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In- vitro Anti-Cancer Screening of *Solanum indicum*, *Rhus succedanea*, *Rheum emodi* and *Gardenia gummifera* Medicinal Plants in Cancer Cells.

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ABSTRACT

The anticancer activities of *Solanum indicum*, *Rhus succedanea*, *Rheum emodi*, and *Gardenia gummifera* medicinal plants from the methanolic extracts were determined by using *in-vitro* MTT cytotoxicity assay in various cancer cell lines human *non-small cell lung carcinoma* (H1975), prostate carcinoma (PC-3 and DU145), colorectal carcinoma (HCT116) and malignant melanoma (A375). The *in-vitro* cytotoxicity assay revealed that *S. indicum*, *R. succedanea*, *R. emodi* and *G. gummifera* extracts had strongest cytotoxicity with IC₅₀ of 8.48 μ g/ml, 24.5 μ g/ml, 28.24 μ g/ml and 28.61 μ g/ml respectively in DU145 cells and also with IC₅₀ of 11.18 μ g/ml, 11.04 μ g/ml, 11.05 μ g/ml and 23.03 μ g/ml respectively in PC-3 cells. Whereas these extracts exhibited cytotoxicity with IC₅₀ of 9.03 μ g/ml, 7.71 μ g/ml, 15.95 μ g/ml and 11.71 μ g/ml respectively in H1975 cells. Similarly all these extracts showed cytotoxicity with IC₅₀ of 17.58 μ g/ml, 8.87 μ g/ml, 19.31 μ g/ml and 11.67 μ g/ml respectively in HCT116 cells. Finally methanolic extracts of these extracts exhibited cytotoxicity with IC₅₀ 27.94 μ g/ml, 13.13 μ g/ml, 11.05 μ g/ml and 7.816 μ g/ml respectively in A375 cells. Our results revealed that methanolic extract of *S. indicum*, *R. succedanea*, *R. emodi*, and *G. gummifera* had profound cytotoxic effect and further studies to be initiated for potential herbal formulations to be used as an alternative of chemotherapeutic drugs due to less toxic to normal cells.

INTRODUCTION

Cancer is a multi-step disease developed by physical, environmental, metabolic, chemical and genetic factors, which play a direct and/or indirect role in the induction of cancers [1]. About 10 million new cases are diagnosed and over 6 million deaths occur worldwide annually because of cancer [2]. The risk of developing cancer generally increases with age [3]. The chances of surviving the disease vary greatly by the type, location of the cancer and the extent of disease at the start of treatment. Chemotherapy with plant-derived phytochemicals has appeared as an accessible and promising approach to cancer control and management [4].

Several plants have been shown to be sources of therapeutically important agents, beneficial in the cancer therapy. For example, many cancer chemotherapeutic drugs including vinca alkaloids vinblastine and vincristine isolated from Madagascar periwinkle [3, 5], (paclitaxel (Taxol) and docetaxol from *Taxus brevifolia* Nutt [6], podophyllotoxin from roots of podophyllum [7], camptothecin from bark of camptotheca acuminata [8] and many more.

The study shows *Solanum indicum* (Solanaceae) is found throughout south Asia, commonly known as "Begun" in India. The different parts (fruits, leaves, roots) of this plant were traditionally used in the treatment of loss of appetite and anorexia, blood disorders, rhinitis, cough, asthma, sore throat and hiccup, sexual disorders, abdominal pain and worm infestation, pain and fever, inflammation, insomnia, urinary complications, cardiac weakness etc. The fruits, leaves and root of this herb contain wax, fatty acid, alkaloids etc. Diosgenin, lanosterol, sitosterol, solasonnine, solamargine, solanine, solanidine and solasidine already isolated from this plant [9]. Also ethanobotanical study reveals its efficacy against hypersensitivity, diabetes and jaundice [10, 11, 12]. *Rhus succedanea* L. (Anacardiaceae) also found throughout Asia. In Indian ethno medicine, this plant is locally known as Kakrasingi and has been used as ayurvedic remedy for diarrhoea and dysentery [13]. It has been reported to possess antiviral [14], antibacterial [15] and cytotoxic properties [16]. *Rheum emodi* is commonly known as Himalayan rhubarb, is a medicinal herb used in the Indian Ayurvedic system of medicine usually found in India. Anthraquinones are the major constituents of *R. emodi* rhizomes. It is used to treat kidney stones and other liver associated disease like gout and jaundice [17, 18]. It has been proved that the extracts of *R. emodi* possess an antimicrobial activity against *Helicobacter pylori* [19]. *Gardenia gummifera* belongs to family Rubiaceae is a flowering shrub known by different names in India. The gum resin of this plant is called Dikamali [20]. This gum possesses number of medicinal properties which include anthelmintic antispasmodic, anti-epilepticcardiotonic and antioxidant potential [21, 22]. Cycloartanes isolated from the *Gardenia gummifera* is found to possess cytotoxic and anti-HIV activities [23, 24, 25, 26, 27].

A survey of the literature revealed that no studies on the anticancer potential of extracts from these plants have been undertaken on five human cancer cell lines associated with four different cancer: human non small cell lung cancer (H1975), prostate cancer cell line (PC-3 and DU-145), colorectal cancer cell line (HCT116) and skin malignant melanoma (A375). It is found that the same anticancer compound shows variation in the effects for different cell lines. Thus, the use of different cell lines becomes more valuable for the evaluation of anticancer effects.

In the present study we undertook the task to investigate the anticancer activity of crude methanolic extracts of *Solanum indicum* (Fruit), *Rhus succedanea* (Stem), *Rheum emodi* (resin), and *Gardenia gummifera* (resin/stick) in five human cancer cell lines of different histological origin. Interestingly all the extracts showed dose dependent manner in anticancer cells and hemolytic assay revealed that these extract had no effect on erythrocytes. The findings of the study will be significance for the development of new anticancer therapeutic agents.

MATERIALS AND METHODS

Plant materials collection

Plants materials of *Solanum indicum* (Fruit), *Rhus succedanea* (Stem), *Rheum emodi* (Resin) and *Gardenia gummifera* (Resin/sticks) were collected from the Amruta Kesari Depot, Bangalore.

Extraction of plant materials

The air-dried and powdered plant materials (20g of each) were extracted with 250 ml methanol (CH₃OH) by Soxhlet apparatus 60 °C, 8hrs [28, 29]. The crude extracts were filtered and the filtrate evaporated using a rotary evaporator. The dissolved constituents were further dried under pressurized vacuum conditions. Stock solutions were prepared by dissolving the dried residue in Dimethylsulphoxide (DMSO). Extract solutions were stored at -20 °C until use.

Cell Culture

In this study we have used six different cancer cell lines were derived from human *non-small cell lung carcinoma* (H1975), prostate carcinoma (PC-3 and DU145), *colorectal carcinoma* (HCT116) and malignant melanoma (A375) were obtained from the American Tissue Culture Collection (ATCC). H1975, PC-3, DU145 and HCT116 cells were grown in Roswell Park Memorial Institute medium (RPMI-1640) and A375 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were incubated at 37 °C in a 5% CO₂ humidified incubator.

Cytotoxic activity (MTT assay)

The cytotoxic assay detects the reduction of MTT [3-(4, 5-dimethylthiazolyl)-2, 5-diphenyl-tetrazolium bromide] by mitochondrial dehydrogenase to blue insoluble formazan product, which reflects the normal functioning of mitochondria and hence the cell viability. Briefly 5.0×10^4 cells of DU145, PC-3, H1975, HCT116 and A375 were plated in triplicate in 96 well plates with RPMI-1640 or DMEM and incubated for 24 hrs at 37°C. Plant extracts were tested as 0, 2, 4, 8, 16, 32, 64 and 128 µg/ml in serum free RPMI media and incubated for 24 hr in CO₂ incubator at 37 °C. After incubation with plant extracts, the media was removed from the wells and added 100 µl/well of the MTT reagent and incubated for 3-4 hrs. After incubation, the MTT reagent was removed before adding 100 µl DMSO to each well and gently shaken. Plant extracts treated cells were compared to untreated cell control wells. Measure the absorbance at 590nm using a Tecanmicroplate reader. The percentage inhibition was determined using the formula.

$$\% \text{ Inhibition} = 100 - (\text{optical density of sample} / \text{optical density of control}) \times 100.$$

IC₅₀ values were calculated as the concentrations that show 50% inhibition of proliferation on any tested cell^[30, 31, 32, 33].

Isolation of human erythrocytes

Five ml of blood was collected from healthy volunteers in the tubes containing 5.4 mg of EDTA to prevent coagulation and centrifuged at 1000 rpm for 10 min at 4°C. Plasma was removed carefully and the white buffy layer was completely removed by aspiration with a pipette with utmost care. The erythrocytes were then washed for additional three times with 1X PBS, pH 7.4. Washed erythrocytes were stored at 4°C and used within 6 h for the haemolysis assay^[34, 35, 36].

Hemolysis Assay

Haemolytic activity is a method to study the interactions between blood and biomaterials may induce erythrocytelysis. In this study we have explained briefly take 50µl of 10 dilution (100µl Erythrocytes suspension: 900µl 1X PBS) of erythrocytes suspension into 2ml of new eppendorf tube and incubated with 100µl of various concentration of plant extracts (0, 2, 4, 8, 16, 32, 64 and 128 µg/ml) at 37°C water bath for 60-90 min. Here we were using 100µl of 1X PBS as negative control and 100µl of 1% SDS as positive controls and then adjusted the volume of reaction mixture to 1ml by adding 850µl of 1X PBS. Finally centrifuged at 300 rpm for 3-5 min and the resulting hemoglobin insupernatant were measured at 540nm by Tecan micro plate reader. Data Analysis Software to determine the concentration of haemoglobin^[34, 35]. The haemolysis caused by 100 µl of 1% SDS was taken as 100% haemolysis; and the percentage of haemolysis was calculated by the equation

$$\% \text{ Hemolysis} = \frac{(\text{Control} - \text{Sample})}{(\text{Control})} \times 100$$

Statistical analysis

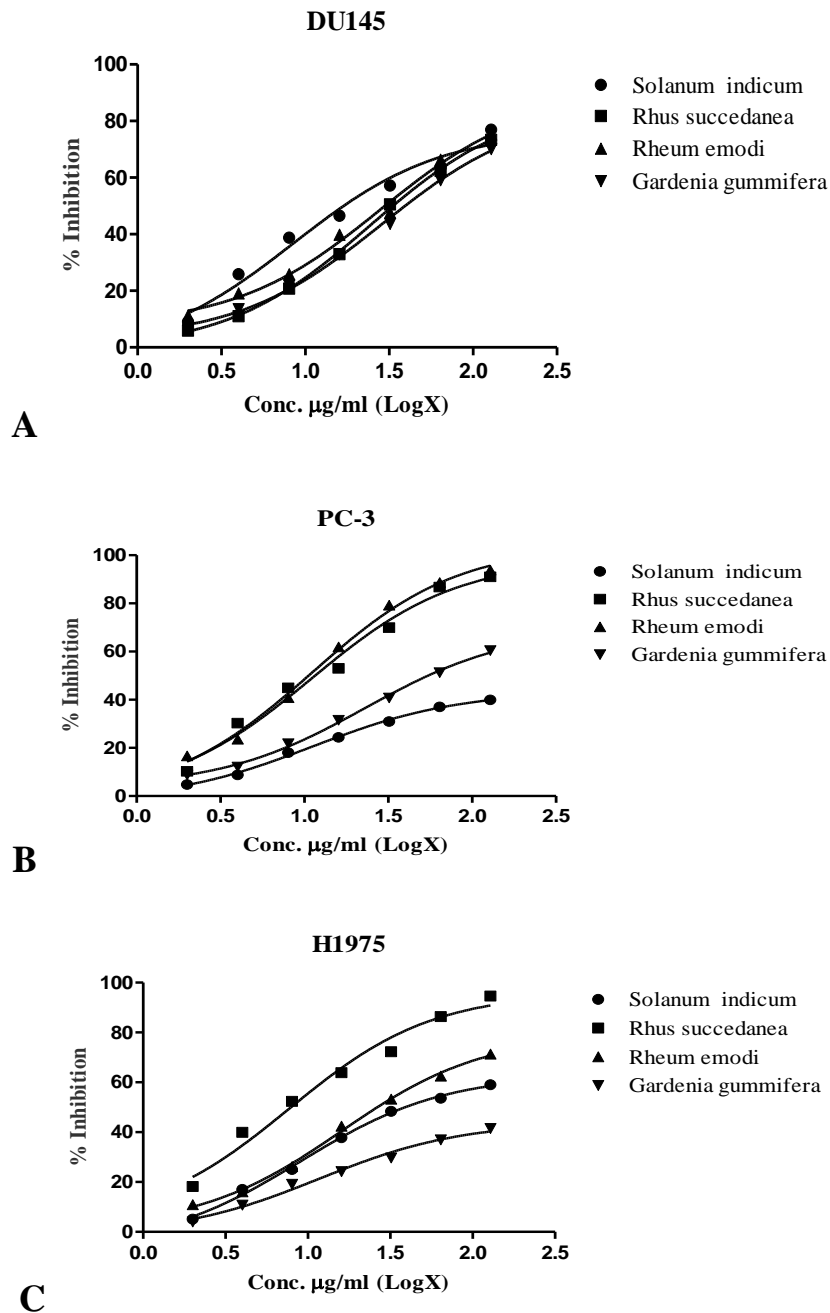
All the assays were performed in triplicates of two to three independent experiments. IC₅₀ values for cytotoxicity tests were derived from a nonlinear regression analysis (curvefit) based on sigmoid dose response curve (variable) and computed using Graph Pad Prism 5 (Graphpad, San Diego, CA, USA)^[37, 38].

RESULTS

Cytotoxic assay

To verify the possible anti-proliferative effect of above plant extracts as a first step toward the development of novel putative anticancer agents, we tested methanol extract of *S.indicum* (Fruit), *R.succedanea* (Stem), *R.emodi* (Resin) and *G.gummifera* (Resin/Stem) on inhibition and proliferation for check their capability to inhibit cell growth or viability on of DU145, PC-3, H1975, HCT116 and A375 cancer cell lines at concentration of 2-128 µg/ml. Proliferation of these cells was significantly inhibited by above extracts in a concentration-dependent manner for 24h, as shown in Figure 1. All the four extracts inhibited 70-75 % growth of DU145 (A) cells and the IC₅₀ of *S.indicum* (8.482 µg/ml), *R.succedanea* (24.5 µg/ml), *R.emodi* (28.24 µg/ml) and *G.gummifera* (28.61 µg/ml). Whereas the *R. succedanea* and *R.emodi*

inhibit 80-90% growth of PC-3 (B) cells and the IC₅₀ of *R succedanea* (11.04 μ g/ml) and *R emodi* (11.05 μ g/ml). But *S. indicum* and *G. gummifera* inhibit 40-50% growth of PC-3 cells and the IC₅₀ of *S.indicum* (11.18 μ g/ml) and *G.gummifera* (23.03 μ g/ml) respectively. In H1975 (C) cells, *S.indicum* inhibit 80-90 % whereas *R.succedanea*, *R.emodi* and *G.gummifera* inhibit 40-65 % of growth and the IC₅₀ of *S.indicum* (9.03μg/ml), *R.succedanea* (7.71μg/ml), *R.emodi* (15.95μg/ml) and *G.gummifera* (11.71μg/ml). About 80-90 % of growth was inhibited in HCT116 (D) cells by all these extracts and it's IC₅₀ of *S.indicum* (17.58μg/ml), *R.succedanea* (8.87μg/ml), *R.emodi* (19.31μg/ml) and *G. gummifera* (11.67μg/ml). Finally *S.indicum* inhibit 75-80 % and remaining extracts inhibit 90-95 % growth of A375 (E) cells and it's IC₅₀ of *S.indicum* (27.94μg/ml), *R.succedanea* (13.13μg/ml), *R.emodi* (11.05μg/ml) and *G.gummifera* (7.81μg/ml). Therefore, our results concluded that methanolic extracts of these plants showed strong and broad-spectrum anticancer activity.



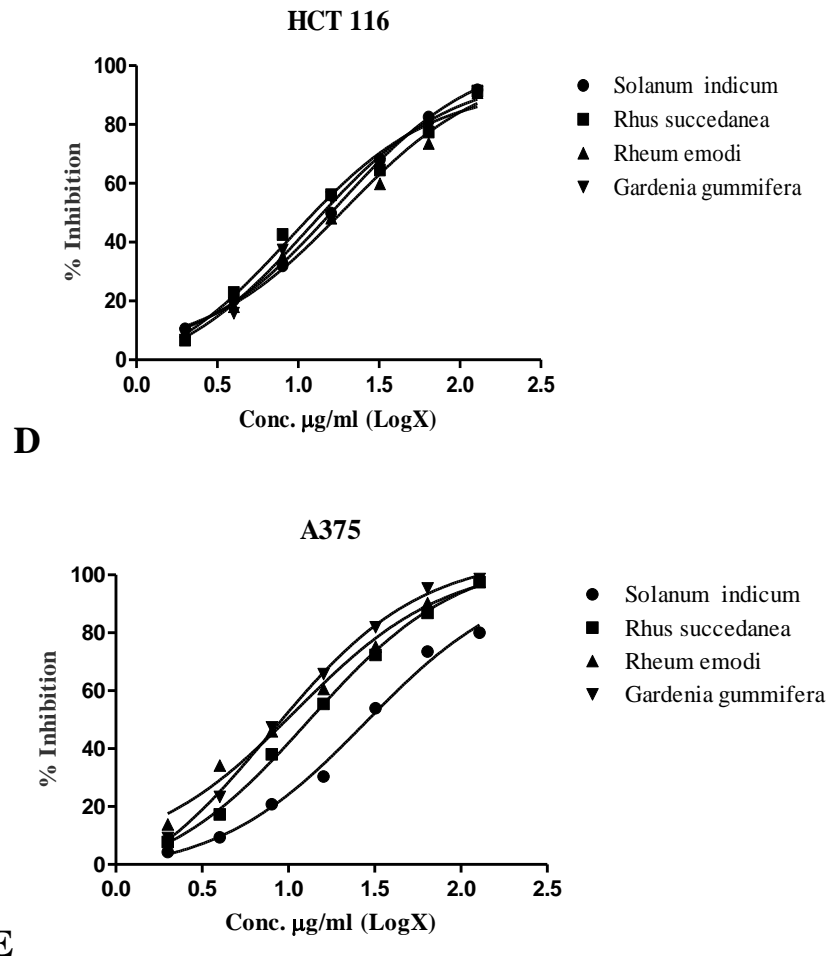


Figure 1 Cytotoxic effect of *Solanum indicum*, *Rhus succedanea*, *Rheum emodi* and *Gardenia gummifera* in prostate cancer cell lines [DU145 (A) and PC-3 (B)], Non-small lung cancer cell line [H1975 (C)], Colorectal cancer cell line [HCT116 (D)] and Skin melanoma cell line [A375 (E)].

Cells were treated with various concentrations (0, 2, 4, 8, 16, 32, 64 and 128 $\mu\text{g/ml}$) of above mentioned plant extracts for 24 hrs grown in a serum free media. The percentage of cell death induced was determined using the MTT assay and each data represents the mean of three independent experiments.

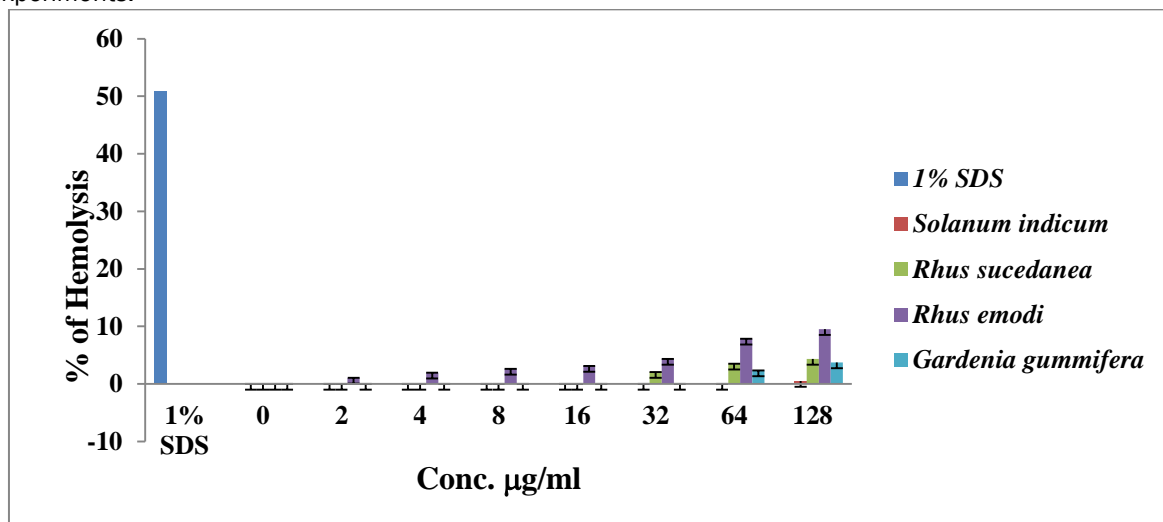


Figure 2 Effect of *Solanum indicum*, *Rhus succedanea*, *Rheum emodi* and *Gardenia gummifera* extracts on hemolysis of human erythrocytes.

Human erythrocytes were incubated with various concentrations (0, 2, 4, 8, 16, 32, 64 and 128 µg/ml) of above mentioned plant extracts at 37°C water bath for 60 min. The absorbance of the resulting supernatant was measured at 540 nm by spectrophotometer (JENWAY 6305 UV/Vis.) to determine the extent of hemolysis. The percentage of haemolysis was calculated by the equation % Hemolysis = $[(A_{Control} - A_{Sample}) / A_{Control}] \times 100$. All analyses are the mean of two independent experiments.

Table 1: IC₅₀ of *S.indicum*, *R. succedanea*, *R.emodi* and *G.gummifera* extracts on DU145, PC-3, H1975, HCT116 and A375 cancer cell lines (expressed in µg/ml).

Sl.No	Name of plants	Parts of plants	DU145	PC-3	H1975	HCT116	A375
1	<i>Solanumindicum</i>	Fruit	8.482	11.18	9.031	17.58	27.94
2	<i>Rhus succedanea</i>	Stem	24.5	11.04	7.712	8.876	13.13
3	<i>Rheum emodi</i>	Resin	28.24	11.05	15.95	19.31	11.05
4	<i>Gardenia gummifera</i>	Resin/Sticks	28.61	23.03	11.71	11.67	7.816

Hemolysis assay

Haemolytic assay method is suited to evaluate the haemocompatibility of biomaterials and medical devices according to the international standard ISO 10993-4:2002. As these methanolic extracts of *S indicum*, *R succedanea*, *R emodi* and *G gummifera* showed anticancer activity in above mentioned human cancer cells, here we have tested 0-128 µg/ml of *S. indicum*, *R.succedanea*, *R. emodi* and *G. gummifera* extracts effect on human erythrocytes as shown in figure 2. None of the concentrations of the *S.indicum* extracts showed any visible hemolysis activity; *R.emodi* extracts showed 2-9.5% hemolysis in a concentration dependent manner but *R. succedanea* and *G. gummifera* extracts showed very minimum level of (< 4%) hemolysis at the concentration of 64-128 µg/ml.

DISCUSSION

This study evaluated the cytotoxic activities of methanol extracts of *S. indicum*, *R.succedanea*, *R. emodi* and *G. gummifera* in DU145, PC-3, H1975, HCT116 and A375 cancer cell lines and with its IC₅₀ shown in the table 1. The different parts (fruits, leaves, roots) of *S. indicum* were traditionally used in the treatment of various disorders as mentioned in the above section. In our study *S. indicum* extract inhibited the growth of DU145, PC-3, H1975, HCT116 and A375 cancer cells and with the IC₅₀ concentrations of 8.48, 11.18, 9.03, 17.58 and 27.94 µg/ml respectively (Fig 1 and Table 1). Bioactive component of Indiosides isolated from *S. indicum* have dose-dependent inhibitory effect on proliferation of Bel-7402 cells, and can induce cell apoptosis through mitochondria-dependent pathway^[39]. Solavetivone 1, a component of *S. indicum* exhibited cytotoxic to OVCAR-3 cells^[40]. Bioactives of beta-Sitosterol (SI-0), beta-sitosterol glucoside (SI-1), dioscin (SI-2), methyl protoprosapogenin A of dioscin (SI-3), methyl protodioscin (SI-4) and protodioscin (SI-5) were isolated from the whole plant of *Solanum indicum* L and showed cytotoxicity on seven cancer cell lines. The ans SI-3, 4 and 5 showed a tumor inhibitory effect in vivo in C6 glioma cells and in addition SI-2 had an inhibitory effect on the DNA synthesis of C6 glioma cells at 10 micrograms/ml^[41].

R. succedanea L, plant is locally known as Kakrasingi and in Indian ethno medicine it has been used as ayurvedic remedy for diarrhoea and dysentery^[13]. It also has been reported to possess antiviral^[14], antibacterial^[15], and cytotoxic properties^[16]. Here *R. succedanea* extract exhibited the growth of DU145, PC-3, H1975, HCT116 and A375 cancer cells and with the IC₅₀ concentrations of 24.5, 11.04, 7.71, 8.87 and 13.13 µg/ml respectively (Fig 1 and Table 1). Two new antioxidative and cytotoxic compounds, 10'(Z), 13'(E), 15'(E)-heptadecatrienylhydroquinone (1) and 10'(Z), 13'(E)-heptadecatrienylhydroquinone (2), as well as the known 10'(Z)-heptadecenylhydroquinone (3), were isolated from an EtOH extract of the sap of *Rhus succedanea* exhibited antioxidative and cytotoxic activities against five cancer cell lines^[42].

R. emodi extract had cytotoxic effect on the growth of DU145, PC-3, H1975, HCT116 and A375 cancer cells and with the IC₅₀ concentrations of 28.24, 11.05, 15.95, 19.31 and 11.05 µg/ml respectively (Fig 1 and Table 1). Methanolic extract of *R. emodi* showed concentration-dependent cytotoxicity in MDA-MB-435S and Hep3B cell lines due to presence of phenolic compounds such as β-Resorcylic acid, Daidzein-8-O-glucoside (puerarin), Daidzein, Quercetin and Flavonol in the *R. emodi*^[43]. Emodin (1) is the major bioactive compound of several herb species, which belongs to anthraquinone class of compound,

was isolated from the roots of *R emodi* exhibited antiproliferative activities against HepG2 and MDA-MB-231 cells [44].

Finally *G.gummifera* extract also inhibited the growth of DU145, PC-3, H1975, HCT116 and A375 cancer cells and with the IC₅₀ concentrations of 28.61, 23.03, 11.71, 11.67 and 7.816 µg/ml respectively (Fig 1 and Table 1). The gum of *G.gummifera* possesses number of medicinal properties which include anthelmintic, antispasmodic, anti-epileptic, cardiotonic and antioxidant potential mentioned in the above section. Dikamaliartane-A, a Cycloartane isolated from gum resin, *G. gummifera* had good *in vitro* and *in vivo* anti-cancer activity in HeLa (cervical cancer) and MCF-7 (breast cancer) cell lines [45].

Overall our results suggested that the presence of bioactive constituents in the methanolic extracts of *S. indicum*, *R.succedanea*, *R. emodi* and *G. gummifera* significantly inhibit the growth of prostate, lung, colorectal and skin carcinoma and induce cell apoptosis through mitochondria-dependent pathway. However we can conclude that all these extracts not only inhibit specific type of cancer cell growth but also inhibit the most type of cancer cell growth.

The hemolysis assay determine whether the methanolic extracts of *S. indicum*, *R.succedanea*, *R. emodi* and *G. gummifera* crosses the red blood cell (RBC) membrane, interacting with hemoglobin and initiates a series of reactions, resulting in RBC lysis (haemolysis) [34]. Except *S. indicum*, remaining extracts exhibited concentration dependent hemolysis activity shown in the figure 2. Most characteristic property of bound phenolic compounds, saponins and other bioactive constituent are their ability to cause haemolysis and since there are presence in these extracts showed haemolysis effect.

According to the American National Cancer Institute, the IC₅₀ value to consider a crude extract promising for development of anti-cancer drug(s) is lower than a limit threshold of 30 µg/ml [43]. In our study all *S. indicum*, *R.succedanea*, *R. emodi* and *G. gummifera* extracts showed significant IC₅₀ value less than the 28 µg/ml in above different types of cancer cell growth. All these extracts can thus serve as potential source for anti-cancer compounds.

CONCLUSION

In conclusion, the results obtained indicated that the methanolic extract of *S. indicum*, *R.succedanea*, *R. emodi* and *G. gummifera* not only inhibit specific type of cancer cell growth but also inhibit the most type of cancer cell growth. Medicinal plants are natural products and may have therapeutic potentials; however, being natural does not make them automatically safe. Further work is progress in our laboratory to evaluate their formulation effective both *In-vitro* and *In-vivo* anticancer potential and in-depth mechanistic aspects.

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