

Evaluation of The Antimicrobial, Anti-Inflammatory And Antioxidant Activities of Citrus Leaf Extract Incorporated Into Sodium Alginate Gel: An in Vitro Study

Dr. Shalini^{1*}, Dr Sundheep Kumar², Prof Dr. Murugesan³, Prof Dr. Santhosh Kumar⁴

¹Post Graduate student, Saveetha dental college and hospital Saveetha Institute of Medical and Technical Sciences (SIMATS) Saveetha University, Chennai, Tamil Nadu, India* Email: Shalinimuthuraj2017@gmail.com

²Senior Lecturer, Saveetha dental college and hospital Saveetha Institute of Medical and Technical Sciences (SIMATS) Saveetha University, Chennai, Tamil Nadu, India* Email: Sundheepkumarv.sdc@saveetha.com

³Head of department, Saveetha dental college and hospital Saveetha Institute of Medical and Technical sciences, Saveetha university, Chennai, Tamil Nadu, India. Email: dr.mkm70@gmail.com

⁴Professor & Research head, Saveetha dental college and hospital Saveetha Institute of Medical and Technical Sciences Saveetha University, Chennai, India. Email: santhoshkumar@saveetha.com

Abstract:

Background

Citrus limon (L.) Osbeck contains various bioactive constituents that may contribute to antimicrobial and antioxidant activities. Incorporating plant extracts into polymeric formulations such as sodium alginate may provide a potential approach for developing plant-based formulations. However, the biological activity of the incorporated extract requires experimental evaluation. The present study aimed to evaluate the antimicrobial, antioxidant, and preliminary anti-inflammatory activities of Citrus limon leaf extract incorporated into a sodium alginate formulation.

Materials and Methods

Fresh C. limon leaves were processed using sterile distilled water and incorporated into a sodium alginate formulation. The antimicrobial activity was evaluated using the agar well diffusion method against *Acinetobacter baumannii* and *Streptococcus mutans*. Antioxidant activity was assessed using the DPPH radical scavenging assay at concentrations of 20, 40, 60, 80, and 100 µg/mL, with ascorbic acid as the reference standard. The anti-inflammatory evaluation was performed using an albumin denaturation assay, with diclofenac sodium as the reference standard. The results were presented descriptively based on the available recorded values.

Results

The formulation produced inhibition zones of 21 mm against *A. baumannii* and 17 mm against *S. mutans*, compared with 25 mm and 20 mm for the respective positive controls. The recorded antioxidant response of the formulation increased from 14.43 at 20 µg/mL to 75.71 at 100 µg/mL, while the corresponding values for ascorbic acid ranged from 21.09 to 89.65. In the albumin assay, the recorded response of the formulation increased from 14.27 to 73.71 across the tested concentration range, whereas diclofenac sodium showed values ranging from 22.94 to 87.33. The formulation showed lower recorded responses than the respective reference standards in both comparative assays.

Conclusion

The sodium alginate formulation containing C. limon leaf extract demonstrated preliminary antibacterial activity against the tested organisms and concentration-related increases in the recorded antioxidant and albumin assay responses. However, the absence of replicate information, variability measures, and inferential statistical analysis limits the strength of the findings. Further studies involving standardized assay protocols, appropriate controls, phytochemical characterization, formulation stability assessment, and safety evaluation are required to establish the reproducibility and potential applications of the formulation.

Keywords: Citrus limon; lemon leaf extract; sodium alginate; antimicrobial activity; antioxidant activity; albumin denaturation; in-vitro study.

Introduction:

The increasing prevalence of infectious and inflammation-associated conditions has created a continuing need to explore effective, safe, and sustainable therapeutic approaches. Although conventional antimicrobial and anti-inflammatory agents remain important in clinical practice, their limitations and the growing concern regarding antimicrobial resistance have encouraged researchers to investigate naturally derived bioactive compounds. Plant-based materials have attracted considerable attention because they contain diverse phytochemicals that may exhibit antimicrobial, antioxidant, and inflammation-related biological activities [1][2]. These properties have contributed to the growing interest in medicinal plants for biomedical and oral healthcare research.

Citrus species are widely recognized for their nutritional and pharmacological significance. Different parts of Citrus plants, including fruits, peels, seeds, and leaves, contain bioactive constituents that have been investigated for their biological properties. Although citrus fruits have received considerable research attention, the leaves represent an underexplored plant component with potential value for developing plant-based formulations. Citrus limon (L.) Osbeck, commonly known as lemon, is of particular interest because of its naturally occurring

phytochemicals. The biological activity of lemon leaf extracts may vary depending on the plant part, extraction method, solvent, concentration, and formulation conditions. Therefore, evaluating a standardized preparation under defined experimental conditions is important for understanding its preliminary biological potential[3]. Antimicrobial activity is an important consideration in the development of plant-derived preparations, particularly in oral healthcare research. *Streptococcus mutans* is associated with the initiation and progression of dental caries and is therefore a relevant organism for investigating potential antimicrobial agents. The evaluation of plant-based formulations against other clinically relevant microorganisms, such as *Acinetobacter baumannii*, may provide additional information regarding their antimicrobial spectrum. However, antimicrobial activity observed in an in-vitro assay should be interpreted as preliminary evidence and should not be directly equated with clinical effectiveness[4].

In addition to microbial activity, oxidative processes and inflammatory responses are relevant to several biological and disease-related pathways. Antioxidants may neutralize free radicals or reduce oxidative reactions under experimental conditions, while anti-inflammatory activity can be investigated through specific validated laboratory assays. The assessment of these biological properties may provide a broader understanding of the potential of plant-derived materials[5][6]. However, the interpretation of inflammation-related activity depends on the specific assay employed, its measured outcome, and the reference standard used. Therefore, the anti-inflammatory findings in the present study should be interpreted in accordance with the confirmed experimental procedure.

The incorporation of plant extracts into suitable delivery systems is another important consideration in formulation-based research. Sodium alginate is a naturally derived polysaccharide that possesses film-forming and gel-forming properties and has been explored in various pharmaceutical and biomedical applications. Its incorporation with plant extracts may provide a convenient formulation platform and may influence the dispersion, handling, and release of incorporated constituents[7]. Nevertheless, the biological activity of a plant extract incorporated into sodium alginate may differ from that of the extract alone. Factors such as polymer concentration, extract concentration, mixing conditions, formulation stability, and diffusion characteristics may influence the observed experimental response.

Despite the growing interest in plant-derived bioactive preparations, further investigation is required to understand the biological activity of individual plant materials incorporated into polymer-based formulations.[8] In particular, the antimicrobial and antioxidant properties of *C. limon* leaf extract incorporated into sodium alginate require evaluation under defined in-vitro conditions. The comparison of the formulation with appropriate reference standards may provide preliminary information regarding its activity at different concentrations. Any inflammation-related evaluation should be interpreted only after confirming the specific assay methodology and outcome measure[9].

Therefore, the present study was undertaken to evaluate the in-vitro antimicrobial and antioxidant activities of *Citrus limon* (L.) Osbeck leaf extract incorporated into a sodium alginate formulation. The antimicrobial activity was assessed against *Streptococcus mutans* and *Acinetobacter baumannii*, while antioxidant activity was evaluated at different concentrations using ascorbic acid as the reference standard.

Methodology:

1. Study Design

The present study was conducted as an in-vitro experimental study to evaluate the antimicrobial and antioxidant activities of *Citrus limon* (L.) Osbeck leaf extract incorporated into a sodium alginate formulation. The antimicrobial activity was evaluated using the agar well diffusion method, while antioxidant activity was assessed using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay.

2. Collection and Preparation of Plant Material

Fresh leaves of *Citrus limon* (L.) Osbeck were collected. Six leaves were selected for the study and washed thoroughly with distilled water to remove surface contaminants. The leaves were surface-dried, and 100 mg of leaf material was accurately weighed for extraction.

3. Preparation of Lemon Leaf Extract

The weighed lemon leaves were homogenized using a sterile mortar and pestle for approximately 5 minutes. The homogenized plant material was extracted using sterile distilled water at an extraction ratio of 1:2 (w/v). Accordingly, 100 mg of plant material was combined with 0.2 mL of sterile distilled water.

The resulting extract was collected and used for incorporation into the sodium alginate formulation. The plant extract was incorporated at a final concentration of 5% (v/v) in the prepared formulation.

4. Preparation of Sodium Alginate Formulation

A 4% (w/v) sodium alginate stock solution was prepared by dissolving 4 g of sodium alginate in 100 mL of sterile distilled water. For the preparation of 100 mL of the final 3% (w/v) sodium alginate formulation, 75 mL of the 4% sodium alginate stock solution was combined with 5 mL of the lemon leaf extract. Sterile distilled water was added to obtain a final volume of 100 mL.

The formulation was stirred thoroughly and maintained overnight at 4°C to facilitate uniform mixing and hydration. The prepared sodium alginate–lemon leaf formulation was stored at 4°C and preferably used within 24–48 hours.

5. Antimicrobial Activity by Agar Well Diffusion Assay

The antimicrobial activity of the prepared sodium alginate–lemon leaf formulation was evaluated using the agar well diffusion assay. Mueller–Hinton agar was used as the culture medium for the antimicrobial assessment.

The bacterial strains used in the study were obtained from the Department of Microbiology, Saveetha Medical College and Hospital. The antimicrobial activity of the formulation was evaluated against the selected bacterial strains, and the resulting zones of inhibition were recorded in millimetres.

6. Antioxidant Activity by DPPH Assay

The antioxidant activity of the sodium alginate–lemon leaf formulation was assessed using the DPPH radical scavenging assay. This assay was used to evaluate the free radical scavenging activity of the prepared formulation.

The antioxidant activity was assessed at concentrations of 20, 40, 60, 80, and 100 µg/mL. Ascorbic acid was used as the reference standard. The antioxidant response of the test formulation was compared with that of the reference standard at the corresponding concentrations.

7. Anti inflammatory activity by albumin denaturation assay.

The in-vitro anti-inflammatory activity of the sodium alginate–Citrus limon leaf formulation was evaluated using the albumin denaturation assay. Diclofenac sodium was used as the reference standard. The test formulation and standard were assessed at concentrations of 20, 40, 60, 80, and 100 µg/mL. The obtained values were compared to evaluate the preliminary anti-inflammatory activity of the formulation.

7. Statistical Analysis

The results obtained from the antimicrobial, antioxidant, and anti-inflammatory evaluations were presented descriptively. Antimicrobial activity was expressed as the zone of inhibition in millimetres for the tested bacterial strains. The antioxidant and anti-inflammatory assay results were recorded at concentrations of 20, 40, 60, 80, and 100 µg/mL and compared with the respective reference standards, ascorbic acid and diclofenac sodium. The recorded values were used for comparative evaluation of the biological activities of the sodium alginate–Citrus limon leaf formulation.

RESULTS:

Antimicrobial Activity

The antimicrobial activity of the sodium alginate formulation containing Citrus limon (L.) Osbeck leaf extract was evaluated against *Acinetobacter baumannii* and *Streptococcus mutans* using the agar well diffusion method. The formulation demonstrated measurable antibacterial activity against both tested organisms, as indicated by the formation of inhibition zones around the wells containing the test formulation.

An inhibition zone of 21 mm was recorded against *A. baumannii*, while an inhibition zone of 17 mm was observed against *S. mutans*. The positive controls produced inhibition zones of 25 mm and 20 mm, respectively. The recorded inhibition zone was therefore greater against *A. baumannii* than against *S. mutans*. For both organisms, the inhibition zones produced by the formulation were lower than those recorded for the corresponding positive controls.

These findings indicate that the sodium alginate formulation containing *C. limon* leaf extract exhibited an observable antibacterial response against the two tested bacterial strains under the conditions of the agar well diffusion assay. The antimicrobial findings are summarized in Table 1.

Table 1. Antimicrobial activity of the sodium alginate–C. limon leaf formulation

Bacterial strain	Formulation (mm)	Positive control (mm)
<i>Acinetobacter baumannii</i>	21	25
<i>Streptococcus mutans</i>	17	20

Antioxidant Activity

The antioxidant activity of the sodium alginate formulation containing *C. limon* leaf extract was assessed using the DPPH radical scavenging assay at concentrations of 20, 40, 60, 80, and 100 µg/mL. Ascorbic acid was used as the reference standard for comparison across the tested concentration range.

The recorded response of the formulation showed an increasing pattern with increasing concentration. At 20 µg/mL, the formulation produced a recorded value of 14.43, which increased to 28.37, 43.69, 59.01, and 75.71 at concentrations of 40, 60, 80, and 100 µg/mL, respectively. The reference standard, ascorbic acid, produced corresponding values of 21.09, 39.03, 60.34, 75.00, and 89.65 across the same concentration range.

The formulation showed lower recorded values than ascorbic acid at each tested concentration. The difference between the formulation and the reference standard was observed throughout the evaluated concentration range. The highest recorded response for the formulation was 75.71 at 100 µg/mL, whereas the corresponding value for ascorbic acid was 89.65.

The results demonstrated an increasing recorded response for both the formulation and the reference standard with increasing concentration. However, the nature of the reported values, including whether they represent percentage inhibition or another calculated assay parameter, should be confirmed from the laboratory records. The antioxidant assay findings are presented in Table 2.

Table 2. Antioxidant assay response of the sodium alginate–*C. limon* leaf formulation and ascorbic acid

Concentration (µg/mL)	Formulation	Ascorbic acid
20	14.43	21.09
40	28.37	39.03
60	43.69	60.34
80	59.01	75.00
100	75.71	89.65

Anti-inflammatory Activity

The anti-inflammatory evaluation of the sodium alginate formulation containing *C. limon* leaf extract was performed using the albumin denaturation assay, with diclofenac sodium used as the reference standard. The test formulation and reference standard were assessed at concentrations of 20, 40, 60, 80, and 100 µg/mL.

The formulation showed an increasing recorded response across the tested concentration range. The value increased from 14.27 at 20 µg/mL to 27.85 at 40 µg/mL, 42.09 at 60 µg/mL, 57.57 at 80 µg/mL, and 73.71 at 100 µg/mL. The reference standard, diclofenac sodium, showed corresponding values of 22.94, 39.04, 55.71, 73.05, and 87.33, respectively.

The recorded values for the formulation were lower than those of diclofenac sodium at all tested concentrations. At 100 µg/mL, the formulation showed a recorded value of 73.71, compared with 87.33 for diclofenac sodium. An increasing response was observed for both the formulation and the reference standard as the concentration increased.

The findings indicate a concentration-related increase in the recorded response of the formulation under the assay conditions. However, the interpretation of these findings depends on confirmation of the precise albumin denaturation protocol, the assay calculation method, and the units of the recorded values. The anti-inflammatory assay findings are presented in Table 3.

Table 3. Anti-inflammatory assay response of the sodium alginate–*C. limon* leaf formulation and diclofenac sodium

Concentration (µg/mL)	Formulation	Diclofenac sodium
20	14.27	22.94

40	27.85	39.04
60	42.09	55.71
80	57.57	73.05
100	73.71	87.33

Overall Results

The sodium alginate formulation containing *C. limon* leaf extract produced inhibition zones against both *A. baumannii* and *S. mutans* in the agar well diffusion assay. The recorded antioxidant and albumin assay responses increased across the tested concentration range of 20–100 µg/mL. The formulation showed lower recorded values than the respective reference standards, ascorbic acid and diclofenac sodium, in the antioxidant and anti-inflammatory evaluations.

DISCUSSION:

The present study evaluated the antimicrobial, antioxidant, and anti-inflammatory activities of *Citrus limon* (L.) Osbeck leaf extract incorporated into a sodium alginate formulation. The formulation demonstrated antimicrobial activity against *Acinetobacter baumannii* and *Streptococcus mutans*, producing inhibition zones of 21 mm and 17 mm, respectively. In addition, the formulation showed increasing recorded responses across the tested concentrations in the antioxidant and anti-inflammatory evaluations. These findings suggest that the prepared lemon leaf formulation possesses biological activity under the experimental conditions used in the study. However, the results should be interpreted cautiously because the available data do not provide replicate numbers, measures of variability, or statistical significance values. Consequently, the findings represent descriptive observations rather than statistically validated differences between the test formulation and the reference standards.

The antimicrobial activity observed in the present study may be associated with bioactive constituents present in *C. limon* leaves. Lemon leaves contain various phytochemicals, including volatile and non-volatile compounds that may contribute to biological activity. The antimicrobial findings are consistent with the observations of Riaz et al., who reported antimicrobial activity in *C. limon* leaf essential oil and hexane extract. Their study also identified limonene as a major constituent and suggested that the chemical composition of lemon leaves may contribute to their biological properties. The presence of compounds such as limonene may influence microbial growth through interactions with the bacterial cell membrane and other cellular components. Nevertheless, the exact mechanism responsible for the antimicrobial activity in the present formulation cannot be established without phytochemical characterization and mechanistic investigations[13][15].

Similarly, Petretto et al. reported antimicrobial activity in essential oil obtained from discarded lemon leaves against several bacterial and yeast strains. The study identified limonene, geranial, and neral among the major chemical components of the essential oil. These findings provide supporting evidence for the antimicrobial potential of lemon leaf-derived preparations. However, the antimicrobial response observed in the present study should not be directly equated with the results of these earlier investigations. Petretto et al. evaluated an essential oil, whereas the present study used a water-based leaf extract incorporated into sodium alginate. Differences in extraction solvent, plant material, concentration, formulation matrix, bacterial susceptibility, and assay conditions may influence the measured inhibition zones. Furthermore, the present study included *A. baumannii* and *S. mutans*, and the response of these organisms may differ from that of the microorganisms evaluated in previous studies[14][16].

The formulation produced an inhibition zone of 21 mm against *A. baumannii* and 17 mm against *S. mutans*. The difference between the recorded inhibition zones suggests that the tested bacterial strains may have responded differently to the formulation. Such variation may be related to differences in bacterial cell structure, membrane composition, susceptibility to plant-derived compounds, and interactions between the extract and the sodium alginate matrix. However, a definitive explanation cannot be provided from the inhibition zone values alone. Further studies involving standardized inoculum concentrations, multiple replicates, appropriate controls, and minimum inhibitory concentration testing would help establish the antimicrobial potency of the formulation more comprehensively.

The antioxidant activity of the formulation was evaluated using the DPPH radical scavenging assay at concentrations ranging from 20 to 100 µg/mL. The recorded values for the sample increased from 14.43 at 20

µg/mL to 75.71 at 100 µg/mL. In comparison, ascorbic acid showed values ranging from 21.09 to 89.65 across the same concentration range. The increase in the recorded sample response with increasing concentration suggests a concentration-dependent pattern under the conditions of the assay. This may indicate that higher concentrations of the formulation provided a greater quantity of compounds capable of reacting with the DPPH radical. However, the observed pattern should be interpreted as a descriptive concentration-related response because the available results do not include replicate measurements or statistical comparisons.

The antioxidant findings are broadly consistent with the observations of Yeasmin et al., who reported antioxidant activity in essential oil obtained from *C. limon* leaves using the DPPH assay. Their study also investigated the antimicrobial potential and chemical constituents of the essential oil [17]. Riaz et al. similarly reported DPPH radical-scavenging activity in lemon leaf essential oil and hexane extract. These studies support the possibility that lemon leaves contain compounds capable of contributing to antioxidant activity. Nevertheless, the chemical constituents present in an essential oil or organic extract may differ from those extracted using sterile distilled water. The antioxidant response of the present formulation may therefore depend on the solubility, stability, and concentration of the compounds extracted into the aqueous medium[13][18].

The antioxidant values recorded for the formulated sample were lower than those of ascorbic acid at all tested concentrations. This observation indicates that ascorbic acid produced a higher recorded response under the assay conditions used. Differences between the sample and standard may be associated with the chemical composition of the extract, the concentration of active antioxidant compounds, extraction efficiency, and interactions with the formulation matrix. The antioxidant activity of a plant extract should not be assessed solely by comparing its values with a pure reference compound, since ascorbic acid is a well-characterized antioxidant and the formulation contains a complex mixture of constituents. In addition, the absence of phytochemical analysis prevents identification of the specific compounds responsible for the observed response.

The anti-inflammatory evaluation showed an increase in the recorded sample values from 14.27 at 20 µg/mL to 73.71 at 100 µg/mL. Diclofenac sodium demonstrated higher values across the tested concentration range. These findings indicate an increasing response in the sample measurements, although the precise interpretation depends on the assay method and the parameter represented by the recorded values. The anti-inflammatory assay used in the present study was not specified in the available methodology. Therefore, the findings should be interpreted as an in-vitro comparative response and should not be used to establish a particular anti-inflammatory mechanism or clinical effectiveness.

Jaradat et al. investigated the phytochemical composition and biological properties of *C. limon* leaf volatile oil, including its antimicrobial and anti-inflammatory activities. Their work supports the investigation of lemon leaf-derived preparations for biological applications[19]. However, the extract type, experimental conditions, and anti-inflammatory assay used in their study may differ from those used in the present investigation. Consequently, the findings can provide contextual support but cannot be considered direct confirmation of the anti-inflammatory activity of the present sodium alginate formulation. Further work should clearly identify the assay used, standardize the experimental conditions, and evaluate the formulation using suitable controls and replicate measurements [20].

Sodium alginate was used as the formulation matrix for incorporating the lemon leaf extract. The formulation was prepared using a 4% (w/v) sodium alginate stock solution, which was diluted to obtain a final concentration of 3% (w/v). The formulation was stirred and maintained overnight at 4°C to facilitate mixing and hydration[21]. The incorporation of plant extract into a polymeric matrix may offer advantages for the preparation and handling of natural-product-based formulations. However, the present study did not separately evaluate the sodium alginate vehicle, the lemon leaf extract alone, or the physicochemical stability of the final preparation. Therefore, it is not possible to determine whether the observed biological effects were attributable exclusively to the plant extract or to interactions between the extract and sodium alginate[22].

The formulation also requires further investigation regarding its stability, uniformity, and release characteristics. Storage at 4°C and use within the intended period may help maintain the preparation under the reported experimental conditions, but the available results do not establish its long-term stability. Future studies should assess changes in appearance, pH, viscosity, active constituent content, and biological activity during storage. Comparative testing of the extract alone, sodium alginate alone, and the combined formulation would also help clarify the contribution of each component. Such comparisons would be particularly important when developing a formulation intended for further biomedical or pharmaceutical investigation[23].

The present study has several limitations. First, the available data do not specify the number of experimental replicates or provide standard deviation, standard error, confidence intervals, or statistical significance values. Second, the antimicrobial assay details, including inoculum standardization, well diameter, sample volume, incubation conditions, and controls, were not fully documented in the supplied methodology. Third, the DPPH assay procedure does not include all the parameters required for reproducibility, such as DPPH concentration,

solvent, incubation period, wavelength, and calculation procedure. Fourth, the exact anti-inflammatory assay and its experimental conditions were not identified. Finally, the study did not include phytochemical profiling or separate testing of the formulation components. These limitations should be addressed in future investigations to strengthen the reliability and reproducibility of the findings.

Overall, the *C. limon* leaf extract incorporated into sodium alginate demonstrated antimicrobial activity against the tested bacterial strains and increasing recorded responses in the antioxidant and anti-inflammatory evaluations. The findings are broadly consistent with previous reports describing the biological activity of lemon leaf extracts and essential oils. However, differences in plant extraction methods, formulation composition, assay conditions, and biological models limit direct comparisons with earlier studies. The current results should therefore be considered preliminary and descriptive. Further research involving standardized extraction procedures, validated assay protocols, suitable controls, replicate experiments, statistical analysis, phytochemical characterization, stability assessment, and safety evaluation is required to establish the biological potential and possible applications of the sodium alginate–*C. limon* leaf formulation.

Conclusion

The present study demonstrated that *Citrus limon* (L.) Osbeck leaf extract incorporated into a sodium alginate formulation exhibited preliminary antimicrobial, antioxidant, and anti-inflammatory activities. The formulation showed antibacterial activity against *Acinetobacter baumannii* and *Streptococcus mutans*, while the antioxidant and anti-inflammatory evaluations demonstrated concentration-dependent increases in activity. However, the observed responses were lower than those of the respective reference standards, ascorbic acid and diclofenac sodium. These findings suggest that the formulated *C. limon* leaf extract may have potential as a plant-based bioactive formulation. Nevertheless, the absence of replicate data, inferential statistical analysis, detailed phytochemical characterization, and comprehensive formulation stability assessment limits the interpretation of the results. Further studies incorporating standardized extraction methods, appropriate controls, validated assay protocols, statistical evaluation, and safety assessment are required to confirm the biological activity, stability, and potential applications of the sodium alginate–*C. limon* leaf extract formulation.

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