

Extraction and Purification of Gallic Acid from *Eucalyptus camaldulensis* Leaves.

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Abstract

Some parameters for optimizing conditions to gallic acid extraction from *Eucalyptus camaldulensis* leaves were studied. Results revealed that maximum yield of gallic acid (GA) was obtained by using 50% ethanol as extraction solvent at 40 °C for 24 hrs. Antioxidant activity of the resulted extract was evaluated against the free radical ABTS, results showed that *E.camaldulensis* is a good antioxidant source since it gave the same IC₅₀ of the standard antioxidant trolox. GA was purified from the mentioned extract by 95% ethanol extraction and adsorption chromatography using sephadex LH-20. HPLC analysis and FTIR spectra confirmed that the purified GA was identical to the standard GA.

Keywords: gallic acid, ethanol, antioxidant, Sephadex LH-20, HPLC.

الخلاصة

تم دراسة بعض العوامل لتحديد الظروف المثلى لاستخلاص حامض الكاليك من اوراق شجرة اليوكالبتوز , وأظهرت النتائج ان اعلى حصيلة من الحامض تم الحصول عليها باستخدام الايثانول 50% كمذيب استخلاص بدرجة حرارة 40 م° لمدة 24 ساعة . تم تقييم الفعالية المضادة للأكسدة في مستخلص اليوكالبتوز ضد الجذر الحر ABTS وتشير النتائج الى ان المستخلص المذكور يعد مضاد اكسدة جيد نظرا لاعطائه فعالية مضادة مماثلة لفعالية مضاد الأكسدة القياسي Trolox . تم تنقية حامض الكاليك من مستخلص اوراق اليوكالبتوز بواسطة الاستخلاص بالايثانول 95% وكروماتوغرافي الإنمصاص باستخدام الهلام Sephadex LH-20. وأثبتت نتائج التحليل بكروماتوغرافي السائل عالي الكفاءة HPLC وطيف الأشعة تحت الحمراء FTIR ان الحامض المنقى مماثلا لحامض الكاليك القياسي.

الكلمات المفتاحية : حامض الكاليك ، ايثانول ، مضاد اكسدة ، سيفادكس ، كروماتوغرافي السائل عالي الاداء .

Introduction

Gallic acid (3,4,5-trihydroxy benzoic acid) is an important organic acid ,has the molecular formula C₆H₂(OH)₃COOH.

Gallic acid is now considered to be one of the most useful chemicals .The present global demand for this acid is about 8000 tons/ year (Lokeswari *et al.*, 2010). However , the global consumption is expected to increase rapidly in the near future due to the various applications of this biomolecule in different fields. It is used in the pharmaceutical industry for the synthesis of antibacterial drug trimethoprim (Kar and Banerjee, 2000) . It is also found to show cytotoxic activity against cancer cells, without harming normal cells (Elvira *et al.*, 2006). Gallic acid is used in food industry as substrate for the chemical synthesis of food preservatives such as pyrogallol and gallate (Gathon *et al.*, 1989) . In chemical industries, this organic acid finds extensive use as an ingredient of developer in photography and printing processes (Pourrat *et al.*, 1987). Additionally, It has varied biological activities including antioxidant, antibacterial, antifungal, etc.

Plants have been considered as a source of medicinal agents. Gallic acid is present in many plants in the form of free acids, esters, catechin derivatives and hydrolysable tannins (Karamac *et al.*, 2009).

The extraction of Gallic acid has a great practical interest because it has several important biological characteristics. Quantitative extraction of phenolic compounds from plant tissues has been a constant challenge to chemists due to various interfering

parameters involved- particle size of the sample ,type (or nature) of the solvent(s) ,time and temperature of extraction, pH of the extraction medium, degradation of compounds during extraction ,etc (Deshpande *et al.*, 1986) .

The aim of the present work was to identify the content of gallic acid in *Eucalyptus camaldulensis* leaves extracts, optimizing the gallic acid extraction and purification of the acid from this plant .

Materials and methods

Fresh leaves of *E.camaldulensis* were collected from Al-Husainia region farms in sacred Karbala .The leaves were cleaned, washed ,shade dried in the laboratory and powdered in an electric grinder.

Optimizing conditions for gallic acid extraction:-

Many parameters of gallic acid extraction from *E. camaldulensis* leaves powder were studied. These include: type of solvent ;(Ethanol, Methanol, Acetone, Water); extraction time (6,12,24,36,48) hrs and temperature (35,40,45) C°.

A portion of 10 gm of powder was extracted with 200 ml of solvent in a reciprocal shaker waterbath at 40 C° and 100 rpm .After 24 hrs of extraction, the mixture was filtered through filter paper. The filtrate was evaporated in an oven with hot air at 40 C°.

Optimization was carried out by varying one parameter at a time, and keeping the other parameters at a constant , during each set of experiment.

Gallic acid determination:-

Gallic acid was determined according to the spectrophotometric method described by Pinto *et al.* (2006) using methanolic rhodanine. Standard gallic acid was used to prepare the calibration curve .

Total phenols determination:-

Total phenolic content (TPC) was determined spectrophotometrically using Folin-Ciocalteu method as described by Budrat and Shotipruk (2008). The (TPC)was expressed as gallic acid equivalents (GAE) in milligrams per gram dry material.

Antioxidant activity determination:-

The capacity of scavenging the ABTS (2, 2'- azinobis-3 EthylbenzoThiazoline 6-Sulfonic acid) free radical was determined according to the method used by Budrat and Shotipruk (2008) . For comparing the antioxidant activity of the extracts ,the reduction of the ABTS radical was measured at 734 nm using spectrophotometer. Concentration of sample producing 50% reduction of the radical absorbance (IC₅₀) was used as an index .The IC₅₀ values for various extracts were found from the plots of percent inhibition (PI) versus the corresponding concentration of the sample. The values of PI were calculated using the following equation:

$$PI\% = [1 - (A_t/A_r)] \times 100$$

Where A_t and A_r are absorbance of test sample and absorbance of the reference, respectively .Trolox was used as a reference compound.

Purification of gallic acid from *E.camaldulensis* leaves extract:-

10 gm of the leaves powder was extracted with 50% Ethanol under the optimal extraction conditions found in this study .The extract was subjected to the following purification steps:

1. Purification with 95% Ethanol :one gram of the extract obtained above was dissolved in 5ml of Ethanol 95% .After well mixing ,the mixture was centrifuged at 5000 rpm for 10 minutes.The filtrate was used in the subsequent purification step.
2. Adsorption chromatography on sephadex LH-20 :A column of sephadex LH-20 (16×1.8) cm was prepared in Ethanol 95% .The adsorption step was carried out according to the method described by Hagerman (2002). Five millilitre of sample

was added to the top of the column and the purification step was achieved by two stages: Firstly, the column was washed with ethanol 95% and the unadsorbed compounds were collected in fractions. This could be confirmed when absorbance at 280 nm approaches to zero. Secondly, Elution, the adsorbed compounds which consist gallic acid were eluted by solvent changing, acetone 70% was used for this purpose.

HPLC analysis:

Standard galic acid and the purified ones from *E.camaldulensis* leaves were analyzed by High Performance Liquid Chromatography (HPLC) according to the method described by Belur and Pallabhanvi (2011) with some modification. ODS column (250 mm× 4.6 mm, 5 µm particle) and UV detector were used for analysis. Mobile phase consisting of methanol, acetic acid and deionized water in the ratio of 15:5:80, respectively. The wave length used for detection was 280 nm.

FTIR Spectra:

FTIR spectra for both standard gallic acid and the purified ones from *E.camaldulensis* leaves, were recorded in KBr pellets.

Results and Discussion

Effect of solvent type on Gallic acid (GA) and total phenol compounds (TPC) extraction

Four solvents were used to study the effect of solvent type on extraction of gallic acid and total phenol from *E. camaldulensis*. Gallic acid is soluble in ethanol, methanol, acetone, and water therefore these solvents are selected for extraction.

Extraction techniques need to take into account the location of phenolic acids in the plant. Most phenolic acid derivatives present in the plant matrix are stored in vacuols and are commonly extracted in alcoholic organic solvents (Robbins, 2003).

Fig.1 shows the amount of GA and TPC using ethanol as a solvent extraction with different concentrations of this solvent (10 -80)%. The extraction yield ranged from (1.948-2.963) mg/gm, for gallic acid and (26.85- 36.15) mg/gm for total phenol. Lowest and highest amounts for both GA and TPC were obtained using (10 and 50)% ethanol, respectively.

It seemed that mixing ethanol with water gave best yields. Water was mixed with organic solvents for two reasons; firstly to increase the solubility of phenols by weakening the hydrogen bonds in aqueous solutions, and secondly to increase permeability of leaves tissue and thus enables a better mass transport by molecular diffusion (Wissam *et al.*, 2012; Jayaprakasha *et al.*, 2001).

Different concentrations of acetone (10-80)% was also used to determine the best concentration in extraction GA and TPC. Results in **Fig.2** showed that with increasing acetone concentration, GA and TPC yields increased. The extraction yield ranged from (2.070 -2.723) mg/gm for gallic acid and (39.825-48.675) mg/gm for total phenol compounds.

Using methanol in extraction, results in **Fig.3** showed that solvent concentration 70% gave highest yield of GA with 2.366 mg/gm and the lowest yield was obtained by using 10% methanol, while it is noticed that TPC yield decreased with increasing methanol concentration, therefore using 10% methanol in extraction gave the highest yield with 50.3 mg/gm.

The present study included using water in extraction of GA and TPC from *E. camaldulensis* leaves. Among the solvents used in the study, water gave the lowest yield of GA with 1.897 mg/gm. This result indicates that water is not efficient in

extracting phenolic acids .Water as polar solvent extracted other undesirable macromolecules such as proteins, polysaccharides, etc..... (Rostagno *et al.*, 2003; Tsao and deng, 2004) .

Deshpande *et al.*(1986) and Tiarks *et al.* (1992). Reported that solvent type can affect the amount of phenolic extracted, the biological activity ,the molecular mass and the type of phenolic compound.

Generally, the results of the present study revealed that ethanol 50% was the most efficient solvent in extracting GA from *E. camaldulensis* leaves .among the four solvents used in this study. Our results were in agreement with those reported by pawar and Surana (2010) who found that aqueous ethanol gave highest yield of GA extracted from *Caesalpinia capetala* wood in compare with aqueous acetone.

Based on the above results, ethanol 50% was chosen as the best extraction solvent and used in further experiments. From the industrialization point of view ,ethanol is a suitable extraction solvent as it possesses a lot of advantages: it is cheap, reusable as well as nontoxic (Galanakis *et al.*, 2010) .

Effect of extraction time on the extraction of gallic acid and phenolic compounds:

Extraction of GA and TPC from *E. camaldulensis* leaves was monitored at various extraction times (6,12,24,36 and 48)hrs .As observed in **Fig.4** GA yield reached its maximum after 24 hrs with 2.963 mg/gm .Beyond this point the yield remained stable with increasing the extraction time .This observation was well explained by Fick's second law of diffusion ,which stated that final equilibrium will be achieved between the solute concentrations in the solid matrix (plant matrix)and in the bulk solution (solvent) after a certain time ,hence an excessive extraction time was not useful to extract more phenolic antioxidants (Silva *et al.*, 2007) .

Therefore, the optimum extraction time appeared to be 24 hrs .As regards total phenol compounds, it is noticed that TPC increased with increasing the extraction time, therefore TPC yield reached to 44.678 mg/gm after 48 hrs of extraction .

Results obtained from this study disagree with those reported by Pawar and Surana (2010) who found that maximum yield of gallic acid extracted from *Caesalpinia capetala* wood was reached after 48 hrs.

Effect of temperature on the extraction of gallic acid and total phenol compounds:

The effect of temperature on GA and TPC extraction was studied. As shown in **Fig.5** best yields of GA and TPC were obtained at 40C° with (2.963 and 47.75) mg/gm, respectively. It seemed that increasing temperature more than 40 C° caused decreasing in both GA and TPC yields. Results obtained in the present study disagree with those reported by Pawar and Surana (2010) who found that 65-70 C° was the optimum temperature for extraction gallic acid from *Caesalpinia capetala* wood.

Increasing temperature could enhance both solubility of solute and diffusion coefficient but beyond certain temperatures phenolic compounds can be denaturated (Spigno *et al.*, 2007) . Additionally, high temperature may encourage solvent loss through vaporization and increase the cost for extraction process from the industrialization point of view (Hismath *et al.*, 2011) .

Antioxidant activity :

Scavenging activity against free radical ABTS was employed to evaluate the antioxidant capacities of *E. camaldulensis* leaves extracts. Antioxidant was represented by IC₅₀ index which is the concentration of sample producing 50% reduction of the radical absorbance.

The IC_{50} values of the solvents (10,30,50,70 and 80)% ethanol, acetone and methanol extracts were (50,50,50,50.75), (50,50,50,25,50) and (75,50,25,25,25) $\mu\text{g/ml}$, respectively (**Fig. 6,7 and 8**), respectively, too. These activities were comparable to that of trolox. Results showed no correlation between gallic acid or total phenol content and antioxidant activity. This result was in agreement with some previous studies. Sengul *et al.* (2009) found similar results in which there were no correlation between total phenolic content of some medicinal plants and antioxidant activity. Results also showed that *E. camaldulensis* leaves extract is a good source of antioxidants since some of the concentrations of the extraction solvents used in this study gave equal IC_{50} to that of the standard (trolox) which was 25 $\mu\text{g/ml}$.

Because radical scavenging involves donation of a hydrogen atom or electron, there might be some other chemical compounds responsible for this action other than phenolics in this species (Jimoh *et al.*, 2008).

Fig.9 shows the effect of various extraction temperatures on scavenging activity of *E. camaldulensis* leaves extracts against ABTS free radical. By using 35°C as extraction temperature, the obtained extract exhibited highest antioxidant activity (IC_{50} = 10 $\mu\text{g/ml}$) while lowest antioxidant activity was obtained from the extract subjected to 40°C extraction temperature. There is also no correlation between either gallic acid or total phenol and antioxidant activity.

The present study included the effect of extraction time on the extraction of antioxidants. As shown in **Fig.10**, IC_{50} at the short extraction times (6 and 12) hrs were 25 $\mu\text{g/ml}$ which is higher than that at the long times (24,36 and 48) hrs which was 50 $\mu\text{g/ml}$. It is obvious that at short extraction times no degradation occurs to the antioxidant compounds.

Purification of gallic acid:

Gallic acid was extracted from *E. camaldulensis* leaves under the optimum conditions obtained from the present study which included using 50% ethanol as a solvent extraction at 40°C for 24 hrs. The resulted extract was the "crude extract" which was subjected to two purification steps.

Firstly, dissolving the crude extract in ethanol 95% and centrifuging resulted in good purification step by removing the insoluble compounds, while filtrate which contains gallic acid and other tannins gave positive reactions with Folin-ciocalteu. This step was very necessary to the extract to become ready to the subsequent purification step. Generally purification with solvents is a fast method which is very suitable especially when the extracted compounds are unstable (Riviere, 1977).

Secondly, the adsorption step, chromatography on sephadex LH-20 is very useful for separating tannin from nontannin phenolics or for fractionating hydrolysable tannins. Sephadex LH-20 sorbs tannins in alcohol and releases them in aqueous acetone (Hagerman, 2002).

After sample loading on the top of the column, gallic acid and other tannins appeared visible as a brown band of pigments. The column was washed with ethanol 95% to remove all nontannin phenolics. Washing is continued until all the unadsorbed compounds leave the column. This was confirmed by monitoring absorbance at 280 nm until no longer changing and is near base line.

The column was subjected to elution by changing the solvent used in separation, acetone 70% was used instead of ethanol 95%. Elution was continued until the sephadex became white. Elution was stopped after collecting 30 fractions. Gallic acid was appeared in the fractions (4-20) accompanied with positive reactions with Folin-ciocalteu specialized in the quantitative determination of total phenols (**Fig.11**). It has been

noticed that increasing in gallic acid concentration was paralleled with increasing in the total phenols concentration. Finally, 6 mg of pure gallic acid was obtained.

HPLC analysis:

Fig.(12) showed the results of HPLC analysis, in which good resolution was obtained for the two analysed gallic acids. It is noticed that the purified GA is identical to the standard one in HPLC chromatogram. Results also showed the appearance of three peaks: one major and two minors.

Major peaks appeared at (11.250 and 11.069) minutes while the minor peaks were appeared at (12.561 and 12.426) minutes and (13.717 and 13.975) minutes for the purified GA and the standard, respectively.

FTIR Spectra:

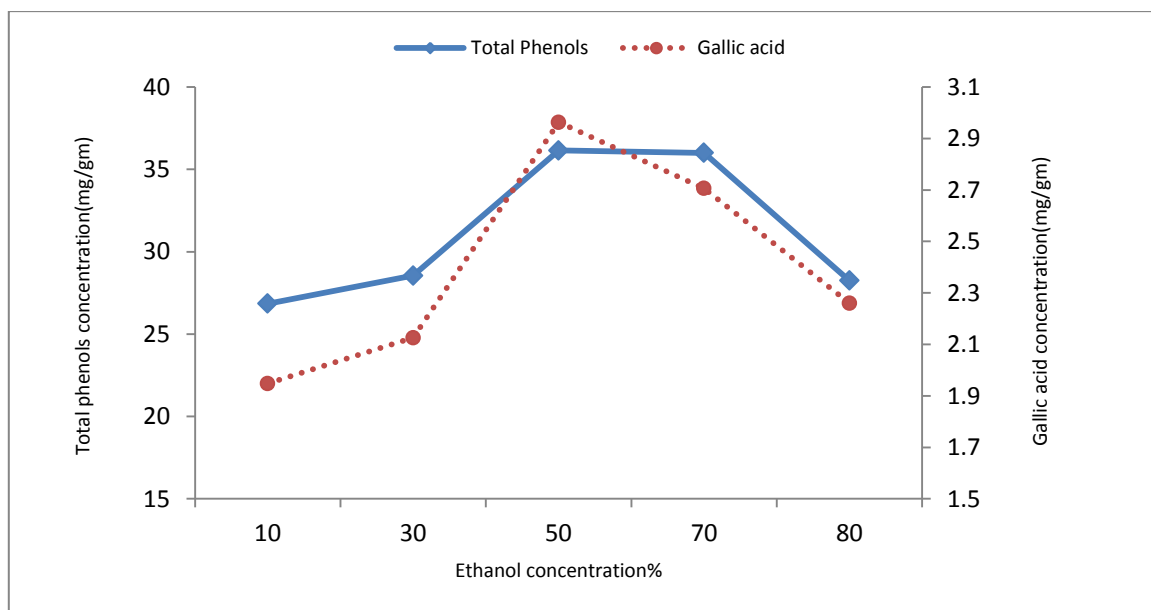
In the FTIR spectrum shown in **Fig (13 a and b)**, Results showed that the spectrum of purified gallic acid was identical to the standard one. The absorption band around 3387 cm^{-1} is attributed to the O-H stretching due to the presence of hydroxyl group and physically adsorbed water molecule (Braterman *et al.*, 2004). The appearance of band around 1454 cm^{-1} is due to CH bending vibration. Bands around 1338 cm^{-1} is attributed to symmetrical stretching of carboxyl group. At 1037 cm^{-1} associated with C-O stretching group and around 744 cm^{-1} is associated with C-C benzene ring vibration (Ghotbi *et al.*, 2009).

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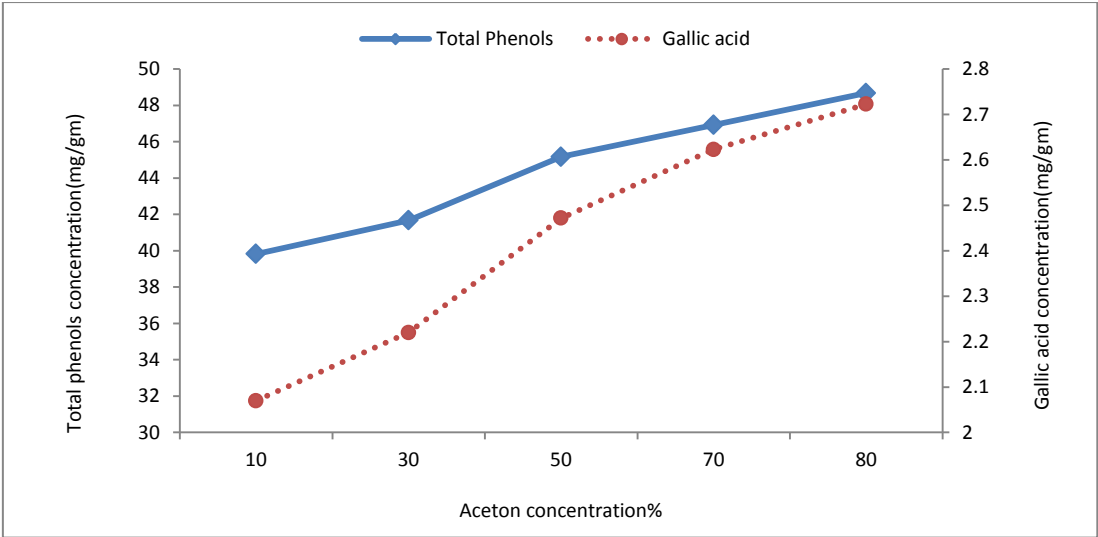
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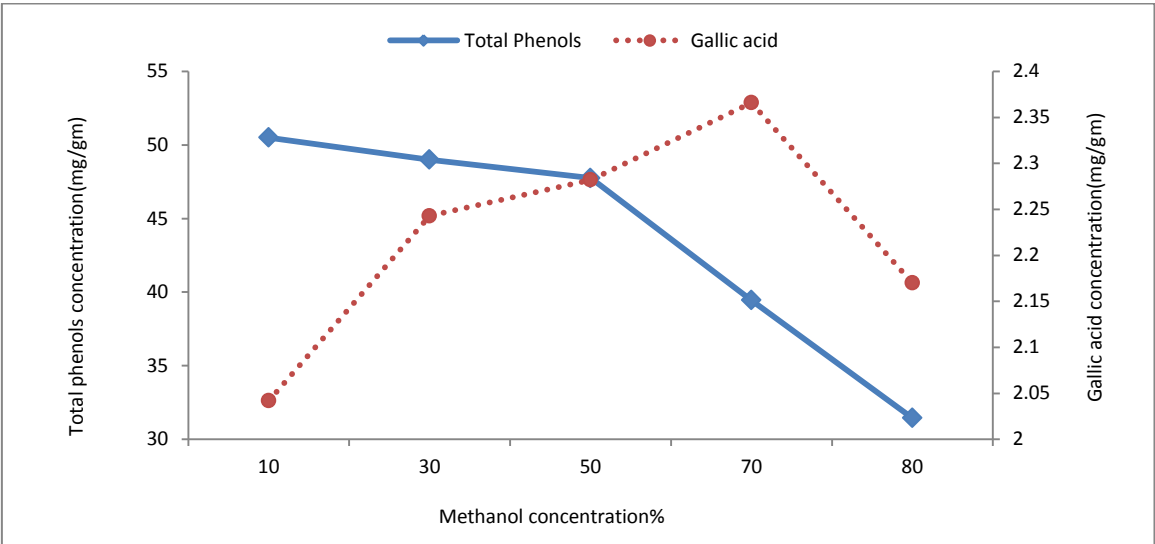
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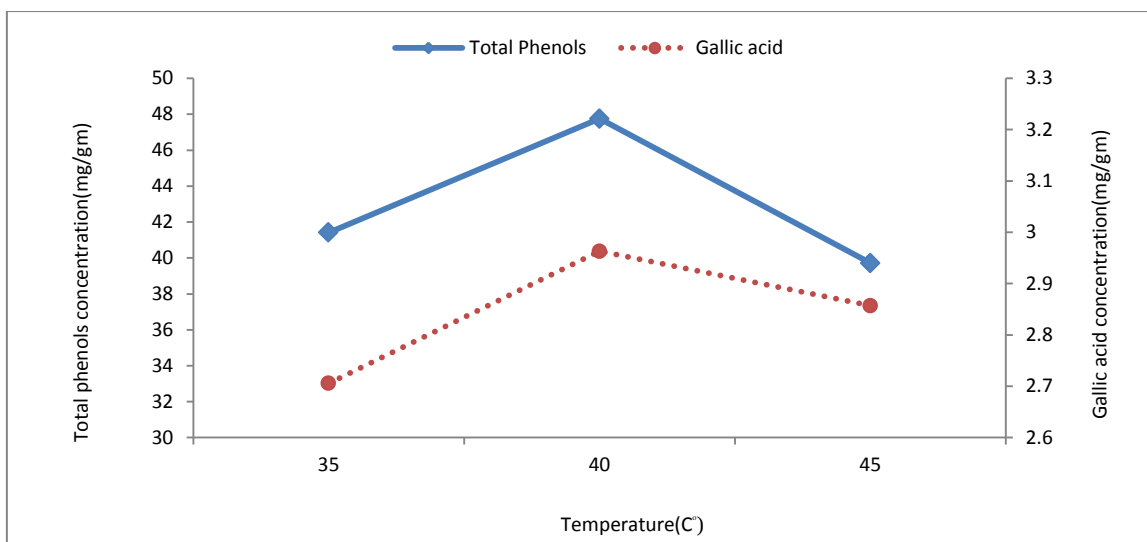
Figure(1): effect of ethanol concentration on extraction of gallic acid and total phenols.



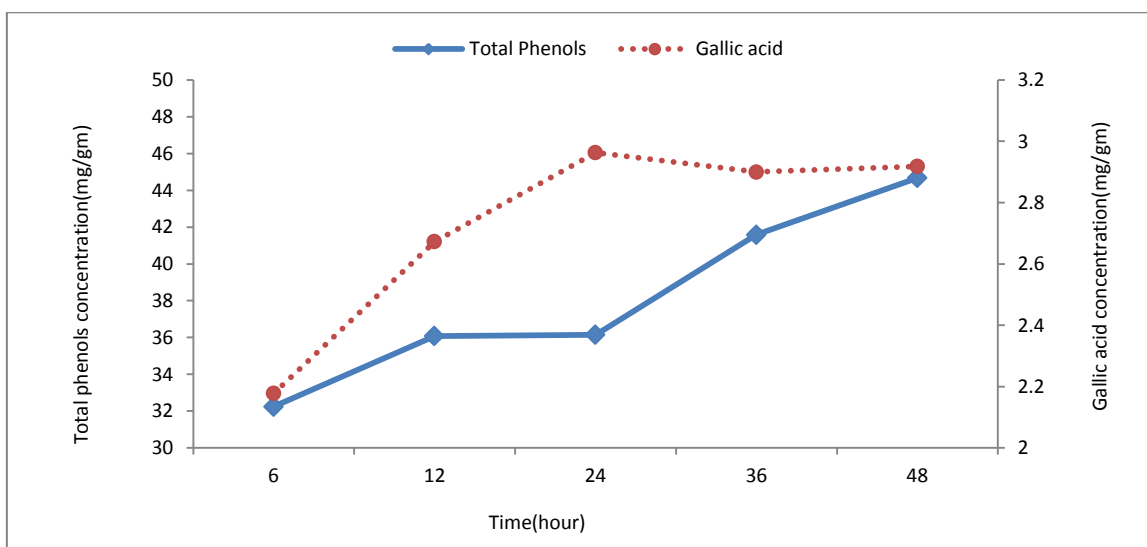
Figure(2): effect of acetone concentration on extraction of gallic acid and total phenols.



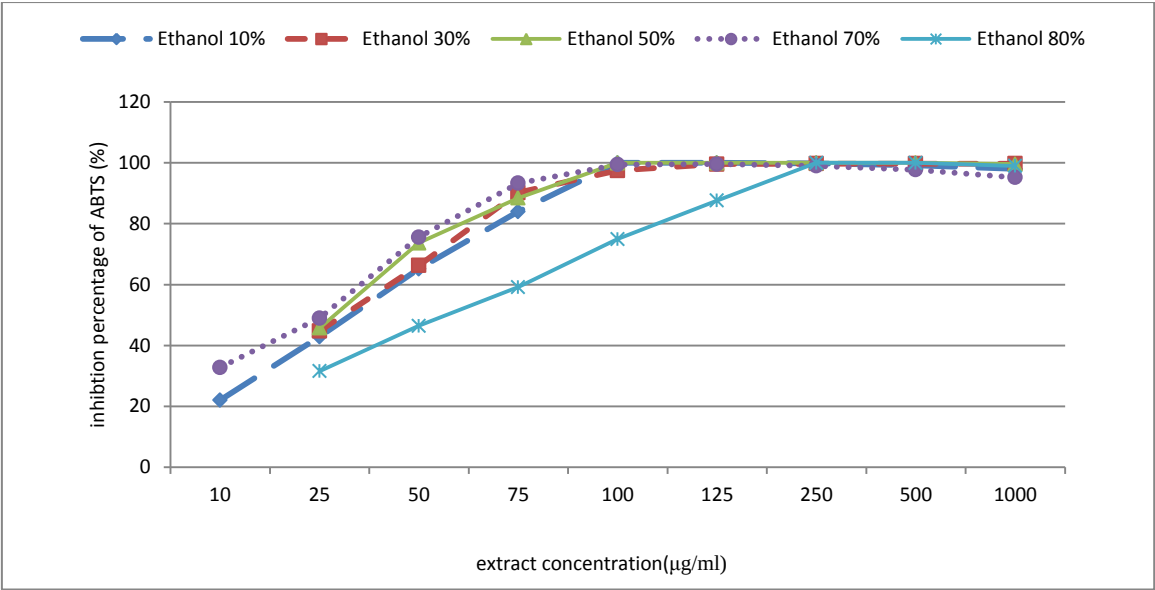
Figure(3): effect of Methanol concentration on extraction of gallic acid and total phenols.



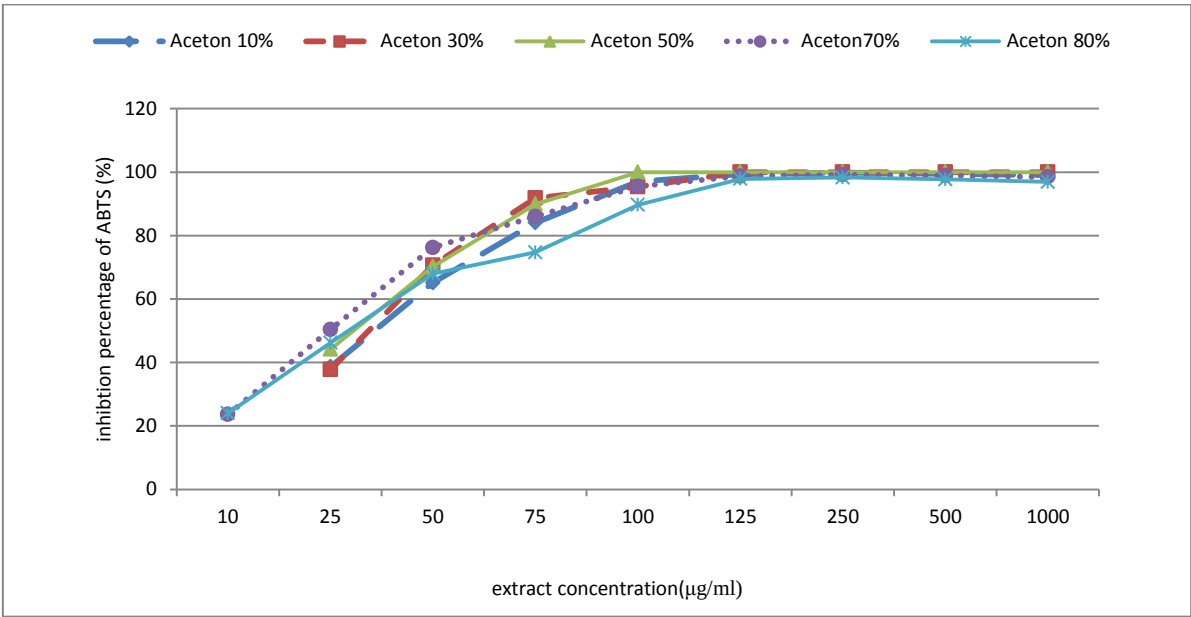
Figure(4): effect of temperature on extraction of gallic acid and total phenols.



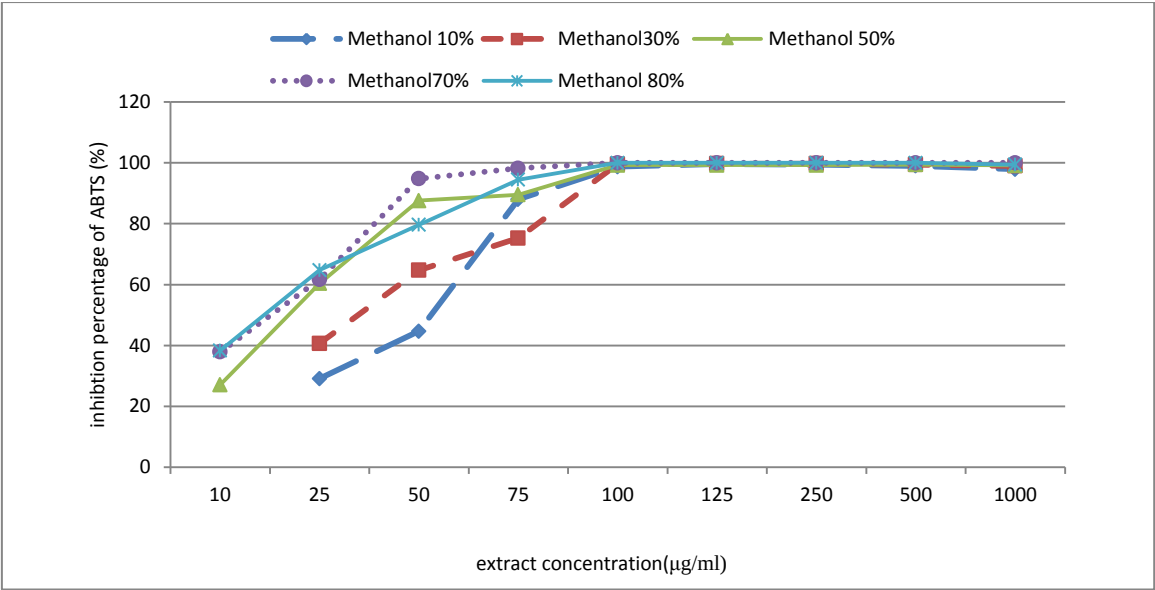
Figure(5): effect of time on extraction of gallic acid and total phenols.



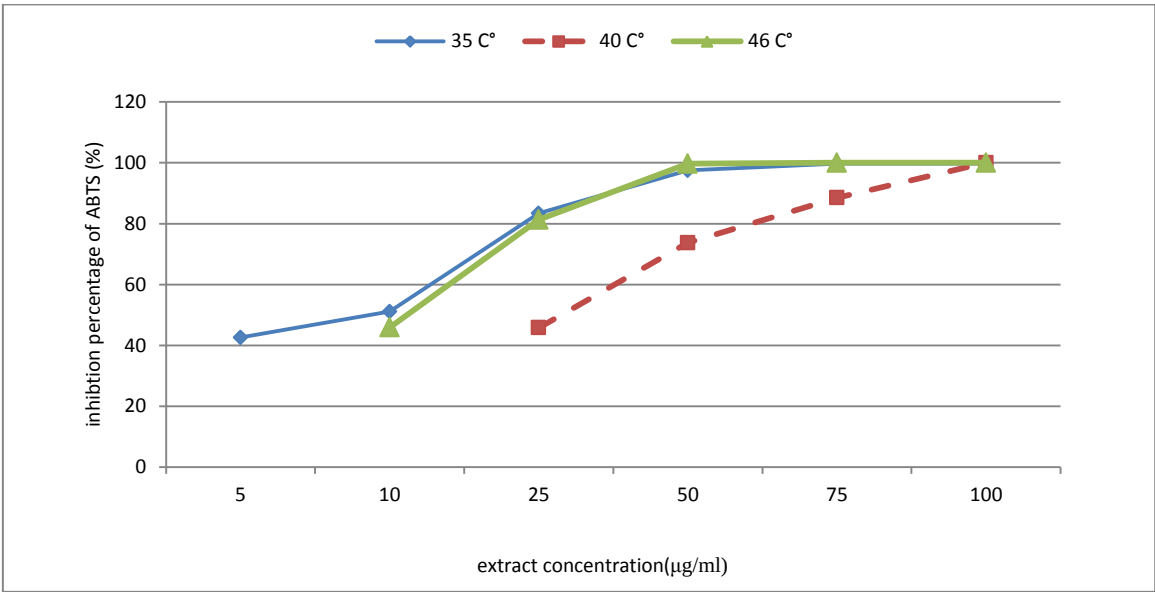
Figure(6): Antioxidant activity of different concentrations of ethanol extract.



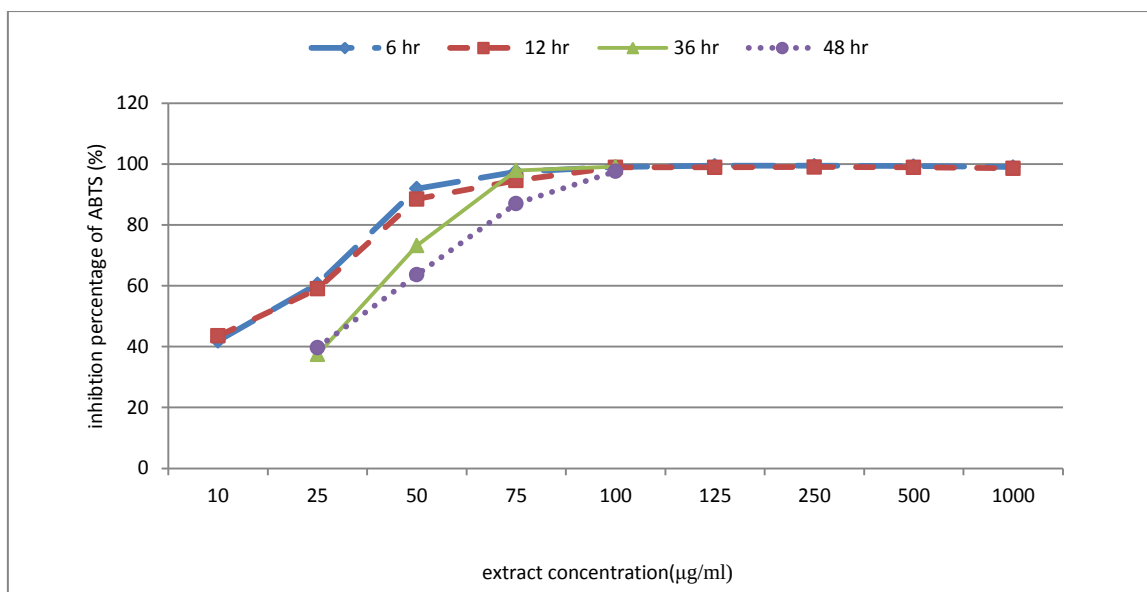
Figure(7): Antioxidant activity of different concentrations of acetone extract.



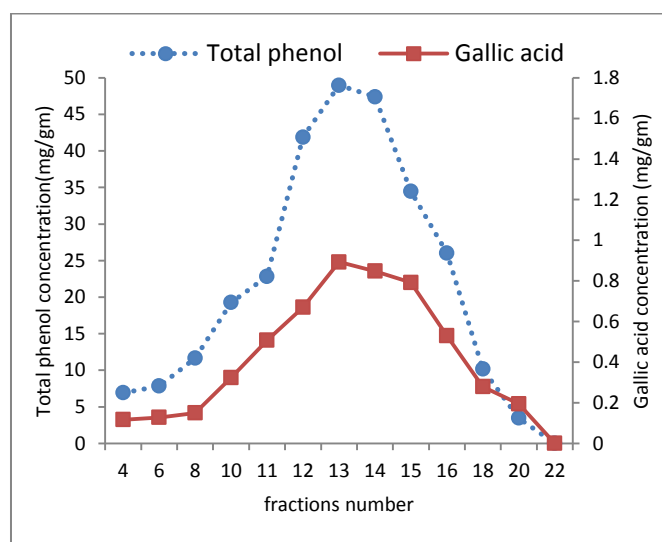
Figure(8): Antioxidant activity of different concentrations of methanol extract.



Figure(9): Antioxidant activity of extracts at different temperatures.



Figure(10): Antioxidant activity of extracts at different incubation times.



Figure(11): Adsorption chromatography of gallic acid and total phenol by sephadex LH-20.

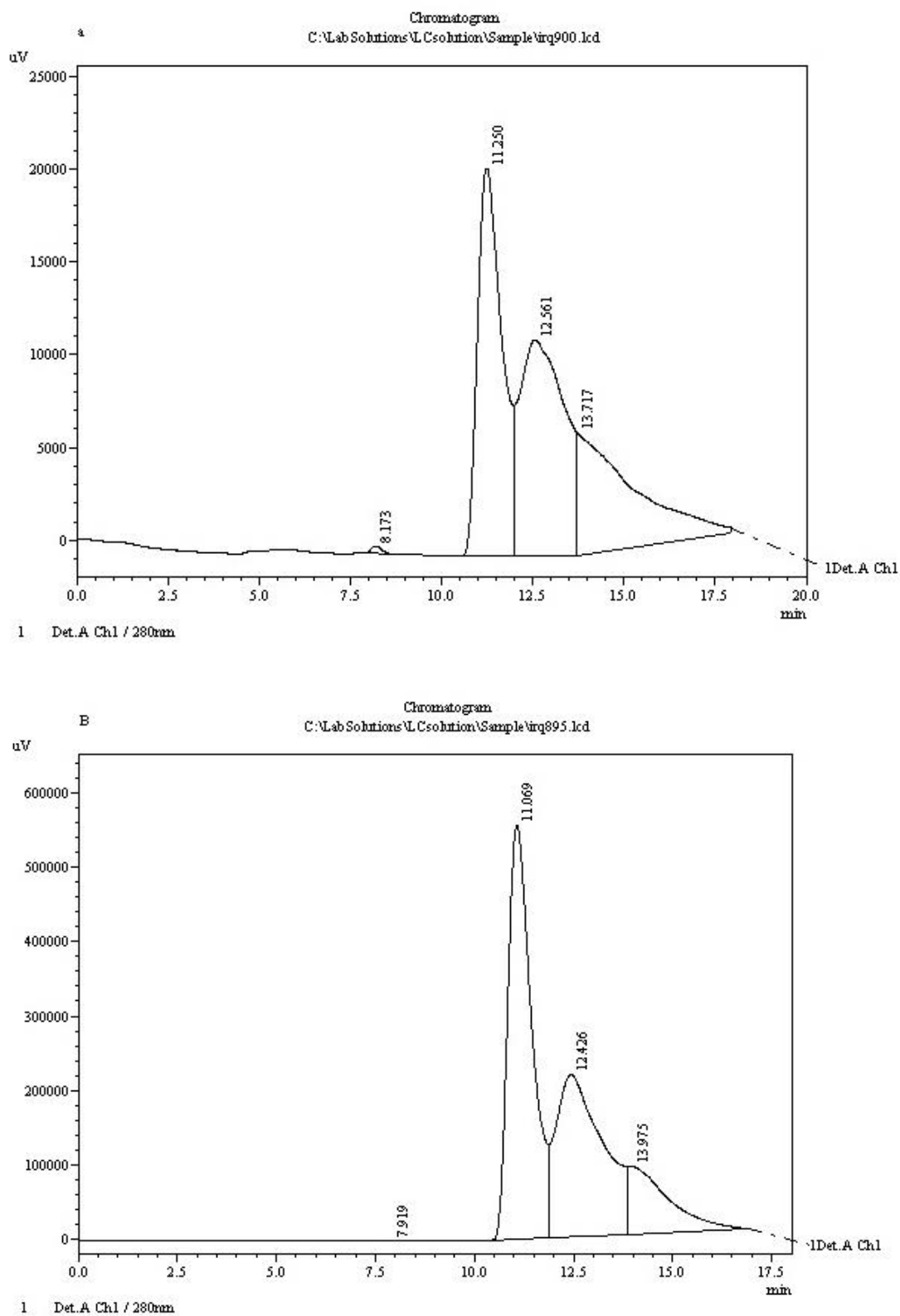
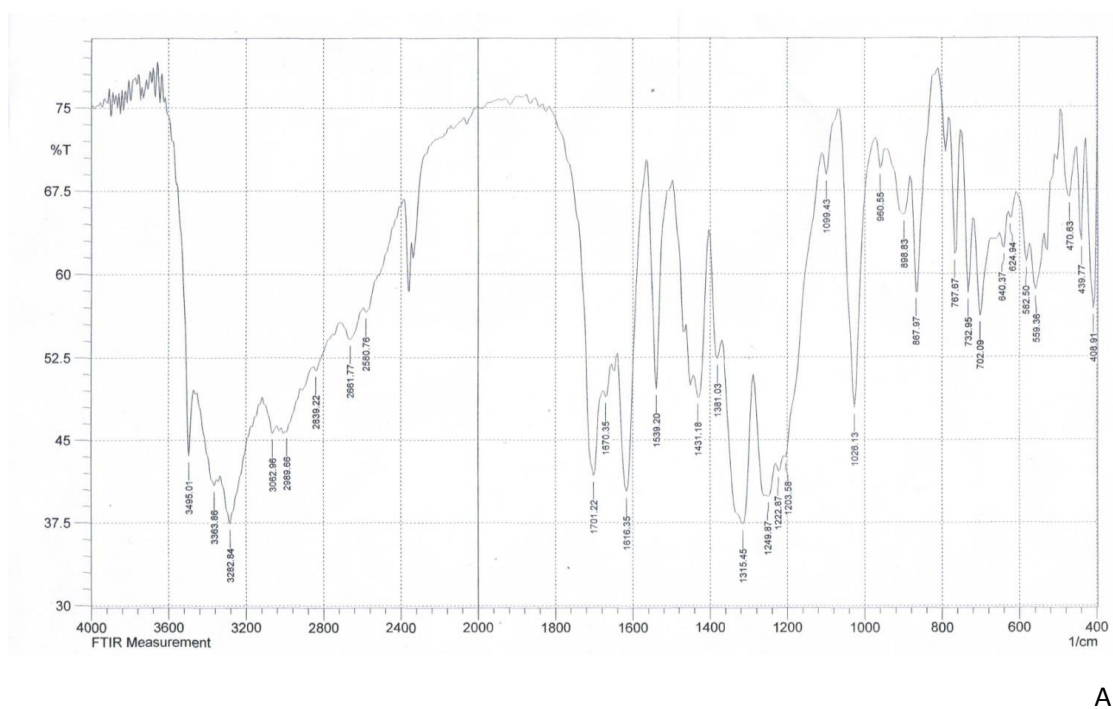


Fig . (12): HPLC chromatogram of purified gallic acid (A) and standard gallic acid (B).



A

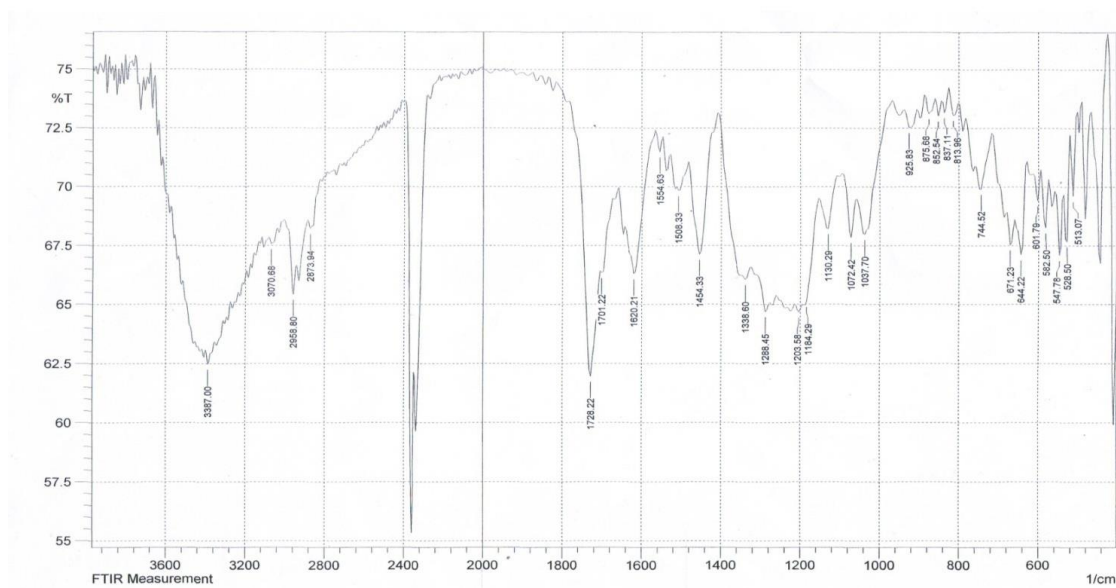


Fig . (13): FTIR spectrum of purified gallic acid (A) and standard gallic acid (B).