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RESEARCH MANUSCRIPT

Evaluation of Antiproliferative effect of *Grewia hirsuta* on HepG2 cell lines

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Abstract

Antioxidant and antiproliferative potential of *Grewia hirsuta* was evaluated in this study. Free radical scavenging potential of the plant was studied by various *in vitro* assays such as DPPH, Phosphomolybdenum, metal chelating and hydroxyl radical scavenging assay. Methanol extract is more powerful in scavenging free radicals, reducing phoshomolybdenum ions and in chelating metal ions. The preliminary phytochemical screening of *Grewia hirsuta* revealed the presence of phenolics, carbohydrates, alkaloids, flavonoids and tannins in high amount. The antiproliferative potential of the methanol extract was studied on HepG2 cell lines by MTT assay. Methanol extract had cytotoxic effect on HepG2 cell lines with IC_{50} value of 15.6 µg/mL. Thus, the study revealed that *Grewia hirsuta* could be considered as a significant source of antioxidant and antiproliferative agents.

Keywords: Antioxidant, antiproliferative, *Grewia hirsuta*, DPPH assay, HepG2 cell lines**.**

Introduction

Oxidation processes are intrinsic in the energy management of all living organisms and are therefore kept under strict control by several cellular mechanisms (Block *et al.*, 1992). However, the excessive production of free radicals and the unbalanced mechanisms of antioxidant protection result in the onset of numerous diseases and accelerate ageing. In the past decade, a number of epidemiological studies have confirmed that intake of exogenous antioxidants is effective in preventing or suppressing such diseases (Singh and Downing, 1995; Zhenbaoa *et al.,* 2007). Several commercially available synthetic antioxidants such as butylated hydroxyanisole, butylated hydroxytoluene (BHT) and tert-butylhydroquinone (TBHQ) are currently in use but their possible toxic properties for human health and environment are inevitable (Fattouch *et al.*, 2007).

Hence, the development of alternative antioxidants from natural origin is the need of the hour. Among dietary antioxidants, phenolics, secondary metabolites from plants are the most abundant natural antioxidants which act as reducing agents, hydrogen donators, free radicals scavengers and singlet oxygen quenchers and therefore, as cell saviours. *Grewia hirsuta* is a shrub or small tree, with branchlets are coarsely gray-brown hairy. Leaf stalks are 2-3 mm long, tomentose. Leaves are lance-shaped, 6-14 cm long, 2-3.5 cm wide, leathery, black-brown when dried, velvety. Lateral basal veins are up to 1/2 as long as leaf blade, lateral veins 4-5 pairs, base narrow, shallowly heart-shaped, margin toothed, tip long pointed or rarely blunt.

Nagbala, as it is called in Ayurvedic lingo, is used for heart disease, cough, wounds and dyspnoea (root); in diarrhoea and dysentery (drupes); heart disease, fever (Kikuzaki and Nakatani, 1993). In the present investigation, an attempt was made to evaluate the antioxidant and antiproliferative potential of *Grewia hirsuta*.

Materials and methods

Collection of plant material: The leaves of *Grewia hirsuta* were collected from Nanmangalam forest, Chennai, and authenticated by Dr. N. Mathivanan, CAS in Botany, University of Madras, Chennai. The collected plant parts were cleaned and shade-dried for 7 d and powdered. Twenty gram of each powdered samples were extracted with 200 mL of methanol under shaking condition (Eloff, 1998). The extracts were decanted into pre-weighed glass flasks. The process was repeated thrice to exhaustively extract the plant material and combined extracts were concentrated in a rotary evaporator (Remi, Mumbai, India). The condensate were weighed and stored for further analysis.

Free radical scavenging activity on DPPH: Antioxidant activity of the extracts was determined in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH according to Koleva *et al.* (2002). Sample extracts at various concentrations was taken and the volume was adjusted to 100 µL with methanol. Methanolic solution (5 mL) of DPPH (0.1 mM) was added and shaken vigorously. The tubes were allowed to stand for 20 min at 27°C. The absorbance of the sample was measured at 517 nm.

Radical scavenging activity was expressed as the inhibition percentage of free radical by the sample and was calculated using the formula:

% DPPH activity = (Control OD–Sample OD/Control OD) × 100.

Phosphomolybdenum assay: Antioxidant activity of samples was evaluated by green phosphomolybdenum complex formation according to Prieto *et al.* (1999). An aliquot of 100 µL of sample solution was combined with 1 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) in a 4 mL vial. The vials were capped and incubated in a water bath at 95°C for 90 min. After cooling, the absorbance of the mixture was measured at 695 nm against a blank. Ascorbic acid (10 mg/mL DMSO) was used as standard. The results were reported as % phosphomolybdenum reduction potential.

Hydroxyl radical scavenging activity: The scavenging activity of the methanol extracts on hydroxyl radical was measured according to Klein *et al.* (1992). Various concentrations (250, 500, 750 and 1000 µg/mL) of extracts were added with 1.0 mL of iron-EDTA solution, 0.5 mL of EDTA solution and 1.0 mL of dimethyl sulphoxide (DMSO). The reaction was initiated by adding 0.5 mL of ascorbic acid and incubated at 80-90°C for 15 min in a water bath. After incubation the reaction was terminated by the addition of 1.0 mL of ice-cold TCA. Nash reagent (3 mL) was added and left at room temperature for 15 min. The reaction mixture without sample was used as control. The intensity of the color formed was measured spectroscopically at 412 nm against reagent blank. The % hydroxyl radical scavenging activity (HRSA) is calculated by the following formula:

$$\% \text{ HRSA} = [(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100$$

Where, $Abs_{control}$ is the absorbance of the control; Abs_{sample} is the absorbance of the extract/standard.

Metal chelating activity: The chelating effect of ferrous ions by the extracts was estimated according to Dinis *et al.* (1994). Hundred µL of the extract was added to 0.05 mL of 2 mM $FeCl_2$. The reaction was initiated by the addition of 0.2 mL of ferrozine (5 mM) and the mixture was shaken vigorously and left at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. The chelating activity of the extracts was evaluated using EDTA as standard. The results were expressed as % metal chelating activity. The ratio of inhibition of ferrozine Fe^{2+} complex was calculated as follows:

% inhibition = (Control OD–Sample OD/Control OD) × 100.

Qualitative and quantitative phytochemical screening: The extracts were subjected to preliminary phytochemical screening to identify the presence of phytoconstituents such as alkaloids, flavonoids, saponins, tannins, phenols, glycosides and steroids according to Harborne (1973).

Estimation of total free phenolics: Total phenolic constituents of plant extracts were estimated by Folin-Ciocalteau's method using Folin-Ciocalteu reagent. The estimation was done spectrometrically at 760 nm and the results were expressed as gallic acid equivalents (GAE) (Sengul *et al*., 2009).

Estimation of total flavonoids: Aluminium chloride method was employed to quantify the total flavonoid content in the plant extracts. The results were expressed as quercetin equivalents (QE) (Chang *et al*., 2002).

Estimation of total alkaloids: Total alkaloid content of the plant extracts was determined according to Sutharsingh *et al*. (2011). Five gram of the sample was filtered and concentrated to one quarter of the original volume on a water bath after treatment with 200 mL of 10% acetic acid in ethanol. Concentrated NH_4OH was added drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitate was collected and washed with dilute NH_4OH, filtered and weighed.

Estimation of total saponins: Powdered sample (20 g) was treated with 100 mL of 20% aqueous ethanol, heated over a hot water bath for 4 h at about 55°C with continuous stirring. The mixture was filtered and the residue re-extracted. The combined extracts were reduced to 40 mL over water bath at about 90°C and the concentrate was transferred into a separating funnel and 20 mL of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated and 60 mL of n-butanol was added to the combined extracts and washed twice with 10 mL of 5% aqueous NaCl. The remaining solution was heated in a water bath, dried in an oven to a constant weight and the saponin content was calculated as percentage (Sutharsingh *et al*., 2011).

Thin layer chromatography: The plant extract was loaded on pre-coated silica plates which were then developed using methanol, chloroform in the ratio of 1:9. The spots were identified both in the UV light and in the iodine chamber. Then R_f value was calculated as the ratio of distance travelled by the solute to the distance travelled by the solvent (Eloff, 1998).

Bioautography: The extract which showed DPPH inhibition of more than 90% was examined by thin layer chromatography (TLC) bioautography.

The plant extract was applied to pre-coated thin layer chromatography sheet and run with the developing solvent mixture then allow it for dry. After drying the plates, it was dipped in 0.2% DPPH reagent in methanol or ethanol and was left for 30 min at room temperature. The plates were observed under white light. Antioxidant activity was confirmed when the DPPH purple color changed to yellow (Saleh *et al*., 2010).

Results and discussion

Radical scavenging activity of leaf extracts: The DPPH radical is widely used to evaluate the free-radical scavenging capacity of antioxidants (Zhenbao *et al*., 2007). From the dose dependent response curve, DPPH radical scavenging activity of different plant extracts of *Grewia hirsuta* was observed. At a concentration of 200 µg, the scavenging activity of methanol extract reached 95%. The methanol extract of *Grewia hirsuta* showed good antioxidant and free radical scavenging activity (Fig. 1). In a similar study, the DPPH radical scavenging activity of solvent fractions of *Cassia tora* was studied in which, ethyl acetate extract showed the highest scavenging activity with an EC_{50} value of 70.1 µg/mL (Zhenbao *et al*., 2007).

Hydroxyl radical scavenging activity: Hydroxyl radical is a form of ROS and is associated with arthritis, cytotoxic, mutagenic and genotoxic involved in disease pathogenesis (Naithani *et al*., 2011). The hydroxyl radical is one of representative reactive oxygen species generated in the body and these radicals are produced through various biological reactions; one of the common reactions is the Iron (II) based Fenton reaction. According to Koo *et al.* (2004), Genipin, active principal of Gardenia possesses anti-inflammatory and is a specific hydroxyl radical. The radical scavenging capacity may be attributed to phenolic compounds in methanol extract with the ability to accept electrons, which can combine with free radical to decrease hydroxyl radical (Fig. 2).

Metal chelating activity: Transition metal has played a pivotal role in the generation of oxygen free radicals in living organisms. Chelating agents may inactivate metal ions and potentially inhibit the metal-dependent processes (Finefrock *et al*., 2003). Presence of transition metal ions in a biological system could catalyse the Haber–Weiss and Fenton-type reactions, resulting in generation of hydroxyl radicals (OH). However, these transition metal ions could form chelates with the antioxidants, which result in the suppression of OH generation and inhibition of peroxidation of biological molecules. Metal ion chelating activity of an antioxidant molecule prevents generation and the consequent damage. Metal ion chelating capacity plays a significant role in antioxidant mechanisms since it reduces the concentration of catalyzing transition metal in LPO (Sudha *et al.,* 2011).

Fig. 1. DPPH radical scavenging activity of *G. hirsuta* methanolic extract.

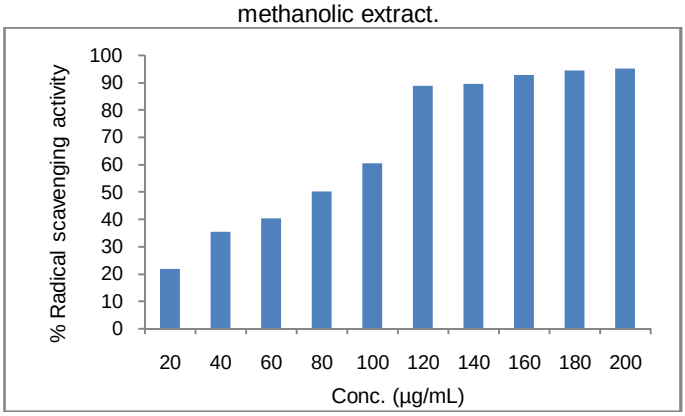


Fig. 2. Hydroxyl radical scavenging activity of *G. hirsuta* methanolic extract.

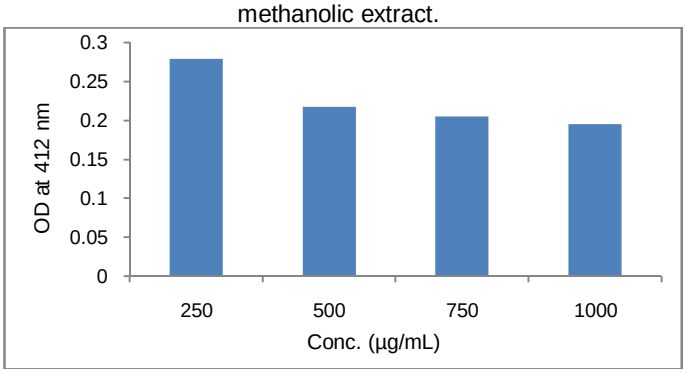


Fig. 3. Metal chelating activity of *G. hirsuta* methanolic extract.

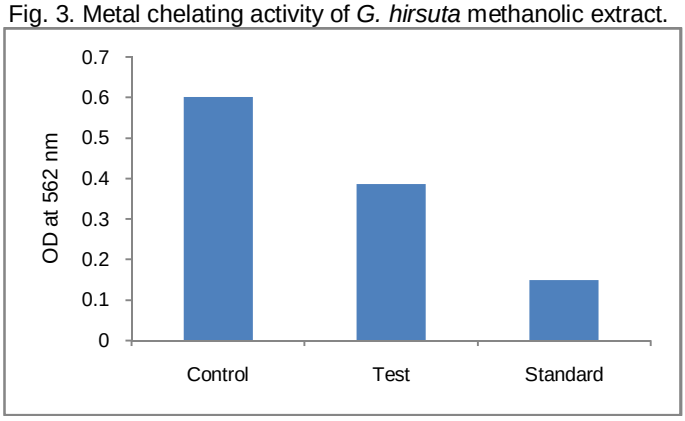
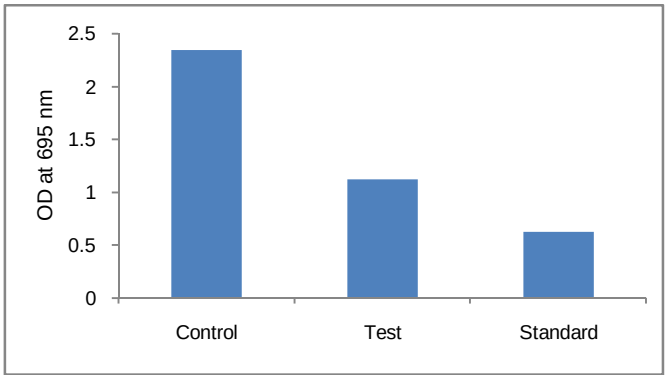


Fig. 4. Phosphomolybdenum reducing activity of *G. hirsuta* methanolic extract.



In this assay, the presence of chelating agents in the extracts of *Grewia hirsuta* disrupts the ferrozine-Fe^{2+} complex formation, thus decreasing the red color. The metal ion scavenging effects of methanol extract is depicted in Fig. 3. It is reported that chelating agents are effective as secondary antioxidants because they reduce the redox potential, thereby stabilizing the oxidized form of the metal ion.

Phosphomolybdenum assay: DPPH scavenging assay detects antioxidant such as flavonoids and polyphenols, whereas phosphomolybdenum assay usually detects antioxidant such as ascorbic acid, some phenolics, atocopherol and carotenoids (Harini *et al.,* 2012). The assay is based on the reduction of Mo (IV) to Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex. The phosphomolybdenum method is quantitative since the antioxidant activity is expressed as the number of equivalents of ascorbic acid. The methanol extract of *Grewia hirsuta* was used to determine their antioxidant capacities by the formation of green phosphompolybdenum complex. The results indicate that the methanol extract is more powerful antioxidant in the reduction of phosphomolybdenum complex (Fig. 4).

Qualitative phytochemical analysis: The preliminary phytochemical screening of *Grewia hirsuta* revealed the presence of phenolics, carbohydrates, alkaloids, flavonoids, tannins and steroids in high amounts followed by glycosides in trace. The saponins, proteins and amino acids are absent (Table 1).

Quantitative phytochemical analysis: The major phytochemicals present in the selected plant extracts were phenols, flavonoids, alkaloids and tannins were quantified. The results of total phenol content, alkaloids, saponins and flavonoids are given in Table 2.

Thin layer chromatography: The chromatogram developed with 10% methanol in chloroform revealed the presence of five major compounds at R_f value of 0.43; 0.58; 0.76; 0.86; 0.95 as visualized under iodine vapour and UV illumination.

Autobiography: The specific compound (band) which has antioxidative properties shows Rf value of 0.76, which was chosen as effective compound, scraped and partially purified. The purity of the compound was checked by TLC with 10% methanol in chloroform.

Anticancer activity: In oncology research and clinical practice many anti-proliferative assays are used in the assessment of cancer types of individual patients. Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay has been described as rapid, simple and reproducible method, widely used in the screening anticancer drugs and to measure the tumor cell proliferation (Edmondson *et al.*, 1988; Fotakis and Timbrell, 2006).

Table 1. Qualitative phytochemical estimation.

Compound	Result
Alkaloids	++
Carbohydrates	+++
Glycosides	+
Saponins	-
Proteins and amino acids	-
Phenolic compounds	+++
Steroids	++
Flavonoids	++
Tannins	++

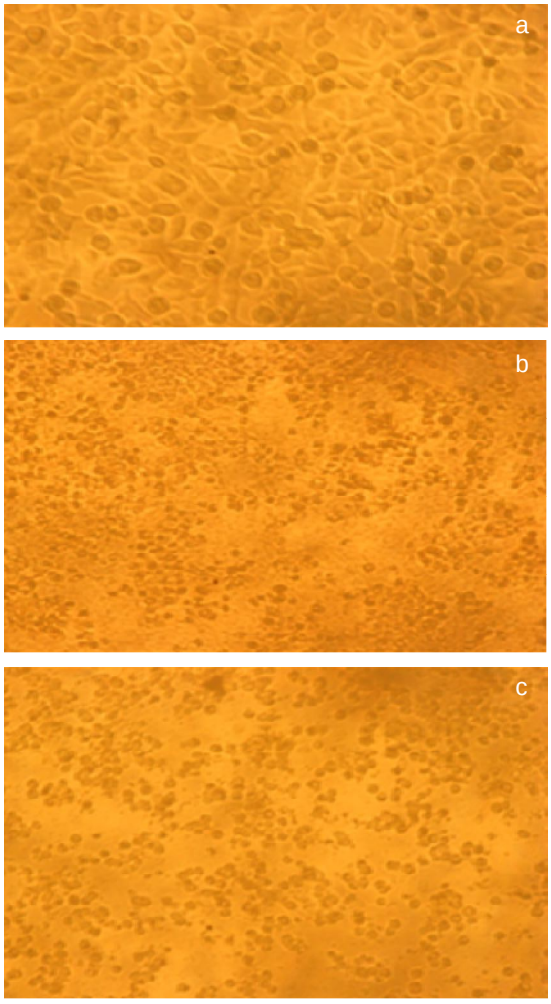
(+) Present in minor amount; (++) Present in moderate amount; (+++) Present in higher amount; (–) Not detectable.

Table 2. Quantitative phytochemical estimation.

Bioactive compound	Result
Total phenols	259.63 µg GAE/g sample
Alkaloids	0.0384 mg/g sample
Saponins	0.116 mg/g sample

GAE–Gallic Acid Equivalent; QE–Quercetin Equivalent.

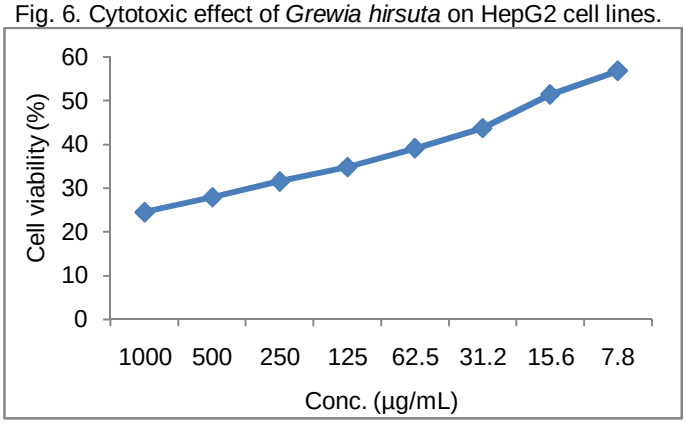
Fig. 5. Cytotoxic effect of *Grewia hirsuta* on HepG2 cell lines.



a. Normal HepG2 cell line
b. Toxicity-10 mg/mL
c. Toxicity-2.5 mg/mL

Hence, in the present investigation, the anti-proliferative property of the selected plant was revealed using this assay. Cell cycle arrest is a common feature of cells that are undergoing terminal differentiation and defective proliferation (Al-Qubaishi *et al*., 2011). The anticancer activity of the methanol extract was subjected for MTT assay (Fig. 5). The extract had an IC_{50} value of 15.6 µg/mL which showed cell viability of 50.4%. From the result, it is clear that methanol extract of *Grewia hirsuta* has cytotoxic effect on HepG2 cell lines (Fig. 6).

Fig. 6. Cytotoxic effect of *Grewia hirsuta* on HepG2 cell lines.



Conclusion

The present investigation suggests that *Grewia hirsuta* possesses significant antioxidant and anti-proliferative potential. Hence, we can conclude that with further mechanistic studies, the plant can be considered as an efficient source of antioxidant and anti-proliferative agents.

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