ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Pharmaceutical Properties of *Emblica officinalis* and *Phyllanthus emblica* Extracts.

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ABSTRACT

In the present study, bioactivity of *Emblica officinalis* and *Phyllanthus emblica* leaves and fruit extract with methanol as the solvent was evaluated. Antimicrobial activity against nine clinical pathogens, viz. *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus* sp., *Proteus mirabilis, Shigella dysenteriae*, *Salmonella* sp., *Klebsiella pneumoniae*, *Serratia marcescens* and *Pseudomonas aeruginosa* was determined. The methanol extraction of *Emblica officinalis* and *Phyllanthus emblica* leaves and fruits were performed using Soxhlet apparatus. The antimicrobial analysis showed that the extracts were more effective against Gram negative bacteria than Gram positive bacteria. Antioxidant activity was performed by DPPH assay followed by cytotoxicity against MG63 osteosarcoma cell lines which was found to be profoundly significant. The leaf extract of both *Emblica officinalis* and *Phyllanthus emblica* was proved to be more effective than the leaf extract. The extracts were analyzed through thin layer chromatography (TLC) and maximum R_f value was observed with chloroform:ethyl acetate: acetic acid. GC-MS analysis of extracts was also done to identify the biologically active compounds. Molecular docking studies were performed for the small amla extract compound against bacterial topoisomerases to validate the *in vitro* results.

Keywords: Antimicrobial activity, antioxidant activity, phytochemical analysis, thin layer chromatography, GC-MS.

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ISSN: 0975-8585

INTRODUCTION

Emblica officinalis (*Phyllanthu semblica* Linn.), commonly known as Indian gooseberry or amla belongs to *Euphorbiaceae* family. Amla is native of tropical and subtropical parts of Asia including India, China, and Thailand. The leaves are simple and light green in color closely attached along the branchlets. The fruits are spherical, smooth, and light greenish-yellow in color. It has high content of vitamin C and constitute of phyllemblin, gallic acid, ascorbic acid, tannins etc. [1]. Due to the presence of polyphenol and tannin in *P. emblica* fruit the vitamin C is highly stable [2]. *Emblica officinalis* are useful in treating diabetes, asthma, jaundice, cough, inflammation etc. It is highly valued in Indian traditional medicines [3]. *Emblica officinalis* also has antioxidant, antibacterial, and antimicrobial properties. Antioxidants are the compounds that inhibit oxidation of lipids and other molecules by inhibiting the oxidative chain reaction which can thus help in preventing or repairing the damage caused by the oxygen to the body [4] and protect from damage by free radicals [5]. Bioactive molecules or phytochemicals are found to be useful for treating various diseases. Antibacterial compounds from plants appear to have a potential approach to contain antibiotic resistance and can help in managing disease. The antimicrobial properties of plant can be of great significance in therapeutic treatments. In the present study, methanol extract of leaves and fruit of two species of *Emblica officinalis* was prepared and the extracts were tested for antimicrobial, antioxidant, anticancer studies.

MATERIAL AND METHODS

Sample collection

The healthy leaves and fruits of *Emblica officinalis* and *Phyllanthus emblica* were collected from the VIT University nursery, Vellore, Tamil Nadu, India. The leaves were washed and air dried. The fruits were washed and cut into pieces and air dried. The air dried leaves and fruits were ground into coarse powder and stored in air tight containers for further processing.

Preparation of extracts

The extraction was carried out by Soxhlet extraction method. The finely powdered sample of *Emblica officinalis* and *Phyllanthus emblica* leaves and fruits was extracted in a Soxhlet apparatus using methanol as the solvent system not exceeding its boiling point. The extracts were then filtered using Whatman Filter paper No.1 and dried under reduced pressure using rota-vapour. The dried extracts were stored at 4 ℃ and were further dissolved in 5% dimethyl sulphoxide (DMSO).

Phytochemical analysis

Test for proteins

Xenthoprotein test was done for proteins. Few drops of nitric acid were added along the wall of test tube to the 1ml of extract. Formation of yellow color indicated the presence of proteins.

Test for carbohydrates

About 1ml of Fehling A and Fehling B solution were added to the extract. This was heated for 30 min and observed for the formation of brick red color which confirmed the presence of carbohydrates.

Test for resins

About 5ml of distilled water was added to the extract and observed for the turbidity.

Test for saponins

About 0.1g of sample was mixed with 5ml of distilled water and allowed to boil. Then the mixture was filtered and 2 drops of olive oil was added in 1ml of filtrate. The mixture was shaken and formation of emulsion and froth was observed. The 1ml filtrate was diluted by adding up to 4ml of distilled water. The mixture was shaken vigorously and observed for the stable froth.

Test for flavonoids

The filtrate was prepared by boiling the mixture of 0.5g of sample and 10ml of ethyl acetate for 1 min. Then the mixture was filtered and 4ml of filtrate was shaken with 1ml of 1% ammonium chloride solution. Formation of yellow color in the presence of ammonium solution indicates the presence of flavonoids.

Test for phenols

About 1ml of extract was mixed with 1ml of distilled water and warmed. To this 2ml of ferric chloride solution was added. Formation of green or blue color confirms the presence of phenols.

Test for glycosides

About 0.5ml of extract was taken in test tube and 1ml of glacial acetic acid was added to it containing traces of ferric chloride. To this solution 1ml of concentrated sulfuric acid was added. Formation of reddish brown color was observed in between the two layers. In the presence of glycosides upper layer turned bluish green.

Thin layer chromatography

TLC was performed on precoated silica gel TLC plates to identify the retention factor (R_f). TLC was used to separate the phytochemical components present in the extract. Different ratio of solvents were used at following ratio methanol:chloroform (20:80), toluene:ethylacetate:aceticacid:formic acid (20:45:20:5), chloroform:hexane:acetic acid (50:50:1) and chloroform:ethylacetate:acetic acid (50:50:1). About 0.2 ml of aliquot was applied on TLC plates and immersed inside the solvent system. TLC plates were observed in ultraviolet chamber using 400nm long wavelength. The R_f values were calculated and based on the R_f values the components present in the extract were determined.

GC-MS analysis

The methanolic extract of the fruit and leaf of *Emblica officinalis and Phyllanthus emblica* was analyzed with gas-chromatography mass spectrometry to obtain the number of compounds and molecular weight of the compounds present in extracts. GC-MS analysis was carried out on a GC Clarus 600 Perkin Elmer system comprising of auto sampler and gas chromatograph interfaced to mass spectrometer. The software adopted to handle mass spectra and chromatograms was a Turbo-Mass ver-5.2.

Antimicrobial assay

The methanol extracts of *E. officinalis* and *P. emblica*was were analyzed for antimicrobial activity against the pathogens by agar-well diffusion inhibition test. Muller-Hinton agar plates were prepared and then the clinical pathogens were swabbed onto the plates. Four wells were aseptically punctured by using sterile borer and different concentrations (25µl, 50µl, 75µl and 100µl) of extracts were loaded into the wells. The plates were incubated at 37ºC for 24h and the zone of inhibition was measured around the wells.

Antioxidant assay

The extracts of *E. officinalis* and *P. emblica* were analyzed for free radical scavenging activity by 2, 2-diphenyl-1-picrylhydrazyl (DPPH). Different concentrations of the extracts were prepared and mixed with 0.5 mM DPPH solution. The reaction was allowed to occur at 37°C for 30 min in dark. The reduction of the DPPH radical was determined by measuring the absorption at 517 nm. The radical scavenging activity (RSA) was calculated as a percentage of DPPH discoloration using the equation % RSA = $[(A_{DPPH}-A_S)/A_{DPPH}]\times100$, where A_S is the absorbance of the solution when the sample extract has been added at a particular level, and A_{DPPH} is the absorbance of the DPPH solution[6].

Anticancer activity assay

The methanolic extract of the fruit and leaf of *Emblica officinalis* and *Phyllanthus emblica* was tested for *in vitro* cytotoxicity, using MG63 osteosarcoma cell lines by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay[7]. RMPI 1640 media was used to grow cell lines. These cells were inoculated into the 96-well micro-titer plate in triplicate. Then plant extracts were introduced into the plate. The cell lines were harvested by trypsinization, each sample was replicated 3 times and the cells were incubated at 37°C in 5% humid CO2 incubator for 24 h. MTT was added in each well after the incubation period and the cells were incubated for another 2-4 h until purple colored complex was clearly visible under a microscope. Flowingly, the medium together with MTT (190 µL) were aspirated from the wells, DMSO (100 µL) was added and the plates shaken for 5 min. The absorbance for each well was measured at 540 nm in a micro-titer plate reader and the percentage cell viability (CV) was calculated manually using the formula:

$$CV = \frac{Avg.abs.of\ sample\ wells}{Avg.abs.of\ control\ wells} \times 100\%$$

To determine the changes in cells, the morphological observation of cells treated with extract of the fruit and leaf of *Emblica officinalis* and *Phyllanthus emblica* was done from cytotoxicity study. Changes such as membrane blebbing, formation of apoptotic bodies, shrinking of the cells, chromatin condensation were observed in predicting the apoptotic mechanism for cell death.

Molecular Docking Studies

Since the extracts showed impressive antimicrobial results, molecular docking studies were performed to support the effect of compounds present in the extracts. The docking of the compound with the enzyme of the pathogens results in halting the infective nature of the pathogen. Thus, better zone of inhibition can be explained in terms of better binding energy of the ligand (compound) with the protein (enzyme). In this study, topoisomerases were taken into consideration as the target molecule for the ligand to bind. Depending on the antimicrobial results, extract showing best zone of inhibition was considered for the validation with molecular docking studies. Thus, the docking was performed with the topoisomerases of the susceptible organism with recorded good zone of inhibition was observed.

The crystallographic structures used in the work were modeled using I-TASSER online server for modeling and the PDB files of the ligand were obtained from CORINA INTERACTIVE online server. The active sites of the protein were deduced from FTSite-binding site prediction server. Among all the binding sites being predicted, the first and the best site was used for the docking studies. Ligand-protein docking was performed using AutoDock 4.0 software. The program starts with ligand, detecting the root (rigid set of atoms) of the ligand along with the rotatable group of atoms (branches) are connected to the rigid root. This was followed by setting the total number of torsions possible for the ligand to bind. This program is capable of finding the favorable dockings in protein banking site using the Lamarckian genetics algorithm (LGA) to form a set of possible conformations. The program was also used to prepare protein by omitting water molecules, adding polar hydrogens as well as kollman charges followed by merging the non-polar hydrogens. AutoDock 4.0 was then used to compute the grid parameters where the protein was placed in a grid box size of 60 Å × 60 Å × 60 Å points with a grid spacing of 0.375. The procedure was performed using AutoDock 4.0 with 20 docking runs.

RESULTS AND DISCUSSION

Phytochemical analysis

The different phytochemical tests revealed the presence of carbohydrates, flavonoids, glycosides and phenol in both fruit and leaf extract of *Phyllanthus emblica.* The phytochemical tests with the *Emblica officinalis* fruit extract showed the presence of flavonoids, carbohydrates and saponins whereas with leaf extract of *Emblica officinalis* showed the presence of flavonoids, carbohydrates, glycosides, phenol and resins (Table 1). The phytoconstituents present in *Emblica officinalis* were earlier reported to be present in many medicinal plants also [8,9] which confirms the results of present study. In the present study, the fruit extracts of both *Emblica officinalis* and *Phyllanthus emblica* were proven to be more effective than the leaf extract in the relation of antimicrobial action of phytochemicals from the respective extracts [10].

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Table 1. Antimicrobial activity of methanolic extract from *Emblica officinalis* and *Phyllanthus emblica*.

S. No.	Pathogen name	Zone of inhibition (mm)															
		Emblica officinalis leaves				*Emblica officinalis* fruit				*Phyllanthus emblica* leaves				*Phyllanthus emblica* fruit			
		*25	50	75	100	25	50	75	100	25	50	75	100	25	50	75	100
1	*Staphylococcus aureus*	-	-	-	-	-	-	-	-	22	24	27	29	21	23	25	26
2	*Escherichia coli*	14	18	23	23	17	18	19	26	-	-	-	-	21	22	24	24
3	*Enterococcus* sp.	-	-	-	-	13	18	20	20	23	23	25	25	-	22	24	24
4	*Proteus mirabilis*	-	17	20	25	18	18	20	23	20	22	24	28	20	24	24	25
5	*Shigella dysenteriae*	-	-	-	17	18	21	20	23	15	20	21	24	21	22	23	23
6	*Salmonella* sp.	-	-	-	-	9	13	17	19	-	-	-	-	20	23	25	26
7	*Klebsiella pneumoniae*	11	16	18	20	14	19	22	25	-	-	-	-	18	21	23	25
8	*Serratia marcescens*	12	15	16	17	11	12	14	17	16	20	20	23	19	21	23	26
9	*Pseudomonas aeruginosa*	12	15	16	18	11	21	24	27	22	24	25	28	22	24	26	26

*all concentrations are in µl of 50 mg/ml extract.
'-' means no activity

Thin Layer Chromatography

The phytochemicals determined in the extracts by the phytochemical tests were analyzed by thin layer chromatographic method. The four different solvents were used in different concentration ratios to analyze the presence of bioactive compounds in *Emblica officinalis* and *Phyllanthus emblica* extracts. The major spots were observed on the silica coated TLC plates. The R_f values were calculated and developed spots were recorded for both the leaf and fruit extracts of *Emblica officinalis* and *Phyllanthus emblica.* The maximum R_f value was observed with the solvent system chloroform: ethyl acetate: acetic acid (50:50:1) (Table 2).

Table 2.Phytochemical analysis of *Emblica officinalis* and *Phyllanthus emblica*extract.

S. No.	Phytochemicals	Phytochemical profile			
		Emblica officinalis leaves	*Emblica officinalis* fruit	*Phyllanthus emblica* leaves	*Phyllanthus emblica* fruit
1	Proteins	-ve	-ve	-ve	+ve
2	Carbohydrates	-ve	+ve	+ve	+ve
3	Resins	+ve	-ve	-ve	+ve
4	Saponins	-ve	+ve	-ve	+ve
5	Flavonoids	-ve	-ve	+ve	+ve
6	Glycosides	+ve	-ve	+ve	+ve
7	Phenols	+ve	-ve	+ve	+ve

GC-MS analysis

The GC-MS analysis was very effective in revealing the biologically active compounds present in the extract. The big amla leaves extracts showed the presence of mainly esters and amines as shown in figure 1. and benzene, indole and oxides extract (Figure 2).The GC-MS analysis of the small amla leaves sample revealed biologically active compounds to be alkanes, amide and ketones (Figure 3).The extract of small amla fruit didn't show much activity due to presence of only alkanes (Figure 4).The biologically active components of *E. officinalis* and *P. emblica* methanol extracts resulted in effective antimicrobial activity. The sophisticated analytical techniques identified methyl esters which were also responsible for inhibition activity against pathogenic microorganisms.

ISSN: 0975-8585

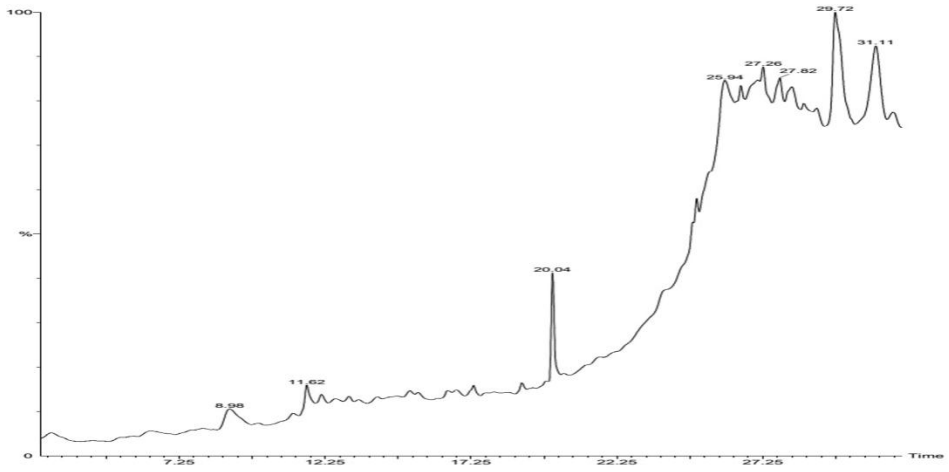

Figure 1: GC-MS analysis of methanolic extract of big amla leaves (BA1).

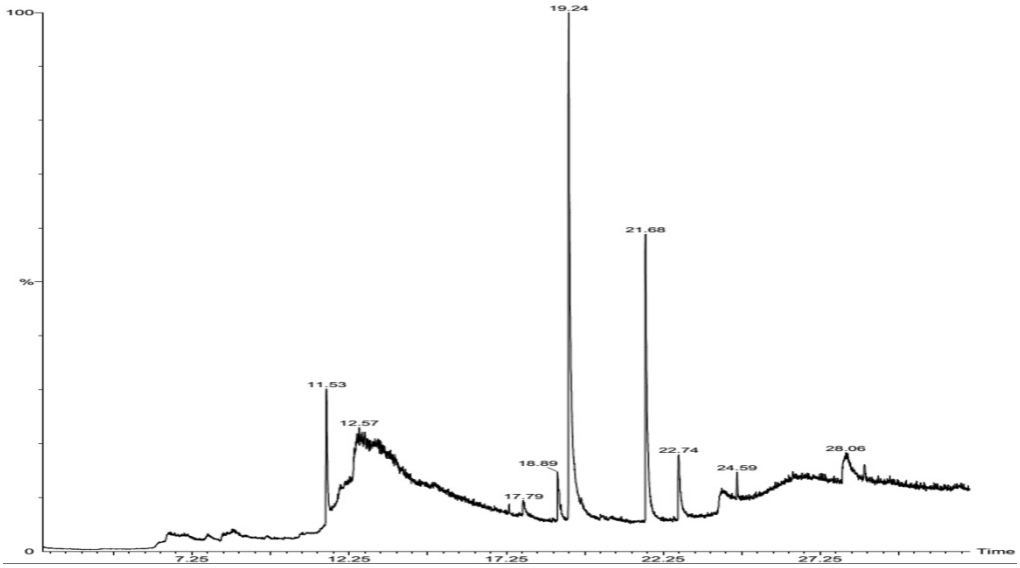

Figure 2: GC-MS analysis of methanolic extract of big amla fruit (BA2).

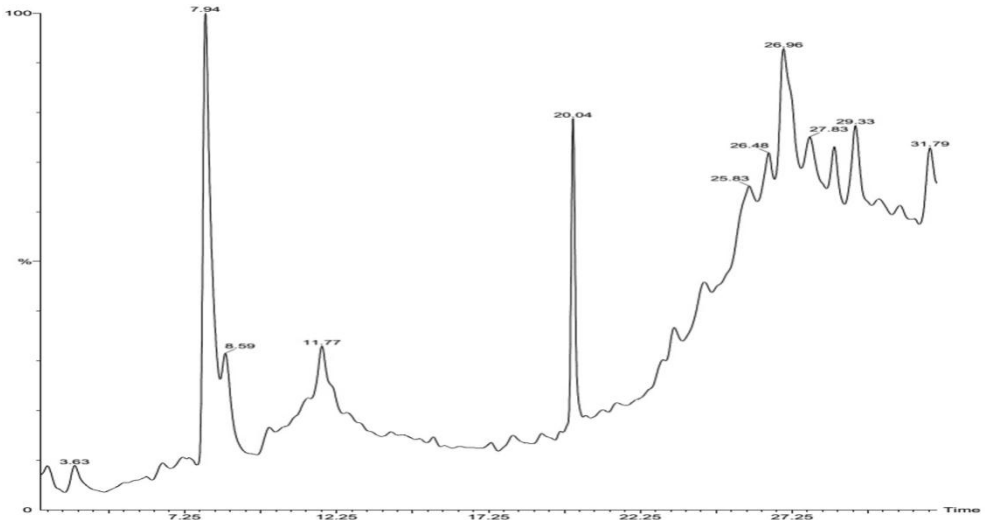

Figure 3: GC-MS analysis of methanolic extract of small amla leaves (SA1).

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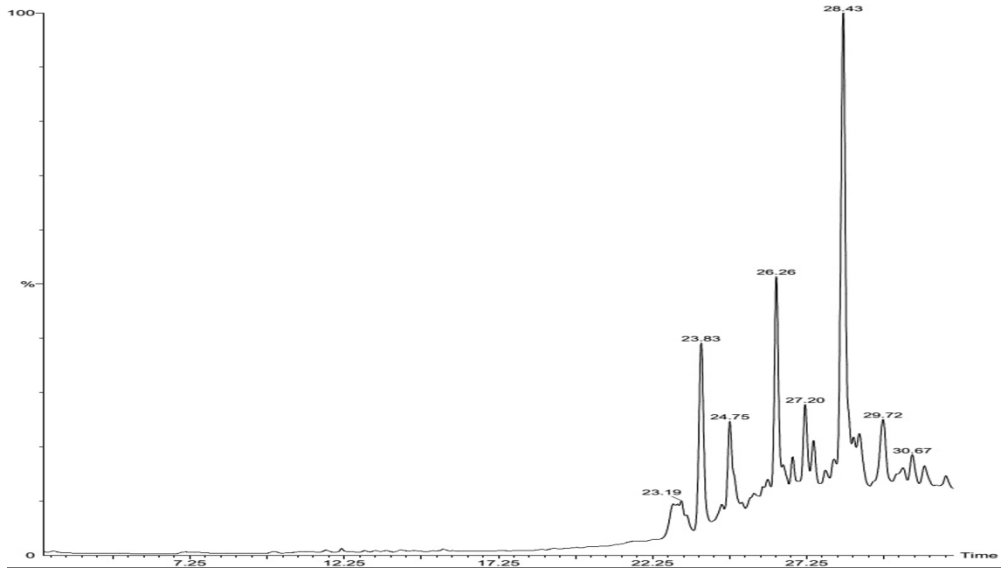

Figure 4: GC-MS analysis of methanolic extract of small amla fruit (SA2).

Antimicrobial activity

The results of the study showed that the leaves and fruit extracts of *Emblica officinalis* and *Phyllanthus emblica* indicates the presence of effective antimicrobial activity, which confirms its use against infection. The analysis of antimicrobial activity was based on measurement of inhibition zones formed around the wells. *Emblica officinalis* leaves extract showed good inhibition effect against *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis* whereas *Emblica officinalis* fruit extract showed good antimicrobial activity against *Proteus mirabilis*, *Shigella dysenteriae*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa. S. aureus* and *Enterococcus* sp. were most sensitive of all the pathogens for *Emblica officinalis* whereas *Escherichia coli*, *Salmonella* sp., *Klebsiella pneumonia* were the most sensitive of all pathogens against leaves extract of *Phyllanthus emblica.* The plant extract showed maximum antimicrobial activity for Gram negative bacteria as compared to Gram positive bacteria [11]. Table 3 summarizes the antimicrobial effect of *Emblica officinalis* and *Phyllanthus emblica* against various pathogens.

Table 3. TLC Analysis of *Emblica officinalis* and *Phyllanthus emblica*extracts.

S. No.	Solvent system	Ratio	Rf values			
			*Emblica officinalis*leaves	*Emblica officinalis*fruit	*Phyllanthus emblica*leaves	*Phyllanthus emblica* fruit
1	Chloroform: hexane: acetic acid	50:50:1	0.73	0.68	0.65	0.72
2	Chloroform: ethyl acetate: acetic acid	50:50:1	0.82	0.77	0.93	0.86
3	Methanol: chloroform	20:80	0.67	0.83	0.88	0.80
4	Toluene: ethyl acetate: acetic acid: formic acid	20:45:20:5	0.73	0.84	0.87	0.78

Antioxidant activity

The antioxidant activity of methanolic extract of *Emblica officinalis* and *Phyllanthus emblica* were determined by using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) which showed a promising radical scavenging ability of DPPH in a concentration dependent manner. A deep purple color with a maximum absorption was exhibited by a freshly prepared DPPH solution and this purple color disappeared in the presence of antioxidant in the mixture. In this method the purple chromogen is reduced to pale yellow hydrazine by the antioxidant compounds in the extract. The number of reduced molecules of DPPH by measuring the decrease in the absorbance at 515-528 nm [12] and free radical scavenging activity was analyzed by the discoloration of the DPPH solution [13]. Fig 5 shows the antioxidant activity measured by DPPH assay.

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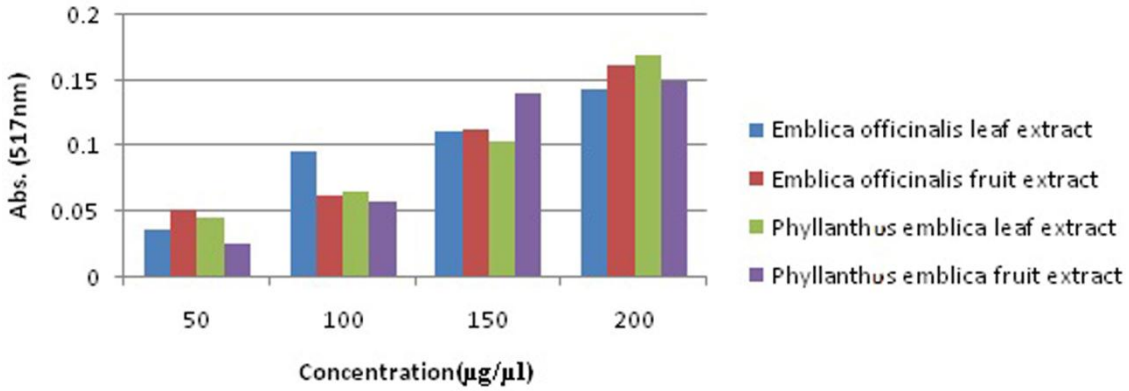

Figure 5: Graph showing antioxidant activity of methanol extract of *Emblica officinalis* and *Phyllanthus emblica* measured by DPPH assay.

Anticancer activity

The anticancer of the extracts of the fruit and leaf of *Emblica officinalis* and *Phyllanthus emblica* on MG63 osteosarcoma cell lines was evaluated through micro-culture tetrazolium assay (MTT). The cell lines and the extract were taken in different concentrations with control. The cell deterioration was observed under phase contrast microscope (Figure 6). There was more death of cell line or cell deterioration observed with increase of concentration of extract. The cells indicated the most prominent effects after exposure to the *E. officinalis* and *P. emblica* extract. The presence of apoptotic bodies could also be seen in the extract treated cells. There was enlargement of the cells at high extract concentration and the cells became to shrink and showed signs of detachment from the surface of the wells denoting cell death. Best activity was observed with small amla leaves extract as there was great amount of deterioration in the MG63 osteosarcoma cell lines. With further purification and processing the small amla leaf extract can be used for anticancer studies.

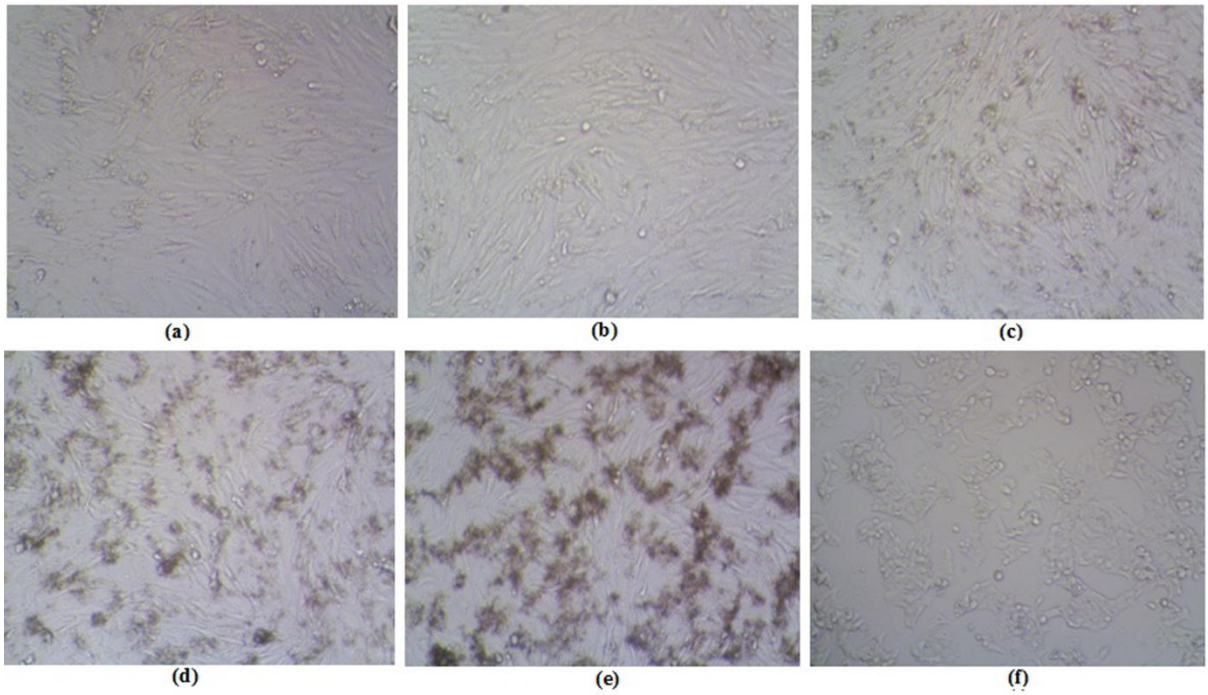

Figure 6: Cell deterioration at different concentrations of samples in µg/ml, (a) 12.5 (b) 25 (c) 50 (d) 100 (e) 200 (f) control, observed under phase contrast microscope.

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Molecular Docking

Based on the antimicrobial results, it was clearly seen that small amla leaf sample was most potent against *Proteus mirabilis* and *Pseudomonas aeruginosa* with a zone of inhibition of 28 mm diameter. The GC-MS results suggests the presence of 1,3-dimethyl-2,4,5-trioxoimidazolidine (DTI) which could be the protein responsible for halting the pathogenesis of the clinical pathogens. To prove this the docking studies were performed between the protein and the ligand. The TORSDOF was used in calculating the change in free energy caused by the loss of torsional degrees of freedom upon binding. It is the total number of possible torsions in the ligand minus the number of torsions that can only rotate hydrogens. The result of docking studies shows that DTI binds to the topoisomerase of *Proteus mirabilis* at LEU239 position with a binding energy of -3.92 kcal/mol indicating that the active compound is efficient having strong antibacterial activity. The 2D and 3D structure of the docking result of *Proteus mirabilis* topoisomerase with ligand is depicted in figure 7. The bond length was found to be 3.06 Å between LEU239 of the protein and O4 (oxygen at fourth position) of the ligand. Similarly, *Pseudomonas aeruginosa* topoisomerases was found to form 2 bonds with the ligand with bond length of 2.83 and 2.84 Å (Figure 8). The oxygen at second position (O2) of the ligand formed bonds with GLN267 and VAL268 respectively and the binding energy was deduced to be -3.76 kcal/mol. This amount of binding energy shows prominent docking between the protein and the ligand indicating good antibacterial effects against the pathogen.

Figure 7: (a) 3D docked complex of *Proteus mirabilis* topoisomerase with ligand (DTI) (b) 2D Ligplot of the complex.

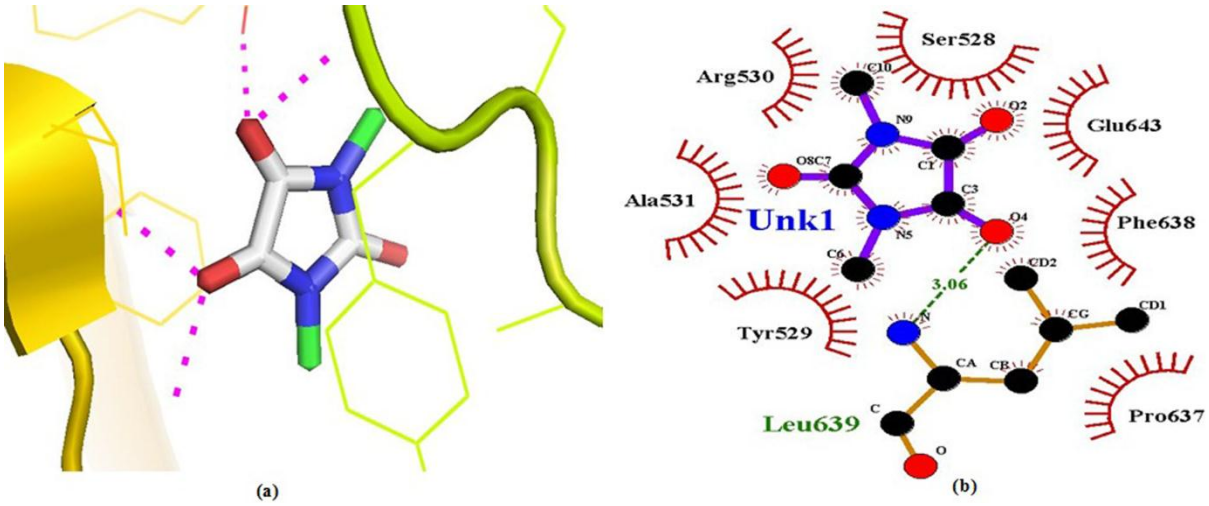
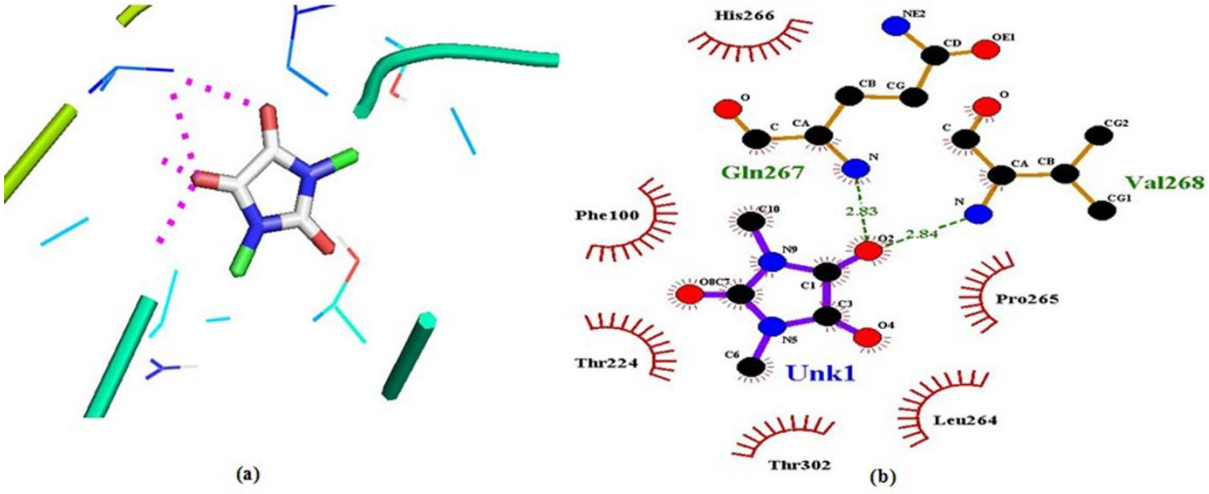


Figure 8: (a) 3D docked complex of *Pseudomonas aeruginosa*topoisomerase with ligand (DTI) (b) 2D Ligplot of the complex.



ISSN: 0975-8585

CONCLUSION

The present study confirmed that the extracts of *Emblica officinalis* and *Phyllanthus emblica* have great potential for antimicrobial activity against various clinical pathogens. They also possess good antioxidant activity. The phytochemical analysis revealed that the fruit extracts of both *Emblica officinalis* and *Phyllanthus emblica* were proven to be more effective than the leaf extract against antimicrobial activity. Furthermore, molecular docking studies confirmed the effectiveness of small amla leaf extracts which showed the maximum antibacterial effects. Thus, it can be further considered as an effective drug against clinical pathogens.

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