PHYTOCHEMICAL ANALYSIS AND ANTIFUNGAL POTENTIAL EVALUATION OF *Phyllanthus emblica*

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ABSTRACT: The Present research work was conducted to examine phytochemical analysis and antifungal activity of *Phyllanthus emblica*. Extraction was performed through maceration method using different solvents and analyzed for the occurrence of secondary metabolites. Positive results were obtained for Saponins, Tannins, Falvonoids and Cardiac glycosides whereas negative results observed for Alkaloids, Anthraquinones and Phlobotanins. Antioxidant activity of plant extracts was also checked which showed strong results for Antioxidant activity as compared to commercially available standard antioxidants. In total antioxidant assay methanol extract of plant possesses high antioxidant potential showing the value 0.44 ± 0.134^{a} which was closer to the standard BHT (Standard) 0.479 ± 0.24^{a}. In case of DPPH assay chloroform extract value (0.213 ± 0.041) was found closer to BHT (Standard) when compared, whose value is 0.190 Antifungal activity of plant was carried out against fungal strains (*Botrytis cinerea* and *Aspergilus niger*), zones of inhibition were measured and compared with the commercially available standard antifungal discs (Itraconazole and Fluconazole). The highest value against *B. cinerea* was observed for chloroform extracts 20.83 nm, whereas in case of *A. niger* distilled water showed maximum value of 16.5 nm. Based on the results obtained in the present study, *Phyllanthus emblica* fruits are rich in phytochemical constituents and possess potent antifungal activity against pathogenic fungi and can be used in treating various diseases caused by these fungal species.

Keywords: Phytochemical analysis, antioxidant activity, zone of inhibition.

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INTRODUCTION

Herbal drugs have been playing a significant role in treatment of various diseases mostly in rural and remote hilly areas where more than 70% of world's population relies on herbal medicines (Feroz *et al*., 2013). According to WHO 80% of population use traditional medicine (World Health Organization, 2011).

Phytochemicals are bioactive compounds, mostly act in plant defense system against diseases. They are of two types, primary metabolites and secondary metabolites. Chlorophyll, sugars, amino acids and proteins are primary metabolites while phenolic compound, terpenoids, tannins and flavonoids include in category of secondary metabolites. Medicinally important plants have phytochemical constituents which are useful in treating human diseases (Abushouk *et al*., 2017). Only a small number of plants are analyzed phytochemically among 250,000-500,000 plant species, therefore phytochemical screening of plants is necessary to discover the new resources of bioactive compounds can be beneficial to use to cure bacterial and fungal diseases of crops which are economically important because of their antimicrobial properties (Chugh *et al*., 2012).

A significant portion of foodstuffs and agricultural products in world over is destroyed by fungi by producing mycotoxins (Alberts *et al*., 2017). Toxic effects of fungi include genotoxicity, carcinogenicity, nephrotoxicity, hepatotoxicity and reproductive disorders in humans (Walker *et al*., 2018). Human population is affected by mycotoxins present in contaminated grains and cereals particularly in Asia and Africa. Plant extracts of many higher plants contain substances that kill fungi, bacteria and insects (Uddin *et al*., 2017) These plant metabolites can be used as better substitutes because they have minimal environmental impact and fewer side effects (Satish *et al*., 2007). Many compounds isolated from plants have proven antifungal activities i.e. phenols, flavonoids, xanthones, alkaloids, coumarins, quinones, saponins, lectins, polypeptides, terpenoids and essential oils (Arif *et al*., 2009).

Phyllanthus emblica (AmLa) is used in Ayurveda as edible. It is rich in phytochemicals as fixed oils, tannins, glycosides, essential oils, phosphatides and fatty acids. Plant extracts possess several medicinal and pharmacological properties like antioxidant, anti-bacterial, anti-viral (HIV, AIDS, HERPES VIRUS, CMV), anti-inflammatory, antipyretic, anti-allergic, analgesic, chemoprotective and antimutagenic activitesb (Gaire and Subedi, 2015).

In present study, phytochemical analysis and antifungal activity of *Phyllanthus emblica* (AmLa) was carried out, presence of secondary metabolites i.e. Saponins, Tannins, Falvonoids and Cardiac glycosides, Alkaloids, Anthraquinones and Phlobotanins was checked. For Antioxidant analysis two types of assays were used (DPPH radical scavenging assay and total antioxidant assay. Antifungal potential was checked against two strains (*Botrytis cinerea* and *Aspergilus niger*) and results were compared with commercially available standard antifungal discs (Itraconazole and Fluconazole).

MATERIAL AND METHODS

Collection and preservation of plant sample: Plant sample was gathered from dry-salter/local herbalist of Phool Nagar, Punjab, Pakistan. *P emblica* L. fruits were preserved in amber colored jar at 4'C at normal conditions after drying and grinding. 15 g of fruit powder was soaked in 60mL of different series of polar and non-polar solvents such as, (Petroleum ether, chloroform, distilled water and methanol) for 7 days using maceration extraction method.

Figure 1: Phool Nagar Map
(www.google.com)

Phytochemical analysis: Phytochemical analysis was done to identify constitutes qualitatively by using standard procedures of Parekh and Chanda, (2007) and Edeoga *et al*. (2005) described in following:

Alkaloids: Alkaloids are identified by adding 4mL of 1% HCl to 0.25 g of plant powder (fruit) which is warmed at 42ºC and filtered. Then added 6 drops of Mayor's reagent (Prepared by dissolving mixture of mercuric chloride (1.36 g) and of potassium iodide (5.00 g) in 100mL water) to 1 mL filtrate separately. Crème color precipitates identified the existence of alkaloids in the filtrate.

Saponins: Plant powder (0.5g) and 5mL of distilled water were boiled in order to detect saponins. It was then cooled and vigorously shaken for persistent froth formation to identify saponins.

Anthraquinones: Boiling and filtration of 0.5g plant powder and 1% HCL were done in this protocol. Two mL of benzene solution was poured to the filtrate and shaken vigorously. 10% Ammonium hydroxide was added after removal of Benzene layer. Anthraquinones are detected by Pink, violet or red color formation.

Coumarins: To detect the presence of coumarins, 0.5 g of soaked plant powder was taken in a test tube and covered with filter paper which was soaked in 0.1 N

NaOH. Then for few minutes test tube was boiled using water bath. After that filter paper was observed under ultraviolet light. Coumarins were detected by yellow fluorescence in UV light.

Terpenoids (Liebermann-Burchard reaction): 2 mL of plant extract was made in Chloroform. Successive filtration was done after dissolving extract in 2mL of chloroform. Filterate was mixed with equal concentration of acetic acid and 1 drop of concentrated H_2SO_4. Blue-green ring detected terpenoids existence.

Flavonoids: To identify flavonoids, petroleum ether was utilized to wash 5g of plant fruit powder. 20 mL of 80% of ethanol was added to defatted residue to dissolve it and then filtered. Filterate (3mL) and 1% KOH (4mL) was then mixed. Formation of dark yellow color indicated the occurrence of flavonoids.

Tannins: For the detection and presence of tannins, 10 mL of distilled water and 0.25g of plant fruit powder was boiled and then filtration was done. Filtrate was mixed with 1% $FeCl_3$. Tannins were detected due to the occurrence of Brownish green or a blue-black coloration.

Phlobotannins: Plant fruit powder 0.25 g and 5 mL of 1% aqueous hydrochloric acid were mixed and boiled. Deposition of red precipitates indicated the incidence of phlobotannins.

Cardiac glycosides: Plant fruit powder (0.5 g), glacial acetic acid (1 mL) and few drops of 1% $FeCl_3$ were mixed. A layer was formed after addition of 1 mL of concentrated H_2SO_4. Formation of green-blue color showed the occurrence of cardiac glycosides.

Antioxidant activity of plants: To evaluate antioxidant activity of the plant extracts DPPH assay and Total antioxidant assay were performed. The details of the methods are described comprehensively:

DPPH radical scavenging activity: Free radical scavenging activity of plant extract was checked to find out the antioxidant present in plants which offer resistance against oxidative stress by scavenging the free radicals was evaluated by adding stable DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical to prepared extracts by using the methodology of (Erasto *et al*., 2004). Dissolution in extraction solvent dimethyl sulphoxide (DMSO) was done by adding 0.5mg/mL quantity of each of dry extract.

0.2 mg/mL DPPH in DMSO was poured in the test compound in equal volume. It was then well mixed and placed for 30 minutes in dark. Absorbance at 517 nm wavelength was checked for the evaluation of DPPH scavenging activity. The same procedure was conducted for the replicates as well.

DPPH radical scavenging activity of Butyl hydroxyl toluene (BHT) and Alpha tocopherol was comparatively assayed. Three replicates were used for each test and arithmetic means were calculated.

Determination of total antioxidant capacity: All the extracts were analyzed for total antioxidant capacity (Prieto *et al*., 1999). 1.9mL of reagent solution (0.6M sulphuric acid, 4mM ammonium molybdate, and 28Mm sodium phosphate) was combined with 0.1mL of each solution (0.5mg/mL). The reaction mixture was incubated for an hour at 95°C and then set to cool. Absorbance was tested at 695nm in comparable with the blank. The antioxidant activity of Beta hydroxyl toluene (BHT) (0.5mg/mL) was also estimated for contrast using single value.

Antifungal assay

Procurement and culturing of fungal strains *Aspergilus niger* and *Botrytis cinerea:* Selected fungal strains were *A. niger* and *B. cinerea* (Plant pathogen). Test fungal strains (pure cultures) were produced from the FFCB (First Fungal Culture Bank) University of the Punjab, Lahore. The fungal cultures were made on (malt extract agar) medium MEA. Formation of media was done by following the methodology of Johnnsen (1940). The slants were prepared according to the methodology of Qadeer *et al*., (1990).

Antifungal activity of Plant extracts: Antifungal activity of plant extract was done by Agar well diffusion method Farreira *et al*., (1996) and Ortega *et al*., (1996). For comparison, commercially available standard antifungal discs i.e. Itraconazole and Fucanozole were used against *B. cinerea* and *A. niger* respectively.

Statistical analysis: In present study randomized complete block design was used. For statistical analysis Duncan's multiple range tests was applied to check the probability <p> values, using Costat (5%level of significance) CS 6204 W.exe. (Alfanso *et al*., 1985).

RESULTS

Table 1: Phytochemical analysis of *P.emblica* L. fruit extract.

Indication	Alkaloid	Saponin	Anthraquinones	Coumarins	Terpenoids	Flavonoids	Tannins	Phlobotanins	Cardiac glycosides
Cream/ Orange Precipitates	-	-	-	-	-	-	-	-	-
Persistent Froth	-	+++	-	-	-	-	-	-	-
Pink/Red Froth	-	-	-	-	-	-	-	-	-
Yellow Fluorescence	-	-	-	+	-	-	-	-	-
Blue green ring	-	-	-	-	+++	-	-	-	-
Dark yellow color	-	-	-	-	-	++	-	-	-
Brownish green color	-	-	-	-	-	-	-	-	-
Red precipitate	-	-	-	-	-	-	-	-	-
Blue green color	-	-	-	-	-	-	-	-	+++

Total Antioxidant Assay: To check antioxidant activity of the plant, total antioxidant assay was performed. Results showed that methanol extract of plant possesses high antioxidant potential showing the value 0.44 ± 0.134[a] which was closer to the standard BHT (Standard) whose value were 0.479 ± 0.24[a], so, it can be used in place of the commercially available antioxidant.

Table-2: Antioxidant evaluation of various extracts of *P. emblica* Linn. by Total Antioxidant Assay.

Plant Specimens	Absorption at 695 (nm)			
	Petroleum ether	**Chloroform**	**Methanol**	**Distilled water**
E. officinalis	0.236± 0.047[b]	0.323±0.075[ab]	0.44 ± 0.134[a]	0.32±0.052[ab]

Standards	α-Tocopherol: 0.513	BHT: 0.476	Blank: 0.026

Antioxidant assay by DPPH radical scavenging activity: The DPPH radical scavenging activity was also performed to check antioxidant potential of plant quantitatively. Chloroform extract value (0.213 ± 0.041) was found closer to BHT (Standard) when compared, whose value is 0.190 therefore it can be used in place of commercially available antioxidant.

Table-3: Antioxidant evaluation of various extracts of *P. emblica* Linn. by DPPH radical scavenging activity.

Plant Specimens	Absorption at 517 (nm)			
	Petroleum ether	**Chloroform**	**Methanol**	**Distilled water**
E. officinalis	0.615 ± 0.043[a]	0.213 ± 0.041[b]	0.293 ± 0.064[b]	0.739±0.135[a]

Strandards	α-Tocopherol: 0.095	BHT: 0.190	Blank: 0.026

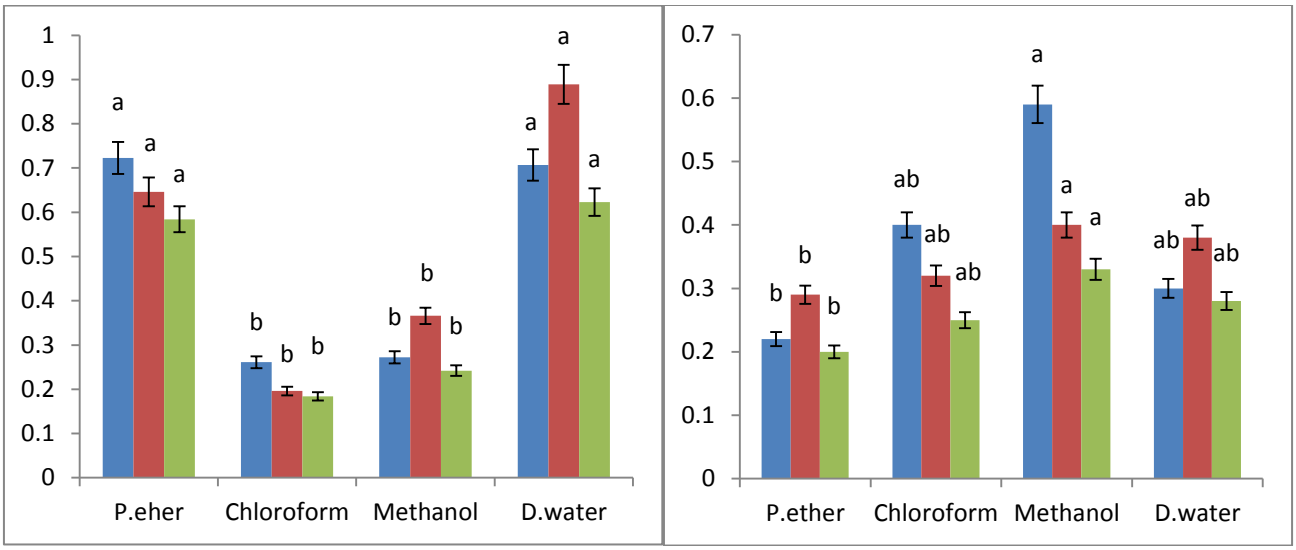


Fig-2: Antioxidant values of *P. emblica* Linn. fruits extract in different solvents by total antioxidant assay(right) and DPPH radical scavenging activity(left).

Antifungal analysis: Antifungal activity of plant extracts was checked using four different solvents against two fungal species (*B. cinerea* and *A. niger*). Zone of inhibition was measured which showed that plant possesses high antifungal activity. The maximum value for the zone of inhibition was shown in chloroform extract against *B. cinerea* i.e. 20.83 ± 1.755[a]. The maximum value against *A. niger* was shown by distilled water extract i.e. 16.5 ± 1.322[a]. Zones of inhibition measured against these strains and the results were compared with the commercially available standard antifungal discs (Itraconazole and Fluconazole).

Table-4: Inhibitory zone (mm) produced by *P. emblica* Linn. fruit extracts against *B. cinerea* and *A. niger.*

Plant extracts	Zone of inhibition (mm) produced against fungal strains	
	B. cinerea	***A. niger***
Pet. ether	18.75 ± 1.767[a]	0 ± 0[c]
Chloroform	20.83 ± 1.755[a]	11 ± 1[b]
Methanol	16.266 ± 1.266[b]	16.133 ± 1.001[a]
D. water	20.166±1.258[a]	16.5 ± 1.322[a]

Each value is an average of three replicates, ± denotes standard deviation among replicates and number followed by different letters at 0.5% level of Significance **(p≤0.05)**

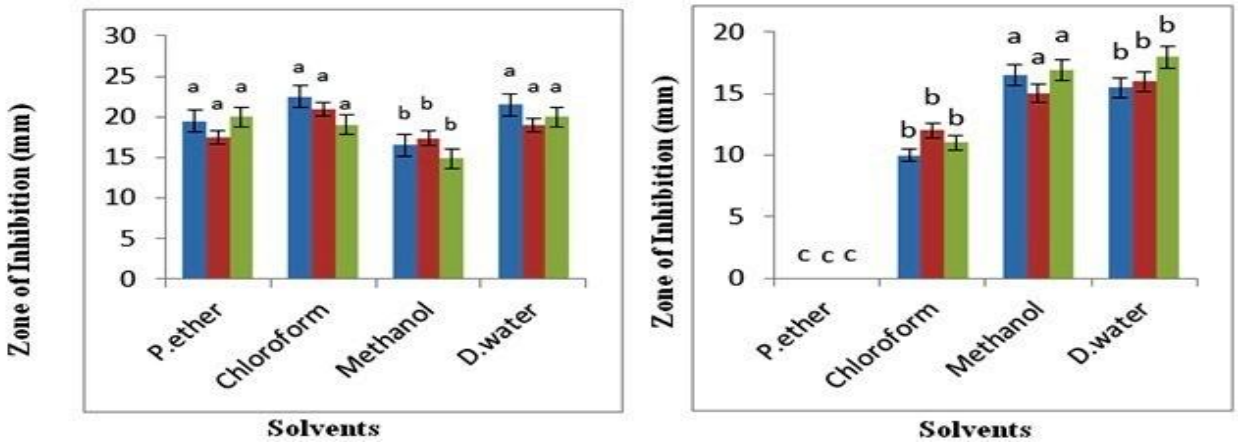


Fig 3: Inhibitory zone (mm) produced by *P. emblica* Linn. fruit extracts in different solvents against *A. niger* (right) and *B. cinerea* (left).

Table-5: Zone of inhibition by various standard discs against Fungi

Strains	Zones of inhibition (mm)	
	Fluconazol	**Itraconazol**
B.cinerea	-	59.5 ± 0.866^{a}
A.niger	28.333 ± 0.577^{b}	-

Each value is an average of three replicates, ± denotes standard deviation among replicates and number followed by different letters at 0.5% level of Significance **($p \leq 0.05$)**

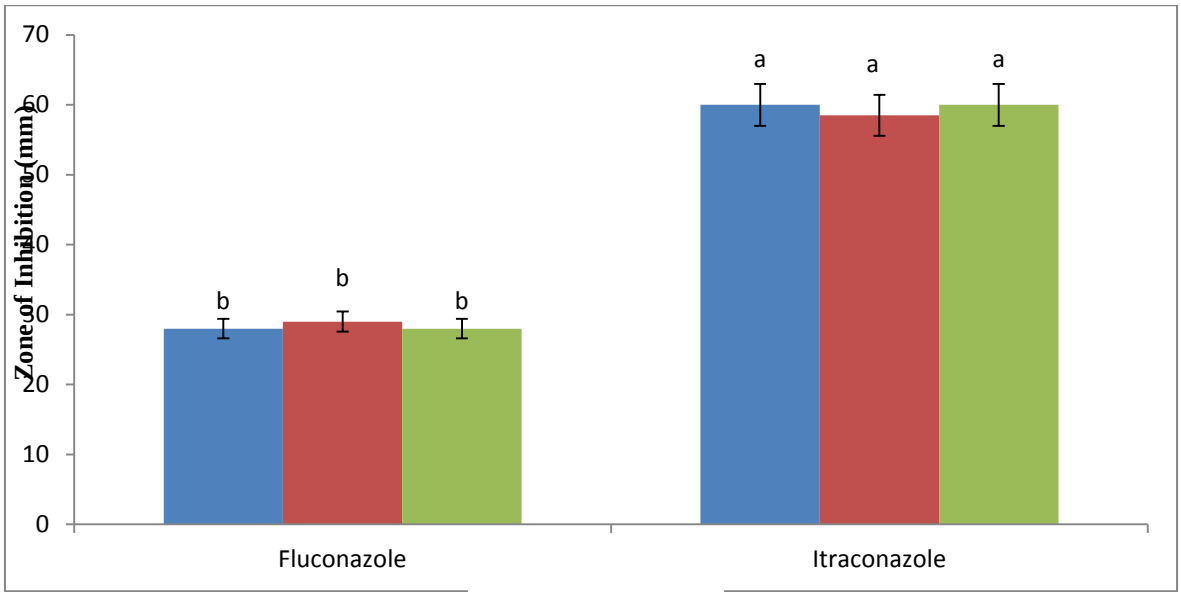


Fig 5: Inhibitory zone (mm) produced by strains against standard discs.

Table 6: Inhibitory zone (mm) shown by various solvents against strains

No. of Obs.	Zone of inhibition (mm)							
	Pet. Ether		**Chloroform**		**Methanol**		**Dist. Water**	
	B. cinerea	***A. iger***	***B. cinerea***	***A. iger***	***B. cinerea***	***A. iger***	***B. cinerea***	***A. iger***
1	2	2	4	5	3	5	2	6
2	4	4	3	7	5	6	5	5
3	2.5	7	3.5	5	4	3	4	8
Mean	3.25 ± 1.06	4.33±2.51	3.5 ± 0.5	5.66±1.15	4 ± 1	4.66±1.52	4.5 ± 0.70	6.33±1.52

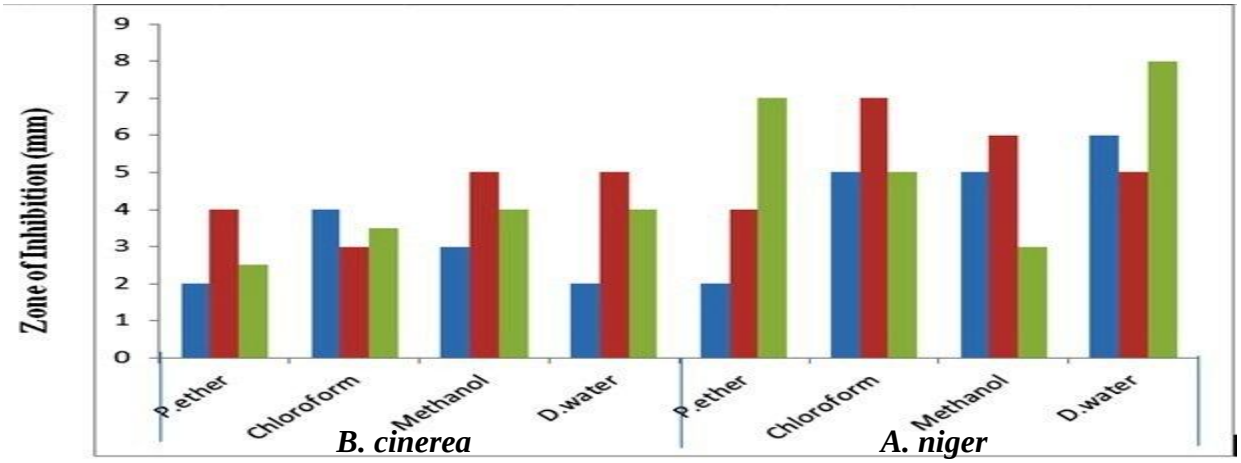
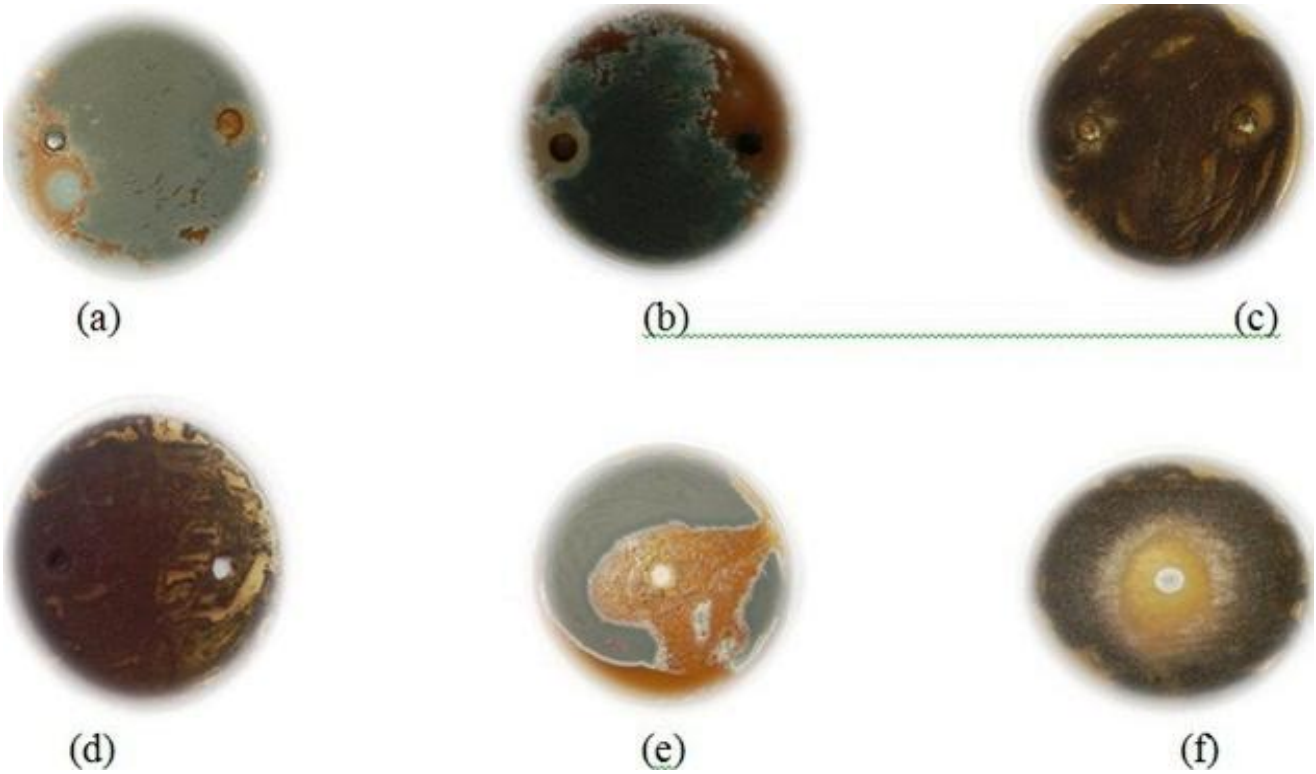


Fig 6: Inhibitory zone (mm) produced by various solvents against strains.



Plates: (a) Antifungal Activity of *P. Emblica* Linn. Fruits against *B. Cinerea* in Chloroform (left) and Methanol (right) Extracts. (b): Antifungal Activity of *P. Emblica* Linn. Fruits against *B. Cinerea* in Petroleum ether (left) and Distilled water (right) Extracts. (c): Antifungal Activity of *P. Emblica* Linn. fruits against *A. niger* in Chloroform (left) and Methanol (right) Extracts. (d): Antifungal Activity of *P. Emblica* Linn. Fruits against *A. niger* in Petroleum ether (left) and Distilled water (right) Extracts. (e): Zone of inhibition shown by *B. niger* against Itraconazole. (f): Zone of inhibition produced by *A. niger* against Fluconazole.

DISCUSSION

In the present investigation *P. emblica* L. fruit extracts showed negative results for alkaloids because orange precipitates were not formed. In other study Phytochemical screening of *Cryptolepis sanguinolenta* (Lindl.), *Morinda lucida* Benth and *Voacanga Africana* Stapt which are antimalarial plant species was done that confirmed the presence of alkaloids in the plant organs. *V. Africana* showed highest contents of alkaloids among

the three plant species (Ameyaw and Duker-Eshun, 2009).Current study showed negative results as compared to literature which showed high alkaloid content.

Production of froth in fruits extracts of *P. emblica* L. showed that saponins were present in plant specimen. In other study *Citrus aurantifolia* (Christm.) *Swingle, C. limon* (L.), *C. paradisii Macf.* (Grapefruit), *C. reticulata Blanco* (Mandarin/Tangerine), C. *grandis Osbeck* (Shaddock/Pummelo), and *C. sinensis* (L.) *Osbeck* (Sweat orange) commonly cultivated in Southern Nigeria were investigated for presence of saponins. Every plant parts of these Citrus species contained saponins (Ezaeabara *et al*., 2014). Hence there was some relation between researches.

In anthraquinones analysis formation of white color froth gave negative results in current study, whereas positive results showed pink, red or violet color froth formation. In ancient red dyes evaluation of the anthraquinones (qualitatively) derivatives was carried out by acid hydrolysis 0.2 to 2.0mg of textile fiber. Important minor constituents, such as kermesic acid in cochineal, were identified due to high sensitivity of the method (Wouters, 2005). The results obtained were contradicts to the previous work.

Phyllanthus emblica L. extracts were found rich on terpenoids In other study selected medicinal plant leaves were washed, dried and ground to fine powder. To find out Phytochemical constituents aqueous extracts of leaf samples were selected. Results showed terpenoids presence in these medicinal plants (Wadood et *al*., 2013).

In present research work a dark yellow colour in the fruits extract of *P. emblica* L. indicated the presence of flavonoids. In past researches to separate and identify flavonoids by using methanolic and ethanolic extract of coriander (*Coriandrum sativum* L.) seeds, Revesred-phase high performance liquid chromatography (RP-HPLC) method with UV/VIS detection was carried out (Rajeshwari and Andallu, 2011). The results were same as the above research work.

In the present investigation indication of blue green coloration in fruits extract of *P. emblica* L. showed that cardiac glycosides were present in respective plant samples. Phytochemicals are dependable sources for the treatment of different health problems. The literature revealed that phytochemicals screening of 20 different medicinal plants, which were collected from different regions of the province Khyber Pakhtunkhwa, Pakistan. In most of the samples all the phytochemicals i.e reducing sugars, glycosides were present (Khan et *al*., 2011). The results of present work were same as obtained by Khan.

In the current activity, Total Antioxidant Assay was used, table 2 showed antioxidant activity of various extracts of *P. emblica* Linn. In different solvents i.e. petroleum ether, chloroform, distilled water and methanol. *P. emblica* L. methanol extract value i.e. 0.44 ± 0.134^{a} is closer to the standard BHT whose value is 0.479 ± 0.24^{a}, So it can be used in place of the standard chemical. Previous studies showed that Antioxidant properties were assessed by total antioxidant assay, free radical scavenging activity, lipid peroxidation assays and metal chelating methods. Free radical scavenging activity was carried out with DPPH (diphenyl picryl hydrazyl) radicals, α-tocopherol and butylated hydroxy toluene (BHT) were used as standard antioxidants to compare results (Singh *et al*., 2010).

The maximum value for the zone of inhibition was observed for chloroform extract against *B. cinerea* that is 20.83 ± 1.755^{a}. The results of the present findings were same as the results of (Saravanan *et al.,* 2012) as they investigated the antimicrobial activity of *C. roseus*. The knowledge of extent and mode of action for antifungal activity of specific compounds, present in the plant extracts, may lead to the successful utilization of such natural compounds for control of pathogenic fungi. The antifungal activity is because of secondary metabolites present in plant extracts such as alkaloids, saponins, flavanoids and others. They investigated that Chloroform extracts of leaves showed maximum activity against fungal and bacterial strains tested.

The present work revealed that in majority of cases minimum value for zone of inhibition is produced by petroleum ether extracts i.e. against the fungal strain *B. cinerea* minimum value of zone of inhibition is shown by Petroleum ether extracts against *A. niger*. (Chengyao *et al*., 2017) tested that petroleum ether extract of *Psoralea corylifolia* seeds. Maximum antifungal activity was recorded in *Aspergillus flavus oryzae* which is 93.5%. The results are antagonistic to the results of present investigation.

The results indicated that plants are antifungal in nature as they have produced some values for the zone of inhibition against fungal strains, while some of them were rather less antifungal. To compare zones of inhibition against fungi like *B.cinerea* and *A.niger*, different antifungal discs were used i.e. Itraconazole and Fluconazole.

Recommendations: Present study revealed that plant is rich in phytochemicals and have antifungal potential which suggest the use of plant in herbal medicines. Further studies can be carried out on isolation, identification and characterization of bioactive compounds of plant.

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