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Thilagavathi P
Post Graduate Student, Department of Microbiology, Valliammal College for Women, Chennai, Tamil Nadu, India

Rekha A
Assistant Professor, Department of Microbiology, Valliammal College for Women, Chennai, Tamil Nadu, India

Soundhari C
Associate Professor, Department of Microbiology, Valliammal College for Women, Chennai, Tamil Nadu, India

Correspondence
Soundhari C
Associate Professor, Department of Microbiology, Valliammal College for Women, Chennai, Tamil Nadu, India

Probiotic and anticancer activity of fermented rice water

Thilagavathi P, Rekha A and Soundhari C

Abstract
Objective: Cancer is a major health concern and it remains one of the main causes of death among human being. Free radicals are known to be the influencing factors for many diseases. The excessive free radical production and low antioxidant defense is caused by oxidative stress, which is harmful and also strongly associated with cancer development. Consuming overnight fermented rice water is a very old practice followed in Tamil Nadu, India. The present study was attempted to investigate the health benefits of Fermented rice water.
Methods: The cooked fermented white rice water (0 hour, 24 hours) samples were used in the study. Isolation, identification of organism was done by morphological tests like gram staining, motility, endospore staining and catalase. The antibacterial activities were performed using agar disc diffusion method against *Escherichia coli, Salmonella typhi, Staphylococcus aureus, Klebsiella punemoniae*. Phenolic compounds were separated using chloroform solvent from fermented white rice water. The separated fractions were used for further studies, and their total phenolic content was determined by qualitative and quantitative methods. The compounds present in the (0 and 24 hours) samples were further analyzed using GC-MS assay.
Results: 0 hour fermented cooked rice water contained ten compounds whereas 24 hours fermented rice water contained twenty three compounds. The fermented cooked rice water was capable of inhibiting proliferation of hepato cellular carcinoma cells with IC_{50} of 125 µg/ml inhibiting 49.41% of liver cancer cells.
Conclusion: The study reports the fermented cooked rice water as natural therapeutic agents with antimicrobial, antioxidant and anticancer activities. Daily intake of this fermented rice water in our diet is beneficial in many ways and would be a natural source of phenolics for common man in developing countries.

Keywords: Fermented rice water, total phenol, antioxidant, anticancer activity, HepG2

Introduction
Cancer is a significant health concern and a major cause of mortality worldwide. Cancer is reported by the American cancer society as the second leading cause of death and is expected to exceed cardiovascular disease in a few years [1]. Genetic defects only account for nearly 5-10% of all cancer cases, whereas 90-95% due to the environment and lifestyle such as race, body mass index, physical activity, smoking and fat consumption [2]. To overcome the failure of chemotherapy there is an urgent need to find an alternative anticancer agent.
The major predisposing factor of a number of diseases, such as coronary heart disease, degenerative disease, and cancer are the free radicals [3]. Oxidation is an important biological process in living organisms but excessive free radical production is harmful to cells, it leads to DNA damage and cancer development. The elimination of destructive free oxygen radicals is done by antioxidants. Many synthetic antioxidants have been reported to cause severe side effects. Thus, alternative antioxidants from a natural source are more preferable. Rice contains antioxidant activity that helps in protecting the body from cell damage caused by free radicals [4].
In diet, the phenolic compounds are essential non-nutrients with poor solubility and less bioavailability. During consumption, these insoluble phenolic compounds undergo transformation by various enzymes in the human intestinal tract and the micro flora in gut also enhances the production of phenols [5]. Phenol compounds inhibit oncogenic signaling and reduce the cell proliferation and death. They also enhance the ROS levels, tumor suppressor proteins and differentiate and transform the cancerous into normal cells.
Epidemiological evidence suggests that human consumption of whole grains foods reduce the incidence of cancer [2] one of the most consumed grains are rice (*Oryza sativa*) and it is

composed of phyto chemicals and nutrients which fights against cancer and increases the immunity.

Fermentation is one of the major methods of adding nutritional values to food. The fermented rice also possesses "probiotics" activity. The lactic acid bacteria present in fermented rice breakdown the anti-nutritional factors in the rice resulting in an improved bioavailability of micro-nutrients and minerals such as iron, potassium and calcium. These activities are enhanced by the phenolic compounds which are p-hydroxybenzoic acid derivative and syringic acids and hydroxycinnamics derivatives present in fermented rice [6]. Consumption of fermented cooked rice water is an indefinitely ancient tradition in Tamil Nadu, India. Hence the present study was done to investigate whether the cooked fermented white rice water has any health benefits like probiotic, antioxidant and anti cancer activity.

Methodology

Preparation of rice sample

The raw white rice (150 gm) was cooked with water for 30 mins, excess water was drained and allowed to cool at room temperature. The cooked white rice (100 gm) was soaked in 500 ml of sterile distilled water [rice: water (1:5) and stored in a container. Overnight fermentation was carried out at room temperature.

Collection of fermented rice water sample

The fermented white rice water sample was collected 0 hour and 24 hours. 50 ml of 0 hour and 24 hours fermented rice water was pipetted out separately into clean glass beakers to characterize the quantitative and qualitative content of phenols. 2 ml from each of the fermented rice water was transferred into clean eppendroff for further organism isolation process for every 24 hours.

Isolation of Lactobacillus

1 ml of fermented rice water sample was taken and plated on MRS agar medium and incubated at 37˚ C for 24 hours. A loopful of selected colonies were restreaked on both Nutrient agar and MRS agar medium plate by quadrant streaking method and incubated aerobically at 37˚ C for 24 hours and observed for the growth of colonies.

Identification of Lactobacillus species

The identification of genus *Lactobacillus* isolates grown on MRS agar was performed with the help of selected following tests [7]:

- Microscopic Examination (Gram staining)
- Biochemical tests - Catalase, Endospore stain and Motility test

Extraction of phenolic compounds

Phenolic compounds from fermented white rice water sample were extracted using chloroform at a ratio of 1:1 (w/v), by the method described by Souza, Oliveira, Rocha and Furlong (2010) with modification[8]. 50 ml of fermented rice water sample was subjected to magnetic stirring at room temperature for 1 hour with chloroform and the extract was kept in the separating flask overnight. The separated chloroform and the samples were collected in two different glass jars for further use.

Determination of total phenolic content

Qualitative analysis

1 ml of sample was taken, to that 3 ml of 10% lead acetate solution was added. Formation of bulky white will confirm the presence of phenol [9]. This was adopted from Raman *et al.,* 2006

Quantitative analysis

The Folin-ciocalteu spectrophotometric method was used for determination of total phenolic content in the fermented rice water sample. 1 ml of fermented rice water sample and 9 ml of distilled water was taken in a 25 ml of volumetric flask. To this mixture, 1 ml of Folin- ciocalteu phenol reagent was added and mixed well. After 5 minutes, 10 ml of 7 % sodium carbonate solution was added and the volume was made to 25 ml with distilled water. And this mixture was incubated with different concentrations of gallic acid (20, 40, 60, 80, and 100 µg/ml) at room temperature for 90 minutes. A standard curve was developed and the absorbance values for both the test and standard solutions were measured using UV/Visible spectrophotometer at 550 nm. Total phenol content was expressed as mg of GAE/gm of sample [10].

Antibacterial activity assay

The antibacterial activity of fermented rice water was determined using standard disc diffusion method on Muller Hinton agar (MHA) medium. 20 µl of sample was added over the disc which was placed on MHA agar medium. And the plates were incubated at 37˚ C for 24 hours. Then the antimicrobial activity was determined by measuring the diameter of zone of inhibition.

Antioxidant activity

Ferric Reducing Antioxidant Power (FRAP)

Ferric to ferrous ion reduction at low pH causes a coloured ferrous- tripyridyltriazine complex form. FRAP values were obtained by comparing the absorbance change at 593 nm test reaction mixtures with those containing ferrous ion in known concentrations. Each sample dilution was tested in triplicates, based on the results a mean absorbance was calculated. FRAP value of sample (µM) = (change in absorbance of sample from 0 to 4 minutes/change in absorbance of standard from 0 to 4 minutes) x FRAP value of standard (1000 µM) [11].

Gas Chromatography-Mass Spectrometry

In order to identify the chemical constituents, present in the extracts of "0 hours fermented rice water" and "24 hours fermented rice water", the extracted fractions were subjected to GC-MS.

In vitro assay for anti cancer activity

Anticancer activity of fermented rice extracts was evaluated on hepatocellular carcinoma cells (HepG2) obtained from the National Centre for Cell Sciences, Pune (NCCS). The cells were cultured in DMEM medium, supplemented with 10% fetal bovine serum, penicillin (100 µg/ml), and streptomycin (100 µg/ml). The cells were maintained under 95% humidified incubator at 37° C in a 5% CO_2. *In vitro* anti cancer activity was estimated by MTT assay. The rate of absorbance was measured at 570 nm using UV-Spectrophotometer. Measurements were performed and the concentration required for a 50% inhibition (IC50) was determined graphically. The percentage of cell viability was calculated using the following formula:

Cell viability % = ODt of treated cells / ODt of control cells × 100

Graphs were plotted using the percentage of Cell Viability at Y-axis and concentration of the sample at X-axis. Using Cell control and sample control in each assay, the cell viability assessments were compared [12].

Results

Isolation of Lactobacillus from fermented rice water

Colonies observed on the surface of MRS agar petri plates and different types of colony morphology (i.e., color, size, margin and shape of the colony) were seen. After repeated sub culturing, the *Lactobacillus* was identified based on the morphological examination. Gram's Positive, catalase negative, nonmotile, non sporing rods were obtained.

Determination of phenol

Qualitative analysis

A bulky white was formed when the lead acetate solution was mixed with the 0 and 24 hours fermented rice water sample. This indicated that the samples were containing phenolic compounds.

Quantitative Analysis

In this study, the total phenolic compound (TPC) contents were estimated using modified Folin-Ciocalteu method and it was expressed as mg of gallic acid equivalents (GAE) per g. The absorbance was measured at 550 nm for 0 hour and 24 hours fermented rice water samples. The results shown that the 24hrs sample had increased TPC of 3µg/mL compared to 0 hrs which had 2.0 µg/mL.

Antibacterial activity

The 24 hours fermented rice water sample showed antibacterial activity against both Gram positive and Gram-negative bacteria. The zone of inhibition was seen only for *Staphylococcus aureus* (15mm), and *Klebsiella pneumoniae* (13mm). However, *E. coli* was not found to be inhibited (table 1)

Table 1: Antibacterial activity of 24 hours fermented rice water extract

Organism	Concentration of the extract(µg/ml)	Zone of inhibition(mm)	Standard antibiotic(mm)
Escherichia coli	20	Nil	32 mm
Klebsiella pneumonia		13 mm	15 mm
Staphylococcus aureus		15 mm	34 mm
Salmonella typhi		Nil	17 mm

Antioxidant activity

Ferric Ion Reduction Potential (FRAP)

The reducing ability of fermented rice water sample was in the range of 110 and 105 µm of Fe (II)/g for 0 and 24 hours of fermented rice water sample. The antioxidant potentials of fermented rice water sample were estimated from their ability to reduce 2, 4, 6-tripyridyl-s-triazine (TPTZ)-Fe (III) complex to TPTZ-Fe (II) (Table 2).

Table 2: The Antioxidant activity of given sample using FRAP assay method

Name of sample	Absorbance at 593 nm		FRAP (µm)
	0 min	4 min	
0 hour fermented rice water extract	0.003	0.025	110
24 hour fermented rice water extract	0.007	0.028	105

GC-MS Analysis

Twenty three compounds were found in the GC MS analysis of the fermented rice after 24 hours whereas, only ten compounds were identified by GC-MS analysis in zero hour, that was before fermentation. The active principles with their retention time (Rt), molecular formula, molecular weight and concentration percentage (area %) are represented in Figs (1) and Tables (3).

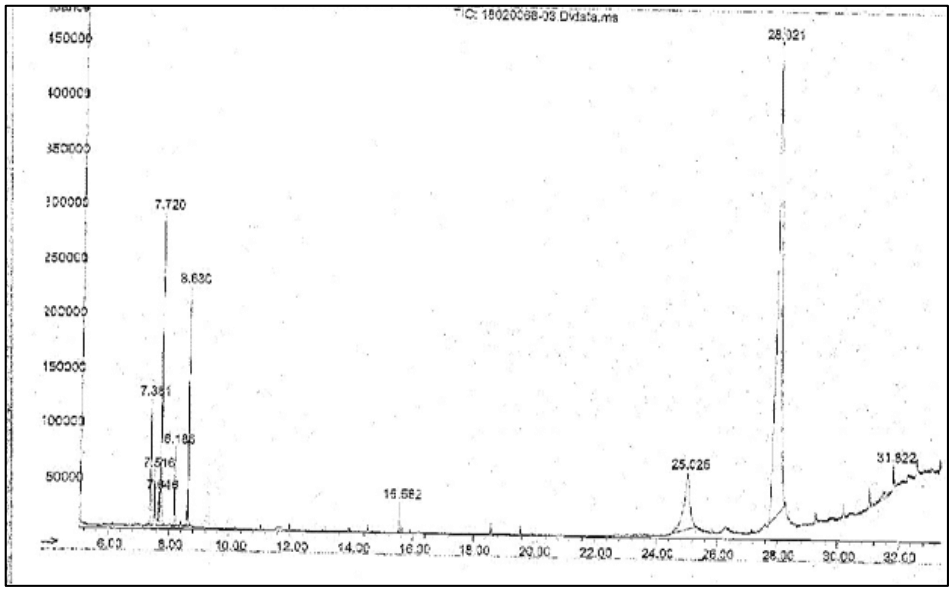

Fig 1: Compounds identified in the 0 hours fermented rice water by GC-MS

Table 3: Compounds identified in the 0 hours fermented rice water by GC-MS

No	Rt	Area %	Compounds name
1	7.382	2.59	(+) -3- Carene
2	7.515	1.17	(+) -4- Carene
3	7.649	0.77	Benzene, 1-methyl-3-(1-methylethyl)-
4	7.720	6.75	D-Limonene
5	8.187	1.73	.gamma, -Terpinene
6	8.630	5.59	Cyclohexene, 1-methyl -4- (1-methylethylidene)-
7	15.563	1.03	Diethyl phthalateP
8	25.025	10.76	Benzaldehyde, 2,4,5-trimethyl-
9	28.020	68.64	Propanamide, 2,2-dimethyl –N- (4-methylphenyl)-
10	31.820	0.97	Tetrasiloxane, decamethyl-

The major compounds which were reported in the fermented rice after 24 hours and identified by GC-MS were 1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester at Rt 19.592 (39.28%), silane, dimethyldecycloxyhexadecyloxy 3a-Methyl-6-((4-methylphenyl) sulfonyl) at Rt 33.373 (17.73%), Hexadecanamide at Rt 23.492(6.79%), Hexadecane at Rt 15.663(2.84%), squalene at Rt 28.416(2.63) Figs (2) and Tables (4).

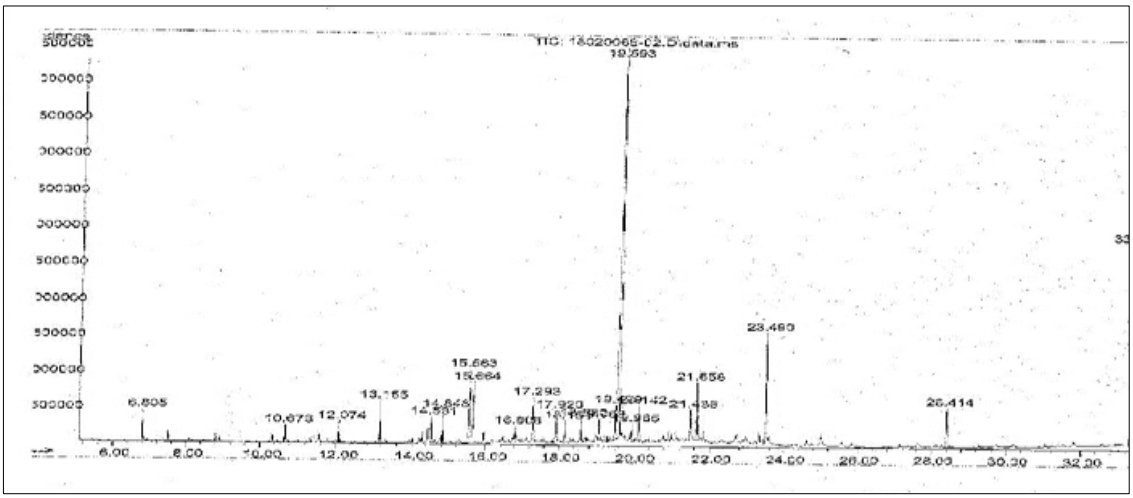

Fig 2: Compounds identified in the 24 hrs rice water by GC-MS

Table 4: Compounds identified in the 24 hrs fermented rice water by GC-MS

No	Rt	Area%	Compound name
1	6.806	1.52	Phenol
2	10.673	0.75	Ethanol, 2-phenoxy-
3	12.073	0.94	Pentadecane octane, 5-ethyl-2-methyl-dodecane,
4	13.154	1.90	Tetradecane
5	14.530	1.21	Phenol, 2,4-bis(1,1-dimethylethyl)
6	14.849	1.67	Pentacosane
7	15.563	4.06	Diethyl phthalate
8	15.663	2.84	Hexadecane
9	16.806	1.12	Hexadecane
10	17.292	2.39	Tridecane, 3-methyl-
11	17.920	1.42	Octadecane
12	18.163	1.12	Isopropyl myristate
13	18.582	1.28	1,2-Benzenedicarboxylic acid,
14	19.063	1.12	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione
15	19.501	1.94	Octadecane
16	19.592	39.28	1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester
17	19.963	0.92	Eicosane
18	20.140	1.99	3-Acetylphenanthrene
19	21.487	2.18	Hentriacontane
20	21.659	3.19	Hexadecanamide
21	23.492	6.79	Hexadecanamide
22	28.416	2.63	Squalene
23	33.373	17.73	Silane, dimethyldecycloxyhexadecyloxy 3a-Methyl-6-((4-methylphenyl) sulfonyl)

Anticancer activity

The fermented cooked rice water extract showed a potent anticancer activity against HepG2 cells, which could inhibit the proliferation of HepG2 cells till a concentration of 62.5µg/ml. The fermented cooked Rice water extract inhibited the proliferation of HepG2 cells significantly in a dose dependent manner from 1000 µg/ml (29.68%), 500 µg/ml (35.26%), 250µg/ml (42.76%) and 125 µg/ml (49.41%) respectively compared to positive control.

The IC_{50} value of the fermented rice water extract after 24 hours was found to be 49.41% at 125 µg/ml concentration. It shown that rice water extract showed a significant anticancer effect (Table 5)

Table 5: Anticancer effect of sample on HepG2 cell line

S. No	Concentration (µg/ml)	Dilutions	Absorbance (O.D)	Cell viability (%)
1	1000	Neat	0.277	29.68
2	500	1:1	0.329	35.26
3	250	1:2	0.399	42.76
4	125	1:4	0.461	49.41
5	62.5	1:8	0.512	54.87
6	31.2	1:16	0.584	62.59
7	15.6	1:32	0.648	69.45
8	7.8	1:64	0.701	75.13
9	Cell control	-	0.933	100

Discussion

Cancer is caused by both exogenous factors (such as poor diet, smoking, radiations etc.) and endogenous factors (inherited mutants, radiation and immune conditions). According to the online database by the International Agency for Research on Cancer (IARC), 14.1 million new cancer cases were reported in 2012 worldwide. The global incidence of cancer is expected to increase to 22.2 million by 2030 [13]. Natural product research for cancer treatment is one new strategy due to its effectiveness against cancer cells and the fact it is harmless to normal cells [14].

Production of free radicals is part of metabolism. The accumulation of excessive free radicals leads to oxidative stress, this may occur because of imbalance between oxidants and antioxidants. This free radical may damage cells and leads to numerous diseases [15].

Experimental studies have shown that phenolic compounds in particular exhibit anticancer properties; the entire mechanism involved is as yet unclear. The mechanisms that have been proposed in the context of the anticancer effect of phenolic compounds include their ability to scavenge free radicals, their ability to induce xenobiotic metabolism, their ability to regulate gene expression, and their ability to modulate DNA repair, apoptosis and cell signaling, such as cell replication and invasion [16].

Phenolic compounds are highly beneficial to human beings in many ways due to their antioxidant activities. Phenols are highly available in various sources such as plants, vegetables, fruits. Cereals, legumes, soybeans, coffee, tea, rosemary and thyme were reported as a rich source of phenolic acids [17]. Others reported that herbs like thyme, oregano; fruits like berry, apple, pears and citrus family also contain phenolic acids. Among vegetables potatoes, red cabbage and cherry tomatoes are highly rich in phenolic acid. But the cost of these is a major concern for common man in developing countries.

There are several reports available for fermented rice bran and unfermented rice water contains phenolic acids which acts as an antioxidant and also effective on various cancer cell lines. But there are no current literature available related to our research.

Microorganism found in the fermented rice water was *Lactobacillus*, which was isolated and characterized. The antimicrobial activity of 24 hour fermented rice water was determined using disc diffusion method against intestinal bacteria. Fermented rice water extract showed complete inhibition of *Klebsiella pneumoniae* as that of standard and *Staphylococcus aureus* showed moderate inhibition. The significant finding is that the fermented rice water extract did not inhibit *Escherichia coli* which are a normal intestinal flora. Justifying that consumption of fermented rice water will not disturb the normal microflora.

The level of phenolic compounds in the chloroform extract of the (white) cooked fermented rice water is given in graphical representation. The total phenolic content was determined using Folin-ciocalteu method and expressed as mg of gallic acid equivalents (GAE) per g. The total phenolic content of 0, 24 and 48 hours are gradually increasing like 2.0 µg/ml, 3 µg/ml and 8 µg/ml. Gradual increase were observed for 72 hours of fermentation, the decreased in the phenolic content after that period probably resulted degradation. Other studies reported that, phenolics are found in plants which are powerful chain-breaking antioxidants with the ability to stabilize lipid peroxidation [18].

The demonstration of FRAP assay showed reducing antioxidant power capacity. The antioxidant activity is because of the mechanisms such as prevention of chain inhibition, the binding of transition metal ion catalysts, the decomposition of peroxides, reductive capacity and free radical scavenging [18].

The GC-MS results showed that 0 hours fermented rice water had 10 compounds whereas 24 hours fermented rice water sample had 23 compounds. Three major compounds of 24 hours sample were 1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester at Rt 19.592 (39.28%), silane, dimethyldecycloxyhexadecyloxy 3a- Methyl-6-((4-methylphenyl) sulfonyl) at Rt 33.373 (17.73%), Hexadecanamide at Rt 23.492(6.79%), Hexadecane at Rt 15.663(2.84%), squalene at Rt 28.416(2.63). The present study revealed that 1, 2-Benzenedicarboxylic acid, mono (2-ethylhexyl) ester are known to be the main compound which has an anticancerous activity against HepG2 cancer cell line. Hexadecane acid has reported to have antibacterial, antifungal, and antioxidant activities.

Human hepatocellular carcinoma cell HepG2 is one of the most pharmaceutical frequently used experiment models for *in vitro* studies on liver cancer. Anticancerous activity revealed that fermented rice water is capable of inhibiting proliferation of hepatocellular carcinoma with IC_{50} = 125 µg/ml where 49.41 % of liver cancer cells were inhibited. Hence cooked fermented rice water can be considered as potentially important source of original bioactive anticancer compounds, these compounds are effective for variety of clinical application without causing any side effects.

This study revealed that fermented rice water may be effective when consumed without creating any microbial imbalance of normal flora in the gut. Cooked fermented rice water can be considered as a probiotic, as probiotics are defined as substances that when administered gives a health benefit to the host. The present study has also revealed that cooked fermented rice water has potential anticancer effect on liver cancer cell lines (HepG2).

Conclusion

The systemic investigation revealed that cooked fermented rice water could be a good source of natural therapeutic agents with antimicrobial, antioxidant and anticancer activities. The phenolic acids present in fermented rice water are rich in antioxidant, antibacterial and anticancerous substances. They also acts against pathogenic microflora present in human intestine without disturbing the normal microflora. Daily intake of this fermented rice water in our diet is beneficial in many ways and would be a natural source of phenolics for common man in developing countries.

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Conflict of interest: None declared.

Authors Contribution

The actual laboratory work was carried by Thilagavathi. P supervised by Ms. Rekha. The manuscript was designed by the authors and carefully reviewed by corresponding author.

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