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A COMPARATIVE STUDY OF PHYTOCHEMICALS, ANTIOXIDANT POTENTIAL AND FREE RADICAL SCAVENGING ACTIVITY OF *Psidium guajava* AND *Malus domestica*- An *in vitro* study

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ABSTRACT

The ethanolic extract of *Psidium guajava* and *Malus domestica* were qualitatively assayed for the presence of various phytochemicals. Total phenols, tannins and flavanoids in both the fruit extract were quantitatively determined. The antioxidant activity of both the fruit extract and the combined fruit extract was studied using the four complementary methods: (i) Free radical scavenging activity (ii) Nitric oxide scavenging activity (iii) Superoxide anion scavenging activity (iv) Ferric reducing antioxidant power assay (FRAP). These results indicated that *Psidium guajava* and *Malus domestica* possess independent antioxidant activity but when combined they showed more potent antioxidant activity. HPTLC finger print of the fruit extract showed the high amount of quercetin in *Malus domestica* than *Psidium guajava*.

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INTRODUCTION

Phytochemicals are naturally occurring, biologically active chemical compounds present in plants. More than 4000 compounds have been discovered. Many of these phytochemicals may help to protect cells against oxidative damage caused by free radicals.^[1] Most important action of these chemicals is similar to that of the antioxidants which react with free radicals. Free radicals can cause cell injury and cell death. The oxidative stress deregulates cellular functions and leads to cardiovascular dysfunction, neurodegenerative diseases, gastroduodenal pathogenesis and metabolic dysfunction.^[2]

In this study we choose two different edible fruits namely *Psidium guajava* and *Malus domestica* and compared their antioxidants and free radical scavenging activity. *Psidium guajava* L. commonly named guava, is a perennial fruit tree in subtropical and tropical areas. It is native to South American countries and was introduced to India by the Portuguese during 17th century *Psidium guajava* has high nutritional content and is especially rich in vitamin C.^[3]

Table 1: Botanical classification of *Psidium guajava* and *Malus domestica*

Biochemical Classification	<i>Psidium guajava</i>	<i>Malus domestica</i>
Kingdom	<i>Plantae</i>	<i>Plantae</i>
Division	<i>Magnoliophyta</i>	<i>Magnoliophyta</i>
Class	<i>Magnoliopsida</i>	<i>Magnoliopsida</i>
Order	<i>Myrtales</i>	<i>Rosales</i>
Family	<i>Myrtaceae</i>	<i>Rosaceae</i>
Genus	<i>Psidium</i>	<i>Malus</i>
Species	<i>guajava</i>	<i>Domestica</i>

MATERIALS AND METHODS

Plant Material

Red apples and white Guava were obtained from local market without any external defects. Fresh fruits were

Malus domestica is a medium sized tree belonging to the *Rosaceae*. It is commonly known as Apple. Apples are significant source of flavanoids in people diet in U.S and Europe. Apples ranked second for total concentration of phenolic compounds, and perhaps more importantly, apples had the highest portion of free phenolics when compared to other fruits.^[4, 5]

No reports are available for the comparative antioxidant capacities of these fruits. It was therefore planned to study the phytochemicals present in both the fruits extracts. The antioxidant capacity was determined to investigate which fruit extract had the maximum antioxidant and free radical scavenging activity. This study may enable further research to be carried out to separate the phenolic compounds and study their mechanism of action in preventing the various pathological diseases like cardiovascular and cancer. The Botanical classifications of the two plants are given in the **Table1**.

selected for analysis. Analysis was carried out on whole apple and guava and the results were expressed in terms of fresh weight. 10g of apple, 10g of guava and 5g of apple + 5g of guava tissue with skin were

homogenized with 100ml of absolute alcohol separately using warring blender. The extract was refrigerated for 72 hours and filtered through four layers of muslin cloth and the residue was re-extracted under the same condition with 100ml of absolute ethanol and the organic layer was allowed to evaporate and the dry residue was dissolved in alcohol. These ethanolic extracts were used for the qualitative and quantitative analysis of phytochemicals and for the determination of radical scavenging activity, ferric reducing capacity and antioxidant capacity.

PHYTOCHEMICAL ANALYSIS

QUALITATIVE ANALYSIS

Chemical tests were carried out on the ethanolic fruit extracts using standard procedures to identify the constituents. [6-8]

Test for flavonoids

5ml of dilute ammonia solution were added to a portion of the fruit extract followed by the addition of concentrated sulphuric acid. Appearance of yellow coloration indicates the presence of Flavonoids. The yellow coloration disappears on standing.

Test for Carbohydrates

To 0.5ml of the extract 1ml of Benedict's reagent was added and boiled for 5 minutes. Appearance of red colour indicates the presence carbohydrate.

Test for Saponin

To 0.5 ml of the extract 0.5ml of the distilled water was added and vortexed for 10 minutes. The formation of foams indicates the presence of saponins.

Test for Phenols

5ml of Folin Ciocalteu reagent and 4ml of aqueous sodium carbonate were added to 0.5ml of extract. Appearance of blue color indicates the presence of phenols.

Test for Tannins

About 0.5g of fruit extracts were boiled in 10ml of distilled water in 15ml test tube and then filtered. A few drops of 0.1% Ferric chloride was added and observed for Brownish or a blue- black color formation. This indicates the presence of tannins.

Test for Phlobatannins

Fruit extracts were boiled with 1% aqueous Hydrochloric acid. Formation of red precipitate indicates the presence of phlobatanins.

Test for Terpenoids

5ml of each extract were mixed in 2ml of Chloroform and 3ml Concentrated sulphuric acid was carefully added to form a layer. A reddish brown colouration at the interface indicates the presence of terpenoids.

Test for Cardiac glycosides

5ml of each extract was treated with 2ml of glacial acetic acid containing one drop of Ferric chloride solution. This was layered with 1ml of Concentrated sulphuric acid. A brown ring at the interface indicates the presence of a deoxysugar which is characteristics of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer, a greenish ring may form just spreading gradually throughout the layer.

QUANTITATIVE ANALYSIS

DETERMINATION OF TOTAL PHENOLS

Total phenols were determined by Folin Ciocalteu reagent. [9] 5ml of Folin Ciocalteu reagent and 4ml of aqueous Sodium carbonate were added to 0.5ml of extract. After 15 mins of incubation at room temperature, the absorbance was read at 765nm. The standard curve was prepared using chlorogenic acid. Total phenols were expressed in terms of chlorogenic acid equivalents (mg CAE/100g FW).

DETERMINATION OF TANNINS

Tannins were determined by the method of Peri and Pompei. [10] Tannins were precipitated by adding lead acetate solution. The concentrations of the reagents required for completely precipitating the tannins was dissolved in a known volume of 10% ethanol and the concentration of tannins was estimated by the Folin Ciocalteu method. The concentrations of tannins were expressed in terms of Chlorogenic acid equivalents (mg CAE/ 100g FW).

DETERMINATION OF TOTAL FLAVONOIDS

Total flavonoids were determined by Aluminium chloride colorimetric method Chang. [11] 0.5 ml of plant extract was mixed with 1.5 ml of ethanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water. It was kept at room temperature for 30 minutes; the absorbance of the

reaction mixture was measured at 415 nm. The calibration curve was prepared using catechin. The values were expressed as mg/100g.

DETERMINATION OF FREE RADICAL SCAVENGING ACTIVITY

Free radical scavenging activity was determined by the method of Koleva.^[12] Different concentrations of fruit extracts were added to an equal volume of alcohol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH). After 15 minutes at room temperature, the absorbance was recorded at 517 nm. The experiment was repeated for three times. BHT (butylated hydroxytoluene) was used as standard control. Radical scavenging activity towards DPPH was estimated from the following equation.

$$\text{Abs control} - \text{Abs sample}$$

$$\% \text{ inhibition of DPPH} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs Control}} \times 100$$

Where Abs control is the absorbance of DPPH solution without extracts.

DETERMINATION OF NITRIC OXIDE RADICAL SCAVENGING ACTIVITY

Nitric oxide scavenging activity was measured spectrophotometrically.^[13] Sodium nitroprusside in phosphate buffered saline was mixed with different concentrations of the extract dissolved in ethanol and incubated at 25°C for 30 minutes. A control without the test compound but with an equivalent amount of ethanol was taken. After 30 minutes, 1.5 ml of incubated solution was removed and treated with Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% naphthyl ethylene diamine dihydrochloride). The absorbance of the chromophore formed during diazotization of the nitrite with sulphanilamide and subsequent coupling with naphthylethylene diamine dihydrochloride was measured at 546 nm.

DETERMINATION OF SUPEROXIDE ANION SCAVENGING ACTIVITY

Superoxide anion scavenging ability was carried out by employing Nitrobluetetrazolium reduction assay.^[14] A reaction mixture containing 0.4µl of sodium pyrophosphate, 25 µl of phenazine methosulphate, 25 µl of nitroblue tetrazolium and 100 µl of NADH was

mixed with varied concentration of extracts and incubated for 90 seconds at 30°C. The purple coloured chromogen formed was measured spectrophotometrically at 560 nm.

FERRIC REDUCING ANTIOXIDANT POWER ASSAY

The ferric reducing power of the fruit extracts was determined by using the potassium ferricyanide-ferric chloride method.^[15] 2ml of extract was added to 2.5 ml of potassium ferricyanide and the mixture was incubated at 50°C for 20 min. Then 2.5ml of Trichloroacetic acid was added to the mixture, which was then centrifuged at 650 x g for 10 mins. To the supernatant (2.5ml) distilled water (2.5ml) and 0.5 ml ferric chloride was added. The absorbance was read at 700 nm. Higher absorbance indicated greater reducing capacity which is calculated as follows.

$$\text{RP} = [\text{Am} / \text{Ac}] \times 100$$

Am = Absorbance of reaction mixture

Ac = Absorbance of control mixture (distilled water instead extract).

CHROMATOGRAPHIC CONDITION FOR HPTLC FINGERPRINT

The given sample was dissolved in 90 % ethanol and made upto 5 ml. 5 µl and 10µl of the solution was applied on Merck Aluminium plate pre – coated with Silica gel 60 F 254 of 0.2 mm thickness using Linomat IV applicator. The plate was developed in Chloroform: Methanol: Formic acid (90: 5.0: 5.0). The plate was scanned at 254nm and 366 nm using Deuterium lamp in Camag HPTLC instrument provided with Win Cats 1.4.4 version software.

STATISTICAL ANALYSIS

All determinations were conducted in triplicate and the experiment was run in duplicate. The values are expressed as mean ± SD. Statistical analysis was done by student 't' test and "P" values were arrived at to assess the statistical significance of change observed. P value less than 0.02 was considered non-significant.

RESULTS & DISCUSSION

Table- 2 Shows the various phytochemicals present in *Psidium guajava* and *Malus domestica*. It was observed from the table that the ethanolic extract of *Psidium*

guajava contained higher concentration of phenols and flavonoids when compared with *Malus domestica*. The presence of tannins, phlobatannins, terpenoids,

carbohydrates and cardiac glycosides were also noted in both the extracts. However the presence of saponin was noted only in the ethanolic extract of *Psidium guajava*.

TABLE – 2: Qualitative analysis of phytochemicals present in *Psidium guajava* and *Malus domestica*

Contents	<i>Psidium guajava</i>	<i>Malus domestica</i>
	(Ethanol)	(Ethanol)
Carbohydrates	+	+
Flavonoids	+++	++
Phenols	+++	++
Tannins	++	+
Saponins	+	-
Terpenoids	+	+
Phlobatannins	+	+
Cardiac glycosides	+	+

Table- 3 Shows the total phenolic content of *Psidium guajava* and *Malus domestica*. It was observed from the table that *Psidium guajava* contains nearly three times the phenolic content of *Malus domestica*. It was observed from the table that *Psidium guajava* contains

high amount of phenols (274mg/100g FW) while *Malus domestica* contains (70mg /100g FW). It has been reported by Wada and Ou ^[1] that these phytochemical protect the cells from oxidative damage caused by free radicals.

TABLE –3: Total phenol contents in the ethanolic fruit extract of *Psidium guajava* and *Malus domestica*

S.No	Ethanolic fruit extracts	Total phenol content (mg / 100 g FW)
1.	<i>Psidium guajava</i>	274 ± 10
2.	<i>Malus domestica</i>	70 ± 6

Values are expressed as Mean ± SD for 6 different preparations.

Table-4 Shows the total flavonoids content in *Psidium guajava* and *Malus domestica*. It was noted from the table that *Psidium guajava* contains (47.9mg/ 100g FW)

nearly two times more of flavonoid content than *Malus domestica* (28.2 mg/100g FW).

TABLE –4: Total Flavonoids content in the ethanolic fruit extracts of *Psidium guajava* and *Malus domestica*

S.No	Ethanolic fruit extracts	Total flavonoid contents (mg / 100 g FW)
1.	<i>Psidium guajava</i>	47.9 ± 2.1
2.	<i>Malus domestica</i>	28.2 ± 1.2

Values are expressed as Mean ± SD for 6 different preparations.

Table -5 shows the tannin content of *Psidium guajava* and *Malus domestica*. It was observed from the table

that the tannin content of *Psidium guajava* is significantly higher when compared to *Malus domestica*.

TABLE – 5: Levels of tannins in the ethanolic fruit extract of *Psidium guajava* and *Malus domestica*

S.No	Ethanolic fruit extracts	Total tannin content (mg / 100 g FW)
1.	<i>Psidium guajava</i>	94.5 ± 5
2.	<i>Malus domestica</i>	81.1 ± 7

Values are expressed as Mean ± SD for 6 different preparations.

Table-6 Shows the Nitric oxide radical scavenging activity of ethanolic extracts of *Psidium guajava* and *Malus domestica* extracts separately and in combinations. It was observed from the figure that with increase in concentration of *Psidium guajava* there is

increase in the Nitric oxide scavenging activity. . It could be observed that increase in the concentration of *Psidium guajava* and *Malus domestica* resulted in the inhibition of NO formation.

TABLE-6: Nitric oxide scavenging activity of the ethanolic fruit extracts of *Psidium guajava* and *Malus domestica*

Extract conc (mg / ml)	<i>Psidium guajava</i>	<i>Malus domestica</i>	<i>Psidium guajava</i> + <i>Malus domestica</i>
10	68.2 ± 1.3	68.7 ± 1.5	73 ± 1.7
20	69.7 ± 1.7	72 ± 1.9	83 ± 2.1
30	77.4 ± 2.3	75 ± 2.5	85 ± 2.7
40	81.5 ± 3.1	80 ± 4.2	87 ± 3.5
50	86.2 ± 3.8	90 ± 4.7	92 ± 4.2

Values are expressed as Mean ± SD for six different preparations.

Table-7 Shows the ferric reducing capacity of ethanolic extract of *Psidium guajava* and *Malus domestica* extracts separately and in combinations. The high reducing capacity is noted in both *Psidium guajava* and *Malus domestica* could be due to their high

concentration of phenols and flavonoids. It is observed from the table that *Malus domestica* showed the maximum reducing capacity than that of *Psidium guajava*. This could be due to the high concentration of quercetin in *Malus domestica*.

TABLE-7: Ferric reducing antioxidant power of the ethanolic fruit extracts of *Psidium guajava* and *Malus domestica*

Extract conc (mg / ml)	<i>Psidium guajava</i>	<i>Malus domestica</i>	<i>Psidium guajava</i> + <i>Malus domestica</i>
10	38.1±1.3	41.2±2.2	41±2.7
20	54.93±2.5	56.27±3.1	57.40±3.1
30	74.35± 3.2	76.42±3.7	77.10±3.7
40	75.8±3.8	80.72±3.8	85.12±3.9
50	77.87±4.1	95.21±4.2	96.12±4.4

Values are expressed as Mean ± SD for six different preparations

Table-8 Shows the superoxide anion scavenging activity of *Psidium guajava* and *Malus domestica* extracts separately and in combinations. It is observed from the table that increasing concentration of the extract inhibited the formation of SO anions. SO scavenging activity of *Malus domestica* was more when compared with *Psidium guajava*. The combined extracts of *Psidium guajava* and *Malus domestica* were not that effective in scavenging SO as compared to *Malus domestica* alone.

TABLE-8: Superoxide radical scavenging activity of the ethanolic fruit extracts of *Psidium guajava* and *Malus domestica*

Extract conc mg / ml	<i>Psidium guajava</i>	<i>Malus domestica</i>	<i>Psidium guajava</i> + <i>Malus domestica</i>
10	33.9 ± 1.2	53.6 ± 1.5	37.08 ± 2.1
20	36.6 ± 1.5	65 ± 1.7	52.1 ± 2.7
30	41.2 ± 1.9	76 ± 1.9	65.1 ± 3.1
40	70.5 ± 2.2	83 ± 2.1	78.7 ± 3.5
50	85 ± 2.7	92 ± 2.8	88.1 ± 3.9

Values are expressed as Mean ± so for six different preparations

Figure- 1 Shows the free radical scavenging activity of various concentrations of *Psidium guajava* and *Malus domestica* extracts in separately and in combinations. At 50mg concentration *Psidium guajava* and *Malus*

domestica individually showed the same % of inhibition as that of the combined extracts with respect to time intervals.

Fig 1: Free radical scavenging activity of 50 mg of ethanolic extract of *Psidium guajava* and *Malus domestica*

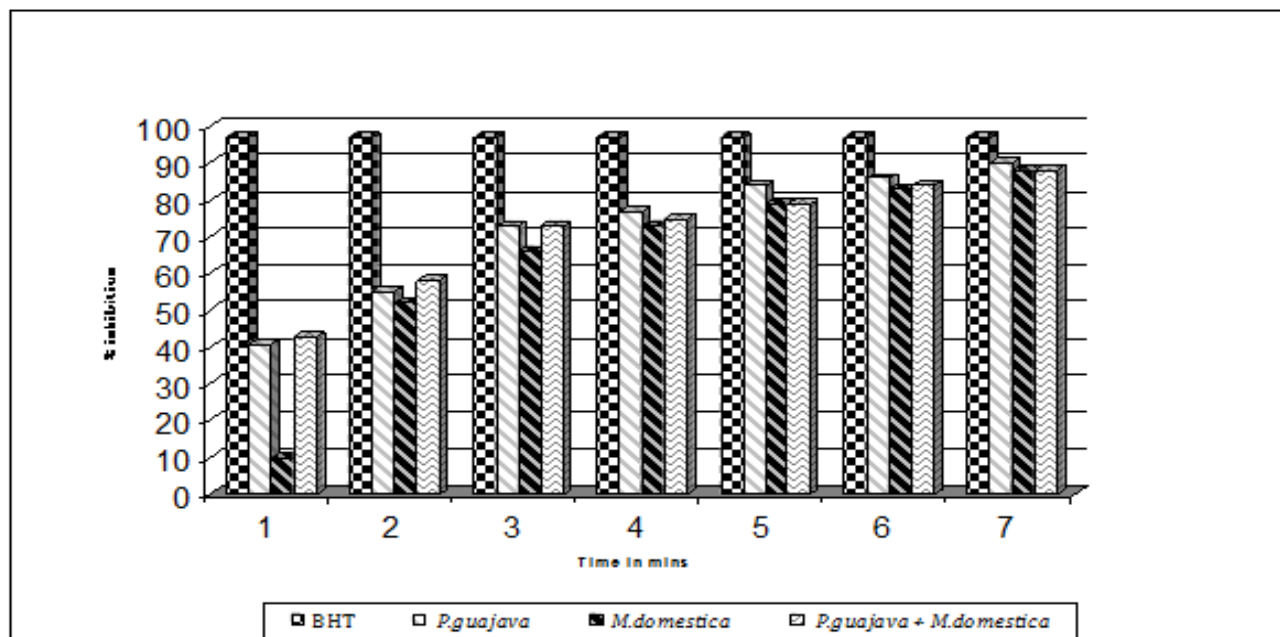


Figure 2 & 3 shows the HPTLC and TLC analysis of ethanolic extracts of *Psidium guajava* and *Malus domestica*. It was observed from the graph that the ethanolic extracts of *Malus domestica* contained high

concentration of Quercetin when compared to *Psidium guajava*. However they were other peaks present in graph and their presence could not be detected due to the want of other standards.

Fig 2: HPTLC analysis of ethanolic extracts of *Psidium guajava* and *Malus domestica*

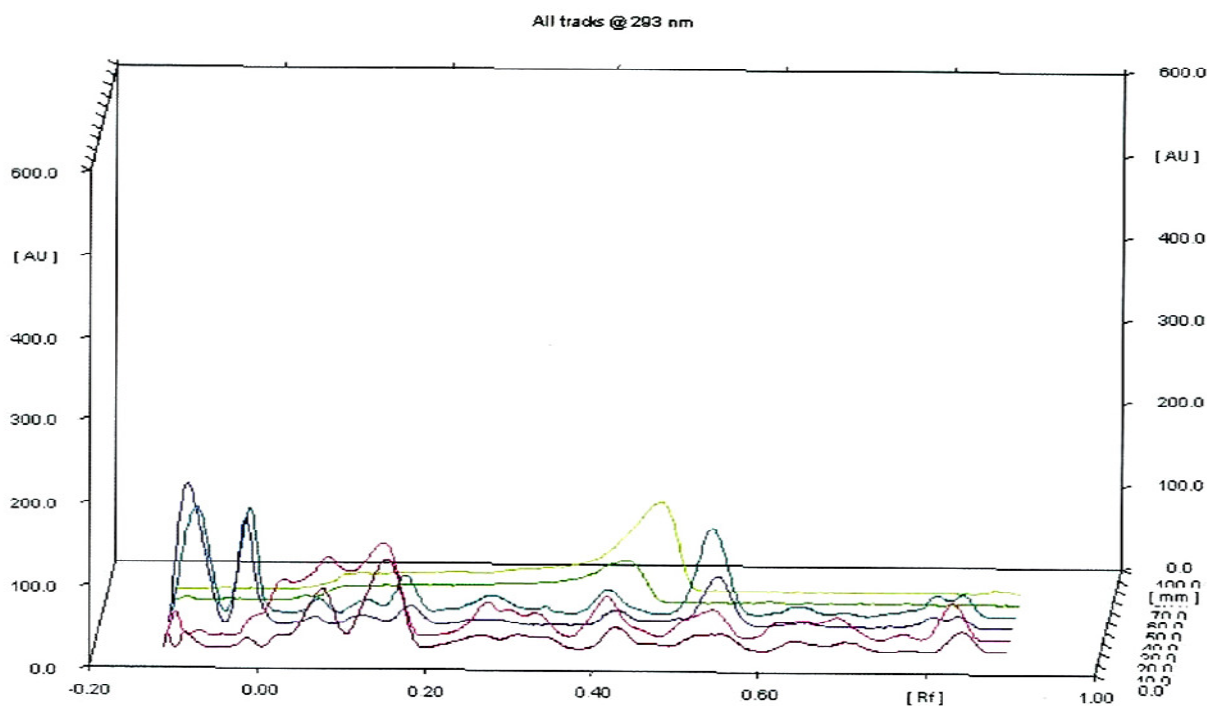
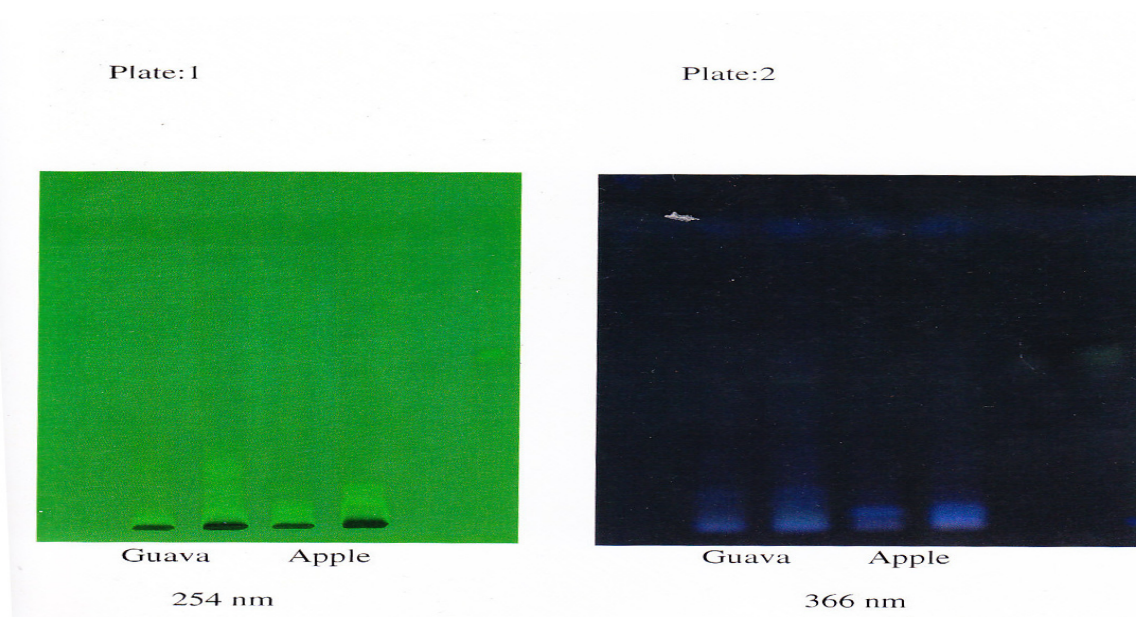


Fig 3: TLC analysis of ethanolic extracts of *Psidium guajava* and *Malus domestica*



CONCLUSION

There is a growing awareness of the potential health benefit of diets rich in fruits and vegetables and nutritional guidelines indicate that an increase in the consumption of foods rich in antioxidant nutrients may decrease the risk of Chronic Heart Disease and certain cancers. Epidemiological studies have reported a reduction in the incidence of Chronic Heart Disease with moderate daily fruit consumption. This effect has been ascribed to the low molecular weight phenolics in many plant based foods which can act as antioxidants because their extensive conjugated π -electron systems allow ready donation of electrons or hydrogen atoms from the hydroxyl moieties to free radicals. Research into the alleged health benefits of phenolic and polyphenolic compounds continues to have considerable potential however many questions remain to be answered. It was observed from our results that combined extracts of both the fruits and *Psidium guajava* individually showed higher free radical scavenging activity. Hence it would be advocated that consuming both *Psidium guajava* and *Malus domestica* together had more desirable effect than either of them separately. These interesting results clearly indicate that combined extracts of both fruits may be beneficial for human consumption.

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