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Study of Phytochemicals in Non-Polar and Polar Solvent Extracts of Root, Bark and Leaf of *Limonia acidissima* and their Anti-bacterial Efficacy

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Abstract:

Limonia acidissima is a tropical fruit belonging to the family Rutaceae. The study was conducted to evaluate the phytochemicals and antibacterial efficacy of root, bark and leaf of *Limonia acidissima* against MDR species. Among the three plant parts, methanolic extract of root and leaf possessed high amount of carbohydrate. The protein content was more in methanolic extract of root and methanolic extract of bark and leaf is rich with alkaloids. Petroleum ether and acetone extract of root showed good antibacterial activity against *S. aureus*. The findings of present study revealed that leaf, root and bark parts of *Limonia acidissima* demonstrate the presence of potential extractive active substances and their antibacterial potential.

Key words:
Antibacterial activity, *Limonia acidissima*, MDR Species, Phytochemicals.

Introduction:

From prehistoric days to recent society, human beings depend on nature for running their life effortlessly. Plants remain a vital source of drugs & it has remained virtually unchanged since time immemorial. *Limonia acidissima* belonging to family Rutaceae and is a Moderate sized deciduous tree grown throughout India. It is a large tree growing to 9 meters (30 ft.) tall, with rough, spiny bark (Ghosh P. *et al* 1982). This plant is known for its phytochemical profile which attributes many medicinal properties like antimicrobial, antifungal, astringent and anti-inflammatory. Plant parts such as stem, leaves, flower, root and fruit used as insecticides, anti-rodent in animal husbandry(Waterman, P.G. and Grundon, M.F., 1983). Alkaloids, flavonoids (Sunitha *et al.,* 2013), steroids, saponins, glycosides, phenols and tannins, gum and mucilage, fixed oils and fats present in *Limonia acidissima* plant parts (Saima Y. *et al.,* 2000). Some are coumarin (AdikaramNKB et al. 2007) and tyramine derivatives (Parthasarathi G. *et al.,* 1991). Leaves are aromatic and astringent, an oil of leaves mixed with a pinch of black pepper used as a carminative (Shermin *et al.,* 2012). Larvae of *Culexquinque fasciatus, Anopheles stephensi* and *Aedesa egypti* can be effectively controlled byacetone extract of the dried leaves of *Limonia acidissima* with LC_{50} of 129.24, 79.58 and 57.23 ppm, respectively (Rahuman A. A. *et al.,* 2000).Due to many adverse effects of drugs of medical discipline and their high cost, the

traditional medicines are being used all over the world. Many pathogenic bacteria are developing resistance to antibiotics and hence their effective management is not possible with modern day's medicines. Therefore, alternate therapy for the management of MDR pathogens is empirical. The existing studies deals with the qualitative analysis of phytochemicals in both non-polar and polar solvents and study their antibacterial efficacy against MDR pathogens.

Material and methods:

Chemicals: Petroleum Ether (60-80 ^{0}C) (PE), Chloroform (CF), Acetone (AT), Methanol (ME).

Collection of plant materials: The parts root, bark and leaves of *Limonia acidissima* were collected from tree located in Nagpur region.

MDR Isolates: *Staphylococcus aureus, Escherichia coli, Pseudomonas sp., Klebsiella sp., Proteus sp. and Acinetobacter baumannii.* The cultures were obtained from the fellow researchers working in our laboratory for their Ph. D. program.

Preparation of Solvent Extract by Maceration process: Leaf, root and bark of *Limonia acidissima* were collected separately, cleaned and air-dried in shed. After complete drying, plant parts were then grounded to fine powder in mortar and pestle. 10 g of dried herb powder of different parts were then added to separate conical flasks containing 100 ml of solvent (Petroleum ether, Chloroform, Acetone, Methanol) and kept in shaking incubator for 8 day for maceration. Then filtrate is obtained by filtration using nylon mesh and extracts were concentrated by evaporation.

Preparation of Extract for Phytochemical Analysis: The solvent extract obtained through maceration process and then evaporated to dryness on water bath. The powdered extracts obtained were then subjected to qualitative tests for the identification of various plant constituents like alkaloids, flavonoids, steroids, saponins, glycosides, phenols, gum and mucilages, fixed oil and fats and tannins (Raaman N., 2006).

Preparation of inoculums: Inoculum turbidity of 6-8 hrs. actively growing culture was adjusted according to 0.5 McFarland standards which were then used as inoculums which correspond to size of 1.5×10^8 cfu/ml.

Antimicrobial Activity of root, bark and leaf Extract:

1. Hi-sensitivity test agar medium maintained at 45-50 ^{0}C was poured in sterile petri plate and allow to solidify at room temperature.
2. A 0.1 ml of inoculum was inoculated on to the solidified agar medium by micropipette using sterile tips under aseptic preparation and inoculums was spread carefully by sterile spreader to ensure uniform distribution of inoculum on the agar surface.

3. Four equidistant wells per plates were cut with the help of sterile metal borer (10mm). 100 µL of each of the Nonpolar and polar extract i.e. PE, CF, AT, ME of leaf, root and bark of *Limonia acidissima* was transferred to the different wells with the help of micropipette.
4. Plates were kept undisturbed in a refrigerator for 1-2 hours for diffusion of substances into the media.
5. After diffusion, Plates were incubated at 35±0.5^{0}C for 24 hrs. and zone of inhibition was measured after incubation.

Phytochemical Screening:

The phytochemicals were tested qualitatively by the methods proposed by N. Raaman (Raaman N., 2006).

Detection of Alkaloids: 50 mg extract is stirred with few ml of dilute HCL and filtered. The filtrate is subjected to following test.

1. **Mayer's test:** To a few ml of filtrate, add one drop of Mayer's reagent. A white or creamy precipitate indicates positive test.
2. **Wagner's test:** To a few ml of filtrate, add few drop of Wagner's reagent. A reddish-brown precipitate indicates positive test.
3. **Hager's test:** To a few ml of filtrate, add 1 or 2 ml of Hager's reagent. A prominent yellow precipitate indicates positive test.

Detection of carbohydrates: 100 mg extract is dissolved in 5 ml of water and filtered. The filtrate is subjected to the following test.

1. **Molisch's test:** To 2 ml of filtrate, add 1ml of Molisch's reagent. A violet ring indicates positive test
2. **Fehling's test:** To 1 ml of filtrate is boiled on water bath with 1 ml each of Fehling's A & B solution. A red precipitate indicates positive test.
3. **Barfoed's test:** To 1 ml of filtrate, add 1 ml Barfoed's reagent and heated on boiling water bath. A red precipitate indicates positive test.
4. **Benedict's test:** To 0.5 ml of filtrate, add 0.5 ml of Benedict's reagent and heated on boiling water bath. A characteristic coloured precipitate indicates positive test.

Detection of Glycosides:

1. **Legal's test:** 50 mg of extract is dissolved in pyridine, sodium nitroprusside solution is added and made alkaline using 10% sodium hydroxide. Pink colour indicates presence of glycoside.

Detection of Proteins and amino acids: 100 mg extract is dissolved in 100 ml of distilled water and filtered. The filtrate is subjected to the following test.

1. **Millon's test:** To 2 ml of filtrate, add few drops of Millon's reagent. A white precipitate indicates positive test.
2. **Biuret test:** 2 ml of filtrate is treated with 1 drop of 2% copper sulphate solution. To this, 1 ml of ethanol (95%) is added, followed by excess of potassium hydroxide pellets. Pink colour in ethanolic layer indicates presence of proteins.

3. **Ninhydrin test:** 2 drops of ninhydrin solution is added to 2 ml of filtrate. A characteristic purple colour indicates the presence of amino acid.

Detection of Saponins: 50 mg is diluted with distilled water and made upto 20 ml. A suspension is shaken in a graduated cylinder for 15 min. A 2cm layer of foam indicates the positive test.

Detection of Phytosterols:

Libermann- Burchard's test: 50 mg extract is dissolved in 2 ml of acetic anhydride. To this, 1 or 2 drops of conc. Sulphuric acid is added along the side of test tube. An array of colour change shows presence of phytosterols.

Detection of fixed oils and fats: Few drop of 0.5N alcoholic potassium hydroxide solution are added to small quatity of extract along with drop of phenolphthalein and heated on water bath for 2 hrs. Formation of soap or partial neutralisation of alkali indicates presence of fixed oils and fats.

Detection of Phenolic compounds and tannins:

1. **Ferric chloride test:** 50 mg is dissolved in 5ml of distilled water, add few drops of neutral 5% ferric chloride solution. A dark green colour indicates presence of phenolic compounds.
2. **Gelatin test:** 50 mg is dissolved in 5ml of distilled water and 2ml of 1% solution of gelatin containing 10% sodium chloride is added to it. White precipitate indicates positive test.
3. **Lead acetate test:** 50 mg is dissolved in distilled water, add 3 ml of 10% lead acetate solution. A bulky white precipitate indicates positive test.
4. **Alkaline reagent test:** An aqueous solution of extract is treated with 10% ammonium hydroxide solution. Yellow fluorescence indicates presence of flavonoids.

Detection of Gum and Mucilages: 100 mg extract is dissolved in 10 ml of distilled water; add 25 ml of absolute alcohol with constant stirring. White and cloudy precipitate indicates positive test.

Result and discussion:

Qualitative phytochemical analysis of Petroleum Ether (60-80 ^{0}C) (PE), Chloroform (CF), Acetone (AT) and Methanol (ME) extracts of root, bark and leaves of *Limonia acidissima*is given in Table 1.

Almost all the parts of *L. acidissima* are rich in phytochemicals. Petroleum ether, chloroform, acetone and methanol extract of root, bark and leaf shows presence of alkaloids. All the solvent extract of root, bark and leaf shows presence of carbohydrates except petroleum ether extract of root and bark. Chloroform extract of bark contain higher amount of glycoside. Protein is present in almost all the non-polar and polar solvent extract of root, bark and leaves but not in chloroform extract of root and acetone extract of bark and leaves. Saponins and phytosterols were found in chloroform, acetone and

methanol extract of root, bark and leaves and only in petroleum ether extract of leaf. Petroleum ether extract of root, bark and leaf and only methanolic extract of leaf possessed fixed oils and fats. Phenolic compound and flavonoids were detected in petroleum ether, chloroform, acetone and methanolic extract of root, bark and leaf except petroleum ether extract of bark. Only Petroleum ether extract of root and bark and chloroform extract of leaf shows presence of gum and mucilages.

The antibiogram study of clinical isolates of *Staphylococcus aureus* (70.37%), *Escherichia coli* (61.76%), *Pseudomonas sp.* (80%), *Klebsiella sp.* (72.72%), *Proteus sp.* (61.76%) and *Acinetobacter baumannii* (87.87%) revealed their multidrug resistances are used for evaluating antibacterial efficiency of extracts. The results were interpreted as Sensitive, Intermediate and Resistant from interpretative chart (Table 2) proposed by Johnsons (Johnson, T. And C. Case, 1995).The results showing antibacterial activity of various extracts are given in Table 3.

Petroleum ether extract of leaf exhibited antimicrobial activity against *Klebsiella sp.* and root extract exhibited against *Klebsiella sp.*, *Staphylococcus aureus* and *Pseudomonas* sp. whereas no activity was exhibited by bark extract.Chloroform extract of leaf demonstrated activity only against *Acinetobacter baumannii* and root and bark extract showed no activity against any of the other test organism. Acetone and methanol extracts of leaf have not exhibited any activity against any of the test organism. Polar solvents acetone and methanol showed antimicrobial activity against MDR test organism. Acetone and methanol extract of bark showed fairly good activity against only *E.coli*. Rahuman A. A. *et al.,* 2000has studied effect of acetone extract of the dried leaves of *Limonia acidissima*on the Larvae of *Culexquinque fasciatus, Anopheles stephensi* and *Aedesaegypti* and found to be effective against them with LC_{50} of 129.24, 79.58 and 57.23 ppm, respectively (Rahuman A. A. *et al.,* 2000). Thomas A. and Ponnammal N.R. (2005) did phytochemical analysis and studied antibacterial activity of only methanolic extract of different plant parts of *Limonia acidissima*and their results found in agreement with our results (Thomas A. and Ponnammal N.R.. 2005). Very limited work has been done on non-polar and polar extracts of this plant parts particularly against MDR pathogens. Our study exploits the potential use of phyto constituents of *Limonia acidissima*for antibacterial activity and other medicinal use.

Table 1: Phytochemical analysis of various extracts of *Limoniaacidissima*:

Sr no.	Phytochemical test	ROOT				BARK				LEAF			
		PE	CF	AT	ME	PE	CF	AT	ME	PE	CF	AT	ME
1	Alkaloids	+	+	++	+	+	+	++	++	+	+	+	++
2	Carbohydrates	-	+	+	++	-	+	++	+	+	+	+	++
3	Glycosides	-	+	-	+	-	+++	-	-	+	+	-	+
4	Proteins & amino	+	-	++	+++	+	+++	-	++	+	+	-	+
5	Saponins	-	+	+	+	-	+	+	+	+	+	+	+
6	Phytosterols	-	+	+	+	-	+	+	+	+	+	+	+
7	Fats	+	-	-	-	+	-	-	-	+	-	-	-
8	Phenolic compound	+	+	+	+++	-	+	+	++	+	+	+	+
9	Gum and Mucilages	+	-	-	-	++	-	-	-	-	+	-	-

+++ High, ++ Medium, + Low and — Absence

Table 2: Zone evaluation table

Category	Diameter of zone of inhibition (mm)
Resistant (R)	10 or less
Intermediate (I)	11 or 15
Sensitive (S)	16 or more

Table. 3- Antibacterial activity of various extracts of root, bark and leaf of *Limonia acidissima*

Sr No.	Organisms	Control				Diameter Zone of inhibition, mm											
						ROOT Extracts				BARK Extracts				LEAF Extracts			
		PE	CF	AT	ME	PE	CF	AT	ME	PE	CF	AT	ME	PE	CF	AT	ME
1	*E.coli*	-	-	-	-	-	-	-	-	-	-	14 (I)	16 (S)	-	-	-	-
2	*Klebsiella sp.*	-	-	-	-	15 (I)	-	-	15 (I)	-	-	-	-	19 (S)	-	-	-
3	*S. aureus*	-	-	-	-	18 (S)	-	17 (S)	12 (I)	-	-	-	-	-	-	-	-
4	*Proteus sp.*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	*Pseudomonas sp.*	-	-	-	-	19 (S)	-	12 (I)	15 (I)	-	-	-	-	-	-	-	-
6	*Acinetobacterbaumannii*	-	-	-	-	-	-	-	-	-	-	-	-	-	21 (S)	-	-

(–) – No inhibition, (I) - Intermediate, (S) - Sensitive

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