In vitro anti-cancer and anti-oxidant activity of different fractions of *Diospyros peregrina* unripe fruit extract

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ABSTRACT

Background: *Diospyros peregrina* (Ebenaceae) is a medium-sized evergreen tree having ethnomedicinal significance as an aphrodisiac, astringent, bactericide and for the treatment of diarrhoea, cholera, dysentery, fever, malaria and diabetes. **Objectives:** To evaluate the anti-oxidant and anti-cancer activity of different extracts and fractions of matured unripe fruits of *Diospyros peregrina* on various *in vitro* models. **Materials and Methods:** Anti-oxidant and free radical scavenging activity of various extracts and fractions were determined by DPPH, ABTS and Alkaline DMSO methods. *In vitro* cytotoxicity of different extracts and fractions of matured unripe fruits of *Diospyros peregrina* was evaluated on MCF-7 and Hep-G2 cell lines by MTT and SRB methods. **Results:** Among the extracts and fractions studied for anti-oxidant activity n-butanol fraction (NBF) and aqueous extract (AE) showed potential scavenging effect against DPPH, ABTS and superoxide free radicals. *In vitro* cytotoxicity assays of extracts and fractions by MTT and SRB methods revealed that dichloromethane fraction (DCMF) exhibited maximum cytotoxicity on both MCF-7 and Hep G2 cell lines. **Conclusion:** The higher anti-oxidant potential of n-butanol fraction (NBF) and aqueous extract (AE) may be due to the presence of phenolic compounds, flavonoids or flavonoid glycosides. Dichloromethane fraction (DCMF) exhibited maximum cytotoxicity on both MCF-7 and Hep G2 cell lines. The cytotoxic activity of DCM fraction may be attributed due the presence of triterpenoids.

Keywords: Anti-oxidant, *Diospyros peregrina*, free radicals scavenging, *in vitro* cytotoxicity.

INTRODUCTION

Most of the modern researches on herbal plants are based on traditional medicine. The property of being non-toxic and quite safer for human consumption make plants an attractive candidate for drug development and research.

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Plant molecules have always shown compatibility with normal cells through natural resistance or tolerance against them. This is the reason why plant compounds are best suited as drugs. Approximately 60% of the anti-cancer and anti-infective agents that are commercially available are of natural product origin.[1] Ancient literatures also mention the use of herbal medicine in the treatment of age-related diseases including memory loss, osteoporosis, diabetic wounds, immune and liver disorders; for which no modern medicine or only palliative therapy is available.

Diospyros peregrina (Ebenaceae) is a medium-sized evergreen tree that grows in coastal West Bengal, all along

Western Ghats and Bangladesh. This plant has ethnomedicinal significance as an aphrodisiac, astringent, bactericide and for the treatment of diarrhoea, cholera, dysentery, fever, malaria and diabetes.[2] The maceration of matured unripe fruit is successfully used in costal West Bengal for treatment of diabetes. The alcoholic extract of stem barks of this plant has been reported to possess hypoglycaemic, diuretic and anti-cancer properties. The bark also possesses anti-viral and anti-protozoal activity.[3]

Different phytochemicals have been isolated from leaf and bark which include ß-sitosterol, betulin, betulinic acid, oleanolic acid, lupeol and gallic acid.[4] Mature fruits contain glucose, protein, and about 50% pectin. Amongst secondary metabolites *D. peregrina* fruit contain soluble tannins, flavanoids, peregrinol, hexacosane, hexacosanol, β-sitosterol, betulinic acid, and lupeol.[5–8] It has been known that diabetes is associated with low levels of anti-oxidants and many plants show anti-diabetic activity due to its anti-oxidant property. Further, an extensive literature survey did not afford any information regarding its anti-cancer effect and thus the present study was undertaken to investigate the *in vitro* anti-cancer and anti-oxidant activity of different fractions of matured unripe fruits of Diospyros *peregrina* extract.

MATERIALS AND METHODS

Plant material

The matured unripe fruits of *Diospyros peregrina* were collected from Manipal, Udupi (District), Karnataka, India, in the month of September 2010 and were authenticated by Botanist Dr. Gopalakrishna Bhat, Professor of botany, Poorna Prajna College, Udupi, India. A Herbarium specimen bearing voucher No: PP970 has been deposited in Manipal College of Pharmaceutical sciences, Manipal, India.

Extraction and fractionation

The shade dried coarsely powdered matured unripe (0.35 kg) was subjected to soxhlet extraction using methanol (1.5 L) for 12 h. Solvent was evaporated under vacuum to obtain methanol extract (ME) (yield 11.8% w/w). The crude methanol extract so obtained was further fractionated using Petroleum ether (40°C – 60°C), Dichloromethane and n-butanol, and later solvents were evaporated from each fractions at reduced pressure to afford petroleum ether fraction (PEF) (yield 1.2% w/w), dichloromethane fraction (DCMF) (yield 1.8% w/w) and n-butanol(NBF w/w) (yield 2.4% w/w) fraction. The aqueous extract (AE) was obtained by heating the powder of dried fruit at 60°C (500 g) with three volumes of distilled water for 48 h. The thick viscous solution obtained was strained through a muslin cloth and was evaporated using a rotary evaporator afforded aqueous extract (AE) (yield 12.4% w/w). The two extracts and three fractions (ME, PEF, DCMF, NBF, AE) so prepared were used for investigating *in vitro* anti-cancer and anti-oxidant activity.

Phytochemical analysis

Preliminary phytochemical analysis of crude methanol extract revealed the presence of tannins, flavonoids, terpenoids, phenolic compounds and sugars.

In vitro antioxidant studies

The extracts and fractions were tested for its free radical scavenging property using different *in vitro* methods. All the experiments were performed thrice and results are averaged. Ascorbic acid was used as the standard for comparison.

DPPH radical scavenging activity

The assay was carried in a 96 well microtiter plate.[9,10] Briefly, 100 µl of various concentrations of extract/fractions or standard in DMSO and 100 µl 0f DPPH (200 µM) solution was added to each well. The plates were incubated at 37°C for 30 min without exposing to light and absorbance was measured with microplate reader using 540 nm filter. DPPH radical-scavenging activity was calculated using the following equation:

$$\% \text{ Inhibition} = (A0 - A1) / A0 \times 100$$

Where A0 was the absorbance of the control (without extract) and A1 was the absorbance in the presence of the extract.

Scavenging of superoxide radical by alkaline DMSO method

To the reaction mixture containing 1 ml of alkaline DMSO (1 ml of DMSO containing 5 mM NaOH in 0.1 ml of water) and 0.3 ml of extract/fractions or standard (in DMSO), 0.1 ml of NBT (1 mg/ml) was added to give a final volume of 1.4 ml. The absorbance was measured at 560 nm.[11]

Scavenging of ABTS radical

To 50 ml of 2 mM ABTS in distilled water, potassium persulphate (17 mM, 0.3 ml) was added. It was left to

stand at room temperature overnight in dark before usage. To 0.2 ml of various concentrations of extracts/fractions or standard in DMSO, 1.0 ml of DMSO and 0.16 ml of ABTS solution were added to make a final volume of 1.36 ml. Absorbance was measured after 20 min at 734 nm.[12,13]

In vitro anti-cancer activity studies

The effect of extracts (ME, AE) and fractions (PEF, DCMF, NBF) of matured unripe fruits of *Diospyros peregrina* on the viability of cancer cells was determined by MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) and SRB (Sulforhodamine B) assay on MCF-7 (Human breast cancer cells – epithelial) and Hep G2 (Human liver cancer cells) cell lines. CTC_{50} (Cytotoxicity) values can be derived graphically from dose response curves.

Determination of mitochondrial synthesis by micro culture tetrazolium (MTT) assay

The monolayer cell culture was trypsinized using TPVG (Trypsin – Versine – Glucose) and the cell count was adjusted to 1.0×10^5 cells/ml using medium containing 10% new born calf serum. Each well of the 96 well plates was seeded with 10^5 cells. After 24 h, when a partial monolayer was formed, the supernatant was flicked off and 100 µl of (500 to 31.25 µg/ml) test extracts were added to the cells in microtiter plates. The plates were then incubated at 37°C for 72 h in 5% CO_2 atmosphere, and microscopic examination was carried out and observations recorded every 24 h. After 72 h, the drug solutions in the wells were discarded and 50 µl of MTT (MTT: prepared in Hank's Balanced Salt Solution without phenol red [(HBSS-PR), 2 mg/ml, Sigma Chemicals] was added to each well. The plates were gently shaken and incubated for 3 h at 37°C in 5% CO_2 atmosphere. The supernatant was removed and 50 µl of 1-propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader (ELISA Reader, Bio-tek) at 540 nm.[14–17] The percentage growth inhibition was calculated using the following formula.

$$\% \text{ cell inhibition} = 100 - \left(\frac{\text{Mean absorbance of test}}{\text{Mean absorbance of control}} \right) \times 100$$

Determination of total cell protein content by *Sulforhodamine* B (SRB) assay

To each well of the 96 well microtiter plates, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once and 100 µl of different drug concentrations (500 to 31.25 µg/ml) prepared in the maintenance medium were added to the cells in microtiter plates. The plates were then incubated at 37°C for 3 days in 5% CO_2 atmosphere, and microscopic examination was carried out and observations recorded every 24 h. After 72 h, 25 µl of 50% trichloroacetic acid was added to the wells gently such that it forms a thin layer over the drug dilutions to form an overall concentration of 10%. The plates were incubated at 4°C for one hour. The plates were flicked and washed five times with tap water to remove traces of medium, drug and serum, and were then air-dried. The air-dried plates were stained with SRB dye (0.4% prepared in 1% acetic acid, Sigma Chemicals) for 30 min. The unbound dye was then removed by rapidly washing four times with 1% acetic acid. The plates were then air-dried. 100 µl of 10 mM Tris base was then added to the wells to solubilize the dye. The plates were shaken vigorously for 5 min. The absorbance was measured using microplate reader (ELISA Reader, Biotek) at a wavelength of 540 nm. The percentage growth inhibition was calculated using the same formula as that of MTT. Cisplatin (1.25–15 µg/ml) was used as reference standard.

RESULTS AND DISCUSSION

DPPH radical scavenging activity

DPPH is usually used as a substrate to evaluate anti-oxidant activity. DPPH assay is based on the measurement of the scavenging ability of anti-oxidant towards the stable DPPH radical. The method is based on the reduction of purple colored methanol solution of DPPH in the presence of hydrogen donating anti-oxidants, by the formation of yellow colored non radical form of DPPH. Lower the absorbance higher the free radical scavenging activity. *In vitro* DPPH radical scavenging activity of extracts (ME, AE) and fractions (PEF, DCMF, NBF) of matured unripe fruits of *Diospyros peregrina* are expressed in terms of IC_{50}, and shown in Figure 1. The extracts and fractions (ME, PEF, DCMF, NBF, AE) were able to reduce purple colored DPPH to yellow colored picryl hydrazine. Among the extracts and fractions n-butanol fraction (NBF) and aqueous extract (AE) showed maximum anti-oxidant activity with IC_{50} of 16.45 and 31.4 µg/ml, respectively. Other moderately active extracts were methanolic extract (ME) and dichloromethane fraction (DCMF) with IC_{50} of 43.41 and 55.89 µg/ml respectively. The DPPH radical scavenging activity of n-butanol

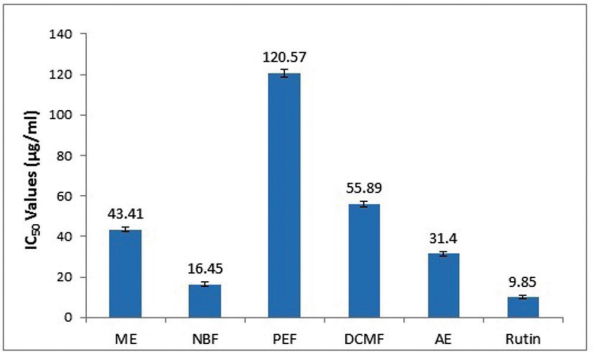

Figure 1. DPPH free radical scavenging activity of different extracts and fractions of unripe fruits of *Diospyros peregrine.*

fraction (NBF) was comparable with that of the standard rutin (IC_{50} 9.85 µg/ml)

ABTS radical scavenging assay

The ABTS assay is based on the ability of anti-oxidants to inhibit the absorbance of radical cation, ABTS at 734 nm. In this assay, the ABTS radical which is a blue green chromogen, is generated in a stable form using potassium persulphate. ABTS radical scavenging activity of extracts (ME, AE) and fractions (PEF, DCMF, NBF) of matured unripe fruits of *Diospyros peregrina* are expressed in terms of IC_{50}, and shown in Figure 2. The extracts and fractions (ME, PEF, DCMF, NBF, AE) were able to prevent the generation of ABTS radical, measured by decreases in absorbance of reaction medium at 734 nm. Among the extracts and fractions n-butanol fraction (NBF) and aqueous extract (AE) showed maximum ABTS radical scavenging activity with IC_{50} of 12.68 and 37.67 µg/ml respectively. Methanolic extract (ME) showed moderate activity with an IC_{50} of 59.85 µg/ml. The ABTS radical scavenging activity of n-butanol fraction (NBF) was better than that of the standard rutin (IC_{50} 14.45 µg/ml).

Alkaline DMSO superoxide scavenging activity

The scavenging activity of extracts against superoxide radical generated in NaOH– alkaline DMSO– NBT system, resulting in the formation of the blue formazan was studied. In this method, superoxide radical is generated by addition of sodium hydroxide to dimethyl sulfoxide (DMSO). The generated superoxide remains stable in solution, which reduces nitro blue tetrazolium into formazan dye at room temperature. Superoxide scavenger capable of reacting inhibits the formation of a red dye formazan. Superoxide radical scavenging activity of extracts (ME, AE) and fractions (PEF, DCMF, NBF) of matured unripe fruits of *Diospyros peregrina* are expressed in terms of IC_{50}, and shown in Figure 3. Among the extracts and fractions n-butanol fraction (NBF) and aqueous extract (AE) showed maximum superoxide radical scavenging activity with IC_{50} of 135.99 and 213.47 µg/ml respectively. Other extracts and fractions were found to be less effective in scavenging superoxide radical.

In vitro cytotoxicity assays

In vitro cytotoxicity of extracts (ME, AE) and fractions (PEF, DCMF, NBF) of matured unripe fruits of *Diospyros peregrina* were determined by MTT and SRB assays on HeLa, and Hep G2 cell lines. The cytotoxicity is expressed as CTC 50 and is summarized in Table 1.

Determination of mitochondrial synthesis by micro culture tetrazolium (MTT) assay

Among the extracts and fractions studied for cytotoxicity on MCF-7 cells dichloromethane fraction (DCMF) showed maximum cytotoxicity with IC_{50} of 37.22 µg/ml.

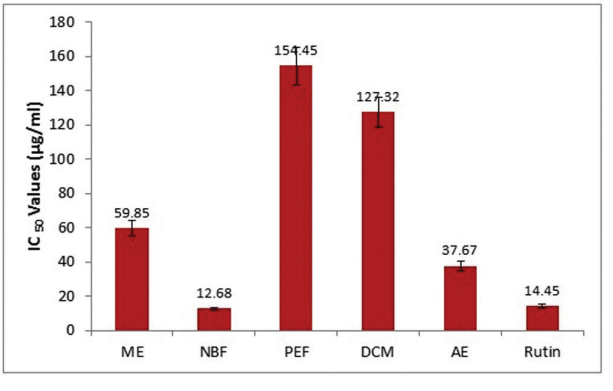

Figure 2. ABTS radical scavenging activity of different extracts and fractions of unripe fruits of *Diospyros peregrina.*

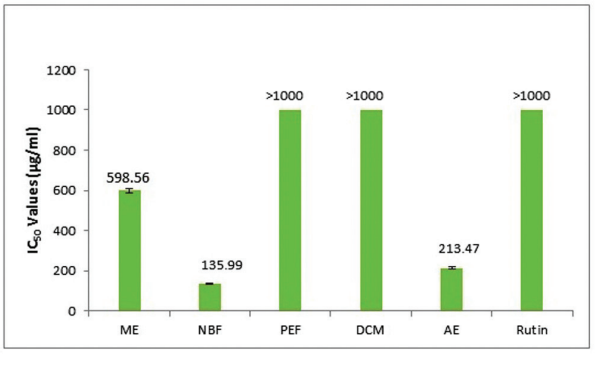

Figure 3. Alkaline DMSO superoxide radical scavenging activity of different extracts and fractions of unripe fruits of *Diospyros peregrina.*

Table 1 CTC_{50} values of different extracts and fractions of unripe fruits of *Diospyros peregrina* by SRB and MTT assay

Sample	CTC_{50} ± SEM* µg/ml SRB Method		CTC_{50} ± SEM* µg/ml MTT Method	
	MCF-7	Hep G2	MCF-7	Hep G2
ME	49.35 ± 1.21	73.75 ± 1.43	53.32 ± 1.15	79.95 ± 1.34
PEF	191.23 ± 1.03	203.15 ± 2.31	167.24 ± 2.23	188.02 ± 2.76
DCM	32.64 ± 0.87	58.54 ± 0.61	37.22 ± 0.91	65.37 ± 1.18
NBF	52.68 ± 1.71	74.25 ± 1.14	61.27 ± 1.11	88.75 ± 1.47
AE	>500	>500	>500	>500
Cisplatin	3.78 ± 0.42	3.85 ± 0.54	3.09 ± 0.62	3.4 ± 0.72

**Average of three determinations*

While n-butanol fraction (NBF) and methanolic extract (ME) exhibited moderate cytotoxicity with IC_{50} 61.27 and 53.32 µg/ml respectively on MCF-7 cells. Against the Hep G2 cells dichloromethane fraction (DCMF) showed maximum cytotoxicity with IC_{50} of 65.37 µg/ml. n-butanol fraction (NBF) and methanolic extract (ME) exhibited moderate cytotoxicity with IC_{50} 88.75 and 79.95 µg/ml respectively on Hep G2 cells.

Determination of total cell protein content by Sulforhodamine B (SRB) assay

By SRB assay dichloromethane fraction (DCMF) showed maximum cytotoxicity with IC_{50} of 32.64 µg/ml on MCF-7 cells. While n-butanol fraction (NBF) and methanolic extract (ME) exhibited moderate cytotoxicity with IC_{50} 52.68 and 49.35 µg/ml respectively on MCF-7 cells. Against the Hep G2 cells dichloromethane fraction (DCMF) showed maximum cytotoxicity with IC_{50} of 58.54 µg/ml. n-butanol fraction (NBF) and methanolic extract (ME) exhibited moderate cytotoxicity with IC_{50} 74.25 and 73.75 µg/ml respectively on Hep G2 cells.

CONCLUSION

The results from various free radicals scavenging systems revealed the aqueous extract (AE) and n-butanol fraction (NBF) of matured unripe fruits of *Diospyros peregrina* has significant anti-oxidant activity. The anti-oxidant potential of these extracts may be due to the presence of phenolic compounds, flavonoids or flavonoid glycosides as revealed by phytochemical analysis. As evidenced from MTT and SRB cytotoxicity assays of extracts and fractions from matured unripe fruits of *Diospyros peregrina* dichloromethane fraction exhibited maximum cytotoxicity on both MCF-7 and Hep G2 cell lines. The cytotoxic activity of DCM fraction may be attributed due the presence of triterpenoids. Hence further studies are needed to evaluate the *in vivo* anti-oxidant and anti-cancer potential of these extracts in various animal models.

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