American Journal of Analytical Chemistry, 2016, 7, 254-259
Published Online March 2016 in SciRes. http://www.scirp.org/journal/ajac
http://dx.doi.org/10.4236/ajac.2016.73022

Study of Phytochemical Screening and Antimicrobial Activity of *Citrus aurantifolia* Seed Extracts

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Received 8 January 2016; accepted 12 March 2016; published 15 March 2016

Abstract

***Citrus aurantifolia*, the family Rutaceae, which consists of 150 genera and 900 species, is gaining grounds as important source for treatment in complementary medicine, and commonly called limone. Chloroform, methanol and ethanol extract exhibited significant antimicrobial activity and highlighted the biological monitoring of activity from the methanol extract of seeds. Phytochemical screening of the diethyl ether, methanol and aqueous extracts of seeds confirmed the presence of alkaloids, saponins, sterols and triterpenes, carotenoids, coumarins, tannins and carbohydrates. Chloroform extract was fractionated by sing liquid solid column chromatographic technique and column fractions eluted with chloroform and methanol. The antibacterial secondary metabolites were achieved by fractionation of the active. The seed extracts were monitored analytically by TLC preparative and some chemical analyses of seeds have been done such as determination of ash, nitrogen, protein content.**

Keywords

***C. aurantifolia*, Antimicrobial Activity, Limone, Phytochemical Screening, Liquid Solid Column Chromatographic**

1. Introduction

C. aurantifolia the family Rutaceae is rich in volatile oils and terpenoids, glycosides and alkaloids. Many of them are with remarkable antimicrobial activities. The seeds of *C. Aurantifolia* species showed a wide range of biological activities [1].

The total extracts of *C. aurantifolia* seeds were tested against Gram positive and Gram negative bacteria, and

How to cite this paper: Mohammed, R.M.O. and Ayoub, S.M.H. (2016) Study of Phytochemical Screening and Antimicrobial Activity of *Citrus aurantifolia* Seed Extracts. *American Journal of Analytical Chemistry*, **7**, 254-259.
http://dx.doi.org/10.4236/ajac.2016.73022

the three extracts of methanol, chloroform and ethanol exhibited significant activity and the methanolic extract gave the highest activity and was selected for further phytochemical investigations.

The results of antibacterial activity, phytochemical screening and chemical analysis of *C. aurantifolia* seeds are reported in the present work.

2. Materials and Methods

The *C. aurantifolia* seeds were collected from fruits purchased from the local market and were identified at the medicinal and Aromatic Plants Institute, the National Centre for Research, Khartoum, Sudan.

The following microorganisms were used for assessment of antimicrobial activity:

1) *Bacillus subtilis* (Gram positive) NCTC 8236.
2) *Staphylococcus aureus* (Gram positive) ATCC 25923.
3) *Escherichia coli* (Gram negative) ATCC 25922.
4) *Proteus vulgaris* (Gram negative) ATCC 6380.
5) *Klebsiella* spp. (Gram positive) ATCC 53657.
6) *Shigella* spp. (Gram positive) NCTC 4837.

Media: Agar, Nutrient Borth**.**

2.1. Qualitative Analysis of Chemical Constituents

The diethyl ether, methanol and water extracts were used for the phytochemical screening.

2.1.1. Preparation of Extracts

Twenty five gram of powdered seeds were extracted separately in a Soxhlet apparatus and after removal of solvents. The percentage yields were determined the extracts were used for phytochemical screening, antimicrobial activity, chromatographic analysis for isolation and identification of active constituents [2].

2.1.2. Sterols and Triterpenes

The diethyl ether extract (10 ml) was dissolved in 0.5 ml acetic anhydride, chloroform (0.5 ml) and concentrated sulphuric acid (2 ml) was placed at the bottom of the test tube by means of a pipette [2].

2.1.3. Carotenoids

The ether extract (10 ml) was evaporated to dryness and saturated solution of antimony trichloride in chloroform (3 drops) was added (Carr price's reactions) [2].

2.1.4. Coumarins

3 ml of extract added water. After cooling was divided is two test tubes contained the reference, and the aqueous solution of the second tube was made alkaline with 0.5 ml of ammonia solution (10%) [2].

2.1.5. Tannins

The methanol and water extracts (0.5 - 1 ml) were diluted water (1 - 2 ml) and 2 - 3 drops of diluted solution of ferric chloride were added [2].

2.1.6. Reducing Sugars

The methanol extract (1 ml) was diluted with distilled water (1.2 ml) and Fehling's (1 and 2) reagent (0.5 - 1 ml) solutions were added and heated [2].

2.1.7. Alkaloid Salts

20 ml of the methanol extract were transferred to a beaker and evaporated on boiling water-bath. 5 - 10 ml of hydrochloric acid (10%) were added to the residue. The alkaloids became salts of mineral acids from the aqueous solution. The alkaloids were liberated as free bases with the help of ammonia solution (10%) and then extracted with ether or chloroform. The solution 1.5 ml 2% hydrochloric acid solution. The acid solution was then divided into three test tubes: one was the reference and in the other two test tubes, 2 - 3 drops of Mayer's or Bertrand's reagent were added [2].

2.1.8. Saponins

2 ml of the aqueous extract added 2 ml of distilled water and shaken, vigorously [2].

2.2. Chromatographic Analysis

The extracts were screened in different solvent systems by thin layer chromatography and the active extracts were fractionated by liquid solid chromatography.

2.2.1. Column Chromatography

The column was packed with about 100 g of silica gel using the wet packing procedure with chloroform. The three grams of chloroform extract were placed on top of column and eluted with the solvent chloroform, followed by increasing amounts of methanol in chloroform and finally with pure methanol [3].

2.2.2. Thin Layer Chromatography

The compositions of fractions collected from the column were monitored by the following solvent systems:

Chloroform/methanol (8.5:1.5) and

Toluene/ethyl acetate/formic acid (5:4:1).

The fractions were further purified by preparative TLC and detection with UV-light and 1% vanaillin in conc. sulphuric acid [3].

2.3. Assessment of Antimicrobial Activity

Microbiological techniques followed were those described by Atlas [4] and [5].

2.4. Organic Matter

Two-grams of each sample were put in a previously weighed porcelain crucible. The crucible was placed in a muffle furnace at 550˚C for six hours, transferred to a desiccator and then weighed. The ash weight was determined and its percentage was calculated as follows:

Weight (g) of (crucible + sample) before ignition = x

Weight (g) of (crucible + sample) after ignition = y

Organic matter% = [(x − y)/(Sample weight (g))] × 100

2.5. Nitrogen and Crude Protein Content

2.5.1. Nitrogen

The procedure used is a two-stage process in which the released gum samples are digested in hot concentrated sulfuric acid and the ammonia using sodium hydroxide, is neutralized using standard acid (AOAC; 1984) [6].

A. Digestion

Sample + (H_2SO_4 conc. + catalyst) → $(NH_4)_2SO_4$

$(NH_4)_2SO_4$ + (1) Alakali (2) steam → $2NH_3 + H_2BO_3$

B. Neutralization

$NH_3 + B_3BO_3 \rightarrow NH_4 + H_2BO_3$

The borate anion equivalent to the ammonia produced is back titrated with standard HCl (0.02 mol·dm^{-3}).

$B_2BO_3 + HCL \rightarrow H_3BO_3 + Cl^-$

Accurately weighed 0.2 g of gum sample, One gram of powdered was added to mixture of potassium sulfate and cupric sulfate (10:1). The contents transferred to a steam distillation unit, 20 ml of 40% sodium hydroxide solution were added, and distillation was carried out with steam. The distillate was collected in 10 ml of boric acid solution (2%) to which methyl red indicator were added and titrated against O.OIN-BCL. The same procedure was carried out for a blank.

$$N\% = \frac{(M_1 - M_2) \times N \times 14}{S.W \times 1000 \times 100}$$

where:

M_1: ml of HCl that neutralized the sample distillate.

M_2: ml of HCl that neutralized the blank distillate.
N: the normality of HCl titrate (0.01).
14: each ml of HCl is equivalent to 14 mg nitrogen.
$S.W$: sample weight (0.2 g).
1000: conversion factor from gram to mg [6].

2.5.2. Protein

Crude protein % = N% × 6.6 [6].

3. Results and Discussion

The extract of *C. aurantifolia* seeds with chloroform, methanol and ethanol was screened against Gram positive and Gram negative bacteria. The antimicrobial activity was significant and gave grounds to the extract in **Table 1**. Phytochemical screening of extracts is presented in **Table 2**.

Table 1. Results of antimicrobial activity of the chloroform, methanolic and ethanolic extracts of *C. aurantifolia* seeds.

Organism	Concentration (mg/ml)	Inhibition zone (mm) of extract		
		96% ethanol	Chloroform	Methanol
Bacillus subtilis	1.00	24	18	24
	0.75	22	17	22
	0.50	16	15	20
	0.1	11	11	14
Staphylococcus aureus	1.00	28	15	35
	0.75	28	20	20
	0.50	16	15	25
	0.1	12	12	15
Escherichia coli	1.00	20	13	20
	0.75	18	17	23
	0.50	14	14	18
	0.10	11	13	11
Proteus vulgaris	1.00	22	20	27
	0.75	18	18	18
	0.50	18	15	20
	0.10	12	13	14
Klebsiella spp.	1.00	15	14	16
	0.75	14	15	18
	0.50	14	13	15
	0.1	10	11	11
Shigella spp.	1.00	20	14	20
	0.75	17	13	11
	0.50	12	12	15
	0.1	10	11	11

The chloroform extracts of *C. aurantifolia* seeds fractionation by using a column packed with silica gel and elution with chloroform and methanol. Biological monitoring of activity gave several active fractions of six fractions which have been isolated in a pure state by preparative TLC. Fraction 1 and 2 elution were with chloroform pure, fraction 3 elution by chloroform:methanol (95:5), fraction 4 elution by chloroform:methanol (90:10), fraction 5 elution by chloroform:methanol (75:25) and fraction 6 elution by chloroform:methanol (50:50). The result was shown in **Table 3**.

Chemical analyses of the seeds have been done such as determination of ash content (0.27%), nitrogen content (3.22), and protein content (21.25%). All trials were restored three times, according to the average result in **Table 4**.

In **Figure 1**, TLC of six fractions chloroform extract of *C. aurantifolia* Stationary phase Silica gel 60 precoated plates, thickness 20 mm the mobile phase by used N-butanol:glacial acidic acid:water (5:4:1) Detection

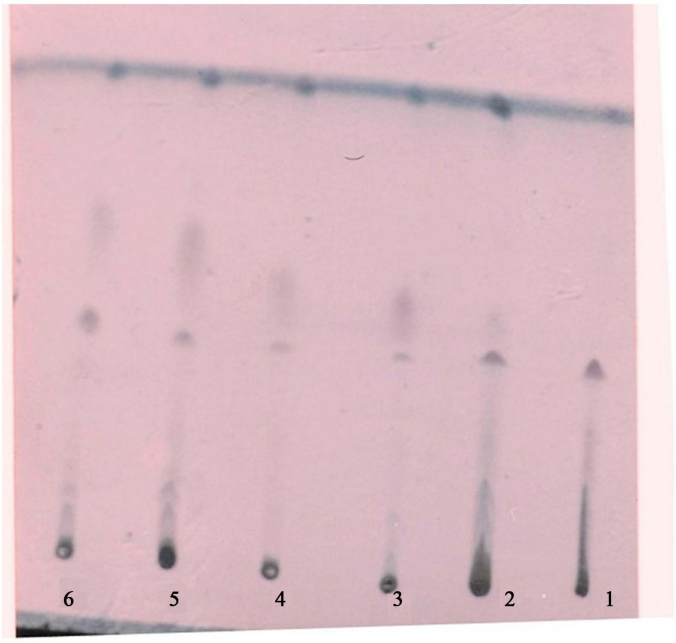

Figure 1. TLC of column fractionation of the chloroform extract of *C. aurantifolia* seeds: Stationary phase = Silica gel 60 precoated plates, thickness 0.20 mm. Mobile phase: Toluene/ethyl acetate/formic acid (5:4:1). Detection: UV-light (366 nm); 1% vanillin in conc. H_2SO_4.

Table 2. Results of the general phytochemical screening of the *C. aurantifolia* seeds.

Class of compound	Extract		
	Diethyl ether	Methanol	Water
Sterols and triterpenes	+	−	−
Carotenoids	+	−	−
Coumarins	−	+	+
Tannins	−	+	+
Reducing sugars	−	−	+
Alkoloidal salts	−	−	+
Saponins	−	−	+
Carbohydrates	−	−	+

Positive result: (+); Negative result: (−).

Table 3. Evaluation of antimicrobial activity of column fractionation of the chloroform extract of *C. aurantifolia* seeds.

Organism	Fraction/inhibition zone (mm)					
	1	2	3	4	5	6
Bacillus subtilis	–	–	12	11	20	20
Staphylococcus aureus	–	–	13	–	13	20
Escherichia coli	–	–	35	–	16	18
Proteus vulgaris	–	–	15	–	14	45
Shigllia sp.	–	13	50	12	14	14

Table 4. Chemical analysis of *C. aurantifolia* seeds.

Item	Content (%)
1-Ash	0.27
2-Nitrogen	3.22
3-Protein	21.25

by UV-light (366 nm) and 1% vanillin in conc. H_2SO_4.

4. Conclusion

Some recent studies reported important uses of seed extracts of *C. aurantifolia* in complementary medicine and we recommend more studies on the seeds composition of the Sudanese varieties.

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