Pharmacognosy Magazine
ISSN: 0973-1296
Vol 4, Issue 16, Oct-Dec, 2008

PHCOG MAG.
An official Publication of Phcog.Net

PHCOG MAG. : Research Article
Antioxidant, antimicrobial and cytotoxicity properties of the methanolic extract from *Grewia tiliaefolia* Vahl

Yadav Vivek Ramshankar[a], Pandit Vinay[a] and P. Vijayan[b]

[a]*Department of Pharmaceutics, Al-Ameen College of Pharmacy, Hosur Road, Bangalore – 560027*
[b]*Department of Pharmaceutical Biotechnology, J.S.S. College of Pharmacy, Rockland, Ooty.*
Author for Correspondence : vivekyadav78@yahoo.co.in

ABSTRACT

The whole, fresh barks of *Grewia tiliaefolia* Vahl. (Tiliiacea), were extracted with methanol. All obtained extracts were evaluated for their antioxidant, antimicrobial and cytotoxicity properties. Two different methods (disc diffusion and agar dilution methods) were employed to evaluate the antimicrobial activities of plant extracts against *B. Sterarothermophilus, B. Aerogeuosa, B. Coagulans, K.Pneumoniae, L. Licjmani, Shigell* and *P. Cepacia*. The minimum inhibitory concentration (MIC) values of *Grewia tiliaefolia* methanolic extract was found to be 125 µg/ml against *B. Sterarothermophilus,* 31.25 µg/ml against *L. Licjmani* and 62.5 µg/ml against *P. Cepacia*. The antioxidant potential was evaluated using following in vitro methods: Nitric oxide radical inhibition activity (superoxide anion) and scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. The antioxidant activities of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals (13.65 µg/ml IC_{50} value) and superoxide anion radicals (44.0 µg/ml IC_{50} value) and reducing power. Cytotoxicity study was done using Vero and HEp-2 cell line. CTC_{50} values were found to be 205 ± 13.23 µg/ml and 345 ± 12.65 µg/ml. The results showed that *Grewia tiliaefolia* extract had great potential as antioxidant, antimicrobial and very good anticancer agent.

KEY WORDS: Minimum inhibitory concentration; Natural antioxidants; Super oxide radical inhibition, DPPH; Antimicrobial activity

INTRODUCTION

Efforts are being made to develop antimicrobial and very good cytotoxic agents from natural sources. In the past, plants have provided lead anticancer compounds like vincristine and taxol. Several members of the species Grewia (Tiliaceae) are being used traditionally for the treatment of a large number of disease conditions[1]. Among them Grewia villosa is reported to possess anticancer activity[2]. Grewia tiliaefolia bark is being used in traditional medicine[1], and so far no investigation has been carried out for its antimicrobial property. Three tri-terpenoids, viz., betulin, friedelin and lupeol were isolated from its bark[3]. In view of these reports and the ethnomedical uses of Grewia tiliaefolia bark, we studied the in vitro antimicrobial, antioxidant properties as well as the cytotoxicity of the extract.

Grewia tiliaefolia Vahl (family Tiliiacea), commonly known as dhamni, is considered as an important fruit crop in tropical countries. Plant is moderated sized to large tree found in the sub-Himalayan tract from Jammu to Assam and in central, western and southern Indian hills. The wood has emetic properties and employed in powder form as an antidote to opium poisoning. Stem bark also used as semen coagulant & cardio vascular activity[4]. The fruit wall comprises of three layers exocarp, mesocarp and endocarp. Due to extensive cross linking between phenolics, lignin and polysaccharides, the mesocarp becomes hard and fibrous.

MATERIALS AND METHODS

Plant material

The whole, fresh bark of *Grewia tiliaefolia* was collected from Hosanagar, Shimoga, Karnataka, authenticated by Survey of Medicinal Plants and Collection Unit, Ootacamund, shade dried to get constant weight.

Chemicals

Analytical grade chemicals were used in sample preparation and all the solvents for extraction purpose were standard grade, purchased from Merck. Muller Hilton Broth and Muller Hilton Agar and Phosphate buffer saline were bought from Hi-Media, India (Mumbai). Dimethylsulfoxide (DMSO), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Sodium nitroprusside, Naphthyl ethylene diamine dihydrochloride (NEDD), Sulphanilic acid (0.33%w/v) reagent and L-ascorbic acid were

obtained from Sigma Chemical Co. (St. Louis, USA).

Extraction

The whole, fresh involucral bracts were cut into pieces and shade dried, powdered and extracted (170 g) with 50% methanol (750 ml) in a Soxhlet extractor for 20 h. The extract was evaporated to dryness at 37^0C under reduced pressure to yield a dark brown solid (32 g, 18.82%). Repeatedly extraction was done with Methanol (90%, v/v) by using Soxhlet distillation apparatus till clear color less methanol as indicate all component of bark get extracted. All obtained extract, was evaporated to dryness and used for all investigations.

Antimicrobial activity and preparation of inoculums

Stock cultures were maintained at 4^0 C on slopes of nutrient agar. Stock cultures are the type culture obtained from cell line center Pune, India. Active cultures for experiments were prepared by transferring a loop full of cells from the stock cultures to test tubes of Mueller–Hinton broth (MHB) and incubated without agitation for 24 h at 37^0 C or 25^0 C. The cultures were diluted with fresh MHB to achieve optical densities corresponding to 10^6 colony forming units (CFU/ml).

Antimicrobial susceptibility test

The disc diffusion method was used to screen the antimicrobial activity[5]. No uniformity in the quotation of the references The MHA plates were prepared by pouring 15 ml of molten media into sterile petri plates. The plates were allowed to solidify for 5 min, 0.1% inoculum suspension was spread uniformly, and the inoculums were allowed to dry for 5 min. Twenty microliters of crude plant extract were loaded on 6 mm sterile disc. The loaded disc was placed on the surface of medium, the compound was allowed to diffuse for 5 min, and the plates were kept for incubation at 37^0 C for 24 h. Streptomycin discs was taken as positive controls. At the end of incubation, inhibition zones formed around the disc were measured with a transparent ruler in millimeters. Through agar diffusion layer plate methods, the diameter of the inhibition zone ranges from 16–21 mm (not including the diameter of the paper discs) suggests strong antimicrobial activity, 11–15 mm indicates moderate antimicrobial activity and the diameter less than 10 mm shows weak antimicrobial activity.

Determination of the MIC and MBC

MIC and MBC of the extract was determined by the method of broth dilution. Aliquots of 5 ml of LB media were placed into tubes before the extract solution was added to the tubes to the final concentration of 50 µg/ml. Next, the concentration of the extract was adjusted ranging from 0.1 - 12.8 µg/ml by adding different volumes of the LB media. All above samples were autoclaved at 121^0 C and cooling before the test bacteria suspension was added into to the inoculum size of 106 cfu/ml and then incubated at 37^0 C for 24 h. Another diluted solution without adding any bacterium was prepared as control sample. The turbidity of all samples was determined at 540 nm. The determination was based on the same turbidity of the two subjects of the same concentration, i.e., the minimum inhibitory concentration at which no bacteria grew in the culture media was defined as MIC.

After the MICs were determined, the samples showing no increases in turbidity were streaked on appropriate agar plates to check bacterial survival and the MBC was determined as the lowest concentration at which the test samples killed the bacteria.

DPPH radical scavenging or hydrogen donating method

The effect of the extract from bark of *Grewia tiliaefolia* Vahl on DPPH radical was estimated according to the procedure described in literature (Brand-Williams, Cuvelier, & Berset, 1995). For each concentration tested, the percentage of DPPH left at the steady state was calculated as follows: %DPPH = $[DPPH]_T/[DPPH]_{T=0}$, where T is the time require to reach the steady state. The antioxidant property was measured in terms of amount of methanolic extract necessary to reduce the initial DPPH concentration by 50% (EC_{50}).

The assay was carried out in a 96 well microtitre plate. To 200µl of DPPH solution (100 µM DPPH dissolved in methanol), 10µl of each of the test sample or the standard solution was added separately in wells of the microtitre plate. The final concentration of the test and standard solutions used were in the range of 15.625µg/ml - 1000µg/ml. The plates were incubated at 37°C for 30 minutes and the absorbance of each solution was measured at 490nm, using ELISA reader against the corresponding test and standard blanks and the remaining DPPH was calculated. Methanol was used as blank. Ascorbic acid was taken as a standard antioxidant. The absorbance of the DPPH radical without any antioxidant was measured as control.

The plot of scavenging activity on DPPH radical was done and IC_{50} value (concentration of sample to scavenge 50% of the DPPH radicals) was calculated.

Superoxide anion radical-scavenging activity

The superoxide anion radical-scavenging activity was assessed by the method of Yu et al. (2006) with a slight

modification. Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH, interact with oxygen to produce nitrite ions which were measured by using a modified Griess-Ilosvany method.

The reaction mixture (6ml) containing sodium nitroprusside (10mm w/v, 4ml), PBS (1ml) and extract (1ml) in DMSO was incubated at 25ºC for 150 minutes. After incubation, 0.5ml of the reaction mixture containing nitrate was removed, 1ml of sulphanitic acid reagent (0.33% in 20% glacial acetic acid) was added, mixed well and allowed to stand for 5minutes for completion of diazotisation, then 1ml of Naphthyl ethylene diamine dihydrochloride was added, mixed and allowed to stand for 30 minutes in diffused light. A pink coloured chromophore was formed. The absorbance of these solutions was measured at 540nm against corresponding blank solution. IC_{50} value is the concentration of the sample required to inhibit 50% nitric oxide radical. Vitamin C was used as a control. All the tests were performed in triplicate. The scavenging of the superoxide anion radicals was calculated by the following equation:

$$\text{Scavenging activity (\%)} = \frac{A_0 - A_1}{A_0} \times 100$$

Where A_0 is the absorbance of the control (without sample) and A_1 is the absorbance of the mixture containing sample. The plot of scavenging activity on superoxide anion radicals was done and IC_{50} value (concentration of sample to scavenge 50% of superoxide anion radicals) was obtained.

Cytotoxicity Study (CTC_{50}) using cell line

The fraction isolated was investigated for cytotoxicity properties in two cell lines. Vero (a normal African green monkey kidney cell line) and HEp-2 (a human larynx epithelial carcinoma cell line) obtained from Pasteur Institute of India, Coonoor. The cultures were maintained in Dulbecco's modified eagles medium (DMEM) containing 10% inactivated new born calf serum (PAA Laboratories, Austria) and were grown in 25 cm^2 tissue culture flasks (Tarsons Products (P) Ltd., Kolkota) till confluent and used for cytotoxicity assays. Confluent monolayer were washed with serum free DMEM medium and trypsinized. The cells were resuspended in DMEM medium with 10% inactivated newborn calf serum and cell population adjusted to 10^5 cells/ml and 0.1 ml of cell suspension was seeded per well in to 95 well microtitre plates (Tarsons Products (P) Ltd., Kolkotta) and incubated for 24 h at 37^0 C, 5% CO_2 in a humidified atmosphere, during which a partial monolayer forms. The cells were then exposed to different concentration [ranging from 1000 µg/ml to 15.6 µg/ml, prepared by two fold serial dilution using maintenance media (DMEM medium containing 2% inactivated new born calf serum) from a stock solution prepared in dimethyl sulfoxide] of methanolic extract of *Grewia tiliafolia*. Control wells received only maintenance media. The plates were incubated at 37^0 C in a humidified incubator with 5% CO_2 for a period of 72 h. Morphological changes were examined using an inverted tissue culture microscope (Olympus Optical Co. Ltd., Japan. Model 1x70) at 24 h time intervals. At the end of 72 h cellular viability was determined by Trypan blue dye exclusion assay[10] and the percentage inhibition and CTC_{50} value which is the concentration of the sample tolerated by 50% of the cultures exposed were calculated.

Statistical analysis

All the experiments were done in triplicate. The triplicate data were subjected to an analysis of variance for a completely random design using statistical analysis software, SPSS 10.0. The significance level was fixed at 0.05 for all statistical analysis.I think that the authors means that a P-value < 0.05 is considered as statistically significant different. Standard deviations were expressed in each table (and in the tables?). Means $\pm$ SD are presented in tables and figures

RESULTS AND DISCUSSION

Extract of active component from Grewia tiliaefolia Vahl

Crude methanolic extract may contain thousands of compounds including flavones, proanthocyanidin and even soluble polysaccharides. The recovery rate reaches up to optimum by 90 % methanol extractant, amounting to 17.6 g/(100 g dried), which is hereby considered as the most effective extractant. Ninty-five percentage methanol extractant characteristic of strong permeability exerts marked effects of solubility and extraction on the effective water and fat soluble components such as volatile oil, flavones, saponins, alkaloids, coumarins, sesquiterpenes and esters, hence an it can be considered as an ideal extractant.

Antimicrobial activity of Grewia tiliaefolia Vahl

The disc diffusion method was used to determine the antimicrobial activity of methanolic extract. The antimicrobial activity on pathogenic strains of Gram-positive, Gram-negative bacteria of methanolic extract was evaluated in the present study. Inhibition zones of bacteria by methanolic extract and streptomycin were measured. Streptomycin is a well-known chemical with

Table 1 : Antimicrobial activity of methanolic extract of Grewia tiliaefolia Vahl

Samples	Diameter of inhibition zone (mm)*						
	B. Sterarothermophilus	*B. Aerogeuosa*	*B. Coagulans*	*K.Pneumoniae*	*L. Licjmani*	*Shigell*	*P. Cepacia*
Methanolic extract	16.4 ± 0.34	14.4 ± 0.63	14.6 ± 0.24	13.7 ± 0.45	21.3 ± 0.47	12.4 ± 0.4	18.6 ± 0.83
Streptomycin	15.3 ± 0.25	18.6 ± 0.53	19.2 ± 0.34	13.5 ± 0.33	20.2 ± 0.98	17.8 ± 0.3	16.5 ± 0.45
	Minimum Inhibitory Concentration (µg/ml)						
Methanolic extract	125	> 250	> 250	> 250	31.25	> 250	62.5
Streptomycin	150	125	62.5	125	62.5	62.5	125

** The diameter (mm) was the mean ±SD of the three independent experiments (not including the 6 mm diameter of the paper disk). The values with different letters in the same line were significantly different from the values for zone diameters with superscript a and from one another ($p < 0.05$) according to Duncan new multiple range test.*

Table 2 : Comparison of IC_{50} value of methanolic extract of Grewia tiliaefolia Vahl and standard antioxidants

Samples	DPPH radical scavenging (µg/ml)	Superoxide anion radical-scavenging activity (µg/ml)
Methanolic extract	13.65	44.00
Ascorbic Acid	19.17	46.23

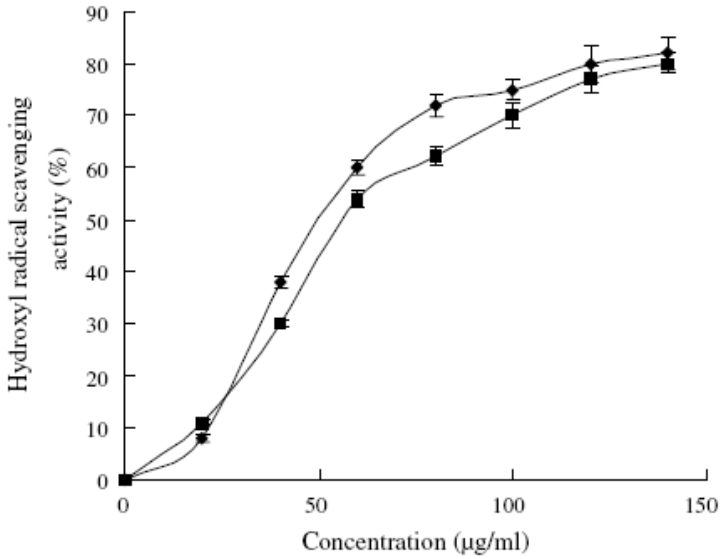

Figure 1. Scavenging activities of methanolic extract of Grewia tiliaefolia Vahl and vitamin C on hydroxyl radicals. (■) vitamin C; (▲)extract. The error bars are not explained

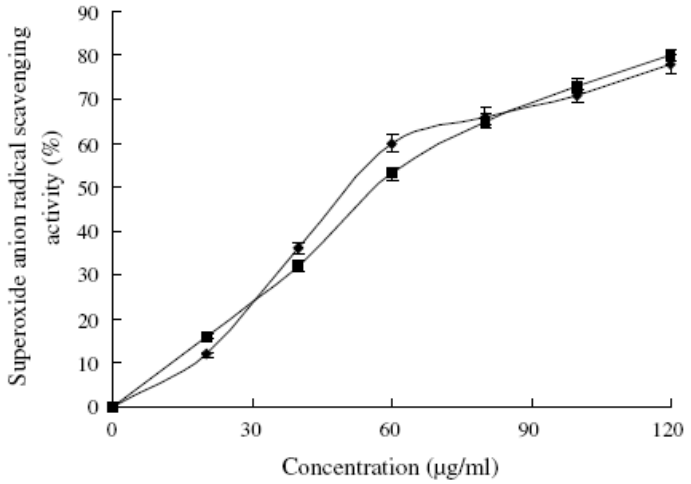

Figure 2. Scavenging activities of methanolic extract of Grewia tiliaefolia Vahl and vitamin C on hydroxyl radicals. (■) vitamin C; (▲)extract.

pronounced antimicrobial potential. As shown in Table 1. Streptomycin was used as a positive control because it has been commonly employed as the antibiotic for Gram-positive and Gram-negative bacteria. Methanolic extract showed good antimicrobial activity against *L. Licjmani* compared to other microorganisms.

DPPH radical scavenging or hydrogen donating method

DPPH radical is commonly used as a substrate to evaluate antioxidant activity; it is a stable free radical that can accept an electron or hydrogen radical to become a stable molecule. The reduction of DPPH radical was determined by the decrease in its absorbance at 490 nm induced by antioxidants. Figure 1 shows a dose-dependend effect of methanolic extract of *Grewia tiliaefolia* Vahl on the ability to quench DPPH radicals. The DPPH radical-scavenging activity of *Grewia tiliaefolia* Vahl at high concentration was significantly higher ($P < 0.05$) than that of vitamin C, a commercial antioxidant used in the food industry. This indicated that methanolic extract of *Grewia tiliaefolia* Vahl was a good antioxidant with strong DPPH radical-scavenging activity.

Superoxide anion radical-scavenging activity

Superoxide anion is a reduced form of molecular oxygen, by receiving an electron. It is also an initial free radical formed from mitochondrial electron transport systems. Mitochondria generate energy using four electron chain reactions, reducing oxygen to water. Some of the electrons escaping from the chain reaction of mitochondria directly react with oxygen and form superoxide anion. It plays an important role in the formation of other reactive oxygen species, such as hydrogen peroxide, hydroxyl radical, or singlet oxygen in living systems (Kulisic, Radonic, & Katyalinic, 2005). The effects of PTL on superoxide anion radicals were determined and the results are shown in Figure 2. *Grewia tiliaefolia* Vahl had a significant scavenging activity on the superoxide anion radicals in a dose-dependent manner. Compared with vitamin C, methanolic extract of *Grewia tiliaefolia* Vahl showed insignificant difference ($P > 0.05$) with regard to superoxide anion radical-scavenging activity.

IC_{50} values for antioxidant activities

Concentration of sample at which the inhibition percentage reaches 50% is the IC50 value. IC_{50} value is negatively related to the antioxidant activity, as it expresses the amount of antioxidant needed to decrease the radical concentration by 50%. The lower the IC_{50} value, the higher is the antioxidant activity of the tested sample. The IC_{50} values of methanolic extract of *Grewia tiliaefolia* Vahl for DPPH radical, hydroxyl radical and superoxide anion radical-scavenging activities are summarized in Table 2. IC_{50} values of methanolic extract of *Grewia tiliaefolia* Vahl for scavenging activities on hydroxyl radicals, superoxide anion radicals and DPPH radicals were 13.64 and 40.0 μg/ml which were significantly lower ($P < 0.05$) than those of the control, vitamin C. The IC_{50} value of extract for DPPH was slightly higher than that of the control, vitamin C, possibly due to the weaker liposolubility of extract than vitamin C. According to the results in Table 2, a conclusion could be drawn that *Grewia tiliaefolia* Vahl possessed strong antioxidant activity.

Cytotoxicity Study (CTC_{50}) using cell line

Concentration of sample at which the inhibition percentage reaches 50% is the viability of cell culture. CTC_{50} value is directly related to the cytotoxicity activity, as it expresses the amount of extract toxic to 50% of normal cell. The higher the CTC_{50} value, the higher is the cytotoxic activity of the tested sample. CTC_{50} values were found to be 205 ± 13.23 µg/ml and 345 ± 12.65 µg/ml, for Vero and HEp-2 cell lines, respectively. The results of cytotoxicity studies and CTC_{50} indicated that the tested samples are cytotoxic only when culture is exposed to very high concentration. High concentrations of any compound, under normal conditions are cytotoxic to cell culture. Hence results show that moderate cytotoxicity activity against these cell lines.

CONCLUSIONS

Strong scavenging activities on hydroxyl radicals, superoxide anion radicals and DPPH radicals were found for *Grewia tiliaefolia* Vahl. The analyses of lipid peroxidation inhibition activity and reducing power also indicated that *Grewia tiliaefolia* Vahl possessed good antioxidant activity. Meanwhile, the proliferation inhibition activity of methanolic extract *Grewia tiliaefolia* Vahl on *B. Sterarothermophilus, B. Aerogeuosa, B. Coagulans, K.Pneumoniae, L. Licjmani, Shigell* and *P. Cepacia* were investigated in this study. The results confirmed that it had good antimicrobial activity against *L. Licjmani* > *P. Cepacia* > *B. Sterarothermophilus*. Based on CTC_{50} values extract is moderately cytotoxic against for Vero and HEp-2 cell lines. From the above results, it appears important to develop natural antioxidants and bacterial inhibitors from *Grewia tiliaefolia* Vahl bark and this may be a good way for extensively utilizing the *Grewia tiliaefolia* Vahl bark for antimicrobial and diabetic treatment as it having a very good free radical scavenging activity.

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Phcog Mag. Vol 4, Issue 16, Oct-Dec, 2008

Submitted on : 16th June, 2008
Revised on : 20th August, 2008
Accepted on: 20th August, 2008

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