Muntingia Calabura – Antibacterial and Antifungal study of Isolated Pharmaceutically active compounds

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**ABSTRACT**

The extraction and isolation of biomolecules was carried out from the active fractions of *Muntingia calabura* which were exhibited stronger antibacterial activity. Among all the extracts, methanol and ethyl acetate extracts of *Muntingia calabura* stem bark showed strong antimicrobial activity. Bioassay guided fractionation of methanol and ethyl acetate extracts acquired from hard woodstem of *Muntingia calabura* was done using silica gel column chromatography (100-200) to identify the active fractions and to eliminate the non- active fractions. Hexane and ethyl acetate were used as eluent in different ratios.

**Keywords -** Muntingia Calabura, Isolation, Antimicrobial activity

 **I. INTRODUCTION**

Resistance gained by the microorganisms towards several contemporary antibiotic drugs makes an exponential raise of health problems during the chemotherapeutic treatment of infectious diseases. Therefore, serious ponder for new antimicrobial substances from medicinal plants have been extensively exploited for the control of pathogencity of the microorganisms. In this persistent, an immense importance given to the scientists round the globe for the development of mew antimicrobial substances potential to solve the problems caused by the microorganisms. Explorations of indigenous plants hold a great strength for the development of natural drugs especially efficient antibiotics[1].

Generally, antibiotics exhibits inhibition mechanism of the cell synthesis by which cells can’t proliferate and subsequently occur for death[2]. Emergences of bacterial resistance to several antibiotics are mainly due to genetic mutations and consecutive selection of resistant mutants present in large amounts in soil, plants, animals and humans[3]. Obstacles due to development of multi-resistant drug organisms and as well as adverse effects shown by the modern drugs have motivated the researches to show renewed interest on plants for the significant source of new medicines[4].

Over the past 60 years, antibiotics played a crucial preventive area of infectious diseases inflicted by bacteria and other microorganisms. Antimicrobial chemotherapy has come in a big way to raise. The average life span during Twentieth Century. Inspite of the same, emergence of disease-causing microorganisms with the property of resistance towards antibiotic drug therapy caused grave health problems like wound infections, tuberculosis, gonorrhea, septicemia, pneumonia, and childhood ear infections. Bacteria has developed different ways to resist antibiotics drugs. Nowadays, about 70 percent of the bacterial strains have caused acquired infections in hospitals, are at the least, offering resistance to some of the antibiotics used for treatment at the preliminary level.

Microbial development of resistance and economic incentives have made way to continuous research and development of new antibiotics for maintaining a pool of effective drugs. Perhaps, there must be gene pool in nature especially for bacterial resistance towards several antibiotics. Most of the microorganisms considered to be producers of specific antibiotic exhibit mechanism of resistance again its own antibiotic.

Upon review, it was noticed that resistance against penicillin by certain strains of staphylococci were immediately identified after introducing the similar resistance to chloramphenicol, streptomycin and tetracycline which was also observed during 1946. Continually over the years, same scenario when very known bacterial pathogenic strains have acquired resistance against one or more antibiotics in clinical use.

Numerous evidences have been begun to accumulate as the points of bacterial resistance that, bacteria involve in the exchange of its own genes responsible for drug resistance between strains and within the species. For example, genes of antibiotic-resistance from Staphylococci are carried on plasmids which could have been transmitted to other bacterial strains such as Streptococcus, Bacillus, and Enterococcus in the form of DNA segment or transposons for successful horizontal gene transmission (HGT). Several mechanisms have been elucidated for exponential raise of antibiotic resistance. These mechanisms may be based on chemical modification of the antibiotic or render its inactivation so that antibiotic loses its affinity towards ribosomal attachment and subsequent inhibition of microbial protein synthesis.

Apart from bacterial drug resistance, the antifungal resistance has been seemed to increase in the past decade[5]. There are several reasons for this resistance however; very little attention was paid to the study on fungal resistance[6].The failure of immune system towards broad range of antibiotics is due to frequent and often indiscriminate use of broad spectrum antibiotics and the frequent usage in hosting intravenous devices as well as the advent of chronic immune suppressive infections like candidasis etc[7]. These developments and corresponding increase in fungal infections have intensified the necessity for safe, more efficacious agents which are affordable and can combat serious fungal infections.

**Table 1.1: Examples of method of resistance towards various types of antibiotics.**

|  |  |
| --- | --- |
| Antibiotic | Method of resistance |
| β-lactams, Aminoglycosides, Chloramphenicol | Enzymatic cleavage or modification to inactivate antibiotic molecule |
| β-lactams, Erythromycin, Lincomycin | Eliminates or reduces binding of antibiotic to cell target |
| Chloramphenicol | Reduced uptake into cell |
| Tetracycline | Active efflux from the cell |
| Sulfonamides, Trimethoprim | Metabolic bypass of inhibited reaction |
| Sulfonamides, Trimethoprim | Overproduction of antibiotic target (titration) |

Worldwide approximately, 130 drugs extracted from higher plants or modified further synthetically are currently in use[8]. Clinically, important pathogens fetch the interest of scientists to carry on research and development programs for the innovation of newer broad spectrum antimicrobial agents due to its multi drug resistant property[9]. Some, other factors which can be considered for the discovery of new drugs are the less availability and high cost of new generation antibiotics necessitates which were seek for the substances from alternative medicine that are claimed antimicrobial activity.

A number of studies have been reported, dealing with screening of medicinal plants that attribute for antimicrobial activities[10]. Reportedly, Nigerian folk remedies and their constituent plants are reported to exhibit antimicrobial activity[11]. Evidently, Moskalenko[12] screened Far Eastern ethno-medicinal plants for the determination of antibacterial activity. Apart from these, there are several medicinal plants extracts are proved with significant antimicrobial activity have been also reported in different traditional literatures [13].

Indonesian medicinal plants possess antimicrobial activities against tested microbial species. It has been reported that, certain medicinal plants of turkey exhibited significant antibacterial activities on different types of Streptococcus species and other strains[14].

**II. MATERIALS AND METHODS**

**A. Antimicrobial studies**

**Preparation of sample/test solution for antimicrobial activity**

A concentration of 200 mg/ml of each isolated compound of different plant parts was prepared in DMSO (which did not influence the microbial growth),

**Microorganisms used**

The following Gram positive and Gram-negative microbial strains were used for testing the antimicrobial property of isolated compounds and plant extracts. These cultures were procured from various sources and updated on their suitable media.

**Bacterial cultures/ Test organisms**

The cultures are procured from Microbial Type Culture Collection Centre, Institute of Microbial Technology (IMETCH, Chandigrah) and few of them are obtained from Department of Microbiology, Kakatiya University, Warangal, Telangana, India.

Micrococus luteus KUCC 09

Bacillus cereus KUCC 23

Bacillus subtilis KUCC 17

Escherichia coli KUCC 03

**Human pathogens**

Klebsiella pneumoniae KUCC 11

Proteus vulgaris KUCC 21

Salmonella paratyphi A KUCC 18

Staphylococcus aureus ATCC

These bacterial cultures were obtained from Kakatiya Medical College (KMC), Warangal, Andhra Pradesh, India.

**Fungal Species**

The following fungal species and dermatophytes were used to assess the antifungal activity of isolated compounds. These were obtained from Kakatiya Medical College, Warangal, Andhra Pradesh` and Fungal Culture Collection Laboratory, Department of Microbiology, Kakatiya University, Warangal, Andhra Pradesh.

 Candida albicns KUCC 23

 Microsporum gypscum KUCC 31

Aspergillus fumigates KUCC 25

Aspergillus flavus KUCC 24

Aspergillus niger KUCC 29

**C. Media used for the assay**

The following different specific growth media were used to culture the microorganisms.

**Media for bacterial cultures**

**Nutrient agar (NA)**

 Peptone 5.00g

 Beef extract 3.00g

 Sodium chloride 5.00g

 Agar 20.00g

 Distilled water 1000 ml

**Nutient Broth (NB)**

 Peptone 5.00g

 Beef extract 3.00g

 Sodium chloride 5.00g

Distilled water 1000 ml

**Double strength Nutrient Agar (DsNA)**

 Peptone 10.00g

 Beef extract 10.00g

 Sodium chloride 5.00g

 Agar 20.00g

 Distilled water 1000 ml

**Media for fungal cultures**

**Sabourad’s Dextrose Agar Medium** (SDA)

 Peptone 10.00g

 Dextrose 40.00g

 Agar 20.00g

 Distilled water 1000 ml

 pH 6.8

**Asthana and Hawker’s Medium** (AH)

 Glucose 5.00g

 KNO3 3.50g

 KH2po4 1.75g

 Mgso4  0.75g

Agar 20.00g

Distilled water 1000 ml

A medium without agar was employed as broth medium.

**III. CULTIVATION TECHNIQUES**

**A. Slant preparation**

Agar slants were prepared by dispensing 10 ml of aliquots of molten medium into 30 ml test tubes and sterilized. The test tubes were held at 30o angle and allowed to set.

**B. Plate preparation**

Using sterile technique, 20 ml aliquots of sterile molten medium were transferred to sterilized petridishes. upon solidifying, the plates were used for the assay.

**C. Sub- culturing**

Subcultures were prepared by transferring loopful of inoculums from culture slants to freshly prepared agar slants. These were incubated in the desired conditions. Bacteria-double strength nutrient agar, 370C for 24hrs; Dermatophytes – Sabourad’s Dextrose Agar medium, 220C for 5-7 days in dark; Fungi – Asthana and Hawker’s medium, room temp, 5-7days.

**IV. ANTIBACTERIAL ACTIVITY**

**A. Preparation of inoculums**

Four to five well isolated colonies was transferred into 5ml of nutrient broth. The broth cultures are incubated at 370C for 24 hrs until a slight visible turbidity appeared. The turbidity of broth cultures is adjusted to obtain a half of MC Farland standard (1x108 to 5x108 cfu/ml). This was used as starter culture for the assay.

**B. Antimicrobial assay by well diffusion method**

The antimicrobial assay was carried out through the well diffusion or agar cup plate method.

**C. Agar cup plate method**

A standardized value 1 to 2 x 107 cfu/ml equals to 0.5 MC Farland standard of bacterial culture was placed onto the surface of sterile nutrient agar media plate and evenly distributed using a sterile glass spreader. The wells of 8mm were cut using a sterile cork borer. The wells were filled with 50, 100 and 150 µg/mL of isolated compounds. The plates were incubated aerobically at 370C for about 24 hrs. The inhibition zones were measured using a ruler. The results were compared with the positive and negative control well containing only DMSO and 10 mg/ml of streptomycin respectively.

**V. ANTIFUNGAL ACTIVITY**

**A. Preparation of spore suspension**

From the fresh cultures, spores were collected and transferred in a test tube containing sterilized distilled water (fungi) and Sabourad’s dextrose broth (dermatophytes). The spore suspension thus obtained was used for testing antifungal activity.

**B. Antifungal assay**

The antifungal property of isolated compounds was screened by agar well diffusion method. The nutrient agar medium plates were inoculated with test organisms. The plates were allowed to solidify and followed by punching 7 mm wells with sterile cork borer. The wells are then filled with 0.05 ml to 50 µl of the test compounds. Plates were incubated at 300C for 72 hrs. The zones of inhibition were measured and recorded.

**C. Evaluation of antimicrobial activity**

In context to bacterial and fungal resistance exhibited by human pathogenic organisms, the current studies have been emphasized on antibacterial (**Table 1.2**) and antifungal activities (**Table 1.3**) of isolated compounds from Muntingia calabura extracts.

**Table 1.2: Antibacterial activity of flavones isolated from the Muntingia calabura extracts**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Bacteria** | **Compound-1** | **Compound-2** | **Compound-3** | **Compound-4** | **Streptomycin** |
| **50** | **100** | **150** | **50** | **100** | **150** | **50** | **100** | **150** | **50** | **100** | **150** | **50** | **100** | **150** |
| **Gram negative** |
| Klebsiella pneumonia | 08 | 14 | 18 | 07 | 12 | 15 | 06 | 11 | 14 | 07 | 10 | 15 | 10 | 12 | 24 |
| Proteus vulgaris | 10 | 18 | 25 | 10 | 16 | 20 | 08 | 12 | 15 | 10 | 14 | 19 | 12 | 13 | 26 |
| Salmeonella paratyphi | -- | -- |  | -- | -- |  | -- | -- | -- | -- | -- | -- | 08 | 10 | 22 |
| Escherichia coli | 12 | 19 | 26 | 10 | 15 | 22 | 07 | 11 | 14 | 08 | 13 | 17 | 12 | 14 | 28 |
| **Gram positive** |
| Micrococcus luteus | 10 | 16 | 20 | 08 | 13 | 17 | 06 | 10 | 13 | 07 | 12 | 16 | 09 | 12 | 23 |
| Bacillus subtilis | 08 | 15 | 22 | 08 | 12 | 18 | 06 | 11 | 14 | 08 | 13 | 16 | 10 | 13 | 25 |
| Bacillus cereus | 07 | 15 | 20 | 08 | 13 | 17 | 09 | 11 | 13 | 10 | 12 | 15 | 10 | 13 | 20 |
| Staphylococcus aureus | 08 | 14 | 19 | 07 | 12 | 15 | 07 | 10 | 12 | 08 | 11 | 14 | 09 | 12 | 22 |

**Compound concentrations in µg/mL, Compound-1:** 5,8-dihydroxy-6,7,4ʹtrimethoxy flavones**,** **Compound-2**: 6,4ʹ dihydroxy 3ʹ propen chalcone, **Compound-3:** 7-(alloxy)-2-Phenyl-4H-Chro men-4one, **Compound-4:** 7-hydroxy-4-oxo-2-phenyl-4H-chromen-8-carbaldehyde

 **Table 1.3: Antifungal activity of flavones isolated from the Muntingia calabura extracts**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Fungi** | **Compound-1** | **Compound-2** | **Compound-3** | **Compound-4** | **Nystatin** |
| **50** | **100** | **150** | **50** | **100** | **150** | **50** | **100** | **150** | **50** | **100** | **150** | **50** | **100** | **150** |
| **Dermatophytes** |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| Candida albicans | 03 | 08 | 13 | 02 | 05 | 07 | 01 | 02 | 02 | 07 | 09 | 12 | 10 | 14 | 21 |
| Microsporum gypscum | 02 | 05 | 09 | 02 | 04 | 07 | 02 | 02 | 04 | 05 | 08 | 10 | 09 | 13 | 18 |
| Aspergillus fumigatus | -- | -- |  | -- | -- |  | -- | -- | -- | -- | -- | -- | 08 | 13 | 16 |
| Aspergillus flavus | 05 | 09 | 14 | 04 | 07 | 11 | 03 | 05 | 07 | 04 | 07 | 10 | 09 | 10 | 24 |
| Aspergillus niger | 04 | 07 | 12 | 06 | 08 | 11 | 04 | 05 | 07 | 05 | 08 | 11 | 07 | 12 | 26 |

**Compound concentrations in µg/mL, Compound-1:** 5,8-dihydroxy-6,7,4ʹtrimethoxy flavones**,** **Compound-2**: 6,4ʹ dihydroxy 3ʹ propen chalcone, **Compound-3:** 7-(alloxy)-2-Phenyl-4H-Chromen-4one, **Compound-4:** 7-hydroxy-4-oxo-2-phenyl-4H-chromen-8-carbaldehyde

**VI. RESULTS AND DISCUSSION**

The antibacterial activity of the compounds which were found to isolated. All the compounds failed to inhibit the growth of Salmonella typhi. Among the compounds tested, the high antibacterial activity was noted by compound 1, and followed by compound 2. The highest zone of inhibitions 25, 26, and 20, 22 was recorded at 150 µg/mL of the compound 1and compound 2 was noted against Proteus valgaris, and E. coli respectively in Gram positive strains. On the other hand, compound 1 and 2 also inhibited the growth of Bacillus subtilis and Bacillus cereus and Micrococus leutus. The high zone of inhibitions noted at 150 µg/mL of compound 1 and 2 against Bacillus subtilis and Bacillus cereus and Micrococcus leutus are 20, 22, 20 and 17, 18, 17 respectively. The results are shown in table 1.2. The hyper fungal reaction was also found associated with compound1 and 2. The results are shown in table 1.3. The outcomes of antibacterial and antifungal activity were compared with the known standards streptomycin and nystatin at 10 µg/mL.

In conclusion, all the compounds exhibited the antibacterial activity in a concentrated dependent manner. All the compounds are need for further studies such as clinical trials for the use of these compounds as antibacterial and antifungal drugs.

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