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

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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 posses’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. The family is an important resource in traditional medical practices for many human diseases, many of these indications are related to treating infections. The efficacy of the plants may be due to the presence of different classes of antimicrobial secondary metabolites.

*Anogeissus leiocarpus*, is an African evergreen tree of genus *Anogeissus* belonging to this family with different uses in traditional medicine. It mainly used in treating skin diseases and infections, wounds infections, sore feet, boils, cysts, syphilitic and diabetic ulcers. The plant was found to exhibited potent antibacterial and antifungal activity against several pathogenic microorganisms.

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.

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.

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

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 silviculture 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. MIC value is the least concentration before the spectrophotometer transmission reading is the same as or more than the initial reading. 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\textsuperscript{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\textsuperscript{44}.

The leaves extract and fractions inhibited the inoculum initial reading 0.04 at 680nm to 0.03, 0.02, 0.03, 0.02 after a week inoculated in 10 mg/ml alcoholic extract, chloroform, ethyl acetate and petroleum ether fractions respectively and to 0.03, 0.02, 0.03 when 5mg/ml is used. In comparison to the inoculum growth reading up to 0.23 in the negative control and inoculum inhibition reading to 0.03 in 5mg/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\textsuperscript{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\textsuperscript{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 at 10mg/ml. The result was compatible with the MIC of antimicrobial agents reported by Banso \textit{et al.}, 1999\textsuperscript{50}; Prescott \textit{et al.}, 2002\textsuperscript{51} and Mann \textit{et al.}, 2008b\textsuperscript{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 \textit{Anogeissus}\textsuperscript{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\textsuperscript{56, 57}.

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

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

CONCLUSIONS

The \textit{M. mycetomatis} fungus was susceptible to \textit{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, stelbenoid and flavonoids in the extracts respectively. The ellagic acid derivatives were the most potent, followed by stelbinoids and finally the flavonoids.

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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

<table>
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<tr>
<th>Compound Peak</th>
<th>(Rt) (min)</th>
<th>M-H (m/z)</th>
<th>CID M⁺ Main Fraction ions (m/z)</th>
<th>Compound Name</th>
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<td>2</td>
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<td>552</td>
<td>481, 301, 275, 271, 243</td>
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<tr>
<td>3</td>
<td>8.8</td>
<td>541</td>
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<td>4</td>
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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

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<th>(R_t) (min)</th>
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<th>Compound Name</th>
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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

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<td>16.9</td>
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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 $\lambda_{\text{max}}$ 254, 280, 300-380nm.

Figure 3(c): RP-HPLC-DAD Chromatogram of *A. leiocarpus* roots chloroform fraction recorded at $\lambda_{\text{max}}$ 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.
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* stem 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.
Figure: 4 (p) MS/MS (m/z) and assigned structures of compounds (30 & 31) in the chloroform fraction of *A. leiocarpus* roots