Biotechnology and Biochemical Analysis Techniques to isolate Vanillin derivatives for Plum Fruit Tree (I)

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Abstract: This application studied some biochemical and technological techniques to identify, isolate and purified some of biological 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 extract the molecules. Then recovered the excess solvent by evaporation and condensation. The sample directly transformed to the flash chromatography technique (FCC) and monitored by Thin layer chromatography (TLC) to isolate m-methoxy-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 elucidated by spectroscopic tools (UV-Vis, ATR-IR, $^1$H-NMR, $^{13}$C-NMR, and Mass spectrometry).

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

1. Introduction:
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 medicinal 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\(^{(1)}\). The Plant products are considered the main source of pharmaceutical agent since long time\(^{(2-3)}\). Tree plum is one of the most important plant that possessing medical applications\(^{(4)}\). Some applications of the plant were used it’s extract as chest pain, throat and urinary infection\(^{(5)}\). It’s also for it’s diuretic, anthelmintic, demulcent, antidiarrheal, anti-inflammatory, and anti-arithmetic activities\(^{(6)}\). In addition to antimicrobial activity against various pathogenic microorganism\(^{(9,10)}\) protective ulcer\(^{(11)}\) and wound healing activity\(^{(12)}\). The leaves of the plum tree also have potent antioxidant activity and can show anticancer activity too\(^{(13-15)}\). On the other hand medicinal plants consider as the major sources of Modern and traditional medicine in the hall world\(^{(16)}\). Recently methanolic extract of cordia leaves tested against human prostate carcinoma cell line by Md.Azizur Rahman and coworkers\(^{(17)}\), 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\(^{(18,19)}\). Vanillic 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.

2. Experimental Section

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

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Apparatus:
The following instrumentations were used in this application: Freeze Dryer (Crust Alpha 1-4 LD with Vacuum Pump RZ6 up to 4-10⁻⁴mbar), 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 partial 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(20)

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 lyophilizer 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 (18-20). 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-NMRfigures (3-10) respectively in addition to melting points measurement in Table (2).
3. Results and Discussion
The results of isolated molecules were represented in both Tables (1, 2) and figures (3-10). 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 (vanilic acid) and EV (ethyl Vanillate) 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. For instance, 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. The $^1$H-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 (5). 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-CH$_3$) protons. On the other hand, the ethyl ester group clearly observed as methylene (2H,CH$_2$, q) at $\delta=4.18$-$4.29$ ppm, and the methyl (3H,CH$_3$, t) at $\delta=1.37$-$1.34$ ppm respectively figure (6).

$^{13}$C-NMR spectrum of both VA and EV in CDCl$_3$ proved the that are purified with 95% as shown in the figure (7, 8) 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 (9,10).

Figure (1a): Plum Tree with Collected outgrowth 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 H₂O | (2) C₂H₅OH | (3)CH₃CO₂C₂H₅ | (4) (C₃H₇)₂O |
|---------|------------|------------|---------------|---------------|
| 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/8 gm | 123 mg/8 gm |

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](image1.png)                               | Structure     | ![Structure](image2.png)              |
| 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 (3): FT-IR spectra of Extract sample (1) Vanilline Acid

Figure (4): FT-IR spectra of second isolated molecule (2) Ethyl Vanillate
Figure (5): $^1$H-NMR spectra of Vanillic acid (VA) after purification

Figure (6): $^1$H-NMR spectra in CDCl$_3$ of Ethyl Vanillate (EV) after purification
Figure (7): $^{13}$C-NMR spectra in CDCl$_3$ for Vanillic acid (VA) after purification.

Figure (8): $^{13}$C-NMR spectra in CDCl$_3$ for Ethyl Vanilate (EV) after purification.
Figure (9) Mass Spectra of Vanillic acid (VA) after purification

Figure (10): Mass spectra of Ethyl Vanilate (EV) after purification
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