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Antioxidant and Antiproliferative Effects of Flavonoids from *Emilia sonchifolia* Linn on Human Cancer Cells

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Abstract: In this study we have looked into a fairly less studied, common edible plant- *Emilia sonchifolia* Linn (ES), for possible antioxidant and antitumor activity. We have isolated the flavonoid fraction from the whole plant and the antioxidant property was assessed by measuring its capacity to inhibit Cu^{2+} induced lipoprotein oxidation in human serum and superoxide production *in vitro* in comparison with Quercetin. The lag time for ES fraction was 60 minutes, whereas for Quercetin it was 72 min. The inhibitory concentration 50% (IC_{50}) values obtained for superoxide production was 48 μg (Flavonoid fraction of ES) compared to 32 μg for Quercetin. Based on these results, the efficiency of the plant product to inhibit the proliferation of human cancer cells were evaluated. Treatment of the cancer cells with the flavonoid fraction of ES showed morphological changes characteristics of apoptosis, confirmed by Ethidium bromide-Acridine Orange staining.

Key words: *Emilia sonchifolia* linn, flavonoid fraction, antioxidant, antitumor, reactive oxygen species, apoptosis

INTRODUCTION

Emilia sonchifolia Linn (ES) (Family: Compositae) is a variable annual weed commonly found in India. The plant is edible, the stem and leaves are cooked and eaten as vegetable in India and other Asian countries. They have a slightly acidic taste with a touch of bitterness and a delicate flavour. The plant is astringent, sweet, thermogenic, antipyretic, ophthalmic and antiasthmatic (Nayar *et al*., 1956). Aqueous and methanolic extracts of ES leaves have been shown to progressively reduce rat paw edema induced by sub plantar injections of albumin, which suggest that these extracts might have anti-inflammatory principles (Muko and Ohiri, 2000). Very little information is available indicating the cytotoxic and antitumor property of the extract (Shylesh and Padikkala, 2000). The present study was conducted to explore the antioxidant and antitumor activities of the flavonoid fraction of ES by cell culture studies.

MATERIALS AND METHODS

Extraction, Isolation and Estimation of flavonoids from *Emilia sonchifolia* Linn: Extraction and isolation of the flavonoid fraction of ES was done as described elsewhere with slight modification (Gayathri Devi *et al*., 2006). Fresh plant materials were collected, authenticated by an expert and an herbarium specimen (KUBH No. 1089) has been deposited in the Department of Botany, University of Kerala, India. The plant was washed thoroughly and shade dried. The dried plant material (1 kg) was then crushed and taken in a round-bottomed flask. 85% ethanol was added such that it covered the material and refluxed in a water bath for 2 days at 60°C. The extract was decanted, filtered and evaporated in a rotor evaporator to remove the alcohol. The extract was cleared of low polarity contaminants such as fats, terpenes, chlorophyll, xanthophyll etc. by repeated extraction with petroleum ether (60-80°C), benzene and ethyl acetate respectively. Ethyl acetate extract contained bulk of flavonoids and this

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extract was evaporated in vacuum and its dried weight was 75 g. It was identified as flavonoid by the method described by Eskin *et al.* (1978) using Quercetin as reference.

***In vitro* antioxidant activity of flavonoid fraction of *Emilia sonchifolia* Linn:**

Cu^{2+} induced lipoprotein oxidation in human serum: The antiperoxidative effect of the flavonoid fraction of ES was compared with that of Quercetin, as a reference antioxidant (Sigma Chemical Company, St Louis, MO, USA). Human serum was used for serum oxidation assay as per the procedure reported earlier (Hodyson *et al.*, 1999). Serum was diluted in PBS and ES (Flavonoid fraction) were added. Oxidation was initiated by adding copper sulphate. The absorbance was read at 234 nm every 20 min for 4 h. The lag time for lipoprotein diene formation was measured from the plot of absorbance against time.

Superoxide production: The ability of the plant extract to inhibit the reduction of nitroblue tetrazolium by superoxide generated by the reaction of photo-reduced riboflavin and oxygen was assayed as reported by Winterbourn *et al.* (1975). To the known dilutions of flavonoid fraction of *Emilia sonchifolia*, EDTA, NBT, Riboflavin and PBS were added. The initial optical density was read at 560 nm. All the tubes were kept in uniform illumination for 15 min and the optical density was taken again at 560 nm. The percentage inhibition of superoxide production by the flavonoid fraction was calculated by comparing with the optical density of control.

Cell culture: The cervical cancer cell (Bu25TK) and epidermoid carcinoma cell line (A431) used for the study were procured from National Centre for Cell Science (NCCS), Pune, India and ovarian cancer cells (AS4, NEO) from Dr. Lois A Anaab, (NIEHS, USA). All cell lines were cultured in Dulbecco's Modified Eagles Medium (DMEM) with 10% Fetal Bovine Serum (FBS) and antibiotics (100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin) at 37°C in CO_2 incubator. DMEM, antibiotics and FBS were purchased from Sigma Chemical Company, St Louis, MO, USA.

***In vitro* growth inhibitory assay:** Cell growth assays were done as described elsewhere with slight modification (Srinivas *et al.*, 2003). Cells were seeded on 96 well plates (5000 cells/well), cultured for a day and then treated with different concentrations (25, 50, 75, 100 μg mL^{-1}) of ES flavonoid fraction for 48 h at 37°C. After incubation, medium was removed and 3-(4-5 dimethylthiozol-2-yl) 2-5 diphenyl tetrazolium bromide (MTT) [(5 mg mL^{-1}) Amersham Biosciences, USA] was added and the cells were further incubated for 3 h. After the incubation, MTT lysis buffer (20% SDS in 50% dimethyl formamide) was added to solubilize the formazan crystals formed. The plates were kept protected from light overnight at 37°C. The colour developed was quantitated in an ELISA plate reader (BioRad systems, USA) at a test wavelength of 570 nm and a reference wavelength of 630 nm. Cell viability was expressed as percentage over the control. The experiments were repeated four times.

Assessment of apoptosis: Characteristic apoptotic morphological changes were assessed by fluorescent microscopy, with acridine orange-ethidium bromide staining method (Garban and Bonavida, 1999). Cells (5×10^5 cells mL^{-1}) were cultured in 12 well plates. The cells were treated with the flavonoid fraction for 6 h. Then the medium was removed and after washing once with PBS, the cells were covered with 100 μL of ethidium bromide/Acridine Orange mixture (1:1, 4 μg mL^{-1} each). After a short period of time, the cells were immediately washed once with PBS and viewed under a fluorescent microscope (TE-Eclipse 300, Nikon).

Statistical analysis: All statistical calculations were carried out with Statistical Package for Social Sciences (SPSS) software program (version 10.0 for Windows). The values are expressed as the mean±SE. The data were analysed using analysis of variance (ANOVA) and significant difference of means was determined using Duncan's multiple range tests at the level of $p<0.05$ (Steel *et al.*, 1997).

RESULTS AND DISCUSSION

The antioxidant potency of the isolated flavonoid fraction was determined by the studies on Cu^{2+} induced lipoprotein oxidation in human serum and superoxide production. The lag time for the serum oxidation curve was calculated by plotting a graph (Fig. 1) between absorbance and time. The lag time (Table 1) values provide an indication of protection of serum lipoproteins against oxidation (Hodyson *et al.*, 2000). The lag time values indicate that flavonoid fraction (60 min) was as effective as Quercetin (72 min). An increase in the lag time and decrease in the slope of the serum oxidation curve

Table 1: Lag time to lipoprotein diene formation

Sample	Lag time (min)
Control	10
Quercetin	72
Flavonoid fraction of ES (50 μg)	36
Flavonoid fraction of ES (100 μg)	60

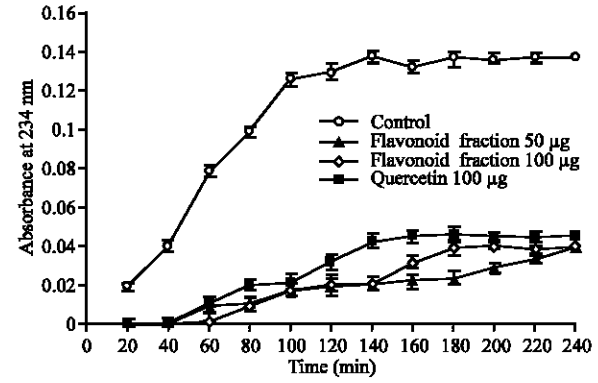

Fig. 1: Cu^{2+} induced lipoprotein oxidation in human serum

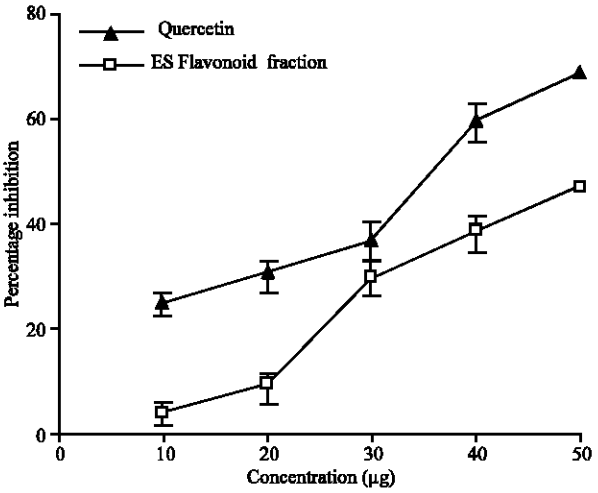

Fig. 2: Percentage inhibition of superoxide production

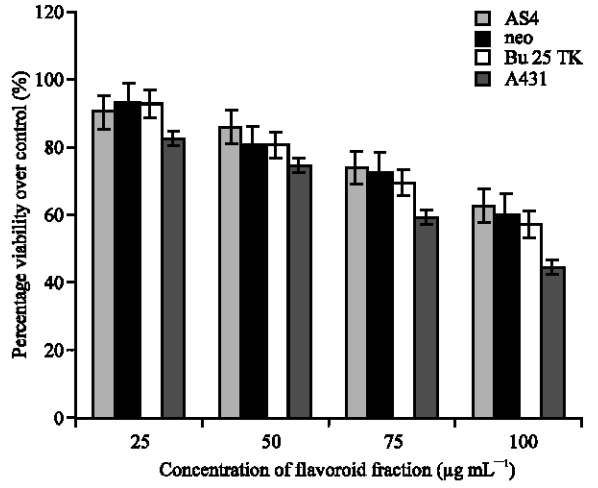

Fig. 3: Mean viability of human cancer cells treated with flavonoid fraction of *Emilia sonchifolia* Linn

indicates better antioxidant property. Our results are supported by the observations made by Pedraza-Chaverrí *et al.* (2004).

Superoxide production was inhibited by the flavonoid fraction of *E. sonchifolia* in a concentration dependent manner with 50% inhibition (IC_{50}) produced by 48 µg (Fig. 2), while for Quercetin it was 32 µg. Lower the concentration required for 50% inhibition, higher the antioxidant property of the compound.

The active principles isolated from the whole plant of *Emilia sonchifolia* Linn are Kaempherol-3 D galactoside, quercitrin, quercetin, rutin, ursolic acid, n-hexacosanol and triacontane (Rastogi and Mehrotra, 1998a). Senkirkine and doronine were reported to be present in the aerial parts (Rastogi and Mehrotra, 1998b). Isolation of sitosterol, stigmasterol, palmitic acid and triacetonoic acids were also reported (Gao *et al.*, 1993). The antioxidant properties of ES might be due to the presence of Quercetin, quercitrin and rutin, the main flavonoid component of the plant.

ES (flavonoid fraction) was tested for cytotoxicity against human cancer cells namely A431, Bu25TK, AS4, NEO. It caused a clear concentration dependent inhibition of growth of the cancer cell lines, which are in agreement with the results reported by Srinivas *et al.* (2003). Growth inhibitory effect was monitored by determining the viability of the cells based on their ability to reduce MTT (Fig. 3). From the graph it is quite evident that ES is most effective on the skin carcinoma cells (A431), this may be due to the wound healing property of ES as described in traditional Indian and Chinese medicine (Nayar *et al.*, 1996).

The phenotypic characteristics of flavonoid fraction of ES treated cells were evaluated by microscopic inspection of overall morphology. The treatment of the cells with 200 µg mL^{-1} ES flavonoid fraction for 24 h resulted in the formation of nuclear condensation, which was clearly evident in light microscopy. The morphological characteristics of flavonoid fraction of ES treated cells and controls are shown in Fig. 4.

To confirm whether the cytotoxic effects induced by the flavonoid fraction of ES in these cells involve apoptotic changes, cells were examined for any nuclear condensation. Upon treatment of cells with different concentrations of flavonoid fraction of ES, nuclear condensation was visible in the cells at 6 h (Fig. 5A and 5B) (data in agreement with MTT data). Present results support the notion that apoptosis is a potential mechanism by which the flavonoid fraction of ES exerts

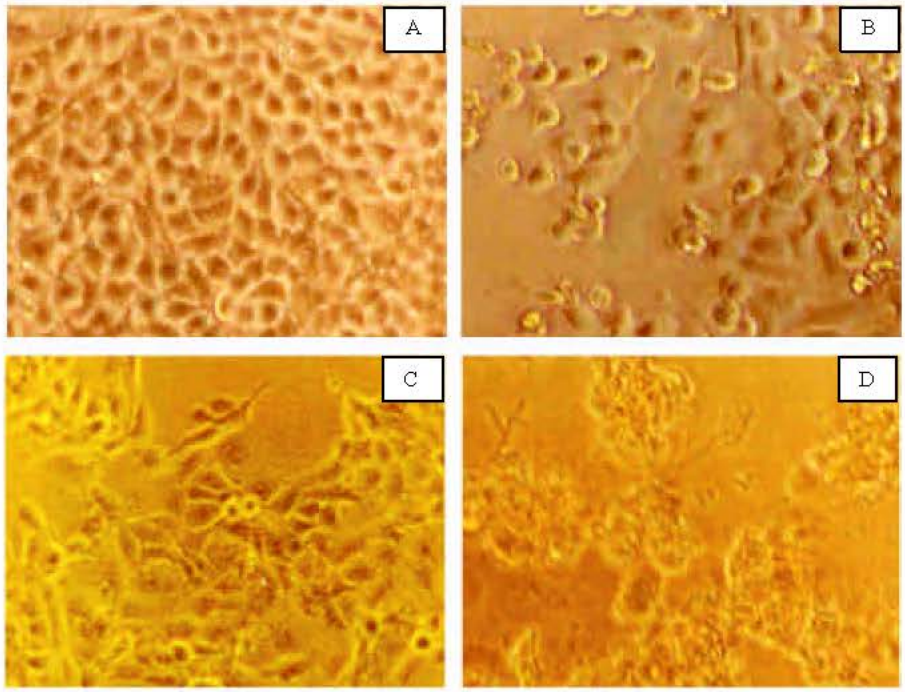

Fig. 4: Morphological changes induced by ES in cancer cells. Human cancer cells, Bu 25TK (Cervical cancer) and A431 (Skin cancer), were seeded in 96-well plates. After 24 h, 100 µL of DMEM containing 5% fetal bovine serum was added without (control) or with 50 µg ES (flavonoid fraction). A-Bu 25TK untreated cells, B-Bu 25 TK cells treated with ES, C-A431 untreated cells, D-A431 cells treated with ES. Cells were visualized in an inverted microscope after 48 h and photographed. The experiment was repeated two times with similar results

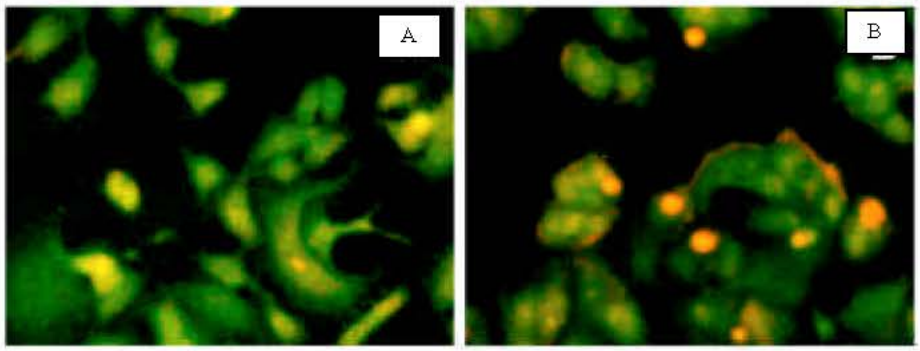

Fig. 5: Changes in nuclear morphology of Neo cells induced by ES. Cells were seeded in 12-well plates and then treated with or without ES for 24 h. After washing with phosphate-buffered saline, the cells were stained with a mixture of acridine orange-ethidium bromide. The cells were viewed under an inverted fluorescent microscope and photographed as described under Materials and Methods. The experiment was repeated two times with similar results. A- Untreated Neo cells and B- ES treated Neo cells

its antiproliferative effect, which are supported by the observations made by Srinivas *et al*. (2004) and Oommen *et al*. (2004).

From this study, it can be concluded that flavonoids from *E. sonchifolia* possess potent anticancer effect which may be due to its excellent antioxidant ability. Further studies are still needed to understand the various mechanisms regulating the antiproliferative effects and apoptosis induced by the ES fraction. However, the recent trend worldwide is to identify therapeutics from natural

sources mainly because most of them are largely free from adverse effects. This work assumes significance, since it aims at identification of natural therapeutics and to translate traditional wisdom to a scientific platform.

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