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Biological potential and Isolation of chemical constituents from *Prosopis cineraria* (L.) Druce leaves

Suman and Rajvir Singh

Abstract
Phytochemical studies of leaves of *Prosopis cineraria* (L.) Druce resulted the isolation of 1-Heptacosanol, 1-nonadecanol, stigmasterol, nonacosane-15-one, 1-tetracosanoic acid and β-sitosterol. Fresh leaves of *Prosopis cineraria* were collected, shadow dried and then extracted with methanol. The extract was fractionated into different solvents and concentrated under reduced pressure to obtain viscous mass. Interaction of protein with phenolics of extract/ fractions was carried out and hexane & acetone fraction exhibited highest protein binding capacity i.e. 3.53±0.04 and 3.39±0.05% respectively at 10µg/µl concentraction.

Keywords: phytochemical, *Prosopis cineraria* (L.) druce, leaves, protein interaction, protein binding capacity

Introduction
Plants have been a valuable source of fuel, wood, shelter, timber, medicine and food for people as well as animals. They possess bioactive components which are attractive site for drug development. Plants are natural laboratories and unique chemists which synthesize numerous compounds belonging to various categories such as terpenoids, alkaloids, glycosides, phenolics, tannins etc. having diverse molecular structure and uses. These compounds interact with other entity in environment in a multiplex way. So, the study of compounds produced by the plants which stimulate or suppress the biological system of other plants and animals is important for understanding the biochemical interaction of one chemical to others. *Prosopis* is the one of most useful and wonder species of family *Leguminosae* [1]. It is commonly known as Khejri, Jandi, Sangri and Janti in Rajasthan, Kandi in Sindhu and Sumri in Gujrat [2]. It is the state tree of Rajasthan which is endemic to hot deserts of India and grows mainly in different parts of India and Arabia which are dry and arid. In the developing world, *Prosopis* species generally known as a vegetable and a medicinal plant. Extract of leaves of *P. cineraria* is used for the treatment of boils and blisters, including mouth ulcers in livestock. For cure of dyspepsia, fresh leaf juice of jandi mixed with lemon juice is a famous therapy. Leaves of *Prosopis* have high nutritional value as well as medicinal value, commonly known as "Loong". The smoke of leaves is beneficial for the cure of eye problems [3]. In veiw of various medicinal properties and activites shown by leaves of this plant, the present study involves the isolation and characterization of compounds from leaves of *Prosopis cineraria* and bioevaluation of various extract/ fractions for phenolic interaction with protein.

Experimental
Prosopis cineraria leaves were used for isolation and characterization of various phytochemical components. The present investigation was undertaken at Department of Chemistry, CCSHAU, Hisar, Haryana. All the chemicals used in the present work were LR grade. Folin-ciocalteau reagent, catechin and Bovine Serium Albumin (BSA) were obtained from Himedia Laboratories Pvt. Ltd., Nashik, Mumbai. Gallic acid, tannic acid, Ponceau S, aluminium chloride, bismuth nitrate, disodium sulphide, butylated hydroxy anisole (BHA), sodium hydroxide 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, dessicator, mixer grinder, pestle mortar, hot air oven, conical flask, round bottom flask, capillary tubes, funnel, melting point apparatus were used.

Methodology of extraction, fractionation and isolation

Leaves of *Prosopis cineraria* were collected from the Kheda village of Siwani Tehsil of Bhiwani district and university, CCS Haryana Agricultural University, Hisar, India. The leaf samples were shadow-dried, crushed and then dipped in hexane for two hours to remove chlorophyll content. After completion of two hours, hexane was removed and distilled while the leaves were extracted using hot methanol by refluxing method for eight hours. The extractives were concentrated over water bath under reduced pressure to obtain a crude extract which was divided into two parts. One major part was mixed with silica gel (60-120 mesh size) and used to fill the column. The remaining part was further fractionated with different polarity solvents viz. hexane, benzene, chloroform, ethyl acetate, acetone and water. These fractions and methanolic extract were used for determination of phenolic interaction with protein (Protein binding capacity (PBCs). The column was eluted with increasing polarity of various solvents i.e. hexane, benzene, ethyl acetate and methanol and their mixtures. The column chromatography of the leaves of *Prosopis cineraria* afforded six compounds labeled as I to VI.

Compound I was obtained as white crystalline on elution with benzene: hexane (1:19). It does not respond to colour reaction for phenolic compounds. It was crystallized from chloroform as a solid (40mg), melting point 140-141ºC. R_f value of the compound was found to be 0.65 in benzene: hexane solvent. The molecular formula $C_{27}H_{56}O$ and molecular weight 396 was deduced from its GC-MS. IR (KBR, $V_{max.}cm^{-1}$): 3291, 2955, 2917, 2849, 1472, 1462, 1062, 730, 719. ^{1}H NMR (δ, $CDCl_3$): 0.88 (t, J=7.0 Hz, 3H,-CH_3), 1.25 (br, 48H, 24× -CH_2), 1.55 (br, 2H, 1× -CH_2), 3.64(t, J=7.0 Hz, 2H, 1×-CH_2 – OH).GC-MS (m/z, % intensity): 364(2.5), 306(2), 250(3), 160(12), 145(18), 130(36), 115(68), 97(100).

Compound II was a colorless needle shaped crystalline solid (30mg) with melting point 62-64ºC (Lit. mp. 62-63ºC) [4]. It was obtained on elution with benzene: hexane (1:19) and recrystallized from benzene: hexane (1:1) with R_f value 0.54. The molecular formula $C_{19}H_{40}O$ was deduced from its LC-MS with molecular weight 284. IR (KBR, $V_{max.}cm^{-1}$): 3292, 2917, 2849, 1462, 1061, 719.^{1}H NMR (δ, DMSO-d_6): 0.87(t, J=7.5 Hz, 3H,1×-CH_3), 1.24-1.43(m, 34H, 17×CH_2), 2.54(s, 1H, 1×-CH_2-OH), 3.41(t, J=8.0Hz, 2H, 1×-CH_2-OH).LC-MS (m/z, % intensity): 202(8), 139(40), 107(100), 85(11).

Compound III was a colorless crystalline solid obtained on elution with benzene: hexane (1:3) and recrystallized from benzene, 20mg, melting point 168-169ºC (Lit. mp 169.5) [5]. R_f value was found to be 0.44 in ethyl acetate: benzene (1:14). The molecular formula $C_{29}H_{48}O$ deduced from its GC-MS having molecular mass 412. IR (KBR, $V_{max.}cm^{-1}$): 3456, 3216, 2945, 2844, 1499, 1396, 1240, 1051, 759.^{1}H NMR (δ, $CDCl_3$): 0.75(s, 6H, 1×C_{29}- $CH_3$and C_{26}- CH_3), 0.97(s, 6H, 1×C_{24}-CH_3 and 1×C_{27}-CH_3), 1.16 (s, 3H, 1×C_{28}-CH_3), 1.22 (s, 3H, 1×C_{19}-CH_3), 3.78(d, J=4.0Hz, 1H, 1×C_3-CH), 4.38 (s, 1H, 1×C_{23}-CH), 4.87(m, J=16.0Hz, 1H, 1×C_{22}-CH), 5.02 (m, J=4.0Hz, 1H, 1×C_6-CH), 7.27 (s, 1H, 1×-OH). GC-MS (m/z, % intensity): 412(35), 389(28), 345(31), 270(21), 241(18), 211(33), 199(87), 87(97).

Compound IV was white solid compound with melting point 82º-84ºC (Lit. mp. 82ºC) [6] was obtained on elution with benzene: hexane (1:1) and recyrstallised from benzene, 80mg. Its R_f value in ethyl acetate: benzene (1:14) was found to be 0.43. The molecular formula $C_{29}H_{58}O$ and molecular mass 422 was deduced from its LC-MS analysis. IR (KBR, $V_{max.}cm^{-1}$): 2954, 2917, 2849, 1710, 1463, 1292, 897, 719.^{1}H NMR (δ, $CDCl_3$): 0.88, (t, J=8.0 Hz, 6H, 2× -CH_3), 1.25 (br, J=8.0 Hz, 44H, 22× -CH_2), 1.63(m, J=8.0 Hz, 4H, 2× -CH_2CH_2CO), 2.35(t, J=8.0 Hz, 4H, 2× -CH_2-CO). LC-MS (m/z, % intensity): 415(65), 316(29), 202(18), 139(25), 119(4), 85(11).

Compound V was obtained on elution with ethyl acetate: benzene (1:19) as a green crystalline solid, 30mg, melting point 84-86ºC. The R_f value for this compound was found to be 0.70 in ethyl acetate solvent. The molecular formula $C_{24}H_{48}O_2$ was deduced from m/z 368 by its GC-MS. IR (KBR, $V_{max.}cm^{-1}$): 3287, 2916, 2849, 1730, 1641, 1175, 1048, 719.^{1}H NMR (δ, DMSO-d_6): 0.87(t, J=8.0Hz, 3H, 1× -CH_3), 1.24- 1.58(m, 42H, 21× -CH_2), 3.44(t, J=8.0Hz, 2H, 1× -CH_2-CO), 3.75(s, 1H, 1× -CO-OH). GC-MS (m/z, % intensity): 354(75), 280(30), 220(25), 146(20), 72(100).

Compound VI was white crystalline solid (30mg) obtained with ethyl acetate: benzene (1:1). It was recrystallized from ethyl acetate, melting point 135-136ºC (Lit. mp. 136-137ºC) [4]. Its R_f value was found to be 0.67 with pure ethyl acetate solvent. It gave positive response of Liebermann Burchard reaction giving green coloration indicated the presence of steroid. The GC-MS and elemental analysis responded the molecular formula $C_{29}H_{50}O$ was deduced from molecular mass 414. IR (KBR, $V_{max.}cm^{-1}$): 3406, 2931, 2849, 1649, 1456, 1365, 1019,799.^{1}H NMR (δ, DMSO-d_6): 0.68(t, J=8.0Hz, 3H, 1×C_{28}-CH_3), 0.81(d, J=8.0Hz, 6H, 1×C_{26}-CH_3, and 1×C_{27}-CH_3), 0.86(t, J=8.0Hz, 3H,C_{29}-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), 4.75-4.82(m,1H, C_3-CH-OH), 5.32(br, J=4.0Hz, 1H=CH). GC-MS (m/z, % intensity): 353.9(95), 339.9(15), 280.1(70), 220.2(35), 146.4(25), 72.7(100).

Biological Potential/ Phenolic interaction with protein

The protein phenol interaction of various fractions of leaves of *Prosopis cineraria* was determined by using spectrophotometric method [7]. Tannic acid (50µg/µl) was used as a reference standard for plotting calibration curve. 0.25g of each plant fraction (1µg/µl) was dissolved in 5ml of methanol. From these solutions 2µl, 4µl, 6µl, 8µl and 10µl were applied in three replications on Whatmann No. 1 filter paper as a spots. The spots were allowed to dry and then sprayed with BSA solution until the paper was completely wet. After 30 minutes the filter paper was thoroughly washed three times with acetate buffer to remove unbound BSA. The paper was strained with Ponceau S dye three times. The strained strips were washed in 0.2% acetic acid v/v until elution of colour from the strips ceased. The strips along with blank were air dried and strained area were cut into small pieces and kept in separate test tubes. The colour of strained cut pieces was eluted with 3ml of 0.1N NaOH followed by addition of 0.3ml of 10% acetic acid. After centrifugation the colour in supernatant liquid was measured at a wavelength 525nm. The amount of protein binding capacity was calculated by using linear regression equation obtained from the standard curve of Tannic acid. The protein binding

capacity was measured as Mean±Standard Deviation (S.D.) and represented as µg/µl tannic acid of dry extract [7].

Statistical analysis: Triplicates of each sample were used for statistical analysis and the resulting values are expressed as mean±standard deviation (S.D). One way and two way analysis of variance (ANOVA) was carried out to assess any significant differences between the means ($p<0.05$) in Online Statistical Analysis (OPSTAT). All other measurements and calculations were carried out in Microsoft Excel 2007.

Results and Discussion

Compound I (1-heptacosanol): The compound was obtained as white crystalline solid on elution with benzene: hexane (1:19). It does not respond to colour reaction for phenolic compounds. It was recrystallized from chloroform as a solid (40mg), melting point 140-141ºC. R_f value of this compound I was found to be 0.65 in benzene: hexane solvent system. The IR spectrum of the compound I displaced a peak at 3291 cm^{-1} suggesting the presence of a hydroxyl group. The GC-MS of this compound indicated its molecular weight 396 and molecular formula to be $C_{27}H_{56}O$. The ^1H NMR spectrum of the compound I in $CDCl_3$ showed a triplet at δ 3.64 with J value 7.0 Hz, integrating the two protons hinted the presence of methylene group positioned at alpha to hydroxyl group.A broad peak observed at δ 1.55 for two protons suggested the presence of a methylene group β to a hydroxyl group. Another broad signal integrating fourty eight protons at δ 1.25 which could be due to twenty four methylene groups. A triplet was shown at δ 0.88 (J= 7.0Hz) integrating to three protons suggesting the presence of terminal methyl group (CH_2-CH_3). The observed spectroscopic and analytical, data were identical to literature data [8] for 1- heptacosanol. The mass fragmentation pattern and elemental analysis suggested that the compound **I** therefore settled as 1- heptacosanol.

Compound II (1- Nonadecanol): The colorless needle shaped crystalline (30mg) compound was isolated from benzene: hexane (1:19) solvent system. It was recrystallized from benzene: hexane (1:1), melting point 62-64ºC (Lit. mp. 62-63 ºC) [4]. Its R_f value was found to be 0.54. The IR spectra of the absorption peak at 3292 cm^{-1} showed the presence of hydroxyl group. Another absorption peaks at 2917, 2849, 1462, 1061 and 719cm^{-1}.LC-MS data analysis of the compound II indicated its molecular formula and molecular mass to be $C_{19}H_{40}O$ and 284 respectively.

The ^1H NMR spectra of the compound II exhibited a triplet at δ 0.87 with coupling constant (J= 7.5 Hz) assigning three protons of terminal methyl group. Other methylene groups appeared as multiplet of broad signal in the range of δ 1.24-1.43 integrating thirty four protons of seventeen methylene groups. A singlet appeared at δ2.54 of one proton exhibited the presence of hydroxyl group. A triplet at δ 3.41 with J value 8.0Hz assignable to two protons of methylene group attached to hydroxyl group. All the above discussed spectral data were in agreement with the literature of 1- Nonadecanol [4]. So, the compound II can be identity as 1-Nonadecanol.

Compound III (Stigmasterol): The colorless compound III was obtained on elution with benzene: hexane (1:3). Fractions (1-10) were repeatedly separated by silica gel column chromatography and pooled together by observing TLC results. It was crystallized from pure benzene, 20mg. The purity of compounds was determined by Thin Layer Chromatography under estimation of its R_f value (0.44). Its melting point was found to be 168-169ºC (Lit. mp 169.5) [5]. The IR absorption spectrum at 3456 and 3216 cm^{-1} confirmed the presence of –OH functionality in its compound. MS analysis of compound III showed its molecular mass 412 with molecular formula $C_{29}H_{48}O$.

The ^1H NMR spectra of compound III in $CDCl_3$ exhibited a singlet at 0.75δ indicating the presence of six protons of two methyl groups and another six protons of two methyl groups could picked up singlet at 0.97δ. Two singlets of three protons appeared at 1.16 δ and 1.22δ for two methyl grouped at C_{28} and C_{19} positioned. A doublet at 3.78δ with coupling constant (J=4.0Hz) integrating for one proton assignable to –CH moiety attached to –OH group at C_3 position. A singlet at 4.38δ was assignable to H at C_{23} positon. A multiplet centered at 4.87 δ with J= 16.0 Hz integrating one proton of methylene group at C_{22} position. Another multiplet of methylene group at 5.02δ with coupling constant J=4.0Hz assignable to one proton at C_6 positioned. A singlet of representing one proton at 7.27δ confirmed the presence of hydroxyl group in it. All the above spectral data is in agreement for the following structure with literature data [9] of stigmasterol. This compound is the report of isolation and characterization from leaves of *Prosopis cineraria.*

Compound IV (Nonacosane-15-one): The white solid compound IV with melting point 82-84ºC was obtained on elution with benzene: hexane (1:1) and it was recrystallized from benzene (80mg) solvent. Its R_f value in ethyl acetate: benzene (1:14) was found to be 0.43. The appearance of absorption band at 1710cm^{-1} confirmed the presence of carbonyl group. Other absorption band at 2954, 2917, 2849, 1463, 1292, 897 and 719 cm^{-1}. LC-MS analysis suggested the molecular mass of the compound to be 422 having molecular formula $C_{29}H_{58}O$.

The ^1H NMR spectrum of the compound IV in $CDCl_3$ showed no signal in aromatic region indicating the aliphatic nature of the compound. A triplet at 2.35 δ integrating four protons with coupling constant J=8.0Hz could be due to methylene proton alpha to keto group. A broad signal at 1.25 δ representing forty four protons was assigned to twenty two methylene groups. A triplet centered at 0.88 δ with coupling constant J= 8.0Hz integrating for six protons aliphatic to terminal methyl groups. Thus, based upon above information, the compound IV could be characterized as Nonacosan-15-one. A complete agreement of the spectral and literature data of this compound Nonacosan-15-one [6] helped us to identify this compound.

Compound V (1- Tetracosanoic acid): It was obtained on elution with ethyl acetate: benzene (1:19) as a green solid, 30mg, melting point 84-86ºC. The R_f value for this compound V was found to be 0.70 in ethyl acetate solvent. The absorptions at 3287, 2916 and 2849cm^{-1} in its IR spectra confirmed the presence of hydroxyl group and methyl group. Other absorptions appeared at 1730, 1641, 1175, 1048 and 719cm^{-1}. GC-MS of the compound V proposed its molecular mass to be 368 and deduced molecular formula $C_{24}H_{48}O_2$.

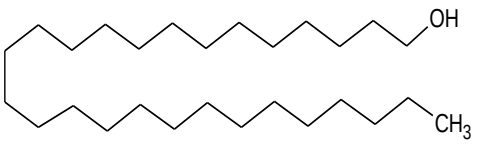
^1H NMR spectra of the compound V in DMSO-d_6 showed a triplet for three protons of terminal methyl group at 0.87δ with coupling constant 8.0Hz. There was a multiplet for forty two protons in the range of 1.24- 1.58 δ indicating the presence of twenty one methylenes. A spectrum exhibited a triplet at 3.44 δ with J value 8.0 Hz for two protons of CH_2 group attached to –COOH group. A singlet centered at 3.75δ for one proton of –OH group confirmed the presence of OH

group in PL-V compound. All these above spectral data helped us to identity the compound, V to be 1- tetracosanoic acid. From the literature survey it seems that this is the first report of isolation and characterization of 1- tetracosanoic acid from the leaves of *Prosopis cineraria.*

Compound VI (β-sitosterol)

The white crystalline compound VI was obtained on elution with ethyl acetate: benzene (1:1). It was recrystallized from ethyl acetate solvent, 30mg, melting point 135-136ºC (Lit. mp. 136-137ºC) [4]. It responded to Liebermann-Burchard reaction and gave positive test of green coloration indicating the presence of steroid. The IR spectra of this compound VI showed a peak at 3406cm^{-1} indicating the presence of OH group. Other absorption peaks at 2931, 2849, 1649, 1456, 1365, 1019 and 799cm^{-1}. The LC-MS and elemental analysis suggested the molecular formula and molecular mass to be $C_{29}H_{50}O$ and 414.

^{1}H NMR spectral details of the compound VI in DMSO-d_6 exhibited a broad signal at 5.32δ for one proton with coupling constant J=4.0Hz assignable to olefinic proton. Appearance of a multiplet ranged 4.75-4.82δ integrating to one proton could be a proton at α- position to a hydroxyl group. Another multiplet in the range of 2.17- 2.38δ representing twenty nine protons hinted the presence of eleven methylene and seven methines. A singlet centered at 1.22δ representing three protons could be due to methyl group at C_{19} position. A doublet centered at δ 0.94 with coupling constant J=7.0 Hz integrating three protons was assignable to methyl group at C_{21} position and a triplet represented at δ 0.86 with J value 8.0Hz integrating three protons suggested the presence of a methyl group at C_{29} position. A doublet centered at δ 0.81 with J= 8.0Hz integrating for six protons indicated presence of two methyl groups positioned at C_{26} and C_{27}. A singlet at δ 0.68 with coupling constant (J=8.0Hz) integrating three protons which was hinted methyl group at C_{28} position. A complete agreement of the spectral data of the compound VI with literature data of β-sitosterol [4] established the identity of the compound VI to be β-sitosterol.



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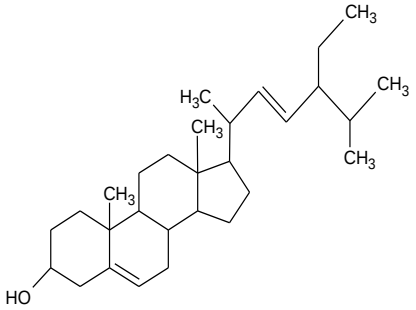

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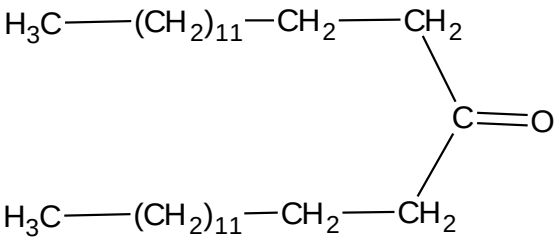

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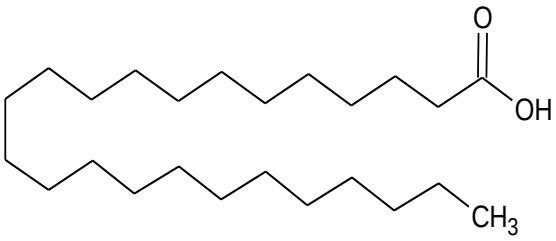
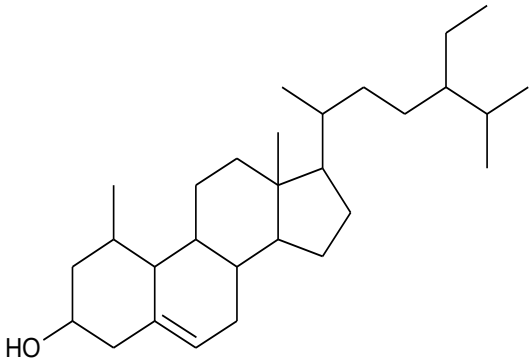


V



VI

Structure of compounds I to VI

Phenolic interaction with protein of leaves of *Prosopis cineraria*

The data shown in table 1 reveals that hexane and acetone fraction of leaves of *Prosopis cineraria* exhibited highest protein binding capacity i.e. 3.53±0.04% and 3.39±0.05% at highest concentration (10µg/µl). Methanolic extract also exhibited activity 3.22±0.01% nearly same as hexane and acetone extracts at highest concentration. Benzene fraction was found to possess minimum activity (0.96±0.03%) at lower concentration while hexane fraction has shown maximum (3.53±0.04%) at higher concentration. At moderate concentration (06µg/µl), ethyl acetate and chloroform fractions showed moderate activity i.e. 1.96±0.03% and 1.83±0.02%. Very less activity was shown by benzene extract at 10µg/µl concentration. Water extract has shown 2.93±0.04% activity at 10µg/µl test concentration. At 4µg/µl test concentration methanol, water and acetone fractions were found to have moderate activity values i.e. 2.46±0.04%, 2.47±0.03% and 2.48±0.005% respectively. Protein binding capacity of various extracts of leaves of *Prosopis cineraria* were collected in the order as hexane> acetone> methanol> water> chloroform> ethyl acetate> benzene. The step wise step increasing value of phenolic interaction with protein showed that nutritional value and digestibility of leaves of *Prosopis cineraria* goes increase respectively [7].

Table 1: Phenolic interaction with protein of various fractions of leaves of *Prosopis cineraria*

Sr. No.	Fractions	Concentrations (µg/µl)					
		00	02	04	06	08	10
1.	Hexane	1.86±0.03	2.61±0.05	2.91±0.04	3.04±0.04	3.37±0.04	3.53±0.04
2.	Benzene	0.96±0.03	1.17±0.06	1.29±0.05	1.50±0.05	1.62±0.05	1.73±0.05
3.	Chloroform	1.19±0.04	1.52±0.04	1.72±0.03	1.83±0.02	1.94±0.06	2.84±0.07
4.	Ethyl acetate	1.39±0.03	1.70±0.02	1.85±0.04	1.96±0.03	2.19±0.03	2.39±0.05
5.	Acetone	1.75±0.02	2.27±0.03	2.48±0.05	2.62±0.03	2.95±0.03	3.39±0.05
6.	Methanol	1.85±0.03	2.25±0.01	2.46±0.04	2.71±0.07	2.82±0.05	3.22±0.01
7.	Water	1.72±0.05	1.96±0.04	2.47±0.03	2.59±0.04	2.86±0.04	2.93±0.04
Factors		SE(d)			CD at 5%		
Concentration		0.026			0.052		
Compound		0.024			0.048		
Conc.×Compound		0.062			0.127		

Phenolic interaction with protein was expressed in percentage, All values are mean ± standard deviation, Values are mean of three replicates (n=3) and µg/µl means microgram per microlitre.

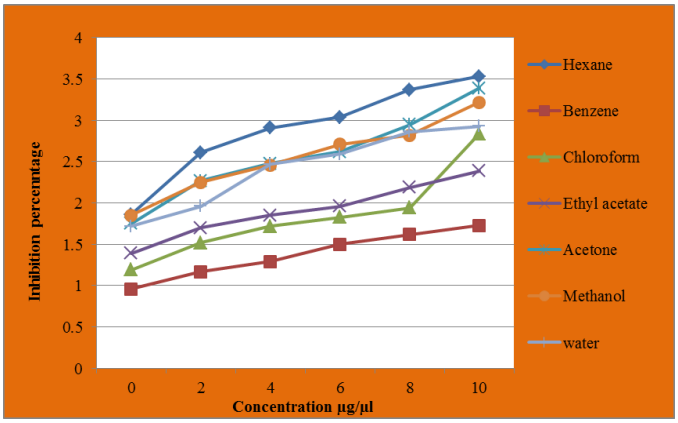


Fig 1: Comparative analysis of Phenolics interaction with protein of various fractions of leaves of *P. cineraria*

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