RESEARCH PAPER

Antioxidant property of nano-emulsion prepared from rhizome extract of *Acorus calamus*

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Abstract
OPMDs, especially Erosive lichen planus, oral submucous fibrosis, and nonhomogeneous leukoplakia, show an increased tendency for malignant transformation. This increased rate of malignant transformation might be due to the generation of free radicals, which induce cell mutation. These free radicals are countered by systemic antioxidants. Due to the lack of absorption at the site where it is needed, there is a need for the topical route of administration. It is predicted that 20% of the systemic antioxidant is taken into action at the particular site of the oral cavity. So, there is a need for the topical route of administration to achieve the complete degeneration of free radicals. The study aims to formulate the *Acorus calamus* nano-emulsion and check for antioxidant properties. The main objective of the current study is to formulate the nano-emulsion of the *Acorus calamus* and check for the antioxidant properties by DPPH and ABTS assays. The nano-emulsion was prepared by mixing 10 mg of powder with an oil-in-water emulsion containing 10 mL of water, 60 mL of palm oil, and 30 mL of Tween 20. Further, the mixture was sonicated for 60 min, and the milky white formation indicates the emulsion formation. With the increase in the volume of the nanoemulsion of the *Acorus calamus*, the particle size distribution also increases to 250 nm when the volume reaches 100%, and then it gradually decreases to 450 nm. *Acorus calamus* nano-emulsion has antioxidant properties by inhibiting the action of free radicals. Nano-emulsion of the *Acorus calamus* has higher penetration when the size is less than 100 microns into the human epithelial cells. Therefore, it is more useful in the management of OPMDs when applied topically.

Keywords: *Acorus calamus*; Antioxidant property; Nanoemulsion; Free radicals
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1. Introduction

Oral cancer is the sixth most common cause of death in an individual, which interferes with day-to-day activities and greatly affects the quality of life. The multifactorial etiology of oral cancer, an epithelial-originated illness, includes genetic, behavioral (tobacco/areca nut/cigarette/alcohol), and microbial variables, which frequently differ between geographic locations or ethnic groups [(Aghbari et al. 2017)]. The normal mucosa has been shown to undergo several histological alterations when oral cancer advances. These alterations include carcinoma in situ, dysplasia, hyperplasia, and mouth cancer. Habitual variables are the main elements that determine the progression of oral cancer since altering underlying habits can stop or decrease the disease's growth, especially when it's benign or dysplastic. More frequently than not, precancerous disorders might start with the development of abnormalities in the oral mucosa, or atypia [(Aguirre-Urizar 2011)]. On the other hand, in terms of identifying the malignancy potential of various premalignant precursors in oral cancer, oral epithelial dysplasia (OED) is classified as an intermediate stage.

OPMDs are defined as “any oral mucosal abnormality that is associated with a statistically increased risk of developing oral cancer” [(Aguirre-Urizar et al. 2020)]. A variety of clinical characteristics are found in OPMDs, including color variations (white, red, and mixed), morphological modifications (plaque/island, smooth, grooved, wrinkled, granular, atrophic), and sizes affecting various oral cavity anatomical regions [(Alaizari et al. 2017)]. According to epidemiological research, 4.47% of people worldwide may have OPMDs. According to Melo et al. (2018), the bulk of these occurrences are documented among the South Asian community. Males are a commonly impacted demographic, most likely because they use alcohol and smoke more frequently than women. According to estimates from several studies, the frequency of OPMDs varies among populations and is mostly related to lifestyle (Al-Hashimi et al. 2007). Uma Maheswari et al conducted a study on saliva as a diagnostic marker in the diagnosis of OPMDs. They found that expression of salivary miRNA has proved that salivary miRNA can be used as a biomarker, as there is an expression of both reference genes [mir_16p] and target genes [mir_21p and mir_31p] in both controls and OPMDs. Another study shows that

advancements in treatment modalities were made based on the different grades of OPMDs.

OPMDs, especially Erosive lichen planus, oral submucous fibrosis, and nonhomogeneous leukoplakia, show an increased tendency for malignant transformation. This increased rate of malignant transformation is due to the generation of free radicals, which induce cell mutation. These free radicals are countered by systemic antioxidants. Due to the lack of absorption at the site where it is needed, there is a need for the topical route of administration. It is predicted that 20% of the systemic antioxidant is taken into action at the particular site of the oral cavity. Dhanvanth et al conducted a systematic review on topical mode of application in the management of OPMDs and found that a 90% complete clinical response was achieved when applied topically, other than systemic mode of administration [(Dhanvanth and Maheswari 2022)].

Acorus calamus, a well-known medicinal plant used in traditional medicine for the management of liver and hepatobiliary carcinomas, has the botanical name of vacha. A wide variety of metabolites have been previously used in the management of cancers. Rhizome of *Acorus calamus* has properties such as anticancer properties, antioxidant properties, as well as neuroprotective properties by inhibiting the action of calcium channels, which are mainly responsible for the pain conduction pathway [(Al-Hashimi et al. 2007)]. By inhibiting the generation of free radicals, the mucosa of the patient returns to its normal morphology after a particular period. There is a need to check for the cytotoxic and antioxidant properties. The study aims to check the antioxidant properties of the *Acorus calamus* nano-emulsion.

2. Materials and methods

2.1. Materials

Tween 20 was purchased from Loba Chemie, India. Palm oil was purchased from a natural plant company in Chennai, India. The extract was collected from the *Acorus calamus* rhizome by using the Soxhlet apparatus.

2.2. Preparation of nano-emulsion

The extraction of bioactive compounds was carried out using a Soxhlet apparatus in which 10 g of the dried sample was subjected to aqueous extraction. The obtained extract was evaporated, and the resulting powder was collected through rotary evaporation. The nano-emulsion was prepared by adding the 10 mg powdered extract into 10 mL of water. Following this, 60 mL of palm oil and 30 mL of Tween 20 were incorporated to form an oil-in-water emulsion. The mixture was then ultrasonicated for 60 min using an ultrasonic processor. The formation of the milky white solution was observed, indicating the successful formation of the nano-emulsion.

The nanoemulsion formulation was characterized using various analytical techniques. The size distribution of the droplets was determined through the dynamic light scattering (DLS) method, and the stability of the emulsion was assessed using a zetasizer. Further confirmation of the nano-emulsion formulation was conducted using field emission scanning electron microscopy (FE-SEM) analysis.

2.3. DPPH Assay

To evaluate the antioxidant potential of the *Acorus calamus*, the DPPH assay was employed, leveraging its principle of color alteration upon interaction with antioxidants. When an antioxidant reduces DPPH, an inert radical molecule with a purple color, it becomes yellow. After producing a 0.1 mM DPPH radical solution, different amounts of *Acorus calamus* nanoemulsion were added. The decrease of DPPH radicals was determined through the measurement of transmittance at 517 nm after a 30-minute incubation interval. The positive control was Trolox.

2.4. ABTS Assay [(2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic)]

ABTS was reacted with an oxidizer to produce ABTS reactive ions ($ABTS^{\bullet+}$), which were used to assess the antioxidant capacity. After the addition of antioxidant compounds, the $ABTS^{\bullet+}$ was reduced, resulting in the generation of a non-radical molecule (ABTS-H) and the mixture's eventual color change. By mixing an $ABTS^{\bullet+}$ suspension with potassium persulfate, a mixture with ABTS radical cations was generated. After adding *Acorus calamus* nano-emulsion in different doses, the antioxidant capacity of the particles was evaluated by measuring a reduction in transmittance at 734 nm.

3. Results

3.1. Characterization of nano-emulsion

The zeta potential (Fig. 1a) is used to determine whether the nano-emulsion prepared is dimensionally stable or not. The SEM image (Fig. 1b) shows the arrangement of the particles within the nano-emulsion. Fig. 1c indicates that the zeta potential value increases when the particle size is 32 nm, with a normal range of less than -30 to +30 mV, indicating an unstable value. However, the nano-emulsion of the *Acorus calamus* is -32, which shows that the nano-emulsion is dimensionally stable.

Fig. 1. a) Zeta potential, b) SEM image, and c) particle size distribution of prepared nanoemulsion.

3.1. DPPH Assay

The particle size distribution was determined by using the dynamic light scattering method. The dynamic light scattering (DLS) method is a technique that can be used to determine the size distribution profile of small particles in suspension or polymers in solution. In the scope of DLS, temporal fluctuations are usually analyzed using the intensity or photon autocorrelation

function (also known as photon correlation spectroscopy – PCS or quasi-elastic light scattering – QELS). Fig. 2 shows that with the increase in the volume of the nanoemulsion of *Acorus calamus*, the particle size distribution also increases to 250 nm when the volume reaches 100%, and afterwards it gradually decreases to 450 nm.

3.2. ABTS Assay

Here, the positive control was chosen as Trolox. According to ABTS inhibition percentage, similar to Trolox, which is a well-known positive reagent in the inhibition of free radicals, the nano-emulsion prepared has the same property of inhibition of free radicals from 10 mL to 100 mL. After reaching 100 mg/L, the $ABTS^{\bullet+}$ inhibition is also increased to a value of 60-80%. It shows that the nano-emulsion has a tendency the inhibit free radicals. Fig. 3 shows that the values are quite similar on the *Acorus calamus* nanoemulsion prepared compared with Trolox, thereby showing a better penetration and antioxidant property by inhibiting free radical generation.

4. Discussion

The classical definition of oxidative stress is a redox imbalance with an excess of oxidants or a deficiency in antioxidants. Oxidants can come from a variety of sources, most of which are extremely reactive and unstable. Most oxidants come from chemical or enzymatic reactions that produce superoxide anion, hydroxyl, peroxyl (RO_2*), alkoxy (RO*), hydroperoxyl (HO_2*), or nitric oxide (NO) [(Manach et al. 2004)]. These can then be converted into secondary very reactive oxygen species (ROS) and reactive nitrogen species (RNS), such as hydrogen peroxide, hypochlorous acid (HOCl), hypobromous acid (HOBr), and peroxynitrite ($ONOO^-$) [(Koolen et al. 2013)]. One of the main compounds in the *Acorus calamus* is alpha-asarone, which has also been identified in the rhizome, where it is present in concentrations of 5.5% and 9.7%, respectively, based on prior research [(Koolen et al. 2013)]. Nevertheless, there was no published literature study on the amount of alpha-asarone in leaves. As the polarity of the extraction solvent dropped, the amount of alpha-asarone rose. Hexane leaf and rhizome extracts had the greatest significant ($p<0.05$) alpha-asarone levels since it is the least polar solvent, followed by methanol and water. This observation may be the result of the alpha-asarone molecule's low polarity behavior. Since methyl and ether, two of the functional groups found in the alpha-asarone structure, are nonpolar and the least polar, respectively.

Kumar et al. [(Nandakumar et al. 2013)] tested the *Acorus calamus* rhizome in rats for antimicrobial properties, and they found that *Acorus calamus* has a better antibacterial effect when used systemically in rats, and they observed that bacterial cells were excreted in the feces of rats. Malik et al. [(Shailajan et al. 2015),(Liu et al. 2008)] emphasize that the *Acorus calamus* has an active component of α-asarone, which is an essential marker, and it has antioxidant properties. In this current study, the extract was prepared using an oil-in-water emulsion process, and the prepared emulsion was extracted using the cold extraction method. After the cold extraction, the extract was converted into a nano-emulsion by the reverse micelle method. Reverse miscella is a process of adding water and surfactant to the oil. As the nanoemulsion is hydrophobic, the nanoemulsion present was soaked inside the head

It's possible that the *Acorus calamus* naturally produces more complex, less hydrophilic phenolic chemicals in its rhizome and more hydrophobic phenolic compounds in its leaves. Long carbon side chains are typically seen in complex or high molecular weight phenolic compounds [(Rajaulla Naikwadi 2024)]. As the length of the hydrophobic carbon side chain that is connected to the benzene ring increases, the hydrophilicity of phenols decreases. Because *Acorus calamus's* rhizome naturally has a larger concentration of complex phenolic compounds than hydrophilic solvents like water and methanol, it is conceivable that this is the cause of the hydrophobic solvent hexane's higher affinity for the phenolic compounds in the rhizome.

When the rhizome was crudely extracted using the hydrophobic solvent hexane, the rhizome showed a considerably greater total phenolic content. However, when methanol—a hydrophilic solvent—was employed as the extraction solvent, the leaf showed substantially more total phenolic content. This observation may also be explained by the fact that the *Acorus calamus's* rhizome naturally contains a higher concentration of complex (and therefore more hydrophobic) phenolic compounds. As a result, hydrophobic solvents like hexane were better suited for extracting phenolic compounds from the rhizome, whereas hydrophilic solvents like methanol were better suited for extracting phenolic compounds from the leaf.

The nano-emulsion has a head, body part, and tail. The head part consists of a hydrophobic head part, and the tail is a hydrophilic part. There, the nano-emulsion was trapped inside the particle. After the formulation of the emulsion, it is checked for both DPPH and ABTS assays. On further results, the DPPH assay shows that the nano-emulsion of *Acorus calamus* shows a well-known scavenger of free radicals. Thereby, it prevents the generation of free radicals and inhibits cell mutation. ABTS assay shows that *Acorus calamus* nanoemulsion can scavenge or inhibit the free radical action at the site where it is involved. It prevents cellular mutation and is involved in the treatment of

cancers and premalignant lesions. Nanoemulsion of the *Acorus calamus* has higher penetration when the size is less than 100 microns into the human epithelial cells. The limitations of the study are that it shows some amount of variation during the study. This current study is more useful to evaluate the ability of *Acorus calamus* nanoemulsion to scavenge the free radical generation and prevent pre-malignant disorders.

5. Conclusion

Acorus calamus nanoemulsion has antioxidant properties by inhibiting the action of free radicals. The nano-emulsion of the *Acorus calamus* has higher penetration when the size is less than 100 microns into the human epithelial cells. So it is more useful in the management of OPMDs when it is applied topically. The topical route of application is the prescribed one in the management as well. As there is a smaller number of studies and evidence, there is a need for more studies to evaluate the pharmacological actions and cytotoxic activity of *Acorus calamus* nanoemulsion.

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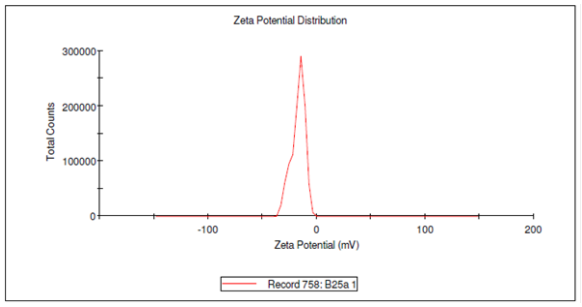
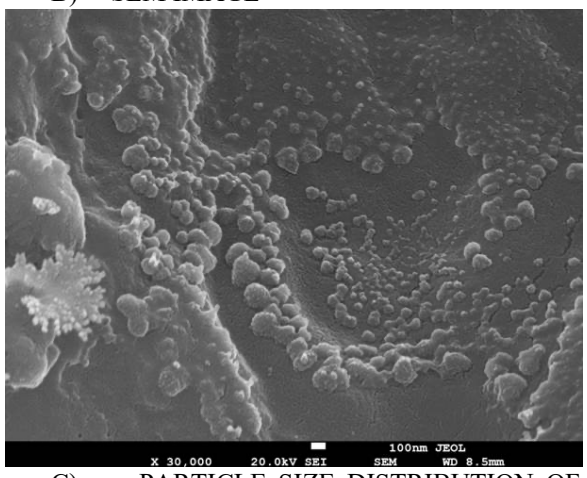
FIG 1 a) Zeta potential, b) SEM image, and c) particle size distribution of prepared nanoemulsion.

A) ZETAPOTENTIAL OF ACORUS CALAMUS



B) SEM IMAGE



C) PARTICLE SIZE DISTRIBUTION OF PREPARED NANOEMULSION

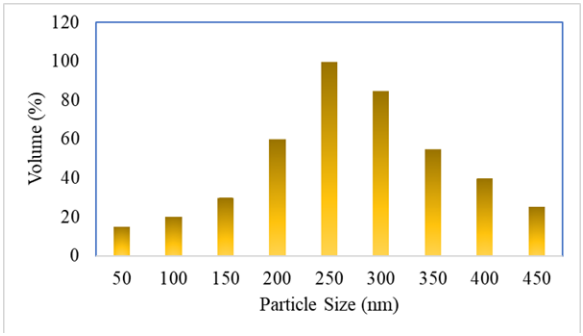
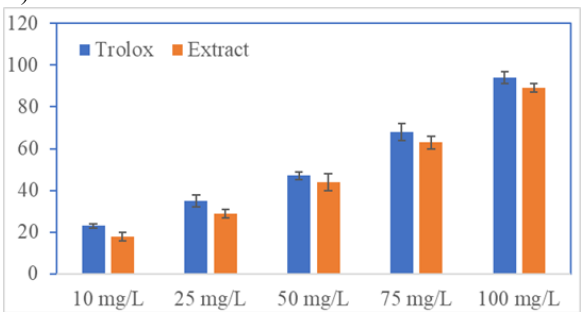


Fig. 2. Graphical representation of (a) DPPH activity and (b) ABTS activity of prepared nanoemulsion.

A) DPPH ASSAY



B) ABTS ASSAY