Antimicrobial Activity, Toxicity and Phytochemical Screening of Four Medicinal Plants Traditionally Used in Msambweni District, Kenya

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Abstract
This study was designed to evaluate the antimicrobial activity, toxicity and phytochemical composition of organic and aqueous crude extracts of Zanthoxylum chalybeum Engl. (Rutaceae), Adansonia digitata L. (Bombacaceae), Launaea cornuta (Hocht. ex Oliv. & Hern) C.Jeffrey (Compositae) and Grewia trichocarpa Hochst. ex A.Rich (Tiliaceae) traditionally used by local communities of Msambweni District in Kenya. Aqueous and organic [Chloroform: Methanol (1: 1)] crude extracts were evaluated for their in vitro antimicrobial activity against Methicillin resistant Staphylococcus aureus (MRSA), Bacillus cereus, Escherichia coli, Pseudomonas aeruginosa and Candida albicans using broth dilution and disc diffusion methods. Toxicity was determined using Brine-shrimp larvae (Artemia salina L. nauplii) assay. The crude extracts were screened to determine the presence of flavonoids, alkaloids, saponins and sesquiterpene lactones using standard techniques. It was observed that the organic crude extracts from all the species tested except L. cornuta exhibited dose dependent activity against B. cereus, MRSA, P. aeruginosa and C. albicans. None of the crude extracts showed any inhibition against E. coli. Adansonia digitata and Grewia trichocarpa both had LC$_{50}$>1000 µg/ml and were shown to be non-toxic to Brine shrimp larvae unlike those of Z. chalybeum and L. cornuta which both had LC$_{50}$<500 µg/ml and were considered to be toxic. Phytochemical screening of the crude extracts showed that alkaloids, flavonoids, sesquiterpene lactones and saponins were present in the four plants tested. The study has shown that A. digitata and Z. chalybeum possess promising antimicrobial activity against microbes of health importance and could lead to the isolation of new and potentially effective antimicrobial compounds.

Keywords: Medicinal plants; Antimicrobial activity; Brine shrimp lethality test; Phytochemical analysis; Msambweni district; Kenya.

1. Introduction
A two-fold increase in microbial resistance against antibiotics has developed over the last forty years in both medical and livestock sector (Daboor and Haroon, 2013). Microbial infections have caused a big burden of diseases and bacteria are listed in the first position among common microorganisms responsible for opportunistic diseases associated with HIV/AIDS (Rathee et al., 2012). Increased antibiotic resistance has become a global concern, coupled with the problem of microbial persistence, thus highlighting the need to develop novel antimicrobial drugs that are not only active against drug resistant microbes, but more importantly, kill persistent micro-organisms and shorten the length of treatment (Mariita et al., 2010). According to World Health Organisation (2001), 80% of African and Asian populations depend on traditional medicine from plant biodiversity for primary health care because plant derived medicines are relatively cheaper and safer compared to the synthetic alternatives. Many commercial drugs used in modern medicine were derived from plants following ethno-botanical and ethno-medical knowledge (Arokriyaraj et al., 2012). The World Health Organisation (WHO, 2001) has advocated traditional medicine as a safe remedy for ailments of microbial and non-microbial origin. Plant derived secondary metabolites such as alkaloids, flavonoids, phenolics, saponins, and tannins protect plants from invaders such as fungi, bacteria, viruses, and nematodes. It is estimated that among the traditional medicinal plants, only 12,000 or less than10% have undergone pharmacological evaluation (Mariimuthu et al., 2011). There is therefore a need to carry out more research on traditional medicinal plants for new antimicrobial agents as alternative to available antibiotics as they may prove to be effective against resistant pathogens to avoid the threat of post antibiotic era (Mariimuthu et al., 2011). Biological activity of plants is attributed to the class and concentration of phytochemical constituents which makes some plant extracts exhibit a variety of activities (Wang et al., 2010). Phytochemical screening of plant extracts especially those which have been used in traditional medicine is therefore essential so as to identify phytochemical constituents in the plants that are responsible for a given bioactivity. The current study investigated the antimicrobial activity, toxicity and phytochemical composition of four plants traditionally used in Msambweni ethnomedicine.
2. Materials and Methods

2.1. Collection of plant materials: Various plant parts depending on the ethnopharmacological knowledge (Nguta et al., 2010a, b) were collected from Msambweni District in Kenya. The plants were identified by a taxonomist from the University of Nairobi and voucher specimens deposited in the Nairobi University herbarium. Voucher numbers (in parenthesis) are: A. digitata (JN01), G. trichocarpa (JN02), L. cornuta (JN03) and Z. chalybeum (JN04). The plants parts were air dried at room temperature, chopped into small pieces and ground into powder. Collected plant parts used in the study were: A. digitata stem bark, G. trichocarpa roots, L. cornuta leaves and Z. chalybeum root bark.

Preparation of crude extracts: Aqueous and organic crude extracts were prepared by cold maceration, whereby 50 grams of the powdered plant material was extracted in 500 mls of distilled water and chloroform-Methanol mixture (1:1) respectively. The plant material was exhaustively extracted over 48 hrs period. The aqueous extracts were filtered, frozen and lyophilized into a dry powder. Organic extracts were filtered and concentrated with a rotary evaporator to dry powder. The recovered extracts were stored at 4°C for further use.

2.2. Microorganism suspension standardization: Bacillus cereus (ATCC11778), Pseudomonas aeruginosa (ATCC27853), Escherichia coli (ATCC25922) and Candida albicans (ATCC10231) were obtained from the Department of Public Health, Pharmacology and Toxicology, University of Nairobi while Methicillin Resistant Staphylococcus aureus (MRSA) was obtained from the Centre for Microbiology Research (CMR), Kenya Medical Research Institute (KEMRI). The fungal suspension was standardized according to the Clinical and Laboratory Standards Institute (CLSI, 2008). The yeast was grown in Sabouraud agar for 72 hours and standardized with sterile saline to turbidity equivalent to 0.5 McFarland scale (approximately1-2×10^8 CFU/ml). Bacteria were grown and standardized according to (CLSI, 2009). This was done by growing them in Muller–Hinton agar for 24 hours and standardizing them with sterile saline to turbidity equivalent to 0.5 McFarland scale (approximately1-2×10^8 CFU/ml) and stored at 4°C. The antibacterial and antifungal activity was determined using Agar well diffusion test and broth dilution method (Kaloyou et al., 2012).

2.3. Disc diffusion: To determine susceptibility, four concentrations (200 mg/ml, 100 mg/ml, 50 mg/ml and 25 mg/ml of crude extracts) were prepared in dimethylsulfoxide (DMSO) and water for organic and aqueous extracts respectively. Sterile antibiotic assay discs (Whatman, 6 mm) were impregnated with 100μl of the reconstituted extract and dried completely under sterile conditions in a laminar flow. Each disc was gently pressed down to ensure complete contact with the agar inoculated with 1ml of test microorganisms (bacteria and fungi). Extracts were tested in triplicate, (Kaloyou et al., 2012). Dimethylsulphoxide (DMSO) and water saturated assay discs were used as negative control. Gentamicin and Amphotericin B were used as positive control for bacteria and fungi respectively. The plates were incubated at 37°C for 18-24 h for bacteria and 30°C for 72 hrs for fungi. Inhibition zones were recorded as the diameter of growth free zones.

2.4. Determination of minimum inhibitory concentration (MIC): Broth dilution method was used to determine minimum inhibitory concentration (MIC). One mL of 24 h culture of test organisms (107 CFU/mL) adjusted to McFarland turbidity standard were incubated in serial dilution ranging from 10 to 200 mg/mL of plant extracts in DMSO for organic extracts and distilled water for aqueous extracts at 37°C for 24 hrs for bacteria and 30°C for 72hrs for fungi. The concentration of the lowest dilution with no detectable bacterial growth was considered as MIC. Absence of growth was confirmed by absence of turbidity and by inoculating into agar.

2.5. Acute toxicity testing: Toxicity of the extracts was evaluated using Brine shrimp assay (Musila et al. 2013). Brine Shrimp eggs were hatched in sea water for 48hrs at room temperature. Ten larvae were transferred to 5mls of 10μg/ml, 100μg/ml and 1000μg/ml of the crude extracts dissolved in sea water for aqueous extracts and DMSO for organic extracts. These were incubated at room temperature for 24hrs and live larvae were counted. DMSO and sea water were used as controls for organic and aqueous extracts respectively. The tests were done in triplicate. LC_{50} was determined by Finney’s probit analysis (Finney, 1971).

2.6. Thin Layer Chromatography (TLC): Crude plant extracts were screened for flavonoids, alkaloids, sesquiterpene lactones and saponins (Harbourne, 2002).

2.7. Data analysis: Statistical analysis of antimicrobial activity was done using SPSS (statistical analysis software). ANOVA was used to determine whether there were significant differences in the mean diameter of inhibition zones in various concentrations. Dunnett test was used for multiple comparisons of inhibition. The significance level used in the analysis was 0.05 (Alpha Level ≤ 0.05). In toxicity testing,
3. Results

3.1. Disc diffusion technique

The results of antimicrobial screening of the extracts of test plants are shown in Table 1 and Table 2. The organic crude extracts of all plants showed larger inhibition zones compared to the aqueous crude extracts. All the plants tested showed antimicrobial activity by inhibiting one or more microorganisms. Among these, *A. digitata* and *Z. chalybeum* organic extracts showed promising activity against Methicillin Resistant *S. aureus* (MRSA) and *B. cereus* respectively.

Table 1: Antimicrobial activity of aqueous and organic extracts.

<table>
<thead>
<tr>
<th>Plant name</th>
<th>Concentration (mg/ml)</th>
<th>C.albicans</th>
<th>E.coli</th>
<th>P.aeruginosa</th>
<th>B.cereus</th>
<th>S.aureus</th>
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<tbody>
<tr>
<td><em>A. digitata</em> L.</td>
<td>200</td>
<td>9.7</td>
<td>0</td>
<td>9.17</td>
<td>9.17</td>
<td>8.67</td>
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<td></td>
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<td>8</td>
<td>9</td>
<td>7.5</td>
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<td></td>
<td>50</td>
<td>0</td>
<td>0</td>
<td>7.5</td>
<td>8.5</td>
<td>0</td>
</tr>
<tr>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td><em>Z. chalybeum</em> Engl.</td>
<td>200</td>
<td>8.17</td>
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<td>10.5</td>
<td>10.67</td>
<td>9.33</td>
</tr>
<tr>
<td></td>
<td>100</td>
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<td>0</td>
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<td>7.67</td>
<td>12.5</td>
</tr>
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<td>7</td>
<td>0</td>
<td>8</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>7.67</td>
<td>7.00</td>
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<tr>
<td><em>L. cornuta</em> (Hocht.ex.Oliv.)C.Jeffrey</td>
<td>200</td>
<td>0</td>
<td>0</td>
<td>10.5</td>
<td>0</td>
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<tr>
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<td>100</td>
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<td>9.17</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>G. trichocarpa</em> Hochst. ex A.Rich</td>
<td>200</td>
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<td>0</td>
<td>11.67</td>
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<td>0</td>
<td>7.33</td>
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<td>0</td>
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</table>

Tables 1: Minimum Inhibitory Concentration

<table>
<thead>
<tr>
<th>Plant name</th>
<th>Minimum inhibitory concentration (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C.albicans</td>
</tr>
<tr>
<td><em>A. digitata</em> L.</td>
<td>&gt; 200 70</td>
</tr>
<tr>
<td><em>Z. chalybeum</em> Engl.</td>
<td>&gt; 200 40</td>
</tr>
<tr>
<td><em>L. cornuta</em> (Hocht.ex.Oliv.)C.Jeffrey</td>
<td>&gt; 200 80</td>
</tr>
<tr>
<td><em>G. trichocarpa</em> Hochst. ex A.Rich</td>
<td>&gt; 200 80</td>
</tr>
</tbody>
</table>

3.3. Brine-Shrimp lethality test

*A. digitata* aqueous crude extract had the highest LC<sub>50</sub> value of 3988 followed by *G. trichocarpa* with 1488 while *Z. chalybeum* exhibited the least LC<sub>50</sub> value of 293.5. *A. digitata* organic crude extract showed the highest LC<sub>50</sub> value of 5626 followed by *G. trichocarpa* with 5602. *L. cornuta* showed LC<sub>50</sub> value of 141.3 while *Z. chalybeum* recorded the lowest LC<sub>50</sub> value of 39.2 as shown in table 3.
3.4. Phytochemical screening of secondary compounds

Alkaloids and flavonoids were present in all the aqueous extracts while saponins were only found in L. cornuta only. Sesquiterpene lactones were absent in all the aqueous extracts. Alkaloids, flavonoids, Sesquiterpene lactones and saponins were present in all the organic extracts. The number of + shows the intensity of colouration while – shows absence of the compounds in the respective plants.

Table 4: Phytochemical screening of aqueous and CHCl3: MeOH crude extracts.

<table>
<thead>
<tr>
<th>Plant name</th>
<th>Alkaloids</th>
<th>Flavonoids</th>
<th>Sesquiterpene lactones</th>
<th>Saponins</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. digitata L.</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Z. chalybeum Engl.</td>
<td>++</td>
<td>+</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>L. cornuta (Hocht.ex.Oliv.)C.Jeffrey</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>G. trichocarpa Hochst. ex A.Rich</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>

4. Discussion

The current study was designed to investigate the antimicrobial activity, toxicity and phytochemical screening of four medicinal plants used in Msambweni District, Kenya. From this study, it is clear that the chloroform: methanol (1:1) solvent extracts of all the plant were more potent than those of aqueous extracts against all the tested microbes. A. digitata organic extract was active against all the microbes except the E. coli. The highest activity of A. digitata recorded was against MRSA followed by P. aeruginosa and B. cereus while the inhibition of C. albicans was moderate. These results are in agreement with Staden et al, (2011) who reported that A. digitata has considerable antimicrobial activity against Bacillus subtilis, S. aureus and C. albicans. This signifies that, A. digitata extract has potential novel antimicrobial compounds for management of diseases caused by the above microbes.

L. cornuta aqueous extract did not show any inhibition against the tested microbes at any concentration. However, the organic extract showed moderate activity against P. aeruginosa and MRSA and failed to inhibit B. cereus, E. coli and C. albicans. This is in agreement with findings from Nagata et al. (2011) who reported that leaves and roots of L. cornuta are used as antimicrobial by people of Suba district-Kenya particularly those living with HIV/AIDS to cure opportunistic diseases.

Z. chalybeum showed antimicrobial activity with the highest activity being observed against B. cereus whereby at 200 mg/ml, there was no significant difference in terms of inhibition zones (mm) with that of standard antibiotic (Gentamicin, 0.04 mg/ml) (P ≥ 0.05). Z. chalybeum organic extract also showed high activity against P. aeruginosa and moderate activity against MRSA and C. albicans but failed to inhibit the growth of E. coli at all concentrations. These results are in agreement with studies by Adesina’s (2005) findings that the ethanol extract of Z. chalybeum has antibacterial activity against B. cereus and P. aeruginosa and antifungal activity against C. albicans.

G. trichocarpa organic extract (CHCl3: MeOH) showed considerable antimicrobial activity against four of the five tested microbes; MRSA, B. cereus, P. aeruginosa and C. albicans and failed to show any inhibition against E. coli. The aqueous extract of G. trichocarpa did not inhibit any of the tested microbes. The organic extract showed high activity against P. aeruginosa and moderate activity against C. albicans, B. cereus and MRSA. This is the first report on antimicrobial activity of G. trichocarpa.

The organic extracts were more active than aqueous extracts. Noting that the traditional herbal remedy preparation from these plants is by use of water, it is a paradox that the aqueous extracts were inactive in this study. This may be due to the absence or insufficient and ineffective concentration of the antimicrobial constituents in the aqueous extracts. These findings are in agreement with the studies of Koua et al., (2011) who reported that inactivity of aqueous extracts of Striga hermonthica may be due to absence or insufficient and effective concentration of the antimicrobial agents of S. hermonthica extracts. It is well known that patients using the traditional herbs take in large amounts of the concoctions and hence may eventually consume sufficient...
amounts of the curative drugs to elicit healing.

The study on toxicity of A. digitata against Brine-shrimp confirmed that both the aqueous and organic extract of A. digitata were non-toxic since both extracts had LC50 > 1000 µg/ml, an observation that is in agreement with that of Musila et al., (2013). A. digitata has also been shown to be non-toxic on mice from previous studies. For instance, LD50 of aqueous extract of the fruit pulp of A. digitata on mice was found to be over 8000 µg/ml (Ramadan et al., 1994). This corroborates the results obtained in this study on the non-toxicity of stem bark of A. digitata on Brine shrimp larvae. The non-toxicity of A. digitata stem implies that the plant may be used to inhibit the growth disease causing bacteria and fungi without eliciting any toxicity on the patients. Toxicity study of L. cornuta on Brine shrimp from this study showed that both the aqueous and organic extracts (CHCl3: MeOH) were toxic particularly the (CHCl3: MeOH) ones. Similarly high levels of toxicity of stem bark of L. cornuta on Brine shrimp larvae has also been reported by Nguta et al. (2011) and Musila et al. (2013).

The study on toxicity of Z. chalybeum found that both the aqueous and organic (CHCl3: MeOH) extracts of Z. chalybeum were highly toxic against brine shrimp larvae. Nguta et al. (2011) while investigating toxicity of aqueous extracts of the leaves, stem bark and root bark of Z. chalybeum on Brine shrimp larvae obtained LD50<500 µg/ml for the three plant parts. This confirms the results obtained in this study and implies that Z. chalybeum may not make safe antimicrobial herbal remedies. This calls upon cautious use of the plant through dose adjustment amongst communities using this plant for preparation of herbal decoctions. Similarly, toxicity of Z. chalybeum methanolic root bark extract on human normal fetal lung fibroblast cells has also been reported by Kamuhabwa et al. (2000).

Toxicity study showed that both the organic and aqueous extracts of G. trichocarpa were non-toxic to brine shrimp larvae. This is the first report on the toxicity studies of G. trichocarpa.

The organic extract of G. trichocarpa contained alkaloids, flavonoids, saponins and sesquiterpene lactones while the aqueous extract contained only alkaloids and flavonoids. There is no available report from the literature on the antimicrobial activity, toxicity and phytoconstituents of Grewia species except on Grewia hexaminta which is said to contain triterpenoid compounds (Raghunathaiyar, 1996). This could be the first time to report on G. trichocarpa phytochemistry.

Thin layer chromatography (TLC) study of A. digitata stem bark shows that the organic extract contained alkaloids, flavonoids, saponins and sesquiterpene lactones while the aqueous extract lacked both saponins and sesquiterpene lactones and contained alkaloids and flavonoids only. This is in agreement with Musila et al. (2013). In addition, A. digitata stem bark contains medicinal compounds which are largely classified under saponins, alkaloids and flavonoids such as lupeol acetate, β-sitosterol, scopeolit, friedelin, betulinic acid and adansonin while the fruit pulp of A. digitata is rich in procyanidins (Sidibe & Williams, 2002; Shahat, 2006). Alkaloids, flavonoids and sesquiterpenes have been reported to be potent plant secondary metabolites with broad spectrum of bioactivities (Mazid et al., 2011). The higher antimicrobial activity of A. digitata on MRSA could be attributed to the presence of large amounts alkaloids. Alkaloids are pharmacogenically active basic principles of flowering plants, (Das et al., 2010). The observed activity of these compounds in A. digitata is in line with that of Karou et al. (2006) who demonstrated that the Indoloquinoline alkaloid causes cell lysis and morphological changes of S. aureus. Secondary metabolites screening in this study found that both aqueous and organic (CHCl3: MeOH) extracts of L. cornuta contained flavonoids, alkaloids, saponins with sesquiterpene lactones lacking in the aqueous extract. These results are in agreement with (Musila et al., 2013). Elsewhere in Ali et al. (2003), the genus Launaea is characterized for flavonoids, triterpenes, sesquiterpene lactones, coumarins and steroids. Various species of Launaea such as, L. arborescens, L. mucronata, L. nudicaulis and L. capitata contain various types of flavones such as luteolin, apigenin and flavone glycosides such as apigenin 7-O-glucoside, vitexin, luteolin 7-O-glucoside and luteolin 7-O-rhamnoside besides others (Christian & Christian, 2010). Organic extract (CHCl3: MeOH) of Z. chalybeum was found to contain alkaloids, flavonoids, saponins and sesquiterpene lactones while the aqueous extract contained only alkaloids and flavonoids. Similar results were obtained by Musila et al. (2013). Z. chalybeum has been reported to contain alkaloids which have antibacterial and cytotoxic activity (Chrian et al., 2011). Zanthoxylum species contains various compounds such as alkaloids, aliphatic and aromatic amides, lignans, coumarins, sesquiterpene lactones and sterols (Cheng et al., 2011; He et al., 2002).

Biological activity is attributed to the presence of various secondary metabolites in plants (Mazid et al., 2011). Not only their presence, but also the quantity of the phytochemical constituents in a given plant extract determines the extent of extracts’ bioactivity. In addition, presence of more than one class of secondary metabolites in a given plant extract will also determine the nature and extent of extract’s biological activity (Wang et al., 2010). The presence of flavonoids in the crude extracts facilitated the antimicrobial activity as flavonoids are known to complex with the extracellular and soluble proteins and bacterial cell wall of bacteria. More flavonoids may also disrupt microbial membrane. The sesquiterpene lactones are known to be active against bacteria, protozoa and viruses through membrane disruption. (Cowan, 1999).
5. Conclusion
The current study shows that Z. chalybeum and A. digitata inhibit the growth of B. cereus and MRSA respectively with no significance difference (P≥0.05) with that of standard antibiotic (Gentamicin). The crude extracts of A. digitata and G. trichocarpa proved to be non toxic on Brine shrimp larvae with LC_{50}>1000 μg/ml while Z. chalybeum and L. cornuta were found to be highly toxic with LC_{50}<500 μg/ml. Flavonoids, alkaloids, saponins and sesquiterpene lactones were present larger amounts in organic extracts of A. digitata, G. trichocarpa, L. cornuta and Z. chalybeum compared to their aqueous extracts. Alkaloids were present in high amount while sesquiterpene lactones were present in low amounts in all the extracts. The antimicrobial activity, toxicity and phytochemistry of crude extracts G. trichocarpa is being reported for the first time. This could be a start point for further drug research of this species on a wide diversity of microbial pathogens.

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References


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