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Original Article

In-Vitro Effect of *Kalanchoe daigremontiana* and Its Main Component, Quercetin against *Entamoeba histolytica* and *Trichomonas vaginalis*

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Abstract

Background: Parasitic infections represent one of the main public health problems in humans according to the WHO. Therefore, the need has arisen to find new treatments that can be used as an alternative cure to parasitosis. We aimed to investigate the in-vitro effects of the methanolic extract of *Kalanchoe daigremontiana* as well as its main component, quercetin against *Entamoeba histolytica* and *Trichomonas vaginalis*.

Methods: For this purpose, the in-vitro activity of the methanol extract of *K. daigremontiana* also its main component, quercetin, against trophozoites of *E. histolytica* and *T. vaginalis* was evaluated, using the microassay technique. Furthermore, the antioxidant activity was determined. Finally, the cytotoxic and cytoprotective capacity was determined using the hemolysis technique.

Results: The IC_{50} indicated that quercetin significantly ($P < 0.05$) inhibited the growth rate of the trophozoite stage of *E. histolytica* and *T. vaginalis* in comparison to the methanolic extract of *K. daigremontiana* (KalL). Also, quercetin significantly ($P < 0.05$) was a better antioxidant as compared with the positive control. In the evaluation of cytotoxicity effects, it could be observed that KalL as compared with quercetin exhibited more cytotoxicity against human erythrocytes. Quercetin significantly ($P < 0.001$) exhibited better cytoprotective activity compared to KalL.

Conclusion: Both *K. daigremontiana* methanolic extract and quercetin alone demonstrated high antiparasitic activity against *E. histolytica* and *T. vaginalis*. However, the in-vivo efficacy of *K. daigremontiana* and quercetin also requires to be evaluated using an animal model.

Introduction

Parasitic diseases are widely distributed throughout the world and constitute one of the main public health problems that affect developing countries, the WHO considers parasites to be the main cause of morbidity, affecting a large part of the population (1). In Latin America, parasitic diseases have a high prevalence due to multiple factors such as immunological, genetic, physiological, and nutritional factors that, according to socioeconomic and cultural conditions, favor the presence of these diseases (2). There are several protozoa of clinical importance such as *Entamoeba histolytica* (3) that can affect the human intestine, and *Trichomonas vaginalis*, which is the parasite that causes the most common non-viral sexually transmitted disease worldwide (4).

The first-line treatment against these parasites is metronidazole, among other nitroimidazoles; however, the existence of strains resistant to these drugs is the main cause of treatment failure, added to the low absorption and inactivation of metronidazole by the body's flora (5). Therefore, it is important to look for new drugs that are more effective and stimulate the innate defense mechanisms of the hosts. The therapeutic use of medicinal plants and natural products for the treatment of diseases is a fundamental basis for the discovery and development of new active molecules since they contain metabolites with diverse biological properties (6,7).

Kalanchoe daigremontiana has a wide variety of biological properties, such as antiviral and antibacterial activity (8), which is derived from the presence of different types of flavonoids, with quercetin being the main component (9). Flavonoid quercetin is known to be active, anti-inflammatory, antibacterial, and primarily antioxidant (10). Furthermore, in other studies quercetin has been shown to have high antigiardial and leishmanicidal activity *in-vitro* (11,12).

We aimed to investigate the in-vitro amebicidal and trichomonicidal effects of *K. daigremontiana* as well as its main component, quercetin against *E. histolytica* and *T. vaginalis*.

Materials and Methods

Ethical statement

The study with human erythrocytes was carried out under the approval of the Ethics Committee of the Universidad Autónoma de Nuevo León (UANL), (Registration Number HI11002) and the consent of healthy donors, following the provisions of the Official Mexican Technical Standard NOM-253-SSA1-2012.

Chemicals used

2,2'-Azobis (2-methylpropionamidine) dihydrochloride (AAPH), Absolute methanol (MeOH), Metronidazole, Quercetin, Dimethylsulfoxide (DMSO), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and Ethylenediaminetetraacetic acid (EDTA), and Vitamin E were purchased from Sigma-Aldrich (Merck®Chemical. USA) and Fetal Bovine Serum (FBS) were purchased from Invitrogen®, USA. All chemicals and solvents were analytical grades.

Parasitological strains and plant material

E. histolytica strain HM1-IMSS and *T. vaginalis* strain GT15 IMSS:0989 and the PEHPS medium were provided by the Northeast Biomedical Research Center of the Mexican Institute of Social Security. The leaves of *K. daigremontiana* were collected in March 2018 in the city of Monterrey [25°42'16"N & 100°23'29"W], México. The taxonomic identification of the plant was carried out in the Botany Laboratory of the College of Biological Sciences of the UANL (Voucher No.29130).

Preparation of the methanolic extract

The dried plant (350 g) was ground and extracted by the maceration method with absolute-MeOH for 120 h, in-room temperature. The solvent was removed in a rotary evaporator (Heidolph®, Germany) and the extract was concentrated to dryness. Finally, the extract obtained was labeled KalL (methanolic extract of *K. daigremontiana* leaf) and stored at 4 ºC until use. The percent extraction yield was calculated with the following formula:

$$\% \text{ Yield} = \left(\frac{\text{Final Weight}}{\text{Initial Weight}} \right) \text{X } 100$$

Activity against E. histolytica and T. vaginalis

The activity of methanolic extract and quercetin, against *E. histolytica* and *T. vaginalis*, was performed using the microassay technique (13). In amber vials, concentrated solutions of the crude methanolic extract or quercetin (1 mg/mL) in 0.5% w/v DMSO were prepared. These solutions were sterilized with 0.22 µm pore nylon membrane filters (Millipore, Germany). Serial dilutions were made from the concentrated solution, with sterile deionized water.

The concentrations of the working test solutions to determine the IC_{50} were deposited in sterile 1-milliliter capacity vials, which had a suspension of *E. histolytica* trophozoites at a concentration of 2 x 10^4 trophozoites/mL at the logarithmic phase, in PEHPS medium added with 10% FSB that was incubated at 36 °C for 72 h. For *T. vaginalis*, 1 x 10^5 trophozoites/mL were deposited at logarithmic phase, in PEHPS medium added with 10% FBS, and incubated at 37 °C/24 h. After the incubation time, the vials were cooled in ice-water for 20 min to finally determine the number of trophozoites with the help of a Neubauer chamber. Metronidazole at a concentration of 1 µg/mL was used as a positive control and medium as a negative control. The results for each parasite were analyzed using the Probit statistical method with a 95% confidence interval. The results were estimated as the percentage of growth inhibition compared to the untreated controls.

Antioxidant Activity

The method of reduction of the DPPH radical was used (14). The treatments were evaluated at concentrations of 20 to 2500 µg/mL. The DPPH was prepared to 125 µM in methanol, 100 µL of each sample was taken, and 100 µL of DPPH was added. The samples were incubated at room temperature, 30 minutes protected from light. The absorbance (Abs) at 517 nm was measured using a spectrophotometer Genesys20 (Thermo-Fisher Scientific, USA). As a positive control (C+), a solution of vitamin E was used and as a Blank control MeOH; the reduction percentage was calculated using the formula:

$$\% \text{ Reduction} = \left(\frac{\text{AbsBlank} - \text{AbsTreatment}}{\text{AbsBlank}} \right) \text{X } 100$$

Cytotoxicity assay

The evaluation of cytotoxicity was determined by hemolysis of a suspension of human blood erythrocytes (15). For the evaluation of the cytotoxicity, the red cell suspension was incubated with different concentrations of the extract or quercetin (50 to 1,000 µg/mL) in 2 mL tubes (Eppendorf®, Germany), for 30 min at 37 °C protected from light, these were labeled as treatments (Tr). As a Blank, a solution of erythrocytes without treatment was used, the positive control (C+) consisted of erythrocytes without treatment with sterile distilled water to produce osmotic hemolysis. The degree of hemolysis was determined by spectrophotometric readings at 540 nm. The readings were recorded as the absorbance (Abs) obtained by each treatment (AbsTr) and finally, the percentage of hemolysis was calculated using the formula:

$$\% \text{ Cytotoxicity} = \left(\frac{\text{AbsTr} - \text{AbsBlank}}{\text{AbsC} +} \right) \text{X } 100$$

Cytoprotection assay

For this, a suspension of erythrocytes was obtained in the manner described in the cytotoxicity assay (15). To evaluate the cytoprotective effect of the extract and quercetin, the previously obtained red blood cell suspension were incubated with different concentrations of the extract or quercetin (50 to 1,000 µg/mL) plus AAPH (150 mM) at 200 rpm (5 h/37 °C), these were classified as treatments (Tr). As a blank of hemolysis, the PBS with the suspension of erythrocytes without AAPH was used. As a C+, the erythrocyte solution with AAPH was used (16). The percentage of cytoprotection was calculated using the formula:

$$\% \text{ Cytoprotection} = 1 - \left(\frac{\text{AbsTr} - \text{AbsBlank}}{\text{AbsC+}} \right) X\,100$$

Statistical analysis

All tests were performed in triplicate. The one-way analysis of variance (Anova) test was used to determine if there was a significant difference between the concentrations evaluated. The post-hoc Tukey test was used to determine the difference between the means in the treatments evaluated. The half-maximal inhibitory concentration (IC_{50}) and the median effective concentration (EC_{50}) were determined by the Probit test, with a 95% confidence interval. Analyzes were directly determined by Probit test in IBM-SPSS software (version 21). Differences were considered significant at $P < 0.05$. Also, the results were expressed as their average and standard deviation.

Results

Activity against trophozoites of E. histolytica and T. vaginalis

Table 1 presents the results obtained in KalL and quercetin *in-vitro* tests on the growth of trophozoites of *E. histolytica* and *T. vaginalis*. The flavonoid quercetin is more effective than the *K. daigremontiana* methanol extract against *E. histolytica* and *T. vaginalis*, however, the reference drug proved to be more effective, presenting IC_{50} of 0.17 and 0.09, respectively.

Antioxidant capacity

Antioxidant capacity was evaluated by the DPPH radical uptake capacity method. The EC_{50} in which a treatment induces an average response after a time of exposure showed that the amount of sample necessary to decrease the concentration of DPPH by 50%, showed the following results Quercetin (1.1 µg/mL) < Vit E (16.9 µg/mL) < KalL (19.2 µg/mL). Quercetin is more effective compared to vitamin E (Table 1).

Table 1: Half inhibitory activity (IC_{50}) and radical scavenging activity DPPH (EC_{50}) of KalL and quercetin. Data are expressed as the mean ± SD ($P < 0.05$). Different letters within the same column are significantly different analyzed through the Tukey test.

Treatments	IC_{50} (µg/mL)		EC_{50} (µg/mL)
	E. histolytica	*T. vaginalis*	DPPH assay
KalL	70.71 ± 3.08^{c}	105.27 ± 5.19^{c}	19.2 ± 2.5^{b}
Quercetin	44.48 ± 3.92^{b}	21.17 ± 2.60^{b}	1.1 ± 0.1^{a}
Metronidazole	0.17 ± 0.03^{a}	0.09 ± 0.01^{a}	ND
Vitamin E	ND	ND	16.9 ± 1.4^{b}

ND: Not determined

Cytotoxicity and cytoprotection assays of the methanolic extract and quercetin by the hemolysis test

The data in Table 2 show the ability of KalL and quercetin to hemolyze (cytotoxic activity) and to prevent hemolysis in the presence of

AAPH (cytoprotective activity). In the case of KalL, hemolysis determined at 1,000 µg/mL resulted in 7.18%, which decreased as the concentration of the extract decreased, since at 50 µg / mL it was 0.41%, compared to the blank control ($P < 0.05$), which had no detectable hemolysis. Regarding cytoprotection, KalL showed low activity between each of its evaluated concentrations. KalL presented cytoprotection inversely proportional to the concentration, with 100 µg/mL the most effective and 1,000 µg/mL the least effective with 3.46 and 0.34% cytoprotection against hemolysis induced by AAPH respectively (Table 2). The blank control had no detectable cytoprotection. Quercetin did not present significant cytotoxicity, also, it turned out to be highly cytoprotective ($P<0.001$), only significant differences were found in some of the concentrations evaluated by the Tukey test.

Table 2: Evaluation of the cytotoxicity and cytoprotection of methanolic extract of *K. daigremontiana* and Quercetin. Values are shown as the median ± SD ($P < 0.05$) of the % cytotoxicity or % cytoprotection, plus the standard error (SE). Different letters within the same column are significantly different analyzed via the Tukey test (*$P < 0.05$. **$P < 0.001$) and compared with C+.

Treatments	***Cytotoxicity***		***Cytoprotection***	
(µg/mL)	KalL	Quercetin	KalL	Quercetin
C+	100.0 ± 0.0	100.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Untreated (Blank)	0.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
50	0.41 ± 0.1^{a}**	0.0 ± 0.0^{a}**	2.96 ± 0.2^{a}	89.2 ± 2.0^{c}*
100	0.49 ± 0.4^{a}**	0.0 ± 0.0^{a}**	3.46 ± 0.6^{a}	90.5 ± 1.8^{c}*
200	0.79 ± 0.4^{a}**	0.1 ± 0.0^{ab}**	2.69 ± 0.2^{a}	94.3 ± 3.1^{b}*
400	2.18 ± 0.7^{b}**	0.1 ± 0.0^{ab}**	1.97 ± 0.1^{b}	96.8 ± 3.6^{b}**
600	4.13 ± 1.1^{c}**	0.2 ± 0.0^{ab}**	1.22 ± 0.0^{b}	100 ± 0.0^{a}**
800	5.37 ± 1.6^{c}**	0.4 ± 0.1^{ab}**	0.55 ± 0.1^{c}	100 ± 0.0^{a}**
1,000	7.18 ± 1.0^{d}**	0.7 ± 0.1^{b}**	0.34 ± 0.1^{c}	100 ± 0.0^{a}**
SE	0.9	0.1	0.5	1.7
P-Anova	< 0.001	< 0.001	< 0.05	< 0.001
F	3.86	3.87	42.04	35.90

Discussion

Several studies (14,17) have been carried out to find new natural sources of compounds to treat diseases that affect man (18,19), however, parasitosis is among the most neglected diseases since the development of new therapies and drugs has received very little attention (15). In this study, the role of the leaves of *K. daigremontiana* and its main component quercetin, against trophozoites from *E. histolytica* and *T. vaginalis* were shown to exhibit an effect against the viability of both parasites (Table 1). Moreover, quercetin was found to possess a high antioxidant activity compared to the evaluated control (Vitamin E). Low cytotoxicity was observed for raw extracts (16), which gives added value to the use of this plant (Table 2). These results were those expected due to the different components that plants of the genus *Kalanchoe* possess, such as polyphenolic compounds, and flavonoids such as quercetin, which can potentiate the effect against different etiological agents. These results are in the same line as previous studies that show that the group of plants belonging to the Crassulaceae family, specifically of the *Kalanchoe* genus, have different biological activities both *in-vitro* and *in-vivo* (20).

The determination of the mean inhibitory concentration against *E. histolytica* and *T. vaginalis* by the Probit test, showed that KalL has an IC_{50} of 70.71 and 105.27 µg/mL respectively, (Table 1), while quercetin presented

IC_{50} of 44.48 and 21.17 and metronidazole presented IC_{50} of 0.17 and 0.09 µg/mL respectively. It is important to mention that although there are no reports of the use of *K. daigremontiana* extracts against the parasites studied, there are some studies evaluating extracts of this plant family against other microorganisms, viruses, including parasites among which are *Leishmania amazonenis*, *Leishmania chagasi* has even been found to have insecticidal activity against *Bombyx mori* (20,21). Those works report crude extracts with lethal concentrations of 500, 400, and 16 µg/mL respectively. This allows us to suppose that there are metabolites in *K. daigremontiana* that inhibit the growth of several protozoa and that these molecules can be solubilized in a wide range of solvent polarity, among the molecules that meet the criteria for antiparasitic capacity, isolated metabolites have been reported in *K. pinnata* such as quercetin and quercitrin that have shown leishmanicidal activity with IC_{50} less than 1 µg/mL (22).

The DPPH assay is considered representative and has been developed to evaluate the capacity of free radical capture. The EC_{50} of 19.2, 1.1, and 16.9 µg/mL was determined for KalL, quercetin, and vitamin E respectively. All three treatments showed antioxidant behavior, with quercetin being the most effective compared to vitamin E. KalL and vitamin E statistically had similar antioxidant activity. The ability to capture free radicals by flavonoids has been extensively studied and proven (14,23).

Hemolysis can be defined as the alteration of erythrocytes and occurs when erythrocytes are damaged, and the erythrocyte cell membrane disintegrates and the hemoglobin molecule leaves the cell (24). Cytotoxicity and cytoprotection studies, such as hemolysis caused by extracts, subfractions or natural metabolites, are performed to validate safety against cells at different concentrations (25). KalL and quercetin were not significantly ($P \leq 0.05$) hemolytic compared to the positive control. As shown in table 2, the extract did not present high toxicity, being approximately 7.4% in its highest concentration and with almost no toxicity in the lowest concentration and at concentrations below 0.2 g/mL (Table 2). It was also observed that quercetin behaved as the evaluated blank since it did not present significant hemolytic activity. However, it did show significant ($P < 0.001$) cytoprotective activity in comparison with KalL and in comparison, with the positive control which caused 100% of the hemolysis (Table 2).

In a study where the hemolytic activity of quercetin-loaded nanoparticles was evaluated, it was observed that all nanoparticles showed a favorable compatibility, and no significant hemolytic effect was observed either by the free quercetin or by the nanoparticles. In addition, antioxidant activity was demonstrated, which increases depending on the amount of quercetin released by the nanoparticles (26). There are reports with this technique of plant extracts with a high protective effect such as the crude extract of *Argemone mexicana* with 99% inhibition of hemolysis and 2.8% of hemolysis at 1,000 µg/mL (15). As shown in Table 2, the KalL extract showed statistically the same protection against oxidative damage compared to the vitamin E. Therefore, the extract of *K. daigremontiana* did not cause significant hemolysis and did not present protection on the erythrocytes.

Some phenolic compounds such as quercetin have the ability to decrease the antioxidant activity of certain radicals such as DPPH, and have even been shown to have better activity than vitamin E (14). Quercetin inhibits the enzyme nitric oxide synthase (iNOS); sarcoplasmic Ca^{2+}-ATPase; has a cell membrane stabilizing effect; prevents adriamycin-induced cardiomyopathy; prevents some diabetic cardiovascular complications and lowers serum cholesterol levels (23). This could be explained by the fact that glycosylated flavonols such as quercetin are the main flavonoids found in the genus *Kalanchoe* (8), this observation could be a consequence of the activity of flavonoids as antioxidants, being effective in metal chela-

tion, and maintaining redox homeostasis, better than the reference free radical scavengers (27).

Considering the impact of amebiasis and trichomoniasis on public health and the emerging number of metronidazole resistant isolates, it is important to develop alternatives for the treatment of these infections (5). Natural products represent a promising source of biomolecules, and the ethnopharmacological approach rescues the knowledge of medicinal plants, this ancient wisdom in combination with pharmacological studies represents a high value in the search for new, safe, and accessible biocomponents (28). This pioneering study showed relevant results on the activity of the methanolic extract of *K. daigremontiana* and its fractions against trophozoites from *E. histolytica* and *T. vaginalis*.

Conclusion

The methanol extract of *K. daigremontiana* and particularly its main component, quercetin exhibited potent activity against *E. histolytica* and *T. vaginalis in-vitro*. Also, further clinical studies are required to assess the exact effect and mechanisms of action of *K. daigremontiana* and quercetin in animal models as a new therapeutic agent against amebiasis and trichomoniasis.

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Conflict of interest

The authors declare that there is no conflict of interest.

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