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Research Article

Growth Inhibition Studies on Bacteria Isolated from Pus and Urine Samples by extracts of Mulberry Fruit (*Morus alba* L.)

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Abstract

In the present study pathogenic bacteria belonging to five species (*E. coli, Klebsiella, Staphylococcus, Pseudomonas* and *Streptococcus*) were isolated from pus and urine samples and preliminary identified by morphological and biochemical characteristics. Growth inhibition studies were carried out by testing antibacterial activity of extracts from mulberry fruits (*Morus alba*). Two types of fruits were selected- raw and ripe and two extracts- ethanolic and methanolic were tested. Agar well diffusion method was initially used for testing the antibacterial activity and extracts were further tested for determination of minimum inhibitory concentration (MIC). Qualitative phytochemical analysis was also performed on extracts. Broad spectrum antibacterial activity was observed for both the extracts against all the bacteria tested, with strong antibacterial effect expressed by methanolic extracts of ripe mulberry fruit. All the extracts expressed lowest antibacterial activity against *K. pneumoniae* whose growth was least inhibited.

Keywords: Agar Well Diffusion; MIC, Mulberry; Phytochemicals

Introduction

One of the oldest foods known to mankind are fruits. In ancient literature there are numerous references to fruits describing their magic and divine properties. According to Vedas fruits are the foods of Gods and Quran describes the fruits as gifts and heavenly fruits of God [1]. In India, medicinal plants are a backbone of traditional systems of medicine. The value of medicinal plants as a potential source of bioactive compounds is acknowledged by many pharmacological studies [2]). White mulberry (*Morus alba*) is a medium sized fast growing tree with a height of 10-20 meters tall. It is known to live more than 100 years but grown in landscapes, it has a life span of only 25-50 years. The tree produces small, edible fruits known as mulberries. The fruits grow up to 1-2cm long and are dark purple in color changing to black upon ripening. Some mulberry varieties produce white fruits [3]. The mulberry tree is now extensively planted and widely naturalized throughout the warm temperate regions of the world. It has been widely grown from Indian subcontinent. Mulberries have been used from a long time in traditional medicine to improve eyesight, prevent diabetes, lower blood pressure, strengthening joints, and in treatment of fever [4].

Antibiotic resistance is an immense growing problem in treating infections. The irregular and uncontrolled use of numerous antibiotics resulted in occurrence of antimicrobial resistance which became a global health issue [5]. In last 30 years there has been a rise in reports on inappropriate use of antimicrobial agents and spread of bacterial resistance among microorganisms [6]. Another cause of drug resistance in microorganisms is genetic due to: a) horizontal gene transfer by plasmids, b) bacteriophages and transposons, c) recombination of foreign DNA in bacterial chromosome and d) mutation in chromosomal segments. Numerous antibiotic resistant bacteria have been discovered in past decades, some of them are methicillin resistant *Staphylococcus aureus* (MRSA), multidrug resistant *Pseudomonas aeruginosa, Serratia marcescens,* beta lactamase resistant Entercococci and vancomycin resistant Enterococci (VRE) [7,8]. In developing countries, the poverty is high, hygienic conditions are substandard and adulterated drugs are in circulation of medical practices, in these circumstances drug resistance is a serious health issue.

Natural products have been used globally in traditional medicine since ages and pre-existed the introduction of modern drugs

and antibiotics. Thousands of plant species have been tested against numerous gram-positive and gram-negative bacterial strains. But very few of these medicinal plant extracts have been tested for clinical trials in humans and animals to know the safety and efficacy [9]. There is continuous search for plants containing antimicrobial substances due to their use as remedies for many infectious diseases [10]. Plants have abundant amounts of secondary metabolites like tannins, alkaloids and flavonoids which have been detected to have antimicrobial properties [11]. WHO specifies medicinal plants as the best source for obtaining variety of drugs [12]. Keeping in view the rising global issue of antimicrobial resistance, this work was undertaken with an objective to search for potential antibacterial substances in *Morus alba* fruits and testing their activity against pathogenic bacterial strains isolated from pus and urine samples.

Materials and Methods

Isolation of Pathogenic Bacterial strains:

Bacterial strains were isolated from pus and urine samples collected from pathological laboratory of King Khalid University Hospital, King Saud University, Riyadh.

1. Pus samples were processed as per the method of Cheesbrough, 2000 [13]. The samples were suspended in sterile peptone water, a ten-fold dilution of the suspension was made in peptone water and 0.1 ml aliquot portion was inoculated on Nutrient agar and MacConkeys agar plates by spread plate method and incubated at 37^0C for 24-48 hrs. The isolated colonies were sub-cultured on respective media by streak plate method to obtain a pure culture. The pure cultures were stored at 4^0C in a refrigerator for further identification.
2. Urine samples were initially screened for presence of urine infection by Urine Dip Slide method (Thermo Scientific, UK). Samples with equal or more than 10^4 CFU/ml were interpreted as positive for urine infection and less than 10^2 CFU/ml were interpreted as negative for urine infection. The urine samples which were identified as positive for UTI **(**Urinary Tract Infection) were further subjected to isolation of bacteria. Streak plate method was used for isolation of pure cultures for which loop full of urine samples were streaked on Mac Conkey agar, Blood agar and Nutrient agar plates (Thermo Scientific, UK) and incubated at 37^0C for 24 hrs. After incubation well isolated colonies were selected and stored at 4^0C in a refrigerator for further identification.

Identification of bacterial strains

The bacterial cultures isolated from pus and urine samples were identified and characterized on the basis of morphological, cultural, physiological and biochemical characteristics [14]. A presumptive identification was performed by Gram staining, oxidase activity, motility, catalase production, acid production in glucose, oxidation-fermentation (OF) test, indole, methyl red, voges-proskauer test (VP) and hydrogen sulfide production. The bacterial isolates were identified with the help of Bergey's Manual of Systematic Bacteriology [15].

Collection of fruits

Fresh white mulberry (*Morus alba*) fruits were purchased from Kothapet Fruit Market (Latitude: N 17° 22.1767' Longitude: E 78° 32.1107'), Hyderabad, Telangana, India. Raw and ripe fruits were collected separately and were transported to lab within one hour of collection. The fruits were washed in running tap water to remove the dirt; surface sterilized with 70% alcohol and then rinsed with sterile distilled water. The fruits were verified and identified as mulberry (*Morus alba*) by Dr. B. Srinivas Rao, Grape Research Station, Hyderabad, India.

Phytochemical extraction method

Surface sterilized fruits were dried in an oven at 50^0C for 48 hrs, grinded into a fine powder by a waring blender (Waring Commercial, USA). The powdered material was stored in sterile screw capped test tubes in refrigerator at 4^0C. The powdered samples were subjected to organic solvent extraction by the method of Gupta., *et al*. [16]. Two extractants methanol and ethanol (95%) were used for the phytochemical extraction, 10g each of raw and ripe berry fruit air dried powder was thoroughly mixed with 100 ml of each solvent. The mixtures were first filtered through muslin cloth and then through Whattman's filter No. 1. The filtrates were then concentrated by complete evaporation of solvent at room temperature to yield the pure extract. Stock solutions of crude extracts were prepared by mixing the weighed quantity of dried extracts with appropriate solvent to obtain a final concentration of 100 mg/ml. Each solution was stored at 4°C sterile screw capped glass tubes until use.

Antibacterial assay

Agar well diffusion assay [17] was used to test the antibacterial activity of fruit extracts. Petri dishes (90mm) containing 18 ml of Mueller Hinton agar were seeded with 100µl inoculum (approximately 10^8 CFU/ml) of each bacterial strain in Mueller Hinton broth. Wells of 8mm diameter were cut using a sterilized cup-borer, 100µl of each fruit extract was poured in the wells and the plates were incubated at 37°C for 18-24 hrs. The assay for each extract was performed in triplicate under strict aseptic conditions. Solvents- ethanol and methanol were also used as negative controls during the study. Along with fruit extracts, commercially available standard antibiotics (Ciprofloxacin, Erythromycin, Gentamycin, Nalidixic acid, Norfloxacin, and Tetracycline) were also tested to check the sensitivity of isolated bacterial pathogens. After incubation period, the antibacterial activity of each extract was noted and expressed in terms of the mean diameter of inhibition zone (DIZ) in mm ± SD.

Determination of minimum inhibitory concentration

Minimum inhibitory concentration (MIC) of both methanolic and ethanolic extracts was determined by two-fold serial dilution broth method [18]. A stock solution of extract was serially diluted in tubes (2ml volume) with Mueller Hinton broth to obtain a concentration of 2.0, 1.0, 0.5, 0.25, 0.12, 0.06% (w/v). 100 µl of inoculum (5 x 10^5 CFU/ml) of each bacterial isolate was added in each tube. The tubes were then kept at 37°C for an overnight incubation. Following incubation, the MIC was calculated as the lowest concentration of the extract inhibiting the visible growth of bacteria.

Phytochemical analysis

Qualitative phytochemical analysis of the methanolic extracts was performed as per the methods described by Harbone [19] and Kolkate., *et al*. [20]. Flavonoids, polyphenols, anthocyanins, steroids, alkaloids, terpenoids, saponins and carbohydrates were tested.

Statistical analysis

All experiments were set in triplicates and results obtained were expressed as Mean ± SD.

Results and Discussion

In the present study pus (30) and urine samples (50) were collected from pathological laboratory of King Khalid University Hospital, King Saud University, Riyadh. A total of 80 samples were collected and processed for isolation of pathogenic bacteria. Pus samples were directly processed for isolation of bacteria. Urine samples were initially tested for urine infection and positive samples were processed for isolation of bacteria. Altogether 50 bacterial cultures were isolated in pure form, 21 from pus samples and 29 from urine samples, the details are shown in Figure 1. *E. coli* was the predominant bacteria isolated, followed by *P. aeruginosa*, *S. pyogenes*, *S. aureus*, and *K. pneumoniae*.

The isolated bacterial cultures were identified by studying different morphological and biochemical characteristics as mentioned in materials and methods. The identification characteristics were cross checked with those of standard manuals [14,15]. Based on the results the isolated bacterial strains were identified as *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Streptococcus pyogenes* (Table 1).

Identity of isolates	Sugar Fermentation					Gram Nature	Cat	Oxi	Coag	Ind	MR	VP	Cit	Urease	NO_3	H_2S
	Glu	Suc	Lac	Man	Fru											
E. coli	+	-	+	+	+	-	+	-	-	+	+	-	-	-	+	-
S. aureus	+	+	+	+	+	+	+	-	+	-	+	-	-	-	+	+
K. pneumoniae	+	+	+	+	-	-	+	-	-	-	-	+	+	+s	-	-
P. aeruginosa	+	-	-	+	-	-	+	+	+	-	+	-	-	+	+	-
S. pyogenes	+	+	+	-	+	+	-	-	-	-	+	-	-	-	-	-

Table 1: Identification of Bacterial Isolates Based on Biochemical Characteristics.

Glu-glucose; Suc-sucrose; Lac-lactose; Mn-mannitol; F-fructose; s-slow reaction; Cat- catalase; Oxi-oxidase; Coag-coagulase; In-indole; MR-methyl red; VP-voges proskaeur; Cit-citrate; NO_3-nitrate reduction; H_2S-hydrogen sulphide.

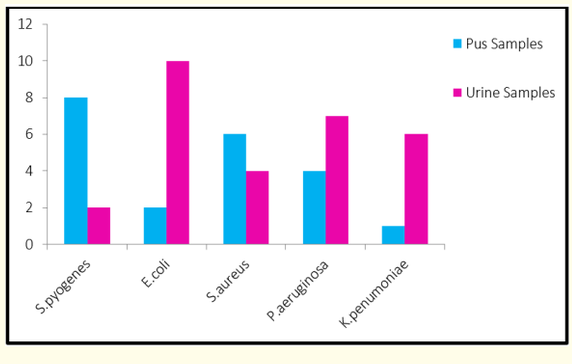

Figure 1: Bacterial Isolates from Pus and Urine Samples.

The bacterial strains after identification studies were further tested for antibacterial activity of mulberry extracts by agar well diffusion method and also the resistance pattern to commercial antibiotics was tested with 6 antibiotics. The results revealed that isolated bacterial strains showed resistance to most of the antibiotics especially tetracycline, gentamycin and nalidixic acid. The resistance pattern is shown in Table 2. The other antibiotics were effective to inhibit the bacterial growth up to certain extent. Antimicrobial resistance is a global problem which is increasing at a fast rate and these results also indicate the same issue. One of the studies reports similar antimicrobial resistance pattern of *E. coli, K. pneumoniae, Enterobacter* and *Proteus* species isolated from urine samples [21]. Antibiotic resistance pattern was also reported by Thipperudraswamy., *et al*. [22] for *Streptococcus pyogens* isolated from clinical samples.

Results obtained for antibacterial activity by agar well diffusion method indicates broad antibacterial spectrum by both raw and ripe mulberry fruit extracts. In general it was observed that meth-

Standard Antibiotics	Diameter of Inhibition Zone in mm				
	E. coli	*S. aureus*	*K. pneumoniae*	*P. aeruginosa*	*S. pyogenes*
Ciprofloxacin	+	-	+	-	+
Erythromycin	-	+	+	-	+
Gentamycin	+	+	-	+	-
Nalidixic acid	+	-	+	+	-
Norfloxacin	-	+	-	-	-
Tetracycline	+	+	+	-	+

Table 2: Antibacterial activities of commercially available standard antibiotics.

R- Resistant; S- Sensitive

anolic extracts of raw and ripe mulberry fruit extracts expressed good antibacterial activity on all isolated bacterial species. The results are depicted in table 3 and 4. The analysis of ethanolic extracts of raw mulberry fruit indicates highest antibacterial activity against S. pyogenes with a DIZ value of 12.66 ± 1.15mm followed by *S. aureus, E. coli, P. aeruginosa* and *K. pneumoniae* with DIZ values of 11.33 ± 1.52, 10.33 ± 1.52, 9.33 ± 0.57 and 8.0 ± 1.0 respectively. The lowest activity was recorded against *K. pneumoniae.*

Bacterial Isolate	Diameter of Inhibition Zone (mm)	
	Ethanolic Extract	Methanolic Extract
S. pyogenes	12.66 ± 1.15	13.00 ± 1.00
E. coli	10.33 ± 1.52	13.00 ± 1.73
S. aureus	11.33 ± 1.52	14.00 ± 1.0
P. aeruginosa	9.33 ± 0.57	9.66 ± 0.57
K. penumoniae	8.00 ± 1.00	9.33 ± 0.57

Table 3: Antibacterial Activity of Ethanolic and Methanolic extracts of Raw Mulberry.

Bacterial Isolate	Diameter of Inhibition Zone (mm)	
	Ethanolic Extract	Methanolic Extract
S. pyogenes	11.10 ± 1.00	11.33 ± 1.15
E. coli	11.66 ± 1.52	14.33 ± 1.15
S. aureus	19.00 ± 1.00	15.33 ± 1.52
P. aeruginosa	10.33 ± 0.57	12.33 ± 1.52
K. penumoniae	7.00 ± 1.00	9.66 ± 0.57

Table 4: Antibacterial Activity of Ethanolic and Methanolic extracts of Ripe Mulberry.

Methanolic extracts of raw mulberry fruit expressed higher antibacterial activity than ethanolic extracts as shown in table 3. The pattern of antibacterial activity observed was slightly different with highest activity against *S. pyogenes* and *E. coli* with an equal DIZ value of 13.00mm and lowest activity was recorded against *K. pneumoniae*. The DIZ values were in the range of 9.33 ± 0.57 and 13.0 ± 1.0mm respectively. The antibacterial effect of methanolic extracts of four wild berries was also studied by Ayachi., *et al*. [23] on 7 Salmonella species isolated from poultry samples and *E. coli* ATCC 25922. A moderate antibacterial effect was observed on *E. coli* ATCC 25922 but there was no effect on all 7 strains of Salmonella.

Ethanolic extracts of ripe mulberry fruits expressed good antibacterial activity against all the bacterial isolates. Particularly the ripe fruit extracts were more effective against *P. aeruginosa* by expressing a high antibacterial activity than raw fruit extracts. When the pattern of antibacterial activity was compared with raw mulberry extracts following variation was observed- highest antibacterial activity was recorded against *S. aureus* with a DIZ value of 19.0 ± 1.0, followed by *E. coli, S. pyogenes, P. aeruginosa* and *K. pneumoniae* with DIZ values of 11.66 ± 1.52, 11.10 ± 1.0, 10.33 ± 0.57 and 7.0 ± 1.0 respectively. Lowest activity was observed on *K. pneumoniae* which was similar to other extracts. The antibacterial activity of aqueous extracts of three mulberry fruit varieties- *Morus alba*, *Morus nigra* and *Morus rubra* in boiled water was reported by Dimitrova., *et al*. [24]. The researchers have tested the antibacterial activity on two gram positive and two gram negative bacterial species- *L. monocytogenes* and *S. aureus*; *E. coli* and Salmonella. Good antibacterial activity was recorded against all the bacteria tested except Salmonella species. The results obtained in our study are in line with this report highlighting the broad spectrum antibacterial activity of *Morus alba* fruit.

Methanolic extracts of ripe mulberry fruits expressed more antibacterial activity than ethanolic extracts. The pattern of antibacterial activity observed was similar to ethanolic extracts with highest activity against S. aureus and lowest against *K. pneumoniae* with DIZ values in range of 9.66 ± 0.57 and 15.33 ± 1.52. The results are shown in Table-5. In one of the study in Egypt by Mohamed Salem., *et al*. [25], antibacterial activity of Morus alba extracts from sapwood, heartwood and bark was studied on 7 ATCC strains of *B. cereus, B. subtilis, S. aureus, E. coli, P. aeruginosa, S. marcescens* and *A. tumefaciens*. Highest activity was recorded against *E. coli* followed by *S. aureus, B. subtitlis, B. cereus, S. marcescens* and lowest activity was observed for *A. tumefaciens*.

Subsequent to agar well diffusion assay, minimum inhibitory concentration (MIC) of the extracts was studied and the results obtained revealed following findings. The methanolic extracts of both raw and ripe mulberry fruits were more effective when compared with ethanolic extracts and inhibited the bacterial growth at low concentration. In case of raw mulberry, methanolic extracts inhibited the growth of *S. pyogenes* and *S. aureus* at a minimum concentration of 0.125, followed by *E. coli* at 0.25% and *P. aeruginosa* at 0.50% (w/v) respectively. The growth of *K. pneumoniae* was inhibited at slight higher concentration of 1.0% (w/v). The results are highlighted in figure 2 (A to E). On the other hand ethanolic extracts were able to inhibit the growth of only *S. pyogenes* at 0.125% and other bacterial strains were inhibited at a concentration of 0.25 to 1.0% (w/v). Similar to methanolic extracts, growth of *K. pneumoniae* was inhibited at high concentration of 1.0% (w/v).

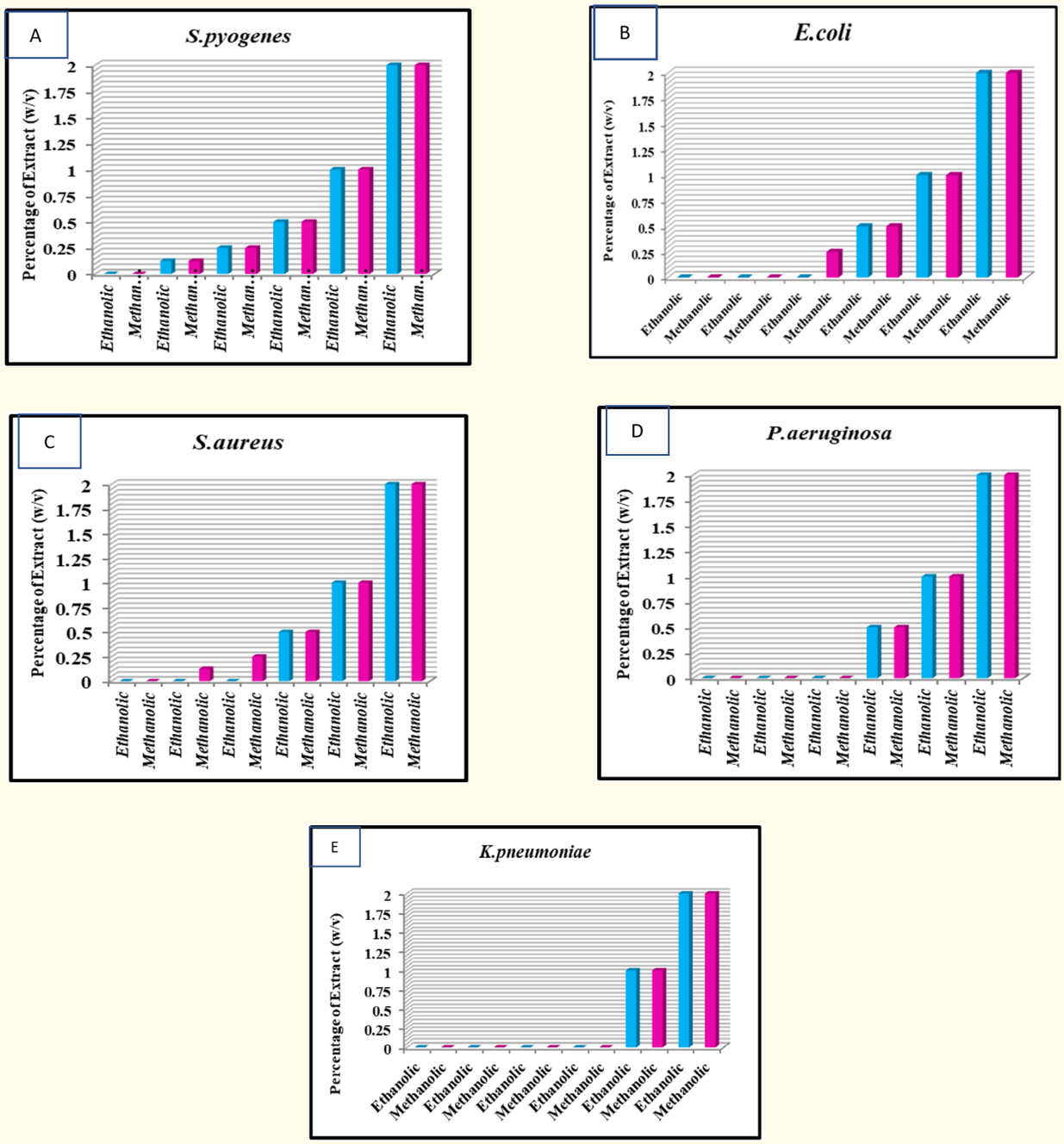

Figure 2: MMIC of Ethanolic and Methanolic extracts of Raw Mulberry Fruit (% w/v):

A- S. pyogenes, B-E. coli, C-S. aureus, D-P. aeruginosa and E- K. pneumoniae

Ripe mulberry fruit extracts in general were effective against all the bacterial isolates. The effectiveness was more for *P. aeruginosa* against which methanolic extracts of ripe fruit expressed good antibacterial activity higher than raw fruit extracts by inhibiting growth at low concentration. Ethanolic extracts at a concentration of 0.125% inhibited the growth of *S. aureus*; as the concentration increased to 0.25% growth of *S. pyogenes* was inhibited and at a concentration of 0.5% (w/v) *E. coli* and *P. aeruginosa* were inhibited. Growth of *K. pneumoniae* was inhibited at a high concentration of 1.0% (w/v) which was similar to raw fruit extracts. The results are shown in figure 3 (A to E). Methanolic extracts of ripe mulberry fruits were found to be more effective than ethanolic extracts by inhibiting the growth of bacteria at low concentration. At a concentration of 0.125% *S. aureus* was inhibited, followed by *S. pyogenes, E. coli* and *P. aeruginosa* at a concentration of 0.25% (w/v). The growth of *K. pneumoniae* was inhibited at a higher concentration of 0.50% (w/v) which was similar to that observed with all extracts. Determination of MIC values of *Morus alba* sapwood, heartwood and bark extracts against 7 pathogenic strains was also studied by Mohammed Salem., *et al*. [25]. Among the three studied parts, bark expressed strong antibacterial growth with an MIC of 8µg/ml.

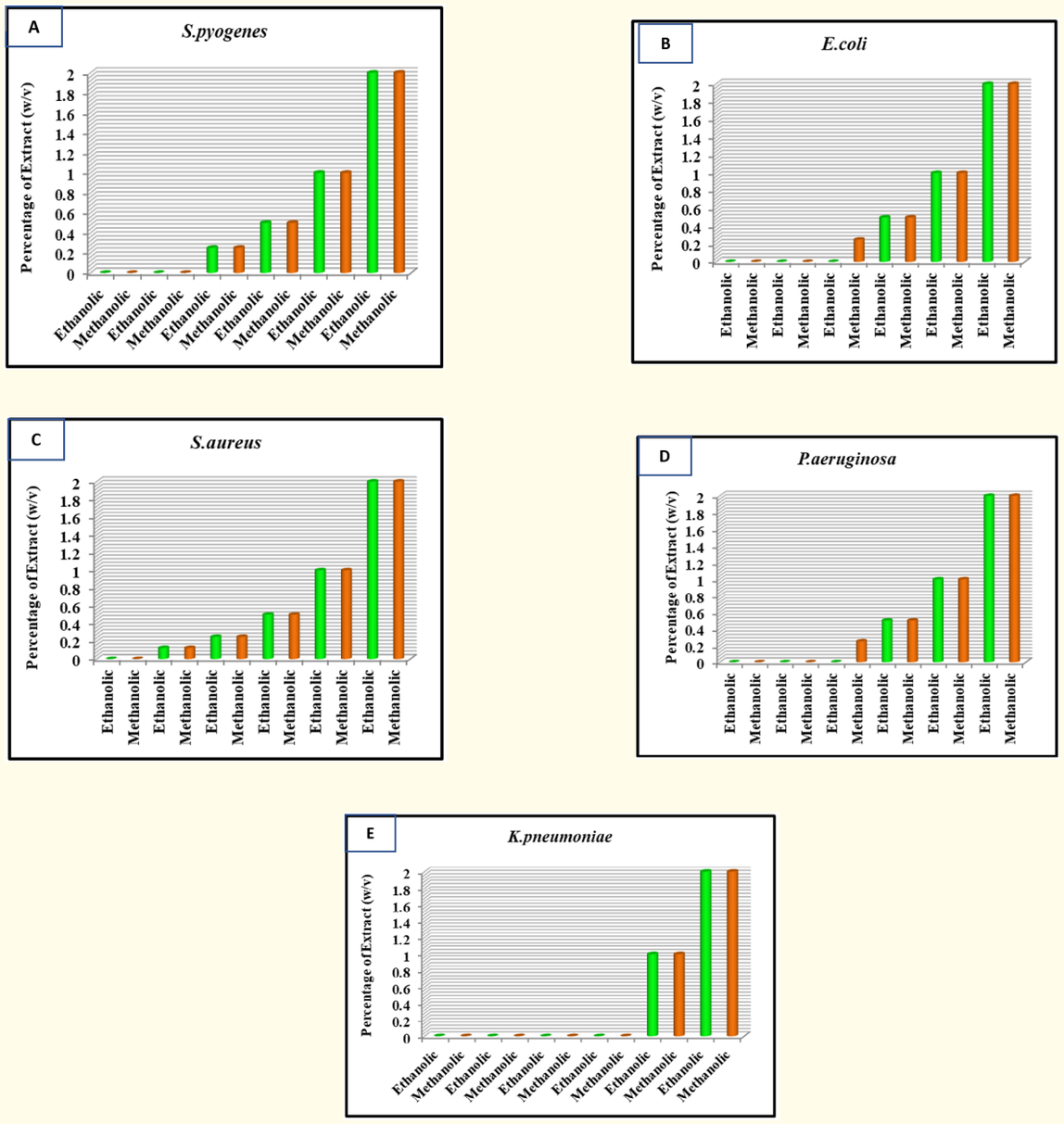

Figure 3: MIC of Ethanolic and Methanolic extracts of Ripe Mulberry Fruit (% w/v):

A- S. pyogenes, B- E. coli, C- S. aureus, D- P. aeruginosa and E- K. pneumoniae

Morus alba fruit extracts were also tested for the presence of phytochemicals by qualitative tests (Table 5). Methanolic extracts were used for this analysis which shows that flavonoids, alkaloids, polyphenols, steroids etc. are present in these fruits which may be responsible for the antibacterial effect recorded against bacterial species studied.

Plant	Phytochemical	Detection
Morus alba fruit	Flavonoid	+
	Polyphenols	+
	Antocyanins	+
	Terpenoids	+
	Alkaloids	+
	Steroids	-
	Carbohydrates	+

Table 5: Phytochemical Analysis of *Morus alba* fruit.

Conclusion

Based on the results obtained in this study it is clear that *Morus alba* fruits have the potential to inhibit the growth of different pathogenic bacterial species by expressing broad spectrum antibacterial activity. These fruits can be a good source for isolation of antibacterial substances which can be used for treatment of infections caused by these types of bacteria. However, more detailed research is required for isolation, identification and characterization of the bioactive ingredients responsible for this activity and to use it for treatment of bacterial infections.

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