

Full Length Research Paper

Evaluation of anticancer activity of *Debregeasia salicifolia* extract against estrogen receptor positive cell line

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Crude methanol extract and fractions of *Debregeasia salicifolia* stem were examined for their anticancer activity. To determine anticancer activity, different concentrations of crude extract were tested on MCF-7 cancer cell line by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. *D. salicifolia* extract showed a significant antiproliferative activity and a dose dependent effect was observed. Minimum inhibition of 25.31% was shown by extract at concentration 10 µg/ml and maximum inhibition (99%) was observed at 500 µg/ml. Hexane, chloroform, ethyl acetate, methanol and aqueous fractions were also tested at a concentration of 200 µg/ml. Hexane, chloroform and ethyl acetate fractions appeared to be most active with 90 to 99% activity. These results indicate the possible potential use of *D. salicifolia* as antineoplastic agent. Preliminary phytochemical screening revealed the chemical composition of *D. salicifolia* extract containing flavonoids, anthraquinones and tannins. Anticancer properties of *D. salicifolia* can be linked with the presence of these chemicals.

Key words: Anticancer, cytotoxic, *Debregeasia salicifolia*, MCF-7 cell line, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, phytochemical.

INTRODUCTION

Medicinal plants have been used as remedies for human diseases for centuries. The reason for using them as medicine lies in the fact that they contain chemical components of therapeutic value (Nostro et al., 2000). The medicinal value of plants lies in some chemical substances (usually secondary metabolites), that produce a definite physiological action on the human body. The most important of these bioactive compounds of plants

are alkaloids, flavanoids, tannins and phenolics (Edeoga et al., 2005).

Cancer is the cause of more than six million deaths each year in the world. In 2001, about 1,268,000 new cancer cases and 553,400 deaths were reported in the United States (Izevbigive, 2003). For a long time, plants are being used in the treatment of cancer (Hartwell, 1982). According to an estimate, 50% of breast cancer and 37% of prostate cancer patients use herbal products (Richardson, 2001).

Breast cancer incidence in Pakistan is the highest reported in any South-Central Asian country. It is the most frequent malignancy in women, where it accounts for 38.5% of all female cancers. About half (43.7%) of all breast cancers are found to be in advanced stage on detection (Soliman et al., 2006). The search for anti-cancer agents from plant sources started in the 1950s

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Abbreviations: DMEM, Dulbecco's modified eagle medium; DMSO, dimethyl sulfoxide; FBS, foetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

and resulted in the discovery and development of the vinca alkaloids, vincristine, and the isolation of the cytotoxic podophyllotoxins (Reddy, 2003; Pezzuto, 1997). More than 60% of currently used anticancer agents are derived in one way or another from natural sources (Cragg and Newman, 2000; Balunas and Kinghorn, 2005).

Biological active components from plants are significant and important source of new drugs that are likely to lead to new and better treatments for breast cancer. As chemotherapy destroys the normal cells along with cancer cells, sometimes cancer cells can develop resistance to treatment through mutations (Wiseman and Spencer, 1998). Therefore, screening for the abundance of biodiverse components is important for research before the forests are lost to deforestation. Overall, this research may lead to new breast cancer chemotherapeutic agents with novel structures and/or mechanisms of action.

Debregeasia salicifolia is a small tree found in Asia and Africa. In Pakistan, it is found in Swat, salt range and Murree hills, usually near springs and water courses (Ali and Nasir, 1972). Aerial parts of plant are used to make a paste that is mixed with mustard oil and used as antifungal for curing skin rashes, dermatitis and eczema. It is also used as hedge plant. *D. salicifolia* has strong antibacterial activity. Its activity was reported along with the isolation and structure elucidation of several compounds (Akbar and Malik, 2001). A new triterpene, 3 β -19 α -dihydroxy-30-norurs-12-ene was isolated from the methanol extract of the plant. Other isolated compounds include lupeol, β -sitosterol, stigmasterol, oleanolic acid, uvaol, ursolic acid, pomolic acid, pomolic acid methyl ester and tormentic acid (Akbar and Malik, 2002).

As a result of its effective ethnobotanical use, this study was undertaken to investigate the antiproliferative activity of the plant against MCF-7 cell line by using different concentrations of crude extract and fractions. Beside this, its phytochemical properties were also investigated.

MATERIALS AND METHODS

Collection of plant material

The fresh plant material was collected from Peer Sohava Islamabad Pakistan and the plant sample was identified by a taxonomist.

Preparation of extract

The fresh plant material was harvested, rinsed under tap water and air dried under shade for 14 days and grinded. The powder material was soaked in methanol for 14 days at room temperature. The mixture was then filtered using a clean muslin cloth and then, Whatman No1. filter paper. The filtrate was then evaporated to dryness using a rotary evaporator attached to a vacuum pump. Extract was stored at 2 to 8°C till further use.

Fractionation of crude extract

Crude methanolic extract of *D. salicifolia* was suspended in water

and then fractionated with organic solvents in order of increasing polarity to get hexane, chloroform, ethyl acetate, methanol and water soluble fractions (Bibi et al, 2010).

Preparation of different concentrations

Different concentrations of crude extract and fractions were prepared by dissolving the extract in DMSO and then diluting it with RPMI medium under sterile conditions.

Cytotoxicity/anticancer assay

MCF-7 breast cancer cell line was used for the determination of cytotoxic activity. Cells were maintained in DMEM (Dulbeccos modified eagles medium) supplemented with FBS (foetal bovine serum) and penicillin/streptomycin-L-glutamine and cultured in a humidified atmosphere of 5% CO₂ 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 μ l of RPMI 1640 medium. Then various concentrations of the crude extract were added to the cells in 100 μ l medium. Cells were incubated for 24 h with test extract concentrations. Each concentration was tested in triplicate.

MTT assay was used to determine cell viability. The MTT assay is a laboratory test which measures changes in colour for measuring the activity of enzyme that reduce MTT to formazan, giving a purple colour. Yellow MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) reduce to purple formazan in living cells (Mosmann, 1983).

After 24 h incubation, 10 μ l MTT reagent was added to each well and incubated for additional 4 h. Then 100 μ l of DMSO solution was added to each wells to solubilize the formazan crystals. The plates were read for optical density at 570 nm, using a plate reader. By using optical density, percentage inhibition of MCF-7 cells was calculated.

Phytochemical screening of the plant material

Phytochemical screening was carried out on the crude extract for the presence of tannins, alkaloids, cardiac glycosides, flavonoids, terpenoids and saponins (Fatima et al., 2009).

Test for alkaloids

Mayer's reagent

Mercuric chloride (0.3555 g) was dissolved in 60 ml of water and 5 g of potassium iodide was dissolved in 20 ml water. Two solutions were mixed and volume was made up to 1000 ml with distilled water.

Dragendorff's reagent

Solution A: Basic Bismuth nitrate (1.7 g) and 20 g of tartaric acid was dissolved in 80 ml of distilled water.

Solution B: Potassium iodide (16 g) was dissolved in 40 ml of distilled water.

Solution A and B were mixed in a ratio of 1:1. Plant extract (0.5 to 0.6 g) was mixed with about 8 ml of 1% HCl, warmed and filtered. 2 ml of filtrate were treated separately with Mayer's reagent and Dragendorff's reagent. Turbidity or precipitation was observed to indicate the presence of alkaloids.

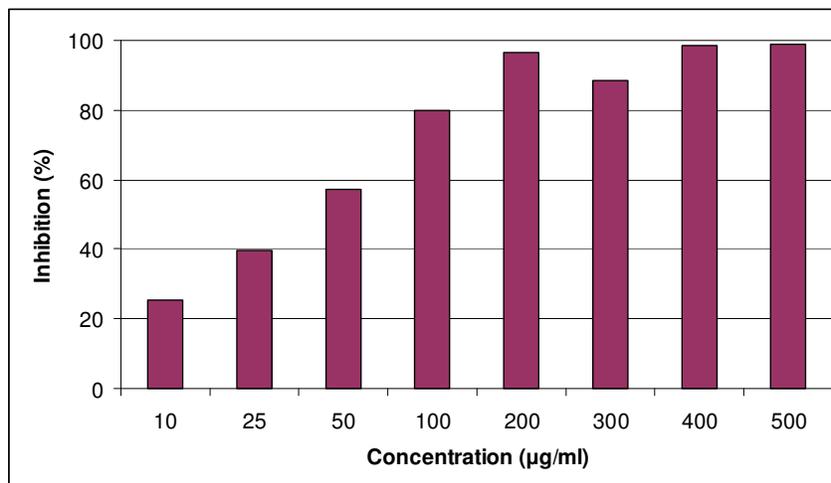


Figure 1. Anticancer activity of *D. salicifolia* crude extract.

Test for anthraquinones

About 1 g of plant extract was boiled with 6 ml of 1% HCl and filtered. The filtrate was shaken with 5 ml of benzene. The benzene layer was removed and then 10% NH_4OH was added. Formation of pink, violet or red colour in alkaline phase was observed for the presence of anthraquinones.

Test for coumarins

Moistened plant extract (0.5 g) was taken in a small test tube and covered with filter paper moistened with 1 N NaOH. The test tube was placed for few minutes in boiling water. Then the filter paper was removed and examined in UV light for yellow fluorescence to indicate the presence of coumarins.

Test for flavonoids

Prepared plant extract (0.5 g) was shaken with pet ether to remove the fatty materials. The defatted residue was dissolved in 20 ml of 80% ethanol and filtered. The filtrate was used for the following test:

- 1) 3 ml of filtrate was mixed with 4 ml of 1% AlCl_3 in MeOH in a test tube. Formation of yellow color was observed to indicate the presence of flavonols, flavones and/or chalcones.
- 2) 3 ml of the filtrate was mixed with 4 ml of 1% KOH. A dark yellow color was observed to indicate the presence of flavonoids.

Test for saponins

Plant extract (0.5 g) was dissolved in boiling water in a test tube, allowed to cool and shaken to mix thoroughly. Froth appears indicating the presence of saponins.

Test for tannins

About 0.5 g of plant extract was boiled in 20 ml of distilled water in a test tube and then filtered. 0.1% FeCl_3 was added to the filtrate. Appearance of brownish green or blue black coloration showed the

presence of tannins.

RESULTS AND DISCUSSION

Cytotoxic activity of plant extracts

The effect of crude methanol extract and fractions of the stem of *D. salicifolia* on the growth of MCF-7 cell line was investigated by the MTT assay. *D. salicifolia* was highly active against MCF-7 cell line. The maximum percentage inhibition value obtained for *D. salicifolia* was 99% at extract concentration $\mu\text{g/ml}$ (Figure 1). The results show dose dependent response. Minimum percentage inhibition was observed at extract concentration of 10 $\mu\text{g/ml}$ that was 37.9%. Results for cytotoxic activity of crude *D. salicifolia* extract are summarized in Table 1.

These results are in accordance with Valko et al. (2006) who investigated the cytotoxicity effects of water extracts from leaves and branches of *Philadelphus coronarius* (Hydrangeaceae), against A431 cells (human skin carcinoma cell line) and the human breast adenocarcinoma cell line with various doses. Highest toxic effects were observed against MCF-7 cell line. A431 were sensitive but their sensitivity was less in comparison with MCF-7 cell line.

Similar results were also reported by Ahmed et al. (2009), who studied effects of different concentrations of *Caralluma tuberculata* crude extract against MCF-7 cell line. Maximum growth inhibition shown by the crude extract was 82% at a concentration of 500 $\mu\text{g/ml}$.

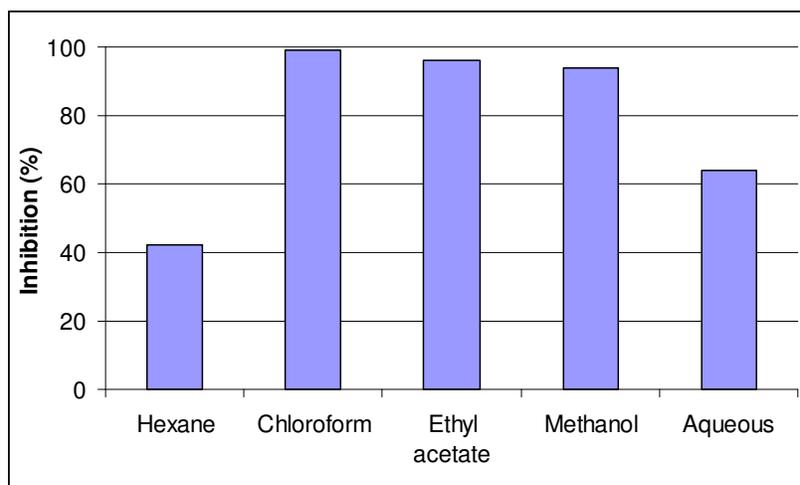
Results obtained from the fractions of *D. salicifolia* extract are presented in the Table 2. The results indicate that chloroform and ethyl acetate fractions are the most potent anticancer fractions with 99.9 and 98.9% inhibition (Figure 2).

Table 1. Effects of different concentrations of crude *D. salicifolia* extract against MCF-7 cell line.

Plant extract concentration ($\mu\text{g/ml}$)	Average absorbance	Actual absorbance	Percentage inhibition (%)
10	0.201 \pm 0.006	0.1286	25.31
25	0.1762 \pm 0.007	0.1038	39.72
50	0.146 \pm 0.003	0.0736	57
100	0.106 \pm 0.007	0.0336	80
200	0.078 \pm 0.004	0.0056	96.7
300	0.0923 \pm 0.003	0.0199	88.44
400	0.0748 \pm 0.003	0.0024	98.6
500	0.073 \pm 0.02	0.0015	99

Table 2. Effect of different fractions of *D. salicifolia* crude extract against MCF-7 cell line.

Fractions	Average absorbance	Actual absorbance	Percentage inhibition
Hexane	0.0986 \pm 0.018	0.0137	90.3
Chloroform	0.085 \pm 0.003	0.0001	99.9
Eth. Acetate	0.0864 \pm 0.004	0.0015	98.9
Methanol	0.2017 \pm 0.025	0.1168	17.33
Aqueous	0.1093 \pm 0.007	0.1089	59.02

**Figure 2.** Anticancer activity of feactions of *D. salicifolia* crude extract fractions.

An ursine type nortriterpene has already been isolated from the chloroform fraction of the *D. salicifolia* stem. This compound was active as an antibacterial agent (Akbar et al., 2001).

Phytochemical screening

Preliminary phytochemical screening of the crude extract of *D. salicifolia* revealed the presence of different phytochemical classes as shown in the Table 3. It contains

flavonoids, anthraquinones and tannins. These compounds have potentially significant applications against human pathogens (El-Mahmood et al., 2008). The presence of glycoside moieties like saponins, cardiac glycosides, anthraquinones and flavonoids are of pharmacognostic importance. These compounds are known to inhibit tumor growth and also serve to protect against gastrointestinal infections. Presence of these components is associated with the utilization of plant in ethnomedicine (El-Mahmood, 2009). Herbs that have tannins as their component are astringent in nature and

Table 3. Phytochemical constituents of *D. salicifolia*.

Phytochemical test	Results		
	Crude extract	Chloroform fraction	Ethyl acetate fraction
Alkaloids	-	-	-
Tannins	+	-	+
Saponins	-	-	-
Coumarins	-	-	-
Flavonoids	+	+	-
Antraquinines	+	-	-

+Yes and -No.

are used for treating intestinal disorders such as diarrhea and dysentery, thus exhibiting antibacterial activity (Akinpelu and Onakoya, 2006). Tannins have wide use in traditional medicine in the treatment of wounds and to stop bleeding (Nguyi, 1988).

Phytochemical analysis for fractions indicated the presence of flavonoids in chloroform fraction and tannins in ethyl acetate fraction. Anthraquinines were absent in all the fractions. The loss of some phytochemical component may occur due to fractionation. These results are in accordance with Babayi et al. (2004) who reported the absence of saponin steroids, saponin glycosided and phenols in the fractions of *Terminalia catappa* although they were originally present in crude extract.

Several plant species rich in flavonoids are reported to reduce disease risk and have therapeutic properties. This observation is of particular importance since flavonoids are ingredients of many vegetables and fruits. Their consumption can reduce the cancer risk (Ferguson et al., 2004; Kanadaswami et al., 2005). Antiproliferative activity recorded in the present study is in accordance with this finding, since the phytochemical evaluation indicated the presence of flavonoids in the crude methanol extract.

Literature data prove that triterpenes and flavonoids are biologically active against different strains of bacteria and many human cancer cell lines (Havsteen, 2002; Mahato et al., 1992; Min et al., 2000). Cytotoxic activity shown by *D. salicifolia* extracts can be attributed mainly to triterpenes and flavonoids. Ursolic acid and oleanolic acid are reported to be present in the plant (Akbar and Malik, 2002). They belong to the pentacyclic triterpenoid acid group. These compounds are also reported to have strong anti-tumor activity. Their activity on HCT15 cells has been studied. The effect of ursolic acid is stronger than that of oleanolic acid. The possible mechanism of action is that both drugs have an inhibitory effect on tumor cell proliferation through cell-cycle arrest (Li et al., 2002). Percentage inhibition shown by hexane fraction is also significant and is 90.3%. The results indicate that some of the active compounds present in *D. salicifolia* stem are of low polarity. Boonsri et al. (2006) also reported isolation of some anthraquinones from the roots

of *Cratoxylum formosum* as antibacterial and cytotoxic agents. These compounds were tested against three human cancer cell lines and found to be active anticancerous agents.

Aqueous fraction showed 59% inhibition, indicating the water solubility of some of the active compounds. As more than one fraction shows the significant activity, it gives a clue about the active compounds present in the *D. salicifolia* extract. Plant extract has more than one active compound with different nature and solubility in different solvents. The methanol fraction was least active with 17.3% of inhibition.

The results obtained from the phytochemical analysis and the cytotoxic activity of this plant revealed that further investigations may lead to the development of potent anticancer agents from *D. salicifolia*.

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