



## Phytochemical Levels and Antioxidant Activity of Traditionally Processed Indian Herbal Mixture (*Acorus calamus*, *Curcuma aromatica* and *Zingiber officinale*)

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### ABSTRACT

Oxidative stress can cause disruptions in normal mechanisms of cellular signalling leading to cell damage, resulting in neurodegenerative disorders such as Alzheimer's disease. In Siddha and Ayurveda systems of medicine, formulations like *Vallarai Ney*, *Pirami Ney* and *Brahmi Gritham* are used to treat such oxidative stress-induced neurodegenerative disorders and memory loss. In these formulations, milk and ghee treated *Curcuma aromatica*, *Acorus calamus* and *Zingiber officinale* are the main ingredients. The present study was designed to investigate the scientific rationale for the use of milk and ghee treated herbs. Each of these herbs and their mixtures was treated with milk and ghee independently as well as in combination and subjected to chemical and *in vitro* antioxidant evaluations. The results obtained reveal that the total phenolic content (641.861 mg GAE/L) and free radical scavenging activity (84.88%) of milk treated sample of *Acorus calamus* were higher than other two herbs. Ghee treated sample of herbal mixture exhibited lesser malondialdehyde (MDA) content (0.04 nM/100 g) and showed higher inhibition of lipid peroxidation (99.65%) on goat brain homogenate model. LC-MS/MS study revealed the presence of Taxifolin-3-glucopyranoside, Velutin, and Methyl digallate in *A. calamus*, 3-Hydroxy-3,4-dimethoxyflavone in *Z. officinale* and Cinnamic acid in *C. aromatica*. Ghee treated herbal mixture exhibited lesser nanoparticle size and enhanced the stability when compared to individual ingredients. The study showed that milk increased the release of antioxidants in the selected herbs and ghee enhanced the nanoparticle formation and their stability.

**Keywords:** *Curcuma aromatica*, *Acorus calamus*, *Zingiber officinale*, Milk, Ghee, Antioxidant activity.

### Introduction

The human body is composed of different types of cells that together create tissues and subsequently organs. These cells are made up of various biomolecules like proteins, lipids, and carbohydrates. Cells undergo redox reactions in various metabolic pathways leading to the production of free radicals at a normal rate. A disturbance in these redox reactions causes overproduction of free radicals which in turn damage all components of cells leading to oxidation of lipids, denaturation of proteins, DNA injury, inflammation, tissue damage and subsequent cellular apoptosis.<sup>1</sup> Oxidative stress is the primary cause of many chronic diseases such as Alzheimer's disease, Parkinson's disease, atherosclerosis, cancer, diabetics, rheumatoid arthritis, post-ischemic perfusion injury, myocardial infarction, cardiovascular diseases, chronic inflammation, stroke, aging and other allied disorders. Antioxidants are the molecules which neutralize the free radicals. But at high concentration of these free radicals, the naturally available enzymatic and non-enzymatic antioxidants like glutathione, vitamin C and E, selenium, glutathione peroxidase,

superoxide dismutase and catalase present in our body are not enough to neutralize them. Cells exposed to oxygen continuously and incomplete oxidation during electron transport chain generates reactive oxygen species (ROS) radicals.<sup>2</sup> Their production can occur in two ways: enzymatic and non-enzymatic reactions. Enzymatic reactions include prostaglandin synthesis, mitochondrial respiratory chain, phagocytosis and cytochrome P450 system. Non-enzymatic generation of ROS involves reactions of oxygen with organic compounds. ROS production is also initiated by ionizing radiation. The non-enzymatic process occurs in the mitochondria during oxidative phosphorylation (i.e., aerobic respiration). Oxidative stress is essentially an imbalance between the production of free radicals and the ability of the body to counteract or detoxify their harmful effects through neutralization by antioxidants.<sup>1</sup> Oxidative stress inactivates the metabolic enzymes and damages the essential cellular components, oxidizes the nucleic acids which lead to neurological diseases such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis.

Antioxidants are the molecules that are capable of inhibiting or preventing the oxidation of biomolecules. The free radicals produced by oxidation process can start chain reactions that damage cells. Antioxidants terminate these chain reactions by removing free radicals and their intermediates, and by inhibiting other oxidation reactions. Our body naturally consists of antioxidants to control the oxidative reactions. The antioxidants in our body are of two types namely enzymatic and non-enzymatic antioxidants.<sup>3</sup> Enzymatic antioxidants are superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSHPx). Non-enzymatic antioxidants are vitamin C, vitamin E, and glutathione (GSH). These antioxidant molecules donate hydrogen to nullify the oxidative effects. But at high concentration of free radicals, the naturally available enzymatic and non-enzymatic antioxidants present in our body are not enough to neutralize the free radicals. Apart from enzymatic and non-enzymatic antioxidants, there are

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a few types of natural antioxidants available like polyphenols, flavonoids, carotenoids, etc., which can be administered through diet or drug. Recent research studies are indicating that the synthetic antioxidants used in pharmaceutical industries could have carcinogenic effects on human cells, thus fueling an intense search for new, natural and efficient antioxidants. Hence in the present research work, antioxidant herbs like *Curcuma aromatica*, *Acorus calamus*, and *Zingiber officinale* were selected and evaluated for their antioxidant potentials. According to our traditional systems of medicine such as Ayurveda and Siddha, these herbs were used after treatment with milk and ghee. Hence, the effect of milk and ghee treatment on the antioxidant activity of the selected herbs was investigated using chemical and *in vitro* assays.<sup>4</sup>

*Acorus calamus* (Local name: Vasambu) is a wetland perennial monocot plant. Leaves and rhizomes of this plant have been medicinally used to treat ailments like fever, asthma, bronchitis, and cough and mainly for gastrointestinal problems such as gas, bloating, colic, and poor digestive function. Some of the active constituents and essential oil were identified and characterized from the leaves and rhizomes.<sup>5</sup> The rhizome of *A. calamus* relieves stomach cramps, dysentery, and asthma and are also used as insecticide, tonic, and stimulant.<sup>6</sup> The compound phenylpropanoid  $\beta$ -asarone was isolated from the alcoholic extract of the rhizome by using bioassay-guided fractionation and was found to possess anthelmintic and antibacterial activities. Phongpaichit *et al.*<sup>7</sup> have evaluated and proved the antimicrobial activity of *A. calamus* on various microorganisms including bacteria, yeasts, and filamentous fungi. The compound Epieudesmin from *A. calamus* showed antineoplastic activity against the murine P388 lymphocytic leukemia cell line and several human cancer cell lines (BXP-3, MCF-7, SF268, NCI-H460, KM20L2, and DU-145).<sup>8</sup> George *et al.*<sup>9</sup> showed that the compound Galgravin present in *A. calamus* can prevent the neuronal death and to stimulate the neurite growth and also showed neuroprotective activity using *in vitro* models for Alzheimer's and Parkinson's disease.

Ginger is consumed as a delicacy, medicine, or herb. The rhizome of the plant *Zingiber officinale* is used for the treatment of flatulence, colic, diarrhoea, spasm, cold and influenza; as an appetite stimulant, a narcotic antagonist, and an anti-inflammatory agent. A recent study showed that it has antioxidant, anticancer, anti-inflammatory, anti-apoptotic, anti-hyperglycemic, anti-hyperlipidemic and anti-emetic actions. Main constituents are sesquiterpenoids with (-)-zingiberene. Sesquiterpene lactones are natural products responsible for its anti-inflammatory activity.<sup>10</sup> The antiplatelet effect of 6-gingerol was also mainly due to the inhibition of thromboxane formation.<sup>11</sup> Shen *et al.*<sup>12</sup> indicated that ginger constituent 6-Shogaol inhibit arachidonic acid metabolism and thus prostaglandin synthesis.

The rhizome of wild turmeric (*Curcuma aromatica* Salisb.) is extensively used as an aromatic medicinal cosmetic in India.<sup>13</sup> In Ayurvedic literature, it is mentioned as a remedy for various diseases related to skin, cardiovascular and respiratory system. For the last few decades, research works have been done to establish the pharmacological potential of *C. aromatica* and its extracts. Previous studies have revealed the medicinal properties of this plant such as anti-inflammatory, wound healing, anti-melanogenic, antioxidant, free radical scavenging activity, anti-tumour, anti-cancer, anti-repellent, anti-tussive, antiplatelet and anti-nephrotoxic activities.<sup>14</sup> According to Jantan *et al.*,<sup>15</sup> curcumin from *C. aromatica* was the most effective antiplatelet compound as it inhibited platelet aggregation with IC<sub>50</sub> value of 37.5  $\mu$ g/mL.

Even though the selected herbs were proven to be useful in the management of many diseases including arthritis, cancer, cardiovascular diseases and neurodegenerative disorders, the scientific basis behind the use of milk and ghee treatment practised in Siddha and Ayurveda systems is not yet studied. This study is intended to establish the scientific rationale for the use of milk and ghee treatments of the selected herbs.

## Materials and Methods

### Sample preparation

Dried samples (Rhizomes of *Acorus calamus*, *Curcuma aromatica*, and *Zingiber officinale*) (Figure 1) were purchased from the local herbal market in Thanjavur. The materials were coarsely powdered using a hammer and made into fine powder using a domestic grinder.

### Treatment with milk

Powdered samples of each herb and their mixture (5 g) were taken, and 50 mL of raw milk was added and kept in a shaker at 200 rpm for 30 min.

After shaking, the samples were centrifuged at 2000 rpm for 10 min, and both the supernatant and residue were obtained. To the residue, 50 mL of distilled water was added and kept at room temperature for 2 h. Then, the contents were centrifuged at 2000 rpm for 10 min and the supernatant obtained was analyzed further.

### Treatment with ghee

Powdered samples of each herb and their mixture (5 g) were taken, and 50 g of ghee was added and heated at 60°C for 30 min. Then it was centrifuged at 2000 rpm for 10 min, and both supernatant and residue were obtained. To the residue, 50 mL of hexane was added, mixed well and centrifuged at 2000 rpm for 10 min. The supernatant was discarded, and to the residue, 50 mL of water was added and kept at room temperature for 2 h. Then it was centrifuged at 2000 rpm for 10 min, and the supernatant was collected and used for the analysis.

### Combination of treatments

Powdered samples of each herb and their mixture (5 g) were taken, and 50 mL of raw milk was added and kept in a shaker at 200 rpm for 30 min. After shaking, the sample was centrifuged at 2000 rpm for 10 min, and both the supernatant and residue was obtained. To the residue, 50 g of ghee was added and heated at 60°C for 30 min. Then it was centrifuged at 2000 rpm for 10 min. The supernatant and residue were obtained. To this residue, 50 mL of hexane was added, mixed well and centrifuged at 2000 rpm for 10 min. The supernatant was discarded, and to the residue, 50 mL of water was added and kept at room temperature for 2 h. Then it was centrifuged at 2000 rpm for 10 min, and the supernatant was obtained and analyzed further.

### Aqueous extract

Powdered samples of each herb and their mixture (5 g) were taken in 50 mL of water and soaked for 2 h. Then the contents were centrifuged at 2000 rpm for 10 min, and the supernatant was collected and used for further analysis.

### Determination of total phenolic content

The Folin-Ciocalteu reagent was used to quantify the total phenolic level in the prepared extracts.<sup>16</sup> Sample (100  $\mu$ L) was taken, and 250  $\mu$ L of Folin-Ciocalteu reagent was added. Then 1 mL of 5% Sodium carbonate was added slowly. A reagent blank with distilled water was prepared. This reaction mixture was kept in the dark at room temperature for 30 min. Then the absorbance against the reagent blank was determined at 750 nm with a UV-Visible spectrophotometer. Total phenolic content was calculated and expressed as mg Gallic Acid Equivalents (GAE)/L.

### DPPH radical scavenging activity

The antioxidant effect was evaluated using the DPPH radical scavenging assay.<sup>17</sup> To 100  $\mu$ L of each sample, 900  $\mu$ L of DPPH solution (5 mg in 100 mL of Methanol) was added. In control, 100  $\mu$ L of respective solvent was added instead of the sample. After incubation in the dark for 30 min at room temperature, the absorbance was measured at 520 nm with a UV-Visible spectrophotometer. The radical scavenging activity was calculated, and the results were expressed in percentages.

### Lipid peroxidation inhibition capacity

Goat brain tissue was collected freshly from the local slaughterhouse in Trichy and homogenized by taking 0.5 g of tissue and 5 mL of PBS. Then 0.3 mL of this homogenate and 0.3 mL of the samples were taken and freshly prepared 3% H<sub>2</sub>O<sub>2</sub> was added. This mixture was incubated at room temperature for 10 min. Then 1.5 mL of TBA reagent (0.375 g was dissolved in 10% TCA) was added and heated in a water bath at 80°C for 15 min. The contents were centrifuged at 2000 rpm for 10 min, and the supernatant was collected, and its absorbance was measured at 540 nm using a UV-Visible spectrophotometer.<sup>18</sup> Standard and control samples were prepared by using BHA and PBS reagents, respectively. Blank was prepared without H<sub>2</sub>O<sub>2</sub>. Based on absorbance, the MDA content of brain homogenate was calculated and expressed in nM/g tissue.

### Zeta size and potential analysis

Ghee treated sample (0.5 g) was mixed with 1 mL of Tween-20 and 9 mL of distilled water. The mixture was vortexed for 1 min and homogenized at 2500 rpm for 30 min. Homogenized sample (1 mL) was kept in an ultrasonic bath for 1 h.<sup>19</sup> From the ultra-sonicated sample, 20  $\mu$ L was taken in an Eppendorf tube, and 1980  $\mu$ L of distilled water was added and analyzed for zeta size and potential using ZETASIZER NANO-ZS 366 nm

*Acorus calamus* rhizome*Zingiber officinale* rhizome*Curcuma aromatica* rhizome**Figure 1:** Raw material of selected herbs.

He-Ne LASER-MAL511755 (Model: ZEN3600, Make: Malvern Instruments).

#### LC-MS/MS analysis

The powdered rhizome of each sample (5 g) was mixed with 50 mL of water and raw milk (1:10, w/v) and kept in a shaker at 2000 rpm for 30 min. Then it was filtered and lyophilized, and 10 mg of the lyophilized sample was dissolved in 10 mL of distilled water and filtered using SPE cartridges to eliminate impurities. From the filtered sample, 1 mL was taken and mixed with 1 mL of acetonitrile to precipitate the proteins.<sup>20</sup> Then it was centrifuged at 4000 rpm at 4°C for 10 min, and the supernatant was collected and filtered through a membrane filter and analyzed in LC-MS/MS (BRUKER DALTONICS Private Limited, Model: UHPLC, microTOF-QIL.) for detecting major chemical constituents.

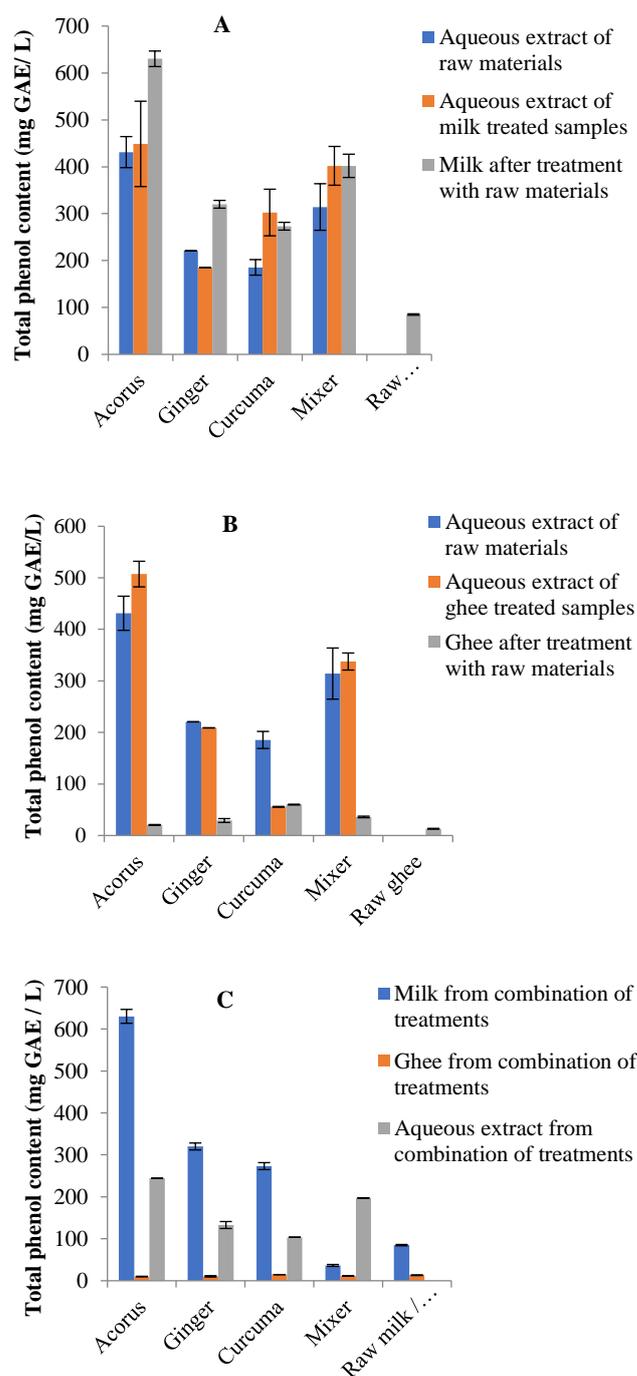
## Results and Discussion

### Total phenolic content

In this experiment, the Folin-Ciocalteu reagent (a phosphomolybdic and phosphotungstic acid complex) reacts with electrons released from phenolic compounds present in the sample to give a blue coloured compound which is measured colourimetrically at 760 nm. Total phenolic content of the investigated herbs indicated that the supernatant of *A. calamus* (630.16 mgGAE/L), exhibited a higher level of total phenolic content followed by its residue (448.78 mgGAE/L) and its aqueous extracts (431.22 mgGAE/L) (Figure 2). In the case of ghee treated herbs, the supernatant of *A. calamus* (20.59 mgGAE/L), *Z. officinale* (29.10 mgGAE/L), *C. aromatica* (59.95 mgGAE/L) and herbal mixture (36.01 mgGAE/L) exhibited lesser total phenolic content when compared with their respective residues. Similarly, the total phenolic content of supernatant of milk treated *A. calamus* (630.16 mgGAE/L), *Z. officinale* (320.05 mgGAE/L), *C. aromatica* (273.24 mgGAE/L) and herbal mixture (401.97 mgGAE/L) have higher values when compared with both supernatant and residue. Among these three treatments, milk treated samples of *A. calamus* exhibited remarkable total phenolic content. Milk treatment appears to be effective in releasing total phenols in all the selected herbals when compared to aqueous extraction and ghee treatment. The selected herbals are administered as whole drugs so that both supernatant and residue fractions were ingested together with milk so that the total phenolic levels would be available at higher content.

### Antioxidant activity

This assay is based on the theory that a hydrogen donor is an antioxidant. DPPH is one of the few stable and commercially available organic nitrogen radicals. The antioxidant effect is proportional to the disappearance of DPPH in test samples. DPPH shows a high absorption maximum at 520 nm (purple). The colour turns from purple to yellow followed by the formation of stable molecule DPPH-H upon absorption of hydrogen from an antioxidant. This reaction is stoichiometric in relation to the number of hydrogen atoms absorbed. Antioxidant activity of milk treated samples were found to be 88.37% in *A. calamus*, 84.88% in *C. aromatica*, 90.12% in *Z. officinale* and 89.53% in the herbal mixture (Figure 3). All the ghee treated samples revealed lesser antioxidant effect when compared to the residue (11.27% in *A. calamus*, 9.86% in *C.*

**Figure 2:** Total phenolic contents of milk treated (A), ghee treated (B) and milk + ghee treated (C) samples.

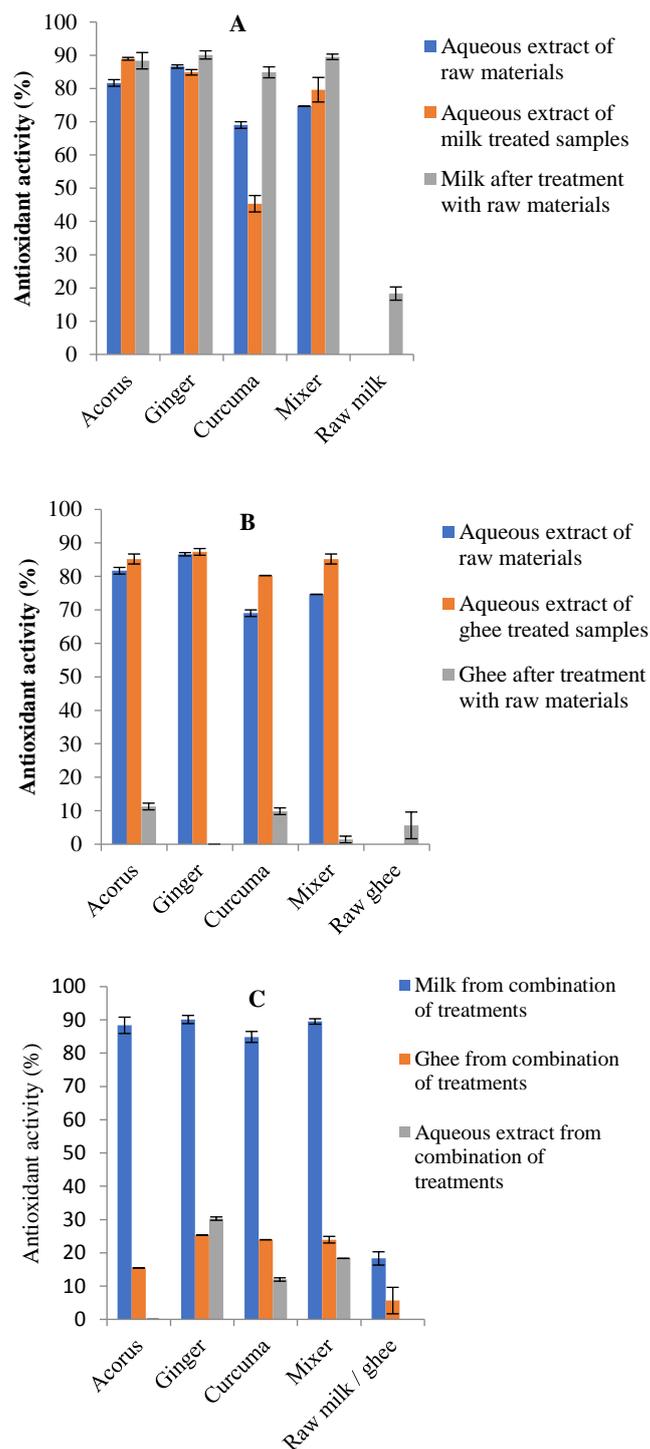
*aromatica*, 0% in *Z. officinale* and 1.41% in the herbal mixture). But, when the herbals were treated with both milk and ghee, samples exhibited higher antioxidant activity (88.37% of *A. calamus*, 45.35% of *C. aromatica*, 90.12% of *Z. officinale*). Between milk and ghee treatment, treatment with milk exhibited the highest antioxidant potential among the investigated herbals and their mixture. The low antioxidant activity of ghee treated samples might be due to the difference in polarity of the phytochemicals.

#### Lipid peroxidation inhibition capacity

Lipid peroxidation is the oxidative degradation of lipids induced by free radicals. It is the process in which free radicals "steal" electrons from the lipids in cell membranes, resulting in cell damage. This process proceeds by a free radical chain reaction mechanism. The ultimate end product of lipid peroxidation is malondialdehyde (MDA). In this assay, the inhibition capacity of a sample is indirectly measured from MDA concentration. The inhibition capacity of the sample is inversely proportional to MDA concentration present in the final reaction mixture. Analysis of lipid peroxidation in goat brain homogenate model using TBARS assay revealed the existence of low levels of MDA (An oxidative stress biomarker) in supernatant of milk treated *Z. officinale* (0.04 nM/g tissue, 99.65% of lipid peroxidation inhibition) followed by residue of milk treated sample of *C. aromatica* (0.44 nM/g tissue, 96.15% of lipid peroxidation inhibition) (Figure 4). In ghee treated *Z. officinale* (-0.76 nM/g tissue, 106.64% of lipid peroxidation inhibition) and herbal mixture (-0.96 nM/g tissue, 108.39% of lipid peroxidation inhibition), very little or no level of MDA was noticed, which indicated the effectiveness of these samples in preventing oxidative stress. In combination with milk and ghee treatments, both supernatant and residue of *Z. officinale* (1.44 nM/g tissue, 87.41% lipid peroxidation inhibition and -0.56 nM/g tissue, 104.93% lipid peroxidation inhibition, respectively) showed remarkable reduction in the level of MDA when compared to other samples. Among the investigated herbals, aqueous extract of *Z. officinale* and herbal mixture exhibited maximum antioxidant activity. In the *in vitro* DPPH radical scavenging assay, milk treated herbals exhibited better antioxidant activity than the biological assay (Goat brain homogenate), in which ghee treated samples revealed promising antioxidant potential. Among the different treatments employed, the combination of milk and ghee showed maximum inhibition of lipid peroxidation, which forms the scientific basis for the implementation of these processing steps in the traditional system of Indian medicine. Besides, the study also suggested that ghee can facilitate the drug entry through the cell membrane, as maximum inhibition of lipid peroxidation was noticed in ghee treated samples.

#### LC-MS/MS Analysis

LC-MS/MS is an analytical chemistry technique widely used to identify the phytochemicals present in biological samples. LC-MS/MS couples the physical separation capabilities of liquid chromatography (LC) with mass analysis capabilities of mass spectrometry (MS). The individual components separated from the mixture by liquid chromatography is analyzed by the mass spectrometry to provide structural identity with high molecular specificity and detection sensitivity. Electrospray ionization (ESI) is an interface, which plays a vital role in transferring the separated components from LC to MS ion source. LC-MS/MS study revealed the presence of Taxifolin-3-glucopyranoside, Velutin, and Methyl digallate in *A. calamus*, 3-Hydroxy-3,4-dimethoxyflavone in *Z. officinale* and Cinnamic acid in *C. aromatica* (Figure 5). The observed antioxidant activity of the investigated plants might be attributed to these phytochemicals because phenolic hydroxyl groups present in these compounds are suitable H-donating antioxidants. Studies carried out by Meng *et al.*<sup>21</sup> revealed that flavones and phenolic contents extraction are low in milk. Hence, the water extract was subjected to LC-MS/MS analysis. Phytosome technology used in this process employs a hydrophilic environment (milk, in the present study) which enhances the therapeutic efficacy of the molecules present in water extract. The phytosome technology protects the valuable components of the herbal extract from destruction by digestive secretions and gut bacteria.<sup>22</sup> Phytosomes also transfer the phytochemicals from a hydrophilic environment into the lipophilic environment of the enterocyte cell membrane and into the cells and finally reaching the blood. Different phytoconstituents such as flavonoids, terpenes and saponins can form a reversible complex with phospholipids. Traditional medical practitioners have used this technology knowingly or unknowingly for enhancing the bioactivities of herbal formulations, such as antioxidant activity. Results obtained in the *in vitro* antioxidant assays also supported this assumption due to the higher activity observed in the milk treated herbal extracts. But,

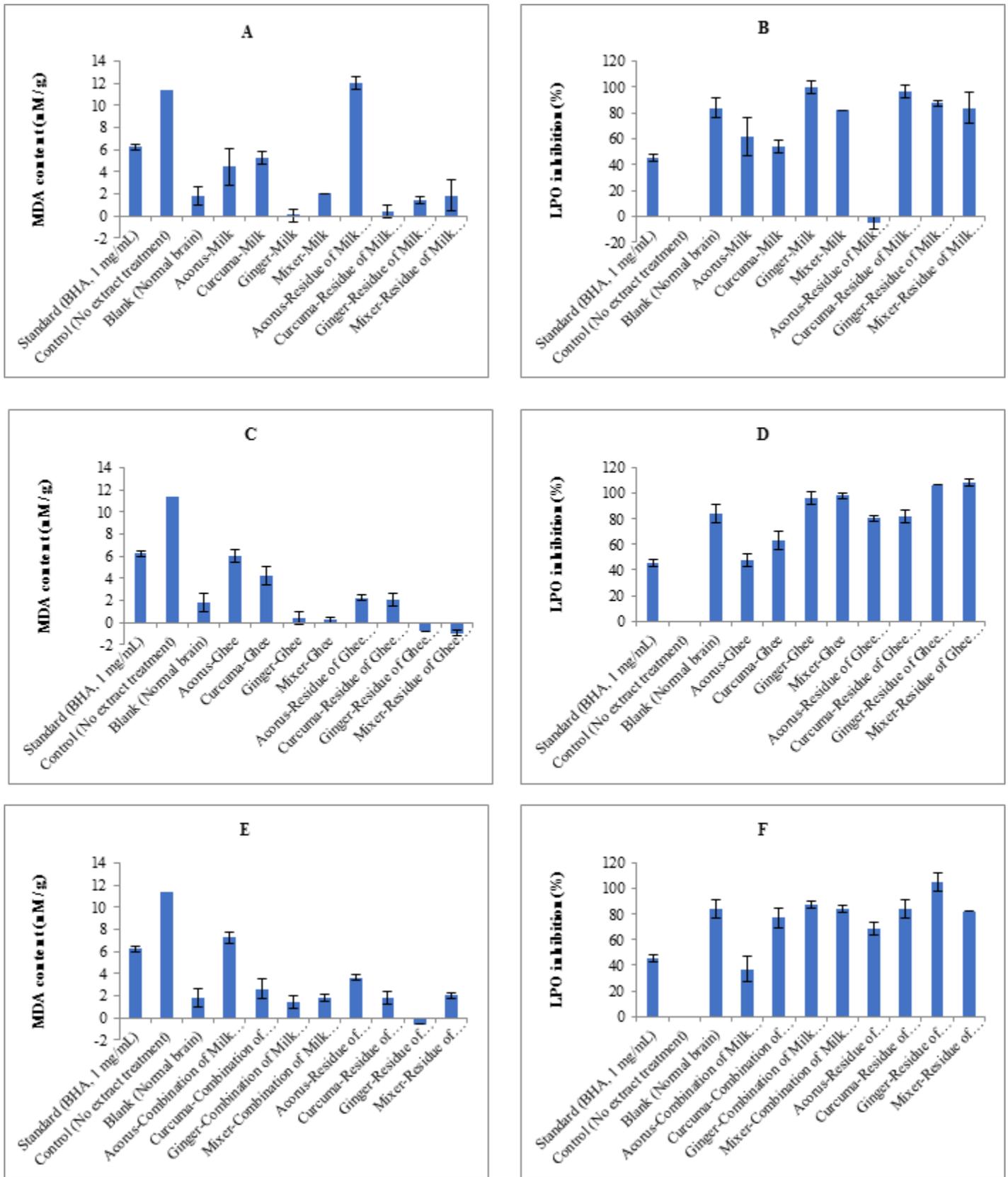


**Figure 3:** Antioxidant activity of milk treated (A), ghee treated (B) and milk + ghee treated (C) samples.

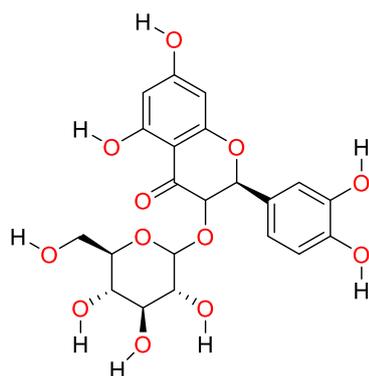
ghee treated samples exhibited maximal inhibition of lipid peroxidation in goat brain homogenate model.

#### Zetasizer Analysis

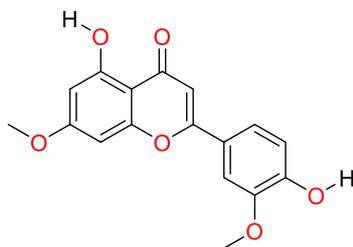
Zeta size analyzer is used to determine the size and potential of micro/nanoparticles. The principle of this instrument is dynamic light scattering with NIBS (non-invasive backscatter optics). Besides potential and size, zeta analyzer can also be used to determine translational diffusion, the mobility of particles, viscosity and viscoelastic properties of protein and polymers solutions. The stability of colloidal dispersions can be analyzed using the zeta potential. The magnitude of zeta potential is directly proportional to the electrostatic repulsion between the similarly charged particles in the distribution. At low potentials, the attraction between the molecules increases causing flocculation of dispersion.



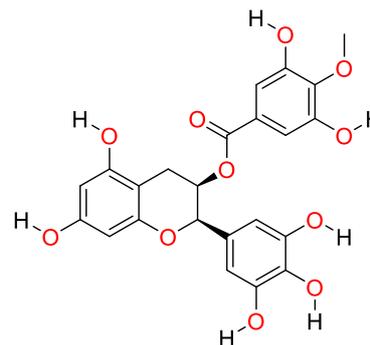
**Figure 4:** Malondialdehyde (MDA) level of goat brain homogenate and lipid peroxidation inhibition capacity of milk treated (A & B), ghee treated (C & D) and milk + ghee treated (E & F) samples.

*Acorus calamus*

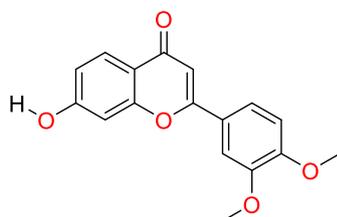
Taxifolin-3-glucopyranoside



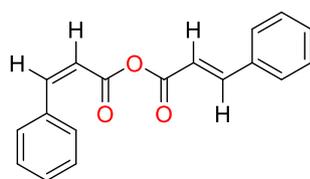
Velutin



Methyl digallate

*Zingiber officinale*

3-Hydroxy-3',4'-Dimethoxyflavone

*Curcuma aromatica*

Cinnamic acid

**Figure 5:** Chemical structures of compounds identified from the LC-MS/MS analysis of aqueous extract of milk treated herbals.

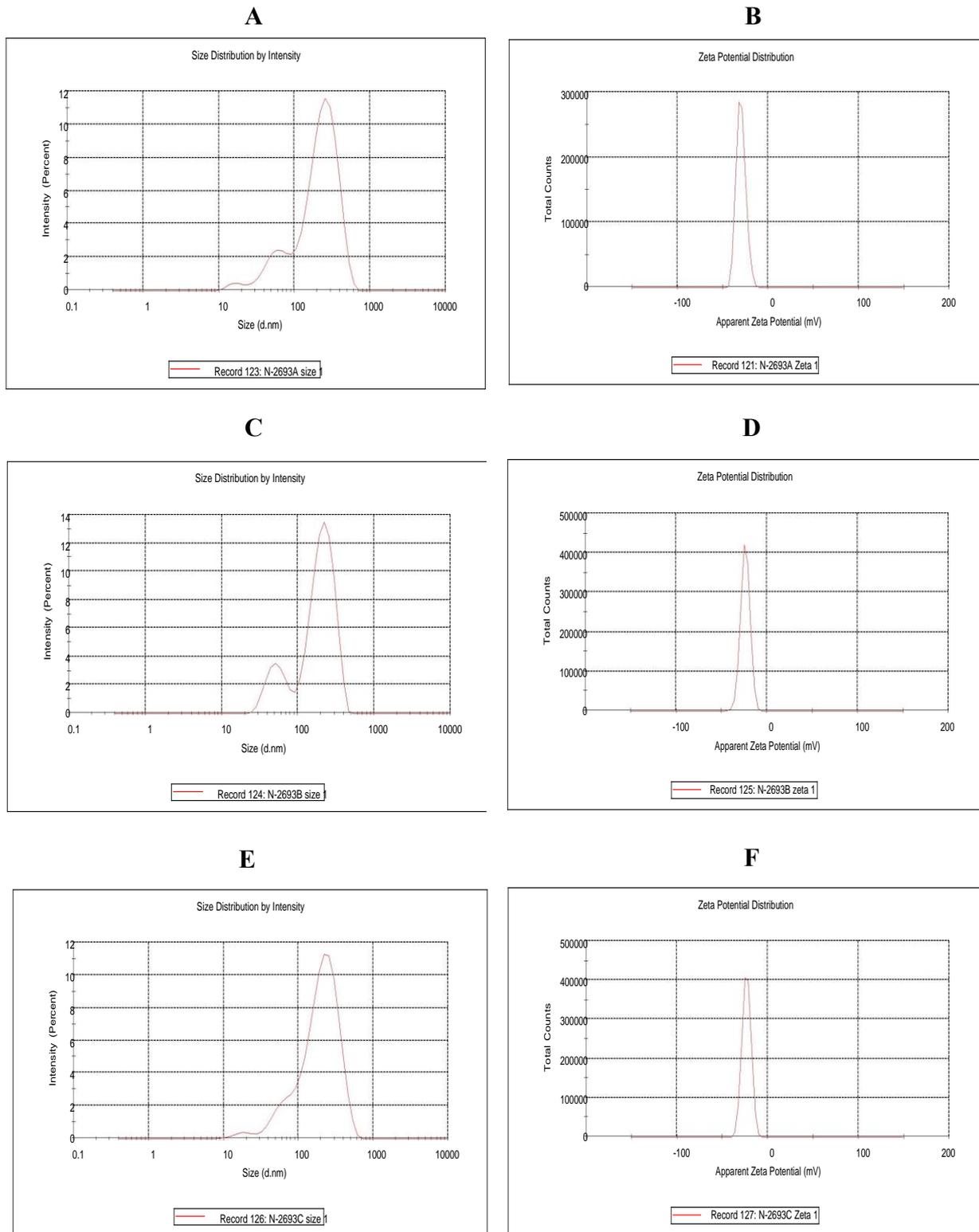
Therefore, colloids with high potential (negative or positive) and small size are more electrically stabilized than the colloids with low potential and larger sized molecules. Zetasizer analysis is used to determine the presence of particles in the nanometer range and the herbal medicine's stability in drug delivery. In the present study, it was observed that the ghee treated extracts of the selected herbs individually as well as in combination has a particle size range from 167 to 268 nm and zeta potential ranging from -23.4 to 36.1 mv (Figure 6). The results suggested that ghee in the formulations enhances efficient delivery of the phytoconstituents of these herbals due to the formation of nanoparticles. Zeta potential results also further suggest good stability of the herbal extracts and mainly the mixture (-36.1 mv) often used in traditional formulations. The data of the zeta analysis further depicted that the treatment of the therapeutically essential herbs with ghee is to facilitate maximum drug absorption and drug delivery because ghee treatment has proven to convert herbal extracts into particles of the nano size.

**Conclusion**

The study showed that milk treated herbal samples showed a high level of phenolic compounds and antioxidant activity when compared to ghee treated samples and aqueous extracts of the respective herbal sample. But in TBARS assay using goat brain homogenate model, ghee treated samples were more effective in inhibiting lipid peroxidation when compared to milk treated and aqueous extracts. The reason for this observation between the chemical *in vitro* and the biological assay may be due to the difference in polarity of the samples with the reaction mixture. Hence, we can conclude that milk treatment enhances the antioxidant property and ghee treatment stabilizes the Indian traditional herbal drug.

**Conflict of interest**

The authors declare no conflict of interest.



**Figure 6:** Zeta-size and zeta-potential of ghee treated *Acorus calamus* (A & B), *Curcuma aromatica* (C & D) and *Zingiber officinale* (E & F).

#### Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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