In vitro efficacy testing of citronella grass oil against *Tritrichomonas foetus* trophozoites

Evaluación *in vitro* de la eficacia del aceite de citronela contra trofozoítos de *Tritrichomonas foetus*

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Abstract

Tritrichomonas foetus, a sexually transmitted parasitic protozoan, causes abortion in cattle. Nitroimidazoles, such as metronidazole, treat bovine trichomonosis, but their use is precluded. Plant extracts might have antiparasitic effects. This study aimed to assess *Cymbopogon nardus* oil activity against *T. foetus* as an alternative to metronidazole. *T. foetus* trophozoites were incubated in culture medium containing serial dilutions of *C. nardus* oil. Cytotoxicity was assessed 24 hours later. *C. nardus* oil killed *T. foetus* cells. Half maximal effective concentration (EC50) was 0.4 µg/mL. These findings suggest that *C. nardus* oil could be exploited for discovery and compound isolation of plant-derived phytopharmaceuticals for bovine trichomoniasis and other protozoan diseases.

Keywords
disease • venereal • trichomonas • trichomoniasis • antimicrobial • dose-response

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Resumen

Tritrichomonas foetus es un protozoo parásito de transmisión sexual que provoca abortos en el ganado. Los nitroimidazoles, como el metronidazol, pueden ayudar en el tratamiento de la tricomonosis bovina, pero no se aconseja su uso. Los extractos de plantas pueden tener un efecto antiparasitario. El estudio actual tuvo como objetivo evaluar la actividad del aceite de *Cymbopogon nardus* contra *T. fetus* como alternativa al metronidazol. Se incubaron trofozoítos de *T. fetus* en medio de cultivo con diluciones seriadas de aceite de *C. nardus*. La citotoxicidad se evaluó a las 24 horas. El aceite de *C. nardus* mató las células de *T. fetus*. La concentración efectiva media máxima (CE50) fue de 0,4 µg/ml. Estos hallazgos sugieren que el aceite de *C. nardus* podría servir para el descubrimiento y aislamiento de fitofármacos para el tratamiento de la tricomoniasis bovina y de otras enfermedades protozoarias.

Palabras clave

enfermedad • venérea • trichomonas • trichomoniasis • antimicrobiano • dosis-respuesta

Introduction

Tritrichomonas foetus is a parasite protozoan that causes bovine trichomonosis (2). This venereal disease causes premature abortion and extended intercalving seasons. Nitroimidazoles are antibiotics used to treat infections such as trichomonosis (10). However, strain resistance and potential toxicity to meat consumers limit their use.

Bovine trichomonosis vaccines are not available and culling TF-infected animals is recommended. Between 2015 and 2019, more than 3,800 *T. foetus*-carrying bulls were culled in the United States (11). Every year in La Pampa, Argentina, more than 300 diagnosed bulls must be slaughtered (13). So far, testing and elimination programs have rarely succeeded in their purpose of eradicating *T. foetus* from cattle populations (13, 16).

New therapies for *T. foetus*-infected animals consider plant extracts as alternatives to commercial drugs. Extracts obtained from black tea, green tea, grape and pomegranate inhibit pathogenic trichomonads (9). Among natural extracts, essential oils are commonly used in traditional medicine and some studies have addressed their effect against protozoa (1, 5).

Citronella essential oil (EO) is extracted from the perennial herb *Cymbopogon nardus* (7). *C. nardus* is cultivated in tropical and subtropical regions, including northern Argentina. The oil has several biological activities, including insect-repellent, fungicidal, and bactericidal properties (7, 9, 12). To the best of our knowledge, the anti-trichomonas activity of this compound has not yet been investigated (4).

This study aimed to investigate the efficacy of *C. nardus* essential oil against the parasite *T. foetus in vitro,* in comparison with metronidazole. Increasing concentrations of *C. nardus* essential oil should significantly decrease *T. foetus* trophozoites viability.

Materials and methods

Plant material was obtained from existing monoculture plantations from El Soberbio, Misiones, Argentina (27°17'43.76" S, 54°11'46.84" W). Approximately 1,000 g of aerial parts were hydrodistilled. The chemical composition of *C. nardus* oil was determined by capillary gas chromatography on a 2014 Shimadzu GC equipped with a FID detector and a capillary column (30 m × 0.25 mm internal diameter) J&W INNOWax 19091 N-213. Nitrogen was used as carrier gas. Samples (1 µl) were diluted with acetone and injected at a 50:1 ratio. Operating conditions considered oven temperature maintained at 60°C for 8 min and gradually raised by 3°C per min, reaching 180°C, and kept for 5 min. Temperature at injection and detector ports was 250°C. *C. nardus* oil main components were citronellal (40.1%), citronellol (12.7%) Geraniol (25.3%), limonene (3.7%), trans-PMD (3.6%), cis-PMD (2.6%) and isopulegols (0.3%).

The clone *T. foetus* B1 obtained from a cow with pyometra, was used for conducting parasite-killing assays (14). Trophozoites were maintained in trypticase-yeast-maltose (TYM) medium supplemented with 1 g/L streptomycin, 1,000,000 IU/L ampicillin and 10% v/v heat-inactivated horse serum. Cultures were maintained at 37°C in sterile 2.0 mL tubes filled with media and tightly capped. Subcultures were made in 2-3 days intervals maintaining cell concentrations below 8 10^5 trophozoites/mL.

Pure cultures in antibiotic-free medium were seeded at a density of 5.10^5 *T. foetus* cells/mL in 10-fold triplicate serial dilutions containing *C. nardus* oil between 0-800 ug/mL or metronidazole (Sigma Aldrich, M1547) dilutions from 0 to 100 ug/mL. Vehicles and drugs diluted in water or 70% ethanol accounted for 1% of final volume. Incubation proceeded for 24 h at 37°C under microaerophilic conditions. All experiments were performed three times. Compound toxicity was assessed by Trypan blue exclusion on hemocytometer slides. GRmetrics' 4-parameter non-linear regression model was used to calculate IC50 and EC50 values as well as to generate dose-response curves (3). *T. foetus* growth rates were plotted against logarithmically transformed drug concentrations.

Results

Figure 1 shows best-fitted dose-response curves depicting *T. foetus* B1 cells response to *C. nardus* oil and metronidazole control. Over 24 hours, mock-treated cells doubled 4.6 times. At the same time, the vehicle (0.7% ethanol) had no visible effect on cell division or morphology. In contrast, treatment with metronidazole (positive kill control) produced a standard S-shaped curve, indicating strain sensibility. The EC50 for metronidazole was 0.016 µg/mL (16 µM). The concentration at which relative cell count was 0.5 of maximal value (IC50) was also 0.016 µg/mL (table 1, page 112).

C. nardus oil also exhibited dose-dependent inhibition, with all cells killed after 24 hours at high oil concentrations. The EC50 value for *C. nardus* oil was 0.391 µg/mL (table 1, page 112). Oil concentration required to achieve a growth rate of 0.5 was 0.65 µg/mL (table 1, page 112). Fitted curves explain over 95% of observed variation (figure 1).

Untransformed growth rate *versus* log-transformed concentration showing effects of *C. nardus* oil compared to metronidazole. Standard curves were built with Grmetrics. GR: growth rate.

Tasa de crecimiento no transformada *versus* concentración logarítmica transformada para mostrar el efecto del aceite en comparación con el metronidazol. Las curvas estándar se construyeron con Grmetrics. GR: tasa de crecimiento.

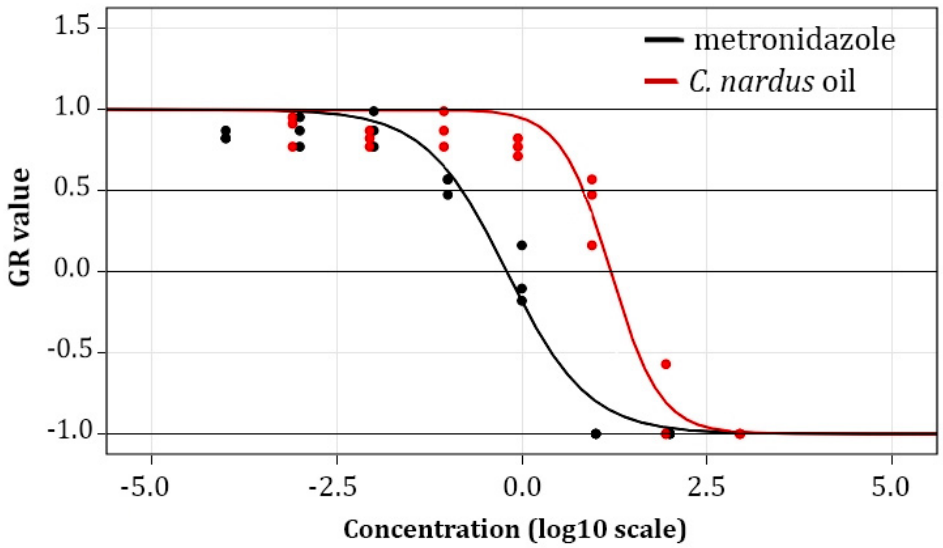

Figure 1. Dose-response curve for *Cymbopogon nardus* oil effect on *Tritrichomonas foetus.*

Figura 1. Curva dosis-respuesta del efecto del aceite de *Cymbopogon nardus* sobre *Tritrichomonas foetus.*

Table 1. *Cymbopogon nardus* oil inhibition values.
Tabla 1. Valores de inhibición del aceite de *Cymbopogon nardus*.

	r^2	GR50	IC50	EC50	h	*p*
Oil	0.96	6.78	0.391	0.391	0.337	2.47 e-08
Metronidazole	0.97	0.15	0.016	0.016	0.419	4.83 e-10

r^2 coefficient of determination indicating goodness of fit; GR50, concentration at which the effect reaches a growth rate value of 0.5 based on interpolation; EC50, the concentration at which relative cell count was 0.5 of maximal values; IC50, the concentration at half-maximal effect; h, Hill coefficient of the fitted (traditional) dose-response curve and the p-value of a F-test comparing curve fit to a horizontal line fit. Concentrations are expressed in ug/mL.

Se indican: r^2, el coeficiente de determinación para indicar bondad de ajuste de la curva; GR50, la concentración a la cual el efecto alcanzó un valor de tasa de crecimiento de 0,5 basado en la interpolación de la curva ajustada; EC50, la concentración a la que el recuento relativo de células fue 0,5 de los valores máximos; IC50, la concentración a la mitad del efecto máximo; h, el coeficiente de Hill de la curva dosis-respuesta ajustada (tradicional) y el valor p de una prueba F que compara el ajuste de la curva con un ajuste de línea horizontal. Las concentraciones se expresan en ug/mL.

Discussion

Several plant compounds have *in vitro* anti-trichomonal activity (6). Data presented here indicate that *C. nardus* oil can kill *T. foetus* in a dose-dependent manner. *C. nardus* oil could be used for local treatment. Additionally, *C. nardus* oil could constitute a basic resourse for plant-derived drugs for protozoan diseases. The main constituents of *C. nardus* oil (geranial, citronellol and elemol) show activity against *Trypanosome brucei* (9). In addition, several components have been found active against fungal strains (citronellal and linalool), Gram-positive and Gram-negative bacterial species (elemol, citronellol, citronellal) (7, 8, 15).

Conclusion

C. nardus oil affects growth and viability of *T. foetus in vitro*. *C. nardus* oil constitutes a useful resource for the discovery of plant-derived drugs for treating bovine trichomonosis and other protozoan diseases.

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Conflict of interests

The authors declare that there is no conflict of interest.