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The antibacterial effect of phenolic monoterpene-containing essential oils: xenohormetic significance and new approaches.

DEGREE FINAL PROJECT

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A mis padres, hermana y amona, que me apoyan, y han hecho que sea lo que soy hoy en día.

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1. Abstract

The xenohormesis is the process by which an organism takes advantage of molecular stress responses originally formed in another organism, in order to obtain health benefits. Carvacrol and thymol are the main phenolic monoterpenes, which are very common among *Origamus* and *Thymus* genera and they are formed during bacterial infections, showing antibacterial properties that could be beneficial for consumers. Consequently, phenolic monoterpenes might support xenohormesis hypothesis.

On the other hand, the rates of antibiotic multi-resistant bacteria are increasing each year, rising death rates and economical costs. Therefore, phenolic monoterpenes could bring us an alternative against this problem. For this reason, the antimicrobial activities of two commercial phenolic monoterpene-containing essential oils obtained from *Thymus vulgaris* and *Origanum compactum* were tested against avian strains of *Pseudomona aureginosa*, *Escherichia coli*, *Bacillus subtillis* and *Staphylococcus aureus* using disk diffusion assay. Gram-negative bacteria showed more resistance. Moreover, combinations of those essential oils with streptomycin and penicillin were tested against *Escherichia coli* and a second *Pseudomona aureginosa* strain. Results showed a slightly increase on the inhibition halo in comparation with the essential oil alone. Altogether, results indicate that this synergy may vary depending on the bacteria strain, oil composition and the antibiotic.

Additionally, a bibliography research was performed in order to associate the antibacterial mechanism of phenolic monoterpenes and its xenohormetic significance. Results indicate that thymol and carvacrol show antibacterial properties due to a combination of membrane disruption, biofilm and motility reduction, and ATPases and efflux pump inhibition. In relation with this, phenolic monoterpenes might be a good representation of this process since they are induced by plant bacterial infections and at the same time, show antimicrobial activity that might be beneficial for consumers. Indeed, this match between original cause of synthesis, and effect, perhaps compose a subtype of xenohormesis, *direct* or *original stress related xenohormesis*.

Key words: phenolic monoterpenes, xenohormesis, antibacterial.

2. Introduction

2.1 What is xenohormesis?

2.1.1 Background

Plants have been known for its benefits to human health for millennia. Their pharmacological properties explain why one third of top 20 drugs on the market are plant-derived molecules. Actually, it gets little attention the reason why plant kingdom produces this number of beneficial substances for superior animals [1].

What is known, is the ability of plants for producing secondary metabolism products to respond environmental stress. These changes include variations in photoperiod, light intensity, water and salt concentration, as well as biological pathogens. These metabolites include phenolics, alkaloids, terpenoids and other molecular groups like jasmonates and salicylic acid [2]. However, this biosynthetic response is not only found in plants but also in bacteria, fungi and algae [3].

For example, sialic acid modulates the synthesis of jasmonic acid and it also inhibits prostaglandin synthesis in mammals due to the binding with COX-1 and COX-2, modulating immune responses such as inflammation. This shared signaling interaction and mechanism was firstly defined as ''phylogenetic spionage'' since herbivores would benefit from a plant defense mechanism [4].

2.1.2 Examples of interaction and consequences

Even though the ''phylogenetic spionage'' clarifies some aspects of this plant-animal interaction, the definition is limited since there are some compounds that produce a benefit to consumers despites not having a chemical homologue [1]. Polyphenols are a good example because this group of plant-stress induced molecules that do not have a biosynthetic analogue in animals but tend to be beneficial for them thanks to the interaction with diverse receptors. Consequently, it has been described some direct polyphenol-receptor interactions [5].

As a response to this evidence, the xenohormesis hypothesis was proposed back in 2008. The word xenohormesis comes from the term *xenos*, meaning stranger in Greek, and *hormesis*, the health improvements of stress responses [1]. The objective is to postulate that xenohormesis is the processes by which an organism takes advantage of molecular stress responses originally formed in another organism, typically plants, in order to obtain health, well-being and adaptability benefits [5]. Therefore, xenohormesis describes an inter-specie hormesis proposing that animals and fungi have evolved maintaining the ability to interact with stress-induced molecules, from the simplest phenolic acids, to the more complex marine compounds from other types of species [1, 3].

Additionally, human health has not been commonly related to the global economy but the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) that emerged in Wuhan on late 2019, has caused a major health and economic crisis over the world due to its high transmissibility and health care collapse [6]. Thereupon, human health and economy highly depend on the ability to find new and effective drugs to combat those emergencies. The understanding of why plants synthesize beneficial compounds for health would be beneficial because it does not get much attention and could help minorizing the losing favor of plant derived drugs in front of new chemical entities [1].

2.2 Phenolic monoterpenes

2.2.1 Characteristics

Phenolic monoterpenes or monoterpenic phenols, are a group of monoterpenoid compounds that include Carvacrol, 5-iso-propyl-2-methylphenol), and thymol (2-isopropyl-5-methylphenol) as the main entities of this subgroup [7, 8, 9].

2.2.1.1 Chemical structure and physical properties

Phenolic monoterpenes are composed by a single phenolic ring formed from the bonding of two isoprene molecules that include three functional group substituents [7]. The isomers, carvacrol and thymol, contain the hydroxyl group attached at the C-1 position but the methyl and isopropyl group position varies among them. Carvacrol possesses the methyl group attached at the C-2 position and the isopropyl group attached to C-5 position, meanwhile thymol has a C-5 methyl group and a C-2 isopropyl group as it can be seen in **Figure 1**. [8, 9]

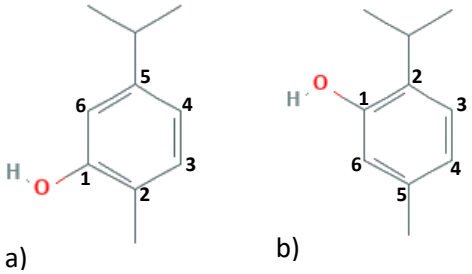

Figure 1. Chemical structure of carvacrol (a) and thymol (b) with numbered carbon atoms of the phenolic ring [10,11].

2.2.1.2 Natural source

Thymol and carvacrol are abundant in the essential oil of two culinary herbs, obtained mainly from oregano (*Origanum vulgare L.*) and thyme (*Thymus vulgaris L.*) [10] and in other plant species from the same genera, as well as others such as *Coridothymus, Thymbra, Satureja* and *Lippia*. [11].

2.2.1.3 Biosynthesis

Both phenolic monoterpenes and phenolic monoterpene-containing essential oils are formed in response to bacterial infections [12] and blue light-mediated stress [13]. However, the composition and quantity of essential oils varies strongly between plant populations and accessions [11].

Carvacrol and thymol are often described as phenols, but they are not related to phenolic synthesis [11], instead, a not validated biosynthetic pathway mediated by the precursor γ-terpinene and the intermediate p-cymene was firstly proposed, and latter elucidated. The formation of thymol and carvacrol first includes a monoterpene synthase that forms γ-terpinene, followed by a cytochrome P450 oxidation causing aromatization. The reaction is promoted by allylic alcohol intermediates and originating side products like p-cymene. Furthermore, a proposal of thymol synthases was made, being CYP71D178, CYP71D179 and CYP71D82 the thymol synthases while CYP71D180 and CYP71D181 were proposed to be two carvacrol synthases. [10]

2.2.2 Biological properties

Carvacrol and thymol have shown different but similar bioactive properties such as antibacterial, antifungal and antiviral effects, as well as other potential pharmacological activities like anti-inflammatory, anticancer and hepatoprotective. For this reason, it is not very surprising that thymol and carvacrol containing essential oils have taken part of different traditional medicines [14, 15].

On the other hand, the number novel antimicrobial approved drugs have decreased over the last decades [1] and the increase of difficult or impossible to treat bacteria is becoming a mayor health crisis with higher infection mortality rates and health costs [16]. Therefore, plant derived products such as phenolic monoterpenes-containing essential oils provide antibacterial activity can be useful as an alternative against multi-resistant bacteria [10]. In addition, the use of essential oils can induce an increase of the antimicrobial activity of antibiotics [17].

3. Hypothesis and objectives.

The global health and economic crisis caused by the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has made us aware about the importance of finding new and effective drugs to combat those emergencies. Moreover, the sum of multidrug-resistant bacteria infections is increasing globally every year, and the high number of untreatable infections is becoming a real problem, making the World Economic Forum of Global Risks to report antibiotic resistance as one of the mayor threats to human health. Unfortunately, the number of novel antimicrobial approved drugs have decreased over the last decades, and the increment of difficult or impossible to treat bacteria, rises infection mortality rates and health costs.

On the other hand, plant derived products such as phenolic monoterpenes, carvacrol and thymol, and phenolic monoterpenes-containing essential oils obtained from plants, could be useful as an alternative against multi-resistant bacteria due to its different antimicrobial properties. At the same time, xenohormesis hypothesis can help explaining and understanding the significance of this plant-animal interaction. Xenohormesis is the processes by which an organism takes advantage of molecular stress responses originally formed in another organism. For example, Thyme and Oregano plants originate the antimicrobial phenolic monoterpene-containing essential oils in response to a stress originated from bacterial infections, and they might later help humans or animals that consume those producers against bacterial infections.

Thereupon, xenohormesis hypothesis would explain why molecules obtained by plant stress mediated pathways, such as carvacrol and thymol, can be useful for humans. The beneficial effects of this compounds on consumers might be related or not to the initial stress that made the producer biosynthesize them. Therefore, there might exist **two types of xenohormesis, direct and indirect. The antimicrobial activity of phenolic monoterpenes after their consumption might be an example of xenohormesis, which could be denominated as *direct* or *original stress related xenohormesis*.**

Subsequently, the main objective is to corroborate the antibacterial effect of phenolic monoterpenes and phenolic monoterpene-containing essential oils as an example of direct xenohormesis.
In order to assess the established assumption, specific objectives are proposed:

1. To evaluate the antimicrobial effect of phenolic monoterpene-containing essential oils and its possible benefits against drug resistant bacteria.

2. To associate phenolic monoterpenes with xenohormesis hypothesis by grouping different molecular basis that are involved on its antimicrobial effects, and its relationship with xenohormesis hypothesis.

4. Materials and methods

4.1 Antimicrobial activity assays

Disk diffusion assays using phenolic monoterpene containing essential oils were performed following Mith et al. method [18], in order to confirm the antibacterial effect of phenolic monoterpenes against different avian bacteria previously isolated at *Avian health center of Catalonia and Aragon-CESAC* (Reus, Spain).

4.1.1 Essential oils

Phenolic monoterpene containing-essential oils provided by *MyCosmetik* (Chasselay, France) were screened for antimicrobial activity. Two types of oils were tested, a thymol based oil (TEO) obtained from *Thymus vulgaris,* composed of thymol 54,7% and carvacrol 3,8% (% v/v), and a carvacrol-rich essential oil (OEO) originated from *Origanum compactum*, containing carvacrol 53,23% and thymol 8,59% (% v/v). These essential oils were stored at ambient temperature before use.

4.1.2 Bacterial strain

In order to assess the antibacterial effects of the essential oils tested, four strains of pathogenic or nonpathogenic avian bacteria were used in the study. Two of them were adequacy characterized, being *Escherichia coli* WDCM 00012 CECT 516 and *Staphylococcus aureus* WDCM 00034 CECT 435. The other two bacteria were strain uncharacterized *Pseudomona aeruginosa* and *Bacillus subtilis*, obtained from previously analyzed and isolated avian samples. The first *Pseudomona aeruginosa* (1) was employed during the preliminary disk diffusion assay and the second (2), was studied during the antibiotic and essential oil combination disk diffusion assay. Strain election was performed with the aim to study two gram-positive bacteria, *Staphylococcus aureus* and *Bacillus subtillis*, and two gram-negative bacteria, *Escherichia coli* and *Pseudomona aeruginosa*.

4.1.3 Preliminary disk diffusion assay

A previous determination of the antibacterial effects of Thyme and Oregano essential oils was performed in quadruplicate by using paper disk diffusion method. Both essential oils were diluted using ethanol at the following concentration rates 1/1, 1/2, 1/4, 1/10, 1/20 and 1/40 (v/v).

Then, 20 μL of each dilution was administered to 6 mm diameter paper disks. In addition, paper disks inoculated with just 20 μL of ethanol were also prepared. All of the disks were later dried for 45 minutes at 36,5°C.

Bacteria were plated using a standard inoculum of each bacteria strain, that was previously elaborated with PBS buffer solution with the aim to get a 0,5 McFarland standard number. Then, each inoculum was diluted by 1/100 with PBS in order to get $1,5x10^6$ CFU/mL standardized bacteria concentrations.

Consequently, 200 µL of each bacteria inoculum were administered and scattered on the medium surface. Mueller-Hinton agar medium (MHA) was used for growing *Pseudomona aeruginosa* and *Escherichia coli*, and trypticase soy agar (TSA) medium was employed for *Bacillus subtlis* and *Staphylococcus aureus.*

One of each essential oil dilution paper disk was placed on the agar medium plus the control disk for one and all bacteria. In addition, two more agar plates were used for each strain, one as a bacterial growth control and the other one was employed in order to locate two additional paper disks with 20 µL of streptomycine 50 mg/mL and peniciline 1U/mL. Agar plates were later incubated at 36,5°C for 24h.

After incubation, the diameter of the inhibition halo of each paper disk was measured and a zone of diameter > 7 mm was considered as positive.

4.1.4 Essential oil and antibiotic combination disk diffusion assay

A combination of streptomycin plus oregano or thyme essential oils and penicillin together with those essential oils was administered to paper disks, in order to check if there is an increase of the antibacterial effect versus the more resistant *Escherichia coli* and *Pseudomona aeruginosa* in quadruplicate. New paper disks contained 10 µL of streptomycine 50 mg/mL and penilicile 1U/mL used on the previous study plus 10 µL of essential oil dilution. In the case of *Escherichia coli*, 10 µL of thyme or oregano essential oil 1/20 was used in order to obtain a final 1/40 (v/v) oil dilution, as this is the minimum concentration that showed a positive result on both essential oils on preliminary test. *Pseudomona aeruginosa* showed resistance against both essential oils, thereupon, a secondary *P. aeruginosa* strain and 10 µL of maximun oil concentration was used together with each antibiotic, obtaining a final 1/2 (v/v) concentration on both essential oils.

Mueller-Hinton agar plates were plated with 200 µL $1,5x10^6$ CFU/mL of each resistant bacteria inoculum and scattered. Plates were later incubated at 36,5°C for 24h. Different paper disk controls were employed, one containing just ethanol and the rest carrying each essential oil or antibiotic final dilution. After incubation, the diameter of the inhibition halo of each paper disk was measured and compared.

4.2 Bibliography research

The bibliography research was performed taking on count all the authors and publications until March 2021. The following databases were screened: PubMed, Google Scholar and Web of Science. No restrictions were placed on the publishing language or dates in order to cover as much as possible the thematic. Different combinations of terms were used to search literature such as ‘’xenohormesis’’, ‘’phenolic monoterpenes’’, ‘’carvacrol’’, ‘thymol’’, ‘’properties’’, ‘’antibacterial’’, ‘’antibiotic’’, ‘’mechanisms’’, ‘’biosynthesis’’ and so on. In addition, the chemical data base PUBCHEM was also consulted with the expressions ‘’carvacrol’’ and ‘’thymol’’.

5. Results and discussion

5.1 Preliminary disk diffusion assay.

The antibacterial effect of phenolic monoterpene-containing essential oils against *Bacillus subtilis*, *Staphylococcus aureus* WDCM 00034 CECT 435 and *Escherichia coli* WDCM 00012 CECT 516 is summarized in **Table 1**. The average values of inhibitory diameter of inhibition and standard derivations were calculated for 1/10, 1/20, 1/40 (v/v) essential oil concentration including diameter of 6 mm paper disk. The measures of 1/1, 1/2 and 1/4 (v/v) oil concentrations were excluded since they showed shared inhibition halos in almost all plates and they were impossible to measure. *Pseudomona aeruginosa 1* values were also excluded since no antimicrobial activity was shown as it is appreciated in **Figure 2**.

Table 1. *Inhibitory diameters of Thymus vulgaris and Origanum compactum essential oils against Bacillus subtilis, Staphylococcus aureus WDCM 00034 CECT 435 and Escherichia coli WDCM 00012 CECT 516 using paper disk diffusion method.*

	Bacillus subtlis				*Staphylococcus aureus*				*Escherichia coli*			
	Control[1]	1/40[1]	1/20[1]	1/10[1]	Control[1]	1/40[1]	1/20[1]	1/10[1]	Control[1]	1/40[1]	1/20[1]	1/10[1]
Essential oil	Diameter of inhibition (mm)[2]											
Thymus vulgaris	-[3]	11±0.8	15.3±2.22	25.8±3.3	-[3]	9.3±0,9	12.5±2.4	19.25±2.1	-[3]	10±0.5	13±2.2	17±3
Origanum compactum	-[3]	9.5±0.8	13.8±1.4	21.7±2.7	-[3]	8.3±0.5	11,7±2.5	19,7±1.7	-[3]	8.3±0.9	10.7±2.2	16±2

[1] Concentrations employed (1/40, 1/20, 1/10) are represented as v/v. Paper discs without essential oil were used as controls.
[2] Values are average diameter of inhibition halo ± standard derivation of four replicates. The diameter of the 6 mm paper disk is also considered. [3] Paper discs with <7 mm diameter were rejected as antimicrobial activity was not shown.

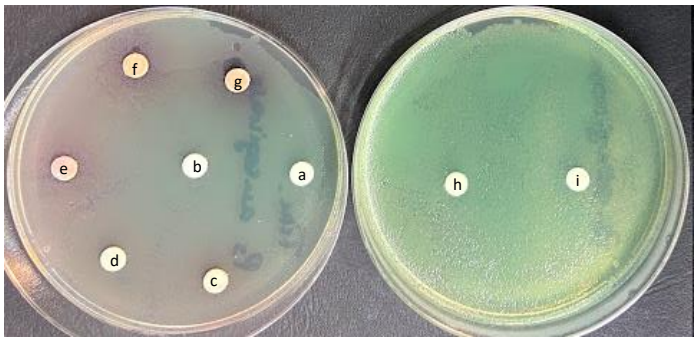

Figure 2. *Pseudomona aeruginosa* 1 plate during preliminary disk diffusion assay after 24h incubation against *Thymus vulgaris* essential oil. Letters (a) to (g) are discs in order: control, 1/40, 1/20, 1/10, 1/4, 1/2, 1/1 (v/v) concentrations. Letters (h) and (i) are discs containing penicillin 1U/mL and streptomycin 50 mg/mL respectively.

Even though essential oils are not pure substances, the fact that *Origanum compactum* essential oil contains a mayor carvacrol concentration and *Thymus vulgaris* essential oil a higher thymol concentration, as it is said in section 4.1.1, could bring us an idea of what characteristics or differences on the antimicrobial effects these compounds have.

Additionally, disk diffusion method is frequently used to screen the antimicrobial activity of plant extracts, but it contains limitations giving quantitative data because of the hydrophobic nature of essential oils that do not spread out properly over the agar medium [19]. Even with the relative hydrophilia of phenolic monoterpenes, some values have an elevated standard derivation, therefore, this assay could bring us an idea of the antimicrobial activity of phenolic monoterpene-containing essential oils, but we must be concerned about its limitations.

The essential oils of *Thymus vulgaris* and *Origanum compactum* showed permanently strong antibacterial effect against all tested bacteria in all different measured concentrations (1/40, 1/20, 1/10 v/v). As it was expected, the diameter of inhibition halo increases at higher concentrations, meaning that the concentrations of the not performed measures (1/4, 1/2, 1/1 v/v) would have had bigger inhibition zones. This explains why higher essential oil concentrations shared inhibition halos.

It is important to mention that *Escherichia coli* is the least sensible bacteria to *Thymus vulgaris* essential oil. *Bacillus subtilis* seems to be the most sensible strain as the rest of the bacteria had slightly smaller inhibition halos. *Staphylococcus aureus* is the second most sensible bacteria against this essential oil. This agrees with a similar previous study performed by Gedikoğlu et al. [20], where *Staphylococcus aureus* and *Bacillus* cereus appeared to be more sensible than *Escherichia co*li against a *Thymus vulgaris* essential oil originally obtained in Turkey.

On the other hand, *Origanum compactum* essential oil appeared to be less effective against *Escherichia coli* and *Bacillus subtilis,* the most affected bacteria. This result matches with the Bouyahya et al. conclusions [21], where *Origanum compactum* essential oil exhibited significant antibacterial effect on both bacteria, being *Bacillus subtilis* the most affected bacteria. Same authors affirmed that the antibacterial mechanism of *Origanum compactum* essential oil against *Escherichia coli* was originated by a cell wall and membrane disruption [21], just as it was described before [22, 23]. Moreover, this type of essential oil demonstrated to reduce the formation of *Bacillus subtilis* biofilms, a quorum sensing regulated process [21], giving us an idea of which are the antimicrobial processes that tend to occur on each bacterium.

The fact that *Thymus vulgaris and Origanum compactum* essential oils showed the lowest antimicrobial response against *Escherichia coli,* indicates that Gram-positive positive bacteria might tend to be more sensible to phenolic monoterpene-containing essential oils than Gram-negative *Escherichia coli,* as it was previously described [24]. Consequently, the less permeable Gram-negative bacteria cell wall seems to make them less susceptible against essential oils because of the presence of lipopolysaccharides [25].

In contrast*, Pseudomona aeruginosa 1* demonstrated to be resistant against both essential oils and antibiotics as no inhibition halo can be appreciated in **Figure 2**. However, a reduction of *Pseudomona aeruginosa 1* biomass growth is suspected as a lighter green tone appears on the essential oil containing plate. This may be related to a decrease on biofilm production as it has been described before [26].

Moreover, *Pseudomona aeruginosa* has been defined as a protected bacterium from carvacrol and thymol components when embedded in biofilms due to a reduced oil diffusion, growth rate decrease, and the production of enzymes able to degrade the antimicrobial substances [27]. Nevertheless, no quantitative test on biomass production was performed during this project.

5.2 Essential oil and antibiotic combination disk diffusion assay

The antibacterial effect of phenolic monoterpene-containing essential oils in combination with streptomycin 50mg/mL and penicillin 1U/mL against *Pseudomona aeruginosa* 2 and *Escherichia coli* WDCM 00012 CECT 516 is summarized in **Table 2** and **Table 3**. Paper disks with neither essential oil or antibiotic were used as controls and essential oil or antibiotic only containing disks are also included. The average values of inhibitory diameter of inhibition and standard derivations were calculated taking on count the diameter of 6 mm disks.

This assay reveals that neither penicillin nor streptomycin showed an antimicrobial effect against the two tested bacteria. A slightly synergism is appreciable on *Origanum compactum* essential oil plus penicillin or streptomycin against *Escherichia coli* that can be seen on **Figure 3**. This result partly coincides with Palaniappan et al. [28] studies, since penicillin was demonstrated to have synergy with carvacrol and thymol against *Escherichia coli* N00 66 using the checkerboard assay. However, our study did not attempt that thymol synergism with *Thymus vulgaris* essential oil as Gallucci et al. [29] also did against *Escherichia coli* obtained from a clinical isolate, conversely, this last article revealed no synergism with carvacrol as Palaniappan et al. [28] and we did.

On the other hand, our results indicate that *Thymus vulgaris* essential oil and penicillin increases the antibacterial effect against *Pseudomona aeruginosa* 2. This fact can be comparable with El-Hosseiny et al. [30] studies, as they demonstrated that piperacillin, a type of ureidopenicillin, in combination with thyme essential oil solved the antibiotic resistance and showed synergism. In addition, it is important to take on count that both *Thyme vulgaris* and *Origanum compactum* essential oils showed antimicrobial activity against Pseudomona *aeruginosa* 2, in contrast with *Pseudomona aeruginosa* 1 as it can be appreciated in **Figure 3**.

Altogether, this evidence reveals that phenolic monoterpenes-containing essential oils have great antimicrobial activity, and they might increase the antimicrobial effect of antibiotics. This could later reduce the antibiotic resistance problem. However, this synergy may vary depending on the bacteria strain, if a pure substance or essential oil is employed or the antibiotic type. There so, further studies are needed.

Table 2. *Inhibitory diameters of the antimicrobial combination of Thymus vulgaris essential oil plus streptomycin and penicillin against Pseudomona aeruginosa 2* and *Escherichia coli WDCM 00012 CECT 516 using paper disk diffusion method.*

	Control[1]	STR[2]	PEN[2]	TEO[2]	STR + TEO[2]	PEN + TEO[2]
	Diameter of inhibition (mm)[3]					
Pseudomona aeruginosa 2	-[4]	-[4]	-[4]	8,7±2,1	9±1,5	10±2,1
Escherichia coli	-[4]	-[4]	-[4]	9,7±2,1	10±0,9	9,5±0,9

[1]Paper discs without essential oil or antibiotic were used as controls. [2](STR) streptomycin 50mg/mL, (PEN) penicillin 1U/ml, TEO (*Thymus vulgaris* Essential Oil 1/2 v/v for *P. aureginos*a and 1/40 v/v for *E. coli*). [3]Values are average diameter of inhibition halo ± standard derivation of four replicates. The diameter of the 6 mm paper disk is also considered. [4] Values with <7 mm diameter were rejected.

Table 3. *Inhibitory diameters of the antimicrobial combination of Origanum compactum essential oil plus streptomycin and penicillin against Pseudomona aeruginosa 2* and *Escherichia coli WDCM 00012 CECT 516 using paper disk diffusion method.*

	Control[1]	STR[2]	PEN[2]	OEO[2]	STR + OEO[2]	PEN + OEO[2]
	Diameter of inhibition (mm)[3]					
Pseudomona aeruginosa 2	-[4]	-[4]	-[4]	8,3±2	9,5±2.1	8±1,6
Escherichia coli	-[4]	-[4]	-[4]	7,7±1,6	10,25±0,9	9±1,6

[1]Paper discs without essential oil or antibiotic were used as controls. [2](STR) streptomycin 50mg/mL, (PEN) penicillin 1U/ml, OEO (*Origanum compactum* Essential Oil 1/2 v/v for *P. aureginosa* and 1/40 v/v for *E. coli*). [3]Values are average diameter of inhibition halo ± standard derivation of four replicates. The diameter of the 6 mm paper disk is also considered. [4] Values with <7 mm diameter were rejected.

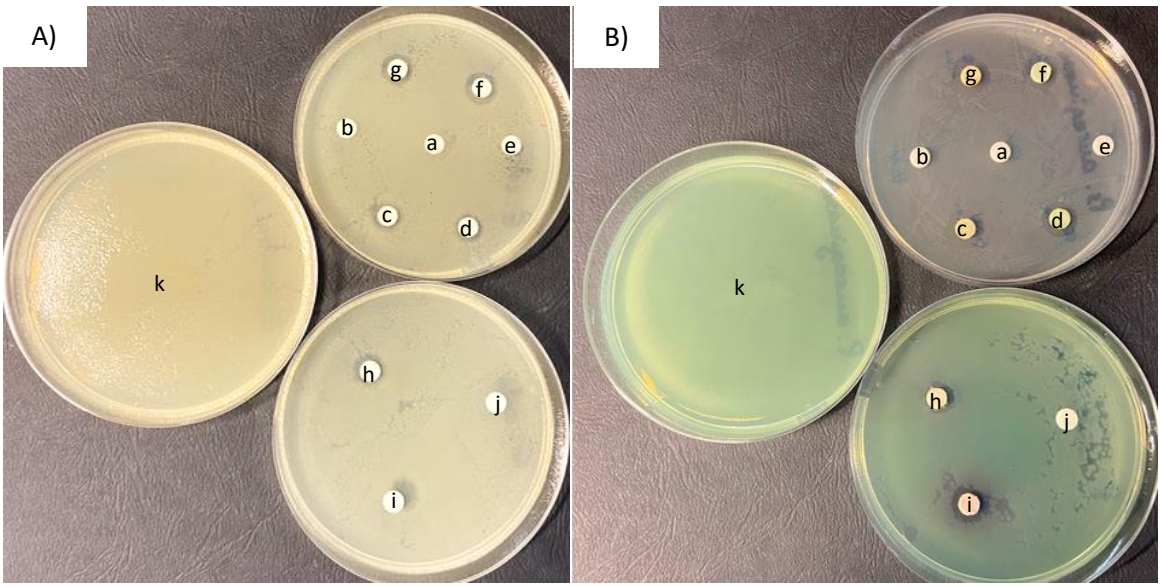

Figure 3. A) *Escherichia coli* and B) *Pseudomona aeruginosa* 2 plate during antimicrobial combination assay after 24h incubation against *Thymus vulgaris (TEO) or Origanum compactum (OEO)* essential oils 1/2 v/v for *P. aureginosa* and 1/40 v/v for *E. coli*, with penicillin (PEN) 10 U/mL or streptomycin (STR) 50 mg/mL. Disk letters: (a) control disk, (b) PEN, (c) PEN + OEO, (d) PEN + TEO, (e) STR, (f) STR + TEO, (g) STR + OEO . Letters (h), (i) and (k) are OEO, TEO and growth control respectively.

5.3 Bibliography research

Results and discussion of the bibliography research were grouped into different sections below, including the mechanisms that provides the antibacterial effects of phenolic monoterpenes, and its significance and approaches related with the xenohormesis hypothesis.

5.3.1 Antibacterial effects of phenolic monoterpenes

5.3.1.1 Membrane disruption

The chemical structure of carvacrol and thymol, presenting a hydroxyl group and a system of delocalized electrons was proposed to generate antimicrobial activities [31]. However, different studies have demonstrated that thymol and carvacrol presented direct antibacterial activities against *Escherichia coli* by direct disruption of the cytoplasmatic membrane due to an increase of its permeability, potential depolarization and reactive oxygen species generation [32,33]. Phenolic monoterpene would also generate distortions in the structure of the cell, provoking an expansion and later destabilization of the membrane, denaturalization of enzymes and channels, as well as an alteration of the proton bomb driving force promoted by pH and electric potential variations [34].

Alternatively, thymol and thymol containing-essential oils obtained from *Lippia sidoides* were reported to increase the efficacy of the antibiotic gentamicin against *Klebsiella pneumoniae* and *Pseudomona aeruginosa* by reducing the minimum (MIC) inhibitory concentration by 32 and 4 times, respectably. The combination of gentamicin and thymol reduced the MIC by 4 times and TCEO-gentamicin by 16 times against *Staphylococcus aureus*. Even though more types of drug antibiotic like penicillin G and neomycin were used, this more effective combination was not successful in all tested resistant bacteria and antibiotic types [34]. There so, phenolic monoterpenes would compensate the reduction of the outer membrane permeability and the consequent antibiotic entry limitation of the more resistant gram-negative bacteria, obtained by the downregulation of porins and the replacement of more-selective channel [35].

5.3.1.2 Motility reduction

Bacteria with reduced flagellin synthesized are aflagellate and nonmotile, cells without flagella have been shown to be significantly less able to attach to epithelial tissue and to be less invisible. In concordance with this, carvacrol can inhibit flagellin synthesis *Escherichia coli* 0157:H7 [36]. The reduction in motillity was also generated in *Listeria monocytogenes* [37] and *Campylobacter jejuni* [38]. Nevertheless, another study revealed that the suppression of motility was reversible after stress removal and nutrient replacement [39].

5.3.1.3 Efflux pump inhibition

The overexpression of efflux pump generates drug resistance due to an antibiotic transport out of the cell, and its inhibitions seems to be a good strategy to impulse the antibacterial potency against multi-drug resistant bacteria [35, 39]. An evidence was generated by using phenolic monoterpenes against *Staphylococcus aureus* IS-58 strain when researchers concluded that the antimicrobial activity was not related to the efflux pump inhibition [40]. However, an inhibition of the efflux pump mechanism in addition to a morphological disruption was reported against Mycobacterium tuberculosis with the use of carvacrol [41]. The inhibition of efflux pumps by phenolic monoterpenes in E. Coli 0157:H7 was also demonstrated without induction of antibiotic resistance nor virulence [39].

5.3.1.4 Inhibition of biofilm formation

Phenolic monoterpene-containing essential oils demonstrated to have a great biofilm mass and metabolic rate reduction against *Salmonella enteriditis* at a sub-minimum inhibitory concentration. The eradication of 48h preformed biofilms occurred in a dose dependent manner [42]. The biofilm reduction was also evidenced with methillin-resistant *Staphilococus aureus,* a bacteria that causes mayor nosocomial infections. Thymol showed to inhibit the biofilm formation and the natural biofilm by a reduction in the synthesis of polysaccharide intracellular adhesin and the liberation of extracellular DNA. Also, the cotreatment of thymol and vancomycin evidenced more effectiveness than the single treatment of vancomycin against a murine infection model [43].

Furthermore, E. coli O157:H7 with thymol and carvacrol in sublethal concentrations, showed to produce 31 to 37% less amount of biofilm mass. In addition, RT-qPCR results demonstrated an increase in the expression of tnaA and bssS genes, two negative regulator of biofilm formation. Other altered genes, such as flhCD, fliAZ abd flgM are also related to biofilm reduction [44].

5.3.1.5 Inhibition of ATPasas

An ATPase inhibition as a carvacrol effect was demonstrated to occur in some pathogens such as *Escherichia Coli* and *Listeria monocytogenes* [45]. In another study related to *E. coli*, carvacrol could promote a complete inhibition of F_1F_0 ATP synthase fractions, meanwhile thymol, showed a residual inhibition [46]. In any case, carvacrol seems to be able to dissipate the proton motive force since its structure might act as a proton exchanger causing an ATP reduction and subsequent cell death [47].

5.3.1.6 Amphipathic nature contribution

The previous studies stablished that the bactericide effects of thymol and carvacrol are be produced by a combination of membrane disruption, biofilm and motility reduction, inhibition of ATPases or efflux pump. However, this bactericidal effect might depend more on physicochemical properties of the phenolic monoterpene molecules. For example, El abed et al. [48] stipulated that the amphipathic nature of carvacrol and thymol can hypothetically explain those seen effects, because its relative hydrophilia could permit its diffusion though the polar polysaccharide matrix into the bacteria membrane, meanwhile its hydrophobic part allows molecules to interact with different components, perturbating the lipidic bilayer. Consequently, a denaturalization of enzymes and channels, cytosolic pH and electric potential variations would occur, and they might be later related with the rest of the bactericide effects of phenolic monoterpenes mentioned before.

5.3.2 New approaches for xenohormesis hypothesis

5.3.2.1 Xenohormesis and phenolic monoterpenes

At the present, xenohormesis hypothesis remains unknown and forgotten. An evidence of this, are the only 30 bibliographical results between 2004 and 2021 of the term ''xenohormesis'' when applied on the PubMed database. For this reason, no restrictions were imposed during the bibliography research. Consequently, there is only one result obtained from Google Scholar, when the terms ''xenohormesis'' and ''carvacrol'' are combined [49]. This result provides a review published back in 2010, where authors grouped different plant derived products, mentioning the original cause that led to its biosynthesis, and the beneficial effects that provides to consumers. Carvacrol is referred as a substance with antimicrobial activity and minimal systemic toxicity generation, among other substances such as curcumin and resveratrol. On the other hand, thymol is not mentioned on the review.

5.3.2.1 Different forms of xenohormesis

It is noticeable that in almost all the mentioned xenohormetic molecules on the previous review [49], the external stress that generates its synthesis and the original effect, differs from the benefits for the consumers. An example of this type of molecules are polyphenols, a set of environmental stress-mediated plant molecules. These phenolic compounds generate resilience on plants but on the other hand, they also modulate metabolism on superior animals due to diverse signaling mechanisms, provoking a set of health effects on insulin resistance, obesity, inflammation, hypertension, oxidative stress, lipid abnormalities, neurotoxicity and so on [5, 49, 50, 51]. This type of xenohormesis could be defined as *non-original stress related* or *indirect xenohormesis*.

Another example of *indirect xenohormesis* might be gingsenosides, a group of phytosteroids produced manly by the *Panax* genus. Gingsenosides are synthesized under certain biotic and abiotic stress conditions, and have certain pharmaceutical benefits for consumers, including inmunomodulation, anti-hyperglycemic, anti-obesity, neuroprotection, anti-aging, anti-fatigue, and anti-cancer activities [52]. This group of beneficial effects, together with the polyphenol ones, mismatch with the original stress that produced its synthesis on plants and its positive effects to superior consumers.

This set of unpaired interactions could be explained by the fact that evolution has favored the maintenance of enzyme-binding and other types of capacities of sensing stress-induced molecules, as this defense mechanisms are encountered in animal and fungi, just as Howitz et al. postulated [1]. Additionally, it has been suggested that biosynthetic pathways come from a common antecessor for all living beings [53]. This would explain why some metabolic products are produced by similar synthetic pathways. For example, fatty acid oxidation plays a role in the wound responses mediated by prostaglandins and jasmonic acid in animals and plants, respectably [4]. These biosynthetic compounds generate similar responses of defense, herbivore resistance in the case of plants, and inflammation and other immune responses in animals [1].

Nevertheless, even though most xenohormetic molecules do not coincide with the synthetic cause on plant and effects on animals, there are other few examples on which the original plant stress response and effect, is linked with the benefit of consumer. For instance, Geraldes et al. [54] described mycosporine-like aminoacids as a group of molecules synthetized by cyanobacteria among other aquatic organisms that are natural UV-absorbing compounds synthetized in response to sun light stress. Consequently, they show a xenohormetic significance as they can be useful for obtaining a natural sunscreen.

The example of phenolic monoterpenes is similar to mycosporine-like amioacids. Carvacrol and thymol synthesis has been demonstrated to be induced by bacterial infections [12] and they show antimicrobial activity, as it is confirmed on the previous antibacterial activity assays using phenolic monoterpene-containing essential oils and also on bibliography research. Consequently, the antimicrobial benefit has a xenohormetic significance, as it would be beneficial for consumers, for instance, for food preservation or antibiotherapy improvement, among other beneficial effects. Therefore, the cause of synthesis matches with the antibacterial effect of phenolic monoterpenes. For this reason, phenolic monoterpenes are a good example of *original stress related* or *direct xenohormesis*.

It is important to know that phenolic monoterpenes have other types of beneficial effects not related with antibacterial activity [14], and that those effects might differ from the cause of synthesis, indicating that the differences between *direct* or *indirect xenohormesis* could be produced by the cause and effect rather by the molecule type by itself. For this reason, a molecule might provide a *direct* and *indirect xenohormetic significance* depending on the relationship between the producer and the consumer.

6. Conclusions

1. Phenolic monoterpenes endorse the xenohormesis hypothesis since they are synthetized in response to stress caused by bacterial infections and generate beneficial effects for consumers.

2. Since the purpose of the phenolic monoterpenes antimicrobial effect is the same for the plant and for the consumers, we could talk about a *direct* or *original stress related xenohormesis*.

3. Carvacrol and thymol-containing essential oils show great antimicrobial activity, but Gram-negative bacteria seems to be less susceptible against phenolic monoterpenes.

4. Phenolic monoterpenes might be helpful against drug-resistant bacteria by increasing the antimicrobial effect of antibiotics, but this synergy may vary depending on essential oil, antibiotic and strain type. Further studies are needed.

The xenohormesis hypothesis tries to explain why this stress induced molecules of producers are helpful for consumers, even if it is not very well known. Phenolic monoterpenes are a good representation of this process since they are induced by plant bacterial infections and at the same time, show antimicrobial activity. Indeed, this match between original cause of synthesis, and effect, perhaps compose a new subtype of xenohormesis, direct or original stress related xenohormesis, that might differ from a mayor subgroup, called non-original stress related or indirect xenohormesis. Moreover, phenolic monoterpenes and phenolic monoterpene-containing essential oils show substantial antimicrobial properties that might be useful for future clinical approaches. Carvacrol and thymol could be an alternative to actual antibiotherapy treatments and as an adjutant for the antibiotics employed. This could potentially lessen the clinical and economical problem of antibiotic multi-resistant bacteria.

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