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Isolation and identification of pineapple extract (*Ananas comosus*) through different analytical techniques and evaluation of antimicrobial activity.

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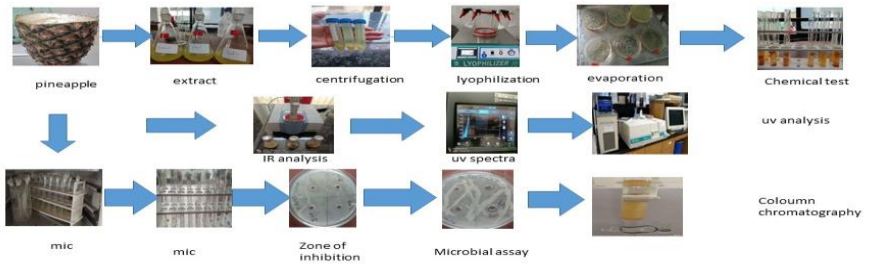
Abstract:

Pineapple is a versatile fruit and it has different pharmacological properties it is gaining much importancein the medicinal field. India is a sub-tropical country and people are suffering from different microbiologicalinfections. As a result, they are taking different synthetic antibiotics from pharmacy shops without knowing theproper course duration and adverse effects. So they are not properly cured and get again infected and becomedrug-resistant. So we are searching for herbal antibiotics that will have good antibacterial activity, fewer sideeffects, and be cost-effective. For this search, we have isolated the different phytoconstituents through column Chromatography and identified them through UV visible spectroscopy and FT-IR. We have investigated the minimum inhibitory concentration and antimicrobial activity. The extract showed different phytochemical andpositive results against different microbes.

Key Words-*Annanas comosus*, Pineapple extract, Isolation, column chromatography, Lyophilization, FT-IR, antimicrobialactivity.



Introduction

People are taking different synthetic antibiotics and also taking different synthetic drugs from the medicine shop.But they are not taking this medicine properly means from time to time, they are not following the dosage and not completing the course as a result different drug resistance is occurring and also different side effects are occurring due to different chemical drug intake so we are searching for the herbal antimicrobial activity of the pineapple extract. From the literature review we found that this drug having different amino acid, so amino acid having particular activity of the bacterial inhibition. Different people have been suffering from different problems so, if we make a herbal preparation from this enzyme, and actually that is a natural enzyme, it will be beneficial for us in the future.

It is derived from the Spanish word pina meaning pine cone that was used in 1398. 300 years later it was called pineapple in order to identify the fruit individually. It was discovered on the island of Guadalupe in 1493 by Europeans. It was treated as a luxurious fruit because of the sort of prestige that it had. The skin of pineapple is used to make alcohol, animal food and vinegar. Pineapple is considered the third most important fruit in the worldwith a projected production of 27.8 million tons for 2020. The pineapple, also known as *Ananas comosus belongs* to the family Bromeliaceae. It is the leading edible member of the family Bromeliaceae, grown in several tropical and subtropical countries including Philippines, Thailand, Indonesia, Malaysia, Kenya, India, and China. It has been used as a medicinal plant in several native cultures. Pineapples as a fruit have effective juice and a vibrant tropical flavor that balances the tastes of sweet and tart.

The pineapple is an herbaceous perennial, which grows to 1.0 to 1.5 m (3 ft 3 in to 4 ft 11 in) tall, although sometimes it can be taller. The plant has a short, stocky stem with tough, waxy leaves. When creating its fruit, itusually produces up to 200 flowers, although some large-fruited cultivars can exceed this. Once it flowers, the individual fruits of the flowers join together to create a multiple fruit. After the first fruit is produced, side shoots(called 'suckers' by commercial growers) are produced in the leaf axils of the main stem. These suckers may be removed for propagation, or left to produce additional fruits on the original plant. Commercially, suckers that appear around the base are cultivated. It has 30 or more narrow, fleshy, trough-shaped leaves that are 30 to 100 cm (1 to 3+½ ft) long, surrounding a thick stem; the leaves have sharp spines along the margins. In the first yearof growth, the axis lengthens and thickens, bearing numerous leaves in close spirals. After 12 to 20 months, the stem grows into a spike-like inflorescence up to 15 cm (6 in) long with over 100 spirally arranged, trimerous flowers, each subtended by a bract.

The ovaries develop into berries, which coalesce into a large, compact, multiple fruit. The fruit of a pineapple isusually arranged in two interlocking helices, often with 8 in one direction and 13 in the other, each being a Pineapple is a type of fruit that is high in nutrients. One of the pineapples that are popular is honey pineapple.This type of pineapple has a delicious and refreshing taste. The pulp of honey pineapple has a thicker pulp thanpineapples in general, is more extensive than regular pineapples, and has a slightly yellowish colour to orange.Pineapple fruit contains lots of nutrients, including vitamin A, calcium, phosphorus, magnesium, iron, sodium,potassium, dextrose, sucrose (cane sugar), and the enzyme bromelain, which is a 95% mixture of cysteineproteases that can hydrolyse protein (proteolysis) and resistant to heat.

Apart from the pulp, pineapple peel also brings many benefits. Pineapple pulp contains many nutrients, and pineapple peel is known to have antioxidants helpful in warding off free radicals. Pineapple peel also contains nano cellulose which is helpful in the pharmaceutical, food and medical industries. Nanocellulose from pineapplepeel has been used as a tooth-strengthening material.

Extensive studies have been done with bromelain to explore its clinical properties. Bromelain is a chief protease enzyme found in pineapple plants (*Ananas comosus*). It has been known chemically since 1876 and was identifiedfor the first time by Marcano in 1891. The investigation and isolation of bromelain has been started since 1894. It was first therapeutically supplemented

in the year 1957. Sulfhydryl proteolytic enzymes are the chief constituents of bromelain. Bromelain is abundant in the stem and fruit of pineapple plants and it can also be isolated in small amounts from pineapple waste such as core, leaves, peel etc.

Experimental Section

Preparation of Extract:

The pineapples were purchased from the local market of Barasat. The herbarium was prepared and sent to BotanicalGarden of India, Shibpur, Howrah-711103 for identification. The specimen number was BRN/SS/PNP/0345. The pineapple peels were cut off into slices. Then, take the pineapple core and cut it into small pieces. These small pieces were given into the mixer grinder for crushing. After complete crushing, the liquid extract was filtered through the gauze. The filtrate of pure pineapple juices is collected in a container. I preparedthree solution types in 100 ml of pure juices and added 100 ml of methanol. ii) Take 100 ml of pure juices and add 100 ml of ethanol. iii) take 100 ml of pure juices and add 100 ml of acetone. After 2 days,the solutions are evaporated in three petri dishes at 37 °C centigrade. After complete evaporation, the final product is taken, and methanol, ethanol, and acetone are added.

Identification test of phytoconstituents present in pineapple:

Test for alkaloids:

I took 3 ml of Pineapple Solution with a few drops of 1% HCl, heated it in a steam bath, and then added a few drops of Mayer & Wagner's reagent. Turbidity indicates the presence of an alkaloid.

Test for steroids:

I dissolved 2mL of Pineapple Solution into Chloroform and added 2mL of concentrated Sulphuric acid tothe mixture; the red color developed, indicating the presence of steroids.

Test for terpenoids:

Take 1 ml of Pineapple Solution, add 0.5 mL of acetic anhydride (acetic acid), and then a

fewdrops of concentrated Sulphuric acid. The appearance of Bluish bluish-green precipitate confirmed the presence ofterpenoids (Mynott et al. 1996).

Test for phlobotannins:

2 ml of Pineapple Solution was hydrolyzed using 1% HCl, and the mixture was boiled for a few minutes.The deposition of a red precipitate indicates the presence of phlobatannins.

Test for glycosides:

Dissolve 2 ml of Pineapple Solution into Chloroform, add 2 ml of Acetic acid into the mixture, then heatand cool. A few drops of sulphuric acid were added, and the color changed from blue to green, confirmingthe presenceof glycosides.

Test for amino acids:

I took 1 mL of Pineapple Solution and treated it with a few drops of Ninhydrin reagent. The developmentof a purple color confirmed the presence of amino acids (Neumayer et al. 2006).

Test for saponins:

2 ml of pineapple Solution was shaken vigorously with 5 ml of distilled water and heated in a water bath;stable foam was formed, indicating the presence of saponins.

Test for tannins:

2 ml of Pineapple Solution was taken with 2 ml of distilled water and stirred; then, a few drops of Ferric chloride solution were added. A green precipitate appeared, showing the presence of tannins.

Identification test of amino acids:

Ninhydrin Test:

At first, a few drops of the sample and a few drops of Ninhydrin reagent were taken. Then, the solution was heated for five minutes. If the result is blue, it confirms that alpha amino acids are present.

Lead sulphide test:

Take 1 ml sample, add a few drops of sodium hydroxide, and put marble chips in the solution. Then, heat the solution for 5 to 10 minutes. It was then cooled by running water. After that, add a few dropsof 10% lead acetate solution. If black residue comes, then it will be Cysteine

(Heinicke et al. 1972).

Millon's test:

Take a 1ml sample, add a few drops of Millon's reagent, and shake the solution. Then add a few drops of Concentrated Nitric Acid. If the red color shows, then it will be tyrosine.

Histidine test:

First, take a 2 ml sample, add 5% bromine and 33% acetic acid solution drops, and place it for 10 minutes.Then, boil 2 ml of ammonium carbonate for 5 minutes. If a blue color is shown, then it will be histidine (Neumayer et al. 2006).

Identification test of carbohydrates:

Molisch's test: To the test solution, add a few drops of alcoholic α-naphthol, then add a few drops of concentrated sulphuric acid through the sides of the test tube. Purple to violet, a color ring appears at thejunction.

Benedict's test: Take 5 ml of Benedict's reagent. Add 8 drops of pineapple juice solution. Boil over a flame or ina boiling water bath for 2 minutes. Let the solution cool down.

TLC STUDIES:

Thin Layer Chromatography is a technique used to isolate non-volatile mixtures. The experiment is conducted ona sheet of aluminum foil, plastic, or glass coated with a thin layer of adsorbent material. The material usually used is aluminum oxide, cellulose, or silica gel. Thin layer chromatography wasperformed with variouscrude extracts such as hexane, chloroform, ethyl acetate, and methanol of *Ananascomosus* (Heinicke et al. 1972).

TLC studies for enzymes:

For each extract, the solvent ratio was 1:1 of acetone and water, methanol, and water. The TLC plate should be 8 cm long, and spots should be above 1 cm. A Silica gel plate is used to separatethe compounds.Based on the band that appears in the TLC plate, the Retention Factor (Rf) value can be calculated.

Rf value=Distance traveled by the compound/Distance traveled by solvent front.

TLC for saponins:

To detect the saponins in the extract, we take chloroform, glacial acetic acid, methanol, and water as a standard ratio of 64:32:12:8. We take the ratio of solvent as 16:8:3:2. After preparing the solvent, we makethe TLC chamber. Then, spotting was done in a TLC plate through a capillary

tube.

TLC for flavonoids:

To detect the saponins in the extract, we take Ethyl acetate: Formic acid: Glacial acetic acid: Water as a standard ratio of 25:2.75:2.75:6.5. After preparing the solvent, we make the TLC chamber. We were then spotted in a TLC plate through the capillary tube—estimation of proteolytic activity on gelatin under UV-VIS spectroscopy.

Sample: Methanolic and Ethanolic extract of pineapple juice.

Methodology for TLC of Pineapple extracts:

Prepare the TLC chamber and plate:

Obtain a TLC chamber with a lid. An inexpensive chamber can be made using a beaker and watch glass.Cut a piece of filter paper (or two) so that when placed in the chamber, the filter paper fits inside the chamber and is flat on the bottom but not obscuring your view of the inside.

Saturation: The filter paper keeps the chamber saturated with vapors so when the eluent rises on the plate,it doesn't quickly evaporate but continues to climb and undergo the chromatography. If the eluent evaporated, movement would stop, but it could also change the local composition of a mixed eluent and affect the results.

The stationary phase applied to the plate is made to dry and stabilize by us. We use sample spots; thin marks are made at the bottom of the plate with the help of a pencil. We take six TLC papers for methanolicand ethanolic extracts for three mobile phases. We Apply sample solutions to the marked spots (Secor etal. 2005). We pour the mobile phase into the TLC chamber, and to maintain equal humidity, we place moistened filter paperin the mobile phase. We Place the plate in the TLC chamber and close it with a lid.It is kept so that the sample faces themobile phase. We immerse the plate for development. Remember to keep the sample spots well above the level of the mobilephase. Do not immerse it in the solvent. Then,wait till the development of spots. Once the spots are developed, please take out the plates and dry them. Thesample spots can be observed under a UV light chamber. We also take water as a sample and these three solutions as a mobile phase (Chobotova et al. 2010).

Isolation through column chromatography

Chemicals: hexane, ethyl acetate, chloroform, acetone, methanol.

Apparatus: Round bottom flask, Chromatography column, Pipettes.

Preparation of Solution: prepare four types of solution with pineapple juices: a) Ethanolic solution withhexane and ethyl acetate (in 1:1 ratio), b) Ethanolic solution with chloroform and acetone (in 1:1 ratio), c)Methanolicsolution with methanol and ethyl acetate (into 1:1 ratio) d)

Methanolic solution with chloroformand acetone (into1:1 ratio)

Preparation of the column:

The wet pack method is used to prepare the column. The chromatography column is made with a plastic tip with a frit,a one-way stopcock, and a plastic funnel. The column is filled with enough silica gel to get therequired height.The dry silica gel is poured into a beaker, and hexane (pet ether) is added.

The mixture is swirled and then poured into the column. The column is tapped so air is not trapped as thesilica gel settles. Then, it is added with a small amount of sand after settling the silica gel. The column should not contain air bubbles and should be homogeneous. Then, the solvent level is allowed to drop to the levelof the silica gel sand intersection (Harrach et al. 1995).

Running the column:

The first solution (Ethanolic solution with hexane and ethyl acetate) is added directly to the prepared column.Then, the stopcock is opened, and the liquid level falls to the top of the silica gel, or the solution ispassed throughthe silica gel, divided into various fractions, and collected in the test tube. The first fraction(elutes) is collected in the test tube, and in seconds, one is collected in another test tube. In the same way,all the solutions are running, and the elutes are collected in various test tubes for further testing (Fissore et al. 2023).

Analysis through FTIR:

Analysis by Fourier Transform Infrared (FTIR) spectroscopy is a powerful technique used to identify andcharacterize chemical compounds based on their unique infrared absorption spectra. Here's a general overview of how to perform analysis using an FTIR spectroscopy machine. The machine was purchasedfrom a certified company, BRUKER, and the machine model is ALPHA II. Three solvents were aqueous, methanol, and ethanol (Harrach et al. 1995; Fissore et al. 2023).

Sample Preparation: Prepare your sample for analysis by placing it on an FTIR-compatible sample holder. Ensure that the sample is uniformly spread or dispersed for accurate measurements.

Instrument Setup: Turn on the FTIR spectrometer and allow it to warm up according to the manufacturer'sinstructions. Align the optical components of the instrument, including the source, beam splitter, and detector, asper the instrument manual. Set the appropriate measurement parameters, such as the desired wavelength range and resolution.

Baseline Correction: Perform a baseline correction by acquiring a background spectrum. Place an emptysample holder or a blank matrix pellet in the spectrometer and record a spectrum. This accounts for any environmental, instrument, or matrix material contributions, allowing you to focus on the sample's spectralfeatures.

Sample Measurement: Place your prepared sample holder or pellet into the FTIR spectrometer and recordthe sample spectrum. Ensure that the sample is properly aligned with the light beam. Acquire the spectrum by allowing the instrument to collect the infrared light transmitted through or reflected by the sample.

Data Analysis: Analyze the obtained FTIR spectrum using appropriate software or data analysis tools. Compare the observed peaks and their intensities with reference databases, such as spectral libraries or known standards, to identify functional groups and specific compounds present in your sample. Various software packages are available to assist with spectral interpretation and identification.

Interpretation and Reporting: Interpret the FTIR spectrum by assigning characteristic peaks to specific chemical bonds or functional groups. Describe the identified compounds or molecular structures based onthe spectral information. Compile your findings in a report or document for further analysis or presentation.

It is important to note that FTIR spectroscopy is a versatile technique that can be used for various sampletypes, such as solids, liquids, gases, and thin films. The sample preparation and measurement techniquesmay also vary depending on the specific instrument and sample requirements. Consult the instrument

manual and relevant literature for detailed instructions and guidelines specific to your analysis (Tysnes etal. 2001).

Antimicrobial Activity of Pineapple Extract:

Preparation of the Extract:

First of all, we collected fresh pineapple from the Barasat market, and then we cut the pineapple into pieces.Then, we collected the pineapple juice through the mixer grinder and shifted it through cotton gauze. Afterthat, we centrifuged the juice at 10000 rpm at a centrifugal machine purchased from Vinayak Enterprises(BWU/LC/1).

Lyophilization and Drying of the Centrifuged Powder:

After centrifugation, we collect the centrifuged powder and decant it from the centrifugal tube. Then, welyophilize the powder in a Lyophilizer machine purchased from Bio Technical Resources Certified Company, CE Approved (BWU/LPZ/1). The Lyophilization is done at -37.5PSI.

Extraction of the Lyophilized Powder:

Then, we collected the powder and mixed it with Ethanol, Methanol, and Aqueous 5mg/ml in three conicalflasks. Then, keep the solution in the fridge at -20ºC for 3 days in intermediate shaking. Then, these 3 solutions evaporate in a water bath at 37°C for 3 days. After evaporation, the 3 solutions are wrapped withfoil paper and kept for future use (Barth et al. 2005; Dong et al. 2014).

Antimicrobial Activity of Pineapple Extract:

Microorganisms

Three potent foodborne pathogens were used in the experiment, including gram-negative bacteria (*Salmonella typhi* NCTC 786), gram-positive bacteria (*Bacillus subtilis* ATCC 6633), and gram-negativebacteria (*Pseudomonas aeruginosa* ATCC 9027). These were obtained from the Central Drugs LaboratoryOffice of the Director Government of India 3, Kyd Street, Kolkata-700016.

Antimicrobial Testing:

Agar disc diffusion assay

The antimicrobial activity of the PPE was tested against three foodborne, waterborne, and airborne pathogens using the agar disc diffusion method according to the process of the Central Drugs Laboratory.Within 15 min after the bacterial suspension preparation, a sterile cotton swab was dipped into the suspension and swabbed on Mueller-Hinton agar (MHA). The PPE was prepared at different concentrations (0.0337, 0.0675, 0.1349, 0.2699 and 0.5398 mg/L). Then, the PPE or the standard positivecontrol (20 mL) of each microorganism or negative control (methanol) was individually impregnated ontoa disc (6 mm diameter) and dried. The Discs were placed on the microorganism lawn on MHA and incubated at 37℃ for 18 s. Then, the inhibition zone was measured (mm.) (Bazvand et al. 2014).

Broth dilution assay

The broth dilution assay was used to determine the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of the PPE regarding the method of Central Drugs Laboratory. The PPE was mixed with the microbial suspension to get the desired final concentrations (0.5398-0.0042 g/ml) in the test tubes (2-8), and the tubes (1 and 10) were the sterile control and the growth control respectively. The MIC (the concentration where the first inhibition was observed) and MBC (the concentration where no growth was observed) were observed after incubation at 37 C for 24 hours (Ajayi et al. 2022). *Salmonella typhi, Bacillus subtilis,* and *P. aeruginosa* are all bacteria inhibited by the above concentrations (aqueous, ethanol, and methanol).

Results and discussion

Different phytochemical present in pineapple is presented in **table 1**. Where it shows pineapple extract is having Alkaloids, Flavonoids, Steroids, Amino acid, Glycosides.

Different amino acid in pineapple is presented in **table 2.** Where it shows pineapple extract is positive for xanthoproteic test which does not contain phenylamine, tyrosine, tryptophan.

The Folin's Mc Carthy Sullivan's test is positive and shows presence of methionine.

The histidine test produces positive test for tryptophan and Millon's test produces positive results for the presence of tyrosine.

Different carbohydrate identification test in **table 3** was positive like Molisch's test and Benedicts test that shows presence of glucose, fructose, sucrose.

Rf Value of different TLC methods in **table 4** shows presence of amino acid and compared with the standard.

UV visible data for acetone, ethanol and methanol in **table 5** shows absorbance of flavonoid and glycosides.

UV analysis of fraction collected through column chromatography from **table 6 and 7** shows presence of different component isolated form different solvent mixture and compared with standard of plant secondary metabolite.

UV spectrum from **fig 1 and 2** shows the absorbance for flavonoid and glycoside and FTIR spectrum from **fig 4,5,6** shows different functional group which is analyzed in FTIR finger print analysis.

From **table no 8** the broth dilution assay we got the minimum inhibitory concentration for three different extracts and three different bacteria. The following data given in the below table**.** Methanolic extract shows 3,5,7,10 mg/ml againstthe Salmonella typhi Acetone extract shows 10 mg/ml against the *Salmonella typhi*. Ethanolic extract shows 5,7,10 mg/ml against the *Salmonella typhi*. Methanolic extract shows 5,7,10 mg/ml against the *Bacillus subtilis*. Acetone extract shows 7,10 mg/ml against the *Bacillus subtilis*. Ethanolic extract shows 3,5,7,10 mg/ml against the *Bacillus subtilis*. Methanolic extract shows 10 mg/ml against the *Bacillus subtilis*. Acetone extract shows 10mg/ml against the *Bacillus subtilis*. Ethanolic extract shows 3,5,7,10

mg/ml against the Bacillus subtilis.

Different zone of inhibition from table no 9 was expressed with standard and ***Salmonenella typhi, Bacillus subtilis, P. aeruginosa***

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Conclusion

From the above experimental work, it was concluded that the pineapple extract has different phytochemicals. The different phytochemicals were identified through TLC and chromatography. The different phytochemicals have different antibacterial activity.

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