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Research Article

Pharmacological properties of *Raphanus sativus* leaves and development of fermented beverage

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Abstract

Primary goal of the present study was to develop viable food product especially, fermented food from *Raphanus sativus* leaves and to evaluate its pharmacological actions. They contains probiotics, including lactic acid bacteria, known for their diverse health-promoting benefits. Fermented beverage made using *Lactobacillus species* present in the gut, has many nutritional values as well as rich in probiotics when compared with the commercially available probiotic drinks and it helps in prevention and treatment of wide range of diseases and deficiencies. Hydro-alcoholic extract of *R. sativus* were tested for the antithrombotic activity and shows 62.99% clot lysis. Its anti-inflammatory activity against albumin denaturation and membrane stabilization was found to be 76.55% with IC₅₀ of 57.85µg/ml and 68.46% with IC₅₀ of 62.93µg/ml, respectively. The antioxidant activity by DPPH, H₂O₂, LPO and total reducing potential methods exhibited IC₅₀ values 13.97, 49.40, 69.47 and 3.02% µg/ml respectively. Moreover, it has antimicrobial activity against wide range of microbes. Fermented beverage developed from *R. sativus* leaves using *L. plantarum* along with *L. acidophilus* was tested for micro and macro nutrients. The results of in vitro activities prove that leaves of *R. sativus* have therapeutic effects and act as a natural source of antithrombotic, anti-inflammatory, antioxidant and antimicrobial agent.

Keywords

Raphanus sativus leaves, antithrombotic, anti-inflammatory, antioxidant, antibacterial, antifungal, fermented beverage

Abbreviations

DMRT – Duncan's multiple range test; DPPH – 2,2-diphenyl-1-picrylhydrazyl; LPO – lipid peroxidation; MHA – Muller Hinton agar; RBC – red blood cell; TBA – 2-thiobarbituric acid

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Introduction

Plants are organic biomolecule producers with a wide range of applications in the development of drugs. Because of the serious adverse consequences of chemical medications, plants with medicinal properties are in high demand around the world. The advancement of science has demonstrated that the use of plants in medicinal approaches is scientifically valid due to the bioactive component constituents in plants, which paved the path for future discovery of plant-derived therapeutic advancements. In order to tackle this problem while offering fundamental health care to every individual, the world is looking for comprehensive methods and more inexpensive, conveniently readily available, and physiologically appropriate traditional medical systems (WHO 2013).

Raphanus sativus (Fig. 1) - a vegetable crop is commonly known as radish which belongs to *Brassicaceae* family.



Figure 1: *Raphanus sativus* leaves

Because of high nutritional value, its tap roots have been consumed worldwide in various edible form such as salads and pickles (Goyeneche et al. 2015). Leaves (Gul et al. 2024) and sprouts (Hernández et al. 2023) also have been claimed to have nutritional and therapeutic importance, in addition to the roots. This species has been scientifically demonstrated to have numerous pharmacological properties. Numerous studies have reported its antioxidant (Baenas et al. 2016), antimicrobial (Singh and Kumar 2019) anticancer characteristics (Pocasap et al. 2017). Moreover, it has also been shown to be effective in treating anxiety-reduction (Siddiq et al. 2018), hypertension, counteract gastric issues, and

potential antitussive (Sham et al. 2013) as well as neuroprotective actions (Do et al. 2021).

Furthermore, studies found that the presence of numerous bioactive phytochemicals have been accountable for these pharmacological actions. *R. sativus* is high in beneficial substances such as glucosinolates and its corresponding isothiocyanates, including sulforaphene and other phytochemicals (Baenas et al. 2016). Furthermore, calcium and flavonoid content was found to be abundant in its leaves than roots (Goyeneche et al. 2015). Due to many beneficial effects of *R. sativus*, utilization of it to produce a viable food product is an excellent choice. Probiotics includes a wide range of bacteria (Lactic acid bacteria), yeasts, and filamentous fungi when consumed regularly helps to fight against wide range of diseases (Voidarou et al. 2021). The purpose of the study is to evaluate antioxidant activity, anti-inflammatory activity, antithrombotic activity and antimicrobial activity of hydro alcoholic extract of *R. sativus* leaves and to develop Fermented beverage from leaves of *R. sativus* using *L. plantarum* and *L. acidophilus*.

Materials and Methods

Plant collection and extraction. The fresh leaves of *R. sativus* were collected from the Krishnagiri, Tamil Nadu. The authentication certificate number is No.GRD/2021/112. The leaves were washed, dried and homogenized to fine powder. Crude extract was prepared by Soxhlet extraction method using five different solvents petroleum ether, chloroform, aqueous, methanol and hydro-alcohol.

Phytochemical screening. Freshly prepared crude extracts were qualitatively tested for the presence of phenols, glycosides, alkaloids, flavonoids, tannins, terpenoids, saponins, oils, gums as described by the method of Sofowora (2008).

In vitro antithrombotic activity. *In vitro* clot lysis activity of test drug was carried out according to the method of Prasad et al. (2006) with minor modifications. Briefly, venous blood drawn from the healthy volunteers was distributed in different pre weighed sterile micro centrifuge tube (0.5 ml/tube) and incubated at 37°C for 45 min. After clot formation, serum was completely removed without disturbing the clot and each tube having clot was again weighed to determine the clot weight. Here, as a standard Streptokinase is used as a non-

thrombolytic control. Difference obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis.

***In vitro* anti-inflammatory activity**

Inhibition of albumin denaturation. Methods of Mizushima & Kobayashi (1968) and Sakat et al. (2010) followed with minor modifications. The reaction mixture will be consisting of hydro-alcoholic extract of *R. sativus* leaves, 1% aqueous solution of bovine albumin fraction.

Membrane stabilization test

Preparation of red blood cells (RBCs) suspension is done using the methods of Thenmozhi et al. (1989); Saket et al. (2010). Denaturation of proteins will be well documented cause of inflammation. According to Duncan's Multiple Range Test (DMRT), the values are to be followed by different subscripts and checked for significant difference at $P < 0.05$, SE-standard error of the mean.

***In vitro* antioxidant activity**

DPPH radical scavenging activity. DPPH radical scavenging activity was carried out by the method of Molyneux (2004). To 1 ml of 100 μ M DPPH solution in methanol, equal volume of the test sample in ethanol of different concentration was added and incubated in dark for 30 min. The change in coloration was observed in terms of absorbance using a spectrophotometer at 514 nm.

H₂O₂ radical scavenging activity. Hydrogen peroxide radical scavenging activity of the test sample was estimated by the method of Ruch et al. (1989). A solution of hydrogen peroxide was prepared in phosphate buffer (pH 7.4). 200.0 μ l of sample containing different concentration was mixed with 0.6 ml of H₂O₂ solution. Absorbance of H₂O₂ was determined 10 minutes later against a blank solution containing phosphate buffer without H₂O₂.

LPO radical scavenging activity. TBA reactive species were used as a measure of the LPO inhibition (Ohkawa et al. 1979). Plant extracts (0.1 ml) were mixed with 0.5 ml egg yolk homogenate (10%) and volume made up to 1 ml with distilled water. LPO was induced by adding 0.05 ml of ferrous sulphate. The absorbance was measured at 532 nm.

Total reducing potential. The reducing power was determined according to a described procedure (Oyaizu 1986). The test sample was spiked with phosphate buffer and 1% potassium ferricyanide. The absorbance at 700 nm was detected and the higher the absorbance represents the stronger the reducing power.

***In vitro* antimicrobial activity**

Test microorganisms. Totally ten bacterial strains (five Gram positive bacterial strains *Enterococcus faecalis*, *Staphylococcus aureus*, *Micrococcus luteus*, *Bacillus Subtilis*, *Streptococcus epidermidis*; five Gram negative bacterial strains *E. coli*, *Salmonella typhi*, *Proteus vulgaris*, *Shigella dysenteria*, *Klebsilla pneumonia*) and one fungal strain (*Aspergillus flavus*) were used throughout investigation. All the microbial cultures were obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. The young bacterial broth cultures were prepared before the screening procedure.

Antibacterial assay. The well diffusion method was used to screen the antimicrobial activity. *In vitro* antimicrobial activity was screened by using Muller Hinton Agar (MHA) obtained from Himedia (Mumbai). The MHA plates were prepared by pouring 15 ml of molten media into sterile Petri plates. The plates could solidify for 5 min and 0.1% inoculums suspension was swabbed uniformly, and the inoculums could dry for 5 min. Wells were cut and 20 μ l of the different concentration of test drug were added. The plates were then incubated at 37°C for 24 h. The antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the well (NCCLS 1993). Chloramphenicol disc was used as a positive control.

Antifungal assay. To test the antifungal activity, methods of well diffusion plates on agar was used and the fractions of different concentration of plant extract were dissolved in 70% ethanol. Sabouraud Dextrose Agar was used for the process. *Aspergillus flavus* was grown in sabouraud dextrose broth and the inhibition zones were determined for the evaluation of antifungal action.

Preparation of fermented beverage (Fig. 2).

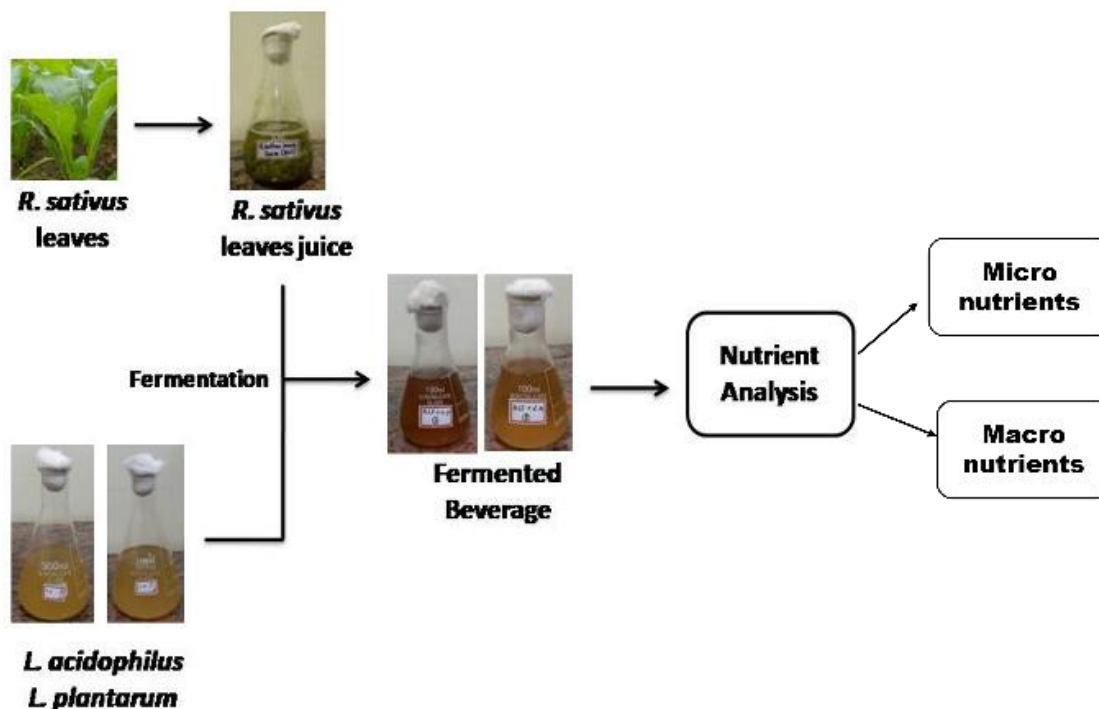


Figure 2: Development of fermented beverage using *R. sativus* leaves

Microbial inoculum preparation. *Lactobacillus plantarum* and *Lactobacillus acidophilus* were purchased from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India.

Preparation of *R. sativus* leaf extract. Leaves of *R. sativus* were directly collected from local farmers in Krishnagiri, Tamil Nadu. 10 kg of *R. sativus* leaves were collected. The part of shredded *R. sativus* leaves was blended and made into juice and it is used for fermentation with *L. acidophilus*, *L. plantarum* serially diluted inoculum.

Fermentation under controlled pH. Seed culture was prepared with microbial strains and inoculum. Cultivation was carried out at 37°C at the agitation speed of 200 rpm, in a 7l Bioflo 415 bioreactor (New Brunswick Scientific Ltd.). Culture pH was maintained at neutral pH 7.0 by the automated control system of bioreactor with the addition of acid or base.

Nutritional analysis of fermented beverage. Calcium, sodium, potassium, iron, magnesium, total protein, total carbohydrate and energy of fermented

beverage were analyzed by the Official Methods of Analysis (AOAC, 1990).

Statistical analysis. The biochemical parameters studied were subjected to statistical analysis using Sigma Stat statistical package (Version3.1). The experimental results were expressed as mean \pm SD. The results obtained for the various parameters analyzed in the four major phases and the salient findings made during the study are presented in the next chapter.

Results and Discussion

Phytochemical screening. Estimates show that a large number of people in many developing countries heavily rely on traditional herbs and traditional practitioners to resolve their primary health need (Beik et al. 2020). The result of qualitative phytochemical screening of *R. sativus* leaves extracts showed the presence of most bioactive compounds like alkaloids, flavonoids, steroids, phenol, tannins, saponins and carbohydrates (Table 1). As maximum number of phytochemicals showed positive results in

the hydro-alcoholic extract of *R. sativus* leaves, it is preferred for all in vitro activities.

In vitro antithrombotic activity. Already existing anti-thrombotic medicines like streptokinase, warfarin and heparin lacks specificity that leads to the increase risk of haemorrhage and undesired effects. Thus, many attempts are ongoing around the world to produce newer and particular thrombolytic agents. The plant based one is more of a natural and least invasive type with lower cytotoxicity and undesirable side effects (Anjum et al. 2022; McFadyen et al. 2017). The hydro-alcoholic extract of *R. sativus* leaves may serve as a promising thrombolytic agent.

The results showed a remarkable clot lysis activity at the range of 62.99% (Table 2 and Fig. 3) when compared to the control added only with phosphate buffer. This indicates that the degradation effect of hydro-alcoholic extract of *R. sativus* leaves towards

human blood clot is mainly due to its fibrinolytic activity. Even though the clot lysis activity of *R. sativus* leaves extract is lower as compared to streptokinase (86.17%). The satisfactory amount of flavonoid content in the plant extract is responsible for a significant and positive effect with thrombolytic activities (Uddin et al. 2016). Flower extract of *Mirabilis jalapa* also exhibited highest clot lysis characteristic action of $53.81 \pm 0.52\%$ in comparison to $41.74 \pm 0.34\%$ clot lysis illustrated by streptokinase (Harun-Or-Rashid et al. 2023).

In vitro anti-inflammatory activity. Inflammation is a defence measure occurs when infectious microbes invade the body (Germolec et al. 2018). The non-steroidal anti-inflammatory drugs are frequently used all over the world. However, due to the negative impacts of those standard drugs, the necessity of organic anti-inflammatory agents is increasing.

Table 1. Phytochemical screening of *R. sativus* leaves

Phytochemical	Extracts				
	Pet. Ether	Chloroform	Aqueous	Methanol	Hydro alcohol
Alkaloids Mayer's tests, Wagner's test	+	ND	+++	+++	++
Flavonoids Lead acetate test, H ₂ SO ₄ test	ND	+	++	+++	+++
Steroids Liebermann-Burchard test	+++	++	++	+++	+++
Phenols Ferric chloride test, Lead acetate test	ND	ND	++	++	+++
Saponin Froth test	ND	ND	+++	++	+++
Tannin Ferric chloride test	+	ND	+++	+++	++
Carbohydrates Benedict's test	ND	ND	+++	++	+
Glycosides Borntrager's Test	ND	ND	ND	ND	ND
Oils & Resins Spot test	ND	ND	ND	ND	ND

Note: +++ = high, ++ = moderate, + = low, ND = Not detectable

The anti-inflammatory activities present in *R. sativus* leaves observed in the extract could be associated with the presence of acceptable quantity of flavonoid compounds in it (Uddin et al. 2016).

In order to compare its potential anti-inflammatory action, the standard drug, Aspirin was used as positive control.

Table 2: *In vitro* Antithrombotic activity of hydro-alcoholic extract of *R. sativus* leaves

Sl. No.	Weight of empty tube A, g	Weight of tube with clot B, g	Weight of clot C (B-A), g	Weight of tube with clot after lysis D, g	Weight of lysis E (B-D), g	% of clot lysis	Average % of clot lysis
1	0.997	1.246	0.249	1.094	0.152	61.04	
2	0.956	1.190	0.234	1.063	0.127	54.27	
3	0.973	1.210	0.237	1.048	0.162	68.35	62.99%
4	0.939	1.238	0.299	1.073	0.165	55.18	
5	0.981	1.224	0.243	1.039	0.185	76.13	

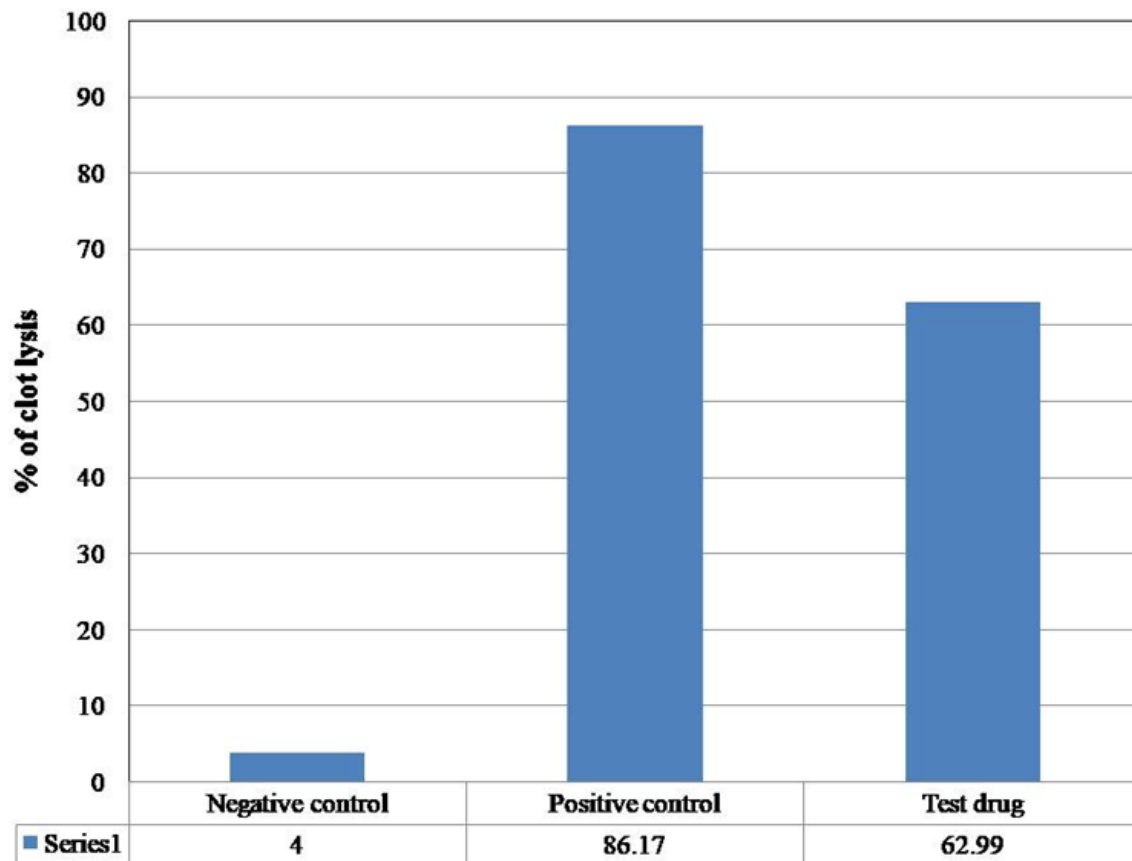


Figure 3: Antithrombotic activity of hydro-alcoholic extract of *R. sativus* leaves in comparison with distilled water and streptokinase

Note: *Negative control*: Phosphate buffer: 4% clot lysis activity; *Positive control*: Streptokinase: 86.17% clot lysis activity; *Test drug*: Hydro-alcoholic extract of *R. sativus* leaves: 62.99% clot lysis activity

Inhibition of albumin denaturation. In this assay, in order to examine anti-inflammatory effects, protein extracts as well as peptide hydrolysates were tested for their capacity to suppress heat-induced denaturation of albumin. The results of in vitro anti-inflammatory activity against albumin denaturation showed that hydro-alcoholic extract of *R. sativus* leaves was effective in inhibiting heat induced albumin denaturation with maximum inhibition of 76.55%, IC₅₀ of 57.85 µg.ml⁻¹ was observed. Aspirin was used as the standard drug, showed the maximum inhibition of 87.73% and IC₅₀ value of 46.38 µg.ml⁻¹ (Fig. 4). Moreover, in the case of *Barringtonia racemosa* extracts, anti-inflammatory activity was evaluated by employing albumin denaturation inhibitory assay and it revealed more than 50% inhibition (Osman et al. 2016).

Membrane stabilization test. The results of hydro-alcoholic extract of *R. sativus* inhibited the heat induced hemolysis of RBCs to varying degree. It showed the maximum inhibition of 68.46% at 100 µg.ml⁻¹ and the IC₅₀ was 62.93 µg.ml⁻¹. Aspirin showed the maximum inhibition of 80% and its IC₅₀ value was 28.45µg/ml (Fig. 4). Furthermore, in another study, human RBC membrane stabilization technique has been employed in order to evaluate the anti-inflammatory action of *Gendarussa vulgaris*. It's both aqueous as well as ethanolic extracts at various concentrations showed significant stabilization towards RBC membranes. The result outcome showed that the leaf extract of *G. vulgaris* had considerable anti-inflammatory activities at various doses, with 300 mg.ml⁻¹ showing the highest efficacy (Saleem et al. 2011).

In vitro antioxidant activity. Radical scavengers may protect cell tissues from free radicals, thereby preventing diseases such as cancer, cardiovascular disease, neurological disease, pulmonary disease, nephropathy and etc.

DPPH radical scavenging activity. The radical scavenging activity of the hydro-alcoholic *R. sativus* leaves extract determined by employing DPPH in a dose dependent manner. A DPPH radical scavenging activity is the capacity of the compound to give away electrons that will counteract the DPPH free radical thus avoiding oxidative stress (Afsar et al. 2018). The colour of DPPH fades when an antioxidant is present in the medium due to

conversion of free radicals to a colourless product (i.e.2, 2-diphenyl-1-hydrazine) resulting in a decrease in absorbance. The result shows maximum inhibition of 96.19% at 100 µg. ml⁻¹ was observed, IC₅₀ value was 13.97 µg.ml⁻¹ (Fig. 5). Ascorbic acid used as standard antioxidant, showed the maximum inhibition of 98.41%, IC₅₀ value was 7.17 µg.ml⁻¹. Similar research found that methanolic extract of *Mirabilis jalapa* flowers showed IC₅₀ value 13.70 ± 0.32, in comparison to ascorbic acid with IC₅₀ 9.98 ± 0.42 µg.ml⁻¹ (Harun-Or-Rashid et al. 2023).

H₂O₂ radical scavenging activity. The scavenging ability of hydro-alcoholic extracts of *R. sativus* leaves on H₂O₂ in a dose dependent manner is compared with Ascorbic acid as standard. Maximum inhibition of 81.68%, IC₅₀ value of 49.40 µg.ml⁻¹ was observed (Fig. 5). Ascorbic acid showed the maximum inhibition of 90.15% value of 33.05 µg.ml⁻¹ was observed. Moreover, methanol as well as petroleum ether extracts of *Hibiscus tiliaceus* leaves was also found to be H₂O₂ scavengers (Surana et al. 2022).

LPO radical scavenging activity. The LPO (lipid peroxidation) radical scavenging activities of the total extracts of the hydro-alcoholic extract of *R. sativus* leaves were studied. The sample showed LPO radical scavenging activity in a dose dependent manner, maximum inhibition of 68.82%, IC₅₀ value of 69.47 µg.ml⁻¹ was observed (Fig. 5). Ascorbic acid used as standard drug showed the maximum inhibition of 85.31%, IC₅₀ value of 43.52 µg.ml⁻¹.

Total reducing potential. Fig. 5 shows the total reducing powers of the hydro-alcoholic extracts of *R. sativus* examined as a function of their concentration. Sample shows maximum inhibition of 3.0259% at 100 µg.ml⁻¹ was observed from hydro-alcoholic extract of *R. sativus* leaves. The standard drug ascorbic acid showed the maximum inhibition of 3.2315% at the concentration of 100 µg.ml⁻¹. Similarly, previous research illustrated that the leaf extract of medicinal plant, *Aurea helianthus* exhibited higher reducing potential as it has the capability to reduce ferric into ferrous ion. Result outcome indicates that it exhibited better reducing power of the antioxidants activity due to the presence of higher concentration and the absorbance increased dependents on concentration (Kim et al. 2017).

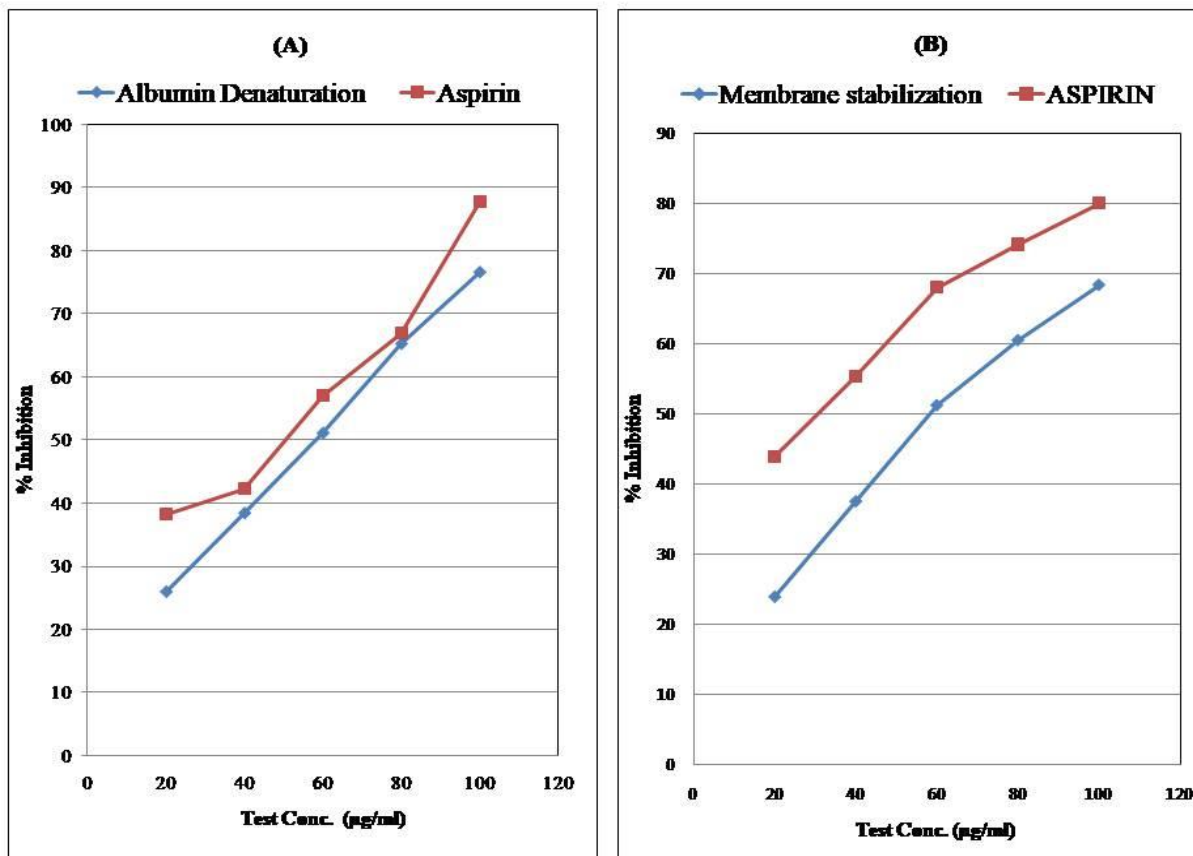


Figure 4: Graphical representation of anti-inflammatory activity of hydro-alcoholic extract of *R. sativus* leaves. (A) albumin denaturation (B) membrane stabilization

In vitro antimicrobial activity. The antimicrobial activity of plant extracts was done at different concentrations and was analyzed in the disc diffusion technique. Fig. 6 shows the results of the antimicrobial assay against a range of microorganisms. It is found that hydro-alcoholic extract of *R. sativus* leaves contained substances toxic to broad spectrum of panel of Gram positive and Gram-negative bacteria and a fungus. The results indicate that concentration of the extract is directly proportional to the zone of inhibition. Flavonoids along with polyphenols present in plant having antioxidant activity exhibits potential antibacterial activity (Bouarab-Chibane et al. 2019). *Streptococcus epidermidis*, *Proteus vulgaris*, *Aspergillus flavus*, *Klebsiella pneumonia*, *Micrococcus luteus*, *Staphylococcus aureus*, *Enterococcus faecalis* shows maximum zone of inhibition. Plant based antimicrobials have gigantic therapeutic potential because they can serve the purpose with lesser side effects when compared

with synthetic antimicrobials. The same technique is employed to evaluate the antimicrobial action of *Mirabilis jalapa* flowers extracts against certain gram positive and negative bacteria. It illustrated substantially higher inhibitory zone diameters against those bacteria ($p < 0.05$) (Peiris et al. 2022; Harun-Or-Rashid et al. 2023).

Nutritional analysis of fermented beverage. Mineral deficiencies are more commonly seen in the developing countries. To prevent the deficiencies from causing, plant-based drinks can be used as a supplement.

Protein and carbohydrate supplementation has been proposed as an effective dietary strategy to increase skeletal muscle mass and improve overall body physical performance (Kanter 2018). It has significant importance for the health care usage of *R. sativus* in phytotherapy.

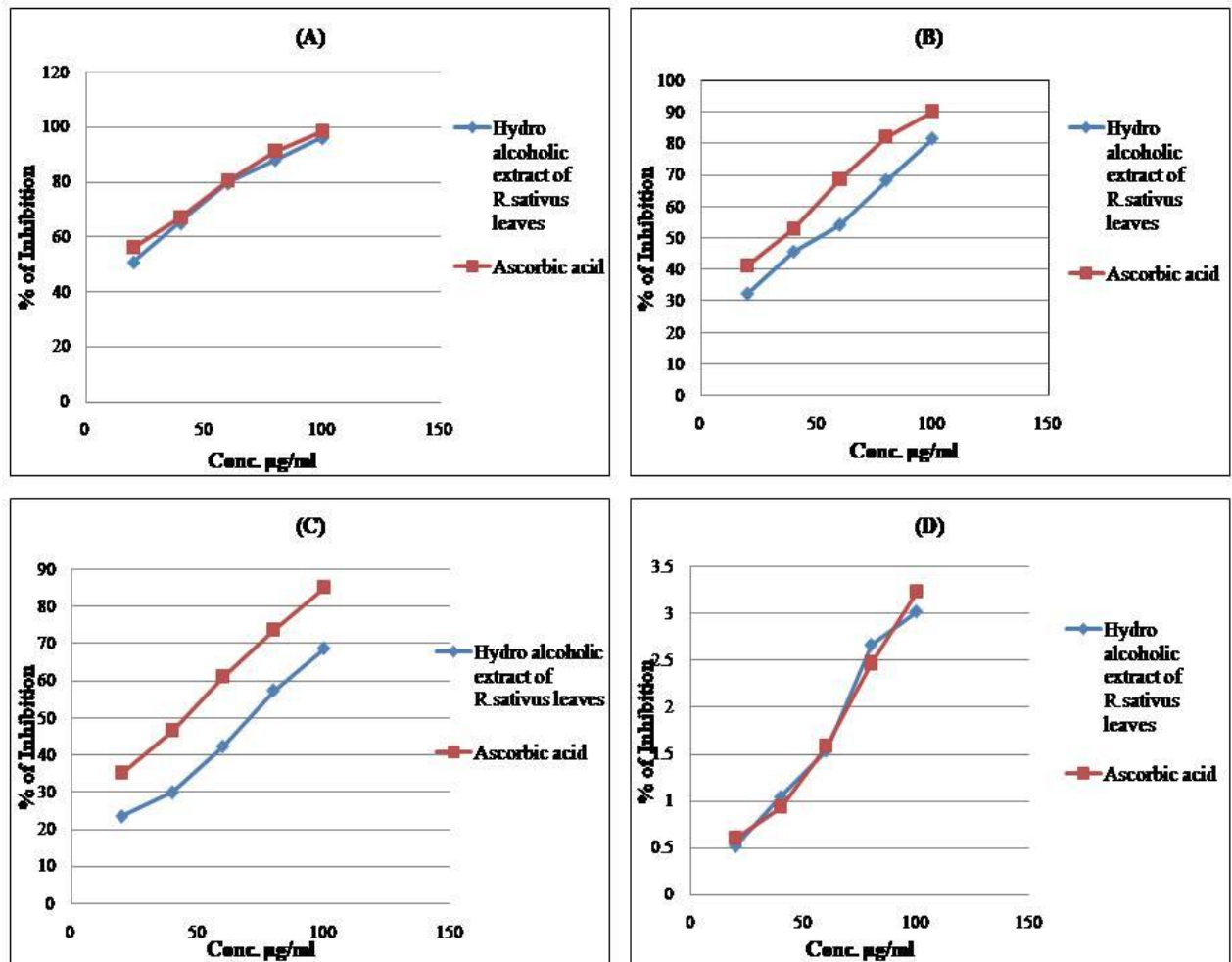


Figure 5: Graphical representation of antioxidant activity of hydro alcoholic extract of *R. sativus* leaves (A) DPPH radical scavenging (B) H₂O₂ radical scavenging (C) LPO radical scavenging (D) reducing potential

Fermented beverage made from *R. sativus* was evaluate for the nutrition content and comparative study was made with the commercially available Dairy based probiotic drink –Yakult and Plant based probiotic drink – Kevita. The sodium, potassium,

iron, magnesium and calorie content of fermented beverage were found to be more when compared with Yakult and Kevita. The calcium, protein content of fermented beverage was found to be greater than Kevita (Table 3).

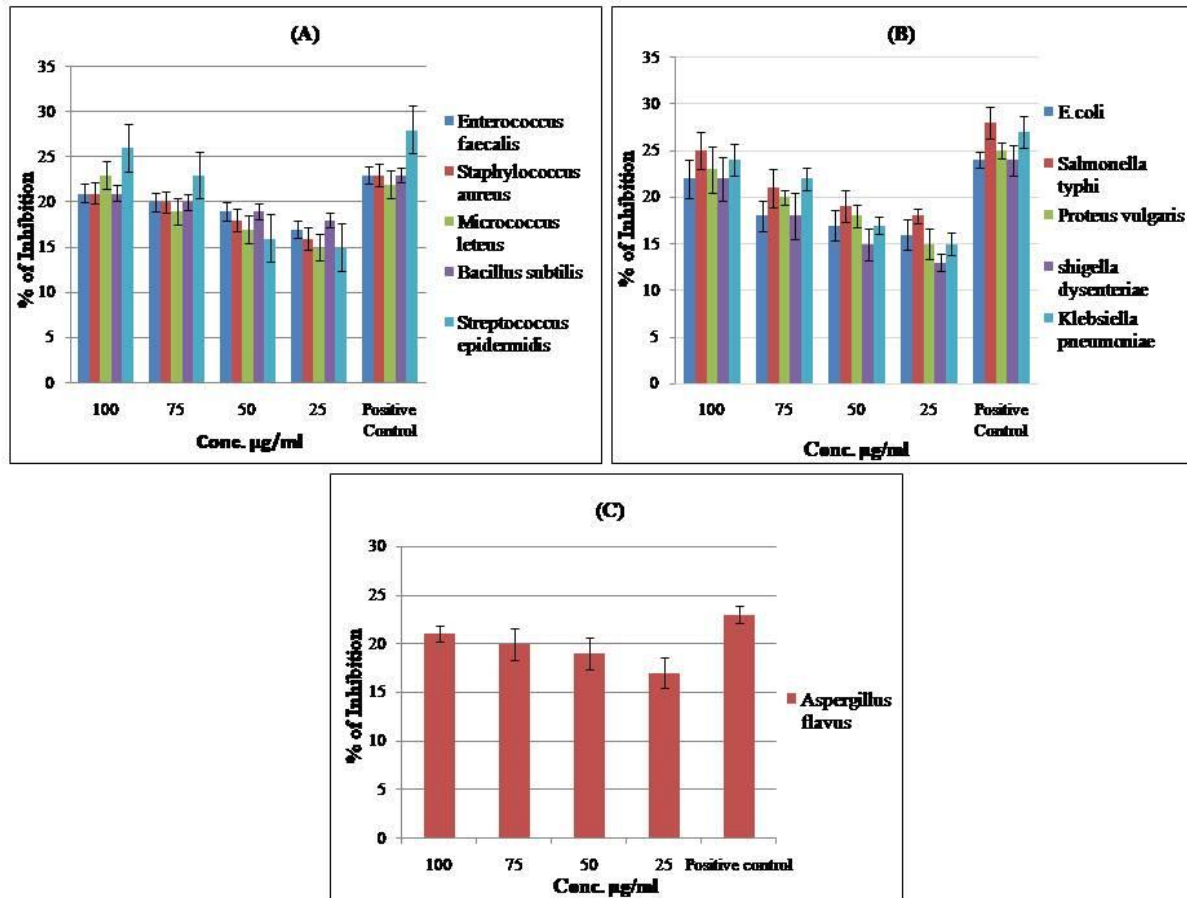


Figure 6: Antimicrobial property of hydro-alcoholic extract of *R. sativus* leaves: (A) Gram positive bacteria (B) Gram negative bacteria (C) Fungi

Table 3: Nutritional Analysis of Fermented Beverage from *R. sativus* leaves & comparison with Yakult, Kevita

S. No	Test parameter	Fermented beverage from <i>R. sativus</i> leaves	Yakult – Dairy based drink	Kevita – Plant based drink
1.	Calcium as Ca	39.91 mg.100 ml ⁻¹	50 mg.100 ml ⁻¹	ND
2.	Sodium as Na	52.87 mg.100 ml ⁻¹	18.75 mg.100 ml ⁻¹	3.33 mg.100 ml ⁻¹
3.	Potassium as K	75.68 mg.100 ml ⁻¹	62.5 mg.100 ml ⁻¹	ND
4.	Iron as Fe	0.91 mg.100 ml ⁻¹	0 mg.100 ml ⁻¹	ND
5.	Magnesium as Mg	14.52 mg.100 ml ⁻¹	ND	ND
6.	Protein	0.17 g.100 ml ⁻¹	1.25 g.100 ml ⁻¹	0 g.100 ml ⁻¹
7.	Carbohydrate	0.82 g.100 ml ⁻¹	15 g.100 ml ⁻¹	3.6 g.100 ml ⁻¹
8.	Energy	3920 cal.100 ml ⁻¹	77 cal.100 ml ⁻¹	13.3 cal.100 ml ⁻¹

Note: ND – Not detectable

Conclusions

The plants have emerged as a good source of ethno medicines. The evaluation of in vitro pharmacological activity of *R. sativus* leaves provide a valid tool that it could serve as a natural source of antithrombotic, anti-inflammatory, antioxidant and antimicrobial agent. Thus *R. sativus* leaves possess many health benefits which help in prevention and treatment of many diseases and act as a possible supplement in the food and pharmaceutical industries. To the best of our knowledge, this is the first report on the utilization of *R. sativus* leaves as a fermented beverage. It is rich in probiotics, nutrients (Fe, Ca, Na, K, Mg, protein and carbohydrate) when compared with commercially available Products. The probiotics maintains gut microbiota, prevents and cures variety of diseases. The macro and micro nutrients when consumed regularly prevents from mineral deficiencies. As this fermented beverage is plant-based product even people who are allergic to dairy can consume. Further study is needed to identify, isolate and quantify the lead active components present in the leaves of *R. sativus*. In vivo activities are needed to further characterize the pharmacological activity of its leaves.

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Author Contributions

Conceptualization, D.P.R. and E.E.; methodology, D.P.R., A.S., and S.E.; formal analysis D.P.R., E.E., A.S., M.S., and C.N.; investigation, C.N., and M.S.; resources, D.P.R., E.E., A.S., and S.E.; data curation, D.P.R., and A.S.; writing – original draft preparation, D.P.R., A.S., M.S., and C.N.; writing - D.P.R., E.E., A.S., and S.E., review and editing, C.N., and M.S., supervision, C.N., and M.S.; All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding authors.

Conflicts of Interest

The authors declare no competing interests.

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