Original Article

A comparative Study of *In vitro* Susceptibility of Madurella mycetomatis to Anogeissus leiocarpous Leaves, Roots and Stem Barks Extracts

Ikram Mohamed Eltayeb*[1], Abdel Khalig Muddathir[2], Hiba Abdel Rahman Ali[3] and Saad Mohamed Hussein Ayoub[1]

[1]Department of Pharmacognosy, Faculty of Pharmacy, University of Medical Sciences and Technology, P. O. Box 12810, Khartoum, Sudan
[2]Department of Pharmacognosy, Faculty of Pharmacy, University of Khartoum, Khartoum, Sudan
[3]Commission of Biotechnology and Genetic Engineering, National Center for Research, Khartoum, Sudan

Address for Correspondence

Department of Pharmacognosy, Faculty of Pharmacy, University of Medical Sciences and Technology, P. O. Box 12810, Khartoum, Sudan

E-mail: kramela_07 @yahoo.com

ABSTRACT

Objective: *Anogeissus leiocarpus* leaves, roots and stem bark are broadly utilized as a part of African traditional medicine against numerous pathogenic microorganisms for treating skin diseases and infections. Mycetoma disease is a fungal and/ or bacterial skin infection, mainly caused by filamentous *Madurella mycetomatis* fungus. The objective of this study is to investigate and compare the antifungal activity of *A. leiocarpus* leaves, roots and stem bark against the isolated mycetoma pathogen, *M. mycetomatis* fungus.
Methods: The alcoholic crude extracts, and their petroleum ether, chloroform and ethyl acetate fractions of *A. leiocarpus* leaves, roots and stem bark were prepared and their antifungal activity against the isolated *M. mycetomatis* fungus were assayed according to the NCCLS antifungal modified method and MTT assay compared to the Ketoconazole, standard antifungal drug. The most bioactive fractions were subjected to chemical analysis using LC-MS/MS chromatographic analytical method.
Results: The results demonstrated the potent antifungal activity of *A. leiocarpus* extracts against the isolated pathogenic *M. mycetomatis* compared to the negative and positive controls. The chloroform fractions showed higher antifungal activity among the other extracts, while the bark chloroform fraction was found to be the highest one. The chromatographic analysis of the chloroform fractions showed the presence of important bioactive compounds such as ellagic and flavellagic acids derivatives, known for their antifungal activity and toxicity to the filamentous fungi, steilbenoid compounds known as phytoalexins secondary metabolites with potent antifungal activities

and the antimicrobial agents, flavonoids
Conclusion: These studies present that the *A. leiocarpus* extracts posse's potent antifungal activity against mycetoma causing pathogen compared to the ketoconazole standard drug and the highest activity was found to be in the stem bark of the plant.

Keywords: *In vitro*, Susceptibility, *Madurella, mycetomatis, Anogeissus, leiocarpus*, Leaves, Roots, Stem barks, extracts.

INTRODUCTION

Combretaceae is a family of flowering plants, widely distributed in the tropical climates of Africa, Asia and South America. It incorporates 20 genera and around 600 species of shrubs, trees (evergreen or deciduous) or woody lianas[1-3]. The family is an important resource in traditional medical practices for many human diseases[3-8], many of these indications are related to treating infections[7]. The efficacy of the plants may be due to the presence of different classes of antimicrobial secondary metabolites[4, 5, 9].

Anogeissus leiocarpus, is an African evergreen tree[10] of genus *Anogeissus* belonging to this family with different uses in traditional medicine[8,11-19]. It mainly used in treating skin diseases and infections, wounds infections, sore feet, boils, cysts, syphilitic and diabetic ulcers[13,20-23]. The plant was found to exhibited potent antibacterial and antifungal activity against several pathogenic microorganisms[24-30,19, 31, 22, 32].

Mycetoma is a chronic granulomatous subcutaneous and deep tissues skin disease or a number of skin infections caused by numerous fungi (eumycetoma) primarily *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 the infected limbs[33, 23, 34].

In Sudan mycetoma is a serious common disease leading to the loss of numerous limbs. The rate of mycetoma infections in Sudan has not changed and around 400 new cases are found in clinics and outpatient centers every year[35, 33, 23, 34].

There is no potent and effective drug for treating mycetoma infection. Ketoconazole is the favored antifungal medication utilized for mycetoma treatment[36,23,34]. Adequate treatment requires a prolonged antifungal drug combined with extensive surgical treatment[33, 23, 34]. Meager data is available for susceptibility of *M. mycetomatis* to the plants secondary metabolites[37].

The present paper reported the results of comparative study and activity assessment of alcoholic leaves, barks and roots extracts of the plant and their chloroform fractions and ethyl acetate fractions against *M. mycetomatis*. Emphasis has been laid on the fungal susceptibility to the different metabolites occurring in different morphological parts of the same plant used in traditional therapy for the infections treatment.

EXPERIMENTAL

Plant Material

The leaves, roots and stem barks of *A. leiocarpus* were collected from El

Damazeine region in Sudan; their botanical identities were authenticated in the siliviculture department, Faculty of Forestry, University of Khartoum, Sudan. The voucher specimen was deposited at Department of Biochemistry, Commission of Biotechnology and Genetic Engineering, National Centre for Research, Sudan. Barks and roots were chipped using saw mill, and the plant materials were air dried under shade at room temperature, ground to a coarse powder using electric grinder. While the leaves were ground into powder utilizing mortar and pestle.

Preparations of the Extracts

Plant powdered materials were extracted by maceration over night in 80% alcohol. Alcoholic extracts were fractionated using solvents with gradually increasing polarities; petroleum, chloroform and ethyl acetate). The obtained fractions were concentrated by evaporation of the solvents under reduced pressure using a rotary vacuum evaporator.

Madurella mycetomatis Collection

M. mycetomatis Isolated fungus was collected from mycetoma research center at Soba hospital, Sudan. The black grains were exuded from open sinuses and surgical biopsy from the lesion, freed from tissues and carried by forceps in sterile container with saline.

Culture and Preparation of Fungal Suspension

The isolated grains were washed several time with saline solution and were firstly cultured in blood agar media,and then sub--cultured in sabouraud dextrose agar and incubated at 37°C for 8 days.

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

Anti Fungal Procedure

NCCLS Antifungal Modified Assay

One ml of RPMI medium containing serially diluted extracts (10-0.31mg/ml) were placed in sterile test tubes, then 1ml of prepared suspension was added. Tow set of control tubes were used in the experiment, one is growth control tubes(-ve) contained 1ml of RPMI medium without any treatment and 1ml of prepared suspension, and the other one was standard drug (+ve) control tubes contained 1ml of RPMI medium with serially diluted ketoconazole (5-0.31mg/ml). The optical density of the prepared growth control suspension was measured prior incubation using a spectrophotometer at 680 nm red filter and reported as initial reading. Then all test tubes were incubated at 37°C for a week then, the optical density was measured at 680 nm[38, 39]

MIC value is the least concentration before the spectrophotometer transmission reading is the same as or more than the initial reading[37]. It the least concentration, when, there is no any growth of inoculated tested organisms had been seen[40].

MTT Assay

The assay is a quick sensitive colorimetric method; utilize tetrazolium salt as indicator of microbial metabolism for evaluation of cell death[41]. This method is actually the reduction of yellow MTT salt [tetrazolium salt (3-{4, 5-dimethylthiazole-2-yl}-2, 5-diphenyl tetrazolium bromide)]

into the green blue or violet blue formazan by the mitochondrial dehydrogenase, show just in the living cells and henceforth discharged into the supernatant. The color intensity is directly proportional to the living cell numbers in the culture. One drop of the indicator was added to all tested tubes after measuring the final optical density by a spectrophotometer[42,43].

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

LC-MS/MS system was equipped with:

HPLC column (RP-C18) and UV detector (Diode array DAD) adjusted at 320 – 380 nm, coupled with Finnigan LCQ ion trap mass spectrometer with the Electrospray Ionization (ESI) interface at negative ion mode for compounds detection. Collision induced dissociation (CID) experiment was performed for fragmentation of glycosides and elucidation of compounds structures.

RESULTS AND DISCUSSIONS

The results of antifungal NCCLS method are shown in figure 2 (a, b, c). The optical density reading of the fungal suspension indicated the susceptibility of fungus to the extracts. The susceptibility was compared to the controls, one with ketoconazole standard drug (positive control) and the other without drug (negative control). The optical density reading of the inoculum at 680nm was set at 0.04, as the initial reading.

The results showed that, all extracts and fractions inhibited the fungal growth with different degree. The extracts had potential antifungal activity against *M. mycetomatis* contrasted with the ketoconazole standard medication. The chloroform fractions of the three parts of the plant showed higher activity than alcoholic extracts and ethyl acetate fractions. In addition to the stem bark chloroform fraction was found to be the most active fraction. The results were compatible with the results of other related *Anogeissus spp* (*Anogeissus latifolia*) against skin disease organisms[44].

The leaves extract and fractions inhibited the inoculum initial reading 0.04 at 680nm to 0.03, 0.02, 0.03, 0.02after a week inoculated in 10 mg/ml alcoholic extract, chloroform, ethyl acetate and petroleum ether fractions respectively and to 0.03, 02, 0.03, 0.03 when 5mg/ml is used. In comparison to the inocuulm growth reading up to 0.23 in the negative control and inoculum inhibition reading to 0.03 in5mg/ml ketoconazole positive control. Chloroform fraction was found to be the most potent. The results justified the traditional uses of the leaves decoction for treatment of skin diseases and infections.

The stem bark extract and fractions showed higher activity than the leaves. The initial inoculum optical density reading was inhibited to 0.02, 0.01, 0.03 when inoculated for a week in 10 mg/ml alcoholic extract, chloroform and ethyl acetate fractions consequently and to 0.02, 0.02, 0.03 in 5mg/ml. Chloroform fraction was found to be the most potent among the all extracts and fractions of the three parts of the plant, and it inhibited the inoculum reading up to 0.01 after a week inoculation in 10mg/ml. It is noteworthy to add that these findings were in agreement with the uses of stem bark decoctions in treatment of skin diseases in African traditional medicine. The results were compatible with the current literature of the stem bark extracts against skin disease caused by other organisms[20].

The root extract and fractions showed less activity than the leaves and stem bark extracts and fractions. The inoculum initial reading was inhibited to 0.03, 0.04, 0.02 when inoculated for a week

in 10 mg/ml alcoholic extract, ethyl acetate and chloroform fractions consequently and to 0.03, 0.04, 0.03 in 5mg/ml.

These findings showed that, the extracts of the stem bark were more potent than the leaves and roots extracts while the leaves extracts were more potent than the roots extracts. These results supported the traditional healer's use of the stem barks more than leaves and root; the leaves more than root in the treatment of skin disease[45, 46, 20, 47, 48, 49, 27, 24].

The MIC values of the extracts compared to the MIC of the control drug (5mg/ml), was found to be 2.5mg/ml in alcoholic extracts of the three parts. The MIC of the ethyl acetate and chloroform fractions of both bark and leaves were found to be 5mg/ml and 0.62mg/ml respectively. While in the root MIC of the chloroform fraction was found to be 2.5mg/ml, and the ethyl acetate fraction is active at10mg/ml. The result was compatible with the MIC of antimicrobial agents reported by Banso *et al*., 1999[50]; Prescott *et al*., 2002[51] and Mann *et al*., 2008b[27].

In the MMT results, the tetrazolium color changed represented the fungal viable and growth. The results of the extracts against the fungus in compared to the ketoconazole, standard antifungal drug showed that, the tetrazolium salt color in the fungal suspension started to change at the concentration of 0.62mg/ml after a week inoculation in alcoholic leaves extract. In the ethyl acetate, chloroform and petroleum ether fractions the color started to change at 5mg/ml, 0.31mg/ml and 1.25mg/ml respectively.

In the stem barks extracts the color started to change at the concentration of 0.62mg/ml and 1.25mg/ml of alcoholic extract and ethyl acetate fraction respectively. In the chloroform fraction there was no color change up to the concentration of 0.31mg/ml.

In the roots extracts the tetrazolium color started to change at the concentration of 1.25mg/ml, 5mg/ml and 0.62mg/ml in alcoholic extract, ethyl acetate and chloroform fractions, respectively. The tetrazolium color changed at 0.31mg/ml in the ketoconazole drug.

The LC-MS/MS analysis of the chloroform fractions with the higher activity are shown in figure 3 (a, b, c) and table 1(a, b, c).

The LC-MS/MS analysis of the leaves chloroform fraction identified fifteen compounds of ellagic acid derivatives, flavonoids and steilbenoids. These findings are reported for the first time with regard to the reported results about the abundance of ellagic, flavellagic acid derivatives and flavonoids in the other member of the genus Anogeissus[52, 53, 44, 54, 55].

Seven ellagic and flavellagic acids derivatives were identified in the chloroform stem bark fraction in agreement with reported chemistry of this part of the plant[56, 57].

Nine compounds were identified in the root chloroform fraction. These results are mentioned for the first time in the *A. leiocarpus* root, in addition to the reported results about the abundance of ellagic, flavellagic acid and flavonoids derivatives in other *Anageissus* species[52, 44, 54].

The results of chromatographic analysis were compatible with the toxicity of ellagic acid against fillamentus fungi presented by Scalbert,(1991)[58], in addition to that, the steilbenoid compounds were known as phytoalexins secondary metabolites with potent antifungal activities[59,60,61,62] and the flavonoid antimicrobial agents[63].

CONCLUSIONS

The *M. mycetomatis* fungus was susceptible to *A. leiocarpus* extracts which showed potent antifungal activity against

mycetoma causing pathogen compared to the ketoconazole standard drug. None of the extracts was found to enhance fungal growth. Advanced hyphenated techniques LC/DAD-MS/MS revealed the presence of ellagic acid derivatives, stelbenoids and flavonoids at different concentrations in the aforesaid extracts. The ellagic acid derivatives in the chloroform stem bark fraction were found to be the highest in concentration, hence the highest toxicity against the *M. mycetomatis* filamentous fungus. The compounds in leaves chloroform fraction with activity next to the stem bark were found to be also ellagic acid derivatives but with less concentration than that in the chloroform stem bark fraction, in addition to antifungal stelbenoids compounds. The moderate and least concentrations of ellagic acid derivatives in the leaves and root fractions enabled by the antifungal stelbenoids to exert better activity followed by the antimicrobial favonoids in these fractions against the fungus. According to these findings which are based on the results obtained it could be concluded that, the activity against *M. mycetomatis* was proportional to the concentration of ellagic acid derivatives, steilbenoid and flavonoids in the extracts respectively. The ellagic acid derivatives were the most potent, followed by stelbinoids and finally the flavonoids.

ACKNOWLEDGEMENTS

The authors are grateful to the Management and Staff members of the Mycetoma Research Center at Soba Hospital, Sudan for providing the *M. mycetomatis* isolates. Many thanks to Mr. Elkhassein, University of Sciences and Technology, Sudan, for his technical encouragement to carry out this study. Our deepest thanks go to Prof. Nickoli Kuhnert group, School of Engineering and Science, International University, Bremen, Germany for assisting in running the Tandam Mass Spectra. Deepest appreciation to Mr. Ashraf Hatium Osman, Lecturer at the University of Medical Science and Technology, Sudan, for his valuable, kind helping and assistance. The authors express many thanks and appreciation to the University of Medical Science and Technology, Sudan, for providing support and encouragement to carry out the present work.

REFERENCES

1. Watson L and Dallwitz MJ. The families of flowering plants: descriptions, illustrations, identification, and information retrieval .1992; *http://delta-intkey.com/* (accessed 5 October 2011).
2. Michael A. "Combretaceae" A dictionary of Plant Sciences.1998; *http://www.encyclopedia.com* (accessed 5 October 2011).
3. Angeh JE, Huang X, Sattler I, Swan GE, Dahse H, Hartl A and Eloff JN.Antimicrobial and anti-inflammatory activity of four known and one new triterpenoid from Combretumimberbe (Combretaceae). *Journal of Ethnopharmacology* 2007; 110(1): 56-60.
4. Eloff JN, Famakin JO and Katerere DRP.Combretumwoodii (Combretaceae) leaf extracts have high activity against Gram-negative and Gram-positive bacteria. *African Journal of Biotechnology* 2005; 4 (10): 1161-1166.
5. Eloff JN, Famakin JO and Katerere DRP. Combretumwoodii (Combretaceae) leaf extracts have high activity against Gram-negative and Gram-positive bacteria. *African Journal of Biotechnology* 2005; 4 (10): 1161-1166.
6. Mann A, Amupitan JO, Oyewale AO, Okogun JI and Ibrahim K. An ethnobotanical survey of indigenous flora for treating tuberculosis and other respiratory diseases in Niger State, Nigeria. *J. Phytomed. Therap* 2007; 12: 1-12.
7. Eloff JN, Katerere DR and McGaw LJ.The biological activity and chemistry of the southern African Combretaceae. *J. Ethnopharmacol* 2008; 119(3): 686-699.

8. Mann A, Banso A and Clifford LC. An antifungal property of crude plant Extracts from Anogessiusleiocarpus and Termina liaavicennioides. *Tanzania Journal of Health Researc* 2008; 10(1): 34-38.
9. Eloff JN, Angeh I and McGaw LJ. A plant antifungal product from Melianthuscomosus (Melianthaceae) leaf extracts. *Planta. Med* 2006; 72: 982.
10. Rocquet C, Reynaud R, Dr Sousselier L, Soliance and France. Innovative Global "Age-Defying" Strategy, Active Ingredients. *Cosmetic Science Technology* 2007; 119-125.
11. Mustofa VA, Benoˆıt-Vical F, Pellissier Y, Kone-Bamba D and Mallié M. Antiplasmodial activity of plants extracts used in West African traditional Medicine. *Journal of Ethnopharmacology* 2000; 73: 145-151.
12. Vonthron-Sénécheau C, Weniger B, Ouattara M, TraBiF, Kamenan A, Lobstein A, Brun R and Anton R. In vitro antiplasmodial activity and cytotoxicity of ethnobotanically selected Ivorian plants. *Journal of Ethno-pharmacology* 2003; 87: 221-225.
13. Okpekon T, Yolou S, Gleye C, Roblot F, Loiseau P, Bories C, *et al*. Antiparasitic activities of medicinal plants used in Ivory Coast. *Journal of Ethnopharmacology* 2004; 90: 91-97.
14. Chaabi M, Benayache S, Vonthron-Sénécheau C, Weniger B, Anton R and Lobstein A. Antiprotozoal activity of saponins from Anogeissusleiocarpus (Combretaceae). *Planta Med* 2006; 72: 7.
15. Agaie BM, Onyeyili PA, Muhammad BY and Ladan M. Acute toxicity effects of the aqueous leaf extract of Anogeissusleiocarpus in rats. *African Journal of Biotechnology* 2007; 6(7): 886-889.
16. Adejumobi JA, Ogundiya MO, Kolapo A and kunade MB. Phytochemical composition and in vitro antimicrobial activity of Anogeissusleiocarpus on some common oral pathogens.*Journal of Medicinal Plants Research* 2008; 2(8): 193-196.
17. Mann A, Amupitan JO, Oyewale AO, Okogun JI and Ibrahim K. 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* 2009; 1(2): 6.
18. Arbab AH. Review on Anogeissusleiocarpus a potent african traditional drug. *International Journal of Research in Pharmacy and Chemistry* 2014; 4(3): 496-500.
19. Mann A, Yusuf A and Daniyan S. TLC analysis and bioactivity screening of the stem bark extract of Anogeissusleiocarpus against multi-resistant Staphylococcus aureus and quantification of its phytoconstituents. *Research Journal of Pharmaceutical, Biological and Chemical Sciences* 2014; 5(2): 187- 203.
20. Adeleye IA, Ogunniyi, AA and Omonigbehin EA. Antimicrobial activity of some local herbs on common skin pathogens. *Bioscience Research Communication* 2003; 15(3): 231-236.
21. Agaie BM and Onyeyili PA. Anthelmintic activity of the crude aqueous leaf extracts of Anogeissusleiocarpus in sheep. *African Journal of Biotechnology* 2007; 6(13): 1511-1515.
22. Elsiddig IME, Muddather A, Ali HA and Ayoub SMH.A comparative study of antimicrobial activity of the extracts from root, leaf and stem of Anogeissusleiocarpous growing in Sudan. *Journal of Pharmacognosy and Phytochemistry 2015; 4(4): 107-113.*
23. Elsiddig IME, Muddather A, Ali HA and Ayoub SMH. In vitro susceptibility of Madurellamycetomatis to the extracts of Anogeissusleiocarpus leaves. *International Journal of Biological, Biomolecular, Agricultural, Food and Biotechnological Engineering*2015; 9 (12): 1039- 1050.
24. Ibrahim MB, Owonubi MO, Onaopo JA. Antibacterial effect of extract of leaf, stem and root bark of Anogeissusleiocarpus on some bacterial organisms.*J. Pharm. Res. Dev* 1997; 2(1): 20-23.
25. Batawila K,Kokou K,Koumaglo K,Gbéassor M, de Foucault B,BouchetPh and Akpagana K. Antifungal activities of five Combretaceae used in Togolese traditional medicine. *Fitoterapia* 2005; 76: 264-268.
26. Sanogo R. Antifungal and Antioxidant Activities of 14 plants used in the treatment of sexually transmitted infections. *Afr. J. Trad, Complem. Alter. Med* 2005; 2(2): 177- 205.

27. Mann A,YahayaY, Banso A and Ajayi GO. Phytochemical and antibacterial screening of Anogeissusleiocarpus against some microorganisms associated with infectious wounds. *African Journal of Microbiology Research* 2008; 2: 60-62.
28. Mann A,Amupitan JO, Oyewale AO, Okogun JI, Ibrahim K,Oladosu P, *et al*. Evaluation of in vitro antimycobacterial activity of Nigerian plants used for treatment of respiratory diseases. *African Journal of Biotechnology* 2008; 7(11): 1630-1636.
29. Mann A,Amupitan JO, Oyewale AO, Okogun JI and Ibrahim K. Antibacterial activity of terpenoidal fractions from Anogeissusleiocarpus and Terminalia-avicennioides against community acquired infections. *African Journal of Pharmacy and Pharmacology*2009; 3(1): 22-25.
30. Mann A. Evaluation of Antimicrobial Activity of Anogeissusleiocarpus and Terminaliaavicennioides against infectious diseases prevalent in hospital environments in Nigeria. *Journal of Microbiology Research* 2012; 2(1): 6-10.
31. Gbadamosi IT and Ogunsuyi AO. An appraisal of the potency of roots of Anogeissusleiocarpus (DC.) Guill. & Perr. And Terminaliaglaucescens Benth. in the management of E. coli related infections. *Journal of Applied Biosciences* 2014;78: 6646-6653.
32. Eltayeb IM, Ali HA, Muddather A and Ayoub SMH. Antioxidant activity and cytotoxic studies of anogeissusleiocarpousroot, leaf and stem. *American Journal of Research Communication* 2016; 4(3): 52- 67.
33. Gumaa SA. The aetiology and epidemiology of mycetoma. *Sudan medical journal*1994; 32(2): 14-22.
34. Eltayeb IM, Ali HA, Muddather A and Ayoub SMH.Madurellamycetomatissusceptibility to Anogeissusleiocarpusstem bark extracts. *American Journal of Research Communication* 2016; 4(3): 68- 86.
35. Mahgoub ES.Medical treatment of mycetoma.*Sudan medical journal*1994;32(2): 88-97.
36. Mahgoub ES and Gumaa SA. Ketoconazole in the Treatment of euomycetoma due to M. mycetomatis.*Trans. Roy. Soc. Med. Hyg.*1984; 78: 376-379.
37. Van de Sande WWJ, Lujendijk A and Ahmed AO.Testing of the in-vitro susceptibility of madurellamycetomatis 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* 2005; 49:1364-1368.
38. National Committee for Clinical Laboratory Standards. 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,Pa; 2002.
39. Ahmed AO, Van de Sande WW, Van Vianen W,Belkum, Van Alex,Fahal AH, *et al*. In-vitro Susceptibility of Madurellamycetomatis 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*2004; 48(7): 2742-2746.
40. Van de Sande WWJ,Fahal A,Verbrugh H, and Belkum Al. Polymorphisms in Genes Involved in Innate Immunity Predispose TowardMycetoma Susceptibility. *Journal of immunology* 2007; 179: 3065-3074.
41. Muraina IA, Picard JA and Eloff JN. Development of a reproducible method to determine minimum inhibitory concentration (MIC) of plant extract against a slow-growing mycoplasmas organism. *Phytomedicine*2009;16(2-3): 262-264.
42. Kuo-Ching Wen, I-Chen Shih, Jhe-Cyuan Hu, Sue-Tsai Liao, Tsung-Wei Su, and Hsiu-Mei Chiang. Inhibitory Effects of Terminaliacatappa on UVB-Induced Photodamage in Fibroblast Cell Line.*Evid Based Complement Alternat Med*2010; 2011: 904532.
43. Ten-Ning C, Guan-Jhong H, Yu-Lin H,Shgh-Shgun, H,Heng-Yua C, Yuan-Shium C. Antioxidant and Antiproliferative Activities

of Crossostephiumchinenis. *The American Journal of Chinese Medicine* 2009; 37(4): 797-814.
44. Govindarajan R,Vijayakumar M,Rao, Ch.V, Shirwaikar A,Pushpangadan P and Mehrotra S. Healing potential of Anogeissuslatifolia for dermal wounds in rats. *Acta. Pharmaceutica* 2004; 54(4): 331-338.
45. Asuquo, B.I. Identification and Characteristics of Local Chewing Stick. B.Sc. Dissertation submitted to the University of Ibadan 1996; 34-37.
46. Malcolm SA and Sofowora EA. Anti-microbial activity of selected Nigerian folk remedies and their constituent parts. *Journal of National product*1969;32: 512- 526.
47. Andary C, Doumbia B, Sauvan N, Olivier M, Garcia M. Anogeissusleiocarpa (DC.) Guill.andPerr. In: Jansen, P.C.M. and Cardon, D. (Editors). PROTA 3: Dyes and tannins/Colorants ettanins [CD-Rom]. PROTA, Wageningen, Netherlands2005; 13.
48. Neuwinger HD. *African traditional medicine.A dictionary of plant use and application.* Germany: Med. Pharm. Press Stuttgart; 2000.
49. Dweek AA. Plant for Africa.Part 2. 1996; *Http://www.dweekdata.co.uk/ Published papers.*(accessed 5 October 2011).
50. Banso A,Adeyemo SO and Jeremiah P. Antimicrobial properties of Vernoniaamygdalina extract. *Journal of Applied Science and Management* 1999; 3: 9-11.
51. Prescott LMH, Arley JP and Klein D. *Microbiology (International Edition)*, 5th ed.Published By McGraw Hill Book Company ; 2002.
52. Reddy KK,Rajadurai S and NayudammaY. Studies on Dhava (Anogeissuslatifolia) Tannins: Part III. Polyphenols of bark, sapwood and heartwood of Dhava. *Indian. J. Chem* 1965; 27: 308-310.
53. Deshpande, VH,Patil AD, Rama Roa AV and Venkataraman.Methylellagic acid and methylflavellagic acid from Anogeissuslatifolia bark, *Ind. J. Chem* 1976; 14B: 641-643.
54. Govindarajan R,Vijayakumar M,RaoChV, Shirwaikar A,Rawat AKS,Mehrotra S and Pushpangadan P. Antioxidant potentialof Anogeissus.*Biol. Pharm. Bull* 2004;27(8): 1266-1269.
55. Pradeep HA, Khan S,Ravikumar K, Ahmed MF, Rao MS, Kiranmai M, *et al*. Hepatoprotective evaluation of Anogeissuslatifolia: In vitro and in vivo studies. *World. J. Gastroenterol* 2009; 15(38): 4816-4822.
56. Nduji AA and Okwute SK. Co-occurrence of 3,3-tri-0-methylflavellagic acid and 3,3-di-0-methylellagic acid in the bark of Anoigessusschimperii. *Phytochemistry*1988;27(5): 1548-1550.
57. Adigun JO,Amupitan JO and Kelly DR.Isolation and investigation of antimicrobial effect of 3, 4, 3'- tri-O-methylflavellagic acid and its glucoside from Anogeissusleiocarpus. *Bull. Chem. Soc. Ethiopia* 2000; 14(2): 169-174.
58. Scalbert A. Antimicrobial properties of tannins. *Phytochemistry*1991;30:3875-3883.
59. Langcake P. Disease resistance of Vitis spp. and the production of the stress metabolites resveratrol, e-viniferin, a-viniferin and pterostilbene. *PhysiologicalPlant Pathology* 1981; 18: 312–226.
60. Alessandro M, Marco SD,Osti F and Cesari A. Bioassays on the activity of resveratrol, pterostilbene and phosphorous acid towards fungal associated with esca of grape vine. *Phytopathol. Mediterr* 2000; 39: 357-365.
61. Suh N, Paul S, Hao X, Simi B, Xiao H, Rimando AM and Reddy BS . Pterostilbene, an active constituent of blueberries, suppresses aberrant crypt foci formation in the azoxymethane-induced colon carcinogenesis model in rats. *Clinical Cancer Research* 2007; 13 (1): 350-355.
62. Rimando AM and Suh N. Biological/chemopreventive activity of stilbenes and their effect on colon cancer. *Planta Med* 2008; 74(13): 1635-1643.
63. Cowan MM. Plant Products as Antimicrobial Agents. *Clinical microbiology reviews* 1999; 12 (4): 564-582.

Table 1(a): RP-HPLC data (Peak NO. & Rt.), and MS/MS data (molecular weight {*m/z*} & main fractions {*m/z*}) and structure assignment of the leaves chloroform fraction

Compound Peak	(R_t) (min	M-H (*m/z*)	CID M^n Main Fraction ions (*m/z*)	Compound Name
1	6.8	541	425, 377, 301, 275, 271, 229, 201,173	Di- hydroxyl, Tri-methoxy-ellagic acid-7-O-β-glucoside
2	8.5	552	481, 301, 275, 271, 243	Di- hydroxy, Tri-methoxy-ellagic acid-7-O-β-glucoside derivative
3	8.8	541	459, 425, 377, 301, 275, 271, 257, 227, 185,117	Di- hydroxyl, Tri-methoxy-ellagic 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- hydroxy, Tri-methoxy-ellagic acid-7-O-β-glucoside derivative
6	12.4	628	623, 552, 481, 301, 275, 271, 243,187	Di- hydroxyl, Tri-methoxy-ellagic acid-7-O-β-glucoside derivative
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	Quercetin 3-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

Table 1(b): RP-HPLC data (Peak NO. & Rt.), and MS/MS data (molecular weight {*m/z*} & main fractions {*m/z*}) and structure assignment of the barks chloroform fraction

Compound Peak	(R_t) (min)	M-H (*m/z*)	CID M^n Main Fraction ions (*m/z*)	Compound Name
16	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
17	20.2	521	506, 385, 342.8, 249, 249, 155, 155, 155, 113	3,4,3'-Tri-methoxy-flavellagic acid-4'-β-D-glucoside
18	20.2	384.8	248.6, 154.7, 152.7, 112.9, 112.9	Flavellagic acid derevative
19	21.6	359.0	343.8,32914,314,300, 285,	Hydroxy, Tetra- methoxy -ellagic acid
20	22.5	329	314, 299, 299, 285, 271, 271	3, 3'-Di-methoxy-ellagic acid
21	25.7	343	328, 313, 313, 298, 298, 285, 270	Tri-methoxy-ellagic acid
22	26.2	359	344, 329, 314,314,300, 285	Tetra-methoxy-ellagic acid

Table 1(c): RP-HPLC data (Peak NO. & Rt.), and MS/MS data (molecular weight {*m/z*} & main fractions {*m/z*}) and structure assignment of the roots chloroform fraction

Compound Peak	(R_t) (min)	M-H (*m/z*)	CID M^n main fraction ions (*m/z*)	Expected compound
23	6.3	466	301, 275, 271, 229, 2001,185	Hydroxy, methoxy-ellagic acid -O- glucoside
24	12.5	443	312.7,252.7, 222.7, 168.7, 168.7, 150.7, 124.8, 124.8	Hydroxy, Trimethoxy- flavone derevative
25	14.3	274.8	257, 229, 200.7, 185.8, 172.7	Chalcone
26	15.2	433	300, 284, 257, 243, 229, 185, 172	ElLagic acid- arabinoside
27	16.9	491	327.7, 312.7, 312.7, 297.7, 297.7, 297.7, 297.7, 284.7, 269.7	Hydroxy, Trimethox- flavone derevative
28	19.6	315	300, 272, 244, 216	Isorehamentein
29	20.2	603	521, 506, 343, 328	Tri-methoxy-flavellagicacid-O-β-glucoside derivative
30	21	613	329, 314, 314, 297, 271	Methoxy- isorehamentein derivative

31	22.4	328	299, 285, 271	Di-hydroxy, dimethoxy-flavones

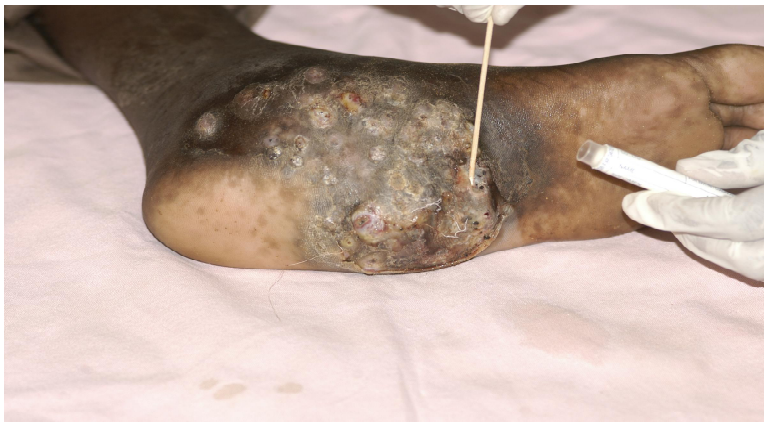


Figure 1: Mycetoma pathogen collection

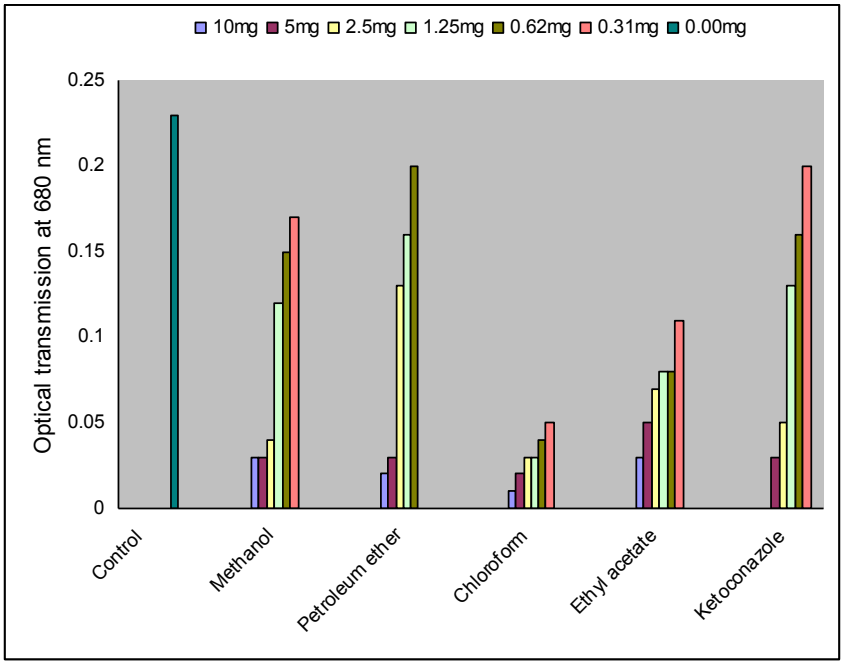


Figure 2(a): *In vitro* susceptibility of *M. mycetomatis* to alcoholic extract, petroleum ether, chloroform and ethyl acetate fractions of *A. leiocarpus* leaves

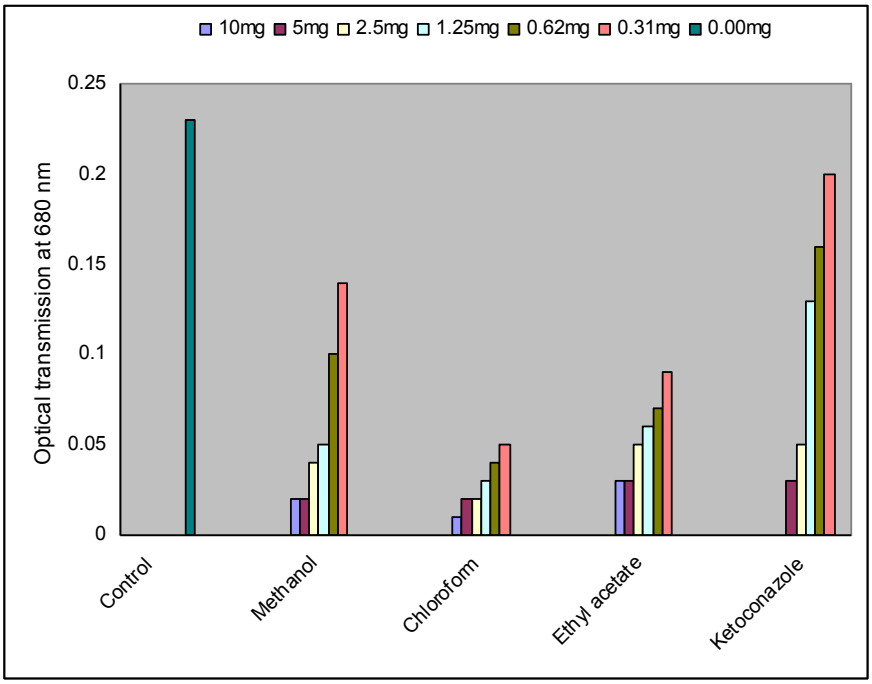


Figure 2(b): *In vitro* susceptibility of *M. mycetomatis* to alcoholic extract, chloroform and ethyl acetate fractions of *A. leiocarpus* stem barks

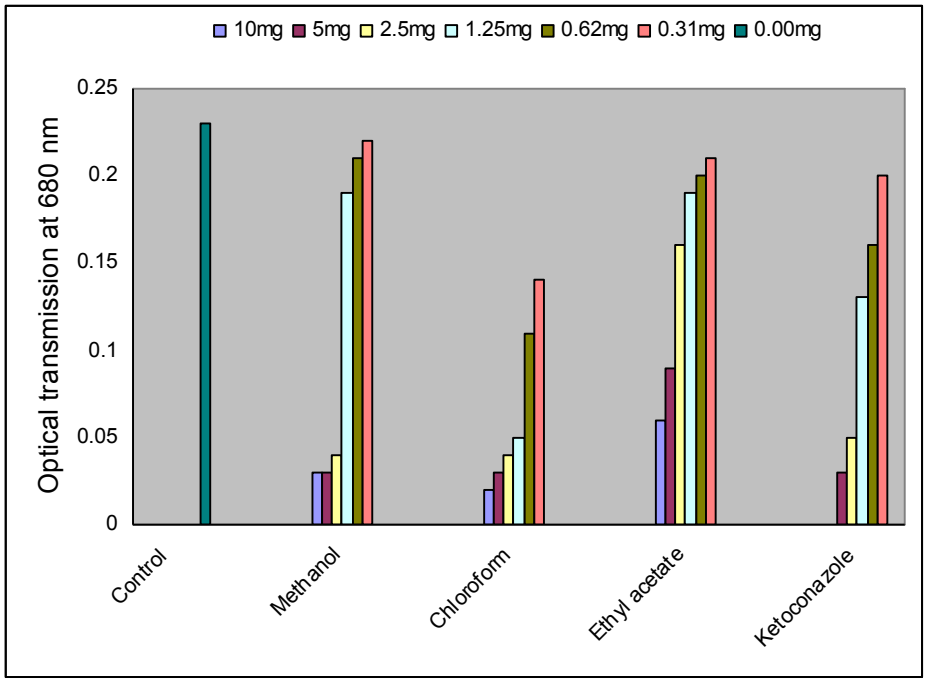


Figure 2(c): *In vitro* susceptibility of *M. mycetomatis* to alcoholic extract, chloroform and ethyl acetate fractions of *A. leiocarpus* roots

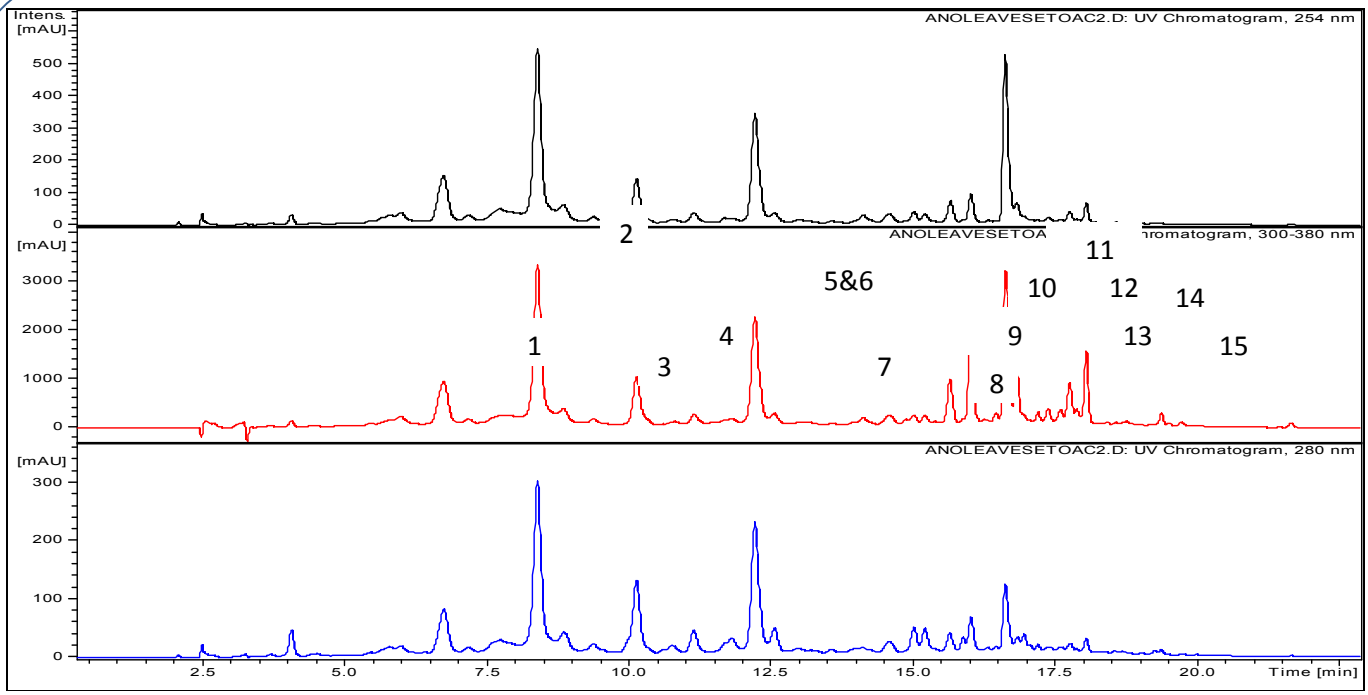


Figure 3(a): RP-HPLC-DAD Chromatogram of *A. leiocarpus* leaves chloroform fraction recorded at λmax 254, 280,300-380nm

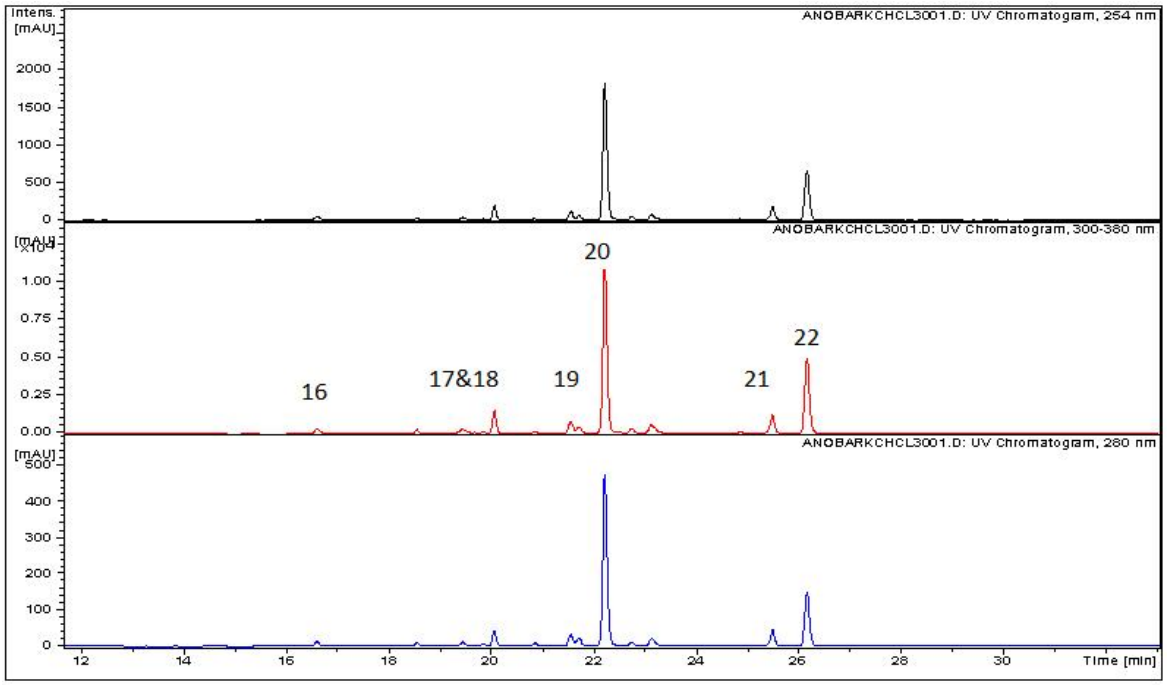


Figure 3(b): RP-HPLC-DAD Chromatogram of *A. leiocarpus* barks chloroform fraction recorded at λmax 254, 280,300-380nm

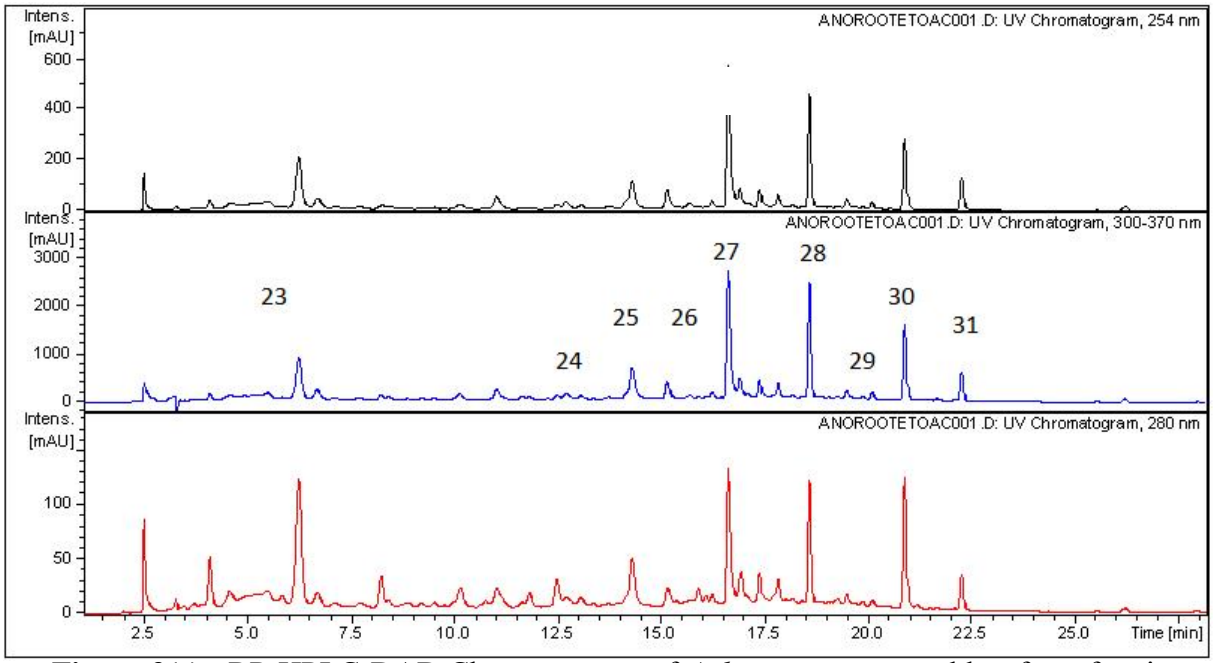


Figure 3(c): RP-HPLC-DAD Chromatogram of *A. leiocarpus* roots chloroform fraction recorded at λmax 254, 280,300-380nm

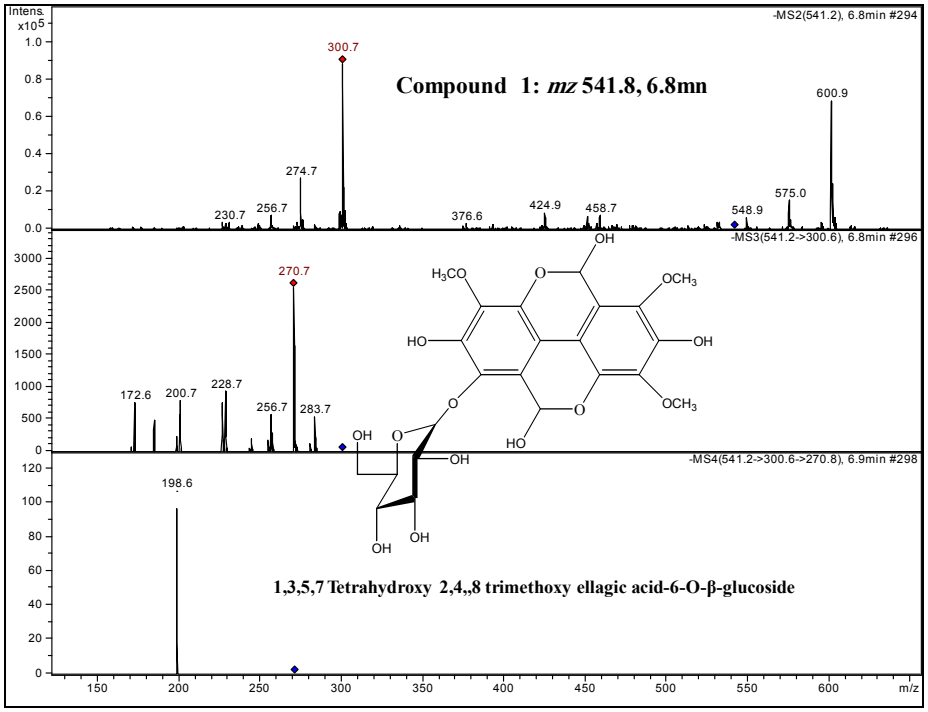


Figure: 4 (a) MS/MS (m/z) and assigned structure of compound (1) in the chloroform fraction of *A. leiocarpus* leaves

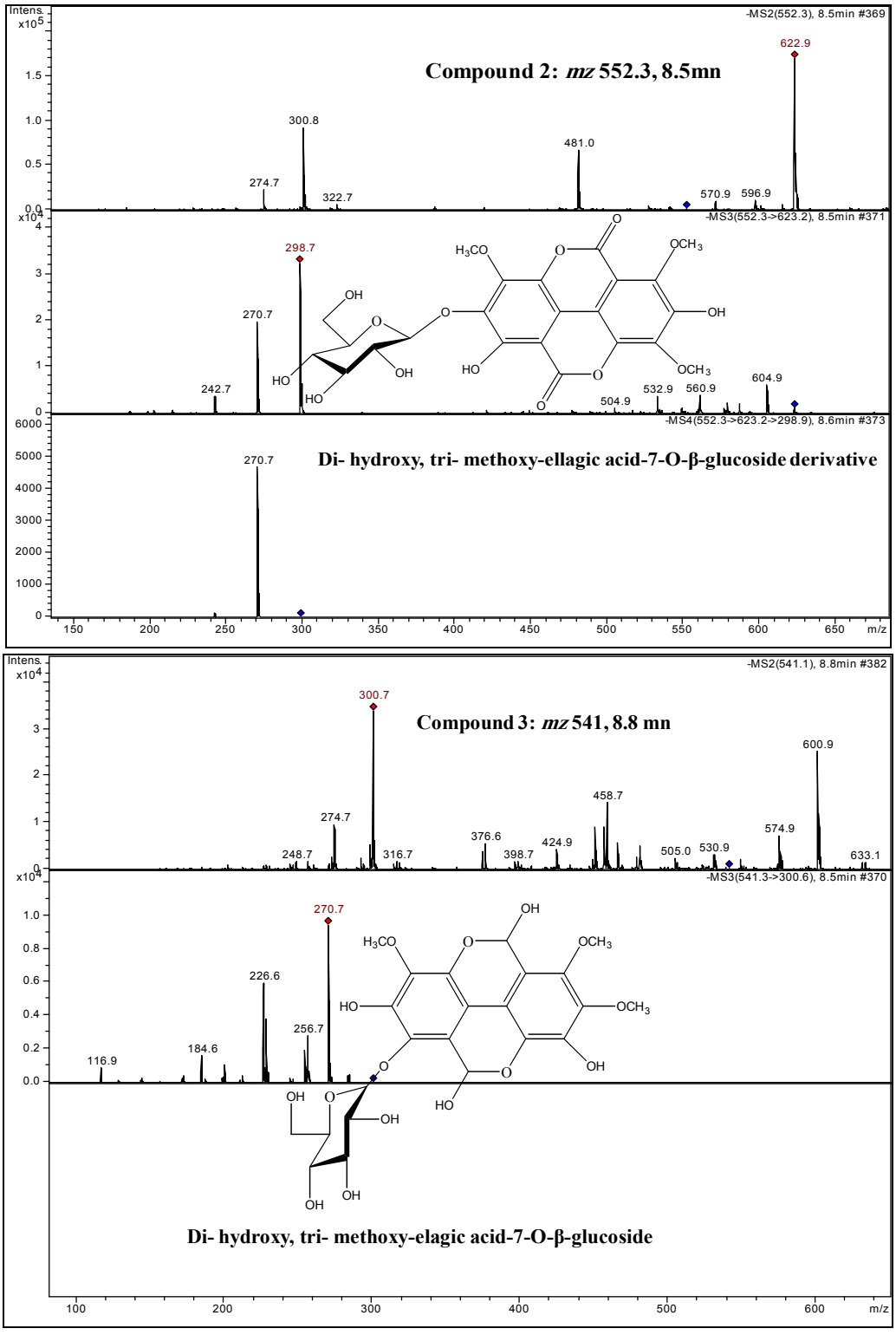
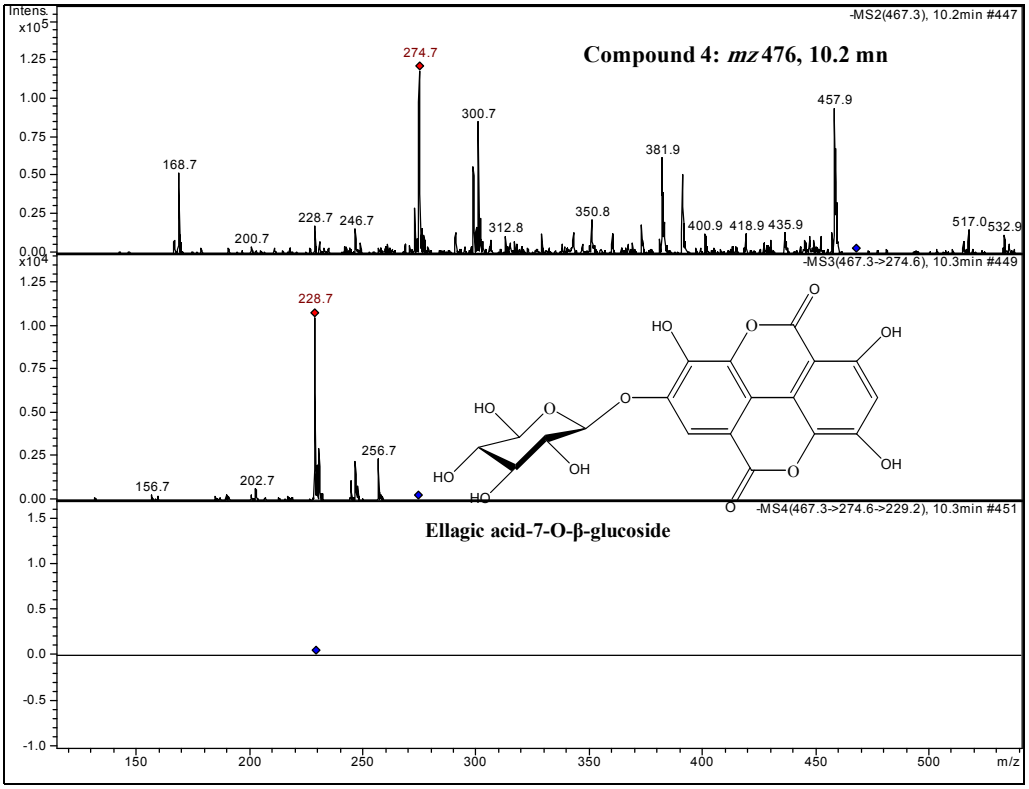


Figure: 4 (b) MS/MS (m/z) and assigned structures of compounds (2 &3) in the chloroform fraction of *A. leiocarpus* leaves



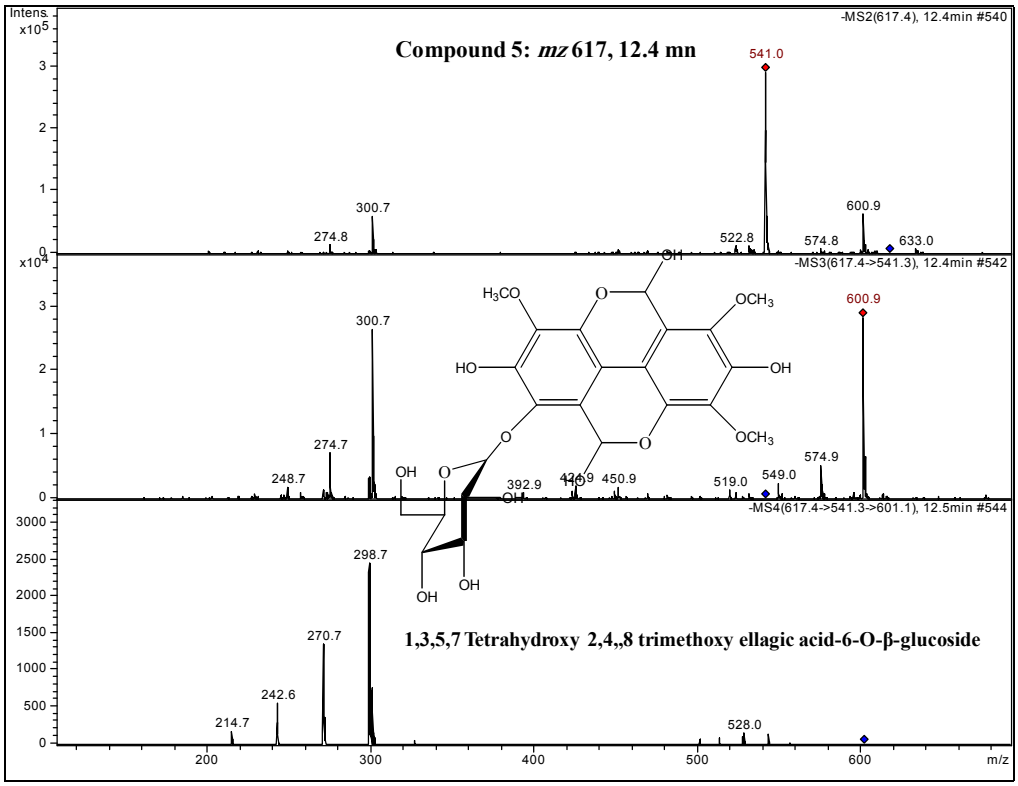


Figure: 4 (c) MS/MS (m/z) and assigned structures of compounds (4 & 5) in the chloroform fraction of *A. leiocarpus* leaves

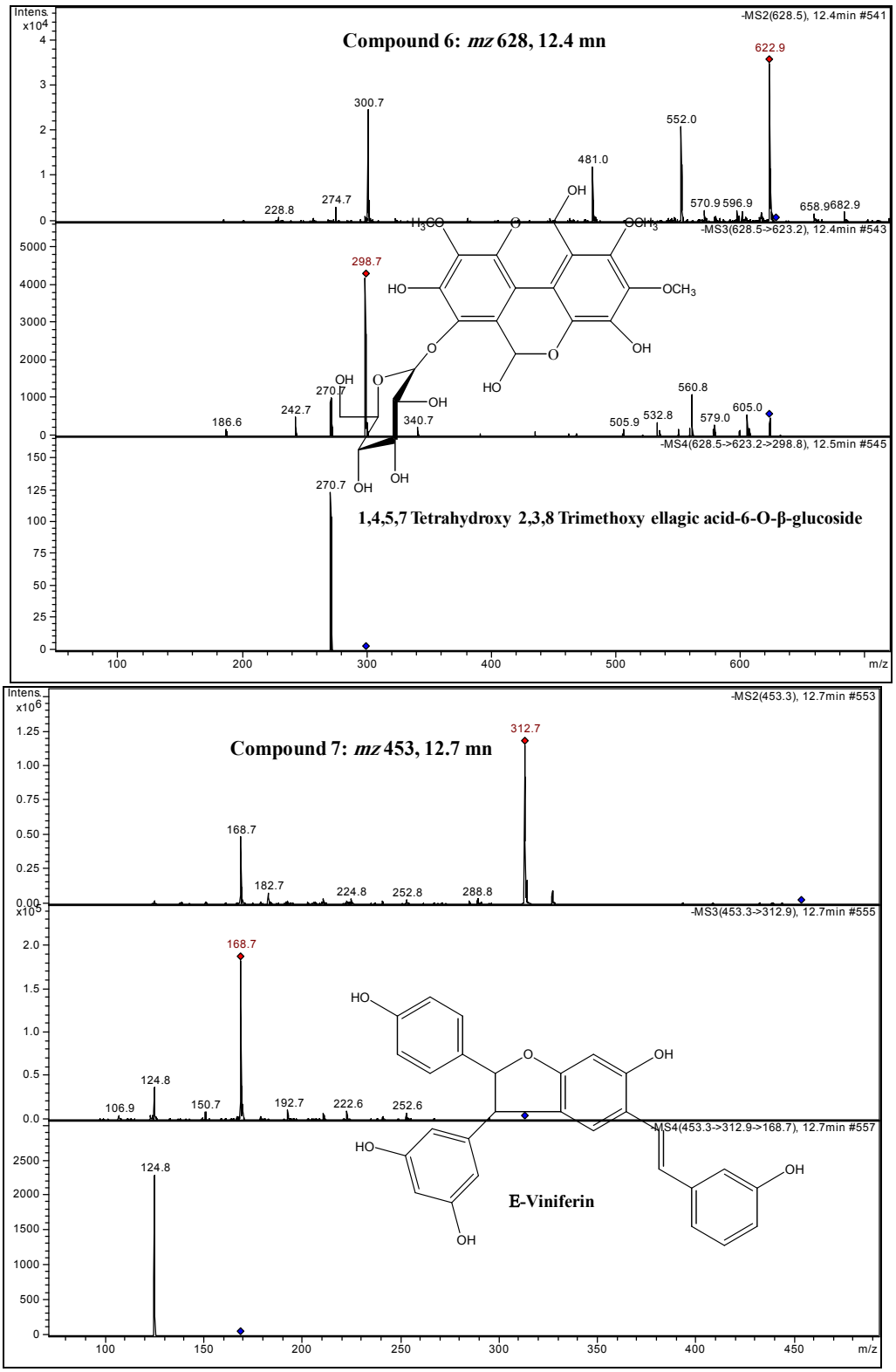


Figure: 4 (d) MS/MS (m/z) and assigned structures of compounds (6 & 7) in the chloroform fraction of *A. leiocarpus* leaves

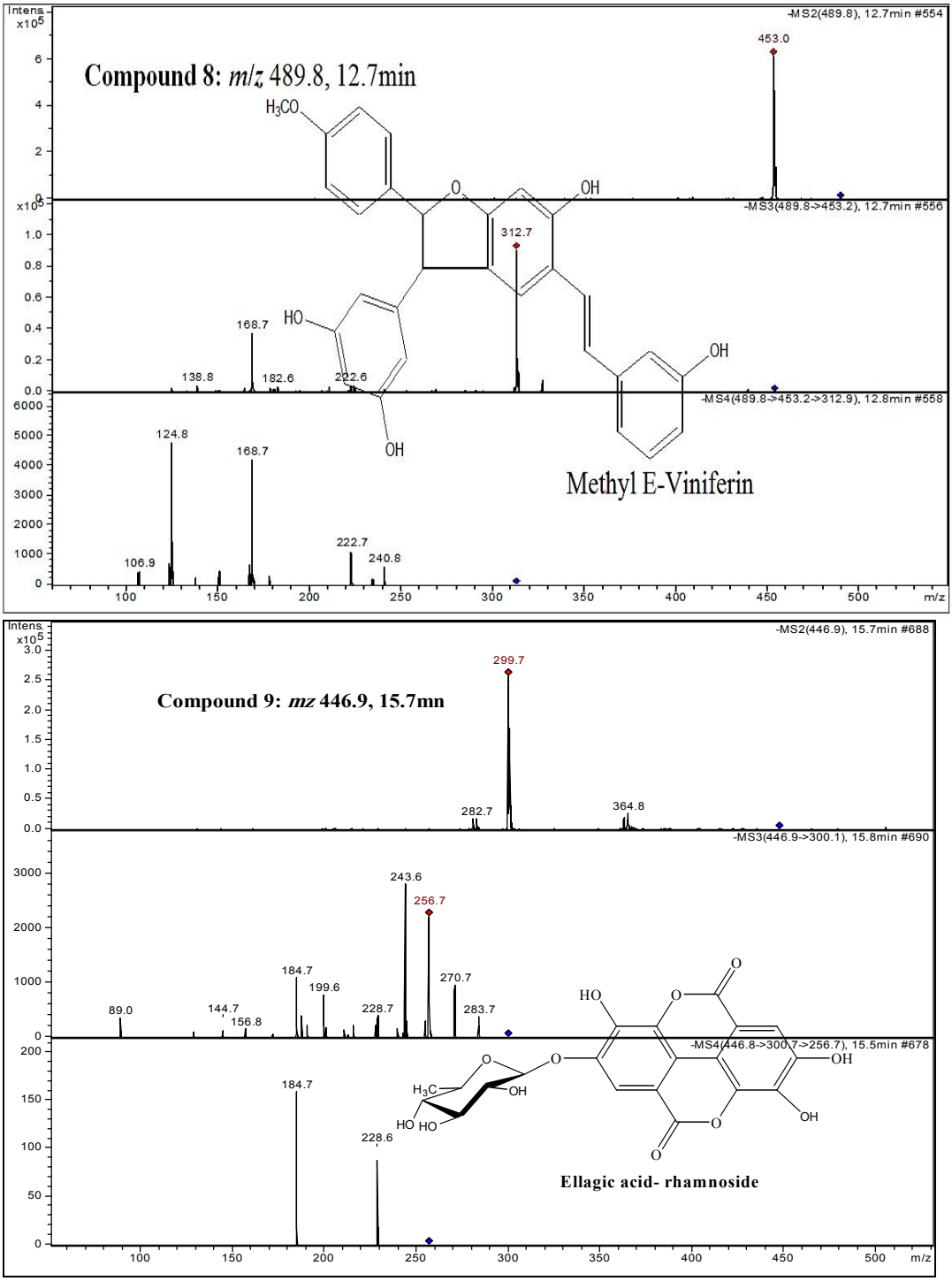


Figure: 4 (e) MS/MS (m/z) and assigned structures of compounds (8 & 9) in the chloroform fraction of *A. leiocarpus* leaves

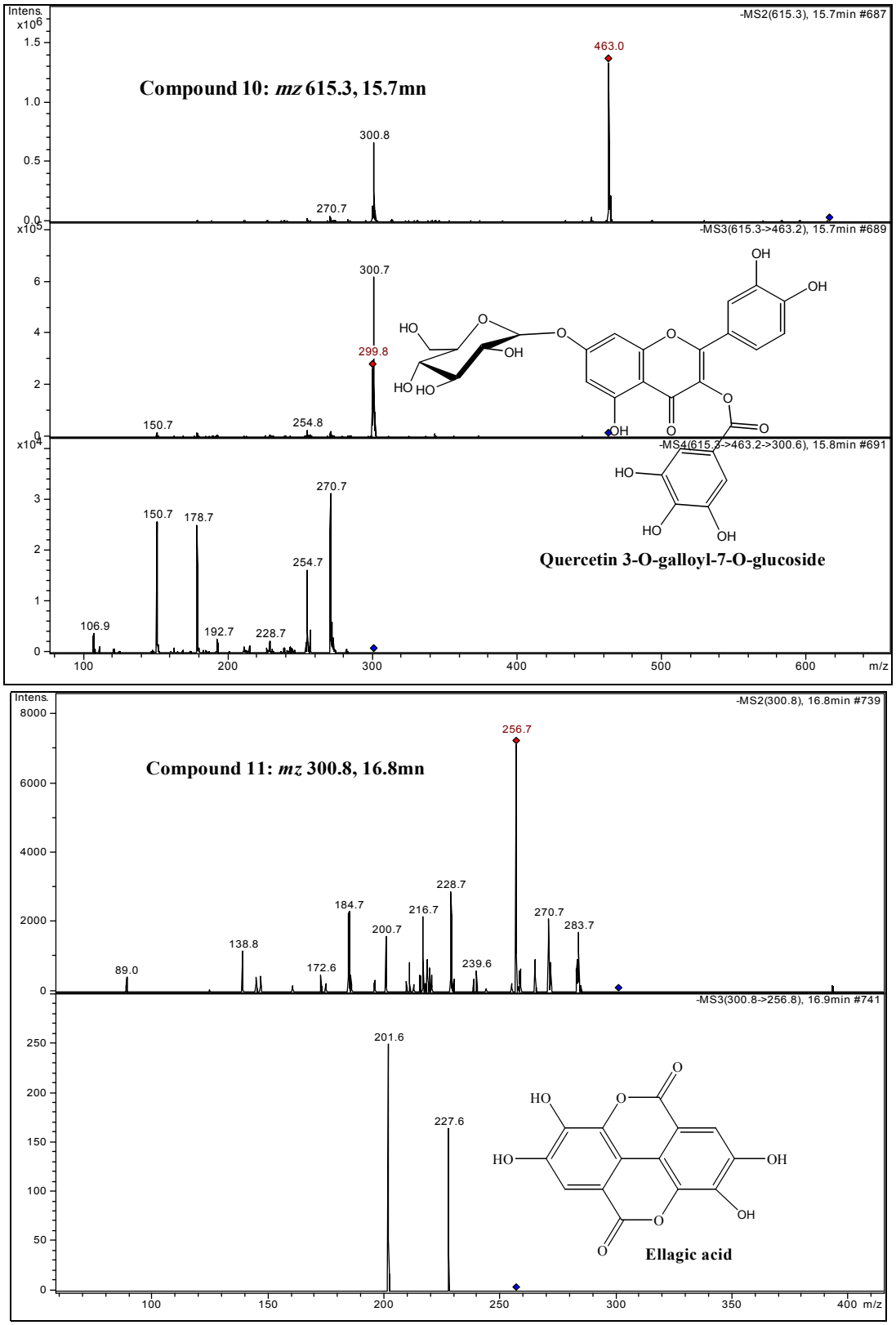
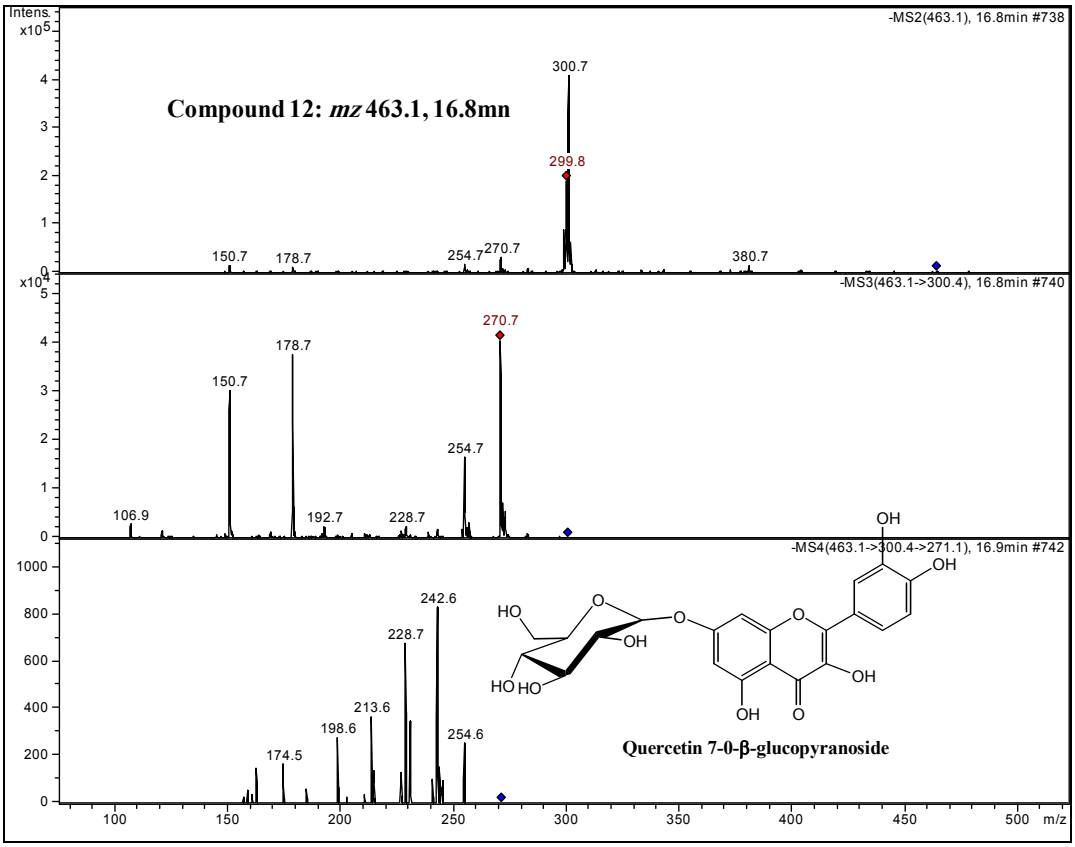


Figure: 4 (f) MS/MS (m/z) and assigned structures of compounds (10 & 11) in the chloroform fraction of *A. leiocarpus* leaves

ISSN 2321 – 2748



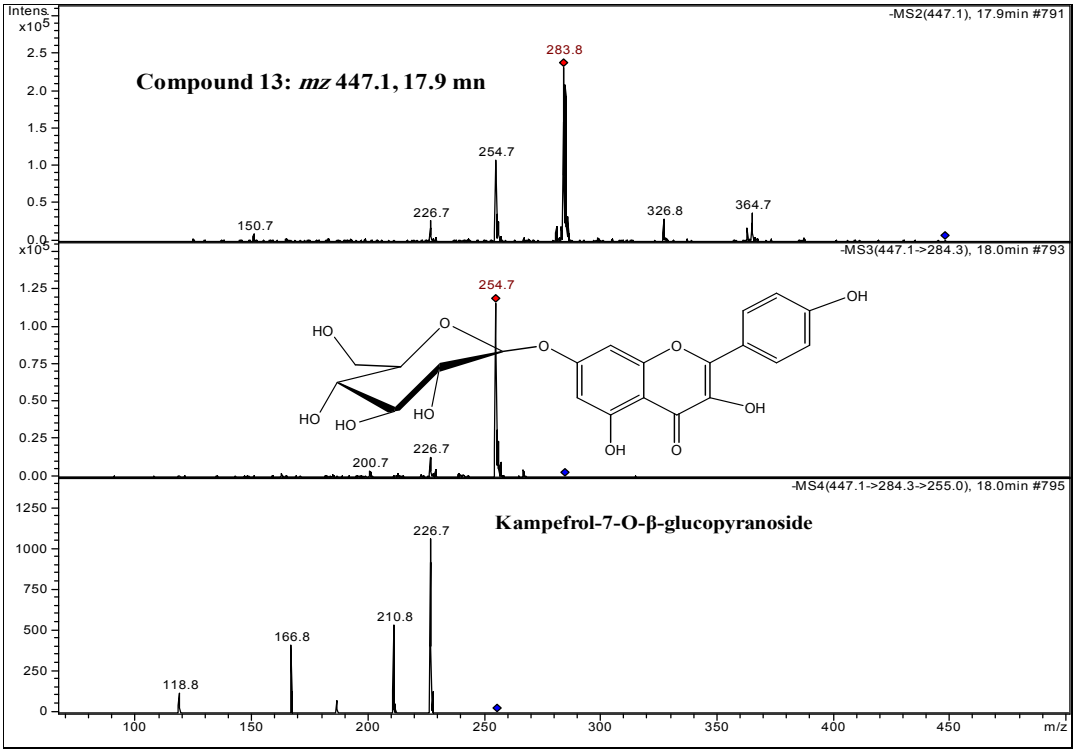
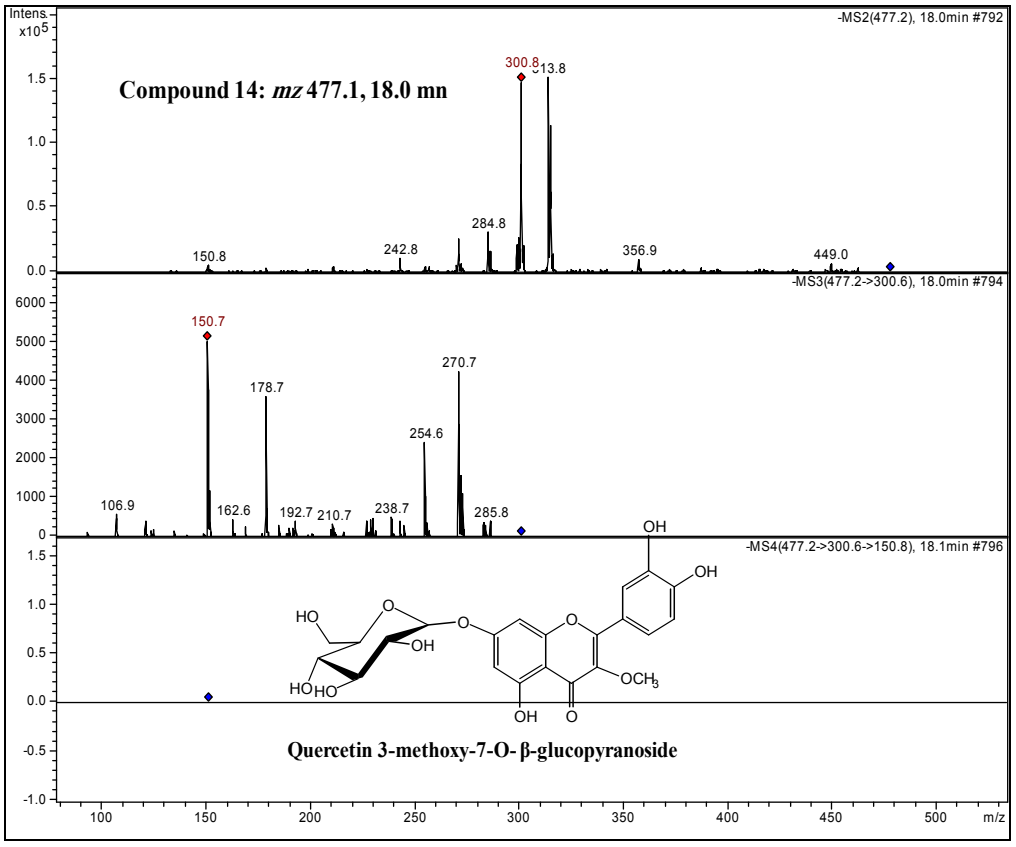


Figure: 4 (g) MS/MS (m/z) and assigned structures of compounds (12 & 13) in the chloroform fraction of *A. leiocarpus* leaves



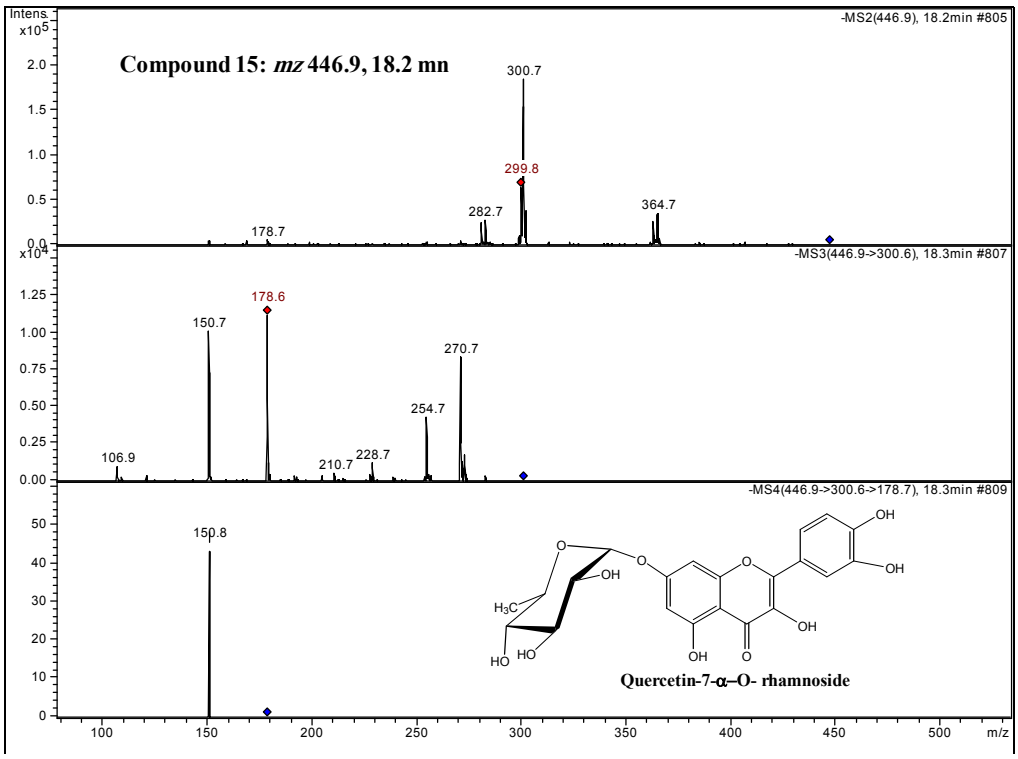
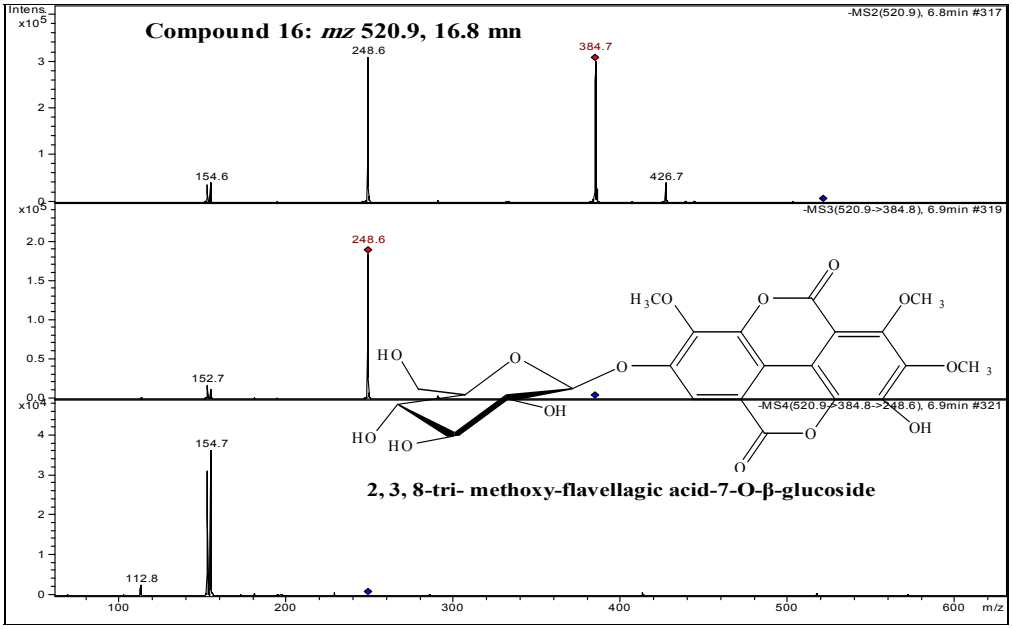


Figure: 4 (h) MS/MS (m/z) and assigned structures of compound (14 &15) in the chloroform fraction of *A. leiocarpus* leaves



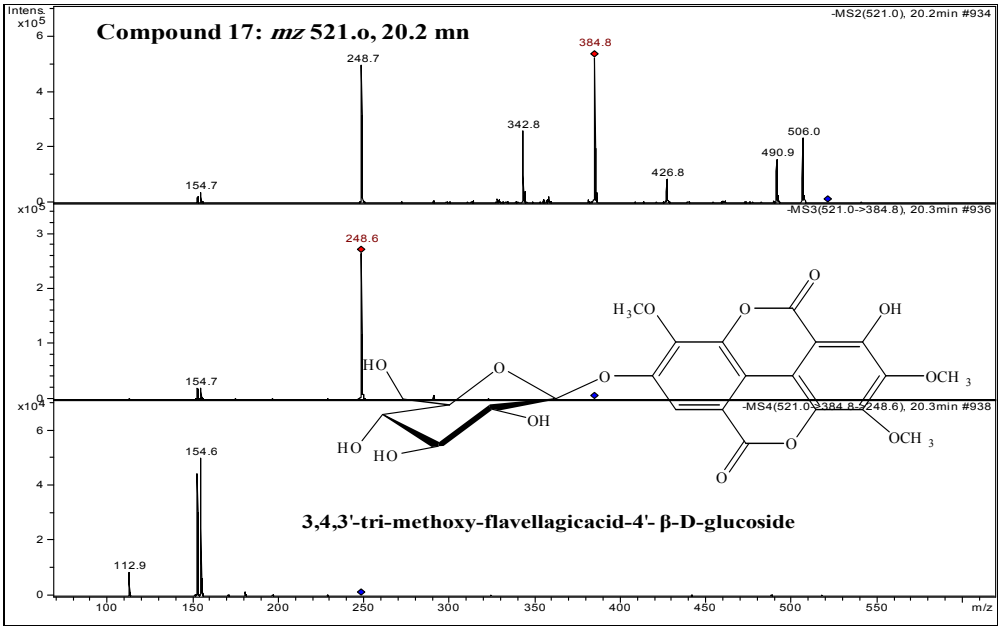
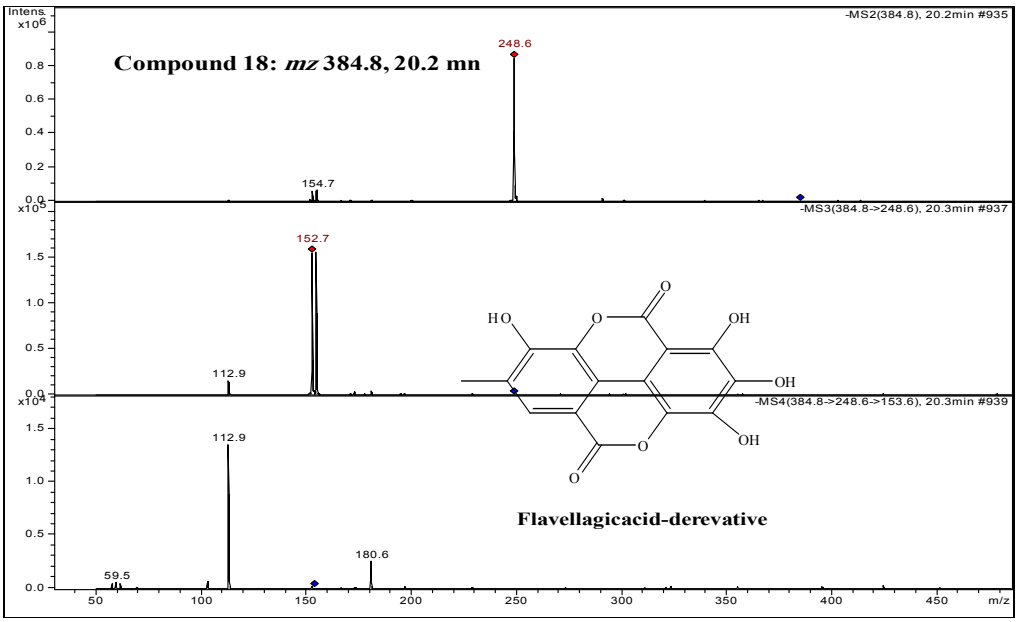


Figure: 4 (i) MS/MS (m/z) and assigned structures of compounds (16 & 17) in the chloroform fraction of *A. leiocarpus s*tem barks



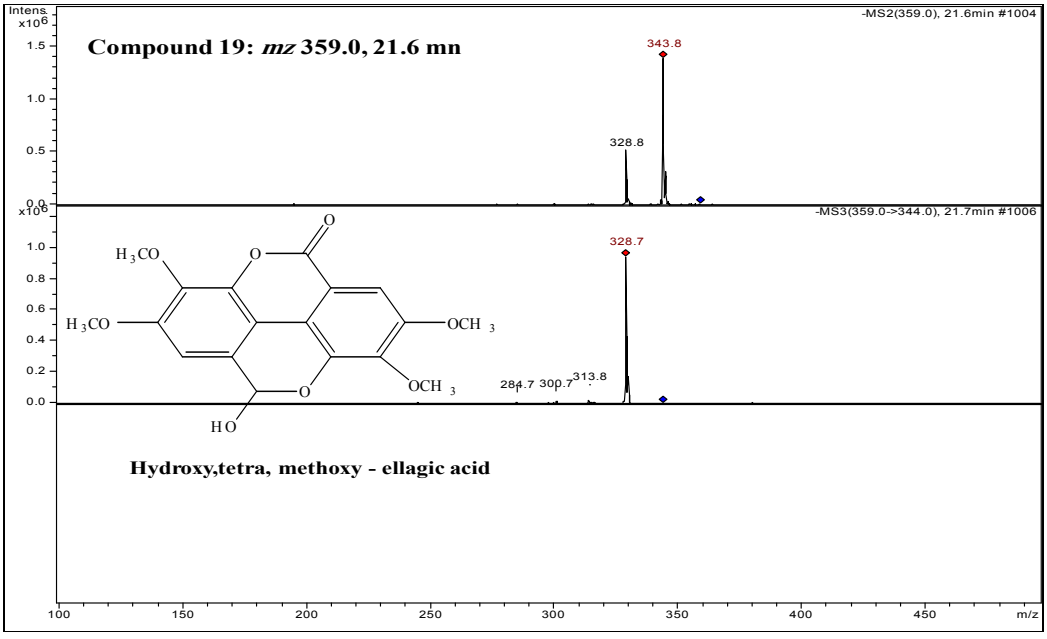
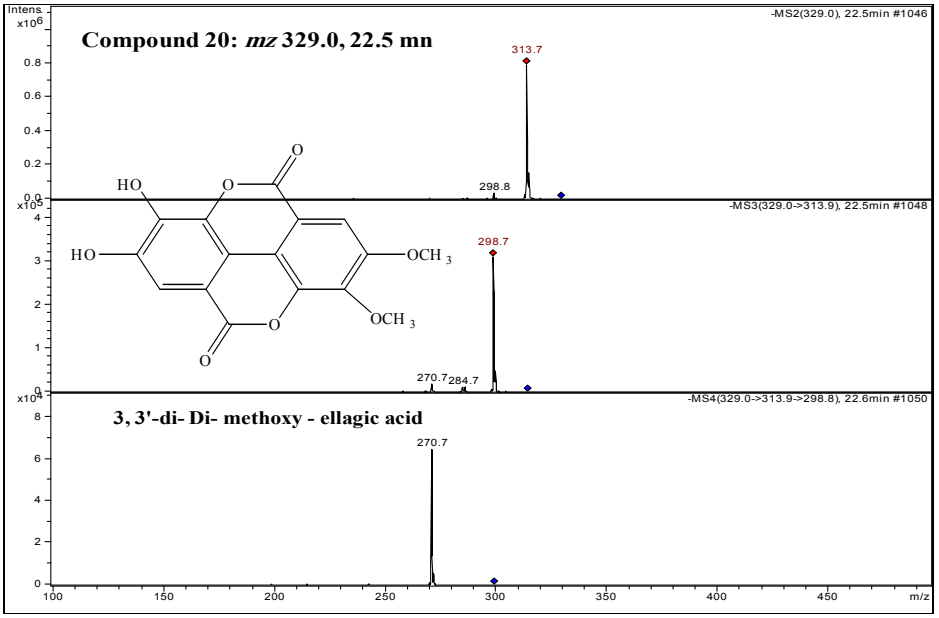


Figure 4 (j) MS/MS (m/z) and assigned structures of compounds (`18 & 19) in the chloroform fraction of *A. leiocarpus* stem barks



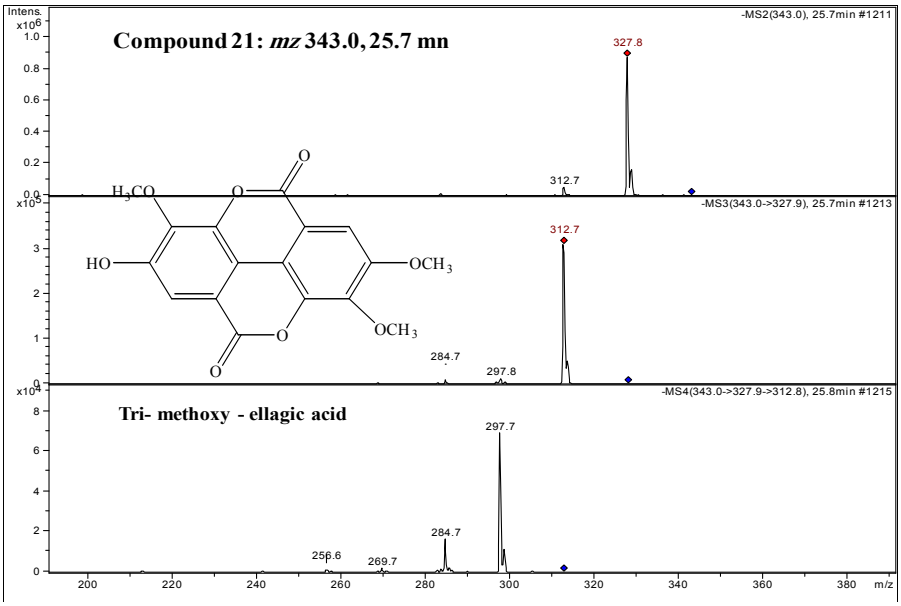
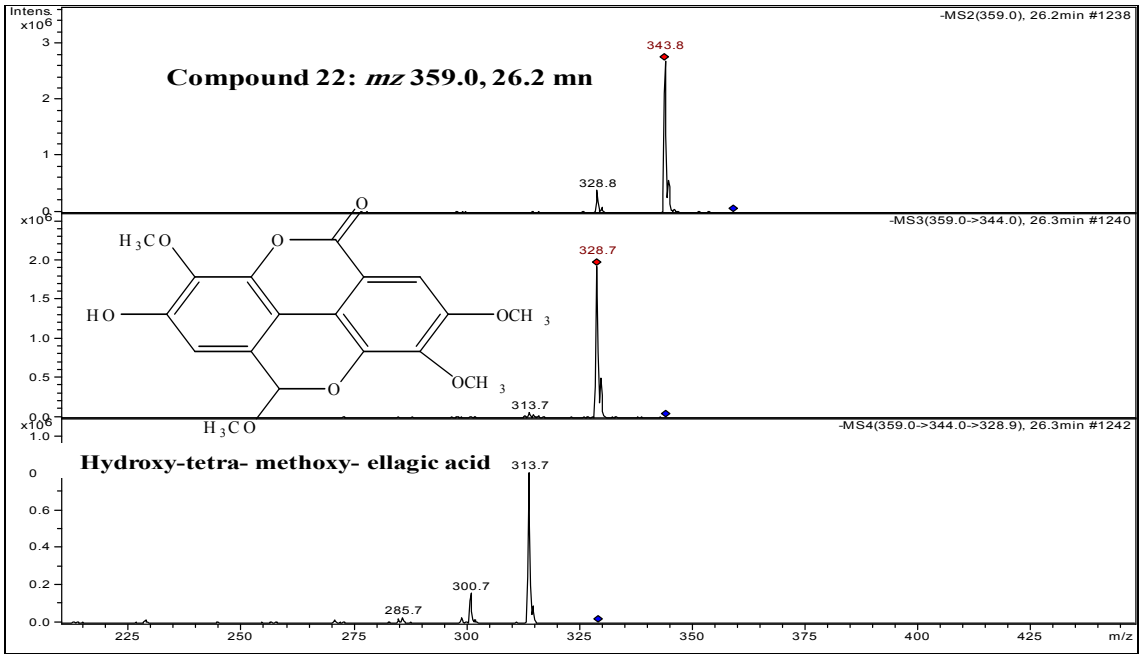


Figure: 4 (k) MS/MS (m/z) and assigned structures of compounds (20 & 21) in the chloroform fraction of *A. leiocarpus* stem barks



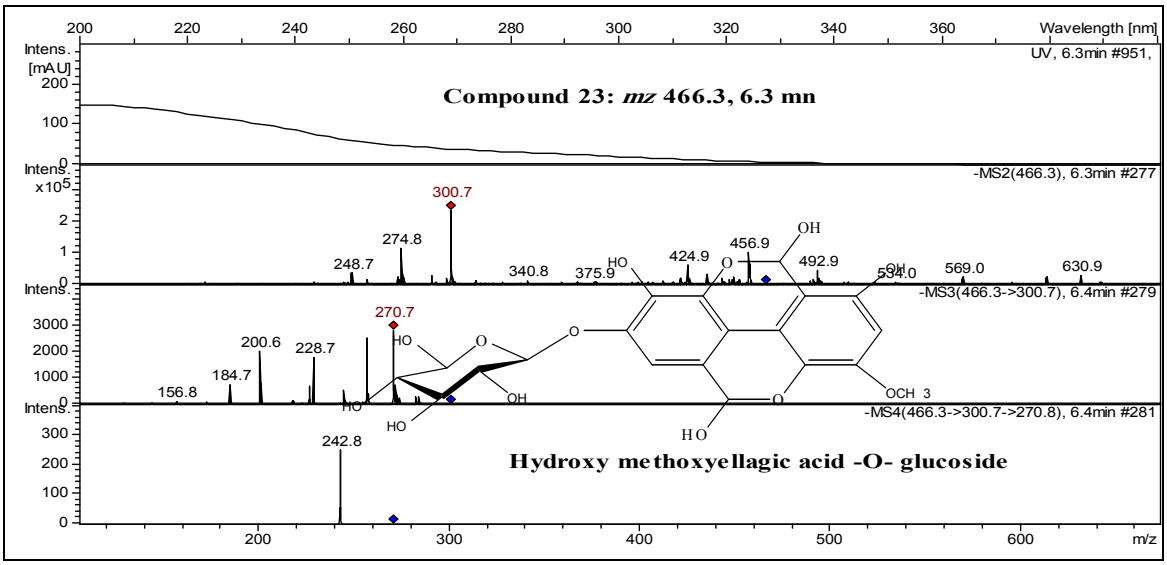


Figure: 4 (l) MS/MS (m/z) and assigned structures of compounds (22) in chloroform fraction of stem bark and (23) in the root of *A. leiocarpus*

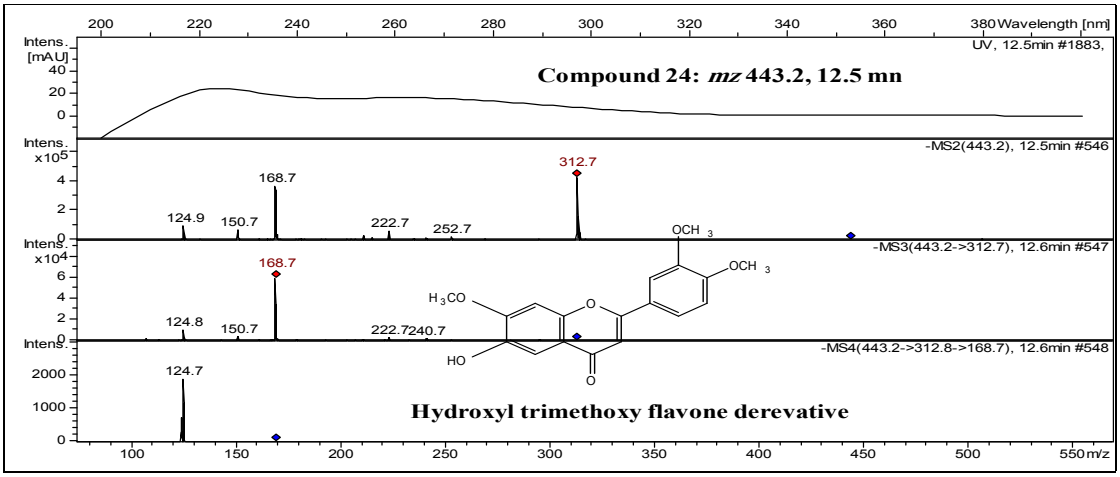
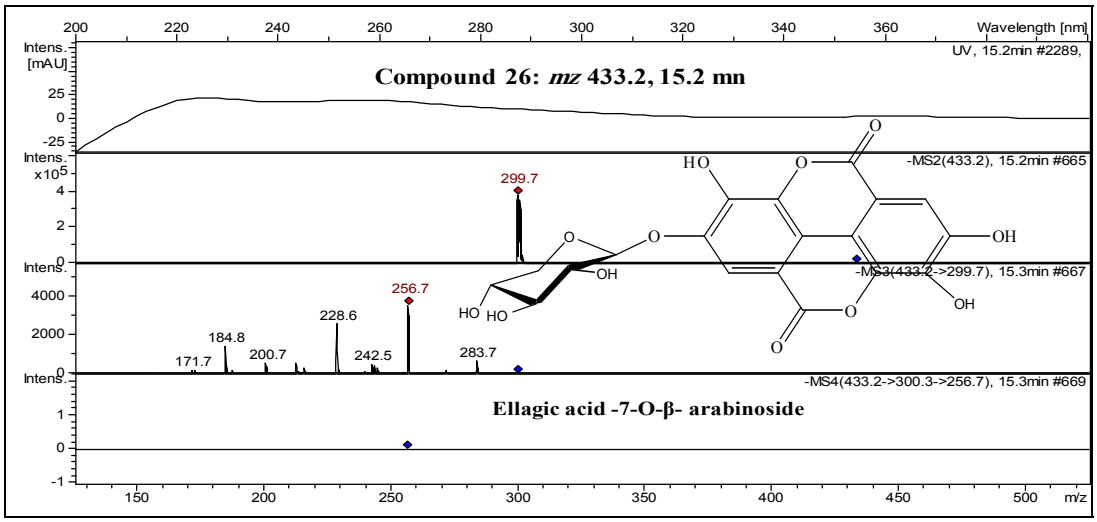


Figure: 4 (m) MS/MS (m/z) and assigned structures of compounds (24 & 25) in the chloroform fraction of *A. leiocarpus* roots



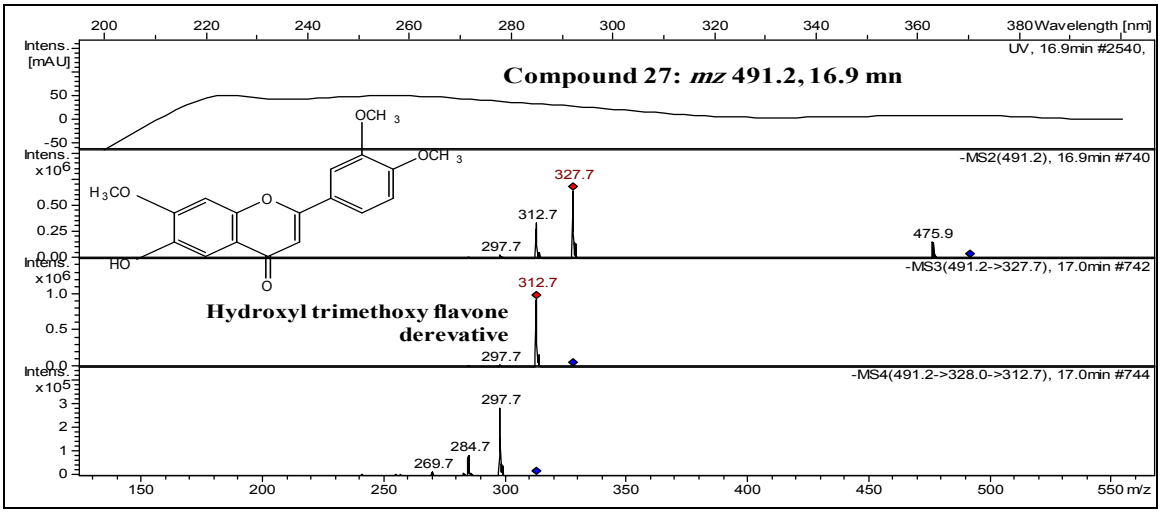
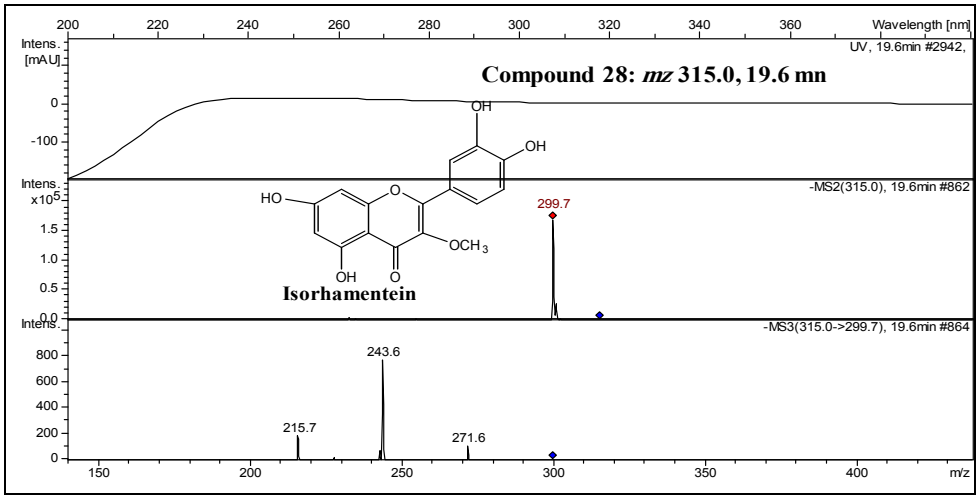


Figure: 4 (n) MS/MS (m/z) and assigned structures of compounds (26 & 27) in the chloroform fraction of *A. leiocarpus* roots



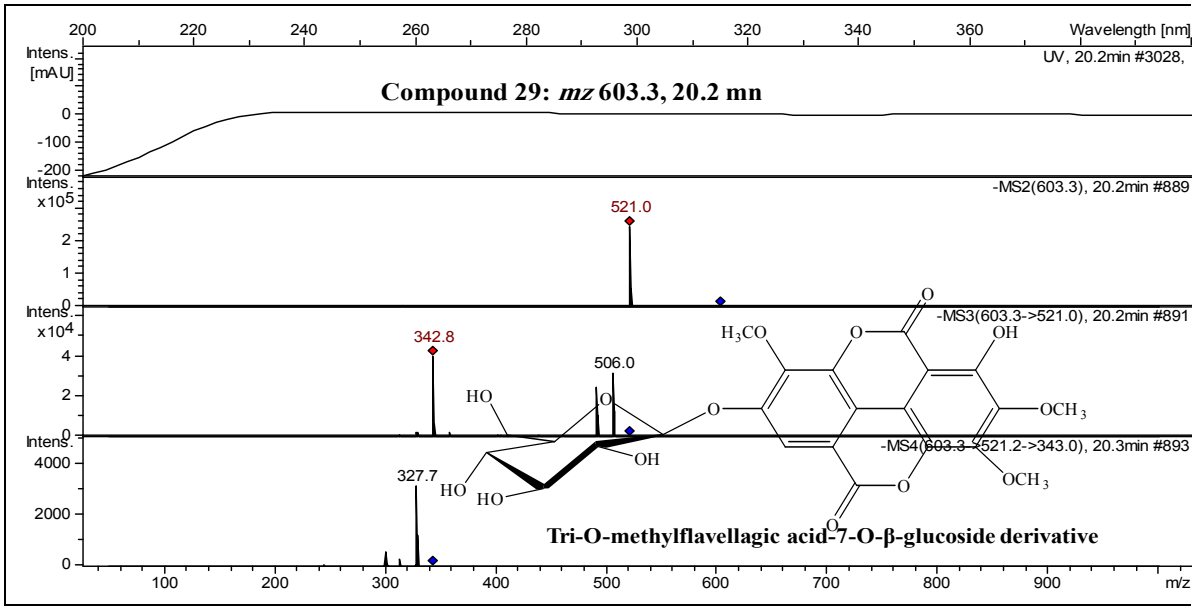
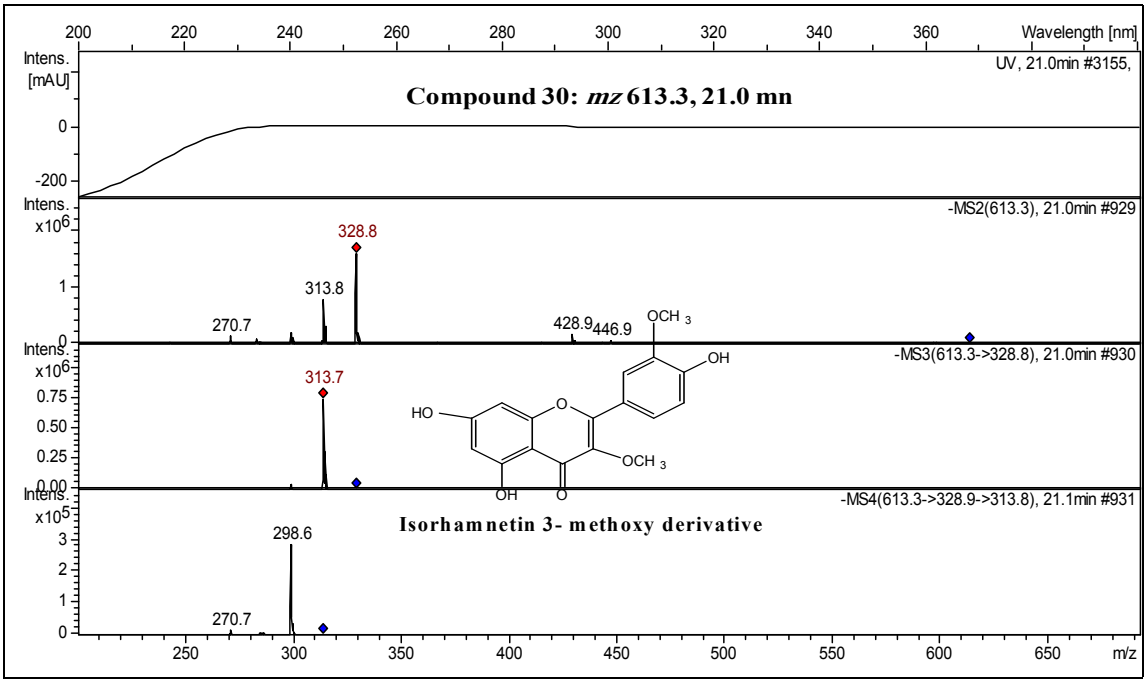


Figure: 4 (o) MS/MS (m/z) and assigned structures of compounds (28 & 29) in the chloroform fraction of *A. leiocarpus* roots

 ISSN 2321 – 2748



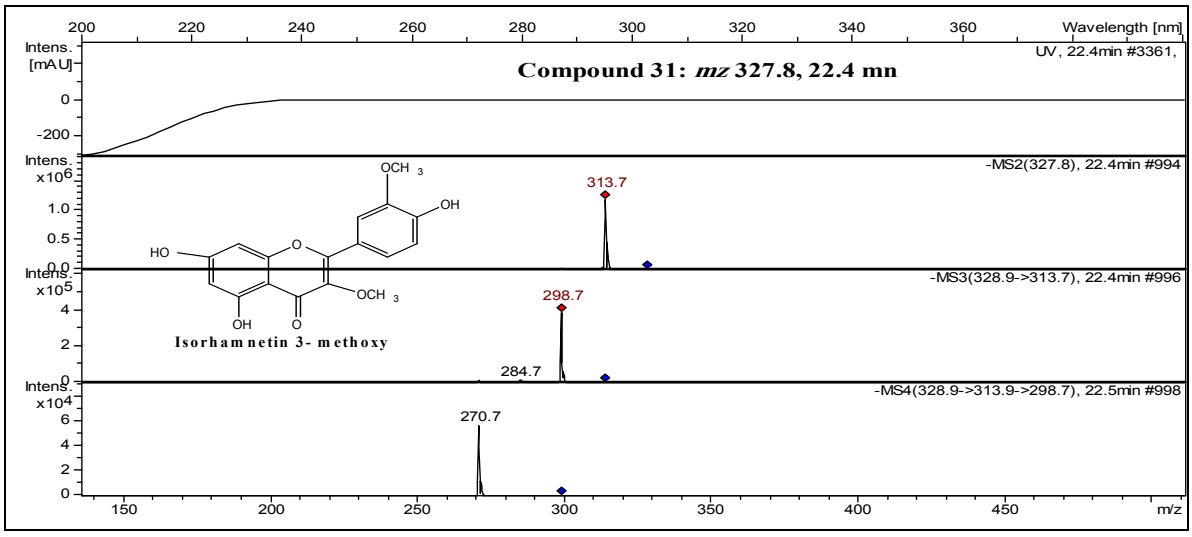


Figure: 4 (p) MS/MS (m/z) and assigned structures of compounds (30 & 31) in the chloroform fraction of *A. leiocarpus* roots