SCREENING OF PETROLEUM ETHER AND METHANOL EXTRACTS OF *JATROPHAPODAGRICA* LEAVES FOR ANTIBACTERIAL ACTIVITY WITH SUPPORTING *IN SILICO* STUDIES

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ABSTRACT

OBJECTIVE

*The aim of this study was to screen the antibacterial activities of methanol and petroleum ether extracts of *Jatropha podagrica* leaves.*

METHODS

*The leaves were collected, dried, ground and extracted using Soxhlet apparatus with methanol and petroleum ether as solvents. The plant extracts were tested for antibacterial activity against bacterial pathogens using the well diffusion method viz. *Escherichia coli*, *Klebsiella sp.*, *Pseudomonas sp.*, *Bacillus sp.*, *Proteus sp.* Further, Thin layer chromatography (TLC) analysis was done for the methanol and petroleum ether extracts. GC-MS analysis was also conducted to gain the knowledge of the compounds that present in the extracts which can be responsible for the antimicrobial potential of the extracts. Furthermore to validate the antibacterial results molecular docking studies were performed using Auto Dock 4.0 followed by interpretation of the results with PyMol and Ligplot plus softwares.*

RESULTS

The results obtained suggested that these extracts have significant antibacterial properties and can be used to make herbal applications.

KEYWORDS: Antimicrobial, GC-MS, Molecular Docking & AutoDock 4.0

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INTRODUCTION

Jatropha podagrica (family- Euphorbiaceae) is also known by various names such as Buddha belly plant, bottle plant shrub, gout plant etc. The plant contains the toxic curcin, making it poisonous. It is also known for its incredible ability to attract a variety of butterflies wherever it is grown. The need of the hour is to have natural antibacterial preparations by screening of various plant parts. The stem bark of *Jatropha podagrica* Hook is said to have a several secondary metabolites (Papalia et al., 2017). Also acetone extract of the roots exhibited an MIC of 0.39 mg/ml in the work done by Mongalo et al. (Aiyelaagbe 2011). Aiyelaagbe et al. (2018) tested the extracts from *Jatropha curcas* for cytotoxicity activity and they were highly active against L5178y mouse lymphoma cells and HeLa human cervix carcinoma cells, showing it as a potent anticancer drug.

Jatropha species are used in a traditional medicine for various diseases including skin infections, sexually transmitted diseases such as gonorrhoea, jaundice and fever. Different parts of the plant are also used for

antipyretic, diuretic, choleric and purgative effects (Mahesh and Satish 2008). Various medicinal and pesticidal properties, including antimicrobial, antitumour and insect antifeedant activities, have also been attributed to this plant (Basaran and Gorkem 2012; Sharma 2012). Thus, it can be a potent natural source of remediation against several pathogenic bacteria. So this study aimed at investigating the properties of leaves of this plant. Though there are varying degree of work conducted about medicinal properties of *Jatropha* sp., bioinformatics studies are unexploited. Keeping this as a main focus, following investigation has been carried out.

MATERIALS AND METHODS

Collection of Plant Material

The leaves of *Jatrophapodagrica* were collected in sterile plastic bags from the nursery of VIT University, Vellore campus. Immediately sample was brought to the lab for processing. Further they were washed with tap water followed by sterile distilled water and then kept for drying at a room temperature. Dried leaves were coarsely powdered using mixer grinder and now the sample was ready for extraction.

Preparation of the Methanol and Petroleum Extracts

Completely dried and powdered leaf sample, about 25 g (Upadhyay et al., 2010) was packed in a Soxhlet apparatus and extracted with methanol followed by petroleum ether (300 ml each) as the solvent system. The obtained extracts were vacuum dried and kept at 4 °C for further analysis. Two extracts were obtained at the end of the extraction.

TLC Analysis

Thin layer chromatography was performed on a sheet of aluminium foil, which was coated with a thin layer of adsorbent material i.e. aluminium oxide (Nema et al., 2012). The composition of the developing solvent used for methanol extracts was toluene: ethyl acetate: methanol in the ratio of 4:0.5:0.5. Similarly for petroleum ether extracts the solvent system developed was hexane: diethyl ether in a ratio of 9:1. The chromatograph thus, obtained was visualized under UV light after suitable treatment with iodine vapors. The formula used to calculate the R_f value is;

$$R_f \text{ value} = \text{Distance travelled by Analyte} / \text{Distance travelled by solvent front}$$

GC-MS Analysis

To know the compounds present in the extracts they were subjected to GC-MS (Perkin Elmer Clarus 680 gas chromatographic instrument equipped with a mass spectrometer detector Clarus 600 model) and an Elite-5MS (30.0 m, 0.25 mm ID, 250 μ m df column). The flow rate was 1 ml/min with helium as the carrier gas. Total run time was 30 min with a set of temperature program of 60 °C for initial 2 min followed by an increase from 10 °C to 300 °C with a hold time of 4 min. The temperature of injector was maintained at 300 °C and the ion trap was operated at 70 eV with a scan range of m/z from 50 to 600. A sample of 1 μ l was injected in split mode (10:1). The intermediate and an end product were identified based on the Wiley registry of mass spectral data.

Antimicrobial Assay

Mueller Hinton agar plates were used as the growth medium for the bacterial cultures (Adwan and Mhanna 2008). The methanol and petroleum ether crude extracts were dissolved in dimethylsulfoxide (DMSO) (Kalek and Mohamed 2012). The bacterial cultures were spread-plated on the Muller Hinton agar (MHA) plates. Wells were cut and marked as 4

different concentrations- 50, 100, 150 and 200 µg/ml (Arnold et al., 2006). The crude extracts reconstituted with DMSO were filled into the wells according to the different concentrations. In addition, ciprofloxacin antibiotic disc was used as a positive control. The plates were incubated at 37 °C for 24-48 hrs. Results were obtained in the form of a diameter of zone of inhibition.

Synergistic Effect

To check the potent synergistic effects of the extracts 4 antibiotic discs ofloxacin, tetracyclin, streptomycin, ciprofloxacin were impregnated (Guex et al., 2009; Kiefer et al., 2009) with the methanolic and petroleum ether extracts. The bacterial strains *Escherichia coli*, *Klebsiella* sp., *Pseudomonas* sp., *Bacillus* sp., *Proteus* sp. were spread on MH Agar with the help of cotton swabs. The discs were kept on the agar plates and the plates were further incubated at 37 °C for 24-48 hrs. Zone of inhibitions were obtained by disc diffusion method (Kirby-Bauer method), by the synergistic effect of the antibiotic and plant extracts (b). Also separate control plates were prepared using only antibiotic discs minus plant extracts (a). Thus, the percent increase in zone of inhibition was calculated as;

$$F_m = \% \text{ increase in zone of inhibition} = (b-a)/a \times 100$$

In-Silico Studies

Protein Homology Modelling

In the present work, topoisomerases of clinical pathogens namely *Pseudomonas aeruginosa*, *E. coli* and *Klebsiella pneumonia* were selected as the route of pathogen inhibition. The experimental 3D structure of it has not been solved till date. Thus, homology modeling was done using SWISS-MODEL (Laskowski et al., 1993; Abeysinghe and Weeraddana 2011; Tomi et al., 2013; Biasini and Bienert 2014). The FASTA sequence of the respective topoisomerases was retrieved from NCBI database and secondary structure was predicted using build model tool of SWISS-MODEL. Best model amongst all the structures generated was chosen on the basis of Ramachandran plots acquired using PROCHECK (Morris and Goodsell 1998).

3D Structure of Ligand and Binding Site Prediction

The structure of ligands-PDB obtained from GC-MS results was sketched using online 3D structure conversion server Corina Interactive and the 3D molecular model was obtained in the form of PDB directly. *In-silico* prediction of the active sites of the various topoisomerases was done using Q-site finder server. It is an energy based method of prediction of protein-ligand binding sites. The ligand binding pockets in all the molecules were selected based on the interaction energy between the protein and the Van der Waals probe. This binding site residue information was used for molecular docking studies.

Molecular Docking

In this study, AutoDock 4.0 package software was used to investigate the affinity of ligand to the binding pocket of topoisomerases. The docking of all the three topoisomerases of the selected microorganisms (on the basis of antimicrobial results) was executed with four compounds resulted in GC-MS data of both methanol and petroleum ether extracts. During docking the grid parameters were 60×60×60 Å with points separated by 0.375 Å. Also the X, Y and Z coordinates were specified as -33.549, 4.414 and 4.356 for *Pseudomonas aeruginosa* topoisomerases. For *Escherichia coli* topoisomerase grid parameters were 60×60×60 Å with 0.375 Å point separation and X, Y, Z coordinates being -23.99, 9.57 and -4.268

respectively. Furthermore, *Klebsiella pneumonia* topoisomerase was found at coordinates -24.34, 9.556, -6.164 being X, Y and Z following the grid parameters 60×60×60 Å separated by 0.375 Å.

Lamarckian Genetic Algorithm was employed as the docking algorithm with 20 runs and the compounds were ranked based on the docking energy. Further, PyMol (Molecular Graphics system) software was used to visualize and analyze various docked structures.

RESULTS AND DISCUSSIONS

TLC Analysis

Different active ingredients of the extracts were separated by TLC analysis. The results obtained after visualization under UV light are given in figure 1 and the R_f value is listed in table 1.

Figure 1: TLC plates of (i) Petroleum Ether Extract and (ii) Methanol Extract.

Table 1: R_f value of the Extracts in TLC Plate.

Methanol Extract	
Spot Color	R_f Value
Lighr brown	0.822
Light blue	0.661
Dark green	0.612
Light green	0.532
Yellow	0.355
Grey	0.15
Dark brown	0.13
Petroleum Ether Extract	
Spot Color	R_f Value
Orange	0.807
Light yellow	0.385
Grey	0.096

GC-MS Analysis

After the analysis the chromatogram showed the presence of 1,6-diphenylhex-5-en-1-one in methanol extract and

2,4-Bis-(1,1-dimethylethyl)-5-methyl-phenol in petroleum ether extract (Figure 2). Also gamma sitosterol was present in both the extracts. These compounds could be one of the causes for the antibacterial activity of the extracts. To ensure this further assays and docking studies were done.

Figure 2: GC-MS of *Jatropha podagrica* (a) Methanol Extract (b) Petroleum Ether Extract.

Microbial Analysis using well Diffusion Method

Pathogenic bacterial cultures were placed on MH agar plates. Wells were dug at 4 different concentrations viz. 50, 100, 150 and 200 µg/ml in which the extracts were filled. The plates were incubated at 37°C for 18-24 hrs. After the incubation was over, zone of inhibition was measured using a ruler and reported in mm scale (Table 2). The petroleum ether extract did not yield any significant results with *Bacillus* sp. Similarly, both the methanolic and petroleum ether extract did not show effective impact on *Proteus* sp. and *Klebsiella* sp.

Table 2: Antimicrobial Assay of the Extracts

Bacterial Strain	Zone of Inhibition	
	Methanol Extract	Petroleum Ether Extract
<i>Proteus</i> sp.	2 mm	3 mm
<i>Klebsiella</i> sp.	16 mm	17 mm
<i>Bacillus</i> sp.	10 mm	7 mm
<i>Pseudomonas</i> sp.	19 mm	17 mm
<i>E. coli</i>	17 mm	17 mm

Synergistic Effects (Disc Diffusion Method)

The impregnated antibiotic discs showed better susceptibility results against the control antibiotics. Impregnated ofloxacin disc showed an increase in the inhibition by 66.66% with methanol extract and 83.33% with petroleum ether extract against *E. coli*. Similarly against *Klebsiella* sp. petroleum ether extract impregnated tetracycline disc showed an increase in sensitivity by 75%. Results of the enhancement in the antibacterial activity of the discs after combination with the extracts is given in table 3.

Table 3: Synergistic Effect of the Extracts

Bacterial Strain	Antibiotic Disc	Inhibition Zones in mm (b)		Control (a)	F _m = % Increase in zone of Inhibition = (b-a)/a x100	
		Methanol	Petroleum Ether Extract		Methanol	Petroleum Ether Extract
<i>Proteus vulgaris</i>	Ofloxacin	23	19	16	43.75	18.75
	Tetracyclin	26	17	24	8.3	29.16
	Streptomycin	11	15	9	22.22	66.66
	Ciprofloxacin	22	24	20	10	20.00
<i>Klebsiella pneumonia</i>	Ofloxacin	12	11	7	71.43	57.14
	Tetracyclin	13	14	8	62.50	75.00
	Streptomycin	12	14	8	50.00	75.00
	Ciprofloxacin	11	10	6	83.33	66.67
<i>Bacillus subtilis</i>	Ofloxacin	19	15	18	5.55	16.60
	Tetracyclin	30	31	26	15.38	19.23
	Streptomycin	9	18	11	18.18	63.63
	Ciprofloxacin	18	27	18	0	50.00
<i>Pseudomonas aeruginosa</i>	Ofloxacin	21	23	13	61.54	76.92
	Tetracyclin	15	16	10	50.00	60.00
	Streptomycin	12	11	7	71.42	57.14
	Ciprofloxacin	20	23	14	26.92	64.28
<i>E. coli</i>	Ofloxacin	20	22	12	66.66	83.33
	Tetracyclin	26	25	18	18.75	38.89
	Streptomycin	16	18	11	18.18	63.64
	Ciprofloxacin	28	30	19	12.5	57.89

In silico studies

Protein Homology Modelling

Three homology based models were obtained for each topoisomerase. Among the three models, model 01 for *Pseudomonas aeruginosa* topoisomerase was found to be most appropriate based on the Ramachandran plot made using PROCHECK analysis, which suggests that the protein has acceptable stability as most of the phi-psi angles of topoisomerase are distributed within the allowed regions. It has 92.8% of residues in most favored region, 6.5% residues in additional allowed regions and 0.2% in not allowed regions.

Klebsiella pneumonia topoisomerase models were screened with Ramachandran plot using PROCHECK server and model 1 was found to show the stability with 85.8% residues in most favored regions, 12.9% in additional allowed regions and 0% in not allowed region. Similarly, for *E. coli*, topoisomerase model 1 was selected as the best model on the basis of Ramachandran plot. It showed 85.8% of residues in the most favored regions, 13% of residues in additional allowed regions and 0.1% in disallowed regions.

Ligand Binding Site Prediction and Molecular Docking

Among all the sites obtained from Q-site finder server, best site was obtained on the basis of binding site of docking. Docking studies are the computational techniques for the exploration of the possible binding modes of a ligand to a given receptor, enzyme or other binding site. In this study, we used AutoDock 4.0 to evaluate the binding energy of ligands inside the known 3D structure of target enzyme. AutoDock 4.0 software consists of two main programs, autogrid that pre-calculates grid maps of interaction energies for various atom types of ligand with a macromolecule and autodock, which performs the docking of the ligand to the specified grids. Considering the well-obtained *in vitro* results, it was a worthwhile

to go for docking to study the binding inside the active site of topoisomerases, potential target for antibacterial agents and support the *in vitro* results. The docking analysis was done for the ligands with the target topoisomerase of the above mentioned bacteria using this software.

In silico results revealed that out of all the 4 compounds selected for the docking studies, only three could show binding with the topoisomerases. DPH was able to bind to topoisomerases of *P. aeruginosa* with binding energy of -7.09Kcal/mol, while BDP showed binding with *P. aeruginosa* as well as *K. pneumonia* with binding energies of -4.19 and -2.31 Kcal/mol. Similarly GS was able to dock with *E. coli* and *K. pneumoniae* topoisomerases with the highest binding energies of -8.69 and -6.83 Kcal/mol respectively. The docked result of secondary structure of *P. aeruginosa* topoisomerases with DPH and BDP are given in figure 3a and 4a and the hydrogen bond interactions were found with Thr-224 and Phe-100.

Figure 3: (a) Docked Complex of DPH and *P. Aeruginosa* Topoisomerase (b) 2D Ligplot of the Complex.

Figure 4: (a) Docked Complex of BDP and *P. Aeruginosa* Topoisomerase (b) 2D Ligplot of the Complex.

Furthermore, 2D plot of hydrogen bond forming amino acids with the target ligand is depicted in figure 3b and 4b. *K. pneumonia* topoisomerase docking with BDP and GS are given in figure 5a and 6a respectively, showing interactions

with Arg-326 and Val-261. Also 2D plot is shown in figure 5b and 6b representing the hydrogen bond formation with bond distance of 2.79 Å and 2.81 Å with BDP and GS respectively. GS was the only compound found to dock with topoisomerases resulting in high binding energies than other compounds. Figure 7a portrays docking results for *E. coli* topoisomerase between the GS and topoisomerase forming bond with Glu-637 followed by figure 7b illustrating 2D plot of the docked complex with a bond distance of 2.89 Å.

Figure 5: (a) Docked Complex of BDP and *K. Pneumoniae* Topoisomerase (b) 2D Ligplot of the Complex.

Figure 6: (a) Docked Complex of GS and *K. Pneumoniae* Topoisomerase (b) 2D Ligplot of the Complex.

Figure 7: (a) Docked Complex of GS and *E. Coli* Topoisomerase (b) 2D Ligplot of the Complex.

CONCLUSIONS

It is evident that the methanol and petroleum extract of *Jatrophapodagrica* have a number of active components (as seen from the TLC analysis and GC-MS analysis). Further the microbial analysis suggests that both the petroleum ether extract as well as methanol extracts have a significant antibacterial property against the bacterial pathogen species of *Pseudomonas*, *Klebsiella* and *E. coli*. Also, the methanol extract especially has shown inhibitory effect on the growth of *Pseudomonas*, and *E. coli*. The result suggests that the leaves of *Jatrophapodagrica* contain some bioactive compounds which have antibacterial properties. Moreover the *in silico* studies have supported the antibacterial activity by providing a sufficient binding results. Thus, the extract can be used as an ingredient for herbal medicine in near future.

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