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Original Article

CYTOTOXIC AND ANTIOXIDANT PROPERTIES OF FRACTIONS ISOLATED FROM *FERONIA ELEPHANTUM*

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ABSTRACT

Objective: The leaves of *Feronia elephantum* are widely used in folk medicine in India to treat various ailments. In the present communication we report the cytotoxic and antioxidant properties of extracts and fractions of *F. elephantum* leaves.

Methods: The leaves of *F. elephantum* were extracted with hexane and ethyl acetate and checked for cytotoxic properties against human A549 lung adenocarcinoma cancer cell line. The active extract was subjected to column chromatography and fractions were bio-assayed. The active fraction was subjected to GC-MS analysis.

Results: Hexane extract exhibited good cytotoxic activity against A549 lung cancer cell line compared to ethyl acetate extract. Hexane extract showed 77.3% activity at the dose of 500 µg/mL with IC_{50} (50.6%) value of 125 µg/mL. Hexane extract was also tested at different time intervals of 12h, 24h, 48h and 72 h. The activity gradually increased. The active hexane extract was subjected to column chromatography. Based on thin layer chromatography profiles, similar fractions were combined to give 9 fractions. When the fractions were bio-assayed fraction 5 showed maximum cytotoxic activity. Fraction 5 was tested against A549 lung cancer cell line at different time intervals; the activity gradually increased. Fraction 5 was used to test the antioxidant properties using DPPH analysis. Maximum antioxidant activity was observed at 1000 µg/mL (71.63 ± 0.15). Active fraction 5 was identified using GC-MS. It showed the presence of Estragole (50.82%), trans-anethole (p-propenylanisole, anise camphor) (14.98%) and Caryophyllene (9.22%).

Conclusion: The results showed that hexane extract of *F. elephantum* could be probed further in drug discovery programme.

Keywords: *Feronia elephantum,* Cytotoxicity, Antioxidant, GC-MS.

INTRODUCTION

Feronia elephantum (*F. elephantum*) Correa (Synonyms: *Feronia limonia*, *Limonia acidissima, Schinus limonia*) belongs to Rutaceae family. It is commonly called Wood apple. It is also called elephant apple, monkey fruit, curd fruit and *kathbel* in India. The wood apple is native to India, Ceylon (Srilanka), Southeast Asia, Malaya and Penang Island. In India, the fruit was traditionally a "poor man's food" until processing techniques were developed in the mid-1950's. The pulp is brown, mealy, odorous, resinous, astringent, acid or sweetish, with numerous small, white seeds scattered through it. The fruit is used as a liver and cardiac tonic and when unripe, as an astringent means of halting diarrhea and dysentery; it is effective to treat hiccups, sore throat and diseases of the gums. The pulp is poultice; it is applied in bites and stings of venomous insects, as is the powdered rind. Juice of young leaves is mixed with milk and sugar candy and given as a remedy for biliousness and intestinal troubles of children. Oil derived from the crushed leaves is applied on itch and the leaf decoction is given to children as an aid to digestion. Leaves, bark, roots and fruit pulp are all used against snakebite. The spines are crushed with those of other trees and an infusion is taken as a remedy for menorrhagia. The bark is chewed with that of *Barringtonia* and applied on venomous wounds. The plant contains stigmasterol, bergapten, marmesin, aurapten, bergapten, isopimpinellin and coumarins [1]. Plant showed repellent, diabetic, hepatoprotective, antibacterial and antifungal activities. [2-5].

Cancer is still a major health problem in both developing and developed countries. Cancer, known medically as a malignant neoplasm, is a broad group of various diseases, all involving unregulated cell growth. There are over 200 different known cancers that afflict humans. Every year at least 200,000 people die worldwide from cancer related to their workplace. According to World Health Organization (2008) [6] 7.8 million deaths (13% of all deaths) were recorded due to cancer around the world. Deaths from cancer worldwide are projected to continue to rise to over 13.1 million in 2030. Lung cancer occurs when there is uncontrolled cell growth in one or both the lungs. Lung cancer is the leading cause of cancer related deaths in Taiwan, the United States and other countries. To date the induction of cell death or the inhibition of cell growth using cisplatin, tubulin-binding agents, DNA-damaging agents, and UV radiation have been used to treat lung cancer. Clinically, however, these anticancer agents may result in serious adverse effects including neutropenia, peripheral neuropathy, nephrotoxicity, and chemotherapy resistance. Thus the development of novel chemotherapeutics for lung cancer is warranted. In the present communication we report the cytotoxicity and antioxidant activity of *F. elephantum* leaves extracted with hexane and ethyl acetate.

MATERIALS AND METHODS

Plant material

The fresh leaves of *F. elephantum* were collected from different places in Tamil Nadu in February 2011. The name of plant species was confirmed by taxonomist at Entomology Research Institute (ERI) and voucher specimens were deposited at Loyola College, Chennai, India.

Extraction of plant material

The extracts were prepared by cold percolation method. The leaves were dried under shade and ground into fine powder using electric blender. 1 kg of powder was soaked in 1500 mL of hexane and ethyl acetate sequentially for 48 h with intermittent shaking. The leaf extract was filtered through Whatman No. 1 filter paper and collected in a conical flask. The filtrate was dried at 40°C using

vacuum rotary evaporator; the dry weights of the hexane and ethyl acetate extract yields were 6.2 and 7.5 g respectively. The extracts were stored at 4°C for further use.

Column chromatography

The total active hexane extract was chromotographed over silica gel (Merck 60-120 mesh) packed with hexane. The column was successively eluted with petroleum either, petroleum either-hexane mixtures with increasing amounts of hexane. Based on thin layer chromatography (TLC) profiles, similar fractions were combined to give 9 fractions.

Cell line

Human A549 lung adenocarcinoma cancer cell was obtained from National Institute of Cell Sciences, Pune, India.

Cell Culture

Cell line was maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum and 2mM L Glutamine, along with antibiotics (about 100 IU/mL of penicillin, 100 µg/mL of streptomycin) with the pH adjusted to 7.2 at 37ºC. RPMI 1640 was purchased from Himedia Chemicals, Mumbai, India. Fetal Bovine Serum (FBS) was purchased from Gibco Life Technologies, India. Trypsin, Methylthiazolyl diphenyl-tetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from SISCO Research Laboratory, Mumbai. All other chemicals and reagents were obtained from Sigma Aldrich Co., USA.

Cytotoxic properties

The cytotoxicity was determined according to the method of Balachandran et al., (2013) [7] with some changes. Cells (5000 cells/well) were seeded in 96 well plates containing medium with different concentrations of hexane and ethyl acetate extracts respectively. The cells were cultivated at 37 °C with 5% CO_2 and 95% air in 100% relative humidity. After various durations of cultivation the solution in the medium was removed. An aliquot of 100 µL of medium containing 1 mg/mL of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) was loaded in the plate. The cells were cultured for 4 h and then the solution in the medium was removed. An aliquot of 100 µL of DMSO was added to the plate which was shaken until the crystals were dissolved. The cytotoxicity against cancer cells was determined by measuring the absorbance of the converted dye at 540 nm in an ELISA reader. Cytotoxicity of each sample was expressed as IC_{50} value. The IC_{50} value is the concentration of test sample that causes 50% inhibition of cell growth averaged from three replicate experiments. Growth inhibition was calculated from the following formula:

(% Cytotoxicity) = {[1−(A/B)]x100}

Where A is the absorbance of treated cells and B is the absorbance of control cells.

2, 2-diphenyl-1-picryl hydrazyl (DPPH) radical scavenging assay

DPPH radical scavenging activity of fraction 5 was determined based on the method of Balachandran et al., (2013) [7]. 40 µL of various concentrations (125-1000 µg/mL) of fraction 5 were added to ethanolic solution of DPPH (0.1 M, 2960 µL). The absorbance of reaction mixture was measured at 517 nm after 30 minutes of incubation in the dark at room temperature. The free radical scavenging activity was calculated as follows:

$DPPH^{\cdot}$ scavenging activity = $[(A_C - A_S / A_C) \times 100]$,

Where A_C is the absorbance of the control and A_S is the absorbance of the extract / standard (Butylated hydroxytoluen). The experiment was run in triplicate and the result was reported as mean ± Standard deviation.

Gas Chromatography-Mass Spectrometry

Gas Chromatography-Mass Spectrometry (GC-MS) analysis was performed with a SHIMADZU-QP2010 with helium as a carrier gas with a linear velocity flow on a Resteck-624 ms column (30m x 0.32 mm id, 1.8 µm film thickness). Column flow rate was 1.491 ml/min. The oven was programmed to rise to 45°C (4 min) isotherm, and then to 175 leads to 240°C at a rate of 10°C/min and 25°C/min respectively. Injector and detector temperatures were 140°C. The identification of the constituents was performed by computer library search and retention indices.

Statistical analysis

Antioxidant and cytotoxic activities of extracts and fractions were statistically analyzed by Duncan multiple range test at P =0.05 with the help of SPSS 11.5 version software package.

RESULTS AND DISCUSSION

The leaves of *F. elephantum* were extracted with hexane and ethyl acetate sequentially and the yields were 6.2 and 7.5 g respectively. After extraction the hexane and ethyl acetate extracts were tested against human A549 lung adenocarcinoma cancer cell line. Hexane extract showed good cytotoxic activity *in vitro* against A549 lung adenocarcinoma cancer cell line compared to ethyl acetate extract. Hexane extract showed 77.3% activity at the dose of 500 µg/mL with IC_{50} (50.6%) value of 125 µg/mL (Table 1). The ethyl acetate extract did not show any cytotoxic activity.

Table 1: Cytotoxic properties of hexane and ethyl acetate extracts of *F. elephantum* leaves against A549 lung adenocarcinoma cancer cell line

Concentration (µg/mL)	Hexane extract		Ethyl acetate extract	
	%	Mean±S.D	%	Mean±S.D
15.63	17.9	1.012±0.00784	8.1	1.131±0.00354
31.25	25.8	0.913±0.00751	11.7	1.087±0.00269
62.5	32.7	0.828±0.00635	19.3	0.993±0.00214
125	50.6	0.608±0.01257	25.9	0.912±0.00387
250	61.9	0.469±0.00601	33.1	0.823±0.00439
500	77.3	0.279±0.01250	38.5	0.757±0.00501

Data are mean ± SD of three independent experiments with each experiment conducted in triplicate. Positive control 9.80±0.43µm (Cisplatin). Hexane extract was also tested at different time intervals. At 12th h it showed 51.7% activity at the dose of 500 µg/mL with IC_{50} value of 500 µg/mL. At 24th h it showed 70.3% activity at the dose of 500 µg/mL with IC_{50} (64.7%) value of 250 µg/mL. At 48th h it showed 74.9% activity at the dose of 500 µg/mL with IC_{50} (51.3%) value of 125 µg/mL. At 72 h it showed 73.4% activity at the dose of 500 µg/mL with IC_{50} (52.4%) value of 125 µg/mL (Table 2). The results showed that the activity gradually increased at different time intervals.

The active hexane extract was chromotographed over silica gel column. Based on thin layer chromatography (TLC) profiles, similar fractions were combined to give 9 fractions. When the fractions were bio-assayed fraction 5 showed maximum cytotoxic activity against A549 lung adenocarcinoma cancer cell line compared to other fractions. Fraction 5 was examined and confirmed as an essential oil. Fraction 5 was tested against A549 lung adenocarcinoma cancer cell line at different time intervals. At 12th h it showed 61.5% activity at the dose of 50 µL/mL with IC_{50} value of 25 µL/mL. At 24th h it showed 75.5% activity at the dose of 50 µL/mL with IC_{50} value of 12.5 µL/mL. At 48th h it showed 77.4% activity at the dose of 50µL/mL with IC_{50} value of 12.5 µL/mL. At 72 h it showed 77.6% activity at the dose of 50 µL/mL with IC_{50} value of 12.5 µL/mL (Table 3). All the concentrations used in the experiment decreased the cell viability significantly ($P<0.05$) in a concentration dependent manner. Yousefzadi et al., (2011) [8] reported the in vitro

cytotoxic activity of the essential oil from ripe fruits of *Dorema ammoniacum* against two human cancer (MCF and SW480) and two normal (HFSF and HFLP) cell lines were more sensitive. Suhail et al., (2011) [9] reported that more abundant high molecular weight compounds, including boswellic acids, were present in *Boswellia sacra* essential oil prepared at 100°C hydro distillation. All three human breast cancer cell lines were sensitive to essential oil treatment with reduced cell viability and elevated cell death, whereas the immortalized normal human breast cell line was more resistant to essential oil treatment. *Zanthoxylum rhoifolium* essential oil was extracted and its cytotoxic effects were studied against HeLa (human cervical carcinoma), A-549 (human lung carcinoma), HT-29 (human colon adenocarcinoma), Vero (monkey kidney) cell lines and mice macrophages; it showed good cytotoxic activity [10].

Table 2: Cytotoxic properties of hexane extract from *F. elephantum* leaves against A549 lung adenocarcinoma cancer cell line at different time interval

Concentration (µg/mL)	12 h		24 h		48 h		72 h	
	%	Mean±S.D	%	Mean±S.D	%	Mean±S.D	%	Mean±S.D
15.63	12.3	1.187±0.00321	16.7	1.025±0.00394	18.6	1.002±0.00521	19.3	1.035±0.00250
31.25	19.7	1.087±0.00258	25.1	0.923±0.00452	25.9	0.911±0.00395	26.4	0.906±0.00358
62.5	30.2	0.945±0.00451	31.4	0.845±0.00711	34.1	0.811±0.00562	34.9	0.801±0.00416
125	36.3	0.863±0.00981	46.2	0.628±0.00325	51.3	0.599±0.00641	52.4	0.586±0.00535
250	46.5	0.725±0.00754	64.7	0.434±0.00647	64.1	0.421±0.00874	66.3	0.415±0.00657
500	51.7	0.654±0.00732	70.3	0.365±0.00635	74.9	0.332±0.00683	73.4	0.327±0.00713

Data are mean ± SD of three independent experiments with each experiment conducted in triplicate. Positive control 9.80±0.43µm (Cisplatin).

Table 3: Cytotoxic properties of fraction 5 (essential oil) from *F. elephantum* leaves against A549 lung adenocarcinoma cancer cell line at different time interval

Concentration (µL/mL)	12 h		24 h		48 h		72 h	
	%	Mean±S.D	%	Mean±S.D	%	Mean±S.D	%	Mean±S.D
1.563	20.5	1.077±0.00354	16.9	1.125±0.00398	17.8	1.113±0.00847	18.5	0.1.103±0.00890
3.125	28.6	0.967±0.00269	36.3	0.863±0.00613	36.9	0.854±0.00561	37.9	0.841±0.00569
6.25	38.3	0.835±0.00285	46.5	0.725±0.00596	46.9	0.718±0.00469	47.7	0.708±0.00421
12.5	45.1	0.743±0.00632	50.4	0.672±0.00545	52.6	0.642±0.00354	52.9	0.638±0.00391
25	53.9	0.623±0.00541	67.1	0.445±0.00836	69.4	0.415±0.00821	69.9	0.408±0.00487
50	61.5	0.521±0.00452	75.5	0.332±0.00847	77.4	0.306±0.00746	77.6	0.301±0.00563

Data are mean ± SD of three independent experiments with each experiment conducted in triplicate. Positive control 9.80±0.43µm (Cisplatin).

Antioxidant activity of fraction 5 was assessed and compared with the standard (Butylated hydroxytoluen). The radical scavenging activity of fraction 5 at different concentrations is shown in Table 4. The radical scavenging activity of fraction 5 was maximum at 1000 µg/mL (71.63 ±0.15); it was comparable with Butylated hydroxytoluen at 1000 µg/mL (91.24 ± 1.96). Senthilkumar and Venkatesalu, (2013) [11] reported the fruit pulp essential oil of wood apple *Feronia limonia* for its chemical constituents, in vitro antioxidant and antimicrobial activities. Totally, 50 constituents were identified by GC–MS analyses and thymol (52.22%) was identified as the major chemical compound. The antioxidant activity showed that the essential oil had good antioxidant activity. The antimicrobial activity showed that *Staphylococcus aureus* and *Bacillus cereus* were the most sensitive organisms and also moderate activity was recorded against all the fungal strains tested. Amiri, (2012) [12] reported the antioxidant activity of *T. kotschyanus, T. eriocalyx* and *T. aenensis* subsp *lancifolius.* The polar sub-fraction of *T. daenensis* sub-sp *lancifolius* was found to show higher activity than others in DPPH assay. The nonpolar sub-fraction of *T. eriocalyx* showed good antioxidant activity. Khelifa et al., (2012) [13] studied the *Ocimum basilicum* essential oil for its *in vitro* antioxidant activity using DPPH assay. It showed that 50% of DPPH was scavenged, by 83.54 mg/mL. This was lower than that of vitamin E (22.0 mg/mL). Tenore et al., (2011) [14] and Oliveira et al., (2012) [15] reported the DPPH radical scavenging potential of thymol rich essential oil from *Satureja montana*.

Table 4: Antioxidant activity of fraction 5 (essential oil) using DPPH

DPPH µL/mL	Essential oil	µg/mL	BHT
1000	71.63 ± 0.15	1000	91.24 ± 1.96
500	59.28 ± 0.17	500	80.62 ± 1.91
250	43.16 ± 0.13	250	70.55 ± 1.02
125	31.62 ± 0.15	125	61.57 ± 0.35

Active fraction 5 was identified using GC-MS analysis. GC-MS analysis showed the presence of Estragole (50.82%), trans-anethole (p-propenylanisole, anise camphor) (14.98%), Caryophyllene (9.22%) and germacra-1(10),4(15),5-triene, (-)- (5.96%)(Table 5 and Fig 1). Muthulakshmi et al., (2012) [20] reported the bioactive components of leaves and bark of *F. elephantum* using GC-MS analysis; they showed 18 components from leaves and 14 components from bark. The prevailing compounds in the ethanol extract of leaves of *F. elephantum* were 7–Norbornadienyl t-butyl ether (17.26%), 2–isopropyl–5-methyl–1– heptanol (11.40%), 1–Octanol,2–butyl (8.47%), Phenol, 4–[2–(dimethylamino)–ethyl]–(4.56%), 2,3–Dimethylquinolin-4(1H)–one (3.58%), Ethyl iso–allocholate (1.63%). The ethanol extract of *F. elephantum* bark contained 2–Propenenitrile, 3–(3.4-dimethoxyphenyl)–(60.72%); it was the major component followed by phenol, 4–(3-hydroxy-I-propenyl)-2-methoxy–(9.35%), 3-(2-NAcetyl–N-methylaminoethyl) indol (1.15%), cholesta–8,24–dine–3–ol, 4–methyl–(3a'- 4a')–(0.86%).

Rahman et al., (2012) [21] reported the cytotoxic and antibacterial activity of essential oil isolated from *Clausena suffruticosa* Leaf. The GC-MS analysis showed that estragole (58.23%) was the major compound. Coutinho et al., (2010) [22] also reported the antibacterial effect of estragole against *B. cereus, S. typhi* and *S. aureus*.

The radical scavenging activity of essential oil can be credited to the presence of its major phenolic compounds, particularly thymol and their recognized impact on lipid oxidation [16]. The antioxidant activity of phenolic compounds is related to the hydroxyl groups linked to the aromatic ring, which are capable of donating hydrogen atoms with electrons and stabilizing free radicals [17, 18]. Guimarães et al., (2010) [19] reported the comparative study between the antioxidant properties of essential oils and phenolic extracts from *Cistus ladanifer* leaves, *Citrus latifolia* fruit peels, *Cupressus lusitanica* foliage and *Eucalyptus gunnii* leaves.

The antioxidant properties of phenolic extracts (expect that of *C. latifolia*) were excellent and they better were than those obtained from the essential oils extracts and even the standard BHA.

Table 5: Composition of essential oil (fraction 5) of *F. elephantum* leaves as analyzed by GC-MS

Peak number	compounds	Retention time(min)	Relative (%)
1	gamma.-Terpinene	5.101	0.55
2	4-thujanol, stereoisomer	5.324	0.17
3	alpha.- terpinolen	5.527	0.25
4	beta.-Linalool	5.747	0.82
5	L-trans-Pinocarveol	6.469	0.25
6	Pinocarvone	6.816	0.30
7	(-)-4-Terpineol	7.070	1.66
8	Estragole	7.471	**50.82**
9	p-Propenylanisole	8.134	0.45
10	trans-anethole (p-propenylanisole, anise camphor)	8.705	**14.98**
11	.beta.-Elemene,	9.981	0.45
12	Methyleugenol	10.181	2.74
13	Caryophyllene	10.472	**9.22**
14	alpha.-Caryophyllene	10.905	1.02
15	germacra-1(10),4(15),5-triene, (-)-	11.254	**5.96**
16	1,3,3-Trimethyl-2-(2-methylcyclopropyl)-1-cyclohexene	11.401	0.78
17	beta.-cadinene	11.654	0.52
18	(11E)-12-Cyclopropyl-11-dodecen-1-ol	11.955	0.48
19	Elemicin	12.000	0.11
20	4-Isopropyl-1,7-dimethyl-2,7-cyclodecadien-1-ol	12.431	0.63
21	Caryophyllene oxide	12.538	3.20
22	2,5,9-trimethyl-4,8-cycloundecadien-1-one	12.846	0.20
23	3-Ethyl-3-hydroxyandrostan-17-one	13.125	0.40
24	Tetracyclo[6.3.2.0(2,5).0(1,8)]tridecan-9-ol, 4,4-dimethyl-	13.173	0.91
25	alpha.-Cadinol	13.354	0.47
26	Aromadendrene oxide-(2)	13.715	1.26
27	7-Methyl-4-(1-methylethylidene)bicyclo[5.3.1]undec-1-en-8-ol	13.781	0.08
28	Phytol	17.871	1.30

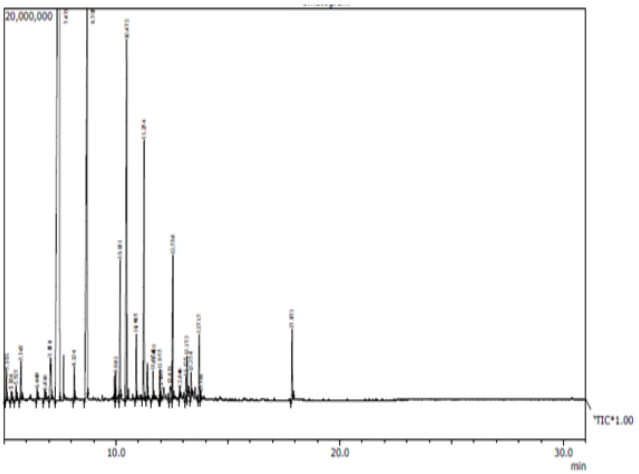

Fig. 1: GC-MS analysis of the essential oil from *F. elephantum* leaves.

CONCLUSION

Hexane extract of *F. elephantum* exhibited good cytotoxic activity against A549 lung cancer cell line compared to ethyl acetate extract. The active hexane extract was subjected to column chromatography and fraction 5 showed maximum cytotoxic activity. Fraction 5 was analysed for its antioxidant property using DPPH assay; it showed maximum antioxidant activity at 1000 µg/mL (71.63 ± 0.15). Active fraction 5 was subjected to GC-MS analysis; the major compound was estragole (50.82%). This can be probed further in drug discovering programme.

CONFLICT OF INTERESTS

Declared None

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