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Characterization and Evaluation of Medicinal Activity of Three Phenolic Compounds From Iraqi *Juglans regia* L. Cortex Against Pathogenic Fungi Causing Intestinal Inflammatory in Children

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ABSTRACT— Juglans regia L. is considered as one of medical plants hasing biochemical activity to treat various diseases resulting from infection by some pathogenic micro-organisms. The current research was achieved to estimate and evaluate the medicinal activity of three phenolic compounds mixture containing 4-hydroxybenzaldeyhde, 5-(3-Hydroxypropyl)- 2,3-dimethoxy phenol and vanillic acid which were isolated and identified from Juglans regia L. cortex against four pathogenic fungi causing intestinal inflammatory in children. The phenolic mixture recorded values of inhibition zone diameters equal to 21, 30, 37, 51,52 and 52 mm at concentrations of 5, 10, 25, 50, 75, and 100 mg/ml respectively against growth of the pathogenic fungus represented by Candida albicans while the same concentrations gave inhibition values were 32, 39, 44, 46, 50 and 51 mm respectively towards Candida globate. In regard to Candida krusei, the phenolic mixture concentrations showed diameters of inhibition equal to 35, 41, 46, 53, 53 and 53 mm respectively while the values of same concentrations recorded inhibition values were represented by 25, 30, 35, 38, 52 and 52 mm against Candida tropicalis growth. The active phenolic metabolites compounds recorded a higher medicinal activity than some antibiotics used against these pathogens.

KEYWORDS: *Juglans regia*, 4-hydroxy benzaldehyde 5-(3-Hydroxypropyl)- 2,3-dimethoxy phenol, vanillic acid, Pathogenic fungi, medicinal activity.

1. INTRODUCTION

Medicinal plants are considered as important sources to produce the biochemical natural products in secondary metabolism from various metabolic pathways catalyzed by multi - enzymes. These biochemical compounds are represented by phenols (flavonoids, phenolic acids, tannins, glycosidic phenols), alkaloids, steroids, terpenes, essential oils and glycosides [1,2]. The medical activity of these chemical compounds comes from existence of different functional chemical groups such as hydroxyl, phenolic, imine and carboxylic groups. So the plant which has these active chemical compounds is considered as medicinal plant. As a result, the abundance of these secondary metabolites leads to treat various diseases caused by infection with different pathogenic micro-organisms such as bacteria and fungi [3,4,5]. Juglans regia (walnut) is considered as one of necessary sources for food in the worldwide especially in China, and this plant belongs to Juglansdecease family. Also all parts of this plant such as leaves, stems, barks, fruits, seeds, flowers and pericarps indicate an important source for providing various therapies to treat various diseases which infect human being [6,7]. Different chemical compounds such as polyunsaturated fatty acids, tocopherol, phenols, hormones and melatonin were isolated, purified and identified from Juglans regia and also these biochemical metabolites were carried out as benefit healthy substances and antioxidants compounds [8,9]. Also some studies were acheived to investigate the effects of Juglans nigra green husk on

pathogenic fungi represented by Candida albicans in laboratory rat's females and this proved that using of 4% of Juglans nigra has killed significantly Candida albicans in rats after one week and this treatment was compared with clotrimazole. A research ensured the biochemical activity of various compounds abundant in this medicinal plant (10). Also the different parts of *Juglans regia* were carried out as antioxidants and also the study proved and evaluated the antioxidant activity of various active chemical compounds isolated from walnut plant and this biochemical metabolite were done for scavining assay of 2,2-diphenyl-1-picrylhydazyl (DPPH) radical. The antioxidant activity was measured for the concentrations ranged between (25.32-79.35%), also the total phenolic compounds amount was 119.8 mg and tanninic compounds amount was 2.645% [11]. Pathogenic fungus is a micro-organism lives in various positions in human and animal body also it is present on surfaces of different plants, in meat and also it is greatly abundant in the air. These pathogens cause multi-infections leading to get the disease resulting from various disorders in metabolic pathways. There are many classes of pathogenic fungi are represented by Candida albicans, Candida Krusei, Candida tropicals, Candida glabrata, Candida parapsilosis, Candida dubliniensis, Candida lusitaniae, Candida auris, Candida antarctica, Candida oleophila and Candida humilis [12,13]. The current research was achieved to determine, investigate and evaluate the antifungal activity of mixture of three phenolic compounds isolated from Iraqi Juglans rigia cortex.

2. Materials and Methods

Juglans regia L. (walnut) cortexes were collected and gotten from local market of Al-Ashar quarter at Basrah Governorate in Iraq. This medicinal plant was taxonomied in biology department in education college for pure sciences at university of Basrah. Then the plant cortexes were washed by cold distilled water, dried in shadow at room temperature, ground as powder and finally placed in dark plastic containers for multi-usings.

2.1 Culture medium

Sabourad dextrose agar (SDA) and potato dextrose agar (PAD) were carried out in this study as culture media. These media were chemically prepared according to scientific information determined by manufacturing company [12].

2.2 Pathogens isolates

The pathogenic fungi strains represented by *Candida albicans*, *Candida Krusei*, *Candida tropicals* and *Candida glabrata* were isolated and characterized in fungi laboratory at college of marine science in university of Basrah - Iraq.

2.3 Isolation of phenolics from Juglans regia

Defatted fifty grams of *Juglans regia* cortex powder were mixed with 250 ml of hydrochloric acid (2% v/v) and the mixture was put in the waterbath at 90 ºC for eight hours. Then the mixture was filtered by Buchner funnel and the precipitate was removed. After that the filtrate volume was measured then a volume of diethylether was added to filtrate with the same volume of filtrate. The contents were put in waterbath at 30ºC for five minutes then the mixture was concentrated by using rotary evaporator finally the phenols were collected with weight equal to 1.8 g [14].

2.4 Preliminary qualitative analysis tests

Various qualitative reagents were used to detect on phenolic compounds abundant in phenols extract such as ferric chloride (1% v/v) for phenols, lead acetate (1% v/v) for tannins and alcoholic potassium hydroxide (5N) for flavonoids [15,16]. Molisch's reagent for carbohydrates, Benedict's reagent for glycosides

Dragendroffe's reagent for alkaloids mercuric chloride (5% m/v) for saponin and ninhydrin (1% w/v) for amino acid [17,18].

2.5 Thin layer chromatography of phenolic extract

The thin layer chromatography (TLC) technique was carried out to separate all phenolic compounds and insurance of their purity. Sixty-five µl of phenols was toulerenced on the surface of a glass plate (4 * 12 cm) covered with silicagel. The eluent system (butanol-acetic acid-water) was used with ratio equle to (4: 2: 0.5) as mobile phase to separate each phenolic compound alone. After that, glass plate was dried by hair dryer than various spots were developed by using UV-lamp at 233 nm, iodine vapur and ferric chloride (1% w/v) and then rate of flow (Rf) values were calculated for separated compounds [19].

2.6 Gas Chromatography-Mass spectroscopy technique of phenolic extract

The active phenolic compounds existing in phenols extract of *Juglans regia* cortexes were separated and identified by using Gas Chromatography- Mass Spectrum (GC-MS) instrument type simadzu GC-MS-QP-zolo ultra system has automatic sampler CTC analysis combi PAL robotic arm. This characterization of phenolic compounds was established in laboratories of Basrah oil company –ministry of oil in Iraq.

2.7 Column chromatography technique of phenolic extract

The phenolic compounds separated by thin layer chromatography, were separated alone by column chromatography method. In this technique, a column is made of glass was carried out with optimization conditions were 1 cm radius, 50 cm length, silicagel was stationary phase and butanol-acetic acid-water (BAW) solvent was used as mobile phase with ratio equal to (4: 1: 0.8). The separation time was 50 minutes with the volume of each separated compound was 3 ml. After that TLC was carried out for each phenolic compound then Rf values were measured for all phenols [20].

2.8 Infra-red spectroscopy of phenolic compounds

Furrier- transformation infra-red (FT-IR) spectra for all phenolic compounds isolated and separated from Juglans regia cortexes were recorded using Shimadzu FT-IR-8400-S spectrophotometer made in Japan. All phenols samples were carried out by mixing each phenolic compound with potassium bromide (KBr) as a disc. The measurements of all spectra were achieved in spectral range (4000-500 cm-1).

2.9 Medicinal activity and minimal inhibitory concentration of phenolic compounds against pathogens

Series concentrations of phenolic compounds mixture isolated and identified from *Juglans regia cortexes*. were prepared equal to 5 ,10,25, 50, 75 and 100 mg/ml and they were applied for against growth of the pathogenic fungi represented *Candida albicans, Candida Krusei, Candida tropicals* and *Candida glabrata* by using disc diffusion procedure. The medicinal activity was carried out against pathogenic fungi by using Petri-dishes having SDA and PDA culture media then they were put in autoclave at 118 ºC for 18 minutes and 1.5 atm pressure. The various concentrations of phenolic compounds were treated with fungi existing in Petri dishes which after that were autoclaved at 25 ºC for eight days. Distilled water with volume equal to 6 ml was mixed with pathogens for formation of fungal suspension then all dishes were autoclaved at 25 ºC for 3-6 days. Finally, the values of inhibition zone diameters were calculated [21].

2.10 Medicinal activity of some antibiotics against pathogens

Various antibiotics were represented by ketoconazole, miconazol and flouconazol were carried out against growth of same pathogenic fungi by the same method of medicinal activity of phenolic compounds mixture belonging to Juglans regia cortex. Also the inhibition zone diameters values were measured [21].

3. Result

Phenols are active metabolites abundant in different medicinal plants. In the current study, phenolic compounds were isolated from *Juglans regia* cortexes with yield equal to 1.8 g with extraction percentage equal to 3.6 %. Table (1) indicates the results of preliminary qualitative analysis of phenolic extract.

Table (1) Preliminary qualitative analysis of phenols isolated from Juglans regia cortex

Test	Chemical result	Indications	Conclusions
Ferric chloride (1%)	+	Bluish-green colour was formed	Phenols are present
Lead acetate (1%)	–	No white to brown precipitate	No tannins
Ethanolic potassium hydroxide (5N)	–	No yellow precipitate	No flavonoids
Molisch	–	No violet ring	No carbohydrates
Benedict	–	No red precipitate	No glycosides
Dragendroff	–	No orange precipitate	No alkaloids
Mercuric chloride (5%)	–	No white precipitate	No saponin
Ninhydrin (1%)	–	No violet colour	No amino acids

From table (1), the results ensured attendance of phenols only but flavonoids, tannins, carbohydrates, glycosides, alkaloids, saponin and amino acids were not found.

The results of thin layer chromatography (TLC) of phenols showed presence of three green spots developed by ferric chloride (1%), so this status proves presence of three phenolic compounds with Rf values are 0.3, 0.6 and 0.82 also and these compounds have a high purity. The developing of the phenolics was done by iodine vapour which showed of brown spots but by using UV-lamp, the spots were lights violet. The developing of phenolic compounds by ferric chloride (1%) showed formation of bluish-green spots as in table (2).

Table (2): results of thin layer chromatography of phenols isolated from *Juglans regia* cortex

Eluent system	Reagent	Spots No.	Result	Rf values	Indication and conclusion
Butanol Acetic acid water (4: 2 : 0.5)	Eyes	3	Light brown spots	0.3 0.6 0.82	Pure compounds
	I2-vapour	3	Brown spots	0.3 0.6 0.82	Attendance of organic compounds
	UV-lamp	3	Light violet spots	0.3 0.6 0.82	Attendance of double bond conjugated system
	Ferric chloride (1%)	3	Bluish-green spots	0.3 0.6 0.82	Attendance of phenolic compounds

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Gas chromatography – mass spectroscopy (GC-MS) results showed abundance of three peaks and this statement means presence of three phenolic compounds in phenolic extract, then mass spectra were recorded for all these active chemical compounds. The mass spectra indicate and ensured presence of the compounds represented by 4-hydroxy benzaldeyhde, 5-(3-Hydroxypropyl)- 2,3-dimethoxy phenol and vanillic acid. Figures (1,2 and 3) show the mass spectra and fine chemical structures for 4-hydroxy benzaldeyhde, 5-(3-Hydroxypropyl)- 2,3-dimethoxy phenol and vanillic acid.

Figure (1): Mass spectrum of 4-hydroxy benzaldeyhde

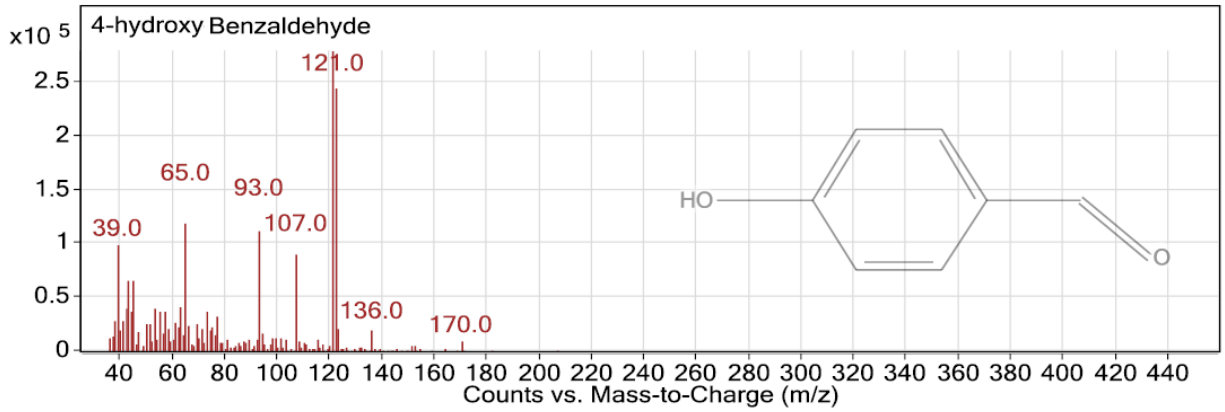


Figure (2): Mass spectrum of 5-(3-Hydroxypropyl)- 2,3-dimethoxyphenol

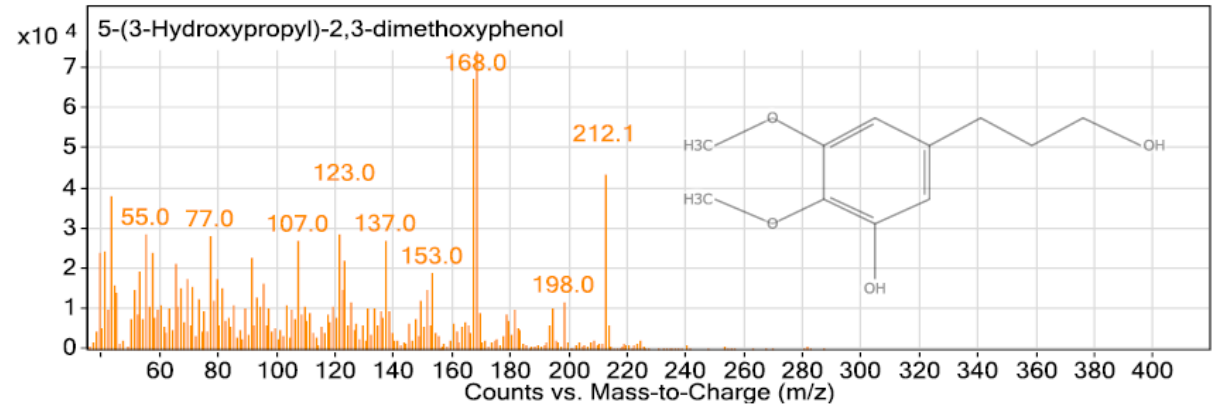
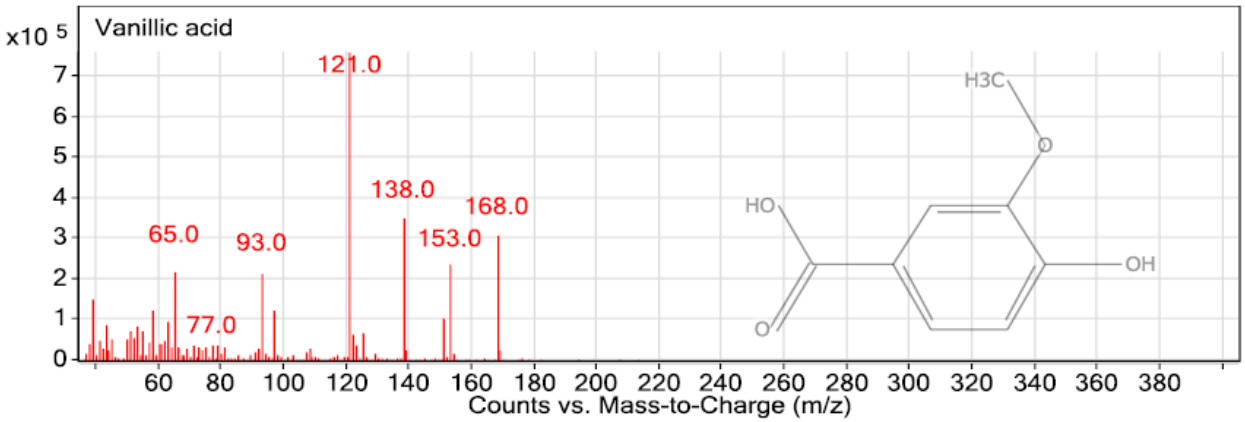


Figure (3): Mass spectrum of vanillic acid



Gas chromatography technique was applied for separation each phenolic compound alone then the FT-IR spectra of 4-hydroxy benzaldeyhde, 5-(3-Hydroxypropyl)- 2,3-dimethoxy phenol and vanillic acid were recorded as in figures (4,5 and 6) respectively.

Figure (4): Forrier transformation infrared (FT-IR) spectrum of 4-hydroxy benzaldeyhde

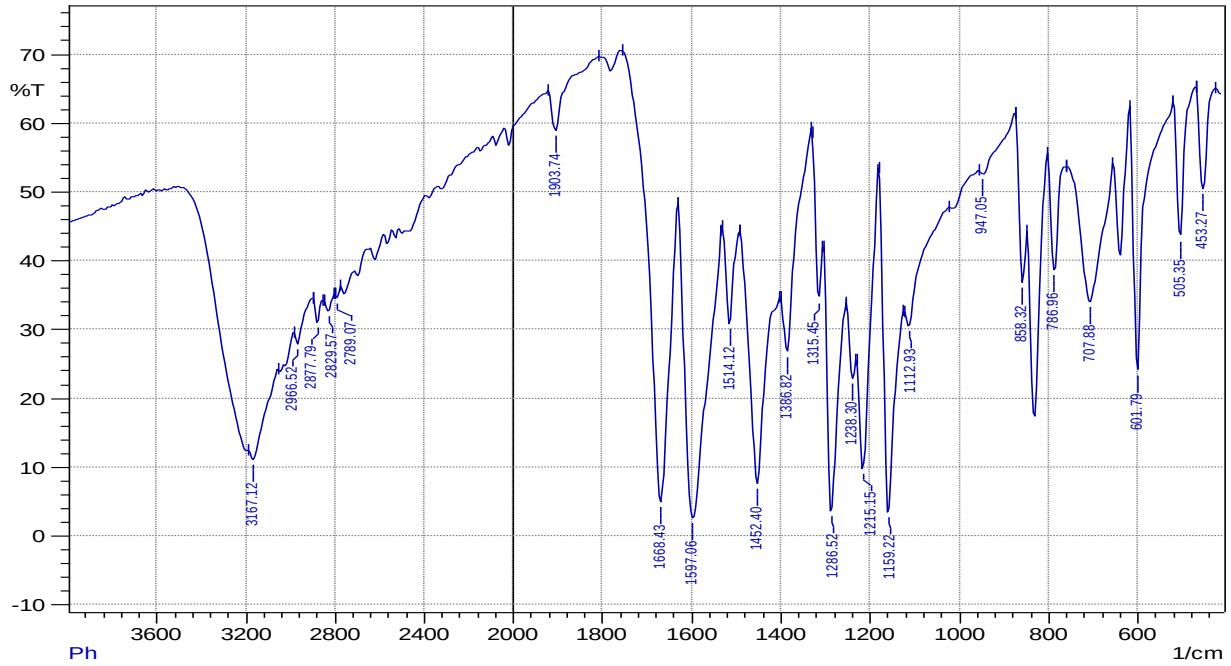


Figure (5): Forrier transformation infrared (FT-IR) spectrum of 5-(3-Hydroxypropyl)- 2,3-dimethoxyphenol

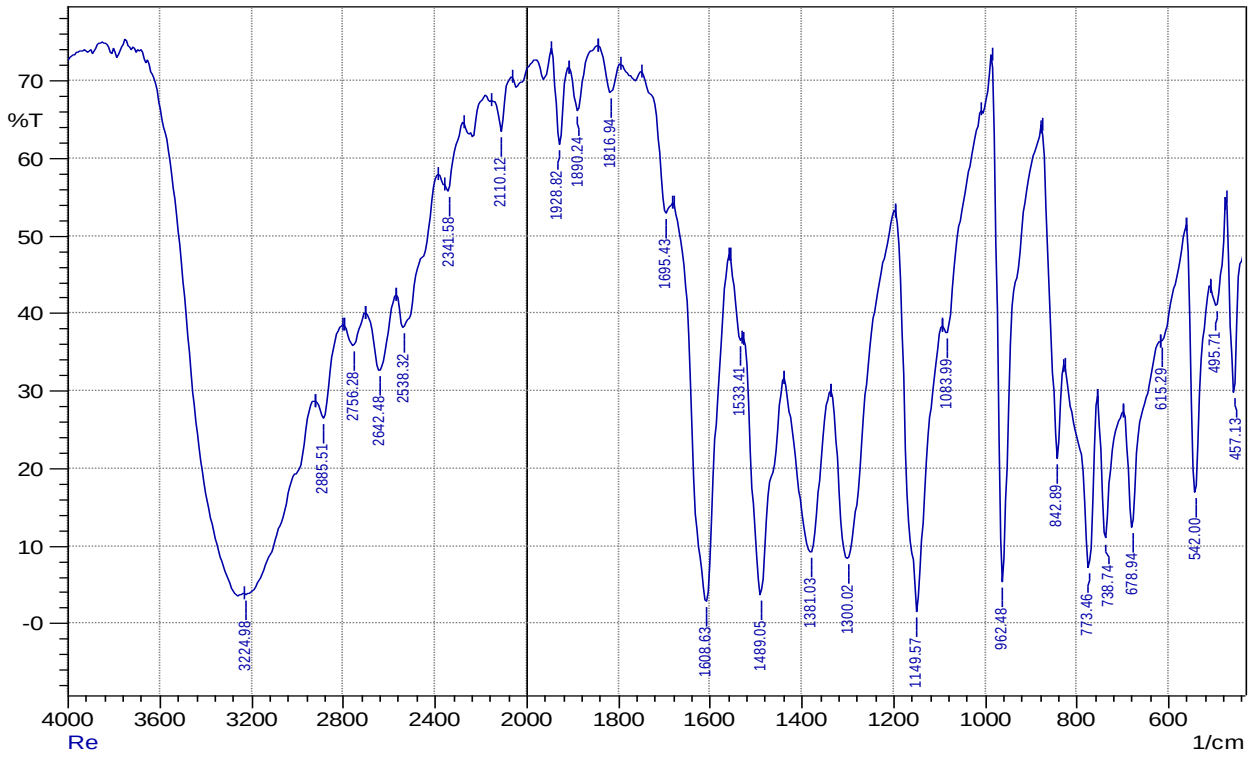


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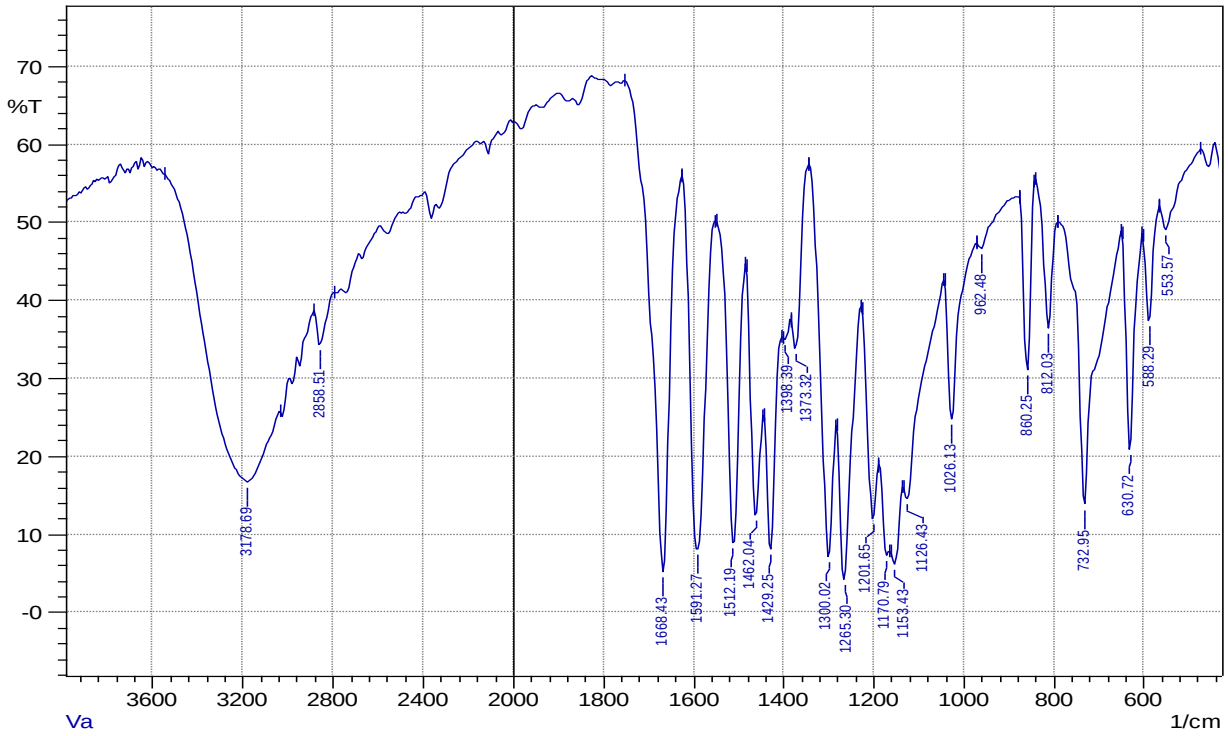
Figure (6): Forrier transformation infrared (FT-IR) spectrum of vanillic acid



All phenolic compounds were used as active chemical compounds against pathogenic fungi like *Candida albicans, Candida Krusei, Candida tropicals* and *Candida glabrata* which cause various intestinal inflammatory in children. The medicinal and antifungal activity of phenolics mixture of *Juglans regia cortexes* is indicated in table (3).

The inhibition zone diameters of phenolics were recorded to be 21, 30, 37, 51, 52 and 52 mm at concentrations 5, 10, 25, 50, 75, and 100 mg/ml respectively against growth of *Candida ablicans* whereas the same phenols concentrations showed values of inhibition zone diameter equal to 32, 39, 44, 46, 50 and 51 mm towards *Candida glabrata* fungus. Concerning *Candida krusei*, the concentrations of phenolics gave inhibition zone diameters were represented by 35, 41, 46, 53, 53 and 53 mm while the same concentrations showed inhibition values equal to 25, 30, 35, 38, 52 and 52 mm against growth of *Candida tropicalis* fungus.

Table (3) inhibition zone diameter of phenolics mixture of Juglans regia cortex against pathogenic fungi causing intestinal inflammatory in children

phenolics mixture conc. mg/ml	inhibition zone diameter (mm)			
	Candida ablicans	*Candida glabrata*	*Candida krusei*	*Candida tropicalis*
5	21	32	35	25
10	30	39	41	30
25	37	44	46	35
50	51	46	53	38
75	52	50	53	52
100	52	51	53	52

Table (4) : represents inhibition zone diameters of some antibiotics represented by ketoconazole, miconazole and fluconazole against the same pathogenic fungi used in the current study.
The maximum inhibitory dose (200 mg) of ketoconazole drug recorded values of inhibition diameters equal to 41, 38, 42 and 37 mm against Candida ablicans, Candida glabrata, Candida krusei and Candida tropicalis respectively but miconazole drug at maximum dose (400 mg) showed diameters values were 37 , 32 , 38 and 40 mm against the same fungi respectively. While the maximum inhibitory dose (150 mg) belonging to fluconazole drug gave inhibitions diameters with values represented by 35, 36, 41 and 37 mm towards the same pathogens.

Table (4): inhibition zone diameters at maximum inhibitory dose of some antibiotics used for treatment of intestinal inflammatory caused by pathogenic fungi in children.

Antibiotic type	Maximum inhibitory dose (mg)	inhibition zone diameter (mm)			
		Candida ablicans	*Candida glabrata*	*Candida krusei*	*Candida tropicalis*
ketoconazole	200	41	38	42	37
miconazole	400	37	32	38	40
fluconazole	150	35	36	41	37

4. Discussion

The extraction percentage of phenolic extract from Juglans regia cortex was 3.6% so this parentage is considered to be somewhat a very good. Various studies showed isolation of phenols from many medicinal plants with good yield [8,12]. Preliminary qualitative analysis ensured presence of phenolics only in phenol extract and this case proves the purity of these active chemical compounds and also no other active metabolites were detected. Thin layer chromatography proved existence of three green spots therefore this status insures attendance of three phenolic compounds having various Rf values depending on molecular weight, polarity, stereochemistry and inter hydrogen bonding [22].

Gas chromatography – mass spectroscopy was used suessfully for insurance of the number of phenolic compounds existing in phenols extract belonging to Juglans regia L. cortex, therefore the separation and characterization by mass spectrum proved presence of three phenolics are 4-hydroxy benzaldeyhde 5-(3-Hydroxypropyl)- 2,3-dimethoxy phenol and vanillic acid. From these results and indications of peaks in the mass spectra of each phenolic compound, the fine chemical structure of each phenol was accurately identified. So this information corresponded with the of TLC results.

In addition, the using GC-MS technique illustrated excellent markers concerning the chemical structure of all phenolic compounds [23]. Also each active phenol was separated alone by gas chromatography and they were purified then FT-IR spectra were recorded for all phenolic. Various functional and structural groups were characterized in these spectra such as hydroxyl (-OH), aromatic C=C, C-C, C-OH, carboxyl, carbonyl and C-H. Therefore, presence of these chemical groups in each spectrum is an indication for identity the phenolic compounds [24]. Also the absorption bands belonging to functional and structural groups have feature of starching or bending vibration for example the starching vibration of hydroxyl group containing hydrogen bonding between phenolic groups. Also the bending vibration of C=C group of phenyl ring [25].

All phenolics are 4-hydroxy benzaldeyhde 5-(3-Hydroxypropyl)- 2,3-dimethoxy phenol and vanillic acid showed an excellent and high medicinal activity towards growth of all pathogenic fungi represebted by *Candida albicans, Candida krusei, Candida tropicals* and *Candida glabrata*. The maximum inhibitory

concentration of each active phenol had the highest ability to kill most of these fungi and this biochemical state can be explained by destructing the membrance and wall of pathogenic fungi cells and decomposition of proteins. This means all phenols isolated from *Juglans regia* L. cortexes have changed the chemical structure of all components of living cell belonging to various fungi [26, 27]. Various studies proved that phenols have a great activity against growth of different micro-organisms including fungi because these active chemical compounds have multi-hydroxyl groups which have the high ability for bonding with all hydrogens in chemical structure of proteins.

Therefore, this statement leads to cleavage sulphuric and hydrogen bonds existing in tertiary structure belonging to protein in the living cell of all fungi [28, 29]. The antifungal activities are explained by capability of phenolic compounds to bind chemically with various enzaymes of living cell of micro-organisms including fungi and this biochemical process leads to inhibit the activity of biological system of these pathogens. Phenols have the great ability to destruct many enzymes in different pathways especially biochemical anabolism of proteins [21, 27] .Some biochemical studies ensured the medicinal efficacy of phenolic metabolites against pathogenic micro-organisms including fungi because these active compounds destruct and inhibit the metabolism of nucleic acids represented by DNA and RNA through hydrogen bonding [30,31].

5. Conclusion

The existing research has ensured and proved the great medicinal activity of phenolic compounds represented by 4-hydroxy benzaldeyhde, 5-(3-Hydroxypropyl)- 2,3-dimethoxy phenol and vanillic acid isolated and identified from Juglans regia cortexes to inhibit the biological growth of pathogenic fungi represented by *Candida albicans*, *Candida krusei*, *Candida tropicals* and *Candida glabrata*. The maximum inhibitory concentration of each phenol had the high capability to kill most these micro-organisms causing intestinal inflammatory in children. Also the phenolic compounds showed more activity than antibiotics used in this study so these active metabolites can be carried out for treatment all diseases caused by these pathogenic fungi.

6. Acknowledgment

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