Chapter 2

Chemical examination of Piper colubrinum Link
Chemical examination of *Piper colubrinum* Link

2.1 Introduction

The genus *Piper* belongs to the family Piperaceae, which is represented by more than 700 species distributed throughout the tropical and subtropical regions of the world\(^1\). Several species occur in Kerala, among which the most important economic being black pepper (*Piper nigrum*). Most of the *Piper* species are reputed in the Indian Ayurvedic system of medicine for their medicinal properties\(^2\). Due to their economic and medicinal importance, several *Piper* species have been investigated worldwide for their chemical constituents and pharmacological properties and several reviews have appeared on these subjects\(^3\)\(^-\)\(^6\). A comprehensive review by Parmar *et. al.* lists the secondary metabolites isolated from *Piper* species up to June 1996\(^1\).

2.2 Chemistry of genus *Piper* - an overview

An extensive survey on the phytochemical literature has been carried out as a part of the present work and the trends in the phytochemistry of the genus is overviewed here. The secondary metabolites isolated from the genus are broadly classified as

1. Amides
2. Lignans
3. Terpenes
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4. Kawapyrones

5. Flavonoids

6. Miscellaneous compounds

A brief and general discussion is intended for the above groups, while a complete compilation of flavonoids is given for a comparison of the compounds from the studied plant.

2.2.1 Amides

Amides are the largest class of compounds isolated from *Piper* species and about 160 amides have been reported from the genus. These are further grouped into isobutyl amides, piperidine amides, pyrrolidine amides and miscellaneous amides depending on the amine part of the molecule. A brief account of the amides and structures of some of the representative compounds are given in the following sections.

2.2.1.1 Isobutyl amides

Forty isobutyl amides have been reported from *Piper* \(^1, 7-34\). Majority of these amides are aromatic amides of \(\Delta^{2,4}\) fatty acids. \((2E, 4E)-N\)-isobutyldecadienamide [pellitorine (1)]\(^{16-22}\), guineensine (2)\(^{23-27}\) and piperlonguminene \(^{28-33}\) have been isolated from several species.

![Structure of isobutyl amide](image-url)
2.2.1.2 Piperidine amides

Thirty-four piperidine amides have been isolated from several *Piper* species. Piperine (3), the pungent principle of black pepper occurs in 24 species \(^1,^{35-37}\). Two piperidine amide dimers (4) have been isolated from *P. peepuloides* \(^38\).
2.2.1.3 Pyrrolidine amides

Pyrrolidine amides (5) occur mainly in *P. trichostachyon* and *P. brachystachyum*, although these are reported from other species.\(^1,15, 39-41\). Dimers of pyrrolidine amides (6) have been isolated from *P. peepuloides*\(^41\).

![Structure of Pyrrolidine Amide (5)](image)

(5)

![Structure of Pyrrolidine Amide (6)](image)

(6)

2.2.1.4 Miscellaneous amides

Several amides other than isobutyl, piperidine and pyrrolidine amides have been isolated from *Piper* species. These include pyridone alkaloids\(^36, 42, 53\), aristolactams\(^36, 45\) (8), dioxoaporphines\(^46-49\) (9), tetrahydropyridine alkaloids\(^43\), benzyl isoquinoline alkaloids\(^44\) and optically active aurantiamide\(^50, 51\) and
auranamide. Recently, two alkaloids possessing cyclobutane ring named pipercyclobutanamides A and B have been isolated from *P. nigrum*.

![Chemical Structure 1](image1)

![Chemical Structure 2](image2)

![Chemical Structure 3](image3)

### 2.2.2 Lignans

Lignans are the second largest class of compounds occurring in *Piper* species and about 125 lignans have been reported from the genus. Lignans are
composed of two C₆, C₃ units linked at the β-carbons of the side chain. The lignans from *Piper* may be divided into nine groups. Only brief accounts of the recently isolated lignans are given here and no attempt is made for compiling all the reported compounds.

### 2.2.2.1 1,4-Diaryl-2,3-dimethyl cyclobutane lignans

Three isomers of this lignan (10) are recorded from two species namely, *P. cubeba* ⁵² and *P. sunatranum* ⁵⁵.

![Chemical structure of 1,4-Diaryl-2,3-dimethyl cyclobutane lignan](image)

### 2.2.2.2 3,4-Dibenzyl-γ-butyrolactol lignans

Most of these lignans (11) are reported from *P. clusii* ⁵⁶ and *P. cubeba* ⁵⁴, ⁵⁷. However, (-) cubebin has been reported from *P. cuneifolium*¹, *P. lacunosum*¹, *P. ribesoides*¹⁷, *P. trichostachyon*⁵⁸ and *P. nigrum*⁵⁹.
2.2.2.3 \( \gamma \)-Butyrolactones

Lignans of this group (12), reported so far from the genus are from \( P. \) cubeba\textsuperscript{57, 60-62}. (-) Hinokinin and (-) yatein occur in \( P. \) clusii also\textsuperscript{1}.

2.2.2.4 2,3-Dibenzylbutan-1, 4-diol lignans

These lignans (13) co-occur with the corresponding dibenzybutyrolactols and lactones. These are found in \( P. \) guineense \textsuperscript{1}, \( P. \) clusii \textsuperscript{56}, \( P. \) cubeba \textsuperscript{57} and \( P. \) trichostachyon \textsuperscript{58}. 
2.2.2.5 2,5-Bisaryl-3, 4-dimethyltetrahydrofurans

Tetrahydrofuran lignans (14) have been reported from *P. schmidtii* ⁶³-⁶⁴, *P. solmsianum* ⁶⁵ and *P. wallichii* ⁶⁶. (-)Grandisin was isolated from *P. solmsianum* ⁶⁵ while (+) grandisin from *P. polysiphorum* ⁶⁷.

2.2.2.6 2,6-Bisaryl-3,7-dioxo-[3,3,0]-bicyclooctane lignans

Lignans of this basic skeleton occur in many *Piper* species ⁶⁸-⁷⁵. Among these (+) sesamin (15) has been reported from 10 species.¹
2.2.2.7 Benzofuran lignans

*P. rignellii* \(^76\) and *P. aequale* \(^77\) are rich sources of benzofuran lignans (16), although these have been reported from *P. futokadsura* \(^1\), *P. wallichii* \(^1\), *P. hancei* \(^1\), *P. schmidtii* \(^1\), *P. capens* \(^1\), *P. clarkii* \(^6\), *P. interruptum* \(^1\) and *P. magnibacum* \(^78\).

2.2.2.8 1,2-Diarylpropanes

This group of lignans (17) has been recorded from several *Piper* species \(^79-83\) and generally co-occurs with benzofurans.
2.2.2.9 Miscellaneous lignans

Besides the above mentioned groups of lignans, a few lignans of some unique structures have also been reported from several *Piper* species and these belong to miscellaneous lignans.

![Chemical structure of a lignan](image)

(17)

2.2.3 Terpenes

Terpenes form an important class of compounds in *Piper*. Most of the terpenes isolated from *Piper* species are either monoterpenes or sesquiterpenes. These constitute the volatile oils from different plant parts. About 90 constituents belonging to monoterpenes, oxygenated monoterpenes, sesquiterpenes and oxygenated sesquiterpenes have been characterized from *Piper* species by GC-MS analysis of volatile oils. Camphene, 3-carene, caryophyllene, caryophyllene oxide, 1,8-cineol, cubebene, ρ-cymene, α-elemene, α-humulene, limonene, linalool, myrcene, α-phellandrene, β-phellandrene, α-pinene, β-pinene, sabinene, α-terpinene, γ-terpinene, α-thujene and terpenolene are encountered in most of the *Piper* species. A
few uncommon sesquiterpenes have been reported from the genus namely, 1,5-epoxy salvial-4 (14)-ene from *P. obliquum*, α-selenene and β-selenene from *P. fimbriulatum* 85, ishwarol from *P. amalago* 130 and capentin from *P. capens* 131. The volatile oil from *P. pierrie* contained two rare esters namely, α-methyl benzylcinnamate and methyl benzylcinnamate 88. Iswarane and trans-anesole, two unusual sesquiterpene hydrocarbons were identified as the major constituents of *P. fulvescens* leaves 150.

Transphytol (18) is the only diterpene isolated from the genus *Piper* 91-93. Triterpenes reported from the genus are β-amyrin 1, friedelin 64, 94, epifriedelanol 94, ursolic acid 95 and ursolic acid-3β-acetate 95.

![Chemical Structure](image)

(18)

### 2.2.4 Kawapyrones and piperolides

![Chemical Structure](image)

(19)
Kawapyrones (19) are \( \delta \)-lactones having styryl and dihydrostyryl substituents. These were originally derived from kawa-kawa \((P. \text{methysticum})\) and known as kawa lactones/pyrones. Besides \(P. \text{methysticum}\) \(^1, 47, 97-102\), the only \(Piper\) species that has yielded kawalactones is \(P. \text{sanctum}\)\(^1\). Two 6-substituted 5,6-dihydropyran-2-ones have been isolated from \(P. \text{reticulatum}\) \(^96\).

![Chemical Structure of Kawapyrones](image)

Piperolides (20) are cinnamalidone butenolides. Only five piperolides have been recorded from \(Piper\) species namely \(P. \text{fadyenii}\) \(^103\), \(P. \text{sanctum}\) \(^104-106\), \(P. \text{aduncum}\) \(^107\) and \(P. \text{hispidum}\) \(^107\).

### 2.2.5 Flavonoids

Flavonoids are a group of natural pigments with benzopyrone skeleton and occur as glycosides or aglycones. The flavonoids isolated from \(Piper\) species may be classified into flavones, flavanones, chalcones and dihydrochalcones.
2.2.5.1 Flavones

Although flavones (21) are ubiquitous in nature, only a few flavones have been reported from Piper species. Most of the flavones isolated are tri or tetra-oxygenated as shown in Table 2.1. Flavonols are 3-hydroxyflavones. The flavonols isolated from Piper species are indicated in Table 2.2.

Mass spectra of flavones give intense molecular ion peaks. The molecular fragments produced by retro-Diels Alder reaction are important in determining the distribution of substituents between A and B-rings.

In $^1$H NMR spectrum the aromatic protons of flavones are observed between $\delta$ 6.0-8.0. H-6 and H-8 generally resonate in the ranges $\delta$ 6.0-6.4 and $\delta$ 6.3-6.9 respectively. H-3 protons also give singlets in this region. Chelated hydroxyl gives a one-proton sharp singlet between $\delta$ 12.7-12.9 and methoxyl protons give singlet (3H) in the range $\delta$ 3.8-3.9. The signals of B-ring protons are generally observed at lower field than those of A-ring protons.

$^{13}$C NMR resonances of flavone nuclear carbons occur in the range $\delta$ 90-185. Carbonyls and oxygenated aromatic carbons resonate at low field ($\delta$ 130-185) where as the resonances for the hydrocarbon and other aliphatic carbons are observed in the range $\delta$ 0-110.
2.2.5.2 Flavanones

Flavanones (22) are 2,3-dihydroflavones. The flavanones except sakuranetin isolated from *Piper* species are oxygenated only in A-ring. Sakuranetin isolated from *P. aduncum* has a hydroxyl group in the B-ring. The flavanones isolated from *Piper* species are indicated in Table 2.3.

2.2.5.3 Chalcones

Chalcones (23) are characterised by the presence of a three carbon bridge consisting of an α, β-unsaturated carbonyl unit between the phenyl rings. Most
of the chalcones from *Piper* are oxygenated at C-4' and C-6' positions. A few unusual monoterpane substituted dihydrochalcones, adunctins (24), were isolated from *P. aduncum* by Orjala *et al.* The chalcones isolated from *Piper* species are indicated in *Table 2.4*.

![Chemical structure of adunctin (24)](image)

### 2.2.6 Miscellaneous compounds

This group of compounds includes sterols, cyclohexanes, aromatic compounds and aliphatic compounds.

#### 2.2.6.1 Sterols

β-Sitosterol occurs in 40 *Piper* species. β-Sitosterol palmitate is reported from *P. betle* leaves. Other sterols reported from the genus are campesterol, cholesterol, cholestanol, daucosterol and stigmasterol.
2.2.6.2 Cyclohexanes

Seven oxygenated cyclohexanes namely, crotepoxide (25), pipoxide, pipoxide chlorohydrin, piperonal A, piperonal B, acetyl piperonal A and (+) zeylenol are known to occur in *Piper* species. Among these, crotepoxide is the most common constituent and has been reported from 11 *Piper* species.

![Chemical Structure of (25)](image)

2.2.6.3 Aromatic compounds

Many aromatic organic acids such as benzoic and cinnamic acid derivatives have been isolated from *Piper* species. Recently, several phenylpropanoids (26), prenylated benzoic acids (27), alkenylphenols, stilbenes and hydroxycinnamic acid esters have been recorded from the genus.
2.2.6.4 Aliphatic compounds

Aliphatic compounds isolated from *Piper* include hydrocarbons, alcohols, acids, esters \(^{14}\), aldehydes and ketones \(^{1,136}\). n-Triacontane, n-triacontanol, stearic acid and palmitic acid are very common in *Piper* species. Four 1-(3,4-methylenedioxyphenyl) alkanes having linear 10, 11, 12 and 14 carbon atoms were isolated from the roots of *P. darrience* \(^{136}\).

2.2.7 Biological activity studies

Several compounds from *Piper* are associated with various biological activities \(^{1}\). *P. piscatorum* yielded an isobutyl amide piperovatine with piscicidal properties\(^9\). \(N\)-[7-(3',4'-methylenedioxyphenyl)-2(\(Z\),4(\(Z\)-
heptadienoyl]pyrrolidine from *P. hispidum* showed antifungal activity against *Cladosporium sphaerospermum* \(^{38}\). Methyl taboganate and 2,2-dimethyl-6-carboxy-chroman-4-one methylester from *P. dilatatum* possessed antifungal property against *Cladosporium cucumerinum*, a plant pathogenic fungus \(^{127}\). Piperine is reported to have antipyretic, anti-inflammatory and analgesic activities \(^{137}\). Pipericide, dihydropipericide and guineensine isolated from *P. nigrum* were shown to be toxic to male adult adzuki bean weevils \(^{138}\). Aduncamide from *P. aduncum* exhibited antibacterial activity against *Bacillus subtilis* and *Micrococcus luteus* \(^{139}\). Eugenol isolated from *P. betle* exhibited antifungal activity against *Aspergillus flavus* \(^{141}\). Dillapiole isolated from *P. aduncum* showed molluscicidal activity against *Biomphalaria glabrata* \(^{142}\). Several benzoic acid derivatives from *Piper* have shown antibacterial and molluscicidal activities \(^{142}\). Crotepoxide has been reported to be an antitumour principle \(^{143}\).

The alkenylphenols from *P. gibbilimbum*, namely, gibbilimbol A \([(E)-4-(4-decenyl)phenol]\), gibbilimbol B \([(E)-4-(3-decenyl)phenol]\), gibbilimbol C \([(E)-4-(4-octenyl)phenol]\) and gibbilimbol D \([(E)-4-(3-octenyl)phenol]\) possessed antibacterial and cytotoxic activities \(^{144}\). 2E, 8E-N-9-(3,4-methylenedioxyphenyl)- nonadienoylpiperidine isolated from *P. nigrum* was reported to cause dilation of heart in rabbits \(^{140}\). Antifungal amides against *Cladosporium sphaerrospermum* have been isolated from *Piper hispidum* and *Piper tuberculatum* \(^{145}\). 1,3-benzodioxole-5-(2,4,8-triene-methylnonanoate), 1,3-
Chapter 2

benzodioxole-5-(2,4,8-triene-isobutynonanoate), myristicin, asarinin, sesamin and fargesin isolated from *P. mullesua* possessed antifeedant activity against the fourth instar larvae of *Spilarctia obliqua*[^68]^[146]. Very recently, amides and prenylated benzoic acids with antifungal activity have been isolated from *P. tuberculatum*, *P. arboreum*[^147] and *P. lanceaefolium*[^162]. Lanceaefolic acid methylester and pinocembrin chalcone isolated from *P. lanceaefolium*[^162] displayed antifungal activity against *Candida albicans* with a minimal inhibitory concentration value of 100 μg/ml in both the cases. 3,4-dihydroxyphenyl-ethanol glucoside and 3,4-dihydroxy-6-(ethylamino)benzamide isolated from green berries of *P. nigrum* inhibited growth of food-borne bacteria namely, *Salmonella typhimurium*, *Staphylococcus aureus*, *Bacillus cereus* and *Escherichia coli*[^151].
Table 2.1: Flavones from *Piper*

<table>
<thead>
<tr>
<th>No.</th>
<th>Compound</th>
<th>Source</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5,7-Dimethoxyflavone</td>
<td><em>P. canninum</em></td>
<td>110</td>
</tr>
<tr>
<td>2</td>
<td>5-Hydroxy-7-methoxyflavone</td>
<td><em>P. falconeri</em></td>
<td>111</td>
</tr>
<tr>
<td></td>
<td>(Tectochrysin )</td>
<td><em>P. manii</em></td>
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</tr>
<tr>
<td></td>
<td></td>
<td><em>P. sylvaticum</em></td>
<td>74</td>
</tr>
<tr>
<td>3</td>
<td>5-Hydroxy-7,4'-dimethoxyflavone</td>
<td><em>P. falconeri</em></td>
<td>111</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P. khasiana</em></td>
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<td></td>
<td><em>P. manausense</em></td>
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<tr>
<td></td>
<td></td>
<td><em>P. manii</em></td>
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</tr>
<tr>
<td></td>
<td></td>
<td><em>P. peepuloides</em></td>
<td>1,117</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P. sylvaticum</em></td>
<td>112</td>
</tr>
<tr>
<td>4</td>
<td>5-Hydroxy-7,3',4'-trimethoxyflavone</td>
<td><em>P. peepuloides</em></td>
<td>1,117</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P. sylvaticum</em></td>
<td>74</td>
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<td>5,3'-Dihydroxy-7,4'-dimethoxyflavone</td>
<td><em>P. auritum</em></td>
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<td>6</td>
<td>5,4'-Dihydroxy-7,3'-dimethoxyflavone</td>
<td><em>P. clarkii</em></td>
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<tr>
<td></td>
<td>(velutin)</td>
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<tr>
<td>7</td>
<td>5,7,4'-Trihydroxyflavone-8-C-glucoside</td>
<td><em>P. clarkii</em></td>
<td>114</td>
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<tr>
<td>8</td>
<td>Marginatoside</td>
<td><em>P. marginatum</em></td>
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<tr>
<td>9</td>
<td>6-C-β-D-Galactopyranosyl-acacetin-7-O-glucoside</td>
<td><em>P. brachystachium</em></td>
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<td>10</td>
<td>6-C-β-D-Glucopyranosyl-acacetin-7-O-glucoside</td>
<td><em>P. brachystachium</em></td>
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</tr>
<tr>
<td>11</td>
<td>5-Hydroxy-7-methoxy-8-C-β-D-glucosyl flavone (kaplanin)</td>
<td><em>P. lhotzkyanum</em></td>
<td>115</td>
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</table>
### Table 2.2: Flavonols from *Piper*

<table>
<thead>
<tr>
<th>No.</th>
<th>Compound</th>
<th>Source</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3,5-Dihydroxy-7,4'-dimethoxyflavone</td>
<td><em>P. sylvaticum</em></td>
<td>116</td>
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<tr>
<td>2</td>
<td>3,5,7,3', 4'-Pentahydroxyflavone-3-O-glucoside (Isoquercitrin)</td>
<td><em>P. nigrum</em></td>
<td>117</td>
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<tr>
<td>3</td>
<td>3,5,7,3'-Tetrahydroxy-4'-methoxyflavone-3-O-β-D-rutinoside (Isorhamnetin-3-O-β-D-rutinoside)</td>
<td><em>P. nigrum</em></td>
<td>117</td>
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<tr>
<td>4</td>
<td>3,5,7,4'-Tetrahydroxyflavone-3-O-arabinoside-7-rhamnoside (Kaempferol-3-O-arabinoside-7-rhamnoside)</td>
<td><em>P. nigrum</em></td>
<td>117</td>
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<tr>
<td>5</td>
<td>3,5,7,4'-Tetrahydroxy flavone-3-O-β-D-glucoside</td>
<td><em>P. nigrum</em></td>
<td>117</td>
</tr>
<tr>
<td>6</td>
<td>3,5,7,3', 4'-Pentahydroxyflavone-3-O-β-D-galactoside (Quercetin 3-O-β-D-galactoside)</td>
<td><em>P. nigrum</em></td>
<td>117</td>
</tr>
<tr>
<td>7</td>
<td>Quercetin-3-O-β-D-rutinoside</td>
<td><em>P. nigrum</em></td>
<td>117</td>
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<tr>
<td>8</td>
<td>Quercetin-3-O-β-D-rhamnoside</td>
<td><em>P. nigrum</em></td>
<td>117</td>
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<tr>
<td>9</td>
<td>Rhamnetin-tri-O-glucoside</td>
<td><em>P. nigrum</em></td>
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Table 2.3: Flavanones from *Piper*

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<th>No.</th>
<th>Compound</th>
<th>Source</th>
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<tbody>
<tr>
<td>1</td>
<td>5,7-Dihydroxyflavanone</td>
<td><em>P. hostmannianum</em></td>
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<td></td>
<td></td>
<td><em>P. steerni</em></td>
<td>119</td>
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<tr>
<td>2</td>
<td>6-Hydroxy-5,7-dimethoxy flavanone</td>
<td><em>P. hispidum</em></td>
<td>120</td>
</tr>
<tr>
<td>3</td>
<td>8-Hydroxy-5,7-dimethoxy flavanone</td>
<td><em>P. hispidum</em></td>
<td>120</td>
</tr>
<tr>
<td>4</td>
<td>5-Hydroxy-7-methoxy-6,8-dimethylflavanone</td>
<td><em>P. hostmannianum</em></td>
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<tr>
<td>5</td>
<td>5-Hydroxy-7-methoxyflavanone (pinostrobin)</td>
<td><em>P. methysticum</em></td>
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<tr>
<td></td>
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<td><em>P. aduncum</em></td>
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<tr>
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<td></td>
<td><em>P. fadyenii</em></td>
<td>107,12</td>
</tr>
<tr>
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<td><em>P. hispidum</em></td>
<td>1</td>
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<tr>
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<td><em>P. steerni</em></td>
<td>107</td>
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<td>121,</td>
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<td></td>
<td>119</td>
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<tr>
<td>6</td>
<td>5,4’-Dihydroxy-7-methoxyflavanone</td>
<td><em>P. aduncum</em></td>
<td>124</td>
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<tr>
<td></td>
<td>(sakuranetin)</td>
<td><em>P. lhotzkyanum</em></td>
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</tr>
<tr>
<td>7</td>
<td>5,7,8-Trimethoxyflavanone</td>
<td><em>P. hispidum</em></td>
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</tr>
<tr>
<td>8</td>
<td>7-Hydroxy-5-methoxy-flavanone (alpinetin)</td>
<td><em>P. methysticum</em></td>
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<td></td>
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<td>123</td>
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Table 2.4: Chalcones and dihydrochalcones from *Piper*

<table>
<thead>
<tr>
<th>No.</th>
<th>Compound</th>
<th>Source</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2′,3′-Dihydroxy-4′,6′-dimethoxychalcone</td>
<td><em>P. hispidum</em></td>
<td>120</td>
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<td>2</td>
<td>2′-Hydroxy-4,4′,6′-trimethoxychalcone (Flavokawain A)</td>
<td><em>P. methysticum</em></td>
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<td>2′-Hydroxy-4′,6′-dimethoxychalcone (Flavokawain B)</td>
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<td>124-126</td>
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<td><em>P. dilatatum</em></td>
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<td>4,2′-Dihydroxy-4′,6′-dimethoxychalcone (Flavokawain C)</td>
<td><em>P. methysticum</em></td>
<td>122, 124-126</td>
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<td><em>P. murrayanum</em></td>
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<td>2′-Hydroxy-2,4′,5′-trimethoxychalcone</td>
<td><em>P. hispidum</em></td>
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<td>2′,4′-Dihydroxy-6′-methoxychalcone (alpinetin chalcone)</td>
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(Table 2.4 contd...)  

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<td>(asebogenin.)</td>
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<td>15</td>
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<td>19</td>
<td>Piperadunctin C</td>
<td><em>P. aduncum</em></td>
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2.3 Present work

*Piper colubrinum* Link (*Piperaceae*) is a multiple disease resistant species, native of Brazil. *P. colubrinum* is valuable for its unique genetic make up, which renders it resistant to the *Phytophthora*-nematode complex causing *Phytophthora* foot-rot disease in black pepper. Hence *P. colubrinum* has immense potential as the donor parent in breeding programme for the improvement of the cultivated species *P. nigrum*. A thorough literature survey revealed that no phytochemical investigation of this plant has been done. Hence a systematic chemical investigation was undertaken to study the chemistry of *P. colubrinum* leaves.
2.4 Experimental details

2.4.1 Materials and methods

Melting points are uncorrected. Silica gel (60-120 mesh) of E-Merck was used for column chromatography. Silica gel-G containing 13% calcium sulphate as binder was used for TLC. The plates were dried at room temperature for 12 h, activated in an air oven at 120 °C for 20 minutes. Samples for analysis were routinely dried under vacuum. UV spectra were recorded on Shimadzu UV-160A spectrometer. IR spectra were recorded as KBr pellets on Shimadzu FT-IR-8101A spectrometer. $^1$H NMR spectra were recorded using Brucker 300 MHz spectrometer in CDCl$_3$, CD$_3$COCD$_3$ or DMSO-d$_6$ and $^{13}$C NMR spectra were recorded at 75 MHz. Chemical shifts are in ppm (δ values) using TMS as internal standard. EI mass spectra were recorded by means of direct insertion probe at ionisation energy of 70 eV.

2.4.2 Extraction

The leaves of *P. colubrinum* were procured from the Indian Institute of Spices Research farm at Calicut. A voucher specimen of the sample is available at the herbarium of I.I.S.R Calicut. Shade dried and powdered leaves (1 kg) were extracted successively with hexane (60-80 °C), chloroform and methanol in a soxhlet apparatus for 30h in each case. The extracts were separately concentrated to dryness under reduced pressure. The dark green residue obtained in each case was subjected to column chromatography over silica gel.
The dark residue from hexane extract of the leaves showed three prominent spots on TLC. Their separation and purification are discussed below.

2.4.3 Chromatographic separation of hexane extract

The dark green residue (20 g) from the hexane extract of the leaves was dissolved in 50 ml of hexane and transferred to a column of silica gel (500 g) set up with hexane. The column was eluted successively with hexane, hexane-ethyl acetate (95:5), hexane-ethyl acetate (90:10), hexane-ethyl acetate (80:20), hexane-ethyl acetate (50:50) and finally with ethyl acetate (100%). Fractions of 100 ml were collected and concentrated. These fractions were monitored by TLC and similar fractions were combined and grouped as shown in Table 2.5.

Groups I-II

No crystalline compound could be obtained from these fractions by repeated crystallisation in different solvents. Hence it was not examined further.

Group III

The fractions, 25-41 were combined and concentrated. The white solid separated was filtered and recrystallised from ethyl acetate. It was designated as compound I (23 mg), Rf 0.84 (hexane: ethyl acetate 95:5).

Group IV

TLC examination of these fractions in different solvent systems revealed non-homogeneous behaviour and no crystalline compound could be isolated by repeated crystallisation. Hence further isolation was discontinued.
Group V

The fractions, 49-56 when concentrated gave a white solid, which was recrystallised from ethyl acetate. It was designated as compound II (21 mg), R_f 0.56 (hexane: ethyl acetate, 80:20).

Groups VI-VIII

The fractions from these groups showed heterogeneous behaviour on TLC and no crystalline material could be isolated by repeated crystallisation.

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<td>25-41</td>
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<td>Hexane-ethyl acetate 50:50</td>
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<tr>
<td>Ethyl acetate (100%)</td>
<td>96-102</td>
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<td>III</td>
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</table>

Table 2.5: Fractionation of hexane extract
Chapter 2

Group IX

The fractions, 96-102 upon concentration yielded a white solid, which was separated and recrystallised from ethyl acetate to give compound III (9 mg), $R_f$ 0.54 (chloroform: methanol 90:10).

Compound I

Compound I was crystallized from ethyl acetate as white solid, (23 mg), $R_f$ 0.84 (hexane – ethyl acetate 95:5), mp 92 °C

IR $\nu_{\text{max}}$ (KBr):

3410 (OH), 2928, 2858, 1710(CO), 1471, 939, 724 cm$^{-1}$

EIMS (70eV): m/z (rel. int.)

Relative abundance below 10% not given.

452[M$^+$], 424, 407, 392, 382, 369(15), 354, 340, 326, 99(21), 87(11), 85(35), 73(91), 57(93), 55(51), 43(100), 41(45).

$^1$H NMR (300 MHz, CDCl$_3$): $\delta$ (ppm)

2.37 (2H, t, $J$ = 7.3 Hz), 1.63 (4H,m), 1.25 (50H,br s), 0.86 (3H, t, $J$ = 6.6 Hz)

$^{13}$C NMR (75MHz, CDCl$_3$): $\delta$ (ppm)

**Chapter 2**

**Compound II**

Compound II was obtained as colourless needles from ethyl acetate, (21mg), Rf 0.56 (hexane-ethyl acetate, 80:20), mp 136 °C, undepressed on admixture with an authentic sample of β-sitosterol. It gave positive Liebermann-Burchard reaction (violet-blue-green) for steroids.

Acetylation:

Compound II (10 mg) was taken in pyridine (1ml) and treated with freshly distilled acetic anhydride (1ml) and left at room temperature for 48 h. The reaction mixture was worked up in the usual manner. The residue was separated and recrystallised from methanol as colourless needles (8mg), mp 125 °C.

**Compound III**

White amorphous powder (9 mg), Rf 0.54 (chloroform: methanol, 90:10), mp 278 °C.

IR ν<sub>max</sub> (KBr):

3440(OH), 2980, 2815, 1470, 1465, 1385, 1381, 1070, 1023 cm<sup>-1</sup>

FAB-MS:

599(M<sup>+</sup> + Na)

EIMS 70eV: m/z (rel. int.):

Relative abundance below 10% not given.

414(10), 396(11), 383(12), 255, 213, 192, 182, 136(35), 92 (100).

<sup>1</sup>H NMR (300 MHz, DMSO-d<sub>6</sub>): δ(ppm)
5.33 (1H, m, H-6), 4.87 (1H, br s, H-6’α), 4.43 (2H, br d, H-1’, H-6’β), 4.22(1H, d, J = 8 0 Hz, H-3’), 3.63(1H, m, H-3), 3.51(1H, m, H-4’), 3.04(1H, m, H-2’), 3.01( 1H, m, H-5’), 2.84-1.00 (CH₃), 0.93 (3H, s, H-19), 0.87 (3H, d, J = 6.3 Hz, H-26), 0.80 (3H, br s, H-21) , 0.78 (3H, br s, H-29), 0.76 (3H, d, J = 6.3 Hz, H-27), 0.65 (3H, s, H-18).

^13C NMR (75MHz, DMSO-d₆): δ(ppm)


2.4.4 Chromatographic separation of chloroform extract

The residue (12 g) from the chloroform extract of *Piper colubrinum* leaves indicated two yellow spots on TLC. The residue was dissolved in diethyl ether, adsorbed on 20 g silica gel and the powder was chromatographed on a silica gel (500 g) column. The column was eluted with hexane-ethyl acetate (95:5), hexane-ethyl acetate (90:10), hexane-ethyl acetate (80:20), hexane-ethyl acetate (70:30), hexane-ethyl acetate (50:50) and ethyl acetate (100%). Fractions of 100 ml were collected, concentrated and similar fractions as monitored by TLC were combined and grouped as indicated in Table 2.6
Chapter 2

Groups I-III

These fractions, when concentrated yielded a waxy material, which showed heterogeneous nature on TLC. No crystalline material could be isolated from these fractions by repeated crystallisation. Hence it was not examined further.

Group IV

The fractions, 29-50 were combined and concentrated to give a yellow solid. It was separated and recrystallised from ethyl acetate as pale yellow needles (96 mg). It was designated as compound IV, $R_f$ 0.80 (chloroform : methanol 95:5), mp 285 °C.

Group V

The fractions, 51-65 were combined and concentrated to give a yellow solid, which was separated and recrystallised from ethyl acetate. It was designated as compound V (47 mg), $R_f$ 0.6 (chloroform : methanol, 95:5), mp 258 °C.

Groups VI-VII

These fractions did not give any crystalline material upon repeated crystallisation from different solvents and suggested waxy nature. It was not examined further.
Table 2.6: Fractionation of Chloroform extract

<table>
<thead>
<tr>
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<tbody>
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<td>Hexane</td>
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<td>Hexane-ethyl acetate 95:5</td>
<td>11-20</td>
<td>II</td>
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<td>Ethyl acetate (100%)</td>
<td>78-89</td>
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</table>

**Compound IV**

Compound IV was obtained as yellow needles (96 mg) from ethyl acetate, R$_f$ 0.80 (chloroform:methanol, 95:5), mp 285 °C.

UV $\lambda_{max}$ nm

MeOH: 268, 335nm

AlCl$_3$: 276, 300, 345, 380

AlCl$_3$ + HCl: 277, 300, 340, 380

NaOAc: 267, 336

NaOAc+H$_3$BO$_3$: 267, 336
IR $\nu_{\text{max}}$ (KBr):

3439(OH), 2928, 1667(CO), 1606,1498 cm$^{-1}$

EIMS (70eV): m/z (rel. int.):

Relative abundance below 10% not given.

284[M]$^+$ (75), 255 (28), 241(32), 212, 167, 166, 157, 138 (42), 118(50),
95(100), 69(90).

$^1$H NMR (300 MHz, acetone-$_d_6$): $\delta$(ppm)

12.99 (chelated OH), 9.62 (OH), 7.96 (2H, d, $J$ = 8.6 Hz, H-2', 6'), 7.02 (2H, d, $J$ = 8.6 Hz, H-3', H-5'), 6.71(1H, s, H-8), 6.69 (1H, s, H-3), 6.34 (1H, s, H-6),
3.94 (3H, s, OCH$_3$)

$^{13}$ C NMR (75MHz, DMSO-$_d_6$): $\delta$(ppm)

181.83 (C-4), 165.04 (C-7), 163.98 (C-2), 161.21 (C-4'), 161.10 (C-9), 157.14
(C-5), 128.47 (C-2', 6'), 120.95 (C-1'), 115.88 (C-3', 5'), 104.57 (C-10), 102.92
(C-3), 97.86 (C-6), 92.60 (C-8), 55.95 (OCH$_3$)

**Compound V**

Compound V was obtained as yellow amorphous powder (47 mg) from ethyl acetate, $R_f$ 0.60 (chloroform : methanol, 95:5), mp 258 $^\circ$C.

$^\circ$UV $\lambda_{\text{max}}$ nm

MeOH: 255, 266sh, 348nm

AlCl$_3$: 272, 295sh, 345,380sh

AlCl$_3$ +HCl: 277, 300sh, 340, 380sh
Chapter 2

IR $\nu_{\text{max}}$(KBr):

3415, 2921, 1664, 1606, 1452, 1042 cm$^{-1}$

EIMS 70eV: m/z (rel. int.):

Relative abundance below 10% not given.

300 (100) [M]$^+$, 272 (33), 258 (21), 168 (18), 138 (14), 133 (11), 95 (30), 69 (30), 43 (29).

$^1$H NMR (300 MHz, acetone-d$_6$): $\delta$(ppm)

12.99 (chelated OH), 7.98 (OH), 7.96 (OH), 7.0 (1H, d, $J = 8.1$Hz), 7.52 (1H, m) and 7.48 (1H, d) merged and appeared as a doublet, 6.69 (1H, br s), 6.63 (1H, br s), 6.33 (1H, br s), 3.94 (3H, s)

$^{13}$C NMR (75MHz, DMSO-d$_6$): $\delta$(ppm)

181.72, 165.02, 164.16, 161.10, 157.11, 149.77, 145.68, 121.27, 119.00, 115.87, 113.38, 104.55, 102.93, 97.85, 92.48, 55.93.

2.4.5 Methanol extract of _Piper colubrinum_ leaves

Methanol extract of _Piper colubrinum_ leaves did not show any interesting spots on TLC. Hence it was not examined further.
2.5 Results and discussion

2.5.1 Hexane extract

The dark brown residue from the hexane extract of *P. colubrinum* leaves yielded three compounds. The bar diagram for their separation is given in **Chart 2.1**.

![Chart 2.1](image)

**Structure of compound I**

Compound I was obtained as white solid from ethyl acetate, mp 92°C. The mass spectrum displayed molecular ion peak at m/z 452 corresponding to the molecular formula C\textsubscript{30}H\textsubscript{60}O\textsubscript{2}. IR spectrum indicated carbonyl and hydroxyl absorptions at 1710 and 3410 cm\(^{-1}\) respectively. A two-proton triplet centered on \(\delta 2.37\) (\(J = 7.3\) Hz) in the \(^1\)H NMR spectrum was indicative of the methylene...
protons adjacent to the carbonyl group. It showed a methyl triplet at δ 0.86 (3H, t, J = 6.6 Hz) for the terminal methyl. A broad singlet at δ 1.25 (50 H) and a multiplet centered on δ 1.63 (4H) were assigned to the methylene protons. The absence of (M+15) ion together with the consecutive loss of 14 and 28 mass units suggested it to be a straight chain aliphatic compound 155, 156. Its mass spectrum was identical with that of n-triacontanoic acid reported in literature (lit. mp 94 °C) 157. Hence it was identified as n- triacontanoic acid, whose structure is given below.

$$\text{CH}_3(\text{CH}_2)_{28}\text{COOH}$$

**Structure of compound II**

Compound II was recrystallised as colourless needles from ethyl acetate, mp 136 °C. It was identified as β-sitosterol (28) by direct comparison of its mp (m mp 136 °C) 163, super imposable IR and co-TLC with an authentic sample of β-sitosterol. Prepared its acetate, mp 125 °C and was found to be identical with the melting point of β-sitosterol acetate (lit. mp 127 °C) 159.

![Structure of compound II](image)

(28) R=H  (29) R=Glc
Structure of compound III

Compound III was obtained as white amorphous powder from chloroform-methanol, mp 278°C. It gave positive reaction to Liebermann-Burchard test for steroids and Molish test for sugars. Its IR spectrum showed the presence of strong absorptions at 3401 and 1070 cm\(^{-1}\) characteristic of a glycoside. The FAB mass spectrum gave a quasimolecular ion at \(m/z\) 599 (\(M^+ + Na\)), which suggested the molecular weight to be 576. EI mass spectrum of compound III showed a molecular fragment at \(m/z\) =396 due to the loss of glucose moiety from the molecular ion. The \(^{13}\)C NMR spectrum showed 34 carbon atoms in the molecule. An anomeric signal at \(\delta\) 100.29 indicated the presence of a single monosaccharide moiety. \(^{13}\)C-DEPT NMR spectrum revealed the presence of six methyl, 12 methylene and 14 methine carbon atoms. Four methine carbon resonances at \(\delta\) 70.62, 77.26, 73.99, 77.26 and one methylene carbon at \(\delta\) 61.62 were assigned to C-2', C-3', C-4', C-5' and C-6' respectively. The olefinic signals at \(\delta\) 140.97 and \(\delta\) 121.74 corresponded to C-5 and C-6 of sterol moiety. \(^1\)H NMR spectrum displayed 2 tertiary methyls [\(\delta\) 0.65 (3H, s, H-48) and \(\delta\) 0.93 (3H, s, H-19)] and four secondary methyls [\(\delta\) 0.87 (3H, d, \(J = 6.3\) Hz, H-26), \(\delta\) 0.80 (3H, br s, H-21), \(\delta\) 0.78 (3H, br s, H-29) and \(\delta\) 0.76 (3H, d, \(J = 6.3\) Hz, H-27)]. The protons of the sugar moiety were observed between \(\delta\) 3.05
and δ 4.88. All other peaks in the $^1$H NMR spectrum were identical with that of β-sitosterol reported earlier$^{159}$.

<table>
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<th>$\delta_\text{C}$</th>
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<td>36.00</td>
<td>1'</td>
<td>101.29</td>
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</table>

Hydrolysis of compound III with 10% sulphuric acid gave an aglycone, which was identified as β-sitosterol by direct comparison with an authentic sample of β-sitosterol. Hence compound III was identified as sitosterol-3-O-β-D-glucopyranoside (29), (lit.mp 283-286 °C)$^{163}$. 
2.5.2 Chloroform extract

Two crystalline compounds obtained from the chloroform extract of *Piper colubrinum* leaves were designated as compounds IV and V. The bar diagram for their separation is given in Chart 2.2.

![Diagram](https://via.placeholder.com/150)

**Chart 2.2**

**Structure of compound IV**

Compound IV was recrystallised as yellow needles from ethyl acetate, mp 285 °C. The mass spectrum indicated molecular ion peak at the m/z 284 corresponding to the molecular formula C₁₆H₁₂O₅. It gave positive reaction to Mg-HCl test for flavonoids. IR spectrum showed carbonyl and hydroxyl absorptions at 1667 cm⁻¹ and 3439 cm⁻¹ respectively. ¹³C NMR spectrum indicated 16 carbons in the molecule. A sharp one-proton singlet at δ 12.99 in
the \(^1\)H NMR spectrum was characteristic of a chelated hydroxyl (OH-5) and a three-proton singlet at \(\delta 3.94\) revealed the presence of a methoxyl group. A pair of doublets at \(\delta 7.96\) (2H, d, \(J = 8.6\) Hz) and \(\delta 7.02\) (2H, d, \(J = 8.6\) Hz) were characteristic of H-2', H-6', H-3' and H-5' respectively of the 1,4-disubstituted aromatic ring system. \(^{108}\) The one-proton singlet at \(\delta 6.69\) was assigned to H-3 of the flavone. Two one-proton broad singlets at \(\delta 6.34\) and \(\delta 6.71\) were assigned to the meta-coupled protons at H-6 and H-8 respectively. \(^{109}\) The molecular fragments at m/z 118, 166 and 167 in the mass spectrum, formed by the retro Diels Alder fragmentation of flavones, suggested that the methoxyl group was in ring A and the hydroxyl in ring B. \(^{108}\) The UV, IR, \(^1\)H NMR, \(^{13}\)C NMR and mass spectra of compound IV were identical with that of 5,4'-dihydroxy-7-methoxy-flavone reported earlier (lit. mp 286°C). \(^{160,108,109}\) Hence compound IV was identified as 5,4'-dihydroxy-7-methoxy-flavone. This is the first report of 5,4'-dihydroxy-7-methoxy-flavone from Piper genus.

![Chemical structure](image)
Structure of compound V

The compound V was obtained as yellow amorphous powder from ethyl acetate mp 258 °C. The mass spectrum of compound V displayed molecular ion peak at m/z 300 which accounted for the molecular formula C_{16}H_{12}O_{6}. IR spectrum indicated hydroxyl and carbonyl absorptions at 3415 cm\(^{-1}\) and 1664 cm\(^{-1}\) respectively. It gave positive reaction to Mg-HCl test for flavonoids. \(^{13}\)C NMR spectrum indicated sixteen carbon atoms in the molecule. \(^{1}\)H NMR spectrum showed a sharp one-proton singlet at \(\delta\) 12.99 for a chelated hydroxyl (H-5) and a three-proton singlet at \(\delta\) 3.94 for a methoxyl group. It also showed two hydroxyls at \(\delta\) 7.98 and \(\delta\) 7.96. The one-proton singlet at \(\delta\) 6.63 was assigned to H-3. The one-proton broad singlets at \(\delta\) 6.33 and \(\delta\) 6.69 were assigned to the meta-coupled protons H-6 and H-8 respectively. The one-proton
mutiplet at $\delta$ 7.52 (1H, H-6') $\varepsilon$, $\delta$ 7.48 (1H, H-2') and $\delta$ 7.0 (1H, d, $J = 8.1$ Hz, H-5') were assigned to the protons of 1, 3, 4-trisubstituted benzene ring. The typical retro Diels Alder fragments at m/z 133, 134, 166 and 168, in the EI mass spectrum revealed that the methoxyl group was in ring-A and two hydroxyls in ring B of the flavone. The IR, UV, $^1$H NMR, $^{13}$C NMR and mass spectra of compound V were identical with that of 5, 3', 4'-trihydroxy-7-methoxy flavone reported earlier (lit. mp 261°C). Hence compound V was identified as 5, 3', 4'-trihydroxy-7-methoxy flavone (31). This is the first report of this flavone from the genus *Piper*. 

![Flavone Structure](image.png)
Scheme 2.2: Mass spectral fragmentation of V
Chapter 2

2.6. References


Chapter 2


