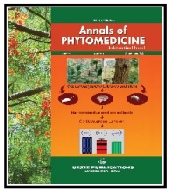
Annals of Phytomedicine 11(1): 472-484, 2022

DOI: http://dx.doi.org/10.54085/ap.2022.11.1.55

Annals of Phytomedicine: An International Journal
http://www.ukaazpublications.com/publications/index.php

Print ISSN : 2278-9839 Online ISSN : 2393-9885



Original Article : Open Access

Isolation, characterization and nutraceutical bioactive composition of bark of *Prosopis cineraria* (L.) Druce

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Article Info

Article history
Received 25 January 2022
Revised 13 March 2022
Accepted 15 March 2022
Published Online 30 June 2022

Keywords
Prosopis cineraria (L.) Druce
Phytochemical
Extract
Phenols
Flavonoids

Abstract

Phytochemical investigation of bark extracts of *Prosopis cineraria* (L.) Druce (Khejri) resulted the isolation of 24-methylenecycloartan-3-one, lupeol, 5, 7, 4'-trihydroxy-3'-methoxy flavanone and β-sitosterol. Extract/fractions of bark of *P. cineraria* also evaluated various activities, *i.e.*, total phenols, flavonoids and mineral contents and protein binding efficiency (PBE)/interaction of phenolics with protein. It was resulted that methanol fraction of bark extract contained largest amount of total phenolic contents, *i.e.*, 842.71 ± 1.43 mg $GAEg^{-1}$ while acetone fraction contained largest quantity of total flavonoid contents, *i.e.*, 371.28 ± 0.99 mg CEg^{-1}. Mineral contents were also estimated by different methods and results that *P. cineraria* possess maximum N content (2700 ± 0.06 mg/100 g) in methanol fraction, followed by P content (712 ± 0.02 mg/100 g) in acetone fraction, K content (517 ± 0.01 mg/100 g) in benzene fraction, Fe content (365.40 ± 0.03 mg/100 g) in chloroform fraction, Zn content (14.23 ± 0.03 mg/100 g), Cu content (13.30 ± 0.02 mg/100 g) and Mn content (06.00 ± 0.05 mg/100 g) in aqueous fraction. From various fractions, the data was examined that highest protein binding efficiency was exhibited by ethyl acetate fraction, *i.e.*, 4.08 ± 0.04 % at 10 µg/ml concentration.

1. Introduction

India is well known for their traditional use in the field of herbal medicines and about 60% of the world populations recommended as alternative system of medicines (Singh *et al*, 2020). Plants and herbs have been used as a contributor for sustain human life quality from thousands of year which provide abundant natural antioxidant and important for human health (Malik *et al.*, 2020). But, these plants have always been a valuable source of natural product for the treatment of various diseases (Newman and Cragg, 2005) and these sources like timber, wood, fuel, shelter, food and herbal medicines that play significant role for living better life of the people and animals. It also develops unique family of chemicals to protect themselves from various microbes. Among the secondary metabolites produced by the plants, the phenolics are water soluble containing one or more phenolic group, *i.e.*, phenolic hydroxyl groups called as polyphenolic compounds and derived from the common building block skeleton, *viz.*, phenyl propanoid unit C_6-C_3 (Hollman, 2001). Beside simple phenolic acids, the greatest number of plant phenolic compounds belongs to the flavonoids show neutroprotective properties by inhibiting the development of reactive oxygen species (Das *et al.*, 2021) which represent an important group of secondary metabolites. In addition, these simple compounds, a major class of phenolics which relates to tannins is defined as water soluble polymeric phenolics that precipitate proteins (Haslam, 1989).

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The great Indian desert commonly known as the 'Thar', which includes some portion of Northwest India, contributes 90% of the Indian arid zone and 61.8% State of Rajasthan (Khandelwal *et al.*, 2015). In these deserts, a promising multipurpose tree species popularly found is *P. cineraria*. This species is synonymous with *P. spicigera*, is a boon to the people due to its myriad virtues. It is a flowering tree which contains approximately forty-four species (Mohammad *et al.*, 2013). It is native species of the deserts of Western and South Asia, including Afghanistan, Iran, India and Pakistan. The plant, *P. cineraria* is the one fruitful species of family Laguminosae, and subfamily Mimosaceae, which is famous by various names, *i.e.*, khejri, jandi, sangri and janti in Rajasthan and kandi in Sindhu and sumri in Gujarat (Khandelwal *et al.*, 2016). It is more famous State tree of Rajasthan and also known as 'Wonder or Golden tree' and 'King of Desert' (Tarachand *et al.*, 2012). Because of all parts of this tree are very useful so called as Kalp- Taru. Bark of this plant is open crown having thick, rough gray color with deep fissures (Kumar *et al.*, 2011) and sweetish in taste. It is reported that during servers famine of Rajputana in 1868-69, many people's lives were saved by the use of bark as a source of food material because it was ground into the flour and made cakes (Gehlot *et al.*, 2008). It is also considered as antihelminthic, refrigerant and its tonic is used for treatment of various diseases such as cough and common cold, asthma, bronchitis, dysentery, leucoderma, muscle tremors, piles and leprosy (Vyas *et al.*, 2020). So, it has various biological activities and medicinal values. According to World Health Organization (WHO), in about 80 per cent of developed countries individuals only use traditional medicines which give better opportunity for understanding their efficiency, properties and safety aspects (Vaidya *et al.*, 2021). The anti-inflammatory and anticancer activity was possessed by the aqueous extract of bark of

P. cineraria (Robertson *et al*., 2012). For hair fall therapeutic treatment, rubbing of bark ashes of khejri is more popular mend. The bark of this plant most popular remedy for immediate relief after bitten of snakes or scorpion stings (Islam *et al*., 2019 ; Soni *et al*., 2015). Laxative and abortifacient action has been shown by the bark of *P. cineraria* (Sachdeva *et al*., 2014). In this research work, firstly we are reported the isolation and characterization of bioactive phytochemical components and having nutraceutical activity of various fractions of bark extract of plant, *P. cineraria.*

Figure 1: Complete plant of *P. cineraria* (L.) Druce.

2. Materials and Methods

2.1 Experimental chemicals

All the chemicals used in the present work were of LR grade. Folin-ciocalteau reagent, catechin and bovine serium albumin (BSA) were obtained from Himedia Laboratories Pvt. Ltd., Nasik, Mumbai. Gallic acid, tannic acid, ponceau S, aluminium chloride, bismuth nitrate, disodium sulphide, butylated hydroxy anisole (BHA), sodium hydroxide, sodium acetate, sodium silicate, Neseller's reagent, 2, 4-dinitrophenol (DNP), potassium iodide and various solvents like benzene, chloroform, ethanol, ethyl acetate, methanol, hexane and petroleum ether issued in this study were of analytical grade and purchased from CDH, Daryaganj, New Delhi or SD Fine Chem. Limited, Mumbai. The adsorbents used for column and thin layer chromatography were silica gel (60-120 mesh) and silica gel G. Different glassware and equipments like thin layer chromatography (TLC) unit, iodine-chamber, TLC glass plates, soxhlet apparatus, column chromatography, electronic water bath, rotary evaporator, digital balance, weighing balance, desiccators, mixer grinder, pestle mortar, hot air oven, conical flask, round bottom flask, capillary tubes, funnel, melting point apparatus were used.

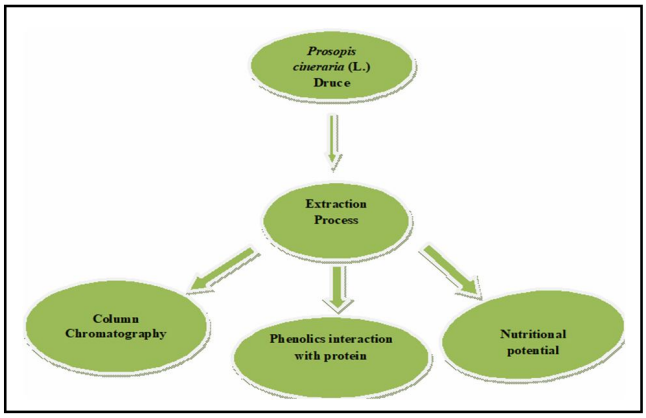
2.2 Collection of materials

Bark of *Prosopis cineraria* (L.) Druce were procured in the month of July-August from campus and outside area of CCS HAU, Hisar, Haryana, India. The plant was identified and authenticated by Botanist, Dr. R. M. Kadam, Department of Botany, Mahatma Gandhi Mahavidhyalaya, Latur, Maharashtra, India by Voucher Specimen Number - DI 15. The collected sample of bark of this plant was washed thoroughly with water and shadow dried. These samples were then chopped into small pieces and kept in air tight containers for further use.

2.3 Extract/fractions preparation

The shadow-dried and chopped pieces of bark were extracted by refluxing method with methanol solvent in eight hours. The process repeated thrice, solvent was removed by distillation process to get extractives. The extractive was pooled together and evaporated under rotary evaporator to get crude extract. These extract was further fractionated into various solvents, *i.e.*, hexane, benzene, chloroform, ethyl acetate, acetone and aqueous and then evaporated to get crude mass, stored in a cooled place till use.

Flow chart of universal experimental procedure



2.4 Phytochemical preliminary analysis

The methanol extract of *P. cineraria*, bark part were taken for qualitative phytochemical analysis, using standard parameters. These parameters help in the identification of various types of phytoconstituents.

2.5 Column chromatography

P. cineraria, bark (methanol extract) was mixed with silica gel (60-120 mesh size) and subjected to column chromatography. The glass column (1000 × 40 mm size) was packed with hexane silica gel slurry. A much portion of bark extract was introduced into the column and then eluted with solvents of increasing polarity, *i.e.*, hexane, benzene, ethyl acetate, acetone, methanol and their mixtures. Each obtained elution was monitored by thin layer chromatography (TLC). These column chromatography results the isolation of four chemical compounds labeled as I to IV.

2.6 Estimation of total phenol contents (TPC)

The TPC of various bark fractions of *P. cineraria* was determined by using folin-ciocalteau reagent method (Singleton and Rossi, 1965). 2.5-100 µg/ml gallic acid (reference standard) used for calibration curve. Bark extract (1ml in 1 mg/ml) was mixed in 1 ml of 1N folin ciocalteau reagent, then added 1ml of 20 % sodium carbonate standard solution. By using distilled water made final volume up to 10 ml, then absorbance was measured at 725 nm wavelength using UV-Visible spectrophotometer. The estimation

of total phenolic contents was measured by using linear regression equation, obtained from standard curve of gallic acid against blank and calculated as Mean ± standard deviation (SD) which was expressed as mg/g gallic acid equivalent (GAE) of dry extract (Wali *et al*., 2019).

2.7 Estimation of total flavonoid contents (TFC)

The TFC was estimated by aluminum chloride colorimetric assay (Marinova *et al*., 2005) and catechin (standard reference) used for calibration curve. 10 mg catechin was dissolved in 100 ml distilled water, and then diluted in various concentrations, *i.e*., 2.5, 5, 10, 20, 40, 60, 80 and 100 µg/ml. Each fraction (1 ml) and catechin standard were mixed separately in 4 ml of distilled water, then add 0.3 ml 10 % aluminum chloride in a 10 ml volumetric flask. After 5 min, added 2 ml of 1 M sodium hydroxide solution, then made final volume with distilled water up to mark of flask. At 520 nm absorbance was measured on UV-Visible spectrophotometer against blank. The total flavonoid content was measured from linear regression equation, which was obtained from calibration curve and calculated as Mean ± standard deviation (SD), expressed as mg/g CE (catechin equivalent) of dry extract.

2.8 Estimation of mineral contents

Micronutrients such as Fe, Cu, Zn and Mn, *etc*., were estimated by atomic absorption spectrophotometer. The samples were digested by wet oxidation process. 0.5 g of each fraction was taken, added 20 ml of diacid mixture (HNO_3 : $HClO_4$ – 4:1) and kept overnight. Next day, the samples were digested on hot plate, then cool and made final volume up to 25 ml using distilled water. The reading was taken on AAS against a blank solvent and mineral contents was determined by using the following formula, expressed as mg/100 g of the extract:

$$\text{Mineral content} = (Abs_{Sample} - Abs_{Blank}) \times \text{Dilution factor}$$

For estimation of nitrogen, phosphorus and potassium, the bark fractions were digested by same above procedure but instead of 20 ml HNO_3 : $HClO_4$ – 4:1, 10 ml of H_2SO_4 : $HClO_4$ – 4:1 diacid mixture was used. The nitrogen content was determined by colorimetric (Nesseler's reagent) method (Lindner, 1944). Each digested plant sample (0.2 ml) was taken in 25 ml volumetric flask then added 0.5 ml of 10% sodium hydroxide and 1 ml of 10% sodium silicate in it. For color development, 1 ml of Nesseler's reagent was added to each flask, and then made volume up to the mark by distilled water. The absorbance was noted at 440 nm using spectronic 20 UV-Visible spectrophotometer against a blank. The concentration of nitrogen was determined using standard curve of ammonium sulphate which expressed in mg/100 g (Rafael *et al*., 2013). The analysis of phosphorus content was done by using vanadomolybdo-phosphoric yellow color method (Koenig and Johnson, 1942). Plant digested sample 2 ml was taken in a volumetric flask, added 1-2 drops of 2, 4-dinitrophenol and then added ammonia solution till yellow color was developed. After color development, added HCl drop wise till it become colorless, then added 5 ml of ammonium molybdate-vandate solution in each flask and final volume was made up to mark by distilled water. The absorbance was recorded at 440 nm using blue filter on spectronic 20 spectrophotometer against a reagent blank. The phosphorus content (mg/100 g) was determined by using the standard plot of phosphate solution. The estimation of potassium content in acid digest of plant sample was done by taking reading on flame photometer against a reagent blank. The concentration of potassium (mg/100 g) was determined by using the standard curve of potassium chloride.

2.9 Estimation of protein binding capacity/protein phenol interaction

The protein binding efficiency of bark extract/fractions of *P. cineraria* was estimated by spectrophotometric method, using 50 µg/ml tannic acid (reference standard) for calibration curve. (1 µg/ml) plant fractions (0.25 g) dissolved in 5 ml methanol and from each solution 2 µl/ml, 4 µl/ml, 6 µl/ml, 8 µl/ml and 10 µl/ml applied in triplicates on Whatsmann No. 1 filter paper as a spots. These were allowed to dry, and then spray with BSA (Bovine Serium Albumin) solution until paper was totally wet. After 30 min, the filter papers were washed thoroughly thrice with acetate buffer to remove unbound BSA, and then paper was again strained with ponceau S dye three times. The strained strips were washed with acetic acid (0.2%) v/v until elution of color from the ceased. The strips were air dried along with blank, then strained area cut into small pieces and kept in separate tubes. The color of strained pieces was eluted with 0.1 N NaOH (3 ml), followed by addition of 10 % acetic acid (0.3 ml), then centrifuge the solution, after centrifugation the color in supernatant was measured at 525 nm wavelength. The total binding capacity was calculated by using linear regression equation, and measured as Mean ± standard deviation (SD) and expressed as µg/ml tannic acid of dry extract (Dawra *et al*., 1988).

2.10 Statistical data analysis

The experimental work was carried out in triplicates and the results were calculated and expressed as Mean ± standard deviation (SD). All the measurements of both one and two-way of variance analysis (ANONA) with mean (p<0.05) in (OPSTATE) were carried out in Microsoft Excel 2007.

3. Results

3.1 Preliminary phytochemical evaluation

The freshly prepared methanol extract of bark of *P. cineraria* was examined for detection of various phytochemical components by using standard phytochemical methods. The extracts were tested for saponins, tannins, carbohydrates, cardiac glycosides, alkaloids, flavonoids, terpenoids and fat and fixed oil, phytosterols, proteins, amino acids and anthraquinone glycosides were shown in Table 1 (Pathak and Kumar, 2017 ; Kumari and Solanki, 2021).

3.2 Isolation, characterization and chemical structure of phytochemical compounds

Plants have more capability to synthesize a large amount of phytochemicals which possess important role in their significant biological functions. Many of these compounds, get beneficial effect on human health and useful for treating various diseases (Musini *et al*., 2013). Isolation and characterization of various compounds are described below:

Compound-I-24-methylenecycloartan-3-one

Compound-I was white solid (20 mg) with melting point 111-113ºC. It was obtained from the elution with benzene: hexane (1:19) and recrystallized from benzene: hexane (1:1), having R_f value 0.47. The molecular formula $C_{31}H_{50}O$ was shown from m/z

439.361[M+H]$^+$ by GC-MS. ^{1}H NMR (δ, $CDCl_3$) : 0.80 (s, 3H, 1×- CH_3), 0.84- 1.02 (m, 16H, 8×-CH_2), 1.18-2.42 (m, 7H, 7×-CH), 4.56 and 4.69 (dd, 2H, 1× =CH and 1×-COCH). IR (KBr, $V_{max,}$ cm^{-1}) : 2918, 2850, 1704, 1644, 1453, 1113, 870, 721. GC-MS (m/z, % intensity) : 427.8 (55), 340.0 (35), 220.2 (20), 146.4 (16), 72.7 (95).

Table 1: Short list of preliminary phytochemical parameters on methanol extract of bark of *P. cineraria*

Phytochemical parameters	Chemical test(s)	Results
Saponins	Frothing test	+
Tannins	Ferric chloride test	+
Carbohydrates	Fehling's test, Tollen's reagent test	+
Cardiac glycosides	Keller-Killiani test	–
Anthraquinone glycosides	Hydroxyanthraquinine test	+
Alkanoids	Hager's test	+
Flavonoids	Alkaline reagent test	+
Terpenoids	Salkowski test	+
Phytosterols	Liebermann-Burchard's test	+
Proteins	Ninhydrin test	+
Amino acids	Millon's test	–
Fats and fixed oil	Copper sulphate test	–

+ sign shows the presence while – sign shows the absence.

Compound II - Lupeol

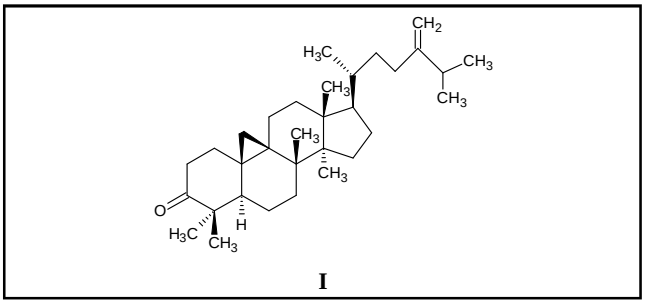
The white niddles compound-II was obtained from the elution with solvent system of benzene: hexane (1:3), recrystallized from pure benzene, 3.3 mg, their melting point was 214-215ºC (Lit. mp. 215-216 ºC) (Jamal *et al*., 2008). Its R_f value was found to be 0.33 in ethyl acetate: benzene (1:19). The IR spectra of this compound-II gave absorption peak at 3408 cm^{-1} confirmed the presence of hydroxy group in it. The molecular formula $C_{30}H_{50}O$ and molecular mass 426 were deduced from its GC-MS (Soni *et. al*, 2015). ^{1}H NMR (δ, $CDCl_3$): 0.76, 0.79, 0.82, 0.92, 0.96, 1.02, 1.18 (s, 21H, 7×-CH_3), 1.25-1.72 (m, 24H, 12×-CH_2), 3.49 (dd, 1H, J= 8.0Hz, 1×C_3-OH), 4.58, 4.69 (s, 2H, 1 × C_{29a} and C_{29b} =CH_2). IR (KBr, V_{max} cm^{-1}): 3408, 2918, 2849, 1680, 1462, 1379, 1053, 801. GC-MS (m/z, % intensity): 426.0 (54), 354.0 (60), 339.9 (25), 280.2 (70), 220.3 (50), 206.3 (15), 146.5 (45), 72.7 (100).

Compound III - 5, 7, 4'-trihydroxy-3'-methoxy flavanone

The brown color compound-III was obtained with elution of ethyl acetate : benzene (1:3) and recrystallized from pure methanol, 25 mg, melting point 223-224ºC (Lit. mp. 224-226ºC) (Kulkarni, 2012). The absorption peaks at 3433 cm^{-1} and 1656 cm^{-1} resulted the presence of hydroxyl and keto groups. The molecular formula $C_{16}H_{14}O_6$ was deduced from its GC-MS analysis with molecular mass 302. ^{1}H NMR (δ, DMSO-d_6): 2.70 (dd, 1H, J= 4.0 Hz, 1 × C_2 - CH_2 - axial position), 3.31 (dd, 1H, J = 4.0 Hz, 1 × C_4- CH_2 - equatorial position), 4.06 (s, 3H, 1 × $C_{3'}$ - OCH_3), 4.44 (dd, 1H, 1 × C_1 - CH_3). 5.78 (s, 2H, 1 × C_6 and C_8 - CH_3), 6.69 (s, 2H, 1 × $C_{5'}$ and $C_{6'}$ - CH_3), 6.94 (s, 1H, 1 × $C_{2'}$ - CH_3). 7.34, 8.51, 8.91 (s, 3H, 1 × C_5, 1 × C_7, 1 × $C_{4'}$ CH_3 - OH). IR (KBr, $V_{max,}$ cm^{-1}): 3433, 1656, 1520, 1440, 1259, 1141, 793. GC-MS (m/z, % intensity): 280.2(100), 220.3(5), 146.5(25), 72.7(90).

Compound IV - β- sitosterol

The compound- IV (Soni *et. at*., 2015) was white crystalline and eluted with ethyl acetate: benzene (1:1). It was recrystallized from pure ethyl acetate solvent with R_f value 0.67. Its melting point was 135-136ºC (Lit. mp. 136-137ºC) (Alemu *et al*., 2015). The presence of steroid from Liebermann Burchard reaction test due to indication of green color. The absorption peak at 3406 cm^{-1} indicating the presence of hydroxyl group. The LC-MS and elemental data responded the molecular formula $C_{29}H_{50}O$ was deduced from molecular mass 414. ^{1}H NMR (δ, DMSO-d_6): 0.68 (t, J= 8.0 Hz, 3H, 1 × C_{28}-CH_3), 0.86 (t, J=8.0Hz, 3H,C_{29} -CH_3), 0.81(d, J=8.0Hz, 6H, 1 × C_{26} -CH_3,, & 1 × C_{27} - CH_3), 0.94 (d, J= 7.0Hz, 3H, C_{21}-CH_3), 1.22 (s, 3H, C_{19} -CH_3), 2.17-2.38 (m, 29H, 11× -CH_2 and 7 × -CH), 5.32 (br, J= 4.0Hz, 1 H=CH), 4.75-4.82 (m, 1H, C_3 – CH-OH). IR (KBr, $V_{max,}$ cm^{-1}): 3406, 2931, 1649, 1456, 1365, 1019, 799. GC-MS (m/z, % intensity): 353 (24), 325 (20), 280 (15), 220 (18), 146 (10), 72 (100).



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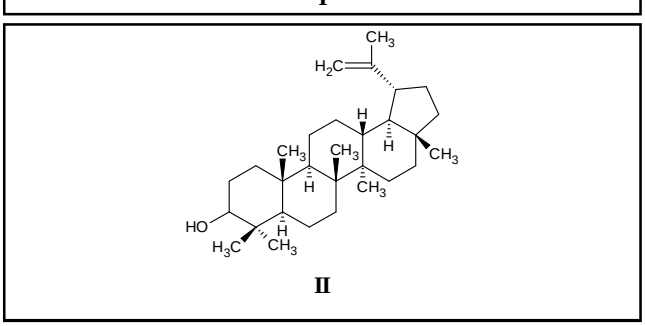

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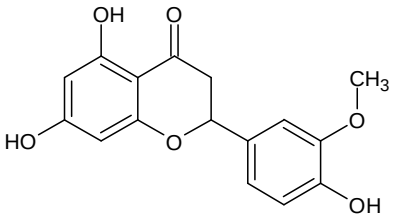
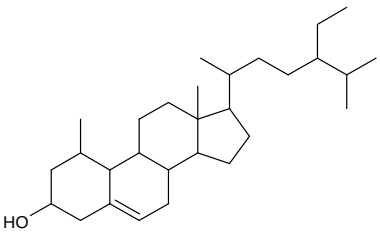


III



IV

Figure 2: Structure of chemical compounds (I-IV) isolated from bark of *P. cineraria*.

3.3 Total phenolic contents (TPC)

Antioxidant compounds play vital role to catalyze the redox reactions in various biochemical processes of reactive oxygen species (ROS). These antioxidant compounds mainly includes polyphenols, phenolic acids, *etc*., are found in plants (Bhatt *et al*., 2019). But, in broad spectrum, these phenolics are present in the form of carbon based aromatic compounds which play vital role in the health of human beings and it possessed biochemical activities. The secondary metabolites, *i.e*., natural antioxidants are ability to scavenge free radicals and active oxygen species. These metabolites present in plants includes carotenoids, flavonoids, cinnamic acids, benzoic acids, tocopherols, *etc*., are very essential components. They also contribute towards antioxidant potential of various plant materials. Therefore, the different fractions of *P. cineraria* bark extract was screened for total phenolic contents (Table 2). The TPCs of various fractions of *P. cineraria* is expressed in terms of gallic acid equivalent (GAE) and calculated using the linear regression equation obtained from standard plot of gallic acid.

3.4 Total flavonoid contents (TFC)

Flavonoids are the most important group of polyphenolics in human diet which is usually found in plants and these are the largest group of naturally occurring phenolic compounds and effective antioxidant. Thus, the total flavonoids content of different fractions of *P. cineraria* bark was determined and expressed in terms of catechin equivalent (CE). The TFCs were calculated using the linear regression equation obtained from the standard plot of catechin.

3.5 Mineral analysis in bark extract of *P. cineraria*

All the living systems require inorganic compounds and minerals to sustain normal life and better survival in environment. Livestock derive most of their dietary nutrients from the feed. *P. cineraria* is one of the chief indigenous tree of the plains and the most important source of medicinal and nutritional areas which play vital role in many healing benefits and feeding processes. Therefore, due to the most valuable property of this plant, it has been decided to determine the micronutrients from various fractions of *P. cineraria*. The results revealed that *P. cineraria* possessed the highest nitrogen (N) content, followed by phosphorus (P), potassium (K), iron (Fe), zinc (Zn), copper (Cu), and manganese (Mn) content (Rafeal *et al*., 2013 ; virginia, 1986). Minerals are the most important part of human diet. It play significants role in many physiological and metabolic functions. The data are presented in Table 3.

Table 2: Total phenol contents (TPC) and total flavonoid contents (TFC) of bark fractions of *P. cineraria*

Sr. No.	Extract/fractions	TPC (mg GAE/g)	TFC (mg CE/g)
1.	Hexane	264.80 ± 0.66	204.91 ± 0.37
2.	Benzene	119.34 ± 0.79	242.48 ± 0.45
3.	Chloroform	233.63 ± 0.88	286.52 ± 0.51
4.	Ethyl acetate	407.44 ± 0.76	334.24 ± 0.98
5.	Acetone	590.80 ± 0.82	371.28 ± 0.99
6.	Methanol	842.71 ± 1.43	271.08 ± 0.86
7.	Water	399.31 ± 0.77	305.18 ± 0.70

All the values are Mean ± S.D.

Milligrams of gallic acid equivalent per gram - mg GAE/g

Milligrams of catechin equivalent per gram - mg CE/g

3.6 Protein phenol interaction/protein binding capacity (PBC) of bark of *P. cineraria*

Protein binding capacity of different extracts of *P. cineraria* plant was determined by using different fraction of solvents. PBCs are expressed in terms of tannic acid. The PBCs were calculated by using the following linear regression equation obtained from the standard plot of tannic acid. The data presented in Table 4 revealed the binding capacity of protein from bark fractions of *P. cineraria* at different test concentrations.

Table 3: Mineral contents (mg/100 g) of various fractions of bark of *P. cineraria*

Sr. No.	Extract/ fractions	Iron (Fe)	Copper (Cu)	Zinc (Zn)	Manganese (Mn)	Nitrogen (N)	Phosphorus (P)	Potassium (K)
1.	Hexane	67.00 ± 0.01	00.10 ± 0.01	05.02 ± 0.07	00.95 ± 0.07	750 ± 0.04	375 ± 0.07	382 ± 0.09
2.	Benzene	78.60 ± 0.02	0.050 ± 0.04	08.76 ± 0.11	01.50 ± 0.02	950 ± 0.09	412 ± 0.06	517 ± 0.01
3.	Chloroform	365.40 ± 0.03	00.10 ± 0.20	08.68 ± 0.01	02.10 ± 0.05	1675 ± 0.01	362 ± 0.01	171 ± 0.07
4.	Ethyl acetate	45.55 ± 0.21	00.15 ± 0.08	07.92 ± 0.03	03.60 ± 0.09	1500 ± 0.08	225 ± 0.04	463 ± 0.06
5.	Acetone	26.10 ± 0.07	00.15 ± 0.01	08.91 ± 0.01	02.60 ± 0.01	1212 ± 0.09	712 ± 0.02	222 ± 0.02
6.	Methanol	92.10 ± 0.13	00.16 ± 0.10	07.24 ± 0.09	02.40 ± 0.10	2700 ± 0.06	287 ± 0.01	265 ± 0.01
7.	Water	322.30 ± 0.05	13.30 ± 0.02	14.23 ± 0.03	06.00 ± 0.05	ND	450 ± 0.06	245 ± 0.05
SE(d)				0.760				
CD at 5%				1.647				
CV%				0.655				

All the values are Mean ± S.D.

ND- Not determined

Table 4: Protein binding capacity (PBC) of *P. cineraria*, bark extract/fractions

Sr.No.	Extract/fractions	Concentrations (µg/ml)					
		0	2	4	6	8	10
1.	Hexane	1.84 ± 0.03	2.20 ± 0.02	2.39 ± 0.04	2.63 ± 0.04	3.21 ± 0.03	3.39 ± 0.06
2.	Benzene	2.30 ± 0.04	2.53 ± 0.01	2.69 ± 0.03	2.83 ± 0.06	3.05 ± 0.05	3.51 ± 0.05
3.	Chloroform	1.28 ± 0.03	1.54 ± 0.03	1.72 ± 0.04	1.87 ± 0.02	2.08 ± 0.03	2.44 ± 0.05
4.	Ethyl acetate	2.54 ± 0.01	2.72 ± 0.05	2.98 ± 0.02	3.51 ± 0.03	3.83 ± 0.04	4.08 ± 0.04
5.	Acetone	2.14 ± 0.03	2.22 ± 0.01	2.52 ± 0.05	2.86 ± 0.02	2.95 ± 0.03	3.16 ± 0.05
6.	Methanol	1.52 ± 0.02	1.74 ± 0.05	1.98 ± 0.03	2.27 ± 0.04	2.39 ± 0.07	2.95 ± 0.04
7.	Water	1.63 ± 0.04	1.83 ± 0.04	2.15 ± 0.04	2.73 ± 0.05	2.84 ± 0.05	3.41 ± 0.03
	Factors		SE(d)			CD at 5%	
	Concentration		0.010			0.019	
	Compound		0.009			0.018	
	Conc.×Compound		0.024			0.048	

Protein binding capacity was expressed in percentage

µg/ml means microgram per millilitre

4. Discussion

Phytochemicals and nutritional evaluation

4.1 Phytochemical screening evaluation

Phytochemical screening resulted the conformation test of presence of tannins, saponins, carbohydrates, alkaloids, flavonoids, terpenoids, anthraquinone glycosides, proteins and phytosterols and absence of cardiac glycosides, amino acid and fat and fixed oil.

4.2 Evaluation of phytochemical compounds

Compound-I is white solid (20 mg) was eluted from benzene: hexane (1:19) and recrystallized from benzene: hexane (1:1) with R_f value 0.47 in 1:3 benzene: hexane solvent. The absorptions at 1704 cm^{-1}confirmed the presence of >C=O group. The molecular formula $C_{31}H_{50}O$ was deduced from its GC-MS with molecular mass m/z 438. The ^1H NMR spectra of this compound-I in $CDCl_3$ displayed a broad singlet at 0.80δ indicating the presence of three protons of methyl group. Twenty four protons of another eight methyl group could be picked up as a multiplet in the range of 0.84-1.02 δ and seven methine protons appeared in the range of 1.18-2.42 δ as a multiplet. Two doublet at 4.56 and 4.69 integrating one proton each were assignable protons of =CH and –COCH moieties, respectively. From spectral data analysis is in right assignment with literature data of 24-methylenecycloartan-3-one (Alsaadi and Al-Maliki, 2015) and it appears that this compound is the first reported and isolated as 24-methylenecycloartan-3-one from bark of *P. cineraria.*

Compound-II is white niddle (3.3 mg) shape that was eluted from benzene: hexane (1:3), recrytallized from benzene pure solvent system with R_f value 0.33. The IR spectra gave absorption peak at

3408 cm^{-1} confirmed the presence of hydroxy group in it. The molecular formula $C_{30}H_{50}O$ and molecular mass 426 were deduced from its GC-MS. The ^{1}HNMR spectra of this compound – II in $CDCl_3$ exhibited singlet at 0.76, 0.79, 0.82, 0.92, 0.96, 1.02 and 1.18 δ integrating twenty one protons of each seven methyl groups. A multiplet in range of 1.25-1.72 δ indicating twenty four protons of twelve methylene groups. A doublet of doublet appeared at 3.49δ indicating one proton of hydroxyl group at C_3 position. Two singlets at 4.58δ and 4.69δ indicating one proton each appeared at C_{29} axial and equatorial position of vinylic protons. The all above spectral details about this compound showed the compound to be lupeol. An overall agreement related to this compound with literature data established the identity of the compound to be lupeol (Soni *et al*., 2015; Islam *et al*., 2019).

Compound-III was eluted from ethyl aceate: benzene (1:3) as a brown solid (25 mg) and recrystallized from methanol. The absorption peaks at 3433 $cm^{-1}$and 1656 cm^{-1} indicating the presence of hydroxyl and keto group from spectral data. The molecular formula $C_{16}H_{14}O_6$ was deduced from its GC-MS analysis with molecular mass 302. ^{1}HNMR spectra of the compound – III in **DMSO-d_6** exhibited one doublet at 2.73 δ and 3.31 δ (J=16.0Hz) integrating two protons present at C_3 position. A singlet peak at 3.97 δ of three protons which indicate methoxy group present at $C_{3'}$ position. Another doublet of one proton with coupling constant J=16.0Hz observed at C_2 position. A singlet centered at 5.77δ integrating two protons positioned at C_6 and C_8. Another singlet at 6.71δ of two protons presented at $C_{5'}$ and $C_{6'}$ position. At 6.94δ also a singlet of one proton assignable at $C_{2'}$ position. At 8.91 δ value a broad singlet centered on one proton of hydroxyl group. From all above spectral information related to this compound could be characterized as 5, 7, 4'-trihydroxy-3'- methoxy flavanone. The compound would be first time report of 5, 7, 4'-trihydroxy-3'-methoxy flavanone from literature survey of bark of *P. cineraria*.

Compound-IV is white crystalline in nature which was eluted from ethyl acetate: benzene (1:1), recrytallised from pure ethyl acetate solvent. A green coloration of Liebermann - Burchard reaction test showed the presence of steroid in this compound. At peak 3406 cm^{-1} examined the presence of hydroxyl groups also from IR spectra. The GC-MS and elemental analysis suggested the molecular formula and molecular mass to be $C_{29}H_{50}O$ and 414. ^{1}H NMR spectral data in DMSO-d_6 gives a broad single spectra of one proton at 5.32 δ, with coupling constant (J=4.0Hz) assign olefinic proton. At range 4.75-4.82 δ give multiplet of one proton, which at α-position to be hydroxyl group. Another multiplet at range of 2.17-2.38 δ represented twenty nine protons hinted the presence of eleven methylene and seven methin. A singlet of three protons at 1.22 δ gives methyl group at C_{19} position. A doublet at δ value 0.94, with J = 7.0 Hz integrating three protons of methyl group at C_{21} and at δ 0.86, triplet of three protons suggested methyl group at C_{29} position. Again a doublet centered at δ 0.81 with J= 8.0 Hz showing six protons of two methyl groups showed at C_{26} and C_{27}. At δ 0.68, triplet showed with coupling constant (J= 8.0Hz) of three protons of methyl group centered at C_{28} position. An overall agreement of spectral report of this compound with literature data of β-sitosterol established the identity of the compound – IV to be β- sitosterols (Soni, *et al.* 2015; Islam, *et al.* 2019).

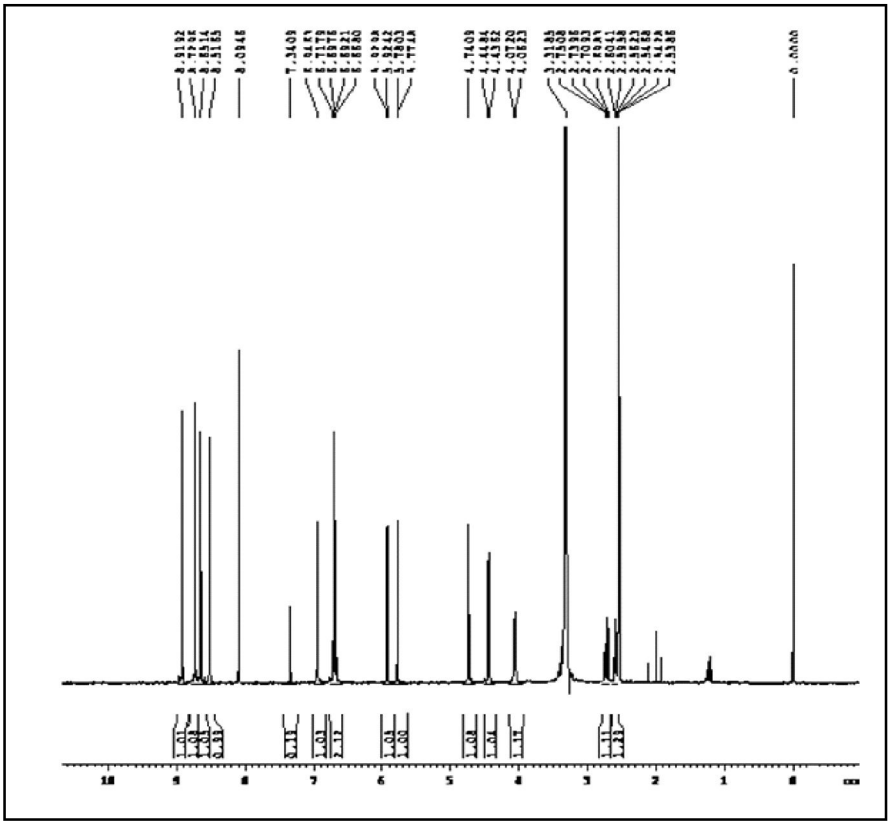


Figure 3: ^{1}H NMR spectrum of Compound-III.

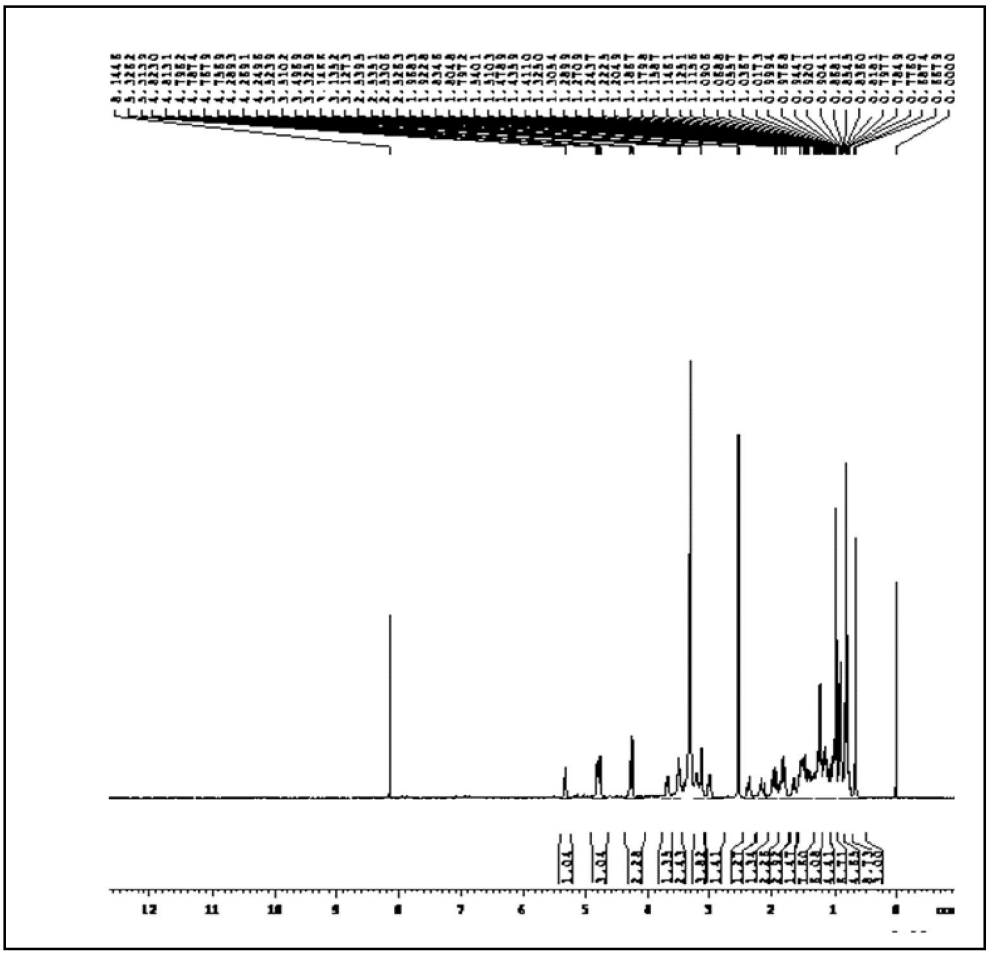


Figure 4: ^{1}H NMR spectrum of Compound- IV.

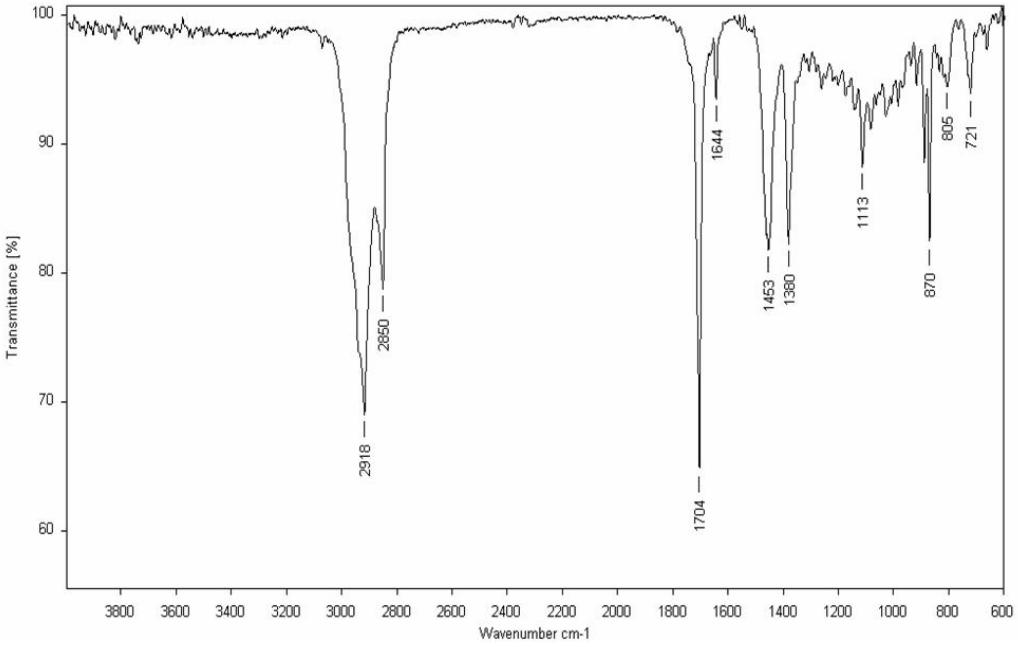


Figure 5: IR spectrum of Compound-I.

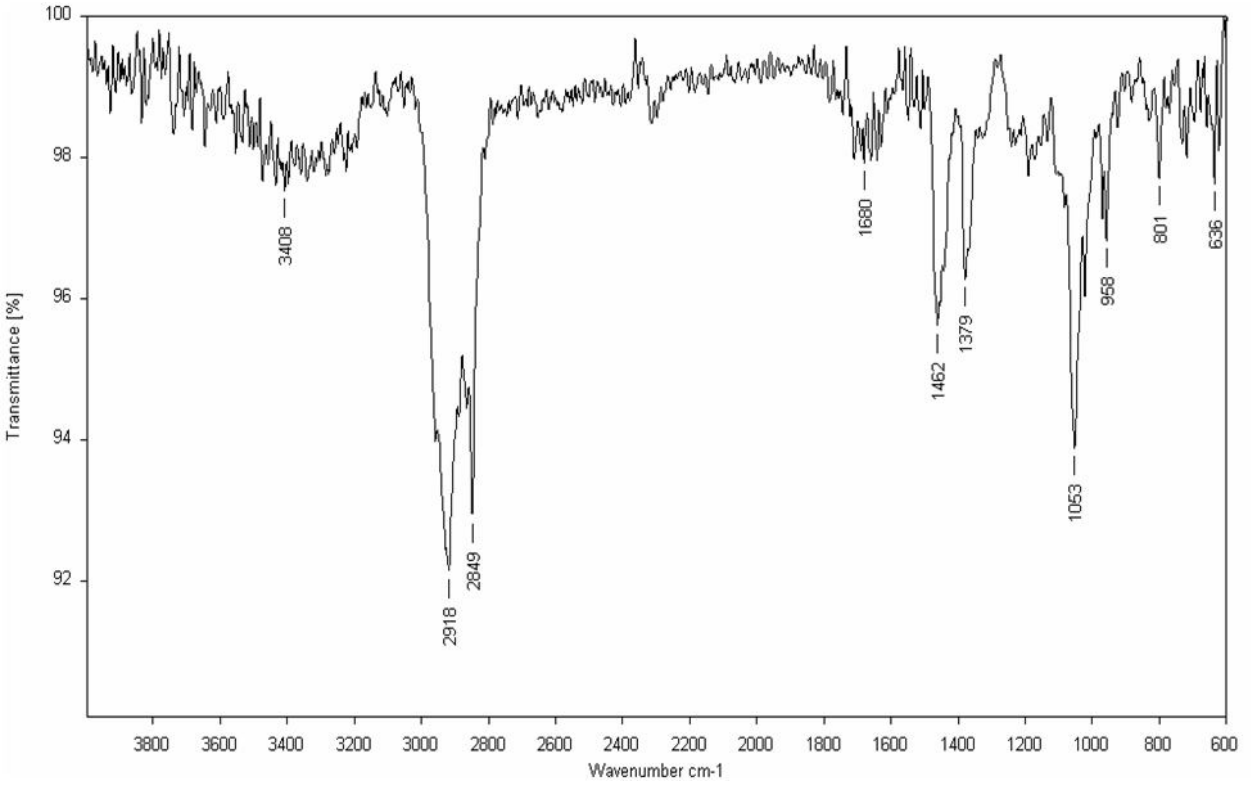


Figure 6: IR spectrum of Compound-II.

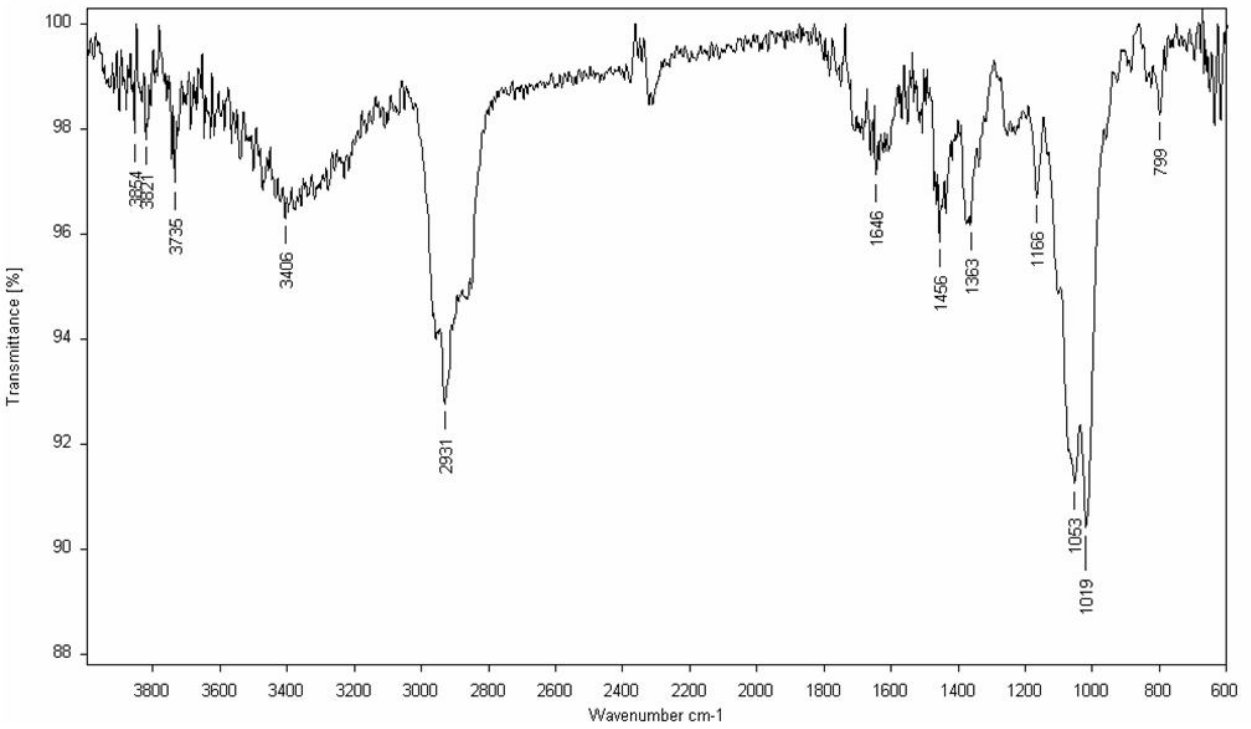


Figure 7: IR spectrum of Compound-IV.

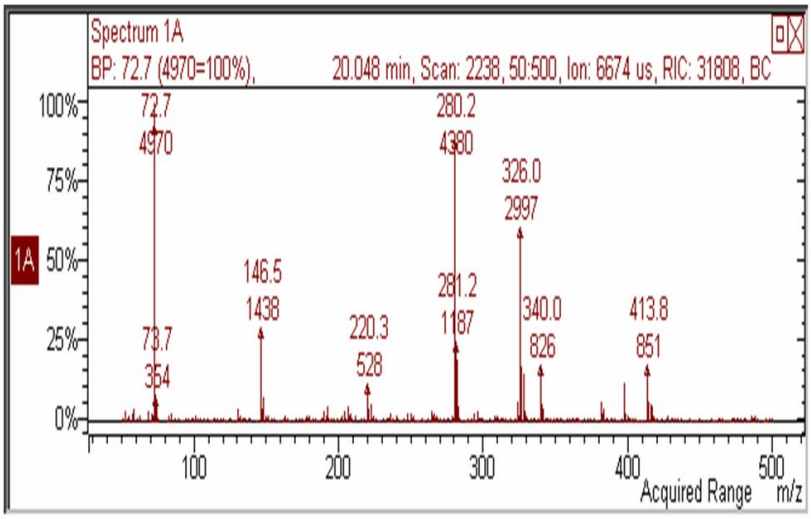


Figure 8: GC-MS of Compound-I.

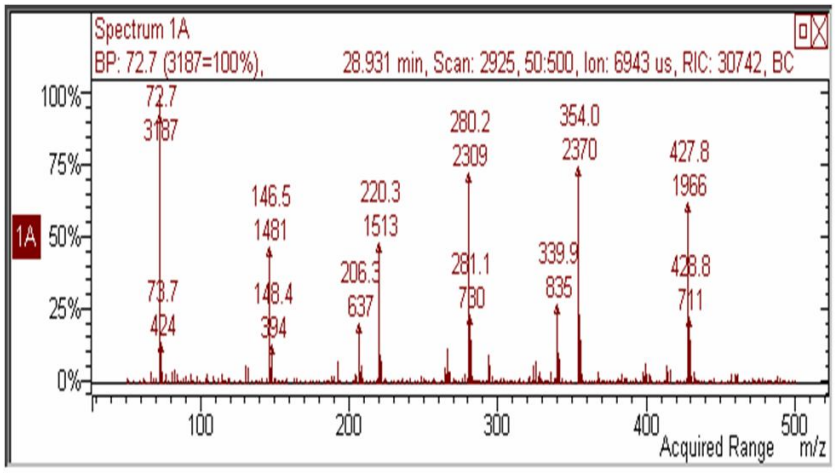


Figure 9: GC-MS of Compound-II.

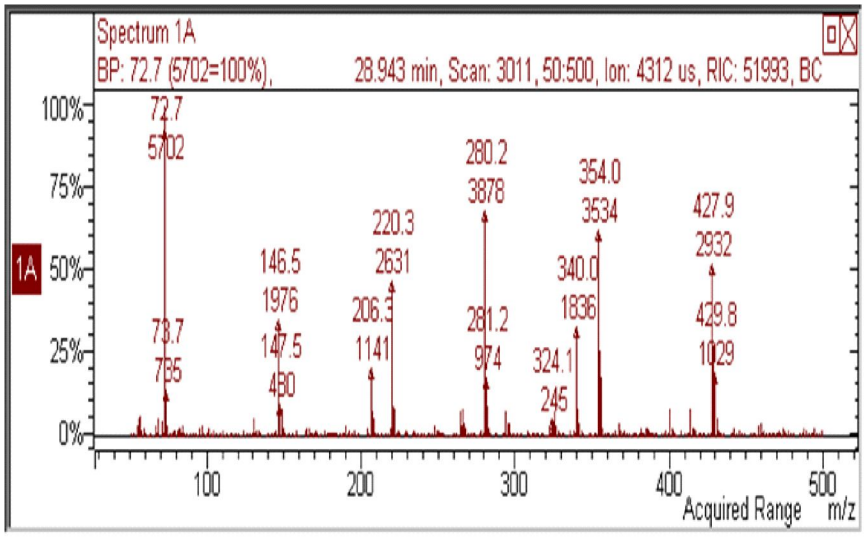


Figure 10: GC-MS of Compound-III.

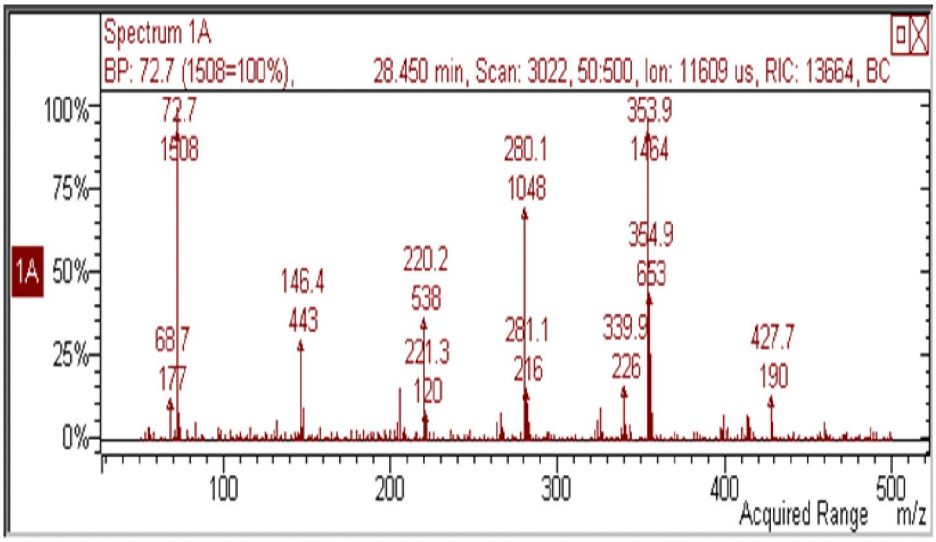


Figure 11: GC-MS of Compound-IV.

4.3 Phytochemical constituents (total phenols and total flavonoids) analysis

Overall results showed that the total phenolic contents are maximum in methanol fraction, *i.e.*, 842.71 ± 1.43 mg/GAEg^{-1} of bark of *P. cineraria* and the acetone fraction of bark contained second highest phenolic contents, *i.e.*, 590.80 ± 0.82 mg/GAEg^{-1}. The amount of total phenols in bark of *P. cineraria* varied from 119.34 ± 0.79 to 842.71 ± 1.43 mg GAEg^{-1}. Various solvent extract/fraction of bark of *P. cineraria* contained total phenols in order: methanol (842.71 ± 1.43 mg GAEg^{-1}) > acetone (590.80 ± 0.82 mg GAEg^{-1}) > ethyl acetate (407.44 ± 0.76 mg GAEg^{-1}) > water (399.31 ± 0.77 mg GAEg^{-1}) > hexane (264.80 ± 0.66 mg GAEg^{-1}) > chloroform (233.63 ± 0.88 mg GAEg^{-1}) > benzene (119.34 ± 0.79 mg GAEg^{-1}).

The present data also revealed the maximum amount of flavonoids was shown in acetone fraction of bark extract, *i.e.*, 371.28 ± 0.99 mg CEg^{-1}. It was followed by ethyl acetate and water fractions, *i.e.*, 334.24 ± 0.98 mg CEg^{-1} and 305.18 ± 0.70 mg CEg^{-1} of flovonoids, respectively. Chloroform, methanol and benzene extracts resulted moderate amount of total flavonoids, *i.e.*, 286.52 ± 0.51, 271.08 ± 0.86 and 242.48 ± 0.45 mg CEg^{-1}, respectively. Hexane fraction of bark extract contained low amount of total flovonoids (204.91 ± 0.37 mg CEg^{-1}).

4.4 Mineral contents analysis

The overall results presented in Table 3 of bark of *P. cineraria* contained highest concentrations of N and P in methanol fraction and acetone fraction (2700 ± 0.10 mg/100 g and 712 ± 0.02 mg/100g), respectively. Iron content was highest in chloroform fraction (365.40 ± 0.03 mg/100 g). The concentrations of Cu, Zn and Mn were found to be maximum in aqueous fraction, *i.e.*, 13.30 ± 0.02 mg/100g, 14.23 ± 0.03 mg/100 g and 06.00 ± 0.05 mg/100 g, respectively. Potassium content in bark of *P. cineraria* varied from 170 ± 0.07 mg/100 g in chloroform fraction to 517 ± 0.01 mg/100 g in benzene fraction. Mineral content of various fractions of bark of *P. cineraria* revealed in order of nitrogen (N) > phosphorus (P) > potassium (K) > iron (Fe) > zinc (Zn) > copper (Cu) >manganese (Mn).

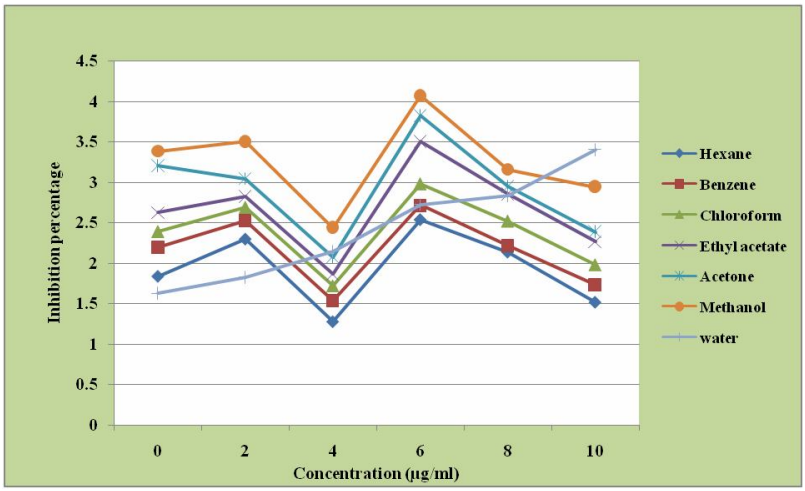


Figure 12: Protein binding capacity (PBC) in bark fractions of *P. cineraria*.

4.5 Comparative analysis of interaction of phenol with proteins of bark fractions of *P. cineraria*

A perusal of the data in Table 4 examined the maximum binding efficiency showed by ethyl acetate fraction, *i.e.*, 4.08 ± 0.04 % at concentration of 10 µg/ml, which was followed by fraction of benzene, *i.e.*, 3.51 ± 0.05 % at 10 µg/ml highest test concentration. Hexane and aqueous fractions exhibited 3.39 ± 0.06 % and 3.41 ± 0.03 % at 10 µg/ml concentrations, respectively. Lowest activity shown by chloroform fraction was 2.44 ± 0.05 % at 10 µg/ml concentration. Acetone fraction also exhibited 3.16 ± 0.05 % at 10 µg/ml concentration. Methanol fraction possessed moderate activity at highest (10 µg/ml) test concentrations, *i.e.*, 2.95 ± 0.04 %. The comparative binding efficiency of bark fractions of *P. cineraria* in terms of their concentrations resulted in order as ethyl acetate > benzene > water > hexane > acetone > methanol > chloroform. The step wise increasing the value of protein binding capacity showed their highest nutritional and digestively power of *P. cineraria*, bark going on increases respectively (Dawra *et al.*, 1988).

5. Conclusion

P. cineraria commonly known as Khejri, has various pharmacological properties which can contribute a lot to the people's health as herbal plant. Various parts of this herbal plant, *P. cineraria*, *i.e.*, bark, stem, leaves, flowers, twigs, fruit pods are used in the treatment of numerous therapeutic effects. Bark of this herbal plant exhibit anti-inflammatory, antidiabetic, nootropic activity and anticancer activity. *P. cineraria* reported many phytoconstituents and various neutraceutical activities by using standard methods. These can help us to choose data of various plant parts for extraction of medically and therapeutically important phytochemicals with greater quantity.

Acknowledgements

We would to like very much thanks the to CCS Haryana Agricultural University, Hisar, India for providing better facilities to undertake the research works and also grateful to SAIF, CIL, UCIL, Panjab University, Chandigarh for providing wonderful spectral analysis facilities.

Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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Citation **Suman, Sheetal, Parvesh Devi, Jyoti Punia, Sumona Kumari and Rajvir Singh (2022). Isolation, characterization and nutraceutical bioactive composition of bark of *Prosopis cineraria* (L.) Druce. Ann. Phytomed., 11(1):472-484. http://dx.doi.org/10.54085/ap.2022.11.1.55.**