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Original Article

Synthesis and Total Phenol Content of New Resveratrol Derivative

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ABSTRACT

This study was conducted with the aim to extract and purify a polyphenolic compound "Resveratrol" from the skin of black grapes *Vitisvinifera* cultivated in Iraq and synthesis new derivative. Chemical investigations and tests for the identifications carried out for the qualification of extracted crystals yield include: general tests for polyphenoles, aromatic unsaturated compounds, spectrophotometric scanning for λ_{max} screening, High Performance Liquid Chromatography (HPLC) comparable with standard and Fourier Transform Infrared (FTIR) to observed groups of compound. Synthesis new derivatives from purified resveratrol were done throughout series of reactions accompanied with FTIR for each derivative. The derivative is (E)-5-(4-(3, 4, 5-trihydroxybenzoyl) oxy) styryl)-1, 3-phenylene bis (3, 4, 5 tri hydroxyl benzoate). The IR spectroscopy result confirms the presence of functional groups in the proposed compounds.

Keywords: Resveratrol, Resveratrol derivatives. HPLC, FTIR, Total phenol.

INTRODUCTION

Resveratrol (3, 4´, 5-trihydroxystilbene) is a phytoalexin found in a wide variety of dietary sources including grapes, plums and peanuts. It is also present in wines, especially red wines and to a much lesser extent in white wines. Its stilbene structure is related to the synthetic oestrogen diethylstilbestrol. Resveratrol exists as cis- and trans-isomers. Trans-resveratrol is the preferred steric form and is relatively stable if it is protected from high pH and light. The synthesis of trans-resveratrol in the plants can be induced by microbial infections, UV radiation and exposure to ozone[1]. A primary impetus for research on resveratrol was initiated from the paradoxical observation that a low incidence of cardiovascular diseases may co-exist with a high-fat diet intake and moderate consumption of red

wine, a phenomenon known as the French paradox[2]. The possible mechanisms by which resveratrol exerts its cardio- and vascular-protection involve inhibition of platelet aggregation, arterial vasodilation mediated by NO (nitric oxide) release, favourable changes in lipid metabolism such as LDL (low-density lipoprotein)–cholesterol oxidation, antioxidant effects, stimulation of angiogenesis , induction of cardioprotective protein expression, and insulin sensitization. Indeed, it reduces the synthesis of certain lipids and eicosanoids that tend to promote inflammation and atherosclerosis; likewise, it suppresses certain cardiac arrhythmias[3]. Some of these effects may be due in part to resveratrol being a phyto-oestrogen, i.e. a plant compound that has biologically similar properties to those of oestrogens[4].

The derivatives of resveratrol associated with the available oral hypoglycemic agents for the treatment of diabetes mellitus are Epsilon-vinifera a resveratrol dimer, Piceatanol an active metabolic of resveratrol found in red wine , Piceid a resveratrol glucoside and Trans-diptoindonesin B a resveratrol trimer[5]. The potential for resveratrol to treat diabetes mellitus type 2 has continued to generate scientific interest[6]. Resveratrol may offer benefits in preventing or managing conditions associated with high blood sugar[7].

The present study aims at syntheses of a new compound derived from resveratrol and determination total phenols compounds.

MATERIALS AND METHODS

Collection of Plants

Local Iraqi black grapes were collected from the local market and classified as *Vitisvinifera*, by the herbarium of the biology Department, College of Science, Baghdad University[8].

The skin was separated from the fruit to be then kept in a dark cool place, till the following steps.

Preparation of Grape Skin Extract

The grape skin extract was prepared; all steps were done away from direct light and extensive stress that led to oxidation of the plant extract. About 500 grams of fresh skin grapes was shaken with 2.5 litters' 99.9% ethyl acetate in cool dark place for 72 hours and give 0.11-0.19 gm. The extract was filtered and the filtrate was dried at 30-40°C by a rotary evaporator to get 1/10 (one tenth) its original volume to be stored at –20°C till the followings steps[8].

Identification of Polyphenols

Phenolic group (C_6H_5-OH) in phenolic compounds, which are colourless but attain colour due to oxidation, are soluble in 5% NaOH solution and insoluble in 5% sodium carbonate solution, and the phenolic groups in the molecule can be determined by the following general tests:

Ferric Chloride Test

The classic procedure for detecting phenolic compound is by means of the intense green, purple, blue or black colours, many of them give in solution when 1% aqueous or alcoholic ferric chloride is added[9].

Liebermann Reaction

Only those phenols which possess a free para position respond to this test. The test includes the additional 1 ml of conc. H_2SO_4 to the phenolic compound in a dry test tube and addition of a few crystal of $NaNO_2$, a blue green or blue- violet colour is immediately formed and then changed to red on dilution[10].

Phthalein Test

It is done by adding phthalic anhydride to the phenolic compound and 2 drops of conc. H_2SO_4 and then heated and after that poured on 10% NaOH solution yield a characteristic colour[10].

Specific Reaction of Benzene (Aromatic Ring)

Aluminium chloride ($AlCl_3$) test (Friedle graft):

To 0.1 gm of the unknown in 1 ml of chloroform add a pinch of anhydrous $AlCl_3$ with a spatula along the sides of the test tube, crystals of $AlCl_3$ become yellow but turn dark orange within few minutes and the chloroform layer is colourless[10].

Specific Test for double Bond

In order to find out unsaturated compound the following two tests are applied[10].

Bromine Decolourisation Test

The unknown resveratrol (0.1g) in 2 ml carbon tetrachloride is added with shaking, then a drop wise of 5% solution of bromine was added. The discharge of reddish brown colour of bromine without evolution of hydrogen bromide represents a positive test for unsaturated compound[10].

The Baeyer Test

This test is applied to supplement the bromine decolourisation test for un-saturation. To a solution of 0.1 g of the compound in 2 ml of water or ethanol add Baeyer's reagent (dilute alkaline $KMnO_4$ solution) drop wise and with continuous shaking. If the purple colour disappears the compound may be unsaturated[10].

Isolation and Purification of Resveratrol

The following steps were followed for the isolation and purification of resveratrol: Liquid portion; Column chromatography (Partial purification); HPLC and FTIR. After each step a process of Liquid portion, Column chromatography (Partial purification) and HPLC procedures were carried on to be, then getting one isolated pure substance detected later by Fourier Transform Infra-Red assay (FTIR)[8].

Column Chromatography (Partial Purification)

Most authors suggested the clean-up of grape products by column chromatography before any procedure of resveratrol assay using different methods such as capillary electrophoreses method[11].

A partial purification of the residue was preceding using open glass column (2.5 x 21) cm filled with silica gel G60 special for column chromatography. The residue was dissolved in 1-2 ml methanol and the mobile phase is benzene: methanol: acetic acid, 20:4:1 (Harbone, 1984). The elution was collected in 100 separated tube each filled with 3ml eluent. All fractions were tested for $FeCl_3$ 1% solution as a colorimetric method for polyphenols identification[9,10]. Only the positive results elution were collected and dried under vacuum by a rotary evaporator.

HPLC Method

Pure resveratrol was identified by HPLC according to [12] using C18 – reverse phase column and an elution system under the following conditions mobile phase: acetonitrile: water, 60:40, flow rate: 0.6 ml/min, Standard concentration: 0.6 mg / ml (exposed to sun light), sample concentration: 0.6 mg / ml and wave length 280 nm.

FTIR Assay

The functional groups in resveratrol structure and their derivatives were detected to be compared with the standard chart. Resveratrol contains many functional

groups: aromatic than these functional groups.

Estimation of total phenolic content

The Folin- Ciocalteau[13] was used to determine the total phenol content.

Gallic acid was used as a standard to produce the calibration curve. Absorbance was measured at 760 nm on a spectrophotometer. Total phenol content was expressed in mg of gallic acid equivalents (GAE)/mL of sample.

Preparation of Resveratrol Derivative

(E)-5-(4-(3, 4, 5-trihydroxyben-zoyl) oxy) styryl)-1, 3-phenylene bis (3, 4, 5-trihydroxybenzoate).

The synthesis of 3, 4, 5-trihydroxy-benzoyl chloride: Thionyl chloride (0.3mol) was added gradually to gallic acid (0.25mol) in a round bottom flask. After addition of thionyl chloride, the mixture was stirred for 4 hour and heated to 80 C˚ for 30 min in water bath. The excess of thionyl chloride was removed by distillation.

The synthesis of esters of Gallic acid: A solution of Resveratrol in ether (50 mL) was added to a solution of 3, 4, 5-trihydroxybenzoyl chloride (0.05 mol) in ether (50 mL). The mixture was heatedon water bath until no further evolution of hydrogen chloride was observed. The mixture was cooled at room temperature and evaporation of solvent yielded the crude ester which was purified by re-crystallization with alcohol (Scheme 1)[14].

RESULTS AND DISCUSSION

Extraction and Purification of Resveratrol

Fresh black grape skin 500gm was hydrolyzed with ethyl acetate and extracted with an organic solvent “Diethyl ether”, then resveratrol was separated with liquid – solid adsorption chromatographic technique by silica gel open column yielded “purified resveratrol".

The yield of the pure resveratrol is about 20 mg for the 500 gm grape skin used; there may be some loss during the processing of extraction[15]. The fresh `skin of black grapes (*Vitisvinifera*) is rich with a non-flavonoid polyphenol, resveratrol and each gram of fresh grape skin contains (50-100) microgram of pure resveratrol[16].

The Extraction Procedure

The procedure for extraction and purification was concluded from different studies. All the processes have been carried out in the dark. In the general process for plant extraction, the grape skin is extracted with 80% ethanol. Alcohol, in any case, is a good all- purpose solvent for preliminary extraction[8].The optimum conditions for the extraction of resveratrol: 80% ethanol for 30 min at 60°C to prevent enzymic oxidation. The trans and cis resveratrol O-D-glycoside (pieced), are acid hydrolyzed with10% conc. HCl on a water bath for (10-30) min[17]. The free aglycon moiety is water insoluble and can be easily taken up with organic solvent such as chloroform[18].

Purification of Resveratrol

Since the grape skin contains numerous amounts of chemical compounds, the extract cleaning up by liquid-liquid partion technique is necessary for separating the compounds according to the different distribution coefficients and the solvent affinity to solutes[19]. Ethyl acetate is a good solvent for resveratrol taking up as viewed in many studies[20].

Chemical Identification of Resveratrol

Resveratrol (pure) was tested for general phenolic tests as shown in Table (1).

High Performance Liquid Chromatography (HPLC) for Resveratrol

The applied HPLC method according to[12] for resveratrol detection uses wave

lengths: 280 nm for resveratrol. All the resultant chromatogram for purified resveratrol. Figure (1). The area under the peak and peak height with corresponding retention times as shown in Table (2). The results emphasize that even if almost all the conditions for extraction and purification occur in dark as much as possible, the compound may be converted from trans configuration to the cis isomer[21,10].

Fourier Transform Infra-Red (FTIR) for Resveratrol

Infrared (IR) spectroscopy was applied for the detection and analysis of any purified compound besides the chemical tests. Since I.R light can be reflected from materials, the loading of even small amounts of any product is needed to enhance the very small spectral signal to be recorded[23]. Fourier Transform Infra-Red (FTIR) spectrophotometers were used for recording spectra in the region 4000 cm^{-1} to 670 cm^{-1} (2.5µm to 15 µm) or in some cases down to 200 cm^{-1}(50 µm) (Figure 2). Fourier transform spectrophotometers used polychromatic radiation and calculate the spectrum in the frequency domain from the original data by Fourier transformation[24]. Resveratrol has many functional groups Phenolic–OH group stretching at wave length 3427.62, Aromatic C–H group stretching at wave length 2928.04-2854.74, Aliphatic C=C at wave length 1464.02 and Aromatic C=C at wave length1689.70 (Table3).

Fourier Transform Infra-Red (FTIR) for Derivative

Derivative has many functional groups: OH broad band group stretching at wave length 3398.18, Aromatic C–H group stretching at wave length 3016.46, Aliphatic C-H out of planer at wave length 756.12, Aromatic C=C at wave length 1610.61, Aliphatic C=C at wave length 1531.53, C=O group at wave length 1703.20 and C-O group at 1188.19. As shown in table (4). Figure (4).

Determination of total phenolic content

The amount of total phenol was determined with the Folin-Ciocalteu reagent. The Folin-Ciocalteu method gives a crude estimation of the total phenolic compounds present in a sample. It is not specific to polyphenols, but many interfering compounds may react with the reagent, giving elevated apparent phenolic concentrations[25]. Since we studied purified extracts it can be assumed that the estimated TP content was real without influence of other compounds that can react with the Folin-Ciocalteu reagent.

Gallic acid was used as a standard compound and the total phenols were expressed as mg/ml gallic acid equivalent (GAE) using the standard curve equation: y = 0.0061x+ 0.0396, where y is absorbance at 760 nm (Figure 4). The data present in table (4) that showed when amount resveratrol and resveratrol derivative concentration increase, the highest amount of phenolic compounds in 50mg/ml of resveratrol derivative when compared with 50mg/ml resveratrol (30.22 and 158.32 mg/ml) respectively. These results agree with[26] when found that total phenol (TP) content in the investigated model solutions of extract resveratrol was 21.98 mg GAE/100ml.

CONCLUSION

The resveratrol and its derivative gave higher phenolic and hydroxyl groups or their synergistic properties to using as safe and potent hydroglycemic and hydrolipidemic agent in future study.

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Table 1. General tests of phenolic compound (resveratrol)

Test	Result
5% NaOH solution	Soluble
5% Sodium carbonate solution	Insoluble
1% Ferric chloride solution	Green colour
Liebermann reaction	+ ve
Phthalein reaction	+ ve (red colour)
Aluminum chloride test (Friedle graft) “for the aromatic ring”	+ve (yellow to orange colour)
Bromine decolourisation test “for the double bond”	+ ve Discharge of reddish – brown colour
Baeyer test “for the double bond”	+ve disappears of the purple color

Table 2. HPLC results for pure extracted and standard resveratrol

Resveratrol	Wave length (nm)	Type isomer	Retention time (min)	Peak area
Standard	280	Cis	11.700	378865
Sample	280	Cis	11.649	312922

Table 3. The IR frequencies region for the functional groups of the standard resveratrol and the extracted pure resveratrol

The Functional Group	I.R Frequencies of Resveratrol Standard	I.R. Frequencies of Extracted Resveratrol
Phenolic–OH group stretching	3294.19	3427.62
Aromatic C–H group stretching	3016.46	2928.04-2854.74
Aliphatic C=C	1589.23	1664.02
Aromatic C=C	1649.50	1689.70

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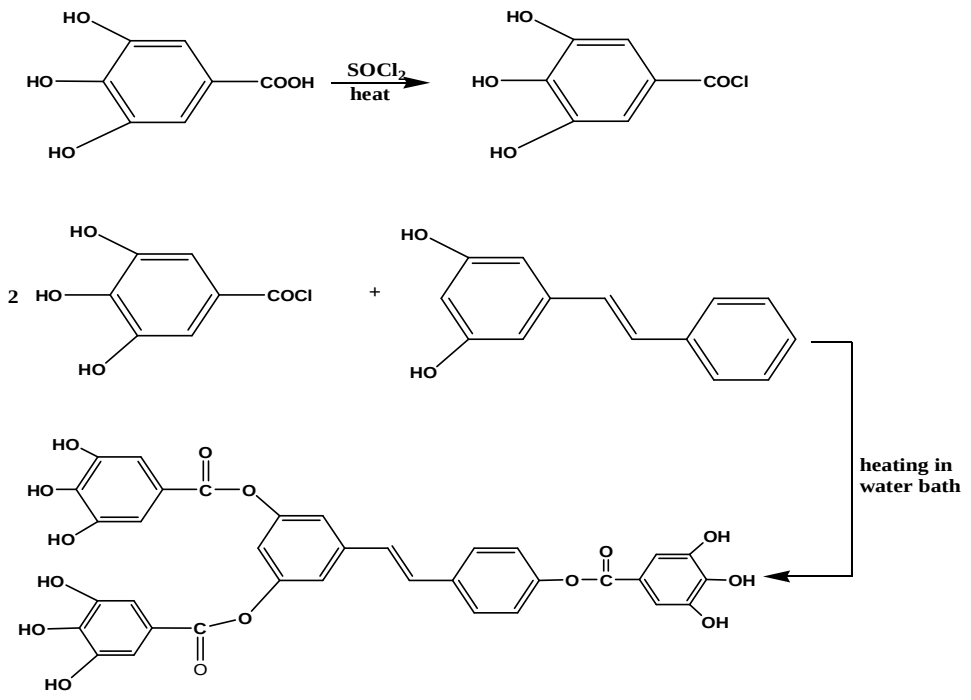
Table 4. The IR frequencies region for the functional groups of the resveratrol derivative

Phenolic –OH group stretching	3398.18
Aromatic C-H group stretching	3016.46
Aliphatic C=C	1531.53
Aromatic C=C	1610.61
C=O	1703.20
C-O	1188.19
Aliphatic C-H (out of planer)	756.12

Table 5. Total Phenolic content of resveratrol and its derivative

Sample	Concentration (mg/ml)	Total phenol (µg/g)
Resveratrol	10	14.85
	25	21.04
	50	30.22
Resveratrol derivative	10	98.91
	25	155.33
	50	158.32



Scheme 1. (E)-5-(4-(3, 4, 5-trihydroxybenzoyl) oxy) styryl)-1, 3-phenylene bis (3, 4, 5 tri hydroxyl benzoate)

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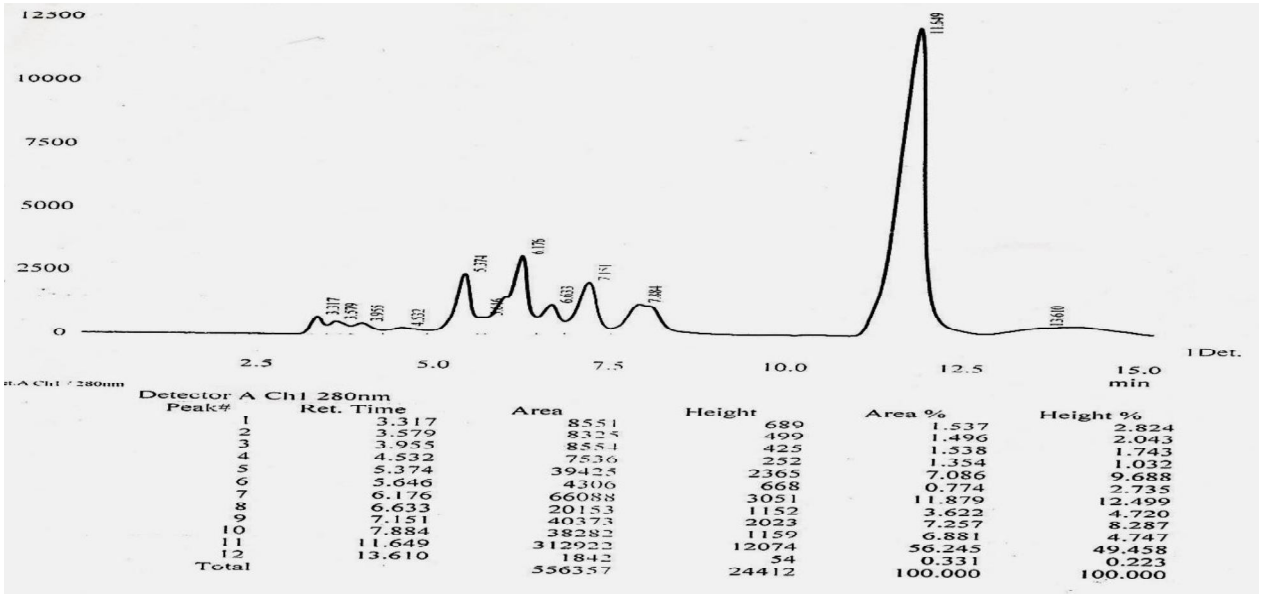

Detector A Ch1 280nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.317	8551	689	1.537	2.824
2	3.579	8324	499	1.496	2.043
3	3.955	8551	425	1.538	1.743
4	4.532	7536	252	1.354	1.032
5	5.374	39425	2365	7.086	9.688
6	5.646	4306	668	0.774	2.735
7	6.176	66088	3051	11.879	12.499
8	6.633	20153	1152	3.622	4.720
9	7.151	40373	2023	7.257	8.287
10	7.884	38282	1159	6.881	4.747
11	11.649	312922	12074	56.245	49.458
12	13.610	1842	54	0.331	0.223
Total		556357	24412	100.000	100.000

Figure 1. Purified resveratrol reading at 280nm

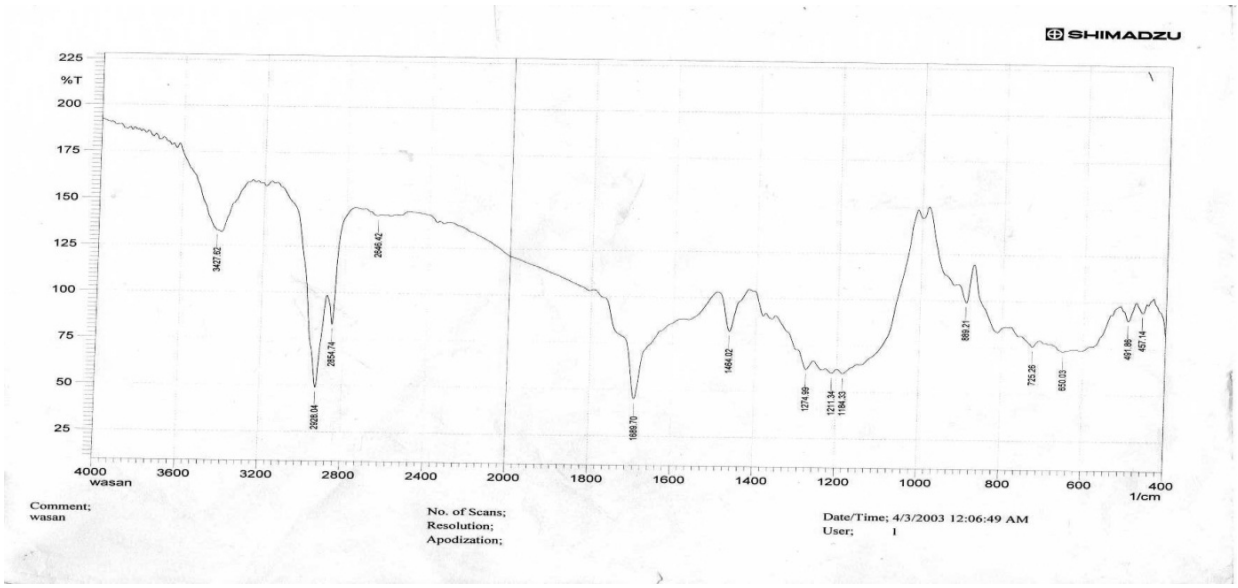

Figure 2. Transform infra-red (FTIR) for resveratrol

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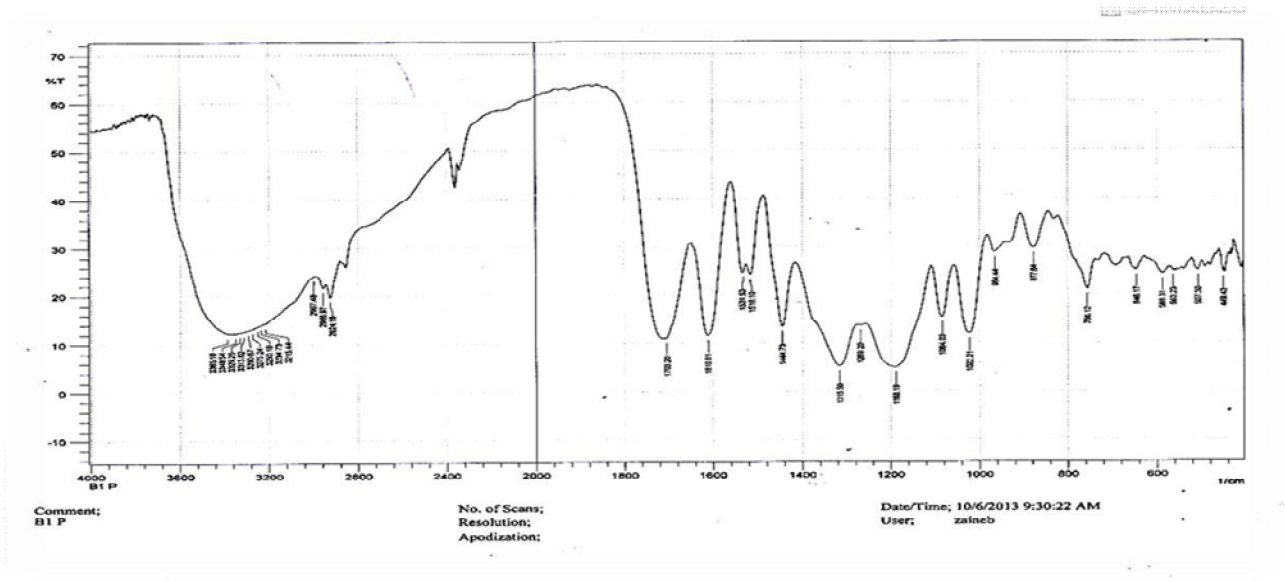

Figure 3. The infrared spectra for Resveratrol derivative

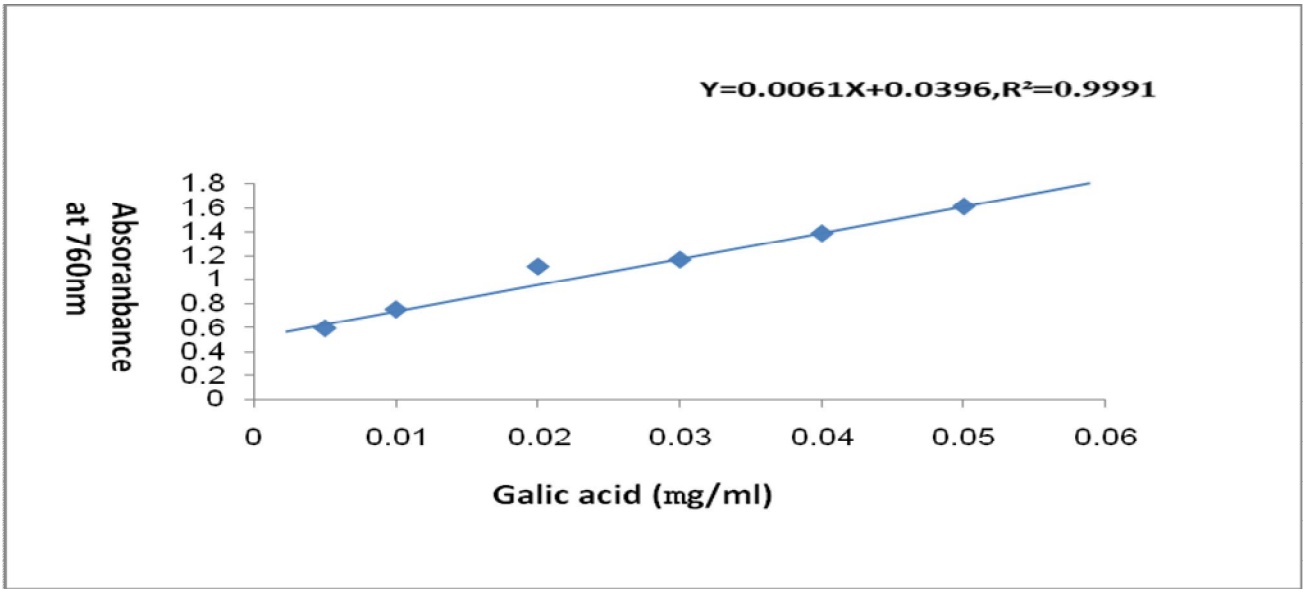

Figure 4. Standard carve of gallic acid