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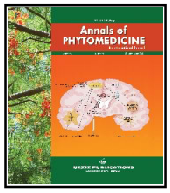

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Phytochemical investigation and evaluation of the antioxidant, antibacterial and antifungal activities of *Stephania japonica* L. leaves extract

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Abstract

Medicinal plants are the most significant sources of natural products and bioactive substances, so many studies are looking for the active principles of these plants for developing new therapeutic alternatives. The *Stephania japonica* L. (Menispermaceae family) species is already used in folk medicine to treat cure fever and pain and as a tonic, as a result, it was chosen to be evaluated concerning its toxicity, antioxidant, antibacterial and antifungal action, which have not yet been scientifically proven. Based on the phytochemical study, the presence of phenolic compounds and flavonoids was detected, and characteristic flavonoids were identified as caempferol, quercetin (rutin) and caempferol-3-O-robinoside-7- rhaminoside. By comparing the retention time of the extract with that of authentic standards, fourteen compounds were identified from the HPLC study. In this sense, the quantification of these compounds and the analysis of their antioxidant capacity brought information about the possible activities of the leaves of *S. japonica*. The evaluation of antioxidant activity indicated that the extract performs free radical scavenging activity. The data obtained in the preliminary survey and by HPLC show that the plant is rich in phytochemicals that are important for human health. The antimicrobial activity of the aqueous extract of *S. japonica* was evaluated in relation to two gram-positive bacterial strains (*Staphylococcus aureus* and *Enterococcus faecalis*) and two gram-negative ones (*Pseudomonas aeruginosa* and *Klebsiella pneumoniae*). In the evaluation of antimicrobial activity, *S. japonica* aqueous extract (SJ-Aq) showed an inhibitory concentration in *S. aureus* and *E. faecalis* strains. The antifungal study included 60 experimental units, 6 concentrations of *S. japonica* aqueous extract, 2 strains of *C. albicans*, and 5 repetitions. The crude aqueous extract concentrations for 2 g of dry *S. japonica* residue were 45, 25, 15, 5, 2.5, and 1.25 mg/ml, respectively. The halos of *C. albicans* were 21.12, 16.08, 9.22, and 7.12 mm at these concentrations. At 45, 25, and 15 mg/ml, the halos of the other strain had average diameters of 10.42, 8.46, and 7.64 mm. The crude aqueous leaf extract abundantly reduces the *C. albicans* strain. Consequently, *S. japonica* leaf extract is a potential candidate for a new drug in the future.

1. Introduction

The search for natural substances that exert biological activity constitutes an innovation strategy in several industrial segments, highlighting the pharmaceutical, food, cosmetics and sanitizing sectors. Bioactive compounds exert antioxidant, antimicrobial, and anti-inflammatory activity, among others. Therefore, morphological, chemical, nutritional and pharmacological studies are developed to understand the characteristics and potential of countless species (Lee *et al.,* 2023). Plants synthesize several chemical compounds with different biological activities, which play an important ecological role, as they can be attractive to pollinating agents, growth regulators and chemical defenders against ultraviolet radiation and attacks by insects, herbivores and microorganisms (Lyu *et al.,* 2020). Among the different classes of bioactive compounds, phenolic compounds stand out for their antioxidant capacity and antimicrobial activity and can be present in all parts of the plant, from the root to the fruit. These compounds have different chemical structures and vary quantitatively and qualitatively within the same plant and in different plants (Abdelhameed *et al.,* 2021; Alam, 2019). The Indian stands out due to the quantity and diversity of native and exotic plant species, many of which are used by native communities in folk medicine. In this way, the in-depth study of the bioactive compounds contained in the different plant species has aroused the interest of the scientific community to determine possible applications, a fact that would help in the sustainable development of the region.

The extraction of bioactive chemicals from plant matrices is a critical step in investigating and developing products containing these substances. A low-cost extractive method could be used to create compounds for use as food additives or nutraceutical products as a product development strategy. The antioxidant capacity and antimicrobial activity of plant extracts rich in bioactive compounds has become the basis of many applications as natural agents for preservation in the food and pharmaceutical industry. Synthetic antioxidants are added to food products to prolong their stability during processing and storage. However, these synthetic substances have been linked with harmful effects on health. Thus, the search for

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antioxidants from natural sources and the identification of these compounds that can replace synthetic ones has received much attention (Dias *et al.,* 2021). Another factor that influences the stability of food products is microbial deterioration. Food contaminated mainly with pathogens is often identified as the main source of food poisoning in humans. In addition, there is a growing development of resistance of microorganisms to commercial antibiotics. In this context, systematic studies to identify bioactive substances from plants can result in the development of new and effective antimicrobial and antioxidant compounds. Oxygen radicals and superoxide anion play an important role in the reactions of the human body. However, disease and deep tissue damage can occur if there is excess production of oxygen radicals during pathophysiological processes or due to adverse environmental factors and there are no antioxidants available *in vivo*. Some flavonoids are able to bind to metal ions, preventing them from acting as catalysts in the production of free radicals. This activity is the result of a set of properties, such as iron chelating activity, free radical scavenging activity, inhibition of cyclooxygenase, lipoxygenase, NADPH-oxidase, xanthine-oxidase and phospholipase enzymes, and stimulation of enzymes with antioxidant activity such as catalase and superoxide dismutase (Seca *et al.,* 2020).

The genus *Stephania* consists of 18 herbaceous or shrubby species that are exclusively found in neotropical regions and restricted to arid or seasonally dry areas of south India (Deepak Kumar Semwal *et al.,*2010). The leaves and roots of some species of this genus are used in Indian folk medicine as antibacterial, anti-inflammatory and antidiarrheal agents (Bombardelli *et al.,* 2002; Silva *et al.,* 2001). *Stephania japonica* L. popularly known as snake vine, slender wiry climber or twining shrub (Senthamarai *et al.,* 2012). This plant is used in popular medicine to treat ailments including inflammation, pain, rheumatism, cancer, bone fracture, and fever (Moniruzzaman *et al.,* 2016). The presence of compounds such as terpenes, lignans and norneolignans has already been reported in the Menisperaceae family (Deepak Kumar Semwal *et al.,* 2010). Some species of the genus *Stephania* are commonly used for medicinal purposes, and given that there have been few studies on the therapeutic properties of the species, *S. japonica*. This work aimed to evaluate the toxicity of the leaf extract of this species as well as its antioxidant potential, antimicrobial, and antifungal properties.

2. Materials and Methods

2.1 Collection of plant material

Leaves of *Stephania japonica* L. (Figure 1) was collected in the Vijayawada rural agricultural fields, 5 km from the municipality of Vijayawada (16º.515099' N, 80º.632095' E), Andhra Pradesh, India. The collected material was taken to a drying oven with forced air circulation (40-45°C) for a period of three to four days. The sample was identified according to the usual taxonomic techniques and deposited in the Herbarium with serial number SJ 1962/17 of Acharya Nagarjuna University, India.

2.2 Preparation of plant extracts

Plant leaves were collected in a bulk, washed under running water, drying in an oven with forced air circulation (60°C/3 h) and ground (5 mm granulometry) (Figure 1). Then, the samples were stored in plastic pots under refrigeration (± 4°C) until the procedure for extracting the bioactive compounds. Plant extracts were obtained through solid-liquid extraction using methods proposed by Abubakar *et al.* (2020). Solid-liquid extraction experiments were carried out in an orbital shaker (Remi Mini Rotary Shaker -RS-12R, India), using in this case water as solvent. The extraction was carried out in Erlenmeyer flasks with a capacity of 500 ml under the following operating conditions: temperature of 30ºC, stirring speed of 150 rpm, extraction time of 18 h, and mass/volume ratio of 30 g *S. japonica* leaves/l of water. The extract obtained was subjected to centrifugation and subsequent vacuum filtration of the supernatant using Whatman No 1. filter paper to separate the solid particles. The volume of extract obtained was 350 ml, which was stored in topaz-coloured glass jars to prevent photo-oxidation of its components and kept frozen at -18 ºC until later use (Figure 1).

Figure 1: ***Stephania japonica*** **L. leaves and leaf powder.**

2.3 Phytochemical analysis

2.3.1 Identification of phenolic compounds through high performance liquid chromatography (HPLC)

According to Proestos *et al.* (2013), high-performance liquid chromatography (HPLC) has been the most used technique in the analysis of phenolic compounds. This analysis found that the *S. japonica* leaf extract is rich in phenolic compounds, corroborating the preliminary survey. As shown in Table 1, fourteen compounds were identified by comparing the retention time of the extract with that of authentic standards.

Chemical characterization of the compounds was carried out by high-performance liquid chromatography - HPLC on a Shimadzu® chromatograph equipped with a UV/visible detector at 280 nm and a Phenomenex® Luna C18 5 µm column at 22°C. The analysis conditions of the samples were as follows: mobile phase consisting of solvents A, B and C (A: 0.1% H_3PO_4 + 0.12 N H_2SO_4; B: MeOH and C: ACN), flow rate of 1.0 ml min^{-1}, sample injected volume of 20 µl, wavelength (l) equal to 280 nm. The identification occurred by comparing the retention time of peaks obtained with the extract and the retention time of authentic standards obtained in Sigma®. The extract and standards were filtered through a 0.22 µm PVDF membrane before injection into the equipment.

2.3.2 Determination of total phenolic content

The determination of the content of phenolic compounds was performed according to the methodology described by Tungmunnithum *et al.* (2018) adapted, using the Folin-Ciocalteu reagent, in a microplate in triplicate. The absorbance reading was performed in an UV reader at 650 nm. For the analysis of the phenolic content, solutions diluted with ethanol at a concentration of 2.5 mg/ml were prepared in triplicate from the crude aqueous extract fraction of the *S. japonica* leaf. For the microplate reaction, 30 µl of distilled water, 80 µl of sample, and 10 µl of FC reagent were added to the wells intended for the samples, and finally, 40 µl of Na_2CO_3 and 40 µl of distilled water completed the volume of the well. Then, blanks were made for all concentrations, which consisted only of 120 µl of distilled water and 80 µl of sample. And finally built, a standard curve with concentrations of 10, 25, 50, 75, 100, 150, 200, 250, 300 and 350 µg/ml, requiring the addition of gallic acid and distilled water in different volumes to obtain the determined concentration, ending with the addition of 10 µl of FC reagent, 40 µl of Na_2CO_3 and 40 µl of water. Using a calibration curve (Figure 3) established from gallic acid standards, total phenolic content may be extrapolated from sample absorbance values.

2.3.3 Total flavonoids

Total flavonoid content was calculated using a modified version of the procedure that Chang *et al.* (2002). The technique is based on measuring the absorbance, at 450 nm, in the UV reader of the complex formed between the flavonoid and the aluminium of the colour reagent, forming yellowish compounds. The flavonoid content was calculated from the calibration curve equation obtained through ten rutin standard dilutions. For the analysis of the flavonoid content, solutions diluted with ethanol at a concentration of 2.5 mg/ml were prepared in triplicate from the aqueous extract fraction of the *S. japonica* leaf. For the reaction in the microplate, 40 µl of ethanol, 100 µl of sample, 4 µl of Aluminium chloride, 4 µl of potassium acetate solution and 52 µl of distilled water were added to the wells intended for samples, completing the volume of the well. Then blanks were made for all concentrations, which consisted of 100 µl of sample, 44 µl of ethanol, 4 µl potassium acetate and 52 µl of distilled water. Finally, a standard curve was constructed with concentrations of 2, 5, 10, 1, 20, 25, 30, 35, 40 and 45 µg/ml, requiring the addition of rutin and ethanol in different volumes in order to obtain the determined concentration, ending with the addition of 10 µl of Folin-Ciocalteu reagent, 40 µl of sodium carbonate and 40 µl of water, 4 µl of aluminium chloride, 4 µl of potassium acetate solution and 52 µl of distilled water. The total flavonoid content could be determined by interpolating the absorbance of the samples against the calibration curve (Figure 4) built with rutin standards (Moro'an *et al.,* 2021).

2.4 Antibacterial activity

The antimicrobial evaluation was carried out using the following microorganisms: *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922 and *Candida albicans* ATCC 90028, purchased from Hi Media, India. The suspension of the microorganisms was carried out as follows. A roast of the colonies to be evaluated was taken and submerged in Mueller-Hinton (MH) broth (HiMedia, India) for bacteria and Sabouraud broth (HiMedia, India) for yeast. The samples were incubated at 37°C for 24 h for bacteria and at 37°C for 48 h for yeast. It was standardized to the 0.5 tube of the MacFarland nephelometer: (1-2) x 10^8 CFU/ml using a UV-Vis spectrophotometer (Elico Model SL-10, India) at a wavelength of 625 nm with absorbance readings between 0.08-0.12. Broth microdilution was performed by determining the minimum inhibitory concentration (MIC), the minimum bactericidal concentration (MBC) using the broth microdilution technique. To achieve this, serial dilutions of the *S. japonica* leaf aqueous extract from 0.012 mg/ml to 30 mg/ml were made in MH broth. MH broth with microorganism was used as a positive control, and MH broth without the microorganism was used as a negative control. To detect the respiratory activity of the microorganism, 0.08% solution of sodium salt of oxidized tetrazolium was used, which generates a red pigment (formazan) in the presence of the microorganism. This procedure was performed as follows: 50 µl of solution of oxidized tetrazolium were added to each inoculated well, mixed using a plate shaker, and incubated at 37°C for 30 min. After this time, the formation of an insoluble red precipitate was observed, which represented the MIC. Where there was no development, the solution remained light in colour, indicating the MBC. To confirm the results, it was determined if the effect was bactericidal or fungicidal by taking a sample of the culture with a loop and sowing it on an MH agar plate that was kept incubated at 37°C for 24 h for the bacteria and for 48 h. h for the case of yeast. Growth on the plate was considered to be indicative of a bacteriostatic or fungistatic effect, while its absence corresponded to a bactericidal or fungicidal effect (Csepregi *et al.,* 2016).

2.5 Determination of antioxidant activity

For this experiment, we used Munteanu *et al. (*2021), method for determining antioxidant activity, which is based on the transfer of electrons that occurs when the purple DPPH is reduced to the yellow diphenyl-picryl-hydrazine by the action of an antioxidant or a radical species, and the resulting decrease in absorbance is measured. The results obtained determined the percentage of antioxidant or free radical scavenging activity. Firstly, solutions diluted in ethanol with a concentration of 400 µl/ml of the crude aqueous extract fraction were prepared in 25 ml volumetric flasks. For the preparation of DPPH, a solution of 0.002 g of DPPH was prepared in ethanol p.a. using a 50 ml flask protected from light. To determine the linearity, a standard curve was constructed with the concentrations 200, 180, 160, 140, 120, 100, 80, 60, 40 and 20 µg/ml, requiring the addition of standard rutin and ethanol in different volumes of in order to obtain the determined concentration, ending with the addition of 100 µl/ml of DPPH. A blank was also prepared for all concentrations, where the 100 µl/ml of DPPH was replaced by ethanol. And a negative control was performed in triplicate by adding 150 µl/ml ethanol and 100 µl/ml DPPH solution. All concentrations were performed in triplicate. The samples were incubated for 30 min after the addition of DPPH. Next, absorbance readings at a wavelength of 490 nm were taken using an UV reader (Bioline Technologies, India). The percentage of antioxidant activity was calculated by determining the IC_{50}.

2.6 Antifungal activity

Preparation of the inoculum of *Candida albicans*.: Two strains provided by the Mycology Laboratory of the Government Medical College, Guntur, Andhra Pradesh, India, were used. A suspension of *C. albicans* was prepared, 5 colony corming units (CFU) ≥1 mm in diameter were taken from Sabouraud dextrose agar (SDA) culture), 24 h, CLSI 13. They were suspended in 5 ml of Sabouraud broth, and with the help of a spectrophotometer (Elico SL 10 Model Spectrophotometer) at a wavelength of 625 nm, they were adjusted to an optical density of 0.12, equivalent to the turbidity of tube No. 0.5 on the scale of McFarland equivalent to a concentration of 1-5× 10^6 CFU/ml. Subsequently, a 1:1000 dilution was made with glucose Sabouraud broth, obtaining a 1-5 × 10^3 concentration. The latter is the one used to sow on the Mueller-Hinton agar plates.

Antifungal activity of the aqueous extract of *S. japonica* against *Candida albicans*. On the seeded plates (5 plates for each strain), 6 perforations of 6 mm in diameter were made, with a punch15, placing 50 µl of each concentration of the crude aqueous extract of *S. japonica* in each well, later they were sealed with parafilm and incubated at 37°C, for 24 h, to measure the inhibition halos of fungal growth. For the negative control, a plate with 6 perforations plus sterile distilled water was used.

2.7 Statistical analysis

To determine the relationship of the *in vitro* antifungal activity of the crude aqueous extract of *S. japonica* on the growth of *C. albicans* fungi, an analysis of variance (ANOVA) was performed with a significance level of $p<0.05$.

3. Results

3.1 Phytochemical assessment

The phytochemical investigation has as its primary purpose to know the chemical constituents of plant species and to evaluate the presence of these compounds, indicating the importance of classes of secondary metabolism responsible for plants' active principles (Greff *et al.,* 2023).

The phytochemical profile of the aqueous extract of *S. japonica* ('SJ-Aq') was obtained through thin layer chromatography and is described in Table 1, where it is possible to observe that 'SJ-Aq' revealed the presence of flavonoids, condensed proanthocyanidins, leucoanthocyanidins and traces of monoterpenes and sesquiterpenes. The presence of alkaloids, saponins, cinnamic derivatives, triterpenes, steroids, coumarins, quinones and hydrolyzable tannins was not detected. The presence or absence of some classes of secondary metabolites is possibly related to natural abiotic factors, such as seasonality, circadian cycle, temperature, water homeostasis, ultraviolet radiation, nutrients, atmospheric pollution, and attack pathogens (Greff *et al.,* 2023). Leucoanthocyanidins and proanthocyanidins are part of a class of flavonoids where it is possible to observe condensed structures (Ramos *et al.,* 2021). Several studies indicate that flavonoids can benefit health, helping combat various diseases. Research has shown that these compounds have anti-inflammatory, antioxidant, antiallergic, hepatoprotective, antithrombotic, antiviral and antitumor activity (Tungmunnithum *et al.,* 2018; Greff *et al.,* 2023)

Table 1: Phytochemical profile of the aqueous extract of *S. japonica* leaves

Classes of secondary metabolites	'SJ-Aq'
Flavonoids	++[1,2]
Dynamic derivatives	
Saponins	-
Alkaloids	+
Triterpenes and steroids	-
Monoterpenes and sesquiterpenes, coumarins	-
Quinones	++
Condensed proanthocyanidins and leucoanthocyanidins	+++[3,4]
Phenol	++

Caption: [1]heterosides of flavonoids 3'-OH and 4'-OH; [2]heterosides of 3'-OH flavonoids; [3] oligomeric proanthocyanidins; [4] polymeric proanthocyanidins; 5 free and polymeric phenolic acids. Caption: (+++) strong; (++) medium; (+) weak; (-) absent.

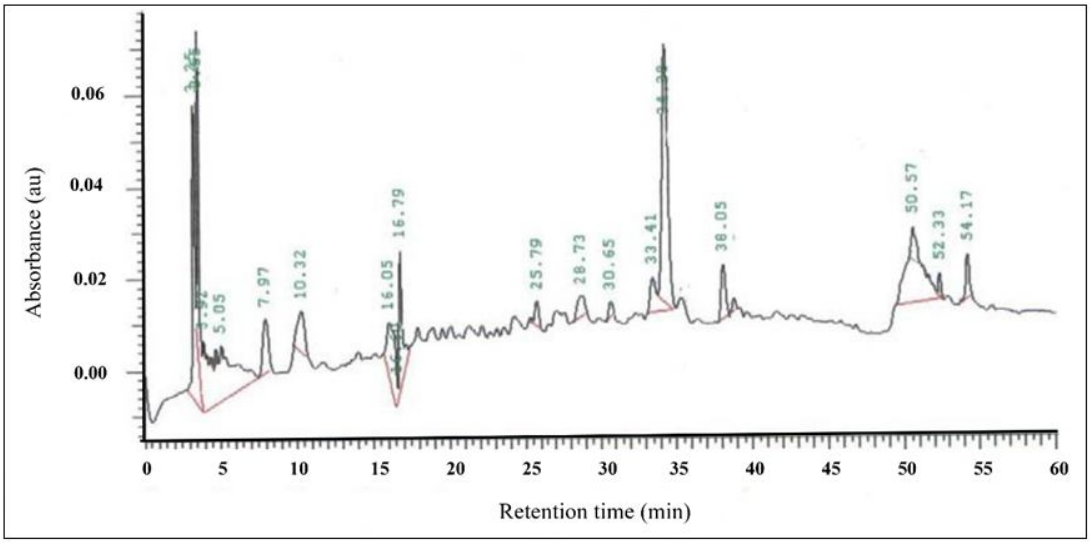

Figure 2: Chromatogram generated from the chromatographic run of the aqueous extract of *S. japonica*.

3.1.1 Phytochemical analysis of 'SJ-Aq' by HPLC

In the chromatographic analysis, the identification of compounds present in the aqueous extract of *S. japonica* leaves ('SJ-Aq') was determined through the chromatogram analysed, as it is indicated for the observation of phenolic compounds, which according to the phytochemical profile of 'SJ-Aq', is present in large amounts. The peaks observed in the chromatogram represent the signal intensity, which is proportional to the concentration of compounds present in the extract. In the 'SJ-Aq' chromatogram (Figure 2), it is possible to observe a more prominent peak with a retention time of approximately 6 min and other smaller peaks at different retention times; however, when these were compared with the peaks of the compounds used as standards, no it was possible to identify which are the major compounds present in 'SJ-Aq', requiring a more detailed study of the compounds of this extract.

Table 2 : Identification of compounds from the retention time of the standards compared to the extract *S. japonica* crude and fractions obtained by HPLC procedure

Compounds identified in *S. japonica* extract	Retention time (Rt) in minutes
Gallic acid	3.55
Chlorogenic acid	3.92
Vanillic acid	5.05
Syringic acid	7.97
Epigallocatechin gallate	16.05
p-coumaric acid	16.79
routine	25.79
Naringin	30.65
Hesperidin	33.41
Myricetin	34.36
Rosmarinic acid	38.05
Morina	50.57
quercetin	52.33
caempferol-3-O-robinoside-7 - rhaminoside	24.17

3.1.2 Determination of total phenolic content and total flavonoid content

For the quantification of the phenolics, the absorbance values found for the different concentrations of the gallic acid standard were used for the construction of the analytical curve. The equation found was $y = 0.0061x + 0.1842$ and $R^2 = 0.9963$ (Figure 3). From this, we can calculate the mean concentration value for the aqueous extract was 75.40 ± 8.04 mg GAE/g (gallic acid equivalents per gram). For the quantification of flavonoids, the absorbance values found for the different concentrations of the rutin standard were used for the construction of the analytical curve. The equation found was $Y = 0.0107x + 0.0501$ and $R^2 = 0.9961$ (Figure 4). From this, the average concentration value for the crude aqueous extract was 0.965 ± 0.12 mg RE/g (rutin equivalents per gram) of sample.

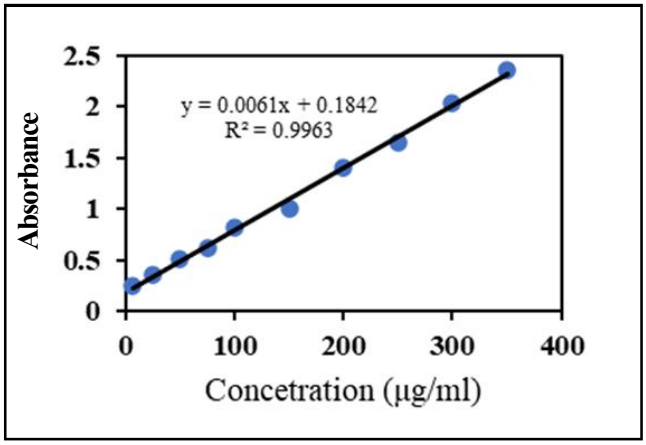

Figure 3: Analytical curve of gallic acid to determine the phenolic content in the crude aqueous extract of the leaves of *S. japonica*.

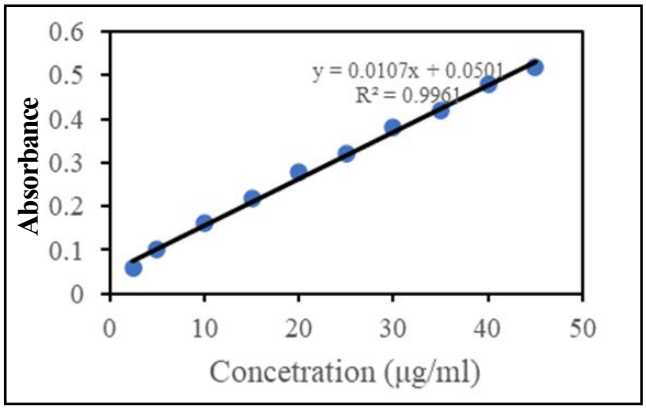

Figure 4: Analytical curve of rutin for determination of flavonoid content in crude aqueous extract fractions of *S. japonica* leaves.

3.2 Determination of antioxidant activity

With the aim of evaluating the ability of the constituents of the crude aqueous extract and fractions of the *S. japonica* leaf to capture free radicals (DPPH), an analysis of their solution with DPPH was carried out. The results were expressed in the percentage of oxidation inhibition; that is, the percentage of antioxidant activity corresponds to the amount of DPPH consumed by the antioxidant (Sethumathi *et al*., 2021). The greater the consumption of DPPH by the sample, the greater its antioxidant activity (AA%) (Mohammed *et al.,* 2022). In the studies by Stagos (2020), where the antioxidant activity was carried out by the method of reducing the DPPH radical of the leaves of two cultivars of *S. japonica*, it was possible to verify a greater antioxidant activity (IC_{50} of approximately 0.020 ± 0.06 µg/ml) than those found in the present study.

It is known that the compounds responsible for the antioxidant activity of plants generally come from secondary metabolism, which is responsible for ecological relationships, adaptation and plant defence mechanisms, and that these metabolites can be produced more under stress conditions (Chaves *et al.,* 2020). Based on the phytochemical study, the presence of phenolic compounds and flavonoids was detected, and characteristic flavonoids were identified: caempferol, quercetin (rutin) and caempferol-3-O-robinoside-7-

rhaminoside. In this sense, the quantification of these compounds and the analysis of their antioxidant capacity (Figure 6) brought information about the possible activities of the leaves of *S. japonica* in values considered satisfactory. Therefore, it can be concluded that hop leaves produced in India have significant levels of phenolic compounds and flavonoids and can also be used to obtain biologically active molecules; this can add value to *S. japonica* leaves.

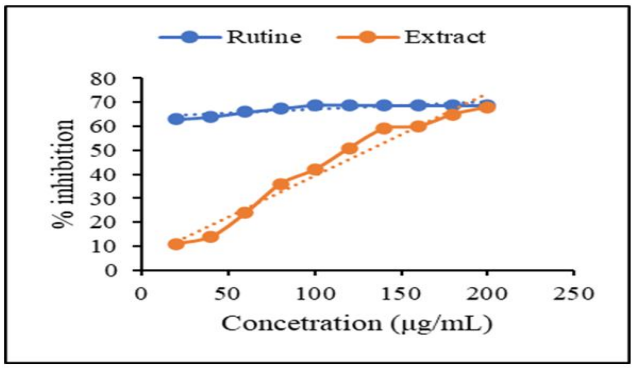

Figure 5: DPPH analytical curve for quantification of the IC_{50} of the aqueous extract of *S. japonica* leaves.

3.3 Evaluation of antibacterial activity

In recent years, the emergence of antibiotic-resistant bacteria has aroused the interest of many researchers in the development of new antimicrobial substances. For this reason, the search for antimicrobials extracted from plants has become an important alternative. Many studies (Yuan *et al.,* 2022) in this area are looking for fewer toxic substances, more effective against bacterial resistance and capable of combating new pathogens. Because of this, the antimicrobial activity of the aqueous extract of *S. japonica* was evaluated in relation to two gram-positive bacterial strains (*Staphylococcus aureus* and *Enterococcus faecalis*) and two gram-negative ones (*Pseudomonas aeruginosa* and *Klebsiella pneumoniae*). The result of this test can be seen in the following table (Table 3).The antimicrobial activity of 'SJ-Aq' was determined from the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC). It is possible to observe that 'SJ-Aq' presented a minimum inhibitory concentration of 0.28 mg/ml for the two strains of gram-positive bacteria tested and a minimum bactericidal concentration of 1.1 mg/ml for *S. aureus* and 0.6 mg/ml for *E. faecalis*. As for gram-negative bacteria, 'SJ-Aq' did not show a minimum inhibitory or bactericidal concentration.

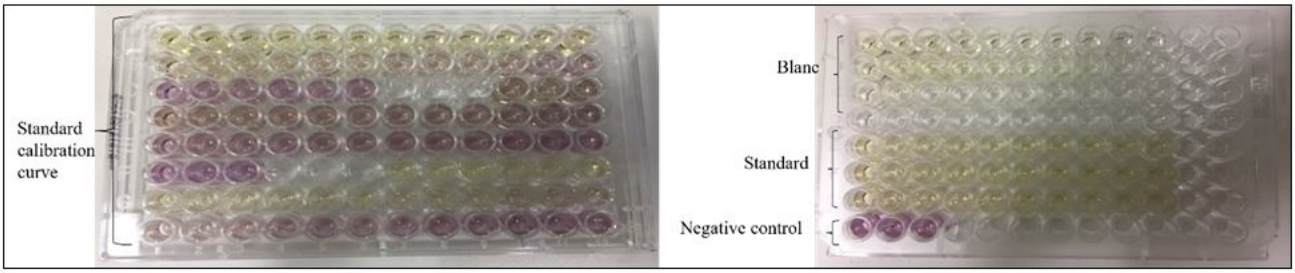

Figure 6: Microplates for the determination of the antioxidant activity of *S. japonica* leaves.

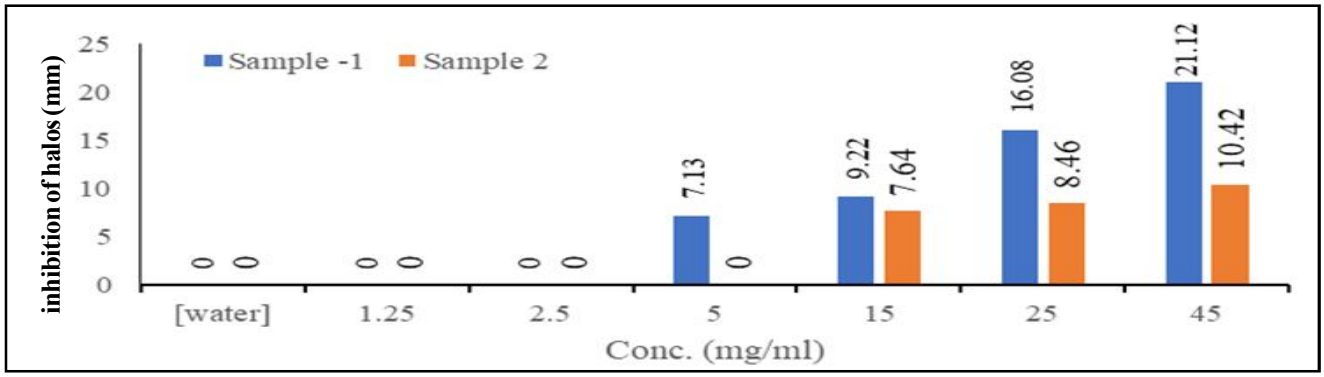

Figure 7: Average inhibition halos in millimetres (mm) of the aqueous extract of *S. japonica* against the *C. albicans* strains and negative control with distilled water [H_2O].

Table 3: Minimum inhibitory concentration and minimum bactericidal concentration of the aqueous extract of leaves of *S. japonica* against gram-positive and gram-negative bacteria

Bacterial strains	MIC (mg/ml)	MBC (mg/ml)
S. aureus	0.28	1.1
E. faecalis	0.28	0.6
P. aeruginosa	0	0
K. pneumoniae	0	0

3.4 Antifungal activity

Figure 7 shows the average inhibition halos (mm) of the 6 concentrations of the aqueous extract of the *S. japonica* leaves against two strains of *C. albicans*.

Of the 6 concentrations evaluated, it is observed that the 45, 25, 15 and 5 mg/ml presented average inhibition halos 21.12, 16.08, 9.22 and 7.13 mm for strain no. 1 and for strain no. 2; the activity was different, the concentrations of 45, 25 and 15 mg/ml presented halos of 7.64, 8.46 and 1 10.42 mm, respectively. Sterile water did not

present an inhibition halo. Figure 8, using the good diffusion method, shows the antifungal activity of the aqueous extract of *S. japonica* leaves and also shows the difference in the sizes of the inhibition halos depending on the concentrations evaluated *in vitro*.

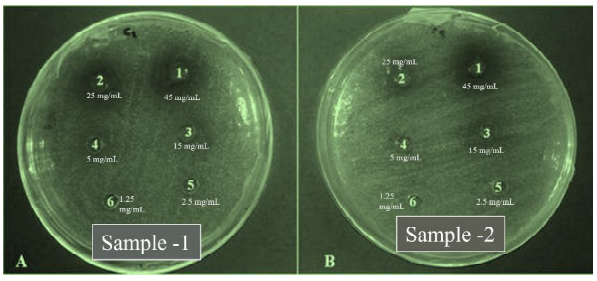

Figure 8: Differences in the inhibition halos in relation to the concentrations of the aqueous extract of *S. japonica* (45 (1), 25 (2), 15 (3), 5 (4),2.5 (5) and 1.25 mg/ml (6), against the 2 strains of *C. albicans* (sample 1 and 2).

4. Discussion

According to Sun *et al.* (2019), high-performance liquid chromatography (HPLC) has been the most used technique in the analysis of phenolic compounds. From this analysis, it was found that the *S. japonica* extract is rich in phenolic compounds, corroborating the preliminary survey. As shown in Table 2, fourteen compounds were identified by comparing the retention time of the extract with that of authentic standards. Abdel-Moneim *et al.* (2017), report that gallic acid and p-coumaric acid are effective against Diabetes mellitus, both in the early stages and in the progression of the disease (Kulathuran Pillai Kumaraswamy *et al*., 2022). The diversity of compounds detected leads to an inference that several compounds of this plant can act synergistically in the control of this human pathology, considering that it is a class of compounds with diverse beneficial actions in the human body (Panda *et al.,* 2022). In the literature, there still needs to be a consensus that defines the antimicrobial activity of extracts obtained from plants. However, many studies classify this activity based on the MIC necessary to inhibit pathogens. Once the MIC and MBC values were obtained, the aqueous extract of *S. japonica* was evaluated for its antimicrobial action according to the MIC values against the studied pathogens, where extracts that present MIC < 0.1 mg/ml have promising antimicrobial potential (good), MIC between 0.1 – 0.5 mg/ml has moderate inhibitory activity, MIC between 0.5 – 1.0 mg/ml has weak inhibitory activity and extracts that have MIC > 1.0 mg/ml are inactive against pathogens (Song *et al.,* 2022). Following these parameters, it is possible to conclude that 'SJ-Aq' showed moderate antimicrobial activity against gram-positive bacterial strains. Several studies demonstrate that compounds extracted from plants that have antibacterial action are more effective against gram-positive than gram-negative strains. This action is related to the structure of the cell wall because gram-negative bacteria have an outer membrane that works as a barrier, preventing the passage of some types of antibiotics, digestive enzymes, detergents and heavy metals in contrast to the simple cell wall structure of gram-positive bacteria. The range of identified compounds demonstrates that *S. japonica* extract can act on carbohydrate metabolism and several factors related to Diabetes mellitus. Abdel-Moneim *et al.* (2017), report that gallic acid and p-coumaric acid are effective against Diabetes mellitus, both in the early stages and in the progression of the disease. The diversity of compounds detected leads to an inference that several compounds of this plant can act synergistically in the control of this human pathology, considering that it is a class of compounds with diverse beneficial actions in the human body.

Many mechanisms of inhibitory action have been attributed to active compounds present in plant extracts. The antimicrobial action of several extracts and plants has already been attributed to the presence of phenolic compounds. Yuan *et al.* (2022) evaluated the antimicrobial activity of isolated flavonoids and found their activity mainly against gram-positive microorganisms. Moreover, according to Górniak *et al.* (2019), flavonoids can also act on the bacterial cell through complexes formed between the proteins and the bacterial cell wall, thus causing its rupture.

Oral *candidiasis* can be present in the hosts as plaque, denture stomatitis, angular cheilitis and systemic mucocutaneous oral manifestations. Likewise, the presence of active resistance genes of *C. albicans* against known antifungals has also been reported (Ali habeeb *et al.,* 2018). In the currently, as part of the solution, new bioactive principles present in plant parts (stem, roots, leaves and seeds) are being sought. *S. japonica*) belongs to the Menispermaceae family; it is an aromatic plant and is used in popular medicine. This study was carried out to evaluate the antifungal activity of *S. japonica* due to its therapeutic properties, which, in agreement with other plant species, such as *Picralima nitride*, which has reported antifungal capacity of aqueous extracts of seeds, due to its ability to inhibit the growth of *Aspergillus flavus*, *Candida albicans* and *Microsporum canis* (Ubulom *et al.,* 2011), and even in aqueous extracts of Olive leaves, with antifungal capacity against. *C. albicans* (Nasrollahi *et al.,* 2015).

The aqueous extract of the *S. japonica* leaves had antifungal activity against *C. albicans*; by the good diffusion method, the average halos for a strain were 21.12, 16.08, 9.22, and 7.13 mm for the concentrations of 45, 25, 15, 5 mg/ml respectively, however, respectively; for the other strain, average halos of 7.64, 8.46 and 10.42 mm were obtained for the concentrations of 45, 25, 15 mg/ml, a directly proportional relationship was achieved between the concentration of the extract and the diameter of the zone of inhibition. This indicates the antifungal capacity of *S. japonica* against *C. albicans*; despite the few studies that report its antifungal activity. They coincide with what was reported by Ramos *et al.* (2020), at a concentration of 200 mg/ml. It obtained a significant reduction of biofilms formed by *C. albicans* after exposure to the extract and with a different methodology to evaluate its antifungal activity, using the microdilution method, determining a MIC of 0.78 mg/ml and a minimum microbicidal concentration (MMC) of 3.13 mg/ml. The bioactive principles of the aqueous extracts in *S. japonica* can not only inhibit the growth of bacteria but can also present antifungal capacity, consistent with studies with aqueous extracts of plants of different species such as *Mimosa pudica* and *Azadirachta indica* (Mahmoud *et al.,* 2011). It was observed that the strains of *C. albicans* used in this research exhibited different degrees of susceptibility to the *S. japonica* extract; even the observed susceptibility to it could be attributed to the inherent resistance factor of the microorganisms (Gupta *et al.,* 2014). Finally, with these data, the extracted crude leaves of *S. japonica* reveal great biological potential by presenting antifungal activity at different doses, representing a therapeutic alternative against

opportunistic infections; although the data is conclusive, work is required to validate this technique, extract their bioactive compounds and allow overtime to be exploited in therapeutic treatment through toothpaste, mouthwashes, root canal irrigation, ointments, among others, and control of some fungal infections. The antifungal activity exhibited by the *S. japonica* leaves extract of the leaves against *C. albicans* will support future research aimed at isolating and characterizing the component(s) responsible for said activity.

5. Conclusion

Phytochemical analysis of the aqueous extract of leaves of *S. japonica* revealed the presence of flavonoids, phenols. These classes of compounds are primarily responsible for the therapeutic properties presented by this species. The antioxidant, antibacterial and antifungal activities found in 'SJ-Aq' are likely linked to its chemical constituents, mainly to its phenolic compounds (flavonoids), since the determined levels of these substances were considerably high. This extract as a medicinal source can be considered safe when administered orally, as it did not show signs of toxicity to the animal organism at the evaluated dose. However, it is necessary to carry out more prolonged studies (sub-chronic and chronic toxicity) to investigate the development of probable damages in the physiological systems resulting from the chronic administration of 'SJ-Aq'. The results obtained in this study make *S. japonica* an up-and-coming source for applications in the pharmaceutical industry. However, further studies must be carried out. The medicinal species in question demonstrates biological effects that motivate the continuity of studies, suggesting for future trials the use of systematic methods of phytochemical investigation aimed at isolating its active principles and its clinical efficacy since clinical studies are indispensable to verify the therapeutic efficiency of herbal products.

Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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