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IN-VITRO EVALUATION OF *CLITORIA TERNATEA* STEM EXTRACT FOR ANTIOXIDANT PROPERTY

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ABSTRACT

Antioxidants are compounds that protect cells against the damaging effects of reactive oxygen species which can neutralise free radicals before they can do harm and may help undo some damage already caused to specific cells. Studies subject that several natural products possessing antidiabetic, antitumor and also flavonoids containing drugs can also act as antioxidants. Ayurveda and various traditional systems of medicines claim that *Clitoria ternatea* (Fabaceae) has diuretic, antidiabetic, antipyretic and brain tonic activity. Acetone and Methanol extracts of *Clitoria ternatea* were screened for antioxidant activity. Phytochemical analysis reveals that the stem contains phytosterols, phenolic compound, flavonoids and carbohydrates. Presence of the above constituents was studied by preliminary phytochemical investigations and TLC. Various *in vitro* models were applied to evaluate anti oxidant property of these extracts. *In vitro* studies include Free Radical Scavenging Capacity (RSC) on DPPH Radicals, Scavenging capacity for hydroxyl radicals, (by measuring the degradation of 2 - deoxyribose with OH radicals generated in Fenton reaction), scavenging capacity for super oxide radicals (NBT reduction assay) and Antioxidant using β - Carotene linoleate model system (β-CLAMS). Also isolate the phytoconstituents responsible for antioxidant activity by preparative TLC method. The methanolic extract showed the maximum free radical scavenging capacity as compared to acetone extract.

INTRODUCTION: *Clitoria ternatea* Linn. (Fabaceae) also known as Aparajita in India is a very common garden flowering plant. A perennial herb with terate, more or less pubescent stems [1, 2, 3]. Plant contains flavonoids. The important flavonoids are quarcetin, Kaemferol, robinin and clitorin. It also contains starch, tannin, resins and anthocyanins [4, 5]. The phytochemical investigations revealed the presence of phytosterols, phenolic compound, flavonoids and carbohydrates. It is used as diuretics, antihelmintic, antidiabetic, antipyretic and brain tonic [6, 7]. It is also useful in eye disease, ulcers, leucoderma, asthma, gonorrhoea, abdominal enlargement, headache, snake bites, ascites and cough etc [8, 9, 10]. Therefore, an effort is made to contribute to establish scientific evidence in this regard.

MATERIALS AND METHODS:

Plant Material: The stem of *Clitoria ternatea* L. was collected from the local areas of Vidyanagar, Gujarat and authenticated by Dr. A. S. Reddy, Department of Botany, Sardar Patel University, Vidyanagar.

Preparation of Extracts: The collected stem of plant was shaded dried, coarsely powdered and powder was extracted exhaustively and successively with various solvents in an increasing polarity viz., petroleum ether (60-80^{o}C), toluene, chloroform, acetone, methanol and water in soxhlet apparatus. Each extract was concentrated to a small volume and allowed to dry. After drying, the respective extracts were weighed and percentage extractive values were determined.

Phytochemical Tests: The qualitative chemical investigations of all the extracts were carried out to check the presence of various phytoconstituents [11]. It revealed the presence of various phytoconstituents such as phytosterols, phenolic compound, flavonoids and carbohydrates.

Antioxidant Activity:

Free Radical Scavenging Capacity on DPPH Radical: For the present study the samples were prepared in different concentrations i.e. 200-1000 µg/ml in AR grade methanol. The samples of above concentrations were mixed with 3 ml of 100 µM of DPPH prepared in AR grade methanol and the final volume made up to 4 ml with AR grade methanol. The absorbance of the resulting solutions and the blank (with same chemicals except sample) were recorded after 15 min at room temperature. The disappearance of colour was read spectrophotometrically at 517 nm using a JASCOV530- Visible spectro-photometer. Radical Scavenging Capacity (RSC) in percent was calculated by following equation:

$$\mathbf{RSC\ (\%) = 100 \times A_{blank} - A_{sample} / A_{blank}}$$

Where;

RSC = Radical Scavenging Capacity
A_{blank} = Absorbance of blank
A_{sample} = Absorbance of sample

From the obtained RSC values of acetone and methanol extracts, the IC_{50} were calculated, which represents the concentration of the scavenging compound that caused 50% neutralisation [12].

Scavenging of Super oxide Radicals:

Nitro blue Tetrazolium Assay: All the reagents were dissolved in phosphate buffer, freshly prepared. Reaction mixture contained riboflavin (20µg), EDTA (12mM), methanol

(12mM), NBT (0.1mg) and drug (variable concentration), in a final volume of 3ml. The reaction mixture was illuminated under light for 5 min. The absorbance was read against a blank (containing buffer solution instead of sample) at 590 nm. RSC in percent was calculated by using the same formula as described in Free Radical Scavenging Capacity on DPPH radicals. From the obtained RSC values the IC_{50} were calculated, which represents the concentration of the scavenging compound that caused 50% neutralisation [13].

Scavenging of Hydroxyl Radical:

Thiobarbituric Acid Reactive Substances (TBARS) Assay: All the reagents were dissolved in phosphate buffer, freshly prepared. Reaction mixture contained deoxyribose (10mM), KH_2PO_4-KOH buffer, pH7.4 (20mM), $FeCl_3$ (10mM), EDTA (1mM), H_2O_2 (10mM), Ascorbate (1mM) and drug (variable concentration), in a final volume of 3 mL. The reaction mixture was incubated for1 hr at 37C.After incubating the reaction was stopped by adding 2 mL of ice cold 0.25N HCl containing 10% trichloroacetic acid 0.5%, thiobarbituric acid and 0.025% BHA (Butylated Hydroxyl Anisole).

Following heating at 80 for 15 min, samples were cooled and centrifuged at 1000g for 10 min; the absorbance of the supernatant was measured at 532 nm. Test compounds were dissolved in 0.05 N NaOH and pH was adjusted to 7.4 with 0.1 N HCl. The absorbance was read against a blank (containing buffer solution instead of sample) at 532 nm. The absorbance was used for calculation of the percentage dissolution of 2-deoxy D-ribose degradation by the sample by using the same formula as described in DPPH method. From the obtained RSC values the IC_{50} were calculated, which represents the concentration of the scavenging compound that caused 50% neutralisation [14].

β- Carotene Linoleate Model System: A solution of β- Carotene was prepared by dissolving 2 mg of β- Carotene in 10 ml chloroform. 2 ml of this solution was pipetted into 100 ml RB flask. After chloroform was removed under vacuum, 40 mg of purified linolic acid, 400 mg of tween 40 emulsifier and 100 ml of aerated distil water was added to shake vigorously. Aliquot (4.8 ml) of this emulsion was added to test tubes containing different concentration of the extracts. As soon as the emulsion was added to each test tube, zero time absorbance was measured on UV visible Spectrophotometer at 470 nm. The tubes were then placed in water bath at 50 ^{o}C and the measurement of absorbance was continued until the colour of β- Carotene disappeared. A blank devoid of β- Carotene was prepared for background correction. From the obtained RSC values the IC_{50} were calculated, which represents the concentration of the scavenging compound that caused 50% neutralisation [15].

Isolation of Flavonoids: Methanol extract was selected for the isolation of flavonoids, as it showed significant antioxidant activity. The isolation was done by Preparative Thin Layer Chromatography. In this the substance of interest was scraped from the layer after detection and subsequently examining it with the aid of a suitable analytical technique. The mobile phase used was Ethyl acetate: Methanol: Water (100:13.5:1). After spotting and developing the plate in the solvent system, it was dried and then observed in UV light. Two spots having green fluorescence of R_f value 0.526 and 0.868 were observed. Both the spots were scrapped, transferred in a

Petri dish and methanol was added. This was then filtered. The filtrate was evaporated on water bath to obtain two compounds i.e. compound M_1 (R_f: 0.526) and compound M_2 (R_f: 0.868).

RESULTS AND DISCUSSION: Antioxidant activity of methanol and acetone extracts of *Clitoria ternatea,* stem part, were performed by using different *in vitro* models such as Free Radical Scavenging Capacity (RSC) on DPPH Radicals, Scavenging capacity for hydroxyl radicals, Scavenging capacity for super oxide radicals (NBT reduction assay) and β-Carotene linoleate model system (β- CLAMS).

Free Radical Scavenging Capacity (RSC) on DPPH Radicals: The results obtained for different concentrations of acetone and methanol extracts were indicated in **Table 1**. Successive acetone and methanol extract showed 50% reduction i.e. IC_{50} value were 758.58 µg and 933.25 µg respectively. Acetone extract showed more significant antioxidant activity in free radical scavenging capacity on DPPH radicals (**Fig. 1 and 2**).

TABLE 1: FREE RADICAL SCAVENGING ACTIVITY ON DPPH RADICALS

Conc. (µg/ml)	Acetone	Conc. (µg/ml)	Methanol
200	11.58	500	33.47
400	24.23	600	35.69
600	42.24	700	35.98
800	51.79	800	39.67
1000	65.12	900	47.73
IC50	758.58	1000	47.96
		1100	50.52
		IC50	933.25

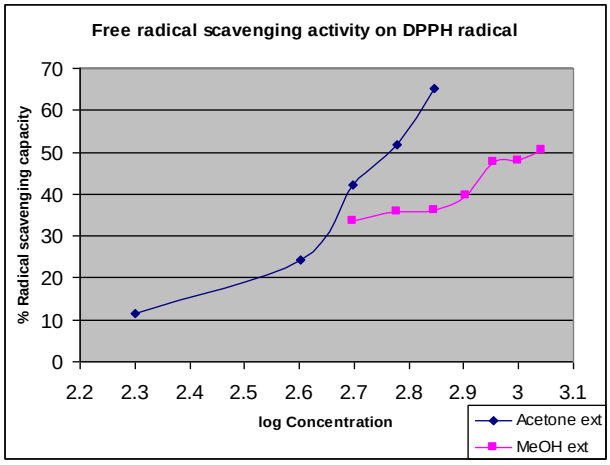

FIG. 1: FREE RADICAL SCAVENGING ACTIVITY ON DPPH RADICALS

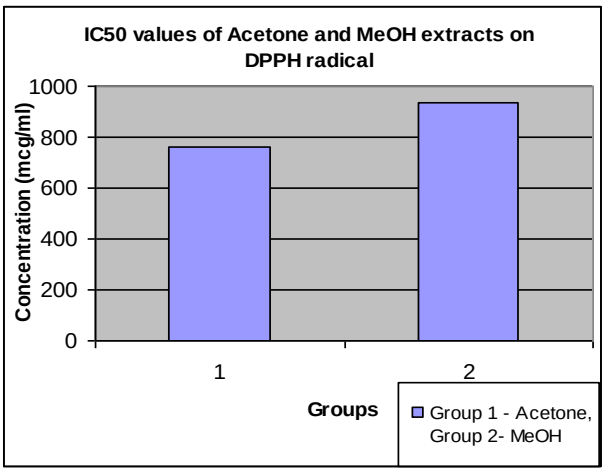

FIG. 2: IC_{50} VALUES OF ACETONE AND MEOH EXTRACTS ON DPPH RADICALS

Scavenging capacity for super oxide radicals: The results obtained for different concentrations of acetone and methanol extracts were indicated in **Table 2**. Successive acetone and methanol extract show 50% reduction i.e. IC_{50} value were 451.85 µg and 288.40 µg respectively. Methanol extract showed more significant antioxidant activity in free radical scavenging capacity on super oxide radicals compared to acetone extract (**Fig. 3 and 4**).

TABLE 2: SCAVENGING CAPACITY OF SUPER OXIDE RADICAL

Conc. (µg/ml)	Acetone	Methanol
100	23.98	22.47
200	37.49	39.46
300	39.86	50.50
400	43.12	52.76
500	51.81	70.76
IC50	451.85	288.40

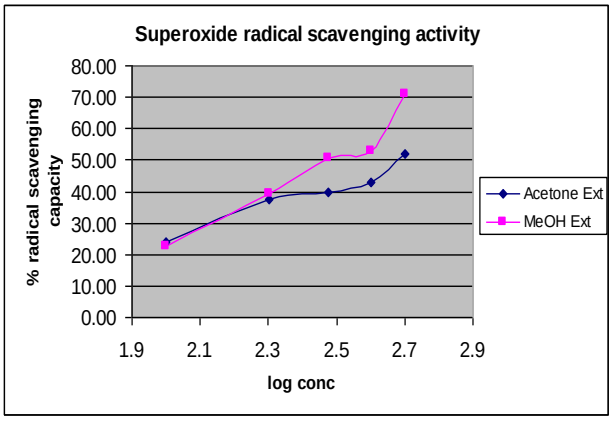

FIG. 3: FREE RADICAL SCAVENGING ACTIVITY ON SUPER OXIDE RADICALS

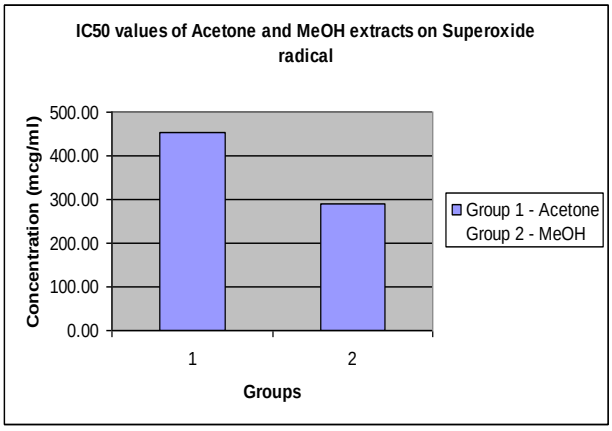

FIG. 4: IC_{50} VALUES OF ACETONE AND MEOH EXTRACTS ON SUPER OXIDE RADICALS

Scavenging capacity for hydroxyl radicals: The results obtained for different concentrations of acetone and methanol extracts were indicated in **Table 3**. Successive acetone and methanol extract show 50% reduction i.e. IC_{50} value were 141.25 µg and 52.48 µg respectively. Methanol extract showed more significant antioxidant activity in free radical scavenging capacity on hydroxyl radicals compared to acetone extract (**Fig. 5** and **6**).

TABLE 3: SCAVENGING CAPACITY FOR HYDROXYL RADICALS

Conc. µg/ml	Acetone	Conc. µg/ml	Methanol
120	37.5	20	0.64
140	50.3	40	32.05
160	59.75	60	58.33
180	68.59	80	77.56
200	76.22	100	81.41
IC50	141.25	IC50	52.48

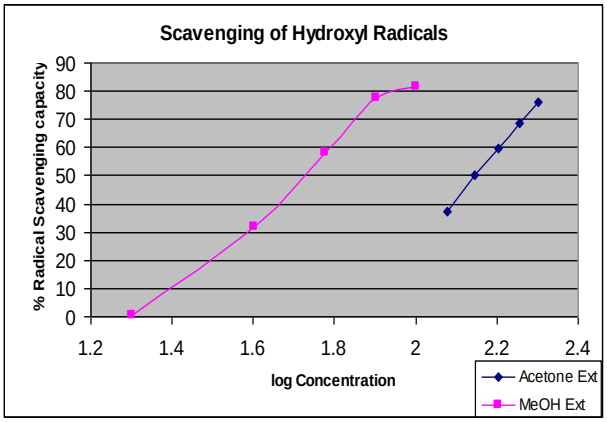

FIG. 5: FREE RADICAL SCAVENGING ACTIVITY ON HYDROXYL RADICALS

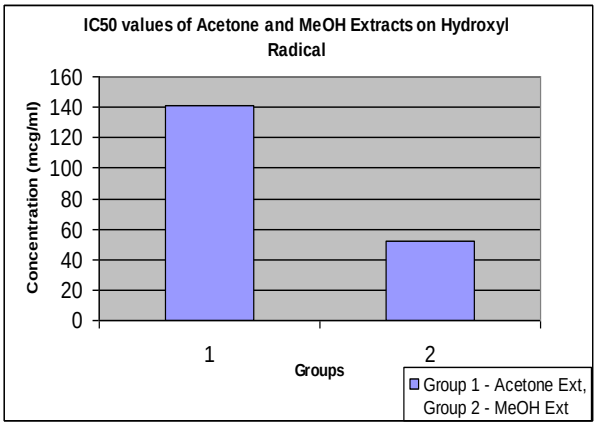

FIG. 6: IC_{50} VALUES OF ACETONE AND MeOH EXTRACTS ON HYDROXYL RADICALS

β- Carotene linoleate model system: The results obtained for different concentrations

of acetone and methanol extracts were indicated in **Table 4**. Successive methanol extract showed 50% reduction i.e. IC_{50} value was 841.39 µg. Methanol extract showed significant antioxidant activity in β- Carotene linoleate model system (**Fig. 7** and **8**). And acetone extract could not respond to antioxidant activity by this method.

TABLE 4: β - CAROTENE LINOLEATE MODEL SYSTEM

Conc. (µg/ml)	Methanol
200	16.32
400	20.4
600	35.37
800	39.45
1000	57.14
IC50	841.39

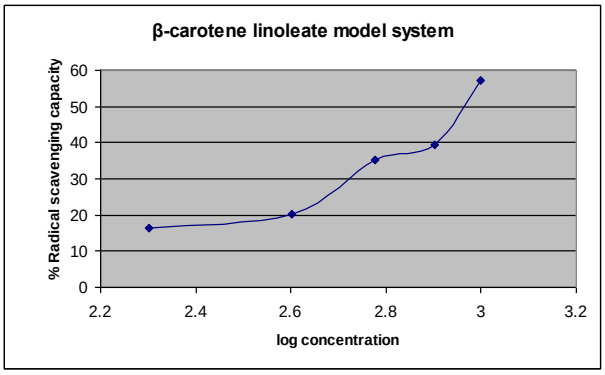

FIG. 7: FREE RADICAL SCAVENGING ACTIVITY ON β - CAROTENE LINOLEATE MODEL SYSTEM

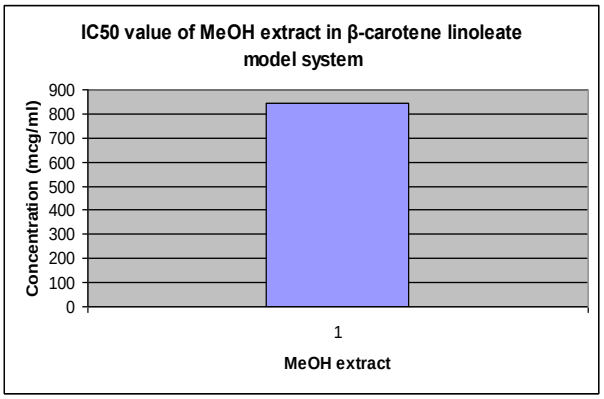

FIG. 8: IC_{50} VALUES OF MEOH EXTRACTS β- CAROTENE LINOLEATE MODEL SYSTEM

Isolation of Flavonoids: The compounds separated by preparative TLC. These compounds responded positively for flavonoids, the yield was 90mg (Rf: 0.526) and 110mg (Rf: 0.868). Both compounds are yellowish brown in colour. TLC studies indicated that, the compounds M_1 and M_2 are single compound and the melting point was found to be 173-175 and 174-178 respectively. UV spectrum of compound (M_1) showed λmax at 214.79 and 314.19 nm and compound (M_2) showed λ_{max} at 214.51 and 281.58 nm. FT-IR of the same compounds indicated broad band at 3421 cm-1, means –OH stretching, C-H band stretching of saturated hydrocarbons at 2958, 2925, 2855 cm-1, chromone carbonyl absorption at 1630 cm^{-1} and strong band showing C=O stretching of –COOH in glucuronide i.e. carbonyl absorption at 1730 cm^{-1}. H-NMR studied of showed that, it has aromatic protons. Mass studies of compounds demonstrated that the molecular weight of (M_1) as 214 and (M_2) as 225.

From the above studies, it can be said that the compounds (M_1 and M_2) may be a flavonoids.

CONCLUSION: Acetone extract showed more significant antioxidant activity in free radical scavenging capacity on DPPH radical while in rest of three methods, methanol extract showed significant antioxidant activity. The methanol extract exhibited statistically highly significant free radical scavenging capacity on Hydroxyl radical (IC_{50} value- 52.48 µg/ml). Since methanolic extract was more effective as antioxidant, it was decided to isolate the flavonoid compounds. The chemical natures of isolated compounds were confirmed by shinoda test, UV scanning, FTIR, NMR, Mass spectroscopy.

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REFERENCES:

1. Shah GL: Flora of Gujarat State, Sardar Patel University, V.V. Nagar 1978; 1: 189-190.
2. Maheshveri JK: Flora of Delhi, CSIR, New Delhi 1963;131
3. Malabadi RB and Natraja K: Journal of Medicinal and Aromatic Plant Sciences 2002; 24(1) :733-737.
4. Rastogi R and Mehlotra BN: Compendium of Indian Medicinal Plants, CDRI, Lucknow, 1995; 1:114-115.
5. Chatterjee A and Chandra PS: The Treatise on Indian Medicinal Plants, CSIR, New Delhi 1992: 2: 79.
6. Kapoor LD: Hand Book of Indian Medicinal Plants, CRC Press 2001; 126-127.
7. Kirtikar KR and Basu BD: Indian Medicinal Plants. Publication by Lalit Mohan Basu, Allahabad, Second Edition, Vol.I, 1981: 802-804.
8. Nadkarni KM: Indian Materia Medica, Popular Prakashan, Mumbai, Second Edition, Vol.I, 1982: 354.
9. The Ayurvedic Pharmacopoeia of India, Govt. of India, Ministry of Health and Family Welfare, Dept of Health. First Edition, Part 1, Vol.1, 130.
10. Sing GK and Dixit VK: Journal of Natural Product. 1998: Vol.61 (11); 1361-1367.
11. Kokate CK: Practical Pharmacognosy, Vallabh Prakashan, Delhi, Edition 3, 1991:107-109.
12. Rajkumar DV and Rao MNA: Dehydrozingerone and isoeugenol as inhibitors of lipid peroxidation and as free radical scavengers, Bio.Chem. Pharmacology; 1993; 46 (11):2067-72.
13. Spencer: Free Radical Scavenging and Antioxidant activity of Plant Flavonoids. Adv.Exp Med Biol 366.1994; 351-376.
14. Raghvan G, Madhvan V, Chandana VR, Shirwaikar A, Rawal AKS, and Mehrotra S: Antioxidant Potential of Anogeissus latifolia. Biol. Pharma. Bull. 2004; 27(8): 1266-69.
15. Yerra RA, Senthi GP, Gupta ML and Kanti U: European Bulletin of Drug Research, **V**ol.13; 2005: 351-376.

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