

## Antibacterial and Antioxidant Potential of *Ziziphus jujube*, *Fagonia Arabica*, *Mallotus phillipensis* and *Hemidesmus Indicus*

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### ABSTRACT

Phytochemicals present in plants are a major source of imparting different medicinal properties to the plant. Four medicinal plants, i.e., *Ziziphus jujube* Mill., *Fagonia arabica* L., *Mallotus phillipensis* (Lam.) Müll.-Arg. and *Hemidesmus indicus* (L.) Schult were evaluated for their antibacterial and antioxidant potentials. Chemical analysis of ethanol and ethyl acetate extract of these plants revealed the presence of various phytochemicals in them. Antibacterial activity of extracts was measured against a *Bacillus* and a *Pseudomonas* spp. Among all of the extracts, *M. phillipensis* ethyl acetate extract gave maximum zone of inhibition (14mm) against *Bacillus* spp. Minimum inhibitory concentration of *M. phillipensis* ethyl acetate extract was 62.5mg/L. *M. phillipensis* extract was found to exhibit the maximum bacterial efflux pump inhibition potential (155%). Due to these antibacterial properties, twelve components of *M. phillipensis* were separated by TLC. Out of these 12, the component showing antibacterial potential was subjected to GCMS analysis which indicated that phthalic acid was the bioactive component responsible for this activity. Antioxidant potential of all extracts was also estimated by various assays where *M. phillipensis* had maximum potential among all. In conclusion, *M. phillipensis* extract had maximum antibacterial and antioxidant potential. The bioactive components isolated from this plant can further be used in pharmaceutical industries.

**Keywords:** Phytochemical Analysis, Antibacterial Activity, Thin Layer Chromatography, Bacterial Efflux pump inhibition activity, Gas chromatography mass spectrometry.

### 1. INTRODUCTION

Plants are considered to be the most abundant creation of nature that provides protection and nutrition to almost all the living things on earth ranging from bacteria to mammals [1]. Ethnobiology is the field of science dealing with plants and animals that were used in primitive times for the benefit of mankind. It was initially described by Edward F. Castetter in University of Mexico as “utilization of plant and animal life by primitive people” [2]. In Pakistan, there are about 6000 higher plants species present due to wide-ranging climatic zones. Out of all of

these plants, about 12% are used medicinally by local practitioners (Pansare) in the crude form as a drug [3].

Plants are found to have antimicrobial properties due to the bioactive components present in them. This antibacterial potential is due to the secondary metabolites produced in the plants. When plants absorb sunlight, they give off plenty of oxygen and secondary metabolites [4]. Plants usually produce secondary metabolites in response to any danger posed to them by surrounding pathogens or any other external stimuli such as environmental change or nutrition deprivation [5].

Research in ethnobotany has also promoted the study of antioxidants in these plants. These antioxidants are responsible for preventing the cells from damage caused by free radicals [6]. Many of these antioxidants are present

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in our body that are derived from fruits or vegetables consumed by us. Reactive oxygen species are known for causing different illnesses, for instance, cancer, diabetes, arteriosclerosis, arthritis, Alzheimer disease and neurodegenerative disease [7]. Antioxidants react with these oxygen species and scavenge them to save the cells from getting deteriorated [8].

Four different plants were used in the present study for their characterization. First plant used was *Ziziphus jujube* commonly known as Unaab [9]. The *Ziziphus* species is utilized as a part of medication for the cure of a few disorders, for example, diabetes, diarrhea, digestive disorders, fever, insomnia, liver complaints, obesity, skin infections, urinary disorders, and weakness [10]. *Fagonia arabica* commonly known as Dhamasa Booti is a tropical herb [11]. This plant is also known to treat different common problems like boils, thirst, leucoderma, vomiting, typhoid, dysentery and asthma [12]. *Mallotus phillypensis* is generally famous as Kameela. The bark decoction of this plant is well-known for treating diseases like meningitis, diarrhea, dysentery, stomachic effect, worm and typhoid [13]. It is also reported to contain anti-allergic properties and bactericidal properties as well as against *Helicobacter pylori* which is a renowned chemo resistant strain [14]. Ushba, i.e., *Hemidesmus indicus*, is official medicine in Indian and British pharmacopeia. This plant is considered as a tonic, diaphoretic, blood purifier, diuretic and demulcent, i.e., relieving inflammation [15]. It is utilized in nutrition deficiency, syphilis, urinary problems, skin diseases and chronic rheumatism. It is usually used in the powder form or as water extract (decoction) [16].

The objective of this research was to identify the phytochemicals, antibacterial and antioxidative potential of four selected medicinal plant extracts that are known to be useful in treating skin infections. Phytochemicals are actually responsible for governing different properties of the plant. Identification of these phytochemicals and their potential can help the pharmaceutical companies to utilize herbs in their medicine and generate better and novel

medicines for the diseases whose medication is still not available. It will help to escape the bacterial resistance issue and also aid in the production of cheaper products.

## 2. Results

Four plants were analyzed for their phytochemical, antibacterial and antioxidant potentials but the plant i.e. *M. phillypensis* that gave good potential of all of these traits was chosen to be separated into different fractions by TLC and the bioactive fraction (having antibacterial potential) was analyzed by GC-MS analysis.

### 2.1 Phytochemical analysis of selected medicinal plants

To check the presence or absence of different phytochemicals in extracts of *Z. jujube*, *F. arabica*, *M. phillypensis* and *H. indicus*, different biochemical tests were performed. The results of these tests are given in table 2.

### 2.2 Antioxidative potential of selected plant extracts

Antioxidants are the molecules that prevent the oxidation of other molecules. Antioxidant activity of any extract is regarded as the potential of its antioxidant constituents to hunt the oxidants in organism thus restraining their activity. In this study, radical scavenging ability, total phenolic content, phosphomolybdate assay, ferric reducing antioxidant potential and total flavonoid content assay were performed to analyze the ability of plant extracts. All the extracts showed some potential. *M. phillypensis* extract gave maximum potential in most of the assays such as, 69.4% Radical scavenging ability, 120GAE $\mu$ g/ml total phenolic content and 7400RE $\mu$ g/ml total flavonoid content. While, *F. arabica* extract gave maximum ferric reducing antioxidant potential i.e. 747AAE $\mu$ g/ml. phosphomolybdate assay revealed almost similar antioxidant potential of *Z. jujube* (553 AAE $\mu$ g/ml), *F. arabica* (552 AAE $\mu$ g/ml) and *H. indicus* (553 AAE $\mu$ g/ml) extracts. The results of all of these assays are summarized in table 1.

### 2.3 Antibacterial activity

Antibacterial activity is termed as the capability of that

compound to constrain the growth of bacteria under its influence. Antibacterial potential of these extracts was confirmed against a gram positive *Bacillus* strain (JQ013099) and a gram negative *Pseudomonas* strain

(KC881030). All the extracts showed variable inhibition of both the strains maximum inhibition zone was given by *M. phillipensis* extract extract i.e. 11mm for ethanol and 14mm for ethyl acetate extract as shown in table 3, figure 1.

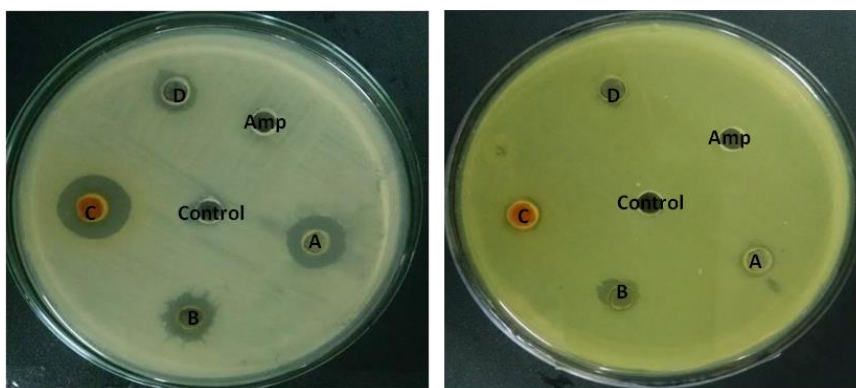
**Table 1: Ferric reducing antioxidant potential, phosphomolybdate assay, total flavonoid and total phenolic content of *Z. jujube*, *F. arabica*, *M. phillipensis* and *H. indicus*.**

Samples		Ferric Reducing Antioxidant Potential (AAE µg/ml)	Phosphomolybdate Assay (AAE µg/ml)	Total Flavonoid Content (RE µg/ml)	Total Phenolic Content (GAE µg/ml)
Ethanol	Control	<b>7.16±5.13</b>	<b>44.13±0.86</b>	<b>8.62±0.28</b>	<b>8.62±0.28</b>
	<i>Z. jujube</i>	677.88±5.54	553.41±0.75	85.86±0.80	85.86±0.80
	<i>F. arabica</i>	747.11±4.67	552.51±0.98	80.33±0.92	80.33±0.92
	<i>M. phillipensis</i>	670.50±3.57	471.75±0.63	120.01±1.21	120.01±1.21
	<i>H. indicus</i>	676.75±3.40	553.05±0.92	46.37±0.69	46.37±0.69
Ethyl Acetate	Control	<b>6.59±2.65</b>	<b>17.44±0.64</b>	<b>2.94±0.11</b>	<b>2.94±0.11</b>
	<i>Z. jujube</i>	206.90±4.33	519.34±0.46	19.37±0.63	19.37±0.63
	<i>F. arabica</i>	524.67±3.40	540.25±0.30	27.24±0.46	27.24±0.46
	<i>M. phillipensis</i>	281.37±4.56	193.22±0.71	15.60±0.51	15.60±0.51
	<i>H. indicus</i>	20.49±4.73	66.12±0.93	9.33±0.17	9.33±0.17

Where, GAE= Gallic acid equivalents, AAE= Ascorbic Acid Equivalents, RE= Rutin Equivalents.

**Table 2: % DPPH radical scavenging potential of *Z. jujube*, *F. arabica*, *M. phillipensis* and *H. indicus* extracts at different concentrations.**

Samples		% DPPH radical scavenging potential of extracts			
		25mg/ml	50mg/ml	100mg/ml	250mg/ml
Ethanol	Control	<b>0.9±0.12</b>	<b>2.4±0.02</b>	<b>4.6±0.98</b>	<b>11.3±1.32</b>
	<i>Z. jujube</i>	5.4±0.09	9.5±0.97	25.1±3.27	52.6±1.44
	<i>F. arabica</i>	4.1±0.21	7.2±0.42	17.6±2.89	35.8±0.75
	<i>M. phillipensis</i>	7.2±0.52	13.5±1.81	28.3±1.74	69.4±1.78
	<i>H. indicus</i>	3.9±0.29	7.5±0.72	15.7±0.82	37.1±2.19
Ethyl Acetate	Control	<b>0.2±0.05</b>	<b>0.7±0.08</b>	<b>1.5±0.03</b>	<b>3.4±0.80</b>
	<i>Z. jujube</i>	1.0±0.23	2.2±0.38	5.1±0.61	12.0±2.42
	<i>F. arabica</i>	2.1±0.71	4.7±0.76	9.8±1.20	23.3±1.78
	<i>M. phillipensis</i>	5.9±0.65	13.4±0.64	25.7±2.32	61.6±2.94
	<i>H. indicus</i>	1.6±0.31	3.3±0.43	6.2±0.51	12.3±1.21



**Figure 1. Antibacterial activity of ethanol extracts against gram positive (left) and gram negative (right) bacteria.**  
 A= *Z. jujube*, B= *F. arabica*, C= *M. phillipensis*, D= *H. indicus*

#### 2.4 Bacterial efflux pump inhibition

Bacterial efflux pumps are the main helpers in imparting resistance to the bacterial cells against the antibacterial agents. Rhodamine is known to promote this activity while reserpine helps to minimize the resistance against antibacterial agents. In this study, bacterial efflux pump inhibition due to the plant extracts was observed. For this, the bacterial efflux pumps were first activated by exposing the cells to Rhodamine and then the inhibition of this resistance mechanism was studied. Reserpine was utilized as a positive control in the current study which showed 30.12% inhibition of the efflux pump. While, *M. phillipensis* ethanol extract was observed to have maximum inhibition potential of efflux pumps i.e. 155%. Results of this assay are mentioned in table 2.

#### 2.5 Minimum inhibitory concentration (MIC) assay

Minimum inhibitory concentration is the concentration of a compound which is minimally required to stop the growth of bacteria completely. *M. phillipensis* extract gave maximum inhibition potential against bacteria. For this reason, MIC of ethanol and Ethyl acetate extracts of *M. phillipensis* were determined against a gram positive and a gram negative strain. *M. phillipensis* ethanol extract resisted the growth of bacteria at minimum concentration of 1.875mg/ml. While for gram negative strain, the minimum concentration to inhibit bacterial growth was 62.5mg/ml.

#### 2.6 Thin layer chromatography (TLC)

Thin layer chromatography is used to separate different compounds present in a mixture depending upon their solubility in the solvent system. *M. phillipensis* (Kameela) was found to have the maximum antibacterial potential. That is why this plant was chosen to analyze the components responsible for its antibacterial potential. Twelve spots from ethanol and seven spots from ethyl acetate extract were separated with the help of TLC.

##### 2.6.1 Antibacterial activity of TLC spots

The compounds separated via TLC were tested for their antibacterial activity independently so that the compound responsible for the activity must be identified. The antibacterial potential of ethanol and ethyl acetate extract spots was tested against a gram-positive strain, and it was found that maximum zone sizes were given by spot 3 ( $R_f$  Value = 0.32) and 4 ( $R_f$  Value = 0.35). Spot 3 gave a diameter of about 3mm while spot 4 gave a 2mm diameter of the inhibition zone against gram positive bacterial test strain.

#### 2.7 GCMS Analysis

GCMS is a chromatographic technique used to identify, separate or quantify the compounds present in a mixture. The purpose of GCMS analysis in this study was to identify bioactive compounds that possessed antibacterial activity in *Mallotus phillipensis* ethyl acetate extract, on the basis of their mass. Analysis of these

compounds showed that spots 3 and 4 both had their maximum peaks at about 31 minutes retention time. Mass

analysis of these compounds revealed them to be phthalic acid in both of the spots as shown in figure 2.

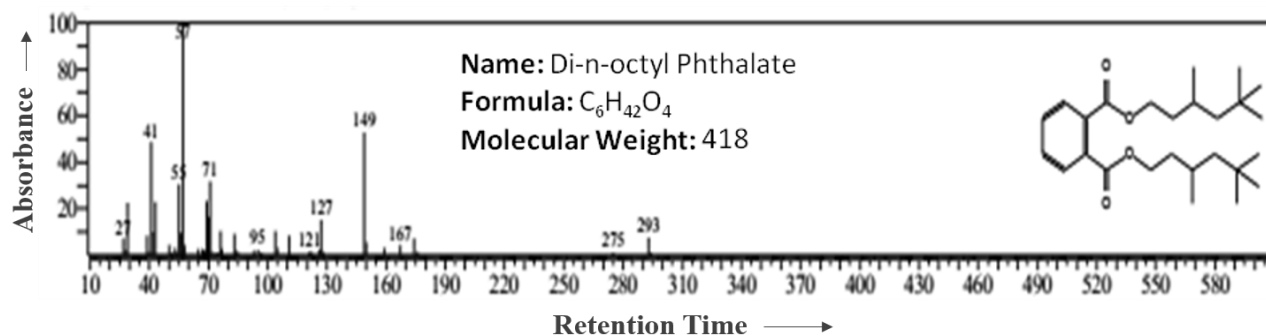


Figure 2: GCMS analysis showing the structure of phthalate isolated from Mallotus philippensis extract.

Table 3: Phytochemical analysis of *Z. jujube*, *F. arabica*, *M. philippensis* and *H. indicus*.

Extracts	Phytochemical Tests										
	Alkaloids	Carbohydrates	Cardiac Glycosides	Flavonoids	Phenols	Phlobatannins	Reducing sugars	Saponins	Steroids	Tannins	Terpenoids
Ethanol	N	-	-	-	-	-	-	-	-	-	-
	A	-	+	+	+	+	-	+	+	+	+
	B	+	+	-	+	-	+	+	+	+	+
	C	+	+	+	-	+	+	+	+	+	+
	D	-	+	+	+	-	-	+	+	+	+
Ethyl Acetate	N	-	-	-	-	-	-	-	-	-	-
	A	-	+	-	-	-	-	-	-	-	-
	B	+	+	+	+	-	+	-	+	-	+
	C	+	+	+	-	-	+	+	+	-	+
	D	-	+	-	+	-	-	-	+	+	+

N= Control, A= *Z. jujube*, B= *F. arabica*, C= *M. philippensis*, D= *H. indicus*

**Table 4: Antibacterial and efflux pump inhibition potential of *Z. jujube*, *F. arabica*, *M. phillipensis* and *H. indicus*.**

Extracts	Antibacterial Activity*		Bacterial efflux pump inhibition% **	
	Gram Positive (mm)	Gram Negative (mm)		
Ethanol	N	<b>01±0.08</b>	<b>00±0.00</b>	<b>18.00±1.15</b>
	A	09±0.12	2.5±0.22	43.21±1.73
	B	07±0.44	02±0.25	75.69±0.57
	C	11±0.59	03±0.12	155.66±2.30
	D	04±0.56	01±0.22	39.40±1.73
Ethyl Acetate	N	<b>08±0.51</b>	<b>01±0.07</b>	<b>5.86±0.57</b>
	A	02±0.35	05±0.03	15.5±2.30
	B	12±0.03	01±0.53	4.06±1.15
	C	14±0.17	02±0.35	5.15±1.73
	D	05±0.28	02±0.48	2.50±2.88

N= Control, A= *Z. jujube*, B= *F. arabica*, C= *M. phillipensis*, D= *H. indicus*

Mean of replicates, ± Standard error of mean

\*Ampicillin (10µg/ml) was used as a standard and all of the extracts were resistant to that antibiotic concentration.

\*\*Reserpine was used as a standard and the %inhibition observed from it was 30.12±1.

### Discussion

Plants are known to be utilized for medicinal purposes for thousands of years [17]. In this study, ethanol and ethyl acetate extracts of some medicinal plants were analyzed for their antibacterial and antioxidant properties. Upon phytochemical detection, ethanol extracts were found to have more phytochemicals in them as compared to the ethyl acetate extracts. This difference in the composition of extracts can be attributed to the fact that different compounds dissolve in different solvents depending upon the polarity of solvent [18]. Ethyl acetate is a solvent that mostly just leaches in to the skin of the plant and removes the chemicals that are present superficially while ethanol is known to rupture the cell membranes and thus extracts the intracellular components of the plants [19].

Carbohydrates, terpenoids and steroids were found in almost each of the plant extracts in both the solvents. Reducing sugars and saponins were present in all of the plant extracts of ethanol while other phytochemicals such as alkaloids, cardiac glycosides, flavonoids, phenols, phlobatanins and tannins were not found in all of the

extracts. This finding was not in accordance to the finding of Fazali and co-researchers, because according to their study alkaloids, flavonoids, terpenoids, phlobatanins, and tannins are present in most of the plants but in our tests these phytochemicals were not found in all of the extracts whereas carbohydrates, terpenoids and steroids were present in almost all of the extracts [20].

According to various studies on phytochemicals, it is reported that they are known to protect the body from diseases that are induced by oxidative stress. This oxidative stress causes the release of different free radicals and other reactive oxygen species in the body, thus causing various harmful diseases. In that case, antioxidants in plants help to scavenge these harmful oxygen species thus reducing the negative effect of oxidative stress related diseases [21].

DPPH free radical scavenging assay was performed on the extracts of selected plants. Ethanol extracts were found to have more antioxidants in them as compared to the ethyl acetate extracts. In both of the solvents *M. phillipensis* extract was having maximum potential. This can be due to

the fact that *M. phillipensis* ethanol extract contained maximum phenolic content in comparison with other extracts. As, according to a research, phenolic compounds act as a free radical terminator thus enhancing the radical scavenging ability of the plant [22].

Extracts in both solvents i.e. ethyl acetate and ethanol were tested for their phenolic content. Ethanol extracts had higher content than ethyl acetate. This relation can be attributed to the fact that ethanol solvent has greater potential to dissolve antioxidants in it [23]. *M. phillipensis* extract showed maximum phenolic content and this observation is in correspondence with the study of Ziaul Haque who has reported the presence of different phenols in *M. phillipensis* plant i.e. mallotophilippinens, bergenin, isorottlerin and rottlerin [24].

Phosphomolybdate assay was done to estimate the total antioxidant capacity of extracts. In ethanol, all the extracts showed almost similar concentration except *M. phillipensis* extract. While in the case of ethyl acetate extracts *Z. jujube* and *F. arabica* showed almost same concentration while *H. indicus* had minimum concentration and *M. phillipensis* had concentration in between these three extracts. According to a previous research there is an inverse relationship between total phenolic content and the antioxidative potential of plant extracts estimated through phosphomolybdenum assay [25]. This previous research is in compliance with current study as, *M. phillipensis* extract showed maximum phenolic content but in phosphomolybdate assay its antioxidative potential was lesser than other extracts.

Ferric reducing antioxidant potential assay was also performed on extracts, just like other antioxidant tests, ethanol extracts showed more potential of ferrous reduction. According to a research conducted by Dudonné et al, there is a noteworthy association among FRAP, total phenolic and DPPH radical scavenging properties. Contrary to Dudonné's research, this relation was not found to be linear in this study [26].

Total flavonoids content of the plants was calculated

by using Aluminum chloride method. When total flavonoids concentration of the extracts was determined, the ethanol extracts showed lesser concentration than the ethyl acetate extracts this observation was similar to the study of Ramammoorthy and Bono who observed greater flavonoid content in ethyl acetate extracts as compared to ethanol extracts [27].

Antibacterial potential of chosen medicinal plants was tested against a gram positive and a gram-negative bacterial strain. All of the extracts showed greater inhibition potential for gram positive strain than gram negative strain. This difference in the antibacterial activity can be due to the fact that gram negative bacteria possess an extra membrane that surrounds the cell wall. This membrane restricts the entry of extracellular compounds from the lipopolysaccharide covering of the cell, thus limiting the potential of chemicals to inhibit bacterial growth. Other than outer membrane, there are some enzymes as well, present in periplasmic membrane that help in breaking down the foreign molecules coming from outside of the cell as a result of which bactericidal effect of chemicals is constrained [28].

Apparently, ethyl acetate extracts showed greater inhibition potential than ethanol extracts. But the zone of inhibition of ethyl acetate control well was 8mm which means if the value of control zone of inhibition is deducted from extract's zone of inhibition, the antibacterial activity due to plant constituent itself would be very low. While in the case of ethanol control well, the zone of inhibition was not much significant which depicted that maximum of the inhibition observed by ethanol extracts was due to the bioactive compounds in them rather than the solvent. As reported by Lou and co-researchers, lesser antibacterial potential of ethyl acetate extracts can be attributed to lower total phenolic content of the ethyl acetate fractions [29]. While, high inhibition capacity of ethanol extracts can be reported due to the better dissolving potential of ethanol [30]. This inhibition of bacterial growth can be due to the presence of flavonoids in plant extracts. Because,

according to a study on antibacterial properties of flavonoids it was reported that flavonoids are a good inhibitor of bacteria. They inhibit the bacterial cell growth by acting on their membranes [31].

Utilizing well diffusion method for antibacterial potential estimation is not always appreciated because this method may show varied results depending upon the penetration power of the constituents. To combat this problem, broth micro-dilution method is a good choice. It gives more detailed results i.e. the quantitative analysis [28]. Tetrazolium salt was used in this study to enhance the bacterial growth sensitivity. As soon as this salt comes in contact with the terminal electron of electron transport chain, it produces color. This terminal electron is liberated only from the electron transport chain being processed by the live cells in the broth. It could be observed in this study that the wells in which concentration of plant constituents was sufficient to inhibit the bacterial growth did not show any change in color. While, the color of broth changed to pink in the wells where phytochemicals were so diluted that they could not produce bactericidal effect [32].

Antibacterial potential of the extracts was also estimated by targeting the efflux pump mechanism of bacteria. Rhodamine dye was used to measure the efflux pump potential of bacteria. In the presence of any efflux pump inhibitor, such as plant extract, the activity of bacterial efflux pumps should be lessened. For this reason, reserpine was used as a standard. Reserpine reduced the efflux of rhodamine dye by 30%. The potential of ethyl acetate extracts was even lesser than the reserpine activity which showed that it was not much active in deactivating the efflux pumps of bacteria. In compliance to this study some researchers also suggest that this activity can be due to lesser potential of ethyl acetate to dissolve phytoconstituents responsible for efflux pump inhibition. While, ethanol being a good solvent gave high activity [33].

Thin layer chromatography of *M. phillipensis* extracts of ethanol and ethyl acetate was performed to separate the different components present in these extracts. When these

TLC plates were developed in respective solvent systems, both of the plates gave a brown band upon treatment with  $H_2SO_4$ . In compliance with a research by Chavan and Amarowicz, dark brown bands demonstrate the presence of sugars in these extracts [34]. While another study demonstrates the presence of different amino acids, if yellowish brown to brown colored bands appear after treatment with ninhydrin spray. These yellowish brown colored bands were observed in both plates indicating the presence of different amino acids [35].

GCMS analysis was carried out to identify the bioactive compounds in *Mallotus phillipensis* that were responsible for imparting antibacterial activity to this plant. The antibacterial activity observed by these spots was lesser than the activity observed when whole plant was subjected to the test. This could be attributed to lesser concentration of the plant components because upon separation by TLC some of the fraction might be lost during scratching or extraction. It could also be due to the synergistic effect of whole plant components as, more than one plant extract's fraction was observed to produce antibacterial effect. Basic component that was identified via GCMS analysis is phthalic acid in both of the spots. Presence of this component in *M. phillipensis* plant has also been reported by other researchers [36].

It can be concluded that, the medicinal plants *Z. jujube*, *F. arabica*, *M. phillipensis*, and *H. indicus* used in this study were found to have antibacterial and antioxidant potential due to the presence of certain phytochemicals in them. *M. phillipensis* was observed to have maximum potential among all extracts. These potential phytochemicals can prove to be pharmacologically beneficial because these phytochemicals can be isolated from crude extracts for manufacturing new synthetic drugs. Future studies should be focused on the detailed analysis of phytochemical constituents of these medicinal plants. Phytochemicals responsible for their medicinal properties should be isolated and modified for pharmacological purpose.



### 3. Methods

#### 3.1 Collection of medicinal plants

Plants known for treating skin infections were purchased from a local store in Lahore, Pakistan. These plants included Unaab (*Ziziphus jujube* Mill.), Dhamasa Booti (*Fagonia arabica* L.), Kameela (*Mallotus philippensis* (Lam.) Müll.-Arg.) and Ushba (*Hemidesmus indicus* L.). These plants were identified by Professor Dr. Sikandar Sultan (Institute of Microbiology and Molecular Genetics, University of the Punjab, Lahore). These plants were analyzed and stored in Institute of microbiology and molecular genetics (University of the Punjab) under the voucher numbers MMG-IM-20, MMG-IM-21, MMG-IM-22 and MMG-IM-23. Whole plant of *F. arabica* and *H. indicus* were used for this study. While, *Z. jujube* flowers and *M. philippensis* seeds were utilized for their analysis.

#### 3.2 Preparation of Plant Extracts

Selected medicinal plants were washed under distilled water, dried and ground into powder form. For the extraction plant to solvent ratio was kept at 1:4. These flasks were left in dark overnight. Next day, these soaked plants were filtered with Whatmann filter paper no. 1 to obtain the extract containing phytochemicals. These extracts were evaporated using Hei-vap-series, Heidolph Germany rotary evaporator. The powdery material obtained after evaporation was stored in dried form and was further diluted as per need.

#### 3.3 Phytochemical screening of selected medicinal plants

The selected medicinal plants were checked for the presence of different phytochemical components in them. The selected medicinal plants were checked for the presence of different phytochemical components in them. Estimation of alkaloids, carbohydrates, cardiac glycosides, flavonoids, phenols, phlobatanins, reducing sugars, saponins, steroids, tannins and terpenoids [37-39].

##### Alkaloids

A mixture of 1ml extract and 1ml 1% HCL was boiled in a water bath. After that, 1ml of Wagner's reagent was

added to the mixture which gave red precipitates indicating positive results.

##### Carbohydrates

In a test tube, 1ml of extract was mixed with 1ml Molisch's Reagent and 1ml conc. sulphuric acid. The mixture was left to stay for few minutes and a red or violet ring at the interface of two layers was observed as a positive result.

##### Cardiac glycosides

Half ml of glacial acetic acid with a drop of ferric chloride was added to 1 ml of extract. To this mixture 0.5ml of conc. sulphuric acid was added. A brown ring development indicated positive result for cardiac glycoside in extracts.

##### Flavonoids

For the estimation of flavonoids in the extract, one ml of extract was mixed with 0.5ml of 20% sodium hydroxide. The mixture turned yellow. These results were cross checked by the addition of dilute HCl which caused the color to fade away.

##### Phenols

Appearance of deep blue or black color when 0.5ml of 5% ferric chloride solution was mixed with 1ml of extract indicated the presence of phenols.

##### Phlobatanins

In a boiling water bath, a test tube containing 1ml extract and 1ml 1% HCl, was placed. The reaction resulted in production of red precipitates which indicated the presence of phlobatanins.

##### Reducing sugars

One milliliter extract was dissolved in 1ml of distilled water in a tube. In another tube, 1ml of Fehling Solution A and B each were added and boiled in water bath. This solution was poured in extract and color change was observed as a positive test indication.

##### Saponins

Saponins presence in extracts was indicated by the stable foam formation when 1ml of extract and 1ml of distilled water were mixed and shaken vigorously.

**Steroids**

One ml of chloroform was mixed with 1ml of extract and 1ml of conc. H<sub>2</sub>SO<sub>4</sub>. Production of red color in the lower layer of chloroform indicated the presence of steroids.

**Tannins**

One milliliter of extract was added in 1ml of distilled water followed by a few drops of ferric chloride solution. Green colored precipitates indicated the existence of tannins in extracts.

**Terpenoids**

One milliliter of extract was mixed in one ml of chloroform and was left to evaporate. One ml of H<sub>2</sub>SO<sub>4</sub> (concentrated) was then added and the mixture was heated for 2 minutes. Formation of grey color indicated the presence of terpenoids in extracts.

**3.4 Antioxidative potential of plant extracts**

Oxygen species produce oxidative stress in the body because number of oxidants increase as compared to antioxidants thus causing harm to biomolecules like proteins, lipids and DNA [24]. Antioxidative properties of plant extracts can be determined by different assays including, DPPH free radical scavenging ability, total phenolic content, phosphomolybdate assay, ferric reducing antioxidant potential and total flavonoids content [40]. In the selected plants, presence of antioxidants was detected by following different assay protocols reported by [41].

**3.4.1 DPPH free radical scavenging ability**

For this assay, a stock solution of DPPH free radical was prepared by adding 24mg of DPPH in 100ml of methanol. From this stock, working solution was prepared by diluting it with methanol until the absorbance of solution became 0.98±0.02 at 517nm. In a test tube, 3ml of this working solution was added along with 100µl of extract and mixed well. These test tubes were placed in dark for 30 minutes. After incubation, the test tubes were taken out and absorbance of solution was taken at 517nm. Percent activity of these extracts was estimated using following formula:

**% Radical scavenging ability =**

$$\frac{\text{Absorbance of Control} - \text{Absorbance of Sample}}{\text{Absorbance of Control}} \times 100$$

**3.4.2 Total phenolic content**

In a test tube 300µl sample was taken and 1ml of respective solvent was further added in it. In that tube, 3.16ml of distilled water was added along with 200µl of Folin Ciocaltaeu reagent and incubated for 8 minutes. After incubation, 600µl of 10% sodium bicarbonate was added and tubes were covered with aluminum foil. Covered tubes were placed in water bath for 30 minutes preset at 40°C. After that, absorbance was taken at 765nm. Same procedure was done for gallic acid standard curve formation for calculating gallic acid equivalent concentration of the phenols in extracts.

**3.4.3 Phosphomolybdate assay for total antioxidant capacity**

Phosphomolybdate reagent was prepared by mixing 28mM sodium phosphate, 4mM ammonium molybdate and 0.6M sulfuric acid. Then 3ml of this reagent was added to 300µl of plant extract and the tubes were covered with aluminum foil. These tubes were incubated for 90 minutes at 95°C. After incubation, absorbance was taken at 765nm. In this method ascorbic acid standard was used to make a standard curve from the concentration of 25mg to 500mg/L. The total antioxidant potential was assessed by this formula: [42]

**Antioxidant capacity (%) =**

$$\frac{\text{Absorbance of Control} - \text{Absorbance of Sample}}{\text{Absorbance of Control}} \times 100$$

**3.4.4 Ferric reducing antioxidant potential**

For this assay, 25ml of 30mM Acetate buffer, 2.5ml of 10mM TPTZ solution and 2.5ml of 20mM Ferric chloride solution were mixed together to make FRAP reagent. Then, 2.85ml of this FRAP reagent was added in a tube containing 150µl of extract. Tubes were put at dark place for 30 minutes and finally optical density was taken at 593nm. Ascorbic acid

was used as a standard and the same process was done for ascorbic acid (50mg- 500mg/L) to make a standard curve. Then the ascorbic acid equivalent (AAE) concentration of extracts was estimated using standard curve.

### 3.4.5 Total flavonoid content

Three hundred microliter of extract and 3.4ml of 30% solvent was mixed in a test tube and 150 $\mu$ l of 0.5M sodium nitrite and 0.3M aluminum chloride each were added in it. The tubes were left at room temperature for 5 minutes. Then, 1ml of 1M sodium hydroxide solution was added and mixed well. Absorbance of these tubes was taken at 506nm. Rutin was used as standard in this test and standard curve was made by using the similar procedure and taking the amount of rutin from 75 to 750mg/L.

### 3.5 Estimation of antibacterial activity of extracts by agar well diffusion method

For this purpose, nutrient broth and Mueller Hinton agar was used. The procedure of bacterial growth and agar well diffusion reported by Dilshad et al was used [43]. Ampicillin (10 $\mu$ g/ml) was used as a standard antibiotic. Ethanol and ethyl acetate were used as negative controls.

### 3.6 Bacterial efflux pump inhibition (EPI)

Efflux pumps are actually the transport proteins which help in transporting toxic substances out of the cell into the extracellular environment [44]. In this study, plant extracts were studied for testing their EPI potential. For this purpose, the method of Sewanu et al was used with minor modifications [45]. Optical density of final supernatant was taken at 527nm and the activity (in percentage) of these extracts was estimated by using following formula.

$$\text{Percent efflux pump inhibition activity} = \frac{1-A_t}{A_o} \times 100$$

Where,  $A_t$  = Absorbance of test

$A_o$  = Absorbance of Control

### 3.7 Minimum inhibitory concentration

Minimum inhibitory concentration estimation is a quantitative assay for determining the minimum extract

concentration that will be able to restrain the bacterial growth [46]. This assay was done by using the method of Klancnik *et al* with some modifications [28]. Penicillin (2 $\mu$ g/ml), erythromycin (2 $\mu$ g/ml), tetracycline (2 $\mu$ g/ml) and chloramphenicol (2 $\mu$ g/ml) were used as standards in this protocol.

### 3.8 Thin layer chromatography

Thin layer chromatography is a method used for separating compounds from extract of any plant [46]. It helps in compound identity, purity and quantification [47]. *M. phillipensis* extract of both solvents were subjected to TLC for identifying the components present in whole extract. Method of Dilshad et al was used here with slight modifications [43]. For ethanol extract, only chloroform was used as a solvent system. While for ethyl acetate extract, chloroform and ethyl acetate were used in combination i.e. 5:1. These developed plates were then observed under high (366nm) and low UV (254nm). The plates were developed in triplicate and treated differently. One plate was treated with sulfuric acid 10% and put in oven for 10 minutes for observing the presence of sugars in extracts. Second plate was treated with ninhydrin solution and heated for observing protein compounds. Third plate was treated with iodine to observe the presence of iodine active compounds.

### 3.9 Antibacterial Activity of Spots

These dried separated components of plants were then subjected to antibacterial activity analysis. For this purpose, disc diffusion assay was conducted. Filter paper discs were autoclaved and put in the tubes containing extract components that were dissolved in a few micro liters of solvent. Mueller Hinton agar plates were spread with bacterial strain. Discs were taken out of tubes and air dried. Dried discs were placed on respective plates and the plates were incubated at 37 $^{\circ}$ C for 24 hours. After incubation period, the plates were analyzed for inhibition zone diameter against bacterial strain. Inhibition zone against bacteria predicted the component responsible for antibacterial activity of that extract.

### 3.10 Gas Chromatography Mass Spectrometry (GCMS)

Gas chromatography mass spectrometry analysis of the spots that had maximum antibacterial activity was done. The protocol of Snageetha et al was followed here [48].

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## مضاد للجراثيم ومضادات الأكسدة المحتملة من زيزيفوس عناب، فاغونيا أرابيكا، مالتوس فيليبينسيس وهيميديسموس إنديكوس

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### ملخص

المواد الكيميائية النباتية الموجودة في النباتات هي مصدر رئيسي لإضافة خصائص طبية مختلفة على النبات. أربعة نباتات طبية، أي زيزيفوس عناب مطحنة، فاغونيا أرابيكا، مالتوس فيليبينسيس (لام.) إم إرمل. - أرح. وهيميديسموس إنديكوس (ل.) تم تقييم شولت لإمكاناتها المضادة للبكتيريا ومضادات الأكسدة. كشف التحليل الكيميائي للإيثانول ومستخلص أسيتات الإيثيل لهذه النباتات عن وجود العديد من المواد الكيميائية النباتية فيها. تم قياس النشاط المضاد للبكتيريا من مقتطفات ضد عصية والزائفة النيابة. من بين جميع المقتطفات، م. فيليبينسيس إيثيل خلات استخراج أعطى أقصى منطقة تثبيط (14 ملليمتر) ضد عصية سب. الحد الأدنى للتركيز المثبط لمستخلص أسيتات الإيثيل فيليبينسيس كان 62.5 ملغم / لتر. تم العثور على مستخلص فيليبينسيس لإظهار أقصى قدر من تثبيط مضخة التدفق البكتيري (155%). بسبب هذه الخصائص المضادة للبكتيريا، تم فصل اثني عشر مكونات م. فيليبينسيس من قبل تلك. من بين هذه 12، تعرض المكون الذي يظهر إمكانات مضادة للبكتيريا لتحليل غمس الذي أشار إلى أن حمض الفثاليك كان المكون النشط بيولوجيا المسؤول عن هذا النشاط. كما تم تقدير إمكانات مضادات الأكسدة لجميع المستخلصات من خلال فحوصات مختلفة حيث كان لدى م. فيليبينسيس أقصى إمكانات بين الجميع. في الختام، لمستخلص م. فيليبينسيس أقصى إمكانات مضادة للبكتيريا ومضادات الأكسدة ويمكن استخدام المكونات النشطة بيولوجيا المعزولة من هذا النبات في الصناعات الدوائية.

**الكلمات الدالة:** التحليل الكيميائي النباتي، النشاط المضاد للبكتيريا، كروماتوغرافيا الطبقة الرقيقة، نشاط تثبيط مضخة التدفق البكتيري، قياس الطيف الكتلي اللوني للغاز.

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