Madurella mycetomatis Susceptibility to *Anogeissus leiocarpus* Stem Bark Extracts

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Abstract

Anogeissus leiocarpus, 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 skin infection, mainly caused by *Madurella mycetomatis* fungus. The present study was carried out *in vitro* to investigate the antifungal activity of *A. leiocarpus* stem bark extracts against the isolated pathogenic *Madurella mycetomatis*, by using the NCCLS modified method, and MTT assay compared to the Ketoconazole standard drug. The bioactive fraction with the highest activity was subjected to chemical analysis implementing different chromatographic analytical methods (TLC, HPLC and LC-MS/MS). The results showed remarkable antifungal activity of the plant extracts against the isolated pathogenic fungus, compared to negative and positive controls. Chloroform fraction showed the highest activity.

The chromatographic analysis of chloroform fraction, with the highest activity showed the accumulation of ellagic and flavellagic acids derivatives, which were previously reported for their toxicity to the filamentous fungi.

Keywords: *Anogeissus*, *leiocarpus*, stem bark, extract, *in vitro*, susceptibility, *Madurella*, *mycetomatis*

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Introduction

Combretaceae is a family of flowering plants, widely distributed in tropical climates of Africa, Asia and South America. The family includes 20 genera and about 600 species of evergreen or deciduous trees, shrubs, or woody lianas (Watson & Dallwitz, 1992; Michael, 1998; Angeh *et al.*, 2007. It is well known for its medicinal uses in Africa and Asia as an important resource in traditional medical practice for many human diseases and infections (Eloff *et al.*, 2005a&b; Angel *et al.*, 2007; Mann *et al.*, 2007; Mann *et al.*, 2008a; Eloff *et al.*, 2008).

Anogeissus is a genus of trees, or shrubs, belonging to this family. They, have long being used in traditional medicine (Mann *et al.*, 2009b and Rocquet *et al.*, 2007). It is widely distributed in Africa and used in traditional medicine for the treatment of many diseases mainly the skin disease (Agaie *et al.*, 2007a; Adejumobi *et al.*, 2008; Okpekon *et al.*, 2004; Vonthron-Sénécheau, 2003; Mustofa, *et al.*, 2000; Chaabi, *et al.*, 2006; Agaie & Onyeyili, 2007; Adeleye *et al.*, 2003). The plant showed strong antibacterial and antifungal activity against many pathogenic microorganisms (Ibrahim *et al.*, 1997; Sanogo, 2005; Batawila *et al.*, 2005; Mann *et al.*, 2008b; Mann *et al.*, 2008c; Mann *et al.*, 2009a; Mann *et al.*, 2009b).

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 (Gumaa *et al.*, 1994).

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 hospitals and outpatient clinics every year (Gumaa *et al.*, 1994; Mahgoub *et al.*, 1994).

Adequate treatment requires prolonged antifungal drugs combined with extensive surgical treatment (Gumaa *et al.*, 1994). Meager data is available for susceptibility of *M. mycetomatis* to the plants secondary metabolites (Van de Sande, *et al.*, 2005).The results of the antifungal activity of the stem bark extracts against *M. mycetomatis* are reported in the present paper.

Materials and Methods

Plant Material Collection and Preparation

A .leiocarpus stem bark was collected from El Damazeine region, Sudan, and identified by taxonomist in the department of siliviculture, Faculty of Forestry, University of Khartoum. The voucher specimen, was deposited in the Department of Biochemistry, Commission of Biotechnology and Genetic Engineering, National Centre for Research. The plant material was chipped using sawmill, dried under shade at room temperature and then ground to a coarse powder using mortar and pestle.

Preparation of the Extract

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

Collection of *Madurella mycetomatis* Fungus

Isolated *M. mycetomatis* fungus was collected from Mycetoma Research Center, Soba hospital. The black grains were exuded from open sinuses and surgical biopsy from the lesion, freed from tissues and carried by forceps in sterile container, then washed with saline for several times.

RPMI 1640 Medium Preparation

RPMI 1640 with L- glutamine medium was 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.

Culture and 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 isolated 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 hyphae were washed for two to three times with RPMI 1640 with L- glutamine medium, and then incubated for 24 hours. The harvested mycelia, was sonicated for 2 minutes until homogenous suspension of mycelia obtained.

Antifungal Procedure

N CCLS Antifungal Modified Assay and 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. The test tubes were incubated at 37°C for a week and after that the optical density was measured spectrophotometerically at 680 nm (NCCLS, 2002; Ahmed, *et al.*, 2004).

MIC value is the least concentration before the spectrophotometer transmission reading is the same as or more than the initial reading (Van de Sande, *et al.*, 2005).

MTT Assay

It is a quick sensitive colorimetric method utilizing tetrazolium salt as indicator of microbial metabolism for evaluation of cell death (Muraina, *et al.*, 2009).

The assay is 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 converts to the violet blue or green blue colored formazan and 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 (Kuo-Ching *et al.*, 2011; Ten-Ning, *et al.*, 2009).

LC-Triple Quadruple Spectrometric Analysis (LC-MS/MS)

LC-MS/MS system was equipped with:

RP-C18 HPLC column and Diode array UV detector (DAD) recorded at 320 – 380 nm for the detection of compounds.

RP-HPLC 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 and elucidation of compounds structures.

Results and Discussions

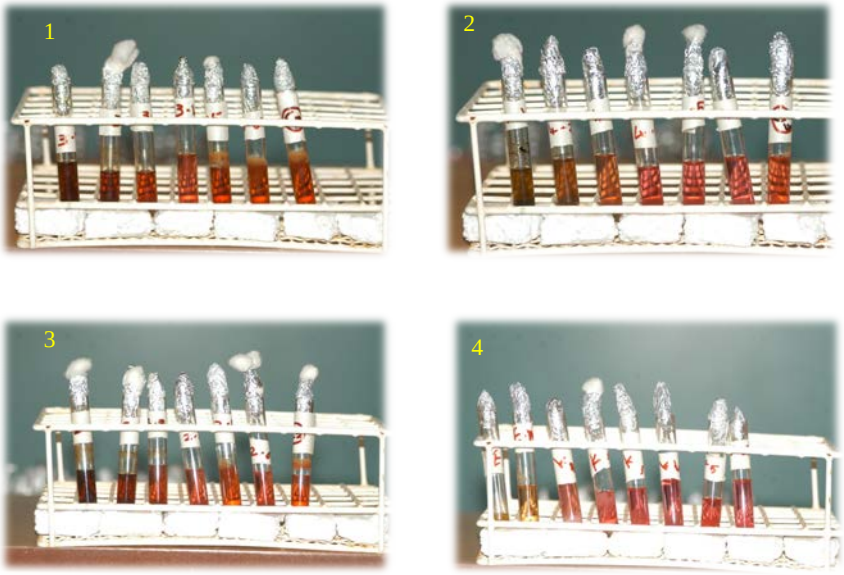


Figure 1: *In vitro* susceptibility of *M. mycetomatis* to *A. leiocarpus* stem bark extracts.

1: methanol; 2: chloroform; 3: ethyl acetate and 4: ketoconazole drug

Alcohol extract and ethyl acetate fraction as shown in figure 1 inhibited the fungus and there was no fungal growth till the concentration of 2.5 mg/ml and 0.62mg/ml respectively. In the chloroform fraction and ketoconazole drug the least concentration inhibited fungal growth.

The inoculum optical density readings of the fungal suspension are shown in Table I and Fig. 2. The initial inoculum optical density reading 0.04 at 680nm was inhibited to 0.02, 0.01, 0.03 after a week inoculated in 10mg/ml alcohol crude extract, chloroform and ethyl acetate fractions respectively. The reading was inhibited to 0.02, 0.02, 0.03 after a week inoculated in 5mg/ml alcohol crude extract, 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.

The results showed that, the extracts possessed high activity against *M. mycetomatis* compared to ketoconazole standard drug. In addition to the chloroform fraction showed the higher activity.

MIC value compared to standard drug (5mg/ml) was found to be 2.5mg/ml, 0.62mg/ml and 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 (MTT assay) of *A. leiocarpus* stem bark extracts compared to ketoconazole standard drug (Fig. 3), showed that, the colour of tetrazolium salt in *M. mycetomatis* suspension inoculated in *A. leiocarpus* bark extracts started to change at the concentration of 2.5 mg/ml, 0.62mg/ml and 2.5mg/ml, in alcoholic extract, chloroform fraction and ethyl acetate fraction respectively, compared to 2.5mg/ml inoculated in the ketoconazole standard drug.

Table 1: Optical density reading (at 680 nm) of *M. mycetomatis* suspension inoculated in *A. leiocarpus* stem bark extracts

Treatment (Extract/ drug)	Concentration	Extract Reading	Reading at zero time (Extract+ Inoculum)	Reading after aweek (Extract+ Inoculum)	Inoculum Reading after aweek (Final reading)
Methanol	10mg	1.78	1.82	0.80	0.02
	5mg	0.86	0.90	0.88	0.02
	2.5mg	0.41	0.45	0.45	0.04
	1.25mg	0.18	0.22	0.23	0.05
	0.62mg	0.07	0.11	0.17	0.10
	0.31mg	0.01	0.05	0.15	0.14
	0.00mg	-	0.04	0.23	0.23
Chloroform	10mg	1.88	1.92	1.89	0.01
	5mg	0.92	0.96	0.94	0.02
	2.5mg	0.44	0.48	0.46	0.02
	1.25mg	0.21	0.25	0.24	0.03
	0.62mg	0.09	0.13	0.13	0.04
	0.31mg	0.02	0.06	0.07	0.05
	0.00mg	-	0.04	0.23	0.23
Ethyl acetate	10mg	1.66	1.70	1.69	0.03
	5mg	0.81	0.85	0.84	0.03
	2.5mg	0.41	0.45	0.46	0.05
	1.25mg	0.19	0.23	0.25	0.06
	0.62mg	0.08	0.12	0.15	0.07
	0.31mg	0.01	0.05	0.10	0.09
	0.00mg	-	0.04	0.23	0.23
	10mg	-	-	-	-
	5mg	0.68	0.72	0.71	0.03
	2.5mg	0.32	0.36	0.37	0.05

Ketoconazole	1.25mg	0.24	0.28	0.37	0.13
	0.62mg	0.10	0.14	0.26	0.16
	0.31mg	0.03	0.07	0.23	0.20
	0.00mg	-	0.04	0.23	0.23

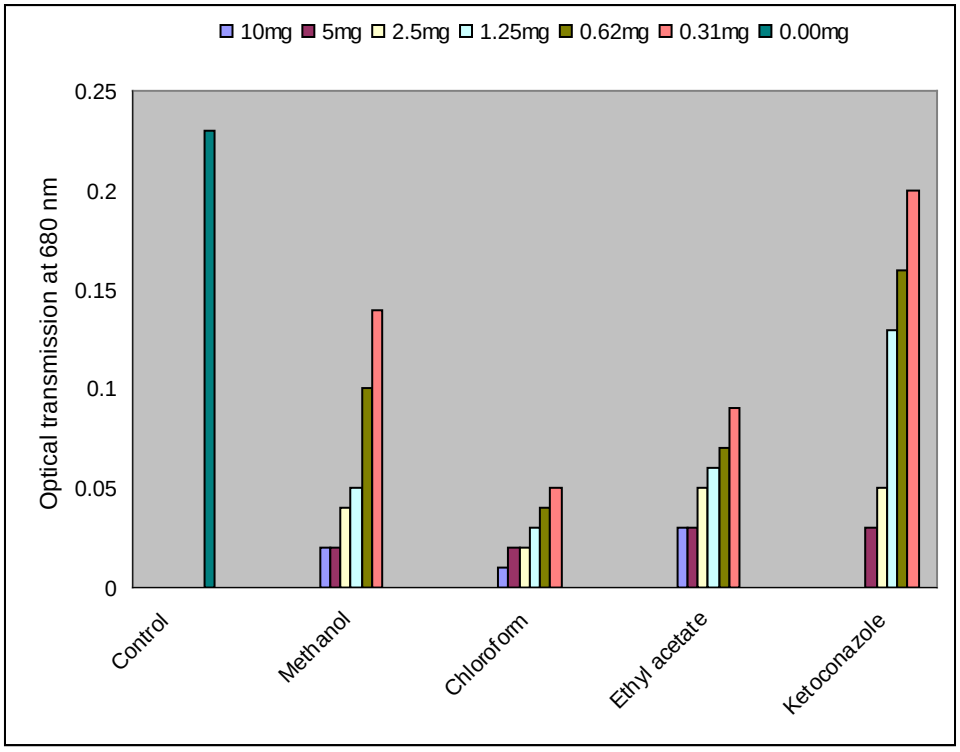


Figure 2: Optical density reading (at 680 nm) of *M. mycetomatis* suspension inoculated in *A. leiocarpus* stem bark extracts

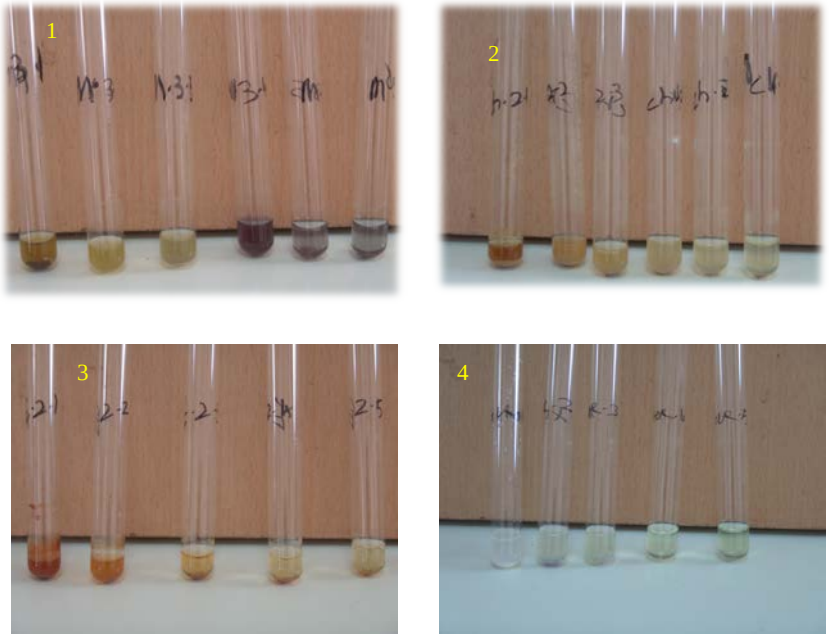


Figure 3: The colour of tetrazolium salt in *M. mycetomatis* suspension inoculated in *A. leiocarpus* stem bark extracts

1: methanol; 2: chloroform; 3: ethyl acetate and

4: ketoconazole drug

These results against *M. mycetomatis* were compatible with the antifungal activity of the plant previously reported against other fungi (Sanogo, 2005; Batawila, *et al.*, 2005; Mann, *et al.*, 2008a). It was also compatible with the activity reported on the plant in the treatment of skin and wound infections caused by other organisms (Adeleye *et al.*, 2003; Mann, *et al.*, 2008b). It added to the activity reported on the other *Anageissus* species in the treatment of skin and wound infections (Govindarajan, *et al.*, 2004).

The LC-MS/MS analysis of the stem bark chloroform fraction with the highest activity clearly demonstrated the accumulation of ellagic acid; ellagic and flavellagic acids derivatives which is compatible with the chemistry of Combretaceae family (Eloff *et al.*, 2008).

These findings are added to the reported results about the abundance of ellagic and flavellagic acid derivatives in the other *Anageissus* species (Reddy *et al.*, 1964; Reddy, 1965; Deshpande, *et al.*, 1976).

The biological and chromatographic results reported in this study were compatible to the published data in the current literature about ellagic acid toxicity to the filamentous fungi (Scalbert, 1991).

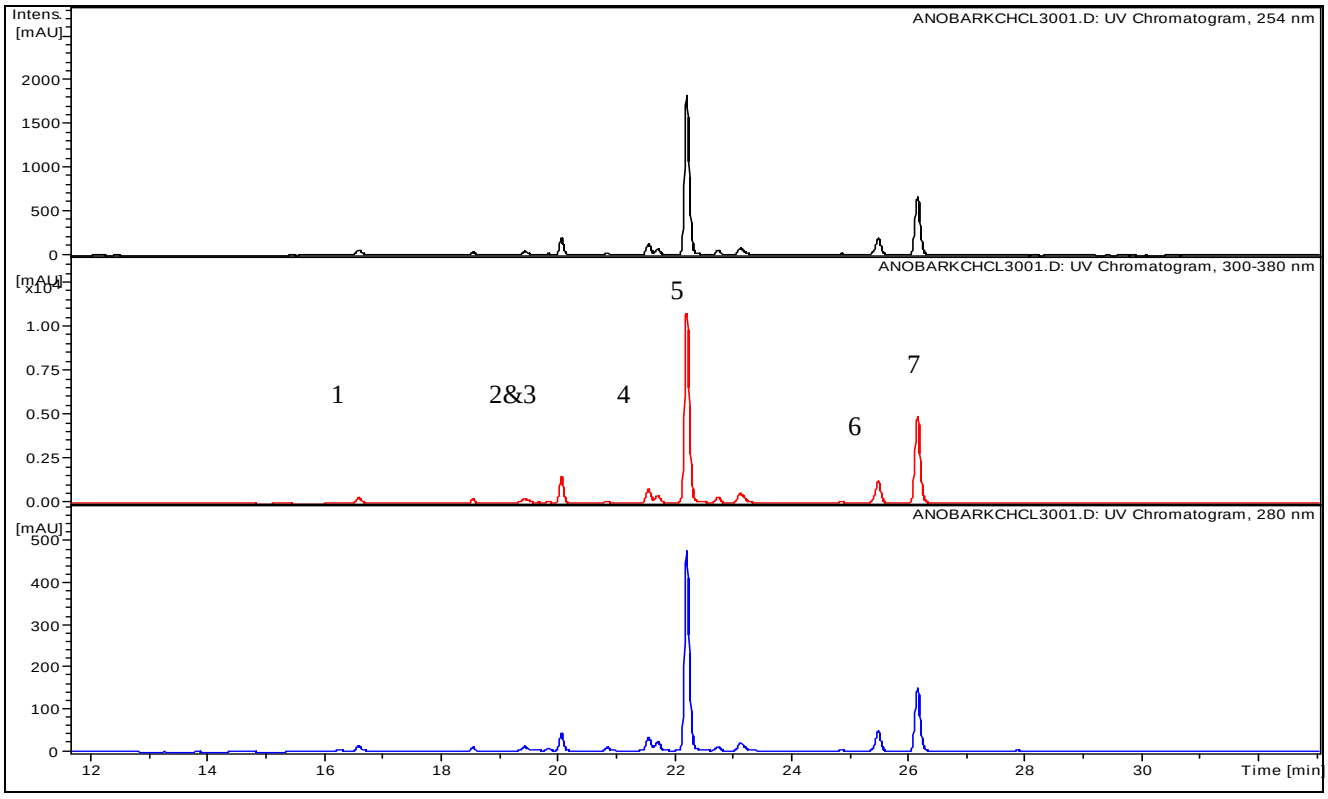
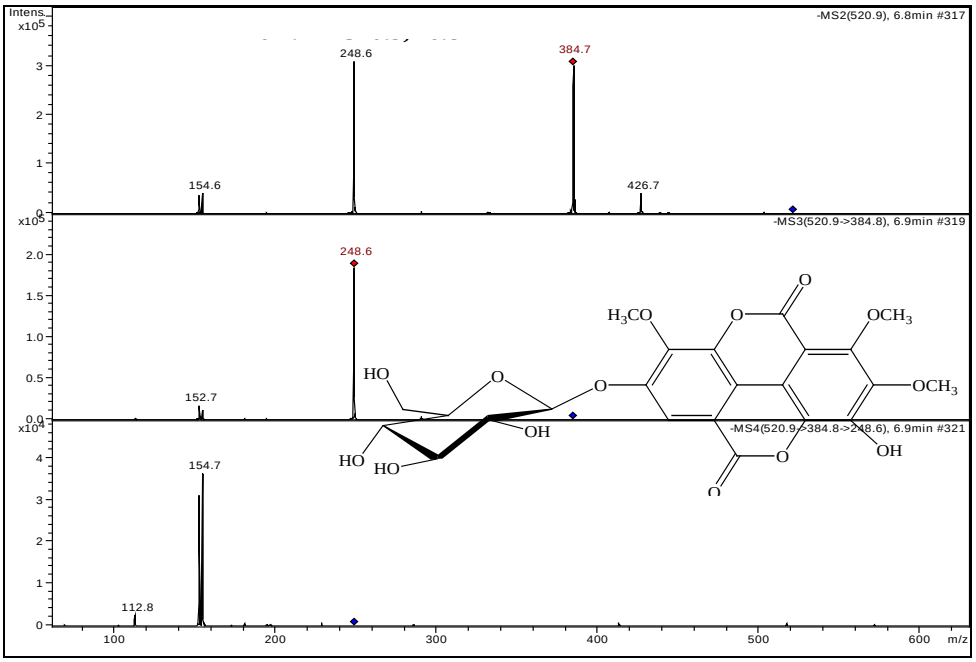


Figure 4: RP-HPLC-DAD Chromatogram of chloroform fraction of *A. leiocarpus* stem bark recorded at λ_{max} 254, 280,300-380nm.

Table 2: RP-HPLC data (Peak NO. & Rt.); MS/MS data (molecular weight {*m/z*} & main fractions {*m/z*}) and assignment structures of bark chloroform fraction

Compound Peak	(R_t) (min)	M-H (*m/z*)	CID M^n Main Fraction ions (*m/z*)	Compound Name
1	6.8	521	506, 491,385, 342.8, 249, 249, 155, 155, 155, 113	2, 3, 8-Tri-methoxy-flavellagic acid-7-β-O-glucoside
2	20.2	521	506, 385, 342.8, 249, 249, 155, 155, 155, 113	3,4,3'-Tri-methoxy-flavellagic acid-4'-β-D-glucoside
3	20.2	384.8	248.6, 154.7, 152.7, 112.9, 112.9	Flavellagic acid derevative
4	21.6	359.0	343.8,32914,314,300, 285,	Hydroxy, Tetra- methoxy -ellagic acid
5	22.5	329	314, 299, 299, 285, 271, 271	3, 3'-Di-methoxy-ellagic acid
6	25.7	343	328, 313, 313, 298, 298, 285, 270	Tri-methoxy-ellagic acid
7	26.2	359	344, 329, 314,314,300, 285	Tetra-methoxy-ellagic acid



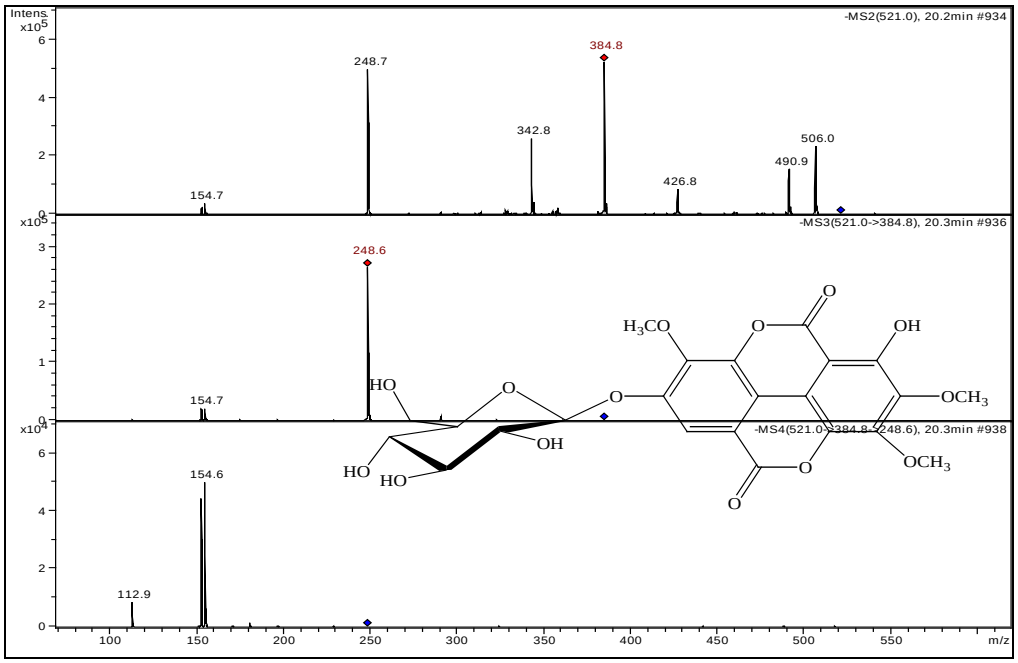
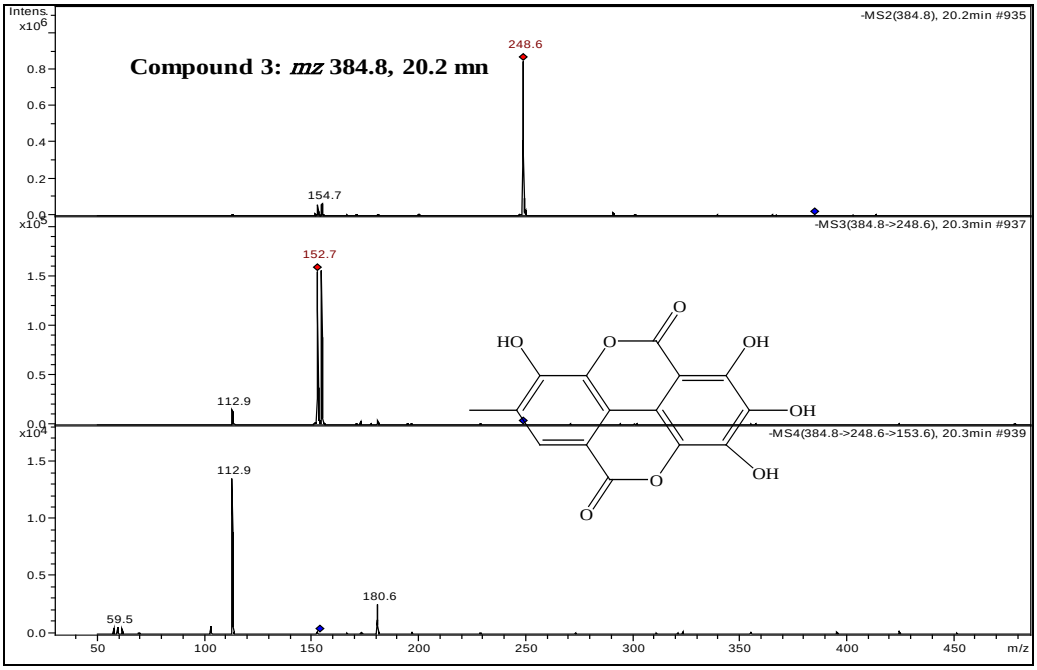


Figure 5 (a): MS/MS (m/z) and assigned structures of compounds (1& 2) in the chloroform fraction of *A. leiocarpus* stem bark extract.



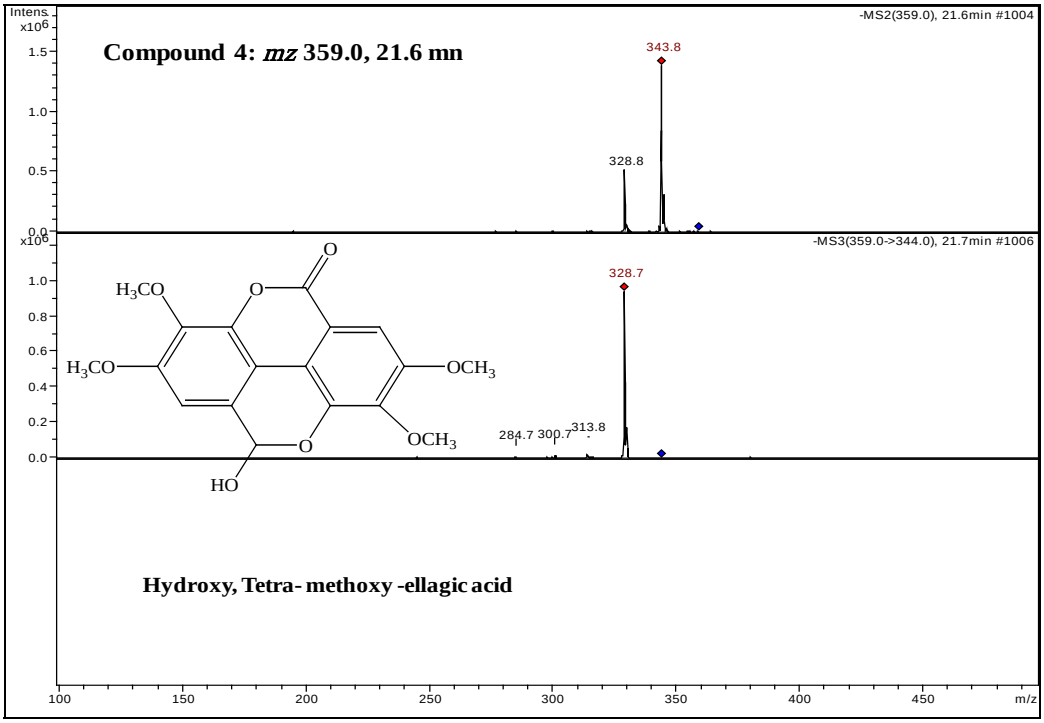
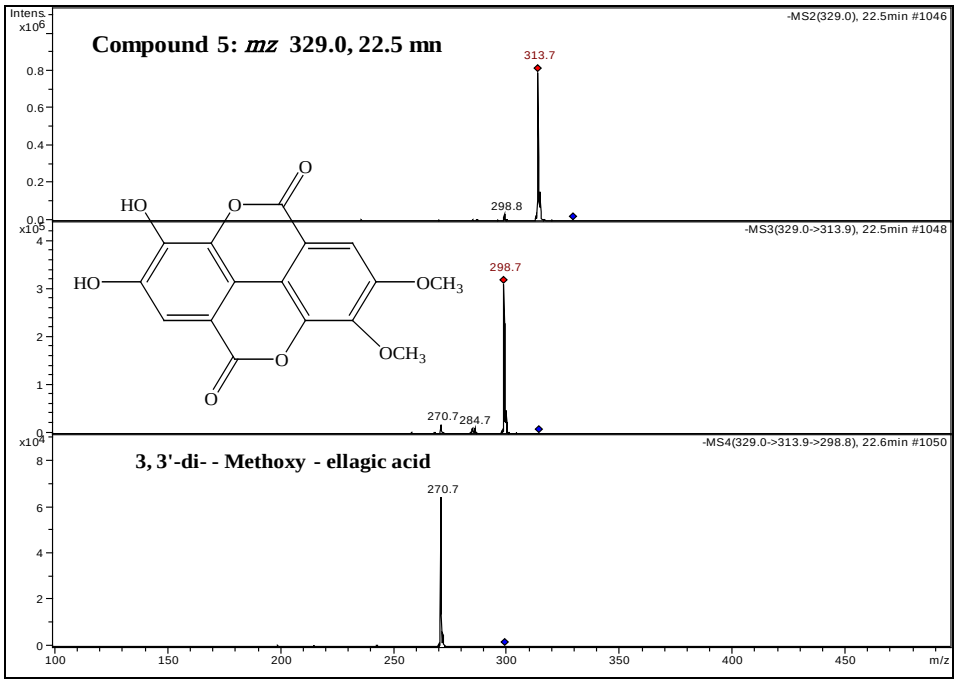


Figure 5 (b): MS/MS (m/z) and assigned structures of compounds (3& 4) in the chloroform fraction of *A. leiocarpus* stem bark extract.



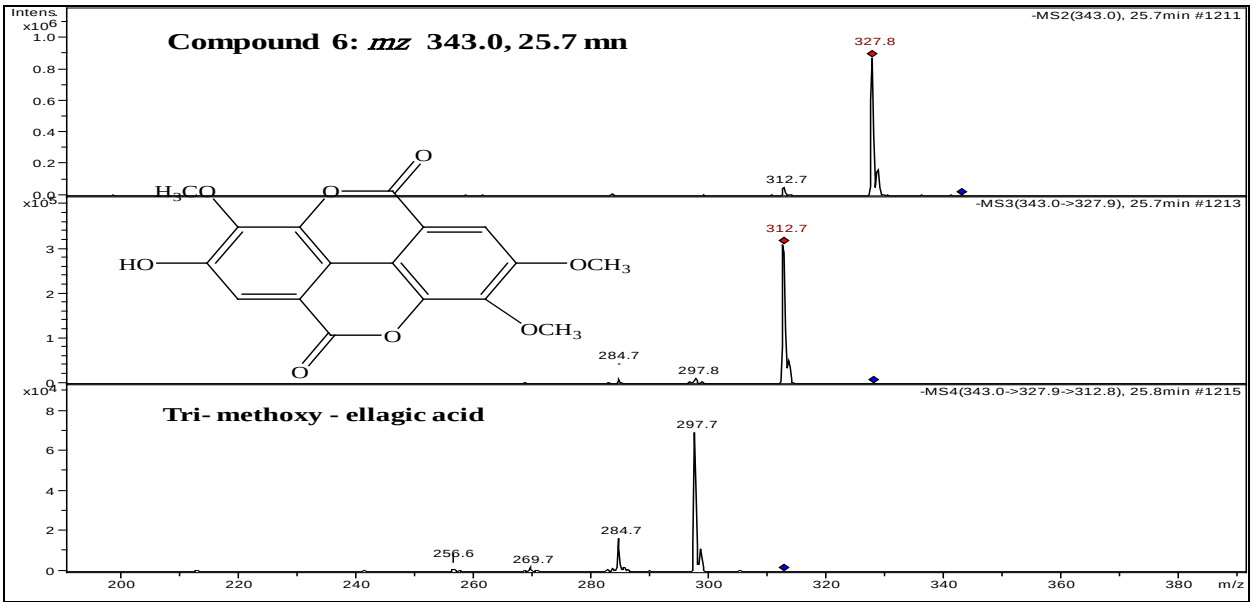


Figure 5 (c): MS/MS (m/z) and assigned structures of compounds (5& 6) in the chloroform fraction of *A. leiocarpus* stem bark extract.

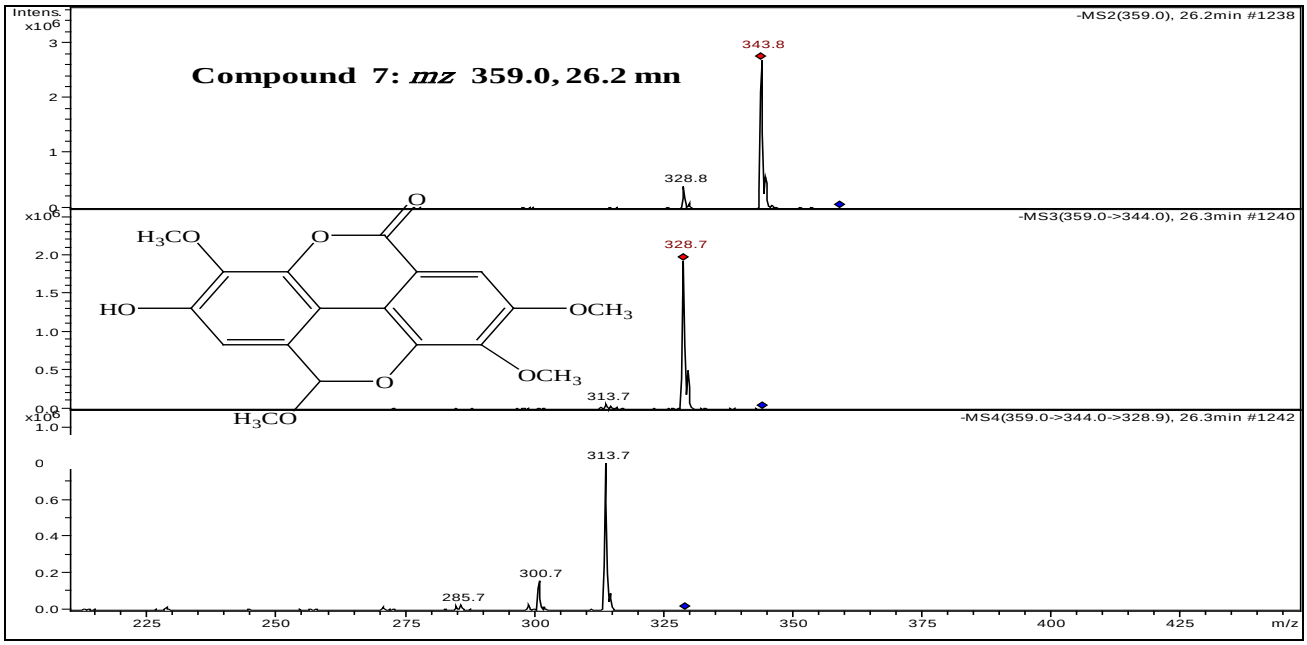


Figure 5 (d): MS/MS (m/z) and assigned structures of compound (7) in the chloroform fraction of *A. leiocarpus* stem bark extract.

Conclusion

In conclusion the results of the *in vitro* susceptibility of *M. mycetomatis* to the *A. leiocarpus* stem bark extracts showed the highest antifungal activity of the extracts against mycetoma causing pathogen. These results confirmed the previous antifungal activity of *A. leiocarpus* (Mann, *et al.*, 2008a), and justifying its traditional uses as a medicinal plant for treatment of skin infections.

References

Adejumobi, J.A.; Ogundiya, M. O.; Kolapo, A.and kunade, M.B., 2008. Phytochemical composition and *in vitro* antimicrobial activity of *Anogeissus leiocarpus* on some common oral pathogens. *Journal of Medicinal Plants Research*, 2(8): 193-196.

Adeleye, I.A.; Ogunniyi, A.A. and Omonigbehin, E. A., 2003. Antimicrobial activity of some local herbs on common skin pathogens. *Bioscience Research Communication*, 15(3): 231-236.

Agaie, B. M. and Onyeyili, P. A., 2007. Anthelmintic activity of the crude aqueous leaf extracts of *Anogeissus leiocarpus* in sheep. *African Journal of Biotechnology*, 6(13): 1511-1515.

Agaie, B. M.; Onyeyili, P.A.; Muhammad, B.Y. and Ladan, M. J.2007a. Acute toxicity effects of the aqueous leaf extract of *Anogeissus leiocarpus* in rats. *African Journal of Biotechnology*, 6(7): 886-889.

Ahmed, A.O.; Van de Sande, W.W.; Van Vianen, W.; Belkum, Van Alex; Fahal, A.H.; Verbrug, H.A.; Irma, A. and Bakker-Woudenber, J.M., 2004. *In- vitro* Susceptibility of *Madurella mycetomatis* to Itraconazole and Amphotericin B assed by a Modified NCCLS Method and a viability based 2,3-bis(2-methoxy 040nitro-5-sulfophenyl)-5-{(phenylamino) carbonyl}-2H-tetrazplium hydroxide (XTT) assay and a modified NCCLS method. *Journal of Antimicrobial Chemotherapy*, 48(7): 2742-2746.

Angeh, JE; Huang, X; Sattler, I; Swan, GE; Dahse, H; Hartl, A and Eloff, JN, 2007. Antimicrobial and anti-inflammatory activity of four known and one new triterpenoid from *Combretum imberbe* (Combretaceae). *Journal of thnopharmacology*, 110: 56-60.

Batawila, K.; Kokou, K.; Koumaglo, K.; Gbéassor, M.; de Foucault ,B.; Bouchet, Ph. and Akpagana, K., 2005. Antifungal activities of five Combretaceae used in Togolese traditional medicine. *Fitoterapia*, 76: 264-268.

Chaabi, M.; Benayache, S.; Vonthron-Sénécheau, C.; Weniger, B.; Anton, R. and Lobstein, A., 2006. Antiprotozoal activity of saponins from *Anogeissus leiocarpus* (Combretaceae). *Planta Med.*, 72: 7.

Deshpande, V.H.; Patil, A.D.; Rama Roa, A.V. and Venkataraman., 1976. Methylellagic acid and methylflavellagic acid from *Anogeissus latifolia* bark, *Ind. J. Chem.*, 14B: 641-643.

Eloff, J. N.; Famakin, J. O. and Katerere, D. R. P., 2005a. *Combretum woodii* (Combretaceae) leaf extracts have high activity against Gram-negative and Gram-positive bacteria. *African Journal of Biotechnology*, 4 (10): 1161-1166.

Eloff, J. N.; Famakin, J. O. and Katerere, D. R. P., 2005b. Isolation of an antibacterial stilbene from *Combretum woodii* (Combretaceae) leaves. *African Journal of Biotechnology*, 4(10): 1167-1171.

Eloff, J.N.; Katerere, D.R. and McGaw, L.J., 2008. The biological activity and chemistry of the southern African Combretaceae. *J. Ethnopharmacol.*, 119(3): 686-699.

Govindarajan, R.; Vijayakumar, M.; Rao, Ch.V.; Shirwaikar, A.; Pushpangadan, P. and Mehrotra, S., 2004b. Healing potential of *Anogeissus latifolia* for dermal wounds in rats. *Acta. Pharmaceutica*., 54(4): 331-338.

Gumaa, S.A.,1994. The aetiology and epidemiology of mycetoma. *Sudan medical journal*, 32(2): 14-22.

Ibrahim, M.B.; Owonubi, M.O.; Onaopo, J.A., 1997. Antibacterial effect of extract of leaf, stem and root bark of *Anogeissus leiocarpus* on some bacterial organisms. *J. Pharm. Res. Dev*., 2(1): 20-23.

Kuo-Ching Wen, I-Chen Shih, Jhe-Cyuan Hu, Sue-Tsai Liao, Tsung-Wei Su, and Hsiu-Mei Chiang, 2011. Inhibitory Effects of *Terminaliacatappa* on UVB-Induced Photodamage in Fibroblast Cell Line. Evid Based Complement Alternat Med.: 904532.

Mahgoub, E.S., 1994. Medical treatment of mycetoma. *Sudan medical journal*, 32(2): 88-97.

Mann, A.; Amupitan, J.O.; Oyewale, A.O.; Okogun, J.I. and Ibrahim, K., 2007. An ethnobotanical survey of indigenous flora for treating tuberculosis and other respiratory diseases in Niger State, *Nigeria. J. Phytomed. Therap.*, 12: 1-12.

Mann, A.; Banso, A. and Clifford, L.C., 2008a. An antifungal property of crude plant Extracts from *Anogessius leiocarpus and Terminalia avicennioides , Tanzania Journal of Health Researc.*, 10(1): 34-38.

Mann, A.; Yahaya,Y.; Banso, A. and Ajayi, G.O., 2008b. Phytochemical and antibacterial screening of *Anogeissus leiocarpus* against some microorganisms associated with infectious wounds. *African Journal of Microbiology Research*, 2: 60-62.

Mann, A.; Amupitan, J.O.; Oyewale, A.O.; Okogun, J.I.; Ibrahim, K.; Oladosu, P.; Lawson, L.; Olajide, I. and Nnamdi, A., 2008c. Evaluation of *in vitro* antimycobacterial activity of Nigerian plants used for treatment of respiratory diseases. *African Journal of Biotechnology*, 7(11): 1630-1636.

Mann, A.; Amupitan, J.O.; Oyewale, A.O.; Okogun, J.I. and Ibrahim, K., 2009a. Antibacterial activity of terpenoidal fractions from *Anogeissus leiocarpus* and

Terminalia avicennioides against community acquired infections. *African Journal of Pharmacy and Pharmacology*, 3(1): 22-25.

Mann, A.; Amupitan, J.O.; Oyewale, A.O.; Okogun, J.I. and Ibrahim, K., 2009b. Chemistry of secondary metabolites and their antimicrobial activity in the drug development process: A review of the genus *Anogeissus*. *Medicinal Plants-International Journal of Phytomedicines and Related Industries*, 1(2): 6.

Michael A., 1998. "Combretaceae" A dictionary of Plant Sciences. http://www.encyclopedia.com/ 2011.

Muraina, I.A.; Picard, J.A. and Eloff, J.N., 2009. Development of a reproducible method to determine minimum inhibitory concentration (MIC) of plant extract against a slow-growing mycoplasmas organism. *Phytomedicine*, 16(2-3): 262-264.

Mustofa,V.A.; Benoˆıt-Vical, F.; Pellissier,Y.; Kone-Bamba, D. and Mallié, M., 2000. Antiplasmodial activity of plants extracts used in West African traditional Medicine. *Journal of Ethnopharmacology*, 73: 145-151.

NCCLS, 2002. National Committee for Clinical Laboratory Standards. 2002. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi. Approved standard NCCLS Document M38-A.9. National Committee for Clinical Laboratory Standards, Wayne,USA.

Okpekon, T.; Yolou, S.; Gleye, C.; Roblot, F.; Loiseau, P.; Bories, C.; Grellier, P.; Frappier F.; Laurens, A. and Hocquemiller, R., 2004. Antiparasitic activities of medicinal plants used in Ivory Coast. *Journal of Ethnopharmacology*, 90: 91-97.

Reddy, K.K.; Rajadurai, S.; Sastry, K.N.S. and Nayudamma, Y., 1964. Studies on dhava tannins: Part I. The isolation and constitution of a gallotannin from dhava (*Anogeissus latifolia*). *Australian Journal of Chemistry*, 17(2): 238-245.

Reddy, K.K.; Rajadurai, S. and Nayudamma,Y., 1965. Studies on Dhava (*Anogeissus latifolia*) Tannins: Part III. Polyphenols of bark, sapwood and heartwood of Dhava. *Indian. J. Chem*., 27: 308-310.

Rocquet, C.; Reynaud, R.; Dr Sousselier, L.; Soliance and, France, 2007. Innovative Global “Age-Defying” Strategy, Active Ingredients. *Cosmetic Science Technology*: 119-125.

Sanogo, R., 2005. Antifungal and Antioxidant Activities of 14 plants used in the treatment of sexually transmitted infections. *Afr. J. Trad, Complem. Alter. Med.*, 2(2): 177- 205.

Ten-Ning, C.; Guan-Jhong, H.; Yu-Lin, H.; Shgh-Shgun, H.; Heng-Yua, C.; Yuan-Shium, C., 2009. Antioxidant and Antiproliferative Activities of

Crossostephiumchinenis. The American Journal of Chinese Medicine, 37(4): 797-814.

Van de Sande, W.W.J.; Lujendijk, A. and Ahmed, A.O., 2005.Testing of the *in-vitro* susceptibility of *madurella mycetomatis* to six antifungal agents by using the sensititer system in comparison with viability based 2,3-bis(2-methoxy040nitro-5-sulfophenyl)-5-{(phenylamino) carbonyl}-2H-tetrazplium hydroxide (XTT) assay and a modified NCCLS method. *Journal of Antimicrobial Chemotherapy*, 49:1364-1368.

Vonthron-Sénécheau ,C. ; Weniger, B.; Ouattara, M. ; Tra Bi, F.; Kamenan, A.; Lobstein , A.; Brun,R. and Anton, R., 2003. In vitro antiplasmodial activity and cytotoxicity of ethnobotanically selected Ivorian plants. *Journal of Ethnopharmacology*, 87: 221-225.

Watson, L. and Dallwitz, M.J., 1992. The families of flowering plants: descriptions, illustrations, identification, and information retrieval. Version: 2009. http://delta-intkey.com/.