e - ISSN - 2249-7722
Print ISSN - 2249-7730

International Journal of Phytotherapy

www.phytotherapyjournal.com

RP-HPLC ANALYSIS AND ANTIMICROBIAL SCREENING OF *CLAUSENA EXCAVATA* BURM. F. (RUTACEAE)

C Guntupalli[1*], M Ramaiah[2], GS Kumar[3]

[1]Department of Pharmacognosy, Mother Teresa Pharmacy College,
Kothuru, Sathupally, Khammam Dist-507303, A.P, India.
[2]Department of Phytochemistry & Pharmacognosy, Hindu College of Pharmacy,
Amaravathi Road, Guntur – 522002, A.P., India
[3]Department of Pharmaceutical chemistry, Gitam Institute of Pharmacy, Gitam University,
Gandhinagar Campus, Rushikonda Visakhapatnam-530045, A.P., India.

ABSTRACT

Clausena excavata Burm. F.(Rutaceae) has long been associated with medicinal benefits in folk medicine, particularly in the treatment of cancer and its related disorders in the eastern region of Thailand. The present study evaluates the antimicrobial and antifungal activity of petroleum ether, chloroform, methanol and ethanol and aqueous extracts of *C. excavate* against various microorganisms. In addition to that analytical reversed phase high performance liquid chromatography (HPLC) of the crude methanolic leaf extracts of *C. excavata* was carried out in order to qualitatively assess the number of constituents present in each fraction. Separation was achieved by using ACE-5-C18 (250 x 4.6 mm) with a flow rate of 1.5 ml/min, with the UV detection at 254 nm. The tests were made using *Staphylococcus aureus* and *Staphylococcus pyogens* and dermatophytes; *Trichophyton rubrum* and *Trichophyton mentagrophytes*, *Epidermatophyton floccusum* and *Candida albicans* by using an agar incorporation technique. The antifungal activity (MIC values) of methanolic extract of *C.excavata* against against *T.rubrum* and *T. mentagrophytes* was found to be 62.5 µg/ml and 31.2 µg/ml. The crude extracts showed no significant activity against *S. aureus* and *S. pyogens* when compared with that of the standard drugs (ciprofloxacin and gentamycin, <3.91 µg/ml). The antifungal activity of the methanolic leaf extract of *C. excavata* was comparable with the standard drug, thus confirming that it might be a good source of antifungal. Following the extensive chromatographic separation techniques major components from the methanolic leaf extract of *C.excavata* were separated and chemically to be confirmed by spectroscopic methods.

Key words: Crude extract, Anti-microbial, Antifungal, RP-HPLC.

INTRODUCTION

Ethnobotanical screening of species of the Rutaceae family in several continents has shown that they have important medicinal properties. *Clausena excavata* is a wild shrub of the Rutaceae family that is distributed in South Asia. It is also cultivated in some areas and grows up to 1.5 m high. It has been used in folk medicine for the treatment of cancer and several disorders in the eastern parts of Thailand [1- 4]. Its leaves and stem are also used to treat colic, cough, headache [3], rhinitis, sore, wounds, and yaws, and it is used for detoxification in some countries [3]. The leaves are particularly used in [5], dysentery, and antiplatelet aggregation [6]. The plant

Corresponding Author:- **Dr. Chakravarthi Guntupalli** Email: chakra_varthi123@rediffmail.com

is also reported to have insecticidal, tonic, and vermifugal properties [6]. *C. excavata* has been reported to contain carbozole alkaloids, coumarins, limnoids, [4] etc. The sap of the leaves is applied on the affected area to treat all kinds of muscular pain [3]. A decoction of the root is given to patients with malaria [5]; an infusion of the stem is given in patients with colic. The leaves and stem bark are also used locally as a diuretic, tonic, astringent, and antinociceptive agent [3]. The coumarins derived from the leaves have been found to have inhibitory effects on tumor progression [7-8].

Several pharmacologically active constituents of plants belonging to the family Rutaceae have been identified-phenylpropanoids [9], methoxyflavones [10], furoquinoline alkaloids [11], coumarins [12], and shown to play a part in the development and pathophysiology of neurodegenerative disorders (e.g., Alzheimer disease, Parkinson disease, multiple sclerosis, and Down syndrome), inflammation, viral infections, autoimmune pathologies, and digestive system disorders such as gastrointestinal inflammation acridone alkaloids [13]. However, the chemical components of species of this family are yet to be fully elucidated. We chose to study the phytochemical properties of extracts of *C. excavata* because it is known to have antioxidant properties. Cellular damage arising from reactive oxygen species (ROS) has been and ulcer [14]. We therefore focused our investigation on the *invitro* anti-microbial and anti-fungal activities of extracts. Often in crude plant extracts, the compounds of interest are not present in very high concentrations; HPLC has been by far the most useful tool for the separation of complex mixtures of components present in crude plant extracts. Reversed phase HPLC on octadecylsilane (ODS or c18) has been recognized as the most common applicable of bonded phases for this purpose [15]**.** For the past twenty years High Performance Liquid Chromatography (HPLC) and Nuclear Magnetic Resonance Spectroscopy (NMR) have been the primary tools used by natural product chemist for the isolation and identification of the compounds [16-19]. Reversed phase HPLC (RP-HPLC) consists of a non-polar stationary phase and an aqueous, moderately polar mobile phase. RP-HPLC allows polar components elute first whereas retention time is quite longer for molecules which are non-polar in nature.

The aim of the present investigation was to separate, and identify the major chemical constituents of *C. excavata* using analytical reversed phase high performance liquid chromatography followed by semi-preparative and preparative HPLC modes for isolation of compounds and further explore the antimicrobial and antifungal potential of the extracts and isolated constituents.

MATERIALS AND METHODS

Plant material

Leaves of *C. excavata* were collected from the northern regions of Malaya in June-July 2005, and their identity was was further authenticated by Bowen, from the Department of Pharmacognosy, University of Sunderland, and a voucher specimen sample was deposited in the herbarium. The collected plant materal was dried well under shade and powdered using electric blender.

Crude extract preparation

Soxhlet extraction

The powdered leaf material (100 g) was successively extracted with petroleum ether, hexane, chloroform, dichloromethane, ethanol, and water using Soxhlet extractor. The solvents were partly removed *in vacuo* below 50° C and freeze-dried to give extracts. The solvents used were hexane, petroleum ether, chloroform, dichloromethane, methanol, ethanol and water.

Test microorganisms used

The strains of bacteria and yeast used were obtained from the Microbial Type Culture Collection (MTCC), Sunderland Royal hospital, University of Sunderland. All the organisms used were pure cultures, preserved as stab cultures at a temperature of 4^0 C. We tested two Gram-positive bacteria Staphylococcus *aureus* and *Streptococcus pyogens* and four yeast cultures, *Candida albicans*, *Trichophyton rubrum*,*Trichophyton mentagrophytes* and *Epidermatophyton floccusum.* The tests were performed in triplicates for each microorganism evaluated.

The following reagents, equipment and organisms were used for anti-microbial assay. Disposable 30 ml plastic universal containers were used, sterile petri dishes, purified water (pH 7), dimethyl sulphoxide (GC) 99+% (Sigma-Aldrich EEC Number 200-664-3). Batch no 0041018 was used as a solvent control, a vortex mixer was used for the homogenization of extract solution, automatic multichannel pipettes Eppendorff, sterile tips 200 µl, sterile plastic 5 ml and 10 ml pipettes, Nutrient Agar (plates) and nutrient broth (liquid culture) were used for the growth of the inoculum, Gentamicin 40 mg/ml DBL Mayne Pharma, Warwickshire U.K. Exp. June 07 Code 2732B, batch no. P012732 was used as a standard drug. Griseofulvin (97 %) Acros Organics, 16084/3, C.A. S. 126-07-8 was used as standard drug.

ANTIMICROBIAL ACTIVITY

The antimicrobial activity of extracts was carried out using the agar diffusion method [20]. A suspension with 0.1 ml of the test microorganism (0.1 ml of 1.5 × 10 8 CFU ml-1) was spread on the surface of the Müeller Hinton Agar solid media (MHA) for bacteria and Sabouraud Dextrose Agar for the fungi in Petri dishes (15 ×90 mm) by spread plate method. This test was performed

on sterile 6 mm filter paper disks as recommended by the National Committee for Clinical Laboratory Standard [21-22]. An aliquot of each extract (100 mg/ml) was sterilized by 0.22 µm membrane filtration (TPP), and the filter paper disks were then impregnated with an aliquot of 5 µl. Afterward, the disk impregnated with extracts was placed on the plates inoculated with the test microorganisms. The plates were incubated at 37°C for 24 h for bacteria and 30°C for 48 h for fungi.

ANTIFUNGAL ACTIVITY

The antifungal activity was tested by disc diffusion method [23]. The potato dextrose agar plates were inoculated with each fungal culture (10 days old) by point inoculation. The filter paper discs (5 mm in diameter) impregnated with 100 µg mlG1 concentrations of the extracts were placed on test organism-seeded plates. Methanol was used to dissolve the extract and was completely evaporated before application on test organism-seeded plates. Blank disc impregnated with solvent methanol followed by drying off was used as negative control. The activity was determined after 72 h of incubation at 28°C.

Determination of minimum inhibitory concentration (MIC)

The MIC values were determined by Two-fold Broth Micro dilution Method Serial two-fold dilutions of the sample was made in Mueller–Hinton Broth containing 0.5 % Tween 80 for the bacteria and Sabouraud Dextrose Broth with 0.5 % Tween 80 for the fungi in 96-well micro titer plates. Fresh microbial suspensions prepared from overnight-grown cultures in the same media were added to give a final concentration of 5×105 organism's ml-1. Controls of medium with microorganisms or sample alone were included. The microplates were incubated at 37 °C for 24 h for the bacteria and 30 °C for 48 h for the fungi. The first dilution with no microbial growth was recorded as the MIC.

HPLC Studies on *Clausena excavata extract*

Instrumentation

The HPLC system used consisted of a Shimadzu LC-10 AD pump, Shimadzu SPD-6AV variable wavelength UV/ Vis detector, and using low dead volume connections, and a A1-450 Dionex integrator (all supplied by Dyson Instruments, Hetton-le-Hole, Tyne & Wear, UK). Injections were manually using a Rheodyne 7125-injection valve (Anachem, Luton, Beds., UK) fitted with a 20 µl, 50 µl, 1 ml and 2 ml loops for analytical, semi-preparative and preparative scale up, respectively.

Materials

ACE-5-C18 (250×4.6mm I.D.), ACE-5-C 18(250×7mm I.D.), ACE-5-C 18(250×22 mm I.D.) Luna NH_2 column (250mm × 4.6 mm) supplied by Hichrom Limited,(Reading, Berkshire, UK) were used during course of the study. Mobile phases were prepared by using HPLC-grade methanol, HPLC far-UV grade (Sigma-Aldrich, Poole, and Dorset UK). Water was distilled and doubly de-ionized using and Elgostat option 3 (Vivendi Water Systems, High Wycombe, Bucks., UK), [Methanol-water (90:10, v/v,)] with 0.002 M ammonium formate was used as mobile phase.

RESULTS

The data for antibacterial and antifungal activities for the crude extracts of *Clausena excavata* [Table 1] against different Standard drugs [Table 2] are shown in tables. The antimicrobial activities assay was performed for the crude extract against two strains of bacteria and four strains of yeast.

Table 1. MIC values for three Rutaceous crude extracts by agar dilution method

Plant	Solvent for extraction	MIC values in µg/ml of microorganisms						
		C.a*	P.a*	S.a*	S.p*	T.r*	T.m*	E.f*
Clausena excavate Leaves	Hexane	500	250	250	250	500	500	500
	petroleum ether	250	1000	1000	500	125	250	250
	chloroform	1000	1000	125	250	1000	>1000	1000
	methanol	250	1000	1000	500	62.5	62.5	250
	ethanol	500	1000	1000	500	<500	<500	500
	Aqueous	500	1000	1000	500	<500	<500	500
	hexane	1000	>1000	500	500	500	1000	1000
	CE1 (Fraction)	1000	250	250	250	125	62.5	>1000
	CE2 (Fraction)	>1000	1000	1000	500	>1000	>1000	>2000
	CE3 (Fraction)	>1000	1000	125	250	>1000	>1000	>1000
	CE4 (Fraction)	>1000	1000	1000	500	125	125	1000
	CE5 (Fraction)	>1000	1000	1000	500	>1000	>1000	500
	CE6 (Fraction)	500	1000	1000	500	>1000	500	500

C. a - Candida albicans; P. a* - Propionibacterium acne; S. a* - Staphylococcus aureus, S. p* -Streptococcus pyogenes; T. r* - Trichophyton rubrum*; *T. m* - Trichophyton mentagrophytes*; *E. f* - Epidermatophyton floccusum*

Table 2. MIC values of standard drugs, by agar dilution against selected microorganisms

Standards	microorganisms MIC in µg/ml						
	t.r*	***T.m****	***E.f****	***C.a****	***S.a****	***S.p****	***P.a****
Ketaconazole	0.04	5	10	<3.91	-	-	-
Miconazole	0.08	0.63	1.25	-	-	-	-
Griseofulvin	1.25	20	20	-	-	-	-
amphotericin b	2.5	5	5	-	-	-	-
Ciclopirox	5	5	5	-	-	-	-
Ciclopirox olamine	5	10	5	-	-	-	-
Ciprofloxacin	-	-	-	-	<3.91	<3.91	-
Gentamycin	-	-	-	-	<3.91	<3.91	-
Erythromycin	-	-	-	-	-	-	0.0625

T. r - Trichophyton rubrum*; *T.m*-Trichophyton mentagrophytes*;
E. f - Epidermatophyton floccusum;* *S. a* - Staphylococcus aureus*;
S. p -Streptococcus pyogens;* *C. a* - Candida albicans*

Figure 1. Analytical reversed phase HPLC profile of methanolic leaf extract of *Clausena excavata* with a mobile phase of methanol-water (20:80, v/v) C_{18} 250×4.6 mm; conc 1. 5 mg/ml

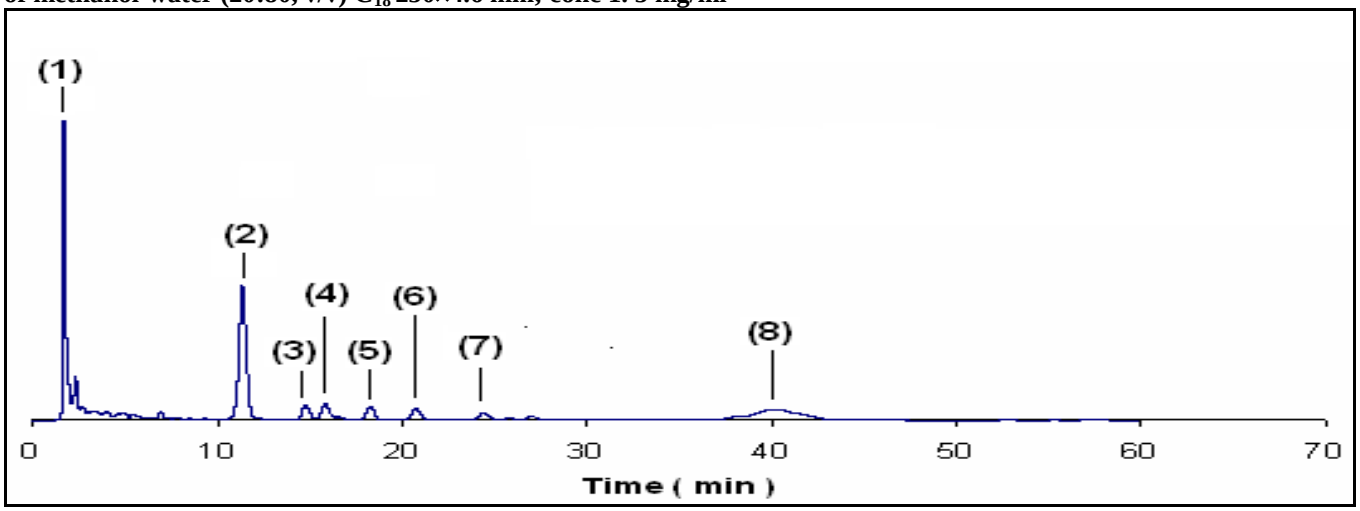
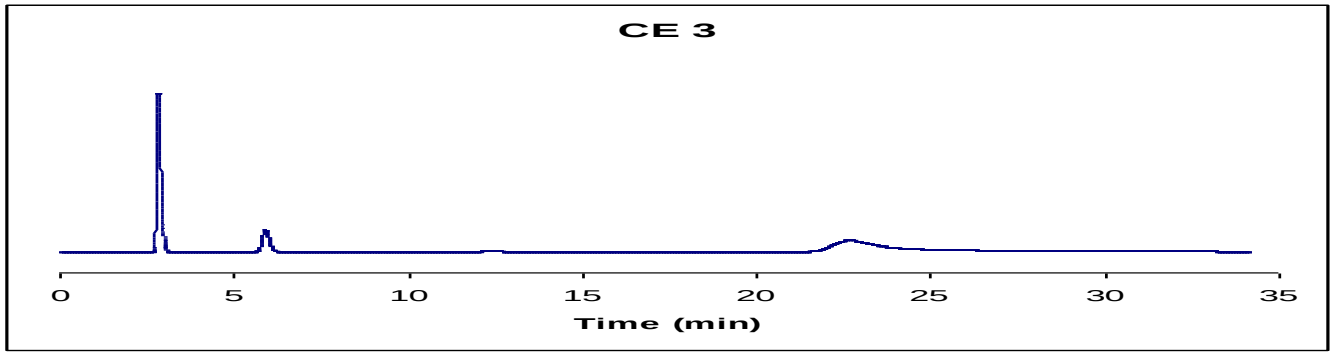


Fig 2. HPLC profile of semi purified fraction (CE 3) of *Clausena excavata* with a mobile phase of methanol-water (90:10, v/v) with 0.002 M ammonium formate LUNA NH2 C18 (250 X 4.6 mm) sample concentration 0.5 mg/ml, injection volume 20 µl , flow rate: 1ml/min; λ_{max} 254 nm



HPLC analysis of *C. excavata*

The samples of *C.* excavata chromatographic separations were carried out on an analytical scale. From the analytical method developed (Figure 1), the retained peaks were collected using the preparative column (250 x 22 mm i.d.) and also using the same conditions with the scaled up parameters (sample concentration 10 mg/ml, injection volume 10 ml and flow rate 9.5 ml/min).

Experimental Parameters

Analytical reversed phase HPLC profile (Figure 1.) of methanolic leaf extract of *Clausena excavata* was carried out using the following Experimental Parameters. Sample preparation was done with methanol-water (20:80, v/v) with a concentration of the sample: 1.5 mg/ml, using isocratic method. Mobile Phase used was methanol-water (20:80, v/v) with 0.1% Tri Fluoro Acetic acid, with flow rate of 1.5 ml/min at a wavelength of 245 nm and the total Analysis run time for the above extract 70 min. The chromatograms show the purity of the fractions and by comparison with standard flavonoids (Rutin and Chrysin) it appears that all fractions contain neutral compounds or those with a single phenolic group (t_r ~ 3 min) and the compounds with phenolic groups (CE 5). CE 5 probably contains one more phenolic group than other fractions. The longer retained peaks in CE3 (Figure 2) and CE 5 almost certainly contain compounds with carboxylic acid groups.

DISCUSSION

A total of six different solvent plant extracts and six semi-purified fractions of *C. excavata* were tested for *in-vitro* efficacy, using an agar incorporation method to determine the minimum inhibition concentration (MIC), against the *streptococcus aureus* and *staphylococcus pyogens* and dermatophyte species; *T.rubrum*, *T.mentagrophytes*, *E. floccusum* and *C. albicans*. The results are tabulated in table 1. The MIC value for crude methanolic extracts o was found to be 62.5 µg/ml and 31.2 µg/ml against *T.rubrum* and *T.mentagrophytes*, whereas other extracts *did* not show any activity against dermatophytes and two bacterial strains the isolated fractions CE1 and CE4 also displayed significant activity against *T.rubrum* and *T.mentagrophytes.* Based on the results and literature reports, it may be possible that the ant-fungal activity of may be due to the presence of compounds in these fractions. In order to support the above, chromatographic separations were carried out on methanolic extract of *C. excavata* an analytical scale. From the analytical method developed (Figure 1), the retained peaks were collected using the preparative column (250 x 22 mm i.d.) and also using the same conditions with the scaled up parameters (sample concentration 10 mg/ml, injection volume 10 ml and flow rate 9.5 ml/min).

The analytical reversed phase high performance liquid chromatography of the methanolic crude extract as carried out in order to determine number of constituents present in the extract. The reversed phase HPLC of the crude extracts was carried out using a ACE-5-C18 column (250mm × 7 mm) at room temperature, using the mobile phase [methanol-water (70:30 %, v/v, with 0.1 % TFA)] at a flow rate of 1.5 ml/min, with the inject on volume of 20 µl. The detection UV trace measured at 245 nm. Sample was prepared in methanol-water (20:80, v/v), concentration of the sample used: 1.5mg/ml, method: isocratic and the analysis stop time were 60 minutes. The fractions exhibiting peaks at similar retention times were combined to isolate the pure compounds by preparative methods and the fractions were collected evaporated and subjected to the spectral analysis. Flavanoids have been reported to possess a variety of pharmacological activities [25] including analgesic, anti-inflammatory, antimicrobial, anti-histaminic, anti-cancer and anti-diabetic. These reports gave further encouragement to proceed to biological testing of the extracts, with emphasis to be given on anti-oxidant activity [26-29] and anti-microbial evaluations.

CONCLUSION

The main general conclusion that can be made is that the separation was achieved using analytical RP-HPLC and Microbiological studies were also carried out and for it was possible to attribute the anti-fungal activity to the major constituents that had been found in the literature [29-30]. Importantly, while there had been limitations in the initial analytical and preparative approaches, these were gradually improved upon as a result of parallel studies on analytical methodology.

As mentioned above, the interesting biological activity found could, in part, be attributed to the flavonoids present in them. The MIC results of anti-dermatophyte activity indicated that methanolic leaf extract of C.excvata has significant inhibitory effect on *T.rubrum and T.mentagrophytes* in comparison to the As these studies were sample limited, it was not possible to test individual components but it is possible that the anti-dermatophytic activity of *C. excavata* might arise from the flavonoid nature of some of the constituents. Coumarins are considered as phytoalexins since plants produce them as defence substances when wounded or attacked by other organisms. Furthermore, coumarin compounds (angelicin isolated both from) have been suggested as possible medicinal agents of importance in the case of hyper proliferative skin diseases like psoriasis. It is suggested that suitable future work should be focused on the anti-dermatophytic activity of isolated fractions. It is also logical to extend the testing of the extracts and isolated compound for other biological activities [30] like ant-inflammatory, analgesic and anti-psoriatic [31]. However further work needs to b done for isolation and characterization of anti-fungal constituents.

COMPETING INTERESTS

The authors declare that they have no competing interests associated with this study.

REFERENCES

1. Manosroi A, Saraphanchotiwitthaya A. In-vivo immunomodulating activity of wood extracts from *Clausena excavata Burm.f. J. Ethnopharmacol*, 102(1), 2005, 5-9.
2. Manosroi A, Saraphanchotiwitthaya A. Immunomodulatory activities of Clausena excavata Burm.f.wood extracts. *J. Ethnopharmacol*, 89(1), 2003, 155-160.
3. Rahman MT, Alimuzzaman M, Shilpi J A, and Hossain M F. Anti-nociceptive activity of ethanolic extract of Clausena excavata leaves. *Fitoterapia*, 73(7-8), 2002, 701-3.
4. Shiow CH, Pei LW, Tian SW. Carbazole alkaloids from Clausena excavata and their biological activity. *Phytochemistry*, 43(1), 1996, 133-40.
5. Randrianarivelojosia M, Rebarison H. Plants traditionally prescribed to treat tazo (malaria) in the eastern region of Madagascar. *Malaria Journal*, 2(1), 2003.
6. Wu Tian-Shung, Huang Shiow-Chyn. Chemical and antiplatelet aggregative investigation of the leaves of Clausena excavate. *Phytochemistry*, 32(2), 1993, 449-51.
7. Tian-Shung Wu, Shiow-Chyn H, Pei-Lin Wu. Carbazole-pyranocoumarin dimer and binary carbazole alkaloid from Clausena excavate. *Tetrahedron Lett*, 37(43), 1996, 7819-22.
8. Masuda T, Inazumi A, Yamada Y, William G. Padolina W.G, Hiroe Kikuzaki H, Nakatani N. Anti-microbial phenylpropanoids from Piper sarmentosum. *Phytochemistry*, 30, 1991, 3227-8.
9. Tian-Shung Wu, Shiow-Chyn Huang, Pei-Lin Wu, Che-Ming Teng. Carbazole alkaloids from Clausena excavata and their biological activity. *Phytochemistry*, 43(1), 1996, 133-40.
10. Simonsen HT, Adsersen A, Smitt U W, Strasburg D, Jaroszewski JW. Methoxyflavones from Melicope borbonica and M. obscura. *Biochem. Syst. Ecol.*, 31(3), 2003, 327-30.
11. Bowen IH, Osborne SJ. Phytochemical studies in the genus Maclurodendron (Rutaceae): furoquinoline alkaloids. *J. Pharm. Pharmacol*, 50, 1998, 233 – 37.
12. Bowen I H, Perera K P W C. Alkaloids, coumarins and flavonoids of Micromelum zeylanicum. *Phytochemistry*, 21(2), 1982, 433-37.
13. Bowen IH, Patel YN. Acridone alkaloids from *Pleiospermium alatum. Phytochemistry*, 25(2), 1986, 429-31.
14. Repetto MG, Llesuy SF. Antioxidant properties of natural compounds used in popular medicine for gastric ulcers. *Braz. J.Med. Biol. Res*, 35(35), 2002, 523-4.
15. Claessens HA, Vanstraten MA. Review on the chemical and thermal stability of stationary phases for reversed-phase liquid chromatography. *Journal of Chromatography A*, 1060, 2004, 23-41
16. Queiroz EF, Wolfender JL, Atindehou KK, Traore D, Hostettmann K. On-line identification of the anti-fungal constituents of *Erythrina vogelii* by liquid chromatography with tandem mass spectrometry, ultraviolet absorbance detection and nuclear magnetic resonance spectrometry combined with liquid chromatographic micro-fractionation. *Journal of Chromatography A*, 974, 2002, 123-134
17. Wolfender JL. Liquid chromatography /ultra violet / mass spectrometric and liquid chromatography / nuclear magnetic Resonance spectroscopic analysis of crude extracts of Gentianaceae Species. *Phytochemical analysis*, 8, 1997, 97-104.
18. Bobzin SC. Application of liquid chromatography-nuclear magnetic resonance spectroscopy to the identification of natural products. *Journal of Chromatography B*, 748, 2000, 259- 267.
19. Hostettmann K, Wolfender JL, Ndjoko K. Liquid chromatography with ultraviolet absorbance-mass spectrometric detection and with nuclear magnetic resonance spectroscopy: a powerful combination for the on-line structural investigation of plant metabolites. *Journal of Chromatography A*, 1000, 2003, 437- 455.
20. Balakrishnan N, Bhaskar VH, Jayakar B, Sangameswaran B. Antibacterial activity of *Mimosa pudica*, *Aegle marmelos* and *Sida cordifolia. Phcog Mag*, 2, 2006, 198-9.
21. National Committee for Clinical Laboratory Standards: Performance standards for antimicrobial disk susceptibility tests. Approved standard M2-A8. 8 th ed. Wayne, PA: National Committee for Clinical Laboratory Standards, 2003.
22. Wang YC, Hsu HW, Liao WL. Antibacterial activity of *Melastoma candidum* D. *Don. Food Sci Technol*, 26, 2008, 01-6.
23. Kuster RM, Arnold N, Wessjohann L. Anti-fungal flavonoids from *Tibouchina grandifolia*. *Biochem Syst Ecol*, 37, 2009, 63-5.
24. Kumaraswamy Y, Byers M, Cox PJ, Sarkar S D. Isolation, structure elucidation, and biological activity of flavone 6-C-Glycosides from Alliaria petiolata. *Chemistry of Natural Products*, 2004, 122-28.
25. Havsteen B. Flavonoids, a class of natural products of high pharmacological potency. *Biochemical Pharmacology*, 32, 1983, 1141-1148
26. Emel Şahin, Saadet Gümüşlü. Alterations in brain antioxidant status, protein oxidation and lipid peroxidation in response to different stress models. *Behav Brain Res*,155(2), 2004, 241-48.
27. Chatterjee SN, Sanjiv A. Liposomes as membrane model for study of lipid peroxidation. *Free Radical Bio Med*, 4(1), 1998, 51-72.

28. Ilya II. A relay model of lipid peroxidation in biological membranes. *Journal of Free Radical Bio Med*, 1(4), 1985, 247-53.
29. Sakagami H, Satoh K. Prooxidant action of two anti-oxidants: ascorbic acid and gallic acid. *Anticancer Research*, 17(1A), 1997, 21-24.
30. Kroes BH, Van den Berg AJJ, Quarles Van Ufford HC, Van Dijk H. Anti-inflammatory activity of Gallic acid. *PlantaMedica*, 58, 1992, 499-504.
31. Luisa M, Eleonora Lo P, Giulia M, Christine M, Francarosa B, Giuseppe F *et al*., Synthesis of angelicin heteroanalogues: preliminary photobiological and pharmacological studies Il. *Farmaco*, 53, 1998, 602-10.