University of Wah
Journal of Science and Technology

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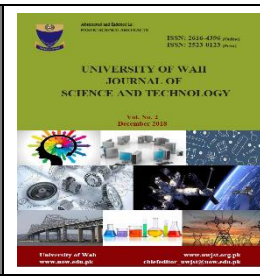

Determination of Antimicrobial Activity of Mulberry Fruit Extracts against Water-borne Microbial Pathogens Isolated from Different Water Samples

Shumaila Naz, Muhammad Idrees, Shehzadi, Zeenat Haq, Andleeb Sultana and Syeda Rida Batool.

***Abstract*—Mulberry (*Morus* sp.) is being used worldwide due to its nutritional values and medicinal properties. In the current study, antimicrobial activities of Ripened and Un-Ripened Black and White mulberry fruit extracts are tested against microbial pathogens isolated from different water samples. Aqueous fruit extracts show significant antimicrobial activity with 35mm zone of inhibition by Ripened White Toot (RWT) against *V. cholera* and 32mm zone of inhibition by Un-Ripened Black Toot (URBT) against *M. luteus*. All mulberry extracts are considerably active against *E. coli* and *B. thuringiensis*. Present findings indicate that mulberry fruit extracts have medicinal potential and will be an excellent choice for the development of alternative antimicrobial drug.**

Index Terms—Antimicrobial activity, antibiotics, *Morus nigra* L., *Morus alba* L., bacterial strains.

I. Introduction

PLANTS have been used as crude drugs and healing agents since pre-historic times [1]. Around 25% of modern medicines are acquired from plants [2]. Almost 65-80% of the world population of developing countries depends on plants for their medicinal needs [3-4]. In flourished countries the preparations of herbal medicines are popular. Recommendation for registration of such medicines exists in developed countries including Germany, France, Italy and the United States [6].

Manuscript received June 14, 2017: accepted August 28, 2018.

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Medicinal plants contain synergistic combinations of active compounds which can be used against common pathogens with low or no side effects. In Pakistan more than 500 species of flowering plants are used in traditional medicine [7-9]. The numbers of plant species which have been used worldwide for medicinal purposes are approximately more than 50,000 [10]. In the past years, worldwide the mass production and growth of chemically synthesized drugs have revolutionized health care. However, in developing countries large portions of the people still depends upon herbal medicines and traditional practitioners for their health concerns. Ninety percent of population in Africa and up to 70% in India relies on traditional medicine. In China, traditional medicine accounts for around 40% of all health care delivered and more than 90% of general hospitals have units for traditional medicine [11].

Due to the hot and humid environment in the region, our local environment remains on the great risk of water-borne diseases. The diseases associated with the contamination of water are believed to be transmitted through water borne pathogens. The widespread water borne infectious diseases caused by protozoan, bacteria and viruses includes diarrheal diseases, Amoebiasis, Microsporidiosis, Giardiasis, Cyclosporiasis, Cryptosporidiosis, *E. coli* Infection, Cholera, Typhoid fever, Leptospirosis, Dysentery, Salmonellosis, Legionellosis, *M. marinum* infection, Campylobacteriosis, Botulism, Vibrio Illness, Otitis Externa, SARS, Hepatitis A, *Poliomyelitis* (Polio) and *Polyomavirus* infection [12-15].

Many efforts have been made for the development of new antibiotics against resistance pathogens [16-17]. Bacteria resistant to antibiotics are actively increasing in aquatic environment. In general, physicians are using wide

range of antibiotics for treatment against infections. The noticeable reason is that most of the bacterial strains are now become resistant to one or more of antibiotics and some may be resistant to all available antibiotics [18].

Mulberry plant (commonly called Toot in local language) is one of conventional herbs which are used in medicine due to its chemical composition and pharmacological function. *Morus alba* L. (white mulberry) is native to Eastern and Central China, Japan, and India. It has a long history of medicinal use in Chinese herbal medicine. According to Chinese pharmacopoeia the leaves, stems, barks, roots and fruits of *M. alba* L. were active ingredients in medicinal preparations [19].

Morus nigra L. (black mulberry) is native to Western Asia. It is widely spread through-out all regions from the tropics to the sub-arctic and from sea level to altitudes as high as 4000 m [20]. The fruits of black mulberry are famous for their flavor and nutritional qualities particularly due to carotene [21]. In different parts of the world, this is used as vegetable and is cultivated for fruit production in European countries. The leaves of mulberry are used as infusion in Asian countries (most common in Japan and Korea). Leaves of Mulberry are also used as powder, tea and juice in Japan [22].

In recent years, several studies have been carried out for the treatment of bacterial infections through alternative drugs. The current study is the assessment of antibacterial properties of *Morus alba* L. and *Morus nigra* L. against pathogenic bacterial strains isolated from different water samples. There is a great potential in this area of research because it will contribute to promote the commercial use of these plants as antimicrobial agent.

II. Materials And Methods

A. Collection of Water Samples

Water samples are collected in sterilized falcon tubes (50 ml) from different areas of Punjab which includes Attock City, Kala Chitta Pahar, Gulistan Colony, Officer's Colony Wah Cantt and Taxila.

B. Determination of Different Properties of Water Samples

Water qualities like type of water, pH, optical density and bacterial count are determined. For the growth of bacterial culture, nutrient agar media (Oxoid) is used. One ml of each collected water sample is spread with the help of sterilized glass spreader. Same process is followed for each sample and incubated the plates overnight at 37 ^{0}C using Electro-thermal Incubator (DNP5092, SERICO). Bacterial count (CFU/ml) is determined by means of the surface viable counting Technique [23].

C. Screening of Bacterial Colonies

Streak plate method is used to isolate and purify bacterial cultures obtained from tested water samples. The streaking method is repeated until pure isolated colonies are obtained.

The Gram's staining and standard biochemical tests including coagulase, urease, catalase, oxidase, citrate utilization, sugar fermentation, indole and motility test are performed for the phenotypic identification of isolated bacterial strains [24-29].

D. Antibiotic Sensitivity by Disc Diffusion Method

Sensitivity and resistance of identified bacteria is checked against standard antibiotics such as Ciprofloxacin 5 µg (CIP5), Gentamicin 10 µg (CN10), Kanamycin 30 µg (K30), Chloramphenicol 10 µg (C10), Sulphamethoxazole-trimethoprim 25 µg (SXT20), Cephalexin 30 µg (CL30), Cefotaxime 30 µg (CTX30), Ofloxacin 5 µg (OFX5), Ampicillin 10 µg (AMP10), Tetracyclin 30 µg (TE30) and Ceftazidime 30 µg (CAZ30) by disc diffusion method.

E. Preparation of Plant Extracts

The fresh fruits of *Morus* sp. are collected from the University Campus. Fresh fruits are washed with distilled water. 20 g of fresh fruit material is grinded with the help of mortar and pestle. 200 ml distilled water is added in the grinded fruits and incubated in the shaking incubator at 100 rpm for 8 hours. The aqueous extracts are then filtered through Watman No. 1 (Schleicher and Schuell 125 mm Cat No. 1001 125) filter paper. The different types of Mulberry fruit extracts i.e. Ripened Black Toot (RBT) as shown in *Fig. 1*, Un Ripened Black Toot (URBT), Ripened White Toot (RWT) as shown in *Fig. 2* and Un Ripened White Toot (URWT) are prepared and passed through bacterial filter for sterilization.

F. Antimicrobial Activity of Plant Extracts by well Diffusion Method

To determine the antimicrobial activity the well diffusion assay for RBT, URBT, RWT, and URWT is performed on Muller Hinton agar plates. 40 µl of each fruit extract was poured in respective well under sterile conditions. Plates are incubated at 37 ^{0}C for 24 hours. Zones of inhibition for each bacterial sample are determined.

In addition, the antimicrobial properties of mulberry extracts are further evaluated by determining the growth curves of bacterial isolates in presence and absence of the extracts. For this purpose optical density of bacterial cultures is measured at 600 nm by using spectrophotometer at different time intervals ranges between 2 - 26 hours.

Fig.1. Ripened fruit of M. nigra L. (RBT).

III. Results And Discussion

All the water samples are soft water having pH ranges 7.0 – 8.5. Different properties of these water samples are summarized in Table I.

TABLE I
SUMMARY OF DIFFERENT PROPERTIES OF WATER SAMPLES

Sampling Site	pH	*OD at 600nm	Density g/ml	Bacterial count **CFU/ml
Officer's colony	8.53	0.050	2.27	32
Taxila	7.50	0.045	2.31	960
Gulistan	8.25	0.049	2.32	800
Kala Pahar	8.36	0.050	2.31	228
Attock	8.28	0.046	2.32	52
Chitta Pahar	7.18	O.049	2.37	1200

*OD: Optical Density; **CFU: Colony Forming Units

Sample analysis shows the presence of wide range of bacteria. Total 65 bacterial colonies are isolated and purified for further investigations. Bacterial identification results confirm the presence of *Clostridium* sp., *Listeria monocytogene*, *Legionella pneumophila*, *Micrococcus luteus*, *Proteus mirabilis*, *Serratia* sp., *Aeromonas hydrophila*, *E. coli*, *Corneybacterium* sp., *Salmonella enteric*, *Bacillus subtilis*, *Moraxella catarrhalis*, *Bacillus megaterium*, *Bacillus thuringiensis*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Actinomyces sp.*, *Vibrio cholera*, *Bacillus anthracis*, *Salmonella typhi* and *Streptococcus pneumonia*.

Antimicrobial susceptibility of isolated bacterial strains against commercially available sterilized antibiotic discs (CIP5, CN10, K30, C10, SXT20, CL30, CTX30, OFX5, AMP10, TE30 and CAZ30) is determined by disc diffusion method. The zone of inhibition is interpreted according to CLSI [30]. The *Clostridium* sp. and *M. luteus* show resistance against SXT20, CAZ30, CTX30 and AMP10. The *L. monocytogene* is resistant against SXT20 and OFX5. The *L. pneumophila* is resistant against C10, SXT20, CTX30 and AMP10. *Proteus mirabilis* show resistance against SXT20 and AMP10. The *Serratia* sp. shows resistance against SXT20. The *A. hydrophila* is resistant against C10, SXT20 and AMP10. *E. coli* is resistant against CAZ30. The *Corneybacterium* sp is resistant against C10, SXT20 and CAZ30. The *B. subtilis* shows resistance against SXT20. The *M. catarrhalis* and *S. pneumonia* are resistant against C10 and SXT20. The *B. megaterium* and *B. thuringiensis* are resistant against C10, SXT20 and AMP10. The *S. aureus* and *Actinomyces sp* were resistant against SXT20 and CAZ30. The *B. anthracis* shows resistance against AMP10 while *S. typhi* shows resistance against TE30 and CTX30 respectively as shown in Table II.

In the present study RWT and URBT extracts shows maximum antimicrobial activities with 35 mm zone of inhibition against *V. cholera* and 32 mm against *M. luteus*, respectively. All fruits extract of mulberry (URBT, RBT, URWT, and RWT) show antimicrobial activity against *M. luteus*, *E. coli*, *B. subtilis*, *B. anthracis* and *B. thuringiensis*. In case of *L. pneumophila*, *P. mirabilis*, *Serratia* sp., *A. hydrophila*, *M. catarrhalis*, *B. megaterium*, *S. pyogenes* and *S. typhi* these extracts show no antibacterial activities.

Current work show that *E. coli* is sensitive to all standard antibiotics except CAZ30. All mulberry extracts are also effective against *E. coli* and show zone of inhibition in RBT (20 mm), URBT (22 mm), RWT (19 mm) and URWT (17 mm). Antibacterial activity of mulberry extracts is even better in case of *B. thuringiensis* RBT (13 mm), URBT (23 mm), RWT (10 mm) and URWT (23 mm). The *B. thuringiensis* show resistance against standard antibiotics C10, SXT20 and AMP10 while it exhibits significant zone of inhibition against all mulberry extracts as shown in Table II and *Fig. 3*. URBT (32 mm) and RWT (35 mm) show greater zone of inhibition than all standard antibiotics used in this study except K30 (35 mm), OFX5 (32 mm) and TE30 (32 mm).

Present findings reveal that antimicrobial effects of mulberry fruit extracts vary in different bacterial species. Further investigations on phytochemical constitutions present in mulberry fruit extracts can determine and compare their antimicrobial properties. Antibacterial activity present in fresh fruit juice of mulberry against the Gram-positive and Gram-negative bacteria has been also observed in some previous studies. Maximum zones of inhibitions against *B. subtilis* (18.46 mm) and the minimum zone of inhibition against *E. coli* (9.98 mm) are reported in [24]. Among the Gram- positive species, Bacillus species show highest zones of inhibition while for Gram- negative bacteria, had higher inhibition than *S. typhimurium* or *E. coli* [31]. Current Results reveal that *E. coli* shows maximum zones of inhibition against all fruits extracts of mulberry i.e., RBT (20 mm), URBT (22 mm), RWT (19 mm), and URWT (17 mm) as shown in *Fig. 3*.

Growth patterns of *M. catarrhalis*, *B. megatrium*, *S. pyogenes*, *S. typhi*, *P. mirabilis*, *V. cholera*, *E.coli*, *M. luteus* and *B. thuringiensis* are studied. The growth curves show two types of patterns: mulberry fruit extracts significantly inhibited the bacterial growth of some bacteria as compared to the control while the same extract enhances the growth of other bacteria as compared to control. The difference of response in different types of bacteria might be related with species specific effect of these extracts. It is found that *M. catarrhalis* growth was inhibited in the presence of RBT mulberry extracts as compared to control as shown in *Fig. 4*. In case of *B. megatrium* maximum growth inhibition is observed in URWT while growth inhibition was minimum in case of RWT as compared to control as shown in *Fig. 5*. *S.*

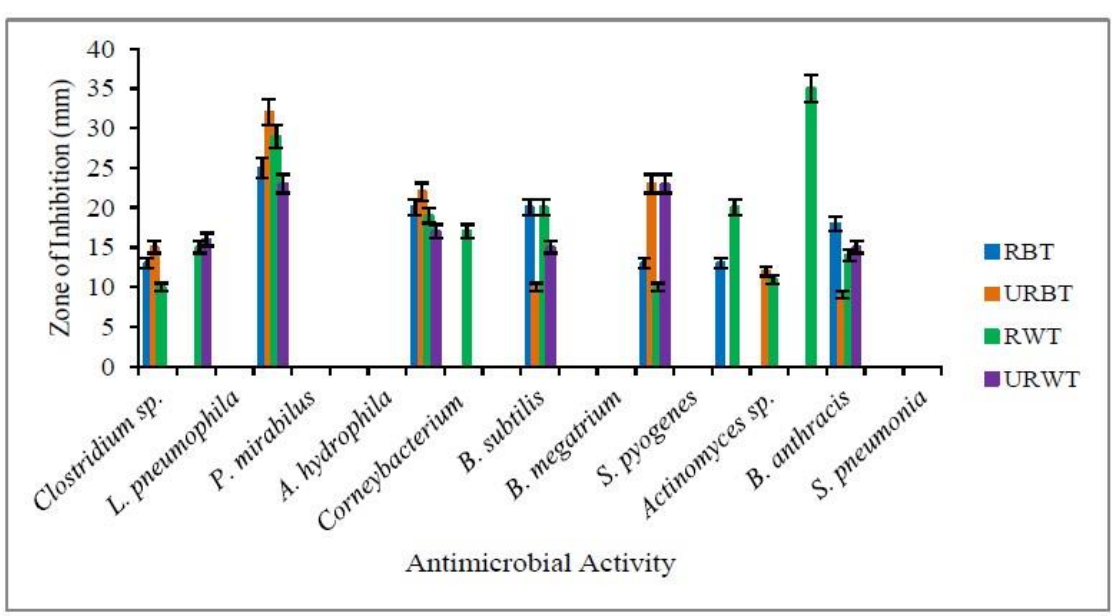
Fig.2. Ripened fruit of M. alba L. (RWT).

pyogenes show enhanced growth in presence of mulberry fruits extracts as compared to the control. Maximum growth is observed in URBT as depicted in *Fig. 6*. Growth of *S. typhi* is inhibited by almost all fruit extracts of mulberry as compared the control while RWT shows significant growth inhibition as shown in *Fig. 7*. In case of *P. mirabilis* maximum growth inhibition is observed in RBT as depicted in *Fig. 8* whereas in *V. cholera*, it is observed in RWT and is shown in *Fig. 9*. *E. coli* in the presence of URBT show maximum inhibition of growth as shown in *Fig. 10*. *M. luteus* also exhibites growth inhibition in all fruit extracts of mulberry but maximum inhibition in growth is found in presence of RBT as depicted in *Fig. 11*. *B. thuringiensis* growth is inhibited when grown with

TABLE II
SUMMARY OF ANTIBIOTIC SENSITIVITY OF BACTERIAL ISOLATES OF DIFFERENT WATER SAMPLES WITH THEIR MEAN ZONE OF INHIBITION

	Zone of inhibition (mm) Against Antibiotics										
Bacterial Species	CIP 5	CN 10	K 30	TE 30	C 10	SXT 25	CAZ 30	CL 30	CTX 30	0FX 5	AMP 10
Clostridium sp.	27	15	16	28	15	0	0	16	0	17	0
L. monocytogens	26	27	16	17	13	0	19	22	21	0	13
L. pneumophila	20	10	20	16	0	0	0	22	10	19	0
M. luteus	25	14	15	25	16	0	0	16	0	17	0
P. mirabilus	18	14	16	20	15	0	20	19	10	24	0
Serratia sp.	18	10	16	10	20	0	25	23	9	23	13
A. hydrophila	20	10	24	15	0	0	22	19	10	22	0
E.coli	25	13	16	14	16	24	0	25	12	27	29
Corneybacterium	24	17	20	18	0	0	0	24	14	21	9
S. enteric	30	15	16	26	20	23	20	23	9	23	13
B. subtilis	26	10	23	15	15	0	20	26	17	26	25
M. catarrhalis	16	15	17	19	0	0	25	30	26	17	33
B. megatrium	20	10	24	13	0	0	21	19	10	22	0
B. thuringiensis	21	10	22	14	0	0	24	21	11	26	0
S. pyogenes	20	10	15	20	11	12	12	22	15	18	20
S. aureus	25	14	21	16	15	0	0	19	12	20	10
Actinomyces sp.	12	12	15	10	11	0	0	20	15	20	10
V. cholera	30	25	35	32	25	15	20	25	32	35	15
B. anthracis	11	15	18	20	23	16	15	25	30	20	0
S. typhi	22	16	13	0	10	9	14	16	0	19	23
S. pneumonia	20	15	10	10	0	0	20	25	18	24	17

Abbreviations: CIP5: Ciprofloxacin 5 µg, CN10: Gentamicin 10 µg, K30: Kanamycin 30 µg, TE30: Tetracyclin 30 µg, C10: Chloramphenicol 10 µg, SXT20: Sulphamethoxazole-trimethoprim 25 µg, CAZ30: Ceftazedime 30 µg, CL30: Cephalexin 30 µg, CTX30: Cefotaxine 30 µg, OFX5: Ofloxacin 5 µg and AMP10: Ampicillin 10 µg.



Abbreviations: Ripened black toot (RBT), Un-ripened Black Toot (URBT), Ripened White Toot (RWT) and Un-Ripened White Toot (URWT)

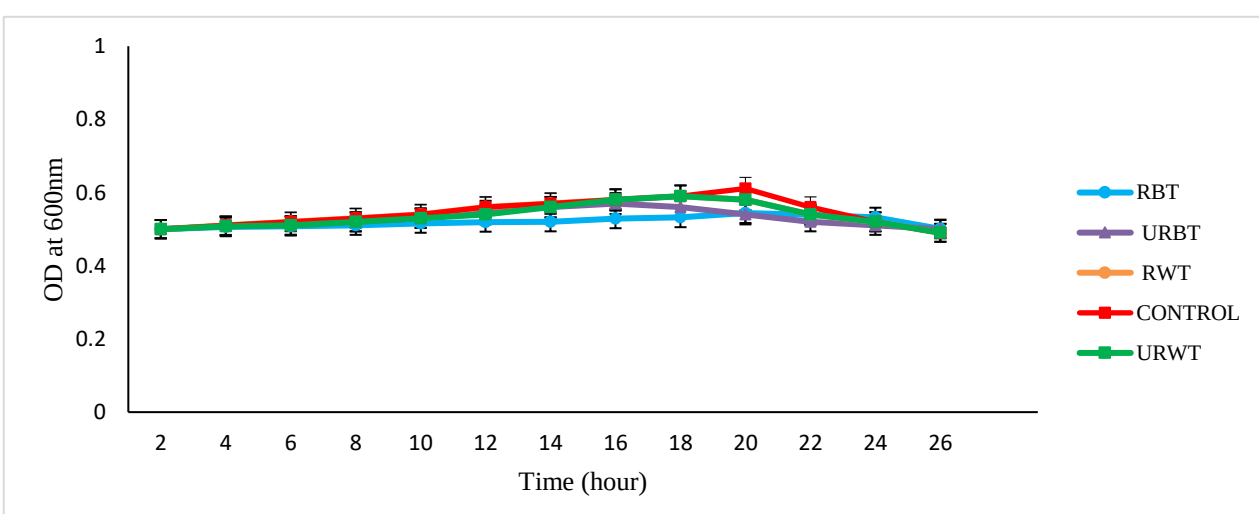
Fig.3. Antimicrobial activities of mulberry fruit extracts against isolated bacterial strains.

URBT as compared to the control as shown in *Fig. 12*.

The mulberry extracts are known to have many active ingredients which inhibit bacterial growth. The phytochemistry, pharmacologically active constituents and nutritional profile of *M. alba* revealed its importance. It is studied in [32] that mulberry extracts are rich in phytochemicals and have antimicrobial properties against harmful pathogens. Kuwanon G (purified from methanolic extract of *M. alba*) showed antimicrobial activity against dental caries associated *Streptococcus mutans* [32]. Mulberries also have antimicrobial chemicals such as kuwanon C, mulberrofuran G and albanol B [33]. It is also reported in [34-35] that phyto-constituents isolated from the aqueous and ethanolic extract of *M. alba* have antibacterial and antifungal activities against oral pathogens such as *Streptococcus mutan*. These findings along with present work indicate that Mulberries extracts have antimicrobial properties against various bacterial species.

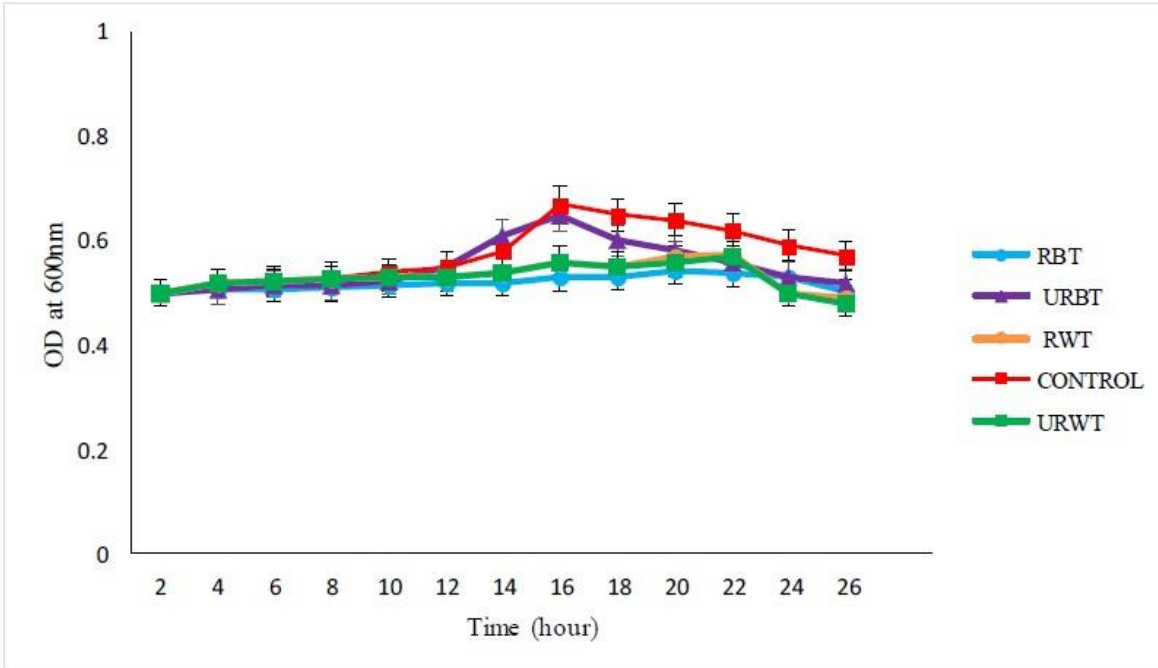
IV. Conclusion

The search of bioactive compounds from plants is considered to be the important paramount to control diseases caused by water-borne pathogens. Presented results indicate that mulberry fruit extracts are effective against *M. Luteus*, *E. coli* and *B. thuringiensis*. Previous studies reported that mulberry fruit extracts contain high phenolic contents, amino acids, vitamins, flavonoids, steroids, tri terpenes and other trace elements [36-37]. In the light of current findings it is concluded that mulberry fruits extracts have a great potential for future discovery of potent antimicrobial agents of plant origin. There is a great potential in this area of research because it would contribute to promote the commercial use of these plants.



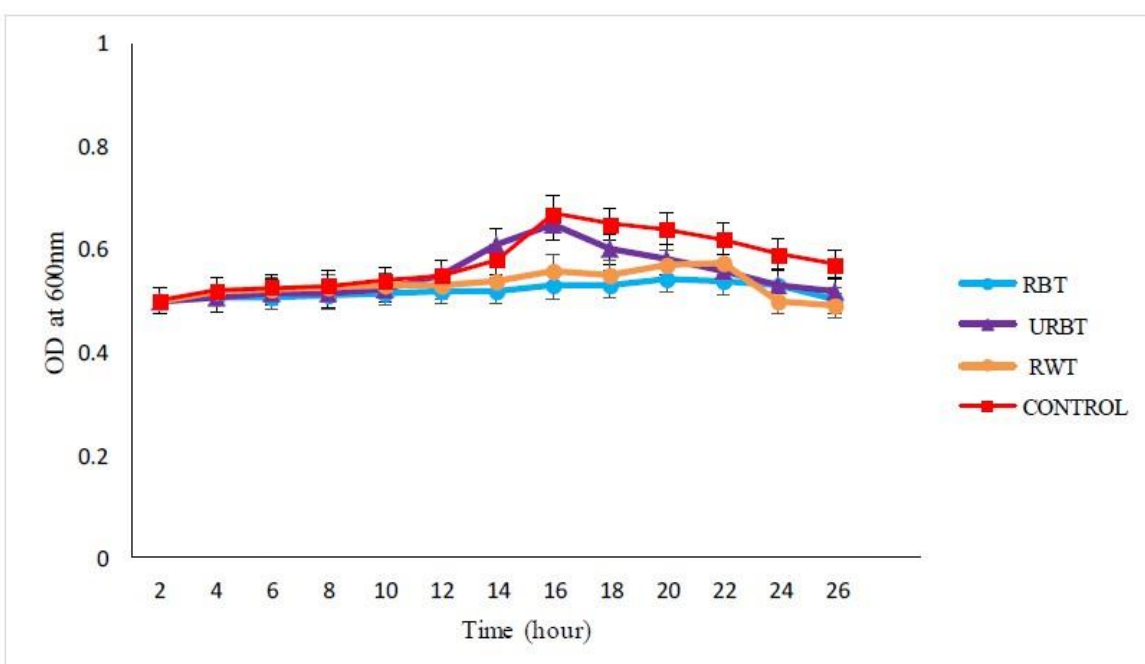
Abbreviations: Ripened black toot (RBT), Un-ripened Black Toot (URBT), Ripened White Toot (RWT), Un-Ripened White Toot (URWT), Optical Density (OD)

Fig. 4. Growth pattern of M. catarrhalis against mulberry fruit extracts.



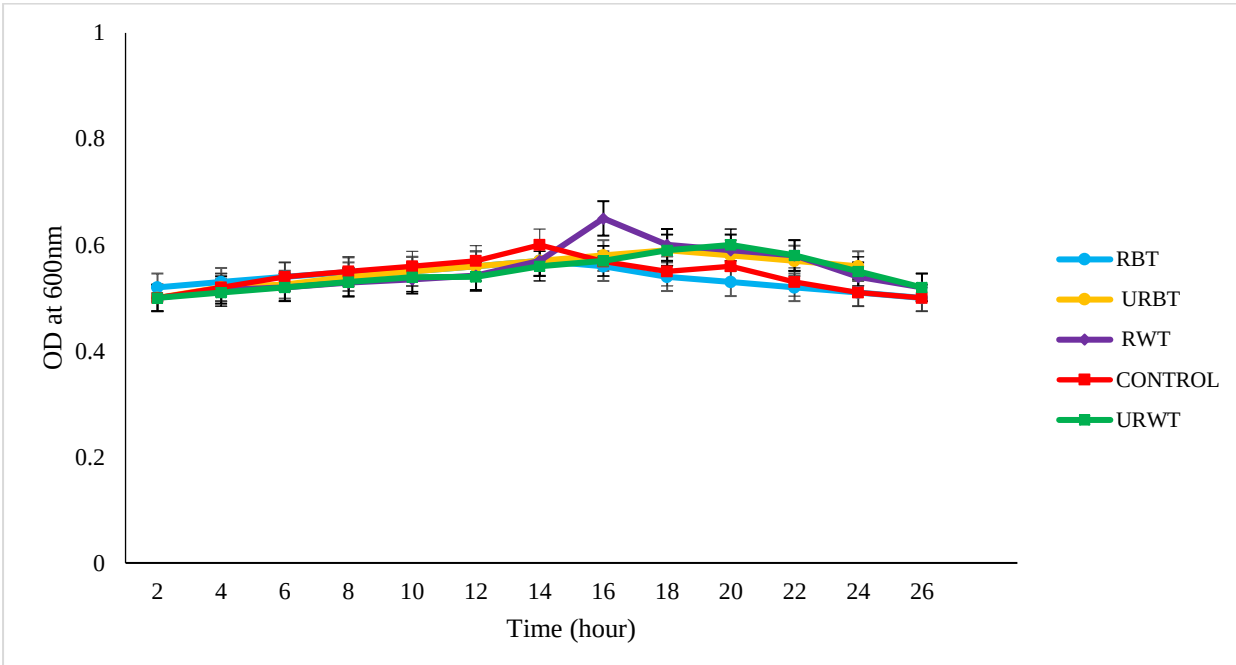
Abbreviations: Ripened black toot (RBT), Un-ripened Black Toot (URBT), Ripened White Toot (RWT), Un-Ripened White Toot (URWT), Optical Density (OD)

Fig. 5. Growth pattern of B. megatrium against mulberry fruit extracts.



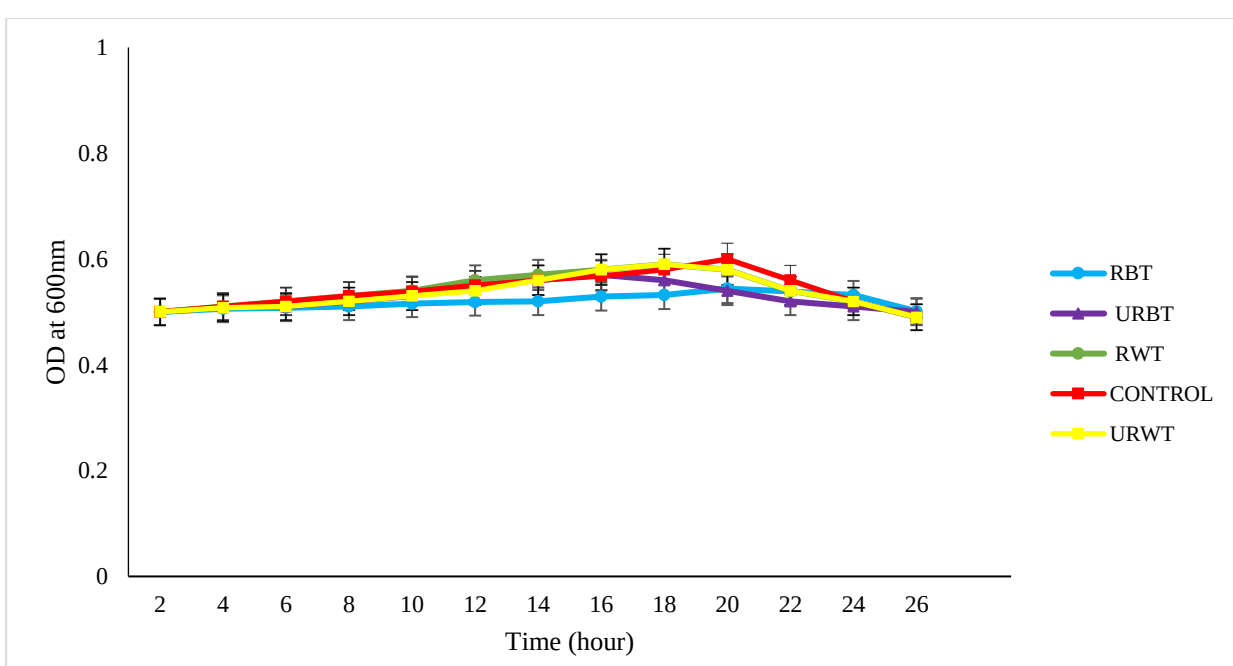
Abbreviations: Ripened black toot (RBT), Un-ripened Black Toot (URBT), Ripened White Toot (RWT), Optical Density (OD)

Fig. 6. Growth pattern of isolated S. pyogenes against mulberry fruit extracts.



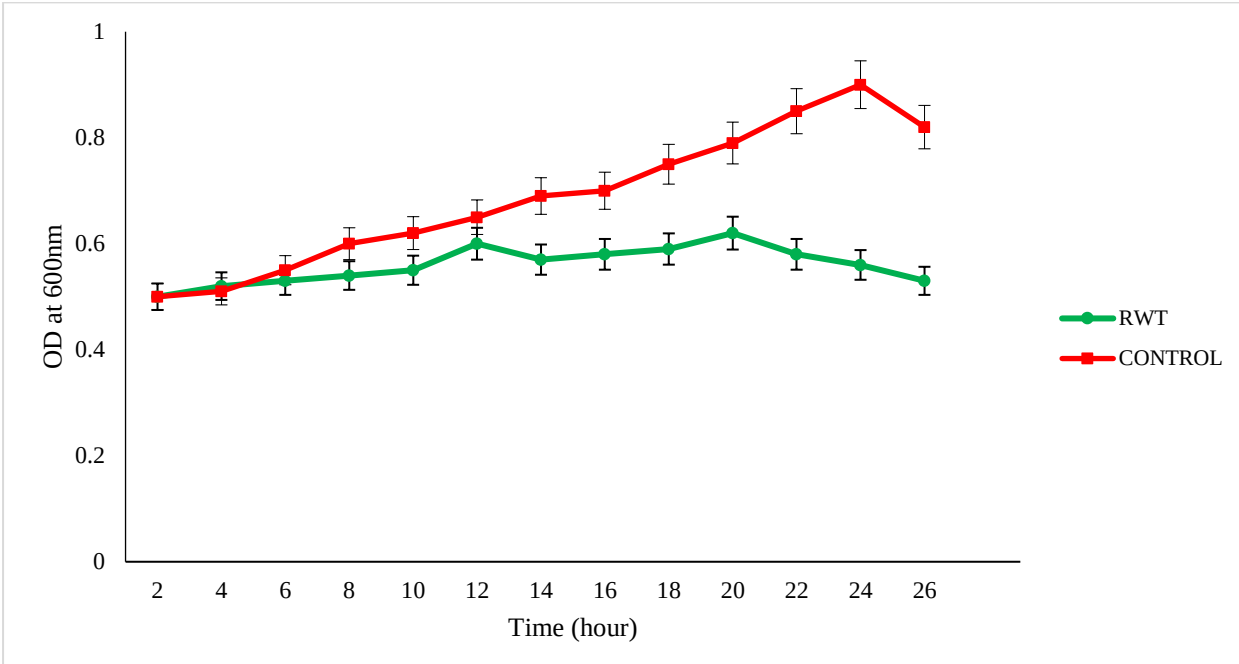
Abbreviations: Ripened black toot (RBT), Un-ripened Black Toot (URBT), Ripened White Toot (RWT), Un-Ripened White Toot (URWT), Optical Density (OD)

Fig. 7. Growth pattern of isolated S. typhi against mulberry fruit extracts.



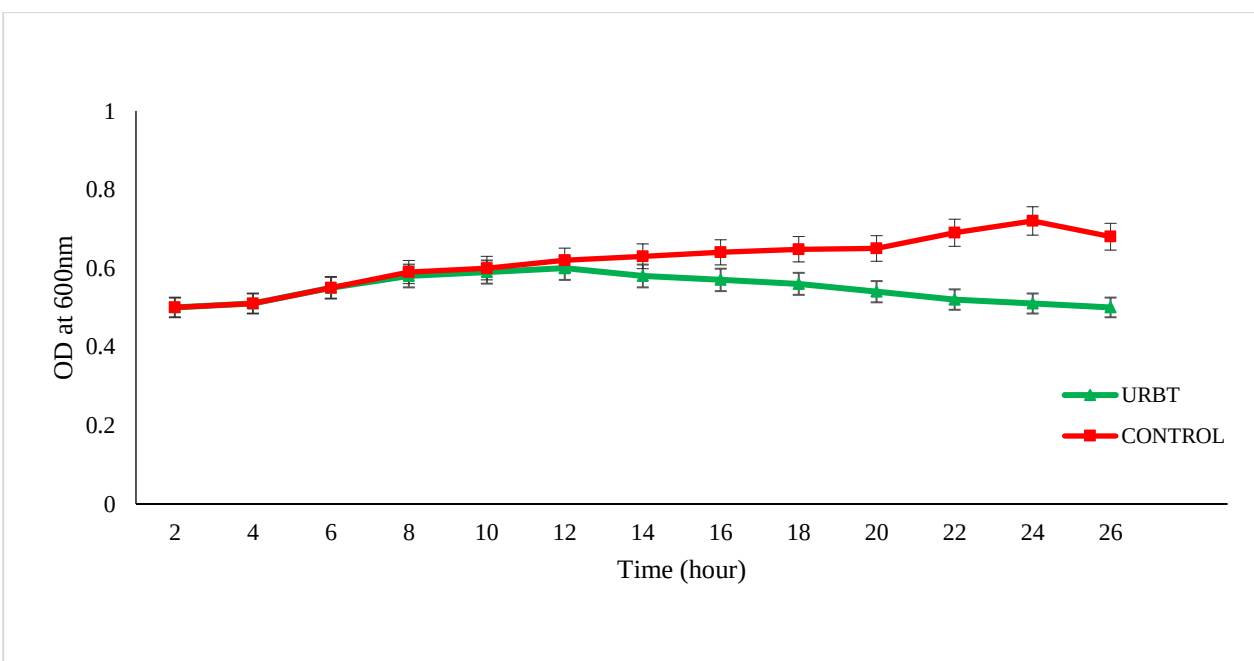
Abbreviations: Ripened black toot (RBT), Un-ripened Black Toot (URBT), Ripened White Toot (RWT), Un-Ripened White Toot (URWT), Optical Density (OD)

Fig. 8. Growth pattern of isolated P. mirabilis against mulberry fruit extracts.



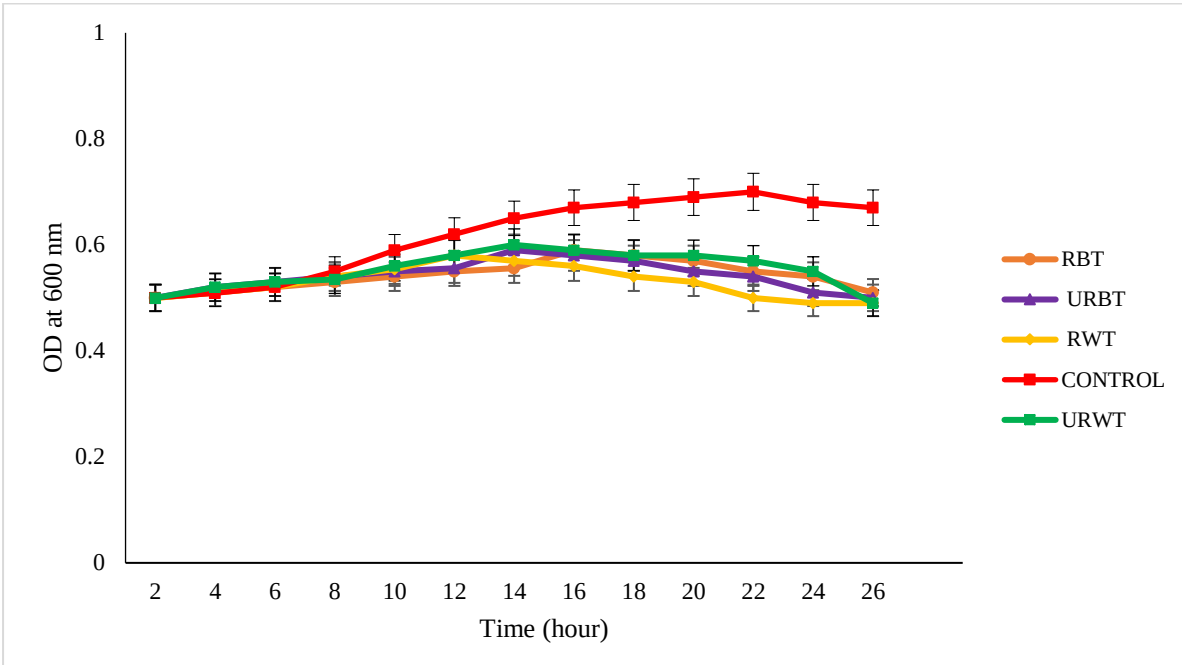
Abbreviations: Ripened White Toot (RWT), Optical Density (OD)

Fig. 9. Growth pattern of V. cholera against mulberry fruit extracts.



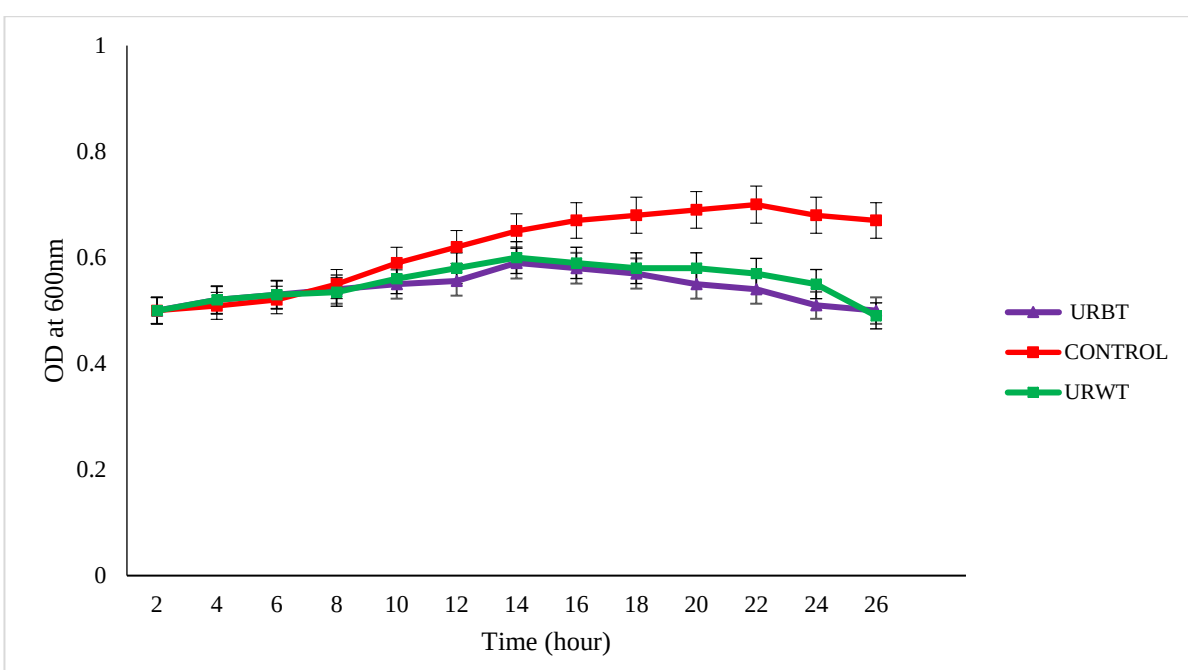
Abbreviations: Un-ripened Black Toot (URBT), Optical Density (OD)

Fig. 10. Growth pattern of E.coli against mulberry fruit extracts.



Abbreviations: Ripened black toot (RBT), Un-ripened Black Toot (URBT), Ripened White Toot (RWT), Un-Ripened White Toot (URWT), Optical Density (OD)

Fig. 11. Growth pattern of M. luteus against mulberry fruit extracts.



Abbreviations: Un-ripened Black Toot (URBT), Un-Ripened White Toot (URWT), Optical Density (OD)

Fig. 12. Growth pattern of B. thuringiensis against mulberry fruit extracts.

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