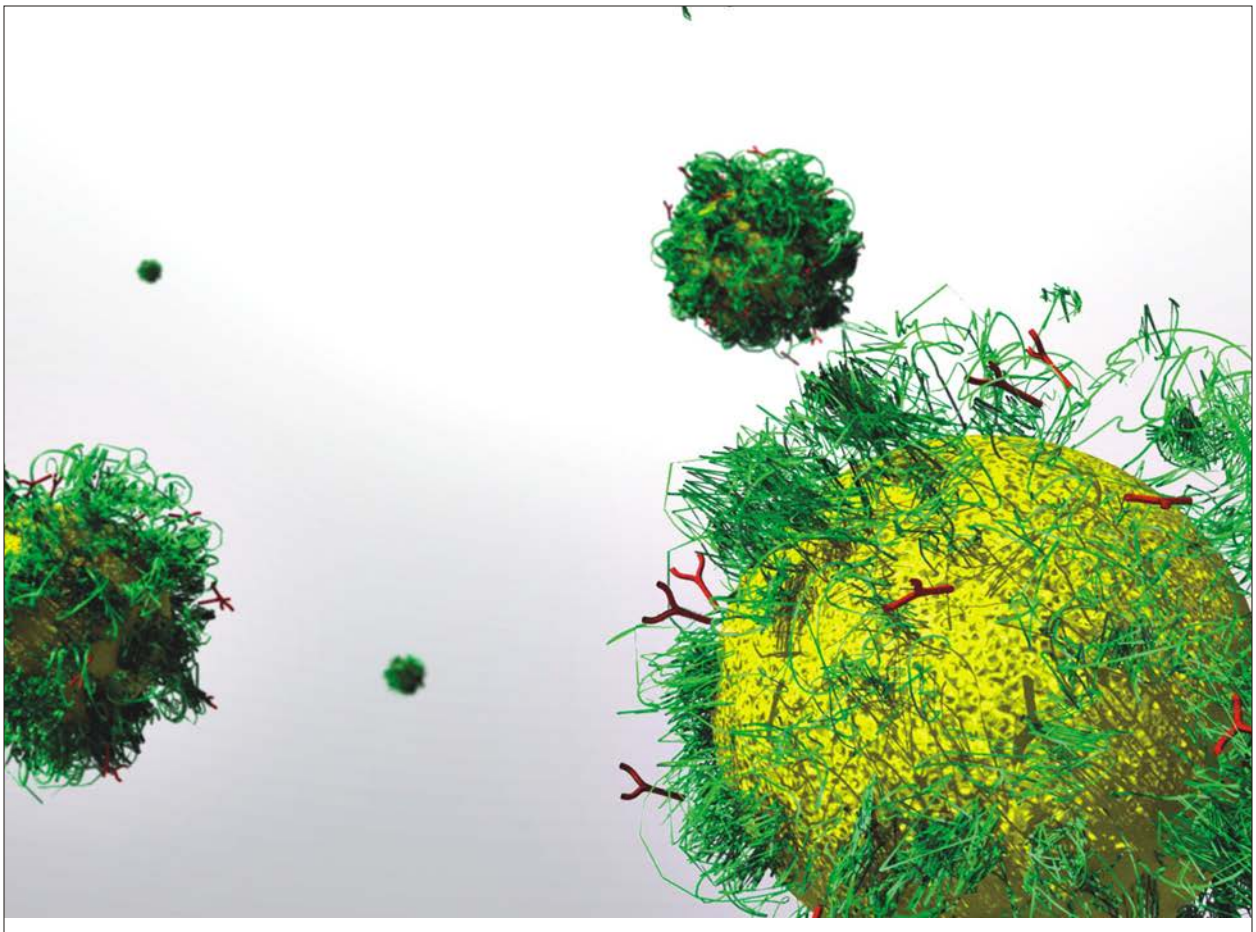

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Research Article
Antioxidant Activity of Polyphenolic Flavonoid of Stem of *Nicotiana tabacum*

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Abstract

Background and Objective: Phytochemical constituents of herbal extracts has shown positive response of antioxidant activity. The purpose of the present study was to investigate the antioxidant activity of polyphenolic flavonoids in the ethanolic extract of stem of *Nicotiana tabacum*. **Materials and Methods:** Flavonoids contents were determined by both thin layer chromatography and aluminium chloride colorimetric method, where as phenolic content was determine by Folin-Ciocalteau method. Antioxidant activity was observed by determining the electron transfer (DPPH free radical scavenging), enzymatic biochemical assay (SOD and GST activity) and nonenzymatic biochemical assay (GSH content and lipid peroxidation). **Results:** Flavonoid and phenolic content were found to be present in the ethanolic extract i.e., 12.5±0.1322 mg QE g^{-1} of extract and 1133.25±0.02 mg QE g^{-1} of extract, respectively. It was found from the present study that 0.4 mg mL^{-1} of ethanolic extract has shown highest level of SOD, GST, GSH and MDA content. The HPLC analysis of ethanolic extract has also revealed the presence of flavonoids in them as compared to standard quercetin. **Conclusion:** This can be concluded from the present study that ethanolic extract of stem of *Nicotiana tabacum* has shown the presence of polphenolic flavonoids and antioxidant level can be utilized as traditional herbal remedy.

Key words: Flavonoids, phenolic content, HPLC, antioxidant activity, enzymatic and nonenzymatic biochemical assay

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Plants are valuable source of natural products that maintain the human health. As herbal medicines which are being described in ayurvedic for use of uplifting the general health of common people[1]. Medicinal plant consist of phytochemical constituents i.e., secondary metabolites that have therapeutic and pharmaceutical efficacies. Secondary metabolites that possess antimicrobial, antiinflammatory, antioxidant, antistress and antinociceptive activities are flavonoids, terpenoids, saponins, alkaloids, soluble phenolic compound and many more. Each and every part of plant has different secondary metabolites present in them and has different antimicrobial activity[2]. Holistic approach of ayurveda in regard to preventive promotive and curative measures is well established in relation with health and disease[1]. Researchers are using extracts of plant to analyze the pharmaceutical activities of hidden secondary metabolites in them[3]. Bioactive compounds that are present in plants are gaining attentions as eco-friendly alternative to synthetic active compounds or agents[4]. As far as the neurological disease are concerned, bioactive compound has shown effective antioxidant activity against free radicals. Free radicals are Reactive Oxygen Species (ROS) that generated due to electromagnetic radiation, cosmic radiation, UV-light, ozone, cigarette smoke and low wavelength electromagnetic radiation. These free radicals affects macromolecule including protein, DNA and lipid causing to cellular or tissue damage[5]. Oxidative stress released free radicals and pose major endogenous damage in the biological system[6]. To suppress these free radicals, scavenging has been initiated by bioactive compound that has antioxidant activity. Antioxidant bioactive compound found to be present in the form of secondary metabolites such as flavonoids, terpenoids and phenolic compounds that balance pro-oxidant and antioxidants. Antioxidant activity of a natural extracts can be determined by electron transfer assay, enzymatic and non enzymatic biochemical assay. These electron transfer assay can be determined by the DPPH free radical scavenging where electrons get transfer and oxidized the free radical. Enzymatic biochemical assay are the specific enzyme activity that determines superoxide dismutase (SOD), catalase (CAT) and glutathione-s-transfer (GST), whereas nonenzymatical biochemical assay are concerned they comprises the estimation of glutathione content and lipid peroxidation[7].

Nicotiana tabacum is a perennial herbaceous plant that belongs to a family of Solanaceae. *Nicotiana tabacum* is commonly known as tobacco[1]. All parts of it are sticky and are covered by shorts viscid-glandular hairs which exude yellow secretion containing nicotine. It was also found that 20% of tobacco resources are discarded as processing waste such as its stem that has no use but recent studies proved that it has antioxidant and antimicrobial activity in its aqueous and methanolic extract[8]. Discarded leaves of *Nicotiana tabacum* has valuable bioactive compound such as polyphenols. Nicotine associated with zinc has shown antimicrobial activity against both Gram-positive bacteria as well as Gram-negative bacteria[9]. Tobacco consists of 30-40% of vegetable oil and biodiesel. Citric acids of tobacco can be used for the production of dyes and varnishes. It was also observed from the previous studies that stem of *Nicotiana tabacum* has 0.005% of nicotine in the production of briquettes[10]. As far as the antioxidant activity of leaves of *Nicotiana tabacum* is concerned, flavonoids of leaves has shown SOD activity, DPPH activity and ABTS radical scavenging at concentration of 600 μg mL^{-1}.

The purpose of the present study was to investigate the antioxidant activity of flavonoids that were found to be present in the ethanolic extracts of stem of *Nicotiana tabacum*. Flavonoids were determined by thin layer chromatography i.e., qualitative assay and quantification of flavonoids were observed by aluminium chloride colorimetric method. Antioxidant activity was assayed by electron transfer, enzymatic and nonenzymatic biochemical assay.

MATERIALS AND METHODS

Plant samples: Plants of *Nicotiana tabcum* were collected from the Khari Baoli, Kucha Challan, Chandni Chowk and Delhi. The plant was identified by Dr. Kumud Bala. Tobacco plant consisting leaves associated with stem were separated and then allowed to wash with distilled water to remove dirt and soil particles. Stems were dried in shaded area and then grounded with an ordinary grinder to form powder and used throughout the study.

Preparation of antioxidant extracts: Ethanolic extract was prepared by dissolving 10 g of grinded powder of stem in the 50 mL and 100 mL of ethanol. Conical flasks of extract was covered with cotton plugs to avoid evaporation and allowed to boil for 1 h at 100°C. The extract was placed in shaking incubator for 24 h at 250 rpm. After 24 h shaking, extract was filtered with muslin cloth and after that with filter paper i.e., 2 times. The filtered extract was allowed[8] to store at 4°C.

Determination of flavonoids

Qualitative estimation (Thin layer chromatography): The TLC was performed on the 20×20 cm plates precoated with

0.2 mm layers of silica gel 60 F254 (Merck). Plates were allowed to bake for 1 h. Volume of 20 µL standard quercetin (1 mg mL^{-1}) loaded along with same amount of ethanolic extracts of stem of *Nicotiana tabacum*. Ethyl acetate-methanol-water in the ratio of 50:3:10 mobile phase was run in order to determine the flavonoid glycoside. The TLC plates were observed under UV illuminator after spraying with 10% $AlCl_3$ solution. Their retardation factor (R_f) was measured[11,12].

Quantitative estimation (Aluminium chloride colorimetric method): To quantify number of flavones in the ethanolic extracts of stem of *Nicotiana tabacum* was determined by aluminium chloride colorimetric method. About 0.1 mL of extracts or quercetin standard 10-100 µg mL^{-1}, 1.5 mL methanol, 0.1 mL aluminium chloride (10%), 0.1 mL of potassium acetate solution (1 M) and 2.8 mL of distilled water were added and mixed well. Sample blank was prepared by replacing aluminum chloride with distilled water and absorbance was measured at 417 nm. The standard calibration plot was made to determine the concentration of flavonoids in the extract. The concentrations of flavonoids in the extract were calculated from the calibration plot and were expressed in mg QE g^{-1} of extract[8,12].

Determination of phenolic content: The total phenolic content in extracts was determined by Folin ciocalteau method. About 0.1 mL of extracts or quercetin standard (10-100 µg mL^{-1}) and 0.1 mL of Folin-Ciocalteu reagent (0.5 N) was added and incubated at room temperature for 30 min. About 2.5 mL of 20% saturated sodium carbonate is added in to the solution and further incubated for 30 min. After incubation the absorbance was measured at 760 nm against the blank reagent. The standard calibration plot was made to determine the concentration of phenolic component in the extract. The concentrations of phenolic component in the extracts were calculated from the calibration plot and were expressed in mg QE of phenol g^{-1} of extract[13,14].

Antioxidant activity: Antioxidant activity of ethanolic extract of stem of *Nicotiana tabacum* was determined by the electron transfer assay, enzymatic biochemical and non-enzymatic biochemical assay.

Electron transfer assay: The DPPH free radical scavenging was assayed in order to determine the oxidants which were reduced by transfer of electron from an antioxidant (oxidized). This assay was based on the degree of color change of the oxidant by oxidation.

Dot blot scan assay: Rapid screening of antioxidant substance in ethanolic extract of stem of *Nicotiana tabacum* was done by qualitative test i.e., dot blot and DPPH staining. Each diluted ethanolic extracts was loaded on a TLC plate. Drop of ethanolic extract was loaded in increasing concentration simultaneously, as control spots of 4 mM DPPPH stain was added and dried. A purple background of DPPH stain was decolorized in white spot at the location where radicals being scavenged[8,15].

DPPH free radical scavenging: The antioxidant activity of ethanolic extracts was measured in terms of hydrogen donating or radical scavenging ability by using the stable radical DPPH (1,1-diphenyl-2 picrylhydrazyl). About 3.8 mL of methanol was taken as blank, whereas control consists of 3 mL of methanol, 0.3 mL of 0.4 mM DPPH solution. Reaction mixture was prepared by mixing 3 mL of methanol, 0.5 mL of ethanolic extracts of stem of *Nicotiana tabacum* and 0.3 mL of 0.4 mM DPPH solution. Solution such as blank, control and reaction mixture were allowed to incubate in the dark for 30 min. The color of the reaction mixture fades as compared to the control and the reduction was observed by the decrease in the absorbance at 517 nm. The results were compared with the positive control i.e., standard quercetin. The percentage inhibition of the DPPH radical was measured by using the following formula[16]:

$$\text{Percentage of inhibition} = \frac{\text{Absorbance of control-Absorbance of reaction mixture}}{\text{Absorbance of control}} \times 100$$

Enzymatic biochemical assay: Enzymatic biochemical assay were determined by superoxide dismutase activity (SOD) and glutathione-s-transferase activity (GST) in the ethanolic extract of stem of *Nicotiana tabacum* by standardized protocol. Superoxide dismutase activity was defined as 1 U enzyme concentration for 50% inhibition at absorbance of 560 nm of chromogen produced in 1 min under assay. The SOD activity was expressed in specific enzyme activity as unit of SOD per minute per milligram of protein[17]. Glutathione-s-transferase activity was determined in the ethanolic extract of stem of *Nicotiana tabacum* to observe the detoxification of compounds that were involved in the reducing free radical damages in blood cells. The GST assay was also a specific enzyme activity that was based on the glutathione conjugate to 1-chloro-2, 4-dinitrobenzene (CDNB) as substrate and was measured at absorbance of 340 nm. Enzyme specific activity was expressed in micro moles of CDNB-GSH conjugate formed per minute per milligram of protein[18].

Nonenzymatic biochemical assay: Nonenzymatic biochemical assay consist of glutathione content and malondialdehyde content (MDA). The MDA content was determined in order to estimate the lipid peroxidation of thiobarbituric acid reactive substances. Lipid peroxidation was determined by breaking down of polyunsaturated fatty acids where the lipid peroxidation levels were expressed in nano moles of malondialdehyde formed per gram of extract by observing spectrophotometrically at 532 nm[19]. Glutathione content (GSH content) was determined in the ethanolic extract of stem of *Nicotiana tabacum* by using dithiobis nitrobenzoic acid (DTNB) and was expressed in the microgram per milligram of protein[20].

HPLC analysis: Retention time of ethanolic extract of stem of *Nicotiana tabacum* was determined by using HPLC system. The column used for chromatographic separation was C18 10 µm 100A (250×4.6 mm). A wavelength of 254 nm was set and the sample was injected. The chromatographic separation was accomplished using mobile phase ethanol, filtered through filter using vacuum pump and also standard quercetin. Mobile phase was pumped at a flow rate of 1 mL min^{-1} at room temperature. Using the optimized chromatographic condition baseline was recorded[21].

Statistical analysis: To estimate the accuracy of the experimental data, each experiment was performed in triplicates and the result was expressed as Mean±Standard Deviation of three replications. The $p<0.05$ was regarded as significant.

RESULTS

Determination of flavonoids: Flavonoids were determined in the ethanolic extracts of stem of *Nicotiana tabacum* by both thin layer chromatography method and aluminium chloride colorimetric method. It was observed from the present study that ethanolic extract has shown the presence of flavonoid glycosides with R_f of 0.77, whereas compared to standard quercetin it has shown R_f of 0.90 as shown in Fig. 1. Total flavonoid count was also determined in the ethanolic extract of stem by aluminium chloride colorimetric method. By standard curve of quercetin ($y = 0.011x$, $R^2 = 0.987$) as shown in Fig. 2. It was found from the present study that ethanolic extract of stem of *Nicotiana tabacum* has revealed the number of flavones i.e., 12.5±0.1322 mg QE g^{-1} of extract.

Determination of phenolic content: Phenolic content in the ethanolic extract of stem of *Nicotiana tabacum* was determined by the Folin-Ciocalteau method. By standard curve of quercetin ($y = 0.003x$, $R^2 = 0.629$) as shown in Fig. 3. Ethanolic extract of stem of *Nicotiana tabacum* has shown the presence of phenolic content i.e., 1133.25±0.02 mg QE g^{-1} of extract.

Antioxidant activity

Electron transfer assay

Dot blot scan assay: This was found from the present study that increasing concentration of ethanolic extract has decolorized the violet spot of DPPH from yellow to white. This revealed that ethanolic extract of stem of *Nicotiana tabacum* has total antioxidant capacity as shown in Fig. 4.

Fig. 1(a-b): Thin layer chromatography of (a) Quercetin (1 mg mL^{-1}) and (b) Flavonoid glycosides of ethanolic extract of stem of *Nicotiana tabacum*

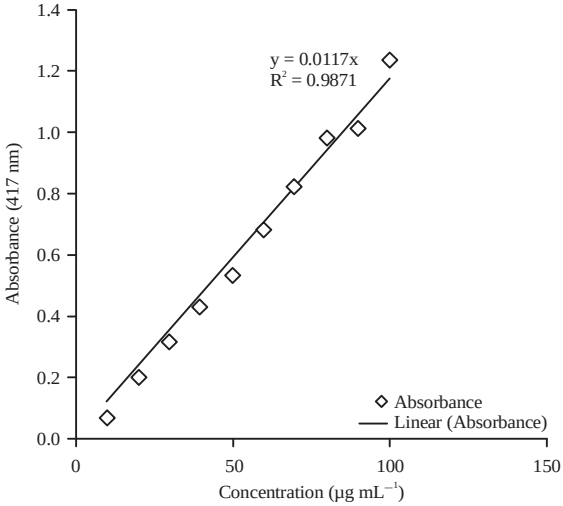

Fig. 2: Caliberation curve of quercetin standard (1 mg mL^{-1}) for total flavonoid count

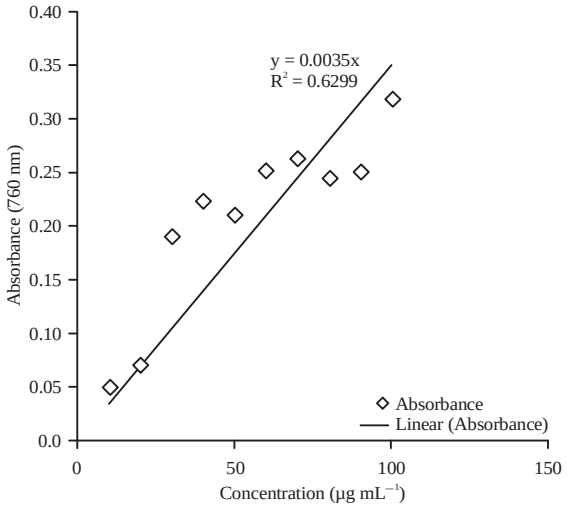

Fig. 3: Calibration curve of quercetin standard (1 mg mL^{-1}) for total phenolic count

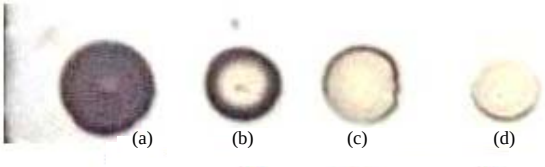

Fig. 4(a-d): Dot blot scan assay of antioxidant compound in ethanolic extracts of stem of *Nicotiana tabacum* where (a) 40 µL, 4 mM DPPH, (b) 10 µL ethanolic extract+30 µL 4 mM DPPH, (c) 20 µL ethanolic extract+20 µL 4 mm DPPH and (d) 30 µL ethanolic extract+10 µL 4 mM DPPH

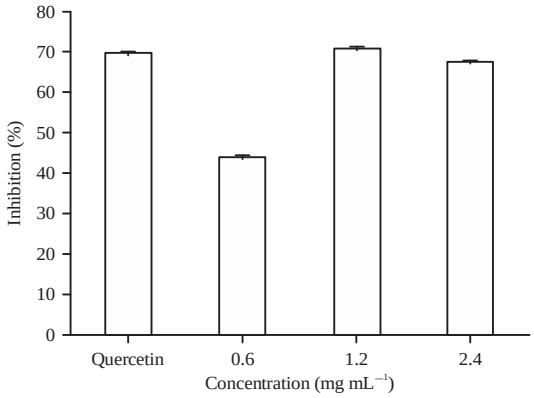

Fig. 5: DPPH free radical scavenging of ethanolic extract of stem of *Nicotiana tabacum* at increasing concentration

DPPH free radical scavenging: Ethanolic extract of stem of *Nicotiana tabacum* has revealed the free radical scavenging by oxidizing oxidants. It was found that 1.2 mg mL^{-1} of ethanolic extract has shown maximum percentage if inhibition i.e., 70.94% as given in Fig. 5, whereas 2.4 mg mL^{-1} and 0.6 mg mL^{-1} has shown 67.56 and 43.82% of inhibition as calculated by the formula.

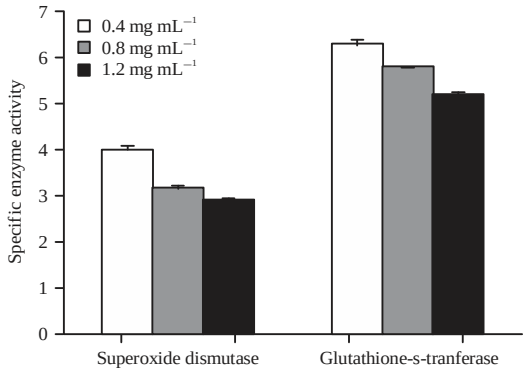

Fig. 6: Enzymatic biochemical assay of ethanolic extract of stem of *Nicotiana tabacum*

Enzymatic biochemical assay: Specific enzyme activity such as superoxide dismutase and glutathione-s-transferase was determined in the ethanolic extract of stem of *Nicotiana tabacum*. As the concentration increases superoxide dismutase activity found to be decrease i.e., 0.4 mg mL^{-1} of extract has shown 4.00±0.1 U min^{-1} mg^{-1} of protein, where as compared to concentration of 1.2 mg mL^{-1} it has shown minimum SOD activity i.e., 2.982±0.001 U min^{-1} mg^{-1} of protein in the ethanolic extract of stem of *Nicotiana tabacum* as shown in Fig. 6. As far as the GST activity was concerned, from the present study it was found that at 1.2 mg mL^{-1} of concentration of extract has shown 5.22±0.01 µmol of CDNB-GSH conjugate formed per minute per milligram of protein, whereas 0.4 mg mL^{-1} concentration of ethanolic extract of stem has shown maximum specific enzyme activity i.e., 6.29±0.02 µmol of CDNB-GSH conjugate formed per minute per milligram of protein. About 0.8 mg mL^{-1} of ethanolic extract of stem has also shown GST and SOD enzyme activity i.e., 5.80±0.01 µmol of CDNB-GSH conjugate formed per minute per milligram of protein and 3.19±0.005 U min^{-1} mg^{-1} of protein, respectively.

Nonenzymatic biochemical assay: The GSH content and MDA content were determined in the ethanolic extract of stem of *Nicotiana tabacum* in order to determine the total glutathione content as well as lipid peroxidation (MDA content). This was found from the present study 0.4 mg mL^{-1} of ethanolic extract has revealed the presence of 365.85 µg of glutathione mg^{-1} of protein. As far as the lipid peroxidation was concerned, MDA content was found to be maximum in 1.2 mg mL^{-1} of ethanolic extract i.e., 0.182±0.001 µmol mg^{-1} protein, where

as 0.4 and 0.8 mg mL^{-1} of ethanolic extract has also shown the presence of lipid peroxidation i.e., 0.111 ± 0.001 µmol mg^{-1} protein and 0.167 ± 0.001 µmol mg^{-1} protein, respectively as shown in Fig. 7.

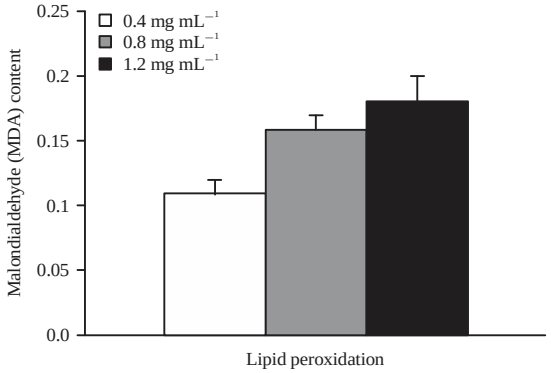

Fig. 7: Nonenzymatic biochemical assay of ethanolic extract of stem of *Nicotiana tabacum*

HPLC analysis: Flavonoids were determined by HPLC analysis where condition used led to a good separation of the peak which could be identified in the chromatogram (Fig. 8). Retention time of quercetin in ethanolic extract of stem of *Nicotiana tabacum* found to be 2.723 min at 254 nm where as compared to the standard quercetin, it has shown retention time of 2.632 min as given in Table 1 and 2.

DISCUSSION

Free radicals found to be present in either oxygen derived (ROS) or nitrogen derived (RNS), where the reactive oxygen species includes superoxide anion, hydrogen peroxide,

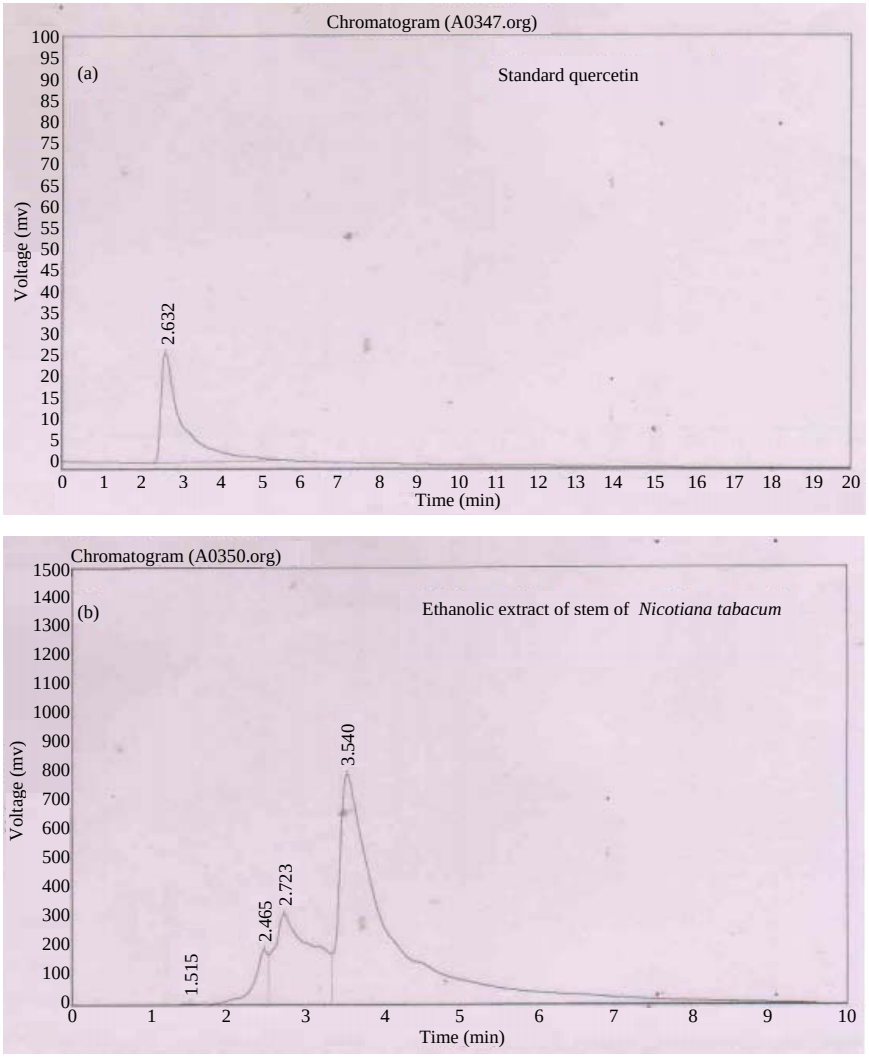

Fig. 8(a-b): HPLC chromatogram of (a) Standard quercetin and (b) Ethanolic extract of stem of *Nicotiana tabacum* at 254 nm

Table 1: Retention time of standard quercetin peak

Peak	Ret time	Height	Area	Concentration
1	2.632	25797.672	958658.688	100%

Table 2: Retention time of quercetin present in the ethanolic extract of stem of *Nicotiana tabacum*

Peak	Ret time	Height	Area	Concentration
1	2.723	309742.156	10852737	22.4289%

peroxyl radical and reactive hydroxyl radicals. There are such secondary metabolites or substances that are found to be present in the extracts of plants that have shown antioxidant activity such as flavonoids which are polyphenolic compounds. The purpose of the present study was to investigate the antioxidant activity of ethanolic extract of stem of *Nicotiana tabacum*. This was found from the present study that flavonoids found to be present in the extract i.e., 12.5 ± 0.1322 mg QE g^{-1} of extract, whereas phenolic content found to be 1133.25 ± 0.02 mg QE g^{-1} of extract. As compared to the previous study, 838 mg QE g^{-1} of extract found to be present in the 80% ethanolic extract of stem of *Nicotiana tabacum*[8]. Flavonoid glycosides were also found to be present in the ethanolic extract of stem of *Nicotiana tabacum* as compared to the standard quercetin. Phenolic content was found to high in the ethanolic extract which revealed the presence of polyphenolic flavonoids in them. Antioxidant activity was also determined by both ET assay and biochemical assay (enzymatic and nonenzymatic). It was found from the present study that 1.2 mg mL^{-1} of ethanolic extract has shown the presence of antioxidant capacity i.e., 70.94% of inhibition. Whereas 0.4 mg mL^{-1} of ethanolic extract has shown maximum specific SOD and GST enzymatic activity. As compared to previous studies, SOD activity was found to be 4.05 ± 0.104 U min^{-1} mg^{-1} protein in the methanolic extract of stem of *Nicotiana tabacum*[8]. Glutathione content was also found to high at 0.4 mg mL^{-1} of extract where as MDA content was also observed in the 0.4 mg mL^{-1} of extract that has shown the minimum presence of lipid peroxidation. As far as the previous study was concerned, MDA content found to be high in the aqueous extract of stem of *Nicotiana tabacum*[8]. As far as HPLC chromatogram was concerned, ethanolic extract has shown the presence of polyphenolic flavonoids i.e., quercetin with concentration i.e., 22%.

CONCLUSION

Authors would like to conclude from the present study that antioxidant activities of polyphenolic flavonoids were found to be present in the ethanolic extract of stem of *Nicotiana tabacum*. This was also observed that 0.4 mg of ethanolic extract has shown highest enzymatic and non-enzymatic biochemical levels. Ethanolic extract of stem of *Nicotiana tabacum* has revealed the presence of flavonoids, phenolic content and antioxidant levels that can be utilized as traditional herbal remedy. Future work includes purification of bioactive compound in the ethanolic extract of stem of *Nicotiana tabacum* to use it as an antioxidant agent against neurological disease.

SIGNIFICANCE STATEMENTS

- Present study describes the presence of antioxidant activity in the tobacco stem
- This study also indicates that stem of tobacco which was discarded as waste can be a good source of therapeutic agent
- Purification of polyphenolic flavonoid can be formulated in tablets and utilized as antioxidant drug

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