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ANTIMICROBIAL AND ANTIOXIDANT ACTIVITIES OF *TRIGONELLA FOENUM-GRAECUM* ESSENTIAL OIL FROM THE REGION OF SETTAT (MOROCCO)

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Abstract

During the past decade, natural bioactive chemicals derived from plants have known an increasing interest for scientific purposes and a variety of other applications including the pharmaceutical and food industries. Fenugreek (*Trigonella-foenum graecum*) is a leguminous plant that is one of the most traditional and promising therapeutic herbs. Fenugreek seeds have been extensively studied for their ability to treat diabetes, inflammation, and cancer. In this work, the chemical composition of *T.foenum graecum* seed essential oil was determined using gas chromatography coupled with mass spectrometry (GC-MS). The two general classes of polyphenols have been studied also, one is the total phenolic content (TPC), and the other is total flavonoid content (TFC), then the antioxidant activity was determined using the following assays: antiradical (DPPH and ABTS), phosphomolybdenum, reducing power (FRAP and CUPRAC), and ferrous chelating assays. The antimicrobial activity was tested against *Listeria monocytogenes,Escherichia coli, Bacillus subtilis, Yersinia enterocolitica, Enterococcus faecium, Staphylococcus aureus,* and *Candida albicans.* The *T. foenum-graecum* essential oil was GC-MS analyzed, conducted to the identification of 18 different chemical compounds. Methyl palmitate (20.54%), Decane, 5, 6-bis (2, 2-dimethylpropylidene)-, (5E, 6Z)- (20.18%), Dihydro methyl jasmonate (13.47%) were found to be major compounds. Furthermore, fenugreek seeds' essential oil showed high antioxidant activity, but it varied according to the used method. The TPC and TFC of the oil were 30.74 mg GAE/g and 24.67 mg RE/ g respectively. As a result, this research suggests that *T.foenum greacum* might be exploited in pharmaceuticals, considering the high content in phenolic and flavonoids present in its essential oil and the considerable antimicrobial properties against human pathogen strains. The results of this work suggest that the *Trigonella foenum-graecum* essential oil may be evaluated as a source of biological agents for food product development and pharmaceutical formulations.

Keywords: *Trigonella-foenum graecum; extraction; antioxidant activity; antibacterial activity.*

Introduction

Essential oils' antibacterial and antioxidant properties have lately been used in a variety of applications, including pharmaceuticals, complementary and alternative medicine, and food preservation [1]. Essential oils are liquid combinations of volatile chemicals extracted from aromatic herbs by steam distillation, which is the most common method. They are what a plant's "essence" is composed of, and they frequently have pleasant scents [2]. In essence, essential oils are employed as multi-functional additives like anti-fungal, anti-microbial agents, as well as flavoring, oxidative stability in food, and other cosmetic products [3]. A synthetic antioxidant such as butylhydroxyanisole (BHA) and butylhydroxytoluene (BHT) present relevant toxicological risks and are thought to be harmful to human health [4]. As a result, natural antioxidants may be preferable to chemical antioxidants because they do not contain chemicals and have long been known for protecting against cell damage from free radical attacks. Natural antioxidants are free from chemicals. Fenugreek, or *Trigonella foenum-graecum*, is a native annual plant to the countries along the Mediterranean's eastern coast and widely cultivated in India, Egypt, and Morocco [5], Because of its outstanding antioxidant, antibacterial, and antifungal properties, fenugreek essential oils and extracts are shown to be particularly beneficial in food preservation [6]. Fenugreek, a member of the leguminous family, is one of the most traditional and promising therapeutic plants. Because of its dietary and therapeutic benefits as a herbal cure, this plant has been commonly utilized for over 2500 years [7]. Because of the presence of polyphenols and flavonoids, this plant can operate as an effective antioxidant source, this research focused on the analysis of the polyphenols contents, antimicrobial, and antioxidant activities of Trigonella foenum-graecum essential oil from Settat region in Morocco. The antioxidant activities of this essential oil was determined by DPPH, ABTS, FRAP, CUPRAC, metal chelating and phosphomolybdenum. The antibacterial activity was tested against *Yersinia enterocolitica, Bacillus subtilis, Listeria monocytogenes, Enterococcus faecium, Staphylococcus aureus,* and *Escherichia coli,* and the antifungal activity was tested against strains of *Candida albicans.*

Methods

Plant material

The *Trigonella foenum-graecum* seeds, previously dried in the field, were collected from the region of Settat (33.0003° N, 7.3659° W) Morocco in June 2020.

Essential oil extraction process

The healthy and mature seeds of *Trigonella foenum-graecum* were ground and hydrodistillation of the essential oil was conducted using a Clevenger type extractor. Anhydrous sodium sulfate was used to dry the essential oil (Na_2SO_4). Finally, and for preventing it was kept at −4 °C to prevent the compounds' degradation for thorough tests.

GC–MS analysis of the essential oil

GC–MS analyses were carried out with a SHIMADZU GC-14B supplied with an FID detector and an L.M-5 (30m × 0.25mm ×0.3mm) capillary column. The components were identified by comparing the retention time of the mass spectra with their equivalents found in the literature data.

Test organisms

Antimicrobial activity was evaluated on seven clinical pathogenic organisms (bacteria and fungi). Gram-positive included *Bacillus subtilis* (CECT 4071), *Listeria monocytogenes* (CECT 911), *Enterococcus faecium* (CECT 4932), and *Staphylococcus aureus* (CECT 976). The other organisms, *Escherichia coli* (CECT 431), and *Yersinia enterocolitica* (CECT 4315) are Gram-negative as well as the yeast *Candida albicans*.

Antimicrobial activity

The antibacterial and antifungal activity of Fenugreek essential oil against the Gram-positive and Gram-negative bacteria and the pathogenic fungi *Candida albicans* were determined using the dilution technique described by Maadane et al. (2017) [8], with minor modifications. For bacteria, the culture medium used was Müller-Hinton agar, whereas Sabouraud Dextrose agar was used to grow the fungi. The incubation of bacteria was at 37 °C for 24 h, while 48 h at 28 °C for the fungi.

Minimum Inhibitory Concentrations (MIC) were determined by visually assessing the lowest concentrations of extracts at which no bacterial/fungal growth was seen in the agar and broth dilutions. The MIC was found by testing the essential oil in DMSO (Dimethyl sulfoxide) solvent for different concentrations ranging from 100 up to 3.125 µl / ml against all microbial strains. For each bacterial strain, the process was repeated, and duplicate sets were prepared.

By re-culturing (subculturing) broth dilutions that inhibit bacterial growth, Minimum Bactericidal Concentrations (MBC) were obtained. Fresh agar plates were used to streak the broth dilutions. The incubation was at 37°C for 24 to 48 hours. The MBC was determined to be the lowest broth dilution of antimicrobial that inhibits the organism from growing on the agar plate.

Determination of polyphenols contents: total phenolic and total flavonoid

Total phenolic content (TPC) in Fenugreek essential oil was estimated by the Folin–Ciocalteu assay as described iby Maadane et al. (2015) [9] with minor modifications. The samples absorbance was measured at 765 nm. The reference standard was gallic acid equivalent (GAL). The results obtained as mg GAE/g are reported as milligrams of gallic acid equivalents per gram of the dry sample.

Total flavonoids content (TFC) from *Trigonella greacum-foenum* essential oil was determined according to He et al. (2018) [10], which led to the formation of the complex flavonoid–an aluminum complex that absorbs at 510 nm. The flavonoids content is expressed as mg Rutin equivalents per gram of each dry sample (mg RE/g). All samples were analyzed in triplicate.

In vitro antioxidant activities

A single antioxidant test model should not be used to determine antioxidant activity. In practice, a variety of in vitro test techniques are used to assess antioxidant activity in the samples of interest. Another consideration is that antioxidant test models differ in several ways. As a result, comparing one procedure to another is difficult. In general, doing in vitro antioxidant studies with free radical traps is rather simple. In comparison to other free radical scavenging methods, the DPPH approach is therefore quick, simple in terms of the number of stages and the used chemicals, and economical. The ABTS decolorization method, on the other hand, works for both lipophilic and hydrophilic antioxidants. In this work, the techniques used to evaluate the antioxidant activity were antiradical (DPPH and ABTS), phosphomolybdenum, reducing power (FRAP and CUPRAC), and ferrous chelating assays. Antioxidant activity was expressed using Trolox equivalents. While for the metal chelating test, EDTA was used as a reference chemical.

DPPH antiradical assay

The DPPH stable radical was used to test the essential oil-free radical scavenging activity. The reduction of the DPPH radical absorbance was read at 518nm. When DPPH is reduced, its purple color changes to yellow. As a result, a low absorbance value indicates a better antioxidant activity [9]. To calculate the percentage of inhibition of free radical DPPH, the following formula is used:

$$I(\%) = \frac{A_{blank} - A_{sample}}{A_{blank}} \times 100$$

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound.

ABTS antiradical assay

The radical scavenging activity of the essential oil of *T. foenum* was assayed with ABTS assay (2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) described by Kontek et al. (2021) with minor modifications [11]. The ABTS radical solution was prepared by mixing ABTS and potassium persulfate ($K_2S_2O_8$). Thereafter, the fenugreek essential oil was added to the ABTS solution. After the incubation in a dark place for 2 h, the absorbance was read at 734 nm. The blank in this method was Methanol. The free radical-scavenging capacity was calculated by the following equation:

$$ABTS\ Radical\ scavenging\ (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

$A_{control}$ represents the absorbance of the mix ABTS and methanol solution excluded the sample. The

A_{sample} represents the absorbance of the ABTS mixed with the sample.

FRAP ferric reducing antioxidant power assay

The FRAP assay is based on the complex ferric ion-TPTZ (2, 4, 6-tri (2-pyridyl)-1, 3, 5-triazine) reduction by antioxidants. The molecular bond of $Fe^{2+}$to the ligand creates a dark blue color.The absorbance is measured to determine the quantity of the reduced iron, that have a strong correlation with the concentration of antioxidants present in the essential oil. Results obtained as mg TE/ g extract, are reported as Trolox equivalents per 1 g of dry sample [12]. Briefly, The FRAP reagent consisted of acetate buffer, ferric (III) chloride, and TPTZ, which was added to wells containing the Fenugreek essential oil and methanol. After a 40 min incubation at 37°C, the colored product ferrous tripyridyltriazine complex absorbance nm was measured at 593. The absorption modification in the solution was compared to the Trolox standard curve prepared in methanol to get the FRAP values.

Cupric reducing antioxidant capacity (CUPRAC)

The cupric reducing antioxidant capacity was determined according to the method of Benner et al. (2018) [13]. In this assay, the solution of copper (II) chloride (0.01 M) was added to the ammonium acetate buffer solution and neocuproine solution. Different concentrations of the essential oil and standards were added to this mixture then measured at 450 nm after 1 h of incubation. Results obtained as mg TE/ g extract, are reported as Trolox equivalents per 1 g of dry sample

Metal (Fe^{2+}) chelating activity

The chelating effect was evaluated using the ferrozine assay provided by Zhang et al. (2019). The IC_{50} value, which is described as the concentration (g/mL) necessary to chelate 50% of the ferrous iron contained in the solution tested, was used to demonstrate metal (Fe^{2+}) chelating activity. The lower the IC_{50} value, the better the chelating activity.

The reference for the positive control for metal chelating activity was Ethylenediaminetetraacetic acid (EDTA). The following formula was used to calculate the chelating antioxidant activity for Fe^{2+} :

$$Metal\ chelating\ rate\ \%(\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

$A_{control}$ refers to the absorbance of the control reaction and A_{sample} represents the absorbance of the sample.

Phosphomolybdenum method

To assess total antioxidant activity, a phosphatemolybdenum test based on the reduction of phosphatemolybdenum (VI) to phosphatemolybdenum (V) by the antioxidant compounds present in the plant essential oil.

The total antioxidant activity (TAA) was determined as reported by Renugadevi et al. (2018) [15]. Essential oil samples were mixed with the phosphomolybdenum reagent. The mixture was then incubated for 90 minutes at 95°C. At 695 nm, the absorbance was then measured.

Results

Extraction of the essential oil

Hydrodistillation of Fenugreek seeds essential oil from conducted using Clevenger extractor, yielded 0.79 % (v/w) of the oil.

Analysis and chemical compound identification

GC–MS analysis of the tested essential oil has proven to have 18 different components with an estimated total value of 86.03 %. Table 1 represents the compounds along with their percentage of composition and retention time (RT). The results obtained indicate that the major compounds are methyl palmitate (20.45%), (5Z, 6Z)-5,6-bis (2, 2,-dimethylpropylidene) decane (20.18%), Dihydro methyl jasmonate (13.47%), and the minor compounds were 2-tridecanone (0.22%), linalool (0.75%). The physiochemical composition of the extracted oil obtained in this work differed significantly from the past research, which could be explained by the variation of the type of extraction method and the solvents used. For example, Weisany et al. (2017) [16] had Dillapiole as the essential oil major component, accounting for nearly 91% of the essential oil chemical composition.

Antimicrobial activity

All of the data on the antibacterial activities of *T. foenum-graecum* against different microbial strains

are expressed in terms of MIC, MBC, and ratio MBC/MIC. Table 2 shows all the values obtained. Essential oil of *T. foenum-graecum* shows interesting activity against all strains tested, as MICs vary from 40.47 to 62.37 µL/mL and MBCs vary from 60.47 to 159.37 µL/mL and according to the MBC/MIC report, the *T. foenum-graecum* essential oil activity has bactericidal effects against all the strains studied, except the bacteriostatic effect observed against *Candida albicans*.

Determination of polyphenols contents: total phenolic and flavonoid of *Trigonella foenum-graecum* seed oil

The total phenolic content (TPC) was evaluated using the Folin-Ciocalteu reagent. Results obtained as GAE are reported as gallic acid equivalent. Comparing to the results shown in Table 3 and Table 4, the amounts of TPC we determined were higher 30.74 mg/g than the result obtained by Bhanger et al. (2008) [17]. The method used to determine the total flavonoid content of *Trigonella foenum-greacum seed* oil in this study is the spectrophotometric method. Total flavonoid content was measured using Rutin as a reference. In Table 3, the results expressed a total flavonoid content of 24.67 mg RE/g and it's higher than the amount Mandegary et al. (2012) [18] had determined (Table 3). These results confirmed fenugreek seed oil's high antioxidant capacity. Plants with high levels of polyphenols compounds have greater antioxidant activity.

Antiradical activity assays DPPH· and ABTS^{+}

DPPH* and ABTS^{+}* determined the free radical scavenging activity of *T.foenum graecum* essential oil. The results are summarized in Table 4 and reported as Trolox Equivalents (mg TE/g) and the amount of antioxidant activity of the essential oil is given based on the 50% inhibition concentration (IC_{50}). In the presence of a hydrogen-donating antioxidant, both DPPH· and ABTS$^{+\cdot}$ assess the reduction of radical solutions. As indicated in Table 4, the antioxidant activity evaluated by ABTS assay (78.22 mg TE/g) was stronger than the DPPH assay (54.17 mg TE/g). The antioxidant activity of most plant compounds appears to be greater against ABTS radicals than against DPPH radicals. This is related to the ABTS assay's great sensitivity in determining antioxidant activity, which speeds up the development process and thus leads to higher antioxidant activity [19].

According to the results, free-radical-scavenging activity is strongly correlated with the phytochemical components of the essential oil analyzed. Furthermore, earlier studies have demonstrated that phenolic compounds contribute directly to medicinal plants' antioxidant activity. Thus, The values of free radical-scavenging assays have shown a high antioxidant activity due to the presence of methyl palmitate identified by GC-MS analysis that is known for its antioxidant and biological activities [20].

FRAP and CUPRAC methods

CUPRAC and FRAP assays were used to assess power reduction that usually indicates an electron donation, which is an important mechanism to evaluate the antioxidant activity of medicinal plants. Cupric ions are reduced to cuprous ions in the CUPRAC assay, whereas ferric ions are reduced to ferrous ions in the FRAP assay in the presence of a radical scavenging agent. So, we decided to examine the reducing power of *T. foenum graecum* essential oil using FRAP and CUPRAC assays. CUPRAC measures the reduction of Cu^{2+} - neocuproine to Cu^{+}, while FRAP is a simpler, faster, and accurate test that investigates the reduction of Fe^{3+} to Fe^{2+}. Both FRAP and CUPRAC assays showed great reducing power (58.31 mg TE/g for FRAP, 108.33 mg TE/g for CUPRAC). Still, the antioxidant capacity of the oil by CUPRAC assay was stronger than that by FRAP assay. The reducing power of the essential oil matched the phenolic content values, demonstrating a significant correlation between phenolic content and reducing power (FRAP and CUPRAC) tests [19].

Metal chelating and phosphomolybdenum assays

The phosphomolybdenum assay determined the essential oil's total antioxidant activity. In this procedure, the formation of Mo (V) green phosphate components is caused by the antioxidant found in *T.foenum graecum* EO that reduces Mo (IV) to Mo (V). The phosphomolybdenum assay result (2.18 mmol TE/g), revealed a weak correlation with other (CUPRAC, DPPH·, FRAP,) antioxidant tests. The evident differences between the assay results

are caused by the transfer of electrons/hydrogen from antioxidants that differ depending on the antioxidant's structure [21]. To further determine the antioxidant activity, the chelating activity was assessed against Fe^{2+} and reported as EDTA equivalents Metal (Fe^{2+}). In this assay, *T. foenum* showed good Fe^{2+} chelating ability (IC_{50} = 19.21 mg EDTAE/g) in comparison with the work of Bhanger et al. (2008) [17] (1.021±1.7 mg EDTAE/g).

Conclusion

This study is a detailed and comprehensive research about the phytochemical composition, antimicrobial and antioxidant activities of *Trigonella foenum-graecum* essential oil plant. The T.*foenum-graecum* seeds essential oil was extracted by the Clevenger technique. The GC-MS analysis reported that methyl palmitate is abundant in fenugreek essential oil, which is known for its wide use in food, pharmaceutical, cosmetic, and industrial applications. In addition, the plant essential oil showed significant antioxidant capacity, as the main chemical compositions of the oil namely, methyl palmitate, Dihydro methyl jasmonate, and other components with lower percentages have been proven to be effective in reducing free radicals (ABTS, $DPPH^{\cdot}$), reducing power (CUPRAC, FRAP) and in the chelating assay due to their natural antioxidant effects, while the phosphomolybdenum assay revealed a weak correlation with other ($DPPH^{\cdot}$, FRAP, CUPRAC) antioxidant assays. Thus, according to this study results, we can conclude that *Trigonella foenum-graecum* seed oil could be beneficial in the treatment of many chronic diseases and conditions. This study provides an important reference for further research aimed at discovering lead phyto-pharmaceuticals to control wellness human and health.

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Table 1. Chemical composition of the essential oil of the seeds of *Trigonella foenum-graecum.*

Peak	RT	Compounds	%
1	26.74	1-Carboxysalsoline	1.85
2	28.87	cis-Calamenene	2.05
3	29.21	Linalool	0.75
4	32.87	5-Fluoro-1,1,3,3-tetramethyl-1,3-dihydroisobenzofuran	4.32
5	34.34	Dihydro methyl jasmonate	13.47
6	36.14	Decane, 5,6-bis(2,2-dimethylpropylidene)-, (5Z,6Z)	20.18
7	37.66	Murolan-3,9(11)-diene-10-peroxy	2.45
8	38.44	Terpineol	0.81
9	38.62	cis-verbenol	0.47
10	41.85	2-Pentadecanone, 6,10,14-trimethyl	1.64
11	44.07	Methyl palmitate	20.45
12	44.36	2,8-diethoxy-1,7-diazatricyclo[7.3.0.03,7]dodeca-2,8-diene	4.75
13	44.74	Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl)	3.21
14	45.09	2-tridecanone	0.22
15	45.99	Ethyl palmitate	2.43
16	49.91	6-Octadecenoic acid	1.12
17	50.72	Methyl stearate	3.14
18	55.87	Oxiraneoctanoic acid, 3-octyl-, methyl ester, cis-	2.72
Total identified compounds (%)			**86.03**

Table 2. Biological parameters of the antimicrobial tests of *Trigonella foenum-graecum* essential oil.

Microbial strains	MIC (µL/mL)	MBC (µL/mL)	MBC/MIC (µL/mL)
Bacillus subtilis	57.7	87.7	1.52
Enterococcus faecium	42.3	67.3	1.59
Escherichia coli	59.46	79.46	1.34
Listeria monocytogenes	70.46	82.69	1.17
Staphylococcus aureus	40.47	60.47	1.49
Yersinia enterocolitica	52.5	62.5	1.19
Candida albicans	62.37	159.37	2.56

Table 3. Comparison of polyphenols content of *Trigonella foenum-graecum* essential oil in different studies

Parameters of activities	Value	Reference
TPC	5.75±0.002 mg GAE/g	[17]
TFC	2.827±0.22 mg RE/ g	[18]
Chelating activity	1.021±1.7 mg EDTAE/g	[17]

Table 4. Polyphenols content and the antioxidant activity of Fenugreek essential oil.

Parameters of activities	Value
Total phenolics content	30.74 mg GAE/g
Total flavonoids content	24.67 mg RE/ g
ABTS	78.22 mg TE/g
DPPH	54.17 mg TE/g
FRAP	58.31 mg TE/g
CUPRAC	108.33 mg TE/g
Phosphomolybdeum	2.18 mmol TE/g
Chelating	19.21 mg EDTAE/g