Phytochemical screening, antioxidant and antibacterial potential of ethanolic extracts of *Cassia, Laccifer and Rosa-damascena*

I. Akbar and **R. Batool**

Department of Microbiology and Molecular Genetics, University of the Punjab, Quaid-e-Azam Campus, 54590, Lahore, Pakistan

Various medicinal plants describe in Unani medicine play a vital role against a range of diseases. Herbs as a whole or their extracts have substantial medicinal value. In this study, ethanolic extracts of three medicinal plants including *Cassia angustifollia Vahl* (Sana kay patay), *Laccifer lacca ken* (Lakh-dana) and *Rosa-damascena* (Gul-e-Surkh) were used to evaluate their phytochemical constituents, antioxidant, and antibacterial activity. Phytochemical examination was carried out for all the extracts as per standard methods. DPPH assay revealed that the antioxidant activity of *Cassia* and *Rosa-damascena* was greater than that of *Laccifer.* Antimicrobial activity of extracts at three different concentrations (100, 75 and 50mg/ml) against Gram positive (*Bacillus*; KC 881030) and Gram negative (*Pseudomonas*; KC 1031) bacteria was determined with absolute ethanol and ethyl acetate as a control. All the plant extracts showed promising zones of inhibition. *Rosa damascena* was selected for further studies and its minimum inhibitory concentration was determined using micro-titter plate assay. The MIC value of *Rosa damascena* was 3.125mg/ml and 1.562mg/ml against gram positive and gram negative bacteria, respectively. In order to perceive the presence of different phytochemicals, the ethyl acetate extract of *Rosa damascena* was subjected to TLC. Appearance of ten different bands on TLC plate indicated the presence of various phytochemicals such as carbohydrates, flavonoids, tannins and terpenoids. Out of these ten, only two spots (1 and 3) showed antibacterial activity with *Rf* value of 0.12 and 0.56. Further study is directed to evaluate the structure analysis of these bioactive compounds so that they can be utilized in pharmaceutical industry.

Keywords: *Rosa-damascena;* phytochemical screening; antioxidant; antimicrobial; TLC

1. Introduction

It is a well-known fact that human beings are using plants for food, medicine, clothing or wood since ancient times [1]. Medicinal plants are in use for centuries for relief from illness. There are two types of medicinal plants. Type 1st includes those plants used by the physicians in developing countries to relieve pain of the native people. These plants are usually used in the form of crude extract. Second type of plants are used by the pharmaceutical companies to get bioactive compounds for the manufacturing of synthetic drugs [2]. The use of herbs as medicine was started several thousand year ago [3]. Although plants are widely used for the curing of several infectious and non-infectious diseases but their exact value is still unknown. World health organization has made efforts to assess this number. It is estimated that about 80% out of 4000 million people use medicinal plants for health care purposes [4].

Conflicting to the common belief, drugs obtained from higher plants occupy an important place in modern medicinal system. About 130 drugs, which were extracted from medicinal plants or further modified, are now prepared synthetically for economic reasons. Medicinal plants are rich in antimicrobial compounds and potent source for many drugs. They are used worldwide for medicinal purpose [5]. The potent components found in plants are phytochemicals [6]. These phytochemicals are the secondary metabolites of the medicinal plants. Their medicinal properties are due to the presence of chemical substance. These substances have physiological action on the biological systems. Some valuable phytochemical constituents are alkaloids, tannins, saponins, glycosides, flavonoids, phosphorus and calcium [7]. Plants do not need these secondary metabolites as they are not required for their primary functioning but are important for the secondary role as they provide health benefits and antimicrobial properties [8]. The demand of novel drug for the treatment of infectious diseases is increasing day by day due to the resistance and side effects of synthetic drugs [9]. Drug developers are focusing on medicinal plants to get bioactive compounds from them which can be used as antibiotics [10].

Considering the importance of medicinal plants, we screened the extracts of *Cassia angustifolia Vahl*, *Laccifer Lacca Kerr*, and *Rosa-damascene*. *Cassia angustifolia Vahl* belongs to the family Fabaceae. It is also known as “Senna” or “Sana k patay” in different regions of the world. Extracts of *Cassia* are used in Unani medicine to treat gastrointestinal infections [11]. *Cassia* is a chief source of important anthraquinone glycosides known as sennosides. These sennosides are extensively use as a laxative in pharmaceutical industry. The laxative effect is attributed to the anthraquinone glycosides. It is also used as antioxidant, antifungal, a febrifuge in splenic enlargements, cholera, jaundice, gout, rheumatism, foul breath, etc. [12].

Laccifer Lacca kerr commonly known as “lakh-dana” and it is widely uses for lowering of blood cholesterol in combination with other medicinal plants. This animal origin drug is use for homeostatic, anti-obesity, anti-inflammatory, stomachic, detergent, kidney tonic, emmenagogues. *Laccifer* is also used for hyperlipidemia, renal, hepatic and spleen disorder, jaundice, backache, cough, asthma, haemoptysis, epilepsy, chicken pox, ulcerations, worm infestation and palpitation [13] *Rosa-damascena* is recognized as “Gul-e-Surkh”, and “Gul-e-Mohammadi”. It is an

ornamental plant and members of the genus Rosa, family Rosaceae [14]. The petals of *Rosa-damascena* have been reported to have blood purifying properties [15]. The flowers of *Rosa-damascena* have astringent, analgesic, anti-inflammatory, antidepressant, antibacterial, diuretic and anti-HIV activity. It is also used as a mild laxative [16]. It contains bioactive compounds such as citronellol, geraniol, nerol, linalool, which are antibacterial in nature [17]. Rosa is used for the treatment of menstrual bleeding, digestive problems, depression, grief, nervous stress, tension, skin problem and headache [18]. Flavonoids present in the petals of *Rosa-damascena* have been reported to strong resistance to UV radiations (254nm). This property of the petals makes to them use in sunblock's creams [19]. Flavonoids also have antioxidative action which can protect the DNA from oxidative damage [20]. These plants are reported to have antibacterial and antioxidant activity, so, we correlate the phytochemical screening with antibacterial and antioxidant activity of the extracts.

2. Materials and methods

2.1 Plant material

The authenticated leaves of *Cassia*, granules of *Laccifer* and flowers of *Rosa-damascena* were collected from Multan, Pakistan. The shade dried material was powdered and stored at room temperature till further use (Table 1).

Table 1 Selected medicinal plants

Plant	Selected Plant	Common Name	Plant Part used
A	*Cassia angustifolia*	Afsanteen	Leaves
B	*Laccifer Lacca kerr*	Lakh dana	Granules
C	*Sphaeranthus indicus*	Gul-e-surkh	Flowers

2.2 Preparation of extract

Fifteen grams of the each plant powder was dissolved in 60ml of absolute Ethanol / Ethyl acetate. After 48 hours, mixture was filtered via Whatman filter paper No. 1. The obtained residues was evaporated to dryness and further processed in rotary evaporator to make it more concentrated. The resultant filtrate was stored at room temperature till further use.

2.3 Phytochemical screening

Following chemical tests were performed to screen the different phytochemical present in all the selected extracts.

2.3.1 Alkaloids

To 1 ml of the extract, added 1 ml of 1% hydrochloric acid (HCl), boiled and 1ml of Wagner's reagent to the mixture. Red precipitates formation indicated the presence of alkaloids [21].

2.3.2 Anthraquinone

Mixed 0.5 ml of the extract with 5ml of chloroform and chloroform layer was pipetted out into another test tube. Equal volume of ammonia was added. Color change of the resulting solution was observed [22].

2.3.3 Carbohydrates

Molish test: To 2 ml of the test extracts, at first few drops of alcohoholic α-naphthol were added. Then, through the sides of test tube few drops of concentrated sulphuric acid were mixed it. Purple to violet color ring appeared at the junction indicated the presence of carbohydrates.

2.3.4 Cardiac glycosides

Keller Kelliani's test: Mixed 1ml of plant extract with 0.5 ml of glacial acetic acid containing one drop of ferric chloride solution and added 0.5 ml of concentrated H_2SO_4. A brown ring at the interference indicated the presence of cardiac glycosides [22].

2.3.5 Flavonoids

Alkaline reagent test: One ml of the extract of selected medicinal plant was taken in a test tube and few drops of NaOH solution were added. An intense yellow color was appeared in the test tube and solution became colorless on addition of a few drops of diluted hydrochloric acid which indicated the presence of flavonoids.

2.3.6 Proteins

Boiled of 3 ml extracts in a water bath. Coagulation of proteins was the indication of presence of proteins [23].

2.3.7 Saponins

Froth formation test: One ml extract of selected medicinal plant was mixed with 1 ml of distilled water in a test tube and placed it in stand by condition for 10 minutes. Formation of stable foam indicated the presence of saponins.

2.3.8 Steroids

One ml of plant extract was mixed with one ml of chloroform and sulphuric acid (H_2SO_4) respectively. Formation of red layer in lower chloroform indicated the positive result [24].

2.3.9 Tannins

Tannins in plant extracts was determined by mixing 1ml of plant extract with few drops of ferric chloride solution. Green precipitation indicated the presence of tannins [24].

2.3.10 Terpenoids

One ml of the extract was mixed with 1ml of chloroform and let it evaporate. Then, concentrated sulphuric acid (1 ml) was added and put the test tube on water bath for two minutes. Grey color of the mixture indicated the presence of terpenoids [24].

2.4 DPPH Assay

The free radical scavenging activity of the extracts were measured in term of hydrogen scavenging ability using the stable radical DPPH (1,1- diphenyl-2-picryl hydrazyl). The stock solution was prepared by dissolving 24g of DPPH reagent in 100 ml of methanol. Working solution was prepared by picking 21 ml from stock and dissolving it into 10 ml of methanol. 100µl of the sample was mixed with 3 ml of the working solution. After incubation of 30 minutes, absorbance of the mixture was taken at 517nm. Decrease absorbance of DPPH working solution indicated the DPPH radical scavenging ability of the antioxidants present in these ethanolic extracts [25]. The percent radical scavenging activity (%RSA) of the extracts was determined

2.5 Antibacterial Activity

The extracts were evaluated for antibacterial activity using agar well diffusion method. It is a qualitative assay which is used to screen the *in-vitro* antibacterial activity of a pure compound or of a crude extract [26]. Three different dilutions (100mg/ml, 75mg/ml and 50mg/ml) of the plant extracts were made in absolute ethanol. Similarly, 100 mg of the extract of selected medicinal plant was dissolved in 1 ml of ethyl acetate. All these dilutions of extracts were subjected to evaluate antibacterial activity of the selected extracts against Gram positive (*Bacillus*; KC 881030) and Gram negative (*Pseudomonas*; KC 1031) bacteria. Absolute ethanol and ethyl acetate were used as a control. The zones of inhibition were measured in millimeters.

2.6 Minimum Inhibitory Concentration (MIC) of *Rosa-damascena*

A micro-dilution technique using 96-well microtiter plate with tetrazolium salt was performed to measure the minimum inhibitory concentration of *Rosa-damascena* extract with five standard antibiotics (Tetracycline, Ampicillin, Streptomycin, Chloramphenicol and Novobiocin). Hundred milligrams of the *Rosa-damascena* extract was dissolved in 1ml of ethanol. Similarly, solutions of five standard antibiotics at concentration 2mg/ml were also made in ethanol. Nutrient broth was prepared, autoclaved and allowed it to cool. Inoculated the 10ml of nutrient broth in a test tube with a loop full of test organisms and incubated it overnight. Next day, Absorbance of the inoculated culture was set 1 at 600nm. 96-well micro-titer plate was opened in laminar flow hood near flame and labeled it. 50µl of nutrient broth was added in all the wells. The 50µl of the plant extract was added in 1^{st} well of the 1^{st} row and serially diluted till 10^{th} well. 50µl from the 10^{th} well was discarded. Then 50µl of the test organism was added up to 11^{th} well. 11^{th} well was positive control and contained 50µl of nutrient broth plus 50µl of test organisms while 12^{th} well was negative control and contained 50µl of nutrient broth only. Same procedure was done with antibiotics and incubated the plate at 37^0C for 24 hours. After incubation 20µl of the tetrazolium salt at the concentration of 0.1g/100ml was added and placed it at room temperature for 20 minutes. Presence of bacterial growth was indicated by the color change (pink color) of the media [27].

2.7 Thin Layer Chromatography (TLC)

Thin layer chromatography is used to separate different components present in the mixture, depending upon their solubility in solvent system being used. Extract of *Rosa- damascena* was spotted on one inch base line marked silica coated aluminum TLC plate (Merck) and allowed for drying. The TLC plate was placed in the tank with chloroform: methanol and few drops of water (5:1: few drops) solvent system. After drying it was stained by spraying it with 10% H_2SO_4 and incubated at 100^0C for 15 minutes. The brown-black spots on TLC plate indicated the presence of various components in the crude extract. TLC plate was also marked under short ultraviolet (254nm) and long ultraviolet light. The component showing UV absorbance and fluorescence were marked and scanned [28].

2.8 Extracting of components from TLC plate and Antibacterial activity of selected spots

Marked spots were scratched from the TLC plate in respective tubes containing ethyl acetate to dissolve the extract components in it. After 24 hours, the extracts from TLC plate were filtered and the filtrate was dried at room temperature.

3. Results

3.1 Phytochemical Screening

Phytochemical screening of the extract of *Cassia angustifolia Vahl* confirmed the presence of alkaloids, carbohydrates, cardiac glycosides, protein, saponins, steroids and tannins. Extract of *Laccifer lacca kerr* was positive for alkaloids, anthraquinones, carbohydrate, cardiac glycoside, phlobatannins, protein, steroids and terpenoids. Phytochemical analysis of the extract of *Rosa-damascena* revealed the presence of alkaloids, anthraquinones, carbohydrate, cardiac glycosides, flavonoids, phlobatannins, saponins, steroids, tannins and terpenoids (Table 2).

3.2 DPPH Assay

Antioxidants are natural or synthetic chemical compounds that hinder the autoxidation, a method by which antioxidants combine with oxygen in the air at room temperature. Antioxidation potential of any medicinal plant extract is the ability of its antioxidant components to scavenge the free radicals in the organisms consequently limiting their activity. Antioxidative activity of the ethanolic extracts of selected medicinal plants was assessed in comparison with DPPH (2, 2-diphenyl 1-1 picrylhydrazyl). All the extracts showed antioxidative ability (23-92%). Maximum antioxidant activity (92%) was showed by *Rosa-damascena* while antioxidant activity of *Cassia angustifolia Vahl* was 81%. Minimum antioxidant activity (23%) was exhibited by extract of *Laccifer lacca kerr* (Table 2).

Table 2: Phytochemical analysis and antioxidant activity of extracts of *Cassia*, *Laccifer* and *Rosa damascena*

Extracts	Phytochemical										Antioxidant Activity	
	Alkaloids	Anthraquinone	Carbohydrates	Cardiac Glycoside	Flavonoids	Protein	Saponins	Steroids	Tannins	Terpenoids	Absorbance at 517nm	(%age)
A	+	+	+	-	-	+	+	+	+	+	0.160	81±0.12
B	+	+	+	+	-	+	-	+	-	+	0.753	23±0.45
C	+	+	+	+	+	+	+	+	+	+	0.140	92±0.32

(+) = indicates presence, (-) = indicates absence. Where, A= Cassia B= Laccifer, C= Rosa

3.3 Antibacterial Activity

Antibacterial activity of *Cassia angustifolia Vahl, Laccifer lacca kerr* and *Rosa-damascena* against gram positive and negative bacteria was determined by agar well diffusion assay with absolute ethanol and ethyl acetate as a control. Ethanolic and ethyl acetate extracts at all selected concentrations inhibited the bacterial growth variedly. Ethanolic extracts showed zones of inhibitions ranging from 17-7mm. Extracts of all the selected plants gave larger zones of inhibitions against gram positive bacteria than gram negative. Maximum zone of inhibition (17mm) was shown by *Rosa-damascena* against *Bacillus* (Table 3). Ethyl acetate extracts also gave zones of inhibition against test organisms ranging from 4 to 14mm (Figure 1).

Table 3 Antibacterial activity of ethanolic extracts against *Bacillus.*

Test Organism	Extracts	Zones of Inhibitions (mm) at three different concentrations (mg/ml)			
		100	***75***	***50***	***Control***
Bacillus	***A***	16±0.50	15±0.15	13±0.35	6±0.40
	B	14±1.30	13±0.30	11±0.81	5±0.21
	C	17±0.78	12±0.83	11±0.41	5±0.37
Pseudomonas	***A***	13±0.04	11±0.64	10±0.30	5±0.38
	B	9.5±0.71	8±0.42	7±0.32	4±0.20
	C	14±0.23	12±0.95	11±0.22	5±0.25

Mean of triplicates, ± standard error of the mean. *Where, A= Cassia, B= Laccifer, C= Rosa*

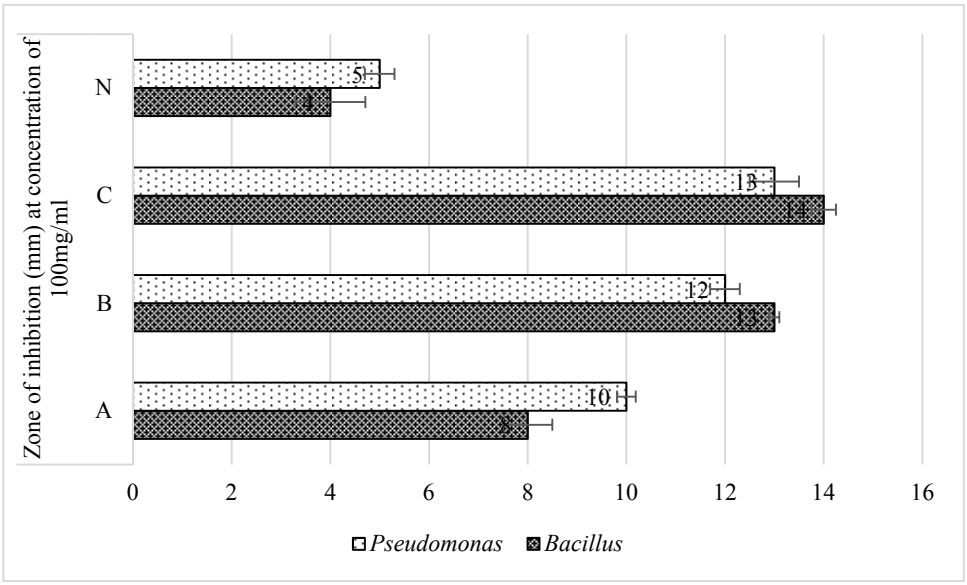

Fig. 1 Antibacterial activity of ethyl acetate extracts against *Bacillus* and *Pseudomonas.* Mean of triplicates, ± standard error of the mean. *Where, A= Cassia, B= Laccifer, C= Rosa* and N= Control

3.4 Minimum Inhibitory Concentration (MIC) Determination

All selected medicinal plant extracts were found to be effective as antimicrobial agents, but due to excellent antioxidant and antibacterial potential *Rosa-damascena* extract was selected for further studies and its minimum inhibitory concentration value was determined. Ethanolic extract of *Rosa-damascena* inhibited the bacterial growth till 3rd well for *Bacillus* and up to 4th well for *Pseudomonas*. Minimum inhibitory concentration values of *Rosa-damascena* extract were compared with MIC values of five standard antibiotics. MIC of *Rosa-damascena* was found to be 3.125 mg /ml and 1.562 mg/ml for *Bacillus* and *Pseudomonas*, respectively (Table 4).

Table 4 MIC values of *Rosa-damascena* extract against *Bacillus* and *Pseudomonas*

Bacterial strain used	Plant	Minimum inhibitory concentration of antibiotics used (mg/ml)				
	A	***B***	***C***	***D***	***E***	***F***
Bacillus	3.125	62.5	250	125	62.5	31.25
Pseudomonas	1.562	125	500	500	250	500

Mean of triplicates, ± standard error of the mean. A= Rosa-damascena (mg/ml), B= Tetracycline; C= Ampicillin; D= Streptomycin; E= Chloramphenicol and F= Novobiocin.

3.5 Thin layer chromatography

Rosa-damascena was selected for the analysis of antibacterial compounds by TLC due to significant antibacterial and antioxidative properties. Multiple spots of *Rosa- damascena* ethyl acetate extract was placed on a TLC plate and was run in chloroform: methanol: water (5:1: few drops). The reason to use ethyl acetate extract instead of ethanolic extract is that ethyl acetate has been reported for dissolving bioactive compounds more efficiently than ethanol. Later on, TLC plate was dried and treated with 10% sulfuric acid to detect the presence of various phytochemicals (Figure 2). From this crude extract, ten spots were selected. These selected constituents were tested for their antibacterial activity. Only spot 1, with *Rf* value of 0.12 and spot 3 with *Rf* value of 0.56, exhibited the bacterial growth inhibition against *Bacillus* with a zone of 1.3mm and 1.1mm, respectively (Figure 3).

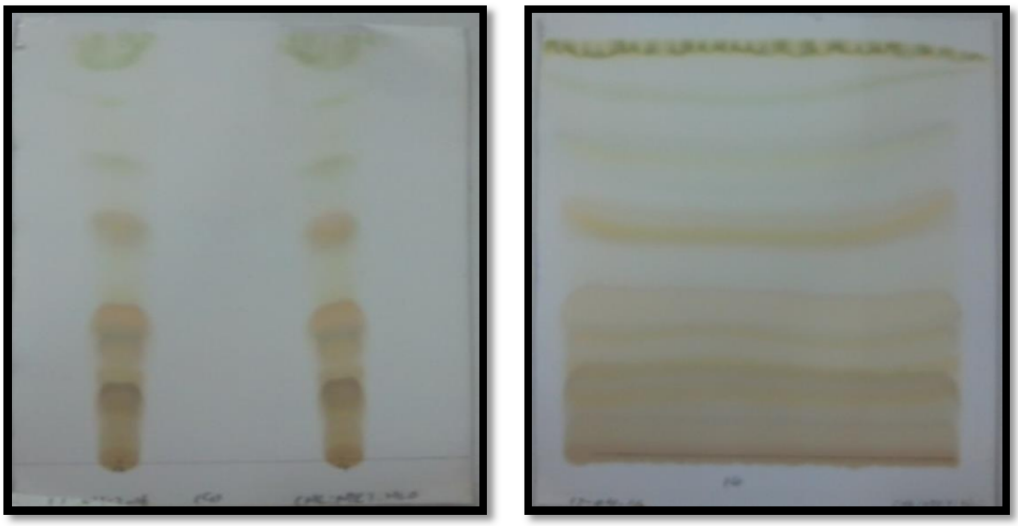

Fig. 2 (Left) TLC plate showing components of extract by developing the plate in chloroform: methanol: water (25:5: few drops) solvent system and H_2SO_4, (Right) without treatment of H_2SO_4.

Fig. 3 Antibacterial activity of *Rosa-damascena* partially purified components. Out of 10 components, only component 1 and 3gave antibacterial activity against *Bacillus.*

4. Discussion

Medicinal plants are prime source of medication in the developing countries [29]. Around 80% of the population of developing countries uses traditional medicinal plants for therapeutic purposes [30]. The natural biological compounds which are derived from medicinal plants are copious source of bioactive compounds. Roughly more than 500, 000 plant species are present worldwide, but only 1% has been investigated phytochemically. That's why; there is great potential for discovering novel bioactive compounds [31].

In this study, *Cassia angustifolia Vahl, Laccifer lacca kerr* and *Rosa-damascena* were selected for the screening of various phytochemicals and to explore the properties subjected to these constituents. These selected medicinal plants have been reported for antimicrobial, antioxidant and several biological activities. Since, several phytochemicals possess antibacterial activity so, the study was conducted to correlate phytochemical screening with antibacterial activity. Ethanol (solvent) was selected for the separation of bioactive components present in medicinal plants. As the extraction process largely depends on solvent being used, ethanol has higher extraction activity as compared to other solvents. It is more efficient in breaking the cell wall and in the release of polyphenols [32]. It can easily penetrate into the cellular membranes to extract the intracellular ingredients from the plant [33]. These crude extracts had different chemical compositions and this difference is due to the fact that different compounds dissolve in different solvents depending upon the polarity of the solvent [34]. Phytochemicals are the secondary metabolites of plants which are usually physiologically active on biological system [35]. These phytochemicals are known to have therapeutic activities like antibacterial, antifungal and antioxidant [19]. The phytochemical tests showed that extracts of *Cassia angustifolia Vahl, Laccifer lacca kerr* and *Rosa-damascena* contained alkaloids, anthraquinone, carbohydrates, cardiac glycosides, steroids, terpenoids and other chemicals being selectively present [19; 23; 36;37]. Reactive oxygen species (ROS)

produce during biochemical reactions are the major source of various chronic and degenerative diseases. These ROS can be blocked by antioxidants which scavenge the free radicals and abolish their damaging effects. Natural antioxidants from plant raise the antioxidant capacity of the plasma and also diminish the risk of cancer, stroke and cardiac arrest etc. [19]. DPPH assay was carried out for the selected medicinal plant extracts to check their free radical scavenging ability. The greater antioxidant potential of the theses extracts can be subjected to the presence of phenolic compounds in the crude extracts as these compounds are potent antioxidants [38].

Encouraging inhibition of the bacterial growth against the tested organisms was observed for ethanolic extract followed by ethyl acetate. The inhibition potential of the ethanolic extract can be attributed to the fact that bioactive compounds required to inhibit the bacterial growth tend to dissolve more in polar solvent than the non-polar ones. So, ethanolic extracts has higher antibacterial activity than the other solvents [39]. The MIC values of the ethanolic extract of *Rosa-damascena* are too high to be active against pathogenic and non-pathogenic bacteria. It is necessary to take into consideration that these values are of crude extract not for pure compounds. A plant extract contains various components all of which are not bioactive ones. Another reason is traditional medicinal practitioners use plant extract solely without combining them with any bioactive compound [40]. The MIC value also varies with the size of inoculum, incubation temperature and duration and method use for determining MIC values [41]. For the separation of different phytochemical components of the extract, thin layer chromatography (TLC) technique was used. TLC confirmed the presence of flavonoids in the extracts [28].

In conclusion, traditional medicinal plants which are used to reduce blood cholesterol level including *Cassia angustifolia Vahl, Laccifer lacca kerr* and *Rosa-damascena* are found to have antibacterial and antioxidant potential. Minimum inhibitory concentration values of *Rosa-damascena* suggests that it is a potent source of natural bioactive components and could be pharmacologically beneficial.

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