There are numerous references in the literature concerning the use of various Arctostaphylos species as medicinal plants. One of the species, A. uva-ursi, was listed in the United States official compendia from 1820 until 1946 as a urinary antiseptic. Many species indigenous to the Pacific Northwest were employed by the Indians for a variety of uses, ranging from consuming the ripe fruits as a food to utilizing the leaves in urinary tract and other infections. Early settlers in the West consequently used the plants for the same purposes.

Phytochemical investigations of the genus Arctostaphylos have not been extensive although several members of the family, Ericaceae, yield compounds restricted only to the family. A thorough review of the literature revealed that there has been
little phytochemical investigation of species other than *Arctostaphylos uva-ursi*. Therefore, the purpose of this study was to thoroughly investigate two other *Arctostaphylos* species, viz., *A. columbiana* and *A. patula*, for organic components. The development of newer methods of extraction, isolation and identification, as well as, a screening of both plants for possible biological activity was also pursued.

A new extraction solvent mixture was utilized in order to extract all of the components of interest in one extraction procedure. The residue from the extraction was then separated into two fractions; one containing sterol and triterpenoid compounds; and the other, phenolic components.

The sterol and triterpene fraction of *A. patula* yielded the following isolated compounds; β-amyrin, β-sitosterol, ursolic acid, uvaol, and nonacosane. Only ursolic acid and uvaol were previously shown to exist in the genus. The identical fraction of *A. columbiana* was screened chromatographically for the same components and all except nonacosane were identified in this manner. Nonacosane was also isolated from *A. columbiana*.

A separation of the components of the phenolic fraction was attempted using several standard methods. However, the method utilized for the identification of these compounds consisted of
thin-layer chromatography and ultra-violet analysis. A relatively crude mixture was chromatographed along with a standard, both spots were eluted from the plate, scanned on a spectrophotometer, and co-spotted in three different solvent systems. This procedure proved the presence of the following compounds in both species; arbutin, ellagic acid, gallic acid, hydroquinone, hyperin and quercetin. The presence of o-pyrocatechuic acid, found in other members of the genus and family, could not be confirmed in these species.

A screening for antibacterial and antifungal properties was conducted on crude plant extracts as well as the compounds found to be present in both plants. Some extracts of both plants demonstrated more antibacterial and antifungal activity than did any of the pure compounds. Further investigation is warranted in this area.

Generally, the results obtained demonstrated the applicability of the new extraction scheme devised for the screening of hitherto uninvestigated plants. Eleven compounds were identified in both species, three of which had not been demonstrated in the genus previously. Another compound, assumed to be widespread in the family, was found to be absent in A. columbiana and A. patula.
PHYTOCHEMICAL INVESTIGATION OF ARCTOSTAPHYLOS COLUMBIANA PIPER AND ARCTOSTAPHYLOS PATULA GREENE

by

GEORGE HARMON CONSTANTINE, JR.

A THESIS
submitted to
OREGON STATE UNIVERSITY

in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

June 1966
dates thesis is presented May 5, 1966

Typed by Gwendolyn Hansen
ACKNOWLEDGEMENTS

The author wishes to express his thanks to Dr. Leo A. Sciuchetti for his advice and guidance during the course of the research and the preparation of the thesis.

He acknowledges Dr. Philip Catalfomo for his interest and advice throughout the research stages and his constructive criticism in the preparation of the thesis.

Thanks go to Dr. Kirti Sheth for his valuable assistance in the laboratory experimentation and in the final proofreading of the thesis; to Dr. David French of Reed College for his interest and assistance throughout the research; to Dean Charles O. Wilson for his assistance and cooperation; and to the U. S. Public Health Service for partial financial support of the research.

Special thanks are extended to my wife, Nancy, whose understanding and devotion made this work possible and also to my parents for their encouragement.
# TABLE OF CONTENTS

**GENERAL INTRODUCTION**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Review of the Literature</td>
<td>2</td>
</tr>
<tr>
<td>Botanical Classification and Description</td>
<td>2</td>
</tr>
<tr>
<td>Ethnobotany</td>
<td>5</td>
</tr>
<tr>
<td>Materia Medica of the genus <em>Arctostaphylos</em></td>
<td>8</td>
</tr>
<tr>
<td>Phytochemistry</td>
<td>11</td>
</tr>
</tbody>
</table>

**EXPERIMENTAL**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triterpenes and Related Components</td>
<td>18</td>
</tr>
<tr>
<td>Introduction</td>
<td>18</td>
</tr>
<tr>
<td>Methodology</td>
<td>20</td>
</tr>
<tr>
<td>Collection and Preparation of Plants</td>
<td>20</td>
</tr>
<tr>
<td>Preliminary Investigation</td>
<td>21</td>
</tr>
<tr>
<td>Extraction and Purification</td>
<td>22</td>
</tr>
<tr>
<td>Fractionation</td>
<td>24</td>
</tr>
<tr>
<td>Fraction IV, <em>A. patula</em> and <em>A. columbiana</em></td>
<td>24</td>
</tr>
<tr>
<td>Fraction V, <em>A. patula</em></td>
<td>29</td>
</tr>
<tr>
<td>Fraction VI, <em>A. patula</em></td>
<td>35</td>
</tr>
<tr>
<td>Fraction III, <em>A. columbiana</em></td>
<td>36</td>
</tr>
<tr>
<td>Results and Discussion</td>
<td>36</td>
</tr>
<tr>
<td>Phenolics and Related Components</td>
<td>39</td>
</tr>
<tr>
<td>Introduction</td>
<td>39</td>
</tr>
<tr>
<td>Methodology</td>
<td>43</td>
</tr>
<tr>
<td>Preliminary Investigation</td>
<td>43</td>
</tr>
<tr>
<td>Fractionation</td>
<td>45</td>
</tr>
<tr>
<td>Determination of Compounds Present</td>
<td>48</td>
</tr>
<tr>
<td>Arbutin</td>
<td>48</td>
</tr>
<tr>
<td>Ellagic acid</td>
<td>50</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>53</td>
</tr>
<tr>
<td>Hydroquinone</td>
<td>54</td>
</tr>
<tr>
<td>Hyperin</td>
<td>55</td>
</tr>
<tr>
<td>o-Pyrocatechuic acid</td>
<td>57</td>
</tr>
<tr>
<td>Quercetin</td>
<td>58</td>
</tr>
<tr>
<td>Results and Discussion</td>
<td>61</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Extraction Scheme for Sterols and/or Triterpenes.</td>
<td>25</td>
</tr>
<tr>
<td>2.</td>
<td>Structures of Ursolic Acid, β-amyrin, β-sitosterol, and Uvaol.</td>
<td>28</td>
</tr>
<tr>
<td>3.</td>
<td>Fractionation of Phenolic Components.</td>
<td>47</td>
</tr>
<tr>
<td>4.</td>
<td>Structures of Arbutin, Hydroquinone, Hyperin and Quercetin</td>
<td>49</td>
</tr>
<tr>
<td>5.</td>
<td>Structures of Ellagic, Gallic and o-Pyrocatechuic acids.</td>
<td>51</td>
</tr>
</tbody>
</table>
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Co-spotting</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.</td>
<td>Co-spotting of Ursolic acid and Fraction IV</td>
<td>27</td>
</tr>
<tr>
<td>II.</td>
<td>Co-spotting of β-amyrin and Column Fraction 4</td>
<td>31</td>
</tr>
<tr>
<td>III.</td>
<td>Co-spotting of β-sitosterol and Column Fractions 4' - 6'</td>
<td>33</td>
</tr>
<tr>
<td>IV.</td>
<td>Co-spotting of Uvaol and Column Fractions 6 and 7</td>
<td>34</td>
</tr>
<tr>
<td>V.</td>
<td>Co-spotting of Standards and <em>A. columbiana</em> Fraction III</td>
<td>37</td>
</tr>
<tr>
<td>VI.</td>
<td>Number of Chromatographic spots from Fractions I and II</td>
<td>44</td>
</tr>
<tr>
<td>VII.</td>
<td>Co-spotting of Standard and Eluted Arbutin</td>
<td>50</td>
</tr>
<tr>
<td>VIII.</td>
<td>Co-spotting of Standard and Eluted Ellagic Acid</td>
<td>52</td>
</tr>
<tr>
<td>IX.</td>
<td>Co-spotting of Standard and Eluted Gallic Acid</td>
<td>54</td>
</tr>
<tr>
<td>X.</td>
<td>Co-spotting of Standard and Eluted Hydroquinone</td>
<td>56</td>
</tr>
<tr>
<td>XI.</td>
<td>Co-spotting of Standard and Eluted Hyperin</td>
<td>57</td>
</tr>
<tr>
<td>XII.</td>
<td>Co-spotting of Standard and Eluted Quercetin</td>
<td>59</td>
</tr>
<tr>
<td>XIII.</td>
<td>UV Maxima of Eluted Compounds, Eluted Standards and Pure Standards</td>
<td>60</td>
</tr>
<tr>
<td>XIV.</td>
<td>Antibacterial-Antifungal Studies</td>
<td>67</td>
</tr>
</tbody>
</table>
Introduction

The use of various Arctostaphylos species as medical plants is well known. One of the species, A. uva-ursi, was listed in the first United States Pharmacopoeia in 1820 and remained in the National Formulary until 1946. Many species indigenous to the Pacific Northwest were employed by the Indians for a variety of uses, ranging from the consumption of the ripe fruits as food to the use of the leaves in infectious conditions. Early settlers in the West consequently made similar use of the plants.

The genus Arctostaphylos (Ericaceae) has never undergone an extensive phytochemical investigation. However several members of the family yield compounds restricted only to it. The only species investigated phytochemically has been A. uva-ursi and the majority of these investigations were completed without the aid of modern analytical tools.

Organic components previously identified in the genus have been restricted to two broad chemical classes, the triterpenes and the phenolic derivatives. In fact, three of the compounds first identified within the genus still retain common names derived from the plant source, viz. arbutin, uvaol, and ursolic acid. The genus
has been found to be devoid of alkaloids although a toxic substance has been identified in other members of the family.

The present study, the phytochemical investigation of *A. columbiana* and *A. patula*, will concern itself with the extraction, isolation, and identification of organic components within the plants. Neither plant has been submitted to such an investigation and the information gained would be a contribution to the phytochemistry of the genus.

The same data would also be of value to the chemotaxonomy of the genus. It is generally accepted that similar components are present at the genus level, but one cannot assume this to be true with the genus *Arctostaphylos* because of the lack of information on species other than *A. uva-ursi*.

A knowledge of the organic components within the plants may also explain some of the reported biological activity of plants within the genus.

**Review of the Literature**

**Botanical Classification and Description**

The accepted classification of the Ericaceae family is that of Drude (1889) in Engler's *Pflanzenfamilien* (23). The Ericaceae, commonly called the heath family, contains 99 genera and
approximately 1350 species. It includes various shrubs and sub-shrubs of the tribes **Vaccinieae** (huckleberries, blueberries, and cranberries), **Gaultheriae** (wintergreen), **Rhododendroneae** (rhododendrons and azaleas) and **Arbutae** (manzanitas).

Recently Watson (122) has provided evidence to support the soundness of the traditional **Arbutae** tribe (**Arbutus**, **Arctostaphylos**, and **Arctous**). He states "...the tribe is unquestionably sound, and appears to be one isolated from the family as a whole." All of the **Arbutae** have broad flat leaves, and bud scales. The inflorescences are leafless and racemose, and the corolla is urceolate. In all respects they resemble the **Vaccinoid** genera. However, the **Vaccinieae** have a typical chromosome number of N = 12 while the **Arbutae** have a basic chromosome number of N = 13.

The genus **Arctostaphylos** contains approximately 50 species, ten of which Peck (91) states are indigenous to Oregon. They are described as follows:

Erect or prostrate shrubs with smooth brown bark, finally deciduous, and thick evergreen leaves; flowers small, in terminal panicles, the peduncle and pedicels white or colored and with scale-like bracts; sepals five and distinct; corolla urceolate, the lobes very short; ovary five to ten chambered with one ovule to a chamber; fruit globose or depressed, drupaceous but berry-like, dry at maturity, the nutlets often united in twos or threes.
A columbiana and A. patula are differentiated from the other Arctostaphylos species by being bushy shrubs never depressed or creeping. A. columbiana has herbage, at least when young, canescent and glaucous with branchlets more or less bristly-hirsute. A. patula has herbage which is deep green, not canescent or glaucous, spreading branches, young branchlets and inflorescence or glandular puberulent and leaves broadly ovate or orbicular.

Howell (52) also attempted to differentiate Arctostaphylos species on the placement of stomata on leaf surfaces, i.e.,
a.) primitive species would have stomata restricted to the lower surface, b.) derived species (more xerophytic) would have equal stomata on the upper and lower surfaces, and c.) crossed species of (a.) and (b.) would have fewer stomata on the upper surface. According to these criteria then, A. patula would be a derived species and more xerophytic whereas A. columbiana resulted from a fertile cross of two other species.

A classification dilemma has been encountered with A. patula. Jeppson (59) first described an ecological factor which he used to differentiate some species of Arctostaphylos, this factor being the behavior of some plants in reference to fire. Some species adapt an enlargement of the root-crown from which young sprouts arise after destruction of the stems by fire, other plant species
are killed.

Adams (2) described A. Parryanna as a species not forming root-crowns but layering, i.e., lateral branches rooting where they touch the soil and A. patula as a species possessing an enlarged root-crown. Weislander and Schreiber (125) had reported one year earlier that A. Parryanna var. pinetorum should be the designation for the layering species killed by fire. A recent United States Department of Agriculture handbook (118) verifies the presence of both species in Oregon.

The conflict resides in the fact that the species differentiation is based solely on an ecological factor and classical taxonomists do not take these factors into consideration when examining herbarium specimens. It is not the purpose of this study to resolve this conflict. However, the phytochemical investigation of A. patula may produce results which could be of value in differentiating two separate species, i.e. A. patula and A. Parryanna.

Ethnobotany

The Indians of North America, particularly those of the West, have used two Arctostaphylos plants, A. uva-ursi and A. glauca for many non-medicinal purposes. Before the introduction of tobacco, the leaves of A. uva-ursi were pulverized and smoked.
Later they were used to stretch the small supplies of tobacco available. French (27) states that their use in tobacco was for flavoring. The Chehalis tribe stated that if one swallows the smoke of kinnikinnick (*A. uva-ursi*), it produces a drunken feeling (34). Reagan (98) reported, "Some years ago an Indian of the Quileute tribe was intoxicated by smoking *A. uva-ursi* leaves and danced in the fire barefooted until the soles of his feet were burned to a crisp and his feet deformed for life. Some years previous another old Indian became drunk on the narcotic inhaled while smoking the leaves of this plant. . . ." He also stated that it was smoked as a medicine and in religious ceremonies but did not elaborate on these uses.

The following Indian medicinal uses of *A. uva-ursi* were reported by Chestnut (14); a.) the Calpella Indians made a tea of the leaves to cure severe colds, b.) the Little Lake Indians boil the leaves until the extract is yellowish-red and use it as cleansing wash for the body and head, c.) the same tribe also uses the leaves to check diarrhea, d.) some old Concow women chew the leaves into a cud and place the mass on sores for healing purposes, younger people grinding the leaves up in water before applying.

Standby (106) reported that the California Indians made "manzanita cider" from the crushed ripe fruit of *A. tomentosa*. 
He also stated that the fruit was an important article of food, being eaten fresh, or dried and ground and stirred into water to form a pinole or cooked as a mush. Palmer (90) had earlier stated that all the western Indians relish the fruit of *A. tomentosa* in whatever way it is prepared but reported no therapeutic effects for it. Densmore (20) noted that the berries of *A. uva-ursi* were cooked with meat as a seasoning by the Chippewas of the Midwest.

The Thompson Indians of British Columbia used a concoction of the leaves and stems as a wash for sore eyes and a diuretic, whereas, a decoction of the root was used for blood spitting (108).

The varied uses of *Arctostaphylos* species among the Indians illustrates the wide variety of uses a genus may have among various tribes. It must not be overlooked however that some tribes used the plants empirically for the same distresses that confronted civilized man (see Materia Medica). Except for the isolated reports of erratic behavior following the swallowing of uva-ursi smoke, the genus is non-toxic. This fact is substantiated by the wide use of the drupes and seeds as a food and a beverage. In fact, Morton (79) incorporates *Arctostaphylos* species into one of the principle wild food plants of the United States in her recent paper.

Wild animals have also fed upon various *Arctostaphylos* species without deleterious effects. Deer have been noted to
browse on *A. patula* as well as *uva ursi* (71). Deer mice, *Peromyscus maniculatus*, have eaten quantities of *A. patula* berries (58) and chipmunks and mantled squirrels have been known to eat the flowers and seeds of the same species (114).

Although the **Arbutae** have been found non-toxic, other members of the family have not been as fortunate. Various species of the tribes **Rhododendronaceae** and **Andromedeae** have been involved. The toxic principle being variously named; asebotoxin, andromedol, andromedotoxin, and grayanotoxin, the last being the term most widely used. Iwasa *et al.* (57) have elucidated a structure for grayanotoxin and Kakisawa *et al.* (64) have recently investigated its stereochemistry. Hardikar (42) and Woud (130) have investigated it pharmacologically. A historical review of the compound has been completed by Schindler (101).

**Materia Medica of the genus Arctostaphylos**

The genus *Arctostaphylos* has long been cited as one possessing medicinal properties, particularly *A. uva-ursi*. The early Greeks and Romans reportedly used the plant. Galen's use (131-201 A.D.) of it is also mentioned (96). According to Grieve (35), there are records that it was used by the 13th century Welsh "Physicians of Myddfai".
A. uva-ursi was admitted to the London Pharmacopoeia in 1764 and in the first United States Pharmacopoeia in 1820. In 1828 Rafinesque (96) best described the therapeutic activity in the nomenclature of her era as "... a tolerable palliative in nephritis, gravel, calculous, disury, strangury, acting as an astringent, useful even when other remedies fail." Woodville (129) reported that uva-ursi had almost entirely fallen into disuse until about the middle of the eighteenth century when DeHaen raised its stature in Europe. Griffith (36) contended however that it is doubtful that the ancients used it at all and gives De Haen full credit for the discovery of its usage in kidney and bladder diseases.

Aside from its previously cited uses, Millspaugh (78) states "... in later years it (uva-ursi) has been called attention to as an uterine excitant, very useful in prolonged parturition. ..." He also stated that large amounts of tannins in the leaves were used extensively in Iceland, Sweden, and Russia for tanning fine grains of leather. Often referred to as the California manzanita, A. glauca was also cited for a wide variety of symptoms and distresses. Culbreth (19) outlined the following uses "... cystitis, gravel, chronic nephritis, urethritis, incontinence, dysuria, strangury, uterine hemmorhage, gleet, leucorrhea, mennorrhagia, urinary calculi, bronchitis, diarrhea, and cardiac dropsy."
Potter (93) recommended its dosage as 30 grains of the powdered leaves, in an infusion or decoction. *A. glauca*, has been found by Flint (26) to be used for much the same purpose as uva-ursi. Many investigators later reported on the medicinal value of *A. glauca* but none of the uses were different than ascribed to uva-ursi.

Wood (128) reported an hitherto uncited use of uva-ursi leaves as a "serviceable" drug in diabetes. Stuhr (111) stated that the leaves were astringent and diuretic, Hatcher (45) citing its use as an urinary antiseptic, Shoemaker (103) reporting that it was used in various infections of the urinary tract while Osol (89) made mention of its usage such as, "... leaves are highly esteemed in diarrhea and gonorrhea." Recently Borkowski (8) found that preparations of uva-ursi leaves inhibited diuresis [see Kreitmair (69)]. More recent work on extracts of Ericaceous plants has revived interest in possible components of therapeutic value. Watanabe et al. (121) found that the family (Ericaceae) showed strong inhibitory action on the normal growth of fibroblasts.

The increasing emphasis on cancer chemotherapy induced Ueki et al. (116) to screen 117 plants against Ehrlich ascites tumor. Water, methanol, acetone, and ether extracts of *A. uva-ursi* were found to be effective inhibitors in this screen.
Phytochemical investigations of the genus *Arctostaphylos* were begun soon after the mention of the medicinal usage of the plant.

According to Wehmer (123), Kawalier in 1852 was the first to isolate pure arbutin from *Arbutus officinalis* (=*Arctostaphylos uva-ursi*) and thus its name. By 1874 arbutin was also reported in other Ericaceae; *Chimaphila, Calluna, Ledum, Vaccinium, Epigaea* and *Gaultheria* (74). Chemically, arbutin is hydroquinone β-D-glucoside. It forms colorless bitter needles and is sometimes referred to as ericolin in the older literature. However, in 1939 Dieterle and Dorner (21) found "ericolin" to be impure arbutin and recommended that the name ericolin be stricken from the literature. A recent review of the evaluation of arbutin containing plants has been published by Kraus (68). Extensive work on the quantitative determination of arbutin has been done in Europe (15, 25, 28, 66, 131) where it is still mentioned as a domestic drug and discussed in modern pharmacognosy texts (110). Kreitmair (69) recently reviewed the medicinal uses of arbutin.

Rosenthaler (100) isolated methylarbutin (p-methoxyphenyl β-D-glucoside) from *A. uva-ursi*. Very recently, three galloyl esters of arbutin have also been identified in *A. uva-ursi* (12).
The flavonoids, isoquercitrin and hyperin, have been isolated by Nakamura (80) and Kawaguchi (123), respectively.

The phenolic derivatives, gallic and quinic acid have also been isolated (65). Ibrahim (56) recently identified α-pyrocatechuic acid in uva-ursi and other Ericaceae.

As previously mentioned, "tannins" have long been known to exist in the leaves of uva-ursi. Hermann (48) has found that "A. uva-ursi tannin" consists of gallic acid, ellagic acid, and glucose. Britton and Haslam (12) very recently have found the tannin from the leaves of A. uva-ursi to consist of penta-to hexa-α-galloyl-β-D-glucose. The terminology of the term "tannin" is presently being extensively evaluated.

Goldstein and Swain (31) proposed that the term tannin be reserved for those phenolic compounds of sufficiently high molecular weight (7500) which form reasonably strong complexes with proteins and other polymers under suitable conditions of concentration and pH. The terminology used herein will restrict itself to this definition.

Plant phenolics have also been implicated in the antibiotic and antitumor activity of plant extracts. Kabadi and Hammerlund (63) have made a preliminary identification of the antibacterial principle "madronin" from Arbutus menziesii (Ericaceae) and have found it
to consist of a mixture of gallotannin, catechol tannin, and the various decomposition products of gallic acid, \( m \)-digallic acid and possibly trigallic acid, catechol, phloroglucinol, and glucose. Cole and Buchalter (18) have investigated aqueous plant extract fractions for their antitumor components and conversely Jones et al. (60) have screened selected flavonoids for their cytotoxicity.

Aside from the phenolic derivatives, one other class of compounds has been reported in \( A. \text{uva-ursi} \). Tromsdorff, according to Wehmer (123), isolated in 1854 the pentacyclic triterpenoid ursolic acid. Later investigations found ursolic acid to be widespread not only in the Ericaceae (109) but also in at least twenty different families of the dicotyledons (92). It has been found in concentrations as high as ten percent in cranberry (\( V\text{accinium} \)) skins (81) and is generally found in the wax-like coating of leaves and fruits. The compound has undergone considerable chemical investigation (104, p. 114-134).

Uvaol, the corresponding pentacyclic alcohol of ursolic acid, was isolated by Orr et al. (87).

The function of the pentacyclic triterpenes in plants is not known, although a few physiological studies have provided some interesting insights. Brieskorn and Polonius (11), while working on \( S\text{alvia} \) species, found that ursolic acid was formed from
germanicol only during the vegetative period and that oleanolic acid was formed during the remainder of the time. Earlier, Brieskorn et al. (9) found an inverse relationship between the ethereal oil and triterpene content as growth of plants progresses. Drozdz (22) demonstrated a correlation between saponin content and time of flowering in four genera. He stated that the highest saponin content occurs before the beginning of flowering and that the lowest values appear at the time of flowering. Nicholas (84) confirmed an increase in concentration with age and also found that leaves and flowers contained the highest concentrations of triterpenes. However, Hajkova and Scacilikova (37) found that Vinca minor had the highest concentration of ursolic acid in the roots. Also, the seasonal variation in concentration of ursolic acid paralleled that of the oncolytic alkaloids.

Ursolic acid was at one time recommended as an excellent emulsifying agent (17). It is non-toxic orally in humans at a dose of 20mg/Kg/day (76). Earlier, Winterstein and Stein (127) found that the sodium salts of ursolic and oleanolic acid were toxic to fish. Ursolic acid has been screened for deoxycorticosteriod, cortisone, estrogenic, and androgenic activity. It was found to be inactive in any of these categories although it produced significant sodium retention (124). This might suggest that ursolic acid
should be neglected. However in the light of recent investigations of pentacyclic triterpenoids this may not be advisable. Glycyrrhetinic acid, asiatic acid, and escin are all compounds of the same chemical classification i.e., pentacyclic triterpenes, and have been found to have therapeutic applications.

Glycyrrhetinic acid, a triterpene from licorice root, *Glycyrrhiza glabra*, has been patented in England as an antitussive (33). It was previously employed as a vehicle for cough preparations. It has also been found to possess adrenal cortical activity, it increases plasma levels of hydroxycorticosteroids, produces hypertension in nephrectomized rats, and it also has cortisone-like anti-leukemic activity (76).

Asiatic acid, from *Centella asiatica*, is a pentacyclic triterpene sapogenin of the alpha-amyrin group and the first of the group to be introduced into therapeutics. It is indicated in the treatment of all atonic and torpid sores as well as an adjuvant in the treatment of lesions in leprosy where association with sulfonamides and sulfones produces spectacular results in leprosy infections (1). Incidentally, this was the primary empirical use of the plant among the natives of Madagascar, Ceylon and India (1). Huettenrach (53) has also shown that asiaticoside, as well as two triterpenoids isolated from *Polyporus* species, possess antibiotic
Escin, from the horse chestnut *Aesculus hippocastanum*, is a member of the \(\beta\)-amyrin group of pentacyclic triterpenes. Pharmacological studies show that it inhibits swelling and edema and the formation of exudation during inflammation caused by foreign bodies (119). Lorenz and Marek (72) have also shown that it reduces swelling in anaphylactic tissue reactions. It is useful in thrombophlebitis and other inflammatory conditions. Another plant triterpenoid, \(\beta\)-sitosterol has received considerable investigation. Hassan *et al.* (44) have investigated its effectiveness as an estrogen in mice, Haskins (43) has demonstrated its capabilities as a "stress" component when added to fungus cultures, and Eli Lilly and Company has long marketed a mixture of sitosterols (Cytellin\(^\circledR\)) as a preventative in cholesterol absorption.

The plants concerned with in this study, *A. columbiana* Piper and *A. patula* Greene, were collected in western Oregon and investigated since they are of widespread occurrence in the area and are taxonomically related to plants which at one time were used for their medicinal activity.

The primary purpose of the study was to investigate both plants phytochemically. The knowledge gained from this information should be of value to the chemotaxonomy of the genus as well as the
family. A biological screening of the plants was also conducted in order to gain insight into the reported physiological activity of some of the members of the genus.
EXPERIMENTAL

Triterpenes and Related Components

Introduction

The advances in methodology for the separation of natural products have been invaluable in the field of polycyclic compounds such as the sterols and triterpenes. The methods employed previously to identify the presence of these compounds have been dependent upon the isolation of each compound individually. In so doing, other components of the same chemical classification were oftentimes lost. Indeed, most of the polycyclic compounds are not found as single entities within a plant species but exist as a mixture with other biogenetically related components.

Generally, there has not been a single method developed that is suitable for the isolation of all polycyclic components from a plant source. Huneck and Snatzke (54) have isolated sterols from a hexane extraction but had to use another (ether) extraction to remove triterpenes. Kondo et al. (67), in contrast, removed triterpenes with methanol and neglected the possibility of sterols. Lawrie, McLean and Paton (70) found it necessary also to employ two extraction steps to remove all of the polycyclic components of Sambucus nigra. Nicholas (83) isolated β-sitosterol, oleanolic
acid, and ursolic acid from an ethanol extract, but required a ten step shake-out fractionation in order to obtain pure fractions for chromatographic identification. Pourrat and Hommouda (94) attempted to develop a method for the extraction of only triterpenes and thus neglected the extraction of the non-polar steroidal components. Ullsperger and Ullsperger (117) patented a method for the extraction of triterpene acids but limited themselves to only that group. Methods employed for the isolation of steroidal sapogenins have been modified for use with non-sapogenins. The most widely publicized of these methods is that of Wall et al. (120), used as part of the survey for precursors of cortisol within the plant kingdom. The need for an improved extraction procedure was definitely indicated since none of the above methods were found to be capable of extracting all of the polycyclic compounds present in a plant.

In the past, an extraction procedure which removed all of the polycyclic compounds at one time would not have been advantageous since the separation of such complex mixtures possessed inherent difficulties. Today, however, modern methods of chromatographic separation and identification have been established and complex mixtures are no longer impossible to separate. Column and thin-layer chromatography have been authenticated as useful techniques
by Randerath (97), Neher (82), Stahl (107), and Tschesche, Duphorn, and Snatzke (115).

Ursolic acid and uvaol, the only triterpenoids known to exist in Arctostaphylos species to date, have been found to occur in several other plants in combination with one or more of the following: α-amyrin, β-amyrin, β-sitosterol, betulin, epifriedelin, friedelin, lupeol, oleanolic acid, and tigogenin. The majority of these components are removed from plant tissues with various non-polar solvents such as petroleum ether, hexane, or Skelly B, (a mixture of low molecular weight hydrocarbons but predominately hexane). Ursolic acid itself has been more easily extracted with more polar solvents such as chloroform, ether, and methanol. Therefore, an extraction system which could remove all possible polycyclic components had to be developed.

Methodology

Collection and Preparation of Plants

A. columbiana and A. patula were gathered in Marion County, Oregon in the vicinity of the Detroit reservoir during the fall of 1965. Specimens of both plants were authenticated by Dr. K. L. Chambers of the Oregon State University Herbarium and vouchers were placed therein. The plants were allowed to air dry on
greenhouse benches for no less than two weeks after which time they were divided into their various plant parts, i.e. leaves, roots, and stems. Each group was then ground to a coarse powder in an Abbé mill. The powdered plant parts were then stored in air-tight plastic bags in the dark until time for analyses.

Preliminary Investigation

Five-Gm. samples of each plant part from both plants were subjected to a selective solvent extraction using successively petroleum ether (90-95°C), chloroform, (U.S.P.), alcohol, (U.S.P.), and distilled water. Each fraction was screened by ascending paper partition chromatography using the solvent system butanol-acetic acid-water (4:1:5, organic phase) and butanol-pyridine-water (2:1:1). Detection of triterpenes and/or sterols was made by spraying the dried sheets with antimony trichloride (10% in chloroform, w/w) and heating for five minutes at 110°C. Positive results (pink to red) were obtained in each fraction of plants in at least one solvent system except for the aqueous fractions; they being negative. The results were verified by testing samples of each extract with Lieberman-Burchard (L-B) reagent (acetic anhydride-sulfuric acid, 4:1, successively) and anisaldehyde (0.5 ml. of anise aldehyde in 50 ml. of glacial acetic acid and 1 ml.
of sulfuric acid). Both of these reagents are widely accepted as methods of detecting the steroid nucleus. The L-B test is generally regarded as positive when green, blue, red, pink or purple colors are produced. The anisaldehyde test produces red to pink colors. Most saturated steroids or triterpenes of the friedelane group are not detected by the L-B test. The chemical reaction mechanisms for the color production have been investigated by Brieskorn and Hofmann (10).

Extraction and Purification

The classical approach to the screening of plants with the use of successive solvents suffers from many disadvantages, the most important being time consumption. In contrast, other methods of extraction for specific types of compounds are generally too restricted in their range of extraction, as previously mentioned. Therefore, a method was devised using solvents of diverse polarity in a mixture which would extract as many components as possible in one extraction procedure. Such a method would decrease the amount of time spent in extraction and yet would remove compounds of diverse solubilities.

Experimentation demonstrated that a 50% mixture of Skelly B and methanol was best suited to this need. Although immiscible
at room temperature, the mixture is readily miscible when heated to boiling, ca. 60–65°C.

Sterols and/or triterpenes generally are present in higher concentrations in the leaves of a plant. This was also ascertained by the preliminary screening. Therefore, it was decided to extract these components from the leaves.

One Kg. of *A. columbiana* leaves and 1.3 Kg. of *A. patula* leaves were extracted for five days in a Soxhlet apparatus with not less than six liters of the extraction solvent. The extracts were concentrated to dryness with the aid of a Rotavapor flash evaporator\(^1\) at a temperature of 30°C and with water suction. The green residue was then redissolved in Skelly B and water (1:1) and placed in a large separatory funnel. On standing, a copious green interphase developed which was removed along with the organic phase and washed at least three times more with water. The brown aqueous fraction (II) was set aside for later examination of phenolic constituents.

The organic phase plus the interphase (III) was again concentrated to dryness and saponified according to the method of Huneck and Snatzke (54). It involves using methanol-benzene-potassium hydroxide in a ratio of 6:2:0.5 and refluxing for at least two hours. Acidification and refrigeration of this mixture

\(^1\) Type KR, Rinco Instrument Company, Greenville, Illinois.
yielded a copious quantity of a green-yellow precipitate which was designated fraction IV. The mother liquor was then subjected to liquid-liquid extraction for 48 hours using hexane as the lighter solvent. The hexane soluble components were designated as fraction V. The extraction scheme is presented in Figure 1.

Fractionation

Fraction IV, *A. patula* and *A. columbiana*. The Lieberman-Burchard and anisaddehyde reactions for this fraction gave strongly positive tests, i.e., red to immediate purple and red, respectively. Thin-layer plates (20 x 20 cm.) were prepared according to the standard method utilizing a Desaga apparatus. A chloroform solution of this fraction in chloroform-acetone (9:1) yielded only one spot with Rf 0.14, identical with ursolic acid run on the same plate. Standard solutions for all known compounds were in a concentration of 5 mg./10 ml.

The precipitate was dissolved in hot chloroform in order to remove inorganic salts, filtered, and concentrated to dryness. The light yellow powdery material was difficult to recrystallize although various authors reported obtaining sharp needles from ethanol. Plouvier (92) admitted the formation of a gelatinous

---

2C. De Saga, Heidelberg, Germany: U. S. Representative; Brinkman Instruments Co., Cantique Road, Westburg, New York.
Figure 1

Extraction Scheme for Sterols and/or Triterpenes.
product (geleé) after which needles were obtained upon slow evaporation of the solvent. Pourrat and LeMen (95) resorted to sublimation to obtain a purer product. Chloroform, petroleum ether, dichloromethane, or methanol did not yield needles and only after much difficulty were needles finally obtained from an ethanolic solution.

Fraction IV from *A. patula* demonstrated the presence of both ursolic acid and uvaol on a TLC plate (chloroform-methanol, 9:1). Advantage was taken of the presence of the carboxylic acid group in ursolic acid to separate the two compounds. A one-Gm. sample of fraction IV of *A. patula* was dissolved in 5% aqueous potassium hydroxide (25 ml.) and shaken out with anhydrous ether (25 ml. x 5). The ether phase on drying yielded an orange-yellow solid whereas the aqueous fraction after acidification and washing, contained a yellow-gold solid. Both fractions, the ethereal and aqueous, chromatographed (chloroform-methanol, 9:1) as uvaol and ursolic acid, respectively.

The melting points were determined on a Thomas Hoover (silicone bath) melting point apparatus with open end capillaries and are uncorrected. Fraction IV (needles) melted at 277-280°C, standard ursolic acid obtained from Dean A. Uhl, University of Wisconsin and recrystallized five times from ethanol melted at

---

225°C. Literature values vary from 224°C (3) to 291°C (38). A mixed melting point of fraction IV and standard ursolic acid melted at 240°C.

To verify the presence of ursolic acid a co-spotting experiment was conducted and the results are to be found in Table I. The information obtained from the melting points, mixed melting points, and chromatographic co-spotting confirmed the presence of ursolic acid (Figure 2) in fraction IV of both A. columbiana and A. patula.

**Table I. Co-spotting of Ursolic acid and Fraction IV**

<table>
<thead>
<tr>
<th>Compound</th>
<th>R_f values^a</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BE^b</td>
<td>CA^b</td>
<td>HBE^b</td>
</tr>
<tr>
<td>1. Ursolic acid</td>
<td>0.14</td>
<td>0.13</td>
<td>0.04</td>
</tr>
<tr>
<td>2. IV (A. columbiana)</td>
<td>0.13</td>
<td>0.12</td>
<td>0.04</td>
</tr>
<tr>
<td>3. Co-spot (1 and 2)</td>
<td>0.13</td>
<td>0.12</td>
<td>0.04</td>
</tr>
<tr>
<td>4. IV (A. patula)</td>
<td>0.13</td>
<td>0.13</td>
<td>0.04</td>
</tr>
<tr>
<td>5. Co-spot (1 and 4)</td>
<td>0.13</td>
<td>0.12</td>
<td>0.04</td>
</tr>
</tbody>
</table>

^aDetected with antimony trichloride (10% in chloroform w/w) and heated at 110°C for 5 minutes.

^bBE, benzene-ethyl acetate (4:1); CA, chloroform-acetone (9:1); HBE, heptane-benzene-ethanol (50:50:0.5).
Figure 2

Structures of Ursolic acid, β-amyrin, β-sitosterol and Uvaol.
Fraction V, *A. patula*. Only portions of fractions III were subjected to saponification as the need arose. Quantitatively, *A. columbiana* would yield 1.5 Gm. of V for each 20 Gm. of III saponified whereas *A. patula* would yield 1.5 Gm. per 30 Gm. saponified. TLC (chloroform - acetone, 9:1) demonstrated the presence of spots corresponding to β-amyrin, β-sitosterol, and uvaol, all of which were spotted on the same plate.

Column chromatography was deemed to be the best method to separate these components. Eight grams of fraction V from *A. patula* were thoroughly mixed with 10 Gm. of activated alumina 4 and placed in a hexane slurry. A 300 Gm. alumina column (50 x 240 cm.) prepared with hexane. All columns used were of glass construction supplied with a sintered glass plate and teflon stop-cocks. Eluting solvents (analytical grade) and their volumes were as follows:

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Composition</th>
<th>Volume (ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Hexane</td>
<td>1000</td>
</tr>
<tr>
<td>2.</td>
<td>Hexane - Benzene (1:1)</td>
<td>1000</td>
</tr>
<tr>
<td>3.</td>
<td>Benzene</td>
<td>3000</td>
</tr>
<tr>
<td>4.</td>
<td>Benzene-Chloroform (7:3)</td>
<td>3000</td>
</tr>
<tr>
<td>5.</td>
<td>Benzene-Chloroform (1:1)</td>
<td>4000</td>
</tr>
<tr>
<td>6.</td>
<td>Chloroform</td>
<td>2500</td>
</tr>
<tr>
<td>7.</td>
<td>Chloroform-Methanol (1:1)</td>
<td>2000</td>
</tr>
<tr>
<td>8.</td>
<td>Methanol</td>
<td>2000</td>
</tr>
</tbody>
</table>

4Matheson, Coleman, and Bell, East Rutherford, New Jersey.
Each fraction was concentrated to approximately ten ml. and examined chromatographically (chloroform - acetone, 9:1). The following results were obtained; Fractions 2 and 3 each had a weak pink component of Rf 0.82, fraction 4 had a spot comparable to \( \beta \)-amyрин, fraction 5 had three spots comparable to \( \beta \)-amyрин, \( \beta \)-sitosterol, and uvaol, fractions 6 and 7 each had a spot comparable to uvaol, and fraction 8 had a spot comparable to ursolic acid.

Fraction 1 did not yield a positive antimony trichloride spot on TLC but upon complete drying did yield 1.1 Gm. of white lustrous product. This material was recrystallized from acetone three times and gave a sharp melting point of 64\(^\circ\)C. Nonacosane \((C_{29}H_{60})\) has been previously extracted from plants and has the same solubility and melting point as fraction 1. Therefore, fraction 1 was tentatively identified as nonacosane.

Fractions 2 and 3 were combined, dried, and yielded 0.7 Gm. of additional nonacosane. The presence of another component in these fractions was suspected due to the development of a chromophore on the TLC plate. However, the mother liquors from the recrystalizations of nonacosane would not render any crystalline material, only a yellow-orange oily residue.

A semi-solid yellow residue (0.8 Gm.) was obtained from fraction 4 upon drying. Two recrystallizations from chloroform-methanol and three recrystallizations from petroleum ether
(light) yielded β-amyrin, melting point 194-196°C, standard β-amyrin, melting at 192-196°C, and a mixed melting point of 194-196°C. Literature values of β-amyrin range from 198-200°C. Co-spotting of the isolated product was performed to verify the results and are presented in Table II. The information obtained from melting points, mixed melting points, and chromatographic co-spotting confirmed the presence of β-amyrin (Figure 2) in *A. patula*.

Table II. Co-Spotting of β-amyrin and Column Fraction 4

<table>
<thead>
<tr>
<th>Compound</th>
<th>$R_f$ values$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BE$^b$</td>
</tr>
<tr>
<td>1. β-amyrin</td>
<td>0.50</td>
</tr>
<tr>
<td>2. Fraction 4</td>
<td>0.51</td>
</tr>
<tr>
<td>3. Co-spot (1 and 2)</td>
<td>0.51</td>
</tr>
</tbody>
</table>

$^a$Detected with antimony trichloride (10% in chloroform, w/w) and heated at 110°C for 5 minutes.

$^b$BE, benzene-ethyl acetate (4:1); CA, chloroform-acetone (9:1); HBE, heptane-benzene-ethanol (50:50:0.5).

$^5$Nutritional Biochemicals Corporation, Cleveland, Ohio.
Fraction 5 (0.7 Gm.) was thoroughly mixed with two Gm. of alumina (MCB), taken up in hexane, and placed upon a 100-Gm. alumina column (30 x 240 cm.) in order to obtain pure β-sitosterol. Eluting solvents were the same as those used in the previous column but the volumes consisted of four 125-ml. fractions of each solvent. Poor separation was again attained. The chloroform fractions contained more β-amyrin but the second chloroform-methanol fraction again contained all three components, i.e., β-amyrin-β-sitosterol, and uvaol. It was deemed necessary therefore to increase the polarity between chloroform and methanol more slowly. Ether was chosen as the most likely solvent with intermediate elutive properties.

The remaining 360 mg. fraction (second chloroform-methanol fraction) was placed upon a third column (100 Gm., 30 x 240 cm.) and eluted with the following:

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Composition</th>
<th>Volume (ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1'</td>
<td>Hexane</td>
<td>250</td>
</tr>
<tr>
<td>2'</td>
<td>Chloroform</td>
<td>2000</td>
</tr>
<tr>
<td>3'</td>
<td>Chloroform:ether (9:1)</td>
<td>125x4</td>
</tr>
<tr>
<td>4'</td>
<td>Chloroform:ether (3:1)</td>
<td>125x4</td>
</tr>
<tr>
<td>5'</td>
<td>Chloroform:ether (1:1)</td>
<td>125x4</td>
</tr>
<tr>
<td>6'</td>
<td>Ether</td>
<td>125x4</td>
</tr>
<tr>
<td>7'</td>
<td>Ether-Methanol (1:1)</td>
<td>125x4</td>
</tr>
<tr>
<td>8'</td>
<td>Methanol</td>
<td>500</td>
</tr>
</tbody>
</table>

Fractions 4' through 6' yielded a single chromatographic spot comparable to β-sitosterol (chloroform-methanol, 9:1).
These fractions were combined and yielded 200 mg. of yellow semi-solid residue. Fine white crystals were obtained from three recrystallizations from ethanol, melting at 135°C. Standard β-sitosterol\(^6\) melted from 133-135°C, a mixed melting point was not depressed, 133-135°C, and the literature value for β-sitosterol is reported at 135°C. Co-spotting was performed to verify the isolated product as β-sitosterol (Table III). The information obtained from melting points, mixed melting points, and chromatographic co-spotting confirmed the presence of β-sitosterol (Figure 2) in A. patula.

Table III. Co-spotting of β-sitosterol and Column Fractions 4'-6'

<table>
<thead>
<tr>
<th>Compound</th>
<th>(R_f) values(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BE(^b)</td>
</tr>
<tr>
<td>1. β-sitosterol</td>
<td>0.41</td>
</tr>
<tr>
<td>2. Fractions 4'-6'</td>
<td>0.42</td>
</tr>
<tr>
<td>3. Co-spot (1 and 2)</td>
<td>0.42</td>
</tr>
</tbody>
</table>

\(^a\)Detected with antimony trichloride (10% in chloroform, w/w) and heated at 110°C for 5 minutes.

\(^b\)BE, benzene-ethyl acetate (4:1); CA, chloroform-acetone (9:1); HBE, heptane-benzene-ethanol (50:50:0.5).

\(^6\)Nutritional Biochemicals Corporation, Cleveland, Ohio.
Fractions 6 and 7, chloroform and chloroform-methanol, from the initial column, yielded one spot comparable to uvaol. Drying of this fraction yielded 0.8 Gm. of a product which was recrystallized first from chloroform-methanol and then ethanol. The fine white crystals melted at 222-224°C. Standard uvaol was unobtainable and had to be synthesized from ursolic acid using a modified method of Nystrom and Brown (86), see below. Synthesized uvaol melted at 223-224°C. A mixture of the synthetic and natural uvaol gave a melting point of 223-224°C. Literature values show a melting point of 223°C. Chromatographic co-spotting results are shown in Table IV. The information obtained from melting points, mixed melting points, and chromatographic co-spotting confirmed the presence of uvaol (Figure 2) in A. patula.

Table IV. Co-spotting of Uvaol and Column Fractions 6 and 7

<table>
<thead>
<tr>
<th>Compound</th>
<th>( R_f ) values(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BE(^b)</td>
</tr>
<tr>
<td>1. Uvaol</td>
<td>0.32</td>
</tr>
<tr>
<td>2. Fractions 6 and 7</td>
<td>0.33</td>
</tr>
<tr>
<td>3. Co-spot (1 and 2)</td>
<td>0.33</td>
</tr>
</tbody>
</table>

\(^a\)Detected with antimony trichloride (10% in chloroform, w/w) and heated at 110°C for 5 minutes.

\(^b\)BE, benzene-ethyl acetate (4:1); CA, chloroform-acetone (9:1); HBE, heptane-benzene-ethanol (50:50:0.5).
Synthesis of uvaol was carried out by reducing ursolic acid to the corresponding alcohol with the aid of lithium aluminum hydride (LiAlH₄). Three grams of the acid were dissolved in 80 ml. of tetrahydrofuran (THF). One and one-half (1.5) Gm. of LiAlH₄ in 100 ml. of THF was placed in a 250-ml. 3-necked flask fitted with a reflux condenser, dropping funnel and mechanical stirrer. The opening was protected with a CaCl₂ drying tube until the reaction was completed. The acid solution was added to the LiAlH₄ solution via the dropping funnel at such a rate as to produce a mild reflux. It required approximately 15 minutes to add 80 ml. of solution. The solution was then refluxed one hour, the reflux halted and the flask was cooled. Rather than adding water, as the authors did, the reaction was halted with the addition of ethyl acetate. Ether was added to the cooled mixture and filtered and the process was repeated three times. The ethereal solution was washed with a saturated solution of NaSO₄ and 1N HCl and then taken down to dryness (ca. 1.7 Gm.). The product was recrystallized from CHCl₃-MeOH three times, melting at 223-224°C.

Fraction VI, A. patula. The dark green residue was examined chromatographically with standards (chloroform-acetone, 9:1) and found to contain only more ursolic acid and uvaol.
Fraction III, A. columbiana. The methods for isolating single components from fraction V of A. patula demonstrated the value of column chromatography. Rather than repeating the entire procedure for fraction V of A. columbiana the decision was made to determine the presence of the same components, none others being detected previously, with the use of TLC.

One hundred milligrams of fraction III was dissolved into 10 ml. of hot methanol-chloroform and used as the fraction containing all of the polycyclic compounds present in A. columbiana. Standards were also run on the plates alone and in combination with fraction III as an equal volume mixture. In so doing, if the \( R_f \) of a compound suspected in A. columbiana were not the same as the standard in three different systems, another spot with a different \( R_f \) would have developed. The results of this experiment are seen in Table V. No new spots were detected in any of the three systems in the co-spotted fractions. Therefore, the presence of \( \beta \)-amyrin, \( \beta \)-sitosterol, ursolic acid, and uvaol was confirmed in A. columbiana.

Results and Discussion

The methods proposed for the extraction and identification of polycyclic compounds from A. columbiana and A. patula appear to
TABLE V. Co-spotting of Standards and *A. columbiana* Fraction III

<table>
<thead>
<tr>
<th>Solvent systems</th>
<th>Rf values&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Co-spot (p-amyrin &amp; III)</th>
<th>Co-spot (β-sitosterol &amp; III)</th>
<th>Co-spot (Ursolic acid &amp; III)</th>
<th>Co-spot (Uvaol &amp; III)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Fraction III</td>
<td>β-amyrin</td>
<td>β-sitosterol</td>
<td>Ursolic acid</td>
</tr>
<tr>
<td>BE&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td>0.14</td>
<td>0.14</td>
<td></td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.32</td>
<td>0.32</td>
<td></td>
<td>0.32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.38</td>
<td>0.38</td>
<td></td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.48</td>
<td>0.48</td>
<td></td>
<td>0.48</td>
</tr>
<tr>
<td>CA&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td>0.08</td>
<td>0.08</td>
<td></td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.33</td>
<td>0.33</td>
<td></td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.42</td>
<td>0.42</td>
<td></td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.54</td>
<td>0.54</td>
<td></td>
<td>0.54</td>
</tr>
<tr>
<td>HBE&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td>0.10</td>
<td>0.10</td>
<td></td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.18</td>
<td>0.18</td>
<td></td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.29</td>
<td>0.29</td>
<td></td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.43</td>
<td>0.43</td>
<td></td>
<td>0.43</td>
</tr>
</tbody>
</table>

<sup>a</sup>Detected with antimony trichloride (10% in chloroform, w/w) and heated at 110°C for 5 minutes.

<sup>b</sup>BE, benzene-ethyl acetate (4:1); CA, chloroform-acetone (9:1); HBE, heptane-benzene-ethanol (50:50:5).
be reliable, dependable and applicable for screening other plants for similar components. The utility of the method remains in the functionality of the initial steps wherein one solvent is capable of extracting substances of diverse polarity. It extracted a straight chain hydrocarbon as well as sterols and triterpenes which have previously required a multi-stage extraction procedure.

Column adsorption chromatography was found to be highly functional and separated components in a greater yield than could have been attained with preparative paper chromatography.

The organic compounds were verified as being present by the use of TLC. However, it cannot be overemphasized that $R_f$ values are only comparable when determined on the same plate with standards. Wide fluctuations occur due to numerous factors and only under strict standard conditions should one attempt to compare $R_f$ data determined at different times. Nevertheless, the method obviates the need for large quantities of material which had to be isolated in the past and subjected to thorough chemical scrutiny before positive identification could be made. Some of these previous methods are outlined by Steiner and Holzen (109). However, these procedures (chemical) are required if compounds of unknown structure are found to be present.

The compounds found in the triterpene/sterol fraction are
not new and could be identified by melting points, mixed melting points, \( R_f \) data, and co-spotting. A fifth compound, nonacosane, was also isolated from both plants in relatively large quantities. It had been previously found in the Ericaceae, in cranberry pomace (75), but never in the genus *Arctostaphylos*.

All of the compounds are quite widespread throughout the plant kingdom and have been the subject of much biosynthetic work in the past five years. Emphasis on the triterpenoids has been great enough to warrant the publication of an extensive book on the subject (7). Nevertheless, a complete biogenetic pathway with all intermediates has not yet been established for any of the triterpenoids nor has there been any concerted investigation concerning the function of these compounds in plants.

**Phenolics and Related Components**

**Introduction**

Plant phenolic compounds represent a heterogeneous group of chemicals. They range from the relatively simple six carbon monomers to complex polymers which have a molecular weight in the thousands and are of unknown structure. Generally, most authors divide the phenolics into two classification; 1) the \( \text{C}_6 - \text{C}_3 - \text{C}_6 \) flavonoid compounds, including the condensed tannins, and 2)
the C₆ - C₃ and C₆ - C₁ compounds and their derivatives, including the hydrolyzable tannins. The diversity of these compounds multiplies the difficulties encountered in their research.

Compilations by Geissman in 1955 (29) and in 1962 (30) are standards for classical chemical techniques. Hillis (49), Goodwin (32) and Harborne (40) concern themselves more with the biochemical approaches and are excellent sources of information concerning recent techniques of isolation and identification. Robinson's (99) treatment, although brief, is valuable as a preliminary source of information. The chemotaxonomical data obtained from phenolic compounds and their derivatives has been well established and is best cited in works by Alston and Turner (4), Swain (112), and Hegnauer (47).

Methods of extraction for plant phenolic components vary greatly, depending on the type of compound sought and the source from which it is taken. Generally, most investigators prefer to remove non-phenolic, non-polar substances from the plant before trying to obtain the phenolic derivatives. Solvents used for the preliminary extraction of phenolics are generally methanol, ethanol or water.

The fractionation of simple phenolics is generally attained by partition between water and immiscible organic solvents, i.e.
ethyl acetate, ether, t-butyl alcohol, etc. Both the pH of the aqueous phase and the successive use of more or less polar solvents are employed to obtain relatively pure compounds. The fractionation of flavonoid compounds is somewhat more difficult since their solubility is dependent upon the nature of the glycoside and the degree of methylation.

Purification and identification techniques have greatly improved during the past decade due mainly to the increased use of all phases of chromatography. Previously, the classical technique of color reactions was extensively used to identify constituents. Clark and Nord (16) and Geissman (29) both discuss the classical color reactions. Some of the information gained from these classical techniques have since found applicability in chromatographic identification.

Chromatography of phenolic extracts has been well established as an essential technique. Seikel (102) stated in 1964 that paper chromatography was the most important method for separation. Since that time works by Randerath (97) and Stahl (107) illustrate the applicability of thin-layer chromatography (TLC) to this area of investigation.

Column chromatography has also received wide attention and a variety of agents have been used either for separating the
components of a mixture or for a preliminary purification procedure to remove polymers, i.e. tannins, lignans, etc. Several agents have been investigated. To date, polyamide and silica gel appear to be the best suited for isolating components from a mixture. Endres and Hörmann (24) have recently reviewed the value of polyamide in preparative and analytical chromatography and Hanson and Zucker (39) were able to separate eighteen phenolic components eluted from a silica gel column.

Characterization of flavonoid compounds is often done by spectrophotometric measurements. The combination of paper chromatography, to separate the components of an extract, and spectral data are invaluable in identifying and characterizing flavonoid compounds according to Geissman (29).

This portion of the phytochemical investigation of _A. columbiana_ and _A. patula_ was concerned with establishing the presence of several compounds known to exist in _A. uva-ursi_. Thin-layer chromatography and ultra-violet spectrophotometry were used as the essential techniques in determining the presence of the compounds being sought. TLC was used for separation and identification and UV was used for additional verification of the following compounds: arbutin, ellagic acid, gallic acid, hydroquinone, hyperin, quercetin, and o-pyrocatechuic acid.
The method of approach was to use fraction II from the previously cited extraction scheme and to obtain all of the above named components from it. Initial purification of fraction II was to include liquid-liquid fractionation. Hydrolysis, both acidic and basic, was also to be performed. The fractions were then to be chromatographed (TLC) with standards and the spots with the same \( R_f \) value as the standards removed from multiple channel plates. These eluates were then to be examined spectrophotometrically in appropriate dilutions. The eluates were then to be concentrated to dryness, reconstituted and co-spotted in three solvent systems. This approach would verify the presence of the suspected compounds without the need for a lengthy extraction procedure for each component from both plants.

The spectral analysis had to be altered slightly since it was found early in the investigation that the standards which were chromatographed and standards which were not would have slightly shifted maxima. Therefore both the chromatographed and non-chromatographed standards were recorded.

**Methodology**

**Preliminary Investigation**

Fraction II was assumed to contain all of the compounds that
were previously identified in *A. uva-ursi*. To verify this however, the marc from the initial Skelly B-methanol (1:1) extraction was again extracted for not less than four days in a Soxhlet apparatus with methanol (U.S.P.). The residue from this extraction, fraction I, was then compared chromatographically with fraction II. The results of this comparison are seen in Table VI and indicate that fraction I did not contain any components which were not already present in fraction II.

Table VI. The Number of Chromatographic Spots from Fractions I and II

<table>
<thead>
<tr>
<th></th>
<th>BMA&lt;sup&gt;b&lt;/sup&gt;</th>
<th>CEF&lt;sup&gt;b&lt;/sup&gt;</th>
<th>TEF&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. columbiana</em></td>
<td>5</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td><em>A. patula</em></td>
<td>5</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Fraction II</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. columbiana</em></td>
<td>6</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td><em>A. patula</em></td>
<td>8</td>
<td>11</td>
<td>11</td>
</tr>
</tbody>
</table>

<sup>a</sup>Detection with UV light, followed by 1% alcoholic ferric chloride.

<sup>b</sup>BMA, benzene-methanol-acetic acid (10:2:1); CEF, chloroform-ethyl acetate-formic acid (5:4:1); TEF, toluene-ethyl formate-formic acid (5:4:1).
Fractionation

Fraction II from both plants, *A. columbiana* (114 Gm.) and *A. patula* (153 Gm.) consisted of a dark brown viscous residue with an extremely bitter taste. The first step in the preliminary purification procedure was to submit fractions of each plant to liquid-liquid extraction. Samples (ca. 20 Gm.) were dissolved in warm distilled water (150 ml.) and were extracted for 24 hours each with ethyl acetate, U.S.P. (100 ml.) and then ether, U.S.P. (100 ml.). Each of the fractions was concentrated to near dryness (ca. 5 ml.) and designated fraction A (ethyl acetate), fraction B (ether) and fraction C (aqueous).

Extracts (fraction II) of both plants were also submitted to acidic and basic hydrolysis. Acidic hydrolysis was conducted by refluxing samples in 2N hydrochloric acid, C.P. for one hour. The refluxing flask was then cooled and the acid solution was then extracted for 24 hours with ether, U.S.P., on a liquid-liquid extractor. This was then concentrated to dryness, redissolved with 5 ml. of methanol and labeled fraction D.

Basic hydrolysis was conducted by refluxing the samples with 5% aqueous potassium hydroxide for one hour, cooling, acidifying, and extracting for 24 hours with ether, U.S.P., on a liquid-liquid extractor. This was then concentrated to dryness, redissolved with 5 ml. of methanol and labeled fraction F. The
remaining aqueous fractions from both the acidic hydrolysis and
the basic hydrolysis were labeled fraction E and G, respectively.
Figure 3 demonstrates more clearly the complete fractionation
scheme.

Further fractionation was not attempted by chemical means.
Each of the fractions was subjected to TLC in several solvent
systems along with standards (5mg./10ml.) and sprayed with
various reagents. In this manner, each of the suspected components
would be located and eluted from a second plate which was spotted
in several channels with the fraction in which the compound was
located. Although the majority of the compounds were located in
fraction A, the other fractions were of aid in verifying the presence
of the compound. For example, a compound containing neither
an ether nor an ester linkage would undoubtedly be found in fractions
A, D, and F. If a compound were a glycoside then it should be
found mainly in fraction D and possibly some of the aglycone in
fraction A.

The identified spot and the standard were then removed from
the second plate with the aid of a "vacuum-zone extractor" originally
described by Mathews (77). Hot methanol (5 ml.) was then used to
elute the compounds from the silica gel in the extractor. These
5-ml. solutions were diluted and then spectrophotometrically
Figure 3

Fractionation of Phenolic Components.

Fraction II

1) Water
2) Ethyl acetate

Organic (A)
Aqueous

1) HCl
2) Reflux
3) Ethyl ether

Organic (D)
Aqueous (E)

1) KOH
2) Reflux
3) Ethyl ether

Aqueous (G)

Organic (B)
Aqueous (C)

Ethyl ether

Organic (F)
Aqueous (G)
examined in a Beckman DB spectrophotometer,\textsuperscript{7} model 1401. The solutions were then concentrated to dryness, dissolved in not more than 1 ml. of methanol and co-spotted in three TLC systems.

Determination of Compounds Present

Arbutin (Figure 4). Chemically, arbutin is hydroquinone β-D-glucoside and has been found in five widely separated families, but is of erratic occurrence in each. As mentioned previously, this compound was first isolated from \textit{A. uva-ursi} and hence its trivial name.

A preliminary screening of fraction A of both plants in benzene-pyridine-acetic acid (BPA, 36:9:5) indicated the presence of arbutin after spraying with Millon's reagent (5 Gm. of mercury in 10 Gm. of fuming nitric acid and diluted with 10 ml. of water). The R\textsubscript{f} of standard arbutin\textsuperscript{8} and spots from both fractions were 0.06. The standard arbutin gave a typical yellow coloration with Millon's reagent whereas the plant extracts were slightly brownish. Another plate was prepared using at least five separate channels for each fraction, i.e. \textit{A. columbiana}, \textit{A. patula}, and standard arbutin, and developed in the same system. The zones

\textsuperscript{7}Beckman Instruments Company, Fullerton, California.

\textsuperscript{8}City Chemical Corporation, New York, New York.
Structures of Arbutin, Hydroquinone, Hyperin and Quercetin.
corresponding to arbutin, detected by spraying one channel of each fraction, was eluted with the extractor and processed as previously described. Co-spotting was performed in three systems (Table VII) and the UV spectra were recorded (Table XIII).

Table VII. Co-spotting of Standard and Eluted Arbutin

<table>
<thead>
<tr>
<th>Compound</th>
<th>Rf values&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BMA&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1. Arbutin</td>
<td>0.11</td>
</tr>
<tr>
<td>2. <em>A. columbiana</em> arbutin</td>
<td>0.11</td>
</tr>
<tr>
<td>3. Co-spot (1 and 2)</td>
<td>0.11</td>
</tr>
<tr>
<td>4. <em>A. patula</em> arbutin</td>
<td>0.11</td>
</tr>
<tr>
<td>5. Co-spot (1 and 4)</td>
<td>0.11</td>
</tr>
</tbody>
</table>

<sup>a</sup>Detected with Millon's reagent.

<sup>b</sup>BMA, benzene-methanol-acetic acid (10:2:1); CAW, chloroform-acetic acid-water (35:50:17.5); TEF, toluene-ethyl formate-formic acid (5:4:1).

Although arbutin is often found to be present in conjunction with its methyl ester, methyl arbutin, this fact could not be substantiated in this investigation.

**Ellagic acid** (Figure 5). The chemistry of ellagic acid and its ether derivatives has been recently reviewed by Jurd (62). It
Ellagic acid

Gallic acid

o-Pyrocatechuic acid

Figure 5

Structures of Ellagic, Gallic, and o-Pyrocatechuic acids.
is formed by the oxidative coupling of two molecules of gallic acid, is found free and combined with sugars, and has been detected in at least forty plant families.

Table VIII. Co-spotting of Standard and Eluted Ellagic Acid

<table>
<thead>
<tr>
<th>Compound</th>
<th>( R_f ) values(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BAW(^b)</td>
</tr>
<tr>
<td>1. Ellagic acid</td>
<td>0.03</td>
</tr>
<tr>
<td>2. \textit{A. columbiana} ellagic acid</td>
<td>0.03</td>
</tr>
<tr>
<td>3. Co-spot (1 and 2)</td>
<td>0.03</td>
</tr>
<tr>
<td>4. \textit{A. patula} ellagic acid</td>
<td>0.03</td>
</tr>
<tr>
<td>5. Co-spot (1 and 4)</td>
<td>0.03</td>
</tr>
</tbody>
</table>

\(^a\)Detected with 1% alcoholic ferric chloride.

\(^b\)BAW, butanol-acetic acid-water (4:1:1); BAW, butanol-acetic acid-water (4:1:5, organic); BMA, butanol-methanol-acetic acid (10:2:1).

Standard ellagic acid\(^9\) showed very little mobility on thin-layer plates even in aqueous systems by means of which it has been previously separated on paper chromatograms. Since toluene-ethyl formate-formic acid (TEF, 5:4:1) appeared to move all

\(^9\)City Chemical Corporation, New York, New York.
compounds of fraction A from the application site, leaving only ellagic acid, it was removed from a plate developed in this system. Ellagic acid itself can be detected with many sprays but was detected in this phase with 5% methanolic phosphomolybdic acid, yielding a grey color. Co-spotting was then performed employing three systems (Table VIII) and UV spectra were recorded (Table XIII).

**Gallic acid** (Figure 5). One large group of hydrolyzable tannins consists of those which yield gallic acid on hydrolysis. Gallic acid, 3,4,5-trihydroxy benzoic acid, has been identified in hundreds of species of plants. It exists freely or more often in many chemical combinations, either with itself, as di- or trigallic acid, in combinations with sugars, as di- or trigalloylglucose, or in combinations with catechins, as 3-galloyl-catechin.

Standard gallic acid had an $R_f$ value of 0.66 in chloroform-acetic acid-water (CAW, 35:5:17.5), and gave a typical purple color with 1% alcoholic ferric chloride spray and a grey-purple color with 5% methanolic phosphomolybdic acid spray after mild heating. Fraction A and D, from both plants demonstrated the presence of a compound with the same $R_f$ value and color reactions. A second plate was developed in the same system with multiple

---

10J. T. Baker Chemical Company, Phillipsburg, New Jersey.
channels of fraction A from both plants and standard gallic acid. Co-spotting of the eluted compounds was then performed in three systems (Table IX) and UV spectra were recorded (Table XIII). Only the spectra of the non-chromatographed standard showed a sharp peak whereas the other compounds displayed a wide band of up to five millimicrons in width.

Table IX. Co-spotting of Standard and Eluted Gallic Acid

<table>
<thead>
<tr>
<th>Compound</th>
<th>( R_f ) values(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BMA(^b)</td>
</tr>
<tr>
<td>1. Gallic acid</td>
<td>0.20</td>
</tr>
<tr>
<td>2. \textit{A. columbiana} gallic acid</td>
<td>0.19</td>
</tr>
<tr>
<td>3. Co-spot (1 and 2)</td>
<td>0.20</td>
</tr>
<tr>
<td>4. \textit{A. patula} gallic acid</td>
<td>0.19</td>
</tr>
<tr>
<td>5. Co-spot (1 and 4)</td>
<td>0.19</td>
</tr>
</tbody>
</table>

\(^a\)Detected with 5% methanolic phosphomolybdic acid.

\(^b\)BMA, benzene-methanol-acetic acid (10:2:1); CEF, chloroform-ethyl formate-formic acid (5:4:1); TEF, toluene-ethyl formate-formic acid (5:4:1).

\textbf{Hydroquinone} (Figure 4). According to Harborne and Simmonds (41) hydroquinone is perhaps the most widely distributed of the simple plant phenols, having been found in the Ericaceae,
Rosaceae, Proteaceae and the Compositae.

Standard hydroquinone\textsuperscript{11} was chromatographed along with fractions A and D from both plants to screen for its possible presence. Benzene-dioxane-acetic acid (BDA, 90:25:4) demonstrated an identical spot (yellow with Millon's reagent) with the standard in fraction D of both plants but only in \textit{A. columbiana} fraction A. \textit{A. patula} fraction A did not appear to contain hydroquinone in the free form. A second system of chloroform-acetic acid-water (CAA, 35:50:17.5) did demonstrate, however, the presence of hydroquinone in all four samples, i.e. fraction A and D of both plants. A third plate was run in the latter system (CAA) with multiple channels using fraction A of both plants and the standard. Co-spotting was then performed in three different systems (Table X) and the UV spectra were recorded (Table XIII). Broad bands were obtained for all spectra.

Hyperin (Figure 4). Quercetin-3-galactoside, hyperin, is of limited occurrence in the plant kingdom. As mentioned previously, it has been isolated from \textit{A. uva-ursi} and from only five species of other unrelated families (46).

Standard hyperin\textsuperscript{12} was chromatographed along with fraction A

\textsuperscript{11} Matheson, Coleman and Bell, East Rutherford, New Jersey.

\textsuperscript{12} Obtained through the courtesy of Prof. Dr. L. Hörhammer, München, Germany.
from both plants in toluene-ethyl formate-formic acid (TEF, 5:4:1) and was detected at $R_f$ 0.02 with 5% methanolic phosphomolybdic acid. Several channels of each were chromatographed on a second plate and eluted from it in the manner described. Co-spotting was performed on these fractions (Table XI) and the UV spectra were recorded (Table XIII). An interesting fact was noted in the spectrum of hyperin. Geissman (29) reported three maxima, 362.5, 312, and 258 μm whereas Hattori (46) only reports two, 359 and 254 μm. Although there was an extremely small shoulder at 310 in the standard hyperin not run on the plate, no such shoulder or peaks were evidenced in those samples which were chromatographed.

Table X. Co-spotting of Standard and Eluted Hydroquinone

<table>
<thead>
<tr>
<th>Compound</th>
<th>$R_f$ values&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BMA&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1. Hydroquinone</td>
<td>0.38</td>
</tr>
<tr>
<td>2. &lt;i&gt;A. columbiana&lt;/i&gt; hydroquinone</td>
<td>0.38</td>
</tr>
<tr>
<td>3. Co-spot (1 and 2)</td>
<td>0.38</td>
</tr>
<tr>
<td>4. &lt;i&gt;A. patula&lt;/i&gt; hydroquinone</td>
<td>0.38</td>
</tr>
<tr>
<td>5. Co-spot (1 and 4)</td>
<td>0.38</td>
</tr>
</tbody>
</table>

<sup>a</sup>Detection with 5% methanolic phosphomolybdic acid.

<sup>b</sup>BMA, benzene-methanol-acetic acid (10:2:1); CEF, chloroform-ethyl formate-formic acid (5:4:1); TEF, toluene-ethyl formate-formic acid (5:4:1).
Table XI. Co-spotting of Standard and Eluted Hyperin

<table>
<thead>
<tr>
<th>Compound</th>
<th>R_f values&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BMA&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1. Hyperin</td>
<td>0.10</td>
</tr>
<tr>
<td>2. A. columbiana hyperin</td>
<td>0.09</td>
</tr>
<tr>
<td>3. Co-spot (1 and 2)</td>
<td>0.10</td>
</tr>
<tr>
<td>4. A. patula hyperin</td>
<td>0.09</td>
</tr>
<tr>
<td>5. Co-spot (1 and 4)</td>
<td>0.10</td>
</tr>
</tbody>
</table>

<sup>a</sup>Detection with 5% methanolic phosphomolybdic acid.

<sup>b</sup>BMA, benzene-methanol-acetic acid (10:2:1); CAW, chloroform-acetic acid-water (35:50:17.5); TEF, toluene-ethyl formate-formic acid (5:4:1).

_0-Pyrocatechuic acid_ (Figure 5). Acid hydrolyzed leaf extracts of at least seven genera in the Ericaceae have been shown to contain _0-pyrocatechuic acid_, known chemically as 2,3-dihydroxybenzoic acid (56). There are only four other reports of its natural occurrence in species of _Populus_, _Asclepias_, _Calotropis_ and _Vinca_ (41).

A standard sample<sup>13</sup> of the acid was chromatographed and R_f values were obtained in the following solvent systems: BAW (4:1:5), R_f 0.67; BPA (36:9:5), R_f 0.32; BDA, (90:25:4); R_f 0.25; CEF (5:4:1), R_f 0.58; TEF (5:4:1), R_f 0.44; and BMA (80:16:8),

<sup>13</sup>Obtained through the courtesy of Dr. R. K. Ibrahim, Alexandria, Egypt.
R_f 0.28. Spots are readily detectable with mild heating as a
definite pink color and also with 1% alcoholic ferric chloride. In
several systems standard catechol^ had the same R_f as the standard
o-pyrocatechuic acid, but could be readily differentiated by its dark
grey color with mild heat. In none of the above mentioned six
systems was a compound found, from fraction D of either plant,
that possessed the same R_f value as standard o-pyrocatechuic acid.
Therefore, o-pyrocatechuic acid was assumed to be absent in
A. columbiana and A. patula.

Quercetin (Figure 4). The flavonol quercetin (5, 7, 3', 4'
flavonol) is of such widespread nature that Bate-Smith (6) has
remarked that it is of more importance when it is not found in a
plant rather than when it is found. In fact, Swain and Bate-Smith
(113) have cited a frequency of occurrence of 62% for quercetin in
the leaves of dicotyledons.

Fraction A was investigated for the presence of quercetin
since fraction D would have contained quercetin as a degradation
product of one of the glycosides, hyperin or isoquercitrin.

Standard quercetin^ was best separated from other compo-
nents in fraction A in BPA (36:9:5), giving a blue-purple color

---

^Matheson, Coleman and Bell, East Rutherford, New Jersey.

with 5% methanolic phosphomolybdic acid or a tan color with 1% alcoholic ferric chloride (Rf 0.36). A second multiple channel plate was run in the same system and the spots were eluted off as previously described. Co-spotting was performed on these fractions (Table XII) and UV spectra were recorded (Table XIII).

Table XII. Co-spotting of Standard and Eluted Quercetin

<table>
<thead>
<tr>
<th>Compound</th>
<th>Rf values\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BMA\textsuperscript{b}</td>
</tr>
<tr>
<td>1. Quercetin</td>
<td>0.36</td>
</tr>
<tr>
<td>2. \textit{A. columbiana} quercetin</td>
<td>0.36</td>
</tr>
<tr>
<td>3. Co-spot (1 and 2)</td>
<td>0.36</td>
</tr>
<tr>
<td>4. \textit{A. patula} quercetin</td>
<td>0.36</td>
</tr>
<tr>
<td>5. Co-spot (1 and 4)</td>
<td>0.36</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Detection with 5% methanolic phosphomolybdic acid.

\textsuperscript{b}BMA, benzene-methanol-acetic acid (10:2:1); CAW, chloroform-acetic acid-water (35:50:17.5); TEF, toluene-ethyl formate-formic acid (5:4:1).

Quercetin-3-glucoside, isoquercitrin, could not be substantiated in either plant due to the inavailability of the standard material. However, a yellow spot which was neither quercetin nor hyperin, was evidenced in fraction \textit{A} in the following systems: BPA (36:9:5), Rf 0.14; CAW (35:50:17.5), Rf 0.46; and BAW
TABLE XIII. UV Maxima of Eluted Compounds, Eluted Standards and Pure Standards\textsuperscript{a, b}

<table>
<thead>
<tr>
<th>Compound</th>
<th>A. columbiana</th>
<th>A. patula</th>
<th>Standard</th>
<th>Pure Standards\textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Arbutin</td>
<td>282</td>
<td>283</td>
<td>283</td>
<td>282</td>
</tr>
<tr>
<td>2. Ellagic acid</td>
<td>254</td>
<td>254</td>
<td>253</td>
<td>252</td>
</tr>
<tr>
<td>3. Gallic acid</td>
<td>272</td>
<td>273</td>
<td>273</td>
<td>272</td>
</tr>
<tr>
<td>4. Hydroquinone</td>
<td>290</td>
<td>290</td>
<td>290</td>
<td>289</td>
</tr>
<tr>
<td>5. Hyperin</td>
<td>360,255</td>
<td>360,255</td>
<td>360,255</td>
<td>360,310\textsuperscript{d}, 255</td>
</tr>
<tr>
<td>6. Quercetin</td>
<td>370,252</td>
<td>370,253</td>
<td>370,252</td>
<td>372,255</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Narrow slit setting, scan beginning at 400 m\(\mu\), chart speed of 40 m\(\mu\)/ minute.

\textsuperscript{b}Samples in analytical grade methanol.

\textsuperscript{c}Not chromatographed.

\textsuperscript{d}Only an inflexion.
Results and Discussion

Several phenolic components were found to be present in *A. columbiana* and *A. patula* which were known to be present in *A. uva-ursi*. Separation and identification was accomplished by the use of TLC and verification was conducted with UV spectra and co-chromatography.

Arbutin, ellagic acid, gallic acid, hydroquinone, hyperin and quercetin were definitely established in both plants. Methyl-arbutin and isoquercitrin appeared to be present but could not be substantiated. The presence of *o*-pyrocatechuic acid could not be substantiated in either plant. The lack of this compound in these plants is chemotaxonomically significant since it was assumed to be present throughout the family (56).

The methodology employed did demonstrate the applicability of TLC and UV spectra for the relatively rapid screening of various phenolic components from partially purified plant extracts. Previous investigators have had to use two dimensional paper chromatography in aqueous systems in order to elucidate the presence of phenolic compounds, which is often a more lengthy procedure than the one described herein. The procedure could be altered slightly
if an investigator wished to further identify suspected components. This could be easily accomplished by using larger glass plates on a preparative scale and obtaining working quantities of suspected components.
ANTIBACTERIAL-ANTIFUNGAL SCREENING

Methodology

Osborne (88) in 1943 was the first to screen higher plants for antibiotic activity. A total of 2300 species were tested. Since then numerous investigators have screened plants against various microorganisms in order to obtain more efficacious antibiotics. The review by Skinner (105) in 1955 is the most complete compilation of all work done in this area to date. In 1959, Nickell (85) carried out an extensive literature survey of all plants which were reported to contain antimicrobial activity.

A general antibiotic screen of Arctostaphylos species has not been performed. The expressed juices of A. patula have been reported to be effective against Mycobacterium tuberculosis but no mention of the plant part or the concentration of the extract was cited (5). Arctostaphylos uva-ursi was found to be effective against Bacillus subtilis, Escherichia coli and Staphylococcus aureus, but again, no mention was made of the part used, the extraction solvent, or the concentration (121). Therefore, it was deemed necessary to screen crude extracts of both A. columbiana

16 The author is grateful to Mrs. D. Bickford and Mr. F. Campbell for their assistance in this phase of the investigation.
and *A. patula* as well as the compounds found to be present in both plants.

**Preparation of Samples**

Two different solvent systems were used to extract components from the ground leaves of both plants for the preparation of crude extracts. The first was the Skelly B-methanol (1:1) mixture and the second was a 70% ethanol mixture. The latter was used in order to verify the extraction capabilities of the former as pertaining to antibiotic compounds. Many previous investigators have also used more polar solvents for extraction. In fact, Kabadi and Hammarlund (63) used water to extract an antibacterial substance, "madronin", from *Arbutus menziesii* (Ericaceae).

Cold extraction was conducted by shaking a five-Gm. sample of plant material in 150 ml. of the appropriate solvent on a reciprocal shaker for at least 24 hours. The extracts were then filtered through a Büchner funnel and concentrated to dryness in vacuo. Samples were then stored under refrigeration and aliquots were drawn from these extracts as needed.

The known compounds employed were obtained from the sources previously cited and were placed in solution shortly before being used.
Experimental methods

The small tube method of Catalfomo and Schultz (13) was used exclusively in order to evaluate the antimicrobial activity of the fractions and compounds under investigation. The bacterial and fungal species as well as the method of preparation were identical to those cited in their work.

Solutions of all the fractions and compounds tested were prepared in 70% ethanol in the appropriate dilutions. Some of the compounds were not entirely soluble in this solvent and were therefore mixed into as fine a suspension as possible before placing in the tubes. The previous workers had shown that the presence of surface crystals did not affect the growth of the organisms in this technique. Blanks were designated as those tubes which contained the respective microorganisms along with the solvent, and controls were those tubes which contained the microorganisms only.

Readings of the tubes were taken at 24, 48, and 72 hours. However, the 48 hour readings are those reported. The earlier readings were insignificant since the microorganisms had not established reasonable growth even in the control tubes. The later readings were insignificant as far as their deviation from the 48 hour readings.
Results and Discussion

The antibacterial-antifungal studies performed on extracts of *A. columbiana* and *A. patula* as well as ten compounds shown earlier to exist in these plants are reported in Table XIV.

The results obtained for all the crude extracts demonstrated definite fungicidal activity against *Trichophyton mentagrophytes*. Crude extracts from *A. columbiana* (Skelly B-methanol) also demonstrated activity against the other fungus, *Candida albicans*. Surprisingly none of the *A. patula* extracts was as effective against *C. albicans*. In contrast, *A. patula* (Skelly B-methanol) extracts were antibacterial against *E. coli* but none of the *A. columbiana* extracts was as effective.

Among the quinoid compounds, hydroquinone demonstrated antibacterial properties against both bacteria whereas arbutin was effective only in a higher concentration against *S. aureus*.

The flavonoids, quercetin and hyperin, demonstrated contrasting results. Quercetin displayed equivocal results against all but *S. aureus* whereas the galactoside, hyperin, had no such property. It would be of interest to further investigate this phenomenon to determine if the 3-hydroxyl group is required for activity.

The phenolic carboxylic acids, gallic and ellagic acid, demonstrated diverse results. Gallic acid possessed some activity
Table XIV. Antibacterial-Antifungal Studies

<table>
<thead>
<tr>
<th>Compound and Concentration</th>
<th>E. coli</th>
<th>S. aureus</th>
<th>C. albicans</th>
<th>T. mentagrophytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. columbiana&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:100 ±&lt;sup&gt;c&lt;/sup&gt;</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>-</td>
</tr>
<tr>
<td>1:500 ±</td>
<td>±</td>
<td>+</td>
<td>±</td>
<td>-</td>
</tr>
<tr>
<td>A. columbiana&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:100 +</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1:500 +</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A. patula&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:100 +</td>
<td>+</td>
<td>+</td>
<td>±</td>
<td>-</td>
</tr>
<tr>
<td>1:500 +</td>
<td>+</td>
<td>+</td>
<td>±</td>
<td>-</td>
</tr>
<tr>
<td>A. patula&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:100 -</td>
<td>-</td>
<td>+</td>
<td>±</td>
<td>-</td>
</tr>
<tr>
<td>1:500 -</td>
<td>-</td>
<td>+</td>
<td>±</td>
<td>-</td>
</tr>
<tr>
<td>Arbutin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:100 +</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1:500 +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hydroquinone</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:100 ±</td>
<td>±</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1:500 ±</td>
<td>±</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Quercetin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:100 ±</td>
<td>±</td>
<td>+</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>1:500 ±</td>
<td>±</td>
<td>+</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>Hyperin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:100 +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1:500 +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Gallic acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:100 ±</td>
<td>±</td>
<td>-</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>1:500 +</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>±</td>
</tr>
</tbody>
</table>

<sup>a</sup>dried 70% ethanol extract.
<sup>b</sup>dried Skelly B-MeOH (1:1) extract.
<sup>c</sup>+, growth; -, no growth; ± equivocal growth.
Table XIV. --Continued

<table>
<thead>
<tr>
<th>Compound and Concentration</th>
<th>E. coli</th>
<th>S. aureus</th>
<th>C. albicans</th>
<th>T. mentagrophytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ellagic acid 1:100 ±</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ellagic acid 1:500 ±</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>β-amyrin 1:100 ± ±</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>β-amyrin 1:500 ± ±</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>β-sitosterol 1:100 +</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>β-sitosterol 1:500 +</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>Ursolic acid 1:100 +</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ursolic acid 1:500 +</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Uvaol 1:100 +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Uvaol 1:500 +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

against all organisms except C. albicans. In contrast, ellagic acid had only equivocal activity against E. coli.

The triterpenes and sterols were somewhat difficult to evaluate. The precipitates of the compounds upon the surface of the tubes made observations of bacterial growth difficult in early readings. However, at the 48 hour reading the precipitates of all the compounds were more discernable from bacterial colonies. The triterpene, β-amyrin, demonstrated the widest spectrum of
activity whereas uvaol demonstrated no activity. Controls as well as blanks showed a luxuriant growth of the microorganisms.

Generally, screening of the known compounds did not warrant their further investigation for antimicrobial activity. The fact that the crude extracts and not any of the isolated compounds possessed a strong inhibitory activity against \textit{T. mentagrophytes} suggests the presence of an antifungal compound or compounds in these extracts. The antifungal activity of \textit{A. columbiana} (Skelly B-methanol) against \textit{C. albicans} and the antibacterial activity of \textit{A. patula} (Skelly B-methanol) against \textit{E. coli} also appears to warrant more investigation.
GENERAL RESULTS AND DISCUSSION

The isolation and identification of organic compounds from plant sources is extremely challenging and demands the use of the most recent techniques available. Early works in phytochemistry were most often spent with the identification of only one compound from a plant source whereas today it is not uncommon to have several compounds identified.

The methodology employed in this study has taken advantage of the recent advances in phytochemistry for the elucidation of the compounds present. A new extraction technique was utilized for the first time whereby one polar-non-polar solvent mixture was used to remove all the compounds of two different chemical classes, i.e., triterpene/sterols and phenolic derivatives. Multi-channel TLC procedures separated adequate quantities of pure phenolics which were then extracted from the plate and identified by means of UV spectrophotometry and co-chromatography. This technique of identification has been previously unrecorded. The screening of plant extracts and pure plant components for antimicrobial activity was the first report of the new small tube method used in this way.

The results obtained in this investigation give credit to these aforementioned techniques. Several compounds were found to exist in the genus Arctostaphylos which had not been previously known
to be present. Also, the presence of several other components known to exist in a closely related species (A. uva-ursi) was substantiated.

Ursolic acid and uvaol were found to be present in both A. columbiana and A. patula as assumed. Also found to be present in the species for the first time were the triterpene, β-amyrin, the sterol, β-sitostosterol, and the hydrocarbon, nonacosane. All of the compounds were isolated from A. patula and their presence was verified in A. columbiana by chromatographic procedures, with the exception of nonacosane which was also isolated from A. columbiana.

The presence of the phenolic compounds and their derivatives was determined by the use of TLC and UV spectra. The method developed herein was effective and rapid for the detection of six compounds. Arbutin, ellagic acid, gallic acid, hydroquinone, hyperin, and quercetin were definitely established to be present in both plants. Methyl arbutin and isoquercitrin also appeared to be present but could not be substantiated. Although o-pyrocatechuic acid was recently found to exist in acid hydrolyzed extracts of A. uva-ursi and other Ericaceae this investigation could not show it to exist in either A. columbiana or A. patula. Six different solvent systems (TLC) were used for screening the acid hydrolyzed
extracts and none had a positive o-pyrocatechuic acid spot. This fact could be of considerable chemotaxonomic importance.

Crude extracts of both plants as well as samples of the compounds shown to exist in the plants were screened for antibacterial (E. coli and S. aureus) and antifungal (C. albicans and T. mentagrophytes) activity. The most interesting result obtained was the fungicidal activity of all the crude extracts against T. mentagrophytes. None of the pure compounds screened were as fungicidal as the crude extracts and the active constituent therefore still remains unknown. Skelly B-methanol (1:1) extract of A. columbiana was also strongly fungicidal to C. albicans and the same solvent extracted a bacteriacidal principle (to E. coli) from A. patula. Of the pure compounds tested arbutin, gallic acid, and hydroquinone were the only agents which demonstrated more than equivocal toxicity toward any of the organisms. However, equivocal growth should not be looked upon as an entirely negative result. Compounds should be further tested against different organisms and under varying conditions.

Several unresolved questions have arisen during the course of the investigation and demand more attention. The difficulty encountered with the recrystallization of ursolic acid is puzzling and would be a challenging encounter for one interested in the problem
of crystal formation. The presence of more phenolic substances in *A. patula* than in *A. columbiana* extracts (Table VI) and the absence of o-pyrocatechuic acid in both the plants would be of great interest from the chemotaxonomical point of view. The antifungal agent present in both species has been shown not to be any of the phenolic compounds tested and thus appears to be worthy of further investigation.

Generally, the results demonstrated the applicability of phytochemistry to studying the chemotaxonomy of plants. Knowledge of the types of compounds which exist in one species is invaluable in determining the presence of chemically related compounds in another species of the same genus. The results obtained herein should be a definite aid to further study of the genus. The method of approach to phytochemical investigations are varied but the methodology utilized in this investigation can be of value to other workers interested in this area of research.
CONCLUSIONS

The presence of eleven organic compounds has been established in _A. columbiana_ and in _A. patula_.

A thorough library investigation revealed the nature of all the organic compounds which were known to exist in the genus to date. This information greatly aided the investigation in that a more thorough examination of the plant for specific compounds was accomplished.

Neither _A. columbiana_ nor _A. patula_ had been previously screened phytochemically. Of the eleven compounds identified, three had not been demonstrated in the genus previously. Two other compounds could not be definitely established as being present. One other compound shown recently to exist in _A. uva-ursi_ and other Ericaceae could not be demonstrated in either of the species investigated.

Antimicrobial screening revealed that the crude extracts contained a definite antifungal activity and none of the isolated compounds from these extracts showed similar activity.
BIBLIOGRAPHY


84. ---. Biosynthesis of sclareol, β-sitosterol, and oleanolic acid from mevalonic acid-2-C\(^{14}\). *Journal of Biological Chemistry* 237:1481-1484. 1962.


117. Ullsperger, E. and H. Ullsperger. Extraction of triterpenic 
(Abstracted in Chemical Abstracts 60:10482c. 1963)

118. U. S. Department of Agriculture. Key to important woody 
plants of eastern Oregon and Washington. Washington, D. C. 

119. Vogel, Gunther and Horst Ubel. Das Therapeutisch Wirksame 
Prinzip der Rosskastanie (Aesculus hippocastunum). 

120. Wall, M. E. et al. Steroidal sapogenins. I. Extraction, 
isolation and identification. Journal of Biological Chemistry 

121. Watanabe, T. Antibiotic components of plants. Annual Reports 
of the Takeda Research Laboratory 14:92-111. 1955. 
(Abstracted in Chemical Abstracts 50:5986i. 1956)

122. Watson, Leslie. The taxonomic significance of certain 
anatomical variations among Ericaceae. Journal of the 
Linnean Society (Botany) 59:111-125. 1964.


on the excretion of Na and K in rats. Journal of the 
American Pharmaceutical Association, Scientific Edition, 

Arctostaphylos. Madrono 5:38-47. 1939.

126. Winter, A. G. and L. Willcha. Unter suchungen über 
Antibiotica aus höheren Pflanzen. Naturwissenschaften 
39:45-46. 1952.

Saponinreihe. VIII. Über Ursolsäure. Zeitschrift fur 

