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FREE RADICAL SCAVENGING ACTIVITY OF NEW COUMARIN COMPOUNDS ISOLATED FROM *CEROPEGIA JUNCEA* (ROXB.)

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ABSTRACT
Plants are the source of natural products which act as models for new pharmacologically active compounds. Coumarin and its derivatives were all considered as phenylpropanoids. Many phenolic compounds were reported to be effective in therapeutic properties. Among them coumarin, is a compound found naturally in plants with broad biological functions. The plant *Ceropegia juncea* (Roxb.) is extracted and isolated for coumarins and were evaluated for their anti-oxidant activities (*in vitro* and *in vivo*). The results showed the percentage of scavenging activity of test coumarin on free radical generation. Coumarin scavenges DPPH radical in a dose dependent manner (1.0-300 µg/ml).

KEYWORDS: Coumarin, Ceropegia, Antioxidant, Free radical scavenging.

INTRODUCTION
Traditional knowledge of medicinal plants has always been used as a valuable guide in the quest for new medicines. The pharmacological actions of crude drugs from medicinal plants were determined by the nature of their constituents. Alkaloids, Tannins, Phenols, Flavonoids, Steroids, Coumarins etc, were some of the chemical constituents found in plants responsible for the desired therapeutic properties.

Different structures of coumarin and their derivatives were reported by many authors. Number of coumarin compounds has been identified from natural sources. Among different derivatives, Hydroxy coumarins exhibit wide range of biological properties. Based on the importance of these dynamic therapeutic properties of coumarins, a modest attempt has been made to study the structural and functional aspects of coumarins in the plant *Ceropegia juncea* (Roxb.). The plant was extracted for the analyses of coumarin and studied their structure and biological activity. Coumarins, being the natural products, possess wide range of pharmacological, biochemical and therapeutical properties (Hoult and Paya, 1996). Coumarins have attracted the attention in recent years because of their diverse pharmacological properties (Irena Kostova, 2005) and these diverse properties are mainly due to their structural variability and types of substitutions in their basic structure (Patel and Natwar, 2011). The evaluation of anti-oxidant activity of specific chemical scavengers is of particular value for their potential use in preventing the damage of cells induced by free radicals. The reducing capability of the compound may serve as significant indicator of its potential anti-oxidant activity and leads to the development of new drug molecules in future. Several natural products with a coumarinic moiety have been reported to have multiple biological activities. Pari and Rajarajeswari (2011), investigated the anti-oxidant activity of coumarin in streptozotocin(STZ)-nicotinamide(NA) induced type 2 diabetic rats. Iranshahi et al. (2009), evaluated the anti-oxidant, anti-inflammatory and lipoxygenase inhibitory activities of the prenylated coumarin umbelliprenin. Hashem (2007) investigated free radical scavenging activity of coumarins isolated from Tecoma radicans. Patel and Natwar (2011), studied in vitro, the anti-oxidant activity of coumarin compounds by DPPH, Super oxide and nitric oxide free radical scavenging assays.

MATERIALS AND METHODS
Anti-Oxidant Test: (DPPH Method) *in vitro* and *in vivo* test (Estimation of Vitamin C and Vitamin E)
Chemicals
- DPPH reagent (0.3mM solution of DPPH radical) solution in Methanol (90%)
- Catechin as Standard (2mg of catechin in 10 ml methanol)
- Blank (DPPH reagent without sample)
- Samples were prepared in Methanol

In the present study, an attempt has been made to study the biological activity of the eluted compound of the plant sample. Tests were carried out to determine the Anti-oxidant activity, of the eluted compounds. All the procedures were done as per the standard reference with slight modifications in optimized conditions of the procedure as required.

***In vitro* Anti-Oxidant activity (DPPH Method)**

For evaluating the radical scavenging activity of the test compounds, DPPH assay was carried out. The stable 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) was used for determination of free radical-scavenging activity of the compound (Nabavi *et al.*, 2008a; Ebrahimzadeh *et al.*, 2008a; Ghasemi *et al.*, 2009). Free radical scavenging capacity was evaluated on the basis of the scavenging activity of DPPH by measuring the reduction of absorbance at 517 nm. The radical scavenging activity of samples corresponds to the intensity of quenching of DPPH. All experiments were carried out in triplicate. The results were expressed as percentage of inhibition (Nickavar *et al.*, 2006).

Procedure

- The sample was prepared into 1.0,5.0,10.0,50.0,100,200,300µg/ml concentrations with methanol.
- 1 ml of DPPH solution was mixed with 2.5 ml of different concentrations of each extract (Sample).
- Catechin as standard was prepared into different concentrations (1.0-300 g/ml) and 1ml of DPPH reagent was added to 2.5ml of standard.
- Methanol 90% (1 ml) plus each sample solution (2.5ml) was used as blank.
- The mixture was shaken vigorously and then incubated for 30 min in darkness at room temperature.
- The change in absorbance was measured at 517 nm using a UV spectrophotometer. Radical scavenging activity was expressed as the inhibition percentage.
- The inhibition of free radicals by DPPH in percentage (%) was calculated by using the following equation:

Percentage Inhibition (%) = (A control-A sample/A control) X 100
A control = absorbance of the control (catechin)
A sample = absorbance of reaction mixture (in the presence of sample)

Based on their absorbance % of inhibition is calculated. By plotting the Graph between % of inhibition and Concentration, IC_{50} value was calculated.

***In vivo* Anti-Oxidant activity**

Animals: Male albino rats weighing 250gms body weight were taken for two different compounds (coumarins). The experimental protocol was approved by the institutional Ethical committee - (A.E.C No: AP1547/PO/a/11/CPCSEA).

Experimental animals

The rats were divided into two groups comprising six animals in each group.

Control: Rats received no test drug
Group 1: Rats received test compound 1 (coumarin)
Group 2: Rats received test compound 2 (coumarin)
For estimation of non-enzymatic activity of coumarin

The animals were starved overnight, for 12hours, before the treatment with the test drug. The test drug (100ppm) was administered orally. After 24 hours, non-enzymatic antioxidants were estimated in plasma and liver tissue cells of rats. For the determination of vitamin E level in the liver, tissues were weighed and lipids were extracted by the method of Folch *et al*. (1957). A Chloroform-methanol mixture ($CHCl_3$: MeOH, 2:1; v/v) was used. For the estimation non-enzymatic antioxidants, tissues were minced and homogenized (10% w/v) in 0.1 M phosphate buffer (pH 7.0) and centrifuged for 10 minutes and the resulting supernatant was used for anti-oxidant assays.

Estimation of Vitamin-C

Vitamin C was estimated by the method described by Omaye *et al.* (1979). 0.5 ml of plasma or tissue homogenate was mixed thoroughly with 1.5ml of 6% TCA and centrifuged for 10min at 3500rpm. After centrifugation, 0.5ml of the supernatant was mixed with 0.5ml of DNPH reagent and allowed to stand at room temperature for an additional 3hrs. 2.5ml. of 85% sulphuric acid was added and allowed to stand for 30 minutes. The absorbance was read at 530nm. A set of standards containing 10-50 g of ascorbic acid were taken and processed similarly along with a blank. Ascorbic acid values were expressed as mg/dl in plasma.

Estimation of Vitamin E (α–Tocopherol)

Vitamin E was determined by the method of Baker *et al.* (1951). 0.1ml of plasma or lipid extract was added to 1.5ml of ethanol and 2ml of petroleum ether and mixed and then subjected to centrifuge at 3000rpm for 10minutes. The supernatant was evaporated to dryness at 80°C. 0.2ml of 2, 2-1-dipyridyl solution and 0.2ml of ferric chloride solution was added and mixed well. This was kept in dark for 5 min and 2 ml of butanol was added. The absorbance was read at 520 nm. Standards of α–tocopherol in the range of 10-100g. The values were expressed as M/mg in tissues.

RESULTS AND DISCUSSION

1. **Compound -1. (E)-2-cyano-3-(7-hydoxy-4-methyl-2-oxo-2H-chromen-8-yl)-N-(4-methylbenzyl) acrylamide**

The results showed the percentage of scavenging activity of test coumarin compound 1 on free radical generation. Coumarin scavenges DPPH radical in a dose dependent manner (1.0-300 µg/ml). The DPPH activity was studied and compared with catechin as standard. The change in absorbance was measured at 517 nm using UV

spectrophotometer. By increasing the concentration of the sample, the percentage of inhibition increased significantly. 1.0 µg/ml of sample showed 11.28% of inhibition and the absorbance was recorded at 0.912 at 517 nm UV. 300.0 µg/ml of sample showed 97.62% of inhibition and the absorbance was read at 0.006 at 517 nm UV. The value of IC_{50} was 82.258µg/ml. The concentration of the test sample showed a correlation with % of inhibition. Graphs were constructed against the concentration of sample and % of inhibition. Based on the observations, the test compound 1 showed free radical scavenging activity.

2. Compound-2. 7-hydroxy-4-methyl-8-(2-oxo-3-p-tolylpropyl)-2H-chromen-2 one

In this the compound exhibits anti-oxidative property with DPPH test. By increasing the concentration of the sample from 1.0 to 300.0µg/ml, the % of inhibition also increased represents the free radical scavenging activity of the test compound 2. The absorbance of 300.0 µg/ml sample concentration determined 0.019 at 517 nm UV. The IC_{50} value was 82.258 µg/ml. The percent of inhibition of the compound is represented in graph.

Table 1: Anti- Oxidant activity of Compound-1.

S.No.	Concentration of Sample of µg/ml	Absorbance of Sample	Absorbance of Control	% of Inhibition
1	1.0	0.192	1.028	11.28
2	5.0	0.813	0.961	15.46
3	10.0	0.672	0.854	21.29
4	50.0	0.386	0.807	52.14
5	100.0	0.099	0.693	85.63
6	200.0	0.026	0.406	93.48
7	300.0	0.006	0.253	97.62

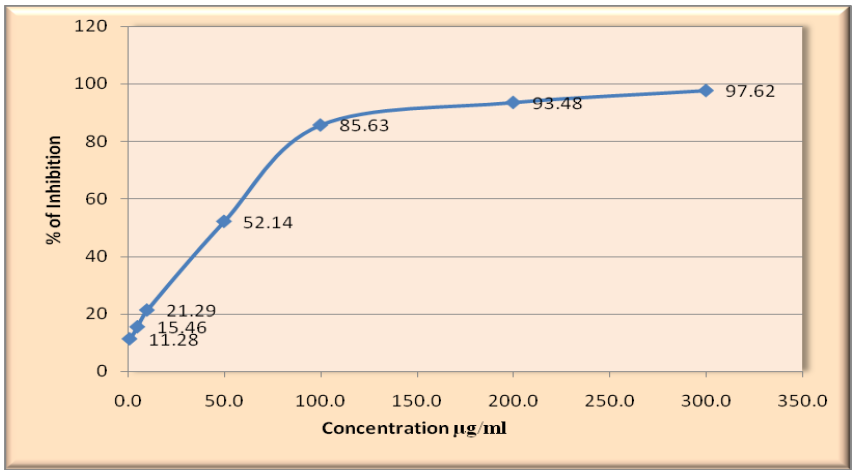

Fig. 1: Free radical scavenging activity of compound-1.

Table 2: Anti- Oxidant activity of Compound-2.

S.No.	Concentration of Sample of µg/ml	Absorbance of Sample	Absorbance of Control	% of Inhibition
1	1.0	0.192	1.028	12.81
2	5.0	0.813	0.961	16.54
3	10.0	0.672	0.854	28.67
4	50.0	0.386	0.807	54.52
5	100.0	0.099	0.693	87.73
6	200.0	0.026	0.406	92.11
7	300.0	0.006	0.253	92.49

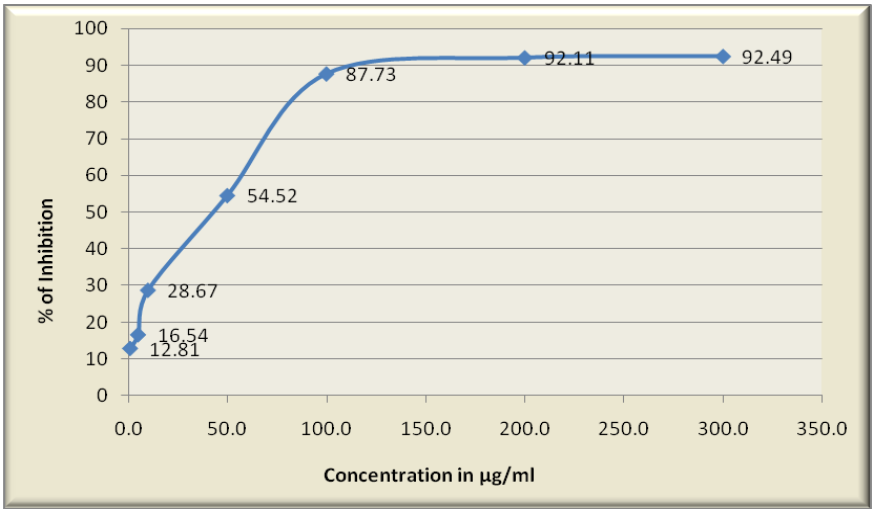

Fig. 2: Free radical scavenging activity of compound 2.

***In vivo* non enzymatic anti-Oxidant activity**

Effect of test compound 1 on vitamin E and vitamin C in liver cells and plasma respectively

The changes in vitamin E in plasma of normal control and experimental animals (albino mice) were represented. Oral administration of the test coumarin compound to the rats led to significant increase in the levels of vitamin E in the liver tissues when compared to control animal. The results were tabulated.

The changes in vitamin C in plasma of normal control and experimental animals (albino mice) were represented. Vitamin C levels were significantly increased in the rats treated with the test coumarin compound in the plasma when compared to control.

Table 3: Effect of test compound -1 on vitamin E and vitamin C in liver cells and plasma respectively.

RAT (100 PPM)	VITAMIN E(mg/dL)	VITAMIN-C(mg/dL)
1	1.48	1.79
2	1.42	1.83
3	1.40	1.75
4	1.51	1.77
5	1.53	1.81
6	1.44	1.73
CONTROLE	1.388	1.64
AVG	1.463	1.78

Table 4: Effect of test compound -2 on vitamin E and vitamin C in liver cells and plasma respectively.

RAT (100 PPM)	VITAMIN E(mg/dL)	VITAMIN-C(mg/dL)
1	1.56	1.96
2	1.56	1.88
3	1.49	1.90
4	1.50	1.93
5	1.48	1.94
6	1.55	1.89
CONTROL	1.45	1.802
AVG	1.518	1.916

The hydroxyl coumarins are typical phenolic compounds and therefore act as potent metal chelators and free radical scavengers (Irena Kostova, 2005). The pharmacological, biochemical properties and therapeutic applications of Coumarins depend upon their pattern of substitution (Irena Kostova, 2005; Patel and Natvar, 2011). Several beneficial pharmacological effects are implicated to have 4-Methyl Coumarins (Takeda, 1981; Deana *et al.*, 1983). DPPH radical scavenging assay was used extensively in natural antioxidant studies for its simplicity and high sensitivity. The antioxidant effect was proportional to the disappearance of DPPH in the test samples.

1. (E)-2-cyano-3-(7-hydoxy-4-methyl-2-oxo-2H-chromen-8-yl)-N-(4-methylbenzyl) acrylamide

From the results, the radical scavenging activity was expressed at the inhibition percentage. The absorbance was read at 517 nm using UV. The % of inhibition showed an increase in reading which resulted in the

scavenging activity. It was observed that with increment in the dosage concentration of the sample, the % of inhibition also increased.

2. 7-hydroxy-4-methyl-8-(2-oxo-3-p-tolylpropyl)-2H-chromen-2-one

In this compound, the % of inhibition is increased indicating anti-oxidative property. The sample concentration at 300µg/ml showed 92.49% of inhibition. There is no much variation found between the two compounds. This compound showed free radical scavenging activity as the absorbance of the sample decreased.

***In vivo* test: Non-enzymatic anti-oxidant test**

The non-enzymatic antioxidants like vitamin C, and vitamin E plays a vital role in quenching free radicals. Vitamin C is a hydrophilic antioxidant in plasma because it disappears faster than other antioxidants when plasma is exposed to reactive oxygen species (Frei, 1991). Ascorbic acid (Vit. C) acts as oxidation–reduction catalyst which can reduce, and thereby neutralize, reactive oxygen species such as hydrogen peroxide (Padayatty *et al.*, 2003).

Vitamin E is a membrane stabilizer (Machin and Bendich, 1987) and it interrupts the chain reaction of lipid per oxidation by reacting with lipid peroxy radicals and protects the cell structure against damage. Oral administration of (100ppm of drug) coumarin to the mice showed that both vitamin C and E increased significantly in the liver and plasma cells.

From the above observations, it can be seen that the two coumarin compounds isolated form Ceropegia jucea (Roxb). showed a correlation between the percent of inhibition and the dosage concentrations of the test compound. It might be due to the fractional functional groups present in the compound, showing the free radical scavenging activity. The radical scavenging activity of samples corresponded to the intensity of quenching DPPH. It was found that the free radical- scavenging activities of extract increased with increasing concentration of the drug. The ability to control the rate of production and the amount of hydroxyl radicals may prove useful for examining the cytotoxic effects of hydroxyl radicals generated in biological systems (Irena Kostova, 2005; Kachur *et al.*, 1998). The present findings show similarities with other works (Rehakova *et al.*, 2008).

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