



**Research Article**

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## Evaluation of antibacterial activity of *Morus nigra* & *Citrus limon*

Rizwan Ahmad<sup>1</sup>, Aziz Ahmed<sup>1</sup>

<sup>1</sup> Assistant Professor and Researcher, Mewar University, Gangrar, Chittorgarh, Rajasthan- 312901, India

### ABSTRACT

The present study was carried out to find out the antimicrobial activity of aqueous, acetone and ethyl acetate extract of *Morus nigra* and *Citrus limon*. Antimicrobial analysis was done by using agar well diffusion method against bacterial pathogens. Ethyl acetate extract of *Morus nigra* exhibited the maximum zone of inhibition against *Staphylococcus aureus*, while ethyl acetate extract of *Citrus limon* shows maximum zone of inhibition against *Bacillus* & *Staphylococcus aureus*. Minimum inhibitory concentrations (MIC) value was determined by using micro broth dilution method. The phytochemical analysis showed the presence of tannins, flavonoids, saponins, terpenoids, and cardiac glycosides.

**Keywords:** Antimicrobial, Ethyl acetate extract, Pathogen, Phytochemical analysis, Secondary metabolites.

### INTRODUCTION

Various antibiotics are available in the market to treat the bacterial infectious diseases by working on various targets to inhibit pathogen growth. Gram-positive and gram-negative bacteria can be inhibited by antibiotics viz. Chloramphenicol, Nalidixic acid, Rifampicin and ampicillin, either by blocking protein, DNA, RNAs or peptidoglycan synthesis respectively.<sup>[1]</sup> However, the development of bacterial resistance to antibiotics enforces the search for new antibacterial agents.<sup>[2][3]</sup> WHO emphasized research for natural components from herbal medicines to find new antibacterial agents.<sup>[4]</sup>

A large portion of the world population, especially in developing countries, depends on the traditional system of medicine to treat a variety of diseases. Several hundred genera of plants are used medicinally. The World Health Organization (WHO) reported that 80% of the world population relies chiefly on traditional medicine and major part of the traditional therapies, which involve the use of plant extracts or their active constituents.<sup>[5]</sup> Due to indiscriminate use of antibiotics, microorganisms have developed resistance to many antibiotics and that has created immense clinical problems in the treatment of infectious diseases.<sup>[6]</sup>

In the present work, some selected plants are screened for their potential antibacterial activities.

### Necessity of work

- Infectious diseases continue to be the major concern for health institutions, pharmaceutical companies and governments all over the world.
- Increasing trends of multidrug resistance among emerging and reemerging bacterial pathogens to the available modern drugs or antibiotics.
- Plants are the potential source of natural antioxidants. Beta-carotene, ascorbic acid and alpha tocopherol are the widely used antioxidants. Free radicals and other reactive species present in the body can be generated both endogenously and exogenously.
- These synthetic antioxidants also show low solubility and moderate antioxidant activity.

### Objectives of the Study

- Preliminary phytochemical analysis of dried leaf powder of selected plant species.
- Quantitative phytochemical estimation of different extracts of the selected plant species.
- Evaluation of antimicrobial activity of plant extracts against selected Gram-positive and Gram-negative bacteria *in-vitro*.

**\*Corresponding author:**

**Rizwan Ahmad**  
Assistant Professor and  
Researcher, Mewar University,  
Gangrar, Chittorgarh, Rajasthan-  
312901, India  
Email:  
rizwansaifi.12345[at]gmail.com

## MATERIAL AND METHOD

### Experimental

#### Evaluation of Anti-microbial

The 50% ethanol extracts of 285 plant materials were screened for 61 biological activities and revealed effective anti-bacterial, and a wide range of pharmacological, activities.<sup>[7]</sup>

#### Present scenario of plants as antimicrobials

\*It is estimated that today, plant materials are present in, or have provided the model for 50% of Western drugs.<sup>[8]</sup>

\*Many commercially proven drugs used in modern medicine were initially used in crude form in traditional or folk healing practices, or for other purposes that suggested potentially useful biological activity.

\*The primary benefits of using plant derived medicines are that they are relatively safer than synthetic alternatives, offering profound therapeutic benefits and are more affordable.<sup>[9,10]</sup>

\*Much of the exploration and utilization of natural products as antimicrobials arise from microbial sources, and the discovery of penicillin led to later discoveries of antibiotics.<sup>[11]</sup>

\*Plant-based antimicrobials have enormous therapeutic potential. Their actions often beyond symptomatic treatment of the disease.<sup>[12]</sup>

#### Extraction

Traditional healers prepare a wide range of healing juices, crude extracts, paste and tincture from various herbs by using a water extract. Water or alcohol (methanol/ethanol) are mainly used for a large number of crude extract/library preparations (dry powder soaking or suspension, mechanical shaker, distillation of essential oils), sequential grinding (alkaloids, steroids, triterpenoids), gradient centrifugation (lectins and polypeptides) and acid hydrolysis (phenols) for a specific time frame.

A variety of extractants are used for their ability to solubilize antimicrobials and also other factors from plants.<sup>[13]</sup>

#### Material and Reagent

##### 1. Collection of powdered leaves sample

Collection of powdered leaves sample of *Morus nigra* and *Citrus limon* were collected from wild source in Ghaziabad, Uttar Pradesh, India in March, 2013. These leaves sample were authenticated by Dr. Neetu Singh, Department of Biotechnology, Mewar Institute of Management, Ghaziabad comparing with the specimen no. 34 available at Department of Biotechnology, Mewar Institute of Management, Ghaziabad. The roots were dried at room temperature and powdered.

##### 2. Microbial strains

The microbial strains will be obtained from Institute of Microbial Technology (IMTECH) Chandigarh, India. The reference strains of bacteria have to be cultured in nutrient broth (Hi-media,) at 37°C, subcultured regularly (every 30 days) and stored in nutrient agar slants at 4°C as well as at -20°C by preparing suspensions in 20% glycerol.

**Table 1:** Bacterial strains selected for antimicrobial study

Gram (+) bacteria	Gram (-) bacteria
<i>Bacillus subtilis</i>	<i>Escherichia coli</i>
<i>Staphylococcus aureus</i>	<i>Pseudomonas aeruginosa</i>

##### 3. Culture media

*Nutrient agar, Nutrient broth, Yeast Peptone Dextrose (YEPD), Mueller Hinton broth (MHB).*

##### 4. Chemicals

The chemicals used are as follows: Acetone, Agar, Aluminium Chloride, Antibiotics discs, Barium Chloride, Beef extract, BHT, Bismuth subnitrate, Chloroform, Dextrose, DMSO, Ethanol, Ethyl acetate, F C Reagent, Ferric Chloride (Anhydrous), Gallic acid, Glacial acetic acid, Magnesium ribbon, Malt extract, Mercuric Chloride, MHA, Peptone, Potassium iodide, Quercetin dehydrate, Sodium acetate, Sodium bicarbonate, Sodium chloride, Sulphuric acid, Swabs, Yeast extract.

All chemicals used were analytical grade. All the chemicals used were procured from Merck Specialties Pvt. Limited (India), Sisco Research Laboratories Pvt. Ltd. (SRL) (India), Ranbaxy Laboratories Ltd. (India), Central Drug House Pvt Ltd (CDH) (India), HiMedia Laboratories Limited (India), and Ranbaxy Fine Chemicals Limited (RANKEM) (India).

##### 4. Equipments

Soxhlet extractor apparatus, Rotary evaporator, Vacuum pump, Spectrophotometer.

##### Preparation of leaf extracts

Fresh and mature leaves from selected medicinal plant species, collected randomly from the local region of Uttar Pradesh. Plant leaves were washed three times by running tap water, dried and powdered for further use.

##### Aqueous Extraction:

- 25 gm of air dried powder was placed in hot distilled water and boiled for 30 min.
- Kept undisturbed for 24hr.
- Filtered off using sterile filter paper (Whatman no. 1) into a clean conical flask.
- Aqueous solvent was removed under pressure using a rotatory vacuum evaporator at 60°C.
- The dried residue of crude extract was resuspended in 20% DMSO.
- And then stored in dark bottles at 4°C.

##### Solvent Extraction:

- Dried powdered leaves were extracted with solvent (95% (v/v) ethanol/acetone) using soxhlet extractor.
- 25 gm powder was put in soxhlet thimble and into a soxhlet thimble tube.
- 250 ml of solvent (95% ethanol/acetone) was added to soxhlet flask then extracted at 40°C until the extract was clear or about 12 hr.

•Solvent was removed under pressure using rotatory evaporator at 40°C.

•The dried residue of crude extract was resuspended in 20% DMSO

•Stored in dark bottles at 4°C.

**Preliminary phytochemical analysis:**

Qualitative phytochemical analysis of dried leaf powder of selected plants was done by using methods of Harbone JB.<sup>[14]</sup>

**Determination of Minimum inhibitory concentrations (MIC):**

Minimum inhibitory concentrations (MIC) was determined by the tube dilution method. The MIC was determined using the tube dilution method. A two- fold serial dilution of the extract and fractions was carried out aseptically to give varying concentrations ranging from 30 mg/ml-0.23435 mg/ml. Each of the dilutions was inoculated with 0.1 ml of the standardized inoculum and the tubes were incubated at 37°C for 24 hours and microbial growth observed as turbidity in tubes was looked out for. The lowest concentration that showed no growth was considered the MIC. The whole procedure was replicated using Ampiclox and Zosyn at concentrations ranging from 0.5 mg/ml-0.0004 mg/ml. A positive control tube containing broth with organisms without plant extract and a negative control tube containing extract without organisms were also incubated along. The MIC was taken as the least concentration that inhibited the growth of the test organisms.<sup>[15]</sup>

**RESULTS & DISCUSSION**

Phytochemical research based on ethanopharmacological informations is generally considered an effective approach to the discovery of antinfective agents from higher plants.<sup>[16]</sup>

Preliminary phytochemical analysis of *Morus nigra* indicates the presence of tannins and terpenoids. However, *Citrus lemon* shows presence of tannins, flavonoids and cardiac glycosides. For Alkaloids, both plant extracts shows negative Wagner’s, Mayor’s and Dragendorff’s test (table 2).

**Table 2:** Preliminary phytochemical analysis of screened medicinal plants

Phytochemical constituent	Plant name		
	<i>M. nigra</i>	<i>C. lemon</i>	
Tannins	+	+	
Saponins	-	-	
Flavonoids	-	+	
Steroids	-	-	
Cardiac glycosides	-	+	
Terpenoids	+	-	
Alkaloids	Wagner’s test	-	-
	Mayor’s test	-	-
	Dragendorff’s test	-	-

(-) Not present, (+) Sparingly present, (++) Present, (+++) Highly present

The total phenolic and flavonoid were estimated by UV spectroscopy and were shown in table 3.

The presence of zones of inhibition on the seeded agar plates showed that the plant extract possesses antibacterial activity on the tested organisms which included both Gram positive and Gram negative organisms. Although the zones of inhibition were lower than that exhibited by the standard drug gentamycin, this could be due to the fact that the plant extract is crude and contains other constituents that

do not possess antibacterial property. Also the ability of the extract to diffuse through the gel may be hindered because of large molecules (stearic hindrance). At higher concentrations of the extract, the zones of inhibition with the standard drug were comparable.

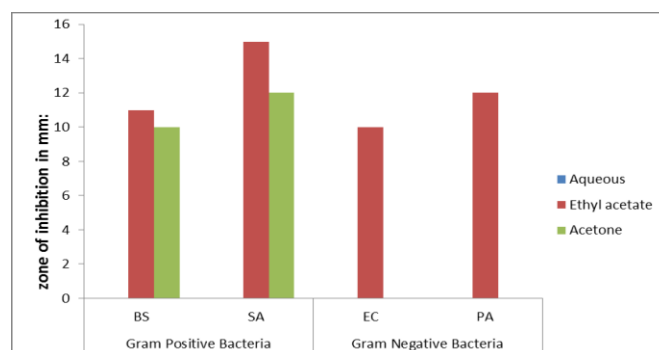
**Table 3:** Quantitative estimation of total phenolics and flavonoids by UV spectroscopy

Sr. No.	Name of plant extract	Total Phenolics <sup>a</sup>	Total Flavonoids <sup>b</sup>
1.	<i>M. nigra</i> (Aq)	6.86	-
2.	<i>M. nigra</i> (Et ac)	8.26	-
3.	<i>M. nigra</i> (Ac)	8.61	-
4.	<i>C. lemon</i> (Aq)	6.41	0.29
5.	<i>C. lemon</i> (Et ac)	7.25	0.45
6.	<i>C. lemon</i> (Ac)	7.78	0.35

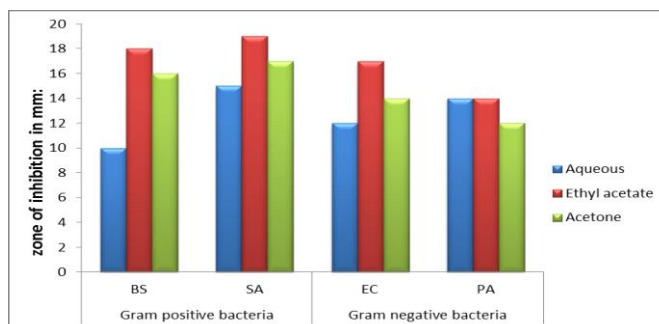
<sup>a</sup>Expressed as mg gallic acid/g of dry material; <sup>b</sup>Expressed as mg quercetin/g of dry material

Generally, the antibacterial activity of the extract against *Escherichia coli*, *Streptococcus aureus*, and *Pseudomonas aeruginosa* agrees with earlier works.<sup>[17-20]</sup>

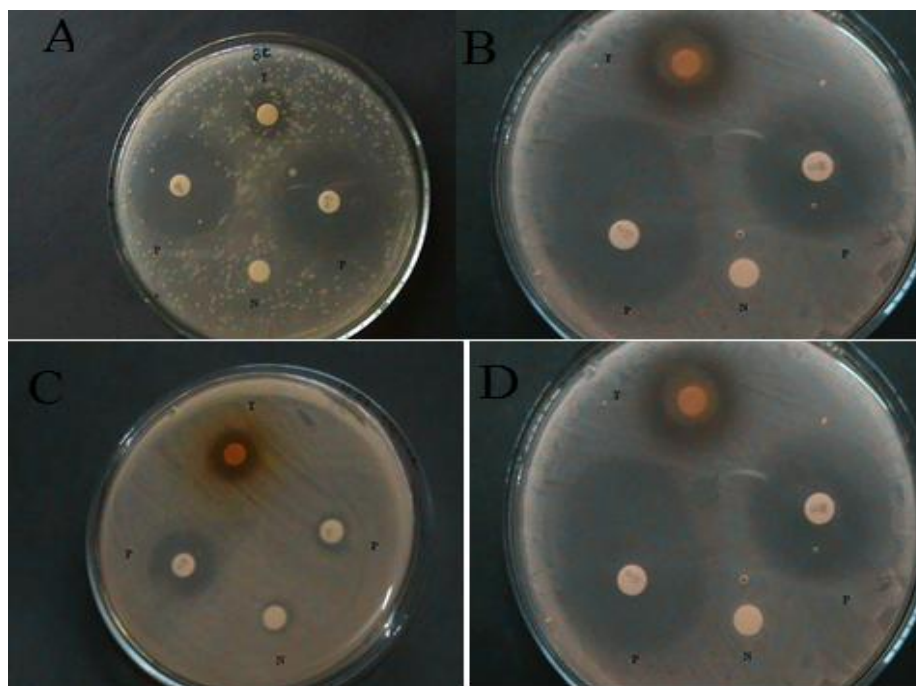
Aqueous extracts of *Morus nigra* showed no activity against all the selected bacterial strains. However, ethyl acetate extract of *Morus nigra* showed noticeable activity against all the selected bacterial strains, whereas acetone extracts of *Morus nigra* were found active against only gram positive bacteria (fig. 1). Aqueous extract of *Citrus lemon* showed min zone of inhibition against all the selected gram positive and negative bacteria (table 4, fig. 2). Ethyl acetate extract of *Citrus lemon* showed min. zone of inhibition against *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa* & *Streptococcus aureus*. On comparing MIC concentration of different plant extracts with gentamycin (10 µg/ml), it was observed that ethyl acetate extract of *Morus nigra* gave best result against *Streptococcus aureus*, whereas ethyl acetate extract of *Citrus lemon* gave its best activity against *Bacillus subtilis* & *Streptococcus aureus* (fig. 3). In case of gram negative bacteria, the ethyl acetate extract of *Morus nigra* worked best against both *Escherichia coli* & *Pseudomonas aeruginosa*, while all the plant extracts of *Citrus lemon* showed noticeable properties against *Escherichia coli* & *Pseudomonas aeruginosa*.



**Figure 1:** Antimicrobial activity of aqueous, ethyl acetate, and acetone leaf extracts of *Morus nigra*



**Figure 2:** Antimicrobial activity of aqueous, ethyl acetate, and acetone leaf extracts of *Citrus lemon*



**Figure 3:** A; Acetone extract of *M. nigra* against *B. subtilis*, B; Ethyl acetate extract of *M. nigra* against *B. subtilis*, C; Aqueous extract of *C. lemon* against *E. coli*, D; Aqueous extract of *C. lemon* against *P. aeruginosa*

**Table 4:** Antimicrobial activity of aqueous, ethyl acetate, and acetone leaf extracts of plants (Zone of inhibition in mm)

Plants	Extracts	Gram positive bacteria		Gram negative bacteria	
		BS	SA	EC	PA
<i>M. nigra</i>	Aqueous	-	-	-	-
	Ethyl acetate	11	15	10	12
	Acetone	10	12	-	-
<i>C. lemon</i>	Aqueous	10	15	12	14
	Ethyl acetate	18	19	17	14
	Acetone	16	17	14	12
<b>Gentamycin (10µg/ml)</b>		22	30	21	34

\*Values are mean inhibition zone (mm), (-) no inhibition, SA-*S.aureus*, BS-*B. subtilis*, EC-*E.coli*, PA-*P.aeruginosa*.

## CONCLUSION

Preliminary phytochemical analysis revealed the presence of tannins and terpenoids. The other secondary metabolites like cardiac glycosides flavonoids, steroids, saponins, etc. were present in trace amounts in some of the plants which could be correlated to antimicrobial properties.

The exact nature and mode of action of these active constituents is quite obscure at this stage. Further work may, however, reveal whether these components act as intracellular bacterial enzyme inhibitor, or impair the cell wall synthesizing system of the cell, or any other biological reaction impairment which causes cessation of growth or death of bacterial cells. Thus, here, it is also not possible whether; these extracts are bactericidal or bacteriostatic in nature.

The potential for developing antimicrobials from higher plants appears rewarding as it will lead to the development of a phytomedicine to act against microbes. Plant-based antimicrobials have enormous therapeutic potential as they can serve the purpose with lesser side effects than that which are often associated with synthetic antimicrobials.

## Future Prospectus

- Providing information about the presence or absence of various plant metabolites that are responsible for their antimicrobial activity, this could be used in future for their isolation from crude leaf extracts.
- Providing information on antibacterial activity of plants, to be used as basic pharmacological data for the clinical treatment in future.
- Providing new sources of antimicrobial agents that can be used against microorganisms causing different types of infections.
- It may also help in the discovery of new chemical classes of antibiotics that could serve as selective agents for the maintenance of human health and may provide biochemical tools for the study of infectious diseases.

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