CHAPTER 4

RESULTS AND DISCUSSION

4.1 The percentage yield of medicinal plant extracts and essential oils

4.1.1 Extraction yield

The dried aerial parts of *A. indica*, and fresh leaves, stems and fruit of *C. javanicum* and *B. retusa* were separately ground and extracted at room temperature with hexane, chloroform and methanol respectively. The percentage yields of the crude extracts were calculated based on a dry weight (Table 4.1).

Table 4.1 The amount and percentage yields of the extracts from Three medicinal plants**:** *A. indica, C. javanicum* and *B. retusa*

Crude extract of three medicinal plants	weight (g) and % yield
1. *A. indica* aerial parts 1.00 kg in various solvents :	
Hexane extract	9.50 (0.95)
Chloroform extract	10.20 (1.02)
Methanol extract	22.40 (2.24)
2. *C. javanicum* leaves 286.9 g in various solvents :	
Hexane extract	1.12 (0.39)
Chloroform extract	2.92 (1.02)
Methanol extract	13.55 (4.72)
C. javanicum stems 561.4 g in various solvents :	
Hexane extract	0.84 (0.15)
Chloroform extract	1.40 (0.25)
Methanol extract	5.16 (0.92)
C. javanicum fruits 202.2 g in various solvents :	
Hexane extract	0.38 (0.19)
Chloroform extract	2.61 (1.29)
Methanol extract	5.07 (2.51)

Table 4.1 (continued)

Crude extract of three medicinal plants	weight (g) and % yield
3. *B. retusa* leaves 616.7 g in various solvents :	
Hexane extract	13.51 (2.19)
Chloroform extract	4.81 (0.78)
Methanol extract	21.23 (3.44)
B. retusa stems 507.4 g in various solvents :	
Hexane extract	0.50 (0.09)
Chloroform extract	2.54 (0.50)
Methanol extract	17.24 (3.40)
B. retusa fruits 24.3 g in various solvents :	
Hexane extract	0.09 (0.39)
Chloroform extract	0.40 (1.68)
Methanol extract	0.81 (3.36)

The methanol extracts from all medicinal plants gave the highest percentage yields. Most of the chloroform extracts gave moderate percentage yields, whereas the hexane extracts gave low yields, except *B. retusa* leaves extracts.

4.1.2 Percentage yields of essential oils

The fresh aerial parts of *A. indica* (1 kg) and fresh leaves and fruits of *C. javanicum* (1 kg for leaves and 390.1 g for fruits) and *B. retusa* (1 kg for leaves and 1 kg for fruits) were cut into small pieces, then the chopped leaves of each plant were subjected to hydrodistillation. The essential oil of each medicinal plant was obtained and the percentage yield was calculated. Results are shown in Table 4.2.

Table 4.2 The percentage yields of the essential oils from three medicinal plants**:** *A. indica, C. javanicum* and *B. retusa*

The essential oil from medicinal plant	weight (g) and %yield
A. indica aerial parts	0.028 (0.0028)
C. javanicum leaves	0.030 (0.0030)
C. javanicum fruits	0.011 (0.0028)
B. retusa leaves	0.013 (0.0013)
B. retusa fruits	0.026 (0.0026)

4.2 Determination of antioxidant activities of the essential oils and medicinal palnt extracts

4.2.1 ABTS method

A series of standard solutions containing: 0.5-2.5 mg mL^{-1} of Trolox was prepared. The absorbance of each solution was measured at 734 nm. The % inhibition of each concentration was calculated (Table 4.3). The % inhibitions were plotted against standard trolox concentrations. Linear calibration curve was obtained over the concentration range of 0.5-2.5 mg mL^{-1} with the R^2 of 0.9953 (Figure 4.1). This calibration curve was used for the ABTS screening method.

Table 4.3 Percentage inhibition of Trolox concentration serie for ABTS screening method

Concentation of Trolox (mg/mL)	% inhibition
0.50	13.84
1.00	37.79
1.50	59.53
2.00	79.84
2.50	96.17

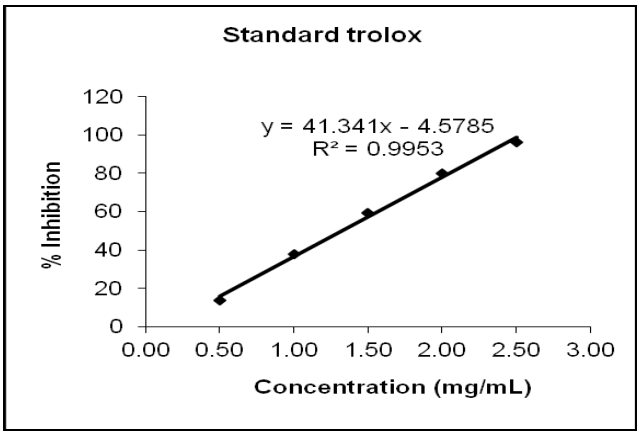

Figure 4.1 Concentration-response curve for the absorbance at 734 nm for $ABTS^{\bullet+}$ as a function of concentration of standard Trolox

The antioxidant activities of the essential oils and the crude extracts of *A. indica*, *C. javanicum* and *B. retusa* in various solvents: hexane, chloroform and methanol were determined by using ABTS free radical-scavenging method. The absorbance of each sample was measured at 734 nm and the percentage inhibition was calculated by reference to the calibration curve of standard Trolox (Figure 4.1). Results are presented in Table 4.4 and Table 4.5.

Table 4.4 Antioxidant activities of the essential oils by ABTS assay

Essential oils	**IC_{50} (mg mL^{-1})**
1. *A. indica* aerial parts	8.18±0.0050
2. *C. javanicum* leaves	12.96±0.0066
C. javanicum fruits	0.71±0.0006
3. *B. retusa* leaves	1.12±0.0010
B. retusa fruits	1.79±0.0005
4. Reference standard: Trolox	1.32±0.0010

All of the essential oils possessed antioxidant activity. The essential oil of *C. javanicum* fruits exhibited the highest antioxidant activity followed by *B. retusa* leaves, *B. retusa* fruits, *A. indica* aerial parts and *C. javanicum* leaves respectively.

Table 4.5 Antioxidant activities of medicinal plant extracts by ABTS assay

Medicinal plant extracts	**IC_{50} (mg mL^{-1})**
1. *A. indica* aerial parts in various solvents :	
-Hexane extract	6.13±0.010
-Chloroform extract	6.31±0.020
-Methanol extract	6.37±0.020
2. *C. javanicum* leaves in various solvents :	
-Hexane extract	8.53±0.009
-Chloroform extract	3.72±0.009
-Methanol extract	0.67±0.007
C. javanicum stems in various solvents :	
-Hexane extract	32.55±0.005
-Chloroform extract	19.47±0.001
-Methanol extract	1.41±0.001
C. javanicum fruits in various solvents :	
-Hexane extract	43.46±0.005

Table 4.5 (continued)

Medicinal plant extracts	**IC_{50} (mg mL^{-1})**
C. javanicum fruits in various solvents :	
-Chloroform extract	11.56±0.001
-Methanol extract	5.58±0.009
3. *B. retusa* leaves in various solvents :	
-Hexane extract	16.62±0.0010
-Chloroform extract	9.85±0.0030
-Methanol extract	1.22±0.0030
B. retusa stems in various solvents :	
-Hexane extract	37.76±0.0110
-Chloroform extract	30.49±0.0210
-Methanol extract	0.56±0.0050
B. retusa fruits in various solvents :	
-Hexane extract	58.41±0.0350
-Chloroform extract	32.55±0.0050
-Methanol extract	0.29±0.0050
4. Reference standard: Trolox	1.32±0.0050

The methanol extracts from all medicinal plants gave the highest antioxidant activity. The results indicated that the methanol extract of *B. retusa* fruits exhibited the highest antioxidant activity (IC_{50} of 0.29 mg mL^{-1}), followed by the methanol extract of *B. retusa* stems (IC_{50} of 0.56 mg mL^{-1}), the methanol extract of *C. javanicum* leaves (IC_{50} of 0.67 mg mL^{-1}), the methanol extract of *B. retusa* leaves (IC_{50} of 1.22 mg mL^{-1}), the methanol extract of *C. javanicum* stems (IC_{50} of 1.41 mg mL^{-1}), the chloroform extract of *C. javanicum* leaves (IC_{50} of 3.72 mg mL^{-1}), the methanol extract of *C. javanicum* fruits (IC_{50} of 5.58 mg mL^{-1}) and the hexane extract of *A. indica* aerial parts (IC_{50} of 6.13 mg mL^{-1}), respectively. Most of the chloroform extracts gave moderate antioxidant activity, whereas the hexane extracts gave low antioxidant activity, except *A. indica* aerial parts extracts.

4.2.2 DPPH method

A series of standard solutions containing: 0.02-0.12 mg mL^{-1} of Trolox, were prepared. The absorbance of each solution was measured at 540 nm and the % inhibition of each concentration was calculated. Results are presented in Table 4.6.

The percentage inhibitions were plotted against the standard Trolox concentrations as illustrated in Figure 4.2. This calibration curve was used for DPPH screening method.

Table 4.6 The percentage inhibition of each concentration of the Trolox standard solution

Concentation of Trolox (mg/mL)	% inhibition
0.02	14.62
0.04	25.98
0.06	36.07
0.08	50.34
0.10	61.01
0.12	73.66

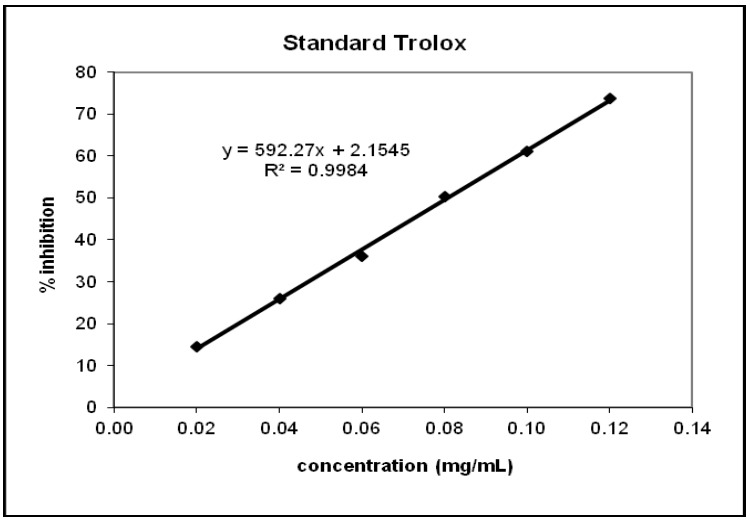

Figure 4.2 Calibration curve for the absorbance at 540 nm of DPPH method as a function of the concentration of Trolox standard solution

The antioxidant activities of the essential oils and medicinal plant extracts were also evaluated by the DPPH method. The absorbance of each sample was measured at 540 nm and the percentage inhibition was calculated by reference to the calibration curve of standard Trolox (Figure 4.2). The results are summarized in Table 4.7 and Table 4.8.

Table 4.7 Antioxidant activities of the essential oils by DPPH assay

Essential oils	IC_{50} (mg mL^{-1})
1. *A. indica* aerial parts	67.62±0.0010
2. *C. javanicum* leaves	27.05±0.0015
C. javanicum fruits	Inactive
3. *B. retusa* leaves	Inactive
B. retusa fruits	Inactive
4. Reference standard: Trolox	0.08±0.0010

The results showed the essential oil of *C. javanicum* leaves exhibited the highest antioxidant activity (IC_{50} of 27.05 mg mL^{-1}), followed by *A. indica* aerial parts (IC_{50} of 67.62 mg mL^{-1}), respectively.

Table 4.8 Antioxidant activities of medicinal plant extracts by DPPH assay

Medicinal plant extracts	IC_{50} (mg mL^{-1})
1. *A. indica* aerial parts in various solvents :	
-Hexane extract	6.19±0.010
-Chloroform extract	5.70±0.050
-Methanol extract	7.79±0.020
2. *C. javanicum* leaves in various solvents :	
-Hexane extract	2.72±0.015
-Chloroform extract	1.26±0.001
-Methanol extract	0.21±0.011
C. javanicum stems in various solvents :	
-Hexane extract	22.13±0.009
-Chloroform extract	11.40±0.011
-Methanol extract	0.76±0.011
C. javanicum fruits in various solvents :	
-Hexane extract	33.33±0.005
-Chloroform extract	22.45±0.001
-Methanol extract	3.56±0.005
3. *B. retusa* leaves in various solvents :	
-Hexane extract	5.54±0.015
-Chloroform extract	6.92±0.013
-Methanol extract	0.52±0.031
B. retusa stems in various solvents :	
-Hexane extract	29.88±0.011
-Chloroform extract	10.46±0.009
-Methanol extract	0.12±0.003

Table 4.8 (continued)

Medicinal plant extracts	IC_{50} (mg mL^{-1})
3. *B. retusa* fruits in various solvents :	
-Hexane extract	36.23±0.009
-Chloroform extract	29.98±0.011
-Methanol extract	0.17±0.005
4. Reference standard: Trolox	0.08±0.001

Table 4.7 indicated that the DPPH method is not suitable for the determination of antioxidant capacities of the essential oils. All the medicinal plant extracts exhibited antioxidant activities (Table 4.8). The methanol extracts from all medicinal plants (except *A. indica* aerial parts) gave the highest antioxidant activity. The methanol extract of *B. retusa* stems exhibited the highest antioxidant activity (IC_{50} of 0.12 mg mL^{-1}), followed by the methanol extract of *B. retusa* fruits (IC_{50} of 0.17 mg mL^{-1}), the methanol extract of *C. javanicum* leaves (IC_{50} of 0.21 mg mL^{-1}), the methanol extract of *B. retusa* leaves (IC_{50} of 0.52 mg mL^{-1}), the methanol extract of *C. javanicum* stems (IC_{50} of 0.76 mg mL^{-1}), the chloroform extract of *C. javanicum* leaves (IC_{50} of 1.26 mg mL^{-1}), the hexane extract of *C. javanicum* leaves (IC_{50} of 2.72 mg mL^{-1}), the methanol extract of *C. javanicum* fruits (IC_{50} of 3.56 mg mL^{-1}), the hexane extract of *B. retusa* leaves (IC_{50} of 5.54 mg mL^{-1}), the chloroform extract of *A. indica* aerial parts (IC_{50} of 5.70 mg mL^{-1}), the hexane extract of *A. indica* aerial parts (IC_{50} of 6.19 mg mL^{-1}), the chloroform extract of *B. retusa* leaves (IC_{50} of 6.92 mg mL^{-1}) and the methanol extract of *A. indica* aerial parts (IC_{50} of 7.79 mg mL^{-1}), respectively. Most of the chloroform extracts gave moderate antioxidant activity, whereas the hexane extracts gave low antioxidant activity, except *A. indica* aerial part extracts and *B. retusa* leave extracts.

4.2.3 Total phenolic contents

The total phenolic contents of crude extracts of *A. indica*, *C. javanicum* and *B. retusa* in various solvents: hexane, chloroform and methanol were investigated. The absorbance of each sample was measured at 750 nm. Total phenolic contents of the test samples were expressed as % gallic acid (w/w) of dry plant material by comparison with the gallic acid standard curve (Figure 4.3). All samples were analyzed in three replications. Results are presented in Table 4.9.

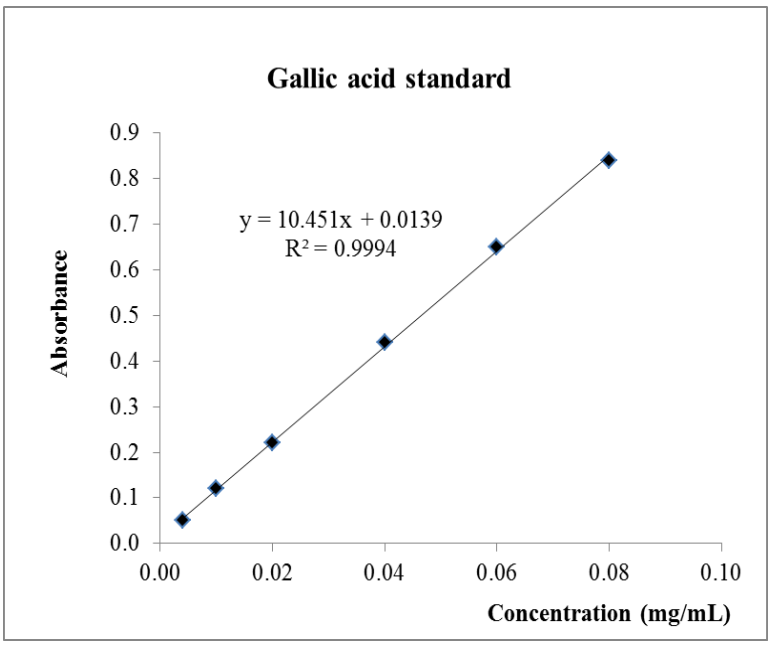

Figure 4.3 Calibration curve of gallic acid

Table 4.9 Total phenol contents of medicinal plant extracts

Medicinal plant extracts	Total phenolic content[a]
1. *A. indica* aerial parts in various solvents :	
-Hexane extract	0.36±0.0010
-Chloroform extract	1.34±0.0010
-Methanol extract	1.74±0.0020
2. *C. javanicum* leaves in various solvents :	
-Hexane extract	0.22±0.0010
-Chloroform extract	1.08±0.0010
-Methanol extract	3.00±0.0020
C. javanicum stems in various solvents :	
-Hexane extract	0.38±0.0001
-Chloroform extract	1.20±0.0005
-Methanol extract	3.32±0.0010
C. javanicum fruits in various solvents :	
-Hexane extract	0.68±0.0005
-Chloroform extract	1.00±0.0010
-Methanol extract	1.46±0.0012
3. *B. retusa* leaves in various solvents :	
-Hexane extract	0.26±0.0010
-Chloroform extract	0.54±0.0010
-Methanol extract	0.44±0.0020

Table 4.9 (continued)

Medicinal plant extracts	Total phenolic content[a]
3. *B. retusa* stems in various solvents :	
-Hexane extract	0.26±0.0005
-Chloroform extract	0.94±0.0020
-Methanol extract	0.54±0.0005
B. retusa fruits in various solvents :	
-Hexane extract	0.20±0.0001
-Chloroform extract	0.48±0.0005
-Methanol extract	1.40±0.0015

All results are the mean of triplicate measurements ± standard deviation (SD).
[a]: Expressed as % gallic acid (w/w) of dry plant material.

The methanol extracts from all medicinal plants (except *B. retusa* stems and leaves) gave the highest total phenolic content of the extracts. The results showed the methanol extract of *C. javanicum* stems exhibited the highest total phenol contents of the extracts (3.32 % gallic acid (w/w) of extract), followed by the methanol extract of *C. javanicum* leaves (3.00 % gallic acid (w/w) of extract), the methanol extract of *A. indica* aerial parts (1.74 % gallic acid (w/w), the methanol extract of *C. javanicum* fruits (1.46 % gallic acid (w/w) and the methanol extract of *B. retusa* fruits (1.40 % gallic acid (w/w), respectively. Most of the chloroform extracts gave moderate total phenol contents of the extracts, whereas the hexane extracts gave low total phenol contents of the extracts, except *B. retusa* stems and leaves.

4.2.4 Total flavonoid contents

The total flavonoid contents of crude extracts of *A. indica*, *C. javanicum* and *B. retusa* in various solvents: hexane, chloroform and methanol were investigated. The absorbance of each sample was measured spectrophotometrically at 430 nm. Linear calibration curve of standard quercetin was constructed. Total flavonoid contents of the test samples were expressed as % quercetin (w/w) of dry plant material by comparison with the quercetin standard curve (Figure 4.4). All samples were analyzed in three replications. Results are presented in Table 4.10.

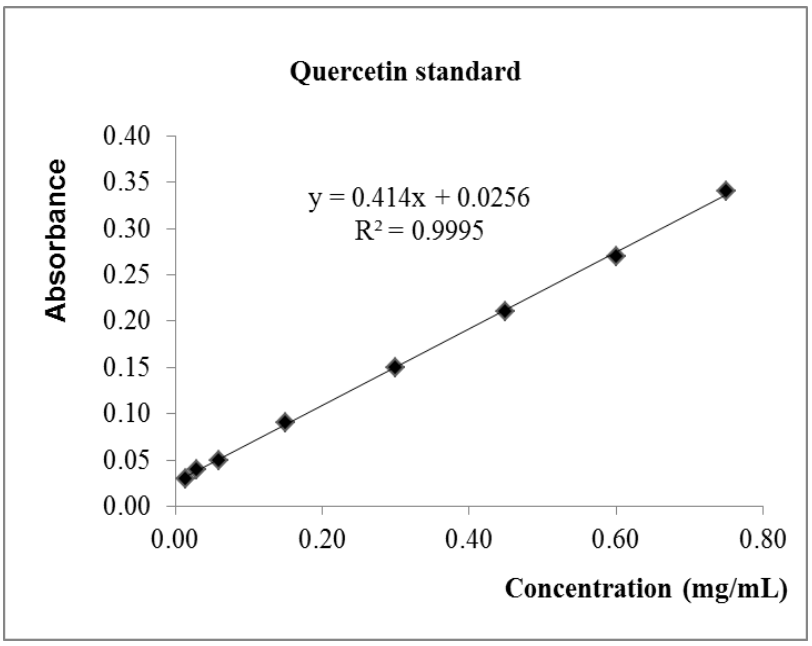

Figure 4.4 Calibration curve of quercetin.

Table 4.10 Total flavonoid contents of medicinal plant extracts

Extracts	Total flavonoid content[a]
1. *A. indica* aerial parts in various solvents :	
-Hexane extract	19.06±0.0010
-Chloroform extract	22.44±0.0005
-Methanol extract	11.80±0.0010
2. *C. javanicum* leaves in various solvents :	
-Hexane extract	16.64±0.0010
-Chloroform extract	20.98±0.0010
-Methanol extract	18.08±0.0020
C. javanicum stems in various solvents :	
-Hexane extract	16.64±0.0005
-Chloroform extract	12.78±0.0020
-Methanol extract	14.22±0.0020
C. javanicum fruits in various solvents :	
-Hexane extract	12.78±0.0010
-Chloroform extract	8.42±0.0010
-Methanol extract	13.26±0.0020
3. *B. retusa* leaves in various solvents :	
-Hexane extract	14.70±0.0010
-Chloroform extract	15.18±0.0005
-Methanol extract	19.06±0.0012

Table 4.10 (continued)

Extracts	Total flavonoid content[a]
3. *B. retusa* stems in various solvents :	
-Hexane extract	12.78±0.0005
-Chloroform extract	12.30±0.0023
-Methanol extract	7.94±0.0012
B. retusa fruits in various solvents :	
-Hexane extract	11.32±0.0020
-Chloroform extract	14.70±0.0010
-Methanol extract	14.70±0.0010

All results are the mean of triplicate measurements ± standard deviation (SD).
[a]: Expressed as % quercetin (w/w) of dry plant material.

The results revealed that the chloroform aerial parts extract of *A. indica* gave the highest flavonoid content, whereas the hexane extract of *A. indica* showed higher flavonoid content than that of the methanol extract. For *C. javanicum*, the chloroform leaf extract, the hexane stem extract and the methanol fruit extract showed higher flavonoid contents than those of the other solvent extracts. For *B. retusa*, the methanol leaf extract, the hexane stem extract and the methanol and chloroform fruit extract gave the highest flavonoid contents.

4.3 Determination of biological activities

4.3.1 Antimicrobial activity

4.3.1.1 Antibacterial activity

The antibacterial activities of *A. indica*, *C. javanicum* and *B. retusa* extracts were determined using agar well diffusion method. The antibacterial studies were carried out *in vitro* against Gram-positive and Gram-negative organisms. The crude extract exhibited antibacterial activity against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, using gentamicin as reference standard. Results are presented in Table 4.11. It is indicated that the hexane, chloroform and methanol extracts of *A. indica* Linn. aerial part showed antibacterial activities against *S. aureus* and *P. aeruginosa*, but did not inhibit *E. coli*. Only methanolic extract of *C. javanicum* leaves exhibited antibacterial activities against *S. aureus*, *E. coli* and *P. aeruginosa* respectively, but the methanolic extract of *C. javanicum* stems

possessed antibacterial activity against *S. aureus.* The chloroform extract of *C. javanicum* fruits exhibited antibacterial activity against *P. aeruginosa*. The methanolic extract of *C. javanicum* fruits showed antibacterial activity against *S. aureus* and *E. coli,* but the hexane extract of *C. javanicum* fruits did not inhibit *S. aureus, E. coli* and *P. aeruginosa*. The chloroform extract of *B. retusa* (L.) A. Juss. fruits showed inhibition zones against *S. aureus, E. coli* and *P. aeruginosa*, but the methanolic extract showed antibacterial activities against *S. aureus* and *P. aeruginosa*. The hexane extracts of *B. retusa* leaves, stems and fruits showed no inhibition zone against *S. aureus, E. coli* and *P. aeruginosa*. The chloroform extracts of *B. retusa* leaves and stems showed no inhibition zone against *S. aureus, E. coli* and *P. aeruginosa*. The methanolic extracts of *B. retusa* leaves exhibited antibacterial activity against *S. aureus* and *P. aeruginosa*. The methanolic extracts of *B. retusa* stems exhibited antibacterial activity against *S. aureus*.

4.3.1.2 Antifungal activity

The antifungal activities of *A. indica*, *C. javanicum* and *B. retusa* extracts were also evaluated by using agar well diffusion method. The inhibitory zones were measured. The crude extract exhibited antifungal activity against *Aspergillus flavus, Candida albican* and *Trichophyton mentagrophyte,* using ketoconazole as reference standard. Results are presented in Table 4.12. It is indicated that the hexane, chloroform and methanol extracts of *A. indica* Linn. aerial parts showed antifungal activity against *T. mentagrophyte*, but the chloroform and methanol extracts showed inhibition zones against *A. flavus* and *C. albican* respectively. The methanolic extracts of *C. javanicum* stems, *B. retusa* leaves and stems exhibited antifungal activity against *T. mentagrophyte*, but all of the extracts of *C. javanicum* leaves showed no inhibition zone against all fungi tested. The hexane and chloroform extracts of *C. javanicum* stems, *B. retusa* leaves and stems showed no inhibition zone against all fungi tested. The hexane, chloroform and methanol extracts of *B. retusa* fruits showed antifungal activity against *C. albican*, but all extracts did not inhibit *A. flavus* and *T. mentagrophyte*. The most active antimicrobial plant was *A. indica*.

Thus the antimicrobial activity of various medicinal plant extracts in family Euphorbiaceae, offers an option to the pharmaceutical industry of new natural medicine sources with activity against these bacterial and fungal strains that represent an important public health problem.

4.3.2 Minimum Inhibition Concentration (MIC)

The minimum inhibition concentrations (MIC's) was determined using inhibitory concentrations in disc diffusion method. It is done by carrying out the diffusion test with discs of different concentration of the plant extracts similar to the concentration used in the sensitivity tests against the bacteria strains mention earlier. The lowest concentration that inhibit the growth of bacteria was noted and considered as the MIC value for each of the bacteria strain. The MIC values of the selected active essential oils were investigated. The most active essential oil was *C. javanicum* fruits which showed antibacterial activity against all of three bacterials: *S. aureus*, *E. coli* and *P. aeruginosa* with the MIC value of 10 mg mL^{-1}. The essential oil of *A. indica* aerial parts exhibited antibacterial activity against *S. aureus*, *E. coli* and *P. aeruginosa* with the MIC values of 10, 20 and 20 mg mL^{-1}, respectively. The essential oil of *B. retusa* leaves exhibited antibacterial activity against *S. aureus*, *E. coli* and *P. aeruginosa*, with the MIC values of 20, 20 and 10 mg mL^{-1} respectively. The essential oil of *B. retusa* fruits showed antibacterial activity against *S. aureus*, *E. coli* and *P. aeruginosa*, with the MIC values of 10, 10 and 50 mg mL^{-1} respectively. Results are shown in Table 4.13.

4.4 Determination of cytotoxic activity

The cytotoxic activity of the selected active extracts and the selected active essential oils of *A. indica*, *C. javanicum* and *B. retusa* were performed using the resazurin microplate assay (REMA). The essential oil of *A. indica* aerial parts showed anticancer activity against all of three cancer cell lines: KB-Oral cavity cancer, MCF7-Breast cancer and NCI-H187-Small cell lung cancer with the IC_{50} values of 24.23, 33.64 and 49.39 µg mL^{-1}, respectively. The essential oil of *C. javanicum* leaves also showed anticancer activity against all of three cancer cell lines: KB-Oral cavity cancer, MCF7-

Breast cancer and NCI-H187-Small cell lung cancer with the IC_{50} values of 47.16, 40.23 and 49.95 µg mL^{-1}, respectively. Only the methanol leaf extract of *B. retusa* exhibited anticancer activity against all of three cancer cells: KB-Oral cavity cancer, MCF7-Breast cancer and NCI-H187-Small cell lung cancer with the IC_{50} values of 21.24, 17.81 and 24.24 µg mL^{-1}, respectively. The methanol aerial part extract of *A. indica* showed anticancer activity against only NCI-H187-Small cell lung cancer with an IC_{50} values of 25 µg mL^{-1}. The hexane stem extract of *C. javanicum* showed anticancer activity against two cancer cell lines: KB-Oral cavity cancer, and NCI-H187-Small cell lung cancer with the IC_{50} values of 49.53 µg mL^{-1} and 47.72 µg mL^{-1}, respectively. The methanol fruit extract of *B. retusa* also exhibited anticancer activity against two cancer cell lines: KB-Oral cavity cancer, and NCI-H187-Small cell lung cancer with the IC_{50} values of 32.46 µg mL^{-1} and 8.21 µg mL^{-1}, respectively. The hexane fruit extract of *C. javanicum* showed anticancer activity against only NCI-H187-Small cell lung cancer with an IC_{50} of 29.11 µg mL^{-1}. Ellipticine and doxorubicine were used as reference standards. The results are shown in Table 4.14.

The cytotoxicity of the selected active extracts and the selected active essential oils of *A. indica*, *C. javanicum* and *B. retusa* were performed using Green Fluorescent Protein (GFP) detection methodology. All selected extracts and selected essential oils of *A. indica*, *C. javanicum* and *B. retusa* were non-cytotoxic against primate cell line (Vero cell). Ellipticine was used as reference standards. Results are shown in Table 4.14.

Table 4.11 Antibacterial activities of medicinal plant extracts

Medicinal plant extracts	Conc. (mg mL^{-1})	Diameter of inhibition zone (mm)[a]		
		S. aureus	*E. coli*	*P.aeroginosa*
1. *A. indica* aerial parts in various solvents :	50			
-Hexane extract		10	-	19
-Chloroform extract		11	-	17
-Methanol extract		10	-	17
2. *C. javanicum* leaves in various solvents :	50			
-Hexane extract		-	-	-
-Chloroform extract		-	-	-
-Methanol extract		25	15	14
C. javanicum stems in various solvents :				
-Hexane extract		-	-	-
-Chloroform extract		-	-	-
-Methanol extract		18	-	-
C. javanicum fruits in various solvents :				
-Hexane extract		-	-	-
-Chloroform extract		-	-	11
-Methanol extract		20	23	-
3. *B. retusa* leaves in various solvents :	50			
-Hexane extract		-	-	-
-Chloroform extract		-	-	-
-Methanol extract		10	-	14
B. retusa stems in various solvents :				
-Hexane extract		-	-	-
-Chloroform extract		-	-	-
-Methanol extract		15	-	-
B. retusa fruits in various solvents :				
-Hexane extract		-	-	-
-Chloroform extract		10	12	12
-Methanol extract		17	-	14
4. Gentamicin	75 µg mL^{-1}	35	27	27

[a] : Inhibition zones are the mean cork borer (9 mm) diameter

(-) : No inhibition zone

Table 4.12 Antifungal activities of medicinal plant extracts

Medicinal plant extracts	Conc. (mg mL^{-1})	Diameter of inhibition zone (mm)[a]		
		A. flavas	*C. albican*	*T. mentagophyte*
1. *A. indica* aerial parts in various solvents :	50			
-Hexane extract		-	-	15
-Chloroform extract		11	-	14
-Methanol extract		-	13	15
2. *C. javanicum* leaves in various solvents :	50			
-Hexane extract		-	-	-
-Chloroform extract		-	-	-
-Methanol extract		-	-	-
C. javanicum stems in various solvents :				
-Hexane extract		-	-	-
-Chloroform extract		-	-	-
-Methanol extract		-	-	11
C. javanicum fruits in various solvents :				
-Hexane extract		-	-	20
-Chloroform extract		-	11	17
-Methanol extract		-	-	27
3. *B. retusa* leaves in various solvents :	50			
-Hexane extract		-	-	-
-Chloroform extract		-	-	-
-Methanol extract		-	-	10
B. retusa stems in various solvents :				
-Hexane extract		-	-	-
-Chloroform extract		-	-	-
-Methanol extract		-	-	18
B. retusa fruits in various solvents :				
-Hexane extract		-	17	-
-Chloroform extract		-	21	-
-Methanol extract		-	14	-
4. Ketoconazole	250 µg mL^{-1}	25	37	16

[a] : Inhibition zones are the mean cork borer (9 mm) diameter

(-) : No inhibition zone

Table 4.13 MIC values of the essential oils of three medicinal plants.

Concentration of essential oils (mg mL^{-1})	Diameter of inhibition zone (mm)[a]		
	S.aureus	*E. coli*	*P.aeroginosa*
1. *A. indica* aerial parts			
50	8	7	7
40	7	7	7
30	7	7	7
20	7	7	7
10	7	-	-
MIC (mg mL^{-1})	10	20	20
2. *C. javanicum* fruits			
50	8	7	7
40	7	7	7
30	7	7	7
20	7	7	7
10	7	7	7
MIC (mg mL^{-1})	10	10	10
3. *B. retusa* leaves			
50	8	8	8
40	8	8	8
30	8	7	7
20	7	7	7
10	-	-	7
MIC (mg mL^{-1})	20	20	10
B. retusa fruits			
50	8	8	7
40	7	8	-
30	7	8	-
20	7	8	-
10	7	7	-
MIC (mg mL^{-1})	10	10	50

[a] : Inhibition zones are the mean diameter of disc 6 mm

(-): Represent that negative test

Table 4.14 Cytotoxic activities of medicinal plant extracts and essential oils

Test samples	KB-Oral cavity cancer		MCF7-Breast cancer		NCI-H187-Small cell lung cancer		Cytotoxicity activity (Vero cells)
	Activity	IC_{50} ($\mu g\ mL^{-1}$)	Activity	IC_{50} ($\mu g\ mL^{-1}$)	Activity	IC_{50} ($\mu g\ mL^{-1}$)	
1. *A. indica* aerial parts in various solvents :							
-Methanol extract	Inactive	-	Inactive	-	Active	25.00	non-cytotoxic
-Essential oils	Active	24.23	Active	33.64	Active	49.39	non-cytotoxic
2. *C. javanicum* leaves in various solvents :							
-Chloroform extract	Inactive	-	Inactive	-	Inactive	-	non-cytotoxic
-Methanol extract	Inactive	-	Inactive	-	Inactive	-	non-cytotoxic
-Essential oils	Active	47.16	Active	40.23	Active	49.95	non-cytotoxic
C. javanicum stems in various solvents :							
-Hexane extract	Active	49.53	Inactive	-	Active	47.72	non-cytotoxic
-Methanol extract	Inactive	-	Inactive	-	Inactive	-	non-cytotoxic
C. javanicum fruits in various solvents :							
-Hexane extract	Inactive	-	Inactive	-	Active	29.11	non-cytotoxic
-Chloroform extract	Inactive	-	Inactive	-	Inactive	-	non-cytotoxic
-Methanol extract	Inactive	-	Inactive	-	Inactive	-	non-cytotoxic
-Essential oils	Inactive	-	Inactive	-	Inactive	-	non-cytotoxic
3. *B. retusa* leaves in various solvents :							
-Methanol extract	Active	21.24	Active	17.81	Active	24.24	non-cytotoxic
-Essential oils	Inactive	-	Inactive	-	Inactive	-	non-cytotoxic
B. retusa stems in various solvents :							
-Methanol extract	Inactive	-	Inactive	-	Inactive	-	non-cytotoxic
B. retusa fruits in various solvents :							
-Methanol extract	Active	32.46	Inactive	-	Active	8.21	non-cytotoxic
-Essential oils	Inactive	-	Inactive	-	Inactive	-	non-cytotoxic

4.5 Analysis of the essential oils

4.5.1 The essential oil of *A. indica* aerial parts

Fresh aerial parts of *A. indica* were homogenized and hydrodistilled for 3 h to yield a pale yellow oil of 0.0028 %. The essential oil was analysed by means of GC and GC-MS. Identification of the oil constituents was performed by comparison of mass spectra with literature data (NIST, NISTREP). Twenty two compounds were identified and are listed in Table 4.15. A typical total ion chromatogram of the essential oil of *A. indica* is presented in Figure 4.5.

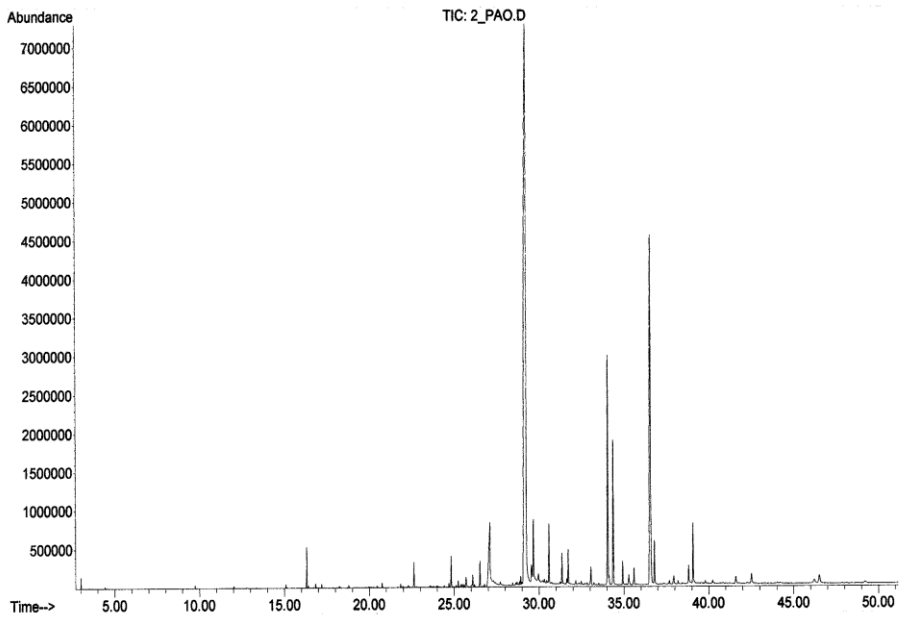

Figure 4.5 Total ion chromatogram of *A. indica* aerial parts essential oil

Table 4.15 Volatile components in aerial parts of *A. indica*

Peak	Compound	RT	RA%	RI_{exp}	RI_{lit}	References
1	Toluene	3.01	0.16	767.49	794	70
2	Linalool	9.75	0.07	1104.48	1097	71
3	Benzeneethanal	17.20	0.08	1435.72	-	-
4	Tridecanal	18.79	0.05	1514.15	1510	71
5	Tetradecanal	20.76	0.11	1615.88	1613	71
6	2-Pentadecanone	24.83	0.69	1844.90	1694	72
7	Cyclotetradecane	25.49	0.07	1884.54	-	-
8	Ethyl linoleate	25.59	0.07	1890.51	-	-
9	Isophytol	26.52	0.58	1948.12	1944	73
10	Benzyl (dideuterated) methyl ether	28.71	0.11	2089.20	-	-
11	Phytol	29.25	47.49	2125.65	1949	73
12	2-Propenoic acid	32.16	0.10	2331.15	-	-
13	3-Eicosene	33.24	0.05	2411.60	1994	73
14	9-Tricosene	34.06	7.05	2475.64	2281	72
15	Pentacosane (CAS)	34.38	3.96	2500.16	2500	71
16	Phthalic acid	34.93	0.57	2544.10	-	-
17	Cyclotetracosane	35.29	0.32	2572.85	-	-
18	Heptacosane	36.80	1.23	2698.25	2700	74
19	Cyclooctacosane	37.68	0.15	2773.96	-	-
20	Nonacosane (CAS)	39.08	1.61	2898.21	2900	74
21	Bis-(octylphenyl)-amine	39.79	0.09	2958.86	-	-
	Total		64.76			

RA: relative area (peak area relative to total peak area).
RI_{exp}: retention indices relative to n-alkanes (C_7-C_{30}) on HP-5MS column.
RI_{lit} : retention indices from literature data.

Twenty two components, represent in 64.76 % of the total essential oil, were identified and are listed in the order of their elution on the HP-5 capillary column used for GC-MS analysis (Table 4.15). The major components of the essential oil were identified as phytol (47.49%), 9-tricosene (7.05%), pentacosane (3.96%), nonacosane (1.61%), and heptacosane (1.23%). The minor components were 2-pentadecanone (0.69%), isophytol (0.58%), phthalic acid (0.57%), cyclotetracosane (0.32%), toluene (0.16%), cyclooctacosane (0.15%), tetradecanal (0.11%), benzyl (dideuterated) methyl ether (0.11%), 2-propenoic acid (0.10%), bis-(octylphenyl)-amine (0.09%), benzeneethanal (0.08%),

linalool (0.07%), cyclotetradecane (0.07%), ethyl linoleate (0.07%), tridecanal (0.05%), and 3-eicosene (0.05%). Benzyl(dideuterated)methyl ether is not a natural component, it may be a contaminant. Phthalic acid is probably contaminant later than an essential oil component. It is a plasticizer contaminant.

Most of the components of the essential oil are lipidic derivatives eg. ethyl linoleolate, 2-propenoic acid which are used as flavour and fragrance agents, essential ingredient in making soaps and shampoos. But phytol and iso-phytol are acyclic terpenoids. Phytol is a diterpene. It can be used as anticancer, antiviral and antipyretic agents (75,76). It is also used as a fixer in perfumery. Iso-phytol is used in manufacturing synthesis of vitamins E and K.

4.5.2 The essential oils of *C. javanicum* leaves and fruits

Fresh leaves and fruits of *C. javanicum* were homogenized and hydrodistilled for 3 h to yield a pale yellow oil of 0.0030 % in leaves and a pale geen oil of 0.0028% in fruits. The essential oil was analysed by means of GC and GC-MS. Identification of the oil constituents was performed by comparison of mass spectra with literature data (NIST, NISTREP) that yielded < 90% matches were identified as unknowns or with those in the literature. Ten compounds (leaves) and nine compounds (fruits) were identified. The results are shown in Table 4.16 (leaves) and Table 4.17 (fruits), respectively. Typical total ion chromatograms of the essential oils of *C. javanicum* are presented in Figure 4.6 and Figure 4.7.

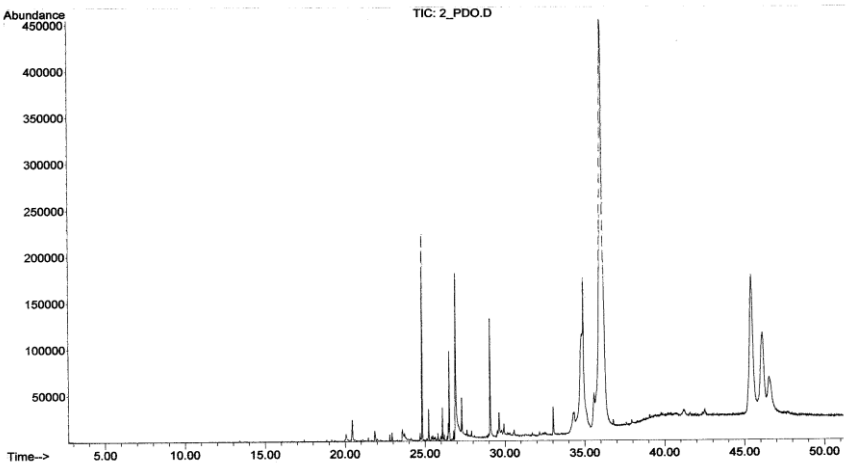

Figure 4.6 Total ion chromatogram of *C. javanicum* leaves essential oil

Table 4.16 Chemical composition of the leaves essential oil of *C. javanicum*

Peak	Compound	RT	RA%	RI_{exp}	RI_{lit}	References
1	Tetradecanoic acid	25.2	0.56	1867.16	1758.2	77
2	Pentadecanone	26.6	1.00	1953.19	1845.0	78
3	Isophytol	28.4	4.80	2068.85	1944.0	79
4	Hexadecanoic acid	28.9	26.77	2101.64	1972.0	80
5	Methyl linoleate	30.7	0.57	2225.82	2092.0	81
6	Trans-phytol	31.0	24.64	2247.23	2111.0	79
7	Ethyl linoleolate	31.6	32.12	2290.01	-	-
8	Neophytadiene	32.5	0.28	2356.51	2114.0	82
9	Dodecanoic acid	34.2	0.45	2486.23	2489.0	83
10	Hentriacontane	48.0	1.51	-	3100.0	84
	Total		92.60			

RA: relative area (peak area relative to total peak area).
RI_{exp}: retention indices relative to n-alkanes (C_7-C_{30}) on HP-5MS column.
RI_{lit} : retention indices from literature data.

Ten components, represent in 92.60 % of the total essential oil, were identified and are listed in the order of their elution on the HP-5 capillary column used for GC-MS analysis (Table 4.16). The major components of the essential oil were identified as ethyl linoleolate (32.12%), hexadecanoic acid (26.77%), trans-phytol (24.64%) and iso-phytol (4.80%). The minor components were hentriacontane (1.51%), pentadecanone (1.00%), methyl linoleate (0.57%), tetradecanoic acid (0.56%), dodecanoic acid (0.45%) and neophytadiene (0.28%).

Most of the components of the essential oil are lipidic derivatives eg. ethyl linoleolate, hexadecanoic acid, methyl linoleate and dodecanoic acid which are used as flavour and fragrance agents, essential ingredient in making soaps and shampoos. But trans-phytol and iso-phytol are acyclic terpenoids. Trans-phytol is a diterpene. It can be used as anticancer, antiviral and antipyretic agents (75,76). It is also used as a fixer in perfumery. Iso-phytol is used in manufacturing synthesis of vitamins E (85) and K (86). Vitamin E is the collective name for a group of fat-soluble compounds with distinctive antioxidant activities (87). Vitamin K cycle could act as a potent antioxidant activity (88).

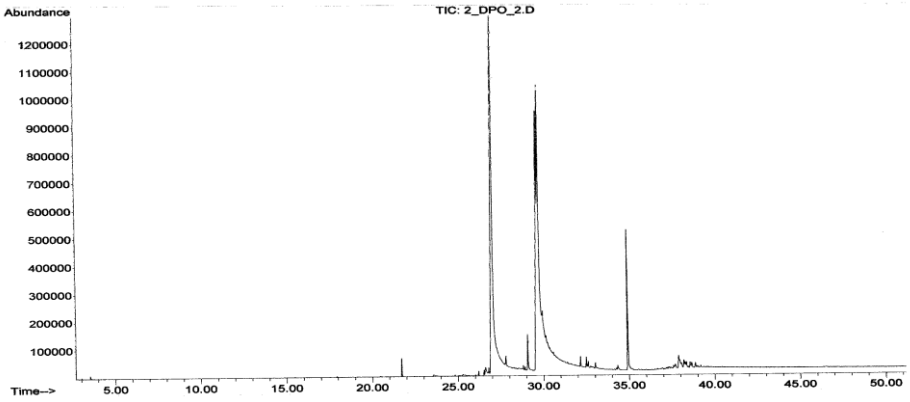

Figure 4.7 Total ion chromatogram of *C. javanicum* fruits essential oil

Table 4.17 Chemical composition of the fruits essential oil of *C. javanicum*

Peak	Compound	RT	RA%	RI_{exp}	RI_{lit}	References
1	Isophytol	26.52	0.14	1948.06	1944	79
2	Hexadecanoic acid (CAS)	27.08	34.42	1983.35	1972	80
3	9, 15-Octadecadienoic acid	28.82	0.12	2096.07	-	-
4	Phytol	29.08	1.19	2113.95	1974	89
5	Linoleic acid	29.69	21.68	2155.74	-	-
6	9, 12, 15-Octadecatrien-1-ol	29.78	21.00	2162.17	-	-
7	2-Propenoic acid	32.15	0.31	2330.71	-	-
8	1-Eicosene	34.33	0.21	2496.06	-	-
9	Phthalic acid	34.93	4.64	2544.02	-	-
	Total		83.71			

RA: relative area (peak area relative to total peak area).
RI_{exp}: retention indices relative to n-alkanes (C_7-C_{30}) on HP-5MS column.
RI_{lit} : retention indices from literature data.

Nine components, represent in 83.71 % of the total essential oil, were identified and are listed in the order of their elution on the HP-5 capillary column used for GC-MS analysis (Table 4.17). The major components of the essential oil were identified as hexadecanoic acid (34.42%), linoleic acid (21.68%), 9, 12, 15-octadecatrien-1-ol (21.00%), phthalic acid (4.64%) and phytol (1.19%). The minor components were 2-propenoic acid (0.31%), 1-eicosene (0.21%), isophytol (0.14%) and 9, 15-octadecadienoic acid (0.12%).

4.5.3 The essential oil of *B. retusa* leaves

Fresh leaves and fruits of *B. retusa* were homogenized and hydrodistilled for 3 h to yield a pale yellow oil of 0.0013 % in leaves and violet-light brown oil of 0.0026% in fruits. The essential oil was analysed by means of GC and GC-MS. Identification of the oil constituents was performed by comparison of mass spectra with literature data (NIST, wiley7n.l) that yielded < 90% matches were identified as unknowns or with those in the literature. Eleven compounds (leaves) and sixteen (fruits) were identified. The results are shown in Table 4.18 (leaves) and Table 4.19 (fryits). Typical total ion chromatograms of the essential oils of *B. retusa* are presented in Figure 4.8 (leaves) and Figure 4.9 (fruits).

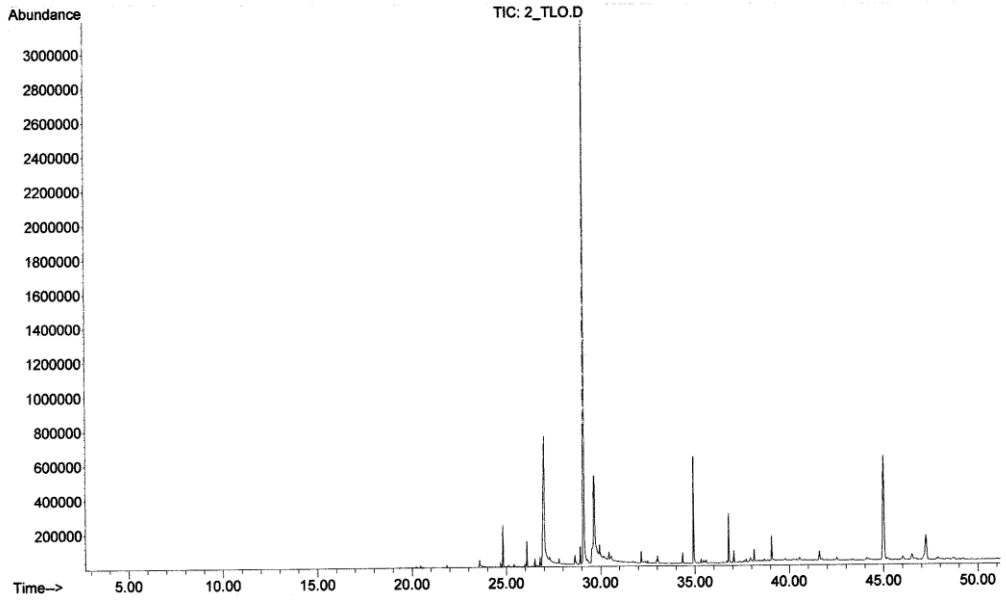

Figure 4.8 Total ion chromatogram of *B. retusa* leaves essential oil

Table 4.18 Chemical composition of the leaf essential oil of *B. retusa*

Peak	Compound	RT	RA%	RI_{exp}	RI_{lit}	References
1	Tetradecanoic acid	23.58	0.4	1772	1767	90
2	Isophytol	26.52	0.3	1948	1944	77
3	Dibutyl phthalate	26.79	0.4	1965	-	-
4	Phytol	29.12	33.4	2117	1949	77
5	2-Propenoic acid	29.94	0.9	2173	-	-
6	Pentacosane (CAS)	34.35	0.5	2498	2500	73
7	Phthalic acid	34.92	5.2	2544	-	-
8	Heptacosane	36.79	2.3	2698	2700	78
9	Pentamethoxyflavone	38.13	0.6	2814	-	-
10	Nonacosane	39.06	1.2	2897	2900	78
11	6, 13-dimethoxy-2, 3, 9, 10-tetramethylpentacene-1, 4, 8, 11-tetrone	47.24	3.4	-	-	-
	Total		48.6			

RA: relative area (peak area relative to total peak area).
RI_{exp}: retention indices relative to n-alkanes (C_7-C_{30}) on HP-5MS column.
RI_{lit} : retention indices from literature data.

Eleven components, represent in 48.6 % of the total essential oil, were identified and are listed in the order of their elution on the HP-5 capillary column used for GC-MS analysis (Table 4.18). The major components of the essential oil were identified as phytol (33.4%), phthalic acid (5.2%), 6, 13-dimethoxy-2, 3, 9, 10-tetramethylpentacene-1, 4, 8, 11-tetrone (3.4%), heptacosane (2.3%) and nonacosane (1.2%). The minor components were 2-propenoic acid (0.9%), pentamethoxyflavone (0.6%), pentacosane (0.5%), tetradecanoic acid (0.4%), dibutyl phthalate (0.4%) and isophytol (0.3%). Dibutyl phthalate is probably contaminant later than an essential oil component. It is a plasticizer contaminant.

The most prominent compound found in the leaf essential oil of *B. retusa* was phytol (33.4%). It is an acryelic diterpene alcohol, which has been reported to have anticancer activity (91) and also possessed antibacterial activity (92). It can be also used as a precursor for the production of synthetic form of vitamin E (85) and vitamin K1 (86). Pentamethoxyflavone (in the leaf oil) has been found to exhibit cytotoxicity activity (93).

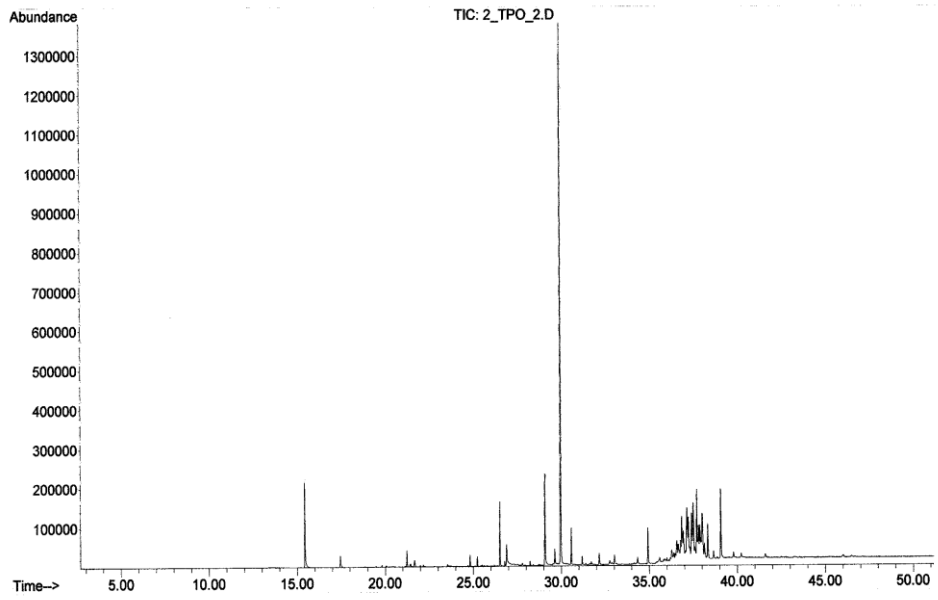

Figure 4.9 Total ion chromatogram of *B. retusa* fruits essential oil

Table 4.19 Chemical composition of the fruit essential oil of *B. retusa*

Peak	Compound	RT	RA%	RI_{exp}	RI_{lit}	References
1	Diacetin	15.43	4.3	1353	-	-
2	γ-Eudesmol	21.24	0.7	1642	1632	71
3	α-Eudesmol	21.67	0.4	1665	1654	71
4	Hexahydrofarnesyl acetone	24.82	0.5	1844	1835	94
5	Isobultyl phthalate	25.23	0.4	1869	-	-
6	Isophytol	26.52	2.7	1948	1944	73
7	Hexadecanoic acid	26.90	1.5	1972	1966	90
8	Phytol Isomer	29.07	4.8	2114	1974	89
9	Dibutyl sebacate	29.97	25.6	2175	-	-
10	Citric acid	31.19	0.4	2261	-	-
11	2-Propenoic acid	32.15	0.6	2331	-	-
12	Phthalic acid	34.92	1.9	2544	-	-
13	Erucylamide	37.88	2.5	2791	-	-
14	Eicosane	37.95	1.2	2797	2000	73
15	Tricosane	39.06	3.9	2897	2300	73
16	Bis-(octylphenyl)-amine	39.79	0.3	2959	-	-
	Total		51.7			

RA: relative area (peak area relative to total peak area).
RI_{exp}: retention indices relative to n-alkanes (C_7-C_{30}) on HP-5MS column.
RI_{lit} : retention indices from literature data.

Sixteen components, represent in 51.7 % of the total essential oil, were identified and are listed in the order of their elution on the HP-5 capillary column used for GC-MS analysis (Table 4.19). The major components of the essential oil were identified as dibutyl sebacate (25.6%), phytol isomer (4.8%), diacetin (4.3%), tricosane (3.9%), isophytol (2.7%), phthalic acid (1.9%), hexadecanoic acid (1.5%), and eicosane (1.2%). The minor components of the essential oil were γ-eudesmol (0.7%), 2-propenoic acid (0.6%), hexahydrofarnesyl acetone (0.5%), isobultyl phthala (0.4%), α-eudesmol (0.4%), citric acid (0.4%), and bis-(octylphenyl)-amine (0.3%). Isobutyl phthalate and bis-(octylphenyl)-amine are probably plasticizer contaminants.

The main compound found in the fruit essential oil was dibutyl sebacate. (25.6%). It is a dibutyl ester of sebacic acid. This compound is used as a plasticizer for film coating of tablets, beads, and granules. It is also used as pharmaceutical excipient in both aqueous and solvent based formulation. Alpha-eudesmol is a

sesquiterpenoid, which is present in the fruit essential oil. This compound is a potent nonpeptidergic compound which blocks the presynaptic omega-Aga-IVA-sensitive Ca^{2+} channel with relative selectivity (95). Hexahydrofarnesyl acetone (in fruit oil) has been found to exhibit antibacterial activity against *S. aureus* (96). The fatty acids, tetradecanoic acid and hexadecanoic acid which are present in the leaf and fruit essential oils have been reported to possess antibacterial activity against *S. aureus* (97).

4.6 Isolation of the bioactive extracts

4.6.1 Isolation of *A. indica* constituent

Isolation and purification of fraction PA21 by column chromatography yielded compound PA21.9*. After recrystallization with methanol, white crystalline powder was obtained (Scheme 3.10).

4.6.1.1 Isolation of PA21.9* (white crystalline powder 11 mg)

The isolated PA21.9* compound was characterized by NMR spectroscopy. The ^{1}H NMR and ^{13}C NMR spectra of PA21.9* were analysed using a Bruker AVANCE 400 NMR spectrometer, operating at 400 and 100 MHz in deuterated methanol (CD_3OD), respectively. The chemical shifts were recorded in ppm by reference to TMS signal. The structure elucidation of the isolated compound was L-quebrachitol as crystalline white powder (Figure 4.10), with the molecular formula $C_7H_{14}O_6$ (Figure 4.11) as deduced from ^{13}C-NMR and HR-ESI-MS data. Analysis of the ^{1}H-NMR, ^{13}C-NMR, and HMBC spectra revealed:

- ^{1}H-NMR (CD_3OD, 400 MHz): δ 4.11 (t, 1H, J 3.36 Hz, H_{eq}-1); 3.32 (dd, 1H, J 9.20, 3.12 Hz, H-2); 3.61 (t, 1H, J 9.12 Hz, H_{ax}-3); 3.55 (t, 1H, J 9.12 Hz, H_{ax}-4); 3.68 (dd, 1H, J 9.20, 3.12 Hz, H_{ax}-5); 3.94 (t, 1H, J 3.38 Hz, H-6); 3.45 (s, 3H, H–OMe), (Table 4.20).

- ^{13}C NMR (100 MHz, CD_3OD): δ 69.49 (C1); 82.61 (C2); 73.95 (C3); 74.87 (C4); 72.49 (C5); 73.52 (C6); 58.03 (Me), (Table 4.20).
- HR-ESI-MS: m/z 195.1270 $[M+1]^+$; calculated mass 195.1268.

The COSY spectrum of this compound indicated the following spin systems: the hydrogen at δ_H = 4.11 (1H, t, H-1) was coupled to the hydrogen at δ_H = 3.32 (1H, dd, H-2) and the hydrogen at δ_H = 3.94 (1H, t, H-6). The hydrogen at δ_H = 3.32 (1H, dd, H-2) was coupled to the hydrogen at δ_H = 4.11 (1H, t, H-1) and the hydrogen at δ_H = 3.61 (1H, t, H-3). The hydrogen at δ_H = 3.61 (1H, t, H-3) was coupled to the hydrogen at δ_H = 3.32 (1H, dd, H-2) and the hydrogen at δ_H = 3.55 (1H, t, H-4). The hydrogen at δ_H = 3.55 (1H, t, H-4) was coupled to the hydrogen at δ_H = 3.61 (1H, t, H-3) and the hydrogen at δ_H = 3.68 (1H, dd, H-5). The hydrogen at δ_H = 3.68 (1H, t, H-5) was coupled to the hydrogen at δ_H = 3.55 (1H, t, H-4) and the hydrogen at δ_H = 3.94 (1H, t, H-6). The hydrogen at δ_H = 3.94 (1H, t, H-6) was coupled to the hydrogen at δ_H = 4.11 (1H, t, H-1) and the hydrogen at δ_H = 3.68 (1H, dd, H-5) (Table 4.20).

The HMBC spectrum of this compound indicated the following spin systems: the proton at δ_H = 4.11 correlated with C-2 (δ_C = 82.61) and C-3 (δ_C = 73.52); the proton at δ_H = 3.32 correlated with C-3 (δ_C = 73.52), the proton at δ_H = 3.61 correlated with C-2 (δ_C = 82.61) and C-4 (δ_C = 74.87); the proton at δ_H = 3.55 correlated with C-6 (δ_C = 73.52); the proton at δ_H = 3.68 correlated with C-4 (δ_C = 74.87); the proton at δ_H = 3.94 correlated with C-1 (δ_C = 69.49), C-2 (δ_C = 82.61), C-4 (δ_C = 74.87) and C-5 (δ_C = 72.49); and the methoxy proton at δ_H = 3.45 correlated with C-2 (δ_C = 82.61) (Table 4.20 and Figure 4.12).

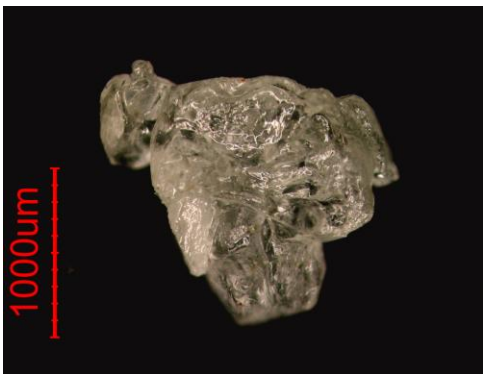

Figure 4.10 Crystalline white powder of *L*-quebrachitol

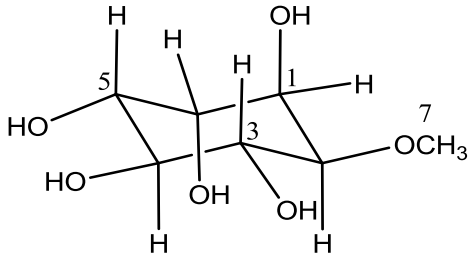

Figure 4.11 Chemical structure of *L*-quebrachitol

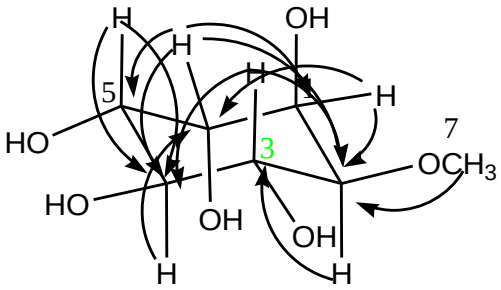

Figure 4.12 Key HMBC correlations of *L*-quebrachitol

Table 4.20 ^{1}H-NMR data of *L*-quebrachitol in CD_3OD

Position	^{1}H	^{13}C	COSY	HMBC (H→C)
1	4.11t (3.36)	69.49	H-2, H-6	C-2, C-6
2	3.32dd (9.20, 3.12)	82.61	H-1, H-3	C-3
3	3.61t ((9.12)	73.95	H-2, H-4	C-2, C-4
4	3.55t ((9.12)	74.87	H-3, H-5	C-6
5	3.68dd (9.20, 3.12)	72.49	H-4, H-6	C-4
6	3.94t (3.38)	73.52	H-1, H-5	C-1, C-2, C-4, C-5
7	3.45s	58.03		C-2

L-quebrachitol is the 2-methyl ether of L-chiro-inositol. *L*-chiro-inositol is a cyclitol whose isomers occur in various plant sources (i.e., Proteaceae (98) and Apocynaceae (99).

L-quebrachitol has currently been used as a starting material for the synthesis of a wide variety of bioactive materials, most notably inositol. *L*-inositol exists in the form of *L*-(-)-2-*o*-methyl-chiro-inositol in 1% of rubber serum and can be used as starting material for synthesis of optically active organic compounds such as antibiotics and anticancer drug (100). Recently, novel antioxidant and anticancer functions of inositol hexaphosphate, a naturally occurring component of plant fiber, have been discovered (101).

4.6.1.2 Determination of L-Quebrachitol by HPLC

A high performance liquid chromatographic method was developed for the determination of *L*-quebrachitol which was isolated from the aerial parts of *A. indica*. In order to achieve optimum separation, various experimental conditions were investigated.

4.6.1.2.1 Optimization of the experimental conditions

4.6.1.2.1.1 The optimum wavelength

The maximum absorption wavelength of *L*-quebrachitol (16 µg/mL) was measured by scanning the standard solution at 200-300 nm by means of a photodiode array detector (DAD). The maximum absorption wavelength of *L*-quebrachitol was found to 206 nm as shown in Figure 4.13.

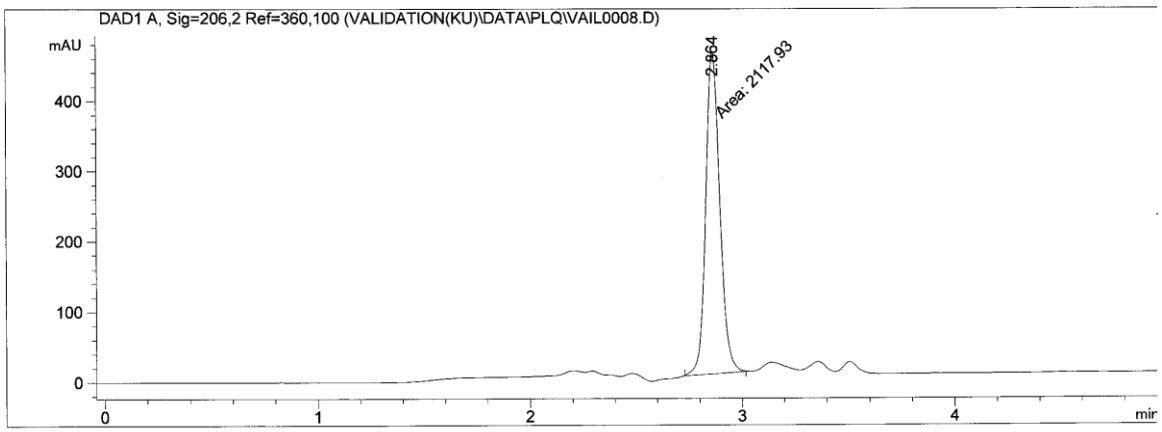

Figure 4.13 The optimum wavelength of *L*-quebrachitol.

4.6.1.2.1.2 Selection of mobile phase and flow rate

During the development of the RP-HPLC method ten different compositions of mobile phases as shown in Table 4.21 were tested. For the analysis of standard solution of *L*-quebrachitol using the mobile phase compositions No. 1-8, the flow rates were kept constant at 1.0 mL/min with DAD detection at 206 nm. The *L*-quebrachitol peaks appeared at the retention time in the range of 2.857-2.864 min. Results showed that best resolution was obtained when using methanol:acetonitrile:tetrahydrofuran (79.5:20.4:0.1 v/v) as mobile phase. Further analysis of *L*-quebrachitol was also performed by varying the flow rate to 0.5 mL/min and 0.3 mL/min respectively. Analytical peaks were obtained at retention time of 9.442 min, but best resolution, well defined peak and maximum peak area were obtained when using

methanol:acetonitrile:tetrahydrofuran (79.5:20.4:0.1 v/v) as mobile phase with the flow rate of 0.3 mL/min.

Table 4.21 Selection of mobile phase and flow rate of standard *L*-quebrachitol

No.	Mobile phase composition (methanol:acetonitrile:tetrahydrofuran, v/v)	Flow rate (mL/min)	Retention Time (min)	Area*
1	70 : 30 : 0.0	1.00	2.858	2067.41
2	80 : 20 : 0.0	1.00	2.858	2083.03
3	75: 25 : 0.0	1.00	2.864	2114.94
4	75 : 24 : 1.0	1.00	2.857	1043.63
5	75 : 24.7: 0.3	1.00	2.858	2052.85
6	75: 24.9 : 0.1	1.00	2.858	2110.64
7	77 : 22.9: 0.1	1.00	2.860	2094.12
8	79.5 : 20.4 : 0.1	1.00	2.857	2087.16
9	79.5 : 20.4 : 0.1	0.50	5.715	4144.63
10	79.5 : 20.4 : 0.1	0.30	9.442	7004.15

*Detection with photodiode array detector at 206 nm

4.6.1.2.2 Method Validation

4.6.1.2.2.1 Sensitivity

The sensitivity of the assay was determined in terms of limit of detection (LOD) and limit of quantitation (LOQ). The limits of detection and quantitation were calculated from the standard deriation (SD) of the chromatographic response and the slope of the curve (S) using the following equations:

LOD = 3.3 (SD/S)

LOQ = 10 (SD/S)

After the chromatographic analysis of *L*-quebrachitol at 206 nm, the lowest amount of *L*-quebrachitol that could be detected (LOD) and quantified (LOQ) were found to be 3.91 µg mL^{-1} and 13.02 µg mL^{-1} respectively.

4.6.1.2.2.2 Linearity

The calibration curve of *L*-quebrachitol was established with different concentrations in the range of 3.91-1000 µg mL^{-1}. Each solution was injected three times, and the areas of peaks, measured at 206 nm and were integrated. Regression equation was established by plotting the peak areas versus the concentrations of L-quebrachitol. The linearity was evaluated by linear regression analysis, which was calculated by least-squares regression. Results showed that the average regression equation of *L*-quebrachitol over the concentration range of 3.91-1000 µg mL^{-1} was y = 4.57138X + 16.16790 with a slope of 4.57138, an intercept of 16.16790 and a correlation coefficient (r^2) value of 0.9998 (Figure 4.14).

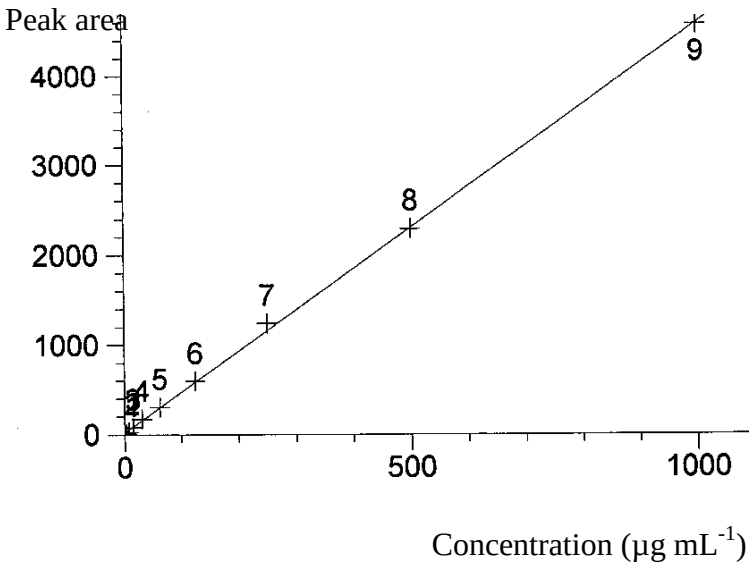

Figure 4.14 Linearity of *L*-quebrachitol standard response (peak area) over the concentration range of 3.91-1000 µg/mL by means of DAD1 A; Sig=206, 2; Ref=360, 100; R^2 = 0.99977; Residual Std. Dev. = 33.63777; y=mx+ b (the y-error bars represent the standard deviation of the response at each concentration).

4.6.1.2.2.3 Precision

The precision of the method was established by using solutions of three different concentrations, 125 µg mL^{-1}, 250 µg mL^{-1} and 500 µg mL^{-1} of *L*-quebrachitol standard solution. Each was analyzed three times (n = 3) on the same day, and the relative standard deviation (RSD) was calculated to ascertain intraday precision. The studies were also repeated on three different days to establish interday precision. Results are presented in Table 4.22.

Table 4.22 Precision study for L-quebrachitol.

L-quebrachitol (µg/mL)	Intraday precision (n=3)	Interday precision (n=3)
	%RSD	%RSD
125	0.24	0.30
250	0.14	0.36
500	0.07	0.33

4.6.1.2.2.4 Accuracy

The accuracy of the proposed RP-HPLC method was also investigated. Known quantities of *L*-quebrachitol (100 µg/mL) was added to previously analyzed sample (*L*-quebrachitol) and analyzed using the proposed method. The percentage recoveries for triplicate determinations were in the range of 93.46-93.82% with the %RSD of 0.29-2.28% (Table 4.23).

Table 4.23 Recovery study of *L*-quebrachitol.

No.	Amount of standard *L*-quebrachitol added (µg mL^{-1})*	Amount found (µg mL^{-1})	% Recovery	% RSD
1	100	93.46	93.46	2.28
2	100	93.78	93.78	1.71
3	100	93.82	93.82	0.29

*****100 µg/mL of standard *L*-quebrachitol added into 100 µL of sample solution

4.6.1.2.2.5 System Suitability

System suitability test for the determination of *L*-quebrachitol was carried out. The testing parameters such as theoretical plates, tailing factor, resolution, asymmetry and reproducibility (RSD) for retention time and area of six replicates were determined and compared against the specifications set for the method. Results are presented in Table 4.24.

Table 4.24 System suitability for RP-HPLC determination of L-quebrachitol.

Property	Values
Retention time (min)	9.442
Area	7004.148
Tailing factor (T)	1.4
Resolution (R)	23.6
Asymmetry (A_S)	3.3
Theoretical plates (N)	12788
Inraday precision (RSD)	0.33%
Interday precision (RSD)	0.15%

Figure 4.15 showed the HPLC chromatogram of *L*-quebrachitol standard, 1000 µg/mL, using Restek® - C_{18} column (5 µm, 4.6 mm x 250 mm), column tempearature 25ºC, the mobile phase consisted of methanol:acetonitrile: tetrahydrofuran (79.5:20.4:0.1 v/v) at flow rate of 0.3 mL/min with photodiode array detection at 206 nm. A well resolved peak of *L*-quebrachitol was obtained at the retention time of 9.442 min.

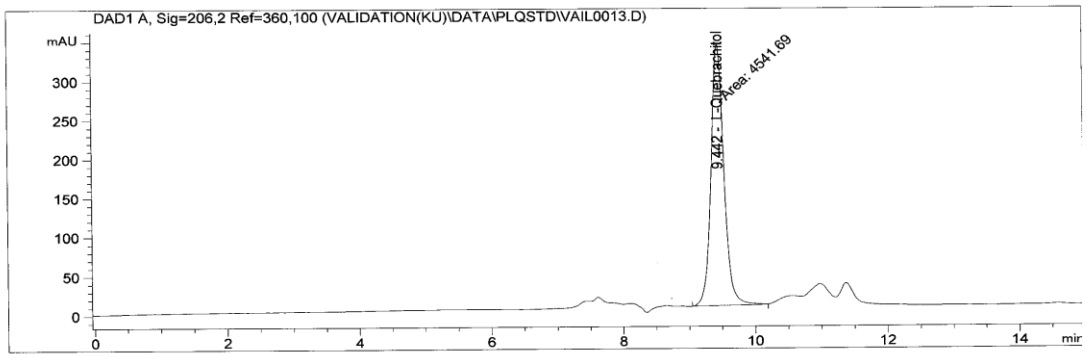

Figure 4.15 HPLC chromatogram of L-quebrachitol standard, obtained using a mobile phase composed of methanol:acetonitrile:tetrahydrofuran in 79.50 : 20.40 : 0.10 (v/v), at a flow rate 0.3 mL/min, column temperature 25 ºC and DAD operated at 206 nm.

4.6.1.2.3 Method application

The proposed RP-HPLC method was successfully applied to the determination of *L*-quebrachitol which was isolated from the aerial part of *A. indica*. This sample was analyzed by the standard addition method. Results are presented in Table 4.25 and Figure 4. The amount of L-quebrachitol in 100 µg/mL sample solution was found to be 62.8 µg. The percentage yield of *L*-quebrachitol in *A. indica* was 0.63%. There is no interference present in the analysis, because only one peak at a retention time of 9.442 min corresponding to that of *L*-quebrachitol was observed in the chromatogram.

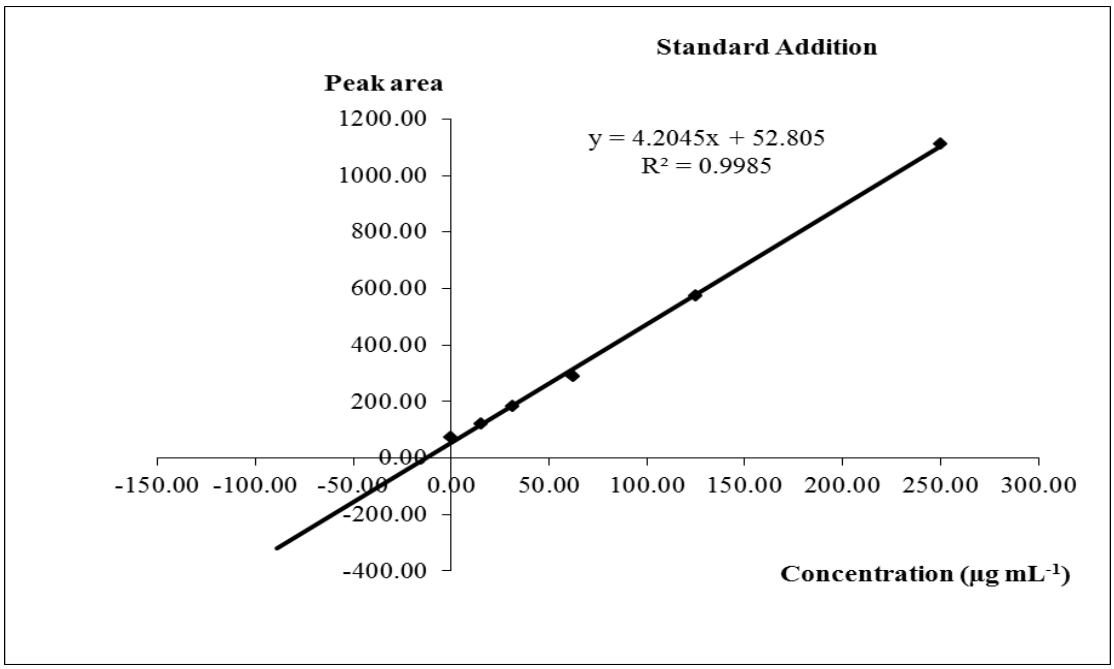

Figure 4.16 Standard addition curve for the determination of *L*-quebrachitol

Table 4.25 Determination of L-quebrachitol by standard addition method.

Solution No.	Concentration of *L*-quebrachitol standard added (µg/mL)	Area
1	0.00	73.68
2	15.63	119.64
3	31.25	182.99
4	62.50	289.57
5	125.00	575.62
6	250.00	1111.89

4.6.2 Isolation of *C. javanicum* constituent

4.6.2.1 Isolation of DfH7.2* (white needle crystalline 2.2 mg) [Scheme 3.15]

DfH7.2* 2.2 mg was dissolved in hexane and analysed by means of GC and GC-MS. Identification of DfH7.2* fraction constituents was performed by comparison of mass spectra with the literature data (NIST, NISTREP), that yielded < 90% matches were identified as unknowns. Six compounds were identified and are listed in Table 4.26. A typical total ion chromatogram is presented in Figure 4.17.

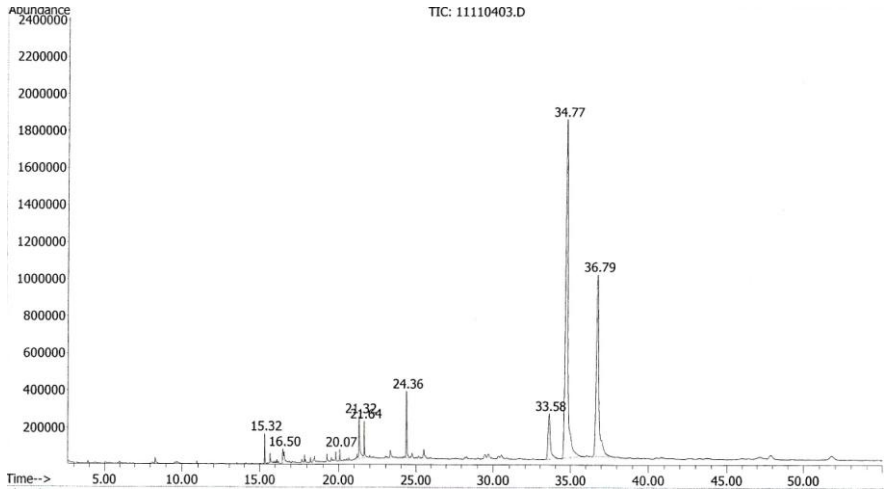

Figure 4.17 Total ion chromatogram of DfH7.2* fraction

Table 4.26 Chemical composition of DfH7.2* fraction

Peak No.	Compound	R.T (min)	%RA	MW
1	2-Pentadecanone	15.32	0.82	226.40
2	Hexadecanoic acid	16.50	0.36	256.43
3	Bis(2-ethylhexyl)phthalate	21.64	1.34	390.56
4	Ergost-5-en-3-ol, (3.beta)-	33.58	6.95	400.37
5	Stigmasterol	34.77	53.97	412.69
6	Gamma-Sitosterol	36.79	30.36	414.71

Six compounds were identified and are listed in the order of their elution on the HP-5 capillary column used for GC-MS analysis (Table 4.26). The major components of the fraction were identified as stigmasterol (53.97%), gamm-sitosterol (30.36%) and ergost-5-en-3-ol, (3.beta)- (6.95%). The minor components were bis(2-ethylhexyl)phthalate (1.34%), 2-pentadecanone (0.82%), and hexadecanoic acid (0.3%). Bis(2-ethylhexyl)phthalate is a contaminant later than an essential oil component. It is a plasticizer contaminant.

4.6.2.2 Isolation of DfH7.3.2* (white needle crystalline 0.8 mg) [Scheme 3.15]

DfH7.3.2* 0.8 mg was dissolved in hexane and analysed by means of GC and GC-MS. Identification of DfH7.3.2* fraction constituents was performed by comparison of mass spectra with the literature data (NIST, NISTREP), that yielded < 90% matches were identified as unknowns. Two compounds were identified and are listed in Table 4.27. A typical total ion chromatogram is presented in Figure 4.18.

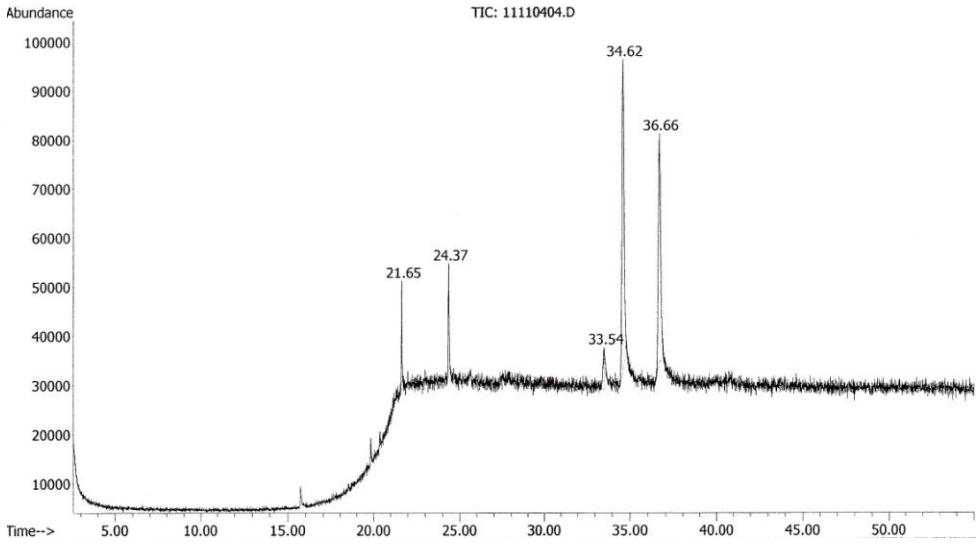

Figure 4.18 Total ion chromatogram of DfH7.3.2* fraction

Table 4.27 Chemical composition of DfH7.3.2* fraction

Peak No.	Compound	R.T (min)	%RA	MW
1	Stigmasta-5, 22-dien-3-ol, (3.beta., 22E)-	34.61	46.31	412.70
2	Gamma.-Sitosterol	36.66	37.59	414.71

Two compounds were identified and are listed in the order of their elution on the HP-5 capillary column used for GC-MS analysis (Table 4.27). The major components of the fraction were identified as gamm-sitosterol (36.66%) and stigmasta-5, 22-dien-3-ol, (3.beta., 22E)- (34.61%).

4.6.2.3 Isolation of DfH7.3.3*, DfH7.3.4*, DfH7.3.5* and DfH7.4* (white needle crystalline 0.5, 0.2, 0.4 and 2.4 mg) [Scheme 3.15]

The isolated compounds DfH7.3.3*, DfH7.3.4*, DfH7.3.5* and DfH7.4* were characterized by NMR spectroscopy. The ^{1}H NMR and ^{13}C NMR spectra of DfH7.3.3*, DfH7.3.4*, DfH7.3.5* and DfH7.4* were analysed using a Bruker AVANCE 400 NMR spectrometer, operating at 400 and 100 MHz in deuterated chloroform ($CDCl_3$), respectively. The chemical shifts were recorded in ppm by reference to TMS signal. The structure elucidation of the isolated compound was stigmasterol as crystalline white powder with the molecular formula $C_{29}H_{48}O$ (Figure 4.19). This structure was deduced from the analysis of the ^{1}H-NMR spectra revealed:

^{1}H-NMR spectra of this compound displayed signals for olefinic proton signals at δ 5.35 (1H, *brd*, J = 5.2 Hz, H-6), 5.16 (1H, *dd*, J = 15.1, 8.6 Hz, H-22) and 5.02 (1H, *dd*, J = 15.1, 8.6 Hz, H-23) (Table 4.28), which the proton signals revealed the basic skeleton of a steroid pattern.

From these data, the structure of this compound was assigned as stigmasterol by the above evidences and by comparison of spectroscopic data with those reported values. Stigmasterol was reported in many plants such as *Ambroma augusta* (102,103).

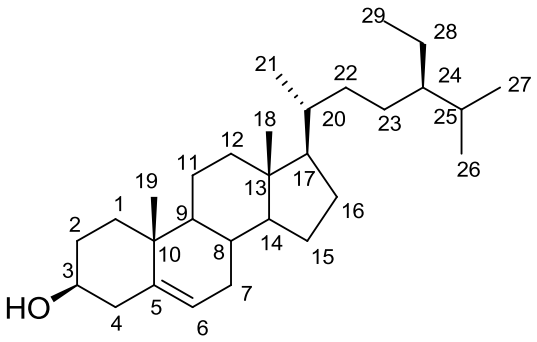

Figure 4.19 Chemical structure of stigmasterol

Table 4.28 ^{1}H-NMR spectral data of stigmasterol in $CDCl_3$

Position	Compound DfH7.4* δ ^{1}Ha (Multiplicity, *J* in Hz)	Stigmasterol[104, 105] δ ^{1}H (Multiplicity, *J* in Hz)
1		
2		
3	3.45 (m)	3.52 (m)
4		
5		
6	5.28 (d,J=5.2)	5.36 (d,J=5.1 Hz)
7		
8		
9		
10		
11		
12		
13		
14		
15		
16		
17		
18	0.79 (s)	0.695 (s)
19	1.09 (s)	1.01 (s)
20		
21		1.02 (d, J=6.4)
22	5.09 (dd,J=15.3,8.6)	5.16 (dd,J=15.0,8.4)
23	4.95 (dd,J=15.1,8.6)	5.02 (dd,J=15.0,8.4)
24	1.52 (*m*)	
25		
26	0.86 (*d*, *J* = 6.5)	0.852 (d, J=8.0)
27		0.835 (d, J=5.3)
28		
29		0.82 (t, J=6.0 Hz)

4.6.3 Isolation of *B. retusa* constituent

4.6.3.1 Isolation of TfM8.5* (white crystalline powder 8.1 mg) [Scheme 3.26]

The isolated compound TfM8.5* was characterized by NMR spectroscopy. The ^{1}H NMR and ^{13}C NMR spectra of DfM8.5* were analysed using a Bruker AVANCE 400 NMR spectrometer, operating at 400 and 100 MHz in deuterated methanol (CD_{3OD}), respectively. The chemical shifts were recorded in ppm by reference to TMS signal. The structure elucidation of the isolated compound was benzoic acid as amorphous crystalline white powder, with the molecular formula $C_7H_6O_2$ (Figure 4.20) as deduced from the analysis of ^{1}H-NMR spectra revealed:

- ^{1}H-NMR (CD_3OD, 400 MHz): δ 8.14 (dd, J 1.6, 8.8 Hz, H-2); 7.47 (t, , J 7.6 Hz, H-3); 7.61 (tt, J 1.6, 7.6 Hz, H-4); 7.47 (t, J 7.6 Hz, H-5); 8.14 (dd, J 1.6, 8.8 Hz, H-6), (Table 4.29).

From these data, the structure of this compound was assigned as benzoic acid by the above evidences and by comparison of spectroscopic data with those reported values. Benzoic acid have been reported in many plants (106).

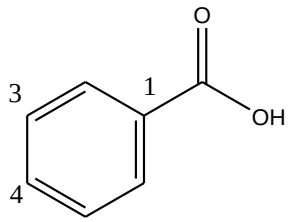

Figure 4.20 Chemical structure of benzoic acid

Table 4.29 ^{1}H-NMR and ^{13}C-NMR spectral data of benzoic acid (compound: DfM8.5*) in $CDCl_3$

Position	Compound DfM8.5* δ_H (*mult.*, *J* (Hz))	Benzoic acid δ_H (*mult.*, *J* (Hz))
1		
2	8.01 (*dd*, *J*=1.2, 8.0)	8.13 (*dd*, *J*=1.6, 8.8)
3	7.46 (*t*, *J*=7.6)	7.47 (*t*, *J*=7.6)
4	7.59 (*tt*, *J*=1.2, 7.6)	7.61 (*tt*, *J*=1.6, 7.6)
5	7.46 (*t*, *J*=7.6)	7.47 (*t*, *J*=7.6)
6	8.01 (*dd*, *J*=1.2, 8.0)	8.13 (*dd*, *J*=1.6, 8.8)
7		