

Biotechnology and Biochemical Analysis Technique to isolate Vanillin derivatives for Plum fruit Tree (I)

Alaa J.Mahrath^a , Tariq H.Al-Mgheer^a , Enaam I. Yousif^b

a) Babylon University College of medicine Biochemistry Department

b) Baghdad University College of education chemistry Department

Abstract :

It's well known that Green Chemistry techniques consider one of the best choice in the field of Medical chemistry research . alternative medicine depends mainly on herbs and medical plants in the hall world which in turn have a lot of benefits in the field of medical treatments through what they have different types of cyclic organic molecules This application studied some biochemical and technological techniques to identify ,isolate and purified some of biochemical molecules from fruits of Plum tree . The study involved drying the sample with freeze dryer for 24 hrs . Then samples were performed with Soxhlet apparatus by protic solvent (PSI) , aprotic solvent (ASI) ,and aprotic-protic solvent interaction(PASI) were used to extracted the molecules . Then evaporate the excess solvent and directly transformed to the flash column chromatography technique (FCC)and monitored by Thin layer chromatography (TLC) to isolate m-methoy-p-hydroxy benzoic acid (**vanillic acid**) or (**VA**) and m-methoxy-p-hydroxy ethyl Benzoate (**Ethyl Vanillate**) or **EV** in about 95% pure state. These molecules **AV and EV** elucidate by spectroscopic tools like (UV-Vis , ATR-IR ,¹H-NMR and GC-Mass spectrometry) .

Key words : plum tree , Vanilinic acid ,flash chromatography , purification .

*Corresponding author e.mail : ajmbioorg@gmail.com , or alajm68@uobabylon.edu.iq

Introduction:

The Plant products are consider the main source of pharmaceutical agent since long time ⁽¹⁻²⁾.Tree plum is one of the most important plant that possessing medical applications ⁽³⁾. Some applications of the plant were used it's extract as chest pain , throat and urinary infection⁽⁴⁾ . It's also for it's diuretic ,anthelmintic , demulcent ,antidiarrheal , anti-inflammatory ,and anti-arthritic activities ⁽⁵⁾ ,anti-diabetic⁽⁶⁻⁷⁾ in addition to antimicrobial activity against various pathogenic microorganism^(8,9)

,protective ulcer⁽¹⁰⁾ and wound healing activity⁽¹¹⁾ .the leaves of the plum tree also have potent antioxidant activity and can show anticancer activity too ⁽¹²⁻¹⁴⁾. On the other hand medicinal plants consider as the major sources of Modern and traditional medicine in the hall world ⁽¹⁵⁾.Recently methanolic extract of cordia leaves tested against human prostate carcinoma cell line by Md.Azizur Rahman and coworkers ⁽¹⁶⁾.according to all of these applications the investigation of the fruit plum became part of our researches. Phenolic compounds or Vanillin derivatives have widely used as antioxidant activity in addition ant-cancer and pharmacological properties^(17,18) . Vaniliic acid and it's derivatives are one of the most flavoring and scent agent that producing nice odors .They are mainly present in many kind of plants .Therefore the aim was trying to Extraction the derivatives of these molecules , isolation and identification them by using Green chemistry principles as possible .

Experimental Section :

Chemical Reagents :

All the reagents and chemicals (ethanol ,ethyl acetate , ether, silica gel ,magnesium sulphate and alumina) used were of analytical grade and also double distilled water (DI) was used in extraction process .

Apparatus :

The following instrumentations were used in this application .

Freeze Dryer (Crust Alpha 1-4 LD with Vacuum Pump RZ6 up to $4 \cdot 10^{-4}$ mbar) , Soxhlet extraction , pH meter , UV-Vis spectrometer(Specord,PC 200 plus KARL KOLB) ,Shaker water bath ,Thin layer chromatography(aluminum oxide 60,size 20×20 cm fluorescent indicator) , Flash Column chromatography (class column 30 × 500 nm,packed with silica gel with partical size 230-400 mesh,1 gm/25 gm with vacuum pumb ROCKER 300 at 50 kpa) , E-graph image ATTO with Wise UV M20) Electro thermal melting point apparatus (stuart) uncorrected , ATR mode with FT-IR spectroscopy (Bruker Tensor 27) with KBr Disk and ATR unite , Gas Chromatography with-Mass spectrometry (shimadzu GC Mass QP2010 Ultra with ion source temperature 150 °C ,ionization mode SCI , DI Temperature 350°C with detector gain : 0.69 KV) . Proton and carbon magnetic resonance (¹H-NMR ,¹³C-NMR) Bruker spectroscopy 300 MHz with tetramethylsilane (TMS)as standard with dimethyl sulfoxide with CDCl₃ .

Collection of plant sample

The plum fruits were collected from Plum tree for experimental purpose in the zone of Al Mahaweel , Hilla city –Babylon district during June 2016 and stored immediately after collection at 10 °C in darkness place .

Preparation of plant sample Extract⁽¹⁹⁾

Step one : Extraction of the sample

About 250 gm of plum fruits **fig(1a)** were washing and drying under dark and reduce pressure for 3 days by laypholyzer then stored in dark at room temperature .The dry sample crashed by blinder then 8 g powder of fruit sample transferred by thimble into the Soxhlet extraction apparatus for 1 to 2 hours according to the kind solvent ,**fig (1b)** .Different solvent were used via this apparatus such as number (1) DI water , (2) Ethanol , (3) ethyl acetate ,and (4) Ether . Different collected product were solvated in each solvent , The crude extract concentrated under rotary evaporating and lympolizer specifically to produce 760 , 656 ,370 and 123 mg respectively **Table (1)**.

Step two :Examine fruit Extraction in TLC :

The crude extract solvated again in ethanol with the following ratio (10 ,20 , 30 , 50 , 70 ,90 %) respectively . and tested in thin layer chromatography (**TLC**) with the following description (aluminum silica 60 ,size 20×20 cm with fluorescent indicator) . the best mobile phase result of the target products was the highest ratio **90% ethanol with 10% ethyl acetate** . more than two spots in component number (2). UV light view develop the TLC plate after drying at about 267 nm **figure (2)** .

Step three : Isolation of the Target molecules by FCC :

The crude that separated in method 1 directly packed in column chromatography with vacuum to accelerated the separation (reducing time for separation) (FCC). The column description was (30 × 500)mm size ,backed with silica gel (220 -380) mesh . the best mobile phase for elution was mixed (Ethanol : ethyl acetate) with the following ratio 4:1 .Ten fractions were collected from the FCC and Tested again with TLC to collect the similar products . the collected samples concentrated again ,Tested with TLC and drying under laypholyzer to produce while the second one was mp = 208-212 °C respectively . this methodology used according to reference ⁽¹⁸⁻¹⁹⁾ . Further purification of the isolated molecules were done by Gas chromatography with Mass analysis (GC-Mass) in College of science Al-Mustansiriah University .

Step Four : Elucidation of the isolated molecules from step three by Spectroscopic Analysis:

Full characterization by spectroscopic Instrumentations such as UV-vis , FT-IR ,¹H-NMR , ¹³C-NMR ,figures (1,2,3) respectively in addition to melting points measurement in **Table (2)**.

Results and Discussion :

The results of isolated molecules were represented in both Tables (1,2) and figures (2,3 ,4). the molecules were tested after separation and purification by spectroscopic techniques as follows : UV-Vis spectra shown the absorption bands at 297 and 312 nm for each **VA** and **EV** respectively . FT-IR spectroscopy with (KBr-disk) and ATR unit of the two isolated molecules confirmed the functional groups structures and matched with the published data base figure **(3a ,4a)**²⁰. For instant Two strong absorption bands for the carbonyl groups at 1727 and 1689 cm^{-1} that belong to (Ester and Carboxylic acid) respectively. Also the two different absorption band at 3484 and 3418 cm^{-1} refer clearly to (OH) phenolic groups in **VA and EV** .while the OH group in vanillic acid appeared as a broad absorption at about (3210-2630) cm^{-1} . the aromatic group also present clearly in the range of absorption at 1552 - 1418 cm^{-1} for both compounds **fig (3a,4a)**.The ¹H-NMR spectroscopy in CDCl_3 for **VA** (1) appeared the following peaks : (dd,2H, aromatic) at $\delta=6.90-6.92$, 7.32-7.36 ppm ,and (s,1H, aromatic) at $\delta=7.51$ ppm , three protons (3H,s, OCH_3) appeared at $\delta= 3.89$ ppm belong to methoxy group , and (b,1H, phenolic OH) at $\delta=9.65$ ppm, **figure (3b)** .The second molecule **EV** proved by the following peaks . three aromatic protons (3H) appeared at $\delta= 7.77$ ppm (dd) , at $\delta= 7.52$ ppm (dd) ,and 7.16 ppm (s) protons . the phenolic proton appear shielded at $\delta= 6.46$ ppm compared with phenolic group at **VA** .Sharp signal observed at $\delta=3.81$ ppm which belong to methoxy group (**3H,O-CH₃**) protons. On the other hand the ethyl ester group clearly observed as methylene (2H,-**CH₂** , q) at $\delta=4.18-4.29$ ppm , and the methyl (**3H,CH₃** , t) at $\delta=1.37-1.34$ ppm respectively **figure(4b)**.

¹³C-NMR spectrum of both **VA** and **EV** in CDCl_3 proved the that are purified with 95% as shown in the **figure (3C and 4C)** respectively . Signal of carbonyl group for **VA(C=O)** appeared at $\delta = 168.18$ and 168.61 ppm , while Carbon of methoxy group (C-O) appeared at $\delta = 53.09$ and 56.51 ppm , plus the rest of spectrum .On the other hand Mass spectroscope give with no doubt by presence of molecular ion peak (m/z)=[168 and 198] for both **VA and EV** respectively **figure (3d ,4d)** .



Figure (1a) : Plum Tree with Collected outgrow th in Al Mahaweel sector .



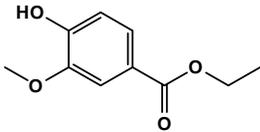
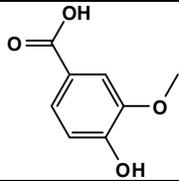
Figure (1b): Extract process for sample

Figure (2) : sample after testing TLC .

Table (1): the optimum condition of sample Extract .

Solvent	(1) DI H ₂ O	(2) C ₂ H ₅ OH	(3)CH ₃ CO ₂ C ₂ H ₅	(4) (C ₂ H ₅) ₂ O
Time	120 mins	65 mins	42 mins	24 mins
Volume solvent	350 ml	350 ml	350 ml	350 ml
Evaporation Solvent by rotary	At 55 °C/ 40 kPa	45 °C/ 70 kPa	50 °C /85 kPa	35 °C/ 90 kPa
Drying by Freeze dryer	24 hrs at -50 /0.04 mbar	10 hrs -30 /1.2 mbar	8 hts -20/40 mbar	4 hrs -10 /80 mbar
Mass of extract	760 mg / 8gm	656 mg/8 gm	370 mg/ 8gm	123 mg/ 8gm

Table (2): Physical properties of Isolated Molecules from Plum Fruit.

Compound Name	Ethyl Vanillate / Ethyl-3-methoxy-4-hydroxy Benzoate	Compound Name	Vanillic acid / 4-hydroxy Anisic Acid
Structure		Structure	
Molecular weight	196.202 g/mol	Molecular weight	168.147 g/mol
Solubility	Methanol	Solubility	Water
Melting point	43-45 °C /45-46 °C lit	Melting point	208-210 °C/ 211-212 °C lit
Boiling Point	292 /293 °C lit	Boiling Point
Molecular Formula	C₁₀H₁₂O₄	Molecular Formula	C₈H₈O₄
Color	Light yellow	Color	Milk

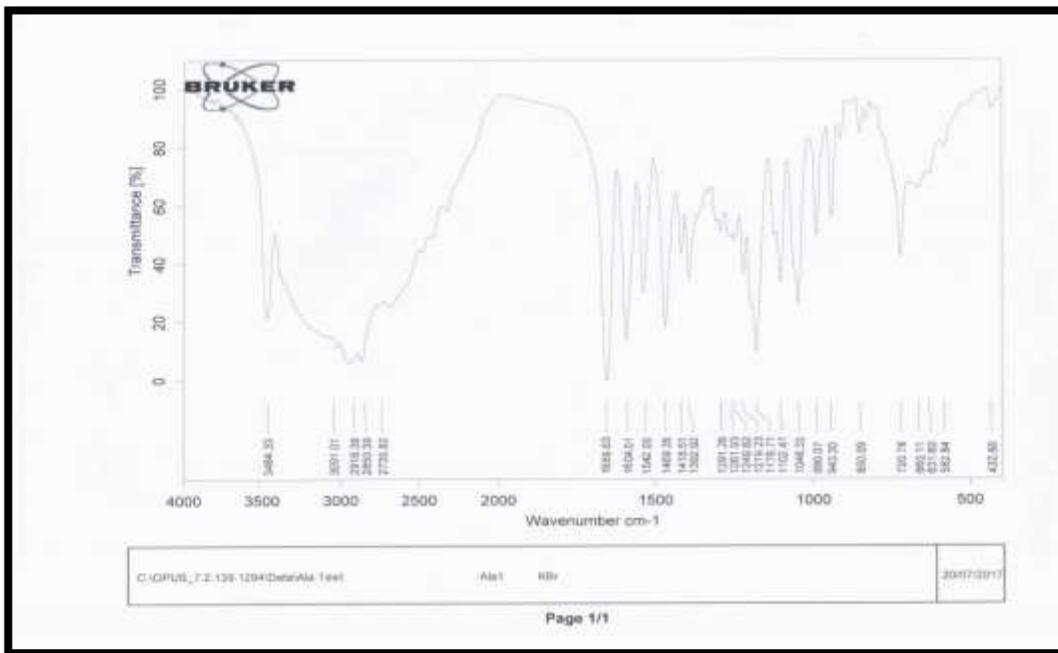


Figure (3a) FT-IR spectra of Extract sample (1) Vanillic Acid

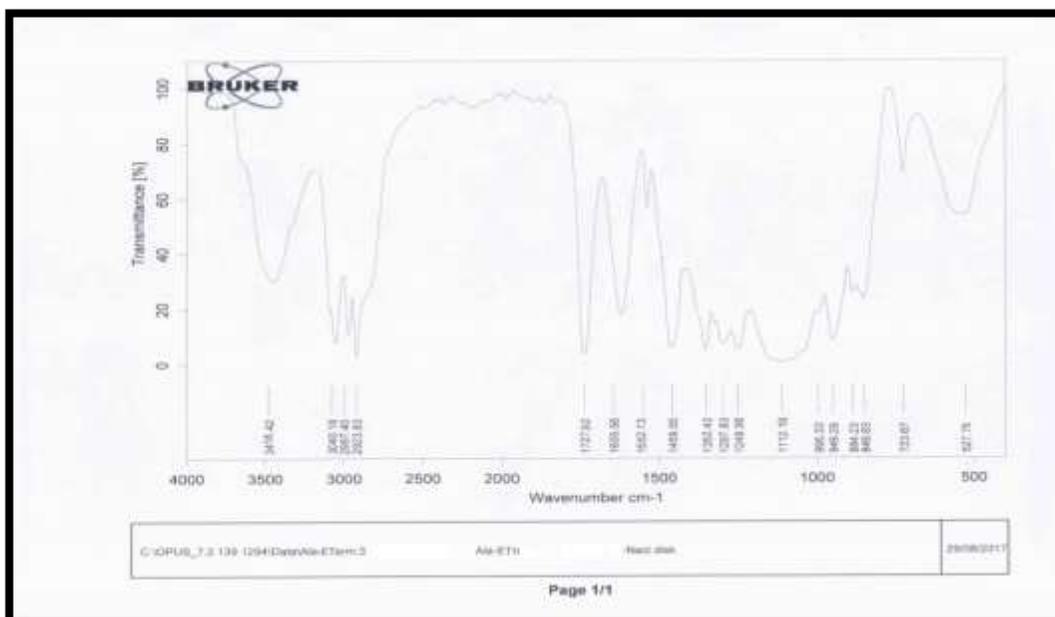
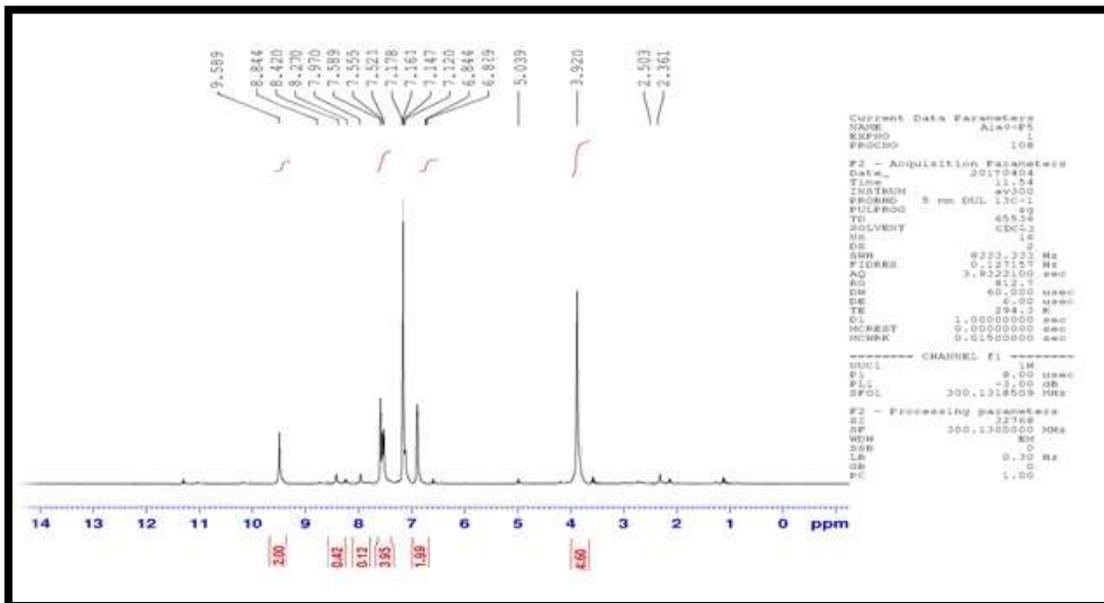


Figure (4a): FT-IR spectra of second isolated molecule (2) Ethyl Vanillate



Figure(3b): ¹H-NMR spectra of VA after purification

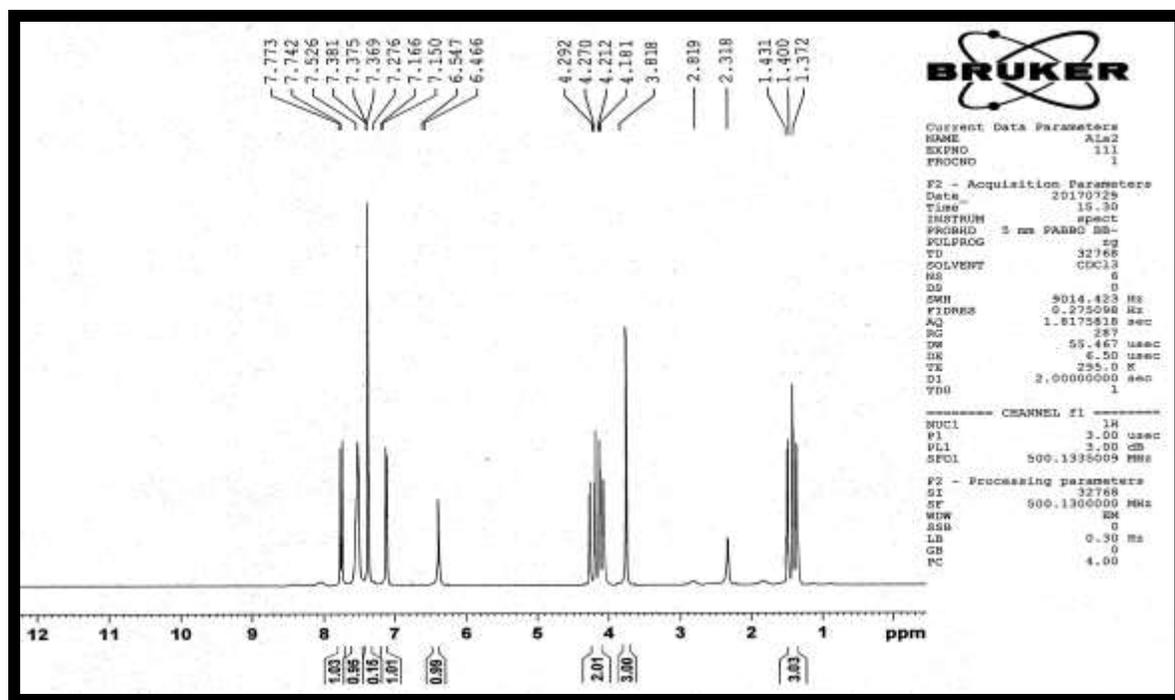


Figure (4b): ¹H-NMR spectra in CDCl₃ of EV after purification

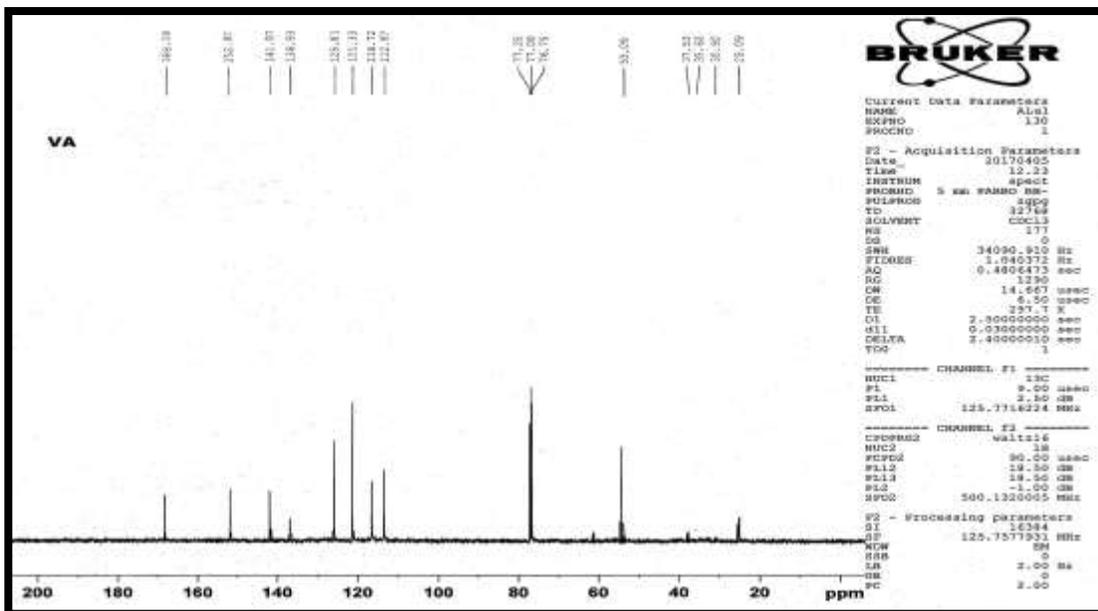


Figure (3C): ^{13}C -NMR spectra in CDCl_3 for VA after purification.

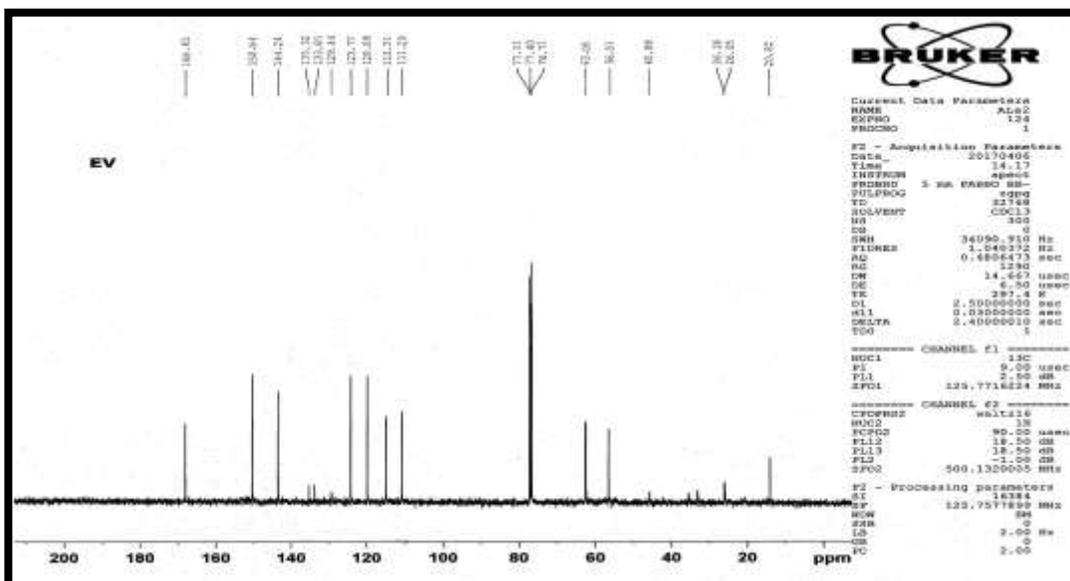


Figure (4C) : ^{13}C -NMR spectra in CDCl_3 for EV after purification.

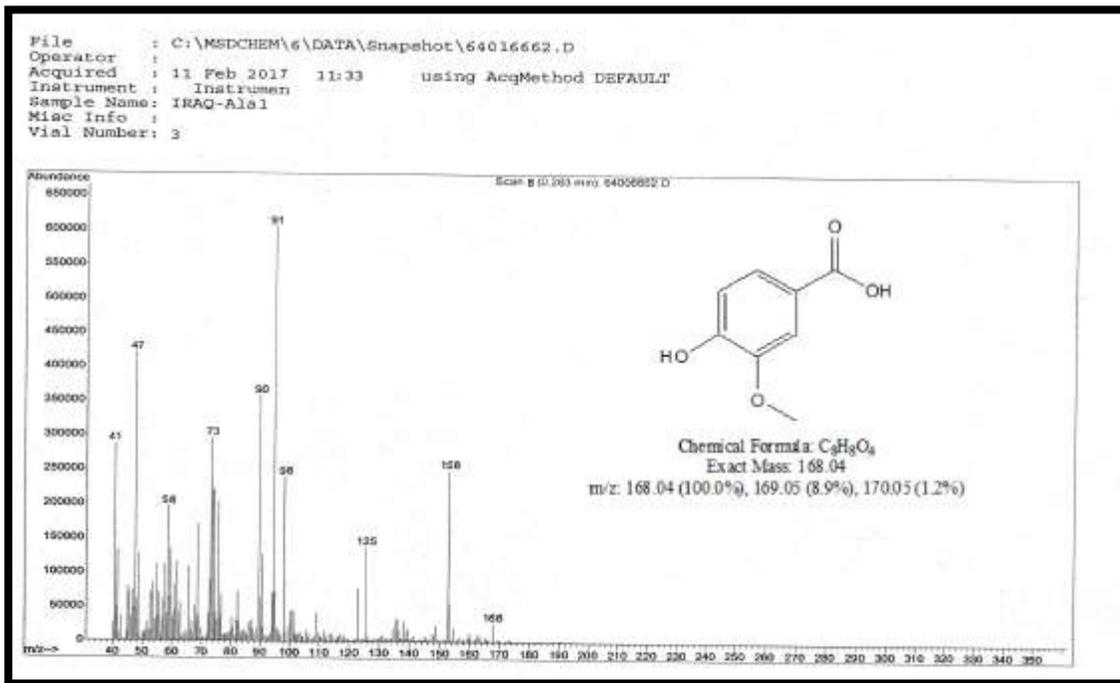


Figure (3d) Mass Spectra of VA after purification

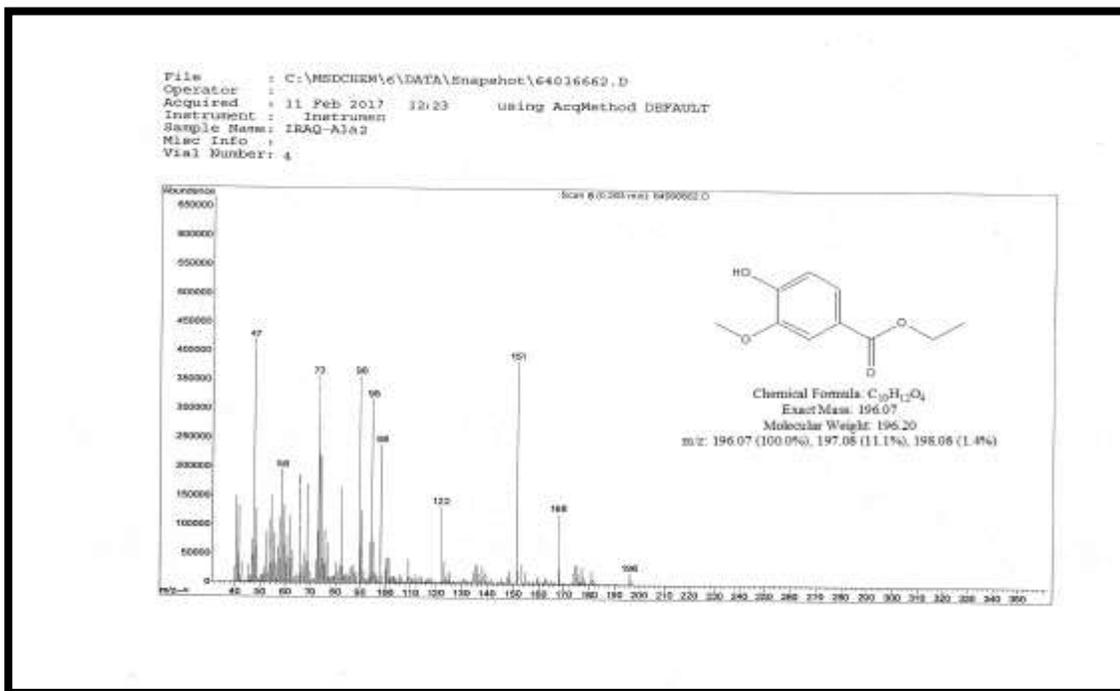


Figure (4d) : Mass spectra of EV after purification

References:

1. Bandow J.E. , Brotz H, Leichert L.O. , Labischinski H. and Hecker M.: 2003 . Proteomic approach to Understanding Antibiotic Action Antimicrobial agent and Chemotherapy ,47: 948.
2. Yadav P. and Singh D.;2012 . Madhuca longifolia (Sapotaceae) :a review of it's traditional uses , phytochemistry and pharmacology. International Journal of Biomedical Research ,3,7:291.
3. Prasad G. Jamkhande , Sonal R. Barde , Shailesh L. Patwekar . 2013 , plant profile phytochemistry and pharmacology of cordia Asian Pac J. Trop Biomed; 3 , 12, 1009.
4. Chopra A. and Doiphode V.;2002 Ayurvedic medicine core concept therapeutic principles and current relevance. Medical Clinics of North America, 86: 75 .
5. Ficarra R. , Ficarra P., Tommasaini S., Barbera R. , and Rapisarda A. ; Leaf extract of some Cordia species ; analgesic and anti-inflammatory activity as well as their chromatographic analysis , Farmaco 1995: 50:245 .
6. Misha A. Garg GS. Ant diabetic activity of fruit pulp of Cordia dichotoma in alloxan induced diabetic rats, Int J. Pharm. Sci. Res. 2011; 2:2314 .
7. Rahman MA, Akhtar J. Phytochemistry and pharmacology of Traditionally used medicinal plant cordia dichotoma , Curr. Trends Biotechnol pharm. 2016 ;10 :180.
8. Bhawana P., Bhagyashree D., Sheetal S. , and Varsha C.;2014, Estimation of Elemental Content of Cordia Myxa and it's antimicrobial activity against pathogenic microorganisms . Indian Journal of science research 4, 1 ,39 .
9. Rawat S. , Saini R. , Sharma A. , Phytochemical Study and antimicrobial activities of Cordia dichotoma, International Research of Journal Pharm ; 2013 ;4 :12;53 .
10. Ganjare AB, Nirmal SA, Rub RA, Patil AN, Pattan SR. Use of Cordia dichotoma bark in the treatment of ulcerative colitis. Pharm Biol 2011; 49(8): 850-855.
11. Kuppast IJ, Nayak PV. Wound healing activity of Cordia dichotoma forst F. fruits. Indian J Nat Prod Resour 2006; 5(2): 99-102
12. Rahman MA, Hussain A. Anticancer activity and apoptosis inducing effect of methanolic extract of cordia dichotoma against human cancer cell line. Bangladesh J. Pharmacol. 2015 ;10 :27.
13. Nariya PB, Bhalodia NR, Shukla VJ, Acharya R, Nariya MB. In vitro evaluation of antioxidant activity of cordia dichotoma Bark Ayu. 2013; 34 :124.
14. Rahman MA, Hussain A. phytochemical and analytical evaluation of cordia dichotoma Linn , leaves . Pharmacogn J. 2015 ;7:58 .
15. Suri RK , Chaudhari DC, Jaffer R. Commercially important medicinal plant from forest , J Ecol Bot Phytochem. 1992: 3; 129 .
16. Md. Azizur Rahman , Sahabiada , and Juber Akhtar. Evaluation of anticancer activity of Cordia leaves. Journal of Traditional and complementary Medicine ; 2017;7:315.
17. Prasad G. Jamkhande , Sonal R. Barde , Shailesh L. Patwekar , Priti S. Tidke . Plant profile , phytochemistry and pharmacology of Cordia dichotoma :A review . Asian Pacific Tropical Biomedicine. 2013 :3(12)1009.
18. Alaa J. Mahrath , Mahmood S. Magtoof . Isolation and identification of green pigment from waste pineapple peels. Chromatography International Labmate online 2015.11: 12 .

19. Alaa J.Mahrath ,Ghafil S.Hassan ,Ehab K.Obaid ; Isolation and characterization of Terpenoid derivatives from medicinal plant.Int.J. Chem.Sci.;2015:13 :2,983.
20. Alaa J. Mahrath , Saadon A. Aowda , Sabah N. Kamil ; Synthesis and spectroscopic studies of some new oxazepine derivatives throughout [2+5] cycloaddition reactions (III), Res Chem Intermed.2012, DOI 10.1007/s11164-012-0877-2.