World Journal of Pharmaceutical Sciences
ISSN (Print): 2321-3310; ISSN (Online): 2321-3086
Published by Atom and Cell Publishers
Available online at: http://www.wjpsonline.org/
Original Article

Physico-phytochemical analysis & Estimation of total phenolic, flavonoids and proanthocyanidin content of *Persea americana* (avocado) seed extracts

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Received: 15-02-2017 / Revised: 29-03-2017 / Accepted: 31-03-2017 / Published: 01-04-2017

ABSTRACT

The *Persea americana* is a valuable and important fruit, used in folklore medicine and research studied also exhibited that they have many pharmacological activities such as antioxidant, Anti-inflammatory, antihypertensive, hepatoprotective, hypolipidemic, and recently amoebicidal activities and various other biological activities. Maintaining quality standards of drugs is the need of today, because of its increasing use.
The present study was designed to evaluate the standard profile of the seeds of avocado. The extracts obtained by using different solvents. Phytochemical, physiochemical and TLC analysis were then carried out and loss on drying, total ash content, the yield of acid insoluble ash, the yield of alcohol soluble ash, the yield of water soluble ash were found to be 9.28, 2.53, 2.19, 45.26 and 5.79%w/w respectively. Phytochemical analysis like phenols, flavanoids, terpenoids, steroids, alkaloids, saponin, carbohydrates, protein, amino acids, phytosterols were found to be present in different seed extract of *Persea americana*. TLC system for analysis was shown to be Chloroform:Methanol:Formic acid was used in the ratio of (60:40:2) respectively. The bands found in this method and its Rf values 0.86cm indicate the presence of β –carotene (0.92cm), which is a tetraterpenoid. The results significantly validate the use of *Persea americana* and help to many researchers about quality and purity of avocado seeds.

Keywords: Avacado, flanvoids, physio-phytochemicals analysis, Phenol, TLC.

INTRODUCTION

The fruit of *Persea americana* of family Lauraceae is one of the most widely cultivated varieties of avocado pear in tropical and subtropical areas and eaten in many parts of the world [1]. In recent years, research has focused on various parts of the plants. The fruit, in particular, has been shown to possess various medicinal properties. The edible fruit pulp contains up to 33% oil rich in monounsaturated fatty acids [2], that are believed to modify the fatty acid contents in cardiac and renal membranes and enhance the absorption of α/ß carotene and lutein [3]. The carotenoid content has been reported to play the significant role in cancer risk reduction [4]. Other properties of the oil include wound healing [5], and hepatoprotection [6]. Phytochemical screening of the leaf extract of *P. americana* revealed the presence of flavonoids which were powerful antioxidants capable of scavenging free radicals by donating a hydrogen atom or electron to stabilize the radical species. The metabolic study of the aqueous leaf extract of *P. americana* in a rat model showed the presence of phenolic acids [7]. which were metabolites of flavonol degradation by intestinal microflora [8]. The objective of the study was to optimize the extraction for maximum active ingredient and minimum interfering content. The polyphenol Vitamin C, carotenoids, vitamin E are compounds with antioxidant effects that help to protect cells from free radical harm. These compounds also have anti-inflammatory effects that may help prevent atherosclerosis or the thickening and hardening of the arteries associated with heart disease. Reducing sodium and maintaining an adequate intake of potassium can help to guard against high blood pressure, heart disease and stroke [9]. The plant is widely used in traditional medicine as a treatment for a toothache, intestinal parasites, diarrhea, dysentery, skin treatment, menorrhagia, stomachache, and bronchitis [10]. The oil from the seed is reportedly used [11-12] for weight loss in obese people. The aqueous extract of the *P. americana* seed has been found to have glycemic and anti-hypertensive properties, with effects on some biochemical indices [13]. The leaf possesses anti-inflammatory and analgesic activities [14]. In

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traditional medicine in southwestern Nigeria, the leafy part of the avocado is usually used to prepare infusions for older people in order to enhance their memory, but there is a dearth of information on the biochemical rationale underlying its use in the management or treatment of Alzheimer disease.

MATERIALS AND METHODS

Collection and authentication of plant material

The *Persea americana* (avocado) fruits were procured from bigbazar, Indira nagar Lucknow, Uttar Pradesh, India, in the month of November in 2016. The plant specimen was authenticated by Associate Prof. Dr. Mohd Khalid and Assistant Prof. Dr. Muhammad Arif Faculty of Pharmacy, Integral University Lucknow (Reference no.: IU/PHAR/HRB/16/07).

Preparation of plant extract

The fruit was washed with distilled water to remove dirt and soil material. The seed of the fruit was separated from the edible portion, chopped into small pieces by a table knife, and dried under shade at room temperature, ground to coarse powder, evenly packed in a Soxhlet extractor, and successively extracted by Soxhlet extraction method at a temperature not exceeding 45–55◦C, using different solvents in ascending order of their polarity, namely, pet ether, chloroform, acetone, ethanol and water to achieve different crude extracts. 20 grams of the powdered material was used in Soxhlet extraction using 200 ml of each solvent. The appearance of colorless solvent in the siphon tube was taken as the termination of extraction. The different crude extracts obtained were dried under reduced pressure and controlled temperature and the percentage yield was calculated. The Pet ether extract (PEE), chloroform extract (CE), acetone extract (AE), ethanol extract (EE), and water extract (WE) were kept in an airtight container in a refrigerator below 4◦C and used for phytochemical analysis.

PHYTOCHEMICAL ANALYSIS

The preliminary phytochemical investigation was performed for the presence or absence of plant constituents like alkaloids, glycosides, carbohydrates, phenolic compounds, tannins, flavonoids, terpenoids, saponins, sterols, proteins and resins in different seed extract of *Persea americana*.

***Detection of alkaloids*:** Extracts were dissolved individually in dilute Hydrochloric acid and filtered. Dragendroff's Test: Filtrates were treated with Dragendroff's reagent (solution of Potassium Bismuth Iodide). Formation of red precipitate indicates the presence of alkaloids.

***Detection of glycosides*:** Extracts were hydrolysed with dil. HCl, and then subjected to test for glycosides.Modified Borntrager's Test: Extracts were treated with Ferric Chloride solution and immersed in boiling water for about 5 minutes. The mixture was cooled and extracted with equal volumes of benzene. The benzene layer was separated and treated with ammonia solution. Formation of rose-pink color in the ammoniacal layer indicates the presence of anthranol glycosides.

***Detection of carbohydrates*:** Extracts were dissolved individually in 5 ml distilled water and filtered. The filtrates were used to test for the presence of carbohydrates. Molisch's Test: Filtrates were treated with 2 drops of alcoholic α-naphthol solution in a test A & B solutions. Formation of red precipitate indicates the presence of reducing sugars.

***Detection of saponins*:** Foam Test: 0.5 gm of the extract was shaken with 2 ml of water. If foam produced persists for ten minutes it indicates the presence of saponins.

Detection of tannins: Gelatin Test: To the extract, 1% gelatin solution containing sodium chloride was added. Formation of white precipitate indicates the presence of tannins.

Detection of phytosterols: Salkowski's Test: Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of Conc. Sulphuric acid, shaken and allowed to stand. An appearance of golden yellow color indicates the presence of triterpenes.

Detection of phenols: Ferric Chloride Test: Extracts were treated with 3-4 drops of ferric chloride solution. Formation of bluish black color indicates the presence of phenols.

Detection of flavonoids: Alkaline Reagent Test: Extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow color, which becomes colorless on the addition of dilute acid, indicates the presence of flavonoids.

Detection of terpenoids: Copper acetate Test: Extracts were dissolved in water and treated with 3-4 drops of copper acetate solution. Formation of emerald green color indicates the presence of diterpenes. The extract was treated with 2ml chloroform and 2ml of concentrated sulphuric acid. Formation of red color indicates the presence of Terpenoids.

Detection of proteins and amino acids: Ninhydrin Test: To the extract, 0.25% w/v ninhydrin reagent was added and boiled for few minutes. Formation of a blue color indicates the presence of amino acid.

Detection of coumarin glycoside: Alcoholic extract made alkaline and then cover the test tube containing sample with filter paper moistened with dilute NaOH solution place the covered test tube on a water bath for several minutes remove the paper and expose to UV light. It shows the blue or green presence of coumarin glycosides.

PHYSICO-CHEMICAL ANALYSIS

Estimation of ash value: Ash value was determined by the method described by Choudhry, (1996). The ash remaining following ignition at 450°C of crude drug was determined by three different methods, which measure total ash, acid-insoluble ash, water-soluble ash [15], and alcohol soluble ash.

Total ash: Indian Pharmacopoeia 1996 and WHO prescribes methods for determination of ash values. About 2-3 gm of an air dried crude drug was placed in the tarred silica crucible and was incinerated at a temperature not exceeding 450°C until free from carbon, cooled and weighed to get the total ash content.

Acid insoluble ash: Acid insoluble ash is the residue obtained after boiling the total ash with dilute hydrochloric acid and igniting the remaining insoluble matter. This measures the amount of silica present, especially as sand and siliceous earth. Ash was boiled with 25 ml of hydrochloric acid for 5 minutes. The insoluble matter was collected on ashless filter paper, washed with hot water and ignited at a temperature not exceeding 450oC to a constant weight.

Water soluble ash: Water soluble ash is the difference in weight between the total ash and the residue after treatment of total ash with water. It is a good indicator of either previous extraction of water-soluble salts in the drug or in corrected preparation. Ash was dissolved in distilled water and the insoluble part collected on an ashless filter paper and was ignited at 450C to a constant weight. By subtracting the weight of insoluble part from that of ash, the weight of the soluble part of ash was obtained.

Alcohol soluble ash: The powdered seed (5gm) samples were macerated with 100 ml of alcohol in a Stopperd flask with frequent shaking during first 6 hrs and allowed to stand for 18 hrs. It was filtered after 24 hrs. 25 ml of the filtrate was evaporated in a tarred dish at 105°C and weighed. Alcohol soluble extractive values were calculated. The experiment was repeated twice, and the average value was taken [16].

Estimation of loss on drying: This parameter determines the amount of moisture as well as volatile components present in a particular sample. The powdered drug sample (10 g) was placed on a tarred evaporating dish and dried at 105°C for 6 hrs and weighed. The drying was continued until two successive reading matched each other or the difference between two successive weighing was not more than 0.25% of constant weight [17].

Estimation of extractive value: According to Indian Pharmacopoeia 1996, British Pharmacopoeia 1980 and WHO guideline the determination of water soluble and alcohol soluble extractives is used as a means of evaluating crude drugs which are not readily estimated by other means. The extraction of any crude drug with particular solvent yields a solution containing different phytoconstituents that are such extractive value provides an indication of the extent of polar, medium polar and nonpolar components present in the plant material [18].

Determination of total phenolic content: Total phenolic components in the different extracts of *Persea americana* seed were determined with Folin Ciocalteu reagent using gallic acid as a standard [19]. The aliquots of the different extracts were taken in a test tube and made up to the volume of 1 ml with distilled water. Then, 0.5 ml of Folin-Ciocalteu reagent (1:1 with water) and 2.5 ml of sodium carbonate solution (20%) were added sequentially in each tube. The tubes were vortexed, allowed to stand in dark for 1 h with intermittent shaking and absorbance was measured at 760 nm. The phenolic content from calibration curve prepared by repeating the operation using 1 ml of gallic acid solution at concentrations (10, 20, 30, 40, 50, 60 µg/ml) in distilled water. The concentration of total phenolic compounds in the extracts was determined by comparing the absorbance of the extract samples to that of the gallic acid standard solutions. All samples were run in triplicate. The total phenolic content of the extract was then calculated as µg of Gallic acid equivalents per ml (10mg) of seed extract (µg GAE/ml).

Determination of Total Flavonoid (TF) Content: Total flavonoid content was estimated by aluminum chloride colorimetric method [20]. Suitable aliquot of each extractive were added in 10 ml volumetric flask containing 4 ml distilled water

and mixed with 0.3 ml of 5% sodium nitrite and after 5 min add 0.3 ml of 10% aluminum chloride. At 6th minute add 2 ml 1M-NaOH and made up the volume 10 ml with distilled water. Then the mixtures were allowed to stand at room temperature for 30 min with intermittent shaking and absorbance was measured at 367 nm. The calibration curve was prepared by preparing rutin solutions at concentrations 10, 20, 30, 40, 50 and 60 µg/ml in distilled water. Total flavonoid contents were calculated using a standard calibration curve, prepared from rutin. The flavonoid contents were expressed as µg of rutin equivalent per ml (10mg) of extract (µg rutin/ml).

Determination of Proanthocyanidin:
Proanthocyanidin content was determined by the vanillin-H2SO4 assay as described by Chang et al. [21]. A volume, 1.0 ml aliquots of avocado seed extract (3 mg in 10 ml absolute methanol) were mixed with 2.5 ml of 1.0% (w/v) vanillin in absolute methanol and then with 2.5 ml of 25% (v/v) sulfuric acid in absolute methanol to undergo vanillin reaction with polyphenols in avocado extract. The blank solution was prepared in the same procedure without vanillin. The vanillin reaction was carried out in 26°C water bath for 15 minutes. The absorbance at 510 nm was read and the results were expressed as (+)- catechin equivalent by a calibration method [22].

Thin layer chromatography (TLC) of Persea americana seed extract: Thin layer chromatography (TLC) performed on pre-coated 20cm × 20cm and 0.25mm thickness. The plates were prepared using silica gel G for TLC were left overnight for drying and activation at 100° c for 1 hr. TLC fingerprint of extracts were analysed. The mobile phase solvent system Chloroform:Methanol:Formic acid was used in ratio of (60:40:2). Anisaldehyde-suphuric acid reagent was used as detecting agent. Different bands were obtained and corresponding Rf values were compared and calculated taking β- carotene as the standard which is a source of tetraterpenoids using the formula- Rf=distance travelled by solute /distance travelled by solvent.

Statistical analysis: The results obtained were expressed as mean ± standard error of mean (SEM) and subjected to one-way analysis of variance (ANOVA). Least significance difference test was used to determine the significant difference between the samples.

RESULTS

Preliminary phytochemical analysis: The results of the preliminary phytochemical investigation showed the presence of phenols, flavanoids, terpenoids, steroids, alkaloids, saponin, carbohydrates, protein, amino acids, phytosterols in a different prepared seed extract of *Persea americana* (Table No.1).

Table No. 1: Phytochemical analysis seed extract of *Persea americana*

S.NO.	Parameters	Pet ether	Chloroform	Acetone	Ethanol	Water
1	Dragendorff's Test	-	+	-	+	-
2	Foam Test	-	+	-	+	+
3	Molisch's Test	-	-	-	+	+
4	Gelatin Test	-	-	-	-	-
5	Ninhydrin Test	-	-	-	+	-
6	Borntrager Test	-	-	-	+	-
7	Terpenoids Test	-	+	+	+	+
8	Ferric Chloride Test	-	-	-	+	-
9	Flavanoids Test	-	-	+	+	-
10	Steroids Test	+	+	-	-	-
11	Coumarins	-	+	-	+	-

Key, (+) = Present, (-) = Absent

Estimation of ash value: The results of ash value of *Persea americana* are mentioned in Table no. 2. Total ash, acid insoluble, alcohol soluble ash and water soluble ash values of *Persea americana* were found to be 2.53, 2.19, 45.26 and 5.79% w/w respectively with respect to air-dried crude drug.

Estimation of loss on drying: The loss on drying of *Persea americana* seed had been determined. It was found to be 9.28% w/w.

Estimation of extractive value: Extractive value for the extracts PEE, CE, AE, EE, WE was found to be 2.6%w/w 5.9% w/w , 8.3% w/w, 19.7% w/w and 13.2% w/w respectively.

Estimation of total phenolic, flavonoid and proanthocyanidin contents of P. americana seed extract: The total phenolic, flavonoid and proanthocyanidin contents in the ethanolic and water extract of *P. americana* seed was found to be 289±0.62, 243±0.19 µg /ml equivalent to gallic acid, 49.6±0.02, 37.2±0.47 µg/ml equivalent to rutin and 13.7±0.01, 9.3 ±0.61 µg/ml equivalent to catechin respectively (Figure 1, Figure 2 and 3).

Thin layer chromatography (TLC) of ethanolic seed extract of Persea americana: In the present study the most suitable TLC system for analysis was shown to be Chloroform:Methanol:Formic acid was used in the ratio of (60:40:2) respectively. The bands found in this method and its values indicate the presence of β –carotene, a tetraterpenoid.

Table No. 2: Physico chemical analysis seed extract of *Persea americana*

S. NO.	Parameter		*Persea americana* (% W/W)
1	Total ash content		2.53
2	Acid insoluble ash		2.19
3	Alcohol soluble ash		45.26
4	Water soluble ash		5.79
5	Loss on drying		9.28
Extractive value		Pet ether extract (PEE)	2.6
		Chloroform extract (CE)	5.9
		Acetone extract (AE)	8.3
		Ethanol extract (EE)	19.7
		Water extract (WE)	13.2

Values are expressed as mean ± SEM. (n = 3).

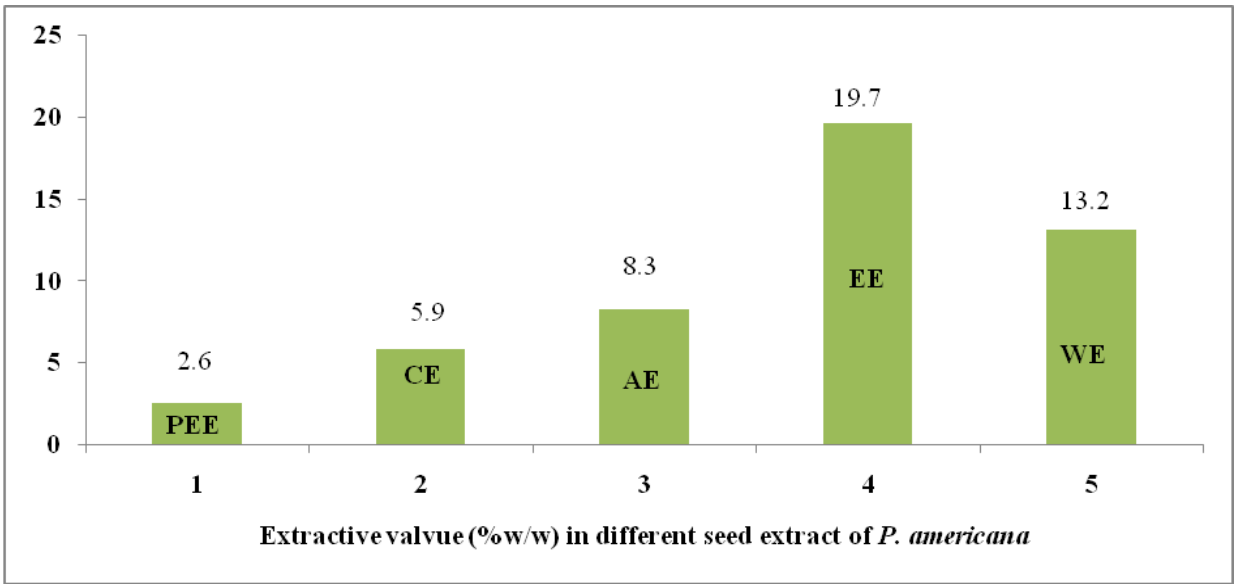


Table No.3: Total phenol, flavonoid and proanthocyanidin content of avocado seed extract

Extracts	Total phenol (µg GAE/ml)	Total flavonoid (µg rutin/ml)	Total proanthocyanidin (µg catechin/ml)
Ethanol	289±0.62	49.6±0.02	13.7±0.01
Water	243±0.19	37.2±0.47	9.3 ±0.61

All values are expressed as mean ± SEM. (n = 3).

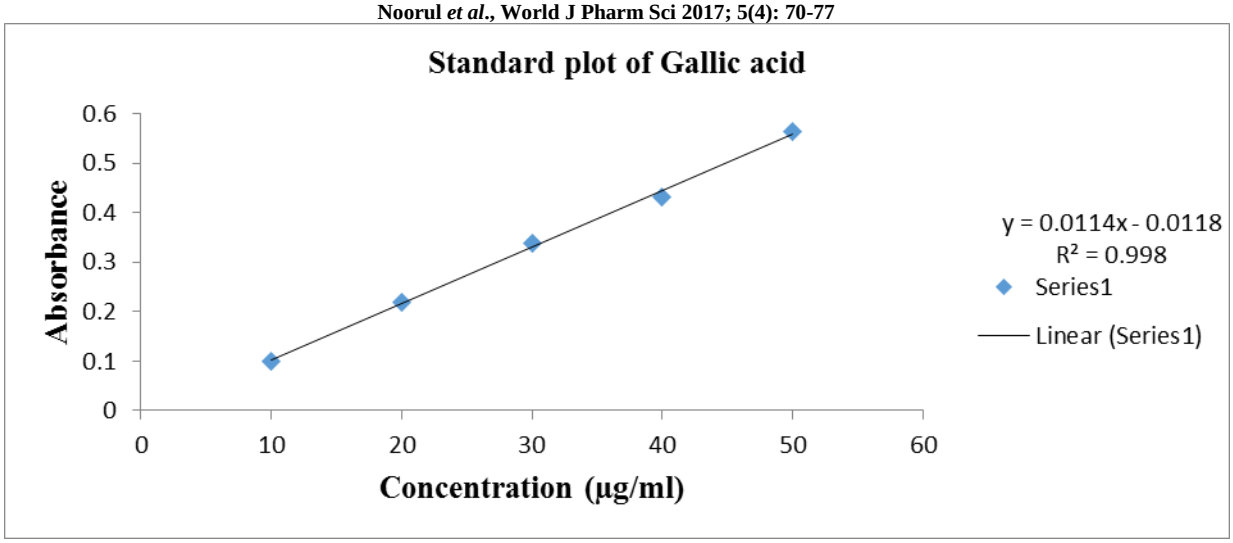


Figure 1: Gallic acid calibration curve for estimation of total phenolic content.

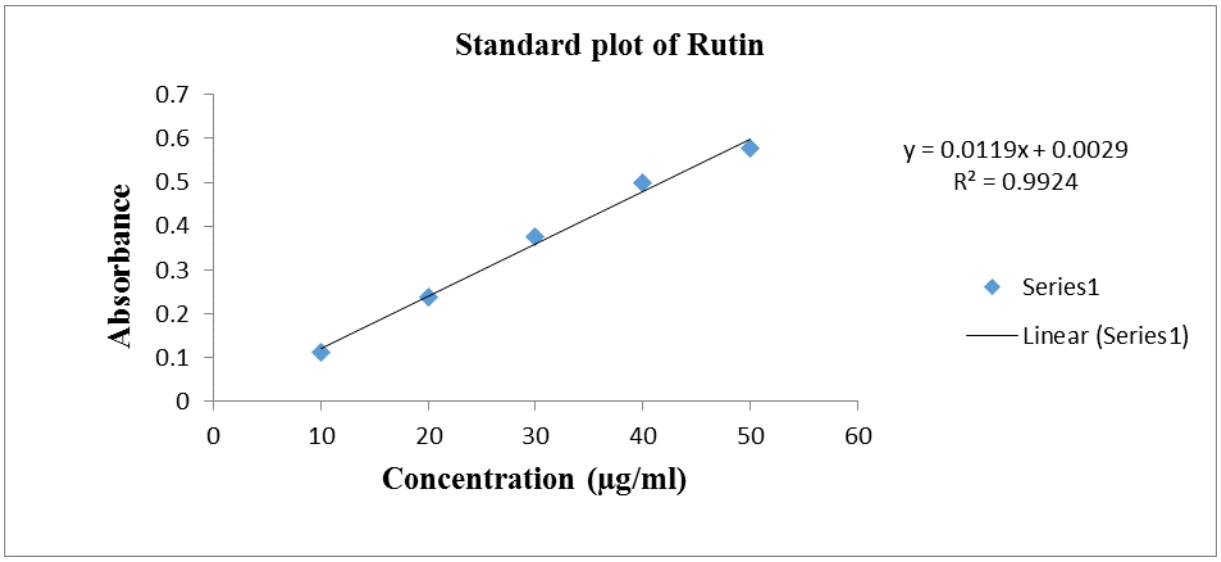


Figure 2: Rutin calibration curve for estimation of total flavonoid content

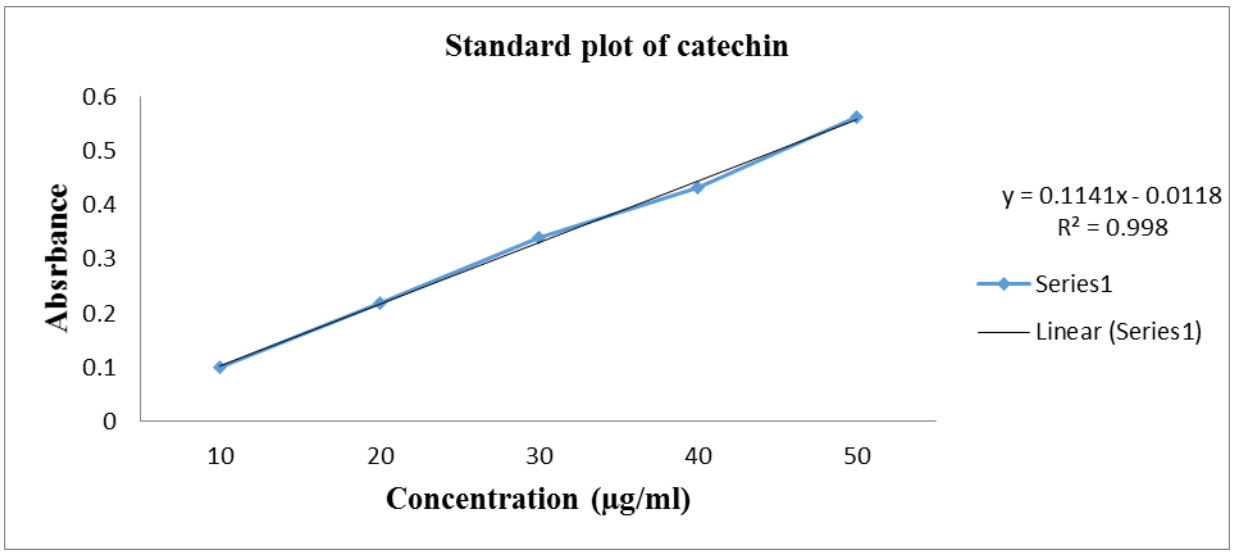
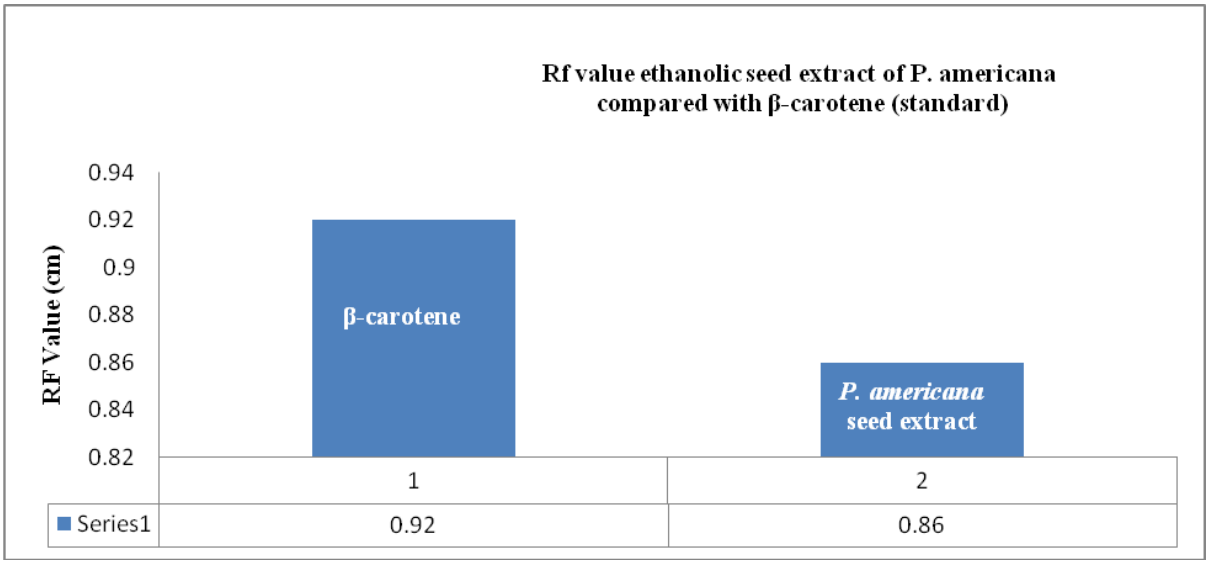


Figure 3: Catechin calibration curve for estimation of total proanthocyanidin.

Table No. 4: *Rf* value ethanolic seed extracts of *Persea americana*

S. No.	Name of extract	RF Value (cm)
1	β-carotene (standard)	0.92
2	Ethanolic seed extract	0.86



DISCUSSION & CONCLUSION

To ascertain "new drug" the presence of any particular primary or secondary metabolite in the extracts of the plant which is having a clinical significance "qualitative phytochemical analysis" is very important information. In any case, if any significant bioactive natural product is present, it is necessary to separate that compound from the mixture of compounds by using a suitable chromatographic technique. *Persea americana* are rich sources of bioactive phytochemicals [23]. The different phytochemicals tests performed on the seed extracts of *Persea americana* showed the presence of phenols, flavanoids, terpenoids, steroids, saponin, alkaloid (Table No.1) abundance in ethanol comparable to other solvents used like water, ethyl acetate, chloroform extracts were not acceptable for further analysis. The TLC experiment on ethanolic seed extract was successful with a mobile phase of methanol: chloroform and the presence of the single prominent band coinciding with beta-carotene. Avocado seeds extract contain considerable amounts of phenols, flavonoids, and proanthocyanidin making it a good source for extraction of antioxidants. Ethanol solvents used were more efficient in extracting the antioxidant component of avocado compared to water or the diluted solvents [24]. The medicinal values of the plant depend on the phytochemicals such as alkaloids, flavonoids, phenolic compounds and other nutrients like as amino acid, proteins, which produce a definite physiological action on the human body [25]. This study examined the antioxidant contents of different seed extracts of avocado. Table no.4 revealed the presence of phenols, flavonoids, and proanthocyanidin in the seed extracts of avocado. The presence of these antioxidants has protective and therapeutic implications for man. Phenols are major contributors to the antioxidant capacity of plants. The phenol content is highest in the ethanol extract (289±0.62µg GAE/ml) and lowest in the water extract (243±0.19 µg GAE/ml). Flavonoids are potent water-soluble super antioxidants and free radical scavengers. They prevent oxidative cell damage, have strong anticancer activity and protect against all stages of carcinogenesis [26]. The flavonoid content is highest in the ethanol extract (49.6±0.02µg rutin/ml) and lowest in the water extract (37.2±0.47µg rutin/ml). The proanthocyanidins were highest in the ethanol extract (13.7±0.01µg catechin/ml) compared to the water extract. The seed of avocado has been reported to contain higher concentrations of flavonoid compared to the skin, pulp, and leaves [27]. This may explain the various biological activities, such as anti-carcinogenic, anti-inflammatory, hepatoprotective, antioxidant, antihyperlipidemic and antiatherosclerotic activities of various parts of avocado. The results of the study indicate the phenolic and flavonoids compounds are responsible for the scavenging and antioxidant activities of ethanolic seed extract of *Persea americana* (EPA).

CONFLICT OF INTERESTS

The Authors declare that they have no conflict of interest.

ACKNOWLEDGEMENT

The Authors are thankful to the Faculty of Pharmacy, Integral University Lucknow (India), for providing the necessary facilities to carry out this research.

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