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Evaluation Of Antioxidant, Anti-Inflammatory Properties Of Juglans Regia L. And Coffea Canephora L. Combination Extract Capsule- In Vitro Study

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Abstract

Background: *Plant-derived bioactive compounds have gained increasing attention as potential therapeutic agents due to their antioxidant and anti-inflammatory properties. Juglans regia L. (walnut) and Coffea canephora L. (robusta coffee) are rich in polyphenols, flavonoids, and other phytoconstituents known to modulate oxidative stress and inflammation. This study evaluated the antioxidant and anti-inflammatory activities of a novel combination extract capsule of J. regia and C. canephora prepared through standardized extraction and encapsulation techniques.*

Materials and Methods: *Aqueous extracts of J. regia kernel and C. canephora green beans were homogenized, condensed, lyophilized, and formulated into gelatin capsules containing 500mg of the combination extract. Antioxidant potential was assessed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, while anti-inflammatory activity was determined using the albumin denaturation method. Both assays were conducted at concentrations of 20, 40, 60, 80, and 100 µg/ml, with ascorbic acid and diclofenac sodium serving as standards, respectively.*

Results: *The extract exhibited a concentration-dependent increase in both antioxidant and anti-inflammatory activities. Maximum antioxidant activity was observed at 100 µg/ml with 69.9% inhibition, compared to 84.8% for ascorbic acid. Similarly, the anti-inflammatory assay showed 74.4% inhibition at 100 µg/ml, which was comparable o diclofenac sodium (87.6%).*

Conclusion: *The combination extract of J. regia and C. canephora demonstrated significant in vitro antioxidant and anti-inflammatory activities, supporting its potential role as a natural therapeutic agent for conditions involving oxidative stress and inflammation, including Oral Submucous Fibrosis (OSMF). Further in vivo and clinical studies are warranted to validate these findings.*

Keywords: *Juglans regia, Coffea canephora, antioxidant, anti-inflammatory, DPPH assay, albumin denaturation, Oral Submucous Fibrosis*

INTRODUCTION:

Natural products have long played a central role in medicine, serving as sources for therapeutic agents with diverse biological activities. Phytochemicals from nuts, seeds, and beverages exhibit antioxidant, anti-inflammatory, antimicrobial, and anticancer effects and are increasingly valued for their safety, accessibility, and multi-target potential compared to synthetic drugs, which may carry toxicity or resistance risks (Newman and Cragg, 2020). Interest has therefore grown in plant-derived molecules that modulate oxidative stress and inflammation, two interconnected processes central to chronic disease pathogenesis (Reuter et al., 2010; Liguori et al., 2018).

Oxidative stress arises from excessive reactive oxygen species (ROS) generation relative to antioxidant defenses, leading to lipid peroxidation, DNA strand breaks, and protein damage. Persistent oxidative imbalance promotes cardiovascular, metabolic, neurodegenerative, and oral potentially malignant disorders. Inflammation, while protective in acute settings, becomes pathological when prolonged, contributing to fibrosis, tissue degeneration, and carcinogenesis (Medzhitov, 2008). Importantly, these two processes amplify each other: ROS activate NF-κB signaling, inducing pro-inflammatory cytokines such as TNF-α and IL-6, while inflammatory mediators further increase ROS production (Mittal et al., 2014). Agents with both antioxidant and anti-inflammatory actions therefore hold promise for disease prevention and management (Pisoschi and Pop, 2015).

Juglans regia L. (walnut), a member of the Juglandaceae family, is widely consumed and recognized for its nutritional and medicinal properties. Its kernels, husks, and leaves contain bioactive compounds including ellagic acid, catechins, tannins, tocopherols, and polyunsaturated fatty acids (Carvalho et al., 2010; Martínez et al., 2010). Several studies confirm strong antioxidant activity of walnut extracts. Rusu et al. (2020) demonstrated significant radical scavenging in DPPH assays, while Fukuda et al. (2003) reported improved antioxidant enzyme activity in animal models following walnut supplementation. Anti-inflammatory potential has also been documented. Abdulalsalam et al. (2017) showed 62.7% inhibition of albumin denaturation by walnut kernel extract at 400 µg/ml, while other studies reported suppression of cyclooxygenase activity and reduced expression of TNF-α and IL-6 (Anderson et al., 2001). Such findings position walnuts as potent sources of antioxidant and anti-inflammatory phytoconstituents.

Coffea canephora L. (robusta coffee), another plant of high nutritional and pharmacological relevance, contains higher levels of chlorogenic acids and caffeine compared with *Coffea arabica* (Farah and Donangelo, 2006). Chlorogenic acids are known radical scavengers and metal chelators that contribute to antioxidant potential. Negri et al. (2016) demonstrated strong DPPH activity of green robusta beans, proportional to chlorogenic acid concentration. Anti-inflammatory effects have also been observed. Chandra et al. (2012) reported that green coffee extract inhibited egg albumin denaturation with an IC_{50} of ~40 µg/ml, significantly outperforming diclofenac sodium, which showed an IC_{50} of 625 µg/ml. These findings emphasize the therapeutic promise of robusta coffee beyond its role as a stimulant.

Combining *Juglans regia* and *Coffea canephora* offers a logical synergistic approach. Walnut polyphenols and flavonoids, together with coffee chlorogenic acids and caffeine, may act additively to scavenge ROS, inhibit pro-inflammatory enzymes, and stabilize proteins. Herbal pharmacology often demonstrates that mixtures of bioactives exert superior biological effects compared with isolated compounds due to enhanced bioavailability, stability, and multi-target interactions (Williamson, 2001). Such synergy could be particularly valuable in Oral Submucous Fibrosis (OSMF), a chronic progressive oral condition strongly linked to areca nut chewing. OSMF is characterized by epithelial atrophy, submucosal fibrosis, restricted mouth opening, and malignant potential (Tilakaratne et al., 2006). Pathogenesis involves persistent oxidative stress, collagen cross-linking, and cytokine-mediated inflammation, making antioxidants and anti-inflammatory agents important therapeutic options.

Several herbal antioxidants including lycopene, curcumin, aloe vera, and spirulina have been trialed in OSMF with encouraging results (Warnakulasuriya and Ranganathan, 2023). However, standardization, reproducibility, and translation from in vitro to clinical success remain challenging. Developing a standardized walnut–coffee capsule using lyophilization and encapsulation may provide controlled dosing, enhanced stability, and improved compliance, thus addressing some of these limitations.

In vitro assays remain critical first steps in evaluating such formulations. The DPPH assay provides a reliable measure of radical scavenging ability, while the albumin denaturation method reflects anti-inflammatory potential by testing protein stabilization under stress conditions (Pisoschi and Pop, 2015). Both assays are widely accepted in validating plant extracts prior to advancing to animal or clinical models. By applying these methods, the present study investigates whether a combined *Juglans regia* and *Coffea canephora* capsule demonstrates significant antioxidant and anti-inflammatory effects comparable to standard agents such as ascorbic acid and diclofenac sodium.

In summary, oxidative stress and inflammation are central to numerous chronic diseases, including OSMF. Walnuts and robusta coffee are both rich in bioactive compounds with proven antioxidant and anti-inflammatory activities. Yet their combined effects in a capsule formulation remain largely unexplored. This study seeks to address this gap by systematically preparing and testing a lyophilized walnut–coffee combination capsule in vitro. The results may provide valuable evidence to support its further development as a natural therapeutic strategy for oxidative and inflammatory conditions.

MATERIALS AND METHODS:

The present in vitro study was designed to evaluate the antioxidant and anti-inflammatory properties of a combination extract capsule containing *Juglans regia* L. and *Coffea canephora* L. The methodology included the preparation of the combined capsule formulation, followed by assessment of its bioactivity using the DPPH radical scavenging assay for antioxidant activity and the albumin denaturation assay for anti-inflammatory activity. All procedures were performed under controlled laboratory conditions to ensure reproducibility and accuracy.

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Capsule Preparation

The formulation of the combined herbal extract began with the preparation of separate aqueous extracts of *Juglans regia* L. (walnut kernel) and *Coffea canephora* L. (green coffee bean). For each extract, 30 g of finely powdered sample was dissolved in 150 ml of sterile boiled water maintained at 100°C in individual 250 ml beakers. The mixtures were subjected to homogenization at 1000 rpm using a high-speed overhead stirrer for 45 minutes to ensure uniform dispersion of the plant material. After homogenization, the extracts were filtered through a muslin cloth to remove coarse residues, yielding clear aqueous extracts.

The filtrates from both plants were then combined in a 500 ml beaker and subjected to condensation on a magnetic stirrer at 60 °C for 2 hours, reducing the volume by approximately 50%. The condensed mixture was subsequently frozen at –80 °C for 24 hours before being lyophilized at 0.01 mbar pressure to obtain a stable powdered extract. The final lyophilized powder was carefully weighed and encapsulated into gelatin capsules containing 500 mg of the combined *Juglans regia* and *Coffea canephora* extract. Formulation and preparation were carried out under controlled laboratory conditions, ensuring standardization and quality consistency throughout the process.

Figure 1: *Juglans regia* L. powdered extract

Figure 2: *Coffea canephora* L. powdered Extract

Figure 3: *Juglans regia* L. aqueous extract

Figure 4: *Coffea canephora* L. aqueous Extract

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Figure 5: Homogenization of *Juglans regia* L. aqueous extract using a high-speed overhead stirrer.

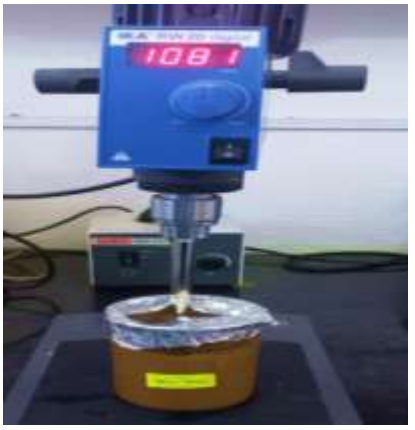

Figure 6: Homogenization of *Coffea canephora* L. aqueous extract using high-speed overhead stirrer.

Figure 7: Filtration of *Juglans regia* L & *Coffea canephora* L. using muslin cloth.

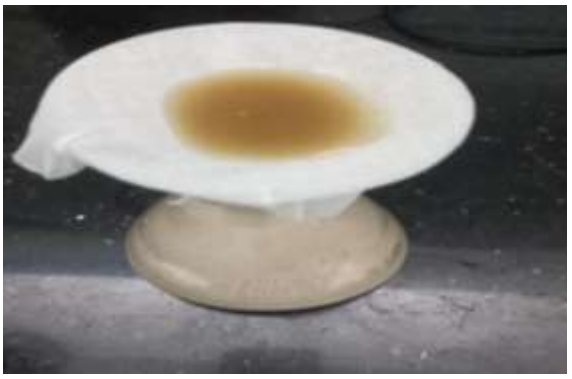

Figure 8: Condensation of *Juglans regia* L & *Coffea canephora* L.

Figure 9: Lyophilization (freeze drying) of *Juglans regia* L. & *Coffea canephora* L.

Figure 10: *Juglans regia* L. & *Coffea canephora* L. combination extract.

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Figure 11: *Juglans regia* L. & *Coffea canephora* L. combination extract capsule

Antioxidant Activity: DPPH Assay:

The antioxidant potential of the combination extract was assessed using the standard 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. The extract was tested at concentrations of 20, 40, 60, 80, and 100 µg/ml, with ascorbic acid used as the reference standard. The principle of the assay is based on the reduction of the DPPH radical, a stable free radical with a characteristic absorbance, by antioxidant molecules, resulting in a measurable decrease in absorbance.

Absorbance values were recorded spectrophotometrically, and the percentage of radical scavenging activity was calculated by comparing the absorbance of the test sample with that of the control. All measurements were performed in triplicate to ensure reliability.

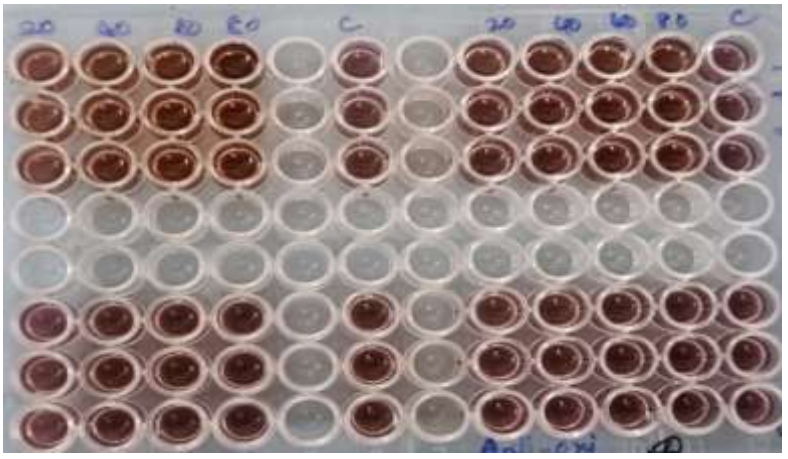


Figure 12: DPPH radical scavenging assay of *Juglans regia* L. *and Coffea canephora* L. extract at varying concentrations in 96-well microplate

Anti-inflammatory Activity: Albumin Denaturation Assay

The anti-inflammatory activity of the extract was evaluated using the protein denaturation method with egg albumin. The test was conducted at concentrations of 20, 40, 60, 80, and 100 µg/ml of the combination extract. Diclofenac sodium was used as the standard drug for comparison. Protein denaturation, a common mechanism in inflammation, was induced by heating albumin under controlled conditions. The ability of the extract to inhibit denaturation was quantified spectrophotometrically by measuring changes in absorbance, with higher inhibition values corresponding to stronger anti-inflammatory potential.

The percentage inhibition of protein denaturation was calculated for each concentration of the extract and compared with the standard diclofenac sodium. All assays were performed in triplicate to minimize variability and ensure reproducibility of results.

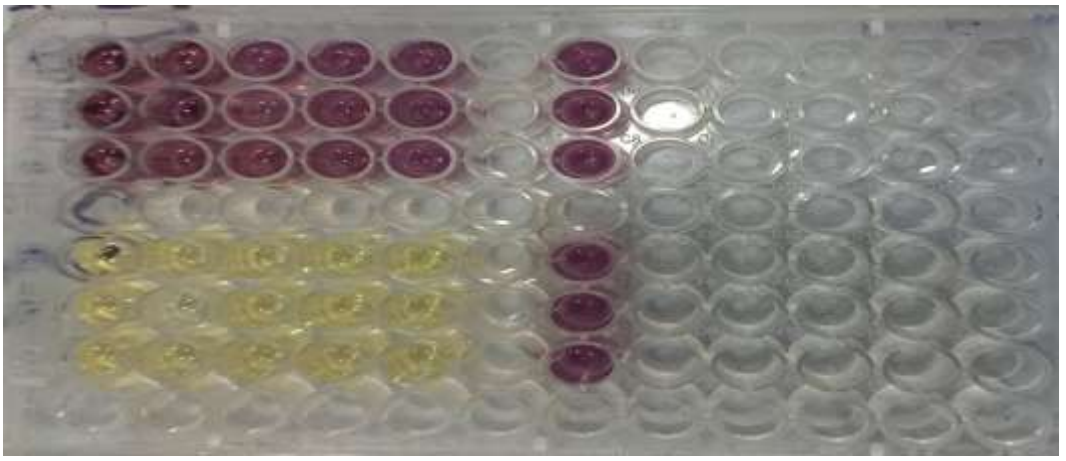


Figure 13: Evaluation of anti-inflammatory activity of *Juglans regia* L. and *Coffea canephora* L. extract using albumin denaturation assay in 96-Well microplate

RESULTS:

The antioxidant and anti-inflammatory activities of the *Juglans regia* L. and *Coffea canephora* L. combination extract capsule were evaluated using the DPPH radical scavenging assay and the albumin denaturation assay, respectively. Both assays demonstrated a concentration-dependent increase in activity, confirming the bioactive potential of the combined extract.

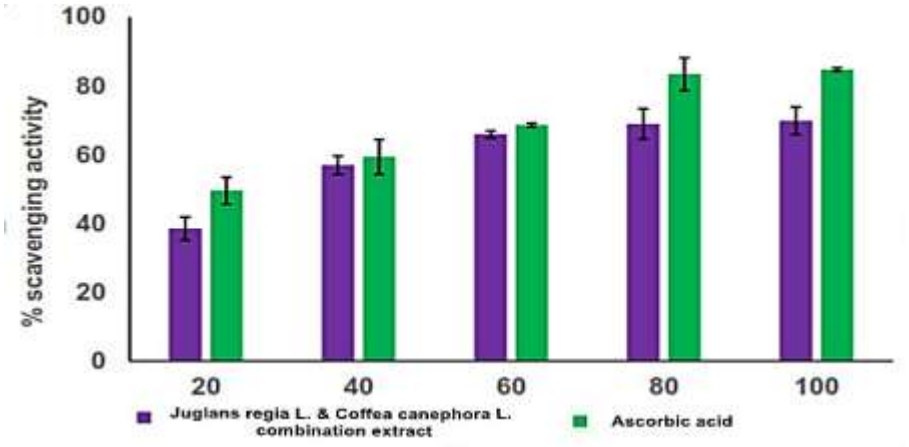
In the DPPH radical scavenging assay, the extract exhibited progressive free radical inhibition across the tested concentrations of 20–100 µg/ml. At the lowest concentration (20 µg/ml), the scavenging activity was modest but increased steadily with dose escalation. The maximum radical scavenging was observed at 100 µg/ml, where the extract achieved 69.9% inhibition. This activity was close to that of the standard antioxidant ascorbic acid, which demonstrated 84.8% inhibition at the same concentration (Graph 1). These findings confirm that the walnut–coffee combination extract possesses strong antioxidant capacity, attributable to its polyphenolic and flavonoid constituents.

The albumin denaturation assay also revealed notable anti-inflammatory activity of the extract. A dose-dependent inhibition of protein denaturation was observed, with the highest activity recorded at 100 µg/ml, where the extract demonstrated 74.4% inhibition. The standard anti-inflammatory drug diclofenac sodium showed 87.6% inhibition under identical conditions. At lower concentrations, the extract exhibited proportionally lower inhibition; however, a clear concentration-response relationship was evident across the tested range (20–100 µg/ml) (Graph 2). The findings indicate that the combination extract is effective in preventing protein destabilization, thereby reflecting potential anti-inflammatory activity.

Statistical analysis of triplicate assays confirmed the reproducibility of results, with values expressed as mean ± SD. Both assays demonstrated statistically significant increases in activity with increasing concentration of the extract ($p < 0.05$), and the results, although slightly lower than those of the respective standard controls, remained within a comparable range.

Inference

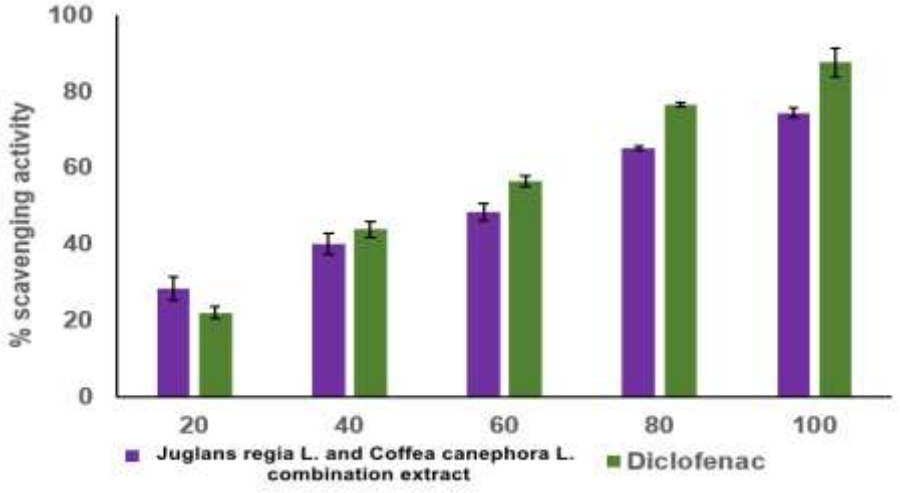
The antioxidant activity of the *Juglans regia* L. and *Coffea canephora* L. combination extract was measured at concentrations of 20, 40, 60, 80, and 100 µg/ml using the DPPH assay. The results demonstrated a concentration-dependent increase in free radical scavenging activity for both the sample (*Juglans regia* L. and *Coffea canephora* L. combination extract) and the standard (Ascorbic acid). The maximum percentage of inhibition was observed at 100 µg/ml, with the extract showing 69.9% activity and the standard showing 84.8%.



Graph 1: *Antioxidant activity of Juglans regia L. and Coffea canephora L. combination extract – formulation with standard DPPH (2,2- diphhenyl-1-picrylhydrazyl) assay*

Inference

The anti-inflammatory activity of the *Juglans regia* L. and *Coffea canephora* L. combination extract was assessed at concentrations of 20, 40, 60, 80, and 100 µg/ml using the Albumin Denaturation method. The results indicate a concentration-dependent increase in anti-inflammatory activity for both the sample and the standard drug (Diclofenac sodium). The extract showed progressive improvement in percentage inhibition, with the maximum activity observed at 100 µg/ml (74.4%), which was comparable to the standard (87.6%).



Graph 2: *Anti-inflammatory activity of Juglans regia L. and Coffea canephora L. combination extract- Formulation with standard Albumin denaturation method*

DISCUSSION:

This study demonstrated that the combination extract capsule containing *Juglans regia* L. and *Coffea canephora* L. possesses significant antioxidant and anti-inflammatory activity in vitro. The extract exhibited a clear concentration-dependent effect in both assays, with maximum values approaching those of the reference standards. At 100 µg/ml, the capsule showed 69.9% radical scavenging activity in the DPPH assay compared to 84.8% for ascorbic acid, and 74.4% inhibition of albumin denaturation compared to 87.6% for diclofenac sodium. These findings confirm that the synergistic formulation of walnut and coffee retains potent bioactivity and provides promising evidence for its therapeutic potential.

The antioxidant results obtained in this study are in agreement with earlier reports on *Juglans regia*. Walnut kernels are known to contain polyphenols such as ellagic acid, catechins, and tannins, which contribute substantially to

radical scavenging capacity (Carvalho et al., 2010; Martínez et al., 2010). Rusu et al. (2020) showed that walnut kernel extracts displayed strong antioxidant activity in DPPH assays, findings that closely parallel the present results. Similarly, Fukuda et al. (2003) identified antioxidative polyphenols from walnuts, further substantiating the role of walnut-derived phenolics in neutralizing reactive oxygen species. These reports provide strong support for the contribution of walnut constituents to the antioxidant activity of the present extract.

The anti-inflammatory findings also correlate well with prior investigations of walnut extracts. Abdulalsalam et al. (2017) reported that walnut kernel extract achieved 62.7% inhibition of protein denaturation at 400 µg/ml, confirming its role in preventing protein destabilization under stress conditions. Anderson et al. (2001) further demonstrated that walnut polyphenolics inhibited oxidative modification of human plasma and low-density lipoprotein, thereby reducing inflammatory mediator activity. The comparable protein-stabilizing effect observed in this study is therefore consistent with previous literature, suggesting that walnut-derived bioactive compounds contribute meaningfully to the anti-inflammatory potential of the capsule.

The contribution of *Coffea canephora* to the observed results is also evident. Robusta coffee contains higher levels of chlorogenic acids and caffeine than arabica, compounds known for their radical scavenging and anti-inflammatory properties (Farah and Donangelo, 2006). Negri et al. (2016) demonstrated that green robusta beans exhibited strong DPPH radical scavenging activity proportional to chlorogenic acid content, findings consistent with the concentration-dependent antioxidant effects seen in the present study. With respect to inflammation, Chandra et al. (2012) showed that green coffee extract significantly inhibited albumin denaturation, with an IC_{50} of approximately 40 µg/ml, which was even lower than that of diclofenac sodium. Although that study examined *Coffea arabica*, the similarities in phytochemical composition provide a plausible explanation for the anti-inflammatory effects observed in the present robusta-based extract. Together, these studies support the dual antioxidant and anti-inflammatory potential of coffee-derived compounds, which align with the present findings.

The combined formulation of walnut and coffee is particularly important because of the likelihood of synergistic interactions between their phytochemicals. Williamson (2001) emphasized that whole-plant mixtures often produce superior bioactivity compared to isolated constituents because of enhanced bioavailability, stability, and complementary mechanisms of action. In the present extract, walnut-derived polyphenols and flavonoids may have worked in concert with coffee chlorogenic acids and caffeine to exert additive or synergistic effects on free radical scavenging and protein stabilization. The near-comparable activities to standard drugs highlight the therapeutic potential of such natural combinations.

The clinical relevance of these results can be particularly appreciated in the context of Oral Submucous Fibrosis (OSMF), a chronic progressive oral disorder associated with areca nut chewing and characterized by inflammation, fibrosis, and a high risk of malignant transformation (Tilakaratne et al., 2006). Oxidative stress and persistent inflammation are central to the pathogenesis of OSMF, with reactive oxygen species and inflammatory cytokines contributing to epithelial atrophy, fibroblast activation, and collagen cross-linking. Therefore, antioxidants and anti-inflammatory agents have been investigated as potential therapies. Clinical studies have evaluated several herbal products such as lycopene, curcumin, spirulina, and aloe vera, all of which demonstrated improvements in burning sensation, mouth opening, and mucosal health (Warnakulasuriya and Ranganathan, 2023). The present results add to this body of evidence by confirming that a walnut–coffee capsule formulation also exerts strong antioxidant and anti-inflammatory effects, suggesting its potential role as a natural therapeutic agent in the management of OSMF.

CONCLUSION:

The present in vitro study demonstrated that the combination extract capsule of *Juglans regia* L. and *Coffea canephora* L. possesses significant antioxidant and anti-inflammatory activities. The extract exhibited a clear concentration-dependent effect, with maximum DPPH radical scavenging activity of 69.9% and inhibition of albumin denaturation of 74.4% at 100 µg/ml, results that were close to the respective standards ascorbic acid (84.8%) and diclofenac sodium (87.6%). These findings suggest that the synergistic interaction of walnut-derived polyphenols and flavonoids with coffee chlorogenic acids and caffeine contributes to the strong bioactivity observed. The near-comparable efficacy of the extract to established reference agents highlights its potential as a natural therapeutic option for managing oxidative stress and inflammation-related conditions, including Oral Submucous Fibrosis.

Limitations and Future Scope:

The study was restricted to in vitro assays, which cannot fully replicate in vivo conditions. The exact molecular mechanisms and long-term stability of the extract were not explored. Further studies should validate these findings in animal models and clinical trials, investigate underlying mechanisms, and develop optimized formulations to enhance bioavailability and stability.

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