

**CHAPTER 4**  
**ISOLATION AND IDENTIFICATION OF PHYTOCONSTITUENTS FROM LG.**

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Osteoprotective potential of LG was confirmed by biochemical, histoarchitecture and calcium metabolism. However, to understand further about the composition of the plant, the study was extended with as aim to isolate and identify the phytoconstituents from LG.

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**INTRODUCTION:**

LG is a medium sized evergreen tree, up to 25 m high and 1.5 m in girth and a clean bole of around 5 – 6 m. It is found throughout India ascending up to an altitude of 1400 m in the Himalayas (Sukh Dev, 2006). *Litsea* has been described as promoter of longevity, promoter of semen generation and emollient. Sap of fresh bark or its decoction is prescribed as a remedy for diarrhoea, dysentery, rheumatism, and as an aid to longevity. In addition, in current usage, a paste prepared by grinding bark with water is used as a plaster in cases of sprain, bruises, wounds, inflammation, back pain, rheumatic and gouty joints, bone fractures etc. It has analgesic, antiseptic and emollient effects (Devi and Meera, 2010).

Methanol extract of LG yields 11 -13 % dark brown semi solid paste. Several alkaloids, mostly of aporphine group, have been isolated from the trunk bark, leaves, stems, and mention is made of boldine, laurotetanine, actinodaphnine and cassythicine (Tewari *et al.*, 1972). In the leaves plus stems they occur to the extent of 0.1%, and the main constituent is boldine. Attention is drawn to sebiferine, another constituent alkaloid which belongs to the morphane group. From leaves, a number of flavonoids (major ones being quercitrin, and kaempferol-3-glucoside) have been isolated. From the bark, water soluble mucilage composed chiefly of arabinose (major) and xylose has been isolated (Sukh Dev, 2006).

Methanol extract of bark showed highly significant antibacterial action against *micrococcus luteus* (Sukh Dev, 2006). In another investigation, methanol extract demonstrated to be as effective as chloramphenicol against sixteen tested organisms (Mandal *et al.*, 2000). Bark extract has useful antifungal activity against several organisms (Sukh Dev, 2006). Enough research data shows that aporphine alkaloids have highly significant antioxidant activity. LG is found to be rich in these alkaloids. Apart from this, Ethanolic extract of the bark also showed aphrodisiac effect in male rats (Sukh Dev, 2006).

Though most of the biological activities had been carried out on the bark extract, very scanty data is available regarding the phytochemical constituents present in the bark, and their effects on pathogens. Thus, the present study was undertaken to characterize and isolate the phytochemicals from this plant and check for their efficacy against pathogens. As most of the studies indicated that alkaloids are abundant in the bark of *L. glutinosa*, alkaloids were further subjected for analysis for their phytochemical and biological analysis.

## MATERIALS AND METHODS

### Plant Material

The bark powder of *L. glutinosa* was purchased from local drug market and checked for any type of bacterial or fungal infection. The dried powdered plant material (20gm) was extracted successively with methanol by Soxhlet's apparatus for 72 hours at a temperature not exceeding the boiling point of the solvent. The filtrate was evaporated at 60°C on water bath to yield semi solid paste. This paste was later stored in -20° C.

### Test for various phytochemicals.

#### Flavonoid:

Test for flavonoids were carried out as described previously (Shastri *et al.*, 2010; Devmurari *et al.*, 2010).

#### Terpenoids:

Test for terpenoids were carried out as described previously (Daneil, 1991).

#### Phenol:

Test for phenols were carried out as described previously (De *et al.*, 2010).

#### Tanin:

Test for tannins were carried out as described previously (Daneil, 1991).

#### Saponin:

Test for saponins were carried out as described previously (Daniel, 1991).

#### Glycosides

Test for glycosides were carried out as described previously (Kodangala *et al.*, 2010; Sahu *et al.*, 2010).

#### Test for Cardiac Glycosides

Test for cardiac glycosides were carried out as described previously (Daneil, 1991).

#### Alkaloids:

Tests for alkaloids were carried out as described previously (Kam *et al.*, 2001). Briefly, 20 mg of plant crude extract was added to 10 ml methanol and placed in a sonic bath to dissolve. The extract was then filtered using a Wattman No.1 filter paper; 2 ml of filtrate was taken and mixed with 1% HCl. Three different tests were performed for Alkaloids.

1. Mayer's test: To 1 ml of mixture, 6 drops of Mayer's reagent, was added leading to the formation of a yellowish creamish precipitate.
2. Wagner's Test: To 1 ml of mixture, 6 drops of Wagner's reagent was added forming bronish red precipitates indicating the presence of alkaloids.

3. Dragendroff's test: To 1 ml of mixture, 6 drops of Dragendroff's reagent was added, forming an orange precipitate indicating the presence of alkaloids.

**Isolation and TLC of Alkaloid:**

Alkaloids were isolated by the method described previously (Daneil, 1991). Briefly, 20gm bark powder was soaked in 100 ml 10% ammonical ethanol for 24 hours. Filtrate was collected and concentrated to yield a semi solid paste, which is further subjected to TLC and analysis of biological activity. TLC was carried out on silica gel G using Chloroform-Methanol-tetraethylamine system developed at the ratio of 75:22:3. After running the sheet for 45 min in a chromatographic chamber on a 20 cm long precoated silica plate, the plate was visualized in uv for the separation of bands. Separated bands were cut from the plate and extracted in methanol for analysis of absorption maxima and GC MS.

**Absorption Maxima:**

For carrying out absorption maxima analysis, 8 fractions obtained through TLC were dissolved in methanol and scanned from 200 to 1000 nm using Perkin Elmer automatic analyzer.

**GC MS analysis:****Gas chromatographic analysis**

GC/MS analysis was carried out using Perkin Elmer auto system XL with turbo mass system equipped with PE 5 MS 30m X 250 micron silica capillary. Injector and detector temperatures were 250°C and 300°C, respectively. The temperature started from 70 ° C for 5 min and then rose to 290°C at the rate of 10° C per minute. Helium was used as carrier gas. The MS was taken at 70eV. Scanning speed was 0.84 scans s<sup>-1</sup> and the scanning period was from 40 to 550 s. sample volume was kept 3 µL.

**Biological activity**

Minimum inhibitory concentration (MIC) of *LG* methanolic extract and alkaloid fraction isolated was determined against seven different strains shown in table and compared with standard drug Amoxicillin using broth dilution method.

Bacterial species	Strain
<i>E.coli</i>	MTCC 443
<i>S.typhi</i>	MTCC 98
<i>Cl.tetani</i>	MTCC 449
<i>Vi.cholerae</i>	MTCC 3906
<i>S.pyogenus</i>	MTCC 443
<i>E.faecalis</i>	MTCC 459
<i>P.vulgaris</i>	MTCC 744

Minimal fungicidal concentration against *C. albicans* and *A. clavatus* organisms were compared with standard drugs nystatin by same method.

Fungal species	Strain
<i>C.albicans</i>	MTCC 227
<i>A.clavatus</i>	MTCC1323

## RESULTS

The result of phytochemical screening of plants gave positive results for alkaloids, flavonoids, glycosides, phenols, tannins and saponins. However, these preliminary tests were negative for terpenoids, amines and anthocyanins. Our results supported previous study conducted by Xie *et al.*, (2000) who reported the presence of alkaloids in this plant. Results are presented in Table 4.1.

Further, methanolic extract of plant and alkaloids were checked for their antimicrobial and antifungal activity. It has been shown that most of anti microbial (Table 4.2, figure 1) and antifungal activity (Table 4.3, Figure 2) of the plant was because of the alkaloids fraction of it. Alkaloid fractions were found to be 100% more potent than total methanolic extract against all pathogens except *V. cholerae*. It has also been noticed that this plant is a powerful anti microbial agent, supporting the results observed previously (Mandal *et al.*, 2000) and its use in diarrhoea and dysentery (Yang *et al.*, 2005; Sukh Dev, 2006).

### TLC of alkaloids:

TLC of alkaloids resulted in 8 *uv* active bands. These bands are shown in the table 4 according to their Rf values (Table 4.4).

### UV SCANS of alkaloids:

*uv* scan of alkaloid fraction revealed that all most of the constituents are colorless and not having any absorption in the visible range (Figure 3). All the fractions showed absorption maxima in the range of 200 – 300 nm. Fraction A was found to be having absorption in visible region, and it yielded a yellow crystalline powder. Rest all fractions yielded a white crystalline powder. These results supported the study of Yang *et al.*, (2005) who isolated two new aporphine alkaloids from *Litsea* bark and reported one's absorption maxima at 218, 282 and 306, while the other compound has absorption maxima at 278 and 306.

### GC MS Analysis

GC spectrum analysis of methanolic extract of the plant is presented in Figure 4.4. MS analysis of the total methanolic extract showed presence of various phytochemicals listed in Table 4.5. GC MS analysis of Alkaloid sub fractions revealed presence of various phytochemicals listed in Table 4.6 and presented in figure 4.5.

### DISCUSSION

Our study showed that *LG* bark contains various phytochemicals including alkaloids, steroids, triterpenoids, saponins, and tannins. The dried bark powder yielded 6.66% yield with methanol. Alkaloids gave 1.12% yield, present in most abundant quantity. These results supported the study of Mandal *et al.*, (2000), who observed similar results. As indicated in the table, plant extract was found to be potent antibacterial agent. These results were similar with Mandal *et al.*, (2000), who carried out similar study using zone inhibition method using *LG* methanolic extract. In addition, our study proved that Alkaloids are having significant antibacterial activity; furthermore antibacterial property of *Litsea* can be attributed to its alkaloid content. These were results were more profound when antifungal activity was carried out, indicating that alkaloids were responsible behind the antifungal property of *L. glutinosa*. However, the potency of the plant as antifungal agent was not as effective as antimicrobial agent. Our study is the first to report antifungal analysis of *LG* bark extract and its alkaloid content.

When absorption maxima was calculated on autoanalyzer, it was found to be co incident with the results of Yang *et al.*, (2005). GC MS analysis revealed the presence of various phytochemicals, including quinoline derivatives. Yang *et al.*, (2005) has reported two aporphine alkaloids from this plant, and we observed similar structured compound in GC MS analysis. This plant was found to contain Oleic acid which is reported to have variety of biological effects, including hypotensive effect (Teres *et al.*, 2008). Eicosanoids are signalling molecules formed during intracellular signalling of inflammation, suggesting that this plant may have a role in inflammation. Apart from

these, we also observed various phytoestrogens in our plant like Pregene derivative and Androsta-triones. Phytoestrogens are proven osteoprotective agents by so many workers (Cassidy *et al.*, 1993; Gardner *et al.*, 1998; Fang *et al.*, 2003; Yang *et al.*, 2006). Thus, this plant may also have a protective role in prevention of bone loss in osteoporosis. This plant is also reported to have aphoridisiac effect in male rats by increasing the ejaculation latency (Sukh Dev, 2006). Our analysis showed the presence of various testosterone derivatives in it, thereby supporting its aphrodisiac effect. This plant was also found to contain coumarin and cinnamic acid like flavonoids derivatives and, cinnamolaurine and crinamine like alkaloids which are considered to be having pharmaceutical property (Hahlbrock and Sheel, 1989). Many piperizine derivatives are established pharmacological drugs, and *LG* GC MS revealed various piperizine derivatives. Piperizine and piperidine derivatives are used in synthesis of various pharmaceutical drugs. Piperizine citrate is a standard drug used in the treatment of helminth infection. Presence of various piperizine derivatives justifies the recent study which showed that this plant is having antihelminthic property (Pattari *et al.*, 2010). Piperidine derivatives have been isolated from numerous natural alkaloids and they are ubiquitous building blocks in the synthesis of pharmaceuticals. Presence of these many important phytochemicals appeal for further analysis of this plant in various pathological conditions.

Our study suggested that *LG* is rich in alkaloids, which contribute towards its biological activity as an anti microbial and antifungal agent and justifying its use in treatment of Diarrhoea and dysentery. Presence various phytoestrogens and other alkaloids suggest that consumption of this plant can be helpful in treating osteoporosis and it can be worth exploring this plant for other pharmacological interventions. GC MS analysis showed probable phytochemical constituents of the plants. However, NMR analysis is required to understand the exact molecular structure for further elucidating the mode of action of the molecules.

Table 4.1: Phytochemical analysis of LG

Fraction		Test	Presence/Absence
Alkaloids		Mayers	+
		Wagners	+
		Dragondorff's	+
Flavonoids		N-lead acetate	-
		Zinc dust	-
		NaOH	+
		H <sub>2</sub> SO <sub>4</sub>	-
Terpenoids		Chl+H <sub>2</sub> SO <sub>4</sub>	-
Amines		Na-Nitro	-
		Dragondorff's	-
		Ehrlich	-
Glycosides	cynogenic	Molisch	+
		Cold H <sub>2</sub> SO <sub>4</sub>	+
	cardiotonic	Kedde	-
		Keller	-
Phenols		N-FeCl <sub>3</sub>	+
		FeSO <sub>4</sub>	+
Anthocynins		Na acetate	-
		Na <sub>2</sub> CO <sub>3</sub>	-
Tannins		Gelatin	+
		K <sub>2</sub> Cr <sub>2</sub> O <sub>7</sub>	+
		Iodine	-
		Lead acetate	-
Saponin		Water	+
		Lead acetate	+

**Table 4.2** The antimicrobial activity of *LG* fractions, compared with standard drug amoxicillin.

Strain	LG MET	LG ALK	MOX
<i>E.coli</i>	200	250	100
<i>S.typhi</i>	250	200	100
<i>Cl.tetani</i>	500	250	100
<i>Vi.cholerae</i>	100	150	250
<i>S.pyogenus</i>	200	150	100
<i>E.faecalis</i>	500	250	250
<i>P.vulgaris</i>	100	100	100

**Table 4.3** Anti fungal activity of fractions of *L. gluinosa* compared with standard drug Nystatin.

	LG MET	LG ALK	NYSTATIN
<i>C. albicans</i>	500	250	100
<i>A. Clavatus</i>	1000	500	100

**Table 4.4** Rf values of various alkaloid fractions.

Band	Rf value
A	0.93
B	0.71
C	0.62
D	0.61
E	0.58
F	0.42
G	0.34
H	0.18

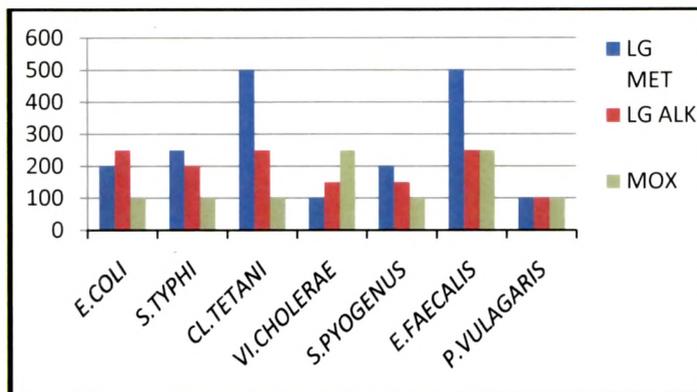
Table 4.5 GC MS scan of plant and its fractions

Fraction	Phytochemical showing maximum probability of occurrence in molecular mass comparison
Total methanolic extract	Oleic acid, tricosene, erucic acid, tetra decanoic acid, pyrrolidinone, piperidine, eicosanoic acid,
Alkaloid fraction	Eicosane, Pieprizine, pyridine, Androsta-trione, pregnene, thio coumarin, tetrahydroisoquinoline, crinamine, Androstane,

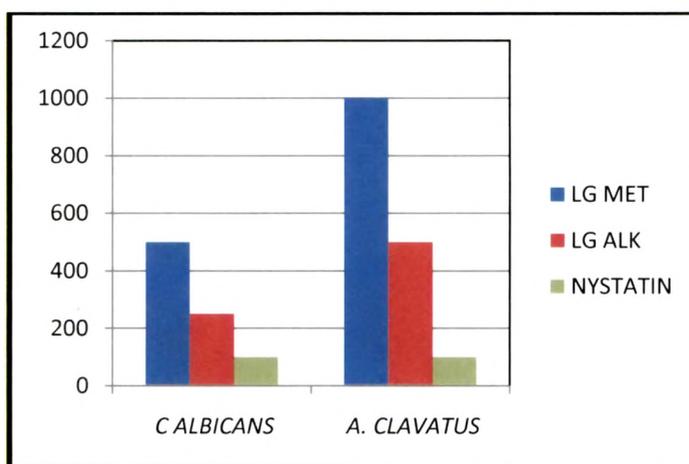
Table 4.6: subfractions of alkaloids

A	Dichloro acetyl phenyl piperazine, cinnamolaurine, Androsta 1, 4 diene trione, 3 $\alpha$ acetyl 20 keto 11 pregnene, tetrahydroisoquinoline, flourocinnamic acid, crinamine, Androstane 3 17 diol, dihydroandrostarone,
B	4, 4, 6 triemethyl Thiocoumarin, coumarin, gestonorone,
C	Eicosane, Oleic acid, Palmityl ester, dimethoxy tetrahydroisoquinoline, aminophenyl flouro cinnamic acid,
D	Eicosane, Dodecane, Hepta decane.
E	Trimethyl Dodecane, Penta Decane.
F	Tricosane, Hepta decane,
G	Benzene dicarboxylic acid diundecyl ester, tridecanoic acid, diacyl ester.
H	Hepta decanoic acid hydroxyl propanediyl ester, Heptacosane.

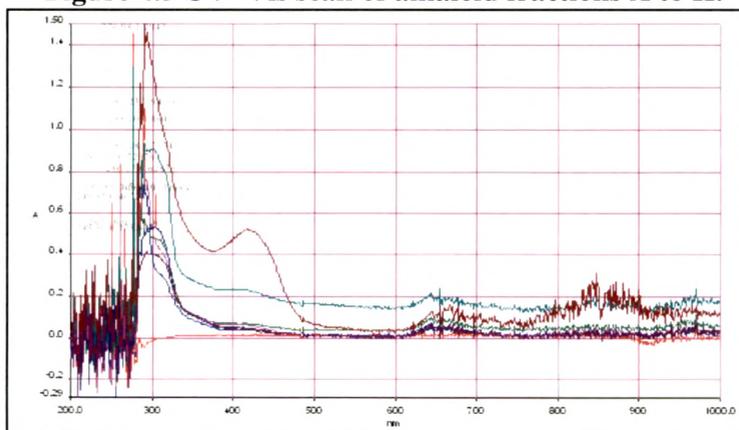
**Figure 4.1 Antibacterial activity of LG and its alkaloids.**



**Figure 4.2 Antifungal activity of LG and its alkaloids**



**Figure 4.3 UV- Vis scan of alkaloid fractions A to H.**



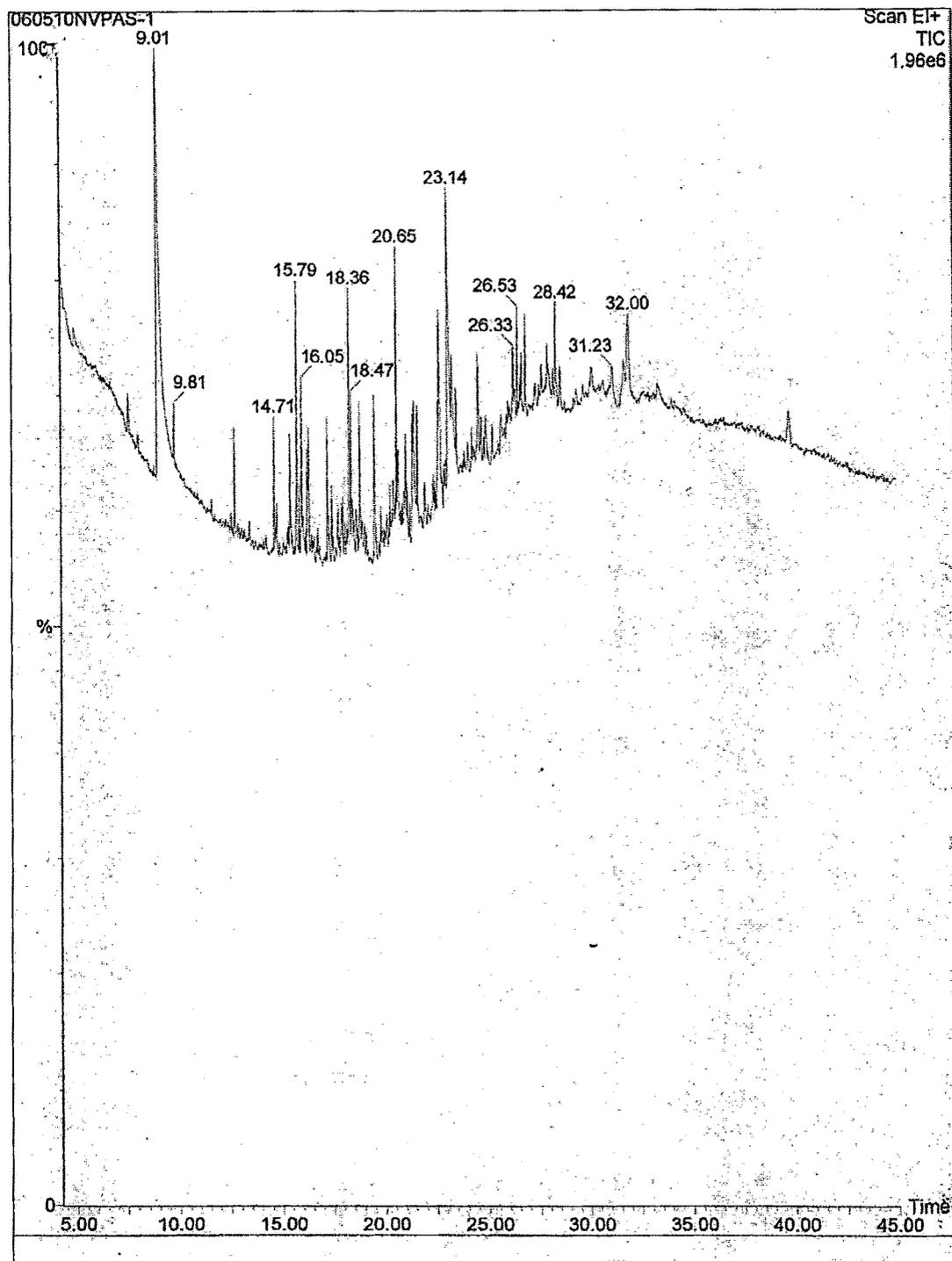


Figure 4.4 GC MS Scan of methanolic extract of plant

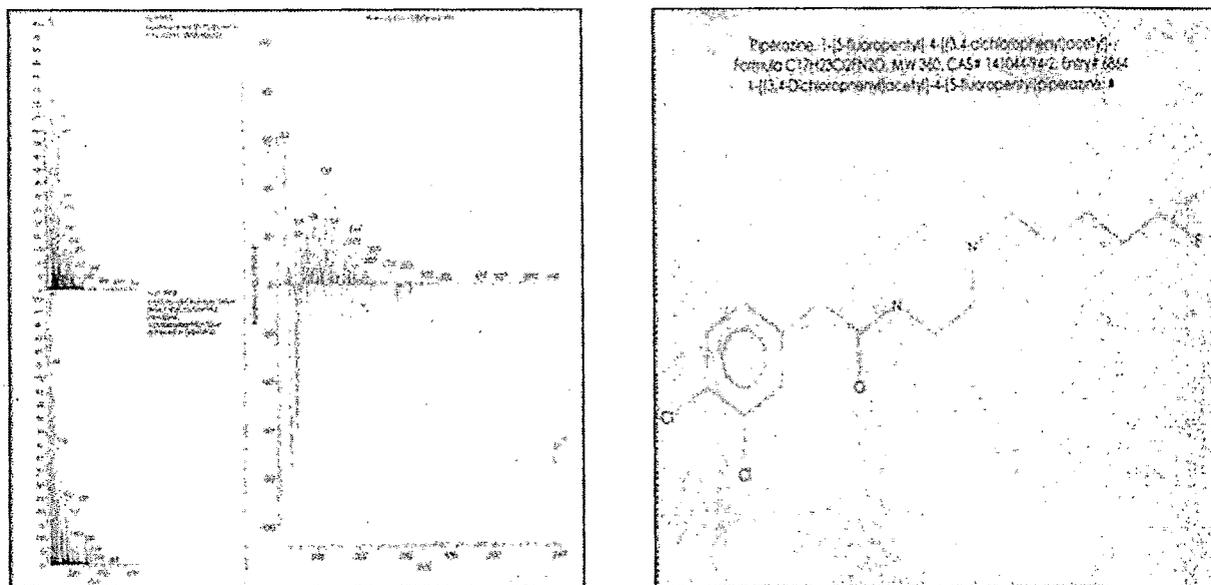


Figure 4.5a MS analysis and probable presence of Piperazine derivatives

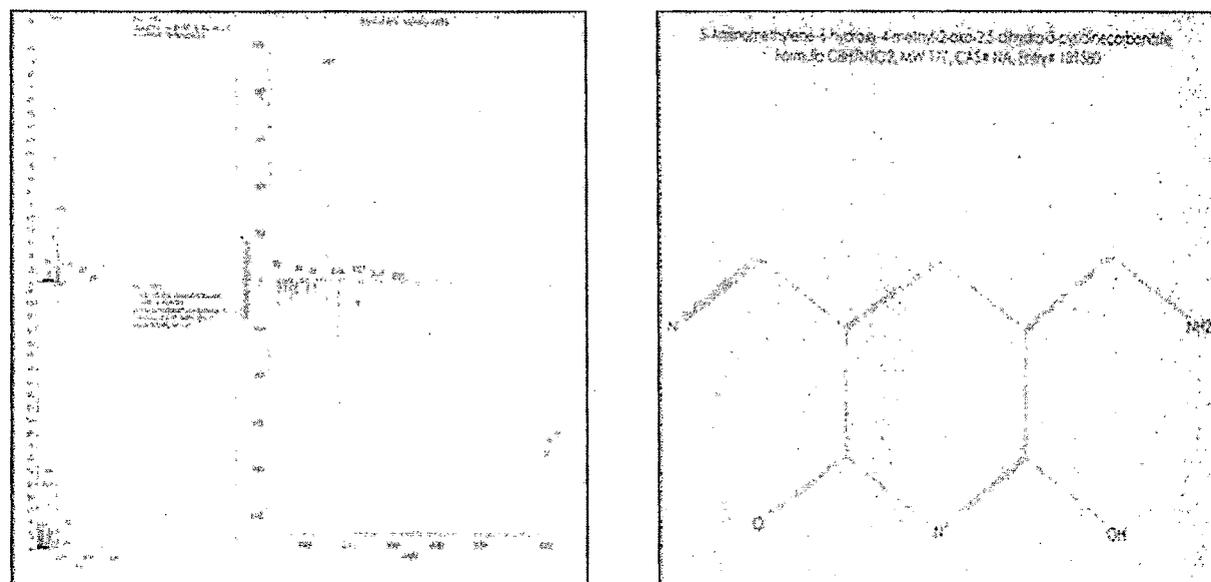


Figure 4.5b MS analysis and probable presence of Piperazine carbonyl

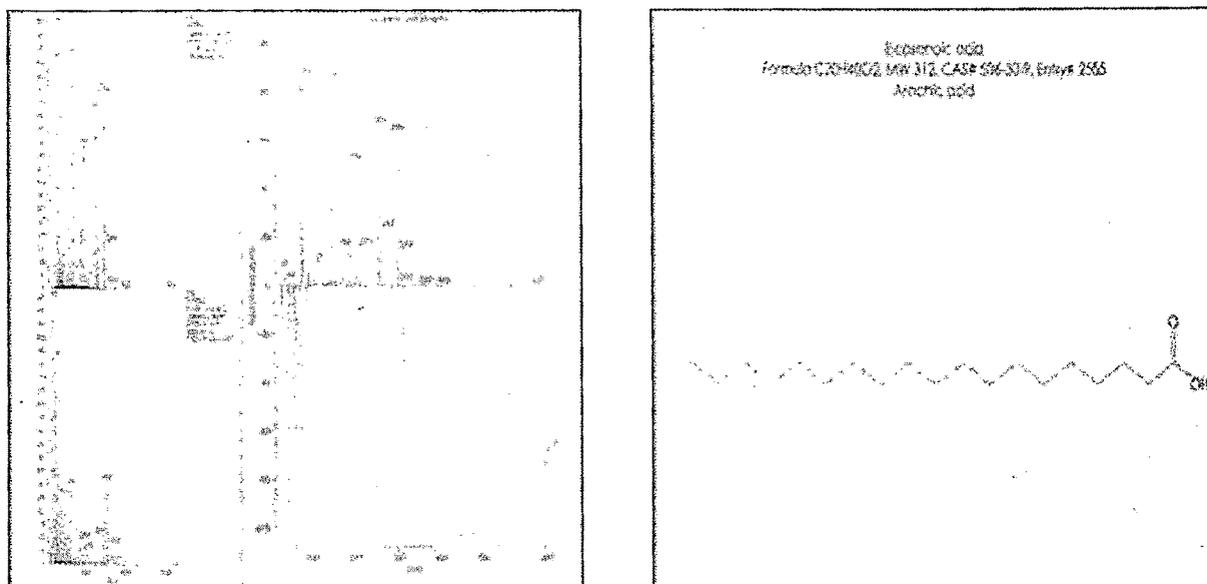


Figure 4.5c MS analysis and probable presence of Eicosanoic acids

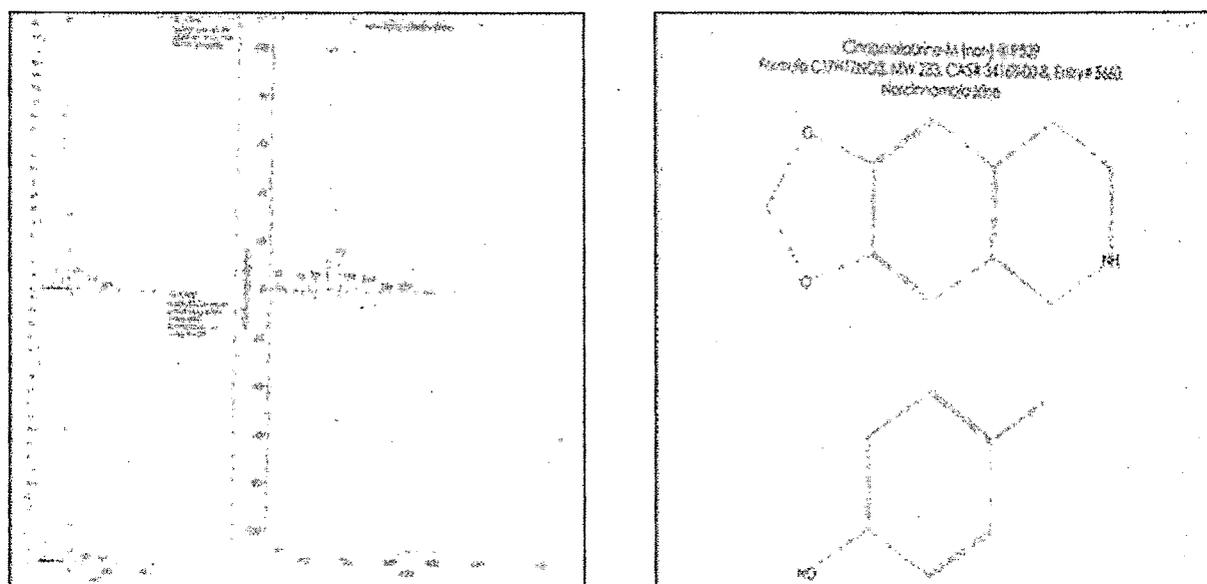


Figure 4.5d MS analysis and probable presence of Cinnamoyl-L-histidine derivatives

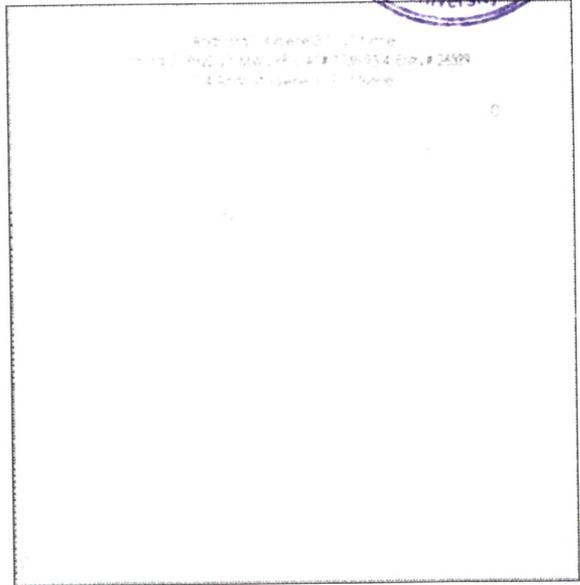
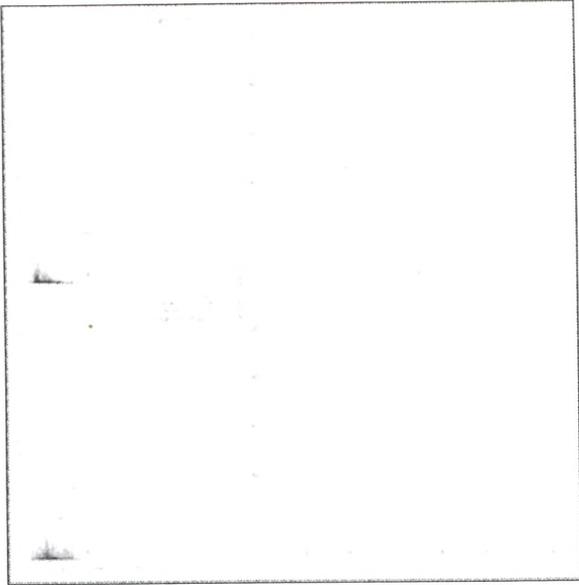


Figure 4.5e MS analysis and probable presence of Androstane derivatives

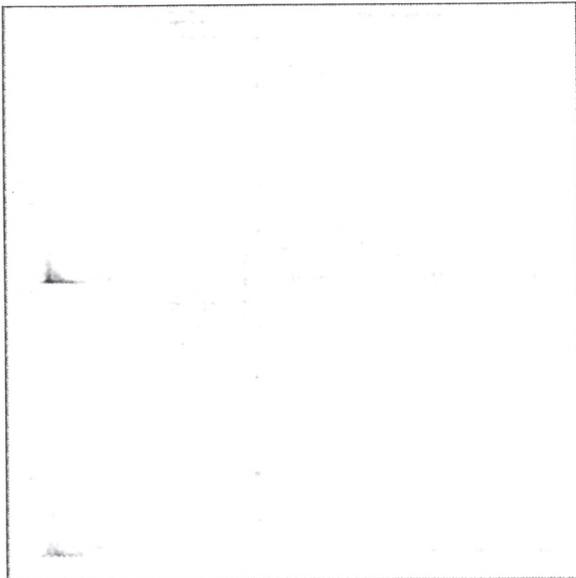


Figure 4.5f MS analysis and probable presence of Pregnane derivatives

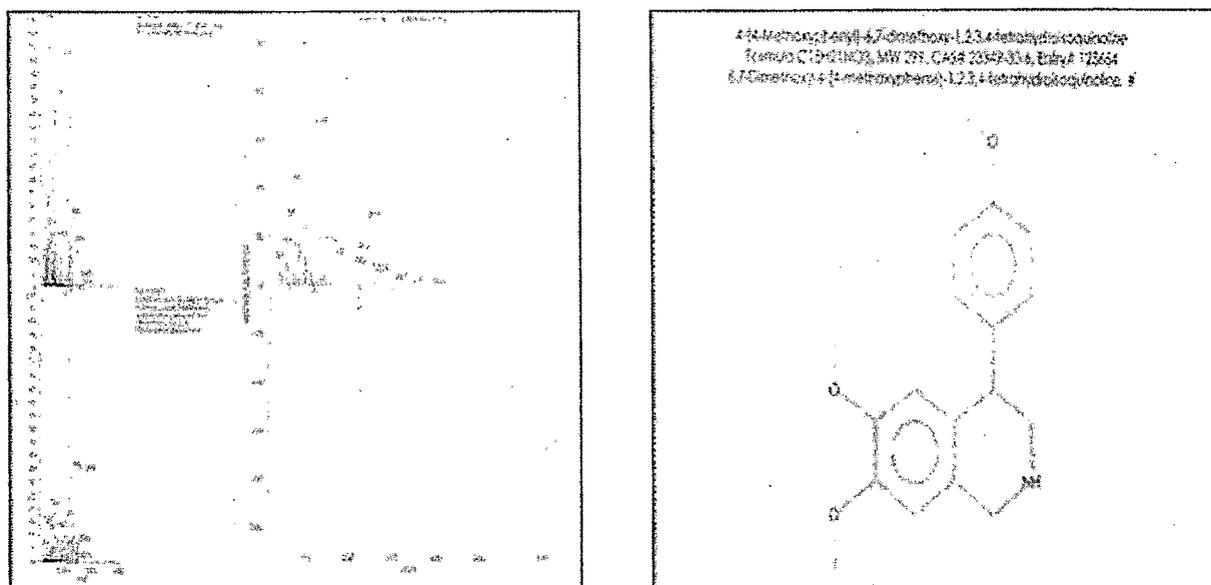


Figure 4.5g MS analysis and probable presence of Quinoline derivatives

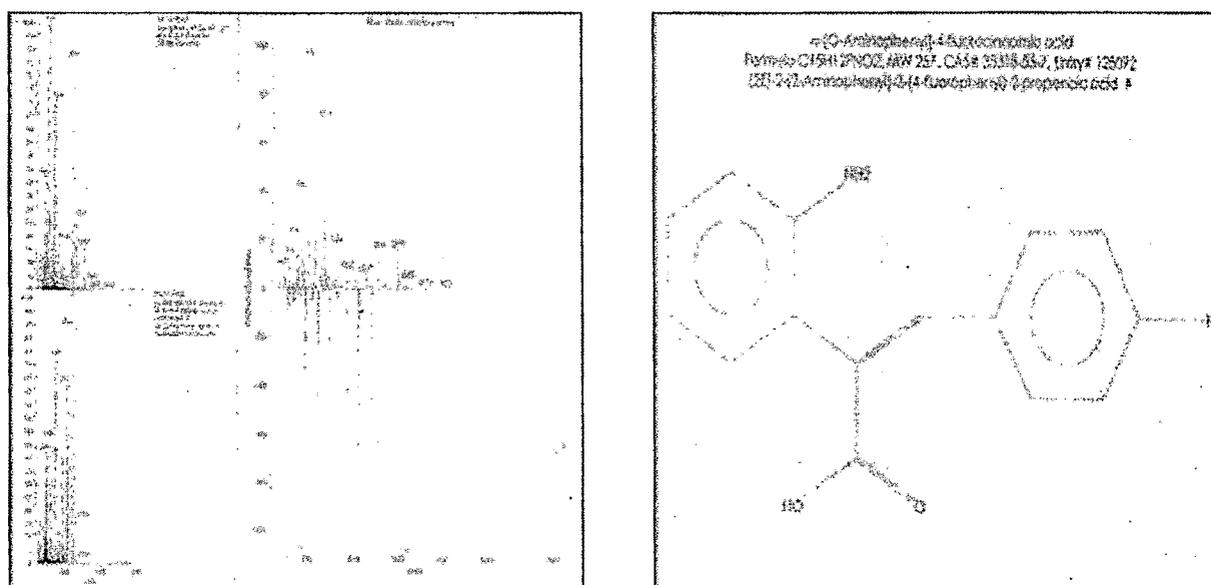


Figure 4.5h MS analysis and probable presence of Cinnamic acid derivative carobnitrile

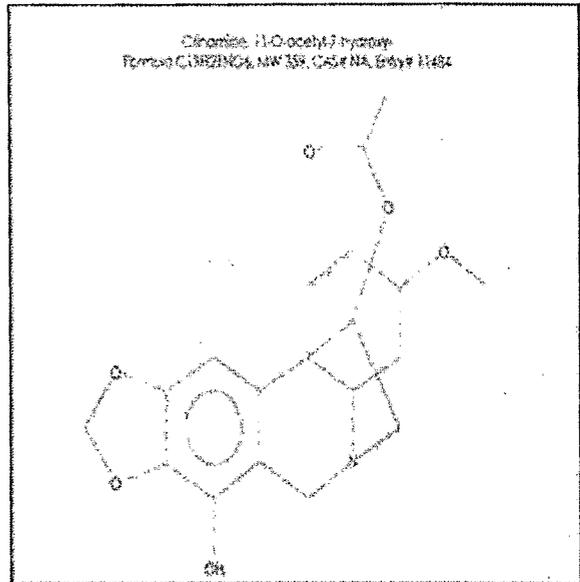
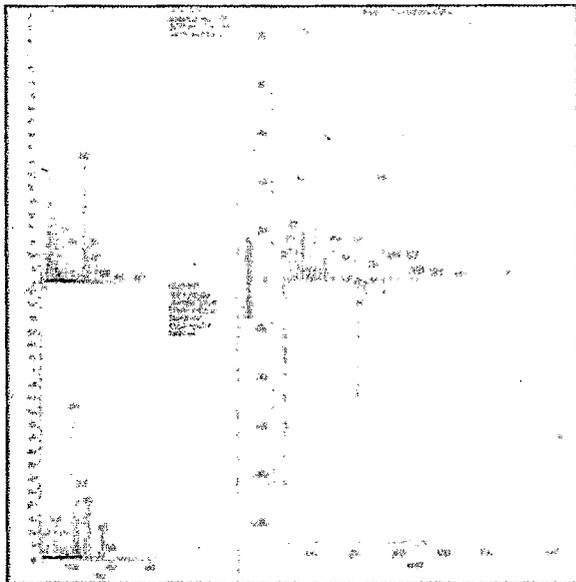


Figure 4.5i MS analysis and probable presence of Crinamine derivatives

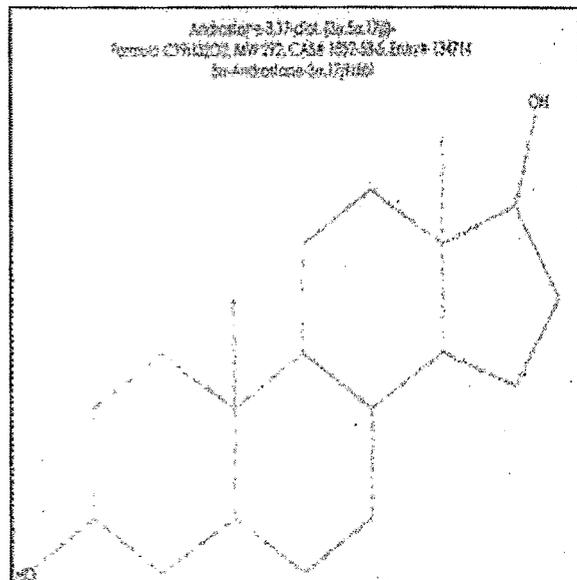
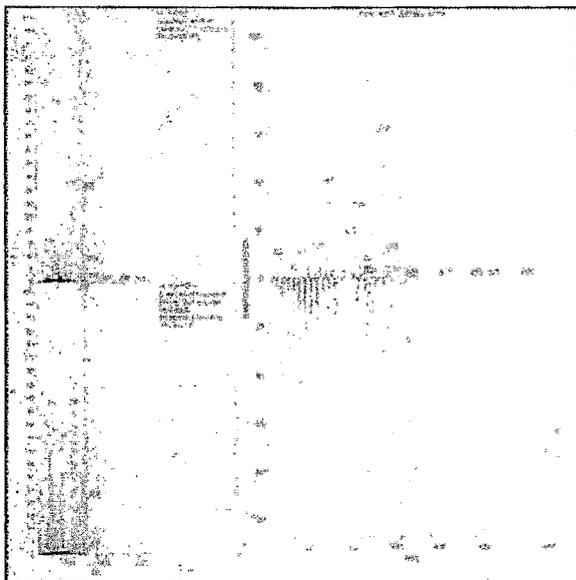


Figure 4.5j MS analysis and probable presence of Androstan

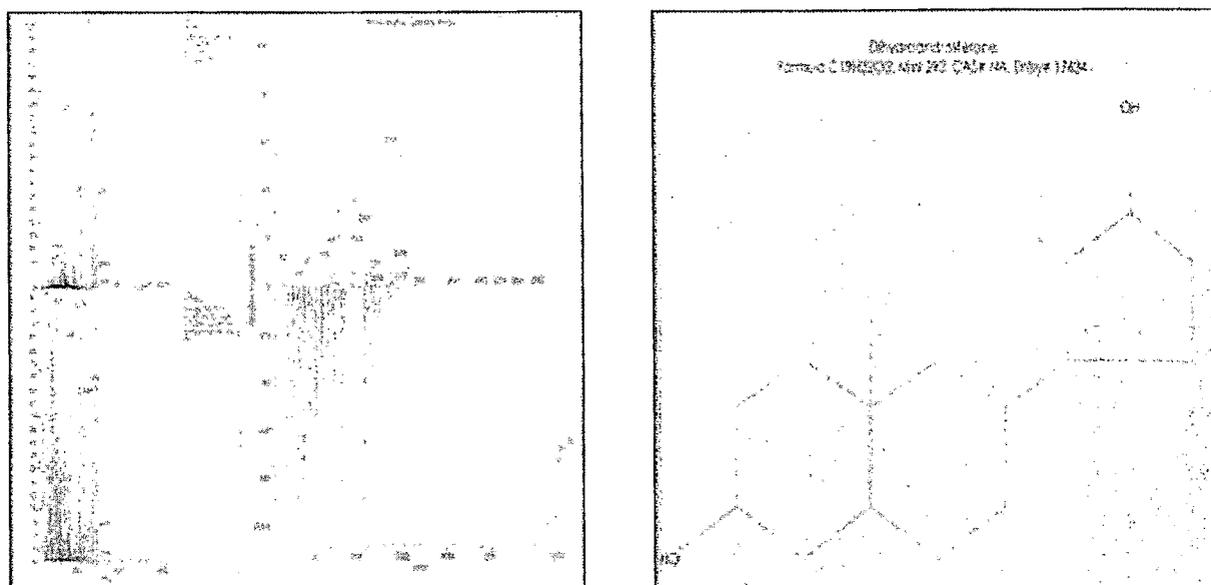


Figure 4.5k MS analysis and probable presence of Dihydroandosteron derivatives

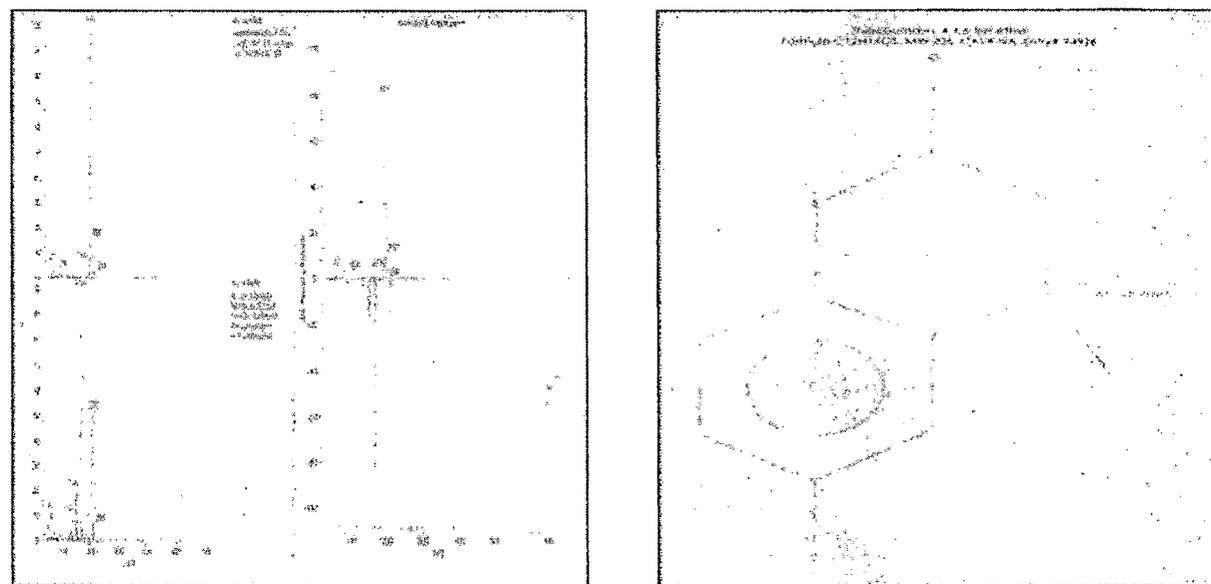


Figure 4.5l MS analysis and probable presence of Thiocoumarin

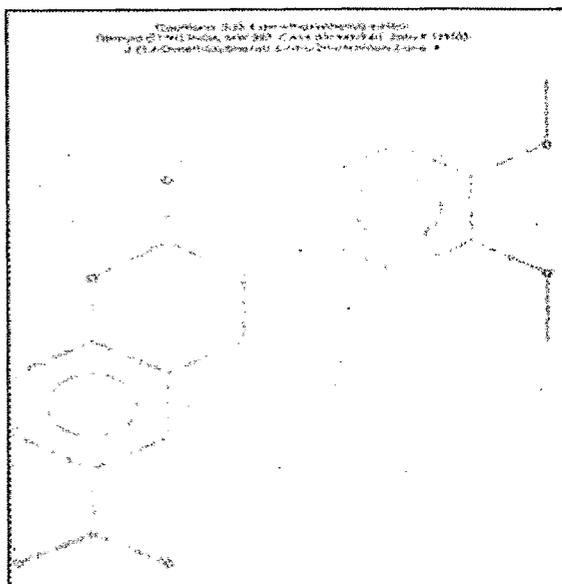
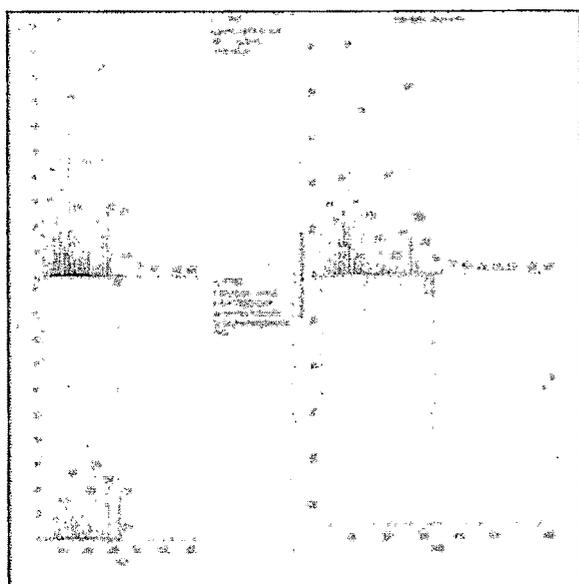


Figure 4.5m MS analysis and probable presence of cinnamonyl derivatives

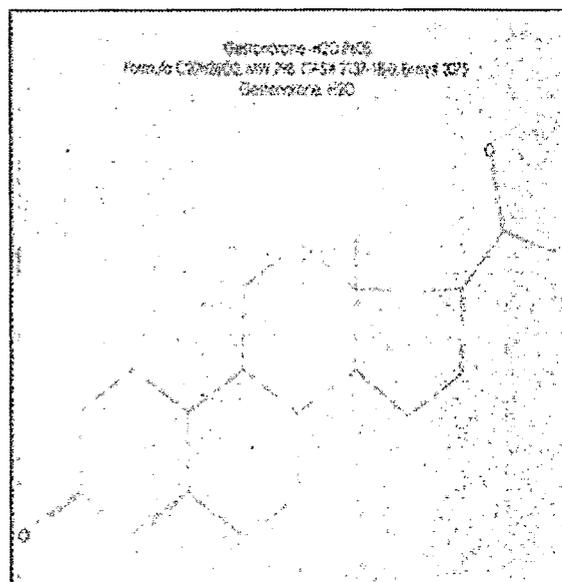
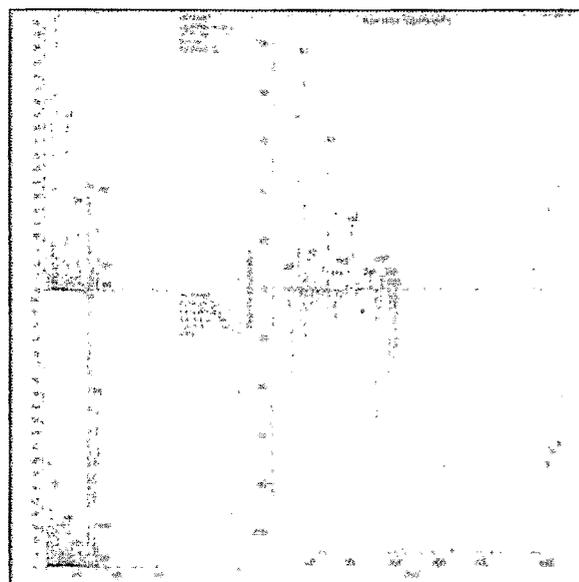


Figure 4.5n MS analysis and probable presence of Gestonorone