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International Journal of Current Research
Vol. 8, Issue, 11, pp.42354-42358, November, 2016

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLE

PHYTOCHEMICAL SCREENING AND ESTIMATION OF TOTAL PHENOLS, TOTAL FLAVANOIDS AND EVALUATION OF *IN VITRO* ANTI OXIDANT AND ANTI INFLAMMATORY ACTIVITIES OF VARIOUS EXTRACTS OF *CLITORIA TERNATEA* ROOT

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ARTICLE INFO

Article History:
Received 26th August, 2016
Received in revised form
10th September, 2016
Accepted 14th October, 2016
Published online 30th November, 2016

Key words:

Total phenols,
Flavonoids,
In vitro antioxidant,
Anti inflammatory.

ABSTRACT

The aim of the present study was to investigate the Phyto -constituents present within the different extracts of *Clitoria ternatea* root and to estimate total phenols, total flavonoids and anti oxidant, anti-inflammatory activities. Phytochemical studies confirmed the presence of carbohydrates, proteins, alkaloids, glycosides, resins, steroids, saponins, phenols, flavonoids were analyzed using spectro photometric technique, based on Folin - Ciocalteau reagent and aluminium chloride colorimetric assay, respectively. Gallic acid was used as for total phenols and Rutin for total flavonoids. Furthermore, the antioxidant, anti-inflammatory activities were determined by using two in vitro methods namely, 2, 2-phenyl,1-picrylhydazyl (DPPH), Total antioxidant capacity, protein denaturation, HRBC membrane stabilization methods. In these methods, ascorbic acid and Ibuprofen were used as reference compounds. The total phenols and flavonoids were found to be more in methanolic extract. The result clearly indicates that the methanolic extract of *Clitoria ternatea* Root of the study shows effective antioxidant and anti-inflammatory properties.

Citation: Neelamma, G., Vanitha, B., Sai Suresh Nehru, Rajesh Kumar and Durai Swamy, B., 2016. "Phytochemical screening and estimation of total phenols, total flavanoids and evaluation of in vitro anti oxidant and anti inflammatory activities of various extracts of Clitoria ternatea root", *International Journal of Current Research*, 8, (11), 42354-42358.

INTRODUCTION

Free radicals can be defined as molecules containing one or more unpaired electrons in an atom orbits. To this group belong reactive oxygen, nitrogen and chloride species which are all of the normal byproducts of metabolism considered as endogenous source and also derived exogenously (Pauwels *et al.,* 2007). Oxidative stress occurs due to the imbalance between the production and the elimination of oxygen species (ROS), like superoxide, hydroxyl, alkoxyl radical and hydrogen peroxide. Inflammation is a normal protective response shown by living tissues against the injury caused by physical trauma, noxious chemicals or microbial agents (Robert, 2009). It is one of the most common reason for which people try to find medical attention. Thus anti-inflammatory are among the most commonly prescribed medication in clinical practice. *Clitoria ternatea* Linn. is an attractive perennial climber with conspicuous blue or white flowers. It belongs to the family Fabaceae and commonly known as "butterfly pea" and "shankhapuspi". It is traditionally used to treat various ailments (Sivarajan and Balachandran, 1994; Kokate, 1999).

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The plant is native to south-east Asia and distributed in tropical Asia including India, the Philippines and Madagascar (Anonymous, 1998). Roots, seeds and leaves of C. ternatea are commonly used in the Ayurvedic system of medicine. Extracts of his plant have been used as an ingredient in the Ayurvedic 'Medhya Rasayana' as a rejuvenating recipe used for treatment of neurological disorders and are considered to enhance the intellect (Sharma and Dash, 1998). Phytochemical screening of medicinal plants is very important in identifying new sources of therapeutical and industrial importance. Phytochemical analysis of methanol extract of *Clitoria ternatea* roots confirmed the presence of tannins and resins and certain other constituents. The present study deals with the phytochemical analysis of the whole plant and seed extracts are used for stomatitis, piles, sterility in females, hematemesis, insomnia, epilepsy, psychosis, leucorrhea and polyurea (Yoganarasimhan, 2000). The roots are bitter, refrigerant, laxative, intellect-promoting, diuretic, anthelmintic, tonic and are useful in dementia, hemicrania, burning sensations, leprosy, inflammation, leucoderma, bronchitis, asthma, pulmonary tuberculosis, ascites, fever, otalgia, hepato pathy and as a cathartic (Nadakarnik, 1976). The root, stem and flower are also used for the treatment of snake bite and scorpion sting. C. ternatea has been shown to have number of pharmacological activities such as possessing anxiolytic,

antidepressant, anticonvulsant, anti stress (Jain *et al.,* 2003), sedative, antipyretic, anti inflammatory, analgesic (Devi *et al.,* 2003), Anthelmintic and anti-microbial activities. The extract of C. ternatea has been shown to improve learning ability, enhance memory, increase apical and basal dendritic branches, and increase acetylcholine content and acetyl cholinesterase activity in rats. The plant contains several secondary metabolites such as kaempferol and its glucoside–clitorin, taraxerol and a lactone aparajitin (Shoib Brand *et al.,* 1995). Seeds contain Sistosterol, hexacosanal, and anthoxanthin.

MATERIALS AND METHODS

Plant material collection

The whole plant of *Clitoria ternatea* was collected from Nilgiris district region of Tamil Nadu, India. The plant was botanically authenticated at Tami Nadu agricultural University, Coimbatore.

Chemicals and reagents

The chemicals were used DPPH, Ascorbic acid, FC reagent, Diclofenac sodium, Ibuprofen, Egg albumin, and other chemicals and solvents procured from Merck India and are of AR Grade.

Preparation of Plant extract

The Root of *Clitoria ternatea* were air and shade dried at room temperature for two weeks and made in to fine powder using electric grinder and sieved. Then 10g of plant powder was packed in Whatmann filter paper. A successive solvent extraction was performed with solvents like Petroleum ether, Chloroform, Ethyl acetate, Methanol, water by using Soxhlet techniques. The extracts were evaporated by rotary evaporator and the residue was used for further studies.

Phytochemical screening

Phytochemical analysis revealed that methanolic extract of the plant shows most of test positive. The detailed analyses of tests were tabulated (Table 1).

Estimation of Total phenolic content (Shoib Brand *et al.,* 1995; Mishra *et al.,* 2012)

The total phenolic content was estimated by Folin Ciocalteu method as described by Singleton *et al.,* (1965) with slight modifications. The extract (1 mg. mL^{-1}) was mixed with 10 ml of methanol, 1.6 ml of sodium carbonate (20%) and 2 ml of Folin Ciocalteu reagent. The mixture was allowed to stand in a water bath for 30 min at 40°C. The content of total phenolic compounds was expressed as mg of Gallic acid equivalents per g dry matter (mg GAE. g^{-1}DM). The absorbance was measured at 750 nm using a UV-Vis spectrophotometer Biomate v1.06. All the experiments were run in triplicate. The mean values and standard deviations were calculated using the Microsoft Excel software (Microsoft Corporation, Redmond, WA).

Determination of total flavonoids (Stratil *et al., ?*)

Aluminum chloride colorimetric method was used for flavonoids determination. One ml of Extracts or standard solution of Rutin (500µg/ml) was added to 10 ml volumetric flask containing 4 ml of H_2O. To the above mixture, 0.3 ml of 5 % $NaNO_2$ was added. After 5 min, 0.3 ml 10 % $AlCl_3$ was added. At 6th min, 2 ml 1 M NaOH was added and the total volume was made up to 10 ml with H_2O. The solution was mixed well and the absorbance was measured against reagent blank at 510 nm. Total flavonoid content of extracts was expressed as total mg Rutin /g of extract.

Formula:

$$X = \frac{A.m_0.10}{A_0.m}$$

Where:
X = flavonoid content, mg/gm plant extract,
A =the absorption of plant extract solution,
A_0 =the absorption of standard rutin solution,
m =the weight of plant extract, g,
m_0 =the weight of rutin in solution in ml.

Determination of Antioxidant activity (DPPH radical scavenging activity) (Mishra *et al.,* 2012; Singleton and Rossi, 1965; Mizushima and Kobayashi, 1968)

Radical scavenging activity against the stable DPPH radical was determined spectrophotometrically. The various concentrations (10-100ug/ml) of each extract prepared in methanol were added to 1 ml of 0.1 mM DPPH solution. An equal amount of methanol and DPPH was added to the control. In case of blank DPPH was replaced by methanol. The mixture was shaken vigorously and incubated for 30 minutes in dark at room temperature and then absorbance was recorded at 517 nm. Ascorbic acid was used as positive control with an increase in concentrations.

Estimation of total antioxidant capacity by Phospho molybdenum method (Sangita Chandra *et al.,* 2012)

100 µl of extract is dissolved in 1 ml of TAC reagent. Blank is maintained with distilled water replacing the TAC reagent. Absorbance is seen at 695 nm. (Gudda Darangavvanahally et al, 2004, Pongtip Sithisarn *et al,* 2005).

Evaluation of *in vitro* anti-inflammatory activity (Protein denaturation method) (Shinde *et al.,* 1999)

The reaction mixture (5 ml) consisted of 0.2 ml of egg albumin (from fresh hen's egg), 2.8 ml of phosphate buffered saline (PBS, pH 6.4) and 2 ml of varying concentrations of different extracts of *Artemisia vulgaris* so that final concentrations become 15.625, 31.25, 62.5, 125,250, 500, 1 000 µg/mL. Similar volume of double-distilled water served as control. Then the mixtures were incubated at (37±2) ºC in a BOD incubator for 15 min and then heated at 70ºC for 5 min. After cooling, their absorbance was measured at 660 nm (UV-Vis Spectrophotometer (Biomate absorbance. The percentage inhibition of protein was calculated by using the following formula: % inhibition = $100\times (A_t / A_c - 1)$

Where:

A_t = absorbance of test sample.
A_c = absorbance of control.

The extract/drug concentration for 50% inhibition (IC_{50}) was determined by plotting percentage.

In vitro Anti-inflammatory activity by HRBC membrane stabilization method (Sangita Chandra *et al.*, 2012; Shinde *et al.*, 1999)

HRBC membrane stabilization method has been used to study the anti-inflammatory activity. Blood was collected from the healthy volunteers and mixed with sodium citrate (pre coated tube).The blood was centrifuged at 3000rpm and packed cells were washed with iso-saline (0.85%, pH 7.2). The assay mixture contains 50µl phosphate buffer [pH 7.4, 0.15 M], 100µl hypo saline [0.36 %], 25µl HRBC suspension[10 % v/v]) with 25µl of plant extracts of various concentrations (31.25, 62.5, 125, 250, 500, 1000µg/ml), standard drug diclofenac sodium (31.25, 62.5, 125, 250, 500, 1000 µg/ml) and control [distilled water instead of hypo saline to produce 100 % hemolysis] were incubated at 37ºC for 30 min. The hemoglobin content in the suspension was estimated using Multimode Micro-plate Reader (TECAN (infinite M200 PRO) at 560 nm. The percentage haemolysis produced in the presence of distilled water was taken as 100% Percentage of HRBC membrane stabilization or protection was calculated using the formula% Stabilization = 100 – [(Optical Density of Drug) / (Optical Density of Control) × 100].

RESULTS AND DISCUSSION

Phytochemical Screening

Phytochemical screening shows methanolic extracts possess high amount of phytochemical presences in leaves of plant. This shows why methanolic extracts have great potency for antioxidant and anti inflammatory activity.

Table 1. The phytochemical analysis of Petroleum Ether (PE), Chloroform (CHL), Ethyl Acetate (EA), Methanol (ME) and Aqueous (AQE) extracts of *Clitoria ternatea* root

S.no	Chemical test	Pe	Chl	Ea	Me	Aqe
1	Carbohydrates	+	+	+	+	+
2	Proteins	-	_	-	-	-
3	Amino acids	-	-	-	-	-
4	Steroids	+	+	+	-	+
5	Flavonoids	+	+	-	+	+
6	Glycosides	+	+	-	+	+
7	Alkaloids	+	+	-	-	+
8	Tannins and Phenolics	-	+	+	+	-

Table 2. Total phenolic content of various extracts of *Clitoria ternatea* root

S.No	Extracts	Absorbance (750nm)	Phenolic Content (µg/ml)
1	Pet ether	0.252	15.37± 0.001
2.	Chloroform	0.230	12.62± 0.007
3	Ethyl acetate	0.306	22.12± 0.001
4.	Methanolic	0.980	97.37± 0.001
5.	Aqueous	0.485	44.5 ± 0.004

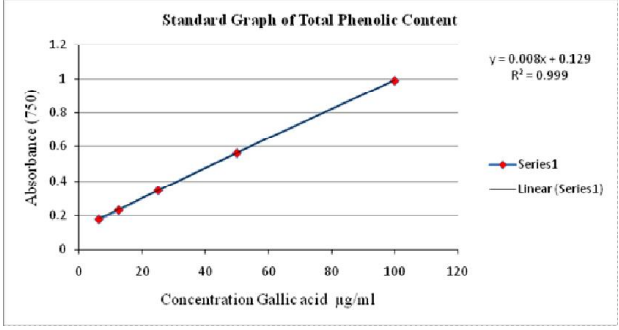
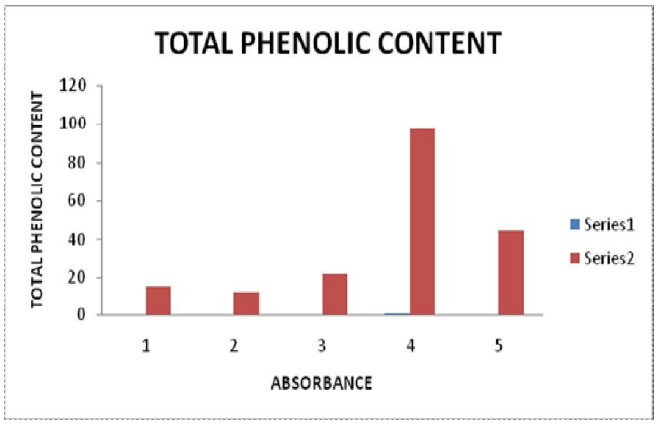
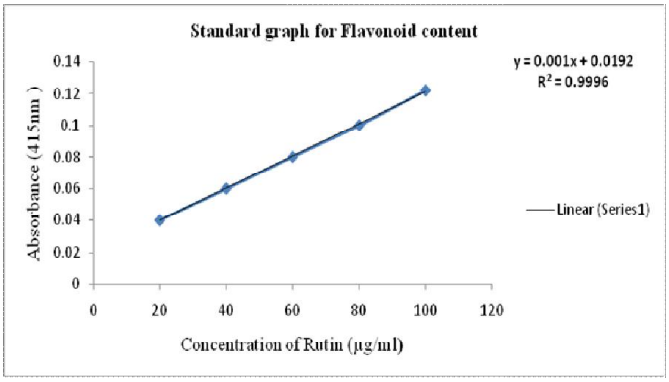
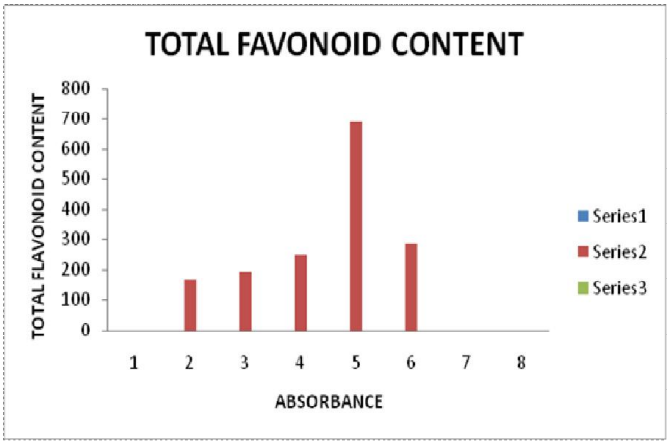


Table 3. Total Flavonoid Content of different extracts of *Clitoria ternatea* root

S.No	Extracts	Absorbance	Total flavonoids (µg/ml)
1	Pet-ether	0.185	165.8±0.0015
2	chloroform	0.214	194.8±0.003
3	Ethyl acetate	0.271	251.8±0.052
4	Methanol	0.709	689.8±0.031
5	Aqueous	0.307	287.8±0.002



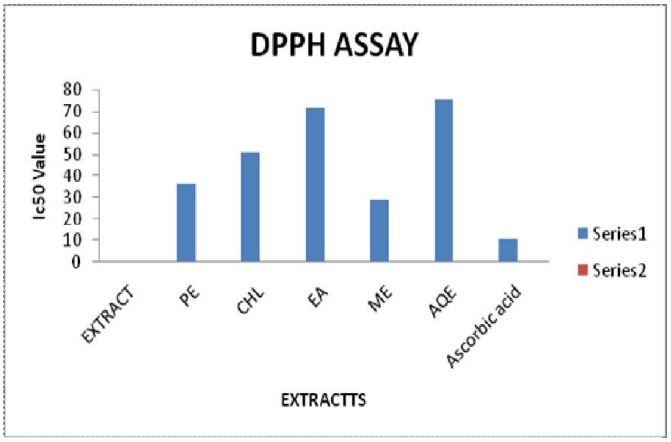




Evaluation of anti oxidant activity (DPPH assay)

Table 4. The IC_{50} values of the various extracts of *Clitoria ternatea* root by DPPH Radical scavenging antioxidant assay

S.No.	Extracts	IC_{50} Values (µg/ml)
1	Petroleum ether	36.25±1.464
2	Chloroform	51.25±1.281
3	Ethyl Acetate	71.5±0.901
4	Methanol	28.7±0.793
5	Aqueous	75.5±1.040
Standard	Ascorbic acid	10.51



Total anti oxidant capacity

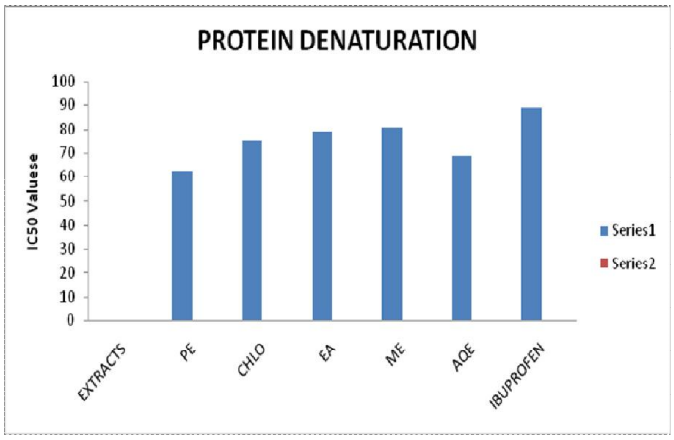
Table 5. The results of Total Antioxidant Capacity of different extracts of *Clitoria ternatea* root

S. No.	Extracts	TAC (mM equivalent of Ascorbic acid)
1.	Petroleum ether	0.222±0.001
2.	Chloroform	0.451±0.04
3.	Ethyl acetate	0.188±0.02
4.	Methanol	0.155±0.002
5.	Aqueous	0.221±0.003

Evaluation anti inflammatory activity

Table 6. The results of Protein Denaturation Method

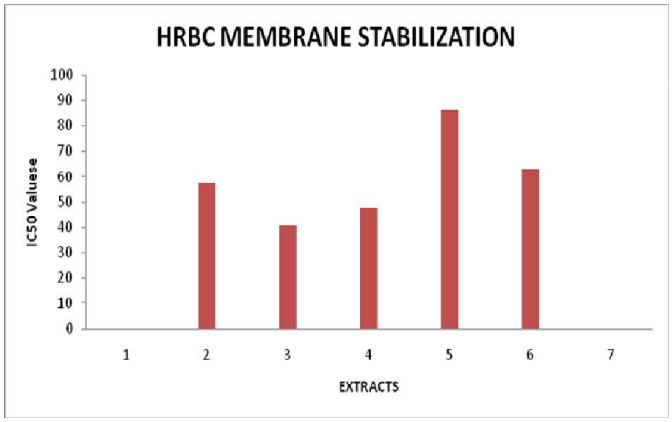
S.NO	EXTRACTS	IC_{50} Values
1	Petroleum ether	62.5±0.763
2	Chloroform	75±1.040
3	Ethyl acetate	28.7±0.793
4	Methanol	80.62±0.788
5	Aqueous	68.5±0.901
Standard	Ibuprofen	88.75±1.289



In vitro Anti-inflammatory activity by HRBC membrane stabilization method

Table 7. IC_{50} values of anti-inflammatory activity on different extracts of *Clitoria ternatea* root

S.NO	EXTRACT	IC_{50} Value
1	Petroleum ether	57.5±1.040
2	Chloroform	40.6±1.266
3	Ethyl Acetate	47.5±0.763
4	Methanol	86±1.258
5	Aqueous	63±0.763



The total phenolic content for *Clitoria ternatea* Root extracts were estimated by Folin Ciocalteu's method using Gallic acid as standard. The reagent is formed from a mixture of phospho tungstic acid and phosphomolybdic acid which after oxidation of the phenols, is reduced to a mixture of blue oxides of tungsten and molybdenum. The blue coloration produced has a maximum absorption in the region of 750 nm and proportional to the total quantity of phenolic compounds originally present. The Gallic acid solution of concentration (10-100 ppm) conformed to Beer's Law at 750 nm with a regression coefficient (R2) = 0.9999.The plot has a slope (m) = 0.0087 and intercept = 0.1294. The equation of standard curve is y = 0.0087x + 0.1294. The total flavonoid content for *Clitoria ternatea* root extracts were measured with the aluminium chloride colorimetric assay using Rutin as standard. Aluminium chloride forms acid stable complexes with the C-4 keto groups and either the C-3 or C-5 hydroxide group of flavones and flavonols In addition it also forms liable complexes with ortho di hydroxide groups in A/B rings of flavonoids. The Rutin solution of concentration (100-1000 ppm) conformed to Beer's Law at 510 nm with a regression co-efficient (R2) = 0.9996. The plot has a slope (m) = 0.001 and intercept = 0.0192. The equation of standard curve is y = 0.001x + 0.0192.

DPPH scavenging activity

All plant different extract were measured by the ability to scavenge DPPH free radicals and Total antioxidant capacity was compared with the standard ascorbic acid. It was observed that methanol extract of the *Clitoria ternatea* root shows higher activity than the other extracts of the plant. This showed that the extracts have the proton donating ability and possibly act as primary antioxidants; could serve as free radical inhibitors or scavenging.

Inhibition of protein denaturation

The anti-inflammatory potential by membrane stabilization and protein denaturation was found to be high in methanol extract whereas. These studies provide an indication for membrane stabilization and protein denaturation as an additional mechanism of *Clitoria ternatea* root for anti inflammatory effect (Table 5 and 6).

Conclusion

Antioxidant and Anti- inflammatory potentials of different extract obtained from Root of *Clitoria ternatea* were evaluated. The methanolic extracts were found to possess more

radical scavenging activity, anti-inflammatory, antioxidant, as determined by protein denaturation, membrane stabilization, scavenging effect on the DPPH, reducing power than the other solvent extracts. In general, results indicated that the extracts possess potent bioactivities in the present study, it is found that the methanolic extract of root of *Clitoria ternatea* contains a substantial amount of phenolics and flavonoids, and it is the presence of phenolics compounds may be responsible for their marked antioxidant activity. The result also shows a prominent binding of the ligand with anti-inflammatory targets. Thus, it can be concluded that methanolic extract of *Clitoria ternatea* root can be used as an antioxidant, anti-inflammatory agent. Anti-inflammatory potentials of the selected extracts were depicted clearly in protein denaturation, membrane stabilization assay. The other extracts also shows some anti-oxidant and anti- inflammatory activities.

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