effect decreased [20]. The TAN toxic effect in water is regulated mainly by pH, temperature, salinity and oxygen [39,18,10,15]. One pH variation of a 25% can affect significantly the ammoniac concentration [34]. In the experiments it was less than 9, so it would not favor the formation of the most toxic fraction, which is ammonia. This can explain the mortality at low concentration in experimental groups with a pH variation of ± 0.2(Table 2).

Other important topic that has effect on ammoniaconcentration is the feeding activity. Feeding the fish, it increases the ammoniac level in plasm, in comparison to the observed level in water, nevertheless in this experiments the fish survived [40]. These results can explain that the fish has glutamine synthesis capacity. Other important factor is the fish capacity for resist the ammoniac concentration under stress conditions [41], then in this scenario [18], showed that the fish during exhaustive exercise and stress incremented its ammonia production and are more sensible to external ammonia. This can be explained by the cortisol increment that is positively related to the glutamine synthesis.

They reported that in *Rachycentron canadum* juveniles erratic and lethargic swim arised when the fish were exposed to ammoniac concentration of 0.80 $NH_3$ $L^{-1}$ [38]. Similar results to this study were observed in the fifth experimental group exposed to 0.605 $NH_3$ $L^{-1}$, that has lethargic swim after 24 h. This experimental group was followed by fourth treatment (exposed to 0.325 $NH_3$ $L^{-1}$) and third (exposed to 0.175 $NH_3$ $L^{-1}$) after 48 and 72 hr respectively. The *E. maclovinus* acute toxicity observed value was of 0.413 $NH_3$ $L^{-1}$.

## 5. CONCLUSION

This study presents the first record of ammonia toxicity in marine species of Chile. In conclusion these results would serve of basis for the intensive culture of this species.

The results of the present experiments demonstrate that ammonia could be problematic at relatively low levels for the intensive rearing of juvenile *E. maclovinus*; under normal culture conditions the ammonia excreted by *E. maclovinus* will react with sea water and will transform into ammonium ion that is less toxic to fish ($NH_4$<1.5 mg $L^{-1}$), therefore the limit concentration of this ion, particularly for recirculation systems that require the removal of nitrogenous compounds [40].

This study, together with the activation oxygen consumption (when fed), and calculated for their different sizes, are necessary to determine the carrying capacity that should be established for the management of the *E. maclovinus* cultivation.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

## REFERENCES

1. SA R, Gavilán M., Rioseco MJ, Llancabure A, Vargas-Chacoff L, Augsburger A, Bas F. Dietary protein requirement of Patagonian blennie (*Eleginopsmaclovinus*, Cuvier 1830) juveniles. Aquaculture. 2014;428-429,125-134.
   Available:http://dx.doi.org/10.1016/j.aquaculture.2014.02.017.
2. Oyarzun R, Martinez D, Soto-davila M, Muñoz JLP, Dantagnan P, Vargas-and Chacoff L. Effects of ration level on growth performance, body composition, intermediary metabolism and serum parameters in juvenile Patagonian blennie *Eleginops maclovinus*. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology. 2019;230:122-130.
   Available:https://doi.org/10.1016/j.cbpa.2019.01.013
3. Oyarzún R, Paredes R, Saravia J, Morera FJ, Muñoz JLP, Ruiz-Jara I, Mancera JM, Vargas-Chacoff L. Stocking density affects the growth performance, intermediary metabolism, osmoregulation, and response to stress in Patagonian blennie Eleginops maclovinus. Aquaculture. 2020;515:734465.
   Available:https://doi.org/10.1016/j.aquaculture.2019.734565.
4. Calvo J, Morriconi E, RAE GA, Roman NA. Evidence of protandry in a subantarctic notothenid, *Eleginops maclovinus*, (Cuv. and Val., 1830) from the Beaglechannel, Argentina. Journal of Fish Biology. 1992;40(2):157-164.
   Available:http://dx.doi.org/10.1111/j.1095-8649.1992.tb02563.x. CARTER, C.G.
5. Venerus LA, Cedrola PV. Review of marine recreational fisheries regulation in Argentina. Marine Policy. 2017;81:202-2010.
   Available:https://doi.org/10.1016/j.marpol.2017.03.007
6. Anbleyth-Evans J, Araos-Leiva F, Ther Rios F, Segovia-Cortes R, Haussermann V, Aguirre-Muñoz C. Toward marine democracy in Chile: examining aquaculture ecological impacts through common property local ecological knowledge. Marine Policy. 2020;113:1036090.
   Available:https://doi.org/10.1016/j.marpol.2019.103690
7. Licandeo RR, Barrientos CA, González MT. Age, growth rates, sex change and feeding habits of notothenioid fish *Eleginops maclovinus.* Environmental Biology of Fishes. 2006;77(1):51-61.
   Available:http://dx.doi.org/10.1007/s10641-006-9054-z
8. Pavéz H, Pequeño G, Bertrán C, Vargas L. Limnetic Feeding of the “Róbalo” *Eleginops maclovinus* (Valenciennes, 1830) in the Valdivia river, Chile. Interciencia. 2005;30:120-125.
9. Rakocy JE, Losordo TM, Masser MP. Recirculating aquaculture tank production systems and overview of critical considerations. Southern Regional Aquaculture Center Publication. 1992; 454:1-8.
10. Gerber R, Wepener V, Smit NJ. Application of multivariate statistics and toxicity indices to evaluate the water quality suitability for fish of three rivers in the Kruger National Park, South Africa. African Journal of Aquatic Science. 2015;40(3):247-259.
    Available:http://dx.doi.org/10.2989/16085914.2015.1073139
11. Zhou Z, LI H, Song C, Cao X, Zhou Y. Prevalence of ammonia-oxidizing bacteria over ammonia-oxidizing archea in sediments as related to nutrient loading in Chinese aquaculture ponds. Journal of Soils and Sediments. 2017;17(7):1928-1938.
    Available:http://dx.doi.org/10.1007/s11368-017-1651-2
12. Silva MJS, Costa FFB, Leme FP, Takata R, Costa DC, Mattioli CC, Luz RK, Miranda-Filho KC. Biological responses of neotropical freswhater fish *Lophiosilurus alexandri* exposed to ammonia and nitrate. The Science of the Total Environment. 2018;616-617,1566-1575.
    Available:http://dx.doi.org/10.1016/j.scitotenv.2017.10.157
    PMid:29102194

13. Verbeeten BE, Carter CG, Purser GJ. The combined effect of feeding time and ratio on growth performance and nitrogen metabolism of greenback flounder. Journal of Fish Biology. 1999;55(6);1328-1343.
Available:http://dx.doi.org/10.1111/j.1095-8649.1999.tb02079.x
14. Shrivastava J, Sinha AK, Cannaerts S, Blust R, Boeck G. Temporal assessment of metabolic rate, ammonia dynamics and ion-status in common carp during fasting: A promising approach for optimizing fasting episode prior to fish transportation. Aquaculture. 2017;481:218-228.
Available:http://dx.doi.org/10.1016/j.aquaculture.2017.09.008.
15. Xiang L, Sun T-F, Mo C-H, LI Y-W, CAI Q-Y, LI H. Related environmental problems and research progresses of quaternary ammonium compounds (QACs). *Huaxue Jinzhan*. 2016;28: 727-736.
16. Eddy FB. Ammonia in estuaries and effects on fish. Journal of Fish Biology. 2005;67(6):1495-1513.
Available:https://doi.org/10.1111/j.1095-8649.2005.00930.x
17. Yuen K IP, SHIT F. CHEW. Ammonia Production, Excretion, Toxicity, and Defense in Fish: A Review. Front Physiol. 2010;1:134.
DOI: 10.3389/fphys.2010.00134
18. Randall DJ, Tsui TKN. Ammonia toxicity in fish. Marine Pollution Bulletin. 2002;45(1-12):17-23.
Available:http://dx.doi.org/10.1016/S0025-326X(02)00227-8
PMid:12398363.
19. Atland A. y Bjerknes V. Calidad de agua para el cultivo de smolts en Chile. Santiago de Chile: NIVA Chile; 2009.
20. Foss A, Imsland AK, Roth B, Schram E, Stefansson SO. Interactive effects of oxygen saturation and ammonia on growth and blood physiology in juvenile turbot. *Aquaculture*. 2007;271(1-4): 244-251.
Available:http://dx.doi.org/10.1016/j.aquaculture.2007.06.025.
21. Beaumont M, Butler P, Taylor E. Plasma ammonia concentration in brown trout in soft acidic water and its relationship to decreased swimming performance. The Journal of Experimental Biology. 1995;198(Pt 10):2213-2220.
PMid:9320125.
22. Miranda-Filho KC, Wasielesky JR, Maçada AP. Efeito da amônia e nitrito no crescimento da tainha *Mugil platanus* (Pisces, Mugilidae). Revista Brasileira de Biologia. 1995;55:45-50.
23. Wajsbrot N, Gasith A, Krom MD, Popper DM. Acute toxicity of ammonia to juvenile gilthead seabream *Sparus auratus* under reduced oxygen levels. Aquaculture. 1991;92:277-288.
Available:http://dx.doi.org/10.1016/0044-8486(91)90029-7.
24. Adams MB, Powell MD, Purser GJ. Effect of acute and chronic ammonia and nitrite exposure on oxygen consumption on growth of juvenile big bellied seahorse. Journal of Fish Biology. 2001;58(3):848-860.
Available:http://dx.doi.org/10.1111/j.1095-8649.2001.tb00535.x
25. Wicks B, Joensen R, Tang Q, Randall J. Swimming and ammonia toxicity in salmonids: The effect of sub lethal ammonia exposure on the swimming performance of coho salmon and the acute toxicity of ammonia in swimming and resting rainbow trout. Aquatic Toxicology. 2002;59(1-2):55-69.
Available:http://dx.doi.org/10.1016/S0166-445X(01)00236-3. PMid:12088633.
26. Russo RC, Thurston RV. Toxicity of ammonia, nitrite and nitrate to fishes. In: D.E. BRUNE and J.R. TOMASSO, eds. Aquaculture and water quality. Baton Rouge: World Aquaculture Society. 1998;58-89.
27. Franson M. *Métodos Normalizados para el análisisde aguas potables y residuales*. Madrid: Díaz de Santos Editores. 2002;1816.
28. Person J, Ruyet L, Galland R, Roux A, Charotis H. Chronic ammonia toxicity in juvenile turbot (*Scophthalmus maximus*). Aquaculture. 2007;154:155-171.

29. Weirich CR, Riche M. Acute tolerance of juvenile Florida pompano, *Trachinotus carolinus* L., to ammonia and nitrite at various salinities. Aquaculture Research. 2006;37(9):855-861.
Available:http://dx.doi.org/10.1111/j.1365-2109.2006.01502.x.
30. Solorzano L. Determination of ammonia in natural waters by the phenolhypochlorite method. Limnology and Oceanography. 1969;14:799-801.
31. Handy RD, Poxton MG. Nitrogen pollution in mariculture: toxicity and excretion of nitrogenous compounds by marine fish. Reviews in Fish Biology and Fisheries. 1993;3(3):205-241.
Available:http://dx.doi.org/10.1007/BF00043929
32. Thurston RB, Russo RC. Acute toxicity of ammonia to rainbow trout. *Transactions of the* American Fisheries Society. 1983;112(5):696-704.
Available:http://dx.doi.org/10.1577/1548-8659(1983)112<696:ATOATR>2.0.CO;2.
33. Thurston RV, Meyn EL. Acute toxicity ofammonia to five fish species from the northwest United States.Bozeman: Bioassay Laboratory, Montana State University, 13 p. Technical Report, no. 1984;84-4, Fish.
34. US Environmental Protection Agency – EPA, 1998. Guidelines for risk assessment. Washington: EPA, 114.
35. Hucheng J, Xiaohui C, Wenji B, Longlong F, Quin Q, Liquiang, Minghua W. Comparison of bacterial communities in channel catfish *Ictalurus punctatus* culture ponds of an industrial ecological purification recirculating aquaculture system. Aquaculture Research. 2020;51(6): 2432.2442.
Available:https://doi.org/10.1111/are.14587
36. Fang L, Bai X-L, Liang X-F, He S, Guo X-Z, LI L, LI B, Shen D, TAO Y-X. Ammonia nitrogen excretion in Mandarin Fish (*Siniperca chuatsi*) and Grass Carp (*Ctenopharyngon idellus*) fed practical diets: the effects of water temperature. Aquaculture Research. 2017;48(3):836-843.
Available:http://dx.doi.org/10.1111/are.12927.
37. Paudel SR. Nitrogen transformation of engineered aquaponics with water celery (*Oenanthe javanica*) and koi carp (*Cyprinus carpio*): effects of plant to fish biomass ratio. *Aquaculture.* 2020;520.
Available:https://doi.org/10.1016/j.aquaculture.2020.734971
38. Rodrigues RV, Schwarz MH, Delbos BC, Sampaio LA. Acute toxicity and sublethal effects of ammonia and nitrite for juvenile cobia *Rachycentron canadum.* Aquaculture. 2007;271(1-4):553-557.
Available:http://dx.doi.org/10.1016/j.aquaculture.2007.06.009.
39. Houlihan DF, Owen SF. Protein synthesis, nitrogen excretion and long-term growth of juvenile *Pleuronectes flesus.* Journal of Fish Biology. 1998;53:272-284.
dx.doi.org/10.1111/j.1095-8649.2005.00930.x
40. Wicks BJ, Randall DJ. The effect of sub lethal ammonia exposure on fed and unfed rainbow trout: The role of glutamine in the regulation of ammonia. Comparative Biochemistry and Physiology. 2002;132(2):275-285.
Available:http://dx.doi.org/10.1016/S1095-6433(02)00034-X. PMid:12020644.
41. Popović NT, Strunjak-Perović I, Barišić J, Kepec S, Jadan M, Beer-Ljubić B, Matijatko V, Palić D, Klobučar G, Babić S, Kljusurić JG, Čož-Rakovac R. Native Prussian carp (*Carassius gibelio*) health status, biochemical and histological responses to treated wastewaters. Environmental Pollution. 2016;218:689-701.
Available:http://dx.doi.org/10.1016/j.envpol.2016.07.063. PMid:27524256.

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He is a marine biologist (1989), an Industrial Civil Engineer (2015) and a Ph.D. in Environmental Sciences (1994) from the University of Concepción (Chile). He works at the Catholic University of Temuco as an associate professor and is director of the Centre for Environmental Studies. He has 25 years of experience in undergraduate and graduate teaching, scientific research and environmental advice, specifically in the analysis and interpretation of Eco toxicological bioassays, ecological risk assessment, impact studies and environmental monitoring in the areas of aquaculture, agriculture, pulp of paper and mining. He has specialized in the modelling and statistical analysis of environmental data, particularly in multivariate analysis for the analysis of environmental data. He has taught numerous national and international courses in ecological risk assessment. He is a member of scientific societies related to the evaluation of ecological risk: Society of Limnology of Chile and Society of Toxicology and Environmental Chemistry (SETAC).



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# Genetic Variability, Coefficient of Variance, Heritability and Genetic Advance of Some Traditional Rice Cultivars of Assam

**Jnandabhiram Chutia[1*], S. P. Borah[2] and Bhaben Tanti[2]**

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## ABSTRACT

Rice is the staple food crop for India as well as entire Asia Pacific region. But this crop has been constantly exposed to various biotic and abiotic stresses effecting the yield pattern. The prominent among the abiotic stress is water stress. This paper tries to evaluate the effect of drought stress on the various physiological parameters taken under consideration during the study. The results has been validated using methods to understand the impact of the drought sustaining characters on the Genetic Variability, Coefficient of Variance, Heritability and Genetic Advance.

*Keywords: Rice; drought stress; traditional varieties; coefficient of variabilities and heritability.*

## 1. INTRODUCTION

Rice is the staple food for more than 65percent of the Indian people. India has the largest area under rice in the world (44.6 million hectare) and ranks next to China in production [1]. It has been observed that rice has been the main food for more than half of the world's population and rice yield losses thus pose a major threat to global food security [2]. Rice being a high water-consuming crop and grown 51% under irrigated condition [3], is susceptible to a wide range of abiotic stresses including water stress i.e., both excess or deficient water in soil as water is required during the various stages of growth and development of the plant [4,5,6,7]). Rice breeding programs have widely focussed on understanding the plant's response to various abiotic and biotic stresses to enhance yield [8].

Levitt's classification of drought resistance (1980) linked adaptive strategies to plant water relation parameters. Drought avoidance and tolerance were defined as the ability to survive or thrive at high and low water potential. Osmotic adjustment constituted the major strategy of dehydration avoidance and stomatal behaviour was associated with water saving and water spending strategies. Thus, the ability to plant or crop water deficit became fundamental to research on the response of plant communities to water stress [9].

Rice is the principal crop of Assam state and is grown in an area of 2.58 million hectares, occupying about 80% of the total cultivated area. Assam is one of the richest repositories of diverse rice germ plasm. Therefore, collection and conservation of both wild and cultivated rice followed by their evaluation and utilisation has to be undertaken (CRRI Bulletin, 1996).

Rice genotypes are known to vary widely in their responses to abiotic stresses, and breeding stress tolerant rice cultivars is the most efficient and economical way to overcome the problems.

There are at least three obvious reasons for slow progress in genetic improvement of rice abiotic stress tolerance. First, past efforts in germplasm screening identified few accessions with high levels of abiotic stress tolerance for use in breeding programs. second, rice tolerance to different abiotic stresses is often complex in nature and its genetic basis, as for most quantitative traits, remains poorly understood. Third, in most areas of the rainfed environments, multiple abiotic stresses coexist.

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Thus, desirable rice varieties for rainfed environments must have tolerances to multiple stresses in addition to good yield potential and acceptable grain quality. Domestication, artificial selection and intensive breeding of crop varieties by human have resulted in the narrowed genetic base in many crops, which renders modern crop varieties more vulnerable to biotic and abiotic stresses [10].

Traditional landraces account for about 70% of the total rice collections in the gene banks worldwide and many of them are known to have different tolerances of abiotic stresses, but plant breeders have been reluctant to utilise these landraces, largely for three reasons [11]. First, slow but consistent progress can be achieved even within the narrow genetic base of many breeding populations. Secondly, outstanding commercial genotypes tend to be destroyed in crosses involving unadapted exotic parents. Thirdly, selection of germplasm as parents of the breeding programs by breeders has been largely based on the phenotype. In view of this observation this study was undertaken to correlate the physiological response to genetic traits.

## 2. MATERIALS AND METHODS

For the study of genotypic variabilities for stress tolerance in traditional rice varieties of Assam, 12 different varieties were selected. These rice varieties were collected from different parts of Assam, locally known as *Beriabhanga* (BES), *Borah* (BOS), *Jahinga*(JHS), *Kolijoha* (KJS), *Laodubi* (LAS), *Leseri* (LES), *Patteseri* (PAS) , *Solpuna* (SOS), *Bengunguti* (BES), *Kesamani* (KES), *Sakuakumal* (SAS) and *Rangadaria* (RAS).

These 12 rice varieties were grown in the field during the *khariff* (May to November) season of 2004, 2005, 2006 and 2007. The present investigation was comprised of three interlinked phases. The stress induced behaviour was studied under three different moisture regimes i.e., normal irrigated condition, unirrigated water stress upland condition and potted simulated drought condition. Standard field preparation methodology was followed comprising of randomized block design. Twenty five days old seedlings were grown and the drought cycle of 10-days duration (i.e., 101-110 days after sowing) was followed. The artificial drought condition was initiated by polyethylene glycol-6000 (PEG-6000) and an osmotic potential(ψs) of 0.15bar, 0.25bar & 0.56 bar was maintained [12].

## 3. RESULTS AND DISCUSSION

### 3.1 Studies on Genetic Components of Variation for Drought Sustaining Characters in 20 Rice Varieties

Collected data were analyzed for determining the genotypic variances for all the characters considered under study in the experimental varieties. Following components of genetic variance were estimated - (i) Seed Germination, (ii) Total plant height (up to the tip of the flag leaf), (iii) Length of the flag leaf, (iv) Flag leaf angle, (v) Panicle length, (vi) Green leaf duration and (vii) Total number of seeds (both fertile and sterile).

The genotypic and phenotypic coefficient of variabilities was calculated according to the formulae of Burton [13]. The heritability in broad sense was calculated according to Johanson et al., [14,15]. The different formulas are as follows:

i. Genotypic variance = $\frac{V - E}{N}$,

Where, V is the mean of squares of lines (varieties), E is the mean square residual and N is the number of replications.

i. Phenotypic variance = Genotypic variance + E,

Where, E is the mean square residual.

ii. Genotypic coefficient of variability (GCV) =√Phenotypic variance x 100 / x

iii. Phenotypic coefficient of variability (PCV) = √Genotypic variance x 100/ x

Where, x is the mean of particular character under study.

iv. Heritability = Genotypic variance x 100 / Phenotypic variance

### 3.2 Germination Index under Artificial Drought Induced Condition

The results of the germination index (GI) under 0.15bar are presented in the Table 1, which represents the mean values along with the standard error (SE). From the table of analysis of variance of the studied plant character (Table 1), it is evident that each one of the varieties under 0.15 bar and the Ψs-0.25bar and Ψs - 0.56bar differed significantly from each other (Table 1).

### 3.3 Discussion on Various Traits

It is evident from the results that LAS (19.57 ± 0.36) showed the highest GI followed by Borah (17.58 ± 0.43) in contrary to it RAS (1.60 ± 0.53) and BES(0.86 ± 0.12) showed the least GI. In Table 1 the GIunder osmotic potential (Ψs) - 0.25bar for Ψs - 0.25bar is presented with significant differences from the Ψs-0.15bar and Ψs - 0.56bar. The result showed that Borah (16.28 ± 0.9) and Laodubi (15.75 ± 1.4) has the highest GI. While RAS (2.05 ± 0.8), KES (1.51 ± 0.4) and BES (1.48 ± 0.4) showed the least GI.

Under osmotic potential (Ψs) - 0.56bar the GI of all the 12 varieties is presented in Table 1 with significant differences from the Ψs-0.15bar and Ψs - 0.25bar. The tabulated results showed that LAS (4.13± 0.27) and BOS (2.04± 0.22) have the highest GI. SAS (0.16± 0.02) and RAS (0.13± 0.01) have the lowest germination index.

It has been observed that significant variation between the varieties existed which is relevant for breeders as breeders look for traits that are suitable for screening rice germ plasm for characters affecting plant water relations under drought conditions. Simulation of drought stress by polyethylene glycol (PEG) indeed induced drought stress on the plant [16] and the results showed the significant deviation from the control ones and continues to increase with the increasing solute potential (Ψs). The study further revealed a positive correlation between germination index (GI) in PEG initiated drought and the whole plant behaviour under the simulated drought conditions. This was evident from the results exhibited by LAS, LES and PAS. Thus GI can be taken as a highly accurate maturity parameter for drought sustenance. Usually breeding programs emphasises direct selection for grain yield under stress but indirect selection for carefully selected secondary traits like flag leaf angle, green leaf duration, panicle length etc can also be helpful in improving selection response by calculating genetic correlation and heritability within a breeding population. Length of leaf is negatively related to osmotic stress while reduction higher osmotic stress is due to its negative correlation with leaf angle [17]. The longer leaves have wide angle while the shorter ones have smaller angles. The leaf angle is a character usually associated with plasticity in leaf rolling when internal water deficit occurs. The leaves of modern semi dwarf varieties have relatively small leaves with acute angle often folded into a tube form [18]. In LAS, LES , PAS and BES, the exceptionally long and drop leaves with larger leaf angle were more susceptible to rolling due to their ability to intercept relatively more radiant energy, greater extensibility and might help conserve water in plant tissues which follows the idea advocated by Chang et. al. [19]. The notion of the change in shape and leaf angle have always been enumerated as a powerful means of riding transpiration rate by plants experiencing water deficit [20,21].

Grain yield under stress environment is the primary trait for selection in breeding for drought tolerance. Drought effect on seed yield is due to the relation with duration of watering from flowering until physiological maturity [22]. There is a significant difference in the number of spikelets in stressed and well-watered plants. Deficient in water requirements causes incomplete and delayed panicle extension as well as degenerated spikelets. These results support the earlier findings of Turner et al., 1986.

**Table 1. Mean value of various plant characters under three different water regimes**

| Variety | Culture condition Drought induced by PEG-6000Ψs- bar | Parameter Studied | Culture condition | Parameters studied | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Rate of germination (GI ± SE) | | Plant height (cm ± SE) | Length of the flag leaf (cm ± SE) | Flag leaf angle (degrees ± SE) | Green leaf duration (days ± SE) | Panicle length (cm ± SE) | Total Seeds (fertile & sterile) (numbers ± SE) |
| | Ψs -0.15 | 19.57±0.36 | Control | 120.41 ± 0.42 | 35.55±0.29 | 30.38 ±0.33 | 137.93 ±0.52 | 23.51±0.29 | 145±0.15 |
| | Ψs -0.25 | 15.75± 1.4 | Upland | 114.51±0.29 | 21.62 ±0.52 | 70.25 ±0.25 | 117.75± 0.38 | 23.51+0.29 | 117.73±0.26 |
| LAS | Ψs -0.56 | 4.13 ± 0.27 | Potted | 85.51 ± 0.51 | 14.73 ±0.37 | 59.89 ±0.42 | 97.92± 0.08 | 13.49±0.25 | 24.63±0.47 |
| | Ψs -0.15 | 17.58± 0.43 | Control | 118.52 ±0.52 | 50.66 ±0.33 | 80.26 ±0.27 | 136.85 ±0.46 | 17.85 ±0.15 | 121.92 ±0.08 |
| | Ψs -0.25 | 16.28 ±0.9 | Upland | 145.55 ±0.29 | 33.51 ±0.51 | 74.88 ±0.48 | 114.85 ±0.45 | 23.85 ±0.45 | 82.40 ±0.41 |
| BOS | Ψs -0.56 | 2.04 ±0.22 | Potted | 74.85 ±0.46 | 18.65 ±0.33 | 50.84 ±0.45 | 96.79 ±0.21 | 11.75 ±0.38 | 14.55 ±0.55 |
| | Ψs -0.15 | 9.37± 0.53 | Control | 112.45±0.29 | 29.92±0.08 | 60.32±0.33 | 133.85±0.45 | 22.38±0.38 | 136.51±0.29 |
| | Ψs -0.25 | 5.77±0.6 | Upland | 133.51±0.52 | 26.73±0.45 | 60.25±0.25 | 113.85±0.46 | 20.38±0.38 | 108.73±0.26 |
| JHS | Ψs -0.56 | 0.97±0.12 | Potted | 62.43±0.30 | 23.45±0.29 | 53.56±0.30 | 95.91±0.08 | 11.69±0.17 | 11.42±0.43 |
| | Ψs -0.15 | 12.94±0.40 | Control | 128.78±0.40 | 21.76±0.24 | 60.55±0.56 | 135.51±0.29 | 20.85±0.15 | 116.78±0.22 |
| | Ψs -0.25 | 8.80±0.9 | Upland | 119.6±0.31 | 27.55±0.29 | 79.98±0.56 | 112.783±0.40 | 19.59±0.30 | 47.96±0.04 |
| BES | Ψs -0.56 | 1.50±0.25 | Potted | 55.48±0.29 | 11.73±0.27 | 65.51±0.52 | 96.88±0.12 | 18.98±0.57 | 6.81±0.56 |
| | Ψs -0.15 | 6.38±0.52 | Control | 121.66±0.33 | 33.51±0.52 | 90.51±0.51 | 134.85±0.45 | 22.75±0.38 | 98.88±0.11 |
| | Ψs -0.25 | 2.51±0.6 | Upland | 132.45±0.45 | 28.95±0.54 | 70.59±0.30 | 108.75±0.38 | 18.98±0.02 | 87.99±0.01 |
| PAS | Ψs -0.56 | 0.33±*0.05* | Potted | 86.55±0.29 | 21.74±0.26 | 52.92±0.51 | 73.87±0.27 | 21.52±0.24 | 6.62±0.32 |
| | Ψs -0.15 | 10.34±0.31 | Control | 110.71±0.36 | 26.85±0.45 | 79.94±0.53 | 133.75±0.38 | 22.38±0.31 | 100.40±0.4 |
| | Ψs -0.25 | 0.76±0.2 | Upland | 97.7±0.54 | 24.45±0.29 | 85.55±0.29 | 105.85±0.46 | 16.70±0.30 | 95.86±0.46 |
| LES | Ψs -0.56 | 0.3±0.03 | Potted | 71.41±0.42 | 23.88±0.48 | 70.23±0.40 | 71.74±0.25 | 17.77±0.23 | 4.743±0.38 |
| | Ψs -0.15 | 6.4425±0.49 | Control | 120.88±0.12 | 26.55±0.29 | 80.55±0.29 | 131.75±0.38 | 22.75±0.25 | 132.77±0.22 |
| | Ψs -0.25 | 4.3±0.9 | Upland | 112.41±0.30 | 25.51±0.29 | 70.29±0.30 | 110.65±0.33 | 23.84±0.15 | 74.51±0.52 |
| KJS | Ψs -0.56 | 0.67±0.06 | Potted | 84.81±0.43 | 21.41±0.42 | 55.45±0.45 | 75.85±0.15 | 19.55±0.29 | 4.51±0.52 |
| | Ψs -0.15 | 6.45±0.51 | Control | 114.49±0.49 | 40.81±0.43 | 80.52±0.52 | 130.75±0.38 | 22.86±0.14 | 98.99±0.01 |
| | Ψs -0.25 | 4.08±0.9 | Upland | 139.52±0.29 | 23.48±0.29 | 70.28±0.28 | 108.95±0.53 | 22.42±0.30 | 55.67±0.51 |
| SOS | Ψs -0.56 | 0.91±0.06 | Potted | 75.85±0.15 | 24.85±0.15 | 59.72±0.27 | 75.25±0.21 | 12.56±0.22 | 3.48±0.48 |
| | Ψs -0.15 | 0.86± 0.12 | Control | 121.74±0.38 | 25.81±0.52 | 75.55±0.29 | 119.73±0.37 | 23.58±0.30 | 126.40±0.30 |
| | Ψs -0.25 | 1.48±0.4 | Upland | 73.45±0.45 | 23.51±0.52 | 59.37±0.32 | 101.88±0.48 | 20.70±0.30 | 56.44±0.29 |
| BES | Ψs -0.56 | 0.74±0.01 | Potted | 55.92±0.51 | 20.43±0.43 | 45.88±0.48 | 66.246±0.25 | 9.723±0.36 | 2.99±0.57 |
| KES | Ψs -0.15 | 1.72±0.26 | Control | 103.51±0.52 | 17.92±0.08 | 69.78±0.40 | 120.85±0.45 | 21.38±0.31 | 125.77±0.52 |
| | Ψs -0.25 | 1.51±0.4 | Upland | 80.88±0.12 | 20.05±0.5 | 81.85±0.46 | 104.85±0.45 | 19.39±0.40 | 75.55±0.55 |
| | Ψs -0.56 | 0.69±0.02 | Potted | 57.77±0.23 | 15.85±0.15 | 59.65±0.33 | 70.90±0.10 | 18.92±0.51 | 2.79±0.41 |

| Variety | Culture condition Drought induced by PEG-6000Ψs- bar | Parameter Studied | Culture condition | Parameters studied | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Rate of germination (GI ± SE) | | Plant height (cm ± SE) | Length of the flag leaf (cm ± SE) | Flag leaf angle (degrees ± SE) | Green leaf duration (days ± SE) | Panicle length (cm ± SE) | Total Seeds (fertile & sterile) (numbers ± SE) |
| | Ψs -0.15 | 2.27±0.54 | Control | 111.92±0.08 | 29.73±0.37 | 69.85±0.15 | 119.92±0.51 | 20.72±0.36 | 146.37±0.32 |
| SAS | Ψs -0.25 | 3.42±0.6 | Upland | 87.59±0.30 | 25.85±0.35 | 80.41±0.30 | 101.77±0.40 | 18.66±0.33 | 72.73±0.27 |
| | Ψs -0.56 | 0.16±0.02 | Potted | 60.55±0.29 | 22.78±0.22 | 65.7±0.53 | 68.92±0.08 | 16.80±0.18 | 1.76±0.39 |
| | Ψs -0.15 | 1.60±0.53 | Control | 139.51±0.29 | 31.41±0.30 | 40.52±0.52 | 117.92±0.51 | 27.40±0.30 | 150.51±0.52 |
| RAS | Ψs -0.25 | 2.05±0.8 | Upland | 87.76±0.52 | 23.55±0.29 | 70.25±0.26 | 105.73±0.37 | 24.59±0.30 | 63.65±0.33 |
| | Ψs -0.56 | 0.13±0.01 | Potted | 64.55±0.55 | 23.84±0.15 | 50.51±0.52 | 72.27±0.29 | 9.85±0.46 | 1.43±0.43 |

**Table 2. Mean sum of squares for various plant characters in 12 traditional rice cultivars grown under three different water regimes**

| Source of variation | Degree of freedom | Character studied | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Germination Index | Plant height | Flag leaf length | Flag leaf angle | Green leaf duration | Panicle length | Seed number |
| Variety | 11 | 196.40** | 1154.932** | 163.01** | 555.36** | 571.383** | 15.16** | 1240.633* |
| Replication | 2 | $0.00^{NS}$ | $0.194^{NS}$ | $1.0275^{NS}$ | $0.675^{NS}$ | $0.037^{NS}$ | $0.23^{NS}$ | $0.285^{NS}$ |
| Culture condition | 2 | 562.90** | 24770.58** | 982.11** | 2238.785** | 22164.48** | 534.565** | 77155.4** |
| Error | 92 | 6.59 | 438.9389 | 57.25663 | 221.2238 | 156.0795 | 12.2341 | 454.1803 |

**Table 3. Estimates of different genetical parameters in 12 different rice varieties**

| Plant character | Mean ± SE | Range | Genotypic variance | Phenotypic variance | Genotypic coefficient of variability GCV% | Phenotypic coefficient of variance PCV% | Heritability % |
|---|---|---|---|---|---|---|---|
| Germination index | 6.276667 | 0.14 -19.85 | 63.266699 | 69.86 | 168.9656 | 177.5532 | 90.56062 |
| Plant height | 99.36111 | 56 - 139 | 238.6643 | 677.6032 | 15.5481 | 26.19819 | 35.22184 |
| Flag leaf length | 25.22222 | 15 -51 | 35.25112 | 92.50775 | 29.08998 | 47.12442 | 38.10613 |
| Flag leaf angle | 65.96296 | 80 - 45 | 111.3787 | 332.6025 | 15.9993 | 27.64793 | 33.48704 |
| Green leaf duration | 106.01 | 66 - 137 | 138.4345 | 294.514 | 11.09886 | 16.18859 | 47.0044 |
| Panicle length | 19.37963 | 09 - 24 | 0.975301 | 13.2094 | 5.095934 | 18.75409 | 7.383385 |
| Seed number | 69.96296 | 01 - 150 | 126.1508 | 716.3311 | 23.14235 | 38.25505 | 36.59632 |

The panicle length is characterised by early initiation, early flowering, longer growth duration, heavier tiller and greater fall leaf area [23]. In some cases panicle remained within the boot leaf sheath even after rewetting. This could be the major cause for high proportion of unfilled spikelets panicle [24,25]. In the present study (Table 1) it was found that the reduction in grain yield was maximum in the variety LAS (23.78±0.40 no. of grains) under irrigated potted condition. On the contrary the same variety exhibited a yield of 117.58±0.30 and 145.81±0.18 no of grains under unirrigated upland and irrigated rained condition respectively. This convicts us that the grain yield under stress was limited due to a decreased production and translocation os assimilates as the sink size is not effected [26,25]. The results (Tables 1,2) clearly indicates that water deficit during booting to anthesis initiation is more detrimental than anthesis stage stress. These observations support our idea that selection for yield under reproductive-stage is effective in rice, and that choice of donor is very important in breeding drought tolerant rice.

**Table 4. Mean and range performance of the traits among the *O sativa* accession**

| Traits | Condition | Minimum | Maximum | Average performance |
|---|---|---|---|---|
| Seed germination | Ψs- 0. 15 bar | 0.86 | 19.57 | 10.21 |
| Index | Ψs - 0.25bar | 0.76 | 16.28 | 8.52 |
| Total plant height | Upland | 73.45 | 145.55 | 109.47 |
| | Potted | 55.48 | 86.55 | 71.01 |
| Flag leaf length | Upland | 20.05 | 33.51 | 26.78 |
| | Potted | 11.73 | 24.85 | 18.29 |
| Flag leaf angle | Upland | 59.37 | 85.55 | 72.46 |
| | Potted | 50.50 | 70.23 | 60.36 |
| Green leaf | Upland | 101.77 | 117.75 | 109.76 |
| duration | Potted | 66.24 | 97.92 | 82.08 |
| Panicle length | Upland | 18.98 | 24.59 | 21.78 |
| | Potted | 9.72 | 21.52 | 15.62 |
| Seed no | Upland | 55.56 | 111.73 | 83.64 |
| | Potted | 1.76 | 24.63 | 13.19 |

**Table 5. Potential varieties as per the different traits**

| NO | chanracters | Culture condition | Potential varieties |
|---|---|---|---|
| 1 | Seed germination | Ψs- 0. 15 bar | LAS, BOS |
| | Index | Ψs - 0.25bar | LAS, BOS, LES |
| 2 | Total plant height | Upland | LAS, BOS, JHS, PAS, SOS |
| | | Potted | BOS, JHS, PAS, LES, KJS, SOS, BES, RAS |
| 3 | Flag leaf length | Upland | LAS, BOS, BES, PAS, LES, KJS, SOS, KES, SAS, RAS |
| | | Potted | LAS, BOS, BES, LES |
| 4 | Flag leaf angle | Upland | LAS, BOS, JHS, BES, PAS, LES, KJS, SOS, RAS, SAS, KES |
| | | Potted | LAS, BOS, JHS, BES |
| 5 | Green leaf | Upland | LAS, BOS, JHS, BES |
| | duration | Potted | LAS, BOS, JHS, BES |
| 6 | Panicle length | Upland | LAS, BOS, JHS, BES, KJS, SOS, LES, PAS, SAS, KES |
| | | Potted | BES, PAS, KJS, KES |
| 7 | Seed no | Upland | LAS, JHS, LES |
| | | Potted | LAS |

Following the concept of variation in a population is due to both factors i.e. genetics and environmental where as genetic variability is the only heritable from generation to the next generation so the heritability alone does not give an idea about the expected gain in the next generation but it has to be considered in conjunction with the genetic advance. The characters those exhibit maximum heritability and high genetic advance as percentage of mean could be used as powerful tool in selection process such characters are controlled by the additive genes and less influenced by the environment [27]. Basing on this presumption the broad sense heritability was sensible enough for all the recorded simulated drought conditions. The heritability percentage ranged from 9% to 90% in the different traits highest been in the GI and the lowest being in the panicle length. Consistency in heritability was observed in the plant height, flag leaf length and flag lea angle i.e., 35.2%, 38.1% and 33.48% respectively while a relatively higher heritability was observed in green leaf duration (47%) which can be substantiated for a higher seed number after bring into work certain other relevant plant breeding techniques (Tables 3 & 4). Thus the results give us ample opportunity to identify some potential donors (Table 5) for the crop improvement which in the present day context can be used as an direct selection trait for most of the yield attributing traits since it exhibited high h=genetic variability and high range go of variation. A high PCV and GCV for the characters studied indicates us that environmental influences on the expression of these traits were relevant in the context of development of a drought resistant variety of the indigenous cultivar of *O. sativa,* Linn.

## 4. CONCLUSION

The leaves of modern semi dwarf varieties have relatively small leaves with acute angles often folded into a tube form. In the traditional rice varieties like LAS, LES, PASandBES, the exceptionally long and droopy leaves with larger leaf angle were more susceptible to rolling due to their ability to intercept relatively more radiant energy, greater extensibility and might help conserved water in plant tissues also worked on rice and advocated the role of larger leaf angle in conservation of water in plant cells. Thus it can be substantiated that long and droopy leaves (*i.e*., with higher leaf angle) results into leaf rolling decreasing the area intercepting radiation resulting into decrease in rate of transpiration [28] as leaf rolling is an adaptive response to water deficit in rice [29].

In the study the grain yield under stress was limited due to decreased production and translocation of assimilates as the sink size is not affected [26,25]. The results clearly indicate that water deficit during booting to anthesis initiation is more detrimental than anthesis stage stress. Ingram, (1989) also reported reduction in grain production due to moisture stress during booting stage to flowering or early grain filling stage in rice. These observations support the hypothesis that selection for yield under reproductive-stage drought stress is effective in rice, and that choice of donor is very important in breeding drought-tolerant rice.

A positive correlation between germination index (GI) in PEG initiated drought and the whole plant behaviour under three water regimes were observed in the present investigation. This was evident from the results exhibited by LAS, LES AND PAS with higher germination index while these same varieties showed good response to other drought sustaining characters under three water regimes. Thus the determination of germination index (GI) can be used just as an easy and reliable parameter for measuring drought sustenance among the traditional rice cultivars of Assam.

The low germination rate in JHS, PAS and KJS as observed in the present study was due to the osmotic stress induced by PEG 6000 which had mark effect in both shoots and roots parameters. The reduction in seed germination may be due to the less availability of free water to the seeds during early hours of inhibition, thus leaving the hydrolytic enzymes inactive. Inhibition of germination at higher osmotic potential may possibly be attributed to moisture deficit in the seed be- low the threshold requirement for germination. The reduction in shoot and root growth is important as PEG induced stress affects root volume and root length [10]. The reduction of root volume under induced osmotic stress originates not only from growth inhibitions but also from a loss of turgidity.

Tolerance to abiotic stresses is very complex at the whole plant and cellular levels. Putting these observations under consideration the subsequent phases of analysis was done so as to establish the complexity of interactions between stress factors and physiological phenomena affecting plant growth

and development. In correlation to this it has been observed that the different components of genotypic variation for six drought-sustaining characters in seven experimental rice varieties indicates that plant height and seed number have less environmental influences with high GCV as 187.16 and 35.99 respectively with high heritability.

From this screening procedure it has been LAS, LES, BES and BOS cultivars were screened out as the best drought sustaining variety among the ones considered in this investigation. This paves the way for further investigation into the inherent characters of drought sustenance characters of the traditional cultivars of Assam.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

## REFERENCES

1. Sarkar RK, Reddy JN, Sharma SG, Ab-delbagi MI. Physiological basis of submergence tolerance in rice and implication for crop improvement. Current Science. 2006;9(7):899-906.
2. Ahmadi N, Audebert A, Bennett MJ, Bishopp A, de Oliveira AC, Courtois B, Diedhiou A, Diévart A, Gantet P, Ghesquière A. The roots of future rice harvests. Rice. 2014;7-29.
3. Tuong T, Bouman B. Rice production in water-scarce environments. In Water Productivity in Agriculture: Limits and Opportunities for Improvement; CABI: Wallingford, UK; International Water Management Institute (IWMI): Colombo, Sri Lanka. 2003;1:13–42.
4. Akula R, Ravishankar GA. Influence of abiotic stress signals on secondary metabolites in plants. Plant Signal. Behav. 2011;6:1720–1731.
5. Kim Y-H, Hwang S-J, Waqas M, Khan AL, Lee J-H, Lee J-D, Nguyen HT, Lee I-J. Comparative analysis of endogenous hormones level in two soybean (*Glycine max* L.) lines diering in waterlogging tolerance. Front. Plant Sci. 2015;6:714.
6. Mouna Lamaoui[1], Martin Jemo[1,2], Raju Datla[3], FaouziBekkaoui[1]*. Heat and Drought Stresses in Crops and Approaches for Their Mitigation. Frontiers in Chemistry. 2018;6:1-20.
7. Kim Y, Seo C-W, Khan AL, Mun B-G, Shahzad R, Ko J-W, Yun B-W, Park S-K, Lee I-J. Exo-ethylene application mitigates waterlogging stress in soybean (*Glycine max* L.). BMC Plant Biol. 2018;18:254.
8. Kumar A, Verulkar S, Dixit S, Chauhan B, Bernier J, Venuprasad R, Zhao D, Shrivastava M. Yield and yield-attributing traits of rice (*Oryza sativa* L:) under lowland drought and suitability of early vigor as a selection criterion. Field Crops Res. 2009;114:99–107.
9. Kumar A, Chippa BR, Singh K. Plant water relations under water stress. Plant productivity under environmental stress. Agro botanical publishers India, Bkaner. 1994;1-10.
10. Tanksley SD, McCouch SR. Seed banks and molecular maps: unlocking genetic potential from the wild. Science. 1997;277:1063–1066.
11. Duvick, Donald N. Crop breeding in the 21st century. Journal of Crop Science. 2005;14(1-2):9-21.
12. Zhu JK. Plant Salt Tolerance. Trends in Plant Science. 2001;6(2):66-71.
13. Burton GW. Quantitative Inheritance in Grasses. Proceedings of 6th Intern, Grasslands Congress. 1952;1:277-283.
14. Johnson HW, Haward CL, Khan AR. Geno-typic, Phenotypic Correlations in Soyabean and Their Implication in Selection. Agronomy Journal. 1955;47(10):477-483.
15. Johnson HW, Robinson HF, Comstock RE. Es- timates of Genetic and Environmental Variability in Soyabean. Agronomy Journal. 1955;47(7):314-318.
16. Jiang Y, Macdonald SE, Zwiazak JJ. Effects of Cold Storage and Water Stress on Water Relations and Gas Exchange of White Spruce (Piceglauca) Seedlings. Tree Physiology. 1995;15(4):267-273
17. Singh S, Singh TN. Significance of leaf rolling in rice during water stress. Indian J. Plant Physiol. 1999;5(3):214-218
18. Turner NC. Role of Shoot Characteristics in Drought Resistance of Crop Plants," In: Drought Resistance in Crops with Emphasis on Rice, IRRI. 1982;115-134.

19. Chang TT, Loresto GC, Tagumpay O. Screening Rice Germplasm for Drought Resistance. Sabard Journal. 1974;6:9-16.
20. Parker JS. Drought resistance mechanisms In: water deficits and plant growth. T.T. Kozlowaskai ed, Vol.I Academic Press, New York. 1968;195-234.
21. Begg JE, Turner NC. Crop water deficits. Advances in agronomy. 1976;28:161-217.
22. Midaoui MEl, Serieys H, Kaan F. Effects of osmotic and water stresses on root and shoot morphology and seed yield in sunflower (*Helianthus annuus* L.) genotypes breed for morocco or issued from introgression with H. argophyllus T. & G. and H. debilis Nutt. HELIA. 26. Nr. 2003;38:1-16.
23. Saikia L, Mahanta TC. Effect of dates and sequential planting of tillers on yield of rice under post-flooded situation of Assam. Indian J. Agron. 1996;41(2):221-225.
24. Turner NC, O'Toole JC, Cruz RT, Namuco OS. Response of seven diverse rice cultivars to water deficit. In: Stress development, canopy temperature, leaf rolling and growth. Field Crops Res. 1986;13(3):257-271.
25. Ahmed MR. Influsence of water deficit on water relations, growth and yield of dry seeded rice. Ph. D. Thesis. Institute of graduate studies, Central Luzon State University, Munoz, Neuva, Ecija, Phillipines; 1992.
26. Chaturvedi GS, lngram KT. Growth and yield of lowland rice in response t shade and drainage. Philippines J Crop Sci. 1989;14:61-67.
27. Association of Official Seed Analysis (AOSA). Seed Vigor Testing Handbook, Contribution No. 32 to the handbook on Seed Testing; 1983.
28. Bates LS, Walden RP, Teare ID. Rapid Deter- mination of Free Proline for Water-Stress Studies. Plant and Soil. 1973;39(1):205-207.
29. Singh DP. Water Deficit Stress in Stress Physiology. New Age Publishers, New Delhi. 2003;64-79.

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***Chapter 12***
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# Utilization of Lignocellulosic Residues (Corn-cob and Pawpaw Fibre) as Substrates for Production and Optimization of Cellulase from *Penicillium* sp.

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## ABSTRACT

Lignocellulosic residues are abundant in agricultural and forest industries in various countries where they remain under exploited. They have useful potential in microbial systems in the secretion of cellulases The present work investigated theutilization of lignocellulosic residues (corn-cob, CC and pawpaw fibre, PF) as substrates for production and optimization of cellulase from *Penicillium* sp.A total of nine fungal isolates were gotten from compost soil and were screened for cellulolytic activity using Standard Methods. Cellulase activity was determined by the DNS method on Congo red agar plate. Out of the nine isolates, Isolate CPF-1 was selected and identified as *Penicillium* sp. based on its cultural and morphological characteristics. The influence of basic fermentation parameters on enzyme production in solid state fermentation and the effects of some physicochemical parameters on crude cellulase activity/stability were studied. The data obtained from the study revealed that the optimal pH and temperature values for the production of crude cellulase by the *Penicillium* sp. were pH 5 and 30°C, respectively; with maximum cellulase activity of 37.32 IU/mL. Optimum cellulase productivity of 15.787 IU/mL was obtained with CC as the substrate while 2.141 IU/mL was obtained with PF substrate after 1 h of fermentation. The cellulase produced was most stable at pH 5 and temperature of 40°C. $Fe^{2+}$ and $Co^{2+}$ stimulated cellulase activity whereas the other ions inhibited the enzyme activity. This study has revealed the potentials of corn-cob and pawpaw fibre as substrates for cellulase production by *Penicillium* sp. through solid state fermentation (SSF); with corn-cob as the most suitable substrate. Considering that these substrates are cost-effective and abundant as well renewable, they present cheaper substrate alternatives for potential large-scale cellulase production and reduction in environmental pollution problems.

*Keywords: Cellulase; Penicillium sp.; lignocellulosic biomass; solid state fermentation.*

## 1. INTRODUCTION

Cellulases are the hydrolytic enzymes which breakdown cellulose into glucose during the growth of microorganisms on cellulosic materials. They are inducible and are secreted by variety of microorganisms. Cellulolytic enzyme system involves three major components which include endoglucanases, exoglucanases and ß-glucosidases. Cellulases belong to the glycoside hydrolase family and catalyse hydrolysis of glyosidic linkages, depolymerizing cellulose to fermentable sugars [1]. These fermentable sugars are used in many industrial processes such as biofuel and other chemical productions [2,3]. Recent interest by researchers to produce cellulase from cheaper substrate is justifiable. Industrially, cellulases are used in various economically relevant processes. They are widely applied in biotechnology industry and other industries. Cellulases have a potential

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application in biotechnology and industry such as, starch processing, alcoholic beverage, malting and brewing, clarify of juice, pulp bleaching, textile industry, laundry, biomass refining and animal feed.

Fungi with cellulolytic abilities are widespread in the soil and comprised mostly soft rot fungi. The following fungi have been implicated in cellulose secretion: *Aspergillus niger, A. nidulans, A. oryzae, A. terreus, Fusarium solani, F. oxysporum, Humicola insolens, H. grisea, Melanocarpus albomyces, Penicillium brasilianum, P. occ1itanis, P. decumbans, P. fumigosum, Trichoderma reesei, T. longibrachiatum, T. harzianum, Chaetomium cellulyticum, C. thermophilum, Neurospora crassa, Thermoascus aurantiacus, Mucor circinelloides, P. janthinellum, Paecilomyces inflatus, P. echinulatum, Trichoderma atroviride*; brown rot fungi: *Coniophora puteana, Lanzitestrabeum, Poria placenta, Tyromyces palustris, Fomitopsis* sp.; and white rot fungi: *Phanerochaete chrysosporium, Sporotrichum thermophile, Trametes versicolor, Agaricus arvensis, Pleurotus ostreatus, Phlebia gigantean* [4].

Cellulases obtained from aerobic fungi are particularly preferred in the industry. This is because they are extracellular, adaptive in nature, and usually secreted in large quantities during exponential phase. These aforementioned features distinguish aerobic fungal cellulases from bacterial as well as from anaerobic fungal cellulases. Anaerobic fungal cellulasesexist as tight multi-enzyme complexes, often membrane-bound as cellulosomes, from which it is difficult to recover individual active enzyme species; hence, economically less important [5,6]. There are widespread reports of cellulolytic enzyme production from aerobic fungi. Some of the aerobic fungi reported for efficient cellulase production include members of the following genera: *Aspergillus* [7]*, Penicillium* [8], and *Trichoderma* [9]. Fungal cellulases are structurally simple, with functionally distinct modules or domains [10].

The choice of substrate is an essential consideration in cellulase production. Different substrates have been employed in the production of cellulase, and they range from pure cellulose [11] to diary manure [12]. Recently, agro residues have been used as carbon sources in cellulase fermentations. Lignocellulosic biomass and other readily available substrates offer great opportunity in producing cellulose by microorganisms at very reduced process cost. Many lignocellulosic feedstocks which is currently under exploited utilized have been reported for the production of cellulases by microorganisms as substrates. Lignocellulosic residue consists of cellulose, hemicelluloses, lignin, water, protein and other compounds.Amongst them include wheat bran, rice bran, corn cob [13], corn straw, corn stalk and husks, sugarcane bagasse and cassava peels [14,15,3], saw dust [8] among others. Hydrolysis of lignocellulosic material is usually achieved through mechanical treatment, steam explosion, ultrasonic pretreatment, acid pretreatment, alkaline pretreatment followed by enzyme hydrolysis.

Two techniques mainly used for cellulose production are submerged fermentation (SmF) and solid state fermentation. The fermentation technology used can greatly determine the success of the enzyme production. Solid state fermentation (SSF) by fungi is a preferable production route for cellulase as it offers lower cost and enables the production of cellulase with higher titre [16]. Other advantages of SSF include maximum productivity, ease of technique, low capital investment, low energy requirement and less water output, better product recovery, and lack of foam build-up. Moreover, SSF has been reported to be most appropriate cellulase production process for developing countries [17,18].

Furthermore, improved cellulase production requires the optimization of the fermentation conditions. Microbial cellulases are inducible in nature and their production can be influenced by several factors such as pH, temperature, agitation, substrate concentration, type of nitrogen and carbon source etc. Various metal ions and chemical compounds may also influence cellulase activity. However, metal ions such as $Hg^{2+}$, $Cu^{2+}$, $Zn^{2+}$, $Mg^{2+}$, $Fe^{3+}$, $Mn^{2+}$, $Ag^{+}$, $Mn^{2+}$, $K^{+}$, may be slightly or completely inhibitory to cellulase, whereas other metal ions such as $Ca^{2+}$, $Na^{+}$ and $Co^{2+}$ stimulate the activity of cellulase [19,20,6]. This study investigated the production and optimization of cellulase from *Penicillium* sp. using corn-cob and pawpaw fibre as substrates.

## 2. MATERIALS AND METHODS

### 2.1 Lignocellulosic Substrate Collection, Processing, Comminution and Pretreatment

The PF and CC used in this study were collected from the Department of Chemistry, University of Port Harcourt, Nigeria and processed using the method described by Ezebuiro et al. [15] with slight modification. In brief, the biomasses were washed and oven-dried at 130°C for 5 h. The dry biomasses were further grinded with an electric blender (Philips blender HR2001, Japan), filtered with a 60 Mesh (0.250 mm) sieve, stored in screw-cap glass containers and labelled accordingly for subsequent use.

Pretreatment was achieved by soaking method. Ten milligram (10 g) of each of the processed substrate was suspended in 200 mL of distilled water in a beaker and allowed to soak overnight. After soaking, each substrate was boiled for 5 min in 250 mL beaker to break the bonds of cellulose subunits, resulting in the release of simple sugars.

### 2.2 Isolation and Screening of Fungi for Cellulolytic Activity

The soil samples that served as source of fungus used in this study were collected from the bottom of decaying agricultural waste dumps within University of Port Harcourt, Choba, Nigeria. These soil samples were mixed together and crushed into small particles in a mortar. One gram of the soil mixture was suspended in 9 mL of physiological saline in a 250 mL beaker and stirred properly. The suspension was filtered with Whatman™ No 1 filter paper (Whatman, UK) and the resulting filtrate used for the isolation of cellulolytic fungi and yeasts.

The modified mineral medium of Nwodo-Chinedu [21] was used for the screening the fungal isolates. The medium contained: $KH_2PO_4$, 1.3 g; $NH_4Cl$, 0.5 g; and $MgSO_4.7H_2O$, 0.2 g in 1000 mL of distilled water. However, the medium was modified by adding $NaNO_3$, 1.5 g; and yeast extract, 0.5 g to supply nitrate and mineral elements, respectively. For each of the pretreated substrates, 2 g was taken and suspended in 25 mL of the mineral medium in 250 mL beaker (pH 5.0). The content was transferred to 100 mL conical flask with 5 mL of the soil filtrate added and mixed properly. A control flask (containing mineral medium without inoculum) was also set up. All culture flasks and the control were incubated at room temperature until growth of fungi appeared.

Serial dilution was performed according to the method described by Nanhini and Josephine [22]. Nine millilitres (9 mL) of normal saline (0.85% NaCl in distilled water) was first dispensed into each clean test tube; the test tubes and their contents were sterilized in an autoclave at 121°C (15 psi) for 15 min. To prepare stock solution, 10 g of the dry soil sample was dissolved in 90 mL of sterile normal saline; from this stock solution $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$ and $10^{-6}$ dilutions were made. Aliquots (0.1 mL each) from $10^{-3}$ to $10^{-6}$ dilutions were plated out onto sterile mineral salt agar, with 2 g of each substrate as sole source of carbon (the media were supplemented with ampicillin [100 µg/mL] to suppress the growth of bacteria). The inoculated plates were incubated at room temperature for 6 days until growth of fungi developed. Distinct colonies on the agar plates were transferred to freshly prepared PDA slants and stored at 4°C for further studies.

All nine isolates recovered from the soil samples were screened for cellulase production by spot-inoculating each of them on agar plates containing either CC or PF as sole carbon source. The plates were incubated for 5 days at room temperature. Thereafter, the inoculated plates were flooded with 0.1% (w/v) Congo red for 30 min. The plates were destained with 1M NaCl. Cellulase-producing isolates produced clear zones around the cultures, indicating cellulase production [3]. The diameter of the halo zones produced was measured with a metre rule and recorded. The best isolate with the highest zone of clearance was chosen for further studies.

### 2.3 Determination of Enzyme Activity

The method of Miller [23] was employed in determining the enzyme activity assay. One millilitre of crude enzyme filtrate was added to 1 mL of 0.1% (w/v) carboxymethyl cellulose in a test tube. Sodium

acetate buffer (0.1 mL, pH 6.5) was pipetted into the mixture and then incubated in a Water Bath (HH – W420, China) at 35$^{o}$C for 30 min. After incubation, 3 mL of the DNS reagent was added to terminate the reaction. The tube was boiled for 5 min and monitored for colour development. Optical density readings were taken at 546 nm and the sugar released extrapolated from the standard glucose curve. The result obtained was then expressed as cellulase activity (U/mL) defined as the enzyme that produced 1 µM of glucose per min under the assay conditions.

## 2.4 Protein Determination

Protein determination method used was a modification of Lowry et al. [24] method. In this method, colour formation was observed; colour formation arises from the reaction of alkaline copper with the protein and the reduction of phosphomolybdate by tyrosine and tryptophan present in the protein. Precisely 0.2 mL of bovine serum albumin (BSA) working standard was pipetted into 5 mL test tubes and made up to 1 mL using distilled water. About 4.5 mL of Reagent I (48 mL of 2% $Na_2CO_3$ in 0.1 N NaOH, 1 mL of 1% NaK Tartrate in $H_2O$, and 1 mL 0.5% $CuSO_4.5H_2O$ in $H_2O$) was added and incubated for 10 min. After incubation, 0.5 mL of Reagent II (1 part Folin-Phenol [2 N]:1 part water) was added and incubated for 30 min. The absorbance was measured at 660 nm and the standard graph plotted. The amount of protein present in the given sample was estimated from the standard graph. Another test tube with 1 mL distilled water served as blank.

## 2.5 Determination of the Effect of Temperature on Cellulase Production

The influence of different incubation temperatures (30, 40, 50, 60, 70 and 80°C) on the production of cellulase by the isolate was studied. This was done by incubating the culture medium at different temperatures and assaying the culture broth for cellulase activity.

## 2.6 Determination of the Effect of Initial pH on Cellulase Production

The influence of different initial pH (4, 4.5, 5, 5.5, 6, 6.5, 7, 8, and 9) on the production of cellulase by the isolate was studied by adjusting the pH of the culture medium and assaying the culture broth for cellulase activity.

## 2.7 Determination of the Effect Metal Ions on Cellulase Production

The influence of different cations, in their salt forms ($CaCl_2$, $MgSO_4$, $ZnSO_4.7H_2O$, Co $(NO)_2.6H_2O$, $HgCl_2$ and EDTA crystalline salt), on the production of cellulase by the isolate using PF and CC as substrates were examined. Each solution of the compound at 10 mM concentration was prepared with distilled water. The reaction mixture contained 1 mL of 1% (w/v) CMC in 1 mL of 0.05 M citrate buffer of pH 6.5 and 1 mL of salt of the respective cations or EDTA. Reaction mixtures were equilibrated to room temperature for 45 min and then inoculated with 0.5 mL of appropriate crude enzyme (from *Penicillium* sp.). The inoculated enzyme-substrate mixture was incubated at 50°C in a Water Bath for 30 min and 1 h, respectively. After incubation, the enzyme-substrate mixture was cooled for 12 min and cellulase activity determined by DNS method [23].

## 2.8 Evaluation of the Effect of pH and Temperature on Cellulase Stability

Effect of pH on enzyme stability was performed as described by Nizamudeen and Bajaj [25] and Ponnuswamy and Prakash [26]. For pH stability study, 0.5 mL of the crude enzyme was pre-incubated without substrate; then, the buffer was adjusted to appropriate pH values at 50°C for 30 min and 1 h, respectively after which cellulase activity was determined.

To determine thermal stability of the enzyme, 0.5 mL of the crude cellulase was incubated without substrate at increasing temperatures of 30, 35, 40, 50, 60, 70 and 80°C for 30 min and 1 h, respectively and then assayed for cellulase activity.

### 2.9 Identification of the Cellulase-Producing Fungus

Benson et al. [27] fungal slide culture technique and the Colour Atlas for clinical fungi identification were used to identify the cellulase-producing fungus. The isolate was inoculated on the edge of sterilized 5 $cm^2$ block of PDA medium, cut out with sterile scalpel and placed on alcohol-sterilized glass slide. The inoculated agar block was covered with cover-slip and placed on top of the short piece of glass rod (8.9 cm) inside a petri dish whose bottom was lined with Whatman™ No 1 Qualitative filter paper. The filter paper was moistened with 5 mL of sterile distilled water to provide humid environment to the culture. The plate was covered and incubated at 30°C for 4 days. After incubation, the agar block culture was removed; the slide and cover-slip were stained with lactophenol cotton blue solution and examined under x40 and x10 objective lenses. Characteristics such as the presence or absence of conidia, phialides, septation and colour of mycelia were noted and used to identify the isolate.

## 3. RESULTS

### 3.1 Pawpaw Fibre (PF) and Corn-cob (CC) Collection, Processing, Comminution and Pretreatment

The PF and CC after processing and pretreatment generated usable substrates of size, 0.250 mm.

### 3.2 Isolation and Screening of Fungi for Cellulolytic Activity

Out of the nine fungal isolates screened for cellulolytic activity, CPF-1 was selected based on its ability to produce halo zones around the colonies when flooded with Congo red dye for 30 min and destained with 1M NaCl. The isolate also showed the highest activity when assayed for cellulase production.

### 3.3 Evaluation of the Effect of Temperature, pH and Metal Ions on the Production of Cellulase and Release of Reducing Sugar

Effects of temperature, pH, and metal ions on the production of cellulase by *Penicillium* sp. utilising either CC or PF as substrates are presented in Fig. 1. *Penicillium* sp. released highest concentration of sugar (19.871 µg/mL) from corn-cob at pH 5.0 during hydrolysis of the substrate. The temperature optimum for cellulase production by *Penicillium* sp. using corn-cob as sole source of carbon was 30°C. At this temperature, *Penicillium* sp. yielded the highest glucose concentration of 3.2435 µg/mL. At higher temperatures (40 to 70°C), the release of glucose depreciated slightly for both substrates.

### 3.4 Evaluation of the Effects of pH and Temperature on Crude Enzyme Stability

Effects of pH and temperature on the stability of the cellulase secreted by *Penicillium* sp. are presented in Fig. 2. From the figure, the cellulase was stable over a pH range of 4.5 to 6; being most stable at pH 5 with corn-con as the substrate. For temperature, the cellulase was stable over a temperature range of 30 to 80°C, with the highest stability observed at 40°C when corn-cob was used as substrate.

### 3.5 Protein Determination

Table 1 presents the protein concentration of the crude enzyme obtained from *Penicillium* sp. when grown on CC and PF substrates, respectively. From the table, the isolate yielded more protein with CC substrate than with PF.

### 3.6 Isolation and Identification of Organisms

The cellulolytic fungus selected for this study was isolate CPF-1 and was identified based on cultural and morphological features and microscopically, under x40 objective lens. *Penicillium* sp. produced

blue-green to ash colonies on PDA plates (Plate 1). Microscopically, it had septate hyphae, branched conidiophores and brush-like conidial head.

**Table 1. Protein estimation of the crude enzyme secreted by *Penicillium* sp. with different substrates**

| Substrate | $OD_{745nm}$ | Protein concentration ($\mu gmL^{-1}$) |
|---|---|---|
| Blank | 0.00 | 0.00 |
| Pawpaw | 0.068 | 3.573 |
| Corn-cob | 0.4025 | 22.104 |

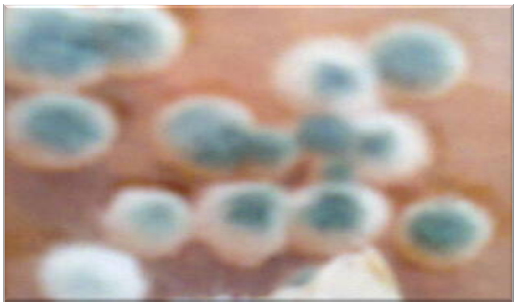


**Plate 1. Cultural morphology of *Penicillium* sp. on PDA medium supplemented with corn-cob as sole carbon source**

*Colonies were greenish in colour with radiated ring on PDA plate; microscopic examination revealed branched conidiospores of long chains on conidia*

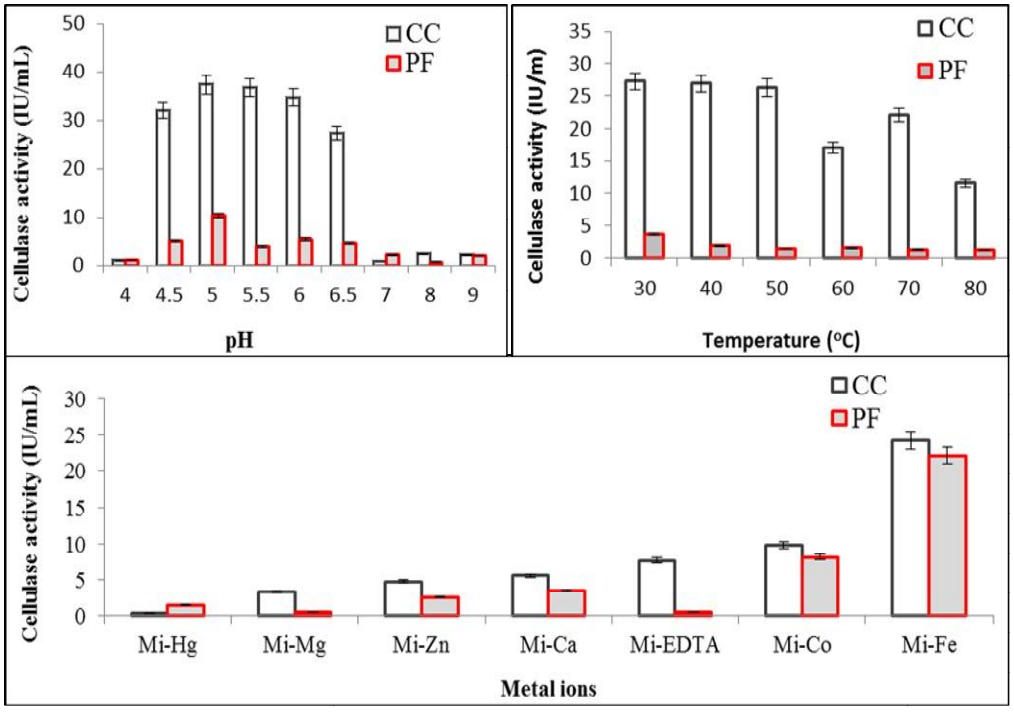



**Fig. 1. Effects of pH, temperature, and metal ions on the activity of cellulase secreted by *Penicillium* sp. using different substrates**

*Legend: Mi-Hg = $HgCl_2$; Mi-Zn = $ZnSO_4$; Mi-Ca = $CaCl_2$; Mi-EDTA = EDTA; Mi-Mg = $MgSO_4$; Mi-Co = $Co(NO_3)_2.6H_20$; Mi-Fe = $FeSO_4.7H_2O$; CC = Corn-cob; PF = Pawpaw fibre*

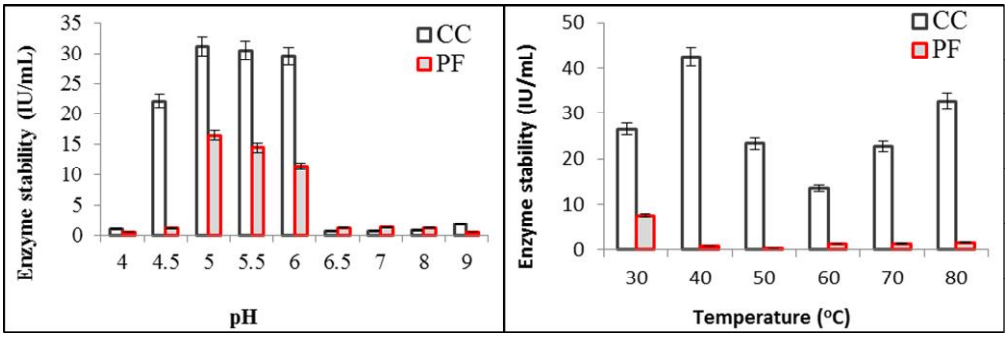



**Fig. 2. Effects of pH and temperature on the stability of cellulase secreted by *Penicillium* sp. using different substrates**

*Legend: CC = Corn-cob; PF = Pawpaw fibre*

**Table 2. Optical density, sugar released, and cellulase activity from solid state fermentation of CC and PF substrates by *Penicillium* sp.**

| Parameters | CC substrate | | | PF substrate | | |
|---|---|---|---|---|---|---|
| | $OD_{546}$ | Sugar (µg $mL^{-1}$) | CMCase (IU $mL^{-1}$) | $OD_{546}$ | Sugar (µg $mL^{-1}$) | CMCase (IU $mL^{-1}$) |
| **pH** | | | | | | |
| 4.0 | 0.025 | 0.602 | 1.034 | 0.037 | 0.911 | 1.565 |
| 4.5 | 0.613 | 15.759 | 27.085 | 0.035 | 0.859 | 1.476 |
| 5.0 | 0.773 | 19.871 | 34.150 | 0.007 | 0.124 | 0.213 |
| 5.5 | 0.758 | 19.497 | 33.508 | 0.023 | 0.537 | 0.923 |
| 6.0 | 0.725 | 18.647 | 32.048 | 0.034 | 0.833 | 1.432 |
| 6.5 | 0.319 | 8.180 | 14.059 | 0.019 | 0.434 | 0.745 |
| 7.0 | 0.021 | 0.486 | 0.835 | 0.022 | 0.524 | 0.901 |
| 8.0 | 0.035 | 0.859 | 1.476 | 0.017 | 0.395 | 0.679 |
| 9.0 | 0.048 | 1.194 | 2.052 | 0.034 | 0.821 | 1.410 |
| **Temperature** | | | | | | |
| 30 | 0.1275 | 3.2435 | 5.574 | 0.048 | 1.181 | 2.030 |
| 40 | 0.044 | 1.0905 | 1.276 | 0.044 | 1.078 | 1.853 |
| 50 | 0.0205 | 0.4855 | 0.8345 | 0.061 | 1.529 | 2.628 |
| 60 | 0.032 | 0.782 | 1.344 | 0.022 | 0.524 | 0.901 |
| 70 | 0.031 | 0.756 | 1.2995 | 0.033 | 0.795 | 1.367 |
| 80 | 0.033 | 0.8075 | 1.388 | 0.040 | 0.988 | 1.698 |
| **Metal ions** | | | | | | |
| $HgCl_2$ | 0.011 | 1.240 | 0.412 | 0.036 | 0.885 | 1.521 |
| $MgSO_4$ | 0.076 | 1.916 | 3.293 | 0.013 | 1.916 | 0.502 |
| $ZnSO_4$ | 0.109 | 2.767 | 4.756 | 0.061 | 1.529 | 6.628 |
| $CaCl_2$ | 0.128 | 3.256 | 5.596 | 0.08 | 2.019 | 3.47 |
| EDTA | 0.176 | 4.494 | 7.724 | 0.014 | 0.318 | 0.547 |
| Co $(NO_3)_2.6H_2O$ | 0.224 | 5.731 | 9.850 | 0.187 | 4.777 | 8.210 |
| $FeSO_4.7H_2O$ | 0.550 | 14.135 | 24.293 | 0.502 | 12.898 | 22.166 |

*Values represent mean of fermentations at 30 min and 1 h, respectively.*
*Legend: $OD_{546}$ – optical density at 546 nm*

## 4. DISCUSSION

This study investigated the production and optimization of cellulase from *Penicillium* sp. using to lignocellulosic residues: corn-cob (CC) and pawpaw fibre (PF) as substrates. Corn-cob, also known

as cob of corn, is the central core of an ear of maize (*Zea mays*). It refers to the part of the ear on which the kernels grow. The chemical composition of corn-cob has been previously reported by Harini and Kumaresan [28]. Corn-cob can also be described as an agricultural lignocellulosic residue with high cellulosic content [28]. Similarly, paw paw fibre is a lignocellulsic derived from paw paw (*Carica papaya*). Proximate chemical analysis of paw paw has been reported by Awe et al. [29]. These substrates were chosen because of their high carbohydrate (especially cellulose) content and their availability.

Corn-cob (CC) used for cellulase production in this study is available locally and has been extensively reported [30-32] for use in cellulase production. However, there is dearth of information on the use of paw paw fibre (PF) in cellulase production. One of the uniqueness of this research is in its examination of locally available but scarcely researched substrate such as paw paw fibre for cellulase production. Although, corn-cob yielded more cellulase when compared to paw paw fibre as substrate, research on different pretreatment methods for paw paw fibre prior to solid state fermentation may yet improve cellulase production from paw paw fibre.

The effect of pH on cellulase production by *Penicillium* sp. was studied. The optimum pH for cellulase production was pH 5 while the fungus was able to secrete cellulase at pH range of 4.5 to 6.5. Similar optimum pH for cellulase production has been reported in other studies. Deshmukh et al. [33] reported pH 5 as the optimum pH for cellulase production by *Aspergillus niger subsp. awamori*. Similarly, Matsakas et al. [34] reported optimum pH of 5.5 by *Myceliophthora thermophilia* utilizing pure cellulose. Ja'afaru and Fagade [35] reported maximum cellulase production at pH 5.0 by *A. niger* YL128; although they also reported significant levels of cellulase with other pH. Either low or high pH values may inactivate cellulase and thus affect its production. There is strong evidence that optimum pH for cellulase production varies amongst different organisms. pH 4.0 to 5.5 was reported for *A. terreus* by Garg and Neelakantan [36] and Pushelkar et al. [37]. pH 4.5 to 5.0 for *Rhizopus oryzae* and pH 5.0 to 6.0 for *Sclerotium rolfsii* have been reported by Darmwal [38] and Amadioha [39], respectively.

Temperature plays important role in the production of cellulase by microorganisms. The optimum temperature range for cellulase production in this study was between 30 and 50°C. Temperatures above 50°C were not suitable in enhancing cellulase production and activity. The result obtained in this study is similar to the findings of Ja'afaru and Fagade [34] who reported 30°C as optimum temperature for cellulase production by *A. niger* YL128. Their isolate was also able to secrete cellulase at a temperature range of 30 to 50°C. Ja'afaru and Fagade [34] reported that temperatures below 30°C and above 50°C may not be conducive for mycelial growth and cellulase synthesis. However, the result of this finding contradicts Matsakas et al. [33] report of highest cellulase activity at 65°C by *M. thermophilia*. More so, different cellulase-producing fungi thrive at different optimum temperatures.

The result obtained from the effect of various metal ions ($Hg^{2+}$, $Mg^{2+}$, $Zn^{2+}$, $Ca^{2+}$, EDTA, $Co^{2+}$, and $Fe^{2+}$) on the production of cellulase by *Penicillium* sp. showed that $Fe^{2+}$ and $Co^{2+}$ stimulated cellulase production; whereas, the other metal ions inhibited cellulase production. These findings are similar but not totally in agreement with the reports by Bagga et al. [40], Yan et al. [41], Dutta et al. [19], and Nazir et al. [42]. They reported that metal ions such as $Hg^{2+}$, $Cu^{2+}$, $Zn^{2+}$, $Mg^{2+}$, $Fe^{3+}$, $Mn^{2+}$, $Ag^{+}$, $Mn^{2+}$, and $K^{+}$ are slightly or completely inhibitory to cellulase production, whereas metal ions such as $Ca^{2+}$, $Na^{+}$, and $Co^{2+}$ stimulate the activity of cellulase. The difference is that $Ca^{2+}$ was not able to stimulate cellulase production in this present study. Meanwhile, Sajith et al. [6] reported differences in metal ions preference for cellulase production by different fungal isolates. Furthermore, Deshmukh et al. [33] reported that $Ca^{2+}$, $Mg^{2+}$, $Zn^{2+}$, $Pb^{2+}$, and $Fe^{2+}$, all had positive influence on cellulolytic activity; although they used *Aspergillus niger* as the fermenting fungus.

## 5. CONCLUSION

The outcome of this work revealed that corn-cob (CC) and pawpaw fibre (PF) present huge potential as low-cost substrates for cellulase production by *Penicillium* sp. through solid state fermentation (SSF). This finding is particularly interesting considering that these substrates are readily available

and inexpensive for large-scale cellulase production by the fungus. Moreover, the optimum cellulase productivity of 15.787 IU/mL and 2.141 IU/mL obtained with CC and PF as substrates indicate that these substrates may be suitable for large-scale cellulase production. In addition, the study revealed that pH, temperature, and metal ions influenced the ability of *Penicillium* sp. to secrete cellulase while utilising CC and PF as substrates. The cellulase was stable over broad range of temperature, which is an important characteristic for industrial scale production of cellulase. Thus, the two lignocellulosic biomass (pawpaw peels and corn cob) has shown a good alternative to formulate a simple inexpensive culture medium for cellulase production by *Penicillium* sp. The use of these cost-effective and environmentally sustainable lignocellulosic residues as alternative feedstocks for enzyme production will help in ameliorating the pollution problems posed by their indiscriminate disposal in the environment.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

## REFERENCES

1. Behera SS, Ray RC. Solid state fermentation for production of microbial cellulases: Recent advances and improvement strategies. Int J Biol Macromol. 2016;86:656-669.
2. Bozell JJ, Petersen GR. Technology development for the production of biobased products from biorefinery carbohydrates—the US Department of Energy's "Top 10" revisited. GreenChem. 2010;12:539–554.
3. Ire FS, Ezebuiro V, Ogugbue CJ. Production of bioethanol by bacterial co-culture from agro-wastes impacted soil through simultaneous saccharification and co-fermentation of steam exploded bagasse. Bioresour Bioprocess. 2016;3:26.
   DOI: 10.1186/s40643-016-0104-x
4. Kuhad RC, Gupta R, Singh A. Microbial cellulases and their industrial applications. EnzymeRes. 2011;1-10.
   DOI: 10.4061/2011/280696
5. Mathew AP, Thielemans W, Dufresne A. Mechanical properties of nanocomposites from sorbitol plasticized starch and tunicin whiskers. J ApplPolSci. 2008;109:4065-4074.
6. Sajith S, Priji P, Sreedevi S, Benjamin S. An overview on fungal cellulases with an industrial perspective. J Nutr Food Sci. 2016;6:461.
   DOI: 10.4172/2155-9600.1000461
7. Amaike SC, Lynn J, Sibert JS, Srikrishnan S, Phatale P, Feldman T, Guenther JM, Hiras J, Tran YTA, Singer SW, Adams PD, Sale KL, Simmons BA, Baker SE, Magnuson JK, Gladden JM. Expression of naturally ionic liquid-tolerant thermophic cellulases in *Aspergillus niger.* PloS One. 2017;12(12).
   Available:http://doi.org/10.1371/journal.pone.0189604
8. Prasanna HN, Ramanjaneyulu G, Reddy BR. Optimization of cellulase production by *Penicillium* sp. 3 Biotech. 2016;6:162.
   DOI: 10.1007/s13205-016-0483-x
9. Andrade JP, Bispo ASDR, Marbach PAS, Nascimento RP. Production and partial characterization of cellulases from *Trichoderma* sp. IS-05 isolated from sandy coastal plains of northeast Brazil. Enzyme Res. 2011;1-7.
   DOI: 10.4061/2011/167248
10. Bayer EA, Chanzy H, Lamed R, Shoham Y. Cellulose, cellulases and cellulosomes. Curr Opin Struct Biol. 1998;8:548-557.
11. Szabo IJ, Johansson G. Optimized cellulase production by *Phanerochaete chrysosporium*: Control of catabolite repression by fed-batch cultivation. JBiotechnol. 1996;48:221-30.
12. Wen Z, Liao W, Chen S. Production of cellulose by *Trichoderma reesei* from dairy manure. Bioresour Technol. 1999;96:491-499.
13. Okoye IG, Ezugwu AL, Udenwobele DI, Eze SOO, Anyawu CU, Chilaka FC. Production and partial characterization of cellulases from *Aspergillus fumigatus* using two distinct parts of corn cob as carbon sources. Nig J Biotechnol. 2013;26:50-59.

14. Behera B, Parida S, Dutta S, Thatoi H. Isolation and identification of cellulose degrading bacteria from mangrove soil of Mahanadi River Delta and their cellulase production ability. Am J Microbiol Res. 2014;2(1):41-46.
15. Ezebuiro V, Ogugbue CJ, Oruwari B, Ire FS. Bioethanol production by an ethanol-tolerant *Bacillus cereus* strain GBPS9 using sugarcane bagasse and cassava peels as feedstocks. J Biotechnol Biomater. 2015;5:213.
DOI: 10.4172/2155-952X.1000213
16. Yoon LW, Ang TN, Ngoh GC, Chua ASM. Fungal solid-state fermentation and various methods of enhancement in cellulase production. Biomass Bioenergy. 2014;67:319–338.
DOI: 10.1016/j.biombioe.2014.05.013
17. Zeng HZ, Chen ZW. Air pressure pulsation solid state fermentation of feruloyl esterase by *Aspergillus niger*. Bioresour Technol. 2009;100:1371-1375.
18. Souza PM, Magalhaes PO. Application of microbial amylase in industry- a review. Braz J Microbiol. 2010;41:850-861.
19. Dutta T, Sahoo R, Sengupta R, Ray SS, Bhattacharjee A, Ghosh S. Novel cellulases from an extremophilic filamentous fungi *Penicillium citrinum*: Production and characterization. J Ind Microbiol Biotechnol. 2008;35:275-282.
20. Nazir A, Soni R, Saini H, Manhas R, Chadha B. Purification and characterization of an endoglucanase from Aspergillusterreus highly active against barley β-glucan and xyloglucan. World J Microbiol Biotechnol. 2009;25:1189-1197.
21. Nwodo-Chinedu S, Okochi VI, Smith HA, Omidiji O. Isolation of cellulolytic microfungi involved in wood-waste decomposition: Prospect for enzymatic hydrolysis of cellulosic wastes. Int J Biomed Health Sci. 2005;1(2):41-51.
22. Nandhini B, Josephine MR. A study on bacterial and fungal diversity in potted soil. Int J Curr Microbiol Appl Sci. 2013;2:1-5.
23. Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Annu Chem. 1972;31:426-428.
24. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein Measurement with the Folin Phenol Reagent. J Bio Chem. 1951;193:265-275.
25. Nizamudeen S, Bajaj B. A novel thermo-alkali tolerant endoglucanase production using cost-effective agricultural residues as substrates by a newly isolated *Bacillus* sp. NZ.Food Sci. Biotechnol. 2009;47:435-440.
26. Ponnuswamy V, Prakash VSG. Purification and characterisation of carboxymethyl-cellulose from *Bacillus* sp. isolated from a paddy field. Polish J Microbiology. 2012;61:51-55.
27. Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J, Wheeler DL. GenBank. Nucleic Acids Res. 2008;36:D25-30.
28. Harini S, Kumaresan R. Production Of cellulase from corn-cobs by *Aspergillus niger* under submerged fermentation. Int J Chemtech Res. 2014;6(5):2900-2904.
29. Awe S, Eniola KIT, Ka yode-Ishola TM. Proximate and mineral composition of locally produced paw paw and banana wine. Am J Res Commun. 2013;1(12):388-397.
30. Ojumu TV, Solomon BO, Betiku E, Layokun SK, Amigun B. Cellulase production by *Aspergillus flavus* Linn isolate NSPR 101 fermented in sawdust, bagasse and corncob. Afr J Biotechnol. 2003;2(6):150-152.
31. Ijaz A, Anwar Z, Zafar Y, Hussain I, Muhammad A, Irshad M, Mehmood S. Optimization of cellulase enzyme production from corn cobs using *Alternariaalternata* by solid state fermentation. J Cell Mol Biol. 2011;9(2):51-56.
32. Xiong L, Huang C, Peng W-F, Tang L-R, Yang X-Y, Chen XF, Chen X-D, M, L-L¸ Chen Y. Efficient cellulase production from low-cost substrates by *Trichoderma reesei* and its application on the enzymatic hydrolysis of corncob. Afr J Microbiol Res. 2013;7(43):5018-5024.
DOI: 10.5897/AJMR12.1761
33. Deshmukh S, More S, Aranganathan V, Sullia SB, Pachauri P. Kinetic study and characterization of cellulase enzyme from isolated *Aspergillus niger subsp. Awomori* for cellulosic biofuel. J Sci Ind Res. 2018;77:55-60.
34. Matsakas L, Antonopoulou I, Christakopoulos P. Evaluation of *Myceliopthora thermophila* as an enzyme factory for the production of thermophilic cellulolytic enzyme. Bio Resources. 2015; 10(3):5140-5158.

35. Ja'afaru MI, Fagade OE. Optimization studies on cellulase enzyme production by an isolated strain of *Aspergillus niger* YL128. Afr J Microbiol Res. 2010;4(24):2635-2639.
36. Garg SK, Neelakantan S. Effect of cultural factors on cellulase activity and protein production by *Aspergillus terreus*. Biotechnol Bioeng. 1981;23:1653-1659.
37. Pushelkar S, Rao KK, Menon K. Production of beta-glucosidase by *Aspergillus terreus*. Curr Microbiol. 1995;30:255-258.
38. Darmwal NS. Studies on cellulolytic fungi for cellulase and protein production and their associative effect with *Azospirillum lipoferum* on plant growth. Ph.D Thesis, Indian Agricultural Research Institute, New Delhi; 1986.
39. Amadioha, AC. Production of cellulolytic enzymes by *Rhizopus oryzae* in culture and Rhizopus-infected tissues of potato tubers. Mycologia. 1993;85:574-578.
40. Bagga PS, Sandhu DK, Sharma S. Purification and characterization of cellulolytic enzymes produced by *Aspergillus nidulans*. J Appl Bacteriol. 1990;68:61-68.
41. Yan TR, Lin CL. Purification and characterization of a glucose-tolerant beta-glucosidase from *Aspergillus niger* CCRC 31494. Biosci Biotechnol Biochem. 1997;61:965-970.
42. Nazir A, Soni R, Saini H, Manhas R, Chadha B. Purification and characterization of an endoglucanase from *Aspergillus terreus* highly active against barley ß-glucan and xyloglucan. World J Microbiol Biotechnol. 2009;25:1189-1197.

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***Chapter 13***
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# A Critical Review on Effects of Metosartan on Testes Tissue

**Eswari Beeram[1,2*]**

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## ABSTRACT

Metosartan induces so many deleterious effects in male Wistar rats treated with drug and RNase. It causes pre maturation of sperms and reduces the sperm count as well as reduces viable sperm count. It induces the x-chromosome desynapsis leading to testicular carcinoma. The effects are so profound in case of drug than compared to the enzyme RNase. Metosartan induces apoptosis in testicular tissue mainly through intrinsic pathway which is mainly due to genotoxic agents. The drug mainly inhibits RNase A in rat testes and has positive effect on Mitosis. Metosartan mainly effects chromatin compaction thus increasing access to nucleases which should be confirmed in invivo. RNase A effects the assembly of nuclear pore complex after mitosis but it does not inhibit the synthesis of NPC.

*Keywords: Metosartan; RNase A; desynapsis; netosis; sperm count; pre maturation; apoptosis.*

## 1. INTRODUCTION

Metosartan is one of the antihypertensive drugs taken for study to assess reproductive potential in male rats. It mainly consists of two components metoprololsuccinate and telmisartan which induces ds breaks in DNA. Metoprolyl known to affect sperm motility so, metosartan was taken to assess reproductive potential and to study drug interactions with DNA. The collapse of chromatin is observed during apoptosis which requires fragmentation of genome in the cells [1]. DNase digestion of chromatin leads to degradation of linker DNA leaving the nucleosomes. As RNA is also a component of chromatin along with DNA and protein, and RNase a treatment also causes disruption of chromatin [2].

Ion exchange chromatography is one of the column chromatographic techniques that aid in separation of proteins based on their charge. Cation exchange chromatography is used to separate cationic proteins and anion exchange chromatography to separate anionic proteins. In my study DEAE and carboxy methylcellulose were used for separation of RNase A from rat testes [3]. Cytochrome C is a water-soluble protein of molecular weight 12 KDa. It exists mainly in a reduced form which prevents apoptosis and acts as electron carrier in electron transport chain. NADPH regulates the reduced form of Cyt C through regeneration of glutathione. Nucleotides like NADPH binds to Cyt C and prevent its binding to APAF1 inhibiting apoptosis Cyt C is a heme protein that gives maxima between 390-600 nm in oxidized form metosartan was known to cause apoptosis by the release of Cyt C from testes and sperm recorded using UV Visible spectrophotometry [4].

## 2. EFFECTS OF METOSARTAN ON TESTES

Metosartan induces double-stranded breaks in testicular DNA which are minimized by RNaseA treatment to some extent. Study of testicular tissue using aniline blue staining resulted in pre maturation in sperms compared to RNase A. Histological examination in deep freeze condition and fresh examined tissue showed the development of yolk sac tumor in testis tissue whereas RNase

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treatment resulted in suppression of tumor compared to combined effect of both RNase A and metosartan as the drug reversibly inhibits metosartan through both competitive and non-competitive inhibition [5].

The drug maximally inhibits RNase A for 3 hrs. and not more than 4 hrs. The i/ki ratio is about-1.6 which indicates inhibition of RNase A by metosartan. From the recent reports of mine RNase A in rats was successfully isolated from the testis with molecular weight of 24 kDa. The isolated enzyme was subjected to agarose gel electrophoresis followed by metosartan treatment resulted in no catalytic activity of the enzyme with regard to cleavage of substrate RNA. Telmisartan is the component of metosartan responsible for inhibition of RNase A whereas Metoprolol is responsible for cytotoxic activity of metosartan associated with testes [6].

The drug-induced the nuclear pore enlargement in testes through association with epigenetic regulation of chromatin dynamics. The metosartan has reducing property of disulfide bonds which may lead to collapse or disassembly of chromatin of mature sperms leading to male infertility. In hypertensive patients, the damage is minimal with regarding to ds breaks and DNA fragmentation but the presence of apoptosis is observed with agarose gel electrophoresis as a measure of DNA fragmentation index. The drug also caused aspermia in hypertensive rats treated with metosartan.

Metosartan induced apoptosis in testes tissue treated *in-vivo* with the drug through oral gavage and also caused desynapsis of the x-chromosome in sperms leading to genetic aberrations in the offspring [7]. Long term analysis of drug showed no histological aberrations in the testes tissue compared to *in-vitro* which caused the adenocarcinoma of the testes tissue. The reason may be due to direct exposure to the drug at higher concentrations. RNase caused apoptosis of the cells but the profound effect was seen in drug-treated rats compared to normal rats. Isolation of enzyme was done by DEAE and Carboxymethyl cellulose resins prepared in the laboratory using buffers. As DEAE cellulose exchanges the anions which result in separation of RNase of Testes and to elute first itself as it is a basic protein. MALDI-TOF mascot results showed expression of Cyt c and death protein-6 in the testes [8].

RNase A-induced Netosis in testes tissue which is inhibited by treatment with metosartan which is due to non-availability of RNase A. The induction of Netosis with RNase A is due to immune responsive reactions seen in rats as the enzyme is from bovine pancreas. The neutrophils in-filtered form the extracellular traps seen in Netosis induced by the RNase A. The drug may prevent the immunogenic reactions by sequestering the antigen in rats. SEM examination of testes tissue helps in identification of Netosis induced by RNaseA [9].

RNaseA activity showed peak activity in two fractions isolated by ion-exchange chromatography. HPLC identification and qualitative analysis showed and identified RNase A is present in first fraction. The number of viable sperms was decreased with metosartan compared to RNase and metosartan. The fractions isolated showed contamination in the form of RNA which is degraded after treatment with pure RNase A. The cytotoxic effect of metosartan is mainly due to its direct effect on DNA of sperms as sperm cells lack DNA repair mechanisms to counteract the damage induced by the drug [10].

Recent studies on Coriandrumsativum, Menthaspicata and Camellia. sinensis (Green tea) protection against ssbreaks induced by metosartan green tea showed maximum protection compared to C.sativum and M.spicata. C.sativum induced foci in both presence and absence of metosartan and M.spicata also showed some amount of ss breaks induction but the green tea C.sinensis showed 0% ss breaks formation in both presence and absence of metosartan.

Metosartan effect on chromatin compaction requires studies on interaction of metosartan with chromatin decompaction proteins like Histonedeacetylases and cell cycle studies on metosartan with long term treatment showed increased apoptosis which is compensated with increased cell division (more number of cells in S-Phase) compared to control. Histological examination was not effected with long term treatment in rat testes but Q –banding showed nucleosomes dissociated from DNA which represents DNA decompaction in testes samples [11].

**Table 1. Effect of metosartan on male fertility and genome integrity in male wistar rats**

| S. no | Objectives studied | Metosartan treated | RNase A treated | Both RNase A and metosartan treated | Relation with Metosartan |
|---|---|---|---|---|---|
| 1. | Histology studies | Normal | Normal | Normal | +ve |
| 2. | Cell cycle | More cells with n Ploidy | Not known | More cells with both 2n and 4n ploidy | -ve |
| 3. | Sperm count | High in invitro sperm but resulted in Aspermia in long term | Not known | decreased | -ve |
| 4. | Sperm viability | decreased | Not known | No dead sperms | -ve |
| 5. | Apoptosis | Profoundly increased | arrested | Minimum | -ve |
| 6. | Ss DNA breaks | Profoundly increased | less | Less due to non availability of metosartan in presence of RNaseA | -ve |
| 7. | Inhibition studies | - | - | Inhibition of RNase A by metosartan | -ve effect on RNase A |
| 8. | Release of cyt C | observed | Not known | arrested | -ve |
| 9. | Mitochondial viability in testes cells | >30’ | Not known | Up to 10’ | +ve with respect to M+R treated group |
| 10. | Sperm morphology | Acrosome reacted sperms | Not known | Release of sperm DNA | -ve |
| 11. | Genome integrity in testes and sperms | Greatly affected | Less affected | Induction of apoptosis is seen in *in vivo* | -ve |

## 3. DISCUSSION AND CONCLUSION

RNase A is found to be expressed in pancreas and secreted into digestive track to degrade the RNA present in the food. There are reports regarding protection of chromatin collapse by RNase H2. So, identification of RNase present in testes is a crucial thing as the drug intake disturbs chromatin integrity. Epididymis consists of RNase 10 which belongs to RNaseAsuperfamily. So, experiments were conducted in our lab for isolation, identification and characterization of RNase present in testes. Rat testis RNase A is isolated by ion-exchange chromatography and further resolved by HPLC ino two different Ribonuclease and MALDI- TOF MS was used for confirmation and to know the expression of proteins in the fraction separated by ion-exchange chromatography. SDS-PAGE and Immunofluorescence have confirmed the presence of RNase A in testis and its location are intracellular in testis and extracellular in pancreas.

In addition to the confirmation of RNase A the protein profile from MALDI showed the expression of Cyt C which is both somatic and germ cell origin. Programmed cell death protein 6 was another protein present in the fraction that is specific to Rattus mitochondria. As treatment of testes in combination with RNase A and metosartan showed less chromatin cleavage compared with RNaseA treated sample indicates maybe the drug inhibits the RNase A. So, studies based on Enzyme kinetics and Enzyme inhibition by U.V spectroscopy it was confirmed that enzyme was inhibited by the substrate Metosartan and was found to be negative modulator of RNase A and shows positive cooperatively.

*In-vivo* analysis of drug metosartan on enzyme RNase A is necessary as *in-vivo* involves complex environment compared to the *in-vitro* analysis as many metabolic reactions are going simultaneously and detoxification of the drug in the liver also to be considered while performing the study. The half-life of drug telmisartan was found to be 24 hrs. and for Metoprolol it was found to be 3-4 hrs in the body. So, *in-vitro* experiments can be considered along with *in-vivo* studies. Metosartan treatment of adult rats showed reduced sperm count, sperm viability and mitochondrial viability also. In addition to these quality parameters, analysis of chromatin integrity with aniline blue staining showed extensive DNA fragmentation in metosartan treated group compared to the combined treatment of drug metosartan and RNase A.

Comet assay and agarose gel electrophoresis further confirmed the DNA fragmentation by presence of cleaved fragment in agarose gel electrophoresis and the presence of DNA ds breaks by comet assay. Analysis of release of Cyt C from mitochondria showed profound apoptosis in drug-treated rats but release of Cyt C was not seen in RNaseA+metosartan treated rats. Release of Cyt C was high in both testes and sperm of rat mitochondria compared to normal rats and its release causes activation of intrinsic pathway of apoptosis by activating caspase 9 and formation of apoptosome complex which finally leads to death of the tissue. So, *in-vivo* analysis has shown drug treatment results in apoptosis in both testes tissue and sperm cells.

## COMPETING INTERESTS

Author has declared that no competing interests exist.

## REFERENCES

1. Kozako T, Soeda S, Yoshimitsu M, Arima N, Kuroki A, et al. Angiotensin II type 1 receptor blocker telmisartan induces apoptosis and autophagy in adult T-cell leukaemia cells. FEBS Open Bio. 2016;6: 442-460.
2. Funao K, Matsuyama M, Kawahito Y, Sano H, Chargui J, et al. Telmisartan is a potenttarget for prevention and treatment in human prostate cancer. Oncol Rep. 2008;20:295-300.
3. Pinter M, Jain RK. Targeting the renin-angiotensin system to improve cancer treatment: Implications for immunotherapy. SciTransl Med. 2017;9:1-11.
4. Grahovac J, Srdic-Rajic T, Santibanez FJ, Pavlovic M, Cavic M, et al. Telmisartan induces melanoma cell apoptosis and synergizes with vemurafenib in vitro by alteringcell bioenergetics. Cancer Biol Med. 2019;16:247-263.

5. Attia YM, Elalkamy EF, Hammam OA, Mahmoud SS, El-Khatib AS. Telmisartan, an AT1receptor blocker and a PPAR gamma activator, alleviates liver fibrosis induced experimentally by Schistosomamansoni infection. Parasit Vectors. 2013;6:1-12.
6. Beeram E. Long term studies on effects of metosartan on testes and sperms of male Wistar rats. Int J Curr Adv Res. 2019;8:18593-18596.
7. Beeram E. Effect of metosartan on male reproductive potential in normal and hypertension induced rats. J Drug Deliv Ther. 2018;8:400-406.
8. Beeram E, Kedam T. Apoptosis of testis tissue by drugmetosartan through activation of intrinsic pathway by the release of Cyt C from mitochondria. Int J Chem Stud. 2018;6:614-617.
9. Na Z, Qiao X, Hao X, Fan L, Xiao Y, et al. The effects of beta-blocker use on cancer prognosis: A meta-analysis based on 319,006 patients. Onco Targets Ther. 2018;11:4913-4944.
10. Philips C, Paramaguru R, Mahadevan P, Ravindranath J, Augustine P. Metoprolol-induced severe liver injury and successful management with therapeutic plasma exchange. Cureus. 2017;9:e1209.
11. Eswaribeeram. Long term studies on effects of metosartan on testes and sperms of male wistarrat.s. IJCAR. 2019;8(5):8593-18596.
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# The First Tissue Culture in *Dalbergia melanoxylon*: Problems and Limitations

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## ABSTRACT

The first attempt dared on *Dalbergia melanoxylon* tissue culture was conducted from 2010 to 2013 in the Botany Laboratory University of Dar es Salaam. A total of 500 seeds were sterilized at different concentration of reagents and inoculated at different strength of the Murashige and Skoog medium for germination to obtain disease free explants for callus induction trials. A total of 400 nodal segments obtained from germinated seeds were sterilized at different concentration of reagents and inoculated at different hormonal combinations to induce callus formation for seedling multiplication. Results from this tissue culture attempt set a foundation for tissue culture success in *Dalbergia melanoxylon* on the future research. Only 19.8% of seeds inoculated in half strength of Murashige and Skoog medium germinated within 7 days while only 6.8% of seeds inoculated in full strength germinated within 6 days. A total of 1% of inoculated *Dalbergia melanoxylon* fragments in Murashige and Skoog media supplemented with hormone combination at 2.0 mg/l BAP + 0.5 mg/l NAA developed callus after16 days from the inoculation day. The final weight of the callus at the last record was 0.62 g. The colour of callus was green and friable in nature. Other hormonal combinations in this case did not induce callus. These results suggested that the problems which affect seed germination in the natural environment are also reflected on germination in the Murashige and Skoog medium and in callus induction. Vulnerability to fungal attack is a limitation for successful callus induction and germination in the culture room. More research under improved sterile conditions is needed to improve callus percentage for seedling multiplication.

*Keywords: Dalbergia melanoxylon; tissue culture; callus induction; explants; murashige and skoog; dared.*

## 1. INTRODUCTION

*Dalbergia melanoxylon* Guill & Perr (Family Leguminosae and subfamily Papilionoidae) [1] grows under a wide range of conditions including semi-arid, sub-humid and tropical lowland areas. It is often found on dry, rocky sites but is most frequent in mixed deciduous forests and savannahs of the coastal region [2,3]. Initially was reported that seed harvested between 8 - 12 weeks from their maturity germinated to 35% and via-bility was 57% while the seeds harvested 16 weeks and above germinated to 21% and viability was 37%. The problem and limitation is that *Dalbergia melanoxylon* seeds lose their viability few weeks from their maturity on the tree [4,5].

In the follow-up findings was reported that juvenile stem cuttings rooted to 72% by IBA treatment compared to 20% rooting of the older stem cuttings. The reported limitation for using the method for propagation of the species is that it involves huge harvesting of roots from the existing plants which is considered to be deforestation [6].

Different plant species are reported to respond differently to different tissue culture media in different sterili-zation conditions as these plants differ anatomically and physically [7]. The application of tissue

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culture techniques has allowed the massive propagation of plants under aseptic and regulated conditions [8,9].

Socio-economic importance including local and commercial use of the *Dalbergia melanoxylon* wood to individuals as well as international with at the mid of 1900's was a reason for researchers to start investigations on the species especially on the ethnobotany and silviculture of the species [10] and [11].

Little knowledge obtained for ethnobotany and silviculture of the species in this period increased awareness of traders to work on the Blackwood and expensive timber of *D. melanoxylon* leading to the rapid depression of the abundance of the *D. melanoxylon* in the late 1900's [12]. One of the problems noted from the mid to the late 1900's was the slow rotation time of the species to the harvestable age and difficult of the seed to germinate un-der natural conditions. The rotation time was reported to be 70 to 100 years and seed germination under natural conditions was reported to be below 50%. These periods were limited by lack of tissue culture technology for multiple productions of the species in the controlled environments. As a result of this limitation the gap between harvesting and natural regeneration of the species became a threat to the future existence of the species and to date the species is classified in the Lower/Risk near threatened in Tanzania, threatened in Kenya and extinct in Burkina Faso as reported in the Red list category [12]. An understanding of seed ecology is essential for developing effective management programs for problematic weed species. Weeds are usually most susceptible to control methods, making control strategies targeted to early life stages highly effective [13,14,15].

In recent years efforts to improve propagation of *D. melanoxylon* started and were directed on how to improve seed germination, rooting of the cuttings, rotation time (growth rate), seedling multiplication and investigations on factors affecting seed germination in the natural environments [16-23,5].

It was reported that 56% of the species seedlings in the forest originate from root suckers. The problem of using this method for propagation of the species is a destructive practice of the existing plants and the limitation is that seedlings from root suckers do not develop a tap root which is important for growth of the plant [24].

It was reported that available moisture content is the main factor affecting seed germination in the natural environment. Seeds are vulnerable to high moisture level and vulnerable to fungal and insect attack. As a result of this vulnerability, seeds tends lose their viability rapidly as they mature from their mother plants [25].

Again was reported two ectomycorrhiza species and one endomycorrhiza species to be associated with *D.melanoxylon* but also the association influenced 100% rooting of the softwood stem cuttings of the species in thenonmist propagator [26].

Finally were reported two findings in two different investigations. The first investigation reported seeds to germinate to 21% in the soil compared to 19.8% for seed germinated in the Murashige and Skoog (MS) medium in the culture room. Germination in the MS was hindered by fungal contamination of seeds in the medium as a limitation. The problem here is that *D. melanoxylon* is still not domesticated and therefore have a lot of born in fungal disease. The second investigation reported root cuttings to root to 37% and softwood cuttings of less than 15 gm by weight rooted to 100% in the non-mist propagator [27] and [28]).

Reported findings from ethnobotany to rooting trials did not give a sustainable propagation methods of *D. melanoxylon* because germination is still limited by low seed viability in the natural environment, root suckersseedlings are limited by lack of tap roots, rooting of the stem cuttings are limited by deforestation of the existing plants by root harvesting. Tissue culture in *D. melanoxylon* therefore was conducted as an alternative to sustainable means of propagating the species. The main concern of this paper therefore is to report the first attempt in *D. melanoxylon* tissue culture, findings, Problems and limitations in conducting *D. melanoxylon* tissue culture.

## 2. MATERIALS AND METHODS

A total of 2 kg of *Dalbergia melanoxylon* seeds from Bwawani collection was purchased from TTSA in September 2010. Other 4 pots bearing 4 days old *D. melanoxylon* seedlings from Dakawa seed collection were purchased from TTSA on March 2012 and taken to the tissue culture laboratory at the Botany Department University of Dar es Salaam. Seeds were used for raising disease- free shoots in Murashige and Skoog media for callus induction trials while the purchased young seedlings were used for callus induction trials. Preparation and ad-ministration of tissue culture and culture materials were conducted according to recommended standards.

### 2.1 *Dalbergia melanoxylon* Seed Sterilization and Inoculation

A total of 500 seeds were used in this experiment in 10 replicates having 50 seeds in 5 Petri dish per replicate. The purpose was to compare full and half strength MS in effecting seed germination. This experiment was con-ducted hand to hand with callus induction trial phase I during October 2010-October 2011 because obtained seedlings were used for callus induction trial. About one hundred and fifty (150) seeds were sterilized using 70% ethanol for 30 minutes and 2.6% sodium hypochlorite for 30 minutes and inoculated in full strength MS at pH 5.8 in 15 Petri dish (3 replicates) . Another 100 seeds were sterilized in 35% ethanol for 20 minutes and 2.6% sodium hypochlorite for 20 minutes and inoculated in half strength MS at 5.8 pH in 10 Petri-dish (2 replicates). Other 150 seeds were sterilized in 70% ethanol for 10 minutes and 2.6% sodium hypochlorite for 10 minutes and inoculated in full strength MS at pH 5.8 in 15 petri dish (3 replicates) while the last 100 seeds were sterilized in 35% ethanol for 10 minutes and 2.6% sodium hypochlorite for 10 minutes and inoculated in half strength MS at 5.8 pH in 10 Petri dish (2 replicates). The cultures were incubated in a culture room at 28˚C under a photoperiod of 16 hours at 3000 lux light intensity provided by cool white fluorescent tubes.

### 2.2 Callus Induction Trials from Seed Germinated Seedlings (Phase I)

A total of 20 nodal explants of *Dalbergia melanoxylon* per replicate were used as experimental material in phase I. Ten (10) replicates with 20 Petri-dishes each replicate and one node segment in each petridish was conducted. All shoots were collected from 2 to 4 days old germinated seeds. These shoot ex-plants were surface sterilized with 35% ethanol for 20 minutes and rinsed with sterilized water three times. Explants were then treated with 2.6% sodium hypochlorite solution for 20 minutes and rinsed with sterilized water three times for inoculation in full strength MS medium at pH 5.8 in 10 replicates during October 2010 -October 2011. The MS media used in all callus induction trials were industrial to avoid measurement errors and to save time.

Three replicates used 10 minutes, four replicates used 20 minutes and other three replicates used 30 minutes to sterilize explants in 35% ethanol and 2.6% sodium hypochlorite solution. The cultures were incubated in a culture room at 28˚C under a photoperiod of 16 hours at 3000 lux light intensity provided by cool white fluorescent tubes. Different ratios of hormones (BAP + NAA, BAP + IAA, BAP + IBA, KIN + NAA) were used to initiate callus. Definitions for hormones abbreviated are given as: BAP is benzyl amino purine, NAA is 1-naph-thaleneacetic acid, IAA is indole-3-acetic acid, IBA is indolebutyric acid and KIN is kinetin.

### 2.3 Callus Induction Trials from Seed Germinated Seedlings (Phase II)

In the callus induction trial phase II conducted between 17 March 2012 and 30 April 2012 using seedlings bought from TTSA (plate 8), only BAP + NAA hormone combination was used since it was the only combination that had induced at least 0.5% callus in phase I. Under this phase a total of 20 nodal explants of *Dalbergiamelanoxylon* per replicate were used as experimental material. Ten (10) replicates with 20 Petri-dishes each replicate and one node segment in each petridish was conducted. All shoots were collected from 2 to 4 days old seedlings. These shoot ex-plants were surface sterilized with 35% ethanol for 20 minutes and rinsed with sterilized water three times. Explants were then treated with 2.6% sodium hypochlorite solution for 20 minutes and rinsed with sterilized water three times for inoculation in full strength MS medium at pH 5.8. Three replicates used 10 minutes,

four replicates used 20 minutes and other three replicates used 30 minutes to sterilize explants in 35% ethanol and 2.6% sodium hypochlorite solution. The cultures were incubated in a culture room at 28˚C under a photoperiod of 16 hours at 3000 lux light intensity provided by cool white fluorescent tubes.

## 3. RESULTS

### 3.1 *D. melanoxylon* Seed Germination in MS Media

A total of 19.8% of seeds inoculated in half strength of MS medium germinated within 7 days after sterilization of 20 minutes in 35% ethanol and 20 minutes in 2.6% sodium hypochlorite while only 6.8% of seeds inoculated in full strength of MS medium germinated within 6 days after sterilization of 30 minutes in 70% ethanol and 30 minutes in 2.6% sodium hypochlorite (Plate 1, Table 1 and Fig. 1).

### 3.2 Results of Callus Induction Trials in Phase I and II

Among the four types of hormonal combinations and their concentrations on 200 shoot segments of *Dalbergia melanoxylon,* callus induction in phase I was observed only in 1 inoculated shoot segment with hormone combination comprising of BAP and NAA. It was observed that only 0.5% of inoculated *D. melanoxylon* seedling fragments in MS media supplemented with hormone combination at 2.0 mg/l BAP + 0.5 mg/l NAA developed callus after16 days from the inoculation day. The final weight of the callus at the last record was 0.62 g. In this induction ex-plants were surface sterilized in 35% ethanol for 20 minutes and 2.6% sodium hypochlorite solution for 20 minutes. The color of callus was green and friable in nature. Other hormonal combinations in this case did not induce callus production (Plate 2, Table 2, Table 3 and Fig. 2).

In the callus induction trial phase II also 1 nodal segment among 200 nodes developed callus using hormone combination at 2.0 mg/l BAP + 0.5 mg/l NAA in 16 days from the inoculation day. This was also 0.5% callus of the inoculated ex-plants making a total of 1% callus from the total of 400 nodes inoculated in phase I and phase In this induction ex-plants were surface sterilized in 35% ethanol for 20 minutes and 2.6% sodium hypochlo-rite solution for 20 minutes. Growth of callus in phase II was observed increasing in size from day four to day eight (Plate 3, Table 4 and Fig. 3). The weight of callus at the last record was 0.62 g. The color of callus was green and friable in nature.
II.



**Plate 1. Representative for germinated seeds in MS media**

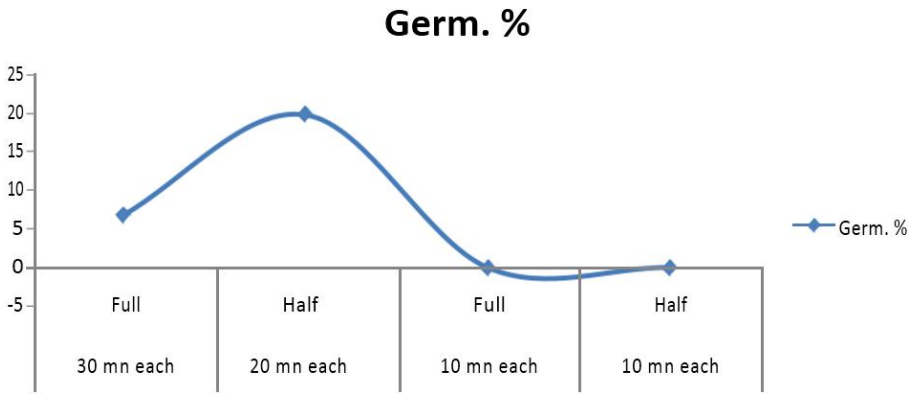



**Fig. 1. Effect of sterilization reagent and duration on seed germination**

**Table 1. Summary for sterilization conditions and germination percentage in the MS**

| #seeds | Sterilization type | Sterilization duration | MS strength | Germination % |
|---|---|---|---|---|
| 150 | 70% ethanol & 2.6% sodium hypochlorite | 30 minutes each | Full strength | 6.8 |
| 100 | 35% ethanol & 2.6% sodium hypochlorite | 20 minutes each | Half strength | 19.8 |
| 150 | 70% ethanol & 2.6% sodium hypochlorite | 10 minutes each | Full strength | 0,0 |
| 100 | 35% ethanol & 2.6% sodium hypochlorite | 10 minutes each | Half strength | 0.0 |

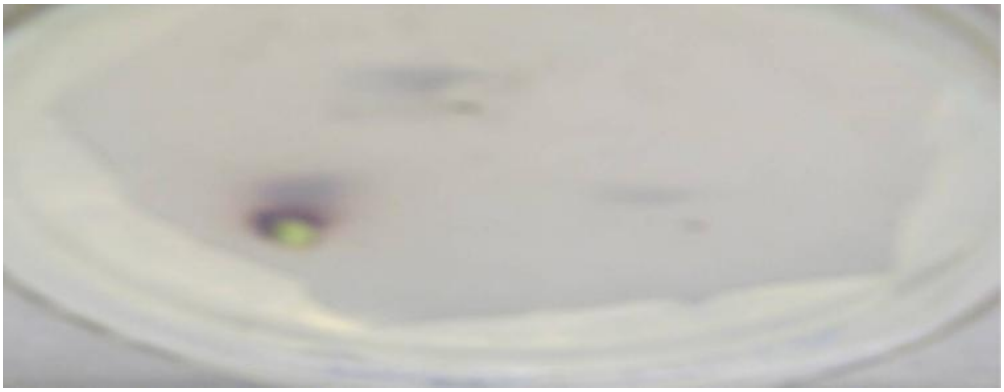


**Plate 2. Two days old callus (Phase I)**

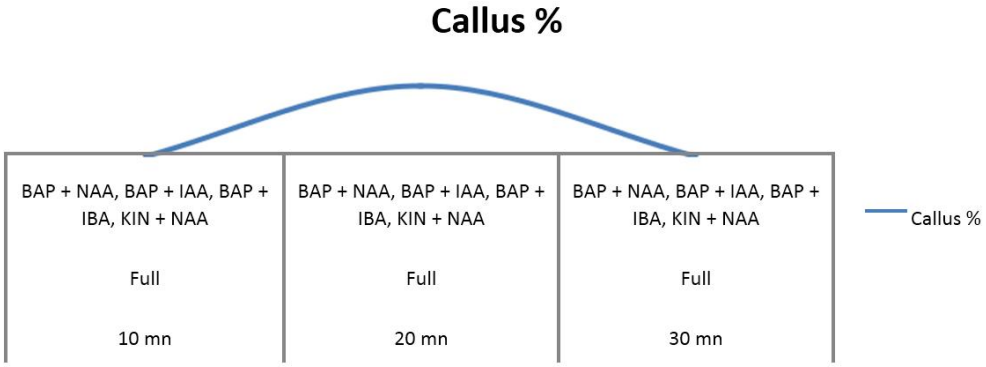



**Fig. 2. Callus induced with hormone concentration (Phase I)**

**Table 2. Summary of sterilization, inoculation conditions against callus percentage (Phase I)**

| #shoot segments | Reagent conc. | Time | MS strength | Hormone combination | Callus % |
|---|---|---|---|---|---|
| 60 | 35% ethanol & 2.6% sodium hypochlorite | 10 mn | Full | BAP + NAA, BAP + IAA, BAP + IBA, KIN + NAA | 0.00 |
| 80 | 35% ethanol & 2.6% sodium hypochlorite | 20 mn | Full | BAP + NAA, BAP + IAA, BAP + IBA, KIN + NAA | 0.5 |
| 60 | 35% ethanol & 2.6% sodium hypochlorite | 30 mn | Full | BAP + NAA, BAP + IAA, BAP + IBA, KIN + NAA | 0.00 |

## 4. DISCUSSION, CONCLUSION AND RECOMMENDATION

### 4.1 Callus Induction in *Dalbergia melanoxylon*

Seeds, nodes, shoot and root tips are efficient ex-plant source for many plant callus initiation and shoot regeneration. This idea was adopted in this study expecting that *D. melanoxylon* ex-plants can easily develop callus by tissue culture. Protocols for tissue culture differ from one plant species to another. Some plant species can respond to many media combinations, others can respond to only one media combination while others do not respond to any media combination. Different cellular and morphological structures in different plants have lead to different preparation and sterilization protocols in different plants species.

Most domesticated plant species have been subjected to genetic transformation such as chromosomal and DNA recombination in such a way that they can be easily accessed and sterilized for tissue culture. *D. melanoxylon* is one of the forest plant which is neither genetically transformed nor domesticated and therefore is colonized by various microorganisms hence is difficult to keep in aseptic conditions for tissue culture. For such forest plants it needs so many trials of sterilizing materials such as ethanol and sodium hypochlorite. Some treatment combinations of *D. melanoxylon* ex-plants in this study did not generate callus or seed germination in the media because there were either no viable seeds, sterilization was not enough or sterilization killed the seed embryos since the extent of sterilizing and ratios for ethanol and sodium hypochlorite and the hormonal combination ratios for *D. melanoxylon* was not known. For plant which is subjected to tissue culture for the first time such as *D. melanoxylon*, the conditions and parameters needs many trials to be known including hours of light exposure of inoculated materials, hours in darkness, quantity and quality of light intensity, temperature and type of ex-plant which can respond easily. All these have lead to difficulties for *D. melanoxylon* callus induction in this study.

### 4.2 Sterilization of *D. melanoxylon* Ex-Plants

Two different sterilization ratios were interchangeably used for *D. melanoxylon* seeds and node tips and they gave different inoculum results. When sterilized in 35% ethanol for 30 minutes and 2.6% sodium hypochlorite for 30 minutes, most of cultures remained uncontaminated but did not produce callus.

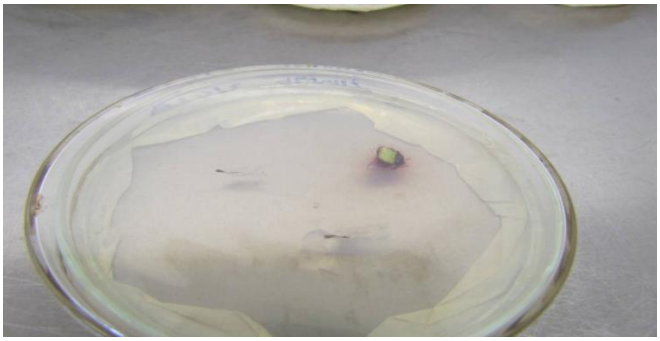


**Plate 3. Four days old callus (Phase II)**

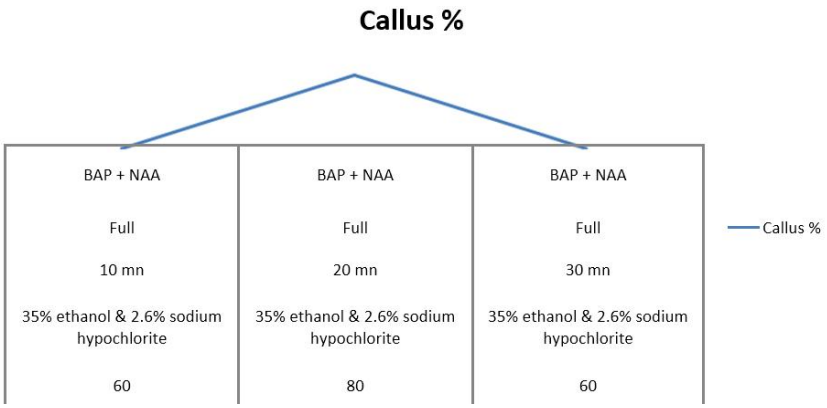



**Fig. 3. Callus induced with hormone concentration (Phase II)**

**Table 3. Effect of different concentration of hprmones in MS medium for induction of callus from the shoot tip segments of *Dalbergia melanoxylon* Phase I (NB: Gn.F means Green and friable)**

| Growth regulator | Concentration (mg/l)/petridish | Days to callus formation | % of Explants responded | Type of callus |
|---|---|---|---|---|
| | 1.5 + 0.5 | -- | -- | -- |
| | 1.5 + 1.0 | -- | -- | -- |
| BAP + NAA | 2.0 + 0.1 | -- | -- | -- |
| | 2.0 + 0.5 | 16 | 0.5 | Gn.F |
| | 2.0 + 1.0 | -- | -- | -- |
| | 1.5 + 0.5 | -- | -- | -- |
| | 1.5 + 1.0 | -- | -- | -- |
| BAP + IBA | 2.0 + 0.1 | -- | -- | -- |
| | 2.0 + 0.5 | -- | -- | -- |
| | 2.0 + 1.0 | -- | -- | -- |
| | 1.5 + 0.5 | -- | -- | -- |
| | 1.5 + 1.0 | -- | -- | -- |
| BAP + IAA | 2.0 + 0.1 | -- | -- | -- |
| | 2.0 + 0.5 | -- | -- | -- |
| | 2.0 + 1.0 | -- | -- | -- |
| | 1.5 + 0.5 | -- | -- | -- |
| | 1.5 + 1.0 | -- | -- | -- |
| KIN + NAA | 2.0 + 0.1 | -- | -- | -- |
| | 2.0 + 0.5 | -- | -- | -- |
| | 2.0 + 1.0 | -- | -- | -- |

**Table 4. Summary of sterilization, inoculation conditions against callus percentage (Phase II)**

| #shoot segments | Reagent conc. | Time | MS strength | Hormone combination | Callus % |
|---|---|---|---|---|---|
| 60 | 35% ethanol & 2.6% sodium hypochlorite | 10 mn | Full | BAP + NAA | 0.00 |
| 80 | 35% ethanol & 2.6% sodium hypochlorite | 20 mn | Full | BAP + NAA | 0.5 |
| 60 | 35% ethanol & 2.6% sodium hypochlorite | 30 mn | Full | BAP + NAA | 0.00 |

Three interpretation can be given to these results: one is successful sterilization but which killed the ex-plants, second is successful sterilization but there was no viable ex-plant in the trial, lastly is that some other necessary conditions are not attained such as light intensity or temperature. Other

sterilization trials were subjected to 70% ethanol for 30 minutes and 2.6% sodium hypochlorite for 30 minutes. In this trial inoculums also did not get contaminated but also did not produce callus or germinate the seed meaning that sterilization was successful but killed the ex-plants or sterilization was successful but there was no viable ex-plant in the trial or any other necessary condition was not attained. When only10 minutes was used to sterilize *D. melanoxylon,* ex-plants in either 35% ethanol and 2.6% hypochlorite or 70% ethanol and 2.6% sodium hypochlorite all inoculums became contaminated meaning that this duration does not keep the ex-plants in aseptic conditions. Full strength MS is more recommended for callus induction in woody plants which are difficult to give callus. However, 70% ethanol sterilization is not recommended for shoot segments since they have delicate young dividing cells.

When 20 minutes was used to sterilize *D. melanoxylon* ex-plants in either 35% ethanol or 70% ethanol at least 19.8% of inoculated ex-plants germinated and a total of 1% ex-plants developed callus meaning that this duration did not kill cells or embryo of all ex -plants but also sterilized successful most of the viable ex-plants. This range of concentrations and duration for sterilizing *D. melanoxylon* ex-plants is in agreement with [11] who re-ported that “some plant species can respond to many media combinations, others can respond to only one media combination while others do not respond to any media combination”.

A protocol was obtained from this research for inducing callus from *D. melanoxylon* shoots. The callus producing protocol was from a 4 days old *D. melanoxylon* seedlings. Seedlings which were below 4 days old were too fragile and easily dried out when inoculated, where as seedlings above 28 days old did not develop callus. Hormone combination comprising of benzyl amino purine (BAP) and naphthalene acetic Acid (NAA) induced at least 1% callus formation. When full strength MS media was used, callus formation was induced as opposed to half strength MS media. Sterilization of 35% ethanol for 20 minutes and 2.6% sodium hypochlorite solution for 20 minutes of shoot segments produced sterile environment for callus formation. Other sterilization concentrations could not induce callus formation in this particular study. Such a new species in tissue culture needs as many trials as possible to conclude appropriate ratios for vitamins, hormones, type and pH level as well as duration of sterilization of ex-plants. *D. melanoxylon* to date is not a domesticated plant as most of the successful plants in tissue culture such as tomato and banana. Most of the wild woody plant species are difficult to regenerate using tissue culture.

Ninety percent (90%) of D. melanoxylon seeds inoculated for germination trial in the laminar flow were at-tacked by fungus before germinating. This was probably due to the fact that the seeds of the species have seed borne fungal infections but this should be investigated further to justify. The fungus attacks were also observed on seeds inoculated in soil media. Tissue culture experiments require unlimited time. Success of micropropagation in cassava by DNA recombination, sisal by meristematic tissue culture, cashew nuts by callus tissue culture and banana by suckers micropropagation came after genetic transformation and to some of them domestication and hence became easily assessed in tissue culture. If researchers could have genetically transformed *D. melanoxylon* today, getting a callus induction and other protocols of the species could not be so difficult since a species could be easily assessed in tissue culture. So genetic transformation was ideal for *D. melanoxylon* before getting into mass production of planting materials of the species but this is a good start to the world of science.

### 4.3 Problems and Limitations in Conducting *D. melanoxylon* Tissue Culture

Being a forest plant as a problem, *D. melanoxylon* is easily attacked by fungal disease in the process to germination and callus induction in the culture room. This was the most limitation of the experiment to obtain recommended germination percentage and callus percentage. To improve callus induction needs techniques to over-come fungal contaminations in the culture room.

## COMPETING INTERESTS

Author has declared that no competing interests exist.

## REFERENCES

1. Tack CH, Nomenclature of East African timbers. Nairobi: East African Timber Advisory Board; 1962.
2. Redhead JF, Temu AB. Valued timber but neglected tree: Mpingo (*Dalbergia melanoxylon*)," Tanzania Association of Foresters Newsletter. 1981;2:8-9.
3. Musimba N, Kimatu JN, Mweu B, Mburu MWK, Nguluu S. Germination effects of purposive bruchid screening of african ebony (*Dalbergia melanoxylon*) seeds. The Arid and Semi-Arid Region of South Eastern Kenya. Current Research in Agricultural Sciences. 2015;2(2):60-66.
4. Amri E. Effect of timing of seed collections an provenances on seed viability and germination capacity of *Dalbergia melanoxylon*. Botany Research Journal. 2008;1:82-88
5. TTSA. Handling of seeds of *Dalbergia melanoxylon* (African Blackwood) seed. 1995;9
6. Amri E. Effect of age of the donor plant, IBA treatment and cutting position to the rooting ability of stem cuttings in *Dalbergia melanoxylon*. New Foresters. 2010;38:183-194. Available:http://dx.doi.org/10.1007/s11056-009-9163-6
7. Arumugam A, Gopinath K. *In-Vitro* callus development of different explants used for different medium of terminalia arjuna. Asian Journal of Biotechnology. 2011;3:564-572. Available:http://dx.doi.org/10.3923/ajbkr.2011.564.572
8. Costa AS, Arrigoni-Blank MF, Blank AF, Mendonza AB, Amancio VF, Ledo AS. Estabelecimento de alecrim-pimenta *in vitro.* Hortic. Bras. 2007;25:068–072.
9. Muñoz-Miranda LA, Rodríguez-Sahagún A, Acevedo Hernández GJ, Cruz-Martínez VO, Torres-Morán MI, Lépiz-Ildefonso R, Castellanos-Hernández OA. Evaluation of somaclonal and ethyl methane sulfonate-induced genetic variation of mexican oregano (*Lippia graveolens* HBK). Agronomy. 2019;9(4);166.
10. Bryce JM. The commercial timbers of tanzania. forest division, ministry of agriculture and co-operatives, Moshi; 1967.
11. Gamborg OL. Plant tissue culture. biotechnology. Milestones. *In Vitro* Cellular and Developmental Biology Plant. 2002;38:84-92. Available:http://dx.doi.org/10.1079/IVP2001281
12. IUCN. Red List of Threatened Plants. The IUCN Species Survival Commission, Royal Botanical Garden, Edin-burgh; 2008
13. Batlla D, Benech-Arnold RL. Predicting changes in dormancy level in weed seed soil banks; Implications for weed management. Crop Protection. 2007;26:189–197.
14. Bond W, Grundy AC. Non-chemical weed management in organic farming systems. Weed Research. 2001;41:383–405
15. Humphries T, Chauhan BS, Florentine SK. Environmental factors effecting the germination and seedling emergence of two populations of an aggressive agricultural weed; *Nassella trichotoma*. PloS one. 2018;13(7):e0199491.
16. Kumar C, Singh AK, Nehar S. *In-Vitro* propagation and callus formation from seedling of terminalia arjuna roxb. An International Quarterly Journal of Life Sciences.2010;2:423-429.
17. Mbuya LP. Useful trees and shrubs for Tanzania. Swedish International Development Authority. Regional Soil Conservation Unit, Morogoro; 1994.
18. Msanga HP. Different types of trees for plantations in different regions of Tanzania. MNRT Morogoro Tanzania; 1999.
19. Murashige T, Skoog F. A revised medium for rapid growth and bio-assays with tobacco tissue cul-ture. Physiologia Plantarum., 1962;15:473-497.
20. Nshubemuki L. *Dalbergia melanoxylon*: Valuable wood from a neglected tree. NFT Highlights. Nitrogen Fixing Tree Association, Hawaii. 1993;1993-1995.
21. Redhead JF, Temu AB. Valued timber but neglected tree: Mpingo (*Dalbergia melanoxylon*) Tanzania Association of Forester, Newsletters. 1981;2:8-9.
22. Sharman H. Investigation in the sustainable management of tropical timber species using dalbergia mela-noxylon as a case study. Unpublished MSc Thesis, University of Edinburgh. 1995;68-69.
23. Trigiano RN, Gray DJ. Plant tissue culture, development and biotechnology. CRC Press, Boca Raton. 2010;186.

24. Washa BW. Dependence of *Dalbergia melanoxylon* natural population on root suckers regeneration. Journal of African Affairs, Hankuk University of Foreign Studies. 2008;24:177-198.
25. Washa BW, Nyomora AMS. The effect of moisture and seed treatment on the in-situ and ex-situ regeneration of *Dabergia melanoxylon* (African Blackwood) in Pugu Forest Reserve. HURIA Journal. 2012;10:56-70.
26. Washa BW, Nyomora AMS, Lyaruu HV. Improving propagation success of *D. melanoxylon* (African Blackwood) in Tanzania. II: Rooting Ability of Root and Stem Cuttings of *Dalbergia melanoxylon* (African Black-wood) in Tanzania. Tanzania Journal of Science. 2012;38:43-53.
27. Washa. Seedling production techniques in *D. melanoxylon*. International Journal of Plant and Forestry Sciences. 2014;1:1-9
28. Washa. Effective cutting type in the rooting of *Dalbergia melanoxylon* in Tanzania. International Journal of AgriScience. 2014;4:256-259.

**Biography of author(s)**



**Dr. Washa B. Washa**
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He was born in 1965 in Tanzania, has three remarkable periods in his profession experience. One is a period of Teaching Biology and Chemistry in Secondary schools of Tanzania in which professionals and experts of various discipline were produced through his teaching. He taught about 7 secondary schools during this period from 1994-2007. Second is a Hardship period of BSc and MSc studies without a study leave through the Open University of Tanzania from 2000-2004 for BSc and 2005-2007 for MSc. His childhood life in forestry environments was a source for him to take an MSc in plant biology particularly the African Blackwood (Dalbergia melanoxylon. Third is his contribution to the science of Botany through investigations in Dalbergiamelanoxylon from 2005-2016. Has been the first researcher in the world to test tissue culture in Dalbergia melanoxylon and the second researcher to write a Review of the Literature of this species in 2014. He is a Senior Lecturer at Mkwawa University College of Education, Botanical sciences Department in Plant physiology, plant Anatomy, Mycorrhizae and plant Tissue culture from 2008 To date.

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# Evaluation of Anti-fertility Activity of *Ruta montana* L. Areal Part Extractin Albino Rats

**Mounira Merghem[1*] and Saliha Dahamna[1]**

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## ABSTRACT

*Ruta montana* L. a herb from the family *Rutaceae*, is known in Algeria as “mountain rue” or “Fidjel” and has been used in folk medicine as an antioxidant, hypoglycemic, antirhematic, antihelmintic, antiepileptic and antipyretic. The objective of the present study is to evaluate the effect of the aqueous extract of *Ruta montana* L. on the fertility in adult male and female rats. Forty healthy adult rats of both sexes were divided into 4 Groups of 10 rats each. During the treatment period, 100, 300 and 600 mg/kg/day of the extract were orally administered to Groups I, II and III respectively, while the control group received distilled water and served as control. The daily administration was carried out for a period of 90 days. The results did not show any significant change in ovaries weight. However, a significant decrease in testis, epididymis, and seminal vesicles weights was detected, as well as a reduction in the number and the motility of spermatozoids in rats treated with the doses of 300 and 600 mg/kg. A dose of 300 mg/kg and 600 mg/kg of aqueous extract of *Ruta Montana* L. was able to cause a decrease in sexual organs weight of male rats and in the number and the motility of spermatozoids.

*Keywords: Ruta montana L.; fertility; sperm motility; sperm counts; histological alterations.*

## 1. INTRODUCTION

The use of plants in the management of diseases has been reported since antiquity, and has continuously grown over time. Various medicinal plant extracts have been tested for their anti-fertility activity in both males and females. The beginnings of the medicinal plants’ use were instinctive, as is the case with animals [1,2]. Some of these plants had spermicidal effects, other caused reduction in the sperm counts, altered the mobility of the sperm, caused testicular change and altered hormone levels [3]. Members of this genus have been used since antiquity in traditional medicine [4] and credited with a long list of medicinal uses, including hypoglycaemic, abortive, antirheumatic, intestinal and hepatic diseases, male sterility, antihelminthic, antiepileptic and antipyretic [5,6].

*Ruta montana* L. a herb from the family *Rutaceae*, is known in Algeria as “mountain rue” or “Fidjel” [7]. This plant is used in folk medicine as an antioxidant [8], hypoglycemic [9], antirhematic [10], antihelmintic, antiepileptic and antipyretic. It is also used in the treatment of intestinal disorders, hepatic diseases [11], and vitiligo [12]. *Ruta montana* L. contains various active principles such as flavonoids, alkaloids, essential oils, coumarins, phenols, saponins lignans, and triterpenes [13]. In Algerian folk medicine, *Ruta montana* is used against child fevers and as an abortive drug [14]. In the present studyextract of *Ruta montana* was tested to investigate a possible effect of this plant on Fertility potential inAlbino rats.

## 2. MATERIALS AND METHODS

### 2.1 Plant

*Ruta montana* L. was collected in October, from Beni aziz region, in the North-East of Algeria, it was identified by Pr. Laouar H. (Department of Ecology & Plant Biology, Faculty of Nature and Life

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Sciences, university Ferhat Abbas, Sétif 1). A voucher specimen was kept at the herbarium of the laboratory of botany of the same faculty.

## 2.2 Animal

Male and female *Albino Wistar* rats were used in this study. The rats were purchased from Pasteur Institute (Algiers, Algeria). These animals were kept in the animal house, at a temperature of 20°C and under a natural photoperiod cycle. The animals were housed in plastic cages (5 rats per cage) and had free access to standard commercial diet and tap water.

## 2.3 Preparation of Plant Extract

The aerial parts of *Ruta montana* L. were cleaned, dried in shade for 2 weeks at room temperature and ground into fine powder using an electric grinder. The aqueous extract was prepared according to the method described by Mbiantcha et al. [15], with some modifications. Briefly, 100g of *Ruta montana*L.powder was mixed with 1L of boiled distilled water (100°C) and was placed at room temperature during 72h, The resulting mixture was filtered using Wattman filter paper n°3 and then evaporated in rotary vacuum evaporator at 45°C.

## 2.4 Experimental Design

*AlbinoWistar* rats of both sexes, weighting between 150 and 200 g, were divided into four groups of 10 rats each (5 females and 5 males):

**Control group:** Rats received normal saline solution daily for 90 days by oral route.

**Group I:** Received aqueous extract of *Ruta montana*L.at the dose of 100 mg/kg of body weight, daily for 90 days.
**Group II:** Received aqueous extract of *Ruta montana*L. at the dose of 300 mg/kg of body weight, daily for 90 days.
**Group III:** Received aqueous extract of *Ruta montana*L. at the dose of 600 mg/kg of body weight, daily for 90 days.

At the end of the treatment period, animals were fasted overnight with free access to water ad libitum. They were subsequently anesthetized with diethyl ether and sacrificed. After that, testes, epididymis, seminal vesicles and ovaries were dissected out, washed with saline solution then fixed in 10% formalin for histopathological examination.

## 2.5 Organ's Weight

After the sacrifice ofanimals, testes, epididymis, seminal vesicles and ovaries were carefully removed and weighed individually. Organ weights were expressed in mg/100 g of body weight(absolute organ weight).

## 2.6 Sperm Suspension

The epididymis of each rat was placed in 1 mL of ringer buffer. The spermatozoa were obtained by making small cuts in caudal epididymis and diluted by adding 9 mL of ringer buffer (1/10) and then incubated at 37°C for 10 minutes. This suspension was used to evaluate the motility and counts of sperm.

## 2.7 Sperm Count and Motility

A sample of sperm suspension was taken and the number of sperm was counted using a haemocytometer under the light microscope. Four squares were counted in triplicate. The count was

expressed as 2500/mm of suspension. Sperm motility (%) was also assessed immediately by counting both motile and immotile spermatozoa and calculated by the following formula:

Number of motile sperm x 100/total number of motile and immotile sperm

## 2.8 Histopathological Examination

Organs of treated and control rats were taken out. They were weighed and examined for gross lesions. Similar samples were fixed in 10% formalin solution, dehydrated in graded (70-90%) alcohol, cleared in xylene, and placed and embedded in paraffin wax. To perform the histology of tissues, 5-6 µm sections were prepared using Microtome (Leica, RM 2145). Then these sections deparaffinated in xylene, passed through 70% to 90% alcohol, and stained with hematoxylin and eosin (H&E). The slides prepared by this process were observed under light microscopy [16].

## 2.9 Statistical Analysis

The results are expressed as the mean value ± standard deviation. One-way analysis of variance (ANOVA) followed by the Tukey test was performed to assess differences between groups. Differences were considered significant at $p < 0.05$. Statistical analyses were performed with the software GraphPad Prism 5®.

## 3. RESULTS AND DISCUSSION

Several lines have suggested that some herbal drugs can be used as anti-fertility agents in mice, rats, rabbits and even humans [17]. Although many reports have showed that the use of plant extract can affect reproductive physiology of the animal.

In the present study, and in order to evaluate the effect of the chronic treatment of the aqueous extract of *Ruta Montana* L. on the fertility of both sexes, 4 groups of rats were used: a control group and 3 treated groups (100, 300, 600 mg/kg).

## 3.1 Effect of *Ruta montana* L. Aqueous Extract on Rat's Organ Weight

Organ's weight is an important index of physiological and pathological status in human and animals. Absolute and relative organ weights of treated rats are shown in Table 1. The administration of the aqueous extract of *Ruta montana* L. did not cause any statistical difference in ovaries weight. Whereas, the treatment with the doses 300 and 600 mg/kg caused a significant decrease in testis, epididymis and seminal vesicles weight, when compared to the control group. Moreover, gross examination of internal organs of all the rat's revealed no detectable abnormalities.

The obtained results showed a significant weight reduction in testes, epididymis and seminal vesicles at the doses 300 and 600 mg/kg when compared to the control group (Table 1). This weight reduction was dose dependent. Generally, reductions in internal organ weight are simple and sensitive indices of toxicity after exposure to toxic substances [18]. The significant reduction of testis weight is known to be mostly related to the number of spermatids and spermatozoa present in the tissue [19]. Such reduction in the weight of reproductive organs is indirectly supports the reduced availability of androgen [20]. It is known that the accessory sex organs are androgen-dependent target organs and manifest differential sensibility to androgens for maintenance of their structure and function. It is also known that, any change in circulating androgens would affect the internal microenvironment of epididymis and thereby lead to alteration in sperm motility and metabolism [21]. Androgen deprivation not only suppresses spermatogenesis, leading to low sperm concentration, but also alters the epididymal milieu which renders it hostile for maturation and survival of the spermatozoa [22,23]. Testosterone, an important androgen, plays a pivotal role in maturation, spermatogenesis and the maintenance of accessory sex organs [24]. The structural and functional integrity of reproductive tissues depends on the circulating androgen [25] and therefore, any small change in testosterone content may result in reductions in weights of reproductive organs.

## 3.2 Effect on Sperm Count and Motility

The results of the sperm count and motility were presented in Table 2. It was clear that animals treated by the aqueous extract of *Ruta montana* L. (100, 300, 600 mg/kg) showed evidence of a dose-dependent toxicity on epididymidal sperm parameters. The sperm count and motility were significantly decreased in group II and group III when compared to the control one.

Sperm characteristics are important reproductive indices as they account for male fecundity. The aqueous extract at the doses of 300 and 600 mg/kg produced a significant reduction in the sperm count and motility. Two possible hypotheses may be proposed to explain this reduction. One hypothesis is that the principle active of the extract may alter the pituitary gonadotropins hormones: luteinizing hormone (LH) and follicle stimulating hormone (FSH) [26]. It is well known that the weight, size and the secretary function of testes, epididymis and seminal vesicles are closely regulated by androgens hormones [27]. The production of the sperm cells (spermatozoa) and testosterone in testis are mainly regulated by the follicle stimulating hormone and Luteinizing hormone, which are released from the interior pituitary [28]. FSH stimulates spermatogenesis in the sertoli cells, while LH stimulates the production of testosterone in leydig cells of testis [29]. Low levels of these hormones decrease endogenous testosterone secretion from the testis depriving developing sperm of the signal required for normal maturation and also it suppress testicular steroidogenesis and spermatogenesis [26] since the pituitary-testicular axis is a central regulatory conduit for testicular function that culminates in the production of spermatozoa [30]. Besides hormonal alteration, the alternative hypothesis is that the principles active may induce oxidative stress in testicular tissue and stored germ cells leading to generation of free radical products, as they exert a detrimental effect on spermatogenesis [31].

## 3.3 Effect of *Ruta montana* L. Aqueous Extract on Organ's Histology

Histological examination of testes of control group showed normal histological structure of seminiferous tubules associated with complete spermatogenic series as demonstrated in Fig. 1C. Testes of rats given 100 mg/kg of*Ruta montana* L. aqueous extract had similar histological appearance as the control rats (Fig. 1 D1) with active spermatogenesis. However, testes of rats of group II and III (treated with 300 and 600 mg/kg of *Ruta montana* L.) revealed degeneration of seminiferous tubules with absence of sperm in tubular lumen as shown in Fig. 2 D2 and Fig. 2 D3.

**Table 1. Effect of chronic oral administration of aqueous extract of *Ruta montana* L. on rats organs weight**

| | Relative organ weight | | | |
|---|---|---|---|---|
| | **Control** | **Group I (100 mg/kg)** | **Group II (300 mg/kg)** | **Group III (600 mg/kg)** |
| Testis | 0,58±0,07 | 0,48±0,13 | 0,23±0,15* | 0,19±0,10** |
| Epididymis | 0,22±0,00 | 0,19±0,04 | 0,11±0,02** | 0,07±0,00** |
| Seminal vesicle | 0,90±0,43 | 0,55±0,26 | 0,13±0,05* | 0,16±0,11* |
| Ovaries | 0,04±0,01 | 0,04±0,00 | 0,03±0,00 | 0,04±0,007 |

*Each value represents the mean ± SD (n =5).* p<0.05; ** p<0.01*

**Table 2. Effect of *Ruta montana* L. aqueous extract on sperm count and motility**

| | **Control** | **Group I (100 mg/kg)** | **Group II (300 mg/kg)** | **Group III (600 mg/kg)** |
|---|---|---|---|---|
| Count (2500/mm$^3$) | 13.21±1.99 | 9.66±2.22 | 5.4±0.04** | 1±0.00*** |
| Motility (%) | 63.73±2.25 | 60.05±2.08 | 19.42±4.30*** | 0.00±0.00*** |

*Each value represents the mean ± SD (n=5); ** P < 0.01, *** P < 0.001 compared with control*

Epididymis of the control group and the group I (100 mg/kg) showed normal tubules with spermatozoa in the lumen (Fig. 3C and D1). Whereas, the treatment with higher doses (300 and 600 mg/kg) caused several damages in epididymis tubules with absence of sperm (Fig. 4 D2 and D3). On the other hand, no histological changes were observed in ovaries of females of all treated groups when compared to the control group (Figs. 5 and 6).

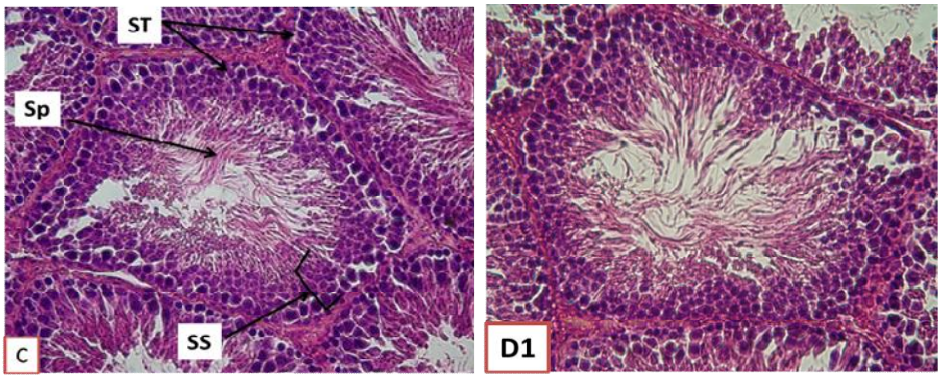



**Fig. 1. Histological sections of testes of control group (C) and group I (D1) treated with 100 mg/kg of *Ruta montana* L. aqueous extract after 90 days of treatment(HE X 200)**
*Showing normal histological structure of semiferous tubules associated with complete spermatogenic series and spermatozoa in the lumen. ST: Seminiferous tubules; Sp: Sperm; SS: Stages of spermatogenesis*

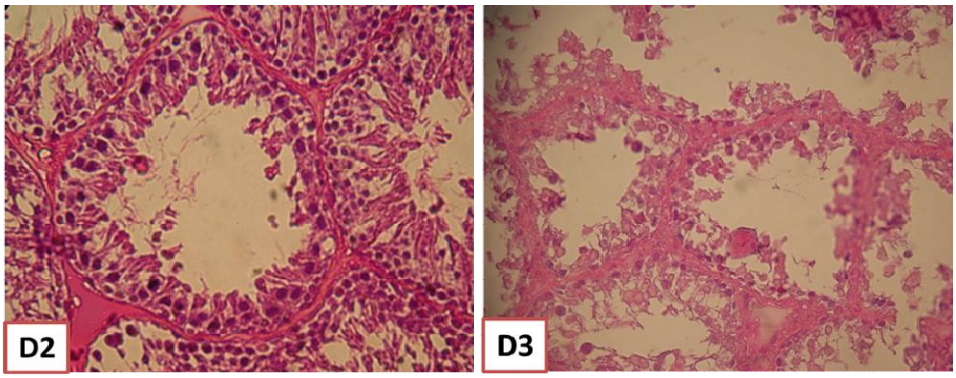



**Fig. 2. Histological sections of testes of group II (D2) and group III (D3) treated with 300 mg/kgand 600 mg/kg respectively of *Ruta montana* L. aqueous extract after 90 days of treatment**
**(HE X 200)**
*Showing degeneration of seminiferous tubules with absence of sperm in tubular lumen*

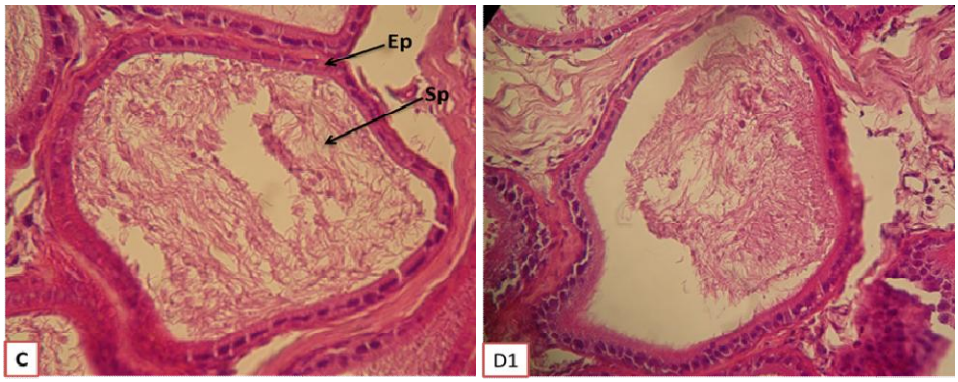



**Fig. 3. Histological sections of the epididymis of control group (C) and group I (D1) treated with 100 mg/kg of *Ruta montana* L. aqueous extract after 90 days of treatment(HE X 200)**
*Showing normal tubules that contained spermatozoa in the lumen.Ep: Epethelial cells; Sp: Sperm*

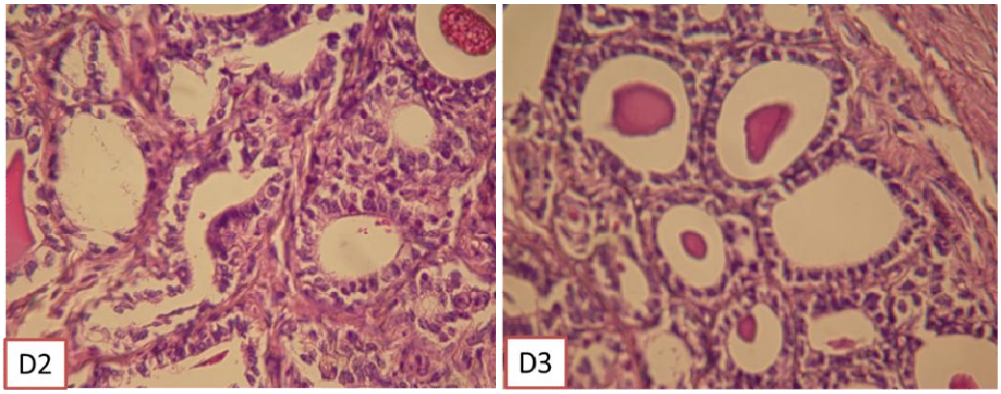



**Fig. 4. Histological sections of the epididymis of group 2 (D2) and group 3 (D3) treated with 300 mg/kg and 600 mg/kg respectively of *Ruta montana* L. aqueous extract after 90 days of treatment (HE X 200)**
*Showing damage in epididymal tubules and absence of sperm*

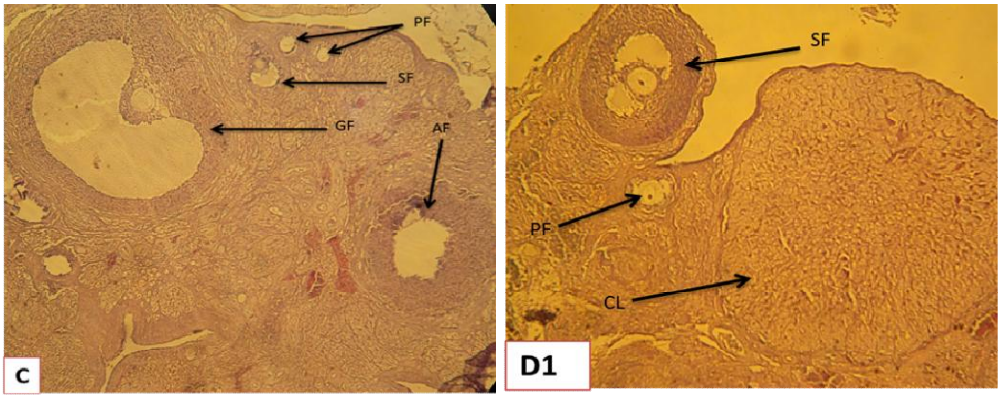



**Fig. 5. Histological sections of Ovary of control group (C) and group I (D1) treated with 100 mg/kg of *Ruta montana* L. aqueous extract after 90 days of treatment (HE X 200)**
*Showing normal structure. PF: Primary follicle; SF: Secondary follicle; GF: Grafian follicle; AF: Atresia follicle; CL: Corpus luteum*

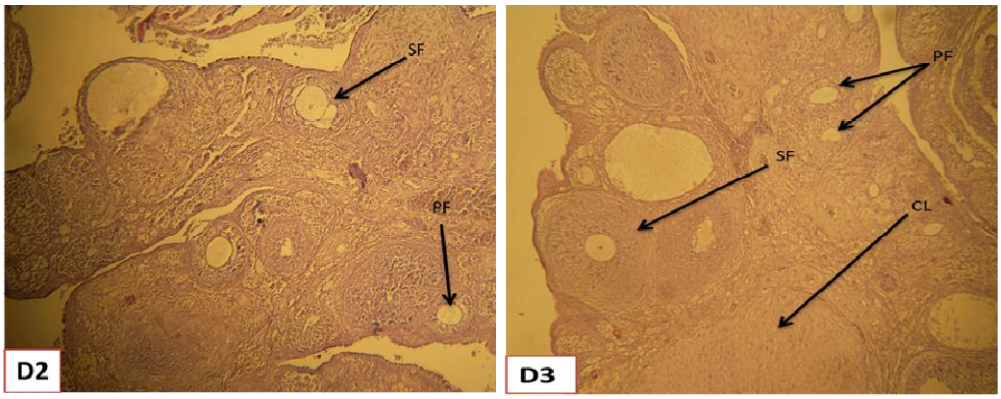



**Fig. 6. Histological sections of Ovaries of group II (D2) and group III (D3) treated with 300 mg/kg and 600 mg/kg respectively of *Ruta montana* L. aqueous extract after 90 days of treatment (HE X 200)**
*Showing normalstructure.PF: Primary follicle; SF: Secondary follicle; CL: Corpus luteum*

## 4. CONCLUSION

It was concluded that the oral administration of aqueous extract of *Ruta montana* L. in rats at 100 mg/kg did not caused any change in weight of reproductive organs. However, the doses of 300 and 600 mg/kg caused a decrease in testis, epididymis, and seminal vesicles weights and reduction in the number and the motility of spermatozoids.

## ETHICAL APPROVAL

As per international standard or university standard written ethical approval has been collected and preserved by the authors.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

## REFERENCES

1. Stojanoski N. Development of health culture in Veles and its region from the past to the end of the 20th century. Veles: Society of science and art. 1999;13–34.
2. Petrovska BB. Historical review of medicinal plants' usage. Pharmacognosy Reviews.2012;6(11):1.
3. Alagammal M, Sakthidevi G, Mohan VR. Anti-fertility activity of whole plant extracts of *Polygala rosmarinifolia* Wight & Arn against male albino rats. Journal of Advanced Pharmaceutical Sciences. 2013;385-393.
4. Pollio A, De Natale A, Appetiti E, Aliotta G, Touwaide A. Continuity and change in the Mediterranean medical tradition: Ruta spp. (Rutaceae) in Hippocratic medicine and present practices. J Ethnopharmacol. 2008;116:469–482.
5. Di Stasi LC, Hiruma CA, Guimaraes CM. Medicinal plants used in Brazilian Amazon. Fitoterapia. 1994;65:529–540.
6. Khadhri A, Bouali I, Belkhir S, Mokded R, Smiti S, Falé P, Araújo MEM, Serralheiro MLM. In vitro digestion, antioxidant and antiacetylcholinesterase activities of two species of Ruta: Ruta chalepensis and *Ruta montana*. Pharmaceutical Biology.2017;55(1):101-107.
7. Bellakhdar J. La pharmacopée marocaine traditionnelle. Maroc: Ed Ibis Press; 1977.
8. Djeridane A, Yousfi M, Nadjemi B, Boutassouna D, Stocker P, Vidal N. Antioxidant activity of some algerian medicinal plants extracts containing phenolic compounds. Food Chemistry. 2006;97:654–660.
9. Ziyyat A, Legssyer A, Mekhfi H, Dassouli A, Serhrouchni M, Benjelloun W. Phytotherapy of hypertension and diabetes in oriental Morocco. J Ethnopharmacol. 1997;58:45–54.
10. Boukef K. Les pratiques parallèles de soins: Aspects sociologiques et éthiques Comité National D'éthique Médicale. 13eme Conférence Annuelle, Dec 4; Sousse, Tunisie; 2009.
11. Bnouham M, Mekhfi H, Legssyer A, Ziyyat A. Ethnopharmacology forum medicinal plants used in the treatment of diabetes in Morocco. Int J Diabetes Metabolism. 2002;10:33–50.
12. Milesi S, Massot B, Gontier E, Bourgaud F, Guckert A. *Ruta graveole* L.: A promising species for the production of furanocoumarins. Plant Science. 2001;161:189–99.
13. Kara Ali W, Ihoual S, AbidliN. Antioxidant and MDR reversal activity in resistant human ovarian cancer cells of methanolic extract from *Ruta montana*located in the North of Algeria, Der Pharma Chemica. 2016;8(12):215-223.
14. Bellakhdar J, Claisse R, Fleurentin J, Younos C. Repertory of standard herbal drugs in the Moroccan pharmacopoeia. J Ethnopharmacol. 1991;35:123–143.
15. Mbiantcha M, Kamanyi A, Teponno RB, Tapondjou LA, Watcho P, Nguelefack TB. Analgesic and anti-inflammatory properties of extracts from the Bulbils of *Dioscorea bulbifera* L. var sativa

(Dioscoreaceae) in Mice and Rats. Evidence-Based Complementary and Alternative Medicine. 2011;1-9.

16. Bensalem-Bendjelloul M. Techniques histologiques théorie et pratique. Office des publications universitaires. 1998;6-41.
17. Koneri R, Saraswati CD, Balaraman R, Ajeesha EA. Antiimplantation activity of the ethanolic root extract of *Momordica cymbalaria* Fenzl in rats. Indian Journal of Pharmacology. 2007;39:90-96.
18. Teo S, Stirling D, Thomas S, Hoberman A, Kiorpes A and Khetani V. A 90- day oral gavage toxicity study of D-methylphenidate and D, Lmethylphenidatein Sprague-Dawley rats. Toxicology. 2002;179:183-196.
19. Gupta RS, Chaudhary R, Yadav RK, Verma SK, Dobhal MP. Effect of saponins of *Albizia lebbeck* (L.) benth bark on the rerproductive system of male albino rats. J. Ethnopharmacol. 2005;96:31-36.
20. Zeherea MN, Reddy SP, Reddy SP, Ravindra and Saraswati BP. Antispermatogenic and androgenic activities of *Momordica charantia* (Karela) in Albino rats. J. Ethnopharmacol. 1998; 61:9-16.
21. Khan PK, Awasthy KS. Cytogenetic toxicity of neem. Food Chem. Toxicol. 2003;41:1325-1328.
22. Setty BS. Regulation of epididymal function and sperm maturation-endocrine approach to fertility control in male. Endocrinology. 1979;74:100-117.
23. Rao MV. Effect of alcoholic extract of *Solanum xanthocarpum* seeds in adult male rats. Indian J. Exp. Biol. 1988;26:95-98.
24. Keel AB, Abney T. Influence of bilateral cryptorchidism in the mature rat; alteration in testicular function and serum hormonal level. Endocrinology. 1980;107:1226-1233.
25. Chinoy NJ, Sheth KM, Seethalakshmi L. Studies on reproductive physiology of animals with special reference to fertility control. Comp. Physiol. Ecol. 1982;7-325.
26. Kusemiju TO, Osinubi AA, Noronha CC, Okanlawon AO. Effect of *Carica papaya* bark extract on the testicular histology of male SD rats. Nig. Quat. Jour. of Hosp. Med. 2010;20(3):133-137.
27. Almasad MM, Qazan WSH, Daradka H. Reproductive toxic effects of Artemisia herba alba ingestion in female Spague-Dawley rats. Pak J Biol Sci. 2007;10(18):3158-3161.
28. Steinberger E. Hormonal control of mammalian Spermatogenesis. Physiology Review. 1971;5111:1-22.
29. Wannang NN, Jimam NS, Gyang SS, Bukar BB, Gotom S. Effects of *Cucumis metuliferus* E Mey. Ex Naud (Cucurbitaceae) fruit extract on some male reproductive parameters in adult rats. African Journal of Pharmacy and Pharmacology. 2008;2(3):048-051.
30. Cheng CY, Wong EW, Yan HH, Mruk DD. Regulation of spermatogenesis in the microenvironment of the seminiferous epithelium: New insights and advances. Mol. Cell Endocrinol. 2010;315(1–2):49–56.
31. Ghosh D, Das UB, Ghosh S, Mallick M, Debnath J. Testicular gametogenic and steroidogenic activities in cyclophasphamide treated rats: A correlative study with testicular oxidative stress. Drug Chem Tox. 2002;25:281-291.

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