Publications
Pharmacognostic Evaluation of the Leaves of Kirganelia reticulata Baill. (Euphorbiaceae)

S. D. Shruthi1 • Y. L. Ramachandra1* • S. Padmalatha Rai2 • Prashant Kumar Jha3

1 P.G. Department of Studies and Research in Biotechnology & Bioinformatics, Kuvempu University, Shankaraghatta – 577 451, Karnataka, India
2 Department of Biotechnology, Manipal Life Science Center, Manipal University, Manipal-576104, India
3 Quality Control Laboratories, ALN Rao Memorial Medical College, Koppa, Chikmagalur-577126, Karnataka, India

Corresponding author: *yprpub@gmail.com

ABSTRACT

The leaves of Kirganelia reticulata Baill. (Euphorbiaceae) are known to have many uses in Indian ethnomedicine. Establishment of a pharmacognostic profile of the leaves will assist in standardization for quality, purity and sample identification. Evaluation of the fresh, powdered and anatomical sections of the leaves were carried out to determine the macro- and micromorphological characters, and quantitative, qualitative and phytochemical profiles. The results of the study could be useful in setting some diagnostic indices for the identification and preparation of a monograph of the plant.

Keywords: fluorescence, pharmacognosy, physicochemical, phytochemical studies, quantitative leaf microscopy

INTRODUCTION

After decades of serious obsession with the modern medicinal system, people have started looking at the ancient healing systems like Ayurveda, Siddha and Unani. This is because of the adverse effects associated with synthetic drugs (Thomas et al. 2008). Plants have been associated with the health of mankind from time immemorial. In the past sickness was viewed as a punishment from the god’s and hence was treated with prayers and rituals which included “magic proportion” prepared from local herbs (Sandhya et al. 2010). Herbal drugs play an important role in health care programs especially in developing countries. However a key obstacle, which has hindered the acceptance of the alternative medicines in the developed countries, is the lack of documentation of research work carried out on traditional medicines and stringent quality control (Dahanukar et al. 2000). With this backdrop, it becomes extremely important to make an effort towards standardization of the plant material to be used as medicine.

Kirganelia reticulata (Synonym: Phyllanthus reticulatus; Bengali name: Panjuli; Family- Euphorbiaceae) is a large, often scandent, shrub. The plant grows throughout tropical areas of India, Bangladesh, China, and the Malay Islands (Kirtikar and Basu 1980; Ghani 2003). The biological work performed so far on this plant showed hypotensive effects and its folkloric use in gastric complaints including colic, constipation etc. and chemical studies demonstrated the presence of octacosanol, teraxerol acetate, friedelene, teraxerone, betulin, sitosterol etc. (Rav et al. 1964; Joshi et al. 1991). The leaves are employed as a diuretic and cooling medicine. The juice of the leaves is used to care diarrhoea in infants. The stems are used to treat sore in eyes and the powdered leaf is used in sores, burns, suppurations and chafing of the skin (Chopra et al. 1956). The bark is used to treat rheumatism, dysentery and venereal diseases (Yoganarasimhan 1996). The plant is used for a variety of ailments, including smallpox, syphilis, asthma, diarrhoea, bleeding from gums (Nandkarni 1982; The Wealth of India 2005). It is also claimed to have antidiabetic activity in tribal areas, which has been validated by Kumar et al. (2008).

The antibacterial potential of the leaf extracts of this plant has been evaluated recently (Shruthi et al. 2010). We now report on the pharmacognostic profile and confirm the ethnopharmaceutical claim of the plant.

Herbs show a number of problems when quality aspect is considered. This is because of nature of the herbal ingredients and different secondary metabolites present therein. It is also due to variation in the chemical profile of herbs due to intrinsic and extrinsic factors like growth, harvesting, geographical source, storage and drying etc (WHO 2002).

To ensure reproducible quality of herbal medicines, proper control of starting material is utmost essential, the first step towards it is authentication followed by creating numerical values of standards for comparison (Agarwal 2005). Some drugs of plant origin in conventional medical practice are not pure compounds but direct extracts or plant materials that have been suitably prepared and standardized (Donald 1986). The World Health Organisation (WHO) has recommended the use of artemisinin derivatives from Artemisia annua (Composite), a Chinese herb with established pharmacognostic data, as a first line drug in the treatment of malaria (WHO 2001, 2002) and identification of samples.

Pharmacognostical parameters like macroscopy, quantitative leaf microscopy, fluorescence, physicochemical and phytochemical studies are few of the basic protocol for standardization of herbals. Hence, in the present work establishment of the pharmacognostic profile of the leaves of K. reticulata is carried out; which will assist in standardization, can guarantee quality, purity and it can also be used to prepare a monograph for the proper identification of the plant.

MATERIALS AND METHODS

Fresh plant materials were collected in winter season locally from Bhadra Wild Life Sanctuary, Karnataka (Southern India) in December 2009. The taxonomic identification of the plant was confirmed by Dr. Y. L. Ramachandra, Department of Biotechnology, Kuvempu University, Shankaraghatta (Voucher specimen number YLR429).

Received: 28 July, 2010. Accepted: 21 August, 2010.
Macroscopy

The following macroscopic characters for the fresh leaves were noted: size and shape, colour, surfaces, venation, presence or absence of petiole, the apex, margin, base, lamina, texture, odour and taste (Wallis 1985; Evans 1996).

Microscopy

The outer epidermal membranous layer (in fragments) were cleared in chloral hydrate, mounted with glycerin and observed under a compound microscope. The presence/absence of the following was observed: epidermal cells, stomata (type and distribution) and epidermal hairs (types of trichomes and distribution). The transverse sections of the fresh leaves through the lamina and the midrib were also cleared, mounted and observed (African Pharmacopoeia 1986).

Quantitative investigation

Quantitative leaf microscopy to determine palisade ratio, stomata number, stomata index, vein – islet number and veinlet termination number were carried out on epidermal strips. Other physicochemical parameters determined for the powdered leaves were moisture content, total ash, acid – insoluble ash, water – soluble ash, alcohol and water-soluble extractive values (British Pharmacopoeia 1980).

Fluorescence analysis

Powdered leaf material was subjected to analysis under ultra violet light after treatment with various chemical and organic reagents like alcohol, 50% sulphuric acid, 10% sodium hydroxide, 50% nitric acid and water (Kokate 1994).

Phytochemical investigation

Chemical tests were employed in the preliminary phytochemical screening for various secondary metabolites such as carbohydrates, alkaloids, phytosterols, glycosides, saponins, flavonoids, proteins, tannins and gum (Brain and Turner 1975; Ciulei 1981; Harborne 1992; Evans 1996).

RESULTS AND DISCUSSION

Morphological characteristics of the leaf

Leaves have alternate arrangement, small or moderate sized, distichious, thin, stipular and lanceolate (Gamble 1921). Leaves are 2.5-5 cm long and 0.7-1.5 cm broad, coriaceous, oblong and elliptic in shape. The apex of leaves is acute with acute or subcordate base. The ventral side of leaves are dark-green in colour while dorsal side being light-green. 6-8 pairs of nerves are prominently raised on dorsal side. Leaves are pubescent along the veins otherwise glabrous. The margin of leaves is emarginated to undulated (Fig. 1). It is having pungent odour and has a characteristic bitter taste.

Microscopic characteristics of the leaf

The outline of transverse section is dorsiventral type. The detailed transverse section shows epidermis (both upper and lower) cover the section both in lamina and midrib portion. The upper epidermis is covered with cuticle especially in lamina portion. The lamina portion exposes that palisade cells, covers half to one third portion of lamina. The bundles of vascular are well developed and exposed in midrib portion while those of primitive type are spreaded over the lamina portion, too. The surface preparation shows the anomocytic type of stomata. The trichomes are scarcely found on the top of section along the midrib. The epidermis (both upper and lower) is followed by hypodermis, composed of collenchymatous cells in midrib. In lamina portion, the palisade parenchyma cells are in continuation with upper epidermis from above to downward while spongy parenchyma cells are followed by lower epidermis from downward to upward. These mesophyll cells (Palisade and spongy parenchyma cells) are filled with chlorophylls and at places are interrupted with vascular cells and secretory cells. In midrib portion below the hypodermis, ground tissue is present. Clusters of calcium oxalate crystals were found in parenchyma cells of this region. Vascular bundles are found in between the ground cells where xylem elements are followed by phloem elements. The vascular bundles are capped with fibres of sclerenchyma (Fig. 2).

The quantitative determination of some pharmacognostic parameters is useful for setting standards for crude drugs. The palisade ratio, stomata number, vein islet, vein termination numbers and the other parameters determined in the quantitative microscopy (Table 1), are relatively constant for plants and can be used to differentiate closely related species. The physical constant evaluation of the drugs is an important parameter in detecting adulteration or improper handling of drugs. The moisture content of the drug is not too high, thus it could discourage bacterial, fungi or yeast growth, as the general requirement for moisture content in crude drug is not more than 14% (African Pharmacopoeia 1986). Equally important in the evaluation of crude drugs, is the ash value and acid-insoluble ash value determination. The total ash is particularly important in the evaluation of purity of drugs, i.e. the presence or absence of foreign

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Range</th>
<th>Mean*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palisade ratio</td>
<td>9-13</td>
<td>11.26 ± 0.42</td>
</tr>
<tr>
<td>Stomata number Upper surface</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Stomata number Lower surface</td>
<td>59-84</td>
<td>72.58 ± 2.76</td>
</tr>
<tr>
<td>Stomata index Upper surface</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Stomata index Lower surface</td>
<td>26.2-35.7</td>
<td>32.23 ± 1.21</td>
</tr>
<tr>
<td>Vein islet number</td>
<td>20-25</td>
<td>22.67 ± 0.52</td>
</tr>
<tr>
<td>Veinlet termination number</td>
<td>35-39</td>
<td>37.14 ± 0.39</td>
</tr>
</tbody>
</table>

* Mean value of 10 counts

### Table 2 Fluorescence behaviour of Kirkanglea reticulata.

<table>
<thead>
<tr>
<th>Treatment as such</th>
<th>Daylight</th>
<th>UV light</th>
</tr>
</thead>
<tbody>
<tr>
<td>Powder (P)</td>
<td>Pale green</td>
<td>Fluorescent yellowish green</td>
</tr>
<tr>
<td>P + water</td>
<td>Brownish green</td>
<td>Fluorescent orangish green</td>
</tr>
<tr>
<td>P + Alcohol¹</td>
<td>Olive green</td>
<td>Fluorescent orange</td>
</tr>
<tr>
<td>P + 10% NaOH²</td>
<td>Blood red</td>
<td>Fluorescent red</td>
</tr>
<tr>
<td>P + 50% HNO₃³</td>
<td>Orangish yellow</td>
<td>Fluorescent green</td>
</tr>
<tr>
<td>P + 50% H₂SO₄⁴</td>
<td>Orangish green</td>
<td>Greenish brown</td>
</tr>
</tbody>
</table>

inorganic matter such as metallic salts and/or silica (Fig. 3). The fluorescence analysis observed in day/visible light and UV light when treated with different chemical reagents, is depicted in Table 2. Different chemical compounds such as alkaloids, tannins, flavonoids, phytosterols, glycosides among others were detected, which could make the plant useful for treating different ailments and having a potential of providing useful drugs of human use. By virtue of their photosynthetic machinery, leaves serve as a sink for several metabolites and as an important source of several bioactive compounds (Sujan et al. 2009; Murti et al. 2010).

Empirical knowledge about medicinal plants plays a vital role in primary health care and has great potential for the discovery of new herbal drugs. These findings may be useful to supplement existing information with regard to the identification and standardization of *K. reticulata*, even in the powdered form of the plant drug, to distinguish it from substitutes and adulterants. These studies also suggested that the observed pharmacognostic and physicochemical parameters are of great value in quality control and formulation development. In conclusion, the present study may be useful to supplement information with regard to its identification and standardization, and in carrying out further research and revalidation of its use in the Ayurvedic System of Medicine.

**ACKNOWLEDGEMENTS**

The laboratory facility of ALN Rao Memorial Medical College, Koppara, Chikmagalur is gratefully acknowledged.
REFERENCES


Braun KR, Turner TD (1975) Practical Evaluation of Phytopharmaceuticals, Wright-ScienceTechnica, Bristol, 144 pp


Chopra RN, Nayar SL, Chopra IC (1956) Glossary of Indian Medicinal Plants (2nd Edn.), CSIR, New Delhi, India


Gamble JS (1921) Flora of the Presidency of Madras (Vol II), Published under the authority of Secretary of State for India in Council, 1294 pp

Ghosh A (2003) Medicinal Plants of Bangladesh, Chemical Constituents and Uses (2nd Edn.), Asiatic Society of Bangladesh, 345 pp


Nandkarni KM (1982) Indian Materia Medica (Vol 2), Publisher, City, 948 pp

Rav MRR, Siddiqui HII (1964) Screening of Indian plants for biological activity. Indian Journal of Experimental Biology 2, 49


The Wealth of India (2005) National Institute of Science Communication and Information Resources (Vol 7), Council of Scientific and Industrial Research, New Delhi, 34 pp


Vasasawasimbi SN (1996) Medicinal Plants of India (Vol 1), Interline Publishing Pvt. Ltd., Bangalore, Karnataka, 275 pp
ANTIBACTERIAL POTENTIAL OF LEAF EXTRACTS FROM *KIRGANELIA RETICULATA* BAILL

**Shruthi SD**, Ramachandra YL, Padmalatha Rai S and Veena Shetty A

1P.G. Department of Studies and Research in Biotechnology & Bioinformatics, Kuvempu University, Shankaraghatta – 577 451, Karnataka, India

2Department of Biotechnology, Manipal Life Science Center, Manipal University, Manipal-576104, India

3Department of Microbiology, K. S. Hegde Medical Academy, Mangalore, India.

Email: ylrrpub@gmail.com

**ABSTRACT**

The in vitro antibacterial activity of crude methanolic, chloroform and hexane extracts of the leaves of *Kirganela reticulata* (Euphorbiaceae) were investigated. Susceptibility of some Gram-negative organisms (*Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi*) and Gram-positive organism (*Staphylococcus aureus*) were tested. Agar well diffusion and broth dilution methods were used to determine the minimum antibacterial activity against all the tested microorganisms. The extracts exhibited antibacterial activities with zones of inhibition ranging from 9.07 - 30.10 mm, 8.17 - 24.57 mm and 5.60 - 14.67 mm for methanol, chloroform and hexane extracts respectively. Screening of crude extracts showed notable minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) at concentrations of 100 to 6.25 mgml⁻¹. The organisms were more sensitive to the methanolic extract of the leaves, where as extracts from other solvents like chloroform and hexane showed moderate to weak activity respectively. Similar results have been showed in MIC and MBC.

**Key Words:** Antibacterial, Gram-negative organism, Gram-positive organism, Leaf extracts.

**INTRODUCTION**

The use of plant compounds to treat infections is an age-old practice in a large part of the world, especially in developing countries, where there is dependence on traditional medicine for a variety of diseases (1, 2). Many pharmacognostical and pharmacological investigations are carried out to identify new drugs or to find new lead structures for the development of novel therapeutic agents for the
treatment of human diseases such as cancer and infectious diseases (3). In India, large segments of the population still rely on folk medicine to treat serious diseases including infections, cancers and different types of inflammations. The increased prevalence of antibiotic resistant bacteria due to the extensive use of antibiotics may render the current antimicrobial agents insufficient to control some bacterial diseases and hence research for identifying novel substances that are active against human pathogens is an urgent need (4). The antimicrobial compounds produced by plants are active against plant and human pathogenic microorganisms (5). The substances that can either inhibit the growth of pathogens or kill them and have no or least toxicity to host cells are considered candidates for developing new antimicrobial drugs. It is expected that plant extracts showing target sites other than those used by antibiotics will be active against drug-resistant microbial pathogens. However, very little information is available on such activity of medicinal plants (6, 7).

*Kirganelia reticulata* Baill. (Synonym-*Phyllanthus reticulatus* Poir.) is a large, often scendent, shrub of the family Euphorbiaceae. The plant grows throughout tropical areas of India, Bangladesh, China, and the Malay Islands (8). The leaves and bark are used as astringent and diuretic. Juice of leaves is used for the treatment of diarrhea in children (9). The bark showed significant antiviral (10) and antiplasmodial activity (11). The antibacterial potential of the aerial parts of this plant has been evaluated (12). Although *K. reticulata* has traditionally been used in the treatment of many ailments in India, no scientific report is available to date to validate these folkloric uses. We now report on the antibacterial activities of different extracts and confirm the ethnomedicinal claim of the plant.

**MATERIALS AND METHODS:**

**Plant materials**

Fresh leaf materials of plant were collected in winter season locally from Bhadra Wild Life Sanctuary, Karnataka (Southern India) in December 2009. The taxonomic identification of the plant was confirmed by Dr. Y. L. Ramachandra, Department of Biotechnology, Kuvempu University, Shankaraghatta (Voucher specimen number YLR429).

**Extraction**

Freshly collected leaves of *K. reticulata* were shade-dried and then powdered using a mechanical grinder. The shade dried leaves were pulverised and subjected for successive extraction using hexane, chloroform and methanol (LR grade, Merck, India) separately using soxhlet apparatus. The extracts were evaporated to dryness under reduced pressure using a Rotavapor (Buchi Flawil, Switzerland). A portion of the residue was used for the antibacterial assay.

**Source and maintenance of organisms**

Stock cultures of Gram-negative organisms (*Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi*) and Gram-positive organism (*Staphylococcus aureus*) were obtained and confirmed at the research laboratory of the Department of Microbiology, K. S. Hegde Medical Academy, Mangalore, Karnataka. They were maintained on Mueller-Hinton Agar (Himedia, Mumbai) slope at 4°C and sub-cultured into Mueller-Hinton broth by a picking-off technique (13). Twenty-four hour old pure cultures were prepared for use each time.

**Bacterial susceptibility testing**

In vitro antibacterial activity of the crude extracts was studied against Gram-negative and Gram-
positive bacteria by the agar well diffusion method (14). The extracts were dissolved in 10% aqueous dimethylsulfoxide (DMSO) to a final concentration of 100 mg/ml. Pure DMSO was taken as the negative control and 0.05% Ciprofloxacin as the positive control. Mueller-Hinton Agar (Himedia, Mumbai) was used as the bacteriological medium. It was prepared according to the manufacturer's instruction, autoclaved and dispensed at 20 ml per plate in 12 x 12 cm petri dishes. Set plates were incubated overnight to ensure sterility before use. Suspension of micro-organisms was made in sterile normal saline and adjusted to 0.5 Macfarland standards (108 Cfu/ml) (15). Each labelled medium plate was uniformly inoculated with a test organism by using a sterile cotton swab rolled in the suspension to streak the plate surface in a form that lawn growth can be observed. A sterile cork borer of 5mm diameter was used to make wells on the medium. 100µl of the various extract concentration and control compound were dropped into each, appropriate labeled well (16, 17). The inoculated plates were kept in the refrigerator for 1 hour to allow the extracts to diffuse into the agar (16). The Mueller Hinton Agar plates were incubated at 37°C for 24 hours. Antibacterial activity was determined by measuring the diameter of zones of inhibition (mm) produced after incubation.

**Determination of Minimum Inhibitory Concentration (MIC)**

To measure the MIC values, micro-broth dilution method was used (18). The reconstituted extracts was serially diluted 2-fold in Mueller-Hinton broth medium to obtain various concentrations of the stock, 100, 50, 25, 12.5, 6.25, 3.125, 1.562, 0.781 mg/ml and were assayed against the test organisms. The minimum inhibitory concentration was defined as the lowest concentration able to inhibit any visible bacterial growth (19, 17).

**Determination of Minimum Bactericidal Concentration (MBC)**

Equal volume of the various concentration of each extract and Mueller Hinton broth were mixed in micro-tubes to make up 0.5ml of solution. 0.5ml of McFarland standard of the organism suspension was added to each tube (17). The tubes were incubated aerobically at 37°C for 24 hours. Two control tubes were maintained for each test batch. These include tube-containing extract without inoculum and the tube containing the growth medium and inoculum. The MBC was determined by sub culturing the test dilution on Mueller Hinton Agar and further incubated for 24 hours. The highest dilution that yielded no single bacterial colony was taken as the Minimum bactericidal concentration (20).

**Statistical analysis**

The results of the experiment are expressed as mean ± SE of three replicates in each test. The data were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's multiple pairwise comparison tests to assess the statistical significance. $P<0.05$ was considered as statistically significant, using software ezANOVA ver. 0.98. The data is presented in Tables 1 and 2.

**RESULTS AND DISCUSSION:**

All the three extracts of the plant tested showed varying degree of antibacterial activities against the test bacterial species (Table 1). The antibacterial activities of the methanol extracts compared favourably with the standard antibiotic (Ciprofloxacin) and have appeared to be broad spectrum as its activities were independent on Gram reaction. The inhibition zone for *Pseudomonas aeruginosa* was much high (14.67 - 30.10 mm), which is followed by
Salmonella typhi (13.07 - 27.90 mm), Staphylococcus aureus (9.10 - 16.83 mm), where as Escherichia coli showed much less (5.60 - 9.07 mm) comparably. The methanol extract (inhibition zone 9.07 - 30.10 mm) was found to be more effective than the chloroform extract (inhibition zone 8.17 - 24.57 mm) against all the organisms. The hexane extract showed low antibacterial activity with inhibition zones ranging between 5.60 and 14.67 mm for different bacteria tested. Ciprofloxacin, which was used as a positive experimental control against all bacterial strains assayed, produced a zone of inhibition of 10.17 to 31.83 mm, while no inhibitory effect could be observed for DMSO used as negative control.

The minimum inhibitory concentration (MIC) of the methanol extract for different organisms ranged between 6.25 and 25.0 mgml⁻¹, while that of the chloroform extract ranged between 12.5 and 50.0 mgml⁻¹. Also the MIC of Ciprofloxacin control ranged between 3.125 and 6.25 mgml⁻¹ (Table 2). The minimum bactericidal activity (MBC) of the extract for different bacteria ranged between 12.50 and 50.0 mgml⁻¹ for the methanol extract and for the chloroform extract ranged between 25.0 and 100.0 mgml⁻¹ (Table 2). Hexane extract was not much active against any of the organisms at the concentrations tested. Generally, the methanol extract was more active than other extracts. This may be attributed to the presence of soluble phenolic and polyphenolic compounds (21).

A correlation was found between the antibacterial activity observed by agar diffusion assay and MIC, MBC determination which was the same case observed with Ramzi et al. (22). The pronounced effect of methanol extract against these organisms may be due to its stronger ability to extract some of the active properties of these plants like phenolic compounds, saponin, bryophyllin and other secondary metabolites which are reported to be antimicrobial (23, 24). Where as unnoticeable effect of hexane extract may be as a result of loss of some of the plant's active principle when drying or the inability of the solvents to dissolve some of the active principles of this plant (25). However, our results reveal that the crude extracts contain certain constituents like flavonoids which are known to be synthesized by plants in response to microbial infection. Hence, it is apparent that they have been found to be effective antibacterial substances against a wide range of microorganisms.

CONCLUSION:

This study not only show the scientific basis for some of the therapeutic uses of this plant in traditional medicine, but also confirms the fact that ethnomedical and disinfectant formulation as well as in chemotherapy if the active principle can be isolated (28). The anti-pseudomonal and anti-staphylococcal activities of the effective extracts of this plant can be further explored. The present results will form the basis for selection of plant species for further investigation in the potential discovery of new natural bioactive compounds. Further studies which aimed at the isolation and structure elucidation of antibacterial active constituents from the plant has been initiated.

ACKNOWLEDGEMENT:

The laboratory facility of Central Research Lab, AB Shetty Memorial Institute of Dental Sciences, Nitte University, Mangalore is gratefully acknowledged.
REFERENCES:


Tables and Figures:

**Table 1: Antibacterial activities profile of three extracts from the leaves of K. reticulata.**

<table>
<thead>
<tr>
<th>Test bacteria</th>
<th>Methanol Extract</th>
<th>Chloroform Extract</th>
<th>Hexane Extract</th>
<th>Ciprofloxacin</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>16.83 ± 0.54**</td>
<td>13.47 ± 0.18**</td>
<td>9.10 ± 0.21**</td>
<td>18.13 ± 0.20</td>
</tr>
<tr>
<td><em>Pseudomonas</em></td>
<td>30.10 ± 0.40*</td>
<td>24.57 ± 0.20**</td>
<td>14.67 ± 0.23**</td>
<td>31.83 ± 0.15</td>
</tr>
</tbody>
</table>

International Journal of Pharma Research and Development – Online
www.ijprd.com
<table>
<thead>
<tr>
<th>Test bacteria</th>
<th>Methanol (mg/ml⁻¹)</th>
<th>Chloroform (mg/ml⁻¹)</th>
<th>Hexane (mg/ml⁻¹)</th>
<th>Ciprofloxacin (mg/ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC</td>
<td>MBC</td>
<td>MIC</td>
<td>MBC</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>12.5</td>
<td>25.0</td>
<td>25.0</td>
<td>50.0</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>6.25</td>
<td>12.5</td>
<td>12.5</td>
<td>25.0</td>
</tr>
<tr>
<td><em>Salmonella typhi</em></td>
<td>6.25</td>
<td>12.5</td>
<td>12.5</td>
<td>25.0</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>25.0</td>
<td>50.0</td>
<td>50.0</td>
<td>100.0</td>
</tr>
</tbody>
</table>

The values are the mean of triplicates ± S.E. * P<0.05, ** P<0.01 compared to standard.

**Table 2:** The MIC and MBC regimes of the extracts of the leaves of *K. reticulata.*
Pharmacophore
(An International Research Journal)
Available online at http://www.pharmacophorejournal.com/

Original Research Paper

IN VITRO ANTIBACTERIAL ACTIVITIES OF KIRGANELIA RETICULATA BAILL. AGAINST METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS

Shruthi SD\textsuperscript{1,3\ast}, Padmalatha Rai S\textsuperscript{2}, Ramachandra YL\textsuperscript{1}

\textsuperscript{1}P.G. Department of Studies and Research in Biotechnology and Bioinformatics, Kuvempu University, Shankaraghatta – 577 451, Karnataka, India

\textsuperscript{2}Department of Biotechnology, Manipal Life Science Center, Manipal University, Manipal-576104, India

\textsuperscript{3}P.G. Department of Biotechnology, the Oxford College of Science, Bangalore, Karnataka, India

ABSTRACT

Methanol, chloroform and hexane extracts from leaves of \textit{Kirganelia reticulata}, used in Indian ayurvedic medicine for the treatment of several ailments of microbial and non-microbial origin were evaluated for potential antibacterial activity against methicillin-resistant \textit{Staphylococcus aureus} (MRSA). Antibacterial activity and biofilm production of crude extracts against MRSA (ATCC 25923) isolated from clinical specimen was studied. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of the methanol, chloroform and hexane extracts were in the range of 12.5 to 50.0 mg/ml and 25.0 to 100.0 mg/ml, respectively. Amongst the evaluated extracts, the methanolic extract showed the strongest antibacterial effect as well as biofilm inhibition. Micro plate screening used for detection of biofilm formation by \textit{Staphylococci} is a quantitative model to study its adherence level and has been a sensitive method.

Keywords: Antibacterial, biofilm, leaf extracts, methicillin-resistant \textit{Staphylococcus aureus}, MIC, MBC.
INTRODUCTION

The antimicrobial compounds produced by plants are active against plant and human pathogenic microorganisms. The substances that can either inhibit the growth of pathogens or kill them and have no or less toxicity to host cells are considered candidates for developing new antimicrobial drugs. There is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action because there has been an alarming increase in the incidence of new and re-emerging infectious diseases. Another big concern is the development of resistance to the antibiotics in current clinical use. In recent years, drug resistance to human pathogenic bacteria has been commonly reported from all over the world. The drug-resistant bacteria and fungal pathogens have further complicated the treatment of infectious diseases in AIDS and cancer patients. Moreover, excessive budget is currently spent on import of antibiotics manufactured abroad. Therefore, antibacterial activity of local medicinal plants should be studied to provide alternative and locally available antibacterial regimens.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is now common in many areas of the world. The frequencies of infections and outbreaks due to MRSA have continued to increase. It is often multidrug resistant and therapeutic options are limited. MRSA is a type of *Staphylococcus* that is resistant to the antibiotics that are often used to treat *staphylococcal* infections. *Osteomyelitis* is one such infection which is particularly difficult to treat. The options for treatment of infections caused by these microorganisms are limited: the sensitivity of clinical strains to quinolones, clindamycin, co-trimoxazole and rifampicin is variable, and the sensitivity is often limited to glycopeptides, which must be administered by the parenteral route. Novel drugs for the treatment of methicillin-resistant *staphylococcal* infections, such as quinupristin–dalfopristin and linezolid, have recently been introduced in clinical practice. However, none has been fully investigated in clinical studies on the treatment of *osteomyelitis*.

*S. aureus* cause disease through the production of virulence factors. They are the part of our normal flora, but they can cause fatal diseases as a result of the expression of multiple virulence factors. These factors include adhesins, exotoxins, enterotoxins, hemolysins, and leukocidin, as well as proteases that enable the bacteria to spread within the host. Strains defective in their ability to form a biofilm or produce toxins show diminished virulence, suggesting that a novel approach for therapy development would be to interfere with the production of virulence factors. TRAP is a membrane-associated 167-amino acid residue protein that is highly conserved among *Staphylococci*. When TRAP is not expressed or not phosphorylated, the bacteria do not adhere, do not form a biofilm, do not express toxins, and do not cause disease. TRAP expression is constitutive, but its phosphorylation is regulated by RAP and reaches peak levels in the mid-exponential phase of growth, followed by activation of *agr* and induction of SQS2 components. RAP is a 277-amino acid residue protein that activates the *agr* system by inducing the phosphorylation of TRAP. RAP is an ortholog of the 50S ribosomal protein L2 that is secreted by *S. aureus*.

*Kirganelia reticulata* Baill. (Synonym-*Phyllanthus reticulatus* Poir.) is a large, often scandent, shrub of the family *Euphorbiaceae*. The plant grows throughout tropical areas of India, Bangladesh, China, and the Malay Islands. The leaves and bark are used as

http://www.pharmacophorejournal.com/
astrangent and diuretic. Juice of leaves is used for the treatment of diarrhea in children. The bark showed significant antiviral and antiplasmodial activity. The antibacterial potential of the aerial parts of this plant has been evaluated. The bark is used to treat rheumatism, dysentery and venereal diseases. The plant is used for a variety of ailments, including smallpox, syphilis, asthma, diarrhoea, bleeding from gums. It is also claimed to have antidiabetic activity in tribal areas, which has been validated by Kumar et al. The antibacterial potential of the leaf extracts of this plant has been evaluated recently.

The medicinal plant, which have been used as folk medicine for several diseases, were selected for this research to study antibacterial activity and biofilm inhibition of their crude methanolic, chloroform and hexane extracts against meticillin-resistant Staphylococcus aureus. In India, multi drug resistance has clearly emerged as a serious problem with MRSA. Hence, the basis of the study is in order to overcome this and to prove the folkloric claims of the plant.

MATERIALS AND METHODS

Collection of material and preparation of extracts

Fresh leaf materials of plant were collected in winter season locally from Bhadra Wild Life Sanctuary, Karnataka (Southern India) in December 2009. The taxonomic identification of the plant was confirmed by Dr. Y. L. Ramachandra, Department of Biotechnology, Kuvempu University, Shankaraghatta (Voucher specimen number YLR429). Freshly collected leaves of K. reticulata were shade-dried and then powdered using a mechanical grinder. The shade dried leaves were pulversed and subjected for successive extraction using hexane, chloroform and methanol (LR grade, Merck, India) separately using soxhlet apparatus. The extracts were evaporated to dryness under reduced pressure using a Rotavapor (Buchi Flawil, Switzerland). A portion of the residue was used for the further activities.

Microorganisms and Antibacterial activity

The MRSA strains used in this study were clinical isolates from patients presenting with symptoms of S. aureus associated diseases. The isolates were identified as S. aureus according to colonial and microscopic morphology, positive catalase and coagulase production. All the isolates were tested for meticillin resistance. The disk diffusion method outlined by the National Committee for Clinical Laboratory Standards (NCCLS) was used with a 1 µg oxacillin disk (Oxoid). Zone sizes were read after incubation at 35°C for 24h. Isolates with zone sizes ±10 mm were considered meticillin resistant. The antibacterial activity was determined by the well diffusion method according to NCCLS. Three to five identical colonies from each agar plate were lifted with a sterile wire loop and transferred into a tube containing 5ml of tryptic soy broth (TSB). The turbidity of each bacterial suspension was adjusted to reach an optical comparison to that of a 0.5 McFarland standard, resulting in a suspension containing approximately 1 to 2 x 10⁸ CFU/ml. Mueller-Hinton agar plates were inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking 2 more times, rotating the plate approximately 60° each time to ensure even distribution of the inoculum. As a final step, the rim of the agar was also swabbed. After allowing the inoculum to dry at room temperature, 6 mm diameter wells were bored in the agar. Each extract was checked for antibacterial activity by introducing 50 µl of a 100 mg/ml concentration into triplicate wells. The extracts were dissolved in 10% aqueous
dimethylsulfoxide (DMSO) to a final concentration of 100 mg/ml. Pure DMSO was taken as the negative control and 0.05% Ciprofloxacin as the positive control. The plates were allowed to stand at room temperature for 1 h for extract to diffuse into the agar and then they were incubated at 37°C for 18 h. Subsequently, the plates were examined for bacterial growth inhibition and the inhibition zone diameter (IZD) measured to the nearest millimeter.

**MIC and MBC determination**

To measure the MIC values, micro-broth dilution method was used. The reconstituted extracts was serially diluted 2-fold in Mueller-Hinton broth medium to obtain various concentrations of the stock, 100, 50, 25, 12.5, 6.25, 3.125, 1.562, 0.781 mg/ml and were assayed against the test organism. The minimum inhibitory concentration was defined as the lowest concentration able to inhibit any visible bacterial growth. Equal volume of the various concentration of each extract and Mueller-Hinton broth were mixed in micro-tubes to make up 0.5 ml of solution. 0.5 ml of McFarland standard of the organism suspension was added to each tube. The tubes were incubated aerobically at 37°C for 24 h. Two control tubes were maintained for each test batch. These include tube-containing extract without inoculum and the tube containing the growth medium and inoculum. The MBC was determined by sub culturing the test dilution on Mueller-Hinton agar and further incubated for 24 h. The highest dilution that yielded no single bacterial colony was taken as the minimum bactericidal concentration.

**Biofilm production**

*Staphylococci* are also a common cause of infections related to bacterial biofilm formation on implanted devices. Isolates from fresh agar plates were inoculated in respective media and incubated for 18 h at 37°C in stationary condition and diluted 1 in 100 with fresh medium. Individual wells of sterile, polystyrene, 96 well-flat bottom tissue culture plates (Tarsen, Kolkata, India) wells were filled with 0.2 ml aliquots of the diluted cultures and only broth served as control to check sterility and non-specific binding of media. The tissue culture plates were incubated for 18 h at 37°C. After incubation content of each well was gently removed by tapping the plates. The wells were washed four times with 0.2ml of phosphate buffer saline (PBS pH 7.2) to remove free-floating planktonic bacteria. Biofilm formed by adherent sessile organisms in plate were fixed with sodium acetate (2%) and stained with crystal violet (0.1% w/v). Excess stain was rinsed off by thorough washing with deionized water and plates were kept for drying. Adherent *Staphylococcus* cells usually formed biofilm on all side wells and were uniformly stained with crystal violet. Optical density (OD) of stained adherent bacteria was determined with a micro ELISA auto reader (LISA Plus, Micro plate reader, Aspen Diagnostics Pvt. Ltd, Delhi) at wavelength of 570 nm. These OD values were considered as an index of bacteria adhering to surface and forming biofilms. Experiment was performed in triplicate and repeated three times, the data was then averaged and standard deviation was calculated. To compensate for background absorbence, OD readings from sterile medium, fixative and dye were averaged and subtracted from all test values. The mean OD value obtained from media control well was deducted from all the test OD values.

**Statistical analysis**

The results of the experiment are expressed as mean ± SE of three replicates in each test. The data were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's multiple pairwise comparison tests to assess.
the statistical significance. P≤0.05 was considered as statistically significant, using

RESULTS AND DISCUSSION

The results of antibacterial activity of all the crude leaf extracts of plant revealed that methanol extract showed good antibacterial activity against MRSA used. Whereas chloroform extract showed moderate effect on MRSA strain. On the other hand, the crude hexane extract was weakly effective against the cocci as judged by the zones of inhibition. The MIC and MBC values obtained for the extracts against the MRSA varied from one another. For instance, the MIC values of 25.0 and 50.0 mg/ml were obtained for chloroform and hexane extracts respectively, while the corresponding MBC values were 50.0 and 100.0 mg/ml. The MIC and MBC values of 12.5 and 25.0 mg/ml were recorded for methanol extract. Hence, this extract was bacteriostatic at lower concentrations and bactericidal at higher concentrations as revealed by MIC and MBC values. The inhibition levels of biofilm ranged from OD values 0.26, 0.18 and 0.17 for methanol, chloroform and hexane extracts respectively; whereas standard showed 0.30. Mathur et al.12 explains the relationship between OD values and biofilm inhibition. The capacity is strong if OD value is >0.240, moderate if 0.120-0.240 and weak if <0.120. With reference to this, our methanol extract has got very strong inhibitory capacity which is followed by chloroform and hexane extracts.

Medicinal plants constitute an effective source of both traditional and modern medicines, herbal medicine has been shown to have genuine utility and about 80% of rural population depends on it as primary health care. Over the years, the World Health Organization advocated that countries should interact with traditional medicine with a view of identifying and exploiting aspects that provide safe and effective remedies for ailments of both microbial and non-microbial origins (33). The results of the study indicated that the medicinal plant Kirganelia reticulata commonly used by traditional medical practitioners to cure venereal diseases and a variety of ailments, including smallpox, syphilis were active against hospital strains of MRSA. Previous studies by Shruthi et al.26 indicated that the crude extracts of these plants were effective against S. aureus. The present study correlate to those findings but the only area of concern is that while those studies only dealt with the effect of crude extracts on S. aureus, while this study focused on the effect of crude extract on the MRSA, along with determination of both MIC and MBC values and biofilm inhibition of the extracts. It is worthy of note that traditional medical practitioners used this plant extracts solely without combining with other plant extracts for the treatment of microorganism associated skin and respiratory diseases.

The MIC value of active plant extract obtained in this study were lower than the MBC values suggesting that the plant extracts were bacteriostatic at lower concentration and bactericidal at higher concentration. The methanolic extract exerted greater antibacterial activity than corresponding chloroform and hexane extract at the same concentrations. These observations may be attributed to two reasons; firstly, the nature of biological active components (saponins, tannins, alkaloids and anthraquinone) which could be enhanced in the presence of methanol. It has been documented that tannins, saponins and alkaloids are plants metabolites well known for antimicrobial activity.34 Secondly, the stronger extraction capacity of methanol could have produced greater number of active constituents responsible for antibacterial activity.31 Our investigation further revealed that methanol

http://www.pharmacophorejournal.com/ 127
extract showed good inhibition of biofilm formation by clinical isolates of MRSA. Biofilms are highly resistant to antibiotic treatment.\textsuperscript{35,36,37,38,39} Infections may result in longer hospitalization time, or need for surgery, and they can even cause death. The spread of drug-resistant strains of \textit{Staphylococci} and the ineffectiveness of treatments in cases of biofilm-related infections underscore the necessity to find new modes of prevention and effective alternatives to antibiotic treatment.

Table 1: Antibacterial, MIC, MBC and Biofilm inhibition values of \textit{Kriganelia reticulata}

<table>
<thead>
<tr>
<th></th>
<th>Antibacterial (mm)</th>
<th>MIC (mg/ml)</th>
<th>MBC (mg/ml)</th>
<th>Biofilm OD at 570 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol Extract</td>
<td>17.60±0.13\textsuperscript{**}</td>
<td>12.5</td>
<td>25.0</td>
<td>0.26±0.01\textsuperscript{**}</td>
</tr>
<tr>
<td>Chloroform Extract</td>
<td>14.60±0.19\textsuperscript{**}</td>
<td>25.0</td>
<td>50.0</td>
<td>0.18±0.01\textsuperscript{**}</td>
</tr>
<tr>
<td>Hexane Extract</td>
<td>10.54±0.12\textsuperscript{**}</td>
<td>50.0</td>
<td>100.0</td>
<td>0.17±0.01\textsuperscript{**}</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>19.65±0.11\textsuperscript{**}</td>
<td>6.25</td>
<td>12.5</td>
<td>0.30±0.01\textsuperscript{**}</td>
</tr>
</tbody>
</table>

The values are the mean of triplicates ± S.E. * P<0.05, ** P<0.01 compared to standard.

CONCLUSION

Our results therefore offer a scientific basis for the traditional use of plant \textit{Kriganelia reticulata} as a potential phytotherapeutic agent. The antimicrobial activities could be enhanced if the active components are purified and adequate dosage determined for proper administration, which is therefore employed in our further studies.

REFERENCES


http://www.pharmacophorejournal.com/


http://www.pharmacophorejournal.com/
PHYSICAL AND ANTIOXIDANT ANALYSIS OF LEAF EXTRACTS FROM *KIRGANELIA RETICULATA* BAILL.

SHRUTHI SD1,2*, RAJESWARI A1, GOVARDHANA RAJU K1, PAVANI A1, VEDAMURTHY AB1 and Ramachandra YL2

1P.G. Department of Biotechnology, The Oxford College of Science, Bangalore- 560 102, Karnataka, India; 2Department of P.G. Studies and Research in Biotechnology, Kuvempu University, Shankaraghatta - 577 451, Karnataka, India. Email: sdsshruthi@gmail.com

Received: 08 Feb 2012, Revised and Accepted: 27 Mar 2012

ABSTRACT

In the present study we carried out a systematic record of the phytochemical and antioxidant properties of the medicinal plant *Kirkangelia reticulata*. The different solvent extracts of *Kirkangelia reticulata* leaves were screened for their *in vitro* phytochemical and antioxidant activity. Leaves were extracted with solvents of different polarities like aqueous, ethanol, methanol, chloroform, acetone and hexane. The distributions of the main active principles such as alkaloid, flavonoids, phenols, steroids tannins etc present in the plant were analyzed. It was also focused to determine the total phenol and flavonoid content present in the extracts. Extracts showed promising results for total antioxidant capacity and reductive capability when compared with standard drug. The ethanolic extract was found to possess excellent phytochemical and antioxidant activities. The antioxidant property may be attributed to the presence of flavonoids and phenolics present in the drug. The ability of the crude extracts of *Kirkangelia reticulata* towards reduction, presence of phenol, flavonoid and antioxidant is an indication of its broad spectrum potential which may be employed in the management of various diseases.

Keywords: Antioxidant, *Kirkangelia reticulata*, Phytochemical, Total phenolics, Total flavonoids

INTRODUCTION

The plants that possess therapeutic properties or exert beneficial pharmacological effects on the animal body are generally designated as "Medicinal plants". Medicinal plants are the local heritage with global importance. World is endowed with a rich wealth of medicinal plants. These plants have made a good contribution to the development of ancient materia medica. Drugs of herbal origin have been used in traditional systems of medicine such as Unani and Ayurveda since ancient times. The drugs are derived either from the whole plant or from different organs like leaves, stem, bark, root, flower, seed etc. These medicines are safer and environment friendly.

*Kirkangelia reticulata* (*Phyllanthus reticulatus*) belongs to family Euphorbiaceae, grows through tropical areas of India, China, Bangladesh and Malay islands. It is a monoeocious scendent shrub present in hill areas and plain lands. The juice of these leaves is used to cure diarrhoea in infants. The leaves are employed as diuretic and cooling medicine. The stems are used to treat sore eyes and the powdered leaf is used in sores, burns, suppurations and chafing of the skin. The bark is used to treat rheumatism, dysentery and veneral diseases. The bark is also used for a variety of ailments including small pox, syphilis, asthma, diarrhoea, bleeding from gums. The biological work performed so far on the plant showed hypotensive effects in gastric complaints including colic, constipation etc and chemical studies demonstrated the presence of octacosanol, telexer acetate, berulint, sitosterol etc. It is believed to have antidiabetic activity in tribal areas. The antibacterial potential of the leaf extracts of this plant has been evaluated recently. Pharmacognostic parameters like microscopy, quantitative leaf microscopy, fluorescence, physicochemical properties are studied. Different chemical compounds such as tannins, flavonoids, glycosides, and alkaloids serves as a sink for several bioactive compounds.

Phytochemicals are chemical compounds, occur naturally in plants. Phytochemicals have been used as drugs for millennia. Phytochemicals in fruits and vegetables may reduce the risk of cancer, possibly due to dietary fibers, polyphenol antioxidants and anti-inflammatory effects. An antioxidant is a molecule capable of inhibiting the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Antioxidants are widely used as ingredients in dietary supplements and have been investigated for the prevention of diseases such as cancer, coronary heart disease and even altitude sickness. These compounds have many industrial uses, such as preservatives in food and cosmetics and preventing the degradation of rubber and gasoline. Flavonoids are also found to be powerful anti-oxidants and researchers are looking into their ability to prevent cancer and cardiovascular diseases.

So the present study is focused towards the identification of secondary metabolites present, estimation of flavonoids, phenolics and antioxidants present in the plant. Hence, an effort has been made to explore the above properties of herbal extracts which proves the potency of the plant as a source of natural antioxidants or nutraceuticals with application to reduce oxidative stress with consequent health benefits.

MATERIALS AND METHODS

Fresh leaf materials of *Kirkangelia reticulata* plant were collected in summer season in and around HSR layout, Bangalore, Karnataka (Southern India). The taxonomic identification of the plant was confirmed and processed for further investigations.

Extraction of plant material

The collected leaves were washed thoroughly with distilled water. Cleaned leaves were then air-dried in shade at room temperature (26°C) for 2 weeks, after which it was ground to uniform powder. About 1gm of dry powdered plant materials were soaked in 10 ml of aqueous, ethanol, methanol, chloroform, acetone and hexane (LR grade, Merck, India) at room temperature for 48 h. The extracts were filtered first through a Whatmann filter paper No. 42 (125mm) and then through cotton wool. The extracts were concentrated using rotary evaporator (Buchi Flawil, Switzerland). The percentage yield of extracts ranged from 7–19% (w/w).

Phytochemical screening

Phytochemical screenings were performed for carbohydrates, alkaloids, phytosterols, glycosides, deoxy sugars, saponins, phenolics, tannins, flavonoids and gums using standard procedures. Carbohydrates: The presence of carbohydrates was determined by Benedict’s method. Alkaloids: Mayer’s test was performed to test alkaloids. Phytosterols: Phytosterols present was tested by Salkowski’s method. Glycosides: The presence of glycosides was determined by Legal’s test.
Deoxy sugars: Keller-Kiliani test was used for determination of Deoxy sugars

Saponins: Saponins were determined by Froth method

Phenolics and tannins: Ferric chloride test was conducted to determine the presence of it.

Flavonoids: Lead acetate test was used for flavonoids.

Gums test: Presence of gum was determined by Borax method

Determination of total phenolics

The total phenolic content in the extract was determined with Folin-Ciocalteu's reagent (FCR). 0.5ml of extract was mixed with 2ml of FCR reagent and 2ml of sodium carbonate. The tubes were kept at room temperature for 90 mins. Absorbance of sample was measured against the blank at 750 nm using a spectrophotometer. A calibration curve was constructed using gallic acid as standard (20mg/100ml in distilled water, working standard taken as 10ml stock made up to 100ml with distilled water). The phenolic content was expressed in terms of milligrams of gallic acid.

Determination of total flavonoids

1ml of test sample was taken and 4ml of water was added. 0.3ml of Sodium nitrate, 0.3ml of aluminium chloride was added. After 6min incubation at room temperature, 2ml of NaOH was added to the reaction mixture, made the volume to 10ml by adding distilled water. The absorbance of the solution was measured at 510nm using a spectrophotometer. Catechin hydrate was used as standard (20mg/100ml in distilled water). Total flavonoids were expressed as catechin equivalents in milligrams.

Determination of total antioxidant capacity

The antioxidant activity of the extract was determined by the phosho molybdenum method. The 0.3ml of extract was combined with 3ml of reagent solution (0.6M sulphuric acid, 4mM ammonium molybdate, 28mM sodium phosphate). The reaction mixture was incubated at 95°C for 90 min and cooled to room temperature. The absorbance of the solution was measured at 695nm using a spectrophotometer against blank. Ascorbic acid was used as standard (1mg/ml in distilled water). The total antioxidant capacity was expressed as the number of equivalents of ascorbic acid (AAE).

Total reductive capability (Fe\(^{2+}\) - Fe\(^{3+}\) transformation)

Different concentrations of extracts were mixed with 2.5ml of 0.2M phosphate buffer (pH 6.6). 2.5ml of 4% potassium ferri cyanide and 2.5ml of 10% trichloroacetic acid, which is then centrifuged at 660rpm for 10 min. The upper layer of the solution was mixed with 2.5ml distilled water and 0.5ml of 0.1%FeCl\(_3\). The amount of iron (II) ferri cyanide complex formed was determined by measuring absorbance of the reaction mixture at 700nm in spectrophotometer. Increased absorbance indicates increased reductant capability. Fe (III) reduction is often used as an indicator of electron donating activity. Quercetin was used as standard (25mg/ml in distilled water).

Statistical analysis

All the experiments are carried out in triplicates. The values were expressed as mean ± SEM. Statistical analysis of data was performed using ANOVA followed by student t-test to study the differences amongst the means. Values of P < 0.05 were considered as statistically significant.

RESULTS AND DISCUSSION

The phytochemical analysis carried out on the plant revealed the presence of several medicinally active constituents. Ethanolic extract has shown to contain large number of secondary groups such as carbohydrates, alkaloids, phenols, flavonoids, saponins, gums, glycosides etc. The results of which are shown in table 1. It may be attributed to the reason that the stronger extraction capacity of ethanol could have extracted a greater number of constituents. These compounds are known to be biologically active and hence aid in the investigation of several activities. These observations therefore support the use of K. reticulata in herbal cure remedies. Alkaloids were detected together with flavonoids; this may be responsible for the antioxidant activity observed in the crude extracts. According to van Beek et al. 15, who studied a large number of plant extracts against various activities, ethanolic extracts always show positive effect. The preliminary phytochemical evaluation revealed the presence of several secondary metabolites which are known to possess various pharmacological effects. In last four decades, the scientists are keen to evaluate many plant drugs used in medicinal folklore, due to their specific healing properties, health action and non toxic effects16.

Although similar to alcohol, phenolics have unique properties and are not classified as alcohol; since the hydroxyl group is not bonded to saturated carbon atom. They have higher acidities due to the aromatic ring's tight coupling with the oxygen and a relatively loose bond between the oxygen and hydrogen. Some phenols are germicidal and are used in formulating disinfectants where as others possess estrogenic or endocrine disrupting activity. So far as plant phenolics constitute one of the major groups of compounds acting as primary antioxidants or free radical terminators, it was reasonable to determine their total amount in the selected plant extracts17.

The content of total phenols in the extracts expressed in gallic acid equivalents (GAE) varied between 1.79±0.02, 1.69±0.03 and 0.59±0.02 mg/g in ethanol, acetone and hexane extracts respectively (Fig 1). The phenols contain hydroxyls that are responsible for the radical scavenging effect mainly due to redox properties. According to our study, the high phenolic content in ethanol extract can explain its high free radical scavenging activity.

Flavonoids are readily ingested by humans and they seem to display important anti-inflammatory, anti-allergic and anti-cancer activities. They are also found to be powerful anti-oxidants and researchers are looking into their ability to prevent cancer and cardiovascular diseases. Flavonoids are most commonly known for their antioxidant activity.

Flavonoids acting as a chain breaking antioxidant impairs with the formation of free radicals in the process of formation of intracellular substances throughout the body, including collagen, bone matrix and tooth dentine. The quantitative determination of flavonoids in plant extracts shows that they are good source of flavonoids. High quantity of it was found to be 0.6±0.02 mg/g in ethanol extract which is followed by acetone 0.4±0.01, whereas in hexane extract it was recorded to have the least value of 0.1±0.01 mg/g (Fig 2).

A variety of intrinsic antioxidants (SOD, CAT, peroxidase, reduced glutathione) are present in the organism, which protect them from oxidative stress, thereby forming the first line of defense. Furthermore, the antioxidant activities of putative antioxidants have been attributed to various mechanisms; among these are prevention of chain initiation, binding of transition metal ion catalyst, decomposition of peroxides, prevention of continued hydrogen abstraction, and radical scavenging. As stated before, numerous polyphenols are known to possess excellent antioxidant effects, especially in vitro and the amount of polyphenols present in a plant extracts has been suggested to correlate with the antioxidant activity. For example, Chinnici et al.17 and Leontowicz et al.18 found good correlations between total polyphenols and the total antioxidant activity. In our study, however, same correlation was found, showing that total antioxidant present in ethanol extract was high 0.5±0.01, followed by acetone 0.4±0.05 and hexane 0.4±0.03 extracts compared to standard as shown in fig 3.

The reducing power can serve as a significant reflection of the antioxidant activity. The reducing properties are generally associated with the presence of reductones. Gordon19 reported that the antioxidant action of reductones is based on the breaking of the free radical chain by the donation of a hydrogen atom. Increasing absorbance at 700 nm indicates an increase in the reducing activity. Ethanolic and acetone extracts showed highest reductant activity such as 0.3±0.04 and 0.32±0.03, whereas hexane extract 0.12±0.02 which was not active considerably. But all the extracts comparatively showed very lesser reducing activity than the standard Quercetin. The results of which are shown in fig 4.

609
However, more detailed analytical information on the constituents mediating the observed biological effects is needed prior to the promotion or development of effective and safe foods for human consumption.

Table 1: Phytochemical investigations of *Kirganeila reticulata*

<table>
<thead>
<tr>
<th>Secondary Metabolites</th>
<th>Extracts</th>
<th>Aqueous</th>
<th>Ethanol</th>
<th>Methanol</th>
<th>Chloroform</th>
<th>Acetone</th>
<th>Hexane</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrates</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Phytosterols</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Deoxy sugars</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Saponin</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Phenol</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tannin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Proteins</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Amino acids</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gum</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glycosides</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Antraquinone glycosides</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

'+'-present, '-'-absent

![Graph 1](image1.png)

**Fig. 1:** Total phenolic content present in the leaf extracts of *Kirganeila reticulata*. Values are expressed in Mean ± SE, n=3.

![Graph 2](image2.png)

**Fig. 2:** Total flavonoid content present in the leaf extracts of *Kirganeila reticulata*. Values are expressed in Mean ± SE, n=3.
CONCLUSION
The systemic research for useful bio actives from the plants is now considered to be a rational approach in nutraceuticals and drug research. The results of phytochemical analysis comprehensively validate the presence of therapeutically important and valuable secondary metabolites. Along with, it confirms the ethnobotanical claim of the plant to be a potential antioxidant. Hence, the plant contains good store of antioxidants and essential metabolites to support its efficiency to be a drug. It can be recommended as dietary supplement there by the nutritive potential indulged by the plant is yet to explore.

REFERENCES


Isolation, characterization, antibacterial, antihelminthic, and in silico studies of polyprenol from Kirganelia reticulata Baill

S. D. Shruthi, S. Padmalatha Rai & Y. L. Ramachandra
Your article is protected by copyright and all rights are held exclusively by Springer Science +Business Media New York. This e-offprint is for personal use only and shall not be self-archived in electronic repositories. If you wish to self-archive your work, please use the accepted author's version for posting to your own website or your institution's repository. You may further deposit the accepted author's version on a funder's repository at a funder's request, provided it is not made publicly available until 12 months after publication.
Isolation, characterization, antibacterial, antihelminthic, and in silico studies of polyrenol from *Kriganelia reticulata* Baill

S. D. Shruthi · S. Padmalatha Rai · Y. L. Ramachandra

Received: 8 March 2012 / Accepted: 20 October 2012 © Springer Science+Business Media New York 2012

**Abstract** Microbial and helminthes infections are the most common health problems in India; in developing countries they pose a large treat to public. These infections can affect most population in endemic areas with major economic and social consequences. In vitro antibacterial activity of a phytoconstituent, polyrenol isolated from the leaves of *Kriganelia reticulata* was screened against Gram-negative and Gram-positive bacteria. The minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC) of the purified antibacterial agent were also determined. The present study also undertook evaluation of antihelminthic activity of polyrenol at different concentrations using *Pheretima posthuma* as a test worm. The bioassay involved the determination of paralysis time and death time of the worm. The polyrenol showed antibacterial activity against different bacterial strains with MIC 6.25–25.0 mg/ml and MBC 12.50–25.0 mg/ml, and possesses better glucosamine-6-phosphate synthase inhibition in molecular docking studies with minimum docking energy and better ligand efficiency when compared to the standard. The antihelminthic results of the present study indicated that polyrenol significantly demonstrated paralysis and caused death of worm in dose-dependent manner; showing comparable results with the standard in docking process. Hence, the isolated compound polyrenol is suggestive of being a good antibacterial and antihelminthic agent.

**Keywords** Glucosamine-6-phosphate synthase · *Kriganelia reticulata* · Molecular docking · Polyrenol · Tubulin protein

**Introduction**

Human beings have been in constant exposure to pathogenic bacteria for many decades. The increasing interest on traditional ethnomedicine may lead to discovery of novel therapeutic agents. Many of the plant species have been documented pharmacologically and clinically in the world, which are endowed phytochemicals with marked activity on human pathogenic bacteria (Khan et al., 2002).

*Kriganelia reticulata* Baill. (Synonym: *Phyllanthus reticulatus* Poir.) is a large, often scattend, shrub of the family Euphorbiaceae. The leaves and bark are used as astringent and diuretic. Juice of leaves is used for the treatment of diarrhea in children (Ghani, 2003). The bark showed significant antiviral (Renuka et al., 1998) and antiplasmodial activity (Omulekoli et al., 1997). The antibacterial potential of the aerial parts of this plant has been evaluated (Direkbusarakom et al., 1998). The bark is used to treat rheumatism, dysentery, and venereal diseases (Yoganarasimhan, 1996). The plant is used for a variety of ailments, including smallpox, syphilis, asthma, diabetes, and bleeding from gums (Nandkarni, 1982; The Wealth of India 2005). It is also claimed to have antidiabetic activity in tribal areas, which has been validated by Kumar et al. (2008). The antibacterial potential of the leaf extracts of this plant has been evaluated recently (Shruthi et al. 2010a, b).
Plant Polyprenols are biologically active and have low toxicity. PP has been proved to be able to lower tumor cell resistance to chemotherapy in vivo. Polyprenol opens up possibilities for targeted regulation of drug resistance. Polyprenol named ficaprenol-11 is isolated from methanolic extract of Leucaena leucocephala (Chen and Wang, 2010).

For a long time, plants have been an important source of natural products for human health. The antimicrobial properties of plants have been investigated by a number of studies worldwide and many of them have been used as therapeutic alternatives because of their antimicrobial properties (Adriana et al., 2007). Thus, searching not only for improved versions of existing drugs but also for new drug targets has become an urgent need. The key enzyme γ-glutamyl: δ-fructose-6-phosphate amidotransferase, known under the trivial name of glucosamine-6-phosphate synthase (EC 2.6.1.16) is responsible for the synthesis of glucosamine-6-phosphate (GlcN-6-P) from δ-fructose-6-phosphate and γ-glutamine. This enzyme is the first in the pathway leading to the formation of UDP-N-acetylglucosamine (UDP-GlcNAc), a product that is present in all types of organisms, but is used by these organisms in different ways (Bates and Pasteurak, 1965; Imada et al., 1977). In prokaryotes it is used to build macromolecules important for the cell wall assembly, such as chitin, mannanproteins, and peptidoglycan. In mammals, UDP-GlcNAc is utilized for biosynthesis of glycoproteins and mucopolysaccharides (Wojciechowski et al., 2005). In spite of the fact that glucosamine-6-phosphate synthase is present in all kinds of cells, it may be exploited as a target for potential antimicrobial drugs and selective toxicity can be achieved (Chmara et al., 1984). Glucosamine-6-phosphate, the product of this enzyme, is indispensable for microbes as well as for human cells, yet the consequences of its deficiency in both species are very different. It has been shown that even a short time inactivation of GlcN-6-P synthase in bacteria is lethal for the pathogen by inducing morphological changes, agglutination, and lysis, while in mammals depletion of the aminosugar pool for a short time is not lethal, because of the much longer lifespan of mammalian cells, long half lifetime of GlcN-6-P synthase, and rapid expression of the mammalian gene encoding this enzyme (Bates et al., 1966; Chmara and Borowski, 1986; Milewski et al., 1986).

Helminth infections are commonly found in community and being recognized as cause of much acute as well as chronic illness among the various human beings as well as cattle’s. More than half of the population of the world suffers from various types of infection and majority of cattle’s suffers from worm infections (Chaturvedi et al., 2009). Development of new efficient and low-toxic anti-helmintic preparations is a current and important problem of modern pharmacology (Kosulina et al., 1997). Tubulin protein being involved in many cellular functions is a crucial drug target for nematodes. It has been demonstrated by several investigators that β-tubulin protein of the worm could be one of the major targets for the anthelmintic activity of the drug (Anderson et al., 2001). Microtubules present in helminthic parasites as well as in other eukaryotic organisms, which are hollow cylindrical in shape, are constructed from linear chains or protofilaments of repeating subunits of α and β-tubulin. Lower eukaryotes such as protozoa and helminthes have different sensitivities to microtubule perturbing agents in contrast to higher eukaryotes (Wampande et al., 2007). Existing anti-tubulin drugs such as Albendazole (ABZ), Mebendazole (MBZ), Benzimidazole (BZs), and Flubendazole are commonly used drugs with broad spectrum of activity against a range of different parasites which are also widely used for the treatment of nematode infection with low bioavailability (Merino et al., 2005). Tubulin has been exploited as a target for anthelminthic (Hugdaul and Morejohn, 1993; Coulaud and Rossignol, 1984; Henriques et al., 2008) and anti-protozoal compounds.

The objective of this study was to investigate the antibacterial and anthelmintic effects of polypropenol isolated from traditionally proven plant and compare the mode of interactions existing, in the hunt of better therapies against microbial diseases and provide scientific evidence to folkloric claim of the plant.

Materials and methods

Plant materials and preparation of extract

Fresh leaf materials of K. reticulata were collected in winter season locally from Bhadra Wild Life Sanctuary, Karnataka (Southern India) in Dec 2011. The taxonomic identification of the plant was confirmed by Dr. Y. L. Ramachandra, Department of Biotechnology, Kuvempu University, Shankaraghatta (Voucher specimen number YLR429). Collected leaves were shade-dried and then powdered using a mechanical grinder (Sieve No. 10/44). Then subjected for successive extraction using hexane, chloroform, and methanol (LR grade, Merck, India) separately using soxhlet apparatus. The extracts were evaporated to dryness under reduced pressure using a Rotavapor (Buchi Flawil, Switzerland). Obtained chloroform extract was used for isolation of polypropenol (PP). A portion of the PP was used for the antibacterial and anthelmintic assays.

Isolation of polypropenol

The chloroform soluble fraction was fractionated by column chromatography (CC) over silica gel (60–120 mesh) using n-hexane and ethyl acetate mixtures of increasing polarities to give 50 fractions, collecting each 25 ml. Preparative thin
layer chromatography (stationary phase-silica gel F254, mobile phase –30 % ethylacetate in hexane, thickness of plates 0.5 mm) of fractions 18–20 afforded compound polypropen. The PP was characterized by subjected to IR, NMR, MASS spectral analysis, and TLC (ethylacetate:hexane, 3:7) with iodine vapor as the detector.

Microorganisms and antibacterial activity

Stock cultures of Gram-negative organisms (Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi) and Gram-positive organism (Staphylococcus aureus) were obtained and confirmed at the research laboratory of the Department of Microbiology, Kuvempu University, Karnataka. They were maintained on Mueller–Hinton Agar (Himedia, Mumbai) slope at 4 °C and sub-cultured into Mueller–Hinton broth by a picking-off technique (Aneja, 2003). Twenty-four hour old pure cultures were prepared for use each time.

In vitro antibacterial activity of the polypropen was studied against Gram-negative and Gram-positive bacteria by the agar well diffusion method (Nair et al., 2005). The compound was dissolved in 10 % aqueous dimethylsulfoxide (DMSO) to a final concentration of 100 mgml⁻¹. Pure DMSO was taken as the negative control and 0.05 % Ciprofloxacin was taken as the positive control. Mueller–Hinton Agar (Himedia, Mumbai) was used as the bacteriological medium. It was prepared according to the manufacturer’s instruction, and autoclaved and dispensed at 20 ml per plate in 12 × 12 cm petri dishes. Set plates were incubated overnight to ensure sterility before use. Suspension of microorganisms was made in sterile normal saline and adjusted to 0.5 Macfarland standards (108 Cfu/ml) (NCCLS, 2000). Each labeled medium plate was uniformly inoculated with a test organism using a sterile cotton swab rolled in the suspension to streak the plate surface in a form that lawn growth can be observed. A sterile cork borer of 5 mm diameter was used to make wells on the medium. 100 µl of the polypropen concentration and control compound were dropped into each, appropriate labeled well (Atata et al., 2003; Shahidi Bonjar, 2004). The inoculated plates were kept in the refrigerator for 1 h to allow the compounds to diffuse into the agar (Atata et al., 2003). The Mueller–Hinton Agar plates were incubated at 37 °C for 24 h. Antibacterial activity was determined by measuring the diameter of zones of inhibition (mm) produced after incubation.

MIC and MBC determination

In order to measure the MIC values, micro-broth dilution method was used (Abu-Shanab et al., 2006). The polypropen concentration was serially diluted twofold in Mueller–Hinton broth medium to obtain various concentrations of the stock, 100, 50, 25, 12.5, 6.25, 3.125, 1.562, and 0.781 mgml⁻¹ and was assayed against the test organism. The minimum inhibitory concentration was defined as the lowest concentration able to inhibit any visible bacterial growth. Equal volume of the polypropen concentration and Mueller–Hinton broth were mixed in micro-tubes to make up 0.5 ml of solution. 0.5 ml of McFarland standard of the organism suspension was added to each tube (Shahidi Bonjar, 2004). The tubes were incubated aerobically at 37 °C for 24 h. Two control tubes were maintained for each test batch. These include tube containing polypropen without inoculum and the tube containing the growth medium and inoculum. The MBC was determined by sub culturing the test dilution on Mueller–Hinton agar and further incubated for 24 h. The highest dilution that yielded no single bacterial colony was taken as the minimum bactericidal concentration (Akinyemi et al., 2005).

Antihelmintic activity

The antihelmintic activity of polypropen was evaluated as per the method reported by Dash et al. (2002). Indian adult earthworms Pheretima posthuma collected from moist soil and washed with normal saline to remove all fecal matter were used for the study. The earthworms of 4–6 cm in length and 0.3–0.4 cm in width were used for all experimental protocol due to its anatomical and physiological resemblance with intestinal roundworms parasite of human beings (Vigar, 1984). Because of easy availability, earthworms have been used widely for the initial evaluation of antihelmintic compounds in vitro (Jain and Jain, 1972; Martin, 1997; Suresh et al., 2002). Polypropen and the standard drug piperazine citrate (SD Fine Chemicals Ltd., Mumbai) were dissolved in 0.5 % DMSO in normal saline (v/v) and used for evaluation. Various concentrations (50, 100, 150, and 200 mgml⁻¹) of polypropen were tested in the bioassay, which involved determination of time of paralysis and time of death of the worms. Piperazine citrate was included as the standard reference and saline water as control. The antihelmintic assay was carried as per the method of (Ajaiyeoba et al., 2001) with minor modifications. Six groups of animals with three earthworms in each group, each earthworm were separately released into 20 ml of different concentrations in normal saline, Group I earthworm were released in 20 ml normal saline in a clean petriplate and were maintained as control. Group II, III, IV, and V earthworms were released in 50, 100, 150, and 200 mgml⁻¹ of polypropen in 20 ml of normal saline, respectively. Group VI earthworms were released in normal saline containing the standard drug piperazine citrate (50 mgml⁻¹). Time for paralysis was noted when no movement of any sort could be observed except when the
worms were shaken vigorously. Death was concluded when the worms lost their motility followed with fading away of their body colors.

Molecular docking studies

Automated docking was used to determine the orientation of inhibitors bound in the active site of GlcN-6-P synthase and tubulin as targets for antibacterial and antihelminthic activities, respectively. A Lamarckian genetic algorithm method, implemented in the program AutoDock 3.0, was employed. The ligand molecules polypropenol, ciprofloxacin, and piperazine citrate were designed and the structure was analyzed using ChemDraw Ultra 6.0. 3D coordinates were prepared using PRODRG server (Ghose and Crippen, 1987). The protein structure file (PDB ID: 1XFF for GlcN-6-P synthase and 1SA0 for tubulin) was taken from PDB (www.rcsb.org/pdb) and was edited by removing the heteroatoms and adding C terminal oxygen (Binkowski et al., 2003). For docking calculations, Gasteiger–Marsili partial charges (Gasteiger and Marsili, 1980) were assigned to the ligands and ion-polar hydrogen atoms were merged. All torsions were allowed to rotate during docking. The grid map was centered at particular residues of the protein which was predicted from the ligplot and were generated with AutoGrid. The Lamarckian genetic algorithm and the pseudo-Solis and Wets methods were applied for minimization, using default parameters (Vidya et al., 2011).

Statistical analysis

The values were expressed as mean ± SEM. Statistical analysis of data was performed using ANOVA followed by student t test to study the differences among the means (Nitha et al., 2007). Values of $P < 0.05$ were considered as statistically significant, using software ezANOVA ver. 0.98.

Results and discussion

The chloroform extract was subjected to column chromatography to furnish orangish red colored waxy mass of PP (Fig. 1). The compound with molecular weight 291 was evidenced by pseudo molecular ions at $m/z$ 293 for [M+H]$^+$ ion in the positive mode APCI-MS and at $m/z$ 291 for [M−H]$^-$ ion in the negative mode APCI-MS. In the $^1$H-NMR spectrum it showed the presence of methyl groups by exhibiting peaks at $\delta$ 0.8 and 0.9. The peak at $\delta$ 1.29 was due to methylene protons, peak at $\delta$ 1.6 and 1.7 were due to methyl groups, and peak at $\delta$ 2.1 and 2.30 were due to $\alpha$-methylene protons of the unsaturated systems. The signals at $\delta$ 2.80 and 3.60 were due to methylene protons attached to oxygen function and set of protons at $\delta$ 4.30, 5.1, and 5.40 were due to unsaturated protons. The $^{13}$C-NMR spectra revealed peaks between $\delta$ 124.34 and 135.00 range, indicating the presence of four (C=C) double bonds. The signals at $\delta$ 51.41 and 61.25 show the presence of two methylene carbon atoms attached to an oxygen atom. The presence of methyl groups was evidenced by the presence of signals at 14.10 and 14.26. The bunch of signals at 16.02, 19.74, 20.58, 22.64, 22.71, and 23.43 represents CH$_2$ group, signals at 32.27, 34.15, 34.44, 37.36, and 39.77 represents CH group, and signal at 51.41 represents carbon atoms attached to unsaturated carbon atoms. From these experimental evaluations the compound was identified to be polypropenol.

The tested compound polypropenol showed varying degree of antibacterial activities against the bacterial species. The activities of PP were compared favorably with the standard antibiotic (Ciprofloxacin) which is a well-known broad-spectrum antibacterial agent. The results obtained for the antibacterial tests by the agar diffusion method are presented in Table 1. The inhibition zone for $P. aeruginosa$ was much high, which was followed by $S. typhi$ and $S. aureus$, whereas $E. coli$ showed much less comparably. Ciprofloxacin, which was used as a positive experimental control against all bacterial strains assayed, produced significant zone of inhibition, while no inhibitory effect could be observed for DMSO, used as negative control. The minimum inhibitory concentration (MIC) values determined by broth dilution method indicated significant antibacterial activity at 6.25 and 25.0 mgml$^{-1}$ (Table 2), whereas the minimum bactericidal activity (MBC) for

<table>
<thead>
<tr>
<th>Test bacteria</th>
<th>Gram strain</th>
<th>Polypropenol</th>
<th>Ciprofloxacin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus aureus</td>
<td>+</td>
<td>9.10 ± 0.21**</td>
<td>18.13 ± 0.20</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>−</td>
<td>14.67 ± 0.23**</td>
<td>31.83 ± 0.15</td>
</tr>
<tr>
<td>Salmonella typhi</td>
<td>−</td>
<td>13.07 ± 0.27**</td>
<td>29.00 ± 0.15</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>−</td>
<td>5.60 ± 0.17**</td>
<td>10.17 ± 0.23</td>
</tr>
</tbody>
</table>

The values are the mean of triplicates ± SE
* $P < 0.05$ are considered significant compared to the standard.
Table 2 The MIC and MBC regimes of polypropen against various bacterial strains (μg/ml)

<table>
<thead>
<tr>
<th>Test bacteria</th>
<th>Polypropen (μg/ml)</th>
<th>Ciprofloxacin (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC</td>
<td>MBC</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>12.5</td>
<td>25.0</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>6.25</td>
<td>12.5</td>
</tr>
<tr>
<td>Salmonella typhi</td>
<td>6.25</td>
<td>12.5</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>12.5</td>
<td>25.0</td>
</tr>
</tbody>
</table>

Table 3 In vitro antihelmintic activity of polypropen against *Pheretima posthuma*

<table>
<thead>
<tr>
<th>Test samples</th>
<th>Concentration (mg/ml)</th>
<th>Time taken for paralysis (min)</th>
<th>Time taken for death (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (normal saline)</td>
<td>50</td>
<td>63.33 ± 0.87</td>
<td>198.33 ± 2.61</td>
</tr>
<tr>
<td>Polypropen 50</td>
<td>51.57 ± 0.47**</td>
<td>81.7 ± 0.52**</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>43.37 ± 1.27**</td>
<td>55.13 ± 0.69**</td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>39.63 ± 1.94*</td>
<td>48.50 ± 0.35**</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>36.17 ± 0.48</td>
<td>44.47 ± 0.43*</td>
<td></td>
</tr>
<tr>
<td>Piperazine citrate</td>
<td>50</td>
<td>32.44 ± 0.56</td>
<td>40.72 ± 0.61</td>
</tr>
</tbody>
</table>

The values are the mean of triplicates ± SE
* P < 0.05 are considered significant compared to the standard

Fig. 2 a Orientation of polypropen in the active pocket of GlcN-6-P synthase.
b Enfolding of ciprofloxacin in active pocket. c Interacting amino acids as predicted from the ligplot.
Table 4 Molecular docking results with glucosamine-6-phosphate synthase

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Binding energy</th>
<th>Docking energy</th>
<th>Inhibitory constant</th>
<th>Internel energy</th>
<th>H-bonds</th>
<th>Bonding</th>
</tr>
</thead>
<tbody>
<tr>
<td>PP</td>
<td>-4.78</td>
<td>-8.42</td>
<td>0.000316</td>
<td>-8.2</td>
<td>2</td>
<td>PP::DRG1:OAT:GPS:A:ARG22:HH21</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PP::DRG1:OAA:GPS:B:ARG202:HH22</td>
</tr>
<tr>
<td>CF (std.)</td>
<td>-10.26</td>
<td>-10.79</td>
<td>3.02e-008</td>
<td>-11.19</td>
<td>2</td>
<td>CF::DRG1:OAB:GPS:A:ARG201:HN</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CF::DRG1:OAA:GPS:B:ARG201:HE</td>
</tr>
</tbody>
</table>

Fig. 3 a Orientation of polypropenol in the active pocket of tubulin. b Enfolding of piperazine citrate in active pocket. c Interacting amino acids as predicted from the ligplot.

documented in Table 4. Practically, PP showed good docking energy and ligand efficiency compared to the standard. The PP was completely enfolded in the entire active pocket of GlcN-6-P synthase (Fig. 2a) as compared to ciprofloxacin (Fig. 2b). The topology of the active site of GlcN-6-Psynthase was similar in both PP and standard, which is lined by interacting amino acids as predicted from the ligplot (Fig. 2c). The earlier investigations (Isupov et al., 1996) noticed that the catalytic nucleophile in glutaminase domain of bacterial glucosamine 6-phosphate synthase, and the nucleophilic character of its thiol group appears to be increased through general base activation by its own alpha-amino group. Similar results are also obtained by Vidya et al. (2011) where they have used plant derived compound as ligand for antibacterial docking studies. By in silico analysis, it seems that PP is promoting the remarkable antibacterial activity through the inhibition of GlcN-6-P synthase. Hence, polypropenol has been proved to be one of the potent antibacterial agents.

Molecular docking of PP into the catalytic site of tubulin was performed using AutoDock3.0. The best docked conformation of ligand-receptor complex was determined based on the lowest interaction energy and binding free energy and compared with the standard drug piperazine citrate (Fig. 3). The binding site residues responsible for hydrogen bond interaction and van der Waal residues are
summarized in the Table 5. PP has shown better conformation (Fig. 3a) than the standard (Fig. 3b) in exhibiting the lowest energy requirement and also one hydrogen bond is formed in both the cases. The interacting amino acids of tubulin are predicted from ligplot (Fig. 3c). These machine learning systems could clearly reduce the cost involved in experimental methods involved in drug discovery pipeline. To best of our knowledge, there are not many reported studies on the prediction of antihelminthic compounds. Marrero-Ponce et al. (2004) used linear discriminant analysis to classify antihelminthic drug-like from non-antihelminthic compounds. In other study robust machine learning model was built to classify and screen compounds active against parasitic nematodes using support vector machine (SVM) algorithm (Khanna and Ranganathan, 2011). Kopp et al. (2009) have recently reported changes in the expression of hookworm nicotinic receptor subunit mRNAs to assess antihelminthic efficacy and receptor function (Williamson et al., 2009). This work also paves the way to an accelerated understanding of the potential development of parasite-specific target based drug.

**Conclusion**

In conclusion, the pyprenol showed remarkable antibacterial activity, in assay conditions, on all microorganisms studied, whereas it has also presented to be having the best antihelminthic activity. It has demonstrated a dose-dependent efficacy in both the activities. More study is required on its effects in vivo and to also evaluate its biosafety and clinical potentials. However, experimental validation of the predicted compound in this direction is needed.

**References**


Henriquez FL, Ingram PR, Muench SP, Rice DW, Roberts CW (2008) Molecular basis for resistance of *cauchamoeba* to tubulins at all major classes of antitubulin compounds. Antimicrob Agents Chemother 52(3):1133–1135


Jain ML, Jain SR (1972) Therapeutic utility of *Ocimum basilicum* var. album. Plant Med 22:56–70

© Springer


© Springer