

Evaluation of anticancer activity of leaves of *Rumex vesicarius* Linn and *Symplocos racemosa* Roxb. by brine shrimp lethality and (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) methods

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Abstract

Introduction: The objective of the present study was to evaluate the anticancer activity of leaves of *Rumex vesicarius* Linn and *Symplocos racemosa* Roxb. **Materials and Methods:** In this study, cytotoxicity was assayed by brine shrimp lethality bioassay (BSL) and (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. **Result:** Different extracts of leaves of plant *R. vesicarius* Linn. and *S. racemosa* Roxb were found to be cytotoxic to HT-29 and PC-3 cell lines. Different extracts of leaves of plant *R. vesicarius* Linn. and *S. racemosa* Roxb showed significant ($P < 0.0001$) cytotoxic effect on HT-29 and PC-3 cell lines in a dose-dependent manner. It also showed significant cytotoxic effect in BSL bioassay. **Conclusion:** The study demonstrated that all the extracts of *R. vesicarius* Linn and *S. racemosa* Roxb show a significant cytotoxic effect on HT-29 and PC-3 cell lines as well as on BSL bioassay in a dose-dependent manner.

Key words: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, brine shrimp lethality bioassay, *Rumex vesicarius* Linn, *Symplocos racemosa* Roxb

INTRODUCTION

Cancer is considered one of the most common causes of morbidity and mortality worldwide. The target of much research has been on the discovery of natural and synthetic compounds that can be used in the prevention and/or treatment of cancer. The natural product of either plant or animal origin that exhibited antitumor activity has been discovered.^[1]

Plants have been a prime source of highly effective conventional drugs for the treatment of various forms of cancer. *Podophyllum peltatum* and *Podophyllum hexandrum* are used in the treatment of Hodgkin's disease, non-Hodgkin's lymphoma, leukemia, bronchogenic carcinoma, and cancers of the ovary and the testis.^[2] Ginger consists of a phenolic compound whose rhizome is used traditionally for its cytotoxic activity through apoptosis in cancer cells.^[3] *Curcuma xanthorrhiza* Roxb consists of xanthorrhizol which is a sesquiterpenoid complex derived from rhizome, which inhibits

the formation and development of tumors.^[4] *Curcuma longa* which consists of curcumin inhibits the growth of cancer cells by preventing the production of harmful eicosanoid. It is used to treat squamous cell carcinoma of the skin and the ulcerating oral cancer.^[5,6] *Ocimum sanctum* inhibits the growth of various cancer cells, particularly breast cancer, and minimizes the side effects of chemotherapy.^[7] *Echinacea angustifolia* is used to treat metastatic carcinoma of the esophagus and the colon.^[8] *Aloe vera* contains aloemodin, which activates the macrophages to fight cancer.^[9] *Chlorella pyrenoidosa* contains a very effective detoxifying agent that protects the body from cancer.^[10] *Nigella sativa* kills cancer cells by binding to asialofetuin (lectin) on the

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surface of cancerous cells. It also reduces the side effects of chemotherapy and radiotherapy.^[11] *Combretum cafferum* contains combretastatin which executes its therapeutic action against cancer by inhibiting blood supply to a tumor.^[12] *Panax ginseng* forest herb that has been used inhibits the growth of cancer by interfering with DNA synthesis.^[13,14]

Rumex vesicarius Linn (Chooka) belongs to perennial herbs to the family *Polygonaceae*. The plant is erect usually with a long tap root. Traditionally, the plant is used as stomachic, diuretic, used for the disorders of the lymphatic and glandular systems, for treating bronchitis, asthma, constipation, dyspepsia, and the diseases of the liver. The plant leaves are rich in ascorbic acid, citric acid, and tartaric acid, and they also contain glycoside, alkaloid, flavonoids, tannins, and phenolic compounds.^[15,16]

Symplocos racemosa Roxb. (Lodhra) belongs to the family *Symplocaceae*, which is a small evergreen tree up to 6 m tall. In the traditional system, it is mainly used for its cardiogenic, antipyretic, anthelmintic, and laxative properties. It is beneficial in billow fever and urinary discharge; pharmacologically, it is used as an antimicrobial, antidiarrheal, spasmogenic, and heart depressant. The plant mainly contains monomethyl pelargonidin glucosides and loturidine, and also contains oxalic acid, phytosterol, ellagic acids, and oleanolic acid.^[17-19]

MATERIALS AND METHODS

Plant Material

The fresh leaves of plant *R. vesicarius* Linn and *S. racemosa* Roxb used in this study were collected at the flowering stage (month: August-November) from the local area of Sangli and Satara, Maharashtra state, India, respectively, and authenticated by Botanical Survey of India, Pune, Maharashtra (BSI/WRC/Iden./2015 dated 4-12-2015).

Extraction

The leaves were separated from fresh stems and dried under shade at room temperature until they become completely dry. After drying, the leaves were subjected to size reduction. The shade-dried coarsely powdered leaves (500 g) were subjected to Soxhlet extraction. (a) *R. vesicarius* Linn. leaves (500 g) were subjected to Soxhlet extraction with 95% ethanol and ethyl acetate to obtain an ethanolic extract of *R. vesicarius* (ERV) and ethyl acetate extract of *R. vesicarius* (EARV), respectively. (b) *S. racemosa* Roxb. leaves (500 g) were subjected to Soxhlet extraction with 95% ethanol and N-hexane to obtain an ethanolic extract of *S. racemosa* (ESR) and N-hexane extract of *S. racemosa* (NSR), respectively. The extracts obtained were subjected to a rotary flash evaporator to remove the excess of solvent,

and the dried extract was stored in a cool place in a tight-pack container for further use.

Preliminary Phytochemical Screening

All the extracts were subjected to preliminary phytochemical screening using the method described by Kokate, Trease, and Evans for the detection of various plant constituents. The test was carried out for the presence or absence of phytoconstituents such as glycosides, flavonoids, saponins, alkaloids, carbohydrates, sterols, phenolic compound, and reducing compounds.^[20-22]

Drugs and Chemicals

All the drugs and chemicals were of analytical grade. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Hi-media Lab; Mumbai), DMSO (Research Lab; Mumbai), Crude sea salt, and 70% alcohol (Research Lab; Mumbai) were purchased. Brine Shrimp cell (CIFE Mumbai), 5-fluorouracil (FU) (Research Lab; Mumbai), ethanol (Research Lab; Mumbai), ethyl acetate (Research Lab; Mumbai), N-Hexane (Research Lab; Mumbai), dried yeast (Bijur Sooper Pvt., Ltd), NaCl (Research Lab; Mumbai), and MTT (Sigma) were also purchased.

Cell Line

HT-29 and PC-3 (National Center for Cell Science, Pune) cells were used.

Instruments/Apparatus

The instruments/apparatus used were a balance (Shimadzu ELB 300), CO₂ incubator (NuAir), micro pipette (Biosystem), hatching chamber, 96-well microliter plate, microplate reader, air pump (aquarium SB-108), and sonicator (Oscar sonicator Model no. Ou-9).

Pharmacological Screening

The anticancer activity is carried out using brine shrimp lethality (BSL) assay and MTT assay methods.

BSL Assay

Material

Artemia salina eggs

The brine shrimp (*Artemia salina*) eggs were procured from the Central Institute of Fisher Education, Seven Bungalows, Andheri (W), Mumbai. Eggs were brown-gray and were very small in size. 100 mg of eggs roughly represent 2.5–3.0 thousands of eggs. The eggs were stored in an air-tight opaque container at room temperature. In this ideal condition, they are viable for many years.

Hatching Chamber

Hatching chamber was fabricated as per the design used by Meyer *et al.* (1982). The chamber was made of glass with an aluminum lid on the top. The chamber was divided into two unequal parts with the help of a fiber sheet which is having a number of holes of 2 mm size. One of the compartments was illuminated with a lamp (40 W), while the other was darkened. Both the chambers were aerated.

Method

Sample preparation

Sample of the extract was prepared by dissolving 10 mg of extract in 20 μ l of DMSO and the volume was made up to 10 ml with distilled water, to get 1000 μ g/ml stock solution. From this stock, a different concentration of extract EARV, ERV, ESR and NSR in the range of 20–500 μ g/ml was prepared. Three replicates were prepared for each dose level. Control vials were prepared by adding an equal volume of distilled water. The standard drug was prepared at a concentration of 20 μ g/ml.

Sea Water

Sea water for hatching of brine shrimp was prepared according to the composition given by Dr. Munil Kumar Sukham (Fish Nutrition), Senior Scientist, the Central Institute of Fisher Education, Seven Bungalows, Andheri (W), Mumbai. The crude sea salt (25 g/L) was dissolved in distilled water and dried yeast 6 mg/L was added in this solution for food of brine shrimp. Before using, it was filtered through a paper.

Hatching of Brine Shrimp Eggs

A volume of 5.0 L sea water was added to the special chamber. 0.20 mg of the eggs was washed with water and then these eggs were sprinkled into the compartment which was darkened. Aeration was provided in the compartment. After 48 h, the phototropic nauplii were collected by capillary from the lighted side and used for bioassay.

Bioassay

The bioassay experiment was performed according to the procedure described by Meyer *et al.* (1982).^[23,24] Nauplii were drawn in glass capillary along with water and ten of such shrimps were transferred to each sample vial containing 4.5 ml brine solution (specific volume brine and yeast suspension after they were counted in the stem of capillary against a lighted background). In each experiment, 0.5 ml of the plant extract (EARV, ERV, ESR, and NSR) was added to 4.5 ml of brine solution at various concentrations of 20–500 μ g/ml. In control vial, 4.5 ml of artificial sea water and 0.5 ml of distilled water were added. Standard

drug was added at a concentration of 20 μ g/ml. The vials were maintained under illumination. After 24 h, survivors were counted using $\times 3$ magnifying glass or against lighted background, and the percentage of deaths and LC₅₀ value were calculated by dose response.

MTT Assay

Sample preparation

A sample of the extract was prepared by dissolving 100 mg of extract in 20 μ l of DMSO and the volume was made up to 10 ml with phosphate-buffered saline (PBS), to get 10,000 μ g/ml stock solution. From this, 100 μ l, 1000 μ l, and 5000 μ l were taken and the volume was made up to 10 ml with PBS. The final concentrations are 100 μ g/ml, 1000 μ g/ml, and 5000 μ g/ml. Three replicates were prepared for each dose. Control vial was prepared by adding an equal volume of 0.5% of DMSO with PBS.

Standard Drug Solution Preparation

A standard drug was prepared by dissolving 100 mg of 5-FU in 20 μ l of DMSO and the volume was made up to 10 ml with PBS to get 10,000 μ g/ml. From this, 200 μ l was taken and the volume was made up to 10 ml with PBS. The final concentration is 200 μ g/ml.

Procedure

Cells were preincubated at a concentration of 1.8×10^6 cells/ml in culture medium for 3 h at 37°C and 5% CO₂. Test wells were incubated with 100 μ l cell line (1.8×10^5 cells/ well) and 100 μ l of test extracts (EARV, ERV, ESR, and NSR) of various concentrations (10 μ g/ml, 100 μ g/ml, and 500 μ g/ml) in respective wells. Standard well were incubated with 100 μ l (200 μ g/ml) of 5-FU and 100 μ l cell line (1.8×10^5 cells/ well). Control wells were incubated with DMSO (0.2% in PBS) and cell line. All samples were incubated in triplicate. Cell culture was incubated for 3 h at 37°C and 5% CO₂ in CO₂ incubators. After the incubation, 10 μ l (5 mg/ml in PBS) of the reconstitute MTT mixture was added to each well using a repeating pipette. After addition of MTT, cells were incubated for 4 h at 37°C in a CO₂ incubator. Formazan crystals were dissolved in 150 μ l of DMSO per well. Before reading the plate, it was mixed gently on an orbital shaker for 1 min to ensure homogeneous distribution of color. Triplicate sample was analyzed by measuring the absorbance of each sample by a microplate reader at a wavelength of 540 nm.^[25]

The cell proliferation or percentage cell survival was calculated from their OD and expressed as percentage of control.

% cell survival = (O.D. of drug treated well \times 100)/O.D. of

control well

Statistical Analysis

The result was expressed as mean value \pm standard error of the mean. The variation in a set of data has been estimated by performing one-way analysis of variance. Individual comparisons of group mean value were done using Dunnett's test. $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSIONS

The presence of various phytoconstituents of the extract was detected by phytochemical screening. The EARV was found to contain alkaloids, flavonoids, tannins, sterols, carbohydrate, and Vitamin C. ERV contains alkaloids, flavonoids, carbohydrate, and Vitamin C. ESR was found to contain cardiac glycoside, flavonoids, alkaloids, tannins, and carbohydrate. NSR contains cardiac glycoside, alkaloids, and steroids.

In the present study, both the plant extracts were found with significant cytotoxic effect in BSL bioassay. EARV shows LC_{50} 47.98 μ g/ml while ERV shows LC_{50} 147.05 μ g/ml. The LC_{50} value of ESR and NSR was found to be 103.95 and 241.54 μ g/ml, respectively. The extract which showed the LC_{50} value <250 μ g/ml was considered active and used for further study (Pisutthanan *et al.*, 2004). Mean percentage death after 24 h for a different extract of both plant extracts is given in Table 1.

Cytotoxic activity of leaves of *R. vesicarius* Linn and *S. racemosa* Roxb on HT-29 and PC-3 cell lines was evaluated by BSL bioassay and MTT assay. MTT assay is a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethylthiazole-2-yl)2,5-diphenyltetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters into mitochondria of cell and reduces to an insoluble colored complex (dark purple) formazan. Reduction of MTT occurs in the metabolically active cell. The level of activity is a measure of the viability of the cell. Percentage cell inhibition of *R. vesicarius* Linn and *S. racemosa* Roxb on HT-29 and PC-3 cell lines is shown in Table 2.

Table 1: Result of BSL assay

Extract	Concentration μ g/ml	Mean percentage death after 24 h	LC_{50} μ g/ml
EARV	20	23.33 \pm 3.33	47.98
	40	40 \pm 0	
	60	56.67 \pm 3.33	
	80	93.33 \pm 3.33	
	100	100 \pm 0	
ERV	60	13.33 \pm 3.33	147.05
	120	36.67 \pm 3.33	
	180	63.33 \pm 3.33	
	240	86.67 \pm 3.33	
	300	100 \pm 0	
ESR	40	3.33 \pm 3.33	103.95
	80	26.67 \pm 3.33	
	120	56.67 \pm 3.33	
	160	83.33 \pm 3.33	
	200	100 \pm 0	
NSR	100	23.33 \pm 3.33	241.54
	200	43.33 \pm 3.33	
	300	66.67 \pm 3.33	
	400	83.33 \pm 3.33	
	500	100 \pm 0	
Std (5-FU)	20	100 \pm 0	-

LC_{50} is lethal concentration 50%, EARV is ethyl acetate extract of *Rumex vesicarius*, ERV is ethanolic extract of *R. vesicarius*, ESR is ethanolic extract of *Symplocos racemosa*, NSR is N-hexane extract of *S. racemosa*, 5-FU is 5-fluorouracil. Values are expressed as (mean \pm SEM). $n=3$ **** $P < 0.0001$ statistically significant when compared with control group by ANOVA followed by Dunnett's test. ANOVA: Analysis of variance, SEM: Standard error of the mean, *R. vesicarius*: *Rumex vesicarius*, *S. racemosa*: *Symplocos racemosa*

Cancer is a growing public health problem whose estimated worldwide new incidence is about 6 million cases per year. A large number of plant, marine, and microbial sources have been tested as leads and many compounds survived the potential leads. Hence, the present study was designed to explore the possible anticancer activity of a different extract of *R. vesicarius* Linn and *S. racemosa* Roxb. In general, anticancer screening involves the use of expensive and sophisticated techniques, namely using human cell lines and grafting. However, rapid, inexpensive, and specific anticancer screening methods are today's need to speed up the cytotoxicity screening. Previous studies have shown that

compounds showing cytotoxicity in brine shrimp bioassay would act as effective anticancer agent. It is found that EARV Linn shows the LC_{50} 47.98 μ g/ml by BSL assay method. Moreover, all the other extracts of both plants also show the LC_{50} <300 μ g/ml. Hence, both the plants have potential to act as an anticancer agent [Figures 1-4].

MTT assay is such a technique, which can play a significant role in preclinical cytotoxicity screening. The MTT assay assesses the functional intactness of mitochondria based on the enzymatic reduction of tetrazolium salt and provides information on cellular metabolism and about cell viability as

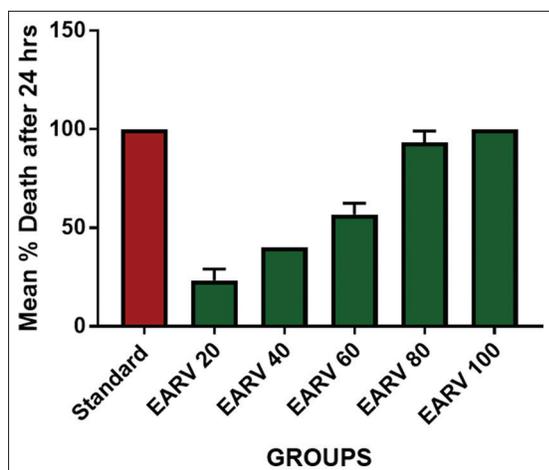


Figure 1: Graphical representation of mean percentage death of brine shrimp after 24 h by ethyl acetate extract of *Rumex vesicarius* (EARV). Mean percentage death of brine shrimp after 24 h by EARV at 20, 40, 60, 80, and 100 μ g/ml and standard

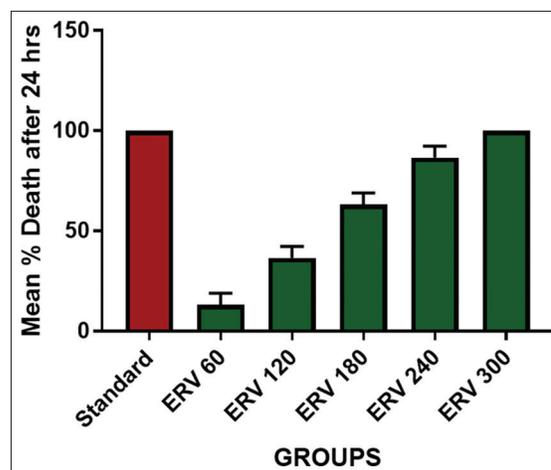


Figure 2: Graphical representation of mean percentage death of brine shrimp after 24 h by ethanolic extract of *Rumex vesicarius* (ERV). Mean percentage death of brine shrimp after 24 h by ERV at 60, 120, 180, 240, and 300 μ g/ml and standard

Table 2: Result of MTT assay

Sample	Concentration (μ g/ml)	Percentage cell viability (HT-29 cell)	Percentage cell viability (PC-3 cell)	IC ₅₀ μ g/ml (HT-29 cell)	IC ₅₀ μ g/ml (PC-3 cell)
Control	-	96.08 \pm 0.3406	94.4 \pm 0.3727	-	-
Std (5-FU)	20	24.24 \pm 0.7791	26.21 \pm 0.6524	-	-
EARV	10	65.25 \pm 0.6191	67.92 \pm 0.4550	54.81 \pm 0.8405	70.90 \pm 1.3080
	100	43.49 \pm 0.6641	45.41 \pm 0.4234		
	500	31.59 \pm 0.3884	33.29 \pm 0.2333		
ESR	10	71.65 \pm 0.3826	69.94 \pm 0.4452	118.66 \pm 7.7153	94.63 \pm 0.8503
	100	51.28 \pm 0.5499	48.99 \pm 0.2938		
	500	37.44 \pm 0.6882	35.65 \pm 0.1495		
ERV	10	73.66 \pm 0.2714	72.69 \pm 0.2621	171.55 \pm 6.899	211.65 \pm 14.4865
	100	55.99 \pm 0.4792	56.96 \pm 0.5424		
	500	39.97 \pm 0.3118	42.60 \pm 0.6372		
NSR	10	78.76 \pm 0.4271	81.00 \pm 0.6302	322.76 \pm 13.015	343.42 \pm 14.9188
	100	60.81 \pm 0.2406	62.85 \pm 0.3435		
	500	45.71 \pm 0.4478	45.58 \pm 0.5260		

IC₅₀ is inhibitory concentration 50%. Values are expressed as mean \pm SEM. $n=3$ **** $P<0.0001$ statistically significant when compared with control group by ANOVA followed by Dunnett's test. ANOVA: Analysis of variance, SEM: Standard error of the mean, MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, EARV: Ethyl acetate extract of *Rumex vesicarius*, ESR: Ethanolic extract of *Symplocos racemosa*, ERV: Ethanolic extract of *Rumex vesicarius*, NSR: N-hexane extract of *S. racemosa*, 5-FU: 5-fluorouracil

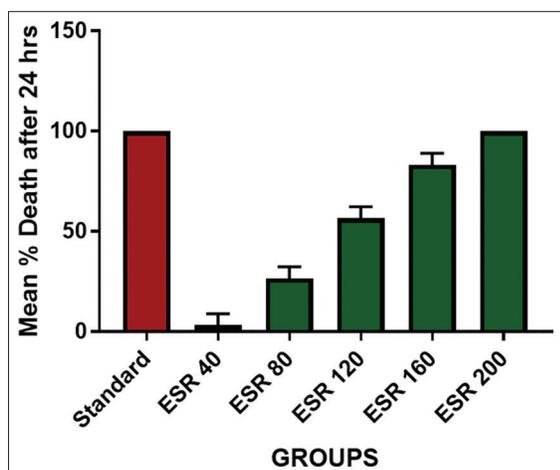


Figure 3: Graphical representation of mean percentage death of brine shrimp after 24 h by ethanolic extract of *Symplocos racemosa* (ESR). Mean percentage death of brine shrimp after 24 h by ESR at 40, 80, 120, 160, and 200 µg/ml and standard

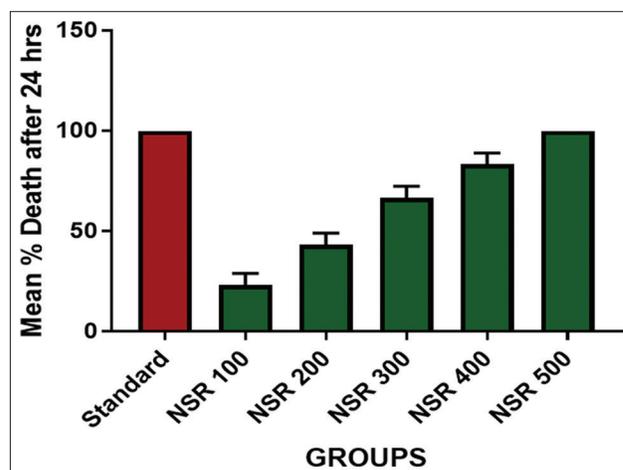


Figure 4: Graphical representation of mean percentage death of brine shrimp after 24 h by N-hexane extract of *Symplocos racemosa* (NSR). Mean percentage death of brine shrimp after 24 h by NSR at 100, 200, 300, 400, and 500 µg/ml and standard

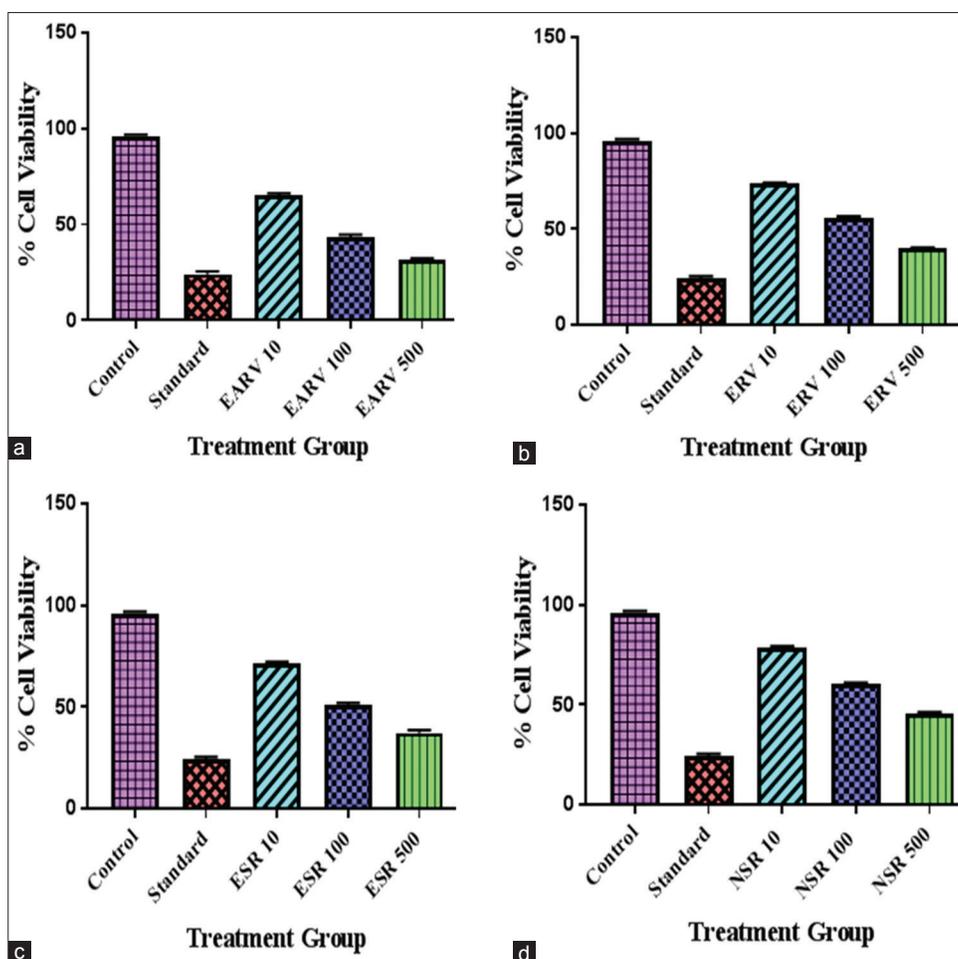


Figure 5: Graphical representation of percentage cell viability of HT-29 cell line (a) Percentage cell viability of HT-29 cell line by ethyl acetate extract of *Rumex vesicarius* at 10, 100, and 500 µg/ml, standard and control (b) Percentage cell viability of HT-29 cell line by ethanolic extract of *R. vesicarius* at 10, 100, and 500 µg/ml, standard and control (c) Percentage cell viability of HT-29 cell line by ethanolic extract of *Symplocos racemosa* at 10, 100, and 500 µg/ml, standard and control (d) Percentage cell viability of HT-29 cell line by N-hexane extract of *S. racemosa* at 10, 100, and 500 µg/ml, standard and control

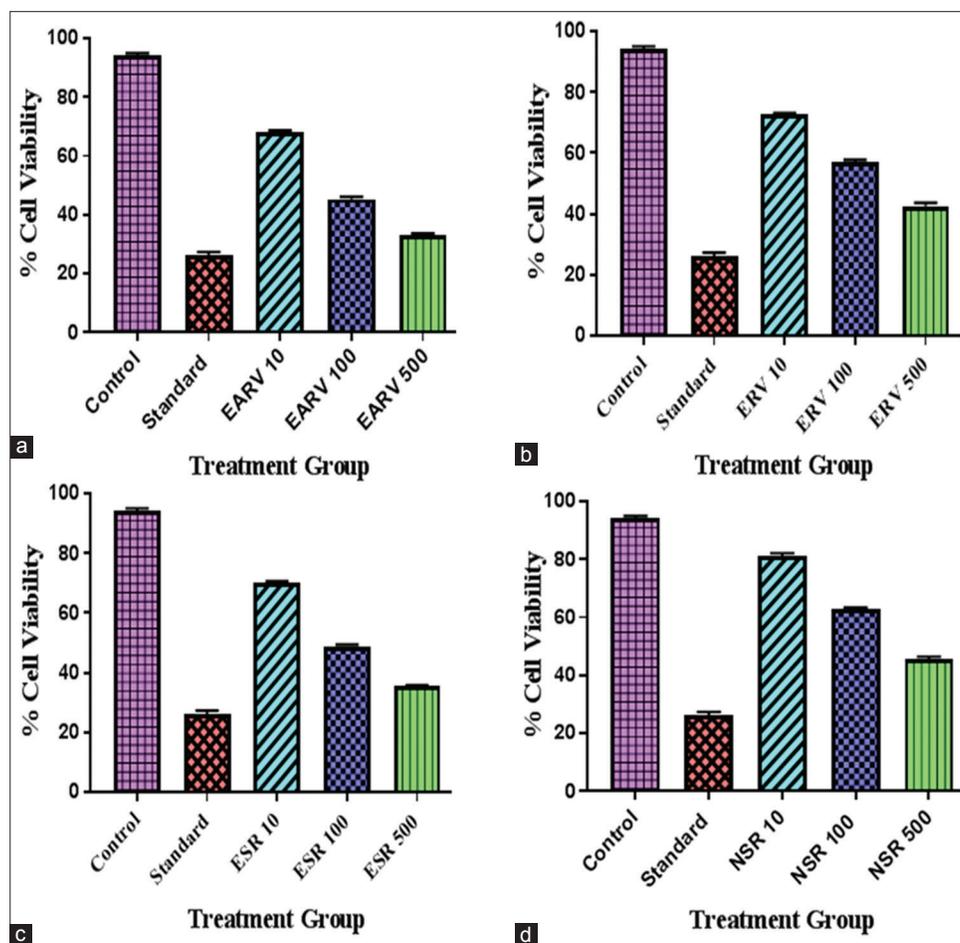


Figure 6: Graphical representation of percentage cell viability on PC-3 cell line (a) Percentage cell viability on PC-3 cell line by ethyl acetate extract of *Rumex vesicarius* at 10, 100, and 500 µg/ml, standard and control (b) Percentage cell viability on PC-3 cell line by N-hexane extract of *S. racemosa* at 10, 100, and 500 µg/ml, standard and control (c) Percentage cell viability on PC-3 cell line by ethanolic extract of *Symplocos racemosa* at 10, 100, and 500 µg/ml, standard and control (d) Percentage cell viability on PC-3 cell line by N-hexane extract of *S. racemosa* at 10, 100, and 500 µg/ml, standard and control

well. All the extracts showed significant ($P < 0.001$) cytotoxic activity of HT-29 and PC-3 cell lines [Figures 5 and 6].

In summary, the cytotoxic effect of leaves of *R. vesicarius* Linn and *S. racemosa* Roxb extract showed the concentration-dependent effect in the culture of HT-29 and PC-3 cells. EARV showed the highest cytotoxicity on both HT-29 and PC-3 cell lines.

The use of some herbs has attracted a great deal of attention as one of the alternative cancer therapies from the point of less toxicity and cost benefits. The result of the present study concluded that leaves of *R. vesicarius* Linn and *S. racemosa* Roxb showed effective anticancer activity in a dose-dependent manner by BSL assay method and MTT. Overall, the order of anticancer activity was found to be EARV>ESR>ERV>NSR on a dose-dependent manner.

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