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ORIGINAL ARTICLE

Obtaining the essential oil of *Syzygium aromaticum*, identification of eugenol and its effect on *Streptococcus mutans*.

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Abstract: Dental caries is a disease which affects the human oral cavity. Currently, the search for active principles of plants with antimicrobial effect seems promising for dental therapy. In this article the activity of the essential oil of *Syzygium aromaticum* (clove) was evaluated with an emphasis on its antimicrobial properties. The oil was obtained by hydrodistillation, characterized by thin layer chromatography and chemical tests. The main compound was identified in the oil obtained from the flower buds and its antibacterial activity against planktonic cells *Streptcoccus mutans* ATCC700611 was assessed by performing serial dilutions, from 15 up to 1000 µg/mL, compared with 0.12% chlorhexidine and dimethylsulfoxide. MIC was also determined. Subsequently, UFC was analyzed and compared with CMR® Test Ivoclar Vivadent. The efficiency in obtaining the oil was 2.20%. By using the CCD technique, a fraction was revealed by UV light, corresponding to eugenol. It had a good response for triterpenoids and flavonoids. It showed greater antimicrobial activity at concentrations of 1000, 500 and 250µg/ml. The MIC and MBC of the oil was 125 to 250µg/mL, respectively. Eugenol was found as an active principle in the oil obtained. Currently, the impact of using plant extracts has favored the evaluation of alternative, effective and biocompatible antibacterial agents for the formulations of oral hygiene products applied to the prevention or treatment of oral diseases.

Keywords: *Eugenol, Streptococcus mutans, Syzygium aromaticum, antimicrobial, minimum inhibitory concentration MIC.*

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INTRODUCTION.

The essential clove oil is derived from the flower buds of *Syzigium aromaticum*(L.) belonging to the family Myrtaceae. Between its chemical components are ß-caryophyllene, which represents 14-21% of its compounds, 10-13% of tannins as well as phenols and sesquiterpenes. The most important component of the oil is phenylpropene, apart from eugenol, which is responsible for the characteristic scent of the plant and its main component. A 49 to 98% of the essential oil is contained in the flower buds[1,2].

The traditional use of clove is supported by its many properties which have been described in numerous scientific reports highlighting its antioxidant, hypotensive, dental analgesic[3], antibacterial[4], antiinflammatory[5] and antifungal[6] activity, apart from the synergistic antimicrobial activity of the essential oil with other plants[7] which allows it to be considered with great potential for dental application[8,9].

The oral cavity is considered as an ecosystem. It allows the development and growth of a wide diversity of microorganisms, favored by its temperature and humidity. *Streptococcus* spp have a "cocci" spherical shape and are grouped in chain or pairs, do not move and are Gram-positive. *Streptococcus mutans* is the main organism which colonizes the surfaces of the teeth after dental eruption. This organism has a tendency to change its morphology, thus it may be found as cocci or coccobacillus. It has a diameter of 0.5 to 0.75µm, is facul-

tative anaerobe α-hemolytic, heterofermentative and has an outer covering called glycocalyx. It is considered as the main isolated organism in carious lesions in humans[10]. It is able to acquire new properties which allow the expression of pathogenicity favoring its virulence in specific environmental conditions. It presents a mechanism of adherence to solid surfaces so it can colonize the oral cavity and form a bacterial biofilm. It can survive in an acidic environment, produce polysaccharides associated with the maturation of the plaque and lactic acid derived from sugar metabolism. It is identified as a primary pathogen because it interacts specifically with other colonizing microorganisms. *Streptococcus mutans* has properties associated with the formation of biofilms, which determine its virulence in the development of dental caries[11]. According to the WHO, caries is the main oral disease causing loss of teeth. The pathogenicity of the organism is centered on the production of acids and the ability to allow bacterial aggregation in biofilm formation[12].

This study evaluated the antimicrobial effect of the essential oil of *Syzigium aromaticum*(L.) against *Streptococcus mutans* in planktonic cells. The minimum inhibitory and bactericidal concentration and the main compound in the oil, which is responsible for its antimicrobial property, were analyzed.

MATERIAL AND METHODS.

Plant material and obtaining the essential oil.

For the purpose of this study, 250g of flower buds of *Syzygium aromatic* "clove", family Myrtaceae, were obtained from recognized places of selling. They were identified in the Herbarium of the Department of Botany at the Faculty of Biological Sciences at the Universidad Autónoma de Nuevo León (U.A.N.L), with registration number 025574.

Obtaining the essential oil.

The dry flower buds were powdered and placed in a 1000mL flask. Then, 500ml of distilled water were added to obtain the essential oil through hydrodistillation, by steam distillation using the glass (pyrex) Clevenger-type trap[13]. Subsequently, it was placed in amber vials with screw cap, dried with sodium sulfate (Na_2SO_4) anhydrous and preserved in refrigeration until its use.

Chemical methods for the identification of functional groups and secondary metabolites.

For the partial identification of compounds present in the essential oil, a preliminary phytochemical analysis was carried out[14]. Dilutions of the oil obtained were prepared by taking 100µl aliquot and solubilizing it in 2ml of methanol (MeOH). They were placed in test tubes and ran various chemical reactions including: Shinoda, Buchard Libermann, coumarins, 10% sodium hydroxide (NaOH) Baljet for sesquiterpene lactones, among others[15].

Chromatographic analysis in the thin layer.

Chromatographic separation was done using Sigma-Aldrich silica gel plates (10x2.5cm). Samples of the oil obtained were taken with a capillary and various standard compounds which were placed on the plate in three replicates were subsequently moved with various eluent systems and dried on silica, judging the presence of the main components in the oil by identifying the fractions revealed with cobalt cloride ($CoCl_2$) and 254nm ultraviolet (UV) light.

Bacterial strain and growth condition.

The bacterial strain of *Streptcoccus mutans* ATCC 700611 was used. It was provided by the Laboratory of Molecular Biology at the Faculty of Odontology, U.A.N.L. It was cultivated in Muller Hinton agar for 24 hours at 37ºC, taking between 4 and 5 colonies of microorganisms to adjust them to the pattern of inoculum of 0.5 on the scale of McFarland, which is equivalent to $1.5x10^6$ CFU. Subsequently, 100µL inoculum was taken for each analysis.

Microbiological testing, minimum inhibitory concentration.

The trials of antimicrobial activity of the oil were performed in Muller Hinton broth. Tubes were prepared with 800µL of media and dilutions of clove oil, starting from a solution of 5mg/ml and assessing increasing concentrations from 15 to 1000µg/ml, taking aliquots of 100µl, comparing them in parallel with the controls. Dimethyl sulfoxide (DMSO) solvent was determined as a negative control and 0.12% v/v chlorhexidine® as positive control. MIC was determined as the lowest concentration

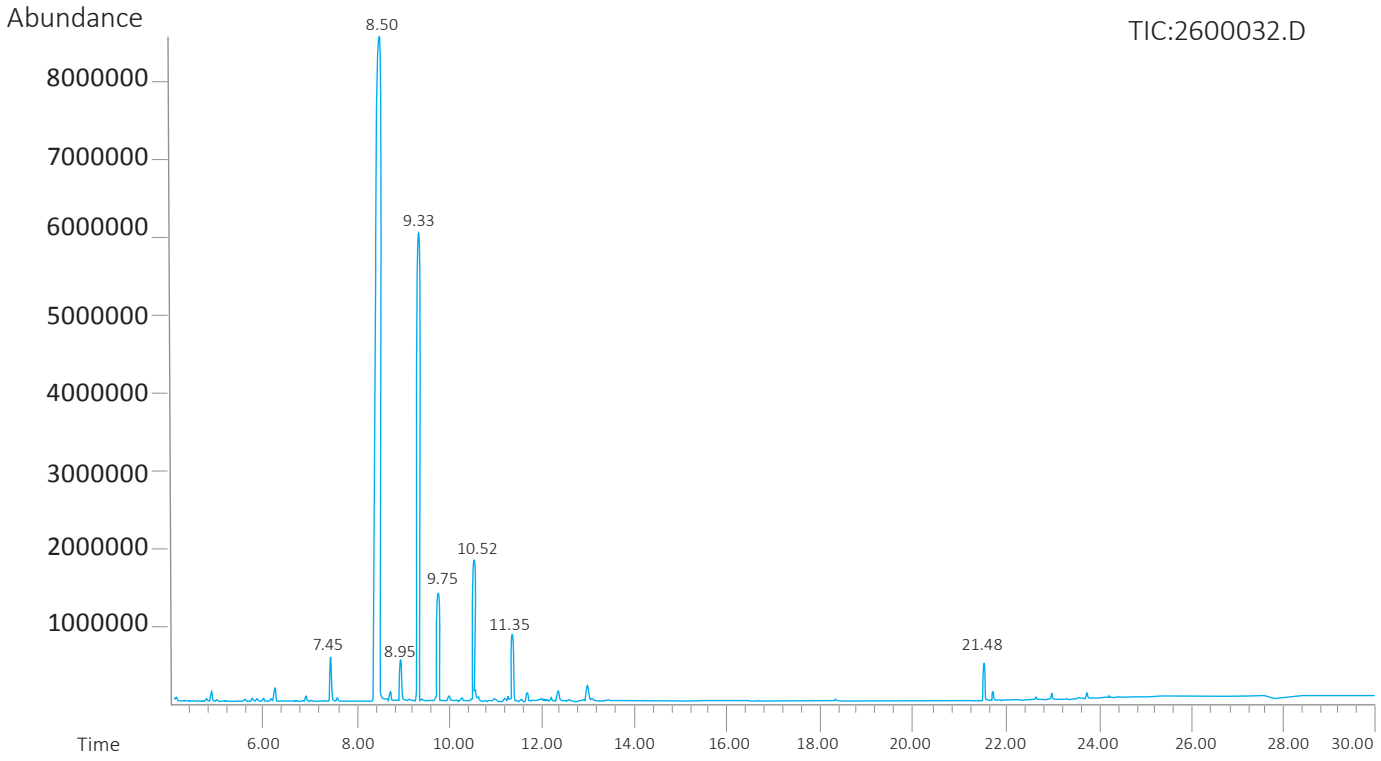

Figure 2. Chromatographic profile of the oil from ***Sysygium aromaticum***. Quantification of the principal component (eugenol), main component in the clove oil by gas chromatography -mass spectrometry.

of oil which can inhibit the growth of a microorganism after simmering for 24 hours at 37°C. Results were qualitatively evaluated and compared.

Determination of the minimum bactericidal concentration.

The minimum bactericidal concentration (MBC) was determined on an aliquot of 25µl of each of the samples tested. They were planted by striated in each sector of the plaque, simmering for 24h to 37ºC. It was done in triplicate. MBC is considered the lowest concentration of the oil needed to kill 99% of the initial inoculum after incubation for 24 hours[15].

The results were compared with the CRT Bacteria test (Ivoclar Vivadent) for counting colony forming units (CFU) at concentrations of 15 to 1000µg/ml, apart from the controls, chlorhexidine, DMSO and growth monitoring.

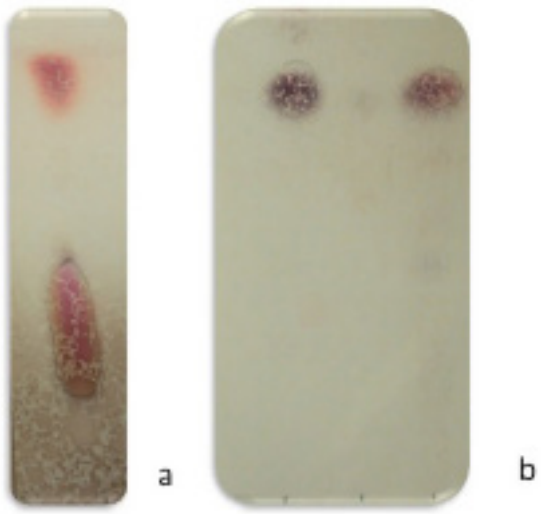

Figure 1. Thin Layer Chromatography (TLC). Comparison between the essential oil of clove obtained, fractions identified (a). Comparison between eugenol and the oil and concordance of R.f 0.65 with mobile phase hexane/acetone 9:1 (b).

Table 1. MIC Results, concentrations of oil from 1000 to 15 µg/ml positive control, chlorhexidine 0.12 %, negative control, DMSO, n=3.

Extract Concentrations	Syzigium aromaticum (clove)						
µg/ml	1000	500	250	125	62	31	15
S. mutans	-	-	-	-	+	+	+
Negative control	+	+	+	+	+	+	+
Positive control	-	-	-	-	-	-	-

(-) without growth; growth (+).

Table 2. Results for MIC, CFU, CMR Test Ivoclar C+ (positive control chlorhexidine 0.12%), C- (negative control, DMSO), CC (Growth Control), CRT® Test.

Oil Concentrations	Count in Petri Dishes		
µg/ml	Repetition 1	Repetition 2	Repetition 3
1000	$< 10^5$	$< 10^5$	$< 10^5$
500	$< 10^5$	$< 10^5$	$< 10^5$
250	$< 10^5$	$< 10^5$	$< 10^5$
125	$< 10^5$	$< 10^5$	$< 10^5$
62	$\geq 10^5$	$\geq 10^5$	$\geq 10^5$
C+	$< 10^5$	$< 10^5$	$< 10^5$
C-	$\geq 10^5$	$\geq 10^5$	$\geq 10^5$
CC	$\geq 10^5$	$\geq 10^5$	$\geq 10^5$

Table 3. Identification of major components of the oil obtained from *Sysygium aromaticum*, retention time for each compound identified (rt) and the percentage of the area (abundance).

Rt (min)	Compounds found	% Area
4.89	Alfa-Terpinolene	0.43
6.25	P-Alinalisol (estragole)	0.58
6.91	P-allylphenol	0.19
7.45	Thymol	1.41
8.50	**Eugenol**	**67.5**
8.73	Alfa-copaene	0.28
8.95	Vanilin	1.15
9.33	Trans-caryophyllene	1626
9.75	Alfa-humulene	3.14
9.99	Germacrene - d	0.14
1052	Eugenol Acetate	4.16
1136	Caryophyllene B	2.05
12.96	cinnamaldehyde	0.52

Identification of eugenol as the main component in the oil.

A sample of the oil obtained was taken to identify and quantify the presence of eugenol. A 6890N gas chromatograph with split/splitless injector and a 5973 mass selective detector (Agilent Technologies, Germany) with electron-impact ionization source and quadrupole mass analyzer were used. The separation was carried out with a 5MS HP column (30mx0.25mmx0.2µm; Agilent Technologies, Germany). The carrier gas was helium at a flow rate of 1.0ml min -1. The program for the furnace temperature consisted of a start-up phase at 80°C for 1 min, going up 10°C min-1 to 200°C for 3 min at this temperature. Finally, it was increased to a speed of 15°C min-1 until reaching 320°C for 8 min. The injector temperature was 270°C and that of the transfer line 250°C. Data acquisition was carried out in sweep mode in a range of 30-550m/z. The identification of the compounds was carried out using the Wiley 7N.l. database.

RESULTS.

An efficiency of the essential clove oil extraction was 2.20%. In the partial phytochemistry identification through chemical reactions, it tested positive in LibermanBuchard for triterpenes, flavonoids, and ferric chloride test for phenolic OH groups.

In the comparative chromatography using hexane-acetone 9:1 as mobile phase, analogy between relationships of fronts (Rf) was identified between the first fraction and the standard eugenol, corresponding to 0.65, confirming the presence of this compound (Figure 1).

Clove oil showed antimicrobial activity against *Streptococcus mutans* from concentrations of 1000 up to 125µg/ml. MIC was 125µg/ml with bactericidal behavior (Table 1). In 1000µg/ml concentrations, (3 replicates) it was reported $<10^5$ CFU growth, as well as for 500, 250 and 125µg/ml concentrations. Regarding lower concentrations, 62µg/mL presented $\geq 10^5$ CFU, the same as the negative controls (Table 2).

Among the main compounds present in the oil obtained from *Sysygium aromaticum,* eugenol was identified as the

major component (its corresponding retention time and percentage of area are seen in Table 3). Likewise, in the chromatographic profile, this compound was clearly identified by matching the abundance peak. The retention time was 8.50 minutes, representing the main component of clove oil in a 67.5% of the area (Figure 2).

DISCUSSION.

Several studies have reported eugenol as the main compound in clove oil. It is considered as the molecule responsible for the great variety of its activities studied[16,17]. When separating the oil compounds through chromatography, using hexane/acetone 9:1 as mobile phase allowed an efficient chromatographic separation, coinciding with other reports described, and seeing the presence of eugenol in the study sample. In the same way, in comparative chromatography, analogy was identified between the first fraction and the standard (eugenol) with R.f 0.65 (Fig.1 A, b), with agreement between the samples analyzed according to previously described techniques[14].

In this study, the essential oil of *Syzygium aromaticum* was obtained, eugenol was identified as a compound and its antimicrobial activity was assessed, agreeing with what has been reported in several studies[18,19]. Its activity against *Streptococcus mutans* was observed, agreeing with several studies which reported its growth inhibitory activity in oral pathogens[20,21].

It was noted that the essential oil obtained by hydrodistillation presented strong antimicrobial activity, comparable to the concentrations reported in the range of micrograms per milliliter, compared to what has been reported as MIC and MBC for *Streptococcus mutans,* where apart from testing clove oil as an antimicrobial and anti-fungal for *Candida albicans*, a MIC and CMB of 390 and 780µg/mL, respectively, was demonstrated for *Streptococcus mutans*[22]. On the other hand, it has been reported that MIC of the essential oil of clove from India on *Candida albicans* varies from 1000 up to 2500µg/ml, for *Streptococcus sanguis* was 0.31mg/mL. It has also been reported sensitivity to the clove oil for concentrations of 512µg/ml, 0.31 and 0.16mg/mL, respectively, for *Candida albicans, Staphylococcus aureus* and *Actinomyces viscosus*[23]. Therefore, based on the described above and in accordance with the established criteria, the essential oils with MIC between 50 and 500µg/mL are considered to have strong antimicrobial activity. Those with a MIC between 600 and 1500µg/ml are considered to have moderate activity and when MIC is greater than 1500 µg/ml they have low activity. In this sense, the essential oil of clove is considered a substance with a strong antimicrobial activity as it presents a MIC of 125µg/mL. MBC was 250µg/mL in agreement with the criteria for the established antimicrobial activity which considers it as a strong activity[24].

On the other hand, in a study on different essential oils of spices, a eugenol level of 82.6% was reported with respect to the influence of heat on the antioxidant activity in the oil of clove. In this study, it was obtained a 67.5%[25]. Other reports mention the presence of eugenol in the clove oil as a compound with values of 89.2% a slightly higher percentage than in this study[26]. Finally, 77.4% of eugenol has been reported, due to the fact that the extraction was performed with an equipment using CO_2. Then, the decline in eugenol could be related to the system used for the extraction of essential oil[27].

CONCLUSIONS.

In this study, it was demonstrated the capacity of the essential oil of clove obtained by hydrodistillation to inhibit growth of *Streptococcus mutans*. Eugenol was identified as the main component of *Syzygium aromaticum*, in conjunction with the standard of eugenol analyzed with Rf 0.65 and as the compound of greater abundance and with bactericidal activity in a concentration range of 125-1000µg/mL, with MIC 125 µg/mL.

Phytotherapy provides an important field for research, where the active metabolites of plants can be tools which provide advantages in therapies, being a promising field for the search of new alternatives with greater antimicrobial application in the dental practice by using the prin-

ciples derived from plants.

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Obtención del aceite esencial de *Syzygium aromaticum*, identificación del eugenol y su efecto sobre *Streptococus mutans*.

Resumen: La caries dental es una enfermedad que afecta la cavidad oral en los humanos. Actualmente la búsqueda de principios activos de plantas con efecto antimicrobiano representa una promesa en la terapia Odontológica. El presente trabajo, evaluó la actividad, del aceite esencial de *Syzygium aromaticum* (clavo) con énfasis en su propiedad antimicrobiana. El aceite fue obtenido por hidrodestilación, caracterizado por cromatografía en capa delgada y pruebas químicas. Se identificó el compuesto principal en el aceite obtenido de los botones florales y se evaluó su actividad antibacteriana contra células plantónicas de *Streptcoccus mutans* ATCC (700611) realizándose diluciones seriadas; desde 15 hasta 1000µg/mL, comparándose con clorhexidina al 0.12% y dimetilsulfóxido, además se determinó la CMI. Posteriormente, se analizó las UFC, comparándose con el Test CMR® Ivoclar Vivadent. La eficiencia en la obtención del aceite fue de 2.20%. Por la técnica de CCD se identificó una fracción al revelado UV, correspondiente al eugenol. Presentó respuesta positiva para flavonoides y triterpenos. Mostró mayor actividad antimicrobiana a las concentraciones de 1000, 500 y 250 µg/mL. La CMI y CMB del aceite, resultó a 125 y 250 µg/mL respectivamente. Se comprobó la presencia del eugenol como principio activo en el aceite obtenido. Actualmente la proyección del uso de extractos de plantas ha favorecido la evaluación de agentes antibacterianos alternos, eficaces y biocompatibles para su empleo en las formulaciones de productos de higiene bucal aplicados a la prevención o tratamiento de enfermedades orales.

Palabras clave: *Eugenol, Streptococcus mutans, Syzygium aromaticum, antimicrobiano, concentración mínima inhibitoria.*

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