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Preliminary screening of *Nicotiana glauca* extracts for determination of antioxidant activity by different methods

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ABSTRACT

Nicotiana glauca (Solanaceae), or tree tobacco, is found in dry arid climates of North America, Africa and Europe . It has been reported to have both toxic and medicinal properties. The main aim of this study was to analyze the phytochemical screening and quantitative estimation of polyphenols, flavonoids and flavonols of crude extracts from the leaves of *Nicotiana glauca* and to evaluate its *in vitro* antioxidant properties. Three different solvents were used to extract bioactive compounds from powdered leaves of *Nicotiana glauca*: dichloromethane (DCM), ethyl acetate (AE) and n-buthanol (n-BuOH). The three extracts were then subjected to qualitative phytochemical screening using standard procedures. Total phenolics, total flavonoids and total flavonols contents of the extracts were measured by Folin Ciocalteu and Aluminium chloride methods respectively. Furthermore, the antioxidant capacity evaluation was performed using the DPPH, ABTS, DMSO alcalin, Phenantroline, FRAP and CUPRAC methods. The three extracts were then subjected to qualitative phytochemical screening using standard procedures. These methods showed the presence of polyphenols and comarines in all extracts. Moreover, flavonoids, tannins, steroids and quinones were reported in the AE and n-BuOH extracts. In addition, alkaloids were seen to be present in DCM extract, while saponines and phlobatannins were absent in all extracts. The antioxidant capacity evaluation was performed using the DPPH, ABTS, DMSO alcalin, Phenantroline, FRAP and CUPRAC methods.

Results using the DPPH method showed strong free radical scavinging activity for three extracts. This activity decreased with increasing concentration in the following order: n-BuOH>AE>DCM. In other assays, all extracts showed good antioxidant activity which decreased with increasing concentration in the following order: AE > n-BuOH > DCM. Extracts were compared with standards: BHT, BHA, Tanic acid and α-Tocopherol. The antioxidant of these extracts is probably related to polyphenols content (351.55±0.07, 284.98±0.08 and 133.8±0.06 mg/g), flavonoids (105.97±0.04, 164.44±0.07 and 1.18±0.005 mg/g) and flavonols (22.41±0.24, 18.75±0.46 mg/g) in AE and n-BuOH, respectively.

As a conclusion, the results of the present study indicate that *Nicotiana glauca* leaves could be considered as a potential source of natural antioxidants.

INTRODUCTION

Abbreviations: ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid), AE (ethyl acetate), BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene), CUPRAC (CUPric Reducing Antioxidant Capacity), DCM (dichloromethane), DMSO (Dimethylsulfoxide), DPPH (1,1-diphenyl-2-picrylhydrazyl), FRAP (Ferric reducing antioxidant power), NBT (Nitro Blue tetrazolium), n-BuOH (n-buthanol), GAE (gallic acid equivalent), QE (quercetin equivalent), TPC (total phenolics content), TFC (total Flavonoids content), TFoC (total Flavonols content).

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INTRODUCTION

Nicotiana glauca Graham (Solanaceae) or tree tobacco is an evergreen perennial plant [1]. In some Arabic countries, it is known as "Massas". Morphologically, it may be distinguished from tobacco due to the non-pubescent (glabrous) leaves and the yellow flowers. It is a tree-like shrub that may reach a height of 6 m [2], and usually grows in open and disturbed areas, in well-drained deep soils. Even though *N. glauca* is native to South America, it has been introduced to other continents, and, currently, it is wide-spread and also naturalized in the Mediterranean region [3].

Nicotiana glauca is toxic to human and animals and this effect was noticed as early as the 1930's [1,2]. Unlike other members of the *Nicotiana genus*, *Nicotiana glauca* does not contain the alkaloid nicotine. However, it produces a similar compound called anabasine. This is the plant's toxin. Anabasine is more toxic than nicotine and can cause fatal deformities in livestock when the mother ingests even small amounts of the plant material during early pregnancy [4].

Nicotiana glauca extracts are widely used by traditional healers as antibacterial, anti fungal, antiviral and anti-inflammatory medicines [5] and shows remarkable biological activities such as Allelopathic activity. Antifungal activity of acetone extracts of *Nicotiana glauca* against ten fungal phytopathogens was also reported. *Nicotiana glauca* extracts exhibit antibacterial and cytotoxic effects [1,2]. The main aim of this study was to analyze the phytochemical screening and quantitative estimation of polyphenols, flavonoids and flavonols of crude extracts from the leaves of *Nicotiana glauca* and to evaluate its *in vitro* antioxidant properties.

MATERIALS AND METHODS

Plant collection and extraction

The leaves of *Nicotiana glauca* were collected from Hodna (M'sila, central Algeria), during spring 2016. The plant was identified by Dr. Rebbas Khellaf, Department of Biology, Faculty of Sciences and University of M'sila.

One kilogram of *N. glauca* powdered leaves was extracted three times with a mixture of (MeOH: H_2O) (70:30) at room temperature. The extract was filtered, then the filtrate was collected and concentrated under reduced pressure at 40 to 50°C. Combined aqueous extracts were subsequently partitioned initially with dichloromethan (DCM) to remove chlorophylls, followed by ethyl acetate (AE) and finally with n-buthanol (n-BuOH). The extractive solutions were evaporated to dryness under vacuum to obtain 3.09 g (DCM), 6.08 g (AE) and 33.94 g (n-BuOH) extracts. The three extracts were used in this study.

Phytochemical screening

Qualitative assay for the presence of plant secondary metabolites such as polyphenols, flavonoids, tannins, phlobatannins, coumarines, terpenoids, steroids, alkaloids, saponin and quinones were carried out on the three extracts (DCM, AE and n-BuOH) of the *Nicotiana glauca* leaves. The presence or absence of the phytochemicals was carried out using standard methods [6-9].

Determination of total phenolics (TPC)

The content of total phenolics was evaluated by using the Folin-Ciocalteu method [10], with some modifications. Twenty microliters of each extract were mixed with 100 µl (1:10) diluted Folin-Ciocalteu reagent and 75 µl sodium carbonate solution (7.5%) in wells of a 96-well microplate. After 2 h in darkness at room temperature, the absorbance was measured at 740 nm in the microplate reader [11]. Total phenolics content was expressed as mg gallic acid equivalent (mg GAE)/g extract. The following equation based on the calibration curve was used:

$$y = 0.0034x+0.1044 \; ; \; R^2 = 0.9972,$$

where x was the absorbance and y was the gallic acid equivalent (mg/g). Measurements were done in triplicate.

Determination of total flavonoid contents (TFC)

The aluminum chloride colorimetric method was applied to determine total flavonoid content following the procedure employed by Topçun G [12]. Herein, 50 µL of each extract, 130 µL methanol, 10 µL of 1 M potassium acetate and 10 µL of 10% $AlCl_3$ were added to each well of a 96-well plate. The mixture was then homogenized and incubated for 40 minutes. The absorbance of the solution was measured at 415 nm in a microplate plate reader [13]. Total flavonoid contents were calculated from a quercetin calibration curve:

$$y = 0.0048x, R^2 = 0.997,$$

where x is the absorbance and y is the quercetin equivalent, and the quantity was expressed as miligram quercetin equivalent (QE) in a gram of dried powder (mg QE/g). Measurements were done in triplicate.

Determination of total Flavonols content (TFoC)

The flavonol content was ascertained based on the method used by Kumaran [14]. In undertaking this, 50 µL of each plant extract was mixed with 50 µl of $AlCl_3$ prepared in water (20 mg/ml). Then 150 µl of sodium acetate solution prepared in water (50 mg/ml) was added. The mixture was incubated for 2.5 h. The absorbance was measured at 440 nm in a microplate plate reader [15]. Total flavonol content was calculated as mg quercetin (mg QE/g) by using the following equation based on the calibration curve:

$$y = 0.0071x + 0.0225; R^2=0.9986,$$

where x is the absorbance and y is the quercetin equivalent. Measurements were done in triplicate.

In vitro antioxidant capacity

DPPH radical scavenging assay

The ability of the extracts to scaving the DPPH radical was investigated by the method described by Blois M.S [16]. Stock solution of the whole plant extracts was prepared to the concentration of 1 mg/ml in methanol. The DPPH was freshly prepared by liquefying 40 mg DPPH in 1000 ml

of methanol to obtain a 1.00±0.01 unit of absorbance. Before keeping it in the dark for 30 min, approximately 40 µl of sample (or standard antioxidant) at different concentration (0.781, 1.562, 3.125, 6.25, 12.5, 25, 50 µg/ml) were mixed up with 160 µL of the DPPH solution, the absorbance was recorded at 517 nm [17-18-19]. Methanol (40 µL)/ DPPH solution (160 µL) was used as blank to avoid interferences due to the sample's color. The annihilation activity of free radicals was calculated in % inhibition according to the following formula:

$$\% \text{ of Inhibition} = (A_{control} - A_{sample})/A_{control} \times 100$$

BHT and BHA were used as positive standards; triplicate measurements were performed for each extract.

ABTS radical scavenging assay

The ABTS radical cation scavenging activity was performed as described by Re R [20], with slight modifications. The ABTS+radicals were produced by the reaction between 7 mM ABTS in water and 2.45 mM potassium persulfate, while stored in the dark at room temperature for 12 h. Prior to use, the solution was diluted to generate an absorbance of 0.700±0.025 at 734 nm. Free radical scavenging activity was assessed by mixing 40 µl of the test sample (or standard antioxidant) at different concentrations (3.125, 6.25, 12.5, 25, 50,100, 200 µg/ml) with 160 µl of ABTS working solution. The decrease in the absorbance was measured after 10 min [21]. Methanol (40 µL)/ABTS solution (160 µL) were used as blank. The percentage inhibition was calculated according to the following formula:

$$\% \text{ of Inhibition} = (A_{control} - A_{sample})/A_{control} \times 100$$

BHT and BHA were used as positive standards, and triplicate measurements were performed for each extract.

Superoxide DMSO alcalin assay

Superoxide radical scavenging activity of the extracts was assessed using the alkaline DMSO method according to the method described by Elizabeth and Rao [22] with slight modification. In this method, the concentration of superoxide in an alkaline DMSO system corresponds to the concentration of oxygen dissolved in DMSO. In tightly stoppered vessels, the superoxide radical is stable for more than 24 h; however, in open vessels, its concentration rapidly decreases. Briefly, the superoxide radical was generated in a non-enzymatic system [23].

To the reaction mixture containing 40 µL of extract (or standard antioxidant) at different concentrations (3.125, 6.25, 12.5, 25, 50,100, 200 µg/ml) and 130 µL of alkaline DMSO (100 mL DMSO containing, 20 mg NaOH in 1 mL water), 30 µL NBT (1 mg/mL solution in H_2O) was added and mixed. The absorbance was measured at 560 nm using a microplate reader. The decrease in the absorbance at 560 nm with antioxidants indicated the consumption of the generated superoxide [24]. The blank control was prepared in the absence of extract and by adding 40 µL Methanol in order to obtain the final volume of 200 µL.

Tannic acid and α-tocopherol were used as positive standards, and triplicate measurements were performed for each extract.

Phenantroline assay

The Phenanthroline assay was used to determine the reducing capacity of plant extracts, according to the method of Szydlowska-Czerniaka [25]. Herein, 10 µl of various concentrations of the sample (or standard antioxidant) at different concentrations (3.125, 6.25, 12.5, 25, 50,100, 200 µg/ml), ferric chloride (50 µl, 0.2%) and 0.5% 1, 10-phenanthroline solution (30 µl) were mixed and made up to the volume of 110 µl with methanol. The reaction mixture was then incubated at 30°C in the dark for 20 min and the absorbance of the orange red solutions was measured at 510 nm against a blank control [21-26]. The blank control was prepared in absence of extract and by adding 10 µL Methanol in order to obtain the final volume of 200 µL. BHT and BHA were used as positive standards, and triplicate measurements were performed for each extract.

Reducing ability (FRAP assay)

The reducing power of each extract was determined by applying the method described by Oyaizu M [27] with some modifications. Briefly, 10 µl of the extracts (or standard antioxidant) at different concentrations (12.5, 25, 50,100, 200, 400, 800 µg/ml) were mixed with 40 µL phosphate buffer (pH 6.6), then mixed with 50 µL of 1% potassium ferricyanide [$K_3Fe(CN)_6$], and the mixture was incubated at 50°C for 20 min. Trichloroacetic acid (10%; 50 µl) was added to the reaction, the mixture was centrifuged at 3000 rpm for 10 min, and then mixed with 40 µl of distilled water and 10 µl of $FeCl_3$ solution (0.1%), and the absorbance was measured at 700 nm. The high absorbance of the reaction mixture indicates greater reducing power [28-29]. The blank control was prepared in absence of extract and by adding 10 µL Methanol in order to obtain the final volume of 200 µL.

Tannic acid, α-tocopherol and Ascorbic acid were used as positive standards, and triplicate measurements were performed for each extract.

Cupric reducing activity (CUPRAC assay)

In order to determine the antioxidant activity of extracts using CUPRAC, the method described by Öztürk M [30] was used. Herein, 40 µL of extract solution (or standard antioxidant) at different concentration (3.125, 6.25, 12.5, 25, 50,100, 200 µg/ml), 60 µL of 1 M NH_4CH_3COO (pH 7), 50 µL of 7.5 mM neocuproine and 50 µL of 10 mM $CuCl_2$ were mixed in a 96-well plate with a total volume of 200 µL. Incubation of the mixture was carried out for 1 h, and the absorbance was recorded at 450 nm using a microplate reader [13-31]. The blank control was prepared in absence of extract and by adding 40 µL Methanol in order to obtain the final volume of 200 µL.

BHT and BHA were used as positive standards and triplicate measurements were performed for each extract.

Statistical Analysis

All data are presented as means ± SD for three replicates for each prepared sample. Linear regression was applied to calculate IC_{50} values using Excel 2007.

RESULTS AND DISCUSSIONS

Phytochemical screening

As shown in Table 1, preliminary phytochemical screening of various secondary metabolites in various leaf extracts (DCM, AE and n-BuOH) of *N.glauca* exhibited the presence of polyphenols, flavonoids, tannins, coumarins, terpens, steroids, alkaloids and quinones. However, all these chemicals were not extractable in one solvent.

Table 1. Preliminary phytochemical screening of *N. glauca* extracts

Phytochemicals	*Nicotiana glauca* extracts		
	(DCM)	(AE)	n-BuOH
Polyphenols	++	++	+++
Flavonoids	–	++	+++
tannins	–	++	++
Phlotatannins	–	–	–
Coumarins	+++	+	++
terpenes	+++	–	–
Steroids	–	++	++
alkaloids	+++*	–	–
Saponosides	–	–	–
Quinones	–	++	+++

Symbol (+++) indicates presence in high concentration, symbol (++) indicates presence in moderate concentration, symbol (+) indicates presence in trace concentration and symbol (–) indicates absence of the respective phytochemical.
*Formation of reddish brown precipitate [6-8].

Alkaloid, Terpens, Coumarins were present in high concentration in DCM extract, weak presence of coumarins was observed in the AE and n-BuOH extracts, while polyphenols were found almost in each extract. Flavonoids and tannins were present in the AE and n-BuOH extracts. Steroids and quinones were reported in AE and n-BuOH extracts. Remarkably, Phlobatannins and saponins were not present in any of the extracts.

Determination of total phenolics, flavonoids and flavonols content

Table 2 shows that the ethyl acetate extract contains high amount of phenols (351.55±0.07 mg/g), while BUOH contains (284.98±0.08 mg/g) and DCM had lower phenolic content with (133.8±0.06 mg/g). Total phenolic content of *Nicotiana glauca* in different solvents was observed, however, ethyl acetate extract was found to contain more phenolic content then any other extract.

Total flavonoid varied from 1.18±0.005 to 164.44±0.07 mg/g in the extracts of *N. glauca*. Herein, n-BUOH and AE extracts contain the highest flavonoid concentration at (164.44±0.07 mg/g) and (105.97±0.04 mg/g), respectively, while the lowest (1.18±0.005 mg/g) was given by the DCM extract. The highest content of total flavonol was obtained in AE extract (22.41±0.24 mg/g) then in n-BuOH extract (18.75±0.46 mg/g), while the absence of these compounds was detected in DCM extract.

Table 2. Total phenolic content (mg $GAE.g^{-1}$), flavonoid content (mg $QE.g^{-1}$) and flavonol contents (mg $QE.g^{-1}$) of various extracts of *Nicotiana glauca* leaves

Extract	TPC (mg GAE /g)	TFC (mg QE/g)	TFoC (mg QE/g)
DCM	133.80 ±0.06	01.18 ±0.005	–
AE	351.55 ±0.07	105.97 ±0.04	22.41 ±0.24
n-BuOH	284.98 ±0.08	164.44 ±0.07	18.75 ±0.46

In vitro antioxidant capacity

In this study, the antioxidant activity is known to increase proportionally with phenolics levels [32] because the methods used to determine the antioxidant activities and the phenolics content have the same mechanism through electron transfer in the reaction [13]. The analysis of the antioxidant activity of leaves of *Nicotiana glauca* harvested in Algeria was performed using six methods: DPPH, ABTS, DMSO alcalin, Phenantroline, FRAP and CUPRAC.

DPPH radical scavenging assay

Using DPPH, the scavenging activity of *N. glauca* leaves extracts and standards decreased with increasing concentration ranging from 0.781 to 50 µg/ml. The results were used to determine the concentration required to obtain the 50% DPPH radical scavenging effect (IC_{50}). A lower IC_{50}value indicates higher scavenging activity [5]. The DPPH and its % inhibition of DCM, AE and n-BuOH extracts showed that the IC_{50} values were 47.17±0.67 µg/ml, 9.31±0.92 µg/ml and 7.40±0.41 µg/ml, respectively. When the results are compared to the activity of standard compounds, only the AE and n-BuOH extracts showed higher activity, Herein, IC_{50} for BHA = 5.73±0.41 µg/ml and for BHT, IC_{50} = 22.32±1.19 µg/ml (Table 3).

Table 3. Results of different *in vitro* antioxidant assay of *Nicotiana glauca* extracts

Extract	IC_{50} µg/ml			Concentration of $A_{0.5}$ µg/ml		
	DPPH	ABTS	DMSO alcalin	Penantroline	FRAP	CUPRAC
DCM	47.17 ±0.67	17.51 ±1.23	3.33 ±0.22	96.33 ±1.30	115.83 ±1.83	44.26 ±0.80
AE	9.31 ±0.92	5.39 ±0.64	2.32 ±0.14	46.43 ±0.77	19.06 ±0.50	13.78 ±0.26
n-BuOH	7.40 ±0.41	12.04 ±0.43	3.04 ±0.16	81.98 ±2.16	35.71 ±3.50	21.24 ±1.30
BHT	22.32 ±1.19	22.32 ±1.19	–	9.62 ±0.87	–	9.62 ±0.87
BHA	5.73 ±0.41	5.73 ±0.41	–	3.64 ±0.19	–	3.64 ±0.19
Tanic acid	–	–	< 3.125	–	5.39 ±0.91	–
α-tocopherol	–	–	< 3.125	–	34.93 ±2.38	–

Values are obtained from regression lines with 95% coincidence level – indicates values not determined

ABTS radical scavenging assay

In the ABTS method, maximum activity was exhibited by the AE extract followed by the n-BuOH extract, then the DCM extract. This ranged from 3.125 to 200 µg/ml

concentration. The IC_{50} value of AE was found to be 5.39±0.64 μg/ml, 12.04±0.43 for n-BuOH and 17.51±1.23 for DCM extract, as compared to that of BHT IC_{50} = 1.81±0.10 and of BHA IC_{50} = 1.29±0.30).

Superoxide DMSO alcalin asssay

As shown in Table 3, the Superoxide radical scavenging activity of Nicotiana glauca extracts (DCM, AE and n-BuOH) showed high antioxidant assay, in comparison to Tannic acid and α-Tocopherol in all different concentrations, and ranged from 3.125 to 200 μg/ml. In this assay, the activity of DCM extract was lower than that of the other extracts and standards at all concentrations, with an IC_{50} of 3.33±0.22 μg/ml. The AE and n-BuOH extracts exibited approximately similar IC_{50} values to that of Tanic acid and α-Tocopherol.

Phenantroline assay

The results from a series of concentrations ranged from 3.125 to 200 μg/mL. The IC_{50} value of DCM, AE and n-BuOH extracts was found to be 96.33±1.30; 46.43±0.77 and 81.98±2.16 μg/ml, respectively, as compared to standard BHT value (9.62±0.87 μg/ml) and BHA value (3.64±0.19 μg/ml).

Reducing ability (FRAP assay)

The reducing power of DCM, AE and n-BuOH extracts increased with increasing concentration, ranging from 12.5 to 800 μg/ml. Their IC_{50} values were 115.83±1.83; 19.06±0.50 and 35.71±3.50 for DCM, AE and n-BuOH extracts, respectively, as compared to standards with an IC_{50} of 5.39±0.91 and 34.93±2.38 for Tannic acid and α-Tocopherol, respectively.

Cupric reducing (CUPRAC assay)

In this assay, a higher absorbance indicates higher antioxidant activity in different concentrations ranging from 3.125 to 200 μg/ml. BHT and BHA were used as references and showed an IC_{50} value of (9.62±0.87 and 3.64±0.19 μg/ml) for BHT and BHA. IC_{50} values were 44.26±0.80; 13.78±0.26 and 21.24±1.30 for DCM, AE and n-BuOH extracts respectively.

In assessing the results obtained for the antioxidant activity of extracts, it was noticed that in using the DPPH method, the antioxidant activity of n-BuOH extract was higher than DCM and AE, this activity decreased in the following order: n-BuOH>AE>DCM, whereas using the ABTS, DMSO alcalin, Phenantroline, FRAP and CUPRAC methods, the antioxidant activity of the AE extract was higher than DCM and n-BuOH. Here, the antioxidant activity decreased in following order: AE>n-BuOH>DCM. AE and n-BuOH extracts are, therefore, more potent than DCM extract. The antioxidant potential of this species could be attributed to the presence of highest concentration of phenols, flavonoids and flavanols in the AE and n-BuOH extracts, while DCM extract contains considerably smaller amounts of phenols and flavonoids. These phytochemicals are the major constituents in most plants reported to possess free radical scavenging activity [5].

CONCLUSION

This study was designed to investigate the phytochemical characterization and to evaluate the *in vitro* antioxidant activity of *Nicotiana glauca* leaves extracts as assessed by applying different methods. Preliminary screening of phytochemicals revealed the presence of various compounds such as flavonoids, tannins, terpenoids, alkaloids, steriods, coumarins and quinones. Furthermore, AE and n-BuOH extracts were found to show the strongest antioxidant activity as compared to the DCM extracts. The prominent, antioxidant activity may be due to presence of higher contents of total phenolics and flavonoids contents in these extracts. However, further studies are needed to understand the underlying mechanisms of antioxidant action and to isolate the compound(s) responsible for such activity.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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