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Research Article

SUPERCRITICAL FLUID EXTRACTION AND EVALUATION OF ANTIOXIDANT ACTIVITY OF FLAVONOIDS FROM *NYCTANTHES ARBOR-TRISTIS* L. LEAVES.

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ABSTRACT

Objective: Supercritical fluid extraction (SFE) represents an efficient and environmentally friendly technique for isolation of phytoconstituents from different plant sources. The objective of this work was to evaluate the supercritical fluid (SCF) extraction of flavonoid from *Nyctanthes arbor-tristis* L. at different operational conditions and evaluate its *in vitro* free radical scavenging activity.

Methods: The parameters studied for supercritical fluid (SCF) extraction were pressure, temperature, CO_2 Flow rate, Co-solvent percentage. Quantitative analysis of total flavonoids was estimated using spectrophotometric method. Further, *in vitro* free radical scavenging activity of SFE extract was evaluated by 1, 1-diphenyl-2-picryl-hydrazil (DPPH) radical scavenging method. Ascorbic acid was used as the reference.

Results: The optimum extraction condition occurred at temperature 40°C, pressure 24.51 MPa, CO_2 Flow rate 2ml/min and co-solvent 8.25%. The extraction yield obtained under optimized SFE conditions was 69.85%.

Conclusion: SFE extract exhibited promising antiradical effects in a concentration dependent manner. Linear regression analysis was used to calculate IC_{50} value. Results showed that, the extract exhibited significant DPPH radical scavenging activity with IC_{50} value of 62.42±0.69 µg/mL.

Keywords: *Nyctanthes arbor-tristis*, Flavonoids, Supercritical fluid extraction

INTRODUCTION

Plants produce a vast array of natural products, including secondary metabolites, some of which exhibit pharmacological activities. Since these active compounds in herbal plants usually are present in low concentrations and they are usually very complex, researchers are focused on the development of more effective and selective extraction methods for their recovery from the raw materials. Flavonoids are a group of polyphenolic compounds found in various sources of fruits, vegetables and plants. One class of natural products, ubiquitous in vascular plants, is the flavonoids, which are made up of over 8000 compounds comprising 12 subclasses including flavones, flavanones and flavanols [1, 2]. The medicinal and pharmacological activities of flavonoids against inflammation, allergies, viruses, cancer and other ailments are well documented [1, 3].

Nyctanthes arbor-tristis L. is a hardy shrub belongs to the family Oleaceae. The leaves of the plant are used in the treatment of inflammations, dyspepsia, helminthiasis, pruritus, dermatopathy, fever [4]. Several studies have been carried out to investigate the bioactivity of this plant. The alcoholic extract of the leaves of *N. arbor-tristis* was evaluated for its anti-inflammatory activity [5]. It was also found to posses analgesic, antipyretic and ulcerogenic activities [6]. It has also been reported to possess hepatoprotective, immunostimulant, anti-leishmanial, anti-viral and anti-fungal activities [7]. Leaf extracts possessed good in vitro antioxidant activities [8].

Traditionally, the extraction of bioactive compounds from herbs is been performed by steam distillation and organic solvent extraction using percolation, maceration and Soxhlet techniques. However, there are drawbacks associated with classical extraction techniques such as consumption of large amounts of solvents, time consuming and considerable waste products treatment [9]. Supercritical fluid extraction (SFE) could be an environmentally beneficial alternative to the conventional organic solvent extraction of these compounds. In addition, the SFE processes are fast, selective and the products are free of residual solvents.

SFE is a rapidly developing method to produce bioactive compounds by pure technology, under mild conditions. The unique characteristic of this system is usage of gases above their critical point to extract selective soluble components from a raw material [10]. Carbon dioxide (CO_2) is the most widely used solvent in SFE, since it is physiologically harmless, environmentally safe, non-explosive, and readily available and it can be easily removed from products [11]. In recent years, several researchers studied the extraction of natural compounds from plant matrix by using supercritical carbon dioxide (SC-CO_2) [12-14]. Plants have played a critical role in maintaining human health and civilizing the quality of human life for thousands of years [15]. Plants are known to be the potential sources of natural antioxidants. With the increasing acceptance of herbal medicine as an alternative form of health care, the screening of medicinal plants for active compounds has become very interesting as they may serve as promising sources with the novel mechanism of action [16].

To date, there are no publications found on flavonoid extraction from *N. arbor-tristis* by supercritical fluid extraction. In the present study supercritical carbon dioxide was employed to extract flavonoid bioactive compounds from *N.arbor-tristis*. Thus the objective of the study was to determine effects of pressure, temperature, CO_2 flow rate and Co-solvent percentage on extraction yield.

MATERIALS AND METHODS

Plant material

Leaves of *Nyctanthes arbor-tristis* were collected from the local garden. The leaves were separated from the stalks, thoroughly washed with tap water and rinsed with distilled water. Leaves were dried at 40°C, powdered and passed through the sieve (mesh size=850µM) to maintain constant particle size. Identification of the species was confirmed at Agharkar Research Institute, Pune.

Chemicals and Reagents

Carbon dioxide (purity 99.99%) was purchased from Rakhangiz Gases, India. Ethanol and $AlCl_3$ (AR grade) and Methanol (HPLC grade) were purchased from Merck (India). Quercetin (purity 99.99%), was purchased from Aldrich Chemical Co. 1, 1-diphenyl-2-picryl-hydrazil (DPPH) and L-ascorbic acid were procured from Sigma Chemicals. All other chemicals, reagents and solvents were of analytical grade available commercially.

Supercritical Fluid Extraction [SFE] Apparatus

The Supercritical carbon dioxide extraction system and components were acquired from JASCO (Japan Spectroscopic Co.). Supercritical

fluid extractor/Chromatograph (900 series), included the following: 100 ml extraction vessel, temperature control unit (JASCO C0-965), high-pressure pump (JASCO-PU-980), automated back pressure regulator (JASCO 880-81) as depicted in **Fig1**. The refrigerating coolant circulator was manufactured by Scinics Co. Ltd. L.R. Grade methanol was used as a coolant and circulated at -5°C for cooling the SC-CO_2 extraction apparatus. Absolute ethanol (95% EtOH) acted as the co-solvent. The independent variables were Pressure (7.84MPa to 29.41MPa), Temperature (35°C to 70°C), CO_2 Flow rate (1.8mL/min to 3.5mL/min) and Co-solvent percentage (6.97% to 13.04%). Before the liquid CO_2 was passed into the extraction vessel, it was compressed to the desired pressure and heated to the specified temperature to reach the supercritical state. The powdered leaves (10 gms) were well mixed with 2.0mm diameter glass beads and placed in the extractor vessel. The introduction of some rigid materials such as glass beads with the ground sample of leaves contributed to maintaining a proper flow rate of CO_2 in the extractor vessel as well as in maintaining the desired permissibility of the particle during extraction process [17, 18]. The supercritical CO_2 flow rate was maintained at 2 mL/min and the dynamic extraction time was fixed to 60 min.

During the dynamic extraction time, CO_2 carrying the crude extract flowed out of the extraction vessel unit and into a collection vessel. The ethanolic SFE extracts for each parameter were stored in amber coloured tubes in a refrigerator (4 °C) until UV-VIS analysis.

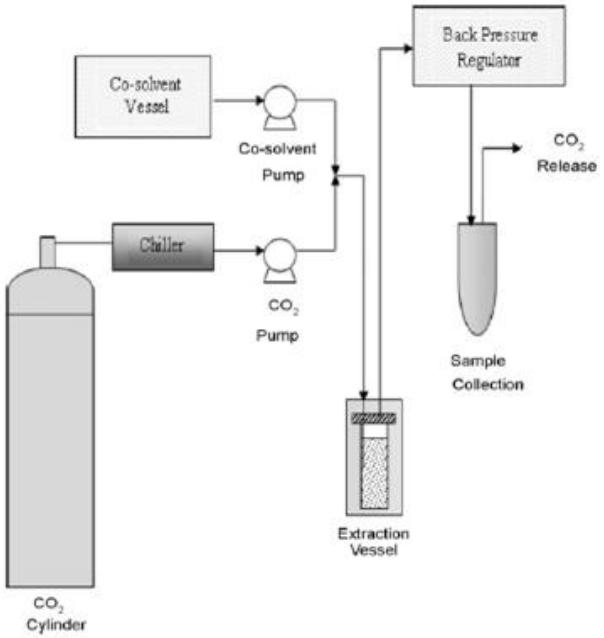

Fig. 1: It shows schematic design of supercritical fluid extraction (SFE) unit

Measurement of Total Flavonoids

Total Flavonoid content of the ethanolic extracts was estimated by Aluminum Chloride Colorimetric method. [19] The principle of this method is that the aluminum chloride forms acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols. [20]

Quercetin was used as a standard to make the calibration curve. 10 mg of quercetin was dissolved in 80% ethanol and then diluted to 25 to 1200 µg/mL. The diluted standard solutions (0.5 mL) were separately mixed with 1.5 mL of 95% ethanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1M potassium acetate and 2.8 mL of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm with JASCO V-550 UV-Visible spectrophotometer. The amount of 10% aluminum chloride was substituted by the same amount of distilled water in the blank. Similarly, 0.5 mL of ethanolic plant extracts were reacted with aluminum chloride for determination of flavonoid content as described above.

Determination of extraction yield

The ethanolic extract, under optimized SFE conditions was collected and the residue of the co-solvent from the extract was removed using a rotary evaporator, under vacuum at 40°C. The extract was then placed in the oven at 40°C and the final constant weight was recorded. The extraction yield of crude extract was calculated as follows:

$$Y_{extract}\,(mg/g) = m_{extract} / m_{herb} \times 100$$

Where $Y_{extract}$ is the % extraction yield; $m_{extract}$ is the crude extract mass (g) and m_{herb} is the extracted herb mass (g)

Free Radical Scavenging Activity evaluated by 1, 1-Diphenyl-2-picrylhydrazyl

The free radical scavenging activity of all of the extracts were evaluated by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) according to the previously reported method with slight modifications [21]. Briefly, a 90µM solution of DPPH in methanol was prepared, and 0.8 ml of this solution was added to 0.2 ml of the solutions of extract in methanol at different concentrations (10 µg/ml - 80 µg/ml). The mixtures were shaken vigorously and allowed to stand at room temperature for 1hr. Then their absorbance was measured at 517 nm using a JASCO V-550 UV-Visible spectrophotometer. Ascorbic acid was used as the reference. Lower absorbance values of reaction mixture indicate higher free radical scavenging activity. The capability to scavenge the DPPH radical was calculated by using the following formula:

DPPH scavenging effect (% inhibition) = [(A0– A1) / A0) × 100]

Where, A0 is the absorbance of the control reaction, and A1 is the absorbance in presence of all of the extract samples and reference. All the tests were performed in triplicate and the results were averaged.

RESULTS AND DISCUSSION

The first step in SFE is to optimize the operating conditions so as to obtain an efficient extraction of the target compounds and reduce the co-extraction of non-targeted compounds. Since various parameters potentially affect the extraction process, the optimization of the experimental conditions is a critical step in the development of a SFE method. In fact, the fluid pressure, temperature and percentage of co-solvent are generally considered

to be the most important factors. Optimization of the suitable extraction conditions in SFE can be carried out step by-step or by using an experimental design. Therefore for complete evaluation of extraction yield, each parameter was performed in triplicates.

Effect of Temperature on the flavonoid extraction

Fig. 2 presents the effect of temperature on total flavonoid content of *Nyctanthes arbor-tristis leaves* in SC-CO_2 at different temperature levels at constant pressure. Density of CO_2 at constant pressure decreases with increasing temperature and hence reduces the solvent power for SC-CO_2. On the other hand the increase of temperature can increase the vapor pressure of the analytes. Therefore the tendency of compounds to be extracted passing through the supercritical fluid increases [22]. A moderate increase in temperature can lead to a large decrease in fluid density, with a consequent reduction in solute solubility [23]. In this study, results showed that the extraction yield increased as temperature was increased from 35°C to 40°C. This can be described to the fact that increasing temperature affects the enhancement of vapor pressure of analytes which is greater than the reduction of density of CO_2. However, a temperature increase from 40 to 70°C caused a decrease in the extraction yield which probably is due to reduction in the density of CO_2.

Effect of Pressure on flavonoid extraction

Fig. 3 shows the effect of pressure on the extraction yield of *Nyctanthes arbor-tristis* leaves in SC-CO_2 at different pressure levels at constant temperature. According to the results, as pressure increases from 7.84MPa to 24.51MPa, the flavonoid content increased. At a constant temperature, increasing the pressure will increase the density of the SC-CO_2. The solvent strength of SC-CO_2 increases with the density of CO_2. As the density increases, the distance between the molecules decreases, therefore the interaction between the analytes and CO_2 increases, leading to greater solubility of the analytes in CO_2 [24]. Therefore the increase in pressure also accelerates mass transfer analytes and solvent in a supercritical extractor vessel system and improves the extraction yield. This suggests that the solubility of flavonoids in SC-CO_2 is proportional to the density of SC-CO_2. In this study, the flavonoid yield increased with increasing pressure to a certain value. Over this range of pressure, increasing fluid density is presumably the main mechanism leading to a higher flavonoid yield. Above this range of pressure, a decreasing flavonoid yield with increasing pressure was observed. The volatility and polarity of extracted analytes might be responsible for the result [25].

Effect of CO_2 Flow rate on flavonoid extraction

In an attempt to investigate the effect of solvent flow rate on the extraction, a set of experiments was carried out at 40°C and 24.51MPa, while the flow rate of CO_2 was varied from 2.0 to 3.5ml/min. **Fig. 4** shows the effect of the solvent flow rate as a parameter. It shows that the flavonoid content increases with increase in supercritical carbon dioxide flow rate, reaches a maximum value, and then decreases with a further increase in the flow rate. The results obtained here can be explained as a trade-off between a mass transfer process and a thermodynamic equilibrium state. The interface gas phase concentration of the solute is a function of the mass transfer coefficient, or solvent flow rate, while an equilibrium state is favored by both high mass transfer rate as well as long residence time [26]. At low flow rates of the solvent, the mass transfer resistance limits the amount of solute transported into the bulk of the solvent and the supercritical carbon dioxide leaves the extractor unsaturated. As the flow rate is increased, mass transfer resistance continues to decrease until the exiting solvent is saturated; and allows equilibrium to be achieved enabling a maximum yield to be attained. Further, increase of the flow rate reduces the residence time causing the system to deviate from equilibrium and the solvent leaves the extractor unsaturated despite the high mass transfer rate [27]. As suggested this is because the amount of solvent that is in excess of what is needed to penetrate the cellular structure of the leaves simply bypasses the extractable leaves [28]. Thus in a supercritical fluid extraction, the optimum solvent flow rate has to be determined. However, it should be noted that the condition of optimum solvent flow rate depends on the nature of the solvent – solute system, the geometry of the extractor, temperature and pressure. [26]

Effect of Co-solvent (Ethanol) percentage on flavonoid extraction

The results of ethanol concentration on the extraction of the flavonoids are shown in **Fig.5.** The yield of flavonoids was studied for different ethanol concentrations (80% and 95%) with a different percentage. Ethanol, added to the supercritical CO_2, increases the polarity of the fluid. The co-solvent basically exerts its effect mainly in two ways: by interacting with the analyte complex to promote rapid desorption into the supercritical fluid and by enhancing the solubility properties of supercritical CO_2 [29]. Various percentage of ethanol used exhibited different effect in changing the fluid polarity and thus resulted in diverse effects on the solubility enhancement of the flavonoids. The optimal extraction yield may be fulfilled when the polarity of the fluid and its flavonoids are coincident. In the present study, the results indicated that the optimal 95% ethanol concentration for extraction of flavonoids of *Nyctanthes arbor-tristis* was 8.25%.

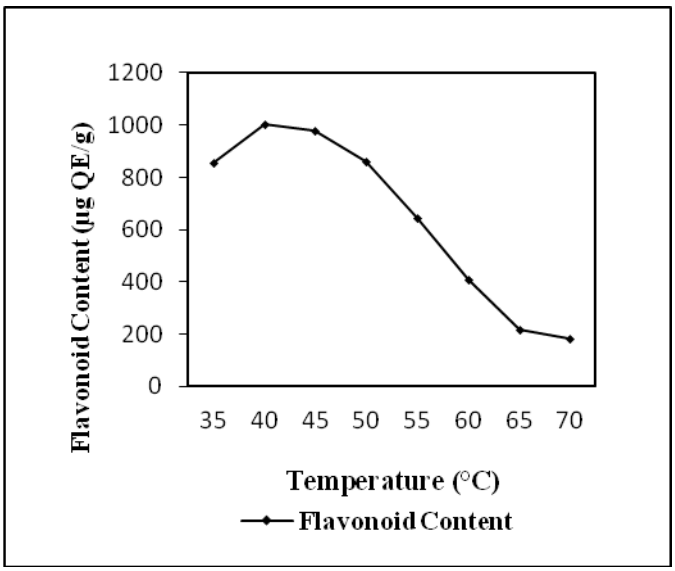

Fig. 2: It shows the effect of Temperature on total flavonoid content

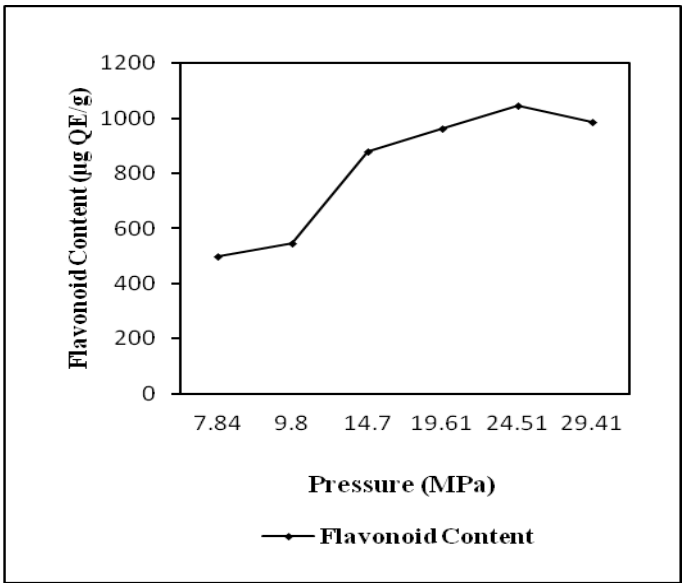

Fig. 3: It shows the effect of Pressure on total flavonoid content

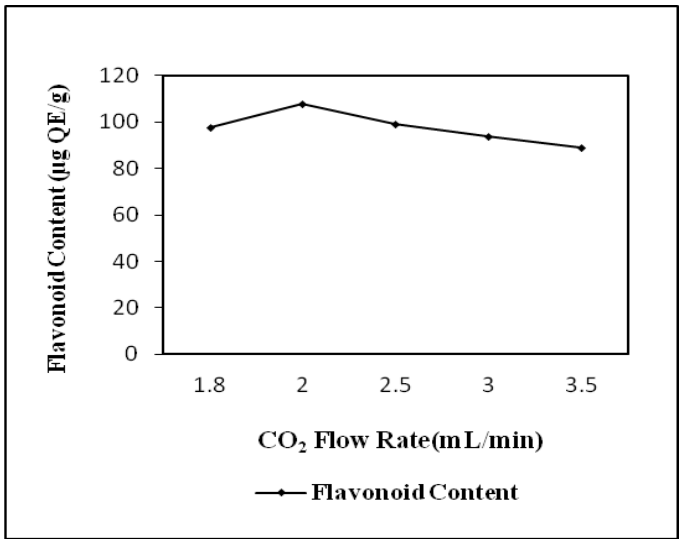

Fig. 4: It shows the effect of CO_2 Flow rate on total flavonoid content

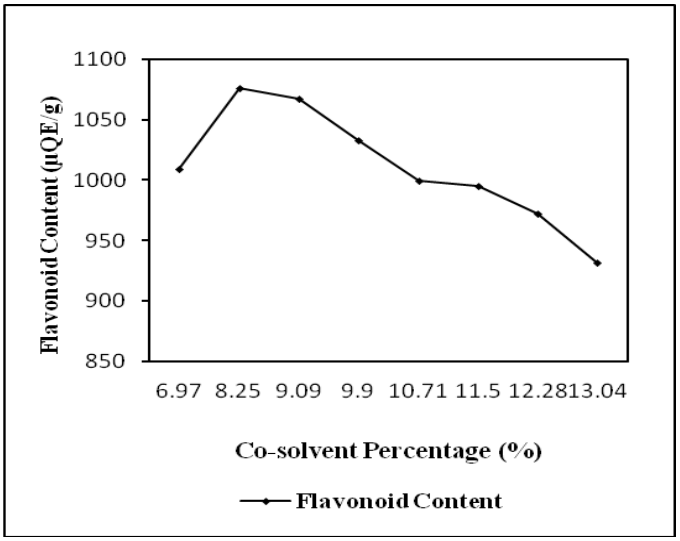

Fig. 5: It shows the effect of Co-solvent percentage on total flavonoid content

Determination of final extraction yield under optimized SFE conditions

The best conditions obtained for the extraction of flavonoids from *N. arbor-tristis* leaves extract were pressure at 24.51MPa, temperature at 40°C, CO_2 flow rate at 2mL/min and Co-solvent at 8.25%. The extract obtained at optimum SFE conditions was dried to obtain final extraction yield. The final extraction yield obtained under optimized SFE conditions is 69.85%.

DPPH radical-scavenging activity

The SFE extract of *Nyctanthes arbor-tristis* was found to be effective free radical inhibitor or scavenger, as well as a primary antioxidant that reacts with free radicals, which may limit free radicals. The results are depicted in **Table 1.** Free radical scavenging activity increased with increasing concentration of the extracts in the range of (30 to 250) µg/ml. Based on the results of this study, it is clear that the test plant extract have powerful *in vitro* free radical scavenging properties against DPPH model in a concentration dependent manner.

Table 1: It shows the percentage free radical scavenging activity of SFE extract by DPPH method

Concentration(µg/ml)	Percentage free radical scavenging activity
10	10.408+ 0.55
20	19.692+ 1.30
35	35.491+ 0.37
45	43.183+ 0.54
65	61.261+ 1.81
80	74.937+ 1.06
IC_{50} Value	62.425+ 0.69

All values in this table represent s the mean + SD (n=3).IC_{50} values, from the data, were calculated by regression analysis.

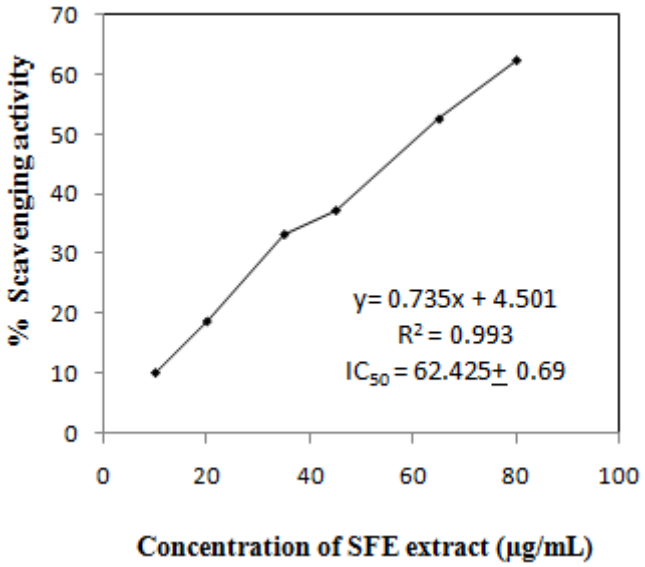

Fig. 6: It shows the effect of SFE extract of *N.arbor-tristis* on 1, 1Diphenyl-2picrylhydrazyl radical scavenging activity

CONCLUSION

The results presented in this work indicated that Supercritical fluid Extraction was feasible for extraction of flavonoids from *Nyctanthes arbor-tristis L*. leaves which is reported to have multiple biological activities. The results show that SFE may be a valuable alternative technique for the extraction of the flavonoids from *N. arbor-tristis L.* The optimum conditions of SC-CO_2 for *N. arbor-tristis* flavonoid compounds are pressure at 24.51MPa, temperature at 40°C, CO_2 flow rate at 2mL/min and co-solvent at 8.25 %. The present study also suggests that the tested plant extract has moderate to potent antioxidant activity and IC_{50} value was found to be 62.42±0.69µg/mL. Thus, further extensive investigations are necessary to find out the active antioxidative principles present in these plants.

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