(Article)

Green nanosuspensions of *Elettaria cardamomum* seeds – Comparative data analysis regarding biological significance of nanosuspensions and ethanolic extract

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Abstract: To establish the efficiency of a plant for a specific ailment, its phytochemical profile is evaluated using different scientific procedures. The objective of the present study was to evaluate the bioactive compounds and biological activities of *Elettaria cardamomum* seed ethanolic extract and nanosuspension. Nanosuspensions were prepared using the nano-precipitation method. The antioxidant potential of seed extracts and nano-suspensions was evaluated by determining the total flavonoid content (TFC), total phenolic content (TPC) and DPPH (2, 2-diphenyl-1-picrylhydrazyl) scavenging activity. The TPC contents found were 358.89 and 126.27 mg GAE/100g (dry weight) in nanosuspensions and extract respectively, while TFC contents; 137.78 and 65.145 mg CE/ 100g (dry weight) were found in nanosuspensions and extract respectively. However, the nanosuspensions and extract showed DPPH radical inhibition as 31.3% and 32.1% respectively. Cytotoxicity and antibiofilm activity was determined using the hemolytic and microtiter dish assays. Hemolytic assay gave 18% and 32% hemolysis using nanosuspension and extract and showed growth resistance against *E. coli* (68.12%) and (64.09%) respectively. The antidiabetic activity was evaluated through alpha-amylase inhibition and antiglycation assays. The cardamom seeds nanosuspension and extract showed significant ($p<0.05$) antidiabetic activities through glycation inhibition (55.77% and 55.15%, respectively) and alpha-amylase inhibition (51.67% and 33.67%, respectively) assays. FTIR was performed for the presence of specific functional groups in nanosuspension which indicated the presence of sulfoxides, fluoro groups, amines, phenols, alkaloids, and alcohols. The study findings suggested that the nanosuspensions and ethanolic seed extract of *Elettaria cardamomum* have exhibited significant biological activities and have the potential for pharmacological uses in the future after further research.

Keywords: Elettaria cardamomum; diabetes mellitus; antioxidant activity;alpha-amylasee.

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1. Introduction

Nanotechnology has been utilized by researchers in the designing of the new drug delivery methods or nanocarriers for appropriate drug delivery to the exact site where needed. This

whole system is worked on a principle that delivers a specified medicine to the specific diseased site for a longer period. This assists in preventing damage to the normal tissues and maintaining the medicine levels in tissue and plasma [1]. In the drug delivery method, when the drug is not soluble in inorganic media and water then nanosuspension is the best option as a fabrication approach. It is useful for compounds having a high dosage and extreme melting points. Nanosuspensions can be employed to increase the solubility of less soluble drugs in lipid and aqueous media. Consequently, the flow of active compounds rises and the maximum level of plasma is achieved rapidly (e.g., intravenous and oral delivery of nanosuspension). It is particularly suitable for various molecules with less permeability, less solubility, or both [2]. Due to local availability, safety, less harmful effects and eco-friendly properties, plants are given more importance according to existing situations. Plants are thought to have healing power in nearly all old civilizations [3,4].

Elettaria cardamomum, commonly known as green or true cardamom, is a medicinal plant that belongs to the *Zingiberacea*e family. *Elettaria cardamomum* is found in various countries like India, Costa Rica, Mexico, Tanzania, Sri Lanka, Indonesia, Guatemala and Nepal. It is also known as ''Hel'' in many gulf countries like Saudi Arabia, Iran, Kuwait, Iraq, United Arab Emirates and other neighborhoods. The name *Elettaria cardamomum* is primarily a botanical name and is aroused from the Tamil word ''*Elettaria*'' which indicates cardamom seed. Among other well-known spices, *Elettaria cardamomum* is generally referred to as the ''Queen of spices'' due to its extensive use in Eastern, Scandinavian, Arab and Western foods for particular aromas [5]. Dried cardamom fruit is composed of cellulose, pigments, steam volatile oil, starch, minerals, fatty oil, pentosans and sugars. The seed comprises 50% of starch while 30% comprises crude fibers. These components differ among all the varieties and depend upon the environmental conditions [6]. *Elettaria cardamomum* has been an excessively utilized herb for its clinical and aromatic properties in treating dry lips, impotence, bronchitis, blood pressure, arrhythmia, gum bleeding, diarrhea, vomiting, toothache, cognitive disorders and more. Cardamom showed anti-cancer and chemopreventive activities, which have been indicated to decrease the mass and diameter of tumors. It was utilized in Egypt due to its clinical characteristics and also in the cure of breast cancer [7,8]. Other important bioactive constituents of cardamom include; cineole, limonene and caffeic acid, which cause the downregulation of various signal-transducing molecules and inhibit the activities of cytochrome P450 and cyclooxygenase-2 [9]. The seed powder is commonly used in the treatment of gastrointestinal disorders and as a digestive, stomachic, breath freshener, anti-emetic and carminative agent [10]. Jahan et al., [11] prepared the nanosuspensions *Elettaria cardamomum* through nanoprecipitation technique. Nanosuspensions of *Elettaria cardamomum* have significantly increased the antiradical effect in comparison to the crude extract. The method addressed sufficiently the problems associated with the formulation and poor solubility. Due to the smaller particle size, nanosuspensions of the plant were useful to scavenge free radicals unlike that of the unrefined extract [11]. Although various parts of *E. cardamomum* have been widely reported for numerous biological activities, however, there is no comparative analysis available until now describing the effectiveness of seed extract and nanosuspension.

2. Materials and Methods

Elettaria cardamomum pods were obtained randomly from the retail market of Faisalabad. The seeds were separated from pods, examined and authenticated at the Department of Botany, University of Agriculture, and Faisalabad, Pakistan. The seeds of *Elettaria cardamomum* seeds were shade dried followed by a tray drier and ground to a powder and stored in an air-tight enclosed vessel or jar. The powdered seeds were soaked with petroleum ether to eliminate the fatty substances and extracted with 95% ethanol using the Soxhlet apparatus, centrifuged and the extract obtained through filtration was kept in a desiccator for further use. Nanosuspensions were prepared using the nanoprecipitation technique. Briefly, 1.5 g of the extract was dissolved in 11.25 mL of acetone and ethanol (3:1). The resulting solution was slowly injected into water (15 mL) which contains polyvinyl alcohol (1.5% w/v) with continuous magnetic stirring at 1000 rpm. The resulting emulsion obtained was then diluted in PVA (30 mL) solution (0.2% w/v in water) in order to minimize coalescence and the mixture was continuously stirred (500 rpm) for 3h at room temperature to allow solvent evaporation and nanosuspension formation. The resulting nanosuspension was freeze-dried at -18°C [12]. Total phenolic content was analyzed as explained by Hussain et al. [13]. Briefly, nanosuspensions and seeds extract (20 µL) were combined with 10% Folin-Ciocalteu (100 µL) reagent and then 3 mL (1%w/v) sodium carbonate was added to this mixture. The mixture was incubated for 2 hours at room temperature. Absorbance was taken at 765 nm. The blue color indicated the presence of phenolic components. Results were evaluated as milligram gallic acid equivalent (GAE) / 100 gram. Aluminium chloride colorimetric assay was used for the determination of phenolic content [13]. For this purpose, 1.25 mL distilled water, 160 µL sodium carbonate and the test sample (50 µL) including nanosuspensions and extract were added to 96 well plates and incubated for 10 minutes. Then, 1mL NaOH, 300 µL (10%) $AlCl_3$ were added and incubated for 5 minutes. Absorbance was taken at 510 nm. Yellow color complex formation indicated the presence of phenolic content. The TFC contents were measured from the standard curve. For radical scavenging analysis [13], about 1 µL sample (nanosuspension, extract) was mixed with 1 mL DPPH (2, 2-diphenyl-1-picrylhydrazyl). DPPH solution was prepared by dissolving 0.004 milligrams DPPH in 15 milliliters methanol. This was then wrapped by aluminum foil for 35 minutes. Absorbance was taken at 520 nm. Colorless complex formation indicated the radical scavenging activity. Percent radical capturing activity was checked by $[A_{(control)} - A_{(sample)} / A_{(control)}] \times 100$. Bovine serum albumin (10 mg) was mixed with 150 µL sample consisting of nanosuspension and extract, 100 µL D-glucose (prepared in phosphate buffer 7.4 pH). The mixture was incubated for 2 days at room temperature. The absorbance was observed at excitation wavelength i.e., 370 nanometers and at emission wavelength i.e. 440 nanometer using spectrophotometer (BMS UV-2600, Japan). The reaction mixture without D-glucose was considered blank. The reference compound utilized was metformin [13]. Percent inhibition was calculated by using this formula: $[A_{(440\ nm)} - A_{(370\ nm)} / A_{(440\ nm)}] \times 100$.

The alpha-amylase inhibitory property of nanosuspensions and extract was analyzed according to the protocol described by Unuofin et al. [14]. Samples and standard acarbose

(100 µL each) were maintained at room temperature for 10 minutes, followed by the addition of 100 µL amylase solution in 0.02 M sodium phosphate buffer. Following pre-incubation, the starch solution was added and incubated for 30 minutes. Then HCL was added, along with it iodine solution was also added in each well and the absorbance was measured at 630 nm against a blank. Percent inhibition was measured by 1-A (control) / A (sample) × 100. To perform the hemolytic analysis, 5 mL blood was taken and centrifuged at 8000 rpm for 5 minutes. The supernatant was discarded and washed the pallet. Washing was done thrice by adding 5mL phosphate buffer saline (PBS) and after last washing 900 mL PBS was added in the remaining debris for dilution purposes. Diluted blood sample (180 µL), Triton X-100 (50 µL), 20 µL PBS, 20 µL nanosuspension and extract were mixed in tubes and incubated for 40 minutes at 37°C. After 15 minutes mixture was centrifuged for 6 minutes. After washing with PBS chilled content was placed in plates and absorbance was taken at 570 nm. In positive and negative control Triton X-100 and PBS were added respectively. Percentage of hemolytic inhibition was obtained by using $A_{(sample)} - A_{(negative\ control)} / A_{(positive\ control)} - A_{(negative\ control)} \times 100$ [13]. To perform biofilm inhibition analysis, the 96-well plate was used containing the 20 µL nutrient broth, 20 µL sample, and 100 µL each of *Escherichia coli and Staphylococcus aureus* bacteria incubated aerobically at 37 °C overnight. Plates were rinsed three times with sterile phosphate buffer saline and shaken the next day to eliminate adhering bacteria. After air drying the plates, crystal violet stain was applied and to solubilize the stain glacial acetic acid was utilized. The absorbance was measured at 630 nm. Ciprofloxacin with nutrient broth was taken as positive control while nutrient broth with bacterial strains was used as negative control [13].

Percent inhibitions were calculated by 100 × [A (control) − A (sample) / A (control)]. For structural characterization by FTIR, the 50 mL whole sample was bireduced through chloroauric solution. Then it was confronted to centrifugation at 10000 rpm for 15 minutes. Pallet was washed three times using 20 mL of deionized water to rinse any isolated undesired enzymes/proteins may be adhered to the particles. The 30 µL materials were then dried and crushed in a potassium bromide pellet mill. Agilent Cary 630 (fig.3.16) was then used in diffuse reflect-array mode with a resolution of 4 cm. A total of 512 scans were performed in order to obtain the requisite acceptable signal/noise ratio [15]. Every measurement was the average of three repeats and an expert data scientist evaluated data using ANOVA (Microsoft Excel 365) to compare means of more than two population means [16].

3. Results

3.1 Antioxidant content and activity

The results of the antioxidant potential of nanosuspensions and extract are presented in table 1. The nanosuspensions of *Elettaria cardamomum* showed higher phenolic content in comparison to that of seed extract. *E. cardamomum* nanosuspensions showed more flavonoid contents as compared to extract. From the findings, it is shown that the nanosuspension and the extract have comparatively less DPPH radical scavenging ability than the standard.

3.2 Antidiabetic activity

The results of glycation and alpha-amylase inhibitory properties are shown in table 1. *E. cardamomum* nanosuspension showed slightly higher antiglycation activity than the seed extract. *E. cardamomum* nanosuspension showed higher alpha amylase inhibitory activity as compared to the inhibitory activity of seed extract.

3.3 Hemolytic analysis

By performing hemolytic analysis less hemolysis was achieved with *E. cardamomum* nanosuspension and higher hemolysis was achieved by using seeds extract (Table 1).

3.4 Biofilm inhibitory analysis

Biofilm formation by pathogenic bacteria is a major problem in the food processing industry. To evaluate the antibiofilm effect of *E. cardamomum* seeds extract and nanosuspensions, *Escherichia coli* and *Staphylococcus aureus* were used (Figure 1 a-d). Maximum reduction of growth was exhibited by nanosuspensions against *E. coli* and no inhibition was found against *Staphylococcus aureus*. Nanosuspension of *E. cardamomum* was better than seed extract in preventing biofilm formation against *E. coli* (Table 1).

Table 1. Different activities of *Elettaria cardamomum* seed extract and nanosuspension

Activities	**Samples**		
	Control	**Extract**	**Nanosuspension**
Antioxidant profile			
TFC (g CE/100 g)	244.44 ± 1.03	65.15 ± 2.23	137.78 ± 1.39*
TPC (g GAE/100g)	750.87 ±3.56	126.27 ± 4.38	358.89 ± 5.00*
DPPH (%)	89.56 ± 0.00	32.13 ± 2.68	31.3 ± 1.88
Antidiabetic profile (% inhibition)			
Glycation (%)	56.91± 5.48	55.15 ± 3.95	55.77± 2.76
Alpha-amylase (%)	82.53 ± 6.37	33.67 ± 0.48	51.67 ± 4.83
Cytotoxic (% inhibition)			
Hemolytic activity %)	96.45± 0.52	32.12 ± 0.3	18.01 ± 0.1

Data are represented as mean ± SE or percentage (n=3). * Significant at $p < 0.05$. Positive controls for; DPPH assay: BHT (Butylated hydroxytoluene), antiglycation assay: metformin, alpha-amylase inhibitory assay: acarbose, hemolysis assay: Triton X-100.

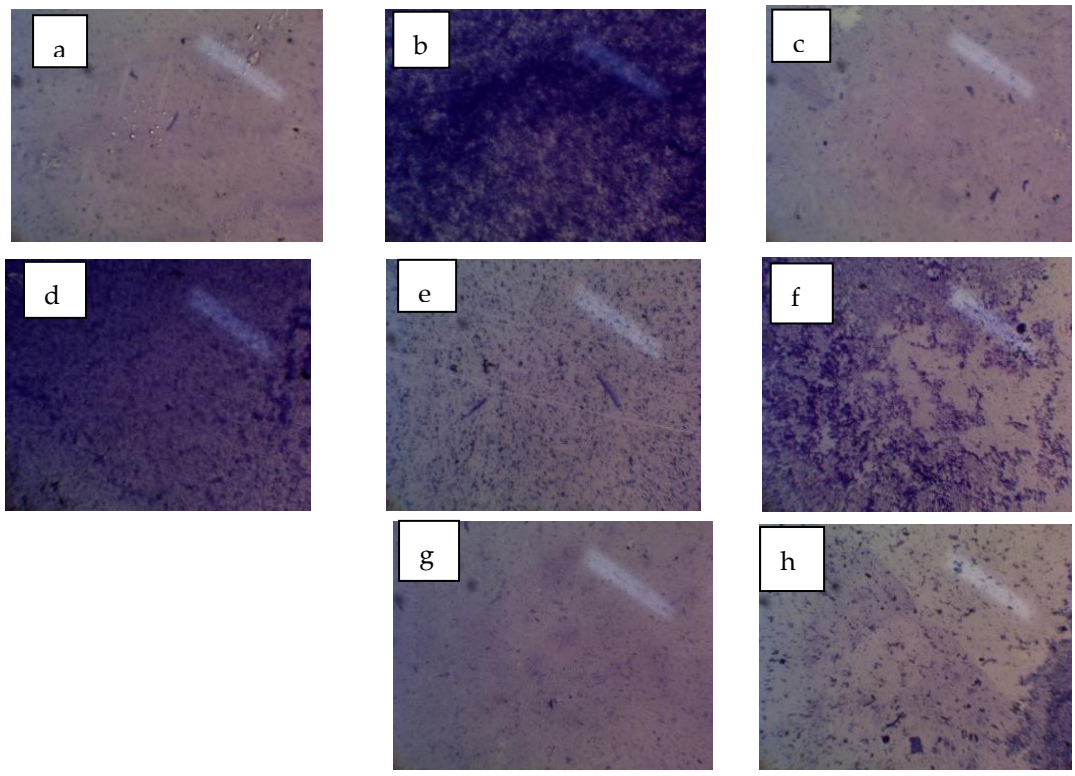

Figure 1. Biofilm inhibition assay

a) *E. coli* positive control, b) *E. coli* negative control, c) *S. aureus* positive control, d) *S. aureus* negative control, e) (Qualitative assay) inhibition of *E. coli* by extract fraction (min.), f) Inhibition of *E. coli* by nanosuspension fraction (max.), g) No Inhibition of *S. aureus* by extract fraction, h) No Inhibition of *S. aureus* by nanosuspension fraction.

3.5 Structural Characterization

FTIR was utilized for the morphological characterization of *E. cardamomum* extract and also for the recognition of biologically active functional groups that exist in the sample. This interferogram obtained by FTIR is the graphical visualization of components recognized in the nanosuspension of *E. cardamomum* (Figure 2, Table 2).

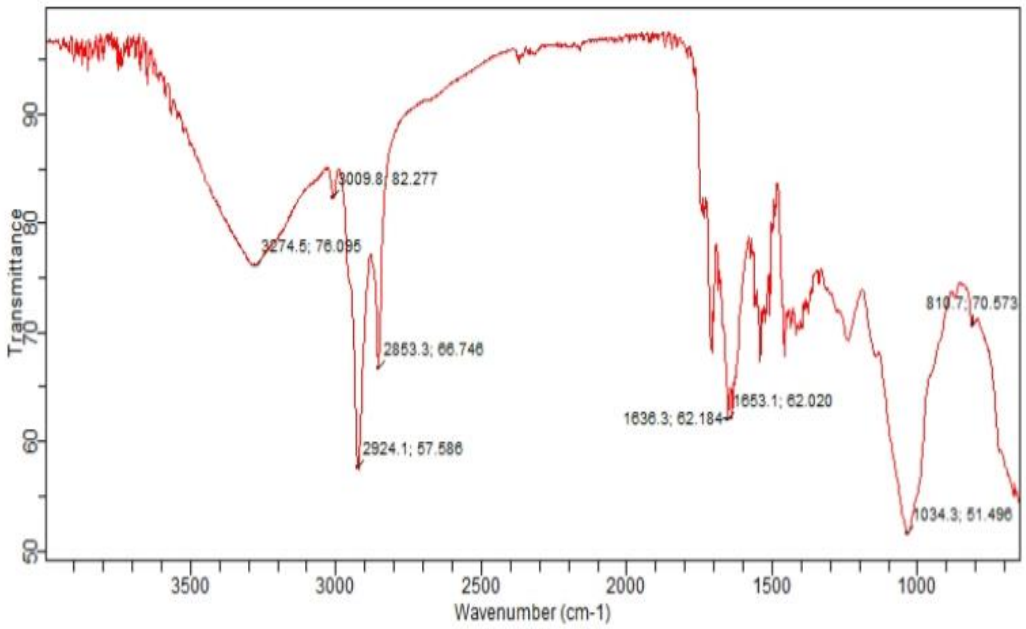

Figure 2. FTIR spectra of *E.cardamomum*

Table 2. FTIR spectrum chart depicting the known functional groups in *E. cardamomum*

Peak number	Absorption	Intensity	Functional Group	Type of vibration	Compound nature
1.	3274.5	Strong	O-H	Stretch	Alcohol
2.	3009.8	Medium	=C-H	Stretch	Alkene (Medial)
3.	2924.1	Medium	C-H	Stretch	Alkane
4.	2853.3	Medium	C-H	Stretch	Alkane
5.	1636.3	Weak	C=C	Stretch	Alkene
6.	1653.3	Weak	C=C	Stretch	Alkene
7.	1034.3	Strong	C-F	Stretch	Alkyl halide
8.	810.7	Weak	C-H	Bend	Aromatic

4. Discussion

Phenolic compounds are commonly present in various plant parts, containing an aromatic ring with several hydroxyl components. These phytoconstituents have been identified to possess excessive antioxidant activities in terms of neutralizing free radicals [17]. Most of the antioxidant activities of *Elettaria cardamomum* are due to phenolic content including polyphenols, flavonoids. During the synthesis of nano-medicines, reduced particle size enhances antiradical ability. Nano-sizing increases the collision of molecules with solvent molecules leading to more solubilized phytochemicals. The DPPH radical scavenging activity has been the most popular method to analyze the antioxidant potential of plant products. The enhanced antioxidant activity may be associated with the presence of various

types of phenolic and flavonoid compounds. The antioxidant activity of quercetin increases due to reduced particle size and increased surface area which results in greater inter-molecular collisions and reactivity [11].

The binding of sugars such as fructose and glucose with lipids and proteins to form ketoamines is a glycation process [18]. Alpha-amylase is a hydrolyzing enzyme carry out the hydrolysis of carbohydrates increasing the level of glucose in the blood. Preceding reports have indicated that cardamom seeds are efficient in lowering blood glucose levels suggesting a potential cure for diabetic patients [19]. Antiglycation activity showed by *E. cardamomum* nanosuspension is because of high flavonoid content and significant antioxidant activities of cardamom. The analysis was carried out to analyze the capability of *E. cardamomum* nanosuspension and extract in impeding the property of the alpha-amylase enzyme. Acarbose is a standard antidiabetic drug that slows down the procedure of disintegration of polysaccharides and prevents hyperglycemia. Although it is well known that phytoconstituents in *E. cardamomum* have an antidiabetic effect [20], it was suggested by Sadowska-Bartosz et al. [21] that various polyphenols and flavonoids attach to proteins and block glycation reactions.

The alpha amylase inhibitory property of *Elettaria cardamomum* might be due to the presence of flavonoids that belong to the family of polyphenols. They play their role by inhibiting the activity of alpha-amylase [22]. Tannins also belong to the family of polyphenols and perform the same role as that flavonoids. Saponins and terpenoids also play a significant role in controlling diabetes. Earlier, aqueous and methanolic extracts of *Elettaria cardamomum* showed varied results due to the different solvents used in the extraction procedure [23].

Research conducted by Din. [24] on essential oil of *Elettaria cardamomum* seeds showed different hemolytic percentages using varying concentrations of essential oil. Bovine erythrocytes and human erythrocytes were used for this purpose and dose-dependent analysis showed almost analogous results of hemolysis for both types of cells. The difference in results might be due to the difference in methodology. Current research showed that nanosuspension causes less hemolysis as compared to extract and these inferences were in agreement with the previous findings [25].

The majority of phytochemicals present in plants are mainly responsible for biofilm inhibition by resisting the initial attachment of the pathogenic bacteria. They might interfere with the expression of genes that are responsible for binding [26]. Previously, the seed oil of *Elettaria cardamomum* did not show antibacterial properties against *E. coli* [27,28]. The present study showed that *E. cardamomum* inhibits the growth of *E. coli*. The difference in results might be due to the use of different solvents i.e. petroleum ether along with ethanol in the above-mentioned research. Moreover, in current research crude ethanolic extract and nanosuspensions were used.

Research conducted by Khan and Haq [29] showed that *E. cardamomum* seeds diethyl ether extract inhibited *S. aureus* growth. However, in the current study, no inhibitory effect of seed extract against *S. aureus* growth was observed.

Fourier transform infrared spectroscopy spectrum was employed to analyze the functional groups of the active constituents that exist in the plant extract. When the sample under evaluation passed through the FTIR, various functional groups were separated on the ratio of the peak value [30]. Table 2 exhibits the particular absorption values as analyzed by the FTIR. Various functional groups existing in the nanosuspension of cardamom were identified. A strong peak at 3274.5 cm^{-1} depicted the occurrence of alcohols. A band present at 3009 cm^{-1} predicted the presence of alkene in the plant sample. The peak at 2853 cm^{-1} corresponds to the existence of alkanes. Similarly, bands at 1636.3 cm^{-1} 1653.1 cm^{-1}, 1034.3 cm^{-1} and 810.7 cm^{-1} depicted the presence of alkene, alkene, alkyl halide and aromatic compounds. From the indication of such chemical groups, the presence of different classes of phenolics and flavonoids is suggested.

5. Conclusions

Elettaria cardamomum nanosuspension and extract both possess antioxidant, antidiabetic, hemolytic and antibiofilm properties. However, more amount of antioxidant contents was found in *Elettaria cardamomum* nanosuspension than in the ethanolic extract. Further research is needed to understand the enhanced biological role of nanosuspensions than the crude extract.

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