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Antioxidant Activity, Total Phenolic and Flavonoid Contents of *Notonia Grandiflora* Wall. Extracts

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Abstract:

The aim of present study was to estimate the total phenolic and flavonoid contents and to investigate *in vitro* antioxidant potential of extracts of *Notonia grandiflora* (Compositae). Antioxidant activity was assessed by using 2,2- diphenyl-1-picryl-hydrazyl (DPPH•) assay, nitric oxide scavenging activity, super oxide free radical scavenging activity and total antioxidant activity. Here, ascorbic acid (ASA) and gallic acid were used as standard antioxidants. The total phenolic and flavonoid contents were also determined and expressed in gallic acid and quercetin equivalent respectively. The result concluded that the ethyl acetate extracts of *Notonia grandiflora* possess more significant free radical scavenging activity than other extracts. Half maximal inhibitory concentration (IC50) of ethyl acetate extract was found to be 48.71 µg/ml for DPPH•; 149.22 µg/ml for nitric oxide scavenging activity; 430 µg/ml for superoxide free radical scavenging activity; and 439.21 µg/ml for total antioxidant activity. The free radical scavenging and antioxidant activities may be attributed to the presence of adequate phenolic and flavonoid compounds. This study revealed that the ethyl acetate extracts of *Notonia grandiflora* has significant antioxidant activity.

Keywords: *Notonia grandiflora*, antioxidant activity, flavonoid, free radical scavenging, phenol.

INTRODUCTION

Radical scavenging antioxidants are mostly important in antioxidative-defence in protecting cells from the injury of free-radical such as superoxide radical (O_2.), Hydroxyl radical (OH) and non-free radical species such as H_2O_2 and singlet oxygen ($^1O_2$). Free radicals and oxidants play an important role for the development of degenerative diseases which affect the survival of the life for the humans. The oxidation induced by Reactive Oxygen Species (ROS) can result in cell membrane disintegration, membrane protein damage and DNA mutation, which can further initiate or propagate the development of many diseases, such as cancer, liver injury, diabetes and cardiovascular disease. This might be due to the continuous exposure to chemicals and contaminants through air, water and food by the humans in the part of their daily lives. The increased production of toxic oxygen derivatives is a universal feature of stress conditions [1,2]. Recent studies showed that several plant products including polyphenolic substances (e.g., flavonoids and tannins) and various plant or herb extracts exert antioxidant actions [3].

Notonia grandiflora is a perennial succulent genus of Asteraceae-Senecioneae commonly known as "Common Fleshy Ragweed". Traditionally, the plant is named as Muyal Kathilai (Tamil). It is commonly found on bare, exposed slopes and rocks of deciduous forests from plains to 1400m [4].

The potential health benefits of *Notonia grandiflora* extracts have not been studied to date. The basic aim of the research was to determine the total phenolic and flavonoid content and *in-vitro* antioxidant activity of aerial part extracts of *Notonia grandiflora* using spectrophotometric methods.

MATERIALS AND METHODS

Collection and Identification of plant material

The fresh aerial parts (stems and leaves) of *Notonia grandiflora* was collected from Tirunellvely district of Tamil Nadu, India in the month of February 2017. It was identified and authenticated by Dr. V. Chelladurai, Research Officer (Botany), Central Council of Research in Ayurveda and Siddha, Government Siddha Medical College, Palayamkottai, Tamilnadu, India.

Preparation of extract

Fresh plant material was washed under running tap water, air dried and powdered. About 50g of coarsely powdered plant materials (50g/250ml) were extracted in a Soxhlet extractor for 8 hours, sequentially with hexane, ethyl acetate, ethanol and water. The temperature was maintained (25°C- 100°C) on an electric heating mantle with thermostat control. The extracts were then concentrated by a rotary evaporator under 40°C and low pressure and finally dried to a constant weight. Dried extracts were kept at 20^0C in air tight containers until further test were carried out [5].

Preliminary qualitative phytochemical analysis

Preliminary qualitative phytochemical analysis was carried out to identify the secondary metabolites present in the various extracts of *Notonia grandiflora* using standard procedures [6,7] and results are showed in Table 1.

Total phenolic content (TPC) determination

Folin-Ciocalteau method was used for the determination of the total phenolic content of the plant extracts using gallic acid as an internal standard with slight modification [8]. 1 ml of the extract (1 mg/ml) was mixed with 2.5 mL of Folin-Ciocalteau phenol reagent. After 5 min, 4 mL of 7.5% Na_2CO_3 solution was added to the mixture and made up to the mark with distilled water. The mixture was

incubated in the dark for 90 mins at room temperature. A set of standard solutions of gallic acid (100,200,400,800, and 1000 µg/mL) were prepared in the same manner as described for the extracts. The absorbance's of the extracts and standard solutions were read against the reagent blank at 750 nm with a UV/Visible spectrophotometer (UV-1800, Shimadzu, Japan). The total phenolic content was determined from the calibration curve and expressed as milligram of gallic acid equivalent (GAE) per gram of the extracts. The determination of the total phenolic in the extract was carried out in triplicate.

Determination of total flavonoid content (TFC)

The total flavonoid content was determined according to the aluminium chloride colorimetric method [9]. Each plant extracts (2 mL, 0.3 mg/mL) in methanol were mixed with 0.1 mL of 10% aluminium chloride hexahydrate, 0.1 mL of 1 M potassium acetate and 2.8 mL of deionized water. After the 40 minutes incubation at the room temperature, the absorbance of the reaction mixture was determined spectrophotometrically at 415 nm. A set of standard solutions of quercetin (100, 80, 60, 40 and 20 µg/ml) were prepared in the same manner as described for the extracts. The absorbance's of the extracts and standard solutions were measured against the reagent blank at 415 nm with a UV/Visible spectrophotometer. The total flavonoid content was determined from the calibration curve and expressed as milligram of quercetin equivalent (QE) per gram of extracts. The determinations of total flavonoid in the extracts and standards were carried out in triplicates.

IN-VITRO ANTIOXIDANT ACTIVITY

DPPH radical scavenging assay

DPPH scavenging activity was measured by the spectrophotometric method [10]. A volume of 1.5 mL of 0.1 mmol/L DPPH solution was mixed with 1.5 mL of various concentrations (12.5 to 200 µg/mL) of leaf extract. The mixture was shaken vigorously and incubated at room temperature for 30 min in the dark. The reduction of the DPPH free radical was measured by reading the absorbance at 517nm by a spectrophotometer. The solution without any extract and with DPPH and methanol was used as control. The experiment was replicated in three independent assays. Ascorbic acid was used as positive controls. Inhibition of DPPH free radical in percentage was calculated by the formula:

DPPH radical scavenging activity (%)=($A_{control}$-A_{test})/$A_{control}$×100

Where, $A_{control}$ is the absorbance of the control and A_{test} is the absorbance of samples.

The antioxidant activity of each sample was expressed in terms of IC_{50} (micromolar concentration required to inhibit DPPH radical formation by 50%), calculated from the graph after plotting inhibition percentage against extract concentration.

Nitric oxide scavenging activity

Nitric oxide was generated from sodium nitroprusside and was measured by the Griess reagent. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitric ions that can be estimated by using Griess reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide. Sodium nitroprusside (5 mmol/L) in phosphate buffer saline (PBS) was mixed with different concentrations of the extract and incubated at 25 °C for 150 min. The samples were added to Griess reagent (1% sulphanilamide, 2% $H_3PO_4$and 0.1% napthylethylenediamine dihydrochloride). The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with napthylethylenediamine was read at 546 nm and referred to the absorbance of standard solutions of ascorbic acid treated in the same way with Griess reagent as a positive control [11]. All the tests were performed in triplicate and the graph was plotted with the mean values. The percentage of inhibition was measured by the following formula:

Radical scavenging activity (%)= ($A_{control}$-A_{test})/$A_{control}$×100

Where $A_{control}$ is the absorbance of the control (without extract) and A_{test} is the absorbance in the presence of the extract/standard.

Superoxide radical scavenging assay

The assay was based on the capacity of the sample to inhibit formazon formation by scavenging the superoxide radicals generated in riboflavin light-NBT system. Each 3ml reaction mixture contained 0.067 mM sodium phosphate buffer (pH 7.6), 0.05ml riboflavin, 0.1 mM EDTA, 0.1 ml NBT and various concentrations (125-2000µg) of sample extracts. Reaction was started by illuminating the reaction mixture with sample extract for 30 minutes. Immediately after illumination the absorbance was measured at 590 nm. The entire reaction assembly was enclosed in a box lined with aluminium foil. Identical tubes with reaction mixture kept in dark served as blank. The percentage inhibition of superoxide anion generation was calculated as: %inhibition = (control OD – sample OD / control OD) ×100

The analysis was performed in triplicate. The sample concentration providing 50% inhibition (IC50) under the assay condition was calculated from the graph of inhibition percentage against sample concentration [12].

Total antioxidant Capacity

The assay is based on the reduction of molybdate-IV (Mo IV) to molybdate-V (Mo V) by the extracts and subsequent formation of green phosphate/Mo V complex in acidic pH. 0.1 ml of the extract (10mg/ml) dissolved in 10% DMSO was mixed with 1ml of the reagent (0.6M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate), incubated at 95^o for 90 min, cooled to room temperature and absorbance was measured at 695nm against a blank. The antioxidant activity was expressed as the number of equivalents of ascorbic acid using standard plot [13].

Statistical analysis: All the experiments were done in triplicate. Values are expressed as mean±SEM

RESULTS AND DISCUSSION

Preliminary qualitative phytochemical analysis:

The present study revealed that the various extracts of *Notonia grandiflora* contained alkaloids, glycosides, flavonoids, phenols, saponins, steroids, tannins and reducing sugars. Compared to all other solvent extracts, ethylacetate extract had higher number of secondary metabolites such as alkaloids, terpenoids, steroids, tannins, flavonoids, phenols, carbohydrates and glycosides with high degree of precipitation (++).

Table 1 Test for Phytoconstituents for different extract of *Notonia grandiflora*

Test for various secondary metabolites	n-Hexane extract	Ethylacetate extract	Ethanol extract	Aqueous extract
Test for alkaloids	±	±	+	±
Test for tannins	+	++	++	+
Test for flavonoids	++	++	+	+
Test for steroids	+	++	++	+
Test for terpenoids	+	++	++	+
Test for carbo-hydrates	±	+	++	++
Test for glycosides	±	++	+	+
Test for oils and fats	-	±	±	-
Test for saponins	±	±	+	+
Test for proteins and amino acids	-	++	++	++
Test for phenols	+	++	+	+

Where; + Positive, ++ Strong positive, ± Trace, - Negative

All the four extracts were found to possess flavonoids, phenols and tannins. Flavonoids have aroused considerable interest recently because of their potential beneficial effects on human health in fighting diseases. Flavonoids have antimicrobial, antiviral, antioxidant and spasmolytic activity [14]. It also helps in managing diabetes induced oxidative stress [15]. Phenolic compounds possessed specific physical, chemical and biological activities that make them useful as drugs. Phenolics were also responsible for the antimicrobial, anti-inflammatory, anti-viral, and anticancer activity [16]. Tannins have amazing stringent properties. They are known to hasten the healing of wounds and inflamed mucous membranes. Terpenoids have been found to be useful in the prevention and therapy of several diseases, including cancer. Terpenoids are also known to possess antimicrobial, antifungal, antiparasitic, antiviral, anti-allergenic, antispasmodic, antihyperglycemic, anti-inflammatory and immunomodulatory properties [15].

Quantitative analysis of phytochemical substances

The highest total phenolics (65.41±0.21mg GAE/g dry wt.) and flavonoids (73.9±0.17mg QE/g dry wt.) was recorded in ethyl acetate extract whereas the contents obtained from ethanolic extract was the least (Table-2).

Table 2: Quantitative analyses of phytochemical substances present in different extracts of *Notonia grandiflora*

Solvents	Total phenolics (mg GAE/g)	Total flavonoids (mg QE/g)
Hexane	25.62± 0.25	62.73±0.30
Ethyl acetate	65.41±0.21	73.9±0.17
ethanol	11.35±0.31	34.92±0.58
aqueous	15.61±0.15	48.3±0.51

Values are means of three analyses of the extract ± standard deviation (n=3)
GAE: Gallic acid equivalent, QE: Quercetin equivalent

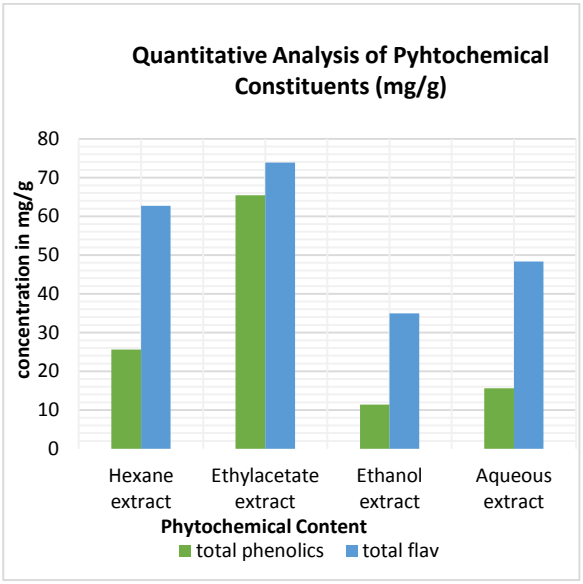

Figure 1: Total Phenolic and Flavonoid content of different extracts

ANTIOXIDANT CAPACITY

From the above four extracts, ethylacetate extract was selected for the determination of antioxidant activity. All the experiments were performed with different concentrations in triplicates. Flavonoids and Polyphenolic compounds present in *N.grandiflora* may contribute significantly to the total antioxidant capacity. Flavonoids play some important pharmacological roles against diseases, such as cardiovascular diseases, cancer, inflammation and allergy [17]. All the extract showed antioxidant activity in concentration dependent manner.

DPPH scavenging capacity

DPPH scavenging capacity of ethylacetate extract of *N. grandiflora* showed 79.07% at 200 µg/ml, whereas, for ascorbic acid (standard) was found to be 96.21 %. The IC50 value of ethylacetate extract and standard were 48.71 µg/ml and 34.30 µg/ml respectively. This indicates that *N. grandiflora* extract is a good potential as a source for natural antioxidants that might be attributed to their hydrogen donating ability [18].

Table 3 Effect of Ethylacetate extract of *N. grandiflora* aerial parts on DPPH assay

Concentration (µg/ml)	% of activity (± SEM)*	
	Ethylacetate extract	Standard
12.5	18.78± 0.01	21.76±0.11
25	37.16 ±0.15	40.04±0.03
50	52.93±0.11	60.36±0.15
100	65.12±0.10	83.43±0.38
200	79.07±0.11	96.21±0.07
IC50: 48.71 µg/ml		**IC50: 34.30 µg/ml**

*All values are expressed as mean ± SEM for three determinations

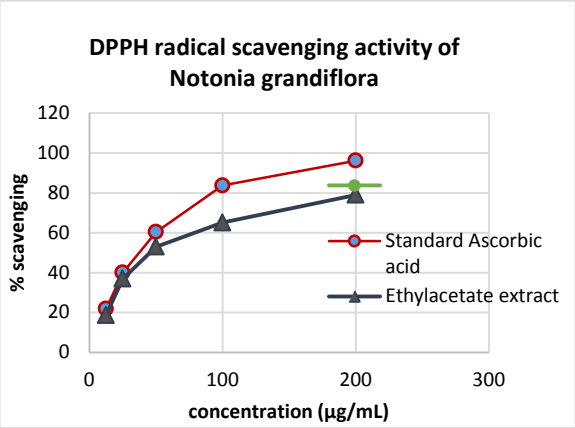

Figure 2: DPPH radical scavenging activity of standard and ethylacetate extract

Nitric Oxide Scavenging

Nitric oxide (NO) is a potent pleiotropic mediator of physiological processes such as smooth muscle relaxation, neuronal signalling, inhibition of platelet aggregation and regulation of cell mediated toxicity [19]. Excess of nitric oxide react with oxygen and generate nitrite and peroxy nitrite anions, which can act as free radicals, resulting in various deleterious effects in the cells. So, the production of nitric oxide should be regulated as much as possible. The extract inhibits nitrite formation by directly competing with oxygen in the reaction with nitric oxide [18]. IC50 value of the ethylacetate extract was found to be 149.22µg/ml and that of ascorbic acid was 102.39 µg/ml. The result indicates that Notonia grandiflora is a potent nitric oxide scavenger in dose dependent manner (fig. 3).

Table 4 Effect of Ethylacetate extract of *N. grandiflora* aerial parts on Nitric Oxide Scavenging assay

Concentration (µg/ml)	% of activity (± SEM)*	
	Ethylacetate extract	Standard
25	19.81± 0.02	31.92±0.01
75	30.27±0.13	43.10±0.10
125	37.26±0.11	54.07±0.03
250	51.25±0.07	64.46±0.40
500	65.2±0.18	68.33±0.32
1000	72.76±0.09	72.48±0.15
IC50: 149.22 µg/ml		IC50: 102.39 µg/ml

*All values are expressed as mean ± SEM for three determinations

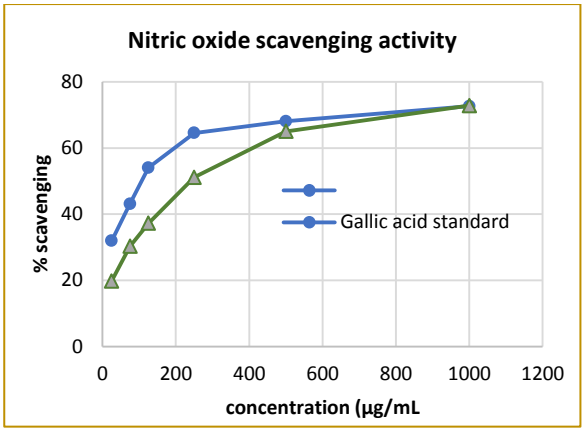

Figure 3: Nitric oxide radical scavenging activity of standard and ethylacetate extract

Superoxide radical scavenging

Superoxide anion is the first reduction product of oxygen and is also very harmful to cellular components [1] Robak and Glyglewski reported that flavonoids are effective antioxidants mainly because they scavenge superoxide anions [20]. As shown in figure 4, the superoxide radical scavenging activities of the plant extract and the reference compound are increased markedly with increasing concentrations. The results suggest that the plant extract is a moderate scavenger of superoxide radical. As shown in figure 4, the IC_{50} values of the plant extract and ascorbic acid on superoxide scavenging activity were 430µg/ml and 254.50 µg/ml, respectively.

Table 5 Effect of Ethylacetate extract of *N. grandiflora* aerial parts on Superoxide radical Scavenging assay

Concentration (µg/ml)	% of activity (± SEM)*	
	Ethylacetate extract	Standard
125	25.73± 0.01	41.14±0.13
250	39.62±0.03	47.43±0.12
500	52.30±0.09	62.66±0.15
1000	65.53±0.08	76.33±0.06
2000	78.14±0.04	90.62±0.20
IC50: 430µg/ml		IC50: 254.50 µg/ml

*All values are expressed as mean ± SEM for three determinations

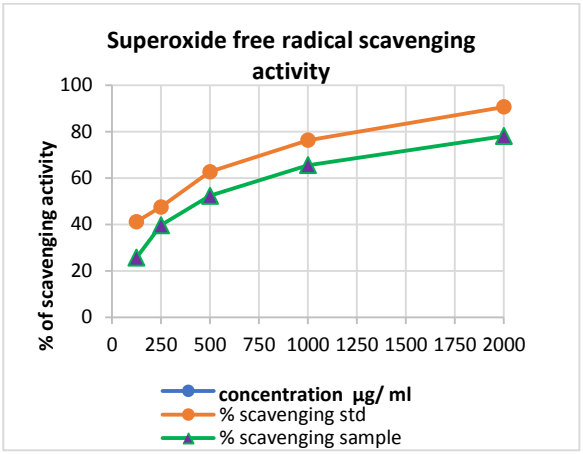

Figure 4: Superoxide radical scavenging activity of standard and ethylacetate extract

Total Antioxidant Capacity

The total antioxidant capacity (TAC) was based on the reduction of Mo(VI) to Mo(V) by the extract and subsequent formation of green phosphate/Mo(V) complex at acid pH. It evaluates both water-soluble and fat-soluble antioxidants (total antioxidant capacity).[2] The results indicate higher TAC (expressed as ascorbic acid equivalent) of the ethylacetate extract compared to standard. Total antioxidant activity of the ethyl acetate extract of *N. grandiflora* showed 87.22% inhibition at 2000 µg/ml, and was similar for ascorbic acid (88.66% at 2000 µg/ml), presented in fig.5.

Table 6 Effect of Ethylacetate extract of *N. grandiflora* aerial parts on Total Antioxidant Capacity

Concentration (µg/ml)	% of activity (± SEM)*	
	Ethylacetate extract	Standard
126	13.88± 0.01	14.55±0.02
251	42.61±0.02	29.46±0.03
500	55.56±0.03	42.81±0.03
1000	66.67±0.02	57.66±0.02
2000	87.22±0.02	88.66±0.02
	IC50: 439.21µg/ml	IC50: 737.39 µg/ml

*All values are expressed as mean ± SEM for three determinations

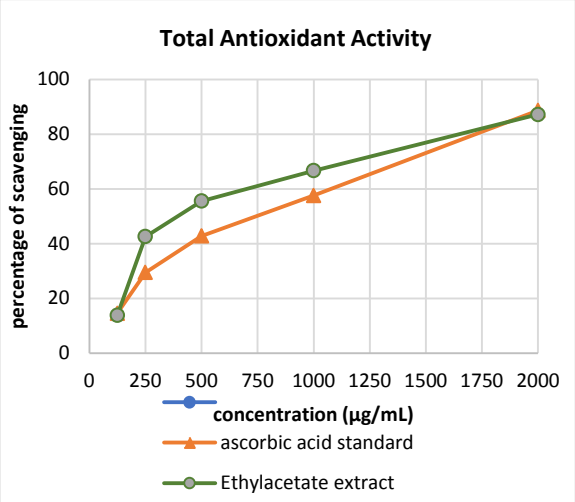

Figure 5: Total antioxidant activity of standard and ethylacetate extract

CONCLUSION

The results obtained in the present study indicated that *Notonia grandiflora* exhibited high antioxidant and free radical scavenging activities against DPPH•, nitric oxide and superoxide radicals. The overall antioxidant activity of *N. grandiflora* might be attributed to its flavanoid, polyphenolic content and other phytochemical constituents. The findings of the present study suggested that *N. grandiflora* could be a potential source of natural antioxidant that would have great importance as therapeutic agents in preventing or slowing the progress of reactive oxygen species and associated oxidative stress related degenerative diseases. Therefore, further studies are needed to isolate, purify, and characterize the antioxidant compounds present in the extract of *Notonia grandiflora*.

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