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Phytochemical Characterization of Active Constituents from Extracts of *Ixora Javanica* D.C Flowers

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Abstract

The investigation of chemical compounds from natural products is fundamentally important for the development of new drugs, especially in view of the vast worldwide flora. The present work is extraction, isolation and characterization of active constituents from *Ixora javanica* D.C flowers. Freshly collected flowers were shade dried, powdered and subjected to continuous soxhilation with various solvents like petroleum ether (60-80°), ethyl acetate and ethanol (70%) of increasing polarity. All the three obtained extracts were concentrated and after preliminary phytochemical investigation, column chromatographed using silica gel.

Three triterpenoidal compounds (Maslinic acid, Ursolic acid and Lupeol), one flavonoidal compound (Formononetin) and two flavonoidal glycoside compounds (Quercetin-3-rutinoside, Quercetin-3-glucoside) have been isolated and further these isolated structures were established by spectral analysis (IR, ^{1}H NMR spectra, ^{13}C NMR and Mass) and direct comparison with authentic samples. This is the first report of occurrence of these compounds from *Ixora javanica* D.C flowers.

Kewords: *Ixora javanica* D.C flowers; Maslinic Acid; Ursolic Acid; Formononetin; Quercetin-3-rutinoside; Quercetin-3-glucoside; Lupeol

Introduction

Natural products once served humankind as the source of all drugs, and higher plants provided most of these therapeutic agents. Today, natural products and their derivatives still represent over 50% of all drugs in clinical use, with higher plant-derived natural products representing 25% of the total [1]. Traditional medicine has served as a source of alternative medicine, new pharmaceuticals, and healthcare products. Medicinal plants are important for pharmacological research and drug development, not only when plant constituents are used directly as therapeutic agents, but also as starting materials for the synthesis of drugs or as models for pharmacologically active compounds [2]. The World Health Organization estimates that 80% of the people in developing countries of the world rely on traditional medicine for their primary health care, and about 85% of traditional medicine involved the use of plant extracts. This means that about 3.5 to 4 billion people in the world rely on plants as sources of drugs [3].

Ixora is a genus of flowering plants in the Rubiaceae family. It consists of tropical evergreen trees and shrubs and holds around 500 species with its centre of diversity in Tropical Asia. Most of the species are grown as ornamental plants. Phytochemical studies of some species like *Ixora coccinea* [4], *I. finalaysonia* [5,6], *I. arborea* [7], etc., indicated the presence of important phytochemicals [8-11] such as lupeol, ursolic acid, oleanolic acid, sitosterol, rutin, lecocyanadin, anthocyanins, proanthocyanidins, glycosides of kaempferol and quercetin. Hence an effort was made to investigate the chemical constituents from another species of Ixora i.e., *Ixora javanica* (*I. j*).

From the literature survey, it was revealed that no substantial work was carried out on *I. javanica* D.C flowers (Figure 1) both in the chemical investigation and pharmacological activities. Hence an effort was made to investigate the chemical constituents of the different extracts of *I. javanica* flowers. Some current drugs are not beneficial in all cases, due to their side effects and potency. Hence search for other alternatives seems necessary and beneficial. This study was aimed to phytochemical investigation of the pure compounds to be used for treating various ailments (like anti-oxidant, hepatoprotective, anti-cancer activities, etc.).

Experimental Procedure

Materials and methods

Solvents: Petroleum ether (60-80°), ethyl acetate, ethanol (70%), chloroform, methanol, ethanol (70%), benzene, etc.

Chemical and reagents: 1. Laboratory grade (L. R) chemicals were used for isolation. 2. Analytical grade (A. R) reagents were used for analytical work.

Absorbents: 1. Silica Gel (ACME Chemical works, Mumbai) was used for TLC. 2. Silica Gel of mesh size 60-120 (Merck, Bombay) was used for column chromatography.

Equipments: All the Melting points were recorded in a Toshiwal electrically heated melting point apparatus and were uncorrected. I.R spectra of the compounds were recorded using Thermo Nicolet Nexus 670 I.R spectrophotometer. ^{1}H NMR spectra were taken on Varian EM-360 (300 MHZ) NMR spectrometer using $CDCl_3$ as solvent. ^{13}C NMR was recorded on Bruker instrument with $CDCl_3$ as solvent at 300 MHz. Mass spectra were recorded on a GC-MS, data on E:/ISO/21184-1.QGD.

General experimental procedures

Collection and authentication *I. javanica* flowers: *I. javanica* D.C flowers were collected from local areas of Hyderabad and authenticated by Dr. B. Bhadraiah, HOD, Department of Botany, Osmania University, Hyderabad. Voucher specimens ((IJ/2012/23) are kept at Malla Reddy College of Pharmacy, Dhulapally, Hyderabad, Telangana, India.

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Figure 1: *Ixora javanica* D.C flowers and leaves.

Preparation of different extracts from flowers of *I. javanica* D.C: The shade dried flowers of *I. javanica* were reduced to fine powder and around 5 kg were subjected to continuous successive extractions with different solvents of increasing polarity like petroleum ether (60-80°), ethyl acetate and ethanol (70%) into 15 batches of each 250-300 gm each in an ethane extractor. These extracts were filtered and air dried. The air dried extracts were weighed and percentage yields of extraction were calculated. The colour and consistency of the extracts were recorded. The organic extracts were dried in vacuum desiccators and the solvents were evaporated in vacuum and extracts were stored in desiccators.

After drying the respective extracts were weighed and percentage yield were recorded (Table 1).

Preliminary phytochemical tests: The preliminary phytochemical studies were performed for testing the different chemical groups present in different extracts. The chemical group tests were performed using different reagents for the identification of different groups. Petroleum ether (60-80°) extract indicated the presence of steroids and triterpenoids whereas ethyl acetate extract gave positive results for the presence of flavonoids, carbohydrates and tri terpenoids and ethanolic extract (70%) showed the presence of steroids, triterpenoids, flavonoids, alkaloids, ethane, carbohydrates, tannins and glycosides (Table 2).

Isolation and characterization of various compounds

Isolation of phytoconstituents from petroleum ether extract: 150 g of silica gel for column chromatography was activated in hot air oven at 110°C for one hour. The petroleum ether was used to build the silica gel in the glass column. The glass wool was fixed at the bottom. The activated silica gel was charged into column in small proportion with gentle tapping after each addition in order to ensure uniform packing. The small quantity of solvent (petroleum ether 60-80°) system was allowed to remain on the top of the column. The air bubbles present in the column were removed by gentle tapping to get a uniform bed of adsorbent.

The concentrated petroleum ether extract (35 g) of *I. javanica* was dissolved in a small quantity of the n-hexane and adsorbed on silica gel and then charged into column. The chromatogram was allowed to develop overnight, taking care to prevent the drying of the column by plugging the open end with adsorbent cotton. The elution of solvent system was started after complete saturation of the column. Column being successively eluted with increasing polarities of n-hexane, n-hexane: ethyl acetate, ethyl acetate. The elution was carried out with n-hexane:ethyl acetate in graded mixture i.e., 98: 2, 96: 4, 94: 6, 92: 8,……up to 50:50 and finally carried out with 100% ethyl acetate. From above elution's, two different fractions were collected (i.e., fractions A and B).

Fraction A was eluted from n-hexane: ethyl acetate (98:2) resulted in a single compound which was confirmed by TLC (n-hexane:ethyl acetate, 8.5:1.5). This elute was collected and concentrated to about 10 ml crude residue substance. It was recrystallized with acetone. The product was designated as compound I-1

Fraction B was eluted from n-hexane:ethyl acetate (84:16), gave a single compound which was confirmed by TLC (n-hexane:ethyl acetate, 8:2). This elute collected was concentrated to crude residue and further recrystalized with acetone. The product was designated as **compound I-2.**

Isolation of phytoconstituents from ethyl acetate extract: The concentrated ethyl acetate extract (33 g) of *I. javanica* was dissolved in a small quantity of the chloroform (20 ml) and adsorbed on silica gel and then charged into column (same as 3.3.1 section). The elution were carried out with chloroform: methanol in graded mixture i.e., 95:05, 90:10, 85:15, and 80:20 …up to 100% methanol. From above elution's three different fractions were collected (fraction C, D and E).

Fraction C was eluted from chloroform: methanol (90:10) and it gave a single spot by using TLC plate in chloroform: methanol (9:1) as mobile phase and its resolution factor was found to be 0.82, the compound was designated as **compound I-3**.

Fraction D was eluted from chloroform: methanol (80:20) and it gave a single spot by using TLC plate in toluene: methanol (4:1) as mobile phase and its resolution factor was found to be 0.61. The product was designated as **compound I-4.**

Fraction E was eluted from chloroform: methanol (80:20) and it gave a single spot by using TLC plate in chloroform: methanol (9:1) as mobile phase and its resolution factor was found to be 0.55. The product was designated as **compound I-5.**

Isolation of phytoconstituents from ethanolic extract: The concentrated ethanolic extract (30 g) was dissolved in a small quantity of the same solvent (chloroform, 20 ml) and adsorbed on silica gel and then charged into column. The column being successively eluted with chloroform: methanol in graded mixture i.e., 95:05, 90:10, 85:15, 80:20,……up to 100% methanol. From above elution's, only one fraction was collected (i.e., fraction F). The concentration of other elutes gave only brown resinous masses which were not processed further.

Fraction F was eluted from chloroform: methanol (90:10) and it gave single spot by using TLC plate in toluene: methanol (4:1) as mobile phase and its resolution factor was found to be 0.81. The product was designated as **compound I-6.**

S No	Extracts	Nature of extract	Colour	Yield (%)
1.	Petroleum ether extract	Fine powder	Yellow orange	8.9
2.	Ethyl acetate extract	Sticky mass	Light yellow	6.7
3.	Ethanol (70%) extract	Sticky mass	Dark green	12.3

Table 1: Percentage yield of different extracts of flowers of *Ixora javanica* D.C.

Extracts	Chemical constituents
Petroleum ether (60-80°) extract	Steroids, triterpenoids
Ethyl acetate extract	Flavonoids, carbohydrates and tri terpenoids.
Ethanolic extract (70%)	Steroids, triterpenoids, flavonoids, alkaloids, saponins, carbohydrates, tannins and glycosides.

Table 2: Results of preliminary phytochemical analysis of different extracts of *I. javanica* flowers.

Results and Discussion

Extraction and preliminary qualitative analysis

The shade dried of flowers of ***I. javanica*** was subjected to successive solvent extraction method by using petroleum ether, ethyl acetate and ethanol (70%). All these extracts were concentrated and calculated for their percentage yield. The yields were found to be 8.9%, 6.7% and 12.3% respectively.

The results of phytochemical investigation of petroleum ether extract shown the presence of sterols, triterpenoids, whereas, ethyl acetate extract shown the presence of flavonoids, triterpenoids and carbohydrates and finally ethanolic extract (70%) indicated the presence of steroids, triterpenoids, flavonoids, alkaloids and glycosides.

Phytochemical characterization of isolated compounds from different extracts of *I. Javanica* flowers

From petroleum ether extract: The two different fractions were obtained from petroleum ether extract.

From fraction A

A yellow orange fine powder was resulted as a single compound from the fraction A. It was designated as **compound I-1,** which was further recrystalized from acetone, with M.P 162-166°C range. IR (KBr) showed absorption band at 3446.24 cm^{-1} of –OH group, a band at 1700.00 cm^{-1} indicated C=O stretching, a peak at 1460.77 cm^{-1} indicated $-CH_2$ bending, a peak at 1376.21 cm^{-1} indicated CH_3 bending and a peak at 1169.43 cm^{-1} indicated C-O stretching.

^1H NMR spectral data, one vinylic portion peak at 5.4 δ ppm, methylene proton peaks appeared as multiplet in between 2.4 to 1.2 δ ppm. Free four $-CH_3$ protons observed as multiplet at 0.99 δ ppm.

^{13}C NMR spectral data exhibited the presence of 30 carbon signals of both aliphatic and cyclic carbons are in their respective ppm. Spectral data matched exactly with that of maslinic acid (Table 3).

Further the structure was confirmed as **"maslinic acid"** (Figure 2) with its molecular ion peak 472 $[M^+]$ (18%) and confirmed with authentic sample.

From fraction B

Whereas fraction B obtained a single triterpenoidal compound, the elution was with n-hexane: ethyl acetate (84:16) which gave a single compound and was confirmed by TLC (n-hexane: ethyl acetate, 8:2). It was designated as **compound I-2,** and was further crystallized from acetone, M.P 284-286°C.

IR (KBr) showed absorption bands at 3430 cm^{-1} which indicated the presence of –OH stretching, 2920.59 cm^{-1} and 2850.90 cm^{-1} defined both CH_2 and CH_3 stretchings, 1742.76 cm^{-1} indicated C=C stretching, 1462.02 cm^{-1} defined $-CH_3$ deformation and a peak at 1163.98 cm^{-1} indicated C-O stretching of secondary alcohol.

In ^1H NMR spectral data, one vinylic proton peak was observed at 5.6 δ ppm, 25 methylene proton peaks appeared as multiplet in between 2.75 to 1.0 δ ppm. Free four CH_3 observed as multiplet at 0.88 δ ppm, methene group of proton peaks appeared as multiplet in between 1.6 δ ppm, proton of carboxylic group was appeared as singlet at 10.3 δ ppm.

^{13}C NMR spectral data exhibited the presence of 30 carbon signals of both aliphatic and cyclic carbons in their respective ppm. The ^{13}C NMR spectral data matched with that of ursolic acid (Table 3). Its identity as **"ursolic acid"** (Figure 3) was further confirmed by mass spectra with its molecular ion peak at 457 $[M+H]^+$ (54%).

From ethyl acetate extract: Three different fractions (C, D, E) were obtained from ethyl acetate extract.

From fraction C

All the collected elutes were combined and subjected for concentration, which was designated as **compound I-3.** This was further recrystallized from methanol, resulted as yellow crystalline needles with M.P 241-243°C.

The IR spectra of compound I-3 showed absorption band for -OH stretching at 3399.14 cm^{-1}, aromatic CH stretching observed at 2925.03 cm^{-1}, C=O stretching absorption peak at 1732.11 cm^{-1}, C=C stretching indicated the absorption peak at 1515.43 and 1459.37 cm^{-1} and finally C-O stretching at 1169 and 1074.54 cm^{-1}.

In ^1H NMR, at 5.42 δ ppm indicated the doublet of four –OH groups, 4.0 to 3.1 δ ppm denoted the –OH groups of sugar moiety.

^{13}C NMR spectral data exhibited the presence of 21 carbon signals of both aromatic and aliphatic carbons in their respective ppm. ^{13}C NMR spectral data matched exactly with that of quercetin-3-glucoside (Table 4). Further the structure was confirmed as **"quercetin-3-glucoside"** (Figure 4) with its molecular ion peak 464 $[M+H]^+$ (20%) and confirmed with authentic sample.

From fraction D

Elutes with chloroform: methanol (80:20) showed the presence of a single compound, which was confirmed by TLC (Toluene: Methanol, 4:1). All the collected elutes were combined and subjected for concentration, which was designated as **compound I-4.**

The IR spectra of compound **I-4** showed absorption band for OH-stretching at 3389.15 cm^{-1}, aromatic C-H stretchings were observed at 2925.03 cm^{-1}, C=O stretching absorption at 1742.20 cm^{-1}, C=C stretching indicated the absorption at 1459.37 and 1374.59 cm^{-1} and finally C-O stretching at 1239.59 cm^{-1}, 1169.00 cm^{-1} and 1074.54 cm^{-1}.

In ^1H NMR, the singlet of aromatic protons was observed at 7.25,

Carbon No.	Compound		
	I.1	I.2	I.6
1	48.2	37.7	22.1
2	76.5	27.1	31.8
3	80.5	74.4	76.5
4	38.3	39.5	44.3
5	55.3	56.5	49.3
6	18.1	19.9	23.9
7	34	33.2	19.9
8	39.9	39.9	62.1
9	50.3	45.7	37.9
10	37.8	37.1	50.5
11	23.7	22.8	41.1
12	129.7	121.9	27.7
13	150.8	140.8	50.9
14	42.9	42.2	38.5
15	29.1	29.1	41.9
16	24.9	25.1	32
17	47.9	50.1	51.9
18	42.8	51.1	52.1

Table 3: ^{13}C-NMR Spectral data of isolated compounds.

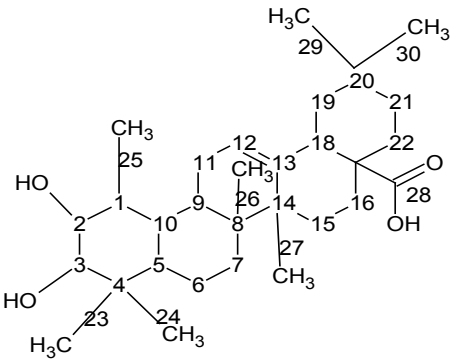

Figure 2: Maslinic acid.

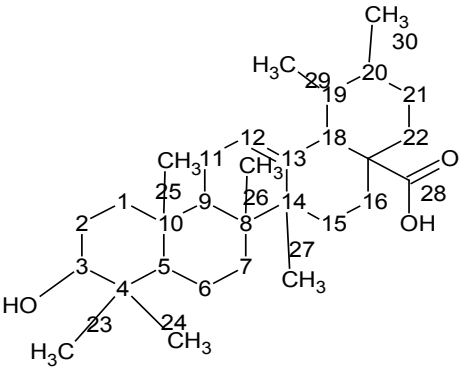

Figure 3: Ursolic acid.

Carbon No.	Compound I.3	Compound I.4	Compound I.5
	C-O	C-O	C-O
1	155.2	153.4	149.5
2	136.1	130.1	119.3
3	179.9	174.5	179.5
4	160.7	161.8	132.2
5	99.5	98.3	115.3
6	173.2	166.5	167.7
7	105.2	94	100.5
8	156.1	156.8	160.3
9	105.3	102.5	121.2
10	124.5	122.8	127.5
1'	142.7	112.3	127.1
2'	143.2	45.9	95.1
3'	126.2	144.5	160.2
4'	125.7	116.2	32.2
5'	125.3	121.8	34.2
6'	-	107.5	-
1"	-	70.4	-
2"	-	70.1	-

Table 4: ^{13}C-NMR Spectral data of isolated compounds.

6.9 and 6.25 δ ppm. Aromatic –OH groups was observed at 3.52 δ ppm. And ^{1}H of CH group in rhamnoglycosyl was observed at 3.48 to 3.97 δ ppm.

^{13}C NMR spectral data exhibited the presence of 27 carbon signals of both aromatic and aliphatic carbons in their respective ppm and the spectral data matched with exactly with that of Quercetin-3-rutinoside (Table 4). Further the structure was confirmed as "**Quercetin-3-rutinoside**" (Figure 5) with its molecular ion peak 610 $[M+H]^+$ (41%).

From fraction E

A light brown coloured compound was obtained by recrystalization with ethanol, M.P 291-293°C. Spectral characterization of IR (KBr) indicated the presence of phenolic –OH absorption at 3405.27 cm^{-1}, 1441 and 1373 cm^{-1} indicated both CH_2 and CH_3 bending, C=C stretching absorption at 1741.23 cm^{-1}, absorption at 1164.18 and 1050 cm^{-1} indicated C-O stretching of secondary alcohol, and absorption at 759 and722 cm^{-1} indicated C-H deformation.

In ^{1}H NMR spectral data, a peak of $-OCH_3$ group appeared as a singlet at 3.63 δ ppm, aromatic –OH group was observed at 5.4 δ ppm, 7H aromatic protons of ring A and B appeared as multiplets at 7.9 to 6.0 δ ppm.

^{13}C NMR spectral data exhibited the presence of 16 carbon signals of both aromatic and aliphatic carbons in their respective ppm. ^{13}C NMR spectral data matched exactly with that of formononetin (Table 4). Hence identified as "**formononetin**" (Figure 6) and was further confirmed by mass spectra with its molecular ion peak at 268 $[M+H]^+$ (16%) and also by Co. chromatography with authentic sample.

From ethanolic extract

A single fraction (F) was obtained from ethanolic extract.

From fraction F

Elutes with chloroform: methanol (90:10) showed the presence of single compound, which was confirmed by TLC (toluene: methanol 4:1). All the collected elutes were combined and subjected for concentration, which was designated as **compound I-6**. This was further recrystalized from methanol, resulted as light brown colour crystalline needles with M.P 218-220°C.

The IR spectra of compound I-6 showed absorption band for –OH stretching at 3421.4 cm^{-1}, aromatic –CH stretching observed at 2919.58 and 2848.80 cm^{-1}, C=C stretching absorption at 1714.15 cm^{-1}, C-O stretching of 2°alcohol indicates the absorption at 1062.98 cm^{-1}.

In ^{1}H NMR, doublet of protons of –C=CH, H-29 1a, 1b were observed at 5.35, 4.83 δ ppm, multiplet of –OH group observed at 3.75 δ ppm and multiplets of 6 $-CH_3$ of 18H were observed at 0.99 δ ppm.

^{13}C NMR spectral data exhibited the presence of 30 carbon signals of both aromatic and aliphatic carbons in their respective ppm. Spectral

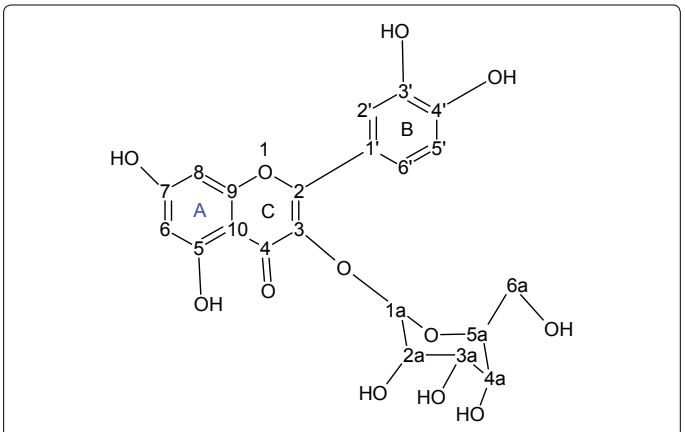

Figure 4: Quercetin-3-glucoside.

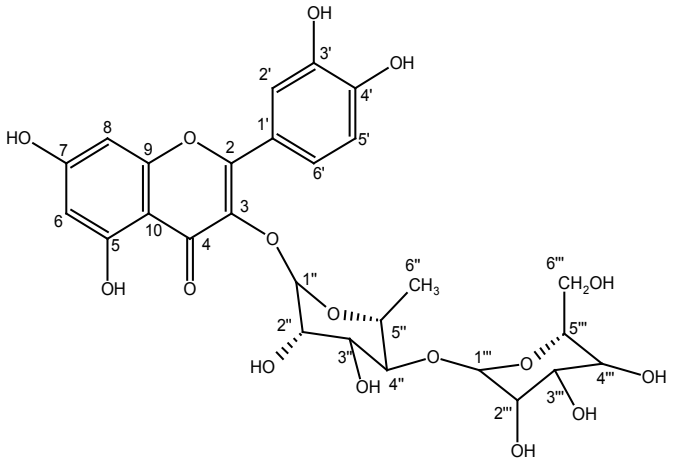

Figure 5: Quercetin-3-rutinoside.

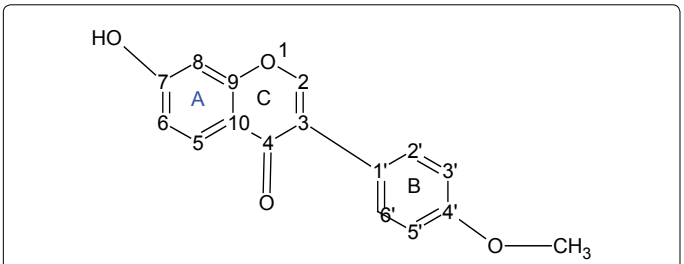

Figure 6: Formononetin.

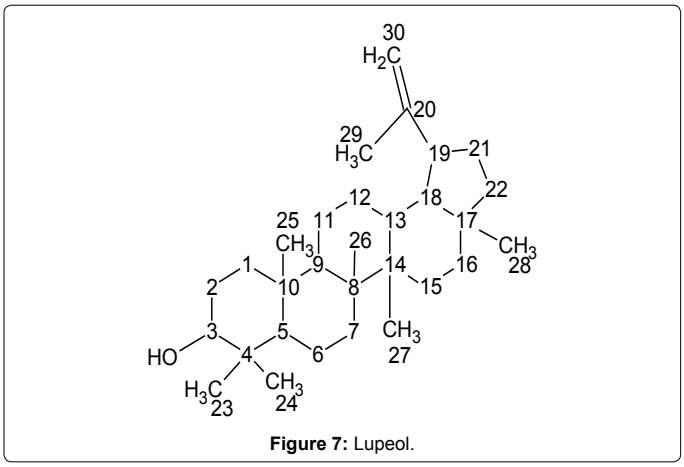

Figure 7: Lupeol.

data was matched exactly with that of authentic sample lupeol (Table 3). Further the structure was confirmed as **"lupeol"** (Figure 7) with its molecular ion peak 426 [M^+] (55%).

Conclusions

Maslinic acid and ursolic acid were isolated from petroleum ether extract by column chromatography using n-hexane: ethyl acetate as mobile phase. Quercetin-3-glucoside, quercetin-3-rutinoside and formononetin were eluted from ethyl acetate extract by column chromatography using chloroform: methanol as mobile phase. Lupeol was isolated from ethanol extract by using chloroform: methanol as mobile phase.

Based on the results, the obtained compounds of *I. javanica* D.C flowers are effective pharmaceutical compounds which will serve as a better alternative to chemical based pharmaceuticals. Further these compounds have to be screened for different activities based on literature available and were to be used for treating various ailments.

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