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Analysis of Glycoside Compound in Apple Family Fruit and Its Detection by Using HPLC Method

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ABSTRACT

The objective of the present study was to evaluate the phytochemical constituents, anti-oxidant activity and high-performance liquid chromatography (HPLC) analysis of glycoside compound from apple and avocado fruit methanolic extract. Preliminary screening involved the qualitative methods to detect the presence of flavonoids, phenols, tannins, steroids, amino acids, saponins, proteins, cardiac glycosides, and alkaloids. Glycoside and flavonoid contents were quantitatively estimated. Antioxidant activity pf methanolic extract of fruits was evaluated by studying ferric reducing antioxidant power assay using the standard procedure. The phytochemical analysis of fruit extracts of apple, avocado, pear, peach has revealed the presence of significant secondary metabolites such as steroids, cardiac glycosides, saponins, tannins, phenols, flavonoids, amino acid, and alkaloids. Quantitatively estimated glycoside content was higher in apple and least in avocado fruit extracts, and the flavonoid content was higher in avocado and least in peach fruit extract. The methanolic extract of apple, avocado, pear and peach fruits had shown significant ferric reducing antioxidant power assay. The fruit extracts were subjected to chromatographic fractionation followed by high-performance liquid chromatography. The major and sharp peaks for apple were found at 4 min while that of avocado lies between 1-2 min by using Gallic acid as standard.

Keywords: Phytochemical screening, HPLC, Apple fruit, Methanolic extract

INTRODUCTION

Numerous studies have confirmed the benefit of phenolic compounds for human health. Studies have shown that different groups of phenolic compounds, as well as their individual components, have a specific effect on different physiological systems. Phenolic compounds in apples have an antiviral effect against Herpes simplex strains and the flu virus [1,2], and they suppress cell proliferation [3], reduce blood levels of triglycerides and low-density lipoproteins [4], and thus are useful in the prevention of cancer and cardiovascular diseases. Polyphenolic compounds protect the gastrointestinal mucosa and may be used as a preventive measure against the adverse effects of anti-inflammatory drugs [5]. Phenolic compounds in apples may be potentially useful in protecting the lungs against damage from cigarette smoke [6] as well as for the prevention of Alzheimer's disease [7]. Many antioxidant compounds can be found in fruits and vegetables including phenolics, carotenoids, anthocyanins, and tocopherols [8]. Approximately 20% of known plants have been used in pharmaceutical studies, impacting the healthcare system in positive ways such as treating cancer and harmful diseases [9]. Plants are able to produce a large number of diverse bioactive compounds. High concentrations of phytochemicals, which may protect against free radical damage, accumulate in fruits and vegetables [10]. Plants containing beneficial phytochemicals may supplement the needs of the human body by acting as natural antioxidants [11]. Various studies have shown that many plants are the rich source of antioxidants. For instance, vitamins A, C, E, and phenolic compounds such as flavonoids, tannins, and lignins, found in plants, all act as antioxidants [10]. The consumption of fruits and vegetables has been linked with several health benefits, a result of medicinal properties and high nutritional value [12]. Antioxidants control and reduce the oxidative damage in foods

by delaying or inhibiting oxidation caused by reactive oxygen species (ROS), ultimately increasing the shelf-life and quality of these foods [13]. Beta carotene, ascorbic acid, and many phenolics play dynamic roles in delaying aging, reducing inflammation, and preventing certain cancers [14]. Increasing the consumption of fruits and vegetables has been recommended by many agencies and health care systems throughout the world [15]. The main objective of this study was to evaluate phytochemical and phenolic properties of different fruit samples which belong to Rosaceae family like apple, avocado, peach and pear using HPLC method.

MATERIAL AND METHODS

For Bacterial Isolation Soil samples were collected from the park near Indira Nagar, Lucknow (U.P.) For Extraction Different fruit samples of the apple family was collected Apple, Avocado, Peach, and Pear from local market of Indira Nagar, Lucknow. Fruit sample was collected from the local market and kept in a refrigerator for freeze drying at -20˚C. Serial dilution is carried out from the soil sample.

Identification of unknown bacterial species

Gram staining

Gram staining technique was developed by Dr. Hans Christaian Gram. This technique is used for the morphological study of unknown bacterial species.

Biochemical test

The biochemical test was according to the methodology by Clarke and Cowan [15,16].

QUALITATIVE ANALYSIS OF THE PHYTOCHEMICALS

3.1: Alkaloids (Mayers's Test)
3.2: Amino Acids (ninhydrin test)
3.3: Flavonoids (Alkalkine Reagent Test)
3.4: Glycosides (Keller-Killian Test)
3.5: Phenols (Ferric Chloride Test)
3.6: Proteins (Xanthoproteic Test)
3.7: Saponins (Froth Test)
3.8: Steroids (Liebermann Burchard Test)
3.9: Tannins (Gelatin Test)

QUANTITATIVE DETERMINATION OF PHYTOCHEMICAL CONSTITUENTS OF FRUITS

Determination of alkaloids

Quantitative determination of alkaloid was according to the methodology by Harborne [17].

Determination of flavonoid

Flavonoid determination was by the method reported by Ejikeme et al. [18] and Boham and Kocipai [19].

Determination of cyanogenic glycoside

Cyanogenic glycoside quantitative determination methodology used in this research is that by Amadi et al. [20] as reported by Ejikeme et al. (2014) [18].

Determination of antimicrobial activity

Antimicrobial activity was determined by agar well diffusion method by Arora and Kaur [21].

Antioxidant test

Ferric Reducing Antioxidant Power (FRAP) the method of Benzie and Strain [22] was followed in determining the FRAP.

High-performance liquid chromatography (HPLC)

Individual polyphenols in extracts were determined by using RP-HPLC-PDA. Analytical consisted of a Varian system

(USA) equipped with a ProStar230 solvent delivery module and a ProStar330 PDA Detector. Compounds were separated on an OmniSpher C18 column (250×4.6 mm inner diameter, 5 µm, Varian, USA) protected with a guard column (ChromSep 1 cm×3 mm, Varian, USA). Mobile phase A was 0.1% phosphoric acid in water and mobile phase B 100% HPLC grade methanol. Gradient was as follows: 0 min 5% B; 0 to 5 min from 5 to 25% B, 5 to 14 min from 25 to 34% B, 14 to 25 min from 34 to 37% B, 25 to 30 min from 37 to 40% B, 30 to 34 min from 40 to 49% B, 34 to 35 min from 49 to 50% B, 35 to 58 min from 50 to 51% B, 58 to 60 min from 51 to 55% B, 60 to 62 min from 55 to 80% B, 62 to 65 min 80% B, 65 to 67 min from 80 to 5% B, 67 to 72 min 5% B; with flow rate=0.8 ml min−1. Injection volume for samples and standards was 20 µl; compounds were separated at room temperature. A 10-min re-equilibration period was used between individual runs. UV–vis spectra were recorded in the wavelength range from 190 to 600 nm. The detection wavelength was 280 nm for procyanidins, monomeric flavan-3-ols, and dihydrochalcones, 320 nm for phenolic acids, 360 nm for flavonols, and 510 nm for anthocyanins. Identification was based on the comparison of retention times and spectral data with those of authentic standards. Furthermore extracts were spiked with polyphenol standards which gave additional information on polyphenol identification**.**

RESULTS

Isolation of bacteria

The bacterial culture was isolated from nutrient agar medium followed by serial dilution after that the plates were incubated at 37˚C for 24 hours. After incubation, the colonies were observed (Figure 1).

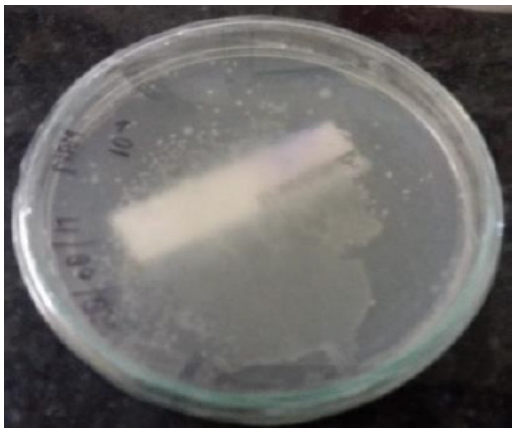

Figure 1: Isolation of bacteria.

Streaking

Streaking was done by taking a single colony of bacteria from pure culture and spread in a zig-zag manner (Figure 2).

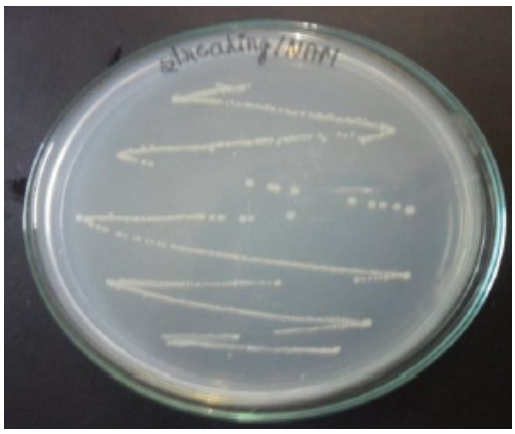

Figure 2: Streaking of bacteria.

Gram's staining

In Gram's staining pink color rod-shaped bacteria were observed in the compound microscope. The bacterias were

gram-negative bacteria. Which could be either of the following like *Escherichia coli*, *Salmonella, Pseudomonas, Enterobacteriaceae* etc. (Figure 3).

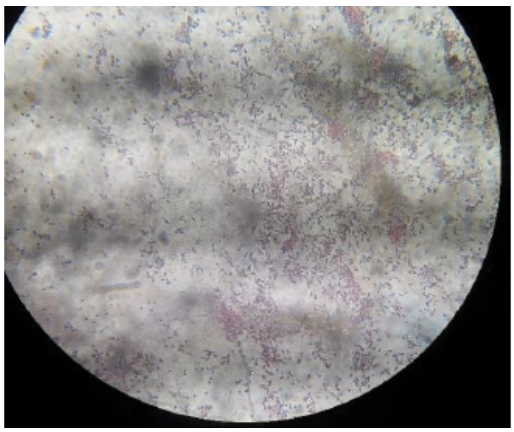

Figure 3: Gram's staining of bacteria pink color rod-shaped bacteria.

IDENTIFICATION OF ISOLATED BACTERIA USING BIOCHEMICAL TEST

Indole test

The bacterial species taken from pure culture shows a positive result for the indole test. This indicates that bacteria were able to produce tryptophanase enzyme after addition of Kovac's reagent which forms a red color ring (Figure 4).

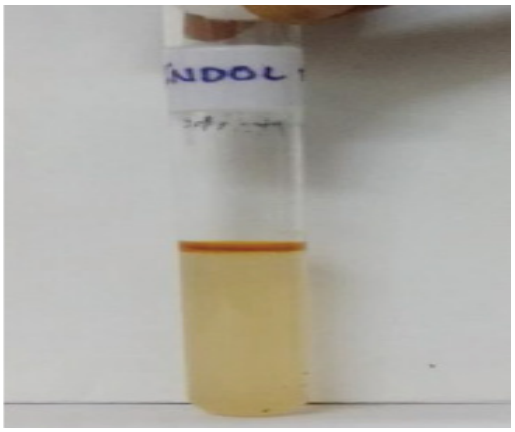

Figure 4: Indole test is positive for the bacteria; there is the appearance of a reddish brown color ring.

Oxidative test

In oxidative fermentation test, acid production is detected in the medium by the appearance of a yellow color. Following are th+e reaction patterns (Table 1).

Table 1: Oxidative Fermentation

Open (Aerobic) Tube	Covered (Anaerobic) Tube	Metabolism
Acid (Yellow)	Alkaline (Green)	Oxidative

Acid production in the open tube (aerobic) and the covered tube (anaerobic) indicates an oxidative result. In this test non-fermenting bacteria that metabolize glucose via oxidative metabolism give an oxidative result (Figure 5).

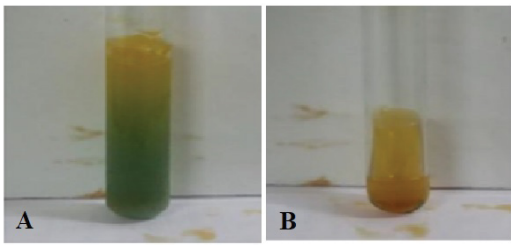

Figure 5: (A) Covered tube (green) is anaerobic in nature which involves oxidation (B) Open tube (yellow) acid is aerobic in nature which involves acid production.

Citrate test

In citrate, test growth was visible on the surface of slant and the medium showed an intense blue color. This indicates that bacteria contains the properties of citrate, hence, the result was positive. The alkaline carbonates and by-carbonates produced as by-products of citrate catabolism raise the pH of the medium to above 7.6 causing the bromothymol blue to change from the original green color to blue (Figure 6).

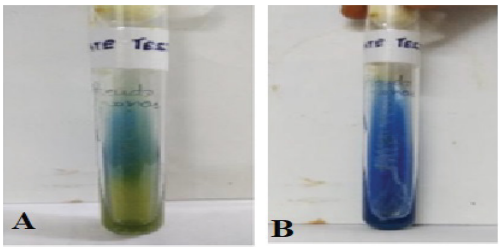

Figure 6: Citrate test shows the positive result which turns green color (A) to intense blue color (B).

Urease test

Organisms that hydrolyze urea rapidly may produce a positive reaction within 1 or 2 hours whereas less active species May 5 or more days. In this test development of an intense magenta color to bright pink color in 5 to 7 days indicates a positive result (Figure 7).

Figure 7: Urease test indicates a positive result; there is the appearance of a bright pink color.

Methyl red test

In this test, the culture medium turns red and forms a ring after addition of methyl red because of a pH at or below 4.4 from the fermentation of glucose which shows a positive result (Figure 8).

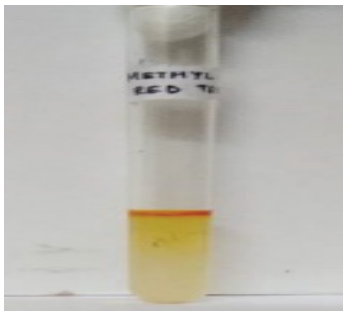

Figure 8: Formation of red ring depicts positive result in the methyl red test Gelatin Hydrolysis.

Gelatin hydrolysis

In this test, the inoculated tube was totally liquefied even after exposure to the cold temperature of the refrigerator at 4˚C which indicates the presence of gelatin in the bacterial culture (Figure 9).

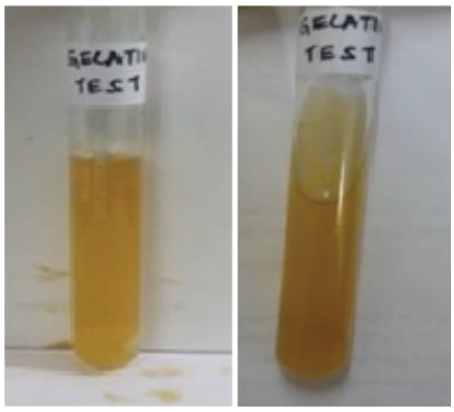

Figure 9: Total liquid-fraction of the media indicates the presence of gelatin.

Triple

Neither lactose/sucrose nor glucose was fermented, both the slant was red. No gas and H_2S was produced. The culture was alkaline(Figure 10).

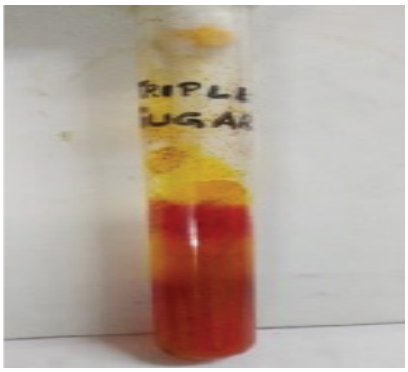

Figure 10: In total sugar iron test both butt and slant is red which indicates little or negligible production of gas and H2S.

Starch hydrolysis

In this test, transparent clear zones were formed around the colonies of bacteria after the iodine's solution which shows the positive result (Figure 11).

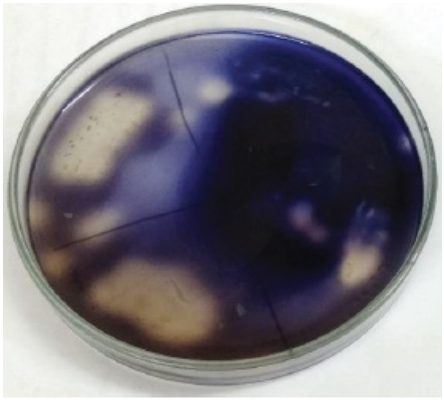

Figure 11: Starch hydrolysis test depicts a positive result by the formation of clear zones around the colonies.

QUALITATIVE ANALYSIS OF THE PHYTOCHEMICALS

Table 2: Qualitative analysis of phytochemicals in fruit sample.

Test	Apple	Avocado	Peach	Pear
Alkaloid	+++	+++	-	+
Amino acid	-	+++	-	-
Flavonoids	-	+++	-	-
Glycosides	+++	+++	+++	+++
Phenols (ferric chloride test)	-	-	-	-
Proteins(Xanthoprotic test)	+++	+++	+++	+++
Saponins (Froth test)	-	+++	-	-
Streoids (Liebermann Burchard test)	+++	+++	+++	+++
Tannins (Gelatin test)	-	-	-	-

Key; +++ = highly present; ++ = moderately present; + = slightly present; - = absent

Alkaloid

In Alkaloid Test, A cream color precipitation was obtained immediately that showed the presence of alkaloids (Figure 12).

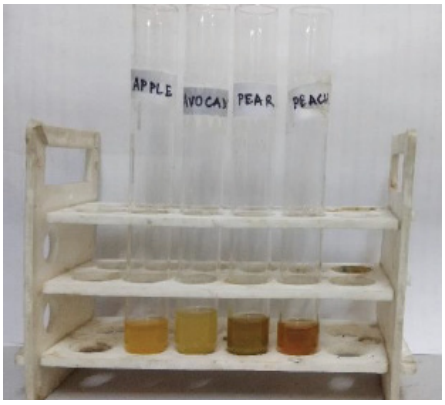

Figure 12: Cream color shows the presence of alkaloid in apple, avocado while a small amount is present in pear and completely absent in peach.

Amino acid

In Amino acid Test, Formation of blue color indicates the presence of amino acid (Figure 13).

Figure 13: Blue color shows the presence of amino acid, which is found in avocado while in apple, pear and peach amino acid is absent.

Flavonoids

In Flavonoids analysis, addition of a few drops of dilute acid that indicated the presence of flavonoids (Figure 14).

Figure 14: Yellow color shows the presence of flavonoid in avocado, while it is absent in apple, pear and peach.

Glycosides

In Glycosides Test, Lower reddish brown layer would indicate a positive test for glycosides (Figure 15).

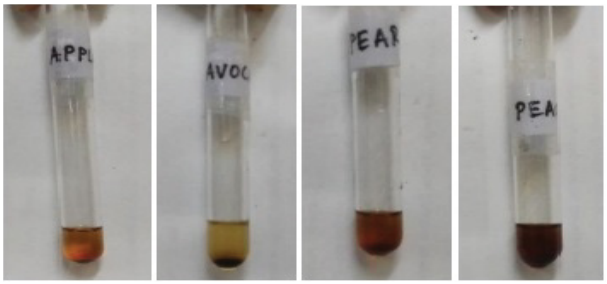

Figure 15: Lower reddish-brown layer shows the presence of glycoside in apple followed by avocado, pear, and peach.

Phenols

In Phenols Test, Formation of bluish black color indicates the presence of phenols (Figure 16).

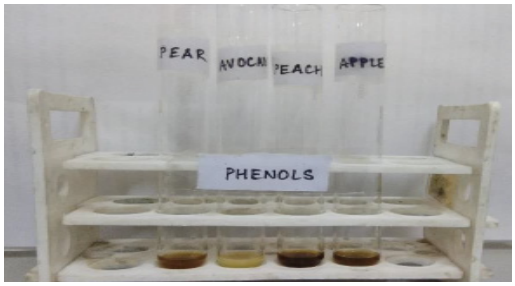

Figure 16: Bluish black color shows the presence of phenols, but in this test, phenol is completely absent in apple followed by avocado, pear, and peach.

Proteins

In Protein Test, Formation of yellow color indicates the presence of proteins (Figure 17).

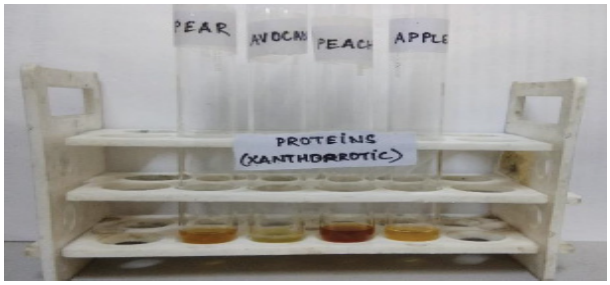

Figure 17: Yellow color shows the presence of proteins, which is highly present in apple followed by avocado, pear, and peach.

Figure 18: Foam formation shows the presence of saponins, which is only formed is avocado while in apple, pear and peach saponins are completely absent.

Saponins

In Saponins Test, Formation of 1cm layer of foam indicates the presence of saponins (Figure 18).

Steroids

The upper layer in the test tube was turns into red and sulphuric acid layer showed yellow with green fluorescence. It showed the presence of steroids (Figure 19).

Figure 19: The upper reddish brown layer and lower yellowish layer shows the presence of steroids, which is highly present is apple, avocado, peach and pear.

Tannins

In Tannins Test, Formation of white precipitation indicates the presence of tannins (Figure 20).

Figure 20: No precipitate formation shows the absence of tannins in apple, avocado, peach, and pear.

QUANTITATIVE ANALYSIS OF THE PHYTOCHEMICALS

Table 3: Quantitative analysis of phytochemicals in fruits.

Test	Apple	Avocado	Peach	Pear
Alkaloid	--	--	--	--
Flavonoid(g/100)	0.007	0.12	0.04	0.06
Glycoside(mg/100)	56.28	12.26	47.926	26.09

Key; -- = Absent

Flavonoid

In Flavonoid Test, It became colorless when an addition of a few drops of dilute acid that indicated the presence of Flavonoids (Figure 21).

Figure 21: In the quantitative analysis of flavonoid the highest amount was found in avocado.

Glycoside

In the quantitative analysis of glycoside, the highest concentration was found in apple (Figure 22).

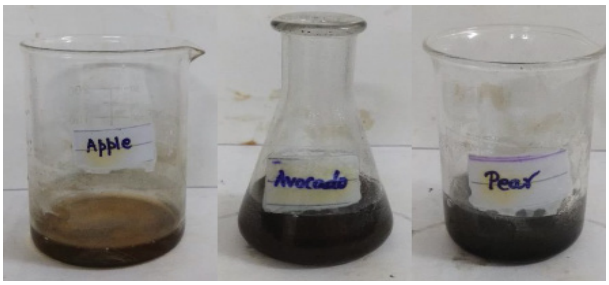

Figure 22: Titrating sample.

Anti-bacterial activity

In the present study the result of the antibacterial activity of fruits extract of apple, avocado, peach, and pear was investigated against bacterial strains; penicillin. The result revealed that the extract exhibited notable antimicrobial activity against bacterial species. Anti-bacterial potentials of fruits were compared according to their zone of inhibition against the pathogenic organism (Tables 4 and 5). The extract of fruit obtained from apple exhibited good anti-microbial activity against the bacterial strain. The extract derived from avocado, pear showed moderate activity while the extract obtained from peach showed low anti-microbial activity against the strain.

Table 4: Zone of inhibition (mm) against bacterial strain.

Sample	Zone of Inhibition (mm)
Apple	3.4
Avocado	3
Peach	2.6
Pear	3

Antioxidant activity

Ferric Reducing Assay (FRAP)

In FRAP test OD is observed depending upon the different concentrations of the samples (Figures 23-25).

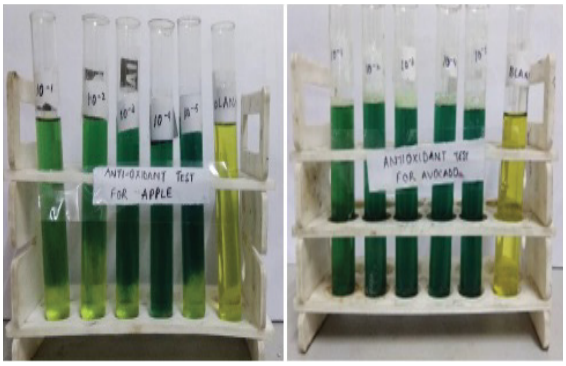

Figure 23: Apple and Avocado.

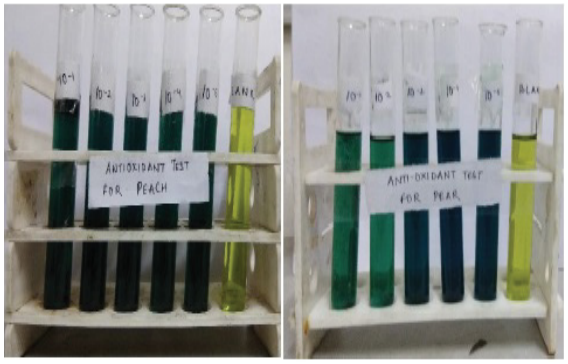

Figure 24: Pear and Peach.

Table 5: Average absorption of fruit extract of FRAP method.

Sample	Absorbance with different concentration				
	1-Oct	2-Oct	3-Oct	4-Oct	5-Oct
Apple	0.16	0.23	0.38	0.46	0.58
Avocado	0.3	0.46	0.52	0.55	0.58
Peach	0.46	0.5	0.53	0.74	0.79
Pear	0.22	0.46	0.65	0.95	1.04

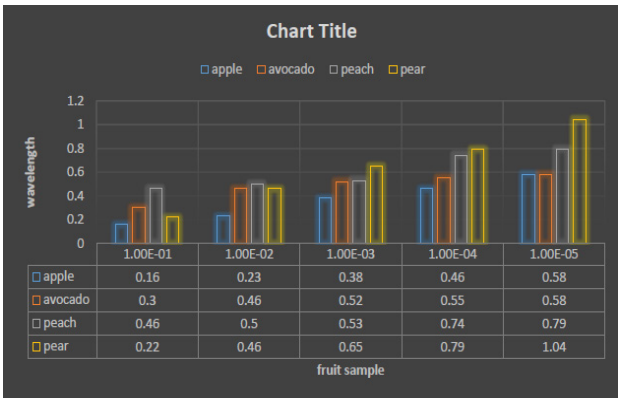

Figure 25: FRAP in different fruit.

Characterization and identification using HPLC

The fruit's extract of apple and peach were used for characterization and identification of Glycosides by HPLC. Separation of glycosides from apple and peach ethanolic extract was carried out using SYS LC-138 (Systronic, India) ODS C18 column (250 × 4.6 mm i.d., 5 µm particle), which was maintained at 25°C. A solvent used for the preparation of the mobile phases was HPLC Grade water. The samples were loaded individually and the chromatogram peaks of both samples were observed at 220 nm at the flow rate of 1 ml/minute. Two peaks were observed in the case of apple sample while a single peak was observed in the case of peach extract. The confirmation of the glycoside was done by comparing the chromatograms of the sample with the chromatogram of standard glycosides (Figures 26a and 26b).

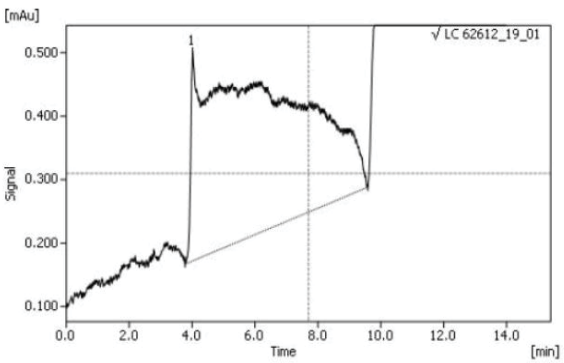

Figure 26a: Chromatogram of Apple sample.

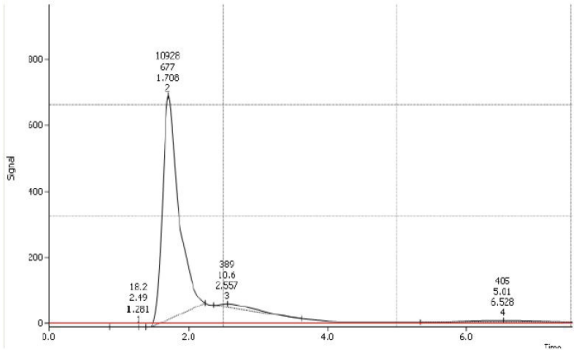

Figure 26b: Chromatogram of Peach sample.

The flow rate was 1 mL/min and the injection volume was 10 µL. The UV detection was performed at 290 nm. The UV spectra of each separated compound from extracts were compared to spectra of standards, which allow positive identification of compounds, based on the spectral match. Using the chromatographic conditions described above, the compound was eluted in less than 10 minutes.

CONCLUSION

The result of the study supports the traditional application of methanolic extracts of fruit which possess phytochemical and pharmacological along with antimicrobial properties that can be used in novel drugs for the treatment of microbial diseases. The phytochemical includes essential nutrients which are required for normal physiological functions. In this study, it is concluded that avocado shows the maximum phytochemical properties in glycosides and flavonoids followed by apple and pear fruit and least shown by peach fruit. In further studies, the fruit extracts were subjected to chromatographic fractionation followed by high-performance liquid chromatography. The major and sharp peaks for apple was found at 4 min while that of avocado lies between 1-2 min by using Gallic acid as standard.

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