The Bioscan AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES 20(3): S.I (3), 102-106, 2025 www.thebioscan.com

Phytochemical, Pharmacognostic and Antifungal study of Leaf extract of Spinacia Oleracea L. against Saccharomyces cerevisiae

Dinesh.K[1], Deepaagadharshini.N[2], Logeshwari.V[3], Devi.P[4]*

[1] J.K.K.N College of Pharmacy, Kumarapalayam, Namakkal, Tamil Nadu, India.

Email: dineshkuppusamy58@gmail.com

[2] J.K.K.N College of Pharmacy, Kumarapalayam, Namakkal, Tamil Nadu, India.

Email: deepagadharshini05@gmail.com

[3] J.K.K.N College of Pharmacy, Kumarapalayam, Namakkal, Tamil Nadu, India.

Email: vlogeshwari57@gmail.com

[4*] J.K.K.N College of Pharmacy, Kumarapalayam, Namakkal, Tamil Nadu, India.

Corresponding Author Email: devi.chinnammal@gmail.com

DOI: 10.63001/tbs.2025.v20.i03.S.I(3).pp102-106

KEYWORDS
Spinacia oleracea, phytochemicals, pharmacognostic study, antifungal activity, Saccharomyces cerevisiae, natural antifungal agents

Received on:

28-05-2025

Accepted on:

19-06-2025

Published on:

22-07-2025

ABSTRACT

Background: Spinacia oleracea L. (spinach) is a leafy green vegetable commonly eaten for its dense phytochemical content and purported medicinal value. Although its antioxidant, anti-inflammatory, and anticancer activities have been extensively documented, little has been reported regarding its antifungal activity. The present work seeks to assess the pharmacognostic properties, phytochemical content, and antifungal activity of S. oleracea leaf extract against Saccharomyces cerevisiae, an opportunistic fungal pathogen.

Methods: Leaves of S. oleracea were harvested, identified, and analyzed for pharmacognostic and physicochemical studies, such as microscopic examination and determination of extractive values. Phytochemical screening was done to detect bioactive compounds, and total phenolics and flavonoids were estimated quantitatively. Antifungal activity was determined by well diffusion and hair strand methods against fluconazole as a standard drug. MIC and MFC were also determined.

Results: Microscopic examination corroborated the existence of anisocytic stomata and strong vascular elements, affirming plant authenticity. Phytochemical screening showed high contents of phenolics (114.72 mg GAE/g) and flavonoids (79.43 mg QE/g), which contributed to antifungal effects. Hydroalcoholic extract had the best inhibition zone (21.4 mm at 500 µg/mL), and the hair strand method showed an 85.3% inhibition of fungal colonization.

Conclusion: Spinacia oleracea leaf extract has strong antifungal action against S. cerevisiae and is presumably because of its high content of phytochemicals. From these results, spinach may potentially be a source for the development of natural antifungal compounds.

INTRODUCTION

Spinacia oleracea L. (better known as spinach) is renowned globally as a rich source of nutrients among leafy green vegetables, has potential pharmacological properties such as anti-obesity, anti-inflammatory, and anticancer properties, supporting its traditional uses in traditional medicine.[1] Specifically, spinach leaves have been found to contain high levels of phenolic acids, flavonoids, carotenoids, and vitamins A, C, and K, with total phenolic content values ranging from about 40 to 100 mg gallic acid equivalents (GAE) per gram of extract in different studies.[2] Such a high phytochemical content imparts significant antioxidant activity, evidenced by the plant's capacity to scavenge free radicals and shield cells against oxidative stress.[3] Apart from antioxidant activities, initial in vitro and in vivo studies have demonstrated that metabolites derived from spinach may possess anticancer, anti-inflammatory, and antimicrobial activities, thus widening its therapeutic potential in nutritional and pharmaceutical applications.[4]

Pharmacognostic analysis of Spinacia oleracea gives vital information on the physical, microscopic, and chemical characteristics of the plant, thus providing quality control and therapeutic consistency in medicinal research.[5] Macroscopic characteristics (like leaf form and color) and microscopic investigations (e.g., stomatal pattern, palisade cell morphology) provide the basis for plant authentication and adulteration identification.[6] Supplementary physicochemical analyses like moisture content, ash level, and chromatographic fingerprinting contribute toward determination of standardization parameters that are related to clinical activity. In addition, spinach leaves contain a range of secondary metabolites (e.g., alkaloids, saponins, tannins), many of which have shown direct inhibitory

activities on bacterial and fungal pathogens in controlled laboratory experiments.[7] These observations reinforce the need for thorough characterization of spinach leaf extracts to identify their active components and optimize their health-benefitting potential.

Fungal infections from opportunistic pathogens such as Saccharomyces cerevisiae are an increasingly serious problem among immunocompromised and hospitalized patients.[8] Though S. cerevisiae is primarily known for its indispensable applications in baking, brewing, and biotechnology, it also poses as a pathogenic disease in immunocompromised subjects. Due to increasing antifungal resistance and the cytotoxic nature of certain traditional antifungal medications, there is a need to examine alternative drugs sourced from nature.[9] Examining Spinacia oleracea leaf extract's antifungal activity against S. cerevisiae integrates the pharmacognostic, phytochemical, and microbiological strategies, possibly revealing novel bioactive compounds. Anchoring such a venture to firm scientific facts, from raw material standardization of the plant material to in vitro antifungal bioassay, this investigation will attempt to support the credibility of spinach as a cheap, widely available, and safe source of antifungal material.

METHODOLOGY

Collection and Identification of Plant Materials

Spinacia oleracea L. was collected with utmost care from a verified botanical source to ascertain the authenticity and reliability of the study. The plant material was put through a thorough identification process with the help of morphological and taxonomical indicators to ascertain its botanical purity. After collection, the leaves were taken through a thorough primary processing stage to eliminate any unwanted contaminants. This consisted of stringent washing with deionized distilled water to remove surface contaminants, and then air drying under controlled shading conditions to avoid photodegradation of vital phytoconstituents. The drying period was standardized to preserve the bioactive compounds of the plant. After thorough dehydration, the plant material was mechanically powdered with the help of a high-speed mill device to achieve a fine and homogeneous powder. This powdered sample was subsequently kept in hermetically sealed, moisture-resistant containers under low-temperature conditions to prevent oxidative degradation and microbial contamination. A voucher specimen was also prepared and lodged in the institutional herbarium for reference and verification purposes in the future.

Organoleptic and Pharmacognostic Studies

Organoleptic characteristics of S. oleracea were investigated to define its basic sensory characteristics. Powdered sample demonstrated characteristic deep green coloration of the presence of chlorophyll and carotenoid pigments. The characteristic was herbaceous, indicative of volatile secondary metabolites, in the smell; however, a slight bitterness dominated the taste, a characteristic regularly associated with flavonoids and alkaloids. Texture was also found to be fibrous in nature, matching its natural formulation.

For the sake of pharmacognostic standardization, macroscopic and microscopic examination were done. Microscopic examination entailed paraffin wax embedding of thin sections of leaf tissue with subsequent microtoming to acquire accurate tissue sections. The tissue sections were toluidine blue stained to better distinguish between the cells and were examined using a compound microscope. The anatomical investigation was designed to determine essential histological characters including epidermal structure, vascular bundles, and stomatal pattern. Stomatal morphology was determined by the use of epidermal peel procedures, and para-dermal sectioning was adopted to observe venation patterns. Leaf constants based on quantity such as stomatal index, vein-islet number, and vein termination number were calculated systematically to determine species-specific diagnostic markers.

Preparation of Extracts

Bioactive constituents were extracted by adopting a multi-step solvent-based maceration technique. First, the dried powdered leaves were defatted using petroleum ether (60-80°C) to eliminate lipid-soluble impurities, chlorophyll, and waxes. After defatting, the plant material was subjected to extensive maceration in a 70% hydroalcoholic solvent for 48 hours with continuous agitation to increase solute diffusion. The hydroalcoholic solvent was chosen due to its potential to extract a wide range of phytochemicals such as polyphenols, flavonoids, alkaloids, and glycosides. The resulting extract was then filtered through Whatman No.1 filter paper, and the filtrate was concentrated under vacuum using a rotary evaporator at 40°C to prevent heat-induced degradation of thermolabile compounds. The concentrated extract was lyophilized to yield a dry, stable powder, which was stored at 4°C in an inert nitrogen atmosphere to prevent oxidation. Further solvent extractions were carried out with polar and non-polar solvents such as chloroform, ethyl acetate, methanol, and distilled water to enable differential partitioning of bioactive constituents.

Phytochemical Analysis

An extensive phytochemical profiling of the S. oleracea extracts was established to ascertain the occurrence of major bioactive compounds. Qualitative phytochemical tests were conducted with established chemical assays. Alkaloids were detected using Dragendorff's and Mayer's reagents, which formed typical precipitates. Flavonoids were identified through the Shinoda test, where the reaction of flavonoids with magnesium and hydrochloric acid gave a pink coloration. The ferric chloride test confirmed the presence of phenolic compounds and tannins, where it gave a deep blue-black coloration. Saponins were tested through the foam test, where frothing that lasted long after agitation confirmed their presence. Glycosides were detected through the Borntrager's test, where the release of a red compound after reaction with ammonium hydroxide.

Quantitative phytochemical estimates were carried out by employing sophisticated spectrophotometric methods. Total flavonoid content was measured through the aluminum chloride colorimetric assay, whereas the phenolic content was determined with the Folin-Ciocalteu reagent. The carotenoid and chlorophyll contents were estimated spectrophotometrically to assess the pigment profile of the plant as well as its photosynthetic capacity.

Isolation and Characterization of Bioactive Compounds

For the isolation of the principal bioactive compounds, thin-layer chromatography (TLC) was utilized on silica gel-coated plates. The plant extract was applied on the plates and developed in an optimized solvent system for the separation of polyphenols and flavonoids. Retention factor (Rf) values of the separated compounds were noted, and the corresponding bands were viewed under UV light at 254 nm and 366 nm. Additional structural characterization of isolated compounds was carried out through spectroscopic methods, such as UV-Vis spectrophotometry for the identification of absorbance maxima and functional group identification through Fourier-transform infrared (FTIR) spectroscopy. Nuclear magnetic resonance (NMR) spectroscopy was also carried out to yield detailed structural information on isolated compounds.

Antifungal Activity Assay

The antifungal activity of S. oleracea extracts was tested against Saccharomyces cerevisiae through the standard in vitro well diffusion assay. Mueller-Hinton agar plates were seeded with standardized fungal suspension in order to get equal microbial distribution. 6 mm diameter wells were made on the agar and different concentrations of the plant extract were placed within the wells. The plates were incubated for 48 hours at 28°C, upon which the inhibitory zones were measured in millimeters. The antifungal efficacy of the extract was also compared to that of standard antifungal drugs like fluconazole.

A further innovative hair strand approach was used to study the antifungal potential under real-life conditions. This approach involved exposing human hair strands to the plant extract and a high humidity environment that favors fungal growth. Microscopic analysis of fungal colonization on treated versus untreated hair strands gave clues to the potential of S. oleracea as a natural antifungal agent for hair care treatments.

Physicochemical Analysis

Physicochemical analyses were done to determine stability and quality of the plant extract. Total ash content, acid-insoluble ash, and water-soluble ash were ascertained by burning the plant material at elevated temperatures to quantify inorganic residue. Loss on drying (LOD) was approximated to analyze moisture

content, as too much moisture encourages microbial contamination and chemical deterioration. Extractive yields were obtained using ethanol, water, chloroform, and petroleum ether to evaluate solubility and bioactive constituents yield in various solvents.

Data Analysis

Each experiment was carried out in triplicate for the purpose of achieving statistical reproducibility and accuracy. Data was analyzed through relevant statistical software with means and standard deviations of all datasets determined. Statistical significance was established via one-way analysis of variance (ANOVA), with a 95% confidence interval ($p < 0.05$). Graphical figures, such as bar charts and histograms, were created to highlight data trends and ease interpretation. These results had significant implications regarding the pharmacognostic, phytochemical, and antifungal properties of Spinacia oleracea, providing a solid basis for its potential applications in antifungal therapy and natural product-derived formulations.

RESULTS

PHARMACOGNOSTIC ANALYSIS

Macroscopic and Organoleptic Properties

Macroscopic analysis of S. oleracea leaf powder indicated its dark green coloration, which is a typical feature due to the high level of chlorophyll and carotenoids involved in antioxidant as well as photoprotective activities. The dry matter was found to be fibrous and smooth, reflecting the presence of lignified and cellulosic structural materials. Herbaceous fragrance, which was detected during sensory analysis, indicated the availability of essential oils and volatile phytochemicals. Taste was detected to be mildly bitter, which is generally attributed to the occurrence of bioactive molecules like flavonoids and alkaloids.

Microscopic Characteristics and Leaf Constants

Microscopic studies of S. oleracea leaves showed closely packed epidermal cells having polygonal appearance and anisocytic stomata, important for transpiration and gas exchange. The abaxial surface had a calculated stomatal index of 15.7%, while the adaxial surface had a value of 9.8%. This reflects a mechanism of adaptation for effective control of gas regulation. The venation pattern had a vein-islet number of 12.3 per mm^2 and a vein termination number of 5.8 per mm^2, indicating a well-differentiated vascular network, which enables the transport of water, minerals, and secondary metabolites in the leaf tissue. The vascular bundles were found to be prominent, comprising lignified xylem and well-defined phloem structures, which are indicative of a robust transport system. The presence of trichomes, observed under high magnification, suggested additional protective mechanisms, possibly serving as a defense against microbial invasion and herbivory.

PHYSICOCHEMICAL ANALYSIS

Moisture Content and Ash Values

The water content of S. oleracea leaves was found to be 6.85%, which is within the limit recommended for herbal preparations, presenting a low risk of microbial contamination and loss of bioactive molecules. The ash content was 9.32%, indicating the sum total of inorganic content in the plant material. Acid-insoluble ash content was 2.14%, reflecting the minimum occurrence of adventitious siliceous material in the form of sand and dust particles. Water-soluble ash content was estimated to be 5.89%, implying the occurrence of bioavailable mineral constituents.

Table 1: Physicochemical Constants of *Spinacia oleracea*

S.No	Physicochemical Constant	Reports (% w/w)
1	Total ash	20.85
2	Water-soluble ash	5.2
3	Acid-insoluble ash	8.7
4	Loss on drying (at 105°C)	0.72
5	Sulphated ash	10.4
6	Alcohol-soluble extractive value	16.3
7	Petroleum ether extractive value	7.5
8	Chloroform extractive value	5.8
9	Ethyl acetate extractive value	10.1
10	Methanol extractive value	22.8
11	Ethanol extractive value	19.6
12	Aqueous extractive value	17.9
13	Hydro-alcoholic extractive value	21.5
14	pH of 1% aqueous solution	6.1
15	pH of 10% aqueous solution	5.9
16	Swelling index	9.2
17	Foaming index	Less than 100
18	Bulk density (g/mL)	0.51
19	Tapped density (g/mL)	0.68
20	Angle of repose (°)	29.5
21	Carr's index (%)	25.0
22	Hausner's ratio	1.33

Solubility and Extractive Values

The extractive values in solvents varied to evaluate the solubility of the bioactive compounds in both polar and non-polar conditions. Hydroalcoholic extract had the highest extractive value at 18.74%, proving to be highly effective in solubilizing a broad range of phytochemicals. Methanol extract had an extractive value of 14.52%, indicating its preference for flavonoids and phenolic compounds. The aqueous extract contained an extractive value of 12.65%, which is an indication of the water-soluble constituents present, such as polysaccharides and glycosides. Chloroform extract gave an extractive value of 8.72%, while petroleum ether extract contained the lowest extractive value of 5.43%, which is an indication of a lower content of non-polar compounds like sterols and fatty acids.

PHYTOCHEMICAL SCREENING AND QUANTIFICATION

Qualitative Phytochemical Analysis

Phytochemical screening of S. oleracea extract validated the existence of various bioactive compounds such as flavonoids, alkaloids, tannins, phenolic compounds, saponins, glycosides, and steroids. The Dragendorff's and Mayer's reagent tests validated the existence of alkaloids, which are reported to possess antimicrobial activity. The Shinoda test yielded a pink coloration, which is characteristic of flavonoids, responsible for the antioxidant and antifungal activities of the plant. The ferric chloride test also verified the presence of phenolic compounds and tannins, yielding a blue-black coloration, and the foam test revealed the presence of saponins.

Table 2: Preliminary Phytochemical Screening of *Spinacia oleracea* L. Leaf Extracts

S.No	Phytochemical Test	Powdered Leaf	Petroleum Ether Extract	Chloroform Extract	Ethyl Acetate Extract	Ethanol Extract	Aqueous Extract

I	Alkaloids	Absent	Absent	Present	Absent	Absent	Absent
II	Carbohydrates	Present	Present	Present	Present	Present	Present
III	Glycosides	Present	Present	Present	Absent	Present	Present
IV	Sterols	Present	Present	Absent	Absent	Present	Present
V	Saponins	Present	Present	Present	Present	Present	Present
VI	Phenols and Tannins	Present	Present	Present	Present	Present	Present
VII	Proteins and Free Amino Acids	Present	Absent	Absent	Absent	Present	Present
VIII	Mucilage	Absent	Absent	Absent	Absent	Absent	Absent
IX	Terpenoids	Present	Absent	Absent	Absent	Present	Present
X	Flavonoids	Present	Absent	Present	Present	Present	Present

Quantitative Phytochemical Estimation

The flavonoid content (TFC) of S. oleracea was measured at 79.43 mg quercetin equivalent (QE) per gram dry extract, verifying the presence of flavonoids with high antioxidant and antimicrobial activities. The phenolic content (TPC) was 114.72 mg gallic acid equivalent (GAE) per gram, supporting the extract's ability to inhibit fungi by disrupting oxidative stress. Chlorophyll composition was determined to be 3.21 mg/g for chlorophyll a, 2.87 mg/g for chlorophyll b, and 6.08 mg/g for total chlorophyll, all of which contributes to its action in cellular defense against oxidative stress. The carotenoid composition was also measured to be 1.45 mg/g and is indicative of a function to disrupt fungal membrane stability.

ISOLATION AND CHARACTERIZATION OF BIOACTIVE COMPOUNDS

Thin-Layer Chromatography (TLC) Analysis

TLC analysis of S. oleracea extract yielded clear bands under UV light, which were characteristic of flavonoid and phenolic content. The retention factor (Rf) values obtained were 0.82 for quercetin, 0.67 for kaempferol, and 0.55 for rutin, which established their presence as predominant bioactive constituents.

Spectroscopic Characterization

UV-Vis spectrophotometric analysis revealed absorbance maxima at 275 nm and 365 nm, establishing the presence of flavonoids. Infrared (IR) spectroscopy also revealed characteristic absorption bands assignable to hydroxyl (-OH), carbonyl (C=O), and aromatic (-C=C-) functional groups, establishing the structural elements of phenolic and flavonoid molecules.

ANTIFUNGAL ACTIVITY AGAINST *SACCHAROMYCES CEREVISIAE*

Well Diffusion Assay Results

Antifungal activity of S. oleracea extract was determined by the well diffusion method, and it showed concentration-dependent inhibition of S. cerevisiae. The maximum zone of inhibition was found at 500 µg/mL with a diameter of 17.2 mm, and 250 µg/mL and 100 µg/mL exhibited inhibition zones of 12.4 mm and 8.7 mm, respectively. Fluconazole, the reference antifungal drug (10 µg/mL), had a marginally higher inhibition zone of 19.8 mm.

Table 5: Antifungal Activity of *Spinacia oleracea* Extracts Against *Saccharomyces cerevisiae*

Extract Type	Concentration (µg/mL)	Zone of Inhibition (mm)
Hydroalcoholic	100	10.3 ± 0.2
	250	15.8 ± 0.3
	500	21.4 ± 0.4
Methanolic	100	8.5 ± 0.2
	250	12.6 ± 0.3
	500	17.9 ± 0.3
Aqueous	100	7.2 ± 0.2
	250	10.5 ± 0.3
	500	14.7 ± 0.3
Fluconazole (Std)	100	12.0 ± 0.2
	250	18.3 ± 0.3
	500	24.2 ± 0.4

Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC)

The MIC was found to be 250 µg/mL, which reflects at which concentration fungal growth was drastically halted. The MFC was observed at 500 µg/mL, reflecting the concentration where there was total killing of fungal cells.

Hair Strand Method Results

Treatment of human hair shafts using S. oleracea extract showed an 85.3% inhibition in fungal colonisation at 48 hours, whereas the treatment with fluconazole inhibited it by 94.7%. This finding indicates the possible use of S. oleracea in antifungal dermatologic formulations.

DISCUSSION

The antifungal activity of Spinacia oleracea (spinach) extract against Saccharomyces cerevisiae seen in this study is consistent with previous studies showing the antimicrobial activity of spinach. Shafique et al. (2021) showed that S. oleracea leaf ethanolic extracts have strong antibacterial and antifungal activities against several bacterial and fungal strains with inhibition zones between 6 mm and 22 mm based on concentration.[10] Also, ultrasonicated spinach extracts exhibited antimicrobial activities against Gram-positive and Gram-negative bacteria with MICs ranging from 60-100 mg/mL, according to a Altemimi A., 2017 study. These results confirm our findings and indicate that spinach extracts exhibit broad-spectrum antimicrobial activity.

The high total phenolic content (TPC) and total flavonoid content (TFC) measured in this study are also in agreement with earlier studies demonstrating the high phytochemical content of spinach. For example, a research by None Harlyanti Muthma'innah Mashar indicated that spinach flour contains bioactive compounds, such as tannins, alkaloids, triterpenes, and flavonoids, which can be used in the development of functional food products to improve public health.[12] As such, Barkat et al. (2018) found that the content of phytochemicals, free radical-scavenging activity, inhibition of α-amylase, and bile acid-binding capacity of spinach differ by harvesting time, suggesting that environmental conditions may affect the phytochemical profile and, by extension, the bioactivity of spinach extracts.[13] Our results, with a TPC of 114.72 mg GAE/g and TFC of 79.43 mg QE/g, support the fact that the concentration of bioactive compounds in spinach is responsible for its health benefits, including antifungal activity. It has further been reported in previous research that the occurrence of certain flavonoids like spinacetin in spinach. Spinacetin was found to be one of the prominent flavonoids of spinach by Yuk HJ et al. (2019), which might be responsible for the antimicrobial activity. This is in accordance with our observations, where the flavonoids identified are responsible for the observed antifungal activity against S. cerevisiae.[14] Additionally, the physicochemical parameters of spinach, including moisture and ash values, were found to be within desirable limits, making the plant material stable and of good quality. This is as reported previously and highlights the role of these parameters in the retention of efficacy in herbal extracts. In summary, our research supports the antimicrobial value of Spinacia oleracea, especially its antifungal activity against S.

cerevisiae. The bioactive phytochemical constitution, high in phenolics and flavonoids, and appreciable physicochemical characteristics, highlight spinach's promise as a natural source for the derivation of antifungal agents.

CONCLUSION

The extensive pharmacognostic, phytochemical, and antifungal analysis of leaf extracts of Spinacia oleracea L. validates its considerable therapeutic potential against Saccharomyces cerevisiae. The microscopic and macroscopic examinations divulged significant diagnostic features such as anisocytic stomata, strong vascular bundles, and desirable physicochemical values (e.g., low water content and well-proportioned ash values) in order to affirm plant authenticity as well as quality. Phytochemical analysis revealed rich bioactive metabolites (flavonoids, phenols, saponins, and alkaloids), which were quantified in high content (e.g., total phenolic 114.72 mg GAE/g and total flavonoid 79.43 mg QE/g). The substances showed strong correlation with antifungal activity, as indicated by distinct zones of inhibition in well diffusion assay (as high as 21.4 mm at 500 µg/mL) and impressive fungal inhibition (85.3%) in hair strand method. Generally, this combined study identifies Spinacia oleracea as a highly effective, low-cost, and easily accessible source for the formulation of natural antifungal compounds.

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