D.R. © TIP Revista Especializada en Ciencias Químico-Biológicas, 9(2):73-77, 2006

EFFECT OF THE ESSENTIAL OIL, INFUSION AND ETHANOL EXTRACT OF *Thymus vulgaris* L., ON THE GROWTH *IN VITRO* OF GROUP A ß-HEMOLYTIC *Streptococcus pyogenes*

[1]Eloy Solano, [1]Carlos Castillejos Cruz, [2]Leticia Álvarez Estrada, [2]Ángel V.M. Arellano Villavicencio, [1]Ma. de la Luz López-Martínez and [1]Ramiro Ríos-Gómez

[1]Herbario FEZA, Carrera de Biólogo, Facultad de Estudios Superiores Zaragoza, UNAM. Apdo. Postal 9-020, Iztapalapa 09230, México, D. F. [2]Instituto Mexicano del Seguro Social. Av. Cuauhtémoc 330, México, D. F. E-mail: [1]solanoec@correo.unam.mx

ABSTRACT

Biological activity of the distilled essential oil, ethanol extract, and infusion of thyme (*Thymus vulgaris* L.) was evaluated on the growth of group A ß-hemolytic *Streptococcus pyogenes*, the primary cause of faryngoamygdalitis. Sensitivity tests and measurements the zones of inhibition *in vitro* was performed. The distilled essential oil showed the greatest effect (inhibition halo of 3.2 cm), superior even to penicillin (2.4 cm). The ethanol extract had less effect, and the infusion showed no effect. The essential oil and the ethanol extract were analyzed by gas chromatography to determine the concentration and purity of their principal components and to compare these to the commercially available pure essential oil of thyme. These analyses allowed us to establish the presence of thymol and to a lesser extent carvacrol, both of which are known to inhibit bacterial growth.

Key Words: *Antimicrobial, faryngoamygdalitis, Streptococcus, Thymus vulgaris.*

RESUMEN

Se estimó un tratamiento alternativo de bajo costo para conocer la efectividad del tomillo *Thymus vulgaris* L., sobre la faringoamigdalitis bacteriana. Al aceite esencial obtenido por destilación, extracto etanólico e infusión de tomillo; se les evaluó su actividad biológica sobre el crecimiento de *Streptococcus pyogenes* ß-hemolítico del grupo A de Lancefield, principal causante de la faringoamigdalitis. Se realizaron pruebas de sensibilidad y se midieron las zonas de inhibición *in vitro*. El aceite esencial destilado, registró el mayor halo de inhibición (3.2 cm), incluso superó a la penicilina (2.4 cm). Con el extracto etanólico la inhibición fue menor y con la infusión no hubo inhibición. El aceite esencial y el extracto etanólico fueron analizados por medio de cromatografía en capa fina y cromatografía de gases para determinar su concentración y pureza en comparación con el aceite escencial puro de tomillo, obteniéndose la presencia de timol y en menor grado carvacrol, agentes activos que producen inhibición en el crecimiento bacteriano.

Palabras Clave: *Antimicrobiano, Faringoamigdalitis, Streptococcus, Thymus vulgaris.*

INTRODUCTION

Faryngoamygdalitis, caused by the ß-hemolytic group A bacteria *Streptococcus pyogenes*, is a major health concern in Mexico. Faryngoamygdalitis often provokes ear and sinus inflammation, pneumonia, and secondary immunological problems such as scarlet fever, glomerulonephritis, and rheumatic fever; the latter can cause lesions in up to 80% of those infected[1,2,3].

The most commonly antibiotic used to treat *Streptococcus* infection is penicillin[4], however its administration can have undesirable toxic effects throughout the body. Furthermore, missuse of penicillin can generate resistance in populations of the target bacteria. Herbal remedies, on the other hand, can provide alternative therapeutic treatments of *Streptococcus* infections. Thyme (*Thymus vulgaris* L.) has been used as an

Nota: Artículo recibido el 20 de septiembre de 2006 y aceptado el 27 de noviembre de 2006.

antiviral and antibacterial agent against the aforementioned diseases. *In vitro* studies of the antimicrobial activity of thyme have reported inhibition of the growth of *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans* and *Pseudomonas aeruginosa*[5]. The antiseptic properties of *T. vulgaris* have been attributed to its essential oil, known to be rich in thymol and carvacrol[6]. In addition, other studies of thymol have reported antihelminthic, antifungal, antibacterial, antiviral, and antispasmodic properties[7-11].

Traditional medicine enjoys wide acceptance in Mexico, and as much as two thirds of the population relies on medicinal plants to resolve health problems. Moreover, it is not uncommon for modern treatments to be abandoned in favor of natural therapies which are often less aggressive and more economically accessible. The present work seeks to evaluate the popular use of thyme as an antimicrobial agent.

MATERIALS AND METHODS

Taxonomic determination of plant material

Three kilograms of fresh plant material were purchased in the Sonora market of Mexico City. This material was positively identified as *Thymus vulgaris* by M. Sc. Carlos Castillejos Cruz and deposited in the FEZA herbarium of the Facultad de Estudios Superiores Zaragoza, UNAM (Voucher No. 5534).

Preparation of the extracts and infusion

Extracts were obtained via distillation of water vapor, maceration and ethanol extraction, and infusion. For the distillation, 300 mL of a saturated NaCl solution was added to 100 g of plant material that had been dried in the shade (~18ºC). After one liter of distillate was obtained, the organic phase was extracted with 25 mL of $CHCl_3$ and dried with 10 g of $CaCl_2$.

For the ethanol extract, 10 g of dried plant material was washed with distilled water, placed in 100 mL of EtOH (50%) for 10 days, and then filtered using a 0.45 µm micropore membrane.

The infusion was prepared by placing 10 g of plant material in one liter of boiling sterile distilled water (93 °C). The infusion was then filtered first with filter paper and then a 0.45 µm millipore filter.

Gas chromatography

A Perkin Elmer model 1022 gas chromatograph was employed for the analyses, and the following conditions were used: temperatures: one 80 °C, oven two 180 °C and oven three 200 °C; detector temperature 200 °C, dragging time 0.5, 6.0 and 4.0 min for each oven, respectively, total development time 22.5 min, range 10 °C/min, dragging gas He, solvent CS_2, column Carbowax 20 m, 0.53 mm D.I., and 0.80 u and an ionization detector with flame. Each sample was dissolved in 1 mL of CS_2; aliquots of 0.5 µL were used for the injection.

Isolation and identification of *Streptococcus pyogenes*

Streptococcus pyogenes was identified and isolated from patients with faryngoamygdalitis at the laboratory of Unidad de Medicina Familiar No. 3, Instituto Mexicano del Seguro Social. Brain heart infusion beef broth was used to culture the pharyngeal exudates. Exudate was obtained from the posterior part of the pharynx using sterile cotton swabs. The cotton swabs were submerged in the culture medium, which was then incubated at 35 ºC for two hours. A sterile blood-agar medium was prepared and left to cool. Once cooled, 25 mL of defibrinated sterile sheep's blood was added. Samples were incubated at 35 ºC for 24 hours in a Gravity Convection Incubator (Model 4 EG).

After incubation, samples were checked for the presence of ß-hemolytic colonies. Colonies were selected and moved to a second blood-agar Petri dish, to which a 0.04 mg disc of bacitracin was added to identify *Streptococcus pyogenes*. These dishes were incubated at 35 ºC for 18 to 24 hours, during which the presence of an inhibition zone was detected, allowing the identification of group A ß-hemolytic *S. pyogenes*.

Determination of optimal volume

The Bauer-Kirby technique[12] was used to determine the optimal volume that inhibits the growth of colonies. Sensitivity tests were performed on blood-agar medium using discs with different volumes of the distillate, ethanol extract, and the infusion.

Culture medium for *Streptococcus* was made dissolving 2.5 g of tripticaseine and soybean phosphate broth in 100 mL of distilled water. This mixture was distributed in 4 mL aliquots to test tubes that were then sterilized. The $BaSO_4$ standard used to adjust the bacterial population density to 10^8 colony forming units (CFU/mL) was prepared with 99.5 mL of H_2SO_4 (0.36N) and 0.5 mL of a 1% $BaCl_2$ solution. Aliquots of 4 to 6 mL were placed in tubes that were then closed and stored in the dark at room temperature.

Ten colonies of *Streptococcus pyogenes* were moved with a platinum loop to test tubes and suspended in tripticaseine broth. The tubes were incubated for 2 to 5 hours, until the turbidity reached that of the $BaSO_4$ standard, which corresponded to half the pattern tube of the McFarland Nephelometer. When adjustment of the inoculate was necessary to approximate the concentration of 10^8 cells/mL, it was diluted with tripticaseine broth or the incubation time was increased.

Sensitivity tests with extracts

Samples were spread uniformly in Petri dishes over a blood-agar medium. After 2 to 5 minutes at room temperature, 7 mm diameter discs of filter paper that had been previously impregnated with 5, 10, 20 or 30 µL of distillate, ethanol extraction, or infusion of *Thymus vulgaris* were distributed on the Petri dishes. A dish with only medium and inoculate and one with only medium were prepared at the same time to be used as positive and negative

controls. Discs impregnated with 20 µL of commercial essential oil were used to establish concentrations, since preliminary trials showed that this volume of essential oil was the most effective at inhibiting bacterial growth. Ten-unit sensi-discs of penicillin were placed over the medium. The Petri dishes were incubated at 37 °C for 24 h. Zones of inhibition were then measured. We realized four independent experiments done in quintuplicate. In order to investigate differences between treatments, an analysis of variance Fisher´s test were performed at 95% confidence. The software STATGRAPHICS Plus Version 5.0 was used for these analysis.

Results and discussion

Gas chromatography

Gas chromatography was performed to elucidate the concentration of thymol and carvacrol, and the resulting chromatograms can be seen in Figures 1, 2, 3 and 4. Table I presents the concentrations of thymol and carvacrol. These data suggest that the methods of extraction used are efficient at separating thymol and carvacrol from thyme plants. As seen in Figure 1, 3 the ethanol extraction and distillation are more efficient in separating thymol but not carvacrol. These results are in accordance with those of Balladin and Oliver[13] who indicate that carvacrol is found in lower concentration in the essential oil of thyme.

The low concentration of thymol present in the ethanol extract and the infusion does not, however, warrant discarding these techniques, since they are the most practical means of extraction for the consumer. Increasing the amount of dry plant material used for extraction might resolve this problem.

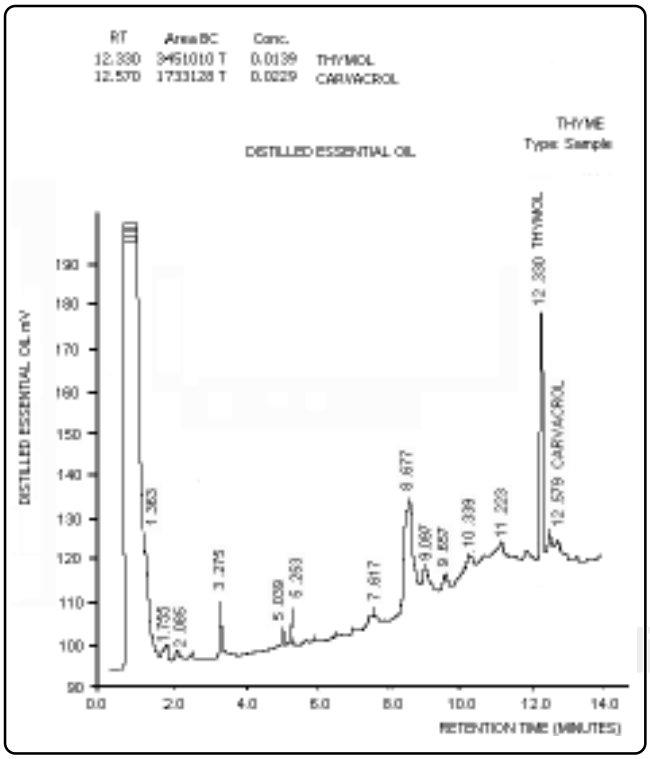

Figure 1. Chromatography of distilled essential oil.

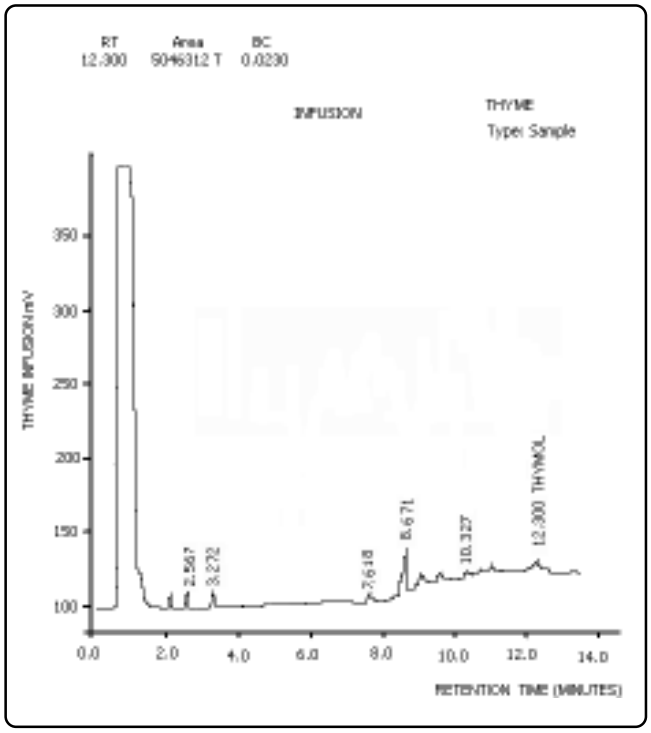

Figure 2. Chromatography of infusion.

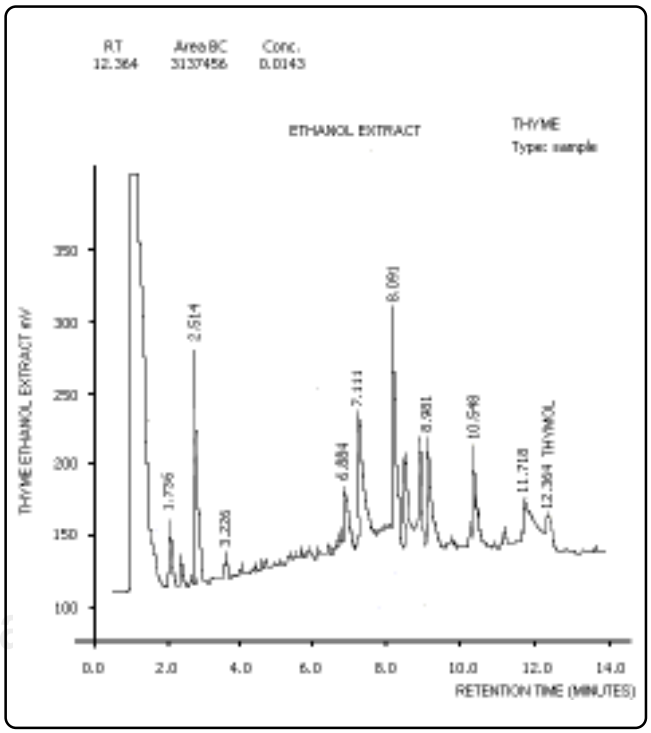

Figure 3. Chromatography of ethanol extract.

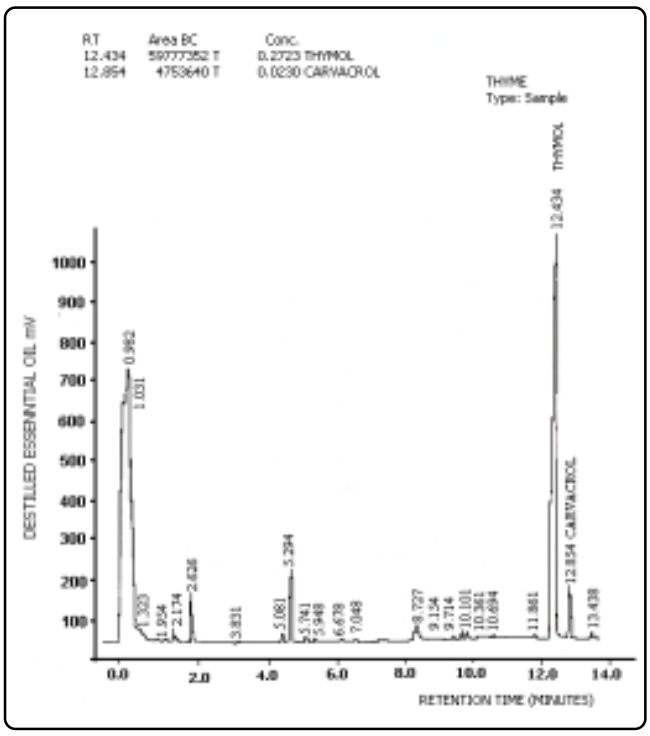

Figure 4. Chromatography of patron oil.

Sample	Thymol (mg/mL)	Carvacrol (mg/mL)
Pure essential oil	0.2723	0.0230
Ethanol extract	0.0143	N. D.
Infusion	0.0230	N. D.
Essential oil destilled	0.0159	0.0229
N.D. Not detected		

Table I. Concentrations of thymol and carvacrol presents in *Thymus vulgaris* L.

Biological Tests

Results of the tests for biological activity are summarized in Table II. The 6 µg of penicillin used in the experiment are equivalent to 10 U, which corresponds to the concentration recommended for Clinical Laboratory Standards Institute and the World Health Organization. As seen in Table II, the essential oil destilled inhibited growth of *Streptococcus pyogenes* at all volumes tested and were statistically significant difference ($p = 0.005$) than penicillin, except for 10 µL. However, the largest halo was observed using a volume of 20 µL, which diminished in size at 30 µL. This phenomenon is called Eagle's "paradoxical effect"; it is thought that this is the result of interference with protein synthesis caused by increased concentrations of β-lactamase.

Upon comparison, the halo of inhibition of the distilled essential oil was greater than penicillin at volumes 20 and 30 µL (Table 2). The halo of inhibition of the ethanol extract was smaller than for all volumes tested. The infusion did not inhibit bacterial growth. It is important to note, however, that the essential oil used is not particularly soluble in water and is very volatile. These factors, in conjunction with the small quantity of material used (20 µL), are perhaps at least partially responsible for the absence of an effect. While the maximum volume used in these analyses was 30 µL, the volume recommended for use in traditional medicine (3 or 4 cups of the infusion daily) corresponds to approximately 800 mL; this suggests that, in spite of the results reported here, infusions should not necessarily be discarded as a treatment for pharyngoamygdalitis. On the other hand, the extraction by distillation did produce the highest quality essential oil with regards to the inhibition of bacterial growth, and the level of inhibition of the distillate and the commercial oil were very similar (halo of inhibition of 3.22 and 3.42 cm respectively).

Conclusions

Extracts of *Thymus vulgaris* L. obtained via distillation contain thymol and carvacrol. The distillate inhibited growth of *Streptococcus pyogenes*, which demonstrates its antibacterial action. Inhibition was not observed using the infusion; however, given that the oil is not water soluble, it might have been retained during the filtration process. Furthermore, the low boiling point of essential oils probably led to volatilization during the extraction process. Ethanol extract is a simple, adequate method for extracting the compounds present in thyme. Though the distillate produced the strongest results, we suggest that, instead of discarding the ethanol extraction, increasing the amount of plant

Volume (µL)	Essential oil destilled halo (cm)		Etanol extract halo (cm)		Infusion halo (cm)		Penicillin (6 mg) halo (cm)		Pure essential oil halo (cm)	
	x	σ_{n-1}	x	σ_{n-1}	x	σ_{n-1}	x	σ_{n-1}	x	σ_{n-1}
5	1.12*	0.0447	0.70	0.00	0.70	0.00	2.46	0.0547	-	-
10	2.46	0.2073	0.70	0.00	0.70	0.00	-	-	-	-
20	3.220*	0.1923	0.75	0.00	0.70	0.00	-	-	3.42*	0.1303
30	2.76*	0.0547	0.75	0.00	0.70	0.00	-	-	-	-
* Denotes a statistically significant difference ($p = 0.005$)										

Table II. Halo of inhibition of the essential oil destilled, ethanol extract, infusion and penicillin on the growth of the *Streptococcus pyogenes* (x = mean, σ_{n-1} = Std. Dev., from four experiments done in quintuplicate).

material used could improve the results. Thymol demonstrates an antibacterial activity that is accentuated in combination with carvacrol and probably other phenol derivatives present in the samples analyzed.

Acknowledgements

The authors thank Dra. Yolanda Ríos Gómez for critical reading of manuscript, Bióls. Ma. del Carmen López and two anonymous reviewers for comments in review.

References

1. Gwaltney, J.M.R. Jr. Virology of middle ear. *Anal. Otol. Rhinl. Laryngol.* **80,** 365 (1971).
2. Stevens, D.L., Tanner, M.H. & Winship, J. Reappearance of scarlet fever toxin A among Streptococci in the Rocky Mountain West: severe Group A Streptococcal infection associated with atoxic shock-like syndrome. *N. Engl. J. Med.* **321,** 1-7 (1989).
3. Stevens, D.L. Streptococcal toxic shock syndrome: spectrum of disease, pathogenesis and new concepts in treatment. *Emerg. Infect. Dis.* **1,** 68-78 (1995).
4. Mascini, E.M., Hanze, M., Schouls, L.M., Verhoef, J. & Van Dijk, H. Penicillin and clyndamycin diffrentially inhibit the production of pyrogenic exotoxins A and B by group A Streptococci. *Int. J. Antimicrob. Agents.* **18,** 395-398 (2001).
5. Cacéres, A.M., Girón, L.R., Alvarado, S. & Torres, F. Screenning of antimicrobial activity of plants popularly used in Guatemala. *J. Etnopharm.* **20,** 223-237 (1987).
6. Didry, N., Dubreuil, L. & Pinkas, M. Activity of thymol, carvacrol, cinnamaldehyde and eugenol on oral bacteria. *Pharm. Acta Helv.* **69,** 25-28 (1994).
7. Knobloch, K. & Strobel, H. Effective concentration of essential oil components to scavenge oxygen radicals and inhibit lipoxigenase turnover rates. *Planta Med.* **59 Supp A,** 669 (1993).
8. Juven, B.J., Kanner, J., Scheved, F. & Weisslowicz, H. Factors the interact with the antibacterial action of thyme essential oil and its active constituens. *J. Appl. Bacteriol.* **76,** 626-631 (1994).
9. Perrucci, S., *et al. In vitro* antifungal activity of essential some aislates of *Microsporum canis* and *Microsporum gypseum*. *Planta Med.* **60,** 184-187 (1994).
10. Mesiter, A.G., Bernhardt, V.G., Christoffel, V. & Buschauer, A. Antiespasmodic activity of *Thymus vulgaris* extracts on isolated guinea-pig trachea: Discrimination between drug an ethanol effects. *Planta Med.* **65,** 512-516 (1999).
11. Flach, A., *et al.* Chemical analisys and antifungal activity of the essential oil of *Calea clematidea*. *Planta Med.* **9,** 836-838 (2002).
12. Bauer, A.W. & Kirby, M.W. Antibiotic susceptibility testing by a standardized single method. *Am. J. Clin. Pathol.* **45,** 493-496 (1966).
13. Balladin, D.A. & Headley, O. Evaluation of solar dried thyme (*Thymus vulgaris* Linné) herbs. *Renewable Energy* **17,** 523-531 (1999).