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SYNERGISTIC ANTIMICROBIAL, ANTIFUNGAL, AND ANTICANCER ACTIVITIES OF METHANOLIC HERBAL EXTRACTS FROM *PIPER BETEL* AND *CALOTROPIS GIGANTEA* LEAVES

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Abstract

Piper betel (Piperaceae) and Calotropis gigantea (Apocynaceae) are well reported for their pharmacological importance, possessing various medicinal properties including anti-inflammatory, antimicrobial, and anticancer effects. The aim of this study was to explore the combined potential of methanolic extracts from Piper betel and Calotropis gigantea leaves for enhanced antimicrobial, antifungal, and anticancer activities alone and in combination. Previous research has shown the presence of phytochemicals with antimicrobial, antifungal, and anticancer properties in various plant extracts, indicating their therapeutic potential. The current study employed the agar well diffusion method to assess antimicrobial activity against Staphylococcus aureus and Escherichia coli, and the Zone Inhibition Method for antifungal activity against Candida albicans and Aspergillus niger. Additionally, the MTT assay was used to evaluate anticancer activity against A549 (lung carcinoma cell line). GC-MS analysis was conducted to identify bioactive compounds present in the extracts. Results revealed promising antimicrobial, antifungal, and anticancer activities of the individual extracts as well as their combination. Percentage yields of the extracts were determined, with methanolic extract of Piper betel leaves yielding 5.8% and Calotropis gigantea leaves yielding 6.3%. In antibacterial and antifungal activities, the combination (1:1) of methanolic extracts of Piper betel and Calotropis gigantea exhibited synergistic efficacy compared to individual extracts. This combination holds promise for the development of medicines targeting bacterial and fungal infections, as well as cancer treatment, given its demonstrated effectiveness and synergistic effects. GC-MS analysis identified compounds with antimicrobial, antifungal, and anticancer properties, supporting the observed biological activities of the extracts. Overall, the study suggests the potential utility of combined herbal extracts for the development of effective therapeutic interventions.

Keywords: Piper Betel, Calotropis Gigantea, Synergistic Activity, Antimicobial, Antifungal, Anticancer Activity, In-Silico Studies, İn Vitro Studies.

1. INTRODUCTION

Piper betel and *Calotropis gigantea* have significant pharmacological importance. Piper betel, also known as Betel leaf, is valued for its medicinal properties such as anti-inflammatory, antiplatelet, and immune modulator effects. It also exhibits antioxidant, antimicrobial, gastroprotective, and antidiabetic activities. *Calotropis gigantea*, commonly known as milkweed, is used in traditional medicine systems for various ailments. It contains a wide range of compounds including alkaloids, flavonoids, terpenoids, and cardiac glycosides. This plant exhibits pharmacological activities such as anti-asthmatic, antioxidant, antibacterial, antiviral, wound healing, anti-inflammatory, hepatoprotective, and hypoglycemic effects. Both plants have been extensively studied for their therapeutic potential and have shown promising results in various areas of medicine.

Phytochemicals with antimicrobial, antifungal, and anticancer activities have been identified in various plants. In the methanolic extracts of *Withania somnifera* (Lingfa &

Ankanagari, 2023), phytochemicals with antimicrobial and anticancer properties were found in the leaf, root, and stem parts of the plant at the reproductive stage. Some of the identified phytochemicals include 2-pentanone, 5-chloro-, benzene, 1,1'-(1,2-ethenediyl)bis[2-methyl-, benzoic acid, 3-methyl-2-trimethylsilyloxy-, trimethylsilyl ester, and acetohydroxamic acid. Ethyl acetate, ethanol, and acetone extracts of *Thymelaea hirsuta*, *Urginea maritima*, and *Plantago albicans* (El-Bondkly et al., 2022) also showed antimycotic and antiproliferative activities against different human cancer cell lines. *Tristemma mauritianum, Crassocephalum bougheyanum,* and *Lavigeria macrocarpa* (Kengne et al., 2023) exhibited antifungal activity against *Candida* spp. and synergistic effects with ketoconazole. Overall, these studies highlight the potential of phytochemicals from different plants for antimicrobial, antifungal, and anticancer applications .

In previous reports, combined herbal extracts have shown antimicrobial, antifungal, and anticancer activities. The combination of *Rosa centifolia L., Curcuma longa L., Rosmarinus officinalis L.*, and *Punica granatum L*. glycolic extracts demonstrated antifungal activity against *Candida spp*. in different concentrations (Meccatti et al., 2023). Extract-extract combinations of *Senna alata*, *Ricinus communis,* and *Lannea barteri* exhibited synergistic effects against various microorganisms, including *Candida albicans* (Donkor et al., 2023). *Tristemma mauritianum, Crassocephalum bougheyanum,* and *Lavigeria macrocarpa* extracts displayed antifungal activity against *Candida* spp., and their combinations with antifungals showed synergistic effects against clinical resistant isolates. Methanol extracts of *Acori gramineri, Rhizoma angelicae, Tenuissimae radix, Cinnamomi cortex, Cinnamomi ramulus, Impatientis semen, Magnoliae cortex, Moutan Cortex radicis, Phellodendri cortex, Scutellariae radix, and Syzygii flos* exhibited antimicrobial activity against *Candida albicans, Staphylococcus aureus,* and *Malassezia* pachydermatis (Yoon & Kim, 2023). Plant-derived bioactive phytochemicals have shown potent antimicrobial activities and synergistic activities alone or in combination with commercially available antimicrobial drugs (Choudhury, 2022).

To check the synergistic effects of combined herbal extracts of *Piper betel* and *Calotropis gigantea* leaves against different microorganisms and cancer cell lines, we hypothesize that the combination of *Piper betel* and *Calotropis gigantea* methanolic extracts would be effectively increased the antimicrobial, antifungal, and anticancer activities compared to individual extracts, indicating an effective strategy for the development of novel therapeutic agents against infectious diseases and cancer.

2. MATERIALS AND METHODS

2.1 Material

The specified chemicals, including 3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), crystal violet, and propidium iodide, were procured from Sigma Chemicals Co. (St. Louis, MO, USA).The materials utilized in this study included Mueller-Hilton Agar (MHA) plates, bacterial cultures of *Pseudomonas aeruginosa* (MTCC3541) and *Staphylococcus aureus*, Whatman No. 1 filter paper discs (5mm), Dimethyl Sulphoxide (DMSO) as a solvent (SRL Chem 28580), Ciprofloxacin (SRL Chem- 78079) for antibacterial activity, Sabouraud dextrose agar (SDA) plates, fungal cultures of *Candida albicans* (MTCC 854) and *Aspergillus niger* (MTCC281), Potato Dextrose Agar (PDA) plates, Amphotericin B (Amphocare) for

antifungal activity, and MCF-7 (Breast cancer cell line) for anticancer activity. All materials were procured from the Akaar Biotechnology Laboratory.

2.2 Plant material collection, authentication and extraction

The *Piper betel* and *Calotropis gigantea* leaves specimens were collected from Greater Noida, Uttar Pradesh, in March 2023. Taxonomic authentication was conducted by Dr. Priyanka Ingle, Scientist C at the Botanical Garden of India Republic, Gautam Budha Nagar, Uttar Pradesh. A voucher specimen (BSI/BGIR/1/TECH./2023/62) has been deposited in the herbarium of the Herbal Research & Development Institute for future reference. Fresh leaves of *Piper betel* and *Calotropis gigantea* were collected. The leaves were washed thoroughly, air-dried, and powdered. Methanolic extraction was performed using the cold compression method (Ranalli et al., 1999).

2.3 GCMS Analysis

10 µl of the sample (50 mg/ml) was placed into a separating funnel and mixed by adding 10 ml of a water and ethyl acetate mixture in a 1:4 ratio (2.5 ml water to 7.5 ml ethyl acetate). The upper layer was collected and then concentrated to 1 ml using a rotary evaporator. Subsequently, 50 µl of N, O-Bis(trimethylsilyl)trifluoroacetamide and trimethylchlorosilane (BSTFA+TMCS) were added, followed by the addition of 10 µl of pyridine. The samples were then heated at 60°C for 30 minutes. To prepare the BSTFA+TMCS solution, a mixture of 99 µl BSTFA and 1 µl TMCS was made to a final volume of 100 µl. The samples were transferred into GC vials and dried using nitrogen gas. Finally, the samples were dissolved in methanol before undergoing GC-MS analysis (Rautela et al., 2018).

2.4 Antimicrobial Assay

The antimicrobial efficacy of individual extracts (MEPB and MECG) and their combination was assessed against both Gram-positive (e.g., *Staphylococcus aureus*) and Gram-negative (e.g., *Escherichia coli*) bacteria using the agar well diffusion method.The antibacterial activity was examined using the Zone Inhibition Method (Kirby-Bauer method) (Al Laham & Al Fadel, 2014; Pacheco et al., 2013; A. A. Singh et al., 2023). To conduct this, Mueller-Hinton agar (MHA) plates were inoculated by spreading with 100 µl of bacterial culture of E. coli (adjusted to 0.5 McFarland Unit - approximately 1.5 X 10^8 CFU/mL) and subsequently discs containing varying concentrations (0,50 ,125 ,250 ,500 ,1000) µg/well were placed. Ten percent of the sample was utilized and serially diluted to achieve the required amount for loading onto the discs. Each plate also included a disc loaded with solvent alone, serving as a vehicle control, while Ciprofloxacin discs (10 µg/well) were employed as positive controls. The plates were then incubated at 37°C for 24 hours, and the clear zones formed around the discs were measured and recorded.

2.5 Antifungal Assay

Antifungal activity of (MEPB and MECG) alone and in combination was assessed against fungal strains such as *Candida albicans and Aspergillus niger* using agar diffusion or broth dilution methods. The Antifungal activity was checked by following Zone Inhibition Method (Kirby-Bauer method) (Al Laham & Al Fadel, 2014; Pacheco et al., 2013; A. A. Singh et al., 2023). The PDA plates were inoculated by spreading with 100 µl of fungal culture, *A. niger or C albicans* (adjusted to 0.5 McFarland Unit - Approx cell density (1.5 X 10^8 CFU/mL) and followed by placing the discs containing

different concentration (0, 50, 125, 250, 500, 1000) µg/well). One disc in each plate was loaded with solvent alone which served as vehicle control and Amphotericin B (50 µg/well) were taken as positive control. The plates of *A. niger or C. albicans* were incubated (Basil Scientific Corp. India- Incubator) at 37 °C for 248 hrs. The clear zones created around the disc were measured and recorded

2.6 Anticancer Assay in A549 (lung cancer cells)

The anticancer activity of individual extracts (MEPB and MECG) and their combination was evaluated against cancer cell lines such as A549 (lung cancer) using MTT assay. Cytotoxicity of the provided samples on A549 cell line (procured from NCCS Pune) was assessed using the MTT Assay. A549 cells (10,000 cells/well) were seeded in a 96-well plate and cultured for 24 hours in DMEM medium supplemented with 10% FBS and 1% antibiotic solution at 37°C with 5% CO2. The following day, cells were treated with varying concentrations (1-1000 µg/mL) of the formulations prepared in incomplete medium. After 24 hours of incubation, MTT Solution (final concentration 250 µg/mL) was added, followed by a further 2-hour incubation. The culture supernatant was then removed, and the cell layer was dissolved in 100 µl Dimethyl Sulfoxide (DMSO). Absorbance was measured at 540 nm and 660 nm using an Elisa plate reader (iMark, Biorad, USA). IC-50 values were calculated using Graph Pad Prism 6 software. Images were captured under an inverted microscope (Olympus ek2) equipped with a camera (AmSope digital camera 10 MP Aptima CMOS). This methodology was conducted following established protocols (Mosmann, 1983).

3. RESULTS

The findings from this study underscore the significant biological activities of methanolic extract of the leaves of *Piper betel* (MEPB) and *Calotropis gigantea* (MECG). These extracts have demonstrated promising effects for antibacterial, antiviral and anticancer activities in different in-vitro biological assays.

3.1 Percentage yield of the extracts:

The percentage yields of methanolic extract of the *Piper betel* (MEPB) leaves was 5.8% (w/w) and for the methanolic extract of *Calotropis gigantea* (MECG) leaves was 6.3% (w/w) which provide essential information as shown in table 1.

Table 1: Extraction of herbal plants and yield percentage (%)

S. No.	Extract name	Weight of Powder (mg)	Weight of Extract(mg)	% Yield
1.	Methanolic extract of *Piper betel* leaves (MEPB)	2000 mg	116	5.8%
2.	Methanolic extract of *Calotropis gigantea* leaves (MECG)	2000 mg	126	6.3%

3.2 Antibacterial Activity

The percentage of inhibition for the antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* for various concentrations (0, 50,125, 250, 500, 1000) µg/well. of methanolic extracts (MEPB and MECG) and their combination (MEPB+MECG) was measured.

Percentage of inhibition for *Staphylococcus aureus*: MEPB: (0, 5.4, 5.8, 7.4, 7.9, 9.6)%, Positive control (Ciprofloxacin) (28%) (Figure 1), MECG: (0, 5.3, 7.3, 8.2, 9.3, 10.7)%, Positive control (Ciprofloxacin) (27%) (Figure 2), MEPB+MECG (1:1): (0, 6.5, 7.9, 9.4, 11.0, 12.8)% Positive control (Ciprofloxacin) (29%) (Figure 3).

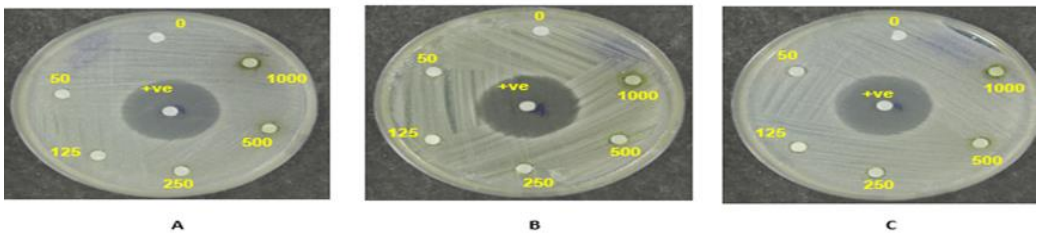

Figure 1: Inhibition zone at different concentrations of MEPB against Staphylococcus aureus

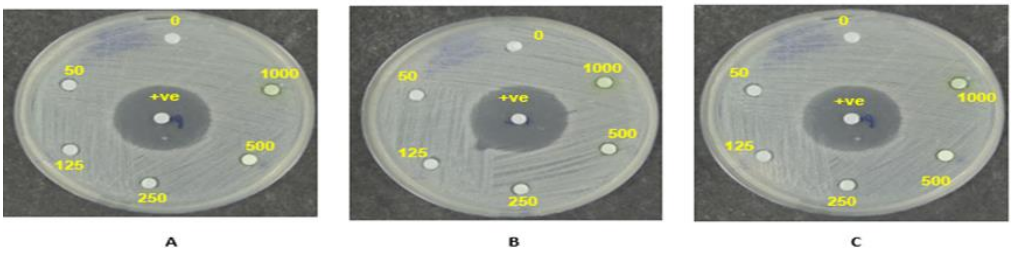

Figure 2: Inhibition zone at different concentrations of MECG against Staphylococcus aureus

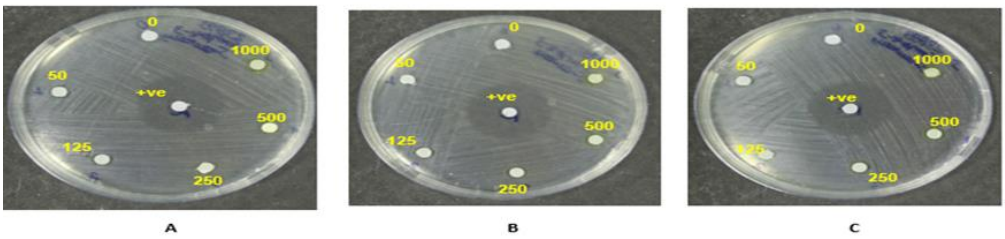

Figure 3: Inhibition zone at different concentrations of MEPB+MECG against Staphylococcus aureus

Percentage of inhibition of MEPB against *Escherichia coli*: (0, 4.9, 6.4, 7.5, 8.6, 8.9)%, Positive control (Ciprofloxacin) (23%) (Figure 4), MECG: (0, 4.9, 5.2, 6.5, 8.4, 8.8)%, Positive control (Ciprofloxacin) (20%) (Figure 5); MEPB+MECG (1:1): (0, 5.2, 6.5, 7.5, 8.5, 9.3)%, Positive control (Ciprofloxacin) (29%) (Figure 6).

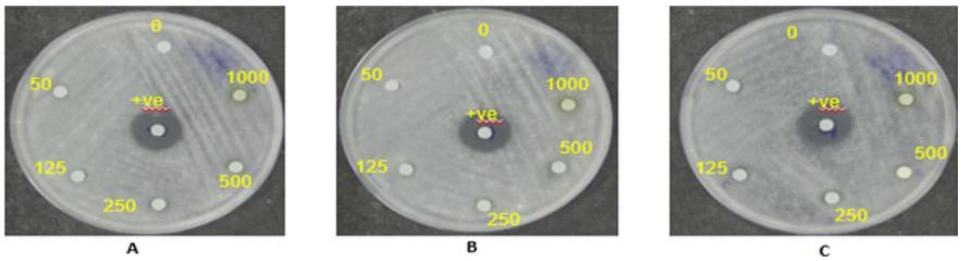

Figure 4: Inhibition zone at different concentrations of MEPB against Escherichia coli

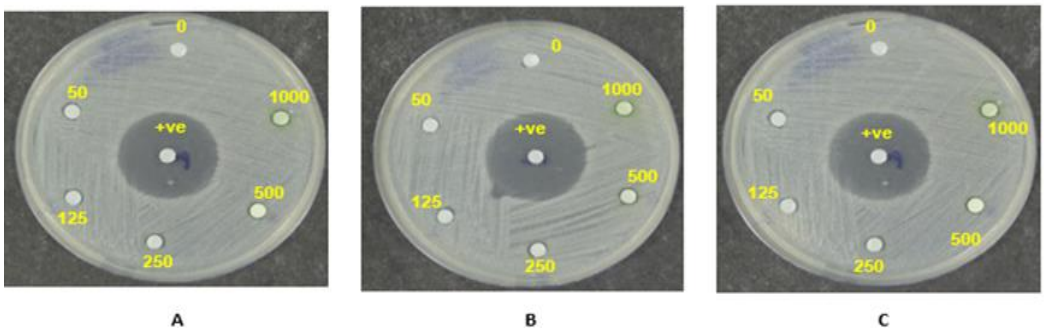

Figure 5: Inhibition zone at different concentrations of MECG against Escherichia coli

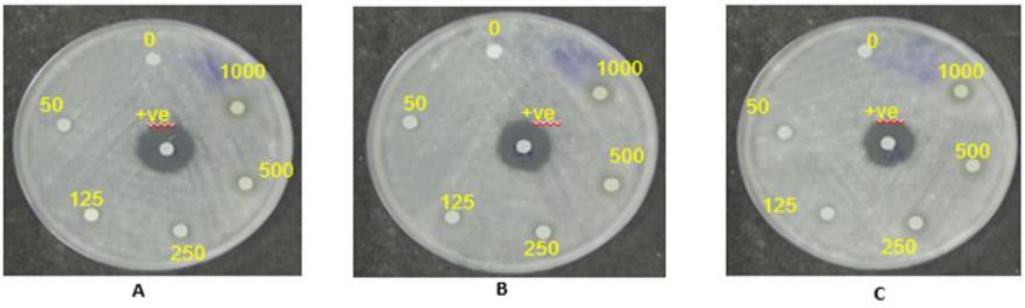

Figure 6: Inhibition zone at different concentrations of MEPB+MECG against Escherichia coli

In antibacterial activities, **Ciprofloxacin** was used as a positive control and denoted as **PC.**

3.3 Antifungal Activity

The percentage inhibition for the antifungal activity against *Aspergillus niger* and *Candida albicans* for various concentrations of methanolic extracts (MEPB and MECG) and their combination (MEPB+MECG) at different concentrations (0,50,125,250,500,1000) µg/well was measured.

For *Candida albicans* MEPB: (0, 5.2, 5.6, 6.5, 7, 7.6)%, Positive control (Amphotericin B) (28%) (Figure 7); MECG: (0, 5.8, 6.4, 7.1, 7.4, 7.5)% , Positive control (Amphotericin B) (27%) (Figure 8), MEPB+MECG (1:1): (0, 5.3, 6.3, 7.2, 8.3, 9.3)% , Positive control (Amphotericin B) (29%) (Figure 9).

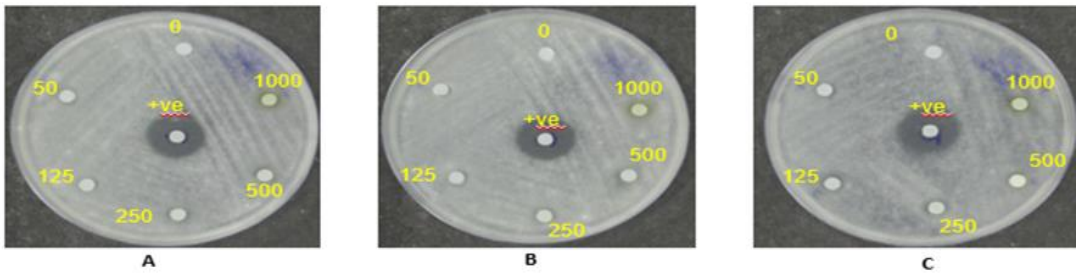

Figure 7: Inhibition zone at different concentrations of MEPB against Candida albicans

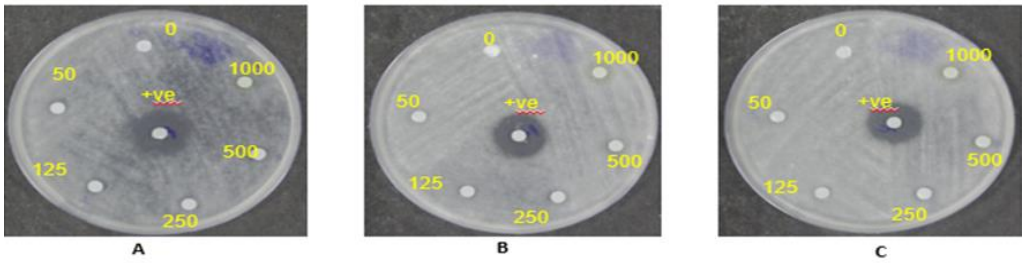

Figure 8: Inhibition zone at different concentrations of MECG against Candida albicans

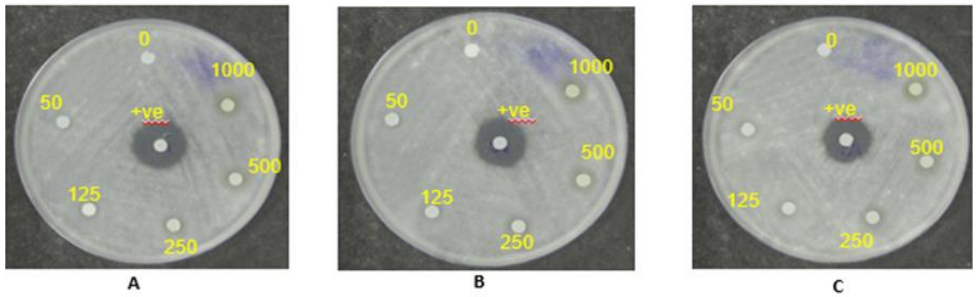

Figure 9: Inhibition zone at different concentrations of MEPB+MECG against Candida albicans

Percentage of inhibition against *Aspergillus niger*, MEPB: (0,4.9, 6.4, 7.5, 8.4, 9.4)% (Figure 10), Positive control (Amphotericin B) (28%) MECG: (0, 5.2, 7, 8.3, 9.1, 10.1)%, Positive control (Amphotericin B) (27%) (Figure 11), MEPB+MECG (1:1): (0, 5.6, 6.9, 7.9, 9, 10.2)%, Positive control (Amphotericin B) (28%) (Figure 12).

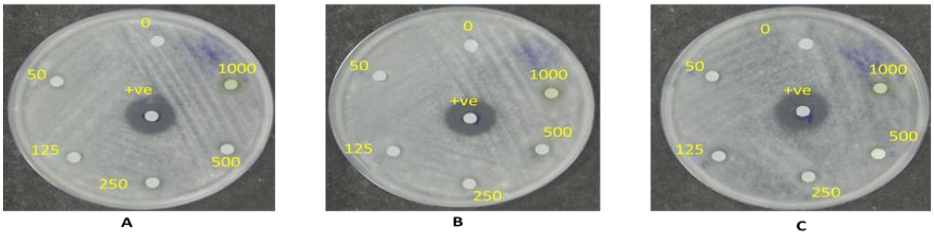

Figure 10: Inhibition zone at different concentrations of MEPB against Aspergillus niger

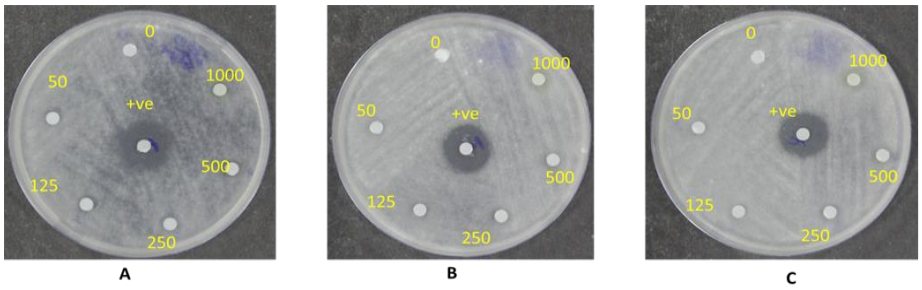

Figure 11: Inhibition zone at different concentrations of MECG against Aspergillus niger

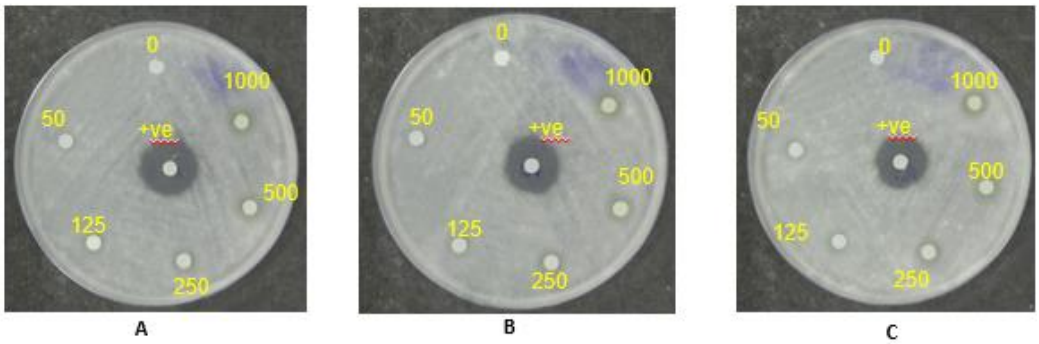

Figure 12: Inhibition zone at different concentrations of MEPB+MECG against Aspergillus niger

In antifungal activities, **Amphotericin-B** was used as a positive control and denoted as **PC.**

3.4. Anticancer activity (MTT Assay)

Cell viability assay was performed in *A549 (Lung carcinoma cell line)* at different concentrations of methanolic extracts (MEPB and MECG) alone and their combination (MEPB+MECG) at (0,1,10,50,100,250,500,1000) µg/ml was measured.

In MTT assay, percentage cell viability inhibition of MEPB: (96.49, 89.42, 81.45, 69.57, 52.52, 41.22, 31.76, 19.87)%, MECG (100.18, 91.68, 82.45, 67.99, 54.08, 41.16, 31.19, 19.79)%; MEPB + MECG (1:1) (99.49, 90.27, 81.65, 68.25, 49.91, 41.19, 31.75, 19.62)% at different concentrations (0,1,10,50,100,250,500,1000) µg/ml, respectively as shown in figures 13, 14 and 15 respectively.

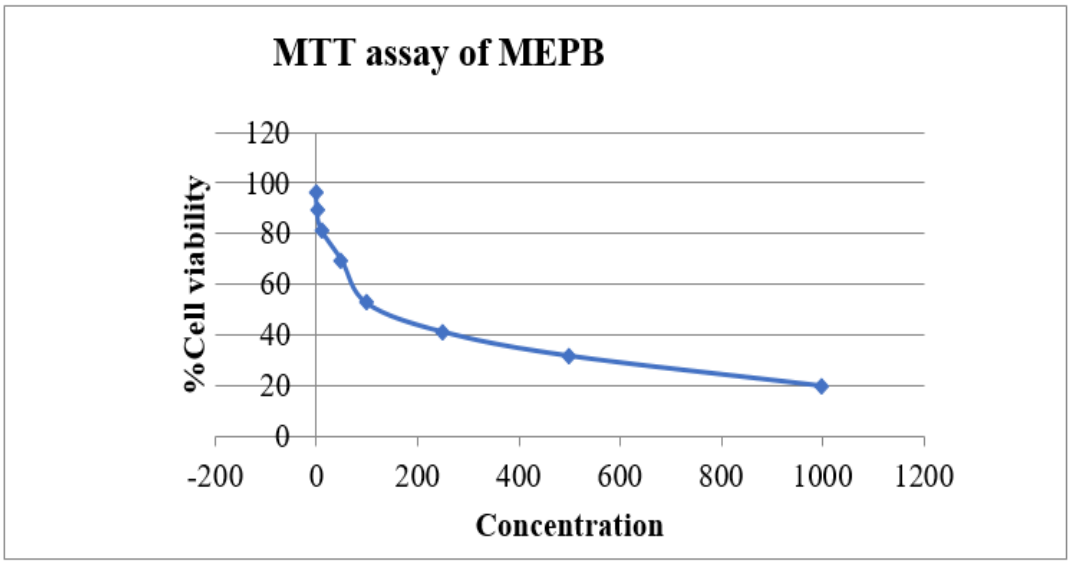

Figure 13: Cell viability assay of MEPB in A549 cell line

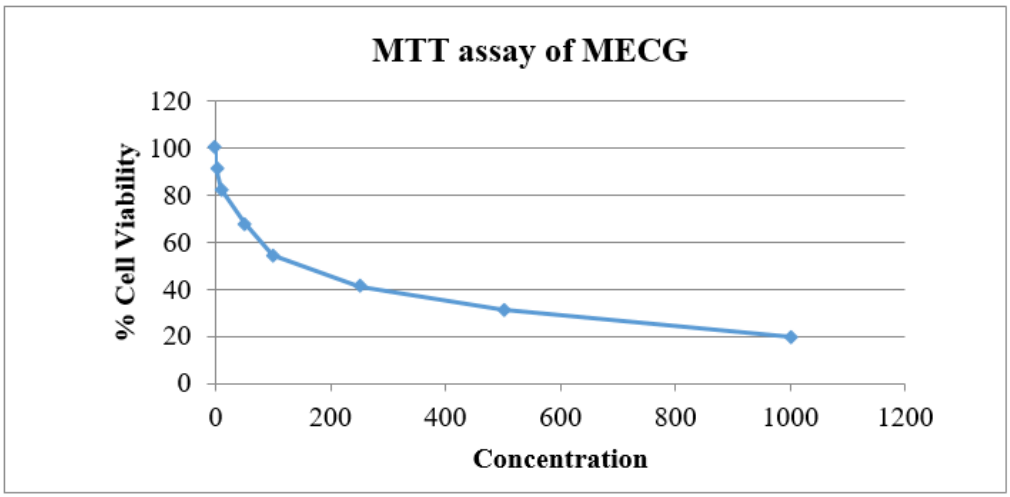

Figure 14: Cell viability assay of MECG in A549 cell line

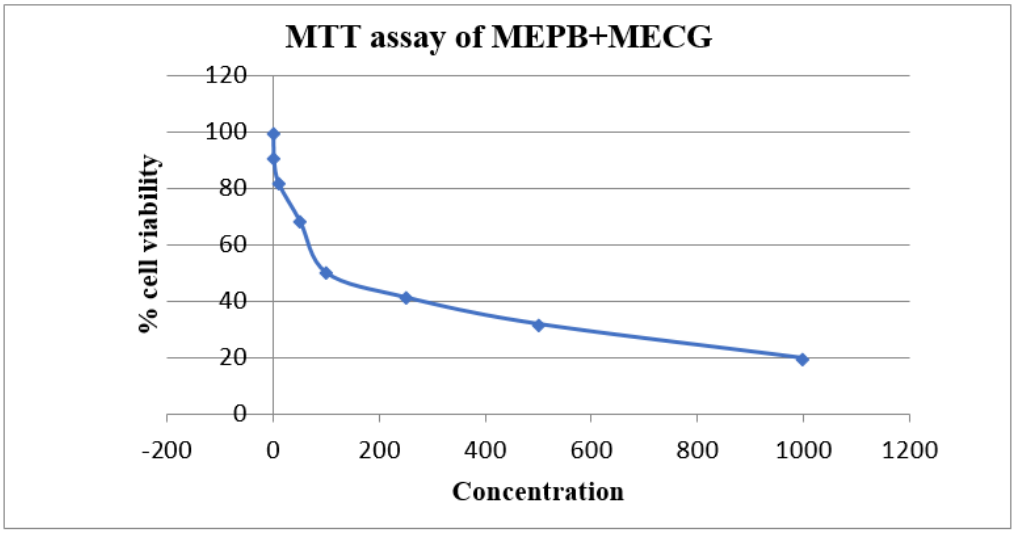

Figure 15: Cell viability assay of MEPB and MECG in A549 cell line

IC_{50} of methanolic extract of the leaves of *Piper betel* (MEPB) was 107.39 µg/ml, *Calotropis gigantea* (MECG) was 114.66 µg/ml, and combination of MEPB and MECG (1:1) was 99.75 µg/ml measured. Four replicates (n=4) were employed to ensure reliability and consistency in evaluating the anticancer activity.

3.5 GC-MS Analysis

Gas Chromatography-Mass Spectrometry (GC-MS) analysis was performed to identify the bioactive compounds present in the methanolic extracts of MEPB and MECG. The analysis confirmed the presence of compounds with antimicrobial, antifungal, and anticancer properties, supporting the observed biological activities of the extracts

The gas chromatography-mass spectrometry (GCMS) analysis revealed the following composition of compounds in MEPB: 2-Tert-Butyl-4-(1,1,3,3-Tetramethylbutyl)Phe at 2.09%, 1,2-Benzenedicarboxylic Acid, Diethyl Ester at 7.93%, 1-(4-Isopropylphenyl)-2-Methylpropyl Acetate at 4.53%, 2-Decen-1-Ol at 1.13%, D-Mannitol, 2,5-Bis-O-(Phenylmethyl)-1,6-Bis- at 0.76%, Propane, 1-Chloro-2,2-Dimethyl at 1.31%, Decanoic Acid, 8-Methyl-, Methyl Ester at 3.34%, Hexamethylcyclohexane at 0.89%, Tetradecanoic Acid, 12-Methyl-, Methyl Ester at 1.14%, 1,2-Benzenedicarboxylic Acid,

Dioctyl Ester at 3.46%, Phenol, 2,4-Bis(1,1-Dimethylethyl)-, Phosphite (3:1) at 13.51%, and Tris(2,4-Di-Tert-Butylphenyl) Phosphate at 59.90%.

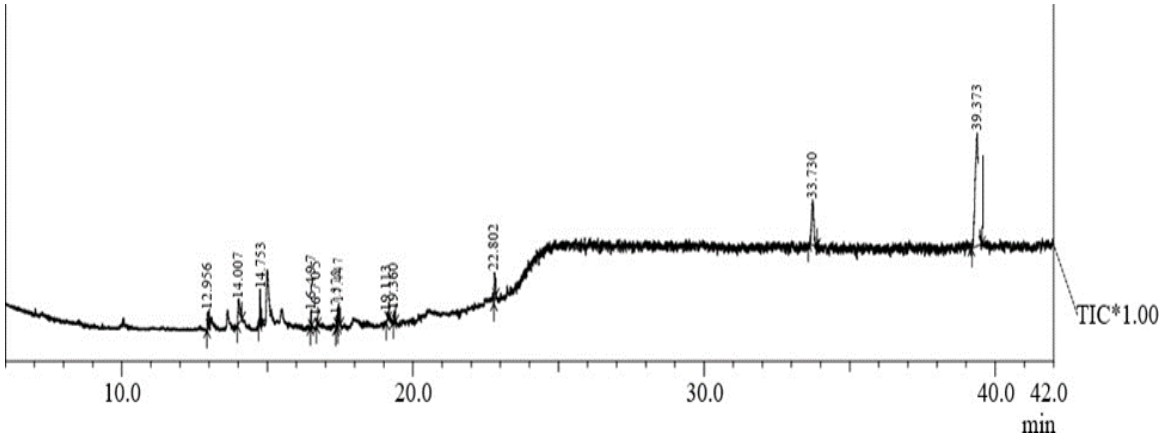

Figure 13: GCMS chromatogram revealed the following composition of compounds in MEPB

The gas chromatography-mass spectrometry (GCMS) analysis revealed the following composition of compounds in MECG: 1.35%: 1,2-Di-Tert-Butylbenzene - 0.44%: Docosane - 1.51%: 1,1-Dicyclopropyl-2-Methyl-1-Pentene - 0.49%: Docosane - 0.16%: 5-Hepten-3-One, 5-Methyl-, (Z)- - 0.85%: Docosane - 2.20%: Phenol, 2,4-Bis(1,1-Dimethylethyl)- - 2.95%: Pentanoic Acid, 1,7,7-Trimethylbicyclo[2.2.1] - 0.69%: 1,3-Cyclohexadiene, 1-Methyl-4-(1-Methylet) - 3.07%: 1,2-Benzenedicarboxylic Acid, Diethyl Ester - 1.21%: 7,9-Di-Tert-Butyl-1-Oxaspiro(4,5)Deca-6,9-Diene-2,8-Dione - 1.64%: Hexadecanoic Acid, Methyl Ester - 4.77%: N-Hexadecanoic Acid - 0.89%: 17-Octadecynoic Acid - 0.76%: Nonanoic Acid, 7-Methyl-, Methyl Ester - 0.11%: (E)-4,4-Dimethylpent-2-Enal - 0.41%: (2r,5r,6r)-2-T-Butyl-5-Isopropyl-5,6-Dimethyl- - 0.19%: (2r,5r,6r)-2-T-Butyl-5-Isopropyl-5,6-Dimethyl- - 1.22%: Cyclohexaneacetic Acid, .Alpha.-Methyl-.Alpha.-Propyl-, Meth - 0.31%: Phosphorochloridic Acid, Hexyl Propyl Ester - 26.57%: Phenol, 2,4-Bis(1,1-Dimethylethyl)-, Phosphite (3:1) - 48.20%: Tris(2,4-Di-Tert-Butylphenyl) Phosphate.

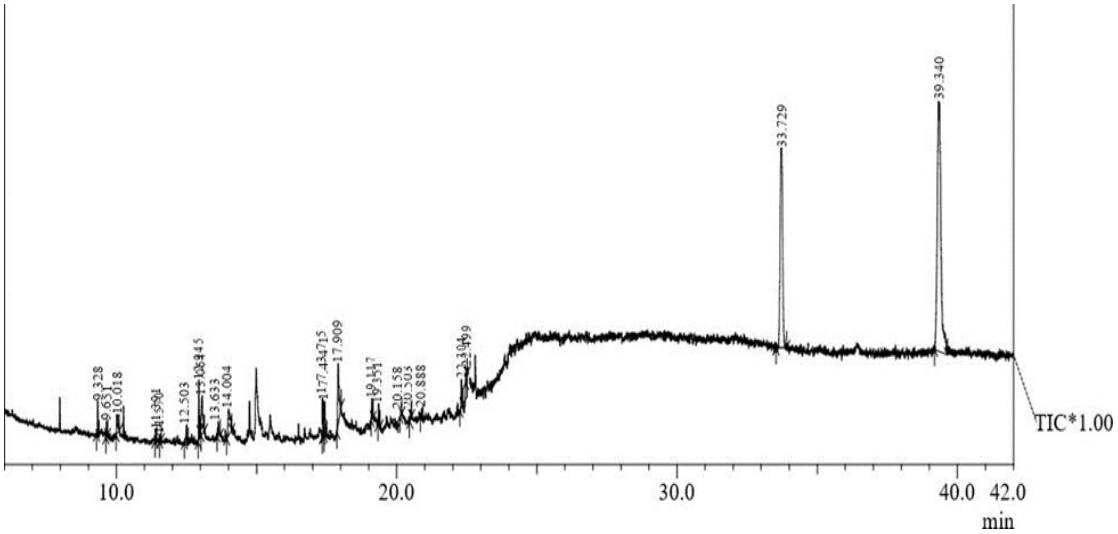

Figure 14: GCMS chromatogram revealed the following composition of compounds in MECG

In GCMS analysis revealed that 2,4-Bis(1,1-Dimethylethyl)-, Phosphite (3:1) and Tris(2,4-Di-Tert-Butylphenyl) Phosphate compounds commonly present in both plant extracts MEPB and MECG and having antioxidant activity as well as anticancer activity. Remaining compounds having antimicrobial, antifungal activity. The combination (1:1) of MEPB and MECG exhibited synergistic efficacy in all tested activities, including antibacterial, antifungal, and anticancer effects. This synergistic activity suggested that combined extracts in the development of novel therapeutic agents for the treatment of bacterial and fungal infections, as well as in cancer.

4. DISCUSSION

The present study aimed to explore the combined potential of methanolic extracts from *Piper betel* and *Calotropis gigantea* leaves for enhanced antimicrobial, antifungal, and anticancer activities alone and in combination. The rationale behind this investigation planned from previous research indicating the presence of phytochemicals with antimicrobial, antifungal, and anticancer properties in various plant extracts (El-Bondkly et al., 2022; Kengne et al., 2023; Lingfa & Ankanagari, 2023).This study aligns with the broader scope of utilizing natural products for therapeutic purposes, leveraging the rich pharmacological potential of traditional medicinal plants.

The methanolic extracts of *Piper betel* and *Calotropis gigantea* leaves exhibited promising biological activities. The antimicrobial assays demonstrated significant inhibition of both Gram-positive *(Staphylococcus aureus)* and Gram-negative *(Escherichia coli)* bacteria. Moreover, the antifungal assays against *Candida albicans* and *Aspergillus niger* also showcased substantial inhibition zones (Al Laham & Al Fadel, 2014; Pacheco et al., 2013; S. Singh et al., 2020). These results are consistent with previous findings on the antimicrobial and antifungal properties of plant extracts, highlighting the potential of *Piper betel* and *Calotropis gigantea* as sources of bioactive compounds with therapeutic implications. Furthermore, the anticancer activity of the extracts was evaluated against the A549 lung carcinoma cell line using the MTT assay.

The MTT assay measures cell viability by assessing metabolic activity, involving the reduction of MTT to formazan crystals. IC50, or half-maximal inhibitory concentration, indicates the concentration of a compound needed to inhibit cell viability by 50%. Plant extracts are categorized as highly active (IC50 < 10 µg/ml), active (10-100 µg/ml), moderately active (100-500 µg/ml), or low activity (IC50 > 500 µg/ml) based on their IC50 values (Moga et al., 2021). The results revealed dose-dependent cytotoxic effects, with the combination of *Piper betel* and *Calotropis gigantea* extracts demonstrating synergistic efficacy in inhibiting cancer cell proliferation.

This synergistic effect is particularly noteworthy as it suggests a potential avenue for the development of novel therapeutic interventions against cancer, leveraging the combined bioactive components of these plant extracts. The GC-MS analysis provided valuable insights into the chemical composition of the extracts, identifying several bioactive compounds with known antimicrobial, antifungal, and anticancer properties (Rautela et al., 2018). Notably, compounds such as 2,4-Bis(1,1-Dimethylethyl)-, Phosphite (3:1) and Tris(2,4-Di-Tert-Butylphenyl) Phosphate were found to be common constituents of both Piper betel and Calotropis gigantea extracts, suggesting their potential contribution to the observed biological activities. These findings supported the observed synergistic effects of methanolic extracts of *Piper betel* and *Calotropis gigantea* leaves. The study highlights the pharmacological significance of

methanolic extracts of *Piper betel* and *Calotropis gigantea* leaves paving the way for their exploration in the development of new therapeutic agents.

5. CONCLUSION

In conclusion, this study demonstrates the synergistic antimicrobial, antifungal, and anticancer activities of methanolic extracts from *Piper betel* and *Calotropis gigantea* leaves. The combination of these extracts showed enhanced efficacy compared to individual extracts, indicating a promising strategy for the development of novel therapeutic agents against infectious diseases and cancer. GC-MS analysis identified bioactive compounds in the extracts, supporting their observed biological activities. These findings underscore the potential utility of combined herbal extracts for the development of effective and safe therapeutic interventions. Further research is needed to explore the mechanisms underlying the synergistic effects and to evaluate the clinical applicability of these combined extracts.

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