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Original Article

Phytochemical, *in vitro* Antioxidant and *in vivo* Safety Evaluation of Leaf Extracts of *Tragia plukenetii*

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ABSTRACT

Objective: To investigate the phytochemical properties, *in vitro* antioxidant and *in vivo* safety profile of leaf extracts of *Tragia plukenetii* (TP). **Methods:** TP leaves were obtained from the south part of India (Guntur District, Andhra Pradesh) and it was extracted with different solvents (Benzene Extract (BE), Chloroform Extract (CE) and methanolic extract (ME)). These TP extracts were analyzed for the *in vitro* antioxidant activity by DPPH reducing power, β-carotene-linoleic acid complex and iron chelation assays followed by *in vivo* acute oral and dermal toxicities using Swiss mice and Wistar rats respectively. **Results:** The present study results revealed ME exhibited an effective and powerful antioxidant activity when compared to a standard antioxidant, Butylated hydroxytoluene (BHT). ME was found to be effective in DPPH, β-carotene-linoleic acid complex and iron chelation assays respectively. *In vivo* acute oral toxicity study revealed that mice treated with up to 5000 mg/kg of BE, CE and ME did not show any signs of toxicity. Furthermore, similarly, acute dermal toxicity study demonstrated that BE, CE and ME did not exhibit any signs of dermal toxicity up to 1000 mg/kg in rats. **Conclusion:** TP extracts possess an excellent antioxidant activity with a devoid of any signs of acute oral and dermal toxicities.

Key words: β-carotene-linoleic acid complex, Dermal toxicity, DPPH assay, Iron chelation, Oral toxicity, *Tragia plukenetii*.

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INTRODUCTION

The use of herbal products, in whole or their aerial parts have been gaining considerable attention as therapeutic or prophylactic measures for many disorders and/or diseases in our daily life throughout the world. Severe adverse effects, higher cost, insufficiency and ineffectiveness of many allopathic drugs have led the researchers to focus more on herbal medicines to combat many diseases including neurological diseases.[1] Oxidative stress owing to the imbalance between Reactive Oxygen Species (ROS) generation and innate antioxidant defense mechanism in our body plays an imperative role in numerous diseases including Alzheimer's,[2] cancer,[3,4] Parkinson's disease,[5] diabetes,[6] acute lung injury,[7] cataracts and cardiovascular diseases such as atherosclerosis and hypertension.[8] The detailed mechanisms of cellular damage mediated through ROS and preventive pathways are well documented in the literature.[9] Plant derived products like flavonoids, anthraquinones, carotenoids, tannins and others protect cellular damage due to their significant free radical scavenging property.[10,11] Recently, phytomedicines are in huge demand in the developed and the developing world, as they could able to combat many diseases.[4] These phytomedicines provide an excellent contribution to modern therapeutics with potential innate natural antioxidants. There is a wide scope for researchers to focus more on the development of natural efficacious antioxidants. Many of the plant derived antioxidants are available in various parts of the world but the specific treatments with these substances are yet to be validated.

Tragia (Family: Euphorbiaceae) is comprised of more than hundred species. Interestingly, most of the research has been done using only five Tragia species, namely, *Tragia involucrate, Tragia cannabina, Tragia spantulata, Tragia plukenetii* and *Tragia benthamii*. The detailed phytochemical and pharmacological properties of these species were reviewed and reported elsewhere.[12] Briefly, the different solvent extracts of different parts of *Tragia plukenetii* (TP) have been reported for antiulcer,[13] antioxidant and antitumor,[14] wound healing, antimicrobial,[15-17] antipyretic, diuretic, antiasthmatic, antispasmodic,[18] antidiabetic, treatment in polio, male impotency and elephantiasis,[19] treatment of uterine complaints and skin irritation,[20] antinociceptive[21] and treatment of gastroenteritis, stomach ache, gonorrhea and tapeworm infestation.[22,23] The above-mentioned virtues are due to the presence of valuable phytochemicals such as sterols, alkaloids, flavonoids,

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glycosides, saponins and tannins. Leo Stanley *et al.* study confirmed the presence of aurones (sulpheretin), chalcones (butein) and flavanones (naringin) as major flavonoids and quercetin, kaempferol, myricetin as minor flavonoids.[24]

In the present study, the different extracts of TP leaves were obtained using benzene, chloroform and methanol and were evaluated for its *in vitro* antioxidant activity and *in vivo* safety using acute oral and dermal toxicity studies. In an earlier study, the antioxidant properties of ethanol extract of TP leaves was reported in cancer bearing mice.[14] However, the chosen TP extracts (benzene, chloroform and methanol) in the present study were not studied and reported in the literature for their antioxidant properties. Moreover, this study compares the different solvent (benzene, chloroform and methanol) extracts of TP leaves for their antioxidant potential and therefore this study results could aid the other researchers to choose an appropriate solvent for extraction and appropriate doses to perform further neuropharmacological studies.[25]

MATERIALS AND METHODS

Chemicals

β-Carotene, Butylated hydroxytoluene (BHT) and linoleic acid were purchased from Hi-Media (Mumbai, India). 2,2-Diphenyl-1-picryl-hydrazyl (DPPH) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Remaining all reagents were analytical grade.

Animals

Swiss albino male mice and Wistar female rats were obtained from the experimental animal center of the Mahaveer Enterprises, Hyderabad, India. All animal experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC), Chalapathi Institute of Pharmaceutical Sciences, Lam, Guntur, India (Approval No: 10/IAEC/CIPS/M. Pharm/2013-2014).

Collection of plant material

TP leaves were collected (from Lam in Guntur District) and authenticated by Prof. Dr. K. Madhava Chetty, Department of Botany, Sri Venkateswara University, Tirupathi. This plant voucher specimen (No.111010) was deposited in the herbarium of Chalapathi Institute of Pharmaceutical Sciences. The collected leaves were dried at 30 ± 5°C and pulverized, using a mechanical grinder. The coarsely ground powder was sieved through a number 40 sieve. The sieved powder was stored in an airtight container at −20°C until extraction.

Preparation of different solvent extracts

The dried and powdered leaf material (50 g) from TP was extracted successively by using a Soxhlet apparatus with benzene, chloroform and methanol. The temperature was kept constant using a heating mantle according to the boiling points of solvents used. In this study, the temperature of 80°C, 62°C and 65°C was kept constant for benzene, chloroform and methanol, respectively. The Benzene Extract (BE), Chloroform Extract (CE) and Methanol Extract (ME) of TP leaves were filtered, concentrated to dryness by using a rotary evaporator (Rotavac, Heidolph, Germany) with reduced pressure.

Phytochemical screening

The preliminary and confirmatory phytochemical studies were carried out on all the extracts of TP (BE, CE and ME) in order to ascertain major groups of chemical constituents by utilizing standard protocols.[26] Chemical tests were carried out on BE, CE and ME using standard procedures to identify the phytoconstituents as described elsewhere.[27,28]

In vitro antioxidant activity

Estimation of radical scavenging activity (RSA) of TP extracts using DPPH assay

The stable 1,1-diphenyl-2-picryl hydrazyl (DPPH) free radical scavenging activity of the BE, CE and ME was determined using DPPH assay as described elsewhere.[29,30] One mL of DPPH solution (0.2 mM) prepared in methanol was mixed with 1 mL of extracts at 125, 250, 500, 1000 and 2000 µg/mL, respectively. At room temperature in dark, the mixture was incubated for 20 min and the absorbance was measured at 517 nm. The ability of BE, CE and ME to scavenge the DPPH radical was calculated and compared with the reference standard, butylated hydroxytoluene (BHT) using the following equation:

$$\% \text{ of DPPH radical scavenging activity} = [(A_0\text{-}A_1)/A_0 \times 100]$$

Where, A_0 is the absorbance of the blank and A_1 is the absorbance of the sample or standard

Antioxidant activity of TP extracts by β-carotene-linoleic acid complex

Antioxidant activity was confirmed by measuring the volatile organic compounds inhibition and the conjugated diene hydroperoxides production from linoleic acid oxidation described by Dapakevicius *et al.*[31] 0.2 mL of Tween® 20 was added to the 0.02 mL of linoleic acid followed by addition of 1 mL *β*-carotene solution (0.2 mg/mL in chloroform). The resulting mixture was subjected to evaporation at 40°C for 10 min by a rotary evaporator to remove chloroform. Then, 100 mL of oxygenated distilled water was supplemented slowly, with vigorous shaking, which will help to form an emulsion. 5 mL of the above emulsion was transferred into different 15 mL test tubes containing 0.2 mL of various concentrations (0.5–20.0 mg/mL) of BE, CE and ME in methanol and the mixture was then gently assorted and placed in a water bath at 50°C for 2 h. The above procedure was repeated for the positive control BHT and a blank. After 2 h incubation, the absorbance of the mixtures was measured at 490 nm. The process of measuring the absorbance was maintained until the *β*-carotene color disappeared. The R (*β*-carotene bleaching rate) was calculated according to the formula:

$$R = \ln (a/b)/ t$$

Where, ln = natural log, a = absorbance at time t (0), b = absorbance at time (120 min).

The antioxidant activity (AA) of TP extracts was calculated in the form of percent inhibition relative to the control by using following formula:

$$AA = [(R_{control} - R_{sample}) / R_{control}] \times 100$$

Chelating effects of TP extracts on ferrous ions

This method was followed as described by Dinis *et al.*[32] Briefly, a varying concentration (0.063–1.0 mg/mL) of extracts (BE, CE and ME) prepared in methanol was used. 0.1 mL of BE, CE and ME were added to 0.1 mL of 2 mM FeCl2 respectively. Subsequently, 0.2 mL of 5 mM ferrozine (3- (3-[2-Pyridyl]-5, 6-diphenyl-1, 2, 4-triazine-4, 4'- disulfonic acid sodium-salt) was added to initiate the reaction and incubated at 37°C for 10 min. After adding 1.5 mL of double distilled H_2O to the mixture, the absorbance was measured at 562 nm. Ultrapure water instead of ferrozine solution was used as a blank and BHT was used as a reference standard. The percentage inhibition of the ferrozine-Fe^{2+} complex formation was determined using the following formula:

$$\% \text{ inhibition of metal chelation} = [(A_{control} - A_{sample})/A_{control}] \times 100$$

Where, $A_{control}$ is the absorbance of the control (ferrozine and $FeCl_2$ solution) and A_{sample} is the absorbance of the test compounds.

In vivo toxicity studies

Acute oral toxicity study of TP extracts in mice

The acute oral toxicity test was carried out using male mice (Swiss albino) weighed between 25 and 32 g. This study protocol was conducted in accordance with the Organization for Economic Cooperation and Development (OECD) Guidelines for the Testing of Chemicals No. 423, adopted December 17, 2001[33] with slight modifications.[34] After acclimatization, the mice were housed in a group of 3 and each treatment group received the extracts (BE, CE and ME) prepared in 0.5% w/v sodium carboxymethyl cellulose (CMC) at the doses of 5, 50, 300, 2000 and 5000 mg/kg B.W in order to find the dose-dependent toxic effect of testing extracts. The vehicle-control received 1 mL/100 g of 0.5% w/v CMC. The first mice of each group were dosed and observed, when no mortality occurred within 48 h, then a further two mice of each group was consequently dosed. After dosing, at different time points (1, 2, 4 and 8 h), animals were observed separately for signs of toxicity on changes in the mucous membranes, skin, eyes, fur, autonomic and circulatory systems, respiratory and central nervous systems (somatomotor activity) and also to change in behavior. The number of surviving animals was recorded after 24 h for 14 days. The body weight of the animals was recorded on day 0, 7 and 14. After 14 days, all mice were euthanized. Histopathological examinations of most important organs such as adrenal glands, heart, kidneys, lungs, spleen, liver and sex organs were performed.

Determination of dermal toxicity upon acute exposure of TP extracts in rats

This study was performed in accordance with OECD Guidelines (No. 402) with slight modifications.[35,36] The preliminary test was performed on Wistar female rats (160-180 g body weight), to observe the dermal toxic effects of this plant extracts upon exposure. A day prior to the actual experiment, the dorsal areas of the trunk of rats were cautiously shaved using depilatory. Each group consisting of ten female rats: control (C), exposed to BE, CE and ME. The extracts at doses of 50, 200 and 1000 mg/kg body weight were applied uniformly on the shaved skin of rats and it was roofed with a thin patch along with a sheet, then the sheet was held with a tape (non-irritating). The surface area exposed was kept at 10% of the total body surface area of the rats. Control rats received vehicle only. Throughout the 24 h exposure period, rats were separately caged. After 24 h, the residual extract or vehicle was cautiously cleaned with distilled water and recorded the sign of local skin reactions such as erythematous edema as per the dermal irritation scoring system (no erythema/ no edema (0), perceptible erythema/ edema (1), well defined erythema/ slight edema (2), moderate to severe erythema/ moderate edema (3) and severe erythema/ edema (4)).

Functional observational battery (FOB)

After dermal irritation scoring, functional observational battery (FOB) was conducted to investigate gross functional deficits due to dermal exposure of extracts as described elsewhere.[37,38]

Exploratory behavior in hole-board test

Followed by FOB, the exploratory behavior was assessed using a hole-board apparatus in all treatment groups. This apparatus consisted of 40 cm square floor with 16 equally spaced holes of 3 cm diameter and mounted on four legs of 25 cm height. Each rat was placed on the center of the hole-board apparatus and the exploratory behavior was monitored. The diameter of the hole has been sufficiently enough for the rats just to poke their nose, but not permissible to allow the entire body of the rats.[39-41] The number of nose poking was scored for 5 min and served as a parameter of exploratory activity.[39,42]

After the hole-board test, rats were housed in their respective groups for 14 days. At the end of 14 days housing, the earlier measured parameters were reexamined to establish reversibility, persistence and/or delayed response of toxicity. Finally, rats were euthanized and the dermal tissues were subjected for necropsy observations and histopathological examinations.

Histopathology

The animals were euthanized at the end of oral and dermal toxicity studies and organs/tissues were isolated. The isolated organs/tissues were fixed at 10% neutral buffered formalin (NBF) and NBF was changed every 24 h with fresh NBF. NBF was used three times to wash the fixture before histopathological studies. The organs/tissues were treated with 70% alcohol followed by 80%, 90% and absolute alcohol respectively for every 2 h intervals. The organs/tissues were kept in fresh absolute alcohol overnight (12 h). Then the organs/tissues were immersed in equal proportion of alcohol and xylene for 3 h followed by treatment with sulfur- free, xylene for 2 h and impregnated with welled paraffin for 2 h. Preparation of block by using the automatic tissue Embedder was used to prepare a block and the block was trimmed with a razor. The block was trimmed into 30 µ section using microtome. The ribbon of the section was transferred to warm water (60°C water bath) and allowed it to stretch as small amount of wrinkling filter one. Satisfactory sections (free from scratches and cracks) selected from tissue ribbons were placed on clean slides coated with albumin solution. These slides were placed in sacks in hot oven to dry. For histology staining, slides with section were placed in a hot air-dry oven at 40°C for 15 min. The dried slides with section were treated with fresh xylene for 5 min followed by absolute ethanol for 3 min, hematoxylin for 15 min and again absolute ethanol for 2 min. Then, the slides were kept contact with 1% acid alcohol for 30 secs and washed with distilled water for 5 min. Finally, 2% eosin was added to the slides for 3 min and distilled water for 5 min followed by 90% alcohol for 1 min and absolute alcohol for 3 min. Then, the slides were treated with xylene, two times for 5 min and air dried. The slides were mounted in the DPX (kept at 40°C to attain adequate viscosity). Cover slips were placed on the slides and allowed to dry overnight, without any air bubble. Finally, the slides were kept for microscopic observation.[36]

Statistical analysis

The data obtained from *in vitro* study are expressed as the mean ± standard deviation (SD); n=3 replicates. The intergroup variations were measured by one-way analysis of variance (ANOVA) using the Graph Pad Prism, version 5.0. The *post hoc* statistical comparisons between treatment groups were made with "Dunnett's test". Results with $P < 0.05$ were taken as statistically significant values.

RESULTS

Extraction yield

The yield of the benzene, chloroform and methanol fractions of TP (BE, CE and ME) was found to be 5.64%, 6.40% and 4.46% w/w respectively.

Phytochemical analysis

Phytochemical analysis of TP extracts (BE, CE and ME) revealed the presence of steroids, triterpenoids, tannins, saponins, glycosides, carbohydrates and amino acids. Preliminary phytochemical screening for BE, CE and ME showed positive results for tannins, anthraquinones, saponins, flavonoids, glycosides, reducing sugars, steroids and proteins. Among the three extracts (BE, CE and ME) of TP, ME was shown good number of phytochemicals. The confirmation phytochemical studies of BE revealed the presence of saponins, tannins and steroids; ME revealed the presence of saponins, tannins, anthraquinones, glycosides, steroids, phenols, alkaloids and carbohydrates; and CE revealed the presence of saponins, tannins, flavonoids and reducing sugars.

Antioxidant Activity

DPPH radical scavenging assay

The DPPH radical scavenging activity of leaves extracts of TP was investigated. **Figure 1a** depicts a steady raise in the percentage inhibition of the DPPH radical by the benzene, chloroform and methanol extracts up to a concentration of 2 mg/mL, after that there was a leveling off with a much slower increase in inhibition. In the presence of 2 mg/mL of the test sample, the DPPH radical inhibition of leaves extracts and the reference standard, BHT was in the following order: BHT (82.13%) > ME (74.95%) > BE (73.69%) > CE (69.57%). The EC_{50} values of BE, CE, ME and BHT were 1.22, 1.26, 1.15 and 0.06 µg/mL respectively (**Figure 1a**).

Antioxidant activity against β-carotene-linoleic acid

Figure 1b shows the antioxidant activities of the BE, CE and ME compared to BHT. At 0.5 to 20.0 mg/mL, the antioxidant activities of BE, CE, ME and BHT ranged from 46.45 to 93.48%, 59.79 to 93.18%, 68.33 to 95.71% and 93.16 to 93.16%, respectively.

Chelating effects on ferrous ions

The ability of ferrous ion chelating by the BE, CE and ME was increased in a dose-dependent manner, as illustrated in **Figure 1c**. The powerful chelating effect (74.19%) was noticed from ME at 1 mg/mL. BE and CE at this concentration (1 mg/mL) showed the lowest chelating effect 54.77, 52.23% respectively. The reference standard, BHT exhibited 49.73% chelation.

Acute oral in vivo toxicity study of TP extracts

This study results revealed that TP extracts up to 5000 mg/kg did not exhibit any signs of toxicity in mice, hence the no-observed-effect level (NOEL) might exceed 5000 mg/kg. The body weight gain observed in 7 and 14th day after acute oral administration of BE, CE and ME (5, 50, 300, 2000 and 5000 mg/kg) was not significantly altered when compared with the vehicle control group (data not shown). Both initial and final body weight of mice treated with the higher dose (5000 mg/kg) of

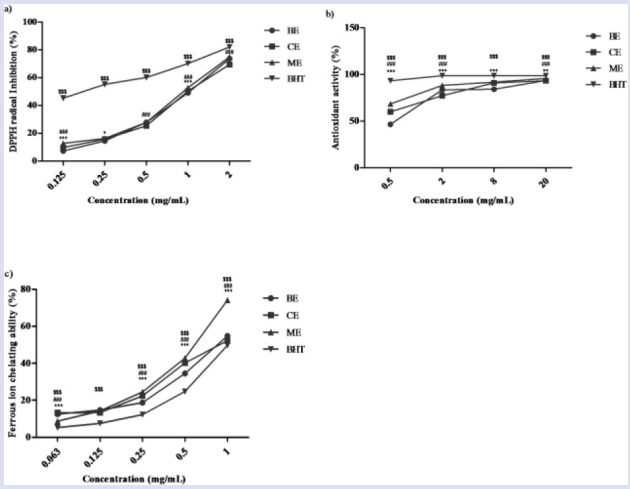

Figure 1: *In vitro* antioxidant activity of TP extracts (BE, CE and ME). (a) DPPH radical scavenging activity, (b) % Antioxidant activity using β-carotene-linoleic acid complex. (c) Chelating effect on ferrous ion, of BHT, BE, CE and ME leaves extracts of TP. Data are representative of three independent experiments. The significance of differences was analyzed by two-way ANOVA followed by Bonferroni multiple comparisons test (a) BE versus ME * p <0.05; *** p <0.001; CE versus ME ### p <0.001; ME versus BHT $$$ p <0.001; (b) BE versus ME ** p <0.01; *** p <0.001; CE versus ME ### p <0.001; ME versus BHT $$$ p <0.001; (c) BE versus ME *** p <0.001; CE versus ME ### p <0.001; ME versus BHT $$$ p <0.001.

Table 1: Absolute organ weights (mg) of mice treated with the BE, CE and ME.

Organ	Control	Extract	Doses				
			5 mg/kg	50 mg/kg	300 mg/kg	2000 mg/kg	5000 mg/kg
Liver	1817.25±10.25	BE	1018.2±25.73	1499.75±6.29	1454.2±14.00	1634.9±25.45	1707.5±230.51
		CE	1322.45±82.09	1319.5±133.64	1363±84.85	1618.45±76.43	1557.5±18.38
		ME	2034.2±49.35	1627.9±14.70	1760.7±6.78	1547.5±4.24	1657.3±18.66
Heart	159.15±5.86	BE	109.2±11.45	98.3±8.62	166.55±6.29	124.65±23.54	142.2±14.28
		CE	99.25±1.06	137.2±14.42	109.35±5.86	93.8±4.66	127.2±7.21
		ME	229.35±8.83	148.05±6.01	165.5±6.78	147.2±7.21	136.4±1.27
Kidney (Right)	215.3±7.49	BE	203.1±5.65	136.7±4.66	231.65±14.77	241±9.89	235.95±24.81
		CE	153.35±13.64	139.55±13.50	152.95±10.53	225.65±22.69	185.95±17.60
		ME	270.35±7.14	179.2±8.06	217.95±3.32	210.95±10.53	175.2±6.96
Kidney (Left)	207.55±1.06	BE	198.3±6.78	129.8±3.25	207.05±4.59	210.45±2.05	239.7±22.34
		CE	102.75±10.25	139.1±20.78	229.8±5.79	184.4±15.27	219.7±5.93
		ME	262.2±2.40	153.4±2.96	225.45±7.28	214.7±13.01	216.9±1.55
Lungs	266.7±2.54	BE	117.95±10.39	227.35±10.11	206.05±7.84	349.35±31.18	245.55±30.61
		CE	243.25±26.51	101.55±6.01	303.8±25.88	275.8±13.15	260.55±9.40
		ME	324.7±21.21	221.8±11.17	247.95±3.74	255.55±16.47	297.65±7.14
Spleen	220.3±25.17	BE	173.25±5.30	136.45±6.29	130.75±14.21	145.1±13.57	228.1±3.67
		CE	156.2±7.49	169.5±3.53	155.7±8.06	263.25±19.30	228.1±3.67
		ME	350.35±5.44	215.1±14.70	229.1±9.19	218.1±10.46	160.15±11.95
Brain	506±14.5	BE	515.15±7.28	366.6±9.05	431.85±11.80	417.85±12.23	556.2±14.99
		CE	362.2±17.25	531.2±29.69	509.1±8.76	505.6±21.63	551.2±22.06
		ME	629.5±7.353	411.95±5.16	509.15±8.98	441.2±6.22	599.2±5.09

Table 2: **Hematological parameters of mice treated with BE, CE and ME.**

Hematological parameter	Control	5 mg/kg			50 mg/kg			300 mg/kg			2000 mg/kg			5000 mg/kg		
		BE	CE	ME	BE	CE	ME	BE	CE	ME	BE	CE	ME	BE	CE	ME
WBC (10^3/mm^3)	10.7±0.63	13.6±0.92	13.5±0.42	14.5±1.97	8.9±1.97	9.15±1.90	5.35±1.48	11.3±3.34	10.9±2.90	10.05±5.7	11±4.52	11.8±2.54	9.9±5.23	9.55±1.34	8.25±1.34	6.1±0.28
Lymphocyte	64.0±0.42	74.9±0.52	75.1±3.81	77.3±3.77	70±2.82	68.6±2.33	73.3±2.61	73.6±4.18	74.6±4.38	76.55±3.3	73.6±1.38	71.6±0.56	71.6±1.97	70.2±0.84	67.9±2.75	74.1±0.07
Monocyte (%)	22.2±0.21	14.4±1.90	15.6±1.20	12.5±1.41	15.4±3.04	15.6±0.35	15.2±0.49	14.4±1.55	15.1±1.34	12.4±3.39	15.1±0.84	17.6±1.20	13.9±1.48	15.9±0.84	16.6±0.91	14.2±0.49
Granulocytes(%)	13.6±0.63	9.8±3.11	12.3±7.42	10.3±2.33	14.9±2.61	12.5±1.97	11.6±2.12	10.5±3.56	10.9±4.24	9.9±1.48	12.8±0.77	16.4±2.12	12.4±1.76	13.2±0.63	12.1±1.55	10.4±0.56
Lymphocytes (10^3/mm^3)	7.25±0.35	3.36±4.01	6.53±1.79	11.2±2.12	4.05±0.21	5.91±0.86	3.9±1.27	1.93±1.80	4.77±0.72	8.2±1.69	5.2±1.83	7.76±0.33	6.14±1.95	3.95±0.77	4.9±0.98	4.15±0.07
Monocytes (10^3/mm^3)	2.4±0.0	0.9±0.19	0.89±0.04	1.75±0.07	0.69±0.13	0.99±0.33	0.75±0.21	1±0.56	1.1±0.21	0.95±0.21	0.75±0.07	1.25±0.42	1.06±0.19	0.75±0.07	1.22±0.44	0.93±0.03
Granulocyte (10^3/mm^3)	1.75±0.07	3.23±3.21	0.8±0.14	1.55±0.21	0.7±0.14	0.65±0.07	0.7±0.0	3.28±3.35	1.15±0.62	1.2±0.56	0.75±0.21	1.1±0.28	1.15±0.49	0.7±0.0	0.59±0.13	0.76±0.05
RBC (10^6/mm^3)	6.91±0.01	8.55±0.63	9.5±1.55	8.69±0.33	7.78±2.65	7.7±1.06	7.46±2.64	9.13±0.10	10.1±0.14	7.91±0.15	7.18±1.80	7.38±0.88	7.03±1.37	9.48±0.38	9.25±1.06	8.3±0.98
HB (g/dL)	9.05±0.07	11.9±1.02	12.7±1.62	12.5±0.21	14.3±1.55	13.7±1.27	11.9±1.76	12.7±0.08	14.3±2.12	11.4±0.14	12.5±1.41	12.9±1.34	11.9±1.06	14.1±1.97	14.9±1.06	12.7±0.28
HCT (%)	32.9±0.84	42.9±5.72	44.5±3.53	45.6±0.84	45.8±2.05	45.5±2.12	38.9±5.86	42.7±7.49	39.5±9.19	43.5±2.05	43.3±6.08	49.5±6.36	37.1±4.17	42.2±4.38	43±18.38	45.5±3.53
MCV (µm^3)	48.0±1.41	52.5±0.70	58±1.41	52.5±0.70	46.5±0.70	47.5±4.94	52.5±0.70	50±7.07	49±14.14	52.3±2.47	52±4.24	50.5±6.36	53.5±2.12	49.5±0.70	44±8.48	50.5±0.70
MCH (pg)	12.6±0.70	16.7±1.20	16.2±2.33	14.5±0.35	18.4±0.70	18.2±0.49	16.7±3.60	15±2.12	15.3±2.12	14.8±0.07	17.0±1.90	16.4±1.20	16.4±3.53	16.2±2.40	15.8±5.23	13.4±0.98
MCHC (g/dL)	28.6±0.70	28.5±0.14	31.4±3.11	27.3±0.14	33.5±3.04	32.6±1.55	31.7±6.08	26.6±4.31	30.6±7.07	26.7±0.63	31.1±3.46	30.6±0.56	32.5±7.77	27.4±4.10	29.3±6.01	25.8±0.91
RDW (%)	16.3±0.49	18.5±0.07	19.2±0.56	16.9±0.77	15.4±2.12	15.1±0.49	15.4±0.14	15.4±4.45	16.7±2.68	16.2±0.42	16.4±2.62	15.4±1.97	16.7±1.55	15.1±1.20	16.1±0.35	15.3±0.49
PLT (10^3/mm^3)	712.5±2.1	571±124.4	603±89.09	505±8.48	656.5±185.7	567.5±201.52	711±14.14	476±32.52	570±28.28	393±32.52	593±103.23	553±183.84	614.5±50.2	624±189.51	640±113.13	649±60.81
MPV (µm^3)	8.3±0.42	7.25±0.49	9±0.28	7.5±0.14	9.15±0.21	9.5±1.41	8±1.14	7.1±0.14	7.65±0.77	7.35±0.35	8.85±1.48	9.5±2.12	8.2±1.83	7.7±0.42	8.05±1.20	7.75±0.63
PCT (%)	0.662±0.07	0.45±0.01	0.594±0.28	0.38±0.16	0.451±0.00	0.866±0.47	0.554±0.05	0.382±0.09	0.427±0.097	0.278±0.07	0.456±0.05	0.996±0.00	0.517±0.106	0.385±0.06	0.43±0.11	0.429±0.17
PDW (%)	1.25±0.35	0.25±0.07	0.35±0.21	0.85±0.77	0.35±0.07	1±0.28	2.25±0.77	0.75±0.21	0.4±0.14	0.195±0.13	0.6±0.28	0.95±0.07	1.65±0.91	0.35±0.21	0.75±0.21	1±0.56

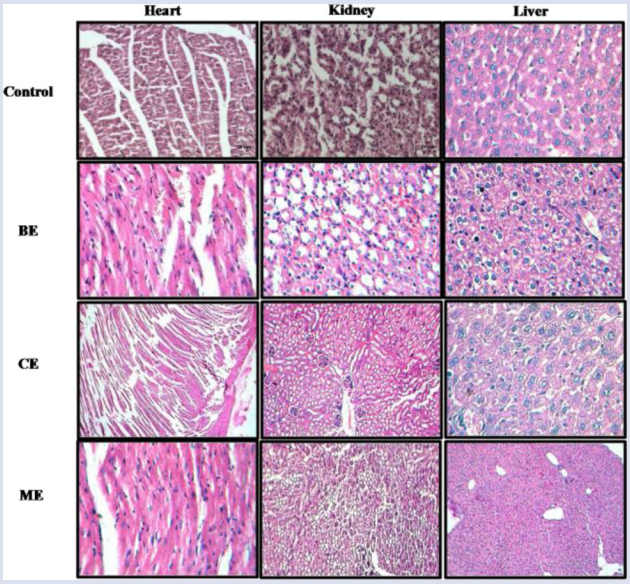

Figure 2: Representative photomicrographs showing histopathological changes in the heart, kidney and liver of mice treated with BE, CE and ME at 5000 mg/kg, p.o.

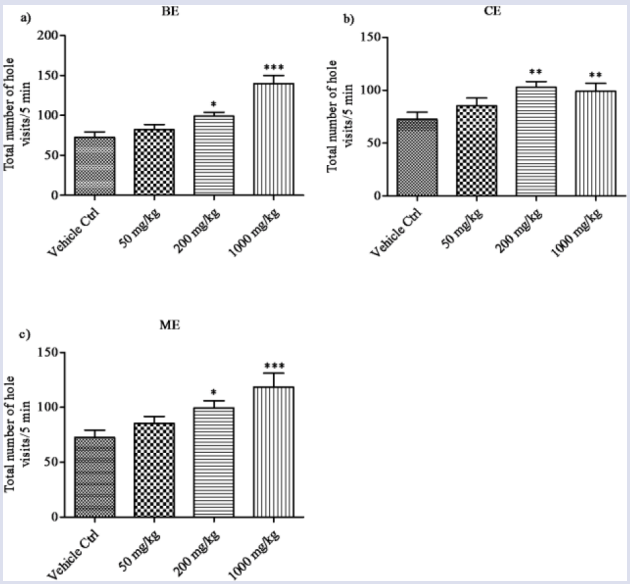

Figure 3: Effect of TP extracts (BE, CE and ME) on the exploratory behaviors of rats in the hole-board test. Total number of holes visited by the rats when treated with BE (a), CE (b) and ME (c). Data are represented as mean ± SEM. The significance of differences was analyzed by one-way ANOVA followed by Bonferroni multiple comparisons test * p <0.05, ** p <0.01, *** p <0.001 versus Vehicle control.

BE, CE and ME was 26.16±0.66; 26.76±0.08, 28.8±1.1; 29.3±1.13 and 28.56±1.30; 30.3±0.50 g respectively. These results implied that the general health of animals treated with TP extracts was not affected. The absolute organ weights (Table 1) and hematological parameters (Table 2) of mice treated with TP extracts are tabulated. The hematoxylin and eosin histopathological staining of heart, liver and kidney of mice treated with 5000 mg/kg BE, CE and ME is shown in Figure 2. These studies revealed no significant abnormal changes in BE, CE and ME treated mice up to the dose of 5000 mg/kg when compared to control.

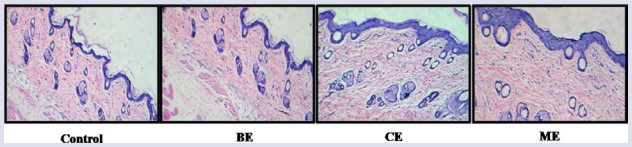

Figure 4: Histopathology of rat skin. Representative microscopic graphs for the rat skin treated with TP extracts (BE, CE and ME) at 1000 mg/kg.

Dermal toxicity and behavioral studies

The TP leaves are used in treating uterine complaints and also in relieving the skin irritation.[24,43] To find the safety of topical application of TP leaves extracts, dermal toxicity study was performed. The total exploratory activity of the treatment groups in the hole-board exploratory test showed a significant dose-dependent increase in the total number of holes visited in 5 min (Figure 3). Moreover, the BE (Figure 3a) and ME (Figure 3c) were shown high exploratory activity when compared with CE (Figure 3b). The hematoxylin and eosin histopathological staining of the skin of the rats treated with 1000 mg/kg of BE, CE and ME is shown in **Figure 4**. These studies revealed no significant abnormal changes in BE, CE and ME treated mice up to the dose of 1000 mg/kg when compared with vehicle control. These results revealed that no physical/superficial toxicity occurred. This suggested TP leaves extracts are safe to apply topically.

DISCUSSION

The present study followed a successive extraction of TP leaves with solvents of increasing polarity from nonpolar to polar solvent to extract a wide range of compounds from the plant material. The polarity of the solvents is mainly determined by two factors like 'dielectric constant' and 'dipole moment'. The polarity of the solvent is directly proportional to 'dielectric constant' and 'dipole moment'. In this study, we used benzene, chloroform and methanol, which has 'dielectric constant' value of 2.2, 4.8 and 32.7 and 'dipole moment' value of 0.0, 1.04 and 1.70, respectively.[44] Benzene was chosen as a strong nonpolar solvent in the present study. Moreover, we earlier reported anticonvulsant activity of these solvent extracts of *Tragia plukenetii* R. Smith leaves against pentylenetetrazole-induced convulsions in mice.[25] Therfore, this study could be a continuation of our earlier report to retrospectively analyse the same solvent extracts in terms of their *in vivo* toxicity and *in vitro* antioxidant activity.

Oxidative stress is one of the major contributors in the occurrence of many diseases. In recent times, numerous herbal drugs with free radical scavenging properties have been used in treating various diseases. The present study was aimed at investigating the *in vitro* antioxidant and *in vivo* safety profile of benzene, chloroform and methanol extracts of TP leaves. Initially, the test extracts were analyzed for antioxidant potential by performing the DPPH, β-carotene-linoleic acid complex and ferrous ion chelating assays (Figure 1a, 1b and 1c). Metals are known to play crucial pathological role in mediating various free radical reactions in addition to their physiological functions. Thus, metal chelating capacity, particularly against transition metals such as iron, copper, zinc and others are considered to be a beneficial action of an antioxidant. In the present study, iron chelating capacity of ME of TP leaves showed higher activity than the referring agent, BHT (**Figure 1c**). The present study could not delineate the actual active principles of TP extracts responsible for antioxidant properties and detailed mechanism of action; however, it is presumed that the polyphenolics present in TP extracts could be

responsible for the antioxidant properties. This is because of the redox properties of polyphenols, which play a major role in quenching singlet and triplet oxygen or decomposing peroxides, absorbing and neutralizing free radicals. Flavonoids and tannins seem to be the most promising polyphenolic compounds in the TP extracts responsible for its antioxidant properties.[24] Further *in vivo* preclinical studies are warranted to elucidate the detailed molecular mechanism of TP extracts, thereby, it can be taken further in novel drug discovery processes. Studies in this direction are currently underway in our laboratory. Acute oral toxicity test was performed to find out the safety dose of test extracts. All TP leaves extracts treated mice were found to be safe up to 5000 mg/kg. Body weight changes (data not shown), hematological parameters (**Table 2**) and absolute organ weights (**Table 1**) and histopathological results (**Figure 2**) were not significantly altered when compared with the control group. The maximum tolerated dose (MTD) of the test extracts (BE, CE and ME) was found to be >5000 mg/kg in mice. The various extracts of TP have been reported for its wound healing, antimicrobial activity and traditionally, it has been used to treat various skin disease.[15] With this inspiration dermal toxicity study on rats was conducted in different doses. There were no abnormalities detected in animals treated with TP extracts. Rats treated with 1000 mg/kg of TP extracts were not shown any histopathological changes. However, rats treated dermally with BE and CE showed a dose-dependent increase in the exploratory activity in the hole-board test (**Figure 3**). Of note, the nervous system function can also be assessed by the general exploratory behavior of the animal. Systemic toxicity alters the functions of the motor, sensory and associative processes of the nervous system.[45] The observed high exploratory behavior in the present study is believed to be due to the mild stimulatory effect of TP extracts on the nervous system. Moreover, these results also suggest the systemic absorption of TP extracts when applied topically. However, extensive work is needed for its therapeutic applicability in different formulations like transdermal patches, buccal delivery and others. Besides, histopathological studies found that no significant changes in the treated rats as compared to control (**Figure 4**).

CONCLUSION

The present work analyzed the *in vitro* antioxidant and *in vivo* oral and dermal toxic effect of leaf extracts of TP. The *in vitro* studies showed that the methanol extract of TP leaves exhibited a potent and significant antioxidant activity which can effectively scavenge free radicals and thereby reduce the risk of oxidative stress. Therefore, it suggests the dietary supplementation of TP leaves may have greater significance in the prevention of oxidative stress related diseases. Moreover, there is a possibility to develop this plant as a nutraceutical or functional food to treat various diseases. Due to its high antioxidant profile, it could also be utilized to treat heavy metal-induced neurodegenerative diseases. Further studies are required to identify the exact molecular mechanisms involved in the antioxidant activity of TP leaves.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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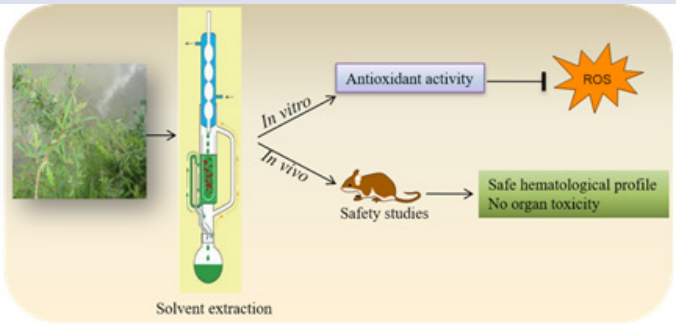
GRAPHICAL ABSTRACT



SUMMARY

- Therapeutic and prophylactic herbal edible products are in the race against many diseases.
- *Tragia plukenetii*, an ancient herbal medicine is widely used to treat many diseases .
- The methanolic extract of *Tragia plukenetii* leaf has been demonstrated for a strong antioxidant activity using a battery of *in vitro* assays.
- The methanolic extract of *Tragia plukenetii* leaf was found to be non-toxic when administered orally as well as dermally in mice and rats, respectively.

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