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ANTI-INFLAMMATORY, CYTOTOXIC AND ANTIOXIDANT EFFECTS OF METHANOLIC EXTRACTS OF *DRYPETES SEPIARIA* (EUPHORBIACEAE)

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Abstract

The aim of this study was to investigate *in vitro* antioxidant, anti-inflammatory and cytotoxic activities of the petroleum ether, ethyl acetate, methanol and aqueous extracts obtained from leaves of *Drypetes sepiaria* (Euphorbiaceae). Total phenolic and flavonoid contents of these crude extracts were determined as gallic acid and quercetin equivalents, respectively. In *in vitro* antioxidant method, methanol extract exhibited higher free radical scavenging activity compared to standard compound, ascorbic acid with IC_{50} of 95.43µg/ml (DPPH) and 67.05µg/ml (ABTS). Methanol extract was able to inhibit inflammation by *in vitro* about 85-90% (HRBC stabilization method) and *in vivo* about 40-45% (Paw oedema method) anti-inflammatory assays compared to standard produced 50.04% at 6h period. In cytotoxicity assay (MTT assay) methanolic extract exhibited IC_{50} of 10µg/ml. In apoptosis (flow cytometric assay), the control group showed normal caspase 3 activity in the SiHa cells which was 0.24%, and increased up to 40% after treatment.

Key words: *Drypetes sepiaria*, Euphorbiaceae, Antioxidant, Anti-inflammatory, Cytotoxicity, Apoptosis

Introduction

The medicinal properties of various plants have been investigated in recent years throughout the globe due to their good pharmacological properties, fewer side effects, and low cost. The genus *Drypetes* comprises approximately 160 species which has been used in the folk medicine of many cultures for many years in the treatment of various pathological conditions (Ngang et al., 2008). The plants that belong to *Drypetes* genus are used for the treatments of sinusitis, swellings, boils, gonorrhoea and dysentery in West and Central Africa (Dalziel, 1937; Irvine, 1961). These plants are extensively used in African folk medicine to treat various diseases such as bronchitis, ailments of the digestive tract, fever, kidney pain and rheumatism (Ata et al., 2011). The plants of this genus have anti-inflammatory and analgesic activity (Wandji et al., 2000; Chungag et al., 2001) and also act as CNS depressants (Sudharshan et al., 2009). It is worth noting that the species was different, although this genus has been used to treat similar disorders. Earlier phytochemical studies on some *Drypetes* plant species including *D. parrifolia*, *D. laciniata*, *D. inaequalis*, *D. armoracia*, *D. gossweileri*, *D. molunduana*, *D. roxburghii* have yielded flavonoids, chalcone glycosides, saponins, tripterpenoids, phenolics, alkaloids, etc. (Nenkep et al., 2008; Fannang et al., 2011; Awanchiri et al., 2009; Wandji et al., 2003; Wandji et al., 2000).

Drypetes sepiaria belongs to the family *Putranjivaceae* (*Euphorbiaceae*). It is an evergreen tree commonly grown in foothills and shrub jungles, and is widely distributed in Srilanka and some places of Tamil Nadu. It is locally known as Kalvirai (Tamil). This plant is used in folk medicine by the tribal people of Western Ghats to treat pain and inflammation. The seeds of this plant are used as a wild edible food by Palliyars (tribal Group) of Western Ghats, India (Arinathan et al., 2007). Recent report on *Drypetes sepiaria* stated that the paste of the roots can be used as an antidote for scorpion bite. The decoction of leaves and seeds of this plant is also noted for reducing rheumatic inflammation (Bharath kumar and Suryanarayana, 2011). As per our literature reports, till date there is no scientific investigations found on species *D. sepiara* on both of its pharmacological properties as well as phytoconstituents present. In view of the ethno-botanical uses of the *Drypetes* genus as described above, the antioxidant, anti-inflammatory activity and cytotoxic activities of different extracts of leaves of *D. sepiaria* were investigated in the present study.

Experimental

Chemicals and Reagents

1,1-dipheny l-2-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), λ-Carrageenan, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were procured from Sigma Aldrich Chemicals, USA. Folin ciocalteau reagent, Sodium dodecyl sulphate (SDS) were procured from Sisco Research Laboratories Pvt. Ltd. Sodium carbonate, Sodium chloride, Ascorbic acid, Gallic acid, Ammonium per sulphate, Aluminium trichloride were

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purchased from SD-Fine Chem limited. Indomethacin and Diclofenac potassium were obtained from Cipla pharmaceuticals. All other chemicals and solvents were of AR grade and obtained from standard sources.

Experimental Animals

Wistar rats (150-200gm) of either sex were obtained from Animal house, VIT University, housed at 20±2^{o}C, with a 12:12 h light and dark cycle and given food and water *ad libitum*. The animals fasted for 12 h before the experiment, with free access to water. All the procedures were approved by the Institute Animal Ethics Committee and the experiments were carried out as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) given on animal experimentation.

Plant material and preparation of extracts

Fresh leaves of *D. sepiara* were collected from Puducherry (Coordinates: 11°58'5"N 79°48'42"E) India, during the month of January, 2011. The plant was authenticated by Professor Angelin Vijaya Kumari, HOD of Biology, Voorhees College, Vellore, Tamil Nadu. The leaves were washed thoroughly with distilled water to remove all contaminated materials. The washed leaves were shade dried to avoid decomposition and pulverised in mixer blender. Sixty grams of the powder was serially extracted with 400 ml of petroleum ether, ethyl acetate, methanol and water in a soxhlet apparatus to obtain petroleum ether (PE), ethyl acetate (EA), methanol (ME) and water (AQ) extracts respectively. The extracts were concentrated in a rotary evaporator and lyophilised, weighed and stored in vacuum desiccators. The total yields of the extracts were 1.38% (PE), 2.69% (EA), 16.22% (ME), and 12.24% (AQ) respectively.

Phytochemical screening of extracts

Preliminary screening of the crude extracts was carried out qualitatively for the presence of alkaloids, saponins, flavonoids, tannins, amino acids, carbohydrates, steroids, phenols, anthraquinones by following standard methods (Edeoga et al., 2005).

Estimation of total phenolics

The amount of total phenolics in the extracts was determined by Folin ciocalteau reagent method explained by (Wangensteen et al., 2004). 20 µl of the test sample (1 mg/mL) 40 µl of folin ciocalteau reagent and 100 µl of Na_2CO_3 (25% w/v) were added and made up to 2 mL and incubated at 45^{o}C in dark for 30 min. In blank, folin ciocalteau reagent was replaced with distilled water and absorbance of developed blue colour was measured at 765 nm using UV-Visible spectrophotometer (Jasco V-670). A calibration curve was prepared using standard solution of gallic acid. The results were expressed in mg of gallic acid equivalents per gram of extract. All determinations were carried out in triplicates.

Determination of total flavonoids

The total flavonoid content was determined by a calorimetric method explained by Ebrahimzadeh et al. (2008) with some modifications. Briefly, 1mL of each extract (1mg/mL) in methanol was mixed with 1 mL 20% aluminium trichloride in ethanol and a drop of acetic acid was added and then diluted with ethanol to 25 mL and left at room temperature for 30 min. The absorption of the reaction mixture was measured at 415 nm using UV-Visible spectrophotometer. A calibration curve was prepared using standard quercitin under same conditions. The results were expressed in mg of Quercetin equivalents per gram of extract. All determinations were carried out in triplicates.

DPPH radical scavenging activity

The scavenging of DPPH radical by the extracts is based on the method described by Mensor et al. (2001) with minor modifications. All the extracts were dissolved in methanol by using sonicator bath. 500 µL of the sample solutions of varying concentrations (10-500 µg/ml) were mixed with 500 µL of freshly prepared methanolic solution of 0.2 mM DPPH. The solution in the test tubes were shaken well and kept in dark for 15 min at room temperature. The reduction in the colour was measured at 517 nm. The control solution consisted of a mixture of 500 µL methanol and 500 µL DPPH. The blank solution contains 500µL of sample and 500µL of methanol. Ascorbic acid was used as a standard (2-10µg/ml). Results were expressed as percentage of inhibition of the DPPH radical. All determinations were done in triplicates. Percentage of inhibition of the DPPH radical was calculated according to the following equation:

$$\text{Inhibition of DPPH (\%)} = (A_c - A_s/A_c) \text{ X } 100$$

Where, A_c = Absorbance of control

A_s = Absorbance of samples (or) standard.

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ABTS radical cation decolourisation assay

This assay was carried out by the method explained by Subhasree et al. (2009). 7 mM of ABTS solution was reacted with freshly prepared 2.45 mM ammonium persulphate solution and kept in dark for 12-16 h to produce a dark coloured solution containing ABTS radical cations. The initial absorbance was measured at 734 nm. This stock solution was diluted to give a final absorbance value of about 0.7 (±0.02). 0.1 mL of different concentrations (10-100 µg/mL) of test samples was mixed with 1 mL of ABTS working standard in a microcuvette. The decrease in absorbance was measured; the final absorbance at seventh minute was noted. Gallic acid was used as a standard at different concentrations (2-10 µg/mL). Results were expressed in percentage of inhibition of ABTS radical. All determinations were done in triplicates.
The percentage inhibition was calculated according to the formula:

$$\text{Inhibition of ABTS (\%)} = (A_c - A_s/A_c) \text{ X } 100$$

Where, A_c = Absorbance of control
A_s = Absorbance of samples (or) standard.

***In vitro* anti-inflammatory activity – HRBC Method**
Preparation of human red blood cells (HRBC) solution

Blood (B +ve) was collected from a healthy human donor not consuming any steroidal drugs for past two weeks. The blood was subjected to centrifugation and the supernatant part was carefully pipetted out with sterile pipettes. The packed cells were resuspended with equal volume of normal physiological saline (pH 7.4) and centrifuged again. The process was repeated four times until the supernatants were clear. A 10% HRBC suspension was then prepared with normal physiological saline and used immediately (Oyedapo and Famurewa, 1995).

Effect of extracts on HRBC stability

4.5 mL of reaction mixture consisting of 2 mL hypotonic saline (0.25% w/v NaCl), 1 mL of sodium phosphate buffer (0.15 M, pH 7.4) and 1 mL of extract were dissolved in normal physiological saline. Then 0.5 mL of 10% HRBC was also added. Two controls were performed, one with 1.0 mL of isotonic saline instead of extract, and the second control with 0.5 mL of isotonic saline instead of red blood cells. The mixture was incubated at 56°C for 30 min. The tubes were cooled under running water for 20 min and the mixture was centrifuged at 3000 rpm. The supernatants were separated and the absorbance of the supernatants read at 560 nm (Mongelli et al., 1997). The percentage membrane stabilising activity was determined using the equation explained by Sadique et al. (1989). The control represents 100% HRBC lysis. Diclofenac potassium was the standard drug used:

$$\text{\% Membrane stability} = 100 - (A_s - A_{c2} / A_{c1}) \text{ X } 100$$

Where, A_s = Absorbance of standard (or) samples
A_{c1} & A_{c2} = Absorbance of control 1 and control 2 respectively.

***In vivo* anti-inflammatory activity – Carrageenan induced paw oedema method**

This assay was based on the method described by Estrada et al. (2006). Wistar rats (150-200gm) of either sex were used. Animals were weighed and randomised in 5 groups (n=6). Before treatment, the average volume of the right paw of each animal was determined using a plethysmometer (Plethysmometer 7140, UGO Basile). All the animals were starved for 12h. To ensure uniform hydration, the rats received 5 mL of water by stomach tube (controls) or the test drug / standard drug dissolved or suspended in the same volume. Immediately after that test, drugs were administered. Group I served as control (V_c) and did not receive any drug. Group II received the standard Indomethacin (10 mg/kg, p.o) and Group III, IV, and V received test drug in three different doses (100, 200, 300 mg/kg, p.o). Thirty minutes later, the rats were challenged by a subcutaneous injection of 0.1 mL of 1% w/v freshly prepared solution of λ-carrageenan in saline into the plantar side of the left hind paw. The paw was marked with ink at the level of the lateral malleolus and immersed in water reservoir of digital Plethysmometer up to this mark to measure the paw volume. The paw volume (V_t) was measured at 2, 4, 6, 12 and 24 h immediately after carrageenan injection in control, extract treated and indomethacin treated groups.

The percentage inhibition of each group was determined using the following formulae:

$$\text{\% Inhibition} = (V_c - V_t / V_c) \text{ X } 100$$

Where, V_c = Mean variation of oedema for the control group
V_t = Mean variation of oedema for treated groups with plant extracts or standard drugs.

Cytotoxic activities
Cell Culture

The HPV16 positive cervical carcinoma cells (designated SiHa) were cultured in a sterile T75 flask containing DMEM medium (Gibco) supplemented with foetal bovine serum (10% v/v) at 37°C in a humidified atmosphere of 5% CO_2 in incubator.

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Cytotoxicity

SiHa cells were exposed to the test compounds (final volume 100µL) in a 96-well microplate for 24h at 37°C in a CO_2 incubator. Two hours prior to completion of treatment duration, cultures were supplemented with 5mg/mL solution of MTT. The microplates were then incubated for 2h at 37°C, and cells were lysed with lysis buffer (20% SDS, 50% Dimethyl Formamide) was added to each well and then incubated overnight at 37°C for solublization of formazan crystals. Absorbance was measured at 570nm with the aid of a 96-well multiscanner autoreader (Biotek, Winooski, Vermont) with the lysis buffer serving as blank (Mahata et al., 2012). The percentage of cell viability was calculated using the formula:

Percentage cell viability = (OD of the experiment samples/OD of the control) × 100

Quantitation of caspase- 3 activity by flow cytometric analysis

The SiHa cell lines were treated with the extracts and then harvested, washed with phosphate buffer saline and incubated with caspase 3. The activity of caspase 3 was measured using the active caspase 3 apoptosis kit (BD Pharmingen, USA) following the manufacturer's protocol. Briefly, 10 µg of extract was incubated with SiHa cells for 12 h and were harvested by pooling. Attached and detached cells were pelleted with centrifugation at 200 × *g* for 5 min at 4°C. The cells were permeabilised, fixed and stained for active caspase 3 (PE-conjugated) as described in manufacturer's protocol (BD Biosciences). The cells were then analysed by fluorescence activated cell sorting (FACS). The number of 10,000 events was acquired and the cells were properly gated for analysis using FACSAria instrument equipped with Flowjo software (Becton-Dickinson Biosciences, San Jose, CA).

Statistical evaluation of data

The results were presented as the mean % ± Standard Error of Mean (SEM). Statistical differences between the treated and control groups were evaluated by one way ANOVA by SPSS version 9.05 software and followed by Dunnetts t-test. The values were considered significant when $p < 0.05$, $p < 0.01$ and $p < 0.001$.

Results and Discussion

Preliminary phytochemical analysis revealed the presence of saponins, anthraquinones in all the extracts, alkaloids in petroleum ether and aqueous extracts, whereas flavonoids and carbohydrates in methanol and aqueous extracts, steroids in petroleum ether and ethyl acetate extracts. These extracts did not show positive report for tannins and amino acids.

The total phenolic and flavonoid content in the different extracts were given in Table 1. Phenolic and flavonoid compounds are expressed in gallic acid and quercetin equivalents. Phenols and flavonoids are very important plant constituents because of their higher scavenging ability. This Folin ciocalteau assay has been extensively used to measure the total phenolics in plant materials for many years and based on electron transfer reaction which measures a sample's reducing capacity (Harput et al., 2011). The results suggest that the phenolic content was found to be high in the methanolic extract (85.0 mg/g) when compared to that of the other extracts. Flavonoid content was high in methanol and aqueous extracts. The phenolic compounds exhibit considerable free radical scavenging activities, through their reactivity as hydrogen or electron donating agents, and metal ion chelating properties (Rice-Evans et al., 1996).

Table 1: Total phenolic and flavonoid content

Extract	TPC (mg of gallic acid equivalents/g of extract)	TFC (mg of quercetin equivalents/g of extract)
Petroleum ether	12.5±0.05	1.2±0.05
Ethyl acetate	42.5±0.1	2.5±0.11
Methanol	85.0±0.56	22.11±0.25
Aqueous	37.5±0.45	20.08±0.09

TPC: Total phenolic content; TFC: Total flavonoid content; Results were expressed in mean ± S.D (n=3)

The DPPH radical scavenging assay is a widely used method to evaluate the free radical scavenging ability. DPPH is a stable free radical with an absorption band around 517-528nm and very useful in the determination of antioxidant activity. This free radical which is purple in colour by accepting a lone pair of electron or hydrogen radical loses its chrompohore and becomes yellow in colour. The radical scavenging ability of all the extracts was represented in Fig. 1. The methanol and ethyl acetate extracts showed most potent and concentration dependent DPPH scavenging activity with IC_{50} values of 95.43µg/mL and 94.1µg/mL respectively. This indicates that compounds that show strongest radical scavenging capacity are of medium polarity. The petroleum ether and aqueous extracts were comparatively less active in this assay with IC_{50} values of 201.54µg/mL and

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188.81µg/mL which may be due to lack of hydrogen donating species. The IC_{50} value of standard ascorbic acid was found to be 3.6µg/mL; the effects of PE and AQ extracts are negligible since their IC_{50} value was comparatively very low. It was found that the radical scavenging activities of all the extracts increased with increasing concentration. Based on the literature reports to our knowledge, antioxidant studies of *D. sepiara* species have not been reported previously. Many plants have been reported to have DPPH scavenging effect by which flavonoids, phenols, steroids and terpenoids may probably contribute to this effect. The DPPH scavenging ability of different extracts is in the order of ME>EA>PE>AQ.

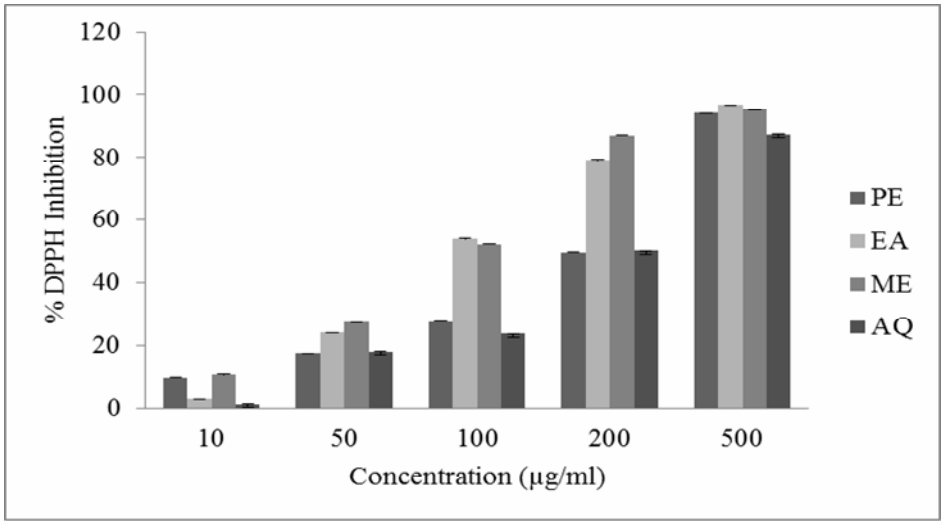

Figure 1: DPPH radical scavenging activity of different extracts obtained from *D. sepiara* leaves. Results are expressed as mean ± S.D (n=3)

ABTS•+ is a stable free radical; the procedure is based on inhibition of the production of the ABTS radical cation. The relative ability of hydrogen donating antioxidants to scavenge ABTS•+ generated in the aqueous phase can be measured spectrophotometrically at 734 nm (Antolovich et al., 2002). The ability of the extracts to scavenge ABTS radical cation was expressed in Fig. 2 at a concentration range of 20-100µg/ml by comparison with standard gallic acid. The ethyl acetate and methanol extracts showed most potent and concentration-dependent ABTS scavenging activity with IC_{50} values of 55.25µg/mL and 67.05µg/mL respectively, whereas the standard gallic acid has an IC_{50} of 8.7µg/mL. The petroleum ether and aqueous extracts showed comparatively low ABTS scavenging activity with IC_{50} values of 102.2µg/mL and 74.8µg/mL. The ABTS radical cation scavenging ability of all the extracts is in the order of EA>ME>AQ>PE.

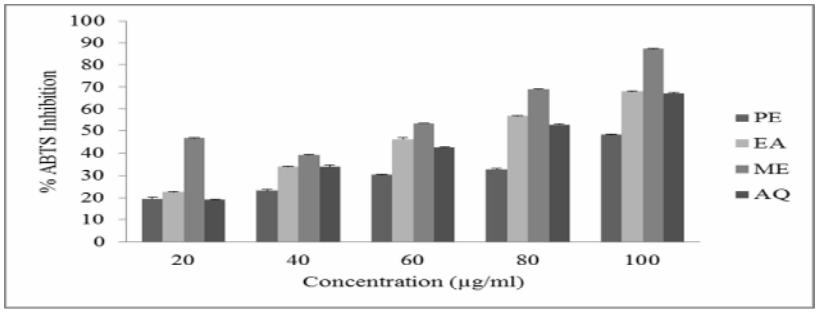

Figure 2: ABTS radical scavenging activity of different extracts obtained from *D. sepiara* leaves. Results are expressed as mean ± S.D (n=3)

***In vitro* anti-inflammatory activity**

The RBC membrane stability test is based on the finding that a number of non-steroidal anti-inflammatory agents inhibit heat induced lysis of erythrocytes, presumably by stabilising the membrane of the cell. The erythrocyte membrane may be considered a model of the lysosomal membrane which plays an important role in inflammation (Weissmann et al., 1969).

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The compounds which prevent the lysis of membrane caused by the release of hydrolytic enzymes contained within the lysosomes may relieve some symptoms of inflammation (Hess and Milonig, 1972). It has been demonstrated that certain herbal preparations were capable of stabilising the red blood cell membrane and this may be indicative of their ability to exert anti-inflammatory activity (Olumayokun et al., 2000).

The mode of action of the extracts or drugs may bind the erythrocyte membranes with subsequent alteration of the surface charges of the cells. This action may prevent physical interaction with aggregating agents or promote dispersal by mutual repulsion of like charges which are involved in the haemolysis of red blood cells. It has been reported that certain saponins and flavonoids exerted profound stabilising effect on lysosomal membrane both *in vivo* and *in vitro*, while tannins and saponins possess ability to bind cations, thereby stabilising erythrocyte membranes and other biological macromolecules (Oyedapo et al., 2010). It was noted that methanolic extract and aqueous extracts which showed positive tests for flavonoids exhibited highest membrane stabilising activity of 90% compared to that of standard Diclofenac potassium which exhibited 87% protection at 500 µg/mL concentration respectively (Fig.3).

Methanolic extracts of leaves of *D. sepiara* has a rich source of phytochemicals and exhibited good antioxidant and *in vitro* anti-inflammatory activity. Thus, this extract was chosen for *in vivo* anti-inflammatory activity and cytotoxicity studies on cervical cancer cell SiHa.

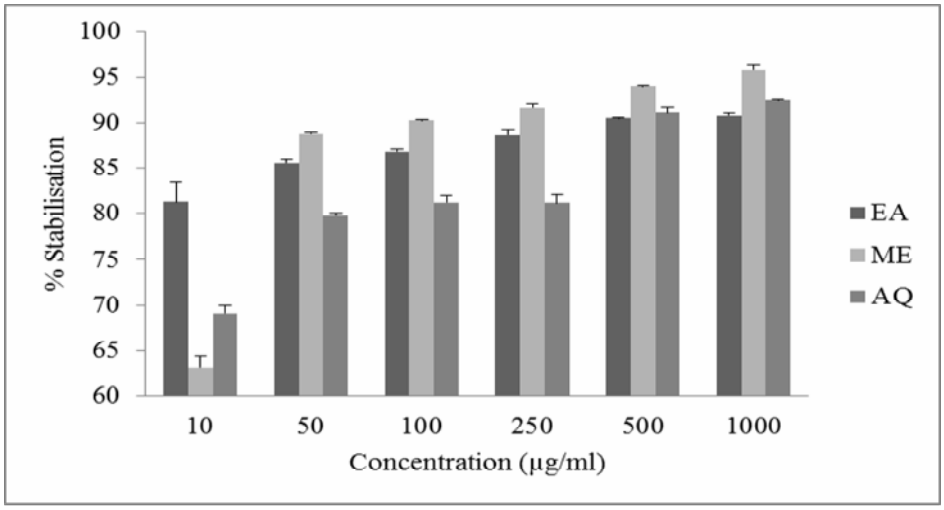

Figure 3: Effect of *D. sepiara* extracts on human red blood cell (HRBC) stability. Results are expressed as mean ± S.D (n=3)

The anti-inflammatory activity of *D. sepiara* methanol extract of leaves in acute experimental model reported in Table 2 explained that the results are comparable to that of the standard drug Indomethacin. Methanol extract at 200 mg/kg and 300 mg/kg showed a significant dose dependent and maximum inhibition of paw oedema induced by carrageenan in rat by 28.0 % ($p<0.001$) and 33.9 % ($p<0.001$) respectively, whereas Indomethacin at 10mg/kg produced 33.2% of inhibition after 12h of carrageenan injection.

Before injection of carrageenan, the basal values ranged between 0.39 and 0.65 ml (0.50±0.039 ml). Oedema inhibition in the compound or drug treated groups was calculated with reference to the control group values and the percentage of inhibition obtained in each treated group are shown in Table 2 in brackets. Indomethacin a standard drug showed a clear inhibition of a carrageenan induced inflammation compared to that of a control group ($p<0.001$). The methanolic extracts of leaves of *D. sepiara* at oral doses of 100 mg/kg does not show much significant effect and 200 mg/kg and 300 mg/kg showed a significant ($p<0.001$) effect at 6h when compared to that of effect produced by indomethacin. At a concentration of 200 mg/kg, it exhibited a maximum inhibition of 43.3%, and at 300 mg/kg it exhibited 46.2% in carrageenan induced rat paw oedema, whereas indomethacin produced 50.04% of inhibition after 6h of carrageenan injection. The percentage inhibitions are expressed in parenthesis in Table 2. The methanolic extract significantly inhibited the carrageenan induced paw oedema after 6h of challenge. Carrageenan induced rat paw oedema model is a suitable experimental animal model for evaluating the anti-inflammatory effects of natural products. Generally, inflammation occurs through two distinct phases and is biphasic. The first phase (up to 1 h) involves the release of serotonin and histamine and the second phase (after 1 h) is mediated by prostaglandin, the cylooxygenase products, and the continuity between the two phases is provided by bradykinins (Perianayagam et al., 2006).

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Table 2: Effect of ME extracts on carrageenan induced hind paw oedema in rats

Results are expressed as mean ± S.E.M (n=6). Asterisks denote the significant levels in comparison with control values *p<0.05,

Groups		Paw oedema (Volume in mL)					
	Dose (mg/kg)	0 hr	2hr	4hr	6hr	12hr	24hr
Control		0.500±0.039	0.9133±0.026	1.1017±0.03	1.1567±0.04	0.8433±0.075	0.6467±0.061
Indomethacin	10	0.4933±0.040 (1.34%)	0.7667±0.03** (16.0%)	0.6383±0.046*** (42.06%)	0.5783±0.043*** (50.04%)	0.5633±0.037*** (33.2%)	0.5050±0.036* (21.9%)
Methanol extracts	100	0.4933±0.021 (1.34%)	0.898±0.034 (1.64%)	0.9133±0.035*** (17.1%)	0.8317±0.046*** (28.09%)	0.7433±0.057 (11.8%)	0.64±0.038 (1.03%)
	200	0.5050±0.01 (0%)	0.8183±0.02* (10.4%)	0.7967±0.026*** (27.68%)	0.655±0.016*** (43.3%)	0.6067±0.011*** (28.0%)	0.5533±0.01 (14.4%)
	300	0.4933±0.022 (1.34%)	0.756±0.029*** (17.1%)	0.745±0.033*** (32.3%)	0.6217±0.026*** (46.2%)	0.5567±0.019*** (33.9%)	0.5167±0.019* (20.01%)

**p<0.01, ***p<0.001. Percentage inhibition is denoted in brackets.

The Methanol extract was evaluated for the cytotoxicity effects by MTT assay against SiHa cell lines. The results of the tested sample activity were summarised in Fig. 4. The results are demonstrated as percentage viability and represented as mean±standard deviation (n=3). From the results, the methanolic extract showed promising cytotoxic activity towards SiHa cells with IC50 values of 10µg/mL. The extract showed dose dependent cytotoxicity against SiHa cell lines. High cytoxicity of the extract towards cancerous cell represents the presence of some potential cytotoxic metabolite in the extract which could be used for the development of anticancer drugs.

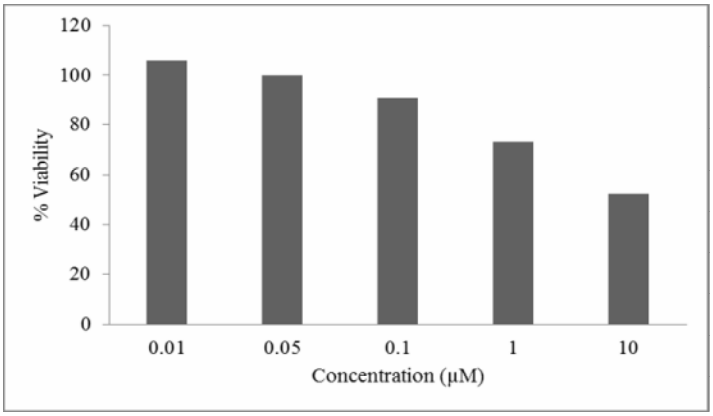

Figure 4: Percentage viability of *D. sepiara* methanolic leaf extract on cervical cancer cells. Results are expressed as mean ± S.D (n=3)

Treatment of SiHa cell lines with methanolic extract of *D. sepiara* at a concentration of 10 µg/ml resulted in the expression of caspase 3. Cytotoxic effect can be due to apoptosis or necrosis. The key biochemical event involved in the induction of apoptosis is activation of caspase 3 which is mediated through proteolytic cleavage of procaspase 3 via upstream caspases (caspase7/9 or caspase 8) (Mahata et al., 2011). SiHa cells were showing characteristic apoptotic bodies even after their treatment. Caspase 3, which has a central role in apoptosis process, was assessed flowcytometrically in order to confirm its status. Results indicate that at 12 hours, caspase 3 was increased 40% which was 0.24% prior to treatment with sample and thereafter it decreased. Results are summarised in Fig. 5.

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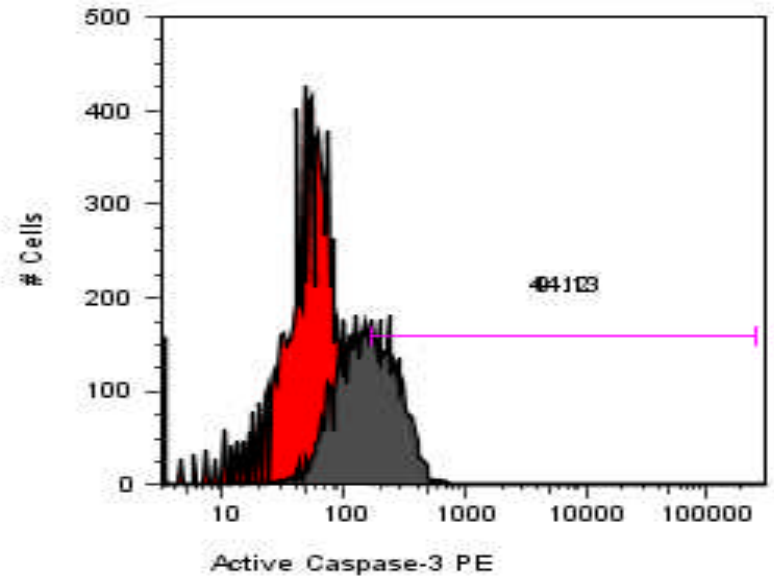

Figure 5: Flowcytometric analysis of SiHa cells for active caspase 3. Percentage in histogram shows proportion of cell with active caspase 3 after berberine treatment

Caspase is present as a proenzyme in un-stimulated cells. Treatment of the cells with anticancer drugs activates the caspase. The caspase could be activated by extra mitochondrial pathway (extrinsic pathway) or intra mitochondrial pathway (intrinsic pathway). Expression of caspase 3 by the treatment of extract is the indicator of cellular apoptosis and could be considered as the mechanism of action of the extract.

Conclusion

In the present study, we focused on the antioxidant, anti-inflammatory and cytotoxic activities of polar and non-polar extracts of *D. sepiara* leaves, for which there is no literature reports till date. On interest, the methanolic extract was able to significantly reduce inflammation induced by carrageenan and is also capable of reducing the cervical cancer cells which were further proven by apoptosis, as evident by increasing amount of caspase 3. The polar extracts also have an ability to scavenge free radicals which play a major role in many metabolisms. These findings suggest that the traditional use of these plants is mostly justified. The further isolation and identification of the individual constituents present in the various fractions are currently under investigation in our laboratory.

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