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Antioxidant Activity of Isolated Protein Mixtures from Seeds of *Azadirachta indica*, *Ocimum sanctum* and *Momordica charantia*

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Authors' contributions

This work was carried out in collaboration between all authors. Author YS designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author JK managed the analyses of the study and managed the literature searches. Author KB reviewed, guided and approved the final manuscript.

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ABSTRACT

Aim: The purpose of study was to investigate the antioxidant activity of primary metabolites such as proteins present in the seeds of *Azadirachta indica*, *Ocimum sanctum* and *Momordica charantia*.
Structure Design: In this work, we have used Protein precipitation method to isolate the protein of seeds of medicinal plants as well as the enzymatic and nonenzymatic biochemical assays has been determined to evaluate the antioxidant activity.
Place and Duration of Study: Study was done in Helix BioGenesis Pvt. Ltd., Noida, India, between December 2016 and April 2017.
Methodology: Proteins were isolated by proteins extraction buffer and purified by acetone precipitation method. Separations of proteins were done by SDS PAGE and by using molecular

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ladder of 250KDa have revealed molecular weight of the proteins of seeds of *Azadirachta indica*, *Ocimum sanctum* and *Momordica charantia*. Antioxidant activity was determined by quantifying the enzymatic biochemical assays i.e. Superoxide dismutase activity (SOD), Catalase activity (CAT) & Glutathione s transferase activity (GST) and nonenzymatic biochemical assay i.e. Lipid peroxidation (MDA Content).

Results: It was observed that CAT activity found to be maximum in the 2.268mg/ml of concentration of proteins of seeds of *Ocimum sanctum*, i.e. 26.06±0.04 µmoles of H_2O_2 oxidized consumed/min/ mg of proteins and GST activity found to be 19.93±0.10 µmole of CDNB-GSH conjugate formed/ min/ mg of proteins. As far as the nonenzymatic biochemical assay is concerned, lipid peroxidation i.e. MDA content was found to be high i.e. 1.14 µ gram of malondialdehyde formed/ mg of proteins in the seeds of *Momordica charantia* at the 1.664mg/ml of concentration.

Conclusion: This can be concluded from the above studies that proteins of seeds of *Azadirachta indica*, *Ocimum sanctum* and *Momordica charantia* have shown antioxidant activity through various test like SOD, CAT, GST and MDA content therefore we suggest that these isolated protein mixture can be utilized as neuroprotective agent against neurological disorders.

Keywords: Proteins; seeds; Momordica charantia; Azadirachta indica; Ocimum sanctum; superoxide dismutase; catalase; glutathione s transferase; lipid peroxidation.

1. INTRODUCTION

In recent times, focus on plant research has increased tremendously all over the world and a large body of evidences collected has shown the great potential of medicinal plants used in various traditional systems. The use of plants and plant products can be traced as far back as the beginning of human civilization. The earliest mention of medicinal use of plants in Hindu culture is found in "Rigveda", which is said to be have written in 4500-1600 BC and is supposed to be the oldest repository of human knowledge [1]. Plants have been an important source of medicine and has helped human in the maintenance of health for thousands of years [2]. Medicinal plants, medicinal herbs, or simply herbs have been identified and used from prehistoric times. According to the World Health Organization estimates, up to 80 percent of people still rely mainly on traditional remedies such as herbs for their health needs due to better cultural acceptability, fewer side effects and better compatibility with the human body [3]. Its civilization is very ancient and the country as a whole has long been known for its rich resources of medical plants [4].

Azadirachta indica has been extensively used in various alternative medical specialties such as Ayurveda, Unani and Homeopathic medicine. *Azadirachta indica* (neem) is a tree which belongs to the Meliaceae family and is considered to be a holy medicinal tree found in India [5]. It is also known as "Indian *Azadirachta indica*/margosa tree" or "Indian Lilac" and "Persian Lilac". It possesses a wide range of biological activities, such as anti-inflammatory, anti-malarial, anti-microbial, anti-viral, anti-fungal, anti-pyretic, anti-oxidant, analgesic, immune-stimulant, anti-fertility, anti-acne, anti-hypoglycemic, anti-cancer and nematicidal properties [6]. In Sanskrit, it is called "Arishta" which means "reliever of sickness" and hence considered "Sarbaroganibarn" [7,8]. *Azadirachta indica* is a common plant which is cultivated in various parts of India for religious and medicinal reasons [6,9,10]. *Azadirachta indica* is considered to be a "village dispensary" since every part of the tree including the leaves, bark, and seeds have medicinal properties. US National Academy of Sciences recognized the importance of neem tree in 1992 and entitled *Azadirachta indica* as "a tree for solving global problems" [11].

Medicinal properties of tulsi i.e. *Ocimum sanctum*. Linn have also been a part of many civilizations for years [6,11]. This medicinal herb is considered as a sacred plant by the Hindus in the Indian subcontinent and Asians too. Scientific explorations of traditional belief of medicinal properties of *Ocimum sanctum* have got momentum mostly after the middle of the 20th century. Scientific evidences are available on various medicinal aspects i.e. anti-microbial, adaptogenic, anti-diabetic, hepato-protective, anti-inflammatory, anti-carcinogenic, radioprotective, immune modulatory, neuroprotective, cardio protective, mosquito repellent etc [12]. Most of these evidences are based on *in-vitro*, experimental and a few human

studies. *Momordica charantia* Linn belongs to the Cucurbitaceae family and commonly known as Bitter Gourd (BG), bitter melon, karela and grows in the humid and subtropical regions of the world. It is inherent in Asia and now widely cultivated in all parts of the world due to its dietary assessment in its immature or ripened fruits. The BG plant is modified to a wide variation of climates however its best production is in warm areas [13]. It is a curative vegetable which has been used conventionally for diabetes treatment. It is a ground-breaking plant for its adaptability as foodstuff and therapeutic applications. Contemporary scientific proof has proved karela, an auspicious, anti-diabetic, anti-cancer, anti-microbial, anti-hepatotoxic, anti-oxidant, anti-viral, anti-ulcerogenic and larvicidal activities [14,15].

The purpose of the present study was to observe the antioxidant activity of proteins mixtures of seeds of *Azadirachta indica*, *Ocimum sanctum* and *Momordica charantia* by determining the enzymatic biochemical assay (Superoxide dismutase activity, Catalase activity & Glutathione S Transferase activity) and nonenzymatic biochemical assay (Lipid peroxidation).

2. MATERIALS AND METHODS

2.1 Source of Sample Selection

Seeds of *Azadirachta indica*, *Ocimum sanctum* and *Momordica charantia* were collected from the Khari Baoli, Kucha Challan, Chandni Chowk, Delhi. Further they were washed with distilled water to remove dirt and soil particles. Seeds were then dried in shaded area and then grounded with an ordinary grinder to form powder and used throughout the study.

2.2 Proteins Extraction and Precipitation

For the isolation of proteins, crude extract was prepared by dissolving the 1 gm powder of respective seeds in the proteins extract buffer consisting of 50 mM Tris HCl, 100 mM NaCl, 1% Triton X-100, 5% Glycerol, 5 mM EDTA, 1%SDS, Proteinsase K, 10 mg/ml PVPP and 5mM β-mercaptaethanol. Solutions were allowed to incubate for 10 min in ice box and then centrifuged at 10,000 rpm for 20 min. Crude extracts were obtained by collecting the supernatant from the resultant cake after the centrifugation. For the precipitation of proteins, acetone precipitation was used, where crude extract were precipitated with 1:4 volume of chilled acetone and then incubated for 1 hr at -20°C. After incubation, precipitate was obtained after centrifugation at 10,000 rpm for 10 min at 4°C and was allowed to dissolve in double distilled water.

2.3 SDS PAGE Analysis

From every seed, 20 µl of precipitated proteins were mixed with in equal ratio of proteins loading dye containing 125 mM Tris HCl, 10% Glycerol, 10% SDS, 5 mM β-mercaptaethanol & bromophenol blue and mixture was heated for 3min at 95°C. Samples and Molecular ladder were allowed to run onto 12% SDS PAGE with constant voltage of 80V. Each electrophoresis experiment was terminated when the tracking dye reached 1 cm above sealing part of the gel cast. Coomassie brilliant blue staining method was used for visualization of proteins bands as well as the marker of 250 KDa [16].

2.4 Quantification of Proteins

Proteins were quantified in the 100 µl of precipitated proteins by Lowry's method by using Floin Ciocalteau Reagent [17] and amount of proteins were determined against standard curve of 1 mg/ml of BSA as given in Fig. 1.

2.5 Statistical Analysis

To estimate the accuracy of the experimental data, each experiment were performed in tripli cates and the results were expressed as Mean±Standard Deviation of three replications. The $p<0.05$ was regarded as significant.

2.6 Antioxidant Activity

2.6.1 Enzymatic biochemical assay

Superoxide dismutase enzyme activity was determined in the precipitated proteins of seeds of *Azadirachta indica*, *Ocimum sanctum* and *Momordica charantia* by Nitroblue tetrazolium assay. Superoxide dismutase activity was expressed as one unit of enzyme activity, which was defined as the enzyme concentration required for inhibition of the absorbance at 560 nm of chromogen produce by 50% in 1 min under assay conditions and expressed as specific activity in a unit of SOD per min per mg of proteins [18]. Catalase enzyme activity was

determined by using 10 mM Hydrogen peroxide as a catalase enzyme buffer. Catalase activity was expressed in specific enzyme activity as µmoles of H_2O_2 oxidized consumed per min per mg proteins [19]. Glutathione s transferase enzyme activity was used to observe the detoxification, based on glutathione conjugation to 1-chloro-2, 4-dinitrobenzene (CDNB) as a substrate and measured spectrophotometrically at 340 nm. The specific enzyme activities of the precipitated proteins of seeds were expressed as µmole of CDNB-GSH conjugate formed per min per mg proteins [20].

2.6.2 Nonenzymatic biochemical assay

Lipid peroxidation was measured in the precipitated proteins of seeds of *Azadirachta indica*, *Ocimum sanctum* and *Momordica charantia* by thiobarbituric acid assay. Thiobarbituric acid reactive substances were measured spectrophotometrically at 532 nm in precipitated proteins of seeds on the principle of formation of Malondialdehyde (MDA) by breaking down of polyunsaturated fatty acids where the levels of lipid peroxidation were expressed as µ grams of malondialdehyde formed per mg of proteins [21,22].

3. RESULTS AND DISCUSSION

Antimicrobial peptides have been generously studied as part of innate response elicited by most living forms. They were found to be present in any parts of plant such as roots, seeds, leaf, flowers and stem. Antioxidant activity of many peptides has also been studied that has shown their relevant efficacies. The following study is an initial screening in the field of antioxidant activity of proteins of seeds of *Ocimum sanctum*, *Azadirachta indica* and *Momordica charantia* by determining the enzymatic and nonenzymatic biochemical assay. The purpose of the present study was to find the antioxidant activity of proteins of seeds of *Azadirachta indica*, *Ocimum sanctum* and *Momordica charantia*. Precipitation of proteins was done by acetone which has shown the best results. Lowry method was used to determine the proteins in the seeds against 1 mg/ml of BSA standard curve with equation ($y=0.023x$, $R^2=0.7671$) as given in the Fig. 1. It has shown the presence of proteins i.e. 567µg/ml of seeds of *Ocimum sanctum*, 897µg/ml of seeds of *Azadirachta indica* and 416µg/ml of seeds of *Momordica charantia*. SDS PAGE was used to separate and purify the proteins by preparing the 12% gel, from where it was found that different bands were observed in all the seeds and they were compared against standard molecular ladder as given in the Figs. 2, 3, 4. Enzyme activity and non enzymatic content were observed in the precipitated proteins in the seeds of *Ocimum sanctum*, *Azadirachta indica* and *Momordica charantia* as given in Tables 1, 2, 3, respectively. To determine the antioxidant activity, enzymatic biochemical assays were done in order to determine the specific enzyme activity. Superoxide dismutase enzyme activity was determined, where it was found that by increasing the concentration of proteins of seeds of *Ocimum sanctum* has shown maximum enzyme activity at 2.268 mg/ml with activity of 0.34±0.02 Unit of SOD /min/ mg of proteins as shown in Fig. 5, whereas seeds of *Azadirachta indica* at 3.588 mg/ ml has shown 50% of inhibition i.e. 1.11±0.09 Unit of SOD /min/ mg of proteins as shown in Fig. 7. This was estimated from the above studies that maximum amount of enzyme activity found to be present in the 2.268 mg/ml of precipitated proteins of seeds of *Ocimum sanctum*, whereas compared to the previous studied, specific enzyme activity was found to be 0.12 Units/mg in seeds of *Ocimum sanctum* [23]. Precipitated proteins of seeds of *Momordica charantia* have shown 2.39±0.03 Unit of SOD /min/ mg of proteins more than that of *Ocimum sanctum* and *Azadirachta indica* as depicted in Fig. 9. Catalase enzyme activity was also studied that determine the activity of precipitated proteins of seeds of *Azadirachta indica*, *Ocimum sanctum* and *Momordica charantia*. Previous studies has shown minimum activity of polyphenol oxidase i.e. 0.295 U/g in the seeds extract of *Momordica charantia* [24], whereas present study has shown the maximum amount of SOD, CAT and GST activity in the precipitated proteins of seeds of *Momordica charantia*. Catalase enzyme activity was found to be maximum in the 1.664 mg/ ml of seeds proteins of *Momordica charantia*, whereas compared to the previous studies, minimum amount of catalase enzyme activity found to be present in the seeds extract of *Momordica charantia* [24]. This was found that by increasing the concentration of proteins i.e. 2.268 mg/ ml of proteins of *Ocimum sanctum* seeds has shown 26.06±0.04 µmoles of H_2O_2 oxidized consumed/ min/ mg of proteins as given in Fig. 5, where as 3.588 mg/ml of proteins of *Azadirachta indica* and 1.664 mg/ml of proteins of *Momordica charantia* has shown 4.28±0.05 and 2.43±0.07 µmoles of H_2O_2 oxidized consumed/ min/ mg of proteins, respectively as depicted in Figs. 7, 9. Glutathione s transferase activity was determined

to find the detoxification of proteins, where as it was found that proteins of seeds of tulsi has shown the maximum enzyme activity at 2.268 mg/ml with enzyme activity i.e. 19.93±0.10 as µmole of CDNB-GSH conjugate formed/ min/ mg of proteins as given in Fig. 5. Proteins of seeds of *Ocimum sanctum* and *Azadirachta indica* has also shown the GST enzyme activity at 3.588 mg/ml of seeds of *Azadirachta indica* and 1.664 mg/ml of seeds of *Momordica charantia* with an enzyme activity of 5.85±0.09 and 18.00±0.03 µmole of CDNB-GSH conjugate formed/ min/ mg of proteins, respectively as given in Figs. 7, 9. Lipid peroxidations of proteins of seeds were also observed and their MDA content levels were determined. This was found from this MDA content that with respect to the increasing concentration of proteins, MDA content levels were also found to be increased. 2.268 mg/ml of proteins of seeds of *Ocimum sanctum* has shown the MDA content of 0.014±0.002 µ gram of malondialdehyde formed/ mg/ proteins as shown in Fig. 6, where as 3.588 mg/ml of proteins of seeds of *Azadirachta indica* has shown 0.37±0.015 µ gram of malondialdehyde formed/ mg of proteins as given in Fig. 8. 1.664 mg/ml of proteins of seeds of *Momordica charantia* has shown lipid peroxidation with MDA content of 1.14±0.10 µ gram of malondialdehyde formed/ mg of proteins as given in Fig. 10. Lipid peroxidation inhibition activity was observed in the methanolic extract of *Azadirachta indica* i.e. 179.15±3.21 µg/ ml [25], whereas compared to the present studies minimum amount of MDA content with respect to lipid peroxidation found to be present in the proteins of seeds of *Azadirachta indica* i.e. 0.37±0.015 µ gram of malondialdehyde formed/ mg of proteins.

Table 1. Enzyme activity and nonenzyme content of precipitated proteins of seeds of *Ocimum sanctum* at difference concentration

Proteins concentration (mg/ml)	Enzyme activity			Nonenzyme content
	Superoxide dismutase activity	Catalase activity	Glutathione S transferase activity	Lipid peroxidation (MDA content)
0.567	0.08±0.008	6.51±0.41	6.64±0.55	0.0035±0.001
1.134	0.17±0.002	13.03±0.019	13.29±0.052	0.0071±0.0015
2.268	0.34±0.024	26.06±0.048	19.93±0.10	0.014±0.002

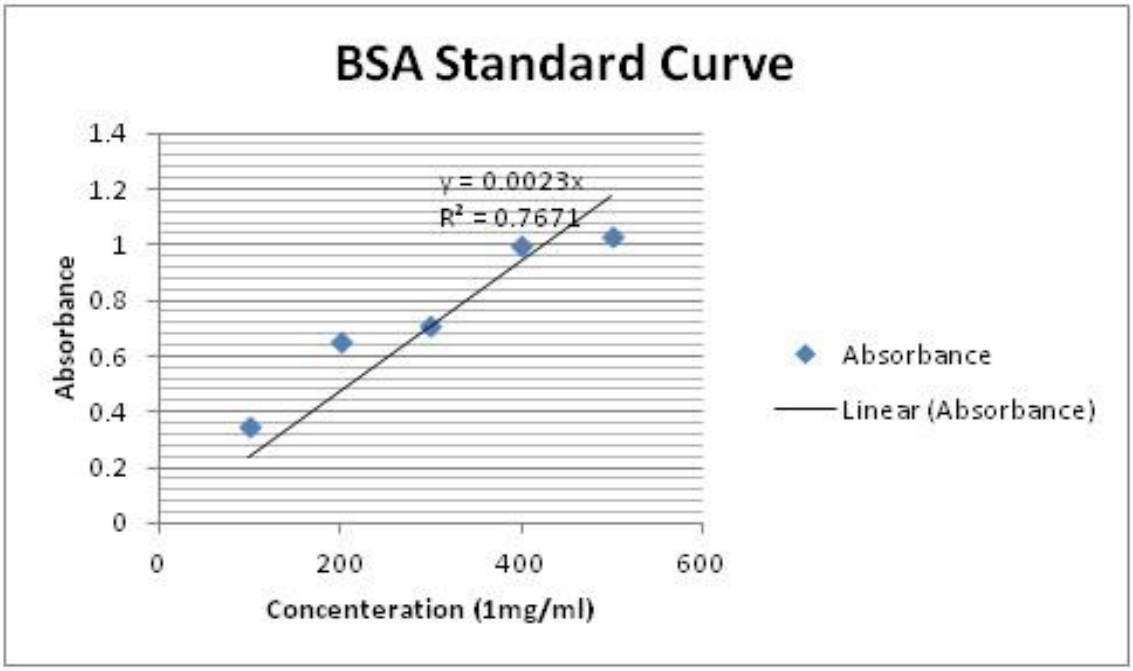

Fig. 1. Standard curve of BSA (1 mg/ml)

Table 2. Enzyme activity and nonenzyme content of precipitated proteins of seeds of *Azadirachta indica* at difference concentration

Proteins concentration (mg/ml)	Enzyme activity			Nonenzyme content
	Superoxide dismutase activity	Catalase activity	Glutathione S transferase activity	Lipid peroxidation (MDA content)
0.897	0.27±0.064	1.07±0.17	3.964±0.09	0.09±0.002
1.794	0.55±0.03	2.14±0.04	7.92±0.17	0.18±0.006
3.588	1.11±0.09	4.28±0.05	15.85±0.09	0.37±0.015

Table 3. Enzyme activity and nonenzyme content of precipitated proteins of seeds of *Momordica charantia* at difference concentration

Proteins concentration (mg/ml)	Enzyme activity			Nonenzyme content
	Superoxide dismutase activity	Catalase activity	Glutathione S transferase activity	Lipid peroxidation (MDA content)
0.416	0.59±0.008	0.60±0.031	4.50±0.05	0.286±0.03
0.832	1.19±0.096	1.21±0.08	9.004±0.04	0.57±0.036
1.664	2.39±0.031	2.43±0.07	18.008±0.031	1.14±0.1

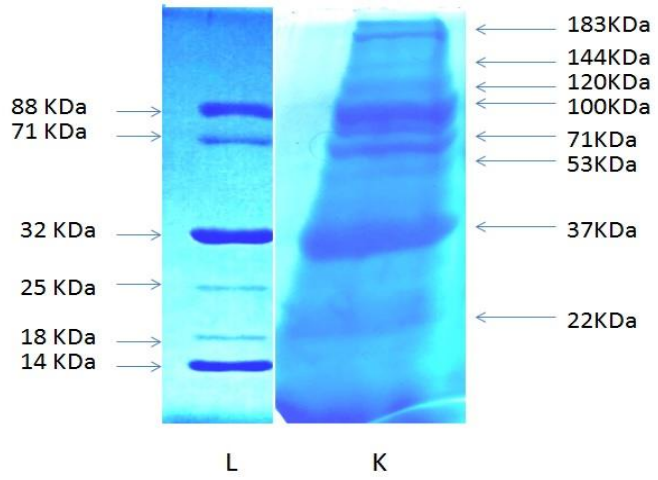

Fig. 2. SDS PAGE analysis of (L) Ladder and (K) precipitated protein of seeds of Karela

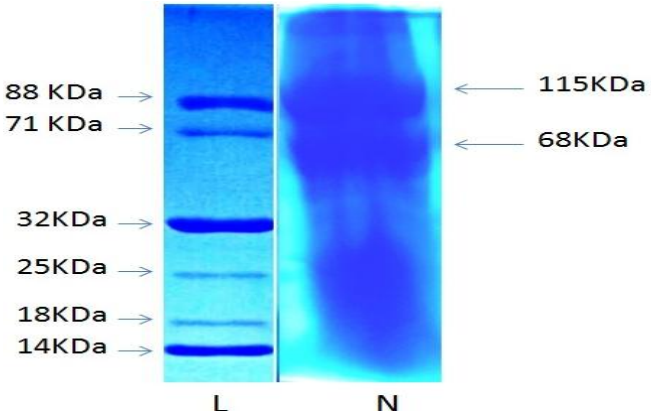

Fig. 3. SDS PAGE analysis of (L) Ladder and (N) precipitated protein of seeds of Neem

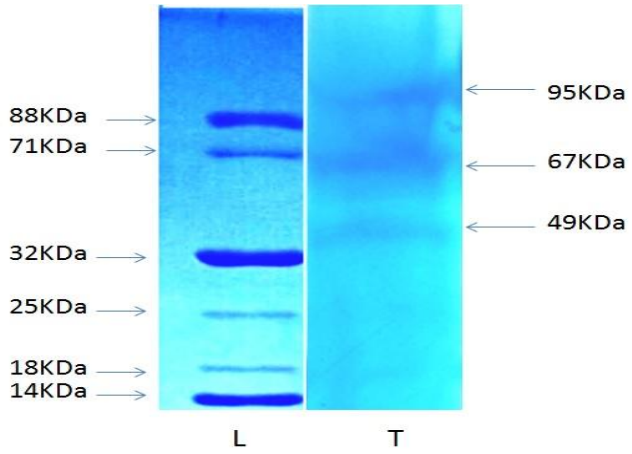

Fig. 4. SDS PAGE analysis of (L) Ladder and (T) precipitated protein of seeds of Tulsi

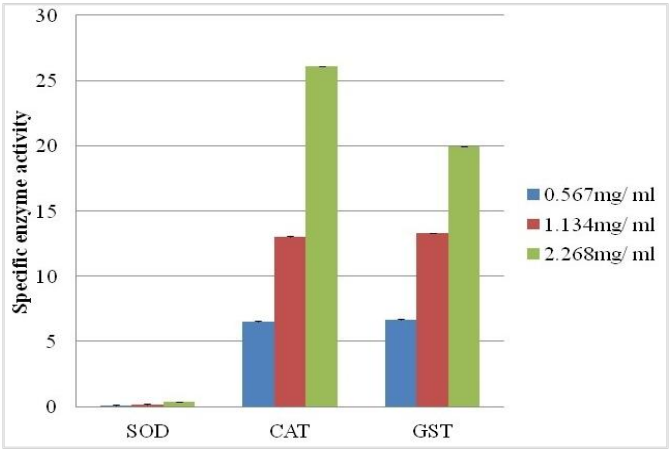

Fig. 5. Enzymatic biochemical assay of precipitated proteins of seeds of *Ocimum sanctum*

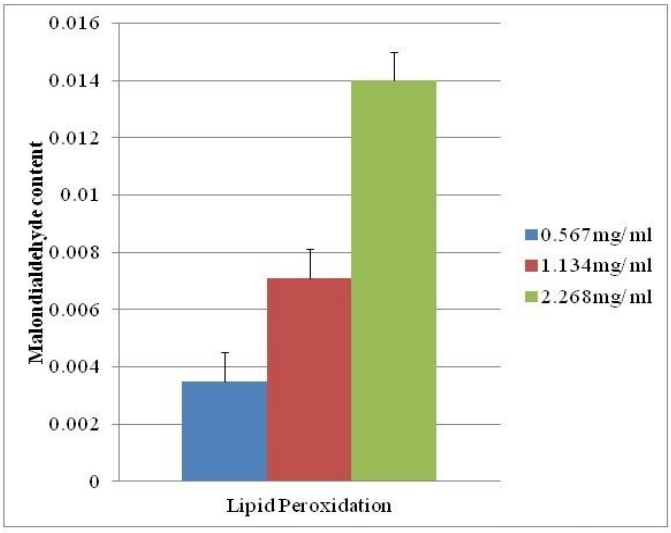

Fig. 6. Nonenzyme biochemical assay of precipitated proteins of seeds of *Osmium sanctum*

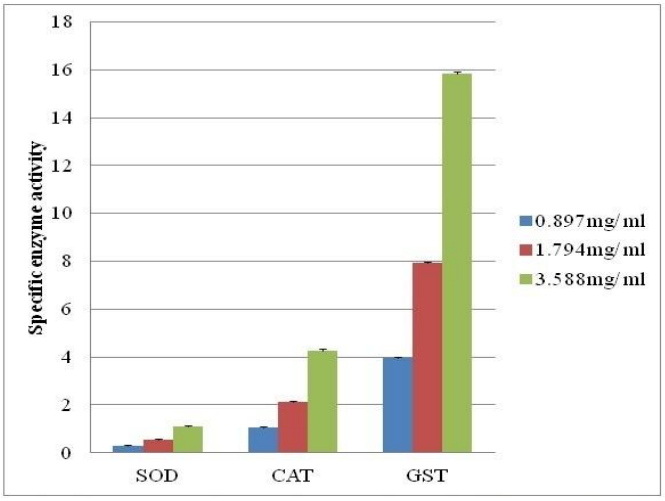

Fig. 7. Enzyme biochemical assay of precipitated proteins of seeds of *Azadirachta indica*

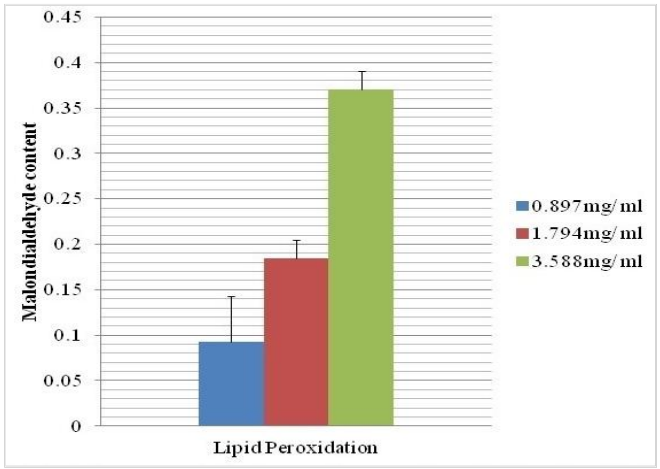

Fig. 8. Nonenzyme biochemical assay of precipitated proteins of seeds of *Azadirachta indica*

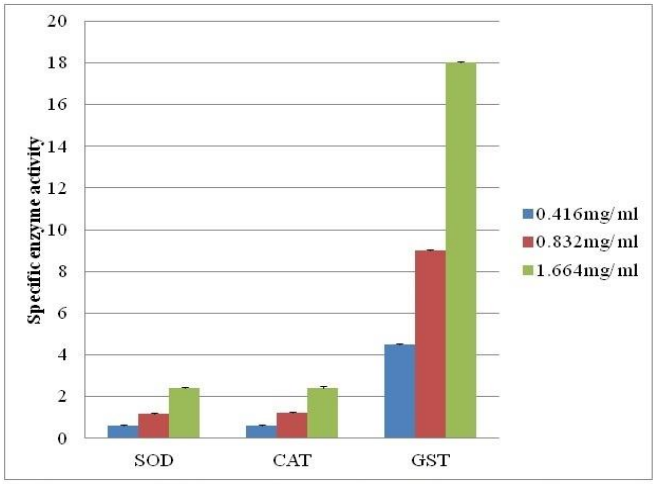

Fig. 9. Enzyme biochemical assay of precipitated proteins of seeds of *Momordica charantia*

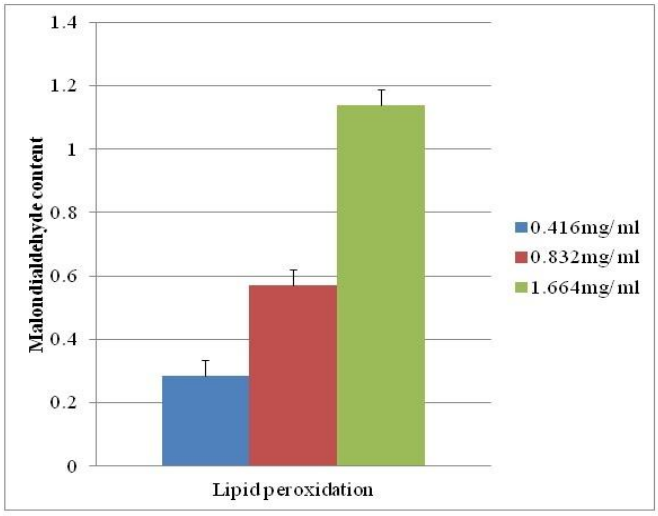

Fig. 10. Nonenzyme biochemical assay of precipitated proteins of seeds of *Momordica charantia*

4. CONCLUSION

We authors would like to conclude from the present studies that seeds of *Ocimum sanctum*, *Azadirachta indica* and *Momordica charantia* found to be the source of proteins. These proteins has shown maximum amount of antioxidant activity as revealed by their enzymatic biochemical assay such as superoxide dismutase, catalase & glutathione s transferase activity and nonenzyme biochemical assay i.e. lipid peroxidation with respect to the concentration. This present study revealed us the presence of proteins that are responsible for the antioxidant activity. These studies suggest us to use these mixtures of proteins as a neuroprotective agent. Future work includes the purification of the peptides from these sources such seeds of *Azadirachta indica*, *Ocimum sanctum* and *Momordica charantia* as to use it as neuroprotective agents against neurological disorders.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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