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Phytochemical Investigations on the Hydro-alcoholic Stem Fractions of *Viburnum* Linn. Species

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The genus *Viburnum* Linn. under the family-Caprifoliaceae (formerly) and Adoxaceae (recently) have been reported to contain about 17 species in India and been reported to possess remarkable biological activities against smooth muscle related troubles and microbial infections (protozoal and bacterial in origin). Three species were collected from Nilgiri hills, Tamilnadu, India and the aqueous ethanolic extracts of the stems of these species were tested for their antibacterial spectrum, which exhibited a significant activity against *S. aureus*, *V. cholerae* and *E. coli*, at a concentration of 800 µg/mL. In particular, *V. erubescens* (one of the species) was far more potent against *P. aeruginosa* at all concentrations (25-800 µg/mL), when compared to the other species as well as the reference (gentamycin, 25-800 µg/mL). To explore the chemical nature of the constituents responsible for antibacterial activity, an experiment was employed to isolate the phyto-constituents from the aqueous ethanolic stem extracts. A preliminary organic analysis on the alcoholic stem extracts showed the presence of phenolic compounds such as flavonoids, tannins, anthocyanins, phenolic acids and their derivatives (glycosides) as their principal constituents, which may be attributed to a mild and the pronounced antibacterial ativities of the extracts. In order to unfold the chemistry of constituents of the extracts, the residues were fractionated in a column chromatography with solvents of increasing polarity followed by a gradient elution of components by methanol-water mixtures of decreasing polarity. About three phenolic compounds were isolated in pure form and spectroscopically characterized (UV, IR, NMR and Mass) to be ferulic acid, arbutin and chlorogenic acid from *V. punctatum*, *V. coriaceum* and *V. erubescens*, respectively.

Key Words: *Viburnum*, MIC, Ferulic acid, Arbutin, Chlorogenic acid, *E. coli*, *P. aeruginosa*.

INTRODUCTION

The genus *Viburnum* Linn. species under the family Caprifoliaceae (formerly) and Adoxaceae (recently) includes about 200 species distributed throughout the world and about 17 of them have been reported in India. Their growth is favoured at an altitude from 1500-2500 ft and are frequently seen in Himalayan tracts, Nilgiri hills and Coimbatore[1,2].

Viburnum Linn. species have been reported to contain sesquiterpenes[3], triterpenes and phytosterols; phenolic compounds and their glycosides such as: tannins, flavonoids and anthocyanins, irridoid glycosides on their stem, root and leaves and investigated to posses uterine sedative, diuretic, cardiovascular stimulant, antimicrobial, antiinflammatory, antinociceptive, antispasmodic, antiasthmatic and astringent activities[4]. In the late 1960s and early 1980s, the magnitude of scientific investigations on the genus *Viburnum* Linn. were voluminous in regard to some phytochemical aspects of constituents from the stems, root barks and leaves of these species[5-7]. However, the number of species exploited for studies and areas of investigations were limited. After a couple of decades, some more *Viburnum* species appeared for having been investigated of their phytochemical and pharmacological characteristics. The typical examples are: iridoid aldehydes and their glycosides in *Viburnum luzonicum*[8] and their cytotoxic effect; vibsane type diterpene from *Viburnum awabuki*[9]; irdoid glycosides from *Viburnum tinos*; antinociceptive and antiinflammatory activities of *Viburnum lanata*[10] and *Viburnum opulus*[11] and an iridoid glucoside from *Viburnum rhytidophyllum*[12]. Recently a detailed pharmacognostical studies have been carried out on a few of the species which deserves a noteworthy in this section, since the same species have been screened for their antibacterial spectrum[13,14].

In addition to the above, a questionnaire and a verbal enquiry have been recently conducted to the local dwellers, tribal and the herbalists of Nilgiri hills and Coimbatore hills,

Tamilnadu, India, about the ethnopharmacological status of some *Viburnum* species, has also revealed that the leaves, stem bark and root barks of mature plants had been reliably in usage to the non-pregnant uterus[15], the GIT related ailments and are also in application as an ideal healing aid against inflammation[16], infections by protozoal and bacterial strains, as well as one of the best home remedies. Some of the above have also been scientifically proven. In view of the above, the current study is aimed at a preliminary detection, isolation and characterization of some phenolic compounds from 75 % v/v hydro alcoholic stem fraction of *V. punctatum*, *V. coriaceum* and *V. erubescens* after an antibacterial screening on the alcoholic stem extracts of all the three species.

The phenolic compounds of plant origin are versatile in biological activities. Their presence in plants, probably may be due to one or all of the following purposes: (a) feed deterrents against cattle; (b) (pathogenic) defence against microbial attack; (c) as a precursors for biosynthesis or as metabolic end products of plant metabolism; (d) pH-dependent colouring agents, especially in floral organs and leaves; (e) as the building blocks of polymeric phenolic molecules of heavy molecular weight such as tannins, procyanidins and lignans and (f) as antioxidants (oxidation-reduction process).

Isolation of phenolic compound by virtual solvent extraction process is supposed to be a highly tedious process, because of its high magnitude of reactivity with other co-molecules of the plants such as proteins (astringent effect) and carboxylic acids to form esters during extraction, in addition to their delicate nature of decomposition in presence of heat, acids, alkali and inorganic elements such as heavy metals.

Phenols, cresols, xylenols and halogenated phenolic derivatives are most powerful antimicrobials (often referred to be "Disinfectants" which are unsuitable for oral administration in the living beings). In this context, the phenols of plant origin are remarkably suiting for application in living system besides an advantage that the desired activity is achieved at a very low concentrations, being parasitotrophic rather than organotrophic.

EXPERIMENTAL

The stems of *V. punctatum*, *V. coriaceum* and *V. erubescens* were collected (flowering season, June-August) from Nilgiri hills, Tamilnadu, India and authentificated by Dr. V. Chelladurai, Ex. Professor, (Botany), Medicinal plant survey for Siddha, Government of India, as *Viburnum punctatum* Buch.-Ham.ex D. Don (VP), *Viburnum coriaceum* Blume (VC) and *Viburnum erubescens* Wall.ex DC (VE). Herbarium of the specimens (labeled V181, VC131 and VE131 for VP, VC and VE, respectively) was submitted to the museum of the Department of Pharmacognosy, Nandini Nagar Mahavidyalaya College of Pharmacy.

Successive extraction and preliminary organic screening: The materials were dried in the sun and then the shade for about 15 days. About 1.25 kg of stems of each *V. punctatum*, *V. coriaceum* and *V. erubescens* were powdered separately using a mechanical grinder in to a moderately coarse powder. The powdered specimens were extracted in a Soxhlet apparatus for about 15-18 h, successively with petroleum ether (60-80 °C), chloroform and 75 % aqueous ethanol. The percentage extractives of the test samples were determined as per the conventional procedures[17-19].

Antibacterial activity of ethanolic stem extracts: The following factors were employed for susceptibility of microbes: Solvent used-dimethyl sulfoxide; standard antibiotic used-gentamycin; concentrations screened-25, 50, 100, 200, 400, 800 µg; sample preparation-10 mg sample was dissolved in 1 mL of DMSO; stock sample concentration-10 mg/mL; Method-Agar diffusion method; Bacteria analyzed-*Staphylococcus aureus*, *Vibrio cholerae*, *Escherichia coli*, *Pseudomonas aeruginosa* (biochemically identified and supplied by BioGenics Ltd, Karnataka, India); media used: peptone 10 g, NaCl 10 g and Yeast extract 5 g, Agar 20 g in 1000 mL of distilled water.

Initially, the stock cultures of bacteria were revived by inoculating in agar broth and grown at 37 ºC for 18 h in an incubator. The agar plates of the above media were prepared and wells were made in the plate. Each plate was inoculated with 18 h old cultures (100 µL, 10^{-4} cfu) and spread evenly on the plates. After 20 min, the wells were filled with of test compounds (25, 50, 100, 200, 400 and 800 µg). The control wells for gentamycin were also prepared same as above described. All the plates were incubated at 37 ºC for 24 h and the diameter of inhibition zone were noted[20]. The results were then tabulated in terms of the concentration dependant zone of inhibition and minimum inhibitory concentration[20].

Isolation of ferulic acid from VP1 fraction of 75 % ethanolic stem extract of *V. punctatum*: The alcoholic stem extract of *V. punctatum* was stored at room temperature for 7 days which showed sediment (pale brownish) at the bottom of the container. The *V. punctatum* (VP) extract was filtered to separate the pale brownish solid and was labelled-VP1 (solid 5.5 g) and VP2 (brownish ethanolic layer).

Physico-chemical features of VP1: VP1 was insoluble in absolute ethanol and methanol, benzene and chloroform; sparingly soluble in acetone; moderately soluble in ethylacetate, hot water and hot methanol and soluble in hot aqueous ethanol (75 and 80 % v/v) and ethanolic solution (80 %) showed λ_{max} at 273 nm under UV (characteristics to long chromophore, Ph-C=C-C).

About 5 g of the sediments (VP1) was refluxed with dilute HCl for *ca.* 3 h and then cooled at room temperature. The mixture was filtered and washed repeatedly with double distilled water. The precipitate was adsorbed on neutral alumina on a boiling water bath and then packed in to a column containing slurry of neutral alumina in acetone. The column was then eluted with acetone (1-5 fraction), benzene (6-10), ethyl acetate (11-20) and methanol: water mixture (21-40) gradiently (50 mL/fraction and 2 mL/min) (Table-1).

Isolation of arbutin from ethanolic stem fractions of *V. coriaceum*: 75 % ethanolic stem extract of *V. coriaceum* (VC) yielded on storage about 6.25 g of reddish orange deposits (labeled VC1) and a brownish clear ethanolic layer. The residue of the later on evaporation, yielded about 35.5 g of dark brown residue (labeled-VC2).

VC2 (the filterate labeled, after separating of VC1-a sediment) was evaporated under reduced pressure to yield 35.5 g of residue. About 20 g of the residue was blended with

TABLE-1
ISOLATION OF FERULIC ACID FROM VP1 FRACTION OF ETHANOLIC EXTRACT OF *V. punctatum*

Fractions	Mobile phase solvent(s)	Residue (+/–)	TLC (Precoated and preparative)	No. of spots	R_f value
1-5*	Acetone	+	Benzene: ethyl acetate (1:1)	2	0.64; 0.49
6-10	Benzene	–	–	–	–
11-20*	Ethyl acetate	+++	BAW (4:1:5)	1	0.88
21-25	Methanol:Water (5:1)	+	–	–	–
26-30	Methanol:Water (4:2)	+	–	–	–
31-35*	Methanol:Water (3:3)	++	BAW (4:1:5)	2	0.29; 0.31
36-40	Methanol:Water (2:4)	++	BAW (4:1:5)	2	0.34; 0.41

50 mL/fraction; 2 mL/min; 50 cm × 3.5 cm glass column; Neutral alumina as adsobent. Iodine vapour and methanolic $FeCl_3$ solution (0.2 %), locating agent; TLC (silica gel-G); – = residue absent; + = negligible quantity; ++ = moderate quantity; +++ = considerable quantity, + = gave a positive test for phenolic compounds with dilute methanolic ferric chloride solution. *gave a positive test for phenolics with alcoholic ferric chloride solution.

TABLE-2
ISOLATION OF ARBUTIN FROM VC2 FRACTION OF ETHANOLIC EXTRACT OF *V. coriaceum*

Fraction	Mobile Phase	Residue	TLC profile	No. of spots	R_f values
1-3	Diethyl ether	–	–	–	–
4-6	Acetone	+	EtOAc-CH_3OH-H_2O (100:13.5:10)	3	0.65, 0.38, 0.72
7-9	Acetone-EtOAc (1:1)	–	EtOAc-CH_3OH-H_2O (100:13.5:10)	–	–
10-13	Ethyl acetate	+	EtOAc-CH_3OH-H_2O (100:13.5:10)	3	0.76, 0.56, 0.45
14-16	Ethyl acetate-$CHCl_3$ (1:1)	–	EtOAc-CH_3OH-H_2O (100:13.5:10)	–	–
17-19	$CHCl_3$	–	EtOAc-CH_3OH-H_2O (100:13.5:10)	–	–
20-24	Methanol-Water (30:70)	+	EtOAc-CH_3OH-H_2O (100:13.5:10)	2	0.52, 0.34
25-29*	CH_3OH-H_2O (40:60)	++	Alcoholic $FeCl_3$ as locating agent,	1	0.41
30-34*	CH_3OH-H_2O (60:40)	+++	50 % ethanol as locating agent	1	0.44
35-39*	CH_3OH-H_2O (80:20)	++	EtOAc-CH_3OH-H_2O (100:13.5:10)	2	0.41, 0.38
40-44*	CH_3OH-H_2O (90:10)	++	EtOAc-CH_3OH-H_2O (100:13.5:10)	3	0.48, 0.55, 0.35

50 mL/fraction, 2 mL/min, 3.5 cm × 50 cm dimension, Slurry of alumina in diethyl ether, – = residue absent; + = negligible quantity; ++ = moderate quantity; +++ = considerable quantity. EtOAc = Ethyl acetate, *Phenolic test positive.

TABLE-3
ISOLATION OF CHLOROGENIC ACID FROM ETHANOLIC EXTRACT OF *V. erubescens*

Fractions	Mobile phase solvent (s)	Residue (+/–)	TLC Profile	No. of spots	R_f value
1-3	Acetone	+		–	–
4-7	Benzene	+	Absolute ethanol as	–	Tailing
8-12*	Ethyl acetate	+++ (VE3)	developing agent,	1	0.46
13-17*	Ethyl acetate:Methanol (1:1)	+++ (VE4)	0.2% alcoholic $FeCl_3$	2	0.41; 0.44
18-21	Methanol (absolute)	++	as locating agent	3	0.81; 0.79; 0.54
22-25	Water	++		2	0.40; 0.49

50 mL/fraction; 2 mL/min; 50 cm × 3.5 cm column; Silica gel-G for column chromatography grade (slurry in acetone), – = residue absent; + = negligible quantity; ++ = moderate quantity; +++ = considerable quantity, *fraction gave a positive for phenolics.

100 g of neutral alumina on a boiling water bath to obtain a uniformly dispersed mixture. A slurry of alumina in diethyl ether was prepared and poured into a glass column (3.5 cm × 50 cm dimension) in such a way that no cracking of alumina and trapping of air bubbles occurred, followed by addition of the sample-adsorbent mixture (at a ratio of 1:5).

The content of the column was fractionated with diethyl ether (1-3); acetone (4-6); acetone-ethyl acetate (1:1 ratio) 7-9; ethyl acetate (10-13); ethyl acetate-chloroform (1:1) 14-16; chloroform (17-19) and finally with methanol-water gradient elution, with mixtures of decreasing polarity methanol-water mixture (30:70 volumes); (40:60), (60:40), (80:20); and (90:10) each of five fractions *viz.*, 20-24, 25-29, 30-34, 35-39 and 40-44, respectively. The yield of the fractions, 25-34 and 30-40 was appreciable and so were evaporated under reduced pressure to collect the respective residues *i.e.*, (25-29) and (30-34) separately (Table-2).

Isolation of chlorogenic acid from ethanolic stem fractions of *V. erubescens*: The alcoholic stem extract was dark brownish and turbid which on filteration, yielded about 1.5 g solid residue (yellowish brown) which was insufficient for handling. So, the filterate was selected and then concentrated under reduced pressure to get a brownish residue (VE1) (28 g). The residue was adsorbed on a paper bulb and placed in a small scale soxhletor and was fractionated successively with diethyl ether, ethyl acetate and absolute ethanol for about 3 h. The last two fractions gave a positive test for phenolic compounds. The ester fraction was selected and labelled to be VE2 (Table-3).

Ethyl acetate soluble constituents (VE2): Silica gel-G for column chromatography was mixed with three of its volume of acetone and triturated well to result an uniformly dispersed and pourable slurry. The slurry was poured in to a column (3.5 cm × 50 cm) in such a way that no air bubbles be trapped.

The residue of ethyl acetate fraction was adsorbed on silica gel-G (column chromatography grade) at a ratio of 1:5 and stirred well placing on a boiling water bath. The sample-adsorbent mixture was then placed in to the column containing slurry of silica gel in acetone. The column was eluted with acetone (1-3 fractions), benzene (4-7), ethyl acetate (8-12), ethyl acetate:absolute methanol (1:1) (13-17), absolute methanol (18-21) and finally with distilled water (22-25).

RESULTS AND DISCUSSION

Percentage extractives and selection of extracts: Petroleum ether, chloroform and 75 % aqueous ethanolic stem extracts of *V. punctatum*, *V. coriaceum* and *V. erubescens* yielded about 10.59, 11.20 and 34 g; 11.01, 13.0 and 45.5 g; and 34, 45.5 and 31.5 g, respectively as their phyto-constituents. A preliminary organic screening of alcoholic fraction assisted with paper chromatography and TLC gave a positive test for phenolic compounds such as: tannins (Gold beater's test), flavonoids (Shinoda's test, Zn-HCl, Fluorescent test under UV-254-quenching due to conjugate system and UV peak maxima), condensed tannins (using paper chromatography), simple phenolic acids (by TLC aided detection), anthocyanins (pH dependent colour change and red colouration of the alcoholic extracts) and glycosides of a few or all of the phenolic compounds (hydrolysis test).

Antibacterial activity: Agar diffusion method (Walker 2000) was employed to screen the antibacterial potentials against about six gram-positive and about six gram-negative strains (Biogenics, Karnataka, India and Biochemically recognized) at a random dose level of 100 µg residue of each of VCEE, VEEE and VPEE. An observation made after 18 h revealed that about five (incubated at 37 °C) strains of gram positive type and two strains of gram negative failed to show a reduction in growth or in number and so omitted from the study. However the susceptibility of microbes in growth was negative for about 4 among 12 strains of bacteria [*S. aureus,* gram positive and *V. cholerae*, *E. coli* and *P. aeruginosa*, gram negative]. The 4 strains were exposed to various concentrations of ethanolic residues of VCEE, VEEE and VPEE (25, 50, 100, 200, 400 and 800 µg in DMSO) keeping gentamycin at the same concentrations as standard. The antibacterial potentials of the test drugs were documented in comparison to the reference (Table-4).

TABLE-4
ANTIBACTERIAL ACTIVITY OF ETHANOLIC STEM EXTRACTS OF *V. coriaceum*, *V. erubescens* AND *V. punctatum*

Bacterial strains	MIC in µg (ZOI in cm)			
	VCEE	VEEE	VPEE	Gentamycin
S. aureus	400 (0.1)	200 (0.4)*	200 (0.3)	25 (1.6)
V. cholerae	400 (0.1)	800 (0.2)	50 (0.2)*	25 (1.3)
E. coli	200 (0.2)	25 (0.2)*	400 (0.2)	25 (0.9)
P. aeruginosa	25 (0.4)*	25 (0.4)	25 (0.3)	25 (0.2)

Values are represented in terms of cm (in parenthesis) of respective concentration (µg) of samples, *Effective test sample among others, VCEE = Ethanolic extract of *V.coriaceum,* VEEE = Ethanolic extract of *V. erubescens,* VPEE = Ethanolic extract of *V. punctatum,* MIC = Minimum inhibitory concentration, ZOI = Zone of inhibition,

Minimum inhibitory concentration (MIC) of VCEE, VEEE and VPEE against *S. aureus* were 400, 200 and 200 µg respectively, against the standard (gentamycin 25 µg). The zone of inhibition at 800 µg of VPEE showed about 1.2 cm in diameter, which was less than one half of the response shown by the standard. Moreover, zone of inhibition of the standard was about 1.6 cm at 25 µg dose level itself.

VPEE at 50 µg concentration exhibited a reduction in growth of *V. cholerae*, however, 800 µg test substance failed to suppress the growth at least equivalent to the MIC of the standard drug. However, VPEE was more effective than the rest of the samples.

Against the strain *E. coli*, no marked suppression in growth was observed with all the three test samples. But, VEEE showed (MIC, 25 µg) an appreciable antibacterial activity, when compared to that of the rest of the test samples.

Against the strain *P. aeruginosa*, all test samples exhibited an appreciable reduction in growth, which was either equivalent or greater than that of the standard at all concentrations (25-800 µg). VCEE, in particular was far more potent than that of the standard.

The probable mechanism of action of *in vitro* antimicrobial activity of the extracts in reference to isolated compounds may be due to the following reasons:

The phenolic compounds are highly reactive, when proteins are in vicinity to the former and may lead to inactivation is incident by denaturation of proteins.

Metabolism in bacterial cells is catalyzed by a large number of enzymes which are essential and vital for the survival of the microbe and to pass their genes to spread in number. The phenolic compounds form hydrogen bonding with those enzymes leading to suppression of growth or destruction of microbes.

The glycosides of ferulic acid, chlorogenic acid and hydroquinone glucosides (arbutin), on hydrolysis in the cytoplasm of microbe, liberate respective phenols and their phenyl propenes leading their binding on the receptors (molecular space on enzyme) resulting deactivation.

The receptor specific action of *ortho*, *para* substituted phenols are greater than that of simple phenols which cannot bind and cover more space to incident a receptor-drug legands on the target molecules of the microbial enzymes (probably, NH_2 domains of amino acids of peptides).

Fractionation of phenolic compound by column chromatography

Isolation of ferulic acid and spectral characterization: Fractions (11-20) were added together and evaporated under reduced pressure and then stored in a refrigerator for 24 h. A mixture of whitish needles and prisms were observed. The sample was insoluble in cold alcohol (ethanol and methanol); sparingly soluble in hot alcohol and freely soluble in ethyl acetate and solvent ether. The crystals were warmed with ethyl acetate and then transferred to beaker containing 1 g of activated charcoal in 25 mL ethyl acetate.

The mixture was filtered to get the filterate. The filterate was warmed on a boiling water bath to yield about 95 mg of crystals and then repeatedly crystallized from benzene to yield pure crystals. The crystals melted between 168-172 ºC. The

homogeneity of the substance isolated was confirmed by ascending TLC, BAW (4:1:5) as developing solvents, iodine vapour and 0.2 % ferric chloride solution in methanol as locating agent (R_f - 0.88).

The substance was further subjected to IR, ^1H NMR, ^{13}C NMR and Mass spectroscopic analysis. The following are the spectral datas of the isolated compound [ferulic acid ($C_{10}H_{10}O_4$, m.p. 168-172 ºC)]: IR (KBr, ν_{max}, cm^{-1}): 3495 (-OH aromatic alcohol stretching); 3022 (aromatic-H stretching); 2984 (=C-H alkyl stretching); 1685 (>C=O carboxylic acid); 1521 (-C-C- aromatic ring); 1216 (-C-O- carboxylic acid, deformation); 1028 (-C-O methoxy group). ^1H NMR (CD_3OD) 300 MHz, TMS, δ ppm: 11.213 (singlet, -COOH carboxyl proton); 7.762, 7.630 (doublet, ethylene *gem* proton); 6.653, 6.506, 6.312 (multiplet, Ar-H aromatic proton); 4.968 (-OH hydroxyl proton); 3.766 (singlet, $-OCH_3$ methoxy proton). ^{13}C NMR ($CDCl_3$) 75 MHz, TMS, δ ppm: 167.85 (-COOH carboxyl carbon); 148.96, 147.80, 144.35 (Ar aromatic carbon); 125.68, 122.68 (aromatic carbon); 115.52 (ethylene carbon); 54.95 ($-OCH_3$ methyl carbon). MS, m/z, [chemical fragments], (relative abundance %): 194 [$C_{12}H_{16}O_7$] M^+, (0.5 %) molecular ion peak; 177 [$C_{12}H_{16}O_7$-CH_3^+] (2 %); 105 [$C_7H_5O^+$], (9.5 %); 89 [$C_7H_5^+$], (100 %); 77 [C_6H_5]$^\bullet$, (46 %).

Isolation of arbutin and spectral characterization: A pale yellowish fraction, upon evaporation yielded a transparent yellowish, glassy residue. The residue was freely soluble in aqueous ethanol and methanol (50 %) and hot water and soluble in absolute methanol, ethanol and in water at room temperature; insoluble in ethyl acetate, acetone, benzene, ether and carbon tetrachloride. A dilute solution in methanol showed λ_{max} at 225 nm [characteristic of a long chromophore of Ph-C=C-].

The crude compound from 25-29 fractions was labelled to be VC2a and the compound from 30-34 being VC2b. Both the compounds (crude residues) melted between 142-147 °C. Crude compound from fractions 25-29 and 30-34 were subjected to ascending TLC using 50 % aqueous ethanol as developing phase and 0.2 % alcoholic ferric chloride as locating agent.

Both VC2a and VC2b assumed 0.41 and 0.44 as R_f values which were comparable, besides showing a green colour with a spray of 0.2 % alcoholic ferric chloride solution on the chromatograms.

The crude samples (VC2a and VC2b) were added together and dissolved in 50 mL of 50 % aqueous methanol and then added was 1 g of activated charcoal. The whole mixture was stirred with a glass rod thoroughly and filtered. The filterate was then evaporated to get colourless crystals whose homogeneity was screened by TLC, as done before (R_f - 0.44). The crystals were repeatedly crystallized from hot water to yield about 125 mg of pure crystals. The isolated compound was subjected to spectral analysis to confirm the compound to be arbutin ($C_{12}H_{16}O_7$, m.p. 145 °C). The spectral data of the isolated compound as follows: IR (KBr, ν_{max}, cm^{-1}): 3429 (-OH stretching aromatic alcohol); 3022 (C-H aromatic-H stretching); 1522 (-C-C- aromatic ring); 1026-1216 (-C-O stretching). ^1H NMR ($CDCl_3$) 300 MHz, TMS, δ ppm: 6.827, 6.624 (multiplets, Ar-H aromatic proton); 5.589 (singlet, carbon attached to -O- of glycoside); 5.101 (singlet, Ar-OH aromatic hydroxyl proton); 3.910, 3.79, 3.761 (multiplet, glycosidic carbon); 2950.2 (singlet, C-H alkane); 2.011 (singlet, -OH alcoholic hydroxyl proton). ^{13}C NMR ($CDCl_3$) 75 MHz, TMS, δ ppm: 152.10, 151.27 (Aromatic carbon attached to -OH); 119.86, 117.09 (aromatic carbon); 102.26 (glycosidic carbon attached to O-side chain); 76.89, 76.4, 73.8, 70.2 (glycosidic carbon); 61.38 (glycosidic side chain carbon attached to -OH). MS, m/z, [chemical fragments], (relative abundance %): 272 [$C_{12}H_{16}O_7$, M^+], (100 %) molecular ion peak or base peak; 273 [$C_{12}H_{16}O_7$ + H], (13.2 %); 274 [M + 2], (1.4 %); 93 [$C_6H_5O_7^+$]$^\bullet$, (6.6 %); 179 [$C_6H_{11}O_6^+$]$^\bullet$, (6.8 %).

Isolation of chlorogenic acid and spectral characterization: Fractions 1 to 3 were added together and evaporated showing a pinkish residue which was, by volume, insufficient to handle. Fractions 4 to 7 yielded no any residue. Fractions 8 to 12 and 13 to 17 yielded yellowish brown and pale brown residues, respectively. Fractions 13 to 17 were added together and evaporated to get a pale brown residue (thin layer), labelled VE4 (phenolic in nature). Fractions 8-12 were added together and evaporated and labelled to be VE3 [on complete evaporation, VE3 and VE4 transformed in to a thin layer (flakes and shining)].

Small quantity of VE3 was dissolved in ethyl acetate and run on a TLC plate using absolute ethanol as developing phase, silicagel-G as stationary phase. A distinct yellowish to orange spot resulted on the chromatogram against a spray of 3 % methanolic H_2SO_4 as locating agent (R_f - 0.46).

Both VE3 and VE4 flakes were pale brown in colour, sparingly soluble in acetone absolute ethanol, methanol and water; soluble in ethyl acetate and ether; insoluble in benzene and chloroform. The flakes melted between 196-200 °C.

The ethyl acetate fractions 8-12 and ethyl acetate-alcohol (1:1) fractions 13-17 gave greenish brown colour with alcoholic ferric chloride solution.VE4 was also run on a TLC plate as that of VE3 to obtain two distinct spots (R_f - 0.41; 0.44; using 0.2 % ethanolic $FeCl_3$ solution as locating agent). Accounting the proximity among R_f values, both VE3 and VE4 were dissolved in 5 mL of ethyl acetate. The residue of ethyl acetate fraction then was adsorbed on 10 g of silica gel-G (column grade) and stirred well on a boiling water bath and packed in to an analytical column containing slurry of silica gel-G (column grade) in acetone, at 1:2 ratio. The column was of 25 cm long and 1 cm wide.

The column was eluted with 20 mL each of acetone and then with 20 mL ethyl acetate. The later was evaporated to obtain shining and pale brownish flakes which melted at 198°C.

Small quantity of the substance was dissolved in ethyl acetate and run on a TLC using absolute ethanol as developing phase and silica gel-G as stationary phase. The chromatogram showed a distinct spot against the spray of 3 % H_2SO_4 (methanolic) R_f - 0.46 (a dark green spot). The crude sample was crystallized repeatedly from distilled water and stored at 4 °C to get about 95 mg of crystals. The spectral studies resulted the following: IR (KBr, ν_{max}, cm^{-1}): 3395 (-OH aromatic alcohol stretching); 3024 (Ar-H aromatic ring stretching); 2960 (CH=CH stretching); 1217 (C-O stretching); 1650 (>C=O stretching); 1554 (-C=C- aromatic ring). ^1H NMR (CD_3OD) 300 MHz, TMS, δ ppm: 10.998 (singlet, -COOH carboxyl proton); 7.652 (doublet, ethylene CH=CH); 6.689, 6.657, 6.501 (multiplet, Ar-H); 5.101 (doublet, Ar-OH); 3.968, 3.843, 3.298,

3.202 (multiplet, cyclohexane proton); 2.189 (singlet, alcoholic-OH); 1.95, 1.85 (doublet, -CH_2 proton of cyclohexane). ^{13}C NMR (CD_3OD) 75 MHz, TMS, δ ppm: 177.45 (-COOH carboxylic carbon); 166.51 (carboxyl carbon); 147.01, 145.92, 144.76 (aromatic carbon); 122.52, 121.23 (ethylene carbon); 76.33, 67.01, 62.96 [substituted aliphatic ring carbon (cyclohexane)]; 38.73, 35.84 (un-substituted aliphatic ring carbon). MS, m/z, [chemical fragments], (relative abundance %): 354 [$C_{16}H_{18}O_9$, M^+], (2.5 %) molecular ion peak; 355 [$C_{16}H_{18}O_9$ + H], (59 %); 356 [$C_{16}H_{18}O_9$ + $2H^+$], (1 %); 163 [$C_9H_7O_3$], (100 %) parent ion peak; 135 [$C_8H_7O_2$], (7 %).

Conclusion

The aqueous ethanolic (75 % v/v) extracts of some three species of *Viburnum* has revealed the extract of *V. erubescens* as the most significant antibacterial drug when compared to the rest of the species. The phyto-constituents responsible for the impact shown on the subjected microbes have been isolated and characterized to be phenolic acids and their glycosides. However, the antibacterial potentials of extracts may not be attributable to a mere contribution of the isolated compounds alone and is a matter of speculation, since the extracts contains several classes of phenolic compound.

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