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Cytotoxic evaluation and phytochemical screening of an ethnomedicinal plant: *Pterospermum rubiginosum* from southern Western Ghats

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Abstract
Pterospermum rubiginosum is an evergreen tree belonging to the family Sterculiaceae, different solvents like hexane, chloroform, acetone, and carbinol were used for sequential soxhlet extraction of air dried powdered bark, the obtained extracts stored at desiccators for phytochemical screening. The phytochemical analysis revealed the presence of alkaloids, phenolics, flavonoids, saponins, terpenoids, and phenolic acids. The main objective of this article is to evaluate the different phytochemicals present in bark extracts for further detailed study and to enable proper documentation for conserving our traditional knowledge about endemic medicinal plants for the future generations. HPTLC fingerprint analysis of Pterospermum *rubiginosum* bark extract showed the presence of tannins, its derivatives and gallic acid. The cell viability of both methanolic and aqueous extracts was evaluated by MTT assay against lung fibroblast (L929) cell line and LC_{50} Value were found to be 150.32µg/mL and 135.17µg/mL for PRAQ and PRME respectively.

Keywords: antioxidant, L929 cell lines, phytochemicals, *Pterospermum rubiginosum*, Western Ghats

Introduction
Western Ghats is an evergreen forest which covers 1600 kilometers from the Satpura Range of Gujarat, traversing the states of Maharashtra, Goa**,** Karnataka, Kerala and Tamil Nadu; enriched with 4000 species of diverse flowering plants; and out of them 1,500 species (approximately 40%) are extremely endemic to the mountain ranges [1]. This biogeographic area, with an optimal climatic condition, with a moderate temperature and rain fall make this region an excellent home for all living beings [2]. *Agasthya Vanam*, a resource reservoir of several therapeutically and economically important medicinal plants stretches across 77°5′ & 77°40′ East and 8°20′ & 8°50′ North in the Paruthippally range and extends from Neyyar wild life sanctuary to the Peppara wild life sanctuary coming under the beautiful evergreen forest. However, a large number of medicinal plants and natural products used by local inhabitants as well as tribal people of this region are scarcely known to the modern world [3].
According to the World Health Organization more than 75% of the world's population depends on the traditional herbal medicine as primary home remedy. With a view to conservation of folk knowledge, an attempt has been made to study the medico-ethnobotanical aspects of medicinal plants from the Kottoor Reserve forest of *Agasthya Vanam*. There is only limited and scattered scientific record of the tribal remedies in detail, used by the local inhabitants, the *Kani* tribe, a forest dwelling tribe, at Agasthya malai of Western Ghat, Kerala. The word *Kanikar* means 'hereditary proprietor of land' and they live together as an organized group, under a *muttukani* or group leader. The muttukani is very much popular in treating severe diseases and ailments in their own community as well as in the civilized populations. The tribal people obtained the knowledge of treatment, medicinal plants growing conditions such as soil pattern, altitude and its typical use from their ancestors [4]. Indian subcontinent is considered as the 'treasure house of medicinal plants' with well-practised traditional system of treatment using herbs and plant derivatives from the period of shastras and samhitas. Unfortunately, India has not been able to take the advantage of these traditional systems by promoting its wide utilization in the modern human society as leading or alternative medicine [5].

Pterospermum rubiginosum is an evergreen tree, with excellent biological potential, but little known to the outer world and no effective attempts have been made to explore its medicinal values in accordance with the principles of pharmacology and therapeutics. In this study we performed the phytochemical screening, determined the biological and other physico-chemical properties of *P. rubiginosum* and have attempted proper documentation which is really inevitable for the expansion of long established folk proficiency for the sake of mankind [6]. Even the Western Ghats is facing serious threat from deforestation, a lot of animals and medicinal plants strictly endemic to this evergreen forest are at the edge of extinction. Therefore it is very important to study, protect and document the great and extraordinary knowledge of Western Ghats.

Fig 1: a *P. rubiginosum* tree; b. Bark cut for sample collection.

The name Pterospermum is combination of two Greek words, "Pteron" and "Sperma," meaning "winged seed". *Pterospermum rubiginosum* B. Heyne ex (Sterculiaceae) generally known as 'Ellootti', 'Edinjal' in Malayalam and its bark is used by local Kani tribal healers as "Ellooripatta" for its excellent bone regeneration potential. It is an evergreen tropical tree having a height of 25-28m with a pink hard and close grained wood having the bark thickness of 5-6 mm, brown, large flaky or exfoliating; outer bark red and inner cream (Fig.1) are located 60 km, away from Trivandrum headquarters [7]., found up to an altitude of 3000ft at evergreen forests endemic to India especially found in Western Ghats, Karnataka, Tamil Nadu and Kerala [8]. The classification and plant details of *P. rubiginosum* was reported by Sasidharan., (2006) [9] and summarized on (Table 1).

Table 1: Taxonomical classification of *Pterospermum rubiginosum*

Taxonomical classification		**Basic information**	
Kingdom	Plantae	Habit	Tree
Subkingdom	Tracheobionta	Medicinal plant	Yes (Leaves and Bark)
Superdivision	Spermatophyta	Habitat	Evergreen and semi-evergreen forests
Division	Magnoliophyta	Endemic	India (Western Ghats)
Class	Magnoliopsida	Distribution	Southern Western Ghats
Subclass	Dilleniidae	Flowering and Fruiting	November-July
Order	Malvales	Localities	Nadugani Ghats, Mukkali forests, Palakkad, Idukki, Thrissur, Thiruvananthapuram, Wayanad.
Family	Sterculiaceae		
Genus	Pterospermum	Vernacular name	Chittalapolagu, malamthodali, malantotali, ponangka, talavari.
Species	Rubiginosum		

The plants can be identified by its peculiar leaf colour, the upper side of leaves is green and the lower part is pale reddish in colour. Stem bark and leaf of the *P. rubiginosum* have been used traditionally for the treatment of bone fracture and inflammation by tribals of Agasthyavanam region as well as in Wayanad settlements. The inner bark paste of *P. rubiginosum* is applied to the fractured area with the help of bamboo slides like plaster and bandage of modern medicine; bark boiled in water is also provided for consumption to relieve the inflammatory changes at fractured site. The leaf extract mixed with warm oil for massaging is commonly used in inflammation and pain relief treatment procedures [4, 10].

Materials and Methods

After removing exfoliated outer bark; the inner bark of *Pterospermum rubiginosum* was collected from Kottur forest range, Thiruvananthapuram district of Kerala with the help of tribals and shade dried for 3-4 weeks. The plant voucher specimen is kept in the herbarium of Department of Botany, University of Kerala, Thiruvananthapuram, India with a voucher number of KUBH 6189. The organic solvents used for extraction were obtained from Merck, (AR grade). The dried bark was powdered; 500 g of the powdered material was sequentially extracted with hexane, chloroform, acetone, and methanol in a soxhlet apparatus for 72 hrs. Water extraction: 100g powdered sample in 500ml distilled water at 50°C and 500rpm in a magnetic stirrer.

Organoleptic Evaluation

Organoleptic evaluation refers to the examination of the medicinal plant or its components by using organs of senses, commonly colour, odour, size, shape, taste, texture, etc. The organoleptic characters of the *P rubiginosum* bark samples were evaluated accordingly to the standard methods described by Siddiqui and Hakim (1995) [11].

Qualitative screening of phytochemicals

The qualitative phytochemical screening of powdered *P*

rubiginosum bark sample for phytochemical evaluation was carried out using the following standard procedures was summarized on (Table 2) [12-15].

Table 2: Phytochemical screening using standard protocols

Nos	Phytochemicals	Sample preparation		Test protocol
2.1	Tannins (phenolic compounds)	Plant barks extracted in water or alcohol and filtrated the sample.	2.1.a	Ferric chloride test: To the test solution, add 2-3 drops of 5% ferric chloride solution, formation of greenish black/ bluish black colour.
			2.1.b	Lead acetate test: To the test solution, add 1ml of 10% lead acetate, formation of bulky white color precipitate.
2.2	Alkaloids	Bark Extracts in dilute hydrochloric acid and filtrate it.	2.2.a	Dragendroff's Test: Filtrate was treated with Potassium Bismuth Iodide. Formation of red precipitate.
			2.2.b	Hager's Test: Filtrate was treated with Hager's reagent. Presence of alkaloids confirmed by the formation of yellow coloured precipitate.
2.3	Flavonoids	Dissolve extract in alcohol or water	2.3.a	Lead acetate Test: Extracts is treated with few drops of lead acetate solution. Formation of yellow colour precipitate.
			2.3.b	Zinc hydrochloric acid reduction test: To the plant extract, add a pinch of zinc dust and Con: HCl, mix well and keep it for 5mins. Appearance of magenta colour.
2.4	Saponins / Saponin glycosides	Dissolve extract in water and filtrate is used.	2.4.a	Foam Test: In this test 0.5 gm of extract is shaken with 2 ml of water. If foam produced persists for ten minutes it indicates the presence of saponins.
2.5	Glycosides	Dissolve extract in dilute hydrochloric acid for 1hr and the filtrate is used.	2.5.a	Legal's test: To the test solution, 1ml of pyridine and 1ml of alkaline sodium nitroprusside solution (sodium hydroxide) was added; formation of blood red colour indicates the presence of cardiac glycosides.
2.6	Proteins	Dissolve 10mg plant extract in 1 ml of distilled water and filtrate is used	2.6.a	Biuret test: To the test solution add 1ml of 4% sodium hydroxide and few drops of 1% copper sulphate solution, formation of violet or pink colour.
			2.6.b	Xanthoproteic test: To the test solution add one ml of concentrated sulphuric acid; a white precipitate is formed, which turns into yellow on addition of sodium hydroxide, formation of orange precipitate.
2.7	Steroids and triterpenoids	The test solution was prepared by dissolving the extracts in chloroform and the filtrate is used as sample		Salkowski's test: To the test solution few drops of concentrated sulphuric acid was added and allow to stand for some time, formation of red colour at the lower layer indicates the presence of steroids and formation of yellow/ golden yellow colour at the lower layer indicates the presence of triterpenoids.
2.8	Carbohydrates	The sample was prepared by dissolving the plant extract in water, filtrate is used for the following test		Benedict's test*:* The test solution mixed with Benedict's reagent in equal quantity and placed in a boiling water bath for five mins, formation of green, yellow, orange or red color indicates the presence of reducing sugars
2.9	Diterpenes			Copper acetate Test: Dissolve the plant extracts in water and then treat with 3-4 drops of copper acetate solution. Formation of emerald green colour.
2.10	Phlobatannins			About 0.5grams of each plant extracts were dissolved in distilled water and filtered. The filtrates were boiled in 2% HCl, red precipitate.
2.11	Resins:	The test solution was prepared by dissolving extract in the alcohol or water		Acetone-water test: Take 1ml of plant extracts were treated with 1ml of acetone, then add 0.5ml of water and shake well, appearance of turbidity indicates the presence of resins.
2.12	Thiol			To 0.5ml plant extract add enough $(NH_4)_2 SO_4$ to saturate it, then add 2-4 drops of 5% Sodium nitroprusside solution followed by 1 or 2 drops of Con. HNO_3. Presence of magenta colour.
2.13	Coumarin			Add 10% NaOH and chloroform to 0.5ml of the extract, observation of yellow colour.

Physicochemical parameters

Moisture content

5g of plant material in a weighed in a tared chinadish, kept in oven for 18-24hrs at 105°C for drying [16]. After removing from oven, the sample was stored at room temperature in a desiccators and final weight taken after achieving constant weight. The weight loss in sample, regarded as moisture content was calculated as follows:

$$\% \text{ Moisture content} = \frac{(\text{Total weight} - \text{Final weight})}{\text{weight of sample}} \times 100$$

Determination of Ash Content

5g of powdered plant material was taken in porcelain crucible and heated first over a low flame till all the material was completely charred, followed by heating in a muffle furnace for 3-5 hrs at 600°C. The sample was cooled in a desiccator and weighed. It was again heated in muffle furnace for 1 hour, cooled and weighed, until the ash became white or grayish white [16]. The loss in weight of the sample gives the ash content, calculated as follows.

$$\% \text{ Ash} = \frac{(\text{Weight of ash crucible} - \text{crucible})}{\text{weight of sample}} \times 100$$

Water soluble Ash

Total ash was mixed with 25ml of distilled hot water, covered with a watch glass and boiled gently for 5minutes [17]. The insoluble matter was collected on an ash less filter paper, washed with hot distilled water, filter paper was transferred to a tarred silica crucible, incinerated at 450ºC in muffle furnace until ash became white. The crucible was allowed to cool in desiccator and weighed to calculate water insoluble ash. From the soluble fraction, water insoluble fraction was calculated.

Alcohol soluble ash

Ash obtained from total ash was refluxed with 25 ml of methanol, for a minimum of 2hrs and filtered through Whatman ash less filter paper [17]. The insoluble matter was collected and transferred to a tarred silica crucible, incinerated

at 450ºC in muffle furnace for 1hr. The crucible was weighed and the alcohol insoluble ash was calculated. The soluble fraction was obtained from the filtrate, collected after filtering was used to calculate alcohol insoluble fraction.

Acid soluble Ash

The total ash was boiled with 25ml of 2M hydrochloric acid for 5 minutes. The insoluble matter was collected in ash less filter paper, washed with hot water [17]. The solution was transferred to the crucible, ignited by gradually increasing the heat to 650°C for 3hours in a muffle furnace and cooled in desiccators, which was weighed; the acid insoluble ash was calculated on the basis of sample taken initially. The acid insoluble fraction was calculated from acid soluble fraction collected.

Sulphated ash

Ash obtained from total ash was mixed with 2ml of con.H_2SO_4 in a tared china-dish to make a paste like material [18]. The china-dish was then placed in hotplate till white fumes stop originating from the surface of the material. Then it was cooled at room temperature in desiccator. The percentage of sulphated ash was calculated with reference to weight of sample taken initially.

HPTLC analysis

The HPTLC fingerprinting of PRME was performed as follows, load 2µl of sample PRME was loaded as 8mm band length in the 60F254 TLC plate using a Hamilton syringe and Camag Linomat 5 instruments. The samples loaded plates were placed in TLC developing chamber with formic acid in water and acetonitrile as a mobile phase. After the run, the plate was dried to evaporate the solvent and debris from the plates and placed in a photo-documentation chamber to capture the images at White light, UV 254 nm and UV366 nm. The camag TLC visualizer and scanner was used to envision the images obtained. The instrument is directly linked with Software vision CATS version 2.5, for detailed interpretation of results (peak display and peak densitogram) [19].

DPPH Assay

The DPPH assay was performed following the method of Chang *et al*., 2001. DPPH (2.4 mg) was dissolved in 100 mL methanol to prepare the stock solution [20]. The DPPH solution was diluted with methanol to achieve an absorbance of 0.980±0.001 units at 517nm. A 500mL aliquot of the above mixture was mixed with 500mL of the samples at different concentrations (5-250 µg/ mL). The mixture was incubated in the dark for 15 minutes and the absorbance was measured at 517 nm.

Nitric Oxide Scavenging Activity

In nitric oxide scavenging assay, sodium nitroprusside ($5mmolL^{-1}$) in phosphate buffered saline of physiological pH 7.4, was mixed with different concentration of the bark extracts (12.5-200µg/ml) from a stock concentration of 2mg/ml and kept at 25°C for 30minutes [21]. The sodium nitroprusside in aqueous solution spontaneously generates nitric oxide and reacts with oxygen to form nitrite ions that can be estimated using Griess reagent [21]. After incubation, 1.5ml of test solution was discarded and 1.5ml of Griess reagent was added, the bark extracts inhibit nitric oxide synthase and directly capture the nitric oxide from the reaction mixture. The bark samples also reduce the level of nitrite formed between oxygen and nitric oxide generated from sodium nitroprusside. The absorbance was measured at 596 nm and the percentage antioxidant activity calculated using the formula in equation with reference standard as ascorbic acid.

$$\% \text{ inhibition} = \frac{(\text{control-test})}{\text{control}} \times 100$$

***In vitro* cytotoxic assay**

The *In vitro* cytotoxic effects of both methanolic and aqueous fraction were tested by MTT assay [22] on L929 (mouse fibroblast) cell line from subcutaneous connective tissue. The cell line was cultured in tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate and antibiotic solution containing: Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphotericin B (2.5µg/ml). After incubation, growth medium was discarded and freshly prepared stock sample in 5% DMEM were serially diluted (100µg, 50µg, 25µg, 12.5µg, 6.25µg in 500µl of 5% DMEM). Add 100µl of diluted samples to corresponding wells (in triplicates) and incubate in a humidified 5% CO_2 incubator at 37ºC. The viability of cells were determined by direct observation by Inverted phase contrast microscope and followed by MTT cell viability assay.

$$\% \text{ cell viability} = \frac{\text{Mean OD Samples}}{\text{Mean OD of control}} \times 100$$

Results

Organoleptic evaluation of *P. rubiginosum* bark showed its unique colour of brownish red after shade drying, with pleasant odour, characteristic taste, shape, with rough and flexible texture (Table 3).The total yield percentage of *P. rubiginosum* bark by different solvents based on polarity is shown in (Table 4). The successive extractive yield revealed the solubility and polarity of the plant metabolites present in different PR extracts. The maximum total yield of PR bark was obtained in the methanol (12.50%) followed by water (11.46%), acetone (10.73%), chloroform (9.17%) and hexane (2.37%) respectively, which showed a higher yield with increase in solvent polarity used for extraction process.

Table 3: Organoleptic evaluation of *Pterospermum rubiginosum* bark

Characters	Fresh bark	After shade drying
Colour	Pale yellow	Yellowish brown
Odour	Pleasant	Pleasant
Taste	Characteristic	Characteristic
Shape	Twisted	Twisted
Texture	Rough, flexible	Rough, fragile

Table 4: Colour and consistency of different extracts of *Pterospermum rubiginosum* bark

Extract	Colour	Consistency	Solubility	Yield (%)
Hexane	Yellowish-brown	Solid Powder	Water insoluble	2.37%
Chloroform	Yellowish-brown	Solid Powder	Water insoluble	9.17%
Acetone	Red	solid (non sticky)	Water soluble	10.73%
Carbinol	Dark red	solid (non sticky)	Water soluble	12.50%
Water	Yellowish-brown	solid (sticky)	Water insoluble	11.46%

Phytochemical screening

Table 5: Phytochemical screening of *Pterospermum rubiginosum* bark

Secondary metabolites	Name of test	Extracts				
		Water	Carbinol	Acetone	Chloroform	Hexane
Phenolic acids	Lead acetate test	+	++	++	+	+
	$Fecl_3$ test	+	++	++	+	+
Alkaloids	Dragendorff's test	++	++	-	-	-
	Hager's test	++	++	-	-	-
Flavonoids	Lead acetate test	+	++	+	++	+
	Zinc-Hcl test	+	++	-	++	+
	Alkaline test	+	++	+	-	-
Resins	Acetone-water test	++	-	-	-	-
Sapoins	Froth test	-	++	+	-	-
	Form test	-	++	+	-	-
Glycosides	Modified Borntrager's test	-	-	-	-	-
	Legal's test	+	++	-	+	-
Carbohydrates	Benedict's test	++	++	++	+	+
	Xanthoprotic test	+	-	-	-	+
	Biuret test	-	+	+	+	-
Thiol		-	+	++	-	-
Coumarins		-	+	-	-	-
Phlobatannins		+	++	++	++	+
Phytosterols	salkowski's test	+	-	-	-	-
	Libermann Burchard's test	-	+	+	-	-
Diterpenes	Copper acetate test	-	-	-	-	-
Triterpenes	Tshugajeu's test	+	++	+	++	+
(+ presence, - absent, ++ strongly present)						

Phytochemical screening is a simple preliminary tool before elaborated qualitative analysis of unknown medicinal plant extracts, which provides a clear basic idea regarding the chemical nature of phytoconstituents in the sample (Table 5).

Detailed physicochemical investigation of Moisture Content, Total Ash, Sulphated Ash, acid, water, alcohol soluble and insoluble ash fractions showed good results and are summarized in the (Table 6).

Table 6: Physicochemical parameters of *Pterospermum rubiginosum* bark

Parameters	Values in percentage
Moisture Content	10.87±0.12
Total Ash	6.63±0.15
Acid insoluble Ash	0.55±0.04
Acid soluble Ash	6.15±0.05
Water insoluble Ash	5.9±0.06
Water soluble Ash	0.69±0.05
Alcohol insoluble Ash	6.34±0.01
Alcohol soluble Ash	0.23±0.05
Sulphated Ash	0.11±0.05

Values expressed are means of three replicate determinations ± standard deviation

HPTLC

HPTLC profiling of both aqueous and methanolic fractions showed characteristic bands during derivatization with light and dark bluish green fluorescence, which indicated the presence of tannins and flavinoid derivatives, in (Table 7).

Table 7: HPTLC profiling of PRAQ and PRME

Sample ID and volume	No. of peaks	Peak No	Rf	Area%	Assigned derivatives
PRAQ (5µl)	3	1	0.074	37.00	Tannins
		2	0.176	28.56	Tannins
		3	0.548	34.44	Gallic acid
PRAQ (10µl)	7	1	0.077	13.16	Tannins
		2	0.179	10.14	Tannins
		3	0.297	3.16	Betulinic acid derivatives
		4	0.453	9.72	Unknown
		5	0.495	7.58	Unknown
		6	0.544	27.13	Gallic acid
		7	0.931	29.10	Flavonoids
PRME (5µl)	3	1	0.555	31.14	Gallic acid
		2	0.911	60.69	Flavonoids
		3	0.944	8.17	Flavonoids
PRME (10µl)	5	1	0.090	3.03	Unknown

		2	0.477	15.49	Quercetin derivatives
		3	0.555	28.05	Gallic acid
		4	0.900	48.00	Unknown
		5	0.929	5.45	Flavonoids

PRAQ - *Pterospermum rubiginosum* aqueous extract and PRME - *Pterospermum rubiginosum* methanolic extract

Antioxidant assay

Being simple and reliable antioxidant measurement, the DPPH and Nitric oxide assay has been commonly used to evaluate the antioxidant capacity of both methanolic and aqueous extracts of P. *rubiginosum*. Both the PRME and PRAQ showed excellent scavenging activity when compared to the standard ascorbic acid in a linear manner at a concentration range from (12.5, 25, 50, 100, 200 µg/ml) and the results are tabulated in the (Fig. 2).

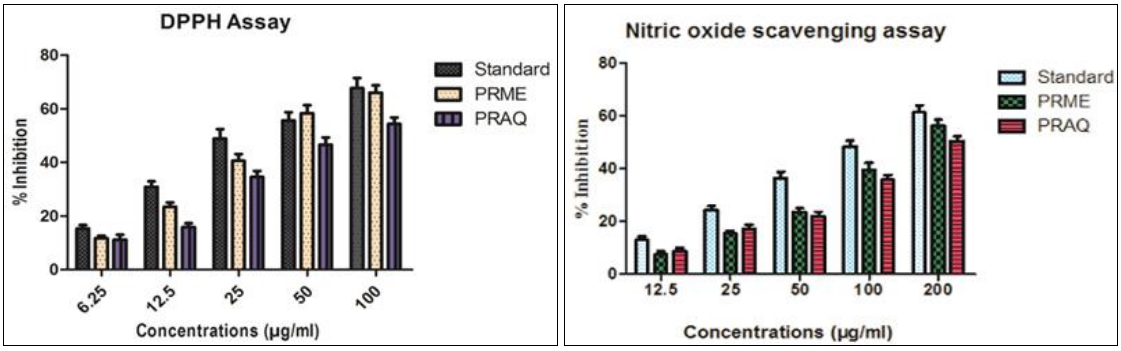

Fig 2: Shows the DPPH and nitric oxide scavenging assay of PRME and PRAQ in a concentration-dependent manner. The PRME extract gave the significant EC_{50} value 43.59 µg/ml followed by aqueous EC_{50} value 90.30 µg/ml. ($p < 0.05$) for DPPH assay. The PRME extract gave the highest EC_{50} value 165.48µg/ml followed by aqueous EC_{50} value 179.372µg /ml for NO scavenging assay. ($p < 0.05$)

MTT assay

The cytotoxic effect of the methanolic and aqueous extracts of *P rubiginosum* bark on mouse fibroblast L929 cell line was studied using the MTT assay. Entire plate was observed after 24 hours of treatment using an inverted phase contrast tissue culture microscope for microscopic cellular evaluation (Olympus, Optika Pro5 CCD camera) and observations were recorded as images, no significant changes in the morphology of the cells, abnormal granulation and vacuolization in the cytoplasm of the cells were not observed [23].

Discussion

The colour and consistency of different extracts of PR bark are shown in the (Table 4), both hexane and chloroform extracts exhibited yellowish brown colour, solid consistency and water insolubility. Only acetone extract was partially soluble in water, the rest of the fractions were water insoluble. The sequential plant sample extraction was performed with soxhlet apparatus using different solvents based on increasing polarity; n-hexane, chloroform, acetone, methanol and aqueous fraction by hot extraction. Hexane and chloroform extracts showed the presence of flavonoids and phenolic acid; acetone extract contain flavonoids, saponins, and carbohydrates. Both methanolic and aqueous extracts were rich in carbohydrates, flavonoids, phenoic acids triterpenes and alkaloids. In addition, methanolic extract showed the presence of glycosides and sapoins; while aqueous fractions exihibited the presence of resins. The phytochemical analysis indicated that methanolic and aqueous fractions contain various phytocompounds with diverse chemical groups. The secondary metabolites are considered to be the key players behind the biological potency of plants; further pharmacological and compound isolation studies will be useful to characterize the bioactive components. Generally plant extracts enriched with flavonoids and phenolic acids show good antioxidant potential irrespective of origin & species [24]. The physicochemical parameters such as moisture content in plant derivatives determine the stability and durability of plant extracts for long term clinical and pre-clinical studies, because they can protect themselves from auto-oxidation and chemical decomposition. The moisture content in *P. rubiginosum* bark was found to be (10.87±0.12%) (Table 6). The quality of powdered crude drugs determined by estimating ash value, (6.63±0.15%) and is as expected for natural products with non-volatile nature. Ash contains only inorganic material of the plant and is an indicator of plant digestibility [25]. The powdered plant material was quantified for proximate composition of alcohol, acid, water soluble and insoluble derivatives of ash as well as sulphated ash. The values were found to be in the order alcohol> water> acid for insoluble ash and acid> water> alcohol for soluble ash respectively, which is important to understand the organic nature and solvent digestibility of plant bark for further interactive elemental studies.

HPTLC profiling of PRME and PRAQ was performed with a suitable solvent system and visualized under visible light, UV 254nm and 366nm. The densitogram showed 18 peaks of Rf values ranging from 0.074, to 0.944 and the chromatogram with rf values has been presented in (Table 7), Sounder and Doss reported [26] the unknown Rf values of tannins (0.07) and gallic acid as (0.55) standard, in our results also found close similarity with the same as the sample PRAQ (5µl) with three peaks with Rf 0.074, 0.176 and 0.548 as tannins, tannin derivative and gallic acid and is same in the sample PRAQ (10µl) and the rest of the peaks with Rf values 0.297 and 0.931 corresponding to betulinic acid derivatives and flavonoids respectively. Out of the 7 peaks, 2 peaks with Rf values 0.453 with area 9.72% and 0.495 with area 7.58 were found to be unknown.

In the case of PRME two samples; PRME (5µl) and PRME (10µl) showed the presence of 3 and 5 peaks respectively with different Rf values of 0.555 corresponding to gallic acid and 0.911, 0.944, 0.929 corresponding to flavinoid derivatives, the Rf value of 0.477 and area of 15.49% corresponds to quercetin derivatives and rest of the peaks seems to be unknown. HPTLC profiling of PRME and PRAQ showed the

presence of tannins, tannin derivative, quercetin derivatives, flavonoids, betulinic acid derivatives, and gallic acid [27]. HPTLC fingerprint provides a brief idea regarding the different class of phytochemicals, which provides a new insight in isolation and characterization of bioactive compounds in bark extract. In addition to of expected peaks, various other characteristic peaks were observed in PRME and PRAQ HPTLC profiles.

DPPH assay is one of the excellent and simple methods used for evaluating the antioxidant capacity of plant extracts because, the DPPH radical is a stable organic free radical, can accept an electron or hydrogen radical to become a stable, leads to the discoloration of test sample from purple to yellow. The results showed that PR extract contains primary antioxidants, which react with free radicals and scavenge them in a linear manner. Free radical scavenging is one of the well-known mechanisms by which antioxidants inhibit lipid peroxidation and protect our body from further damages [28]. In our study the PRME with EC_{50} value 43.59 µg/ml followed by PRAQ fractions 90.30 µg/ml, showed excellent DPPH scavenging capacity, in a dose dependent manner ($p < 0.05$) (Fig. 2). Correlation analysis showed an excellent result for both aqueous and methanolic extract (r = 0.985) when compared to standard (r = 0.995).

Nitric oxide is an important chemical mediator involved in inflammatory mechanisms and in physiological processes, but an increased level will directly lead to vascular damages. Reactive nitrogen species (RNS) are free radicals that are formed due to the interaction of NO with oxygen, which leads to the production of different intermediates such as stable nitrates, NO_2, N_2O_4 and N_3O_4. The singlet oxygen (O_2) and NO radicals are directly involved in lipid peroxidation process and in chronic inflammatory cascades [29] which even leads to severe cytotoxic damages in cancer, Alzheimer's diseases and bone disorders. The results showed a dose response increase in the capacity to quench hydroxyl radicals for all the concentrations studied (Fig. 2). A concentration dependent scavenging of NO radicals by the methanol extract was significant at $p<0.05$ (r= 0.9956, p=0.01), followed by methanolic (r= 0.995) and aqueous (r=0.9938) on correlation analysis. The methanolic fraction showed highest OH scavenging potential (EC50 value of 165.48µg/ml) in a concentration-dependent manner followed by the aqueous fraction 179.372µg/ml.

The percentage viability of untreated cells was considered 100% and no significant decrease in the viable cell population across the treatment, suggesting that the extract had nil cytotoxic effect. The percent viability of the cells at the highest concentration of 100µg/mL was found to be 64.16±0.17 and 68.51±0.21with LC50 Value of 150.32µg/mL and 135.17µg/mL (Fig. 3) for PRAQ and PRME respectively. The mitochondrial dehydrogenase enzyme reduces yellow tetrazolium MTT salt to blue MTT formazan complex in metabolically active cells and is directly proportional to cell viability [30]. The present study showed that natural compounds in PRME and PRAQ significantly ($P<0.01$) inhibited the proliferation of L929 cells in a dose-dependent manner.

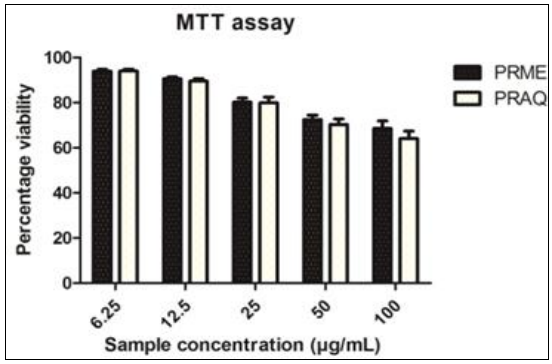

Fig 3: Effect of PRME and PRAQ on L929 cells. The values are represented as mean ± standard of three experiments

From the available limited literature and live experiences of Kani traditional practitioners and based on our laboratory studies; we strongly believe that this plant will be an excellent therapeutic agent for curing bone-related complications in the near future. So, further studies are beneficial to reveal its hidden molecular mechanism, biological potential for bone fracture healing and anti-inflammatory activity.

Conclusion

This article discusses available ethanomedicinal literature of *P rubiginosum*, phytochemical screening showed significant presence of alkaloids, phenolics, flavonoids, saponins, terpenoids, and phenolic acids. The cytotoxicity assay by L929 cell line showed the plant extracts are least toxic to cells up to a concentration of 135µg/mL and further elaborative studies will be useful for isolating and characterising biologically potent compounds to find significance in biological pathways both *in vivo* and *in vitro* experimental conditions to evaluate the wide possibilities for the development of novel therapeutic agents. The demand for alternative drugs with fewest side effects and contraindications are increasing day by day in the modern medication system. It is suggested that our government along with the forest department can take suitable measures to preserve these endemic medicinal plants, its habitat and promote the traditional medicinal knowledge for the up gradation of tribals and for the conservation of traditional precious knowledge and preservation of forest biotic and abiotic resources.

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References

1. Myers N. Threatened biotas: hot spots in tropical forests. Environmentalist 1988;8(3):187-208.
2. Gadgil M. Western Ghats: a life scape. Journal of the Indian Institute of Science 1996;76(4):495.
3. Lal B, Vats SK, Singh RD, Gupta AK. Plants used as ethnomedicine by Gaddis in Kantra and Chamba district of Himachal Pradesh, India, Ethnobiology in Human Welfare, Proc 4th Int Cong Ethnobiol, Lucknow, Uttar Pradesh, India 1994, 143.
4. Vijayan A, Liju VB, Reena John JV, Parthipan B, Renuka C. Traditional remedies of *Kani* tribes of Kottoor reserve forest, Agasthyavanam, Thiruvananthapuram, Kerala, Indian Journal of Traditional Knowledge 2007;6(4):589-594.
5. Patwardhan B, Warude D, Pushpangadan P, Bhatt N. Ayurveda and traditional Chinese medicine: a comparative overview. Evidence-based complementary and alternative medicine: eCAM 2005;2(4):465-473.
6. Vedavathy S. Scope and importance of traditional medicine. Indian Journal of Traditional Knowledge 2003;2(3):236-239
7. *Pterospermum rubiginosum* Heyne in GBIF Secretariat. GBIF Backbone Taxonomy 2017. Checklist Dataset https://doi.org/10.15468/39omei accessed via GBIF.org on 2018-02-22.
8. Nayar TS, Beegam AR, Mohanan NN, Rajkumar G. Flowering Plants of Kerala: A Handbook. Thiruvananthapuram 2006.
9. Sasidharan N. Illustrated manual on tree flora of Kerala supplemented with computer-aided identification. KFRI research report 2006, 282.
10. Sadia Raisa Lamia, Sumaiya Afra, Md Rezaul Hoque, Sajib Paul, Saifuzzaman Sumon. Pharmacological activity investigation of Sterculia coccinea. International Journal of Research in Pharmacy and Pharmaceutical Sciences, 2020:5(1):01-05.
11. Siddiqui S, Hakim MA. Format for the Pharmacopoeial Analytical Standards of Compound Formulation, Workshop on Standardization of Unani Drugs. New Delhi: Central Council for Research in Unani Medicine (CCRUM) 1995, 24-5.
12. Trease GE, Evans WC. Pharmacognosy. 12th edn. Bailliere Tindall, East Bourne, BN213UN 1983.
13. Mukherjee PK. Quality control of Herbal Drugs, an approach to evaluation of botanicals 1st Edn. Business Horizons, New Delhi 2002.
14. Khandelwal KR. Practical Pharmacognosy. Techniques and Experiments. 10th ed. Pune, India: Nirali Prakashan 2006, 149-56.
15. Haborne JB. Phytochemical methods, a guide to modern techniques of plant analysis, 1998; 3rd Edn. Springer (India) private limited, New Delhi.
16. Tripathy S, Mida A, Swain SR. Phytochemical screening and thin layer chromatographic studies of elaeocarpus ganitrus seed the magical electromagnetic bead (rudraksha). IJPBS 2016;6(3):16-24.
17. Pandey VB, Bhattacharya SK. Scientic appraisal of rudraksha (Elaeocarpus ganitrus): chemical and pharmacological studies. J Res. Educ. Indian Med 1985, 47-50.
18. United States Pharmacopoeia-National Formulary. 26th United States Pharmacopoeial Convention, Rockville 2003, 2086.
19. Dinakaran SK, Chelle S, Avasarala H. Profiling and determination of phenolic compounds in poly herbal formulations and their comparative evaluation. Journal of traditional and complementary medicine 2019;9(4):319-327.
20. Chang ST, Wu JH, Wang SY, Kang PL, Yang NS, Shyur LF. Antioxidant Activity of Extracts from Acacia confuse Bark and Heartwood. J Agric. Food Chem 2001;49:3420-3424.
21. Patel A, Patel A, Patel A, Patel NM. Determination of polyphenols and free radical scavenging activity of *Tephrosia purpurea* linn leaves (Leguminosae). Pharmacognosy Res 2010;2(3):152-158.
22. Bahuguna A, Khan I, Bajpai VK, Kang SC. MTT assay to evaluate the cytotoxic potential of a drug. Bangladesh J Pharmacol 2017;12:115-8.
23. Zahri S, Razavi SM, Niri FH, Mohammadi S. Induction of programmed cell death by Prangos uloptera, a medicinal plant, Biological Research 2009;42(4):517-522.
24. Song FL, Gan RY, Zhang YA, Xiao Q, Kuang L, Li HB. Total phenolic contents and antioxidant capacities of selected Chinese medicinal plants. Int. J Mol. Sci 2010;11:2362-2372.
25. Ibrahim J, Ajaegbu VC, Egharevba HO. Pharmacognostic and phytochemical analysis of *Commelina benghalensis* L. J Ethnobot Leaflet 2010;14:610-615.
26. Sounder L, Doss VA. Chromatographic studies on bioactive compounds of ethanolic leaf extract of aerva lanata by high performance thin layer chromatography technique. asian j pharm clin res 2017;10(6):340-345.
27. Sethiya NK, Mishra S. Simultaneous HPTLC analysis of ursolic acid, betulinic acid, stigmasterol and lupeol for the identification of four medicinal plants commonly available in the Indian market as Shankhpushpi. Journal of chromatographic science 2015;53(5):816-823.
28. Halliwell B, Gutteridge JMC. Free radicals in biology and medicine. Oxford: Oxford University Press 2006, 617-783.
29. Valko M, Leibfritz D, Moncol J, Cronin TD, Mazur M Telser M. Free radicals and antioxidants in normal physiological functions and human disease- Review. The International Journal of Biochemistry & Cell Biology 2007;39(1):44-84.
30. Wilson AP, John RW. Animal Cell Culture: A Practical Approach. Oxford University Press 2000.
31. Devi Prasad AG, Shyma TB. Medicinal plants used by the tribes of Vythiri taluk, Wayanad district (Kerala state) for the treatment of human and domestic animal ailments. Journal of Medicinal Plants Research 2013;7(20):1439-1451.