Antioxidant Activity of *Hipophae salicifolia* Defatted Seed Extract and Estimation of Ascorbic Acid Content Using High Performance Thin Layer Chromatography

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ABSTRACT - *Hippophae salicifolia* (Seabuckthorn) is a Himalayan shrub which is well known for its extraordinary medicinal potential due to the presence of various antioxidant components such as vitamin C, Vitamin E, tocopherols, flavonoids etc. as reported in the previous studies. The main concern of the present study was to check the activity of methanolic extract of *Hippophae salicifolia* defatted seeds by H_2O_2 and DPPH method and the estimation of Ascorbic acid content using HPTLC method. Seeds were harvested in the month of July 2018 at DIBER High Altitude Research Station, Auli (Chamoli district) Uttarakhand, India. The seeds were shed dried for 10 days and grinded in mixer. Methanolic extract was prepared by soxhlet apparatus and solvent evaporation was done using Rotary evaporator. FRSA content was reported higher than the ascorbic acid standard in case of H_2O_2 method i.e. 52.54 % and DPPH was also very near to the standard value 76.22 %, ascorbic acid Analyses and its quantification was performed in pre coated aluminium sheet TLC plate (Merck, Silica gel 60, F254) as stationary phase. Samples were sprayed onto TLC plate in the form of bands with Nitrogen (Linomat 5) at the speed of 150 nl/sec. Chromatogram development was done using mobile solvent i.e. Ethanol: Acetic acid (9.5:0.5 v/v). Evaluation (TLC Scanner 4) of developed chromatogram was done at 254 -336 nm (U-V range) using Deuterium and Tungsten lamp. The result showed clear band of ascorbic acid, quantitative estimation was done by standard curve and the values of ascorbic acid in seeds was near about 196 mg / 100 g. Method is reproducible and easy which can be used for herbal and pharmaceutical doses using HS as a source of herbal formulation.

Keywords: Ascorbic Acid, HPTLC, *Hipophae salicifolia*, Methanol.

Abbreviations

MeOH	Methyl Alcohol
ROS	Reactive Oxygen Species
HPTLC	High Performance Thin Layer Chromatography
UV	Ultra violet
FRSA	Free Radical Scavenging Activity
DPPH	2,2 Diphenyl -1- Picryl Hydrazyl
HS	*Hippophae salicifolia*
SDW	Sterile Distill Water
AA	Ascorbic Acid

1. INTRODUCTION

There are number of phytochemicals which are present in different part of the plant and works as defence mechanism for the plant to restrict the pathogen attack and provides a strength against outside harmful organisms. Not only for itself, these phytoconstituents (primary and secondary metabolites) are also very useful for the human being, they play a vital role to control the various degenerative disease. These disease includes aging, diabetes, cancer and so on[1]. The main cause of all these type of disease is the free radical which generates in the body during the chemical reactions and metabolic pathways, it is important to trap these free radical before they harm the cells of the organism. These trapping agents are generally regarded as antioxidant which stabilize or deactivate the free radicals. Vitamin E, vitamin C and beta carotene are among the most widely studied dietary antioxidants[2]. Vitamin C is deliberated as the most significant water-soluble antioxidant in extracellular fluids. It is capable of neutralizing ROS in the aqueous phase before lipid peroxidation is started. Vitamin C also cited as being capable of regenerating vitamin E[3,4].Almost every plant contains vitamin C as an important source of antioxidant, HS is also known for the high content of these vitamins, so the present study

was conducted to examine the potential of HS seed MeOH extract as an antioxidant and the quantitative estimation of vitamin C (Ascorbic acid) using HPTLC.

The plant is deciduous shrub belongs to the family Elaeagnaceae, the leaves of this species are broad (4 to 10 mm (0.39 in)) and green (less silvery), and the berries are yellow in colour. As reported, the plant is stress tolerant and soil stabliser[5]. All parts of the plant have its use in various field being it medicinal, cosmetic industry, fodder additives etc. Different part of the plant has been used to treat various diseases such as cardiovascular diseases, skin and eye ailments, high blood pressure etc[6,7]. Seabuckthorn oil is one of the valuable products from seabuckthorn berries, as berries are good source of antioxidants. After the oil removal almost 80 %(±5) defatted part obtained which can be further used for fodder activities, so the present study was done for examine antioxidant potential of defatted part and also an attempt was made to develop the easy and reproducible method for the quantification of ascorbic acid content in the extract using HPTLC method.

2. MATERIAL AND METHOD

2.1. Plant material and chemical

The *Hippophae salicifolia* seeds were collected in July 2018 from DIBER High Altitude Research Station, Auli (Chamoli district), Uttarakhand, where the plants were cultured under natural environment. Collected seeds were washed thoroughly with sterile distill water, dried in hot air oven at 35˚C and were grounded in mixer grinder to form a powder (REMI, India). All other chemicals (Make: Merck) were procured from the local supplier and used without extra refinement.

2.2. Preparation of HS MeOH extract

50 g of powdered seeds were extracted in 300 ml hexane by soxhlet extraction technique Soxhlet (1879) run for 72 hrs. to remove the oil and lipids from the HS seeds. Again the defatted seed powder was collected from the thimble and dried in oven to remove hexane from it. Obtained powder was again placed in thimble for the preparation of methanolic extract. The excessive solvent was evaporated using rotary evaporator (IKA RV10, Germany) and then lyophilized (LABCONCO) to obtain powder form and stored at 4˚ C for further analyses.

2.3.Free radical Scavenging Activity

2.3.1. BY H_2O_2 Method: -

FRSA activity was estimated using the hydrogen peroxide as a reactive oxygen species. For this purpose, we followed the method of Czochra and Widensk (2002)[8]. 1 ml of extract and standard (Ascorbic acid) was added with 2 ml of H_2O_2 (43 mM of 30% H_2O_2) in the manner of 10,20,30,40,50 µg/mL. 2.4 ml of 0.1 M phosphate buffer (pH 7.4), this assay mixture was incubated for 10 minutes and then absorbance was taken at 230 nm.

FRSA was calculated by using formula

$$FRSA(\%) = [(Vo-V_1) /Vo] X 100$$

where, V_0 = absorbance of control, and V_1 = absorbance of sample

2.3.2. By DPPH Method: -

FRSA of the extract was evaluated by 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) followed by the method of Shen et al. (2010)[9]. 0.1mM solution of DPPH in methanol was prepared and 1mL of this solution was added to 3 ml of the solution of AA and methanolic extract at different concentration (10,20,30,40,50 µg/mL). The mixtures were shaken vigorously and allowed to stand at room temperature for 30 minutes. Then the absorbance was measured at 517 nm using a UV-VIS spectrophotometer. Ascorbic acid was used as the reference. Lower absorbance values of reaction mixture indicate higher free radical scavenging activity. The capability of scavenging the DPPH radical was calculated by using the following formula.

DPPH scavenging effect (% inhibition) = $\{(A_0 - A_1)/A_0)*100\}$

Where, A_0 is the absorbance of the control reaction, and A_1 is the absorbance in presence of all of the extract samples and reference

2.4. HPTLC instrumentation and procedure

For the analysis and quantifying the ascorbic acid content, CAMAG HPTLC (Switzerland) model was used. Lyophilized sample were dissolved with the methanol to form 1mg/ml of the solution, as standard 1mg/ml of Ascorbic acid was also prepared. A silica gel aluminum sheet was used as a stationary phase. Plate was saturated with methanol under the chamber (TTC 20*10) and then activated in hot air oven before loading the sample. Samples were loaded in the form of bands (width 8 mm) with the help of Camag µlit syringe injector using LINOMAT V at the speed of 150 nl/sec with the help of nitrogen. first track position was set at 28 mm and the band gap between the spots were 17.4 mm. TLC Chromatogram was developed in ascending order in twin through glass chamber of 20*10 mm, Solvent (takes almost 20 minutes to flow at the top of the

chromatogram (80 mm), the developed chromatogram was dried in TLC spray cabinet II, Densitometric analysis (TLC Scanner IV) and photo documentation (TLC visualize II) of developed chromatogram was done at 254 - 336 nm (U-V range) using Deuterium and Tungsten lamp. Programming and documentation of HPTLC operated on Vison CATS 2.5 software. Whole HPTLC procedure was operated under standard laboratory conditions {(Temp. 25 ± 2 º C), RH 50 ± 5% (MEXTECH TM-2)}

Sr. No	Chromatography Conditions	System Setup
1.	Stationary Phase	Merck HPTLC Plates Silica gel 60, F254
2.	Plate format	200 X 100 mm
3.	First track	28 mm
4.	Sample solvent	Methanol
5.	Solvent front position	80 mm
6.	Tank	TTC 20 *10
7.	Mobile Phase	Ethanol: Acetic Acid (9.5:0.5 v/v)
8.	Saturation time	20 minutes
9.	Imaging	R254, R366
10.	Lamp	Deuterium & Tungsten

2.5. Calibration curve of Ascorbic Acid

For this purpose, 1mg/ml stock solution of AA prepared in methanol, from this stock solution 200, 400, 600, 800, and 1000 µg/ml (2 µlit. from each) was spotted on TLC plate to obtain concentration in nanogram range. Accuracy and precision of the calibrated curve was validated in terms of Coefficient of variation (CV %) and Correlation coefficient (R %).

3. RESULT AND DISCUSSION:

3.1. Antioxidant potential of HS Extract:

FRSA activity is the basic observation of any herbal drug to know its capability about to trapping the ROS. Antioxidants cannot measure by direct method of counting or any other techniques, it can be just observed by controlling the extent of oxidation in any chemical reaction, almost every plant species has various antioxidant compound these compound show different activity towards differently generated ROS. In the present study, we evaluated antioxidant potential by using two different chemicals i.e. H_2O_2 and DPPH which are highly responsible for generating the free radicals.

Percentage inhibition of free radicals which are generated by hydrogen peroxide was controlled by extract and AA standard are 10.98 and 16.87 when abs taken at lowest concentration (10 µlit) and at higher concentration (i.e. 50 µlit) the inhibition values were 52.54 and 47.61 respectively. This result suggests at lower concentration AA was more effective to control the free radical, but at higher concentration the methanolic extract shows remarkable percentage inhibition which was higher than the AA standard.

Sr No.	Conc. (µlit.)	Inhibition (in %) using HS extract	Inhibition (in %) using AA Standard
1.	10	11.47 ±0.69	16.56 ±1.15
2.	20	22.51 ±0.82	20.70 ±1.59
3.	30	34.93 ±0.40	39.76 ±0.88
4.	40	44.69 ±1.13	46.76 ±0.72
5.	50	53.45 ±0.82	49.15 ±0.09
MSE		0.658	0.828

Table: FRSA Activity (H_2O_2 Method)

Sr No.	Conc. (µlit.)	Inhibition (in %) using HS extract	Inhibition (in %) using AA Standard
1.	**10**	27.35 ±1.65	21.76 ±0.97
2.	**20**	31.47 ±0.47	33.95 ±1.05
3.	**30**	50.19 ±0.59	46.31 ±0.49
4.	**40**	54.08 ±0.62	62.00 ±1.07
5.	**50**	76.07 ±0.27	83.45 ±0.47
MSE		0.707	0.696

Table: FRSA Activity (DPPH Method)

* Each value expressed as mean, ± Standard Deviation (n=3); MSE: Mean Standard Error
* The mean difference is significant at the 0.05 level
(All Statistical analyses done using SPSS 2016 software)

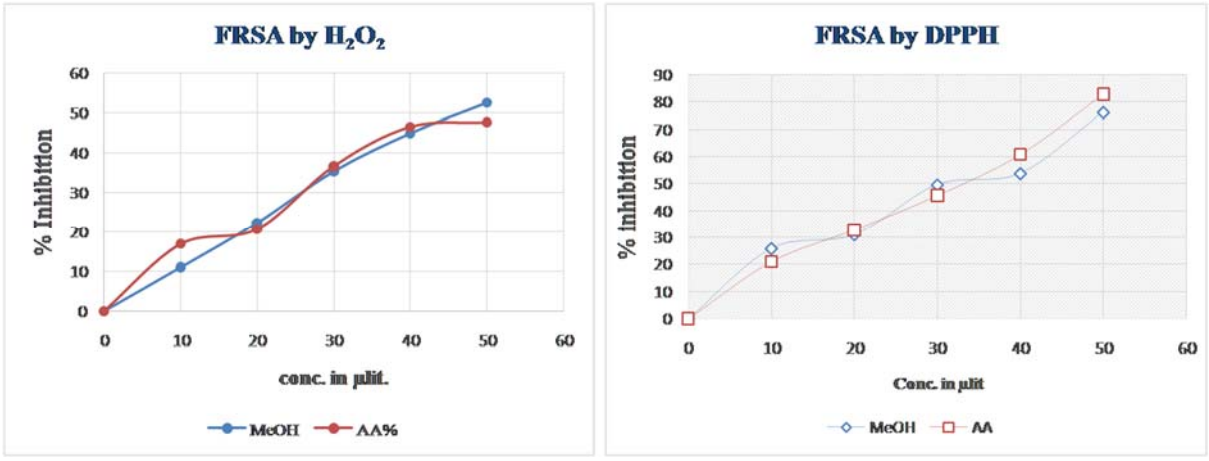


Fig: Graph showing the percentage inhibition control at different concentration

Free radicals which generated by DPPH was also inhibited by methanolic extract in very high amount. At lower concentration of 10 µlit the inhibition percentage was coming near at 25.87% which was higher than the AA standard i.e. 20.97 %. However, at higher concentration value of methanolic extract was lower (76.22%) than the AA standard (82.98%). Still, the value of FRSA activity by both methods showing remarkable and significant results.

3.2. Ascorbic acid content quantification by HPTLC

3.2.1. Selection of extracting solvent

Hippophae salicifolia L. seeds are well known for the lipids present in it, the oil content is rich in its nutritional value, seeds of HS contain almost 8 to 9% of the lipid, for the removal of the oil Hexane was used as a primary solvent. Other polar substance which remains in the defatted seed were extracted using methanol. Obtained extract considered as it contains the highest amount of Ascorbic acid.

3.2.2.Selection for the mobile phase (Developing solvents)

Mobile solvent for TLC chromatogram solvent was used Ethanol: Acetic Acid (9.5:0.5 v/v) which is already cited for ascorbic acid determination previously[10]. The solvent was filtered using Whatmann filter (no. 00), this solvent system was mixed well and poured into TTC for 20 minutes for saturating the chamber. After 20 minutes, dried spotted plate of aluminum sheet was placed into the chamber and run the chromatogram till the solvent rich the 80mm position.

3.2.3 Chromatogram development and imaging

The developed plate was dried for further imaging. For this purpose, plate was analyzed in TLC visualizer II, a band of standard was clearly found, these standard spots (first five) becoming darker as the quantity of standard was increased. Developed chromatogram, was not visible in RT white, but at 254 nm clear spots are confirming the presence of L ascorbic acid in the defatted seed of HS.

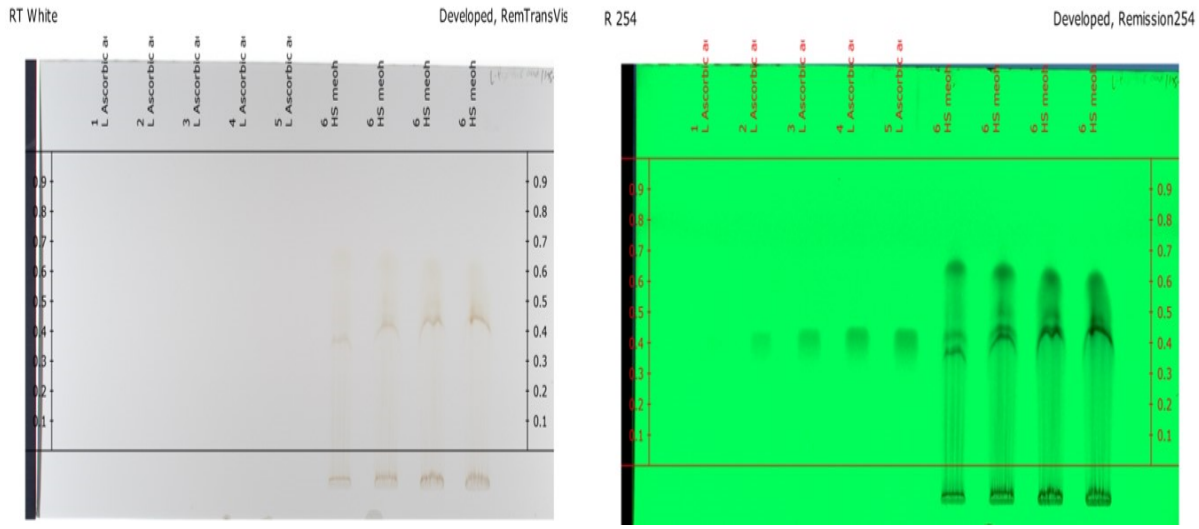


Fig 3: Showing Chromatogram at RT white and at 254 nm.

3.2.4.Scanning of the spots (Standard & extract)

Scanned image of chromatogram shows intense peak at a certain point in the level of 0.4 to 0.5 R_f values when standard was taken for imaging.

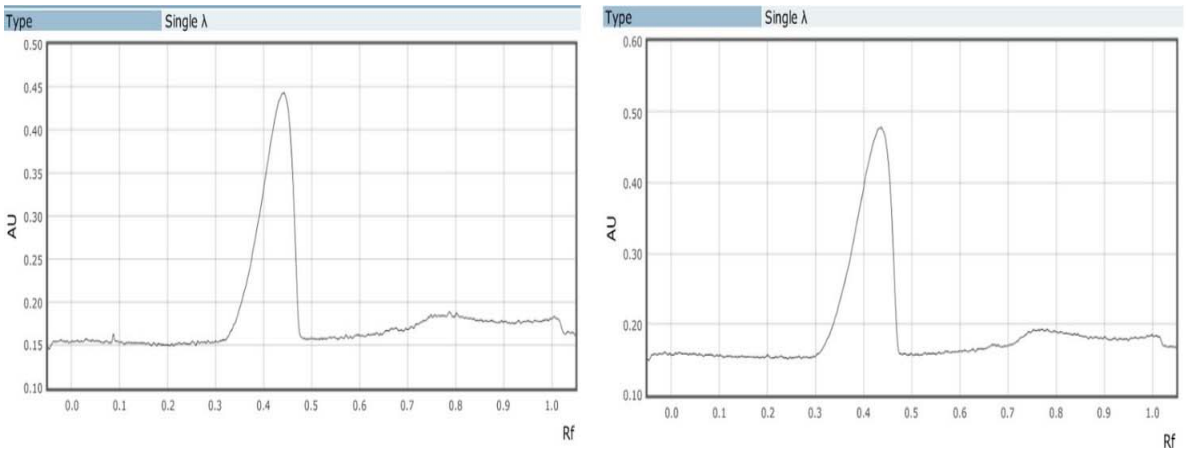


Fig 4: - Showing scanned image of AA standard in two different tracks

In case of extract, there are 3 different peaks confirming the presence of 3 different compound at a different $R_f$value, in these peaks an intense peak near at 0.40-0.50 shows the presence of ascorbic acid in the methanolic extract.

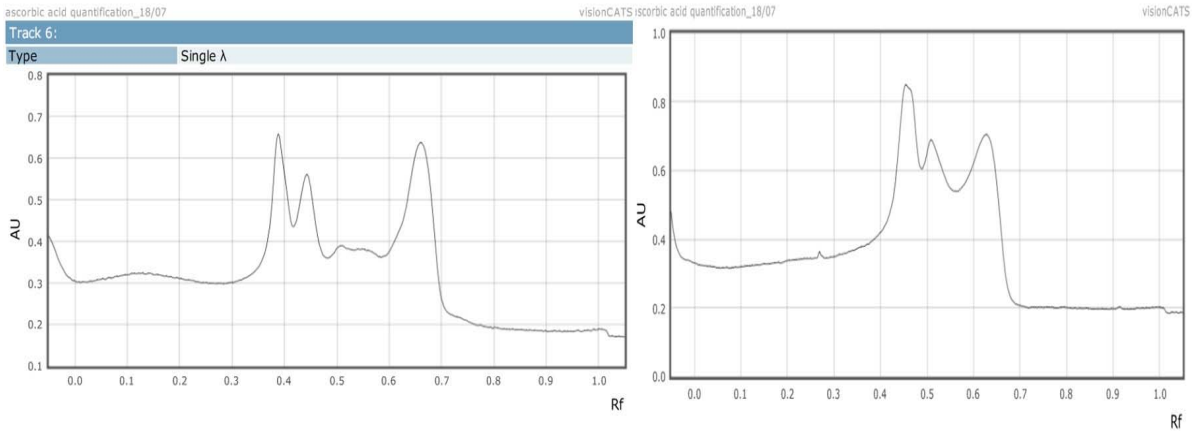
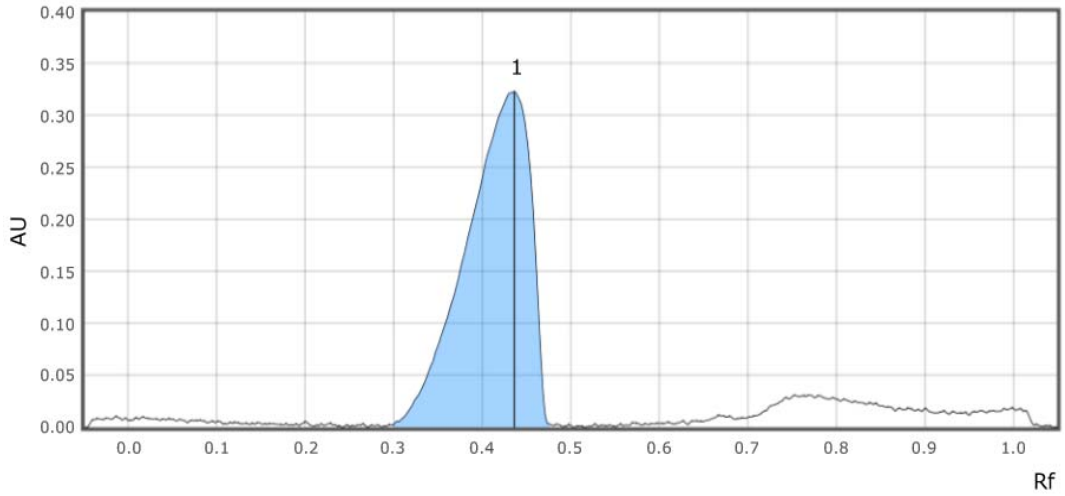


Fig 5: - Showing scanned image of methanolic extract

Table 1: R_f value and Height of the peaks of reference and extract

Sr. No.	Track	Sample	Rf Value		
			Starts at	Max	Height (AU)
1.	1	Ascorbic acid (Reference)	0.438	0.453	0.4434
2.	2	Ascorbic acid (Reference)	0.464	0.492	0.2419
3.	3	Ascorbic acid (Reference)	0.518	0.576	0.2159
4.	6	Ascorbicacid (Sample)	0.462	0.622	0.494



Peak #	Start		Max			End		Area		Manual peak	Substance Name
	Rf	H	Rf	H	%	Rf	H	A	%		
1	0.298	0.0022	0.436	0.3232	100.00	0.479	0.0018	0.02746	100.00	No	ASA

Fig 6: Ascorbic acid standard

Track 6:	
Type	Sample
Vial ID	6
Description	HS meoh
Volume	1.0 µl

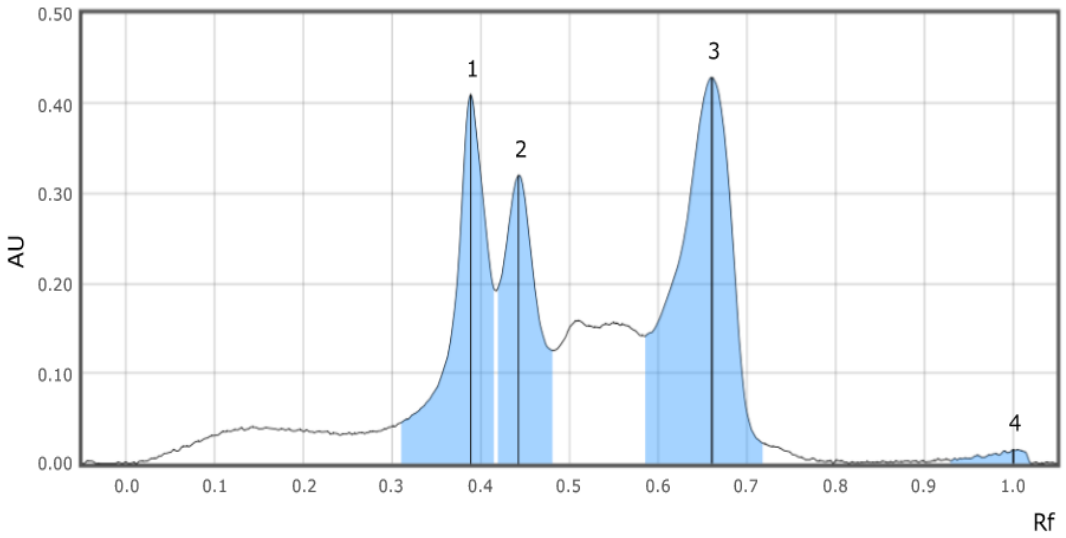


Fig 7: -Ascorbic acid in Defatted HS seed methanolic extract

3.2.5. Calibration curve: Area calibration curve of Ascorbic acid was linear in the concentration range of 06-2.2 µg range. All the values of the curve showed in the table below:

Table 2: Showing Calibration curve data

Sr. No.	Calibration curve data	Obtained values
1.	Regression Mode	Linear -2
2.	Range Deviation	5.00%
3.	Related substances	default
4.	No. of References	3
5.	Calibration Function	$Y = 1.56 * 10^{-7}x - 1.504 * 10^{-2}$
6.	Coefficient of variation	3.758
7.	Correlation coefficient	R = 99.424 %

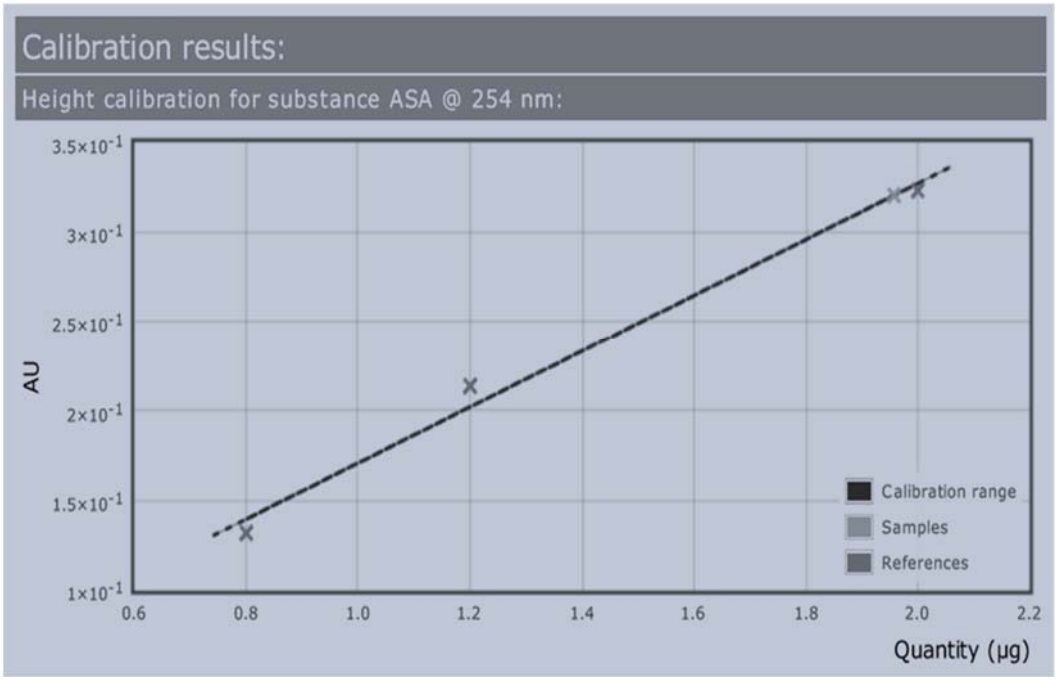


Fig 8: - Calibration curve (Ascorbic Acid Standard)

ascorbic acid quantification_18/07 visionCATS

ASA	(1 sample assignments) @ 254 nm		
Sample '6'	1.958 mg/ml	(CV unavailable)	(1 applications)
Volume: 1.0 µl	1.958 mg/ml	(CV unavailable)	(1 replicas)
Track 6	1.958 mg/ml	1.958 µg	

Statistical analyses from the calibration curve showed the value of ascorbic acid in defatted seed of HS was in the range of 195.8 mg / 100 g.

4.0. CONCLUSION: -

HS is well known Himalayan species, seeds are mainly useful for lipid extraction, however its defatted part also contains high amount of antioxidant as shown in the present article, also a HPTLC method was derived for estimating the amount of Ascorbic acid in seed content. It may be concluded that the defatted part of the HS seed can also be used in pharmaceutical and for the herbal formulations due to its remarkable and significant antioxidant potential.

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REFERENCES

[1] Hertog, M.G.L., Feskens, E.J.M., Hollmann P.C.H., Katan, M.B, Kromhout, D. (1993). Dietry antioxidant flavonoids and risk of coromary heat disease: The Zutphen Elderly Study. Lancet, 342: 1007-1011

[2] Sies, H. and Stahl, W., (1995). Vitamins E and C, beta carotene, and other carotenoids as antioxidants. Am J Clin Nutr; 62(suppl):1315S-21S.

[3] Halliwell, B., (1994). Free Radicals, Antioxidants, and Human Disease: Curiosity, Cause, or Consequence. Lancet;344:721-724.

[4] Briviba, K. and Sies, H., Nonenzymatic Antioxidant Defense Systems (1994). In: Natural Antioxidants in Human Health and Disease. ed. Frei, B. Academic Press: San Diego. Ch 4, p. 107-128.

[5] Pathak R., Goyal S., Kumari A., Dwivedi S.K., (2018). Antioxidant Profiling and Fatty Acid Composition of Lipids Present in HippophaeSalicifolia Grown in Higher Altitude of Uttarakhand Region. World Journal of Pharmaceutical Research. Vol 7, Issue 16. Pp.1286-1297.

[6] Liu B., Wu Z., and Liu, W., (1980). "Preliminary Observation on Curing Effects of Seabuckthorn Fruit Juice for High Blood Cholesterol and Coronary Heart Disease", Acta. Acad. Medicin. Sichua., Vol. 11, pp. 178-182.

[7] Yang B., Kallio H., Tahvonen R., Kalimo K., Mattila L., and Kallio S., (2000), "Effects of Dietary Supplementation of Seabuckthorn (Hippophaerhamnoides) Oils on Fatty Acids in Patients with Atopic Dermatitis", J. Nutri. Biochem., Vol. 11, No. 6, pp. 338-340.

[8] Kumar K., Pathak R., 2016. Phytochemical analysis and assessment of in vitro antibacterial activity of non-polar solvent based Camelina seed extracts. Ind J Plant Physiol. 21(3):255–262.

[9] Shen Q., Zhang B., Xu R., Wang Y., Ding X., Li P., Antioxidant activity in vitro of selenium-contained protein from the se-enriched. Bifodobacteriumanimalis 01. Anaerobe, 2010; 16: 380-386.

[10] Chakraborthy G.,S., (2009). Quantitative estimation of Ascorbic Acid by HPTLC in different varieties of Amla. J. Young Pharm Vol 1/ No. 1:82 – 85.

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