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Polyphenolic composition, antibacterial, modulator and neuroprotective activity of *Tarenaya spinosa* (Jacq.) Raf. (Cleomaceae)

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ABSTRACT

Objective: To evaluate the antibacterial activity and neuroprotective capacity of the ethanolic and aqueous extracts of *Tarenaya spinosa* (*T. spinosa*) as well as to determine and quantify some of its polyphenols by high performance liquid chromatography with diode-array detection (HPLC-DAD). **Methods:** The bacterial *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* strains, grown in Heart Agar Infusion, were tested. The drugs gentamicin, norfloxacin and imipenem were used to evaluate the modulating or antagonistic capacity of the *T. spinosa* extracts. The extract was analysed by HPLC-DAD to determine the main phenolic compounds. For the cell viability tests, individual heads of the *Nauphoeta cinerea* arthropod model were removed, homogenized in Trifluoromethyl ketone and centrifuged afterwards. Subsequently, 20 μL of NaNO$_2$ were added to the biological material, except in the control group, to evaluate the protection capacity of the extracts. The homogenate of the insect heads was incubated for 2 h in tubes containing tetrazolium bromide. **Results:** HPLC-DAD demonstrated that the ethanolic extract of *T. spinosa* presented caffeic acid as the major compound. The ethanolic extract also showed neuroprotective effects at concentrations ≥ 10 μg/mL, while aqueous extract was shown to have a protective effect only at the concentration of 100 μg/mL. The aqueous extract demonstrated a clinically relevant antibacterial activity against the *Staphylococcus aureus* multidrug resistant strain - MDR, with MIC 512 μg/mL. However, when the extracts were associated with gentamicin and imipenem, a synergism was detected against *Staphylococcus aureus* and *Escherichia coli* MDR strains. **Conclusions:** Although it does not present an antibacterial action, the extracts of *T. spinosa* can be used in the pharmaceutical industries since its extracts show modulating action of drugs. Besides, these natural products have neuroprotective capacity.

1. Introduction

Bacteria are organisms that have a very short reproductive cycle, such ability makes these beings easily adaptable to environmental changes, as a result of which, resistance to antibiotics occurs. In this way, indiscriminate antibiotic use favors the selection of resistant pathogens, rendering the use of these drugs ineffective in patient treatment, thus causing the death of many patients worldwide[1]. Therefore, bacterial resistance is a major public health problem worldwide, affecting developed and underdeveloped countries[2].

In the literature, *Pseudomonas aeruginosa* (*P. aeruginosa*), *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) bacteria are registered as the main cause of diseases that affect

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and kill people all over the world. *P. aeruginosa* is one of the major agents causing hospital infections, affecting patients who are immunocompromised due to evasive treatments, as well as being responsible for most respiratory and urinary tract infections. In more rare cases, it can cause pneumonia resulting in the death of 60% of those infected[3].

The *Escherichia* genus (Enterobacteriaceae) is composed by Gram-negative facultative anaerobic bacilli, with species widely distributed in nature and in the human digetive system. Although most *E. coli* strains harmlessly inhabit the colon, a large number of pathogenic strains may cause intestinal diseases such as diarrhea[4,5].

Among the Gram-positive strains, *S. aureus* is the one that has shown the most resistance to penicillin-based antibiotics, being responsible for numerous acute infections, such as pneumonia, osteomyelitis, endocarditis, myocarditis, pericarditis and meningitis, and being considered an opportunistic pathogen which affects patients already debilitated due to treatment session submission in hospital environments[6].

In addition to bacterial resistance, other factors such as poor diet and inadequate food preservation methods have worried government agencies. These are considered a major risk factor for stomach and esophagus cancer due to the presence of nitrates and nitrites, substances found in preserved and smoked foods. These N-nitrous compounds and nitrates induce the formation of tumors through their transformation into nitrite, a destabilized oxide, promoting an increase in free radical production and cellular damage. The stomach cancer risk increases with the consumption of nitrous compounds due to the increase in free radicals, which leads to cell injury reducing mucus production, whose function is to protect the gastric mucosa[7,8].

For the treatment of pathologies such as these, as well as for the search for more efficient drugs in the face of microbial resistance, man has resorted to the use of medicinal plants, which is considered one of the oldest medical practices, often being the only phytotherapic alternative in many communities, due to its easy access as well as for economic and cultural reasons[9,10].

Research that involves therapeutic plant potentials has been of relevant importance for physicians who seek alternatives for the fight against resistant microorganisms[11]. Plant use is encouraged by the World Health Organization, which estimates that approximately 80% of the population in emerging countries use them to treat primary diseases[12]. One of these therapeutic plants is the species *Tarenaya spinosa* (Jacq.) Raf. (*T. spinosa*) popularly known as "mussambê", a herbaceous plant belonging to the Cleomaceae family[13]. Cited in other studies with the name *Cleome spinosa*[14] this species presents a series of medicinal uses for the community as an alternative herbal remedy. It acts as a digestive tonic, and it is used in the treatment of respiratory diseases such as asthma, bronchitis, cough as well as in suppurative otitis, headaches and wounds, among other[15].

In Brazil, all *T. spinosa* parts are used in popular medicine, the roots of which are used to combat cough and asthmatic bronchitis, the flowers have anti-inflammatory potential and the essential oil of their aerial parts presents moderate antimicrobial potential and significant bacterial growth inhibitory activity[16].

With the increasing demand for new drugs due to the increase in microbial resistance, it has become necessary to discover natural products which have a potential for the treatment of these infections and which present low toxicity to patients. In view of this, this study aims to analyze the polyphenolic composition of *T. spinosa* (Jacq.) Raf. (Cleomaceae), its antibacterial and modulator activity as well as its neuroprotective capacity.

2. Materials and methods

2.1. Botanic material

T. spinosa leaves were collected in the period of June 2016 at (09:00 ± 30) min, in the city of Quixelô - CE, under coordinates 6°14'35.54"S and 39°16'14.66"W. The plant material with flowers and fruits was collected, pressed and deposited in the Caririense Dárdano de Andrade Lima Herbarium (URCA), under voucher 7347, identified by Dr. Karina Vieiralves Linhares.

2.2. Ethanolic (EETS) and aqueous (EATS) extract preparation

For EETS, 250 g of shade dried leaves were crushed and packed in vials with 96% ethanol. After 72 h, the material was filtered and concentrated on a rotoevaporator (model Q-344B, Quimis, Diadema, Brazil) and lyophilized acquiring 2.78% yield. As for EATS by infusion preparation, 250 g of dried leaves were added to a flask with 2 L of distilled water at 100 ℃. After 72 h, the material was filtered, frozen and lyophilized to give 3.15% yield. All extracts were refrigerated until the experiments were carried out[17].

2.3. Chemical, apparatus and general procedures

All chemicals were of analytical grade. Acetonitrile, formic acid, gallic acid, ellagic acid, *p*-coumaric acid, caffeic acid and chlorogenic acid were purchased from Merck (Darmstadt, Germany). Quercetin, apigenin, catechin and rutin were acquired from Sigma Chemical Co. (St. Louis, MO, USA). High performance liquid chromatography with diode-array detection (HPLC-DAD) was performed with a Shimadzu Prominence Auto Sampler (SIL-20A) HPLC system (Shimadzu, Kyoto, Japan), equipped with Shimadzu LC-20AT reciprocating pumps connected to a DGU 20A5 degasser with a CBM 20A integrator, SPD-M20A diode array detector and LC solution 1.22 SP1 software.

2.4. HPLC–DAD

T. spinosa extracts at a concentration of 10 mg/mL was injected by means of a model SIL-20A Shimadzu Auto sampler. Separations were carried out using Phenomenex C_{18} column (4.6 mm × 250 mm × 5 μ m particle size). The mobile phase was water with 1% formic acid (v/v) (solvent A) and HPLC grade acetonitrile (solvent B) at a flow rate of 0.6 mL/min and injection volume 50 μL. The composition gradient was: 5% solvent B reaching 15% at 10 min; 30% solvent B at 20 min, 65% solvent B at 30 min and 98% solvent B at 40 min, followed by 50 min at isocratic elution until 55 min. At 60 min, the gradient reached the initial conditions again, following

the method described by Bezerra *et al.*[11] with slight modifications. The sample and mobile phase were filtered through 0.45 μm membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. Stock solutions of standards references were prepared in the acetonitrile: water (1:1, v/v) at a concentration range of 0.030-0.500 mg/mL. Quantifications were carried out by integration of the peaks using the external standard method, at 280 nm for catechin; 254 nm for ellagic and gallic acids; 327 nm for caffeic acid, *p*-coumaric acid and chlorogenic acids; and 366 for quercetin, apigenin and rutin. The chromatography peaks were confirmed by comparing its retention time with those of reference standards and by DAD spectra (200 to 700 nm). All chromatography operations were carried out at ambient temperature and in triplicate.

2.5. Bacterial strains, culture media and drugs used

The bacterial strains tested were clinical isolates. The bacteria used were: *E. coli* (EC-ATCC 25922); *S. aureus* (SA-ATCC25923) and *P. aeruginosa* (PA-ATCC9027).

Bacteria were cultured in Heart Agar Infusion where they remained incubated for 24 h at a temperature of 35 ℃, while replicate individuals to be tested were inoculated into a brain and heart broth, Brain Heart Infusion at concentrations recommended by the manufacturer and incubated under the same conditions as above. Suspensions with bacterial growth were diluted in 10% Brain Heart Infusion until obtaining 10^5 cells/mL (NCCLS, 2000).

The drugs used to evaluate the modulatory or antagonistic capacity of the *T. spinosa* extracts were gentamicin from the aminoglycosides class, norfloxacin, belonging to the fluoroquinolones class and imipenem from the carbapenem class.

2.6. Antibacterial activity

2.6.1. Extract solution preparations

For the initial solution, 10 mg of the extracts were weighed, and the contents were dissolved in 1 mL of dimethyl sulfoxide (DMSO-Merck, Darmstadt, Germany) giving an initial concentration of 10 mg/mL. Thereafter, the solution was diluted in distilled water to reach an extract concentration of 1 024 μg/mL with the DMSO concentration being reduced to 10% followed by 1:1 serial dilutions during the microdilution test for obtaining extract concentrations ranging from 8-512 μg/mL whilst maintaining DMSO at a concentration of 5%.

2.6.2. Minimum inhibitory concentration (MIC) and antibacterial drug modulation

The MIC is defined as the lowest concentration capable of inhibiting the growth of bacterial strains, where standard bacteria and their respective multi-resistant strains were used in the modulation. The MIC assay took place in 10% Brain Heart Infusion using the microdilution methodology using 10^5 CFU/mL suspensions, according to NCCLS protocol (2003). The inoculum contained 100 μL of the suspension with 100 μL of the extract, at concentrations ranging from 8-1 024 μg/mL[18,19]. The modulatory activity of the extracts were evaluated in their presence and absence, under which they were at sub-inhibitory concentrations (MIC/8). The plates were incubated for 24 h at 37 ℃.

After the incubation period, 20 μL of resazurin, a bacterial growth indicator solution, was incubated in each well of the plates and this was allowed to incubate for an additional hour at room temperature. Following this period, a color change from blue to pink caused by the reduction of resazurin indicates that there was bacterial growth[18,20,21], thus contributing to the MIC identification, the lowest concentration capable of inhibiting bacterial growth, perceptible through the unchanged blue coloration.

2.7. Neuroprotective assay

The test was performed using neuronal cells from *Nauphoeta cinerea* (*N. cinerea*). Individual heads from the *N. cinerea* arthropod model were divided into four eppendorfs containing five heads each in triplicates to be assayed with each extract (control, nitrite, EETS and EATS extract groups). The material was homogenized in trifluoromethyl ketone and then centrifuged at a speed of 10 000 rpm for 10 min for supernatant collection. The sodium nitrite ($NaNO_2$) solution was prepared in a 1:10 ratio, where 3 mM $NaNO_2$ were topped up with distilled water to make up 10 mL. From this concentration, 100 μL was withdrawn and 900 μL of H_2O were added to obtain a final volume of 1 000 μL. To prepare the extracts, 0.007 5 g of the extracts were weighed and dissolved in 5 mL of H_2O to form the stock solution. Thereafter, concentrations ranging from 10 to 250 μg/mL were diluted in 1 mL of distilled water.

Subsequently, 20 μL of $NaNO_2$ were added to the neuronal cells from the head of the *N. cinerea*, except in the control group, to evaluate the protection capacity of the extracts. The neuronal cells were then incubated for 2 h, followed by addition of 100 μL of 0.1 mg/mL tetrazolium bromide in all tubes. The material was then allowed to stand for an additional 2 h. A total of 150 μL of the material were removed from each eppendorf and placed in a flat bottom 96-well plate, followed by the addition of 50 μL DMSO. After 10 min, a reading was performed on the spectrometer at 492 and 630 nm, according described by da Silva *et al*[34] .

2.8. Statistical analysis

All tests were performed in triplicates. For the statistical analysis of the data, the mean (± standard deviation) was obtained using the GraphPad Prism 6 software and a one-way analysis of variance (ANOVA), followed by Tukey's test ($P<0.05$). Differences between groups of HPLC-DAD were assessed by an analysis of variance model and Tukey's test. The level of significance for the analyses was set to $P< 0.05$. These analyses were performed by using the free software R version 3.1.1[22].

3. Results

3.1. HPLC–DAD

The HPLC profile of *T. spinosa* extracts was acquired and shown in Figure 1. The both extracts contained other minor compounds in addition to gallic acid [retention time(tR) = 9.82 min, peak 1], catechin (tR = 15.08 min, peak 2), chlorogenic acid (tR = 19.65 min, peak 3), caffeic acid (tR = 24.75 min, peak 4), ellagic acid (tR =

32.12 min, peak 5), *p*-coumaric acid (tR = 36.11 min, peak 6), rutin (tR = 39.22 min, peak 7), quercetin (tR = 52.23 min, peak 8) and apigenin (tR = 70.33 min, peak 9).
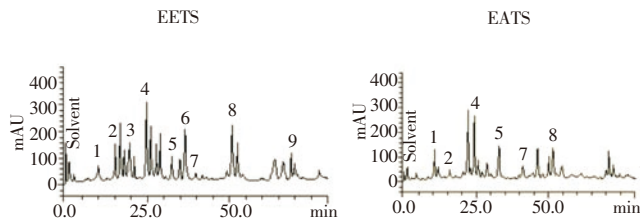
Figure 1. Representative high performance liquid chromatography profile of two extracts of *T. spinosa*.
Gallic acid (peak 1), catechin (peak 2), chlorogenic acid (peak 3), caffeic acid (peak 4), ellagic acid (peak 5), *p*-coumaric acid (peak 6), rutin (peak 7), quercetin (peak 8) and apigenin (peak 9).

The HPLC-DAD phytochemical evaluation showed that the EETS presented 9 compounds analyzed in this study (Table 1), with caffeic acid being the major compound [(7.54 ± 0.01) mg/g], followed by quercetin [(4.19 ± 0.01) mg/g]. From the compounds analyzed, rutin showed the lowest concentration [(0.36 ± 0.01) mg/g]. As for the EATS, three compounds were not present from the compounds analyzed, these being chlorogenic acid, *p*-coumaric acid and apigenin. In addition, this extract presented a major compound at a concentration higher than caffeic acid [(6.49 ± 0.01) mg/g]. However, as it was not one of the compounds analyzed, it was not possible to identify this compound.

Table 1
Components of *T. spinosa* extracts (mg/g).

Compounds	EETS	EATS
Gallic acid	1.49 ± 0.01[a]	2.96 ± 0.03[a]
Catechin	3.07 ± 0.04[b]	0.54 ± 0.02[b]
Chlorogenic acid	3.12 ± 0.03[b]	-
Caffeic acid	7.54 ± 0.01[c]	6.49 ± 0.01[c]
Ellagic acid	2.47 ± 0.02[d]	3.29 ± 0.04[d]
p-Coumaric acid	3.98 ± 0.05[e]	-
Rutin	0.36 ± 0.01[f]	1.41 ± 0.01[e]
Quercetin	4.19 ± 0.01[e]	3.16 ± 0.01[d]
Apigenin	2.43 ± 0.04[d]	-

Results are expressed as mean ± standard deviations (SD) of three determinations. Averages followed by different letters differ by Tukey test at $P < 0.05$.

3.2. Antimicrobial potential

3.2.1. Minimum inhibitory concentration and antimicrobial activity of the T. spinosa extracts
The EETS and EATS presented MICs ≥ 1 024 μg/mL against all the used bacteria. However, when the EATS was tested against multi-resistant *S. aureus* strains, the MIC of the product was 512 μg/mL such that the extract showed antibacterial activity.

3.2.2. Modulatory activity of the T. spinosa extracts
The EETS presented an antagonistic modulating effect on the gentamicin and imipenem antibiotics against the *P. aeruginosa* strains, however it did not modulate norfloxacin effect synergistically or antagonistically (Table 2). It was observed that the EATS

significantly decreased imipenem antibacterial action against this strains, and the same can be observed with gentamicin, but without statistical siginificance ($P>0.05$). However, none effect can be observed against norfloxacin. (Table 2).

Table 2
Antibiotic modulating action of the ethanolic (EETS) and aqueous (EATS) *T. spinosa* extracts against bacterial MDR strains (μ g/mL).

Extracts and drugs		*S. aureus*	*P. aeruginosa*	*E. coli*
Ethanolic extract	Gentamicin	50.79±4.96	1.58±2.88	64.00±1.00
	Imipenem	0.50±1.00	1.58±1.49	203.18±16.46
	Norfloxacin	256.00±1.00	3.17±1.85	128.00±2.00
	EETS	≥1 024	≥1 024	≥1 024
	EETS+ Gentamicin	32.00±3.32[**]	6.35±1.49[**]	64.00±1.00
	EETS+ Imipenem	0.79±2.22	5.04±1.49[**]	1.00±2.00[***]
	EETS+ Norfloxacin	256.00±1.00	3.17±2.22	128.00±2.00
Aqueous extract	Gentamicin	20.15±2.88	2.52±2.22	25.39±1.49
	Imipenem	0.50±1.00	0.79±1.49	4.00±1.00
	Norfloxacin	256.00±1.00	3.17±1.85	3.17±2.26
	EATS	512	≥1 024	≥1 024
	EATS+ Gentamicin	32.00±2.00[**]	5.04±2.22	25.39±1.49
	EATS+ Imipenem	0.79±2.22	5.04±1.49[**]	1.00±2.00
	EATS+ Norfloxacin	256.00±1.00	3.17±1.49	3.17±2.22

[**]Statistically significant with $P<0.01$ value; [***]Statistically significant with $P<0.001$ value.

For *E. coli* strains, the extracts did not modulate antibiotic effects, except for EETS which had a synergistic effect on imipenem effect such that the antibiotic concentration required to inhibit bacterial growth decreased (Table 2). The EETS extract presented a synergistic modulating effect with the gentamicin which was antibacterial against *S. aureus* bacteria, whereas the EATS extract presented an antagonistic effect with the same (Table 2).

3.3. Neuroprotective potential of T. spinosa extracts

The EETS showed neuroprotective effects in cells subjected to NaNO$_2$ at concentrations ≥ 10 μg/mL (Figure 2), whereas EATS showed a protective effect only at the 100 μg/mL concentration (Figure 3).
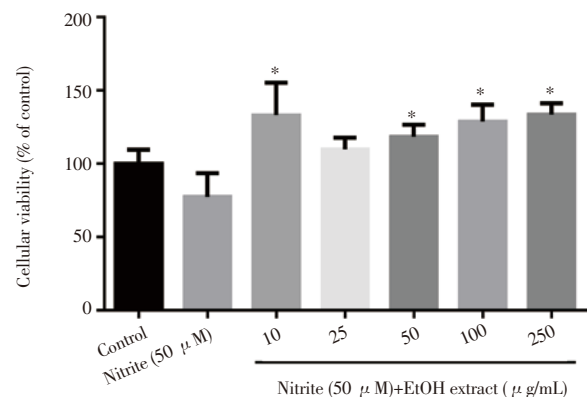
Figure 2. Neuroprotective action of the *T. spinosa* ethanolic extract (EETS) on *N. cinerea* cells subjected to sodium nitrite.
[*]$P<0.01$ statistically significant compared to the positive control (50 μM nitrite).
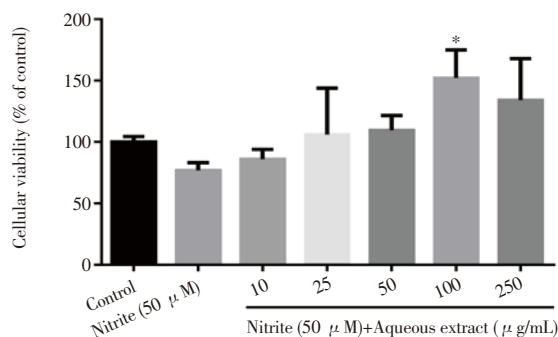
Figure 3. Neuroprotective action of the *T. spinosa* aqueous extract (EATS) on *N. cinerea* cells subjected to sodium nitrite.

$^*P<0.01$ statistically significant compared to the positive control (50 μM nitrite).

4. Discussion

The phytochemical tests carried out by Silva *et al*[23] using leaf extracts made from several solvents with different polarities showed that *T. spinosa* leaves presented the following classes of secondary metabolites: anthracene derivatives, flavonoids, tannins, monoterpenes, sesquiterpenes, diterpenes, triterpenes, steroids, proanthocyanidins, cinnamic acid derivatives, leucoanthocyanidin derivatives and saponins. Since these phytoconstituents originate from the secondary metabolism of plants, they may act in the defense of plants against pathogenic microorganisms, and may thus present biological activities against microorganisms with clinical interest[24]. Although Silva *et al*[23] demonstrated that *T. spinosa* leaves exhibited antibacterial activity, this was justified since in their study the extracts used were prepared with low polarity solvents such as cyclohexane and chloroform, consequently their substance extraction was different from extractions using high polarity solvents, such as water and ethanol[24,25]. For the high polarity solvent extracts in the study of Silva *et al*[23] the antimicrobial activities presented a very high minimum inhibitory concentration, such that it did not present clinical relevance.

Phenolic compounds represent a wide variety of substances which have the characteristic of one or more aromatic rings attached to one or more hydroxyl radicals which can be divided according to the quantity of phenolic rings as well as the structures to which they are attached[26]. The flavonoids are one of the groups from these compounds, which have actions in biological organisms, so that they present antimicrobial, antiviral, cytotoxic, antineoplastic, antioxidant, anti-hepatotoxic, antihypertensive, hypolipidemic and antiplatelet actions[27,28]. In this study, the HPLC assay demonstrated that caffeic acid is the major compound of the extracts. Although this simple phenylpropanoide has a synergistic drug modulating effect, including norfloxacin against *S. aureus* strains[27,28], in our study a synergistic modulatory effect of this antibiotic against the microorganism was not observed[29]. Still in the study by Lima *et al*[29], caffeic acid modulated imipenem effect against *E. coli* bacteria, and this synergistic effect was also observed in our study.

As for the antagonistic action of the extracts with the antibiotics, this is attributed to structural differences in the cell wall of these organisms, since they are surrounded by a hydrophilic surface which acts to prevent the permeability of many substances, including natural compounds[30,31]. A report corroborated such activity, in which organic acids were advocated as being more effective against Gram-positive bacteria than against Gram-negative bacteria[32].

Regarding the neuroprotective action of *T. spinosa* extracts, this effect is explained by the presence of polyphenolic compounds that are reported for presenting pharmacological activities. Adedara *et al*[33], working with Luteolin, a flavonoid commonly found in foods, observed the reversion of neurotoxic damage induced by methylmercury by the reduction of antiacetylcholinesterase activity. Other work of da Silva *et al*[34] with caffeine demonstrated the enhancement of the GSH activity and reducing the lipid peroxidation, indicating the same antioxidative effect.

Several mechanisms corroborate the extracts having an inhibitory action, that is, preventing or delaying microorganismal growth. Factors such as the hydrophobic nature of these components, allow an interaction with the cell membrane lipid layer and the respiratory chain and energy production[35], at other times, attribute the cell with a greater permeability to antibiotics, interrupting cellular activity[36]. These mechanisms may be obtained by combining antibiotics with extracts at sub-inhibitory concentrations and applied directly to the culture medium[37]. However, we can see that, depending on the antibiotic and the bacterium, *T. spinosa* extracts are not effective.

To sum up, we report that while *T. spinosa* does not present antimicrobial activity, its extracts present drug modulating activity. Additionally, the species, which is rich in phenolic substances, possesses neuroprotective action. However, studies which are focused on the isolation and identification of the substances responsible for such activities must be carried out.

Conflict of interest statement

All authors declare that there is no conflict of interest.

Acknowledgements

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