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ANTIOXIDANT POTENTIAL OF CLITORIA TERNATEA LEAF EXTRACTS IN VITRO

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ABSTRACT

Enzymic antioxidants were analyzed in both blue flowered leaf and white flowered leaf of *Clitoria ternatea*. The *in vitro* model used in the study as alternatives to live animals, was goat liver slices. The liver slices exposed to different treatments were incubated for one hour at 37˚C with very mild shaking. The enzymic antioxidant activity was analyzed by using goat liver slices, both blue flowered leaf and white flowered leaf of *Clitoria ternatea* and H_2O_2 as oxidant. The results showed that the white flowered leaves had higher content of all the enzymic antioxidants analyzed than the blue flower.

KEY WORDS: ***Clitoria ternatea,*** **Enzymic antioxidants, Goat liver slices, H_2O_2, FT-IR spectra.**

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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 have become important [1]. Medicinal plants are considered as potential sources of antioxidant compounds. There is an increasing interest in the investigation of naturally occurring antioxidants from plants [2]. 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[3].*Clitoria ternatea* is a perennial twinning herb bearing blue or white flowers as shown in Figure1, concentrating on enzymic antioxidant activity was analyze in both blue and white flowered leaf of *Clitoria ternatea* leaf extracts by using (goat liver slices presence and absence of oxidant H_2O_2 *) in vitro* model.

MATERIALS AND METHODS

The enzymic antioxidants analyzed in the leaves were superoxide dismutase (SOD), catalase, peroxidase, polyphenol oxidase, glutathione reductase and glutathione S-transferase**.**

ASSAY OF SUPEROXIDE DISMUTASE (SOD)

Superoxide dismutase activity was assayed by the method [4] .An accurate amount (0.5g) of leaves of *Clitoria ternatea* was homogenized with 3.0ml of potassium phosphate buffer, centrifuged at 5000 rpm for 10 minutes and the supernatant was used for the assay. The assay mixture contained 1.2ml of sodium pyrophosphate buffer, 0.1ml of PMS, 0.3ml of NBT, 0.2ml of enzyme preparation and water in a total volume of 2.8ml. The reaction was started by the addition of 0.2ml of NADH. After incubation at 30˚C for 90 seconds, the reaction was stopped by the addition of 1.0ml of glacial acetic acid. The reaction mixture was stirred vigorously with 4.0ml of n-butanol. The mixture was allowed to stand for 10 minutes and then centrifuged and measured at 560 nm against butanol as blank.

ASSAY OF CATALASE

Catalase activity in the leaves of white and blue variety of *Clitoria ternatea* was assayed by the method [5] .An exact amount (0.5g) of *Clitoria ternatea* leaves were homogenized in 2.5ml of phosphate buffer. An aliquot (3.0ml) of hydrogen peroxide in phosphate buffer was taken and adjusted at 240nm in a spectrophotometer.

ASSAY OF PEROXIDASE

Peroxidase activity was assayed spectrophotometrically by the method [6]. An accurate amount (0.5g) of *Clitoria ternatea* leaves was homogenized in 2.5ml of 0.1M phosphate buffer, centrifuged and the supernatant was used as the enzyme source. Pyrogallol solution (3.0ml) and enzyme extract (0.2ml) were pipette out into a cuvette. The spectrophotometer was adjusted to read 'zero' at 430nm.

ASSAY OF POLYPHENOL OXIDASES (PPO)

Polyphenol oxidase activity was determined by the method [7] .In this method, catechol oxidase and laccase activities were simultaneously estimated spectrophotometrically. *Clitoria ternatea* leaves (0.5g) were homogenized in the medium, containing Tris-HCl, sorbitol and NaCl, and was made upto 2.0ml. The homogenate was centrifuged and the supernatant was used for the assay. An aliquot of 2.5ml of (0.1M) phosphate buffer and 0.3ml of catechol solution were added into

a cuvette and the spectrophotometer was set at 495nm.

ASSAY OF GLUTATHIONE S-TRANSFERASE

Glutathione S-transferase activity was assayed by the method [8] .An accurate amount (0.5g) of *Clitoria ternatea* leaves were homogenized in 5.0ml of phosphate buffer. The homogenate was centrifuged and the supernatant was used for the assay. The enzyme activity was determined by monitoring the change in absorbance at 340 nm in a spectrophotometer.

ASSAY OF GLUTATHIONE REDUCTASE

This method [9] was adopted for assaying the activity of glutathione reductase. *Clitoria ternatea* leaves (0.5g) were homogenized in 5.0ml of phosphate buffer. The assay system contained 1.0ml of potassium phosphate buffer, 0.1ml of EDTA, 0.1ml of sodium azide, 0.1ml of oxidized glutathione, 0.1ml of the enzyme source and water in a final volume of 2.0ml, and then 0.1ml of NADPH was added. The absorbance at 340 nm was recorded .

ASSAY OF GLUTATHIONE PEROXIDASE

The method [10] was used for the assay of GPx in the tissue homogenate.The reaction mixture (1.0ml) contained 0.4ml of sodium phosphate buffer, 0.1 ml of sodium azide, 0.2ml of reduced glutathione, 0.2ml of tissue homogenate and 0.1ml of H_2O_2. The tubes were incubated at 37˚C for 3 minutes. Add 0.3ml of disodium hydrogen phosphate and 1.0ml of DTNB solution, . The yellow colour developed was read at 412nm in a spectrophotometer.

ANTIOXIDANT STATUS IN VITRO

The *in vitro* model used in the study as alternatives to live animals was goat liver slices. The effect of the exposure of the leaf extracts in the presence or absence of the oxidants was followed in precision-cut goat liver slices, which simulated the *in vivo* environment. Fresh goat liver was obtained .Very thin slices (~1mm thick) were cut from the liver using a sterile scalpel. The slices were taken in sterile Hank's balanced salt solution (HBSS) . The standard oxidant H_2O_2 was used at a final concentration of 200µM. The plant extract (20 µl of the plant extract corresponding to 20mg) was used. The liver slices exposed to different treatments were incubated for one hour at 37˚C with very mild shaking.

The following groups were set up for every assay.

1. Untreated (negative) control
2. H_2O_2 treated (positive) control
3. Group treated with white flowered leaf extract
4. Group treated H_2O_2 and white flowered leaf extract
5. Group treated with blue flowered leaf extract
6. Group treated with H_2O_2 and blue flowered leaf extract

After the addition of the respective agents, the tissue slices were incubated at 37˚C for one hour with mild shaking. After the incubation period, the tissue was homogenized in a Teflon homogenizer with HBSS and the estimation of various parameters indicative of antioxidant potential were carried out

RESULTS

ACTIVITIES OF ENZYMIC ANTIOXIDANTS IN CLITORIA TERNATEA LEAVES

The enzymic antioxidants analyzed in the two different varieties of *Clitoria ternatea* (bearing blue and white flowers) in Table 1. The leaves of *Clitoria ternatea* were found to be good sources of all the enzymic antioxidants analyzed (SOD, CAT, POD, GST and PPO). The results showed that the activities of all the enzymic antioxidants analyzed were found to be more in the white flowered leaves than in the blue flowered leaves

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TABLE 1
ACTIVITIES OF ENZYMIC ANTIOXIDANTS IN Clitoria ternatea LEAVES

Enzyme		Blue flowered leaf	White flowered leaf
SOD (U^{+}/g)		14.97 ± 0.125	15.25 ± 0.261[a]
CAT ($U^{@}$/g)		110.39 ± 1.969	133.63 ± 0.961[a]
POD (U*/g)		68.42 ± 0.848	86.11 ± 1.075[a]
GST ($U^{\#}$/g)		2.060 ± 0.122	6.37 ± 0.126[a]
PPO ($U^{\&}$/g)	Catechol oxidase	0.260 ± 0.010	0.322 ± 0.011 [a]
	Laccase	0.203 ± 0.006	0.232 ± 0.007 [a]

Values are mean ± SD of triplicates
a - Statistically significant (P< 0.05) compared to blue flowered leaves.
+1 unit - Activity of enzyme that gives 50% inhibition of the extent of NBT reduction in 1minute.
@ 1unit - Amount of enzyme required to decrease the absorbance at 240nm by 0.05 units.
** 1unit - Change in absorbance / minute at 430nm.*
 # 1unit - nmoles of CDNB conjugated / minute.
&1unit - Activity of catechol oxidase/laccase which transforms 1μmol of dihydro-phenol to quinine / minute

ENZYMIC ANTIOXIDANTS IN GOAT LIVER SLICES

The enzymic antioxidants analyzed in the goat liver slices were superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione S-transferase (GST).

SOD

Figure 1 represents the activities of SOD in the goat liver slices exposed to H_2O_2 in the presence and the absence of *Clitoria ternatea* leaf extracts. H_2O_2 exposure caused a significant decrease in SOD activity. The administration of goat liver slices with blue flowered leaf extract and white flowered leaf extract caused a significant ($P<0.05$) increase in SOD activity, compared to control group. When the leaves were administered along with H_2O_2, the SOD activity increased significantly ($P<0.05$). Increases in SOD enzyme activity corresponds with enhanced resistance to oxidative stress [18, 19, 20].Thus, SOD plays a major role in first line defense against oxidants in all cellular and extracellular compartments [21] Rice flavonone imparts a protective effect on hepatic injury by increasing the activity of SOD and GPx and scavenging free radicals produced by H_2O_2.

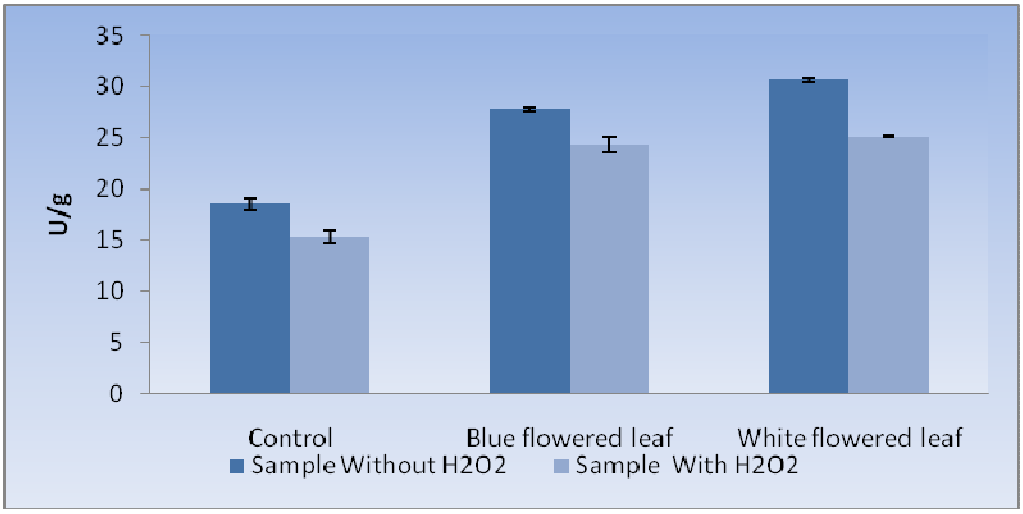

Figure 1
EFFECT OF Clitoria ternatea LEAVES ON SOD ACTIVITY IN H_2O_2 – INDUCED OXIDATIVE STRESS IN GOAT LIVER SLICES

CAT

The activities of catalase in the H_2O_2 treated liver slices in the presence and absence of *Clitoria ternatea* leaf extracts are presented in Figure 2. Oxidant challenge, by way of H_2O_2 treatment, significantly reduced the activity of CAT ($P<0.05$), which effect was nullified by the administration of the *Clitoria ternatea* leaf extracts. Both the leaves, by themselves, significantly ($P<0.05$) increased the CAT activity over the untreated control. A significant reduction was observed in the activity of catalase when the liver slices were treated with H_2O_2.

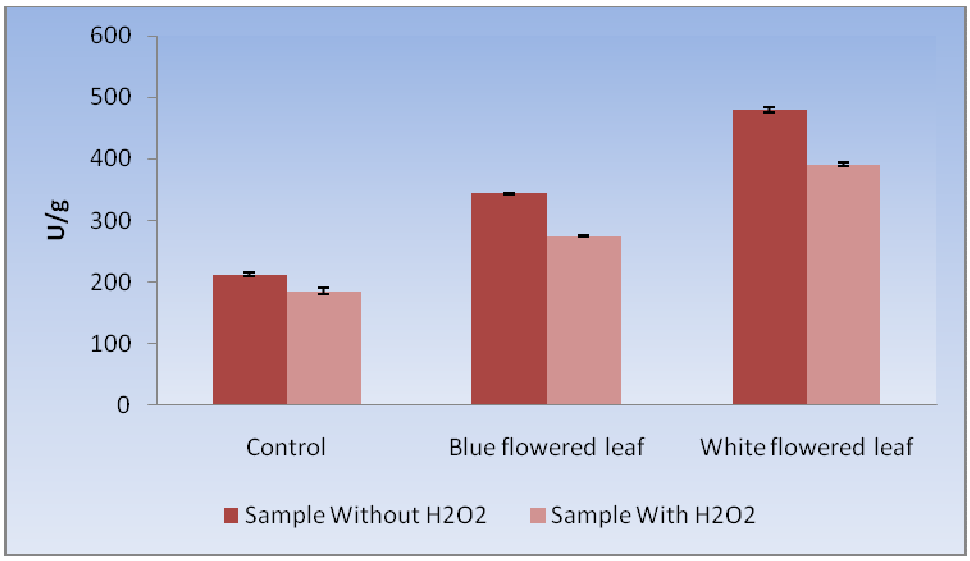

Figure 2
EFFECT OF Clitoria ternatea LEAVES ON CATALASE ACTIVITY IN H_2O_2 - INDUCED OXIDATIVE STRESS IN GOAT LIVER SLICES

GPx

Glutathione peroxidase activity, a well known first line defense against oxidative stress, was assayed in the liver slices exposed to H_2O_2 and / or *Clitoria ternatea* leaf extracts. Figure 3.

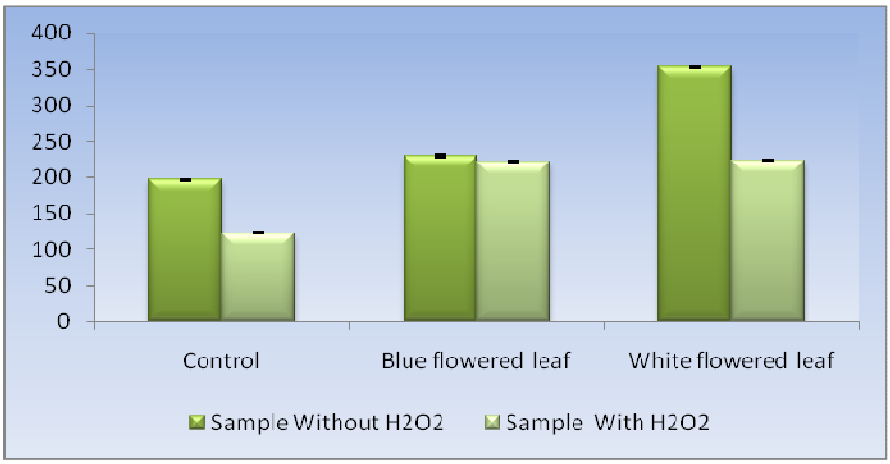

Figure 3
EFFECT OF Clitoria ternatea LEAVES ON GPx ACTIVITY IN H_2O_2-INDUCED OXIDATIVE STRESS IN GOAT LIVER SLICES

The values of GPx activities observed. Oxidative stress induced by H_2O_2 was reflected by a steep decrease in the activity of GPx ($P<0.05$). The treatment with the leaf extracts could revert this effect by significantly increasing the GPx activities. Our results show that *Clitoria ternatea* leaf extracts can bring about an improvement of the activities of catalase in oxidatively stressed liver slices.

GR

Glutathione reductase (GR) is an enzyme with a lot of importance to the antioxidant system, as it is one of the major determinants of the GSH levels, by replenishing the reduced form from its oxidized one. The GR activities observed in various treatment groups in the present study are given in Figure 4.

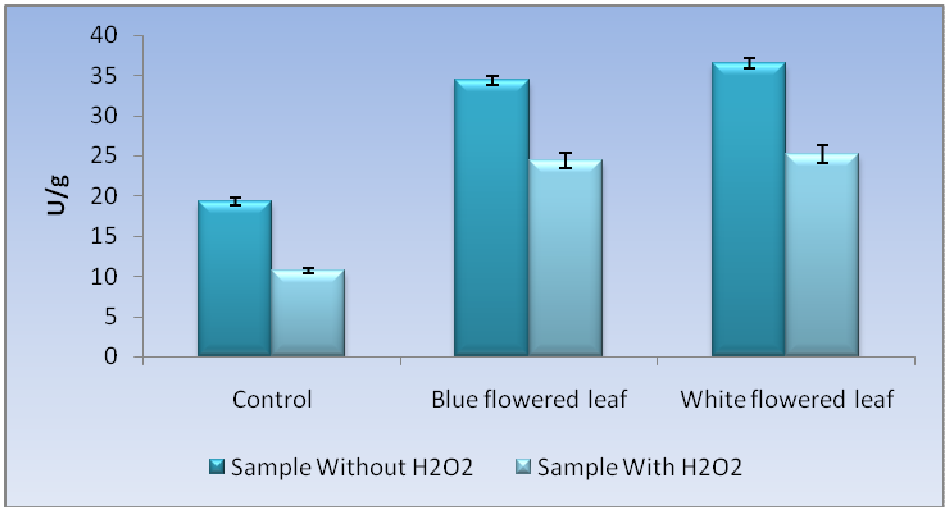

Figure 4
EFFECT OF Clitoria ternatea LEAVES ON GR ACTIVITY IN H_2O_2 - INDUCED OXIDATIVE STRESS IN GOAT LIVER SLICES

GST

GST is a conjugating enzyme, which is intimately involved in glutathione metabolism. The GST activities in the H_2O_2-treated liver slices in the presence and the absence of leaf extracts are expressed in Figure 5.

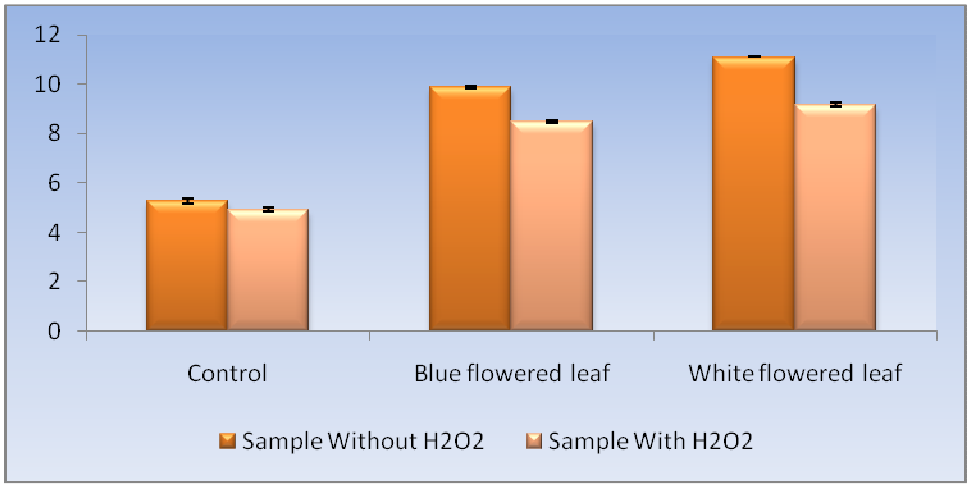

Figure 5
EFFECT OF Clitoria ternatea LEAVES ON GST ACTIVITY IN H_2O_2 - INDUCED OXIDATIVE STRESS IN GOAT LIVER SLICES

DISCUSSION

As the two widely available varieties of *Clitoria ternatea* leaves, namely the leaves obtained from plants bearing blue flowers and those from plants bearing white flowers were analyzed for the activities of enzymic antioxidants. A comparative study of antioxidant levels in different Piper species showed a differential antioxidant status with reference to enzyme antioxidants, environmental stresses and is important in breeding programs [11, 12, 13]. SOD and CAT activities declined in *Triticum aestivium* leaves upon senescence [14].*Coffea arabica* and *Coffea canephora* of green coffee samples has been reported to have high catalase activity [15, 16, 17]. The results showed that the white flowered leaves possessed higher activities of enzyme antioxidants when compared to blue flowered leaves. The results of the present study showed that the blue flowered leaf extract and white flowered leaf extract of *Clitoria ternatea* caused a significant increase in SOD activity when compared to H_2O_2 treated group. The effect of white flowered leaf extract was slightly higher than that of the blue flowered leaf extract. The antioxidant enzymes, SOD and CAT, constitute a mutually supportive team of defense against ROS [22] .Catalase has been shown to be of primary importance in erythrocyte defense against H_2O_2 than the other enzymes [23, 24, 25]. *Catharanthus roseus, Helianthus annunus, Vigna unguiculata* [26, 27, 28] .and increased catalase activity under drought stressed conditions. *Arabidopsis thaliana* increased catalase activity in response to methyl jasmonate [29]. The crude methanolic extract of *Centella asiatica, Indigofera oblongifolia, Artemisia apiacea* increased the activity of GPx in cell line induced lymphoma-bearing mice[30, 31, 32 33**]** Garlic and neem extracts significantly raised the levels of GSH and GPx in gastric carcinogenesis induced by MNNG in experimental rats. Peroxidase-inducing effect was also observed with the administration of

Fiscusbeng alensis, Withiana somnifera in hypercholesterolemic rabbits [34, 35].*Ginkgo biloba* methanolic, *Lawsonia alba* extract significantly increased GPx activities [36, 37] . As a result the present study shows that the *Clitoria ternatea* leaf extracts also improved the GPx activities.

GR also exhibited a significant decrease upon oxidant assault. The leaf extracts (both blue and white flowered) significantly elevated the GR activities from the decreased levels. Glutathione reductase acts together with antioxidant enzymes like SOD, CAT and POD to quench harmful radicals [38] .Glutathione reductase is essential for maintaining high concentration of GSH, another antioxidant in the cell [39, 40. 41] Rubiadin, a major constituent isolated from *Rubia cardiofolia* Linn, significantly prevented the hepatic injury by elevating the activities of GST and GR and scavenging the free radicals produced by oxidant exposure. *Ginkgo biloba* extract and the methanolic extract of *Coscinium fenestratum* stem powder [42, 43] retrieved the decreased activities of glutathione reductase. Our results show that the *Clitoria ternatea* leaf extracts bearing white flowered leaves ha higher induction of enzyme antioxidants than the blue flowered leaves.

The activity of GST decreased significantly upon exposure to H_2O_2 ($P<0.05$). This depletion of GST with the exposure of H_2O_2 was counteracted by the co-treatment with the leaf extracts. The white flowered leaf extract showed significantly higher effect than the blue flowered one. GSTs are catalyze the conjugation of electrophilic xenobiotics with the sulphydryl moiety of GSH, thus providing less toxic and more water soluble derivatives [44, 45] . An alcoholic extract of *Aloe vera* leaves resulted in a significant increase in GST activity in the tissues of diabetic rats [46] . *Spirulina fusiformis*, *Thespesia populnea*, significantly enhanced the activities of SOD, CAT, GPx and GST in the liver of experimental mice in retaliation of genotoxicity by cisplatin and urethane [47, 48] .The activity of GST has been found to be decreased in cancerous conditions in experimental rats [49] .There was marked reduction in the activities of GPx and GST in hyperlipidemic rats [50] .The results observed in the present study also show that the activity of GST, decreased upon exposure to H_2O_2, was counteracted by co-treatment with the leaf extracts. Our results shows that H_2O_2 elicited an oxidative stress ,decreased activities of enzyme antioxidants and *Clitoria ternatea* leaf extracts alterations in the enzymic antioxidants. Among the two, the white flowered leaves rendered slightly higher induction of enzyme antioxidants than the blue flowered leaves.

CONCLUSION

There is a growing awareness regarding the ethical concerns involved in the use of live animals in biological research. In tune with this concern, a global effort has been initiated to formulate and standardize *in vitro* models that will reflect the *in vivo* conditions as accurately as possible. In the present study, the use of liver slices has been employed as an alternative experimental model to minimize the use of live animals. The liver slices were subjected to oxidative stress using H_2O_2 and the effect of the co-administration of the leaf extracts was monitored on the antioxidant status. Enzymic antioxidants were analyzed in the liver slices.

Our results show that the direct exposure of the liver slices to the oxidant H_2O_2 elicited an oxidative stress as reflected by the decreased activities of enzymic antioxidants. The oxidative stress was combated by the *Clitoria ternatea* leaf extracts as reflected by the beneficial alterations in the enzymic antioxidants. Among the two, the white flowered leaves rendered higher induction of enzymic antioxidants than the blue flowered leaves.

The protective effects rendered by the leaf extracts of blue and white flower bearing *Clitoria ternatea* plants under oxidative stress conditions. It is also the best characterized underlying cause of many diseases. *Clitoria*

ternatea is a widely available plant that can be easily cultivated. The present study unearths the potential antioxidant activity of the leaves of these plants, thus validating it as a source of valuable drugs.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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