Chapter 3: Evaluation of the antioxidative and antimicrobial properties of the plants used in rice beer starter culture preparation

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3.1 Introduction

The starters for rice beer fermentation are in the form of dry powder, hard balls or cakes and various plant materials are used in the preparation of these starters. Many of these plants also possess medicinal properties and they affect the quality of rice beer. Out of these plants, leaves of *Artocarpus heterophyllus*, *Cyclosorus extensa*, *Oldenlandia corymbosa* and *Alpinia malaccensis* were found to be with the highest content of total phenolic compounds and antioxidant activity (DPPH radical scavenging activity) based on an initial screening test. All these four plants are native to Southeast Asia and grow abundantly in Assam as wild plants and hence were selected from among the sixty numbers of plants screened.

Cyclosorus is a genus of ferns of the family Thelypteridaceae and is a commonly growing fern and is widely distributed all over Asia. Antibacterial activity of the closely related fern *C. interruptus* against *Staphylococcus aureus* has already been reported [1]. *C. extensa* is found in India, Sri Lanka, Indo-China, Malaysia and Australia. In Assam the leaves are traditionally used in the treatment of pneumonia [2], herpes and skin infection [3]. Three new coumarin derivatives, three new furanocoumarins and a novel dioxocane derivative have also been isolated from the closely related species of *Cyclosorus interruptus* [4]. *Oldenlandia* is a genus of flowering plant belonging to the family Rubiaceae. It is a prostrate to decumbent, annual herb and grows in areas such as roadsides, lawns, open forest, stream sides etc [5], and is a weedy annual herb with ascending or erect 4-angled stems [6]. Its extracts are said to be active against appendicitis, hepatitis, pneumonia, cholecystitis, urinary infection, cellulites and snake bite, skin sores, ulcers, sore throat, bronchitis, gynecologic infections and pelvic inflammatory diseases [7]. Ten new compounds have also been isolated from the whole plants of *O. corymbosa* of Thai origin [8]. *A. heterophyllus*, also known as jackfruit, is a species of tree in the Moraceae family. It grows in many tropical countries of Southeast Asia and is a large evergreen tree which is usually 10-15 m in height [9]. Reports are also available on the wound healing activity of flavonoid rich fraction of ethyl acetate extract of the leaves of *A. heterophyllus* using porcine skin wound healing model [10]. The methanolic extract of *A. heterophyllus* leaves exhibited significant analgesic and immunomodulator effect on Swiss albino mice [11]. The hypoglycemic activity of the leaves of *A. heterophyllus* in normal and streptozocin induced diabetic rats has also been

reported [12]. *A. malaccensis* is a plant in the Zingiberaceae family and is a native of Indonesia and Malaysia. It has a wide distribution, extending from north-east India to Indochina, southwards to Peninsular Malaysia and Java [13]. It is a perennial plant growing widely in the subtropical and tropical regions of Asia. It is a large ginger (2 to 4 m tall) in which leafy stems arise from stout rhizomes just below the ground [14]. Many species of the genus *Alpinia* containing flavonoids, terpenoids [15] and kavalactones provide a variety of medicinal properties [16]. Traditionally *A. malaccensis* is used to cure wounds and sores, to make the voice strong and clear, applied on gastralgia with tympanites, for bathing feverish people and as an anti-vomiting agent [17,18].

The shelf life of food materials and their spoilage can be characterized by various chemical and microbiological means. These help in establishing the causes of spoilage and also in assigning total or remaining shelf life and their acceptability [19]. Several quality aspects may be subjected to changes during beer storage and stability in the form of flavour, colour, foam, and microbiology determines its shelf life [20]. The spoilage in beer may be characterized by various chemical reactions such as etherification, and formation of linear aldehydes, esters, acetal and polyphenols [21]. The factors responsible for such reactions are the beer type, raw materials and exposure to oxygen, light, temperature and time [22]. The application of phenolic compounds extracted from plants in the preservation and extendibility of shelf life of foods and beverages have been reported earlier like in inhibition of lipid oxidation and rancidity by *Glycyrrhiza glabra* extracts in precooked pork patties [23], grapefruit seed extract in the preservation of fresh makgeolli [24] and tea polyphenols in anti retrogradation of cooked starch-based product [25]. The inhibition of lipid peroxidation induced by oxygen in the presence of initiators such as heat, free radicals, photosensitizing pigments, and metal ions in stored products also is an important role of radical-scavenging dietary antioxidants[26]. The recovery of polyphenols using polymeric resins is widely used in industrial process due to high adsorption capacity, easy recovery of adsorbed molecules, relatively low cost, easy regeneration etc [27]. Polymeric plant polyphenols can be processed by solid-phase extraction for the purpose of separation and purification. Amberlite XAD-8 resin has been successfully used for the purification of polyphenols from cassava roots [28]. Studies have been done on the recovery of flavonoids by different Amberlite XAD resins and it was found that XAD-2 was the most suitable resin for the recovery and fractionation of flavonoids from plant extracts [29].

Taking the above points into consideration the following, in this chapter the phytochemical composition and antimicrobial activity of the extracts of the leaves of

Artocarpus heterophyllus, *Cyclosorus extensa*, *Oldenlandia corymbosa* and *Alpinia malaccensis*, which might significantly affect the quality of the alcoholic beverage, was first studied. These four plant species traditionally used in the making of rice beer in Assam have not been studied for their antioxidant properties. Although certain works have been reported by other authors on the bioactivities of the extracts of these plants [30-36], yet detailed work on the antioxidant activities of the constituent polyphenols in the extracts has not been reported so far. Hence the next study was aimed to determine content of various polyphenols and compare the antioxidant potentials of the purified phenolic extracts of these plant leaves. Altogether ten different *in vitro* methodologies of determining antioxidant properties with distinctive mode of operation were employed. Thereafter, it was seen that out of these plants, *Cyclosorus extensa* and *Artocarpus heterophyllus* possess high content of various polyphenols and these phenols were found to have good antioxidant properties. Most beers are expected to have shelf lives of 17-26 weeks at room temperature (75 °F). The shipment and storage of beer under refrigerated conditions is not always economically feasible. Hence, forced aging under augmented temperature can actually predict the actual shelf life of a beer [37]. Hence, an attempt was next made to purify the flavonoid rich fraction from the ethanolic extract of *C. extensa* and *A. heterophyllus* leaves and to see their effects on the storage stability of rice beer under accelerated temperature condition. Moreover, literature survey revealed that no work till now has been reported on the correct choice of solvent vis-a-vis the extraction conditions for obtaining phenolic compounds from *Cyclosorus extensa*. Response surface methodology (RSM) is a multivariate equation solving technique which uses a collection of mathematical and statistical methods to evaluate relationships between a group of quantitative independent variables and one or more responses. Operation variables that may or may not have significant effect in the main response can be identified and optimized using RSM [38-40]. Hence, finally an attempt was made to standardize these parameters via optimization of time and temperature using four different solvents by using RSM based on the response of total phenolic content, antioxidant, antibacterial and antifungal properties.

3.2 Materials and methods

3.2.1 Materials

Leaves of the four plant species viz. *Artocarpus heterophyllus* Lam., *Cyclosorus extensa* (Blume) Ching, *Oldenlandia corymbosa* L. and *Alpinia. malaccensis* (Burm. f.) Roscoe were collected from the botanical gardens of Tezpur University campus, Assam. Taxonomical identification of the collected plant species were done in the Department of Botany, Darrang College, Tezpur, Assam., The young and tender leaves were selected for analysis based on the traditional uses of the plants for starter cake making. The chemicals and solvents used for analysis were of high purity analytical grade and obtained from Sigma-Aldrich (USA), E. Merck (Germany) and HiMedia (India). The HPLC standards and the chemicals viz., α tocopherol, 3,5-di-tert-4-butylhydroxytoluene (BHT), 2-deoxyribose, 1,1-diphenyl-2-picryl-hydrazyl (DPPH), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), nitro blue tetrazoliumchloride (NBT), ethylenediaminetetraacetic acid (EDTA), nicotinamide adenine dinucleotide reduced (NADH), phenazinemethosulfate (PMS), thiobarbituric acid (TBA), linoleic acid and ferrozine were procured from Sigma-Aldrich Corporation (USA). The fungi *Aspergillus oryzae* ATCC 10124, *Aspergillus niger* MTCC 281, *Fusarium oxysporum* MTCC 1755, *Saccharomyces cerevisae* ATCC 9763 and *Candida albicans* MTCC 183 and bacteria *Escherichia coli* MTCC 40, *Staphylococcus aureus* MTCC 3160, *Lactobacillus plantarum* ATCC 8014 and *Bacillus subtilis* ATCC 11774 were kindly provided by the Department of Food Engineering and Technology, Tezpur University, Assam. Rice (*Oryza sativa*) variety "*bora*" was obtained from Assam Agricultural University, Assam. The equipment used during the study were tray dryer (BDI-51, Labotech, India), UV-Vis spectrophotometer (Spectrascan UV2600, Thermo Scientific, USA), incubator (LBI-150M, Labtech, South Korea), incubator shaker (Excella E24R, NBS, USA), water bath (BW-20G, Jeiotech, South Korea), rotary vacuum evaporator (N-1200AS, Eyela, Japan), HPLC system (Ultimate 3000, Dionex, Germany), FTIR spectrophotometer (Spectrum 100, Perkin Elmer, USA), freeze dryer (LDF 5512, Daihan Labtech, Korea), ultrasonic bath (RK 510H, Bandelin, Germany), ultrasonic probe (UW2070, Bandelin, Germany) and Hunter Lab Color Quest (Ultrascan Vis, Hunterlab, USA).

3.2.2 Phytochemical constituents, ATR-FTIR analysis and antimicrobial activity of leaves of *Artocarpus heterophyllus* Lam., *Cyclosorus extensa* (Blume) Ching, *Oldenlandia corymbosa* L. and *Alpinia. malaccensis* (Burm. f.) Roscoe

3.2.2.1 Phytochemical analysis of the leaves

Total phenolic compounds in the plant extracts were determined as per method of Slinkard and Singleton [41] by using Folin–Ciocalteu reagent and were expressed as gallic acid equivalent (GAE). The content of alkaloids was estimated on a % weight basis following the ammonium hydroxide method of Harborne [42]. The tannin content was estimated by Folin-Denis method [43] and a standard curve of tannic acid was used for quantification. Terpenoids was estimated on a % weight basis as per the method described by Ferguson [44]. Total flavonoids content was determined following aluminium chloride method of Zhishen et al. [45] and a standard curve of quercetin was used for quantification. Anthraquinone content was estimated according to the method of Soladoye and Chukwuma [46] and a standard curve of alizarin was used for quantification. Determination of gylcosides was done by modification of the method of El-Olemy [47] and a standard curve of digitoxin was used for quantification. Anthocyanin content in the plant materials was estimated according to Ozsoy et al. [48]. Determination of saponin content was done according to Brunner [49] and quantification was done based on disogenin standard curve. Estimation of phytosterols was done according to the method of Sabir et al.[50] by using Liberman-Burchard reagent and a standard curve of stigmasterol was used for quantification.

3.2.2.2 Preparation of solvent extracts (SEs)

Leaves were washed with deionized water, cut into small pieces and dried in a tray dryer at 45 ºC until complete dryness. The dried leaves were ground to a fine powder and sieved through 50 U.S. mesh size. The powders were extracted with distilled water (H_2O), methanol (MeOH), ethyl acetate (EA) and hexane (Hex) at room temperature in an incubator shaker at 150 rpm for 24 h. An ultrasonic bath was used to enhance the dissolution process at regular intervals. The extracts were filtered through four layers of muslin cloth and centrifuged at 3000 rpm for 10 min and filtered through Whatman No. 1 filter paper in a vacuum assisted filtration unit. The extracts were further dried in a rotary vacuum evaporator at room temperature under reduced pressure. The weight of the dried extracts was recorded

and again dissolved in deionized water to a final concentration of 1000 µg/mL (stock solution). The dried extracts were dissolved in deionized water in order to nullify any effect which other synthetic solvents might have on the desired property. In order to facilitate dissolution of the non-polar extracts like methanol and hexane, an ultrasonic probe was used to bring uniform dispersion.

3.2.2.3 FTIR analysis of the SEs

Attenuated total reflectance (ATR) FTIR spectrophotometric analysis was used to identify the functional group present in the SEs in liquid state. A horizontal ATR accessory which allows direct sample application of liquids was used for obtaining the spectra. A zinc selenide (ZnSe) crystal trough plate at 45º was used as a liquid sampling top-plate. The data were collected in absorbance (A) mode and the selected wavelength ranged from 4000 cm^{-1} to 400 cm^{-1}. A resolution of 4 cm^{-1} was used while the number of scans per sample was 4. Detection was done based on the peak value in the region of infrared radiation and compared with previously reported data and other libraries.

3.2.2.4 Antimicrobial activity of the SEs

The agar well diffusion method of Perez et al. [51] and antifungal activity assay described by Barry [52] was followed for carrying out the assays. Various media were used based on the microorganisms viz. potato dextrose agar for *F. oxysporum* and *C. albicans*, Baird Parker agar base supplemented with egg yolk emulsion and potassium tellurite for *S. aureus*, EMB agar for *E. coli*, MRS agar for *L. plantarum* and modified MYP agar for *B. subtilis*. Cultures (100 µl, 24 h old) with the count adjusted to 10^8 CFU/mL were spread on the respective plates. Two wells were made in all the plates with a sterile cork borer. A volume of 100 µL of the SEs were added to one well and deionized water was used as a negative control. Diffusion of the extracts was allowed at room temperature for 1h in a laminar air flow work chamber. In case of mould, 10 mm diameter circles of 72 h old culture of *F. oxysporum* were dug out using a cork borer and inoculated on the centre of the respective plates and the extracts were allowed to diffuse in the same manner as in case of the other organisms. The plates were incubated for 96 h at 25 ºC for *F. oxysporum*, 48 h at 30 ºC for *C. albicans*, 48 h at 37 ºC for *E. coli*, *S. aureus*, *L. plantarum* and *B. subtilis.* The zones of

inhibition were measured in terms of their diameter with an antibiotic zone scale (HiMedia, India).

3.2.2.5 Determination of minimum inhibitory concentration (MIC)

MIC value of each of the plant extract showing antimicrobial activity against the tested microorganism was determined according to the broth micro dilution method of Basri and Fan [53]. The SEs were serial diluted on antibiotic assay broth (Medium No 3, HiMedia, India) in test tubes to make final concentrations ranging from 10 to 500 µg/mL. Inoculum (100 µl) of bacteria (10^8 CFU/mL) and yeast (10^7 CFU/mL) cultures were added to each tube. Assay broth without inoculation was used as negative control, while broth containing antibiotic was used as positive control. The tubes were incubated at 37 ºC for 24 h for bacteria and 27 ºC for 48 h for yeast. Each extract was assayed in triplicate and 100µL of media from each tube was plated onto agar plates for observation of apparent growth. The MIC value was taken as the least concentration of extract showing no visible growth of the microbes on plating. In order to bring out a comparison, standard antimicrobial agents, viz., streptomycin and chloramphenicol were assessed for their MIC values against the bacteria and fungi respectively.

3.2.3 *In vitro* antioxidant activity of polyphenols purified from leaves of *Artocarpus heterophyllus* Lam., *Cyclosorus extensa* (Blume) Ching, *Oldenlandia corymbosa* L. and *Alpinia malaccensis* (Burm. f.) Roscoe

3.2.3.1 Extraction and purification of phenolic compounds

Extraction and purification of phenolic compounds from the plants were done as per the method described by Tsujita et al. [54]. The freshly plucked plant leaves were freeze dried until a final moisture content of 2 % was obtained and then ground in a laboratory blender to a fine powder and passed through a mesh of 50 US size. Dried powdered samples (500 g) were extracted with 1L of 75 % ethanol in an incubator shaker at 100 rpm (37 ºC for 48 h). The mixture was filtered through four layers of muslin cloth and extracts were centrifuged at 10000xg for 20 min and the supernatant was filtered through Whatman No. 1 filter paper in a vacuum assisted filtration unit. The extract was washed twice with hexane and dried in a rotary vacuum evaporator under reduced pressure and lyophilized. The

lyophilized powder was redissolved in 30 mL of deionised water (DW) in an ultrasonic bath to enhance the dissolution process. This solution was dialyzed against 2 L of DW using a 12000 Da MWCO dialysis membrane (D6066, Sigma-Aldrich, USA). The inner dialyzed materials were concentrated, lyophilized and dissolved with DW(100 mg/10 mL) and applied to a column (300 mm x 20 mm i.d) prepared with 20-60 mesh Amberite XAD-2 resin (RM9218-100G, Himedia, India) equilibrated with DW. The column was washed twice with DW and then eluted with 60% aqueous ethanol. The ethanolic eluate was concentrated, lyophilized and again dissolved in DW (100 mg/10 mL) and applied to a column (300 mm x 20 mm i.d) prepared with Sephadex® LH-20(LH20100, Sigma-Aldrich, USA) equilibrated with DW. This column was washed with DW and eluted with 70 % aqueous acetone to obtain the purified phenolic extracts (PPEs). The PPEs were again concentrated and lyophilized, and various concentrations of the dry powder were made in DW.

3.2.3.2 Estimation of phenolic compounds in the PPEs by HPLC

The eluate from the Sephadex® column were subjected to hydrolysis with 2 N HCl at 100 °C for 1 h and neutralized by the addition of 2 mL of 2 N NaOH. The volume of the mixture was made up to 50 mL and the pH was adjusted to 7.0. This was passed through two C18 Sep Pak® cartridges in series which were pre conditioned with methanol and 20 µL of filtrate was injected for estimation. The quantitative analysis of polyphenols was carried out in a HPLC system with an UV detector at 265 nm. The column used was Acclaim 120® C18 column (5µm beads size; 120 Å; 4.0 x 250 mm, Thermo Scientific) and the column oven temperature was maintained at 30 ºC. The mobile phase used was a mixture of the following two solvents; Eluent A – acidified DW (pH adjusted to 2.64 with dilute HCl) and Eluent B – Acidified DW: acetonitrile (20:80). A gradient run at a constant flow rate of 1.5 mL/min was used for elution [55].

3.2.3.3 Estimation of antioxidant activities

The lyophilized PPEs were separated to two sets of varying concentrations in DW. The first set (S1) contained concentrations in the series of 20, 40, 60, 80, 100 and 120 µg/mL and the second set (S2) contained concentrations in the series of 50, 100, 150, 200, 250 and 300 µg/mL. Both the sets were made based on some preliminary study in relation to their antioxidant acitivites, keeping in mind that a correlation is maintained between the

concentrations used and the activity shown under various assay conditions. A total of ten different techniques for evaluation of antioxidant activities were employed. BHT, α-tocopherol or EDTA were used as reference standards. The reduction in the intensity of colour was measured in a spectrophotometer and the antioxidant activities were calculated according to the Eq. 3.1.

$$\%\ Activity = \frac{(A0-A1)}{A0} \times 100 \qquad \text{Eq. (3.1)}$$

Where, A0 is the absorbance of the control and A1 is the absorbance in presence of the antioxidant compound. All the absorbance readings were taken in a UV-Vis spectrophotometer.

3.2.3.3.1 DPPH free radical scavenging activity assay

DPPH reacts with an antioxidant compound that can donate hydrogen and gets reduced to become a stable diamagnetic molecule. It loses this absorption when accepting an electron or a free radical species, which results in a discolouration from purple to yellow. To 4 mL of S1, 2 mL of 0.1 mM DPPH solution in ethanol was mixed. The tubes were incubated for 10 min in the dark and the absorbance was taken at 517 nm and for blank, PPEs and ethanol were used in place of DPPH solution. The absorbance of the DPPH solution incubated with DW was taken as control [56].

3.2.3.3.2 ABTS radical cation decolourisation assay

This technique is involved in the production of the blue/green ABTS+ chromophore through the reaction between ABTS+ and potassium persulphate. The presence of antioxidant compounds compete with ABTS+ and diminish the colour formation. To 4 mL of S1, 0.54 mL of ABTS solution (7 mM ABTS and 2.6 mM potassium persulphate in 1:1 ratio, allowed to stand in the dark at room temperature for 16 h before use) and 0.5 mL of 100 mM phosphate buffer (pH 7.4) were added and allowed to stand in the dark for 2 hand the absorbance was taken at 734 nm. For control, DW was used instead of the PPEs and the blank contained different concentrations of the sample in buffer and DW [57].

3.2.3.3.3 Hydroxyl radical (•OH) scavenging assay

Hydroxyl radicals are generated by direct addition of iron (II) salts to a reaction mixture containing ascorbic acid and the radicals in turn caused degradation of 2-deoxyribose to products with pink colour. To 0.6 mL of S2, 0.2 mL of phosphate buffer (0.2 M, pH 7.4), 0.4 mL of 500 μM ferric chloride solution, 0.2 mL of 1 mM ascorbic acid solution, 0.2 mL of 1 mM EDTA, 0.2 mL of 10 mM hydrogen peroxide solution and 0.4 mL of 15 mM 2-deoxyribose were added and vortexed. The contents were incubated at room temperature for 60 min and 2 mL of 1% TBA in 0.05 N NaOH and 2 mL of 2.8% TCA were added and followed by keeping all the tubes in a boiling water bath for 30 min. The absorbance of the resulting mixture was read at 535 nm and the mixture containing DW instead of the PPEs was used as control [58].

3.2.3.3.4 Hydrogen peroxide (H_2O_2) scavenging activity assay

To 3.5 mL of S2, 0.6 mL of 40 mM H_2O_2 solution in phosphate buffer (pH 7.4) was mixed and the mixture was incubated in the dark for 10 min and absorbance of the mixture was read at 230 nm. The absorbance of the H_2O_2 solution was taken as control and phosphate buffer without H_2O_2 was used as a blank [59].

3.2.3.3.5 Superoxide anion (O2•-) scavenging activity assay or NBT assay

Superoxide reduces NBT to form blue coloured formazon. Competition for superoxide occurs when an antioxidative substance is added with NBT and decrease in the formation of colour occurs which is a measure of its superoxide scavenging activity. To 2 mL of S2, 1 mL of NBT (100 μM NBT in 100 mM phosphate buffer, pH 7.4) solution and 1 mL of NADH (468 μM in 100 mM phosphate buffer, pH 7.4) solution were mixed, followed by the addition of 100 μL of PMS solution (60 μM PMS in 100 mM phosphate buffer, pH 7.4). The reaction mixture was incubated at 30 ºC for 15 min and the absorbance of the resulting mixture was measured at 560 nm and incubation with DW instead of the PPEs was used as a control [60].

3.2.3.3.6 Nitric oxide radical (NO•) scavenging activity assay

Nitric oxide which interacts with oxygen to produce nitrite ions is estimated using Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to less production of nitrite ions. To 2 mL of S2, 2 mL of 5 mM sodium nitroprusside in 0.5 M phosphate buffer (pH 7.4) and 0.5 mL of chloroform were mixed and the mixture was incubated at 25 ºC for 3 h in presence of a visible polychromatic light source. Incubation mixture (2 mL) was mixed with 2 mL of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediaminedihydrochloride, 1:1 ratio) and incubated at room temperature for another 30 min after which the absorbance of the reaction mixture was read at 546 nm and incubation with DW instead of the PPEs was used as a control (61)[78].

3.2.3.3.7 Ferrous ion (Fe^{2+}) chelating assay

The antioxidants compete with ferrozine for Fe^{2+} ions in free solution and bivalent transition metal ions like Fe^{2+} either acts as catalysts of oxidative processes, leading to the formation of hydroxyl radicals or causes hydrogen peroxide decomposition reactions via Fenton reaction. To 4 mL of S2, ferrous chloride (2 mM, 0.1 mL) and ferrozine (5 mM, 0.2 mL) were added and the absorbance was taken at 562 nm after 20 min room temperature incubation in the dark. DW instead of ferrozine was used as blank and ferrous chloride and ferrozine were used as a control [62].

3.2.3.3.8 Ferric reducing antioxidant power (FRAP) assay

The amount of Fe (II) complex formed is monitored by measuring the formation of ferric-ferrous complex. Reduction of ferric to ferrous ion increases the absorbance indicating the reducing ability. To 2 mL of S2, 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide (1% solution in DW) were added and incubated at 50 ºC for 20 min. This was followed by addition of 1.5 mL of 10% TCA and centrifuged at 3000 x g for 10 min. Supernatant (1 mL) was mixed with 2 mL of DW and 1 mL of ferric chloride (0.1%) and the absorbance was measured at 700 nm and incubation with DW in place of PPEs was used as the blank [63].

3.2.3.3.9 Ferric thiocyanate (FTC) assay

Ferric ions are formed upon reaction of peroxide with ferrous chloride and then unite with ammonium thiocyanate producing ferric thiocyanate, a red-coloured substance. The darker the colour more is the peroxidation value, and is reduced in presence of an antioxidant. A 0.02 M linoleic acid emulsion was prepared in phosphate buffer (0.05 M, pH 7.4) and emulsified by adding equal amount of Tween 20. To 0.2 mL of S2, 2.5 mL of linoleic acid emulsion and 2.3 mL of phosphate buffer (0.2 M, pH 7.0) were added and the mixture was placed in screw capped glass vials, vortexed, and incubated at 37 ºC in the dark. To 0.1 mL of reaction mixture, 4.7 mL of 75% ethanol, 0.1 mL 30% ammonium thiocyanate and 0.1 mL of 0.02 M $FeCl_2$ in 3.5% HCl were added and exactly after 3 min the absorbance was taken at 500 nm. This reading was taken at every 24 h until the absorbance of the control reached a maximum and incubation with DW instead of the PPEs was used as a control [64].

3.2.3.3.10 Thiobarbituric acid (TBA) assay

The secondary product malonaldehyde is formed and binds at low pH and high temperature with TBA to form a red complex that gives an indication of peroxidation. To 0.2 mL of S2, 2.5 mL of 0.02 M linoleic acid emulsion and 2.3 mL of phosphate buffer (0.2 M, pH 7.0) were added. The mixture was placed in screw capped glass vials, vortexed and incubated at 37 ºC in the dark. To 2 mL of the mixture, 2 mL of 20% TCA and 2 mL of 0.67% TBA (prepared in 0.05 N NaOH) were added and the mixture was kept in a water bath at 100 ºC for 10 min, cooled and centrifuged at 3000 rpm for 20 min. Antioxidant activity was measured from the absorbance of the supernatant at 532 nm and DW instead of PPEs was used as a control [65].

3.2.3.4 IC_{50} value calculation

The PPEs' concentration required to bring 50% of scavenging (IC_{50}) was calculated from the interpolation of the curves plotted for percentage inhibition against the respective concentrations. The IC_{50} value specifies that the tested preparation contains 50% of the contents of the inhibitor which is enough to exhibit the activity under study. It is important to accurately measure the optimal concentration of the compounds which needs to be

administered in order to obtain the desired function. These values might be useful l in further processing of these polyphenols in the preparation of functional foods.

3.2.4 Storage study of rice beer under accelerated temperature condition by incorporation of bioflavonoids from *Artocarpus heterophyllus* and *Cyclosorus extensa* leaves

3.2.4.1 Extraction and purification of bioflavonoids

Plant leaves were dried at 45 ºC until complete dryness and grinded in a mechanical grinder and sieved through 50 US mesh. Extraction was done with 70 % ethanol by shaking in an incubator shaker for 24 h with occasional sonication, followed by filtration through Whatman No 1 filter paper. The filtrate was washed with equal volume of carbon tetrachloride and concentrated by drying at 45 ºC under reduced pressure. The residue was dissolved in deionized water. For purification, 20 g of Amberlite XAD – 2 resin was washed with methanol and packed into glass columns (30x2 cm). The columns were washed with 200 ml methanol and then with 100 ml of acid distilled water (pH 2 with 2N HCl). Aliquots (50 ml) were passed through the column and washed with 200 ml of acid distilled water (pH 2) and then with 50 ml of distilled water (pH 5). The absorbed flavonoids were eluted with methanol until no UV absorbance (350 nm) was observed in the eluate. Eluates were collected as 1 ml fractions. The collected eluates were pooled and again dried under reduced pressure. Two different flavonoid extracts were obtained by this process viz. *A. heterophyllus* extract (AH) and *C. extensa* extract (CE).

3.2.4.2 HPLC estimation of flavonoid content

The extracts were diluted with deionized water, filtered through 0.2 µm syringe filter and then passed through two C18 Sep Pak® cartridges in series which were preconditioned with methanol and this filtrate was injected for estimation. The analysis was carried out in a HPLC system (Ultimate 3000, Dionex, USA) and detection was done in an UV detector at 280 nm. The injection volume was 20 µL and the column used was Acclaim 120® C18 column (5 µm beads size; 120 Å; 4.0 x 250 mm) which was maintained at 30 ºC. The compounds were eluted with a gradient elution of mobile phases A (deionized water and 1% acetic acid) and B (methanol and 1% acetic acid). Gradient elution program of time versus %

B was followed (B = 0 min:10 %; 18 min:17.2 %; 30 min:23 %; 40 min:23 %; 53 min: 31.3 %; 65 min: 46 %; 70 min: 55 %; 75 min:100 %; 83 min: 100 %; 85 min: 10 %; 90 min:10 %) (66)[82].

3.2.4.3 Preparation of rice beer

Rice was boiled and cooled to room temperature and then 1 week old culture of *A. oryzae* dissolved in distilled water was added (to a final concentration of 10^5 CFU/g rice). The mould was grown in potato dextrose broth for one week and then centrifuged and washed twice with distilled water. Liquefication was allowed to take place inside sterile glass containers for 5 days at 27 ºC. The mixture was then filtered through several layers of muslin cloth and transferred to a 5.0 L fermentor (Bioflo 115, New Brunswick Scientific, USA) and autoclaved. The wort mixture was then inoculated with 24 h old culture of *Saccharomyces cerevisae* dissolved in distilled water (to a final concentration of 10^7 CFU/mL wort). The yeast was grown in yeast malt broth for one week and then centrifuged and washed twice with sterile distilled water. Fermentation was further carried out under controlled condition for 5 days at 27 ºC and the volumetric alcoholic content [67] at that point was 6.2%. The following combinations of rice beer were prepared in triplicate under aseptic conditions: RBAH – 490 mL fortified 10 mL of with flavonoids (500 µg/mL) from *A. heterophyllus*; RBCE - 490 mL fortified with 10 mL of with flavonoids (500 µg/mL) from *C. extensa*; RBBHT - 490 mL fortified with 10 mL of with butylatedhydroxytoluene solution (500 µg/ml); RBF – filtered through Whatman No. 42 filter paper.; RBC - control without any additive.

3.2.4.4 Storage of the rice beers

All the beers were transferred to 500 mL sterile screw cap glass bottles and storage was done at 32 ºC in an incubator for a period of 8 weeks. Starting from day 0, at regular interval of 1 week, the beers were collected and analysed for the microbial and biochemical changes up to a period of eight weeks.

3.2.4.5 Analysis of the stored rice beers

3.2.4.5.1 Aerobic plate counts (APCs)

All the samples were serial diluted on 0.86% NaCl solution, plated by spread plate technique on plate count agar (M091, Himedia, India) followed by incubation at 37 ºC. The colony forming units (CFU) per mL of sample was calculated for each of the sample (68)[84].

3.2.4.5.2 pH

The undiluted test portions were tested in a digital pH meter (pH510, Eutech Instruments)

3.2.4.5.3 Total phenolic content (TPC)

This was determined according to the method of Bray and Thorpe [69]. The filtered beer samples were treated with Folin-Ciocalteu reagent and the absorbance was taken at 650 nm.

3.2.4.5.4 Radical scavenging activity (RSA)

The antioxidant activity in terms of RSA was measured as per the method of Brand-Williams et al. [56] as described in section **3.2.3.3.1.**

3.2.4.5.5 Total proteins

The samples were filtered and mixed with alkaline copper solution before reacting with FCR. The intensity of blue colour formed after 30 min of incubation was measured colorimetrically at 660 nm. A standard curve of bovine serum albumin was used for obtaining the values [70].

3.2.4.5.6 Thiobarbituric acid reactive substances (TBARS) assay

Beer samples (200 µL) were mixed with 0.8 ml of reagent constituted of 0.37% TBA: 5% TCA: 0.25 N HCl (1:1:1). This mixture was heated for 20 min at 90 °C, cooled to room temperature and centrifuged at 3000xg for 10 min and the supernatant's absorbance was measured at 532 nm. A malondialdehyde–thiobarbituric acid (MDA–TBA) complex molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} was used for calculating TBARS [71].

3.2.4.5.7 Peroxide value (POV)

Beer samples (1 mL) were heated in a water bath at 60 °C for 3 min, and then 30 mL of acetic acid–chloroform solution (3:2 v/v) was added to it and thoroughly agitated for 3 min to dissolve the fat. This was filtered through Whatman No 1 filter paper under vacuum and to the filtrate 0.5 mL of saturated potassium iodide solution was added. This was allowed to stand for exactly five min and then titrated against a 0.01N solution of sodium thiosulfate. POV was calculated and expressed following Eq. 3.2 (S = volume of titration (ml), N = normality of sodium thiosulfate solution, W = weight of sample) [72].

$$POV\left(\frac{\text{meq}}{\text{kg}}\right) = S \times NW \times 1000 \qquad \text{Eq. (3.2)}$$

3.2.4.5.8 Colour measurement

A Hunter Lab Color Quest (Ultrascan Vis, Hunterlab, USA) was used to obtain the colour of the beers in Commission Internationale de I'Eclairage L, a and b (CIELAB) format, and the total change in colour (ΔE) based on L, a and b values was calculated according to the Eq. 3.3 (L_1, a_1, b_1 representing the control and L_2, a_2 and b_2 representing the sample).

$$\Delta E = \sqrt{(L_2 - L_1)^2 + (a_2 - a_1)^2 + (b_2 - b_1)^2} \qquad \text{Eq. (3.3)}$$

3.2.5 Optimization of the extraction of phenolic compounds from *Cyclosorus extensa* with solvents of varying polarities

3.2.5.1 Drying and grinding of plant materials

The plant materials were washed with distilled water, cut into small pieces and dried in a tray dryer at 45 ºC with continuous air flow until a constant weight of the plant samples was obtained. The dried plant materials were ground in a laboratory blender to fine powder and then sieved through a mesh of size 50 US mesh. The powdered samples were immediately transferred to airtight containers and stored at 4 ºC until further use.

3.2.5.2 Experimental design

In the following equation of RSM (Eq. 3.4), x_1 and x_2 are independent variables and the dependant response is denoted by y.

$$y = f(x_1, x_2) + \varepsilon \qquad \text{Eq. (3.4)}$$

Here $\varepsilon$is the experimental error term, which represents any measurement error on the response, as well as other type of variations not counted in f. In case of a curvature in the response surface, a higher degree polynomial called a second-order model is used and is given as Eq. 3.5 [73].

$$y = \beta_0 + \sum_{i=1}^{n} \beta_1 x_1 + \sum \sum_{i<j} \beta_{ij} x_i x_j + \sum_{i=1}^{n} \beta_{ii} x_i^2 + \epsilon \qquad \text{Eq. (3.5)}$$

The central composite design (CCD) is the most popular design for fitting a second-order model. It consists of factorial points (2^k), central points (nc) and axial points ($2k$). Here, the values of n_c, the number of center-point replications, can be chosen so that the CCD can acquire certain desirable properties [73,74]. The total number of design points in a CCD is given by the Eq. 3.6. The CCD is said to be rotatable if the precision of the estimated response surface at some point x depends only on the distance from x to the origin and not on the direction. When the rotatable design is rotated about the center, the variance of $\hat{y}$ will remain same [75].

$$n = 2^k + 2k + n_c \quad \text{Eq. (3.6)}$$

Each of the variables is taken at two levels meaning that each variable has a low and high numeric value. A coded numeric value of -1 and +1 is assigned to represent the variable's low and high values, 0 for the centre points and ± γ for the axial points. The central point or zero point may be defined as the region where the optimal conditions are supposedly met [39]. When the response data are obtained from the test work, a regression analysis is carried out to determine the coefficients of the response model, their standard errors and significance. In addition to the constant (β_0) and error (ε) terms, the response model incorporates linear terms in each of the variables, squared terms in each of the variables and first-order interaction terms for each paired combination. Thus, for the two variables under consideration, the response model is given by the Eq. 3.7.

$$y = (\beta_0 + \varepsilon) + \sum_{i=1}^{2} \beta_i x_i + \sum_{i=1}^{2} \beta_{ii} x_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{2} \beta_{ij} x_i x_j \quad \text{Eq. (3.7)}$$

The β coefficients, which should be determined in thesecond-order model, are obtained by the least squares method. In general Eq. 3.7 can be written in matrix form as given in Eq. 3.8.

$$Y = \beta X + \varepsilon \quad \text{Eq. (3.8)}$$

Where, Y is defined to be a matrix of measured values and X to be a matrix of independent variables. The matrices β and error ε consist of coefficients and errors, respectively. The solution of Eq. 3.9 can be obtained by the matrix approach (Eq. 3.9).

$$\beta = (X' \cdot X)^{-1} X' \cdot Y \quad \text{Eq. (3.9)}$$

Where, X' is the transpose of the matrix X and $(X' \cdot X)^{-1}$ is the inverse of the matrix $X' \cdot X$ [76].

In order to minimise energy cost of the extraction process, time and temperature are important parameters to be optimised. With the increase in working temperature, extraction is enhanced, due to increase in both solubility of solute and the diffusion coefficient, but beyond

a certain value phenolic compounds may be denatured [77]. The experimental runs which were carried out according to the CCRD design were for the two identified design independent variables, namely, extraction time in hours (β_1) and extraction temperature in °C (β_2) and for all the four solvents viz. hexane (Hex), ethyl acetate (EA), methanol (MeOH) and distilled water (H_2O). The extreme values of the variables were X_1 (12 and 48 h) and X_2 (25 and 55 ºC). The central values of the variables were X_1 (30 h) and X_2 (40 ºC). The total numbers of experiments generated were 13 (Eq. 3.5). The responses studied were total polyphenols content, radical scavenging activity, antibacterial activity and antifungal activity.

3.2.5.3 Extraction procedure

Dried powdered samples (10 g) were taken in 250 mL Erlenmeyer flasks and 150 mL of each of the solvents viz. Hex, EA, MeOH and H_2O were added to it. The flasks were shaken in an incubator shaker at 150 rpm and maintained at the specified conditions of time and temperature as given by the design. Following this, the mixture was filtered through four layers of muslin cloth and then centrifuged at 1000xg for 10 min and further filtered through Whatman No. 1 filter paper in a vacuum assisted filtration unit. The removal of chlorophyll from the polar extracts was done by washing with carbon tetrachloride twice in a separating funnel.

3.2.5.4 Estimation of total phenolic compounds (TPC)

The total phenolic compounds in the extracts were analyzed by the method of Slinkard and Singleton [41] by using Folin-Ciocalteu reagent as described in section **3.2.4.5.3**

3.2.5.5 Estimation of radical scavenging activity (RSA)

The free radical scavenging activity was measured as per the method of Brand-Williams et al. [56] as described in section **3.2.4.5.4**

3.2.5.6 Estimation of antibacterial activity (ABA) and antifungal activity (AFA)

The bacterium *Escherichia coli* MTCC 40 and the mould *A. niger* MTCC 281 were used as test organism for assessment of antibacterial [51] and antifungal [52] activities as described in section **3.2.2.4.**

3.2.6 Statistical analysis

Calculation of mean values, standard deviation and plotting of curves were done using the statistical software OriginPro 8.0 (OriginLab Corporation, USA) and results were expressed as average of three replicates. The analysis of variance (ANOVA) Statistical analysis was performed using Statistical Package for Social Sciences (SPSS) software (ver. 20, SPSS, Inc, Chicago, IL, USA) and the Fisher`s least significant difference (LSD) was taken at $p<0.05$. The statistical software Design Expert Ver. 6.0.11 (Stat-Ease Inc., Minneapolis, USA) was used for design of experiments, regression and graphical analyses of the data obtained, and statistical analysis of the model to evaluate the analysis of variance.

3.3 Results and discussions

3.3.1 Phytochemical constituents, ATR-FTIR analysis and antimicrobial activity of leaves of *A. heterophyllus, C. extensa, O. corymbosa* and *A. malaccensis*

3.3.1.1 Various phytochemicals in the leaves

The content of various phytochemicals in the leaf samples are presented in Table 3.1.The highest content of total polyphenols (2.06 %) was found in *O. corymbosa*, followed by *A. malaccensis* and *A. heterophyllus* with no significant difference and lowest (0.62 %) was found in *C. extensa*. The highest content of alkaloids content was recorded in *A. heterophyllus* (6.00 mg/g) and lowest in *A. malaccensis* (2.47 mg/g). No significant difference in content was recorded between *C. extensa* and *O. corymbosa*. The highest content of tannins (1.95 mg/g) was found in *C. extensa* and lowest in *A. malaccensis* (0.75 mg/g). No significant difference in content between *A. heterophyllus* and *O. Corymbosa* was observed. *A. heterophyllus* had the highest content of terpenoids (5.79 mg/g), followed by *O. corymbosa* (3.55 mg/g), *A. malaccensis* (2.50 mg/g) and *C. extensa* (1.71 mg/g). The highest

content of flavonoids was recorded in *O. corymbosa* (6.82 mg/g) followed by *C. extensa* (5.47 mg/g), whereas in both *A. heterophyllus* and *A. malaccensis* the content was below 2.00 mg/g. Anthocyanins which belong to the family of flavonoids were also detected and *A. heterophyllus* and *C. extensa* revealed the same anthocyanin content of 0.23 mg/g, but significant difference between *O. corymbosa* (0.45 mg/g) and *A. malaccensis* (0.59 mg/g) was observed. No significant difference in glycosides content among all the species was observed and ranged from 1.45 to 1.52 mg/g. The anthraquinone content was low in all the plant species and ranged from 0.13 mg/g (*C. extensa*) to 0.18 mg/g (*A. malaccensis*). The highest content of saponins was recorded in *C. extensa* (1.78 mg/g), followed by *A. heterophyllus* (1.49 mg/g), *A. malaccensis* (1.26 mg/g) and *O. corymbosa* (1.12 mg/g). The content of phytosterols was relatively high in *O. corymbosa* (0.70 mg/g) and *C. extensa* (0.57 mg/g), while both *A. heterophyllus* and *A. malaccensis* were at par (0.14 mg/g).

Table 3.1 Content of different phytochemicals in the leaves of the four plant species

Phytochemicals	Concentration in the plant leaves (DM)			
	A. heterophyllus	*C. extensa*	*O. corymbosa*	*A. malaccensis*
Polyphenols (%±SD)	1.40 ± 0.28^{b}	0.62 ± 0.04^{a}	2.06 ± 0.39^{c}	1.05 ± 0.10^{b}
Alkaloids (mg/g±SD)	6.00 ± 1.04^{c}	3.57 ± 0.15^{b}	3.57 ± 0.25^{b}	2.47 ± 0.2^{a}
Tannin (mg/g±SD)	1.16 ± 0.06^{b}	$1.95\ \pm0.15^{c}$	$1.22\ \pm0.02^{b}$	0.75 ± 0.01^{a}
Terpenoids (mg/g±SD)	5.79 ± 0.48^{c}	1.71 ± 0.53^{a}	3.55 ± 0.99^{b}	2.50 ± 0.25^{ab}
Flavonoids (mg/g±SD)	1.35 ± 0.08^{a}	5.47 ± 0.20^{c}	6.82 ± 0.20^{d}	1.93 ± 0.32^{b}
Anthocyanin (mg/g±SD)	0.23 ± 0.001^{a}	0.23 ± 0.002^{a}	0.45 ± 0.004^{b}	0.59 ± 0.011^{c}
Gylcosides (mg/g±SD)	1.46 ± 0.02^{a}	1.45 ± 0.08^{a}	1.52 ± 0.03^{a}	1.52 ± 0.07^{a}
Anthraquinone (mg/g±SD)	0.14 ± 0.001^{b}	0.13 ± 0.002^{a}	0.17 ± 0.001^{c}	0.18 ± 0.002^{d}
Saponins (mg/g±SD)	1.49 ± 0.07^{c}	1.78 ± 0.04^{d}	1.12 ± 0.03^{a}	1.26 ± 0.02^{b}
Phytosterols (mg/g±SD)	0.14 ± 0.02^{a}	0.57 ± 0.04^{b}	0.70 ± 0.03^{c}	0.14 ± 0.04^{a}

Note: DM - dry matter; n = 3; Different superscripted alphabets along the rows represent the LSD at $p<0.05$.

The methanolic extract of *A. malaccensis* leaves was also examined by Sahoo et al. (78)[94] and found the total phenolic content 76.25 mg GAE/g of the extract. In another study by Ahmed et al.[79] the methanolic extract of the closely related species *A. nigra* leaf showed the presence of medicinally active secondary metabolites such as alkaloids,

glycosides, cardiac glycosides, flavonoids, steroids, tannins, anthraquinone glycosides, and saponins. The presence of alkaloids, tannins, glycosides, terpenoids, steroids, flavonoids, and saponins has already been reported in the extracts of *O. corymbosa* by Hussain and Kumaresan [80]. The total phenolic and flavonoid contents of the aqueous extract of *O. corymbosa* were also found to be 11.6 mg/gm and 4.4 mg/gm respectively by Yadav and Agarwala [81]. The presence of polyphenols in fairly high amount is interesting, as it has high potential to be used as anticancer agents, antioxidants, food preservatives, antifibrillogenic agents for the fight against neurodegenerative pathologies, and other functionalized materials [82]. Tannins were found in minimal amount and intake of a small quantity of tannin may be beneficial to human health by affecting the metabolic enzymes, immunomodulation, or other functions [83]. The presence of terpenoids and flavonoids also suggest that these plants may be effective against cancer, malaria, inflammation, coronary heart diseases and a variety of infectious diseases [84,85]. Anthraquinones, glycosides and saponins were significantly less in occurrence in all the species. The phytosterols were also present and have role in inhibition of the absorption of intestinal cholesterol, including recirculating endogenous biliary cholesterol [86].

3.3.1.2 Functional groups detected in the SEs

The results of FTIR peak values and the respective functional groups are presented in Table 3.2 and the spectra are illustrated in Figs. 3.1, 3.2, 3.3 and 3.4. The FTIR spectra indicated the presence of various functional groups and compounds in the tested plant extracts and variations in the peaks of all the extracts were observed. Alkanes and alkyl halides were invariably present in all the extracts. Except for the Hex extracts, phenolic groups were present in all the extracts. Carboxylic acids were present in extracts of H_2O, MeOH and EA of *A. heterophyllus*, H_2O and Hex of *C. extensa*, H_2O, MeOH and Hex of *O. corymbosa* and *A. malaccensis*. The alcohols were present in EA extracts of all the species and the H_2O extracts of *A. heterophyllus*, *O. corymbosa* and *A. malaccensis*. Results revealed that the primary amines were present in the H_2O, MeOH and EA extracts. The alkynes were totally absent from *A. heterophyllus* and were present in H_2O, MeOH and Hex extracts of *C. extensa*, *O. corymbosa* and *A. malaccensis*. The carbonyl groups were present in the H_2O and EA extracts of *A. heterophyllus*, *C. extensa* and *A. malaccensis*, but in case of EA extract of *O. corymbosa* their presence was detected. Aliphatic amines were present in the MeOH and EA extracts of *A. heterophyllus*, MeOH and Hex extracts of *C. extensa* and *O. corymbosa* and

MeOH, EA and Hex extracts of *A. malaccensis*. Esters or ethers were present in the EA extract of *A. heterophyllus*, H_2O and EA extract of *C. extensa*, EA extract of *O. corymbosa*, H_2O and EA extract of *A. malaccensis*. Aromatics were present in the EA and Hex extracts of all the species. The ketones, nitro compounds and aromatic amines were present only in the EA extract of all the species. Aldehydes were found only in the H_2O extract of *A. heterophyllus* and *O. corymbosa*. These functional groups suggest the presence of compounds such as flavonoids, saponins, alkaloids, phenols, glycosides, steroids and naphthoquinone [87]. The occurrence of similar pattern of functional groups has also been reported in the leaf extracts of some other plant species like *Albizia lebbeck* by Bobby et al. [88], *Aerva lanata* by Mariswamy et al. [89] and *Hybanthus enneaspermus* by Anand and Gokulakrishnan [90].

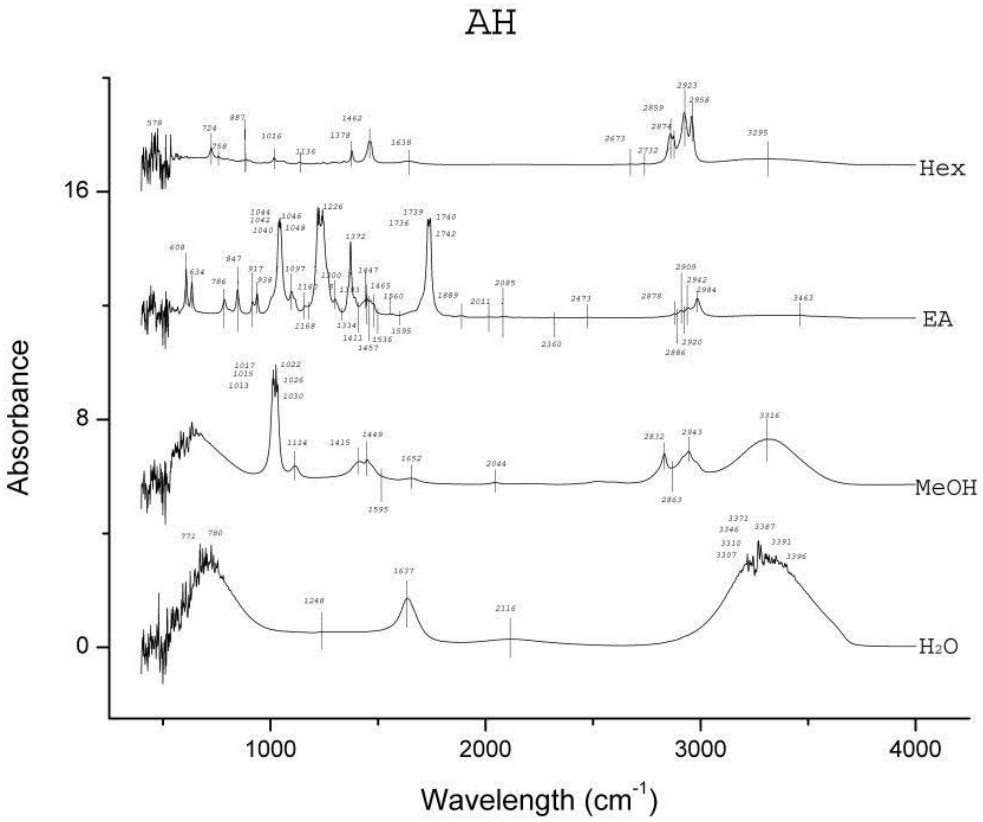


Fig 3.1 FTIR spectra of the four different extracts of *A. heterophyllus* (AH) (Hex: Hexane; EA: Ethyl acetate; MeOH: Methanol; H_20: Water)

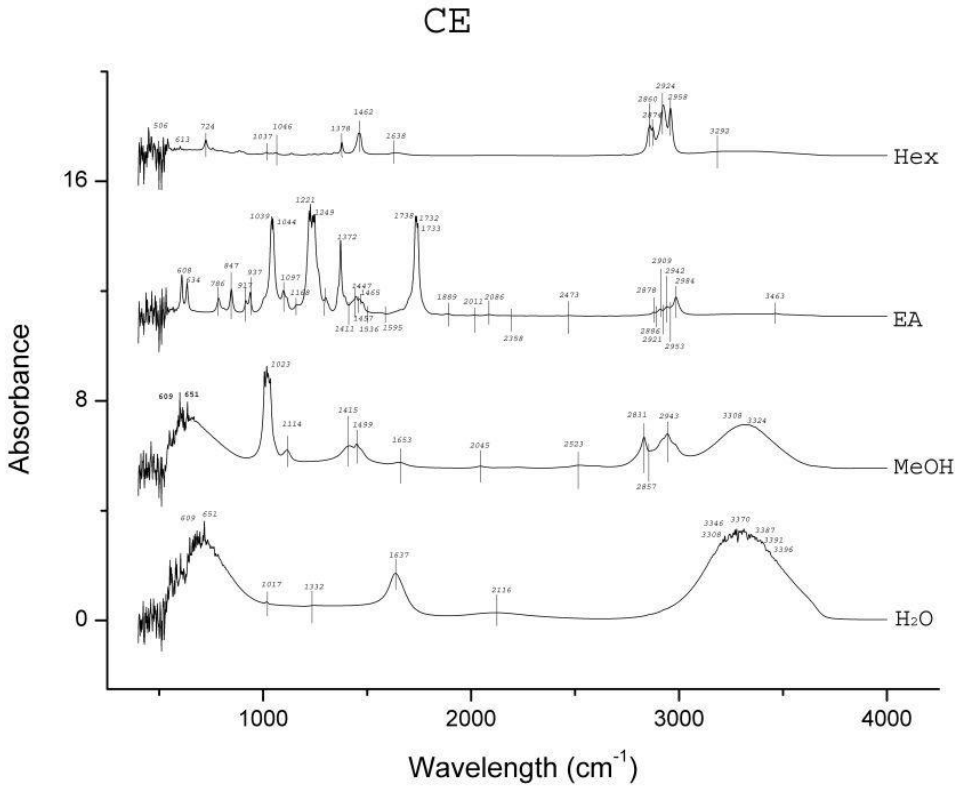


Fig 3.2 FTIR spectra of the four different extracts of *C. extensa* (CE) (Hex: Hexane; EA: Ethyl acetate; MeOH: Methanol; H_20: Water)

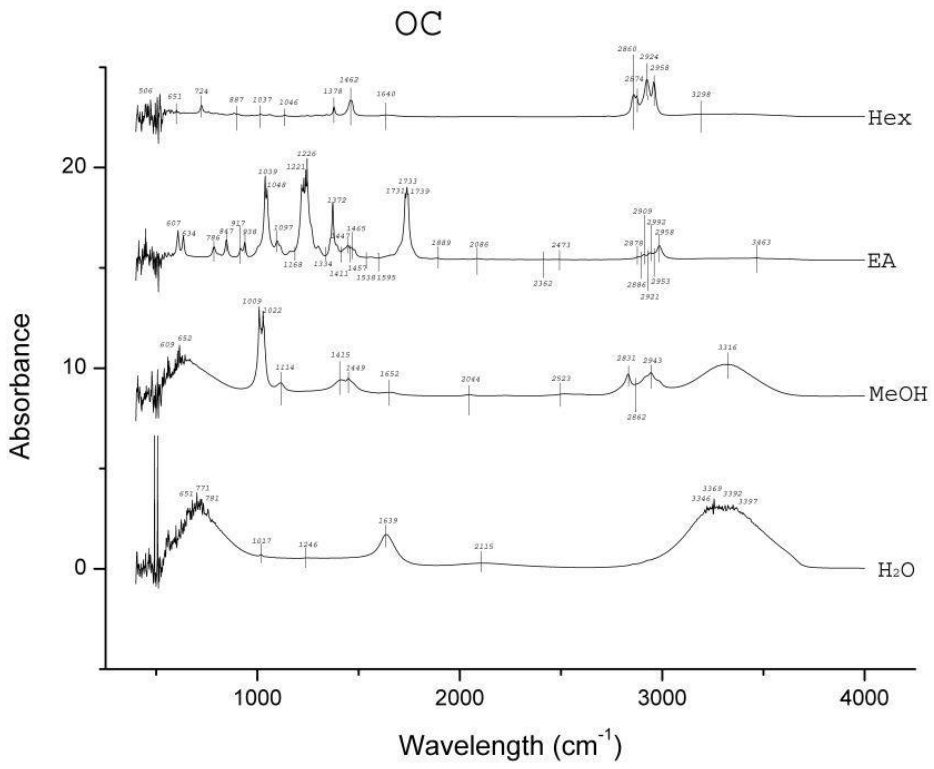


Fig 3.3 FTIR spectra of the four different extracts of *O. corymbosa* (OC) (Hex: Hexane; EA: Ethyl acetate; MeOH: Methanol; H_20: Water)

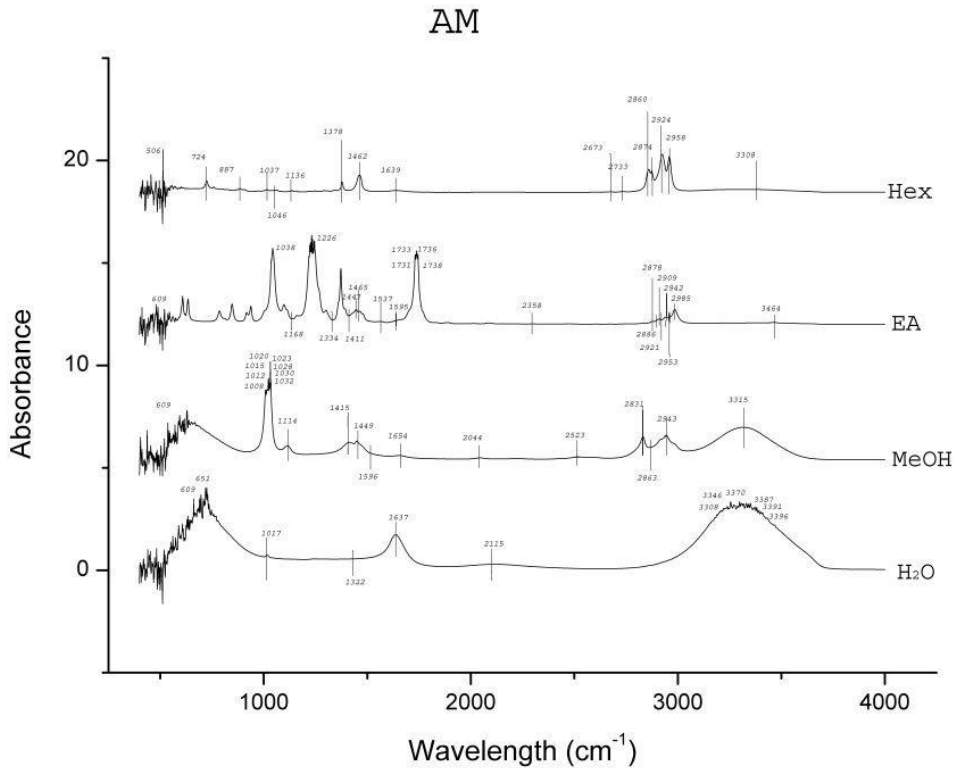


Fig 3.4 FTIR spectra of the four different extracts of *A. malaccensis* (AM) (Hex: Hexane; EA: Ethyl acetate; MeOH: Methanol; H_20: Water)

Table 3.2 The functional groups detected by ATR-FTIR analysis of the water, methanol, ethyl acetate and hexane extracts of the four plant species

Functional groups	Peak ranges (cm^{-1})	*A. heterophyllus*				*C. extensa*				*O. corymbosa*				*A. malaccensis*			
		H_2O	MeOH	EA	Hex	H_2O	MeOH	EA	Hex	H_2O	MeOH	EA	Hex	H_2O	MeOH	EA	Hex
Phenolic groups	3371/ 3369/ 3464/3324/ 3316	√	√	√	x	√	√	√	x	√	√	√	x	√	√	√	x
Carboxylic acids	3397/ 3392/ 2832/ 1046	√	√	√	x	√	x	x	√	√	√	x	√	√	√	x	√
Alcohols/Polyols	3397/ 3387/ 1226	√	x	√	x	x	x	√	x	√	x	√	x	√	x	√	x
Primary amines	3346/ 772	√	√	√	x	√	√	√	x	√	√	√	x	√	√	√	x
Alkynes	3308/ 651/ 613	x	x	x	x	√	√	x	√	√	√	x	√	√	√	x	√
Alkanes	3310/ 2953/ 2886/ 2863/ 2924/ 1457	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Ketones	1637/1739	x	x	√	x	x	x	√	x	x	x	√	x	x	x	√	x
Carbonyl groups	1733/ 1637/ 1249	√	x	√	x	√	x	√	x	x	x	√	x	√	x	√	x
Nitro compounds	1538	x	x	√	x	x	x	√	x	x	x	√	x	x	x	√	x
Aromatics	1462	x	x	√	√	x	x	√	√	x	x	√	√	x	x	√	√
Aromatic amines	1411	x	x	√	x	x	x	√	x	x	x	√	x	x	x	√	x
Aliphatic amines	1389/ 1114/ 1038	x	√	√	x	x	√	x	√	x	√	x	√	x	√	√	√
Esters/ ethers	1311/ 1334	x	x	√	x	√	x	√	x	x	x	√	x	√	x	√	x
Alkyl halides	1168/ 771/ 767/ 609/ 578/ 506	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Aldehydes	781	√	x	x	x	x	x	x	x	√	x	x	x	x	x	x	x

Note: H_2O – water; MeOH - methanol; EA - ethyl acetate; Hex – hexane; √ - Present; x – absent

3.3.1.3 Antimicrobial activity of the SEs

The antimicrobial activities of the SEs are presented in Table 3.3 and wide variation in the activity of the extracts against different pathogens was seen. *C albicans* is a normal commensal of humans but can be pathogenic if a person's immunity is lowered resulting in oral and genital infections and is the causative organism for candidaitis [91]. The H_2O extract of none of the species was found to be effective against *C. albicans*. The MeOH extracts of *A. heterophyllus* and *C. extensa* were also found to have no activity; however, the MeOH extracts of *O. corymbosa* and *A. malaccensis* showed zones of 5.00 mm and 11.25 mm respectively. The EA extract of *A. heterophyllus*, *C. extensa* and *A. malaccensis* revealed inhibition zones of 16, 10 and 14 mm respectively, but EA extract of *O. corymbosa* did not show any activity. On the other hand, the Hex extract of only *A. malaccensis* showed a zone of 15.50 mm. However, was observed that none of the SEs were effective against the mould *F. oxysporum. E. coli* is an important member of the coliform group and most strains of *E. coli* are commonly a part of the normal flora of the gut and are harmless, but some causes serious food poisoning, gastroenteritis, diarrhoea and urinary tract infection [92]. The H_2O extract of only *A. malaccensis* showed inhibition zone of 6.25 mm. In case of MeOH extracts largest zone was shown by *A. malaccensis* (19.50 mm), followed by *C. extensa* (12.75 mm) and *O. corymbosa* (11.50 mm). All the EA extracts showed activity and the highest was shown by *A. heterophyllus* (33.25 mm) and *A. malaccensis* (29.00 mm), while in case of both *C. extensa* and *O. corymbosa* the zones remained in the range of 16 mm. The Hex extract of *A. heterophyllus* showed a zone of 22.00 mm and less activity was shown by *A. heterophyllus* (3 mm) and there was no activity by *C. extensa* and *O. corymbosa*. Some strains of *S. aureus* are a common cause of skin infections, respiratory diseases, and food poisoning [87]. The H_2O extract of none of the species was found to be effective against *S. aureus*. In case of MeOH extracts, *A. heterophyllus* showed a zone of 12.25 mm and 5.00 mm in case of *O. corymbosa*. All the EA extracts were found to be effective, with *A. heterophyllus* showing the largest zone of 23.50 mm followed by *A. malaccensis* (17.50 mm). Both the EA extracts of *C. extensa* and *O. corymbosa* showed zones in the range of 12 mm. The Hex extract of only *O. corymbosa* showed a zone of 5.00 mm. The Gram-positive bacterium *B. subtilis* can form endospore and can survive in extreme environmental conditions of temperature and desiccation. It is also responsible for the formation of biofilms [93]. Even in this case the EA extracts were found to be effective and highest activity was that of *A. heterophyllus* and *A. malaccensis.*

Table 3.3 Antimicrobial activity of the four plant extracts

Plant species	Solvent	Diameter of zone of inhibition (mm ± SD; n=3)						
		F. oxysporum	*C. albicans*	*S. cerevisae*	*E. coli*	*S. aureus*	*L. plantarum*	*B. subtilis*
A. heterophyllus	H_2O	NA	NA	NA	NA	NA	NA	NA
	MeOH	NA	NA	NA	NA	NA	NA	NA
	EA	NA	16.00±0	NA	33.25±2.22	23.50±0.58	NA	18.22±0.42
	Hex	NA	NA	NA	3.00±0.60	NA	NA	NA
C. extensa	H_2O	NA	NA	NA	NA	NA	NA	NA
	MeOH	NA	NA	NA	12.75±0.50	NA	NA	NA
	EA	NA	10.00±0	NA	16.50±0.58	12.00±0.00	NA	12.45±0.24
	Hex	NA	NA	NA	NA	NA	NA	NA
O. corymbosa	H_2O	NA	NA	NA	NA	NA	NA	NA
	MeOH	NA	5.00±0.77	NA	11.50±0.58	5.00±0.77	NA	5.00±0.24
	EA	NA	NA	NA	16.00±1.41	12.75±0.50	NA	10.08±0.55
	Hex	NA	NA	NA	NA	5.00±0.77	NA	NA
A. malaccensis	H_2O	NA	NA	NA	6.25±0.23	NA	NA	NA
	MeOH	NA	11.25±0.50	NA	19.50±1.29	12.25±0.83	NA	10.88±0.41
	EA	NA	14.00±0.00	NA	29.00±2.45	17.50±0.58	NA	18.19±0.48
	Hex	NA	15.50±1.29	NA	22.00±3.37	NA	NA	NA

Note: H_2O – water; MeOH - methanol; EA - ethyl acetate; Hex – hexane; NA – no activity

Substantial activity of the SEs against the tested water and food borne pathogens was observed. The folklore or traditional belief stands that rice beer has the potential to treat various ailments of microbial origin. Hence, the inhibitory effects shown by the SEs justifies the usage of these plant leaves in the rice beer making process. Moreover, this effectiveness also implies their usage in food preservation in general and rice beer in particular. It was interesting to see that none of the SEs could inhibit the growth of the fermenting organisms viz. *S. cerevisae* and *L. plantarum. S. cerevisiae*, also known as brewer's yeast is one of the most notable and well-known species of yeast in fermentation, health and wellness. It is used as a protein supplement, energy booster, immune enhancer, or other vehicle for compound insertion [94]. *L. plantarum* is found in a variety of fermented foods and is a natural inhabitant of the human gastrointestinal tract. It is a potentially probiotic lactic acid producing bacteria [95]. The ineffectiveness of the SEs against these representative beneficial fermentative microorganisms signifies a positive implication of these plants' use in the fermentation process of rice beer. The results also revealed that the EA extracts of all the species exhibited high antimicrobial activity. The FTIR analysis (Table 3.3) also favourably supported the higher number of functional groups in EA extracts as compared to the other solvents. The major peaks shown in the EA extracts were of ketones, alcohols and carboxylic acids in case of *A. heterophyllus* (Fig 3.1), carbonyl group, alcohols and aliphatic amines in case of *C. extensa* (Fig 3.2), carbonyl group and alcohols in case of *O. corymbosa* (Fig 3.3) and carbonyl group, alcohols and aliphatic amines in case of *A. malaccensis* (Fig 3.4). The MeOH extracts of *C. extensa*, *O. corymbosa* and *A. malaccensis* also exhibited antimicrobial properties, especially against *E. coli.* In the MeOH extracts, the major peaks were shown by phenolics in case of *C. extensa*, *O. corymbosa* and *A. malaccensis*; carboxylic acids in case of *C. extensa* and *A. malaccensis*; alkanes in case of *C. extensa* and aliphatic amines in case of *A. malaccensis*. In Hex extracts, substantial activity was exhibited by *A. malaccensis* and the major peaks were shown by the alkanes, alkynes and aromatics.

In the work of Pauline et al. [1] with antibacterial effect of *C. interruptus* extracts against *S. aureus*, it was seen that the highest activity was of the crude acetone extracts at a minimum concentration 31.255 µg/100µl. This was followed by the ethyl acetate and chloroform fractions of the crude extract. In the work of Loizzo et al. [31] the total water extract, ethyl acetate, and aqueous fractions from the leaves of *A. heterophyllus* were evaluated for antibacterial activities by agar diffusion method against *E. coli*, *B. cereus*, and *S. aureus.* They reported the diameters of inhibition 12.2 mm for the total extract, 10.7 mm and 11.5 mm for ethyl acetate and aqueous fractions, respectively. The antibacterial activity

observed for the methanolic extract of the closely related species *A. nigra* leaf at 2 mg/disc was found to be mild as compared to tetracycline (50 mg/disc) [79]. The extract *O. corymbosa* were also found to have antimicrobial activity against both Gram (+) and Gram (-) bacteria by Hussain and Kumaresan [80].

3.3.1.4 MIC values of the SEs

The minimum inhibitory concentrations (MICs) are defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism. MICs are very helpful in determining the *in vitro* activity of new antimicrobials against a wide range of test organisms [96]. The MIC values of the extracts which evinced positive antimicrobial activity are shown in Table 3.4. In case of *C. albicans*, the EA extract of *A. heterophyllus* was found to be the most effective with a MIC value of 75 µg/mL and was followed by the EA extract of *A. malaccensis* with a value of 100 µg/mL. Similar values were observed for the EA extract of *C. extensa* and Hex extract of *A. malaccensis* (120 µg/mL) and MeOH extract of *O. corymbosa* and MeOH extract of *A. malaccensis* (150 µg/mL). For *E. coli* the EA extract of *A. malaccensis* was found to be the most effective with a MIC value of 45 µg/mL. Both the EA extract of *A. heterophyllus* and the MeOH extract of *A. malaccensis* evinced a value of 50 µg/mL, which was followed by Hex extract of *A. malaccensis* (60 µg/mL), EA extract of *O. corymbosa* (70 µg/mL), EA extract of *C. extensa* (80 µg/mL), MeOH extract of *O. corymbosa* (120 µg/mL) and MeOH extract of *C. extensa* (170 µg/mL) respectively. The Hex extract of *A. heterophyllus* (270 µg/mL) and H_2O extract of *A. malaccensis* (350 µg/mL) were found to be the least effective. For *S. aureus* the highest activities were shown by the EA extracts of *A. heterophyllus* (60 µg/mL) and *A. malaccensis* (100 µg/mL). The MIC value of EA extract of *O. corymbosa* and MeOH extract of *A. malaccensis* were similar (130 µg/mL), and followed by MeOH extract of *O. corymbosa* (190 µg/mL), EA extract of *C. extensa* (200 µg/mL) and Hex extract of *O. corymbosa* (280 µg/mL) respectively. In case of *B. subtilis* also, the lowest MIC value was of the EA extracts of *A. heterophyllus* (130µg/mL) and was followed by the EA extracts of *A. malaccensis* (180 µg/mL), while the EA extracts of *C. extensa* and *O. corymbosa* had similar MIC values. In comparison, the effectivity of streptomycin against *E. coli*, *S. arueus* and *B. subtilis* in terms of its MIC value was found to be 20 µg/mL, 50 µg/mL and 30 µg/mL respectively. While, the MIC value of chloramphenicol against *C. albicans* was 25µg/mL. In similar studies, Loizzo et al. [31] also found the MICs of *A. heterophyllus* leaves to range from 221.9 µg/mL

for ethyl acetate fraction to 488.1 µg/mL for total extract against some common food borne pathogens. Sahoo et al. [78] also reported the MIC values of the methanolic extract of *A. malaccensis* to be 2.5 µl/mL, 10 µl/mL, 8.5 µl/mL and 9.5 µl/mL against *S. aureus*, *P. aeruginosa*, *C. albicans* and *A. niger* respectively.

Table 3.4 Minimum inhibitory concentration (MIC) values of the four plant extracts

Plant species	Solvent	Concentration (µg/mL)			
		C. albicans	*E. coli*	*S. aureus*	*B. subtilis*
A. heterophyllus	EA	75	50	60	130
	Hex	--	270	--	--
C. extensa	MeOH	--	170	--	--
	EA	120	80	200	200
O. corymbosa	MeOH	150	120	190	300
	EA	--	70	130	200
	Hex	--	--	280	--
A. malaccensis	H_2O	--	350	--	--
	MeOH	150	50	130	
	EA	100	45	100	180
	Hex	120	60	--	--
Streptomycin		--	20	50	30
Chloramphenicol		25	--	--	--

Note: H_2O – water; MeOH - methanol; EA - ethyl acetate; Hex – hexane

3.3.2 Antioxidant activity of the polyphenols purified from leaves of *Artocarpus heterophyllus, Cyclosorus extensa, Oldenlandia corymbosa* and *Alpinia malaccensis*

3.3.2.1 Content of phenolic compounds in the leaves

The HPLC chromatograms of phenolic compounds in PPEs are illustrated in Fig 3.5 and various concentrations are presented in Table 3.5. Quinic acid ($C_7H_{12}O_6$) was present in *O. corymbosa* (2277.69 ppm) and *A. malaccensis* (1703.75 ppm); however, it was not detected in both *A. heterophyllus* and *C. extensa*. Caffeic acid ($C_9H_8O_4$) was detected in *C. extensa* (47.63 ppm) and *O. corymbosa* (110.68 ppm), and not found in *A. heterophyllus* and

A. malaccensis. Ferulic acid ($C_{10}H_{10}O_4$) was found in *C. extensa* (912.75 ppm) and *A. heterophyllus* (400.48 ppm) and low content was observed in *O. corymbosa* (48.73 ppm) and *A. malaccensis* (15.06 ppm). The highest content of *p*-coumaric acid ($C_9H_8O_3$) was detected in *A. heterophyllus* (90.05 ppm) and lowest in *C. extensa* (5.87 ppm) and not recorded in both *O. corymbosa* and *A. malaccensis*. The highest concentration of chlorogenic acid ($C_{16}H_{18}O_9$) was found in *O. corymbosa* (259.15 ppm) and lowest in *A. heterophyllus* (24.69 ppm) and absent in both *C. extensa* and *A. malaccensis*. Quercetin ($C_{15}H_{10}O_7$) was recorded in all the four plant species and the highest concentration was observed in *O. corymbosa* (2088.74 ppm), followed by *A. heterophyllus* (282.19 ppm), *A. malaccensis* (202.33 ppm) and *C. extensa* (150.26 ppm). Salicylic acid ($C_7H_6O_3$) was t detected in all the four plant species in reasonably high content except in *A. malaccensis* and the highest content was recorded in *A. heterophyllus* (24164.93 ppm), followed by *O. corymbosa* (12534.11 ppm) and *C. extensa* (4412.78 ppm). Naringin ($C_{27}H_{32}O_{14}$) content was found to be highest in C. *extensa* (16020.16 ppm) followed by *A. malaccensis* (4842.14 ppm) and absent in *A. heterophyllus* and *O. corymbosa*. Negligible content of catechin ($C_{15}H_{14}O_6$) was recorded in *O. corymbosa* (8.94 ppm) and *A. malaccensis* (3.14 ppm) and absent in *A. heterophyllus* and *C. extensa*. Catechol ($C_6H_4(OH)_2$) was detected only in *C. extensa* and *A. malaccensis* having concentrations of 74.91 ppm and 36.90 ppm respectively. Resorcinol ($C_6H_6O_2$) was detected only in *C. extensa* (30.91 ppm). Apigenin ($C_{15}H_{10}O_5$) was detected in *A. malaccensis* in high concentration (17785.22 ppm), followed by *A. heterophyllus* (5394.60 ppm), *C. extensa* (4340.50 ppm) and *O. corymbosa* (1080.67 ppm). In the present study gallic acid, vanillic acid, syringic acid and hydroquinone were not detected in all the tested four plant species.

Thus, a wide array of polyphenols was detected in all the tested four plant species. Salicylic acid, a β-hydroxyphenolic acid was found in high amount in all the four species and it has the ability to ease aches, pains and reduce fevers [97]. From the flavonoid group, quercetin, naringin, apigenin and catechin were detected in fairly good amounts. Quercetin is a potential anti-cancer agent, including cell cycle regulation, interaction with type II estrogen binding sites, tyrosine kinase inhibition and has also reports of inhibiting tumour growth [98]. Naringin's beneficiary activities include anti-inflammatory, antioxidant, cardioprotective, lowering of blood glucose, and cholesterol concentrations and improved insulin signalling [99]. Apigeninhas been shown to possess remarkable anti-inflammatory, antioxidant, anticarcinogenic, antimutagenic, antiproliferative and antiprogression properties [100]. Catechin can exert vascular protective effects through multiple mechanisms like antioxidative, anti-hypertensive, anti-inflammatory, anti-proliferative, anti-thrombogenic, and

lipid lowering effects [101]. Caffeic acid and its biosynthesized form ferulic acid were present and they have many physiological functions like antioxidant, antimicrobial, antiinflammatory, antithrombosis, and anticancer activities. They are also effective against coronary disease, lowers cholesterol and increases sperm viability [102]. The cinnamic acid derivatives *p*-coumaric acid and chlorogenic acid were also present at variable amounts. *p*-Coumaric acid has antioxidant properties, decreases peroxidation of low density lipoprotein and has potential to reduce the formation of carcinogenic nitrosamines [103]. Chlorogenic acid has been associated with reduction in risk of cardiovascular disease, type 2 diabetes, Alzheimer's disease, antibacterial and antiinflammatory activities [104]. Quinic acid was present and it possesses DNA repair, immune and antiinflammatory enhancing properties and versatile chiral starting material for the synthesis of new pharmaceuticals [105]. Resorcinol and catechol were also present in small quantities.

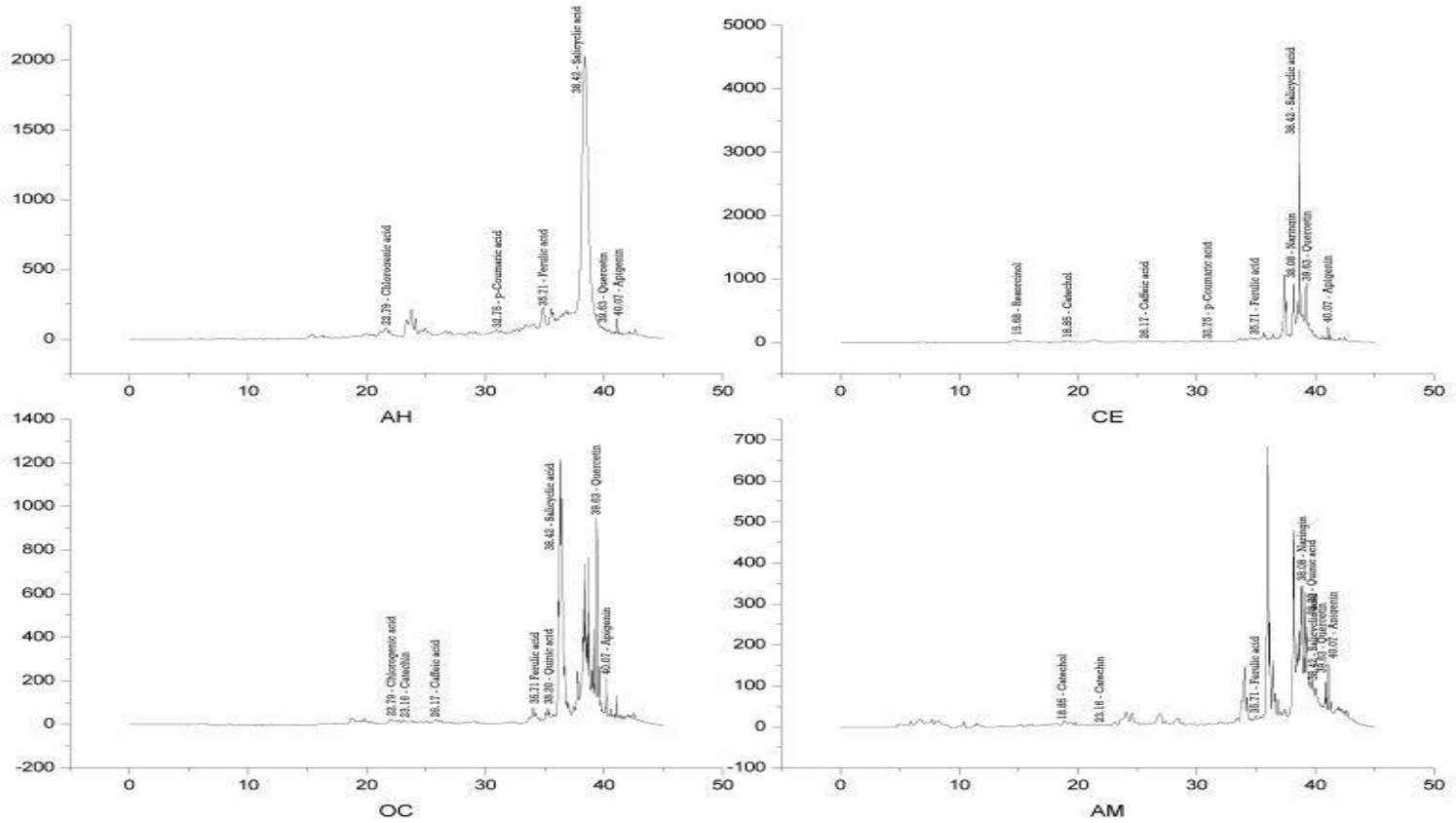


Fig. 3.5 HPLC chromatograms of the PPE for detection of phenolic compounds (AH: *A. heterophyllus*; CE: *C. extensa*; OC: *O. corymbosa*; AM: *A. malaccensis*)

Table 3.5 Phenolic compounds detected by HPLC in the PPE of the four plant species

Phenolic compound	RT (min)	Concentration in the plant leaves (ppm)			
		A. heterophyllus	*C. extensa*	*O. corymbosa*	*A. malaccensis*
Gallic acid	7.16	ND	ND	ND	ND
Hydroquinone	8.91	ND	ND	ND	ND
Resorcinol	15.68	ND	30.91	ND	ND
Catechol	18.85	ND	74.91	ND	36.90
Chlorogenic acid	22.79	24.69	ND	259.15	ND
Catechin	23.16	ND	ND	8.94	3.14
Vanillic acid	25.15	ND	ND	ND	ND
Caffeic acid	26.17	ND	47.63	110.68	ND
Syringic acid	26.43	ND	ND	ND	ND
p-coumaric acid	32.75	90.05	5.87	ND	ND
Ferulic acid	35.71	400.48	912.75	48.73	15.06
Naringin	38.08	ND	16020.16	ND	4842.14
Quinic acid	38.20	ND	ND	2277.69	1703.75
Salicylic acid	38.42	24164.93	4412.78	12534.11	95.26
Quercetin	39.63	282.19	150.26	2088.74	202.33
Apigenin	40.07	5394.60	4340.50	1080.67	17785.22

Note: RT - retention time; ND - not detected

3.3.2.2 DPPH and ABTS scavenging activity

DPPH free radical scavenging activities of the PPEs are shown in Fig. 3.6a and the highest activity was found in *O. corymbosa* (95.09 % at 100 µg/mL) and *C. extensa* (93.39 % at 100 µg/mL) and this result was close to the reference standard BHT (95.33% at 100 µg/mL) and followed by *A. malaccensis* (91.43 % at 100 µg/mL) and *A. heterophyllus* (84.34 % at 100 µg/mL). The IC_{50} values (Table 3.6) of all the PPEs including BHT was below the lowest concentration of the PPEs (20 µg/mL) taken for analysis.

ABTS radical decolourisation activities of the PPEs against the respective concentrations are presented in Fig. 3.6b. At the concentration of 100 µg/mL the highest activity was noted in *C. extensa* (96.8 %) which was even higher than BHT (95.79 %). The activity of *A. heterophyllus* (90.67 % at 100 µg/mL) was also close to *C. extensa* throughout

all concentrations. The IC_{50} values (Table 3.6) of the PPEs were in the order of *A. heterophyllus* (28.64 µg/mL) > *C. extensa* (39.95 µg/mL) > *O. corymbosa* (64.86 µg/mL) > *A. malaccensis* (103.76 µg/mL).

Free radicals play important role in the development of numerous chronic and degenerative diseases and have been implicated in premature ageing [106]. The results of the DPPH free radical scavenging activity assay suggest a good potential of all the four PPEs. DPPH is a stable free radical which has an unpaired valence electron at one atom of nitrogen bridge and the ability to scavenge this free radical is considered an important antioxidant property [107].

3.3.2.3 •OH, H_2O_2, O2•- and NO• scavenging activity

Hydroxyl radical scavenging activities of the PPEs are illustrated in Fig. 3.6c. The scavenging activities of all the four PPEs were close, however, *C. extensa* showed the highest activity of 25.53 % at concentration of 300 µg/mL. The IC_{50} value of α-tocopherol was lower than the lowest experimental concentration (50 µg/mL) and the IC_{50} values (Table 3.6) of all the PPEs were not found within the highest concentration (300 µg/mL) taken for analysis. H_2O_2 scavenging activities assay of the PPEs (Fig. 3.6d) revealed that at the highest concentration of 300 µg/mL, the activity of *C. extensa* (71.23 %) was highest, followed by *A. heterophyllus* (65.76 %), *A. malaccensis* (65.21 %) and *O. corymbosa* (60.31 %). However, their values were lower than the standard α-tocopherol (82.21 %). The IC_{50} values (Table 3.6) of the PPEs were in the order of *A. malaccensis* (146.50 µg/mL) > *C. extensa* (165.76 µg/mL) > *A. heterophyllus* (171.48 µg/mL) > *O. corymbosa* (201.04 µg/mL). Superoxide anion scavenging activities of the PPE are illustrated in Fig. 3.7a and *A. heterophyllus*, *C. extensa* and *A. malaccensis* evinced better results over α-tocopherol (47.30 % at 300 µg/mL), and *A. heterophyllus* was found to be the most potent in the scavenging of superoxide anions. *C. extensa* and *A. malaccensis* also exhibited better results as compared to the reference standard. It was also observed that the IC_{50} values (Table 3.6) of the three PPEs viz., *A. heterophyllus* (120.92 µg/mL), *C. extensa* (298.75 µg/mL) and *A. malaccensis* (293.84 µg/mL) were lower than α-tocopherol. Nitric oxide scavenging activity of the PPE against various concentrations (Fig 3.7b) revealed that the PPEs of *A. malaccensis* was the most effective with an activity of 56.24 % at 300 µg/mL and an IC_{50} value of 52.51 µg/mL. Furthermore, *O. corymbosa* and *A. heterophyllus* also evinced substantial activity and both recorded IC_{50} values of 130.40 µg/mL and 256.93 µg/mL respectively (Table 3.6), and was

lesser than the reference standard BHT, however, *C. extensa* was found to have comparatively lesser activity.

The PPEs were found to scavenge hydroxyl radical, hydrogen peroxide, superoxide anion and nitric oxide radical. Hydroxyl radical is the most reactive species among the oxygen radicals and induces severe damage to nearby biomolecules. It acts by abstracting hydrogen atoms from biological molecules, thereby leading to the formation of sulfur radicals. These radicals are capable of combining with oxygen to generate oxysulfur radicals, which are toxic to biomolecules [108]. Hydrogen peroxide may give rise to hydroxyl radicals inside the cell which can be toxic. The scavenging of H_2O_2 may be attributed mainly to the phenolic compounds which can donate electrons to H_2O_2, thereby neutralizing it to water [107]. A superoxide anion can be generated by a xanthine oxidase/hypoxanthine system. Superoxide radical acts as a precursor of the more reactive oxygen species such as the hydroxyl radical (HO•), peroxynitrite ($ONOO^-$) and singlet oxygen, which are very harmful to cellular components and causes tissue damage and various diseases in the body [109]. Nitric oxide radical is a reactive oxygen species and is implicated in inflammation, cancer and other pathological conditions in the body and can alter the structure and function of many cellular components [110].

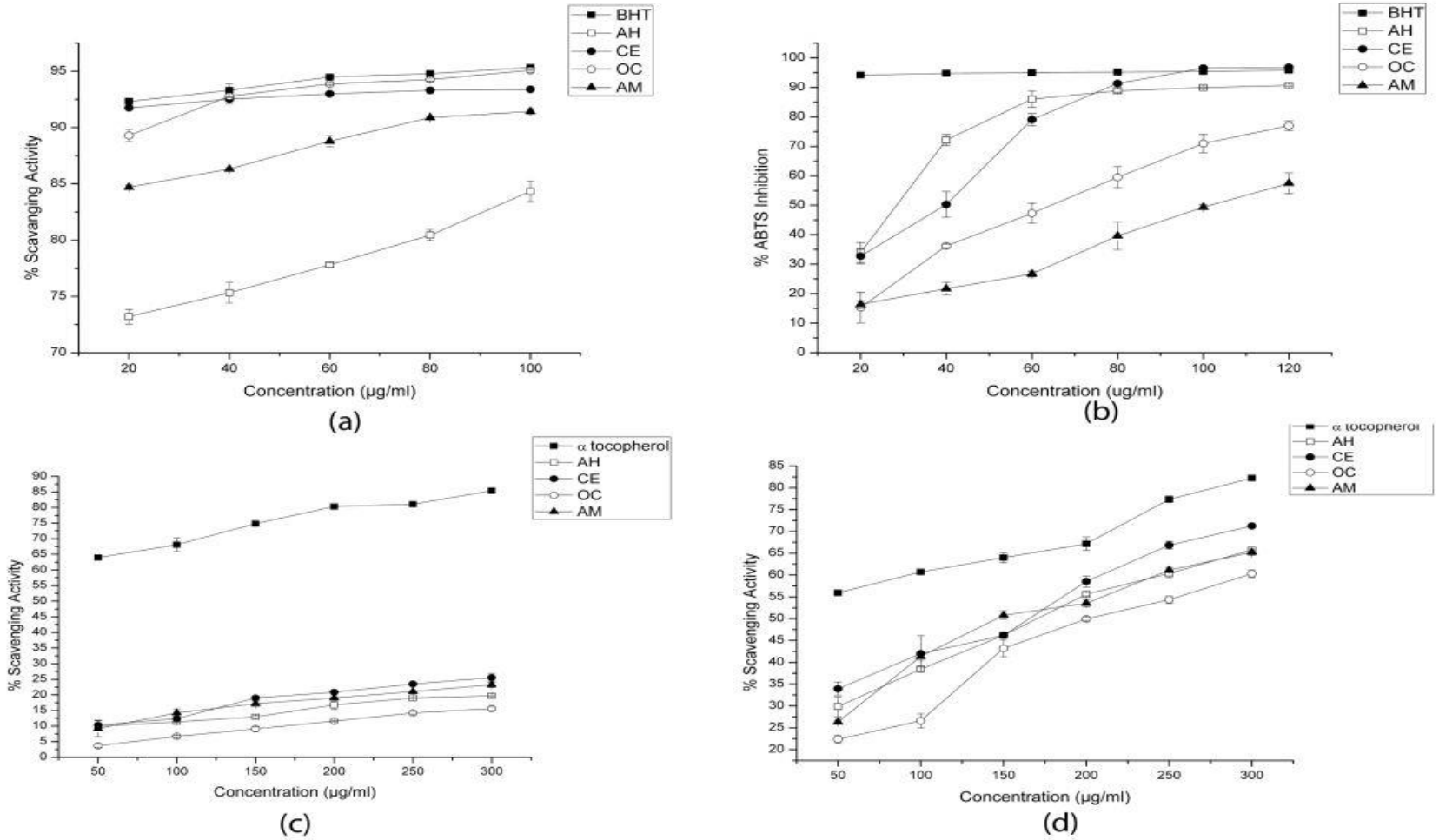


Fig. 3.6 (a) DPPH free radical scavenging activity assay of the PPE; (b) ABTS radical cation decolourisation assay of the PPE; (c) Hydroxyl radical scavenging assay of the PPE; (d) Hydrogen peroxide scavenging activity assay of the PPE. The results are means ± SD (n=3); (AH: *A. heterophyllus*; CE: *C. extensa*; OC: *O. corymbosa*; AM: *A. malaccensis*)

3.3.2.4 Fe^{2+}chelating activity and FRAP assay

Ferrous ion chelating activity of the PPEs against various concentrations (Fig 3.7c) revealed that none of the PPEs were as effective as the positive control EDTA. However, among the PPEs, *C. extensa* produced the highest activity with an IC_{50} value of 230.38 µg/mL (Table 3.6), followed by *A. malaccensis*, *O. corymbosa* and *A. heterophyllus* at the maximum concentration of 300 µg/mL. FRAP assay of the PPEs (Fig 3.7d) revealed that the reducing power of *C. extensa* was almost at par with α-tocopherol. A linear increase in reducing power of all the PPEs with an increase in concentration was observed. In all the concentrations studied, the order of reducing power of the PPEs was *C. extensa* > *A. heterophyllus* > *O. corymbosa* > *A. malaccensis*.

The PPE also exhibited ferrous ions chelating and ferric reducing activities. The ferrous ions may be released in the breakdown of red blood cells, causing the levels of ferrous ion in the body to increase and it may also be implicated in human cardiovascular disease [111]. The reducing power (electron donating capacity) of bioactive compounds is associated with antioxidant activity. Fe(III) reduction is an important mechanism of phenolic antioxidant action, and the transformation of Fe(III) to Fe(II) due to the reductive ability of the four PPEs can be a strong indicator of the antioxidant activity.

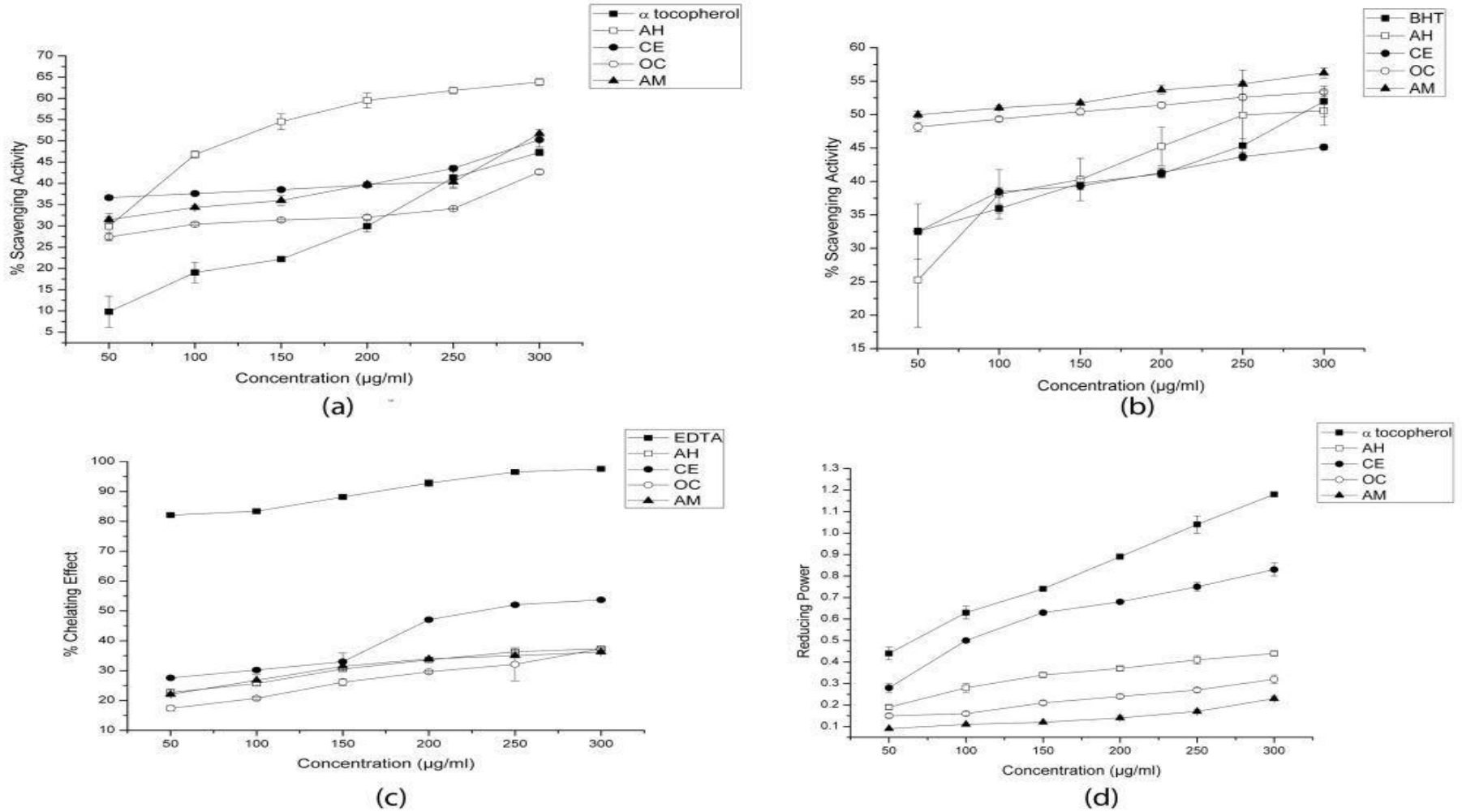


Fig 3.7 (a) Superoxide anion scavenging activity assay of the PPE; (b) Nitric oxide scavenging activity assay of the PPE; (c) Ferrous-ion chelating assay of the PPE; (d) Ferric reducing antioxidant power assay of the PPE. The results are means ± SD (n=3); (AH: *A. heterophyllus*; CE: *C. extensa*; OC: *O. corymbosa*; AM: *A. malaccensis*)

Table 3.6 The IC_{50} values of the PPE under various assay conditions

Assay conditions	IC_{50} values (µg/mL) of PPE				BHT/ α-tocopherol
	A. heterophyllus	*C. extensa*	*O. corymbosa*	*A. malaccensis*	
DPPH scavenging	+	+	+	+	+
ABTS decolourisation	28.64	39.95	64.86	103.76	+
•OH scavenging	-	-	-	-	+
H_2O_2 scavenging	171.48	165.76	201.04	146.50	+
NBT scavenging	120.92	298.75	-	293.84	-
NO• scavenging	256.93	-	130.40	52.51	286.25
Fe^{2+} chelating	-	230.38	-	-	+
FTC inhibition (0 h)	-	-	-	-	-
FTC inhibition (24 h)	+	+	+	+	+
FTC inhibition (48 h)	+	+	+	+	+
FTC inhibition (72 h)	146.72	50.00	103.01	190.04	112.77
TBA inhibition (0 h)	-	-	-	-	-
TBA inhibition (24 h)	-	-	280.06	-	275.00
TBA inhibition (48 h)	+	+	+	+	+
TBA inhibition (72 h)	+	+	+	+	+
TBA inhibition (96 h)	+	+	+	+	+

Note: "+" denotes above 50% activity of all assay concentrations; "-" denotes below 50% activity of all assay concentrations

3.3.2.5 FTC and TBA assay

Ferric thiocyanate assay of the PPEs (Table 3.7) revealed that at 0 h all the PPEs exhibited low activity and their IC_{50} values (Table 3.6) including α-tocopherol were higher than the highest concentration (300 µg/mL) taken for analysis and in addition the highest activity at all concentrations was that of *O. corymbosa* and lowest activity of inhibition was that of the positive control. At 24 h and 48 h, all concentrations of various PPEs and α-tocopherol substantially inhibited the peroxidation of lipid at the highest level. The activity of all the PPE was above 68% and the highest inhibition activity at this period was that of *A. heterophyllus* (92.31 % at 24 h and 84.5% at 48 h, at concentrations of 300 µg/mL). During this period, IC_{50} values of all the PPE and positive control were below the lowest

concentration of the PPEs (50 µg/mL) taken for analysis. However, at 72 h the inhibition of all the PPEs decreased below 80 %, and during this time the best activity was shown by *C. extensa* which had an IC_{50} value of 50.00 µg/mL and was followed by *O. corymbosa* (103.01 µg/mL), BHT (112.77 µg/mL), *A. heterophyllus* (146.72 µg/mL) and *A. malaccensis* (190.04 µg/mL).

Thiobarbituric acid assay of the PPEs (Table 3.8) revealed that at both 0 h and 24 h, the percentage of peroxidation inhibition was found to be low. At 0 h the IC_{50} values (Table 3.6) of all the PPEs including α-tocopherol were higher than the highest concentration (300 µg/mL) taken for analysis. At 24 h, the inhibition activities all the PPEs were below 48 %. However, certain activity was recorded in *O. corymbosa* at 24 h, which had an IC_{50} value of 280.06 µg/mL, and this was close to the IC_{50} value of α-tocopherol (275 µg/mL). From 48 h onwards, high percentage of peroxidation inhibition was observed for all the PPEs. During 48 h, 72 h and 96 h, IC_{50} values of all the PPEs and positive control was found below the lowest concentration of the PPEs (50 µg/mL) taken for analysis and the activity of all the PPE throughout all concentrations was almost at par with the reference standard (α-tocopherol).

Fairly good inhibition of lipid peroxidation was exhibited by the PPEs. Membrane lipids are rich in unsaturated fatty acids and are most susceptible to oxidative processes. Lipids such as linoleic and arachidonic acids are easy targets of peroxidation. Lipid peroxidation is involved in the formation and propagation of lipid radicals, which eventually destroy membrane lipids [64]. The FTC method determines the amount of peroxide at the initial stage of lipid peroxidation and hence these PPEs can be beneficial in combating the threat of lipid peroxidation, both in the body and food materials. The TBA assay is used to measure the secondary product of oxidation such as aldehyde and ketone and this assay revealed that the PPE were found to have positive implications in the prevention of lipid peroxidation.

Table 3.7 Ferric thiocyanate assay showing the inhibition of lipid peroxidation by PPEs

Time	Concentration (µg/ml)	*A. heterophyllus*	*C. extensa*	*O. corymbosa*	*A. malaccensis*	α tocopherol
0 h	50	10.36	4.99	11.45	9.11	0.88
	100	13.11	11.49	27.36	13.23	5.98
	150	13.73	16.23	30.46	24.72	11.83
	200	25.72	18.23	31.34	25.47	13.03
	250	26.72	23.72	37.40	27.47	14.56
	300	29.34	26.72	40.79	28.21	16.28
24 h	50	68.16	80.52	72.12	72.07	72.26
	100	72.85	80.76	77.40	77.86	72.66
	150	79.63	85.11	85.83	80.02	75.92
	200	87.46	85.28	86.91	82.59	78.94
	250	87.93	85.59	87.23	85.95	79.20
	300	92.31	88.32	87.97	86.29	79.58
48 h	50	65.06	72.18	61.92	56.98	70.83
	100	70.37	75.31	69.90	61.75	71.55
	150	76.17	79.93	79.59	71.70	73.06
	200	76.97	81.13	79.90	72.98	74.53
	250	84.04	81.45	82.10	74.38	74.96
	300	84.50	82.57	83.83	82.23	77.36
72 h	50	14.83	50.66	44.51	19.96	43.45
	100	32.17	61.77	49.40	27.64	45.81
	150	51.44	66.77	65.17	32.41	63.61
	200	56.44	69.46	69.36	54.82	64.41
	250	72.19	70.84	70.39	56.00	66.19
	300	77.38	80.83	73.82	75.79	67.64

Table 3.8 Thiobarbituric acid assay showing the inhibition of lipid peroxidation by the PPE

Time	Concentration (µg/ml)	*A. heterophyllus*	*C. extensa*	*O. corymbosa*	*A. malaccensis*	α tocopherol
0 h	50	0.11	3.24	20.02	18.09	21.03
	100	1.34	9.51	21.81	21.59	26.96
	150	4.92	11.74	22.48	22.60	28.75
	200	13.98	19.57	24.83	24.38	37.25
	250	20.69	20.25	25.72	25.84	41.16
	300	28.19	21.25	28.64	28.41	41.16
24 h	50	21.92	33.53	43.35	27.78	16.17
	100	30.85	35.81	43.55	32.34	27.08
	150	33.93	37.30	43.95	37.01	33.13
	200	40.58	38.79	44.94	39.78	38.69
	250	42.56	42.56	45.83	41.67	39.89
	300	45.93	47.32	52.88	44.25	60.32
48 h	50	79.87	68.64	87.65	66.99	85.35
	100	84.55	69.42	90.23	69.17	85.56
	150	84.65	75.25	90.35	72.47	85.68
	200	82.65	76.72	90.56	73.62	85.78
	250	86.04	76.99	90.90	76.06	86.26
	300	91.49	81.39	91.77	77.15	86.89
72 h	50	79.91	74.39	81.93	70.30	76.84
	100	82.41	76.20	83.65	73.91	79.94
	150	84.46	79.41	83.83	77.47	80.19
	200	85.42	80.72	84.18	81.86	83.95
	250	85.74	82.51	82.61	84.96	85.59
	300	87.13	85.01	85.87	84.66	86.47
96 h	50	71.54	74.51	77.49	64.13	75.84
	100	74.57	72.60	79.59	68.56	80.94
	150	76.77	77.03	80.42	70.66	84.33
	200	81.07	78.11	81.92	72.91	85.83
	250	81.09	79.36	82.72	74.83	86.32
	300	83.24	80.63	83.73	76.43	86.30

Some significant studies on these and other related species have also been reported earlier by other workers. Omar et al. [50] examined the antioxidative, hypoglycemic, and hypolipidemic activities of *A. heterophyllus* leaf extracts and concluded that 70 % ethanol and n-butanol extracts exert hypoglycemic and hypolipidemic effects in streptozotocin - diabetic rats through an antioxidative pathway that might be referred to their flavonoid contents. The antioxidant and antibacterial activities of *A. heterophyllus* leaf extracts was tested by Loizzo et al. [31]. They found that the MICs determined by agar dilution method against some food borne pathogens ranged from 221.9 μg/mL for ethyl acetate fraction to 488.1 μg/mL for total extracts. Assays of the extracts revealed significant antioxidant activity through DPPH, ABTS, FRAP, and Fe2+ chelating activity. In another work Chandrika et al. [32] found that the total flavonoid content of *A. heterophyllus* leaf exhibited a non-toxic and significant hypoglycaemic activity in male Wistar rats. Chen et al. [33] on their work with *Cyclosorus acuminatus* found that its flavanone-rich extract had renoprotective properties in diabetic mice via modulating the PPAR signalling pathway and eventually improving the extents of oxidative stress and inflammatory response. Recently, three new chalcone derivatives together with four known chalcones were isolated from the leaves of *Cyclosorus parasiticus* by Wei et al. [34] Some of the compounds exhibited substantial cytotoxicity against six cell lines, especially toward HepG2. Pandey et al. [35] found anticancer activity of the ethanolic extracts of *O. corymbosa* Lam leaves on K562 human leukemia cancer cell line by SRB assay. The ethanolic and aqueous extracts of *O. corymbosa* also revealed antioxidant activity on chromium induced oxidative stress in albino rats [36]. In another study by Sahoo et al. [78] the antioxidant activity of methanol extract of *Alpinia malaccensis* leaf was investigated and the IC_{50} values were found to be 22.5 μg/ml in DPPH, 72.38 μg/ml in NO, 26.23 μg/ml in ABTS and 80 μg/ml in H_2O_2 radical scavenging assays respectively

3.3.3 Storage of rice beer under accelerated temperature condition by incorporation of bioflavonoids from *Artocarpus heterophyllus* and *Cyclosorus extensa* leaves

3.3.3.1 Content of various flavonoids in the purified extracts

Flavonoids and their condensed products, the proanthocyanidins, are a class of readily oxidizable compounds which are capable of preventing the oxidation of other molecules present in beer [37]. The various flavonoids detected in both the plant extracts by HPLC are shown in Table 3.9 and the chromatograms are shown in Fig 3.8. The flavonols (myricetin,

quercetin, kaempferol), flavones (apigenin, luteoline,), flavanones (naringenin) and proanthocyanidins (epigallocatechin, catechin, epicatechin, epigallocatechin gallate) were detected in both the extracts. A total of nine flavonoids were detected in AH in varying amounts, wherein the content of myricetin (251.11 ppm) and epigallocatechin gallate (166.16 ppm) was the highest. Myricetin which is a common natural flavonol occurring in fruits, vegetables, tea, berries, red wine and medical plants is reported to have antioxidant, anticancer, antidiabetic and anti-inflammatory activities. Its ability to protect lipids against oxidation makes it a potential agent to extend the shelf life of foods containing oils and fats [112]. On the other hand, the most common occurrence of epigallocatechin gallate is in green tea (*Camellia sinensis*) and it exhibits several activities like antiinflammatory, antioxidant, immunosuppressive, cancer chemoprevention etc [113].

In case of CE only six flavonoids were detected. However, as compared to AH, their concentrations were relatively higher in CE with kaempferol (1323.22 ppm), luteolin (511.84 ppm) and quercetin (364.36 ppm) being the major ones. Kaempferol is a flavonoid abundant in plants used in traditional medicine such as *Ginkgo biloba* and also found in edible plants like tea, tomato, beans and cabbage. Its pharmacological activities include antimicrobial, antioxidant, neuroprotective, cardioprotective, analgesic and protection against cancer, diabetes, inflammation, diabetes, osteoporosis and allergy [114]. Luteolin is a commonly occurring flavonoid that exists in many types of plants such as carrots, cabbages, onion leaves, celery, broccoli, apple skins, peppers etc. It functions as either an antioxidant or a prooxidant biochemically and it has antiinflammation, antiallergy, anticancer, and antihypertensive activity [115]. Quercetin is the most abundant of the flavonoids and is found in abundance in onions, broccoli, apples and berries. Its potential activities include antiinflammatory, antioxidant, anticancer, antihaemolytic, cardioprotective and inhibitor of the glycoprotein P450 enzyme [116].

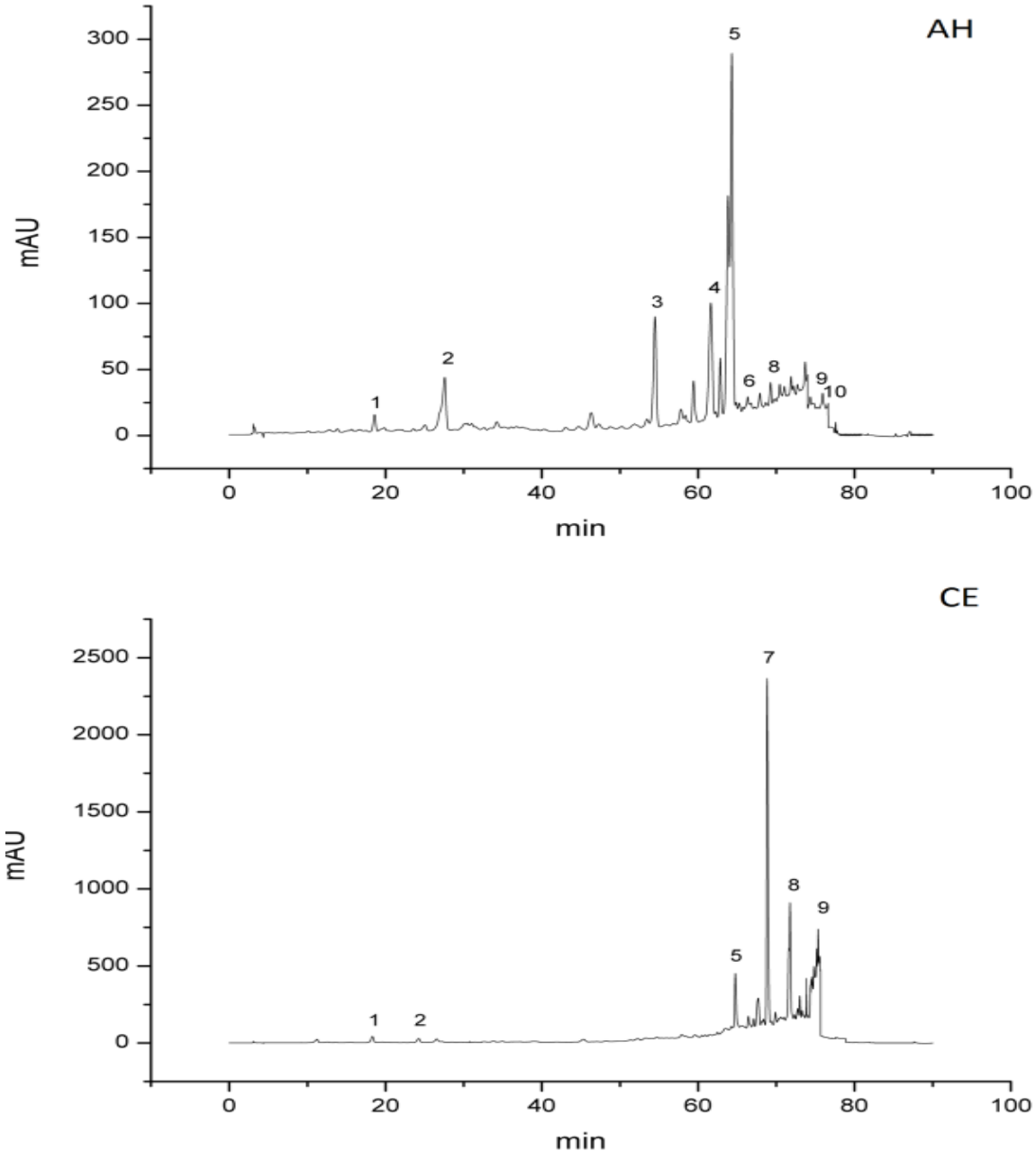
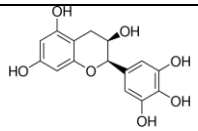
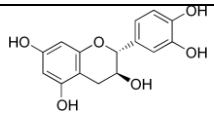
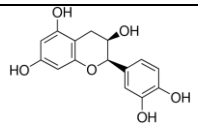
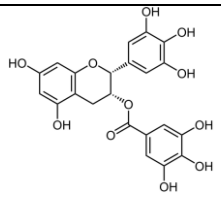
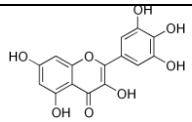
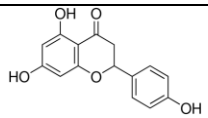
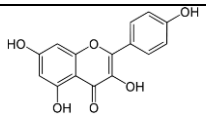
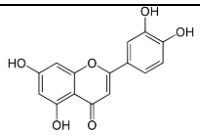
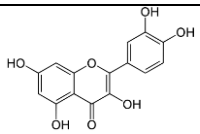
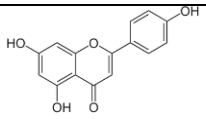


Fig 3.8 HPLC chromatograms of the two plants' bioflavonoid extracts. The detected compounds are 1. epigallocatechin, 2. catechin, 3. epicatechin, 4. epigallocatechingallate, 5. myricetin, 6. naringenin, 7. kaempferol, 8. luteolin, 9. quercetin 10. apigenin

Table 3.9 Content of various flavonoids in the purified plant extracts

Flavonoid	Structure	RT (min)	Concentration (ppm)	
			AH	CE
Epigallocatechin		18.45	12.56	48.47
Catechin		23.14	71.62	39.60
Epicatechin		54.48	95.82	ND
Epigallocatechin gallate		61.78	166.16	ND
Myricetin		64.53	251.11	235.99
Naringenin		66.35	9.13	ND
Kaempferol		68.38	ND	1323.22
Luteolin		71.82	7.97	511.84
Quercetin		75.25	8.61	364.36
Apigenin		77.13	9.43	ND

Note: AH -flavonoid extracts of *A. heterophyllus*; CE - flavonoid extracts of *C. extensa*

3.3.3.2 Effect of storage on various indicator parameters of rice beer

3.3.3.2.1 APC

APC indicates the level of microorganism in a product and it helps to assess the viable bacterial growth or spoilage of a food sample. Microbiological spoilage can occur if the beer becomes infected with a spoilage organism, or if there is a change in the normal metabolism of the brewing yeast. The amount of oxygen and nutrients available determines which organisms are capable of spoilage. The change in APCs with storage of the beers is shown in Table 3.10. The APCs started with low count of 0-3 log CFU ml^{-1} in all the samples except RBF. Their counts were found to increase throughout the storage period. It was more or less consistent in between RBAH and RBCE and in both the beers; it reached 5 log CFU ml^{-1}on the 4^{th} week and ended with counts of 7 log CFU ml^{-1} on the 8^{th} week. Considerably higher counts were observed in RBBHT and RBC where immediately on the 2^{nd} week the counts reached 8 log CFU $ml^{-1}$and on the 5^{th} week the count reached 10 log CFU $ml^{-1}$and remained in that range till the end of storage period. Whereas, in RBF the APCs appeared only on the 2^{nd} week and increased only up to 4 log CFU ml^{-1} on the 8^{th} week. The APCs were less in the supplemented beers as compared to the controls thereby indicating less spoilage. In both RBAH and RBAC the count was less from the beginning which might be attributed to the antimicrobial activity exhibited by the flavonoids e.g. quercetin leads to inhibition of DNA gyrase and epigallocatechingallate inhibits cytoplasmic membrane function. Myricetin, apigenin and rutin have also been found to exhibit antimicrobial properties [117]. BHT was however unable to control the growth and the count was almost equivalent to the control sample after the 5^{th} week. Hence the inhibitory activity of BHT might have degraded with time. Filtration was however found to be more efficient in controlling the microbial load.

Table 3.10 Change of plate counts in the beers during storage

Time (Weeks)	Log CFU ml^{-1}				
	RBAH	RBCE	RBBHT	RBC	RBF
0	0.30 ± 0.00^{a}	0.00^{a}	3.63 ± 0.46^{a}	3.53 ± 0.31^{a}	0.00^{a}
1	2.88 ± 0.14^{b}	3.49 ± 0.20^{b}	8.25 ± 0.35^{b}	8.00 ± 0.39^{b}	0.00^{a}
2	4.12 ± 0.52^{c}	4.18 ± 0.03^{c}	8.35 ± 0.25^{b}	8.14 ± 0.36^{b}	0.61 ± 0.13^{b}
3	4.71 ± 0.29^{cd}	4.69 ± 0.38^{d}	8.37 ± 0.43^{b}	8.32 ± 0.31^{b}	0.85 ± 0.06^{c}
4	5.11 ± 0.06^{d}	5.44 ± 0.33^{e}	9.45 ± 0.42^{c}	9.32 ± 0.38^{c}	1.23 ± 0.04^{d}
5	5.97 ± 0.31^{e}	6.53 ± 0.05^{f}	10.32 ± 0.50^{d}	10.29 ± 0.37^{d}	1.64 ± 0.11^{e}
6	6.49 ± 0.64^{e}	6.58 ± 0.03^{f}	10.33 ± 0.24^{d}	11.41 ± 0.31^{e}	2.05 ± 0.11^{f}
7	6.71 ± 0.50^{ef}	6.83 ± 0.22^{f}	10.38 ± 0.40^{d}	11.47 ± 0.34^{e}	3.01 ± 0.04^{g}
8	7.32 ± 0.15^{f}	7.93 ± 0.56^{g}	10.45 ± 0.30^{d}	11.49 ± 0.37^{e}	4.12 ± 0.11^{h}

Note: Values (n=3)±SD in a column with different superscripted alphabets differs significantly ($p\leq0.05$)

3.3.3.2.2 pH

The pH is a key factor for influencing beer stability and sensory characteristics. According to Taylor [118], when the pH of beer decreases below 4.0, the sense of sharpness, acidity and bitterness increase in intensity. Whereas, above pH of 4.0, sense of mouth-coating, biscuit and toasted characters increase. Enhanced metallic after-palate was also reported for pH values below 3.7 and soapy and caustic notes for pH values above 4.4. The pH of all the beers studied by us remained in the range of 4-5 during the entire storage period (Fig 3.9). However, there was a decrease of pH in all the beers with storage and reached from 4.19 to 4.12 in RBAH, 4.22 to 4.12 in RBCE, 4.25 to 4.12 in RBBHT, 4.26 to 4.11 in RBC and 4.26 to 4.19 in RBF till the completion of storage. The possible causes for the decrease in pH are due to the propagation of yeasts and other organisms in semi aerobic conditions, thereby leading to the production of organic acids and absorption of basic amino acids and primary phosphate [119]. A decline in the rate of pH decrease was observed in both RBAH and RBCE, thereby suggesting a control of microbial growth.

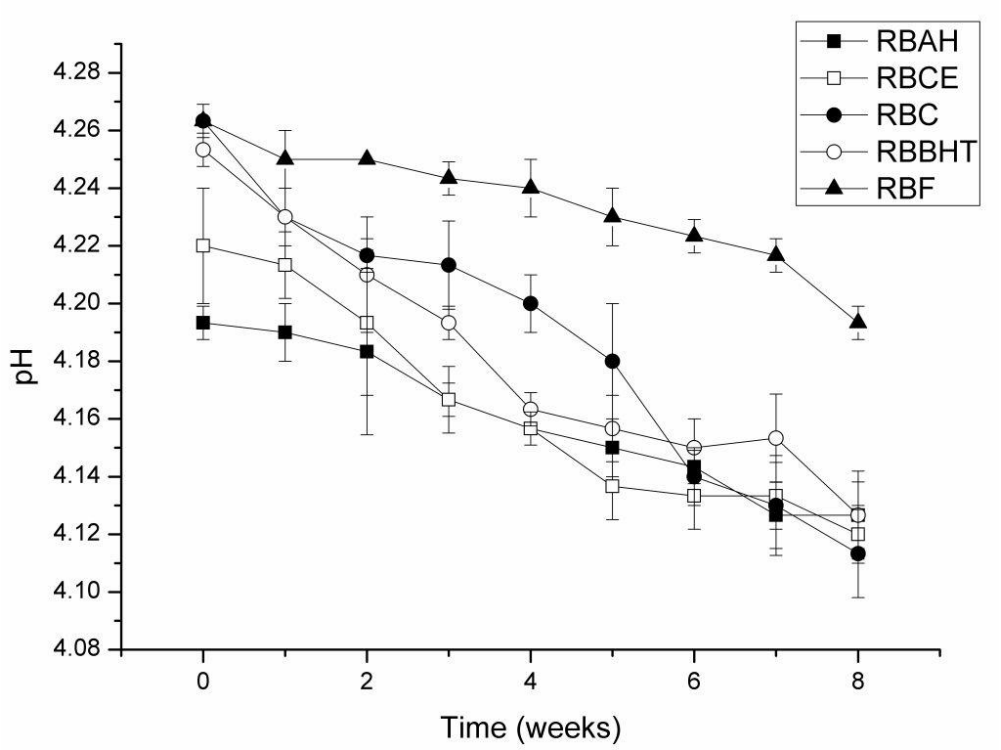


Fig 3.9 Change in pH of the beers (n=3) during storage

3.3.3.2.3 TPC

Polyphenols can react with free radicals to produce relatively stable phenoxyradicals, they chelate transition metal ions and behave as pro-oxidants by transferring electrons to transition metal ions. Polyphenols in beer easily react with ROS and free radicals. In this process, simple polyphenols polymerize to high molecular weight species (tannins), either by acid catalysis, oxidative mechanisms, induction by acetaldehyde formed by yeast or by ethanol oxidation [20]. The TPC in all the beers marked an increase in content ($p \leq 0.01$) with the storage period (Fig 3.10). In RBAH and RBCE it started with an initial content of 159.12 µg/100 ml and 172.66 µg/100 ml, respectively, which gradually went on increasing to 228.09 µg/100 ml and 220.21 µg/100 ml, respectively till the 8th week. In RBC and RBF also it started with initial contents of 168.60 µg/100 ml and 181.05 µg/100 ml, which reached 214.59 µg/100 ml and 229.94 µg/100 ml, respectively on the 8th day. However, the TPC were relatively lower in RBBHT. The increase in phenolic content with storage may be attributed to the conversion of bound polyphenols to free form resulting from secondary fermentation. Otherwise, it is possible that during storage, some compounds were formed that reacts with

Folin–Ciocalteu reagent and enhance total phenolic content detection [120]. The low content of TPC in RBBHT may be due to the degradation of phenolics by BHT.

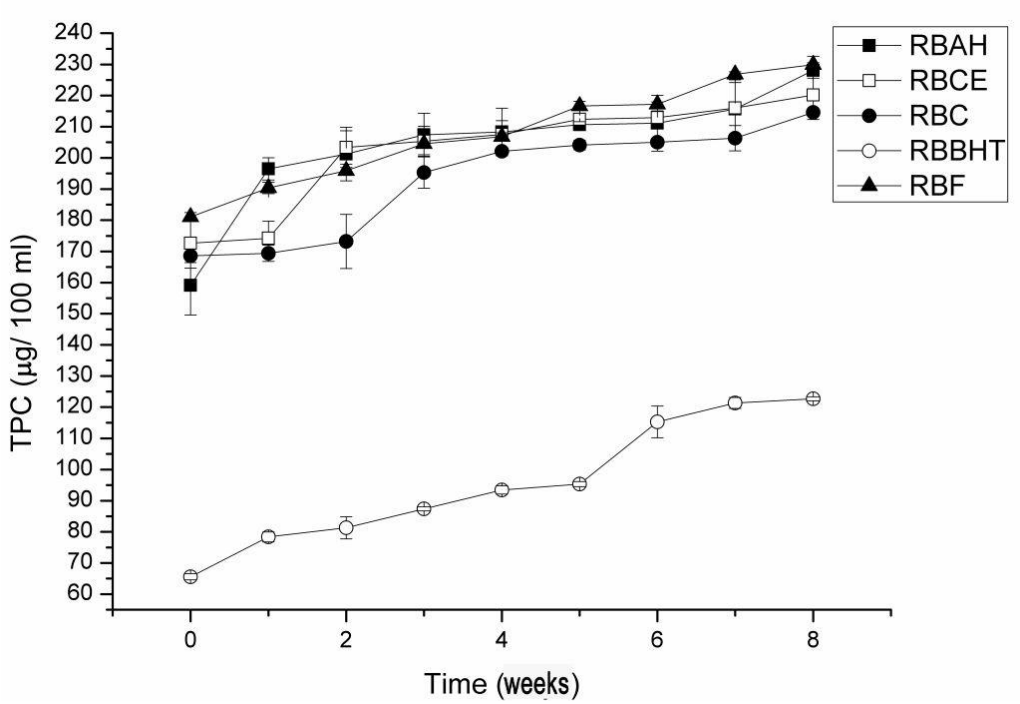


Fig 3.10 Change in total phenolic content (TPC) of the beers (n=3) during storage

3.3.3.2.4 RSA

One of the reasons for the change in flavour of aged beers are the free radicals in the form of reactive oxygen species (ROS) as they have the potential to convert to oxidising radicals [22]. The free radicals react with all kinds of organic molecules in beer, such as polyphenols, isohumulones and alcohols, resulting in various changes in the quality of beer. The free radicals can be formed after a definite time period of aging process, called the ‘‘lag time’’ of free-radical generation. This lag time is dependent on the endogenous antioxidant activity of beer [20]. The RSA in all the samples went on significantly ($p \leq 0.01$) decreasing with time (Fig. 3.11). The highest activity was shown by RBAH which started with an initial activity of 56.31 % and decreased instantly to 32.58 % on the 1st week. Thereafter it again increased till 42.88 % on the 4th week and slowly decreased and ended with 34.68 % on the 8th week. In RBCE the initial value was 21.06 % which reduced to 8.18 % on the 1st week, but thereafter gradually increased till 24.81 % on the 8th week. In RBBHT, activity was recorded till the first week only and thereafter it disappeared. On the other hand, relatively

lesser activity was recorded in RBC and RBF during the entire storage period. The increase in RSA might have occurred due to the formation of new reducing compounds via Maillard reaction [20] and the formation of complex phenolic molecules with higher reducing status. Similar trend in increase of antioxidant activity with storage have been reported by other authors like Klimczak et al. [121] in orange juices stored for 18, 28 and 38 °C for 2, 4 and 6 months and Piga et al. [122] in mandarin juices stored for 15 days at 4 °C. Andersen et al. [123] also tested the potential antioxidative role of phenolic compounds (0.2 mM) in lager beer by ESR spin trapping and found that none of the tested phenolic compounds affected the length of the lag phase or the rate at which the N-tert-butyl-R-phenylnitrone spin adduct was formed once the lag phase had ended.

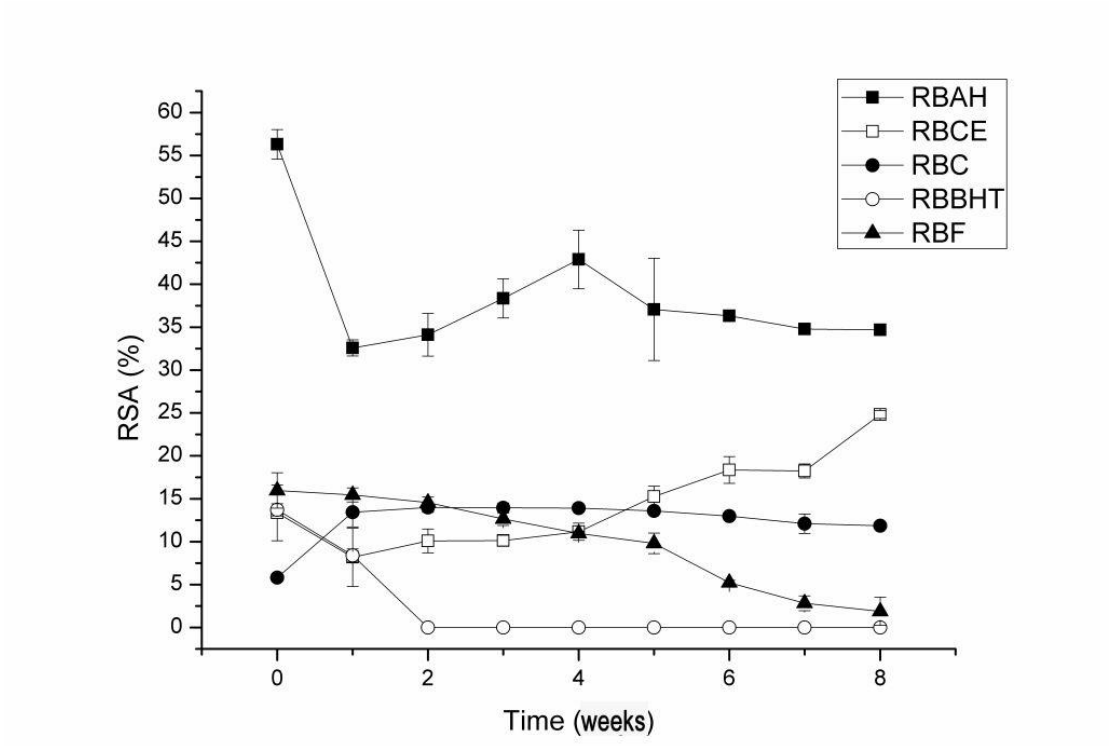


Fig 3.11 Change in radical scavenging activity (RSA) of the beers (n=3) during storage

3.3.3.2.4 Protein content

Proteins play a major role in beer stability and the main cause of beer haze formation. They are divided into two main groups, viz., first proteins and second their breakdown products [124]. In all the beers the protein content was found to significantly ($p \leq 0.01$) increase with the storage time (Fig. 3.12). The highest content was observed in RBAH and RBCE in which the initial contents were 274.03 µg/ml and 223.68 µg/ml, respectively, which

increased to 363.16 µg/ml and 298.25 µg/ml, respectively. The content was more or less consistent in between RBC and RBF throughout the entire period, whereas, in RBBHT the increase was less prominent. The quality and stability of beer are related to the quantity of protein and certain amino acids. The change in content of proteins in all the beers could be related to yeast activity during secondary fermentation and to nonspecific microbial contaminations during storage [125]. In the experiment of Berner [126] also with fermentation of standard hopped wort, yeast strain dependent changes in the immature beer proteome was observed. In one study by Wu et al. [127] fresh Australian lager beer was aged for 12 weeks at 30 °C and the flavour stability of the beer during storage was correlated with the presence of an unknown 10 kDa protein which was active in DPPH reduction and antioxidant activity.

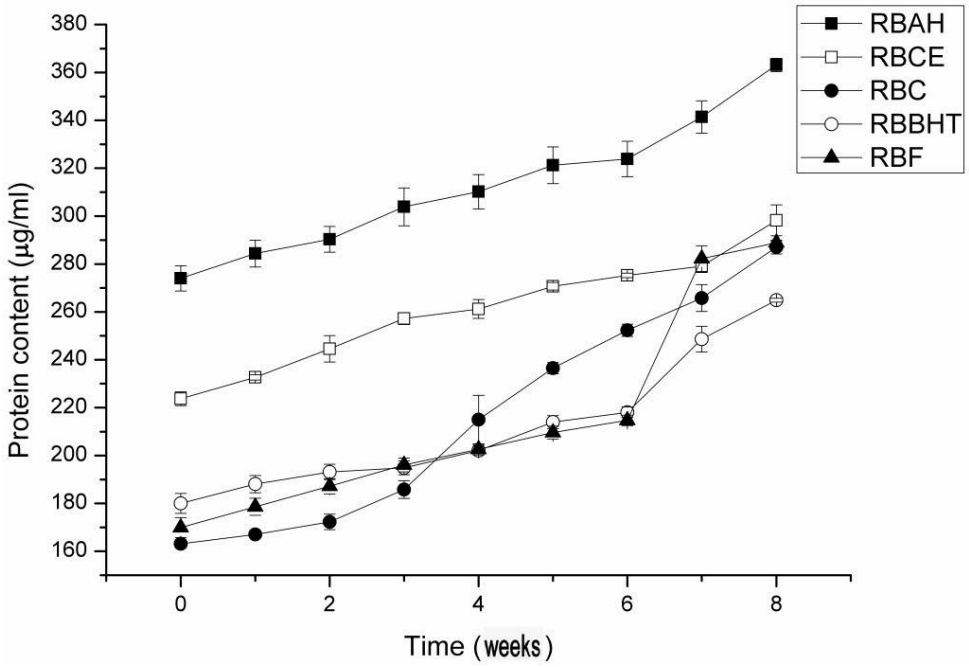


Fig 3.12 Change in protein content of the beers (n=3) during storage

3.3.3.2.5 Colour

The tristimulus measurement and henceforth conversion to L, a and b values for the quantification of beer colour relies upon transmission measurements and this technique may be applied to all beers, no matter how dark they are in colour and without dilution, It gives results which may be compared favourably with those from standard procedure [128]. In

beer, structural rearrangements of flavan-3-ol monomers can cause colour changes during storage. Also the proline-rich proteins and oligomeric flavanoids forms soluble complexes which results in a visible haze [129]. The change in colour (ΔE) obtained (Fig. 3.13) was relative to the reading of 0 week, which was taken as the control. The highest ΔE was observed in RBC in which an immediate change was observed on the 2nd week, and it went on increasing to reach 6.71 on the 7th week and took a sharp rise on the 8th week to reach 12.08. The RBBHT and RBF exhibited similar patterns of change and reached 5.18 and 6.07, respectively on the 8th week. The changes in colour were however relatively less in RBAH and RBCE. The change in colour of beer during storage results due to the formation of coloured molecules derived from the Maillard reaction and the oxidation of polyphenols. Hence, the decrease in colour change in RBAH and RBCE may be due to the prevention of polyphenol oxidation by the bioflavonoids. In the experiment of McMurrough et al. [130] and Vanderhaegen et al. [131] an almost linear increase of colour was observed for samples stored at 40 °C in the absence of oxygen. They concluded that the formation of coloured Maillard products during storage was likely the predominant cause of colour change. Moreover, in the presence of oxygen, the colour increase was rapid during the first days, due to oxidation and subsequent degradation of the polyphenols. In the experiment of Callemien and Collin [129] with storage of lager beers for 1 year at 20 °C, colour evolved more rapidly in the PET bottle, suggesting a key role of oxygen. This was more rapid in beers containing catechin, which was found to be the precursor of less polar products, characterized by a yellow-brown colour.

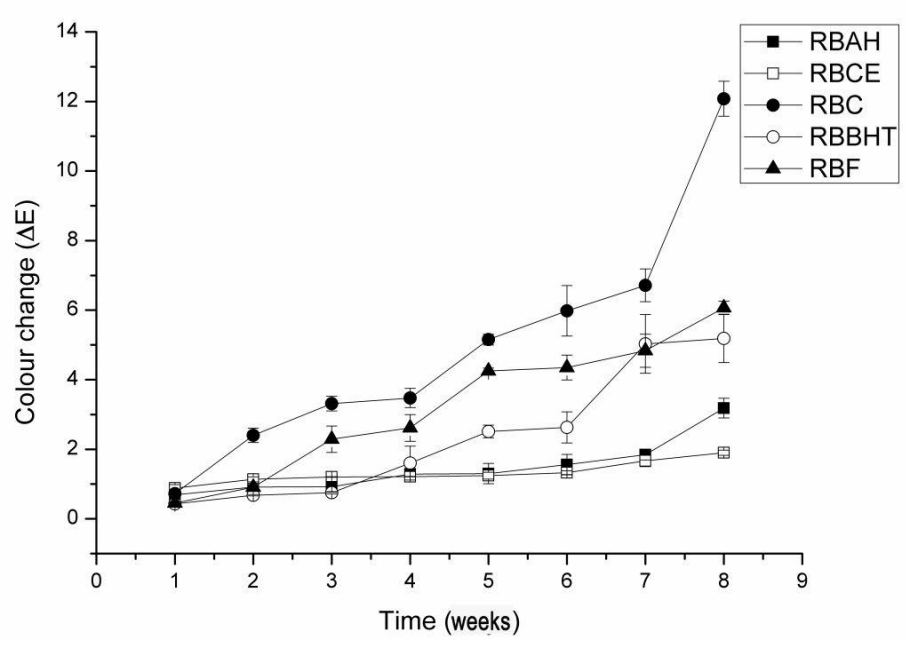


Fig 3.13 Change in colour (ΔE) of the beers (n=3) during storage

3.3.3.2.6 TBARS and POV

Autoxidation of fatty acids is a spontaneous reaction of molecular oxygen with lipids, leading to oxidative deterioration. It proceeds by a free radical chain mechanism involving three steps: initiation step – homolytic hydrogen atom abstraction from a methylene group that leads to alkyl radical (R•) formation; propagation step – formation of peroxy radicals (ROO•) able to react with unsaturated fatty acids and form hydroperoxides (ROOH); termination step – formation of non-radical products by interaction of R• and ROO• [132]. The POV and TBARS are commonly used indices to assess the degree of lipid oxidation and these are shown in Table 3.11. TBARS and POV were not detected in RBAH and RBCE till the 6^{th} week and 5^{th} week, respectively. On the 8^{th} week they exhibited values of 0.17 TBARS and 0.02 POV in RBAH and 0.115 TBARS and 0.021 POV in RBCE. In BHT the peroxidation was observed from the 2^{nd} week where it started with values of 0.085 TBARS and 0.013 POV and achieved highest values of 0.363 TBARS and 0.109 POV on the 8^{th} week. In both RBC and RBF the peroxidation was observed from week 1, wherein it started with values of 0.057 TBARS and 0.013 POV in RBC and 0.022 TBARS and 0.009 POV in RBF and gradually went on increasing. The delay in lipid peroxidation in RBAH and RBCE can be attributed to the prooxidant activity of the flavonoids such as catechin, myricetin, naringenin, quercetin and apigenin [133] which have a low half peak oxidation potential. Two pharmacophores in flavones are responsible for protection against lipid peroxidation viz., a catechol moiety as ring B and an OH group at the 3 position with electron donating groups at the 5 and/or 7 position in the AC-ring [134]. Kong et al. [135] also reported that the polyphenol rich *B. racemosa* leaf extract inhibited the formation of TBARS and lipid hydroperoxides in LDL oxidation assay (IC50 = 73.0 µg/ml). Jiang et al. [23] also investigated the efficacy of the radical-scavenging extract of *Glycyrrhiza glabra* to curtail lipid oxidation in ground pork patties. They found a rise in TBARS values (0.22 mg/kg to 9.3-9.4 mg/kg) in refrigerated control samples after 14 days storage, as compared to patties treated with 0.1 % extract (0.22 mg/kg to 3.4-4.4 mg/kg in).

Table 3.11 Presence of TBARS and POV in the beers during storage

Time (Weeks)	RBAH		RBCE		RBBHT		RBC		RBF	
	T	P	T	P	T	P	T	P	T	P
0	0.00[a]	0.00[a]	0.00[a]	0.00[a]	0.00[a]	0.00[a]	0.00[a]	0.00a	0.00[a]	0.00[a]
1	0.00[a]	0.00[a]	0.00[a]	0.00[a]	0.00[a]	0.00[a]	0.057±0.01[b]	0.013±0.005[b]	0.022±0.004[b]	0.009±0.004[b]
2	0.00[a]	0.00[a]	0.00[a]	0.00[a]	0.085±0.01[b]	0.013±0.005[b]	0.086±0.01[c]	0.018±0.01[bc]	0.064±0.01[c]	0.013±0.003[b]
3	0.00[a]	0.00[a]	0.00[a]	0.00[a]	0.125±0.01[c]	0.015±0.01[b]	0.123±0.01[d]	0.025±0.005[c]	0.113±0.01[d]	0.014±0.005[b]
4	0.00[a]	0.00[a]	0.00[a]	0.00[a]	0.165±0.01[d]	0.026±0.01[c]	0.144±0.01[e]	0.055±0.01[d]	0.135±0.01[e]	0.035±0.01[c]
5	0.00[a]	0.00[a]	0.00[a]	0.00[a]	0.191±0.01[e]	0.028±0.01[c]	0.157±0.02[e]	0.064±0.01[d]	0.146±0.01[e]	0.045±0.01[d]
6	0.00[a]	0.00[a]	0.019±0.001[b]	0.009±0.003[b]	0.266±0.02[f]	0.054±0.01[d]	0.269±0.02[f]	0.123±0.01[e]	0.183±0.01[f]	0.058±0.01[e]
7	0.022±0.003[b]	0.013±0.01[b]	0.108±0.01[c]	0.020±0.01[c]	0.295±0.02[g]	0.067±0.01[e]	0.307±0.01[g]	0.154±0.01[f]	0.195±0.01[f]	0.063±0.01[e]
8	0.17±0.01[c]	0.02±0.004[c]	0.115±0.01[c]	0.021±0.003[c]	0.363±0.02[h]	0.109±.001[f]	0.392±0.02[h]	0.195±.001[g]	0.285±0.01[f]	0.121±0.01[e]

Note: [1]T – TBARS (µg MDA/kg beer); P – POV (meq peroxide/kg beer)

[2]Values (n=3) ±SD in a column with different superscripted alphabets differs significantly ($p \leq 0.05$)

3.3.4 Optimization of the extraction of phenolic compounds from *Cyclosorus extensa* with solvents of varying polarities

3.3.4.1 Statistical analysis and model fitting

The combination of temperature and time for extraction set by the design and the responses of TPC, RSA, ABA and AFA obtained for the whole experiment are shown in Table 3.12. The statistical data representing the analysis of variance (ANOVA) were obtained. The sequential sum of squares, *F*-value and the corresponding coefficient of determination (R^2) and adjusted coefficient of determination (Adj. R^2) are also presented. Variance and regression analysis was carried out to fit the suggested quadratic models and investigated the statistical significance of model factors. The adequacies of the models were investigated by the *F*-values and corresponding *p* values of the regression models. It was observed that, the predicted models for all the response variables were adequately fitted to the observed experimental data ($p \leq 0.001$). The effect of linear, square and interaction of each response variables were also obtained. The accuracy of the fitness of the models was also judged by the lack of fit values for each response and observed no lack of fits ($p > 0.05$) in any response model. Non-significant lack of fit tests also suggested that quadratic models were best fitted for the extraction of bioactive compounds using the four different solvents. Fittness of quadratic models was ascertained by computing the R^2 and Adj. R^2 values. All these values are shown in Table 3.13 and 3.14. Except for the TPC of Hex extracts the R^2 values for all the other responses were above 90 %. The difference between the R^2 and R^2_{adj} values were less than 0.2, implying there are no insignificant terms added to the models. Thus results revealed that the models can establish optimum condition for the extraction of bioactive compounds from the leaves of *C. extensa* using Hex, EA, MeOH and H_2O.

Table 3.12 Response sheet for CCRD experimental design with process variables and experimental results for extraction with different solvents

Run	Factors		Responses															
	Temperature (°C)	Time (h)	TPC (mg/100mL)				RSA (%)				ABA (mm)				AFA (mm)			
			Hex	EA	MeOH	H_2O	Hex	EA	MeOH	H_2O	Hex	EA	MeOH	H_2O	Hex	EA	MeOH	H_2O
1	25	12	0.04	0.35	0.62	0.13	25.38	40.30	60.79	66.13	12.00	14.67	12.67	12.67	12.00	15.13	12.14	14.50
2	40	30	0.00	0.56	0.99	0.20	19.33	47.80	65.69	60.28	12.23	13.50	13.25	13.00	13.00	12.84	15.50	11.50
3	18.79	30	0.11	0.41	0.53	0.09	24.21	40.84	59.66	68.38	13.67	14.33	12.67	12.33	13.50	16.04	14.00	13.50
4	40	30	0.01	0.55	0.97	0.16	18.76	47.11	65.43	60.21	11.98	13.56	13.06	13.20	13.06	12.19	15.22	11.06
5	40	30	0.05	0.62	0.93	0.19	19.56	47.95	64.84	59.32	12.71	14.13	12.98	12.98	13.24	12.72	15.68	11.63
6	25	48	0.10	0.44	0.43	0.18	17.94	50.21	61.47	67.24	12.00	14.00	12.00	12.00	12.48	13.45	14.50	12.50
7	40	30	0.01	0.63	0.90	0.20	19.74	47.53	65.34	60.15	12.05	13.78	12.86	12.94	13.08	11.54	15.55	11.25
8	61.21	30	0.03	0.46	0.82	0.29	23.32	52.86	65.55	63.78	14.87	13.50	12.00	13.67	13.47	12.14	15.50	16.00
9	55	48	0.06	0.39	0.79	0.26	22.49	55.52	66.79	64.07	13.33	15.00	12.00	13.33	12.25	12.01	11.50	14.00
10	55	12	0.00	0.42	0.42	0.29	23.81	51.62	69.81	64.28	12.33	12.67	11.68	13.00	12.00	14.14	15.50	14.50
11	40	4.54	0.00	0.36	0.38	0.21	24.28	46.43	68.38	62.56	11.23	13.67	12.14	12.33	11.50	14.48	11.00	13.74
12	40	30	0.00	0.52	0.82	0.20	18.34	47.02	66.54	60.25	11.88	13.94	12.76	13.06	12.84	12.49	15.00	11.50
13	40	55.46	0.1	0.39	0.75	0.23	15.57	56.15	66.53	65.13	12.00	14.33	12.00	12.33	12.04	11.08	11.00	11.00

Table 3.13 Analysis of variance showing the linear, quadratic interaction and lack of fit of the response variables

Response variables			Source of variation									
			Regression	Quadratic	Linear	Square	Residual	Lack of fit	Pure error	Corr. Total	R^2	Adjusted R^2
Hex	TPC	SS	0.019	0.05	0.01	0.00	0.01	0.003	0.01	0.02	89.88%	82.65%
		F	112.43**	9.22**	8.59**	0.00 ^{ns}	--	0.31 ^{ns}	--	--		
	RSA	SS	107.32	42.05	55.90	9.36	1.98	4.46	1.36	113.13	94.86%	91.19%
		F	28.85***	25.32***	4.88*	1.76 ^{ns}	--	4.38^{ns}	--	--		
	ABA	SS	10.05	7.85	1.95	0.25	0.99	0.56	0.43	11.04	91.05%	84.66%
		F	14.25**	27.81***	1.08 ^{ns}	0.25 ^{ns}	--	1.73 ^{ns}	--	--		
	AFA	SS	4.50	4.20	0.29	0.01	0.48	0.40	0.08	4.98	90.28%	83.35%
		F	13.01**	30.35***	0.31 ^{ns}	0.03 ^{ns}	--	6.40 ^{ns}	--	--		
EA	TPC	SS	0.10***	0.10	0.02	0.03	0.08	0.004	0.08	0.11	91.63%	85.65%
		F	15.33	36.10***	0.11^{ns}	0.31 ^{ns}	--	0.06 ^{ns}	--	--		
	RSA	SS	274.55	29.24	236.28	9.03	0.91	0.25	0.67	275.47	99.66%	99.42%
		F	414.90***	110.46***	30.14***	2.69 ^{ns}	--	0.50 ^{ns}	--	--		
	ABA	SS	3.86	0.18	1.43	2.25	0.31	0.10	0.27	4.24	91.11%	84.76%
		F	14.35***	1.70^{ns}	2.55^{ns}	36.13***	--	0.50 ^{ns}	--	--		
	AFA	SS	23.39	6.17	17.18	0.05	1.20	1.44	1.08	25.91	90.29%	83.36%
		F	13.02**	8.58**	9.83**	0.05 ^{ns}	--	1.78 ^{ns}	--	--		
MeOH	TPC	SS	0.53	0.35	0.10	0.07	0.02	0.03	0.02	0.57	91.90%	86.11%
		F	15.87***	26.07***	1.09 ^{ns}	1.80 ^{ns}	--	2.13 ^{ns}	--	--		
	RSA	SS	95.74	25.01	67.31	3.42	1.76	4.72	1.56	102.30	93.84%	89.44%
		F	21.33***	13.93**	9.69**	0.98 ^{ns}	--	4.04 ^{ns}	--	--		
	ABA	SS	2.98	2.23	0.51	0.25	0.17	0.03	0.14	3.15	94.55%	90.66%
		F	24.29***	45.39***	0.96 ^{ns}	0.92 ^{ns}	--	0.28 ^{ns}	--	--		
	AFA	SS	40.82	29.60	1.11	10.11	0.88	1.30	0.30	42.42	96.23%	93.54%
		F	35.74***	64.79***	0.13^{ns}	2.92 ^{ns}	--	5.71 ^{ns}	--	--		
H_2O	TPC	SS	0.038	0.02	0.03	0.01	0.01	0.004	0.01	0.040	95.89%	92.95%
		F	32.64***	4.55^{ns}	32.09***	3.82 ^{ns}	--	0.49 ^{ns}	--	--		
	RSA	SS	106.35	86.74	19.17	0.44	1.10	1.65	0.66	108.66	97.88%	96.36%
		F	64.49***	131.50***	1.07^{ns}	0.04 ^{ns}	--	3.32 ^{ns}	--	--		
	ABA	SS	2.63	0.78	1.59	0.25	0.06	0.04	0.04	2.70	97.15%	95.12%
		F	47.78***	35.53***	7.20*	2.62 ^{ns}	--	1.16 ^{ns}	--	--		
	AFA	SS	31.17	22.36	8.25	0.56	0.41	0.95	0.21	32.34	96.40%	93.83%
		F	37.53***	67.30***	1.71^{ns}	0.22 ^{ns}	--	6.04 ^{ns}	--	--		

Note: SS - Sequential sum of square;F – F value; *significant at p≤0.05;**significant at p≤0.01;***significant at p≤0.001;^{ns}not significant;

Table 3.14 Estimated regression coefficients of the fitted second order polynomial for response variables

Estimated coefficients		Coefficients						Probability of F value	Probability of lack of fit	Final predictive equation
		β_0	β_1	β_2	$\beta_1\beta_2$	β_1^2	β_2^2			
	TPC	0.014	-0.024**	0.033**	< -0.0001 ^{ns}	0.026**	0.015 ^{ns}	0.0023	0.8210	$Y = 0.014 - 0.024X_1 + 0.033X_2 + 0.026{X_1}^2$
	RSA	19.15	0.22^{ns}	-2.63***	1.53*	2.54***	0.53 ^{ns}	0.0002	0.0938	$Y = 19.15 - 2.63X_2 + 1.53X_1X_2 + 2.54{X_1}^2$
	ABA	12.17	0.42*	0.26 ^{ns}	0.25 ^{ns}	0.92***	-0.41*	0.0015	0.2985	$Y = 12.17 + 0.42X_1 + 0.92{X_1}^2 - 0.41{X_2}^2$
	AFA	13.04	-0.034 ^{ns}	0.19 ^{ns}	-0.057 ^{ns}	0.11 ^{ns}	-0.75***	0.0020	0.0525	$Y = 13.04 - 0.75{X_2}^2$
EA	TPC	0.58	0.011 ^{ns}	0.013 ^{ns}	-0.030 ^{ns}	-0.072**	-0.10***	0.0012	0.9776	$Y = 0.58 - 0.072{X_1}^2 - 0.10{X_2}^2$
	RSA	274.55	141.36***	94.92***	9.03***	0.37 ^{ns}	27.54***	<0.0001	0.7030	$Y = 274.55 + 141.36X_1 + 94.92X_2 + 9.03X_1X_2 + 27.54{X_2}^2$
	ABA	13.78	-0.27*	0.32**	0.75***	0.098 ^{ns}	0.14 ^{ns}	0.0015	0.7044	$Y = 13.79 - 0.27X_1 + 0.32X_2 + 0.75X_1X_2$
	AFA	12.36	-0.99**	-1.08**	-0.11 ^{ns}	0.93**	0.27 ^{ns}	0.0020	0.2904	$Y = 12.36 - 0.99X_1 - 1.08X_2 + 0.93{X_1}^2$
MeOH	TPC	0.92	0.071*	0.088*	0.14*	-0.14**	-0.19***	0.0011	0.2388	$Y = 0.92 + 0.071X_1 + 0.088X_2 + 0.14X_1X_2 - 0.14{X_1}^2 - 0.19{X_2}^2$
	RSA	65.57	2.83***	-0.62 ^{ns}	-0.93 ^{ns}	-1.56**	0.86*	0.0004	0.1056	$Y = 65.57 + 2.83X_1 - 1.56{X_1}^2 + 0.86{X_2}^2$
	ABA	12.98	-0.24**	-0.068 ^{ns}	0.25*	-0.35***	-0.48***	0.0003	0.8398	$Y = 12.98 - 0.24X_1 + 0.25X_1X_2 - 0.35{X_1}^2 - 0.48{X_2}^2$
	AFA	15.39	0.31 ^{ns}	-0.20 ^{ns}	-1.59***	-0.19 ^{ns}	-2.06***	<0.0001	0.0628	$Y = 15.3 - 1.59X_1X_2 - 2.06{X_2}^2$
H_2O	TPC	0.19	0.065***	0.06 ^{ns}	-0.02*	0.02 ^{ns}	0.018**	0.0001	0.7098	$Y = 0.19 + 0.065X_1 - 0.02X_1X_2 + 0.018{X_2}^2$
	RSA	60.04	-1.44***	0.57*	-0.33 ^{ns}	3.14***	2.02***	<0.0001	0.1382	$Y = 60.04 - 1.44X_1 + 0.57X_2 + 3.14{X_1}^2 + 2.02{X_2}^2$
	ABA	13.04	0.44***	-0.043 ^{ns}	0.25**	0.03 ^{ns}	-0.33***	<0.0001	0.4278	$Y = 13.04 + 0.44X_1 + .025X_1X_2 - 0.33{X_2}^2$
	AFA	11.39	0.63**	-0.80***	0.37 ^{ns}	1.76***	0.57**	<0.0001	0.0575	$Y = 11.39 + 0.63X_1 - 0.80X_2 + 1.76{X_1}^2 + 0.57{X_2}^2$

Note: *significant at p≤0.05, **significant at p≤0.01, ***significant at p≤0.001, ^{ns} not significant

3.3.4.2 Effect of the process variables on various responses of the extracts

The values of the coefficients for TPC, RSA, ABA and AFA for Hex, EA, MeOH and H_2O extracts were used for constructing a final predictive equation, neglecting the non-significant cross terms (Table 3.14). To determine the optimal levels of variables for obtaining the maximum TPC, RSA, ABA and AFA for all the extracts, three dimensional surface plots were constructed according to these predictive equations which are illustrated in Figs. 3.14, 3.15, 3.16 and 3.17 for Hex, EA, MeOH and H_2O respectively.

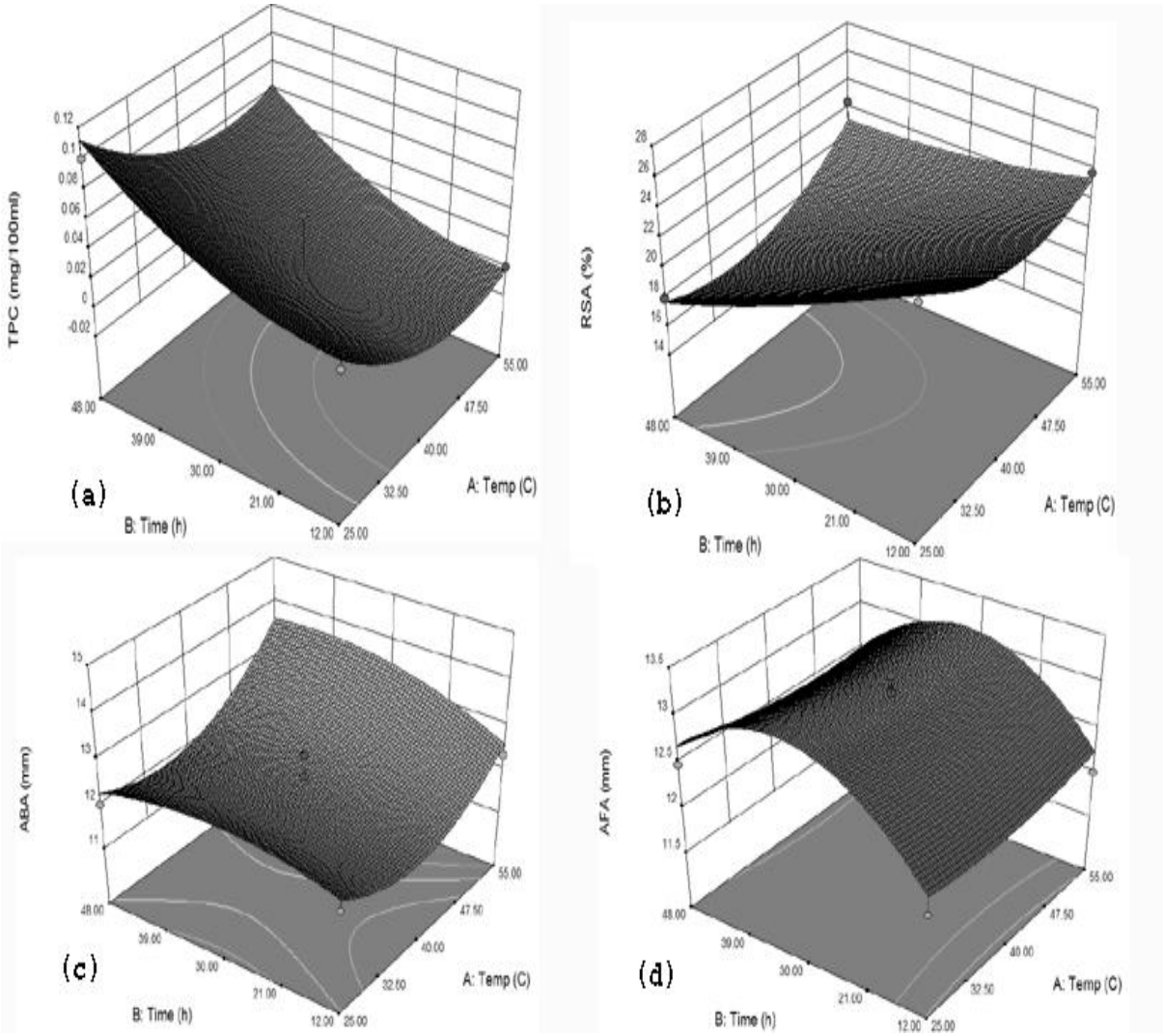


Fig 3.14 a. Effect of time and temperature on the: a. total phenolic content (TPC), b. radical scavenging activity (RAS), c. antibacterial activity (ABA) and d. antifungal activity (AFA) of the hexane (Hex) extracts

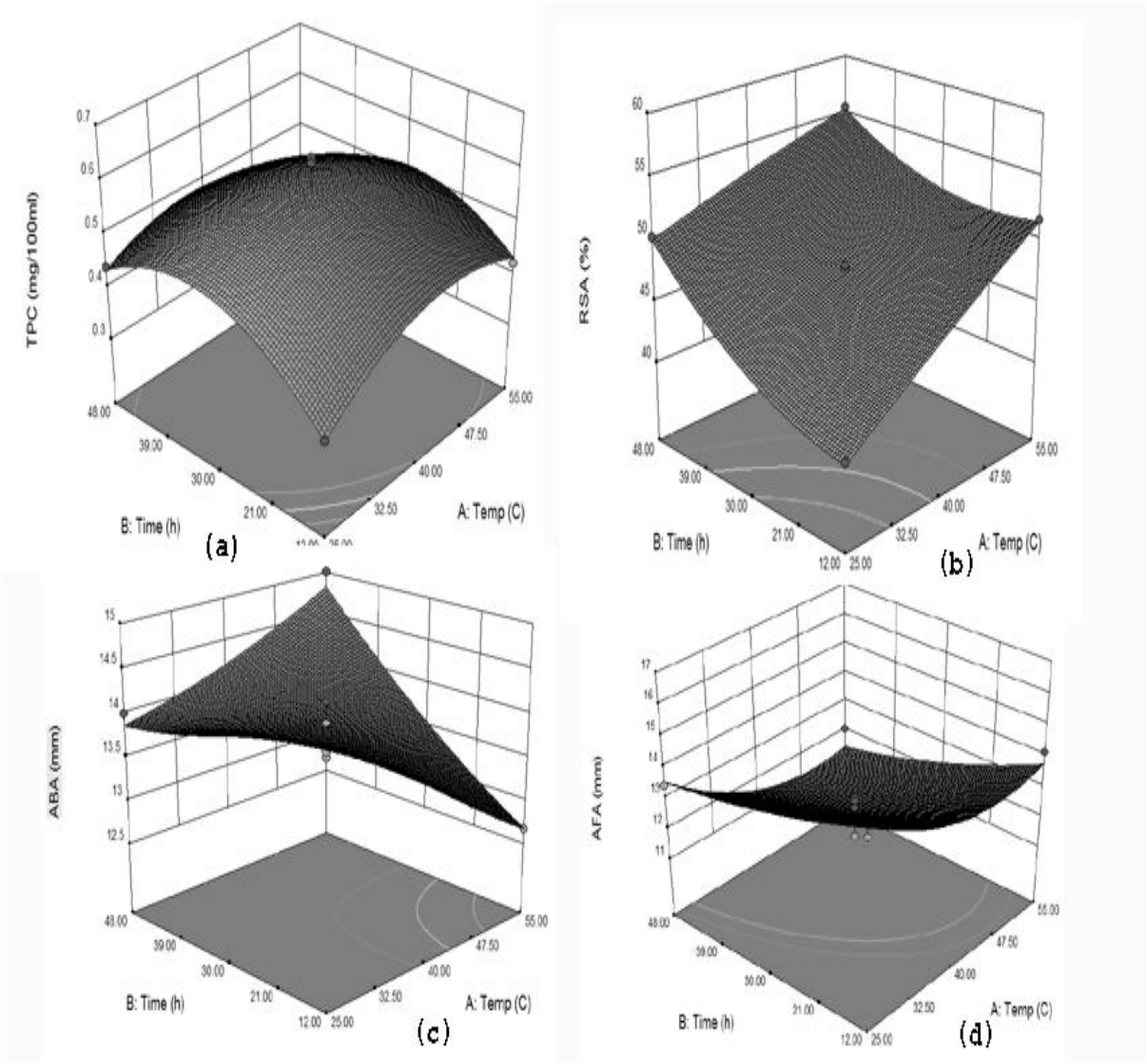


Fig 3.15 a. Effect of time and temperature on the: a. total phenolic content (TPC), b. radical scavenging activity (RAS), c. antibacterial activity (ABA) and d. antifungal activity (AFA) of the ethyl acetate (EA) extracts

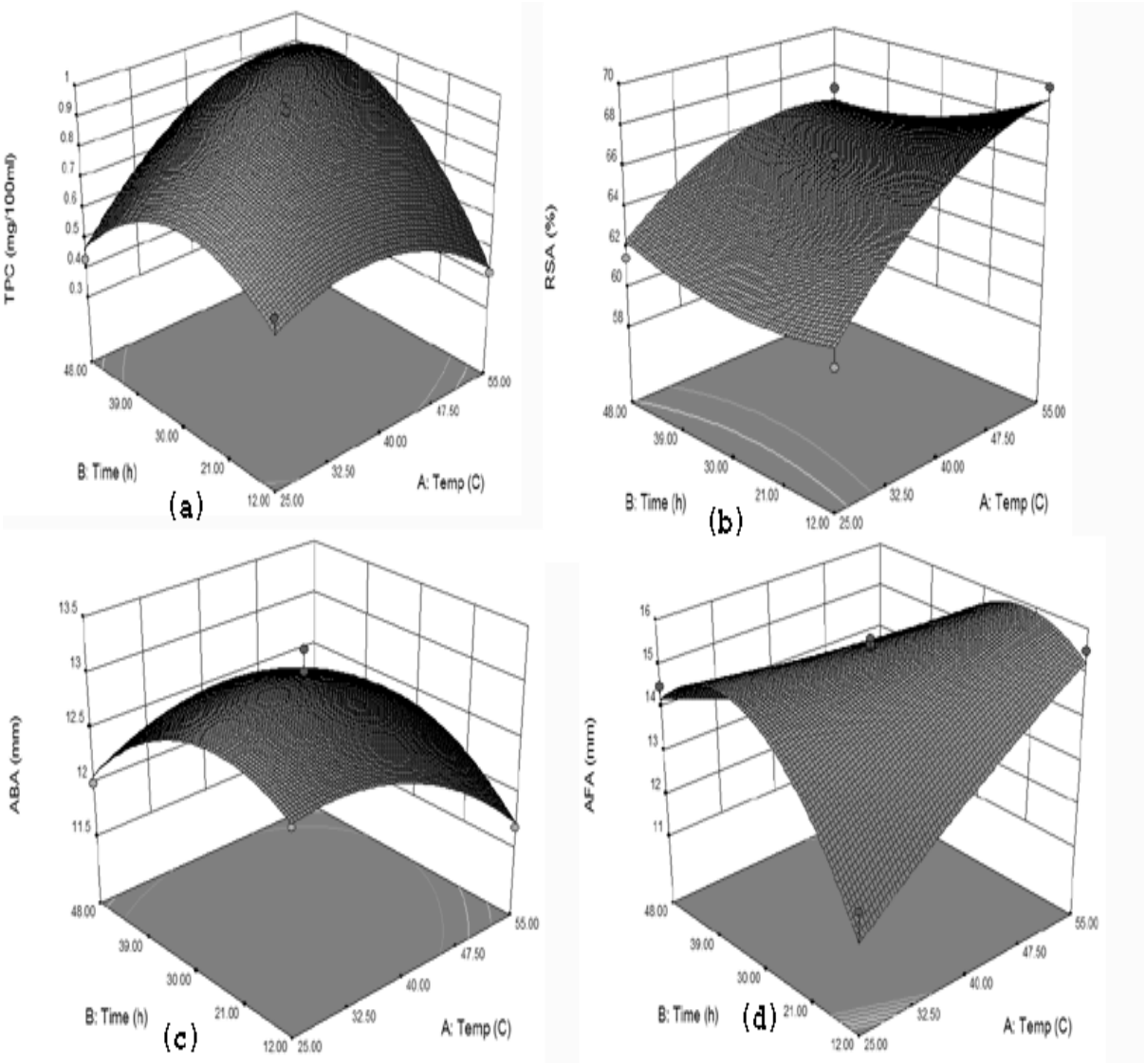


Fig 3.16 a. Effect of time and temperature on the: a. total phenolic content (TPC), b. radical scavenging activity (RAS), c. antibacterial activity (ABA) and d. antifungal activity (AFA) of the methanolic (MeOH) extracts

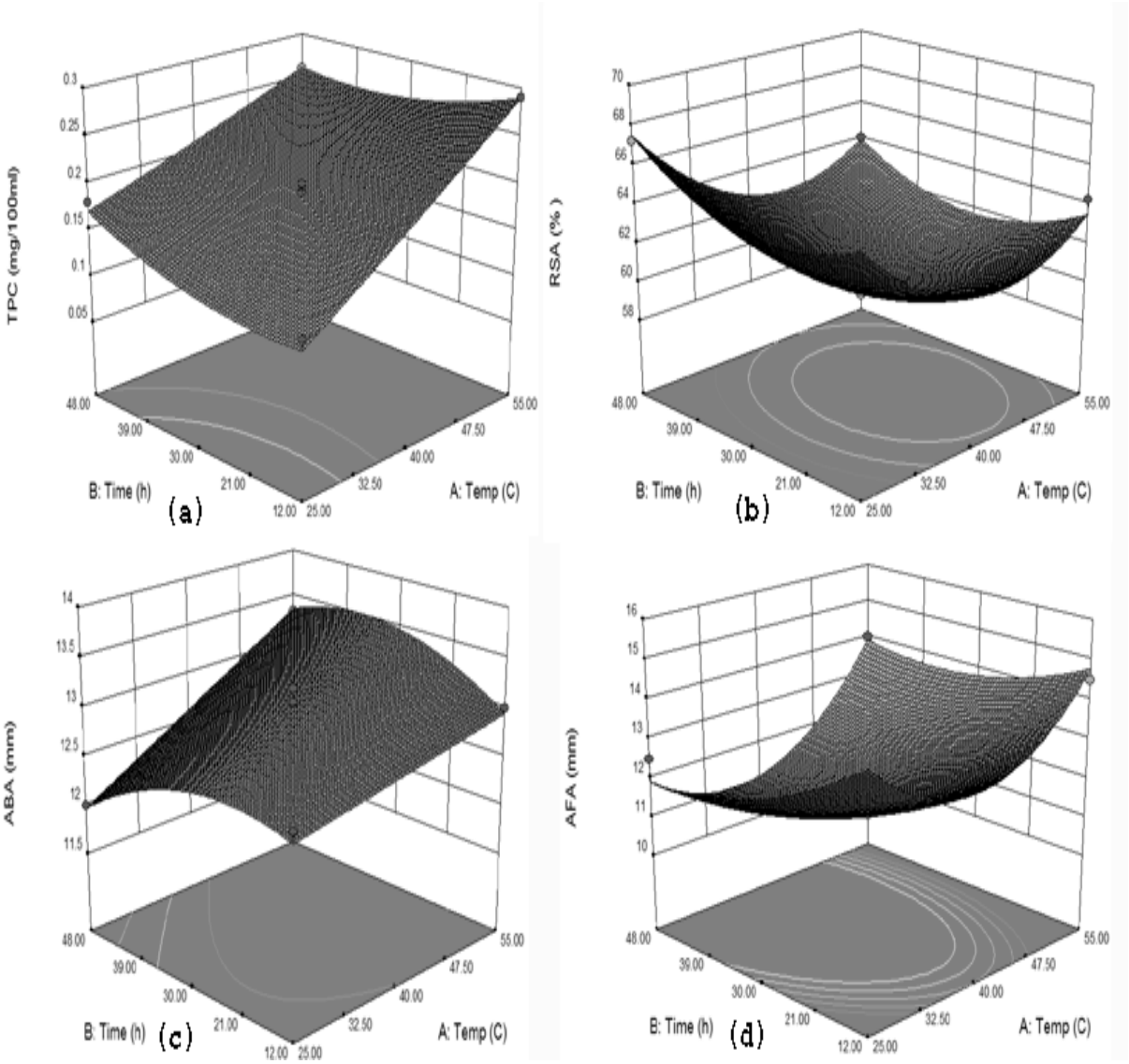


Fig 3.17 a. Effect of time and temperature on the: a. total phenolic content (TPC), b. radical scavenging activity (RAS), c. antibacterial activity (ABA) and d. antifungal activity (AFA) of the aqueous (H_2O) extracts

For all the solvents under study, it was observed that all the response values increased up to a certain level with increase in both time and temperature, keeping the other variables constant. In case of Hex, the TPC increased with increase in time and reached up to 0.4 mg/100 g and it was maximum (0.6 mg/100g) at 30 h and 40 ^{o}C. An increase in temperature also led to a gradual increase in the TPC. The RSA and ABA also increased with increasing time and reached up to 18 % and 12 mm ZOI respectively. The RSA and ABA also increased gradually with increase in temperature. The AFA reached up to 12.5 mm ZOI at a fixed temperature and the maximum AFA (13 mm ZOI) was obtained at 30 h and 40 ^{o}C. In case of EA, the TPC increased up to 0.4 mg/100g at fixed temperature and the maximum TPC of 0.6 mg/100g was at 30 h and 40 ^{o}C. The RSA, ABA and AFA values increased up to 50 %, 14 mm ZOI and 13 mm ZOI respectively at a fixed temperature. Similarly, the increase in temperature at fixed time also led to a gradual increase in all the responses. In MeOH, the TPC reached 0.4 mg/100g at fixed temperature with a maximum value of 0.9 mg/100g at 30 h and 40 ^{o}C. RSA increased with the increase of time and reached 62%. The ABA increased up to 12 mm ZOI at a fixed temperature and reached the maximum of 12.5 mm ZOI at 30 h and 40 ^{o}C. The AFA also reached up to 14 mm ZOI at a fixed temperature. Similarly in H_2O, the TPC, RSA, ABA and AFA increased till 0.15 mg/100g, 66 %, 12 mm ZOI and 12 mm ZOI respectively at fixed temperature. An increase in temperature at fixed time led to a gradual increase in all the responses.

3.3.4.3 Optimization of parameters

The independent variables were optimized numerically using statistical software Design Expert, Ver. 6.0.11. Less time and temperature for extraction would result in incomplete extraction; whereas more time and temperature would lead to waste of time and energy. For this purpose the goals for the variables i.e. extraction time and temperature were kept in range and all the response parameters were set at maximum. The optimal conditions, predicted values and experimental values for various responses are shown in Table 3.15. Numerical analysis revealed that in case of hexane, extraction temperature of 25 ^{o}C for a period of 29.43 h gave an optimized extraction condition with maximum TPC of 0.06 mg/100 mL, RSA of 21.51 %, ABA of 12.67 mm and AFA of 13.18 mm, all with a combined desirability of 0.582. When the experiment was actually performed under the optimized conditions, all the parameters were however found to be lower than the respective predicted values. In case of ethyl acetate, extraction temperature of 28.28 ^{o}C for a period of 41.27 h

gave an optimized extraction condition with maximum TPC of 0.51 mg/100 mL, RSA of 47.73 %, ABA of 13.95 mm and AFA of 13.19 mm, all with a combined desirability of 0.497. In this case also, all the parameters were found to be higher than the respective predicted values under actual optimized experimental conditions. In case of methanol, extraction temperature of 43.95 ºC for a period of 29.61 h gave an optimized extraction condition with maximum TPC of 0.93 mg/100 mL, RSA of 66.23 %, ABA of 12.89 mm and AFA of 15.47 mm, all with a combined desirability of 0.810. In this case also, all the parameters were higher than the respective predicted values under actual optimized experimental conditions. In case of H_2O, extraction temperature of 55.00 ºC for a period of 48.00 h gave an optimized extraction condition with maximum TPC of 0.26 mg/100 mL, RSA of 63.99 %, ABA of 13.36 mm and AFA of 13.93 mm, all with a combined desirability of 0.677. However, under actual experimental optimized conditions, TPC and RSA were lower, while ABA and AFA were higher than the respective predicted values.

Table 3.15 Estimated optimum conditions, experimental value and residual value

Solvent	Parameter	Factors		Responses			
		Temperature (°C)	Time (h)	TPC (mg/100mL)	RSA (%)	ABA (mm)	AFA (mm)
Hex	PV	25.00	29.43	0.06	21.51	12.67	13.18
	EV	--	--	0.058	20.62	11.58	12.67
	RV	--	--	3.33%	4.14%	8.60%	3.87%
EA	PV	28.28	41.27	0.51	47.73	13.95	13.19
	EV	--	--	0.55	49.57	14.39	14.07
	RV	--	--	7.84%	3.86%	3.15%	6.67%
MeOH	PV	43.95	29.61	0.93	66.23	12.89	15.47
	EV	--	--	0.99	68.11	13.81	16.15
	RV	--	--	6.45%	2.84%	7.14%	4.40%
H_2O	PV	55.00	48.00	0.26	63.99	13.36	13.93
	EV	--	--	0.24	61.25	14.26	14.10
	RV	--	--	7.69%	4.28%	6.73%	1.22%

Note: PV: Predicted value; EV - Experimental value; RV - Residual value

3.3.4.4 Effect of the solvents and optimized extraction conditions on TPC

The solubility of all the phenolics, and thereby their tendency to get transferred or diffused into a given solvent is governed by thermodynamics, which is also described by the activity coefficient factor [136,137]. The experimental data correlating the solubility of total phenolics in the four different solvents with varying temperature can also be correlated by the modified Apelblat equation [138,139] which is shown in Eq. 3.10, where *w* is the mass fraction solubility of phenolics and *T* is the absolute temperature. The parameters *A*, *B*, and *C* are the parameters of the equation and can be obtained by fitting the experimental solubility data.

$$\ln(w) = A + B/(T/K) + C\ln(T/K) \qquad \text{Eq. (3.10)}$$

As evident from the experimental data, the solubility of the phenols was more in MeOH, followed by EA, H_2O and Hex. The natural phenols possess a higher solubility preference to solvents with intermediate polarity (alcohols), rather than more polar (water) or less polar (hexane) solvents. This solubility preference can be attributed to the stereochemistry of phenols and the intermolecular forces which occur in between the molecules and the solvents, viz. the hydroxyl groups present in phenols can develop hydrogen bonds with the electronegative oxygen of the alcohols. The higher solubility in polar protic (methanol) instead of polar aprotic solvents (ethyl acetate) can be explained by the fact that the alcohols' hydroxyl groups can develop hydrogen bonds with the oxygen atoms occurring inside phenol molecules [140].

3.3.4.5 Effect of the solvents and optimized extraction conditions on RSA

DPPH is a stable free radical which has an unpaired valence electron at one atom of nitrogen bridge [107] and the sensitivity of DPPH radical is high enough to detect active ingredients at low concentrations and it can also accommodate many samples in a short period [141]. Hence its scavenging is considered as a good parameter in screening assays. Owing to the different antioxidant potentials of compounds with different polarity, both extraction yield and antioxidant chemical activity of extracts are strongly dependent on the solvent [142]. As presented in Table 3.15, in the DHHP assay, the MeOH extracts possessed

the highest RSA, followed by H_2O, EA, and Hex. The overall antioxidant activities of extracts are contributed by the phenolic compounds [143], and a direct correlation was observed in between the phenolic content and the antioxidant activity for all the solvents. The antioxidant activity depends on the type and polarity of the extracting solvent and the isolation conditions, and this may be attributed to the different antioxidant activities of phenolic extracts [144].

Lafka et al. [145] also reported the antioxidant activity of phenolic compounds from winery wastes by DPPH method and found that methanolic and ethanolic extracts possessed the highest RSA. The optimization study by RSM for extraction of antioxidants from black mulberry leaves by Radojkovi et al. [146] found that ethanol (59.47 %), temperature (59.92 °C) and liquid/solid ratio of 20.73 mL/g yielded maximum TPC of 48.5 mg/g dried leaves and minimum IC50 value of 0.023 mg/mL for DPPH activity. Bouterfas et al. [147] also carried out the optimization study with *Marrubium vulgare* L. leaves and found that the maximum total phenolics (293.34 mg /g dry weigh) were obtained with 60 % aqueous methanol at 25 °C for 180 min. Significant effect of various extracting solvents and temperatures on total phenolics and anthocyanin extracts were studied by Rababah et al. [148] and found that methanol and 60 ºC of extraction conditions were the best for extracting phenolic compounds from oregano, thyme, terebinth, and pomegranate.

3.3.4.6 Effect of the solvents and optimized extraction conditions on ABA and AFA

Most strains of *Escherichia coli* are commonly a part of the normal flora of the gut and are harmless, but some causes serious food poisoning [92]. *Aspergillus niger* is a common food contaminant and causes a disease called black mould on certain fruits and vegetables. It is ubiquitous in soil and is commonly reported from indoor environments [149]. All the extracts were found to have variable inhibitory activity against these two indicator microbes. Variability in potency is usually ascribed to differences in the relative amount and composition of phenolic compounds extracted with specific solvents [150]. Phenolic extracts are believed to induce lesions in cell membranes thereby initiating a series of events that leads to microbial cell death [151]. It was observed that under the optimized conditions (Table 3.15), the highest ABA was found in EA extracts and AFA was highest in MeOH extracts. In both the cases H_2O extracts exhibited the second highest activity. These results are favourably supported by explanation that antimicrobial phytochemicals are soluble in moderate polar solvent [152] and activity may be slightly weaker in aqueous extracts due to

lower concentrations of compounds like tartaric acid esters and flavonols than in methanolic extracts [150]. The phenomenon has been reported previously by Bassam et al. [153] who found higher activity of methanolic extracts of aromatic herbs against a range of microorganisms as compared to the aqueous extracts prepared in hot water.

Optimization of the process conditions for extraction of antibacterial compounds (against *B. subtilis* and *E. coli*) with methanol, ethyl acetate, hexane and distilled water from 19 Malaysian flowering plants was carried out by Abdullah et al. [154] and ethyl acetate extracts revealed the highest antibacterial activity and the best conditions postulated from the optimization study were 9.58 h, 300 rpm and 27.35 °C. In another study, the optimum extraction conditions for antimicrobial activities of *Nitellopsis obtusa* and *Chara vulgaris* extracts against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Proteus vulgaris, Saccharomyces cerevisiae,* and *Candida albicans* were found to be solid to solvent ratio 1:15, temperature 85 °C, ethanol concentration 50 %, extraction time 6 h for *N. obtusa*; and solid to solvent ratio 1:15, temperature 85 °C, ethanol concentration 70 %, extraction time 10 h for *C. vulgaris* [155]. Palakawong et al. [150] also optimised the extraction of phenolics with antimicrobial properties against *Escherichia coli, Salmonella typhimurium. Listeria monocytogenes* and *Staphylococcus aureus* from mangosteen bark, leaf and fruit pericarp and found the optimal conditions to be 60 ºC and 1:60 solute to solvent ratio.

3.4 Conclusions

Results revealed the presence of diverse group of phytochemicals in all the four plant species under investigation. All the four plant species exhibited antimicrobial properties and the ethyl acetate fractions produced the most effective antimicrobial properties and also contained wide array of functional groups. Moreover, it was observed that the extracts were more effective against *E. coli* as compared to the other pathogens. The MIC values of some of the extracts, particularly *A. malaccensis* was close to establish antimicrobial agents. It was also seen that all the four species are potentially rich sources of polyphenols and have high antioxidative property. The purified phenolic extracts (PPEs) were found to quench free radicals, act as reducing agents, and chelate transition metals to suppress the initiation of radical formation and inhibit lipid peroxidation. Based on the assay results, it was observed that all the PPEs exhibited varying levels of antioxidant activities. *A. heterophyllus* showed good activity in case of DPPH, ABTS, superoxide anion scavenging activity and FTC assays. *C. extensa* showed good results for ABTS, hydroxyl radical scavenging, H_2O_2 scavenging

activity, ferrous-ion chelating, FRAP and FTC assays. *O. corymbosa* and *A. malaccensis* exhibited good results in case of TBA and nitric oxide scavenging activity assays respectively.

The use of antioxidants as food additives enables protection of food against undesirable oxidative changes. However, synthetic antioxidants which are relatively inexpensive are not preferred due to consumer demand and stringent regulations. In the present study, substantial improvement in the shelf life characteristics of the rice beers stored at 32 ºC was observed with the incorporation of bioflavonoid fractions from the leaves of *A. heterophyllus* and *C. extensa*. The extracts were found to be more effective than the synthetic antioxidant BHT and pre filtration process.

Results also revealed that the total phenolic content, antioxidant activity and antimicrobial activity of the extracts from *C. extensa* leaves varied depending on the type of solvent used. These results reinforced the importance of extractions conditions on total phenolic content, antioxidant activity and antimicrobial activity as a function of changing polarities of the four solvents in the order of distilled water > methanol > ethyl acetate > hexane. Analyses of the response surfaces performed eased to optimize the extraction conditions for bioactive compounds. The experimental data were satisfactorily described by the second-order polynomial model. The optimum conditions of extraction temperature and time for hexane, ethyl acetate, methanol and distilled water were 25 ºC for 29.43 h, 28.28 ºC for 41.27 h, 43.95 ºC for 29.61 h and 55.00 ºC for 48.00 h respectively. Increasing temperature enhanced diffusivity and yields of bioactive compounds in extracts, however, when temperature was too high, the solvents decreased and the yields also decreased. A good correlation in between total phenolic content and antioxidant activity was also observed. Except in case of antibacterial activity, the methanolic extract was found to have the highest activity for all the responses. These optimal conditions will be useful in future studies to carry out the extraction of bioactive compounds with reduced time and also in the development of industrial extraction processes.

Thus, all the tested plants have high potentials for extraction of bioactive compounds and antimicrobial agents, which could be used in the brewing, food and pharmaceutical industries. These extracts could effectively be used as natural antioxidants in food, nutraceuticals, cosmetic and pharmaceutical industries thereby restricting the use of synthetic antioxidants. In addition, they can act as therapeutic or preventive agents against certain degenerative diseases or slowing the oxidative stress in body. All the four plant species are abundantly available throughout the entire Northeastern region of India and has high potential

of industrial application. The results of the present study strongly justify the age old use of these plant species in preparation of rice beer of Northeast India and for traditional management of various ailments as well.

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