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Anti-Aging Potential of Crude Methanolic Extract from *Cocos nucifera* Embryo Using *Caenorhabditis elegans* as Model Organism

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Abstract

Aging is closely associated with oxidative stress, metabolic damage, and the progressive decline of cellular function. Natural plant-based compounds have gained increasing attention as cost-effective and accessible alternatives to commercial anti-aging supplements. Although *Cocos nucifera* contains phenolic compounds and cytokinins with reported bioactivities, the coconut embryo (buwa) remains understudied, and its potential anti-aging effects have not been fully characterized. This study evaluated the phytochemical components and anti-aging properties of *Cocos nucifera* embryo methanolic extract (CEME) using *Caenorhabditis elegans* as a model organism. CEME was analyzed for the presence of cytokinins using chromatographic and mass-spectrometric techniques, and its phenolic content was quantified. Wild-type N2 nematodes were treated with varying concentrations of CEME to assess its effects on lifespan, motility, and stress tolerance under heat-induced conditions. To explore underlying mechanisms, expression of HSP-16.2 and nuclear localization of DAF-16/FOXO were examined using transgenic CL2070 and TJ356 strains expressing GFP reporter constructs. CEME significantly increased the lifespan of C. elegans, with the most pronounced effect observed at 60 mg/mL. Treated nematodes exhibited improved motility in aged worms and demonstrated greater survival under acute thermal stress, indicating enhanced physiological resilience. CEME also induced robust expression of the HSP-16.2 stress reporter and promoted nuclear translocation of DAF-16/FOXO, suggesting activation of conserved stress-response and longevity pathways. These findings imply that the antioxidant constituents of the coconut embryo contribute to its protective and anti-aging effects. This study provides the first integrated evidence of the phytochemical profile and biological activity of coconut embryo methanolic extract, demonstrating its ability to enhance lifespan, stress tolerance, and cellular stress-response mechanisms in C. elegans. These findings support the potential of coconut embryo-derived constituents as candidates for further bioactivity-guided fractionation and mechanistic validation in additional model systems. Further studies are warranted to isolate its active compounds, validate its mechanisms, and assess its applicability in higher organisms.

Keywords: *aging; antioxidant; coconut; cytokinins; lifespan; Cocos nucifera embryo; Caenorhabditis elegans; anti-aging; DAF-16/FOXO; HSP-16.2; stress tolerance; phenolic content*

1. Introduction

Aging is widely recognized as a complex biological process characterized by progressive physiological decline and increasing vulnerability to disease. It reflects the cumulative influence of genetic, molecular, environmental, and lifestyle factors acting over time, leading to visible changes such as wrinkling and reduced sensory function, as well as heightened susceptibility to chronic illnesses including cardiovascular disease, diabetes, and neurodegeneration. Central to this process is the accumulation of molecular damage produced by reactive oxygen and nitrogen species, mitochondrial dysfunction, and impaired cellular quality-control systems. These forms of oxidative and metabolic stress accelerate functional decline and are key drivers of age-associated pathologies.

Although aging cannot be halted, substantial evidence indicates that its rate can be modulated. Interventions that reduce oxidative stress or enhance cellular stress-response pathways can delay functional deterioration and extend lifespan in several model organisms. Natural products, in particular, have attracted significant interest because of their antioxidant, anti-inflammatory, and cytoprotective properties. Phytochemicals such as resveratrol, curcumin, catechins, and fucoxanthin have demonstrated anti-aging activity in multiple biological models by modulating conserved genetic pathways and improving stress resistance.

Cocos nucifera is a widely utilized tropical plant with documented nutritional and therapeutic potential. Its fruit components contain vitamins, minerals, phenolic compounds, and

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phytohormones—including cytokinins—associated with antioxidant, anti-carcinogenic, and antithrombotic effects. Cytokinin-rich coconut water has been examined for various bioactivities, while other coconut plant parts have been shown to possess anti-inflammatory, antimicrobial, antifungal, and antitumor properties. Despite this growing body of literature, the coconut embryo (also known as the coconut apple)—a distinct structure formed within mature fruits—remains poorly studied, with limited information on its phytochemical profile or potential anti-aging properties.

Given the need for natural, accessible, and cost-effective anti-aging interventions, and the paucity of research on coconut embryo extracts, the present study investigated the anti-aging potential of Cocos nucifera embryo methanolic extract (CEME) using Caenorhabditis elegans. This nematode is a widely established model organism for aging due to its short lifespan, transparent body, and highly conserved genetic pathways involved in stress resilience and longevity. Its insulin/IGF-1-like signaling pathway, particularly the DAF-2 receptor and the downstream DAF-16/FOXO transcription factor, provides a robust framework for examining the molecular mechanisms underlying lifespan extension and stress responses.

This study pursued the following objectives:

a. To determine the presence of cytokinins, particularly kinetin, in coconut embryo methanolic extract using chromatographic and mass-spectrometric techniques.
b. To quantify the phenolic content of CEME.
c. To evaluate the effects of CEME on the lifespan of C. elegans.
d. To assess the impact of CEME on the motility of aged worms and on thermotolerance under heat-induced stress.
e. To examine the expression of stress-related reporter proteins (HSP-16.2 and DAF-16/FOXO) in transgenic C. elegans strains following CEME treatment.

2. Review of Related Literature

2.1 Theories and Mechanisms of Aging

Aging is widely recognized as a multifactorial, systems-level process rather than a single linear pathway. Classical and contemporary theories converge on the view that genetic, molecular, cellular, and systemic factors interact to produce heterogeneous aging trajectories among individuals and species. Early integrative accounts emphasized the role of evolutionary trade-offs, gene regulation, cellular senescence, free-radical damage, and neuro-endocrine-immune interactions in shaping the tempo and manifestations of aging, while also identifying calorie restriction as one of the most robust experimental interventions capable of extending functional lifespan and delaying age-related decline (Weinert & Timiras, 1985).

More recent work has attempted to conceptually unify diverse aging hypotheses by focusing on cumulative deleterious change. Gladyshev (2016) proposed the notion of the "deleteriome," defined as the totality of cumulative molecular and cellular damage that accrues over time as an indirect consequence of imperfect biological functions across all levels of organization. In this framework, aging is conceptualized as a progressive decline in organismal fitness due to the rising deleteriome, whose trajectory is adjusted—but not fundamentally caused—by genetic factors, environmental exposures, and stochastic events. Different classical theories (e.g., molecular damage, antagonistic pleiotropy, hyperfunction, incomplete repair) are understood as partial descriptions of how components of the deleteriome accumulate and influence fitness.

Given the complexity of these processes, computational modelling has become an important approach for dissecting aging mechanisms. Mc Auley et al. (2017) reviewed a range of models that simulate DNA damage, oxidative stress, and the dynamics of repair pathways at the cellular level. These in silico models allow researchers to test hypotheses about how random molecular damage accumulates, how repair efficiency changes with age, and how exogenous stressors alter aging trajectories. By integrating experimental data and computational predictions, such modelling efforts support the development of mechanistically informed interventions aimed at mitigating age-related decline.

Taken together, these theoretical and modelling frameworks highlight two themes that are directly relevant to anti-aging interventions in biological model systems. First, oxidative and other molecular damage are central features of the deleteriome, making antioxidant and stress-modulating strategies plausible targets for lifespan and healthspan extension. Second, genetic and environmental modulation of conserved pathways can significantly alter the rate at which damage accumulates, justifying the use of tractable

organisms such as Caenorhabditis elegans to probe these mechanisms experimentally.

2.2 Caenorhabditis elegans as a Model Organism for Aging, Stress and Survival

Over the past several decades, Caenorhabditis elegans has become a cornerstone model in aging research. Johnson (2013) traced more than thirty years of work beginning in the 1980s, when genetic studies in C. elegans transformed aging research from largely descriptive and poorly controlled observations into a field anchored in rigorous experimental genetics. The identification of longevity-modulating mutations—particularly in insulin/IGF-1-like signaling—demonstrated that lifespan can be substantially extended through specific genetic interventions, thus establishing aging as a plastic and experimentally tractable phenotype rather than a fixed, inevitable process.

A central pathway identified in C. elegans aging is the DAF-2 insulin-like signaling cascade. Evans et al. (2008) showed that loss-of-function mutations in daf-2 and its downstream effector age-1 not only extend lifespan but also increase resistance to bacterial pathogens such as Pseudomonas aeruginosa. Interestingly, the enhancement of pathogen resistance is not simply a byproduct of increased lifespan; downstream components of the pathway differentially regulate aging and immunity. For example, pdk-1 and sgk-1 strongly affect lifespan but not pathogen resistance, whereas akt-1 and akt-2 mutations modestly affect lifespan while significantly increasing pathogen resistance. These findings underscore that aging and innate immunity can be uncoupled and are controlled by both shared and distinct branches of the DAF-2 signaling network. The pathway's canonical transcription factor, DAF-16/FOXO, is a key regulator of stress resistance and longevity, and often serves as a readout of pathway activation in studies of natural products and anti-aging interventions.

Methodologically, C. elegans offers robust survival and stress-resistance assays that can be standardized across laboratories. Park et al. (2017) reviewed survival assay protocols that leverage the worm's rapid development, genetic tractability, and ease of cultivation to study aging, stress responses, and pathogen defense. These assays, including pathogen infection models and thermal or oxidative stress paradigms, are amenable to high-throughput formats and automated readouts, enabling efficient screening of genetic mutants and pharmacological agents that affect lifespan and stress tolerance. The use of fluorescent reporters in transgenic strains further allows real-time visualization of stress response genes and apoptotic events.

Beyond classical survival endpoints, C. elegans has also been exploited as a model for exercise and functional healthspan. Laranjeiro et al. (2017) demonstrated that single swimming sessions induce physiological changes in the worm that parallel mammalian exercise, including increased energy expenditure, mitochondrial oxidative shifts, and upregulation of oxidative stress defense genes. Repeated swim exercise extended neuromuscular and intestinal healthspan, improved learning, and protected against neurodegeneration in disease models, with benefits persisting into midlife. The extracellular superoxide dismutase SOD-4 was identified as an important factor for maintaining these long-term exercise-induced benefits, highlighting conserved pathways of stress resistance and tissue maintenance.

Collectively, these studies establish C. elegans as an excellent model for interrogating conserved aging pathways, testing anti-aging interventions, and evaluating both lifespan and healthspan endpoints, including motility, stress resistance, and immune function. The availability of transgenic strains expressing GFP reporters for DAF-16/FOXO, HSP proteins, and other stress markers provides powerful tools for mechanistic insights into how natural products such as plant extracts influence longevity and resilience to stress.

2.3 Natural Products and Phytochemicals with Anti-Aging or Protective Effects

Natural products and plant-derived phytochemicals have attracted substantial interest as candidate anti-aging interventions, in part due to their structural diversity, evolutionary conservation of targets, and generally lower side-effect profiles compared with many synthetic drugs. Lahlou (2013) underscored the enduring role of natural products in drug discovery, noting that modern screening technologies, analytical tools, and computational methods have revitalized this field by facilitating rapid isolation, characterization, and optimization of bioactive compounds from plants and microbes.

From an aging perspective, several reviews converge on the idea that many plant-derived phytochemicals act via antioxidant, anti-inflammatory, and gene-regulatory mechanisms. Mondal et al. (2015) highlighted polyphenols such as curcumin, resveratrol, quercetin, and epigallocatechin gallate as prototypical anti-aging compounds that mitigate oxidative stress and modulate key signaling pathways. Ding et al. (2017) further emphasized that natural products have shown conserved effects on aging pathways across model organisms including C. elegans, Drosophila, and mice, often slowing aging and extending lifespan by targeting common molecular nodes. These compounds hold promise for delaying age-related

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diseases by influencing signaling networks that regulate stress resistance, metabolism, and cellular maintenance.

Several experimental studies in C. elegans support the longevity-promoting potential of plant extracts. Pannakal et al. (2017) reported that a low-molecular-weight polysaccharide from the Ayurvedic herb Chlorophytum borivilianum extended lifespan in both Saccharomyces cerevisiae and C. elegans in a dose-dependent manner and also improved skin-related parameters such as keratinocyte proliferation and hyaluronic acid synthesis, aligning lifespan extension with skin health benefits. Im et al. (2016) showed that Moringa oleifera leaf extract significantly extended C. elegans lifespan and enhanced resistance to thermal, oxidative, and osmotic stress, while improving motility in aged worms. Mechanistically, these effects involved activation of the transcription factors SKN-1 and SIR-2.1, inhibition of insulin/IGF-1 signaling, and crucially, activation of DAF-16/FOXO, linking phytochemical exposure to conserved longevity pathways.

Similarly, Duangjan et al. (2021) demonstrated that Vitis vinifera leaf extract exerted neuroprotective and antioxidant effects in HT22 hippocampal cells and reduced oxidative stress markers in C. elegans. While it did not extend lifespan under normal conditions, the extract increased expression of stress-response genes (e.g., gst-4, sod-3), decreased age-related lipofuscin accumulation, and likely mediated its effects through DAF-16/FoxO signaling. These findings illustrate that some plant extracts may preferentially enhance stress resistance and functional markers of aging (healthspan) even when effects on maximal lifespan are modest.

Beyond direct lifespan studies, other natural product investigations provide relevant mechanistic context. Kabir et al. (2017) discussed tocotrienols—members of the vitamin E family—as bioactive lipophilic compounds with anticancer, neuroprotective, and anti-inflammatory potentials, whose therapeutic impact is currently constrained by poor bioavailability. Johari et al. (2019) and Aryal et al. (2019) both linked high total phenolic and flavonoid contents in plant materials (Pereskia bleo leaves and various wild vegetables, respectively) to strong antioxidant capacities across multiple in vitro assays, reinforcing the central role of phenolics in scavenging free radicals and contributing to protective health effects. Leoncio (2018) added another dimension by demonstrating that tannin-rich plant extracts can interfere with bacterial quorum sensing, reducing virulence and biofilm formation without directly killing bacteria, thus representing a complementary "anti-virulence" rather than antibiotic mechanism.

Taken together, these studies support a general model in which plant-derived compounds and extracts can modulate conserved aging and stress pathways—particularly oxidative stress defenses and DAF-16/FOXO-linked networks—while also providing antioxidant, anti-inflammatory, antimicrobial, and anti-virulence benefits. This literature underpins the rationale for investigating new plant sources, such as the coconut embryo, for anti-aging and stress-protective activity using C. elegans as a sensitive model system.

2.4 Phytochemistry and Biological Activities of Cocos nucifera

Cocos nucifera (coconut) is a highly valued tropical plant with a wide range of traditional and scientifically documented uses. Hooda et al. (2012) reviewed the phytochemical and pharmacological profile of coconut and reported that various plant parts—including fruit, flower, root, leaves, and shell—contain diverse bioactive compounds such as flavonoids, phenols, alkaloids, tannins, saponins, steroids, and terpenoids. These constituents are associated with multiple pharmacological activities, including anti-inflammatory, antioxidant, antimicrobial, antitumor, analgesic, antidiabetic, antihypertensive, hepatoprotective, nephroprotective, and cardioprotective effects. The husk and shell, often discarded as waste, were also noted as rich sources of fibers and bioactive compounds with potential anti-obesity, antidiabetic, and anti-inflammatory properties.

Coconut water, the liquid endosperm of young coconuts, has been particularly highlighted as a natural functional beverage. Prades et al. (2012) described its composition as low-solid (2–5%) but enriched in sugars, minerals, vitamins, amino acids, phytohormones, and antioxidants. Quality is influenced by varietal and agronomic factors, but the beverage is widely used as a natural sports drink, in plant tissue culture, and in skin and hair care applications. The presence of bioactive compounds is believed to underlie its antioxidant, antibacterial, and anti-inflammatory properties, although the specific molecular agents responsible remain incompletely characterized.

There is growing interest in coconut-derived compounds in relation to neurodegeneration and

metabolic disorders. Fernando et al. (2015) reviewed evidence suggesting that dietary coconut—particularly coconut oil rich in medium-chain fatty acids (MCFAs)—may help prevent or ameliorate Alzheimer's disease by providing ketone bodies as an alternative brain energy source, compensating for impaired glucose metabolism. They also noted that phenolic compounds and cytokinin hormones in coconut could inhibit amyloid-β aggregation, a central process in AD pathogenesis. Additionally, coconut's potential role in improving dyslipidemia, insulin resistance, hypertension, and obesity further aligns it with the mitigation of key AD risk factors. While mechanistic and clinical work remains preliminary, these findings point to a neuroprotective and metabolic regulatory potential of coconut components.

Other studies highlight the antimicrobial activities of coconut products. Cholan et al. (2017) demonstrated that green coconut water exhibits dose-dependent antifungal activity against Candida albicans in vitro, with higher concentrations achieving effects comparable to conventional antifungals like amphotericin B or ketoconazole. This activity is likely mediated by bioactive constituents such as saponins, alkaloids, and steroids, which also display antimicrobial effects against other oral microorganisms.

At the level of plant development, Nwite et al. (2017) examined the germination of coconut zygotic embryos and showed that medium composition, growth hormones, sterilization protocols, and physical treatment profoundly affect shoot emergence, rooting, and plantlet survival. Although primarily agronomic, this work underscores the physiological sensitivity of coconut embryos and hints at their distinct biochemical environment during germination and early development.

Finally, the presence of cytokinins in coconut water has been directly demonstrated. Ge et al. (2005) identified kinetin and kinetin riboside—previously thought to be synthetic plant growth regulators—as natural constituents of coconut water using liquid chromatography–tandem mass spectrometry, high-performance liquid chromatography, and capillary electrophoresis. Their detection at very low concentrations suggests that phytohormones in coconut contribute to its biological activity. Given that cytokinins have been linked to anti-aging, anti-carcinogenic, and antithrombotic properties in other contexts, their presence in coconut water and potentially in related tissues provides a mechanistic basis for exploring coconut-derived extracts, including coconut embryo methanolic extract, as candidates for anti-aging and stress-protective applications.

2.5 Cytokinins and Analytical Approaches for Plant Hormone Detection

The investigation of cytokinins in plant materials—and by extension in coconut embryo extracts—critically depends on sensitive and selective analytical methods. Ge et al. (2005) combined LC-MS/MS, HPLC, and capillary electrophoresis to detect kinetin and kinetin riboside in coconut water, achieving very low detection limits (0.02 µM for kinetin and 0.005 µM for kinetin riboside) despite complex sample matrices. Their approach relied on characteristic mass fragments and precise chromatographic separation, illustrating how advanced analytical techniques can reveal phytohormones previously assumed to be purely synthetic.

Subsequent methodological advances have further improved cytokinin analysis in small plant samples. Svačinová et al. (2012) developed a miniaturized pipette-tip solid-phase extraction ("StageTip") method coupled with ultra-high-performance liquid chromatography and fast-scanning tandem mass spectrometry. This technique, using combined reverse-phase and cation-exchange sorbents, allowed >80% recovery of cytokinins from as little as 1–5 mg of Arabidopsis tissue, with detection limits in the femtomole range and quantification over five orders of magnitude. Eighteen different cytokinins—including bases, ribosides, glucosides, and nucleotides—were identified in roots and shoots within a 25-minute run time. The method is fast, cost-effective, and adaptable to other phytohormones.

In the context of the present study, these analytical developments are directly relevant to attempts to quantify cytokinins such as kinetin in coconut embryo methanolic extract. They provide methodological precedents and help explain potential challenges, such as low endogenous concentrations and matrix interference, that may affect detection. They also justify the use of more advanced platforms such as UHPLC-QTOF-MS when conventional HPLC fails to reliably detect or quantify specific cytokinins.

2.6 Research Gap and Rationale for the Present Study

Theoretical and experimental work over the past decades has established aging as a multifactorial process driven by cumulative molecular damage, with oxidative stress and imperfect repair mechanisms playing central roles in the progressive decline of organismal fitness (Gladyshev, 2016; Mc Auley et al., 2017; Weinert & Timiras, 1985). These insights have motivated the search for interventions that modulate conserved aging pathways and enhance stress resistance, with

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particular attention to antioxidant and cytoprotective strategies.

Within this context, Caenorhabditis elegans has emerged as a powerful model organism for aging research. Genetic studies have demonstrated that lifespan can be extended substantially through mutations in insulin/IGF-1-like signaling and related pathways (Johnson, 2013), and that the DAF-2–DAF-16/FOXO axis independently regulates both aging and innate immunity (Evans et al., 2008). Standardized survival, stress-resistance, and functional assays—as well as transgenic strains expressing GFP-tagged stress markers—now enable systematic evaluation of putative anti-aging agents at the levels of lifespan, healthspan, and molecular signaling (Park et al., 2017; Laranjeiro et al., 2017).

Parallel to these developments, a growing body of literature highlights natural products and plant-derived phytochemicals as promising anti-aging candidates. Reviews emphasize that polyphenols and other phytochemicals can attenuate oxidative damage, modulate conserved longevity pathways, and delay the onset of age-related functional decline (Lahlou, 2013; Mondal et al., 2015; Ding et al., 2017). Experimental studies using C. elegans have shown that specific plant extracts—such as polysaccharides from Chlorophytum borivilianum, Moringa oleifera leaf extract, and Vitis vinifera leaf extract—can extend lifespan and/or enhance stress resistance, often through activation of DAF-16/FOXO and other stress-responsive transcription factors (Pannakal et al., 2017; Im et al., 2016; Duangjan et al., 2021). At the same time, in vitro work on diverse plant materials consistently links high phenolic content with strong antioxidant activity, reinforcing the mechanistic importance of phenolics as radical scavengers and modulators of redox balance (Johari et al., 2019; Aryal et al., 2019).

Cocos nucifera occupies a particularly interesting position in this landscape. Multiple plant parts contain phenols, flavonoids, and other bioactive constituents associated with antioxidant, anti-inflammatory, antimicrobial, cardiometabolic, and neuroprotective effects (Hooda et al., 2012; Fernando et al., 2015). Coconut water, in particular, has been characterized as a functional beverage rich in vitamins, amino acids, phytohormones, and antioxidants (Prades et al., 2012), and has been shown to possess antimicrobial and tissue-supportive properties (Cholan et al., 2017). Importantly, cytokinins such as kinetin and kinetin riboside—compounds with reported anti-aging and anticancer potential—have been detected at low concentrations in coconut water using sensitive chromatographic and mass-spectrometric methods (Ge et al., 2005). Recent methodological advances in cytokinin analysis further demonstrate that even trace amounts of such phytohormones can be quantified with appropriate UHPLC–MS/MS approaches (Svačinová et al., 2012).

Despite this rich phytochemical and pharmacological profile, the coconut embryo (buwa) remains comparatively underexplored. Existing work has largely focused on coconut water, oil, and other plant parts, while the embryo is typically treated as a by-product or agronomic subject (e.g., in zygotic embryo germination studies) rather than as a potential source of anti-aging compounds (Nwite et al., 2017). To date, there is:

a. No systematic characterization of the phytochemical profile of coconut embryo methanolic extract (CEME) with respect to phenolic content and the presence or absence of cytokinins such as kinetin using both conventional HPLC and more advanced LC–MS-based techniques.
b. No published evaluation of the anti-aging and healthspan-related effects of CEME in C. elegans, particularly in terms of lifespan extension under normal conditions, motility in aged worms, and resistance to acute stressors such as heat.
c. No mechanistic investigation of whether any observed protective effects of CEME are mediated through canonical stress and longevity pathways, specifically the induction of heat shock proteins (e.g., HSP-16.2) and the nuclear translocation of DAF-16/FOXO in appropriate transgenic C. elegans strains.
d. No integration of phytochemical quantification with functional and genetic readouts in a single experimental framework, which would allow linking constituents (e.g., phenolics, putative cytokinins) with observed lifespan, motility, and stress-resistance phenotypes in a well-established animal model.

The present study was designed to address these gaps by (i) assessing the presence of cytokinins, particularly kinetin, in CEME using chromatographic and mass-spectrometric methods; (ii) quantifying the total phenolic content of the extract; (iii) determining the effects of different CEME concentrations on lifespan, motility, and

thermotolerance in C. elegans; and (iv) examining the expression of stress-related green fluorescent reporter proteins, specifically HSP-16.2 and DAF-16/FOXO, in transgenic strains under heat stress. By combining phytochemical analysis with functional assays and reporter-based genetic readouts, this work aims to provide the first integrated evaluation of the anti-aging and stress-protective potential of coconut embryo methanolic extract in a well-characterized in vivo model.

3. Materials and Methods

This study utilized standard laboratory procedures for the preparation of Cocos nucifera embryo methanolic extract (CEME), phytochemical profiling, and Caenorhabditis elegans–based bioassays. All reagents and solvents were analytical grade. The nematode strains used were wild-type N2, the CL2070 reporter strain expressing HSP-16.2::GFP, and the TJ356 strain expressing DAF-16::GFP for stress-response analyses. Worms were maintained on nematode growth medium (NGM) plates seeded with Escherichia coli OP50 following established culture conditions. Experimental procedures included extract preparation, chromatographic and mass-spectrometric analyses, and functional assays assessing lifespan, motility, thermotolerance, and stress-related protein expression in transgenic strains.

3.1. Collection/ Preparation of sample

Coconut embryos were bought and delivered by a local supplier in Bangkok, Thailand. Coconut embryos were purchased from a local supplier in Bangkok, Thailand. Approximately 3.5 kg of embryos were macerated and soaked in 6 L of 80% methanol for 48 h at 4°C in the dark. The mixture was filtered, and the filtrate was concentrated using a rotary evaporator at 40°C to remove methanol. The pH of the concentrate was adjusted to 8.5 using dilute sodium hydroxide (NaOH), after which liquid–liquid partitioning was performed with chloroform. Equal volumes of extract and chloroform (100 mL each) were combined in a separatory funnel, vigorously mixed, and allowed to separate; the aqueous phase was collected for subsequent processing.

3.2. Processing of Sample/ Rotary Evaporation/ Lyophilization

The sample was reconcentrated in the rotary evaporator and lyophilized at -50 ℃ .The plant extracts were dissolved in Dimethylsulfoxide (DMSO) in the concentration of 100mg/ml, 60mg/ml, and 30mg/ml as stock solution. The solution should be protected from light and are stored at −20℃ for further investigation.

To purify the cytokinins, 10 grams of liquid CEME were placed at the circle inlet of the glass tube with silica gel, and then suitable mixture of solvent methanol/chloroform/distilled water/ formic acid, 12/5/2/1 (Blieski solution) is added repeatedly throughout the process. Each component (by layer) was collected in a separate tube. Representative tubes were analyzed by HPLC for the determination of kinetin.

3.3. Cytokinins Determination by HPLC

Following the procedure of Liya Ge et al., (2005) with some modifications, the cytokinins in the form of kinetin was analyzed with the use of High Performance Liquid Chromatography (HPLC) [16]. The reference standard used was kinetin >99.0% HPLC (SIGMA). Sample and standard were dissolved with an HPLC grade methanol with concentration of 3 mg/ml and 10 ul of this sample was injected in the column RP-CP18, 269 nm wavelength were used. The flow rate was 1.0 ml/min throughout the whole process. Prior to each analysis, the column was washed with mobile phase methanol and acetic buffer 95:5, v/v for 5 minutes. The retention time was 10 minutes. The CEME was quantitatively determined using the formula:

$$\frac{\text{conc. of standard}}{\text{conc. of CEME}} = \frac{\text{peak area of standard}}{\text{peak area of standard}}$$

3.4. C. elegans Culture

Caenorhabditis elegans of N2 wild type, CL 2070 and TJ 356 transgenic strain and Escherichia coli (OP50) were used in this study, the N2 wild type strain were provided by Asst. Prof. Dr. Tewin Tencomnao, Head of Graduate School, Department of Clinical Chemistry, the Faculty of Allied Health Sciences and were maintained by Dr. Lyer Prasanth at Chulalongkorn University Bangkok, Thailand. All other strains were purchased from the Caenorhabditis Genetic Center (University of Minnesota/Department of GCD, 6-160 Jackson Hall / 321 Church Street SE Minneapolis, MN 55455). The transgenic CL 2070 (dvla 70) and TJ 356 (zls356 IV) were used to visualize HSP-16.2 and localization of DAF 16, respectively.

The worms were grown at 15-20 ℃ on nematode growth medium agar plate (NGM) (formulation 0.25% tryptone, 0.3% sodium chloride, 1mM calcium chloride, 1mM magnesium sulfate, 25mM potassium phosphate, 5ug/ml cholesterol, and 1.5% agar), with E. coli as food source. Caenorhabditis elegans bleached, and eggs were collected and grown to have ample supply of age synchronized organisms for the duration of the study. Briefly, gravid adult worm and eggs were recovered by washing the plates with M9 buffer and collected in 15ml test tube, pellet worms by centrifugation for

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2 minutes and discard supernatant, perform the procedure for 3 times or until the solution appeared clear of bacteria, add the desired bleaching solution and agitate for 3-6 minutes (check under microscope for traces of adult worm), stop the reaction by adding M9 buffer to the tube, centrifuge for 1 minute and discard supernatant, wash pellets for 3 times with M9 buffer, add 0.5ml of M9 buffer to the pellet and place eggs to desired plates (Day 0).

3.5. Lifespan Assay

The organism was tested for Lifespan assay for at least 3 times independently at 20℃. Briefly, strain of N2 wild type (var. Bristol) synchronized young adult day 4 worms were transferred to M9 buffer plate (10 worms/plate) containing the control vehicle (medium use in stock solution preparation) and different concentrations of CEME at 30, 60 and 100 mg/ml. Test worms were checked every day and considered dead when they fail to respond to slight tapping of the plate and no body movement. The plates were checked every day until no worms are alive. The 5-fluoro-2-2deoxyuridine (FUdR) an inhibitor of DNA synthesis that blocks egg-hatching were used to stop progeny production and are added in each well.

3.6 Movement Assay

To check the body movement, eight-day old adult worms of N2 wild type strain seeded in with/without the presence of CEME, was transferred to normal NGM plate and was observed for their motility using dissecting microscope for 1 minute. Distance travel (track length) was analyzed and measured in micrometers. The assay was in triplicates.

3.7 Stress Resistance Assay

To test for stress resistance, the age-synchronized worms (N2 Wild Type) (day 4 young adult), were transferred to the M9 buffer with/without various concentrations of CEME for 72 hours. For the stress resistance assay (thermotolerance), the worms were subjected to 37(+/- 1℃) temperature for 2 hours and return to 15-20℃ for 1 hour; the survivals were scored every hour. The survival of worms was determined by provoked movement as previously mentioned. Worms which fail to respond to gentle tapping and no movement was considered dead. Each test will be performed at least 3 times.

3.8 HSP 16.2 and DAF-16 Fluorescence microscopy and visualization

CL2070 mutant strain L1 worms containing a HSP-16.2: GFP reporter was maintained in the presence or absence of CEME at 15-20℃ for 72 hours. Prior to microscopy observation CL 2070 mutants received heat shock at 37℃ (+/- 1℃) for 2 hours and be allowed to recover at 15-20℃ for 4 hours. Transgenic worms were treated with 2% sodium azide to induced paralysis, and mounted on a glass slide.

The age synchronized TJ 356 mutant L1 worms, which express DAF-16: GFP reporter fusion protein was treated with presence or absence of CEME. The nematode was maintained at 15-20℃ for 72 hours, Prior to microscopy observation of TJ 356 strain received heat shock (procedure as previously described) and then the worm was mounted on a glass slide and anesthetized with 2% sodium azide. At least thirty worms per group were imaged on a fluorescence microscope using Infinity 1 software with 10x objective lens and constant exposure time. Distribution of the transcription factor DAF-16: GFP in each worm can be in the nucleus, cytoplasm, or the intermediate region between the nucleus and cytoplasm. Worms were sorted and counted according to localization of DAF-16: GFP. To determine the protein expression levels, photographs of the transgenic worms were taken and analyzed.

3.9. Analysis of Data

The data in this study for lifespan assay, motility and stress resistance assay were plotted using Kaplan-Meier analysis and statistical significance were analyzed by log rank (Mantel-Cox) test. Other data were presented as mean+/- standard deviation as indicated. Statistical significance of differences between the control and treated group were analyzed by student t-test. All experiments were performed in triplicate. Differences with $p<0.05$ were considered statistically significant.

4. Results and Discussion

4.1. Presence of cytokinins (Kinetin) as potential anti-ageing phytohormones present in CEME.

Purified cytokinin from CEME was characterized using Thin Layer Chromatography (TLC) and High-Performance Liquid Chromatography (HPLC) (Shidmadzu Prominence SPD-20A LabSolution System). These methods allowed for the identification of compounds, checking the purity, and monitoring the progress of the purification process by comparing the samples

(CEME) and the standard (HPLC standard kinetin). The TLC result for cytokinin (kinetin) showed an Rf value of 0.84 at 352 nm (Figure 1). This value was similar to the standard (kinetin) which showed an Rf value of 0.84. These measurements were taken as:

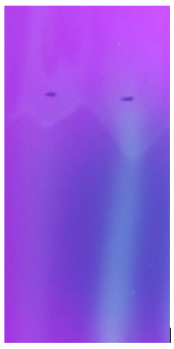
$$\text{Rf} = \frac{\text{distance traveled by the component}}{\text{distance traveled by the dolvent}}$$

$$\text{Standard Rf} = \frac{55\text{ cm (standard)}}{65\text{ cm (solvent)}}$$

$$\text{Standard Rf} = .84$$

$$\text{CEME Rf} = \frac{55\text{ cm (standard)}}{65\text{ cm (solvent)}}$$

$$\text{CEME Rf} = .84$$



(a) (b)

Figure 1. *Kinetin standard and CEME by Thin Layer Chromatography. (a) Kinetin Standard Rf = 0.84; (b) CEME Rf = 0.84*

To confirm the presence of kinetin in CEME, further analysis was performed using a HPLC equipment (Shidmadzu Prominence SPD-20A LabSolution System), where kinetin (Sigma HPLC Grade) was utilized as the reference standard, and methanol (HPLC Grade): 40 mM acetic acid (95:5) as the mobile phase. The retention time for the purified CEME peak was at 6.257 with a peak area of 69660274, while kinetin standard retention time was at 5.834 with peak area of 37036808 (Figures 2 and 3). The quantitative estimation of the purified CEME by HPLC was calculated as follows:

$$\frac{\text{conc. of standard}}{\text{conc. of purified CEME}} = \frac{\text{peak area of standard}}{\text{peak area of purified CEME}}$$

$$\text{conc. of purified CEME} = \frac{3\frac{\text{mg}}{\text{ml}}(37036808)}{69660274}$$

$$\text{conc. of purified CEME} = 1.59\frac{\text{mg}}{\text{ml}}\text{ of purified CEME}$$

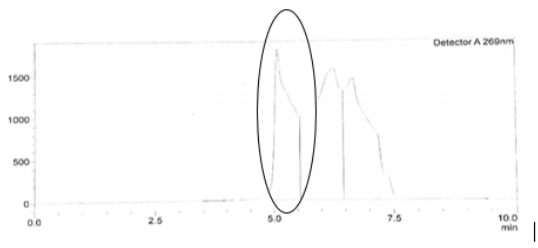


Figure 2. *HPLC Chromatogram of Purified CEME at 269nm Detection*

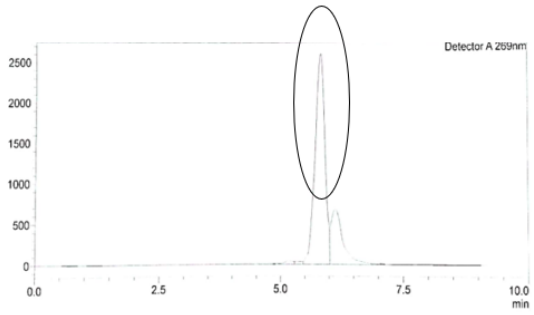


Figure 3. *HPLC Chromatogram of Cytokinin(Kinetin) Standard at 269nm Detection*

Based on the given computation of 1.59 mg of kinetin in every ml of CEME, the presence of cytokinin in the extract is very low. In addition, a large unknown peak was observed in CEME samples and standard (kinetin) chromatograms. This could be due to the interference in the matrix during the running of samples.

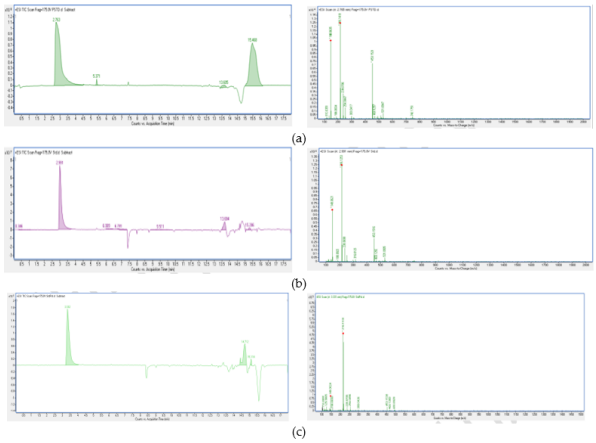


Figure 4. *(a) Trial 1 Kinetin Standard Chromatogram and Spectrum; (b) Trial 2 Kinetin Standard and Spectrum; (c) Trial 3 Kinetin Standard Chromatogram, and Spectrum.*

To overcome the problem in the detection of the presence of cytokinin and other types phytochemicals present in CEME, ultra-high performance liquid chromatography equipped with quadrupole time-of-flight mass spectrometry (UHPLC-QTOF LC/MS) was used. The retention time of the kinetin standard in Trials 1 and 2 as shown in Figures 4 (A, and B) is at 2.763 and 2.991 min with peak areas 2,815,450,778.24 and 775,107,757.22, and concentrations of 1000ppm and 10ppm, respectively. The prominent m/z at this peak for both trials from the MS spectra are 148 and 216, with the 216 ion being the characteristic [M+H]+ ion

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of kinetin. For trial 3, the retention time shifted from around 2.8 min to 3.332 min with a peak area of 215,882,532.91. The prominent ions at the 3.332 min peak for trial 3 are also those with m/z of 148 and 216 Figures 4 (C). For the plant sample, as it can be seen in Figures 5 (A and B). There is no peak present within the retention time expected (around 2.8 min) based on the kinetin standard chromatogram.

In Figure 5 (C), there is still no peak detected around the expected retention time (3.3 min). In Figures 6 (A, B and C), the MS spectra for both the kinetin standard and the plant sample were compared. However, for all trials, the two expected prominent m/z of kinetin were not found in the MS spectra of the samples.

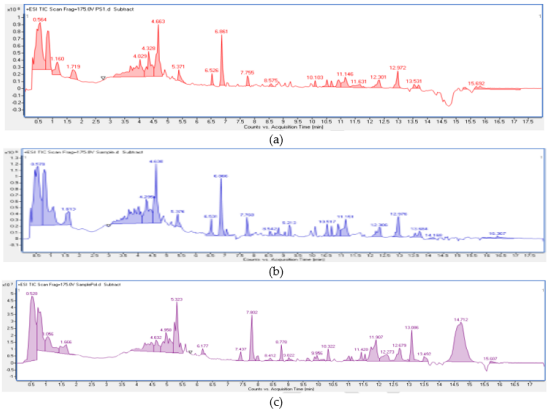


Figure 5. *(a) Trial 1 CEME Sample Chromatogram; (b) Trial 2 CEME Sample Chromatogram; (c) Trial 3 CEME Sample Chromatogram*

Based on the results, it was shown that the target compound, cytokinin (kinetin), is not detected from the plant extract sample (CEME). The result was similar to the observation of (Prades et al., 2012) where the concentration of kinetin is below the detection limit of LCMS [21]. It could suggest that the enzyme activity of coconut fruit probably depends on the factors such as variety, state of cultivation, stage of maturity at harvest, and storage conditions. Results of the UHPLC-QTOF LC/MS showed multiple chromatographic peaks that can be analyzed to identify other compounds that contributed to the prolonged lifespan of C. elegans.

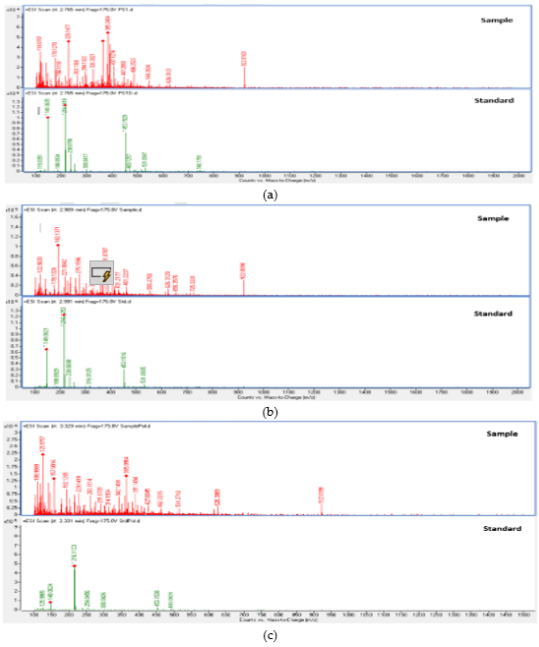


Figure 6. (a, b, c) *Comparison of MS Spectra for all the Trials of Both CEME Sample and Kinetin Standard.*

4.2. The Quantification of Phenolic Content of CEME

The antioxidant activity of which is attributed to the positive relationship with the total phenolic content value. Hence, these plants rich with phenolic compounds can be considered as antioxidants. In this study, phenolic content was measured using the Folin–Ciocalteu reagent in CEME. The results were derived from a calibration curve of the standard and expressed in gallic acid equivalents (GAE) per gram dry extract weight.

The total phenolic content of the CEME extract from coconut embryo is 9.045 mg GAE/g dry weight extract. The presence of phenolic compounds from the data suggests the potential antioxidant properties of CEME that are bioavailable. This is in agreement with other research that showed the antioxidant effects of polyphenols.

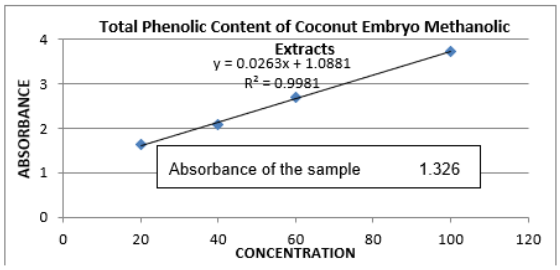


Figure 7. *Result of Phenolic Content of CEME*

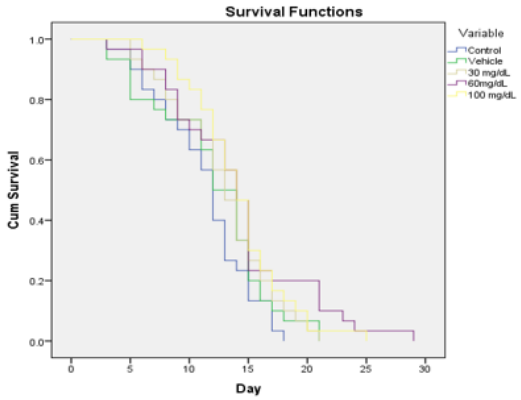
4.3. Effects of CEME in C. elegans

4.3.1. Lifespan of C. elegans

Lifespan curves are created to show the percentages of live worms in populations over time. All concentrations that were tested showed that the lifespan of the C. elegans was extended, wherein concentrations of 30 and 100mg/ml exhibited maximum extension of 20 and 24 days respectively. The 60mg/ml concentration significantly increased the maximum lifespan of the C. elegans by 28 days

Table 1. Effects of CEME in *C. elegans* Lifespan (Days).

Concentration	Maximum	Mean
Control	17d	14.67d
Vehicle	20d	17.67d
30 mg/ml	20d	18.33d
60 mg/ml	28d	24.33d
100 mg/ml	24d	20.0d



(p<0.017), the control exhibited a lifespan of 17 days, while the vehicle exhibited lifespan of 20 days. (Figure 8 and Table 1). This indicates that the solvent used induced no change in mediating the lifespan extension of CEME.

Figure 8. *Effects of CEME on the Lifespan of N2 Wild Type Strain. The Number of worms Used per each Lifespan Assay was 10 and Three Independent Experiments were Repeated (N=3).*

4.3.2. Motility Assay

The data showed that CEME supplementation of 30 and 60mg/ml efficiently increased motility and slowed down reduction in body movements of the C. elegans with maximum distance of 3,600um and 3,800um (travel distance) respectively, compared to the control group mean of 3,500um (travel distance). These results of CEME supplementation significantly increased the movement of aged nematodes (Figure 9), and this revealed that CEME could not only affect the C. elegans lifespan but as well the health span.

4.3.3. Stress Assay (Thermotolerance)

The wild type N2 strain (var. Bristol) of C. elegans was used in the assay. A typical temperature for acute heat stress resistance assays (thermotolerance) is 37°C, for 2 hours, it was place in incubator and subjected with this kind of high temperature, and returned to their normal temperature at 15-20℃ for 1 hour, the survivals were scored every hour, by gentle tapping of the well and if no movement observed the nematode will be considered dead.

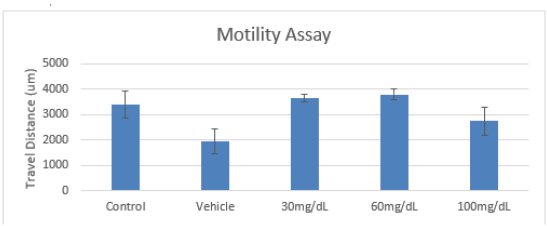


Figure 9. *Effects of CEME on Aging Related Factors in Wild Type N2 Strain .To Check the Body Movement, the Analysis was taken on the 8th Day of Adulthood Assessments were Analyzed by Digital LED Microscope*

Upon completion of the assay, the data showed that the nematode with pretreatments of CEME (30, 60, 100mg/ml) enhanced the survival rates under stress conditions. Among the CEME concentration, the 60mg/ml concentration significantly increased the survival rate of N2 wild type strain of C. elegans by mean of 14.01 hours as compared to the control group with mean of 8.2 hours (t-test p-value: < 0.001). This suggest possible role of the extract in promoting heat tolerance of C. elegans under stress conditions like heat stress, maybe the heat shock proteins (HSPs) of the nematodes may play a major role in the protection of the organism against molecular damage (Figure 10).

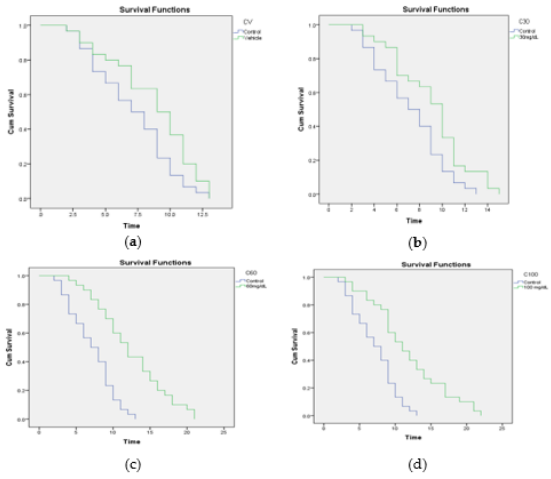


Figure 10. *Effects of CEME on the Stress Resistance of Wild Type N2 Strain. (a) Control and vehicle on the stress resistance; (b) CEME 30 mg/ml concentration on the stress resistance; (c) CEME 60 mg/ml concentration on the stress resistance; (d) CEME 100mg/ml concentration on the stress resistance.*

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4.4 Expression of Green Reporter Fusion Protein in C. elegans

4.4.1. Effect of CEME on Gene Expression of Stress Response (HSP-16.2)

In C. elegans CL2070 transgenic strain, the expression of HSP-16.2 plays a key role in protecting the nematodes against heat stress. Interestingly, the expression HSP 16.2 GFP was significantly elevated that were pretreated with different concentrations of CEME (30, 60, 100 mg/ml) when compared with control worms under heat shock conditions. At concentration of 100 mg/ml, the maximum increased in the expression of HSP 16.2 was observed, compared to the control group ($p<0.001$) (Figure 11).

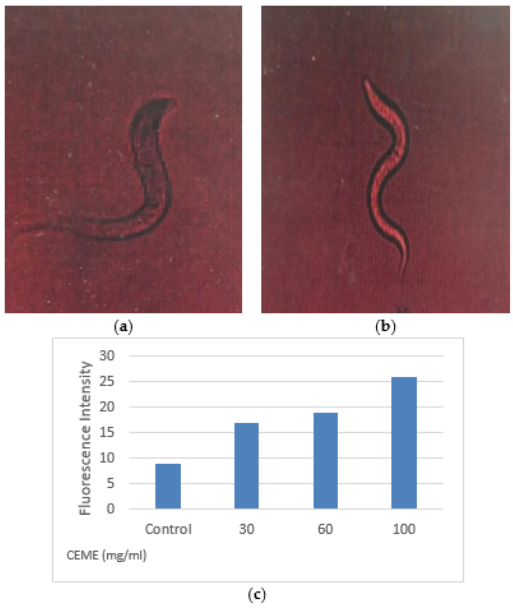

Figure 11. *Effects of CEME on Expression of HSP 16.2 GFP in C.elegans CL 2070. (a) Control (OP 50); (b) CEME Treated (100mg/ml); (c) Data expressed as the mean +-S.E.M. of three independent experiments (N=3). Differences compared to the control were considered significant at p <0.001 by t-test.*

4.4.2 Effect of CEME on Gene Expression in Stress Resistance Properties Mediated By the DAF-16/FOXO Pathway in C. elegans

Forkhead transcription factor which is called FOXO proteins in humans, that were investigated in TJ 356 transgenic strain, the CEME pretreated nematodes (30, 60, 100 mg/ml) enhanced DAF-16 translocation. The CEME concentration of 60 and 100 mg/ml resulted in a high percentage of subcellular localization of DAF-16 compared to the control. This result suggested that the antioxidant effect of CEME was mediated via DAF-16/FOXO pathway. Representative microscopy images from individual worms can be found in Figure 12.

4.5 Discussion

The present study provides the first integrated analysis of the phytochemical profile and biological activity of coconut embryo methanolic extract (CEME) in Caenorhabditis elegans. Although initial chromatographic screening using TLC and conventional HPLC suggested the presence of kinetin-like activity, UHPLC–QTOF–MS failed to detect kinetin or its characteristic mass fragments in the extract. This finding is consistent with previous work reporting that cytokinins in coconut water may occur at extremely low concentrations—often near or below LC–MS detection thresholds—and may vary with cultivar, environmental conditions, and physiological maturity (Ge et al., 2005; Prades et al., 2012). Thus, the lack of detectable kinetin in CEME suggests that the observed biological effects are unlikely to be cytokinin-mediated and may instead arise from other phytochemicals present in the embryo.

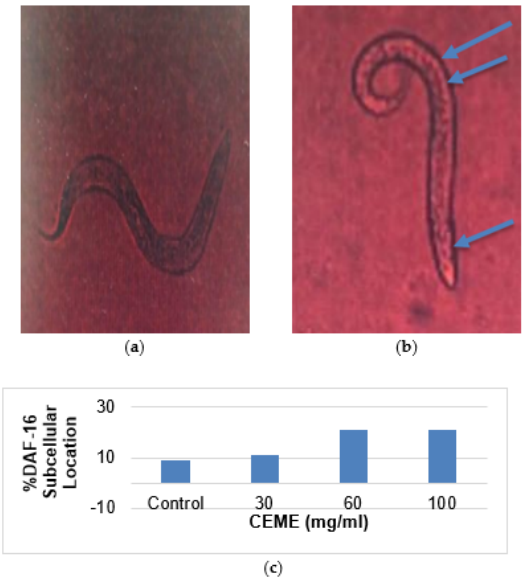

Figure 12. *Effects of CEME on DAF-16 translocation. (a) Image of TJ356 control worm; (b) Image of CEME treated nematodes significantly increase DAF-16 translocation; (c) Data expressed as the mean +-S.E.M. of three independent experiments (N=3). Differences compared to the control were considered significant at p <0.003 by T-test.*

The total phenolic content assay confirmed measurable phenolic constituents in CEME, supporting the likelihood that antioxidant compounds contribute to lifespan extension and improved stress resistance. Phenolics are well-documented modulators of oxidative stress and have repeatedly been shown to enhance longevity and healthspan in C. elegans by reducing reactive oxygen species and influencing stress-responsive pathways (Mondal et al., 2015; Ding et al., 2017). The phenolic content quantified in this study provides a mechanistic basis for the observed enhancements in lifespan, motility, and thermotolerance.

Across functional assays, the 60 mg/mL concentration consistently produced the most pronounced effects, significantly extending lifespan, improving motility in aged worms, and enhancing survival under thermal stress. These findings align with prior studies demonstrating that plant-derived extracts can simultaneously influence lifespan and healthspan, suggesting that CEME targets conserved pathways governing organismal resilience rather than acting solely through antioxidant scavenging (Im et al., 2016; Pannakal et al., 2017).

The genetic assays further strengthen this interpretation. Elevated expression of HSP-16.2 in heat-shocked CL2070 worms indicates activation of molecular chaperones essential for maintaining proteostasis under stress. Similarly, the enhanced nuclear localization of DAF-16/FOXO in the TJ356 strain suggests that CEME influences insulin/IGF-1-like signaling, a central regulator of longevity, stress responses, and metabolic adaptation in C. elegans. Many natural products exert their anti-aging effects through DAF-16 activation, which induces downstream transcription of antioxidant enzymes, heat shock proteins, detoxification components, and longevity-associated genes. The convergence of phenotypic outcomes (improved stress resistance, extended lifespan) and molecular readouts (HSP-16.2 induction, DAF-16 nuclear translocation) supports the conclusion that CEME activates conserved stress-response networks rather than acting through a single compound.

Importantly, the failure to detect kinetin in CEME shifts the mechanistic focus away from cytokinins and toward alternative constituents—likely phenolics, flavonoids, or yet-unidentified metabolites revealed by UHPLC chromatographic peaks. These unidentified compounds may synergistically contribute to the observed effects and warrant further isolation and structure elucidation.

This study has several strengths, including its combined phytochemical, functional, and reporter-based approach and its use of multiple experimental replicates across assays. However, it also carries limitations. The absence of compound isolation prevents identification of specific active constituents, and the lack of polymer-level identification of extract components limits mechanistic clarity. Additionally, while C. elegans provides robust insights into conserved pathways, translation to higher organisms requires caution.

Overall, these findings demonstrate the anti-aging and stress-protective potential of coconut embryo extract and justify deeper phytochemical characterization and mechanistic validation. Future work should isolate the active components responsible for DAF-16 activation, perform dose–response kinetics, and explore comparative effects in other model organisms. The evidence generated here provides a foundation for considering CEME as a natural, accessible, and cost-effective candidate for anti-aging and anti-stress applications.

5. Conclusion and Recommendations

5.1 Conclusion

This study provides evidence that the crude methanolic extract of Cocos nucifera embryo (CEME) possesses anti-aging and stress-protective properties in Caenorhabditis elegans. Although kinetin was not detected by UHPLC–MS, the extract exhibited measurable phenolic content and produced significant biological effects, including lifespan extension, improved motility in aged worms, and enhanced thermotolerance. CEME also activated key molecular pathways associated with stress resistance, as indicated by increased expression of HSP-16.2 and nuclear localization of DAF-16/FOXO in transgenic strains. These results suggest that the protective effects of CEME may be mediated by phenolic constituents or other bioactive compounds that modulate conserved stress-response mechanisms. Overall, the findings support the potential of coconut embryo extract as a natural source of anti-aging and anti-stress bioactivity, warranting further investigation through compound isolation and evaluation in higher model systems.

5.2 Recommendations

Based on the results and methodological limitations of the study, the following recommendations are offered:

a. Future work may evaluate additional extraction methods, such as solvent partitioning, solid-phase extraction, or enzymatic-assisted protocols, to improve detection sensitivity for kinetin and related cytokinins.
b. Investigate biological and environmental factors influencing cytokinin levels in coconut embryos. Subsequent studies may

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examine how maturation stage, developmental phase, harvesting season, soil type, and environmental conditions affect cytokinin production and accumulation within the embryo.

c. Isolate and identify additional phytochemical constituents of coconut embryos. Targeted compound isolation and structural elucidation will help determine which metabolites—other than cytokinins—contribute to the observed anti-aging and stress-protective effects.
d. Assess a broader range of CEME concentrations in biological assays. Evaluating additional doses will aid in determining the optimal concentration for lifespan extension, thermotolerance, and motility improvement in *C. elegans*.
e. Conduct complementary antioxidant assays. Further research may incorporate flavonoid quantification and free radical scavenging assays such as DPPH and ABTS to more comprehensively characterize the antioxidant properties of coconut embryo extracts.
f. Utilize additional *C. elegans* strains targeting downstream stress-response genes. Employing reporter strains for genes such as *sod-3* and *gst-4* will broaden understanding of how CEME influences oxidative stress pathways and may strengthen evidence for its anti-aging potential.

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Conflicts of Interest: The authors declare no conflict of interest.

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