ANTI-SALMONELLA ACTIVITY AND CYTOTOXICITY STUDIES ON THE METHANOLIC LEAF EXTRACT OF SWIETENIA MAHAGONI

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ABSTRACT
Swietenia mahagoni (L.) Jacq.is a medicinal tree species from Meliaceae with numerous pharmacological and ethnomedical activities. The methanolic seed extracts of this plant species which are rich in triterpenoids have shown good activity against Salmonella Typhi and S. paratyphi. Hence, the less explored methanolic extract of the leaf parts of S mahagoni was chosen to study its antibacterial activity against Salmonella Typhi. Disc diffusion and thin layer agar diffusion assays were performed for in-vitro screening of the crude methanolic leaf extract followed by bioautography to detect the bioactive principles in the extract. The methanolic extract showed a good inhibition of 7.25, 8 & 8.5 mm at 500, 750 & 1000 µg/mL (15 µg/well) against S Typhi in thin layer agar diffusion method. The MIC for the crude methanolic leaf extract was determined as 4.37 mg/ml. The bioautography for screening the bioactive components in methanolic leaf extract revealed the presence of 7 bioactive compounds at various Rf provides the reason for the activity of this methanolic leaf extract. Cytotoxic potential of the crude extract was evaluated using Brine Shrimp Lethality Bioassay which showed a very low LC50 value of 13.59 µg/ml. On an overall, the methanolic leaf extract of Swietenia mahagoni tested for its antisalmonella activity showed a good concentration dependent potency against Salmonella Typhi with less cytotoxicity.
INTRODUCTION

Swietenia mahagoni (L.) Jacq. (Meliaceae) is a medicinal tree species with various pharmacological and ethnomedical activities. The extracts of S mahagoni were reported in treating hypertension, malaria and diabetes as folk medicine [1] and possess various therapeutic activities like anti-feedant activity [2], anti-microbial activity [3-6], inhibition of platelet aggregation [7], anti-HIV protease activity [8] and pharmacological activity including anti-inflammatory activity [9].

Various extracts from different parts of this tree have been used to isolate novel compounds and analyze their bioactivity. Some of the compounds purified from the leaves of this tree include several limonoids; swietephragmins (A-G), 2-hydroxy-3-O-tigloylswietenolide and deacetylscomahoganin, Swietenolide A-E, Swiemahogins A & B [10, 11]. The other therapeutically important tetranortriterpenoids like Swietemahonin A-G and fatty acids have also been isolated from this tree species [12].

There are many number of reports on the methanolic seed extract of this plant characterized [3, 6, 15, 16]. The findings from Shahidur et al., [14] has reported the presence of 2 compounds, swietenolide and 2-hydroxy-3-O-tigloylswietenolide with good bioactivity against Salmonella Typhi and S. paratyphi. But there were not many reports on the bioactivity of methanolic leaf extract of this plant except for one from Goun et al.,2003 [3] stating no inhibitory activity of methanolic leaf extract against all the tested organisms (B. subtilis, B. megaterium, E. coli, X. campestris). Exploring the less explored methanolic leaf extract of this plant forms the main objective of this current study.

One of the medically important pathogen belonging to Enterobactericeae family that causes typhoid and diarrhea is Salmonella Typhi (WHO). According to the reports from National Institute of Health (NIH), typhoid is one of the major fevers that affect children and adult with high incidence rate. Recently S Typhi has been shown to acquire multidrug resistance [13] and the treatment requires novel compounds with less toxicity and side-effects. Hence, this in vitro study was aimed at screening the less explored methanolic leaf extract of Swietenia mahagoni for antibacterial activity against Salmonella Typhi using various methods and also to evaluate its cytotoxicity using brine shrimp lethality assay.

MATERIALS AND METHODS

Plant material collection and extraction [4]:

The fresh leaves were collected from, Patna, India in 2004. Authentication of the sample was obtained from Prof (Retd.) P. Jayaraman, Director, Institute of Herbal Botany, Plant Anatomy Research Centre, West Tambaram, Chennai-45. The leaves were washed, sun dried and ground. The ground leaf powder (500 g) was extracted with hexane followed by methanol. The methanolic extract was then concentrated using rotary evaporator and used for biological assays.

Antibacterial Studies

The antibacterial activity of the methanolic leaf extract of Swietenia mahagoni was tested against Salmonella Typhi using both conventional disc diffusion assay and the new and improved thin layer agar diffusion assay. The Minimum Inhibitory Concentration (MIC) was determined using microbroth dilution method. And the bioactive ingredients present in the methanolic extract were detected using bioautography assay.

Disc diffusion assay [17]:

A stock concentration of 10 mg/mL of the methanolic leaf extract of S mahagoni to be tested against S Typhi was prepared in methanol. Fifty microlitres of this extract was loaded on sterile paper disc (Hi-media) to obtain a concentration of 500 µg/disc and air-dried in an aseptic hood. The discs were then placed on the culture streaked Muller Hinton agar (MHA) plates and incubated at 37° C for 17 hrs. The anti-bacterial activity was determined by measuring the respective zone of inhibition in millimeters. The results were then compared with standard antibiotic - Ciprofloxacin (90 µg/disc) and methanol serving as negative control.

Thin Layer Agar Diffusion Assay [18]:

The freshly prepared Muller Hinton Agar (1% agarose for visible clarity) with bearable warmth was seeded with 0.5 OD culture of S. Typhi and a uniform thin layer of the agar (~10-12 mL/plate) was prepared in Tarsons petridish. The medium was allowed for solidification and wells were cut (~2.5 mm diameter) at equal distance. From a stock concentration of 35 mg/mL methanolic extract, the test concentrations of 500, 750 & 1000 µg/mL were prepared in methanol. A test volume of 15 µl from different test concentrations of the methanolic extract was added in respective wells (the ideal concentration being 7.5, 11.25 and 15 µg/well). To compare the activity, 15 µl of the standard antibiotic - ciprofloxacin 90 µg/mL (1.35 µg/well) and methanol were added as controls. The experiment was carried out in duplicates and the plates were incubated at 37° C for 17 hrs. The antibacterial activity of the extract was then determined by measuring respective inhibition zones.
MIC - Microbroth Dilution Method:
The MIC of the methanolic leaf extract of *Swietenia mahagoni* towards *Salmonella Typhi* was determined after finding its sensitivity by agar diffusion assays. In this method, the microbroth dilution technique was utilized where the plant extract was prepared to the highest concentration of 35 mg/mL (stock concentration) and serially diluted (two-fold) to a working concentration ranging from 17.5 mg/mL to 0.06 mg/mL using MH broth in a 96 well titre plate. An inoculum of 0.1 OD S. Typhi was prepared from 16 hrs broth culture and 100 µl of this culture was inoculated into each well containing 100 µl of serially diluted methanolic extract. The positive control was culture inoculated MH broth with the antibiotic ciprofloxacin (250 µg/mL) and negative control was culture inoculated MH broth. To all the wells 10 µl of 0.1% TTC (2, 3, 5- Triphenyltetrazolium chloride) was added finally and the experiment was carried out in triplicates. The microtitre plate was then incubated at 37ºC overnight. The lowest concentration of the extract that has the ability to inhibit visible growth of the microorganism (due to the addition of TTC) after overnight incubation is termed as MIC.

Bioautography [19]:
Bioautography is the method of choice for activity guided fractionation of antimicrobial compounds from plants. This is because it allows the combination of a bioassay in situ and, at the same time, localization of active constituents on the TLC plate employed for the assay. The Bioautography of methanolic leaf extract for *S. Typhi* was performed according to the method described by Hamburger and Cordell (1987) [19] with some modifications. Thin layer chromatographic strip spotted with methanolic leaf extract was developed in a solvent system of hexane: ethyl acetate (6:4). The TLC strip was then air-dried and subjected to a simple agar overlay assay seeded with *S. Typhi* for the detection of bioactive components in the extract. The TLC strip was placed in sterile petriplate and incubated at 37ºC for 17 hrs. After incubation the strip was flooded with 0.1% solution of TTC dye and left undisturbed for the formazan to be produced and then the white inhibition zones were observed against a pink background. The Rf of the inhibition zones were then recorded by comparing with TLC strips visualized under visible light, long UV and Short UV.

Cytotoxicity Screening using Brine shrimp Lethality test (BSLT)
The brine shrimp cytotoxicity assay has been used for preliminary assessment of toxicity, detection of fungal toxins, heavy metals, pesticides and cytotoxicity testing of dental materials. The brine shrimp assay is very useful tool for isolation of bioactive compounds from plant extracts [20]. The method is attractive because it is very simple, inexpensive and low toxin amounts are sufficient to perform the test in reduced volumes.

The cytotoxic properties of the crude methanolic leaf extracts of *Swietenia mahagoni* was tested using brine shrimp lethality bioassay. Brine shrimp cysts of 100 mg were initially soaked in tap water for about an hour and later transferred to 500 mL sea water in a separating funnel. A continuous illumination and strong aeration at 28ºC were provided for better hatching. After 12 hrs, the hatched phototropic nauplii were collected and concentrated in a small vial.

Stock solution of the methanolic leaf extract was prepared by dissolving 10 mg of the extract in 1 mL of methanol to obtain a stock of 10,000 µg/mL. From the stock concentration, five different test concentrations were prepared (500, 250, 100, 50, 10 µg/mL). Form each test concentration, 480µL of extract was added into respective wells of a 24 well culture plate and left to evaporate. The evaporated extracts were re-dissolved in 480 µL (1:5 of total volume) of 0.5 % aqueous DMSO. For control, 480 µL of methanol was evaporated and re-dissolved in 480 µL of 0.5 % aqueous DMSO. Ten brine shrimps were selected at each well using a Pasteur pipette and sea water was added to each well and the volume was made up to 2.4 mL.

The toxicity was determined after 24 hr (nauplii in instar II/III) and 48 h (mainly nauplii in instar III/IV) of exposure. The number of survivors were counted and percentage of dead was calculated. Larvae were considered dead if they did not exhibit any internal or external movement during 10 to 15 seconds of observation.

Potassium dichromate was used as the positive control and bioassay was performed for five different concentrations (1, 5, 10, 20, 30 µg/mL) to evaluate its LC50. From the lethality of brine shrimp, the probits analysis was done for each concentration by using the software “SPSS” version 16. The log concentrations obtained were plotted against corresponding Probits of mortality of potassium dichromate to get LC50 (lethality concentration at 50%) value through regression analysis.

Statistical Analysis
Data were obtained as percentage of mortality of the ten shrimps tested per concentration with triplicates. LC50 values were determined by Probit Analysis by using Statistical Package for Social Sciences (SPSS) version 16.

RESULTS AND DISCUSSION

Antibacterial activity
The prevalence and emergence of multidrug resistance *Salmonella Typhi* in India is a concern and hence there is a need for identifying and isolating novel compounds for the treatment of these multidrug resistant organisms. Hence, this work was initiated as a preliminary screen for crude extract with inhibitory activity towards S Typhi, which can be used for isolating the bioactive components in future. The methanolic leaf extract of *Swietenia mahagoni* was tested against the human pathogen *Salmonella Typhi* for the first time.
Disc diffusion & Thin layer agar diffusion assay

The test concentration of 500 µg/disc showed good inhibition (14mm) of bacterial growth in the disc diffusion assay (figure I.A). The thin layer agar diffusion assay performed for different concentrations of methanolic extract of 500, 750 and 1000 µg/mL showed increase in the zone of inhibition with increase in extract concentration (figure I.B). With respect to the concentration of extract in each well (7.5, 11.25 and 15 µg/well), the zone of inhibition obtained were 7.25, 8 and 8.5 mm in diameter. The inhibition zones for the test concentrations of extract in respective wells clearly shows the sensitivity of this particular thin layer agar diffusion assay over the conventional disc diffusion assay. The antibiotic control ciprofloxacin, 90 µg/mL (1.35 µg/well) showed 15mm inhibition zone and solvent control methanol showed no inhibition against S. Typhi.

Figure I: Anti-salmonella activity of Methanolic leaf extract of Swietenia mahagoni. (A) Disc diffusion assay showing zone of inhibition for methanolic extract against S. Typhi; (B) Thin layer agar diffusion assay for methanolic extract showing dose dependent inhibition; (C) Linearity graph for different concentrations of extract by thin layer agar diffusion assay

The methanolic seed extract tested by Sahgal and co-workers in 2009 [6] has been reported to have inhibitory activity against Salmonella Typhi but there was no linearity in the inhibition zones among the test concentrations. But our findings showed a linearity in inhibition with increase in the extract concentration (figure I.C).

Minimum Inhibitory Concentration (MIC)

The MIC of the methanolic crude extract was determined as 4.37 mg/mL, which showed no pink color formation when observed visually. The lower concentrations of the extract (2.18 to 0.06 mg/mL) showed bacterial growth with pink coloration. The antibiotic control ciprofloxacin 250 µg/mL showed no bacterial growth indicating the susceptibility of S. Typhi.

Bioautography

The methanolic leaf extract loaded TLC strip subjected for bioautography against S. Typhi showed clear inhibition zones from Rf 0.03 to 0.6. The Rf of the inhibition zones where compared with the extract developed TLC strips visualized under visible light, short UV (254nm) and long UV (366nm). The compounds at Rf 0.03, 0.12, 0.20, 0.28, 0.49, 0.59 and 0.6 fluoresced under 366nm and all these compounds showed inhibition in the growth of Salmonella Typhi on culture coated TLC strips (figure II). And the inhibition was observed as white zones against pink background due to flooding of 0.1% TTC.

The results of this assay for screening for bioactive components in methanolic leaf extract of S. mahagoni revealed the presence of 7 bioactive compounds with anti-salmonella potential at various Rf. The reports by Shahidur et al., 2009 [14] on the presence of swietenolide and 2-hydroxy-3-O-tigloylswietenolide in methanolic seed extract substantiates our present results on the strong anti-salmonella activity of methanolic leaf extract. The presence of these potent compounds could have been the reason for good anti-salmonella activity in methanolic leaf extract of this plant.
Figure II: Phytochemical and Bioautography profile for methanolic leaf extract of *Swietenia mahagoni*.

**Cytotoxicity screening- Brine shrimp lethality test (BSLT)**

Brine shrimp larvae have been used as a model in bioassay for evaluating the cytotoxicity of toxic substances. The method has also been applied to plant extracts in order to facilitate the isolation of biologically active compounds (21-23). The potassium dichromate was used as control and the LC$_{50}$ value of potassium dichromate was found to be 3.658 μg/mL (Table I) with 95% confidence limit. Also the lower and upper limits were found to be 1.769 and 5.942 μg/mL respectively.

BSLT at 24 hrs showed proportionate increase in lethality with increasing concentrations. The concentration 500 μg/mL which was the highest concentration showed 100% lethality at both 24 and 48 hrs. For the concentration of 250 μg/mL, 100% lethality was observed at 48 hrs and at 24 hrs it was only 80%. The lower test concentrations also exhibited increased lethality at 48 hrs compared to lethality at 24 hrs, this variation could have been stimulated by external stimuli at 48 hr (III & IV instar). Hence the LC$_{50}$ value was evaluated for 48 hr observation and was found to be 13.59 μg/mL (denoted as ‘x’ inside blue square) as shown in figure III.

**Table I: Brine shrimp toxicity expressed as LC$_{50}$ value.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>LC$_{50}$ (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanolic leaf extract of <em>S mahagoni</em> (48hrs)</td>
<td>13.59</td>
</tr>
<tr>
<td>Potassium dichromate (48 hrs)</td>
<td>3.658</td>
</tr>
</tbody>
</table>

Figure III: Cytotoxicity of methanolic leaf extract of *S mahagoni* - Brine Shrimp.
CONCLUSION

The methanolic leaf extract of *Swietenia mahagoni* was evaluated for its anti-salmonella activity. The extract exhibited activity towards *S. Typhi* at a concentration of 500 µg/disc showing 14 mm inhibitory zone in disc diffusion assay. A linearity in the inhibition zones were observed with increasing concentrations of the extract in thin layer agar diffusion assay. The MIC of the extract was determined as 4.37 mg/mL and the cytotoxic evaluation of the extract with brine shrimp model exhibited a very low LD₅₀ of 13.59µg/mL. The reason for good potency of the methanolic leaf extract towards *Salmonella Typhi* could be due to the presence of seven bioactive components at various R₅ (0.03 to 0.6) revealed in the bioautography profile. On an overall, the methanolic leaf extract of *Swietenia mahagoni* tested for its antimalarial activity showed good potency against *Salmonella Typhi* with less cytotoxicity. The active principles present in the methanolic leaf extract when studied in a systematic manner towards *Salmonella Typhi* might hold a promising future to enter the drug pipeline.

Conflict of interests

The authors declare no conflict of interest.

REFERENCES