

## Radical scavenging activity of *Clitoria ternatea* leaf extracts

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### ABSTRACT

Free radical scavenging assays were performed in both blue flowered leaf and white flowered leaf of *Clitoria ternatea* and analyse their ability to scavenge the oxidants like DPPH, SO, NO, and OH<sup>-</sup> by using different solvents like petroleum ether, butanol, chloroform, ethanol and water. The ethanolic extracts offered the maximum scavenging in all the assays. The effects of white flowered leaves were more efficient than those of blue flowered leaves.

**Key words:** *Clitoria ternatea*, antioxidants, free radical, different solvents.

### INTRODUCTION

Antioxidants act as a defense mechanism that protects against oxidative damage, and include compounds and repair enzymes to remove or repair damaged molecules. However, the natural antioxidant compounds become important (Malpure *et al.*, 2006). Antioxidants can prevent/retard the oxidation caused by free radicals and sufficient intake of antioxidants is supposed to protect against diseases (Celiktar *et al.*, 2007). Nitric oxide radical is a labile compound, which has a brief half life and is rapidly converted to the stable end products nitrite (NO<sub>2</sub><sup>-</sup>) and nitrate (NO<sub>3</sub><sup>-</sup>) (Everekiloglu *et al.*, 2003). One of the plants that deserves attention is *Clitoria ternatea* (Sanskrit-Sankupushpam) belongs to the family *Fabaceae*, is widely used in traditional Indian system of medicine as a brain tonic (Gomez and Kalamani, 2003). *Clitoria ternatea* is a perennial twinning herb bearing blue or white flowers. The present study is the new one concentrating on the antioxidant activity and to analyze the free radical scavenging, biomolecular protecting and antioxidant modulating effects.

### MATERIAL AND METHODS

TLC plates (Silica gel 60 F<sub>254</sub>, Merck), DPPH (0.4mM in methanol), DPPH (0.3 mM in methanol), Methanol, EDTA (0.1M containing 1.5 mg NaCN), Nitroblue tetrazolium (NBT, 1.5mM), Riboflavin (0.12mM), Phosphate buffer (0.06 M, pH 7.6), Dimethyl sulphoxide (DMSO), Phosphate buffered saline (PBS), pH 7.2, Sodium nitroprusside (100mM), Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% naphthylethylene diamine dihydrochloride), Ferric chloride (0.1 mM), EDTA (0.1mM), H<sub>2</sub>O<sub>2</sub> (1mM), Ascorbate (0.1mM), KH<sub>2</sub>PO<sub>4</sub>-KOH buffer (20mM, pH 7.4, Deoxyribose (2.8mM), Thiobarbituric acid (TBA, 1%), All other reagents were of the highest grade available commercially.

#### Solvent extraction

As the initial step in the second phase, the leaves were serially extracted into cold solvents of increasing polarity. The solvents used were petroleum ether, benzene, chloroform, ethanol, methanol and water. The residues of the different solvent extracts were weighed and redissolved in a

known volume of the same solvent in which they were extracted.

#### Free radical and oxidant scavenging effects of the leaves of *Clitoria ternatea*

The leaf extracts were tested for their scavenging activity against the stable free radical DPPH (2,2'-diphenyl-1-picryl hydrazyl). The ability of the leaf extracts to scavenge DPPH was studied in a dot plot rapid screening assay and quantified using a spectrophotometric assay as proposed by Soler-Rivas *et al.* (2000) and Mensor *et al.* (2001) respectively.

#### Rapid screening of antioxidant activity by dot plot assay

Antioxidants react with diphenyl-1-picryl hydrazyl (DPPH) and convert it to diphenyl-picryl hydrazine. The degree of discolouration from purple to yellow colour can be used as a measure of the scavenging potential of antioxidant extract. Aliquots (3 $\mu$ l) of *Clitoria ternatea* extracts were spotted on a TLC plate and allowed to dry. The TLC plate bearing the dry spots was placed upside down for 10 seconds in the solution of DPPH. The spots exhibiting radical scavenging, antioxidant activity showed up as yellow spots in a violet background. The intensity of the yellow colour depends on the amount and nature of the radical scavenger present in the spot.

#### DPPH photometric assay

The ability of the leaf extracts to bleach DPPH can be quantified using a spectrophotometric assay, the extent of scavenging causing a proportionate change in the absorption at 518nm. An exact amount (0.5ml) of the methanolic solution of DPPH was added with 20  $\mu$ l of the leaf extracts in the different solvents and the crude aqueous extract (corresponding to 4mg) and 0.48ml of methanol, and allowed to stand at room temperature for 30 minutes. Methanol served as the blank. After 30 minutes, the absorbance was measured at 518nm and converted into percentage radical scavenging activity as follows:

$$\text{Scavengin activity (\%)} = \frac{[\text{sample}] - A_{518}[\text{blank}]}{A_{518}[\text{blank}]} \times 100$$

#### Inhibition of *in vitro* generation of superoxide and nitric oxide

In the present study, the efficiency of the leaf extracts of *Clitoria ternatea* in inhibiting the *in vitro* generation of superoxide and nitric oxide were studied using the methods elaborated by Winterbourn *et al.* (1975) and Green *et al.* (1982) respectively.

#### Determination of superoxide generation *in vitro*

The extent of superoxide generation was studied on the basis of inhibition of production of nitroblue tetrazolium formazone by the plant extracts measured spectrophotometrically at 560nm. The assay tubes contained 0.02ml of the plant sample (corresponding to 20mg extract) with 0.2ml EDTA, 0.1 ml nitroblue tetrazolium, 0.05ml riboflavin and 2.64ml phosphate buffer. The control tubes were set up without the leaf extracts, where DMSO was added. The initial optical densities of the solutions were recorded at 560nm and the tubes were illuminated uniformly with the fluorescent lamp for 30 minutes.  $A_{560}$  was measured again and the difference in O.D was taken as the quantum of superoxide production. The percentage inhibition by the leaf samples was calculated by comparing with the O.D of the control tubes.

#### Determination of nitric oxide generation *in vitro*

Aqueous solution of sodium nitroprusside spontaneously generates nitric oxide (NO) at physiological pH, which interacts with oxygen to produce nitrite ions, which is measured spectrophotometrically at 546nm. The reaction mixture containing 0.3ml of sodium nitroprusside, 2.68ml PBS and 20 $\mu$ l of leaf extract (20mg) was incubated at 25°C for 15 minutes. Control tubes (100% generation) were prepared without the leaf extracts. After incubation, 0.5ml of the Griess reagent was added. The absorbance of the chromophore formed, indicative of the quantum of NO generated, was read at 546 nm.

#### Determination of hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of the leaf extracts was quantified by the method reported by Elizabeth and Rao (1990). The damage to deoxyribose was quantified as thiobarbituric acid

reactive substances, which is also taken as a measure of hydroxyl radical scavenging activity of the leaf extracts. The reaction mixture (0.1ml) contained 0.1ml of deoxyribose, 0.1ml of  $\text{FeCl}_3$ , 0.1ml of EDTA, 0.1ml of  $\text{H}_2\text{O}_2$ , 0.1ml of ascorbate, 0.1ml of  $\text{KH}_2\text{PO}_4$ -KOH buffer and 20 $\mu\text{l}$  of leaf extracts (20mg) in a final volume of 1.0ml. The reaction mixture was incubated for 1 hour at 37°C. Then 1.0ml of TBA was added and heated in a boiling water bath for 20 minutes. The pink colour produced was measured at 535 nm in a spectrophotometer. Deoxyribose degradation was measured as TBARS and the per cent inhibition was calculated.

#### Statistical analysis

All the parameters studied were subjected to statistical treatment using SigmaStat Statistical package (version 3.1). one way ANOVA, followed by post-hoc analysis using Fisher's LSD was adopted to all the parameters under study to test the levels of statistical significance.

## RESULTS

### Free radical and oxidant scavenging effects of the leaves of *Clitoria ternatea*- dot plot assay

In an effort to understand the nature of the active principles in the leaves, they were serially extracted into solvents of increasing polarity (petroleum ether, benzene, chloroform, ethanol and methanol).

The leaf extracts were tested for their scavenging activity against the stable free radical DPPH by a qualitative rapid dot plot assay and quantified spectrophotometrically.

The picture obtained in the dot plot assay is shown in Plate 1. In this assay, the maximum DPPH scavenging activity was expressed by the ethanolic extract, followed by the methanolic extract. Considerable scavenging effects were also noted with the other extracts

#### DPPH DOT PLOT ASSAY



a. *Clitoria ternatea* - Blue flowered leaf extracts



b. *Clitoria ternatea* - White flowered leaf extracts

PE - Petroleum Ether    B - Benzene    C - Chloroform  
E - Ethanol            M - Methanol    A - Aqueous

### Photometric assay

The radical scavenging effects of *Clitoria ternatea* leaves were also quantified using the photometric assay. To confirm the observations in dot plot rapid screen, the photometric quantification of the extent of DPPH scavenging by different solvent extracts was followed. The results, expressed as per cent scavenging, are represented in Figure 1.

The results revealed that the maximum extent of DPPH scavenging activity was elicited by the ethanol extract, followed closely by methanol extract and then by the aqueous extract. This trend was observed in both the leaves. The white flowered leaves exhibited more DPPH scavenging activity than the blue flowered ones.

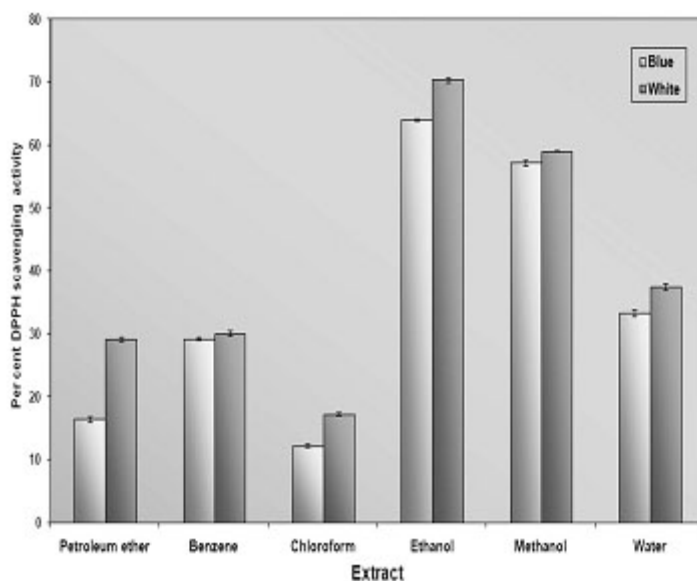


Fig. 1: DPPH scavenging activity of *Clitoria ternatea* leaves

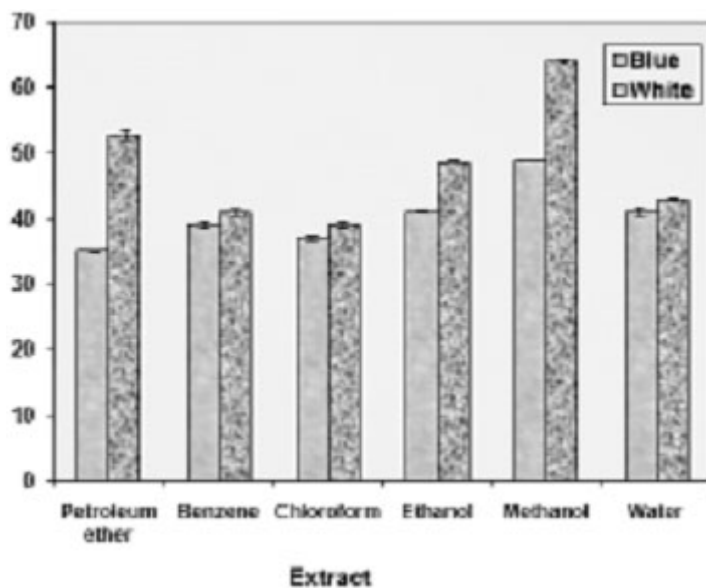


Fig. 2: *Clitoria ternatea* leaves on superoxide generation in vitro

Following this, the extracts were individually tested for their effects on the *in vitro* generation of SO and NO. Figures 2 and 3 shows the per cent inhibition of superoxide and nitric oxide radical generation *in vitro* by the leaves of *Clitoria ternatea* and Figure 4 shows the hydroxyl radical scavenging activity.

Both the varieties of *Clitoria ternatea* leaf extracts exhibited considerable scavenging of both SO and NO generated *in vitro*. The highest inhibitory effect was observed in the ethanolic extract of the leaves, with the white flowered ones faring better over the blue flowered ones.

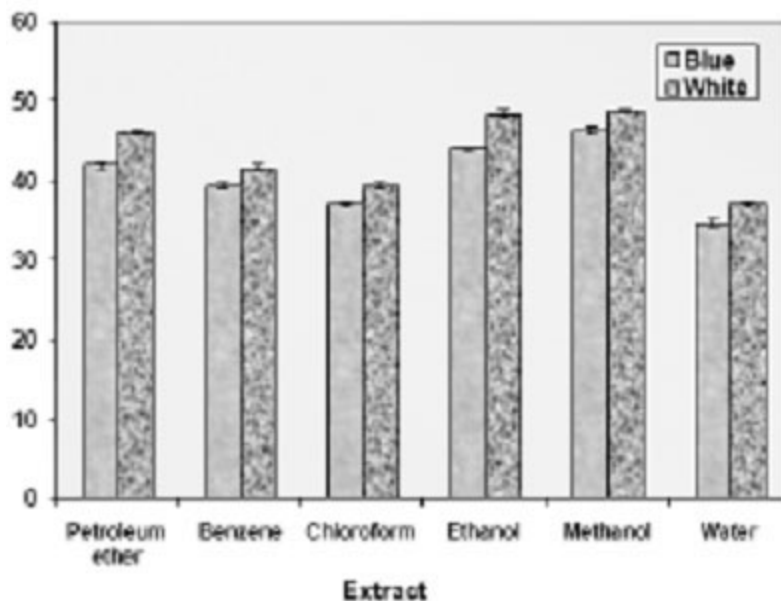


Fig. 3: Effect of *Clitoria ternatea* leaves on nitric oxide generation *in vitro*

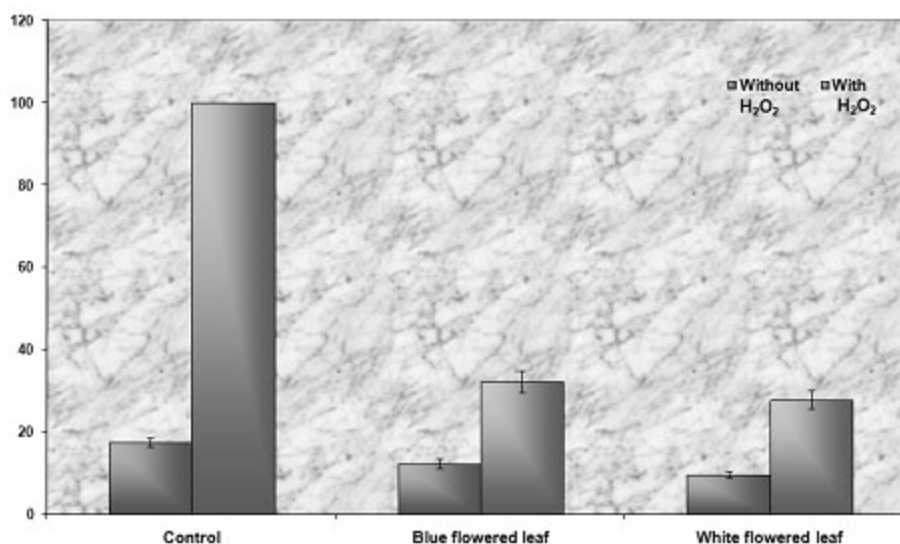


Fig. 4: Hydroxyl radical scavenging activity of *Clitoria ternatea* leaf

The extracts of *Clitoria ternatea* leaves effectively scavenged hydroxyl radicals. It can be observed from the results that white flowered leaves rendered better protection than the blue flowered leaves.

## DISCUSSION

*Clitoria ternatea* leaves, extracted serially into solvents of increasing polarity (petroleum ether, benzene, chloroform, ethanol, methanol and water) were tested for their free radical scavenging activity against DPPH, superoxide ( $\text{SO}^\circ$ ), nitric oxide (NO) and hydroxyl radicals ( $\text{OH}^\circ$ ).

The DPPH scavenging ability of medicinal plants has been attributed to several components, including phenolics (Beppu *et al.*, 2003; Valentao *et al.*, 2003; Bartolome *et al.*, 2004), flavonoids (Shetgiri and D'Mello, 2003) and meroterpenoids (Fisch *et al.*, 2003). The DPPH scavenging activity of selected herbal medicinal plants was found to be related to their antimutagenic activity (Gulcin *et al.*, 2004). Many medicinal plants have been analyzed and reported for their DPPH scavenging activity. Jainu and Shyamaladevi (2005) have shown that a methanolic extract of *Cissus guandangularis* exhibited significant DPPH scavenging effect. In the present study, the maximum extent of DPPH scavenging activity was elicited by the ethanol extract followed by the methanol extract and then by the aqueous extract. The white flowered leaves exhibited more DPPH scavenging activity than the blue flowered ones.

*Clitoria ternatea* leaves were also tested for their scavenging activity against superoxide radical generation. Many reports in the literature associate the  $\text{SO}^\circ$  scavenging activity of plants and their components with strong antioxidant activity. The white flowered leaves exhibited a better radical scavenging activity in ethanol extract than the blue flowered leaves. Superoxides are believed to underlie many of the oxidative changes. Superoxides react with nitric oxide forming peroxynitrite ( $\text{ONOO}^\circ$ ), which rapidly causes oxidative event which rapidly causes oxidative events including LPO, DNA damage and cell death (Cowell and Rusell, 2004). Excessive production or inadequate removal of  $\text{O}_2^{\cdot-}$  has been implicated

in the pathogenesis of many cardiovascular and other diseases including atherosclerosis, hypertension and diabetes (Fukai *et al.*, 2000). Superoxide has been reported to directly initiate lipid peroxidation (Yen and Duh, 1994), if the dismuted superoxide derivative is not immediately scavenged by antiperoxidative enzymes. The present study shows that the  $\text{SO}^\circ$  scavenging action is higher in polar solvent extracts of *Clitoria ternatea* leaves than the non-polar extracts. However, the non-polar solvent extracts also exhibited considerable  $\text{SO}^\circ$  radical inhibition.

Another radical that was studied in the present investigation was nitric oxide (NO). NO, which is the primary RNS, is a key signaling molecule in physiological and pathological conditions. Quercetin, a polyphenolic flavonoid compound reacts strongly with hydrogen peroxide and effectively scavenges NO (Geetha *et al.*, 2005). Similarly, several specific components of plants have been shown to possess potent NO inhibitory activities (Banskota *et al.*, 2003). Nitrogen is taken into the body as nitrates, nitrites, peptides, proteins and amino acids. The metabolites of nitrogen include nitric oxide, higher oxides of nitrogen and peroxy nitrite. Although NO is a free radical, it is probably insufficiently reactive to attack DNA directly. By contrast,  $\text{N}_2\text{O}_3$ ,  $\text{HNO}_2$  and  $\text{ONOO}^\circ$  can nitrate and deaminate DNA and cause strand breakage and mutations (Ishii *et al.*, 2001).

In the present study, both the varieties of *Clitoria ternatea* exhibited considerable scavenging of both SO and NO generated *in vitro*. The highest inhibitory effect was observed in the ethanolic extract of leaves, with the white flowered ones faring better over the blue flowered ones. The  $\text{OH}^\circ$  radical scavenging activity caused the maximum damage to deoxyribose. This damage was very effectively counteracted by the leaf extracts of *Clitoria ternatea*. The white flowered leaves rendered better protection than the blue flowered leaves.

Based on the above results, in order to reap the maximum benefits of the *Clitoria ternatea* leaf extracts, an ethanolic extract of the leaves was prepared and used in the subsequent studies. In all the contents the white flowered leaves were higher than the blue flowered leaves.

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