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Studies on the Antioxidant Activities of Natural Vanilla Extract and Its Constituent Compounds through *in Vitro* Models

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Vanilla extract was prepared by extraction of cured vanilla beans with aqueous ethyl alcohol (60%). The extract was profiled by HPLC, wherein major compounds, viz., vanillic acid, 4-hydroxybenzyl alcohol, 4-hydroxy-3-methoxybenzyl alcohol, 4-hydroxybenzaldehyde and vanillin, could be identified and separated. Extract and pure standard compounds were screened for antioxidant activity using β-carotene-linoleate and DPPH *in vitro* model systems. At a concentration of 200 ppm, the extract showed 26% and 43% of antioxidant activity by β-carotene-linoleate and DPPH methods, respectively, in comparison to corresponding values of 93% and 92% for BHA. Interestingly, 4-hydroxy-3-methoxybenzyl alcohol and 4-hydroxybenzyl alcohol exhibited antioxidant activity of 65% and 45% by β-carotene-linoleate method and 90% and 50% by DPPH methods, respectively. In contrast, pure vanillin exhibited much lower antioxidant activity. The present study points toward the potential use of vanilla extract components as antioxidants for food preservation and in health supplements as nutraceuticals.

KEYWORDS: Vanilla; antioxidant; vanillin; 4-hydroxy-3-methoxybenzyl alcohol; nutraceutical

INTRODUCTION

Vanilla is a tropical climbing orchid cultivated extensively in Mexico, Madagascar, Seychelles, Comoro, and Indonesian islands of Java, Bali, Sulawesi, Lombok, and Papua, and South Pacific islands of Tonga (*1*). Currently, about 60% of the World's vanilla is grown in Madagascar. Vanilla production in India is on the rise with the current annual production of natural vanilla being about 700 tons of green pods (*2*).Vanilla is a very popular flavor. Vanilla extracts are mainly used in several foods like ice cream, chocolate, cakes, and cookies and also in several perfumery and pharmaceutical formulations. They are also shown to exhibit antimicrobial and antimutagenic effects (*3*). Hence, there is immense commercial potential for natural vanilla. The characteristic flavor of vanilla is due to the presence of a large number of aromatic compounds. The principal component of vanilla is "vanillin", which contributes to 1/3 of the flavor, whereas other volatile and nonvolatile compounds contribute to the remaining 2/3 of the flavor in vanilla (*1*). The extract of *Vanilla planifolia* contains not only vanillin but also a range of related phenylpropanoid (C_6–C_3) compounds (**Figure 1**), which combine to give the unique subtlety to natural vanilla flavor (*4*). During the important process of curing the beans, they undergo enzymatic reactions responsible for the development of the characteristic aroma and flavor of vanilla (*5*). Green vanilla beans (pods) contain little free vanillin but aroma precursors such as β-D-glucosides, which come into contact with β-D-glucosidases during the curing process (*6*). The four crucial steps in curing are scalding, sweating, drying, and conditioning.

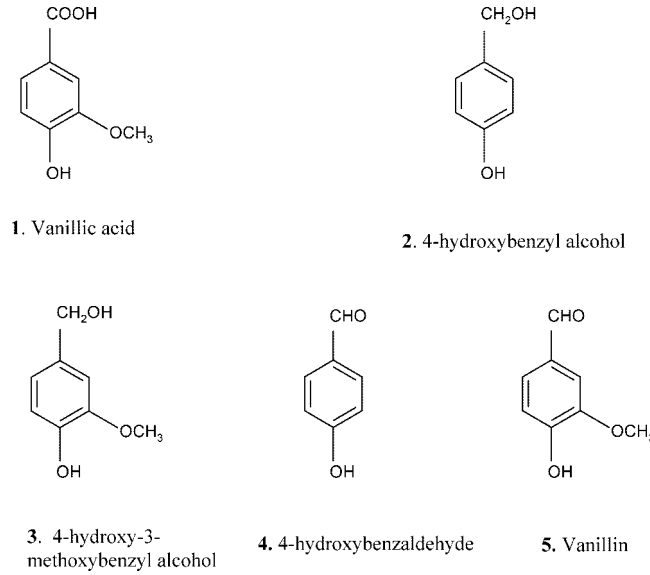

Figure 1. Chemical structures of the major flavor compounds found in cured beans of *Vanilla planifolia.*

The anticlastogenic properties of vanillin have been studied over the last two decades by many researchers (*7*). A recent study (*8*) explored vanillin's ability to reduce chromosomal

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damage caused by X-ray and UV light. The antimutagenic property of vanillin was evident as it significantly reduced the number of micronuclei, and at all dose levels, vanillin reduced the frequency of chromosomal aberrations (*9*). Vanillin has also been found to have anticarcinogenic effects in a family of DNA-PK inhibitors (*10*). Vanillin's antimicrobial properties against yeasts have been studied, and it was found that the structure of vanillin played an important role (*11*).

Despite several reports on vanilla processing, and antimutagenic, anticarcinogenic, and anticlastogenic properties of vanilla, the antioxidant activity of vanilla extracts or its constituents has not been studied. Because there is increased cultivation of vanilla beans in India, larger quantities of natural vanilla extracts might be available for producing added-value products. Therefore, evaluation of its antioxidant properties is of interest primarily to find new sources for natural antioxidants for functional foods and also possible applications of natural vanilla other than as a flavorant. Hence, the present investigation was undertaken to study antioxidant potential of natural vanilla extracts and their constituent compounds to identify the components responsible for antioxidant activity.

MATERIALS AND METHODS

Materials. Linoleic acid, α-α-diphenyl-β-picrylhydrazyl (DPPH), β-carotene, potassium ferricyanide, trichloroacetic acid, BHA (Butylated hydroxyl anisole), and ferric chloride were purchased from Sigma chemicals. All the solvents/chemicals used were of analytical grade and obtained from SD Fine Chemicals, Mumbai, India. Vanilla standards, namely, vanillic acid, 4-hydroxybenzyl alcohol, 4-hydroxy-3-methoxybenzyl alcohol, 4-hydroxybenzaldehyde, and vanillin, were procured from Sigma Aldrich Chemical Co. (St. Louis, MO).

Preparation of Vanilla Extract. Fresh vanilla beans were procured from indigenous vanilla growers and cured by the traditional Bourbon method (*12*), which consists of four steps, i.e., scalding, sweating, drying, and conditioning. The cured beans were made into powder and extracted with aqueous ethyl alcohol (60%). These alcoholic extracts were used for evaluation of antioxidant activity.

Vanillin Estimation by UV-Spectrophotometric Method. Vanillin content was estimated according to AOAC method (*13*). Five grams of sample was weighed and macerated with aqueous alcohol (60%). This was allowed to stand overnight and then decanted into a 250 mL standard flask using Whatman filter paper (no. 1). The residue was ground again with 60% ethanol and filtered and washed by decantation on paper, and this process was repeated twice. The extracts were pooled, and the total quantity was increased to 250 mL with 60% ethanol. Ten milliliters of this solution was diluted to 100 mL with water and mixed. Two milliliters of this solution was diluted with water, and then the volume was increased to 200 mL. In another flask, 2 mL 0.1 N NaOH was added to the similarly diluted vanilla extract, and the volume was increased to 200 mL with water. The absorbance of this sample was measured at 348 nm with neutral solution as a reference blank using a spectrophotometer.

Determination of Polyphenols. Samples were analyzed for total polyphenol content according to the Folin-Ciocalteu method (*14*). A known volume of the extract was dissolved in water. A 0.5 mL aliquot of the resulting solution was added to 0.2 mL of Folin-Ciocalteau reagent, and a saturated solution of Na_2CO_3 (0.5 mL) was added. This was increased to 10 mL with distilled water and incubated at 27 °C for 1 h. Optical density was measured at 765 nm using a spectrophotometer. The concentration was calculated using gallic acid as a standard, and the results were expressed as gallic acid equivalents per gram of extract.

***β*-Carotene-Linoleate Model System.** The antioxidant activity of vanilla standard compounds and vanilla extract was evaluated by the β-carotene method (*15*). β-Carotene (0.2 mg) in 0.2 mL of chloroform, linoleic acid (20 mg), and Tween-40 (polyoxyethylenesorbitan monopalmitate) (200 mg) were mixed. Chloroform was removed at 40 °C under reduced pressure (40 mmHg), and the resulting mixture was diluted with 10 mL of water and mixed well. To this emulsion was added 40 mL of oxygenated water. Four milliliter aliquots of the emulsion were transferred into different test tubes containing 0.2 mL of solutions of vanilla standard compounds, vanilla extracts (50, 100, and 200 ppm), and BHA in ethanol. A control containing 0.2 mL of ethanol and 4 mL of the above emulsion was prepared. The tubes were placed in a water bath maintained at 50 °C, and the absorbance was measured at 470 nm at zero time ($t = 0$). Measurement of absorbance was continued until the color of the β-carotene disappeared in the control tubes ($t = 180$ min) at an intervals of 15 min. A mixture prepared as above without β-carotene served as blank. All determinations were carried out in triplicate. The antioxidant activity (AA) of the extracts was evaluated in terms of bleaching of the β-carotene using the following formula: $AA = 100[1 - (A_0 - A_t)/(A^0_0 - A^0_t)]$, where A_0 and A^0_0 are the absorbance values measured at zero time of the incubation for test sample and control, respectively, and A_t and A^0_t are the absorbance values measured in the test sample and control, respectively, after incubation for 180 min.

Free Radical Scavenging Activity. Free radical scavenging activity was measured by the 1,1-diphenyl-2-picrylhydrazil (DPPH) method (*16*). Different concentrations (50, 100, and 200 ppm) of vanilla standards and extract were taken in different test tubes. The volume was adjusted to 100 μL by adding MeOH. Four milliliters of a 0.1 mM methanol solution of DPPH was added to these test tubes and shaken vigorously. The tubes were then incubated in the dark at room temperature for 20 min. A control sample was prepared as above without extract, and methanol was used for the baseline correction. Changes in the absorbance of the samples were measured at 517 nm. All analysis was run in triplicate and averaged. Radical scavenging activity was expressed as the inhibition percentage and was calculated using the following formula: Radical scavenging activity (%) = (Control OD − Sample OD/Control OD) × 100.

Reducing Power. The reducing power of vanilla extracts and standard compounds was determined according to the method described in the literature (*17*). Different amounts of sample (50–250 μg) in 1 mL of distilled water were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 mL, 1%). The mixture was incubated at 50 °C for 20 min. A portion (2.5 mL) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%) were mixed and the absorbance was measured at 700 nm. All analyses were run in triplicate and results averaged. Increased absorbance of the reaction mixture indicated increased reducing power.

HPLC Profiling of Vanilla Extracts. Many HPLC techniques have been developed during the past two decades focusing on quantification not only of vanillin but also other important chemicals present in vanilla extract (*18–22*). These mainly employ gradient elution and also multisolvent systems. To determine the composition of the vanilla components, a simple HPLC isocratic elution method was developed, which efficiently separated various vanilla constituents, vanillic acid, 4-hydroxy-3-methoxybenzyl alcohol, vanillin, and so forth.

The liquid chromatographic system consisted of a Waters HPLC (Millford, USA) consisting of a Waters μ-Bondapak. C-18 column (300 × 3.9 mm i.d), a Waters automated gradient controller and a Rheodyne injector. Detection was done using a 2487 dual absorbance detector set at a sensitivity of 0.01 AUFS and a wavelength of 278 nm with the data processed by Clarity software. Vanilla beans (5 g) were macerated using aqueous ethyl alcohol 60% and the volume increased to 250 mL. This was repeated thrice for complete extraction. The extract was passed through a membrane filter (Millipore, 0.45 μm). Five milligram samples of each standard were weighed separately, dissolved in ethyl alcohol, and volume increased to 50 mL with Millipore water. Extract and standard compounds (10 μL) were injected into the HPLC. Elution was carried out at a flow rate of 1 mL/min under isocratic condition using a solvent mixture of acetonitrile and water (10:90 v/v). The percentage of respective compounds was expressed as absolute assay as well as in terms of its chromatographic purity based on peak area normalization method. Preparative HPLC analysis was carried out using C-18 (250 × 10 mm i.d.) semipreparative column. Elution was carried out at a flow rate of 3 mL/min under isocratic conditions.

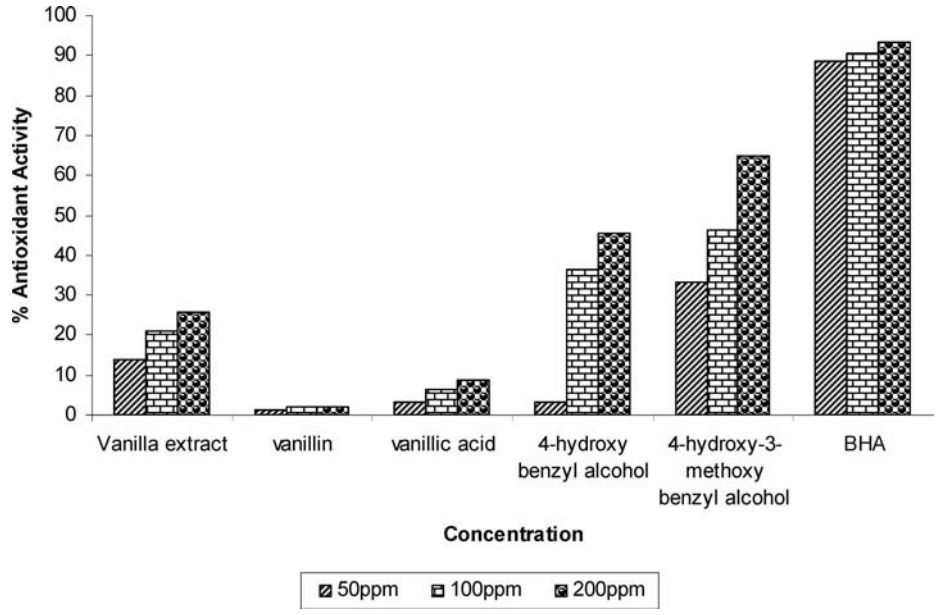

Figure 2. Antioxidant activity of vanilla extract and standards by β-carotene method.

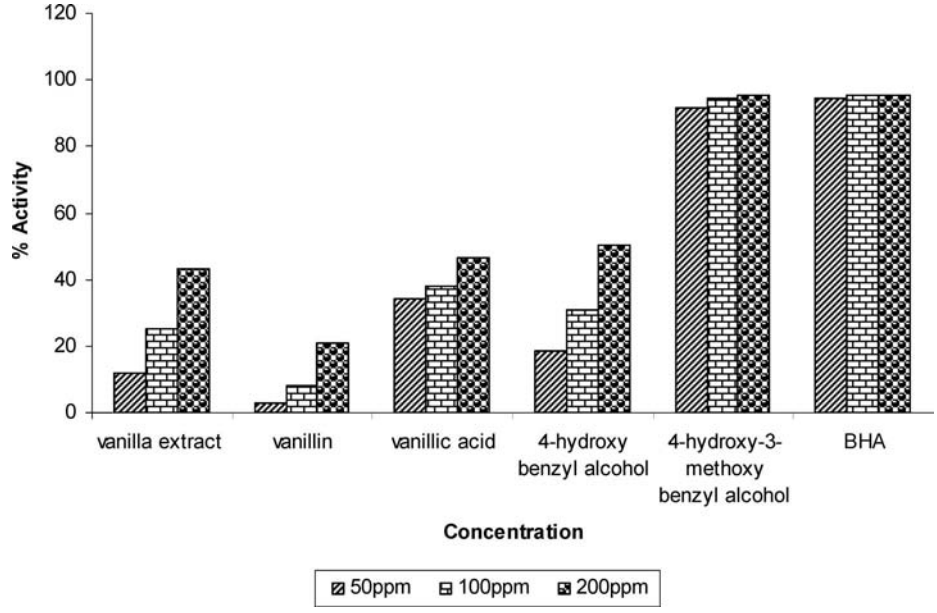

Figure 3. Antioxidant activity of vanilla extract and standards by DPPH method.

Table 1. *F*-ratios (β-carotene-linoleate) of Extract and Standard Compounds

sample	*F*-ratio	*P*-value
extract vs 4-hydroxy-3-methoxy benzyl alcohol	33.98581***	0.00004
extract vs 4-hydroxybenzyl alcohol	2.488763ns	0.13698
extract vs vanillic acid	48.03168***	0.00000
extract vs vanillin	98.29814***	0.00000

*** Extremely significant. ns Not significant.

Statistical Analysis. The data were analyzed by one-way analysis of variance (ANOVA) to study the differences in the antioxidant activity of natural vanilla extract and standard compounds and between the different compounds. Probability level was fixed to $P < 0.05$. The analysis was carried out using Microsoft *Excel XP*.

RESULTS AND DISCUSSION

Vanilla extracts were prepared from vanilla beans cured by the Bourbon method which involved scalding, sweating, drying, and conditioning of fresh vanilla beans followed by size reduction and extraction with aqueous alcohol. They were analyzed for alcoholic strength, total polyphenol and vanillin content. Alcoholic strength was measured by using an alcohol meter and recorded as 35%. Total polyphenols content in vanilla extract was 17%, and vanillin was 2.7 %. Antioxidant and antimicrobial properties of low-molecular-weight phenolic compounds are reported (*23*). In this study, vanilla extracts and standard compounds were evaluated for their antioxidant activity.

***β*-Carotene-Linoleate Model System.** The mechanism of bleaching of β-carotene is a free radical mediated phenomenon resulting from the hydroperoxides formed from linoleic acid. β-Carotene in this model system undergoes rapid discoloration in the absence of an antioxidant. The linoleic acid free radical formed upon the abstraction of a hydrogen atom from one of its diallylic methylene groups attacks the highly unsaturated β-carotene molecules. As β-carotene molecules lose their double bonds by oxidation, the com-

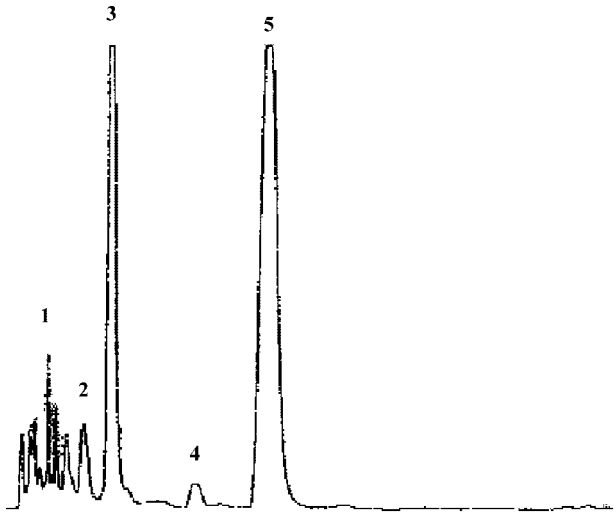

Figure 4. HPLC pattern of vanilla extract. 1: vanillic acid. 2: 4-hydroxybenzyl alcohol. 3: 4-hydroxy-3-methyoxybenzyl alcohol. 4: 4-hydroxybenzaldehyde. 5: vanillin.

Table 2. *F*-Ratios (free radical scavenging activity) of Extract and Standard Compounds

samples	*F*-ratio	*P*-value
extract vs 4-hydroxy-3-methoxybenzyl	195.5589***	0.0000
extract vs 4-hydroxybenzyl alcohol	0.997812ns	0.3347
extract vs vanillic acid	4.866976ns	0.0445
extract vs vanillin	10.17176**	0.0065

*** - Extremely significant ** - Highly significant * - Significant ns - Not significant

pound loses its chromophore and characteristic orange color, which can be monitored spectrophotometrically. The presence of different extracts can hinder the extent of β-carotene bleaching by neutralizing the linoleate free radical and other free radicals formed in the system. The antioxidant activity of vanilla standards and vanilla extract as measured by the bleaching of β-carotene is presented in **Figure 2**. At 200 ppm. vanilla constituents, viz., 4-hydroxy-3-methoxybenzyl alcohol, 4-hydroxybenzyl alcohol, and vanilla extract, exhibited 64%, 45%, and 26% activity in comparison with BHA (93%). IC_{50} values of standard compounds of vanilla were found to be 154 ppm (4-hydroxy-3-methoxybenzyl alcohol) and 220 ppm (4-hydroxybenzyl alcohol), and that of vanilla extract was found to be 385 ppm. Vanillin and vanillic acid were not potent enough to cause 50% inhibition in the β-carotene linoleate method.

The bleaching of β-carotene was found to vary between the different compounds of vanilla. The differences were found to be extremely significant as per analysis of variance (F = 28.76336***). As seen from **Table 1**, the antioxidant activities of the vanilla extract and three of its standard compounds were found to be extremely significant but were similar to that of 4-hydroxybenzyl alcohol.

Free Radical Scavenging Activity. Vanilla standards exhibited good free radical scavenging activities from 20% to 95% (**Figure 3**); the activity increased with increasing concentration. Of the standards, 4-hydroxy-3-methoxybenzyl alcohol exhibited the highest activity followed by 4-hydroxybenzyl alcohol, vanillic acid, and vanillin. 4-Hydroxybenzaldehyde did not show any activity by either method. In the extract, about 43% activity was observed. IC_{50} values of standard compounds of vanilla was found to be 105 ppm (4-hydroxy-3-methoxybenzyl alcohol), 199 ppm (4-hydroxybenzyl alcohol), 216 ppm (vanillic acid), and 480 ppm (vanillin), and that of vanilla extract was 230 ppm. Cinnamic acid and vanillin have been used to preserve the processed cereals and cereal products for protection against lipid oxidation at low levels of 0.01–0.5% (*24*, *25*). Vanilla is also reported to have highest inhibition by the hydrogen peroxide scavenging method compared to six other dessert spices including nutmeg, cinnamon, and so forth (*26*).

The free radical scavenging capacity was found to vary among the different compounds of vanilla. The differences among the compounds were found to be extremely significant as per analysis of variance (F = 91.9804***). As seen from **Table 2**, the free radical scavenging activity values among the vanilla extract and its standard compounds showed variations wherein it was similar to 4-hydroxybenzyl alcohol but was significantly lower than other compounds. From the above results, it is clear by both assays that antioxidant

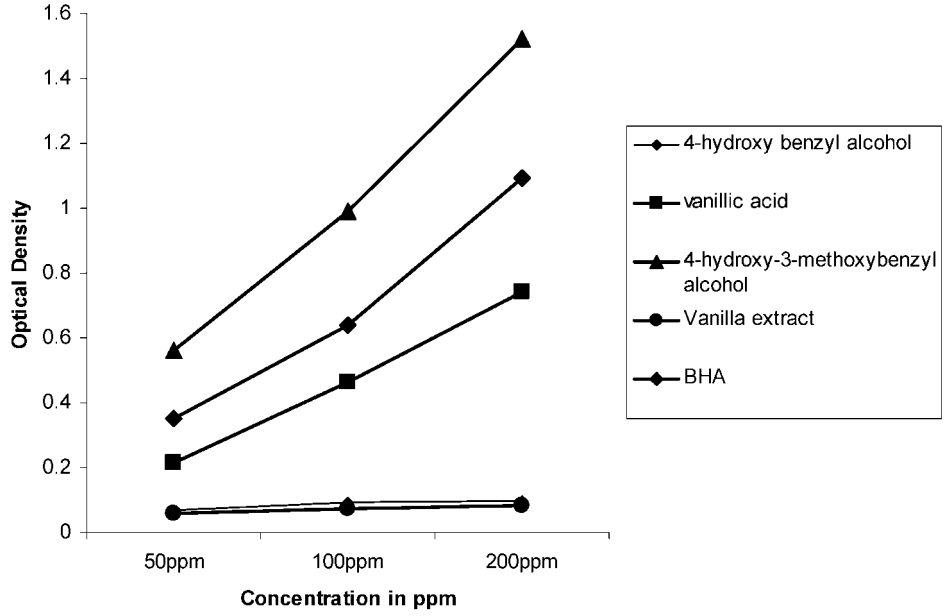

Figure 5. Reducing power of vanilla extract and standard compounds.

activities of different compounds vary to a large extent and the activity of vanilla extract depends largely on the proportion of various compounds present.

Identification by HPLC. The five most important constituents, namely, vanillin (2–2.8%), *p*-hydroxybenzaldehyde (0.2%), vanillic acid (0.2%), *p*-hydroxybenzylmethyl ether (0.02%), and acetic acid (0.02%), were identified in vanilla extracts (*27, 28*). In the present study, the HPLC profile of the vanilla extract showed five major peaks, which were identified as vanillic acid, 4-hydroxybenzyl alcohol, 4-hydroxy-3-methoxybenzyl alcohol, 4-hydroxybenzaldehyde, and vanillin (**Figure 4**).Identification was carried out using the external standard method. Solutions of each standard at various concentration levels were injected into the HPLC system, and the peak areas were recorded. Thus, the calibration curves were prepared and response factors were calculated under the same conditions. Vanilla standard compounds (vanillic acid, 4-hydroxybenzyl alcohol, 4-hydroxy-3-methoxybenzyl alcohol, 4-hydroxybenzaldehyde, and vanillin) in the vanilla extract, based on the comparison of peak areas of each peak with that of authentic samples and from calibration curves, were found to be in the range 2–3%.

Reducing Power. The vanilla constituents and extract were subjected to antioxidant activity by reducing power assay. Antioxidant effect exponentially increases as a function of the development of the reducing power, indicating that the antioxidant properties are concomitant with the development of reducing power (*29*). Reductones are believed not only to react directly with peroxides, but also to prevent peroxide formation by reacting with certain precursors. As seen in **Figure 5**, the reducing power of vanilla constituents increased with increasing concentration from 50 to 200 ppm. 4-Hydroxy-3-methoxybenzyl alcohol showed higher reducing power than BHA. The reducing power of the vanilla constituents followed the order of 4-hydroxy-3-methoxybenzyl alcohol > vanillic acid > 4-hydroxybenzyl alcohol > vanilla extract. Vanillin and 4-hydroxybenzaldehyde showed little activity. The reducing capacity between the constituents was significant (26.97719***). This shows that even in the vanilla extract differences exist between different components as seen in standard compounds. These studies showed that the 4-hydroxy-3-methoxybenzyl alcohol has superior reducing power to BHA.

Conclusions. Vanillic acid, 4-hydroxybenzyl alcohol, 4-hydroxy-3-methoxybenzyl alcohol, 4-hydroxybenzaldehyde, and vanillin were characterized as the major compounds in the vanilla extract by HPLC. Total polyphenol content in the vanilla extract was found to be $17 \pm 0.1\%$. Interestingly, vanilla flavor standards, namely, 4-hydroxy-3-methoxybenzyl alcohol and 4-hydroxybenzyl alcohol, showed appreciable antioxidant activity by both β-carotene-linoleate and DPPH methods. In comparison, vanillin and vanillic acid exhibited much lower antioxidant activity. 4-Hydroxybenzaldehyde showed little activity by any of these methods. In contrast, 4-hydroxy-3-methoxybenzyl alcohol isolated from vanilla extracts exhibited reducing power higher than BHA. The results conclusively demonstrate that natural vanilla extract, containing 35% ethanol—conventionally used as a flavorant—possesses antioxidant activity.

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