



Qualitative & Quantitative Estimation of Bioactive Compounds and Antioxidant Activity in Citrus Hystrix

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Abstract:

Medicinal plants are great importance to the health of individuals and communities. The medicinal value of these plants lies in some chemical substances that produce a definite physiological action on the human body. The objective of the present study was to estimate phytochemical constituents and antioxidant activity of different solvent extract (Water, Iso propyl alcohol, Acetone and Benzene) of dried leaves of medicinally important herbs, citrus hystrix. The Phytochemical screening revealed the extract richness in alkaloids, flavonoids, Saponins, phenols, protein, cardiac glycosides, terpenoids, carbohydrates and quinines. Quantitatively done by total flavonoids 6.85 mg/ml (benzene), 6.00 mg/ml (acetone), carbohydrates 0.115 mg/ml (aqueous), protein 0.787 mg/ml (IPA), 0.487 mg/ml (aqueous) and tannins content 0.841 mg/ml (IPA), 0.218 mg/ml (aqueous), alkaloids 40 mg/g (acetone) Antioxidant activity was established by DPPH assay. Methanol extract of citrus hystrix leaves decreased absorption values at 517 nm indicating that the fractions had hydrogen donating ability or can scavenge free radical. The activity was significant when compared to the standards used Ascorbic Acid and sample. The extracts gave % AA 90% was highest in citrus hystrix.

Key Words: Phytochemical, Antioxidant activity, Citrus hystrix, DPPH and Cardiac glycosides, AA (Antioxidant Activity)

1. INTRODUCTION

There is no doubt that plants are good source of biologically active natural product. Many phytochemicals were possibly been destroyed or removed by modern food processing techniques, including cooking. For this reason, it is believed that industrially processed foods are less beneficial than unprocessed foods. The absence or deficiency of phytochemicals is believed to have contributed to the increased prevalence of the above cited preventable or treatable causes of death in contemporary society. Plants contain too many useless compounds and photochemical approach is too wasteful of time and resource. The complete extraction of product from plant material is rarely achieved by classical methods of extraction and separation of individual compounds was very difficult. Medicinal plants are great importance to the health of individuals and communities. The medicinal value of these plants lies in some chemical substances that produce a definite physiological action on the human body. The most important of these bioactive compounds of plants are alkaloids, tannins, flavonoids and phenolic groups. Many of these indigenous medicinal plants are used as spices and food manufacturing. Approximately 80% of the words population depends exclusively on plants for their health and healing. Whereas, in the developed world, reliance on surgery and pharmaceutical medicine is more useful. But in the recent year, more and more people are complementing their treatment with natural supplements. Furthermore motivation of people towards herbs are increasing due to their concern about the side effects of drugs, those are prepared from synthetic materials. The medicinal plants find application in pharmaceutical, cosmetics, agricultural and food industry. The use of medicinal herbs for curing disease has been documented in history of all

civilizations. With the onset of research, it was concluded that plants contain active principles, which are responsible for curative action of the herbs.

1.1 ANTIOXIDANT AND BIOACTIVE COMPOUNDS

An antioxidant is a molecule that inhibits the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons or hydrogen from a substance to an oxidizing agent. Oxidation reactions can produce free radicals. In turn, these radicals can start chain reactions. When the chain reaction occurs in a cell, it can cause damage or death to the cell. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions. They do this by being oxidized themselves, so antioxidants are often reducing agents such as thiols, ascorbic acid, or polyphenols. A substance or a compound has a biological activity if it has direct effects on a living organism. These effects can both be adverse or beneficial depending on the substance, the dose or the bioavailability. The term food bioactive compound or component (from now 3 referred to as food bioactive) is usually only associated with positive effects on an organism. One definition is that food bioactive affect status of health and therefore have an added biological value beyond their caloric content. However, there is no unifying definition.

1.2 CITRUS HYSTRIX

The Kaffir lime is also known as combava, kieffer, lime, limau purut, jeruk purut. It is a lime native to Indochinese and Malaysian ecoregions in India, Laos, Thailand and adjacent countries. It is used in Southeast Asian cuisine

Kingdom : Planate (Angiosperms)
Order : Spindale's

Family : Rutaceae
Genus : Citrus S

Species : C. hystrix
Binomial name: Citrus hystrix



Figure.1. Citrus Hystrix Plant

2. MATERIALS AND METHODS

2.1 Collection Of Plant Material

Fresh leaves of selected herbs *Citrus Hystrix* were collected from local area in the month of Jan, 2013. The leaves were washed, cleaned and chopped into pieces and dried at 40 °C in thermostatically controlled oven until they attained a constant weight. The samples were then crushed into powder, using mechanical operation, so as to enhance effective contact of solvent with sites on the plant materials.

2.2 Preparation of Plant Extract

50 g of each powdered leaves were placed in conical flask and 100 ml of solvent was added and plugged with cotton. The powder material was extracted with solvent for 24 hrs at room temperature with continuous stirring. After 24 hrs was collected extract by using filtration. The extracts were stored in airtight bottles in a refrigerator for further use.

2.3 Qualitative Estimation

Qualitative Phytochemical analysis were used to detect the presence of compounds like Alkaloids, Flavonoids, Saponins, Tannins, Phenols, Proteins, Cardiac glycosides, Terpenoids, Carbohydrate & Quinones. For the analysis of the presence of the chemical in the extract there are different procedures were carried out.

2.3.1 Test for Phenolic

Take 1 ml of extract add 2ml of 5% aqueous ferric chloride. Formation of blue color indicates the presence of phenol in the extract. Otherwise, phenols are absent.

2.3.2 Test for Flavonoids

Take 2 ml of Extract and add few drops of 20% NaOH, the intense yellow color was formed. Then add 70% dilute HCl, the yellow color get disappear. Formation and disappearance of intense yellow color indicates presence of Flavonoids in the Extract. No yellow coloration indicates absence of Flavonoids in extract.

2.3.3 Test for Tannin

Take 1 ml of extract and add 10% of alcoholic ferric chloride. Formation of brownish blue or bluish black color indicates the presence of tannins in the Extract. Otherwise it shows the absence of tannins in the Extract and leaves ethanol extract.

2.3.4 Test for Saponins

2 ml of Extract are mixed with 6 ml of distilled water. Shake the mixture vigorously. Formation of persistent Foam/bubbles indicates the presence of Saponins in the Extract. Otherwise it shows the absence of Saponins in the Extract and leaves ethanol extract.

2.3.5 Test for proteins

Take 2 ml of extract from each sample and add 1ml of 40% NaOH and few drops of 1% copper sulphate. Formation of violet color indicates the presence of peptide linkage molecule in the extract. Otherwise, proteins are absent in the given extract.

2.3.6 Test for cardiac glycosides

Take 1 ml of extract of each solvent and add 0.5ml of glacial acetic acid and 3 drops of 1% aqueous ferric chloride solution. Formation of brown ring at the interface indicates the presence of cardiac glycosides in the extract. No formation of brown ring indicates negative test.

2.3.7 Test for carbohydrates

Take 1ml of Extract, add few drops of Molisch's reagent and then add 1ml of concentrated sulphuric acid at the side of the tubes. The mixture was then allowed to stand for 2 to 3 minutes. Formation of red or dull violet color indicates the presence of carbohydrates in the extract. Otherwise it shows the absence of carbohydrates in the extract.

2.3.8 Test for quinines

Take 1ml of extract add concentrated Hydrochloric Acid. Formation of yellow precipitate or Coloration indicates the presence of Quinones in the Extract. Otherwise indicates absence of Quinones in the given extract.

2.3.9 Test for alkaloids

Take 2 ml of Extract and add few drops of Wagner's reagent. The Wagner's reagent was prepared by mixing of 0.635 g of Iodine and 1 g of Potassium Iodide in 50 ml of distilled water. Formation of Reddish-Brown precipitate indicates the presence of alkaloids in the extract. Otherwise, alkaloids are absent.

2.3.10 Test for Terpenoids

Take 1ml of extract of each solvent and add 0.5 ml of chloroform followed by a few drops of concentrated sulphuric acid. Formation of reddish brown precipitate indicates the presence of Terpenoids in the extract. Otherwise, negative test.

2.4 Quantitative Estimation

2.4.1 Total flavonoids contents determination

The total flavonoids content was determined using the Dowd method. 5 mL of 2 % Aluminum trichloride (AlCl₃) in methanol was mixed with the same volume of the extract solution (0.4 mg/mL). Absorption readings at 415 nm were taken after 10 minutes against a blank sample. The total flavonoids content was determined using a standard curve with catechins as the standard. Total flavonoids content is expressed as mg of catechins equivalents (CE) / g of extract (RAMAMOORTHY, AWANG BONO, 2007)

REF: RAMAMOORTHY et al, *Antioxidant Activity, Total Phenolic and Flavonoid Content of Morinda Citrifolia fruit Extracts from Various Extraction Processes*, Journal of Engineering Science and Technology, 2007, Vol. 2, No. 1, 70 – 80.

2.4.2 Total carbohydrate content determination

1ml for solvent to estimate the polysaccharide content, add 1ml of 5% phenol to the 1ml of sample solution, and then add 5ml of concentrated H₂SO₄ and measure the absorbance after 10 minutes at 488nm against blank. Then compare it with standard solution of glucose. To prepare Blank, 1ml of distilled water added 1ml of 5% phenol followed by 5ml of Concentrated H₂SO₄. (Manisha Bhatti1, Anjoo Kamboj1*, Ajay Kumar Saluja2, 2013)

REF: Manisha Bhatti et al, *Spectrophotometric Estimation Of Total Polysaccharides In Kalanchoe Pinnatum And Kalanchoe Crenata*, International Journal Of Pharmacy And Pharmaceutical Sciences, 2013, Vol 5, Issue 2

2.4.3 Total protein content determination

- Coomassie Brilliant Blue-50g
- Ethanol (99%)-25ml
- O-Phosphoric Acid -50ml
- Make up volume 500 ml by DW and Filter through Whatman filter paper, store RT.

Standard assay method

1. Prepare Stock of Bovine serum albumin with 2 mg/ml concentration.
2. Required dilutions of BSA Stock (2 mg/ml) are prepared from 0.2 mg/ml to 1.4 mg/ml. Prepare duplicates of each sample.
3. For the calibration curve, dilutions of BSA Stock (2mg/ml) are prepared from 0.2mg/ml to 1.4 mg/ml as shown in the Table 1
4. Add 3 ml of Bradford's reagent to each tube and mix well by inversion or gentle vortex mixing. Avoid foaming, which will lead to poor reproducibility.

5. Incubate for 5-45mins at room temperature

6. Measure the Absorbance at 595nm of the samples and standards against the reagent blank before 1 h after mixing.

2.4.4 Total tannins contents determination

The tannins were determined by slightly modified Folin and Ciocalteu method. Briefly, 1 ml of the sample extract is added with 7.5 ml of distilled water and adds 0.5 ml of Folin Phenol reagent, 1 ml of 35% sodium carbonate solution. The absorbance was Measured at 725 nm. Tannic acid dilutions (0 to 0.5mg/ml) were used as standard solutions. The results of tannins are expressed in terms of tannic acid in mg/g of extract. (Md. Sazzad Hossain 1, Nizam Uddin 1, et al, 2013)

REF: (Md. Sazzad Hossain et al, *Phytochemical, cytotoxic, in-vitro antioxidant and anti-microbial investigation of ethanolic leaf extract of Zizyphus rugosa lam*, IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS), 2013, Volume 6, Issue 5, PP 74-81)

2.4.5 Total alkaloids content determination

1g of the sample was weighed into a 50 ml beaker and 20 ml of 10% acetic acid in Solvent was added and covered and allowed to stand for 4 h. This was filtered and the extract was concentrated on a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitated was collected and washed with dilute ammonium hydroxide and then filtered. The residue is the alkaloid, which was dried and weighed (Gracelin et al.)

REF: D. herin sheeba gracelin* a. john de Britto and p. Benjamin jeya rathna kumar, 2013, *qualitative and quantitative analysis of phytochemicals in five pteris species*, International Journal of Pharmacy and Pharmaceutical Sciences, Vol 5, Suppl 1.

2.5 DPPH [1, 1 -diphenyl-2-picryl hydrazyl] Assay

Free radicals are generally very reactive molecules possessing an unpaired electron which are produced continuously in cells either as byproducts of metabolism or by leakage from mitochondrial respiration. The free radicals produced in-vivo include the active oxygen species such as the superoxide radical O₂⁻, hydrogen peroxide (H₂O₂) and hypochlorous acid (HOCl). Oxygen free radicals have been shown to be responsible for many pathological conditions. Free radicals and Reactive Oxygen Species (ROS) cause DNA damage, lipid peroxidation, protein damage. They are known to be involved in the pathogenesis of a wide variety of clinical disorders such as Cancer, Cardiovascular diseases, inflammatory diseases, asthma and aging. Free radicals like the hydroxyl radical, hydrogen peroxide, super-oxide anion etc. mediate components of the inflammatory response, with production of migratory factors, cyclic nucleotides and eicosanoids. Super-oxide radicals amplify the inflammation process, increasing vascular permeability, adhesion of polymorph nuclear leucocytes to the endothelium and stimulation of platelet aggregation.

2.5.1 Procedure

In brief, a 250 µl total reaction volume contains 200 µl of ethanol / positive control / various concentration of test solution and 50 µl of 0.659mM DPPH. The reaction mixture is incubated at 25° C for 20 minutes, following which the absorbance is read at 510 nm % inhibition is calculated by the formula

$$\% \text{ inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

3. RESULT & DISCUSSION

3.1 Qualitative Estimation

3.1.1 Alkaloids

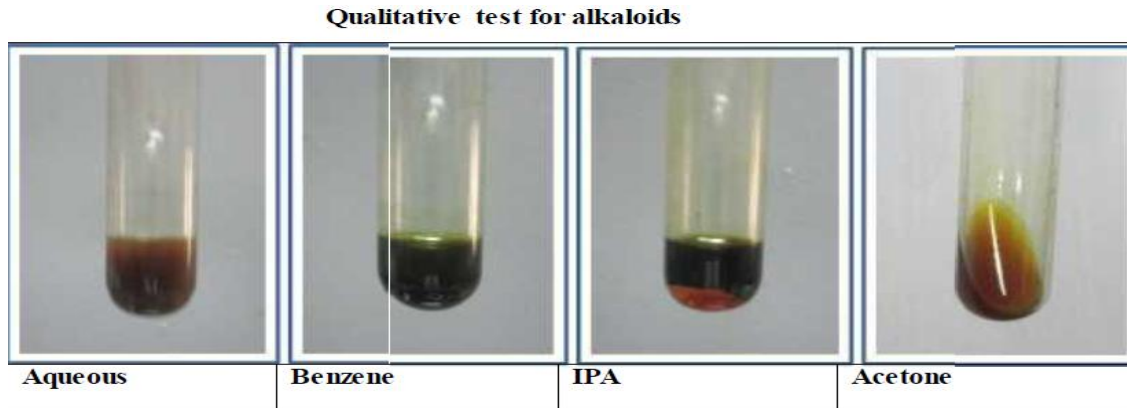


Figure.1. Qualitative test for Alkaloids

3.1.2 Carbohydrates

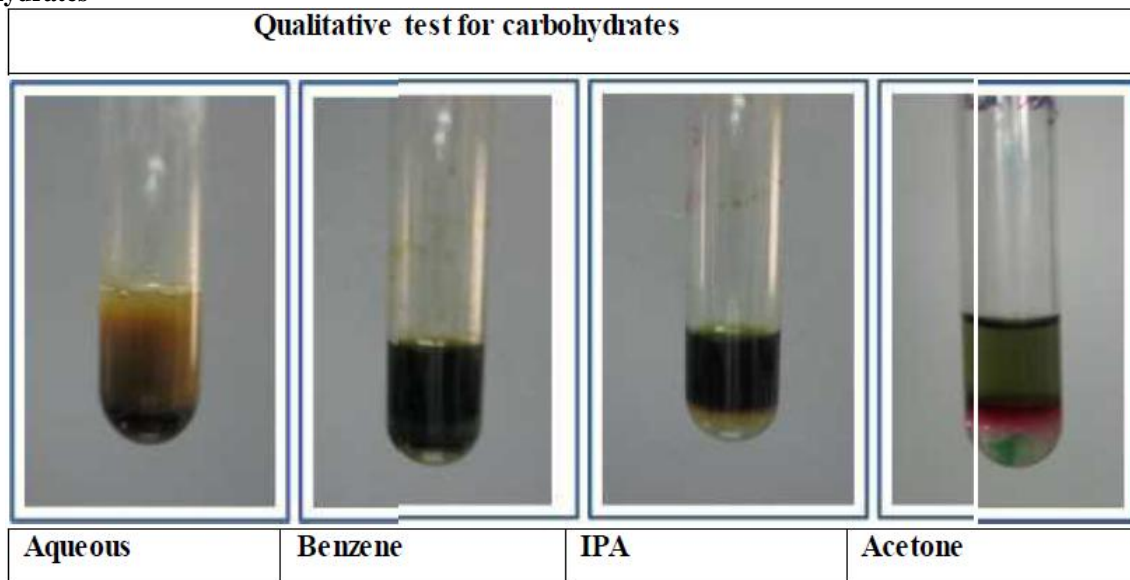


Figure.2. Qualitative test for Carbohydrates

3.1.3 Cardiac Glycosides

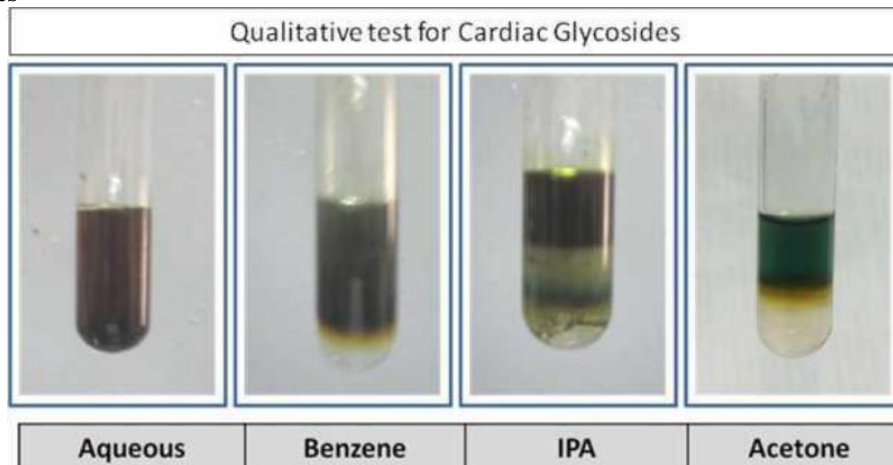


Figure.3. Qualitative test for Cardiac Glycosides

3.1.4 Flavonoids

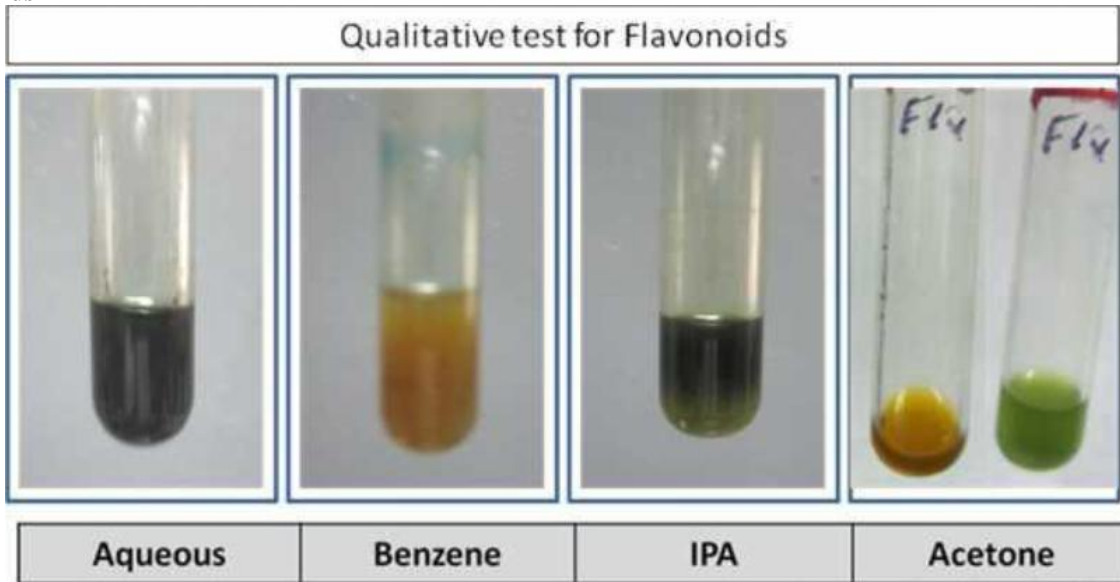


Figure. 3. Qualitative test for Flavonoids

3.1.5 Phenol

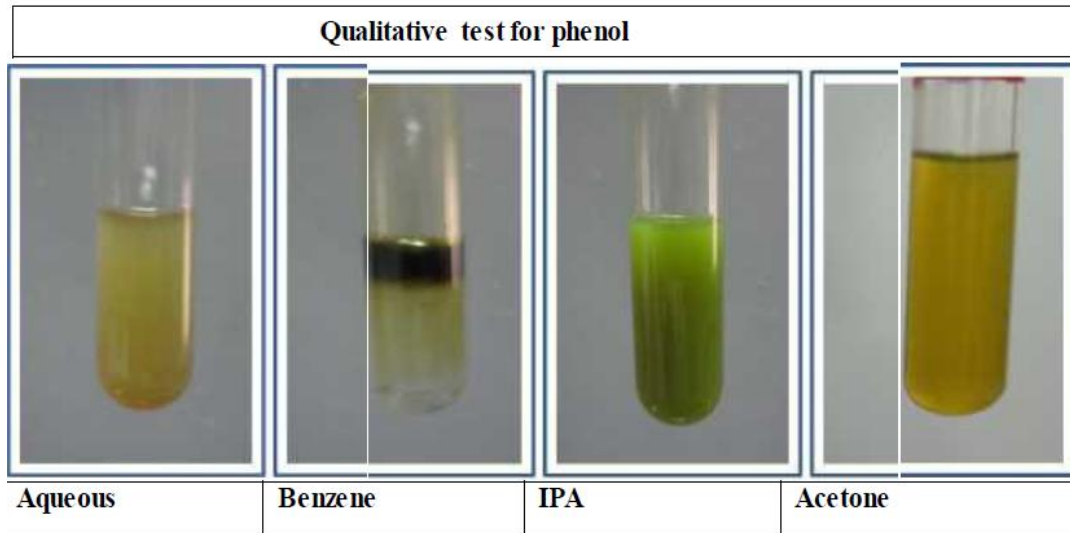


Figure.5. Qualitative test for Phenol

3.1.6 Saponins

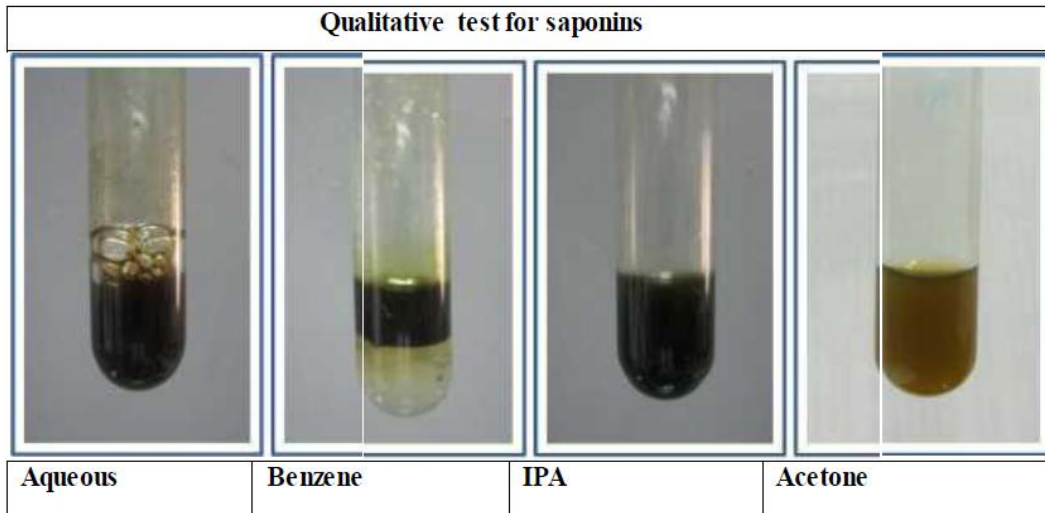


Figure.6. Qualitative test for Saponins

3.1.7 Tannins

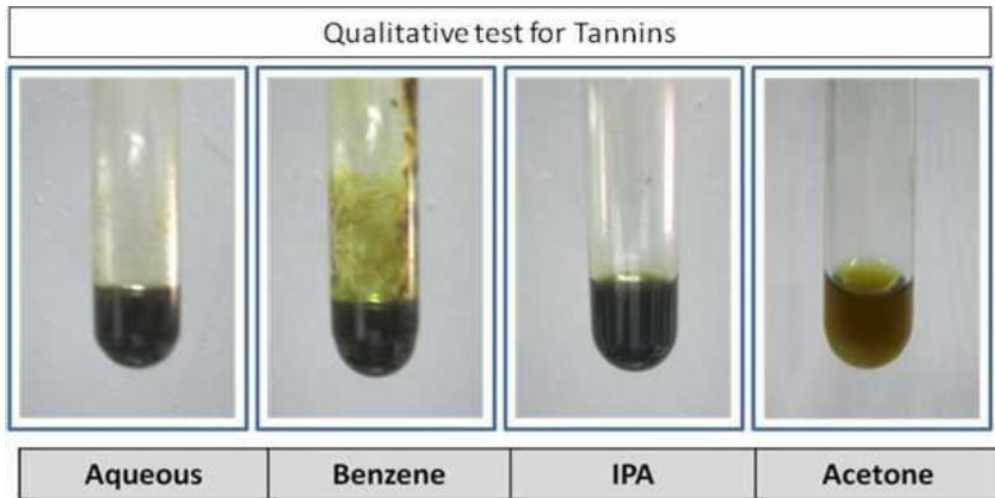


Figure.7. Qualitative test for Tannins

3.1.8 Terpenoids

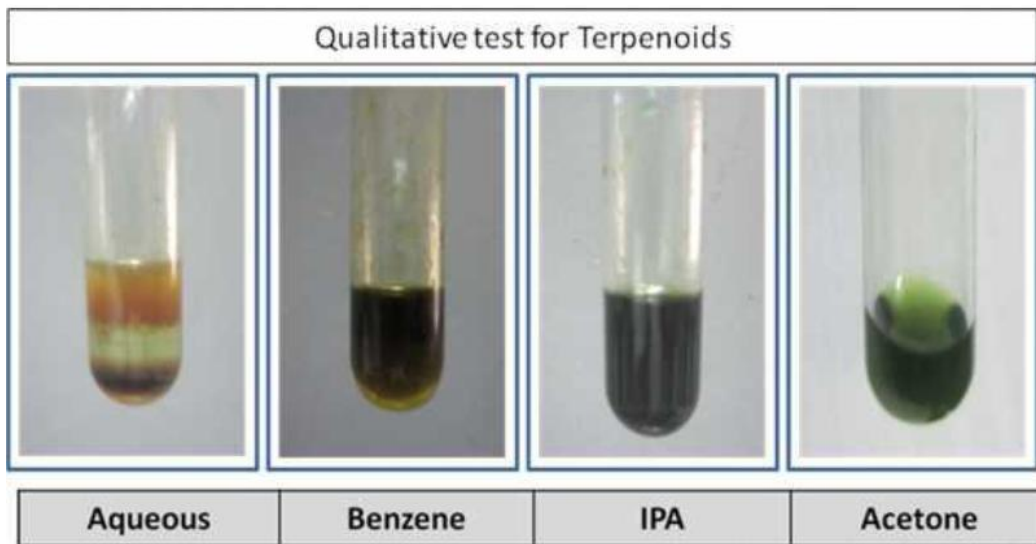


Figure.8. Qualitative test for Terpenoids

3.1.9 Quinones

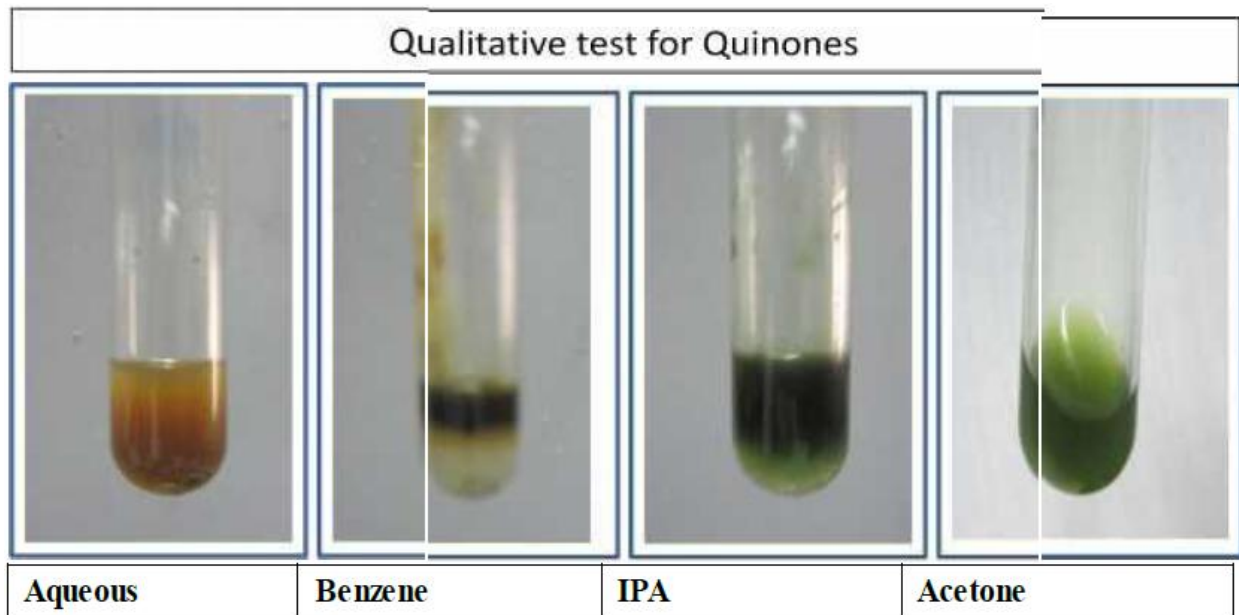


Figure.9. Qualitative test for Quinones

3.1.10 Proteins

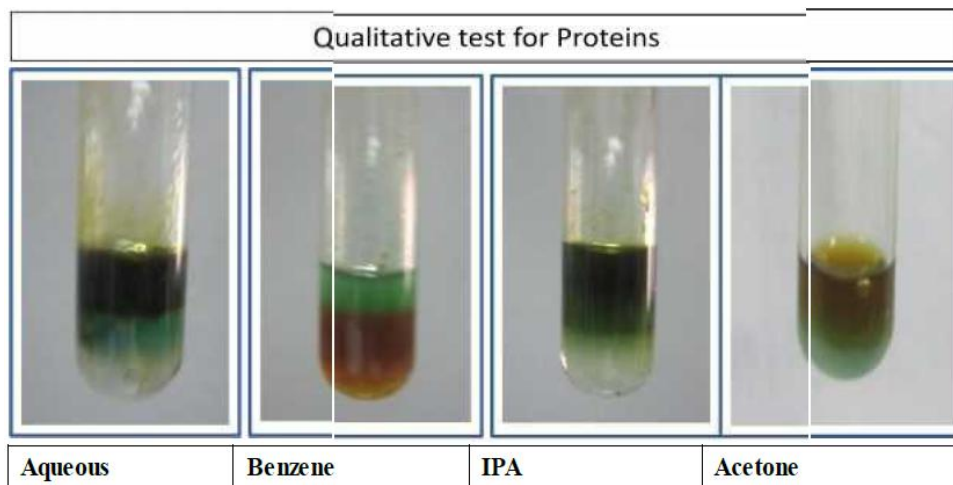


Figure.10. Qualitative test for Proteins

Table .3.1 Overall Qualitative Estimation

Test	Extracts			
	Aqueous	Benzene	IPA	Acetone
Alkaloids	-	-	+	+
Carbohydrates	-	-	-	+
Cardiac Glycosides	-	+	+	+
Flavonoids	-	+	-	+
Phenols	-	-	-	-
Saponins	-	-	-	-
Tanins	+	+	+	-
Terpenoids	+	-	-	-
Quinones	+	-	-	-
Proteins	+	-	+	-

- + = Presence of Compounds, - = Absence of Compounds

3.2 Quantitation Estmiation

3.2.1 Carbohydrates: The phytochemical quantitative estimation by plotting Concentration and Absorbance at 725 nm

from that graph Carbohydrates was found is 0.115 mg/ml in Acetone solvent.

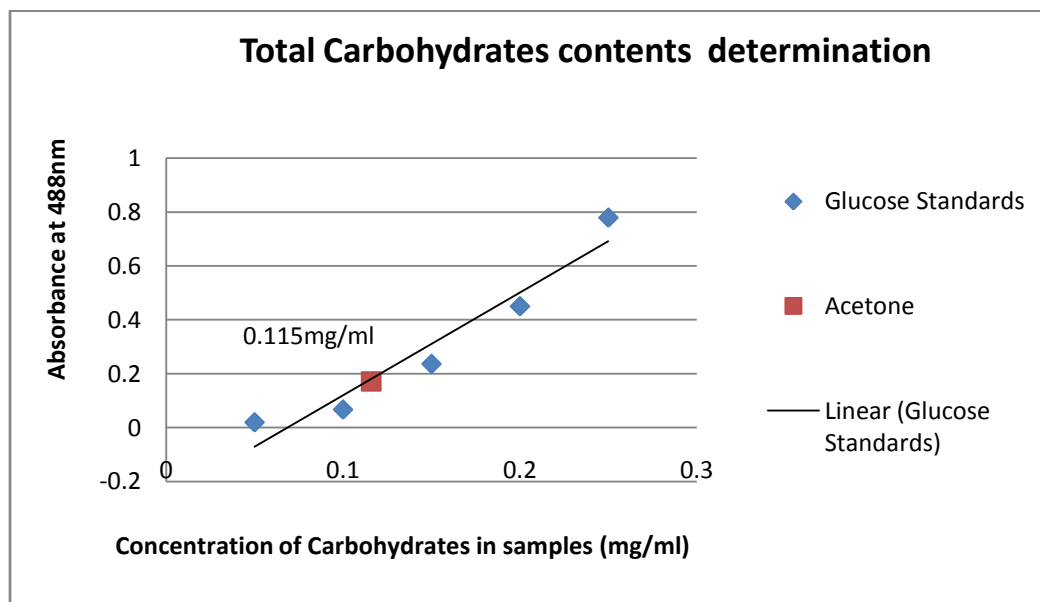


Figure.11. Determination of Carbohydrates

3.2.2 Tannin: From graph we can conclude Tannin was iso propyl alcohol solvent. estimated 0.841 mg/ml in aqueous solvent and 0.218 mg/ml in

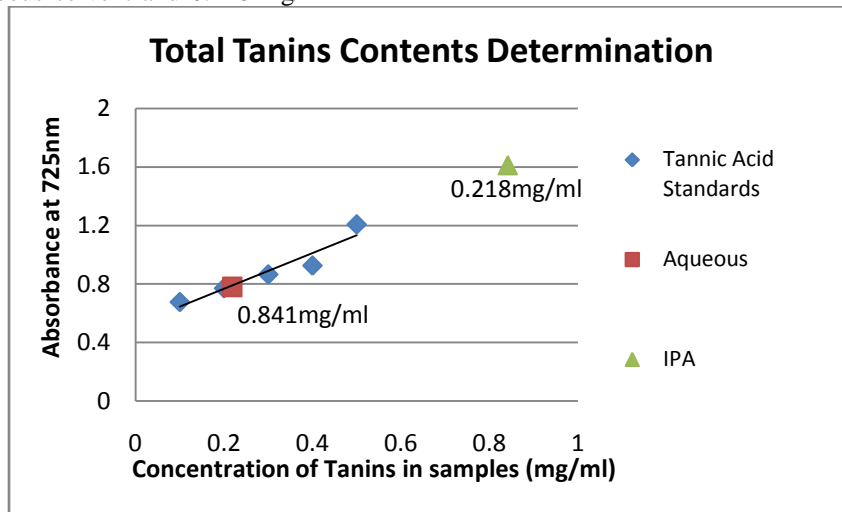


Figure.12. Determination of Tannin

3.2.3 Flvonoids: Flavonoids can be determined by 6 mg/ml in Acetone solvent and 6.85 mg/ml in Benzene solvent that has shown in Fig 3.3

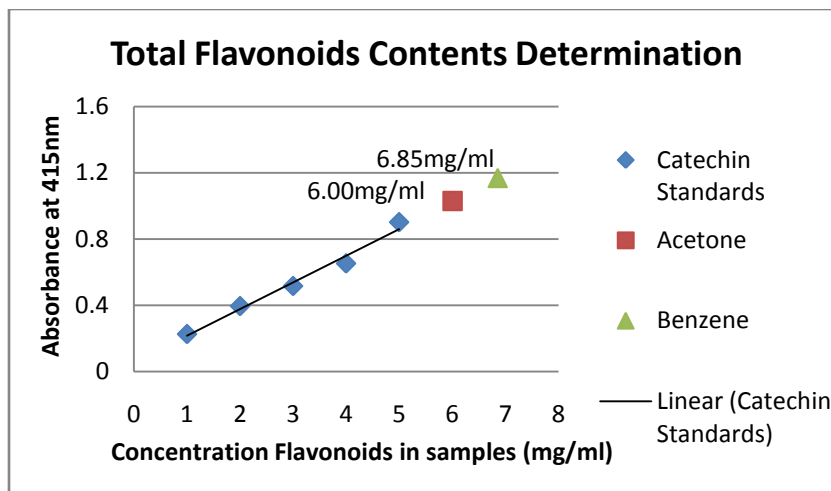


Figure.13. Determination of Flavonoids

3.2.4 Protein

Protein can be determined by 0.484 mg/ml in Aqueous solvent and 0.787 mg/ml in Iso Propyl Alcohol that has shown in Fig in 3.4

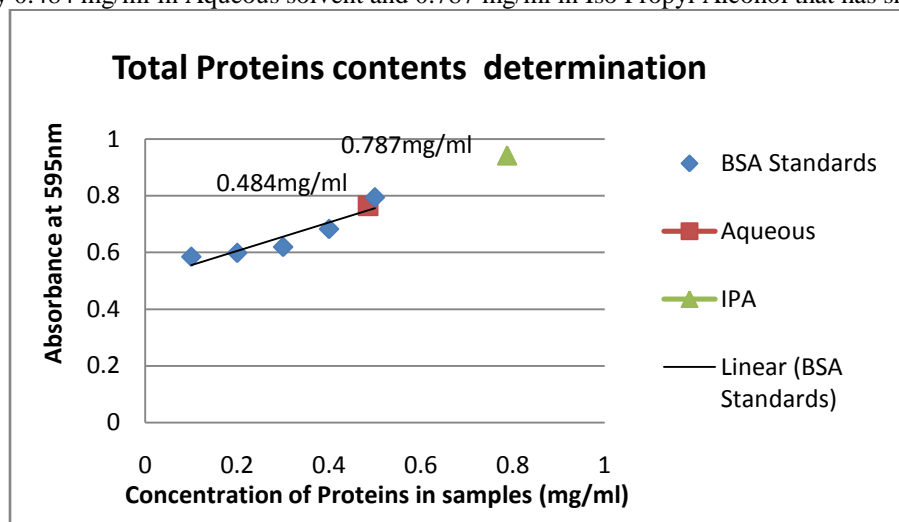


Figure.14. Determination of Protein

The plants studied here can be used as a potential source of drugs. Further studies are going on these plants in order to

isolate, identify and characterized.

3.2.5 Alkaloids

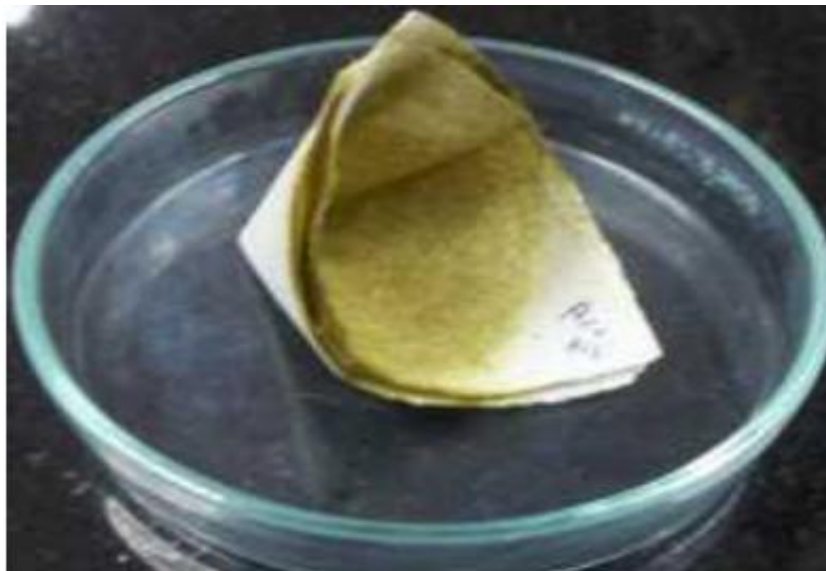


Figure.15. Determination of Alkaloids

Residue obtained after filtration of alkaloid precipitated solution. Residue obtained after filtering was dried and weighed. Dry weight of residue was found to be 40mg/g of Sample.

3.3 Antioxidant Activity

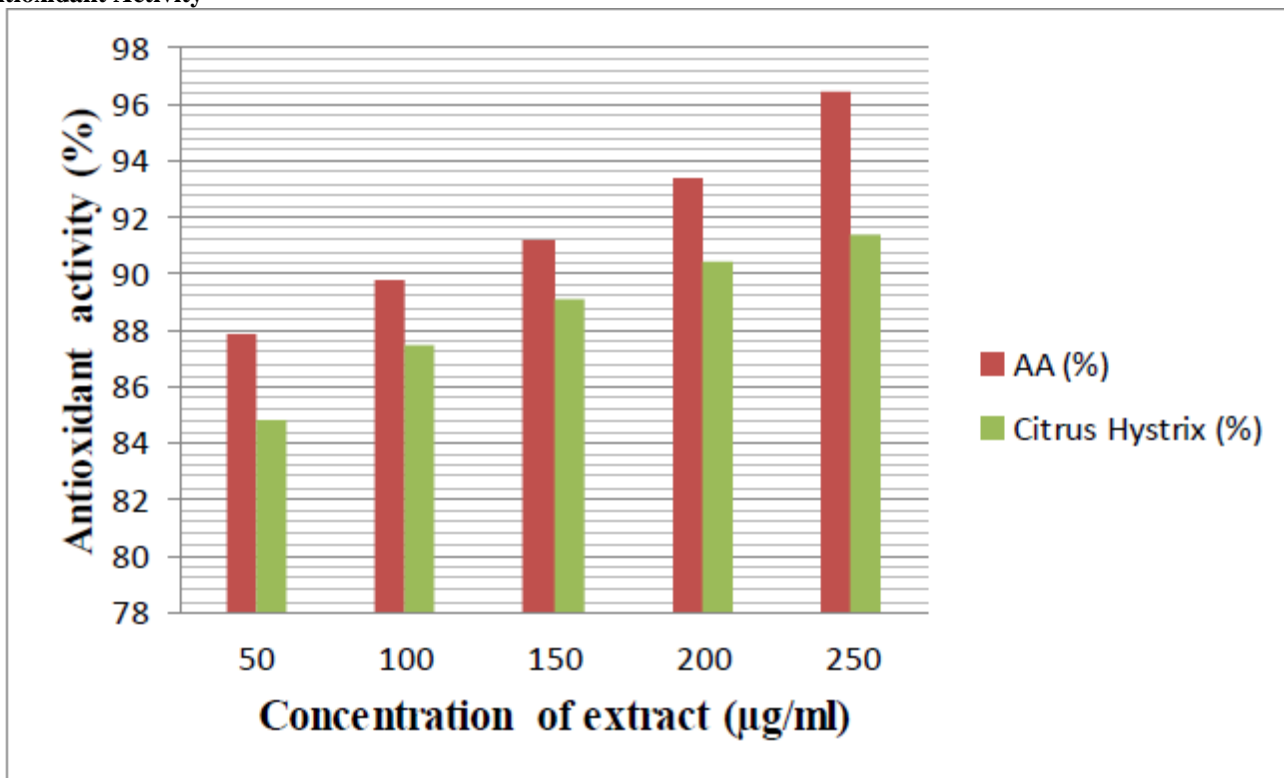


Figure.16. Comparison of Antioxidant Activity

4. CONCLUSION

Many medicinal virtues have been attributed to Indian citrus. The fresh fruit is light, laxative and diuretic. A tablespoonful of each fresh citrus juice and honey mixed together forms a very valuable medicine for the treatment of several ailments. It should

be taken every morning. Its regular to use will promote vigor in the body within a few days. When fresh fruit is not available, dry powder can be mixed with honey. In India, the fruits are taken as liver tonic to enrich blood. Phytochemical screening of (IPA, aqueous, acetone and benzene) extracts of herbs citrus hystrix had revealed the presence of flavonoids, tannins, terpenoids,

alkaloids, cardiac glycosides, proteins, carbohydrates and quinones by positive reaction with the respective test reagent. By using acetone to extract cardiac glycosides, flavonoids, alkaloids and carbohydrates and also using IPA to extract cardiac glycosides, tannins, alkaloids and proteins. Saponins and phenol we can't extract these solvents like IPA, benzene, acetone and aqueous. An AA value is the concentration of the sample required to scavenge 50% of the free radicals present in the system. AA value is inversely related to the antioxidant activity of crude extracts. Lowest AA value and highest activity was found in etanolics extracts of *Citrus Hystrix* compared to ascorbic acid.

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