



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

J. S. JANGWAN, R. P. AQUINO<sup>a</sup>, T. MENCHERINI<sup>a</sup>, P. PICERNO<sup>a</sup> and R. SINGH<sup>\*</sup>

Department of Chemistry, H. N. B. Garhwal Central University SRT Campus, Badshahi Thaul Tehri Garhwal, GARHWAL (U.K.) INDIA

<sup>a</sup>Department of Pharmaceutical and Biomedical Sciences, University of Salerno, ITALY

(Received : 09.01.2015; Revised : 20.01.2015; Accepted : 23.01.2015)

### ABSTRACT

An Iridoid *agnuside* has been isolated from ethanolic extract of *Vitex trifolia* leaves. The compound was characterized using spectroscopic methods including <sup>1</sup>H NMR, <sup>13</sup>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<sub>50</sub> value 0.478 mg/mL was found more effective than chloroform extract with EC<sub>50</sub> value 0.602 mg/mL and n-hexane extract with EC<sub>50</sub> value 0.794 mg/mL. NO free radical scavenging activity for ethanol extract of leaves with EC<sub>50</sub> value 0.524 mg/mL was found more effective than chloroform extract with EC<sub>50</sub> value 0.660 mg/mL and n-hexane extract with EC<sub>50</sub> 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<sup>1</sup>. *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<sup>2</sup>. 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*<sup>3</sup>. 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<sub>50</sub> 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

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<sub>50</sub> values were prepared.

Crude ethanolic extract (50 g) was adsorbed on silica gel and subjected to column chromatographic separation using CHCl<sub>3</sub> followed by increasing proportions of MeOH in CHCl<sub>3</sub> (v/v) as an eluent. Collection of 150 mL of fractions, 200 mg (brown mass) crystallized from 100% MeOH solvent gave sample (1). The <sup>1</sup>H NMR spectrum in CD<sub>3</sub>OD 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-Bondapak C18 column (300 × 7.8 mm i.d) and a U6k injector using MeOH-H<sub>2</sub>O (9:1) as mobile phase (flow rate-2.0 mL/min) giving pure compound (1) t<sub>R</sub> 9.3 min. NMR experiments, a Bruker DRX-600 NMR spectrometer using the Uxnmr software package, NMR 600 MHz for <sup>1</sup>H NMR and 150 MHz for <sup>13</sup>C NMR, solvent CD<sub>3</sub>OD, values relative to TMS reference. Chemical shift expressed in δ (parts per million) values, solvent peak δ<sub>H</sub> 3.34 ppm and δ<sub>C</sub> 49.0 ppm for CD<sub>3</sub>OD; coupling constant (J) are in Hz. 1D- and 2D-NMR experiments were carried out using conventional pulse sequence<sup>4</sup>. 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<sub>2</sub>SO<sub>4</sub> 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.<sup>5</sup> 0.2 mL of extract solution in ethanol (95%) at different concentrations (0.2, 0.5, 0.8 and 1.2 mg mL<sup>-1</sup>) 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:

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

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<sub>50</sub>

EC<sub>50</sub> 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<sub>10</sub> concentration (X-axis) and draw a straight line of best line through plotted points, then use this line to estimate the log<sub>10</sub> concentration associated with a probit of 5.

### NO radical scavenging activity

Nitric oxide was generated from sodium nitroprusside and measured by Griess reaction<sup>6</sup>. 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 diluted with 0.5 mL of Griess reagent (1% sulphanilamide, 2% o-phosphoric acid and 0.1% naphthylethylene diamine dihydrochloride). The absorbance of the chromophore formed during diazotization of nitrite with the sulphanilamide and its subsequent coupling with naphthylethylene 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°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  $H_{2''}-C_{2''}$ ,  $H_{6''}-C_{6''}$ ,  $H_{5''}-C_{5''}$ ,  $H_{3''}-C_{3''}$ ,  $H_3-C_3$ ,  $H_7-C_7$ ,  $H_1-C_1$ ,  $H_{1'}-C_{1'}$ ,  $H_6-C_6$ ,  $H_{10}-C_{10}$ ,  $H_9-C_9$ ,  $H_5-C_5$ . HMBC correlation were established for  $H_{2''}-C_{6''}$ ,  $C_{4''}$ ,  $C=O$ ;  $H_{3'}-C_{5''}$ ,  $C_{1''}$ ,  $C_{4''}$ ;  $H_3-C_5$ ,  $C_1$ ,  $C_4$ ;  $H_7-C_5$ ,  $C_{10}$ ,  $C_6$ ,  $C_8$ ;  $H_{1'}-C_{2'}$ ,  $C_{5'}$ ,  $C_1$ ;  $H_9-C_5$ ,  $C_6$ ,  $C_1$ ,  $C_7$ ,  $C_8$ . The structure elucidation of compound (1) as agnuside (Fig. 1) was performed with the help of UV,  $^1H$ NMR,  $^{13}C$  NMR, 2D experiments (HSQC, HMBC) and ESIMS analysis (Table 1 and Fig. 2-4) and confirmed as reported earlier<sup>7-9</sup>.

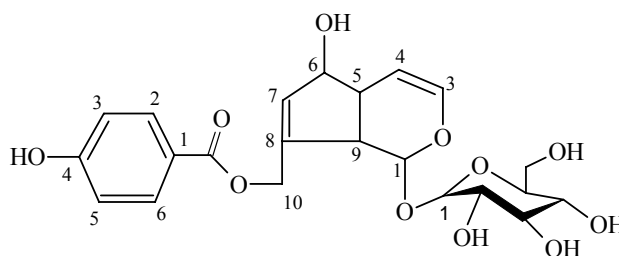


Fig. 1: Structure of compound (1)

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

Position	$\delta_c$	$\delta_H$ (J in Hz) <sup>b</sup>
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)

Position	$\delta_c$	$\delta_H$ (J in Hz) <sup>b</sup>
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* <sup>1</sup>H-<sup>1</sup>H coupling constants (Hz) were measured from the COSY spectra in Hz

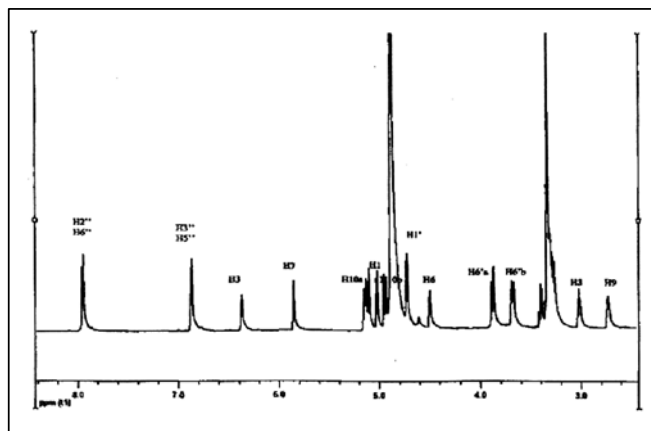


Fig. 2: <sup>1</sup>H NMR spectrum of Compound (1)

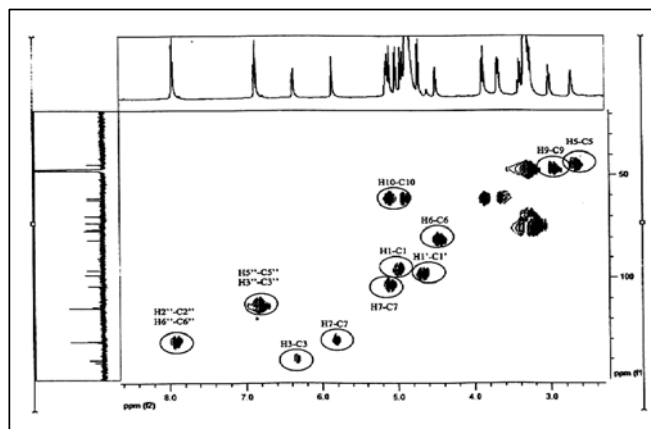


Fig. 3: HSQC spectrum of Compound (1).

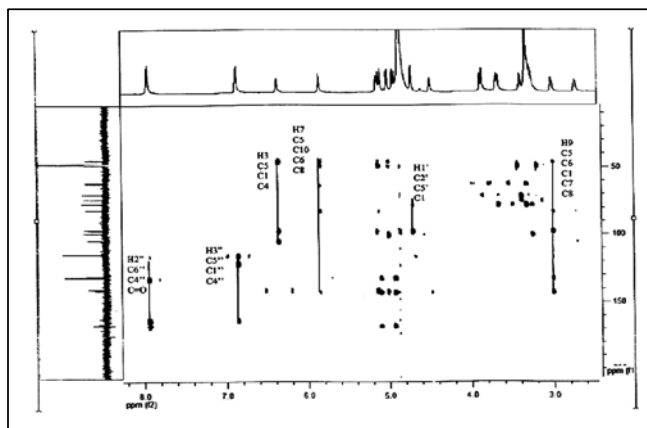


Fig. 4: HMBC spectrum of Compound (1)

### 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<sup>10</sup>. The use of stable DPPH radical has the advantage of being unaffected by side reaction, such as enzyme inhibition and metal chelation<sup>11</sup>.

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<sub>50</sub> 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.

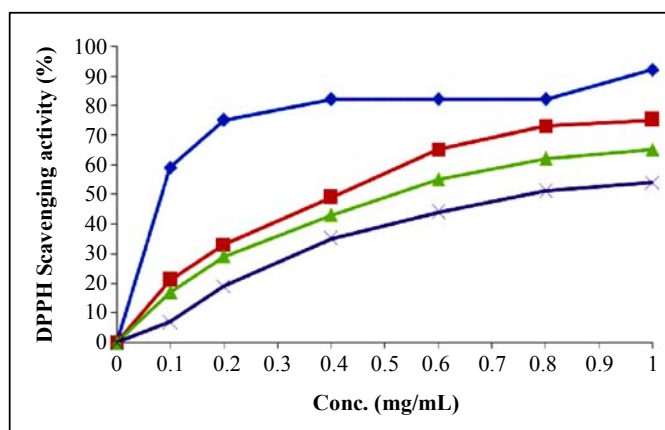
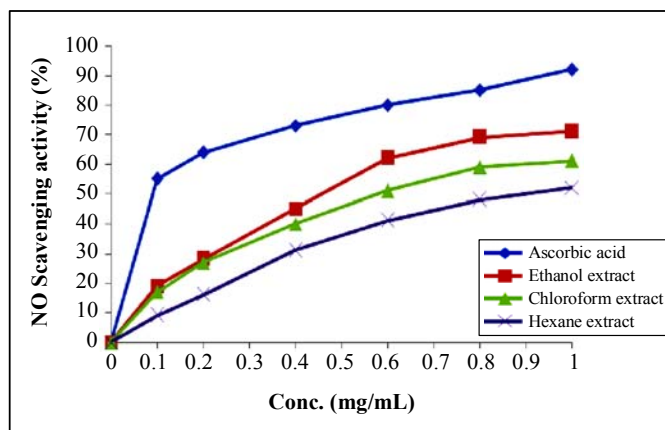


Fig. 5: DPPH scavenging activity % of ethanolic, chloroform and n-hexane extract

### 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<sup>12</sup>. Nitric oxide is very unstable species under aerobic condition. It reacts with oxygen to produce stable product nitrate and nitrite through intermediate NO<sub>2</sub>, N<sub>2</sub>O<sub>4</sub> and N<sub>4</sub>O<sub>3</sub>. High concentration of nitric oxide can be toxic and inhibition of the over production of nitric oxide is an important goal<sup>13</sup>. 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<sub>50</sub> 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<sub>50</sub> 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.

## ACKNOWLEDGEMENT

The authors thank Mr. Naveen Kumar, Research Scholar, Department of Chemistry, HNB Garhwal University, SRT Campus, Badshashi Thaul for their assistance in this work.

## REFERENCES

1. K. R. Kirtikar, B. D. Basu, Indian Med Plants, 1935 (1991).
2. D. K. Holdsworth, Medicinal Plants of Papua New Guinea (South Pacific Commission Technical Paper, Noumea, New Caledonia, **65**, 175 (1977).
3. O. Masateru, S. Hiromi, I. Yasuyuki, M. Koichi and N. Toshihiro, Diterpenoids from Fruits of *Vitex trifolia*, Phytochemistry, **55**, 873-877 (2000).
4. D. G. Davis and A. Bax, Assignment of Complex H NMR Spectra via Two-Dimensional Homonuclear Hartmann-Hahn Spectroscopy, J. Amer. Chem. Soc., **107**, 2820-282 (1985).
5. Y. Pan, K. Wang, S. Huang, H. Wang, X. Mu, C. He, X. Ji, J. Zhang and F. Huang, Food Chem., **106**, 1264-1270 (2008).

6. L. Marcocci, J. J Maguire, M. T. Droy-lefaix and L. Packer, The Nitric Oxide Scavenging Property of *Ginkgo Biloba* Extracts EGB 761, *Biochem. Biophys. Res. Comm.*, **201**, 748-755 (1994).
7. C. K. Sehgal, S. C. Taneja, K. L. Dhar and C. K. Atal, *Phytochemistry*, **21**, 363-366 (1982).
8. S. Suksamrarn, S. Kumarchoen and A. Subksamrarn, *Planta Med.*, **65**, 392-393 (1999).
9. C. K. Sehgal, S. C. Taneja, K. L. Dhar and C. K. Atal, *Phytochemistry*, **22**, 1036-1038 (1983).
10. S. Dash, L. K. Nath, S. Bhise and N. Bhuyan, Antioxidant and Antimicrobial Activities of *Heracleum Nepalense* Don Root, *Trop. J. Pharm. Res.*, **4**, 341-347 (2005).
11. M. Waltasinghe and F. Shahidi, Antioxidant and Free Radical Scavenging Properties of Ethanolic Extracts of Defatted Borage (*Berapa officinalis L.*) Seeds, *Food Chem.*, **63**, 399-414 (1999).
12. G. Ray and S. A. Husain, Oxidants, Antioxidants and Carcinogenesis, *Indian J. Exp. Biol.*, **40**, 1213-1232 (2002).
13. B. S. Wang, J. H. Chen, Y. C. Liang and P. D. Duh, Effects of Welsh Onion on Oxidation of Low-Density Lipoprotein and Nitric Oxide Production in Macrophage Cell Line Raw 264.7, *Food Chem.*, **91**, 147-155 (2005).