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RESEARCH ARTICLE

Combination of *Azadirachta indica* and *Phyllanthus acidus* - A complete Evaluation and Characterization Study

Hemasudha. T. S[1], Yuvarani. S[2], Rajakumari. K[1]*

[1]Department of Bio-Engineering, School of Engineering, Chennai-117, Tamil Nadu, India.
[2]Vels Institute of Science, Technology and Advanced Studies (VISTAS), Pallavaram, Chennai-117, Tamil Nadu, India

*Corresponding Author E-mail: **rajikumari91@gmail.com**

ABSTRACT:

Plants contain many sources in traditional system, Neutraceuticals, Folk and Ayurvedic medicines. Medicinal plants contains a great source of economic value all over the world. The plant extracts are mainly used for pharmaceuticals and for natural therapies. *Azadirachta indica* is commonly known as neem tree or Indian lilac. Amla is commonly known as *Phyllanthus acidus* or *Phyllanthus emblica*. The main objective was to check the characterisation study in the combination plant of *Azadirachta indica* and *Phyllanthus acidus*. The fresh (wet) plant sample of *Neem* and *Amla* was done in Ethyl acetate and Hexane solvents in various concentrations. The present study investigated to explore the preliminary phytochemical constituents, ultrasonication, purification techniques such as TLC and column chromatography of combination plants are used wherelse both the solvents were used. Compare to Hexane, Ethyl acetate gave the best result in characterization studies.

KEYWORDS: *Azadirachta indica*, *Phyllanthus acidus*, Ultrasonication, TLC, Column Chromatography (CC), phytochemical analysis.

INTRODUCTION:

Azadirchta indica, is commonly known as neem, nimtree or Indian Tilac, is a tree in the mahogany. It belongs to the family Meliaceae. The neem leaves, roots, fruit, flowers are used for treating diseases. Neem is the fastest growing tree and the branches are wide and spreaded. Neem tree have been extensively used for many diseases like fever, skin diseases, psoriasis, etc. It is considered in Siddha, Ayurvedha and Unani medicine and it is mainly used for skin diseases[(1)]. The neem leaf is used for birth control and to cause abortions. Neem leaf is used for eye disorders, leprosy, diseases of heart and blood vessels, liver problems, intestinal worms, skin ulcers and diabetes. Neem is also known as the village pharmacy[(2)].

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Phyllathus acidus commonly known as the Otaheite gooseberry, Country gooseberry, Star gooseberry. Gooseberry tree is the tree with edible small yellow berries fruit in the Phyllanthaceae family. Amla juice is considered as best tonic to make you younger. It prevents cancer due to some important polyphenols. Amla helps to control the blood sugar levels and diabetes[(3)].

MATERIALS AND METHODS:

Collection of sample:

The sample *Azadirachta indica* and *Phyllanthus acidus* (Neem and Amla) was collected from our college surroundings of Tamil Nadu, India. The collected sample was cleaned with the tap water to remove the dust and sand particles. Then the sample was washed with freshwater and extracted by using the solvents Hexane (non-polar solvent) and Ethyl acetate (polar solvent)[(4)].

Sample extraction by using solvent:

Hexane and Ethyl acetate are the solvents used for solvent extraction. It is a method of separating the compounds based on their relative solubilities in two immiscible liquids.

Weigh 5g of *Azadirachta indica* (neem) and *Phyllanthus acidus* (Amla) leaves was grinded in mortar and pestle. The grinded sample is transferred to 250ml beaker and 100ml of Hexane is added to the beaker containing the sample. Then the extract was placed in ultrasonicator for cell disruption and cell lysis.

Weigh 5g of *Azadirachta indica* (Neem) and *Phyllanthus acidus* (Amla) leaves was grinded in mortar and pestle. The grinded sample is transferred to 250ml beaker and 100ml of Ethyl acetate is added to the beaker containing the sample. Then the extract was placed in ultrasonicator for cell disruption and cell lysis[4].

Ultrasonication:
In the case of sonication for cell lysis, ultrasound (high-frequency) energy is applied to samples to agitate and disrupt the cell membranes.

5g of wet (fresh) sample dried sample and 5g of dried *Phyllanthus acidus* were crushed together using mortar and pestle. 20ml of solvent (Hexane and Ethyl acetate) were added to the samples to form a homogenized sample. Homogenized sample are transferred to the 250ml beaker then add 80ml of ethyl acetate and hexane to the beaker containing the sample. Ultrasonic probe system, program the extraction process with 72°C temperature. The pulse with the time of 5sec with an interval of 10sec over the duration of 20min. glass beaker containing sample are placed on the platform within the ultrasonic probe system. Glass beaker containing sample levelled up till the ultrasonic probe submerge ¾ into the beaker containing the solution with sample level. Collected ultrasonicated samples are separated into the flacon tubes. Centrifuge the falcon tube at 5000 rpm for 10min. The ultrasonicated samples are stored for further use.

Thin layer chromatography:
Thin layer chromatography is a chromatographic technique used to separate non-volatile mixtures. TLC analysis was carried out using both the solvents. Thin layer chromatography is done for crude extract. Add 16g of silica powder with 4g of anhydrous calcium sulfate powder. Grind the powder with mortar and pestle to form a homogenous form to dissolve in water. Water: powder in the ration of 2:1. Prepare silica powder in a glass beaker and slowly pour the water into the beaker and mix well for silica solution. Slowly pour the silica solution into the TLC glass slide. Allow the slide to dry for 30min until white and smooth surface is visible on the plate. Activation and removal of water is carried out by heating the silica plate in a hot air oven at 120°C for 30min. then cool the plate and keep the slide on the table. By using a microtip add one drop of sample to the TLC glass slide. Prepare TLC chamber placed in a solution using Ethyl acetate: hexane solvent in the ratio of 7:3. The spotted sample should be placed into the TLC sample which is fully submerged in the chamber. The formation of bands develops. Remove the TLC plate when the solvent is about 0.5cm from the top of the plate quickly. Mark the solvent line with a pencil. Observe the formation of band using UV transilluminator. The retention factor (R_f) is calculated after the formation of band is observed under UV transilluminator[5].

$$R_f = \frac{\text{Distance moved by the solute}}{\text{Distance moved by solvent}}$$

Column chromatography:
A long cylindrical glass column fixed firmly to a column-chromatography stand. Plug small amount of cotton to the base of the glass column. Prepare silica column using 30g column silica powder and 90ml solvent using Ethyl acetate: Hexane in the ratio of 7:3. Ensure that the silica gel is packed tightly and exclude any air bubbles. Dried plant sample mixed with solvent. Transfer 5ml of sample to the top of the silica column. Solvents of varying polarity is prepared in the following ratios for Ethyl acetate : Hexane are 10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, 0:10 Solvents of different polarities were passed through the column at a uniform rate.

Fractions collected separately in falcon tubes and labelled for further use.TLC partially separates organic and inorganic materials. This is useful mainly for checking the purity of fractions. One drop of eluents collected from varying polarity is applied on to the TLC plates using a capillary tube. The sample is places 0.5cm above the lower edge of the TLC plate. Plate is kept into the developing chamber containing solvent system prepared using Ethyl acetate: Hexane in the ratio of 7:3. Remove plate when solvent reached upper top edge of the TLC plate. Bands formed on TLC plate visualized under UV cabinet. The spots detected are marked and R_f value is calculated[6].

The formula for RF value is:

$$R_f = \frac{\text{Distance travelled by the sample (cm)}}{\text{Distance travelled by the solvent (cm)}}$$

Further analysis can be carried out for the purified compounds.

Fig 1: CC loaded with ultrasonicated Sample

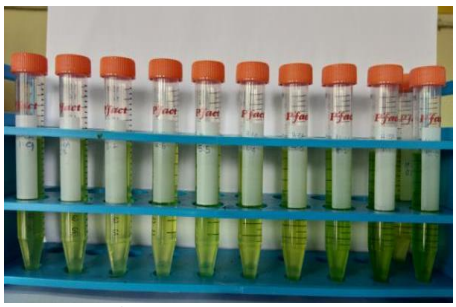


Fig 2: Eluted sample from column chromatography

Phytochemical screening:
Phytochemical screening was carried out by the standard phytochemical assays. The phytochemical screening allows the presence or absence of various chemical compounds like flavonoids, alkaloids, tannins, proteins, carbohydrates, etc., [(7)]

Test for tannin:
To 1ml of plant sample add 1ml of potassium ferrocyanide. Add 1ml of 0.02 M $FeCl_2$ with 0.1 M HCl. To form 0.02M $FeCl_2$ dissolves 0.03ml of $FeCl_2$ with 10ml of water. For 0.1M HCl, dissolves 0.036ml HCl in 10ml water. Formation of blue black precipitate shows the presence of tannins.

Test for anthraquinones:
To 1ml of plant sample add 10ml of benzene solution. Shake the solution for 10minutes and then filter the mixture using Whatman filter paper. To the filtered extract add 10ml of 10% ammonia solution and shake vigorously. Formation of pink or violet or red colour indicates the presence of anthraquinones.

Test for phylobatannins:
To 1ml of sample extract add 1% of HCl. 1% HCl is prepared by dissolving 0.1ml HCl in 10ml of water. Place the mixture of hot plate and mix the content using magnetic stirrer. Formation of red colour precipitates indicates the presence of phylobatannins.

Test for saponin:
To 1ml of the crude purified extract add 5ml of distilled water is added and mix vigorously. Use vortex for vigorous shaking or mixing. Then add few drops of olive oil to the mixture sample. Appearance of foam indicates the presence of saponin.

Test for flavonoids:
To 1ml of the plant extract add 2ml of 2% NaOH solution is added which produces the yellow colour. Then 2 drops of dilute acid was added. Formation of colourless solution indicates the presence of flavonoids.

Test for glycosides:
To the crude plant extract add 2ml of acetic acid and 2ml of chloroform was added. Cool the mixture to room temperature and few drops of concentrated sulphuric acid is added. Formation of grey colour indicates the presence of steroidal plant part of glycosides.

Test for terpenoids:
To the 5ml of plant extract add 2ml of chloroform and wait until the chloroform evaporates. Boil the extract with 3ml of concentrated sulfuric acid. Formation of grey colour indicates the presence of terpenoids.

Test for steroids:
To 0.5ml of sample add 2ml of concentrated H_2SO_4 add 2ml of acetic anhydride. Colour change from violet to blue green colour is observed and indicates the presence of steroids.

Test for carbohydrates:
To a 2ml of the extract, add 1ml of Fehling's solution A and 1ml of Fehling's solution B. Mix the solution and boil the solution in boiling water bath for 10min. Formation of red precipitate of cuprous oxide indicates the presence of carbohydrates [(8)].

Test for protein:
To 1ml of the plant sample add 2ml of NaOH solution and then add 4-5 drops of 1% copper sulphate solution. Heat the solution for 5minutes. Formation of bluish violet colour indicates the presence of protein[(9)].

STERILITY TEST:
Sterility testing in defined as the testing which confirms that the product is free from contamination or the product free from the presence of viable microorganism. Measure 4.6g of agar and transfer it into the 250ml conical flask. The add 200ml of distilled water into a clean and sterile flask. Flame sterilize a clean glass stir rod to stir the medium which it melts. Mix the content constantly and keep the area with sterile condition. Boil the mixture for 1min. remove from heat. Allow the medium to cool then pour the melted agar into each sterile petri plates and cover the lid to the dish. Allow the nutrient agar to solidify. Then add 1ml of sample onto the agar plate and incubate at 37^0C or 90^0F for 48hrs. The bacterial growth should start to become visible in 2-3 days. Then after 48hrs, observe the plate for growth. No growth in the extract after incubation indicates that the extract is sterile.

RESULT AND DISCUSSION:

For fresh (wet) sample analysis:
The extraction method were used for crude extracts of combined fresh *Azadirachta indica* leaves and *Phyllanthus acidus.* Extract gained screened for Thin-Layer Chromatography (TLC) and calculated for retention factor (R_f). Ethyl acetate and Hexane are the solvents used for the extraction (crude and purified extracts).

Ultrasonication is a disruption method used for extracting compounds present within a sample of interest. Using mortar and pestle sample is ground in a polar solvent (Ethyl acetate) and a non-polar solvent (Hexane). Ethyl acetate solvent gives the best result when compared to Hexane solvent in ultrasonication.

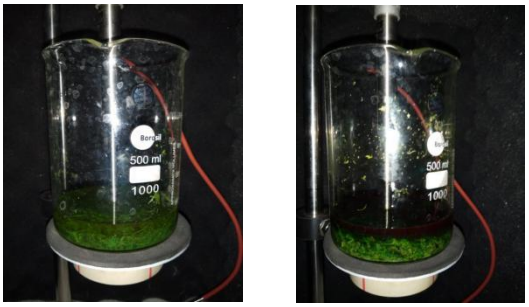


Fig 3: Ultrasonication extract (Hexane and Ethyl acetate)

TLC analysis for the ultrasonicated sample was spotted using capillary tube, 1cm above TLC plate. Sample was separated forming a band which is visualized under the UV cabinet. The movement of sample is directly proportional to the rate of binding of compound to the absorbent. Retention factor was calculated for the crude sample extract of ethyl acetate and hexane. Retention factor of Ethyl acetate and Hexane crude sample was 25mm and 15mm respectively[7][11]. Furthermore, R_f factor of Ethyl acetate is more prominent when compared to hexane and the retention factor value is stated below in table 1. Thus crude Ultrasonicated dry sample of ethyl acetate is selected for further purification method. The purification method carried out is column chromatography.

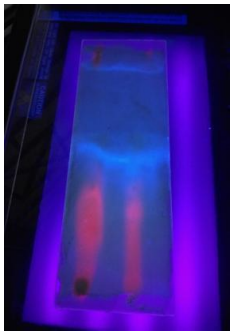


Fig 4: Crude ultrasonicated Ethyl acetate and Hexane sample ran on TLC plate

R_f value for crude Ultrasonicated samples and purified Ultrasonicated wet (fresh) sample.

The purification using column chromatography is ran as stated in section. 5ml of Ultrasonicated dry sample extract is loaded to the top of silica column. Eluents of various concentrations based on polarity dissolves the sample and elutes it through the column. Eluents were collected into sterile falcon tubes, labelled for their respective concentrations. A total of 10 concentrations were eluted.

Table 1: R_f value for crude ultrasonicated sample extract using fresh samples.

Solvent used in extraction (fresh)	Solvent phase	Ratio	R_f value (mm)
Ethyl acetate (Crude)	Ethyl acetate: Hexane	7:3	25mm
Hexane (Crude)	Ethyl acetate: Hexane	7:3	15mm

Collected purified fractions were run on TLC plates prepared as stated in section. Concentrations 10:0, 8:3, 5:5, 8:1 gave satisfactory band separation for dry Ultrasonicated sample.

PHYTOCHEMICAL ANALYSIS:

Phytochemical analysis is a series of methods to screen and identify medicinally active constituent found in plants. The tests carried out are as stated in section. In the present study, phytochemical analysis was carried out for Ultrasonicated ethyl acetate and hexane samples. Phytochemical tests for Ultrasonicated Ethyl acetate and Hexane fresh crude sample is stated in table 2.

Table 2: phytochemical analysis of the crude extract of *Azadirachta indica* (Neem) and *Phyllanthus acidus* (Amla) (presence +, absence -)[10]

Phytochemical analysis	Hexane solvent	Ethyl acetate solvent
Tannins	+	+
Anthra Quinones	-	-
Phlobatannins	-	-
Saponins	+	+
Flavonoids	+	+
Glycosides	+	+
Terpenoids	+	+
Steroids	+	+
Carbohydrates	-	-
Proteins	-	-

All the crude extracts from Ultrasonicated fresh sample in Ethyl acetate and Hexane solvent gave the same result for each test respectively. The phytochemicals present in all the crude samples are Tannins, Flavonoids, Glycosides, Terpenoids, Steroids and saponins[11].

Tannins related studies have reported its ability to exhibit antiviral, antibacterial, anti-tumour activities. Certain tannins are even able to inhibit HIV replication selectivity and are used as diuretic.

Steroids are important compounds as it is related to human sex hormones. Their profound biological activities are highly sought after in medical field. Research has proven that steroidal hormones serve as effective starters to help balance hormones of expectant mothers and breast feeding mother.

Glycosides are compounds known to inhibit Na^+/ K^+ pump. Treatment of congestive heart failure and cardiac arrhythmia uses these glycosides. Controlled dosage of glycosides helps strengthen weakened heart allowing it

to function more efficiently for the amount of therapeutic dose is close to the toxic dose[12].

Terpenoids are total phenols present. This is found in all the plant extracts extracted in this research. Typically plants rich in Terpenoids are used widely in herbal medicines.

Flavonoid was present in the solvent extractions. Compounds of Flavonoids possess anti-allergic, anti-inflammatory, anti-microbial and anti-cancer activities. They possess the ability to modify body's reaction against allergies, viruses and carcinogen, thus it is referred to as naturally available biological response modifier.

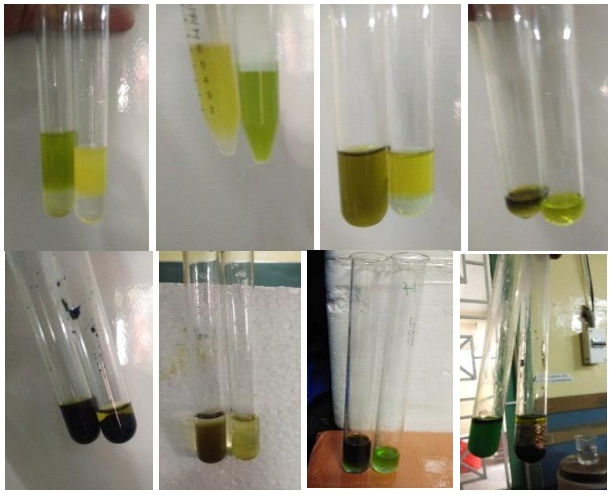
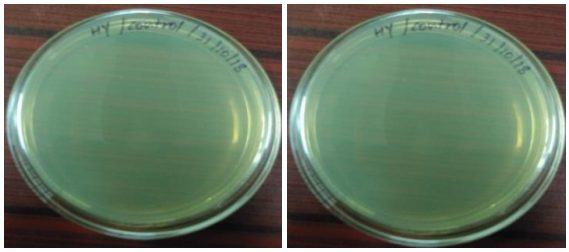


Fig 5: Phytochemical analysis Sterility test:

Sterility test was carried out for the crude extracts and the purified extracts as stated in section. Growth for viable microorganism was observed. Absence of microbial growth. This proves all crude samples are free from contamination and are sterile.



Control **Crude extract**

Fig 6: Sterility test for crude extract

CONCLUSION:

The extraction method of ultrasonication were carried on fresh sample *Azadirachta indica* and *Phyllanthus acidus* plant combination. The plants crude and purified extracts were run on TLC plates for R_f calculation. Furthermore, phytochemical tests and sterility tests were done on the crude sample extracts. It is determined that fresh ultrasonicated Ethyl acetate samples gave the best result when compared to the result amongst all other samples.

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CONFLICTS OF INTERESTS:

The authors declare that they do not have any Conflicts of Interests.

REFERENCES:

1. Khalid et al. Isolation and characterization of an antimalarial agent of the neem tree *Azadirachta indica*. Journal of natural products. 1989 Sep;52(5):922-7.
2. Verma et al. The endophytic mycoflora of bark, leaf, and stem tissues of *Azadirachta indica* A. Juss (Neem) from Varanasi (India). Microbial Ecology. 2007 Jul 1; 54(1):119-25.
3. Devi SS, Paul SB. An overview on Cicca acida (Phyllanthus acidus). Assam University Journal of Science and Technology. 2011 Apr 6;7(1):156-60.
4. Joshi et al. Phytochemical extraction and antimicrobial properties of different medicinal plants: Ocimum sanctum (Tulsi), *Eugenia caryophyllata* (Clove), *Achyranthes bidentata* (Datiwan) and *Azadirachta indica* (Neem). Journal of Microbiology and Antimicrobials. 2011 Jan 30; 3(1):1-7.
5. Susmitha et al. Phytochemical extraction and antimicrobial properties of *Azadirachta indica* (Neem). Global Journal of Pharmacology. 2013;7(3):316-20.
6. Jabeen R. Medicinal plants-a potent antibacterial source against bacterial leaf blight (BLB) of rice. Pak J Bot. 2011 Dec 1;43:111-8.
7. Pandey G, Verma KK, Singh M. Evaluation of phytochemical, antibacterial and free radical scavenging properties of *Azadirachta indica* (neem) leaves. Int J Pharm Pharm Sci. 2014; 6(2):444-7.
8. Yadav et al. Preliminary phytochemical screening of six medicinal plants used in traditional medicine. Int J Pharm Pharm Sci. 2014; 6(5):539-42.
9. Singh B, Ahamad A, Pal V. Evaluation of Antibacterial Activity and Phytochemical Screening of *Azadirachta indica* Leaves Extracts Against Staphylococcus aureus. UK Journal of Pharmaceutical and Biosciences. 2015; 3(4):43-7.
10. Jagessar RC, Hope S. Antimicrobial activity of the uncombined and combined aqueous extract of *Phyllanthus acidus, Sphagneticola trilobata* leaves and *Doliocarpus dentatus's* bark against human pathogenic microorganisms.
11. Ravishankar et al. Preliminary phytochemical screening and in vitro antibacterial activity on Asparagus racemosus root extract. Int J Pharm Chem Biol Sci. 2012; 2: 117-23.
12. Joshi et al. Phytochemical extraction and antimicrobial properties of different medicinal plants: *Ocimum sanctum* (Tulsi), *Eugenia caryophyllata* (Clove), *Achyranthes bidentata* (Datiwan) and *Azadirachta indica* (Neem). Journal of Microbiology and Antimicrobials. 2011 Jan 30;3(1):1-7.