Chapter 6

Extractions of antimicrobial biometabolites and antioxidant extract from different biological sources
6.1 Introduction

Lactic acid bacteria (LAB) are widespread and found commonly in nature, therefore there is an increased interest in this bacterium, for its ability to biopreserve. Several studies have been directed at identifying LAB that produces these bioactive antimicrobial metabolites, which may be used to control the growth of pathogenic and food spoilage microbes in food products.1-3 LAB isolated from different sources have shown probiotic potential4-5 and inhibitory effects on various pathogenic bacteria in the genera Enterococcus, Salmonella, and Staphylococcus.6-8 Production of organic acids, undissociated organic acid molecules, bacteriocins, the competition for adhesion sites and coaggregate with pathogens are some of the several mechanisms, which have been suggested for the antimicrobial activity of lactic acid bacteria towards pathogens.7,9 It has also been suggested that the antimicrobial activity, may also be due to other inhibitory substances.10-11 There are also some reports where several authors have suggested that production of organic acids are the sole reason for the antimicrobial activity of lactic acid bacterial strains.12

Khorisa, a traditional fermented bamboo shoot product made by the indigenous people of Assam in the Northeastern regions of India. Khorisa is made by the lactic acid fermentation of fresh bamboo shoots during the monsoons. The fermented bamboo shoot product has good antibacterial and antifungal properties,13 and the product can be stored and used up to six months to a year, without the addition of food preservatives. LAB isolated from Jiang-sun, a fermented bamboo shoot product from Taiwan, has shown bacteriocin producing capabilities.14 The dominant lactic acid microflora in khorisa might also be responsible for the antimicrobial property.

Antioxidants are molecules that inhibit the oxidation of other molecules. Antioxidants are primarily composed of thiols, ascorbic acid or polyphenols.15 They tend to minimize DNA damage, protein oxidation and lipid peroxidation in living cells caused by free radicals and various redox reactions.16 There is an increased awareness and interest in the use of natural antioxidants. Synthetic antioxidants and preservatives are used in food industries to prolong shelf life of foods. These synthetic additives are found to cause nutrition losses, and produce toxic effects.17 Worldwide there have been
numerous steps to substitute all synthetic antioxidants with natural ones. Many antioxidants are being identified as anticarcinogens and cardio-protective.\textsuperscript{18-20} Neutraceutical properties of antioxidants have sparked immense interest among researchers to search plant sources for identifying antioxidants.\textsuperscript{21-22}

*Garcinia pedunculata* Roxb. (GPR) is a globose shaped fruit with fleshy aril, found mostly in the states of Northeast India. It belongs to the genus *Garcinia* and family Clusiaceae (or Guttiferae). The mature fruit is eaten cooked or raw\textsuperscript{23} and is locally known as “Borthekera” in Assam, a Northeastern State of India. The fruit usually matures during the month of April and is collected, cut into small pieces and sun dried. Dried pieces of the fruit are stored and used by the indigenous people throughout the year. The indigenous people of Northeast India use it for various medicinal uses. The water extract of the dried pellets of GPR are used as antidiarrhoeic and antidysentric\textsuperscript{24} and it is rich in benzophenones, pedunculol, garcinol and cambogin.\textsuperscript{25} High antioxidant activity has been reported in GPR by Gogoi et al.\textsuperscript{26} and Mudoi et al.\textsuperscript{27} GPR is a rich source of secondary metabolites including xanthones, flavonoids, benzophenones, lactones and phenolic acids with wide range of biological and pharmacological activities.\textsuperscript{28-29}

Microwave assisted extraction (MAE) is a new green extraction technique that combines microwave radiation and traditional solvent extraction methods. It is a simple, cheap procedure than solvent extraction method, and also has less polarity limitations for the extractant. It offers higher degree of reproducibility, simplified manipulation, shorter extraction time, lesser use of solvent, and high extraction rate compared to conventional solvent extraction methods.\textsuperscript{30} Conductive and convective processes to heat the sample is used in conventional solvent extraction methods, whereas microwave heating occurs by direct energy transfer to the sample.\textsuperscript{31-32} Microwave heating is volumetric in nature so microwave irradiation efficiently produces internal heating by coupling microwaves with polar components inside the solvent and the sample. According to the cell-wall broken theory,\textsuperscript{33} there are certain solvents which are microwave transparent, while some are microwave absorbing. By using microwave transparent solvents, there is more energy for the plant material to absorb. Cellular
structures contain water, which absorbs the microwave energy. This creates a sudden increase in temperature, and results in the rupture of the cell wall, and release of constituents into the surrounding solvent. Several studies have also used non-polar solvents which are transparent to microwave and in these cases only the sample matrix gets heated leading to release of analytes in a cold solvent. This shows the higher extraction of polyphenolic compounds in acetone, compared to methanol, ethanol or water. Higher extraction of polyphenolic compounds was observed, when solvent polarity was modified by addition of water in the solvent. Microwave extraction shows promising advantages over conventional solvent extraction system and is an efficient method for extracting active biological compounds. Polyphenolic compounds from waste peanut shells, grape seeds, citrus mandarin peels and tea leaves have also been successfully extracted by MAE technique.

The objective of the present work is two folds. First the extractions of secondary metabolites from L. plantarum isolated from khorisa having antimicrobial properties. Various solvents viz., ethyl acetate, petroleum ether, ethanol, chloroform etc. have been used for the purification of antimicrobial biometabolites. Secondly to study the feasibility of microwave-assisted extraction (MAE) for the extraction of antioxidants extract from Garcinia pedunculata Roxb. (GPR). Box–Behnken design (BBD) combined with response surface methodology (RSM) was employed to analyze the interaction among the MAE operating factors. The effect of time of microwave bombardment on micro structural changes in plant material was also observed.

6.2 Materials and methods

6.2.1 Materials

The biometabolites were extracted from previously isolated Lactobacillus plantarum (identified by sugar fermentation and biochemical characterization) form fermented bamboo shoot product of Assam (khorisa). The L. plantarum was cultured in MRS broth (HiMedia Labs., India) under anaerobic condition at 37°C. Indicator bacteria used for antimicrobial assays viz., Escherichia coli MTCC 443, Streptococcus aureus MTCC 740 and Bacillus cereus MTCC 430 were collected from the Dept. of
Food Engineering & Technology and Dept. of Molecular Biology and Biotechnology, Tezpur University (India). They were appropriately sub-cultured and used throughout the study.

For the extraction of antioxidant extract, fresh mature fruits of GPR were collected from Nagaon district of Assam, a Northeastern State of India. The exocarp and the mesocarp of the fruit were used for the purpose of study. They were cut into small pieces, washed and dried in a cross airflow tray drier (IKON, India) at 40 °C for 24 hours. 1, 1-diphenyl-2-picryl hydrazyl (DPPH) and 2, 2'-azino-bis, 3-ethylbenzo thiazoline-6-sulphonic acid (ABTS) were obtained from Merck, India. HPLC grade 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) standard was obtained from Sigma Aldrich, USA. Absolute ethanol used for microwave extraction and all other chemical reagents and solvents used in experiments were of analytical grade purchased from Merck (India), and double distilled water was used throughout the experiment.

6.2.2 Antimicrobial activity and growth kinetics of *L. plantarum*

The *L. plantarum* was tested for antibacterial activity against *Staphylococcus aureus* by agar overlay method in nutrient agar. Nutrient agar was supplemented with dextrose (20g/lt.), polysorbate 80 (1g/lt.), ammonium citrate (2g/lt.), sodium acetate (5g/lt.), magnesium sulphate (0.10g/lt.), manganese sulphate (0.05g/lt.) and dipotassium phosphate (2g/lt.) for proper growth of *L. plantarum*. Final pH of the medium was adjusted to 7±0.5 at 25°C (De Man et al. 1960) and zone of inhibition was noted.

MRS broth (50 ml) was taken in 11 different Erlynmeyer flasks to study the Growth kinetics of *L. plantarum*. Ten flasks were inoculated with 1 ml of 24 h old culture of *L. plantarum* at concentration of 7.68 McFarland, and 1 flask was kept as blank. The flasks were incubated at 37°C at 100 rpm in a shaking incubator (New Brunswick Scientific, USA), till stationary phase was achieved. Each flask was taken out at intervals of 6 h and absorbance was measured in a spectrophotometer (Spectronic 20D+, ThermoFisher Scientific, USA) against blank medium at 600 nm and the growth curve was plotted between absorbance and time.
6.2.3 Production and purification of biometabolites

Submerged aerobic fermentation technique was used for the production of biometabolites using *L. plantarum*. Two sets of 50 ml MRS broth in Erlenmeyer flasks were taken and each was inoculated with 24 hour old culture of *L. plantarum* (1 ml) at concentration of 7.68 McFarland. The flasks were kept for fermentation at 37ºC at 100 rpm till the pre-stationary period in a shaking incubator. The pre-stationary period was decided on the basis of growth curve of *L. plantarum*. The flasks were removed from the incubator and stored at 4ºC for further processing.49

Cell-free crude supernatants (CFCS) were obtained by centrifugation (4000×g, 4ºC, and 20 min) of fermented broth. The supernatant was filtered through a 0.22µm cellulose acetate filter to remove residual cells. As the nature of the metabolite was unknown, three organic solvents viz., chloroform, hexane and petroleum ether were used for the extraction of the unknown biometabolites from the CFCS, on the basis of maximum solubility in solvent phase and maximum antimicrobial activity. CFCS (5ml) was taken in screw cap non-reactive teflon tubes, and mixed with equal quantities 1:1 (v/v) each with chloroform, hexane and petroleum ether. The tubes were kept horizontally for 4 h in a shaker (180 rpm at 25 ºC). After this incubation, the solvent phase was separated and evaporated at 40ºC in a vacuum oven kept at 650mm Hg for 24 h.49 The weight of dried metabolites was noted and it was re-dissolved with 1ml of sterile double distilled water, so that the final concentration of the metabolite was known in g/ml equivalent. This was considered as cell-free purified supernatants (CFPS).

6.2.4 Antimicrobial activity of biometabolites

To neutralize the organic acid function on the antimicrobial activity, 1 ml of CFCS was adjusted to pH 6.5±0.2, using 1M NaOH solution. In order to test the heat sensitivity, 1 ml CFCS of the *L. plantarum* was heated to 100ºC in a water bath for 15 min. Agar well diffusion assay50 with slight modification was used for analyzing antibacterial activity of CFCS, CFPS, organic acid neutralized CFCS and heat treated
CFCS. Nutrient agar plates were prepared by inoculating the molten agar (at ~50°C) with 1:100(v/v) 24 h cultures of the indicator food contaminating strains *E. coli*, *S. aureus* and *B. cereus* at concentration of 6.50 McFarland. Sterile cork borer was used to dig wells of 8 mm diameter in the agar plates. CFCS, CFPS, organic acid neutralized CFCS and heat treated CFPS (100µl each) were loaded on to the wells. Also, 100µl of standard antibiotic tetracycline (25 µg/ml) was used as the positive control, and sterile water was used as the negative control. After loading the plates were kept at 4 °C for 4 hours, for the preparations to diffuse from the wells into the agar. The plates were then placed in an incubator for 24 h at 37 °C. The ability to inhibit growth (−, +, ++, ++++) of the indicator strains was observed.

### 6.2.5 Analysis of organic acid composition in CFCS by HPLC

CFCS was taken and an equal volume of 20% (v/v) trichloroacetic acid was added to remove proteins. After centrifugation (10,000×g, 15 min), 1 ml supernatant was mixed with 5 ml 5 mM H$_2$SO$_4$. Finally, the mixture was filtered through 0.22 µm cellulose acetate membrane filter. Organic acid concentration in the crude extract was measured by high performance liquid chromatography (HPLC, Dionex Ultimate 3000, Germany). Acid separation from CFCS was performed by purifying the CFCS via means of solid phase extraction using Sep-Pak C18 Plus cartridges (Waters, Milford, MA, USA). Organic acids were quantified using UV detection of 210 nm, on Hamilton Organic Acid C18-column and mobile phase was 0.2M sodium sulphate solution, adjusted with methane sulphonic acid to pH 2.68. Standard acids were used for analysis are oxalic acid, tartaric acid, formic acid, pyruvic acid, lactic acid, acetic acid, citric acid, succinic acid and propionic acid (Sigma, USA). Acid identification was performed by comparing the retention times of the samples with that of the standards of organic acid.51
6.2.6 Effect of biometabolites on minimum lethal dose concentration (LD$_{\text{min}}$) and death rate kinetics of test bacterial strains

Two-fold micro broth dilution technique was used to determine the minimum lethal dose concentration using the standard procedure$^{52}$ with slight modification. Nutrient broth (5 ml) was added to each of the tubes. The final concentration of the CFPS chloroform fraction was adjusted to 54, 27, 13.5, 6.75, 3.37, 1.68, 0.84, 0.42, 0.21, 0.10 mg/ml concentration in the tubes accordingly. An inoculums suspension of 20µl for 24 hour old culture of the indicator strains concentration of 6.50 McFarland was added to each of the tubes. Tetracycline (25 µg/ml) was used as positive control, and water was used as negative control. The tubes were incubated at 37 ºC for 24 h, and then visually checked for turbidity (visible bacterial growth). The lowest concentration at which turbidity was inhibited was recorded as the LD$_{\text{min}}$ for respective indicator strain.

Death rates kinetics of test bacterial strains cultured in nutrient broth supplemented with CFPS chloroform fraction equivalent to LD$_{\text{min}}$ of respective pathogen was studied. Nutrient broth (5 ml) was inoculated with 100µl of the test strain concentration of 6.50 McFarland. The tubes were incubated till 24 hours at 37ºC in an incubator (New Brunswick Scientific, USA). The inoculated broth (100µl) was plated on nutrient agar plates at intervals of 0, 2, 4, 8, 10, 12, 16, 20 and 24 h respectively, and incubated at 37 ºC for 36-48 h. The cell count was taken as log cfu/ml.

6.2.7 Optimization of microwave assisted extraction process of antioxidant extract

The extractions were carried out in a domestic microwave oven (Samsung, India). The dried pieces of GPR were crushed and finely ground. The fine grinding was carried out in order to increase the surface area of contact with solvent. Powdered GPR (5 g) was weighed into a flat-bottomed flask. The flask was maintained with varying solvent (absolute ethanol) concentration (50-100%) and solvent to sample ratio (10:1-20:1). It was kept at room temperature for 90 minutes for leaching of the solvent into the sample,$^{36}$ and then microwave-irradiated at 180 Watt on a carousel for defined time
(4–10 min). The suspensions were irradiated with microwaves for 1 min (heating to the desired temperature about 65-70 °C) and cooling to room temperature (30±1 °C) in water bath and this cycle is continued to the pre-set extraction time. The lower microwave power (180 W) was selected for treatment to avoid boiling of solvent; as at higher power excessive boiling and evaporation of solvent was observed. However the flask was covered loosely with polyethylene to avoid evaporative loss. After microwave irradiation, the residues were then filtered and stored at 4 °C.

6.2.8 Experimental design for microwave assisted extraction

A three-variable, three-level Box–Behnken design (BBD) was applied to determine the best combination of extraction process variables for achieving higher antioxidant activity of extract obtained from GPR.53-54 The three independent variables set were solvent concentration (%, X1), solvent to sample ratio (v/w, X2) and irradiation time (min, X3), and each variable set at three levels. The independent variables were coded at three levels and their actual values selected on the basis of preliminary experimental results. Antioxidant activity of extract in terms of DPPH radical scavenging activity (DPPH RSA) and ABTS radical scavenging activity (ABTS RSA) were taken as responses. The coded and uncoded (actual) levels of the independent variables are given in Table 6.3. Experiments were augmented with 5 replications at the center point to evaluate the pure error. RSM was applied to the experimental data using a commercial statistical package, Design-Expert version 6.0.11 (Stat-Ease, Inc., Minneapolis, MN, USA). The experiments were conducted randomly to minimize the effects of unexplained variability in the observed responses as a result of external factors. Regression analysis for the experiment data was performed, and was fitted into the empirical second order polynomial model (Eq. 6.1).

\[ Y = a_o + \sum_{i=1}^{n} a_i x_i + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} a_{ij} x_i x_j + \sum_{i=1}^{n} a_{ii} x_i^2 \]  

(6.1)

Where, \( a_o \), \( a_i \), \( a_{ii} \) and \( a_{ij} \) are the regression coefficients and \( x_i, x_j \) are the coded levels
of independent variables $i$ and $j$. Model adequacy was evaluated using F ratio and coefficient of correlation ($R^2$) represented at 1, 5 and 10 % level of significance accordingly.

### 6.2.9 DPPH radical scavenging activity of antioxidant extract

Free radical scavenging activity was used to measure the total antioxidant activity by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay method. DPPH is a commercial oxidising radical used to be reduced by anti-oxidants. The disappearance of the DPPH radical absorption at a particular wavelength was monitored by the reduction in optical density. To 20µL methanolic extract of GPR, 1.5 ml of DPPH solution (0.025g DPPH in 1000 ml of methanol) was added. The tubes were vortexed (Vortex Shaker, Labtech, Korea) for proper mixture and allowed to react for 45 minutes in a dark environment at room temperature. The control was prepared by adding distilled water instead of GPR extract for baseline correction. Absorption was measured at 517nm in a spectrophotometer (Spectronic 20D+, Thermo Scientific, USA). The Free radical scavenging activity was expressed as inhibition percentage and calculated by using the following equation (Eq. 6.2).\(^55\)

\[
\% \text{ Free radical scavenger activity} = \frac{[(\text{Control absorbance} - \text{Sample absorbance})]}{\text{Control absorbance}} \times 100
\]  
\[\text{Eq. 6.2}\]

### 6.2.10 ABTS radical scavenging activity of antioxidant extract

The method is based on the ability of antioxidant molecules to quench 2, 2’-azino-bis, 3-ethylbenzo thiazoline-6-sulphonic acid (ABTS) radical, a blue-green chromophore with characteristic absorption at 734 nm, compared with that of trolox, a water-soluble vitamin E analog. The addition of antioxidants to the preformed radical cation, reduces it to ABTS radical resulting in decolorization. A stable stock solution of ABTS was prepared by the reaction of a 7 mmol/l aqueous solution of ABTS with 2.45 mmol/l potassium persulfate (final concentration) and allowing the mixture to stand in
the dark at room temperature for 16 h before use.\textsuperscript{56} The ABTS working solution was obtained by the dilution of the stock solution in ethanol to an absorbance of 0.70±0.02 AU at 734 nm. 2 ml diluted ABTS solution was added to the appropriately diluted fruit residue extracts. The contents were mixed well and absorbance was read after 2 min after mixing. The percentage inhibition was calculated against trolox standard curve prepared using 0-2.5 mM trolox and the results were expressed as mg-trolox equivalent /g dry weight.

\textbf{6.2.11 FTIR spectroscopy analysis}

The infrared spectra of untreated GPR powder and MAE treated residue were obtained with a FTIR spectrophotometer (Perkin Elmer, USA). MAE was carried out at optimized values of solvent concentration and solvent to sample ratio; however, irradiation time were varied at three different levels. The equipment was operated with scanning range of 4000–450 cm\textsuperscript{-1} and spectrum of 100. Sample (clear glassy disk) for FTIR analysis was prepared by mixing powder sample with IR grade KBr using a suitable pressure of 12,000 psi.

\textbf{6.2.12 Microscopic observation}

The cellular microstructures were observed for untreated GPR and MAE treated GPR slices, in order to check for damage in the cellular structure (mesocarp and exocarp) and parenchyma, as proposed by the Broken cell wall theory,\textsuperscript{39} which would in turn facilitate the release of chemical substances inside the cells, into the surrounding solvent. Microscopic observations were carried out using a trinocular microscope (DM 3000, Leica Microsystems, Wetzlar, Germany) equipped with a CCD camera (Leica DFC 440C) and Leica Application Suite (LAS) software on thin slices of untreated GPR and MAE treated GPR. The slices were stained with 0.1% methylene blue solution for a short time and then washed to remove the excess stain. Stained slice was covered with cover slip and checked under the microscope at a magnification of 40X.\textsuperscript{57}
6.2.13 Statistical analysis

All the experiments used for biometabolites extraction were performed three times independently and each assay was performed in duplicate. Results were expressed as means ± standard deviation. The level of significance was analyzed by ANOVA (P < 0.05). RSM was applied to the experimental data of MAE using a commercial statistical package, Design-Expert version 6.0.11 (Stat-Ease, Inc., Minneapolis, MN, USA).

6.3 Results and discussion

6.3.1 Antimicrobial activity and growth kinetics of *L. plantarum*

The *L. plantarum* were tested for antibacterial activity against *Staphylococcus aureus* by agar overlay method in nutrient agar. A large zone of inhibition was recorded, which show the strong antimicrobial activity of *L. plantarum* against *S. aureus*. The fermentation growth curve of the strain was studied in order to have an idea of the period when it reaches its stationary phase, as this phase is considered to give maximum yield of secondary biometabolites. The growth curve of *L. plantarum* was plotted between absorbance of MRS broth inoculated and time (Fig. 6.1). Stationary phase was achieved at 24th h of incubation.

![Growth curve of L. plantarum isolated form fermented bamboo shoot. Vertical bars represent standard errors of means, n = 3.](image)

**Fig. 6.1** Growth curve of *L. plantarum* isolated form fermented bamboo shoot. Vertical bars represent standard errors of means, n = 3.
6.3.2 Purification of biometabolite and its antimicrobial activity

Bacterial metabolite was extracted in organic solvents, as it gives higher yields compared to other techniques. Three organic solvents viz., chloroform, hexane and petroleum ether were used for the extraction and concentration of biometabolites from the CFCS, on the basis of maximum solubility in the solvent and antibacterial activity. The yield of biometabolite was found to be highest in the chloroform extract (0.054g/ml) followed by hexane (0.052g/ml). However, petroleum ether fraction shows the lowest yield (0.026g/ml).

CFCS showed antimicrobial activity against all the three test pathogens by the agar well assay (Fig. 6.2). The pH of the CFCS was found to be 4.2 at the pre-stationary phase. When the CFCS was adjusted to pH 6.5, the antimicrobial activity slightly diminished. This might be an indicator of organic acids presence in CFCS. The diminishing of antimicrobial activity upon adjustment of pH might be due to the negation of the inhibition effects of acids upon the test pathogens. The same cause can also be attributed to the slight loss of antimicrobial activity in the CFPS fractions of chloroform and hexane. The purified extracts, most noticeably the chloroform extract showed good inhibition against the test pathogens. The hexane fraction showed positive inhibition against *S. aureus* and *B. cereus*, but did not show any inhibition against *E. coli*. The petroleum ether fraction did not result in positive inhibition of any of the test pathogens. However, the antimicrobial activity totally diminished after heat treatment of the CFPS (Table 6.1).
Fig. 6.2. Inhibition zones of different extracts against (a) *E. coli*, (b) *S. aureus* and (c) *B. cereus*

Table 6.1. Antimicrobial activity of different extracts against test pathogens*

<table>
<thead>
<tr>
<th>Indicator Strain</th>
<th>Inhibition by CFCS (pH 6.5±2)</th>
<th>Inhibition by CFPS</th>
<th>Inhibition by CFPS (Heat Treated (100ºC for 15 min))</th>
<th>Inhibition by Tetracycline (Positive Control)</th>
<th>Inhibition by Water (Negative Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hexane</td>
<td>Chloroform</td>
<td>Petroleum ether</td>
<td>Hexane</td>
<td>Chloroform</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>B. cereus</em></td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Symbols inside the table refer to the size of the inhibition zone diameter observed with growing cells: +, 1 mm; ++, 2 mm; +++>, >2–5 mm; −, absence of an inhibitory zone. CFCS, Cell-free crude supernatants; CFPS, Cell-free purified supernatants
6.3.3 HPLC analysis of organic acids

HPLC analysis confirmed that the main organic acid present in the CFCS was lactic acid with an amount of 2850.50 ppm. Moreover, tartaric acid and formic acid were also found and the content was found to be 625.67 ppm and 245.39 ppm respectively (Fig. 6.3). Relatively low amounts of formic acid were produced by the strain. No oxalic acid, pyruvic acid, acetic acid, citric acid, succinic acid and propionic acid were detected in the CFCS. It is well established that antibacterial activity is shown by organic acids.\(^6^1\) Makras et al.\(^7\) reported that an antimicrobial activity of *Lactobacillus rhamnosus* GG and *L. casei* Shirota was entirely owed to the production of lactic acid. Organic acids, especially succinic, fulfill a barrier effect on pathogenic bacteria.\(^6^2\) In addition, organic acids in the undissociated form enters the pathogenic cell and dissociates it inside cytoplasm, due to decrease of intracellular pH and eventually the accumulation of the ionized organic acid causes the death of the pathogenic bacteria.\(^6^3\)

![HPLC spectrum of organic acids available in CFCS](image)

**Fig. 6.3(a).** HPLC spectrum of organic acids available in CFCS
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Fig. 6.3(b). HPLC chromatograms of standard acids
6.3.4 Minimum lethal dose concentration and death rate kinetics of the CFPS chloroform fraction

The minimum lethal dose concentration of CFPS chloroform fraction against different bacterial strains were reported as 27 mg/ml for *E. coli*, 1.68mg/ml for *S. aureus* and 1.68mg/ml for *B. cereus* (Table 6.2). The CFPS showed strong antimicrobial activity against two indicator strains viz., *S. aureus* and *B. cereus*. The antimicrobial activity against *E. coli* was fairly lower, as higher concentrations of CFPS were required for the inhibition of the test strain. Based on death rate kinetics of enteropathogens used in this study, bacterial viable count after inoculating with the CFPS was less than 1 cfu/ml before 12 h for *E. coli*, 12 h for *B. cereus* and less than 16 h for *S. aureus*. Differences in LD$_{min}$ values of bacteria might be related to differential susceptibility of bacterial cell wall, which is the functional barrier and minor differences present in outer membrane in the cell wall composition.64

Table 6.2. Death rates kinetics of test bacterial strains cultured in nutrient broth supplemented with biometabolite equivalent to LD$_{min}$ of respective pathogen

<table>
<thead>
<tr>
<th>Incubation Time (h)</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>10</th>
<th>12</th>
<th>16</th>
<th>20</th>
<th>24</th>
<th>LD$_{min}$ Values (mg /ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> (Log cfu/ml)</td>
<td>3.48</td>
<td>3.08</td>
<td>2.89</td>
<td>2.21</td>
<td>1.51</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>27</td>
</tr>
<tr>
<td><em>S. aureus</em> (Log cfu/ml)</td>
<td>4.29</td>
<td>3.87</td>
<td>3.70</td>
<td>3.60</td>
<td>3.00</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.68</td>
</tr>
<tr>
<td><em>B. cereus</em> (Log cfu/ml)</td>
<td>6.30</td>
<td>3.00</td>
<td>3.34</td>
<td>2.78</td>
<td>3.00</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.68</td>
</tr>
</tbody>
</table>

6.3.5 Model fitting for microwave assisted extraction process

During microwave assisted extraction process the effect of solvent concentration ($X_1$), solvent to sample ratio ($X_2$) and irradiation time ($X_3$) on DPPH RSA and ABTS RSA of GPR were studied and results are shown in Table 6.3. The coefficient of regression of the intercept, linear, quadratic and interaction terms of the model were calculated using the least square technique. Regression analysis and ANOVA were used for fitting the model and to examine the statistical significance of the terms and the results of ANOVA were given in Table 6.4. The correlation coefficients ($R^2$) for the responses DPPH RSA and ABTS RSA were 0.9297 and 0.9256 respectively. Based on
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$r$-statistics, the regression coefficient significant at 95% probability levels were selected for developing the model given below [Eq. 6.3 & 6.4].

\[
\text{DPPH RSA} = 6.46349 + 0.62942 X_1 + 6.77674 X_2 + 2.01375X_3 - 3.46616E-003 X_1^2 - 0.12634 X_2^2 - 0.70412 X_3^2 - 0.029778 X_1X_2 + 0.056457 X_1X_3 - 0.033600 X_2X_3
\]

\[\text{6.3}\]

\[
\text{ABTS RSA} = -39.78199 + 1.14460 X_1 -0.11886 X_2 +6.32008 X_3 -5.17547E-003 X_1^2 +0.027017 X_2^2 -0.36926 X_3^2 -0.010300 X_1X_2 -0.031777 X_1X_3 -9.70667E-003 X_2X_3
\]

\[\text{6.4}\]

Where, \(X_1, X_2\) and \(X_3\) are independent variables viz., solvent concentration (%), solvent to sample ratio (v/w) and irradiation time (min), and each variable set in the range of 50-100%, 10:1-20:1 and 4–10 min respectively.

\(F\)-value for the lack of fit was insignificant (\(P > 0.05\)) thereby confirming the validity of the model. The model \(F\) value of 10.29 and 9.68 implies that the model is significant. There is only a 0.28% and 0.34% chance that a model \(F\) value this large could occur due to noise. The value of coefficient of variation (C.V.) was 5.73% and 11.42%, suggested that the model was reliable and reproducible. The results indicated that the model could work well for the prediction of antioxidant activity of extract from GPR.

6.3.6 Effect of process variables on DPPH radical scavenging activity

The results given in Table 6.4 show the linear, quadratic and interaction effects of the three factors on the responses. Linear and quadratic term of irradiation time showed the significant effect on DPPH RSA at the 99% and 95% level respectively. However, other terms did not affect significantly on DPPH RSA. The effect of solvent to sample ratio and time on DPPH RSA is shown in Fig. 6.4(a). DPPH RSA was found to decrease with an increase in time of irradiation, but solvent to sample ratio did not have much impact on the DPPH RSA. This result was contrary to the results derived from extraction of polyphenols from \(Camellia oleifera\) fruit hull given by Zhang et al.\textsuperscript{65} However, prolonged exposure to heat owing to increase irradiation time could have resulted in breakdown of antioxidant compounds.\textsuperscript{66} Decrease in solvent concentration did not show any significant change in DPPH activity till 75% concentration. However,
further decrease in solvent concentration resulted in the decrease in DPPH RSA (Fig. 6.4(b)). Antioxidant activity increased slightly with increasing solvent to sample ratio from 1:10 to 1:15 (Fig. 6.4(c)). Beyond this level, the antioxidant activity of extract slightly decreased, which indicated that a greater antioxidant activity of extract could be achieved if the moderate solvent to sample ratio was maintained. Similar results were also reported by Zhang et al. 65

**Table 6.3** Box–Behnken design (BBD) with observed response for DPPH RAS and ABTS RAS.

<table>
<thead>
<tr>
<th>Run No.</th>
<th>Uncoded (coded) process variables</th>
<th>Responses</th>
<th>ABTS RSA (mg-trolox equivalent/g dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Solvent concentration (X1) (%, w/w)</td>
<td>Solvent-Sample ratio (X2) (w/w)</td>
<td>Irradiation time (X3) (min)</td>
</tr>
<tr>
<td>1</td>
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<td>15(0)</td>
<td>10(1)</td>
</tr>
<tr>
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<td>4(-1)</td>
</tr>
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<td>20(1)</td>
<td>4(-1)</td>
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<tr>
<td>17</td>
<td>100(1)</td>
<td>15(0)</td>
<td>4(-1)</td>
</tr>
</tbody>
</table>

All the responses are mean ± SD of three replicates. Numbers in bracket are coded values of process variables.
### Table 6.4 ANOVA for DPPH RSA and ABTS RAS of GPR extract

| Parameters | DF | DPPH RSA | | | | ABTS RSA | | | |
|------------|----|----------|----|----|----------|----------|----|----------|
|            |    | SS       | F value | p value | SS       | F value | p value |
| Model      | 9  | 1668.37  | 10.29   | 0.0028  | 267.04   | 9.68    | 0.0034  |
| X1         | 1  | 16.83    | 0.93    | 0.3659  | 0.37     | 0.12    | 0.7368  |
| X2         | 1  | 53.64    | 2.98    | 0.1280  | 4.43     | 1.44    | 0.2684  |
| X3         | 1  | 1218.43  | 67.65   | < 0.0001| 136.80   | 44.63   | 0.0003  |
| X1^2       | 1  | 19.76    | 1.10    | 0.3297  | 44.06    | 14.37   | 0.0068  |
| X2^2       | 1  | 42.01    | 2.33    | 0.1706  | 1.92     | 0.63    | 0.4545  |
| X3^2       | 1  | 169.09   | 9.39    | 0.0182  | 46.50    | 15.17   | 0.0059  |
| X1X2       | 1  | 55.42    | 3.08    | 0.1228  | 6.63     | 2.16    | 0.1848  |
| X1X3       | 1  | 71.72    | 3.98    | 0.0862  | 22.72    | 7.41    | 0.0297  |
| X2X3       | 1  | 1.02     | 0.056   | 0.8191  | 0.085    | 0.028   | 0.8726  |
| Lack of Fit| 3  | 46.14    | 0.77    | 0.5680  | 16.54    | 4.49    | 0.0904  |
| Pure Error | 4  | 79.93    |         |         | 4.91     |         |         |
| R^2        |    | 0.9297   |         |         | 0.9256   |         |         |
| Adjusted R^2| 0.8394 |         |         |         | 0.8300   |         |         |
| CV (%)     |    | 5.73     |         |         | 11.42    |         |         |

DF: degree of freedom; SS: sum of squares; CV: coefficient of variation

### 6.3.7 Effect of process variables on ABTS radical scavenging activity

ABTS RSA is significantly affected linearly by irradiation time followed by quadratic term of irradiation time and solvent concentration at 99% level (Table 6.4). Also interaction term of solvent concentration and irradiation time had significant effect at 95% level. However, other terms did not show significant effect on ABTS RSA. The effect of solvent to sample ratio and solvent concentration on ABTS RSA is given in Fig. 6.5(a). ABTS RSA was found to increase with decrease in solvent concentration; but, a further decrease in solvent concentration resulted in the reversal of this trend. However, solvent to sample ratio did not have much of an impact on the ABTS RSA. This result was contrary to the results derived for extraction of phenolic compounds from grape seed by Hong et al.\textsuperscript{36} They observed higher extraction of polyphenolic compounds, when solvent polarity was modified by addition of water in the solvent.
Combine effect of solvent concentration and irradiation time had a significant impact on ABTS RSA. Increasing in solvent concentration and irradiation time, ABTS RSA found to be increased (Fig 6.5(b)). The effect of solvent to sample ratio and time on ABTS RSA are shown in Fig. 6.5(c). With respect to solvent to sample ratio, ABTS RSA antioxidant activity did not have any significant effect, but antioxidant activity showed a sharp decrease with increase in irradiation time. However, their combine effect showed the positive impact on ABTS RSA. This could be attributed to the fact that prolonged exposure to heat owing to increase irradiation time could have resulted in breakdown of antioxidant compounds as described earlier.

6.3.8 Optimization of microwave assisted extraction conditions

The process variables viz., solvent concentration, solvent to sample ratio and irradiation time was numerically optimized to find out the conditions for getting the best results from MAE extraction. Optimization was done for obtaining maximum DPPH RSA and ABTS RSA. The optimal conditions of MAE for antioxidant extract obtained were found as solvent concentration of 70.79, solvent to sample ratio of 20:1 and irradiation time of 4.73 minutes. For the validation and adequacy of the model equation, a verification experiment was carried out under the optimized conditions mentioned above. The predicted antioxidant activity of extract was 85.98% DPPH RSA and 19.23 mg-trolox equivalent/g dry weight (ABTS RSA), which was consistent with the practical antioxidant activity of 85.02% DPPH RSA and 19.68 mg-trolox equivalent/g dry weight (ABTS RSA) of extract. The strong correlation between the real and predicted results confirmed that the response model was adequate to reflect the expected optimization.
Fig. 6.4. DPPH RSA during microwave irradiation of GPR as function of (a) solvent to sample ratio and irradiation time; (b) solvent concentration and irradiation time; (c) solvent concentration and solvent to sample ratio

Fig. 6.5. ABTS RSA during microwave irradiation of GPR as function of (a) solvent concentration and solvent to sample ratio (b) solvent concentration and irradiation time and (c) solvent to sample ratio and irradiation time
6.3.9 FTIR spectroscopy of untreated GPR powder and MAE treated residue

Optimized value of solvent concentration (70.79% ~71%) and solvent to sample ratio (20:1) were taken for MAE at different irradiation time (4, 7 & 10 min.). FTIR spectra for the untreated GPR powder and MAE treated residue obtained at these three different conditions were recorded by FTIR spectrometer (Fig. 6.6). The broadband around 3420–3440 cm\(^{-1}\) is characteristic for hydroxyl group indicates the presence of phenolic compounds and the peak intensity get reduced with increasing extraction time. The observed absorption band at 1628 cm\(^{-1}\) indicates N–H bend, shows the presence of primary amines in untreated GPR powder and MAE residue treated for 4 min. However, this peak intensity was fully reduced with 7 and 10 min MAE treatment. Absorption at 2920-2930 cm\(^{-1}\) is characteristics of C-H stretching in aldehydes. Strong band in the region (1200-1000 cm\(^{-1}\)) confirms the presence of esters, aldehydes, ketones, lactones, carboxylic acids, amides, alcohols. A band at 1400 cm\(^{-1}\) is the characteristic for C-C stretch ring, which shows the presence of aromatic compounds in untreated GPR and this peak is absent in all MAE treated samples. Absorption at 1105 cm\(^{-1}\) is the most characteristics of ethers \(i.e.\) C-O stretching in CO- C group. Strong band at 1195 cm\(^{-1}\) and another at 1401 cm\(^{-1}\) gives the presence of phenols. Appearance of strong band at 1628 cm\(^{-1}\) is due to C=O stretching which indicates the presence of quinines and amides. Absorption around 1750–1735 (s) could be due to C=O stretch caused by esters and saturated aliphatic groups. Absorption at around 700 cm\(^{-1}\) may be due to carbohydrate. Samples treated with MAE showed gradual reduction in peak intensities at 4, 7 and 10 minutes respectively, indicating release of antioxidant and cellular components into the surrounding solvents, hereby resulting in absence from the spectra of the residual cellular matter. FTIR study revealed that, the extraction was higher with increase in irradiation time, but antioxidant activity of extract was lower with increase in irradiation time. This could be attributed to loss of antioxidant activity of extract due to prolonged exposure to heat caused by irradiation.
Fig. 6.6. FTIR spectra of GPR obtained from (a) control (untreated GPR powder) (b) MAE treated residue at 70% concentration, 20:1 ratio and 7 min (c) MAE treated residue at 70% concentration, 20:1 ratio and 10 min (d) MAE treated residue at 70% concentration, 20:1 ratio and 4 min

6.3.10 Microscopic observation of untreated GPR powder and MAE treated residue

The morphological changes in the sample during microwave assisted extraction were studied by observing the samples under light microscope. The micrographs of the untreated GPR cross-sectional slices and that of treated slices of GPR irradiated at the optimized condition (Fig. 6.7). There was no visible destruction in the cells for the untreated samples, as shown in Fig. 6.7(a) for exocarp and Fig. 6.7(c) for mesocarp.
After microwave bombardment, there was visible change in the texture of the mesocarp (Fig. 6.7(d)) and breakage in the cells structure in the exocarp in (Fig. 6.7(b)) was observed. This could be attributed to the absorption of microwave energy by the water in the cells and parenchyma, resulting in sudden rise in temperature and internal pressure rise. In MAE, the exocarp of samples was immensely changed and destroyed in Fig. 6.7(b). The higher efficiency could be attributed to action of microwave irradiation, which produces the disruptions of tissues and cell walls leading to a greater contact area between solid and liquid phase, better access of solvent to valuable components. The microscopic imaging confirmed the bursting of cells and disruption in the cellular parenchyma.

**Fig. 6.7.** Light microscope images of (a) untreated GPR exocarp, (b) sample after MAE (exocarp), (c) untreated GPR mesocarp, (d) sample after MAE (mesocarp)
6.4 Conclusion

The study shows that *L. plantarum* isolated from fermented bamboo shoots strongly inhibits the three test pathogens. This study will offer useful information for the improvement of *khorisa* production. This biometabolites could be incorporated in edible film and coating to control the growth of pathogenic microorganism on fruits, vegetables and other food products. The biometabolite might also be used in combination with established antibiotics, and could prove useful in combating emerging drug resistant species of enteropathogens.

MAE results evinced that irradiation time was the major factors which affected the antioxidant activity of extract obtained from GPR. The optimum extraction parameters were obtained and the predicted values for antioxidant activity of extracts were well consistent with the experimental ones. The extraction of antioxidant extract using MAE method used lesser solvent and decreased extraction time compared to conventional solvent extraction methods. The extract exhibited significant DPPH RSA and ABTS RSA. The information obtained from this study would be valuable for further exploitation and application of this resource.
Reference


