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Radical scavenging activity of vanilla (Vanilla fragrans) pods and commercial vanilla essence

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Abstract- Methanolic extract (Me) of vanilla pods and commercial vanilla, hexane (He), benzene (Be), ethyl acetate (Ea), n-butanol (nBu) and aqueous (Aq) fractions of methanolic extract of vanilla pods and commercial vanilla essence were analyzed for radical scavenging activities viz. DPPH radical, hydroxyl radical, superoxide radical and nitric oxide radical scavenging activities to screen the best fraction of the methanolic extract (Me) of the samples that posses the highest free radical scavenging activity. All the fractions and methanolic extract of both the samples have individualized and concentration dependent activities. Methanolic extract, benzene, ethyl acetate and aqueous fractions of vanilla pods showed comparatively better scavenging activities with IC_{50} values ranging from 64-485µg/ml in various assays conducted whereas, of vanilla essence, ethyl acetate, hexane, n-butanol and benzene fractions were better with IC_{50} values ranging from 61-489µg/ml. The correlation between each fractions of samples were carried out using ANOVA at a level of $p< 0.05$ and $p<0.001$.

Index Terms- Methanolic extract (Me), hexane (He), benzene (Be), ethyl acetate (Ea), n-butanol (nBu) and aqueous (Aq) fraction.

I. INTRODUCTION

The irony of life in this planet is that molecule that sustains aerobic life, oxygen, is not only essential for energy metabolism and respiration, but it has been implicated in many diseases and degenerative conditions. At low or moderate concentrations, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are necessary for the maturation process of cellular structures and can act as weapons for the host defense system. When the equilibrium between a free radical / reactive oxygen species formation and endogenous antioxidant defense mechanisms, get disturbed, it can produce oxidative stress (Pani *et al*., 2000). Hence, reactive oxygen species and reactive nitrogen species at low or moderate levels are vital to human health can be fatal at higher levels.

Oxidative stress can result from a decrease in antioxidant levels, e.g. mutations decreasing the levels of Mn-SOD, depletion of dietary antioxidants and other essential dietary constituents (e.g. copper, iron, zinc, and magnesium) (Halliwell and Gutteridge, 2006). Phytochemicals are found ubiquitously in plants, they have demonstrated potent antioxidant activity mainly due to its redox properties, which allow them to act as reducing agents, singlet oxygen quenchers, hydrogen donors, and chelators of metal ions. These phytochemicals have wide range of biochemical and pharmacological functions and present almost in all spices which make them antioxidative, antidiabetic, anti-inflammatory, anticarcinogenic, antilithogenic and antimutagenic agents (Rice- Evans *et al*., 1995).

Vanilla fragrans is one of such dynamic spices with its multibeneficial effects. The plant has its functions ranging from flavouring agent to a potent antioxidant, antimutagen etc. *Vanilla fragrans,* an important spice, an orchid belongs to the family orchidaceae. It is cultivated for its beans, which have a sweet scent aroma and a pleasant flavor. Vanilla is the costliest spice in the spice horizon, the important source of vanillin, which is used to flavor ice-cream, chocolates beverages, cakes, custards and other confectionery and also being exploited in perfumery and medicine.

The chief constituents of vanilla beans, in addition to natural vanillin (1.3-3.8%) are resins, fat, glucose, fructose, about 26 volatile constituents as well as 144 other volatile compounds and moisture, "vanilla sugar" obtained from the beans, is used in the manufacturer of chocolates (Korthou and Verpoorte, 2007). The characteristic aroma of vanilla flavor is due to a wide variety of non-volatile constituents like tannins, polyphenols, free amino acids and resins (Rao and Ravishankar, 2000) and volatile constituents like acids, ethers, alcohols, acetals, heterocyclics, phenolics, hydrocarbons, esters and carbonyls (Klimes and Lamparsky, 1976). The other major constituents of vanilla aroma are vanillin (4 hydroxy 3 methoxy benzaldehyde) (2-2.8%), accompanied by minor amounts of p-hydroxy benzaldehyde (0.2%), vanilla (0.2%), p-hydroxy benzyl ether (0.02%) and acetic acid (0.2%) (Anklam,1993). Vanilla is known for its various health benefits and the heroic action of the plant is because of the presence of its principle constituent, vanillin, which has structural similarities with other antioxidant compounds such as eugenol, zingerone and capsaicin. Vanillin posses antioxidant (Naqeb *et al*., 2010), antineoplastic activities (Mc Cann *et al.*, 2007) and could potentially prevent some types of cancers (Lirdprapamongkol *et al*., 2005). It can inhibit peroxynitrite-mediated reactions (Kumar *et al*., 2004) important in several neurodegenerative diseases such as Alzheimer's and Parkinson diseases.

Recent reports have explained the cholesterol lowering effect of vanilla. The activity is either due to its hypotriglyceridemic effect or its regulatory effect on the genes involved in cholesterol metabolism including low density lipoprotein receptor (LDLR) and 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG Co A reductase-HMGCR) genes. Vanillin has been reported to possess anticarcinogenic activity against a variety of chemical and physical agents (Akagi *et al*., 1995).

Studies have indicated that vanillin has antisickling activity due to its ability to react covalently with sickle celled

hemoglobin. An additional function of vanillin is its antimicrobial activity where the compound exhibited inhibitory activity against bacterial strains like *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus cereus*, *Escherichia coli* and *Yersinia enterocolitica* with lower minimum inhibitory concentration (MIC) (Mourtzinos *et al*., 2009).

Keeping in view of the harmful effects of free radicals on human health, detrimental effects of synthetic drugs as well as synthetic antioxidants, and the medicinal properties especially the antioxidant potential of vanilla, present investigation was undertaken with an objective to screen the most potent free radical scavenging fraction of vanilla pods and vanilla essence and to compare the antioxidant potential of both vanilla pods and vanilla essence.

II. Materials and Methods

2.1. Procurement of vanilla samples and chemicals

Vanilla pods (*Vanilla planifolia)* purchased from spices board of India, Calicut branch, Kerala and commercial vanilla essence purchased from Tharakan and Company, Kottayam, Kerala were used for this study. All the chemicals and solvents were of analytical grade.

2.2. Preparations of methanolic extract and various fractions of methanolic extract of natural vanilla pods and commercial vanilla

Vanilla pods

Vanilla pods were split length wise and the split beans were again cut into finer pieces and extracted with 80% methanol (Me), thrice (1:1, w/v) at room temperature (Petra *et al*., 1999). The combined extract was concentrated by evaporation and the residue was dissolved in water and fractionated successively with the solvents of increasing polarity [hexane (He), benzene (Be), ethyl acetate (Ea),n-butanol (nBu) and water (Aq)]and each extract was evaporated to dryness and weight of each residue was noted. Before use, a small quantity of each fraction was re-dissolved in a suitable solvent at different concentrations (Hashim *et al*., 2005) and diluted further to obtain various concentrations i.e. 100µg-500µg/ml.

Vanilla essence

Vanilla essence after dilution (1:1) with 80% (v/v) methanol, was fractionated successively as mentioned above using hexane (He), benzene (Be), ethyl acetate (Ea), n-butanol (nBu) and each extract was evaporated to dryness and weight of each residue was noted. Various concentrations i.e. 100µg-500µg/ml were prepared. During sequential extraction, separate aqueous layer was not formed and hence, testing could not be possible for aqueous fraction.

2.3. Evaluation of in vitro antioxidant efficacy

2.3.1. Determination of DPPH radical scavenging activity

DPPH radical scavenging activity of vanilla extract was determined according to the method given by Sreejayan and Rao (1996). The absorbance of the test mixture was read at 517nm using, Cyberlab, a double beam spectrophotometer. The percentage scavenging of DPPH radical was calculated by comparing the result of the test with that of control (methanol and 1 ml DPPH) using the formula (Schlesier *et al*., 2002):

$$\text{Percentage scavenging activity} = \frac{\text{(Absorbance of control – Absorbance of test)}}{\text{Absorbance of control}} \times 100$$

2.3.2. Determination of hydroxyl radical scavenging activity

The Hydroxyl radical scavenging activity (HRSA) of the sample was determined by the method given by Klein *et al.*, (1991). The intensity of the color formed was measured at 412 nm against reagent blank using spectrophotometer. The percentage hydroxyl radical scavenging activity was calculated by the following formula:

% HRSA = 1-(Absorbance of sample / Absorbance control) x 100

2.3.3. Determination of superoxide radical scavenging activity

Superoxide radical scavenging activity was measured according to the method of Robak and Gryglewski (1998). Absorbance was measured at 560nm against butylated hydroxy toluene as positive control (0.2mg/ml), which was taken in different volumes (200-1000µl) to obtain different concentrations and treated in a similar way. The percentage scavenging activity was calculated using the formula:

$$\text{Percentage scavenging activity} = \frac{\text{Absorbance of control – Absorbance of test}}{\text{Absorbance of control}} \times 100$$

2.3.4. Determination of nitric oxide radical scavenging activity

Nitric oxide radical scavenging activity was estimated by the method given by Sreejayan and Rao (1997) and Marcocci *et al*., (1994). Nitric oxide (NO) radicals were generated from sodium nitroprusside solution at physiological pH. Absorbance was read at 546nm and percentage scavenging activity was calculated using the formula:

$$\text{Percentage scavenging activity} = \frac{\text{Absorbance of control – Absorbance of test}}{\text{Absorbance of control}} \times 100$$

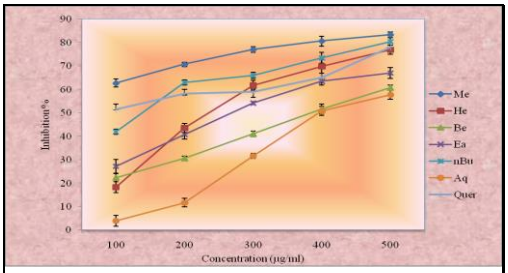
2.4. Statistical analysis

Results are presented as mean ± standard error of means (SEM). Statistical analyses between the experimental samples were carried out using ANOVA. Pre-assigned levels of significant differences were considered at a level of $p < 0.05$ and $p < 0.001$.

III. Results and Discussion

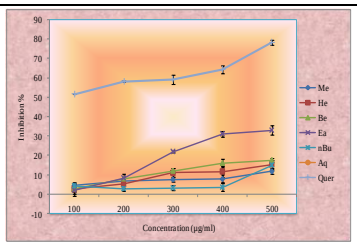
Vanilla pods constitute vanillin, O-vanillin and these compounds have phenolic group – OH, which is responsible for the free radical scavenging activity. The main factor responsible for their radical scavenging ability is the reduction potential or the energy required for the conversion of vanillin/O-vanillin to its oxidized form. The presence of ortho phenolic hydroxyl group would result in intramolecular hydrogen bonding, making the O-

H bond more stretched and hence it breaks easily (Kumar *et al*., 2002).



Me-Methanolic extract, He-Hexane fraction, Be- Benzene fraction, Ea- ethyl acetate, nBu- n-butanol fraction, Aq- Aqueous fraction and Quer- Quercetin
Values are mean ± SEM of three replicates (p<0.001)

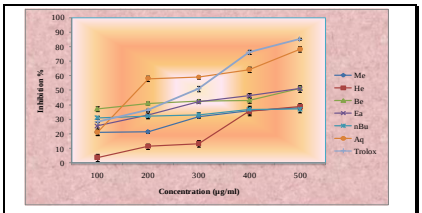
fig. 1. DPPH radical scavenging activity of methanolic extract and various fractions of vanilla pods



Me-Methanolic extract, He-Hexane fraction, Be- Benzene fraction, nBu- n-butanol fraction, Aq- Aqueous fraction and Quer- Quercetin
Values are mean ± SEM of three replicates (p<0.001)

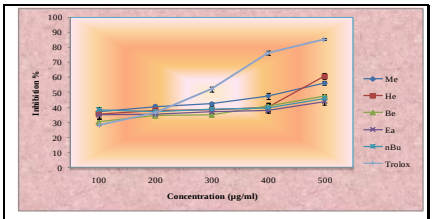
Fig. 2. DPPH radical scavenging activity of methanolic extract and various fractions of vanilla essence

Higher DPPH radical scavenging activity exhibited by vanilla pods is due to different flavonoids present in various fractions as well as in methanolic extract. Higher % activity shown by methanolic extract of vanilla pods than that of quercetin (**Fig.1**) is also due to a number of antioxidant compounds present in vanilla pods viz. catechin, eugenol, tannins, vanillic acid etc. (www.ars_grin.gov/duke) which is probably a result of synergistic action of number of compounds extracted into methanolic extract. As shown in **Fig.2,** ethyl acetate fraction of vanilla essence exhibited better activity than the other fractions, but is lesser than that of quercetin and that of vanilla pods reflecting lesser amount of bioactive compounds in the vanilla essence as a result of processing given to vanilla pods.



Me-Methanolic extract, He-Hexane fraction, Be- Benzene fraction, nBu- n-butanol fraction, Aq- Aqueous fraction
Values are mean ± SEM of three replicates (p<0.05)

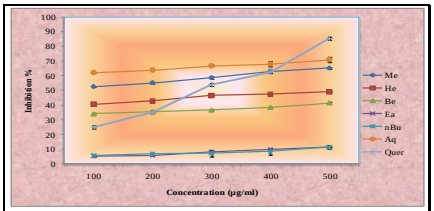
Fig.3. Hydroxyl radical scavenging activity of methanolic extract and various fractions of vanilla pods



Me-Methanolic extract, He-Hexane fraction, Be- Benzene fraction, nBu- n-butanol fraction, Aq- Aqueous fraction
Values are mean ± SEM of three replicates (p<0.05)

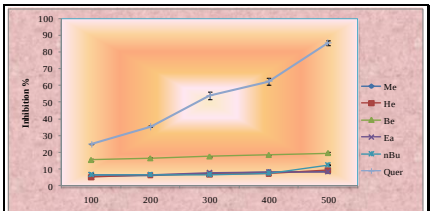
Fig. 4. Hydroxyl radical scavenging activity of methanolic extract and various fractions of vanilla essence

Methanolic extract and all the fractions of vanilla pods as well as vanilla essence exhibited hydroxyl radical scavenging activity in proportion to the concentration (**Fig.3** and **4)**. Among the fractions, benzene and ethyl acetate fractions of vanilla pods exhibited maximum hydroxyl radical scavenging activity with IC_{50} value of 485µg/ml and 489µg/ml respectively, followed by aqueous, hexane fractions, methanolic extract and n-butanol fraction. The trend was different with vanilla essence in which hexane fraction exhibited maximum activity followed by methanolic extract, benzene, n-butanol and ethyl acetate fractions. Both vanilla pods and vanilla essence showed lesser activity than trolox, a commercial standard antioxidant (IC_{50} value 288 µg/ml). The hydroxyl radical scavengers in vanilla are p- hydroxy benzoic acid and vanillin, the scavenging activity of which can be further supported by a study wherein vanillin and p -hydroxy benzoic acid inhibited iron dependent lipid peroxidation in rat brain homogenate, microsomes and mitochondria (Liu and Mori, 1993). The activity exhibited by vanilla essence can be attributed to the cold extraction process which would have released compounds responsible for hydroxyl radical scavenging activity from their complexes.



Me-Methanolic extract, He-Hexane fraction, Be- Benzene fraction, nBu- n-butanol fraction, Aq- Aqueous fraction and Quer- Quercetin
Values are mean ± SEM of three replicates (p<0.05)

Fig. 5. Superoxide radical scavenging activity of methanolic extract and various fractions of vanilla pods



Me-Methanolic extract, He-Hexane fraction, Be- Benzene fraction, nBu- n-butanol fraction, Aq- Aqueous fraction and Quer- Quercetin
Values are mean ± SEM of three replicates (p<0.05)

Fig. 6. Superoxide radical scavenging activity of methanolic extra and various fractions of vanilla essence

Among the various fractions, aqueous fraction of vanilla pods exhibited maximum superoxide radical scavenging activity with an IC_{50} value of 80µg/ml, while benzene fraction of essence had better superoxide radical scavenging activity than the other fractions of vanilla essence (**Fig. 5 and 6)**. Very high IC_{50} values for superoxide radical scavenging activity shown by the fractions of vanilla essence indicate lesser efficiency in scavenging superoxide radicals than that of vanilla pods. Fractions of vanilla pods had better scavenging potential than that of synthetic antioxidant BHT which exhibited an IC_{50} value of 277µg/ml.

The least superoxide radical scavenging activity by vanilla essence is a reflection of lesser amount of phenolics present in essence as compared to vanilla pods conforming that radical scavenging activities depend on the flavonoids present in i.e. vanilla pods, the material under investigation.

Table I: Nitric oxide radical scavenging activity of methanolic extract and various fractions of vanilla pods

Conc (µg/ml)	Me	He	Be	Ea	nBu	Aq	BHT
100	53.1±1.9	54.1±3.5	78.5±2.1	74.9±2.1	70.5±0.5	76.9±0.8	28.3±0.4
200	59.5±0.3	56.7±0.2	81.9±1.1	81.1±0.2	76.6±0.6	1.6±0.4	36.5±0.9
300	68.1±0.3	65.7±0.6	84.8±0.3	82.9±0.5	80.9±0.3	82.3±0.2	52.1±1.7
400	74.8±0.39	74.6±0.36	85.2±0.48	82.8±0.62	81.2±0.2	83.8±0.1	6.5±1.4
500	81.5±0.80	78.4±0.43	85.1±0.45	83.4±0.56	86.1±0.6	85.9±0.7	85.4±0.6
IC_{50} (µg/ml)	94	92	64	67	71	65	288

Values are mean ± SEM of three replicates
Me-Methanolic extract, He-Hexane fraction, Be- Benzene fraction, nBu- n-butanol fraction, Aq- Aqueous fraction

Table II: Nitric oxide radical scavenging activity of methanolic extract and various fractions of vanilla essence

Conc (µg/ml)	Me	He	Be	Ea	nBu	BHT
100	69.4±0.73	81.6±0.34	78.8±0.27	68.3±0.61	71.7±0.80	28.3±0.4
200	77.9±1.43	81.5±0.71	81.9±0.62	81.2±0.16	78.4±0.35	36.5±0.9
300	80.8±0.33	85.8±0.37	83.3±0.56	81.5±0.41	80.9±0.41	52.1±1.7
400	81.5±0.36	85.3±0.62	84.3±0.23	81.6±0.84	82.3±0.86	76.5±1.4
500	81.1±0.15	84.2±1.26	84.1±1.25	83.6±0.41	89.1±0.28	85.4±0.6
IC_{50} (µg/ml)	72	61	63	73	70	288

Values are mean ± SEM of three replicates
Me-Methanolic extract, He-Hexane fraction, Be- Benzene fraction, nBu- n-butanol fraction, Aq- Aqueous fraction

All the fractions and methanolic extract of vanilla pods and vanilla essence exhibited concentration dependent nitric oxide radical scavenging activity ($p<0.001$)(**Table 1 and 2)**.Both vanilla pods and vanilla essence had similar effects with an IC_{50} value ranging from 64-92 µg/ml and 61-73 µg/ml respectively. At 500 µg/ml, n-butanol fraction showed highest activity in both the samples, followed by aqueous, benzene, ethyl acetate, methanolic extract and hexane fractions in case of vanilla pods and hexane, benzene, ethyl acetate and methanolic extract in case of vanilla essence. BHT examined as positive control in the same study, exhibited the radical scavenging activity ranging from 29%-84% with an IC_{50} value of 289µg/ml.

The data obtained in the present study, indicates that vanilla pods and essence obtained from pods possessed better $NO^{\bullet}$ scavenging activity than BHT with lesser IC_{50} value, indicating more potency against $NO^{\bullet}$ radicals. It is assumed that vanillin, a potent radical scavenger reacts with radicals via adduct formation or self-dimerization (Tai *et al*., 2011).

IV. Conclusions

Methanolic extract and all the fractions of methanolic extract of vanilla pods scavenged radicals inan individualized and concentration dependent manner. However, methanolic extract scavenged most efficiently DPPH radical, while benzene and ethyl acetate fractions scavenged most efficiently hydroxyl radicals and nitric oxide radicals and aqueous fraction scavenged most efficiently superoxide radicals.

Different fractions of vanilla essence had shown maximum activity in various assays. With respect to DPPH, ethyl acetate fraction, hydroxyl radical scavenging activity, hexane fraction, nitric oxide radical scavenging, n-butanol fraction, superoxide radical scavenging assay benzene fraction had shown the highest activity.

Methanolic extract and various fractions of methanolic extract of vanilla essence had lesser radical scavenging efficiency indicating lesser biochemicals and phytochemicals than in vanilla pods owing to the loss of phytochemicals during processing.

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