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Antioxidant and antiproliferative activity of methanolic extract of *Grewia tiliaefolia* (Vahl) bark in different cancer cell lines

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Abstract

The importance of botanical, chemical, pharmacological evaluation of plant-derived agents used in the treatment of human ailments has been increasingly recognized in the last decades. Plants continue to be an important source of new drugs even today. The present study was carried out to evaluate the antioxidant and tumour cell suppression potential of methanolic extract of *Grewia tiliaefolia* bark in the *in vitro* model system. The different radical systems comprising of superoxide radical ($O2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$) and nitric oxide radical ($NO^{\cdot}$) systems were studied against different concentration of *G. tiliaefolia* extract. The percentage of radical inhibition and IC50 (concentration of extract required to inhibit 50% radicals) were calculated. The study showed that the methanolic extract was found to have potential antioxidant activity in the *in vitro* model system. The tumour cell suppression or antiproliferative activity was demonstrated in three different cancer cell lines i.e., MCF7 (breast cancer cell line), HepG2 (hepatocancer cell line) and A549 (lung cancer cell line). The Sulphorhodamine B (SRB assay) method was adopted for evaluating the tumour cell suppression activity. The Growth inhibition of 50% (GI50), Total Growth Inhibition (TGI) and lethal concentration 50% (LC50) of *G. tiliaefolia* extract were evaluated against all the three cancer cell lines. The study proved that the methanolic extract was found to have potential tumour cell suppression activity in the selected *in vitro* cell culture system. The efficacy of the extract was highest against MCF7 cells and then followed by HepG2 cells and A549 cells. The further study has to be focused for understanding the molecular level mode of action of test extract.

Key words: antioxidant activity, free radicals, cancer, antiproliferative activity, tumour cells.

Introduction

Cancer is one of the major causes of death in developed countries, together with cardiac and cerebrovascular diseasesas (WHO report, 1998). Cancer is clinically treated by surgery, radiotherapy and chemotherapy. After surgical ablation of progressive cancer, however, metastasized tumour cells continue to progress and this is one of the causes making cancer treatment difficult (Fidler and Kripke, 1997). Radioactive rays and most anticancer drugs damage DNA or suppress DNA duplication to kill tumour cells growing rapidly. At the same time, they also affect normal cells to cause serious adverse effects, such as bone marrow function inhibition, nausea, vomiting and alopecia (Kligerman, 1973).

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The involvement of free radicals in causing various diseases has been documented recently. Free radical initiating auto oxidation of cellular membrane lipids can lead to cellular necrosis and is now accepted to be important in connection with a variety of pathological conditions (Newman *et al.*, 2003). These radicals react with cell membranes and induce lipid peroxidation or cause inflammation, which may result as important pathological mediators in many clinical disorders such as heart disease, diabetes, gout and cancer (Mothana *et al.*, 2006). Reduction of these radicals by antioxidant molecules is crucial to the protection of cells against various disorders (Brand *et al.*, 1995). Nature has provided an excellent storehouse of remedies to cure all the ailments of mankind. Lignans, Flavonoids and other poly phenolic compounds that are widely distributed in plants, have been reported to exert multiple biological effects, including antioxidant and anticancer activity (Manjunatha and Vidya, 2008).

Grewia tiliaefolia is a medium sized tree upto 20 m in height, with a clear bole of 8 m and 65 cm in diameter and grey to blackish brown rough fibrous bark peeling off in thin flakes; leaves simple, alternat. The flowers are yellow, small on thick axillary peduncles and fruits are globose drupes of the size of a pea, 2-4 lobed, black when ripe, seeds 1-2. The bark is astringent, sweet, acrid, refrigerant, oleaginous, expectorant, antipruritic, vulnerary, constipating, emetic, styptic, aphrodisiac and tonic. It is useful in vitiated conditions of pitta and kapha, burning sensation, hyperdipsia, rhinopathy, ulcers, skin diseases, haematemesis and general debility (Warrier *et al.*, 1995).So, considering the clinical importance, the present study was designed to evaluate the antioxidant and antiproliferative activity of *Grewia tiliaefolia* Bark methanolic extract.

Material and methods

Plant Material and Extraction

The bark of *Grewia tiliaefolia* Vahl was collected from forest area of western ghat and was authenticated by Kerala Forest Research Institute, Peechi, Thrissur. A voucher specimen is maintained in the department for future reference. The bark was shade dried, coarsely powdered and extracted using methanol for continuous 10 hours in Soxhlet extraction apparatus. After the completion of the extraction, the solvent was removed using rotary vacuum evaporator and solvent-free dried extract was aliquoted into small sterile vials and stored in refrigerator for further use.

Chemicals

All basic chemicals and solvents were of analytical grade and were obtained from HiMedia Chemicals, Mumbai, India. 2,2-azinobis(3-ethylbenzq-thiozoline-6-sulphonate) (ABTS) was obtained from Sigma Chemicals, USA. The other chemical used were 1,1- diphenyl, 2-picryl hydrazyl (DDPH), sodium nitropursside, sulphanilamide, o-phosphoric acid, napthyl ethylene diamine dihydrochloride, ferrous sulphate ($FeSO_4$), thiobarbituric acid (TBA), trichloroacetic acid (TCA), nitroblue tetrazolium (NBT) and ethylene diamine tetra acetic acid obtained from Merck India. Adherent cell lines MCF7, HepG2 and A549 were received from the National Centre for Cell Science, Pune. Positive control drugs Adriamycinn (Sigma), ellipticine (Sigma), 10% (wt/vol) TCA, 1% (vol/vol) acetic acid, 0.057% (wt/vol) SRB (Fluka).

Scavenging of superoxide radical

The scavenging activity towards the superoxide radical ($O_2^{\cdot-}$) was measured in terms of inhibition of generation of oxygen radicals (Sanchez-Moreno, 2002). The reaction mixture consisted of phosphate buffer (50 mM, pH 7.6), riboflavin (20 µg/0.2 ml), EDTA (12 mM), NBT (0.1 mg/3ml) and sodium cyanide (3 µg/0.2 ml) test compounds of various concentrations of 50-250 µg/ml were added to make a total volume of 3 ml. The absorbance was read at 530 nm before and after illumination under UV lam for 15 min against a control instead of sample. The percentage inhibition was calculated by using the same formula.

$$\text{Inhibition (\%)} = \frac{\text{(control- test)}}{\text{control}} \times 100$$

Scavenging of hydroxyl radical

Hydroxyl radical scavenging activity was measured according to the method of Kunchadny and Rao (1990) by studying the competition between deoxyribose and test extract for hydroxyl radical generated by Fenton's reaction. The reaction mixture contained deoxyribose (2.8 mM), $FeCl_3$ (0.1 mM), EDTA (0.1 mM), H_2O_2 (1 mM), ascorbate (0.1 mM), KH_2PO_4- KOH buffer (20 mM, pH 7.4) and various concentrations of the sample extracts in a final volume of 0.1 ml. The reaction mixture was incubated for 1 h at 37^0C. Deoxyribose degradation was measured as thiobarbituric acid reacting substances (TBARS) and the percentage inhibition calculated.

Scavenging of nitric oxide radical

Nitric oxide was generated from sodium nitro prusside and measured by Griess reaction (Sreejayan and Rao, 1997; Green and Reed, 1998; Marcocci *et al.*, 1994). Sodium nitroprusside (5mM) in standard phosphate buffer solution was incubated with different concentration (50-250 µg/ml) of the methanol extract dissolved in phosphate buffer (0.025 M, pH 7.4) and the tubes were incubated at 25^0C for 5 hr. Control experiments without the test compounds, but with equivalent amounts of buffer were conducted in an identical manner. After 5 hr, 0.5 ml of incubation solution was removed and diluted with 0.5 ml of Griess' reagent (1%sulphanilamide, 2%o-phosphoric acid and 0.1% napthylene diamine dihydrochloride). The absorbance of the chromophore formed during diazotization of nitrite with sulphanilamide and its subsequent coupling with napthylene diamine was read at 546 nm. The experiment was repeated in triplicate.

Sulphorhodamine B Assay (SRB Assay)

The Sulphorhodamine B assay (Vanicha and Kanyawim, 2006; Boydem, 1997) was used for screening anti-proliferative activity of test extract. The cell lines were grown in RPMI 1640 medium containing 10% fetal bovine serum and 2 ml L-glutamine. For present screening experiment, cells were inoculated into 96 well microtiter plates in 90 µl. After cell inoculation, the microtiter plates were incubated at 37^0C, 5% CO_2, 95% air and 100% relative humidity for 24 hrs prior to addition of experimental drugs.

After 24 hrs, one plate of each cell line was fixed in situ with TCA, to represent a measurement of the cell population for each cell line at the time of drug addition (Tz). Experimental drugs were solubilized in DMSO at 400 fold the desired final maximum test concentrations and stored frozen prior to use. At the time of drug addition, an aliquot of frozen concentrate was thawed and diluted to 10 times the desired final maximum. Aliquots of 10µl of these different drug dilutions were added to the appropriate micro-titer wells already containing 90 µl of medium, resulting in the required final drug concentrations of 10, 20, 40, 80 µg/ml. It was prepared six concentrations of positive controls from 160 to 5µg ml^{-1} using twofold serial dilution in 10% (vol/vol) DMSO.

Endpoint Measurement

After compound addition, plates were incubated at standard conditions for 48 hours and assay was terminated by the addition of cold TCA. Cells were fixed *in situ* by the gentle addition of 50 µl of cold 30% (w/v) TCA (final concentration, 10% TCA) and incubated for 60 minutes at 4^0C. The supernatant was discarded; the plates were washed five times with tap water and air dried. Sulforhodamine B (SRB) solution (50 µl) at 0.4 % (w/v) in 1% acetic acid was added to each of the wells, and plates were incubated for 20 minutes at room temperature. After staining, unbound dye was recovered and the residual dye was removed by washing five times with 1 % acetic acid. The plates were air dried. Bound stain was subsequently eluted with 10mM tris base, and the absorbance was read on an Elisa plate reader at a wavelength of 540 nm with 690 nm reference wavelength.

Percent growth was calculated on a plate-by-plate basis for test wells relative to control wells. Percent Growth was expressed as the ratio of average absorbance of the test well to the average absorbance of the control wells.

Using the six absorbance measurements (time zero (Tz), control growth (C), and test growth in the presence of drug at the four concentration levels (Ti), the percentage growth was calculated at each of the drug concentration levels. Percentage growth inhibition was calculated as:

(Ti-Tz)/(C-Tz) x 100 for concentrations for which Ti>/= Tz (Ti-Tz) positive or zero

(Ti-Tz)/Tz x 100 for concentrations for which Ti<Tz. (Ti-Tz) negative.

The dose response parameters were calculated for each test article. Growth inhibition of 50% (GI50) was calculated from (Ti-Tz/(C-Tz) x 100 =50, which is the drug concentration resulting in a 50% reduction in the net protein increase (as measured by SRB staining) in control cells during the drug incubation. The drug concentration resulting in total growth inhibition (TGI) was calculated from Ti=Tz. The LC50 (concentration of drug resulting in a 50 % reduction in the measured protein at the end of the drug treatment as compared to that at the beginning) indicating a net loss of cells following treatment is calculated from (Ti-Tz)/Tz x 100= -50. Triplicates of these plates were made. For each of the experiments a known anticancer drug Adriamycin was used as a positive control to ensure the experimental set is perfect. For each of the experiments column A in 96 well plate is used as a cell control without drug addition. Values were calculated for each of these three parameters if the level of activity was reached; however if the effect was not reached or was exceeded, the values for that parameter were expressed as greater or less than the maximum or minimum concentration tested.

Results

The present study showed that the methanolic extract of *G. tiliaefolia* is found to be having potential free radical scavenging activity against various radical systems. The different radical systems like superoxide radical, hydroxyl radical, nitric oxide radical were used for evaluating the antioxidant activity of the test drug. The *G. tiliaefolia* extract at 50,100, 200, 250 µg/ml concentrations were used against all the radicals. The radical scavenging activity was expressed in terms of % of inhibition as mentioned in the methodological part. The increasing concentration of test extract increases the inhibition %. The activity of the extract is found to be varying according to the chemical nature of radicals. The reason may be the due to the different phytomolecules that are present in the crude extract acts on different way against the radicals.

The IC 50 (Concentration required for 50% inhibition) values were calculated and presented in the Table. 2. The results showed that the methanolic extract of *G. tiliaefolia* is having highest activity against Hydroxyl radical then followed by superoxide radical and nitric oxide radical.

In the present study, the cytotoxic activity of methanolic extracts of *G. tiliaefolia* was evaluated in three different cancer cell lines viz MCF7 (breast cancer cell line), HepG2 (liver cancer cell line), and A549 (lung cancer cell line) considering the clinical importance of the cancer types. The results showed that the methanolic extract of *G. tiliaefolia* is found to have potential antiproliferative activity against the selected cancer cell lines (Table 3-5). The Growth Inhibition of 50% (GI50), Total growth Inhibition (TGI) concentration and Lethal concentration of 50% (LC50) were evaluated on all the three cancer cell lines. Adriamycin was used as the standard drug for the experiment. Among the three cell lines, the *G. tiliaefolia* extract showed highest activity against MCF7, followed by HepG2 and A549.

Table-1. Effect of methanolic extract of *Grewia tiliaefolia* on different antioxidant models.

Concentration (µg/ml)	Free radical scavenging activity (inhibition %)		
	Superoxide radical	Hydroxyl radical	Nitric oxide radical
50	16.42 ± 0.45	27.60 ± 0.69	11.70 ± 0.65
100	29.58 ± 0.78	42.60 ± 0.70	26.42 ± 0.48
150	53.50 ± 0.83	56.42 ± 0.85	34.66 ± 0.83
200	69.80 ± 1.40	69.35 ± 0.94	48.52 ± 1.30
250	80.11 ± 1.38	84.30 ±1.20	61.95 ± 1.55

(Values are expressed mean± SEM, n=3)

Table- 2. IC50 value of methanolic extract of *G. tiliaefolia* on free radical scavenging system.

Name of the Plant	*Free radical scavenging assay IC 50 value (µg/ml)*		
	Superoxide radical	Hydroxyl radical	Nitricoxide radical
Grewia tiliaefolia	150.35	128.40	204.44

Table-3. Antiproliferative activity of *G. tiliaefolia* on MCF7 cancer cell line

Test Drugs	Cytotoxicity assay on MCF7 breast cancer cell line (Concentration in µg/ml)		
	GI50	TGI	LC50
Grewia tiliaefolia	16.40 ± 1.66	76.53 ± 1.41	>80
Adriamycin Standard	< 10	26.40 ± 1.20	59.60 ± 1.38

(Values are Mean ± SEM, n=3)

Table- 4. Cytotoxic activity of *G. tiliaefolia* on HepG2 cancer cell line

Plant Names	Cytotoxicity assay on HepG2 liver cancer cell line (Concentration in µg/ml)		
	GI50	TGI	LC50
Grewia tiliaefolia	18.29 ± 1.32	56.80 ± 3.26	>80
Adriamycin Standard	< 10	15.60 ± 0.55	48.35 ± 1.40

(Values are Mean ± SEM, n=3)

Table- 5. Cytotoxic activity of *G. tiliaefolia* on A549 cancer cell line

Plant Names	Cytotoxicity assay of study drug on A549 lung cancer cell line (Concentration in µg/ml)		
	GI50	TGI	LC50
Grewia tiliaefolia	18.35 ± 1.21	> 80	> 80
Adriamycin Standard	< 10	< 10	36.76 ± 2.40

(Values are Mean ± SEM, n=3)

Discussion

Free radicals are chemical entities that can exist separately with one or more unpaired electrons. The generation of free radical can bring about thousands of reactions and thus cause extensive tissue damage. Lipids, proteins and DNA are all susceptible to attack by free radicals. Antioxidants may offer resistance against oxidative stress by scavenging the free radicals. Superoxides are produced from molecular oxygen due to oxidative enzymes of body as well as via non-enzymatic reaction such as auto-oxidation by catecholamines (Hemmani and Parihar, 1998). The scavenging activity towards the superoxide radical ($O_2^{\cdot-}$) is measured in terms of inhibition of generation of $O_2^{\cdot-}$. In the present study, superoxide radical reduces NBT to a blue coloured formation that is measured at 560 nm. The results showed that the potency of *G. tiliaefolia* against superoxide radical scavenging activity in the model system.

The hydroxyl radical scavenging activity is measured as the percentage of inhibition of hydroxyl radicals generated in the Fenton's reaction mixture by studying the competition between deoxyribose and the extract for hydrogen radicals generated from Fe^{3+}/ascorbate/EDTA/H_2O_2 systems. The hydroxyl radicals attack deoxyribose which eventually results in TBARS formation. In the present *in vitro* study, it is observed that the extract of *G. tiliaefolia* also having hydroxyl radical scavenging activity.Nitric oxide is a free radical generated by endothelial cells, macrophages, neurons etc., and involved in regulation of various physiological process (Lata and Ahuja, 2000).

Excess concentration of Nitric oxide is associated with several diseases. Oxygen reacts with the excess nitric oxide to generate nitrite and peroxy nitrite anions, which act as free radicals (Sainani *et al.*, 1997). In the present study, the methanolic extract of *G. tiliaefolia* showed activity in competing with oxygen to react with nitric oxide and thus the inhibition of generation of anions.

A number of naturally derived agents have been entered in to clinical trials after various pre clinical trials. The discovery of novel antitumour agents from natural sources was largely based on the testing for cytotoxic activity against cancer cell lines grown either *in vitro* or using *in vivo* models (Cragg *et al.*, 2005; Newman *et al.*, 2002). Many of the naturally derived anti-cancer agents originally, discovered using such assays, have been shown to exert their cytotoxic action through interaction with tubulin and include agents such as vinblastine and colchicines (Cassady and Douros, 1980). With the advent of the era of molecularly targeted therapies in the last decade, the anticancer drug development process has undergone a steady and concomitant revolution (Chiruvella and Raghavan, 2010). Recent scientific research in the fields of medicinal chemistry and pharmacology has been increasingly addressed to the isolation and characterization of the active principle from plant extract for the production of chemotherapeutic agents comprised of single compound with multiple targets (Rehman *et al.*, 2001).

The drug discovery approach followed in the present study is based upon the most current trends of drug discovery used by major pharmaceutical companies in the screening of plant material and subsequent fractionation (Harvey, 2000). The simplest antiproliferative assay is to measure the concentration of sample needed to inhibit cell growth by 50% against a single cell line. The major advantage of antiproliferative assay is that all potential mechanisms concerning cellular proliferation are monitored simultaneously. In the present study, SRB assay was carried out for measuring the cytotoxicity in cells in reponse to plant extracts. So, the present study concluded that methanolic extract of *G. tiliaefolia* is found to have potential antioxidant and antiproliferative activity in the selected *in vitro* model systems. The efficacy of the extract may be due to the presence of steroids, alkaloids, flavanoids and polyphenols in the methanolic extract and may be due to synergistic effect. The further study has to be extended for carrying out the mode of action of the plant extract on the cancer cell lines at molecular level.

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