**SYNERGISTIC EFFECT OF POLYHERBAL EXTRACT CONTAINING INDIGENOUS MEDICINAL PLANTS**

**ABSTRACT**

The aim of the present study is synergistic effect of polyherbal containing indigenous medicinal plants, including antioxidant and immunostimulatory effects of polyherbal extracts derived from *Morinda citrifolia*, *Annona muricata*, and *Curcuma caesia*. Synergism, the enhanced biological effect of combined agents over individual ones, plays a pivotal role in polyherbal formulations (PHFs), improving therapeutic efficacy while minimizing toxicity. The selected medicinal plants, known for their rich phytochemical profiles and traditional uses, were extracted using ethanol in a Soxhlet apparatus. Preliminary phytochemical screening revealed the presence of key constituents such as tannins, sterols, glycosides, flavonoids, quinones, terpenoids, and phenols. GC-MS analysis confirmed the presence of bioactive compounds, including turmerone, known for its potent pharmacological activities. Antioxidant capacity was evaluated through in vitro assays, namely DPPH and hydroxyl radical scavenging methods. The results indicated significant free radical scavenging activity, with the combination extracts exhibiting greater efficacy than individual ones, validating synergistic interaction. Further, the phagocytosis assay demonstrated notable immunostimulatory potential, with increased phagocyte index values in treated groups compared to controls. The synergistic effect was quantitatively assessed using fractional inhibitory concentration (FIC) indices, confirming improved antioxidant activity in the combined extracts. Toxicity assessment through brine shrimp lethality bioassay showed low cytotoxicity across tested concentrations, supporting the safety profile of the formulations. This research highlights the therapeutic promise of these polyherbal combinations, especially for developing cost-effective, natural antioxidant and immunostimulatory agents. The study also supports traditional knowledge with scientific validation and encourages further exploration of synergistic herbal therapies in modern medicine.

**Keywords:** Polyherbal formulation, *Morinda citrifolia*, *Annona muricata*, *Curcuma caesia*, Antioxidant activity, immunostimulant, toxicity.

1. **INTRODUCTION**
   1. **Synergistic effect**

The synergistic effect refers to increasing the activity of combined substances over the additive effect, whereby they interact with compounds through poly-herbal combinations. The synergistic effect enhances the antioxidant activity through a combination of several potent herbal plants with high antioxidant activity. Synergy is defined as the interaction of two or more agents to produce a combined effect greater than the sum of their individual effects. In the medicinal research field, however, the understanding of synergy is complicated. Spinella has classified the concept of synergy broadly into two main categories based on the mode of action-pharmacodynamic and pharmacokinetic synergy. The first type of synergy describes two or more agents that work on the same receptors or biological targets, resulting in enhanced therapeutic outcomes through their positive interactions. The second type of synergy results from interactions between two or more agents during their pharmacokinetic processes (absorption, distribution, metabolism and elimination), leading to changes of the agents quantitatively in the body and hence their therapeutic effects.

* 1. **Polyherbal extraction**

The combination of two or more drugs or plants is referred to as Polyherbal Formulations (PHF). A Polyherbal formulation increases the therapeutic effect and shows synergism. It also reduces the adverse effects by reducing the concentrations of single herbs.(Kumar et al., 2022) Polyherbal medicines, which contain two or more herbal ingredients, are often more effective than single drugs because of their complementary and potentiating activities.

The combination of two or more herbal extracts brings about increased therapeutic efficiency, enhanced pharmacological actions, faster relief, and reduced adverse effects as compared to conventional medicine due to a lower dose of administration.( Dev SK et al., 2019) Polyherbal medicines are now widely preferred and used around the world because of their high effectiveness, ready availability, low toxicity, and environmentally friendly nature, and they reduce the time of treatment or the individual cost of anti-inflammatory and antimicrobial drugs,(Thabrew MI et al., 2003) resulting in lower prescription costs. The concept of polyherbal combination has been well established and has achieved remarkable success in allopathic medicine, providing patients with new hope. (Parasuraman S et al., 2014)

* 1. ***Morinda citrifolia***

*Morinda citrifolia L.*, small evergreen trees that belong to the Rubiaceae family, are native to Southeastern Asia and Australia. They are referred to as "noni" on the Hawaiian and Tahitian islands. It is also known as the Indian Mulberry, Ba Ji Tian, Nono or Nonu, and Nhau, Cheese Fruit. Leaves are pinnately veined, opposite and glossy. Blades membranous, elliptic to elliptic-ovate, glabrous. Petioles stout. Stipules are connate or distinct, the apex entire or 2–3 lobed.



**Fig. No:1 *Morinda citrifolia***

It has essentially bioactive components like scopoletin, flavonoids,(Sang S et al 2001) coumarins, anthraquinones, polysaccharides, terpenoids, sitosterol, iridoids, fatty acids, glycosides, octanoic acid, potassium, vitamin C, alkaloids, β-carotene, vitamin A, and linoleic acid are among the components that have been identified from Noni.( Ui Joung Youn & Leng Chee Chang 2017) F**o**lk medicine has been using natural and herbal remedies for ages in all cultures around the world. The ancients noticed that particular food items had unique qualities that might prevent or cure specific illnesses and promote overall health.

It has widely acted as an antibacterial, antiviral, antitubercular, anticancer, anthelmintic, analgesic, hypotensive, and immunological agent. It also possesses smooth muscle stimulatory activity, and histaminergic effects have been made from the roots, stems, bark, leaves, and fruits. Noni was a traditional medicine used to cure wounds, ulcers (Mahattanadul S et al., 2011), bruises, fractured bones, and deep cuts. In most regions of India, the fresh leaf is used as a poultice for sprains and fractured bones.(Vijaykumar pandurang rasal et al., 2008) It can grow in infertile, acidic, and alkaline soils and is at home in very dry to very wet areas. All parts of the plant have traditional and/or modern uses, including roots and bark (dyes, medicine), trunks (firewood, tools) and leaves and fruits (food, medicine).

* 1. ***Annona muricata***

*Annona muricata* L., in the family of Annonaceae, which are native to America, Africa and India. They are referred to as “Mullu seetha” in Tamil. It is also known as prickly custard apple, guanabana, soursop, and durian Belanda. The leaves of the *Annona muricata* are lanceolate, simple and alternate. (Hadibrata et al., 2019)



**Fig. No: 2 *Annona muricata***

It has essentially bioactive components like acetogenins, lichexanthone, a compound in xanthone classes and Annonacin, annonamine, a compound in alkaloids, flavonoids and terpenes. The soursop plant is used in Malaysian ethnobotany as an astringent and a treatment for dermatosis, hypertension, rheumatism(Foong CP & Hamid RA 2012), boils, coughs, diarrhoea, and styptic. Furthermore, molluscicidal and antioxidant activities are present in *Annona muricata* leaf extracts. It acted as an anti-inflammatory agents to treat Rheumatoid arthritis and abscesses. (Rajkapoor B et al., 2007

* 1. ***Curcuma caesia***

*Curcuma caesia* Roxb is a perennial plant, belonging to the family of Zingiberaceae, which is native to India, West Bengal, Orrisa, Madhya Pradesh, Uttar Pradesh. It is also commonly known as black turmeric, black zedoary. The leaves of the Curcuma caseia are short-term leaves that arise from the underground rhizome with large, oblong leaves. The leaves are ovate, pyriform or oblong, ovate, cylindrical rhizomes are branched and brownish in colour.

It has essentially bioactive components like d-camphor, camphene, curcumin, turmerone, and sesquiterpenes.(Angana Borah et al., 2018) It has antibacterial and antifungal properties and laxative properties. (Pandey et al., 2014)



**Fig. No: 3 *Curcuma caesia***

It is used as a tonic for the brain and heart. Rhizomes are useful in treating leukoderma, piles, bronchitis, asthma, tumours, tuberculosis, glands of the neck, enlargement of the spleen and allergic eruptions. Plasters made from leaves are used to treat adenitis, furunculosis, and lymphangitis.

* 1. **Drug Interactions**

Combined extracts of the leaves of *Morinda citrifolia* (noni), *Annona muricata* (soursop), and *Curcuma caesia* (black turmeric) may lead to various drug-herb interactions.

***Morinda citrifolia* with Antiepileptic drugs (Phenytoin)**

*Morinda citrifolia* may induce cytochrome P450 enzymes, particularly CYP2C9, leading to increased metabolism and reduced plasma levels of drugs like phenytoin. ​ Reduced efficacy of antiepileptic drugs, leading to poor seizure control. (Kang YC et al., 2015)

***Annona muricata* with Chemotherapic Agents (Doxorubicin)**

*Annona muricata* leaf extract has shown synergistic cytotoxic effects when combined with doxorubicin in vitro, suggesting potential for enhanced anticancer activity. ​Potential for increased toxicity. (Salsabila, Irfani Aura et al 2021)

***Curcuma caesia* with Anticoagulant and Antiplatelet Drugs (Warfarin, Aspirin)**

Curcuma caesia possesses anti-inflammatory properties that may potentiate the effects of anticoagulant and antiplatelet medications, increasing bleeding risk, thus enhanced bleeding tendency.

***Curcuma caesia* with Antidiabetic agents**

The leaf extract has demonstrated inhibitory effects on α-amylase and α-glucosidase enzymes, which are involved in carbohydrate digestion. This suggests potential blood sugar-lowering effects. (Mutakin, et al., 2022)​

***Morinda citrifolia* and *Annona muricata* with Antidiabetic drugs (Metformin, Insulin, Sulfonylureas)**

Both Annona muricata and Morinda citrifolia have demonstrated hypoglycemic effects by inhibiting enzymes like α-amylase and α-glucosidase, potentially enhancing the effects of antidiabetic drugs, thus increased risk of hypoglycemia. (Agu, K.C et al., 2019)

1. **MATERIALS & METHODS**

**2.1. PREPARATION OF THE EXTRACTS BY USING SOXHLET APPARATUS**

Leaves are dried under the shade and crushed using a blender. Weigh 200 g of the sample. Place the sample in a thimble and insert it into the Soxhlet extractor. Add 200 mL ethanol to the round-bottom flask as the solvent. Assemble the apparatus (extractor, condenser, and flask). Set the heating mantle to maintain the solvent at 45°C. Allow the solvent to circulate through the sample for 3 hours. Collect the extract and store it in a suitable vessel after the process is complete. (Praveen K et al., 2007)

***A device with a tube and a tube on a table

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**Fig. No*.*4 Soxhlet extraction**

**2.2. PRELIMINARY PHYTOCHEMICAL SCREENING OF THE EXTRACTS** (Sekar VD et al., 2018 & Zubaidi S.N et al., 2023)

**Test for Saponins**

2ml of plant extract was taken in a test tube, and 2ml of distilled water was added and shaken well. The mixture was allowed to stand for 15 mins. Formation of a 1cm layer of foam indicates the presence of Saponins.

**Test for Tannins**

1ml of plant extract was taken in a test tube and 2ml of 5% ferric chloride was added to it and observed for the formation dark blue colour.

**Test for Sterol**

2ml of plant extracts was taken in a test tube and 2ml of chloroform was added to it. To this solution, 2ml of conc. Sulphuric acid was added and shaken well. The formation of the chloroform layer appeared red in colour.

**Test for Glycosides**

0.5 ml of sample extract was taken in a test tube and 2ml of glacial acetic acid was added. To this solution, 2 drops of 5% ferric chloride was added. This mixture was under layered with 1ml of conc. Sulphuric acid. Formation of brown ring at the interface, indicates the presence of Cardiac glycosides.

**Test for Flavonoids**

2ml of plant extract was taken in a test tube and few drops of Sulphuric acid was added. Formation of Orange colour, indicated the presence of Flavonoids.

**Test for Alkaloids**

2 ml of sample extract was taken in a test tube, mixed with 2 ml of conc. hydrochloric acid. The mixture was filtered and the filtrate was shaken with few drops of mayers reagent. Formation of green or white precipitate, indicates the presence of Alkaloids.

**Test for Quinones**

1ml of extract was taken in a test tube and add 1ml of conc. Sulphuric acid. Formation of red colour, indicates the presence of Quinones.

**Test for Terpenoids**

0.5ml extract taken in a test tube, 2ml of chloroform was added. To this solution conc. Sulphuric acid was added. Formation of red brown colour, indicates the presence of Terpenoids.

**Test for Phlobatannins**

1ml of plant extract was taken in a test tube and mixed with few drops of 2% hydrochloric acid. Formation of red colour precipitate, indicates the presence of Phlobatannins.

**Test for Phenols**

1ml of plant extract was taken in a test tube and mixed with 2 drops of ferric chloride. Formation of bluish black, indicates the presence of Phenols.

**Test for Carbohydrates**

1ml of plant extract was taken in a test tube and add 1ml of Fehling’s reagent and heated gently. Formation of red colour, indicates the presence of Carbohydrates.

Test tubes with different colored liquids

Description automatically generatedTest tubes with different liquids on a shelf

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**Fig No. 5 Preliminary Phytochemical Screening of the extracts**

* 1. **ANALYTICAL STUDIES**

**GAS CHROMATOGRAPHY MASS SPECTROMETRY**

GC-MS: The combination of GC and MS allows for the identification of unknown substances or contaminants in a sample. GC-MS is used in various fields, including food safety, pharmaceuticals, environmental analysis, and forensic testing. GC-MS offers high sensitivity, accuracy, and precision, making it a valuable tool for analyzing complex mixtures. GC-MS is primarily used for analyzing volatile and semi-volatile compounds, and certain compounds may require derivatization to improve their volatility and stability.

In the QC-2010 method, the column oven was maintained at 50 °C while the injection temperature was set at 250 °C. The injection was performed in split mode, and the flow control was based on linear velocity. The system operated at a pressure of –68.1 kPa with a total flow of 16.2 mL/min, including a column flow of 1.20 mL/min and a linear velocity of 39.7 cm/sec. Additionally, a purge flow of 3 mL/min was applied with a split ratio of 10.

In the QC-QP2020 method, the ion source temperature was set at –200 °C and the interface temperature at –250 °C. A solvent cut time of 3.50 minutes was employed, and the detector gain mode was adjusted relative to the tuning result with a detector gain of +0.00 kV. The threshold was set at 1000, with data acquisition starting at 4 minutes and ending at 40.33 minutes.

**2.4*. INVITRO* STUDIES**

**2.4.1. DPPH RADICAL SCAVENGING ASSAY**

The hydrogen atom donating ability of extractives was determined by the decolorization of methanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH). The antioxidant activity of the samples was determined on the basis of their scavenging activity of the stable 1, 1-diphenyl-2-picryl hydroxyl (DPPH) free radical. DPPH is a stable free radical containing an odd electron in its structure and usually utilized for detection of the radical scavenging activity in chemical analysis. DPPH produces violet/purple color in methanol solution and fades to shades of yellow color in the presence of antioxidants. A solution of 0.1 mM DPPH in methanol was prepared, and 2.4 mL of this solution was mixed with 1.6 mL of extract in methanol at different concentrations. The reaction mixture was vortexed thoroughly and left in the dark at RT for 30 min. (Pal et al., 2011) The absorbance of the mixture was measured spectrophotometrically at 517 nm. ascorbic acid used as standard. Percentage DPPH radical scavenging activity was calculated by the following equation

% DPPH radical scavenging activity= {(Blank− Test)/Blank} ×100

Test tubes with liquid in them

Description automatically generated A group of test tubes in a rack

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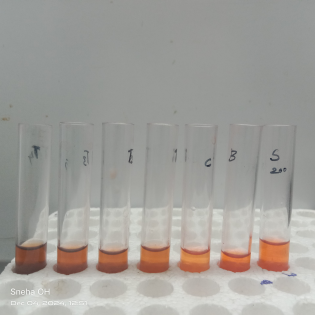
a. b.

**Fig No. 6 a. DPPH Blank, b. DPPH sample**

**2.4.2. HYDROXYL (OH) RADICAL SCAVENGING ASSAY**

The OH radicals scavenging activity was demonstrated with Fenton reaction. The reaction mixture contained, 60 μl of FeCl2 (1 mM), 90 μl of 1–10 phenanthroline (1 mM), 2.4 ml of phosphate buffer (0.2 M, pH 7.8), 150 μl of H2O2 (0.17 M) and 1.5 ml of individual plant extract (1 mg/ml). The reaction was started by adding H2O2. After 5 min incubation at room temperature, the absorbance was recorded at 560 nm. Ascorbic acid (1 mM) was used as reference compound. Inhibition in the formation of Fe (II)-1,10-phenanthroline by hydroxyl radical is taken as quantitative measure of scavenging activity (Pal et al., 2011)

% Inhibition = [(A0-A1)/A0 × 100]

 A group of test tubes with red liquid in them

Description automatically generated

1. b.

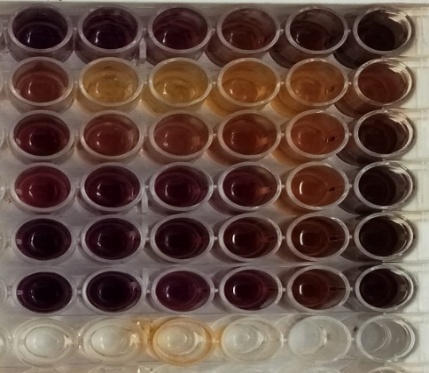
**Fig No. 7 a. Hydroxyl Blank, b. Hydroxyl sample**

**2.4.3. SYNERGISTIC TEST**

**DPPH radical scavenging assay synergistic test**

Two drugs at different concentration were prepared perpendicular to another drug. 100 µL of Two-fold concentration of sample A (200µg) was added to micro titer well containing 100 μl of ethanol and then serially diluted by transferring 100μl to adjacent wells in row and drug B was added to column wells. The first row and column were used as neat. The concentration of samples was 100,50,25,12.5, 6.25 μg/ml wells were loaded with 100μl DPPH. All the wells loaded with DPPH 5 mM and kept incubation under dark condition for 15 min. Least concentration having DPPH radical scavenging activity was recorded and FIC was calculated by the following equation:

A and B is mic at combination; MICA and MIC B alone

A plastic tray with small plastic containers

Description automatically generated with medium confidence A tray of plastic cups with different colored liquid

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a. b. c.

**Fig No. 8 Synergistic comparision of a.) *Annona muricata* Vs *Morinda citrifolia,* b.) *Morinda citrifolia* Vs *Curcuma caesia,* c.) *Curcuma caesia* Vs *Annona muricata***

**2.4.4. PHAGOCYTOSIS ASSAY**

Capillary blood (0.2 ml) was obtained by finger prick method and was placed on a clean grease free glass slide and spread to 1.5×1.5 cm. Blood was allowed to clot at 37° for 25 min. The clot was removed using sterile normal saline. The polymorphonuclear leukocytes were found adhered to the glass surface while the rest of the blood components are washed away. (Jantan I et al., 2019) Slides in duplicates were prepared and used for each dilution of the plant extract. Different dilutions of the ethanol extracts (0.1 ml) were flooded over the PMN layer on the slides, after which the slides were incubated at 37° for 15 min followed by the addition of 100 μl of *C. albicans* cell suspension. The slides were further incubated at 37° for 60 min. After incubation, the film was washed twice with sterile normal saline. The film was fixed with methanol for 5 min. Diluted methylene blue stain was flooded over the film and was left undisturbed for 15 min. The excess stain was removed using HBSS and air dried. The slides were observed under the oil immersion (×100) objective. (Shalu C et al., 2024) The mean number of *Candida* cells phagocytosed by PMNs on the slide were determined microscopically for 100 granulocytes using morphological criteria. This number was taken as the phagocyte index (PI) and was compared with the PI of the control. Immunostimulation (%) was calculated by using the following Equation

Stimulation (%) = PI (test) – PI (control)×100/PI (control)

A close-up of a microscope

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**Fig No. 9 Cells engulfed under the stain Diluted methylene blue**

**2.4.5. BRINE SHRIMP LETHALITY TEST**

The toxicity of compound was tested at various concentrations viz. 50, 100, 250, and 500 μg/mL in seawater. 100 nauplii were used in each test. Three replications were used for each concentration. After 24 h, survivors were counted using microscope and the percentage of the mortality (%M) of each dose was calculated as compared with control. (Mozhiarhasi Sandra sagrin et al., 2019 & Lilybeth F et al., 2013)

Mortality = number of death larvae/ Total larvae tested x 100

Corrected mortality = P-P0/100-P0 X 100

P- sample death rate

P0 -control death rate

A group of small fish under a microscope

Description automatically generated **A close-up of a sea creature

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1. b.

**Fig No. 10 a.) Live healthy brine shrimp larvae, b.) Dead brine shrimp larvae**

1. **RESULT AND DISCUSSION**

**3.1. PRELIMINARY PHYTOCHEMICAL SCREENING OF THE EXTRACTS**

Preliminary phytochemical screening of the extracts revealed the presence of tannins, sterols, glycosides, flavonoids, quinones, terpenoids, and phenols. However, saponins, alkaloids, phlobatannins, and carbohydrates were absent.

**Table No. 1 Preliminary Phytochemical Screening of the Extracts**

|  |  |  |
| --- | --- | --- |
| **S. No.** | **TEST** | **RESULTS** |
| 1. | Saponins | (-) |
| 2. | Tannins | (+) |
| 3. | Sterol | (+) |
| 4. | Glycosides | (+) |
| 5. | Flavonoids | (+) |
| 6. | Alkaloids | (-) |
| 7. | Quinones | (+) |
| 8. | Terpenoids | (+) |
| 9. | Phlobatannin | (-) |
| 10. | Phenol | (+) |
| 11. | Carbohydrates | (-) |

**3.2. GAS CHROMATOGRAPHY-MASS SPECTROSCOPY (GCMS)**

|  |  |  |  |
| --- | --- | --- | --- |
| **Table No. 2 Gas chromatography Mass spectroscopy** | | | |
| **S.NO** | **Retention Time** | **Probable name of the compound** | **% Area** |
| 1. | 13.848 | (6S)-2-methyl-6-(4-methyl phenyl) hept-2-en-4 one (Turmerone) | 3.74 |
| 2. | 17.456 | 1,3-DIPHENYL-1-((TRIMETHYLSILYL)OXY)-1(Z)-HEPTENE | 2.42 |
| 3. | 37.777 | 9-OCTADECENAMIDE | 55.77 |
| 4. | 37.91 | 3-(4-PIVALOXYBUTYL) CYCLOHEXANONE | 3.62 |
| 5. | 38.855 | (4B.ALPHA.,4C.BETA.,5.ALPHA.,8.ALPHA.,8A.BETA.,8B.ALPHA.)-2,4B,5,8,8B,11-HEXAMETHYL-6,7-DIPHENYL-4,4B,5,8,8B,9-HEXAHYDRO-4C,8A- | 5.4 |
| 6. | 38.9 | 4-TERT-BUTYL-3,4-DIHYDRO-2,4-DIPHENYLQUINAZOLINE | 2.24 |
| 7. | 39 | (3. ALPHA.,3A.BETA.,7A.BETA.)-(+-)-3-[(1,2-DIHYDRO-1-METHOXY-2-OXO-3H-INDOL-3-YLIDENE)METHYL]HEXAHYDRO-6-OXOPYRANO[3,4-C]PYRROLE | 4.64 |
| 8. | 39.166 | (3. ALPHA.,3A. BETA.,7A. BETA.) -(+-)-3-[(1,2-DIHYDRO-1-METHOXY-2-OXO-3H-INDOL-3-YLIDENE) METHYL]HEXAHYDRO-6-OXOPYRANO[3,4-C]PYRROLE | 1.24 |
| 9. | 39.225 | 4-TERT-BUTYL-3,4-DIHYDRO-2,4-DIPHENYLQUINAZOLINE | 3.94 |
| 10. | 39.322 | (3. ALPHA.,3A.BETA.,7A.BETA.)-(+-)-3-[(1,2-DIHYDRO-1-METHOXY-2-OXO-3H-INDOL-3-YLIDENE)METHYL]HEXAHYDRO-6-OXOPYRANO[3,4-C]PYRROLE | 5.35 |
| 11. | 39.52 | 4-TERT-BUTYL-3,4-DIHYDRO-2,4-DIPHENYLQUINAZOLINE | 2.53 |
| 12. | 39.563 | ACETAMIDE, N-(ACETYLOXY)-N-[2-CHLORO-3-NITRO-5-(TRIFLUOROMETHYL)PHENYL]- | 1.88 |
| 13. | 39.65 | (3.ALPHA.,3A.BETA.,7A.BETA.)-(+-)-3-[(1,2-DIHYDRO-1-METHOXY-2-OXO-3H-INDOL-3-YLIDENE)METHYL]HEXAHYDRO-6-OXOPYRANO[3,4-C]PYRROLE | 3.69 |
| 14. | 39.706 | 4,4’-BIS(PHENYLTELLURO)AZOBENZENE | 2.12 |
| 15. | 40.035 | 1,3,5,11,13-CYCLOPENTADECAPENTAENE-7,9-DIYNE, 15-(2,4-CYCLOPENTADIEN-1-YLIDENE)-6,11-DIMETHYL-, (E,E,Z,Z,E)- | 1.42 |

GC-MS analysis revealed the presence of several compounds, with 9-octadecenamide showing the highest abundance at 55.77%. Other notable constituents include turmerone, diphenyl-substituted heptenes, quinazoline derivatives, pyranopyrrole analogs, and acetamide derivatives in varying proportions.

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**Fig No. 11 Peak value of GCMS**

**3.3. DPPH RADICAL SCAVENGING ASSAY**

**Table No. 3 DPPH radical scavenging assay**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| S. No | Name of the sample | % Free radical scavenging activity (IC50 values) | | | |
| Dose | | | |
| 25μg | 50μg | 75μg | 100μg |
| 1. | Standard  (Ascorbic acid) | 24 | 44 | 66 | 74 |
| 2. | Test | 28 | 44 | 62 | 82 |

The test sample showed dose-dependent antioxidant activity in the DPPH assay with 82% scavenging at 100 μg, surpassing the standard. Compared to ascorbic acid, it exhibited slightly higher efficacy at higher concentrations.

**Fig No. 12 DPPH Free radical scavenging activity**

**3.4. HYDROXYL (OH) RADICAL SCAVENGING ASSAY**

**Table No. 4 Hydroxyl (OH) radical scavenging assay**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| S. No | Name of the sample | % Hydroxyl (OH) radical scavenging assay (IC50 values) | | | |
| Dose | | | |
| 25μg | 50μg | 75μg | 100μg |
| 1. | Standard  (Ascorbic acid) | 22 | 42 | 60 | 74 |
| 2. | Test | 30 | 46 | 62 | 78 |

In the hydroxyl radical scavenging assay, the test sample showed dose-dependent activity with % scavenging values of 30%, 46%, 62%, and 78% at 25, 50, 75, and 100 μg, respectively. Compared to the standard (ascorbic acid) which showed 22%, 42%, 60%, and 74%, the test exhibited slightly higher activity.

**Fig No. 13 Hydroxyl (OH) radical scavenging activity**

**3.5. SYNERGISTIC TEST**

In the synergistic test, Sample A showed a MIC of 0 with an FIC of 0.125 (B), indicating strong synergy. Samples B and C had MICs of 100 with FICs of 0.375 (C) and 0.625, respectively, suggesting additive to partial synergistic effects.

**Table No. 5 Synergistic Test**

|  |  |  |
| --- | --- | --- |
| **SAMPLE** | **MIC** | **FIC** |
| A | 0 | 0.125 (B) |
| B | 100 | 0.375 (C) |
| C | 100 |  |

**3.6. PHAGOCYTOSIS ASSAY**

In the phagocytosis assay, the phagocytic index increased from 36 at 0 μg to 38, 47, and 52 at 25, 50, and 100 μg concentrations, respectively. This corresponded to a stimulation of 0%, 5.5%, 30.5%, and 44.4%, indicating dose-dependent immunostimulatory activity.

**Table No. 6 Phagocytosis Assay**

|  |  |  |
| --- | --- | --- |
| CONCENTRATION | Phagocytic index | Stimulation (%) |
| 0 | 36 | 0 |
| 25 | 38 | 5.5 |
| 50 | 47 | 30.5 |
| 100 | 52 | 44.4 |

**3.7. BRINE SHRIMP LETHALITY TEST**

In the brine shrimp lethality test, mortality increased with concentration, ranging from 22% at 0 μg to 26%, 28%, and 30% at 50, 100–250, and 500 μg, respectively. The calculated LC₅₀ value was 3709.20 μg, indicating low toxicity of the sample.

**Table No. 7 Brine Shrimp Lethality Test**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Concentration μg** | **Live** | **Dead** | **Mortality** | **p-p0/100-p0\*100** |
| 0 | 78 | 22 | 22 | 22 |
| 50 | 74 | 26 | 26 | 5.1 |
| 100 | 72 | 28 | 28 | 6 |
| 250 | 72 | 28 | 28 | 7.6 |
| 500 | 70 | 30 | 30 | 10.25 |
| LC50 |  | | | |

A light in the dark

AI-generated content may be incorrect. A bright light in the dark

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a. b. c.

A bright light in the dark

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d. e.

**Fig No. 14 Microscopic live and dead larval test, a.) 50μg, b.) 100μg,**

**c.) 250μg, d.) 500μg, e.) control**

1. **CONCLUSION**

A Synergic effect in a polyherbal formulations occurs when the combined effect of multiple herbs is greater than the sum of the effects of each herb when given individually. These can result in more effective treatment with fewer side effects.

The selected three indigenous medicinal plants like *Morinda citrifolia, Annona muricata, Curcuma caesia* has the antioxidant activity as the result of the work.

Plant Authenticated and qualitative analysis was carried out with the Phytochemical Analysis method.

Preliminary phytochemical screening of ethanolic extract of *Morinda citrifolia, Annona muricata, Curcuma caesia* showed the presence of tannins, sterol, glycosides, flavonoids, Quinones, Terpenoids, Phenol.

GC-MS Analysis was performed to identify the phytoconstituents and major constituents were found they are ; 9-octadecenamide, 3-(4-pivaloxybutyl) cyclohexanone, 4-tert-butyl-3,4-dihydro-2,4-diphenylquinazoline,4,4’-bis(phenyltelluro)azobenzene, 3.alpha.,3a. beta.,7a. beta.)- (+-)-3-[(1,2-dihydro-1-methoxy-2-oxo-3h-inylidenemethyl] hexahydro-6-oxopyrano[3,4-c] pyrrole

The DPPH and hydroxyl radical scavenging assays confirmed the test extract's antioxidant potential. It showed higher free radical scavenging activity than ascorbic acid at 100 µg.

The DPPH synergistic test assessed the combined antioxidant effects of two drugs using the Fractional Inhibitory Concentration index. Results showed potential synergy, enhancing free radical scavenging at lower concentration 6.25 μg/ml.

The phagocytosis assay assessed that the plant extract's immunostimulatory effects on polymorphonuclear leukocytes against *Candida albicans*. Engulfed cells under methylene blue stain confirmed the immune response.

In Brine Shrimp Lethality Test the compound's toxicity was evaluated by measuring mortality rate across different concentrations at 24 hours. Mortality increased dose-dependently from 22% at 0 µg to 30% at 500 µg. The LC50 value was determined to be 3709.2 µg/mL. These results indicate the compound has relatively low toxicity.

CONSENT

It is not applicable.

ETHICAL APPROVAL

No need for ethical approval.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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