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RESEARCH ARTICLE

Antioxidant and anticancer activity of *Helicteres isora* dried fruit solvent extracts

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Abstract

Helicteres isora dried fruit solvent extracts were evaluated for their antioxidant and anticancer activity. Acetone fruit extract *H. isora* showed (96.44%) strong antioxidant activity compared to hexane, and iso-propyl alcohol (IPA). Acetone extract exhibited better cytotoxicity against human lung cancer cells (NCI-H460) whereas; acetone and crude protein extracts showed activity against reactive oxygen species.

Keywords: *Helicteres isora,* antioxidant, anticancer, acetone, cytotoxicity, reactive oxygen species.

Introduction

Helicteres isora Linn. is a shrub belongs to the family Sterculiaceae commonly known as 'East Indian Screw tree. The plant genus consists of 45 species distributed in warmer regions of hemispheres; four species are reported to occur in India (Anonymous, 1997). They are a rich source of medicinal compounds with a wide range of properties (Bapalal, 1982), which is also known as Avartani in Ayurveda (Yoganarasimhan, 1996). Tribals of Wyanad, Malappuram and Palghat districts of Kerala, India, used *H. isora* plant extracts for its anticancer properties (Mathew and Unnithan, 1992). Traditionally, the root juice and bark of *H. isora* were used against emphysema and diabetes. It is also used as expectorant, astringent, antigalactagogue, to reduce gripping and a cure for snakebite (Kirtikar and Basu, 1993, Singh *et al*., 1984). *Helicteres isora* fruits are used as astringent, stomachic, vermifuge, vulnerary and useful in bowel gripes (Chopra *et al*., 1956). Cell cycle inhibitory activity of *H. isora* against tsFT210 cell line was reported by Quinchun *et al.* (2005). The present study was aimed to assess the antioxidant and anticancer properties of dried fruit extracts of *H. isora* considering the above facts.

Materials and methods

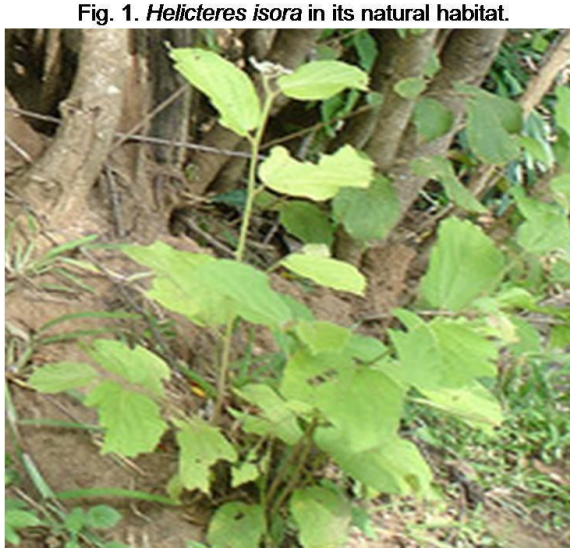
Chemicals: One percent phosphate buffer saline (PBS), fluorescent probe, 2',7'-dichlorfluorescein-diacetate (DCFH-DA), hexane, acetone, isopropyl alcohol (IPA), coomassie brilliant blue G 250 (CBB-G250), ethanol, ortho-phosphoric acid, bovine serum albumin (BSA), mono and dibasic sodium phosphate, phosphate buffer 6.8, 7.2. Analytical grade chemicals supplied by Loba, Hi-Media, S.D. Fine Chemicals, Merck, Qualigens and Sigma Chemicals (U.S.A) were used.

Source of plant material: Helicteres isora fruits were collected during June 2011 to Nov 2011 (Fig. 1). Subsequently, the fresh fruit was collected and washed thoroughly in running tap water followed by distilled water. It was then dried at room temperature and powdered using mechanical pulveriser and subjected for extraction.

Preparation of dried fruit solvent extracts

Acetone extracts were extracted by soaking 250 g powder in 900 mL of acetone for 72 h. The extract was filtered through Whatman No. 40. The above preparation was repeated thrice. Isopropyl alcohol extracts were prepared by soaking 250 g powder in 750 mL of IPA for 72 h. The extract was filtered through Whatman No. 40, The above preparation were repeated thrice. Hexane extracts were prepared by soaking 300 g powder in 750 mL of hexane for 72 h. The extract was filtered through Whatman No. 40, the above preparation were repeated thrice. Acetone, isopropyl alcohol and hexane extracts was condensed in rotary evaporator with constant temperature at 45ºC at 200 rpm and the residue was used for further analysis.

Fig. 1. *Helicteres isora* in its natural habitat.



Extraction and estimation of protein

Protein was extracted using Glass Distilled water and phosphate buffer (pH 6.8 and 7.2). One gram of sample was grinded with 10 mL of respective solvent. Homogenates and sample was filtered and the filtrate was centrifuged at 5000 rpm for 5 min. supernatant was treated with ice cold acetone at 4°C, centrifuged at 10000 rpm for 10-15 min. supernatant was discarded and pellet was dissolved in respective solvent. The dissolved protein was estimated (Bradford, 1976). The samples were prepared in 100 mL of PBS. Dye binding solution (5 mL) was added in each tube, mixed for 5 min where red dye turns into blue due to protein binding and absorbance was read at 595 nm.

Dot plot rapid assay

The rapid screening assay was performed by the method proposed by Soler-Rivas *et al*. (2000) with slight modification. Aliquots of plant extracts (3 µL) were spotted carefully on TLC plates and dried for 3 min. The sheets bearing the dry spots were placed upside down for 10 sec in 0.4 mM DPPH solution and the layer was dried. The stained silica layer revealed a purple background with yellow spots, which showed radical scavenging capacity.

DPPH free radical scavenging activity

The odd electron in the DPPH free radical gives a strong absorption maximum at 517 nm and is purple in color. The color turns from purple to yellow as the molar absorptivity of the DPPH radical at 517 nm reduces from 9660 to 1640 when the odd electron of DPPH radical becomes paired with hydrogen from a free radical scavenging antioxidant to form the reduced DPPH-H (Nagarajan *et al*., 2012). The resulting decolorization is stoichiometric with respect to number of electrons captured. Antioxidant compounds may be water-soluble, lipid soluble, insoluble or bound to cell walls. Hence, extraction efficiency is an important factor in quantification of antioxidant activity of foods. The reaction mixture consists 1 mL of 0.1 mM DPPH in ethanol with various concentrations (500 µg/mL, 250 µg /mL, 125 µg/mL, 64 µg/mL, 32 µg/mL, 16 µg/mL and 8 µg/mL). 1 mL of DPPH and 1 mL of ethanol were used as control and ascorbic acid was used as standard. The mixture was then allowed to react at room temperature for 30 min. Methanol served as the blank and DPPH in methanol, without the extracts, served as the positive control. After 30 min of incubation, the discolouration of the purple colour was measured at 517 nm in a spectrophotometer (Genesys 10-S, USA). The radical scavenging activity was calculated as follows:

% Inhibition = OD control-OD sample/ OD control ×100

The 50% inhibitory concentration (IC_{50}) values were calculated by plotting an x, y scatter trend line with regression equation.

Cytotoxicity determination by MTT assay

Helicteres isora fruit extracts were evaluated for anticancer activity. For the Cytotoxicity/anticancer assay, NCI-H460 human lung cancer cells were used. Cells were maintained in DMEM (Dulbeccos modified eagles medium) supplemented with FBS (foetal bovine serum) and penicillin/streptomycin-L-glutamine and cultured in a humified atmosphere of 5% CO_2 and 95% air at 37°C in Thermo Hera Cell 150 incubator. Cells were seeded in 96 well plates at the density of 5000 cells/well in 100 µL of RMPI 1640 medium. Then various concentrations (10 µg/mL, 20 µg/mL, 30 µg/mL and 40 µg/mL) of the crude extracts and protein samples were added to the cells. Cells were incubated for 24 h with test extract concentrations (Arjun *et al*., 2012). Each concentration was tested in triplicate.

Cell viability test

MTT assay was used to determine cell viability. MTT assay measures changes in colour for measuring the activity of enzyme that reduce MTT to formazan, giving a purple colour. Yellow MTT (a tetrazole) reduce to purple formazan in living cells (Mosmann, 1983). After 24 h incubation, 10 µL of MTT was added to each well and incubated for additional 4 h. Then 100 µL of DMSO solution was added to each well to solubilize the formazan crystals. The plates were read for optical density at 570 nm using a plate reader. By using optical density, the percentage inhibition of NCI-H460 human lung cancer cells was calculated. The percentage viability was calculated as follows:

Cell viability = OD of samples/OD of control × 100.

Measurement of intracellular ROS by Fluorescent probe, 2',7'-dichlorfluorescein-diacetate

Cell suspension (0.2 mL) was taken ($1X10^6$) and incubated with the test sample. Cells (200 mL) were taken after treatment and diluted in 3 mL of PBS and 300 µL DCFH-DA (1 µg/mL) was added. Reading was taken immediately without any delay using spectrofluorometer (excitation-485 nm, emission–530 nm) and incubated for 30 min in dark conditions (Bhosle *et al*., 2005).

Fluorescence microscopy for ROS measurement

Test compound was added to suspend cells ($1x10^6$ cells/mL) in 1 mL warm medium in small petri dishes. After the treatment, DCFH-DA dye (1 µg/mL) was added and incubated at 37ºC for 30 min. Then, the cells were washed once by adding 2 mL of warm PBS and finally the cells were viewed under fluorescence microscope using blue filter (Muthu kumar *et al*., 2012).

Assessment of mitochondrial membrane potential

Approximately $1x10^6$ cells/mL was added in 1 mL medium in dark test tubes and the treated sample was added and incubated at different time intervals (24/48/72 h). 10 µL Rhodamine-123 dye (10 µg/mL) was added and cells were incubated at 37ºC in 5% CO_2 for 30 min and centrifuged at 1200 rpm for 10 min. Supernatant was discarded and 2 mL of warm PBS was added in pellet. Finally it was read at 480 and 550 nm using spectrofluorometer.

Table 1. Protein content of crude and defatted dry fruit powder of *H. isora*.

Experiments	Protein (mg/g)		Protein (%)	
	Crude	defatted material	Crude	defatted material
Hot water (mg/mL)	15	20.04	1.5	2.00
Cold water (mg/g)	25.2	18	2.52	1.8
Phosphate buffer PH (6.8) mg/g	20.04	16.2	2.05	1.62
Phosphate buffer PH (7.2) mg/g	37.2	12	3.72	1.2

Values are means of three replicates and the values in the columns with same letter are not significantly different at p<0.05 level.

Table 2. Antioxidant activity of dried fruit solvent extracts of *H. isora.*

Sample (µg/mL)	Ascorbic acid (µg/µL)	Radical scavenging activity (%)			
		IPA	Acetone	Hexane	Ascorbic acid
10	2	10.56	12.89	17.73	48.99
20	4	13.58	43.73	19.43	55.98
30	6	19.43	59.25	20.56	71.56
40	8	22.82	68.03	29.99	85.99
50	10	29.24	91.95	36.41	91.65
60		36.79	96.44	38.67	

Table 3. Cytotoxicity measured by MTT assay used by various fruit extracts.

Sample (µg/mL)	% Cytotoxicity			
	Acetone	IPA	Hexane	Crude Protein
10	93.7	66.7	12.5	33.4
20	0	78.7	25	53.4
30	0	89.6	37.5	70
40	0	96.2	62.5	90.3

Fluorescence microscopy method

Approximately 1x10^6 cells/mL was added in 1 mL medium in petri dishes and treated sample was added and incubated. 10 µL Rhodamine-123 dye (10 µg/mL) was added and incubated at 37ºC with 5% CO_2 for 30 min and washed with 2 mL of warm PBS. Finally the cells were observed under fluorescence microscope (510-590 nm).

Analysis of apoptotic morphological changes

Approximately 1x10^6 cells/mL was added in 1 mL medium in petri dishes and treated sample was added and incubated. Fifty µL/mL EB/AO was added and incubated at 37ºC with 5% CO_2 for 30 min and washed with 2 mL of warm PBS. Finally the cells were observed under fluorescence microscope (510-590 nm) (Coligan, *et al*., 1995).

$$\% \text{ apoptotic cells} = \frac{\text{Total No. of red cells}}{\text{Total No. of red and green cells}} \times 100$$

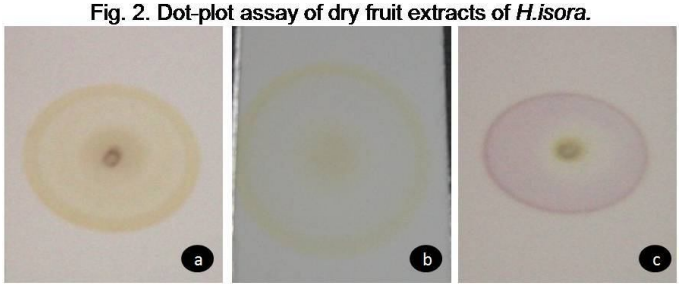
Statistical analysis

The data were subjected to one-way analysis of variance (ANOVA) to evaluate the significant of difference of means of various treatment groups using SPSS statistical software package (Version: 10). The values are presented as mean ± S.D and P<0.05.

Results and discussion

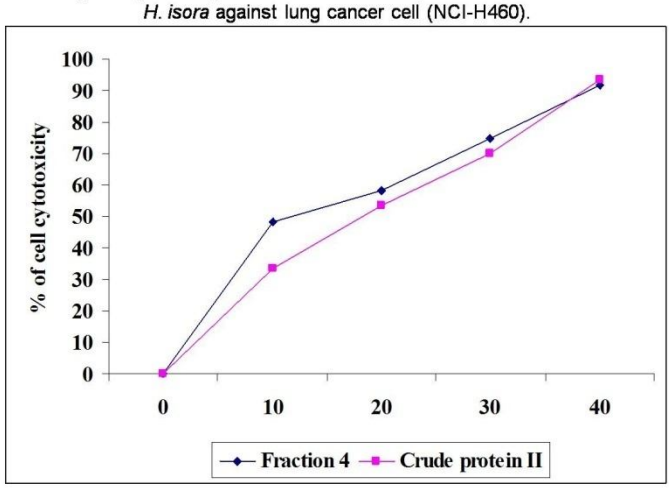
The protein was extracted from the dried fruit powder using water and with different buffers. Protein content was estimated by Bradford method (1976). Protein estimation was done on both crude as well as defatted material. Maximum protein around 3.72% was extracted using the buffer at pH 6.8 (Table. 1).

Fig. 2. Dot-plot assay of dry fruit extracts of *H.isora.*



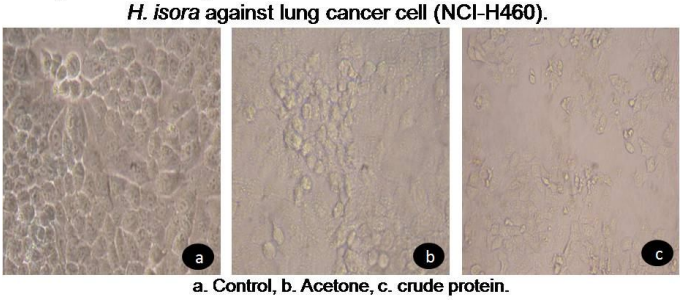
a. Acetone, b. IPA, c. Hexane

Fig. 3. Cytotoxicity of acetone and crude protein fruit extracts of *H. isora* against lung cancer cell (NCI-H460).



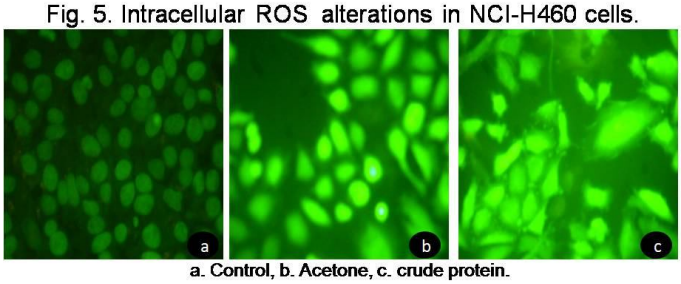
Different dried fruit solvent extracts of *H. isora* was detected in TLC plates by DPPH staining method. For rapid screening, each diluted sample was applied as a dot on a TLC plate that was then stained with DPPH solution. The appearance of white colour in the spots revealed the potential value for the indirect evaluation of the different extracts from *H. isora* (Soler-Rivas *et al*., 2000; Chang *et al*., 2002). The method is typically based on the inhibition of the accumulation of oxidized products since the generation of free radicals is inhibited by the addition of antioxidants. Dot plot assay is performed to assess the presence of any antioxidant activity in the sample. The result revealed the antioxidant potential of the fruit extracts.

Fig. 4. Cell viability of acetone and crude protein fruit extracts of *H. isora* against lung cancer cell (NCI-H460).



a. Control, b. Acetone, c. crude protein.

Fig. 5. Intracellular ROS alterations in NCI-H460 cells.



a. Control, b. Acetone, c. crude protein.

Fig. 6. Measurement of ROS generation.

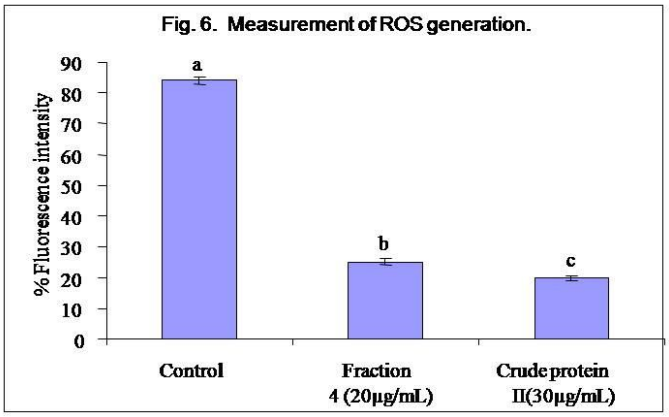
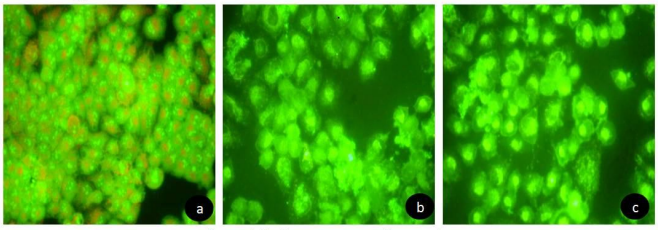


Fig. 7. Mitochondrial membrane alterations in NCI-H460 cells.



a. Control, b. Acetone, c. crude protein.

Different solvents (hexane, acetone, IPA) dried fruit extracts of *H. isora* were used for DPPH radical scavenging activity. Compared to hexane and IPA, It was found that acetone extract (60 µg/mL) of *H. isora* recorded strong radical scavenging activity of 96.44±0.57% whereas, hexane and IPA showed low antioxidant activity (Table 2). According to Suthar *et al*. (2009) the antioxidant activities measured in hot water extract of *H. isora* showed IC_{50} value of 25.12±0.18 µg/mL, which was comparable to that of ascorbic acid (IC_{50} 2.75±0.29 µg/mL).

Hexane, acetone, IPA and crude protein fruit extracts of *H. isora* were used for cytotoxicity/cell viability against human lung cancer cells. Acetone recorded 93.7% (10 µg/mL) whereas IPA recorded 96.2% (40 µg/mL). Crude protein recorded 90.3% (40 µg/mL) and hexane extract recorded 62.5% (40 µg/mL) respectively. All the extracts showed good toxicity to the cancer cells. Among the extracts tested, acetone fruit extract showed excellent cytotoxicity against the cancer cells (Table 3; Fig. 3). It was evident from MTT assay that acetone extract had the best anticancer property as compared to the other extracts supported by Mosmann (1983) in his study. Intracellular ROS was measured using a non-fluorescent probe, 2,7,-diacetyl dicholorofluorescein diacetate (DCFH-DA) that can penetrate into the intracellular matrix of cells where it is oxidized by ROS to fluorescent dichlorofluorescein (DCF). The non-fluorescent DCFH-DA is oxidized by intracellular ROS and forms the highly fluorescent DCF (Philip Jesudason *et al*., 2008) which is measured spectrofluorimetrically (Fig. 5, 6).

Fig. 8. Mitochondrial membrane alteration potential.

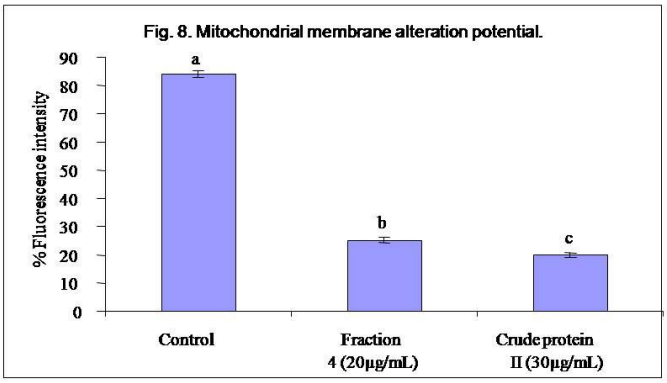
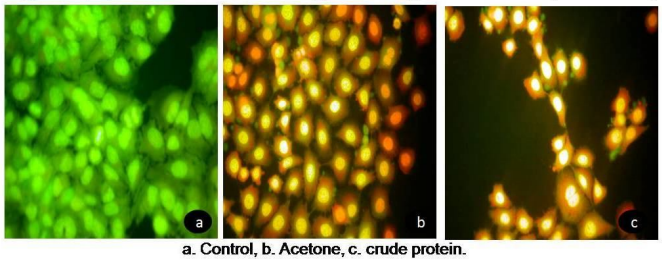


Fig. 9. Apoptotic cell death changes in lung cancer cells (NCI-H460).



a. Control, b. Acetone, c. crude protein.

Measurement of Mitochondrial Membrane potential

Alteration in mitochondrial membrane potential (depolarization) is an indication of early stages of apoptosis. Rhodamine 123 (Rh 123) is lipophilic cationic dye, highly specific for mitochondria. Polarized mitochondria are marked by orange red fluorescence and depolarized mitochondria are marked by green fluorescence (Fig. 7, 8). Apoptotic nuclei exhibiting typical changes such as nuclear condensation and segmentation were stained by AO/EB. *H. isora* extracts, both treated and untreated cells (2 x 10^4 /well) were seeded into 6-well plate and incubated in CO_2 incubator for 24 h and then the apoptotic morphological changes were observed using a fluorescence microscope under blue filter (Fig. 9).

The typical morphological features of apoptosis, including increased cell membrane permeability, cellular shrinkage and granulation in the nucleus, are major consequences of the apoptotic trigger. NCI-H460 cells treated with selected best extracts exhibited apoptotic morphological changes, as detected with AO/EB double staining. The numbers of apoptotic cells, including early-apoptotic (AO+, EB–) and late-apoptotic cells (AO+, EB+) were increased in a dose-dependent manner. After treatment with acridones, an increase in cell membrane permeability was observed, as evidenced by the red fluorescence of EB in the nucleus. At low concentrations, a majority of AO+, EB– cells were detected, indicating apoptotic granulation and an intact cell membrane. As the concentration was increased, late-apoptotic (AO+, EB+) and necrotic (AO–, EB+) cell populations appeared (Réthy *et al*., 2007). This assay helped us to visualize the different stages of apoptosis. Both acetone extract and the crude protein exhibited good anticancer activity.

Conclusion

Antioxidant and anticancer activity was evaluated using various solvent extracts (hexane, IPA and acetone) and crude protein. Dot plot assay conformed presence of antioxidant activity, acetone fruit extract of *H. isora* showed 96.44% strong antioxidant activity compared to hexane, and IPA. Acetone extract exhibited better cytotoxicity against human lung cancer cells (NCI-H460) whereas; acetone and crude protein extracts showed activity against ROS. The investigation revealed the antioxidant and anticancer activity of *H. isora* dried fruit extracts. To conclude, further investigations are necessary to find out the active bio active molecules responsible for inhibitory mechanisms for its anticancer and antioxidant activity.

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