Antioxidant properties of *Cordyline terminalis* (L.) Kunth and *Myristica fragrans* Houtt. encapsulated separately into casein beads

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In the present study, we have developed an encapsulation procedure which retains the antioxidant properties and polyphenol contents from two novel plants stable over a period of one year at room temperature at non-toxic level. Methanolic extracts containing polyphenols were prepared from *Cordyline terminalis* and *Myristica fragrans* under optimized conditions (*C. terminalis* polyphenol extracts – CTPE and *M. fragrans* polyphenol extracts – MFPE). These extracts contained 102.6 and 72.9 mg/g of gallic acid equivalent of polyphenols respectively, when measured using the Folin–Ciocalteu method. The antioxidant activity of CTPE and MFPE was 13.4 and 10 mg/g of ascorbic acid equivalent respectively, when measured using the 2,2-diphenyl-1-picryl-hydrazyl method. CTPE and MFPE were individually encapsulated in sodium-caseinate beads in order to protect the polyphenol content from oxidation, and were stored separately in dark at room temperature. The stability of the encapsulated extracts was studied over a period of 12 months after dissolution of the beads in deionized water. We observed that the polyphenol content and antioxidant activity remained stable in the encapsulated beads compared to the unencapsulated extracts. Sodium-caseinate beads prove to be a promising technique for food supplementation with natural antioxidants.

Keywords: Antioxidant property, casein beads, *Cordyline terminalis*, *Myristica fragrans*, phenolic content.

EPIDEMIOLOGICAL studies consistently indicate that the consumption of fruits, vegetables and herbs rich in phytochemicals lowers the incidence of diseases in humans[1–3]. This beneficial effect is mainly due to the presence of polyphenolic compounds[4,5]. Polyphenols from the plant kingdom represent a wide range of molecules[6]. The basic structure is composed of a benzene ring linked to one or more hydroxyl groups, free or attached with another chemical functional group (e.g. dimethyl ether, ester, sugar). These are aromatic compounds formed from the metabolism of shikimic acid. Structurally they fall into different families, including anthocyanins, coumarins, lignins, flavonoids, tannins, quinones, acids and phenols[6]. This structural diversity results in large variability in the physico-chemical properties influencing the extraction of polyphenols.

The interest in polyphenols has grown considerably because of their high capacity to entrap the free radicals associated with different diseases like cancer, inflammation, chronic diseases and metabolic disorders by balancing the reactive oxygen species (ROS). ROS are by-products of regular cell metabolism and are capable of oxidizing cellular proteins, nucleic acids and lipids[7]. When they are in excess, it leads to coronary heart disease, cellular ageing, mutagenesis, carcinogenesis, etc. possibly through destabilization of membranes, DNA damage and oxidation of low-density lipoproteins[8]. Maintenance of balance between oxidants and antioxidants is of significance for cellular functions. Though the biological system has ways to mitigate ROS, natural antioxidants can be supplemented to remove the excess ROS by inactivating the free radicals. Recently, there has been an increasing interest in determining relevant dietary sources of antioxidant phenolics. Assessment of biological properties of plant extracts remains an interesting and useful task in identifying new sources of natural antioxidants which can be used as nutraceuticals.

All phenolic compounds are highly unstable and rapidly transformed into various reactive products. They are generally prone to degradation on storage. Encapsulation is a technique where a bioactive compound is entrapped into a polymer, thereby protecting it from oxygen, water or other conditions to improve its stability. The encapsulation process not only gives stability to the polyphenols, but also masks their bad taste. Casein is a milk protein which is a biocompatible polymer, edible and easily degradable without any side effects[3]. Casein is organized in micelles form and is designed by nature to stabilize, transport and concentrate mainly calcium and protein for neonates[9]. Stabilization of polyphenols could also be improved by casein encapsulation.

Based on the local availability we have chosen two plants for our studies, *Cordyline terminalis* and *Myristica fragrans*, which are rich in polyphenols. *C. terminalis*, also called Ti plant belongs to the Liliaceae family. Its leaves have been used for forage, and also in medicine for antipyretic, analgesic and antibacterial activity, and in cooking[10]. *M. fragrans*, commonly known as nutmeg belongs to the Myristicaceae family. It is an anti-diarrhoeal, stomachic stimulant, carminative, intestinal catarrh and colic to stimulate appetite, and aromatic[11].

The objective of this study was to encapsulate the polyphenols from these two novel plants into casein beads, with an aim to achieve the sustained release and improved stability over a period of 12 months of storage. To evaluate its stability of polyphenols and antioxidant

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activities of the encapsulated and unencapsulated extracts of *C. terminalis* polyphenol extract (CTPE) and *M. fragrans* polyphenol extract (MFPE) were studied.

2,2-Diphenyl-1-picryl-hydrazyl (DPPH), gallic acid, ascorbic acid, deoxyribose, casein and xanthane were purchased from Sigma-Aldrich (St Louis, MO, USA). Folin–Ciocalteu reagent (FC), sodium carbonate, sodium phosphate, potassium acetate, glycerol, ethylenediamine-tetraacetic acid (EDTA), methanol, ethyl acetate, chloroform, sulphuric acid, trichloroacetic acid (TCA) and hydrogen peroxide reagents were obtained from Qualigens (Mumbai). All other chemicals used were of high quality analytical grade.

The extract preparation was carried out using a modified method of the Montoro *et al.*[12]. *C. terminalis* and *M. fragrans* were collected from Vellore (Tamil Nadu, India) and used for extraction on the same day. *C. terminalis* leaves and *M. fragrans* seeds (100 g) were grounded and mixed with methanol : water (70 : 30), incubated for a week at 4°C, and filtered through a cheese cloth. The filtrate was centrifuged at 7000 rpm for 10 min, and the supernatant was collected. It was subjected to evaporation in Buchi Rota vapour apparatus (R-215, Switzerland), and the extract was lyophilized. The dried powder was stored at 4°C in dark until subsequent analyses.

Total phenol content of the extracts was determined according to the modified method of Slinkard *et al.*[13], using the Folin–Ciocalteu reagent colorimetrically at 725 nm. The methanolic extracts of both plants (0.1–0.3 ml) were mixed with 0.1 ml of Folin–Ciocalteu reagent followed by addition of 0.25 ml of sodium carbonate (25%) and made up to 1 ml using water. After 1 h, the absorbance of the sample was measured at 725 nm against a blank using a double-beam ultraviolet–visible spectrophotometer (Hitachi U-1100; Hitachi Ltd, Tokyo, Japan). Gallic acid was used as a standard for preparing the calibration curve. The phenolic content was expressed as milligram equivalent of gallic acid per gram of the extract. The experiment was done in triplicate.

The antioxidant activity of the extracts was evaluated according to the modified method of Bandoniene *et al.*[14] using the stable DPPH radical. The methanolic extracts of both plants (0.1 ml) were added to 0.5 ml of DPPH of 2×10^{-4} M solution and made up to 1 ml using methanol; the absorbance was read at 515 nm against methanol using a double-beam ultraviolet–visible spectrophotometer (Hitachi U-1100). Simultaneously, the absorbance at 515 nm of the blank sample was measured against methanol. The radical scavenging activities of the tested samples, expressed as percentage inhibition of DPPH, were calculated according to the following formula proposed by Yen and Duh[15].

$$\% \text{ Scavenging of DPPH} = [(A_0 - A_s)/A_0] \times 100,$$

where A_0 is the absorbance of the blank sample at 515 nm at time $t = 0$ min, and A_s is the final absorbance of the test sample. The extract concentration providing 50% inhibition (EC_{50}) was calculated from the graph of percentage scavenging effect against extract concentration.

Encapsulation of the extracts in casein beads was prepared according to the modified method of Dehkharghanian *et al.*[16]. A solution of caseinate beads was prepared by mixing sodium caseinate, glycerol, xanthane and distilled water with extract and without extract as control. Total weight of the solution was 50 g. The solution was stirred for 1 h and centrifuged at 1098 *g* for 10 min at 20°C. After the solution was homogenized, the beads were obtained by dropping the mixture from a burette into 1 litre of 2N hydrochloric acid. The beads were filtered, washed with distilled water and dried at room temperature in the dark. The total weight and diameter of the beads were measured. The beads were stored at room temperature. The average sizes of the beads of CTPE and MFPE were 2.1 ± 0.3 and 2.33 ± 0.1 mm respectively.

The experiment was carried out to standardize the temperature and time limit for the release of polyphenol content from the encapsulated beads of CTPE and MFPE. Beads weighing 50 mg were taken in 1 ml of water and heated at various temperatures (30°C, 40°C, 50°C and 60°C) and at different time intervals (1 h, 4 h and 7 h). The release of polyphenol content was estimated in each case.

Destabilization of the beads was done according to the modified method of Dehkharghanian *et al.*[16]. For this 50 mg of beads was taken in 1 ml distilled water and boiled at 60°C for 7 h, 20% of TCA was added and grounded, filtered and centrifuged at 2000 rpm. This supernatant was used for estimation of polyphenols.

All measurements were obtained in triplicate, and the values were averaged and reported along with the standard deviation (SD). Data were analysed using one-way ANOVA tests and Student's *t*-test to assess the differences between means. All the data were analysed with the SPSS 10.0 statistical software.

Recently, natural plants have received much attention as a source of biologically active substances, including antioxidants, antimutagens and anticarcinogens[17]. The ability of phenolic compounds, including flavonoids, phenolic acids and lignins to act as potential antioxidants has been extensively studied[18,19]. The extraction of phenolic compounds from plant material is influenced by their chemical nature, the extraction method, sample size, time and storage conditions as well as the presence of interfering substances[20].

In the present study, different percentages of methanol (from 0 to 100) were used for the extraction of polyphenols from *C. terminalis* leaves and *M. fragrans* seeds. The results of polyphenol content at different percentages of methanol used are given in Figure 1. The results indicate that maximum amount of polyphenols was extracted in 70% methanol. Therefore, we used 70% methanol for the extraction process. The percentage yield of the extracts

were 6.8 for *C. terminalis* and 8.8 for *M. fragrans* per 100 g dry wt (DW) respectively.

There are reports that the maximum extraction yield and antioxidant activity of the extracts from plants were obtained with ethanol and methanol, and their mixtures with water[21]. The use of an alcoholic solution provides satisfactory results for the extraction process[22]. High yield was achieved using methanol for the extraction of phenols from *C. terminalis* and *M. fragrans* (Figure 1).

Total polyphenolic content of *C. terminalis* and *M. fragrans*, was 106.2 and 72.9 mg gallic acid equivalent (GAE) per gram of extract when extracted with 70% methanol (Figure 2). *C. terminalis* is well known for its medicinal use in New Zealand[10], but no reports are available on the analysis of the polyphenolic content from *C. terminalis* extract. The polyphenolic content of *M. fragrans* was reported by Wojdylo *et al.*[23] and Siddharthan *et al.*[24] as 89 and 19 mg of GAE per 100 g when extracted with 80% methanol. We observed higher polyphenolic content of *M. fragrans*; it was 72.9 mg/g of GAE. Nevertheless, the extract used by Wojdylo *et al.*[23] and Siddharthan *et al.*[24] was prepared with a different protocol compared to ours.

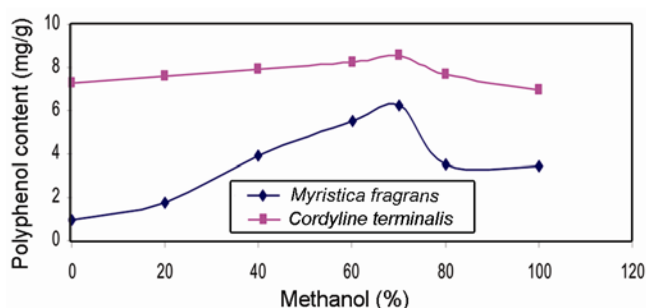

Figure 1. Effect of different percentages of methanol on polyphenol content of *Cordyline terminalis* and *Myristica fragrans*. Polyphenol content at different percentages of methanol used for the extraction of *C. terminalis* and *M. fragrans*. Values are expressed as mean ± SD of gallic acid equivalents, $n = 3$ ($P < 0.001$).

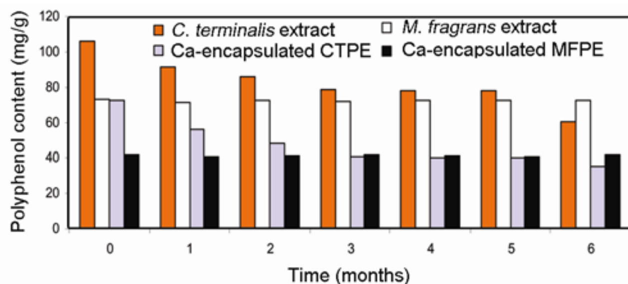

Figure 2. Stability of polyphenol content in unencapsulated and encapsulated methanolic extract of *C. terminalis* and *M. fragrans* for a period of 12 months. Total polyphenol content of unencapsulated and encapsulated methanolic extracts of *C. terminalis* and *M. fragrans* during a period of twelve months of storage. Values are expressed as mean ± SD of gallic acid equivalents; $n = 3$ ($P < 0.001$). CTPE, Casein encapsulated *C. terminalis* methanolic extract; MFPE, Casein encapsulated *M. fragrans* methanolic extract.

MFPE exhibited higher free radical scavenging activity by DPPH. The EC_{50} value was 10 µg of ascorbic acid/g of extract when extracted with 70% methanol. The EC_{50} values of DPPH radical from CTPE and MFPE are given in Figure 3. Whereas Wojdylo *et al.*[23] and Siddharthan *et al.*[24] reported that the antioxidant activity of *M. fragrans* was 3.3 and 0.045 Trolox equivalent (TEAC) mg/100 g DW, when extracted with 80% methanol. No reports are available in the literature for *C. terminalis* on antioxidant activity. In the case of methanolic extract of *C. terminalis*, the EC_{50} value was 0.135 mg/g ascorbic acid equivalent (AAE).

For stability of polyphenols, encapsulation was done with sodium caseinate in a protective polymer such as casein. Casein, the milk protein possess excellent nutritional value and numerous functional properties such as barrier to oxygen transfer, stability to form a complex due to hydrophobic bonds as well as for the cross-linking ability via disulphide bonds or by tyrosine formation[16]. In our work we achieved an encapsulation of 68.1 mg/g (6.81%) for CTE and 57.1 mg/g of casein (5.71%) for MFE.

In the case of encapsulated beads of CTPE and MFPE, by heating 50 mg of beads with 1 ml of deionized water at 60°C for 7 h, the polyphenols were released. This helped in the digestion of casein and released the contents. The results of polyphenol content are shown in Figure 2 and antioxidant activity is shown in Figure 3. We observed a continuous decrease of polyphenol content for a period of three months in the unencapsulated extracts of *C. terminalis* and *M. fragrans*. During the next two months the polyphenol content was stable, but on the sixth month it again decreased. This is in agreement with Montoro *et al.*[12], who reported that flavonoids and anthocyanins are unstable and the extracts cannot be used over three months after their preparation.

Antioxidant activity and polyphenol content were determined every month over a period of six months and subsequently after one year to evaluate whether the

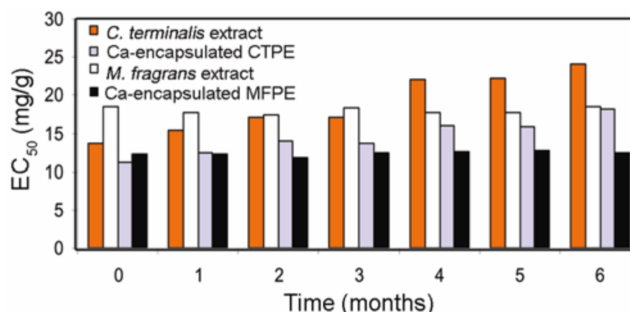

Figure 3. Antioxidant activity of unencapsulated and encapsulated methanolic extracts of *C. terminalis* and *M. fragrans* for a period of 12 months. EC_{50} values of unencapsulated and encapsulated methanolic extracts of *C. terminalis* and *M. fragrans* on free radical scavenging activity during six months of storage. Values are expressed as mean ± SD of ascorbic acid equivalents; $n = 3$ ($P < 0.001$).

encapsulation technique could modify antioxidant power of the loaded extract. The encapsulated CTPE showed the polyphenol content of 72.94 mg/g polyphenols equivalent of gallic acid at day 1. Every month for a period of six months, the polyphenol content was analysed, it was stable with an average mean value of 72.35 mg/g of GAE. Even after one year the polyphenol content remained constant and was 72.38 mg/g of GAE. Whereas the unencapsulated CTPE showed polyphenol content of 106.2 mg/g of GAE at day 1. The polyphenol content was analysed and found to be unstable every month for a period of six months. At the end of the six month the polyphenol content decreased to 60.02 mg/g of GAE. At one year the polyphenol content decreased further and was 52.18 mg/g of GAE.

The polyphenol content of MFPE was encapsulated and observed to be 41.90 mg/g polyphenols equivalent of gallic acid at day 1. Every month for a period of six months, the polyphenol content was analysed and was stable with an average mean value of 41.39 mg/g of GAE. There was no further decrease up to one year. Whereas the unencapsulated MFPE showed polyphenol content 72.48 mg/g of GAE at day 1. Subsequently every month for a period of six months, the polyphenol content was analysed and found to be unstable. At the end of six months the polyphenol content decreased to 35.20 mg/g of GAE. The decreasing tendency continued even after one year to 30.65 mg/g of GAE.

The concentration of antioxidant needed to decrease by 50% of the initial substrate concentration (EC_{50}), is a parameter widely used to measure the antioxidant power[25]. The lower the EC_{50} value, the higher the antioxidant power. The EC_{50} values of antioxidant activity in the encapsulated CTPE and MFPE at 1 day were 13.66 and 11.13 mg/g of AAE. After six months, the antioxidant activity of both the extracts was 24.15 and 18.21 mg/g of AAE respectively, and after one year it was 27.38 and 20.98 mg/g of AAE. The polyphenol content and antioxidant activity values of the encapsulated extracts of *C. terminalis* and *M. fragrans* were stable for a period up to one year.

Encapsulated CTPE and MFPE showed significant antioxidant activity compared to the unencapsulated extracts (Figures 2 and 3). The encapsulated CTPE and MFPE showed good protection against oxidation, and its relatively strong protective effect in casein beads could be attributed to the amphiphilic properties of phenolic constituents. It is generally assumed that an increase in the number of hydroxyl groups in a phenol enhances the hydrogen donor ability and inhibition of oxidation[26]. Thus, the encapsulation technique maintained the antioxidant power of CTPE and MFPE.

To study the effect of temperature and time of incubation on the release of polyphenols from the encapsulated beads of CTPE and MFPE, the beads were heated at different temperatures and at different time intervals. The results are shown in Figures 4 and 5. We observed that the release of polyphenols from the encapsulated beads depends on the temperature and time of incubation. In the case of CTPE and MFPE, maximum amounts of polyphenols were released when the beads were incubated at 60°C for 7 h. At 40°C and 50°C respectively, CTPE and MFPE showed a degradation of their polyphenol content (Figures 4 and 5), suggesting that the correct selection of the temperature and time control is an important step in guaranteeing the stability and amount of the polyphenols in the beads. The results also indicate that the temperature had a significant effect on the total polyphenol content of CTPE and MFPE. The wall material successfully used for encapsulation of polyphenol was protein (sodium caseinate) emulsion, which has been used in green tea polyphenol encapsulation[16]. This is a multipurpose encapsulation process, creating a novel nutraceutical product suitable for a variety of applications in functional food manufacturing. Further studies need to be carried out based on these conditions because time and temperature are the important factors to be considered when it is administrated to animal models for bioavailability studies.

C. terminalis and *M. fragrans* are two novel plants which are rich in polyphenols and antioxidant activity. In this study we formulated the polyphenolic content and antioxidant activity for a period of 12 months, and

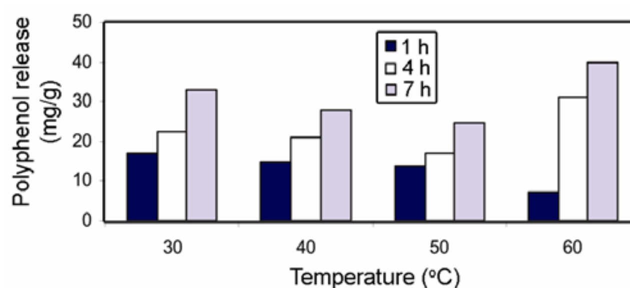

Figure 4. Effects of temperature and time on the release of polyphenols from casein encapsulated *C. terminalis* methanolic extract. Temperature and time dependence on the release of polyphenols from the encapsulated beads of *C. terminalis* extract. Values are expressed as mean ± SD of gallic acid equivalents; $n = 3$ ($P < 0.001$).

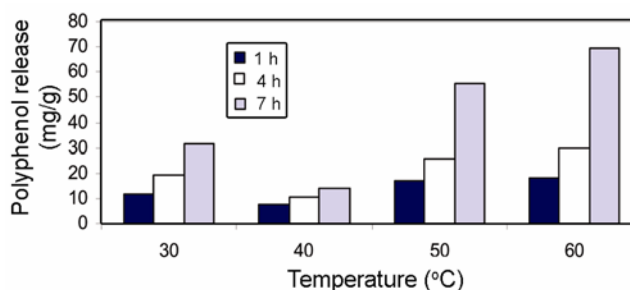

Figure 5. Effects of temperature and time on the release of polyphenols from casein encapsulated *M. fragrans* methanolic extract. Temperature and time dependence on the release of polyphenols from the encapsulated beads of *M. fragrans* extract. Values are expressed as mean ± SD of gallic acid equivalents; $n = 3$ ($P < 0.001$).

extracts were stable in casein beads at non-toxic level. However, it was found to be unstable in uncapsulated extracts for both the plants. Moreover, the encapsulated extracts at room temperature also showed no significant colour change in beads over a period of 12 months. In conclusion, our present study has commercial potential; we are working with an industrial partner under confidential agreement and the data are promising.

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Using bryophytes as a tool to cure European foulbrood disease of honey bee: an eco-friendly approach

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European foulbrood disease is a broad disease in honey bees caused by a bacterium, *Melissococcus plutonius*. By now, various herbal and chemical drugs have been tried to control it. In the present study, the effects of different organic extracts of three different bryophytes and a standard drug (positive control) have been tried to control the bacterium *in vitro* by using agar disc diffusion and micro broth dilution method. All the tested extracts showed good antibacterial

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