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Research Article

Limonia Acidissima: The Phytochemical Analysis and Comprehensive Evaluation of Antimicrobial and Anti-Oxidant Properties

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Abstract

As the microorganisms have developed the inherent ability to develop and adopt a mechanism of resistance against antibiotics. The harmful side effects of antibiotic including their cost of drug development have slowly shifted toward the plant derived phytochemical based medicines. The screening of antimicrobial property of this medicinal plant shows a positive result against the different species of bacteria (*S. aureus, P. aeruginosa* and fungi *(A. Niger, Fusarium*). This study of phytochemicals shows the important part to prevent and protect the plant against the microorganisms as well as phytochemicals to provide the information about the compound which are responsible for the antimicrobial activity like Alkaloids, Phenolics, and Flavonoids etc. This plant has enough amount of Total phenolics and hence, it has a high anti-oxidant activity. The anti-oxidant activity involve in the prevention of plant cell tissue damage. Anti-oxidant activity is measured by DPPH. The *L. acidissima* plant have many applications such as in bale diarrhea, dysentery, used for liver, cardiac tonic and effective for high cough, sore throat.

Keywords: *Wood apple, DPPH, Antimicrobial activity, Anti-oxidant activity, Aqueous extracts (hot and cold extraction); Solvent extraction; Rotary vacuum evaporator; Soxhlet.*

Introduction

Limonia acidissima Linn, syn, *Feronia Lim*onia. It is the wood apple and belonging to the family Rutaceae. It is an important indigenous tree of India known for its medicinal and processing properties [Abdillahi, H. S. *et al*., 2010] [Al-Quran, S. *et al*., 2008] [Alali, F. *et al*., 2005]. Fruits are edible and considered to be a stomachic, astringent, diuretic, cardiotonic the fruits are also used in treatment of tumour, asthma, wounds, cardiac and hepatitis and tonic for the liver and lungs [Al-Bagieh. *et al*., 1992] [Almas, K. *et al*., 2002] [Almas, K. *et al*., 2005]. The leaves are aromatic and carminative and used for the treatment of indigestion and minor bowel infections of children's and also the root sometime given as the antidote to snakebite. Plant has many phytochemicals which are responsible for its Antimicrobial and Anti-Oxidant activities. Phytochemical word derived from the Greek word which mean is 'plant'. It is biologically active compounds, occurring naturally in the plants [Abdel, W. S. *et al*., 1990] [Noumi, E. *et al*., 2011]. The role of phytochemicals in the plant is the protection of plant cell from the UV exposure, pollution, stress, drought and pathogenic attack the chemicals of plant which has the property to fight against the pathogens called as the secondary metabolites and can provide the health benefits to humans [Alali, F. *et al*., 2003] [Malik, S. *et al*., 1987] [Noumi, E. *et al*., 2010]. Study of these phytochemicals proposes that may it reduce the risk of coronary heart disease by preventing the oxidation of low- density lipoproteins (LDL) cholesterol, reducing the synthesis or absorption of cholesterol, normalizing blood pressure and clotting. Medicinal plants are most important in field of pharmaceutical industries for new drug preparation [Ray, A.B. *et al*., 1975]. Medicinal plants are major source of secondary

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metabolites, which are used to treat many infectious diseases, these medicinal plants are have several phytochemical analyses, anti-microbial and anti-oxidant activities [Muhammad, S. *et al*., 2010]. Based on these properties medicinal plants are used in therapeutic purpose and manufacturing of drugs. The value of plants depends on the presence of chemical substances and their role in human body. The phytochemical involve in protection against diseases are alkaloids, flavonoids etc.

On the basis of traditional and pharmacological study the *L. acidissima* plants was selected and explored their antimicrobial potential.

Methodology

Collection of Medicinal Plants

The plant sample of *Limonia acidissima* (leaves& twigs) was collected from the "Medicinal and Aromatic Plant Research Station, Anand Agricultural University, Boriyavi, Gujarat, India."

Preparation of the Plant Extract

The plant parts were washed with distilled water and air dried at 40 ºC by using hot air oven or dried at direct sunlight for 1 or 2 days. Then, make a powder by using grinder. The powder was collected in the zip bag to prevent the moisture.

Plant Sample Extraction (Aqueous)

The plant sample was extracted by using aqueous method and solvent extraction method.

Hot Water Extraction

Take 5 to 10 gm of plant powder sample and add it to in 100 ml of distilled water in a conical flask and incubate to water bath at 80ºC for 2 to 3 hours. After that, the mixture was homogenized and filtered by using muslin cheese cloth and sample was proceed into rotary vacuum evaporator for evaporation of water and sample extraction. The temperature of rotary vaccum evaporator is depends on the boiling point of solvent to be used. After the evaporation, the sample was filtrated (Whatman filter paper) and collected in black screw cap bottle and stored in refrigerator.

Cold Water Extraction

Take 5 to 10 gm of the dried powder sample and added to 100 ml distilled water in a conical flak and incubate into orbital shaker incubator at 140 rpm for overnight. Next day, the mixture was homogenized and filtrated with use of muslin cheese cloth and proceed it in to rotary vacuum evaporator and after that the extract must filtrated with (Whatman filter paper) and the final extract collect in to the black screw cap bottle and stored in to refrigerator.

Plant Sample Extraction (Solvent)

Plant extract is extracted by using Soxhlet extractor with high efficiency to analyse the phytochemicals present in the extract and by using this extract we can perform the different assay. This temperature used in this method is based on the boiling point of solvent. The solvent used for the extraction of plant sample are Methanol (boiling temperature is 64.7ºC or 65ºC) and Hexane (69ºC). Different solvent used because some phytochemicals are dissolved in polar and some are dissolved in non-polar solvent.

Phytochemical Analysis

Test for Coumarins

Take 2 ml of plant extract and add 10 % NaOH, formation of yellow color indicates the presence of coumarins.

Test for Anthocyanin

Take 2 ml of extract and add 2N HCL and few drops of ammonia, if the anthocyanin is present in the sample the pink –red color turning to blue-violet color.

Test for Steroids [Libermann Burchard Test]

Take 1 ml of extract and add 10 ml chloroform and in that add equal amount of H_2SO_4 positive result gives upper layer red, while lower layer yellow with green fluorescence.

Test for Saponins

Take 2 ml of extract and add 6 ml of distilled water and then shaken it vigorously, foam was observed when the sample has saponins.

Test for Terpenoids
2 ml of extract treated with 2 ml of acetic anhydride and then add few drops of H_2SO_4, positive result give blue, green ring formation.

Test for Tannins [Braymer's Test]
Take 2 ml of extract and allowed it to react with 10% alcoholic ferric chloride solution, positive result gives the formation of blue, green color.

Test for Phenolics
Add few drops of extract in to 5% aqueous ferric chloride and when this test is positive deep blue or dark color form.

Test for Flavonoids [Alkaline Reagent Test]
2 ml of extract treated with 1N sodium hydroxide solution and give intense yellow color if, sample has flavonoids.

Test for Alkaloids [Mayer's Reagent]
Add 2 ml of extract with few drops of Mayer's reagent and if, sample contain alkaloids than it will give white creamy precipitates.

Test for Reducing Sugar
Take 0.5 ml of sample and add 5 ml of benedict reagent, boil it in boiling water bath for 1 min if the sample has reducing sugar, the solution form brick red color precipitates.

Test Microorganisms
The bacterial (*S. aureus, P. aeruginosa*) and fungal (*A. niger* and *Fusarium*) strain were collected from Shri Alpesh N. Patel Post Graduation Institute of Science and Research, Anand.

Culture Media and Inoculums
The N-agar media used for the Anti-bacterial activity and PDA media was used for Anti-fungal activity. The bacterial culture was inoculated in the nutrient broth and incubated overnight to allow the growth.

Antibiotics
The antibiotic was used as a positive control in antimicrobial activity. In this activity Ampicillin was used as positive control and the concentration was 10mg/ml. In anti-fungal activity the Fluconazole was used, the concentration was 10 mg/ml.

Antimicrobial Screening
The antimicrobial activity of different parts' extract carried out by agar well diffusion method.

Antibacterial Activity
The Antibacterial activity was carried out by using agar well diffusion method and test microorganisms. The negative control of Antibacterial activity is Methanol, Hexane and distilled water.

Antifungal Activity
The Antifungal activity was carried out by using well diffusion method. In this method the first step is growth of fungus on selective media, make a suspension of it. If fungus is sporulated, then count the spores by hemocytometer and after that make a suspension which is used for the assay. Take an aliquot of 0.1 ml of suspension and spread it on the appropriate media, then make a well. Wells were filled with different parts' extracts, in which for negative control, solvent and distilled water used and for positive control the Fluconazole was used. Incubate the plates in incubator for 6 to 7 days at 25ºC to 30ºC. If the sample has Antifungal activity, then the zone of inhibition was observed after the incubation time.

Anti-Oxidant Activity
The anti-oxidant activity was determined by 2, 2 – diphenyl-1-picrylhydrazyl (DPPH) Radical scavenging method. The anti-oxidant activity of different extract were measured in terms of H^+ donating or radical scavenging ability, using the stable radical DPPH. The different aliquots of extracts and 2 ml of DPPH in each tube were incubate in dark for 15 to 20 minutes and then take O.D at 517 nm.

Total Phenolics
Take 0.5 to 1 gm of plant sample and grind it with mortar and pestle in 10 times volume of ethanol. Centrifuge the homogenate at 10,000 RPM for 20 min, save the supernatant. Evaporate the supernatant to dryness. Dissolve the residue in a known volume of

distilled water (5 ml), pipette out different aliquots into test tubes. Make up the volume in each tube to 3 ml with water, add 0.5 ml of FCR reagent. After 3 min, add 2 ml of 20 % Na_2CO_3 solution to each tube, boil it in the boiling water bath and after that take O.D at 650.

Results and Discussion

Phytochemical Analysis of Plant Extracts

The study of phytochemicals of *L. acidissima* shows that the alkaloids, flavonoids, tannins, saponins, terpenoids, steroids, reducing sugar, Coumarins, Phenolics were present [Deshpan, R. *et al*., 2011] [Kamil, M. *et al*., 1999].

Antimicrobial Activity of Medicinal Plants

In *L. acidissima* the antimicrobial activity was due to many phytochemicals like alkaloids, tannins, flavonoids etc.

The screening of antimicrobial activity of the *L. acidissima* shows the antibacterial activity against the *S. aureus, P. aeruginosa* and there is no antifungal effect observed against *fusarium* [Huang, S.H. *et al*., 2012].

Anti-Fungal Activity of Medicinal Plants

The extraction of the *L. acidissima* gives the antifungal activity against *A. Niger*, and some parts give antifungal activity against the *Fusarium*. The fluconazole antibiotic used as positive control; concentration is 10 mg/ml [Abdel, W. S. *et al*., 1990] [Alali, F. *et al*., 2005].

Anti-Oxidant Activity

The absorbance of Ascorbic acid increase with increase of concentration of ascorbic acid. The radical scavenges activity of ascorbic acid increase after 30 min incubation in dark [Alali, F. *et al*., 2003].

$A_{control} = 0.223$nm

NB: '+' symbol for positive whereas, '-' for negative.

Table 1: Phytochemical analysis of aqueous plant extract

Plant name	Type of extract	Coumarins	Steroids	Saponins	Tannins	Phenolics	Flavonoids
L.acidissima							
Leaves	Hot Cold	+ -	- -	- -	- -	- +	- -
Fruit pulp	Hot Cold	+ +	- -	- +	- +	- +	- +
Fruit rind	Hot Cold	- -	- -	+ +	- +	+ -	- +

Table 2: Phytochemical analysis of Methanol plant extract

Test	Test	Leave s	Fruit pulp	Fruit rind
Steroids	Steroids	-	-	-
Coumarins	Coumarins	-	+	+
Saponins	Saponins	-	-	+
Alkaloids	Alkaloids	-	-	-
Flavonoids'	Flavonoids	+	-	-
Tannins	Tannins	-	-	+
Phenolics	Phenolics	+	-	+
Reducing sugar	Reducing sugar	-	-	+
Terpenoids	Terpenoids	-	-	-

Table 3: Phytochemical analysis of Hexane plant extract

Test	Leaves	Pulp	Rind
Steroids	+	-	-
Coumarins	-	-	-
Saponins	-	-	-
Alkaloids	-	+	-
flavonoid's	-	-	-
Tannins	-	-	-
Reducing sugar	-	-	-

Table 4: Anti-bacterial activity of *L. acidissima* against the *P. aeruginosa* (mm

Leaves	Hot Cold	- -
Pulp	Hot Cold	6 -
Lorinda	Hot cold	12 14

Table 5: Antibacterial activity of aqueous extract (mm)

L. rind	Hot Cold	14 -
L. pulp	Hot Cold	5 -
L. leaves	Hot Cold	5 -

Table 6: Antibacterial activity of methanolic extracts of *L. acidissima*

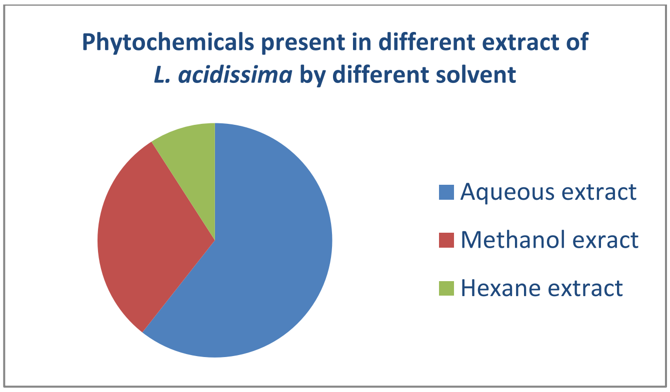
L. acidissima	***S. aureus*(mm)**	***P. aeruginosa*(mm)**
Leaves	13 11	14 15
Fruit pulp	13 09	- -
Fruit rind	- 12	- 12

Table 7: Anti-bacterial activity of Hexane extracts of *L. acidissima*

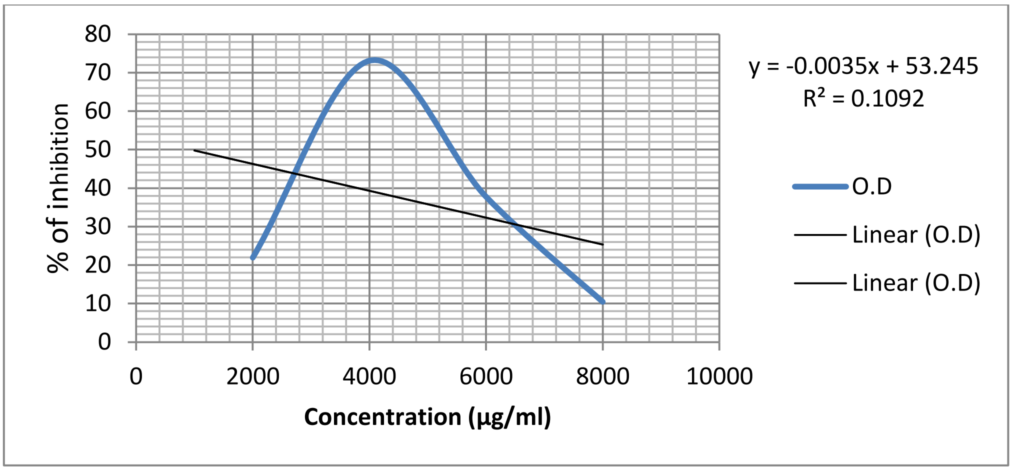
L.acidissima	***S. aureus* (mm)**	***P. aeruginosa* (mm)**
Leaves	12	-
Fruit pulp	10	12
Fruit rind	11	09

Table 8: Antifungal activity of different extracts of *L. acidissima*

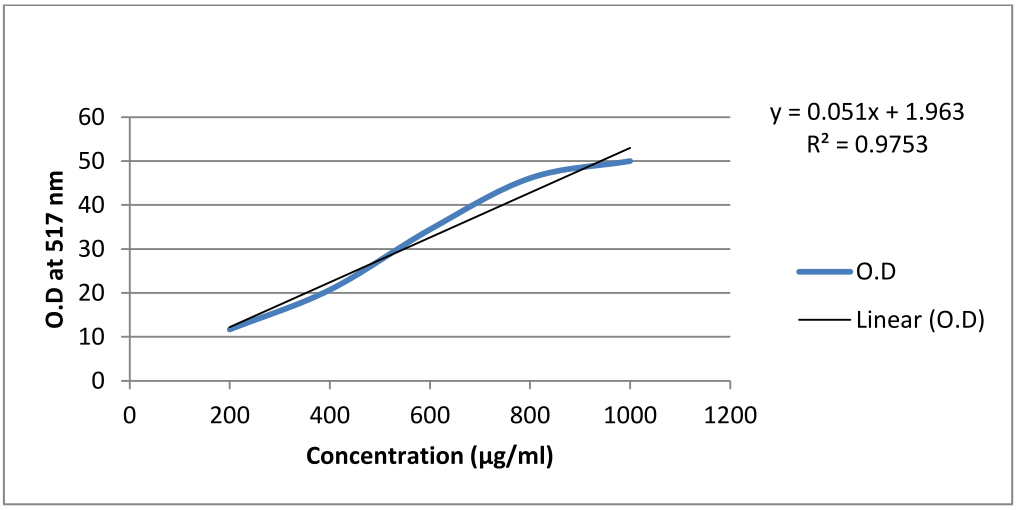
Name of plant *L.acidissima*	**Type of extraction**	***A. niger***	***Fusarium***
Leaves	Hot Cold Methanol hexane	- - - -	- - - -
Rind Pulp	Hot Cold Methanol Hexane Hot Cold Methanol hexane	- - - - - - - -	+ (1mm) - - - - - - -



Graph 1: Phytochemicals of *L. acidissima*



Graph 2: Anti-oxidant activity of *L. acidissima*



Graph 3: Antioxidant activity of *L. acidissima* (Leaves)

	
Image 1: Antimicrobial activity against *S. aureus*	**Image 2:** Antimicrobial activity against *P. aeruginosa*

Image 3: Antifungal activity on *A. niger*

Image 4: Anti-oxidant activity of *L. acidissima* (Leaves)

Image 5: Anti-oxidant activity of *L. acidissima* (Rind)

Conclusion

As the microorganisms had developed the inherent ability to developed and adopted to a mechanism of resistance against antibiotic. The harmful side effect of antibiotics including their cost of drug development had slowly shifted toward the plant derived phytochemical based medicines. The screening of antimicrobial property of *L. acidissima* saw a good result against different species of bacteria (*S. aureus, P. aeruginosa*) and fungi (*A. Niger, Fusarium*). The study of phytochemicals saw the important part to prevent and protect the plant against the microorganisms. Another importance of *Acidissima* provides the information about the compound which was responsible for antimicrobial activity namely, alkaloids, phenolics, flavonoids etc. The anti-oxidant activity involved in the prevention of plant cell tissue damage. Anti-oxidant activity was measured by DPPH. The total phenolics content of this plant was good and there for this plant had an antimicrobial activity. The *L. acidissima* plant have many applications in bale diarrhea, dysentery, used for liver, cardiac tonic and effective for treatment of high cough, sore throat and disease of the gums.

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