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Screening of potential probiotic *Lactiplantibacillus plantarum* from fermented foods and determining its hypocholesterolemic activity

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ABSTRACT

A total of 80 isolates were isolated from local fermented food samples. All isolates were classified as lactic acid bacteria based on their Gram-positive, catalase-negative characteristics. The lactic acid bacterial isolates were evaluated for their ability to produce antibacterial agents against five specific pathogens. Isolates were tested for antibacterial activities. Out of 80 isolates, only 38 showed antibacterial activity. Out of 80 isolates, 38 were isolated from commercial samples, and 42 were isolated from homemade samples. Of 38 isolates from commercial samples, only 21 showed antibacterial activity against selected pathogens, while 17 did not. Among 42 isolates from homemade samples, only 17 showed antibacterial activity against the pathogens chosen, while 21 did not. Among these isolates, five isolates showed broad antibacterial activity. Among the five isolates, isolate CP2 showed the most significant cholesterol reduction (42.6%). Potential hypocholesteric isolates were identified using morphological, biochemical, and 16S rRNA identification methods. The promising isolate was identified as *Lactiplantibacillus plantarum.*

INTRODUCTION:

The International Scientific Association for Probiotics and Prebiotics defines probiotics as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" [1]. Fermented foods are produced by the microbial activity of bacteria, yeast, and mycelial fungi, and their enzymes, and are nutritionally and functionally rich [2]. Microorganisms present in conventional fermented foods, and a few of these microorganisms exhibit potential probiotic properties. The investigation focused on fermented foods and their probiotic microorganisms, with a narrower scope than for other dairy products [3]. Recently, plant-based fermented foods have been viewed as a promising way to include probiotic cultures through vegetable fermentation.[4]. Hypercholesterolemia is a lipid disorder characterised by elevated low-density lipoprotein (LDL) cholesterol, commonly known as "bad" cholesterol. This condition leads to the accumulation of fat in the arteries, a process known as atherosclerosis, which increases the risk of heart attacks and strokes. Atherosclerosis is the primary cause of cardiovascular disease,

which is responsible for more deaths worldwide than any other condition [5].

Lactic acid bacteria are a diverse group of Gram-positive bacteria found in a variety of ecological contexts, including meats, traditional foods, fermented foods, plants, and the digestive tracts of mammals [6,7]. Lactic acid bacteria are the group to which *L. plantarum* belongs. It does this by helping preserve the integrity of the intestinal wall [8], bolstering the immune system [9], and interacting with genes [10]. It has been shown that *L. plantarum*, a probiotic bacterium considered harmless, possesses several immunomodulatory capabilities. These qualities include anticancer, antiallergic, and antiviral activities. The ability of *L. plantarum* to colonise diverse environments is facilitated by its distinct functional genomes, which enable metabolic flexibility [11,12]. The use of food products containing L. plantarum has been shown to provide several health benefits, including reducing the risk of gastrointestinal infections, lowering the risk of inflammatory bowel disease, and boosting the immune system. Furthermore, because of its inherent inhibitory action, this bacterium can limit the growth of foodborne pathogens and extend the shelf life of foods modified through bioprocessing [13, 14]. In addition, this strain has the potential further to enhance the nutritional quality and flavour characteristics of food, reduce the presence of unacceptable chemicals, boost antibacterial and antioxidant activities, and extend the shelf life of food [15, 16].

This study aims to isolate and characterise lactic acid bacteria (LAB) from traditional South Indian fermented foods, with a focus on key probiotic characteristics. Additionally, we evaluated the functional properties of these bacteria to select strains that can effectively reduce cholesterol levels. Candidates meeting the established criteria could potentially serve as novel probiotic strains, paving the way for further research in medical applications.

MATERIALS AND METHODS:

COLLECTION OF SAMPLES

The typical habitats of lactic acid bacteria were selected, and fermented food samples were brought into the laboratory. Commercial and homemade fermented food samples, including buttermilk, jalebi batter, dhokla batter, curd, and pickles, were collected from different locations **(Table No.1)**. Samples were brought to the laboratory in a cooled environment and stored at 4°C.

Table No.1: Screening of LAB from different sources.

Isolation Sources		Samples Screened	Location	Number of isolates
Commercial	Buttermilk	2	Karad and Islampur	8
	Jalebi batter	3	Karad, Kolhapur, and Islampur	9
	Dhokla batter	2	Karad and Islampur	7
	Curd	3	Karad, Kolhapur, and Islampur	8
	Pickles	3	Karad, Kolhapur, and Islampur	6
Homemade	Buttermilk	3	Karad, Kolhapur, and Islampur	8
	Jalebi batter	3	Karad, Kolhapur, and Islampur	9
	Dhokla batter	3	Karad, Kolhapur, and Islampur	8
	Curd	2	Karad and Islampur	9
	Pickles	3	Karad, Kolhapur, and Islampur	8
Total		**27**	**03**	**80**

ENRICHMENT OF SAMPLES:

Each sample of buttermilk, jalebi batter, dhokla batter, curd, and pickles was aseptically weighed at 1 g and homogenised separately in 10 ml of sterile 0.85% saline. 10-fold serial dilutions (10^{-1}–10^{-8}) were prepared for each sample, and 1 ml from the highest dilution was inoculated into MRS broth [17] at pH 5.5–6.2.

ISOLATION OF LACTIC ACID BACTERIA:

Lactic acid bacteria were isolated using MRS medium. Aliquots of 0.1 ml from higher dilutions (10^4 to 10^8) were spread onto MRS plates, which were then incubated at room temperature for 48 hours. White, creamy colonies were randomly selected and purified by three successive transfers to MRS medium. The purity of the cultures was routinely verified through microscopic examination. Gram-positive, catalase-negative isolates were selected and stored at 4°C on MRS agar [18, 19]. The purified and stored isolates were revived by subculturing in MRS broth before use.

DETERMINATION OF ANTIBACTERIAL ACTIVITY OF LAB ISOLATES

LAB isolates were cultured in MRS broth medium and incubated for 24 hours with a 2% inoculum. Cell-free supernatants (CFS) from the LAB isolates were prepared by centrifuging the mixture at 7000 rpm for 10 minutes at 4°C, thereby removing the cells from the MRS broth. CFS was frequently favoured because it effectively isolates diffusible metabolites while excluding live LAB cells, offering a distinct advantage in specific contexts.

The antibacterial activity

The antibacterial activity spectrum of cell-free supernatants from LAB isolates was thoroughly evaluated using the spot-on-lawn method [20] against five distinct pathogens, demonstrating their potential effectiveness **(Table N o . 2)**. A suspension of the test pathogen was prepared in saline to a final cell concentration of approximately 2×107 cells/ml. Overnight-grown cultures were inoculated separately in 5 mL of the corresponding soft agar. The contents of the tubes were mixed thoroughly and poured onto previously prepared MRS agar plates, allowing lawns of pathogen cultures to grow on the medium's surface. Ten microliters of each cell-free supernatant were placed onto the surface of a soft agar plate. After 24 hours of incubation, the plates were examined for the presence of inhibition zones. Clear zones surrounding the spots indicate the antibacterial activity of the isolated bacteria.

Table No. 2: Indicator strains and their growth conditions

Pathogenic microorganisms	**Medium and growth temperature**
Escherichia coli	MacConkey's broth, 37°C
Klebsiella pneumoniae	MacConkey's broth, 37°C
Staphylococcus aureus	Nutrient broth, 37°C
Pseudomonas aeruginosa	Nutrient broth, 37°C
Proteus vulgaris	MacConkey's broth, 37°C

CHOLESTEROL ASSIMILATION

Isolates were grown in a medium containing known cholesterol (usually MRS ± bile salts). After incubation, the residual cholesterol in the supernatant was compared to that of the uninoculated control. The amount of cholesterol that ended up associated with cells was also determined. The percentage of cholesterol reduction was determined with the colourimetric method. The maximum percentage cholesterol-reducing isolate was selected for further study. MRS broth was freshly prepared and supplemented with 0.3% oxgall or sodium taurocholate (which promotes BSH activity and mimics the gut environment). This mixture was incorporated with water-soluble cholesterol or cholesterol stock (1–5 mg/mL in ethanol with 0.5% Tween-80), resulting in a final concentration in the medium of 100 µg/mL. The entire mixture was inoculated with 1% (~10^7 CFU/mL) inoculum of each isolate separately, and all tubes were incubated at 37°C for 24 hrs. After incubation, cells were eliminated by centrifugation at 10,000 × g for 10 min, and the supernatant was collected in a separate empty tube. The remaining cholesterol in the spent

broth was determined calorimetrically using the o-phthalaldehyde method [21]. The aliquots of supernatant were saponified with ethanolic KOH (e.g., 1 ml sample and 1 ml 33% KOH in ethanol, 60° C , 10 min). After cooling, 2 mL of distilled water and 3 mL of hexane were added, and the mixture was vortexed for 1 minute. One millilitre of the hexane layer was transferred to a glass tube and evaporated in a water bath at 65°C. The residue was promptly redissolved in glacial acetic acid. Add ~0.5 mg/mL OPA in acetic acid OPA reagent, then carefully add 1 mL concentrated H_2SO_4 (ice bath; strong exotherm). The mixture was vortexed for 1 minute. Absorbance was measured at 550 nm, and a cholesterol standard curve was processed identically. Calculations

Cholesterol reduction in medium (%)

$$\% \text{ reduction} = \frac{C_0 - C_s}{C_0} \times 100$$

Total removal, including cell-associated (optional)

$$\% \text{ total removal} = \frac{C_0 - (C_s + C_{\text{cell}})}{C_0} \times 100$$

Where,

C_0 = cholesterol in abiotic control (same batch, same timepoint)

Cs = cholesterol in sample supernatant

Ccell = (optional) cholesterol recovered from cell pellet extract

IDENTIFICATION OF THE POTENTIAL LAB ISOLATES

The phenotypic characterisation of potential lactic acid bacteria (LAB) isolates was conducted through various morphological traits. This included examining colony morphology by assessing the shape, size, margin, colour, and opacity on MRS agar. Cell morphology, specifically the shape (cocci or rods) and arrangement (chains or clusters), was observed through Gram staining. Motility tests were performed using the hanging-drop method or motility agar, as LAB are generally nonmotile. Additionally, spore formation was evaluated using the spore staining method. Physiological characteristics were also assessed, including Gram staining to determine the Gram nature of each isolate. The catalase test was conducted, and growth was monitored at various temperatures: 10°C, 15°C, 30°C, 37°C, and 45°C. The isolates' ability to tolerate different concentrations of sodium chloride (NaCl) was tested at 2%, 4%, and 6.5%, as this helps significantly in differentiating species. Furthermore, pH tolerance was evaluated by observing survival rates at low pH levels (e.g., pH 3.0–4.0), which may be important for probiotic potential. Finally, growth was tested in the presence of bile salts (0.3%–0.5%) to assess bile salt tolerance.

Biochemical characterisation was assessed for isolates using sugars (glucose, lactose, sucrose, maltose, mannitol, etc.), gas production from glucose, arginine hydrolysis, proteolytic and amylolytic activity. LAB isolates were tested.

16S RRNA IDENTIFICATION FOR THE ISOLATE:

DNA EXTRACTION AND PURIFICATION FROM BACTERIAL ISOLATES:

DNA extraction from bacterial cultures was performed following the protocol [22]. First, bacterial cultures were centrifuged at 15,000 g for 10 minutes to form a pellet. The pellet was then washed with 400 µl of SET buffer and subjected to a second centrifugation at 10,000 g for 10 minutes. Afterwards, the pellet was resuspended in TE buffer and treated with 100 µl of tris-saturated phenol, followed by centrifugation at 10,000 g for 10 minutes at 4°C. The aqueous phase was collected, mixed with TE buffer and chloroform, and then centrifuged again. The resulting supernatant was treated with RNase and chloroform, and then the DNA was evaluated for purity and yield spectrophotometrically.

POLYMERASE CHAIN REACTION (PCR) AMPLIFICATION*:*

For PCR amplification, universal LAB primers designed from the invariant region of 16S rDNA [23] were used in a 20 µl PCR reaction mixture. This mixture contained GoTaq reaction buffer, DNA polymerase, a nucleotide mix, primers, genomic DNA, and nuclease-free water. The PCR process was conducted in a thermocycler, which applied specific temperatures for denaturation, annealing, and extension. The PCR products were then separated on a 1% agarose gel containing ethidium bromide, with a DNA marker ranging from 100 to 1500 bp serving as a molecular weight standard.

DNA Sequencing:

Sequencing reactions were performed using an automated DNA sequencer, and database searches were conducted on the NCBI website [24].

RESULTS AND DISCUSSIONS:

ISOLATION AND DETERMINATION OF ANTIBACTERIAL ACTIVITIES OF ISOLATES:

Samples were collected from various locations and brought to the laboratory. A total of eighty lactic acid bacteria were isolated from these samples **(Table 1)**. All isolates were classified as lactic acid bacteria based on their Gram-positive, catalase-negative characteristics [25]. The probiotic potential of the isolates was confirmed through antibacterial activity tests, and the most effective isolates were selected for further study. The lactic acid bacterial isolates were evaluated for their ability to produce antibacterial agents

against five specific pathogens **(Table 3)**. Of 80 lactic acid bacteria isolates, only 34 exhibited antibacterial activity to varying degrees. Out of 80 isolates, 38 were isolated from commercial samples, and 42 were isolated from homemade samples. Of 38 isolates from commercial samples, only 21 showed antibacterial activity against selected pathogens, while 17 did not. Among 42 isolates from homemade samples, only 17 showed antibacterial activity against the pathogens chosen, while 21 did not.

Table 3 represents the antibacterial activity of isolates (CB, CJ, CD, CC, CP, HB, HJ, HD, HC, HP) against five test organisms (*E. coli, Klebsiella pneumoniae, Staphylococcus aureus, Pseudomonas aeruginosa*, and *Proteus vulgaris*). Activity is measured as the zone of inhibition (mm) and then categorised into activity levels (+, ++, +++). The pathogen *E. coli* was strongly inhibited by many isolates (zones ≥18 mm in CJ3, CC4, CP2, HC6, and HP6). This indicates that *E. coli* was susceptible to all isolates. Antibacterial activity of isolates against *Klebsiella pneumoniae* varied, with some exhibiting potent inhibition (CJ3 = 19 mm, CC4 = 21 mm, HC6 = 21 mm), while many had little to no effect (e.g., CB5, CB6, CD4). This indicated that Klebsiella *pneumoniae* was selectively inhibited. Almost all isolates inhibited *Staphylococcus aureus* (12–23 mm). Isolate CC4 had the highest inhibition (23 mm). This indicated that LAB were generally effective against Gram-positive bacteria. Antibacterial activity was less consistent; some isolates showed potent inhibition (CJ3 = 15 mm, CC4 = 18 mm, CP2 = 18 mm, HC6 = 18 mm, HP6 = 12 mm), but many isolates had no effect (0 mm). This indicated that *Pseudomonas aeruginosa* was resistant to some strains. Antibacterial activity of the isolates against *Proteus vulgaris* showed that some isolates showed potent inhibition (HP6 = 22 mm, CJ3 = 18 mm, CC4 = 19 mm). Many isolates were inactivated against *Proteus vulgaris* (0 mm).

CJ3 (zones up to 21 mm), CC4 (22–23 mm across pathogens), CP2 (21–20 mm range), HC6 (22–21 mm range), and HP6 (16–22 mm range) isolates showed broad-spectrum, high antibacterial activity and could be strong probiotic or antibacterial candidates. (Fig. 1) Each group was shown the inhibition zones for one isolate. Isolates CC4 and HC6 showed the highest and most consistent inhibition across all pathogens, while others showed slight variation. The five promising LAB isolates were examined for additional probiotic criteria, including cholesterol removal capacity.

Table No. 3: Antibacterial activities of isolates

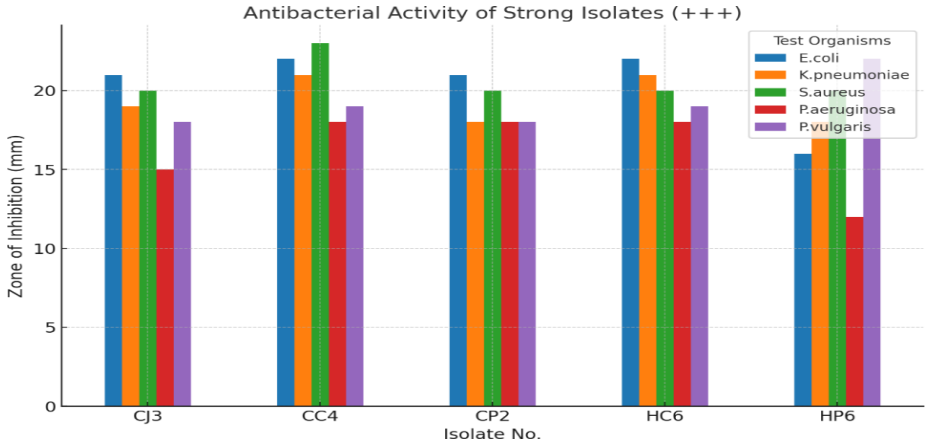
Sr. No.	Isolate No.	*Escherichia coli*	*Klebsiella pneumoniae*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Proteus vulgaris*	Activity Level
		Zone diameter in mm					
1	CB4	16	15	13	0	0	+
2	CB5	10	0	12	10	11	+
3	CB6	13	0	13	11	12	+
4	CB7	14	13	12	12	14	++
5	CB8	18	15	13	14	15	++
6	CJ1	14	20	12	9	0	+
7	CJ3	21	19	20	15	18	+++
8	CJ5	19	0	16	15	14	+
9	CJ6	12	13	14	17	0	+
10	CJ7	0	0	15	0	0	+
11	CD2	10	11	0	8	0	+
12	CD4	12	0	12	0	0	+
13	CD5	15	16	13	14	0	+
14	CC2	16	18	20	12	0	+
14	CC4	22	21	23	18	19	+++
16	CC6	18	17	20	18	14	++
17	CC7	16	18	14	15	14	++
18	CP2	21	18	20	18	18	+++
19	CP3	12	11	19	11	13	++
20	CP4	15	8	12	0	0	+
21	CP6	12	0	12	18	13	++
22	HB3	12	15	17	0	0	+
23	HB4	12	12	12	0	0	+
24	HB5	18	11	15	11	15	++
25	HB8	10	0	17	14	16	+
26	HJ5	14	20	12	9	0	+
27	HJ7	18	18	19	16	12	++
28	HJ8	10	0	17	0	0	+
29	HD2	10	11	0	8	15	+
30	HD4	15	13	18	12	13	++
31	HD5	11	10	18	13	14	++
32	HD7	15	9	19	0	0	+
33	HC5	10	11	0	8	0	+
34	HC6	22	21	20	18	19	+++
35	HC7	10	10	10	0	0	+
36	HP6	16	18	20	12	22	+++
37	HP7	18	11	19	12	16	++
38	HP8	15	14	17	13	17	++

Where: + Mild, ++ Moderate, +++ Strong

A one-way ANOVA comparing inhibition zones across the five pathogens (*E. coli, K. pneumoniae, S. aureus, P. aeruginosa, P. vulgaris*) was applied for **Table 3**. The calculated value of the F-statistic is 6.28, and the p-value is 9.2×10^{-5}. Since $p < 0.001$, there

is a highly significant difference in mean inhibition zones among the pathogens. This means the isolates did not act uniformly—some pathogens are more susceptible, while others are more resistant.

Fig. 1: Antibacterial activity of potent isolates



CHOLESTEROL REMOVAL BY LAB ISOLATES:

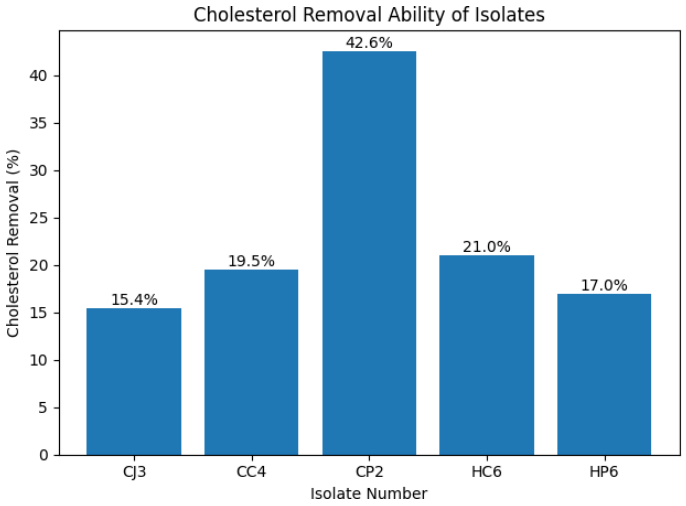
One important risk factor for coronary heart disease is high blood cholesterol. Consequently, lowering blood cholesterol levels is crucial for preventing illness. In one study, five LAB (lactic acid bacteria) isolates were grown anaerobically for 24 hours at 37°C. Their capacity to remove cholesterol was evaluated in vitro using oxgall. All five LAB isolates demonstrated the capability to remove cholesterol from the media, with varying degrees of effectiveness, ranging from 15.4% to 42.6% **(Table 4).**

The CP2 isolate demonstrated a remarkable ability to remove cholesterol from the medium, achieving a 42.6% reduction, which significantly surpassed the performance of the other LAB isolates examined. In contrast, the CJ3 isolate exhibited the lowest cholesterol assimilation rate at just 15.4%. Numerous strains of LAB have previously shown an ability to reduce cholesterol levels in model culture media [26, 27, 28, and 29]. Further research is needed to identify the mechanisms underlying cholesterol removal by these probiotic LAB isolates.

Table No. 4: Cholesterol removal ability of lactic acid bacterial isolates

Sr. No.	Isolate No.	% Cholesterol Removal	Activity level
1	CJ3	15.4%	+
2	CC4	19.5%	+
3	CP2	42.6%	+++
4	HC6	21%	++
5	HP6	17%	+

Fig.2: Cholesterol removal by lactic acid bacterial isolates.



Identification of potential lactic acid bacteria isolates:

The five promising isolated strains (CJ3, CC4, CP2, HC6, and HP6) are Gram-positive, catalase-negative, non-spore-forming bacteria that ferment glucose. All isolates have a rod-shaped morphology. The physiological and biochemical characteristics of the selected LAB isolates, as presented in **Table 5**, align with those described in Bergey's Manual of Determinative Bacteriology [30] for the genera *Lactobacillus* and *Lactococcus*. While some Commercial identification systems can provide reliable genus-level identification, but they are often inadequate for accurate species-level identification [31, 32].

Table No. 5: Physiological characteristics of lactic acid bacteria isolates.

Physiological characteristics		Lactic acid bacteria isolates				
		CJ3	CC4	CP2	HC6	HP6
Gram stain		+	+	+	+	+
Catalase production		-	-	-	-	-
Glucose fermentation		+	+	+	+	+
Growth	15^0°C	+	-	-	-	-
	37^0C	+	+	+	+	+
	45^0°C	-	+	+	-	-
NaCl	2%	+	+	+	+	+
	4%	+	+	+	+	+
	6.5%	-	-	+	-	+

MOLECULAR IDENTIFICATION

Molecular methods are crucial for identifying bacteria and may offer greater accuracy

for lactic acid bacteria (LAB) compared to traditional phenotypic methods. In this study, we successfully amplified and sequenced the 16S rRNA from the total genomic DNA of promising lactic acid bacteria (LAB) isolates to facilitate accurate identification. Utilising a universal primer, we generated a robust PCR product of approximately 1200 base pairs (bp). The sequencing data for the purified 16S rDNA from the isolates were utilised for bacterial identification. We aligned the selected isolate sequences with 16S rRNA sequences from the GenBank database to identify the microorganisms under study. The 16S rDNA sequencing data for the most promising isolate, designated CP2, showed a 99% homology with *Lactiplantibacillus plantarum* .

CONCLUSION

In the present study, 80 lactic acid bacterial strains were isolated from various sources, and five were selected for their high antagonistic activity. These five promising lactic acid bacteria (LAB) isolates demonstrated a considerable ability to remove cholesterol. Among them, the isolate CP2, identified as *Lactiplantibacillus plantarum* and sourced from commercial curd, showed the highest cholesterol removal capability at 42.6%. Given its beneficial probiotic properties, this strain could potentially be used in functional food and health products, particularly those aimed at reducing cholesterol. Further in vivo studies are needed to confirm the hypocholesterolemic effect of the isolated *Lactiplantibacillus plantarum*. Additionally, *in vitro* studies are required to understand the mechanisms by which this promising isolate reduces cholesterol.

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