CHEMICAL CONSTITUENTS FROM *VITEX NEGUNDO* (LINN.) OF NEPALESE ORIGIN

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Abstract: *Vitex negundo Linn.,* belonging to family Verbenaceae, is an aromatic shrub distributed in lower part of Himalayan region of Nepal. In the ayurvedic system of medicine, it is used to control pain, inflammation and other related diseases. It contains many polyphenolic compounds, terpenoids, glycosidic iridoids and alkaloids. Bioactivity guided phytochemical investigation of methanolic extract of leaves of *Vitex negundo* resulted in the isolation of eight compounds under silicagel VLC, CC and preparative TLC. They were identified as negundoside, agnuside, vitegnoside, 7,8 dimethyl herbacetin 3-rhamnoside, 5,3'-dihydroxy—7,8,4'-trimethoxy flavanone, 5-hydroxy-3,6,7,3',4'-pentamethoxy flavone, 5,7 dihydroxy- 6,4' dimethoxy flavonone, and 5 hydroxy-7,4' dimethoxy flavone. The structures of pure compounds were elucidated by MP, R_f values, Co-TLC, Colour reactions (Cerric sulphate spray), mild acid hydrolysis and spectroscopic methods (Mass, UV, IR, ^{1}H, ^{13}C and 2 D NMR). All the isolated compounds were evaluated for their antimicrobial activities. They were found to have significant antibiotic activity against *Bacillus subtilis, Staphylococcus aureus, Micrococcus pyogenes, Pseudomonas aeruginosa and E. coli*. The compounds vitegnoside, and 7, 8 dimethyl herbacetin 3-rhamnoside have MIC 6.25mg/ml.

Keywords*: Vitex negundo;* Verbenaceae; Chemical constituents; Bioassay; Analysis.

INTRODUCTION

Plants and plant derived agents have long history as source of potential chemotherapeutic agents in Ayurvedic and Unani system of medicine. *Vitex negundo* Linn., belonging to family Verbenaceae (which comprises 75 genera and nearly 2500 species), commonly known as simali in Nepali, and also called Five leaved chaste tree (Eng), Nirgandi (Hindi), Nirgundi (Sanskrit), is a deciduous shrub, occur in tropical to temperate regions (up to 2200 m from east to west) grows gregariously in wastelands and is also widely used as a hedge-plant. It is an erect (2–5 m in ht), slender tree with quadrangular branchlets. The leaves have five leaflets in a palmately arrangement, which are lanceolate, 4–10 cm long, hairy beneath and pointed at both ends. The bluish purple flowers are numerous. The fruit is succulent, black and rounded when ripe having about 4 mm in diameter. *Vitex negundo* (Linn.) is one of the common plants used in traditional medicine and reported to have variety of pharmacological activities.[1, 2] Although, all parts of *V. negundo* are used as medicine in the indigenous system of medicine, the leaves are the most potent for medicinal use. The decoction of leaves is used for treatment of inflammation, eye-disease, toothache, leucoderma, enlargement of the spleen, ulcers, cancers, catarrhal fever, rheumatoid arthritis, gonorrhea, sinuses, scrofulous sores, bronchitis and as tonics, vermifuge, lactagogue, emmenagogue, antibacterial, antipyretic, antihistaminic, analgesic, insecticidal, ovicidal, feeding deterrence, growth inhibition and morphogenetic agents[3-12]. Antigenotoxic, antihistamine, CNS depressant activity and anti-fertility effects were reported from the leaves of *V. negundo*[13]. Previous phytochemical studies on *V. negundo* have afforded several types of compounds, such as volatile oils[14-17], lignans[18-19], flavonoids[20-25], iridoids,[26-28] terpenes (triterpenes, diterpenes, sesquiterpenes),[8, 29-31] and steroids[32]. Even though, it constitutes a rare medicinal shrub with high biological value, very little survey research work has been done on this species in our country. Taking into account of these ethnobotanical importances, the chemical constituents of the leaves of *V. negundo* have been investigated.

MATERIALS AND METHODS

Plant material

The leaves of *V. negundo* Linn. were collected in November 2005 from Parbat district and the specimen was identified and authenticated voucher specimen no. NP-125 was deposited at the National Herbarium and Plant Laboratories, Division of Department of Plant Resources, Kathmandu, Nepal.

Analysis of Various Parameters

Different physicochemical parameters like crude protein, carbohydrate, fat, ash the fiber content and others of the air dried *V. negundo* was determined by AOAC methods [33, 34].

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General experimental procedures

Melting points were determined on Electric Sun-Vic melting point apparatus and are uncorrected. The IR spectra (In KBr) were recorded on a 460 Shimadzu spectrometer. The UV spectra were recorded on a Hitachi UV-3200 spectrophotometer (ëmax in nm). Silica gel (Fine G & H as well as 60-120 mesh) were used in VLC and Column chromatography respectively. TLC plates and pre-coated silica gel G-25-UV254 plates were used to check the purity and isolation of the compounds. Visualization of the TLC plates was carried out under UV at 254 and 366 nm and by spraying with cerric sulphate reagent (with heating). The ^{1}H- and ^{13}C-NMR, and other 2D spectra were recorded on Bruker spectrometers operating at 400MHz for ^{1}H and 100MHz for ^{13}C-NMR respectively ä values in ppm downfield from TMS. EI-MS measurements were carried out on micro mass QUATTRO II triple quadrupole mass spectrometer.

Bioassay Guided Extraction, Fractionation and Isolation

The shade dried powdered leaves (1.5 kg) of *V. negundo* were extracted three times, 6 days each, with methanol by percolation. The combined methanolic extract was evaporated in vaccuo affording (303 g) extract. The resulting methanolic extract residue was suspended in water and extracted successively with petroleum ether (63 g), $CHCl_3$ (65 g) ethyl acetate (81g) and methanol (93 g) soluble fractions. This extraction and fractionation were repeated three times to obtain triple amount of extracts and fractions. In bioassay, five different bacteria viz. *Bacillus subtilis, Staphylococcus aureus, Micrococcus pyogenes, Pseudomonas aeruginosa and E. coli* were used as test organisms. The bioassay tests were performed on nutrient agar medium by cup well method[35,36]. The solvent used to prepare the solution of the extract and the fractions were used as a control. Each crude extract and different fractions obtained from the VLC and CC were screened for bioactivity by using the concentration of 50 mg/ml.

On bioassay, the activity was found localized in methanolic fraction. The methanol soluble fraction was vacuum liquid chromatographed (over TLC level fine silica gel) followed by column chromatography (over 60-120 mesh silica gel), eluted with a gradient of Pet ether/$CHCl_3$/EtOAC/methanol (95:05) to methanol/water (95:05 to 95) to afford six fractions (F-1 to F-6). Analysis of each fraction were done by using TLC methods[37]. Re-Column chromatography of fraction F-1 using $CHCl_3$ / MeOH (94:6) afforded compound 1 (15 mg). Purification of F-2 and prep TLC using EtOAC/MeOH (93:7) afforded compound 2 (11 mg) and with EtOAC/MeOH (90:10) gave compound 3 (11 mg). Successive purification of F-3 by repeated column chromatography, eluted with a gradient of MeOH/ H_2O (35:65), afforded F4 (13 mg) and F5 (12gm). Column chromatography and purification of F-4, using $CHCl_3$/MeOH (75:25), afforded 6 (11 mg) and 7(14gm). Repeated column chromatography of fraction F-5, eluted with EtOAc/Acetone/H_2O (70:24:6), yielded compound 8 (13 mg). All the eight compounds were evaluated for antibacterial activity.

RESULTS AND DISCUSSION

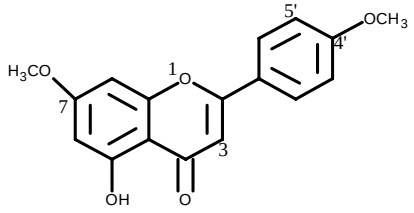
The standardization parameters were given in Table 1. The ash and moisture values were found high. Similarly, the water extract, crude fiber and crude protein were also found much more than expected. The higher fat content showed the plant has more oily substances. Bitterness and presence of alkaloids showed that it has enormous importance.

Table 1: Analysis results of *V. negundo*

SN	Parameters	Quantitative (%)
1	Foreign Matter	1.9-2.0
2	Ash	7.5-8.5
3	Acid insoluble ash	0.3-0.6
4	Ethanol soluble extractive	8.2-10.4
5	Water soluble extractive	16.50-20.55
6	Moisture	15.00-18.70
7	Crude protein	12.22-15.23
8	Crude fiber	25.50-30.50
9	Fat	5.00-9.00
10	Carbohydrate	7.5-10.57
11	Alkaloids	0.5
12	Bitter	5

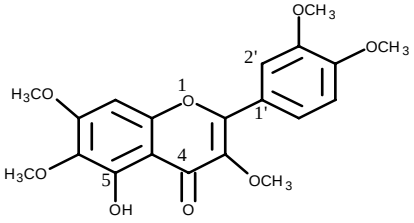
As part of a systematic examination of bioactive constituents, bioactivity directed extraction and isolation of various compounds were done. They were identified as:

5 hydroxy-7, 4' dimethoxy flavone(F1): The compound F1 mp 168-172ºC, R_f = 0.61(1:4, MeOH:$CHCl_3$), $M^{+\cdot}$ at m/z 298 corresponding to the molecular formula $C_{17}H_{14}O_5$ was a yellowish crystalline needle. IR absorption at 3320, 1670, 1625, cm^{-1} was indicative of the presence of aromatic –OH and Carbonyl groups respectively. The ^{1}H NMR spectrum pattern was consistent with a 7 & 4' oxygenated flavone. The singlet 3H at 3.89 and 3.90 were assigned for two methoxy groups attached to C-7 and C-4'. The singlet at ä12.80 was assigned as C-3 -OH group. The doublets at ä 6.35 and 6.47(J=1.6Hz) were assigned as the protons of C-6 and C-8. The other doublets at ä 7.04 and 7.03(J=2Hz) were due to the protons of C-3' & C-5'. The singlet at ä 6.57 was olefin hydrogen at C-2. The other doublet protons at ä 7.63 and 7.84(J=2Hz) were C-2' & C-6' protons. The ^{13}C-NMR values ranging from 55 to 183 indicated the presence of 17 carbons. The value at ä183 was assigned as C=O group. Thus the structure was confirmed as 5 hydroxy-7, 4' dimethoxy flavone [20,23,38-41].



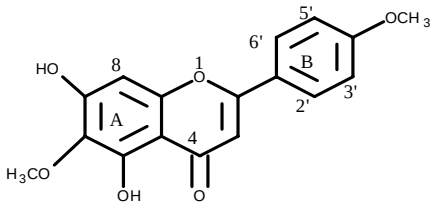
5-hydroxy-7,4'dimethoxy flavone

5-hydroxy-3,6,7,3',4'-pentamethoxy flavone(F2): It was a yellow crystalline material, could be inferred to be a 5-hydroxyflavanone on the basis of the virtually identical UV and ^{1}H NMR spectra. The ^{1}H NMR spectra indicated that each ring A & B contained two methoxy groups with one additional methoxy group at C-3. The R_f value, colour test as well as comparison with authentic literature data confirmed the compound be 5-hydroxy-3,6,7,3',4' pentamethoxyflavone.[20, 21,23,38-41]



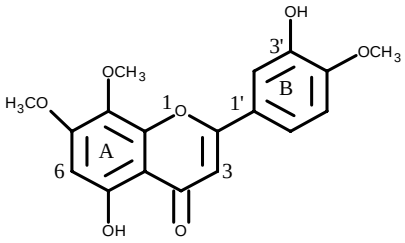
5-hydroxy-3,6,7,3',4'-Pentamethoxy flavone

5,7 dihydroxy- 6,4' dimethoxy flavanone(F3): The compound F3 melting point 179ºC, R_f value 0.6(2:1, Pet ether:EtOAC), $M^{+\cdot}$ at m/z of corresponding to molecular formula was a yellowish needle shaped crystal. It gave positive test for flavanone. IR absorption at 3390, 1650, 3050 cm^{-1}were indicative of the presence of –OH,-C=O, and =C-H groups stretching respectively. The ^{1}H-NMR spectrum was consistent with 6, 7, 4'-oxygenated flavonol. The singlets at 3.97 and 3.90 were assigned for two methoxy groups attached to C-6 and C-4'. The doublets at 8.15(J=8.7Hz) and 7.20(J=8.9Hz) were due to the protons of C-2', C-6' and C-3', C-5' respectively. The singlets at 7.0 and 6.70 were protons of C-3 and C-8. The singlet value at 13.14 was due to 5 and 7 –OH groups. ^{13}C-NMR values ranging from 55 to 183 indicated the presence of carbons. The carbons at 55 and 61 were assigned for two methoxy carbons. The value at 183 was assigned as C=O group. The two alkene carbons at 104 and 163 were C-2 and C-3. The B ring carbons were appeared at 123(C-1'), 128(C-2', C-6'), 115(C-3', C-5') and 162(C-4'). Similarly, the A ring carbons were assigned at 104(C-10), 132(C-6), 152(C-9), 153(C-5), 158(C-7), and 194(C-8). Thus the compound was identified as pectolinarigenin or 5, 7 dihydroxy-6,4' dimethoxy flavanone [21-23,38-41].



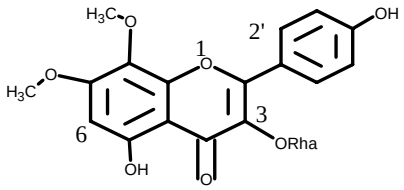
5,7-dihydroxy-6,4'dimethoxy flavanone

5,3'-dihydroxy—7,8,4'-trimethoxy flavanone(F4): It was a light yellow amorphous material exhibited a UV maximum at 289nm which underwent a bathochromic shift of 21nm with $AlCl_3$. No significant change on further addition of HCl indicated the presence of a 5-OH group in flavonone nucleus. In the ^{1}H spectrum, the one proton signal at 11.94 exchangeable with D_2O confirmed a chelated OH group at C-5. The double doublets at ä5.35 (J=12, 4Hz), 3.09(J=16,12Hz) and 2.84(J=16,4Hz) was in conformity with the normal flavanone skeleton. Three methoxy signals were also observed at ä3.77, 3.88 and 3.89 while an exchangeable signal at ä5.72 signified a phenolic group. In the aromatic region, a singlet at ä6.10 indicates the ring A to be trisubstituted, while the three proton multiplet at ä6.91-7.07 could be described to ring B which must be disubstituted [22-23,38-41].



5, 3' dihydroxy-7, 8, 4'-trimethoxy flavanone

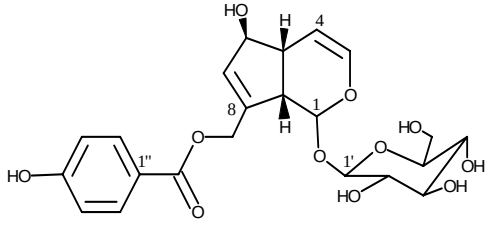
7,8 dimethyl herbacetin 3-rhamnoside (F5): It has mp 189ºC with molecular formula $C_{23}H_{24}O_{11}$ of $M^{+\cdot}$ 476. IR showed a strong absorption band at 1650cm^{-1} for chelated carbonyl and for C-O stretching between 1090 71050cm^{-1}. The bonded phenol gave rise to absorption at 3380cm^{-1} and the band at 1590, 1545, 1480, 800 &740cm^{-1} were assigned as aromatic systems. In UV, two bands seen in MeOH solution, band II 273nm and band I 333nm. The hypsochromic shift of band I showed presence of sugar residue. The glycoside formed a crystalline penta acetate mp 97ºC whose NMR spectrum exhibited a sharp doublet at ä0.8 (J=6Hz) which is a distinguishing feature of rhamnosyl C**H**$_3$. Also, the rhamnosyl C-1" H coupled with the C-2" H (J=2Hz) and appeared at ä 5.5 while other four sugar protons appeared in the region of ä 4.70-5.40. The five CH_3CO resonated as singlets at ä 1.80, 1.90, 2.10, 2.15, & 2.35 and two sharp CH_3O singlets appeared at ä 3.85, &3.65. The resonance for one aromatic H occurred as a singlet at 6.80 and a pair of ortho coupled doublets for four H of an A_2B_2 system could be seen at ä 7.15 & 7.70(J=9.0Hz) and indicated the Para substitution of ring B and only one ring A H. The NMR spectra clearly indicated mono glycosidation in the molecule. Acid hydrolysis of original glycoside yielded an aglycone (mp 262ºC) and rhamnose. Above mentioned data showed the compound to be 7, 8 dimethyl herbacetin 3-rhamnoside[22,24,25,38-41].



7, 8 dimethyl herbacetin 3-rhamnoside

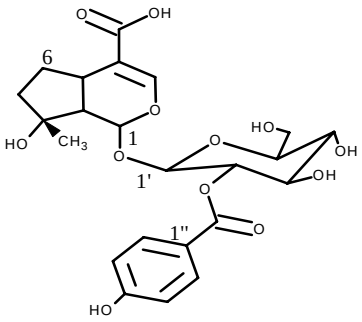
Agnuside (F6): Compound F6 was a light yellow crystalline substance having melting point 149-151ºC with R_f value 0.33(4:1, $CHCl_3$:MeOH). It gave positive molisch test indicating glycoside linkage which undergoes acid hydrolysis. The

mass spectrum showed M-H at m/z at 465 corresponding to the molecular formula $C_{22}H_{26}O_{11}$. The IR spectrum showed the presence of –OH group (3400cm^{-1}), -C=O (1700cm^{-1}) and C=C (1610, 1660). The values at 1160 and 1100cm-1 were assigned as C-O-C and secondary C-OH respectively. In ^{1}H-NMR the multiplets at ä 2.60 and 2.90 were H-5 and H-9. The similar multiplet from ä 3.00-4.00 were assigned as glucose H-2', 3', 4', 5'. The double doublet at ä 5.10 and ä 6.30 (J=6.6), ä 4.2(J=6.6, 1.8 Hz) were respectively assigned as H-4 and H-3. Again the broad singlet at ä 5.80 was olefin H-7. The multiplet at ä 4.40 was H-6 attached to –OH group. The multiplet at ä 4.90-5.00 was H-10. Similar multiplet at ä 4.80 was H-1. The doublet at ä 6.75 and ä 7.80(J=8.4Hz) were H-3'', H-5'' and H-2'', H-6'' aromatic Meta related protons. The ä value at 4.60 was assigned as 6-OH. The ^{13}C-NMR values ranging from ä46-165 indicated the presence of 22 carbons. The ä values at 142, 105 and 134, 144 were C=C. The ä values 64, 65, 72, 73, 78, were the glucose carbons. The aromatic carbons were appeared at ä163, 116,134, and 98. The other carbons at ä 46, 48 and 83 were 5, 9 and 6-C. The above mentioned spectral evidences suggested the compound to be agnuside[26,27,28].



Agnuside

Negundoside (F7): Compound F7 was a crystalline compound melted at 147-151ºC. The compound was found to be a single spotted in TLC with R_f value 0.25(1:9, MeOH: EtOAC). It gives positive wiffering test and molisch test indicating the compound to be an iridoid glycoside. The mass spectrum showed M^+: at m/z 496 corresponding to the molecular formula $C_{23}H_{28}O_{12}$. The UV in MeOH showed ë$_{max}$ at 260 nm. The IR spectrum showed the presence of –OH group (3400) –C=O (1700) and C=C (1640 & 1610cm^{-1}). In ^{1}HNMR the doublet at ä5.50 (J=3) which is due to strong deshielding from either side is H-1 proton. The singlet proton at ä 7.10 was alkene H-3. The multiplet at ä 2.5 and 2.2 were H-5 and H-8. The singlet at ä 1.20 was C**H**$_3$-10. The double doublet at ä 6.90(J=2, 7 Hz) were assigned as H-3'' and H-5'' Meta coupled aromatic protons. Similar doublet at 7.80 (J= 2, 7Hz) were accounted for H-2'' and H-6''. Another doublet at ä 4.70 and 5.00(J=7Hz) could be H-2' and H-1' due to nearby -OR group. Thus the assignment confirmed the presence of aromatic ring and glycosidic group.^{13}C NMR showed the 21 peaks. They were ä 123(C-1), 135(C-2'', 6''), 117(C-3'', 5''), 165(C-4''), 168(C=O), 152 (C-3 double bond), ä 116, 30, 32, 42, 80, 53, 25, 170 were C-4, 5, 6, 7, 8, 9, 10 &11. In the similar way the glycosyl moiety carbons were assigned at ä 98, 84, 76, 72, 80, & 63 respectively from C-1'-C-6'. All the above mentioned spectral values identified the compound as 2'-p-hydroxybenzoylmussaenosidic acid also called iridoid glycoside or negundoside[26,27,28]. The structure of the compound was negundoside.



Negundoside

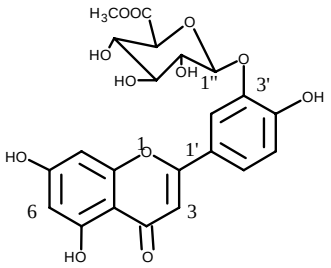
Vitegnoside(F8): Compound F8 was obtained as yellow colored solid. Positive Shinoda and Fiegel's test indicated that compound could be a flavonoid glycoside. The FAB-MS exhibited molecular ion peak $[M+H]^+$ at m/z 477 corresponding to the molecular formula $C_{22}H_{20}O_{12}$. IR absorption bands at 3250, 1746, 1640, and 1498 cm^{-1} indicated the presence of hydroxyl group, ester moiety, conjugated carbonyl, and aromatic ring, respectively. UV spectrum showed ë$_{max}$ at 336, 329, 266, and 209 nm (MeOH). A bathochromic shift (ë$_{max}$ 329, 266, 216 nm) of 7 nm of the band II with NaOAc indicated the presence of free 7-OH group. Addition of NaOMe resulted in the bathochromic shift (ë$_{max}$ 391, 324, 272 nm) of 55 nm of the band I confirmed the presence of free 4'-OH group. The UV spectrum of F8 was unaffected by the addition of NaOAc + H_3BO_3 (ë$_{max}$ 328, 266, 210 nm) which clearly suggested the absence of ortho dihydroxyl system. ^{1}H and ^{13}C NMR spectrum showed the characteristic signal for H-3 proton at äH 6.80 (s); äC 104.1. A sharp singlet observed at äH 12.8 was assigned to the C-5 chelated hydroxyl proton. The aromatic protons were resolved as one ABX system at äH 7.60 (d, J = 1.5 Hz), äC 114.7 for H-2', äH 6.97 (d, J = 8.2 Hz); äC 117.5 for H-5' and äH 7.62 (dd, J = 8.2, 1.5 Hz) for H-6'. The other AX system at äH 6.19 (d, J = 2.0 Hz), äC 99.7 and äH 6.45 (d, J = 2.0 Hz), äC 94.8 was assigned to H-6 and H-8 protons, respectively. A singlet at äH 3.67 (äC 52.9) was assigned to methoxy group of the esterified sugar. In case of 3', 4' dihydroxy flavone system, studies revealed that äC value for 4' appeared downfield than for 3'. Hence äC 145.9, 151.6 were assigned to C-3' and C-4', respectively. The anomeric proton of the sugar appeared at äH 5.21 (d, J = 6.2 Hz; äC 101.6). The coupling constant of the anomeric proton (J = 6.2 Hz) confirmed the â-linkage of the sugar. Using anomeric proton as starting point in ^{1}H–^{1}H COSY spectrum other sugar protons were assigned, respectively. Downfield shift and splitting pattern of H-5 sugar proton with absence of C-6 methylene of glucose indicated presence of carbonyl group at this position. The carbonyl carbon was found at äC 170.0 in ^{13}C NMR spectrum. The HMBC spectrum was utilized to identify position of sugar, a long range correlation between H-1''/C-3' confirmed the attachment of sugar at C-3', other useful correlation between H-5''/C-6'', -OCH3/C-6'', H-2'/C-3', 4', and H-5'/C-1', 3' confirmed the position of methoxyl and carbonyl group. Further acid hydrolysis of F4 followed by Co-TLC with authentic samples glycone (methyl ester of glucuronic acid) and aglycone (luteolin) was confirmed. Further D-configuration of glycone

Table 2: Antimicrobial activity of extract, fractions and pure compounds

Ext./Fract./Pure compounds	Zone of Inhibition(Well 6mm)					MIC(mg/ml)
	Bacillus subtilis	*Staphylococcus aureus*	*Micrococcus pyogenes*	*Pseudomonas aeruginosa*	*E. coli*	*S. aureus*
Methanol ext.	18	19	17	18	19	50
Pet. Ether fract.	7	8	7	7	8	>500
$CHCl_3$ fract.	8	9	8	8	9	350
EtOAC fract.	10	10	11	10	10	250
Methanol fract.	19	19	20	19	19	50
F1	17	17	17	17	15	100
F2	17	18	17	17	15	100
F3	17	18	18	18	15	100
F4	17	18	17	17	16	80
F5	19	20	21	20	20	6.25
F6	19	19	20	19	19	12.5
F7	18	18	18	18	18	12.5
F8	19	20	21	20	20	6.25
Ampicillin	ND	ND	ND	ND	ND	0.03

Ext. = Extract, Fract. = Fraction, F1-F8 = Pure compounds, ND = Not done, Control well = 6mm

was confirmed with optical rotation of acetate derivative [á] $^{30}_{D}$ $+11.4^{0}$ ($CHCl_3$) with those reported in the literature. Thus based on the foregoing evidence, the structure of compound F8 was elucidated as 4', 5, 7-trihydroxy-3'-O-â-D-glucuronic acid-6''-methyl ester, named as vitegnoside.[24,38-41]



Vitegnoside

As part of a systematic biological study, the antibacterial bioassay of the crude extract and different fractions as well as the isolated compounds of the *V. negundo* obtained from VLC and CC showed various range of antibacterial activity against aforementioned five different bacteria. The activities were found increased from crude extract to methanol fraction against all of the selected bacteria (Table 2)[35,36,42,44]. The minimum inhibitory concentration (MIC) of standard drug ampicillin and test compounds was determined against test isolate by broth micro-dilution technique [35,36,43]. The pure compounds were also found to be active against all the bacteria. The MIC values of F5 and F8 found encouraging as compared with standard ampicillin.

In conclusion, eight compounds from *V. negundo* were isolated for the first time in Nepal by activity guided fractionation and found the potent antibacterial activity of isolated compounds. The compounds F5 and F8 were found to be most active at MIC 6.25 mg/ml among all the isolated compounds.

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REFERENCES

1. Baral, S.R. and Kurmi, P.P. 2006. *A Compendium of Medicinal Plants in Nepal*, P 450-451, Pub.: Mrs Rachana Sharma, 281 Maiju Bahal, Chabhil, Kathmandu, Nepal.
2. Kirtikar, K.R.; Basu, B.D. 1935. *Ind. Med. plants* 3.
3. The wealth of India: Raw materials Publication & Information Directorate, CSIR, New Delhi, 1976; Vol. X, pp 520.
4. Balbao, J.G.; Lim-Sylianco, C.Y. Phillip. 1993. *J. Sci.* 122, 1.
5. Bhargava, S. K. J. 1989. *Ethnopharmacol.* 27: 327.
6. Gupta, M.; Mazumder, U.K.; Bhawal, S. 1999. CNS activity of *Vitex negundo* Linn. In Mice *Indian J. Exp. Biol.*, 37: 143.
7. Rimando, A. M.; Inoshiri, S.; Otsuka, H.; Kohda, H.; Yamasaki, K.; Padolina, W. G.; Torres, L.; Quintana, E. G.; Cantoria, M. C. 1987. *Shoyakugaku Zasshi* 41: 242.
8. Chawla, A. S., Sharma, A.K., Handa, S.S. 1992b. Chemical investigation and anti-inflammatory activity of *Vitex negundo* seeds. *J. Nat. Prod.* 55: 163–167.
9. Dharmasiri, M.G., Jayakody, J.R.A.C., Galhena, G., Liyanage, S.S.P., Ratnasooriya, W.D. 2003. Anti-inflammatory and analgesic activities of mature fresh leaves of *Vitex negundo. J. Ethnopharmacol.* 87: 199–206.
10. Alam, M.I., Gomes, A. 2003. Snake venom neutralization by Indian medicinal plants (*Vitex negundo and Emblica officinalis*) root extracts. *J. Ethnopharmacol.* 86, 75–80.

11. Das, B., Das, R. 1994. Medicinal properties and chemical constituents of *Vitex negundo* Linn. *Indian Drugs,* 31:431-435.

12. Tamhankar, C.P., Saraf, M.N. 1994. Antiarthritic activity of *Vitex negundo* Linn. *Indan J. Pharmaceut Sci* 56:158-159.

13. Makwana, H. G. 1993. *Pharmacological and Biochemical studies on Vitex species with special references to their role in inflammatory disorders.* Ph. D. Thesis submitted to Gujarat University, Ahmedabad.

14. Singh, V., Dayal, R., Bartley, J.P. 1999. Volatile constituents of *Vitex negundo* leaves. *Planta Med.* 65: 580–582.

15. Mallavarapu G.R., Ramesh S., Kaul P.N., Bhattacharya A.K., Rajeshwara Rao B. R. 1994. Composition of essential oils the leaves of *Vitex negundo*. *Planta Med,* 60:583-584.

16. Dayal R., Singh V. 2000. A comparative study of volatile constituent of *Vitex negundo* Leaves. *J med Aromat Plant Sci.* 22:639-640

17. Taneja, S.C., Gupta, R.K., Dhar, K.L., Atak, C.K. 1979. The essential oil of *Vitex negundo. Indian perfume*. 23:162.

18. Chawla, A.S., Sharma, A.K., Handa, S.S., Dhar, K.L. 1992a. A lignan from *Vitex negundo* Linn. Seeds. *Phytochemistry* 31: 4378–4379.

19. Azhar-ul-Haq, Malik, A., Anis, I., Khan, S.B., Ahmed, E., Ahmed, Z., Nawaz, S.A., Choudhary, M.I. 2004. Enzyme inhibiting lignans from *Vitex negundo*. *Chem. Pharm. Bull.* 52: 1269–1272.

20. Achari, B.; Chowdhury, U.S.; Dutta, P.K.; Pakrashi, S.C. 1984. Two Isomeric flavonones from *Vitex negundo* Linn. *Phytochemistry*, 23: 703.

21. Banerji, J.; Das, B.; Chakrabarty, R.; Jha, H. 1988. Isolation of 4, 4'-dimethoxy-trans-stilbene & flavonoids from leaves & twigs of *Vitex negundo* Linn. *Indian J. Chem., Sect. B*, 27: 597.

22. Subramanian, P.M.; Misra, G.S. 1979. Flavonoids of *Vitex negundo*, *J. Nat. Products*, 42(5) pp540-542.

23. Banerjee, A.; Chadha, M.S.; Malshet, V.G. 1969. Isolation of 5-hydroxy-3,6,7,3',4',-pentamethoxyflavone from *Vitex negundo*. *Phytochemistry 8,* 511.

24. Misra, G.S., Subramanian, P.M. 1980. Three new flavone glycosides from *Vitex negundo. Planta Med.* 38: 155–160.

25. Banerji, J., Das, B., Chakrabarty, R. 1988. Isolation of 4, 4'-dimethoxy-trans-stilbene & flavonoids from leaves & twigs of *Vitex negundo* Linn. *Indian J. Chem.* 27B: 597–599.

26. Dutta, P.K.; Chowdhury, U.S.; Chakravarty, A.K.; Achari, B.; Pakrashi, S.C. 1983. Studies on Indian medicinal plants—Part LXXV. Nishindaside, A noval irridoid glycoside from *Vitex negundo, Tetrahedron*, 39: 3067.

27. Sehgal, C.K.; Taneja, S.C.; Dhar, K.L.; Atal, C.K. 1982. 2'-p-Hydroxybenzoyl mussaenosidic acid, a new irridoid glucoside from *Vitex negundo, Phytochemistry*, 21: 363.

28. Sehgal, C.K.; Taneja, S.C.; Dhar, K.L.; Atal, C.K. 1983. 6'-p-Hydroxybenzoylmuss-aenosidic acid-an irridoid glucoside from *Vitex negundo* Linn. *Phytochemistry*, 22: 1036.

29. Vishnoi, S.P., Shoeb, A., Kapil, R.S., Popli, S.P. 1983. A furanoeremophilane from *Vitex negundo. Phytochemistry* 22: 597–598.

30. Chandramu, C., Manohar, R.D., Krupadanam, D.G.L., Dashavantha, R.V. 2003. Isolation characterization and biological activity of betulinic acid and ursolic acid from *Vitex negundo* L. *Phytother. Res.* 17: 129–134.

31. Chawla, A.S., Sharma, A.K., Handa, S.S. 1991. Chemical investigation and anti inflammatory activity of Vitex negundo seeds: Part I. *Indian J. Chem.* 30B: 773–776.

32. Mukherjee, K.S.; Badruddoza, S. 1981. Chemical constituents of *Dillenia indica* Linn. And *Vitex negundo* Linn. *J. Indian Chem. Soc.* 58: 97.

33. Helrich, K. 1990. *"Official Methods of Analysis of the Association of Official Analytical Chemists",* Fifteenth Edition, Published by the AOAC, Inc. USA.

34. Association of Official Agricultural Chemists Official Methods of Analysis(AOAC), Washington DC, 1970, 1980 p. 438

35. Baron, E .J., Peterson, L.R. and Finegold, S.M. 1994. *Baily and Scots Diognostic Microbiology,* 9th edn Mosby-year Book, USA.

36. Dingle, J.; Reid, W.W. and Solomonos, G.L. 1953. *The enzymatic degradation of pectin and other polysaccharides, Journal of Science, Food and Agriculture,* 4, pp149-153.

37. Wagner, H., Bladt, S. 1983. *Plant Drug Analysis-A Thin Layer Chromatography Atlas.* 2nd edn. Berlin: Springer Publication, p349-354.

38. Harbone, J.B.; Marbry, T.J. 1982. *The Flavonoids: Advances in Research;* Champan & Hall Ltd: London, p 19.

39. Finar, I. L. 1997. *Organic Chemistry, Vol-2, Stereochemistry and the chemistry of Natural products,* 5th edn, ELBS.

40. Geissman, T.A. 1962. The Chemistry of Flavonoid Compounds Macmillan, New York, PP 317-352.

41. Mabry, T.J.; Markham, K.R. and Thomas, M.B. 1970. The Systematic Identification of Flavonoids, Springer, New York. P 354.

42. Gautam L.N. 2004. *Phytochemical and Biological Studies on Podocarpus neriifolious D. Don.: A Himalayan Conifer of Nepal,* M. Sc. Thesis, Submitted to Central Department of Chemistry, Tribhuvan University, Kathmandu, Nepal

43. Gautam, L.N., Awale, S., Kalauni, S.K., Shrestha, K. and Gewali, M.B. 2005. *Phytochemical and Biological Studies on Podocarpus neriifolious D. Don.: A Himalayan Conifer of Nepal,* ***Scientific World****,* , **3**, 3, pp 22-28

44. Gautam, L.N., Belbase, S., Tamrakar, B. and Shrestha, S. 2007. *"Bio-phyto-chemical investigation of Bergenia ciliata (Haw) sternb.: A rocky Himalayan medicinal plant of Nepal"* for International Conference on Emerging Issues on Research and Development organized by JUSAN, April 4-6 page 6 (Full Paper in CD).

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