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IN VITRO ANTIOXIDANT AND BIOMOLECULE PROTECTIVE ROLES OF PAPEVAR SOMNIFERUM LEAF ETHANOLIC EXTRACT

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ABSTRACT

Extract of leaves of *P. somniferum* was prepared in ethanol and it was evaluated for *in vitro* antioxidant, biomolecule protective test and antibacterial test such as DPPH radical reduction assay Lipid peroxidation inhibitory assay, protein carbonyl assay as well as Antihaemolytic assay. Test extract were proved potent antioxidant and protective agent as it was dose dependently inhibits free radical generation in DPPH system and also protect against molecular and cellular damage caused due to Fenton reagent even using lowest concentrations further more it also proved as potent antibacterial agent against defined pathogenic bacteria.*P. somniferum*was proved as a potent protective agent in all the tests carried out *in vitro*, it can be concluded that there must be develop a suitable therapeutic agent if evaluated at *in vivo* level.

Keywords- *Antioxidants, biomolecule protection, lipid peroxidation, oxidative stress,P. somniferum.*

I. INTRODUCTION

Phenolics are important phyto constituents present throughout in plant body and parts. They play a crucial role as antioxidants during oxidative stress related conditions [1]. Plant contains many compounds as phenolics which possess individual importance on recovering oxidative damage as well as in many disease conditions[2].Phenolics are also important in combating against various infectious diseases and more on against specific pathogenic microorganisms[3]. ***Papaver somniferum***, the **opium poppy** is a species of flowering plant in the family Papaveraceae. It is the species of plant from which opium and poppy seeds are derived and is a valuable ornamental plant, grown in gardens. Its native range is probably the eastern Mediterranean, but is now obscured by ancient introductions and cultivation[4]. Alkaloids are a major component of opium plant that exhibits many medicinal properties. On the other hands their sedative properties plays a key role as analgesics and anaesthetics [5, 6]. The fact behind the use of Ethanol for extraction is that the most of the polar bioactive compounds like alkaloid and flavonoids are dissolve in polar solvent so that the use of Ethanol for plant[7].As mentioned earlier, plant species contains many phenolics that can combat against oxidative cellular damage, the present study was focused on antiperoxidative and molecule protective roles of ethanolic extract of P. somniferum leaves.

II. MATERIALS AND METHODS

Plant material andPreparation of extracts

Fresh Leaves of *P. somniferum*were taken from harvested crops of Opium poppy by opium cultivar fields (**Licenced Farmer Mr. OmprakashChoudhary**) of Village Punyakhedi Dist. Mandsour India and thoroughly washed using tap water then rinsed with distilled water. The leavess were dried for 3 days naturally under shade to avoid any type of hindrance in antioxidant composition. The ethanol extracts were obtained by weighing out a fraction 20 g of the pulverized powdered leaves of the plant and soaking in 100 ml of the 60% ethanol and kept in dark for three days with occasional shaking to take out the extract. The extract was then filtered using Whatman no.1 filter paper. All filtrates were air dried at 28^{o}C for three days to obtain semi dried extracts[8].

Sample Preparations for *In vitro* Studies

A stock solution of concentration of 1000 µg/ml of leaves extract was prepared in 10% ethanol while standard Ascorbic acid was prepared in .1 M phosphate buffer. Then both the solutions were kept on dark narrow mouth bottles and stored at 4° C[8].

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DPPH Radical Scavenging activity

DPPH model system, for assessment of *in vitro* radical reduction activity ofleaves extract was applied according to the methods already described [9] with slight modifications. Briefly, two ml of 0.004% DPPH radical solution along with one ml plant extract solutions ranging from 10 to 100 µg/ml was mixed and vortex thoroughly and then incubated at room temperature in a dark room,to prevent autolysis of DPPH radical, for 30 min. After incubation the O.D. at 517 nm was taken against a control containing methanol and DPPH solution without plant extract while Methanol was used as a blank for baseline correction. The DPPH radical concentration was calculated using the following equation:

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

Inhibition of Lipid Peroxidation In Egg Yolk Homogenate

A slightly modified method for assessment of Lipid peroxidation inhibition of leaf extract was applied using Egg yolk as a lipid rich medium[10]thiobarbituric acid- reactive species (TBARS) assay as previously described [11]. A 10 % V/V Egg homogenate was prepared in phosphate buffer saline (pH 7.4) and 0.1 ml of leaf extract of varying concentrations was mixed separately then the volume was made up to 1 ml, by adding distilled water. To initiate fenton reaction[12]0.05 ml FeSO4 (0.07 M) was added to the above mixture and incubated for 30 min, to induce lipid peroxidation. A subsequent addition of, 1.5 ml of 20% acetic acid and 1.5 ml of 0.8% TBA (w/v) in 1.1% sodium dodecyl sulfate (SDS) and 0.05 ml 20% TCA was done after first incubation. The reaction mixture was vortexed and then heated in a boiling water bath for 60 min. After cooling, 5.0 ml of butanol was added to each tube and centrifuged at 3000 rpm for 10 min. The absorbance of the organic upper layer containing MDA – TBA pink coloured complex was measured at 532 nm.

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

Ex vivo Inhibition of Lipid Peroxidation in Goat Liver

Standard method for estimation of TBARS was used to assay the degree of lipid peroxidation described [13] with slight modifications. From freshly excised liver of goat 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 H_2O_2] 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:

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

Inhibition of Protein Carbonyl in BSA Model System

BSA was oxidised by a Fenton-type reaction [14]. The reaction was carried out in 2ml polypropylene tubes with lids. Briefly plant extracts ranging 10 to 100 µg/ml were added to the medium and, after incubation and protein precipitation by TCA, the mixture was centrifuged (3000g, 4°C, 5 min) and the pellet used for protein carbonyl content determination. This was assayed as a 2,4-dinitrophenylhydrazine (DNPH) derivative by the standardized method[14] with some modifications. After extraction and a second precipitation of the precipitate, the protein pellets were dissolved in 1ml of 6M urea and centrifuged (3000g, 4°C, 5 min). The different spectra of the DNPH derivatives were obtained at 372nm.

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Inhibition of Haemolysis

Goat blood Erythrocytes were subjected as model system to analyse the antihaemolytic activity of opium leaves ethanolic extract cell hemolysis of methanolic extract was examined by the *Ex vivo* described previously[15]. The erythrocytes from Goat 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 amounts of sample (10 – 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 preincubated for 120 min and then 0.5 mL of 0.1 mMH_2O_2 solution prepared in buffer was added. After the final incubation of 120 min, each aliquot was centrifugedfor 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.

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

Antibacterial activity[16]

Test Microorganisms Some common human pathogenic bacteria viz. *E. coli, S. aureus, Streptococcus, P. aeruginosa and Salmonella sp*were considered for in vitro evaluation of growth inhibition potential of flower ethanolic extract.

Culture preparation for antibacterial assay

The cultures were grown on nutrient agar at 37 °C for 18 h and the colonies were suspended in saline (0.85% Nacl) and its turbidity was adjusted to 0.5 Mac Farland standards (108 CFU/mL). This saline culture preparation was used to inoculate the plates. Disc diffusion In the agar disc diffusion method the test compounds, i.e. the flower hydroethanolic extract were introduced into a disc 0.5 mm (hi-media) and then allowed to dry. Thus the disc was completely saturated with the test compound at concentration of 400 µg/mL. Then these discs were placed directly on the surface of Muller Hinton agar plates, swabbed with the test organism and the plates were incubated at 37 °C for 24 h.

Statistical analysis: All the test were conducted as triplicate and were expressed as mean ± S.D.

III. RESULTS AND DISCUSSIONS

Radical scavenging potential on 2, 2 Diphenyl – 1 picrylhydrazyl model system for *P. somniferum*leaf Ethanol extract was assessed by the method already described. Here **Figure 1** represents the Comparative radical Scavenging potential between *P.Somniferum* Leaf extract and Standard Antioxidant Ascorbic Acid. Both, Ascorbic Acid and Test extract showed a Dose dependent increase in percentage inhibition of DPPH radical. Leaf ethanolic extract of *P. Somniferum* inhibited the DPPH radical between lowest (10 µg/ml) and highest (100 µg/ml) as 31.91±0.141 and 89.75±0.8 respectively. Radical reduction assessment of *P. somnifrum* leaves reveals that it contains properties to inhibit DPPH radical generation and also imacts as dose dependent manner. Previous studies conducted on *P. somniferum* seeds indicated its radical reduction properties.[17, 18]Egg yolk Homogenate was used as lipid rich medium to assess the inhibition of Lipid Peroxidation by *P. somniferum* leaf ethanol extract, while using Ascorbic Acid as standard antioxidant. Concentration dependent increase in % inhibitory potentials were observed by both Test extract and Standard as shown in **Figure 2**. Test extract reflects the % inhibition ranges between 57.43±0.44 and 84.48±0.427 at 10 µg/ml and 100 µg/ml respectively. To ensure the inhibitory effects of Test extract against MDA and TBARS generation in tissue system goat liver homogenate was used as model to induce LPO by Fenton reaction. Dose dependent effect was shown in **Figure 3** between Test Extract and Standard. As a maximum inhibitory potential of 82.44 ± 0.438 was recorded at highest concentration of 100 µg/ml for *P. somniferum* leaf ethanol extract. Assessment of *P. somniferum*leaf ethanol extract for inhibition of protein carbonyl formation in BSA System induced by H_2O_2 was shown in **Figure4** as % inhibition of 89.45 ± 0.813 at 100 µg / ml comparatively high from Ascorbic Acid Standard as 73.80 ± 1.021 at same concentration. Antibacterial potentials of Leaf Ethanolic Extract and Ethanol (70 %) were assessed on different pathogens *viz.E.coli, S.aurius, P.aerugenosa and Salmonella*

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at a concentration of 200 µg/ml in **Table 1**. Here also the *P. somniferum* leaf extract reflected the maximum diameter of zone of growth inhibition as 17mm, 18mm, 13mm and 15 mm for E.coli, S.aurius, P.aerugenosa and Salmonella respectively which was followed by Ethanol.

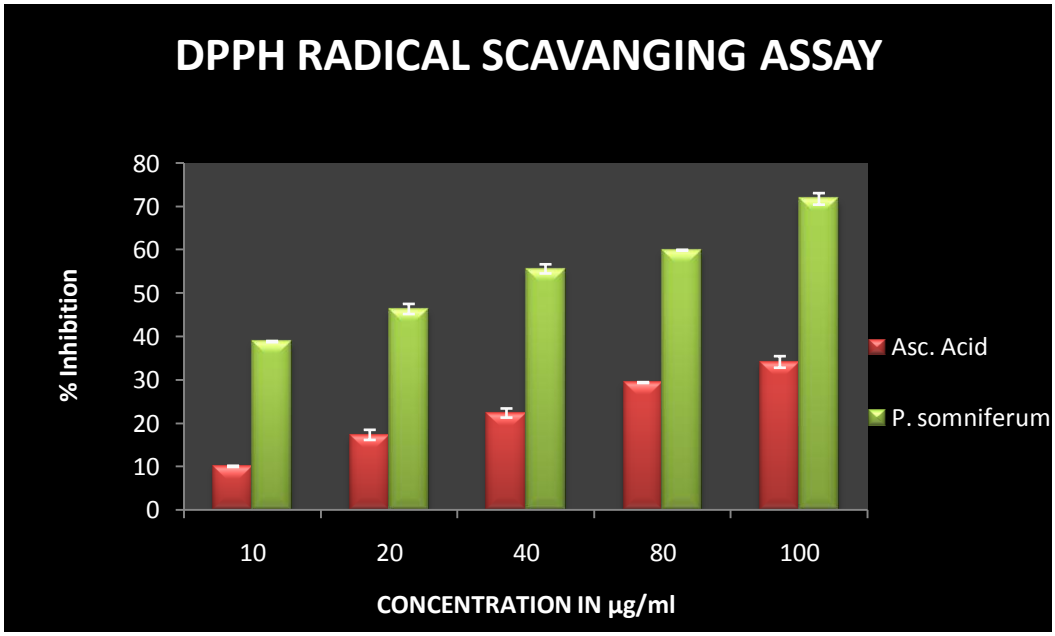

Figure 1: Inhibition of DPPH radical Asc. Acid and P. somniferum at varying conc. (10, 20, 40, 80 and 100 µg/ml). Mean ± S.D. [n= 3]

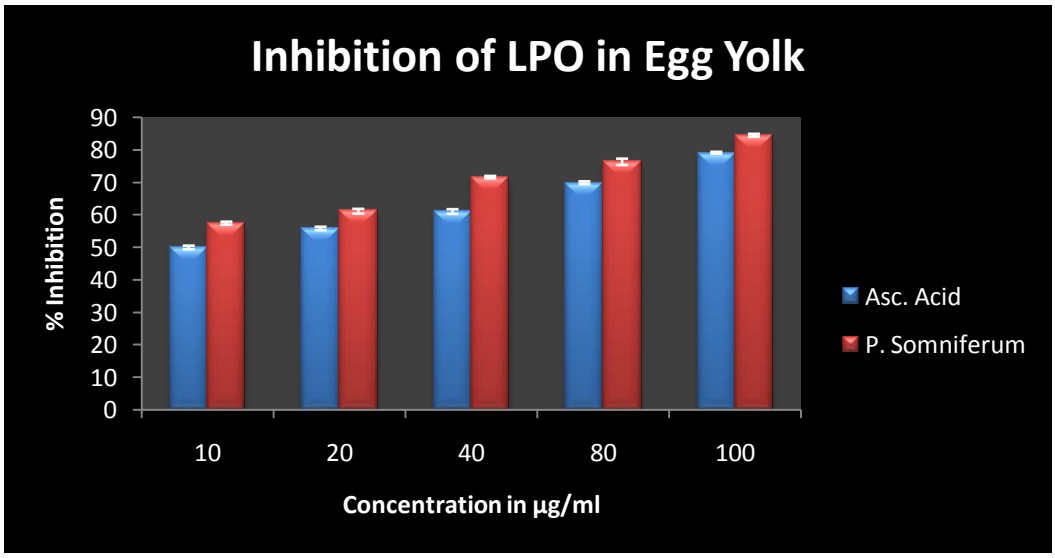

Figure 2: Inhibition of DPPH radical Asc. Acid and P. somniferum at varying conc. (10, 20, 40, 80 and 100 µg/ml). Mean ± S.D. [n= 3]

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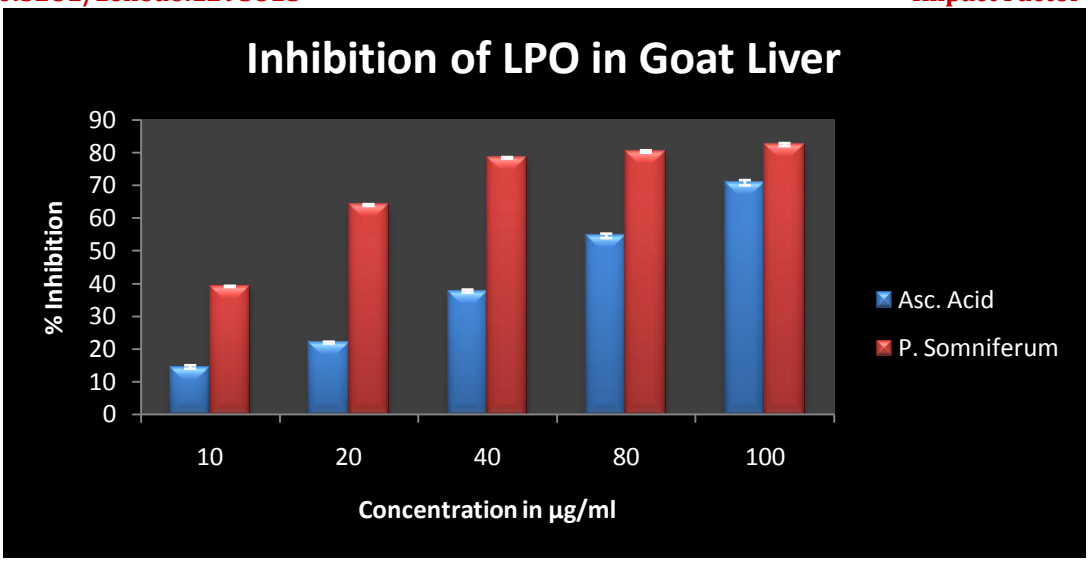

Figure 3: Inhibition of DPPH radical Asc. Acid and P. somniferum at varying conc. (10, 20, 40, 80 and 100 µg/ml). Mean ± S.D. [n= 3]

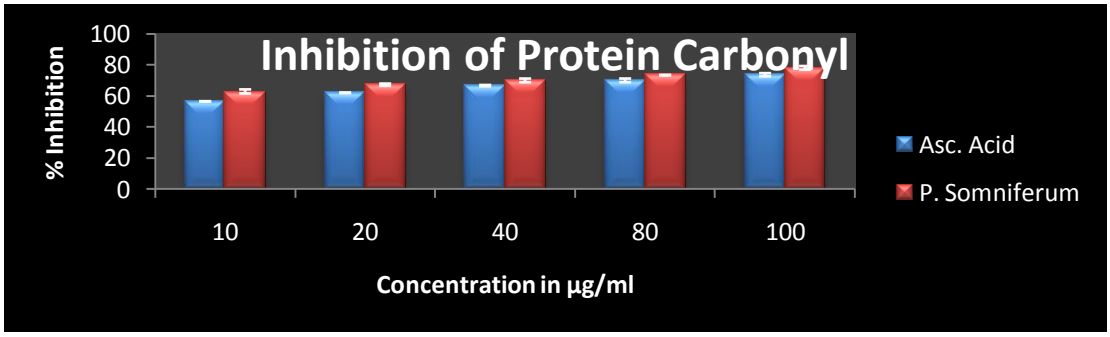

Figure 4: Inhibition of DPPH radical Asc. Acid and P. somniferum at varying conc. (10, 20, 40, 80 and 100 µg/ml). Mean ± S.D. [n= 3]

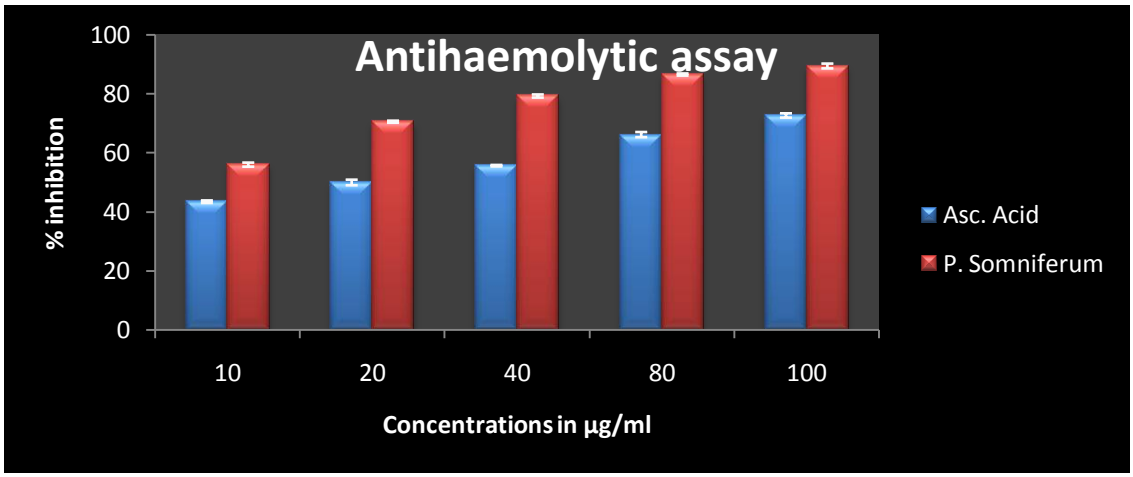

Figure 5: Inhibition of DPPH radical Asc. Acid and P. somniferum at varying conc. (10, 20, 40, 80 and 100 µg/ml). Mean ± S.D. [n= 3]

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Table 1: Inhibition of growth of Test Bacterial culture (mm.) by P. somniferumat 200 µg/ml

S.No.	Bacteria	Control	*Ethanol*	*P. Somniferum*
1	*E.coli*	0	13	17
2	*S. aureus*	0	13	18
3	*P.aerugenosa*	0	11	13
4	*Salmonella*	0	14	15

IV. CONCLUSION

Study concluded the free radical inhibitory and protective role of *P. somniferum* leaf extract as a potent antioxidant agent. The future studies must be focused on isolation of its bioactive components and their individual protective potentials.

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