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In *Vitro* Study of Biological Activity of *Teucrium Davaeanum* Extracts on Strains of Pathogenic Bacteria

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ABSTRACT

The major purpose of this study was to recognize the role of different extraction techniques (decoction, Soxhlet, and soaking) and solvents (water, ethanol, ethyl acetate, ether, and chloroform) on the antimicrobial activities of extracts of *Teucrium davaeanum*. These solvents were examined to determine the conditions for which extracts with a higher content of bioactive compounds. The Kirby-Bauer disk diffusion method was used to assess *in vitro* antimicrobial activity of the different crude botanical extracts against Gram-positive (*Staphylococcus aureus*) and -negative (*Escherichia coli* and *Pseudomonas aeruginosa)*. The antibiotics Gentamycin, Ciprofloxacin, and Azithromycin were used for antimicrobial susceptibility tests. The microbial growth was determined by measuring the diameter of the zone of inhibition and the mean values are presented with ± S.E.M. The ethyl acetate extract showed the highest inhibition diameter of 20±1 mm at a concentration of 0.1 g/mL against *S. aureus*, so it is considered to have higher efficacy than Ciprofloxacin, which showed an inhibition diameter of 15 mm. The isolated essential oil is also showed powerful inhibition effect with a diameter of 12±2 mm against *S. aureus* and 10±2 mm towards *P. aeruginosa* at a concentration of 0.1 g/mL, so it is a higher antimicrobial effect than Gentamycin and Azithromycin, which showed an inhibition diameter of 10 mm. The cold aqueous extract afforded an inhibiting diameter of 7 mm against *S. aureus* at a concentration of 0.1 g/mL, so it is considered to be less effective compared to the antibiotics that used as control. Moreover, hot water showed a low inhibitory effect on *E. coli* with 3±1 mm at a concentration of 0.1 g/mL. The results of applying ethanol extract showed an inhibitory effect on *S. aureus* only with 4±1 mm, otherwise, the results of applying chloroform extract and diethyl ether showed no inhibitory effect. Therefore, the most active extract was ethyl acetate extract, followed by volatile oil and then the cold aqueous extract. This could be attributed to the higher content of bioactive compounds in both extracts of ethyl acetate extract and essential oil.

1. Introduction

The human race has suffered since a long-established time from different diseases which are caused by pathogenic microbes.[1] Plants are considered an important source of active substances that are used in the preparation of many medicines, as it has been scientifically proven that the laboratory-manufactured substance does not perform the same physiological effect as the active one extracted from medicinal plants which have fewer side effects.[1-2]

The plants of the genus *Teucrium* have been used as medicinal plants since ancient times, and some of them

are still used in folk medicine as an anti-inflammatory, anti-convulsing, anti-fever, or anti-abscess.[2] Moreover, many studies showed that *T. davaeanum* has promising biological activity.[3-7] One of these studies showed that the alcoholic extract of *T. davaeanum* that grows in Libya can reduce blood sugar in diabetic mice after daily injection for two consecutive weeks by a significant percentage (60%).[3] Furthermore, another biological study of extracts of some Libyan plants as antimicrobials of the different extracts showed three species belonging to the Labiate family including, *T. davaeanum*, which showed that solvent extracts (ethyl acetate, chloroform, butanol, acetone) and volatile oil extract were indicated an inhibitory effect against Gram-positive *S. Aureus*, *M. phlei*, *B. subtilis*, *E. coli* and *C. albicans*.[4] Likewise, aqueous and alcoholic extracts of aerial parts of *T. davaeanum* were stated to have *in vitro* antimicrobial effects on the growth of *S. aureus*, *Proteus vulgaris*, *P. aeruginosa*, *E. coli*, and *S. pyogenes*.[5]

Furthermore, it was shown that the combination of silver nanoparticles and ethanolic extract of *Teucrium polium* can inhibit the growth of different pathogenic microorganisms: *P. aeruginosa*, *K. pneumoniae*, *S. aureus*, *candida*, and *albicans*.[6]

2. Experimental

2.1 Plant Sample Collection

T. davaeanum were collected from the area of Wadi Atlal (Sirte, Libya). Fresh aerial parts (leaves, flowers, branches) were collected in April 2019 during the flowering time. The aerial parts of the plant were separated, washed, and air-dried in a dark place without resorting to ovens to avoid loss of vehicles.

2.2 Extraction Methods

2.2.1 Preparation of Cold Aqueous (Method of Maceration)

22 grams of dried powder was placed in flask with 300 mL of distilled water for three days at room temperature under a magnetic shaking (Stuart, model: CB161) . Then the filtration was done using Buchner funnel.

2.2.2 Preparation of Hot Aqueous Extract (Decoction)

40 grams of dried plant powder was placed in flask with 500 ml of distilled water, then the mixture is subjected to shaking (Stuart, model: CB161) with heating. Then, the precipitate is separated using a centrifuge at a speed (300 cycles / 15 minutes) and then the mixture is placed in the electric furnace at 35 ˚C until a concentrated extract is obtained, then it is left to cool. The filtration process is carried out using Buchner funnel to left the filtrate which was stored in airtight bottles at 0°C.

2.2.3 Extraction Methods Using Soxhlet

Sample of dried powder plant was weighed and relocated into a Soxhlet glass sample tube which was transferred to the extraction section in the Soxhlet apparatus. A measured volume of used solvent was transferred into the solvent flask and placed on the heating mantle. The cooling water supply to the condensers was opened to ensure continuous recycling of the solvent and temperature selected as per the *Büchi* manual for extraction in the continuous mode. Boiling point temperatures were chosen depending on their boiling points of the solvents shown in Table 1.

The extractions were conducted for 9 h. After the extraction process is completed, the extract solution was allowed to cool to room temperature and then filtered to obtain pure extracts and ensure that there are no impurities. The solvent was removed using a vacuum rotary evaporator. The extracts are left for a whole day to complete the drying process and then grounded into a powder.

Table (1): solvents used in this study arranged according to the order of decreasing polarity.

Solvent	B. p. (˚C)	Density@ 25 ˚C (g/mL)	Relative polarity
Water	100	0.998	1.000
Ethanol	78.5	0.789	0.654
Ethyl acetate	77	0.894	4.3
Chloroform	61.2	1.489	0.259
Diethyl ether	34.6	0.713	0.117

2.2.4 Extraction of Essential Oil

25 grams of the fresh of plant leaves are transferred into a round flask with a capacity of 250 ml, and then a 100 ml of distilled water is added. The contents of the rounded flask are heated at 60°C for 8 hours to extract the volatile

oil. The volume of extracted crude essential oil is 12 mL without drying from aqueous content. This extraction is done according to Clevenger procedure (Fig. 1).[11-12]

Figure (1): Clevenger apparatus.

2.3 Calculations of the Percentage Yield of Botanical Solvent Extracts

Table (2) shows the percentage of yields that were obtained in this study using the following equation:

$$(\text{Yield ratio } \%) = \frac{Real\ yield}{Theoretical\ yield} \times (100)$$

Real yield = Flask weight after extraction - Flask weight before extraction

Theoretical yield = sample weight

Table (2): Yield ratio for each botanical extract.

Extraction	Real yield	Theoretical yield	Yield ratio (%)
Ethanol	0.57	13	4.3
Cold water	0.77	22	3.5
Ethyl acetate	0.26	10	2.6
Chloroform	0.15	10	1.5
Diethyl ether	0.1	10	1
Hot water	0.23	40	0.6

2.4 Bacterial Strains and Growth Conditions

Samples swabs were obtained from patients at Ibn Sina Hospital in Sirte city (Libya) as shown in Table 3. Furthermore, bacteria strains were classified using sensitivity strips and then, these strains were determined by microscopic examination using the characteristics of each class. Each bacteria were cultured overnight (≈16 h) in flasks containing MacConkey agar or Nutrient agar (Bioscience, Ismailia, Egypt) at 37 ◦C.

Sample No.	Kind of sample	Classification of bacteria
1	Inflamed ear swab	*P. aeruginosa*
2	Sore toe swab	*S. aureus (Gr.+ve)*
3	A swab from a sore hand caused by burning gasoline	*S. aureus*
4	Swab for inflamed surgery	*S. aureus*
5	A swab from a burning foot caused by burning coals	*S. aureus*
6	A swab from a stress-inflamed fracture	*S. aureus*
7	A swab from a flaming fingernail	*S. aureus*
8	Vaginal swab	*E. coli*
9	Stool sample of a patient	*E. coli*

Table (3): Type of samples and bacterial classification.

2.5 Preparation of the Extracts Concentrations

Inhibition experiments have been performed as is commonly applied with pharmaceutical powders, so 100 mg of isolated crude herbal extract (organic solvents extracts) was dissolved in 1 mL of WFI (water for injection), and then heated on a water bath to complete solvation. A concentration of 0.1 g/mL is obtained as a standard concentration. More diluted samples were prepared 0.05 g/mL and 0.03 g/mL for each extract. In addition, 1 mL of the volatile oil is dissolved in 9 ml of ethylene glycol and then, a concentration of 0.1 g/mL is obtained.

2.6 Preparing of Plant Extracts Discs

Discs with a diameter of 6 mm were prepared from 3 mm thick chromatographic papers using a circular paper cutter with a diameter of 6 mm. The serial numbering process was carried out by writing a number which is symbolized for each type of botanical extract. Then, the discs were sterilized in an ultra violet germicidal irradiation (UVGI) for 15 minutes. Therefore, each disc was immersed in the botanical extract according to its sequence number. Generally, the processes of immersion, concentration and drying were carried out three times for each botanical extract separately (Fig. 2). Then, the discs were incubated at 37°C for 24 h overnight.

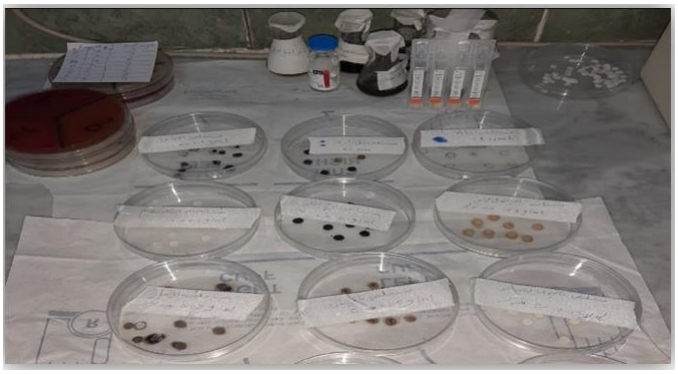

Figure (2): Prepare extract discs to test their efficacy.

2.7 Method for Testing Extracts as Antimicrobials

The method of diffusion through the discs (disc - diffusion modified-Kirby-Bauer method) was used to test the antibacterial activity of the botanical extracts against three bacteria.[7-10] In the procedure, the nutrient media

was cultured with the tested microbes, then the disc containing the test botanical extract was placed on this nutrient agar. The plates were then incubated at a temperature of 37 °C for a period of 24 hours to allow maximum growth of the bacterial strains. Furthermore, reading the diameters of the inhibition zones formed around the discs after the incubation period was measured in millimetres using a ruler.

Figure (3): The antibiotics was used as a control.

2.8 Antibiotics Used in Present Study

The antibiotics (Table 4) Ciprofloxacin, Gentamycin and Azithromycin were used for antimicrobial susceptibility test. Antibiotics were purchased from local medical laboratory (Tripoli, Libya). Standard disc of each antibiotic (500 µg/disc) and blank disc (soaked with solvents followed by evaporation) were used as positive and negative control, respectively.

Table (4): Antibiotics inhibition diameters.

Kind of medicine	Inhibition zone (*S. aureus*)
Ciprofloxacin 500 mg	15 mm
Gentamycin 80 mg	10 mm
Azithromycin 250 mg	10 mm

2.9 Detection of Active Ingredients in Ethyl Acetate Extract

2.9.1 Detection of flavonoids:

A chemical study was carried out for the extract of ethyl acetate to detect flavonoids content by examination under ultraviolet rays, which gave green phosphorous radiation.[13]

2.9.2 Detection of alkaloids:

As stated in (Smolensk et al., 1972), a white substance was precipitated by exposure to wagner`s reagent (Solution of Iodine + KI), which indicate to present of alkaloids in the extract mixture.[14]

3. Results and Discussion

3.1. Plant Selection

Teucrium davaeanum (Fig. 4) is chosen due to it is one of the medicinal plants that grow in the Libya regions and it is available in the local market in abundance and has been used since ancient times because of its effectiveness against infections and urinary tract problems and being rich in many useful compounds that can be used in the preparation of many medicines and formulations.

Figure (4): Arial parts of *Teucrium davaeanum*.

3.2 Antimicrobial Activity

The antimicrobial activity against Gram-positive (*S. aureus*) and -negative (*E. coli* and *P. aeruginosa)* was determined using the Kirby-Bauer disk diffusion technique for the purpose of testing the effectiveness of plant extracts on those bacteria. Moreover, “the Kirby-Bauer disk diffusion method is one of the most widely practiced antimicrobial susceptibility tests (AST)”.[10] The microbial growth was determined by measuring the diameter of the zone of inhibition and the mean values are presented with ± S.E.M (Figs. 4 and 5). Table (5) summarizes the results of applying each extract on the bacteria.

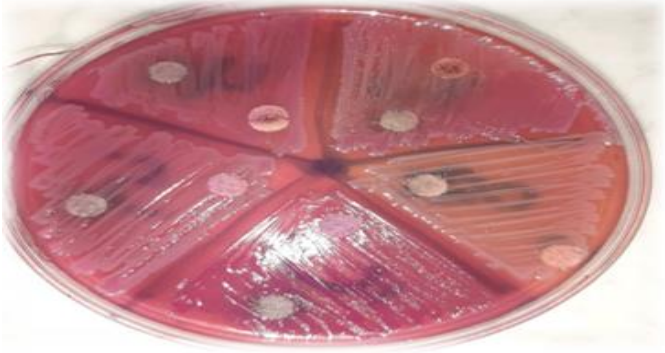

Figure (5): Resistance of bacteria to extracts and their growth on them

Table (5**):** Effect of each extract on microbes

The extract	concentration	Diameter (mm) Type of bacteria *P. aeruginosa*	*S. aureus*	*E. coli*
Ethanol	A	R	4±1	R
	B	R	1	R
	C	R	R	R
Cold water	A	R	7	R
	B	R	2	R
	C	R	R	R
Ethyl acetate	A	6±1	20±1	R
	B	2	13±2	R
	C	R	5	R
Chloroform	A	R	R	R
	B	R	R	R
	C	R	R	R
Diethyl ether	A	R	R	R
	B	R	R	R
	C	R	R	R
Hot water	A	R	R	3±1
	B	R	R	1
	C	R	R	R
Essential oil	A	10±2	12±2	R
	B	10±1	1±5	R
	C	R	R	R

*When A = 0.1 g/mL, B = 0.05 g/mL, C = 0.03 g/ml, R = Resistance

3.3 Assessment of Antibacterial Activity of Botanical Extracts of T. davaeanum

As shown in Table (5) and Fig. (6), the results of applying ethyl acetate extract to S. aureus showed a strong inhibitory effect with the highest average reading of the inhibition zones diameters: 20, 13, 5 mm, respectively, even though this extract had no inhibitory effect on E. coli at the concentrations A, B, and C.

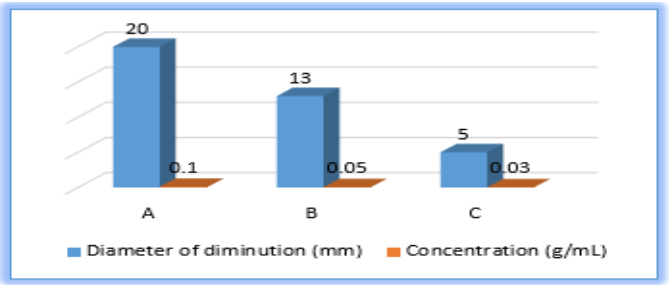

Figure (6): Antibacterial effect of ethyl acetate extract on *S. aureus.*

As shown in Table (5) and Fig. (7), the antibacterial effect of ethyl acetate extract on *P. aeruginosa* is shown an inhibition diameter of 6±1 mm at a concentration of 0.1 g/mL, followed by 2 mm at a concentration of 0.05 g/mL but no effect at all at 0.03 g/mL.

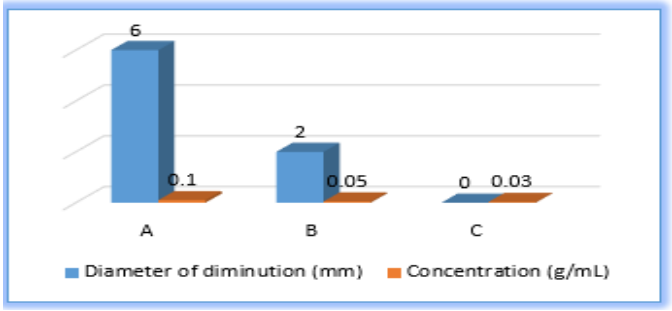

Figure (7): Antibacterial effect of ethyl acetate extract on *P. aeruginosa*.

The results of applying the ethanol extract to the tested microbes showed an inhibitory effect on *S. aureus* (Fig. 8) only at the concentration of 0.1 g/mL gave the highest value of the inhibition diameter of 4±1 mm, followed by 0.05 g/mL with a diameter of 1 mm, while 0.03 g/mL gave no effect.

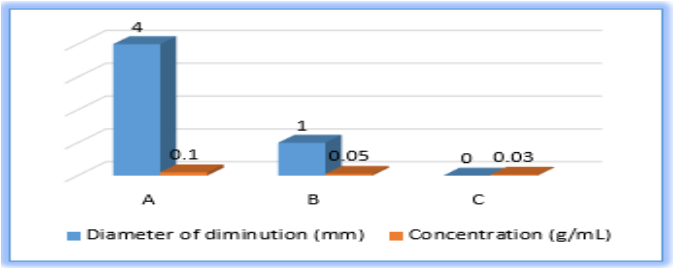

Figure (8): Antibacterial effect of ethanol extract on *S. aureus*.

The results of applying chloroform and diethyl ether extract on the tested microbes showed that there was no inhibitory effect, as shown in Table (5) at concentrations (A, B, C).

The results of applying the hot aqueous extract showed no inhibitory effect on *S. aureus* and *P. aeruginosa* (Fig. 9); while it showed an inhibitory effect on *E. coli* with a value of inhibition diameter was 3 mm at a concentration of 0.1 g/mL and 1 mm at a concentration of 0.05 g/mL, and no effect was shown at a concentration of 0.03 g/mL.

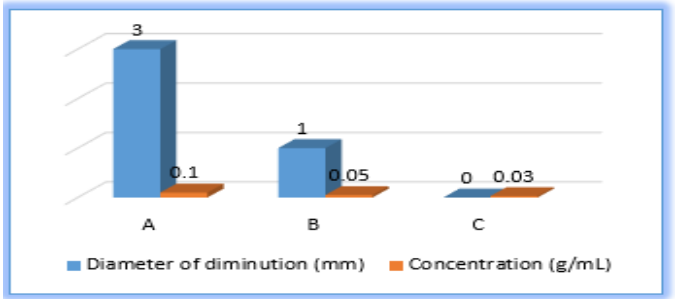

Figure (9): Antibacterial effect of the hot aqueous extract on *E. coli*.

As for the cold aqueous extract, it showed an inhibitory effect on *S. aureus* (Fig. 10). The value of the inhibition diameter was 7 mm at a concentration of 0.1 g/mL, and the value of the inhibition diameter was 2 mm at a concentration of 0.05 g/mL, and there was no effect at a concentration of 0.03 g/mL. This aqueous extract showed no effect on *E. coli* and *P. aeruginosa*.

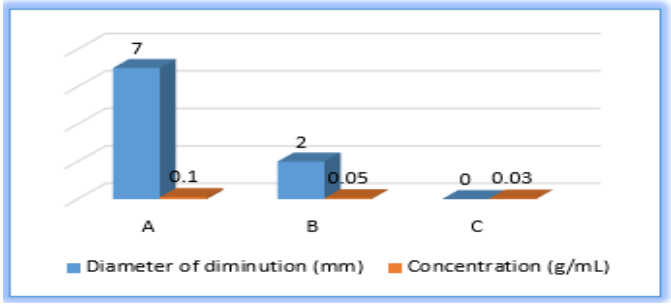

Figure (10): Antibacterial effect of cold aqueous extract on *S. aureus*.

3.5. Extraction of Essential Oil of T. davaeanum by Hydro-distillation Method

The essential oil was separated by the Clevenger method (hydro-distillation) and its percentage was 12% (Fig. 1).[11-12] Its refreshing smell and light texture distinguished it. It was found that it did not dissolve in water. Thus, ethylene glycol was used as a polar solvent to dissolve this oil. 1 mL was taken of the essential oil and dissolved in 9 mL of dilute ethylene glycol. Far along, a concentration of 0.1 g/mL was obtained to test on bacterial strains.

3.6. Antimicrobial Activity of Essential Oil of T. davaeanum

The results of applying the essential oil showed a powerful inhibitory effect against *S. aureus* and *P. aeruginosa* but no activity at all against *E. coli*. The inhibition diameter was 12±2 mm against *S. aureus* and 10±2 mm against *P. aeruginosa* (Fig. 11).

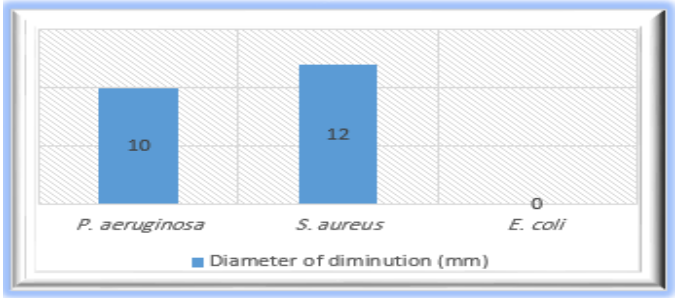

Figure (11): Antibacterial effect of essential oil at concentration of 0.1 g/cm^3.

3.7. Comparison of The effectiveness of botanical extracts of T. davaeanum on bacterial samples

It found that the highest effective was ethyl acetate extract for the Soxhlet extraction method with a maximum inhibition diameter of 20±1 mm at a concentration of 0.1 g/mL against *S. aureus*. Its effectiveness is considered to be higher than Ciprofloxacin, which showed a diameter of 15 mm inhibition against *S. aureus*. Thus, ethyl acetate seemed to be the best solvent used to obtain extracts rich in bioactive compounds with antimicrobial activity. Essential oil, with a diameter of its inhibition was 12±2 mm against *S. aureus* at a concentration of 0.1 g/mL. It is more effective than Gentamycin and Azithromycin, which showed the activity against *S. aureus* with a diameter of 10 mm, then essential oil, with a diameter of 10±2 mm against *P. aeruginosa*, and finally the cold aqueous extract gave a diameter of 7 mm against *S. aureus* at a concentration of 0.1 g/mL is considered less effective compared to the antibiotics used as a guide.

Overall, for Gram-negative, the most susceptible was *p. aeruginosa* (10±2 mm) for essential oil extract followed by ethyl acetate extract (6±1 mm) but, for Gram-positive, the most susceptible was *S. aureus* (20±1 mm) for ethyl acetate extract followed by essential oil extract (12±2 mm).

4. Detection of Active Components in Ethyl Acetate Extract

All of the flavonoids are able to absorb ultraviolet rays, henceforth usually they can be detected by UV detectors. [15] "It is usually detected at 254–280 nm or 340–360 nm for flavones, flavonols and the corresponding glycosides, 520–540 nm for anthocyanins and the corresponding glycosides, 250 nm for chromones".[15]

In this study, both flavonoids and alkaloids are detected in ethyl acetate extract as above-mentioned in previously paragraph number 2.9.

5. Conclusions

The Soxhlet extraction method was selected because of its easiness in process, qualified safety and prospective for upscaling to industrial plant level. Five solvents covering a range of polarities and solubility were chosen for this research. This study showed that ethyl acetate was generally more efficient in the extraction of bioactive content than the other solvents studied. On the other hand, essential oil showed remarkably antimicrobial activity against *S. aureus and Pseudomonas aeruginosa* which was more susceptible than *E. coli*.

Therefore, the effectiveness of both ethyl acetate and essential oil could be attributed to the fact that it is to its content of flavonoids and other bioactive materials that have many functions.

Overall, the results of the current study contributed to establishing which extraction method and solvent could be the best suitable for obtaining bioactive components from the *T. davaeanum* plant. Additionally, it was introduced that *T. davaeanum* extracts can be reflected as a possible commencing of natural antibacterial sources. This is of valued significance, as the results of this work provide some basis for the traditional use of this plant in antimicrobial agents and new insights into the using of crude herbal extracts for the treatment of anti-bacterial infections.

6. Recommendations

Authors suggest that several tasks would perform, including:

I. Continuing the research in testing the effectiveness of extracts of the plant ***T. davaeanum*** on other types of bacteria and fungi.

II. Study of the active ingredients of the ***T. davaeanum*** plant and their biological importance.

III. Work on preparing a formulation of the antibiotics based on herbal extracts or essential oil or ethyl acetate extract of ***T. davaeanum***.

6. Acknowledgments

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