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CHEMICAL CONSTITUENTS OF ETHANOL EXTRACT AND FREE RADICAL SCAVENGING ACTIVITY OF *VITEX TRIFOLIA* LINN

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ABSTRACT

An Iridoid *agnuside* has been isolated from ethanolic extract of *Vitex trifolia* leaves. The compound was characterized using spectroscopic methods including ^{1}H NMR, ^{13}C NMR, ESIMS and 2D-NMR (HSQC, HMBC, COSY) experiments and confirmed by comparison of NMR data with those from the literature. DPPH free radical scavenging activity for ethanol extract of leaves with EC_{50} value 0.478 mg/mL was found more effective than chloroform extract with EC_{50} value 0.602 mg/mL and n-hexane extract with EC_{50} value 0.794 mg/mL. NO free radical scavenging activity for ethanol extract of leaves with EC_{50} value 0.524 mg/mL was found more effective than chloroform extract with EC_{50} value 0.660 mg/mL and n-hexane extract with EC_{50} value 0.851 mg/mL.

Key words: Ethanol extract, Radical Scavenging activity, *Vitex trifolia* Linn.

INTRODUCTION

Vitex trifolia (Common name: three leaved chaste tree) belongs to *Verbenaceae* family. The plant parts like leaves and flower have a great therapeutic potential in Indian system of medicine[1]. *Vitex trifolia* Linn is a tropical shrub widespread in pacific Asian countries such as India, Srilanka, China, Phillipines, and French Poloynesia. Among plants of *Vitex* genus that includes approximately 200 species growing in tropical region. *Vitex trifolia* is employed to cure numerous illnesses. The stems of *Vitex trifolia* are used for the treatment of dysentery in Papua New Guinea[2]. In New Caledonia leaves are used to relieve fever, in Samoans to alleviate rheumatic pain and sprained joints when applied topically in New Caledonia. Abietane-type diterpenes, labdane type diterpenes, rotundifuran, dihydrosolidagenone and abietatriene-2b-ol have been previously isolated from the acetone extract of the fruits of *Vitex* trifolia[3]. Herein, we report the isolation of an iridoid agnuside from the leaves of plant. Ethanol, chloroform and n-hexane extracts of plant leaves were subjected to DPPH and NO free radical scavenging activity and EC_{50} value was calculated. Study aimed at isolation and characterization of plant's medicinally active constituents and ascertaining its free radical scavenging potential.

EXPERIMENTAL

Vitex trifolia leaves (3 Kg) were collected from Mandi (H.P.) and identified for its authentication in

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Department of Botany, HNB Garhwal University, Srinagar Gahwal (U.K.) and then air-dried in shade. After complete drying, extraction was carried out in ethanol and n-hexane for separate batches of leaves (2 Kg). Thereafter, ethanol extract obtained was partitioned with chloroform, concentrated in a rotary evaporator to give extracts of three types. The extracts of the plant were dissolved in ethanol (95%) and series of dilutions that permit the computation of EC_{50} values were prepared.

Crude ethanolic extract (50 g) was adsorbed on silica gel and subjected to column chromatographic separation using $CHCl_3$ followed by increasing proportions of MeOH in $CHCl_3$ (*v/v*) as an eluent. Collection of 150 mL of fractions, 200 mg (brown mass) crystallized from 100% MeOH solvent gave sample (1). The ^1H NMR spectrum in CD_3OD showed that the samples were not pure compounds, although, TLC showed single spot. For this reason, sample was subjected to further purification by RP-HPLC with water 590 series pumping system equipped with water R401 refractive index detector, a μ-Bondapack C18 column (300 × 7.8 mm i.d) and a U6k injector using MeOH-H_2O (9:1) as mobile phase (flow rate-2.0 mL/min) giving pure compound (1) t_R 9.3 min. NMR experiments, a Bruker DRX-600 NMR spectrometer using the UXNMR software package, NMR 600 MHz for ^1H NMR and 150 MHz for ^{13}C NMR, solvent CD_3OD, values relative to TMS reference. Chemical shift expressed in δ (parts per million) values, solvent peak δ_H 3.34 ppm and δ_c 49.0 ppm for CD3OD; coupling constant (J) are in Hz. 1D- and 2D-NMR experiments were carried out using conventional pulse sequence[4]. ESIMS was performed on a Finnigan LQ-Q Deca instrument (Thermoquest, San Jose, CA) equipped with Xcalibur software. Column chromatography was performed on silica gel (Merck) and TLC on Kieselgel 60G (Merck), spot on TLC was visualized by spraying with 20% H_2SO_4 and heating at 120°C for a few minutes.

DPPH radical scavenging activity

Plant extracts were tested for the scavenging effect on DPPH radical according to the method of Pan et al.[5] 0.2 mL of extract solution in ethanol (95%) at different concentrations (0.2, 0.5, 0.8 and 1.2 mg mL^{-1}) was added to 8 mL of 0.004% (w/v) stock solution of DPPH in ethanol (95%). The scavenging activity on the DPPH radical was determined by measuring the absorbance at 517 nm until the reaction reached the steady state, using a UV–visible spectrophotometer. As a positive control, synthetic antioxidant BHT was used. All determinations were performed in triplicate. The DPPH radical scavenging activity (S %) was calculated using the following equation:

$$S\ \% = [(A\ control - A\ sample)/A\ control] \times 100$$

where *A* control is the absorbance of the blank control (containing all reagents except the extract solution) and *A* sample is the absorbance of the test sample.

Calculation of EC_{50}

EC_{50} value is the effective concentration at which DPPH radicals were scavenged by 50% and was obtained by probit analysis. Graph probit values (Y-axis) against $\log_{10}$ concentration (X-axis) and draw a straight line of best line through plotted points, then use this line to estimate the $\log_{10}$ concentration associated with a probit of 5.

NO radical scavenging activity

Nitric oxide was generated from sodium nitroprusside and measured by Griess reaction[6]. Sodium nitroprusside (5 mM) in standard phosphate buffer solution was incubated with different concentration of each extract dissolved in phosphate buffer (0.025 m; pH 7.4) and the tubes are in a fixed light condition. Control set without the test compounds but the equivalent amount of buffer was conducted in an identical

manner. After 1 hr, 0.5 mL of incubated solution was removed and dilted with 0.5 mL of Griess reagent (1% sulphanilamide, 2%o-phosphoric acid and 0.1% napthylethylene diamine dihydrochloride). The absorbance of the chromophore formed during diazotization of nitrite with the sulphanilamide and its subsequent coupling with napthylethylene diamine dihydrochloride (NEDD) was read at 546 nm. Ascorbic acid was used as a standard. EC_{50} value denotes the concentration of tested extract required to quench 50% of the NO radicals released by sodium nitroprusside.

RESULTS AND DISCUSSION

Characterization of Compound (1)

It was crystallized from MeOH as brown crystalline solid, m.p. 224-226^{o}C and it gave positive vaniline hydrochloride test indicating iridoid. The ESIMS of compound at m/z 465 $[M-H]^{-}$, 467 $[M+H]^{+}$ showed molecular weight of the compound to be 464. HSQC correlations H2''-C2'', H6''-C6'', H5''-C5'', H3''-C3'', H3-C3, H7-C7, H1-C1, H1'-C1', H6-C6, H10-C10, H 9-C9, H5-C5. HMBC correlation were established for H2''-C6'', C4'', C=O; H3'-C5'', C1'', C4''; H3-C5, C1, C4; H7-C5, C10, C6, C8; H1'-C2', C5', C1; H9-C5, C6, C1, C7, C8. The structure elucidation of compound (1) as agnuside (Fig. 1) was performed with the help of UV, ^{1}HNMR, ^{13}C NMR, 2D experiments (HSQC, HMBC) and ESIMS analysis (Table 1 and Fig. 2-4) and confirmed as reported earlier[7-9].

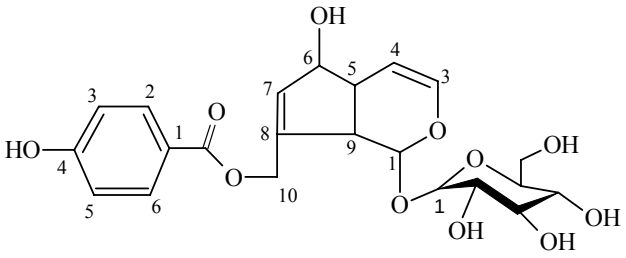

Fig. 1: Structure of compound (1)

Table 1: ^{13}C and ^{1}H NMR (in CD_3OD)^{a} spectroscopic data of Compound (1)

Position	δ_c	δ_H (J in Hz)^{b}
1	97.4	4.99, d (7.7)
2	-	-
3	141.2	6.35, dd (1.8, 6.1)
4	105.5	5.13, dd (4.3, 6.8)
5	46.1	2.70, m
6	82.4	4.48, brd (5.5)
7	131.8	5.81, s
8	143.0	-
9	48.6	2.98, d (7.3)
10	63.0	5.11, d (15.3)
		4.92, d (15.3)

Cont…

Position	δ_c	δ_H (J in Hz)[b]
C=O	168.2	-
1’	99.3	4.66, d (7.5)
2’	74.9	3.21, dd (7.5, 8.5)
3’	77.4	3.38, t (8.5)
4’	71.2	3.31, t (8.5)
5’	78.0	3.26, m
6’	62.4	3.64, dd (12.0, 4.5)
		3.85, dd (12.0, 3.0)
1’’	121.9	-
2’’ and 6’’	102.4	7.94, d (8.5)
3’’ and 5’’	115.5	6.86, d (8.5)
6’’		

a Assignments confirmed by 2D COSY, HSQC, and HMBC experiments.
b ^{1}H-^{1}H coupling constants (Hz) were measured from the COSY spectra in Hz

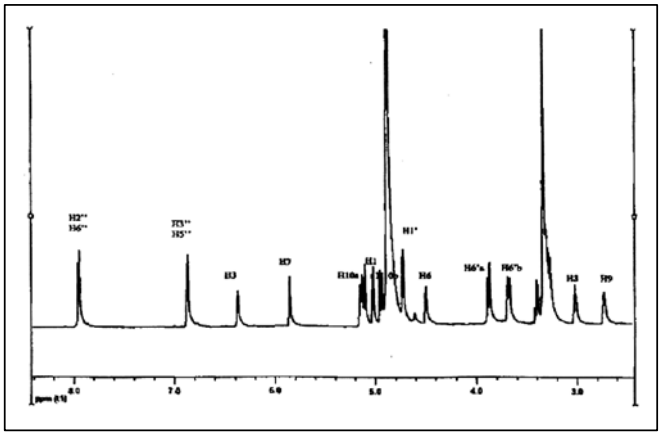

Fig. 2: ^{1}H NMR spectrum of Compound (1)

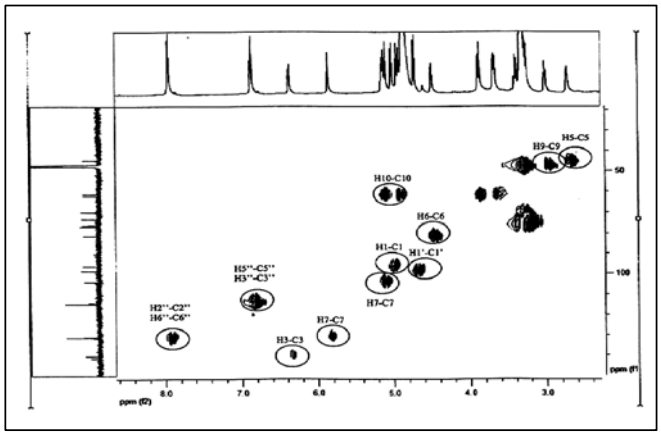

Fig. 3: HSQC spectrum of Compound (1).

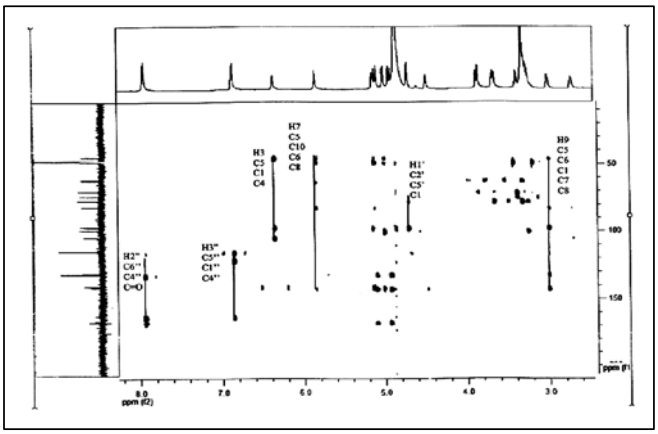

Fig. 4: HMBC spectrum of Compound (1)

DPPH radical scavenging activity

DPPH is a stable free radical and possess a characteristic absorbance at 517 nm, which decreases significantly on exposure to radical scavengers by providing hydrogen atom or electron to become a stable diamagnetic molecule[10]. The use of stable DPPH radical has the advantage of being unaffected by side reaction, such as enzyme inhibition and metal chelation[11].

DPPH radical scavenging activity of extracts increased in a concentration dependent manner (Fig. 5). The ethanol extracts showed the highest DPPH radical scavenging activity, while the weakest scavengers were the n-hexane extracts. On the other hand, the chloroform extracts exhibited stronger DPPH radical scavenging ability than the corresponding n-hexane extract. EC_{50} value, the effective concentration at which DPPH radicals scavenged by 50% were obtained by probit analysis found 0.478 mg/mL, 0.602 mg/mL and 0.794 mg/mL for ethanol extract, chloroform extract and n-hexane extract, respectively.

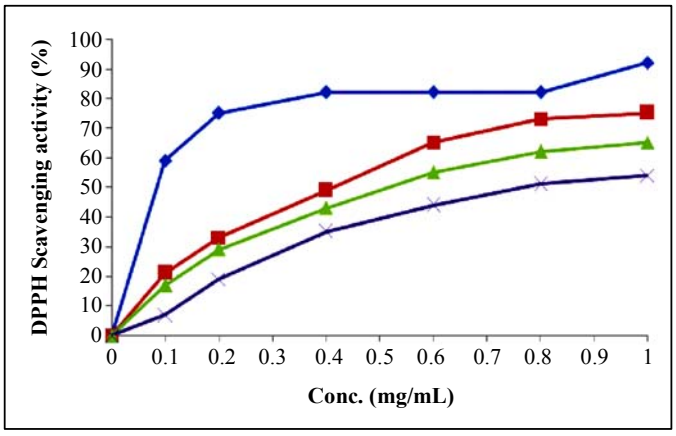

Fig. 5: DPPH scavenging activity % of ethanolic, chloroform and n-hexane extract

NO radical scavenging activity

Nitric oxide is a free radical produced in mammalian cell, involved in the regulation of various physiological processes. However the excess production of NO is associated with several diseases[12]. Nitric oxide is very unstable species under aerobic condition. It reacts with oxygen to produce stable product nitrate and nitrite through intermediate NO_2, N_2O_4 and N_4O_3. High concentration of nitric oxide can be toxic and inhibition of the over production of nitric oxide is an important goal[13]. In the present study, the nitrate

produced by the incubation of solution of sodium nitroprusside in standard phosphate buffer at 25°C was reduced by the ethanol, chloroform and hexane extracts of *Vitex trifolia* leaves exhibited NO-scavenging dose response curve (Fig. 6). The corresponding EC_{50} value for ethanol extract showed better NO scavenging activity than chloroform and n-hexane extract. Our finding suggests that ethanol extract of *Vitex trifolia* inhibited nitric oxide production. Among these extracts from plant leaves the effectiveness in antioxidant was in the descending order ethanol > chloroform > n-hexane extract. EC_{50} value, the effective concentration at which DPPH radicals scavenged by 50% were obtained by probit analysis found 0.524 mg/mL, 0.660 mg/mL and 0.851 mg/mL for ethanol extract, chloroform extract and n-hexane extract, respectively.

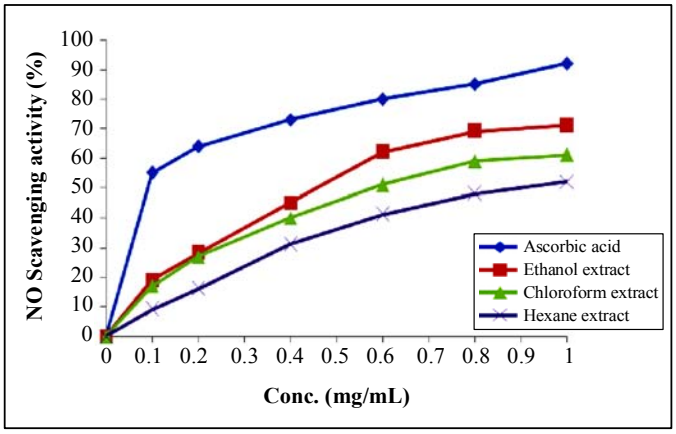

Fig. 6: NO scavenging activity % of ethanolic, chloroform and n-hexane extract

CONCLUSION

Present study confirms the presence of an iridoid agnuside in ethanolic extract of *Vitex trifolia* leaves. Ethanol extract of leaves have considerable free radical scavenging activity, which is higher than chloroform and n-hexane extract against DPPH and NO free radicals which validates *Vitex trifolia* leaves as a potent free radical scavenger.

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