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ORIGINAL ARTICLE

Cytotoxic, antioxidant and phytochemical analysis of Ethyl acetate extract of *Putranjiva roxburghii* wall"

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ABSTRACT
The cytotoxic activity of ethyl acetate extract of bark of Putranjiva roxburghii wall (Euphorbiaceae family) was carried outon five cancer cell lines by using sulforhodamine-B (SRB) assay and antioxidant activity by using 1, 1-diphenyl -2-picryl-hydrazyl radical. Phytochemical analysis of ethyl acetate extract of this plant shows presence of different groups of chemical moieties and it may be responsible for in vitro cytotoxic activity. In vitro activity was investigated against cancer cell lines human breast cancer cell line MDA-MB-231, MCF 7, mouse melanoma cell line B16F10 and B16F1, human melanoma cell line SK-MEL-2.The ethyl acetate extract of bark of Putranjiva roxburghii shows significant in vitro cytotoxic activity withGI50 30.5, 31.2, 37.9 against MDA-MB-231, B16F10, B16F1 and less antiproliferation on the MCF 7, SK-MEL-2. Ethyl acetate extract of bark of Putranjiva roxburghii shows potent antioxidant activity support to treat cancer. The present study is considered to be the first report on cytotoxic activities against cancer cell lines of this plant.
Key Words: *Putranjiva roxburghii Wall**; Euphorbiaceae**; Gas chromatography and mass spectrometry**; cytotoxic activity**, Antioxidant activity*

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INTRODUCTION

Euphorbiaceae family having 220 genera and 4,000 plant species found in various tropical regions of India. In India, following genera of Euphorbiaceae are reported as medicinal plants: Acalypha, Aleurites. Bridelia, Jatropha, phyllantus, Putranjiva, Ricinus [1-3]. Species of Euphorbiaceae have been used by the local population of many countries in folk medicine as remedies against several diseases complains such as cancer, diabetes, diarrhea, heart diseases, hemorrhages, hepatitis, Jaundice, malaria, ophthalmic diseases, rheumatism and scabies etc.

Various parts of *the Putranjiva roxburghii Wall* such as leaf, bark, seed are medicinally useful. The leaves, fruits and the stones of fruits are given in decoction, cold and fevers. The bark and the seeds are useful in the antidotal treatment of snakebite, its leaves and fruits have been traditionally used for the treatment of fever, muscle twisting, arthralgia and rheumatism [6].This plant was effectively used as antinociceptive, antipyretic, anti-inflammatory, antioxidant and to cure hemorrhoids [7].

Putranjiva roxburghii Wall was reported pharmacologically important compounds such triterpenoids, Friedelin, Putranjivanonol and Putranjic acid, Friedelin, Putranjivadione, Friedelanol and Roxburgholonewere isolated from the trunk bark of *Putranjiva roxburghii* [8-10].Roxburghonic acid, a triterpene acid and putraflavone, a biflavonoid, were isolated from the alcoholic extract of the leaves of this plant [11]. Amentoflavone, β-amyrin and stigmasterol determined from *Putranjiva roxburghii* by HPTLC method [12-13]. All published reports mentioned phytochemical, pharmacological studies of this plant effectively use against various diseases.

The present study aims to evaluate the in vitro cytotoxic activity of ethyl acetate extract of bark of *Puntranjiva roxbourghii* Wall against five cancer cell lines, such as human breast cancer cell line MDA-MB-

231, MCF 7, mouse melanoma cell line B16F10 and B16F1, human melanoma cell line SK-MEL-2 and study also focus on analysis of various chemical moiety by preliminary phytochemical analysis. This study is considered to be the first report on*the Putranjiva roxburghii use* against these cancer cell line

MATERIAL AND METHODS

Plant Collection:

The trunk bark of fully grow tree of *the Putranjiva roxburghii Wall* was collected from Tal. Haweli, Pune, Khadaki region of Maharashtra, India in June 2014. The taxon is authenticated from Botanical Survey of India, Pune dated 18/08/2014 with Voucher number BSI/WRC/Cert./2014 and collection no. KKA 01. The herbarium specimen is deposited in the herbarium of Modern college of pharmacy, Nigdi, Pune.

Extraction:

The barks of this plant were collected, air dried and ground to fine powder.Ethyl acetate extract was prepared by using50 gmof bark powder and Soxhlet extraction methodology. The extract was filtered, concentrated and use for preliminary phytochemical analysis and I*n vitro* SRB assay on various cancer cell lines.

Phytochemical Analysis:

The barks of *Puntranjiva roxbourghii* Wall (Euphorbiaceae)used for extraction. Dried and coarsely powdered crude drugs were passed through sieves no. 40 and extraction has been carried out on plant material by soxhlet extraction. Preliminary chemical analysis was performed according to the established protocol mentioned in Rangari V.D., 2002 and Khandelwal K.R., 2005. [14-15]

Cell culture and in vitro cytotoxic activity:

Cancer cell lines, such as human breast cancer cell line MDA-MB-231 and MCF 7, mouse melanoma cell line B16F10 and B16F1, human melanoma cell line SK-MEL-2 were used in the study and the cell lines were preserved at the Tata memorial center(ACTREC) Kharghar, Navi Mumbai.All these cell lines were grown in RPMI 1640 medium containing 10% fetal bovine serum and 2 mm L-glutamine. For the present screening experiment, cells were inoculated into 96 well on the doubling time of individual cell lines. After cell inoculation, the microtiter plates were incubated at 37° C, 5 % CO2, 95 % air and 100 % relative humidity for 24 hrs prior to addition of experimental drugs.

The cytotoxicity activity of ethyl acetate was carried out by using sulforhodamine-B (SRB) assay according to the reported method[16-17].Ethyl acetate and Adriamycin solubilized in dimethyl sulfoxide at 100mg/ml and diluted to 1mg/ml using water and stored frozen prior to use and finally drug concentration was prepared as 10 µg/ml, 20 µg/ml, 40 µg/ml, 80 µg/ml. After compound addition, plates were incubated at standard conditions for 48 hours and the assay was terminated by the addition of cold TCA. Cells were fixed *in situ* by the gentle addition of 50 µl of cold 30 % (w/v) TCA (final concentration, 10 % TCA) and incubated for 60 minutes at 4°C. The supernatant was discarded; the plates were washed five times with tap water and air dried. Sulforhodamine B (SRB) solution (50 µl) at 0.4 % (w/v) in 1 % acetic acid was added to each of the wells, and plates were incubated for 20 minutes at room temperature. After staining, unbound dye was recovered and the residual dye was removed by washing five times with 1 % acetic acid. The plates were air dried. Bound stain was subsequently eluted with 10 mM trizma base, and the absorbance was read on a plate reader at a wavelength of 540 nm with 690 nm reference wavelength. Percent growth was calculated on a plate-by-plate basis for test wells relative to control wells. Percent Growth was expressed as the ratio of average absorbance of the test well to the average absorbance of the control wells * 100.

The monolayer cell culture was trypsinized and the cell count was adjusted to 0.5-1.0 x 105 cells/ml using medium containing 10% new born sheep serum. To each well of the 96 well microtitre plate, 0.1ml of the diluted cell suspension(approximately 10,000 cells) was added. After 24 hours, when a partial monolayer was formed, the supernatant was flicked off, washed once and 100µl of different test compound concentrations were added to the cells in microtitre plates. The plates were then incubated at 37^{o}C for 72 hours in 5% CO_2 incubator and microscopic examination was carried out and observations recorded every 24 hours. After 72 hours, 25µl of 50% trichloroacetic acid was added to the wells gently such that it forms a thin layer over the test compounds to form an overall concentration 10%. The plates were incubated at 4^{o}C for one hour. The plates were flicked and washed five times with tap water to remove traces of medium, sample and serum, and were then air dried. The air-dried plates were stained with 100µl SRB and kept for 30 minutes at room temperature. The unbound dye was removed by rapidly washing four times with 1% acetic acid. The plates were then air-dried. 100µl of 10mmTris base was then added to the wells to solubilize the dye. The plates were shaken vigorously for 5 minutes. The absorbance was measured using a microplate reader at a wavelength of 540nm [16-17].

The percentage growth inhibition was calculated using following formula,

% Growth inhibition = 100 - (Mean OD of individual Test Group/ Mean OD of control Group)X 100

Evaluation of in-vitro antioxidant activity:

DPPH scavenging activity

The percentage radical scavenging activity was investigated by using reported method [18]. 1 ml of extract solution, 5ml of methanolic solution of DPPH were mixed and incubated at 37^0C for 20min. The absorbance was measured against methanol as a blank at 517nm. The absorbance of DPPH was taken as a control. The percent antiradical activity was calculated by using following formula [19].

% antiradical activity = (A Control. – A sample) / A Control X 100

Where A Control: Control absorbance (DPPH).

A sample: Sample/standard absorbance.

RESULTS AND DISCUSSION

Phytochemical analysis:

The detailed phytochemical analysis of the bark reveals that it contains adequate quantity of tannins, saponins, steroids and terpenoids, which shows tremendous pharmacological potential of further studies. (Table 1)

***In vitro* cytotoxic activity:**

Present study focus on in vitro cytotoxic activity against five cancer cell lines and the results suggested that the ethyl acetate extract showed significant anti-proliferative action on Human breast cancer cell line (MDA-MB-231), Mouse melanoma cell line (B16F10), Mouse melanoma cell line (B16F1) (Fig. 1 A/C/D) and moderate inhibition on human breast cancer cell line (MCF-7), Human melanoma cell line (SK-MEL-2) and Adriamycin use as positive control (Figure 1 B/E)GI50 (concentration of drug causing 50% inhibition of cell growth) of Ethyl acetate extract shows30.5, 31.2, 37.9, 57.1 and 63.8 against MDA-MB-231, B16F10, B16F1, MCF-7, SK-MEL-2respectively (Table 2.1 to 2.10)

***In vitro* antioxidant activity:**

In plants various reactive oxygen species were produced and these are key components in plant defensive responses [20]. Most plants constitutively synthesize phenylpropanoids including flavonoids and triterpenoids. While the ethyl acetate extract of bark of *the Putranjiva roxburghii Wall* has significant antioxidant potential. From results (Table 4& Fig. 2), it was evident that an ethyl acetate extract of bark at 80µg/ml concentration shows significant, inhibitory concentration (IC50) 5.36 as compared with ascorbic acid.

Table 1.Phytochemical analysis of ethyl acetate extract of bark of *Putranjiva roxburghii* wall.

Phytoconstituents	*Puntranjiva roxbourghii* Wall
Carbohydrates	+
Proteins	+
Alkaloids	-
Glycosides	+
Steroids	++
Triterpenoids	++
Flavonoids	+
Tannins	+
Volatile oil	-

Table 2. IN-VITRO CYTOTOXICITY ASSAY

Table 2.1 Cytotoxicity assay on MDA-MB-231 (Human origin breast cancer Cell line)

	MDA-MB-231			
	Percentage Control Growth			
	Drug Concentrations in µg/ml			
	Average Values			
	10	20	40	80
UA	67.09±2.4	61.27±4.3	19.51±2.7	-43.1±1.7
EAPR	81.56±4.6	81.96±4.0	17.26±3.1	-27.3±3.3
ADR	-73.7±1.8	-76.3±0.8	-82.7±0.8	-84.4±0.7

Table 2.2 Growth curve: MDA-MB-231 (Human origin breast cancer cells

Sample names	Cytotoxicity assay on cancer cell line MDA-MB-231(Human origin breast cancer cells) (concentration in microgram per millilitre)		
	LC50	TGI	GI50*
UA	NE	53.5	22.9
EAPR	NE	>80	30.5
ADR	<10	<10	<10

Table 2.3 Cytotoxicity on MCF-7 cancer cell line (Human origin breast cancer cell line)

	MCF-7 (Human origin breast cancer cell line)			
	Percentage of Control Growth			
	Drug Concentrations in microgram per millilitre			
	Average Values			
	10	20	40	80
UA	94.25±5.7	83.28±6.0	49.66±4.0	54.49±2.6
EAPR	92.75±5.4	84.55±7.3	53.00±6.5	35.21±2.4
ADR	-47.7±5.4	-31.1±7.3	-38.1±6.5	-29.2±2.4

Table 2.4 Growth curve: MCF-7 (Human origin Breast Cancer Cell Line)

Sample names	Parameters		
	LC50	TGI	GI50*
UA	NE	>80	74.0
EAPR	NE	>80	57.1
ADR	<10	<10	<10

Table 2.5 Cytotoxicity assay on cell line B16F10 (Mouse Melanoma origin)

	MOUSE MELANOMA B16F10 CANCER CELL LINE			
	Percentage of Control Growth			
	Drug concentration in microgram per milliliter			
	Average Values			
	10	20	40	80
UA	87.57±6.0	73.48±4.15	9.05±2.81	81.46±3.6
EAPR	81.46±3.6	73.31±4.9	35.32±2.6	-73.9
ADR	-73.9±1.8	-79.0±1.3	-80.7±1.0	-72.7±3.2

Table 2.6 Growth curve: B16F10 Mouse Melanoma cell line

Sample names	Parameters		
	LC50	TGI	GI50*
UA	NE	53.5	31.4
EAPR	NE	>80	31.2
ADR	<10	<10	<10

Table 2.7 Cytotoxicity assay on Cell Line B16F1 (Mouse Melanoma origin)

	MOUSE MELANOMA B16F1 CANCER CELL LINE			
	Percentage of Control Growth			
	Drug concentration in microgram per milliliter			
	Average Values			
	10	20	40	80
UA	93.5±4.8	67.4±1.3	40.7±2.7	23.4±1.3
EAPR	87.2±3.4	68.3±2.5	43.7±2.5	2.6±0.5
ADR	-53.9±1.7	-53.9±0.5	-52.5±0.2	-25.9±4.6

Table 2.8 Growth curve: cell line B16F1 (Mouse Melanoma)

Sample names	Parameters		
	LC50	TGI	GI50*
UA	NE	>80	44.3
EAPR	NE	>80	37.9
ADR	<10	<10	<10

Table 2.9 Cytotoxicity assay on cancer cell line SK-MEL-2 (Human Melanoma origin)

	SK-MEL-2 (HUMAN MELANOMA CANCER CELL LINE)			
	% Control Growth			
	Drug Concentrations (µg/ml)			
	Average Values			
	10	20	40	80
UA	142.03±16.1	123.03±23.5	93.45±17.9	23.41±19.2
EAPR	152.21±12.0	143.51±17.2	113.68±19.2	10.01±15.8
ADR	-60.4±1.0	-64.7±1.2	-73.9±1.5	-75.9±3.0

Table 2.10 Growth curve: Cell line SK-MEL-2 (Human Melanoma cell line)

Sample names	Cytotoxicity assay on Human Melanoma cell line SK-MEL-2 (concentration in µg/ml)		
	LC50	TGI	GI50*
UA	NE	>80	64.6
EAPR	NE	>80	63.8
ADR	<10	<10	<10

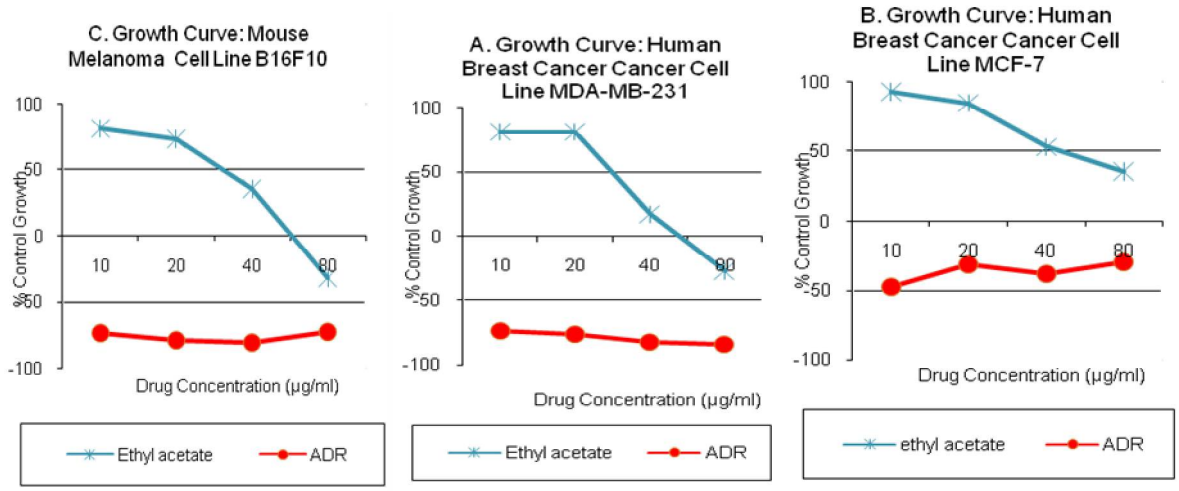
Table 3. GI50 of the antitumor activity of ethyl acetate of bark of *Putranjiva roxburghii* Wall against five cancer cell lines.

Cell lines	GI50
Human breast cancer cell line (MDA-MB-231)	30.5
Human breast cancer cell line (MCF 7)	57.1
Mouse melanoma cell line (B16F10)	31.2
Mouse melanoma cell line (B16F1)	37.9
Human melanoma cell line (SK-MEL-2)	63.8
Adriamycin (Positive control)	<10

Table 4.*In vitro* anti-oxidant activity of ethyl acetate extract of *Putranjiva roxburghii* [IC_{50}, (µg/ml)]

DPPH radical scavenging activity	IC50
Ascorbic acid	2.42±0.003
Ethyl acetate Extract	5.36±0.016

Values are expressed as Mean ± SEM, n=3



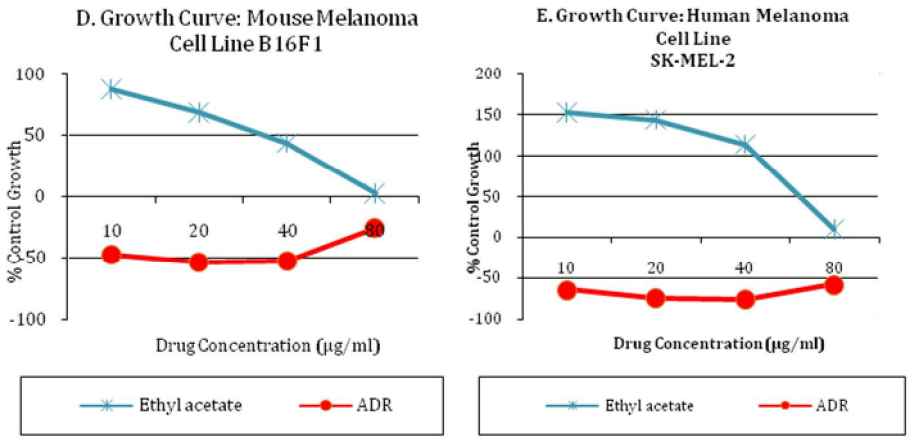


Figure 1 Cytotoxicity activity of Ethyl acetate extract of bark of *Putranjiva roxburghii* Wall
A. Growth curve: Human breast cancer cell line MDA-MB-231 B. Growth curve Human breast cancer cell line MCF-7 C. Mouse melanoma cell line B16F10 D. Mouse melanoma cell line B16F1 E. Human melanoma cell line SK-MEL-2

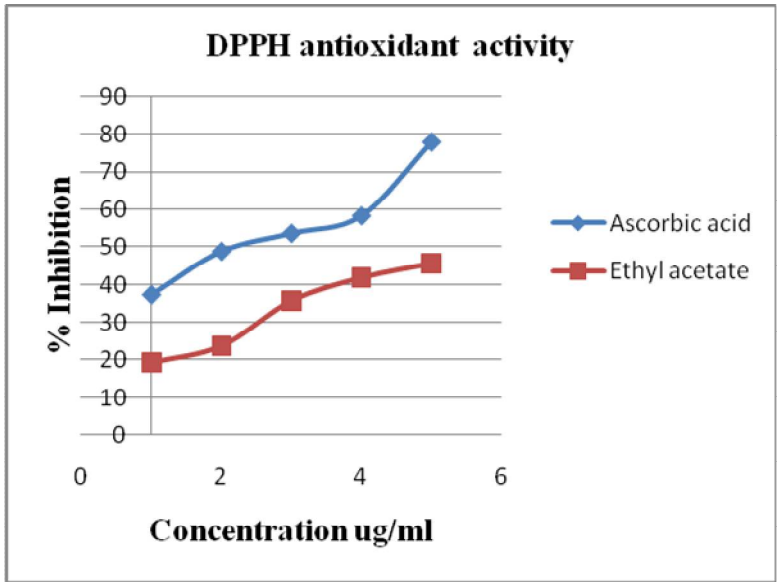


Figure 2. DPPH antioxidant activity Antioxidant activity of ethyl acetate extract of bark of *Putranjiva roxburghii* Wall

CONCLUSION

Medicinal plants having various phytocostituents, many of which biologically active compounds are responsible for exhibiting diverse pharmacological activities[21]. Various analytical methods are use of this GC-MS analysis is one of promising method now a day's use of herbal drug analysis. In present study GCMS analysis of Ethyl acetate extract of bark of *Putranjiva roxburghii* Wall reveals the presence of 15 phytoconstituents, majorly friedelanone and steroidal moieties were interpreted based on retention time and % area of the chromatogram. This preliminary chemical analysis of this plant defiantly helps in analysis of in-vitro cytotoxic activity.

Previous study reports that the methanol extract of seeds of *Putranjiva roxburghii* Wall (Euphorbiaceae) showed cytotoxic activity by using brine shrimp lethality bioassay [22].So thisstudy was the first attempt to perform in vitro cytotoxicity study against five cancer cell lines, such as MDA-MB-231, MCF 7, B16F10, B16F1, SK-MEL-2.The cytotoxic results suggested that the ethyl acetate shows significant antiproliferative activity on MDA-MB-231, B16F10, B16F1 and minimum against MCF-7, SK-MEL-2.Further, the potent antioxidant potential of ethyl acetate extract was investigated and sheds some light on to improve the mechanism of anticancer activity.

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CONFLICTS OF INTEREST
There are no conflicts of interest

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