Chemical Characterization of the Essential Oil of *Syzygium Aromaticum* and its Antimicrobial Activity Against A Probiotic *Lactobacillus Acidophilus*

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Abstract

Eugenol is the main constituent of clove (*Syzygium aromaticum*) essential oil. It is liquid and oily in consistency, light yellow, with a characteristic aroma, slightly soluble in water and soluble in alcohol. In addition, it is considered an essential oil of high antibacterial, antifungal, and antioxidant capability. Studies related to its effect on probiotic bacteria are scanty. The objective of this research was to determine the chemical composition of clove essential oil and the antimicrobial influence of clove extract from clove on the growth of *Lactobacillus acidophillus*. Among the main constituents in clove essential oil, eugenol stands out with 79.47%, β-cariophilene with 7.67% and α-humulene with 2.65%. *L. acidophilus* was inoculated in MRS Sorbitol agar containing 0%, 0.1, 0.50, 1, 2 and 3% essential oil and incubated for 0, 24 and 48 h. Pour plates were incubated anaerobically at 35°C ± 1°C for 48 h. A colony counter was used to enumerate bacteria. The clove extract showed an inhibitory effect against *L. acidophillus* from concentrations greater than 1%, but at concentration less than 1% essential oil did not adversely influence growth of *L. acidophilus.* The inoculation time at 0 h showed significantly highest counts compared to 24 h and 48 h which had counts not significantly different from each other. Although counts were lowered, *L acidophilus* survived the 48 h exposure. Clove essential oil use and *L. acidophilus* probiotic benefit can both be had when essential is used at less than 1%.

Keywords: Clove, Natural Products, Antimicrobial, Eugenol

Introduction

There is increasing interest in bioactive compounds obtained from essential oils from plants to treat microbial infections caused by antibiotic resistance (Cui *et al.*, 2019). On the other hand, essential oils are increasingly booming in order to replace synthetic preservatives in foods in favor of natural alternatives (Simas *et al.,* 2017). Plants are considered as the most important source of chemical compounds that exists (Mesquita and Tavares, 2018).

The essential oil consists of secondary metabolites, including terpenes and sesquiterpenes with biological potential (Cunha *et al.*, 2005). The quantity and composition of essential oils, according to Costa (2008), vary in terms of the genetic and physiological levels of the plant, as well as depending on the conditions of cultivation, harvest and post-harvest treatment as well as environmental factors.

Among the essential oils extracted from medicinal plants, the clove (*Syzygium aromaticum*) stands out. Along with other medicinal plants, this essential oil was used in World War II to heal soldiers wounded in combat (Cunha and Roque, 2013). Clove (*Syzygium aromaticum*) is a plant characterized as one of the aromatic spices best known for its medicinal, antibacterial, antioxidant properties and for its use as a flavoring in foods for human consumption (Rivas *et al.*, 2015).

In its chemical composition, clove has numerous secondary metabolites with biological activity. These compounds include phenolic compounds, flavonoids, hydroxybenzoic acids and hydrokinetic acids, with eugenol being the main constituent with concentrations that can vary between 9.38-14.7 g 100 g^{-1}. Among the phenolic compounds, gallic acid is the major compound (Cortés-Rojas *et al.,* 2014) Chemical composition of clove essential oil, between 80-90% is eugenol, 15% of eugenol acetate and between 5-12% of beta caryophyllene (Alma *et al.*, 2007).

The other important compound found in this essential oil in concentrations of up to 2.1% is alpha humulene. Additonally, other volatile compounds present in lower concentrations are beta pinene, limonene, farnesol, benzaldehyde, 2-heptanone and ethyl hexanoate (Aguilar and Malo, 2013). According to the WHO, clove essential oil is considered safe when consumed in concentrations of 1500 mg kg^{-1} or lower (Gülçin *et al.,* 2012).

Probiotics are live microorganisms that have beneficial effects on the health of humans and animals (Torres *et al.,* 2011). Lactic acid bacteria, which include the Lactobacillus and Bifidobacterium species have been used for food preservation by fermentation for many of years and can potentially have a dual function, acting as agents for food fermentation and health benefits, (Torres *et al.,* 2011, Aryana and Olson 2017). *L. acidophillus* is of great industrial importance, as it is used in various lactic fermentation processes (yogurt and cheese). *L. acidophillus* is also involved in the manufacture of products derived from vegetables. Probiotics must remain viable during processing, storage and transmission through the gastrointestinal tract (da Silva *et al.*, 2015).

Clove is a very common plant and its oil can be easily extracted, it could be used in the manufacture of nutraceutical foods. There are very few reports on the synergistic and antagonistic effects of clove extracts on probiotic bacteria (Pastrana *et al.,* 2017). The objective of this work was to determine whether or not there was antimicrobial activity of clove essential oil, mainly due to eugenol as a major constituent in the growth of *L. acidophillus,*

Material and method:

Essential oil extraction

Approximately 1 kg of cloves, were purchased in the town of Catacamas, department of Olancho (Honduras) was washed with distilled water for the removal of any type of impurity and then placed in a hydro-distillation flask with distilled water in a Clevenger type apparatus where hydrodestilation was performed over a period of 4 hours. It was subsequently dried over anhydrous sodium sulfate and stored in an amber bottle until it was time to perform the analyzes.

Essential oil analysis by gas chromatography

The essential oil was analyzed on a HP 7820A Gas Chromatograph (GC) equipped with a flame ionization detector (FID) using a capillary column (HP5 30 × 0.32 mm × 0.25 microns, Agilent). Column temperature: 50 °C (0 min) to 3 °C min^{-1} to 230 °C. Gun: 250°C Split (1:30). FID Detector: 250 °C. Carrier gas: hydrogen to 3 ml min^{-1}. Vol injection: 1 µl. Essential oil was diluted to 1% in chloroform. Data acquisition software used was Compact EZChrom Elite (Agilent). The quantitative analysis was performed using standard areas from the chromatograms obtained by GC-FID.

Subsequently, to quantify the different constituents, a CG-MS with Column: Rxi-1MS 923 30 m × 0.25 mm × 0.25 microns (Restek) was used. Column Temp: 50ºC (3 min), 3°C min $^{-1}$ to 250°C. Injector: 250°C Split (1:10), GC-MS interface at 250 ºC. MS detector (electron impact at 70 eV) temperature was 250 ºC. Carrier gas: Helium up to 1.5 ml min $^{-1}$. Vol injection: 1 µl. Essential oil was diluted to 0.1% in chloroform. Data acquisition software used was GC-MS Solution (Shimadzu) with NIST11 library. Identification of peaks was made by comparison of the mass spectra obtained by GC-MS spectra with the NIST11 library and also by comparing the Kovats indices calculated by GC-FID and literature data.

Antimicrobial activity of the essential oil of *S. aromaticum* in the growth of *L. acidophilus*.

This research was carried out at the Biotechnology Institute of the National University of Agriculture (Honduras).

MRS broth powder was incorporated into distilled water and autoclave at 121 °C for 15 minutes to make MRS broth. A 10% sorbitol solution, filter sterilized with Nalgene membrane, was prepared. This solution (10%) was added to the MRS broth (90%) to make sorbitol MRS broth. Essential oil at concentrations of 0, 0.1, 0.50, 1, 2 and 3% (w/v) was incorporated into sorbitol-MRS broth. This essential oil sorbitol-MRS broth was inoculated with *L. acidophilus* and incubated at 0, 24 and 48 h. Serial dilutions were prepared. Pour plates were incubated anaerobically at 35 °C ± 1 °C for 48 h. after which colonies were counted.

Statistical Analysis

The data was analyzed in the SPSS software, version 25.0, and means were separated using Tukey test and significant differences were determined at $P<0.05$.

Results and discussion:

Identification of the chemical constituents of *Syzygium aromaticum.* In table 1, the chemical constituents in the essential oil of *S. aromaticum* are presented, a total of 11 constituents being undefined.

Table 1. Identification of constituents not essential oil de *S. aromaticum*.

Number	Constituent	%
1	Eugenol	79.47 ± 0.3
2	Copaene	1.76 ± 0.05
3	α-humulene	2.65 ± 0.04
4	β-cariofilene	7.67 ± 0.10
5	Eugenol acetate	1.32 ± 0.02
6	Cariofilene oxide	0.65 ± 0.03
7	2-heptanone	0.24 ± 0.01
8	Humulene	2.48 ± 0.04
9	Calamene	0.37 ± 0.03
10	calacorene	0.45± 0.04
11	2-etil-hexanoic acid	0.12 ± 0.01
Other		2.82 ± 0.05

*Results in triplicate describe the mean ± standard deviation

In table 1, a total of 11 chemical constituents were identified in the essential oil of *S. aromaticum*, the majority being eugenol with 79.47%, β-cariophilene with 7.67%, α-humulene with 2.65%, humulene with 2.48 % and copaene with 1.76%.

The concentration of eugenol found in this work as a major compound is in accordance with the values indicated by Affonso *et al.,* (2012), who reported concentration of eugenol between 82.47-90.41% in the essential oil of the different parts of the plant. The next major compound found was β-cariofilene. Since eugenol is the major component of the essential oil, it is the one that contributes most to the antifungal and antibacterial action (Pereira *et al.,* 2008).

Inhibitory effect of eugenol of *Lactobacillus acidophilus*.

The eugenol from *S. aromaticum*, showed a significant inhibitory effect against *L. acidophillus* cells from 0.5 to 3%, with 3% having the greatest inhibitory effect. However, concentration of 0.1% was similar to the control (Figure 1).

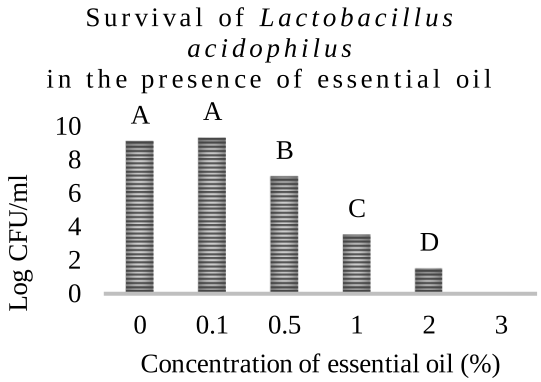

Figure 1. Tukey media grouping between treatments ($p \leq 0.05$)

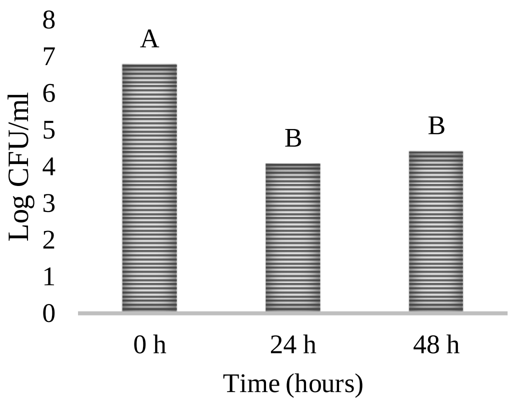

Figure 2. Survival of *L. acidophilus* in the presence of essential oil during different times of inoculation.

In Figure 2, the relationship between inoculation times at 0, 24 and 48 hours with respect to the effect of clove essential oil on the survival of *L. acidophilus* is presented. These results indicate that at 0 hours, the counts were significantly high. However, a greater inhibitory effect was observed between 24-48 hours, without showing significant differences in the optimal quantification times. These results coincide with those provided by Gonzalez (2002), which indicates the effect that eugenol has, the main constituent of the essential oil in the inhibitory effect of fungi and bacteria.

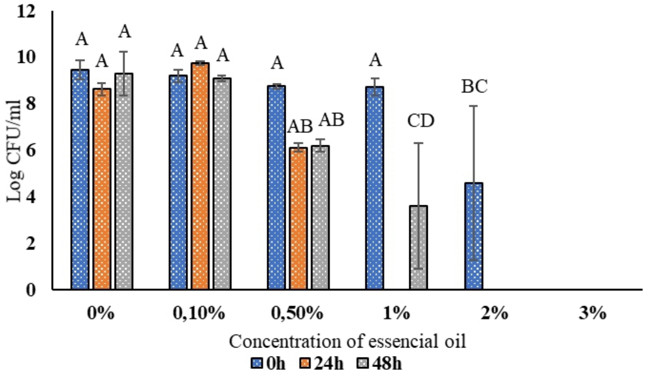

Figure 3. Survival of *L. acidophilus* in the presence of essential oil during different times of incubation.

Clove oil, which contains a high eugenol (79.47%) content, has not only shown antimicrobial activities in *Lactobacillus acidophilus* but also having wide spectra of antimicrobial effects against enterobacteria (Burt and Reinders 2003; Nanasombat and Lohasupthawee 2005). Park et al. (2007), pointed out that clove essential oil has always been used due to its properties as an antimicrobial agent, given its efficiency in inhibiting pathogenic microorganisms, being one of the properties of said oil that has been little explored until now. Since the main inhibitory component of clove oil is believed to be eugenol (Jayatilaka et al. 1995; Della Porta et al. 1998; Bullarman et al. 2006; Sukutta et al. 2008), clove, also, has been report to be effective against molds and yeasts (Lopez-Malo et al. 2002). The essential oil extracted from aromatic plants have been shown to have antibacterial (Gianneanas et al. 2003), antifungal (Janatan et al. 2003), antioxidant activities (Bostoglou et al. 2004). The action of the microbial inhibition of eugenol may be related to the rupturing of the membrane or the inactivation of enzymes and genetic materials (Wendakdoon and Sakaguchi, 1993).

Conclusion

Clove essential oil has different bioactive compounds in its chemical composition, mainly in eugenol as a major constituent. Said constituent gives the essential oil medicinal properties being demonstrated in this work. Essential oil concentration of 0.5% is optimum as it can have essential oil use with *L. acidophilus* presence for probiotic effect.

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