Assessment of bioactive compounds, antibacterial potential and acute toxicity of a volatile *Origanum compactum* essential oil, an endemic plant of northern Morocco

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Abstract: The search for a natural alternative against the resistance of bacterial strains pathogenic to antibiotics remains a necessity to prevent human diseases. In this work the identification of the chemical composition of the *Origanum compactum* essential oil's by GC/ MS revealed the presence of three major compounds: Carvacrol (72.97%), ρ-Cymene (14.5%), and γ-Terpinene (6.01%). The evaluation of the antibacterial activity of this essential oil and two synthetic antibiotics (Piperacillin 'PRL', Ampicillin 'AMP') was carried out by disc diffusion agar and micro-Dilution on agar medium for the determination of the minimum inhibitory concentration, against some strains (*Escherichia coli, Salmonella sp, Klebsiella pneumoniae, Pseudomonas aeruginosa, Streptococcus sp, Staphylococcus aureus*) of clinical origin. The toxicity assessment was conducted in accordance with OECD Protocol 423 (Organization for Economic Cooperation and Development). The results showed a strong antibacterial effect of the volatile essential oil compared to the two antibiotics used. The essential oil is classified in category 5 GHS (harmonized classification system) with LD_{50} greater than 5000 mg/kg. These results suggest the use of this natural product in the treatment and prevention of infectious diseases caused by pathogenic bacteria.

Key words: Medicinal Plants, *Origanum compactum*, essential oil, chemical composition, antibacterial activity, acute toxicity.

ملخص: إن البحث عن بديل طبيعي لمكافحة مقاومة السلالات البكتيرية للمضادات الحيوية يظل ضرورة للوقاية من الأمراض التي تصيب كومباكتوم (*Origanum compactum*) أوريجانوم الإنسان. في هذا البحث، تم تحديد التركيب الكيميائي للزيت الأساسي العطري لنبات بواسطة تقنية GC / SM، عن وجود ثلاثة مركبات رئيسية : كارفاكرول (72.97٪) ، ρ -سيمين (14.5٪) و γ-تربينين (6.01٪). كما تم إجراء تقييم الفعالية المضادة للبكتيريا من طرف هذا الزيت العطري واثنين من المضادات الحيوية الاصطناعية لتحديد التركيز الأدنى "الميكرو أروماتوچرام" و "الانتبيوچرام") بواسطة تقنيات 'AMP' Ampicillin، ('PRL' Piperacillin المثبط ضد السلالات السريرية:*Escherichia coli, Salmonella sp, Klebsiella pneumoniae, Pseudomonas aeruginosa, Streptococcus sp, Staphylococcus aureus* مع مقارنة الطيار الأساسي للزيت قوي تأثير النتائج بينت . أن (OECD)المضادات الحيوية المستخدمة. كما أظهرت نتائج تقييم السمية وفقًا للبروتوكول 423 لمنظمة التعاون الاقتصادي والتنمية تشير هذه النتائج .mg/kg5000 أكبر من DL_{50} (نظام التصنيف المنسق) مع جرعة قاتلة GHSهذا الزيت الطيار يصنف ضمن الفئة 5 .إلى إمكانية استخدام هذا المنتج الطبيعي للعلاج والوقاية من الأمراض المعدية التي تسببها البكتيريات

الكلمات المفتاحية: النباتات الطبية، الزيت العطري، التركيب الكيميائي، النشاط المضاد للبكتيريا، السمية الحادة، أوريجانوم كومباكتوم

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Introduction:

The fight against bacterial attacks is based mainly on the use of antibiotics. However, for several years, the selection of multidrug resistant strains has been observed in human medicine, for which the misuse of antibiotics as a therapeutic measure is largely responsible (Goossens et al., 2005). Among the pathogenic bacteria which have become real problems for human health, we find species belonging to the Pseudomonadaceae, Enterobacteriaceae, and bacteria of the Acinetobacter family which are almost resistant to all prescribed antibiotics (Bouyahya et al., 2016; Chaudhary, 2016).

In order to preserve human health, the discovery of new molecules has become an absolute necessity. The fields of investigation are vast but the exploration of natural resources especially plants appear to be most promising because they have the largest pool of active substances (Hmidani et al., 2021; Khribch et al., 2018). Currently, medicinal and aromatic plants (MAP) represent a considerable and permanent source for the extraction of active substances. In Morocco, more than 71% of respondents use MAP for treatment. Several recent studies have confirmed *in vitro* the antimicrobial activity of secondary metabolites from certain essential oils (Cassella, 2002; Hammer et al., 1999). In this respect, these volatile products obtained from aromatic plants could replace or strengthen the usual synthetic antibiotics.

The genus *Origanum*, belonging to the family of Lamiaceae, has 38 species which are widespread in the Euro-Suberian and Irano-Siberian regions. However, most species, about 75%, are concentrated around the Mediterranean, particularly in the Eastern Mediterranean regions (Elezi et al., 2013; Ruberto et al., 2002). *Origanum compactum* is an endemic plant of northern Morocco. It is widely used in Moroccan popular medicine because of its multiple therapeutic effects. In fact, it is recommended, among other things, in the treatment of diarrhea, respiratory, skin and urinary infections (Bouyahya et al., 2016). According to Bouyahya et al. (2016), the essential oil (EO) of oregano is presented in many books as a natural antibiotic acting on diverse pathogenic germs that attack the human body, while preserving the intestinal flora (beneficial bacteria).

In this context that the herein work was designed to obtain the essential oil of *Origanum compactum*, determine the main volatile compounds by GC / MS analysis, study the acute toxicity and the antibacterial activity of this volatile EO on resistant bacteria to synthetic antibiotics.

Material and methods:

Plant material:

The plants used in this work were harvested during the flowering period in the northwest of Morocco on the outskirts of the region of Kenitra. Before extraction, the harvested plant material was dried in the laboratory at room temperature in an airy place protected from light.

Essential oil:

The essential oil used in this work was obtained by hydrodistillation in a Clevenger type apparatus (Clevenger, 1928), from the aerial part of *Origanum compactum*; an aromatic and medicinal plant well known in the Moroccan traditional pharmacopoeia. The essential oil is stored at 4 °C in the dark, in presence of anhydrous sodium sulfate before it uses.

Phytochemical analysis of essential oil:

The EO was chemically analyzed by gas chromatography coupled to mass spectrometry (GC–MS) on a thermofischer capillary gas chromatograph coupled to the mass spectrometry system (GC ULTRA model S / N 210729). The analytical is performed by injecting 1 µl of essential oil, diluted using hexane as solvent (Ez-Zriouli et al., 2020).

Microbial strains studied:

The evaluation of the antimicrobial activity of *Origanum compactum* was performed against six selected bacterial strains (*Staphylococcus aureus*, *Streptococcus* sp, *Escherichia coli*, *Salmonella* sp, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*). These bacteria are maintained by transplanting onto a Muller Hinton agar nutrient medium that supports growth for 24 hours in the dark at 37°C.

Antibiotics:

The antibiotics used are Piperacillin (PRL), Ampicillin (AMP)

Antibacterial activity test:

In order to assess the antimicrobial activity of the volatile EO of the plant under study, sterile discs of 6 mm diameter Wattman paper soaked with 15 µl of essential oil and antibiotic discs to be tested were placed on the surface of the Muller Hinton agar medium, pre-inoculated by swabbing from standardized bacterial suspensions (10^8 cfu/mL). After that, Petri dishes were incubated at 37°C for 24 hours. The control was carried out under the same conditions

without essential oils or synthetic antibiotics. The experiments were repeated three times to confirm the results.

Strain sensitivity was assessed by measuring the diameters of inhibition zones around each disc. The strain was noted according to the following range (Boutabia et al., 2016; Ponce et al., 2003) :

Not sensitive (-) for diameter less sensitive than 6 mm;

Sensitive (+) for diameter between 9-14 mm;

Very sensitive (++) for diameter between 15-19 mm;

Extremely sensitive (+++) for diameter more than 20 mm.

Minimum inhibitory concentration:

The MIC value is determined by the agar microdilution technique (Bouterfas et al., 2016; Ez-Zriouli et al., 2019). The essential oil to be tested is emulsified in a 0.2% agar solution in order to obtain a homogeneous distribution in the culture medium and improve the contact of the EO with the tested germs, dilutions are prepared at 1/10, 1/25, 1/50, 1/100, 1/200, 1/300 and 1/500 in this agar solution.

1.5 mL of each dilution was added to test tubes each containing 13.5 mL of MH (Muller Hinton) agar medium, autoclaved for 20 minutes at 121°C and cooled to 45°C; to obtain final concentrations of 1/100 to 1/5000 (v/v). The tubes are then shaken well before being poured into Petri dishes. Control tubes, containing only the culture medium supplemented with the 0.2% agar solution alone, were also prepared.

After solidification, the medium is inoculated with bacteria using a calibrated plate to collect the same volume of inoculum, which is presented as a 24-hour culture broth.

The incubation is done at 37°C for 24 hours. Each test is repeated three times. The results give the MIC as the lowest concentration for which no growth was seen with the naked eye.

Statistical analysis:

The normality of the data distribution was analyzed by the Shapiro-wilk test, and the normality is fulfilled by the transformation when necessary. An analysis of variance two-way (ANOVA) was used to assess the Effect comparison of substances (Essential Oils and Antibiotics), bacterial species and their interaction on inhibition area diameter. The

processing averages are compared by the LSD t-Student test to the risk of error of 0.05. Furthermore, a Chi-square Cochran-Mantel-Haenszel test was used to analyze the association between the Essential Oils concentration and bacterial species resistance. The data were analyzed by JMP SAS Pro software (JMP®, Version <14>. SAS Institute Inc.)

Acute oral toxicity study:

The LD_{50} was determined in accordance with the Organisation for Economic Co-operation and Development (OECD) Directive 423. Animals used from the animal farm at the Faculty of Science ibn Tofail University of Kenitra. Adult Wistar female rats 2-2.5 months of age were kept for the test. After the fasting period of (3-4h), the animals were weighed and divided by concentration into 4 lots of 3 rats; the dose to be administered orally was calculated with reference to body weight. The first batch received the initial dose recommended by the OECD Guideline 423, 300 mg/kg orally, the second batch received the dose of 2000 mg/kg, the third batch received the 5000 mg/kg dose and the fourth batch (control group) received orally corn oil. The experiment was repeated twice. For each step, the animals were observed for toxic symptoms. Surviving animals were weighed and kept under observation for 14 days to record possible mortality to find LD_{50}. Moreover, the eventual toxicity of the EO on behaviour; ingestion of food and respiration was recorded, too.

Results and discussion:

Phytochemical analysis of essential oil:

The results of gas chromatography coupled with mass spectrometry (Fig 1) reveal the presence of seven volatile compounds, representing 99.99% of the total percentage of the EO with the major compounds are Carvacrol (72.97%), ρ-Cymene (14.5%) and γ-Terpinene (6.01%) (Tab 1). Our results are in agreement with other researchers who have worked on samples of oregano from different regions. The predominant compound identified was always carvacrol with different percentages 43.97% (Figuérédo et al., 2006), 55.9% (Hammou et al., 2011; Sbayou et al., 2014). On the other hand, in other studies, the results of chemical characterization showed that *Origanum compactum* from two sources (Moroccan forest 'Talambot' and 'Tanaqoub') is rich in thymol (20.49%, 27.49%), carvacrol (16,68%, 25,31%), γ-terpinene (26.11%, 22.65%), and o-cimène (20,68%, 9,76%)(Ghanmi et al., 2015). According to Ez-Zriouli et al. (2019), the variation of the chemical composition could be related to the geographical conditions or to the method of preparation.

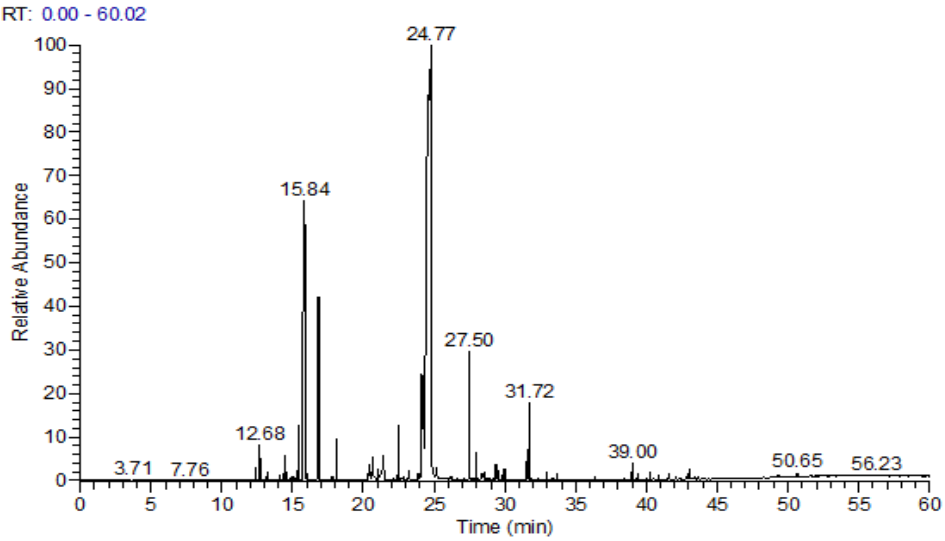

Fig 1. Analysis of essential oil of *origanum compactum* by GC / MS

Tab 1. Chemical composition of the essential oil of *O. compactum*

Chemical Compounds	Percentage (%)	Retention Time (RT) (min)
ß-Myrcene	1.08	15.41
p-Cymene	**14.50**	15.84
γ-Terpinene	**6.01**	16.85
Thymol	0.92	22.46
Carvacrol	**72.97**	24.77
Caryophyllene	2.61	27.50
Caryophyllene Oxide	1.90	31.72
Total	**99.99 %**	

Antibacterial activity test :

The study of the antimicrobial activity of natural substances such as essential oils and synthetic products such as antibiotics on bacterial disease agents is of great interest. In this regard, the antibacterial activity of marketed antibiotics and oregano essential oil was tested *in vitro* by performing disc diffusion method. Thus, the diameters of the inhibition zones allowed establishing an antibiogram (Fig 2). The inhibitory activity recorded differs from one bacterium to another and from one product to another. Indeed, in the presence of the pure essential oil of *Origanum compactum*, the values of the diameters of inhibition recorded are higher (from 1.1 cm to 4.5 cm) compared to those recorded in the presence of synthetic

antibiotics (from 0cm to 2cm) showing consequently the high inhibitory efficacy of *Origanum* EO.

However, the strain *Pseudomenas aeruginosa* shows resistance in comparison with other strains. These results state that our strains react differently to the products tested, demonstrating the mutagenic nature of these strains that allows them to develop antibiotic resistance.

Statistical analysis of variance for the variable "inhibition area diameter" (tab2), shows very highly significant differences for the factors "antibiotics", bacterial species and their interaction ($p < .0001$). The comparison of means for this parameter (tab3) indicates four groups for the bacterial species factor (*Salmonella* sp = *Staphylcoccus aureus* > *Escherichia coli* > *Streptococcus* sp = *Klebsiella pneumoniae* > *Pseudomonas aeruginosa*). Whereas the antibiotics factor reveals three groups (* *Origanum compactum* > AMP 10 > PRL 30*).

Tests carried out on the same clinical isolates to determine the minimum inhibitory concentration (MIC) of the volatile extract, allowed us to say that our product used caused an inhibition of the growth of bacterial strains at low concentration (Fig 3).

Our extract caused growth inhibition of Escherichia coli and salmonella sp from a concentration of 0.02% of EO in the culture medium, at concentration 0.05% the growth of *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* was inhibited as well, and *Streptococcus sp* at concentration 0.1%. Therefore, we can say that Escherichia coli and Salmonella sp strains are the most sensitive to the effect of oil of oregano in the culture medium (MIC=0.02%). These MIC values are lower than those reported in other works, in fact 0.45% of oregano oil was recorded as the average MIC capable of inhibiting the growth of *Pseudomonas aeruginosa* (Maggini et al., 2017).

Studies have found a selective bactericidal effect against pathogenic beneficial bacteria, Lactobacillus and Bifidobacterium less sensitive than the pathogens Salmonella and E. coli to the effect of oregano EO. On the other hand, other works have shown that this EO has a non-selective antibacterial activity against Gram-negative and Gram-positive bacteria(Betancourt et al., 2012).

It's interesting to note that the bacteria with the largest zones of inhibition by the agar diffusion method are not always those with the lowest MIC values (Yousif et al., 2021). This

is because the diameter of the growth inhibition zone is affected by the solubility of the oil in water and the volatility of the oil (Hernández et al., 2005).

The results of Cochran-Mantel-Haenszel Tests (Tab 4), show highly significant evidence for the association between resistance and bacterial species. Moreover, for the same bacterial species, the results showed a highly significant association between the inhibitory efficacy and the essential oil concentration.

The antibacterial activity of the essential oil is dependent on its nature and the strain studied. The study on the chemical composition of the essential oil of compact oregano used in this work has shown its richness in Carvacrol (72.97ρ-Cymène (14.5%), and γ- Terpinène (6,01%). Thus, we suggested that these major compounds are at the origin of the activity recorded in this work. Previous literature research has shown that carvacrol, which is composed mainly of oregano oil, has a broad spectrum of antimicrobial action against bacterial, fungal, mite and insect strains (Chami et al., 2005; Jeong et al., 2008). This inhibitory power is attributed to its hydrophobic character (Khribch et al., 2018).

To date, carvacrol is classified as one of the most effective plant antibacterial agents known (Nazer et al., 2005). Previous studies have shown that this molecule is able to inactivate bacterial strains in the food system as well as in synthetic media. Carvacrol can destabilize the cytoplasmic membrane and acts as a proton exchanger, thereby reducing the pH gradient across the cytoplasmic membrane. As a result the depletion of the ATP pool ultimately leads to cell death (Ultee et al., 2002).

In vitro testing has shown that carvacrol has antibacterial power against *Escherichia coli,* this may be due to the high content of oregano by this active ingredient alongside thymol as the majority phenolic compounds of this EO (Skoula et al., 1999). According to (Satrani et al., 2007), these compounds are often responsible for the antibacterial activity observed, because carvacrol is considered to be biocidal, with its precursor, p-cymene, endowed with an anti-weak bacterial, but probably acting synergistically with carvacrol through membrane expansion, resulting in destabilization of the bacterial membrane (Jamali et al., 2013). This does not preclude the possibility that minority EO compounds may be involved as antagonists or synergists with other active compounds.

Therefore, the activity could be attributed to the presence of other components such as p-cymene, γ-Terpinene, also known for their antibacterial activity. Moreover, studies have

shown that the presence of p-cymene, even in small quantities with carvacrol, acts in synergy by allowing the latter to be more easily transported into the cell (Ultee et al., 2002).

In addition, it has been reported that essential oils rich in phenolic components possess high levels of antimicrobial and cancer protective activity. However, the most abundant compounds are not necessarily responsible for the total activity; the involvement of less abundant constituents must also be considered (Sonboli et al., 2005).

Other works have shown interesting bactericidal properties of oregano EO in relation to the *E.coli* strain (Burt and Reinders, 2003). In parallel (Rhayour et al., 2003) confirmed that oregano and clove Essential Oil caused cell lysis of bacterial strains with rapid mortality on *Bacillus subtilis* and *E.coli*.

As, also seen in the results of this work, Essential Oil acts on both Gram-negative and Gram-positive bacteria. In this context, some researchers concluded that sub-lethal concentrations of carvacrol and thymol inhibit the enzymatic activity of coagulase and lipase in *Staphylococcus aureus*. Cell deformation has been observed in the scanning electron microscope confirming damage to the cell cytoplasmic membrane, which in turn causes disruption of protein secretion (Souza et al., 2013).

The diversity and molecular complexity of essential oils cause membrane alterations by penetrating these molecules into the cells, these molecules cross the lipid bilayer and penetrate inside and interact with intracytoplasmic targets (Cristani et al., 2007).

In addition to the hydrophobicity of molecules coming from essential oil, ensures their passage between membrane phospholipids and subsequently their solubilization in the lipid bilayer. This operation causes the destabilization of the membrane structure and a modification of its permeability to protons, ions and other cellular components (Carson et al., 2002; Souza et al., 2013; Trombetta et al., 2005; Ultee et al., 2002).

However, the strain *P. aeruginosa*, a gram-negative bacterium, remained the least sensitive bacterium to the effect of essential oil. This resistance is due to its membrane structure rich in waterproof lipopolysaccharides to hydrophobic compounds in the presence of permeable agents of the external membrane.

Acute oral toxicity study:

In the 14 days following observation of the four groups of rats treated with doses of 300 mg/kg, 2000 mg/kg, 5000 mg/kg and the control group, no evidence of toxic effects was detected, and no mortality was observed even at the 5000 mg/kg body weight dose. All animals were found to be normal until the end of the observation period, no significant change in behaviour or undesirable pathology was observed except that the appetite of the rats in the treated groups increased compared to the control in addition to their weight (fig 4).

This study determined the toxicological parameters of the volatile extract of oregano administered by gavage.

The estimated LD_{50} value is greater than 5000 mg/kg and therefore the extract is considered to be of low oral toxicity (Kassi Bosson et al., 2018). The absence of deaths at different doses makes it possible to classify the extract into category 5 of the harmonized classification system GHS according to the OECD 423 utility method. Therefore, the doses used in this work could be tolerated by the body is used safely in humans.

Conclusion

According to the present study, phytochemical analysis of oregano oil revealed the presence of three major compounds: Carvacrol (72.97%), ρ-Cymene (14.5%), and γ- Terpinene (6.01%). Furthermore, the extract showed an important antimicrobial effect; it proved to be remarkably active against the tested bacterial strains, exerting a significant inhibitory effect on these strains, the inhibition zones obtained with this active principle exceed those of the reference antibiotics and the MIC values vary between 0.1% and 0.02%. Considering these results, the use of *Origanum compactum* essential oil as a broad-spectrum antibiotic is encouraging.

The acute toxicity evaluation showed that the volatile extract is weakly toxic in vivo by the oral route; LD50 higher than 5000 mg/kg VO which gives it a safety character.

However, in addition to the present toxicity study, the in vivo evaluation of the pharmaco-dynamic characteristics and the biological tolerance by the determination of some biochemical and haematological parameters in animals is also necessary for a better rationalization of the use of this plant.

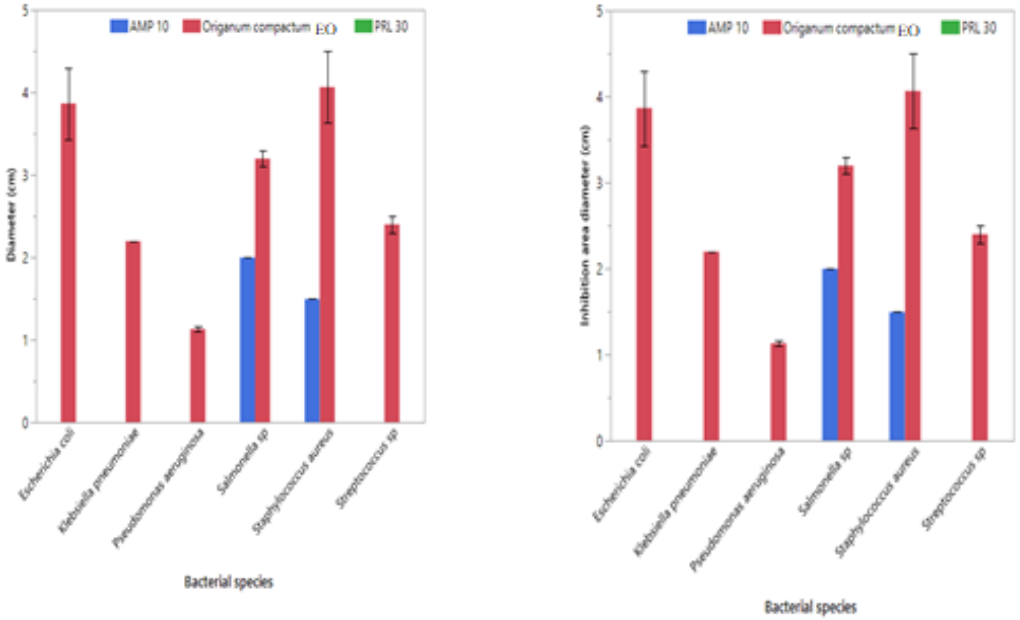

Figure 2. Inhibition area diameter according to antibiotics and bacterial species. Data are presented as mean (±SE). PRL: Piperacillin; AMP: Ampicillin

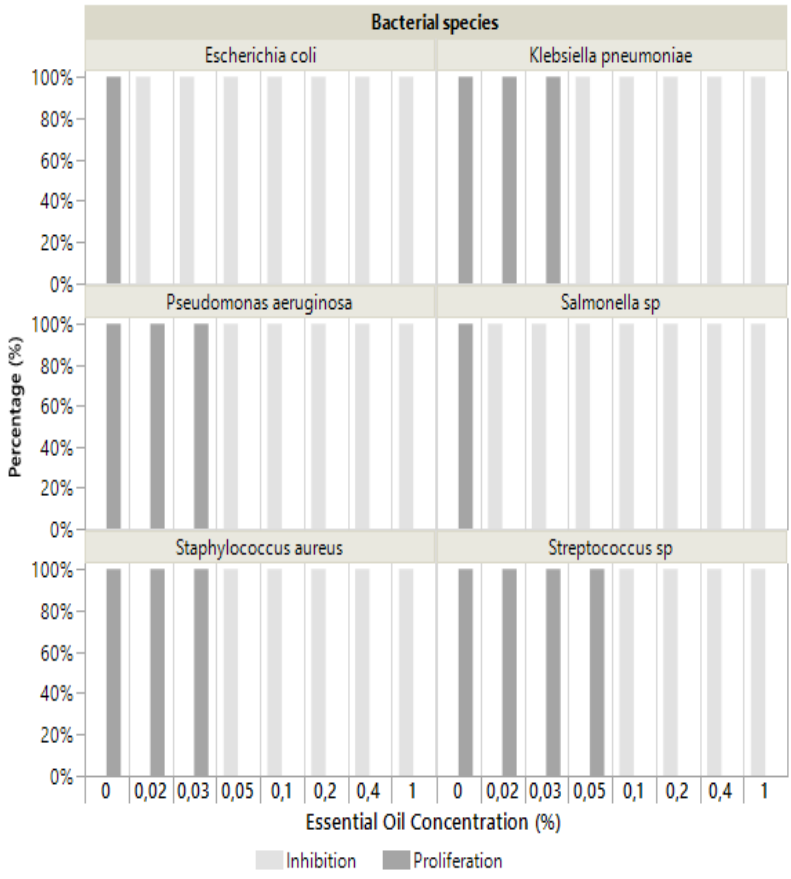

Figure 3. Minimum inhibitory concentration of *Origanum compactum* essential oil

Table 1. ANOVA results to test for inhibition area diameter across substances (antibiotics) and bacterial species. ***: significant at 0.1% level; DF: Degree of freedom

Source of variation	DF	Sum of Squares	Mean Square	F Ratio	P-value
Antibiotics	2	79,23	39,62	599,246	<,0001*
Bacterial species	5	15,73	3,15	47,586	<,0001*
Antibiotics*Bact. Sp	10	15,49	1,55	23,435	<,0001***
Model	17	110,46	6,50	98,281	<,0001***
Error	36	2,38	0,07		
C. Total	53	112,84			

Table 2. Effect comparison of substance (antibiotics), bacterial species and their interaction on inhibition area diameter. Data are presented through the means ± SE.

	Parameters
Bacterial species	**Inhibition area diameter (cm)**
Escherichia coli	1,29±0,66b
Klebsiella pneumoniae	0,73±0,37c
Pseudomonas aeruginosa	0,38±0,19d
Salmonella sp	1,73±0,47a
Staphylococcus aureus	1,86±0,61a
Streptococcus sp	0,80±0,40c
Antibiotics	
AMP 10	0,58±0,20b
Origanum compactum (EO)	2,81±0,26a
PRL 30	0,00±0,00c
Antibiotics x Bacterial species	
AMP 10	
Escherichia coli	0,00±0,00^{e}
Klebsiella pneumoniae	0,00±0,00^{e}
Pseudomonas aeruginosa	0,00±0,00^{e}
Salmonella sp	2,00±0,00c
Staphylococcus aureus	1,50±0,00d
Streptococcus sp	0,00±0,00^{e}
***Origanum compactum* (EO)**	
Escherichia coli	3,87±0,43a
Klebsiella pneumoniae	2,20±0,00c
Pseudomonas aeruginosa	1,13±0,03d
Salmonella sp	3,20±0,10b
Staphylococcus aureus	4,07±0,43a
Streptococcus sp	2,40±0,10c
PRL 30	
Escherichia coli	0,00±0,00^{e}
Klebsiella pneumoniae	0,00±0,00^{e}
Pseudomonas aeruginosa	0,00±0,00^{e}
Salmonella sp	0,00±0,00^{e}
Staphylococcus aureus	0,00±0,00^{e}
Streptococcus sp	0,00±0,00^{e}

For the same parameter, means linked with the same letter were not significantly different at $p = 0.05$ (a, b, c, d, and e) ; represent the classes generated by the comparison of Bonferroni means

Table 3. Cochran-Mantel-Haenszel Tests. *: significant at 0.1% level; DF: Degree of freedom

	Bacterial species			Essential Oil Concentration		
CMH Test	**ChiSquare**	**DF**	**Prob>Chisq**	**ChiSquare**	**DF**	**Prob>Chisq**
Correlation of Scores	11,727	1	0,0006*	82,143	1	<,0001*
Row Score by Col Categories	36,429	5	<,0001*	100,280	7	<,0001*
Col Score by Row Categories	11,727	1	0,0006*	82,143	1	<,0001*
General Assoc. of Categories	36,429	5	<,0001*	100,280	7	<,0001*

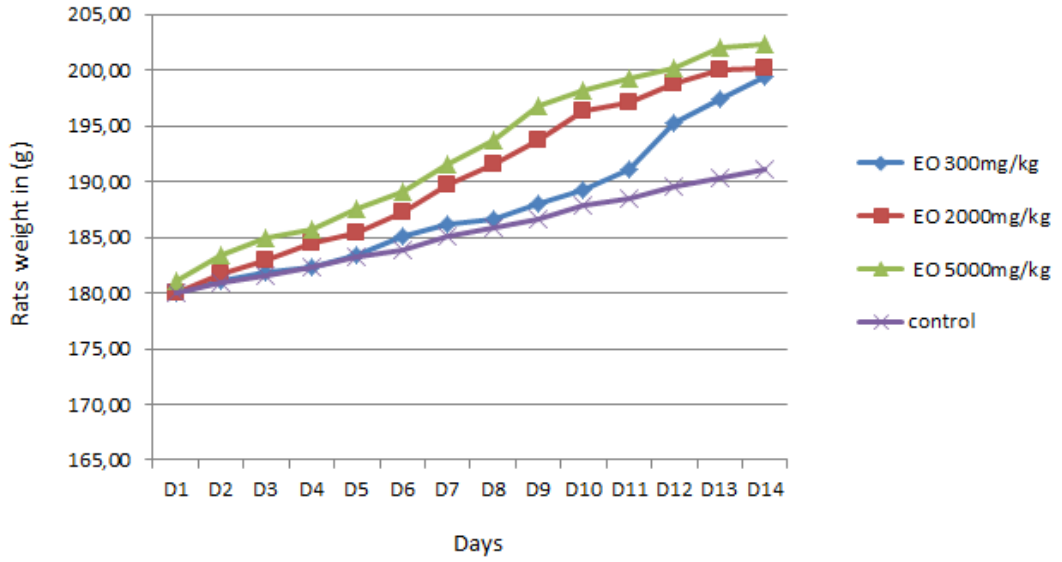

Figure 4. Variation in the mean weight of rats for 14 days after oral administration of 300, 2000 and 5000 mg/kg of oregano compact essential oil.

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