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**Original Article**

# Preliminary Phytochemical Analysis and Antibacterial Activity of Methanol Extracts from *Origanum majorana*, *Rumex nervosus*, and *Withania somnifera*

Jameel M Abduljalil [1,*], Asmaa A AL-Rakhami [2], Thabet M AL-Haj [2], Ali M AL-Rrimy [2], AmatAllah S AL-Wheabi [2]

[1] Microbiology Section, Department of Biological Sciences, Faculty of Applied Sciences, Thamar University, Dhamar, Yemen.
[2] Biochemistry Technology program, Department of Chemistry, Faculty of Applied Sciences, Thamar University, Dhamar, Yemen.

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ABSTRACT

**Introduction and aim:**Most of today's drugs are plant-derived natural products or their derivatives. The objectives of this study were to carry out a primary screening for the phytochemicals and antibacterial activity of methanol extracts of *Origanum majorana*, *Rumex nervosus*, and *Withania somnifera*. **Methods**: Three medicinal plants were collected from Dhamar during April 2018. Primary phytochemical analysis was performed by classical chemical assays.The antibacterial activity of the extracts was evaluated against three opportunistic pathogens; *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* by the disk diffusion agar assay. Synergy was investigated by combining extracts with standard antibiotic disks. **Results**: Steroids, saponins, tannins, glycosides, and anthocyanins were detected in all extracts. Meanwhile, alkaloids and anthraquinones were absent. Phenols and flavonoids were also undetected in the extracts of *W. somnifera* and *R. nervosus* respectively. In terms of antibacterial activity,*W. somnifera* was the most active extract against *S. aureus* and *E. coli*with inhibition zone diameters range from 17 mm to 24 mm at 2 mg/disk. Extracts of *O. majorana* and *R. nervosus* showed no activity against the challenged bacteria. In terms of synergy, extracts of *O. majorana* and *R. nervosus* enhanced the activity of chloramphenicol against *S. aureus* while antagonistic effect was observed when extract of *R. nervosus* combined with fluoroquinolones against *E. coli* and *P. aeruginosa*. **Conclusions**: Only the extract of *W. somnifera* showed good antibacterial activity against the tested bacteria.According to the literature, it is most likely that this is the first report on synergy testing between standard antibiotics and extracts of these medicinal species.

**Keywords:** Methanol extract, *Origanum majorana*, *Rumex nervosus*, *Withania somnifera*, Antibacterial activity.

**Corresponding author ***
Jameel M Abduljalil
Microbiology Section, Department of Biological Sciences, Faculty of Applied Sciences, Thamar University, Dhamar, Yemen.
E mail: Jameel.abduljalil@gmail.com

## 1. INTRODUCTION

Plants have been exploited since ancient history for curative purposes. Nowadays, numerous plants continue to be valuable sources of tremendous amounts of compounds used in medicine[1]. In Yemen, indigenous people have a strong tradition to use medicinal plants for health promotion and treatment of infectious diseases without a scientific background regarding their effects. Other non-infectious diseases and medical conditions such as poisoning (by snakes' bites and scorpions), joints inflammation, gastrointestinal tract disturbance, and even gynecologic conditions are also treated[2].

*Origanum majorana*(English name is Marjoram) (Lamiaceae) is an aromatic plant native to the Mediterranean region and known popularly named "Bardagoosh"[3]. Nowadays, research is investigating the potentials to marjoram essential oil for antimicrobial activity against food-spoilage bacteria and fungi[3-5]. Indeed, numerous reports have found promising results in this field[6, 7]. *Rumex nervosus* (Polygonaceae) is a flowering species native to Arabian Peninsula (known as Othrob) and some African regions[2]. The phytochemical studies on *R. nervosus* are rare and much is still unknown about its chemically active constituents due to its limited distribution. However, its antimicrobial activities have been reported decades ago[8-10].*Withania somnifera* (Solanaceae) is a xerophytic short shrub commonly found in Africa, India, and the Mediterranean under many names such as Ashwaganda, Winter cherry, and Obab[11].*W. somnifera* is a rich source of bioactive secondary metabolites of diverse medicinal properties[12]. In terms of antimicrobial activities, withanolides and flavonoids from *W. somnifera* reported to have significant antibacterial activities[13-17].

Emergence of multi-drug resistant bacteria is one of the biggest concerns in today's world owing to the adverse impacts on the outcome of antimicrobial chemotherapy[18]. As a consequence; screening for novel antimicrobial agents from natural sources is an urgent and promising field to counteract the problem of drug resistance. In the present study, three medicinal plants were collected from Dhamar governorate (Yemen) for preliminary phytochemical analysis and antibacterial activity against three opportunistic pathogens; *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*. Synergistic interactions with a panel of standard antibiotics were also evaluated.

## 2. MATERIALS AND METHODS

### Plant Materials

The healthy aerialparts of *R. nervosus*, and *O. majorana* were collected from Rakhamah village, while*W. somnifera* were collected from Thamar city (Dhamar, Yemen)in April 2018. The identities of the plants were confirmed in Authority of Agricultural Researches, Dhamar.Aerial parts were washed aseptically and dried under shade for five to eight days.

### Tested Bacteria

Bacterial isolates of *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* were obtained from clinical specimens and identified according to standard morphological and biochemical criteria[19] in Microbiology laboratory, Faculty of Applied Sciences, Thamar University.

### Antibiotics

A set of standard disks was chosen for testing their interactions with plant extracts against the challenged bacteria. Amikacin (30 µg), cefotaxime (30 µg), sparofloxacin (5 µg) were purchased from Beacon, India. Amoxicillin (30 µg), chloramphenicol (30 µg), gentamicin (10 µg), ciprofloxacin (5 µg), tetracycline (30 µg), erythromycin (15 µg) were products of HiMedia, India. The laterthree antibiotics were tested only against *P. aeruginosa*.

### Preparation of Plant Extract

Absolute methanol was used as a solvent for extraction. The extract was filtered using Whatman filter paper (No 3) and concentrated at 25° C using a rotary evaporator. Concentrated extracts were stored in dark containers inrefrigerator at 4° C.

#### *Origanum majorana*

Dried powdered leaves and stems (100 g) were macerated with 700 mL of 99.5% methanol for 72 h at room temperature in a dark glass tightly closed bottle[20]. Commercially available oil of marjoram used in the antibacterial assay was purchased from YaseenSpices Co.,Sanaa,Yemen.

#### *Rumex nervosus*

Extraction was performed as described previously[21]. Briefly; 1100 mL of 99.5% methanol was added to 184g of dried leaves in a dark glass bottle and left at room temperature for five days.

#### *Withania somnifera*

Two hundred grams of leaves and stemswere macerated in a dark glass bottle with 900 mL of methanol and left to stand for 24 hours at room temperature[22].

### Preliminary Phytochemical Analysis

Classical chemical tests were carried out on the methanol extracts for the qualitative determination of phytochemical constituents as described previously[23], briefly as follow:

#### Alkaloids

A white creamy precipitate indicated the presence of alkaloids in the extracts after addition of Mayer's reagent (1.36g $HgCl_2$ + 5g KI) to the side of the tube containing 10 mg of extract suspended in 2 mL of methanol.

#### Anthraquinones

Five milligrams of the extract were boiled with 10% HCl for few minutes in a water bath. After it was filtered and allowed to cool, equal volume of $CHCl_3$ was added to the filtrate. Few drops of 10% $NH_3$were added and followed by heat. Development of pink color was considered a positive result.

#### Anthocyanins

Two milliliter of extract were mixed with equal volume of HCl and ammonia ($NH_3$). Formation of a pinkish red to bluish violet coloration was indicative for the presence of anthocyanins.

#### Phenols

Ten milligrams of extract were treated with few drops of ferric chloride solution. Appearance of bluish black color indicated the presence of phenols.

**Glycosides**

About 2 mLof extractwere mixed with 2 mL of glacial acetic acid containing 1-2 drops of 5% of $FeCl_3$. The mixture was poured into test tube containing 1 mL of concentrated $H_2SO_4$. Development of brown ring at the junction of two liquid layers indicated a positive result.

**Flavonoids**

Ten milligrams of each extract were re-suspended in 1 mL of methanol. Appearance of orange color after addition of few drops of $H_2SO_4$ to the extracts indicated the presence of flavonoids.

**Steroids and Terpenoids**

Presence of steroids was detected by Libermann-burchard's test. For short, 2 mL of acetic anhydride were added to 5 mg of the extracts, each with 2 mL of $H_2SO_4$. Blue or green color indicated the presence of steroids and terpenoids.

**Saponins**

About 0.5 mg of the extract was added to 5 mL of distilled water. Formation of froth after shaking the mixture indicated the presence of saponins.

**Tannins**

Tannins were detected by ferric chloride test. Briefly, a greenish colored precipitate indicated the presence of tannins after mixing small amounts of the crude extracts (2 mL) with 2-3 drops of 5% of $FeCl_3$.

**Gum and Mucilage**

Fifty milligrams of extract were dissolved in 5 mL of distilled water followed by addition of 2 mL of absolute alcohol with constant stirring. White or cloudy precipitate indicated the presence of gums and mucilage.

**Determination of the Antibacterial Activity**

The antibacterial activity was evaluated by modified Kirby-Bauer disk diffusion assay[24]. Briefly, Mueller-Hinton (MH) agar (HiMedia, India) plates were inoculated by swabbing the tested bacteria onto the surfaces of the MH agar. Bacterial inoculum was adjusted to match the standard 0.5 McFarland standard solution (equivalent to ≈1x $10^8$ CFU/mL). 200 mg of the extract were mixed with 2 mL of methanol to produce a stock solution of 100 mg/mL. Two-fold serial dilutions were made from the stock to prepare 50, 25, 12.5, and 6.25 mg/mL solutions. Marjoram oil was diluted in 95% ethanol as previously published[6, 25].

Sterile blank paper disks (WhatmanNo. 3) of 6 mm in diameter were impregnated with 20 µL of the targeted concentration[22]. 5 µL of extract were spotted alternately on both sides of the disks and allowed to dry before the next set of 5 µL is spotted to ensure precise impregnation. Impregnation bymarjoram oil was done by the same method employed for extracts. Disks impregnated with 20µL of methanol (and ethanol when marjoram oil was tested) were used as negative control while disks of Gentamicin(10µg) (HiMedia, India) were also used as a positive control. Disks were placed aseptically on surfaces of inoculated plates and incubated at 36±1°C aerobically for 16-18 hours.

**Detection of Synergy with Antibiotics**

Standard antibiotic disks were saturated with 20µL of the concentration 6.25 mg/mL giving a total concentration of approximately 0.13 mg/disk[26].The disks were placed aseptically on MH agar plate pre-inoculated with the test bacteria alongside with non-impregnated standard antibiotic disks. Synergistic or antagonistic interactions were considered when inhibition zone diameters enlarged or decreased by 5 mm or more, respectively, compared to the inhibition zones of corresponding standard non-saturated antibiotic disk[27].

## 3. RESULTS

**Phytochemical Profile**

The phytochemical analysis of extracts was carried out by classical chemical assays. The presence of various phytochemicals is summarized in Table 1. Interestingly, alkaloids and anthraquinones were not detected in any of the extracts tested. Additionally, phenolic compounds and flavonoids were not detected in the extracts of *W. somnifera* and *R. nervosus* respectively.

**Table 1: Qualitative phytochemical analysis of methanol extracts from the selected plants**

| Compound group | *O. majorana* | *R. nervosus* | *W. somnifera* |
|---|---|---|---|
| Alkaloids | – | – | – |
| Anthocyanins | + | + | + |
| Anthraquinones | – | – | – |
| Flavonoids | + | – | + |
| Glycosides | + | + | + |
| Phenols | + | + | – |
| Saponins | + | + | + |
| Steroids | + | + | + |
| Tannins | + | + | + |
| Gums and mucilage | – | – | – |

(+) Detected (–) Not detected

**Antibacterial activity**

Methanol extract of *O. majorana* showed no activity against all challenged bacterial species at any concentration, while oil of *O. majorana* showed weak activity only at high concentrations(Table 2).The most affected bacterial species by methanol extract of *W. somnifera* is *E. coli* (inhibition zone = 24 mm). On the contrary, the extract showed moderate activity against *S. aureus* and no activity against *P aeruginosa* at any concentration (table 2).Meanwhile, the extract of *R. nervosus* was found ineffective against test species at any concentration. Only at the highest concentration (2 mg/disk), *R nervosus* showed weak activity(inhibition zone was 10 mm) against *P. aeruginosa*(results not shown).

**Synergy with selected antibiotics**

The observed activity of β-lactam antibiotics in combination with the extract of *W. somnifera* against *E. coli*(Fig.1) is not different from the activity seen for the extract alone (see table 2) at the tested concentration (0.13 mg/disk).In contrast, *R. nervosus* extract seemed to decrease the activity of Sparofloxacin antibiotic when tested in combination. Other combinations with tested antibiotics against *E. coli* showed indifferent effect and are depicted in Fig.1. Results of combining extracts with antibiotics against *S. aureus* are depicted in Fig.2. Of note, amikacin was antagonized by extract of *W. somnifera*where the inhibition zone diameter decreased from 24 mm for amikacin disk alone to 19 mm when combined with the extract. On the other hand, chloramphenicol activity against *S. aureus* was enhanced by extracts of *R. nervosus* and *O. majorana*(inhibition zones increased from 12 mm to 20 mm and 19 mm) respectively. Other combinations showed indifference in activity when compared to standard antibiotic disks.

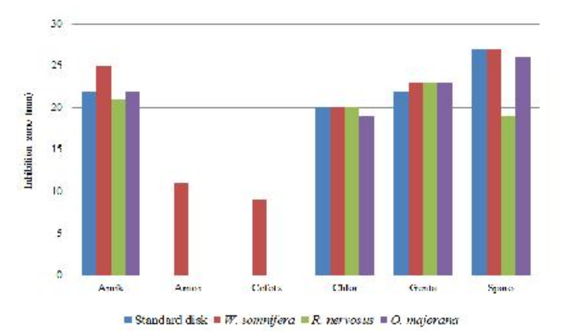


**Fig 1: Inhibition zones of extracts combined with antibiotics against *E. coli*.Amik; Amikacin, Amox; Amoxicillin, Chlor; Chloramphenicol, Genta; Gentamicin, Sparo; Sparofloxacin, Cipro; Ciprofloxacin, Eryth; Erythromycin, Tetr; Tetracycline.**

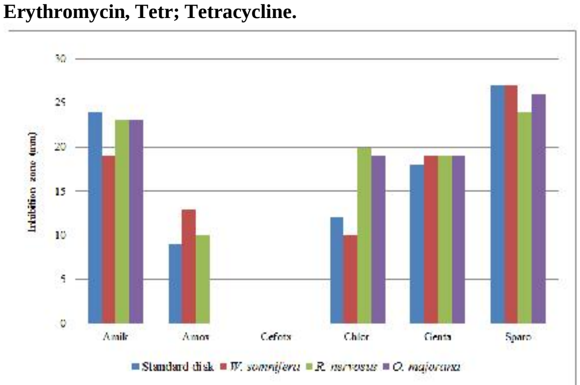


**Fig 2: Inhibition zones of extracts combined with antibiotics against *S. aureus*.Amik; Amikacin, Amox; Amoxicillin, Chlor; Chloramphenicol, Genta; Gentamicin, Sparo; Sparofloxacin, Cipro; Ciprofloxacin, Eryth; Erythromycin, Tetr; Tetracycline.**

**Table 2: The inhibition zones of marjoram oil and *W. somnifera* methanol extract against challenged bacteria**

| Dilution (mg/disk) | Inhibition zones diameters (mm) | | | | | |
|---|---|---|---|---|---|---|
| | Marjoram oil | | | *W. somnifera* extract | | |
| | *S. aureus* | *E. coli* | *P. aeruginosa* | *S. aureus* | *E. coli* | *P. aeruginosa* |
| 100 (2) | 8 | 8 | 8 | 17 | 24 | – |
| 50 (1) | 7 | 7 | 8 | 14 | 20 | – |
| 25 (0.5) | – | 7 | 9 | 10 | 18 | – |
| 12.5 (0.25) | – | – | – | 9 | 12 | – |
| 6.25 (0.13) | – | – | – | – | 11 | – |
| 3.12 (0.07) | – | – | – | – | 10 | – |
| Gentamicin 10 µg | 21 | 20 | 18 | 20 | 20 | 18 |
| Methanol 99.5% | – | – | – | – | – | – |

–; No inhibition

Interestingly, amoxicillin was found to be antagonized by all extracts tested and completely lost its efficacy against *P. aeruginosa* (Fig.3).Additionally, efficacy of sparofloxacin and tetracycline were decreased by the extract of *R. nervosus* and *O. majorana* (inhibition zone dropped from 27 mm to 20 mm and from 33mm to 27 mm respectively). Other combinations with the selected antibiotics showed indifferent interactions with the extracts against *P. aeruginosa* (Fig.3).

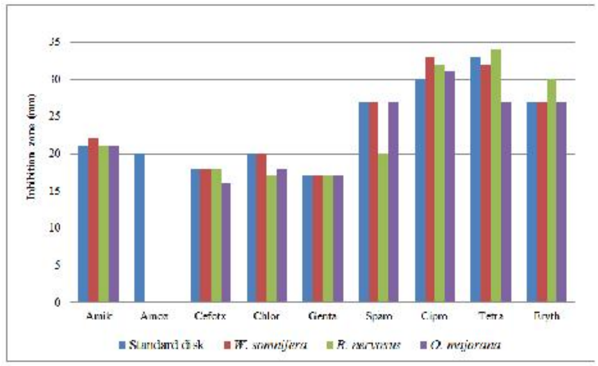


**Fig 3: Inhibition zones of extracts combined with antibiotics against *P. aeruginosa*. Amik; Amikacin, Amox; Amoxicillin, Chlor; Chloramphenicol, Genta; Gentamicin, Sparo; Sparofloxacin, Cipro; Ciprofloxacin, Eryth; Erythromycin, Tetr; Tetracycline.**

## 4. DISCUSSION

The analyzed extracts revealed the presence of major groups of secondary metabolites. Interestingly, Alkaloids were not detected in the extract of *W. somnifera*. However, it is well-documented that *W. somnifera* contains diverse alkaloids of immuno-modulatory, anti-inflammatory, and cytotoxic activity on different tumor cell lines.[28]Thisdisagreementmay be attributed to the detectable level of alkaloids presented in the extracts anddifferences in geographical location[29, 30], physiologicaland phenological status [31, 32],and/or developmental stage and season [33]even in nearby locations[34]. The phytochemical profile of *R. nervosus* obtained in the present study agreed with an Eritrean study[35].Meanwhile, phytochemical findings of *W. somnifera* are in good agreement with Indian studies[16, 17].Detected majorchemical constituents in *O. majorana* are also reported in other recent reports[36, 37].

Extract of*W. somnifera* exhibited a good antibacterial activity against *E. coli* and *S. aureus*(Table 2). Since alkaloids were not detected in the extract of *W. somnifera*, the activity is most likely attributed to the detected phenolic compounds (flavonoids and tannins) steroids and anthraquinones presented in the methanol extracts. The antibacterial activities of phenols and flavonoids from *W. somnifera* have been reported previously [15, 38]. On the other hand, lack of activity against *P. aeruginosa* is expected because this bacterial species is intrinsically resistant to a number of antimicrobial agents [39].

A previous Yemeni study collected *W. somnifera* plant from AL-Hodaydah[40] and another Indian study [13]found a lower activity of ethanol extracts than the activity found in this work against *S. aureus* and *E. coli*. Such higher activity may be attributed to the difference in altitude between the locales alongside with other factors [31]. The challenged bacterial strain employed for evaluation may also account for such differences[29, 32, 41].Additionally, the Yemeni study found *W. somnifera* extract to have weak activity against *P. aeruginosa* (inhibition zones measured 10 mm) while no activity was found herein. The antibacterial activity of *W. somnifera*against *S. aureus* agreed with the findings of a previous study carried out in Kenya [22] and with other study from India[13]. However, the present study found larger inhibition zones (24 mm) and (17 mm) at lower concentration (2 mg/disk) for *E. coli* and *S. aureus* respectively.

The results of marjoram oil activity against *S. aureus* and *E. coli* are in good agreement with a Scottish study [25], however, the oil was reported to have a good efficacy against *P. aeruginosa*. This discrepancy is proposed to differences in methods of oil extraction, species differences, and tested bacterial strains[41].On the other hand, the results of antibacterial activity of methanol extract of*O. majorana* are not in agreement with previously published reports[42, 43].The lack of antibacterial activity of methanol extract of*R. nervosus* observed in this study is consistent with a previous Yemeni study that collected *R. nervosus* from Dhamar [40], who found the 70% ethanol extract to be ineffective against *S. aureus*, *E. coli*, and *P. aeruginosa* even at high concentrations (4 mg/disk). Furthermore, another Yemeni-Japanese study obtained *R. nervosus* from Yemen also found acetone-buffered methanol extracts to lack antibacterial activity against *E. coli* despite using a sophisticated extraction protocol[44].

To the best of authors' knowledge, no published studies have tested the effect of combining extracts from these plant species with standard antibiotics. Only single study had tested the synergistic effect between *W. somnifera* leaves extract with only single standard antibiotic against *E. coli*[45]that found indifferent effect when methanol extract was combined with Tibrim (Rifampicin-Isoniazid combination). The loss of activity of amoxicillin and sparofloxacin against *P. aeruginosa* is most likely attributed to the fact that functional groups of sparofloxacin and amoxicillin may have bound to some constituents from the extracts resulting in larger structures unable to pass the outer membranes of Gram negative bacteria. Indeed, the outer membrane of Gram-negative bacteria is known to confer additional selective permeability and resistance against antimicrobial drugs with large size structures[46].

## 5. CONCLUSION

Scientific characterization of chemical content of medicinal plants and their biological activities is important to authenticate their uses and prevent the potential adverse effects. In the present study, major groups of secondary metabolites were detected in the extracts. Methanol extracts of *W. somnifera* showed a good antibacterial activity against *E. coli* and *S. aureus*, while other extracts showed almost no antibacterial activity. Further studies are recommended to isolate the solitary bioactive compounds presented in the extracts to evaluate their contributions to the antibacterial activity, synergistic effects, and mechanism of action.

## 6. AUTHORS' CONTRIBUTIONS

JMA designed, mentored the study and wrote the manuscript. AAAproofread the manuscript. The first four authors contributed to all laboratory work during the study except phytochemical analysis which JMA didnot participate in.

## 7. ACKNOWLEDGEMENT

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