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Characterization of Chemical Groups and Identification of Novel Volatile Constituents in Organic Solvent Extracts of Cured Indian Vanilla Beans by GC-MS

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Abstract: After saffron, vanilla is second most expensive spice in world and has long history to be used as flavouring agent. Chemical profile of vanilla beans varies considerably with the cultivation area and curing process adopted. As all constituents along with vanillin contribute in characteristic flavour and aroma of vanilla. Present study was aimed to identify phytoconstituents of n-hexane, ethyl acetate and methanol extracts of cured vanilla beans grown in India, by preliminary phytochemical screening and GC-MS. The results confirmed the presence of 19 novel volatile compounds including Ethyl tricyclo[2.2.1.0^{2,6}]hept-3-ylcarbamate, isojasmone, 5-heptyldihydro-2(3H)-furanone, bicyclo [3.1.0]hexane-2-undecanoic acid in these extracts, not reported previously in cured vanilla beans of any origin. Keywords: Vanilla planifolia, cured beans, phytochemical screening, GC-MS analysis.

Key words:

INTRODUCTION

Vanilla is the only orchid that provides an edible fruit to humans. Out of 110 members [1] of vanilla genus, *Vanilla planifolia* is the most common species because of its pod quality and yield. Though native to Mexico, now vanilla is mainly grown in the twenty degree band North and South of the equator that covers mid-Mexico, northern half of South America, Madagascar, the island of Tahiti, Puerto Rico and other moist tropical climes. From early 1990s India has also started commercial production of vanilla, especially in southern states like Karnataka, Kerala and Tamil Nadu approximately on around 2545 hectare land [2].

Like many orchids, the *V. planifolia* is an epiphyte. It is thick waxy vine 1 to 1.5 cm in diameter that clambers up to tree top in a zigzag fashion with length even more than 35 m. 9 to 15 cm long tapered leaves that grow alternately every 4 to 7 cm throughout the stem length. From the underside of each leaf growth may emerge one or several tendrils that anchor the vine steadfastly to the growing surface [3].

Vanilla has been widely used as versatile flavoring agent in food, beverages, pharmaceutical, cosmetics and tobacco industry [4]. Vanilla plant has also been used traditionally, for treatment of several diseases like dysmenorrhea, fever, hysteria, dyspepsia, prevents dental caries and alleviates tooth ache and ulcers [5]. Vanillin has also been reported to have aphrodisiac activity [6] and anti oxidant activity [7].

Interestingly, green vanilla beans are flavorless and develop its characteristic aroma only after, a fermentation process 'Curing', performed on harvested and dried beans. This process includes various chemical and enzymatic reactions that probably break glycosides of flavor compounds present in vanilla beans [8]. Though vanillin is the major flavor constituent of vanilla, but it is believed, that many other compounds present in vanilla beans are also contributing towards distinct aroma and flavor of natural vanilla in compare to synthetic one. As vanilla is one of the most expensive crop in world and the production of cured vanilla beans is quite long and labor intensive process; almost takes 4-5 years to be part of food from field [9]. By considering these factors as well as

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global demand of vanilla flavor, artificial vanilla extracts are made. But synthetic vanilla extract is quite distinguishable, as it lacks many compounds present in natural vanilla and mainly contains vanillin and ethyl vanillin [10].To synthesize better match of natural vanilla, the complete chemical composition of vanilla plant has to be known.

Vanilla is one of most widely used flavor in world and this specific flavor is due to a complex mixture of volatile compounds. For a very long time, research to discover all these volatile constituents is going on in different parts of world. 169 volatile compounds in cured bourbon (Madagascar) vanilla beans have been reported; of which vanillin was most abundant [11]. Currently more than 200 volatile compounds are known to occur in cured bourbon vanilla beans [12]. Organic solvent extraction is mainly used to collect volatiles from vanilla beans [8, 13]. Direct thermal desorption [14,15], solid phase microextraction [10], sorptive stir bar extraction [16] are some other sampling techniques used to isolate volatiles from vanilla beans. GC-MS is one of the most popular techniques to analyze the major constituents of different solvent extracts of vanilla beans. On performing GC-MS and GC-Olfactrometric analysis of aroma compounds in representative organic aroma extracts of cured *V. planifolia* beans [17], 65 volatiles were identified in pentane/diethyl ether extract, out of which 26 were reported aroma active compounds on GC-O analysis. In the same study 54 volatile compounds were reported in diethyl ether extract and 41 volatiles were identified in pentane/ dichloromethane solvent extract on GC-MS study. On the comparative analysis of volatiles in traditionally cured bourbon (cultivated in Madagascar and other islands in the Indian Ocean) and Ugandan (cultivated in Uganda) *V. planifolia* beans by GC-MS technique, total 246 compounds were found in the dichloromethane: water (8:1, v/v) extracts of both vanilla beans [12].The volatile compounds identified in cured vanilla beans belong to different chemical classes' viz. hydrocarbons, aldehydes, ketones, alcohols, phenols, acids, esters/ lactones, ethers and heterocyclics. In GC-O analysis out of 246 compounds 78 compounds were found odor active in extracts of both the vanilla beans, confirming the contribution of various constituents present in vanilla beans towards specific aroma of natural vanilla extract.

Considerable variation in flavor profile of vanilla is observed with different cultivation region, growth conditions, soil composition, fruit maturity and mainly, with the type of processing. In a report, even difference in quantities of major volatile aromatic flavor compounds vanillin, p-hydroxybenzoic acid, p-hydroxybenzaldehyde and vanillic acid in vanilla extracts made from beans of various global origins [18] were observed. Significant deviation was studied in relative amount of aromatic compounds in Indian vanilla beans from the beans of different geographic origin [19]. In present study, to identify the volatiles profile of cured vanilla beans grown in India, organic solvent extracts of different polarity like n-hexane, ethyl acetate and methanol were analyzed by GC-MS technique.

MATERIALS AND METHODS

Plant Material: Cured beans of *V. planifolia* were obtained from Athiyodi dist. Kozhikode (Kerala), in Apr, 2010 through a friend and duly identified by the Authorities of department of Botany, Govt. Arts & Science College, Ratlam (M.P.), India. The collected dried, cured beans were ground by means of a blender to fine powder. Solvents used for extraction purpose, were of analytical grade and obtained from Qualigen, India [20].

Extraction: 350 g of the cured vanilla bean's powder was extracted (72 hours each) using soxhlet apparatus over water bath sequentially with *n*-hexane (250 mL x 4), ethyl acetate(250 mL x 4) and methanol (250 mL x 4)solvents. The extracts obtained were filtered and evaporated to dryness by using a vacuum distillation unit. The final crude extracts 22 g n-hexane, 190 g ethyl acetate and 81 g methanol were kept in refrigerator till further use [21].

Phytochemical Screening: All the prepared extracts were tested for presence of steroids, alkaloids, reducing sugars, phenolic compounds, flavonoids, saponins, flavones and tannins. Phytochemical screening of the extracts was carried out according to standard method (Brindha *et al*.). In each test 10% (w/v) solution of the extract in methanol was taken.

GC-MS Analysis: Instrument and chromatographic conditions*:* GC–MS analysis was carried out using a Hewlett–Packard 5890 gas chromatograph coupled to a Hewlett–Packard 5970A mass spectrometer detector (MSD). Volatiles were separated on fused silica capillary column (25 m, 0.32 mm i.d., 0.25 µm film thickness). The oven temperature was set at 60°C for 2 min, then raised to 220°C at 4°C/min and held at this temperature for

20 min. The on-column injector (injection volume 2 µL) was heated from 20°C to 245°C at 180°C /min and held at this temperature for 90 min. The detector and injector temperatures were 250°C and helium was the carrier gas (1.1 mL/min). 2 µL volumes were injected in split less mode. Peak areas and retention times were measured through electronic integration. The electron impact energy was 70 eV. Electron impact (EI) mass spectra were recorded in the 10–425 amu range at 2 s intervals[22].

Identification and Quantification of Volatiles: Volatile compounds were identified by comparing mass spectra data of samples with those of the NIST (National Institute of Standards and Technology, Gaithersburg, MD, USA) standard reference database number 69. The name, molecular weight and structure of the components of the test materials were ascertained by comparing the mass spectra with the known compounds using computer searches on a NIST Ver.2.1 MS data library. Literature reports already published, also helped in understanding the major categories of compounds present in cured vanilla beans. The quantitative estimation of each peak obtained in GC was made by computer, attached with GC-MS instrument.

RESULTS AND DISCUSSION

Chemical Group Test: Results of different chemical tests on the *n*-hexane, ethyl acetate and methanol extracts of cured vanilla beans ensured the presence of steroids, alkaloids, saponins, tannins and significant presence of phenolics and reducing sugars (Table 1).

In best of our knowledge, present study is first documentation of volatile profiling of vanilla beans grown in India by GC-MS. The chemical profiles of the extracts, the amount (%) of the individual components and gas chromatographic and mass spectral data are summarized in Table 2, 3 and 4. The gas chromatograms with peaks are also given in Figure 1, 2 and 3.

Constituents Identified in *N*-Hexane Extract: 6 compounds viz. vanillin (47.34%), 9-pentacosene (4.75%), cyclopentane (2-decyldodecyl)(5.23%), 1-heneicosene (10.17%), isojasmone (1.14%), 2(3H)-furanone, 5-heptyldihydro(31.37%)were identified in n-hexane extract of cured beans of *V. planifolia*, out of which presence of vanillin in different solvent extracts of vanilla beans, was confirmed in all previous studies. While in vanilla beans, furanone derivatives like dihydro-3-methyl-2(3H)-furanone (α-methyl-δ-butyrolactone), 5-ethyl-2(5H)-furanone (2-hexen-4-olide), 3,4-dimethyl-2,5-furandione have been previously reported [14]. In a study [20], 0.4% hydrocarbon content including various n-alkanes, branched alkanes and alkenes from beans of *V. fragrans* also known as *V. planifolia*, were identified by GC-MS of hexane fraction of unsaponifiable extract, identification of alkenes like 9-pentacosene, 1-heneicosene in present study confirms previous reports further.

Constituents Identified in Ethyl Acetate Extract: The results revealed the presence of 9 different compounds viz., 9-tridecenoic acid,methyl ester(6.95%),9-octadecenoic acid, methyl ester/methyl oleate (3.31%), 9,12-octadecadienoic acid, methyl ester (5.60%), 9,12-octadecadienoic acid (10.29%), bicyclo [3.1.0]hexane-2-undecanoic acid, methyl ester (19.90%), 3,6-decadiene (0.88%), ethyl tricyclo[2.2.1.$0^{2,6}$]hept-3-ylcarbamate (3.86%), pentacosane-2,4-dione (44.87%), cyclohexadecane-1,3-dione(4.34%) in ethyl acetate extract of cured beans of *V. planifolia.* In literature identification of esters like 11-Octadecenoic acid,methyl ester and 10,13-Octadecadienoic acic,methyl ester in vanilla beans is already reported. In an experiment, even long chain aliphatic β-diketones 16-pentacosene-2,4-dione, 18-heptacosene-2,4-dione, 20-nonacosene-2,4-dione, 22-hentriacontene-2,4-dione and 24-tritriacontene-2,4-dione have also been isolated and identified in lipidic fraction of beans having aroma from two species *V. fragrans* and *V. tahitensis* and found absent in *V. madagascariensis* beans, lacking any aroma [21].

Constituents Identified in Methanol Extract: GC-MS analysis of methanol extract of cured beans of *V. planifolia* confirmed the presence of 7 different compounds viz. 5-methyl-5-propylnonane, cyclopentane-1,1'-ethylidenebis-, 2-Hydroxy-3-(2-propenyl)-benzaldehyde,1-Ethyl-3-methyl cyclohexane, pentadecane, cyclotetradecane, Propane-1,1'-[ethylidenebis(oxy)]bis[2-methyl]. Presence of straight chain and branched alkanes in vanilla beans is already confirmed [20], while P-hydroxybenzaldehyde is found as a core compound present in most of the vanilla beans samples [14]. Mostly the ketones and alcohol derivatives of alicyclic compounds are reported in vanilla beans [13, 16, 17], while in present study some new alicyclic hydrocarbons were identified.

Table 1: Presence of possible chemical groups

S.No.	Name of Extract	Steroids	Reducing Sugars	Alkaloids	Phenolics	Flavonoids	Flavone	Saponins	Tannins
1.	*n*-hexane	+	+	+	++	+	-	-	-
2.	Ethyl acetate	+	++	+	++	+	+	+	+
3.	Methanol	+	++	+	++	+	-	+	+

+: Presence; - : Absence; ++: significant presence

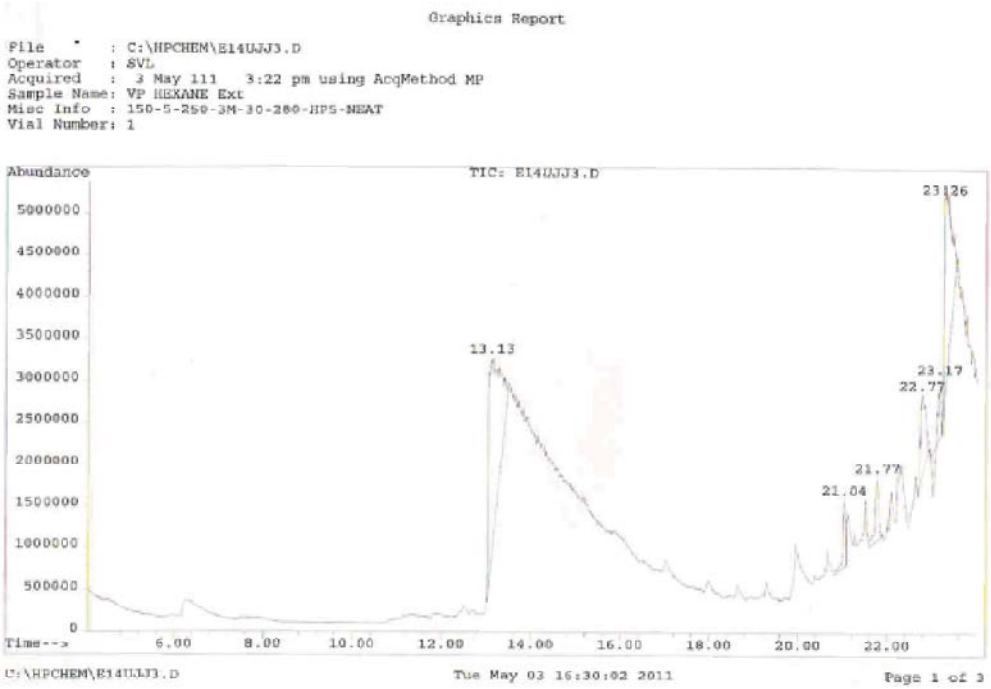

Fig. 1: Gas chromatogram of *n*-hexane extract of cured Indian vanilla beans

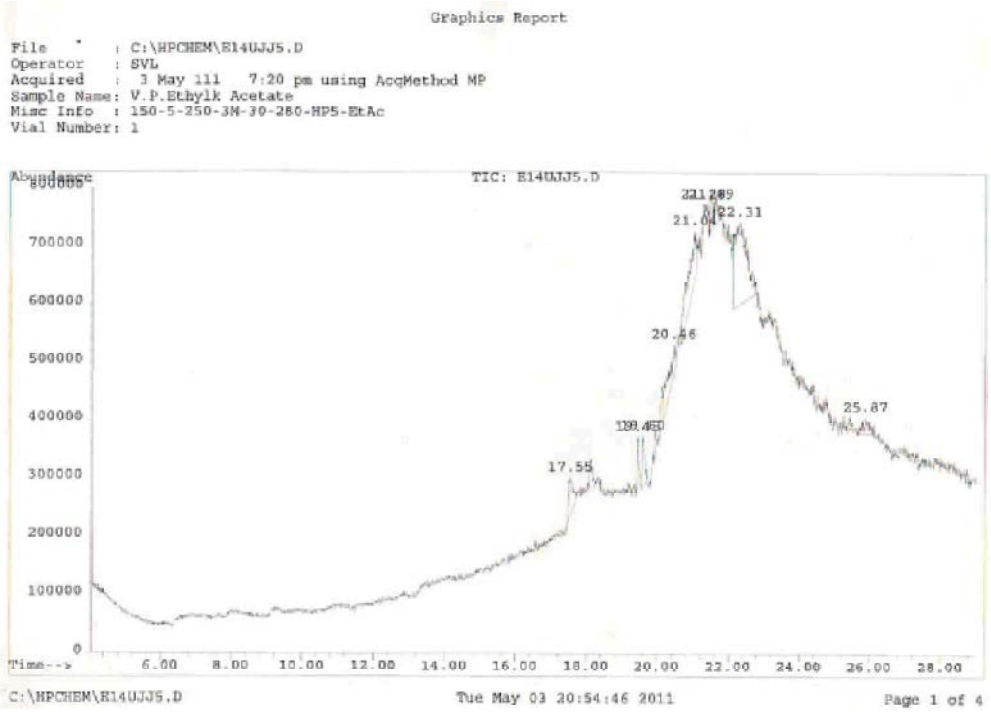

Fig. 2: Gas chromatogram of ethyl acetate extract of cured Indian vanilla beans

Table 2: Description of volatile constituents identified in *n*-hexane extract of cured Indian vanilla beans

Peak	RT	Area %	Compound	Molecular Mass and Formula	Mass fragment ions
1.	13.13	47.34	Vanillin	152, $C_8H_8O_3$	M^+152(100%),151(95.5%),123 (17.7 %), 109 (22.2%)
2.	21.04	4.75	9-Pentacosene	350, $C_{25}H_{50}$	M^+350(2.2%),125(22.2%),111 (48.8%), 97(88.8%), 83(82.2%),69 (75.5%), 57(100%), 43(80%),
3.	21.77	5.23	Cyclopentane(2-decyldodecyl)	378, $C_{27}H_{54}$	M^+378(1.1%),125(26.6%),111 (28.8%),97(44.4%), 83(48.8%), 81(48.8%),71(80%),57(88.8), 43(100%)
4.	22.77	10.17	1-Heneicosene	294, $C_{21}H_{42}$	(M^+-1)293(1.1%), 167(11.1%), 149 (24.4%), 125(33.3%), 111(51.1%), 97(93.3%), 83(97.7%), 71(80%), 57 (100%), 43(57.7%)
5.	23.17	1.14	Isojasmone	166, $C_{11}H_{18}O$	(M^+-1)165(2.2%), 135(15.5%), 123(17.7%), 111(77.7%), 97(100%), 81(51.1%), 71(53.3%), 55 (62.2%), 43(44.4%)
6.	23.26	31.37	2(3H)-Furanone,5-heptyldihydro	184, $C_{11}H_{20}O_2$	(M^+-17)167(1.1%), 138(13.3%), 113(28.8%), 100(77.7%), 85(100%), 69(31.1%),55(35.5%),43(42.2%)

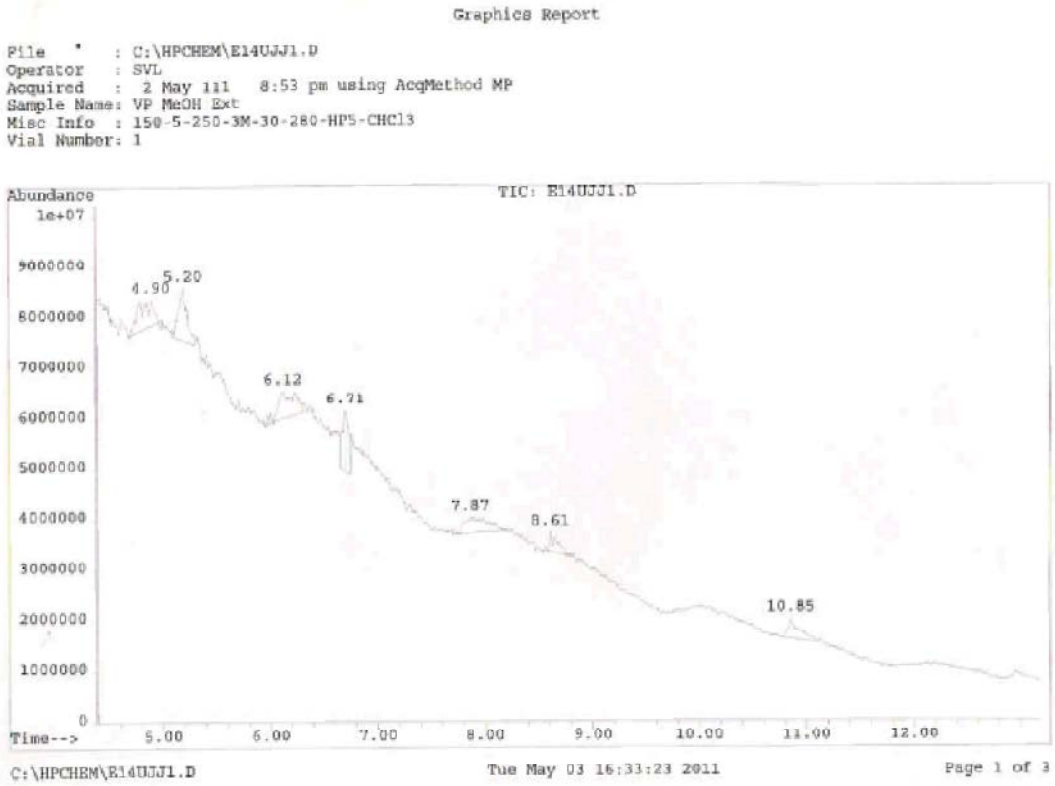

Fig. 3: Gas chromatogram of methanol extract of cured Indian vanilla beans

Table 3: Description of volatile constituents identified in ethyl acetate extract of cured Indian vanilla beans

Peak		RT	Area %	Compound	Molecular Mass and Formula	Mass fragment ions
1.	17.55	6.95	9-Tridecenoic acid,methyl ester	226, $C_{12}H_{23}COOCH_3$	$(M^+$+1)227(11.1%), 152 (82.2%), 151(100%), 143 (20%), 123(13.3%), 87(55.5%), 74(97.7%), 55(44.4%), 43 (31.1%)	
2.	19.44	3.31	9-Octadecenoic acid, methyl ester / Methyl oleate	296, $C_{17}H_{33}COOCH_3$	$(M^+$-15) 281(1.1%), 264(6.6%), 180(11.1%), 152 (11.1%), 123(20%), 111 (22.2%), 110(24.4%), 97 (31.1%), 87(64.4%),83(64.4%), 74(100%), 69(57.7%), 55 (57.7%), 41(48.8%)	
3.	19.60	5.60	9,12-Octadecadienoic acid, methyl ester	294, $C_{17}H_{31}COOCH_3$	M^+294(2.2%), 124(11.1%), 109(40%), 95(28.8%), 81(100%), 67(75.5%), 55 (46.6%), 41(46.6%)	
4.	20.46	10.29	9,12- Octadecadienoic acid	280, $C_{17}H_{31}COOH$	M^+ 280(11.1%), 149(22.2%), 129(22.2%), 121(31.1%), 105(33.3%), 95(53.3%), 91(46.6%), 67(37.7%), 60(44.4%), 57(53.3%), 55(100%), 41(40%)	
5.	21.04	19.90	Bicyclo [3.1.0]hexane-2-undecanoic acid, methyl ester	280, $C_{16}H_{29}COOCH_3$	M^+280(2.2%), 256(8.8%), 197(8.8%),189(11.1%), 161 (11.1%), 149(22.2%), 117 (31.1%%), 105(64.4%%), 95 (60%), 85(48.8%), 83 (48.8%), 81(55.5%), 71(57.7%), 69 (64.4%), 67(73.3%), 57 (53.3%), 55(100%), 43 (97.7%)	
6.	21.28	0.88	3,6-Decadiene	138, $C_{10}H_{18}$	M^+138(1.1%), 111(35.5%), 95(60%), 81(62.2%), 67 (100%), 55(84.4%), 41 (26.6%)	
7.	21.49	3.86	Ethyl tricyclo [2.2.1.0^{2,6}] hept-3-ylcarbamate	181, $C_{10}H_{15}NO_2$	M^+181(11.1%), 168(24.4%), 135(37.7%), 121(66.6%), 109 (93.3%), 95(77.7%), 83(80%), 81(77.7%), 68(84.4%), 55(100%), 39(95.5%)	
8.	22.31	44.87	Pentacosane-2,4-dione	380, $C_{25}H_{48}O_2$	M^+380(6.6%), 280(13.3%), 219(17.7%), 168(22.2%), 149 (26.6%), 123(60%),100 (100%), 87(82.2%), 69(60%), 55(97.7%), 41(24.4%)	
9.	25.87	4.34	Cyclohexadecane-1,3-dione	252, $C_{16}H_{28}O_2$	M^+252(13.3%), 251(13.3%), 168(17.7%), 152(17.7%), 119 (44.4%), 100(73.3%), 85 (68.8%), 69(100%), 55 (46.6%), 43(95.5%)	

As variation in constituents of vanilla beans is observed with cultivation zones; in present study many volatile compounds identified, in cured Indian vanilla beans were different than the compounds already reported in various extracts of cured vanilla beans of global origin. On the basis of comparison with the database of 192 volatile compounds of 10 vanilla beans samples Bourbon-A (Madagascar), Bourbon-B (Madagascar). Balinese-A (Bali), Balinese-B (Bali), Tahiti, Java, Mexico, Tonga, Costa Rica, Jamaica [11, 14, 15], 54 hydrocarbons identified from three vanilla beans species: *V. fragrans*, *V. madagascariensis*, *V. tahitensis* [20], 65 compounds identified in Cured vanilla beans (*V. planifolia* G. Jackson) from the Tuxtepec region of Mexico[17], 246 compounds identified in bourbon and Ugandan cured vanilla beans [12] and all compounds reported in Tanzanian, Bourbon, Indonesian, Ugandan and Tahitian vanilla beans by the new DTD-GC-MS technique [22], most of the compounds like cyclopentane-1,1'-ethylidenebis, 2-hydroxy-3-(2-propenyl)-benzaldehyde, isojasmone, 5-heptyldihydro-2(3H)-furanone, propane-1,1'-[ethylidenebis(oxy)]bis[2-methyl], 9-tridecenoic acid,methyl ester, 9-octadecenoic acid, methyl ester /Methyl oleate, bicyclo [3.1.0]hexane-2-undecanoic acid, methyl ester, 3,6-decadiene, Ethyl tricyclo[2.2.1.0^{2,6}]hept-3-ylcarbamate, pentacosane-2,4-dione,cyclohexadecane-1,3-dione, 5-methyl-5-propylnonane, 9-pentacosene, 1-heneicosene,1-ethyl-3-methylcyclohexane, cyclotetradecane, cyclopentane(2-decyldodecyl), 5-methyl-5-propylnonane are identified first time in cured vanilla beans of any origin.

Table 4: Description of volatile constituents identified in methanol extract of cured Indian vanilla beans

Peak		RT	Area %	Compound	Molecular Mass and Formula	Mass fragment ions
1.	4.90	19.99	5-Methyl-5-propyl nonane	184, $C_{13}H_{28}$	$(M^{+-}15\text{-}H^{+})$168(4.4%),126 (40%), 97(17.7%), 85(57.7%), 71(97.7%), 57(95.5%), 43 (100%)	
2.	5.20	14.51	Cyclopentane,1,1'-ethylidenebis	166, $C_{12}H_{22}$	M^{+}166(20%), 113(15.5%), 97 (100%), 83(33.3%), 69(35.5%), 55(60%), 41 (31.1%)	
3.	6.12	22.16	2-Hydroxy-3-(2-propenyl) -Benzaldehyde	162, $C_{10}H_{10}O_2$	M+162(26.6%), 148(68.8%), 133(100%), 117(73.3%), 105(97.7%), 97(75.5%), 84(22.2%), 69(51.1%), 55(35.5%), 41(17.7%)	
4.	6.71	16.09	1-Ethyl-3-methylcyclohexane	126, C_9H_{18}	M^{+}126(6.6%), 111(24.4%), 97(100%), 85(35.5%), 83(33.3%), 69(37.7%), 55(62.2%), 41(40%)	
5.	7.86	12.30	Pentadecane	212, $C_{15}H_{32}$	M^{+}212(1.1%), 160(22.2%), 145(48.8%), 126(22.2%), 112(22.2%), 85(46.6%),71(75.5%), 57(100%), 43(55.5%)	
6.	8.61	5.17	Cyclotetradecane	196, $C_{14}H_{28}$	M^{+}196(2.2%),125(17.7%), 111(35.5%),97(62.2%), 83(100%), 69 (73.3%), 55(84.4%)	
7.	10.86	9.78	Propane-1,1'-[ethylidenebis (oxy)]bis[2-methyl]	174, $C_{10}H_{22}O_2$	$(M^{+}\text{-}1)$173(1.1%), 160(15.5%) 145(71.1%), 97(31.1%), 85 (51.1%), 71(84.4%),57(100%)43 (73.3%)	

CONCLUSION

Presence of many new volatile constituents in organic solvent extracts of cured vanilla beans grown in India may be a reason of its consistent good quality and need to be explored more by different techniques and even the identification of aroma and oral sensitivity of identified constituents can help in improving the quality of synthetic vanilla further.

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