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Research Article

EXTRACTION, ISOLATION AND STRUCTURAL ELUCIDATION OF FLAVONOID FROM *CHROZOPHORA PLICATA* LEAVES AND EVALUATION OF ITS ANTIOXIDATIVE POTENTIALS

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ABSTRACT

Objective: This investigation involves the extraction, isolation, and characterization of flavonoid from a Euphorbiaceae family plant *Chrozophora plicata* followed by evaluation of its antioxidant principles.

Methods: The dried leaves were subjected to sequential soxhlation with polar and nonpolar solvents. Methanolic extract reveals the presence of large amount of flavonoids. Methanolic extract was subjected to isolation using column chromatographic analysis with solvents such as petroleum ether, chloroform, hexane, ethyl acetate, methanol, and water. Further, the isolated compound was subjected to thin layer chromatography technique and spectral analysis such as infrared, ^{1}HNMR, ^{13}CNMR, mass spectroscopy, and high performance thin layer chromatography (HPTLC) finger printing techniques. The compound was evaluated for *in vitro* antioxidant studies using 2,2-diphenyl-1-picrylhydrazyl (DPPH), NO assay, reducing power assay, H_2O_2 scavenging assay, superoxide anion scavenging assay and β-Carotene linoleate system and *in vivo* antioxidative studies using carbon tetrachloride (CCl_4), and acetaminophen intoxicated rats.

Results: The compound was isolated in methanol:water in the ratio of 80:20 using column chromatographic technique. On the basis of phytochemical, chromatographic, and spectral analysis, the isolated compound was identified as kaempferol and finally with HPTLC finger printing technique it was found that the Rf value of the isolated compound was found to be 0.58 which is nearly similar to the Rf value of standard kaempferol (0.55). Hence, the isolated compound was confirmed as kaempferol and is structurally elucidated as 3,5,7-trihydroxy-2-(4-hydroxyphenyl)chromen-4-one. This compound was isolated for the first time from the *C. plicata* leaves. The *in vitro* antioxidant assay of isolated flavonoid has shown a dose-dependent increase in free radical scavenging activity using DPPH, no assay, reducing power assay, H_2O_2 scavenging assay, superoxide anion scavenging assay, and β-carotene linoleate system. Further, the methanolic extract of *C. plicata* (MECP) leaves was subjected to single dose acute toxicity study for 14 days in female rats on the basis of OECD guidelines 423 and the therapeutically selected doses were 200 mg/kg and 400 mg/kg. *In vivo* antioxidant studies in CCl_4 and acetaminophen intoxicated rats indicated that the MECP leaves have significantly decreased lipid peroxidation in a dose-dependent manner and increased the levels of catalase, superoxide dismutase, and glutathione.

Conclusions: By the above results, it was concluded that the isolated compound from *C. plicata* leaves was confirmed as kaempferol and it possesses significant antioxidative potentials.

Keywords: *Chrozophora plicata* leaves, Flavonoids, Extraction, Isolation, Characterization, Methanolic extract, Antioxidant activity, Carbon tetrachloride, Acetaminophen.

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INTRODUCTION

Herbal drugs have become an essential part of native medical systems across the globe [1]. Traditional people have a long history of using herbal drugs for treatment of certain diseases [2]. Blind dependence on allopathic medicines has been gradually decreased, and majority of the population are leaning toward the herbal drugs hoping their ability to eradicate the diseases from the root and also due to their outstanding safety [3]. The world population depends on the usage of herbal medicines to an extent of three-quarter ratio of individuals [4]. Due to the universal health benefits of flavonoids reported in various ailments, there has been an increasing curiosity in the research of flavonoids from plant sources [5,6]. Flavonoids are believed to possess health beneficial properties as a result of high antioxidant potentials in both *in vitro* and *in vivo* systems [7,8]. Numerous reports have suggested the protective effects of flavonoids against degenerative diseases such as cancers, cardiovascular diseases, and age-related disorders as well as in many infections (bacterial and viral diseases) [9,10]. *Chrozophora plicata* also known as Suryavarta in India is a medicinal plant which is one of the ingredients in the well-known Indian herbal tonic Safi. Safi is considered to be a potent blood purifier. With the above background, this research was planned to extract, isolate and characterize a flavonoid from the leaves of the plant *C. plicata* belonging to the family Euphorbiaceae and to establish its antioxidant activity by *in vitro* and *in vivo* methods. The plant *C. plicata* has been reported to contain flavonoids [11,12]. The leaves of the plant possesses a wide range of medicinal properties such as anti-inflammatory, antiulcer, anthelmintic, hepatoprotective, gastroprotective activities, for skin diseases and in asthma and bronchitis [13]. Since, there is no literature evidence regarding extraction, isolation, characterization, and antioxidant activity of flavonoids from *C. plicata* leaves, this research is essential and justifiable.

METHODS

For this study, the leaves of *C. plicata* plant were collected from the vicinity of the Gajwel (mandal), Medak dist, Telangana, India. Few leaves of *C. plicata* were deposited in a polythene bag. The sample specimen is kept in fresh condition by adding 2% formalin. Plant material was identified and authenticated by Dr. N. Sivaraj, Senior Scientist (Eco Botany), National Bureau of Plant Genetic Resources, Rajendranagar, Hyderabad. The study protocol was approved by Institutional Animal

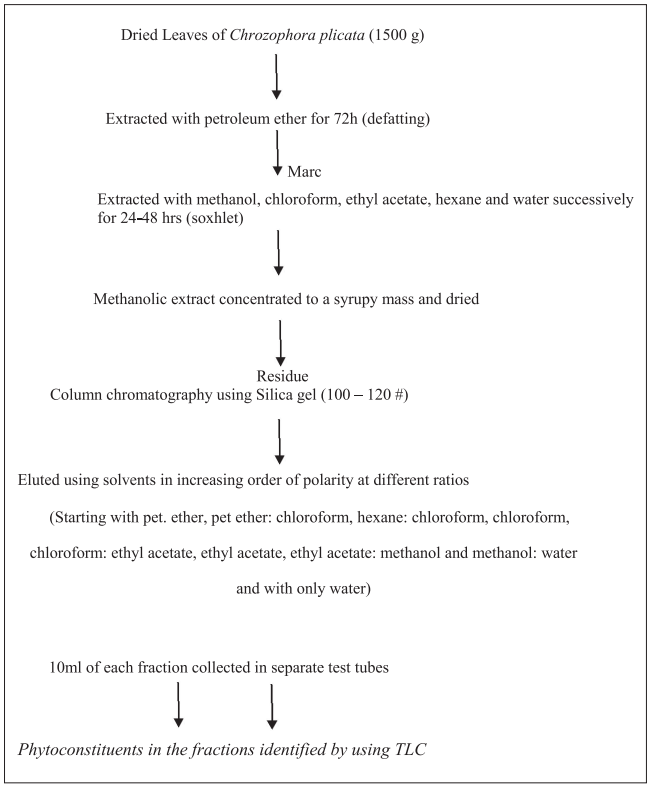

Chart 1: Procedure involved in isolation of flavonoids from chrozophora plicata leaves by using column chromatography

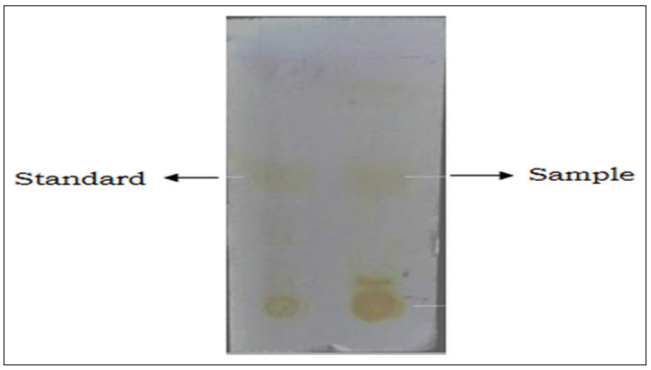

Fig. 1: Thin layer chromatographic analysis of isolated compound from *Chrozophora plicata* leaves and its comparison with standard flavonoid kaempferol

Ethical Committee (IAEC) of Vijaya College of Pharmacy, Hyderabad (1292/ac/09//CPCSEA). The leaves of *C. plicata* were shade dried and mechanically reduced to a coarse powder. The weight of the coarse powder was around 1500 g. The powder was subjected to hot continuous successive extraction in a Soxhlet apparatus with solvents in the increasing order of polarity using petroleum ether, chloroform, hexane, ethyl acetate, methanol, and water under controlled temperature (50-60°C) [14]. Extractives were concentrated below 40°C and further drying was carried out under reduced pressure. The six dried extractives were stored in a dessicator for further evaluation [15]. The extracts were subjected to qualitative tests for detection of phytoconstituents present in it, *viz*., alkaloids, carbohydrates, glycosides, phytosterols, fixed oils and fats, phenolic compounds and tannins, proteins and free amino acids, gums and mucilages, flavonoids, lignins, and saponins [16]. The methanolic extract revealed the presence of a large amount of flavonoids. Hence, methanolic extract of *C. plicata* (MECP) leaves was selected for further isolation and investigations. Silica gel 100-200 mesh was made into a homogenous suspension by shaking with petroleum ether (first eluent). The bottom of the column was plugged with little cotton to prevent the adsorbent pass out, and then the silica gel suspension was poured into the column, set aside for 10 minutes and used. The column 30 cm length and 2.5 cm in diameter were washed with the suitable solvent and dried. The dried column was filled with petroleum ether up to two-third of the column length. Slurry of activated silica gel (column grade 100-200 mesh) prepared using petroleum ether was poured into the column and allowed to settle down, care was taken to avoid any air space or bubble during packing. The silica gel was packed up to three-fourth of the column length, and the solvent level was maintained 5 cm above the silica layer to avoid cracking and air entrapment. A known amount of crude MECP was dissolved in a small quantity of suitable solvent to form clear solution. To this clear solution, 10 g of activated silica gel was added and mixed thoroughly. The solvent was then dried off completely, and the sample adsorbed silica gel was uniformly placed on top of the column, care was taken that the solvent level is always maintained 1cm above the layer of silica gel. After stabilizing a filter paper disc was carefully placed on top of the silica gel.

All the collected fractions were subjected to thin layer chromatography (TLC) and the fractions with similar Rf values were combined. The major fractions were purified by recrystallization. All the fractions collected by column chromatography were subjected to TLC analysis [17,18]. The spots obtained on TLC plate with chrozophora plicata methanolic extract and with standard flavonoid kaempferol is shown in Fig. 1. Benzene:Acetone:Formic acid (5:4:1 v/v/v) is used as the mobile phase. Among all the spots, spots with fraction methanol:water (80:20) were clear and distinct. These fractions were combined and concentrated using Rotavapor apparatus to get a residue and were subjected for purification [19]. The schematic representation of the procedure involved in the isolation of flavonoids from chrozophora plicata leaves is shown in Chart 1. Further, the isolated compound was compared with standard flavonoids. The compound was spectrally characterized using FT-IR, ^{1}HNMR, ^{13}CNMR, mass spectroscopy, and high performance TLC (HPTLC).

Characterization of the isolated compound

To characterize the isolated compound IR, ^{1}HNMR, ^{13}CNMR, mass spectroscopy, and HPTLC finger printing methods are employed. A small quantity of the sample (1-3 mg) was pressed in KBr (3-5 mg) pellets using Fourier transform infrared spectroscopy (Perkin Elmer). The spectrum was recorded with the wave number 4000-400 cm^{-1}. ^{1}HNMR and ^{13}CNMR were recorded on Bruker Avance-300 DRX NMR Spectrometer using dimethyl sulfoxide (DMSO) as solvent. Mass spectroscopy converts molecules into ions and according to their mass and charge the ions can be separated and sorted. The mass spectrometer used for this purpose was SHIMADZU (SHIMADZU ACCUSPOT). HPTLC analysis was performed with CAMAG LINOMAT 5 instrument, and the images were observed at white light, UV 254 nm, and UV 366 nm. The spectras obtained from IR, 1H NMR, 13 C NMR, Mass spectroscopy and HPTLC finger printing techniques are shown in Figs. 2-9.

***In vitro* antioxidant activity of isolated flavonoid from *C. plicata* leaves**

2,2-diphenyl-1-picrylhydrazyl (DPPH) assay[20]

The free radical scavenging activity of isolated flavonoid from MECP leaves was determined using Blois method. To 1 ml of 0.1 mM solution of DPPH in methanol add 3 ml of different concentrations of isolated flavonoid of *C. plicata* leaves (5, 10, 25, 50, and 100 µg/ml). Reference standard used is ascorbic acid (100 µg/ml). All the experiments were performed in triplicate. The percentage inhibition was calculated using the formula.

$$\% \text{ inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

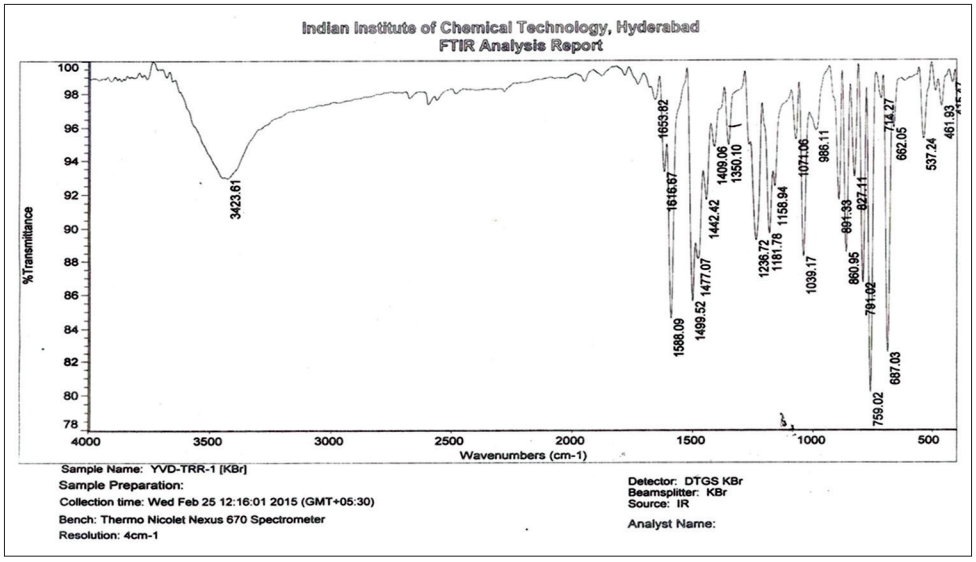

Fig. 2: Infrared spectra of isolated compound from *Chrozophora plicata* leaf

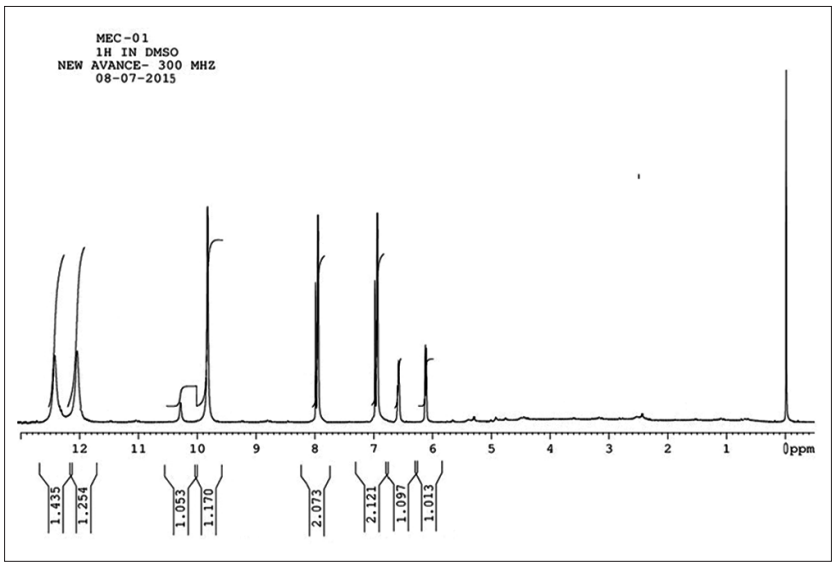

Fig. 3: ^{1}HNMR spectra of isolated compound of *Chrozophora plicata* leaf

Nitric oxide scavenging activity

Sodium nitroprusside (2 ml) in phosphate buffer (pH 7.4) is mixed with 0.5 ml of various concentrations of isolated flavonoid of *C. plicata* leaves ranging from 5-100 µg/ml dissolved in methanol. The mixtures were incubated at 25°C for 150 minutes at room temperature. The same reaction mixture without isolated flavonoid from *C. plicata* served as control. After incubation, 0.5 ml of Griess reagent was added to 0.5 ml of each sample and was incubated for further 30 minutes at room temperature. The absorbance of the chromophore was measured at 546 nm. This experiment was done in triplicate, and the % inhibition was calculated using the following formula,

$$\% \text{ inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Reducing power assay

The reducing power of isolated flavonoid form *C. plicata* leaves was determined by Oyaizu method [21]. 1ml of distilled water containing different concentrations of isolated flavonoid of *C. plicata* leaves (20, 40, 60, 80, and 100 µg/ml) was mixed with phosphate buffer (2.5 ml, 0.2 M, and pH 6.6), potassium ferricyanide (2.5 ml, 1%), and incubated at 50°C for 20 minutes. After incubation, a portion (2.5 ml) of 10% trichloroacetic acid was added and centrifuged at 3000 rpm for 10 minutes 2.5 ml of supernatant solution, i.e., the upper layer was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%) and the absorbance was measured at 700 nm. Sodium metabisulfite was used as reference standard. The test was performed in triplicate. Absorbance is directly proportional to the reducing power. Increase in absorbance of the reaction mixture suggests increase in reducing power. The percentage increase in absorbance was calculated using the following formula,

$$\% \text{ increase in absorbance} = \frac{\text{Control OD} - \text{Test OD}}{\text{ControlOD}}$$

Hydrogen peroxide scavenging activity

The ability of the substance to scavenge hydrogen peroxide was determined according to the method designed by Ruch *et al*. [22,23]. 1 ml of various concentrations of isolated flavonoid from *C. plicata* leaves (50-250 µg/ml) were mixed with 2 ml of 20 mM hydrogen peroxide in phosphate buffer saline (pH7.5) and incubated for 10 minutes. The absorbance was read at 230 nm against phosphate buffer saline blank.

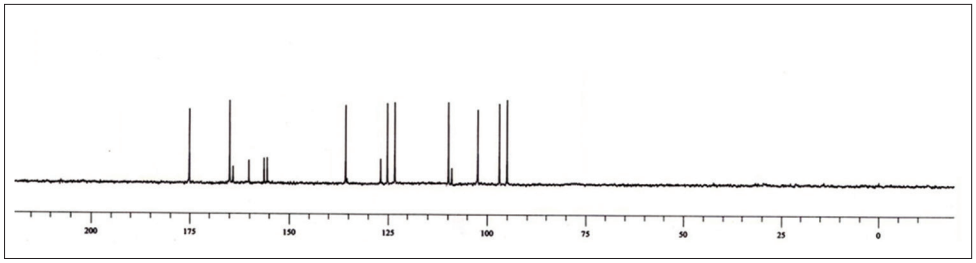

Fig. 4: ^{13}CNMR spectra of isolated compound of *Chrozophora plicata* leaf

The experiment was done in triplicate. The data were represented as % inhibition. Ascorbic acid was used as reference standard.

$$\% \text{ increase in absorbance} = \frac{\text{Control OD} - \text{Test OD}}{\text{Control OD}}$$

Superoxide anion radical scavenging assay

The assay is based on the ability of the isolated compound to inhibit formazon formation by scavenging the superoxide radicals generated in riboflavin-light-NTB system [24,25]. 100 µl riboflavin (2 µm), 200 µl methionine (13 mM), 200 µl ethylenediaminetetraacetic acid (EDTA) (100 µm), and 100 µl NBT (75 µm) and 1 ml of isolated flavonoid from *C. plicata* leaves (100, 200, 300,400, and 500 µg/ml) was mixed and it was then diluted to 3 ml with sodium phosphate buffer. The production of formazon was followed by reading the absorbance at 560 nm after a 10 minutes illumination from a fluorescent lamp. A reaction mixture was kept in identical tubes at dark served as blanks. The percentage inhibition was calculated.

β-carotene linoleate model [26]

The antioxidant activity by β-carotene linoleate model was according to the method of Kartal *et al.* [27]. 2 mg of β-carotene was dissolved in 10 ml of chloroform, and 2 ml of the above solution was transferred into 100 ml round bottom flask. Chloroform was removed under vacuum and then 40 mg of linoleic acid, 400 mg of Tween-40, and 100 ml of distilled water (aerated) were added. 4.8 ml of this emulsion (aliquots) was added to test tubes containing an isolated compound of *C. plicata* leaves (100, 200, 300, 400, and 500 µg/ml) in 2 ml, immediately the zero time absorbance was measured at 470 nm. After incubating the tubes at 50°C for 2 hrs, the absorbance was measured again. A blank was prepared without β-Carotene and absorbance was measured. The same procedure was repeated with butylated hydroxytoluene (BHT).

Antioxidant activity = (β-carotene content after 2 hr of assay/initial β - carotene content) ×100

Acute toxicity studies [28,29]

The Acute toxicity study was performed on the basis of OECD guidelines 423 and fixed dose studies were conducted where the limit dose is 2000 mg/kg body weight of test rats. The study protocol was approved by IAEC of Vijaya College of Pharmacy, Hyderabad (1292/ac/09//CPCSEA). The Procedure was divided into two phases, phase I (observation made on day 1) and phase II (observed the animals for next 14 days). The rats were dosed at 100, 250, 500, 700, 1000, and 2000 mg/kg of MECP leaves. Individual rats were observed for 4 hrs for behavior, autonomic and neurological symptoms or mortality. Body weights were recorded 6 hrs post dosing. From next day onward, each day every 1 hr the behavioral change, toxic signs or mortality was observed in the same animals for next 14 days, and body weights were recorded on 8th and 14th day post dosing. In the absence of lethality, 1/10th and 1/5th of the higher dose was selected as a therapeutic dose.

***In vivo* antioxidant studies**

In vivo, antioxidant studies were conducted in carbon tetrachloride (CCL_4) and paracetamol intoxicated rats. The effect of MECP leaves on antioxidant enzymes (catalase, superoxide dismutase (SOD), and glutathione) and lipid peroxidation was studied.

***In vivo* antioxidant studies of MECP leaves in CCL_4 intoxicated rats [30,31]**

Groups: Animals were divided into seven groups containing six animals each.

- Group I: Untreated control group (1% liquid paraffin 1 ml/kg s.c as vehicle) (-ve control).
- Group II: Hepatotoxin control (+ve control) group (vehicle only for 7 days (s.c) followed by 1 ml/kg b.w CCl4:liquid paraffin (1:1) s.c on 7th day).
- Group III: Standard group (100 mg/kg silymarin) once daily for 7 days (p.o) followed by 1 ml/kg b.w CCl_4:liquid paraffin (1:1) s.c on 7th day.
- Group IV: MECP leaves (200 mg/kg, b.w) daily for 7 days p.o followed by 1 ml/kg b.w CCl_4:liquid paraffin (1:1) on 7th day.
- Group V: MECP leaves (400 mg/kg, b.w) daily for 7 days p.o followed by 1 ml/kg b.w CCl_4:liquid paraffin (1:1) s.c on 7th day.

Effect of MECP leaves against acetaminophen intoxicated rats [32]

- Groups: Animals were divided into seven groups containing six animals each.
- Group I: Untreated control group (-ve control) (2% w/v acacia suspension)
- Group II: Positive control group (toxic control) (vehicle for 7 days + paracetamol 2 g/kg b.w (p.o) on 5th day) [33].
- Group III: Standard group (Silymarin 100 mg/kg b.w daily for 7 days and paracetamol 2 g/kg b.w on the 5th day).
- Group IV: Received MECP leaves (200 mg/kg b.w dose daily for 7 days p.o and paracetamol 2 g/kg b.w p.o on the 5th day).
- Group V: Received MECP leaves (400 mg/kg b.w dose daily for 7 days p.o and paracetamol 2 g/kg b.w p.o on the 5th day).

Isolation of liver

Animals were sacrificed on 8th day by mild ether anesthesia for the isolation of liver. Liver was isolated and washed thoroughly with ice-cold saline solution. The liver weight was recorded after blotting filter paper pads. The liver homogenate was prepared and used for further *in vivo* antioxidant studies.

Lipid peroxidation (malondialdehyde)

1 ml of solution D was added to 500 µl of liver homogenate and boiled for 15 minutes. After cooling, the solution mixture was subjected to centrifugation at 10,000 rpm for 5 minutes. The absorbance of clear supernatant was measured at 532 nm against reference blank. The malondialdehyde content was estimated as thiobarbituric acid reactive substances (TBARS) using the formula, C=A/єt

C= Concentration of sample (TBARS), A= absorbance of sample

Є = Molar extinction coefficient of MDA (1.56×10^5/moles/cm)

t = Path length

Catalase [34,35]

Catalase activity was measured in by mixing 100 µl of 10% liver homogenate with 1.9 ml of phosphate buffer (pH 7), and the absorbance

was measured at 240 nm. Add 1ml of 10 mM H_2O_2 solution and after 1 minute reading was against taken. Catalase activity was calculated using the formula.

$$\text{Catalase unit per ml of sample} = \frac{(\Delta A_S - \Delta A_O) \times 3XDF}{\in \times 2}$$

$$\text{Catalase unit per mg of tissue} = \frac{\text{Catalase unit per ml of sample}}{\text{Mg of tissue per ml}}$$

Where,
ΔA_S: Absorbance difference of sample
ΔA_0: Absorbance of control
DF: Dilution factor
Є: Molar coefficient of H_2O_2

SOD [36]
To 100 µl of 10% w/v liver homogenate, add 1 ml of Na_2CO_3, 400 µl nitro blue tetrazolium and 200 µl EDTA. The absorbance was measured at 560 nm. 400 µl of hydroxylamine hydrochloride was added to initiate the reaction and incubated for 5 minutes at 25°C. The reduction of NBT was recorded after 5 minutes at 560 nm. One unit of enzyme activity of SOD is defined as the concentration of enzyme present in 100 µl of 10% liver homogenate capable of inhibiting the reduction of NBT by 50% under assay conditions and is expressed as units/mg of tissue.

SOD= 1/50 x DF x 1/ 0.1 x 1/mg of tissue/ml

Glutathione [37]
To 1 ml of 10% w/v liver homogenate, add distilled water (1.8 ml) and 2 ml of phosphate buffer (pH 7) and the absorbance was read at 412 nm. 5 minutes after adding 0.2 ml of DTNB reagent, the intensity of yellow color was measured at 412 nm. The amount of glutathione was determined using molar extinction coefficient of 13,060/m/cm and expressed in terms of µ mol/mg of protein. It can be calculated using the following formula, C=A/єt.

RESULTS AND DISCUSSION

Chromatographic techniques for isolation and spectral analysis for characterization of *C. plicata* leaves have not been reported until today according to literature survey. MECP leaves were subjected for isolation using column chromatography. Among all the fractions collected, fractions (80-84) showed identical spots with an Rf values of 0.75, 0.53, 0.26, and 0.17 using Benzene:Acetone:Formic acid (5:4:1 v/v/v) as the mobile phase. Among all these spots, spots with Rf value of 0.75 showed major spot. These fractions were combined and concentrated using Rotavapor apparatus to get a residue and were subjected for purification. The residue showed three minor spots and a major spot in TLC. To the residue, 15 ml of acetone was added and mixed thoroughly and filtered. The procedure was followed twice; the filtrates collected were mixed and subjected for TLC. The filtrate showed two spots one major spot at 0.46 and minor spot at 0.82. To the filtrate, 5 ml of ether was added. Precipitation occurred during addition of ether. The precipitate was filtered and dried and subjected for TLC. The TLC showed single spot at Rf value 0.56 using Benzene:Acetone:Formic acid (5:4:1 v/v/v) as the mobile phase. It was yellowish-green substance designated as the compound. Further, the isolated compound was subjected to TLC and compared with standard flavonoid kaempferol.

Solvent system:Benzene:Acetone:Formic acid (5:4:1 v/v/v).

Spraying agent: Iodine vapors.

Rf values: 0.56 (standard kaempferol), 0.54 (compound).

Spectral characteristics of the isolated compound of *C. plicata* leaf: IR (KBr) spectrum
The data obtained from IR spectral analysis of isolated compound indicates the various functional groups present in the isolated compound. The data are as follows:

FT-IR (KBr) cm^{-1}: 3423.61 (OH), 1653.82 (C=0), 1039.17 (Phenolic C-O), and 1588.09 (aromatic C=C).

^{1}HNMR spectral analysis
Proton NMR spectra (300 MHZ, DMSO-d6) δ (ppm) showed the peaks at δ 12.4(1H, s, OH-5), 12.1(1H, s, OH-7), 10.3 (1H, s, OH-4'), 9.8 (1H, s, OH-3), 7.9 (2H, d, H-2', 6'), 6.9 (2H, d, H-3', 5'), 6.6 (1H, s, H-8), and 6.1 (1H, s, H-6). The δ values at 12.4, 12.1, 10.3, and 9.8 indicate the presence of four OH protons. Protons attached to the C-2' and C-6' positions were assigned a doublet at 7.9. Protons attached to the C-3' and C-5' positions were assigned a doublet at 6.9. Similarly, protons attached to C-6' and C-8' were assigned two singlets at *δ* values 6.6 and 6.1.

^{13}CNMR characterization of isolated compound of *C. plicata* leaf
The ^{13}CNMR spectrum showed signals at δ values 156 (C-2), 136 (C-3), 175 (C-4), 165 (C-5), 97 (C-6), 164 (C-7), 95 (C-8), 160 (C-9), 103 (C-10), 123 (C-1'), 126 (C-2'), 110 (C-3'), 155 (C-4'), 101 (C-5'), and 125 (C-6').

Mass spectral analysis
The molecular ion peak M+ of the isolated flavonoid was observed at m/e 286 and is shown in the Fig. 5.

HPTLC finger printing
MECP leaves and isolated compound from MECP leaves were subjected to HPTLC determination and finally compared with the standard HPTLC of flavonoid kaempferol. 2.0 µl of the test extract, isolated compound, and 2.0 µl of standard flavonoid were dissolved in methanol and loaded as 5 mm band length in the 4×10 silica gel 60F254 TLC plate using Hamilton syringe and CAMAG LINOMAT 5 instrument. The sample loaded plate was then developed with 10 ml of solvent system, Benzene:Acetone:Formic acid (5:4:1) in TLC twin trough developing chamber (after saturated with solvent vapor) and the plate was

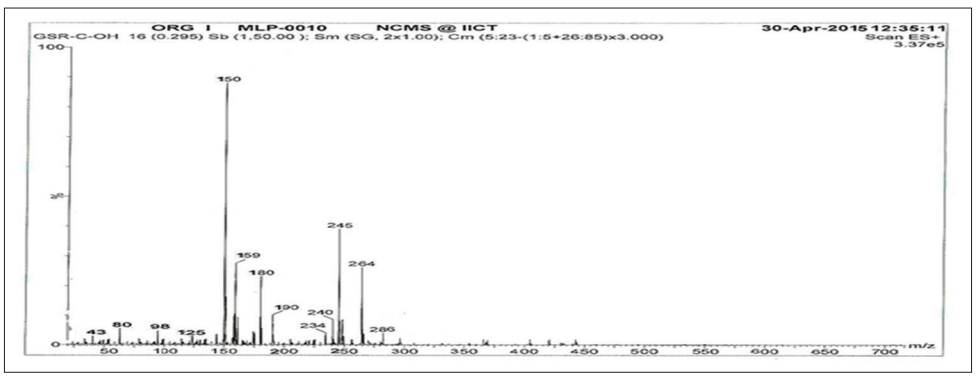

Fig. 5: Mass spectra of isolated compound of *Chrozophora plicata* leaves

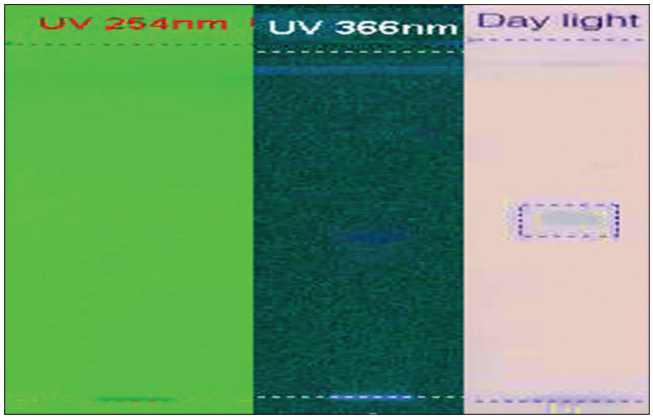

Fig. 6: *Chrozophora plicata* leaf methanolic extract plant sample peak display (scanned at 254 nm)

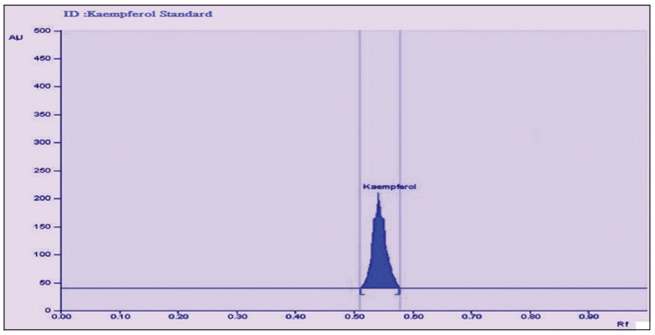

Fig. 7: Kaempferol standard peak densitogram display (scanned at 254 nm)

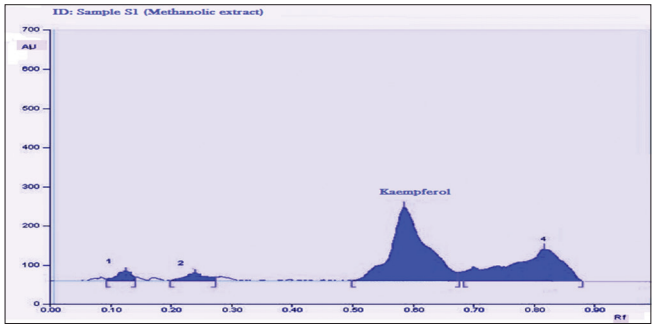

Fig. 8: *Chrozophora plicata* leaf sample (S1) methanolic extract plant sample peak densitogram display (scanned at 254 nm)

developed in the respective mobile phase up to 90 mm. The developed plate was subjected to drying by hot air oven at 100°C to evaporate solvents from the plate. The plate was placed in photo documentation chamber (CAMAG REPROSTAR 3) and images are captured at white light, UV 254 nm, and UV 366 nm. The peak table, peak display, and peak densitogram were recorded.

It is evident from the above HPTLC chromatograms that the standard kaempferol peak is seen at Rf 0.55 and the peaks of *C. plicata* leaf and isolated compound is seen at Rf values 0.56 and 0.58 which are nearly similar to the Rf value of standard kaempferol. This indicates that the isolated compound from *C. plicata* leaves was identified as kaempferol. The spectral data obtained from the FT-IR, ^{1}HNMR, ^{13}CNMR, mass spectroscopy, and HPTLC shows that the isolated compound from *C. plicata* leaves has similar resemblance with kaempferol. Hence, the isolated compound from *C. plicata* leaves was confirmed as kaempferol and its structure is presented below. The structure of the isolated compound is shown in the figure 10.

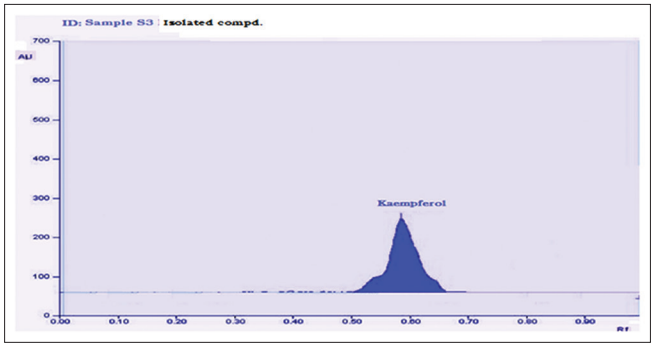

Fig. 9: Isolated compound from *Chrozophora plicata* leaf sample peak densitogram display (scanned at 254 nm)

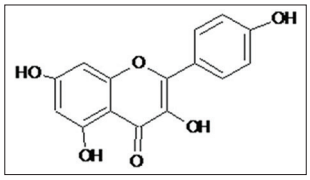

Fig. 10: Structure of isolated flavonoid from *Chrozophora plicata* leaves (3,5,7-trihydroxy-2-(4-hydroxyphenyl)chromen-4-one) (kaempferol)

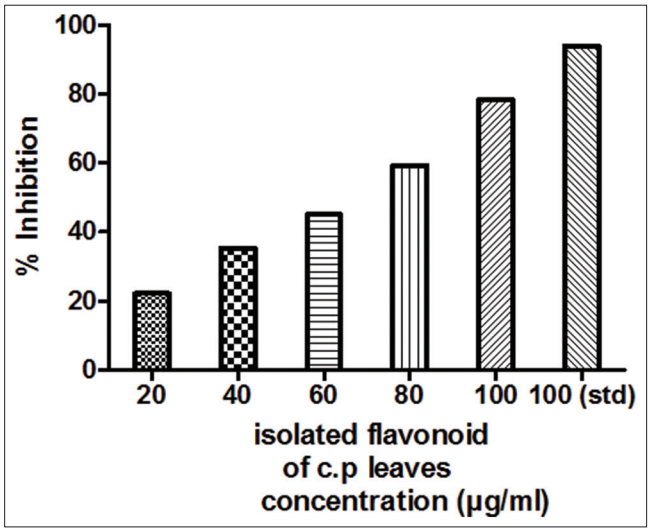

Fig. 11: Graphical representation of 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity of isolated flavonoid of *Chrozophora plicata* leaves

***In vitro* antioxidant study: DPPH (2, 2-Diphenyl-1-picryl-hydrazyl) assay**

In this assay, isolated flavonoid of *C. plicata* leaves has shown a dose-dependent increase in the DPPH radical scavenging activity. Ascorbic acid (100 µg) has exhibited 94.12% activity. However, 100 µg/ml of isolated flavonoid has shown maximum scavenging activity, i.e., 78.27. The results are summarized in Table 1 and graphically depicted in Fig. 11. The inhibitory concentration (IC_{50}) value is also shown in Table 1.

Nitric oxide radical scavenging activity

In this experiment, it is observed that the isolated flavonoid of *C. plicata* leaves has shown a significant dose-dependent increase in the nitric oxide anion scavenging property. Ascorbic acid (100 µg) has shown 84.64% activity. However, 100 µg of isolated flavonoid of *C. plicata* leaves has shown maximum scavenging activity, i.e., 69.76%. The results are shown in Table 2 and graphically depicted in Fig. 12. The IC_{50} value is also shown in Table 2.

Reducing power assay

From the above assay, it is observed that the isolated flavonoid of *C. plicata* leaves has exhibited dose-dependent increase in the reducing property. Standard sodium metabisulfite (100 µg) has shown 92.6% activity. Isolated flavonoid at 20, 40, 60, and 80 µg has shown steady increase in reducing property, but there is a sudden increase in the activity at concentration of 100 µg (70.11%). The results are summarized in Table 3 and graphically depicted in Fig. 13.

Hydrogen peroxide scavenging activity

Isolated flavonoid from *C. plicata* leaves exhibited dose-dependent H_2O_2 scavenging activity. Standard ascorbic acid (100 µg) has shown 77.84% activity. Isolated flavonoid from *C. plicata* leaves exhibited maximum activity at 250 µg (71.28) with IC_{50} value of 166 µg. The results are summarized in Table 4 and graphically depicted in Fig. 13. The results are graphically depicted in Fig. 14. The IC_{50} value is also shown in Table 4.

Superoxide anion scavenging activity

It was observed that the isolated flavonoid from *C. plicata* leaves scavenge the superoxide anion in dose-dependent manner. Ascorbic acid (100 µg) has shown 97.15% activity. However, 500 µg of isolated flavonoid of the leaf extract has shown maximum scavenging activity, i.e., 79.65%. The results are summarized in Table 5 and depicted in Fig. 15. The IC_{50} value is also shown in Table 5.

Antioxidant activity in β-carotene linoleate system

It was found that the isolated flavonoid of *C. plicata* leaves has shown significant dose-dependent antioxidant activity. BHT (100 µg) has shown 95.75% activity. However, 500 µg of isolated flavonoid of leaf extract has shown maximum scavenging activity, i.e., 74.68 but less than that of standard. The results are shown in Table 6 and graphically depicted in Fig. 16. The IC_{50} value is also shown in Table 6.

Table 1: DPPH radical scavenging activity of isolated flavonoid of *C. plicata* leaves

Sample	Concentration (µg/ml)	DPPH free radical inhibition (%)	IC_{50} (µg/ml)
Isolated flavonoid of *C. plicata* leaves	20	22.11±0.004*	67
	40	35.26±0.006*	
	60	45.14±0.002*	
	80	59.35±0.008*	
	100	78.27±0.004**	
Standard (ascorbic acid)	100	94.12±0.003**	

C. plicata: Chrozophora plicata, Data and results are expressed as mean±SEM. *p<0.05, **p<0.01, were considered statistically significant when compared to control. DPPH: 2,2-diphenyl-1-picrylhydrazyl, IC_{50}: Inhibitory concentration

Table 2: Nitric oxide radical scavenging activity of isolated flavonoid of *C. plicata* leaves

Sample	Concentration (µg/ml)	Nitric oxide radical inhibition (%)	IC_{50} (µg/ml)
Isolated flavonoid of *C. plicata* leaves	20	20.26±0.001*	70
	40	27.73±0.005*	
	60	43.47±0.003*	
	80	58.91±0.012*	
	100	69.76±0.007**	
Standard (ascorbic acid)	100	84.64±0.008**	

Data and results are expressed as mean±SEM. *p<0.05, **p<0.01, were considered statistically significant when compared to control. *C. plicata*: *Chrozophora plicata*, IC_{50}: Inhibitory concentration

Acute toxicity studies

Acute toxicity studies of MECP leaves were conducted as per OECD guidelines 423. The methanolic extract was given to female rats at doses 100, 250,500, 700, 1000, and 2000 mg/kg using oral gavage did not reveal any signs of toxicity or adverse effects. The rats were observed twice daily for 14 days did not revealed any drug-related toxic signs

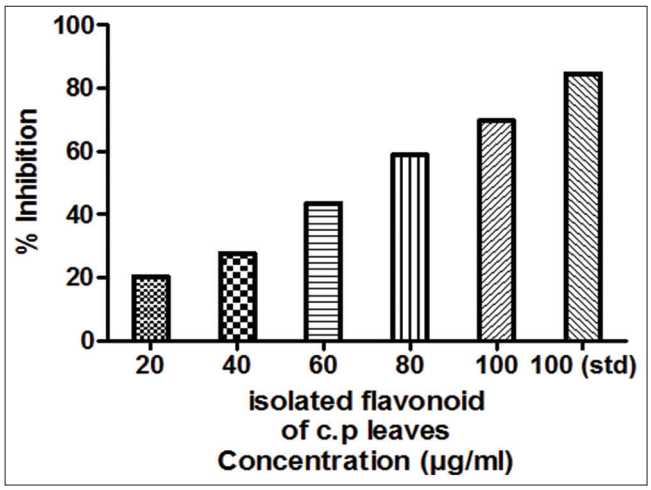

Fig. 12: Graphical representation of nitric oxide scavenging activity of isolated flavonoid of *Chrozophora plicata* leaves

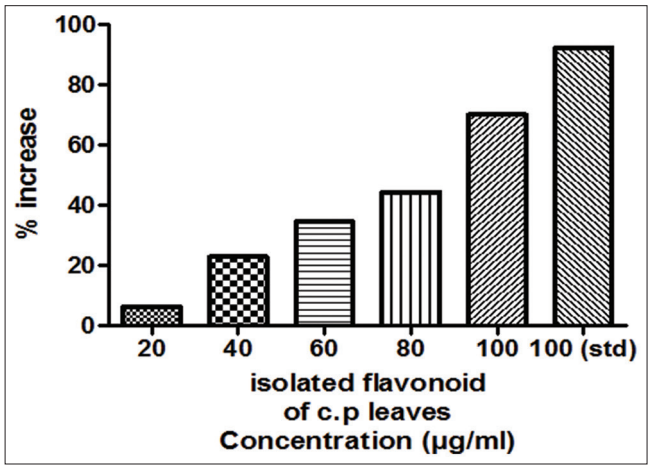

Fig. 13: Graphical representation of reducing power activity of isolated flavonoid from *Chrozophora plicata* leaves

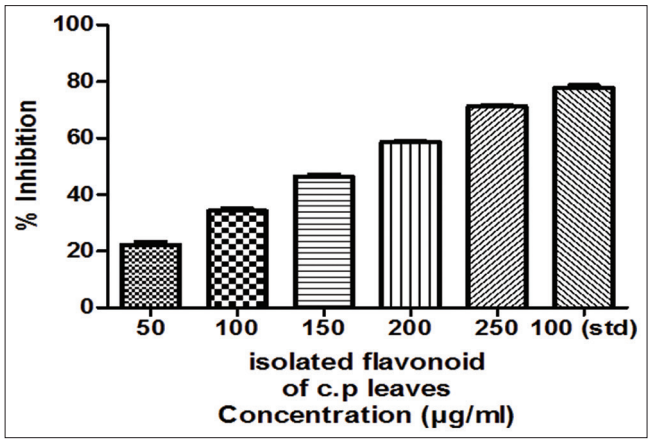

Fig. 14: Graphical representation of hydrogen peroxide scavenging activity of isolated flavonoid from *Chrozophora plicata* leaves

Table 3: Reducing power activity of isolated flavonoid of *C. plicata* leaves

Sample	Concentration (µg/ml)	Absorbance (mean±SEM)	Percentage increase	IC_{50} (µg/ml)
Control	-	0.1625±0.027	-	-
Isolated flavonoid of *C. plicata* leaves	20	0.1728±0.033*	6.25	88
	40	0.1994±0.026*	22.84	
	60	0.2186±0.017*	34.59	
	80	0.2342±0.001*	44.43	
	100	0.2751±0.046*	70.11	
Standard (sodium metabisulfite)	100	0.3054±0.021**	92.26	-

C. plicata: Chrozophora plicata, Data and results are expressed as mean±SEM. *p<0.05, **p<0.01, were considered statistically significant when compared to control. IC_{50}: Inhibitory concentration

Table 4: Hydrogen peroxide scavenging activity of isolated flavonoid from *C. plicata* leaves

Sample	Concentration (µg/ml)	Scavenging of hydrogen peroxide (%)	IC_{50} (µg/ml)
Isolated flavonoid of *C. plicata* leaves	50	22.25±1.1031*	166
	100	34.28±0.916*	
	150	46.41±0.812*	
	200	58.72±0.305*	
	250	71.28±0.526**	
Standard (ascorbic acid)	100	77.84±0.921**	

Data and results are expressed as mean±SEM. *p<0.05, **p<0.01, were considered statistically significant when compared to control. *C. plicata: Chrozophora plicata*, IC_{50}: Inhibitory concentration

Table 5: Superoxide anion scavenging activity of isolated flavonoid from *C. plicata* leaves

Sample	Concentration (µg/ml)	Superoxide anion radical inhibition (%)	IC_{50} (µg/ml)
Isolated flavonoid of *C. plicata* leaves	100	20.86±0.453*	332
	200	32.24±1.003*	
	300	46.30±0.675*	
	400	55.05±0.403*	
	500	79.65±0.712**	
Standard (ascorbic acid)	100	97.15±0.925**	

Data and results are expressed as mean±SEM. *p<0.05, **p<0.01, were considered statistically significant when compared to control. *C. plicata: Chrozophora plicata,* IC_{50}: Inhibitory concentration

Table 6: Antioxidant activity of isolated flavonoid of *C. plicata* leaves in β-carotene linoleate system

Sample	Concentration (µg/ml)	Antioxidant activity (%)	IC_{50} (µg/ml)
Isolated flavonoid of *C. plicata* leaves	100	13.76±1.63*	315
	200	30.75±0.841*	
	300	46.73±0.672*	
	400	64.62±0.57*	
	500	74.68±0.982**	
Standard (BHT)	100	95.75±0.586**	

BHT: Butylated hydroxytoluene, *C. plicata: Chrozophora plicata*, Data and results are expressed as mean±SEM. *p<0.05, **p<0.01, were considered statistically when compared to control. significant, IC_{50}: Inhibitory concentration

or mortality. Hence, the acute oral LD_{50} of the methanolic extract was concluded to exceed 2000 mg/kg body weight. Therefore, 2000 mg/kg

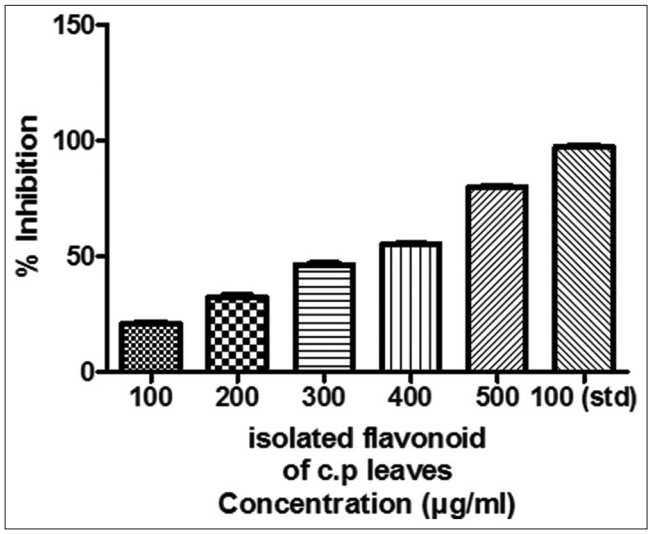

Fig. 15: Graphical representation of superoxide anion scavenging activity of isolated flavonoid from *Chrozophora plicata* leaves

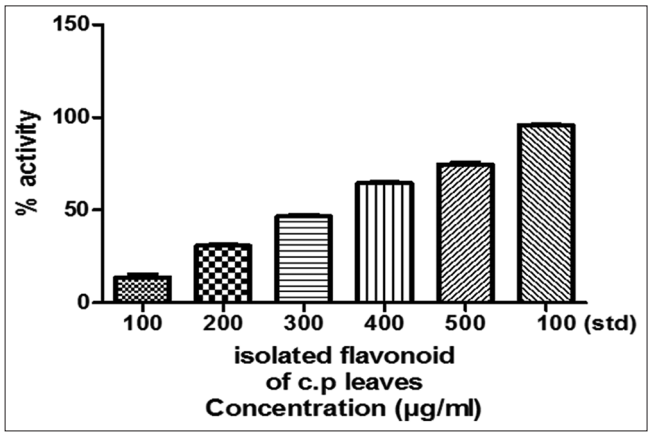

Fig. 16: Graphical representation of antioxidant activity of isolated flavonoid of *Chrozophora plicata* leaves in β-carotene linoleate system

was considered as safest higher dose and 1/10th, i.e., 200 mg/kg and 1/5th of 2000 mg/kg, i.e., 400 mg/kg was selected for the further studies.

***In vivo* antioxidant studies**

CCl_4 treated rats

CCl4 significantly increased the level of lipid peroxidation and reduced catalase, SOD, and glutathione levels in toxicant animals by 52.62%, 44.55%, and 45.22%, respectively. Lipid peroxidation was significantly decreased (p<0.001) with MECP leaves (MECP) at 200 mg and 400 mg/kg. Catalase levels were significantly increased by *C. plicata*

Table 7: *In vivo* antioxidant activity of methanolic extract of *C. plicata* leaves in CCl_4 treated rats

Groups	Lipid peroxidation (µ mol MDA/mg of protein)	Catalase (unit/mg of tissue)	SOD (unit/mg of protein)	Glutathione (µ mol/mg of protein)
Normal control	0.302±0.0113	9.837±0.5132	2.841±0.015	20.31±0.4512
Positive control (CCl_4)	1.365±0.1082	4.628±0.3164	1.726±0.1472	11.14±0.4154
Standard (silymarin) 100 mg/kg	0.4314±0.018***	8.276±0.2516***	2.814±0.1306***	17.39±0.3872***
MECP (200 mg/kg)	1.092±0.0371**	4.731±0.1836	2.164±0.0326**	12.75±0.5186***
MECP (400 mg/kg)	0.7108±0.0521***	6.438±0.1584***	2.653±0.134***	15.14±0.6172***

Values are expressed as mean±SEM. Data compared against positive control group. One-way analysis of variance. **p<0.01, ***p<0.001 were considered statistically significant when compared to control. using Tukey-Kramer multiple comparison test

Table 8: *In vivo* antioxidant activity of methanolic extract of *C. plicata* leaves in acetaminophen-treated rats

Groups	Lipid peroxidation (µ mol MDA/mg of protein)	Catalase (unit/mg of tissue)	SOD (unit/mg of protein)	Glutathione (µ mol/mg of protein)
Normal control	0.3014±0.0104	9.745±0.418	2.856±0.163	21.08±0.012
Positive control (acetaminophen)	1.117±0.054	5.173±0.216	1.789±0.605	12.57±0.673
Standard (silymarin)	0.3912±0.116***	8.216±0.178***	2.736±0.042***	16.85±0.105***
MECP (200 mg/kg)	0.7610±0.027*	6.581±0.169**	2.268±0.085**	13.61±0.513**
MECP (400 mg/kg)	0.5284±0.132**	6.965±0.386***	2.564±0.087***	15.02±0.306***

Values are expressed as mean±SEM. Data compared against positive control group. One-way analysis of variance. *p<0.05, **p<0.01, ***p<0.001 using Tukey-Kramer multiple comparison test

methanolic extract at 400 mg/kg, whereas the levels of catalase were not significantly increased with 200 mg/kg. SOD and glutathione levels were significantly increased with MECP at both 200 mg/kg and 400 mg/kg. Silymarin exhibited significant effect on all the parameters at the dose of 100 mg/kg. The results are depicted in Table 7.

Acetaminophen treated rats

Acetaminophen significantly enhanced the level of lipid peroxidation and decreased the levels of catalase (48.6%), SOD (38.6%), and glutathione (35.8%) in hepatotoxin group. Lipid peroxidation was significantly reduced (p<0.001) with MECP at 200 mg/kg and 400 mg/kg. Catalase levels were significantly increased by MECP at 200 mg/kg and 400 mg/kg (p<0.001). SOD and glutathione levels were increased significantly with IFCP (p<0.01 for 200 mg/kg and p<0.001 for 400 mg/kg). Silymarin exhibited significant effect on all the parameters. The results are depicted in Table 8.

CONCLUSION

In the above research, we conclude that the flavonoid from *C. plicata* leaves was successfully isolated and on the basis of spectral analysis it was confirmed as 3,5,7-trihydroxy-2-(4-hydroxyphenyl)chromen-4-one (kaempferol). Further, *in vitro* and *in vivo* antioxidant studies of isolated kaempferol and MECP leaves reveals that the compound has potent antioxidant property.

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