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Proximate analysis of medicinally important plant *Annona squamosa* seeds

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Abstract
The *Annona squamosa* (Sitaphal; family – *Annonaceae*) is mainly distributed in the regions of America, Australia, Africa, Malaysia and India. The proximate analysis of medicinal plants or its phytoconstituents become a highly complex issue. Keeping in view the traditional, alternative and complementary medicinal uses, sporadic pharmacognostic reports are available on plant. Therefore, it was considered worthwhile to establish a complete proximate analysis of this clinically potential plant. The various proximate analysis parameters of plant were established using well established methods reported in scientific reports. The mean values of total ash, moisture content, protein content, fat content, fiber content and carbohydrates content were found to be 11.02 ± 0.02, 8.23 ± 0.01, 13.32 ± 0.58, 30.21 ± 0.12, 7.28 ± 0.10 and 45.45 ± 0.98 % w/w respectively. The plant has been reported to contain sterols, triterpenoids, fats in *n*-hexane extract; alkaloids, triterpenoids in chloroform extract; saponins, flavonoids, phenols / tannins, proteins in methanol extract and proteins, saponins, carbohydrates in water extract. The mean values of total phenols and flavonoids content in methanol extract of plant seeds were found to be 7.11 ± 0.41 and 2.52 ± 0.10 % w/w respectively. The methanol extract exhibited maximum antioxidant activity (IC_{50} = 64.71 µg/ml) in comparison to rutin (IC_{50} = 5.91 µg/ml). Further, it can be suggested that antioxidant activity of plant may be due to presence of higher amount of total phenols and flavonoids as major bioactive compounds. At last, the research findings of present investigations suggested that various proximate analysis standards for *Annona squamosa* seeds help the various research persons working in the field of medicinal plants for various natural plants related activities such as phytochemical and pharmacological work.

Keywords: ash, antioxidant, custard apple seeds, flavonoid, moisture, protein

Introduction
In the old system of herbal medication, the medications are basically apportioned as fluid or ethanol extricate. The therapeutic plants ought to be real and free from destructive materials like pesticides, heavy metals, and microbial and radioactive pollution. The herbal medicinal plant ought to be single dissolvable extraction once or more than once or watery concentrate or as depicted in the old writings. The concentrate ought to be then checked for pharmacological action in exploratory animal models. The bioactive concentrate ought to be standardized based on active compound. The bioactive concentrate ought to go through restricted safety investigations (Adailkan and Gauthaman 2001; Alam *et al* 1994) [1, 2].
The *Annona squamosa* is belonging to family *Annonaceae*. The plant is mainly distributed in the regions of America, Australia, Africa, Malaysia and India. The plant is commonly known as sitaphal in South India and sharifa in North India. This fruit is popularly known as sweet sop. In India the crushed leaves are applied on ulcers and wounds and a leaf decoction is taken in cases of dysentery. In Aligarh district of Northern India, villagers used to consume a mixture of 4-5 newly grown young leaves of *A. squamosa* along with black pepper for management of diabetes. It is documented that this may ensure up to 80% of the positive results with continued therapy. The bark decoction is given as a tonic and to halt diarrhea. Throughout tropical America, a decoction of the leaves is imbibed either as an emmenagogue, febrifuge, tonic, cold remedy, digestive, or to clarify urine. The leaf decoction is also employed in baths to alleviate rheumatic pain (Mariod *et al* 2010; Ramalingum and Mahomoodally 2014) [12, 13].
This is the most often criticized aspect of herbal medicines. One important fact is that an herbal preparation is administered for its holistic value. Each herbal ingredient in the herbal preparation has an array of chemical constitution with complex molecular formulae. Thus each herbal preparation is a source of poly-pharmacy within itself.
The proximate analysis of medicinal plants or its phytoconstituents becomes a highly complex issue (Verma *et al* 2021) [14]. Despite it is major limitation, pharmaceutical industry strives hard to have in house specifications based on the quantification of marker compounds. Therefore a consensus is being arrived at to incorporate the qualitative finger-printing together with other physicochemical parameters of quality protocols for herbal medicines is an on-going process and this shortcoming could be overcome shortly. Keeping in view the traditional, alternative and complementary medicinal uses, sporadic pharmacognostic reports are available on plant. Therefore, it was considered worthwhile to establish a complete proximate analysis of this clinically potential plant.

Materials and Methods
Plant Material
Keeping in view the traditional, alternative and complementary medicinal uses, sporadic pharmacognostic reports are available on *Parthenium hysterophorus*

Therefore, it was considered worthwhile to establish a complete monograph of this clinically potential plant and implement the following plan of work to achieve the goal.

Fig 1: Powder of custard apple seeds

Solvents, Chemicals and Reagents

The various chemicals, reagents and solvents of analytical grade used in present investigations were purchased from E-Merck Ltd., Mumbai and S.D. Fine Chemicals, Biosar.

Ash Content

About 2 to 3 g of the powdered crude drug was accurately weighed in a silica crucible and incinerated in a muffle furnace at a temperature not exceeding 450ºC until free from carbon. The crucible was taken out, cooled in a desiccator for 30 min and weighed. The incineration was continued until constant weight of ash was recorded in two consecutive weighing. The percentage of ash on dried weight basis of the drug was calculated from reading of the three samples. The percentage of total ash was calculated as: % of total ash = (weight of the ash × 100) / weight of sample (Kumar and Kumar 2016; 2017) [7, 8].

Moisture Content

The moisture content of plant aerial parts was determined separately ***by azeotropic distillation method following the procedure given in*** scientific reports***. The experiment was done in triplicate. Toluene*** (previously saturated with distilled water) ***was used in the determination of moisture content.*** Coarsely powdered plant material (5 g) was taken in round bottom flasks and 200 ml of prepared toluene was added. The flask was heated gently on heating mantle till its contents began to boil. Then, distillation was carried out at the rate of about 2 drops per sec until most of the water had distilled over. Finally, the rate was increased to about 4 drops per sec which was maintained for 5 min (Kumar and Kumar 2016; 2017) [7, 8].

Protein Content

The content of crude protein in the medicinal and aromatic powdered plant material was assessed by well-known analytical approach known as Micro Kjeldahl Technique. The initial step is the transformation of natural nitrogen to ammonium sulphate which includes the absorption of the test material with concentrated Sulphuric acid and known catalyst. The ammonium sulfate was decayed with Sodium hydroxide. The released alkali was refined with 2% boric acid. Titration was done with the nitrogen acquired utilizing 0.05N hydrochloric acid. Phenolphthalein was utilized as a potential indicator. The value of nitrogen acquired was multiplied by a factor 6.25 to give the level of crude protein (Lalitha and Vijayalakshmi 2018) [10].

The cycle of processing started with the expansion of ten gram of sodium sulfate, half gram of copper sulfate and two gram of the test in a Kjeldahl cup. The substances of the apparatus were blended well and warmed for 15-20 min alongside 25 ml of concentrated sulfuric acid in a slanted position. The glass beads were added to the flask to prohibit the back pressure of substances inside the flask. The measure was preceded until greenish shading was framed. The arrangement was permitted to cool. Then, at that point, 100 ml of refined water was added to the arrangement in the Kjeldahl cup. It was shaken well and moved to a 250 ml cone like flask. The all out volume was made up to 250 ml with refined water. The cone like flask containing 10-15 ml of 2% boric acid was put beneath the refining mechanical assembly. In a Micro Kjeldahl steam refining mechanical assembly, 5 ml of the aliquots was added trailed by the expansion of 1 drop of phenolphthalein marker and 10-15 ml of 40 % Sodium hydroxide. The cycle of refining was proceeded for 5-10 min until the arrangement was liberated from alkali. The refined item was titrated against N/10 H2SO4. The percentage of nitrogen was estimated as:

% of Nitrogen = {ml of 0.1 N sulfuric acid consumed × 250 × 0.0014 × 100} / {volume of aliquot used × sample weight in grams}. Further % of crude protein content = % of nitrogen × 6.25.

Crude Fat Content

Unrefined fat was assessed by well-known ether extraction protocol (Lalitha and Vijayalakshmi 2018) [10]. An extraction thimble joined to the Soxhlet extractor was taken in which added 5 gm of the powdered test. A pre - weighed funnel shaped flagon was appended to the extractor. Ether was poured into the extractor. The whole arrangement of device was put in a water shower at 60 - 80ºC. It was additionally associated with the condenser. The extractor was permitted to cool for around 8 h with the assistance of a condenser which provided cool water ceaselessly. The thimble was eliminated from the extractor. Resetting the assembly into distillation setup and warming in a water bath would result in ether recovery from the recipient flagon. After separating the recipient cup, it was dried in a hot air oven at 100ºC for 60 min. It was then, at that point cooled and gauged.

Percentage of crude fat = {weight of crucible with dry residue - weight of crucible with ash} × 100 / sample taken in grams.

Crude Fiber Content

The unrefined fiber was gotten from absorption of tests free from fat utilizing 1.25% of sulphuric acid and sodium hydroxide arrangements individually under standard conditions (Lalitha and Vijayalakshmi 2018) [10].

Percentage of crude fiber = {weight loss after ignition / weight of sample taken} × 100.

Carbohydrates Content
The content of carbohydrates was estimated by minus the complete ash content, unrefined protein, crude fiber and unrefined fat from the whole amount of test sample (Lalitha and Vijayalakshmi 2018) [10].

Percentage of carbohydrates = 100 – (crude protein % + crude fat % + crude fiber % + total ash %).

Phytochemical Screening of Various Extracts
The daylight dried seeds of plant were coarsely powdered with the assistance of processor. The 25 g of powdered seeds were separated with the assistance of notable extraction contraption known as Soxhlet mechanical assembly utilizing solvents (150 ml each) in expanding request of polarity *viz*., n-hexane, chloroform and methanol. The marc of plant was separated with water (150 ml) on a hot plate for 2 h by decoction technology to get ready water extract. Solvents and water from rough concentrates were recuperated under diminished pressing factor utilizing rotating vacuum evaporator to get n-hexane extract, chloroform extract, methanol extract and water extract (Kumar and Kumar 2015) [9] and exposed to phytochemical screening to identify various gatherings of phytoconstituents present (Farnsworth 1966) [3].

Total Phenols and Flavonoids Content
The estimation of total phenols content and flavonoids content was done using the well-known analytical protocols mentioned in scientific reports available online such as Folin Ciocalteu's assay and aluminium chloride respectively (Kumar *et al* 2014) [5]. The formula involved in the calculation part of estimation of total phenols content and flavonoids content are presented as:

Total phenolic content (%w/w) = GAE × V × D× 10^{-6} × 100 / W

Where GAE = gallic acid equivalents (µg/ml); V = total volume of sample (ml); D = dilution factor; W = sample weight (g)

Flavonoid content (% w/w) = QE × V × D × 10^{-6} × 100 / W

Where QE = quercetin equivalents (µg/ml); V = total volume of sample (ml); D = dilution factor; W = sample weight (g)

***In vitro* Antioxidant Profile**
The antioxidant activity of test samples was investigated using the well-known analytical protocols mentioned in scientific reports available online such as *in vitro* DPPH assay (Kumar and Dhobi 2017) [4]. The formula involved in the calculation part of the antioxidant activity is presented as:

% Radical Scavenging Power = [Ac-(As-Ao]/Ac × 100

Where Ac = Absorbance of control (DPPH); As = Absorbance of sample/ standard + DPPH; Ao = Absorbance of sample / standard without DPPH interaction.
The measurements were taken thrice, and scavenging effect was calculated based on the percentage of DPPH scavenged. IC_{50} values of the samples for antioxidant activity were calculated using standard curve of rutin.

Statistics
The data were expressed as Mean ± S.D. The test groups were compared with the standard group using one way ANOVA followed by student Newman Keul's test.

Results and Discussion
Approval of plant material is an imperative essential proceeding using it as assessment material or as prescription part for patients. Hence, it was proposed to set up various proximate analysis standards for *Annona squamosa* seeds to have established solid boundaries to approve the plant. These proximate analysis standards will help the researchers working in this field to pick real plant material for phytochemical and pharmacological work (Kumar and Kumar 2016; 2017) [7, 8].
Debris or ash esteems are useful for determine adulteration with fake, exhausted drugs, and excess of sandy and coarse matter. The event of debris material in plant tests is assessed as total ash. The consolidated combination of carbonates, phosphates and silicates of calcium and magnesium are available in total ash. The calcium oxalates are additionally significant piece of total ash if accessible in plant materials which differ in huge and variable amounts (Kumar *et al* 2012; Madaan *et al* 2010) [6, 11].
The additional measure of moisture presence in plant test goes about as a debasement and can cause decay in the plant material as it propels microbial turn of events. Accordingly, it should be settled and controlled (Kumar *et al* 2012; Madaan *et al* 2010) [6, 11].
Proteins are polymers of amino acids. Twenty unique kinds of amino acids happen normally in proteins. Proteins contrast from one another as indicated by the type, number and arrangement of amino acids that make up the polypeptide spine. Subsequently they have distinctive atomic constructions, nutritional value and physiochemical properties. Proteins are significant constituents of food varieties for various reasons. They are a significant wellspring of energy, just as containing fundamental amino-acids, like lysine, tryptophan, methionine, leucine, isoleucine and valine, which are vital for human wellbeing, yet which the body can't blend. Proteins are additionally the major primary parts of numerous normal food varieties, frequently deciding their general surface, e.g., delicacy of meat or fish items. The proteins isolation are regularly utilized in food sources as fixings on account of their exceptional useful properties, i.e., their capacity to give advantageous appearance, surface or solidness. Ordinarily, proteins are utilized as gelling specialists, emulsifiers, frothing specialists and thickeners. Numerous food proteins are catalysts which are fit for upgrading the pace of certain biochemical responses. These responses can have either a great or negative impact on the general properties of food varieties. Food examiners are keen on knowing the whole amount, type, sub-atomic design and utilitarian properties of the proteins in food varieties (Lalitha and Vijayalakshmi 2018) [10].
The plant parts are wealthy in sugars as they keep up with the energy potential, proteins are viewed as the structure square of cells, fats are the energy suppliers and helps in the retention of fat dissolvable nutrients and crude fiber is vital for improve the processing of food (Lalitha and

Vijayalakshmi 2018) [10]. Fat assumes a significant part in numerous food varieties. Fat add to the kind of food just as it gives surface and furthermore mouth feel to the food. It is a significant segment which gives us most extreme energy such as each gram of food provides 9 Kcal of energy. Additional admission of fat generally prompts heftiness and beneath the level lead to hunger. It feeds the body with all the fundamental unsaturated fat that body cannot integrate and furthermore help in building the body (Lalitha and Vijayalakshmi 2018) [10]. Unrefined fiber comprises to a great extent of cellulose and lignin (97%) in addition to some mineral matter. It addresses simply 60% to 80% of the cellulose and 4% to 6% of the lignin. The rough fiber content is regularly utilized as a proportion of the nutritive worth of poultry and animals takes care of and furthermore in the examination of different food varieties and food items to recognize defilement, quality and amount (Lalitha and Vijayalakshmi 2018) [10]. The various proximate analysis standards for *Annona squamosa* seeds are presented in table 1.

Table 1: The mean values of various proximal analysis parameters of plant seeds

Parameters	Observations
Total ash	11.02 ± 0.02 % w/w
Moisture content	8.23 ± 0.01 % w/w
Protein content	13.32 ± 0.58 % w/w
Fat content	30.21 ± 0.12 % w/w
Fiber content	7.28 ± 0.10 % w/w
Carbohydrates content	45.45 ± 0.98 % w/w

n = 3

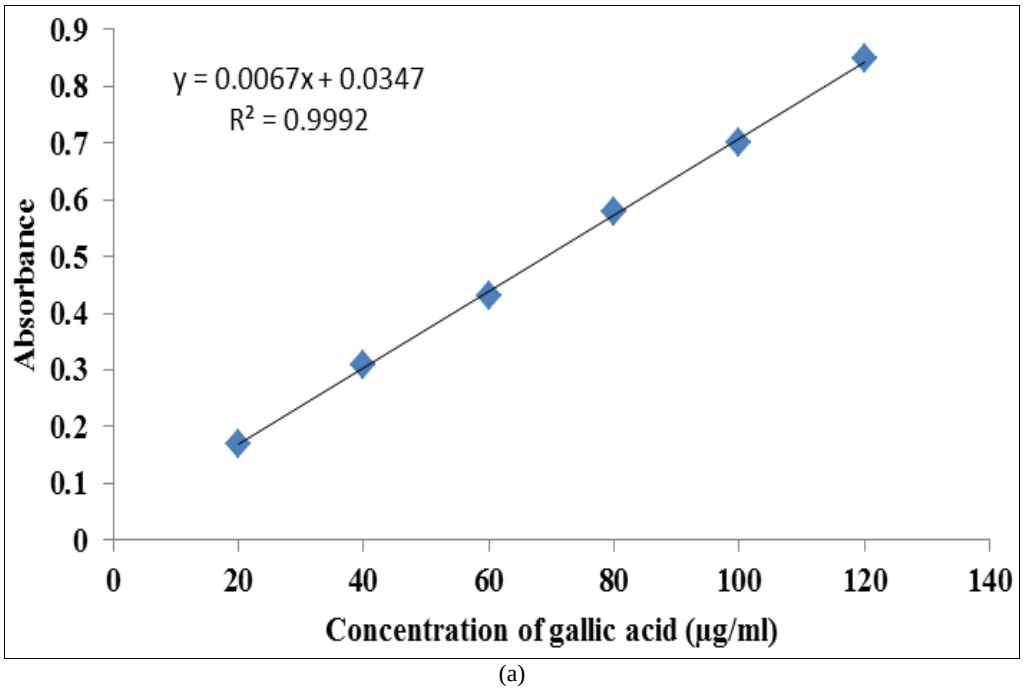
The fundamental phytochemical examinations help to recognizable proof of different classes of essential and auxiliary metabolites present in unrefined extracts. The greater part of these tests is shading responses which are explicit for specific substances or chemicals. These tests incorporate the overall tests for alkaloids, glycosides, tannins, flavonoids, and so on (Madaan *et al* 2010) [11]. The results of fundamental phytochemical examinations of different rough concentrates are displayed in table 2.

Table 2: Phytochemical screening of various extracts of plant seeds

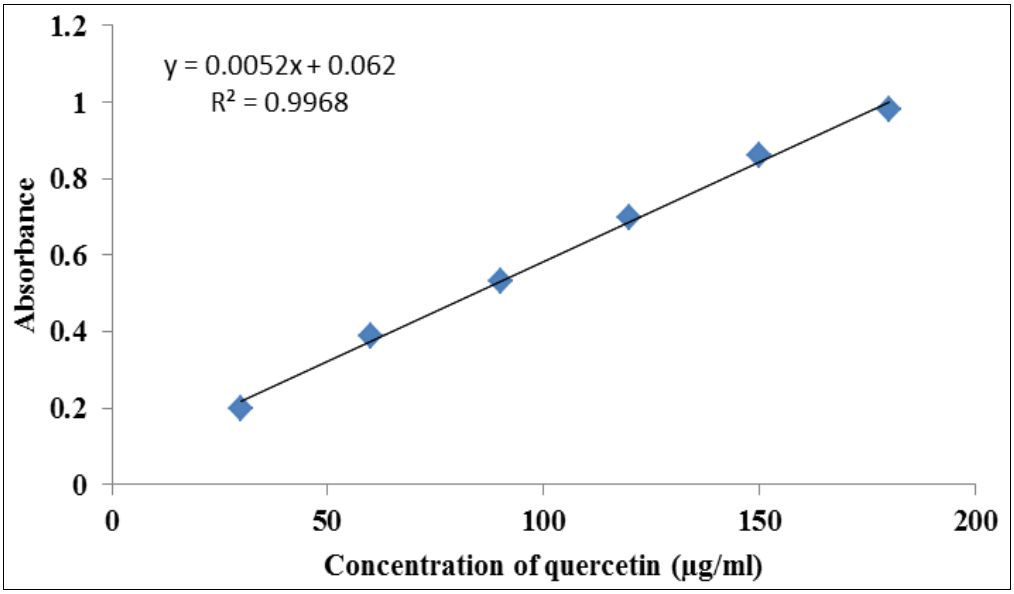
Class of phytoconstituents	n-hexane extract	Chloroform extract	Methanol extract	Water extract
Alkaloids	-	+	-	-
Glycosides	-	-	-	-
Sterols / Triterpenoids	+/+	-/+	-/-	-
Saponins	-	-	+	+
Flavonoids	-	-	+	-
Coumarins	-	-	-	-
Phenols / tannins	-	-	+	-
Carbohydrates	-	-	-	+
Proteins	-	-	+	+
Fats	+	-	-	-

+ Present, - Absent

The preliminary phytochemical profile additionally affirmed that only the methanol extract of plant seeds showed presence of bioactive classes of compounds like phenolic and flavonoids. Hence, it was planned to estimate the total phenolic and flavonoids content in methanol extract of plant seeds. The quantification of total phenol and flavonoid matters in methanol extract of crude was completed based on regression equations (Fig 2) of respective calibration curve. It is clearly evident from table 3 that methanol extract of plant seeds contained a good amount of phenols and flavonoids.



(a)



(b)

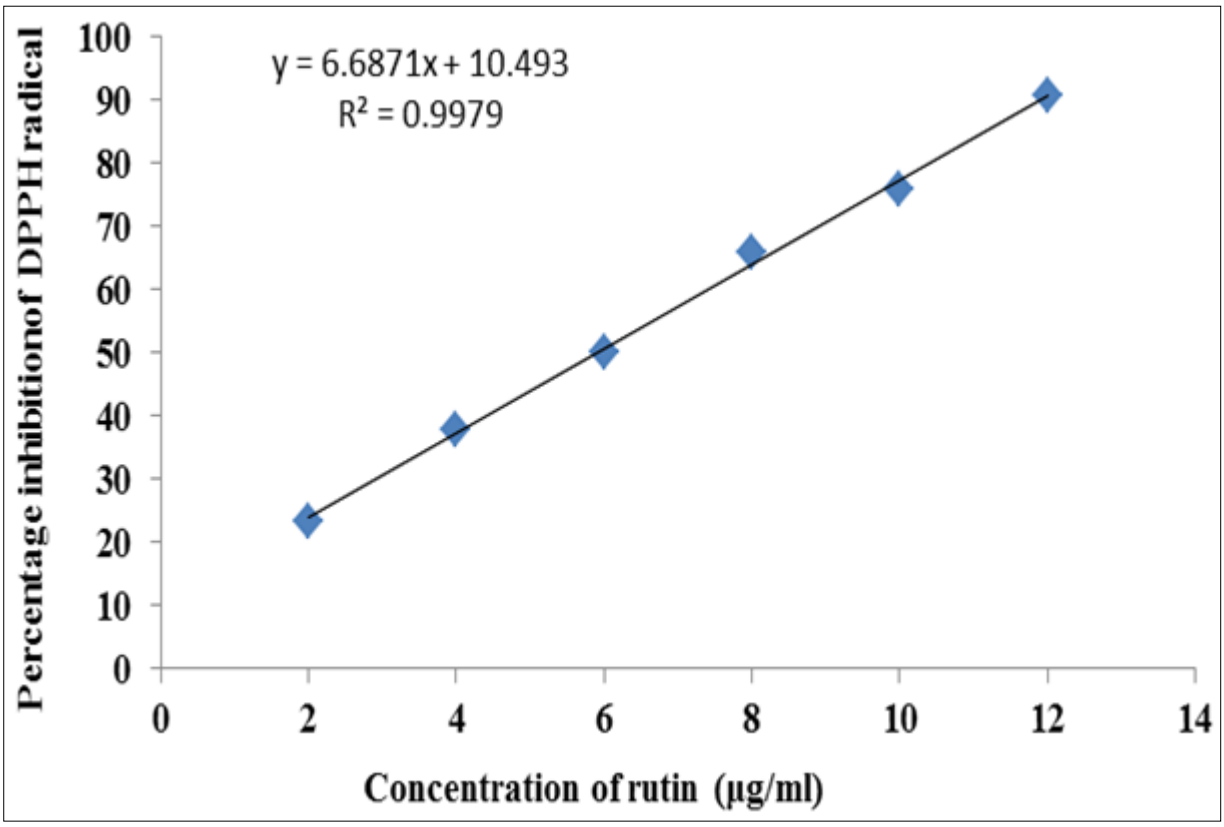
Fig 2: Calibration curve between concentration of gallic acid (a) vs. absorbance and concentration of quercetin (b) vs. absorbance

Table 3: Percentage content of total phenols and flavonoids in methanol extracts of plant seeds

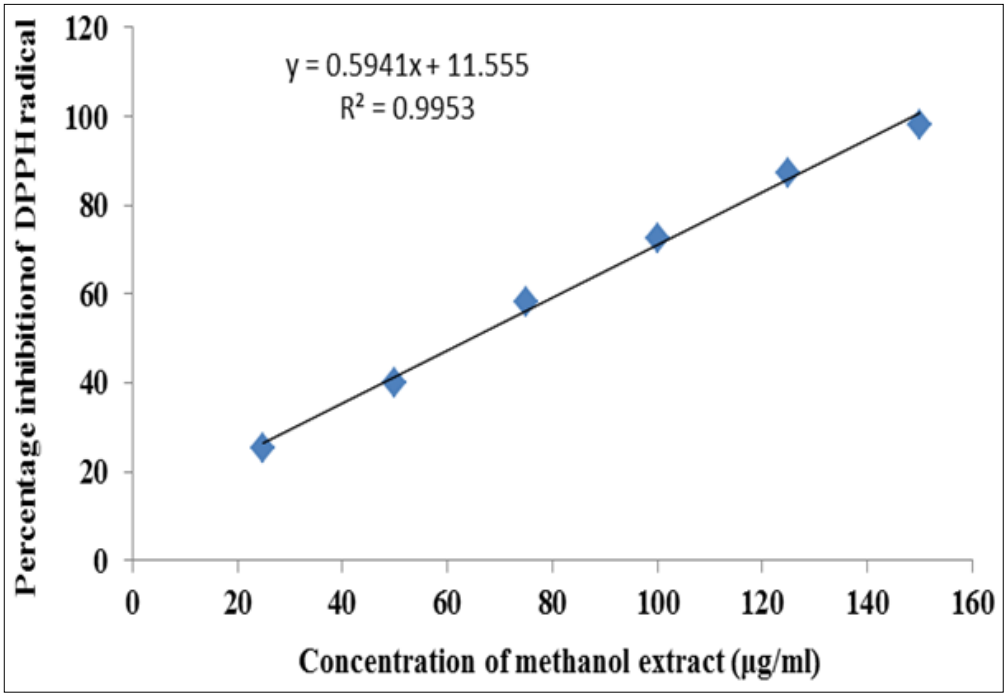
Test sample	Total phenols content (% w/w) Mean^n ± S.D.	Total flavonoids content (% w/w) Mean^n ± S.D.
Methanol extract	7.11 ± 0.41	2.52 ± 0.10

n=3

As the methanol extract of plant contained a good amount of phenolic and flavonoidal type of compounds, thus, it was planned to evaluate antioxidant activity using *in vitro* DPPH assay. The antioxidant activity observations were compared with the rutin standard antioxidant drug. Calibration curve of standard drug and test drug were prepared between % inhibition of DPPH and concentrations of standard/ test (Fig 3). The results presented in table 4 suggested that the methanol extract of plant seeds exhibited maximum antioxidant activity with IC_{50} value of 64.71 µg/ml, whereas standard rutin showed IC_{50} value 5.91 µg/ml.



(a)



(b)

Fig 3: Calibration curve between % inhibitions of DPPH vs. concentration of test sample. (a), rutin; (b), methanol extract plant seeds

Table 4: Antioxidant activity of methanol extract of plant seeds

Treatment	Concentration (µg/ml)	Mean[n] percentage inhibition of DPPH radical ± S.D.	IC_{50} Values (µg/ml)
Rutin	2	23.25 ± 0.140	5.91
	4	37.80 ± 0.225	
	6	50.14 ± 0.458	
	8	65.87 ± 0.110	
	10	75.89 ± 0.654	
	12	90.87 ± 0.870	
Methanol extract	25	25.12 ± 0.321	64.71*
	50	40.14 ± 0.460	
	75	58.40 ± 0.745	
	100	72.35 ± 0.235	
	125	87.10 ± 0.587	
	150	98.12 ± 0.987	

n=3; *P<0.05 vs. Rutin; One way ANOVA followed by Student Newman Keul's test.

Conclusion

At last, the research findings of present investigations suggested that various proximate analysis standards for *Annona squamosa* seeds help the various research persons working in the field of medicinal plants for various natural plants related activities such as phytochemical and pharmacological work. Further, it is suggested that plant contained higher amount of total phenols and flavonoids as major bioactive compounds and these compounds might be responsible for the antioxidant profile of plant.

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