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Assessment of Antioxidant Activity, Total Phenolic, Total Flavonoid Content and Cytotoxic Activity of Methanol Root Extracts of Sudanese *Citrus sinensis*

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ABSTRACT

In the present time the medicinal plants are substantial sources of biologically active materials including phenolics and flavonoids which are a good antioxidant agent. *Citrus. sinensis* species are important because of their medicinal, traditional and economical uses. They are used in Sudan for treatment of various diseases traditionally. In this study the plant material was extracted with different solvents by using Soxhlet apparatus. Antioxidant activity; total phenolic; total flavonoid content; and brine shrimp lethality potential from *C. sinensis* methanol root extract was determined. Three fractions of the methanol extract of the root showed remarkable results in antioxidant activity, total phenolic, total flavonoid content and no genotoxic effect was observed. The total content of phenols and flavonoids of these fractions were strongly associated with the antioxidant activity. Generally, *C. sinensis* root can be used as source of natural antioxidant compounds, hence may also support the plant usage against diseases.

Keywords: *Citrus sinensis*, Sudan, total phenolic, total flavonoid, cytotoxicity.

INTRODUCTION

The family Rutaceae has several species of the genus *Citrus*, showed cytotoxic, painkiller and heal wounds activities. From this genus a several chemical compounds have been identified, mainly flavonoids, lignans, coumarins, sitosterol and acridones [1]. in *Citrus* species three types of flavonoids have been found: flavonols, flavanones and flavones [2]. In Citrus the flavonoids were found as glycosides except the permethoxylated flavones which was found as free aglycones [2].

Citrus sinensis is one of important medicinal plants that are grown throughout the world. This plant is prescribed in many countries as a traditional medicine for prevention diseases. It has been used as an anti-diabetic [3], antimicrobial [4], antifungal [5], hypotensive agent [6] and antioxidant [7, 8]. The plant is a rich source of vitamin E, C, phenols, flavanones and carotenoids [9], which play an important role as antioxidants and inhibitors of free radicals that cause damage to the components of cells, which leads to the occurrence of many diseases, [10]. Many epidemiological studies have confirmed an inverse relationship between eating vegetables, fruits and infections with arterial diseases, cancer and aging, and this is related to their containment of compounds with significant biological activity [11]. The objective of this study was to assess the antioxidant activities, phenolic compounds, flavonoid content and cytotoxicity of fractions of methanol root extract of *C. Sinensis.*

MATERIALS AND METHODS

Chemicals and standards

Petroleum ether, Chloroform, Methanol, Hexane, Silica gel, Acetone, Ethyl acetate, Ethanol, Gallic acid, Quercetin, Folin-Ciocalteu, Sodium carbonate, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), Aluminum chloride, Sodium nitrite and Sodium hydroxide.

Instruments

Rotary evaporator, Jenway 6300 ultraviolet spectrophotometer, Refrigerator and Sensitive balance.

Sample collection

Citrus sinensis roots were collected from Faculty of Agriculture, University of Khartoum. Botanical identification was carried out and authenticated with reference to a herbarium material at the Medicinal and Aromatic Plants Research Institute (MAPRI), Khartoum, Sudan. The collected roots were carefully examined, and the healthy roots were disseminated and dried under shade at room temperature in the laboratory for three weeks and size reduced by mechanical grinder into coarse powder, and stored in a clean closed glassware container free from environmental climatic changes till usage.

Extraction of plant material

Air dried roots of *C.sinensis* (about 600 g) were extracted respectively with petroleum ether (60-80º C), chloroform and methanol for 72 hours in suitably sized Soxhlet apparatus. The extracts were filtered using a filter paper (Whatman No.1), evaporated and concentrated under vacuum using rotary evaporator, then dried in a desiccator. The extracts were stored at 5° C in refrigerator in sterilized bottles before use.

Column chromatography of the root methanolic extract

Column chromatography was used to fractionated the methanol root extract. A glass column was thoroughly washed, dried and packed with 150 g of silica gel (60–200 mesh size) using open column procedure. The methanol extract was mixed with hexane and silica gel and poured onto the column (length 60 cm and diameter 3 cm) and was rinsed with the solvent. Gradient elution method was followed to separate fractions by using pure solvents and solvent mixtures of increasing polarity (i.e. hexane, chloroform and methanol) in varying ratios. Total 175 fractions were collected in 10 ml portion. Similar fractions were pooled together according to thin layer chromatography (TLC).

TLC of fractions

The fractions separated from the column were subjected to thin layer chromatography to detect the presence of phytocompounds. The same Rf values of fractions were pooled together, dried and weight of pooled fractions was measured. The prepared fractions were further analyzed for the presence of antioxidant compounds.

Antioxidant Assay

The capability of free radical scavenging of plant extracts against DPPH was measured using the method of Shimada *et al.* [12] with minor modification. A solution of DPPH was prepared in ethanol and mixed with the plant extracts (dissolved in DMSO) and allowed to incubate in 96-wells plate for half an hour at 37ºC. The concentration of DPPH was kept as (300µM). The absorbance of the reaction mixtures was measured at 517nm using UV spectrophotometer. Inhibition percentage of DPPH radical scavenging activity was calculated according to DMSO treated control group. All the procedures were performed in triplicate.

IC50 Calculations

The IC50 value (half maximal inhibitory concentration) of the extracts and fractions was calculated depending on the percentage inhibition points obtained from the absorption values of various concentration ranging from 0.5-0.0035mg/ml. EZ-Fit Enzyme Kinetic Program was used to estimate the IC50 values.

Determination of total phenolic contents (TPC)

Folin–Ciocalteu method was used to assessment the phenolic content of the plant extracts [13]. 0.5ml of (1 mg/ml) methanolic solution of extract was added to 2.5 ml of 10% Folin-Ciocalteu's reagent diluted in water and were mixed together. After 3 min, (2.5ml) 7.5% sodium carbonate solution was added. The mixtures were allowed to stand at room temperature for 2 hr. For each analysis the samples were repeated and the absorbance value was measured at 765 nm using UV spectrophotometer. Different concentrations of gallic acid as standard (10, 20, 30, 50 and 100 mg/ml) were used to construct a calibration curve, figure (2). The total phenols values were expressed as (mg) of gallic acid equivalent per (g) of dried extract.

Determination of total flavonoid content (TFC)

Estimating of total flavonoid content of extracts was done according to a modified colorimetric assay with aluminum chloride [14]. One ml of each extract was dissolved in 4 ml of distilled water and added to 0.3 ml of sodium nitrite solution (5%), followed by 0.3 ml of aluminum chloride solution (10%). Six min later, 2ml of sodium hydroxide (1 mol/L) was added to the mixture. Immediately, the size of the mixture was supplemented with distilled water to 10 ml. The absorbance was measured at 510 nm against a blank on UV spectrophotometer. A calibration curve was made with different concentrations (20, 40, 60, 80 and 100 mg/ml) of Quercetin standard. The values of total flavonoid content were calculated as (mg) of quercetin equivalent per (g) of dried extract. All the procedures were performed in triplicate, figure (3).

Toxicity testing against the brine shrimp

Eggs of *Artemia salina* can survive for many years if kept in low and favorable temperature conditions.

Brine shrimp eggs, were laid in Seawater for hatching. Therefore, salt water was prepared by dissolving 38 g of salt in a liter of distilled water, where shrimp eggs were placed in it for hatching the larvae within 24-72 hr at room temperature. Brine shrimp larvae was obtained for analysis by placing 50 mg of eggs in hatching container containing 75 ml of artificial Seawater for 48 hr under a fluorescent bulb until hatching. Twenty mg of each extract in 2 ml of methanol was subjected to brine shrimp lethality bioassay for testing the toxicity of the extracts. Three different sizes of each extract solution 500, 50, 5µl was transferred into vials corresponding to 1000, 100, and 10 µg/ml. Dimethyl sulphoxide (DMSO) drop was added to the test and control vials as a soluble catalyst. Then through pipette and by light source where the larvae were attracted, ten larvae was taken and placed in each of the previous vials, then the size was completed with Seawater to 5 ml. Values of LD50 were measured by analyzing the data using Probit analysis [16] on "Finney computer program." The dose at which it could kill 50% larvae (LD50) was determined (table1) [17-19].

RESULTS

Result of chromatographic analysis, antioxidant activity, phenolic, flavonoid compounds and cytotoxic activity of methanolic extract of *C.sinensis* root growing in Sudan are reported in the following tables and figures:

Table 1: The percentage yield, percentage of radical scavenging activity and cytotoxicity of *C. sinensis* root extracts:

NO.	Extract	Yield% (w/w)	%RSA ±SD (DPPH)	LD50	Result
1	Petroleum ether	0.14	10± 0.05	28993.117	Non-toxic
2	Chloroform	0.33	52± 0.03	24980.561	Non-toxic
3	Methanol	1.06	80± 0.02	7861.492	Non-toxic

Key: LD_{50} = Median Lethal Dose.

Table 2: Fractions collected from Column Chromatography of methanol root extracts

NO.	Solvent system	Ratio (%)	Fractions	Pooled fractions	Weight of pooled fractions (mg)
1	n-Hexane	100	F1- F21	PF1	8.9
2	n-Hexane: chloroform	90: 10	F22- F41+F45-F50	PF 2	23.9
3	n-Hexane: chloroform	80: 20	F31- F32	PF 3	7.7
4	n-Hexane: chloroform	70: 30	F42- F44+F51-F60	PF 4	26.6
5	n-Hexane: chloroform	60: 40	F61- F63	PF 5	35.9
6	n-Hexane: chloroform	50: 50	F64- F67	PF 6	133.0
7	n-Hexane: chloroform	40: 60	F68- F75	PF 7	47.6
8	n-Hexane: chloroform	30: 70	F76- F106	PF 8	19.7
9	n-Hexane: chloroform	20: 80	F107- F110	PF 9	26.1
10	n-Hexane: chloroform	10: 90	F111- F113	PF 10	31.8
11	Chloroform	100	F114- F116	PF 11	233.2
12	Chloroform: Methanol	90: 10	F117- F120	PF 12	124.5
13	Chloroform: Methanol	80:20, 70: 30	F121- F125	PF 13	182.2
14	Chloroform: Methanol	60:40, 50: 50,	F126- F134	PF 14	143.5
15	Chloroform: Methanol	40:60, 30: 70	F135- F143	PF 15	102.8
16	Chloroform: Methanol	20:80, 10:90, 0:100	F144- F175	PF 16	91.6

Table 3: Percentage of radical scavenging activity and IC_{50} Value for the fractions from *C. sinensis* methanol root extract:

Fractions of methanol root extract of *C. sinensis*	%RSA ±SD (DPPH)	IC_{50} ±SD mg /ml (DPPH)
1	20 ±**.0**03	-
2	21 ±**.0**04	-
3	03 ±0.04	-
4	12 ±**.0**04	-
5	08 ±0.03	-
6	08±0.01	-
7	72±0.01	0.123 ±0.05
8	66±0.04	0.184 ±0.07
9	70±0.01	0.137 ±0.03
10	32 ±0.02	-
11	32 ±0.02	-
12	41 ±0.09	-
13	16 ±0.09	-
14	14±0.04	-
15	10±0.02	-
16	23±0.09	-
Standard (Propyl Gallate)	91 ±0.01	0.077µg/ml± 0.01

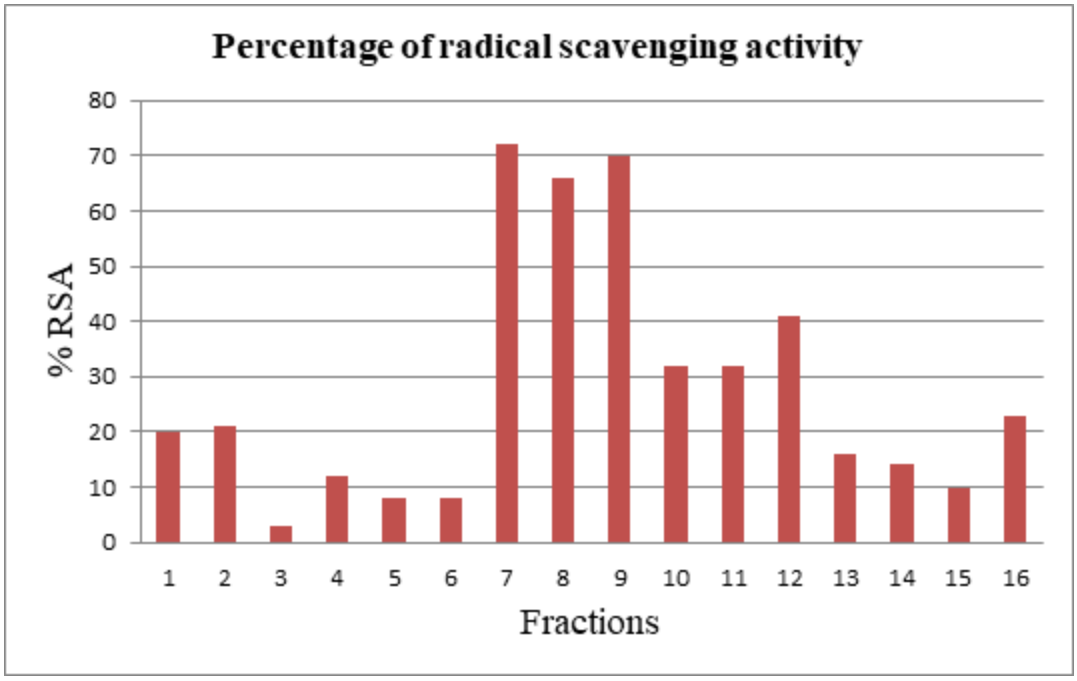

Figure 1: Percentage of radical scavenging activity for the fractions from *C. sinensis* methanol root extract.

Table 4: Gallic Acid (Standard) absorption:

Concentration (mg/ml)	Absorption (Mean) λ max=765 nm
10	0،028
20	0،038
30	0،058
50	0،085
100	0،149

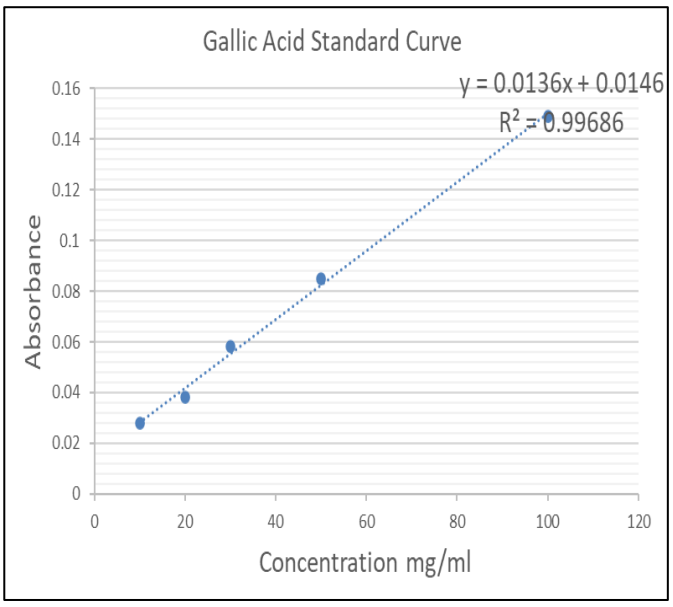

Fig 2: Standard curve of Gallic acid (TPC)

Table.**4** Shows the standard gallic acid absorbance in different concentrations.

Fig.2 shows the curve of the standard gallic acid and slope equation used to calculate phenolic content of the fractions

Table 5: Quercetin (Standard) absorption:

Concentration (mg/ml)	Absorption (Mean) λ max=510 nm
20	0،025
40	0،035
60	0،046
80	0،06
100	0،077

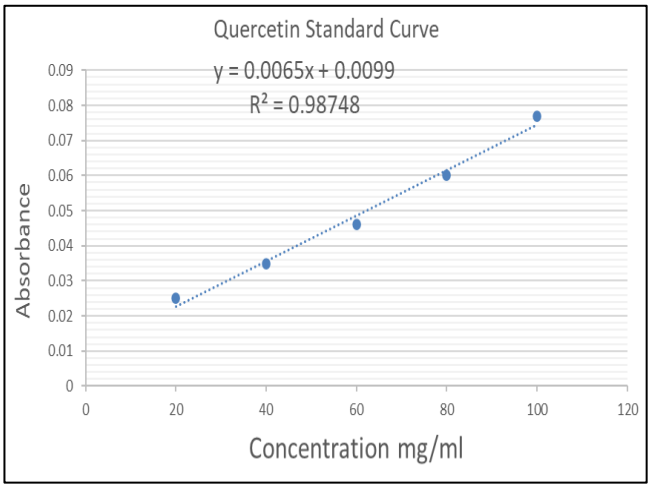

Fig 3: Standard Curve of Quercetin (TFC)

Table.5 Shows the standard quercetin absorbance in different concentrations

Fig.3 shows the curve of the standard quercetin and slope equation used to calculate flavonoid content of the fractions.

Table 6: Total phenolic, total flavonoid content and percentage of radical scavenging activity of the fractions from *C. sinensis* methanol root extract:

Fraction	Total Phenolic content (TPC), (mg GA*/g fraction)	Total Flavonoid content (TFC), (mg of Q**/g of fraction)	%RSA ±SD (DPPH)
3	1.343±.0091	9.469±2.184	03 ±0.04
5	2.359±0.097	14.641±3.872	08 ±0.03
7	9.807±0.093	29.821±1.776	72±0.01
8	6.807±0.337	30.000±3.997	66±0.04
9	7.641±0.293	32.128±0.444	70±0.01
11	3.511±0.093	19.359±0.444	32 ±0.02
13	3.023±0.088	12.734±1.663	16 ±0.09

The results represent Mean ± Standard Deviation. Each experiment was repeated three times; (n =3).

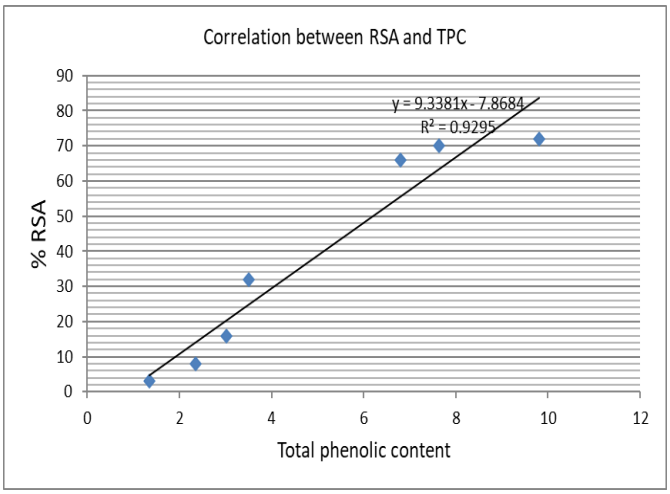

Fig 4: Correlation between radical scavenging activity and total phenolic content (TPC).

Key: RSA= Radicals scavenging activity**,** DPPH= 1,1-diphenyl-2-picryl hydrazyl. IC_{50}= half concentration of inhibition. The lower IC_{50} value indicates the greater overall effectiveness of the antioxidant. Control (PG) = Propyl Gallate.

Key: * Gallic acid (mg) equivalent of total phenolics content (GAE).

** Quercetin (mg) equivalent of total flavonoid content (QE).

DISCUSSION

The percentages yield of *C. sinensis* root extracts were appear in (Table 1) and methanol extract of *C.sinensis* showed the highest antioxidant activity (80± 0.02) among all the extracts.

Fractions (1-16) gained from the column of *C. sinensis* methanol root extract (Table 2) were examined for their *in vitro* antioxidant activity and were subjected for determination of IC50 values only if% inhibition was 50% or more at 0.5 mg/ml in the respective assay. Fractions (7-9) showed strong antioxidant activity. The results of DPPH of fractions are given in (Table 3).

Folin Ciocalteu's procedure was used to assess the total phenolic content of fractions of methanol root extracts using gallic acid as standard [13]. The phenols were oxidized by reducing the mixture of phosphomolybdic acid and phosphotungstic acid to the blue colored of molybdenum and tungsten oxides, where it was proportional to the total content of phenol when absorbed at the wavelength 765 nm. The gallic acid concentrations (10, 20, 30, 50 and 100 mg/ml) were measured at 765 nm to obtain the standard curve equation y = 0.0136x + 0.0146 (Fig. 2)

The total flavonoid content of fractions of methanol root extracts was estimated by the aluminum chloride colorimetric method using quercetin as standard. Different concentrations of the quercetin reagent (20, 40, 60, 80 and 100 mg/ml) were measured at 510 nm to obtain the standard curve equation y = 0.0065x + 0.0099 (Fig. 3).

The phenolic compounds are considered source of antioxidants and free radical scavengers which were showed in, (table 3) and (table 6); it is clearly observed that high correlation between antioxidant activity and the phenolic and the flavonoid content. The results also did not show any cytotoxicity against the brine shrimp (table 1). A remarkable correlation between phenolic content and antioxidant activity (Fig. 4) confirmed that phenolic constituent could be one of the main sources of the antioxidant agents of *C.sinensis* root.

CONCLUSION

This study confirmed that the methanol root extract of *C.sinensis* has high antioxidant, high phenolic and flavonoid content. The results also did not show any cytotoxicity against the brine shrimp. The high content of phenolic and flavonoid compounds of three fractions of methanol root extract of *C.sinensis* was the primary cause of the high antioxidant activity of these fractions. Therefore *C.sinensis* root is a reliable natural source of antioxidant compounds. The active constituents of column fractions could be isolated and identified by application of state-of the art apparatus in the Phytochemical field and could be of significance in human therapy. The presence of these compounds in friction

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