A preliminary chromatographic detection of phenolic compounds from ethanolic stem extracts of Viburnum Linn. species by TLC and PC

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ABSTRACT

The stems of three viburnum species and their alcoholic extracs were subjected Thin layer and paper chromatographic techniques using suitable stationary and developing phases, after a primary organic analysis. With aide of appropriate detecting agents and reference phenolics, presence of a few phenolic compounds was identified based on their Retardation Factors (Rf) values to observe that VP stem extract possessed astragalin, rutin and arbutin; VC stem extract contained amentoflavone, caffeic acid and arbutin and VE stem extract represented the presence of chlorogenic acid and rutin.

KEY WORDS: Viburnum, phenolics, TLC, PC, Arbutin.

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 [Wealth of India].

Viburnum Linn. Species have been reported to contain sesquiterpenes, 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, anti-inflammatory, antinociceptive, antispasmodic, anti-asthmatic and astringent activities [3, 4]. In the late 1960s and early 1980s, the 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 very limited. After a couple of decades, some more *Viburnum* species appeared for having been investigated on 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]; iridoid glycosides from *Viburnum tinos*; antinociceptive and anti-inflammatory activities of *Viburnum lanata* [10], and *Viburnum opulus* [11], and an iridoid glucoside from *Viburnum rhytidophyllum* [12]. And a detailed pharmacognostical studies have, recently, been carried out on a few of the species which deserves a noteworthy here in this section.

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 ethno-pharmacological status of some *Viburnum* species, which revealed that the leaves, stem bark and root barks of mature plants had been reliably in usage to the non-pregnant uterus, the GIT related ailments, and are also in application as an ideal healing aid against inflammation, infections by protozoal and bacterial strains as well as one of the best home remedies. 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: feed deterrents against cattle; pathogenic induction against microbial attack; as a precursors or metabolic end products of plant metabolism; pH-dependent colouring agents, especially in floral organs and leaves; as the building blocks of polymeric phenolic molecules of heavy molecular weight such as tannins, procyanidins and lignans; and 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 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, bases and electropositive inorganic metal ions.

Phenols, cresols, xylenols and halogenator phenolic derivatives are most powerful antimicrobials (often referred to be “Disinfectants” which are unsuitable for use 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. By considering the above facts that the phenolics are very versatile in their biological spectrum of actions and so a search for them in plant kingdom, employing simplest procedures like TLC, PC, deserves a note worthy in the present study.

**MATERIALS AND METHODS**

**Plant Material**
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. The chemicals and reagents subjected for chromatographic analysis were of analytical grade, wherever required. For extraction, solvents of technical grade were used after purification.
Successive Solvent Extraction
The shade dried sample of stems of *V. punctatum*, *V. coriaceum* and *V. erubescens* were ground in to a moderately coarse powder using a mechanical grinder. About 100 g of each sample were separately and successively extracted (19 – 24 h) in a soxhletator with petroleum ether (60 – 80°C), benzene, chloroform, 75% v/v ethanol followed by a digestion of the marc in distilled water for about 24 h at room temperature. The extracts were filtered and labelled. The physical nature of the extracts was noted down followed by determination of their percentage extractives(13)

Qualitative Chemical Analysis
All extracts were tested with suitable chemical reagents to unfold the diverse classes of chemical constituents present, and then the results were tabulated. Non-polar solvent extracts (petroleum ether 60 – 80°C, benzene and chloroform) were tested for the presence of phyto-sterols, triterpenes (pentacyclic), and the chloroform fraction for alkaloids; ethanolic and aqueous extracts were tested for the presence of alkaloids, phenolic compounds such as flavonoids, procyanidine, tannins, and phenolic glycosides, saponins and free reducing sugar.

The hydroalcoholic extracts of VPEE, VCEE and VEEE were involved to preliminary phytochemical analysis using appropriate chemicals and reagents followed by thin layer chromatographic screening. All extracts gave positive test for diverse classes of phenolic compounds such as tannins (Gold beater’s skin test), chlorogenic acid (ester fraction with 3% methanolic H₂SO₄) flavonoids (Shinoda’s test and UV-254 nm) phenolic glycosides (test for sugar and phenolic compounds after exhausting the free sugars from the extracts and then followed by hydrolysis.)

Preliminary detection of various phenolic compounds from alcoholic stem fractions of VP, VC and VE by TLC and Paper chromatography
Detection of phenolic compounds by ascending TLC techniques
About 10 g of powdered stem of VP, VC and VE were, separately, extracted with 100 ml of methanol for about 1 h on a water bath at about 60 – 70°C.

The extract was filtered and 30 µl of the extract spotted on a precoated plate containing silicagel 60F₂₅₄ (Merck) as stationary phase.

The reference compounds were prepared (0.05% w/v in absolute methanol) and spotted against the test solution. [kaemferol-3-*O*-glucoside (astragalatin) (R1), rutin (R2), ferulic acid (R3), catechol (R4), caffeic acid (R5) and chlorogenic acid (R6) (Biogenic, Karnataka)]. The reference compounds, which failed to go in to the solution of absolute methanol, were dissolved in any one of the mobile phases to spot on the chromatogram – the same procedure was adapted to the tests also. For each reference substance, about three chromatograms, one each spotted with VP, VC and VE were developed in appropriate mobile phases.

Mobile Phase (Developing solvents)
Ethyl acetate-formic acid-glacial acetic acid and water with a ratio (100:11:11:27). The first three solvents were mixed together followed by the addition of water gradually with a vigorous shaking; Chloroform-ethyl acetate (60:40): for the separation of the flavonoid aglycones. Remarks: Flavonoid aglycones can also be separated in benzene-pyridine-formic acid (72:18:10) or in toluene-ethyl formate-formic acid (50:40:10) [developing phase in TLC and paper chromatography]; Ethylacetate-formic acid-glacial acetic acid-water(100:11:11:27):The ethyl
acetate, formic acid and glacial acetic acid are mixed first, and then water is added gradually with vigorous shaking. If the ethyl acetate is technical grade, the composition 100:11:11:26 should be used; separation with this mixture shows very slightly difference in \(R_f\) values, but no change in the order of separation. (TLC investigation of flavonoid glycosides); Ethyl acetate-methanol-water (100:13.5:10): for the analysis of arbutin, cardiac glycosides, bitter principles, flavonoids and saponins. Mobile phases to TLC and Paper chromatography: Ethyl acetate-formic acid-glacial acetic acid-ethylmethyl-ketone-water (50:7:3:30:10); Chloroform-acetone-formic acid (75:16.5:8.5); Chloroform-ethylacetate (60:40); Toluene-ethylformate-formic acid (50:40:10); n-butanol-glacial acetic acid-water (4:1:5); Acetic acid-conc.HCl-water (30:3:10); Acetic acid-chloroform (3:2); Benzene-methanol-acetic acid (45:8:4) and Acetic acid (5%)(14)

**Visualizing agents/locating agents**

1. **Natural product reagent** [Intense orange fluorescence at UV-365 nm]: Sprayed with 10 ml of 1% methanolic diphenyl boryloxy ethylamine followed by 8 ml of 5% ethanolic polyethylene glycol-4000 (PEG) (Wagner 1983)
2. **UV-254**: Distinct quenching of fluorescence (without any chemical treatment)
3. **Iodine reagent**: 10 g of Iodine spread on the bottom of a chromatography tank. Yellowish zones upon exposure to Iodine vapour (conjugate double bonds).

**Detection of Arbutin and Amentoflavone in Viburnum Linn. species by TLC**

**Amentoflavone**

About 10 g of powdered stems of VP, VC and VE were extracted under reflex for about 30 min with 250 ml of petroleum ether (60 – 80°C) and filtered. The marc left was then extracted by boiling for about 30 min with 100 ml of methanol, and then filtered. The filtrate was evaporated to about 5 ml, and 50 µl were applied on the chromatogram.

**Reference compounds**

10 ml of 0.025% of w/v of methanolic-Arbutin and 10 ml of 0.1% w/v of methanolic Amentoflavone [Mobile phase: ethyl acetate-methanol-water with a ratio of (100:13.5:10) for both arbutin and amentoflavone] (Wagner 1983).

**Arbutin**

10 g powdered sample of VP, VC and VE were mixed separately with 1 g of CaCO\(_3\) and extracted with 20 ml of 50% ethanol for 30 min under reflex using a water bath. The mixture was filtered and washed with 50% ethanol to produce 20 ml again. 2.5 g of basic lead acetate was added for every 10 ml of 50% ethanolic extract then filtered. 30 µl of the same were directly spotted on the paper chromatogram, with aid of a micropipette (Wagner 1983, Harborne 2005).

**Preliminary detection of rutin by paper chromatography in V.punctatum**

Whatman No. 1 filter paper was cut in to 2.5x55 cm strips. The strips were spotted 8 cm from one end using micropipette with alcoholic solution of the flavonoid pigments. A hand-type hair drier was used to evaporate the spotting solvent. The strips are then placed in the chromatographic chamber by inserting the end of the strips nearest the pigment zone under the glass rod provided with each trough. A portion of the solvent system was placed in borosilicate-glass pie plates located at the bottom of the chamber to ensure a sufficient supply of solvent vapour and thus prevent excessive evaporation of solvent from the paper strips during the chromatographic process. The troughs were then filled with the solvent system and the chamber was closed. The two-phase, two-component systems were prepared by saturating a sufficient amount of the solvent with water and allowing the two-phase system to stand for several hours in...
order to achieve complete separation. The layer rich in organic solvent was used for filling the chromatogram troughs. Development of the chromatograms by the descending techniques was allowed to proceed until the solvent has travelled 35 to 40 cm beyond the starting line. The strips were then removed from the chamber and allowed to air-dry. The flavonoids, if any, were detected in visible and ultraviolet light and then by spraying with alcoholic aluminium chloride, 1%; keeping aqueous sodium carbonate, 1% solution as an alternative (16,17,18).

RESULT AND DISCUSSION

TLC
The R_f value of each reference compound was recorded by ascending TLC as performed for the test samples. VPEE exhibited 4 spots, VCEE showed 4 spots and VEEE 3 spots shown in (Table.1).

A fluorescence quenching zone under UV-254 nm; An intensive orange and a yellow fluorescence were found after spraying PEG-NP reagent on the chromatogram and then observed under UV-365 nm; R_f values resulted by test samples of VP, were 0.95, 0.70; for VC, 0.9; and in case of VE, 0.45, 0.40 which were parallel to the R_f values of reference compounds, astragalin, rutin, ferulic acid, chlorogenic acid and caffeic acid.

Detection of arbutin by TLC
Detection by locating agent was carried out using three types of reagents separately.

Berlin blue reagent (BB)
When 5-10 ml of BB were sprayed on the plate, a blue zone appeared at R_f value 0.40 of test solution, which was comparable/parallel to the R_f value of reference compound mixture a blue zone against day light.

NP-PEG reagent: Yellow brown zones were seen in UV-365 nm, whose R_f value (0.40)
Potassium hydroxide reagent (8 – 10% ethanolic KOH): In UV-365 nm, yellow-green fluorescent zone appeared whose R_f value (0.40).

Table 1. Preliminary detection of various phenolic compounds against reference compounds

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Name of Specimen</th>
<th>No. of spots (Test)</th>
<th>R_f value of test</th>
<th>Reference Compound (R_f value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>V.puntatum</td>
<td>4</td>
<td>0.79*, 0.52, 0.70*, 0.95*, 0.04</td>
<td>R1-Astragalin (0.70), R2-Rutin (0.40)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Retardation flow of test samples found between 0.52 – 9.5</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>V.coriaceum</td>
<td>4</td>
<td>0.90*, 0.35, 0.70, 0.45*</td>
<td>R3-Ferulic acid (0.95), R5-Caffeic acid (0.90),</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Retardation flow of test samples found between 0.3 – 0.7</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>V.erubescnes</td>
<td>3</td>
<td>0.62, 0.88, 0.40*, 0.45</td>
<td>R6-Chlorogenic acid (0.45),</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Retardation flow of test samples found between 0.40 – 0.62</td>
<td></td>
</tr>
</tbody>
</table>

*– R_f values parallel to that of reference substances involved, R1, R2, R3, R5, R6 – reference compounds

Descending paper chromatography for detection of rutin
The paper chromatogram under long UV-365 nm, showed a pale yellow spot (R_f value: 0.15, 0.45 and 0.83) when mobile phases 1, 2 and 3 were used. After a spray of 1% aluminium
chloride solution over the paper chromatogram showed a distinct greenish yellow spot in case of VP (Raphael 1964).

**Mobile phases**
1. Ethyl acetate saturated with water
2. Phenol saturated with water
3. 60% isopropanol and 40% water mixer

**Detection of condensed tannins**

**Paper chromatography (two dimensional and ascending)**
About 100 mg each of ethanolic stem residue of VP, VC and VE were treated separately with 5 ml of 50% methanol (λ_{max} 475 and 560 nm visible region) and filtered. 30 – 40 µl of the filterates were spotted on Whatmann filter paper No.1 with aid of a micropipette.

The paper was subjected to two dimensional chromatography using (Butane-2-ol : Acetic acid : Water) at ratio 14:1:5 followed by 6% acetic acid as developing phase (Harborne 2005).

The paper chromatogram was air dried and exposed to UV-254 nm, which showed dark purple spots indicating the presence of condensed tannins in all three species.

**Detection of amentoflavone by TLC**
The thin layer chromatograms of VP, VC and VE spotted were developed using chloroform-acetone-formic acid (75:16.5:8.5) as developing phase.

The chromatogram of VC showed a distinct fluorescent orange spot (R_f-0.74) upon a spray of KOH reagent under long UV-365 nm.

**CONCLUSION**

The characteristics spots on the chromatograms of the individual species show the probable presence of the following phenolic compounds:

**VP stem extract:** astragalin, rutin and arbutin  
**VC stem extract:** amentoflavone, caffeic acid and arbutin  
**VE stem extract:** chlorogenic acid and rutin

Rutin – A flavonol glycoside is a common constituent of VP and VE, while arbutin with VP and VC. But, amentoflavone, astragalin and chlorogenic acid are not evident as that of the formers. However, the condensed tannins are not species specific (i.e, found in all the three species). This study can be a referential tool for progressing isolation of these phenolic compounds from the species subjected in this study by trial and error methods.

**REFERENCES**


Pelagia Research Library