REVISTA BIO CIENCIAS
http://revistabiociencias.uan.edu.mx
https://doi.org/10.15741/revbio.10.e1348

ISSN 2007-3380

Original Article /Artículo Original

Antioxidant and antibacterial effect of different extracts from *Tithonia tubaeformis.*

Efecto antioxidante y antibacteriano de diferentes extractos de *Tithonia tubaeformis*.

García-Vázquez, L. M.[1], *Ocampo-López, J.*[1], *Ayala-Martínez, M.*[1], *Hernández-Aco, R. S.*[1], *Zaragoza-Bastida A.*[1], *Zepeda-Bastida, A.*[1]*

[1] Área Académica de Medicina Veterinaria y Zootecnia. Instituto de Ciencias Agropecuarias. Universidad Autónoma del Estado de Hidalgo. Av. Universidad Km. 1 Rancho Universitario, CP. 43600. Tulancingo, Hidalgo. México

Please cite this article as/Como citar este artículo: García-Vázquez, L. M., Ocampo-López, J., Ayala-Martínez, M., Hernández-Aco, R. S., Zaragoza-Bastida A., Zepeda-Bastida, A. (2023). Antioxidant and antibacterial effect of different extracts from *Tithonia tubaeformis*. *Revista Bio Ciencias*, 10 e1348 https://doi.org/10.15741/revbio.10.e1348

Article Info/Información del artículo

Received/Recibido: May 12th 2022.

Accepted/Aceptado: March 28th 2023.

Available on line/Publicado: April 21th 2023.

ABSTRACT

The genus *Tithonia* has been extensively studied to determine its bioactive effects of pharmaceutical and nutritional interest; therefore, this study aimed to determine the antioxidant and antibacterial effect of five extracts of *Tithonia tubaeformis*, obtained with ascending polarity solvents, hexane, dichloromethane, ethyl acetate, methanol, and ethanol. In addition, tannins, alkaloids, and total phenols were identified. The antioxidant effect of ABTS, DPPH radicals, and ferric ion reducing power was measured, and the Minimum Inhibitory Concentration of each extract was determined on *Escherichia coli, Salmonella typhimurium, Salmonella enteritidis*, and *Listeria monocytogenes*. The results showed that the five extracts have antioxidant effects on ABTS, DPPH radicals, and ferric ion-reducing potential; however, methanol and ethanol had a statistically significant effect ($p < 0.05$). In addition, methanol, ethanol, and ethyl acetate extracts showed antimicrobial activity; ethanol solvent was the best, with a Minimum Inhibitory Concentration of 1 mg/mL on *Escherichia coli* and 3 mg/mL on *Salmonella typhimurium, Salmonella enteritidis, Escherichia coli*, and *Listeria monocytogenes* respectively. These results suggest that *Tithonia tubaeformis* may be of pharmaceutical and nutritional interest due to the bioactive effects of phytochemicals in its extracts.

KEY WORDS: ABTS, DPPH, Ferric ion, Pathogenic bacteria.

***Corresponding Author:**

Armando Zepeda Bastida. Área Académica de Medicina Veterinaria y Zootecnia. Instituto de Ciencias Agropecuarias. Universidad Autónoma del Estado de Hidalgo. Av. Universidad Km. 1 Rancho Universitario, CP. 43600. Tulancingo, Hidalgo. México.
Tel. 7717172000 Ext 2449. E-mail: azepeda@uaeh.edu.mx

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ISSN 2007-3380

RESUMEN

El género *Tithonia*, ha sido ampliamente estudiado para determinar sus efectos bioactivos de interés farmacéutico y nutricional, por lo tanto, el objetivo del presente estudio fue determinar el efecto antioxidante y antibacterial de cinco extractos de *Tithonia tubaeformis*, obtenidos con solventes de polaridad ascendente, hexano, diclorometano, acetato de etilo, metanol y etanol. Se identificó la presencia de taninos, alcaloides y fenoles totales. Se midió el efecto antioxidante a los radicales ABTS, DPPH y poder reductor al ion férrico, se determinó la Concentración Mínima Inhibitoria de cada extracto sobre *Escherichia coli*, *Salmonella typhimurium*, *Salmonella enteritidis* y *Listeria monocytogenes*. Los resultados obtenidos mostraron que los cinco extractos tienen efecto antioxidante sobre los radicales ABTS, DPPH y potencial reductor al ion férrico; sin embargo, los extractos que presentaron un efecto estadísticamente significativo ($p < 0.05$) fueron metanol y etanol. Los extractos de metanol, etanol y acetato de etilo mostraron actividad antimicrobiana, siendo el solvente de etanol el que mayor actividad antibacterial tuvo, con una Concentración Mínima Inhibitoria de 1 mg/mL sobre *Escherichia coli* y 3 mg/mL sobre *Salmonella typhimurium, Salmonella enteritidis, Escherichia coli* y *Listeria monocytogenes*. Estos resultados sugieren que *Tithonia tubaeformis* puede ser de interés farmacéutico y nutricional por los efectos bioactivos de los fitoquímicos presentes en sus extractos.

PALABRAS CLAVE: ABTS, DPPH, Ion férrico, bacterias patógenas.

Introduction

The *Tithonia* genus is an essential source of several natural compounds, such as lactones, sesquiterpenes, diterpenes, and flavonoids; the most investigated species has been *Tithonia diversifolia*, which exerts anti-inflammatory, analgesic, antimalarial, antiviral activity, antidiabetic, antidiarrheal, antimicrobial, antispasmodic, relaxing vessel, antioxidant, and cytotoxic effects (Chagas-Paula *et al*., 2012). *Tithonia tubaeformis* is a native species of México, located throughout its territory; it grows like a weed on cultivated land, mainly maize, so it is considered waste (Mendoza-Ramírez *et al*., 2021). In traditional medicine, it is used to control gastrointestinal problems and promote digestion; in addition, some farmers use it as feed for livestock (Gheno-Heredia *et al*., 2011); different parts of the plant were used in the feeding of rabbits to evaluate the productive parameters in rabbit fattening (Pérez-Martínez *et al*., 2018); also, the quality of the meat and carcass of rabbits fed with the plant was evaluated (Zepeda-Bastida *et al*., 2019); *Tithonia tubaeformis* extracts showed an antinociceptive effect, on a mouse model (Nawaz *et al*., 2018; 2019); likewise, some phytochemical compounds have been identified such as saponins, tannins, phenols, and flavonoids in methanolic extracts, which are attributed anti-inflammatory activity (Hinojosa-Dávalos *et al*., 2013). These findings suggest that secondary metabolites present in the plant can prevent or delay the development of cardiovascular diseases, diabetes,

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and cancer, due to processes involving reactive oxygen species and may also have a modulating effect on biochemical reactions in signaling cascades necessary for cellular functions (Pretti *et al*., 2018). There are several techniques for the extraction of bioactive compounds, most of which are based on the extraction power of different solvents and the application of heat to the compounds of interest (Azmir *et al*., 2013). This study aimed to evaluate the antioxidant and antimicrobial effect of five extracts of *Tithonia tubaeformis*.

Material and Methods

Reagents and solutions

Hexane (Hx), dichloromethane (DCM), ethyl acetate (AcOEt), ethanol (EtOH), and methanol (MeOH) were acquired from Meyer®. In addition, distilled and deionized water was obtained using an Elix essential water purification system (Merck-Millipore, Germany) 2,2′-Azino-bis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-Tris(2-pyridyl)-s-triazine, Folin ciocalteu, 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), gallic acid, sodium carbonate (Na_2CO_3), bromocresol green ($C_{21}H_{14}Br_4O_5S$), sodium phosphate (Na_3PO_4) chloroform, penicillin, acquired from Merck®, and Agar Mueller Hinton obtained from (DIBICO®, Mexico).

Equipment

Oster® blender (USA), spectrophotometer Jenway model 6300, water bath StableTemp, Cole Parmer, (USA).

Extraction with organic solvents

Tithonia tubaeformis was from corn fields in Teotihuacan, State of Mexico, México (19°41´00´´ N 98°52´00´´ W) between September and October 2018. The stems, leaves, and flowers were used, dehydrated at room temperature in the dark, ground in a blender, and stored in a food-grade brown paper bag in the dark and moisture-free until later use.

Extracts were obtained by maceration (Brusotti *et al*., 2014). Weighed 40 g of vegetable material which remained in contact with the different solvents: Hx, DCM, AcOEt, EtOH, and MeOH, for 24 h at room temperature and in constant agitation; subsequently filtered through four layers of gauze followed by filter paper (Whatman No. 40), the extracts obtained were stored in an amber flask and cooled to 4 °C until use.

Phytochemical analysis

The antioxidant activity was determined through the ABTS technique (Siddhuraju & Becker, 2007) based on the discoloration of the ABTS radical due to the reduction of the electron cation present in the plant sample. A calibration curve was made with Trolox, and for the extracts, 50 mL each were taken and incubated for 30 min at room temperature in darkness with 1,45 mL of the radical ABTS, and absorbance was measured at 732 nm. On the other hand, the antioxidant

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ISSN 2007-3380

effect was determined using the DPPH technique (Brand-Williams *et al*., 1995), based on the ability of an antioxidant to neutralize a radical. The initial concentration of the DPPH compound was determined, and the resulting concentration after the extracts was added transferred 1,95 mL of DPPH solution to 50 mL of each; the absorbance was measured at 517 nm every 10 min for one hour, the time when the concentration of DPPH is reduced by half, and the percentage of inhibition was determined at 30 min. Additionally, for DPPH and ABTS, the IC_{50} measurement was performed.

Reducing the potential of Fe^{+3} to Fe^{+2} was measured (Müller *et al*., 2011). A calibration curve was made with Trolox as a control. The extracts were taken 100 mL of each, were deposited in a test tube with 300 mL of distilled water and 300 mL of FRAP solution (2,5 mL de 2,4,6-Tris(2-pyridyl)-s-triazine with 2,5 mL de $FeCl_3$ in acetate buffer 0,3 M pH 3,6), incubated in a water bath (StableTemp, Cole Parmer, USA) at 37 °C for 30 min, after time, the absorbance at 593 nm was evaluated.

The results obtained from the three assays were analyzed using a completely random design, a one-way variance analysis. A Tukey means comparison test was performed with a $p < 0,05$ in the IBM SPSS Statistics version 20 statistical program.

Total phenols were measured (Singleton *et al*., 1999) based on a colorimetric method that allows the analysis of organic compounds with aromatic rings (phenols, tannic acid, lignin, and proteins); an aqueous extract was prepared using 40 g of the plant in 100 mL of distilled water, then 1,58 mL of the extract was placed in test tubes, 100 μL of Folin ciocalteu reagent was added, stirred and incubated for eight min at light-free ambient temperature, 300 μL of Na_2CO_3 at 20 % (p/v), was added over time, stirred and incubated at 50 °C in a water bath for 15 min, at the end of incubation the absorbance at 765 nm was evaluated.

The presence of alkaloids was measured (Shamsa *et al*., 2010) based on the reaction of alkaloids with nitrogen in their structure, with bromocresol green resulting in the formation of an alkaloid-green bromocresol charge-transfer complex. An extract was made with 20 g of dried plant in 50 mL of boiling distilled water, covered with aluminum, and left for one hour, then filtered with three layers of gauze and filter paper; 50 mL of boiling water was added to the remaining plant material, waited 30 minutes, then filtered with gauze, five mL of extract was transferred to a test tube, added five mL bromocresol green, and five mL Na_3PO_4, was shaken, two mL chloroform were added, then emptied into a separation funnel to recover the chloroformic phase into a 10 mL volumetric flask, the rest of the solution was returned to the tube, and repeated to reach a final volume of 10 mL, the chloroformic phase was measured absorbance at 470 nm.

For tannins determination (Bajaj & Devsharma, 1977), an aqueous extract with 0,8 g of the plant was prepared in 100 mL of distilled water, filtered with medium pore filter paper; in a test, the tube was transferred 250 mL of extract, 100 μL of Folin-Denis reagent, 250 μL of Na_2CO_3 (35 %), and 4,40 mL of distilled water, the mixture was incubated for 30 min at room temperature, and the absorbance was measured at 760 nm.

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Antimicrobial assay

The extracts obtained with the solvents were evaluated for their antimicrobial capacity against *Escherichia coli*, *Salmonella tiphymurium*, *Salmonella enteritidis,* and *Listeria monocytogenes* (CLSI, 2015); 5 concentrations of each extract (10, 7, 5, 3, 1, and 0,5 mg/mL) were adjusted to determine the minimum inhibitory concentration (MIC), which were filtered with spin filters of 0,45 mm pore diameter (Thermo Scientific®). The antibacterial activity of the extracts was determined by the test of discs on agar, for which the bacteria were seeded in a monolayer, adjusting the concentration of these to the standard of 0,5 of the McFarland scale ($1,5x10^8$ UFC/mL) in the petri dish with Muller Hinton solid medium. Discs of impregnated filter paper were placed from each extract to be evaluated with different concentrations, and penicillin 20 mg/mL as a positive control; finally, they were incubated at 37 °C for 48 h. MIC was defined as the lowest concentration of the extract that did not show visible bacterial growth after the incubation period.

Results and Discussion

To evaluate the antioxidant effect, it is necessary to use different methods such as DPPH, ABTS, and FRAP; the results obtained from the different extracts of *Tithonia tubaeformis* are shown in Table 1; methanol had significantly ($p < 0.05$) the highest antioxidant capacity (76,49 %), followed by ethanol, and hexane (67,6 % and 56,14 %, respectively) using the DPPH technique, regarding dichloromethane and ethyl acetate. Regarding the ABTS technique, the results obtained were like the DPPH technique, methanol had the highest percentage of inhibition (79,38 %), followed by ethanol and hexane (76,10 % and 62,57 %, respectively) concerning dichloromethane and ethyl acetate; we can observe this same behavior in the IC_{50} measurement, where we find that methanol (2,10 mg/mL DPPH and 0,80 mg/mL ABTS) and ethanol (6,05 mg/mL DPPH and 2,27 mg/mL ABTS) required a lower concentration to have an antioxidant effect on one half of a free radical; the antioxidant capacity through the ABTS method was lower than the DPPH method, perhaps due to the different reaction mechanisms of the radicals evaluated. The difference between the antioxidant capacity of the extracts may be due to the concentration or type of compound dissolved in each solvent because the percentage of extraction increases progressively along with the polarity of the solvent; this means that the rate of extraction yield is directly proportional to the polarity index of solvents (Zhang *et al*., 2015). Within the *Tithonia* genus, the antioxidant effect has been extensively evaluated; the aqueous extract of *Tithonia diversifolia* can counteract radical chain reactions, preventing oxidative damage of plasma lipids beyond the action of antioxidants naturally present in plasma (di Giacomo *et al*., 2015).

On the other hand, in Table 1, the ferric ion reducing potential (FRAP) of the different extracts obtained from *Tithonia tubaeformis* can be observed; the results showed that the methanol extract, which had significantly ($p < 0,05$), the most excellent reducing power over the ferric ion (137,27 mM/mL), this is related to the oxidative stress induced by metal ions to which significant diseases have been attributed. In contrast, the antioxidant/reducing effect measured with the FRAP technique refers to chelating the Fe^{3+} ion to Fe^{2+}, which could prevent potential damage to

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biomolecules from iron accumulation leading to an increase in free radicals and the development of oxidative stress (Ojo *et al*., 2018); it has been suggested that the presence of monoterpenes and monoterpenes oxygenated in the essential oil of *Tithonia diversifolia* are mainly responsible for the antioxidant-reducing potential (Orsomando *et al*., 2016). Furthermore, extracts obtained with acetone, ethyl acetate, dichloromethane, and butane from the leaves of *Tithonia diversifolia* have been shown to have Fe^{3+} ion-reducing power (Pantoja-Pulido *et al*., 2017).

Table 1.- Antioxidant effect of extracts obtained from *Tithonia tubaeformis.*

Extracts	DPPH		ABTS		FRAP (mM/mL)
	(%)	IC_{50} (mg/mL)	(%)	IC_{50} (mg/mL)	
Hexane	56,14^{ab} ±3.50	11,15	62,57^{ab} ±1,.38	8,29	49,04^{c} ± 2,37
Dichloromethane	35,58^{c} ±11.12	12,54	56,95^{bc} ±5,13	10,79	1,87^{e} ± 1,50
Ethyl acetate	24,16^{bc} ±12.96	25,40	51,14^{c} ± 4,29	13,38	25,05^{d} ± 1,26
Methanol	76,49^{a} ±2.37	2,10	79,38^{a} ± 0,21	0,80	137,.27^{a} ± 1,11
Ethanol	67,60^{a} ±5.64	6,05	76,10^{a} ±1,37	2,27	89,72^{b} ± 4,87

^{abc} Different letters within the rows indicate significant differences between treatments ($p < 0,05$).

The quantification of tannins, alkaloids, and total phenols present in the whole plant of *Tithonia tubaeformis* is shown in Table 2. The results showed that the aqueous extract of *Tithonia tubaeformis* contains 2 % tannins, 3 % alkaloids, and 230 mg EAG/L of total phenols, which suggests that this plant could be of pharmaceutical interest, as these compounds are linked to pharmacological activities such as anti-inflammatory, antimicrobial, and antioxidant effects (da Gama *et al*., 2014). Furthermore, in other investigations (Hinojosa-Dávalos *et al*., 2013; Zhang *et al*., 2015), phenols, alkaloids, steroids, tannins, and coumarins in methanolic and ethanolic extracts of *Tithonia tubaeformis* have been mentioned, which demonstrated inhibition to the DPPH radical. Furthermore, investigations of extracts of the species *Tithonia diversifolia* have been evaluated, showing an antioxidant effect on the radical DPPH (da Gama *et al*., 2014) and the presence of phenols (Ang *et al*., 2019). Therefore, our results obtained from the quantification of tannins, alkaloids, and phenols are consistent with those obtained by other authors since their presence correlates with an antioxidant effect (Nithya *et al*., 2016; Santhosh *et al*., 2022).

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Table 2. Quantification of tannins, alkaloids, and total phenols present in the complete plant of *Tithonia tubaeformis.*

Phytochemical	Concentration
Tannins %	2
Alkaloids %	3
Phenols mg EAG/L	230

The results of the antimicrobial activity of the different extracts obtained from *Tithonia tubaeformis* are shown in Table 3. The extract that had the best antimicrobial activity was ethanol, with a MIC of 1 mg/mL on *Escherichia coli*, 3 mg/mL on *Salmonella typhimurium*, *Salmonella enteritidis, Escherichia coli*, and *Listeria monocytogenes*, followed by ethyl acetate and methanol. Obtained data are consistent with ethanolic and methanolic extracts of *Arnica montana*, which showed an antibacterial effect on *Staphylococcus aureus* with an inhibition zone of 20 mm and 19 mm respectively (Kryvtsova & Koščová, 2020); In addition, extracts of *Olea africana* and *Tithonia diversifolia* have demonstrated antimicrobial effect on *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus,* and *Bacillus subtilis* (Douglas & Jeruto, 2016). The antimicrobial effect has been attributed to phytochemical compounds present in extracts from plant sources, which suggests that some of these compounds can permeate the cell membrane of bacteria through their hydrophilic and hydrophobic action interacting with membrane molecules and leading to cell death (Ju *et al.,* 2019).

Table 3. Antibacterial activity of extracts obtained from *Tithonia tubaeformis.*

Extracts	MIC mg/mL			
	Escherichia coli	***Salmonella typhimurium***	***Salmonella enteritidis***	***Listeria monocytogenes***
Hexane	N/I	N/I	N/I	N/I
Dichloromethane	N/I	N/I	N/I	N/I
Ethyl acetate	3	3	3	5
Methanol	5	5	7	1
Ethanol	1	3	3	3

MIC= Minimum inhibitory concentration

N/I= No inhibition

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Conclusion

Our results suggest that extracts obtained with methanol, ethanol, and ethyl acetate of *Tithonia tubaeformis* are effective as carriers of phytochemicals since they showed an antioxidant and antibacterial effect, antibacterial effect against *Escherichia coli*, *Staphylococcus typhimurium*, *Salmonella enteritidis,* and *Listeria monocytogenes*, this suggests that, in these extracts, the presence of secondary metabolites could be more significant and diverse. However, more research is needed to determine the type of metabolite present in the plant and to which these bioactive effects are attributed.

Author contributions

Work conceptualization García-Vázquez, L. M. and Zepeda-Bastida, A. Methodology development García-Vázquez, L. M., Ocampo-López, J., Ayala-Martínez, M. Experimental validation Hernández-Aco, R. S. Analysis of results García-Vázquez, L. M. and Zaragoza-Bastida A. Wrote and preparation of the manuscript García-Vázquez, L. M., Ocampo-López, J., and Zepeda-Bastida, A. Write, proofreading and edition Zaragoza-Bastida A., Hernández-Aco, R. S. and Zepeda-Bastida, A. All authors read and approved the final manuscript.

Funding

This research did not receive external funding.

Acknowledgments

Thanks to the Consejo Nacional de Ciencia y Tecnología for the scholarship awarded for doctoral studies.

Conflict of interest

The authors declare that they have no conflict of interest.

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ISSN 2007-3380

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