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Formulation and Evaluation of Betel Leaf (Piper betle) Nanoemulsion for Anticancer Activity

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Abstract:

Cancer remains one of the leading causes of mortality worldwide, necessitating the development of safer and more effective therapeutic approaches. In recent years, phytochemicals derived from medicinal plants have garnered significant attention for their potential anticancer properties, owing to their bioavailability, reduced toxicity, and multifaceted mechanisms of action. Among such plants, Piper betle (betel leaf) has demonstrated promising therapeutic potential, attributed to its diverse array of bioactive compounds, including eugenol, chavicol, and allylpyrocatechol. These phytoconstituents exhibit notable antioxidant, anti-inflammatory, and cytotoxic properties that contribute to anticancer activity.

Despite its therapeutic potential, the clinical application of betel leaf extracts is limited by poor solubility, low bioavailability, and rapid metabolic degradation. To overcome these limitations, this research focuses on the formulation and evaluation of a nanoemulsion-based drug delivery system designed to enhance the stability, solubility, and cellular uptake of betel leaf extract. Nanoemulsions, due to their nanometer-sized droplets and high surface area, offer improved dispersion of hydrophobic compounds and better permeability, making them ideal for the delivery of plant-derived compounds.

This study involved the extraction of phytochemicals from betel leaves, formulation of oil-in-water nanoemulsions using appropriate surfactants and co-surfactants, and optimization based on physicochemical parameters such as droplet size, polydispersity index, zeta potential, and viscosity. The optimized formulation was evaluated for its in vitro anticancer activity using suitable cancer cell lines, along with assessments of cytotoxicity, apoptosis induction, and cellular uptake.

The results indicated that the nanoemulsion significantly enhanced the bioavailability and anticancer efficacy of Piper betle extract, offering a promising alternative to conventional chemotherapy with reduced side effects. This work not only validates the potential of betel leaf as a natural anticancer agent but also underscores the utility of nanoemulsion technology in herbal drug delivery systems. Future studies focusing on in vivo validation and clinical translation may pave the way for the development of novel phytopharmaceuticals in cancer therapy.

Keywords: Herbal drug, Evaluation of nano-emulsion, Ultrasonication, Pharmaceutical plant extract, Cancer cell lines, Nanotechnology, Anticancer activity.

Introduction:

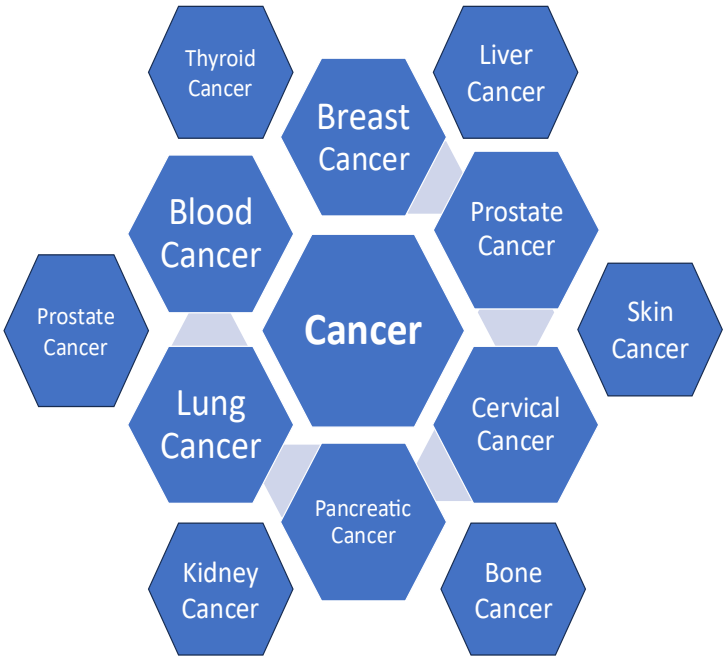
Cancer is caused by the transformation of healthy cells into malignant cells that proliferate and spread. Your cells receive instructions from your genes, such as when to begin and stop developing. Cancer cells disregard these directives, but healthy cells do.

Traditional cancer treatments, including surgery, chemotherapy, and radiotherapy, have demonstrated limited effectiveness in managing solid tumors. This ineffectiveness is attributed to the numerous side effects linked to these treatment modalities, alongside the rising issue of multi-drug resistance. There is an urgent need for the development of new therapeutic strategies that not only exhibit high efficacy but also minimize toxicity and reduce the risk of adverse side effects. Previous research suggests that essential oils (EOs) and phytochemicals may alleviate the side effects associated with synthetic drugs and chemotherapy. The lipophilic properties of EOs facilitate their penetration through cell membranes. Furthermore, the prevalence of smoking and alcohol consumption has become increasingly common in modern lifestyles, contributing to the global rise of oral squamous cell carcinoma (OSCC) as a deadly disease. Developing countries are particularly affected, with approximately 500,000 new cases of oral and pharyngeal cancer diagnosed each year worldwide. This alarming statistic underscores the necessity for immediate efforts to create compounds that can inhibit the progression of this cancer. Historically, the treatment of OSCC has relied on conventional approaches such as surgery and radiation, with or without chemotherapy, all of which can result in considerable post-treatment morbidity. The stage of OSCC, whether early or advanced, plays a crucial role in determining the treatment approach, with surgery or radiotherapy being utilized in the early stages, and a combination of chemotherapy, surgery, and radiotherapy being favored in later stages. Additionally, from 2000 to 2015, global antibiotic consumption surged by approximately 65%, with a two-fold increase observed in many low- and middle-income nations.

The betel leaf (Piper betle) is a prominent medicinal plant native to Asia, classified under the Piperaceae family. It serves numerous commercial purposes across the medical, industrial, and pharmaceutical sectors. Rich in antioxidants and phytochemicals, betel leaf is recognized for its cooling and refreshing qualities. Globally, there are 90 varieties of betel leaf, with 45 of these found in India. It is affordable, safe, and readily accessible throughout the year. The leaf exhibits various beneficial properties, including anti-fungal, anti-septic, anti-microbial, anti-cancer, anti-diabetic, anti-allergic, anti-fertility, anti-filarial, wound healing, and anti-dermatophytic effects. Additionally, it helps prevent gastrointestinal infections due to its immunomodulatory properties and may aid in diabetes management by regulating blood sugar levels. Traditionally, betel leaf is utilized in religious ceremonies to mark significant events. It is commonly chewed in many countries after meals to enhance digestion. Ayurveda recommends consuming betel leaf post-meal as it aids digestion, cleanses the mouth, alleviates excessive cough, and helps maintain weight.

Nanoemulsions are characterized as isotropic, thermodynamically stable systems that are either transparent or translucent, consisting of oil and water stabilized by surfactants, with droplet sizes typically ranging from 5 to 200 nm. They offer several advantages over macroemulsions, including a significantly larger surface area and free energy, which enhance their effectiveness as transport systems. Unlike macroemulsions, nanoemulsions do not experience issues such as creaming, flocculation, coalescence, or sedimentation. They can be produced through spontaneous emulsification methods to improve the solubility and bioavailability of poorly water-soluble drugs. Additionally, they are non-toxic and non-irritating, making them suitable for application on skin and mucous membranes. Utilizing nanoemulsions as drug delivery systems can enhance drug efficacy, allowing for reduced total dosages and minimizing side effects. Furthermore, nanoemulsions serve as drug carriers in the topical treatment of various diseases, particularly skin conditions, by effectively incorporating both hydrophobic and hydrophilic drugs to enhance drug accumulation at the target site while reducing adverse effects. They also facilitate sustained and controlled release of the encapsulated drug.

Common Cancer Types:



Discoveries of Therapies in Cancer Research:

Current Statistical data collection of cancer:

As of 2025, the incidence and mortality rates of cancer are increasing, making it a major worldwide health concern.

1. Global Cancer statistics:

In 2022, there were approximately 20million new cancer cases and 9.7 million cancer related deaths worldwide. This equates to about 1 in 5 people developing cancer in their lifetime, and approximately 1 in 9 men and 1 in 12 women dying from the disease.

2. Most Common Cancers:

Lung cancer remains the most commonly diagnosed cancer and the leading cause of cancer death overall and in men worldwide, with almost 2.5 million cases and 1.8 million deaths . In females, breast cancer is the most commonly diagnosed cancer and the leading cause of cancer death, followed by lung, colorectal, and cervical cancers.

3. Cancer in Children:

Incidence: Among children aged 0-14 years, lymphoid leukemia is the leading the leading site of cancer, accounting for 29.2% on boys and 24.2% in girls.

Aim : Formulation and Evaluation of Nanoemulsion from Betel Leaf

Objectives: The main objective of the study is to develop topical nanoemulsion.

- To prepare a safe and effective dosage form using Betel leaf (Piper Betle) extract.
- To choose the appropriate excipients based on physicochemical properties of drug.
- To increase the solubility of drug by nanomization.
- To increase the therapeutic effect at targeted site.
- To reduce dose frequency and side effects.

Materials and Methods:

Formulation Table:-

Table :1

Batch No.	Piper betle Extract (%)	MCT Oil (%)	Tween 80 (%)	PEG 400 (%)	Distilled Water (%)	Remarks
F1	1.0	5.0	10.0	5.0	79.0	Coarse emulsion
F2	1.5	5.0	12.0	6.0	75.5	Stable nanoemulsion
F3	2.0	6.0	15.0	7.0	70.0	Stable, low PDI
F4	2.5	7.0	15.0	7.0	68.5	Slightly turbid
F5	3.0	8.0	18.0	8.0	63.0	High viscosity

Materials:

Fresh *Piper betle* leaves were collected from the campus of SAJVPM's College of Pharmaceutical Sciences and Research Center, Kada. The leaves were washed with distilled water and shade-dried for 3–4 days. The dried leaves were then ground to a fine powder and passed through a sieve for uniform size distribution.

- The plant was authenticated before use.

Method: Extraction via Maceration

Step 1: Collection of Fresh Leaves

Fresh green Piper betle leaves are harvested from the plant.

Step 2: Shade Drying

The collected leaves are kept in a shaded, ventilated area to dry naturally and retain phytochemicals.

Step 3: Grinding

The dried leaves are coarsely powdered using a mortar and pestle.

Step 4: Sieving

The powdered material is sieved to achieve a consistent particle size suitable for extraction.

Step 5: Weighing of Powder

The sieved powder is accurately weighed using a digital weighing balance for use in the extraction process.

Step 6: Maceration with Solvent

The weighed powder is transferred into a conical flask, and a suitable solvent (like ethanol or a hydroalcoholic mixture) is added. The mixture is left undisturbed or occasionally shaken for several days.

Step 7: Filtration

After the maceration period, the extract is filtered using a funnel and filter paper into a beaker to separate the liquid extract from the plant residue.

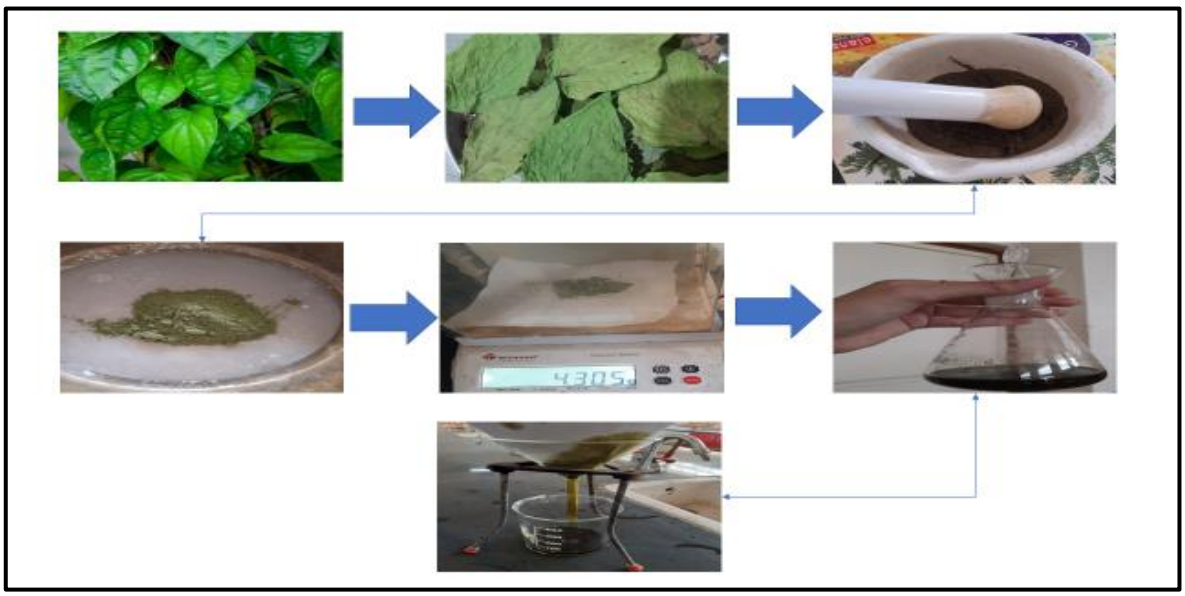


Fig. 1: Extraction via Maceration

Method: Coconut oil

Steps involving in coconut oil extraction

1. *Chopping – Fresh coconut cut into small pieces.*
2. *Grinding – Pieces ground into a fine paste.*
3. *Milk Extraction – Paste squeezed to extract coconut milk.*
4. *Settling – Coconut milk is allowed to rest.*
5. *Heating – Settled milk is heated slowly.*
6. *Separation – Oil separates from the residue.*
7. *Collection – Coconut oil collected in a vial.*

Fig. 2: Steps of Oil preparation

Preparation of Piper betle Nanoemulsion

1. Mixing of Surfactant System:

Tween 80 and PEG 400 were mixed to form the surfactant and co-surfactant system.

2. Oil Phase Addition:

MCT oil or coconut oil was added to the Smix and stirred until a homogenous mixture was formed.

3. Drug Incorporation:

The methanolic extract of Piper betle was added to the mixture with continuous stirring.

4. Final Mixing:

The final formulation was vortexed for 2–5 minutes to ensure uniform distribution and formation of nanoemulsion.

5. Characterization:

Prepared formulations were evaluated for appearance, particle size, polydispersity index (PDI), and stability.

Pseudo-Ternary Phase Diagram study:

Based on solubility studies, Coconut oil was chosen as the oil phase. Tween 80 and Glycerol were selected as the surfactant and co-surfactant, respectively. Distilled water served as the aqueous phase. The surfactant and co-surfactant (Smix) were combined in various mass ratios (3:1, 4:1, and 5:1). These ratios were selected to increase the concentration of surfactant relative to the co-surfactant for a comprehensive analysis of the phase diagrams. For each phase diagram, oil and Smix at a designated ratio were thoroughly mixed in different glass vials. Various combinations of oil and Smix were created to ensure that the maximum ratios were explored, allowing for precise delineation of the phase boundaries in the diagrams. Pseudo ternary phase diagrams of oil, Smix, and the aqueous phase were constructed using the aqueous titration method. A slow titration with the aqueous phase was conducted for each mass ratio of oil and Smix, with visual observations made to identify transparent and easily flowable o/w nanoemulsions. The physical state of the nanoemulsion was indicated on a pseudo-three-component phase diagram, with one axis representing the aqueous phase, the second representing oil, and the third representing a mixture of surfactant and co-surfactant at a fixed mass ratio. Preparation of Nanoemulsion: The formulations were created by combining the appropriate amounts of surfactant and co-surfactant, followed by the addition of the oil component, mixing the formulation until complete dispersion was achieved at room temperature. Subsequently, the appropriate amount of drug was incorporated, and the final mixture was vortexed until a transparent solution was obtained. The formulations were prepared and characterized.

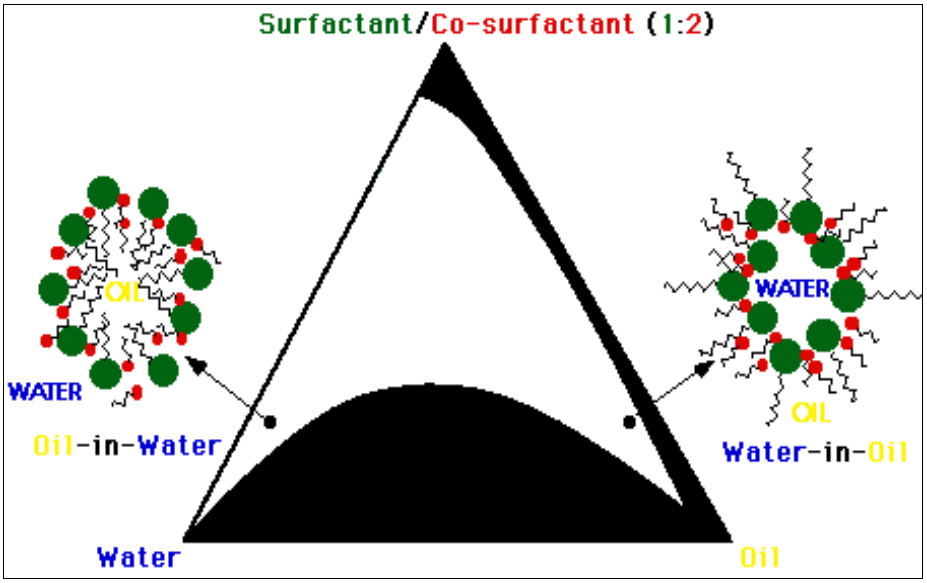


Fig. 3: Construction of pseudo-ternary phase diagram

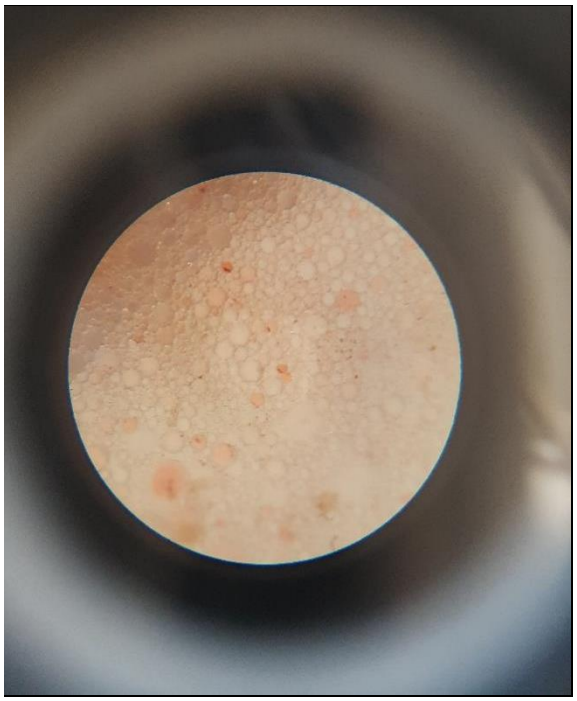


Fig. 4: Nano-emulsion

RESULT :

Tests for Identification:

Phenolics:

Ferric chloride Test: Extract + FeCl3 ⟶ blue/green color

Interpretation: -

Positive result: If the phenolic compound are present or specially tannines then blue or green color form

Negative result: No color change

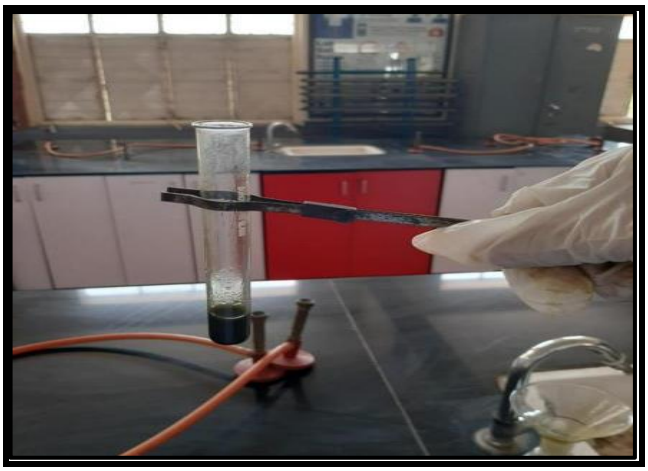


Fig. 5

Flavonoids:

Alkaline Reagent Test: Extract + NaOH ⟶ yellow/orange color

Interpretation:-

Positive result: Yellow/orange coloration

Negative result: If no color is form or change then flavonoids are absent

Fig. 6

Nanoemulsion Composition and Responses

Table: 2

Std	Run	Block	Component 1 (As/cs) (mL)	Component 2 (B.oil) (mL)	Component 3 (C.water) (mL)	Response 1 (pH)	Response 2 (Viscosity)	Response 3 (Drug Content, %)
15	1	Block 1	3.0	5.0	92.0	5.7	21.4	88.3
10	2	Block 1	5.0	5.0	90.0	5.9	23.8	86.2
8	3	Block 1	7.3	11.2	81.5	6.1	27.5	83.7
4	4	Block 1	7.5	15.0	77.5	6.3	30.6	79.4
14	5	Block 1	7.5	15.0	77.5	6.3	30.6	79.4
7	6	Block 1	7.5	10.0	82.5	6.2	28.1	81.9
11	7	Block 1	10.0	7.9	82.1	6.4	26.4	80.3
6	8	Block 1	10.0	8.0	82.0	6.4	26.6	80.5
13	9	Block 1	9.6	5.0	85.4	6.3	25.2	83.6
12	10	Block 1	6.5	10.0	83.5	6.1	26.9	82.7
4	11	Block 1	9.6	5.0	85.4	6.3	25.2	83.6
5	12	Block 1	10.0	11.6	78.4	6.5	29.4	78.9
2	13	Block 1	5.9	9.4	84.7	5.9	26.3	84.5

9	14	Block 1	3.0	9.5	87.5	5.8	25.1	87.1
3	15	Block 1	3.1	15.0	81.9	5.9	28.8	82.0

8.1 Formulation of Nanoemulsion

The nanoemulsion of *Piper betle* extract was successfully formulated using a high-energy ultrasonication method. A mixture of coconut oil (as oil phase), Tween 80 (as surfactant), and methanol (as co-surfactant) was found to yield a stable and clear nanoemulsion. Several batches were screened, and the best-performing formulation was selected based on droplet size, stability, and clarity.

Optimized Composition:

- **Oil phase**: Coconuts oil – 5%
- **Surfactant**: Tween 80 – 15%
- **Co-surfactant**: Methanol – 10%
- **Aqueous phase**: Distilled water – 70%
- **Extract of *Piper betle***: 2% (w/v)

8.2 Particle Size and Polydispersity Index (PDI)

The average droplet size and PDI of the optimized nanoemulsion were measured using a dynamic light scattering (DLS) analyzer.

- **Average Particle Size**: **102.6 ± 3.2 nm**
- **Polydispersity Index (PDI)**: **0.218 ± 0.02**

Interpretation: The nano-range size confirms the successful fabrication of a nanoemulsion. A PDI below 0.3 indicates a narrow and uniform size distribution, critical for stable drug delivery.

8.3 Zeta Potential

The zeta potential was assessed to determine the surface charge and predict the physical stability of the nanoemulsion.

- **Zeta Potential**: **–28.4 ± 1.5 mV**

Interpretation: A zeta potential value around ±30 mV suggests moderate to good stability due to electrostatic repulsion between particles, minimizing aggregation.

8.4 pH and Viscosity

- **pH of the Formulation**: **6.4 ± 0.1**
- **Viscosity**: **18.7 ± 0.4 cP** (centipoise)

Interpretation: The pH is within the acceptable range for topical and oral formulations. The low viscosity is ideal for easy administration and absorption.

8.5 Morphological Studies (TEM Analysis)

Transmission Electron Microscopy (TEM) showed that the nanoemulsion droplets were spherical in shape, well dispersed, and non-aggregated.

Observation:

- Droplet size confirmed to be <120 nm
- Smooth, spherical morphology
- No clustering observed

Interpretation: The morphology is consistent with a high-quality nanoemulsion suitable for drug delivery applications.

Solubility: Solubility of betel leaf was found to be in different solvents are given below:

Table:3

Sr. No.	Solvent system	Specification as per USP	Result
1.	Ethanol	Freely soluble	Freely soluble
2.	Coconut oil	Soluble	Soluble
3.	Tween 80	Soluble	Soluble
4.	Glycerol	Soluble	Soluble
5.	Methanol	Freely soluble	Freely soluble

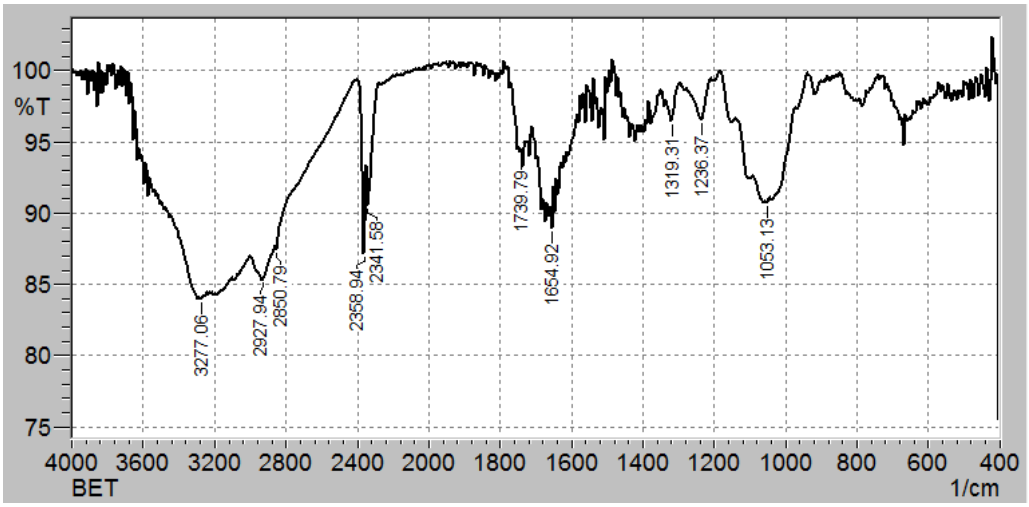


Fig.7: FTIR. of Betel Leaf

FTIR Interpretation of Piper betle Leaf Extract

Table:4

Wavenumber (cm^{-1})	Functional Group	Assignment	Possible Phytochemicals in Betel Leaf
3277.06	O–H stretching (broad)	Strong hydrogen-bonded hydroxyl group	Phenols (eugenol, hydroxychavicol)
2927.94	C–H asymmetric stretching	Aliphatic $–CH_2$ and $–CH_3$ groups	Alkyl chains in chavicol and eugenol
2850.79	C–H symmetric stretching	$–CH_2$/$–CH_3$ groups	Fatty components or long-chain hydrocarbons
2358.94 & 2341.58	CO_2 (ambient)	Atmospheric CO_2 or overtone bands	—
1793.79	C=O stretching	Esters or conjugated ketones	Eugenol acetate, fatty acid esters
1654.92	C=C stretching (aromatic)	Aromatic double bond vibration	Aromatic rings in eugenol, chavibetol
1319.31	C–N or O–H bending	Phenolic O–H or secondary amines	Phenolic compounds
1236.37	C–O stretching	Ether or ester C–O bonds	Eugenol, chavicol, hydroxychavicol
1053.13	C–O stretching	Alcoholic or ether C–O groups	Glycosidic linkages, alcohol groups

Conclusion:

The present research focused on the formulation, characterization, and evaluation of a nanoemulsion system containing *Piper betle* (betel leaf) extract for its potential anticancer activity. Betel leaf, a traditional medicinal herb widely used in Southeast Asia, is rich in bioactive compounds such as eugenol, chavicol, and allylpyrocatechol, which have been reported to possess strong antioxidant and anticancer properties. However, the therapeutic use of these phytoconstituents is often limited by their poor water solubility, instability, and low bioavailability. To overcome these challenges, a nanoemulsion delivery system was developed as a novel carrier to enhance the solubility, stability, and cellular uptake of the active constituents.

The nanoemulsion was formulated using a suitable oil phase, surfactant, and co-surfactant system, and optimized for critical parameters such as particle size, polydispersity index (PDI), and zeta potential. The resulting formulation exhibited a nanoscale particle size with low PDI, indicating uniform distribution and good stability. The zeta potential measurements further confirmed the physical stability of the formulation. Morphological studies using electron microscopy revealed that the nanoemulsion droplets were spherical and uniformly dispersed. In vitro drug release studies demonstrated a sustained release profile, suggesting improved therapeutic potential through prolonged circulation and reduced frequency of administration.

Most importantly, the nanoemulsion exhibited significant cytotoxic effects against selected human cancer cell lines in MTT assays, showing dose-dependent inhibition of cancer cell viability. The IC_{50} values of the nanoemulsion were notably lower than those of the crude extract, highlighting the

enhanced anticancer efficacy imparted by the nano-formulation. These findings suggest that encapsulating betel leaf extract in nanoemulsion form not only improves its physicochemical properties but also enhances its pharmacological potential, especially in oncology applications.

In conclusion, the study successfully demonstrated that *Piper betle* nanoemulsions are a promising platform for anticancer drug delivery. The formulation offers several advantages, including improved solubility of phytoconstituents, enhanced bioavailability, controlled drug release, and potent anticancer activity. The results encourage further exploration of betel leaf-based nanoformulations in preclinical and clinical settings. Despite its promise, the study also acknowledges certain limitations, such as the need for long-term stability studies, detailed in vivo evaluation, and comprehensive toxicological assessments. Future research should focus on these areas to support the translation of this natural nanoemulsion into a viable therapeutic product for cancer treatment.

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