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IN VITRO ANTIOXIDENT ACTIVITY OF METHANOLIC EXTRACT OF ROOT OF CLITORIA TERNATEA

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ABSTRACT

The main objective of the present work is to investigate the in vitro antioxidant activity of methanolic extract of root of *Clitoria ternatea*. 1-1-Diphenyl-2-picryl-hydrazyl (DPPH) radical, superoxide anion radical, nitric oxide radical and hydroxyl radical scavenging were carried out to evaluate the antioxidant potential of the extract. The free radical scavenging activity of methanolic activity of root of *Clitoria ternatea* increase in a concentration dependent manner. The methanolic extract posses statistically significance DPPH free radical scavenging activity. Extract inhibited 58.78±4.48% nitric oxide radical generated from sodium nitroprusside whereas, curcumin in the same concentration inhibited at 87.24 ±4.12%. Moreover, *Clitoria ternatea* extract scavenged the superoxide radical generated by xanthine/xanthine oxidase system. The extract in the dose of 1000 µg/ml inhibited 63.04 ±5.11%. The hydroxyl radical was generated by Fenton's reaction. The amounts of total phenolic compound were determined and 56.98 µg pyrocatechol phenol equivalents were detected in one mg of extract. Therefore, *Clitoria ternatea* could be considered as a potential source natural antioxidant.

KEYWORDS: Clitoria ternatea, antioxidant activity, methanolic extract.

INTRODUCTION

The importance of the reactive oxygen species (ROS) has attracted increasing attention over the last decade. Reactive oxygen species include free radicals such as super oxide anion radicals, hydroxyl radicals, non free radicals such as hydrogen peroxide and singlet oxygen,

along with various forms of activated oxygen. They are involved in various physicochemical processes and disease such as aging, cancer and activity atherosclerosis. Butyl hydroxyl anisole (BHA) and butyl hydroxyl toluene (BHT) are the most the commonly used antioxidants, but both are suspected to cause liver damage. The objective of the present study is to investigate the in vitro antioxidant activity of the root of *Clitoria ternatea* belongs to the family Papilionaceae.

MATERIAL AND METHOD

Plant materials and extraction

The plant material was collected from the local area of Amravti district in Maharashtra. The plant materials were identified and authenticated. The Plant material were air-dried and pulverized in course powder and was loaded in the Soxhlet apparatus for defating with petroleum ether and followed by extraction with methanol. The methanolic extract was subjected to fractionation with ethyl acetate (R1), acetone (R2) and methanol (R3).

Determination of total antioxidant activity

The antioxidant activity of different fractions (R-1, R-2 and R-3) obtained from *Clitoria ternatea* was determined using the thiocyanate method.Various concentration (50, 100, 250 & 500 µg/ml) of different fractions of *C. ternatea* were prepared in methanol and added to a linoleic acid emulsion (2.5 ml, 40 mM, pH 7.0) and phosphate buffer (2.5 ml, 40 mM, pH 7.0). The linoleic acid emulsion was prepared by mixing 0.2804 g linoleic acid with 0.2804 g Tween 20 as emulsifier in 50 ml 40 mM phosphate buffer. The mixture was then homogenized. The final volume was adjusted to 5 ml with 40 mM phosphate buffer and pH 7.0. The mixed samples were then incubated at 37^0 C in a glass flask for 60 hr. to accelerate the oxidation process. 1 ml of incubated sample was removed at 12 hr interval & 0.1 ml 20 mM $FeCl_2$ and 0.1 ml 30% ammonium thiocynate was added. The absorbance of this was measured 500 nm. using spectrophotometer. Alpha tocoferol was used as a reference compound. To eliminate solvent effect, control sample, which contained the same amount of sample added to linoleic acid emulsion in the test sample and the reference compound. All data reported are the average of triplicate analysis. Percentage inhibition of lipid peroxide generation was calculated by using following formula.

$$\% \text{ Inhibition} = \frac{\text{control absorbance- test absorbance}}{\text{Control absorbance}} \qquad \text{(Formula 1)}$$

DPPH free radicals scavenging activity

The free radical scavenging activity of the different fractions of *Clitoria ternatea* was measured using DPPH, employing the method of Blois.1 ml of each fraction of *C. ternatea* and the reference compound in various concentration (50,100, 250 & 500 µg/ml) was added to 1 ml of 0.1mM solution of DPPH in methanol. After 30 min., absorbance was measured at 517 nm using spectrophotometer. 0.01 mM solution of DPPH in methanol was used as control, whereas BHA was used as reference material. All tests were performed in triplicate. Percentage inhibition was calculated by using following formula 1.

Nitric oxide radical scavenging activity

Nitric oxide generated from sodium nitropruside in aqueous solutions at physiological pH interacted with oxygen to produce nitrite ions, which were measured using the Griess reaction. 3 ml of 10 mM sodium nitropruside in phosphate buffer was added to 2 ml of each fraction of *C. ternatea* and reference compound in different concentration (20, 40, 60, 80 &100 µg/ml). The resulting solution were then incubated at 25^0 for 60 min. A similar procedure was repeted with methanol as a blank, which served as control. To 5 ml of the incubated sample, 5 ml Griess reagent (1% sulfanilamide, 0.1% napthylene diamine dihydrochloride in 2% H_3PO_3) was added.The absorbance was measured at 546nm using spectrophotometer. All tests was performed in triplicate. Percentage inhibition of nitric oxide generated was measured by comparing the absorbance values of control and test preparation (Formula 1). Curcumin was used as reference material.

Superoxide anion radical scavenging activity

The method describe by Chang et al. was used to investigate the superoxide anion scavenging activity of different fractions obtained from *Clitoria ternatea*. The reaction mixture was prepared by dissolving Na_2CO_3 (0.53 gm). EDTA and xanthin in 100 ml of distilled water. 10 ml of NBT solution(0.025mM) was added to the reaction mixture. From this 995 µl with xanthin was added to 5µl of each fraction of *C. ternatea* and reference compound in different concentration in distilled water. After 15 min. the absorbance was measured using spectrophotometer at 560 nm. The reaction mixture with xanthin oxidase was used as control, whereas BHT was used as reference compound. All tests were performed in triplicate. Percentage inhibition was calculated by comparing the results of control and tests sample (Formula 1).

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was measured by comparing deoxyribose and *Clitoria ternatea* for hydroxyl radical generated by the ferric ascorbate-EDTA-hydrogen peroxide system (Fenton reaction), using the method of Kunchandy and Rao. The reaction mixture containing (1.0 ml) 100 µl 2-deoxy-2-ribose (28 mM in 20 mM phosphate buffer pH 7.4). 500 µl of each fraction of *C. ternatea*and the reference compound in phosphate buffer (20 mM pH 7.4), 200 µl of 1.04 mM EDTA and 200 µl of $FeCl_3$, 100 µl 1.0mM hydrogen peroxide and 100 µl 1.0 mM ascorbic acid was incubated at 37^0C for 1 hr. 1 ml of 1% thiobarbituric acid and 1 ml of 2.8% trichloroacetic acid were added and incubated at 100^0 C for 20 min. After cooling absorbance was measured by using spectrophotometer at 532 nm. against control preparation containing deoxyribose and buffer. Catechin was used as a positive control. Reaction were carried out in triplicate. Percentage inhibition was determined by comparing the result of test & control samples (Formula 1).

Amount of total Phenolic compounds

Total soluble Phenolic compounds present in the different fractions of *Clitoria ternatea* were determined with the Folin-Ciocalten reagent, according to the methods suggested by Slinkard and Singleton. To 0.1 ml of each fraction of *C. ternatea*(1mg/ml in dist. water), 1 ml Folin-Ciocalten reagent was added. After 3 min. 3 ml 2% Na_2CO_3 was added. Subsequently, the mixture was shaken for 2 hr. at room temperature and absorbance was measured using \spectrophotometer at 760 nm. All tests were performed in triplicate. The concentration of total phenolic compound in sample was determined as µg pyrocatechol equivalents using the following equation obtained from standard pyrocatechol graph

Absorbance = 0.001 X pyrocatechol (µg) + 0.0033

Statistical analysis

Data are mean ± SD of three measurements. Statistical analysis was performed by the student t-test and by ANOVA. $P< 0.05$ was regarded as significant.

RESULTS AND DISCUSSION

Preliminary Phytochemical investigation

Preliminary phytochemical screening of *Clitoria ternatea* showed the presence of sterol, flavonoids, carbohydrates and glycosides.

Total antioxidant activity

In the present study, the antioxidant activity of different fraction (R1, R2, & R3) of *C. ternatea* was determined using the ammonium thiocynate method. All the factions showed antioxidant property in a concentration dependent manner as shown in [Table 1]. Percent inhibition in case of R-3 was more than R-2.The fraction R-1 showed minimum percent inhibition. All the fraction showed less percent inhibition than reference (α-tocopherol). The results indicate that the fractions R2 and R3 of *C. ternatea* significantly ($P< 0.05$) inhibited linoleic acid peroxidation.

DPPH free radical scavenging activity

The DPPH radical is considered to be a model of lipophilic radical. A chain reaction in lipophilic radical was initiated by lipid autoxidation. The scavenging effects of different fractions of *C. ternatea* and BHA on the DPPH radicals are illustrated [Table 2]. R-2 and R-3 had significant scavenging effects on the DPPH radical which increased with increasing concentration from 50-250 µg/ml. The scavenging effect of all the fractions was lower than that of BHA. The fraction R-1 showed negligible percent inhibition. The fraction R-2 and R-3 possess statistically significant DPPH free radical scavenging activity ($P< 0.05$).

Nitric oxide radical scavenging activity

The percentage inhibition of nitric oxide generation by different fraction of *C. ternatea*.[Table 3]. The oxidant activity of all the three fractions was less than curcumin, which was used as reference compound. It was also observed that R-3 inhibits the production of nitric oxide radicals more actively than R-2 and R-1.

Superoxide anion radical scavenging activity

All the fraction of *C. ternatea* were found to possess scavenging effects on superoxide anion, in a concentration dependant manner [Table 4]. R-3 in the dose of 100 µg/ml inhibited the production of super oxide anion radicals by 74.22 ± 3.55% showing strong super oxide radical scavenging activity. However, the activity was lesser than the BHT.

Hydroxyl radical scavenging activity

This was measured by studying the competition between deoxyribose and tast (R1, R2 & R3) compounds for hydroxyl radical generated by Ferric^{3+} acerbate-EDTA- H_2O_2 system. The R-2 and R-3 fractions of *C. ternatea* were capable of reducing DNA damage at all concentrations, whereas R-1 showed negligible or no effect[Table 5]. Catechin was used as a

standard was more effective than the fractions of *C. ternatea* in inhibiting the oxidative DNA damage.

All the fractions showed free radical scavenging activity in different models in the present study. On comparison, it was found that fraction R3 has the highest antioxidant activity. Whereas R1 has the least free radical scavenging activity. The antioxidant activity of these fractions might be due to inactivation of free radical or complex formation with metal ion or a combination.

Table-1: Inhibition (%) of lipid per oxidation in a linoleic acid emulsion by different fractions of methanolic extract of clitoria ternatea and by α-tocopherol (A-Tph)

Quantity of the fractions used in µg		% of inhibition		
	R-1	R-2	R-3	α -Tph
50	3.33 ±5.25	42.12 ±5.24	49.94 ±5.71	61.36 ±4.35
100	6.58 ±2.80	54.56 ±1.32	57.59 ±4.85	67.32 ±5.48
250	9.18 ±6.50	58.97 ±2.86	60.19 ±5.22	72.44 ±2.57
500	11.21±3.95	62.89 ±2.51	63.48 ±3.33	79.47 ±5.12

Table-2-DPPH Free radical scavenging activity of different fractions of methanolic extract of clitoria ternatea and butylated hydroxyl anisole

Quantity of the fractions used in µg		% of inhibition		
	R-1	R-2	R-3	BHA
50	4.46 ±1.32	11.48 ±2.8	14.31 ±5.41	40.57 ±2.85
100	6.85 ±2.51	34.21 ±3.95	39.44 ±5.12	68.48 ±3.33
150	9.77 ±2.86	51.13 ±6.55	45.47 ±2.57	81.19 ±5.22
200	10.12 ±5.24	52.33 ±5.25	52.36 ±4.35	78.94 ±5.78
250	12.41 ±6.62	61.24 ±7.58	61.11 ±4.99	82.59 ±4.26

Table-3-Inhibition percent of nitric oxide radicals different fractions of methanolic extract of clitoria ternatea and curcumin

Quantity of the fractions used in µg		% of inhibition		
	R-1	R-2	R-3	Curcumin
20	5.53 ±3.28	11.53 ±2.96	14.32 ±4.48	53.22 ±3.55
40	9.37 ±2.24	25.63 ±4.58	44.56 ±5.88	75.06 ±2.54
60	14.45 ±3.39	37.78 ±5.18	47.52 ±4.19	78.45 ±2.48
80	18.86 ±4.17	39.65 ±4.28	56.14 ±5.11	82.87 ±5.02
100	21.82 ±4.31	43.23 ±7.47	58.78 ±4.48	87.24 ±4.12

Table-4-Superoxide anion scavenging activity different fractions of methanolic extract of clitoria ternatea and butylated hydroxyl toluene

Quantity of the fractions used in µg		% of inhibition		
	R-1	R-2	R-3	BHT
20	8.75 ±4.28	27.56 ±4.17	50.86 ±5.02	66.04 ±5.11
40	12.23 ±7.47	48.32 ±4.31	57.27 ±4.12	47.68 ±4.48
60	17.58 ±5.11	59.55 ±3.39	65.45 ±2.48	79.52 ±4.19
80	19.43 ±4.5	63.57 ±2.24	70.086 ±2.54	81.39 ±5.88
100	20.54 ±2.96	67.43 ± 3.28	74.22 ±3.55	85.24 ±3.99

Table-5- Hydroxyl radical scavenging activity different fractions of methanolic extract of clitoria ternatea and catechin on deoxyribose damage

Quantity of the fractions used in µg		% of inhibition		
	R-1	R-2	R-3	Catechin
50	19.27 ±4.12	34.23 ±3.28	46.38 ±4.48	52.22 ±3.55
100	21.54 ±3.98	46.23 ±7.47	57.54 ±2.96	67.62 ±4.31
250	23.58 ±2.54	56.29 ±5.88	59.57 ±2.24	69.25 ±4.58
500	25.65 ±2.48	59.52 ±4.19	63.45 ±3.39	70.67 ±5.18
1000	28.47 ±5.04	63.04 ±5.11	65.86 ±4.17	73.97 ±4.28

CONCLUSION

The data presented here indicate that the markad antioxidant activity of *Clitoria ternatea* seems to be due to presence of Flavonoid, which may act in similar fashion as reductones by donating the electron and reacting with free radicals to convert them to a more stable product and terminate free radical chain reaction. Further total phenolic content of extract confirmed that *Clitoria ternatea* contains good amount of phenolics. It is apparent from the present study that the *C. ternatea* not only scavenges off the free radicals but also inhibits the generation of free radicals. It was already reported that naturally occurring phenolic compounds have free radical scavenging properties, due to their hydroxyl groups. Further, phenolic compounds are effective hydrogen donors, which make them antioxidant.

It may be concluded that fractions obtained from methanolic extract of *Clitoria ternatea* have significant antioxidant activity. The antioxidant potential may be attributed to the presence of polyphenolic compounds. These results are encouraging enough to pursue characterization of these fractions.

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