Preliminary studies on anti-cataract activities of *Persea americana* & *Actinidia deliciosa* from chromatographic fractions and HPTLC analysis.

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Abstract - *Persea americana* and *Actinidia deliciosa* are commonly known as Butter fruit and kiwi respectively. Both the fruits show various medicinal effects, including antidiabetic, anticancer, prevention of cardiovascular diseases. Hence the present investigation was carried out to determine the active constituents of the fruits after they were defatted with ethanol and submitted to chromatographic isolation using column chromatography(CC) , thin layer chromatography (TLC) and High pressure thin layer chromatography (HPTLC) finger printing. The active fraction obtained from CC was determined for anticataract effect using goat lens by *In vitro* method. A significant increase in LPO and conjugated dienes was found in Group II opposed to the Groups treated with *P.americana* and *A.deliciosa* of column fractions at different concentration of 100µg, 500µg, 1000µg of fruit samples and standard ascorbic acid. The results were analysed and it was observed that *P. americana* and *A. deliciosa* prevented cataract formation.

Keywords: Column chromatography, TLC, HPTLC finger printing, Anticataract effect, Active constituents.

I. INTRODUCTION

The avocado fruit is low in simple sugars and contains appreciable levels of dietary fibers (Bergh, 1992). The fruits extracts reduce fat absorption, constipation, lower glycemic index and plasma insulin levels, microbial proliferation and controls plasma cholesterol (Kritchevsky and Bonfield C, 1995). It is involved in the regulation of normal intestine performance, risk factors for diabetes, obesity, gall stones, hyper cholesterolemia and heart diseases (Gray, 1995). Avocado contains phytochemicals such as alkanols, terpenoids, glycosides, various furan ring-containing derivatives, flavonoids, and coumarins (Naveh E, *et al.,* 2002). Kiwi fruits, stems and roots are diuretic, febrifuge and sedative. They are used in the treatment of stones in the urinary tract, rheumatoid arthralgia, cancers of the liver and oesophagus (Ferguson AR, 1990). They are rich in bioactive compounds like polyphenols (Park *et al.,* 2006). It contains glucose and fructose and low amount of sucrose (Nishiyama, 2007). Traditionally they have been used to treat different cancers, including those of the digestive system (Ye MH, 1979; Zhi CJ, 1980). Chromatographic fingerprint is a pattern of some common chemical components of pharmacologically active and or chemical characteristics present in the extract. The chromatographic profiles are featured by its integrity, fuzziness or sameness and differences of herbal medicine (Patil and Shettigar, 2010).

The fractions obtained from column chromatography were used to study the HPTL profile and *In vitro* anticataract effect in goat lens. The chemical constituents present in both the extracts may contribute to these effects.

II. MATERIALS & METHODS

Plant materials

Edible fresh materials of *Persea americana* (PA) and *Actinidia deliciosa* (AD) were procured from the Supermarket, Chennai, and authenticated by Dr. J. Jayaraman, Plant anatomy Research Centre, West Tambaram.

Extraction

Fresh PA and AD (1 Kg) were cut to small pieces then grinded with electric mixer for extraction process. Fruit pulps were extracted with pure ethanol for 3 days, by shaking at 100rpm /min. The extraction process is repeated twice for the same sample, until faint green colour appears for PA and light brown for AD. The solvent was distilled using rotatory evaporator. The dried ethanolic extract of both the fruits were utilized for chromatographic separation process and *in vitro* analysis.

Chemicals:

Silica gel G (100-200 mesh), Hexane, Ethyl acetate, Ethanol, Methanol, distilled water, Toluene, Chloroform: Methanol: Formic acid (7:3:1, 9:1:0.5), Toluene: Ethyl acetate (3:7), Vanidilin Sulphate. TLC aluminium sheets silica gel 60 F 254 (Merck), Linomat 5 were obtained from Sigma, Aldrich.

Values are expressed as mean ± SD for 6 different analysis. Statistical analysis was done by using Analysis of Variance (ANOVA) with SPSS16.0. Difference were considered significant at *p< 0.001, **p < 0.01, ***p <0.05.

Chromatographic Separation of Ethanol Extract

50gm of ethanol extract of PA and AD was subjected to column chromatography 4X15 cm in height and 4 cm in diameter packed with silica gel 100-200 mesh as stationary phase. Elution was carried out with Hexane, Hexane / Ethyl acetate, Ethyl acetate, Ethyl acetate/ Ethanol, Ethanol, Ethanol/ Methanol, Methanol/ Distilled water and finally with pure distilled water. Fractions 50 ml each were collected, concentrated and examined by TLC silica gel using solvent systems; Toluene, Chloroform: Methanol: Formic acid(7:3:1, 9:1:0.5). Chromatograms were visualized after spraying with Vanidilin Sulphate. Fractions eluted were concentrated, dried and kept in container with suitable label for further use. The TLC was carried out and the fractions which have similar TLC pattern were pooled.

High Performance Thin Layer Chromatography (HPTLC) Profile

The chromatography was performed on silica gel 60 F254, 10 cm x 10 cm HPTLC plates from Merck. The plates were previously washed with methanol for 30 minutes and dried at room temperature in a fume hood. Before analyses, plates were activated at 120 ºC for 30 min. Samples were applied as bands by means of the Automatic TLC Sampler (ATS) 3 and Linomat III automated spray-on applicator equipped with a 07 µl syringe, both from CAMAG (Muttenz, Switzerland), operated with the following settings band length of 5 mm using Linomat 5 sample applicator set at a speed of 150 nl/sec. Plates were developed up to a migration distance of 50 mm in a CAMAG HPTLC twin-trough chamber equilibrated with the mobile phase for 15 min. A number of solvent systems were tried, for each extract for better resolution and maximum number of spots, but the satisfactory resolution was obtained with solvent Toluene: Ethyl acetate (3:7). The chromatograms were developed in twin trough glass chamber saturated with solvent Toluene: Ethyl acetate (3:7) for 20 minutes. For multiple developments, the plates were run three times up to the distance of 80 mm as maximum. Freeze-dried extract (100 mg) was dissolved in methanol, filtered through cotton and diluted to 50 mL with methanol. This operation was performed in triplicate, and developed three times. The air dried plates were viewed in ultraviolet radiation to mid-day light (Figure 1). Spots were visible without derivatization at 254 and 366 nm Scanning was performed by CAMAG HPTLC Densitometer (Scanner 3) in absorbance mode at both 254 and 366 nm, the extracts were also scanned at 350-600 nm using deuterium and tungsten lamp with slit dimension 6.0 X 0.45 macro. The Rf values and colour of the resolved bands were noted.

Sodium selenite induced cataract

Fresh goat eyeballs were obtained from local slaughterhouse within two hours after killing of the animals and the lenses were isolated. They are preserved and carried to the laboratory at 0- 4°C. The isolated lens were incubated in artificial aqueous humor at 37°C and pH 7.8 for 72 h. Sodium selenite (SS) at a concentration of 30 µM / Kg weight was used to induce cataract . A total of 210 goat lenses were used and divided into twelve experimental groups consisting of 6 in each group

Group I: Artificial aqueous humor alone (Normal control)

Group II: SS 30 µM / Kg W alone (Negative control)

Group III:

A: SS 30 µM / Kg W + FI of PA (100 µg/ml)

B: SS 30 µM / Kg W + FI of PA (500 µg/ml)

C: SS 30 µM / Kg W + FI of PA (1000 µg/ml)

Group IV:

A: SS 30 µM / Kg W + FII of PA (100 µg/ml)

B: SS 30 µM / Kg W + FII of PA (500 µg/ml)

C: SS 30 µM / Kg W + FII of PA (1000 µg/ml)

Group V:

A: SS 30 µM / Kg W + FIII of PA (100 µg/ml)

B: SS 30 µM / Kg W + FIII of PA (500 µg/ml)

C: SS 30 µM / Kg W + FIII of PA (1000 µg/ml)

Group VI:

A: SS 30 µM / Kg W + FIV of PA (100 µg/ml)

B: SS 30 µM / Kg W + FIV of PA (500 µg/ml)

C: SS 30 µM / Kg W + FIV of PA (1000 µg/ml)

Group VII:

A: SS 30 µM / Kg W + FI of AD (100 µg/ml)

B: SS 30 µM / Kg W + FI of AD (500 µg/ml)

C: SS 30 µM / Kg W + FI of AD (1000 µg/ml)

Group VIII:

A: SS 30 µM / Kg W + FII of AD (100 µg/ml)

B: SS 30 µM / Kg W + FII of AD (500 µg/ml)

C: SS 30 µM / Kg W + FII of AD (1000 µg/ml)

Group IX:

A: SS 30 µM / Kg W + FIII of AD (100 µg/ml)

B: SS 30 µM / Kg W + FIII of AD (500 µg/ml)

C: SS 30 µM / Kg W + FIII of AD (1000 µg/ml)

Group X:

A: SS 30 µM / Kg W + FIV of AD (100 µg/ml)

B: SS 30 µM / Kg W + FIV of AD (500 µg/ml)

C: SS 30 µM / Kg W + FIV of AD (1000 µg/ml)

Group XI:

A: SS 30 µM / Kg W + FV of AD (100 µg/ml)

B: SS 30 µM / Kg W + FV of AD (500 µg/ml)

C: SS 30 µM / Kg W + FV of AD (1000 µg/ml)

Group XII:

A: SS 30 µM / Kg W + FVI of AD (100 µg/ml)

B: SS 30 µM / Kg W + FVI of AD (500 µg/ml)

C: SS 30 µM / Kg W + FVI of AD (1000 µg/ml)

Group XIII:

A: SS 30 µM / Kg W + Standard Ascorbic acid (100 µg/ml)

B: SS 30 µM / Kg W + Standard Ascorbic acid (500 µg/ml)

C: SS 30 µM / Kg W + Standard Ascorbic acid (1000 µg/ml)

At the end of the experiment, the lenses were removed from the medium and rolled on filter paper to remove medium, adhering non lens tissue, and vitreous humor.

Preparation of lens homogenate

After incubation, lenses were homogenized with 10 volumes of 0.1M potassium phosphate buffer, pH 7.0. The homogenate was centrifuged at 10,000 g for 1 h and the supernatant was used for estimation of biochemical parameters.

Estimation of Lipid peroxide (LPO) in Lens:

The lens LPO level was determined by the method of Hiroshi Ohkawa, *et al.,* (1979). To 0.2ml of homogenate (sample), 0.2ml of SDS, 1.5ml of acetic acid and 1.5 ml of TBA were added. The mixture was made up to 4ml with water and then heated in an oil bath at 95°C for 60min using glass ball as a condenser. After cooling, 1ml of water and 5ml of n-butanol/pyridine mixture were added and shaken vigorously. After centrifugation at 4000rpm for 10 min, the absorbance of organic layer was measured at 532nm. As an external standard, tetramethoxy-propane was used. The concentration of lipid peroxides was expressed as nmoles of MDA formed/mg protein.

Estimation of conjugated dienes:

Conjugated dienes were assayed by the method of Rao and Recknagel (1968). Lipid peroxidation is associated with rearrangement of the double bonds in polyunsaturated fatty acid leading to the formation of conjugated dienes. The measurement of the formation of the conjugated dienes reflects the extent of lipid peroxidation taking place. To 1ml of the lens homogenate, 5.0 ml of chloroform methanol reagent (2:1v/v) was added, mixed thoroughly, centrifuged for 5 minutes and 3.0 ml of the lower phase evaporated to dryness. To this 1.5ml of cyclohexane was added and the absorbance was read at 233nm against cyclohexane blank. Conjugated dienes are expressed as nmol/dl lens

III. RESULTS & DISCUSSION

Chromatography and HPTLC analysis:

63 fractions were collected from column chromatography for *P.americana* and 80 fractions for *A.deliciosa* collected and they were pooled to four and six fractions according to their TLC profile. The details of collected fractions were given in Fig1, 2. The concentrated fractions obtained from column chromatography were screened for HPTLC analysis. TLC is a rapid technique for the phytochemical evaluation of herbal drugs and it extensively provides qualitative and semi quantitative information of the resolved compounds (Patil & Shettigar, 2010). Flavonoids, Phenolic compounds are effective hydrogen donors and have high antioxidant potential which attributes to scavenge harmful reactive oxygen species (Bors W *et al*., 1990; Takuo Okuda and Hideyuki Ito 2011). Phenolics have antioxidative and antidiabetic effect (Merinal S and Stella Boi VG, 2012).

Biochemical assessment of the lens crystalline:

A marked reduction in the LPO and conjugated dienes in fractions of FII followed by the FIII and FIV at the concentration of 100µg, 500µg, 1000µg of *P.americana* samples compared with untreated sodium selenite Group and standard ascorbic acid group was absenced. A significant increase in LPO and conjugated dienes was found in Group II opposed to the GroupI,VII,VIII,IX,X,XI,XII,XIII of *A. deliciosa* column fractions (Table1,2,3). As the concentration increases LPO and conjugated dienes levels were decreased in both the fruit extracts. Natural flavonoids prevent and affect multiple mechanisms or etiological factors responsible for cataract (Stefek M, 2011).

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Table 1. The levels of lipid peroxides and conjugated dienes in goat lens treated with 100 µg/ml of fruit extracts of *P. americana* , *A. deliciosa* and Ascorbic acid

Groups	LPO (nmol/mg protein)	Conjugated diene(nmol/dl)
Group I	1.17±0.10	0.9±0.08
Group II	2.65±0.2 a*	2.45±0.14 a*
Group III	2.05 ±0.03 b*	1.9±0.03 b*
Group IV	1.37±0.01 c*	1.6±0.06c*
Group V	1.24±0.01 d*	1.2±0.12 d*
Group VI	1.36±0.01 e*	1.24±0.01 e*
Group VII	2.33±0.12 f**	2.11±0.00 f*
Group VIII	2.15±0.1 g*	2.13±0.05 g*
Group IX	1.14±0.10 h*	1. 12±0.12 h*
Group X	1.20±0.12 i*	1.07±0.24 i**
Group XI	1.11±0.04j*	1.42±0.09 j*
Group XII	1.18±0.02 k*	1.49±0.01 k*
Group XIII	1.19±0.09 l*	1.14±0.10 l*

Values are expressed as mean ± SD for 6 goat lens in each group

Statistical significance: * $p < 0.001$, ** $p < 0.01$.

Comparision:

a - as compared with Group I ; b- as compared with Group II ; c- as compared with Group II ; d- as compared with Group II; e - as compared with Group II; f- as compared with Group II; g- as compared with Group II; h- as compared with Group II; i- as compared with Group II, j- as compared with Group II; k- as compared with Group II; l - as compared with Group II

Table 2. The levels of lipid peroxides and conjugated dienes in goat lens treated with 500 µg/ml of fruit extracts of *P. americana* and *A. deliciosa*

Groups	LPO (nmol/mg protein)	Conjugated diene(nmol/mg protein)
Group I	1.15 ± 0.08	1.00±0.04
Group II	2.65±0.17 a*	2.39 ±0.15 a*
Group III	1.57±0.01 b*	1.44±0.02 b*
Group IV	1.23 ±0.02 c*	0.94±0.13 b*
Group V	1.15±0.02 d*	1.16 ±0.02 c**
Group VI	1.24 ±0.15 e**	1.15 ±0.03 e**
Group VII	2.05±0.15 f**	2.0 ±0.02 f**
Group VIII	1.85±0.04 g*	1.00±0.02 g*
Group IX	1.03±0.01 h*	1.0±0.01 h*
Group X	1.01±0.08 i*	0.98±0.09 i*
Group XI	1.03±0.02 j*	0.96±0.02 j*
Group XII	1.1±0.14 k*	0.99±0.08 k*
Group XIII	1.0±0.1 l*	0.97±0.02 l*

Values are expressed as mean ± SD for 6 goat lens in each group

Statistical significance: * p< 0.001,**p < 0.01,***p < 0.05

Comparision: a - as compared with Group I ; b- as compared with Group II ; c- as compared with Group II ; d- as compared with Group II; e - as compared with Group II; f- as compared with Group II; g- as compared with Group II; h- as compared with Group II; i- as compared with Group II ; j- as compared with Group II; k- as compared with Group II; l - as compared with Group II

Table 3. The levels of lipid peroxides and conjugated dienes in goat lens treated with 1000 µg/ml of fruit extracts of *P. americana* and *A. deliciosa*

Groups	LPO (nmol/mg protein)	Conjugated diene(nmol/mg protein)
Group I	1.15±0.17	1.02±0.09
Group II	2.53±0.1 a*	2.25±0.21 a*
Group III	1.07±0.02 b*	1.00±0.06 b*
Group IV	0.99±0.3 c*	0.94±0.50 c*
Group V	1.01±0.09 d*	0.98 ±0.02 d*
Group VI	1.04±0.01 e*	0.99 ±0.01 e*
Group VII	1.99 ±0.02 f***	1.85 ±0.02 f***
Group VIII	1.50±0.1 g*	0.98±0.12 g*
Group IX	1.00±0.2 h*	0.95±0.2 h*
Group X	0.99 ±0.08 i*	0.88±0.02 i*
Group XI	1.02 ±0.4 j*	0.80±0.09 j*
Group XII	1.30 ±0.01k*	1.0±0.02 k*

Group XIII	1.3 ±0.14 l*	1.07±0.06 l*

Values are expressed as mean ± SD for 6 goat lens in each group

Statistical significance: * p< 0.001, **p < 0.01, ***p < 0.05

Comparision: a - as compared with Group I ; b- as compared with Group II ; c- as compared with Group II ; d- as compared with Group II; e - as compared with Group II; f- as compared with Group II; g- as compared with Group II; h- as compared with Group II; i- as compared with Group II ; j- as compared with Group II; k- as compared with Group II; l - as compared with Group II.

Fig 1. Percentage yield of different fractions Of *Persea americana*

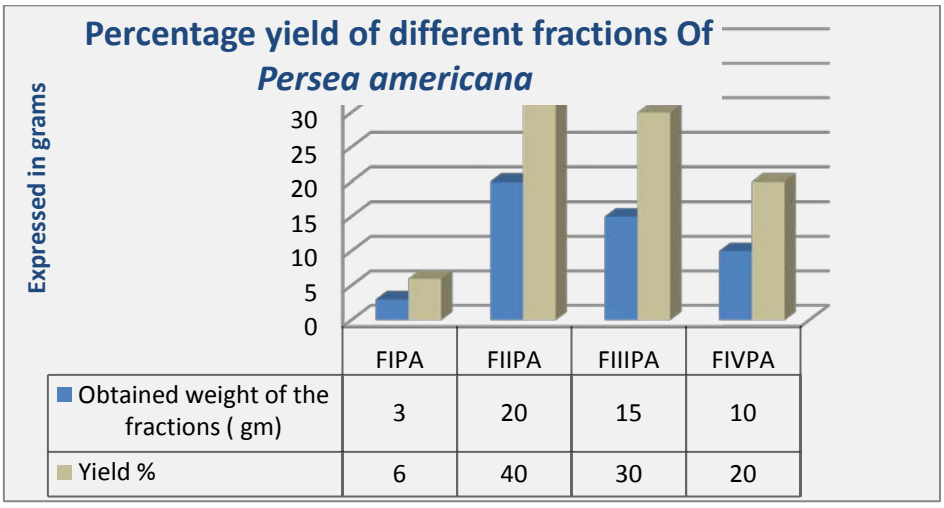


Fig 2. Percentage yield of different fractions Of *Actinidia deliciosa*

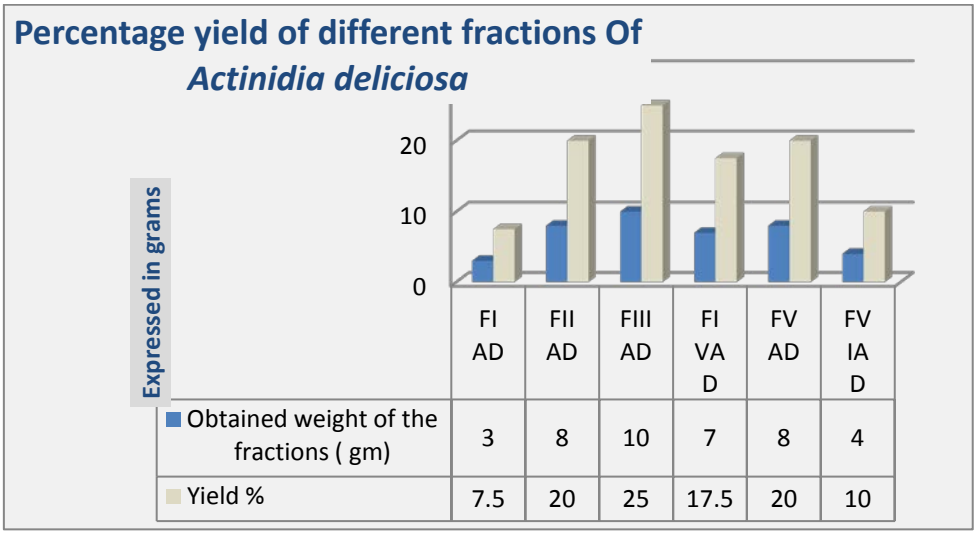
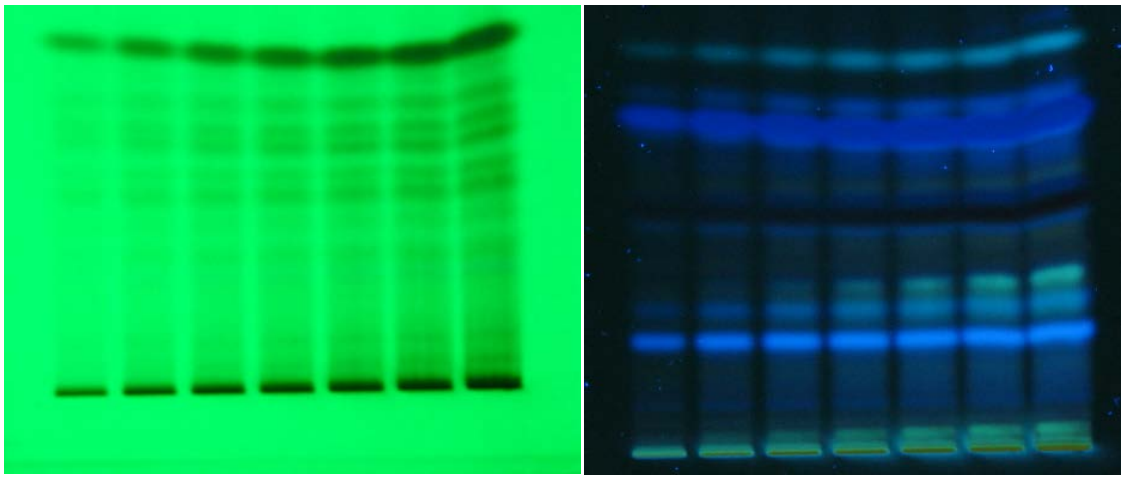


Fig3.HPTLC Analysis of *Persea americana*



A. 254 nm B. 366 nm

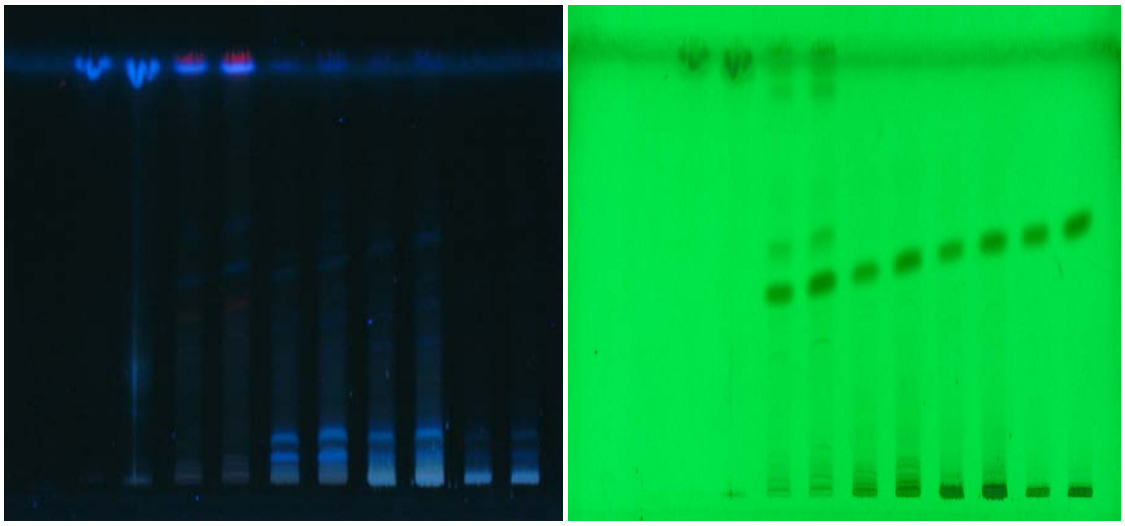
Track 1: 4µl of FIPA

Track 2: 4µl of FIIPA, Track 3: 6µl of FIIPA

Track4: 4µl of FIIIPA, Track 5: 6µl of FIIIPA

Track6: 4µl of FIVPA, Track 7: 6µl of FIVPA

Fig 4.HPTLC Analysis of *Actinida deliciosa*



A. 254 nm B. 366 nm

Track 1: 4µl of FIAD, Track 2: 6µl of FIAD,

Track 3: 4µl of FIIAD, Track4: 6µl of FIIAD

Track 5: 4µl of FIIIAD, Track6: 6µl of FIIIAD

Track 7: 4µl of FIVAD, Track 8: 6µl of FIVAD

Track 9: 4µl of FVAD, Track 10: 6µl of - FVAD

Track 11 4µl of FVIAD, Track 12: 6µl of FVIAD