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Removing Antinutrients from Rapeseed Press-Cake and Their Benevolent Role in Waste Cooking Oil-Derived Biodiesel: Conjoining the Valorization of Two Disparate Industrial Wastes

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ABSTRACT: Valorization of oilseed processing wastes is thwarted due to the presence of several antinutritional factors such as phenolics, tannins, glucosinolates, allyl isothiocyanates, and phytates; moreover, literature reporting on their simultaneous extraction and subsequent practical application is scanty. Different solvent mixtures containing acetone or methanol pure or combined with water or an acid (hydrochloric, acetic, perchloric, trichloroacetic, phosphoric) were tested for their efficiency for extraction of these antinutritive compounds from rapeseed press-cake. Acidified extraction mixtures (nonaqueous) were found to be superior to the nonacidified ones. The characteristic differences in the efficacy of these wide varieties of solvents were studied by principal component analysis, on the basis of which the mixture 0.2% perchloric acid in methanol/acetone (1:1 v/v) was deemed as "the best" for detoxification of rapeseed meal. Despite its high reductive potential, hemolytic activity of the extract from this solvent mixture clearly indicated the toxicity of the above-mentioned compounds on mammalian erythrocytes. Because of the presence of a high amount of antinutritive antioxidants, the study was further extended to examine the influence of this solvent extract on the stability of waste cooking oil-derived biodiesel. Treatment with the extract harbored significant improvement ($p < 0.05$) in the induction periods and pronounced reduction in microbial load of stored biodiesel investigated herein. Thus, a suitable solvent system was devised for removing the major antinutrients from rapeseed press-cake, and the solvent extract can, thereafter, be used as an effective exogenous antioxidant for biodiesel. In other words, integrated valorization of two different industrial wastes was successfully achieved.

KEYWORDS: *rapeseed press-cake, biodiesel, antinutrients, antioxidant*

INTRODUCTION

Currently there is a considerable impetus for the recovery, recycling, and upgrading of wastes. Industries are more and more forced to find an alternative use for the residual wastes, because large volumes of generated biowastes pose potential disposal and pollution problems along with loss of valuable biomass and functional nutrients.[1] In many instances, food processing wastes might have a potential for recycling raw materials or for conversion into useful products of higher value as a byproduct, or even as raw material for other industries, or for use as food or feed/fodder. In this context, valorization of rapeseed meal is gaining special attention due to its high protein and polyphenol contents. Among these, the content of phenolics in rapeseed flour is much higher than in other oleaginous seeds and amounts to about 30 times that in soybean flour.[2] Although plant polyphenols are usually regarded as valuable natural antioxidants, not all phenolics present in foods are beneficial, and some may even be of antinutritional concern. The presence of such high levels of phenolics (6.4−18.4 mg/g rapeseed flour) or tannins (0.2−3% of defatted rapeseed meal)[3] or their oxidized products contributes to the dark color, bitter taste, and astringency of rapeseed products, lowering its palatability and being nutritionally detrimental in the diets of ruminants. Phenolic compounds have been identified as potent inhibitors of enzymes and iron ions in the gastrointestinal lumen, thus making the iron not available for absorption. In addition, adverse effects of sinapic acid (predominant phenolic acid of rapeseed flour) and tannins have been associated with the tainting of eggs, when incorporated into poultry rations.[4,5] Likewise, the presence of phytates, glucosinolates, allyl isothiocyanates (AITC), etc., has thwarted the application of oilseed meal as a functional food/feed additive. Phytates (5−7%) occupy the second largest position among the antinutrients in rapeseed/canola. They remain strongly associated with protein, starch, and fiber matrices and thereby reduce their digestibility. Also, they reduce the bioavailability of minerals in animal bodies mainly by chelation with divalent cations.[6]

At present there are few possibilities for the utilization of these wastes; the residues are thus disposed of in landfills or used as fertilizers. Incineration of oil-cakes has been largely investigated but not strongly pursued due to the low calorific value and high water content.[1] In the past decade interest in the alternative use of agricultural waste streams beyond disposal or fertilization has increased drastically. Spurred by the above-mentioned factors along with high protein content, the oil-cakes were mostly used as animal feed in the past. However, in

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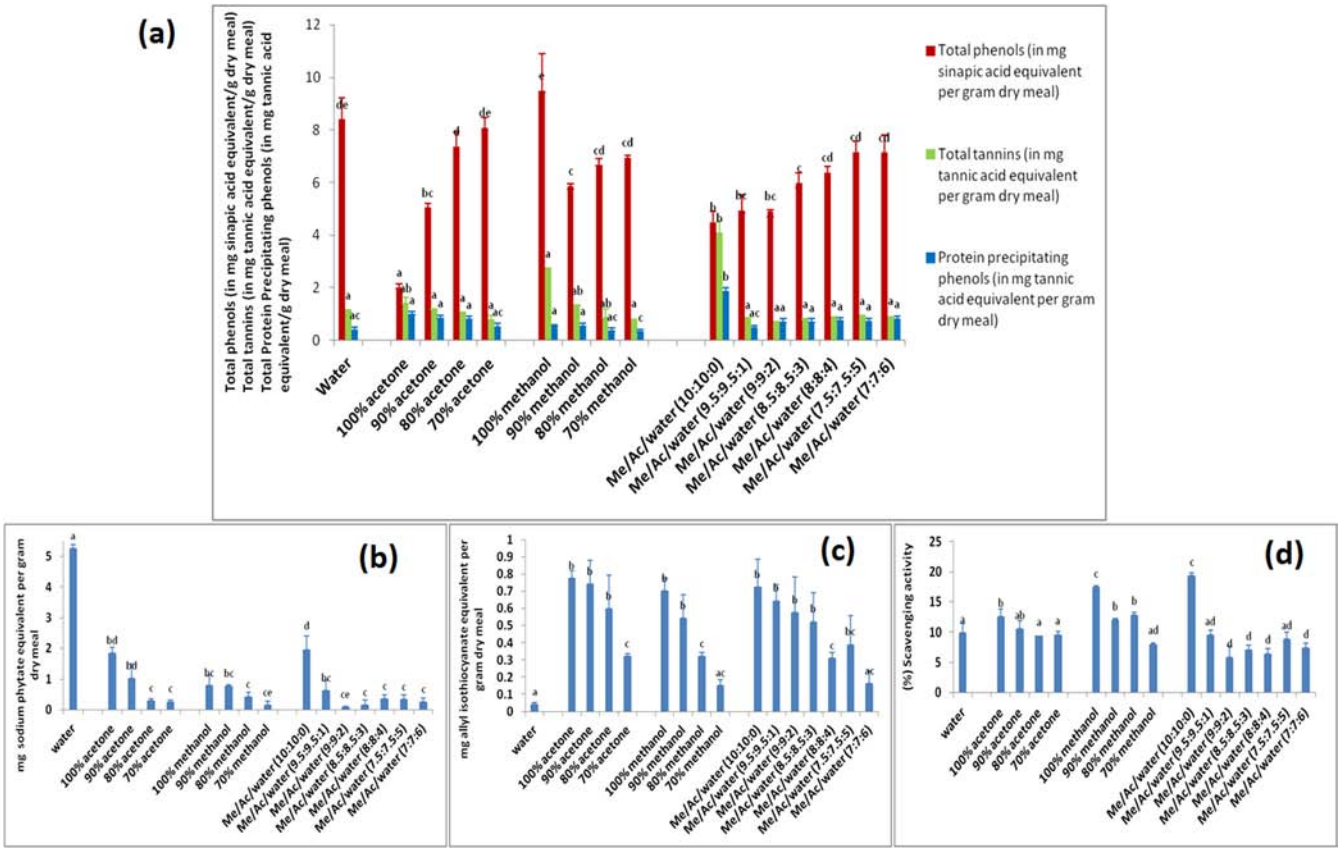

Figure 1. Effect of absolute or aqueous solvent(s) on the yield of (a) polyphenols, (b) phytic acid, (c) allyl isothiocyanate, and (d) DPPH radical scavenging activity.

the past few years, owing to the increasing necessity to take into consideration the ill effects of these antinutritional compounds on human/animal health and animal products, pretreatment of the meal or waste treatment by detoxifying, neutralizing, or destroying the undesirable compounds prior to feeding has been made stringent.[7] Accordingly, to produce rapeseed meal that can be incorporated into feed/food formulations, most of these compounds must be removed. Upgrading of vegetable residues to create a secondary use for the waste products has often been anchored with pretreatment step(s), which have been designated a key stage in the conversion of a biomass to useful products of higher value. Such preprocessing stages must be simple and practically feasible. Certain methods (chemical treatments using alkali or Ca^{2+}/ NH_4^+/Fe^{2+} salts or heat treatment or fermentation)[6,8−11] proposed earlier for detoxifying rapeseed have neither been commercially exploited nor scaled up to practice so far, most probably due to incomplete removal, loss of useful soluble protein, excessive cost, high water consumption, or tedious processing involving cumbersome and error-prone multisteps.[12] Although there is a large body of literature focusing on the phenolic compounds in canola/rapeseed hull, knowledge of extraction of rapeseed phenolics and its other antinutritional compounds from the meal remains fragmentary and diverse. This makes the task of finding a satisfactory method very difficult and urgently needed. Moreover, no systematic attempt appears to have been made to study the simultaneous extractability of these major toxicants from rapeseed meal in a single extraction stage as a function of the composition, concentration, and nature of acidic extraction solvents. The present investigation attempts such a methodical study.

Furthermore, possible application of such an antinutrient-rich extract from rapeseed meal has been explored in this paper. The antioxidative potential of rapeseed antinutrients cannot be overlooked, examination of which has been the subject of numerous publications and scientific papers. Therefore, they may hold much promise for industrial adaptation; particularly, it seems prudent to incorporate these antinutritional antioxidants into nonfood items. In the milieu of exploitation of processing wastes, utilization of waste cooking oil (another industrial waste being generated in bulk quantities annually in several countries) for biodiesel production is obtaining attention. Recently, the demand for biodiesel has increased due to the rise in petroleum prices and the development of government measures such as EU Directive 2003/30/EC on the promotion of the use of biofuels or other renewable fuels for transport. Biodiesel produced from virgin vegetable oils costs much more than petro-diesel; this is a major drawback to the commercialization of biodiesel in the market. Therefore, the use of waste cooking oils (WCO) is one of the more attractive options to reduce the raw material cost. Oxidation stability is one of the major issues affecting the use of biodiesel because of its content of polyunsaturated methyl esters.[13] A minimum Rancimat induction period (IP) of 6 h is defined for biodiesel samples within UNE-EN 14214. Fuels with longer IPs are more stable to oxidation. It is well-known that it is very difficult to meet this limit for biodiesel fuels derived from many common raw materials, unless antioxidants are added to the biodiesel.[13] Petrochemically derived synthetics such as *tert*-butylhydroqui-

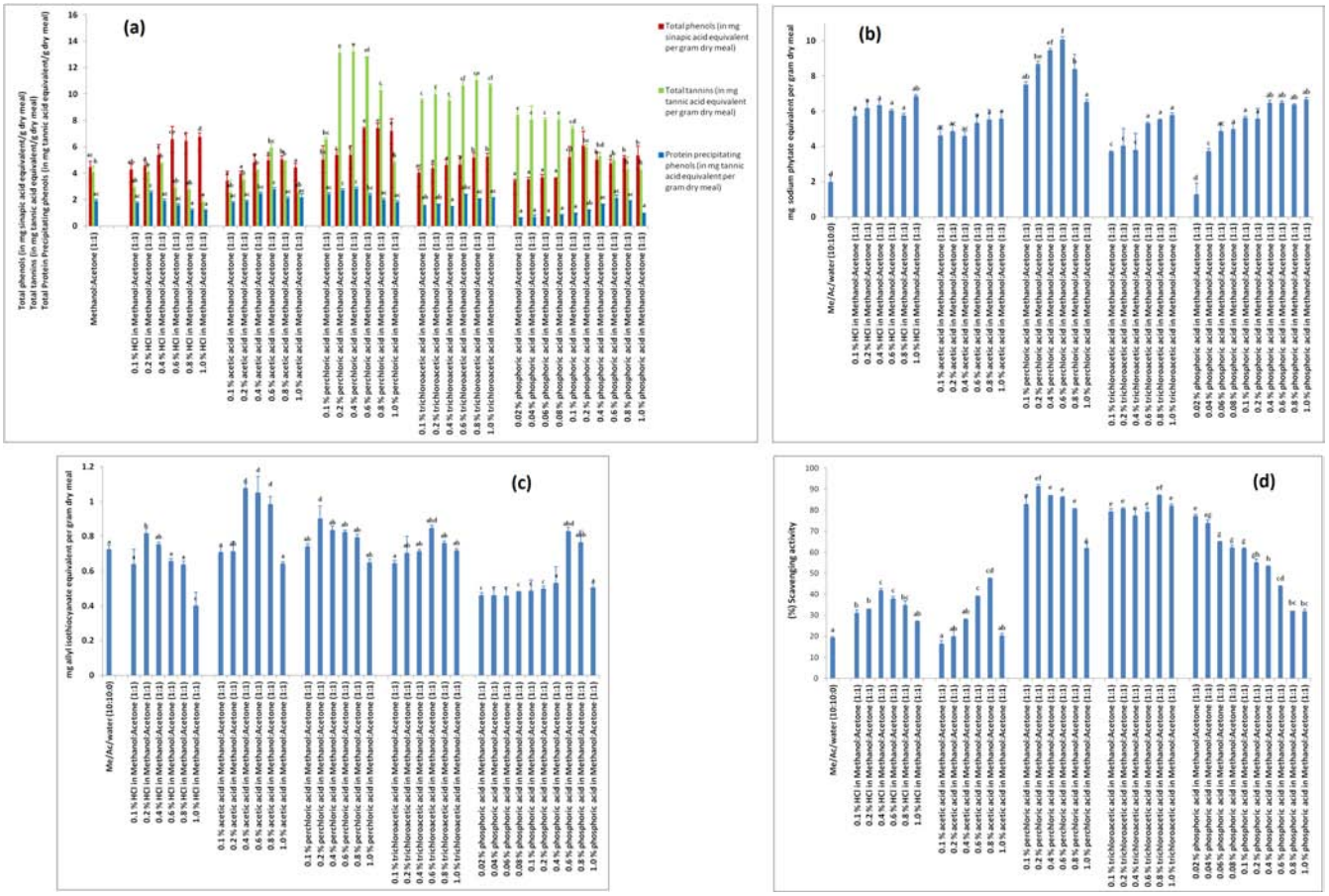

Figure 2. Effect of acidified solvent mixture on the yield of (a) polyphenols, (b) phytic acid, (c) allyl isothiocyanate, and (d) DPPH radical scavenging activity.

none, butylated hydroxytoluene, and butylated hydroxyanisole, although generally more effective at improving IP than natural antioxidants, originate from nonrenewable materials and are expensive for bulk use or even may be insoluble at the desired concentration.[14] Moser[14] used gossypol from cottonseed, and Alexandrino et al.[15] used natural antioxidants from mango seed kernel for increasing the resistance of biodiesels against oxidation. We, therefore, incorporated the rapeseed meal extract (with high reductive potential) into waste cooking oil-derived biodiesel in the pursuit for improving its oxidative and microbial stability, and a comparative study is presented to show the effect of such additive. Thus, in this paper, we attempted to design novel strategies to turn these above-mentioned agro-industrial wastes into value-added products/ingredients via integrated processes.

MATERIALS AND METHODS

Chemicals and Sample Preparation. Rapeseed press-cake was obtained from Assam Khadi & Village Industries Board, Guwahati, India. Press-cake was ground to pass through a 60 mesh size sieve and then stored at −20 °C until use. Waste cooking oil for biodiesel production was collected from a local restaurant situated in the vicinity of Tezpur University campus, Tezpur, India. All solvents and reagents were obtained from E. Merck (India), of either high-performance liquid chromatography (HPLC) grade or analytical reagent grade, and were used without further purification.

Preparation of the Extracts. Meal (1 g) was treated with 10 mL of different solvents (mentioned in Figures 1a and 2a). The suspension was shaken at 200 rpm for 2 h (at 25 ± 1 °C) in an orbital shaker (Sartorius Stedin Biotech, CERTOMAT IS) and then centrifuged (Sigma 3-18K centrifuge) at 5000 rpm for 20 min (at 4 °C). The supernatant (solvent extract) was filtered (Whatman paper no. 4) and stored at −20 °C for further analysis.

Each solvent extract was then dried under vacuum at 35 ± 1 °C. The dried residue was dissolved in 25 mL of Milli-Q water, and its pH was adjusted to neutrality (pH ≈6.8). This neutral solution was filtered through a 0.22 μm MCE syringe filter (BIOFIL) and then either used as-such for phytate and glucosinolate estimation or extracted three times with a diethyl ether/ethyl acetate (1:1 v/v) mixture.[16] The pooled organic phase was finally reduced to dryness under vacuum using a rotary vacuum evaporator (EYELA, model CCA-1110, Japan) at 35 ± 1 °C and was redispersed either in methanol (for quantification of polyphenols and DPPH radical scavenging activity) or in dichloromethane (for AITC estimation).

The best selected solvent extract was dried under vacuum and neutralized according to the same method as described above. An aliquot of this neutralized solution was either dispersed into phosphate buffer solution (PBS, pH 7.4) (for hemolytic activity test) or freeze-dried for using it directly as an additive in biodiesel. Likewise, the residual pellet (meal remaining after solvent treatment) was also dried in a vacuum oven under reduced pressure (150 mmHg) at 35 ± 1 °C for 42 h and then extracted in PBS buffer for 2 h at 25 ± 1 °C using an orbital shaker (200 rpm). This aqueous meal extract was filtered through a 0.22 μm MCE syringe filter (BIOFIL) and used for the hemolytic test.

Quantification of Antinutritional Compounds. The amount of total phenolics (TP) in the extract was determined using the Folin−Ciocalteu reagent and sinapic acid as standard (sinapic acid equivalent, SAE) as described by Szydłowska-Czerniak et al.[17]

Total tannins (TT) and protein precipitable phenols (PPP) were estimated spectrophotometrically according to the protocols devel-

oped by Hagerman and Butler[18] and Makkar et al.,[19] respectively, using tannic acid as standard (tannin acid equivalent, TAE).

Estimation of phytates was done using Wade reagent, according to the method described by Bhandari and Kawabata.[20] Results were expressed as sodium phytate equivalent (SPE) in milligrams per gram dry meal.

Glucosinolate content, in terms of sinigrin equivalent, was determined following the methodology developed by Tsao et al.[21]

For quantification of AITC, the protocol followed by Zhang et al.[22] was used with slight modifications. To the dried solvent extract residue (30−50 mg) were added 1 mL of water and 50 mL of dichloromethane, and then the mixture was sonicated in an ultrasonic water bath (Labotec Inc., UK). AITC was extracted by dichloromethane from the residue for 10 min twice in the ultrasonic condition. The lower phase containing AITC was obtained by centrifugation at 6000 rpm for 10 min. The content of AITC in dichloromethane was determined using ultraviolet spectrophotometry at 247.1 nm (its maximum absorbance).

DPPH Radical Scavenging Activity. One hundred microliters of the sample was mixed with 3 mL of a methanolic solution of DPPH (0.1 mM). After 30 min of incubation in the dark (at room temperature), absorbance was measured at 515 nm. The percentage scavenging was calculated according to the following equation:

$$\%\ \text{scavenging activity} = [\{\text{Abs}_{\text{control}} - \text{Abs}_{\text{extract}}\}/\text{Abs}_{\text{control}}] \times 100$$

$\text{Abs}_{\text{control}}$ is the absorbance of the reagent (without extract) after 30 min, and $\text{Abs}_{\text{extract}}$ is the absorbance of extract after 30 min.

Hemolytic Activity Assay. The hemolytic test was performed following the protocol of Nair et al.[23] with slight modification. Briefly, fresh goat blood from a slaughterhouse was collected in a centrifuge tube containing anticoagulant, trisodium citrate (3.2%), and was centrifuged at 2500*g* for 10 min. The supernatant was discarded, and only the erythrocytes were collected. The erythrocytes were further washed three times with PBS (pH 7.4). A 10% (v/v) suspension of erythrocytes in PBS was prepared; 1.9 mL of this erythrocyte solution was placed in a 2 mL centrifuge tube, and 0.1 mL of sample extract in PBS was added to it. The tubes were then incubated for 1 h at 37 °C. Triton X-100 (0.2%) and PBS were taken as the positive and negative controls, respectively, for comparison. After incubation, the tubes were subjected to centrifugation at 2500*g* for 10 min. Then, 0.2 mL of the supernatant was added to 2.8 mL of PBS for dilution, and finally absorbance was taken at 570 nm in a UV−visible spectrophotometer (CECIL 7400, 7000 series, Aquarius).

Transesterification of Waste Cooking Oil. Biodiesel was prepared by the two-step esterification procedure described by Patil and Deng.[24]

Analytical Methods for Biodiesel. *Characterization of Physical Properties.* Biodiesel, with or without added rapeseed meal extract, was characterized for its physical properties, those being density, kinematic viscosity, cloud point, pour point, flash point, and acid number, employing American Society for Testing and Materials (ASTM) methods (Table 3).

Oxidative Stability. IP (h) was measured in accordance with EN 14112 utilizing a Metrohm USA, Inc. (Riverview, FL, USA) model 743 Rancimat instrument. The flow rate of air through 3 ± 0.01 g of sample was 10 L/h. The block temperature was set to 110 °C. The glass conductivity measuring vessel contained 50 ± 0.1 mL of deionized water. IP was mathematically determined as the inflection point of a computer-generated plot of conductivity (μS/cm) of deionized water versus time (h).

FTIR Analysis. Infrared spectra were measured using an FTIR Nicolet Magna 5PC spectrophotometer (Impact 410, Nicolet) coupled to a PC with Omnic analysis software and having a deuterated triglycine sulfate (DTGS) detector and Nernst Filament as the IR light source. The resolution was 2 cm^{-1} with an average of 30 scans. Spectra were recorded in the range of 4000−400 cm^{-1}.

GC-MS Analysis. The fatty acid methyl esters (FAMEs) content was determined by gas chromatography (model Perkin-Elmer, Clarus-600G), coupled with a mass spectrometer (Clarus-600 mass spectrometer). Separation was performed with an Elite-wax capillary column (0.3 mm internal diameter × 30 m × 0.5 μm film thickness). The oven temperature was increased from 50 to 300 °C at a rate of 5 °C/min, the injector temperature was 280 °C, and the detector temperature was 275 °C. Helium was used as carrier gas at a rate of 1 mL/min linear velocity. The sample (1 μL) was injected into a split mode (split ratio = 50:1) by a Hamilton 75N syringe (ga 26 S/51 mm/pst 2). The MS was operated in electron ionization mode at 70 eV. Individual components (peak identification) were identified by comparing retention time with their reference standards (run under identical experimental conditions) and reconfirmed by mass spectral library of standard compounds (NIST-MS Search 2.0 software, National Institute for Standard Technology). The individual components were quantified by peak area integration, using a built-in data handling software system provided by GC-MS manufacturer (Perkin-Elmer).

Microbiological Analysis. Microbial count was determined using nutrient agar for total viable cell count.[25] Sample (1 mL) was spread in triplicate using the surface plating method; the plates were then incubated at 30 °C for 48 h, after which colony counting was done manually, and the mean reading was recorded.

Statistical Analysis. All analyses were performed in triplicate, and the mean value was calculated. Analysis of variance (ANOVA) and separation of means were carried out by Tukey's HSD test, using SPSS software (version 16.0, SPSS Inc., Chicago, IL, USA) and considered significantly different at $p < 0.05$. A multivariate analysis of the colorimetric and sensory data was analyzed by principal component analysis (PCA) with the Statistica (version 7, StatSoft, Tulsa, OK, USA) software package.

RESULTS AND DISCUSSION

Recently, Khattab et al.[26] and Thiyam et al.[27] analyzed sinapic acid derivatives of rapeseed and found numerous unidentified peaks in the HPLC chromatogram due to lack of authentic standards. A method for simultaneous determinations of all rapeseed polyphenolic compounds and their derivatives is still unknown, and therefore it is difficult, on the basis of available data, to select both appropriate standards and HPLC methodologies for separation and quantitation of each phenol involved.[28] The total polyphenol content of each solvent extract was therefore quantified by the widely employed Folin−Ciocalteu assay, which is generally regarded as an index for overall yield.[17]

Moreover, the crude extract of the meal is expected to contain a series of oligomeric as well as polymeric phenolics (especially tannins), which differ in their specificity toward the reagents used for their determination.[5] This again makes the selection of appropriate methods for quantitation of tannins an intricate task. Lately, attention has been focused on the quantification of tannins based on their property of binding/precipitating proteins, as both the ecological and biological roles of tannins are attributed to its complexation with proteins, and it provides information that cannot be obtained with chemical assays.[18] Besides the condensed tannins, hydrolyzable tannins can also precipitate proteins.[29] Therefore, to avoid complications associated with different classes of tannins and to distinguish polyphenols of nutritional concern from other low-molecular-weight phenolics that also occur naturally in these products, net contents of tannins and protein precipitable phenols were determined by protein precipitation assay because this method is highly correlated with the biological value of tannin-rich food and feed.

Only one-stage extraction is performed here as Cai and Arntfield[30] and Wang et al.[31] found no statistical differences in the amount of extracted rapeseed phenolics with the number of extractions or the extraction time.

Series I: Selection of Suitable Solvent(s) for Simultaneous Extraction of Polyphenols, Phytic Acid, and AITC. Selection of an extraction solvent is the preliminary parameter that should be critically considered before an extraction is started as there is no single universal solvent applicable for all plant matrices. Absolute methanol or acetone or their aqueous mixture is commonly used in rapeseed/canola polyphenol extraction.[2] Preliminary trials were performed using these solvents to determine the most efficient solvent(s) for the simultaneous extraction of TP, TT, PPP, phytates, and AITC from the meal. Usually, the presence of a small amount of water along with acetone and/methanol has been reported to be essential to ensure high phenolics yield.[2] Conversely, a large amount of water, for example, >30%, is undesirable because the protein content is to some degree soluble in the water and so a substantial amount of meal protein will be lost if too much water is used.[32] Therefore, in accordance with the common practice in the literature, aqueous organic solvent having water content >30% was not tested. Figure 1a shows that the extraction of phenolics was dependent on the solvent used, with statistically significant differences ($p < 0.05$) between the values for the sample. This variation is highly due to the relationship with variable degree of phenolic polymerization, solubility of phenolics, type of phenolic present, interaction between phenolics and other constituents in a particular extract, polarity and dielectric constant of the solvent(s) used, etc.[33,34] TP yield in absolute methanol extract (100% methanol) was the highest, which was closely followed by those in the water and 70% acetone extracts (Figure 1a), whereas the highest yield of TT was obtained in a Me/Ac (1:1 v/v) mixture, which was followed by 100% methanol (Figure 1a). Cai and Arntfield[30] also found refluxing with 100% methanol to be very efficient in removing phenolics from rapeseed. An increase in the percentage of water in methanol or acetone or Me/Ac mixture caused a progressive increase in TP yield. One of the possible reasons for this observed increasing trend may be due to the fact that aqueous methanol, due to its polarity, is more effective at extracting polyphenols linked to polar fibrous matrices. On the other hand, acetone/water mixtures are more useful for extracting polyphenols from protein matrices because they appear to degrade the polyphenol−protein complexes.[35] As for TT extraction, irrespective of the presence of methanol or acetone, the yield decreased drastically as the water content in the extraction solvent increased. This can be explained according to the postulations of Dai and Mumper;[36] that is, the extractability of higher molecular weight polymeric phenols decreases as the polarity of the solvent increases. Wide discrepancies are apparent in the literature on rapeseed phenolics and tannin contents,[3] which may be due to the existing differences in the solvent extraction systems employed for their recovery and methods subsequently used for their quantitation;[2] hence, comparison of the present values with the reported data is difficult.

The deleterious effect of tannins depends on the quantity and their protein-precipitating capacity. Naczk et al.[5] reported that tannins isolated from canola and rapeseed hulls exhibit a protein precipitation capacity or reduce protein digestibility by affecting enzyme activities. Therefore, PPP content in the solvent extracts also needs to be determined. The order of PPP extraction efficiency of the tested solvents was similar to that noted for TT, except that acetone-containing solvent extracts performed better than the ones obtained with methanol. Acetone is useful in dissolving the protein-bound phenolic complexes, especially less polar phenolics and highly methoxylated aglycone forms of polyphenols, and therefore considered as a good solvent to extract phenolics from protein-rich samples such as rapeseed meal.[34] Similar to our study, Naczk et al.[5] also found that the PPP correlated well with the TT content. Although the water extract showed a high amount of TP, it exhibited very poor protein precipitating activity, signifying that water is not an appropriate solvent for releasing PPP from the meal. Me/Ac (1:1 v/v) extract showed the highest yield of ∼2 mg TAE/g dry meal, which was found to be quite less compared to that obtained for TP and TT.

Liberation of phytates from the meal into these solvent media was also analyzed. Water extract showed the highest yield of phytic acid (Figure 1b). This is due to the fact that water leaches out a large quantity of meal protein and sugars, onto which the oilseed phytates are mostly attached.[37] Although the use of water for extracting phytic acid seems lucrative, it causes heavy loss of meal protein, which is highly valued in the food and cosmetic industries. Moreover, if any extended application or assay of this water extract is desired, removal of unwanted proteinaceous matter from the extract would require additional processing, thereby making this solvent less attractive for further use. Following the water extract, the Me/Ac (1:1 v/v) extract presented the second highest phytate yield, which in turn is closely followed by the 100% acetone extract (Figure 1b). A general decreasing trend in phytate yield was observed with increasing water content in the organic solvents or their mixtures. This may be due to the release of more phytate-related phospholipids into the organic solvents than in the aqueous ones. Phytates have been reported to show a high correlation with the organic phosphorus content of many fruits, vegetables, crops, etc.[38] Varying the percentage of water in aqueous the Me/Ac mixture gave comparable phytic acid yield ($p > 0.05$), so the presence of water gave no significant improvement.

Brassica and other cruciferae have long been known to contain high levels of undesirable thioglucosides commonly known as glucosinolates. In the current study, glucosinolate content in the meal and the solvent extracts was found to be below the detection level (<0.15 μg sinigrin equiv/g dry meal or /mL of extract), which perhaps may be due to its autolysis by the endogenous myrosinase enzyme liberated alongside during cold-pressing of seeds for oil extraction. A similar observation was noted by Xu et al.[39] in yellow mustard meal. Tsao et al.[40] showed that sinigrin dissolved into aqueous media from ground mustard seed and was very quickly degraded by codissolved myrosinase into AITC. This seems to be the plausible cause for the much reduced glucosinolate content in all of the solvent extracts and the meal. Varying manifestations of toxicity, from depressed weight gain to enlarged thyroids and kidneys to carcinogenesis to death, are observed in rats and other species ingesting glucosinolate hydrolytic products.[41] Consequently, the major hydrolytic product of glucosinolates, that is, AITC, was estimated. AITC yields of different extracts are presented in Figure 1c. It is clear that because of the nonpolar nature of AITC, its solubility and hence the yield were more facilitated in organic solvents, devoid of water, than in the aqueous ones. AITC content perpetually declined in aqueous solvents. This remains true, no matter what the solvent used.

Rapeseed polyphenols, AITC, and phytates have been well-recognized as strong antioxidants. With the hope of finding the antioxidative power of these solvent extracts, DPPH radical

scavenging activity was performed. The model using scavenging of the stable DPPH radical is a widely used assay to evaluate antioxidant activity in a relatively short time compared with other methods. The decrease in absorbance occurs when the DPPH radical accepts an electron or hydrogen from an antioxidant. The radical becomes a stable molecule that is visually noticeable as a color change from purple to yellow. The percent scavenging activities of the crude extracts toward DPPH radical are depicted in Figure 1d. Comparable scavenging activity has been achieved for acetonic and methanolic extracts (except 100% methanol). As can be seen from the results, the highest variations were found in the aqueous Me/Ac (1:1 v/v) extracts. Extract from the Me/Ac (1:1 v/v) mixture showed the highest scavenging activity, which may be ascribed to its high TT and PPP contents.

Series II: Augmenting the Solvent Efficacy Using Hydrochloric Acid (HCl), Acetic Acid (CH_3COOH), Perchloric Acid ($HClO_4$), Trichloroacetic Acid (CCl_3COOH), and Phosphoric Acid (H_3PO_4). Many authors have stated that the most efficient extraction of polyphenols and phytates can be achieved by using acidic solvent. Because the primary focus of this work was to increase the effectiveness of commonly used extraction solvent(s) for removing antinutritional antioxidants from rapeseed meal, the second step of the preliminary study (series II) was the selection of the most appropriate solvent system from series I and then the addition of various concentrations of different acids into it, for further boosting its efficiency to extract TP, TT, PPP, phytates, and AITC from the meal. Finally, enhancement or reduction in DPPH radical scavenging potency of these acidified solvent extracts was also assessed. Me/Ac (1:1 v/v) mixture was selected for series II, as it showed the highest yield of TT, PPP, and scavenging activity and fairly good yield of TP, AITC, and phytates, among all of the tested solvents of series I. Our choice partly agreed with that of earlier workers; Naczk et al.[42] used a mixture of acetone/methanol/water (7:7:6 by volume) to obtain a high amount of phenolics from rapeseed/canola, whereas Krygier et al.[16] employed 70% methanol/70% acetone (1:1) to do the same. On the basis of the implication of Dai and Mumper,[36] concentrations of acids above 1% in Me/Ac (1:1 v/v) mixture were not tested in the current study.

On average, solvent extract containing higher concentration of acids (≥0.6%) gave higher TP yield, especially HCl and $HClO_4$ (Figure 2a). The most efficient solvent was 0.8% $HClO_4$ in Me/Ac (1:1 v/v), presenting ∼7.42 mg SAE/g dry meal, followed by 0.6% $HClO_4$ in Me/Ac (1:1 v/v) and 1% $HClO_4$ in Me/Ac (1:1 v/v) with ∼7.39 and ∼7.21 mg SAE/g dry meal, respectively. Likewise, the TT extractability remarkably increased in $HClO_4$-, CCl_3COOH-, and H_3PO_4-containing solvent systems (Figure 2a). In the case of TT yield, 0.4% $HClO_4$ in Me/Ac (1:1 v/v) proved the most efficient solvent (∼13.18 mg TAE/g dry meal), followed by 0.2% $HClO_4$ in Me/Ac (1:1 v/v) and 0.6% $HClO_4$ in Me/Ac (1:1 v/v) (∼13.07 and ∼12.75 mg TAE/g dry meal, respectively). An increasing trend in TT yield was observed with increasing concentration of CCl_3COOH in solvent mixture (Figure 2a). A low concentration of H_3PO_4, ranging from 0.02 to 0.1%, was found to be suitable for tannin extraction. It can be inferred that acid breaks the molecules liberating the bound phenolic compounds from the plant matrices. The presence of an acid in the solvent increases the extraction capacity as well as the rate of extraction of these desired compounds.[43] However, the acidity of the extraction solvent may cause hydrolysis of some labile phenolic compounds and/or may affect the chemical modification of these compounds, thereby changing their sensitivity to the reagents used in the assays.[36] The latter may be one of the probable reasons for explaining the decreasing trend of TT yield vis-a-vis the perpetually increasing trend of PPP yield in the extracts having ascending concentration of H_3PO_4 (Figure 2a), although TT and PPP were expected to show correlation as predicted by many authors.

As can be seen from Figure 2a, the highest variations in the PPP yield were found in the CH_3COOH- and CCl_3COOH-containing solvent mixtures. The variations may be attributed to polarities of different compounds present in the meal. Lower concentration of H_3PO_4 (<0.2%) was found as not very suitable for extracting PPP. Likewise, very high concentration of acids also gave poor results. This can be attributed to the possible degradation of some labile phenolics under such harsh conditions, or there is a possibility of the formation of insoluble colored decomposition products in the extracts having high acid level,[44] which may not be able to form complexes with soluble protein during the assay. The best result was obtained with 0.2−0.4% $HClO_4$. These results were similar to those obtained with 0.2% HCl and 0.6% CH_3COOH and slightly higher than that obtained for an extraction with Me/Ac (1:1 v/v).

The content of phytic acid in the acidified solvent mixtures increased greatly compared to those in nonacidified solvent systems (Figures 1b and 2b). Usually, phytates are extracted with the aid of an acid. Solvents having $HClO_4$, HCl, and H_3PO_4 were more efficient in phytate extraction than CH_3COOH- and CCl_3COOH-containing ones (Figure 2b). Levels of 0.6, 0.4, and 0.2% $HClO_4$ in Me/Ac (1:1 v/v) were the most efficient solvents, resulting in ∼10.06, ∼9.45, and ∼8.66 mg SPE/g dry meal, respectively. Acid concentration dependent phytic acid yield was observed in the CCl_3COOH and H_3PO_4 acidified solvents; application of higher concentration of acid gave comparatively higher yield. This may be due to the acidic hydrolysis of linkages existing between phytate molecules with other plant components.[45] Phytate contents of solvent extracts containing HCl were comparable between themselves and the CH_3COOH-containing ones.

With regard to the AITC content, the extracts prepared from 0.4−0.8% CH_3COOH contained substantially higher amounts compared to other solvents (Figure 2c). Sinigrin and AITC are potentially prone to degradation if exposed to ambient conditions and pH <5.[40] This may be one of the possible explanations for the decreasing trend in AITC yield noted in the case of acidified solvents, particularly when HCl, CH_3COOH, and $HClO_4$ were used at a concentration of ≥0.2%. Some exceptions were observed in the case of CCl_3COOH- and H_3PO_4-containing solvents; even so, their concentration >0.6% pointed toward pretty much alike decreasing tendency.

The different acidified solvents clearly influenced the antioxidant activity of the extracts (Figure 2d), with statistically significant differences ($p < 0.05$) between them. $HClO_4$- and CCl_3COOH-containing mixtures gave much higher scavenging activity than H_3PO_4-containing ones, which can mainly be attributed to the higher quantities of tannins and phytic acid in these extracts. Moreover, in the extracts obtained with H_3PO_4, the scavenging effect decreased with the increasing acid concentration, indicating the hydrolysis of the antioxidative compounds, whereas the opposite was observed in the extracts having CH_3COOH. For HCl-acidified solvents, a comparatively

higher activity was observed at intermediate acid levels. In HCl- and CH_3COOH-containing mixtures, the scavenging activities were significantly lower compared to the other acidified mixtures. Nevertheless, the DPPH radical scavenging activity of all the tested acidified solvent systems was found to be either much higher or comparable with that of Me/Ac (1:1 v/v). There may be two probable reasons behind the increased antioxidant activity of the acidified Me/Ac (1:1 v/v) extracts than the nonacidified one: the higher dissolubility of phytates, AITC/ and polymeric phenols in acidic media imparted more DPPH radical scavenging, or the use of acids in extraction media hydrolyzed the sinapine and sinapic acid derivatives from the meal into free sinapic acid, which is reported to have very high antioxidant potential.[27]

Principal Component Analysis. To find the most appropriate solvent system for obtaining higher yield of the selected antioxidative phytochemicals (polyphenols, AITC, and phytates) from rapeseed meal concomitantly in a single run, PCA was carried out using data listed in Figure 2. By the cross-validation technique, it was established that three PCs were significant (eigenvalue > 1.0) for explanation of total cumulative variability of 92.24%; PC1, PC2, and PC3 accounted for 42.93, 28.70, and 20.61% of total variability, respectively (Table 1).

Table 1. Eigenvalues of Correlation Matrix and Related Statistics

principal component	eigenvalue	% total variance	cumulative eigenvalue	cumulative (%)
1	2.576	42.928	2.576	42.928
2	1.722	28.698	4.298	71.626
3	1.237	20.614	5.534	92.240
4	0.317	5.282	5.851	97.522
5	0.090	1.506	5.942	99.028
6	0.058	0.973	6.000	100.00

The PCA plots (PC1 × PC2) provided an overview of the similarities and differences between samples and their selected variables. All of the studied parameters (variables) fell in the right-hand area of PC1 (located on the first and second quadrants) (Figure 3a). Being clustered tightly together, the loading plot (Figure 3a) clearly showed that the DPPH radical scavenging activity of the extracts was positively correlated to tannin content (Pearson correlation coefficient, r, = 0.92). Other parameters also exhibited similar close associations among themselves; however, Pearson correlation coefficients were found to be <0.9. To understand more about the relationship between the variables and PCs, eigenvectors were studied.[46] Among the eigenvector values (Table 2), those showing absolute values >0.1 were adopted to explain the projection of the samples on the factor plane.[46] PC1 was positively correlated with all of the studied variables. PC2 showed positive correlation with TP, PPP, phytate, and AITC contents and negative correlation with TT and DPPH scavenging activity. PC3 showed high negative correlation with TP and marginal association with the others.

The score values for the first two PCs (PC1 and PC2) are often used to represent the characteristics of the samples. The score plots (Figure 3b) enabled differentiations among quality characteristics of the extracts tested. It was noticeable that the 0.2, 0.4, and 0.6% $HClO_4$-containing solvent extracts were closely clustered separately from all of the other samples in the first quadrant of the plot, indicating their similarity in overall extraction efficiency. It is also clear that processing with these solvent systems generally results in a shift closer to the studied variables (toward the right-hand side of the plot), that is, these solvent extracts reflect higher amounts of polyhenols, phytates, AITC, and DPPH activity compared to the others. The separated locations of these extracts in the multivariate space clearly revealed differentiations between the large numbers of samples and also helped in drawing a compromise between the partially incommensurable quality traits of these solvent extracts. Of these three solvent systems, the extract from 0.2% $HClO_4$ in Me/Ac (1:1 v/v) was selected for further use because of practical reasons (acids should be used at the lowest possible concentrations) and for its highest DPPH activity. Therefore, the use of a multivariate tool (PCA) enabled us to comprehensively and systematically assess the potentiality of the solvent(s) that need to be used in the processing industries.

Hemolytic Activity Assay. With the anticipation of utilizing such high antioxidative plant extract for food or pharmaceutical or cosmetic application, the hemocompatibility of such an extract was tested, as it would eventually come in direct/indirect contact with blood as soon it was exposed to the host's body. The 0.2% $HClO_4$-containing Me/Ac (1:1 v/v) solvent extract was selected for this assay, as it showed the highest reductive potential (DPPH radical scavenging activity). Because this extract also contains high concentrations of phytates, AITC, and polyphenols and could therefore be considered as

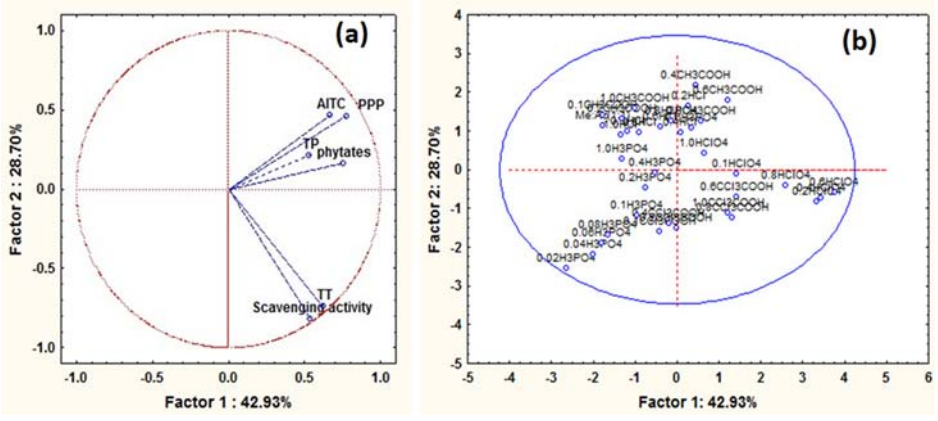

Figure 3. Distribution of (a) variables (loading plot) and (b) solvent extracts (score plot) on the multivariate domain (PC1 × PC2).

Table 2. Eigenvectors of Correlation Matrix

variable	PC1	PC2	PC3	PC4	PC5	PC6
total phenolics (TP)	0.332	0.161	−0.664	0.629	0.103	0.132
total tannins (TT)	0.388	−0.564	0.168	0.001	−0.175	0.688
protein precipitating phenols (PPP)	0.484	0.351	0.328	−0.139	0.709	0.116
phytic acid	0.470	0.122	−0.451	−0.682	−0.284	−0.125
DPPH radical scavenging activity	0.336	−0.626	0.040	0.132	0.196	−0.662
allyl isothiocyanate (AITC)	0.416	0.356	0.468	0.321	−0.580	−0.205

potentially more hemotoxic, its impact was checked by erythrocyte hemolytic assay taking PBS and Triton X-100 as negative and positive controls, respectively (Figure 4). The hemolytic activity of the solvent extract was found to be ~65.89%, which is much higher than that observed for negative control (Figure 4). Thus, the compounds present in the extract were found to be toxic and noncompatible with blood as they caused lysis of the erythrocytes. This hemolytic assay is crucial and provides an obvious reason for removing such harmful phytochemicals from rapeseed meal before it can be used as a food/feed item.

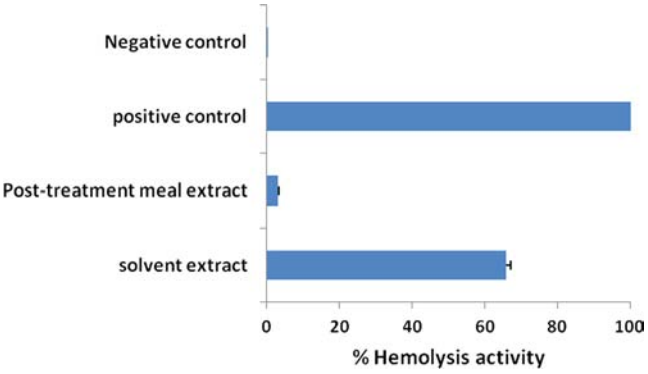

Figure 4. Percent red blood corpuscle hemolytic activity of the 0.2% $HClO_4$-containing solvent extract and its treated meal.

Furthermore, the meal remaining after treatment with the 0.2% $HClO_4$-containing Me/Ac (1:1 v/v) solvent was extracted with PBS buffer, and the buffer extract of treated meal (post-treatment meal extract) was also tested for hemolytic activity to assess the suitability of this solvent-treated meal as food/feedstuff. The activity of the post-treatment meal extract (~3.15%) was much reduced (20.9-fold) compared to that of solvent extract, which again vouched for the fact that the treated meal contained very little or a negligible amount of residual antinutrients and hence can be recommended for feed/food purposes.

Addition of Solvent Extract into Waste Cooking Oil-Derived Biodiesel (Waste Cooking Oil Methyl Esters, WCME). Because of its highest reductive potential and toxicity, the 0.2% $HClO_4$-containing solvent extract was hereafter used as an exogenous additive for WCME. Freshly prepared

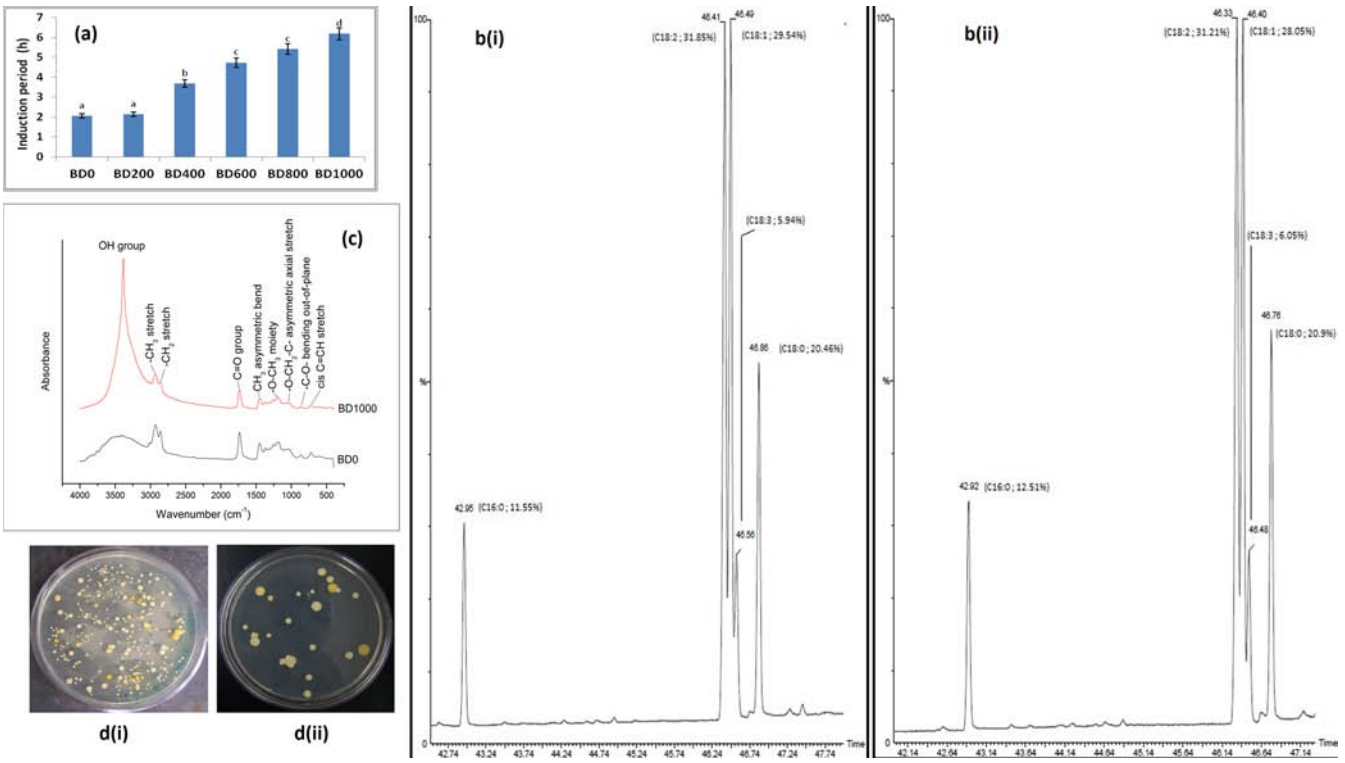

Figure 5. (a) Effect of antioxidant loading on oxidation stability of waste cooking oil biodiesel fuel; (b) GC-MS chromatogram of (i) BD0 and (ii) BD1000; (c) FTIR spectra of biodiesel with or without exogenous antioxidant; (d) microbial load in (i) BD0 and (ii) BD1000 after 90 days of storage.

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biodiesel (BD0) served as control, and those incorporated with various concentrations of the solvent extract (200, 400, 600, 800, and 1000 ppm) were accordingly coded as BD200, BD400, BD600, BD800, and BD1000, respectively. Presented in Figure 5a is the influence of the extract level on the oxidative stability of WCME. The need for addition of antioxidant into WCME is reconfirmed by the value of IP: BD0 did not achieve the minimum limit of a 6 h IP. As anticipated, higher treatment levels in WCME afforded higher IPs ($p < 0.05$). Only at a level of 1000 ppm did the extract improve the oxidation stability to fulfill the EN 14214 specification of 6 h. However, BD400, BD600, and BD800 provided IPs that were compliant with the less stringent 3 h limit specified in ASTM D6751. The antioxidative effect of the meal extract can be attributed to the molecular structures of different compounds present in the extract, especially tannins. They have an aromatic ring with different functional groups at different positions of the ring. The active hydroxyl group can provide protons that combine with free radicals, thus delaying the initiation or slowing the rate of oxidation.[47] Additionally, the oxidative stability of BD0 and BD1000 after 90 days of storage at room temperature (30 °C) was investigated. At $t = 90$ days, the IP of BD0 was found to be 1.42 h and that of BD1000 was 5.6 h. The higher IP value of BD1000 correlated with a slower rate of oxidation over the storage period, relative to that of BD0 ($p < 0.05$). This in turn ascertained the effectiveness of solvent extract as exogenous antioxidant for biodiesel.

Influence of Solvent Extract on Properties of WCME. The fuel properties of BD0 and BD1000 are shown in Table 3 and are compared with ASTM standards. Addition of the exogenous antioxidant to biodiesel did not significantly ($p > 0.05$) affect kinematic viscosity and density. These parameters are directly associated with engine performance and were within the limits given by the specifications valid for biodiesel. As noted in Table 3, the acid value of BD1000 was slightly higher than that of BD0, which can be attributed to the presence of various phenolic acids and phytic acid in the meal extract. Nevertheless, both samples conformed to the ASTM D6751 criterion. Flash point, cloud point, and pour point also did not change significantly ($p > 0.05$). Therefore, it can be presumed that the meal extract as an additive did not have an unfavorable effect on physical quality parameters of WCME.

Table 3. Physical Properties of WCME

fuel property	ASTM method used	ASTM specification[a]	BD0[b]	BD1000[b]
kinematic viscosity (40 °C, $mm^2\ s^{-1}$)	D 445	1.9 to 6.0	4.59a	4.55a
density (15 °C, kg/m^3)	D 5002	860 to 900	887a	882a
flash point (°C)	D 93	100 to 170	156a	152a
cloud point (°C)	D 2500	−3 to 12	2.0a	2.3a
pour point (°C)	D 97	−15 to 16	−1.0a	−1.2a
acid no. (mg KOH g^{-1})	D 664	≤0.5	0.35a	0.46b

[a]Specification as given in Tariq et al.[49] [b]Values followed by different letters in a row are significantly different ($p < 0.05$).

Fatty Acid Profile by GC-MS. FAME compositions of BD0 and BD1000 are shown in Figure 5b. The chromatograms of both BD0 and BD1000 were almost similar, except for minor shifting of the retention time of constituent peaks. The fatty acid profile of BD0 was characterized by a high content of linoleic (31.85%) and oleic (29.54%) acids, along with significant percentages of palmitic (11.55%), stearic (20.46%), and linolenic (5.94%) acids. Much like BD0, BD1000 was principally composed of FAMEs from linoleic (31.21%) and oleic (28.05%) acids, with lesser amounts of palmitic (12.51%), stearic (20.9%), and linolenic (6.05%) acids. Therefore, the overall distribution of FAMEs in WCME was predominated by a high amount of polyunsaturated constituents. The obtained results concurred with previous studies.[14,48] Therefore, the antioxidant influence on fatty acid profile was found to be almost negligible. Polyphenols, phytates, and AITC were not detectable in the chromatogram of BD1000 with the used GC-MS procedure and setup, as their specific derivatizing agents were not used. A GC-MS protocol pertaining to only fatty acid detection was attempted here.

FTIR Analyses. FTIR is lately being used to identify the functional groups and the bands corresponding to various stretching and bending vibrations in biodiesel. Like GC-MS chromatograms, spectra of both BD0 and BD1000 were almost similar (Figure 5c), except for the major difference observed at 3100−3750 cm^{-1}. Moreover, the spectral regions at 2849−3000 cm^{-1} in BD0 have higher intensities than those in BD1000. The appearance of a strong −OH absorption band (within 3400−3600 cm^{-1}) in BD1000 confirms the presence of a large number of −OH groups containing polyphenols.[49]

Impact of Solvent Extract on Microbial Stability of WCME. Analysis of microbial load in biodiesel is not widely studied; however, several microorganisms are able to use mineral oils as energy source in an aqueous environment. Bacterial cultures that have been isolated from the surroundings of an oil field in the Arabian Sea were able to use diesel fuel as sole carbon and energy source,[50] where 39% of the fuels were degraded under aerobic conditions within 8 days. Because of the hygroscopic nature of biodiesel, water gradually accumulates in it during storage. High levels of dissolved water lead to the formation of microdroplets, which culminates in the production of a second phase of free water in the bottom of the tank, a fundamental condition for the proliferation of microorganisms.[51] This leads to important consequences such as filter clogging, the presence of biosediments, and corrosion influenced by microorganisms. Adoption of mitigation measures is required to avoid these degenerative processes in biodiesel.[51] As polyphenols and AITC are known to impart strong antimicrobial activity, we compared the microbial load in BD0 and BD1000 at 0 and 90 days of storage. Freshly prepared BD0 and BD1000 did not show any detectable colony-forming units (CFU). Subsequent to a storage period of 90 days, the total viable count was ascertained to be 22 ± 6 and 281 ± 12 CFU/mL in BD1000 and BD0, respectively (Figure 5d). The number of CFU in BD0 was ∼12.8 times higher compared to BD1000. Thus, it became apparent that the presence of rapeseed meal extract greatly suppressed the growth of micro-organisms in stored biodiesel and delayed its degradation.

The results from this study suggest the 0.2% $HClO_4$-containing Me/Ac mixture (1:1 v/v) as the most effective solvent for extraction of the major antinutrients (polyphenols, phytates, AITC) from rapeseed press-cake. Our results can contribute to the selection of the most efficient extraction solvents to be used when removal of the above-mentioned compounds are to be made. Although the polyphenol-rich solvent extract exhibited excellent DPPH radical scavenging activity, it cannot be recommended for food or drug application due to its high toxicity. From this work, we can conclude that the advantageous qualities of the solvent extract can effectively

be harnessed by using it in suitable nonfood models such as biodiesel. In summary, the solvent extract from rapeseed meal proved to be an effective antioxidant additive for WCME, which is attributable to the myriad phytochemicals present in it. A further survey on the characteristics of performance and exhaust emissions of a diesel engine using the antioxidant-loaded biodiesel should be undertaken.

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Notes

The authors declare no competing financial interest.

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