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Comparative Evaluation of Biological Activities, Bioautography and Spot Screening of Boiled and Macerated Extracts of Medicinal Plants against Zoonotic Pathogens

Rabia Ilyas[1], Saiqa Andleeb[2*], Muhammad Babar[3], Rozina Ghulam Mustafa[3], Irsa Shafique[2], Atiya Zafar[2] and Shaukat Ali[3]

[1]Department of Biotechnology, University of Azad Jammu and Kashmir, Muzaffarabad, 13100, Pakistan.
[2]Department of Zoology, Microbial Biotechnology Laboratory, University of Azad Jammu and Kashmir, Muzaffarabad, 13100, Pakistan.
[3]Department of Zoology, Toxicology Laboratory, University of Azad Jammu and Kashmir, Muzaffarabad, 13100, Pakistan.

Authors' contributions

This work was carried out in collaboration between all authors. Authors Saiqa Andleeb and Shaukat Ali designed the study, performed the statistical analysis, wrote the protocol, wrote the first draft of the manuscript and managed literature searches. Authors RI, MB, RGM, IS and AZ did experiments of the study and literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: There is an escalating requirement for the development of novel drugs for the treatment of zoonotic diseases. Herbal plants have always been the best source of therapeutic agents.
Study Design: Biological activities of medicinal plants.
Place and Duration of Study: Microbial Biotechnology laboratory, Department of Zoology,

**Corresponding author: E-mail: drsaiqa@gmail.com;*

University of Azad Jammu and Kashmir, Muzaffarabad, 13100, Pakistan, between 12 Feb, 2014 and 29 June, 2015.

Methodology: In current research work biological properties of various extracts of medicinal plants i.e. *Atropa acuminate, Atropa belladonna* and *Morus nigra* were investigated. Extracts were prepared through both maceration and boiling extraction methods. Antibacterial activity against zoonotic pathogens such as *Serratia odorifera*, *Enterobacter amnigenus*, *Shigella flexneri, Salmonella* Typhimurium and *Pseudomonas aeruginosa* was assessed using agar well diffusion method. DPPH free radical scavenging method was used to measure antioxidant potential. Total phenolic and flavonoid contents were estimated.

Results: It was found that boiling extracts showed maximum antibacterial activity against all tested pathogens. The results of antioxidant potential revealed the maximum activity in root extracts of *A. belladonna* and all extracts of *M. nigra* while *A. acuminata* showed lowest scavenging activity. Phytochemical analysis indicated the existence of alkaloids, flavonoids, quinones, phenols, tannins, terpenoides, glycosides, steroids and carbohydrates. Thin layer chromatography (TLC) also mentioned the presence of phytochemical constituents. TLC- developed plates have showed the maximum presence of both antioxidant constituents and flavonoids contents in all boiling extracts of *Z. armatum*. Bio-autobiography by agar overlying assay indicated the significant inhibition of tested pathogens.

Conclusion: The current study provides scientific confirmation supporting the therapeutic potency of these medicinal plants for medicinal uses and identifies gaps for future research to facilitate commercial utilization.

Keywords: Antibacterial activity; extraction method; antioxidant potential; thin layer chromatography; TLC-Bioautography.

1. INTRODUCTION

Zoonotic diseases are the major cause of spreading dangerous infections in humans [1]. Zoonotic diseases cause by various infectious agents such as bacteria, viruses, fungi and parasites. Infections transmitted through several routes i.e. penetration of bacteria through direct contact with tissues, blood, urine, fetuses of infected animals, by ingesting contaminated food, drinking water, inhaling contaminated soil and also penetrate through abraded skin. Various bacterial diseases transmitted from animals i.e. calves, dogs, cats, rodents, fishes, chicken, cattle, pigs, sheep, goats, swine etc to humans. The major treatment for bacterial infections of fish and humans is the antibiotics [2]. In spite of this crucial role to treat various infectious diseases, antibiotics also have numerous adverse effects. Development of multidrug resistant pathogens and increase level of new infections is a major cause of failure of antibiotics [3]. Microbial resistance develops due to the limited effective duration of an antibiotic, maltreatment with traditional antibiotics and over recommendation [4].

The elevating level of resistance to various antimicrobial agents is a main hurdle to their use and control over bacterial infection. There is no antimicrobial agent which is fully effective against dormant facultative bacteria [5]. Furthermore in search of antimicrobial prospective, the estimation of the plant extracts along with other plant products has proved that the higher plant presents a probable source of all new prototypes of antibiotics [6]. Numerous studies have also revealed that medicinal plants have several constituents act as powerful antibiotics [7].

The current research work was planned to assess antibacterial activity, phytochemical screening, total phenolics and flavonoids contents, thin layer chromatography (TLC), bioautography of TLC plates and antioxidant potential of selected medicinal plants.

2. MATERIALS AND METHODS

2.1 Medicinal Plants

Rhizome and leaves of *Atropa acuminate* and *Atropa belladonna* were collected during the period of July and August, 2013 from forests of Leepa, Jhelum Valley, Azad Jammu and Kashmir (AJK), Pakistan. Fruits of *Morus nigra* were collected from Kutla Bheri, District Muzaffarabad, AJK, Pakistan. Medicinal plants were identified by Dr. Abdul Rehman Niazi (Botanist) at M. S. Zahoor Memorial Herbarium (LAH), Lahore,

Botany Department, University of Punjab, Lahore, and Pakistan.

2.2 Processing of Medicinal Plants

Collected specimens were washed thoroughly with running tap water to get rid of dust and the other redundant adherent materials. Then air dried at room temperature for two weeks and ground well to make powdered with the help of a mechanical grinder.

2.3 Conventional Extraction Method

Maceration used as conventional extraction method. Each powdered material (10 g) was soaked in 100 ml of different solvents such as ethanol (E), methanol (M), chloroform (Ch) in separate bottles and were kept at room temperature (25±2℃) in extraction bottles for few days to ensure solubility. Crude extract was filtrated using ordinary filter paper and filtrate was subjected for biological studies.

2.4 Boiling Extraction Method

In boiling extraction method each powdered material (10 g) was soaked in 100 ml of different solvents such as ethanol (E), methanol (M), chloroform (Ch) in separate bottles and the mixture was boiled for 1 min at 100℃, cooled down and crude extract was filtrated using ordinary filter paper. Solvents were evaporated at room temperature (25±2℃) and remaining filtrate was subjected for biological studies.

2.5 Test-bacteria

The bacterial pathogens were taken from Microbial Biotechnology Laboratory, Zoology Department, AJK University, Muzaffarabad, Pakistan. These pathogens *Serratia odorifera* (F), *Enterobacter amnigenus* (F_1), *Shigella flexneri* (KL_2), *Salmonella typhimurium* (KL_1) and *Pseudomonas auregnosa* (CH_B) were isolated from spoiled fish and chicken [8].

2.6 Agar Well Diffusion Method

The antimicrobial activity was assessed by agar-well diffusion method [9]. Muller Hinton broth medium (HIMEDIA) and Muller Hinton agar medium (MERCK) were used for bacterial culture. The microorganisms were activated by inoculating a loop full of strain in 25 ml of nutrient broth medium and incubated on a rotary shaker at 37℃ for 24 h. The overnight culture was mixed with freshly prepared Muller Hinton agar medium at 45℃ and was poured into the sterilized Petri dishes. All Petri dishes were kept at room temperature in laminar flow for solidification. In each plate, three wells of 5 mm diameter were made using a 1 ml of sterilized micropipette tip and sterilized needle was used for the removal of agar plug. Approximately 30 µl of each crude extract and control solvent samples were placed in each prepared wells and placed at 37℃ for 24-48 h. The all solvents were also used as a negative control. Microbial growth was determined by measuring the diameter of zone of inhibition after 24 h in millimeter [10]. Diameter of the clear zones (if greater then 5mm) around each well was measured with the help of scale [11]. The results of the sensitivity tests were expressed as (0) for no sensitivity, *(1- 6 mm) for low sensitivity, **(>5-12 mm) for moderate sensitivity and ***(>12-24 mm) for high sensitivity.

2.7 Sensitivity Test

Antibacterial activity of *v*arious groups of antibiotics such as aminoglycosides (Tobramycin 10 µg/ml), Penicillins (Ampicillin 10 µg/ml, Penicillin G 10 µg/ml), Tetracyclines (Tetracycline 10 µg/ml), and Fluoroquinolones (Ciprofloxacin 10 µg/ml) was assessed by agar disc diffusion method used as positive control, like antibacterial activity procedure of medicinal plants, overnight culture was mixed with freshly prepared Muller Hinton agar medium and poured in Petri plates then antibiotics discs were placed by help of sterilized forecep [12]. All antibiotic discs were purchased and made by Oxoid Company. To check Sensitivity clear zone were measured in mm around the disc after 24 h. Antibacterial activity of antibiotics was also calculated as a mean of three replicates and was compared with that of antibacterial activity of solvent extracts.

2.8 DPPH free Radical-scavenging Ability

DPPH free radical scavenging activity was performed according to the reported method of Rubens and Wagner, [13] with some modifications. The absorbance was measured on Spectrophotometer at 517 nm (triplicates for single extracts). The percentage scavenging activity was calculated by formula, %=[(Ao-Ai)/Ao]x100; where Ao is the absorbance of control and Ai (A-B) is the absorbance with extracts.

2.9 Phytochemical Screening

All extracts of *A. belladona, A. acuminata, Z. armatum* and *M. nigra* were tested for the presence of active compounds/phytochemicals such as alkaloids, flavonoids, steroids, saponins, quinones, glycosides, phenols, carbohydrates, protein, amino acids, terpenoides and tannins as described in previous literatures [14-16] (Table 1).

2.10 Thin Layer Chromatography

The major phytochemicals of medicinal plants were further confirmed by TLC using precoated Silica gel 60F264 plates [17]. In order to get better resolution of the components four different screening systems were used such as solvent system A {(CH_3OH, CH_3COOH, H_2O (8, 2 , 10)}, solvent system B {(Acetone; $(CH_3)_2CO$), CH_3COOH, H_2O (8,2,10)}, solvent system C {(CH_3COOH, H_2O (8, 2, 10)} and solvent system D {(Butanol; C_4H_9OH, acetone; $(CH_3)_2CO$), H_2O (10,5,20)}. After that TLC plates were labeled such as extract name and solvent system at upper end and a line was drawn at lower end 3 cm above the base, then a drop of extract was dropped on the center of this line and placed on the beaker containing solvents. Then observed the movement of solvent and extracts above, the developed plates were observed under visible as well as UV light (734 nm). R_f value was calculated for each band as R_f = Distance travelled by the solute / Distance travelled by the solvent.

2.11 TLC Analysis of Antioxidant Constituents

The antioxidant constituents were analyzed using thin layer chromatography (TLC) followed by DPPH (2, 2- Diphenyl-1-picrylhydrazyl) spray technique [18]. After the development of TLC plates, 0.05% of DPPH solution in methanol (100 ml) was sprayed on the surface of developed TLC plates and incubated at room temperature for 10 min. The active antioxidant constituents of medicinal plants were detected as yellowish green spots produced by bleaching of DPPH by resolved bands on the TLC plates with purple background.

2.12 TLC Analysis of Flavonoides Constituents

For detection of flavonoids, TLC-developed plates were spray with a solution containing 1% of aluminum chloride and incubated at room temperature for 10 min. Yellow, brown, and dark green fluorescence in long wavelength UV light (360 nm) indicated positive results.

2.13 TLC Analysis of Different Drugs

Presence of different drugs was also confirmed by spraying 96% ethanolic solution of potassium hydroxide (KOH). After incubation of 10 min, yellow zones indicated positive results.

2.14 Bioautography

Agar overlay assay was used as described by Slusarenko et al. [19]. In bioautography TLC developed plates were used to check antibacterial activity. TLC-developed plates were covered with fresh overnight grown culture of bacterial pathogens (*S. flexneri* and *P. auregnosa*) and plates were incubated for 24 h at 37℃. Then MTT (Thiazolyl Blue Tetrazolium Blue) was used. The inhibited zone of bacterial growth (white/pale yellow color indication) could be seen around the active chromatogram spot under normal light with purple background (inhibition indication).

2.15 Total Phenolic Contents

The total phenols were determined using the Folin–Ciocalteu reagent method described by Zhou and Yu [20] with slight modifications and it was expressed as mg/g gallic acid equivalent using the following equation based on the calibration curve, $y = 0.476x + 0.8$, $R^2 = 0.996$, where y was the absorbance and x was the gallic acid equivalent (mg/g). Reaction mixture was incubated at room temperature for 1 h and absorbance of deep blue complex was measured against a blank at 765 nm.

2.16 Total Flavonoid Contents

Total flavonoid contents of extracts was quantified by the method illustrated by Zou et al. [21] and it was expressed as mg/g rutin equivalent using the following equation based on the calibration curve, $y = 0.333x + 0.069$, $R^2 = 0.999$, where y was the absorbance and x was the rutin equivalent (mg/g). The reaction mixture was incubated at room temperature for 6 min. After incubation, 150 µl (0.15 ml) Aluminum chloride ($AlCl_3$) solution (10%) was added and then 2 ml of Sodium hydroxide (NaOH) solution (4%) was added to reaction mixture. Then distilled water was added immediately to bring

final volume to 10 ml and mixture was thoroughly shaken and kept for 15 min. Absorbance of reaction mixture was measured at 510 nm versus prepared water blank.

2.17 Statistical Analysis

Each experiment was repeated in triplicate and Standard Deviation from absolute data was calculated (http,//easycalculation.com/statistics/standard - deviation.php).

3. RESULTS

3.1 Inhibitory Effect of Medicinal Plants

The antibacterial activity of boiled extracts of rhizome and leaf of *A. acuminata* was more effective as compared to conventional extraction (Table 2). All boiled extracts of *A. acuminata* showed maximum inhibition of *S. flexneri* (13.0±1.73 mm, 17.33±2.88 mm, 13.0±6.92 mm, 12.0±0.0 mm, and 15.0±0.0 mm) except rhizome extract in methanol, which showed moderate inhibition (99.0±0.0 mm). Similarly both polar boiled extracts indicated the maximum inhibition of *P. aeruginosa* as compared to macerated extracts (11.33±1.15 mm, 11.0±0.0 mm, 14.33±1.15 mm, 11.33±0.57 mm. Non polar boiled extract had no effect against all tested pathogens except *S. flexneri*, while macerated chloroform showed maximum inhibition of *S. odorifera* (15.66±3.78 mm, 12.66±0.57 mm).

Results revealed that the inhibitory effect of boiled extracts of *A. belladonna* was more significant as compared to macerated extracts (Table 2). Macerated polar extracts showed moderate inhibition of *E. amnigenus* and *P. aeruginosa* whereas non polar macerated extracts indicated low sensitivity or had no effect (Table 3). Methanolic and ethanolic boiled extracts showed the maximum inhibition of *E. amnigenus* (15.0±0.0 mm, 12.66±0.57 mm, 15.0±0.0 mm, 14.66±0.57 mm), *S.* Typhimurium (11.66±0.57 mm, 11.66±0.57 mm, 12.33±0.57 mm, 10.66±0.57 mm), *P. aeruginosa* (16.0±0.0 mm, 13.0±0.0 mm, 15.33±0.57 mm, 12.66±0.57 mm). On the other hand, only rhizome extracts of polar solvents showed the inhibition of *S. flexneri* with 11.0±1.73 mm and 10.33±1.15 mm. Non polar boiled extracts had no effect against all tested zoonotic infectious agents.

Antibacterial activity results of *M. nigra* revealed that polar boiled extracts had significant effect on bacterial inhibition compared to macerated polar extracts (Table 2). Methanolic and ethanolic boiled extracts showed the maximum inhibition of all tested zoonotic pathogens with range from 11.66±1.15 mm to 18.33±0.57 mm while chloroform boiled extract only showed the inhibition of *S. odorifera (*13.0±0.0 mm) and *P. aeruginosa* (10.66±0.57 mm). On the other hand all macerated extracts showed moderate inhibition of *P. aeruginosa* with 7.33±0.57 mm, 10.0±1.73 mm, 7.66±0.57 mm zone of inhibition. Only solvents used as negative control had no effect against all tested pathogens. Interpretations of spot screening of TLC-developed plates and TLC-bioautography showed the significant use of medicinal plants as antibacterial agent.

3.2 Antibiogram Analysis

Standard antibiotics were used as positive control. It was recorded that all isolated pathogens *viz*., *S. flexneri, E. amnigenus, S.* Typhimurium*, S. odorifera* and *P. aeruginosa* were significantly inhibited by Tobramycin (25.0±0.0 mm, 25.0±0.0 mm, 27.0±0.0 mm, 20.0±0.0 mm, 20.0±0.0 mm), Ciprofloxacin (62.0±0.0 mm, 45.0±0.0 mm, 30.0±0.0 mm, 42.0±0.0 mm, 45.0±0.0 mm), and Tetracycline (25.0±0.0 mm, 25.0±0.0 mm, 20.0±0.0 mm, 15.0±0.0 mm, 0.0±0.0 mm). On the other hand Penicillin G, Trimethoprim and Ampicillin had no effect on the growth of all tested pathogens.

3.3 Antioxidant Potential of Medicinal Plants

Antioxidant potential of medicinal plants were measured using DPPH free radical scavenging method. Results revealed that macerated extracts had greater antioxidant potential compare to boiled extracts (Fig. 1). Boiled leaf extracts of *A. accuminata* showed DPPH potential as methanol (34%), ethanol (19%) and much lower in chloroform (4%) while minimum DPPH scavenging activity of *A. acuminata* rhizome extracts in ethanol, methanol and chloroform were observed as 16%, 14%, 8%, respectively. As compared to boiled extracts, maximum DPPH scavenging potential was recorded in macerated leaf and rhizome extracts i.e. 92%, 85%, 88%, 54%, 54%, and 31%. In case of *A. bellodanna* boiled leaf and rhizome extracts exhibited 42%, 78%, 44%, 85%, 66%,

and 74% DPPH activity while macerated extracts had 45%, 94%, 84%, 95%, 89%, and 57% free radical scavenging potential. Interesting results were recorded in case of *M. nigra*. Both boiled and macerated polar extracts had greater antioxidant activity such as boiled extracts had 98%, 63%, 100%, 97% while non-polar exhibited 99% in case of boiled and 11% in case of macerated, respectively. Presence of antioxidant constituents in all extracts of medicinal plants were also analysed through 0.5% DPPH spray. Yellow zones appeared on the TLC-developed plates along with purple background indicated the presence of antioxidants.

3.4 Qualitative and Quantitative Phytochemical Screening

Qualitative analysis of phytochemical screening revealed that both *A. acuminata* and *A. belladona* rhizome and leaf extracts were rich with tannins, alkaloids, quinones, flavonoids (except AB_RS_c) and phenols. Carbohydrates, terpenoides and glycosides were highest in *A. belladona* and present in some extracts of *A. acuminate.* On the other hand amino acids, saponins and proteins were found in few extracts of both these plants (Table 3). Out of all tested phytochemicals, alkaloids, terpenoides, flavonoids, quinones and glycosides were found in all extracts of *M. nigra*. Amino acids, tannins, carbohydrates, steroids and proteins were existing in all extracts of mulberry except extracts in chloroform while saponins are not present at all (Table 3). The standard calibration curve of gallic acid equivalents for total phenolic content and the standard calibration curve of rutin hydrate equivalent for total flavonoid content were expressed. Phenolic contents were higher in ethanolic extracts of *A. bellodona* rhizome (4.33 mg/100 ml) as compared to leaf extracts. Similarly, phenolic contents were found in greater amount in methanol and ethanol rhizome extracts of *A. accuminata* (5.61 mg/100ml and 5.45 mg/100ml) as compared to chloroform extracts. The flavonoid contents were found in chloroform leaf extracts of *A. accuminata* (1.977 mg/100 ml). Analysis of total phenolic and flavonoid contents revealed the presence of phenolics and flavonoid in fruit extracts of *M. nigra*. It was observed that ethanol extracts of *M. nigra* had high phenolics and flavonoid contents as compared to chloroform.

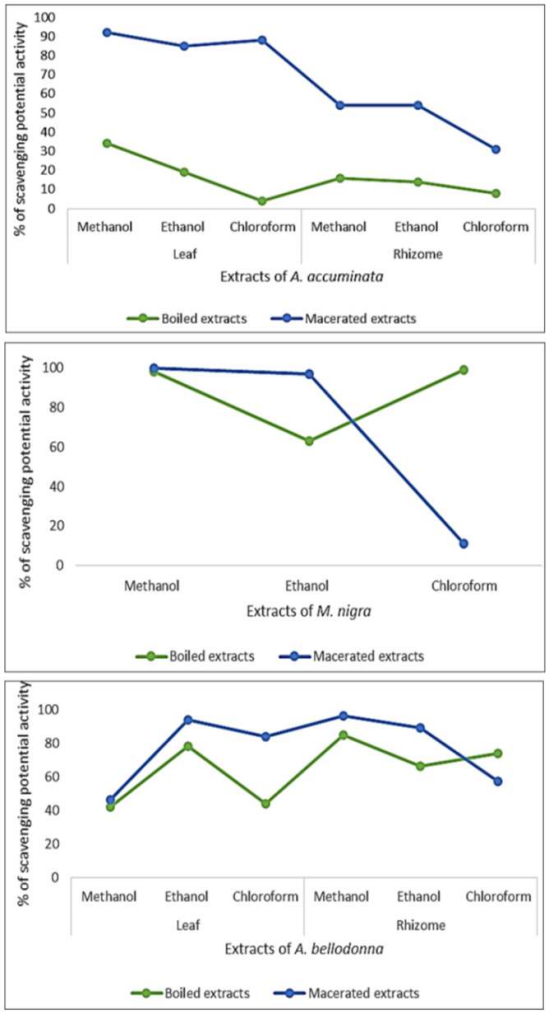

Fig. 1. Antioxidant potential of medicinal plants

3.5 Fingerprinting of Phytochemicals

Thin layer chromatography indicated the presence of various bioactive compounds or phytochemical constituents that are responsible as therapeutic agents. Flavonoids were also observed on the TLC-developed plates. Blue, green, brown and purple color indicating the presence of flavonoid contents in all extracts of medicinal plants through $AlCl_3$ spray Except *A. belladona* rhizome extract in methanol and chloroform. *A. acuminata* rhizome extract in methanol and *M. nigra* extract indicated positive results.

Table 1. Phytochemical screening of extracts of medicinal plant

Phytochemical constituents	Test used	Methodology	Positive indications
Tannins	1. Ferric chloride test 2. Gelatin test	1. 1 ml (extract) + 3 drops of 5% $FeCl_3$. 2. 1 ml (extract) + gelatin solution.	1. Brownish-green, blue black, green or blue green color 2. White precipitates
Saponins		1 ml (extract) + 5 ml water, shaking tubes vigorously.	Copious lather formation
Glycosides	Keller-Killani test	2 ml (extract) + 2 ml glacial acetic acid + 1 drop of 5% $FeCl_3$. This solution was carefully transferred to surface of 1 ml conc. H_2SO_4.	Formation of reddish brown ring at the junction of two liquids
Proteins	1. Ninhydrin test 2. Millon's reagent	1. 1 ml extract + few drops of 0.2% Ninhydrin + heated for 5 min 2. 1 ml (extract) + 2 ml Millon's reagent	1. Blue color 2. Reddish brown color
Carbohydrates	Benedict's reagent test	1 ml (extract) + 2 ml Benedict's solution + heated for few seconds.	Formation of reddish brown color
Free amino acids	Ninhydrin test	1 ml (extract) + added few drops of Ninhydrin reagent	Purple, light brown color
Alkaloids	1. Wagner's Test 2. Mayor' Test	2 ml (extracts) + 1 ml (1% HCL) + 0.5 ml Wagner's reagent/ Mayor's reagents.	Creamish, turbidity, pale yellow, reddish brown and dull white precipitate
Terpenoids	Salkowski test	2 ml (extract) + 1 ml Chloroform + 2 ml concentrated H_2S0_4.	Reddish brown, light brown color ring formed at inter face
Steroids/phytosteroids		1 ml of each extract + 1 ml chloroform + few drops of conc. H_2SO_4	Formation of brown ring indicated the presence steroids and blue brown ring for phytosteroids
Phenols	Folin Ciocalteu Test	1 ml (extract) + few drops of diluted Folin Ciocalteu reagent and aqueous sodium carbonate solution, mixture was allowed to stand for 10 min.	Formation of gray or black color
Flavonoids	1. Ethyl acetate/ammonia Test 2. Ferric Chloride Test	1. 2 ml (ethyl acetate extract), heated over a steam bath (40–50℃) for 5 min, filtrate + 1 ml dilute ammonia. 2. 2 ml (extract)+added few drops of $FeCl_3$.	1. Yellow color 2. Blackish red or dark brown color
Quinones		1 ml of each extract +1 ml of conc. H_2SO_4	Formation of red color

Table 2. Antibacterial activity of extracts of medicinal plants against zoonotic bacterial pathogens

Medicinal plant	Part used	Solvent used	Zone of inhibition (M±SD) recorded against pathogens				
			Enterobacter amnigenus	*Serratia odorifera*	*Salmonella Typhimurium*	*Shigella flexneri*	*Pseudomonas aeruginosa*
			Conventional extraction method				
Atropa acuminata	Leaf	Methanol	7.66±1.15**	5.66±1.15*	8.66±2.30**	0.0±0.0	7.33±0.57**
	Rhizome	Methanol	5.66±1.15*	2.66±0.57*	10.66±2.88**	3.66±0.57*	7.33±1.15**
	Leaf	Ethanol	10.66±0.57**	10.33±0.57**	1.66±0.57*	0.66±0.57	7.66±0.57**
	Rhizome	Ethanol	6.66±0.57**	7.66±1.15**	1.0±0.0*	0.66±0.57	5.66±0.57*
	Leaf	Chloroform	0.0±0.0	15.66±3.78***	11.0±0.0**	0.0±0.0	0.0±0.0
	Rhizome	Chloroform	0.0±0.0	12.66±0.57***	0.66±0.57	0.0±0.0	0.0±0.0
			Boiling extraction method				
	Leaf	Methanol	10.33±0.57**	13.66±2.30***	12.0±0.0***	13.0±1.73***	11.33±1.15**
	Rhizome	Methanol	12.33±1.15***	10.33±0.57***	9.66±0.57**	9.0±0.0**	11.0±0.0**
	Leaf	Ethanol	16.0±0.0***	10.0±0.0**	12.33±0.57***	17.33±2.88***	14.33±1.15***
	Rhizome	Ethanol	10.0±0.0**	5.0±0.0*	18.66±0***	13.0±6.92***	11.33±0.57**
	Leaf	Chloroform	0.0±0.0	0.0±0.0	0.0±0.0	12.0±0.0***	0.0±0.0
	Rhizome	Chloroform	0.0±0.0	0.0±0.0	0.0±0.0	15.0±0.0***	0.0±0.0
			Conventional extraction method				
Atropa belladonna	Leaf	Methanol	8.66±1.15**	4.0±0*	8.0±6.92**	1.33±1.52*	9.33±0.57**
	Rhizome	Methanol	8.66±0.57**	2.66±0.57*	0.0±0.0	8.33±0.57**	9.0±1.73**
	Leaf	Ethanol	5.66±0.57*	3.66±0.57*	1.66±2.08*	0.0±0.0	6.66±1.15**
	Rhizome	Ethanol	5.33±0.57*	9.66±2.30**	0.0±0.0	0.0±0.0	6.33±0.57**
	Leaf	Chloroform	0.0±0.0	1.66±0.57*	1.66±2.08*	0.0±0.0	0.0±0.0
	Rhizome	Chloroform	0.0±0.0	8.33±3.21**	2.66±1.52*	0.0±0.0	0.0±0.0
			Boiling extraction method				
	Leaf	Methanol	15.0±0.0***	6.0±1.0**	11.66±0.57**	9.33±2.08**	16.0±0.0***
	Rhizome	Methanol	12.66±0.57***	9.66±0.57**	11.66±0.57**	11.0±1.73**	13.0±0.0***
	Leaf	Ethanol	15.0±0.0***	10.0±0.0**	12.33±0.57***	9±1.73**	15.33±0.57***
	Rhizome	Ethanol	14.66±0.57***	8.33±2.88**	10.66±0.57**	10.33±1.15**	12.66±0.57***
	Leaf	Chloroform	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	Rhizome	Chloroform	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
			Conventional extraction method				
Morus nigra	Fruit	Methanol	9.0±1.0**	0.0±0.0	0.0±0.0	0.0±0.0	7.33±0.57**
		Ethanol	8.66±2.08**	0.0±0.0	0.0±0.0	0.0±0.0	10.0±1.73**
		Chloroform	0.0±0.0	0.33±0.57	6.0±1.0**	0.0±0.0	7.66±0.57**
			Boiling extraction method				
	Fruit	Methanol	14.0±0.0***	18.33±0.57***	14.0±1.73***	14.33±0.57***	14.0±0.0***
		Ethanol	12.0±0.0***	13.66±2.30***	11.66±1.15**	13.0±0.0***	13.33±2.30***
		Chloroform	0.0±0.0	13.0±0.0***	0.0±0.0	8.0±0.0**	10.66±0.57**

*Growth inhibition was expressed as (0) for no sensitivity, *(1- 5 mm) for low sensitivity, **(>5-10 mm) for moderate sensitivity and ***(>10-20 mm) for high sensitivity*

Table 3. Qualitative phytochemical screening of extracts of medicinal plants

Part used	Plant	Solvent used	Free amino acids	Tannins	Alkaloids	Saponins	Terpenoides	Carbohydrates	Quinones	Glycosides	Steroids	Flavonoids	Proteins	Phenols
Fruit	*Morus nigra*	Methanol	++	++	+++	-	+++	+	+	+++	++	+++	++	+++
		Ethanol	+++	+	+++	-	++	+	+++	++	+	++	+	+++
		Chloroform	-	-	+++	-	+++	-	+	+++	-	+	-	++
Leaf		Methanol	+++	+++	+++	+++	-	+++	++	-	++	+++	+	+++
	Atropa Accuminata	Ethanol	+	+++	+++	++	-	++	++	-	+++	+++	+	+++
		Chloroform	-	+++	+++	-	-	+++	++	-	++	+++		+++
Rhizome		Methanol	+++	+	+++	+++	-	-	+	-	+	+	-	+++
		Ethanol	-	+	+++	-	+	-	+	+	-	++	-	+++
		Chloroform	-	+	+	+++	+++	-	+++	+++	++	+	-	++
Leaf		Methanol	+	+++	+++	-	+++	+++	++	+++	++	+++	-	+++
	Atropa bellodonna	Ethanol	+++	+++	+++	-	+	+++	++	+	+++	+++	-	+++
		Chloroform	-	++	+++	-	+++	+++	++	+++	+	+++	-	+++
Rhizome		Methanol	++	++	+++	-	+++	+	++	+++	++	+	-	+++
		Ethanol	-	++	+++	-	+	+	+++	+	+++	+	-	+++
		Chloroform	-	+	+	-	+++	-	+++	+++	++	-		+++

(+++) indicates found in abundant amount, (++) found in mild amount, (+) found in low amount, and (-) indicates not detected

3.6 Total Phenolic and Flavonoid Content

Phenolic contents were higher in ethanolic and DMSO extracts of *A. bellodona* roots (4.33 mg/100 ml and 4.44 mg/100 ml) as compared to leaf extracts whereas flavonoids are more in DMSO extracts of *A. bellodona* leaf (3.323 mg/100 ml). Phenolic contents were found in greater amount in methanol and ethanol extracts of root of *A. accuminata* (5.61 mg/100 ml and 5.45 mg/100 ml) as compared to other extracts. The flavonoid contents were found in chloroform and DMSO leaf extracts of *A. accuminata* (1.810 mg/100 ml and 1.977 mg/100 ml). Presence of phenolic and flavonoids in fruit extracts of *M. nigra*. It was observed that ethanol and acetone extracts of *M. nigra* had high phenolic and flavonoids contents as compared to chloroform and diethyl ether extracts. On the other hand methanol, diethyl ether and chloroform extracts had low contents of phenolic whereas lack flavonoids content.

4. DISCUSSION

Infectious diseases occurred due to presence of etiological agents. Antibiotics have been appreciated to be the most effective drug to treat infectious diseases. However, microbes have becoming resistant to antibiotics [22]. Approximately 6,000 species of higher plants found in Pakistan, out of which 12% are used medicinally [23]. Broad ranges of medicinal plants have been screened recently for antimicrobial and antioxidant activities [24, 25]. In recent study both conventional (macerated) and boiled extracts of three types of plants e.g. *A. belladona, A. acuminata* and *M. nigra* were analyzed. *A. Belladona* plant have the presence of commercially used pharmaceutical bioactive tropane alkaloids like scopolamine and hyoscyamine which are largely used as antagonists of acetylcholine in both central nervous system and due to this reason this plant have greatest concern [26]. In current study Boiling extracts in methanol, and ethanol were more potent against *E. amnigenus* and *P. aeruginosa* than conventional extracts which were moderately to less effective against these pathogens. Our results were similar with Sultana, [27] who also reported that methanolic and ethanolic extracts of *A. belladona* has showed significant antibacterial activity for pathogenic bacteria specifically *B. subtilis* and *S. epidermidis*. Previous literatures indicated the inhibitory action of herbs against fish bacterial pathogens, as an antibacterial agent [28,29]. Munir et al. [30] also reported that ethanolic extracts of *A. belladonna* has more significant antimicrobial activity against *S. aureus* than *E. coli.*

As a traditional herbal medicine, *A. acuminata* was studied for a variety of biological activities [31]. In recent research boiled extracts showed higher activity of *S. odorifera* and also showed maximum activity for all other pathogens. While conventional Leaf and root extracts in chloroform showed higher antibacterial activity against *S. odorifera* with inhibitory zone (15.66±3.78) and (12.66±0.57). Better mann et al., 2001 have reported that the aerial parts of *A. acuminata* have been used in traditional medicine to treat countless diseases e.g. chicken pox, anxiety, asthma and acute infections.

In current study boiling extracts of *M. nigra* were highly active against *E. amnigenus*, *S. odorifera*, *S. flexneri* and *P. aeruginosa* while *S. Typhimurium* was inhibited by methanolic and ethanolic extracts of mulberry than conventional extracts which showed moderate antibacterial activity against *P. aeruginosa* and *E. amnigenus.* Similarly, Khalid et al. [32] also reported that fresh juices of Mulberry showed good antimicrobial activity both for Gram- positive and Gram-negative bacteria. The bark, stem wood and stem of *M. nigra* also showed antibacterial activity against *P. aeruginosa, B.subtilis*, *S. aureus*, *S. faecalis*, *M. flavus* and *S. abony* [32]. The DPPH radical has been accepted model and widely used to evaluate the free radical scavenging ability of various natural products [33].

In recent work methanolic, ethanolic, and chloroform root extracts of *A. belladona* showed significant DPPH free radical scavenging activity than leaf extracts, indicate that that *Atropa belladonna* has a strong antioxidant potential like the other plants of family Solanaceae [30]. The extracts of medicinal plants possibly contain different type of phenolic compounds, which have different antioxidant capacities [34] Foods rich in antioxidant phytochemicals were important for the prevention of diseases related to oxidant stress i.e. heart and cancer [35]. As compared to *A. belladona* no significant DPPH free radical scavenging activity was observed for *A. acuminata,* only methanolic and DMSO extracts of leaf were moderately active while roots showed minimum scavenging activity. Mehmood et al. [36] have reported that *A. acuminata* contains highly oxygenated triterpenes and tropane alkaloids. According to

Jayakanthi et al. [31] ethanolic extracts of *A. acuminata* was active against the damaging effects of free radical produced by acetaminophen induced hepatotoxicity. Nisar et al. [37] reported that ethanolic extract of *A. acuminata* significantly increase the level of antioxidant enzymes such as superoxide dismutases, *glutathione peroxidase and glutathione reductase.* In current research work fruit extracts of *M. nigra* in methanol, ethanol, and chloroform showed higher antioxidant activity. Similar results were also obtained from findings of Arfan et al. [38]. Who reported that *M. nigra* fruits exhibited greater total antioxidant activities than *M. alba* fruits as determined by ABTS, DPPH, and reducing power assays.

In current study both *A. acuminata* and *A. belladona* root and leaf extracts were rich with tannins, alkaloids, quinones, steroids flavonoids and phenols. Our results are consistent with the previous findings [36]. The results of Shalabi et al. [39] supported our findings that carbohydrates, terpenoides and glycosides were highest in *A. belladona than A. acuminata*. Flavonoids are also shown to inhibit microbes which are resistant to antibiotics Linuma et al. [40].

The plant active substances were soluble in organic solvents so plant extracts obtained more activity than commercial antibiotics [41]. Venkatesh and Chauhan, [42] also reported the presence of tannin in *M. nigra*. Nitra et al. [43] reported that black mulberry fruit contains high amounts of total phenolic, total flavonoids, and ascorbic acid content. Bioautographic analysis of TLC develop plates of these four medicinal plants indicated the conspicuous inhibitory zones against *S. flexneri* and *P. aeruginosa*. It indicated that the presence of secondary metabolites showed antibacterial activity against bacterial pathogens. Bioautography procedure enables the evaluation of plant extracts and essential oils against human and plant pathogens [44,45]. Antifungal and antibacterial compounds of the essential oils were observed on TLC plate using direct bioautography [46]. Our results were agreed with the findings of Nitra et al. [43] who reported that black mulberry fruit contains high amounts of total phenolic, total flavonoids, and ascorbic acid content.

5. CONCLUSION

Recently used plant extracts in comparison with standard antibiotics like Streptomycin, Tobramycin, Gentamycin and Ciproflaxin produced smaller inhibitory zones. However, these plant extracts has advantages over the tested antibiotics because microbes have not yet developed resistance against these extracts and contains higher amounts of phytochemicals. A broad research is needed to better evaluate the potential effectiveness of these crude extracts against zoonotic diseases.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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