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**Beyond the flavour: de-flavoured polyphenol rich extract of
Clove buds (*Syzygium aromaticum* L) as a novel
dietary antioxidant ingredient**

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Abstract

Though kitchen spices constitute an important source of dietary antioxidants, their consumption at a physiologically relevant dose was very often hampered by the unpleasant flavour characteristics. The present paper describes a novel approach to derive stable de-flavoured spice extracts with minimised taste and odour profile suitable for the impregnation into a variety of food and beverage matrices at physiologically relevant doses. A popular kitchen spice, Clove bud (*Syzygium aromaticum L*), having strong flavour and pungency characteristics was selected in the present study to derive a de-flavoured extract with standardised polyphenolic profile (Clovinol) and incorporated in various food. Antioxidant efficacy of Clovinol on healthy human volunteers who check and answer official emails were investigated by analysing their endogenous antioxidant enzymes and extent of lipid peroxidation upon consumption of Clovinol either as capsules or as different food/beverages at 250 mg/serving/day for 30 days. It was observed that Clovinol can be conveniently incorporated in various food matrices without flavour issues and the consumption of such food/beverages may support an effective detoxification process with an average elevation of 33 ±3% in catalase, 66 ±8% in SOD, 56 ±5% in GPx, 167 ±21% in GSH levels and 81 ±11% attenuation in membrane lipid peroxidation level.

Keywords: *Syzygium aromaticum L*, *Clove buds*, *Polyphenols*, *Functional dietary ingredient*, *Detoxification*, *oxidative stress*

1. Introduction

Recent upsurge in the understanding of oxidative stress and its significance in the pathogenesis of various dreadful diseases, has become instrumental in the quest for safe and powerful bioavailable natural antioxidants capable of acting at the cellular and molecular level to mop up the highly reactive species to maintain the genomic stability and plausible prevention of free radical mediated/age-related diseases.¹⁻³ A surplus of studies have demonstrated the safety, efficacy and multi-targeted mechanism of action of various natural antioxidants in ameliorating the oxidative damages, either to delay the onset or to improve the health state in a number of pro-inflammatory disease conditions.^{1,3-5} Though fruits and vegetables are generally established as good natural sources of dietary antioxidants, kitchen spices were shown to possess more medicinal values owing to the presence of a panacea of antioxidant and anti-inflammatory phytochemicals.⁶⁻⁸ Inspired by the extensive use of kitchen spices in traditional systems of medicine, a great deal of research has also been carried out on the bioactive principles and their pharmacological effects.⁷⁻¹⁰ The observational studies have delineated the plausible efficacy of dietary spices to the relatively low incidence of diseases like cancer, Alzheimer's and obesity among people from the Indian subcontinent who consumes high levels of spices as compared to the Western countries. For instance, it was reported that US had 356 colon cancer cases reported and 139 deaths *per* 1 million people in 2000, whereas India had only 40 reported cases of colon cancer and 26 deaths per 1 million people.⁷ However, the unfavourable organoleptic characteristics such as pungency, aroma and colour of spices have seriously limited its transformation as a functional ingredient beyond their global status as food flavourings capable of imparting a hot culinary pungency with characteristic flavour and colour. Thus, the innovative challenge in the development of functional spice ingredients lies in the ability to develop selective extraction and formulation of bioactive molecules of spices with sufficient stability, solubility, compatibility and bioavailability suitable for various food/beverage formats of commercial interest when impregnated at relevant doses per serving.

In this context, we have developed a unique process of extraction, emulsification and microencapsulation to derive de-flavoured phytonutrient-rich spice extracts with minimised taste and odour profile suitable for the impregnation into a variety of food and beverage matrices at physiologically relevant doses (*herein after termed as NutriSPICE™*).

NutriSPICE extracts are regarded as green extracts with no synthetic emulsifiers and are instantly water soluble and stable with standardised levels of bioactive phytonutrient molecules. The present paper describes the NutriSPICE approach on Clove buds (*Syzygium aromaticum L*) to derive water soluble and stable de-flavoured extract of clove with standardised polyphenolic profile (*herein after referred to as 'Clovinol'*) suitable for its use as a functional dietary ingredient. Clove buds are a typical example of aromatic and pungent spice rich in volatile oil [10 to 15% (w/w)] possessing an array of health beneficial pharmacological activities.¹¹ Clove buds were shown to possess highest polyphenol content and antioxidant compounds among the 100 richest dietary sources of polyphenols including fruits and vegetables.¹² Though most of the studies on clove has mainly been carried out on eugenol, a bioactive terpenoid accounting greater than 80% of its essential oil, clove buds were also shown to be consisted of flavonoids, aromatic hydroxy acids, hydrolysable tannins and their glycosylated derivatives.¹³⁻¹⁶ Since plant polyphenols have generally been identified as super active antioxidants and the consumption of polyphenol-rich food and beverage has been well correlated with various health promotion/maintenance functions,^{6, 17-19} the present contribution was aimed at evaluating the effect of consumption of polyphenol-rich NutriSPICE extracts of clove buds, (Clovinol) - fortified food/beverages in modulating the endogenous antioxidant defenses and lipid peroxidation on healthy human volunteers who are in regular use of official emails to carry out their responsibilities as employees and its impact on overall oxidative stress and detoxification process. It has already been established that handling large number of responsible emails is a source of stress at work with elevation in heart rate and blood pressure, which may eventually lead to various health problems.²⁰ To the best of our knowledge, this is the first report on the use of tailored spice extracts with minimized taste and odour profile as functional ingredient and the demonstration of efficacy in human volunteers. Nutritional composition, storage stability, pH stability, temperature stability *In vitro* antioxidant capacity of Clovinol was also investigated in the present study. Optimized doses of Clovinol for impregnation into various food/beverages, without compromising the taste/appearance and the product acceptability, has also been investigated in detail. Hence the ultimate aim of this investigation is to illustrate the potential health benefits and acceptance of spice based nutraceuticals. As an example, one of the very popular kitchen spices, Clove bud having strong flavour and

pungency characteristics was selected to derive a de-flavoured extract with an acceptable level of natural flavour/ aroma and rich polyphenol load, and also to understand its health beneficial effects in human subjects.

2. Materials and methods

2.1. Preparation and Characterization of 'Clovinol'

Dried clove buds were received from a selected farm in Indonesia where clove trees are grown without using any pesticides or chemicals. The samples were identified by an authenticated botanist and a voucher specimen (AK-CLV-011) was deposited at the Herbarium of M/s Akay Flavours & Aromatics Ltd, Cochin, India. A representative sample of 'Clovinol' produced by hydro-ethanolic extraction followed by purification, emulsification and spray drying (Lot No. 01/13 B2 dated July 2013) was obtained from Akay Flavours & Aromatics Ltd, Cochin, India and was used for the present study. Total polyphenols were measured by the standard Folin-Ciocalteu test using gallic acid as the standard.²¹ Characterization of polyphenols was achieved by 1290 infinity Ultra-performance liquid chromatography (UPLC) system coupled with an Agilent 6530 QTOF instrument having a Jet-Stream source (Agilent India Pvt Ltd, Bangalore, India). Ammonium acetate (10 mM) in water (A) and methanol (B) was employed as the mobile phase with a Zorbax Eclipse Plus C18 (3.0 × 100 mm; 1.8 µm) column at 30 °C and 5 µL injection volume. Nutritional composition, polyphenol content and heavy metal ions of Clovinol were analysed prior to the incorporation into the food matrices by following the analytical procedures described in the Association of Analytical Chemists (AOAC).²² The microbial parameters were analysed by the procedures described in U.S. Food and Drug Administration Bacteriological Analytical Manual (FDA-BAM).

2.2. Stability studies of 'Clovinol'

Storage stability studies of Clovinol were carried out using a protocol prepared by following the International Conference on Harmonization (ICH) guidelines.²³ Briefly, the sample packets (10 g) of Clovinol were incubated at 40°C ± 2 °C and 75 ± 5% relative humidity for a period of 6 months in a stability chamber (Remi, Mumbai, India). The samples were withdrawn at 0, 1, 2, 3, and 6 months and analyzed for various physicochemical parameters

such as polyphenol content, total carbohydrate, proteins, moisture content etc. The pH stability of the aqueous solution was checked by preparing 5% (w/w) solutions at pH 2.0, 5.0, and 6.8 using hydrochloric acid and phosphate buffers. 5 mL volumes of the solutions were withdrawn at regular intervals of 2 h for a period of 24 h, and the polyphenol content was checked using Folin–Ciocalteu's reagent method.²¹ Temperature stability was verified by maintaining a 5% aqueous solution of Clovinol at 90 ± 2 °C for 30 min followed by total polyphenol content estimation.

2.3. *In vitro* antioxidant effect of 'Clovinol'

In vitro antioxidant effect and radical scavenging capacity of Clovinol was investigated using a set of well standardised assays. Oxygen radical absorbing capacity (ORAC) was measured on the basis of the capacity of the extract to stabilize the fluorescence signal of a fluorescence probe (fluorescein sodium) over the time which in turn directly related to the capacity of the extract to neutralize the peroxy radicals generated from the decomposition of 2,2'-azobis(2-amidino-propane) dihydrochloride (AAPH) and expressed as Trolox equivalents.²⁴ Cellular antioxidant protection at erythrocyte models (CAP-e assay) was conducted as per the method of Honzel et al,²⁵ by measuring the inhibition of oxidative damage on erythrocytes exerted by Clovinol when treated with peroxy radical generator AAPH. The antioxidant efficacy was reported as CAP-e value which reflects the IC₅₀ of the test substance, as compared to that of a known antioxidant, gallic acid. Superoxide radical scavenging activity of Clovinol was estimated by following the method of Mc Cord and Fridovich,²⁶ by measuring the reduction of nitroblue tetrazolium salt (NBT) by superoxide radicals generated during the photoreduction of riboflavin. Hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and Clovinol for the hydroxyl radicals generated from Fe³⁺/ascorbate/EDTA/H₂O₂ system (Fenton reaction). The hydroxyl radicals attack deoxyribose, which eventually results in the formation of thiobarbituric acid reacting substances.²⁷ The free radical scavenging activities were determined by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) method by Coruh et al.²⁸

2.4. *Animals/human study*

All animal experiments were carried out in strict accordance with the ethical norms approved by the Institutional Animal Ethics Committee, recognized by the Committee for the

Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India (Registration No: 1620/PO/RcBi/S/12/CPCSEA.). Human study was approved by the Institutional Ethical Committee clearance (CKL-IEC/01-2014) and written consent from all individuals was obtained prior to study.

2.5. Lipid peroxidation assay

Freshly excised rat (6 weeks old male Wistar rats weighing 150-200 g) liver was processed to get 10% homogenate in cold phosphate buffered saline, pH 7.4 using glass teflon homogenizer and filtered to get a clear homogenate. The level of lipid peroxidation was measured by the method of Ohkawa et al,²⁹ by measuring the complex formed by the major secondary product of lipid peroxidation, malondialdehyde (MDA) and thiobarbituric acid (TBA). The extent of complex formation was monitored by measuring the absorbance at 532 nm as thiobarbituric acid reactive substances (TBARS).

2.6. Preparation of food/beverages containing 'Clovinol' and sensory analysis

Various food and beverage items (fruit juice, yogurt, honey and plain drinking water) containing 100, 250 mg and 500 mg/serving of Clovinol were prepared as described below and stored in closed opaque high density polyethylene food containers at $4 \pm 0.3^\circ\text{C}$ for two weeks in a refrigerator. The organoleptic properties were assessed by a panel of seven experts who tasted and scored individually. On the basis of sensory results, the following food items containing a dose of 250 mg Clovinol/serving/day were selected for continuous consumption for 30 days and subsequent analysis of the antioxidant status. Mango juice was prepared by homogenizing 50 g mango pulp with 250 mg Clovinol and further diluting with drinking water to 200 mL of serving size. Yogurt and honey were also prepared by mixing with 250 mg Clovinol at respective serving sizes of 50 g and 20 g respectively. Hard gelatine capsules containing Clovinol (250 mg x 1) was prepared with the help of a capsule filling machine (CMD4-20 Tablet Press, Cadmach Machinery Co. Pvt. Ltd., Ahmedabad, India).

2.7. Effect of supplementation of 'Clovinol' as food/beverage or as capsules upon the oxidative stress of official email users – an open label study

Institutional ethical committee clearance and written consent from all volunteers were obtained after explaining the detailed protocol and purpose of the study. Twenty six healthy

human volunteers (twelve male and twelve female) not involved in any medication or health supplementation, having an average BMI of 30 ± 1 and aged between 25 and 40 years, were selected for the study. The human subjects were the employees who were in responsible positions dealing with several official emails per day. Two of the subjects were dropped out of the study due to personal reasons. The subjects were randomly grouped into 4 groups in such a way that each group contains three males and three females, and provided with various Clovinol fortified food items and Clovinol capsules as follows (Fig. 1); Group I (250 mg x 1) Clovinol hard gelatine capsule per day); Group II (200 mL mango juice with 250 mg Clovinol/serving/day), Group III (50 g yogurt with 250 mg Clovinol/serving/day) and Group IV (20 g honey with 250 mg Clovinol/serving/day). All the volunteers had similar physical activity per day and were not allowed to ingest black/green tea during the study period. They were advised to follow the typical south Indian food pattern of around 1800 to 2000 calories per day, comprising breakfast made of rice (having an approximate nutritional composition of fat: 22 to 24%, carbohydrates: 35 to 40%, protein: 27 to 30%; energy 400 to 500 calories), lunch with rice and vegetable/non-vegetable curries (having an approximate nutritional composition of fat: 25 to 30%, carbohydrates: 35 to 42%, protein: 32 to 35%; energy 500 to 580 calories) and dinner with similar nutritional compositions to lunch. The volunteers were advised to consume the food product or capsule daily for 30 days along with the dinner. The blood samples (5 mL) were withdrawn at the beginning and also on the 7, 15 and 30 days of administration and the plasma was separated by centrifugation, kept at $-20\text{ }^{\circ}\text{C}$ for analysis. Haematological and biochemical parameters were also tested at the beginning and on 31st day of supplementation to demonstrate the safety and tolerance. Antioxidant status and lipid peroxidation of the blood was measured at the beginning and at the end of 30 days of supplementation, as a measure of the oxidative stress. Superoxide dismutase (SOD) activity in blood was estimated using the standard procedure by McCord and Fridovich.²⁶ Catalase (CAT) activity in blood was determined by the method of Aebi,³⁰ by measuring the rate of decomposition of hydrogen peroxide at 240 nm and the Glutathione peroxidase (GPx) activity in blood was determined by the method of Paglia & Valentine,³¹ based on the degradation of hydrogen peroxide in the presence of reduced glutathione. The non-protein thiol, glutathione (GSH) activity was measured by the method of Moron et al,³² based on the reaction with 5,5-dithiobis-2-nitrobenzoic acid (DTNB)

reagent. The extent of lipid peroxidation was analysed by the thiobarbituric acid reactive substance (TBARS) test.²⁹

2.8. Statistical analysis

The values are expressed as mean \pm SD. The statistical significance was compared between control and experimental groups by one way analysis of variance (ANOVA) followed by appropriate post hoc test (Tukey's multiple comparison test) using GraphPad InStat software (version 3.05). Data of Clovinol treated animals were compared with untreated animals.

3. Results

3.1. Characterisation of Clovinol

Clovinol was obtained as free flowing powder suitable for handling freely in our ambient conditions of $60 \pm 5^\circ$ RH (relative humidity) and at temperature of $26 \pm 2^\circ\text{C}$. The powder showed instant cold water and hot water ($> 50^\circ\text{C}$) solubility to produce a fully transparent water with no settlement on standing even after 72 h at both ambient and refrigerated conditions (4°C). Clovinol was found to contain 23.6 % gallic acid equivalent of polyphenol content with very faint characteristic aroma and astringency of clove buds; but with no mouth warming effect. Upon tandem mass spectrometric analysis, Clovinol was found to contain various types of polyphenols (flavonoids, phenolic acids, and hydrolysable tannins and their glycosides) as identified by the literature data and reference compounds. When analyzed under positive electrospray ionization, quercetin glucoside (m/z 463), quercetin rhamnoside (m/z 447), Luteolin glucoside (m/z 447.9), apigenin hexosides (m/z 431.6) were identified in addition to the free aglycones as quercetin (m/z 301), luteolin (m/z 288), and apigenin (m/z 271). When analysed in the negative ionization mode, a number of phenolic acids and their derivatives were observed. Gallic acid (m/z 171.1), caffeic acid (m/z 181.3) chlorogenic acid (m/z 354.6), ellagic acid (m/z 301.4), and their derivatives such as the gallic acid ester of caffeic acid (m/z 333.4) were also identified in Clovinol. Further, HPLC analysis employing the respective analytical standards revealed the presence of gallic acid (38.6 mg/g), ellagic acid (18.45 mg/g), catechin (26.6 mg/g), quercetin (11 mg/g), chlorogenic acid (13.7 mg/g) and eugenol (9.4 mg/g) (Fig. 2). The results showed enrichment in the concentration of these polyphenols in Clovinol, when compared to the

clove buds material used. When tested for storage stability under accelerated conditions as specified in ICH guidelines, Clovinol was found to undergo only a 0.5% loss in polyphenol content, with no significant change in other parameters such as colour, appearance, odour, taste, polyphenol content, moisture content, bulk density, carbohydrates, proteins, and microbial status (Table 1), indicating the possibility of convenient storage for two years at ambient conditions of less than 25 °C in closed containers, away from direct light and moisture. An aqueous solution of Clovinol at pH 6.5 showed only 2.3% polyphenol degradation when stored for 30 days under room conditions of $26 \pm 2^\circ\text{C}$. On heating to $90 \pm 2^\circ\text{C}$ for 30 min, the solution showed 4.2% polyphenol degradation, without precipitation or settlement on further cooling to room temperature.

In vitro antioxidant activity of Clovinol

Clovinol has shown significant radical scavenging activity and antioxidant efficacy when subjected to various *in vitro* assays; the IC_{50} values are given in Table 2. Clovinol showed an effective superoxide radical scavenging ability with an IC_{50} value of 12.5 $\mu\text{g}/\text{mL}$, in comparison with the well-studied water soluble antioxidant, vitamin C whose IC_{50} was found to be 1900 $\mu\text{g}/\text{mL}$ under the same conditions of experiment. Hydroxyl radical quenching activity of Clovinol was also found to be better than Vitamin C with respective IC_{50} values of 76 $\mu\text{g}/\text{mL}$ and 1550 $\mu\text{g}/\text{mL}$ respectively. When the radical scavenging capabilities of Clovinol was investigated in comparison with that of Vitamin C in a DPPH assay, the IC_{50} values were found to be 6 $\mu\text{g}/\text{mL}$ and 14 $\mu\text{g}/\text{mL}$ respectively. The measurement of CAP-e value as Gallic Acid Equivalents (GAE) units also has reflected the cellular antioxidant protection effect of Clovinol in erythrocytes, as compared to the known polyphenolic antioxidant, gallic acid. The CAP-e value of Clovinol was found to be 85 CAP-e units/g, and Clovinol has showed a significant inhibition in oxidative damage with an IC_{50} value of 270 $\mu\text{g}/\text{mL}$. ORAC value measurements showed a better antioxidant effect ($9000 \pm 700 \mu\text{mol TE}/\text{g}$). Further investigation on the effect of Clovinol to inhibit the lipid peroxidation in comparison with vitamin C, using incubation of rat liver homogenates with the oxidant chemical species Fe^{2+} , showed an IC_{50} value of 375 $\mu\text{g}/\text{mL}$ and 192 $\mu\text{g}/\text{mL}$ respectively for Vitamin C and Clovinol (Fig. 3).

3.2. Clovinol as a food ingredient

Clovinol was incorporated into various food and beverage matrices like fruit juices, yogurt, honey and plain drinking water at 100, 250 and 500 mg/serving size, without having the solubility or compatibility issues. The above food items were selected in consideration of the possibility for further development as a functional food ingredient for ready-to-cook or ready-to-use products. Because of the wide spread use and increasing demand, ready to use products has become one of the most convenient vehicles for micronutrient delivery. However, no food items involving extensive cooking or baking was tried in the present study owing to the plausible thermal instability of polyphenols. Though the sensory analysis of the organoleptic properties of Clovinol-impregnated various food items was consumption was good at all the three doses/servings, 250 mg/serving/day was selected for the repeated dose 30 days supplementation study to check its effectiveness on oxidative stress and detoxification. Incorporation of Clovinol was found to offer no change to the characteristic taste of the food stuff. On the other hand, it provided a unique mouth feel as an after taste which provided a sense of freshness to the mouth.

3.3. *Effect of 'Clovinol' on oxidative stress when used as a dietary ingredient – an open label investigation on healthy subjects*

The effect of repeated 30 days of consumption of various food/beverage items containing Clovinol at 250 mg/serving/day, on the oxidative stress of healthy human subjects was investigated by measuring the plasma antioxidants status, by measuring the plasma catalase (CAT), SOD, GPx and non-protein thiol (GSH) levels, along with the extent of lipid peroxidation. The observed effect was also compared with the effects observed when Clovinol was administered as hard gelatin capsules (250 mg x 1) to demonstrate the stability and absorption of antioxidants from food/beverages. The results demonstrated a significant reduction in oxidative stress as evident from the elevation in the primary antioxidant defence enzymes CAT (33 ±3%), SOD (66 ±8%), GPx (56 ±5%) and GSH (167 ±21%) as compared to the initial values (Fig. 4). The elevation in endogenous antioxidant enzymes has also found to contribute to a significant inhibition in plasma lipid peroxidation (81 ± 11%) among all the people who consumed Clovinol either as food/beverage or as capsules (Fig. 5). The antioxidant effect from the various food/beverage or capsules was found to be in the order capsules > honey > yogurt > mango pulp with the relative average difference of

±15%, indicating their effectiveness as dietary ingredient. Moreover, repeated dose supplementation of Clovinol for 30 days at 250 mg/serving/day has not shown any behavioural symptoms or adverse effect in any of the subjects indicating its tolerance. The biochemical parameters of the subjects were also found to remain in the normal range without any significant change, indicating its safety upon repeated consumption (Table 3).

4. Discussion

The excess formation of highly reactive free radicals, commonly referred as 'reactive oxygen species' (ROS) has been shown to damage the cellular redox equilibrium resulting in the undesirable oxidation, membrane damage, lipid peroxidation, protein modification, DNA damage, and cell death which in turn may lead to several pathogenic conditions such as atherosclerosis, neurodegenerative disorders, chronic inflammatory diseases, metabolic syndromes, age-related diseases and certain types of cancer.³³ Though the complex *in vivo* antioxidant defenses (SOD, catalase, GPx, GSH etc) usually protects the cellular components, the living conditions such as unhealthy food habits, sedentary or stressful life, smoking, alcoholism, aging, UVA/UVB radiations, environmental pollution, and malnutrition were found to impair the endogenous antioxidant balance. For instance, excess production of ROS was found to activate the stress-sensitive signaling pathways, called mitogen activated protein kinases (MAPK) leading to the formation of gene products responsible for the complications of metabolic diseases.³⁴ Treatment or supplementation with bioavailable antioxidants was shown to restore the redox equilibrium with significant improvement in oxidative stress.³⁵

The antioxidants exert efficacy either by scavenging the reactive oxygen species or by activating the battery of detoxifying/defensive proteins such as SOD, Catalase and GPx. SOD destroys the superoxide radicals by converting it to peroxides which further neutralized by catalase or GPx.³⁶ Catalase is regarded as one of the most efficient antioxidant enzymes since it was not saturated by H₂O₂ at any concentration.³⁷ GPx, on the other hand, reduces a variety of hydroperoxides employing a GSH dependent mechanism, whereby GSH is oxidized to inactive disulfides. Thus, the agents or enzymatic systems (eg., glutathione peroxidase and glutathione reductase) capable of either synthesizing GSH or reducing the disulfides to GSH are of great importance in the cells' inherent ability for detoxification and maintenance

of intracellular redox environment. Moreover, GSH was also shown to participate in the metabolism, immune modulation, gene expression and in the regulation of the activity of sulfhydryl-containing proteins either *via* thiol-disulfide exchange reactions or by the reduction of potentially toxic peroxides.³⁸

In addition to the endogenous antioxidants mentioned above, there exist exogenous antioxidant molecules, comprising vitamins, polyphenols (flavanols, flavonoids, anthocyanins) carotenoids, polyunsaturated fatty acids etc, as dietary antioxidants capable of upregulating the depleted cellular antioxidants for detoxification. Regular consumption of antioxidants at adequate levels and their bioavailability have recognized as two important factors related to the antioxidant supplementation regime. Fruits and vegetables are generally considered as antioxidant rich food components and their daily intake is very much recommended. Though kitchen spices were shown to have many fold high antioxidant effects than fruits and vegetables, their regular consumption at adequate level has always been challenged by their high pungency and aroma. In the present paper, we demonstrate the development of de-flavoured spice extracts (odour and taste minimized, NutriSPICE™), as dietary antioxidant ingredients. Clove buds, a notorious aroma and pungent spice rich in volatile oil, was employed in the present study to demonstrate the deflavoured spice antioxidant extracts as dietary ingredients. Clove was already recognized as one of the best antioxidant botanical in recent studies and even referred to as the 'Champion of Spice'.^{11, 12,}
³⁹ NutriSPICE-clove bud extract (Clovinol) was prepared as a standardized polyphenol rich water soluble extract possessing a very mild flavour and taste suitable for its impregnation in a variety of food/beverage items without taste/aroma issue. Though eugenol from clove was regarded as GRAS (Generally Regarded as Safe)-listed antioxidant principle of Clove buds,⁴⁰ Clovinol used in the present study was rich in water soluble polyphenols belonging to the flavonoids and their glycosides along with phenolic acids, hydrolysable tannins of ellagic acid and gallic acid.¹³ Reports on the pharmacological activities of Clove polyphenols were found to be rare, except the aphrodisiac, antioxidant, carminative, and antispasmodic effects of some non-standardised hydro-ethanolic extracts.^{14,41} Recently, we had reported a polyphenol standardised extract of clove buds and its anti-ulcerogenic activity.¹³ Clovinol used in the present study was found to contain 23.6 gallic acid equivalent of polyphenols with instant water solubility at 500 mg/300 mL concentration with no sedimentation on

storage for one month at 25°C and also at 4°C. The aqueous solution at 5% (w/v) level showed sufficient stability of polyphenols under physiologically relevant pH states during the study period of 24 h. The observed temperature stability of aqueous solution was also found to be satisfactory for pasteurization conditions of dairy and beverage products. The results of the storage stability studies were found to be in agreement with its convenient storage for minimum 12 months and transportation in air-tight containers kept at dark and cool (< 25°C) place protected from moisture and direct sunlight.

Initial *in vitro* antioxidant studies using a set of well-established assays as detailed under section 2.3 revealed the potential plausible antioxidant efficacy of Clovinol due to its strong reactive oxygen and nitrogen species scavenging activity, in comparison with that of Vitamin C. The observed hydroxyl, super oxide, free radical scavenging activities as evident from the IC₅₀ values (Table 2) was found to be physiologically relevant when compared with some of the earlier reports. For instance, green tea polyphenol extract has been reported to show significant antioxidant activities with an ORAC value of 2820 µM TE/g and their mechanism of action was primarily attributed to properties such as radical and oxidant scavenging; metal chelation; inhibition of redox-sensitive transcription factors and pro-oxidant enzymes.⁴² Grape seed polyphenols, yet another widely investigated antioxidant, was reported to have an ORAC value of 3009.2 µM TE/g.⁴³ ORAC value of 9000 ±700 µM TE/g found with Clovinol used in the present study was one of the highest values reported so far. Though there are some concerns regarding the ORAC values and bioavailable antioxidant effects, it is generally one of the best available *in vitro* measures of the antioxidant feasibility of a substance.⁴⁴ Further, the CAP-e assay employing the erythrocyte cells, were reported to be efficient to predict the cell permeability and bioavailability of an antioxidant to provide protection to cells from oxidative damage.²⁵ The CAP-e value reflects the IC₅₀, indicating the dose required to provide 50% inhibition of oxidative damage. Clovinol showed a CAP-e value of 85 units/g, with an IC₅₀ value of 270 µg/mL, indicating its potential *in vivo* antioxidant efficacy. Thus, the *in vitro* studies demonstrate the radical scavenging activity of Clovinol without a pro-oxidant effect. Further, its solubility, stability, mild flavour and regulatory compliance along with the possibility of economical production has tempted us to further evaluate its efficacy as a dietary ingredient to ameliorate the oxidative and/or detoxification, a key step in the pathogenesis of many disease states.

Clovinol was successfully impregnated into various food and beverage systems, including the plain drinking water, at physiologically significant doses of 100, 250 and 500 mg per serving to demonstrate that it can be used as dietary ingredient without the taste/flavour issues. From the sensory evaluation results, a dose of 250 mg/serving was selected for further clinical evaluations. The solubility of Clovinol allowed its easy impregnation into various beverage matrices like mango juice, yogurt and honey without any sedimentation. The taste of clove upon the food consumption depends mainly on the food matrix. In the case of yogurt and honey, addition of Clovinol even at 500 mg/serving had only a little effect as compared to the plain drinking water. Impregnation in matrices like yogurt, juice and honey was found to furnish a very pleasant after taste of clove with a unique mouth feel. It was found that the flavour can be tailored for various food matrices to meet unique taste profiles with varying degrees of clove touch by adjusting the alcohol percentage of the extracting solvent. General acceptability score of various products prepared in this study was > 92% when provided to forty five people.

It has already been demonstrated that the plant polyphenolics can contribute not only to the quality of food by modifying colour, taste, flavour, aroma and shelf-life, but also to the health beneficial pharmacological effects.¹⁸ The effect of green tea catechins, grape procyanidins, and cocoa flavonoids in maintaining the cellular endogenous antioxidant levels has already been established.³ Regular consumption of Clovinol for 30 days was also found to offer a significant ($p < 0.05$) enhancement in the primary antioxidant defence enzymes in human subjects who are in regular use of official e-mails for their responsibilities. It has already been reported that the use of official emails induce more stress among employees especially during the information gathering (reading) and sharing (sending) activities with enhanced blood pressure and heart rate.²⁰ The relative enhancement in SOD and GSH levels were highly significant ($p < 0.001$) with almost two fold improvement as compared to the initial values. The observed levels of upregulation upon Clovinol consumption was found to be 10 to 15% better than some of the early reported effects on green tea extracts.⁴⁵ Modulation of intracellular GSH concentrations has already been related to a number of disease preventing GSH-dependent cellular processes such as detoxification of xenobiotics, glutathionylation of proteins, regulation of redox switching of protein functions and muscle fatigueness,^{46,47} while supplementation of synthetic GSH has

practiced as a dietary regime to upregulate the GSH levels.⁴⁸ The capacity of Clove to naturally enhance the GSH levels to a significant extent assumes importance. The extent of lipid peroxidation, a major factor involved in the development of atherosclerosis,⁴⁹ was found to have an $83 \pm 11\%$ reduction among the subjects who consumed Clovinol containing food. The observed efficacy Clovinol from various food matrices was in found to be in the order capsule > honey > yogurt > mango pulp. The maximum effect of capsules may be due to its direct consumption as compared to the food/beverages which have undergone a production process followed by storage for minimum 14 days. Relatively higher activity in honey can be attributed to the better absorption from the phospholipids in honey. There were no wide variations in the blood pressure and heart rates in these individuals (data not shown), may be due to the selection of healthy volunteers without any previous history of cardiac problems, but there are various studies reported on the hypotensive effect of polyphenols due to their potential antioxidant efficacy.⁵⁰ Considering the already established role of antioxidant defences in maintaining the cell integrity, detoxification, mitochondrial health and cell division,² the supplementation of Clove polyphenolic extract (Clovinol) was found to be beneficial for those who are in regular use of official emails and also may help under varying conditions such as extensive alcoholism, smoking, and other stressful conditions and where high oxidative stress and impaired antioxidant defence system may exist.

5. Conclusions

The present paper describes an attempt to prepare taste and odour minimised standardised phytonutrient extracts of kitchen spices as functional dietary ingredients and to demonstrate its efficacy. The concept and the process have been successfully elaborated by selecting clove - an essential oil rich, highly pungent and strong smelling kitchen spice and subjecting to a solvent extraction, purification, concentration and microencapsulation to provide taste and odour minimised polyphenol rich extract (Clovinol), possessing sufficient water solubility, stability and organoleptic characteristics suitable for easy impregnation into various food/beverage matrices. When supplemented for 30 days at 250 mg/serving/day Clovinol exhibited significant enhancement in endogenous cellular antioxidant enzyme levels among employees who are in constant official email use, indicating its usefulness as antioxidant dietary ingredient capable of managing the oxidative

stress and cellular detoxification process. Thus, the present study, for the first time, points towards the possibility of making use of spices beyond just flavourings; but as a nutraceutical/functional food ingredient without the taste/aroma issues.

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Conflict of Interest

NutriSPICE™ is the registered trademark of M/s Akay Flavours & Aromatics Pvt Ltd, Cochin, India, for the unique range of deflavoured phytonutrient extracts of spices, protected under a patent application.

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Table 1

Storage stability of Clovinol as per an in-house protocol based on ICH guidelines. The samples were incubated at 40 ± 2 °C and 75% \pm 5% RH for a period of six months.

Parameter	0 month	1 month	2 months	3 months	6 months
Appearance	Reddish Brown	Reddish Brown	Reddish Brown	Reddish Brown	Reddish Brown
Odour	Characteristic	Characteristic	Characteristic	Characteristic	Characteristic
Polyphenol content (%)	23.6	23.6	23.6	23.5	23.1
Moisture (%)	1.97	2.1	2.07	2.12	2.1
Bulk density (g mL ⁻¹)	0.39	0.39	0.39	0.39	0.39
Carbohydrates (g per 100 g)	68.90	68.90	68.87	68.89	68.85
Protein (g per 100 g)	4.5	4.4	4.6	4.6	4.5
<u>Microbiology</u>					
Total plate count	<700 cfu g ⁻¹	450 cfu g ⁻¹	350 cfu g ⁻¹	400 cfu g ⁻¹	400 cfu g ⁻¹
Yeast & Mould	<100 cfu g ⁻¹	50 cfu g ⁻¹	40 cfu g ⁻¹	30 cfu g ⁻¹	30 cfu g ⁻¹
Coliforms	<3 MPN g ⁻¹	<3 MPN g ⁻¹	<3 MPN g ⁻¹	<3 MPN g ⁻¹	<3 MPN g ⁻¹
E. coli	Absent/g	Absent/g	Absent/g	Absent/g	Absent/g
Salmonella	Absent/g	Absent/g	Absent/g	Absent/g	Absent/g

Table 2*In vitro* antioxidant effect of Clovinol (IC₅₀) in comparison to Vitamin C

Assay	IC ₅₀ Values	
	Clovinol	Vitamin C
Superoxide radical scavenging (µg/ml)	12.5	1900
Hydroxyl radical scavenging (µg/ml)	76	1550
DPPH (µg/ml)	6	14
Lipid peroxidation inhibition (µg/ml)	192	375
CAP-e (µg/ml)	270	---
ORAC (µM TE/g)	9000 ±700	---

Table 3

Biochemical and haematological analysis of human volunteers before and after 30 days of Clovinol supplementation.

Parameters	Clovinol 250 mg	
	Before	After
<u>Hematology</u>		
Hb (g dL ⁻¹)	15.23±1.59	15.28±1.38
WBC x 10 ³ (mm ³) ⁻¹	8.13±1.77	7.67±1.40
RBC x 10 ⁶ (mm ³) ⁻¹	5.63±0.63	5.47±0.62
Platelet x 10 ⁵ (mm ³) ⁻¹	2.24±0.65	2.39±0.66
Lymphocytes (%)	41.33±4.98	41.50±5.60
Eosinophils (%)	2.70±0.80	2.50±0.80
Neutrophils (%)	54.50±4.90	63.40±5.70
Monocytes (%)	1.50±0.80	0.90±0.80
<u>Biochemical</u>		
SGPT (U L ⁻¹)	40.6±11.30	39.3±15.30
SGOT (U L ⁻¹)	27.9±9.60	24.8±7.70
ALP (U L ⁻¹)	68.6±16.30	67.8±14.60
Total Protein (g dL ⁻¹)	7.50±0.42	7.45±0.35
Bilirubin (mg dL ⁻¹)	0.65±0.41	0.70±0.50
Albumin (g dL ⁻¹)	5.03±0.28	4.69±0.18
Globulin (g dL ⁻¹)	2.56±0.35	2.76±0.35
A/G ratio	1.99:1±0.30	1.73:1±0.20
Cholesterol(mg dL ⁻¹)	191.7±35.40	182.8±30.6
Triglycerides(mg dL ⁻¹)	138.20±47.10	124.70±43.70
HDL (mg dL ⁻¹)	45.3±7.80	47.50±7.90
LDL (mg dL ⁻¹)	120.2±32.3	118 ±33.22
VLDL(mg dL ⁻¹)	29.60±9.4	22.30±7.70
Creatinine (mg dL ⁻¹)	0.77±0.17	0.73±0.13

The values are expressed as mean ± SD for 24 subjects from all groups

Legend to Tables and Figures

Fig. 1- Human study protocol to assess the effect of Clovinol as dietary antioxidant ingredient.

Fig. 2- HPLC profile of (a) Clovinol, and (b) the clove buds used for the study. Peak- 1, Gallic acid; 2, Quercetin; 3, Catechin; 4, Ellagic acid; 5, Chlorogenic acid, and 6, Eugenol. Analysis was carried out on a Shimadzu model LC 20 AT, with an M20A photo diode array (PDA) detector (Shimadzu Analytical India Pvt Ltd, Mumbai, India), fitted with a reverse phase C18 column (250 × 4.6 mm, 3 μm) (Phenomenex, Hyderabad, India). The chromatogram was collected at 254 nm and 1 mL/min flow rate. For recording the HPLC of the raw material, 1g of the clove buds powder was extracted with acetone/water (70/30 v/v) under ultrasonication. The filtrate was made up to 100 mL and 20 μL was injected.

Fig. 3- Effect of Clovinol on peroxidation of membrane lipids in rat liver homogenate (*in vitro*) in comparison to Vitamin C, as measured by TBARS assay. Values are expressed as mean ±SD of 4 samples.

Fig. 4- Relative antioxidant efficacy of Clovinol when supplemented as (250 mg x 1) hard shell gelatin capsule and as food/beverages to healthy human volunteers (a) Catalase; (b) SOD; (c) GPx; and (d) GSH levels. The results are expressed as Mean±SD, where n denotes $p > 0.05$, *denotes $p < 0.05$, # denotes $p < 0.01$ and \$ denotes $p < 0.001$ when compared with initial values on day 0.

Fig. 5- Effect of Clovinol (250 mg) capsule and Clovinol fortified food administration in healthy human volunteers on inhibition of lipid peroxidation measured as MDA levels. The results are expressed as Mean±SD, where # denotes $p < 0.01$ and \$ denotes $p < 0.001$ when compared with initial values on day 0.

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Table 2

In vitro antioxidant effect of Clovinol (IC₅₀) in comparison to Vitamin C.

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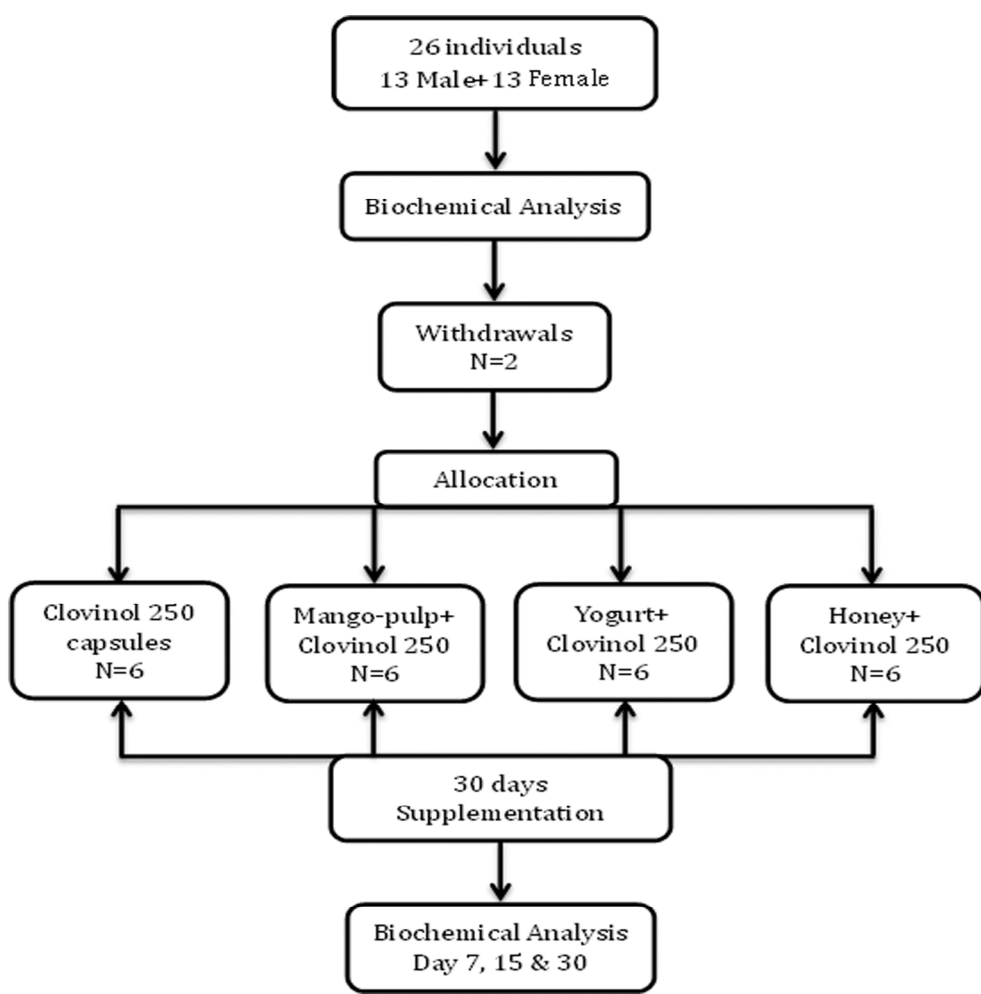


Fig. 1- Human study protocol to assess the effect of Clovinol as dietary antioxidant ingredient. 82x82mm (600 x 600 DPI)

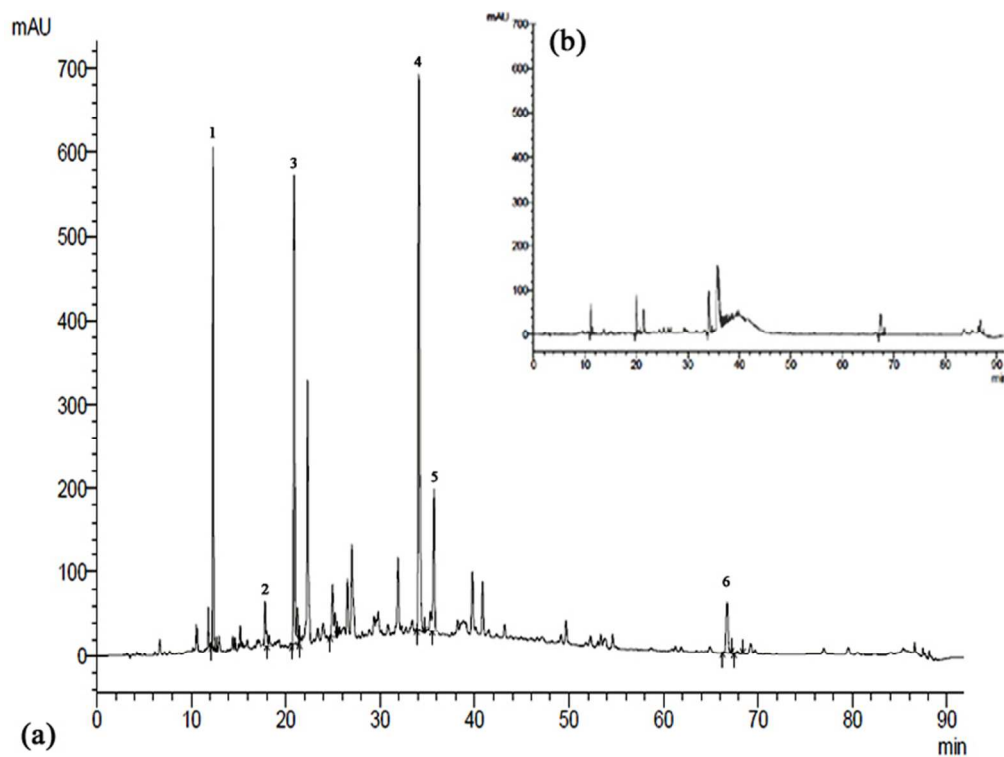


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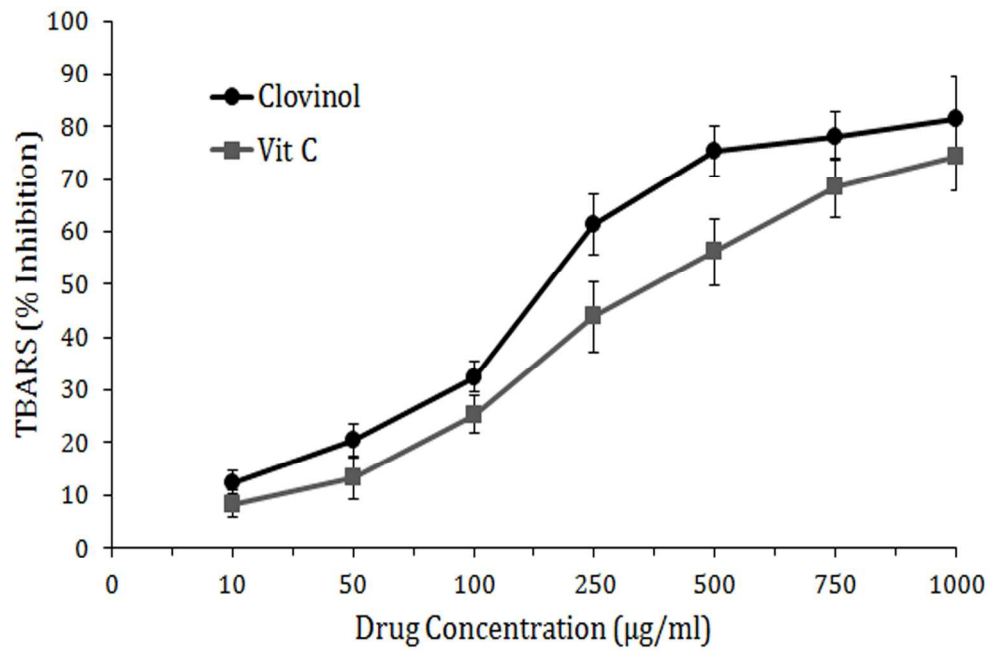


Fig. 3- Effect of Clovinol on peroxidation of membrane lipids in rat liver homogenate (in vitro) in comparison to Vitamin C, as measured by TBARS assay. Values are expressed as mean \pm SD of 4 samples.
101x67mm (600 x 600 DPI)

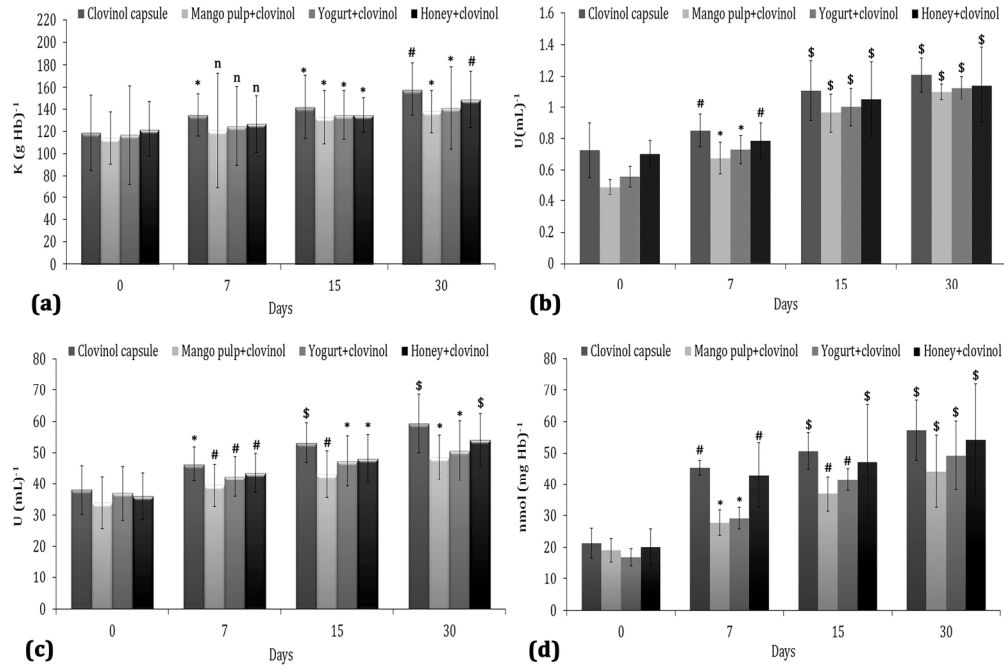


Fig. 4- Relative antioxidant efficacy of Clovinol when supplemented as (250 mg x 1) hard shell gelatin capsule and as food/beverages to healthy human volunteers (a) Catalase; (b) SOD; (c) GPx; and (d) GSH levels. The results are expressed as Mean±SD, where n denotes p >0.05, *denotes p <0.05, # denotes p <0.01 and \$ denotes p <0.001 when compared with initial values on day 0.
81x54mm (600 x 600 DPI)

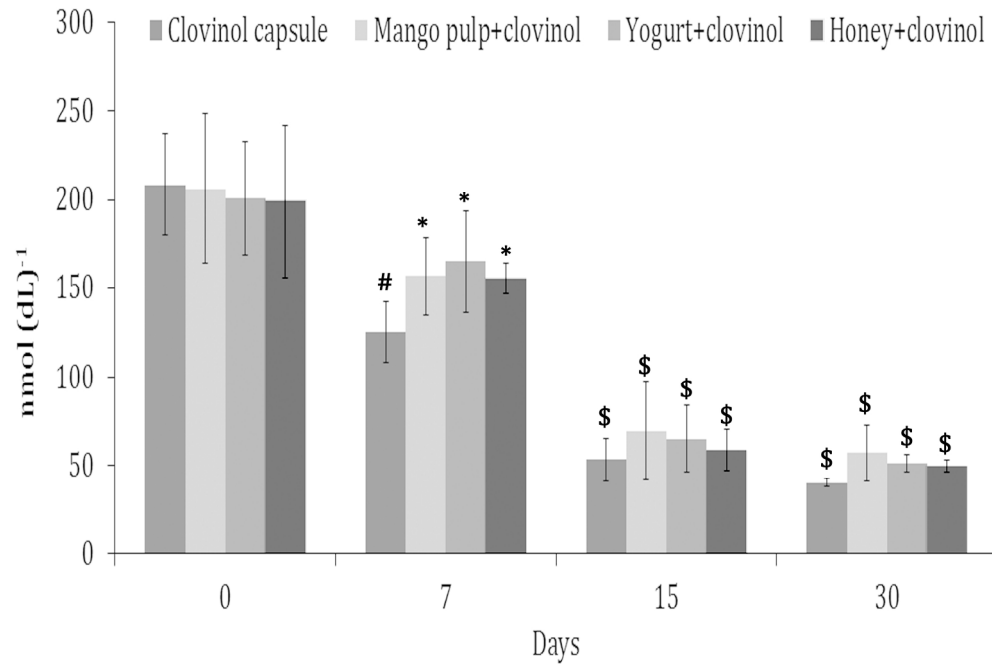


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