International Journal of Biological, Life and Agricultural Sciences
ISSN: 2415-6612
Vol:9, No:12, 2015

In vitro Susceptibility of *Madurella mycetomatis* to the Extracts of *Anogeissus leiocarpus* Leaves

Ikram Mohamed Eltayeb Elsiddig, Abdel Khalig Muddather, Hiba Abdel Rahman Ali, Saad Mohamed Hussein Ayoub

Abstract*—Anogeissus leiocarpus* (Combretaceae) is well known for its medicinal uses in African traditional medicine, for treating many human diseases mainly skin diseases and infections. Mycetoma disease is a fungal and/ or bacterial skininfection, mainly cause by *Madurella mycetomatis* fungus. This study was carried out *in vitro* to investigate the antifungal activity of *Anogeissus leiocarpus* leaf extracts against the isolated pathogenic *Madurella mycetomatis*, by using the NCCLS modified method compared to Ketoconazole standard drug, and MTT assay. The bioactive fraction was subjected to chemical analysis implementing different chromatographic analytical methods (TLC, HPLC, and LC-MS/MS). The results showed significance antifungal activity of *A. leiocarpus* leaf extracts against the isolated pathogenic *M. mycetomatis*, compared to negative and positive controls. The chloroform fraction showed the highest antifungal activity. The chromatographic analysis of the chloroform fraction with the highest activity showed the presence of important bioactive compounds such as ellagic and flavellagic acids derivatives, flavonoids and stilbenoid, which are well known for their antifungal activity.

Keywords*—Anogeissus leiocarpus*, crude extracts and fractions of *Anogeissus leiocarpus*, *in vitro* susceptibility of *Madurella mycetomatis, Madurella mycetomatis*.

I. Introduction

ANOGEISSUS LEIOCARPUS (*Combretaceae*), is an evergreen tree widely distributed in Africa [1], [2] and well known in African traditional medicine for treating many diseases mainly skin diseases and infections, wounds infections, sore feet, boils, cysts, syphilitic and diabetic ulcers [3]-[5].

Leaves are widely used against skin diseases and infections, jaundice, hepatitis, haemorrhoids, respiratory diseases, headache and toothache, as antimalarial, leprotic, laxative and anthelmintic [1], [3], [6]-[10].

A. leiocarpus showed strong antibacterial and antifungal activity against pathogenic microorganisms [11]-[17].

Ikram Mohamed Eltayeb Elsiddig is with the Department of Pharmacognosy, Faculty of Pharmacy, University of Medical Sciences and Technology/ Khartoum/Sudan (corresponding author: phone +249912987518; fax +249/83/224799; e-mail: kramela_07 @yahoo.com).

Abdel Khalig Muddather is with the Department of Pharmacognosy, Faculty of Pharmacy, University of Khartoum/ Khartoum/Sudan (e-mail: muka46@hotmail.com).

Hiba Abdel Rahman Ali is with the Commission of Biotechnology and Genetic Engineering, National Center for Research/ Khartoum/Sudan (e-mail: hibaali@hotmail.com).

Saad Mohamed Hussein Ayoub is with the Department of Pharmacognosy, Faculty of Pharmacy, University of Medical Science and Technology/ Khartoum/Sudan (e-mail: sacitrullus@gmail.com).

Mycetoma is a chronic subcutaneous and deep tissues granulomatous skin disease or a group of skin infections caused by several fungi (eumycetoma) mainly *Madurella mycetomatis* fungus, or by bacteria (actinomycetoma). Progressive destruction of tissues leads to loss of function and impaired the affected site. Serious cases require amputation leading to loss of numerous infected limbs [18].

In Sudan, mycetoma is a serious common disease leading to loss of numerous limbs. The incidence of mycetoma in Sudan has not change and around 400 new cases are seen in hospital and outpatient clinics every year [18], [19].

There are no 100% effective drugs for eumycetoma infection, and adequate treatment requires a prolonged antifungal drug combined with extensive surgical treatment [18].

Meager data is available for susceptibility of *M. mycetomatis* to plant secondary metabolites [20]-[22].

II. Materials and Methods

A. Plant Material Collection and Preparation

A. leiocarpus leaves were collected from El Damazeine region, Sudan, identified by taxonomist in the department of silviculture, Faculty of Forestry, University of Khartoum, andthe voucher specimen, IKR2, May - 2008 was kept in the Herbarium of the Department of Biochemistry, Commission of Biotechnology and Genetic Engineering, National Centre for Research. The plant material was air dried under shade at room temperature, then ground into powder using pestle and mortar.

B. Preparation of the Extract

Powdered leaves were extracted by maceration overnight in 80% alcohol, and then the extract was fractionated by using solvents with increasing polarities: petroleum (PE), chloroform ($CHCl_3$) and ethyl acetate (EtOAc). The solvents were evaporated to dryness under reduced pressure using rotary evaporator.

C. Collection and Culture of Madurella mycetomatis Fungus

Isolated *M. mycetomatis* fungus was collected in mycetoma research center at Soba hospital whereas, black grains were exuded from open sinuses and surgical biopsy from the lesion, freed from tissues and carried by forceps in sterile container (Fig. 1), then washed with saline for several times.

D. RPMI 1640 Medium Preparation

RPMI 1640 with L- glutamine medium, prepared by dissolving 0.3g RPMI 1640 with L- glutamine powder (PM Biomedical Inc. France) and 0.02g MOPHS buffer (3, 4-morpholinopropane sulfonic acid) in one liter distilled water and sterilized by autoclaving at 151bs pressure and121°C for 15 minutes.

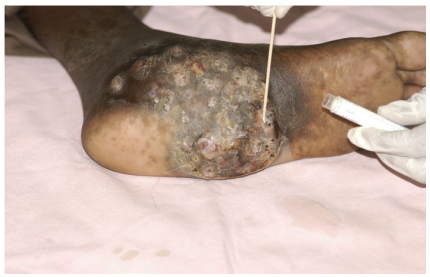

Fig. 1 Mycetoma pathogen collection

E. Preparation of Fungal Suspension

The isolated grains of *M. mycetomatis* were firstly cultured in blood agar media, then subculture in Sabouraud dextrose agar and incubated at 37°C for 8 days.

The isolate strains were subcultured again to maintain pure isolate of hyphae. The subculture of hyphae was repeated for two weeks to maintain pure hyphae which were harvested in mycological peptone (BDH) water broth medium with chloroamphenicol. The harvested mycelia or hyphal was washed for two to three times with RPMI 1640 with L-glutamine medium, then incubated for 24 hours. The harvesting mycelia, was sonicated for 2 mins until homogenous suspension of mycelia obtained.

F. Antifungal Procedure

1. NCCLS Modified Assay for Antifungal Activity and Determination of MIC Value

One ml of RPMI medium containing serially diluted extracts (10-0.31mg/ml) in sterile test tubes, then 1ml of prepared suspension was added. Two sets of control tubes were added to the experiment, one is growth (-ve) control tubes contained 1ml of RPMI medium without any treatment and 1mlof prepared suspension, other is standard drug (+ve) control tubes contained 1ml of RPMI medium with serially diluted ketoconazole (5-0.31mg/ml). The optical density of prepared suspension (growth control) before incubation was measured by a spectrophotometer at 680 nm red filter and taken as initial reading. Then all test tubes were incubated at 37°C for a week. After a week the optical density was measured spectrophotometerically at 680 nm.[20],[21].

MIC value is the least concentration before the spectrophotometer transmission reading is the same as or more than the initial reading [22].

2) MTT Assay

A quick sensitive colorimetric method utilizes tetrazolium salt as indicator of microbial metabolism for evaluation of cell death [23].

This assay based on the reduction of the yellow MTT [tetrazolium salt (3-{4, 5-dimethylthiazole-2-yl}-2, 5-diphenyl tetrazolium bromide)] by the mitochonderial dehydrogenase, present only in the living cells and hence released to the supernatant. MTT salt converted to the violet blue or green blue colored formazan. The colour intensity is directly proportional to the living cell numbers in the culture.

One drop of the indicator was added to the all tested tubes after measuring the final optical density by a spectrophotometer [24], [25].

G. Reverse Phase High Performance Liquid Chromatography (RHPLC)

Reversed-phase HPLC system was equipped with: RP-C18 HPLC column and Diode array UV detector (DAD) recorded at 320 – 380 nm for the detection of compounds.

H.HPLC-Triple Quadruple Spectrometric Analysis (LC-MS/MS)

RP-HPLC was joined with a Finnigan LCQ ion trap mass spectrometer with the Electrospray Ionization (ESI) interface at negative ion mode.

Collision induced dissociation (CID) experiment was performed for fragmentation of glycoside.

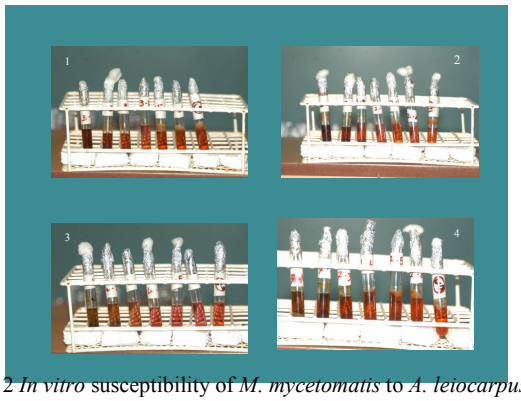

Fig. 2 *In vitro* susceptibility of *M. mycetomatis* to *A. letocarpus* leaf extracts (1: alch; 2: pet; 3: ch; 4: ethy)

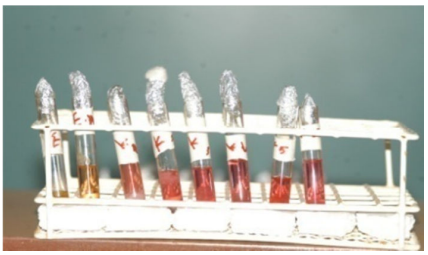

Fig. 3 *In vitro* susceptibility of *M. mycetomatis* to ketoconazole drug

As appeared in Fig. 2, the extracts inhibited the fungal growth compared to the standard drug (Ketoconazole) in Fig. 3. The result was shown in Table I and Fig. 4. The extracts possessed significant activity against *M. mycetomatis* compared to standard drug (ketoconazole). In addition to the chloroform fraction showed the higher activity.

The initial inoculum reading (0.04) at 680nm was inhibited to 0.03, 0.03, 0.02, 0.03 after a week inoculated in 5mg/ml alcohol crude extract, pet. ether, chloroform and ethyl acetate fractions respectively. While in the Ketoconazole (5mg/ml) the inoculum reading was inhibited to 0.03. In the negative control, the inoculum was grown up to 0.23.

MIC value compared to standard drug (5mg/ml), was found to be 2.5mg/ml, 0.62mg/ml 5mg/ml, in alcoholic extract, chloroform and ethyl acetate fractions respectively. The MIC values showed that, the extracts with low activity had high MIC, while with high activity had low MIC in agreement with MIC of antimicrobial agents.

The colorimetric results of MTT assay (Fig. 5) showed that, the colour of tetrazolium salt in *M. mycetomatis* suspension inoculated in *A. leiocarpus* leaf extracts started to change at the concentration of 2.5 mg/ml, 5mg/ml, 0.31mg/ml and 1.25mg/ml in the alcoholic extract, petroleum ether fraction, chloroform fraction and ethyl acetate fraction respectively. These results were compatible with the antifungal activity of the plant previously reported against other fungi [12], [13], [15], it also compatible with the activity reported on this plant in the treatment of skin infection [5]and wound infection [14], [15] cause by other organisms.

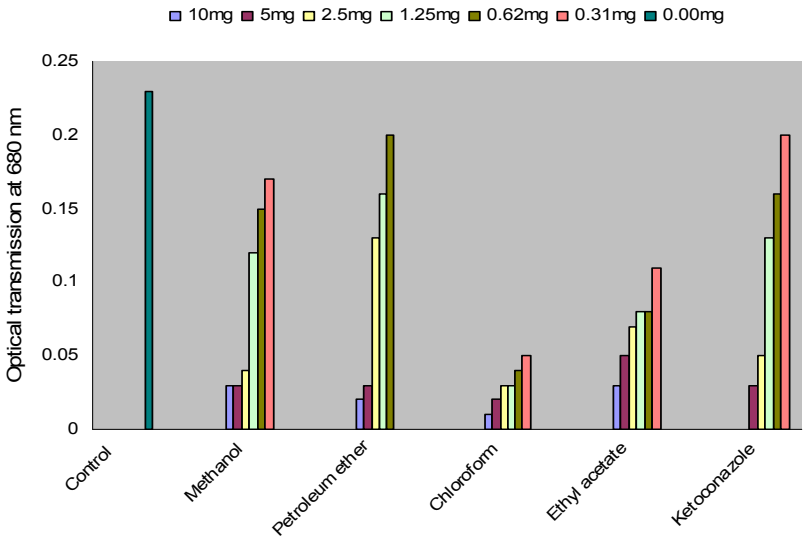

Fig. 4 Optical density reading (at 680 nm) of *M. mycetomatis* suspension inoculated in *A. leiocarpus* leaf extracts

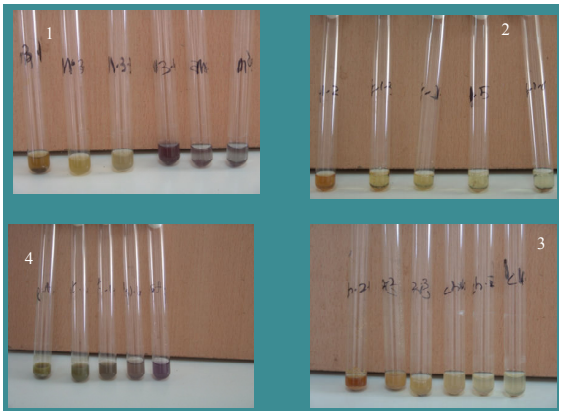

Fig. 5 The colour of tetrazolium salt in *M. mycetomatis* suspension inoculated in *A. leiocarpus* leaf extracts (1: alch; 2: pet; 3:ch; 4:ethy)

The RP-HPLC-DAD analysis (Fig. 6) and the m/z MS/MS data analysis of the leaf chloroform extract (Fig. 7 and Table II) revealed the presence of ellagic acid; ellagic and flavellagic acids derivatives; Quercetin glycosides and stilbenoid compounds which is compatible with the chemistry of the Combretaceae family [26]. These findings are reported for the first time and adds to the reported results about the abundance of ellagic and flavellagic acid derivatives in other *Anageissus* species [27]-[33].

The biological and chromatographical results of this study were compatible to the published data in the current literature, where as the ellagic acid was reported to be toxic to the filamentous fungi [34], flavonoids were known as antimicrobial agent [35] and the steilbenoid compounds were

known as phytoalexins secondary metabolites with potent antifungal activities [36]-[40].

TABLE I
OPTICAL DENSITY READING (AT 680 NM) OF *M. MYCETOMATIS* SUSPENSION INOCULATED IN *A. LEIOCARPUS* LEAF EXTRACTS

Treatment (Extract/ drug)	Concentration	Reading at a zero time	Extract Reading	Reading after a week	Inoculum Reading after a week
Methanol	10mg	1.88	1.84	1.87	0.03
	5mg	0.94	0.90	0.93	0.03
	2.5mg	0.46	0.42	0.46	0.04
	1.25mg	0.23	0.19	0.31	0.12
	0.62mg	0.11	0.07	0.22	0.15
	0.31mg	0.05	0.01	0.18	0.17
	0.00mg	0.04	-	0.23	0.23
Petroleum ether	10mg	1.10	1.06	1.08	0.02
	5mg	0.50	0.46	0.43	0.03
	2.5mg	0.23	0.19	0.32	0.13
	1.25mg	0.10	0.06	0.22	0.16
	0.62mg	0.06	0.02	0.22	0.20
	0.31mg	-	-	-	-
	0.00mg	0.04	-	0.23	0.23
Chloroform	10mg	1.99	1.95	1.97	0.02
	5mg	0.99	0.95	0.97	0.02
	2.5mg	0.50	0.46	0.49	0.03
	1.25mg	0.27	0.23	0.26	0.03
	0.62mg	0.14	0.10	0.14	0.04
	0.31mg	0.08	0.04	0.09	0.05
	0.00mg	0.04	-	0.23	0.23
Ethylacetate	10mg	1.92	1.88	1.91	0.03
	5mg	0.96	0.92	0.95	0.03
	2.5mg	0.48	0.44	0.51	0.07
	1.25mg	0.24	0.20	0.28	0.08
	0.62mg	0.13	0.09	0.17	0.08
	0.31mg	0.06	0.02	0.13	0.11
	0.00mg	0.04	-	0.23	0.23
Ketoconazole	10mg	-	-	-	-
	5mg	0.72	0.68	0.71	0.03
	2.5mg	0.36	0.32	0.37	0.05
	1.25mg	0.28	0.24	0.37	0.13
	0.62mg	0.14	0.10	0.26	0.16
	0.31mg0.00mg	0.07	0.03	0.23	0.20
		0.04	-	0.23	0.23

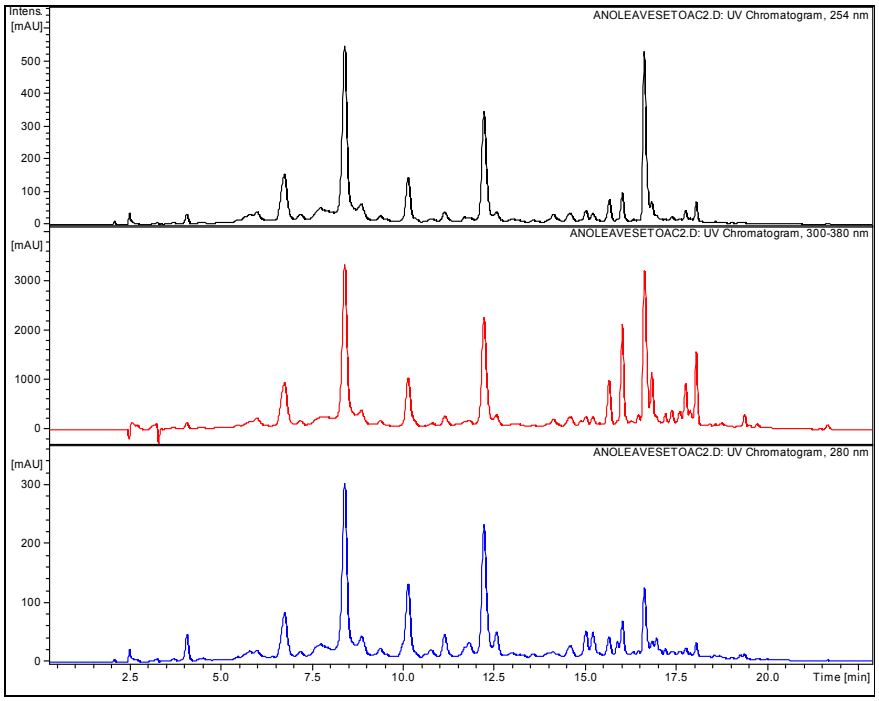

Fig. 6 RP-HPLC-DAD Chromatogram of chloroform fraction of *A. leiocarpus* leaves recorded at λ_{max} 254, 280,300-380nm

TABLE II
RP-HPLC DATA (PEAK NO. & RT_t), MS/MS DATA (*M/Z*) AND ASSIGNED STRUCTURES OF *A. LEIOCARPUS* LEAF CHLOROFORM FRACTION

Compound Peak	(R_t) (min)	M-H (*m/z*)	CID M^n main fraction ions(*m/z*)	Expected compound
1	6.8	541	425, 377, 301, 275, 271, 229, 201,173	Di- hydroxyl-tri-O- methylellagic acid-7-O-β-glucoside
2	8.5	552	481, 301, 275, 271, 243	Di- hydroxyl-tri-O- methylellagic acid-7-O-β-glucosidederevative
3	8.8	541	459, 425, 377, 301, 275, 271, 257, 227, 185,117	Di- hydroxyl-tri-O- methylellagic acid-7-O-β-glucoside
4	10.2	467	458, 436, 419, 401, 382, 351, 313,301, 275, 229	Ellagic acid-7-O-β-glucoside
5	12.4	617	601, 541, 522, 481,301, 299, 275, 271, 243	Di- hydroxyl-tri-O- methylellagic acid-7-O-β-glucosidederevative
6	12.4	628	623, 552, 481, 301,275, 271, 243,187	Di- hydroxyl-tri-O- methylellagic acid-7-O-β-glucosidederevative
7	12.7	453	312.7, 252.7, 222.7, 168.7, 168.7,150.7,124.8,124.8	E-Viniferin
8	12.7	490	453, 312.7, 252.7,222.7,168.7, 168.7, 150.7.8	Methyl E-Viniferin
9	15.7	447	365, 300, 283, 271, 257, 243, 229,170, 185,157,145,89	Ellagic acid-4'-O-β- rhamnoside
10	15.7	615	463, 301,300, 271, 255, 229, 193,178,151,107	Quercetin-3-O-galloyl- 7-O-β-glucoside
11	16.8	301	283, 271, 257, 240, 229, 228, 217, 202, 185, 173,139, 89	Ellagic acid
12	16.8	463	381, 301, 300, 271, 255, 229, 214,179,175, 151,107	Quercetin-7-O-β-glucopyranoside
13	17.9	447	365, 327, 285, 255,227, 211, 201,167,151,119	Kampefrol-7-O-β-glucopyranoside
14	18	477	449, 360, 301, 285, 271, 255, 243, 239, 211,123,179,163, 151,107	Quercetin3-methoxy-7-O- β –glucopyranoside
15	18.2	447	365,301,300,283,271,255,229,211,179,151,107	Quercetin-7-O-β-rhamnoside

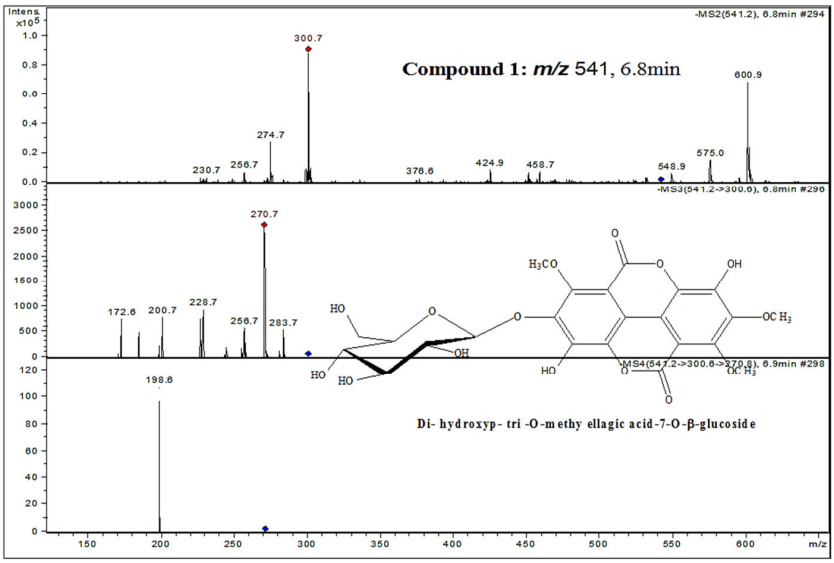

Fig. 7 (a) MS/MS (m/z) and assigned structures of compound (1) in the chloroform fraction of *A. leiocarpus* leaf extract

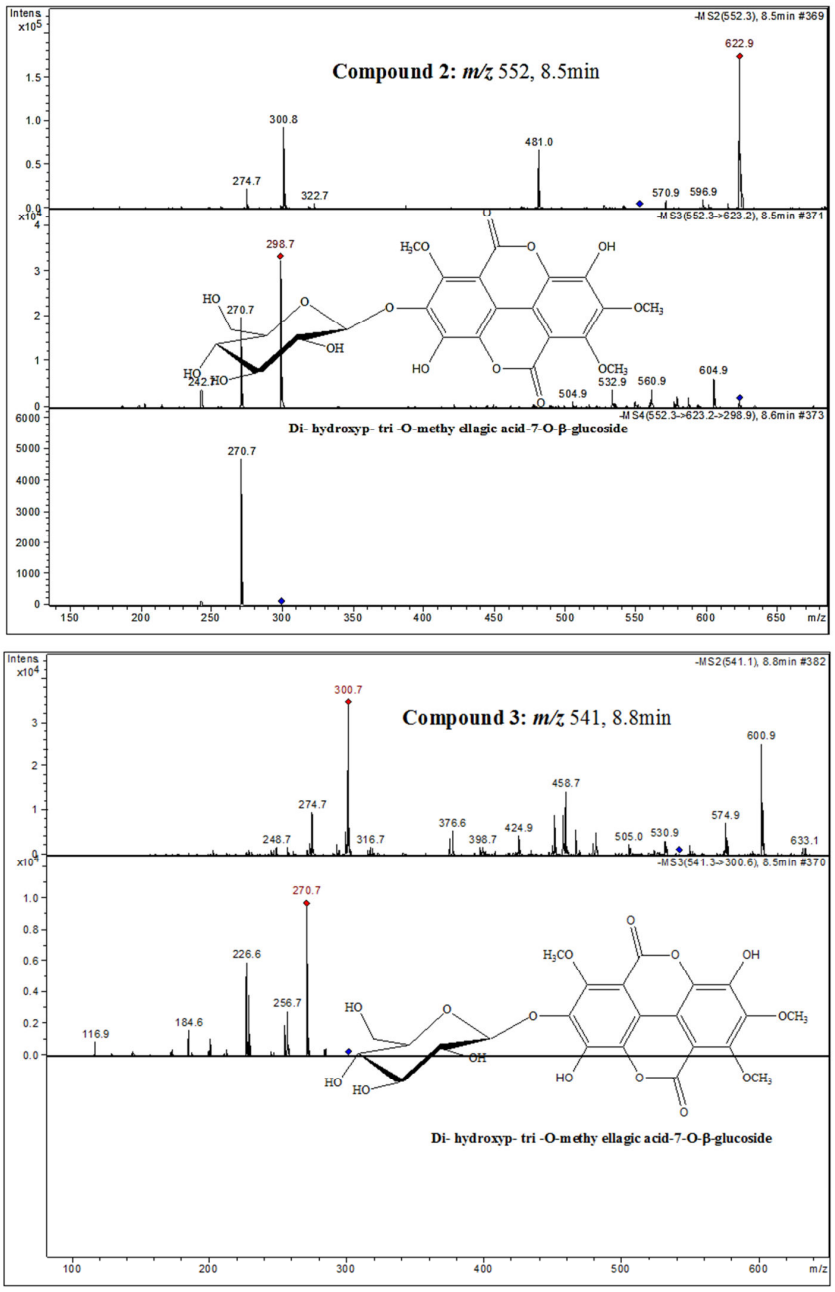

Fig. 7 (b) MS/MS (m/z) and assigned structures of compounds (2&3) in the chloroform fraction of *A. leiocarpus* leaf extract

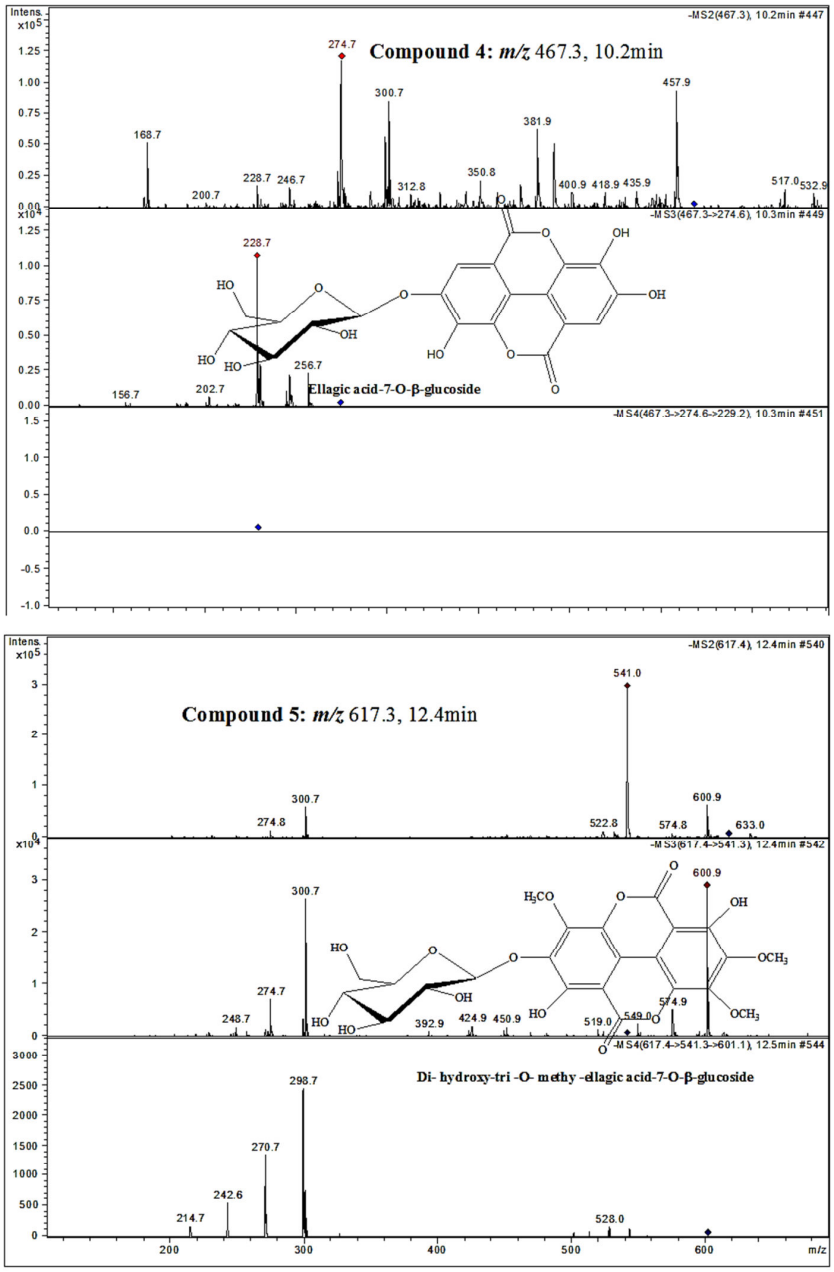

Fig. 7 (c) MS/MS (m/z) and assigned structures of compounds (4&5) in the chloroform fraction of *A. leiocarpus* leaf extract

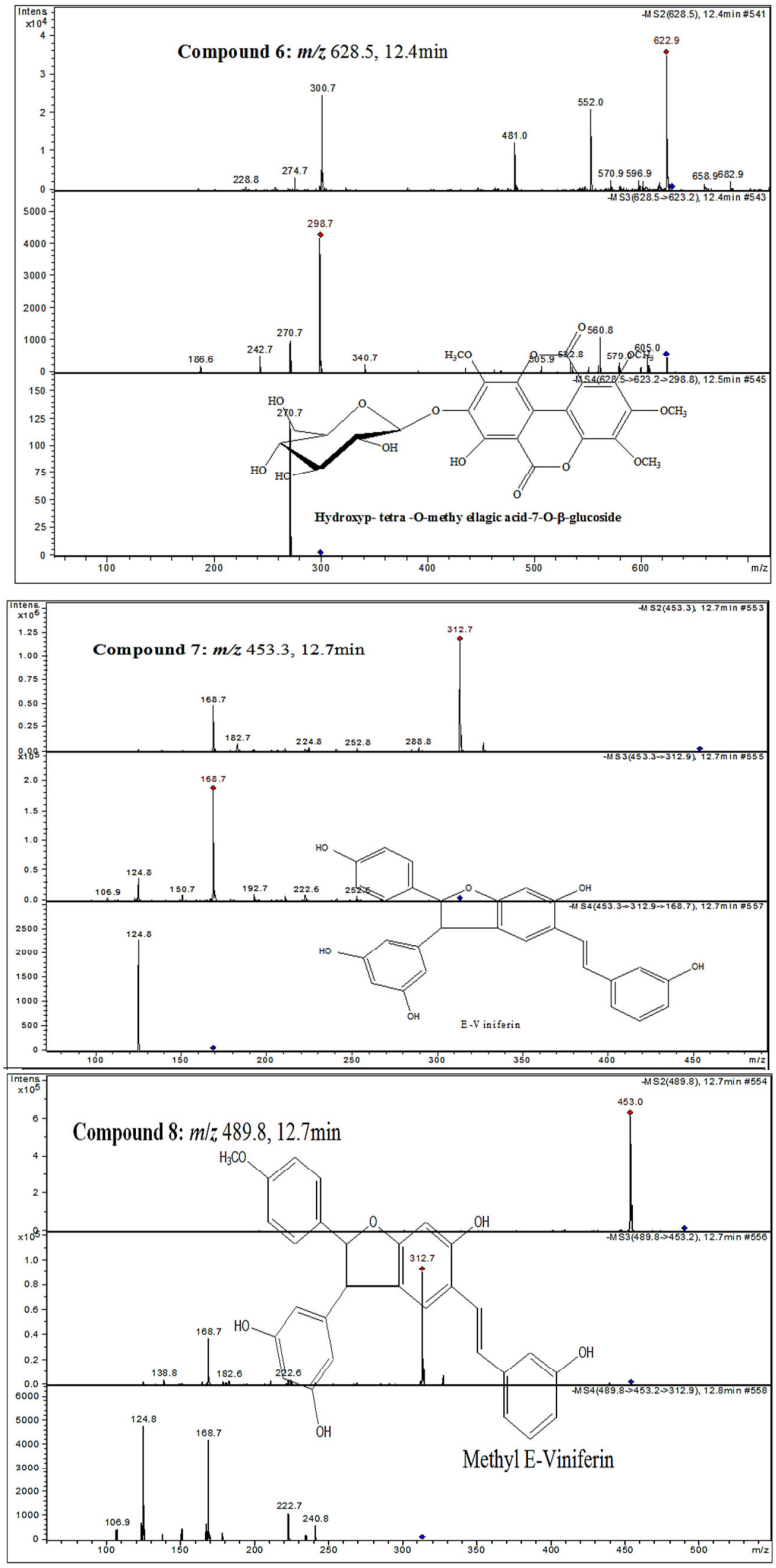

Fig. 7 (d) MS/MS (m/z) and assigned structures of compounds (6, 7& 8) in the chloroform fraction of *A. leiocarpus* leaf extract

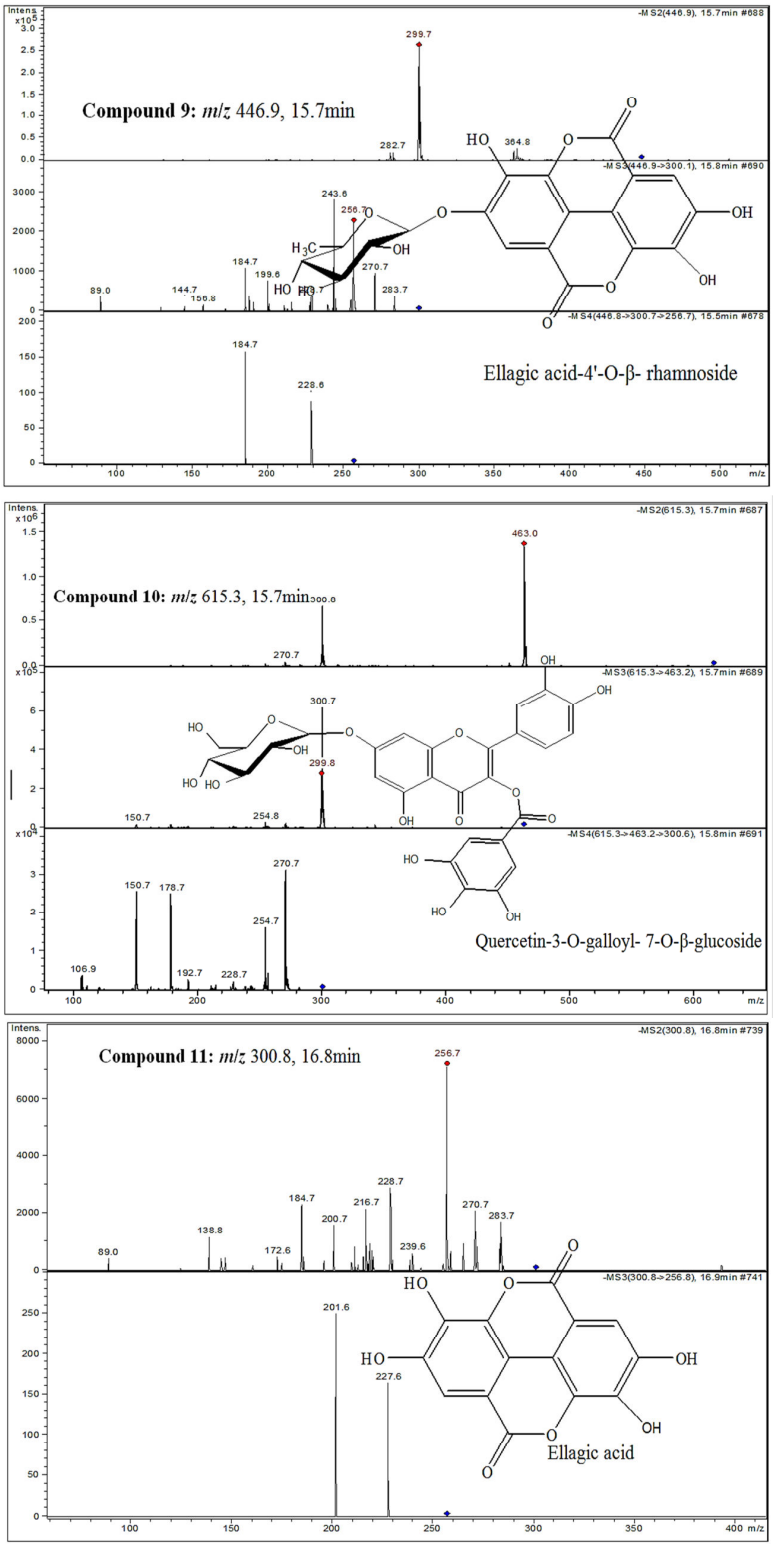

Fig. 7 (e) MS/MS (m/z) and assigned structures of compounds (9, 10& 11) in the chloroform fraction of *A. leiocarpus* leaf extract

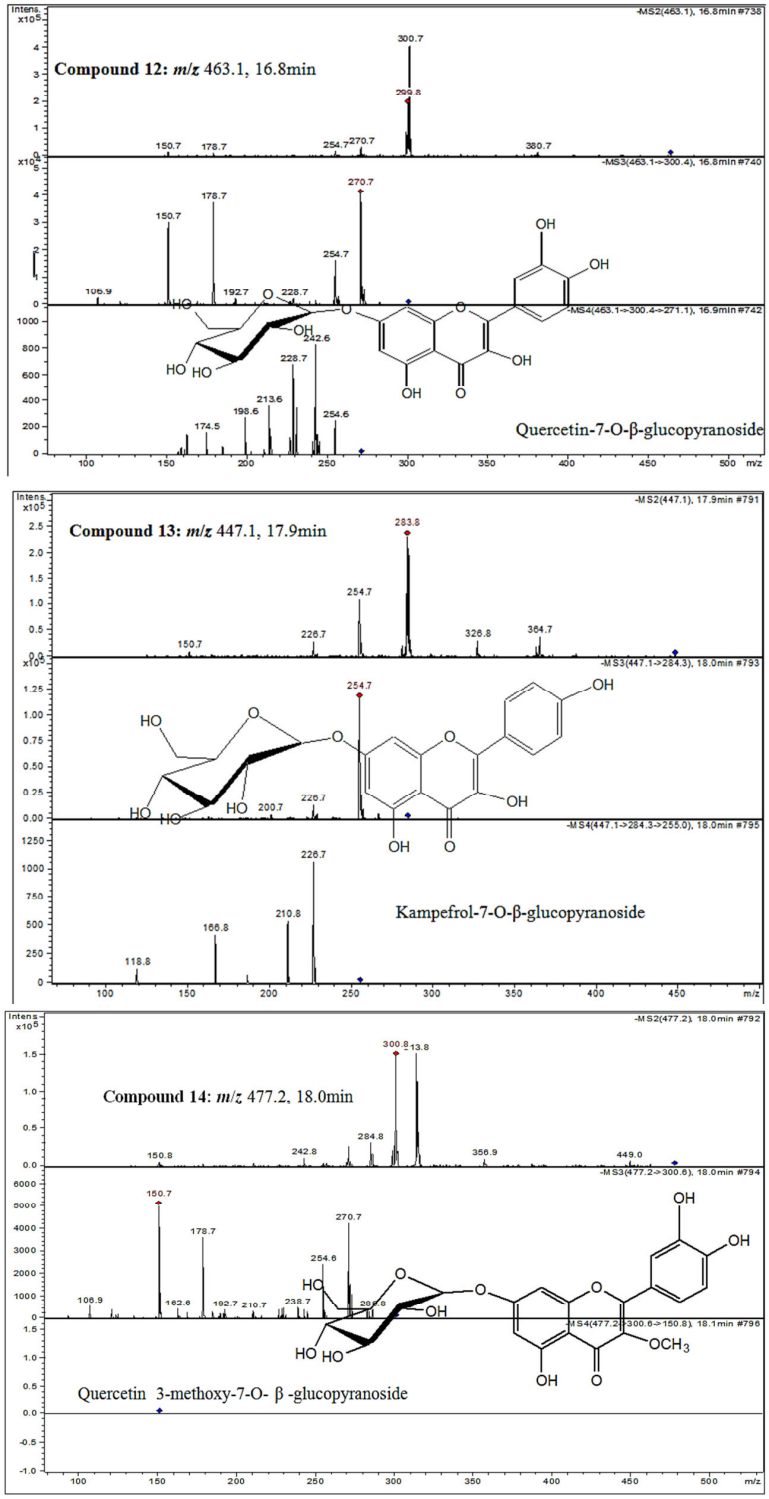

Fig. 7 (f) MS/MS (m/z) and assigned structures of compounds (12, 13& 14) in the chloroform fraction of *A. leiocarpus* leaf extract

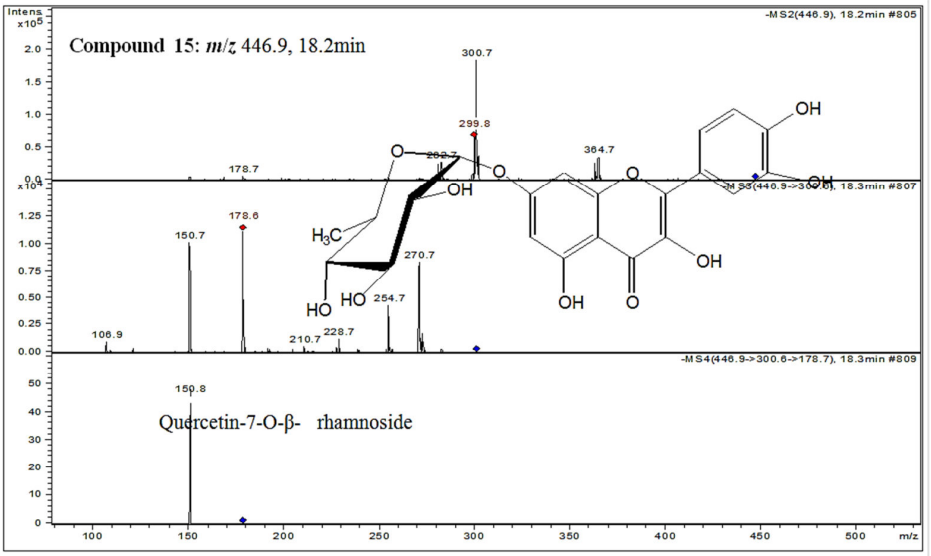

Fig. 7 (g) MS/MS (m/z) and assigned structures of compound (15) in the chloroform fraction of *A. leiocarpus* leaf extract

IV. Conclusions

In conclusion, the results of the *in vitro* susceptibility of *M. mycetomatis* to the *A. leiocarpus* leaf extracts showed the potent antifungal activity of the extracts against mycetoma causing pathogen. These results confirmed the previous antimicrobial activity of *A. leiocarpus* [14] and justifying its traditional uses as a medicinal plant for treatment of skin infections.

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