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Research Article

In vitro Antioxidant Activity, *In vivo* Skin Irritation Studies and HPTLC Analysis of *Cayratia trifolia* (L.) Domin

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ABSTRACT
Oxidative stress and inflammation are constant features of many chronic diseases and complications and it have been linked to carcinogenesis. Antioxidants from natural sources play a paramount role in serving endogenous antioxidants to neutralize oxidative stress. Thus, the aim of this study was to evaluate enzymatic and non-enzymatic antioxidant capacity and anti-skin irritation activity of *Cayratia trifolia* against perchloroethylene (PCE) induced wistar albino rats. The total alkaloid, flavonoid, phenol and tannin and antioxidant assays like, catalase, peroxidase, superoxide dismutase, vitamin C were estimated. Skin irritation was induced through wiping PCE in knee skin of experimental rats. After 30 days the rats were sacrificed and histopathological studies were done. Enzymatic and non-enzymatic antioxidant assays revealed that, the ethanolic extract of *Cayratia trifolia* posseses comparable antioxidant activity with standard drug. The HPTLC analysis shown that, tannin and phenol compounds present in this extract. The histopathological results, when compared with control, PCE induced groups showed mild inflammation whereas ethanolic extract of *Cayratia trifolia* treated groups showed compact inflammation. Based on the results, it can be concluded that, ethanolic extract of *Cayratia trifolia* perhaps a natural source of antioxidants. In future, it may be potential natural resources for pharmacology of functional foods.

Keywords: Medicinal plants, *Cayratia trifolia*, HPTLC analysis, Skin irritation, Perchloroethylene.

INTRODUCTION

Plants are the precious, incredible and traditional sources for the curability of various diseases in the form of medicines[1]. They are the main source of drugs that are being used from the ancient times as an herbal remedies for the health care, prevention and cure of various diseases and ailments[2]. They are rich sources of bioactive compounds and they serve as an important raw material for drug production and have become a target for the search of new drugs[3]. Phytochemicals are the natural bioactive compounds found in plants. The most important of these bioactive constituents of plants are alkaloids, tannins, flavonoids, steroids, terpenoids, carbohydrates and phenolic compounds[4]. These secondary metabolites have been implicated for most of the plant therapeutic activities[5]. The WHO has emphasized the need to ensure the quality of medicinal plant products by using modern controlled techniques and applying suitable standards[6]. HPTLC is a simple, rapid and accurate method for analyzing plant material[7]. HPTLC fingerprint has enhanced resolution and estimation of active constituents is done with reasonable accuracy in a shorter time. The HPTLC method can be used for phytochemical profiling of plants and quantification of compounds present in plants, through increasing demand for herbal products as medicines and cosmetics there is a vital need for standardization of plant products[8].

Skin is the largest human body organ, provides a major interface between the environment and the body and is constantly exposed to an array of chemical and physical environmental pollutants[9]. In addition, a large number of dietary contaminants and drugs can manifest their toxicity in skin[10]. These environmental toxicants or their metabolites are inherent oxidants were directly or indirectly drive the production of a variety of reactive oxidants also known as reactive oxygen species (ROS). ROS are short-lived entities that are continuously generated at low levels during the course of normal aerobic metabolism[11]. The harmful effects and biological damage caused by ROS and RNS is termed oxidative stress and nitrosative stress[12].

Natural antioxidants such as phenols, flavonoids and tannins are increasingly attracting attention because they are having natural qualities of disease-preventing, health-promoting and anti-ageing substances[13]. Antioxidants may serve the task of reducing oxidative damage induced by free radicals and reactive oxygen species under oxidative stress conditions in humans. These conditions can cause DNA and protein damage, lipid peroxidation, cancer, ageing and inflammatory activity[14]. Reactive oxygen species (ROS) such as superoxide anion,

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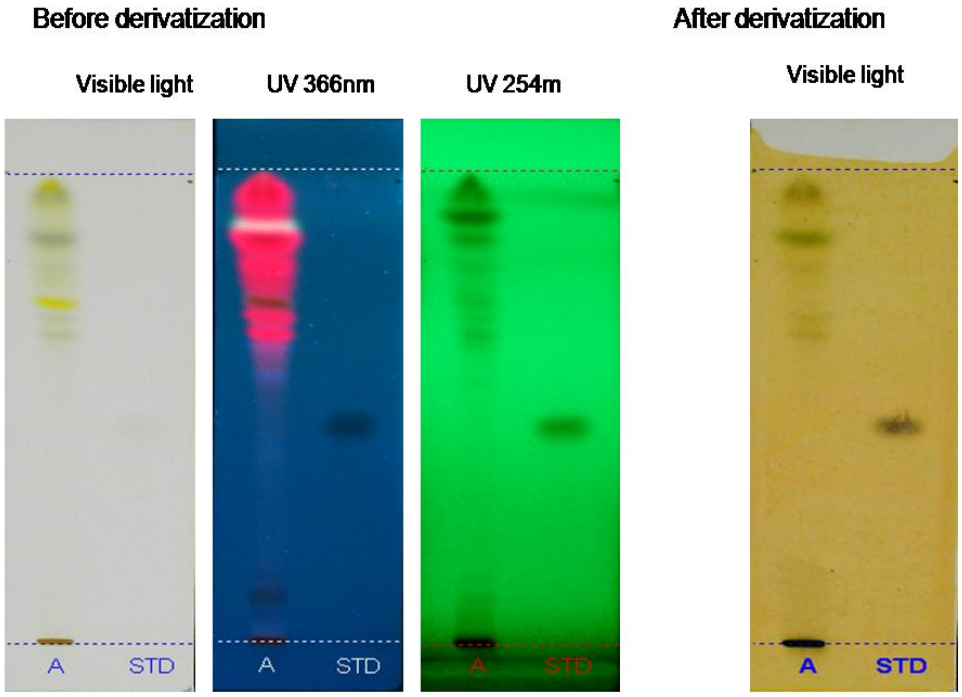


Fig 1. HPTLC Chromatogram of ethanolic extract of *Cayratia trifolia*

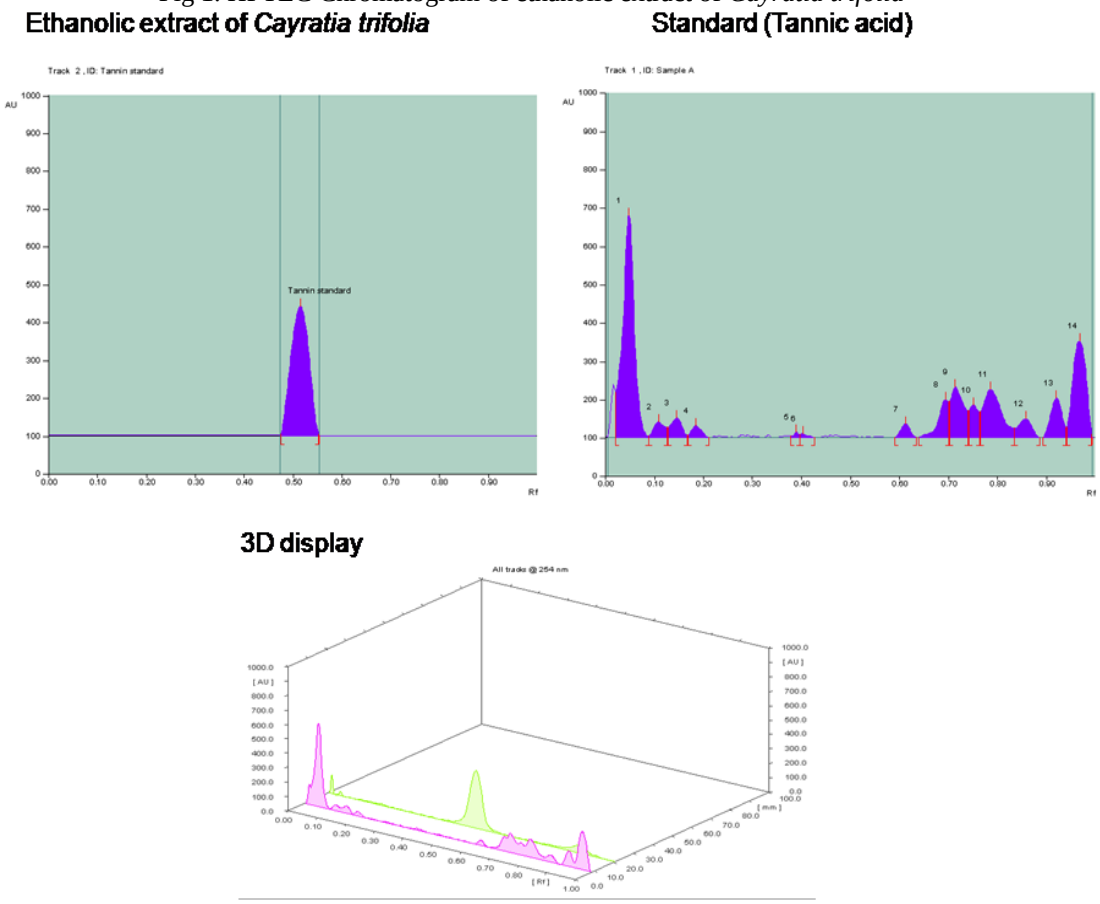


Fig 2. Densitogram and 3D display of Tannin of the ethanolic extract of *Cayratia trifolia*

hydroxyl radical and hydrogen peroxide formed *in vivo* are highly reactive and potentially damage transient chemical species. These are continuously produced in the human body, as they are essential for energy supply, detoxification, chemical signaling and immune function [(12)]. Recently, there has been an upsurge of interest in the therapeutic potential of medicinal plants as antioxidants in reducing free radical induced tissue injury[15].

Cayratia trifolia Linn. Domin syn (Vitaceae) is native of India, Asia and Australia. It is a weak herbaceous climber

Table 1. Quantitative analysis of secondary metabolites in crude ethanolic extract of *Cayratia trifolia*

S.No	Parameters	Content
1	Phenol (mg/g)	80.1±0.21
2	Tannin (mg/g)	98.3±0.47
3	Alkaloid (mg/g)	277.2±0.25
4	Flavonoid (mg/g)	60.4±0.45

Values are expressed as Mean±SD (n=3)

Table 2. Levels of enzymatic & non enzymatic antioxidants present in fresh sample of Cayratia trifolia

S.No	Parameters	Content
1	Catalase (µmole of H_2O_2 consumed/min/mg protein)	7.32±0.03
2	Peroxidase (µmoles/g sample)	10.2±0.25
3	Superoxide Dismutase (units/mg protein)	80.2±0.03
4	Glutathione peroxidase (µg of glutathione oxidized/min/mg protein;)	25.1±0.02
5	Vitamin C (µg/mg of plant tissue)	268.4±0.45

Values are expressed as Mean±SD (n=3)

having trifoliated leaves with (2-3 cm), long petioles and ovate to oblong-ovate leaflets. Flowers are small greenish white brown in color[16]. Fruits are fleshy, juicy, dark purple or black, nearly spherical and about 1 cm in diameter[17]. The whole plant is used as diuretic, in tumors, neuralgia and splenopathy, leucorrhea. The paste of tubers is applied on the affected part in the treatment of snake bite. It is reported to possess antiviral, antibacterial, antiprotozoal, hypoglycaemic, anticancer and diuretic activity etc[18]. This plant also contains kaempferol, myricetin, quercetin, triterpenes and epifriedelanol. The bark extract has been reported to have antiviral, antibacterial, antiprotozoal, hypoglycemic, anticancer, anti-inflammatory and diuretic activities in animal models[19]. The aim of the study is to investigate *in vitro* antioxidants, quantitative analysis, HPTLC fingerprinting analysis profiles and anti-skin irritation activity of *Cayratia trifolia* against perchloroethylene (PCE) induced wistar albino rats.

MATERIALS AND METHODS

Plant collection

Cayratia trifolia was collected from in and around area of Kumbakonam, Thanjavur district, Tamil Nadu, India. The plant was authenticated by Dr. P. Sathyanarayanan, Botanical Survey India, TNAU Campus, Coimbatore. The voucher number is BSI/SRC/5/23/2010-2011/Tech.1527. Fresh whole plant material was washed under running tap water, air dried and powdered[20].

Preparation of ethanolic extract

50 g of powdered plant material was weighed and extracted with 250 ml of ethanol for 72 hours using occasional shaker. The supernatant was collected and concentrated at 40°C. It was stored at 4°C in air tight bottles for further studies.

Preparation of fresh plant extract for *in vitro* antioxidant assays

The fresh plant material was prepared by grinding one gram of *Cayratia trifolia* in 2 ml of 50% ethanol, separately, in a pre-chilled mortar and pestle and the extracts were centrifuged at 10,000 g at 4ºC for 10 minutes. The supernatants were used within four hours for various enzymatic and non-enzymatic antioxidants assays.

Quantitative analysis of secondary metabolites

Determination of total alkaloid

The alkaloid content of sample was determined as described by[21]. 5 g of the sample was weighed into a 250 ml beaker and 200 ml of 10% acetic acid in ethanol was added and covered and allowed to stand for 4 h. The mixture was filtered through Whatman no 1 filter paper and the filtrate concentrated to ¼ of its original volume on a water bath maintained at 90°C. Alkaloid was precipitated from each sample, using a concentrated ammonium hydroxide solution (NH_4OH) and then allowed to sediment. The whole solution was allowed to

Table 3. Retention factor (R_f), height and area of peaks of Tannin

Track	Peak	Rf	Height	Area	Assigned substance
Sample A	1	0.05	579.4	12687.4	Unknown
Sample A	2	0.11	40.9	872.6	Unknown
Sample A	3	0.14	51.1	1102.0	Unknown
Sample A	4	0.18	30.9	586.2	Tannin 1
Sample A	5	0.39	13.9	135.3	Unknown
Sample A	6	0.40	10.4	131.4	Unknown
Sample A	7	0.61	35.9	626.5	Unknown
Sample A	8	0.69	98.9	1962.2	Tannin 2
Sample A	9	0.71	132.3	3208.5	Tannin 3
Sample A	10	0.75	85.7	1461.0	Unknown
Sample A	11	0.79	125.8	4298.9	Tannin 4
Sample A	12	0.86	48.7	1244.4	Tannin 5
Sample A	13	0.92	103.0	2207.3	Unknown
Sample A	14	0.97	252.1	6366.2	Tannin 6
STD	1	0.52	397.5	16766.2	Gallic acid

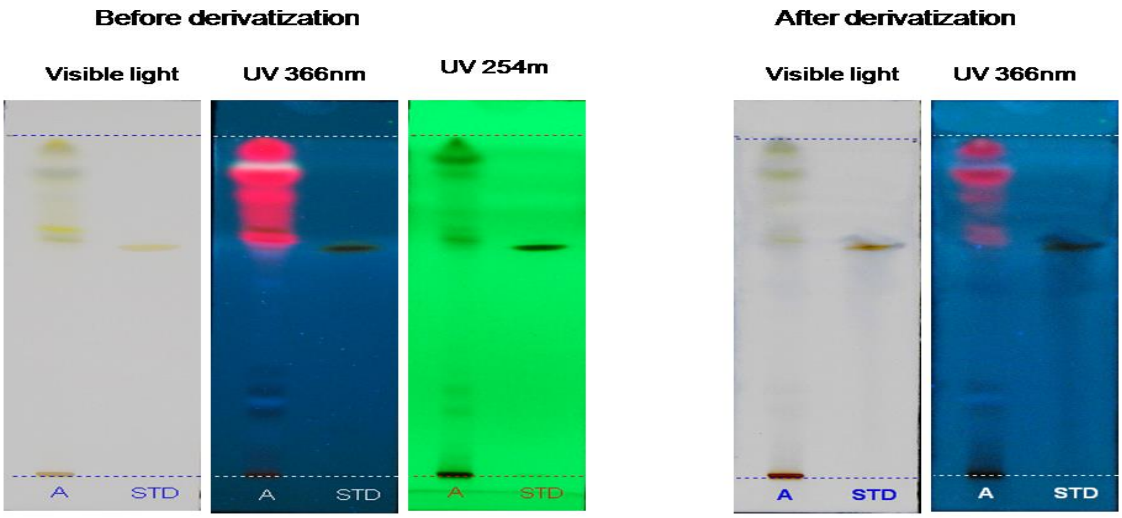


Fig 3. HPTLC Chromatogram of Phenol in ethanolic extract of *Cayratia trifolia*

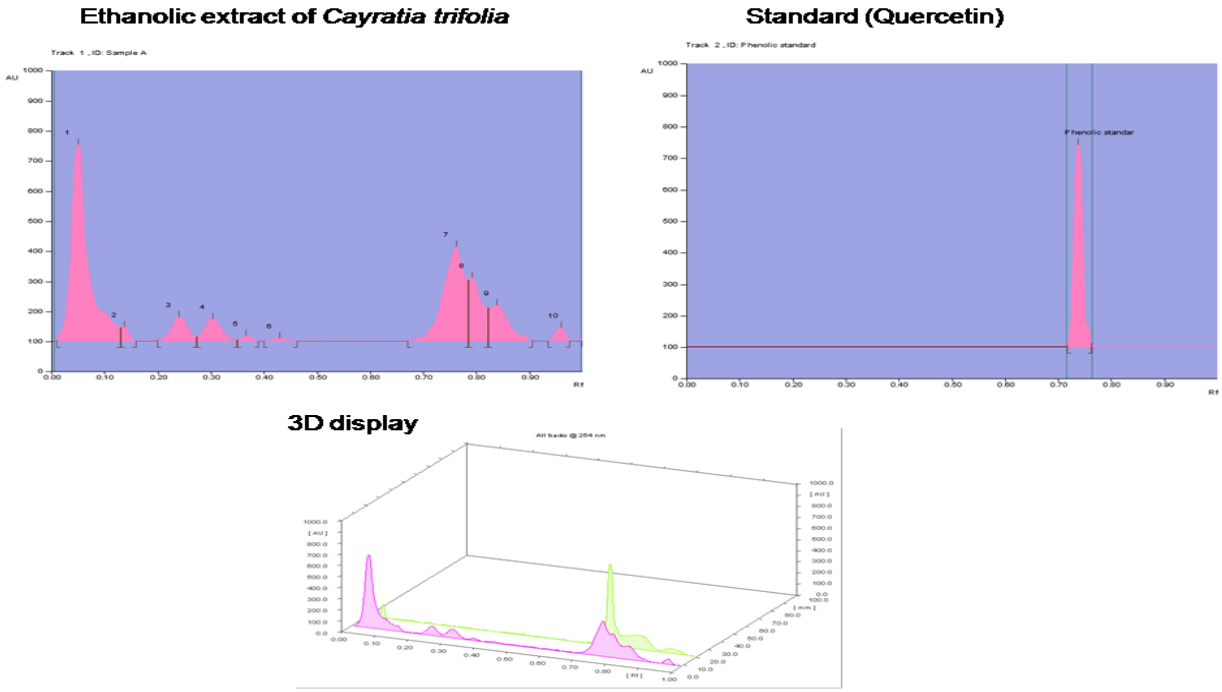


Fig 4. Densitogram and 3D display of Phenols in the ethanolic extract of *Cayratia trifolia*

settle and the precipitated was collected and washed with concentrated NH_4OH and then dried in a hot air oven.

The residue is alkaloid and is calculated thus: (%) Alkaloid = W2-W1 / W x 100,

Where, W1 = Initial weight before drying, W2 = Final weight after drying, W = weight of sample

Estimation of Total Flavonoid

Total flavonoid content was determined using the method of[22]. 0.5 ml of 2% $AlCl_3$ in ethanol solution was added to 0.5 ml of sample solution. After one hour incubation at room temperature, yellow colour was developed. This was measured at 420 nm with UV-Visible spectrophotometer. A standard graph was prepared using the quercetin and the total flavonoid content was expressed as quercetin equivalent (mg/g).

Estimation of Total Phenol

Total phenolic content was carried out by[23]. The sample extract (0.1 ml) was mixed with distilled water (3 ml) and 0.5 ml of Folin-Ciocalteu reagent was added. After 3 minutes 2 ml of 20% sodium carbonate was added and mixed thoroughly. The tubes were incubated in a boiling water bath for exactly one minute. It was then cooled and the absorbance was measured at 650 nm using spectrophotometer against the reagent blank. Standard curve of gallic acid solution (10, 20, 40, 60, 80 and 100 ppm) was prepared using the similar procedure and the results were expressed as mg of gallic acid equivalents (GAEs) per g of extract.

Estimation of Total Tannin

Total tannin content was determined in the method of[24]. 0.2-1.0 ml of standard tannic acid solution was pipetted out in to a series of test tubes. To another test tube 0.5 ml of extract solution was taken. The volumes of all the

Table 4. Retention factor (Rf), height and area of peaks of Phenols

Track	Peak	Rf	Height	Area	Assigned substance
Sample A	1	0.05	654.6	19647.7	Unknown
Sample A	2	0.14	48.5	660.8	Unknown
Sample A	3	0.24	80.8	2345.7	Phenolic 1
Sample A	4	0.30	75.3	2300.2	Unknown
Sample A	5	0.36	18.5	322.8	Unknown
Sample A	6	0.43	11.1	274.3	Unknown
Sample A	7	0.76	313.9	11579.5	Phenolic 2
Sample A	8	0.79	211.9	4821.2	Unknown
Sample A	9	0.84	119.6	4012.0	Phenolic 3
Sample A	10	0.96	45.1	791.5	Phenolic 4
STD	1	0.74	735.8	15586.9	Quercetin

tubes were made up to 3.0 ml with distilled water. 3.0 ml of distilled water was taken as blank. To all the tubes added 2.0 ml of 20% Na_2CO_3 followed by the addition of 0.5 ml of Folin-Ciocalteu reagent and incubated at room temperature for 30 minutes. The absorbance was read against reagent blank at 700 nm. From the standard graph, the amount of tannin present in the sample was calculated.

In vitro antioxidant activity

Assay of Superoxide Dismutase (SOD)

The assay of superoxide dismutase was done according to the method of[25]. In this method, 1.4ml aliquots of the reaction mixture (comprising 1.11 ml of 50 mM phosphate buffer of pH 7.4, 0.075 ml of 20 mM L-Methionine, 0.04ml of 1% (v/v) Triton X-100, 0.075 ml of 10 mM Hydroxylamine hydrochloride and 0.1ml of 50 mM EDTA) was added to 100µl of the sample extract and incubated at 30ºC for 5 minutes. 80 µl of 50 µM riboflavin was added and the tubes were exposed for 10 min to 200 W-philips fluorescent lamps. After the exposure time, 1ml of Greiss reagent (mixture of equal volume of 1% sulphanilamide in 5% phosphoric acid) was added and the absorbance of the color was measured at 543 nm.

Assay of Catalase (CAT)

Catalase activity was assayed by the method of[26]. The enzyme extract (0.5 ml) was added to the reaction mixture containing 1ml of 0.01 M phosphate buffer (pH 7.0), 0.5 ml of 0.2 M H_2O_2, 0.4 ml H_2O and incubated for different time period. The reaction was terminated by the addition of 2 ml of acid reagent (dichromate/acetic acid mixture) which was prepared by mixing 5% potassium dichromate with glacial acetic acid (1:3 by volume). To the control, the enzyme was added after the addition of acid reagent. All the tubes were heated for 10 minutes and the absorbance was read at 610 nm. Catalase activity was expressed in terms of µmoles of H_2O_2 consumed/min/mg protein.

Assay of Glutathione Peroxidase (GPx)

Glutathione peroxidase was assayed according to the method of[27] with slight modifications. The reaction mixture consisting of 0.4 ml of 0.4 M sodium phosphate buffer (pH 7.0), 0.1 ml of 10mM sodium azide, 0.2 ml of 4 mM reduced glutathione, 0.1 ml of 2.5 mM H_2O_2, 0.2 ml of water and 0.5 ml of plant extract was incubated at 0, 30, 60, 90 seconds respectively. The reaction was terminated with 0.5 ml of 10% TCA and after centrifugation; 2 ml of the supernatant was added to 3 ml of phosphate buffer and 1ml of DTNB reagent (0.04% DTNB in 1% sodium citrate). The colour developed was read at 412 nm and the enzyme activity is expressed in terms of µg of glutathione utilized/min/mg protein.

Figure 5- Skin irritation studies of male albino rats

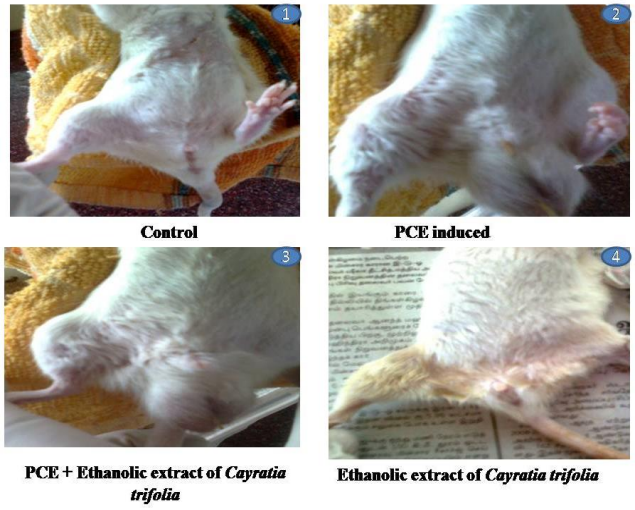
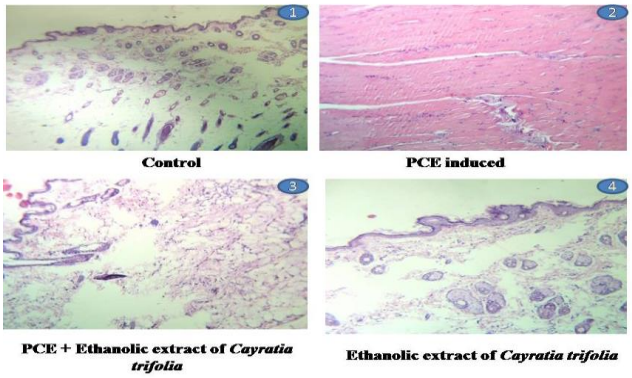


Figure 6- Histopathology studies of skin irritation activity in *Cayratia trifolia* against PCE induced rats



Assay of Peroxidase

The peroxidase assay was carried out by the method of[28]. The reaction mixture consisted of 3ml of buffered pyrogallol (0.05 M pyrogallol in 0.1 M phosphate buffer (pH 7.0)) and 0.5 ml of 1% H_2O_2. To this added 0.1 ml plant extract and O.D. change was measured at 430 nm for every 30 seconds for 2 minutes. The peroxidase

activity was calculated using an extinction coefficient of oxidized pyrogallol (4.5 litres/mol).

Estimation of Vitamin C

Vitamin C assay was determined by the method of[29]. The assay mixture for vitamin C consisted of 0.1 ml of brominated sample extract, 2.9 ml of distilled water, 1 ml of 2% DNPH reagent and 1-2 drops of thiourea. After incubation at 37ºC for 3 h, the orange-red osazone crystals formed were dissolved by the addition of 7 ml of 80% sulphuric acid and absorbance was read at 540 nm after 30 minutes. Vitamin C concentration was expressed in terms of µg/mg plant tissue.

HPTLC analysis

2 µl of ethanolic extract was loaded as 5mm band length in the 3 x 10cm Silica gel $60F_{254}$ TLC plate using Hamilton syringe and CAMAG LINOMAT 5 instrument. The sample loaded plate was kept in TLC twin trough developing chamber and the plate was developed with respective mobile phases (Tannin: Toluene-Ethyl acetate-Formic acid-Methanol at the ratio of 3 : 3 : 0.8 : 0.2, Phenols: Toluene-Acetone-Formic acid at the ratio of 4.5 : 4.5 : 1) up to 90mm. the developed plate was dried by hot air to evaporate solvents from the plate. The plate was kept in photo-documentation chamber (CAMAG REPROSTAR 3) and captured the images at visible light, UV 254nm and UV366nm. Derivatization: The developed plate was sprayed with respective spray reagent (Tannin: 5% Ferric chloride reagent, Phenols: 20% Sodium carbonate solution followed by Folin ciocalteu reagent) and dried at 100°C in Hot air oven. The plate was photo-documented in Visible light and UV 366nm mode using Photo-documentation (CAMAG REPROSTAR 3) chamber. Scanning: After derivatization, the plate was fixed in scanner stage (CAMAG TLC SCANNER 3) and scanning was done at UV 254nm for tannin and phenol.

Statistical Analysis

The results obtained were expressed as mean ±SD. The Statistical comparison among the groups were performed a statistical package program (SPSS 10.0).

In vivo skin irritation studies in albino rats

Experimental animals

Adult male albino rats weighing about 120-150 g were obtained from the animal house of Karpagam University, Coimbatore and used for the study. Rats were housed at constant temperature of 22±5°C with a 12-hour light, 12-hour dark cycle and fed on pellets with free access to tap water. All the experiments were carried out according to the guidelines recommended by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

Experimental designs

Group 1: Control rats

Group 2: PCE induced rats (wiped the rats skin with perchloroethylene dipped cotton)

Group 3: Treated as group II + 30mg/kg (b/w) of ethanolic extract of *Cayratia trifolia* (applied on knee skin)

Group 4: Ethanolic extract alone treated groups (30mg/kg b/w) (applied on knee skin)

Skin irritation was induced by wiping with perchloroethylene (PCE). After 30 days the animals were sacrificed under mild chloroform anesthesia. The knee tissue was excised immediately, cleaned free of extraneous material and perfused with ice cold saline (0.9%) solution, snap-frozen in liquid N_2 and stored at 80ºC until analysis.

RESULTS AND DISCUSSION

Natural antioxidants that are present in herbs and spices are responsible for inhibiting or preventing the deleterious consequences of oxidative stress[30]. Many of the naturally occurring antioxidant compounds like alkaloids, flavonoids were isolated from various medicinal plants[31]. Previous studies revealed that the *Cayratia trifolia* is medicinally important and used in the treatment of various diseases[32]. Total phenol, tannin, alkaloid and flavonoid contents were estimated in ethanolic extract of *Cayratia trifolia* which is showed in table 1. Alkaloid shows highest amount when compared with other secondary metabolites. Edible plant materials containing tannins are known to be astringent, and are used for treating intestinal disorders such as diarrhea and dysentery[33].

The significance of phenol compounds in plants as natural antioxidants and their use as substitutes to synthetic antioxidants in food additives is well known[34]. Flavonoids in plants have strong free radical scavenging properties. The multiple pharmacological properties of flavonoids, such as anti-inflammatory, antibiotic and cardiovascular activities are to a large extent, linked to their polyphenolic and therefore radical scavenging nature and act as primary antioxidants or free radical scavengers[35,36]. It is interesting to note that the phytochemicals alkaloids and flavonoids are commonly associated with various pharmacological activities of natural products[37]. Enzymatic and non enzymatic antioxidant activities were determined in fresh sample of *Cayratia trifolia*. The level of enzymatic antioxidants such as SOD, CAT, GPx, Peroxidase Vitamin C values showed in table 2.

The body posses defense mechanisms against free radical-induced oxidative stress, which involve preventative mechanisms, repair mechanisms, physical defenses and antioxidant defenses. Enzymatic antioxidant defenses include Superoxide Dismutase (SOD), Glutathione Peroxidase (GPx), Catalase (CAT) etc., whereas non-enzymatic antioxidants are ascorbic acid (vitamin C), vitamin E etc. All these act by one or more of the mechanisms like reducing activity, free radical-scavenging, potential complexing of pro-oxidant metals and quenching of singlet oxygen. It is potential to reduce the risks of chronic diseases and prevent disease progression by either enhancing the body's natural antioxidant defenses or by supplementing with proven dietary antioxidants[38].

HPTLC fingerprinting analysis of ethanolic extract of *Cayratia trifolia* was specified. Figure 1 and 2 shows the tannin profile of ethanolic extract of *cayratia trifolia* compared with standard Gallic acid along with peak

densitogram. Ethanolic extract of *Cayratia trifolia* contain six tannin compounds shown in table 3.

Figure 3 and 4 shows the phenol profile of ethanolic extract of *Cayratia trifolia* compared with standard quercetin along with densitogram. Ethanolic extract of *Cayratia trifolia* contain four phenols compounds shown in table 4.

Phenolic compounds are known to be a powerful chain breaking antioxidants and they possess scavenging ability due to their hydroxyl groups[39]. Tannins enhance glucose uptake and inhibit adipogenesis, thus being potential drugs for the treatment of non-insulin dependent diabetes mellitus[40,41]. In past few years tannins have also been studied for their potential effects against cancer through different mechanisms[42].

Figure 5 showed the skin irritation activity of ethanolic extract of *Cayratia trifolia*. Group 1 is treated as control, when compared to the group 1, perchloroethylene induced group (2) shows the hair fall. Perchloroethylene & ethanolic extract treated group (3) exhibited slight changes when compared with control group. Group 4 treated with ethanolic extract of *Cayratia trifolia* shows the good hair growth on rat skin.

The histopathological examination revealed extensive alterations in muscle and skin tissue of PCE induced skin irritation bearing rats. Figure 6 shows the histopathological changes in skin tissue of control and experimental animals. The histopathological observation of skin tissue in control (group 1), showed normal manner. Group 2 skin tissue bearing rats showing mild inflammatory cell Infiltration. The ethanolic extract & PCE treated (group 3) showing minimum inflammatory cell infiltration. However, rats treated with ethanolic extract alone (group 4) animals showed normal morphological appearances with mild solvent aberrations when compared to normal group.

Traditional herbal medicines used by different communities play an important role in alleviating such skin diseases[43]. Recent studies have shown that the polyphenols found in dietary and medicinal plants could inhibit oxidative stress by antioxidant mechanism[44]. Skin lesions resulting from contact with PCE are characterized by irritant reactions, profound erythema, local inflammation, even desquamative dermatitis and toxic epidermal necrosis[45]. SOD activities could represent significant enzyme depletion due to clearance of the free radicals inside the skin and thus indicate a high degree of free radical production and LPO occurrence. The anti-oxidative capacity is apparently damage d in the skin by PCE treatment, which would exacerbate toxic effects due to LPO. Supplementation of the skin with anti-oxidants such as vitamin C and vitamin E to enhance the skin's anti-oxidant capacity proves to be a valid approach, and the protective effect of topically applied anti-oxidants has been subject to intense investigation.

CONCLUSION

Based on all these studies it is suggested that this plant is a potential source of natural antioxidant and possess more secondary metabolites. The *Cayratia trifolia* plant shows the anti-inflammatory activity. Thus, the effective source of *Cayratia trifolia* could be employed in all medicinal preparations to combat diseases associated with oxidative stress including cancer, inflammation, diabetes and skin related disorders.

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