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# Anti-Diabetic Activity of *Amomum Subulatum* Roxb. Fruit Constituents

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**Abstract**

Large cardamom (fruit of *Amomum subulatum* Roxb, Zingiberaceae) commonly known as 'Bari Ilaichi' is used in various disease condition and also described in Ayurveda. Anti-diabetic activity of *Amomum subulatum* Roxb. Seed was evaluated in fructose fed metabolic syndrome in rat. Acetone and methanol extracts were assayed for total phenolic contents by UV method. Presence of protocatechuic acid was estimated by HPTLC method. Oral administration of both *A. subulatum* extracts revealed a significant (P<0.001) increment of serum insulin levels, higher reduction in hyperglycemia when compared to the diabetic control rats (P<0.001). The histological studies of the endocrine region of pancreas of diabetic animals revealed that shrinkage of β cells of islets of langerhans. Animals treated with both extracts of *A. subulatum*, revealed restoration of β-cells. This activity of acetone and methanolic extract might be due to presence of phenolics like protocatechuic acid.

**Keywords:** *Amomum subulatum* Roxb. Protocatechuic acid, HPTLC, anti-diabetic

## Introduction

*Amomum subulatum* Roxb. (Family: Zingiberaceae) commonly known as Large or Greater Cardamom, Moti elaichi. Large cardamom is a tall perennial herb found in Eastern Himalayas and sub-Himalayan region of West Bengal, Assam and Sikkim. The seeds are aromatic pungent, stimulant, stomachic, alexipharmic and astringent. Traditionally, it is used to treat stomach pain, flatulence, belching, indigestion vomiting, malarial disorders, and drunkenness from alcohol Consumption. A. subulatum contains 1, 8-Cineole, a-pinene and β-pinene and geraniol.

## Material and Methods

### Collection and authentication of the fruits and seeds

The fruits of *Amomum subulatum* Roxb. Were collected from local market of Modasa and authenticated by Dr. H. B. Singh Scientist and Head of Raw Materials Herbarium & Museum Dept of National Institute of Science and Communication and Information Resources, New Delhi (NISCAIR) and preserved the herbarium in Smt. R. B. Patel Mahila Pharmacy College, Atkot, Rajkot, and Gujarat.

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**Extraction and Phytochemical Investigations**

*Amomum subulatum* fruit powder was subjected to systemic preliminary phytochemical screening after extraction with acetone and methanol. The extracts were subjected for phytochemical investigation by qualitative chemical tests, TLC, HPTLC and spectral analysis.

**Estimation of Total phenolics and Total Flavonoids of Methanol and Acetone extracts of *A. subulatum Roxb.* by Spectrophotometric method**

**Total phenolics:**

Total phenolic in the Methanolic and Acetone extract was determined using the Folin-Ciocalteu reagent. The reaction mixture was prepared by mixing 0.1 ml of methanol solution (50mg/ml) of extract, 7.9 ml of distilled water, 0.5 ml of the Folin-Ciocalteu's reagent and 1.5 ml of 20% sodium carbonate. After 2h, the absorbance at 750 nm was obtained against blank that had been prepared in a similar manner, by replacing the extract with distilled water. The total phenolic content, expressed as mg gallic acid equivalents per g dry weight of garlic was determined using calibration curve of gallic acid standard.

**Total Flavonoids:**

Total Flavonoids of Acetone and Methanolic extract were estimated according to Gordana Cetkovic (2008) Flavonoids from both extract (0.2 g) were extracted in 2 ml of extraction medium (70% [v/v] methanol, 5% [v/v] acetic acid and 25% [v/v] distilled water) at room temperature for 60 min. The resulting solution was filtered through Whatman paper No. 4 and filtrate volume adjusted to 10 ml. The probes were prepared by mixing: 5 ml of extract, 1 ml of distilled water and 2.5 ml of $AlCl_3$ solution (26.6 mg $AlCl_3 \cdot 6H_2O$ and 80 mg $CH_3COONa$ dissolved in 20 ml distilled water). A blank probe was prepared by replacing $AlCl_3$ solution with distilled water. The absorbance of probes and blank probe were measured immediately at 430 nm. Total flavonoids content, expressed as mg Rutin per g dry weight of extracts, was calculated from a calibration curve using rutin as standard.

**Estimation of Protocatechuic acid by HPTLC in Methanol and Acetone extract of *A. subulatum Roxb***

**Materials:**

Standard Protocatechuic acid was a purchased from LGC Promochem Pvt. Ltd. Bangalore All the chemicals used in the experiments is of analytical grade.

**Experimental condition**

**Sample applicator:** Camag Linomat V Automatic Sample Spotter

**Stationary phase :** precoated silica gel plates 60 F254 (10 cm10 cm with 0.2 mm thickness, E. Merck, Darmstadt, Germany) The plates were prewashed by methanol and activated at 60 °C for 5 min prior to chromatography.

**Solvent system:** Chloroform: Acetic acid (9:1)

**Development chamber:** CAMAG glass twin-through chamber (1010 cm) previously saturated with the solvent for 60 min (temperature 25.2 °C, relative humidity 40%). The development distance was 8 cm. Subsequent to the scanning.

**Scanner:** Camag TLC scanner III in absorbance mode at 254 nm and operated by Win Cats software 4.03 version.

Evaluation was *via* peak areas with linear regression

**Calibration Curve of Standard Protocatechuic acid:** A stock solution of Protocatechuic acid was prepared by dissolving 10mg of compound in ethanol and volume was made up to 10 ml in volumetric flask. From this solution 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 0.8µl spots were applied on plate.

**Estimation of Protocatechuic acid in Alcoholic and Acetone Extract:** To determine content of Protocatechuic acid in methanolic and acetone extract, an accurately weighed 50 mg of extracts were transferred to 10ml volumetric flask separately. Then dissolved in ethanol and diluted up to 10ml with ethanol. The solutions were filtered with what man no. 1 filter paper. Spots of 5 and 10 µl of both the solutions were applied to TLC plate. 0.2, 0.4, 0.6 and 0.8 µl of Protocatachueic acid Standard (1mg/ml) spots were applied on same plate. Peak of Protocatechuic acid in extract solution was identified by matching the Rf with peak obtained in Protocatachueic acid Standard solution.

**Fructose fed Insulin resistance in rats**

**Animals**

Adult male Wistar rats weighing around 180-200g were obtained from zydus healthcare, Ahmadabad, India. The animals were kept in polypropylene cages (three in each cage) at an ambient temperature of 25±20C and 55-65% relative humidity 12±1 hr light and dark schedule was maintained in the animal house till the animals were acclimatized to the laboratory conditions, and were fed with commercially available rat chow and had free access to water. The experiments were designed and conducted in accordance with the institutional guidelines. The study protocol was approved by institutional animal ethical commit, RBPMPC, Atkot, India

**Experimental design**

Adult wistar rat with an initial body weight of 180 to 200g were taken, and divided into nine groups each containing six animals.

Group I: Normal control: They were administered with vehicle (water) for 30 days. They were fed with standard laboratory diet and water *ad libitum.*

Group II: Disease control: They were administered with vehicle (water) for 30 days. They were fed with standard laboratory diet and 10% fructose in water for 30 days.

Group III: Acetone extract of *A. subulatum seeds* (200 mg/kg, p.o.), was administered for 30 days along with 10% fructose in water

Group IV: Acetone extract of *A. subulatum seeds* (400 mg/kg, p.o.), was administered for 30 days along with 10% fructose in water

Group V: Acetone extract of *A. subulatum seeds* (800 mg/kg, p.o.), was administered for 30 days along with 10% fructose in water

Group VI: Methanol extract of *A. subulatum seeds* (200 mg/kg, p.o.), was administered for 30 days along with 10% fructose in water

Group VII: Methanol extract of *A. subulatum seeds* (400 mg/kg, p.o.), was

administered for 30 days along with 10% fructose in water

Group VIII: Methanol extract of *A. subulatum seeds* (800 mg/kg, p.o.), was administered for 30 days along with 10% fructose in water

Group IX: Metformin (200 mg/kg/day, p.o) was administered for 30 days along with 10% fructose in water.

On day 30$^{th}$ blood was collected for haemolytic parameter and at 30$^{th}$ day of experiment animal was sacrificed for histopathological studies.

**Biochemical analysis**

After the 30$^{th}$ day of treatment, blood was collected retro-orbitally from the inner cantus of the eye (under light ether anesthesia) using capillary tubes(micro hematocrit capillaries, mucaps) in fresh vials containing sodium fluoride and sodium oxalate as anti-coagulants agents. Fasting blood glucose level measured by accu chek active glucostrips (Roche Diagnostic India Pvt. Ltd, Mumbai).The serum was separated in a T8 electric centrifuge (remi udyog, New Delhi) at 2000 rpm for 2 min. and Fasting insulin measured by Insulin kit, (Mercodia Insulin ELISA) using spectrophotometer(thermo-electronics India).the procedure involved an equilibrium assay carried out in BSA-borate buffer.

**Histopathology**

On 30$^{th}$ day animal were sacrificed. The pancreatic tissues were dissected out and washed on ice cold saline immediately. A portion of pancreatic tissue was fixed in 10% neutral formalin fixative solution for histological studies. After fixation tissues were embedded in paraffin, solid sections were cut at 5µm and the sections were stained with haematoxylin and eosin.

**STATISTICAL ANALYSIS**

All the grouped data was statistically evaluated via the Graph pad prism version 5 included one-way analysis of various (ANOVA) followed by least significant difference test. P-values of less than 0.05 were considered to indicate statistical significance. All the results were expressed as mean ± S.E.M for 6 animals in each group.

**Results and Discussion**

Table **-1** shows that 15.06% w/w methanol extract having dark brownish black colour with characteristic odour and semisolid consistency, 14.56% w/w Acetone extract having dark brownish black colour with characteristic odour and semisolid consistency were obtained.

Qualitative chemical examinations of methanol and Acetone extracts showed presence of Carbohydrates, Flavonoids, Amino acids, Steroids, Triterpenoids, Glycosides, and Tannins and phenolics (Table-2).

**Estimation of Total phenolics and Total Flavonoids of Methanol and Acetone extracts of *A. subulatum Roxb.* by Spectrophotometric method:**

**Total Phenolics:**

Total phenolic content in the Methanol and acetone extract were found to be 4.494 and 6.223 µg/5mg (0.09% and 0.124%w/w) respectively calculated in terms of Gallic acid by using the calibration curve of Gallic acid (Fig.-1). The phenolic compounds may contribute directly to the anti oxidative action. Hatano et al showed that Phenolic compounds are effective hydrogen donors, which makes them good

antioxidants. Shahidi and Naczkreported that naturally occurring phenolic compounds exhibit antioxidative activity. Diplockhad described that phenols are very important plant constituents because of their scavenging ability due to their hydroxyl groups. Thus, the therapeutic properties of *A. subulatum* Roxb. may possibly be attributed to the phenolic compounds.

**Total Flavonoids:**

The total flavonoids content of methanol and acetone extracts of *A. subulatum* Roxb. Seed was calculated by using calibration of Rutin (Fig.-2) and found to be 2.48% w/w was expressed as equivalent to Rutin.

Fig.3 and table-3 shows that in Acetone extract 8 peaks were observed its Rf and area is shown in table. Peak no 2 at Rf 0.16 was assigned as Protocatechuic acid by matching Rf with standard Protocatechuic acid which is shown in picture of HPTLC plate.

Graph-4 and Table- 4 shows that in Methanol extract 11 peaks were observed its Rf and area is shown in table. Peak no 2 at Rf 0.16 was assigned as Protocatechuic acid by matching Rf with standard Protocatechuic acid which is shown in picture of HPTLC plate.

Concentration of Protocatechuic acid in acetone extract and methanol extract were found to be 10.2 and 8.371 %w/w respectively calculated by regression equation $y = 452.83x + 411.63$, obtained from calibration curve of standard Protocatechuic acid (Fig-9).

Fructose significantly increased serum fasting glucose ($p<0.001$), FIRI ($p<0.001$) as compared to control group, although serum insulin was not found to be increasing significantly. Acetone and methanol extract treatment significantly reduced the serum glucose ($p<0.01$) and FIRI ($p<0.001$) as compared to fructose treated group (Table-5).

**Conclusion-**

The major findings of the study were (1) fructose feeding for 30 days resulted in insulin resistance indicated by FIRI. Fructose feeding for 30 days although increases the serum insulin level but it was not found to be significant. (2) A. subulatum seed extracts treatment for 30 days along with fructose largely prevented the rise in serum biochemical parameters. (3) Metformin as previously reported prevents the rise in serum biochemical parameters. Oxidative stress was also found to be inducing with fructose and was attenuated with A. subulatum seed extracts and Metformin.

Earlier fructose was considered as one of the glucose alternative in diabetic patients. But afterwards it was found that upon chronic usage fructose causes **metabolic syndrome** including insulin resistance. Research in the metabolism of fructose has left more questions about the difference between short-term positive effects, and the negative effects of chronic, long-term use of fructose sugars. The long-term negative effects can include changes in digestion, absorption, plasma hormone levels, appetite, and hepatic metabolism, leading to development of insulin resistance, diabetes, obesity, and inevitably cardiovascular disease.

Fructose is a potent regulator of glycogen synthesis and liver glucose uptake. Therefore any catalytic

improvements are due to hepatic glucokinase and glucose uptake facilitation. However, as mentioned, the beneficial effects do not continue with chronic fructose utilization. Because of its lipogenic properties, excess fructose in the diet can cause glucose and fructose malabsorption, and greater elevations in TG and cholesterol compared to other carbohydrates. Of the key importance is the ability of fructose to bypass the main regulatory step of glycolysis, the conversion of gluctose-6-phosphate to fructose 1,6-bisphosphate, controlled by phosphofructokinase. Thus, while glucose metabolism is *negatively* regulated by phosphofructokinase, fructose can *continuously* enter the glycolytic pathway. Therefore, fructose can uncontrollably produce glucose, glycogen, lactate, and pyruvate, providing both the glycerol and acyl portions of acyl-glycerol molecules. These particular substrates, and the resultant excess energy flux due to unregulated fructose metabolism, will promote the overproduction of TG.

It also been reported that fructose causes the metabolic syndrome through involvement of leptin, adiponectin, and free fatty acids.

With our observation it was found that insulin is not increasing significantly but insulin resistance is increasing. It is in compliance with the previous results.

Amomum subulatum was found to be decreasing the insulin resistance induced by the fructose feeding.

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**Table -1 Physical character of various extracts of *A. subulatum* Roxb. Seeds**

| Extract | % Dry wt. in gms. | Colour | Odour | Consistency |
|---|---|---|---|---|
| Methanol | 15.06 | Dark Brownish black | Characteristic | Semisolid |
| Acetone | 14.56 | Dark Brownish black | Characteristic | Semisolid |

**Table -2 Phytochemical screening of various extracts of *A. subulatum* Roxb seeds**

| Nature | Methanol | Acetone |
|---|---|---|
| Alkaloids | - | - |
| Carbohydrates | + | + |
| Flavonoids | + | + |
| Amino acids | + | + |
| Steroids | + | + |
| Triterpenoids | + | + |
| Saponins | - | - |
| Glycosides | + | + |
| Tanins & Phenolics | + | + |

**S.P.E**=Petroleum Ether, **SCH**. = Chloroform, **SMET**=Methanol**, SAQ=** Aqueous

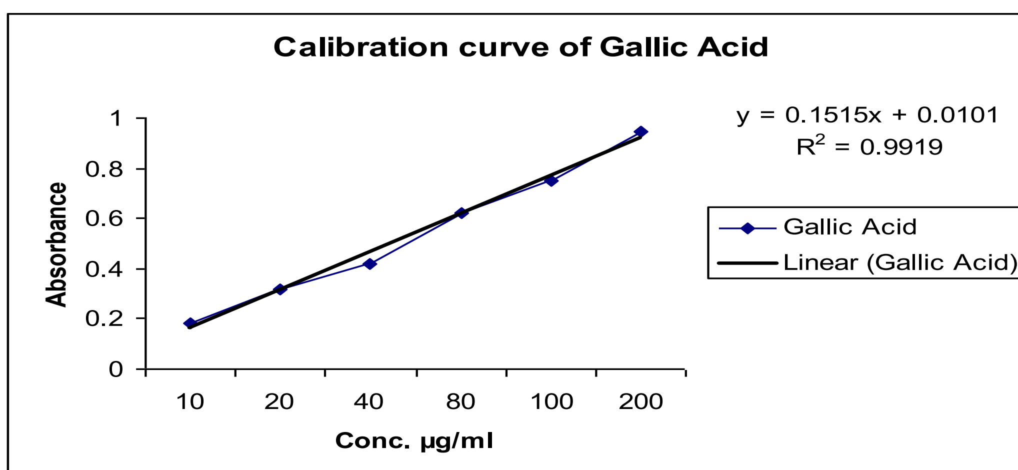


**Fig.1 Calibration Curve of Gallic Acid**

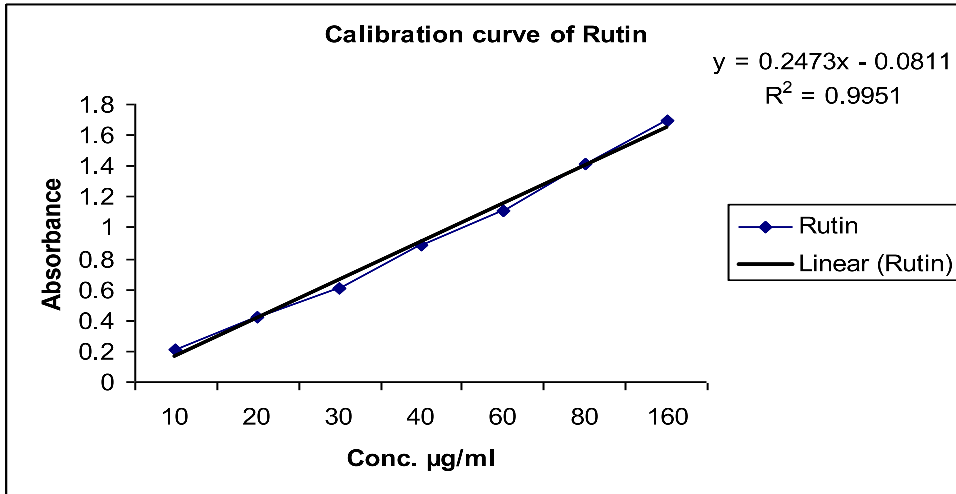


**Fig.2 Calibration Curve of Rutin**

**Estimation of Protocatechuic acid by HPTLC Methanol and Acetone extract of *A. subulatum Roxb.*HPTLC Finger Printing of both extract**

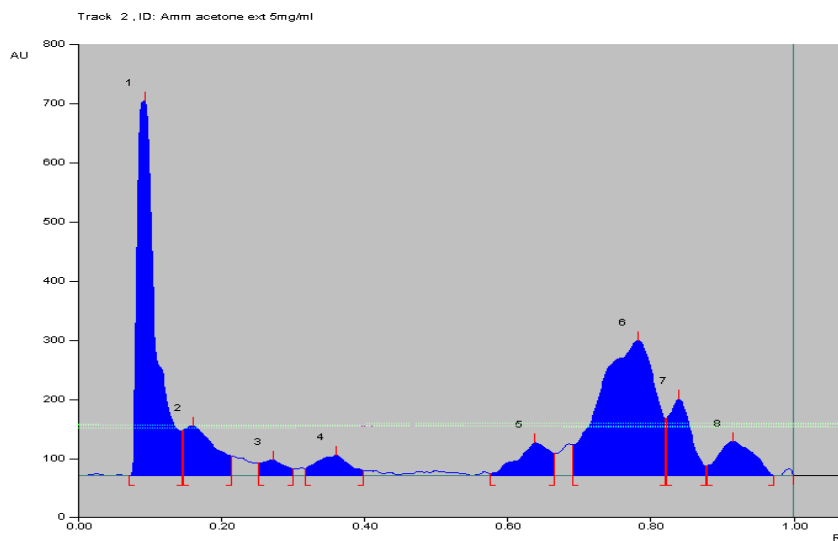


**Fig.-3 Finger** print of Acetone extract of *A. subulatum* (5mg/ml)

**Table -3** Rf and area Acetone extract of *A. subulatum* (5mg/ml)

| Peak No. | Rf | Area |
|---|---|---|
| 1 | 0.09 | 12149.8 |
| 2 | 0.16 | 2734.5 |
| 3 | 0.27 | 657.5 |
| 4 | 0.35 | 1190.8 |
| 5 | 0.64 | 1891.7 |
| 6 | 0.78 | 13094.5 |
| 7 | 0.84 | 2981.1 |
| 8 | 0.91 | 2119.3 |

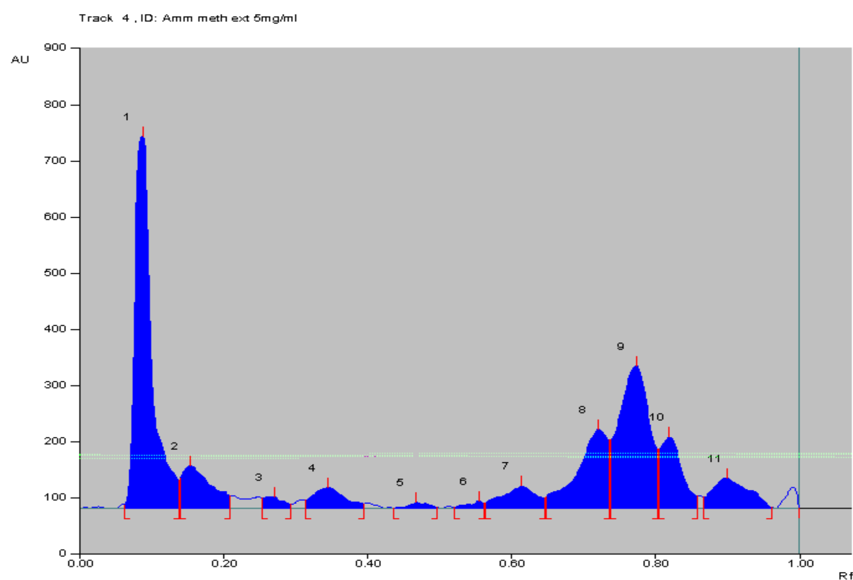


**Fig. -4** Finger print of Methanol extract of A. *subulatum* (5mg/ml)

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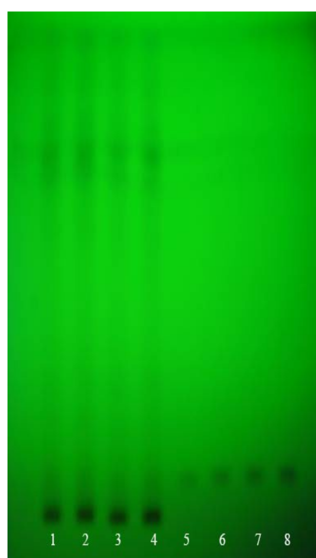
**Table-4 Rf and area of 10µl Methanol extract**

| Peak No. | Rf | Area |
|---|---|---|
| 1 | 0.06 | 11852.6 |
| 2 | 0.14 | 2307.6 |
| 3 | 0.25 | 430.5 |
| 4 | 0.31 | 1227.2 |
| 5 | 0.44 | 209.8 |
| 6 | 0.52 | 205.8 |
| 7 | 9.0 | 1388.7 |
| 8 | 0.65 | 4478.8 |
| 9 | 0.74 | 8049.2 |
| 10 | 0.81 | 2824.1 |
| 11 | 0.87 | 2054.4 |

1.




2. 5µl Acetone extract (5mg/ml)
3. 10µl Acetone extract (5mg/ml)
4. 5µl Methanol extract (5mg/ml)
5. 10µl Methanol extract (5mg/ml)
6. 0.2µl Protocatechuic acid Standard (1mg/ml)
7. 0.4µl Protocatechuic acid Standard (1mg/ml)
8. 0.6µl Protocatechuic acid Standard (1mg/ml)
9. 0.8µl Protocatechuic acid Standard (1mg/ml)

Solvent system Chloroform: Acetic acid (9:1)

Detection at 254nm

**Fig -5 Image of HPTLC plate (254nm) fingerprinting**

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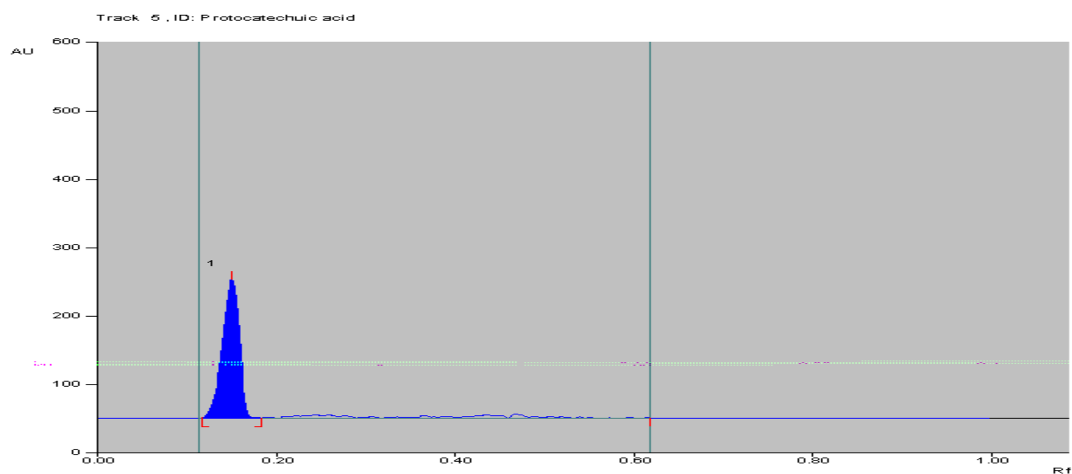



**Fig.-6 Chromatogram of standard Protocatechueic acid (Rf 0.16); mobile phase: Chloroform: Acetic acid (9:1)**

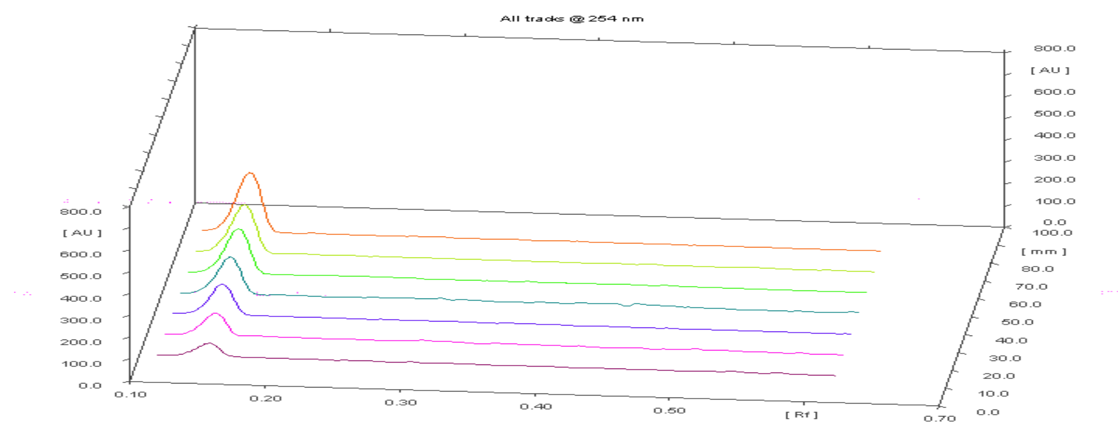
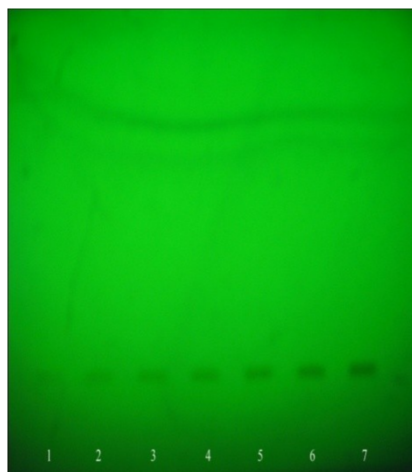



**Fig.-7 Thee dimensional image of calibration spots of Protocatechuic acid (all tracks at 254nm)**




1 - 100 ng of Protocatechuic acid Standard

2 - 200 ng of Protocatechuic acid Standard

3 - 300 ng of Protocatechuic acid Standard

4 - 400 ng of Protocatechuic acid Standard

5 - 500 ng of Protocatechuic acid Standard

6 - 600 ng of Protocatechuic acid Standard

7 - 800 ng of Protocatechuic acid Standard

**Fig 8 Image of HPTLC plate (254nm) for calibration curve**

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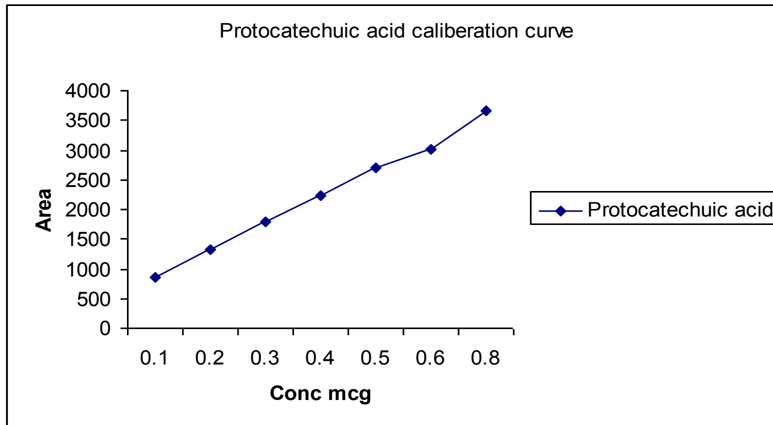


**Fig.-9 Calibration curve of Protocatechuic acid ($R^2$ 0.9963)**

**Table-5 Effect of various extracts of *A. subulatum* seed on fructose fed insulin resistance**

| Parameter | Normal rats (NC) | Diabetic Control rats (DC) | AE200 | AE400 | AE800 | ME200 | ME400 | ME800 | Metformin |
|---|---|---|---|---|---|---|---|---|---|
| Serum Insulin (ng/dl) | 17.99± 0.41 | 19.92± 0.48 | 18.55± 0.37 | 18.33± 0.33 | 18.03± 0.17 | 18.25± 1.59 | 18.66± 1.59 | 18.05± 1.28 | 18.09± 0.41 |
| Blood Glucose (µu/ml) | 78.46± 0.49 | 385.38± 1.02* | 246.27± 1.47** | 194.73± 1.0** | 127.87± 0.56** | 290.59± 2.56** | 197.78± 18.1** | 151.36± 14.3** | 85.59± 1.89 |