**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*
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&amp;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 (CHCl₃) 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 and 121°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 CCCLS 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 1ml of 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 spectrophotometrically 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 mitochondrial 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](image1.png)

**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

<table>
<thead>
<tr>
<th>Treatment (Extract/ drug)</th>
<th>Concentration</th>
<th>Extract Reading</th>
<th>Reading at zero time (Extract+ Inoculum)</th>
<th>Reading after a week (Extract+ Inoculum)</th>
<th>Inoculum Reading after a week (Final reading)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>10mg</td>
<td>1.78</td>
<td>1.82</td>
<td>0.80</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>5mg</td>
<td>0.86</td>
<td>0.90</td>
<td>0.88</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>2.5mg</td>
<td>0.41</td>
<td>0.45</td>
<td>0.45</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>1.25mg</td>
<td>0.18</td>
<td>0.22</td>
<td>0.23</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>0.62mg</td>
<td>0.07</td>
<td>0.11</td>
<td>0.17</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>0.31mg</td>
<td>0.01</td>
<td>0.05</td>
<td>0.15</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>0.00mg</td>
<td>-</td>
<td>0.04</td>
<td>0.23</td>
<td>0.23</td>
</tr>
<tr>
<td>Chloroform</td>
<td>10mg</td>
<td>1.88</td>
<td>1.92</td>
<td>1.89</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>5mg</td>
<td>0.92</td>
<td>0.96</td>
<td>0.94</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>2.5mg</td>
<td>0.44</td>
<td>0.48</td>
<td>0.46</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>1.25mg</td>
<td>0.21</td>
<td>0.25</td>
<td>0.24</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>0.62mg</td>
<td>0.09</td>
<td>0.13</td>
<td>0.13</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>0.31mg</td>
<td>0.02</td>
<td>0.06</td>
<td>0.07</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>0.00mg</td>
<td>-</td>
<td>0.04</td>
<td>0.23</td>
<td>0.23</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>10mg</td>
<td>1.66</td>
<td>1.70</td>
<td>1.69</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>5mg</td>
<td>0.81</td>
<td>0.85</td>
<td>0.84</td>
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</tr>
<tr>
<td></td>
<td>2.5mg</td>
<td>0.41</td>
<td>0.45</td>
<td>0.46</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>1.25mg</td>
<td>0.19</td>
<td>0.23</td>
<td>0.25</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>0.62mg</td>
<td>0.08</td>
<td>0.12</td>
<td>0.15</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>0.31mg</td>
<td>0.01</td>
<td>0.05</td>
<td>0.10</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>0.00mg</td>
<td>-</td>
<td>0.04</td>
<td>0.23</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>10mg</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>5mg</td>
<td>0.68</td>
<td>0.72</td>
<td>0.71</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>2.5mg</td>
<td>0.32</td>
<td>0.36</td>
<td>0.37</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>1.25mg</td>
<td>0.62mg</td>
<td>0.31mg</td>
<td>0.00mg</td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
<td></td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>0.24</td>
<td>0.10</td>
<td>0.03</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.28</td>
<td>0.14</td>
<td>0.07</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.37</td>
<td>0.26</td>
<td>0.23</td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.13</td>
<td>0.16</td>
<td>0.20</td>
<td>0.20</td>
<td></td>
</tr>
</tbody>
</table>

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

**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 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 $\lambda_{\text{max}}$ 254, 280, 300-380 nm.
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

<table>
<thead>
<tr>
<th>Compound Peak</th>
<th>(R&lt;sub&gt;t&lt;/sub&gt;) (min)</th>
<th>M-H (m/z)</th>
<th>CID M&lt;sup&gt;n&lt;/sup&gt; Main Fraction ions (m/z)</th>
<th>Compound Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.8</td>
<td>521</td>
<td>506, 491, 385, 342.8, 249, 249, 155, 155, 155, 113</td>
<td>2, 3, 8-Tri-methoxy-flavellagic acid-7-β-O-glucoside</td>
</tr>
<tr>
<td>2</td>
<td>20.2</td>
<td>521</td>
<td>506, 385, 342.8, 249, 249, 155, 155, 113</td>
<td>3,4,3'-Tri-methoxy-flavellagic acid-4'-β-D-glucoside</td>
</tr>
<tr>
<td>3</td>
<td>20.2</td>
<td>384.8</td>
<td>248.6, 154.7, 152.7, 112.9, 112.9</td>
<td>Flavellagic acid derivative</td>
</tr>
<tr>
<td>4</td>
<td>21.6</td>
<td>359.0</td>
<td>343.8, 329, 14, 314, 300, 285</td>
<td>Hydroxy, Tetra-methoxy-ellagic acid</td>
</tr>
<tr>
<td>5</td>
<td>22.5</td>
<td>329</td>
<td>314, 299, 299, 285, 271, 271</td>
<td>3, 3'-Di-methoxy-ellagic acid</td>
</tr>
<tr>
<td>6</td>
<td>25.7</td>
<td>343</td>
<td>328, 313, 313, 298, 298, 285, 270</td>
<td>Tri-methoxy-ellagic acid</td>
</tr>
<tr>
<td>7</td>
<td>26.2</td>
<td>359</td>
<td>344, 329, 314, 314, 300, 285</td>
<td>Tetra-methoxy-ellagic acid</td>
</tr>
</tbody>
</table>
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.

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

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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.

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