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Antioxidant efficacy of phytochemical extracts from defatted rice bran in the bulk oil system

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Abstract

The antioxidant compounds oryzanols, tocols and ferulic acid were identified in the methanolic extracts of defatted rice bran (DRB) by high pressure liquid chromatography (HPLC). The crude methanolic extract (CME) was partially purified by re-extraction with acetone to give an acetone extract (AE). For further purification of the acetone extract, sequential solvent extraction was employed yielding a lipophilic phase (AE-LP) with hexane and a polar phase (AE-PP) with acetone. The antioxidant potential of the DRB extracts and their phytochemical constituents in bulk oils were evaluated using the Schall oven test (SOT) and differential scanning calorimetry (DSC). The extracts were effective in inhibiting lipid oxidation as assessed by peroxide value, diene value and *p*-anisidine value. The activity of the extracts with respect to the inhibition of primary oxidation products followed the order AE-PP > AE-LP = AE > CME with the activity of AE-PP being equivalent to that of butylated hydroxytoluene (BHT) at a 200 ppm level. However, tertiarybutylhydroquinone (TBHQ) was most active as compared to extracts and pure compounds with AE-PP showing about 45% of the activity of TBHQ at 200 ppm level. Defatted rice bran extracts proved to be effective even at the high temperature employed in DSC. The antioxidant efficacy of AE-PP was close to that of TBHQ and far greater than that of BHT at a 200 ppm level as evident from DSC results. The increase in activity with purification might be due to the enhanced levels of antioxidants in purified extracts compared to CME.

Keywords: Defatted rice bran; Phytochemicals; HPLC; Oryzanols; Tocols; Ferulic acid; Tricin; Soybean oil; Schaal oven test; DSC analysis

1. Introduction

Antioxidant compounds are gaining importance due to their dual role in the food industry as lipid stabilizers and in preventive medicine as suppressors of excessive oxidation that causes cancer and ageing (Namiki, 1990). Although synthetic compounds like butylated hydroxytoluene (BHT), tertiarybutylhydroquinone (TBHQ), utylated hydroxyanisole (BHA) and propyl gallate (PG) are widely used as antioxidants in the food industry, their toxicological aspects together with consumer preference for natural products have popularized the use of natural antioxidants (Branen, 1975). Thus researchers are monitoring various plant materials as possible sources of compounds with the potential to be developed into highly efficient and safe antioxidants with a multitude of biological effects.

Agro-industrial byproducts such as oilseed meals have been reported to be rich sources of natural antioxidants. Thus, phenolic antioxidants in the meals of sesame, soybean, canola, peanut, cotton seed, mustard and rapeseed have been characterized (Wettasinghe, Shahidi, & Amarowics, 2002). In the present study, we have focused on the antioxidant potential of defatted rice bran, the major byproduct of the rice bran processing industry.

Rice (*Oryza sativa* L.) is the main cereal of the orient and sub-tropical regions and accounts for 21%, 14% and 2% of global energy, protein and fat supply, respectively (FAO, 1999). With an annual production of 130 million metric tonnes (MMT) paddy corresponding to 80 MMT rice and 10 MMT rice bran, India is the second largest producer of rice and rice bran in the world after China

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(Rajam, Sobankumar, Sundaresan, & Arumughan, 2005). Rice bran, the major byproduct of the rice milling industry is also the source of a high quality vegetable oil viz. rice bran oil (RBO). Rice bran oil has attracted much medical attention due to its strong hypocholesterolemic properties primarily attributable to its balanced fatty acid composition and high levels of antioxidant phytochemicals such as oryzanols, tocopherols and tocotrienols. Defatted rice bran, the predominant byproduct of RBO extraction is a good source of insoluble dietary fibre, protein, phytic acid, inositol and vitamin B (Hargrove, 1994). Though an aqueous ethanolic extract of defatted rice bran (DRB) was reported to have antioxidant activity in the active oxygen method (AOM) test, the extract was not characterized chemically or phytochemically (Shin, Chang, Kang, & Jung, 1992). In the present study, Schall oven test method (60 °C) and DSC method (150 °C) were used to evaluate the antioxidant potential of DRB extracts and the phytochemical constituents identified in the extracts by HPLC. These two models widely differing in temperatures were employed to evaluate the stability and effect of DRB extracts in protecting edible oil (Refined, bleached, deodorized (RBD) soybean oil) under accelerated oxidation conditions of aeration and heating.

2. Materials and methods

2.1. Materials

Defatted rice bran samples were obtained from rice bran oil industry (M/s. Chakkiyathumooda Solvent Extractions, Ankamali, Kerala, India). Tocopherol and tocotrienol standards were obtained from *E*-Merck, Darmstadt, Germany. Ferulic acid was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Standard compounds of oryzanols, cycloartenyl ferulate and 24-methylene cycloartanyl ferulate were a generous gift from M/s. Tsuno Rice Fine Chemicals, Wakayama, Japan. High performance liquid chromatography (HPLC) grade solvents were purchased from Merck Ltd. (Mumbai, India). All other reagents were of analytical grade. Refined, bleached, deodorized (RBD) soybean oil, without added antioxidants was supplied by soybean oil industry (M/s. Sakti Soya's, Pollachi, Tamil Nadu, India).

2.2. Extraction and enrichment of antioxidants from DRB

Defatted rice bran (10 g) was extracted with methanol (150 ml) for 10 h using a soxhlet extractor. The extract was filtered through a Whatman No. 1 filter paper and the solvent was removed under vacuum/N_2 flow to dryness. The weighed residue was redissolved in methanol to obtain an antioxidant solution of known concentration. The crude methanolic extract (CME) thus obtained was partially purified by re-extraction with acetone to give an acetone extract (AE). The lipophilic and polar compounds of the acetone extract (AE) thus obtained were then separated by sequential solvent extraction. For this, dry AE was re-extracted with hexane (5 ml × 3) to give a soluble fraction enriched in lipophilic compounds (AE-LP) and a residue insoluble in the nonpolar solvent. The above residue was redissolved in acetone to give a fraction enriched in polar compounds (AE-PP) (Renuka Devi, 2005a). Pure compounds were isolated from CME by column chromatography and were identified with the help of UV, IR, NMR and Mass spectral (MS) data (Renuka Devi, 2005b).

2.3. Compositional analysis of DRB extracts

The extracts were analysed for protein, sugar and ash according to AOAC methods (1984). The hexane solubles of the extracts were determined by re-extraction of the respective dry extracts with hexane (5 ml × 3). The total phenolic content (TPC) of the extracts was determined using Folin–Ciocalteau reagent (AOAC, 1984).

2.4. Identification of active compounds in rice bran extracts

2.4.1. Analysis of oryzanols

The analysis was performed using a Shimadzu LC-10A HPLC binary system (Shimadzu Corporation, Kyoto, Japan) with a LC-10 AD model pump, a 7125 model Rheodyne injector (Rheodyne, Rohnert Park, CA, USA) fitted with a 20 μL sample loop, a SPD-10A UV–visible detector, with a C-R7 Ae plus integrator for data acquisition and display. A Waters μ-bondapak TM C_{18} column (4.6 mm i.d. × 25 cm) (Millford, MA) was used in the reversed-phase with the solvent system of acetonitrile, dichloromethane, and acetic acid (88:6:6, v/v/v): methanol, *n*-butyl alcohol, water (90:2:8, v/v/v) in the ratio of 75:25 (v/v) at a flow rate of 1 ml/min. The UV detector was set at 325 nm. All extracts were filtered through polytetrafluoroethylene (PTFE) membrane before injection into the HPLC column. Samples were diluted with the mobile phase and analysed. Peak identification was based on the comparison of retention time (RT) values with authentic standards of cycloartenyl ferulate, 24-methylene cycloartanyl ferulate and a mixture of oryzanol standards. The various forms were quantified based upon the peak area of 24-methylene cycloartanyl ferulate, the major oryzanol component in rice bran (Renuka Devi, Jayalekshmy, & Arumughan, 2000).

2.4.2. Analysis of ferulic acid

Preliminary trials were done using isocratic elution with 20% acetonitrile in water adjusted to pH 2 with trifluoroacetic acid and at a wavelength of 280 nm (Adom & Liu, 2002). Later on, the same HPLC conditions used for oryzanols were followed. The main advantage was the simultaneous detection of both ferulic acid and oryzanols in a single run. Peak identification was based on the comparison of RT values with authentic standard of *trans*-ferulic acid and was further confirmed by spiking studies in which a known amount of pure *trans*-ferulic acid was added to CME and analysed by HPLC. The ferulic acid concentra-

tion of sample extracts was extrapolated from the pure *trans*-ferulic acid standard curve.

2.4.3. Analysis of tocopherols and tocotrienols

The same HPLC system as that used for the analysis of oryzanols and a Shim-pack (LC-NH_2 (M)) column (4.6 mm i.d. × 25 cm) was used in the normal-phase with the solvent system, *n*-hexane/isopropanol (96:4; v/v) and at a flow rate of 1 ml/min. The UV detector was set at 297 nm (Renuka Devi, Suja, Jayalekshmy, & Arumughan, 2000). Peak identification was based on comparison of RT values with authentic standards of tocopherols and tocotrienols. The various vitamin E forms were quantitated based upon the peak areas relative to standard calibration plots by an external standard method. Samples were redissolved in *n*-hexane and 20 μl of the solutions were injected into the HPLC column.

2.5. Oil storage studies

Schall oven test at 60 °C was conducted to evaluate antioxidant extracts of DRB against oxidation during the accelerated storage conditions (Fennema, 1976). The antioxidant extracts were added to fresh, RBD soybean oil (without synthetic antioxidant) at various concentrations. The antioxidant treatments included DRB extracts viz. CME, AE, AE-LP and AE-PP; synthetic antioxidants viz. BHT, TBHQ and pure phytochemicals identified in DRB extracts viz. oryzanols (OYL), ferulic acid (FA) and a 1:1 mixture of α-tocopherol and γ-tocotrienol (T_{mix}) at 100 and 200 ppm levels. The oil samples were drawn at three day intervals over a 15 day storage period and analysed for peroxide value (AOCS methods (1997)), *p*-anisidine value (Jirusova, 1975), and diene value (Wettasinghe & Shahidi, 1999). The RBD soybean oil without added antioxidants was used as a control. The results are expressed as % inhibition = [{(value for antioxidant-value for control)/value for control} × 100].

2.6. DSC analysis

For assessing the induction period for lipid oxidation under aeration and heating, DSC analysis was performed (Tan, Che Man, Selamat, & Yusoff, 2002). A DSC instrument (DSC 821; Mettler Toledo, Schwerzenbach, Switzerland) previously calibrated for temperature (melting points of gallium and benzoic acid) and energy (indium fusion enthalpy) was used. Samples of 10 mg were weighed in an open aluminium pan by a Mettler Toledo AG 204 electronic balance and kept in the sample chamber against an empty aluminium pan. The oil without additives (control) was first studied under a dynamic heating regime from 90 to 200 °C and the temperature of onset of oxidative changes was noted from the DSC curve as the point of inflection. The samples were then analysed isothermally at a temperature of 10 °C below the onset temperature. The isothermal temperature was programmed at 150 °C and purified oxygen (99.8%) was passed through the sample enclosure at 40 ml/min. The flow of nitrogen was 200 ml/min.

The antioxidant treatments included DRB extracts viz. CME, AE, AE-LP and AE-PP; synthetic antioxidants viz. BHT, TBHQ and pure phytochemicals identified in DRB extracts viz. oryzanols, ferulic acid, T_{mix}, β-sitosterol and tricin at 100 and 200 ppm levels. These were prepared by dissolving respective quantities in a minimum amount of acetone (~0.5 mL) and the solvent was purged with nitrogen and were immediately subjected to DSC analysis. The onset time of the oxidation reaction corresponded closely to the intersection of the extrapolated baseline and the tangent line of the exotherm and this induction time (IT) was taken as indicative of the oxidative stability of oil. The results are expressed as % stabilization effectiveness (%SE) = [{(IT antioxidant-IT control)/IT control} × 100] (Yen & Lee, 1997).

2.7. Statistical analysis

All measurements were duplicated on duplicate samples (2 × 2). The results were statistically analysed by analysis of variance (ANOVA) and Duncan's multiple range test (DMRT). Statistical significance was accepted at a level of $P < 0.05$ (Duncan, 1955).

3. Results and discussion

3.1. Chemical and phytochemical composition of DRB extracts

The optimization of the process parameters for the preparation and enrichment of an antioxidant extract from DRB has been based on the yields of TPC and on the yields of oryzanols, tocols, and ferulic acid, the phytochemicals identified in DRB extracts (Renuka Devi, 2005a). The HPLC profiles of oryzanols and ferulic acid of CME and tocols of CME are given in Figs. 1 and 2, respectively.

Compositional analysis of CME showed 7.5% hexane solubles, 24.4% sugar, 22.0% protein and 3.9% minerals. The total phenolic content was 5.5%. Oryzanols, ferulic acid and tocol contents of CME were 7832, 5786 and 146 ppm, respectively. Re-extraction of CME with acetone yielded an acetone extract (AE) which was enriched more than 1.5 fold in TPC, oryzanols, ferulic acid and tocols compared to CME (Table 1). Further purification of the acetone extract employing sequential solvent extraction yielded a lipophilic phase (AE-LP) with hexane and a polar phase (AE-PP) with acetone. AE-LP was enriched in oryzanols and tocols and their quantities were increased by two fold or more compared to those in CME. However, the contents of total phenolics, and ferulic acid were decreased significantly ($P < 0.05$) (Table 1). On the other hand, AE-PP fraction was enriched in TPC and ferulic acid by three fold compared to those in CME with a corresponding decrease in the contents of

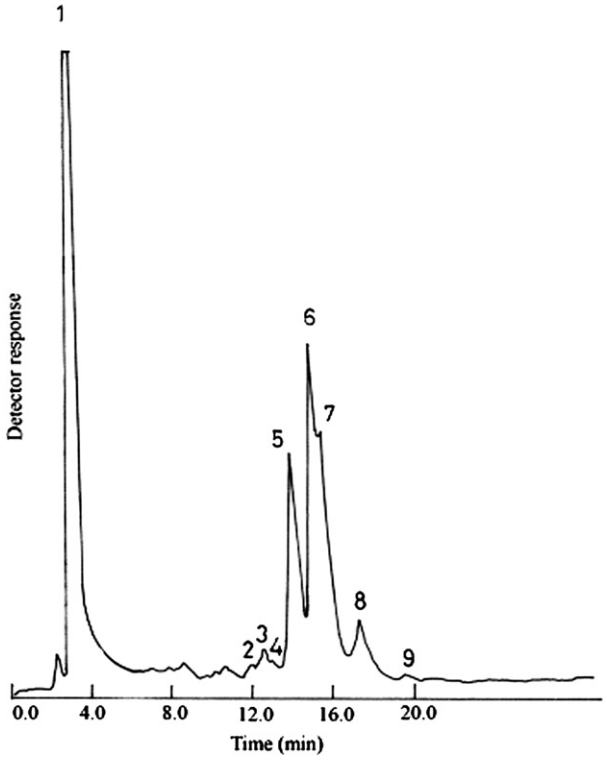

Fig. 1. Reverse- phase HPLC/UV–Visible detection analysis of oryzanols and ferulic acid of defatted rice bran. Peak identification: 1, Ferulic acid; 2, Unidentified; 3, Stigmasteryl ferulate; 4, Unidentified; 5, Cycloartenyl ferulate; 6, 24-methylene cycloartanyl ferulate; 7, Campesteryl ferulate; 8, β-sitosteryl ferulate; 9, Cycloartanyl ferulate.

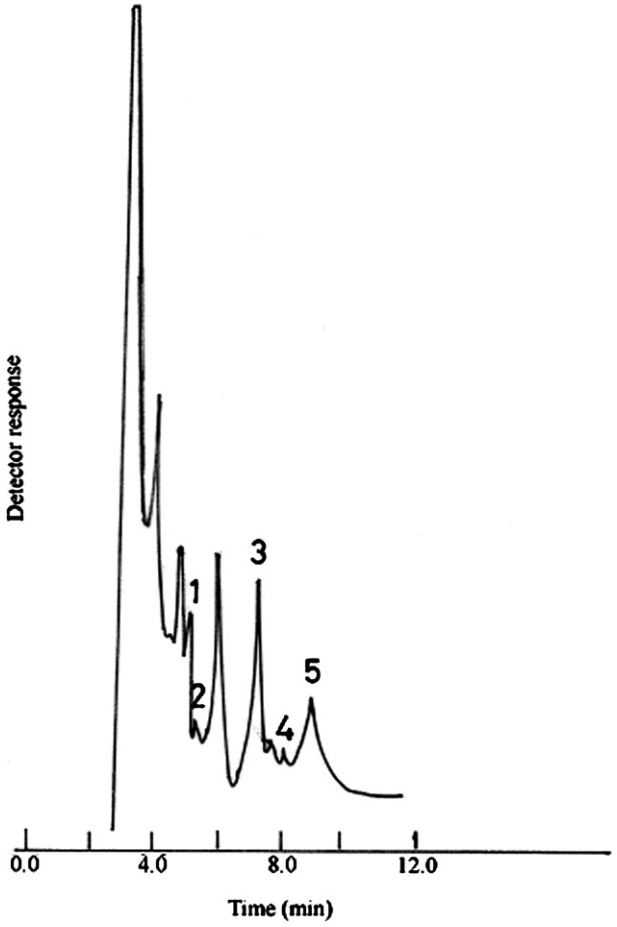

Fig. 2. Normal-phase HPLC/UV–Visible detection analysis of tocopherols and tocotrienols of defatted rice bran. Peak identification: 1, α-T; 2, α-T_3; 3, γ-T_3; 4, δ-T; 5, δ-T_3 (T-tocopherol, T_3-tocotrienol).

oryzanols and tocols (Table 1). Two pure compounds isolated from CME by column chromatography were identified as β-sitosterol and tricin with the help of UV, IR, NMR and MS data (Renuka Devi, 2005b)

3.2. Schall oven test

During 15 days of storage at 60 °C in the dark, soybean oil treated with various DRB extracts, and their pure phytochemical constituents and the synthetic antioxidants had significantly lower peroxide value (PV) than that of the control (Fig. 3). The control reached a maximum peroxide value of 78.9 meq/kg after 15 days of storage from an initial value of 1.8. At the end of the storage period, the order of antioxidant activity expressed in terms of percent inhibition values was TBHQ (86.3) > BHT = AE-PP

Table 1
Phytochemical composition of defatted rice bran extracts

Extracts[B]	Recovery (%)[C]	Phytochemical constituents in the extract[A]			
		TPC (wt %)	Oryzanols (ppm)	Ferulic acid (ppm)	Tocols (ppm)
CME	_	5.5[b] (±.0.2)	7832[b] (±57)	5786[b] (±33)	146[b] (±2)
AE	47.7[c] (±1.8)	10.7[c] (±0.3)	14697[c] (±102)	9204[c] (±66)	260[c] (±3)
AE-LP	26.6[b] (±0.9)	4.8[a] (±0.2)	20469[d] (±129)	944[a] (±8)	347[d] (±4)
AE-PP	19.9[a] (±0.8)	17.7[d] (±0.4)	978[a] (±9)	15858[d] (±113)	51[a] (±1)

[A] Each value in the table represents the mean (± standard deviation) of four analyses from two replications.
[B] CME: crude methanolic extract; AE: acetone extract; AE-LP: acetone extract-lipophilic phase; AE-PP: acetone extract – polar phase.
[C] Recovery percentages are based on CME; for AE-LP & AE-PP, recovery percentages based on AE are 55.8(±1.9) and 41.8(±1.8), respectively.
[a–d] Means within a column with different letters are significantly different ($P < 0.05$) according to Duncan's Multiple Range Test (DMRT).

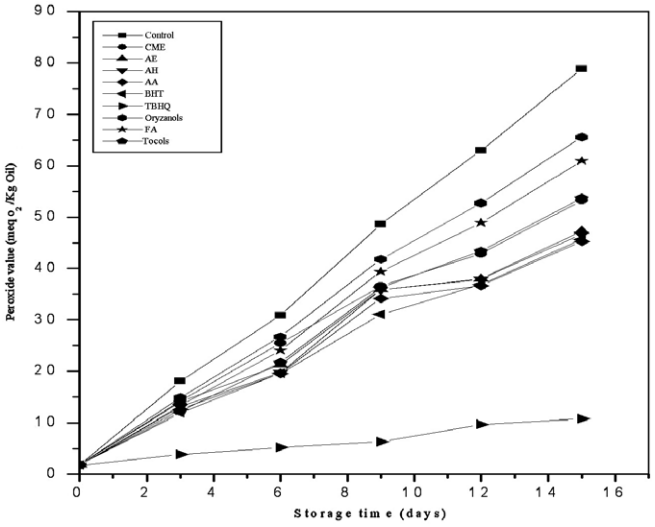

Fig. 3. Effect of rice bran extracts and synthetic antioxidants at 200 ppm levels on the peroxide value of RBD soybean oil by Schaal oven method at 60 °C.

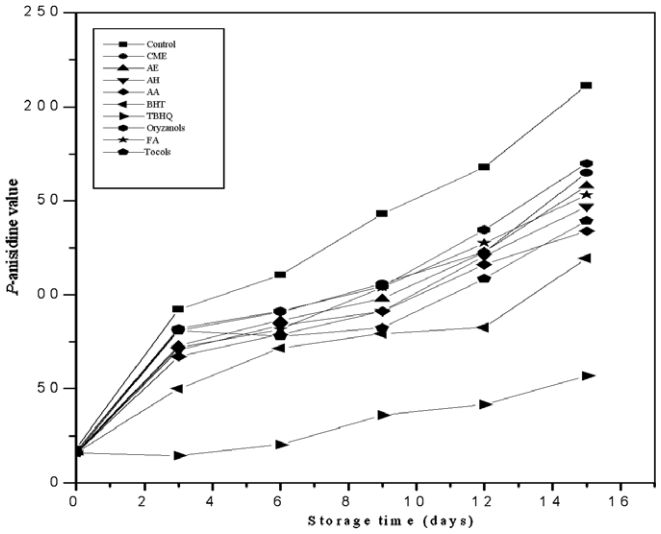

Fig. 4. Effect of rice bran extracts and synthetic antioxidants at 200 ppm levels on the *p*-anisidine value of RBD soybean oil by Schaal oven method at 60 °C.

(42.1) > AE = AE-LP (40.0) > CME = T_{mix} (31.8) > FA (22.7) > OYL (16.8), with the inhibition rates given in parenthesis. The corresponding peroxide values were 10.8, 45.6, 47.3, 53.7, 60.9, and 65.6 meq/kg. The activity of the extracts followed the order AE-PP > AE-LP = AE > CME and the order for the pure compounds was TBHQ > BHT > T_{mix} > FA > OYL. An interesting observation towards the end of the storage period was that all the DRB extracts performed either equally or better than the phytochemical constituents tested which are known to be very good antioxidants. Moreover, the activity of one of the purified fractions viz. AE-PP equaled that of BHT, a widely used synthetic antioxidant in food products. However, TBHQ was the most active as compared to those of extracts or other pure compounds with AE-PP showing about 45% of the activity of TBHQ at 200 ppm level.

Para-anisidine value (*p*-AV) is indicative of the amount of secondary oxidation products produced during oxidative degradation of oils during storage. The *p*-AV of the storage studies of soybean oil are shown in Fig. 4. It is clear from the figure that antioxidant treated samples showed significantly lower *p*-AV($P < 0.05$) compared to that of control during storage. The control reached a maximum *p*-AV of 211.1 from an initial value of 18.0 after 15 days of storage. At the end of the storage period, at 200 ppm levels, the order of antioxidant activity expressed in terms of percent inhibition values was TBHQ (73.1) > BHT (43.4) > AE-PP (36.5) > T_{mix} (33.9) > AE-LP (30.3) > FA (27.4) > AE (25.1) > CME (21.9) > OYL (19.5), with the inhibition rates given in parenthesis. The corresponding *p*-anisidine values were 56.8, 119.3, 133.9, 139.4, 147.0, 153.1, 158.0, 164.8 and 169.9. None of the extracts equalled to the activity of TBHQ, but AE-PP was close to BHT. However all the extracts and pure compounds offered protection in varying degrees either equal to or better than oryzanols.

Conjugated diene value measures conjugation of double bonds before formation of fatty acid hydroperoxides and indicates primary lipid oxidation. Changes in conjugated diene value of the experimental samples are given in Fig. 5. It is evident from the figure that all the antioxidant treated samples showed significantly lower conjugated diene values as compared to that of control during storage. The control reached a maximum D.V of 9.2 from an initial value of 2.9 after 15 days of storage. At the end of the storage period, the order of activity obtained was TBHQ (56.0) > BHT = AE-PP (28.4) > AE = AE-LP (25.2) > CME = T_{mix} (24.3) > FA (22.8) > OYL (19.9), with the inhibition rates given in parenthesis. The corresponding conjugated diene values were 3.9, 6.4, 6.7, 6.8, 6.9, and 7.1. TBHQ was the most potent antioxidant under the experimental conditions studied and all the extracts and pure compounds inhibited diene formation at varying degrees as in the case of PV and *p*-AV.

3.3. Differential scanning calorimetry (DSC)

During oxidation of soybean oil under the standardized DSC conditions of aeration (40 mL/min) and isothermal heating (150 °C), all the antioxidant treatments including samples and standards demonstrated significantly higher induction periods compared to that of the control. For the control, the onset of oxidation was 6.1 minutes. The antioxidant activity expressed as percent stabilization effectiveness values (% SE) followed the order TBHQ > AE-PP > AE-LP > T_{mix} > AE > BHT > CME > tricin > FA > OYL > β-sitosterol at 100 ppm level. At 200 ppm level, the activity order was TBHQ (109.5) > AE-PP (72.5) > AE-LP (70.3) > BHT (59.9) > AE = CME (48.3) ⩾ T_{mix} (47.5) > tricin (43.2) > FA (38.1) > OYL (12.1) > β-sitosterol (7.5), with the activity indices given in parenthesis. The corresponding induction

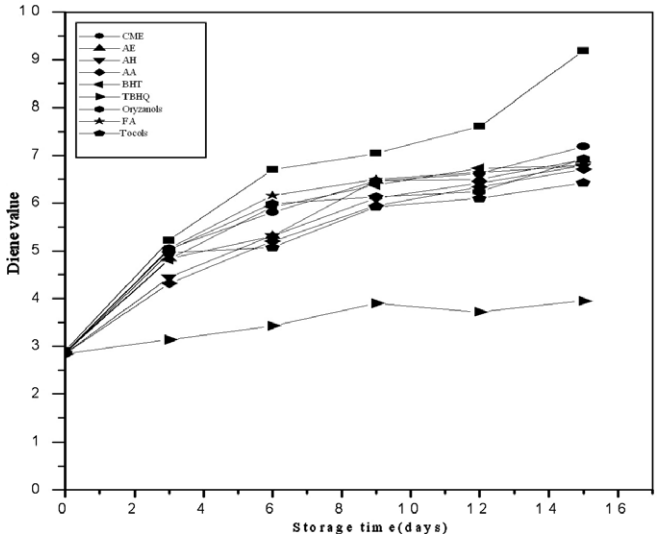

Fig. 5. Effect of rice bran extracts and synthetic antioxidants at 200 ppm levels on the diene value of RBD soybean oil by Schaal oven method at 60 °C.

times at 200 ppm levels were 12.8, 10.7, 10.6, 9.6, 9.2, 9.0, 8.8, 8.3, 7.0 and 6.5 minutes. DSC was especially useful in assessing the antioxidant efficacies of β-sitosterol and tricin which were isolated in small quantities from CME by column chromatography and identified with the help of spectral data (Renuka Devi, 2005b). The fractions AE-PP and AE-LP performed significantly better than BHT and all the extracts were more efficient than tricin, ferulic acid, oryzanol and sterol. The increase in activity with fractionation might be due to the enhanced levels of antioxidants in the resultant fractions compared to CME.

Isothermal DSC has many advantages over other accelerated stabilty methods like SOT, AOM, Rancimat etc. including small sample size (less than 20 mg), minimal sample preparation, simplicity of operation and results in minutes (Tan et al., 2002). For example, oil samples which require 15 days by Schall oven method could be evaluated in less than one hour by DSC. Results of the DSC experiments showed that the DRB extracts were stable at high temperatures and therefore capable of protecting soybean oil against oxidation even at elevated temperatures.

Antioxidant activity of tested samples was found to differ between the accelerated stability methods employed here viz. Schall oven test and DSC. While the order of activity of various extracts and pure compounds by the SOT (PV and DV data) was TBHQ > BHT = AE-PP > AE = AE-LP > CME = T_{mix} > FA > OYL, by DSC it was TBHQ > AE-PP > AE-LP > BHT > AE = CME ⩾ T_{mix} > FA > OYL, at 200 ppm levels. The antioxidant efficacy of AE-PP was close to that of TBHQ and far greater than that of BHT as evident from DSC results. In the case of SOT, the results for AE-PP was comparable or less than that of BHT. Synergistic action and protective effects of proteins, sugars present and other unidentified phenolic compounds at higher temperatures in the case of DSC could be reasons for higher activity for AE-PP fraction.

The greater antioxidant activity of AE-PP fraction compared to that of AE-LP fraction in a bulk oil system may be explained on the basis of the "polar paradox" theory which states that lipophilic antioxidants performed better in emulsions compared to polar hydrophilic antioxidants which were more efficient in bulk oils (Khan & Shahidi, 2000). At identical concentrations AE-PP, AE-LP and AE, performed better than the phytochemical constituents oryzanols, ferulic acid, tricin, sterol and tocols as pure compounds with respect to PV, DV and DSC results. Similar reports on the superior activities of crude extracts compared to the isolated compounds are not uncommon (Xing & White, 1997). The extracts evaluated here also performed either comparable to or even better than some of the currently available natural extracts of plant origin (Duh & Yen, 1997; Lee, Kim, Nam, & Ahn, 2003; Xing & White, 1997). Antioxidant compounds identified and quantitated in the DRB extracts and concentrates are oryzanols, tocols, ferulic acid, tricin etc. which are known to have a multitude of biological effects as well (Kamal-Eldin & Appelqvist, 1996; Lee et al., 1981; Ou & Kwok, 2004; Rukmini & Raghuram, 1991). However, the fact that their concentrations are far lower in the extracts used for evaluation here but their antioxidant efficacy was far greater than that of the individual pure compounds need explanation in the light of synergistic activity and other constituents such as proteins, free amino acids, sugars, and several other unidentified phenolics.

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