3.1

(207)

E-ISSN: 2278-3229

IJGHC, March 2016 – May 2016; Sec. A; Vol.5, No.2, 122-138.

International Journal of Green and Herbal Chemistry

An International Peer Review E-3 Journal of Sciences

Available online at www.ijghc.com

Section A: Green Chemistry

Research Article

CODEN (USA): IJGHAY

Preliminary Studies on Activity Guided Fractionation of the Ethanolic Extract of Dried Flowers of *Aegle Marmelos*

K. D. K. P. Kumari[1*], K. Samarasinghe[2], S. M. Handunnetti[3] and T. S. Suresh[4]

[1]Department of Basic Sciences, Faculty of Allied Health Sciences, General Sir John Kotelawala Defence University, Ratmalana, Sri Lanka.

[2]Department of Pathology, Faculty of Medical Sciences, University of Sri Jayewardenepura, Gangodawila, Nugegoda, Sri Lanka

[3]Institute of Biochemistry, Molecular Biology and Biotechnology, University of Colombo, Colombo, Sri Lanka

[4]Department of Biochemistry, Faculty of Medical Sciences, University of Sri Jayewardenepura, Gangodawila, Nugegoda, Sri Lanka

Received: 4 February 2016; **Revised:** 8 March 2016; **Accepted:** 21 March 2016

Abstract: *Aegle marmelos* is a reputed medicinal plant with many biologically active compounds. Activity guided fractionation of the ethanolic extract of dried flowers of *Aegle marmelos*, based on the anti-inflammatory and hypoglycaemic effect was studied in the present study. The ethanolic extract was partitioned into ethyl acetate and hexane and the fractions were tested for anti-inflammatory and hypoglycaemic effect in rats. The volatile compounds in ethyl acetate and hexane fraction were analyzed by GC-MS. The ethyl acetate fraction showed the highest anti-inflammatory and hypoglycemic effect and it was further separated by column and thin layer chromatography. All the fractions were subjected to phytochemical screening. Alpha-phellandrene and eugenol was the most abundant volatile compound identified in the ethyl acetate and hexane fractions

respectively. The fraction which showed the highest anti-inflammatory effect contained poly phenols and triterpenoids and its FTIR spectrum exhibited the presence of O-H/N-H/C=O/C-Cl and C=C groups. The fraction with the highest hypoglycaemic effect contained coumarins and flavonoids and the FTIR spectrum exhibited the presence of O-H/N-H/C=O and C=C groups. The identified monoterpenes and sesquiterpenes may responsible for the typical aroma of the extracts of dried flowers of *A. marmelos* while triterpenoids, coumarins and flavonoids may responsible for the anti-inflammatory and hypoglycaemic effect of the ethanolic extract of dried flowers of A. marmelos.

Keywords: *Aegle marmelos*, Anti-inflammatory, Hypoglycaemic, Fractionation, Ethanol extract.

INTRODUCTION

Aegle marmelos (L.) Correa (Family: Rutaceae) is a highly reputed medicinal plant in the traditional systems of medicine which is used to treat a wide variety of disorders. This plant, commonly known as the *bael* fruit tree is found in many Asian countries including Sri Lanka, India, Burma, Bangladesh, Egypt, Malaysia, Myanmar, Pakistan, Thailand and Indo-China[1,2]. *Aegle marmelos* is a slow-growing, mid-sized, slender, aromatic, armed tree growing up to 40 or 50 ft tall with short trunk and thick, flaking bark. The older branches are spreading straight and the lower ones are drooping while the young branches are slightly zigzag and compressed. Some branches are spiny and young suckers bear many stiff, straight spines. A clear, gummy sap, resembling gum arabic, exudes from wounded branches and hangs down in long strands, becoming gradually solid. It is sweet at first taste and then irritating to the throat. Leaves are deciduous, alternate, attenuate trifoliate (occasionally digitately 5-foliate) and the leaflets are oval pointed, shallowly toothed, 4-10 cm long, 2-5 cm wide and the terminal one with a long petiole. New foliage is glossy and pinkish-maroon. Mature leaves emit a disagreeable odour when bruised. Fragrant flowers, in clusters of 4 to 7 along the young branchlets, have 4 recurved, fleshy petals, green outside, yellowish inside and 50 or more greenish yellow stamens. The fruit has a pyriform, oval or round shape and it is 5 to 20 cm in diameter. It may has a thin, hard, woody shell or a more or less soft rind, gray green until the fruit is fully ripe, when it turns yellowish[3,4]. Almost every part of this plant has been used in ancient and modern traditional medicine to relieve different diseases such as dysentery, cholera, constipation and diabetes mellitus[1]. Extensive investigations that have been carried out have revealed different parts of *Aegle marmelos* has pharmacological activities such as anti-inflammatory, antipyretic, analgesic, antioxidant, anti-diabetic, anti-diarrhoeal, anti-proliferative and anti-fungal activity[2,5]. It has been found that different parts of this plant contain varied classes of compound such as tannins, alkaloids, coumarins, essential oils and steroids[1]. Different types of phyto-constituents confer specific characteristics and properties to plants. Therefore, the analysis of these constituents would help in determining various biological activities of plants and a new compound detected form a plant material provides unlimited opportunities for new drug. Although extensive investigations done on the other parts of this plant, the medicinal values of the flower is overlooked. The hypoglycaemic and anti-inflammatory activities of the water and ethanolic extracts of the dried flowers of *A. marmelos* have been established. Bioactivity guided fractionation of the ethanolic extract of the flowers is studied in the present study.

EXPERIMENTAL

Ethical clearance: The protocol for animal experiments was approved by the Ethics Review Committee of the Faculty of Medical Sciences, University of Sri Jayewardenepura, Gangodawila, Nugegoda, Sri Lanka (No 432/09). International guidelines and recommendations of Federation of European Laboratory Animal Science Associations (FELASA) were followed for handling of animals. Assays were carried out at the Animal House and the Department of Biochemistry of University of Sri Jayewardenepura, Sri Lanka.

Plant material: The fallen flowers of *A. marmelos* which were collected and sun dried by the farmers were purchased. The plant material was identified by Prof. P. Tissera, Professor of Botany, Department of Botany, Faculty of Applied Sciences, University of Sri Jayewardenepura, Sri Lanka in comparison to the herbarium specimens of the Department. A voucher specimen (USJP FMS 6/2010) has been deposited at the herbarium of the Department of Biochemistry, Faculty of Medical Sciences, University of Sri Jayewardenepura, Gangodawila, Nugegoda, Sri Lanka.

Animals: Healthy adult male, Wistar rats weighing 150 - 200 g were purchased from Medical Research Institute, Colombo 8, Sri Lanka. Rats were housed under standard conditions (230 ± 2 0C, 60 % - 70 % relative humidity and 12 h photo period) and fed with standard diet and water ad libitum.

Preparation of extracts: To prepare the ethanolic extract 25 g of dried flowers was extracted in 350 ml of absolute ethanol using the Soxhlet apparatus and rotary evaporator and freeze dried.

Partitioning of the EEAM: For partitioning, 10 g of freeze dried ethanol extract which was dissolved in 100 ml of double distilled (DD) water (Toyobo, DW 18-111) and mixed thoroughly with 100 ml of HPLC grade hexane (Prolabo, France) in a separating funnel and after 30 min. the water and hexane fractions were collected separately. The remaining water fraction was then mixed thoroughly with 100 ml of HPLC grade ethyl acetate (Prolabo, France) and the water and ethyl acetate fractions were collected separately. The hexane fraction (HF) and ethyl acetate fraction (EAF) were evaporated by the rotary evaporator and used for further experiments. These were subjected to Gas Chromatography – Mass Spectrum analysis (GC-MS) to identify the volatile components and the anti-inflammatory and hypoglycaemic effects were evaluated in rats.

Comparison of the anti-inflammatory effect of the fractions in healthy rats: Carrageenan-induced paw oedema model was used for evaluation of anti-inflammatory effect as described by Buadonpri *et al.*[6], with a few modifications. Initially, the baseline values of the paw volume were taken at the zero hour using a plethysmometer (Letica Scientific Instruments, Barcelona, Spain). Eighteen rats were divided randomly into 3 groups of six animals each. The fractions were orally administered in the following manner: Group 1 received 2.5 ml distilled water as the negative control, Group 2 received a dose of 100 mg/kg hexane fraction and Group 3 received a dose of 100 mg/kg ethyl acetate fraction. After 1 h, 0.1 ml of carrageenan [(1% in normal saline) (Himedia, India)] was injected into the subcutaneous tissue of left hind paw and the paw volumes were measured at 1, 2, 3, 4 and 5 h. Oedema was expressed as the mean increase in paw volume relative to control animals. The percentage inhibition of oedema was calculated by the following equation:

Percentage inhibition of oedema = [(V t – V 0) control – (V t - V 0) treated] x 100

(V t – V 0) control

Where,

V0 - Paw volume at 0 hour

Vt - Paw volume at 1, 2, 3, 4 or 5 hour

Comparison of the hypoglycaemic effect of the fractions in healthy rats: The oral hypoglycaemic effect of the fractions was evaluated using oral glucose tolerance test (OGTT) in healthy Wistar rats[7]. The rats were fasted for 8-10 h and randomly selected in to 3 groups. The Group 1 received 1 ml of distilled water, while the Group 2 and 3 received a dose of 100 mg/kg hexane and ethyl acetate fraction respectively. This was followed by the administration of a glucose load (3 g/kg) after 30 min. Blood was drawn from the lateral tail vein after 2 h under light anaesthesia with anaesthetic ether. Blood was collected in to tubes containing NaF to inhibit the glycolysis. The serum glucose concentrations were measured using glucose oxidase reagent kits (Biolabo, France).

Gas Chromatography – Mass Spectrum analysis (GC-MS) of the fractions: The GC- MS technique was used to identify the volatile phyto-components present in the hexane and ethyl acetate fractions. Volatile compounds in the fractions were isolated by using solid phase micro-extraction (SPME) method. The volatiles were sampled by manual headspace solid phase micro-extraction at 60 °C. The fibre (100 µm PDMS, Supelco) was pierced into the injection port of the GC/MS after 20 min of sampling, and then desorbed at 200 °C for 5 min. Gas chromatography/ mass spectrometry conditions were as follows. A single quadrapole GC equipment with an Dynamax XR detection system (Thermoscientific, USA) was used, with the injector and detector maintained at 200 and 260 °C, respectively. Helium gas (99.99%) was used as the carrier gas at a constant flow rate of 5.7 ml/min. The temperature program was isothermal at 50 °C for 10 min, increased to 240 °C at 15 °C/ min, then held for 10 min. For GC-MS detection, an electron ionization energy system with ionization energy of 65 eV was used and the ion source and quadrupole maintained at 230 and 150 °C, respectively; mass range m/z 10-350. Compounds were identified by matching mass spectra (quality match > 80 %) and retention indices with the spectral library of standard compounds of the machine. The relative percentage amount of each component was calculated by comparing its average peak area to the total area.

Fractionation of ethyl acetate fraction by gel filtration chromatography: The ethyl acetate fraction (1g) was adsorbed to the Sephadex G25 (10 g) gel (Sigma Aldrich, USA) that was packed in a glass column (2 X 25 cm) and eluted with 0.01M phosphate buffer (pH 7.4). The fractions of 2 ml were collected and according to the UV fluorescence (Model UVG 2-58, USA) patterns of TLC (Pre coated, AlugarmxtraSil G/UV 254, Germany), the fractions with similar patterns were pooled together. The combined fractions (named Fraction 1, 2, 3 and 4) were freeze dried and the hypoglycaemic and anti-inflammatory activities of them were compared by administrating a dose of 100 mg/kg of each fraction to each rat.

Subfractionation of the Fraction 1 by column chromatography: As Fraction 1 showed the highest anti-inflammatory effect, it was subjected to further fractionation by column chromatography. The Fraction 1 was spotted on the pre-coated TLC plates (500 µm) and allowed to run in different solvent systems to select the best solvent system for distinct separation. The best separation of the Fraction 1 was observed in the solvent system, dichloromethane: ethyl acetate in the ratio = 7:3 and it was used for the

further fractionation of the Fraction 1 (0.5 g) in a silica column [(20 x 2 cm) (Sigma Aldrich)]. The fractions of 2 ml were collected and according to the UV fluorescence patterns of TLC, the fractions with similar patterns were pooled together. The combined subfractions (named Subfraction 7, 8 and 9) were freeze dried and the anti-inflammatory activity of them was compared by administrating a dose of 50 mg/kg of each fraction to each rat.

Subfractionation of the Subfraction 7 by thin layer chromatography: As Subfraction 7 showed the highest anti-inflammatory effect, it was subjected to further fractionation by thin layer chromatography. The Subfraction 7 was spotted on the pre-coated TLC plates (500 µm) and allowed to run in different solvent systems to select the best solvent system for distinct separation. The best separation of the Subfraction 7 was observed in the solvent system, Hexane: ethyl acetate (1:9) and it was used for the further fractionation of the Subfraction 7 (25 mg) in thin layer chromatography. The sample was streaked on glass plates (20 x 20 cm) that were coated with 1 mm layer of Silica gel GP254 (LOBA Chemie, India) and allowed to run in the saturated solvent system. The separated bands were detected by the fluorescence under the UV light and the Rf values of the bands were determined according to the following equation. The resulted bands (named as Band 1, 2 and 3) were scraped separately along with the silica gel and the compounds in the band were dissolved in distilled water and silica gel was removed by centrifugation. The dissolved bands were freeze dried and the anti-inflammatory activity of them was compared by administrating a dose of 25 mg/kg of each band to each rat. The FTIR spectrum (AVATAR, 320 FT IR, Thermo Nicolet, USA) of the Band 3 was taken in the region 4000-400 cm1 by employing standard KBr pellet technique.

$$\text{(Rf value) Retention factor} = \frac{\text{Distance traveled by the plant extract}}{\text{Distance traveled by the solvent system.}}$$

Subfractionation of the Fraction 3 by thin layer chromatography: As Fraction 3 showed the highest hypoglycaemic effect, it was subjected to further fractionation by thin layer chromatography. It was spotted on the pre-coated TLC plates (500 µm) and allowed to run in different solvent systems to select the best solvent system for distinct separation. The best separation of the Fraction 3 was observed in the solvent system, Toluene: Ethyl acetate (1:1) and it was used for the further fractionation of the Fraction 3 (25 mg) in thin layer chromatography. The separated bands were detected by the fluorescence under the UV light and the resulted bands (named as Band 4 and 5) were scraped separately along with the silica gel. The compounds in the bands were dissolved in distilled water and silica gel was removed by centrifugation. The dissolved bands were freeze dried and the hypoglycaemic activity of them was compared by administrating a dose of 25 mg/kg of each band to each rat. The FTIR spectrum (AVATAR, 320 FT IR, Thermo Nicolet, USA) of the Band 4 was taken in the region 4000-400 cm1 by employing standard KBr pellet technique.

Phytochemical screening of fractions: Chemical tests were done to identify the constituents in the various fractions and TLC bands by the methods previously described by Janarthanan et al., (2012).

Test for tannins: To 1ml of test sample, 2 ml of 5 % ferric chloride (Sigma Aldrich, USA) was added and formation of greenish black color indicated the presence of tannins.

Test for saponins: To 2 ml of test sample, 2 ml of distilled water was added and shaken in a graduated cylinder for 15 min lengthwise and formation of foam was observed.

Test for flavonoids: To 2 ml of test sample, 5 ml of dilute ammonia solution (Sigma Aldrich) was added and followed by addition of concentrated sulphuric acid. The appearance of yellow colouration indicated the presence of flavonoids.

Test for alkaloids: To 2 ml of test sample, 2 ml of concentrated hydrochloric acid was added and then few drops of Mayer's reagent (Sigma Aldrich) was added. Presence of green colour indicated the presence of alkaloids.

Test for anthocyanin: To 2 ml of test sample, 1 ml of 2 N NaOH was added and heated for 5minutes at 100 ℃. Formation of yellow colour indicated the presence of Anthocyanin.

Test for quinones: To 1 ml of test sample, 1 ml of concentrated sulphuric acid was added and the formation of red colour indicated the presence of quinones.

Test for terpenoids: To 0.5 ml of test sample, 2 ml of chloroform was added and concentrated sulphuric acid was added carefully. Red brown colour formation at the interface indicated the presence of terpenoids.

Test for triterpenoids: To 1.5 ml of test sample, 1 ml of Libemann–Buchard Reagent (acetic anhydride + concentrated sulphuric acid) was added and the formation of blue green colour indicated the presence of triterpenoids.

Test for phenols: To 1 ml of the test sample, 2 ml of distilled water was added followed by addition of few drops of 10 % ferric chloride (Fisher Scientific, India) and formation of green colour indicated the presence of phenols.

Test for coumarins: To 1 ml of test sample, 1ml of 10 % sodium hydroxide was added and the formation of yellow colour indicated the presence of coumarins.

Test for glycosides: To 2 ml of test sample, 3 ml of chloroform (Prolabo, France) and 10 % ammonia solution was added. Pink colour formation indicated the presence of glycosides.

RESULTS

Partitioning of the EEAM: The hexane fraction was a whitish power with the yield of 1.23 g from 10 g of the ethanol extract and the ethyl acetate fraction was a yellowish powder with the yield of 2.67 g from 10 g of the ethanol extract.

Comparison of the anti-inflammatory effect of the fractions in healthy rats: The anti-inflammatory effect of the fractions on carrageenan-induced paw oedema is summarized in the Table 1. The EAF exhibited maximum inhibition of 82.7 % at 2 h, while hexane fraction decreased paw oedema with the highest inhibition of 66.1 % at 5 h. The EAF showed the highest anti-inflammatory effect.

Comparison of the hypoglycaemic effect of the fractions in healthy rats: The EAF and HF showed a significant hypoglycaemic effect compared to the Control group. The mean serum glucose concentration of the Control, EAF and HF groups were 8.9 ± 0.03, 6.5 ± 0.9 and 8.4 ± 0.2 mmol/L respectively. The percentage reduction of serum glucose concentration of EAF was 26.9 % while it was 5.9 % for HF. The EAF showed the highest hypoglycaemic effect.

Table 1: Effect of the hexane and ethyl acetate (EAF) fractions on carrageenan induced rat paw oedema in healthy rats.

Treatment–(mg/kg)	Change in paw oedema (ml) ± SEM (Percent Inhibition; %)				
	1h	2h	3h	4h	5h
Distilled water	0.13±0.02	0.12±0.02	0.11±0.02	0.10±0.02	0.10±0.02
Indomethacin -10	0.05±0.02** (54.2)	0.02±0.02** (85.9)	0.03±0.02** (71.5)	0.05±0.02** (60.3)	0.06±0.03* (54.4)
HF- 100	0.12±0.01 (5.1)	0.08±0.01 (40)	0.06±0.01 (44.8)	0.04±0.01 (57.1)	0.03±0.01 (66.1)
EAF-100	0.10±0.01 (27.8)	0.02±0.02** (82.7)	0.03±0.01* (70.1)	0.04±0.01 (57.1)	0.05±0.01 (50.8)

(P<0.05*, P<0.01**) The ethyl acetate fraction showed the highest anti-inflammatory effect.

Gas Chromatography – Mass Spectrum analysis (GC-MS) of the fractions: Fifteen volatile compounds were identified in ethyl acetate fraction (Figure 1 and Table 2) and Alpha-Phellandrene was the most abundant compound. Limonene and p-cymene were also identified as main components.

Table 2: Volatile compounds identified from SPME/GC/MS of ethyl acetate fraction

Peak No.	Retention time	compound	% area
1	16.956	Benzene, 1-fluoro-4-methoxy	1.97
2	17.100	1,2-Dihydro-3-isopropyl-2-oxoquinoxaline	1.14
3	17.184	alpha-Phellandrene	14.26
4	17.306	3-hexan-2-one,3,4-dimethyl	3.46
5	17.404	2- Acetylcyclopentanone	1.32
6	17.754	2-Butanoic acid	1.45
7	17.828	Oxazolidine, 2,2-diethyl	3.57
8	17.860	2-heptanone, 3-methyl	7.49
9	17.921	limonene	10.99
10	17.990	1H-Pyrazole, 3-ethoxy-5-methyl	3.04
11	18.121	3-cyclopentene-1-ethanol, 2,2,4-trimetyl	4.9
12	18.234	p-cymene	10.73
13	18.432	3-Fluoro-o-anisidine	2.17
14	20.937	3-Cyclopentene -1- ethanol, 2,2,4-trimethyl	2.76
15	21.386	Cyclohexanol	1.56

The most abundant compound was alpha-phellandrene

Ten volatile compounds were identified in hexane fraction (Figure 2 and Table 3) and Eugenol was the most abundant compound. The 3-hexanol-4-methyl, Oxirane (2-methylbutyl) and Cyclopentanol-1-methyl were also found in high concentrations.

Table 3: Volatile compounds identified from SPME/GC/MS of hexane fraction

Peak No.	Retention time	compound	% area
1	7.504	1,4-pentadien-3-ol	12.17
2	7.650	3-hexanol, 4-methyl	28.75
3	8.637	Oxirane, (2-methylbutyl)	26.40
4	10.324	n-Hexadecanoic acid	5.78
5	11.249	Eugenol	32.68
6	12.189	Cyclohexane, 1-methylene-4-(methylethyl)	5.81
7	12.266	Oxirane, 2,2,-dimethyl-3-propyl	6.09
8	12.348	1,4-Pentadien-3-ol	6.24
9	12.991	Cyclopentanol, 3-methyl	6.53
10	17.576	Cyclopentanol, 1-methyl	28.15

The most abundant compound was Eugenol.

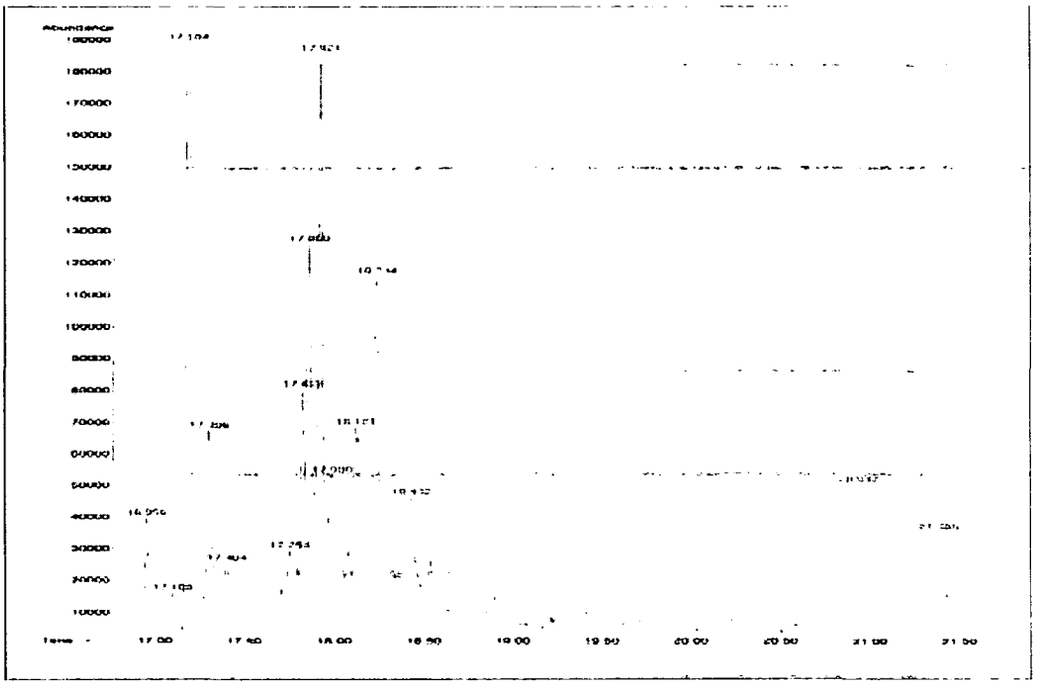

Fig. 1: The GCMS spectrum of the ethyl acetate fraction. Alpha-Phellandrene, limonene and p-cymene were present in high concentrations.

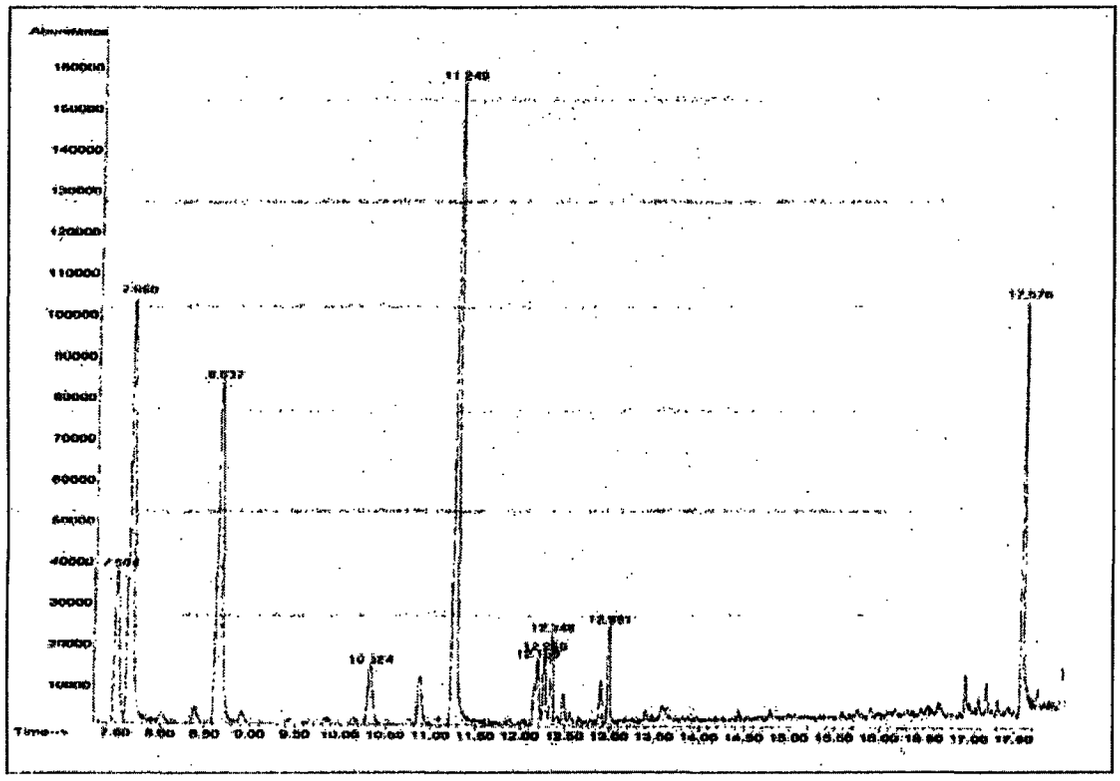

Fig. 2: The GCMS spectrum of the hexane fraction. Eugenol, 3-hexanol-4-methyl, Oxirane (2-methylbutyl) and Cyclopentanol-1-methyl were present in high concentrations.

Fractionation of ethyl acetate fraction by gel filtration chromatography: The fractions were pooled into 4 groups (fraction numbers; 1- 20, 21-36, 37-71, 72-100) and named as Fraction 1, 2, 3 and 4, with the yield of 186, 158, 178 and 170 mg for 1 g of the ethyl acetate fraction. The anti-inflammatory effect of the fractions on carrageenan-induced paw oedema is summarized in the Table 4. The Fraction 1 showed the maximum inhibition of 86.7 % at 2 h while the Fraction 2 exhibited the maximum inhibition of 72.3 % at 5 h. The highest percentage inhibition of Fraction 3 was 76.0 % at 3 h and Fraction 4 was 67.3 % at 4 h. Therefore the Fraction 1 showed the highest anti-inflammatory effect.

The each fraction showed a significant hypoglycaemic effect compared to the Control group. The mean serum glucose concentration of the Control group and the Test groups received Fraction 1, 2, 3, and 4 were 8.9 ± 0.1, 7.3 ± 0.2, 7.7 ± 0.2, 6.9 ± 0.1 and 7.9 ± 0.2 respectively. The percentage reduction of serum glucose concentration of the Fraction 1, 2, 3 and 4 were 18 %, 13.2%, 22.2% and 4.4 % respectively. Thus Fraction 3 showed the highest hypoglycaemic effect.

Table 4: Effect of the Fractions 1, 2, 3 and 4 on carrageenan induced rat paw oedema in healthy rats.

Treatment– (mg/kg)	Change in paw oedema (ml) ± SEM (Percent Inhibition; %)				
	1h	2h	3h	4h	5h

Distilled water	0.24±0.02	0.28±0.04	0.26±0.04	0.26±0.02	0.26±0.02
Indomethacin -10	0.12±0.02**	0.04±0.02**	0.09±0.02**	0.11±0.02**	0.13±0.03*
	(52.8)	(82.1)	(66.2)	(55.1)	(45.8)
Fraction 1-100	0.12±0.02*	0.04±0.01***	0.08±0.02**	0.09±0.03**	0.10±0.02***
	(52.7)	(86.7)	(71.5)	(65.4)	(59.4)
Fraction 2-100	0.21±0.02	0.14±0.03	0.13±0.02*	0.1±0.01**	0.07±0.01***
	(13.7)	(49.4)	(51.3)	(53.3)	(72.3)
Fraction 3-100	0.13±0.03*	0.11±0.03*	0.06±0.02**	0.10±0.01**	0.12±0.04*
	(45.9)	(60.2)	(75.94)	(63.5)	(54.2)
Fraction 4-100	0.20±0.03	0.16±0.02	0.10±0.02**	0.08±0.01***	0.12±0.01***
	(19.2)	(42.2)	(62.0)	(67.31)	(53.5)

(P<0.05*. P<0.01**) The Fraction 1 showed the highest anti-inflammatory effect.

Sub fractionation of the Fraction 1 by column chromatography: Fraction 1 was separated into 3 distinct fractions (fractions 1-20, 21-30, 31-55) which were named as sub fraction 7, 8 and 9. The sub fraction 7 was a slight yellow colour powder with a yield of 56 mg for 0.5 g of Fraction 1, while the sub fraction 8 and 9 were whitish power with the yield of 34 mg and 42 mg for 0.5 g of Fraction 1 respectively. The anti-inflammatory effect of the fractions on carrageenan-induced paw oedema is summarized in the Table 5. The sub fraction 7 showed the maximum inhibition of 67.4 % at 2 h while the sub fraction 8 and 9 exhibited the maximum inhibition of 36.8 % at 2 h and 53.1% at 4 h. The sub fraction 7 showed the highest anti-inflammatory effect.

Table 5: Effect of the Subfractions 7, 8 and 9 on carrageenan induced rat paw oedema in healthy rats.

Treatment– (mg/kg)	**Change in paw oedema (ml) ± SEM (Percent Inhibition; %)**				
	1h	**2h**	**3h**	**4h**	**5h**
Distilled water	0.15±0.03	0.16±0.01	0.15±0.01	0.14±0.01	0.12±0.02
Indomethacin -10	0.09±0.02**	0.03±0.02**	0.07±0.02**	0.05±0.02**	0.06±0.03*
	(55.6)	(86.4)	(71.9)	(64.3)	(53.8)
Subfraction 7- 50	0.13±0.01	0.05±0.02**	0.08±0.02*	0.08±0.02	0.08±0.02
	(11.1)	(67.4)	(51.1)	(40.8)	(31.4)
Subfraction 8- 50	0.14±0.03	0.10±0.03	0.11±0.03	0.10±0.03	0.10±0.03
	(8.9)	(36.7)	(28.3)	(25.9)	(14.3)
Subfraction 9- 50	0.12±0.03	0.12±0.03	0.10±0.03*	0.06±0.02*	0.06±0.02**
	(18.8)	(26.5)	(38.0)	(53.1)	(51.4)

(P<0.05*. P<0.01**) The Subfraction 7 showed the highest anti-inflammatory effect.

Sub fractionation of the Subfraction 7 by thin layer chromatography: The subfraction 7 was separated into 3 distinct bands named as Band 1, 2 and 3 and the Rf values were 0.2, 0.61 and 0.79 respectively. Band 1, 2, and 3 were whitish powder with the yield of 1.3, 2.3 and 2.1 mg per 25 mg of Subfraction 7.

The anti-inflammatory effect of the Bands on carrageenan-induced paw oedema is summarized in the Table 6. The Band 3 showed the highest anti-inflammatory effect by exhibiting the maximum inhibition of 54 % at 2 h. The Band 1 and 2 exhibited the maximum inhibition of 45.5 % at 5 h and 30.9 % at 2 h respectively. The FTIR spectrum exhibited the presence of O-H/N-H/C=O/C-Cl and C=C groups in Band 3 and the details of IR spectrum are presented in Table 7 and Figure 3.

Table 6: Effect of the Band 1, 2 and 3 on carrageenan induced rat paw oedema in healthy rats.

Treatment– (mg/kg)	**Change in paw oedema (ml) ± SEM (Percent Inhibition; %)**				
	1h	**2h**	**3h**	**4h**	**5h**
Distilled water	0.24±0.02	0.25±0.03	0.26±0.04	0.24±0.04	0.24±0.03
Indomethacin -10	0.1±0.02** (51.9)	0.08±0.02** (79.8)	0.1±0.02** (65.7)	0.11±0.02** (58.4)	0.13±0.03* (47.2)
Band 1- 25	0.22±0.01 (8.5)	0.18±0.02 (27.0)	0.17±0.01 (32.7)	0.14±0.01* (41.8)	0.13±0.02* (45.5)
Band 2- 25	0.19±0.03 (18.3)	0.18±0.01 (30.9)	0.19±0.03 (26.8)	0.19±0.04 (21.2)	0.19±0.03 (21.0)
Band 3- 25	0.17±0.03 (27.5)	0.12±0.02* (54.0)	0.14±0.02* (47.1)	0.16±0.02 (36.3)	0.17±0.02 (27.3)

(P<0.05*, P<0.01**) The Band 3 showed the highest anti-inflammatory effect

Table 7: The detailed analysis of FTIR spectrum of Band 3

Wave number (cm^{-1})	Functional group
3440.79	O-H stretch
2922.60	C-H stretch
2851.49	C-H stretch
1742.00	C=O stretch
1633.53	N-H Bend
1464.03	CH_2 bend
1377.90	C-H in a plane bend
1161.22	C-O stretch
1113.98	C-O stretch
668.59	C-CL stretch

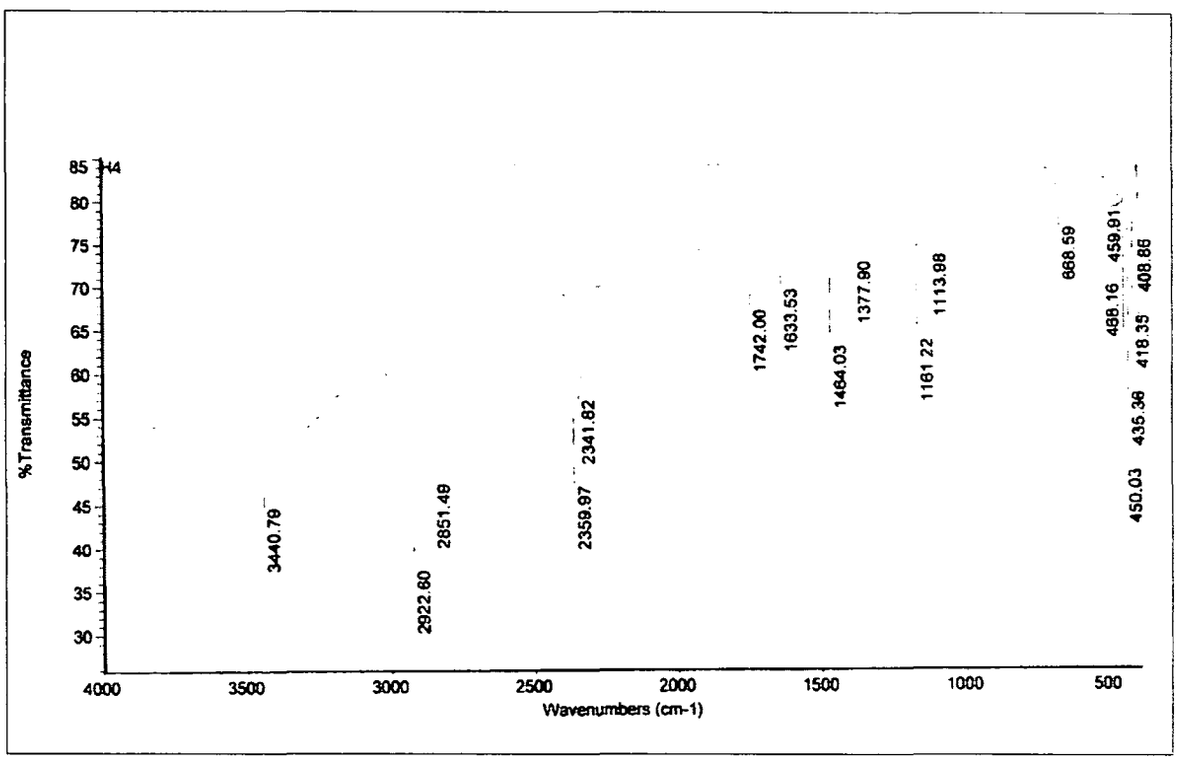

Fig 3: The FTIR spectrum of Band 3

Fractionation of the Fraction 3 by thin layer chromatography: The Fraction 3 was separated into 2 distinct bands named as Band 4 and 5 and the Rf values were 0.23 and 0.41 respectively. Band 4 and 5 were whitish powders with the yield of 1.7 and 1.4 mg per 25 mg of Fraction 3. Each fraction showed a significant hypoglycaemic effect compared to the Control group. The mean serum glucose concentration of the Control group and the Test groups received Band 4 and 5 were 8.7 ± 0.2, 7.2 ± 0.1 and 7.9 ± 0.1 respectively. The percentage reduction of serum glucose concentration of the Band 4 and 5 were 17.2 % and 9.2 %. Thus Band 4 showed the highest hypoglycaemic effect. The FTIR spectrum exhibited the presence of O-H/N-H/C=O and C=C groups in Band 4 and the details of the IR spectrum are presented in table 8 and Figure 4.

Table 8: The detailed analysis of FTIR spectrum of Band 4

Wave number (cm-1)	Functional group
3395.25	OH or NH stretch
1711.69	C=O stretching
1646.31	C=O stretching,
1557.01	N-H Bend
1531.90	N-H Bend
1503.29	N-H Bend

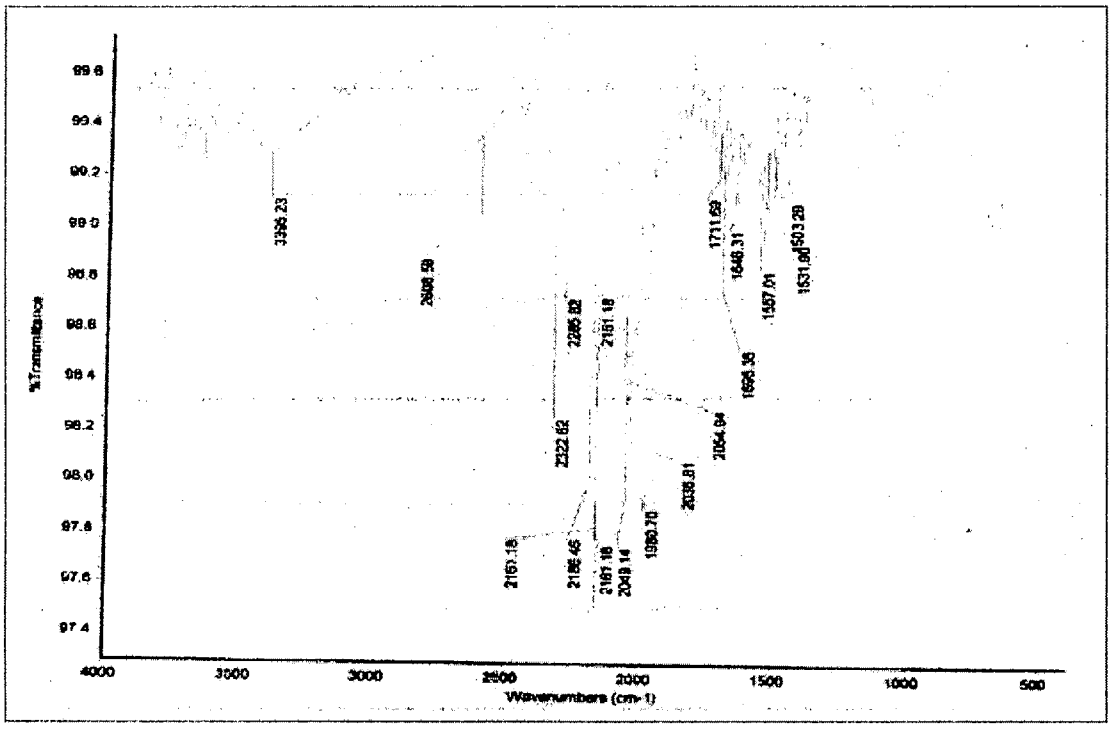

Fig. 4: The FTIR spectrum of Band 4

Phytochemical screening of fractions: The results of qualitative chemical test of fractions and TLC bands are presented in the Table 9 and 10. The Band 3 contained triterpeniods while the Band 4 contained flavonoids and coumarins.

Table 9: The phytochemical analysis of fractions of ethanolic extract of dried flowers of *A. marmelos.*

Test	Ethanolic extract	EAF	HF	Fraction 1	Fraction 2	Fraction 3	Fraction 4
Tannins	+	+	-	+	+	+	_
Saponins	+	_	+	_	_	_	_
Flavonoids	+	+	-	+	+	+	-
Alkaloids	+	-	+	-	-	-	-
Anthocyanin	+	+	-	+	-	-	-
Quinones	+	+	-	-	-	-	+
Terpenoids	+	+	-	+	+	+	+
Triterpenoids	+	+	-	+	+	-	-
Phenols	+	+	-	+	+	+	+
Coumarins	+	+	-	+	-	+	-
Glycosides	-	-	-	-	-	-	-

Table 10: The phytochemical analysis of fractions and TLC bands of ethanolic extract of dried flowers of *A. marmelos*.

Test	Fraction 7	Fraction 8	Fraction 9	Band 1	Band 2	Band 3	Band 4	Band 5
Tannins	+	+	-	-	+	-	-	-
Saponins	-	-	-	-	-	-	-	-
Flavonoids	+	+	-	-	-	-	+	-
Alkaloids	-	-	-	-	-	-	-	-
Anthocyanin	-	-	+	-	-	-	-	-
Quinones	-	-	-	-	-	-	-	-
Terpenoids	+	+	+	-	-	+	-	-
Triterpenoids	+	-	+	-	-	+	-	-
Phenols	+	+	+	+	+	+	+	-
Coumarins	+	-	-	+	-	-	+	-
Glycosides	-	-	-	-	-	-	-	-

DISCUSSION

A variety of techniques can be used to determine and estimate the presence of such phyto-constituents in medicinal plants. Chromatography and spectroscopic techniques are the most useful and popular tools used for this purpose. Using these techniques, an activity guided fractionation of the ethanol extract of dried flowers of *A. marmelos* was carried out. It was found that the hypoglycaemic active fraction contained a mixture of flavonoids and coumarins and the anti-inflammatory active fraction contained triterpeniods. These phytochemicals might be responsible for the hypoglycaemic and anti-inflammatory effect of the test extract and further purification and identification of structures is required to verify these observations.

Triterpenoids are ubiquitously presented in variety of ethnomedicinal plants and *A. marmelos* contain triterpenoids such as lupeol, β and γ-sitosterol[8]. Triterpenoids comprise one of the most interesting groups of natural products due to their diverse pharmacological activities such as immunomodulatory, anticancer, anti-inflammatory, anti-anxiety, antidepressant, memory enhancer, antinociceptive, neuroprotective and other CNS actions[9]. *In vitro* as well as *in vivo* studies done on Lupeol revealed that it is a potent anti-inflammatory, anti-noceptive and anti-cancer agent[10] and Nirmal *et al*[11] reported β-sitosterol isolated from *Nyctanthes arbortristis* leaves showed a significant analgesic and anti-inflammatory activity in various animal models.

The richest sources of coumarins are the plants belonging to family Rutaceae and Umbelliferae. Although coumarins are distributed throughout all parts of a plant, they occur at the highest concentration in the fruits, followed by the roots, stems and leaves[12]. *Aegle marmelos* is reported to contain a number of coumarins such as aegelinol, marmesins, psoralen, xanthotoxin, mermin, skimmianine, imperatorin, scopoletin, umbelliferone and alloimperatorin[13]. Coumarin and coumarin-related compounds have proved to exhibit multiple biological activities including anti-hyperglycaemic, anti-HIV, anti-tumour, anti-

hypertension, anti-arrhythmia, anti-inflammatory, anti-osteoporosis, antiseptic, anti-oxidant and analgesic[14,15]. Pari and Rajarajeswari[16] reported that coumarin exterts a significant antidiabetic effect through the stimulation of the insulin secretion from existing beta cells and alterations in the metabolism of glucose in liver of streptozotocin induced diabetic rats.

Photochemical analysis of various parts of the *A. marmelos* had shown to contain flavonoids such as rutin[8]. Flavonoids are polyphenols that have been reported to exert wide range of biological activities including anti-oxidant, anti-inflammatory, anti-septic, anti-allergic and anti-tumor[17]. Previous studies have demonstrated the hypoglycemic effects of flavonoids using different experimental models and flavonoids can enhance insulin secretion or act as insulin[5,18]. Some other studies have been found flavonoids stimulate glucose uptake in peripheral tissues and regulate the activity and/or expression of the rate-limiting enzymes involved in carbohydrate metabolism pathway[19]. Another study that was undertaken in normal rats for hypoglycaemic effect of flavonoid compounds: boswellic acid, ellagic acid, quercetin and rutin revealed that rutin has more activity when compared to other three flavonoids and these compounds increase glucose uptake by rat hemi-diaphragm[20]. These suggest that flavonoids may be responsible for the hypoglycaemic effect of the test extract and possible mechanism of action could be enhancing peripheral glucose utilization either by direct stimulation of glucose uptake or via the mediation of enhanced insulin secretion.

In the present study the major volatile compounds that have been identified in ethyl acetate and hexane fraction were α-phellandrene and eugenol respectively. Other than that limonene, p-cymene, 3-hexanol-4 metyl, oxirane and cyclopentanol-1-methyl were found in high concentrations. These are monoterpenes and sesquiterpenes which are important contributors to odour of the aromatic parts of the plants.

The analysis of the essential oil obtained from the leaves of *A. marmelos* revealed the major components of oil were limonene, α-phellandrene, β-ocimene and germacrene B[21,22]. Charoensiddhi and Anprung[23] recognized limonene, p-cymene, β-phellandrene and dihydro-β-ionone as the main volatile compounds of the fruit pulp which are important contributors to fruit aroma.

Alpha-phellandrene is a monoterpene and exhibits many biological properties such as analgesic, anti-inflammatory, antinociceptive and a potent anti-leukemic effect[24,25]. Limonene is also a monoterpene that possess potent antioxidant activity and anti-proliferative action[26,27].

Eugenol is one such naturally occurring phenolic compound with potent anti-oxidant effect and it is sometimes called clove oil because it is the active element in cloves which causes the aromatic smell typical of cloves[28]. It is used as antiseptic, analgesic and anti-bacterial agent in traditional medicine in Asia and in dentistry as main ingredient of cavity filling cement. It also makes a good local anaesthetic for temporary relief from toothache pain[29].

The different phyto-constituents in medicinal plants are associated with different pharmacological properties. Therefore purification of the phyto-constituents leads to discover new drugs. The activity guided fractionation of the dried flower extract, revealed there are bioactive compounds with potent anti-inflammatory and hypoglycaemic activity and by further purification and structure elucidation might discover new drugs to treat anti-inflammatory diseases and diabetes mellitus[30].

CONCLUSIONS

The activity guided fractionation of the ethanol extract of the dried flowers revealed the hypoglycaemic active fraction contained a mixture of flavonoids and coumarins and anti-inflammatory active fraction contain triterpenoids.

ACKNOWLEDGEMENTS

We would like to University of Sri Jayewardenepura for providing the financial support through the grants ASP/R/06/2010 and WCUP/PHD/03/12.

REFERENCES

1. P. C. Sharma, V. Bhatia, N. Bansal and A. Sharma, *Nat Prod Rad*, 2007, 6(2). 171-178.
2. D. Gupta, P. P. John, K. Pankaj, R. Kaushik and Yadav, *Int. J. Pharm. Sci. Res.*, 2011, 2 (8), 2031-2036.
3. G. N. Sharma, S. K. Dubey, P. Sharma and N. Sati, *Int. J. Curr. Pharm. Rev. Res.*, 2011, 2(1), 13-22.
4. S. Dhankhar, S. Ruhil, M. Balhara, S. Dhankhar and A. K. Chhillar, *J. Med. Plant. Res.*, 2011, 5, 1497-1507.
5. R. Patel, G. Dipak, C. Manodeep and J. V. Kana, *Int. J. Res. Ayurveda Pharm.*, 2012, 3(2), 159-163.
6. A. Buadonpri, W. Wichititthad, P. Rojsitthisak and P. Towiwat, *J. Health Res.*, 2009, **23(1)**, 11-16.
7. F. E. Amrani, A. Rhallab, T. Alaoui, K. E. Badaoui and S. Chaki, *J. Med. Plants Res.*, 2009, 3 (9), 625-629.
8. M. L. Dhar, M. M. Dhar, B. N. Dhavan, B. N. Mehrotra and C. Ray, *Indian J. exp. Biol.*, 1968, 6, 232.
9. N. P. Yadav and C.S. Chanotia, *Pharm. Rev.*, 2009, 114-150.
10. S. B. Mahato and S. Sen, *Phytochem.* 1997, 44(7), 1185-1236.
11. M. Salee, *Cancer Lett.*, 2009, 285(2), 109–115.
12. S. A. Nirmal, S. C. Pal, S. C. Mandal and A. N. Patil, *Inflammopharmacol*, 2012, 20(4), 219-224.
13. A. Lacy and R. O'Kennedy, *Curr. Pharm. Des.*, 2004, 10, 3797-3811.
14. V. B. Lambole, K. Murti, U. Kumar, P. B. Sandipkumar and V. Gajera, *Int. J. Pharma. Sci. Rev. Res.*, 2010, 5(2), 67-72.
15. N. S. Dighe, S. R. Pattan, S. S. Dengale, D. S. Musmade, M. Shelar, V. Tambe and M. B. Hole, *Arch. Appl. Sci. Res.*, 2010, 2(2), 65-71.
16. P. K. Jain and H. Josh, *J. Appl. Pharma. Sci.*, 2012, 2(6), 236-240.
17. Pari and Rajarajeswari, *Chem. Biol. Interact.*, 2009, 181(3), 292-296.
18. H. K. Sandhar, B. Kumar, S. Prasher, P. Tiwari and M. Salhan, *Inte. Pharm. Sci.*, 2011, 1(1), 25-41.

19. M. Pinent, A. Castell, I. Baiges, G. Montagut, L. Arola and A. Ardevol, *Compr. Rev. Food Sci. F.*, 2008, 7(4), 299-308.
20. S. A. Hussain and B. H. Marouf, *Academia J. Med. Plants*, 2013, 1(2), 031-036.
21. R. Jadhav and G. Puchchakayala, *Int. J. Pharm. Sci.*, 2012, 4(2), 251-256.
22. P. Satyal, K. E. Woods, N. S. Dosoky, S. Neupane and W. N. Setzer, *J. Med. Act. Plants.*, 2012, 1(3), 114-122.
23. R. S. Verma, R. C. Padalia and A. Chauhan, *J. Sci. Food Agr.*, 2013, doi: 10.1002/jsfa.6510.
24. S. Charoensiddhi and P. Anprung, *Int. Food Res. J.*, 2008, 15(3), 287-295.
25. J. J. Lin, *J. Cell Sci. Ther.*, 2012, 3(8), 89.
26. D. F. Lima, M. S. Brandão, J. B. Moura, J. M. Leitão, F. A. Carvalho, L. M. Miúra, J. R. Leite, D. P. Sousa and F. R. Almeida, *J. Pharm. Pharmacol.*, 2012, **64(2)**, 283-292.
27. J. Sun, *Altern. Med. Rev.*, 2007, 12(3), 259-26.
28. A. Djilani and A. Dicko, 2012; http://www.intechopen.com/books/nutrition-well-being-and-health/the-therapeutic-benefits-of-essential-oils accessed on 19/03/2014
29. M. H. Alma, M. Ertas, S. Nitz and H. Kollmannsberger, *Bioresour.*, 2007, 2(2), 265-269.
30. M. Pisano, G. Pagnan, M. Loi, M. E. Mura, M. G. Tilocca, G. Palmieri, D. Fabbri, M. A. Dettori, G. Delogu, M. Ponzoni and C. Rozzo, *Mol. Cancer.*, 2007, 6, 8.

***Corresponding Author: K. D. K. P. Kumari;**

Department of Basic Sciences, Faculty of Allied Health Sciences, General Sir John Kotelawala Defence University, Ratmalana, Sri Lanka.