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Antimycobacterial and cytotoxic activities of extracts from fungal isolates of Lake Magadi

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ABSTRACT

In this study, antimycobacterial and cytotoxic activities of ethyl acetate extracts of fungal isolates from Lake Magadi were evaluated. The extracts were tested against *Mycobacterium madagascariense* (*MM*) and *M. indicus pranii* (*MIP*), and cytotoxicity against brine shrimp (*Artemia salina*) larvae. Fungal strains were identified using sequence comparison of the Internal Transcribed Spacer (ITS) region. Potent antimycobacterial activities against *MM* were exhibited by extracts from *Volutella colletotrichoides*, *Helicoon richonis, Penicillium limosum, P. sacculum, Aspergillus parasiticus* and *A. nomius* strains that exhibited minimum inhibition concentration (MIC) in the range of 0.19 – 12.5 mg/mL. On the other hand, significant antimycobacterial effects against *MIP* was shown by extracts from *V. colletotrichoides*, *H. richonis, A. parasiticus, Fusarium merismoides, A. silvaticus* and *A. fumigatus* strains in the same MIC range. Notable cytotoxic activities of the extracts were from *A. versicolor, A. nomius, P. janthinellum* and *H. richonis* strains with LC_{50} values ranging from 46.60 – 98.12 µg/mL. These results indicate that fungi inhabiting Lake Magadi have the ability to produce bioactive metabolites that could be further explored for potential medicinal agents.
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Keywords: Antimycobacterial, cytotoxic, *Artemia salina*, *Mycobacterium madagascariense*, *Mycobacterium indicus pranii.*

INTRODUCTION

Extremophilic microorganisms thrive in extreme habitats with unusual levels of salinity, pH, temperature, pressures and low water activities. Those inhabiting hypersaline environments have been categorized into two

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groups namely, haloalkaliphiles and halophiles (Kumar and Gummadi, 2009). Haloalkaphiles and halophiles are prokaryotic and eukaryotic microbes adopted to balance their osmotic pressure in order to resist the denaturing effects of salt (Kis-Papo et al., 2000). Hypersaline environments of soda lakes like Lake Magadi are formed in depressions where ground water is rich in carbon dioxide, but poor in magnesium and calcium, leaching sodium from sodium-rich rocks. The absence of these dissolved divalent cations causes carbonate to precipitate. During dry seasons, carbonate salts become more concentrated due to increased evaporation rates. This leads to the formation of natural sodium carbonate/bicarbonate buffered systems with elevated pH values in the range of 9.5 – 11 and salt concentrations reach saturation (Sorokin et al., 2014). This chemical composition put pressure on the biota and only haloalkaliphiles microorganisms thrive in such conditions.

A number of studies on mycobiota inhabiting saline and hypersaline environment have been investigated especially in salterns (Gunde-Cimerman et al., 2000; Gunde-Cinerman et al., 2004; Cantrell et al., 2006; Nayak et al., 2012; Rani et al., 2013; Niknejad et al., 2013), dead sea (Kis-Papo et al., 2000; Molitoris et al., 2000) and desert soils (El-Meleigy et al., 2010) and found to be dominated by *Cladosporium*, *Aspergillus* and *Penicillium* species. Studies on the occurrence of fungi in salt lakes remain very scanty. Thus, studies reported from four salt lakes of Egypt demonstrated the occurrence of *Perconia prolifica, Clavatospora bulbosa. Cirrenalia basiminuta, Panorbis viscosus* and *Ceriosporopsis* as dominant species from Lake Edku, Lake Marriott, Lake Burullus and Lake Quaron (El-Sharouny et al., 2009). Higher fungi such as *Aniptodera chesapeakensis, Dictyosporium heptasporum* and *Savoryella lignicola* were reported from Lake Fuxian, Yunnan China (Cai et al., 2002).

Lake Magadi which is located in the southern part of the Kenyan Rift Valley represents a unique hypersaline environment. It is 2 °S and 36 °E from the equator at an elevation of 600 m above sea level and supplied by a series of alkaline springs (Behr and Rohricht, 2000). The previous studies on Lake Magadi revealed a variety of microorganisms (Kambura 2011, Muruga and Anyango 2013, Kambura et al., 2016). In an attempt to further elucidate the potential of fungal species of Lake Magadi ecosystem, the mycobiota of the lake to evaluate their capacity to produce antimycobacterially active metabolites was investigated. The extracts were also screened for their potential cytotoxic effects.

MATERIALS AND METHODS

Sample collections

Microbial mat, surface water, salt, biofilms, foam on surface water and sediments were sampled from different sites (Figure 1) at the eastern shore of Lake Magadi in July 2014 using grab sampler and soil agar. The samples were transferred into sterile bottles and bags, stored in an insulated box and transported to the Chemistry Department, University of Dar es Salaam (UDSM), where they were placed in a refrigerator. Physicochemical profiles: temperature, conductivity, pH, total dissolved solids and dissolved oxygen were determined at the sampling sites with multi-parameter meter.

Fungal isolation and identification

1 g of fresh wet (sediment and salt) samples and 1 ml of (microbial mat, foam, water and biofilms) samples were suspended in 9 ml sterile saline solution (0.85% NaCl), treated by thermal shock at 60 °C for 6 minutes to minimize the gram negative bacterial growth and then serially diluted to a factor of 10^{-3}. Aliquots (0.1 ml) of each dilution were spread in duplicate onto

selective isolation media, malt extract agar with 10, 20 and 40% v/v of salt water (M1**)**. Malt extract Agar prepared with sterile distilled water (M2**)** was also used. All media were supplemented with rifampicin (100 µg ml^{-1}) to inhibit bacterial growth (Omotayo et al., 2011). The inoculated plates were incubated at 30 °C. Colonies were picked and sub cultured onto new M1 plates and incubated at 30 °C. Pure cultures were sub-cultured onto M1 slants and stored at 4 °C in the fridge at Chemistry Department, University of Dar es Salaam. The isolated fungal strains were identified by studying the ITS 1-5.8-ITS 2 region of rDNA. Amplification involved the use of the following sets of primers which are mainly used for fungi identification; ITS 1-sequence: 5'_ TCCGTAGGTGAACCTGCGG forward primer; and ITS 4-sequence: 5'_ TCCTCCGCTTATTGATATGC reverse primer. The PCR products were sequenced using the Big Dye chain termination method employing an ABI 3730 Genetic analyzer at International Livestock Research Institute (ILRI), Kenya. Each sample was sequenced twice using either forward or reverse primer. BLAST which is an online tool found at the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/) was used to find the region of similarities between the partial sequences of the fungal strains and those found in the Genbank. The evolutionary history was inferred using the neighbour-joining method and the evolutionary distances were computed using the maximum composite likelihood method. Evolutionary analyses of 23 nucleotide sequences were conducted in MEGA 6.

Preparation of fungal crude extracts

The fungal strains were cultivated on 2 L flasks containing malt extract broth with salt composition as described by Baumgarte (2003). The broth was extracted by liquid-liquid extraction method using ethyl acetate (EtOAc). Mycelium was extracted by overnight soaking in EtOAc and then filtered. The filtrate was then concentrated on a rotary evaporator with bath water maintained at 40 °C at 90 rpm (Masalu et al., 2012) to afford the crude extracts.

Antimycobacterial assay

Antimycobacterial screening was carried out at the Institute of Traditional Medicines (ITM), Muhimbili University of Health and Allied Sciences (MUHAS). The two fast growing, non-pathogenic mycobacterial strains *Mycobacterium madagascariense* (*MM*) and *M. indicus pranii* (*MIP*) were used as markers for determination of a potential antimycobacterial efficacy of the fungal isolates extracts. The MIC values of fungal extracts against Mycobacterium strains were determined by two-fold microdilution method, in the sterile flat-bottomed 96 well polystyrene microtiter plates as described by Magadula et al. (2012) and Donkeng-Donfack et al. (2014).

Cytotoxicity assay

Brine shrimp lethality bioassay was carried out on fungal extracts as described by Magadula et al. (2012) and Oloyede et al. (2010).

Statistical analysis

The lethality was calculated from the mean survival larvae shrimps on extracts treated tubes and control. Log probit analysis was used to determine log dose regression lines for mortality in relation to concentrations thereafter LC_{50} values were obtained from best-fit line.

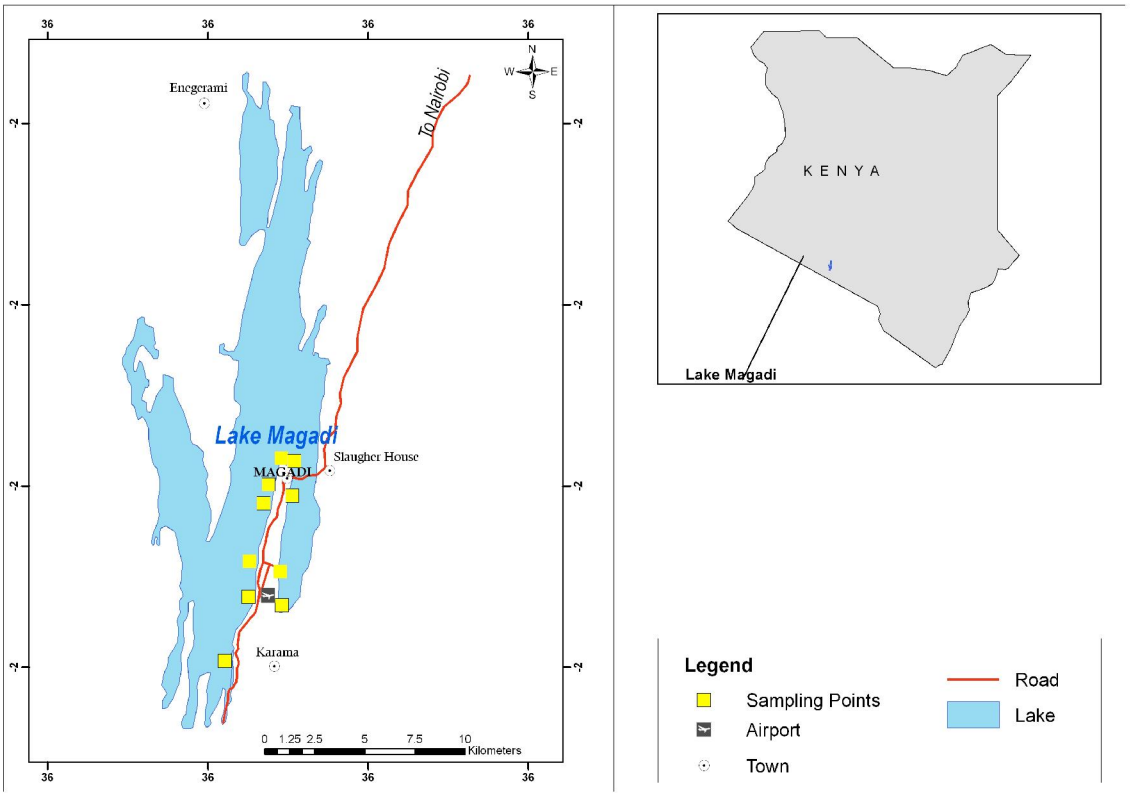


Figure 1: Map of Lake Magadi Basin showing sampling sites.

RESULTS

Physicochemical parameters of sampling points

Physicochemical parameters of each sampling points were determined and reported as mean values. The mean value of salinity as reflected by electrical conductivity ranged from 7.25-10.49 S/m. Other parameters were temperature (45.84 ± 0.51 °C), pH (9.8 ± 2.86), total dissolved solids (3.16 ± 2.17 mg/L) and dissolved oxygen (7.71 ± 0.63 mg/L).

Fungal species composition

A total of 23 fungal strains were isolated and identified from Lake Magadi. Table 1 presents the list of fungal strains isolated, their taxonomy, source of origin and their morphology. As indicated in Figure 2, microbial mat had the highest number of fungal strains (6) followed by the foam on water surface (4) and biofilms (4). Sediment, salt and surface water samples resulted into the isolation of 3 fungal strains each (Figure 2). The fungal strains isolated were identified to belong to genera of *Volutella*, *Aspergillus*, *Fusarium*, *Penicillium*, *Helicoon*, *Neurospora*, *Polyozellus*, *Eupenicillium*, *Sarocladium*, *Teratosphaeria* and *Acremonium*. As in Table 1 and Figure 3, the fungal strains were dominated by *Aspergillus* species (8) identified taxonomically as *Aspergillus ochraceus*, *A. alliaceus*, *A. silvaticus*, *A. fumigatus*, *A. oryzae*, *A. versicolor*, *A. nomius*

and *A. parasiticus* followed by *Penicillium* species (6) identified as *Penicillium glabrum*, *P. limosum*, *P. janthinellum*, *P. decumbens*, *P. charlesii* and *P. sacculum*. All other genera had one species each.

Fungal evolutionary history and phylogenetic relationship

The optimal tree and the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown in Figure 4. The phylogenetic assay based on ITS region of rDNA showed that the 23 fungal strains could be sorted into 5 clusters. Cluster one consisted of *N. crassa*, *F. merismoides*, *V. colletotrichoides* while *S. strictum. H. richonis*, *P. multiplex* and *T. mexicana* were grouped into cluster two, three and four, respectively. Cluster five was divided into 6 sub clusters bearing 72% confidence with *A. alliaceus, A. fumigatus, A. oryzae* and *A. fumigatus* in sub cluster one, two, three and four, respectively. Sub cluster five was further sub classified into 69%, 55% and 61% confidence as sub-sub-clusters which were *P. charlesii*, *P. glabrum*, *P. decumbens*, *P. janthinellum*, *P. limosum, Eupenicillium* sp. and *P. sacculum,* respectively. *A. cellulolyticus* was as an out group. Finally, sub cluster six was further sub classified into 60% sub-sub cluster and 91% sub-sub-sub cluster as *A. nomius*, *A. ochraceus*, *A. versicolor* and *A. silvaticus*. *P. multiplex* showing having the genetically distant divergence at 15.3% against *V. colletotrichoides* at 14.6%. The genetically closely related fungi were *P. glabrum* with *P. charlesii* as well as *A. versicolour* with *A. silvaticus*.

Antimycobacterial assay

The EtOAc extracts exhibited antimycobacterial activities with the minimum inhibition concentration (MIC) values ranging between 0.19 to 6.25 mg/mL (Table 2). Extracts of *V. colletotrichoides* and *H. richonis* exhibited potent activity against *MM* and *MIP* both having the MIC value of 0.19 mg/mL. The extracts from *P. limosum*, *P. sacculum, A. parasiticus* and *A. nomius* showed MIC value of 0.78 mg/mL against *MM* followed by *A. versicolor*, *P. glabrum*, *P. decumbens*, *P. charlesii*, *A. fumigatus* and *F. merismoides* which exhibited MIC value of 3.13 mg/mL against *MM*. No activity against *MM* was detected on extracts of *N. crassa*, *A. oryzae*, *Eupenicillium* species, *S. strictum*, *A. silvaticus*, *T. mexicana* and *P. multiplex*. For *MIP*, good activity was observed with extracts from *A. parasiticus* at MIC value of 0.39 mg/mL and moderate activity was exhibited by extract of *A. nomius*, *A. silvaticus*, *A. fumigatus* and *F. merismoides* at MIC value of 1.56 mg/mL. 16 and 18 fungal extracts were found to be active than isoniazid against *MM* and *MIP*, respectively. However, all the fungal extracts were less active than Ciprofloxacin (<0.046 mg/mL for *MM* and <0.01 mg/mL *MIP*) standard antibiotics (Nkya et al., 2014).

Cytotoxic activities

Cytotoxic activity results for 23 fungal extracts evaluated in this study is given in Table 3. The results showed that LC_{50} of these fungal extracts were diverse, ranging from 46.60 µg/mL to 679.17 µg/mL. Four of the fungal extracts displayed active toxicity (LC_{50} below 100 µg/mL) with *A. versicolor* extract being the most active having an LC_{50} value of 46.6 µg/mL followed *A. nomius*, *H. richonis* and *P. janthinellum* extracts which had LC_{50} values of 60.51, 93.33 and 98.12 µg/mL, respectively. Other extracts had mild lethality activities having LC_{50} values above 100 µg/mL.

Table 1: Fungal strains isolated from Lake Magadi in this study.

Strain no.	Strains	Strain accession no.	Origin	Morphology			
				Colour		Margin	Elevation
				Top	Bottom		
F 01	*Volutella colletotrichoides*	AJ301962	Sediment	Orange	Cream	Filamentous	Flat
F 02	*Aspergillus ochraceus*[a]	AF548065	Foam on water surface	Brown	Brown	Curled	Umbonate
F 03	*Fusarium merismoides*[a]	AF141950	Microbial mat	White	Black	Entire	Flat
F 04	*Penicillium glabrum*	FJ717698	Surface water	White	Cream	Entire	Umbonate
F 05	*Helicoon richonis*	AY856952	Salt	Cream	Cream	Entire	Flat
F 06	*Neurospora crassa*[a]	FJ610444	Biofilms	Cream	Orange	Entire	Flat
F 07	*Polyozellus multiplex*	AY771600	Foam on water surface	Greenish	Brown	Undulate	Crateriform
F 08	*Eupenicillium sp.*	GQ253349	Salt	Grey	Black	Undulate	Convex
F 09	*Aspergillus alliaceus*	AB002071	Microbial mat	Green	Yellow	Entire	Raised
F 10	*Sarocladium strictum*	HM216184	Biofilms	Grey	Black	Undulate	Convex
F 11	*Aspergillus silvaticus*	AF548067	Surface water	Green	Cream	Curled	Umbonate
F 12	*Penicillium limosum*	EF411061	Biofilms	Green	Purple	Entire	Convex
F 13	*Teratosphaeria Mexicana*	GU214604	Sediment	Green	Black	Entire	Flat
F 14	*Penicillium janthinellum*	AB293968	Salt	Grey	Cream	Entire	Flat
F 15	*Penicillium decumbens*	FJ458446	Sediment	Green	Cream	Entire	Flat
F 16	*Penicillium charlesii*	FJ430768	Biofilms	Brown	Black	Entire	Flat
F 17	*Penicillium sacculum*	AB027410	Foam on water surface	White	White	Entire	Umbonate
F 18	*Aspergillus fumigatus*[a]	FJ840490	Microbial mat	Cream	Brown	Entire	Flat
F 19	*Aspergillus oryzae*[a]	HM064501	Microbial mat	Pinkish	Brown	Entire	Flat
F 20	*Aspergillus versicolor*[a]	AF548069	Surface water	Cream	Purple	Undulate	Undulate
F 21	*Aspergillus nomius*	AB008404	Microbial mfat	Green	Yellow	Entire	Raised
F 22	*Aspergillus parasiticus*	D63699	Foam on water surface	Cream	Orange	Entire	Raised
F 23	*Acremonium cellulolyticus*	AB474749	Microbial mat	Cream	Brown	Undulate	Flat

[A] Reported from other hypersaline waters (Cantrell et al. 2006; Gonsalves et al. 2012)

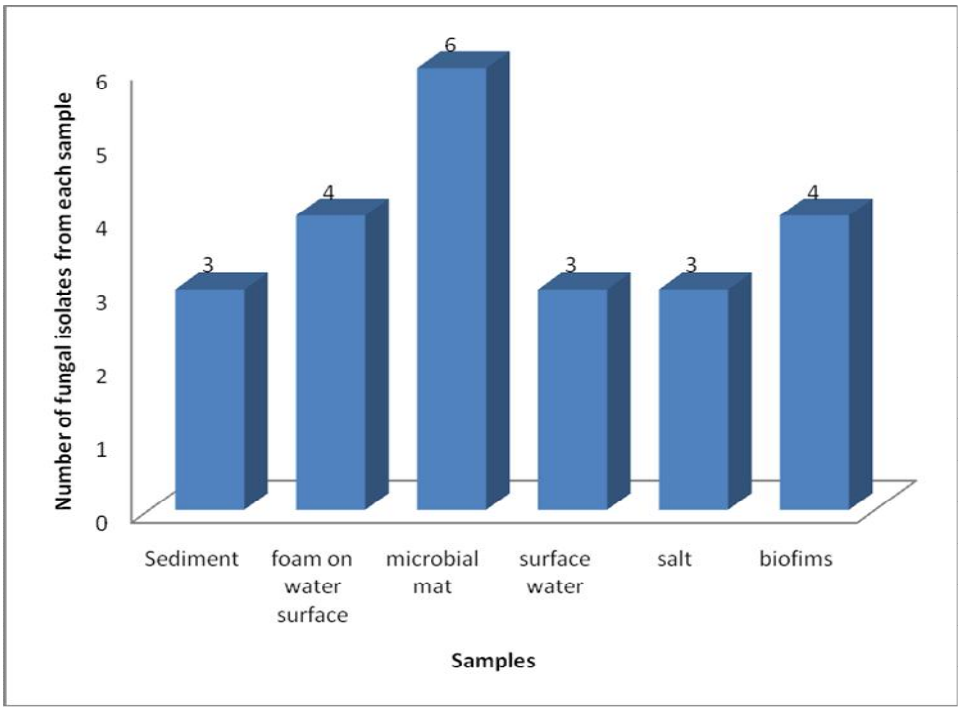


Figure 2: Number of fungal isolates obtained from each sample.

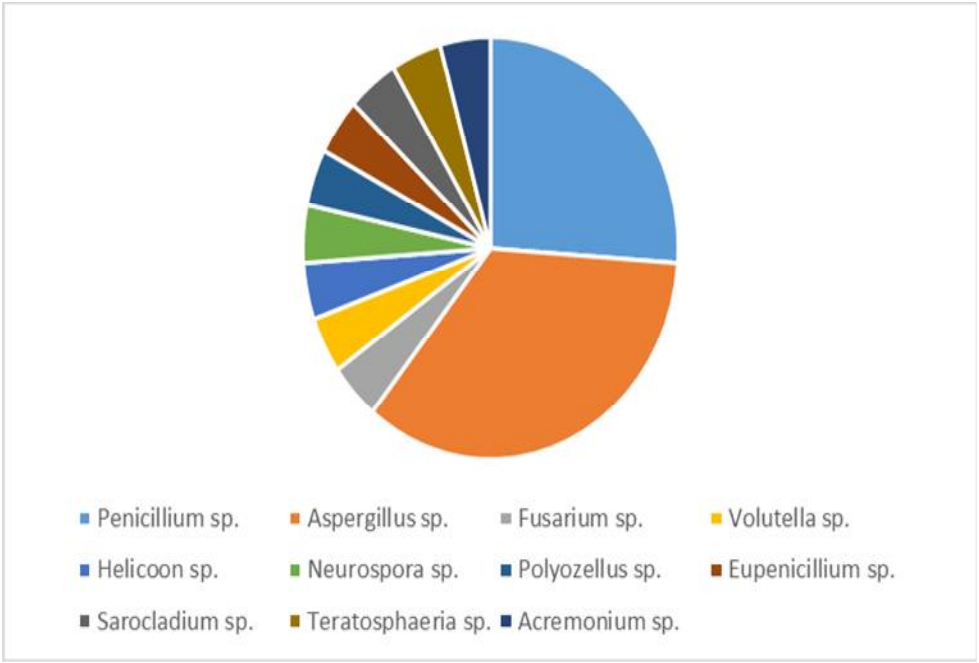


Figure 3: Distribution of various fungal genera in Lake Magadi samples.

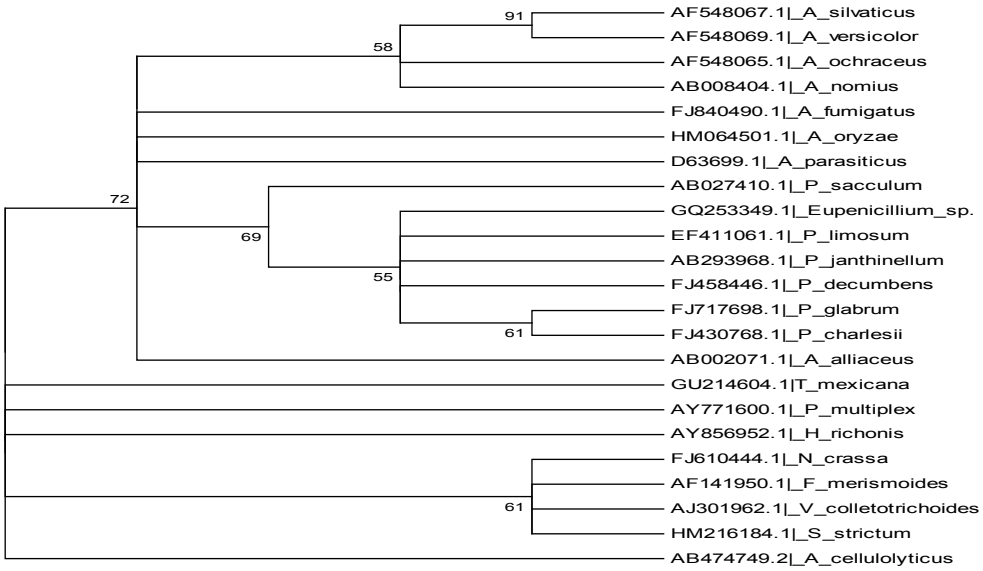


Figure 4: Dendrogram of phylogenetic position of Lake Magadi fungal strains as deduced from sequence of ITS region of the rDNA.

Table 2: MIC results for *M. madagascariense* and *M. indicus pranii* on Lake Magadi EtOAc fungal extracts.

Fungal Species	**Minimum Inhibition Concentration (mg/mL)**	
	M. madagascariense	***M. indicus pranii***
Volutella colletotrichoides	0.19	0.19
Aspergillus ochraceus	6.25	6.25
Fusarium merismoides	3.13	1.56
Penicillium glabrum	3.13	3.13
Helicoon richonis	0.19	0.19
Neurospora crassa	12.5	12.5
Polyozellus multiplex	12.5	3.13
Eupenicillium sp.	12.5	12.5
Aspergillus alliaceus	6.25	12.5
Sarocladium strictum	12.5	3.13
Aspergillus silvaticus	12.5	1.56
Penicillium limosum	0.78	0.78
Teratosphaeria Mexicana	12.5	6.25
Penicillium janthinellum	6.25	6.25
Penicillium decumbens	3.13	6.25
Penicillium charlesii	3.13	3.13
Penicillium sacculum	0.78	12.5
Aspergillus fumigatus	3.13	1.56
Aspergillus oryzae	12.5	12.5
Aspergillus versicolor	3.13	3.13
Aspergillus nomius	1.56	1.56
Aspergillus parasiticus	0.78	0.39
Acremonium cellulolyticus	6.25	6.25
Isoniazid (+ve control)	12.5	12.5

Table 3: Brine shrimp lethality bioassay results for EtOAc extracts from fungal species of Lake Magadi.

Fungal species	Percentage death of nauplii after 24 hrs exposure					LC_{50} µgmL^{-1}	95 % CI µgmL^{-1}	Best-fit line
	240 µgmL^{-1}	120 µgmL^{-1}	80 µgmL^{-1}	40 µgmL^{-1}	24 µgmL^{-1}			
Volutella colletotrichoides	85	45	35	0	0	118.61	101.4-138.7	y=106.6logx-171.1
Aspergillus ochraceus	100	20	0	0	0	117.81	74.6-186.0	y=125.0logx-2089
Fusarium merismoides	10	5	0	0	0	inact.	--	--
Penicillium glabrum	85	25	5	0	0	153.31	124.4-188.9	y=109.4logx-189.1
Helicoon richonis	100	65	25	10	0	98.12	65.8-146.2	y=121.0logx-191
Neurospora crassa	40	15	0	0	0	315.14	183.1-542.3	y=83.7logx-159.2
Polyozellus multiplex	55	5	0	0	0	202.77	119.2-344.8	y=43.0logx-279.9
Eupenicillium sp.	20	0	0	0	0	679.17	164.1-2811.0	y=66.4logx-138.1
Aspergillus alliaceus	90	25	5	0	0	146.05	106.7-199.8	y=115.5logx-200
Sarocladium strictum	30	5	0	0	0	513.77	141.6-1864.6	y=65.0logx-126.2
Aspergillus silvaticus	100	10	0	0	0	152.21	52.8-438.1	y=219.1logx-428.1
Penicillium limosum	100	20	0	0	0	146.88	51.8-416.2	y=215.6logx-417.2
Teratosphaeria mexicana	75	30	15	0	0	158.39	147.4-170.2	y=95.8logx-160.8
Penicillium janthinellum	100	65	30	15	0	93.33	70.5-123.6	y=113.5logx-173.6
Penicillium decumbens	100	20	0	0	0	146.88	51.8-416.2	y=215.6logx-417.2
Penicillium charlesii	0	0	0	0	0	inact.	--	--
Penicillium sacculum	70	50	10	0	0	148.66	141.0-156.8	y=96.6logx-159.9
Aspergillus fumigatus	95	65	15	5	0	107.72	62.1-186.9	y=123.8logx-201.6
Aspergillus oryzae	100	25	10	0	0	131.66	81.8-211.8	y=126.4logx-217.9
Aspergillus versicolor	100	100	55	50	0	46.60	37.1-58.6	y=95.3logx-109
Aspergillus nomius	95	70	60	30	0	60.51	40.4-90.5	y=83.6logx-98.96
Aspergillus parasiticus	100	45	25	0	0	136.80	85.0-220.1	y=126.4logx-220
Acremonium cellulolyticus	5	0	0	0	0	inact.	--	--
DMSO (-ve control)	0	0	0	0	0	inact.	--	--

NB: Inact. = Inactive

DISCUSSION

A total of 23 fungi strains were isolated and taxonomically identified from five different matrices of Lake Magadi. The identified species could be grouped into eleven genera namely, *Volutella*, *Aspergillus*, *Fusarium*, *Penicillium*, *Helicoon*, *Neurospora*, *Polyozellus*, *Eupenicillium*, *Sarocladium*, *Teratosphaeria* and *Acremonium*. These being common strains occurring in salt lakes as reported in other studies elsewhere (Cai et al., 2002, El-Sharouny et al., 2009; Kambura et al., 2016). The results also indicated that six of the twenty three fungal strains were obtained from the microbial mat, being the highest number compared with the other matrices (Figure 2). The occurrence of fungi in microbial mat might have been influenced by oxic levels and photosynthetic organisms that provided protection (Cantrell et al., 2006). Twenty-six percent of the 23 strains isolated in this study namely *A. ochraceus, F. merismoides, N. crassa, A. fumigatus, A. oryzae* and *A. versicolor* have been reported in other hypersaline waters (Cantrell et al., 2006; Gonsalves et al., 2012 and Kambura et al., 2016). The MIC values of *MM* and *MIP*, nonpathogenic microorganisms obtained from the studied fungal extracts closely related to the sensitivity of *Mycobacterium tuberculosis* in other studies on fungal extracts. De Prince et al. (2012) and Gamboa-Angulo et al. (2013) studied extracts from various fungal species for antimycobacterial activity and found them to possess anti-*Mycobacterium tuberculosis* activity. Jouda et al. (2016) isolated various compounds produced by an endophytic fungus of *Penicillium* species harbored in *Garcinia nobilis*, and were found to be active against *Mycobacterium smegmatis*. Therefore, observed antimycobacterial activities from these fungal extracts supports the immensity of the potential of antimycobacterial drug discovery from fungal species. The brine shrimp lethality assay is a quick bioassay for testing extracts which correlates with cytotoxic and antitumor properties (Krishnaraju et al., 2005). The cytotoxicity assay using the brine shrimps revealed that all extracts were less toxic to brine shrimps compared with podophyllotoxin (LC_{50} 2.72 µg/mL, Table 3), a well-known cytotoxic lignin. Our findings correlate with other findings on cytotoxicity of fungal extracts (Lu and Wang, 2009). Based on the observed brine shrimp larvae lethality test, it can be speculated that these fungal extracts could possess some anticancer, larvicidal or pesticidal potential.

Conclusion

In this study, twenty-three fungal species have been found to be present in Lake Magadi. For the first time, the results of these fungal species revealed that their extracts have significant *in vitro* antimycobacterial and cytotoxic activities. These results show that fungi living in the halotolerant environment of Lake Magadi have the ability to produce bioactive metabolites that could be further investigated for active principles for possible development of diseases control agents.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

JJEM and SSN conceived and designed the project. JJEM, RM and EG supervised the work. PM assisted in antimycobacterial and cytotoxic analysis. KDK carried out the work and produced the first draft of the manuscript. All the authors contributed equally to the improvement of the manuscript and agreed on the final version before submission.

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