Journal of Materials and Environmental Science
ISSN : 2028-2508
CODEN : JMESCN

J. Mater. Environ. Sci., 2021, Volume 12, Issue 7, Page 974-983

http://www.jmaterenvironsci.com

Antimicrobial Activity of Xanthotoxin Isolated from *Ruta montana* L. Extract and Effect of Harvesting Time on its Content

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Received 25 Aug 2020,
Revised 07 July 2021,
Accepted 10 July 2021

Keywords
- ✓ *Ruta montana* L.,
- ✓ Xanthotoxin,
- ✓ Furocoumarin,
- ✓ Phytoscreening,
- ✓ Antimicrobial activity.

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Abstract

The present study focused on the phytochemical screening of Algerian *Ruta montana* L. and the evaluation of the antimicrobial activity of xanthotoxin after its isolation from their apolar extract. The results showed that *Ruta montana* L. methanolic extract contained flavonoids, alkaloids, tannins and coumarins; however, the apolar extract was very rich only in coumarins. In this context the isolation and purification of xanthotoxin from *Ruta montana* L. harvested during different seasons was released; it was noted that the highest yield of xanthotoxin (0.45%) was obtained from the plant harvested at the flowering stage (in August); while the lowest yield (0.12%) was obtained in winter. The evaluation of the antimicrobial activity of xanthotoxin was performed by disc diffusion method against three bacterial strains and four fungi after confirmation of its chemical structure on the basis of melting-point and spectroscopic analysis (UV, FTIR, ^{1}H NMR, ^{13}C NMR and 2D NMR). The results of the antimicrobial activity, showed the efficacy of xanthotoxin as an antimicrobial agent from 50 mg against *Staphyloccocus aureus*, *Bacillus subtilis* and *Candida albicans*; however, this product has been shown to be effective against *Pseudomonas aeruginosa* and *Aspergillus* fungi only from 80 mg. The largest inhibition diameters (15-28 mm) were obtained against the fungus *Candida albicans*.

1. Introduction

Furocoumarins are a class of chemical compounds with phototoxic properties, synthesized by plants through the fusion of coumarin to a furan ring; they are found naturally in many plant species, including the *Umbelliferae*, *Rutaceae*, *Leguminosae* and *Mimosae* [1]. Depending on the position of the furan ring, we obtained linear (Psoralen and derivatives) or angular (Angelicin and derivatives) furocoumarin isomers [2], with linear furocoumarins predominating in natural sources [3].Psoralen and its derivatives (linear furanocoumarins) are used as antibacterial [4], antioxidant [5],anticonvulsant [6]and selective anticancer agents, prompting a biological investigation to determine and predict their clinical therapeutic significance [7,8] and they are also very promising in the treatment of other diseases [9]. These secondary metabolites occur naturally in plants, in response to stress and to defend against predators such as fungi, bacteria, and insects [10-13].

Ruta chalepensis, *R. angustifolia*, *R. graveolens* and *R. montana* (*Rutaceae*) have been studied to evaluate their potential for the production of furanocoumarins (psoralen, xanthotoxin, bergapten,

isopimpinellin) [14-18] and dihydrofuranocoumarins (Rutamarin) [19]. The *Ruta* species contained from 4 to 17 mg. g^{-1} dry weight of furanocoumarins; these concentrations were higher than those found in other families known to produce the same compounds (*Moraceae*, *Apiacae* and *Fabaceae*) [20]. *Ruta montana* is a common specie of the genus *Ruta* (Rutaceae) very widespread in the Mediterranean region. Phytochemical screening of *Ruta* species showed the presence of alkaloids, flavonoids, coumarins, tannins, volatile oil, glycosides, sterols and triterpenes as possible active constituents [21, 22].
In this context xanthotoxin was isolated from the *Ruta montana* L. areal parts harvested from Algeria and the effect of season on yield was studied. The isolated xanthotoxin was identified by different spectroscopic methods, and its antimicrobial activity was evaluated against three microbial and four fungi strains.

2. Material and Methods

2.1. Plant and materials

Petroleum Ether 40-60 °C (95%), Methanol (99.7%), Ethanol (98%) were purchased from Sigma-Aldrich (Germany) and dimethyl sulfoxide (99%) from Panreac. The aerial part of *Ruta montana* L. was collected from Setif region (North-East of Algeria) during different seasons in (2017- 2018) (Figure 1). The plant was dried at room temperature and identified at the process Engineering Department, Blida-1 University (Blida, Algeria).

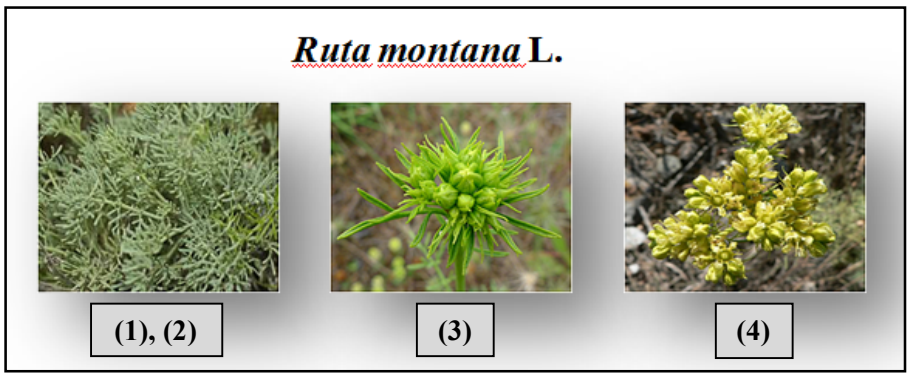

Figure 1: *Ruta montana* L. harvested in December, February (1,2); April (3); August (4).

2.2. Extract's preparation

The air-dried aerial parts of the plant were grounded and extracted successively with petroleum ether (PE) and Methanol using a soxhlet apparatus. All the extracts were separately concentrated using a rotary evaporator.

2.3. Phytochemical screening

Polar and apolar *Ruta montana* extracts were subjected to various phytochemical tests in order to detected the different secondary metabolites present by coloring reactions, precipitation and observations under ultraviolet light.

- Test for alkaloids: 0.5 mL of Mayer's reagent was contacted with 0.5 mL of each extract. The formation of a white or brown precipitate indicates the presence of the alkaloids [23].
- Test for flavonoids: The presence or absence of flavonoids in the extracts was revealed by magnesium test [23]. In a test tube, 1 ml of concentrated HCl was added to 1 ml of the extract, followed by few magnesium turnings. Flavonoids are present if red, orange or pink color appears.

- Test for tannins: 2 ml of each extract was added to 0.5 ml of $FeCl_3$ (1%). The presence of tannins has been revealed by the appearance of a greenish or blue-black color after incubation for 15 min at room temperature [24].
- Test for coumarins:5 ml of each extract dissolved in 1-2 ml of hot distilled water was divided into two parts. Half of the volume was taken as a witness and 0.5 ml 10% NH_4OH was added to the other volume; then from each preparation a spot was put on a filter paper and examined under UV light. The presence of coumarins was indicated by the appearance of intense fluorescence [24].

2.4. General procedures

UV spectrum of xanthotoxin was recorded in methanol with Shimadzu UV-1800 spectrophotometer, and the FT/ IR was registered on BRUKER FT/IR spectrophotometer. The ^{1}H NMR, ^{13}C NMR and 2D-NMR spectra were recorded on a Bruker-Ascendi 400MHz spectrometer in the DMSO-d_6 (Sigma-Aldrich, Germany). Melting points were determined by Fisher- Johns melting point apparatus. Thin layer chromatogram (TLC) was run on plates coated with silica gel (Kieselgel G Merck) and developed with ethyl acetate / cyclohexane (7:3) at ambient temperature.

2.5. Antimicrobial activity

The antimicrobial activity of xanthotoxine was evaluated against three bacterial: *Escherichia coli* (ATCC 10536), *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATTC 2785*)* and four fungi strains: *Candida albicans* (ATCC 10231), *Aspergillus fusarium* (ATTC 4620), *Aspergillus niger* (ATTC 1015) and *Aspergillus brasiliensis* (ATTC 16404) by the disk diffusion method. Solutions of 50, 80, 100 and 150 mg of xanthotoxin in DMSO were prepared and sterilized disks of 6 mm diameter on Whatman paper impregnated with each of these solutions have been deposed on the surface of the agar plates with microorganisms suspension. The inhibition diameters were measured after incubation at 37 °C for 24 and 48 h.

3. Results and discussion

3.1. *Yield and properties of extracts*

The average yields of the polar and apolar extract of *Ruta montana* L. were calculated after evaporation of the solvents. It was noted that extraction with methanol provided a dark extract with an average yield of (13.40%), higher than the dark green extract obtained with petroleum ether (0.60%).

3.2 Phytochemical study

Phytochemical tests of the *Ruta Montana* L. extracts were carried out using specific reagents, discoloration, precipitation reactions and observations under ultraviolet light. The results are reported in Table 1.

Table 1: Phytochemical screening of the *Ruta Montana* L. extracts.

	Alcaloïdes	Flavonoïds	Tanins	Coumarins
Polair extract	++	++	+	++
Apolair extract	-	-	-	+++

Key: +++ (high), ++ (low), + (very low)

The results of the phytochemical tests carried out, showed the presence of different secondary metabolites in this plant. The results obtained revealed the presence of three chemical families in the polar extract, alkaloids, polyphenols (flavonoids and tannins) and coumarins. However, we observe the absence of alkaloids and polyphenols in the apolar extract; on the other hand, coumarins were present in greater quantity in the apolar extract compared to the polar; it was reported by authors that the methanol extract of *Ruta* is richer in secondary metabolites [25].

3.3. Isolation of xanthotoxin and yield

Xanthotoxin was isolated from the apolar extract of *Ruta montana* L. harvested during different seasons. A white precipitate has been crystallized in the apolar extract of *Ruta montana* after cooling the mixture (petroleum ether and extract); the product was filtered, recrystallized from ethanol, the yield was calculated and the product was characterized by the different spectroscopic methods.

Xanthotoxin was obtained with a low yield which varies between 0.12 and 0.45%. The variation of yields as a function of the harvest period represented in figure 2 show that the highest yield (0.45%) was obtained from the plant harvested in August, at the flowering stage; while the lowest yield (0.12%) was obtained in winter.

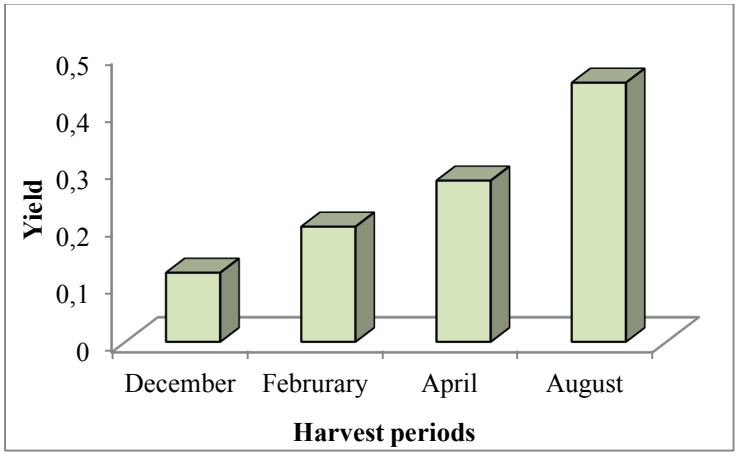

Figure 2: Representation of xanthotoxin yields according to the harvest periods.

Generally, the content of furocoumarins is a function of the sanitary and physiological state of the plant, in particular in certain *Umbelliferae*. It was recorded that parsley (*Petroselinum sativum*) total contents in furocoumarins varies from 20 to 200 ppm depending on the harvest month; Plants harvested in autumn and having undergone the high summer temperatures had the highest concentrations, showing in fact that furocoumarins are produced more intensively when the plant is subjected to certain stressors (water deficit) [26].

Chaudhary et al., [27] recorded levels of total furocoumarins up to 5 times higher for *Apium graveolens* (celery) infested with *Sclerotinia* compared to healthy individuals. The same phenomenon was observed in the parsnip, *Pastinaca sativa* (Johnson et al., 1970) attacked by *Ceratocystis*. It seems that linear furocoumarins (psoralens, 5MOP, 8MOP and 5-8MOP) are particularly involved in the defense of the plant against certain pathogens in *Umbelliferae* and therefore play the role of phytoalexins [10, 28].

3.4. Characterization of xanthotoxin

Xanthotoxin (Figure 3) crystallized as a colorless powder with a melting point of 145 to 147 ° C andR_f $CH_3COOC_2H_5$/ C_6H_{12} (7 :3 V/V)=0,46.

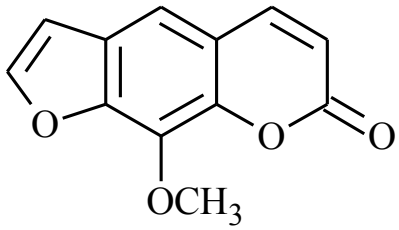

Figure 3: Chemical structure of Xnathotoxin.

In general, coumarins presented UV-Vis spectra with 4 maximum at 210-350 nm [29, 30], therefore the UV-vis spectrum of isolated xanthotoxine, dissolved in methanol (figure 4) characterized by four bands at λ = 205, 244, 293 and 333 nm, indicates the presence of the coumarin ring.

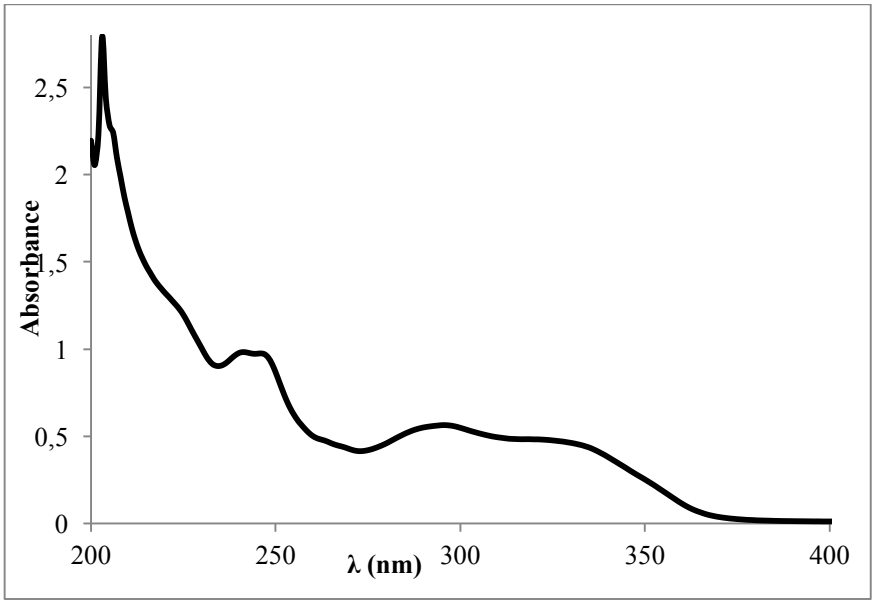

Figure 4: UV-Vis spectrum of xanthotoxin.

The infrared spectrum of xanthotoxin represented in figure 5 is characterized by the presence of a characteristic peak of the =CH aromatic group at 3117 cm^{-1} relative to the benzene ring; the presence of a strong absorption bond at 1705 cm^{-1} is relative to the carbonyl group C = O of the pyrone-carbonyl (α-pyrone), while the strong absorption at 1681 cm^{-1} is due to the frequency of stretching of the double bond C = C of the pyrone nucleus. The two peaks at 1332 and 1150 cm^{-1} are considered to be characteristic to the stretching vibrations of the C-O bond of furan and that of the coumarin ring.The H^1 NMR spectrum of xanthotoxin defined 8 protons. The two doublets at δ: 6.42 and 8.15 ppm (J = 9.5 Hz) are typical of coumarin nucleus in the pyrone ring, and are attributed to the protons H-3 and H-4 of this ring. This is further corroborated by the presence of a singular to a proton at 7.65 relative to the proton H-5. The signal made up of three protons at 4.20 ppm is assigned to a methoxy group located on C-8. The two doublets at 7.15 and 8.13 (J = 2.5 Hz) are typical of furanocoumarins with the benzene ring substituted ortho for furan oxygen and are attributed to the protons H-4 'and H-5' of this ring. The signals obtained were practically identical to those obtained by other researchers [31, 32].

The exact chemical structure of xanthotoxin has been confirmed by the HSQC NMR spectrum (Figure 6) which allows the hydrogen to be assigned to the carbons which are directly linked.

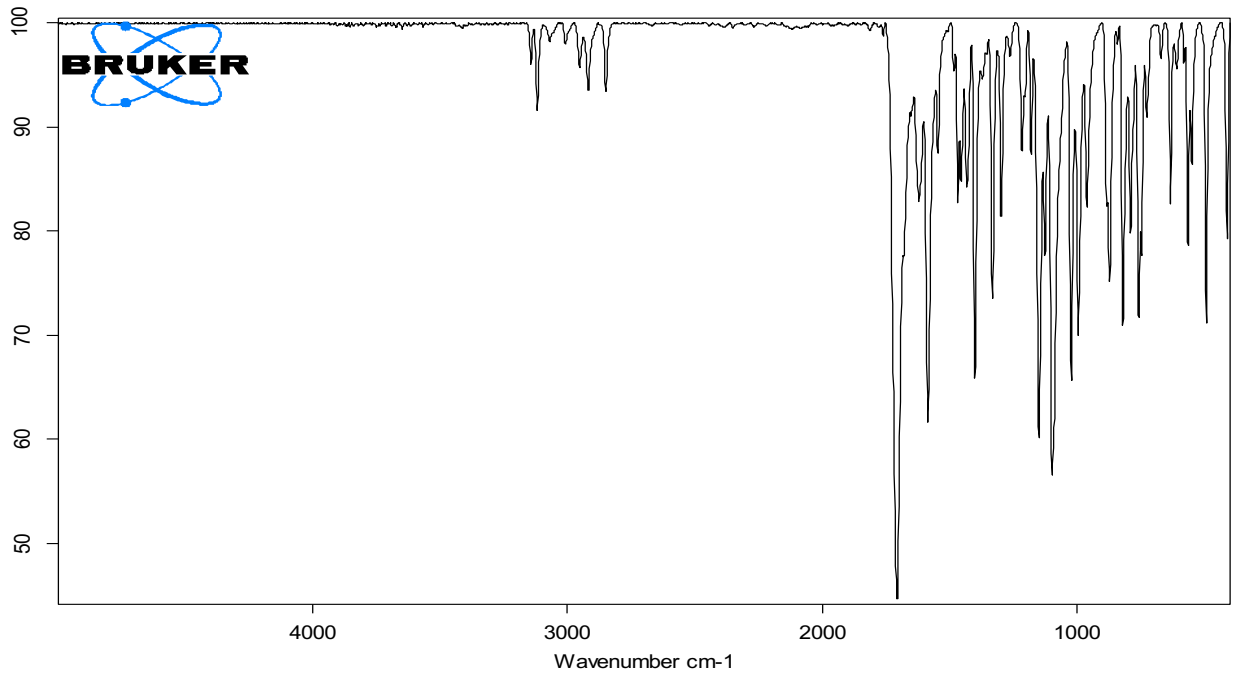

Figure 5: FTIR spectrum of xanthotoxin.

RMN H[1] ($CDCl_3$,400 MHz) δ/ppm: 4.20 (s, 3H, OCH_3), 6,42 (d, *J*=9,5 Hz, 1H, H-3), 7,15 (d, *J*=2,0 Hz, 1H, H-4'),7,65 (s, 1H, H-5), 8,13 (d, *J*=2,0 Hz, 1H, H-5'), 8,15 (d, *J*=10,0 Hz,1H, H-4).
RMN C[13] δ/ppm: 61.58 (9-OCH_3), 107.72 (C-4'), 114.01 (C-5), 114.64 (C-3), 116.92 (C-6), 125.96 (C-4a), 132.51 (C-8), 142.26 (C-8a), 145.72 (C-4), 147.32 (C-2), 147.89 (C-5'), 160, 18 (C-7).

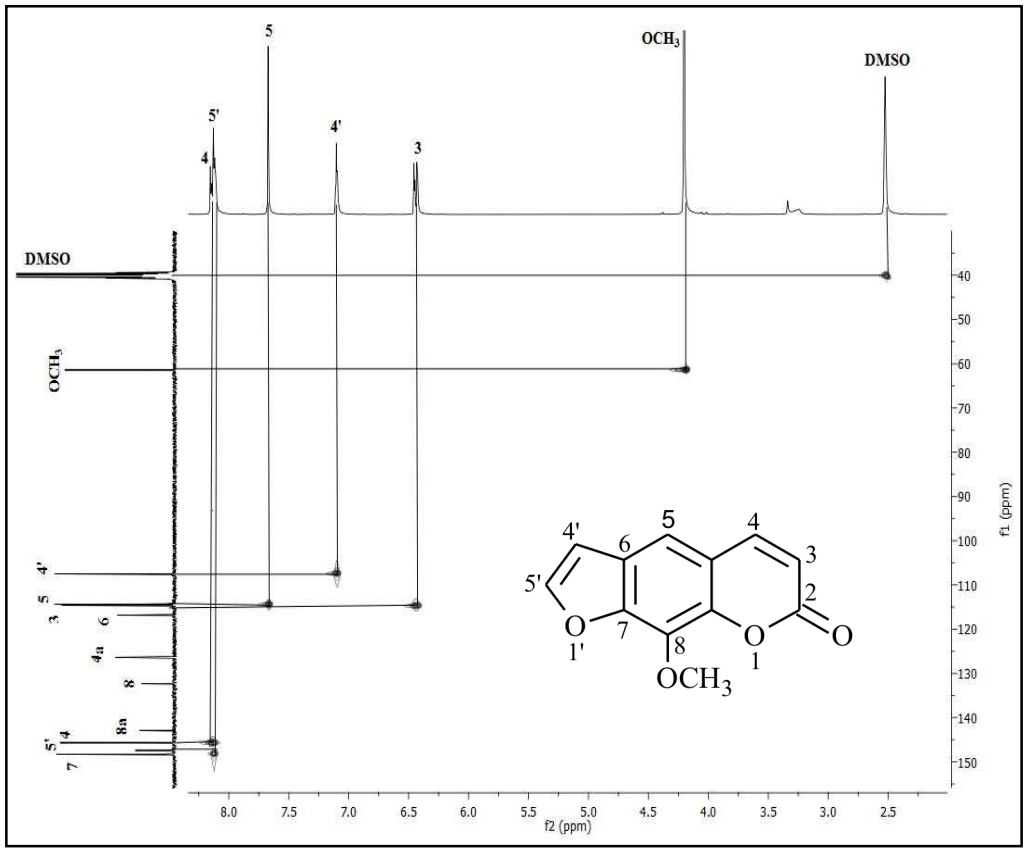

Figure 6: NMR HSQCS spectrum of xanthotoxin.

3.5. Antimicrobial Activity

The results of the antimicrobial activity of xanthotoxin represented in Table 2 show the efficacy of xanthotoxin as an antibacterial against the bacteria *Staphylococcus aureus* and *Bacillus subtilis* from 50 mg and against *pseudomonas aeruginosa* from 80 mg, with inhibition diameters between 11-33 mm. The inhibition diameters increase with increasing concentrations. Fungal growth was significantly inhibited against all *Aspergillus* fungi tested from 80 mg, and from 50 mg against the yeast *Candida albicans,* where the largest inhibition diameters were (15-28 mm). These results are comparable with those obtained with antibiotic references.

Coumarins are high-value secondary metabolites with a broad spectrum of pharmacological properties [33, 34]. Depending on their structure, some coumarins exhibit strong antibacterial and antifungal activity [35, 36, 37]; xanthotoxin and other furocoumarins isolated from *Heracleum mantegazzianum* Bed base & Lever (*Umbelliferae*) have shown strong antimicrobial activity [38].

Table 2: Antimicrobial Activity of xanthotoxin.

Microorganisms	**Xanthotoxin**				**Cephalexine**	**Flazol**
Bacteria	**50 mg**	**80 mg**	**100 mg**	**150 mg**	**20mg**	**20mg**
Staphyloccocus aureus	11	12	20	24	10	12
Bacillus subtilis	16	20	25	33	22	13
Pseudomonas aeruginosa	-	13	18	25	16	07
Fungi						
Aspergillus niger	-	11	14	16	15	10
Aspergillus flavus	-	16	18	22	12	15
Aspergillus Brasiliensis	-	12	15	18	14	10
Candida albicans	15	18	22	28	-	07

Conclusion

The phytochemical screening of the *Ruta montana* L. areal parts revealed that the methanolic extract contain tannin, phenolic compounds, alkaloids and coumarins; on the other hand the apolar extract (from petroleum ether) showed the presence of only coumarins in high contents; from this extract, xanthotoxin was isolated and identified with different spectroscopic methods.

Our results show that the major factor influencing furocoumarins contents, specifically xanthotoxin is the harvest period of the plant; where the highest yield (0.45%) was obtained from the plant harvested in August, while the lowest yield (0.12%) was obtained in December.

The efficacy of xanthotoxin as antimicrobial agent was observed from 50 mg against *Staphyloccocus aureus*, *Bacillus subtilis* and *Candida albicans*; and from 80 mg against *Pseudomonas aeruginosa* and *Aspergillus* fungi; this activity is linked to their photosensitive properties

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