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IN- VITRO ANTIOXIDANT PROPERTIES, ACUTE AND SUBACUTE TOXICITY STUDIES OF *TEPHROSIA CALOPHYLLA* ON ALBINO WISTAR RATS

Vimal John Samuel [1] and A. R. Mahesh [*2]

Department of Pharmacology [1], Department of Pharmaceutical Chemistry [2], College of Pharmaceutical Sciences, Dayananda Sagar University, Bangalore - 560078, Karnataka, India.

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Correspondence to Author:
AR Mahesh

Assistant Professor,
Department of Pharmaceutical Chemistry, College of Pharmaceutical Sciences, Dayananda Sagar University, Bangalore - 560078, Karnataka, India.

E-mail: mahesh-sps@dsu.edu.in

ABSTRACT: The evaluation of acute oral toxicity of chloroform extract of *Tephrosia calophylla* (CETC) and methanolic extract of *Tephrosia calophylla* (METC) and sub-acute toxicity of CETC was done on albino Wistar rats. The animals (female) were divided into four groups containing 3 animals in each group normal control, and 200, 400 and 800 mg/kg of freshly prepared extracts respectively, every 24 h orally for 28 days. At the end of each study, physical parameters and hematological analysis were evaluated. There was no significant difference (p>0.05) observed with control in terms of body weights, food and water intake, hematological parameters, and gross abnormalities. No mortality was recorded. Hence, the study was concluded as oral administration of CETC for 28 days does not cause toxicity. In conventional therapy, various studies have been done to identify antioxidants from the plant sources. Excessive production of free radicals causes direct damage to the biological molecules such as DNA, proteins, lipids, carbohydrates leading to progression and tumor development. Phytochemicals from the plant origin serve as natural antioxidant molecules. Antioxidant potential of CETC & METC was studied by using different *in-vitro* models like DPPH (1,1-Diphenyl–2–picrylhydrazyl) assay, superoxide free radical scavenging activity, and nitric oxide scavenging activity. In the present investigation, CETC & METC showed potent antioxidant activity as indicated by the DPPH, nitric oxide, and superoxide free radical were significantly decreased. Hence, it concluded that the extracts of *Tephrosia calophylla* possess potent *in-vitro* antioxidant activity suggesting its role as an effective free radical scavenger.

INTRODUCTION: The concern related to toxicity testing of drugs is for safety purposes. All drugs included in the research must be devoid of any toxicity. Toxicology is the study of chemical substances affecting the normal processes and interacts with the living system.

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The uses of this information provide safe exposure levels. Toxicological research and testing help to live safely and predict benefit from synthetic and natural substance while avoiding harm [1].

Over the years, the use of herbs in the treatment of illnesses has been very successful, and its historic usage has been useful in drug discovery development [2]. All herbs should be tested individually before including it in research regarding its toxicity. Toxicity testing is conducted to get information on the biological activity and mechanism of action of the drug. Preclinical studies of herbal drugs provide scientific justification for

their traditional use and prove that they are safe and efficacious. Due to the adverse side-effects, and also the development of resistance against synthetic drugs, the uses of plant-derived drugs are becoming popular in developed countries. However, the latest surveys have indicated many medicinal plants also showed adverse effects. This raises concerns about the potential toxic effect resulting from chronic use of such medicinal plants. Therefore, evaluating the toxicological effects of any medicinal plant extract intended to be used clinically or preclinically, is a crucial part of its assessment of potential toxic effects [3].

Free radicals are chemical species, which contain one or more unpaired electrons due to which they are highly unstable and cause damage to other molecules by extracting electrons from them to attain stability. Free radicals are generated as part of the body's normal metabolic process and play a dual role in our body as both deleterious and beneficial species [4]. Excess production of reactive oxygen species (ROS) and a decrease in antioxidant levels may lead to tissue damage and different diseases. Antioxidant plays a major role in protecting our body from disease by reducing the oxidative damage to cellular component caused by ROS [5].

Recent investigations suggest that the plant origin antioxidants with free-radical scavenging properties may have great therapeutic importance in free radical-mediated diseases like diabetes, cancer, neurodegenerative disease, cardiovascular diseases, aging, gastrointestinal diseases, arthritis, and aging process [6].

MATERIALS AND METHODS:

In-vitro Antioxidant Activity:

Collection of Plant Material: The whole plant of *Tephrosia calophylla* was collected from Tirupati, Andhra Pradesh, India in Jan 2011 with Voucher No. VS: TC-101 and shade dried.

Preparation of Extract: The whole plant of *Tephrosia calophylla* was dried, powdered, and extracted with chloroform and methanol by using Soxhlet apparatus.

Animals: Wistar albino male rats (150-180 g) were procured from Sri Raghavendra Enterprises, Bengaluru. All the animals were housed under standard laboratory conditions, maintained on a 12 h light: 12 h dark cycle at the temperature of 26 ± 2 ºC and relative humidity 44-56% and food and water were provided *ad libitum*. Animals were acclimatized for 7 days to laboratory conditions before the test. All experiments were performed in the morning according to current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals. The study protocol was duly approved by the Institutional Animal Ethics Committee (IAEC) (CPCSEA, Reg no: 222/2000/CPCSEA). Studies were performed by the CPCSEA guidelines. The standard orogastric cannula was used for oral drug administration in experimental animals.

Acute Oral Toxicity Studies: Acute oral toxicity study was performed for CETC and METC according to the toxic class method 423 as per organization for economic cooperation and development (OECD) guidelines. Female Wistar rats weighing between 150 and 180 g were used for acute toxicity study to determine lethal dose, 50% of the extract. The dose was administered to three animals for each step. The starting dose level was selected from 50 mg/kg b.w.p.o and was given dose p.o. up to 3000 mg/kg b.w. Drug-treated animals were observed for toxicity signs and mortality. Hence, in the case of chloroform extract at the dose of 3000 mg/kg mortality was observed and for methanolic extract, no mortality was observed at the dose of 3000 mg/kg hence 5000 mg/kg b.w was given. Thus $1/10^{th}$ of toxic (200 mg/kg for chloroform extract and 500 mg/kg for methanolic extract) dose was selected for the further pharmacological study.

Sub-Acute Toxicity Studies: Subacute oral toxicity study was performed according to the Organization of Economic Co-Operation and Development (OECD) guideline 407 for testing of chemicals and World Health Organization guideline. Twelve Albino Wistar rats of both the sexes (male & female) were used for the study. The animals were divided into four groups containing 3 rats in each group (n = 3). Group I treated with vehicle (1% CMC) was kept as control. Groups II, III and IV treated with 200, 400 and 800 mg/kg body weight corresponding to low, intermediate and high dose, respectively for 28 days according

to body weight of each group rats through oral route. Treatment was done once daily for continuous 28 days. Body weight was monitored periodically. At the end of treatment, overnight fasted animals were sacrificed, and blood samples were collected on the 29th day. Hematological parameters (RBC, WBC, Hb) were measured in all treated groups as well as in control group [7].

DPPH Radical Scavenging Activity: 4.3 mg of DPPH (1, 1-Diphenyl -2-picrylhydrazyl) was dissolved in 3.3 ml methanol; the test tubes were covered with aluminum foil to protect from light. For control reading 150 µl DPPH solution was added to 3 ml methanol and absorbance was taken immediately at 517 nm. Different volumes of plant extract like 30 µl, 60 µl, 90 µl, 120 µl, 150 µl were taken, and the volume was made using methanol up to 150 µl uniformly. Each of the samples was diluted with methanol up to 3 ml, and 150 µl DPPH was added. After 15 min the absorbance was taken at 517 nm using methanol as blank on UV-visible spectrometer Shimadzu, UV-1601, Japan. IC_{50} values for HATP were then calculated and compared with value of Ascorbic acid, taking it as a positive control [8].

Super Oxide Free Radical Scavenging Activity: 100 µl riboflavin solution [20 µg], 200 µl EDTA solution [12 mM], 200 µl methanol and 100 µl NBT (Nitro-blue tetrazolium) solution [0.1 mg] were mixed in test tube, and the reaction mixture was diluted with phosphate buffer [50 mM] up to 3 ml. The absorbance of the solution was measured at 590 nm using blank (phosphate buffer) as after illumination for 5 min, and it is taken as control. Different volumes of 50 µl, 70 µl, 100 µl, 125 µl and 150 µl of samples of plant extract were taken and volume was made up to 150 µl by using methanol, to each of sample, 100 µl riboflavin, 200 µl EDTA, 200 µl methanol and 100 µl NBT was mixed in test tubes and it was diluted up to 3ml with phosphate buffer. Finally, absorbance was measured after illumination for 5 min at 590 nm on UV visible spectrometer Shimadzu, UV-1601, Japan. IC_{50} value were calculated and it was compared with ascorbic acid which was used as positive control in this assay [9].

Nitric Oxide Radical Scavenging Activity: Different volumes of plant extract like 50 µl, 60 µl, 70 µl and 90 µl were taken in separate tubes and the volume was uniformly made with methanol uniformly up to 150 µl, and 2.0 ml of sodium nitroprusside (10 mM) in phosphate buffer saline was added to each tube. The solutions were incubated at room temperature for 150 min. The similar procedure was repeated with blank by using methanol, which served as control. After the incubation, to each tube, including control, 5 ml of Griess reagent was added. The absorbance of chromophore formed was measured at 546 nm on UV-visible spectrometer Shimadzu, UV-1601, Japan. Curcumin was used as positive control IC_{50} values were compared by paired t-test [10].

RESULTS:

Acute Oral Toxicity Studies: CETC exhibited mortality at the dose of 2000 mg/kg, and METC did not cause any mortality in rats up to 5000 mg/kg b.w, respectively. None of the doses tested produced any apparent gross effect on general motor activity, muscular weakness, fecal output, feeding behavior, etc. during the period of observation. Thus 1/10th of toxic (200 mg/kg for chloroform extract and 500 mg/kg for methanolic extract) dose was selected for the further pharmacological study.

Sub-acute Toxicity: On administration of CETC and METC for 28 days at 3 dose levels namely 200 (low dose), 400 (mid-dose), 800 (high-dose) mg/kg body weight. No significant changes were observed in body weight, food consumption, or water intake when compared to control groups. No significant difference was observed RBC, WBC, and Hb count.

Effect of CETC & METC on Body Weight: No significant difference was observed in body weight.

TABLE 1: EFFECT OF CETC & METC ON BODY WEIGHT

Treatment	Initial body weight	Final body weight
Control	160	165±1.64
200 mg/kg b.w	145	142±1.26
400 mg/kg b.w	152	145±1.18
800 mg/kg b.w	155	154±0.79

All the values are expressed in mean ± SEM. Six animals in a group (n=6)

Effect of CETC & METC on Food and Water Intake: No observable change was observed in food and water intake.

Effect of CETC & METC on Hematological Parameters: No significant difference was observed in any of the parameters.

Effect of CETC and METC on *in-vitro* DPPH Free Radical Scavenging Activity: The free radical scavenging activity of various extracts is expressed in terms of percentage inhibition. The decrease in the percentage of inhibition shows increased absorbance. The decrease in optical absorbance at 517 nm after addition of the test compounds is measured. The percentage of DPPH radical scavenged for various extracts ranges from 15.60% (chloroform extracts) to a maximum of 88.42% as in methanolic extract. The CETC and METC extract exhibited moderately significant inhibition of DPPH activity, with a 50% inhibition (IC_{50}) at a concentration of 10.2 and 9.2 µg/ml, respectively. The IC_{50} value of methanolic extract was found to be nearer to the IC_{50} value of standard Ascorbic acid. The free radical scavenging is maximum with ascorbic acid > methanol > chloroform, which is given in **Table 3** and **Fig. 1**.

TABLE 2: EFFECT OF CETC & METC ON HEMATOLOGICAL PARAMETERS

Treatment	Hb (gm %)	RBC (10^6cells/cmm^3)	WBC (10^3/cells/cmm^3)
Control	10.42 ± 0.32	2.58 ± 0.11	7.03 ± 0.73
200mg/kg	11.72 ± 0.17	2.22 ± 0.85	7.17 ± 0.62
400mg/kg	11.61 ± 0.28	2.33 ± 0.12	7.23 ± 0.65
800mg/kg	11.85 ± 0.33	2.84 ± 0.11	2.84 ± 0.11

TABLE 3: DPPH FREE RADICAL SCAVENGING ACTIVITY

Conc. of extract/std (mcg/ml)	% Inhibition		
	Ascorbic acid	CETC	METC
2	31.62 ± 0.27	15.60 ± 0.31	31.26 ± 0.35
4	46.32 ± 0.36	28.44 ± 0.16	45.44 ± 0.29
8	91.19 ± 0.45	48.34 ± 0.45	47.36 ± 0.42
16	94.32 ± 0.78	57.80 ± 0.23	58.64 ± 0.47
32	95.48 ± 0.38	61.41 ± 0.25	81.36 ± 0.22
64	96.32 ± 0.30	61.85 ± 0.29	78.91 ± 0.38
128	96.45 ± 056	62.64 ± 047	88.54 ± 0.34
256	97.41 ± 0.43	62.36 ± 0.17	80.36 ± 0.23
512	94.21 ± 0.21	58.46 ± 0.57	88.42 ± 0.42

Effect of CETC and METC on *in-vitro* Superoxide Anion Scavenging Activity: The superoxide anion scavenging activity is expressed in terms of percentage inhibition. METC is found to possess good scavenging activity on superoxide anion at all concentrations. As the concentration increases, scavenging activity also increases. METC at concentrations ranging from 2 - 512 µg/ml inhibited the production of superoxide anion radical by 24.43% to 85.75%. On the other hand, the standard butylated hydroxyl toluene (BHT) showed significant scavenging activity in a dose-dependent manner. The greatest scavenging activity was observed by BHT at the concentration of 512 µg/ml, which effectively depressed the formation of superoxide anion by 96.72%. The maximum inhibition of superoxide anion produced by METC was observed at 512 µg/ml concentration and is 85.75% **Table 4**.

TABLE 4: SUPEROXIDE ANION FREE RADICAL SCAVENGING ACTIVITY

Conc. of extract/std (mcg/ml)	% Inhibition		
	Ascorbic acid	CETC	METC
2	22.98 ± 0.27	13.65 ± 0.31	24.43 ± 0.35
4	33.08 ± 0.36	15.08 ± 0.16	26.32 ± 0.29
8	44.32 ± 0.45	24.04 ± 0.45	30.03 ± 0.42
16	59.23 ± 0.78	27.76 ± 0.23	37.26 ±0.47
32	76.05 ± 0.38	31.64 ± 0.25	55.40 ± 0.22
64	82.91 ± 0.30	39.04 ± 0.29	65.30 ± 0.38
128	91.15 ± 056	45.45 ± 047	81.97 ± 0.34
256	93.80 ± 0.43	54.36 ± 0.17	83.52 ± 0.23
512	96.72 ± 0.21	57.54 ± 0.57	85.75 ± 0.42

The superoxide scavenging activity is least with CETC, which inhibited the production of superoxide radical by 13.65% to 57.54% in within the concentration 2-512 µg/ml respectively. The METC exhibited a moderately significant inhibition of superoxide anion scavenging activity, with a 50% inhibition (IC_{50}) at a concentration of 28.8 µg/ml which is found to be nearer to the IC_{50} value of standard BHT (11.2 µg/ml) whereas CETC shows less significant inhibition (IC_{50}) at 204.8 µg/ml. The free radical scavenging is maximum with BHT > methanol > chloroform, which is given in **Table 4** and **Fig. 2**.

Effect of CETC and METC on *in-vitro* Nitric Oxide Scavenging Method: The Nitric oxide anion scavenging activity is expressed in terms of percentage inhibition. The METC is found to possess good scavenging activity on nitric oxide anion at all concentration. METC as the concentration increased from 2-512 µg/ml inhibition of the production of nitric oxide anion radical also increased by 39.71% to 79.41% respectively. On the other hand, the standard ascorbic acid (ASC) showed a significant increase in scavenging activity in a dose-dependent manner. The greatest scavenging activity of ASC was observed at the concentration of 512 µg/ml, which effectively depressed the formation of nitric oxide anion by 85.04%. METC produced maximum inhibition of superoxide anion was observed at 512 µg/ml concentration and is 79.41% **Table 5**. The superoxide scavenging activity is least with CETC from the concentrations ranging from 2-512 µg/ml (32.14% - 54.14%) respectively. The METC exhibited a moderately significant inhibition of nitric oxide anion scavenging activity, with a 50% inhibition (IC_{50}) at a concentration of 12 µg/ml which is found to be nearer to the IC_{50} value of standard Ascorbic acid (2.6 µg/ml) whereas CETC shows less significant inhibition (IC_{50}) at 166.4 µg/ml. The IC_{50} value of methanolic extract was. The free radical scavenging is maximum with ASC > methanol > chloroform, which is given in **Table 5** and **Fig. 3**.

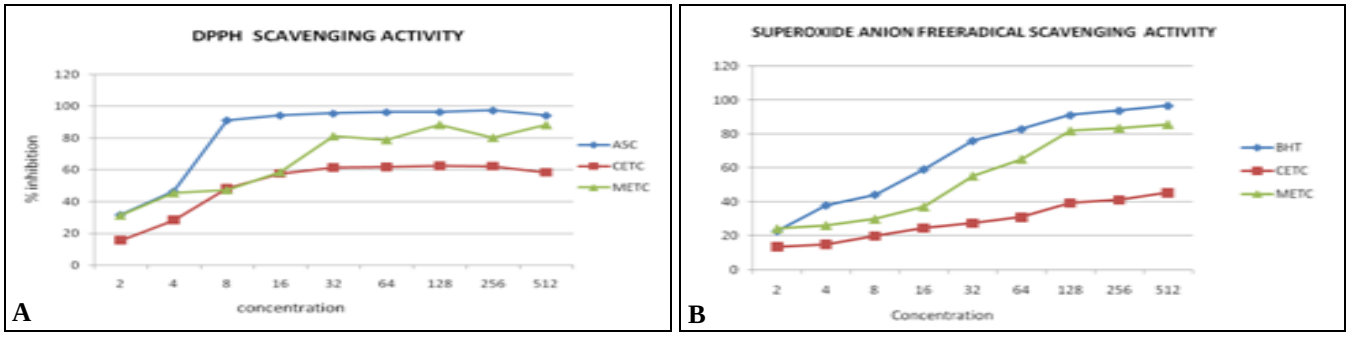

FIG. 1 AND 2: EFFECT OF CETC AND METC ON A) DPPH FREE RADICAL SCAVENGING ACTIVITY B) SUPEROXIDE ANION FREE RADICAL SCAVENGING ACTIVITY

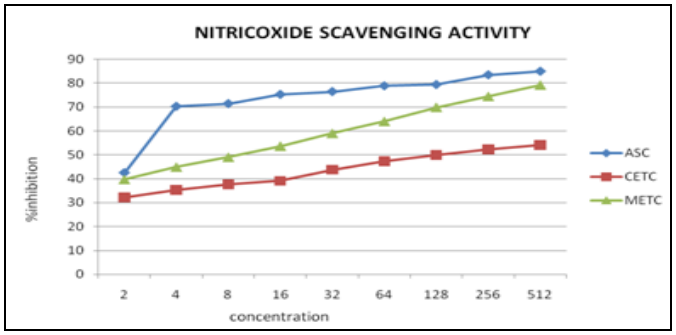

FIG. 3: EFFECT OF CETC AND METC ON NITRIC OXIDE SCAVENGING ACTIVITY

TABLE 5: NITRIC OXIDE SCAVENGING METHOD

Conc. of extract/std (mcg/ml)	% Inhibition		
	Ascorbic acid	CETC	METC
2	42.45 ± 0.27	32.14 ± 0.31	39.71 ± 0.35
4	70.32 ± 0.36	35.32 ± 0.16	44.86 ± 0.29
8	71.46 ± 0.45	37.61 ± 0.45	48.99 ± 0.42
16	75.31 ± 0.78	39.14 ± 0.23	53.64 ± 0.47

32	76.52 ± 0.38	43.78 ± 0.25	58.94 ± 0.22
64	78.93 ± 0.30	47.36 ± 0.29	63.94 ± 0.38
128	79.46 ± 056	49.90 ± 04	69.84 ± 0.34
256	83.54 ± 0.43	52.32 ± 0.17	74.36 ± 0.23
512	85.04 ± 0.21	54.14 ± 0.57	79.14 ± 0.42

DISCUSSION: DPPH is a stable free radical at room temperature and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The reduction capability of DPPH radicals was determined by the decrease in its absorbance at 517 nm, which is induced by antioxidants [5, 11]. The significant decrease in the concentration of the DPPH radical is due to scavenging ability in chloroform and methanolic extracts of *Tephrosia calophylla*.

Superoxide radical known to be very harmful to the cellular component. Superoxide free radical was formed by alkaline DMSO, which reacts with nitro blue tetrazolium (NBT) to produced colored diformazan. The HATP scavenge superoxide radical and thus inhibit formazan formation [12]. Significant scavenging activity of Superoxide radical was observed in chloroform and methanolic extracts of *Tephrosia calophylla.* Nitric acid was generated from sodium nitroprusside and measured by the Griess reduction. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions that can be estimated by the use of Griess reagent.

Scavengers of Nitric oxide compete with the oxygen, leading to reduced production of nitric oxide [13]. Significant scavenging activity of nitric oxide was observed in chloroform and methanolic extracts of *Tephrosia calophylla*.

CETC and METC treated rats was no significant reduction in body weight and a decrease in appetite. There were no obvious hemolytic changes in the serums of extract treated rats. Hence, we suggest that there are no clinical and side effects following the treatment with extracts. Preliminary phytochemical screening of the extract gave positive results for the presence of glycosides, flavonoids, saponins, and coumarins, which could be responsible for the antisecretory and cytoprotective action of this plant. The presence of these phytochemicals may be responsible for the gastroprotective action of the plant extract.

Hence, the present results showed that CETC and METC had no toxic effects and it shows safety with sub-acute toxicity also. Hence, the *Tephrosia calophylla* shows good antioxidant and cytoprotective activity.

CONCLUSION: The current study concludes that both extracts had no toxic effects, and it shows safety with sub-acute toxicity studies. The extracts of *Tephrosia calophylla* possess a potent *in-vitro* antioxidant and cytoprotective activity, suggesting its role as an effective free radical scavenger.

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