ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Free radical scavenging activity of methanolic extract of *Grewia tiliaefolia* bark in various *In-vitro* model systems

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ABSTRACT

Free radicals are reactive molecules involved in many physiological processes and human diseases, such as cancer, ageing, arthritis and liver injury. As a result of which, much attention has been directed towards the characterization of free radical scavenging activity or antioxidant activity of plants and plant extracts. The present study was designed to evaluate the free radical scavenging activity of methanolic extract of *Grewia tiliaefolia* Bark in the specific *in vitro* model systems. The different radical systems comprised of superoxide radical, hydroxyl radicals, nitric oxide radical, DPPH, ABTS, and lipid peroxidatin systems were selected for the present experiment. The extract was used at different concentrations like 50, 100, 150, 200 and 250µg/ml and radical scavenging activity was determined in terms of inhibition %. The IC50 (Concentration required for 50 % inhibition) was calculated for the test drug against each radicals. The study proved that the *G. tiliaefolia* extract found to have potential radical scavenging activity against different radical system. The highest activity was observed against DPPH radicals, and then followed by hydroxyl radical, superoxide radical, ABTS, lipid peroxidation system and nitric oxide radical. The activity may be due to the presence of phytomolecules like lignans, flavanoids, phenols and alkaloids in the crude extract. The further study has to be focused on isolation and characterization of functional molecules of the extract and their individual activity.

Keywords: free radicals, antioxidant activity, *Grewia tiliaefolia*, Growth Inhibition 50%

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ISSN: 0975-8585

INTRODUCTION

Oxygen is essential for the survival of all on this earth. During the process of oxygen utilization in normal physiological and metabolic processes approximately 5 % of oxygen gets univalently reduced to oxygen derived free radicals like superoxide, hydrogen peroxide, hydroxyl and nitric oxide radicals [1-2]. All these radicals known as reactive oxygen species (ROS) exert oxidative stress towards the cells of human body rendering each cell to face about 10,000 oxidative hits per second [3]. When generations of ROS overtakes the antioxidant defense of the cells, the free radicals start attacking the cell proteins, lipids and carbohydrates [4-6] and this leads to a number of physiological disorders. Free radicals are involved in the development of degenerative diseases [7]. They have also been implicated in the pathogenesis of diabetes, nephrotoxicity, liver diseases, cancer, cardiovascular diseases and in the process of ageing. Many plants often contain substantial amounts of antioxidants including polyphenols, flavonoids ant tannins etc., and thus can be utilized to scavenge the excess free radical from human body.

Grewia tialefolia is a medium sized tree upt o 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, alternate, ovate with oblique base, crenate-denate, acuminate, upper surface minutely stellately hairy. The fruits are globose drupes of the size of a pea, 2-4 lobed, black when ripe, and 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 [8].

So, considering the traditional use of this plant, the present study has been designed to evaluate the free radical scavenging activity of methanolic extract of *Grewia tiliaefolia* Bark in various *in vitro* model systems.

MATERIALS AND METHODS

Chemicals and Reagents

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 (FeSO4), thiobarbituric acid (TBA), trichloroacetic acid (TCA), nitroblue tetrazolium (NBT) and ethylene diamine tetra acetic acid obtained from Merck India.

ISSN: 0975-8585

Plant Material and Extraction

The plant material was collected from the Western ghat region of Kerala. The plant was authenticated by the Kerala Forest Research Institute, Peechi. The shade dried bark material was used for the extraction. Nearly 200 gm of coarse powdered bark was taken in the cellulose thimble and exhaustively extracted with methanol using Soxhlet extraction apparatus for continuous 8 hrs. The solvent was removed using rotary vacuum evaporator, and solvent-free extract was aliquoted in different sterile vials and stored in refrigerator until further use.

Scavenging of superoxide radical

The scavenging activity towards the superoxide radical ($O2^{\cdot-}$) was measured in terms of inhibition of generation of O_2 [9]. 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.

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

Scavenging of hydroxyl radical

Hydroxyl radical scavenging activity was measured according to the method of Kunchadny and Rao [10], 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 form sodium nitroprusside and measured by Griess reaction [11,12]. 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.

DPPH radical activity

DPPH scavenging activity was measured by the spectrophotometric method [13]. To a methanolic solution of DPPH (200µM), 0.05 ml of the test compounds dissolved in methanol were added at different concentrations (50-250 µg/ml). An equal amount of methanol was added to the control. After 20 min, the decrease in the absorbance of test mixture (due to quenching of DPPH free radicals) was read at 517 nm and the percentage inhibition calculated by using the formula [14]. The experiment was repeated in triplicate.

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

ABTS radical cation decolorisation assay

In this improved version, $ABTS^{\cdot-}$, the oxidant is generated by persulfate oxidation of 2, 2-azinobis (3-ethylbenzoline-6-sulfonic acid)- ($ABTS^{2-}$) [15]. ABTS radical cation was produced by reacting ABTS solution (7mM) with 2.45 mM ammonium persulphate and the mixture were allowed to stand in dark at room temperature for 12-16 hrs before use. For the study, different concentrations (50-250 µg/ml) of methanolic extract (0.5 ml) were added to 0.3 ml of ABTS solution and the final volume was made up with ethanol to make 1.0 ml. The absorbance was read at 745 nm and the percentage inhibition calculated.

***In vitro* lipid peroxidation assay**

Freshly excised rat liver was processed to get 10% homogenate in cold phosphate buffered saline, pH 7.4 using glass Teflon homogenizer and filtered to get a clear homogenate. The degree of lipid peroxidation was assayed by estimating the TBARS by using the standard method [16] with minor modifications. Different concentrations of the extracts (50-250 µg/ml) in water were initiated by adding 100 µl of 15 mM ferrous sulphate solution to 3 ml of the tissue homogenate. After 30 min, 100 µl of this reaction mixture was taken in a tube containing 1.5 ml of 10% TCA. After 10 min, tubes were centrifuged and supernatant was separated and mixed with 1,5 ml of 0.67 % TBA in 50 % acetic acid. The mixture was heated for 30 min in a boiling water bath. The intensity of the pink colored complex formed was measured at 535 nm. The results were expressed as percentage Inhibition.

RESULTS

The present study showed that the methanolic extract of *Grewia 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, DPPH

ISSN: 0975-8585

radical, ABTS and lipid peroxidation systems 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 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 IC50 (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 DPPH radicals and then followed by Hydroxyl radical, superoxide radical, ABTS, lipid peroxide system and nitric oxide radical.

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	Nitricoxide radical	DPPH radical	ABTS	Lipid peroxidation
50	16.42 ± 0.45	27.60 ± 0.69	11.70 ± 0.65	32.10 ± 0.40	8.38 ± 0.50	14.580 ± 0.63
100	29.58 ± 0.78	42.60 ± 0.70	26.42 ± 0.48	59.40 ± 0.76	21.45 ± 0.67	27.65 ±0.69
150	53.50 ± 0.83	56.42 ± 0.85	34.66 ± 0.83	71.25 ± 1.21	38.32 ± 0.70	36.58 ± 0.73
200	69.80 ± 1.40	69.35 ± 0.94	48.52 ± 1.30	77.65 ±0.94	55.42 ± 0.81	54.30 ± 1.22
250	80.11 ± 1.38	84.30 ±1.20	61.95 ± 1.55	85.44 ± 1.13	76.38 ± 1.37	60.83 ± 0.80

(Values are expressed Mean ± SEM, n=3)

Figure.1. Comparative analysis of free radical scavenging actvity of *G. tiliaefolia* at different concentration.

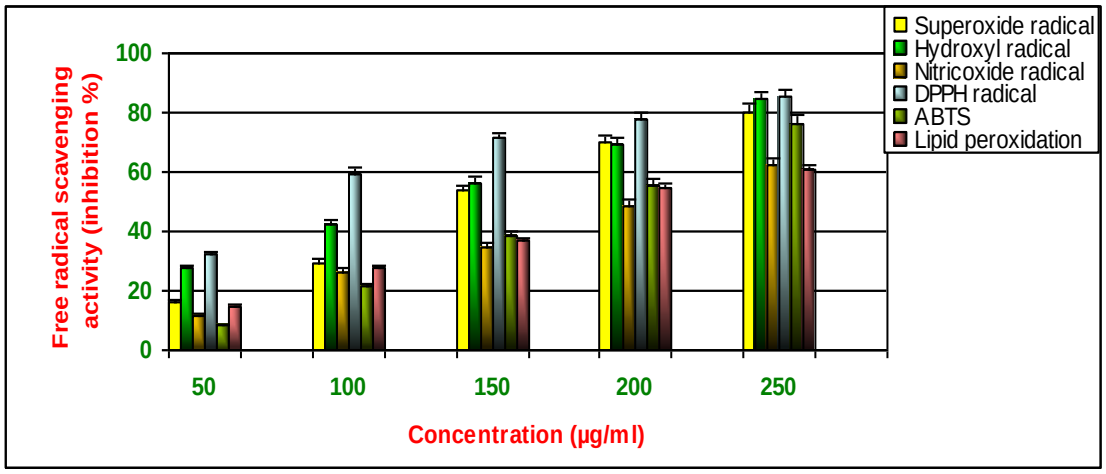

Table. 2. IC 50 value of the methanolic extract of *G. tiliaefolia* on different radical system.

ree radical systems	IC 50 value *(µg/ml) of G. tilaefolia*
Superoxide radical	150.35
Hydroxyl radical	128.40
Nitric oxide radical	204.44
DPPH radical	82.29
ABTS	179.50
Lipid peroxidation	197.04

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. The free radicals are involved in various acute and chronic diseases including cancer, atheroscelerosis, ageing etc [17]. The elevation of free radicals in cancerous conditions has been well documented [18,19].

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 [20]. The scavenging activity towards the superoxide radical ($O2^{\cdot-}$) is measured in terms of inhibition of generation of $O2^{\cdot-}$. 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. There are similar reports for other plants that are carried out in different systems [21]. The probable mechanism of scavenging the superoxide anions may be due to the presence of lignans, phenolic compounds that are present in the extracts and their uptake of generated superoxide in the *in vitro* reaction mixture.

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 [22]. 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 [23]. In the present study, the

methanolic extract of *G. tiliaefolia* showed better activity in competing with oxygen to react with nitric oxide and thus the inhibition of generation of anions.

The 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical was widely used as the model system to investigate the scavenging activities of several natural compounds such as phenolic and anthocyanins or crude mixtures such as the methanol extract of plants. DPPH radical is scavenged by antioxidants through the donation of proton forming the reduced DPPH. DPPH is a relatively stable radical. The assay is based on the measurement of the scavenging ability of antioxidants towards the stable radical DPPH which reacts with suitable reducing agent. The electrons become paired off and solution loses colour stochiometrically depending on the number of electrons taken up. From the present study, it may be postulated that *Grewia tiliaefolia*, has DPPH scavenging activity, by reducing the radical to corresponding hydrazine when it reacts with hydrogen donors in antioxidant principles.

The ABTS assay is based on the inhibition of the absorbance of the radical cation $ABTS^{\cdot+}$, which has a characteristic long wavelength absorption spectrum. The results indicated the methanolic extract of *G. tiliaefolia.*

Lipid peroxidation is the oxidative degradation of polyunsaturated fatty acids and involves formation of lipid radicals leading to membrane damage. Free radicals induce lipid peroxidation in polyunsaturated lipid rich areas like brain and liver [24]. Initiation of lipid peroxidation by ferrous sulphate takes place through hydroxyl radical by Fenton' reaction. The inhibition could be caused by absence of ferryl-perferryl complex or by scavenging the hydroxy radical or the superoxide radicals or by changing the Fe^{3+}/Fe^{2+} or by reducing the rate of conversion of ferrous to ferric or by chelating the iron itself. The present study showed the efficacy of antioxidant activity of methanolic extract of *Grewia tiliaefolia* in the selected model system.

The production of free radicals and the scavenging activity of test extracts can be correlated with the life expectancies. Polyphenols, tannins and flavanoids are very valuable plant constituents in the scavenging action due to their several phenolic hydroxyl groups. However, the phytoconstituents like polyphenol, flavanoids, sterols and triterpenoids present in the plant extracts may be responsible for antimicrobial, antioxidant and free radical scavenging activities as reported by many researchers for various plants [25, 26].

The further study has to be carried out for isolation characterization of functional molecules in the extract and assessing the efficacy in suitable *in vivo* systems.

CONCLUSION

The present study proved the free radical scavenging activity of methanolic extract of *G. tiliaefolia* against various synthetic radicals in the *in vitro* model systems.

ISSN: 0975-8585

ACKNOWLEDGEMENT

The authors are thankful to the sponsoring agency ICAR for the successful completion of the work and extend gratitude to Anna University, Chennai for providing the instrumentation facility.

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