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ASSESSMENT OF ANTIOXIDANT AND PROTECTIVE POTENTIALS OF *OCIMUM TENUIFLORUM LINN.* LEAVES ETHANOLIC EXTRACT: AN *IN VITRO* STUDY.

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Abstract:

Ethanolic extract of Ocimum tenuiflorum leaves was prepared and subjected for preliminary phytochemical screening, antioxidant activity using 1,1 Diphenyl 2 picryl hydrazyl radical scavenging assay, protective roles at various levels as ex vivo lipid peroxidation, Anhti haemolytic assay and inhibition of protein carbonyl. Following ehtanolic extraction O. tenuiflorum exhibit some important phyotchemicals as flavanoids, tannins, terpenoids and coumanrins, which are important from the medicinal and pharmacological point of view. Moreover in O. tenuiflorum reflected potent antioxidant and protective capacity to quench DPPH radical, inhibiting lipid peroxidation, Inhibiting erythrocyte heamolysis and prevention of phenyl hydrazone generation in protein carbonylation in a dose dependent manner even at very small concentrations ranging 1, 5, 10, 25 and 50 µg/ml. All the test results evedent potent antioxidant and protective capacity of O. tenuiflorum at in vitro level.

Key words: Ocimum tenuiflorum, herbs, holy basil, Antioxidant, Lipid peroxidation, protein carbonyl, protective role.

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INTRODUCTION:

Many health complications are directly or indirectly related to "oxidative stress" which is known as the imbalance between oxidants and antioxidants in cellular environments [1]. Changing life style habits may onsets the oxidative stress. The oxidants are reactive oxygen species which are generated as hydrogen peroxide (H2O2) and the superoxide radical anion (O2•−), among others during metabolic processes. Oxidative stress related pathophysiological conditions appears when the level of these reactive oxygen species exceeds [2]. As already known, the redox balance in cellular environments is achieved by some antioxidants, which are categorized in enzymatic and non enzymatic antioxidants. These antioxidants are primarily resides in cells like Superoxide dismutase , Catalase and GPx as enzymatic while bilirubin, albumin, Vitamin C and Vitamin E as non enzymatic antioxidants. Antioxidants have the ability to combat the oxidative stress by capturing the reactive oxygen species through the mechanism of either donating a Hydrogen atom to reactive oxygen species or accepting a free electron from the same [3]. Herbs contain many non enzymatic antioxidants which falls in a common category Phenolic compounds such as flavanoids. alkaloids, Terepinoids, coumanrines etc [4]. From the ancient times in various cultures herbs are being practiced to treat many ailments. These herbs are applied as a whole or in form of extracts, decoctions, powders or ashes based upon the ailment to be treated and its severity [5]. *Ocimum tenuiflorum* or Holy Basil a herb having various medicinal properties with the psychotic effect due to presence of variety of bioactive compounds. It is one of the Sacred Plants in Indian cultures [6]. Since it possesses medicinal activities, *Ocimum tenuiflorum* has a wide historical perspective of applications to treat diseases in ancient ayurvedic literatures. Several ayurvedic formulations contains different preparations from various parts of *Ocimum tenuiflorum* [7]. Different parts of the Tulsi plant can also stimulate digestion, act as analgesics, nervous system stimulants, can have antimicrobial property, strengthen Immune system, diuretic, and anti diebitic activity [8]. In recent years *Ocimum tenuiflorum* is being a center of focus towards it's phytochemistry, antioxidant potential and protective roles. A recent studies revealed the composition of essential oils of *Ocimum tenuiflorum* stem and its antioxidant capacity [9].

Present study was focused at the antioxidant and biomolecule protecting potentials of *Ocimum tenuiflorum* leaf ethanolic extracts in *in vitro* conditions.

MATERIALS AND METHODS:

Healthy plant of *O. tenuiflorum* was purchased from local nursery. All the chemicals and solvents were purchased from Himedia labs and Merk ltd while the spectrophotometric analysis was done in UV-Vis Spectrophotometer Systronics Ltd.

Preparation of extracts

Crude extract of plant material was prepared by cold extraction method [8] with slight modification. Fresh leaves of *O. tenuiflorum* were picked, washed and shed dried for three days to remove complete moisture. Dried leaves were then macerated using a pestle and mortar to obtain a fine powder . From the above, 5 gram of powdered leaves were weighed and mixed with 50 ml of 80% ethanol. The mixture is then allowed for extraction for about 3 days with occasional shaking. After 3 days upper layer of plant extract was collected in Petri dishes. These Petri dishes were then allowed for drying in water bath at 37º C for 24 hrs to obtain dry extract.

Phytochemical Screening

Analysis of Alkaloids

Dried ethanolic extract of *O. tenuiflorum* was subjected for estimation of Alkaloids. Briefly 0.4 gm of dried extract were mixed with 8 ml of 1% HCl and allowed to warm on water bath. After that the mixture was filtered and then few drops of potassium mercuric iodide and with potassium bismuth were added in 2 ml of obtained filtrate. Confirmation of presence of alkaloid was indicated by formation of precipitate or turbidity. [9]

Estimation of Saponins

Emulsion formation criteria was used to estimate saponin in extract.9. Briefly suspension of 20 mg extract in 20 ml distilled water was allowed to boil and then filtered. Then 5 ml of distilled water is mixed in 10 ml of above filtrate and shaken well to develop froth. For observation of emulsion formation some amount of olive oil is mixed with froths which confirmes the presence of saponin. [9]

Test for terpenoids

Qualitative estimation of terpenoids was detected in extract. A sample of 5ml having 1mg /ml extract was prepared in distilled water and 2 ml of chloroform was mixed then 3 ml of concentrated H2SO4 was added to develop the color. Exhibition of reddish brown coloration at the interface confirmed the presence of terpenoids.[9]

Estimation of flavanoids

Presence of flavanoids in extract was detected by standard method [10]. In brief 50 mg of extract was added in 100 ml of distilled water and then filtered. After that 5ml liquid ammonia solution is mixed with 10 ml of above filtrate. Appearance of yellow colour after addition of few drops concentrated sulfuric acid was considered as a confirmation for presence of flavanoids.

Test for tannins
A mixture was prepared by mixing 50 mg of ethanolic extract in 20 ml of distilled water and boiled. Appearance of brownish green or blue-black coloration after mixing few drops of 0.1% FeCl3 confirmed the existence of tannins [10].

Test for coumarins
A sample of extract containing 300 mg/ml was plugged with filter paper dipped in 1 N NaOH and boiled in a boiling water bath for few minutes. Yellow fluorescence of filter paper under UV light confirmed the presence of coumarins. [11]

Analysis Antioxidant activity and protective potentials
DPPH radical scavenging assay
DPPH model system, for assessment of in vitro radical reduction activity of leaves extract was applied according to the methods previously standardized with some modifications [13]. Briefly, two ml of 0.004% DPPH radical solution in methanol added to one ml plant extract solutions having concentrations 10, 25, 50, 75 and 100 µg/ml. A control solution containing one ml methanol in place of plant extract was also prepared. Then above mixture vortex thoroughly and incubated at room temperature along with control solution in dark to prevent autolysis of DPPH radical, for 30 min. Immediately after incubation the O.D. at 517 nm was taken. Methanol was used as a blank for baseline correction. The DPPH radical concentration was calculated using the following equation:

$$\% \text{ INHIBITION} = \frac{\text{O.D CONTROL - O.D SAMPLE}}{\text{O.D CONTROL}} \times 100$$

***Ex vivo* Inhibition of Lipid Peroxidation in Goat Liver**
Estimation of inhibitory potential of *O. tenuiflorum* for the generation of Thiobarbituric acid reactive substances was undertaken by using Standard method with slight modifications [14]. Freshly excised goat liver was purchased from butcher market area of Indore, India and placed in phosphate buffer saline of pH 7.4 till the use. From this goat liver 10× homogenate was made in cold phosphate buffer saline (pH 7.4). Extract was added to 3 ml of homogenate followed by addition of Fenton reagent [100 µl of (15 mM) ferrous sulphate and 100 mM H2O2] to induce Lipid peroxidation. After incubation for 30 min; 0.1 ml of this reaction mixture was mixed with 1.5 ml of 10% TCA. After 10 min of incubation it was filtered and supernatant was added in a tube having 1.5 ml of 0.67% TBA (in 50% acetic acid) and placed in a boiling water bath for 30 min. Concentration of chromogen formed was measured at 535 nm. Inhibition of lipid peroxidation was assessed by using the following formula:

$$\% \text{ INHIBITION} = \frac{\text{O.D CONTROL-O.D SAMPLE}}{\text{O.D CONTROL}} \times 100$$

Inhibition of Haemolysis Goat blood
Erythrocytes were subjected as model system to analyse the antihaemolytic activity of O. tenuiflorum leaf ethanolic extract using previously described standard procedure [15]. Goat blood was collected in 3.8 % Trisodium citrate buffer to avoid its coagulation. Then erythrocytes from this blood were separated by centrifugation and washed with saline or isotonic sodium phosphate buffer (pH 7.4) until the supernatant was colorless. The erythrocytes were then diluted with saline or phosphate buffer to give a 4% suspension. Varying concentrations of sample 10, 25, 50, 75 and 100 µg/ml with saline or buffer were added to 2 mL of the suspension of erythrocytes and the volume was made up to 3.5 mL with saline or buffer. This mixture was pre incubated for 120 min and then 0.5 mL of 0.1 mMH2O2 solution prepared in buffer was added. After the final incubation of 120 min, each aliquot was centrifuged for 5 min at ×1000 g and the extent of hemolysis was determined by measurement of the absorbance at 540 nm corresponding to hemoglobin liberation. Antihemolytic activity was expressed as the inhibition percentage and was calculated using the following formula.

$$\% \text{ INHIBITION} = \frac{\text{O.D CONTROL - O.D SAMPLE}}{\text{O.D CONTROL}} \times 100$$

Inhibition of Protein Carbonyl in BSA Model System
A Fenton-type reaction induced oxidation of Bovine serum albumin was carried out using previously standardized method [16]. Briefly The reaction mixture (1.2 ml), containing sample extract (1, 5,10 ,25 and 50 µg/ml), potassium phosphate buffer (20 mM, pH 7.4), BSA (4 mg ml–1), FeCl3 (50 µM), H2 O2 (1 mM) and ascorbic acid (100 µM) were taken in a 2ml polypropylene tube and incubated for 30 min at 37 ºC. For determination of protein carbonyl content in the samples, 1 ml of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2 M HCl was added to the reaction mixture. Samples were incubated for 30 min at room temperature. Then, 1 ml of cold TCA (10%, w/v) was added to the mixture and centrifuged at 3000 g for 10 min. The protein pellet was washed three times with 2 ml of ethanol/ethyl acetate (1:1, v/v) and dissolved in 1 ml of guanidine hydrochloride (6 M, pH 2.3). The absorbance of the sample was read at 370 nm. The

data were expressed in terms of percentage inhibition, calculated from a control measurement of the reaction mixture without the test sample. Ascorbic acid was used as a standard and the inhibition percentage of protein oxidation of the sample was calculated by the following equation:

$$\% \text{ INHIBITION} = \frac{\text{O.D CONTROL - O.D SAMPLE}}{\text{O.D CONTROL}} \times 100$$

Statistical analysis

All the calculations were done and expressed as mean ± S.D.

RESULTS AND DISCUSSION:

Qualitative determination of Phytochemicals

Qualitative determination of active principle in Extract is depicted by Table 1. *O. tenuiflorum* contain various phytochemicals which plays an essential role in its antioxidant activities. These active components were fractionated using different solvents and different methods. [17]

Table 1: Phytochemical analysis of *O.tenuiflorum* leaf ethanolic extract. (- indicate absent of component, + indicate satisfactory presence while +++ indicates strong presence of component).

S.No.	Test for Active Principle	Test result
1	Alkaloid	-
2	Saponin	-
3	Flavanoid	+++
4	Terpenoids	++
5	Tannins	+
6	Coumarines	+

DPPH radical scavenging activity:

Ability of reduction of DPPH free radical by *O. tenuiflorum* is represented in Fig. 1. Where the highest activity was recorded as 58.59 ± 0.31 at the highest concentration of 50 µg/ml, while comparing with standard Ascorbic acid as 66.06 ± 0.37 at the same concentration. Extract exhibited concentration dependent inhibition of DPPH radical in between the concentration ranging from 1 to 50 µg/ml. IC50 value of extract was recorded exactly at 25 µg/ml as 50.71 ± 0.37. Inhibitory potential of O. tenuiflorum extract using various solvents was described previously. [18],[19].

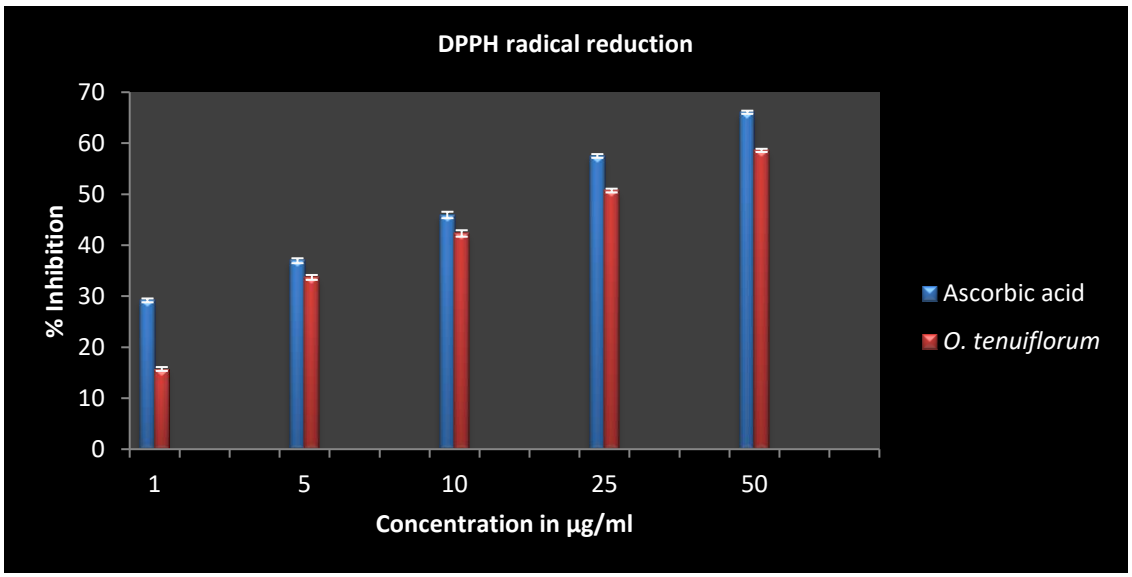

Figure 1: DPPH radical scavenging assay of *O. tenuiflorum* leaf ethanolic extract and Standard antioxidant Ascorbic acid at various concentration (1, 5,10 ,25 and 50 µg/ml). Data expressed as mean ± S.D

Inhibition of Goat liver homogenate Lipid Peroxidation.

Generation of thiobirbituric acid reactive substances is chief criteria for lipid peroxidation. Inhibition of TBARS generation induced by fentoin reaction in Goat liver homogenate was assessed for Extract while inducing and it reflected a concentration dependent increase in Lipid peroxidation inhibitory activity presented in Fig.2. Highest % inhibition was shown as 70.12 ± 0.43 and it is approximately equal to the standard Ascorbic acid 73.93 ± .29 at same concentration. One of a research reported the protective role of O. sanctum on on lipid peroxidation in stress induced by anemic hypoxia in rabbits [20] Another study reported protective role of *O. sanctum* against the lipid peroxidation in *in vitro* and *in vivo* condition [21].

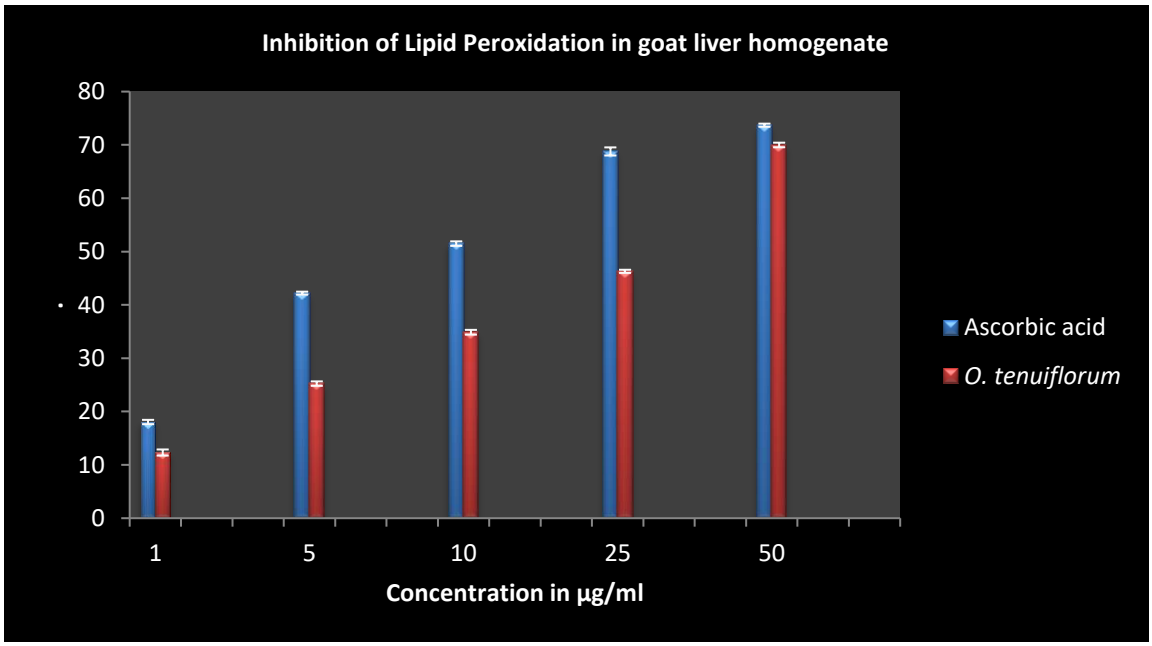

Figure 2: Inhibition of Lipid Peroxidation in goat liver homogenate of *O. tenuiflorum* leaf ethanolic extract and Standard antioxidant Ascorbic acid at various concentration (1, 5,10 ,25 and 50 µg/ml). Data expressed as mean ± S.D

Inhibition of Goat RBC Haemolysis

Protective potential of *O.tenuiflorum* extracts agains H2O2 induced oxidative damage in Goat blood erythrocytes is presented in Fig. 3. A dose dependent increase in the protective ability was recorded by both, Extract and Standard Ascorbic acid between concentration range of 1 – 50 50 µg/ml.

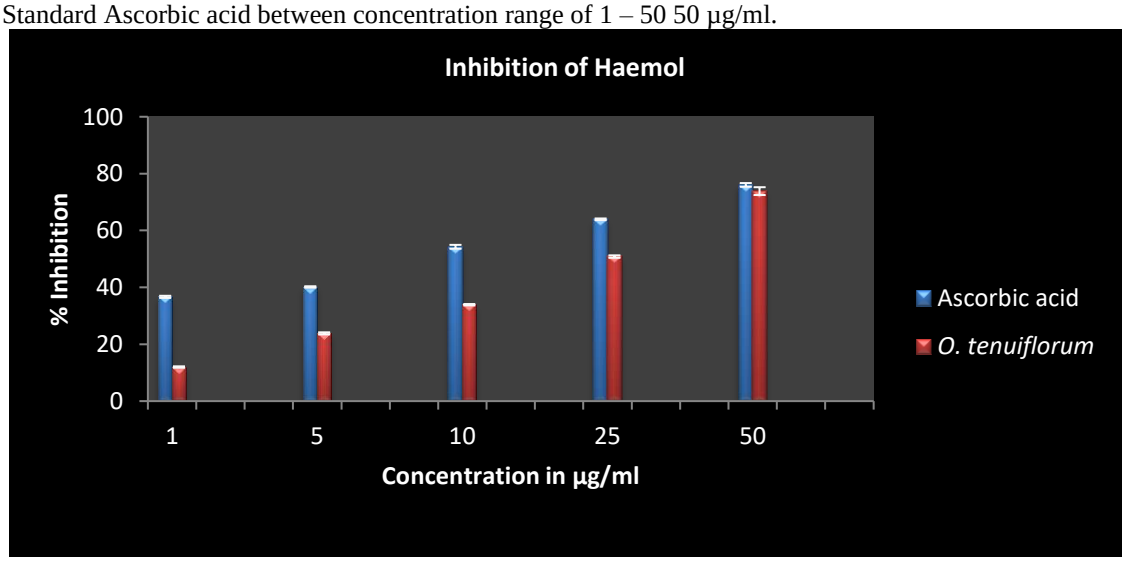

Figure 3: Inhibition of Heamolysis in goat blood of *O. tenuiflorum* leaf ethanolic extract and Standard antioxidant Ascorbic acid at various concentration (1, 5,10 ,25 and 50 µg/ml). Data expressed as mean ± S.D

Inhibition of Protein carbonyl content

Generation of protein derived hydrazones was inhibited in a dose dependent manner while undertaking the assessment of inhibition of protein carbonyl by the extract of *O. tenuiflorum.* Fig. 4 reflected the protective roles of extract in comparison with standard antioxidant Ascorbic acid. At a concentration of 50 µg/ml *O.tenuiflorum* exhibited a % inhibition of 73.52 ± 0.32.

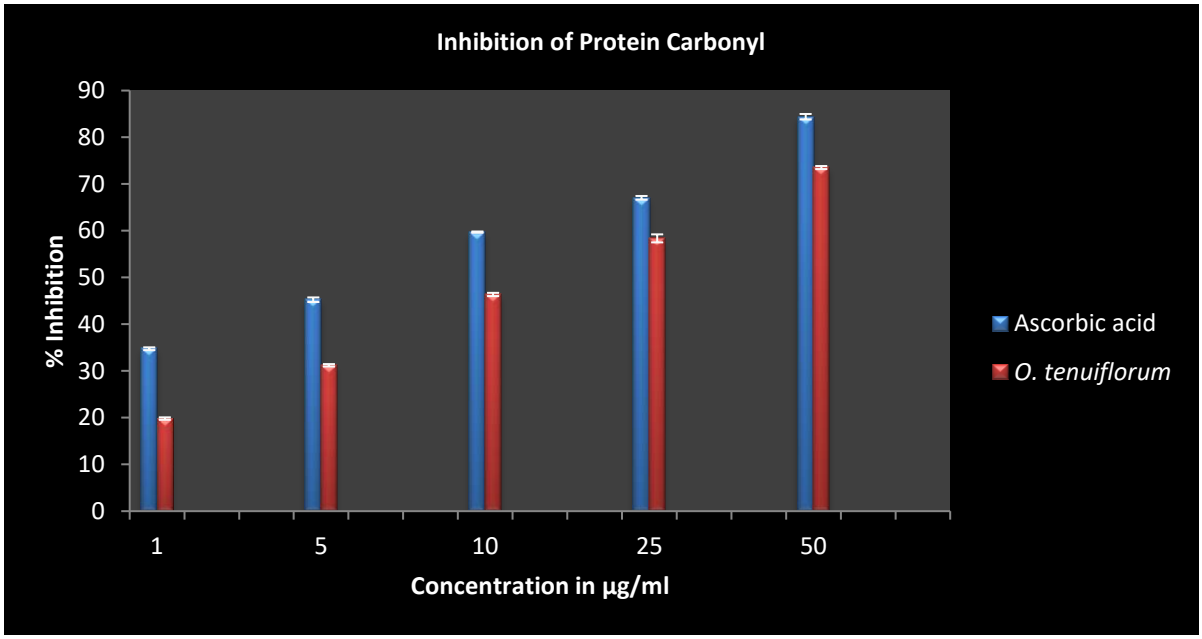

Figure 3: Inhibition of Heamolysis in goat blood of *O. tenuiflorum* leaf ethanolic extract and Standard antioxidant Ascorbic acid at various concentration (1, 5,10 ,25 and 50 µg/ml). Data expressed as mean ± S.D

CONCLUSION:

O. tenuiflorum not only a herb but is a bunch of medicinally active property. As reflected by the present study it has potent antioxidant and protective activity. This herb is practiced since a long time as a chief component in Ayurvedic medicinal preparation. Results of present work suggested more focused studies at molecular level as well as its nanoparticle formation and further researches *in vitro* as well as *in vivo* level.

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