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Research Article

Antioxidant and cytotoxic effects of methanolic extract of *Salicornia brachiata* L. in HepG2 cells

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ABSTRACT

The aim of this study was to determine the antioxidant and anticancer activities of methanolic extract of *S.brachiata*. In this study; total phenolic content was estimated, and free radical scavenging activity was measured by 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) method, 2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS), Ferric Thiocyanate (FTC), and Thiobarbituric acid (TBA). The methanolic extract of *S.brachiata* was tested against HepG2 cells for its inhibitory effect of cell viability study was carried out by MTT assay. The extract showed highest DPPH inhibition percentage of 85% and ABTS inhibitory activity of 75% at 1000 µg/mL. The inhibition percentage of lipid peroxidation was evaluated by FTC and TBA Method. The results showed decreased cell viability and increased growth inhibition at a concentration dependent manner and altered the cell morphology. The inhibition percentage with regard to cytotoxicity was found to be 80% at 1000 µg/mL. The methanolic extract of *S.brachiata* showed better antioxidant and anticancer activity.

Keywords: Antioxidant; Cytotoxicity; Halophyte; MTT assay; *S. brachiata*

INTRODUCTION

Oxidation is crucial to many living organisms for the production of energy to fuel biological processes. Oxygen-centred free radicals and other Reactive Oxygen Species (ROS) that are continuously produced *in vivo*, result in cell death and tissue damage. Oxidative damage caused by free radicals may be related to aging and diseases, such as atherosclerosis, diabetes, cancer and cirrhosis (Halliwell, 1984). Antioxidants are major ingredients that protect the quality of oils and fats by retarding oxidation (Wanasundara *et al.*, 1994). Synthetic antioxidants are used at legal limits to reduce deterioration; rancidity and oxidative discoloration (Dziezak, 1986). There are some serious problems concerning the safety and toxicity of such synthetic antioxidants related to their metabolism and possible absorption and accumulation in body organ and tissues (Tappel, 1995). Therefore, the search for preparation of useful natural antioxidants is highly desirable, which is found to be found in abundance in plant materials

such as oilseeds, cereal crops, vegetables, fruits, leaves, barks and roots, spices and herbs (Ramarathnam *et al.*, 1995). Plants contain high concentrations of numerous redox-active antioxidants, such as polyphenol, flavanoid, carotenoids, tocopherols, glutathione, ascorbic acid and enzymes with antioxidant activity, which fight against hazardous oxidative damage of plant cell components (Schaffer *et al.*, 2007). Phenolics are antioxidants, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers, and it also had metal chelating properties (Kahkonen *et al.*, 1999).

Cancer is one of the most prominent diseases in humans. Currently, there is a considerable scientific and commercial interest in continuing the discovery of new anticancer agents from natural products (Kingham *et al.*, 2003). Anticancer agents are mainly related to their therapeutic role in a damaged system. Under the normal cells in which the DNA or other components are eternally damaged by various causes undergo apoptotic cell death, which is a self-destructive metabolism according to the genetically encoded cell death-signal (Hooper *et al.*, 1999). However, cancer cells are already irreversibly developed and obtain the capability to evade apoptosis by various ways. The aim of anticancer agents is to trigger the apoptosis signaling system in these cancer cells whilst disturbing their proliferation (Bold *et al.*, 1997).

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Salicornia brachiata (Chenopodiaceae) is a stem succulent and leafless euhalophyte with potential ecological, economic and medicinal uses. It was used as a folk medicine to treat a variety of diseases such as constipation, obesity, diabetes and cancers (Park, 2000) and is known to have antibacterial, antifungal and antioxidant activity due to a range of bioactive compounds (Lee et al., 2002). *S. brachiata* contains salt stored in its vacuoles which it absorbs when grown under saline conditions. It was also shown that major phytoconstituents such as 2'-hydroxy - 6, 7-methylenedioxyisoflavone, -2'-hydroxy-6, 7-methylenedioxyisoflavanone and 2', 7-dihydroxy- 6-methoxyisoflavone that are associated with osmoregulation of the plant (Arakawa et al., 1982). Hence, in the present study, an effort was to evaluate the antioxidant and anticancer activity of methanolic extract of *S. brachiata*.

MATERIALS & METHODS

Plant Material

The halophyte, *S. brachiata* belonging to the *Chenopodiaceae* family was collected from the back water areas of Ennore, Chennai, Tamil Nadu. The plant materials were collected and washed with water and shade dried at room temperature over a period of two weeks.

Preparation of Plant Extract

The dry plant material was ground into a fine powder using the mixer grinder. A portion of plant material (50 g) was successively extracted with 500 mL of methanol by using a Soxhlet apparatus at a temperature not exceeding the boiling point of the solvent (Lin et al., 1999). The methanol extracts were filtered using whatman filter paper (No: 1) the pooled extracts were concentrated under rotary evaporator at 40°C. The concentrated extract was prepared and used for the further studies.

Determination of Total Phenol Content

To 125 µL of the diluted sample extracts, 0.5 mL of distilled water and 125 µL of the Folin-Ciocalteu reagents (Dewanto et al., 2002) were added. The mixture was allowed to stand for 6 min and then 1.25 mL of 7% aqueous sodium carbonate solution was added. The final volume was adjusted to 3 mL. The mixture was allowed to stand for 90 min and the absorption was measured at 760 nm against water as a blank. The amount of total phenolics is expressed as gallic acid equivalents (GAE, mg gallic acid/g sample) through the calibration curve of gallic acid. The calibration curve ranged between 20-100 mg/mL.

Scavenging Activity of DPPH Radical

The scavenging activity of the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical was determined by the method described by Marwah et al (Marwah et al., 2007). The reaction medium contained 2 mL of 0.135 mM DPPH violet solution in ethanol and 2 mL of plant

extract (or water for the control). The reaction mixture was incubated in the dark for 15 min. and the absorbance was measured at 517 nm. The assay was carried out in triplicate. The decrease in absorbance on addition of test samples was used to calculate the antiradical activity, as expressed by the inhibition percentage (%IP) of DPPH radical, following the equation:

$$\text{Inhibition percentage (\%IP)} = [(A_c - A_s) / A_c] \times 100$$

Antioxidant Activity by the ABTS⁺ Assay

The 2, 2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) radical cation scavenging activity was measured according to the method described by Re et al (Re et al., 1999). ABTS was dissolved in water to a 7 mM concentration. The ABTS radical cation was produced by adding to the ABTS stock solution 2.45 mM potassium persulphate. The completion of radical generation was obtained in the dark at room temperature for 12–16 h. This solution was then diluted with ethanol to adjust its absorbance at 734 nm to 0.70 ± 0.02. To determine the scavenging activity, 1 mL of diluted ABTS solution was added to different concentration of plant extract (or water for the control), and the absorbance at 734 nm was measured 6 mins after the initial mixing, using ethanol as the blank. The percentage of inhibition was calculated by the equation:

$$\text{Inhibition percentage (\%IP)} = [(A_c - A_s) / A_c] \times 100$$

Determination of Antioxidant Activity with Ferric Thiocyanate (FTC) Method

The antioxidant activity analysis using FTC was performed according to the method reported by Osawa & Namiki (Osawa et al., 1981). Different concentrations of *S. brachiata* extract were dissolved in 0.12 mL of 98% ethanol, and 2.88 mL of 2.51% linoleic acid solution in Ethanol to which 9 mL of a 40 mM phosphate buffer (pH 7.0) was added. The mixture was incubated at 40°C in a dark screw-cap vial. During the incubation, 0.1 mL aliquot was taken from the mixture, and diluted with 9.7 mL of 75% ethanol, followed by the addition of 0.1 mL of 30% ammonium thiocyanate. Precisely, 3 mins after adding 0.1 mL of 20 mM ferrous chloride in 3.5% hydrochloric acid, the absorbance for the red color was measured at 500 nm. The percent inhibition of linoleic acid peroxidation was calculated as:

$$\text{Inhibition of Lipid peroxidation} = 100 - [(\text{absorbance increase of the sample} / \text{absorbance increase of the control}) \times 100]$$

Thiobarbituric Acid Test (TBA)

This assay was performed according to the method reported by Kikuzaki & Nakatani (Kikuzaki et al., 1993). Two milliliters of 20% trichloroacetic acid and 2 mL of TBA solutions were added to 1 mL of the mixture solution containing linoleic acid, which was prepared according to the FTC procedure. The mixture was then placed into a boiling water bath for 10 min. After cooling, the mixture was centrifuged at 3000 rpm for 20

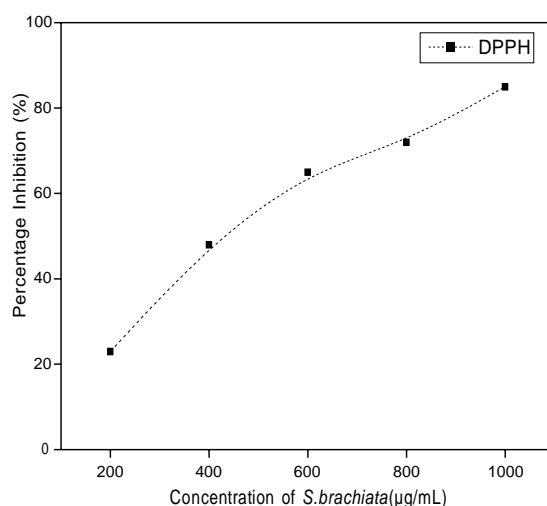


Figure 1: Antioxidant activity of *S.brachiata* was measured by DPPH method

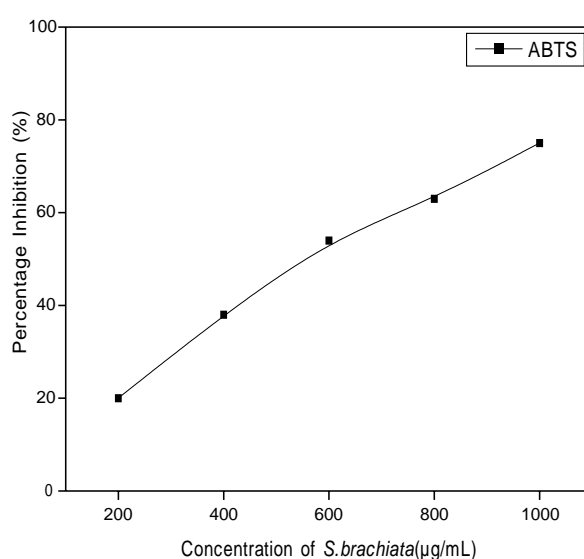


Figure 2: Effect of methanolic extract from *S.brachiata* at various concentrations on ABTS activity

min, and the absorbance of the supernatant was measured at 532 nm.

Cytotoxicity Assay

The cytotoxicity activity of methanol extracts of *S.brachiata* was performed on HepG2 cell lines. The cell viability was measured using MTT assay as a described method of Deepa et al (Deepa et al., 2011). The assay was performed in triplicates of the extracts. The mean of the cell viability values was compared with the control to determine the effect of the extract on cells and % cell viability was plotted against concentration of the plant extract. Cell morphology was observed by optical microscopy. The reductions in cell viability under conditions of co-culture with the tested samples were measured using the MTT assay (Jayakumar et al., 2012). Normal cells were kept as the control. At the end of culture, the yellow tetrazolium MTT solution was added and incubated for 3 h until a purple precipitate was visible. The absorbance of each well was measured at 550 nm. The results were expressed as mean \pm SD of three parallel measurements, and were

analyzed by one-way analysis of variance (ANOVA) with the level of statistical significance set at $p < 0.05$.

RESULTS AND DISCUSSION

The standard curve of gallic acid was prepared, and the total phenolic contents of examined extracts were calculated based upon this standard and presented as gallic acid equivalents (GAE) per gram of dry sample. The total phenolic content was found to be 46.57mg/g of methanolic extracts of *S.brachiata* was shown in Table 1. Generally, extracts that contain a high amount of phenolic content also exhibit high antioxidant activity. Phenolics are very important plant constituents because their hydroxyl groups confer scavenging ability for the plant system (Yildirim et al., 2000). The significance in the phenolics was increased outstandingly due to their prominent free radical scavenging activity. These phenolics are responsible for antioxidant activity and hence, measurement of total phenolic content could be used to relate their antioxidant properties (Katalinic et al., 2006).

Scavenging activity of methanolic extracts of *S.brachiata* is given in the Fig. 1. As seen from Fig. 1,

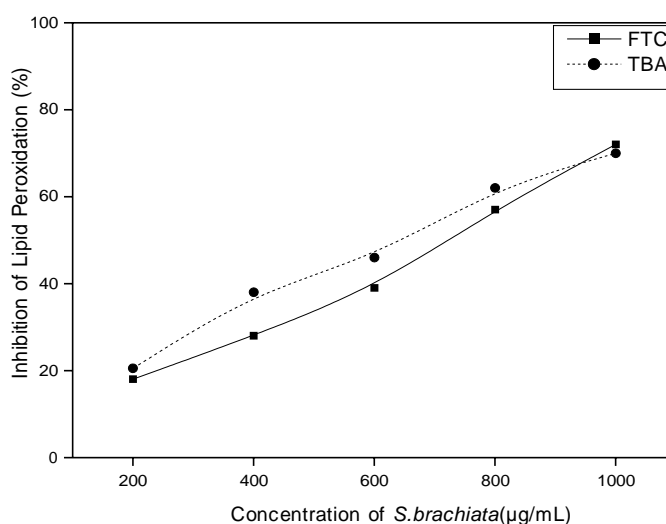


Figure 3: Antioxidant activity by FTC and TBA method of methanolic extract of *S.brachiata*

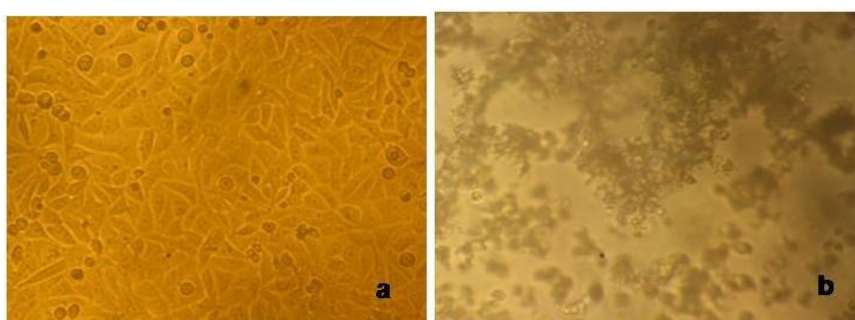


Figure 4: Photomicrograph (Pictorial view) of Normal HepG2 Cells : (a) Normal HepG2 Cells- Control, (b) *S.brachiata* 1000 µg/ml

Table 1: Total phenolic content of the methanolic extract of *S.brachiata*

Plant	Total phenolic content mg/g GAE
<i>S.brachiata</i>	46.57 ± 0.64

Table 2: Determination of cytotoxicity by MTT assay

S. No	Concentration (µg/ml)	Absorbance	Cell viability (%)
1	1000	0.10	20.40
2	500	0.18	36.73
3	250	0.25	51.02
4	125	0.27	55.10
5	62.5	0.31	63.26
6	31.25	0.39	79.59
7	15.625	0.41	83.67
8	7.8125	0.47	95.91
9	Cell control	0.49	100

Cells were treated with extracts in a dose-dependent manner for 48 h; the MTT assay was used to assess cell viability.

the scavenging activity of DPPH was increased. At a concentration of 200 µg/mL, the scavenging ability on DPPH was 23%. However, at 1000 µg/mL, the scavenging activity of *S.brachiata* extracts was 85%. Antioxidants are transfer, an electron or a hydrogen atom to DPPH, thus neutralizing its free radical character (Pan et al., 2008). Free radicals are known to be a major factor in biological damages, and DPPH was used to evaluate the free radical-scavenging activity of natural

antioxidants (Zhu et al., 2001). DPPH, which is a radical itself with a purple color, changes into a stable compound with a yellow colour by reacting with an antioxidant and the extent of the reaction, depends upon the hydrogen donating ability of the antioxidant. The variation of the free radical scavenging activity may be due to the differences in their secondary constituents. The therapeutic potential of natural medicinal plants as an antioxidant in reducing such free radical induced tissue

injury, suggests that many plants have antioxidant activities that can be therapeutically useful (Kanatt *et al.*, 2007).

The ABTS⁺ radical assay is also one of the most commonly used methods to evaluate the antioxidant activity. Figure 2 showed the dose-response curve of ABTS radical scavenging activities of the methanolic extract of the *S.brachiata*. As shown in Figure 2 the radical activity was gradually increased. ABTS scavenging activity at various concentrations from 200, 400, 600, 800, 1000 µg/mL of the extract showed the percentage inhibition ranged from 20% to 75% respectively. The ABTS radical assay is also one of the most commonly used methods to evaluate the antioxidant activity. The method is based on the ability of antioxidant molecules to quench the long-lived ABTS radical cation (ABTS⁺).

Antioxidant activity of the crude extracts was measured using the ferric thiocyanate (FTC) method as given in Figure 3. The inhibition percentage of FTC method was found to be 72% of the *S.brachiata* extract. In the FTC test, this determines the amount of peroxide produced during the initial stages of lipid peroxidation, where the ferric ion was formed upon reaction of peroxide with ferrous chloride. The ferric ion will then unite with ammonium thiocyanate producing ferric thiocyanate, a red-coloured substance. The darker the colour, the higher will be the absorbance. As shown in Figure 3, *S.brachiata* extracts delayed the oxidation of linoleic acid, based on low absorbance values, and exhibited higher antioxidant activity. This reduction is due to the increased level of Malonaldehyde (MDA) compounds from linoleic acid oxidation, which is not stable. Membrane lipids are rich in unsaturated fatty acids that are most susceptible to oxidative processes. Especially, linoleic acid and arachidonic acid are targets of lipid peroxidation (Kikuzaki *et al.*, 1993).

The antioxidation effect of the extracts and standards on the peroxidation of linoleic acid was investigated, and the result was given in Figure 3. The Figure shows the percentage of inhibition as the gradual increase in the antioxidant activity by TBA method. FTC is used to measure the production of peroxide compound at the beginning stage of oxidation while TBA test is used to measure the secondary product of oxidation such as aldehyde and ketone. MDA is one of the TBARS and products of lipid peroxidation, studied widely as a marker of oxidative stress and as an index of lipid peroxidation (Nabavi *et al.*, 2009). The decrease in the MDA level with the increase in the concentration of the extract indicates the role of the extract as an antioxidant. This could be indicated that the amount of peroxidation was greater than that in secondary stage. Secondary product such as MDA is not stable for a long period. It would be turned into alcohol and acid, which cannot be detected by a spectrophotometer (Janero, 1990).

The cytotoxic study results are tabulated in Table 2. As shown in Table 2, the percentage growth inhibition was increased with increasing concentration of the extract. Anticancer activity of the *S.brachiata* extracts was investigated using MTT assay on HepG2 cell lines. The cell morphology was viewed through the microscope, which clearly shows the cells degradation and granulation at higher when compared to control cells are shown in Figure 4. Polyphenolic compounds might inhibit cancer cells by xenobiotic metabolizing enzymes that alter metabolic activation of potential carcinogens (Zhao *et al.*, 2007). The mechanism of action of anticancer activity of phenolics could be by disturbing the cellular division during mitosis at the telophase stage. It was also reported that phenolics reduced the amount of cellular protein and mitotic index, and the colony formation during cell proliferation of cancer cells (Gawron *et al.*, 1992). In this study, the free radical scavenging activity and the protective effects against macromolecular oxidation as well as the cytotoxicity activity of *S.brachiata* of methanolic extracts was examined.

CONCLUSION

The methanolic extract of *S.brachiata* was confirmed to reveal the antioxidant and anticancer activities. Thus, the content of phenolic compounds could be used as an important indicator of antioxidant capacity. The total phenolic content of methanolic extracts of *S.brachiata* was found to be 46.57 mg/g. The antioxidant activity was analysed by using DPPH, ABTS, FTC and TBA method. DPPH method showed the better antioxidant activity when compared to rest of the methods. Methanolic extracts of *S.brachiata* was tested for their cytotoxic activity on HepG2 cell lines by the MTT assay. A dose-dependent inhibition of cell proliferation was observed for the extract tested during this study. The present study explores that the natural and potent antioxidants and anticancer activity of methanolic extract of *S.brachiata*.

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