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Anti-Oxidant and Anti-Inflammatory Properties of Juglans Regia L. and Coffea Canephora Extract Gel: An *In-vitro* Study

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KEYWORDS

Juglans regia L., Coffea canephora, antioxidant, anti-inflammatory, extract, Topical gel formulation, Sustainable herbal therapeutics

ABSTRACT:

Background:

Oral potentially malignant disorders (OPMDs) pose a challenge due to chronic progression, malignant potential, and the limitations of conventional therapies, which often cause side effects and fail to address underlying mechanisms like oxidative stress and inflammation. Juglans regia L. (walnut) and Coffea canephora (coffee bean) are rich in polyphenols, flavonoids, tannins, and caffeine, known for their antioxidant, anti-inflammatory, antimicrobial, and antifibrolytic properties.

Aim:

To evaluate the antioxidant, anti-inflammatory, and antimicrobial properties of a novel topical gel combining Juglans regia and Coffea canephora extracts as a safer and more effective alternative for managing OPMDs.

Materials and Methods:

The aqueous extracts of Juglans regia L. (walnut) and Coffea canephora (coffee) were prepared separately by grinding 10 grams of each respective powder and mixing with 100 ml of distilled water, heated, and then filtered. Condensing solutions were formed by heating 50 ml of each extract to 50-60°C until reduced to 5 ml. These extracts were added to a mixture of Carbopol 940 and carboxymethyl cellulose to prepare the gel. The gel's antioxidant activity was evaluated through 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, hydrogen peroxide (H_2O_2) radical scavenging assay, and the Ferric Reducing Antioxidant Power (FRAP) assay.. Anti-inflammatory properties were assessed via bovine serum albumin and egg albumin denaturation assays, and membrane stabilization assay.

Results:

The Coffee bean and Walnut gel exhibited dose-dependent antioxidant and anti-inflammatory activities across all assays. Antioxidant effects in DPPH, H_2O_2, and FRAP assays were comparable to the standard, with no statistically significant differences ($p > 0.05$). Similarly, in BSA, EA, and membrane stabilization assays, the gel's anti-inflammatory response closely mirrored that of the standard. All results were based on triplicate tests and analyzed using Student's t-test ($p < 0.05$ considered significant).

Conclusion:

Juglans regia L. and Coffea canephora extract gel could be a promising, treatment option for inflammatory and potentially malignant disorders especially, OSMF due to its natural composition, targeted delivery, and potential to address the underlying mechanisms of the disease. This novel treatment modality offers a potential alternative or adjunct to the currently available antioxidant and anti-inflammatory medications.

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Introduction:

In recent years, there has been a growing focus on potentially malignant disorders, particularly oral submucous fibrosis (OSMF), which poses a significant health concern due to its premalignant nature. Alongside, inflammatory disorders have also garnered attention for their implications on oral health. However, current treatment approaches for these conditions primarily revolve around symptom management, often utilizing antioxidants and steroids. Despite their widespread use, these therapies are limited in efficacy and may pose adverse effects, prompting the exploration of alternative treatment modalities. In response to the drawbacks associated with conventional treatments, there has been a noticeable shift in research towards alternative medicine. One such avenue involves investigating natural extracts such as Juglans regia L. and Coffea canephora. These extracts offer promising potential in addressing the underlying mechanisms of OSMF and other oral inflammatory conditions, suggesting a paradigm shift towards more holistic and potentially efficacious treatment approaches. This transition underscores the ongoing quest for safer and more effective therapies to improve patient outcomes in the realm of oral health. The problem with presently available drugs that have antioxidant and anti-inflammatory activities is that they can often have undesirable side effects. For example, nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly used to treat inflammation and pain, but they can cause stomach ulcers, bleeding, and other gastrointestinal problems. Steroids are also effective anti-inflammatory drugs, but they can have serious side effects such as weight gain, osteoporosis, and diabetes. Thus, for better-multifaceted disease management, it is necessary to develop new drugs implying antioxidant and anti-inflammatory activities. In this context, medicinal plants and derivatives have been widely used for therapeutic purposes to heal and protect against several illnesses.

J. regia is a well-known worldwide species of the *Juglans* genus belonging to the family Juglandaceae. It is also known as English walnut, Persian walnut, common walnut, Akhrot, Aksoda, Aksota [1]. It is widely distributed in Southern Europe, Western Asia, Central Asia, Kashmir, Tajikistan, Kyrgyzstan, China, the United States, Turkey, India, Australia, and New Zealand. This plant can live for a duration of 100-200 years, and some species for 1000 years. All parts of *J. regia L*., such as leaves, bark, green husk, shell, seed, and fruit, have pharmacological activities. *J. regia L.* is a source of Vitamin E, monounsaturated fatty acids, omega-3 fatty acids, and arachidonic acids [2]. China is the largest, and the United States is the second-largest producer of walnuts in the world According to the Indian scenario, it is mainly cultivated in Jammu & Kashmir, Arunachal Pradesh, Himachal Pradesh, and Uttarakhand. In the world, there are different varieties of walnuts, such as black walnut (*Juglans nigra*), English or Persian walnut (*J. regia L.),* Butter or white walnut (*Juglans cinerea*). English walnut is not too hard to crack compared to black or white walnut [3]. *J. regia L.* contains various types of chemical constituents such as Juglone, polyphenols, Flavonoids, Terpenoids, Steroids, Ascorbic acid, Gallic acid, Sitosterol, Quercetin, and Omega-3 Fatty acid. *J. regia L.* is reported to be used as an Anti-inflammatory, Diuretic, Anticancer, Laxative, Antidiabetic, Antiatherogenic, Antimutagenic, Antifungal, Antioxidant, Antiseptic, Antibacterial, Antiallergic, Astringent, and Antiulcer. It is useful in traditional medicines for the treatment of Cardiac diseases, Dental plaque, reducing Cholesterol levels, and blood purification, regulating the immune system. The nut of *J. regia L.* is used in the Cosmetic, Food, and Pharmaceutical industry [4]. The main molecules found in J. regia are polyphenols, including phenolic acids (gallic acid, vanillic acid, syringic acid, ellagic acid, caffeic acid, ferulic acid, sinapic acid, chlorogenic acid), flavonoids, and tannins. *J. regia* extracts act on different inflammatory mediators; husk extracts can inhibit the production of nitric oxide (NO) in macrophages, kernel extracts inhibit the activation and the expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) and are involved in the diminution of edemas. Bioactive compounds extracted from natural products can offer specific properties that could act on specific targets and exhibit several

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biological properties for treating particular diseases. The use of medicinal plants has increased in recent years. Therefore, screening biological activities remains an important step in therapeutic virtues research. In vitro assays, including enzyme inhibition assays, are commonly used for biological properties, bioactive natural compounds screening, and also for drug synthesis. Many factors such as environmental conditions, UV radiation, foods, stress, as well as pollutants are all causes of free radical formation in the body. Free radicals can induce many diseases, such as different types of cancer, coronary artery disease, nervous system diseases, lung diseases, and also rheumatoid arthritis [5]. Moreover, they play an important role in tissue aging, including skin aging. It is a never-ending endeavor for researchers to attempt to find new active ingredients to counteract the aging process, especially focusing on antioxidant or anti-free radical capability and also anti-tyrosinase activity; which are involved in the prevention of skin aging and help to generate skin brightening. Numerous Thai plants have been used as healthcare and cosmetic products for many decades. Coffee is one of the economic plants which is widely grown in Thailand. It is a native plant of Africa in the Rubiaceae family and is very popular around the world, especially in Southeast Asia [6]. Coffea arabica (Arabica) is popularly grown in the Northern part of Thailand, while *Coffea canephora* (Robusta) is mostly cultivated in Southern Thailand. They differ in seed shape, smell, and taste. Robusta coffee is a major production in Thailand, with about 80,000-85,500 tons per year, whereas Arabica coffee production is only approximately 800-850 tons per year. Sixty percent of the Robusta coffee is exported and mostly used for instant coffee production. Most of the Arabica coffee is used in roasted and ground coffee for the domestic market.

Previous studies have extensively highlighted the myriad health benefits associated with consuming coffee. It has been demonstrated that regular coffee consumption can potentially mitigate the risk of developing neurodegenerative diseases such as Parkinson's and Alzheimer's, as well as conditions like hypertension, type 2 diabetes, and certain cancers. Moreover, coffee has been found to support liver function, making it a favorable beverage for overall health. Beyond its traditional role as a beverage, coffee beans exhibit remarkable properties that make them promising candidates for cosmeceutical products. Phytochemical analyses have revealed that green coffee beans contain caffeine, caffeic acid, chlorogenic acid, and trigonelline, while roasted coffee beans comprise caffeine, trigonelline, chlorogenic acid, and melanoidin [7]. These chemical constituents not only endow coffee beans with antioxidant properties but also confer additional benefits such as anti-inflammatory effects, inhibition of albumin denaturation, UV radiation protection, and antimicrobial activities [8]. Furthermore, recent research has highlighted the antifibrolytic properties of coffee, suggesting its potential in combating fibrotic conditions. Collectively, these findings emphasize the multifaceted therapeutic potential of coffee beans, positioning them as a valuable resource for dietary, medicinal, and cosmetic applications. Considering the broad spectrum of bioactivities—including antioxidant, anti-inflammatory, and antifibrolytic properties—of *Juglans regia* and *Coffea canephora*, their combination may be particularly beneficial in the management of various inflammatory and oral potentially malignant conditions. This is especially relevant for Oral Submucous Fibrosis (OSMF), where antifibrolytic activity plays a crucial role. Hence, this study aims to evaluate the therapeutic potential of a topical gel formulation incorporating *Juglans regia* and *Coffea canephora* extracts for the management of OSMF.

Materials and methods:

1.Preparation of extract:

To prepare the *Juglans regia L.* and *Coffea canephora* gel, 10 grams of walnut powder and 10 grams of coffee bean powder were weighed using a weighing scale. The walnut powder and coffee bean powder were then transferred into separate conical flasks. Next, 100 milliliters of distilled water were added to each conical flask. The conical flasks were placed in heating mantles and heated for 15 to 20 minutes, or until the extracts became hot to the

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touch. Once the extracts were hot, they were filtered using Muslin cloth or Whatman Grade 1 qualitative filter paper. The filtered extracts were collected in separate containers. Next, 50 milliliters of each extracted solution were heated at 50 to 60 ℃ until it reduced to 5 milliliters, forming a condensing solution. The condensing solutions were labeled separately and accurately. To prepare the gel, 20 grams of Carbopol 940 and CarboxyMethyl Cellulose (CMC) were taken. The Carbopol and CMC were then mixed with 2 milliliters of *Juglans regia L.* condensing solution and 2 milliliters of *Coffea canephora* condensing solution. The resultant extracts were frozen (4degree) in airtight containers to preserve the bioactive compounds and extend their shelf life.

Formulation of the gel:

First, 10 grams of walnut powder and 10 grams of coffee bean powder were weighed using a weighing scale. Then, the walnut powder and coffee bean powder were transferred into separate conical flasks. Next, 100 milliliters of distilled water were added to each conical flask, and the flasks were placed in heating mantles and heated for 15 to 20 minutes, or until the extracts were hot to the touch. Once the extracts were hot, they were filtered using Muslin cloth or Whatman Grade 1 qualitative filter paper. The filtered extracts were collected in separate containers. Next, 50 milliliters of each extracted solution were heated at 50 to 60 degrees Celsius until they were reduced to 5 milliliters, forming a condensing solution. The condensing solutions were labeled separately and accurately. To prepare the gel, 20 grams of Carbopol (940)and CarboxyMethyl Cellulose (CMC) were mixed with 2 milliliters of *Juglans regia* condensing solution and 2 milliliters of *Coffea canephora* condensing solution. The pH adjustment was accomplished by slowly adding sodium hydroxide (NaOH) solution drop by drop into the gel mixture while continuously stirring, until the desired pH range of 5.5 as achieved. After pH adjustment, the prepared gel was stored inamber colored glasss containers 940in the freezer to preserve the bioactive compounds and extend their shelf life.

2. Anti-oxidant activity:

A. DPPH assay:

To assess the antioxidant activity, we initiated the process by preparing a stock solution of 0.1 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) in methanol. For each test, a fresh working solution was created by diluting the stock solution to reach a final concentration of 20 µM in methanol. Subsequently, various concentrations (10, 20, 30, 40, 50 µg/mL) of the resultant extracts from *Juglans regia L.* and *Coffea canephora* were added to 200 µL of the DPPH working solution in a 96-well plate [9]. The plate was then incubated in darkness for 30 minutes at room temperature. Following incubation, we measured the absorbance at 517 nm using a microplate reader, with methanol serving as the blank. The percentage of DPPH scavenging activity was determined using the formula below:

$$\% \text{ DPPH Scavenging Activity} = [(A_{control} - A_{sample}) / A_{control}] \times 100$$

where Acontrol is the absorbance of the control (DPPH solution without the sample), and Asample is the absorbance of the sample (DPPH solution with the green synthesized silver nanoparticles).The positive control group consisted of ascorbic acid (1 mg/mL).

B. H_2O_2 assay:

In our study, we employed the hydroxyl radical scavenging assay to assess antioxidant activity, utilizing the method initially proposed by Halliwell et al. For this purpose, a 1 mL reaction mixture was prepared, containing 100 µL of 28 mM 2-deoxy-2-ribose. Different concentrations of the resultant extracts from *Juglans regia* and *Coffea canephora* (ranging from 10 to 50 µg/mL) were added to the mixture. Additionally, 200 µL of 200 µM ferric chloride, 200 µL of EDTA, and 100 µL of ascorbic acid were introduced. Subsequently, the reaction mixture was incubated for 1 hour at 37 °C, and the optical density was measured at 532 nm against a blank solution. As a positive control, we employed Vitamin E [10]. This method allowed us to evaluate the hydroxyl radical scavenging ability of the *Juglans regia L.* and *Coffea canephora* extracts and determine their antioxidant properties.

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Hydroxyl radical scavenging activity (%) = [(Ablank − Asample)/Ablank] × 100

Where Ablank is the absorbance of the control reaction (without sample), and Asample is the absorbance of the reaction with the sample.

C. FRAP assay:

FRAP (Ferric Reducing Ability of Plasma) solution (3.6 mL) was added to distilled water (0.4 mL) and incubated at 37˚C for 5 minutes. Then this solution was mixed with a certain concentration of the *Juglans regia L.* & *Coffea canephora* (resultant extracts) (80 mL) and incubated at 37˚C for 10 minutes. The absorbance of the reaction mixture was measured at 593 nm. For the construction of the calibration curve, five concentrations of FeSO4,7H2O (0.1, 0.4, 0.8, 1, 1.12, 1.5 mM) were used, and the absorbance values were measured as for sample solutions.

3. Anti-inflammatory activity:

A. Bovine serum albumin denaturation assay:

In our experimental procedure, we initiated by combining 0.45 mL of bovine serum albumin with 0.05 mL of the resultant extracts from *Juglans regia L.* and *Coffea canephora*, each at varying concentrations (ranging from 10 to 50 µg/mL). The pH of the mixture was carefully adjusted to 6.3. Following this, the solution was left at room temperature for 10 minutes, after which it was incubated in a water bath at 55°C for a duration of 30 minutes.As part of our analysis, we utilized diclofenac sodium as a standard reference group, and dimethyl sulfoxide was included as a control. Subsequently, spectrophotometric measurements were taken from the samples at a wavelength of 660 nm [11]

Percentage of protein denaturation was determined utilizing following equation,

$$\% \text{ inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of sample} \times 100}{\text{Absorbance of control}}$$

B. Egg Albumin denaturation assay:

To conduct the Egg albumin denaturation assay, we initiated the procedure by combining 0.2 mL of fresh egg albumin with 2.8 mL of phosphate buffer. Various concentrations (ranging from 10 to 50 µg/mL) of the extracts obtained from *Juglans regia L.* and *Coffea canephora* were introduced into the reaction mixture. The pH was adjusted to 6.3. The solution was then left at room temperature for 10 minutes, followed by a 30-minute incubation in a water bath at 55°C. For reference, diclofenac sodium served as the standard control group, and dimethyl sulfoxide was included as the control. After the incubation period, spectrophotometric measurements were taken from the samples at a wavelength of 660 nm [12].

The percentage of protein denaturation was determined using the following equation:

$$\% \text{ inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of sample} \times 100}{\text{Absorbance of control}}$$

C. Membrane stabilization assay:

The in vitro membrane stabilization assay was a widely used technique for evaluating the membrane-stabilizing properties of natural and synthetic compounds. This assay measured the ability of a compound to stabilize the cell membrane by preventing its disruption and subsequent release of intracellular contents. The materials used included Human red blood cells (RBCs), Phosphate-buffered saline (PBS), Tris-HCl buffer (50 mM, pH 7.4), different concentrations of the resultant extracts from *Juglans regia L.* and *Coffea canephora* (ranging from 10 to 50 µg/mL), a centrifuge tube, and a UV-Vis spectrophotometer [13].

Preparation of RBC suspension:

Fresh human blood was collected in a sterile tube containing an anticoagulant. The blood was then centrifuged at 1000 g for 10 minutes at room temperature to separate the RBCs from other blood components. The supernatant was removed, and the RBCs were washed three times with PBS. Subsequently, the RBCs were resuspended in Tris-HCl buffer to obtain a 10% (v/v) RBC suspension.

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Assay procedure:

One ml of the RBC suspension was pipetted into each centrifuge tube. Then, different concentrations of the resultant extracts from *Juglans regia L.* and *Coffea canephora* were added to each tube. The mix was gently stirred, and the tubes were incubated at 37°C for 30 minutes. Subsequently, the tubes were centrifuged at 1000 g for 10 minutes at room temperature to pellet the RBCs. The absorbance of the supernatant was measured at 540 nm using a UV-Vis spectrophotometer.

The percentage inhibition of hemolysis was calculated using the following formula:

% inhibition = [(OD control – OD sample) / OD control] x 100

where OD control is the absorbance of the RBC suspension without the test compound(s) and OD sample is the absorbance of the RBC suspension with the test compound.

Results:

Significant differences were detected in the antioxidant and anti-inflammatory potency between the standard and Coffee Bean + Walnut gel extract. The six distinct assays yielded the subsequent results:

1. DPPH Radical Scavenging Assay:

DPPH Radical Scavenging Assay showed that the antioxidant activity of the prepared gel under investigation had comparable results with the standard Ascorbic acid which was employed as a positive control (1mg/mL). The scavenging ability of Coffee bean and Walnut gel on DPPH Assay was compared with Different concentrations (10,20,30,40,50 µg/mL). The antioxidant activity of the gel increases with increasing concentration of the gel. This is because a higher concentration of the gel contains more antioxidants, which can scavenge more DPPH free radicals. For 10µg/mL of the gel concentration, the DPPH radical inhibition activity was 63.67%, for 20µg/mL it was 74.04%, for 30µg/mL it was 82.65%, for 40µg/mL it was 86.97% and at its highest concentration of 50 µg/ml tested, the biosynthesized coffee bean + walnut gel extract exhibited the most significant DPPH radical inhibition activity (91.45%). A dose-dependent antioxidant activity that was comparable to the DPPH-scavenging activity of ascorbic acid (Standard) was observed. This suggests that the gel has strong antioxidant properties.

Table 1: Anti-oxidant activity (DPPH Assay)

Concentration is in µg/mL. Values represent percentage inhibition (%).

Concentration (µg/mL)	10	20	30	40	50
Ascorbic acid (Standard)	66.25	78.52	85.63	88.68	93.15
Coffea canephora + Juglans regia extract gel	63.67	74.04	82.65	86.97	91.45

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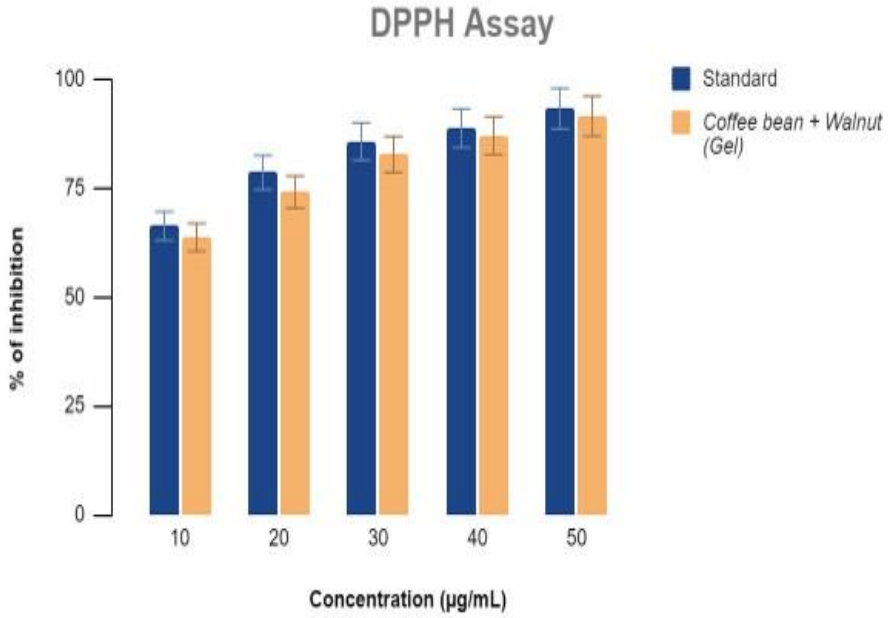

Graph 1: DPPH assay showing antioxidant activity of coffee bean and walnut (gel) extract compared with standard at concentrations of 10–50 µg/ml. Data represent mean ± SD.

2. Hydrogen Peroxide H_2O_2 Assay:

H_2O_2 Scavenging Assay showed that the antioxidant activity of the prepared gel under investigation had comparable results with the standard Vitamin E which was employed as a positive control. The scavenging ability of Coffee bean and Walnut gel on H_2O_2 was compared with Different concentrations (10,20,30,40,50 µg/mL). The antioxidant activity of the gel increases with increasing concentration of the gel. This is because a higher concentration of the gel contains more antioxidants, which can scavenge more H_2O_2 assay. For 10µg/mL of the gel concentration, the H_2O_2 assay activity was 48.8%, for 20µg/mL it was 50.4%, for 30µg/mL it was 62.7%, for 40µg/mL it was 74.3% and at its highest concentration of 50 µg/ml, the biosynthesized coffee bean + walnut gel extract displayed the most robust H_2O_2 scavenging activity (84.5%). A dose-dependent antioxidant activity that was comparable to H_2O_2 scavenging activity of Vitamin-E (Standard) was observed. H_2O_2 Assay. This suggests that the gel has strong antioxidant properties activity here as well.

Table 2: Anti-oxidant activity (H_2O_2 Assay)

Concentration is in µg/mL. Values represent percentage inhibition (%).

Concentration (µg/mL)	10	20	30	40	50
Ascorbic acid (Standard)	51.1	56.9	66.1	78.8	89.9
Coffea canephora + Juglans regia extract gel	48.8	50.4	62.7	74.3	84.5

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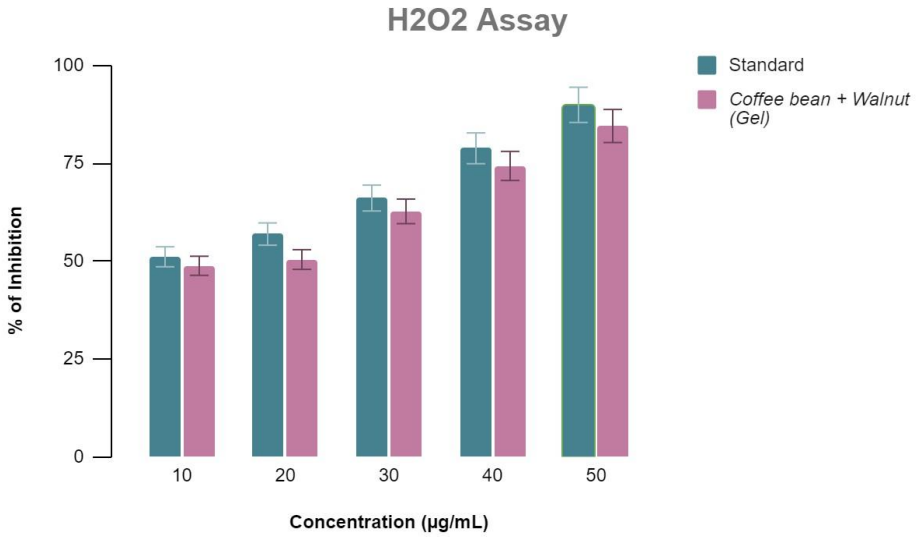

Graph 2: H_2O_2 assay showing antioxidant activity of coffee bean and walnut (gel) extract compared with standard at concentrations of 10–50 µg/ml. Data represent mean ± SD.

3. FRAP assay:

The ferric reducing ability of plasma (FRAP) antioxidant potential of Coffee bean and Walnut gel was determined using the FRAP assay developed by Benzie and Strain in 1996. The antioxidant potentials of the resultant extract were compared to the Standard (FeSO4, 7H2O). It is a colorimetric assay that measures the total antioxidant capacity of a sample. The FRAP value of the gel increases with increasing concentration. The FRAP assay activity of Coffee bean and Walnut gel was compared with FeSO4, 7H2O FRAP Assay activity that was reported (Standard) for different concentrations (10,20,30,40,50 µg/mL). For 10µg/mL of the gel concentration, the FRAP assay activity was 69.34%, for 20µg/mL it was 73.58%, for 30µg/mL it was 79.84%, for 40µg/mL it was 81.49% and at its highest concentration of 50 µg/ml, the biosynthesized coffee bean + walnut gel extract displayed the highest activity (88.93%).The results of the FRAP assay suggest that the *Juglans regia L.* and *Coffea canephora* extract gel has strong antioxidant properties.

Table 3: Anti-oxidant activity (FRAP Assay)

Concentration is in µg/mL. Values represent percentage inhibition (%).

Concentration (µg/mL)	10	20	30	40	50
Ascorbic acid (Standard)	72.98	76.84	81.31	85.84	90.89
Coffea canephora + Juglans regia extract gel	69.34	73.58	79.84	81.49	88.93

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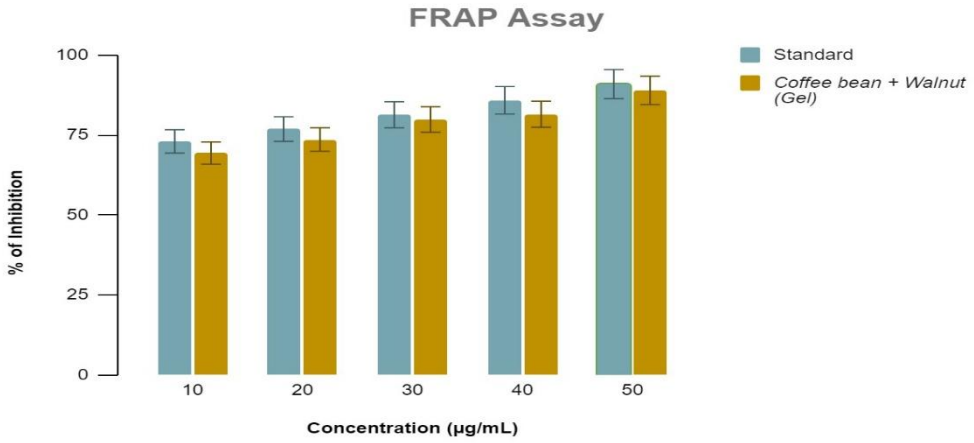

Graph 3: FRAP assay showing antioxidant activity of coffee bean and walnut (gel) extract compared with standard at concentrations of 10–50 µg/ml. Data represent mean ± SD.

4. Bovine serum albumin (BSA) denaturation assay:

The Bovine serum albumin (BSA) assay was employed to measure the anti-inflammatory activity of Coffee bean and Walnut extract gel. The BSA assay activity of Coffee bean and Walnut gel was compared with Diclofenac sodium (Standard) for different concentrations (10,20,30,40,50 µg/mL). For 10µg/mL of the gel concentration, the BSA assay activity was 41%, for 20µg/mL it was 55%, for 30µg/mL it was 67%, for 40µg/mL it was 73% and at its highest concentration of 50 µg/ml, the biosynthesized coffee bean + walnut gel extract displayed the most significant BSA assay activity (79%). The results of the BSA assay suggest that the *Juglans regia L.* and *Coffea canephora* extract gel has strong anti-inflammatory properties.

Table 4: Percentage inhibition of protein denaturation by different concentrations of extract and standard (BSA Assay)

Concentration is in µg/mL. Values represent percentage inhibition (%).

Concentration (µg/mL)	10	20	30	40	50
Coffea canephora + Juglans regia extract gel	41	55	67	73	79
Diclofenac sodium (Standard)	47	60	72	78	84

Footnotes:

The results are expressed as % inhibition of protein denaturation.

Coffea canephora = Coffee bean extract, Juglans regia = Walnut extract.

Standard used: Diclofenac sodium.

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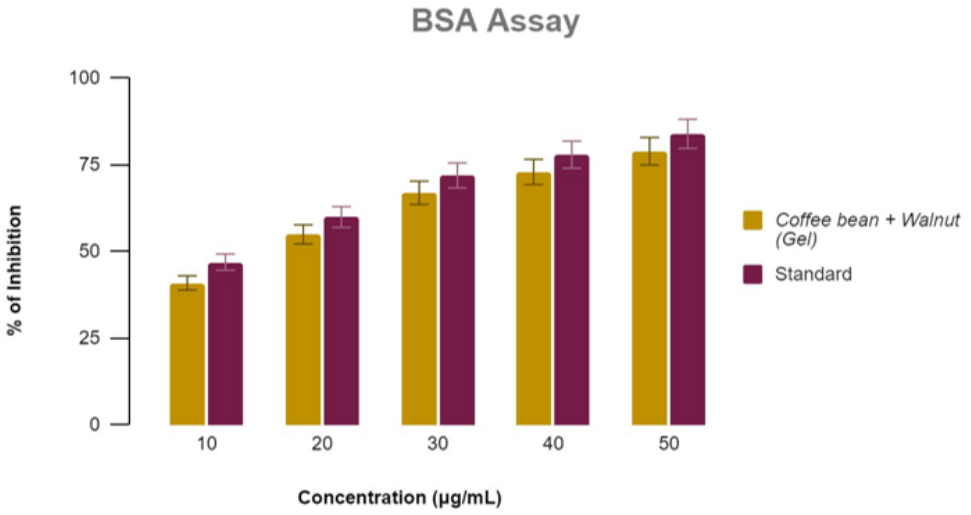

Graph 4: BSA assay showing anti-inflammatory activity of coffee bean and walnut (gel) extract compared with standard at concentrations of 10–50 µg/ml. Data represent mean ± SD.

5. Egg albumin (EA) denaturation assay:

Thermal treatments applied to egg white caused undesirable modifications to their physicochemical and functional properties. The results demonstrated that the Coffee bean and Walnut extract gel, used within a concentration range of 10-50 µg/ml, exhibited a concentration-dependent inhibition of protein denaturation. For 10µg/mL of the gel concentration, the EA assay activity was 50%, for 20µg/mL it was 59%, for 30µg/mL it was 63%, for 40µg/mL it was 68% and at its highest (50 µg/ml), the maximum percentage of inhibition (76%) which was comparable to that of the standard drug (Diclofenac sodium) at 81% (50 µg/ml) used in the study. The results of the EA assay suggest that the *Juglans regia L.* and *Coffea canephora* extract gel has strong anti-inflammatory properties even in this test.

Table 5: Percentage inhibition of protein denaturation (EA Assay)

Concentration is in µg/mL. Values represent percentage inhibition (%).

Concentration (µg/mL)	10	20	30	40	50
Coffea canephora + Juglans regia extract gel	50	59	63	68	76
Diclofenac sodium (Standard)	55	64	69	72	81

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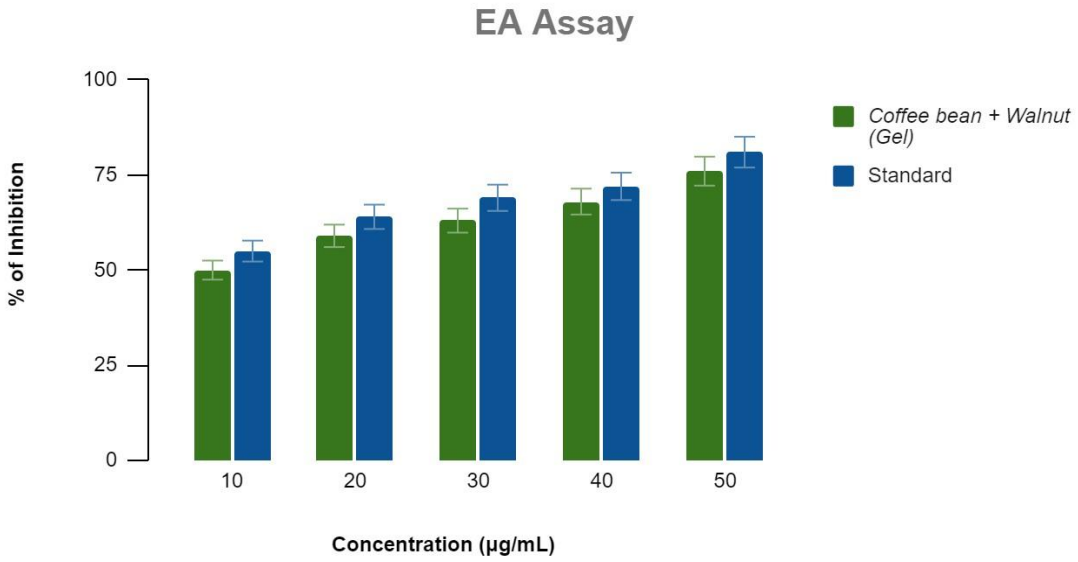

Graph 5: EA assay showing anti-inflammatory activity of coffee bean and walnut (gel) extract compared with standard at concentrations of 10–50 µg/ml. Data represent mean ± SD.

6. Membrane stabilization assay: HRBC (Human red blood cell)

Human red blood cell (HRBC) membranes are similar to lysosomal membrane components, and inhibition of hypotonicity and heat-induced red blood cell membrane lysis was employed as a measure of the potency of the extract's anti-inflammatory activity. The results for the Coffee bean and Walnut extract gel revealed that, for 10µg/mL of the gel concentration, the membrane stabilization assay activity was 52%, for 20µg/mL it was 67%, for 30µg/mL it was 71%, for 40µg/mL it was 78% and at its highest concentration of 50 µg/ml, it displayed the most significant membrane stabilization assay activity (86%). This activity was comparable to NSAIDs (aspirin) [Standard], and it demonstrated significant prevention of the leakage of serum proteins and fluids in a dose-dependent manner. The results of the Membrane stabilization assay suggest that the *Juglans regia L.* and *Coffea canephora* extract gel has strong anti-inflammatory properties.

Table 6: Percentage inhibition of membrane lysis (Membrane Stabilization Assay)

Concentration is in µg/mL. Values represent percentage inhibition (%).

Concentration (µg/mL)	10	20	30	40	50
Coffea canephora + Juglans regia extract gel	52	67	71	78	86
Diclofenac sodium (Standard)	58	70	77	82	89

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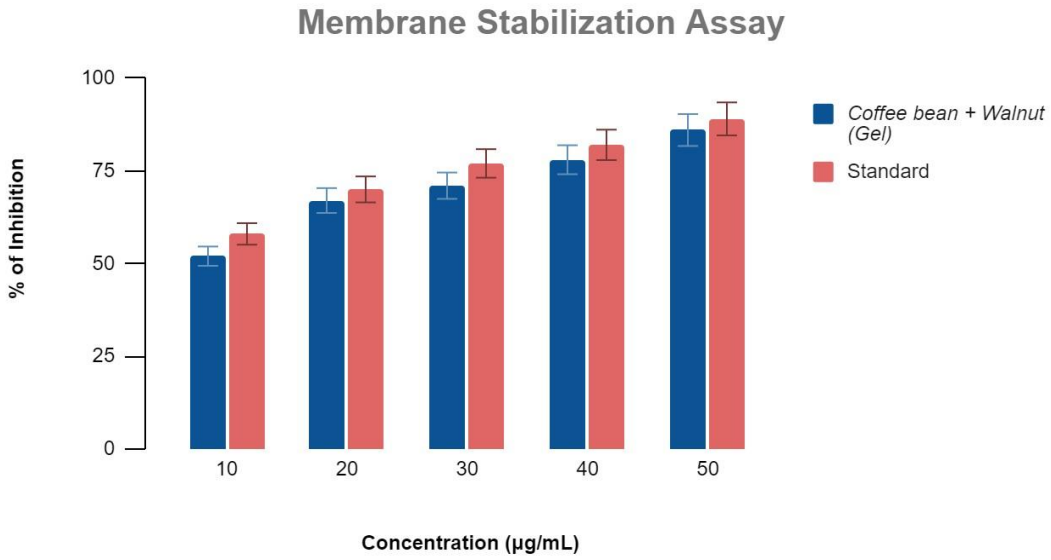

Graph 6: Membrane stabilization assay showing anti-inflammatory activity of coffee bean and walnut (gel) extract compared with standard at concentrations of 10–50 µg/ml. Data represent mean ± SD.

Discussion:

The antioxidant and anti-inflammatory properties of the Coffee bean and Walnut extract gel are likely due to the presence of bioactive compounds in the gel. These bioactive compounds include polyphenols, flavonoids, and tannins. Polyphenols are known for their ability to scavenge free radicals, inhibit lipid peroxidation, and protect against protein denaturation. Flavonoids are known for their anti-inflammatory and antioxidant properties. Tannins are known for their ability to bind to proteins and protect them from denaturation.

The DPPH radical scavenging assay showed that the gel had a strong ability to scavenge free radicals. Free radicals are unstable molecules that can damage cells by oxidizing DNA, proteins, and lipids. The gel's ability to scavenge free radicals suggests that it may be useful for protecting cells from oxidative stress. This was in accordance with studies conducted by Yalcinkaya C. et al [14] wherein DPPH assay in Mirra *(Coffea arabica)* sample was performed using a spectrophotometric method. High antioxidant activity was observed and the results suggested that DPPH was a useful tool for assaying the antioxidant activity in Mirra and it showed that DPPH assay activity showed a good correlation with different antioxidant compounds used in that study.

The hydrogen peroxide H_2O_2 assay showed that the gel was able to scavenge hydrogen peroxide. Hydrogen peroxide is a reactive oxygen species that can cause oxidative damage to cells. The gel's ability to scavenge hydrogen peroxide suggests that it may be useful for protecting cells from oxidative stress This was in accordance with study conducted by Raja B et al [15] wherein the hydroxyl radical scavenging activity was determined by Halliwell's method. In this assay, OH^- is produced by reduction of H_2O_2 by the transition metal (iron) in the presence of ascorbic acid. The generation of OH^- is detected by its ability to degrade deoxyribose to form products, which on heating with thiobarbituric acid (TBA) forms a pink colour, chromogen

The FRAP assay showed that the gel had a strong reducing power. The reducing power of a substance is its ability to donate electrons to another substance. The gel's reducing power suggests that it may be useful for protecting cells from oxidative stress This was in accordance with studies conducted by Simon C, Langley-Evans [16] wherein they compared the

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antioxidant properties of green tea, black leaf and black bagged teas over a range of infusion times from 15 s to 15 min. at the infusion temperature of 90°C black leaf and black bagged teas had similar antioxidant potential (FRAP) across the full timescale of the experiment. Green tea had significantly greater antioxidant potential than the black teas at 15 s, and from 2 to 15 min infusion time. At 15 min, green tea had 2.5-fold higher FRAP than both black teas. Release of antioxidants from the teas was rapid. For black teas maximal antioxidant potential was present in the infusate by 5 min and 82–91% of the maximal FRAP was present at 2 min. Although green tea also released antioxidants into the infusate rapidly, equilibrium was never achieved and the FRAP value was still increasing at 15 min. An initial rapid phase of release resulted in 61% of the 15 min FRAP value being present in the infusate at 2 min.

The bovine serum albumin denaturation assay and egg albumin denaturation assay showed that the gel was able to inhibit protein denaturation. Protein denaturation is the process by which proteins lose their structure and function. The gel's ability to inhibit protein denaturation suggests that it may be useful for protecting cells from inflammation. This was in accordance with studies conducted by Priya M et al [17] wherein the Invitro Anti-inflammatory activity of synthesized compounds were studied using denaturation of protein by Bovine serum albumin (BSA) and results were compared with std Ibuprofen. 3-(4Bromo phenyl)-4-(3H)quinazolinone , 3-(4-methyl phenyl)-4- (3H) quinazolinone exhibited highest invitro anti-inflammatory activity among the synthesized compounds.

The egg albumin denaturation assay is a valuable tool for screening substances for anti-inflammatory activity. The results of the egg albumin denaturation assay showed that the Coffee bean and Walnut extract gel was able to inhibit egg albumin denaturation in a dose-dependent manner. This suggests that the gel has anti-inflammatory activity. This was in accordance with studies conducted by Kumar N. et al [18] wherein egg albumin denaturation method and HRBC membrane stabilization methods were used to estimate anti-inflammatory potential in-vitro. Thermal treatments applied to egg white causes undesirable modifications to their physicochemical and functional properties. The results showed a sigmoidale evolution with negative slope of transmittance due to irreversible loss of solubility. Coagulation and/or precipitation of egg white protein has been observed at around 74°C for 20 min.

The membrane stabilization assay showed that the gel was able to stabilize cell membranes. Cell membranes are essential for cell function and integrity. The gel's ability to stabilize cell membranes suggests that it may be useful for protecting cells from damage. This was in accordance with studies conducted by Kumar N. et al [18] wherein Membrane stabilization leads to prevention of leakage of serum protein and fluids into the tissues during a period of increased permeability caused by inflammatory mediators.

The antioxidant and anti-inflammatory effects of coffee and walnut extracts stem from their rich content of polyphenols and flavonoids. These compounds neutralize free radicals, reduce oxidative stress, and modulate inflammatory pathways by inhibiting enzymes like cyclooxygenase and lipoxygenase, and suppressing pro-inflammatory mediators. This beneficial activity suggests their potential in preventing and treating conditions related to oxidative stress and inflammation.

Overall, the results of the study suggest that the Coffee bean and Walnut extract gel has significant antioxidant and anti-inflammatory activities. The gel may be useful for preventing and treating a variety of conditions, including oxidative stress, inflammation, and chronic diseases.

Conclusion:

In conclusion, the novel topical gel incorporating *Juglans regia L.* and *Coffea canephora* extracts demonstrated promising antioxidant and anti-inflammatory properties, making it a potential alternative or adjunct treatment option for inflammatory and potentially malignant disorders,

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especially OSMF. The gel's natural composition and ability to address the underlying mechanisms of the disease offer a patient-centered treatment option with reduced side effects and improved long-term effectiveness compared to conventional treatments.

Limitations and Future Scope:

The present study was limited to in-vitro assays without in-vivo or clinical validation, and future work should focus on molecular pathway analysis, antifibrolytic and antifungal evaluation, as well as stability and clinical studies to establish the gel's safety and efficacy in OPMD management.

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