

Isolation and Characterization of New Triterpenoid Compound (α -Amyrin) from Dichloromethane Extract of *Spinacia Oleracea*

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Abstract: - The present investigation was aimed to study the new chemical compound from dichloromethane extract of *Spinacia oleracea*. Spinach (*Spinacia oleracea* L.) is widely distributed as a functional food due to its diverse nutritional composition, which includes carbohydrates vitamins and minerals, and to its phytochemicals that promote health and basic nutrition. It's phytochemicals and bioactives, such as glycolipids, flavonoids, carotenoids and phenolic compounds, impart their health benefits. The biological activities contribute to the anti-cancer, anti-oxidant, anti-obesity, hypoglycemic, anthelmintic and hypolipidemic properties of spinach.

Key words: Phytochemicals, flavonoids, β -carotene, antioxidant.

I. INTRODUCTION

Spinach is a leafy green vegetable grown in most parts of the world. Scientifically it is known as *Spinacia oleracea* Linn. (Family-Chenopodiaceae). Plant is sweet, cooling, carminative, laxative, alexipharmic; useful in diseases of blood and brain, asthma, leprosy, biliousness; causes "kapha" (Ayurveda). It has been used in the treatment of urinary calculi. In experiments it has been shown to have hypoglycemic properties. Spinach is a leafy green vegetable grown in most parts of the world. Leaves are cooling, emollient, wholesome, antipyretic, diuretic, maturant, laxative, digestible, anthelmintic, useful in urinary concretion, inflammation of the lungs and the bowels, sore throat, pain in joints, thirst, lumbago, cold and sneezing, sore eye, ring worm scabies, leucoderma, soalding urine, arrest vomiting, biliousness, flatulence. And have been used in the treatment of febrile conditions. Seeds are useful in fevers, leucorrhoea, urinary discharges, lumbago, and diseases of the brain and of the heart (Yunani). Seeds are laxative and cooling. They have been used in the treatment of difficulty in breathing, inflammation of the liver and jaundice. The green plant is given for the urinary calculi^{1,2}.

Spinacia oleracea is very rich in the flavonoids. Various flavonoids reported to be present are quercetin; myricetin; kampefero³, apigenin; luteolin; patuletin; spinacetin⁴. Hepatoprotective activity of *S.oleracea* Linn. Is based on its active constituents, including β -carotene, lutein, zeaxanthine,

flavonoids, vitamin C, p-coumaric acid and micronutrients. Free radical scavenging compounds such as β -carotene and vitamin C can protect DNA from oxidizing radical reactions. β -carotene is a potent free radical quencher, singlet oxygen scavenger, and lipid antioxidant. β -carotene has already been reported to quench not only singlet oxygen but also to scavenge a variety of free radical species. Lutein is effective at inhibiting autoxidation of cellular lipids. Vitamin C is considered to be the most important antioxidant in extracellular fluids. It acts to protect membranes against peroxidation by enhancing the activity of α -tocopherol, the chief lipid soluble and chain breaking antioxidant. P-Coumaric acid derivatives are strong antioxidants and have an ability of scavenge free radicals. As antioxidants, carotenoids are more or less efficient quenchers of reactive oxygen species and excited chlorophyll⁵. When a carotenoid reacts with a radical, the unpaired electron is delocalised across the conjugated double bonds, forming a relatively stable radical⁶. Flavonoids are also involved in protection against herbivores and micrororganisms, both as constitutive agents and as phytoalexins⁷.

II. MATERIAL AND METHODS

The leaves of *Spinacia oleracea* and *Desmodium triflorum* were collected from outfield medicinal garden near to Gwalior (M.P.) that show the green color with rough surface. The plant leaves was washed thoroughly in tap water, dried in shade, finely powdered and used for extraction. Plants were identified by the Dr. Gaurav Nigam, Asst. Professor, Department of Botany, Bundelkhand University, Jhansi (U.P.). A voucher specimen has been deposited in the herbarium at Department of Pharmacognosy for future references. A voucher specimen number for *Spinacia oleracea* is BU/Bot/10/05.

TLC of Dichloromethane extract of Spinacia oleracea:

TLC for the separation of various bioactive compounds from bioactive extract, dichloromethane was developed to find out the probable number of compounds present in them. On the precoated TLC plate, test samples (after dissolving in

respective solvents) were applied in the form of spots with the help of fine capillary. Spots were marked on the top of the plate for their identification. Rectangular glass chambers were used for chromatography. To avoid insufficient chamber saturation and undesirable edge effect, a smooth sheet of filter paper was placed in TLC chamber and was allowed to be in the developing solvent. A number of developing solvent systems were tried during the study. Each time plate was sprayed with Anisaldehyde sulphuric acid and vanillin sulphuric acid and heated at 115°C for 5 minutes. The solvent system in which there is a satisfactory resolution was taken as a final solvent system. Solvent systems; n-hexane:ethyl acetate (72:28) was found to be most satisfactory solvent system. After development of plates, they were air-dried and number of spots, color and R_f values were recorded⁸.

TLC plate-Precoated TLC plate silica gel 60 F₂₆₄

Bioactive extract- dichloromethane extract

Solvent system- n-hexane:ethyl acetate (72:28)

Spraying agents- Anisaldehyde sulphuric acid and vanillin sulphuric acid

R_f value=Distance travelled by solute/Distance travelled by solvent

HPTLC analysis of dichloromethane extract of Spinacia oleracea:

HPTLC fingerprint profile was established for Dichloromethane extract of *Spinacia oleracea*. TLC plate was kept in an ascending mode and activated at 120°C for 1 hour prior to application of sample bands. The sample was dissolved in 10 ml of dichloromethane and sonicated for 10 minutes, filtered and applied on TLC plates (5×10 cm) in 1 tracks (4µl for dichloromethane extract) in the form of band and allowed to equilibrate for 10 minutes. The spotted plate was then dipped in mobile phase and solvent front was allowed to travel about 70-80% distance on the plate vertically. Plate was then removed from chamber and dried.

Plate was then scanned at 254 nm for dichloromethane extract. Number of spots, color, R_f values and % relative areas were recorded⁹.

Isolation of Active compounds from fractions of Spinacia oleracea

Preparation of the column

The slurry of adsorbent (silica gel; 60-120 mesh) was prepared by mixing the adsorbent in the n-hexane and used as stationary phase. It was then poured into glass column (90cm x 3cm) and allowed to settle. The air entrapped was removed by stirring with glass rod. This method of column filling is called as wet filling method. A small amount of sand was kept at the top of the column. Excess of solvent was run off until the level of mobile phase fell to one cm just above the top of the sand layer¹⁰.

Preparation of sample and loading

Dichloromethane extract (10 g) was dissolved in a minimum volume of dichloromethane and adsorbed on silica gel (60-120 mesh), dried and applied on the column to separate possible phytoconstituents.

Selection of mobile phase for separation of phytoconstituents

Active dichloromethane extract (10 g) of *Spinacia Oleracea* was subjected to chromatographic separation by loading out on a glass column. The solvent system was used as n-hexane: ethyl acetate. The gradient elution was followed for the isolation. Initially n-hexane was used as pure solvent and then column was eluted by increasing quantity (95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 65:35, 60:40 and finally 50:50) of ethyl acetate. Total 220 fractions were collected of 20 ml each. All the fractions were monitored simultaneously on a TLC plate using n-hexane: ethyl acetate (72:28) as solvent system. The fractions showing same color and R_f on TLC were pooled together and finally 4 fractions (F1-F4) were obtained.

Characterization and Identification of compounds

UV Spectra [Graph No (2)] of Isolated compound: maximum wavelength were shown at 243 nm.

In α -amyrin, IR spectrum [Graph No (3)] absorption bands were appeared at 3442 cm^{-1} indicating the presence of hydroxyl group, 3056 cm^{-1} (C-H str. in CH_3), 2860 cm^{-1} (C-H str. in CH_2), 1731 cm^{-1} (C=O str.), 1483 cm^{-1} (C-H def. in CH_3), 1359 cm^{-1} (C-H deformation in gem dimethyl), 842 cm^{-1} (=C-H out plane bending).

The ^1H -NMR spectrum [Graph No (4)] shows that H-2 proton appeared at δ 3.47 as a multiplet and H-13 olefinic proton shows a singlet at δ 5.40. Also, eight methyl protons appeared as singlet as well as multiplet at δ 1.13, δ 1.02, δ 1.01, δ 0.96, δ 0.94, 0.88 and δ 0.88 which were quite similar with α -amyrin as mentioned by Saha et al.

The ^{13}C -NMR spectrum [Graph No (5)] has shown recognizable signals at 143.02 and 125.43ppm, which corresponds to double bond at C-12 and C-13. The δ value at 78.56 ppm is due to C-2 β -hydroxyl group. The peaks also showed that the isolated compound had eight methyl group, ten $-\text{CH}_2$ group and four $-\text{CH}$ groups.

The Mass spectrum [Graph No (6)] has shown. On the basis of the molecular mass at $m/z = 426$ and the structural information obtained by NMR analysis, the molecular formula $\text{C}_{30}\text{H}_{50}\text{O}$ was attributed to compound α -amyrin. The results were compared with the available literature and confirmed the presence of α -amyrin. Based on the UV, IR, NMR, Mass analysis, the isolated compound was identified as α -amyrin.

III. RESULT AND DISCUSSION

Chromatographic Studies of Bioactive extract

TLC study has shown the presence of different components present in dichloromethane extract of *Spinacia oleracea* and methanolic extract of *Desmodium triflorum* when the extracts were run in specific solvent system. Before reaching to most optimum solvent system a number of systems were employed.

Table No 1 TLC of dichloromethane extract of *Spinacia oleracea*

S No.	Fractions	Solvent systems	Detecting reagents	Color	No. of spots	R _f value of Spots
1	Dichloromethane	n-hexane: ethyl acetate (72:28)	Anisaldehyde sulphuric acid, heated at 100°C for 5 Min.	Green & yellow	4	0.25, 0.38, 0.50, 0.55

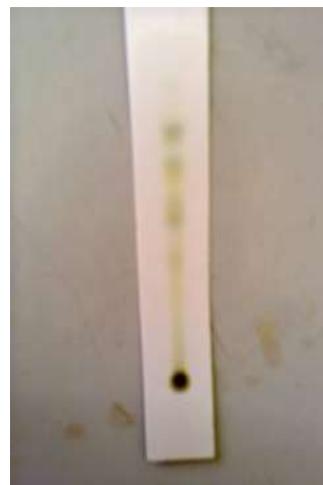
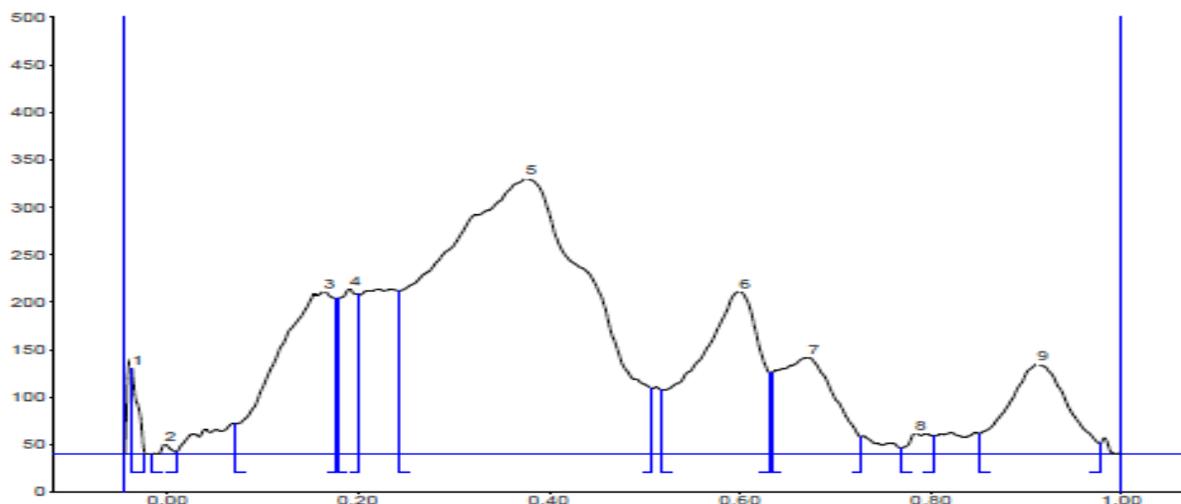


Figure No 1 TLC of dichloromethane extracts

HPTLC Fingerprint Analysis of bioactive Extract



Graph No 1 HPTLC Fingerprint profile of dichloromethane extract

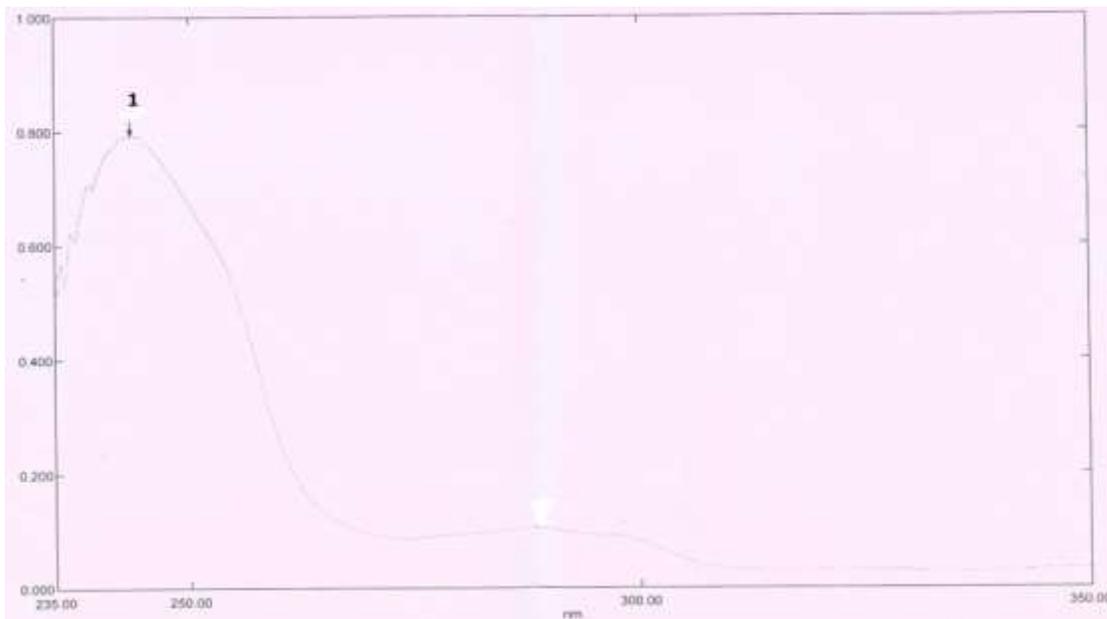
Table No 2 R_f values & relative percentage of compounds from dichloromethane extract

S No.	Volume	Peak	Start R _f values	Start Height	Max Height	Max %	% Area	Assigned substance
1	4 µL	1	-0.04	90.6	90.6	8.02	0.68	Sub 1
2	4 µL	2	-0.02	0.3	11.1	0.98	0.12	Sub 2
3	4 µL	3	0.07	31.8	171.9	15.21	12.20	Sub 3
4	4 µL	4	0.18	164.2	174.7	15.45	4.07	Sub 4
5	4 µL	5	0.24	172.0	290.7	25.71	54.84	Sub 5
6	4 µL	6	0.52	67.3	171.6	15.18	13.35	Sub 6
7	4 µL	7	0.63	77.5	102.2	9.08	7.17	Sub 7
8	4 µL	8	0.77	6.3	22.4	1.98	0.61	Sub 8
9	4 µL	9	0.82	21.8	95.3	8.43	6.96	Sub 9

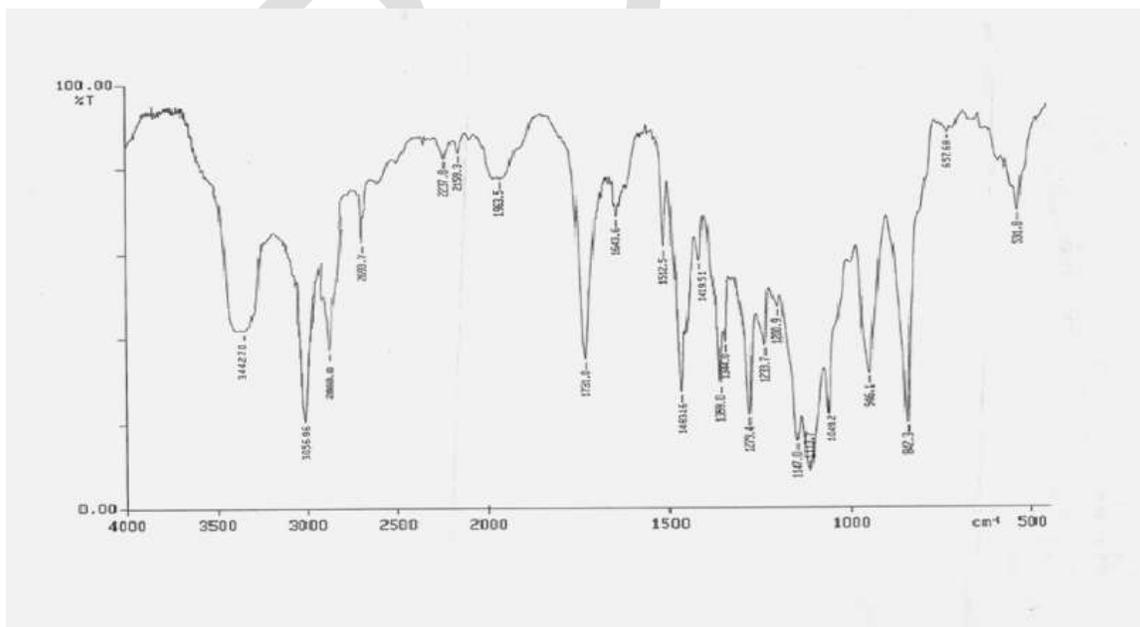
Bioactive dichloromethane extract was then tested chemically to know the presence of different chemical constituents. TLC and HPTLC studies were also performed to know the number of constituents present in extract and to establish finger print profile. In the present investigation, phytochemical screening showed the presence of steroids, terpenoids in dichloromethane extract. TLC findings were in agreement

with the data of qualitative chemical tests and the spots characteristic for steroids, terpenoids and flavonoids were observed.

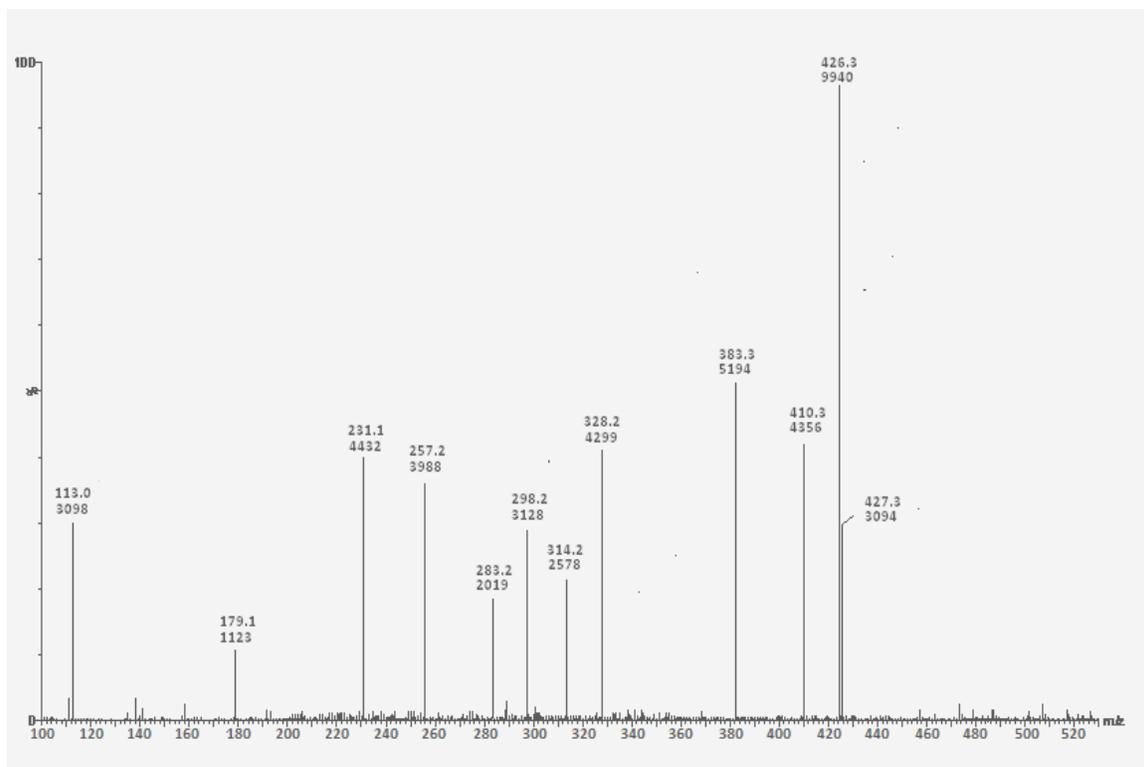
Characterization and Identification of compounds



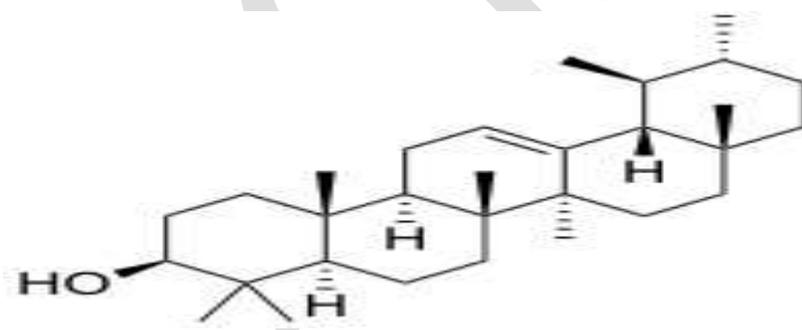
Graph No (2) UV Spectra of α -amyrin



Graph No (3) IR Spectra of α -amyrin



Graph No (6) Mass Spectra of α -amyirin



Structure of α -amyirin

IV. CONCLUSION

In this study, we extracted dichloromethane extract by soxhlet extractor. Then dichloromethane extract purified by TLC, isolated by HPTLC analysis and characterized with spectroscopy methods. The advantage of our method is the isolation and characterization of new compound from *S. oleracea* and it found as α -amyirin a class of triterpene chemical compound. We further studied it for their pharmacological activity.

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