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Anti-*Candida* activity of *Quercus infectoria* gall extracts against *Candida* species

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Abstract

Background:

Galls of *Quercus infectoria* have been traditionally used to treat common ailments, including yeast infections caused by *Candida* species.

Objective:

This study aimed to evaluate the *in vitro* anti-*Candida* activity of *Q. infectoria* gall extracts against selected *Candida* species.

Materials and Methods:

Methanol and aqueous extracts of *Q. infectoria* galls were tested for anti-*Candida* activity against *Candida albicans*, *Candida krusei*, *Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis*. The minimum inhibitory concentrations were determined using the two-fold serial dilution technique of concentrations ranging from 16 mg/ml to 0.03 mg/ml. After 24 h, the minimum fungicidal concentrations were determined by subculturing the wells, which showed no turbidity on the agar plate. Potential phytochemical group in the crude extracts was screened by phytochemical qualitative tests and subsequently subjected to the gas chromatography-mass spectrometry analysis.

Results:

Both methanol and aqueous extracts displayed substantial anti-*Candida* activity and pyrogallol was the major component of both crude extracts.

Conclusions:

Data from current study suggested that *Q. infectoria* gall extracts are a potential source to be developed as anti-candidiasis.

KEY WORDS: *Candida* species, disc diffusion test, microbroth dilution method, phytochemical analysis, *Quercus infectoria* gall extracts

Candidiasis, which holds a potential of life-threatening disease refers to the yeast infections caused by *Candida* species commonly from *Candida albicans* species.[1] However, there has been a recent increase in yeast infections due to the non-albicans species such as *Candida tropicalis*, *Candida glabrata*, *Candida parapsilosis* and *Candida krusei*.[2,3,4] Vaginal infection is one of common manifestation of candidiasis in which around three-quarters of women will be infected in their lives.[5] Vaginal yeast infection usually causes unpleasant smell and itchiness around the vaginal area.[1]

Treatment of candidiasis is available; however, resistance towards many of the antifungal used to treat this condition is increasing. Patients receiving fluconazole are particularly at risk of developing infections due to fluconazole-resistant of *C. albicans* strain.[4] Problems with antifungal resistance and the increasing number of infections caused by non-albicans *Candida* (NAC) have created a huge demand for new effective antifungal therapies.[6]

Quercus infectoria, one of the popular medicinal plants used traditionally in postpartum care and treatment of various ailments. This plant is a small tree or shrub about two meters high and is mainly found in Asia, Greece, and Iran. Its galls are round-shaped abnormal growth found arising on the young branches of the oak tree due to the attack by the stinging gall-wasp *Adleria gallae-tinctoria*.[7] In Malaysia, the galls known as “manjakani” nut, which have been studied over the years and they are the most well accepted “jamu” (health supplement) to be used during postpartum care.[8] The old folk's women believed that “manjakani” is a ‘magical’ fruit that has many usage especially to women. Tannins which constituted for almost 50-70% of *Q. infectoria* galls were reported to demonstrate most of the anti-inflammatory, antibacterial, and antifungal activities.[9,10] Apart from that, a small amount of gallic acid and ellagic acid were also present in the gall extracts.[11]

New alternative treatments to provide safe, cheap and effective antifungal agents are urgently needed. Due to the wide spectrum of anti-microbial properties[9,10] of *Q. infectoria* galls, this plant might be potentially effective to treat the increasing cases of local and systemic candidiasis. Thus, this study was conducted to evaluate the anti-*Candida* potential of methanol and aqueous extracts of *Q. infectoria* galls toward the commonly isolated *Candida* species.

Materials and Methods

Plant material

Quercus infectoria olivier galls [Figure 1] were purchased from the local herbal shop in Kota Bharu, Kelantan and identified based on their physical appearances which were globular in shape, 0.8 cm to 2.5 cm in diameter, green-yellow in color, odor is slight, strongly pungent taste and tuberculated surface.[12] The galls were washed with distilled water, left dry at room temperature before they were crushed and ground prior to the extraction.

Preparation of extracts

The methanol extract was prepared by immersing 100 g of the *Q. infectoria* gall powder in 500 ml of absolute methanol (Merck) for 72 h in 50°C water bath. The mixture was then filtered using Whatman filter paper No 1. The filtrates were concentrated under reduced pressure using a rotary evaporator at temperature of 55°C. The resulting pellet was finally pounded to dryness at 50°C for 48 h to produce a powdered and brown crude extract.

The aqueous extract was prepared by immersing 100 g of *Q. infectoria* powder in gall 500 ml of sterile distilled water for 72 h in 50°C water bath. The mixture was then prefiltered using a coffee filter and then filtered using Whatmann filter paper No 1. The filtrates were concentrated under reduced pressure using a rotary evaporator at a temperature of 80°C. The resulting pellet was freeze-dried at −50°C under vacuum until the pellet produce a fine crystal-like crude extract. The crude extracts were stored in airtight jars at 4°C.

The extracts were dissolved in sterile distilled water to a final concentration of 100 mg/ml for disc diffusion technique and 64 mg/ml for broth microdilution technique. The mixture was left to dissolve on rotary mixer. Extract solutions were sterilized through membrane filter size 0.2 µm. Blank discs (Oxoid) were impregnated with the desired volume of extract solutions to get the final concentration of 1.0, 2.0 and 5.0 mg/disc and allowed to dry in sterile condition.

Microorganisms and preparation of inoculum

Five American Type Culture Collection (ATCC) strains of *Candida* species were used in this study; *C. albicans* ATCC 10231, *C. tropicalis* ATCC 13803, *C. parapsilosis* ATCC 20019, *C. glabrata* ATCC 90525

and *C. krusei* ATCC 6258. Similarly, one archived clinical isolates was selected for each *Candida* species obtained from Hospital Universiti Sains Malaysia (HUSM) Kubang Kerian, Kelantan. Yeasts were subcultured and maintained onto Sabouraud dextrose agar (Oxoid) and potato dextrose agar (Difco) at 35°C for 24 h. The yeast suspension of each strain was prepared at a concentration of 10^6 cells/ml or McFarland equivalent of 0.5 for disc diffusion test and broth dilution assay.

Anti-*Candida* activity

The procedures described here for disc diffusion test and determination of minimum inhibitory concentration (MIC) values are in accordance with standard international recommendations provided by the Clinical and Laboratory Standards Institute.[13]

Screening by disc-diffusion test

The standardized test inoculum was spread in three directions onto the surface of the Mueller Hinton agar using a sterile cotton swab. Extract discs were placed on the inoculated agar surface along with negative and positive control within 15 min of inoculation. Disc impregnated with sterile distilled water was used as negative control, while amphotericin B disc (10 μg) was used as positive control. The test was done in triplicate. All plates were incubated at 35°C for 24 h. The anti-*Candida* activity was observed from the size of the inhibition zone diameter surrounding the disc measured in millimeters (mm).

Determination of minimum inhibitory concentration and minimum fungicidal concentrations values

The MIC values of each extract against the *Candida* strains were determined using a twofold serial microdilution of extracts with concentration ranging from 16 mg/ml to 0.03 mg/ml. Equal volumes of diluted inoculums suspensions were added to the designated test and control wells. All organisms were tested in triplicate. The MIC values were taken as the lowest concentrations of extracts showing no turbidity after 24 h incubation at 35°C. The wells with absence turbidity were subcultured onto Sabouraud dextrose agar and incubated at 35°C for 24 h to determine the minimum fungicidal concentration (MFC) values.

Phytochemical analysis

The phytochemical qualitative tests were carried out for both methanol and aqueous extracts of *Q. infectoria* to screen for the presence of tannin, saponin, phenol, flavonoids and alkaloid using standard procedure.[14,15] These active compounds were commonly reported in plant extract with bioactivity.

Gas chromatography-mass spectrometry analysis

The analysis was carried out at Universiti Pendidikan Sultan Idris, Perak. The gas chromatography (GC) system (Agilent 7890A) was equipped with Triple Axis Detector (Agilent 5975C) and an Autosampler (Agilent 7693). The column temperature was programmed from an initial temperature of 70°C to a final temperature of 280°C with increased time at the 20°C/min. The injector temperature was 280°C and injection volume was 5 μL. The carrier gas was helium (1 mL/min). Identification of phytochemical components was performed by diluting 1 gram of crude extracts into 5 mL of appropriate solvents. Total running time was 48 min.

Statistical analysis

Data entry and statistical analysis were performed using IBM Corp. Released for Windows: IBM SPSS software version 20. Student's *t*-test was used for statistical comparison and $P < 0.05$ were considered as significant.

Results

Screening of methanol and aqueous extracts against all *Candida* species exerted an increasing mean inhibition zone diameter in a concentration dependent manner. At lower concentrations (1.0 mg/disc and 2.0 mg/disc), the extracts inhibited the growth of yeasts weakly (between 7 and 10 mm in diameter) or not at all. Both extracts inhibited all *Candida* species with some strains exhibited greater inhibition zone diameter compared to the positive control at concentration of 5.0 mg/disc [Table 1]. The lowest MIC and

MFC values detected were 0.06 mg/ml as shown in Table 2. Both methanol and aqueous extracts showed relatively similar anti-*Candida* activity. *C. krusei* appeared the most susceptible *Candida* species showing low MIC and MFC values.

Preliminary phytochemical screening was performed to establish the profile of gall extracts for its chemical composition. Both methanol and aqueous *Q. infectoria* gall extracts showed the presence of tannins [Table 3]. The result of GC-mass spectrometry (GC-MS) analysis of the crude extracts of *Q. infectoria* revealed two major compounds scanned by the NISTO5a.L database. The active principle, retention time (RT), molecular weight and molecular structure were presented in Figure 2. 1, 2, 3 - benzenetriol (pyrogallol) in both methanol and aqueous extracts of *Q. infectoria* gall was identified in high percentage, which accounted for 81.66% and 100% of the total respectively.

Discussion

Candida albicans has historically been the predominant species causing *Candida* infections. However, the emergence of NAC with intrinsic resistance to azoles represents a major challenge for empirical, therapeutic and prophylactic strategies in the near future especially in immunocompromised and severely ill-patients.[4] The most common NAC species causing candidiasis include *C. glabrata*, *C. tropicalis*, *C. parapsilosis* and *C. krusei*.[16] Selection of *Candida* species in this study were based on previous reported *Candida* isolates identified in various spectra of candidiasis in HUSM.[17]

The results obtained from this study were in agreement with the previous studies on antibacterial acitivities of *Q. infectoria* gall extracts.[18,19] In our study, the gall extracts exhibited a substantial *in-vitro* anti-*Candida* activity against all tested *Candida* species. In general, both methanolic and aqueous extracts effectively inhibited the growth of the yeasts at the minimum concentration of 5 mg/disc, which is slightly higher than the concentrations of disc extracts used to screen anti-bacterial activity (1 mg/disc or 2 mg/disc) by disc diffusion test.[10]

In this study, we obtained relatively similar MIC values for both ATCC and clinical isolates of all *Candida* species in either methanolic or aqueous extract. Lowest MIC values were observed for both extracts against isolates of *C. krusei* when compared to other *Candida* species. Lower MIC values were also seen for *C. glabrata*. *C. krusei* is intrinsically resistant to fluconazole and demonstrates decreased susceptibility to amphotericin B, thus requiring higher doses to be used for treatment.[20]

Minimum fungicidal concentrations are one of various *in vitro* microbiological parameters used to determine the fungicidal activity of antimicrobial agents. Microbiological definition of bactericidal or fungicidal activity has been taken arbitrarily as a ratio of MFC to MIC of 4 or less.[21] In this study, the MFC values of the extracts ranged from 0.06 to 8.0 mg/ml. The MFC/MIC ratio of aqueous extract against *C. parapsilosis*, clinical isolates of *C. krusei* and *C. glabrata* were >4, which were considered fungistatic. Fungicidal activities were observed in the methanol extract against almost all tested *Candida* species.

The constant increase in the number of immunocompromised individuals has resulted in an ever-growing number of serious fungal infections due to *C. albicans* and *C. krusei*.[22,23] Most serious fungal infections occur in profoundly immunosuppressed individuals, thus it is generally assumed that a cidal activity would be preferable.[24,25] However, a clear advantage of fungicidal activity over fungistatic activity remains elusive.[26] Some example shows that despite being a fungistatic drug, the clinical trials shows effective result in treating candidiasis.[19] A further study required to determine whether there are clinical settings in which a fungicidal drug may provide an improved response when compared with a fungistatic drug in the treatment of fungal infection.[27] The introduction of active antifungal agents with increased potency action offers new hope for improved therapeutic outcomes and has resulted in a need to assess the fungicidal activity of these agents.[28]

A different organic solvent used as extractor might extract different active compound from the plant.[29] However, this characteristic is also relying on the polarity of the solvent and compound to be extracted. Therefore, the bioactivity of each extract might vary toward bacterial and fungal species.[30]

The phytochemical screening revealed the occurrence of tannins in both extracts. Tannins are classified as hydrolysable tannins and condensed tannin.[31] Some hydrolysable tannins have shown to be more reactive

and have stronger inhibitory effects than condensed tannins.[32] Pyrogallol, one of hydrolysable tannin with high molecular weight and glucose esters of phenolic acids[31] was the main bioactive constituent found from both methanol and aqueous extracts in our study. The presence of hydroxyl groups and alpha-beta double bonds in a phenolic compound [Figure 3] play an important role toward antimicrobial activity.[33] Pyrogallol has been reported to have various biological activities such as candidicidal and fungicidal activities.[34] Those activities were possibly triggered by the presence of three hydroxyl groups in the structure,[33] which eventually affects the biosynthesis of cell wall and cell membrane.[35] Changes in the permeability of cell membrane could cause a decrease in cell volume, thus abnormalities may attribute to cell membrane alterations.[36]

Pyrogallol[37] is the extracted compound that potentially possesses anti-*Candida* properties, which can be further explored for detail evaluations in near future. However, the use of isolated bioactive compound in elucidating maximum antimicrobial activity is doubtful because it has been found that the whole herbal extracts are more effective than isolated phytochemicals due to synergistic effect between the phytochemical components.[38] Therefore, whole extract formulation with a standard marker of bioactive compound is a need in order to make use the synergistic effect of existing compounds.

Conclusion

It is intriguing to note that crude extracts of *Q. infectoria* galls hold an anti-*Candida* potential, which might be further explored to be used as an alternative for treatment and control of some fungal infections. Further investigations are needed to develop a standardized *Q. infectoria* gall extract and to understand its mechanism of anti-*Candida* activity.

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Footnotes

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Conflict of Interest: None declared.

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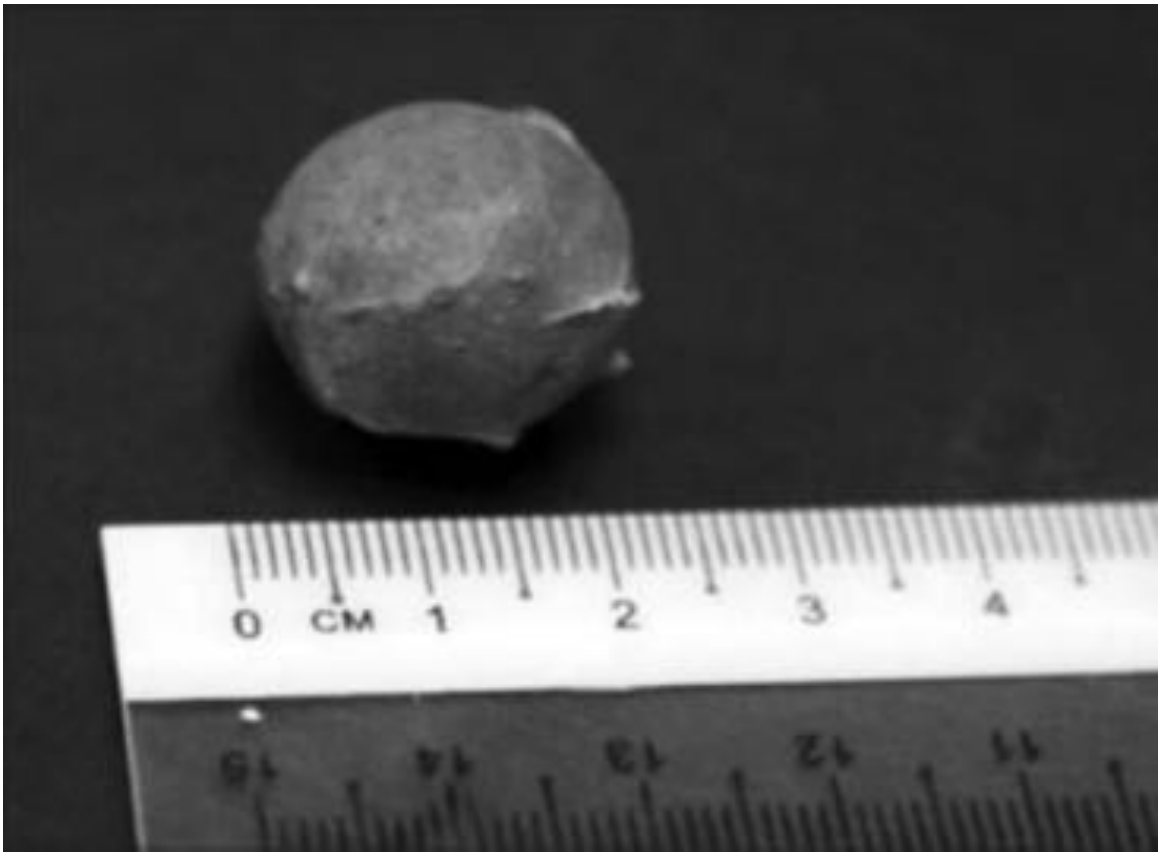
Figures and Tables

Figure 1



Gall of *Quercus infectoria* olivier

Table 1

Species	Inhibition zone diameter (mm±SEM)[†]			
	Positive control (10 µg/disc)	Methanol extract (5.0 mg/disc)	Aqueous extract (5.0 mg/disc)	*P* value[*]
C. albicans ATCC	20.67±0.47	18.00±0.00	16.33±0.47	0.0075
C. albicans CLIS	20.00±0.82	17.67±1.24	16.33±0.94	0.2943
C. glabrata ATCC	19.33±0.47	15.00±1.41	15.33±0.82	0.9999
C. glabrata CLIS	19.67±0.47	12.67±0.94	12.33±0.47	0.6779
C. krusei ATCC	18.67±0.94	21.33±0.94	20.67±0.47	0.4216
C. krusei CLIS	19.33±0.47	21.33±0.47	21.00±0.82	0.6433
C. tropicalis ATCC	19.33±0.47	21.00±0.82	9.33±0.47	0.0001
C. tropicalis CLIS	19.67±0.47	18.33±0.47	15.00±0.00	0.0006
C. parapsilosis ATCC	22.00±0.82	24.00±0.82	23.00±0.82	0.4918
C. parapsilosis CLIS	22.00±0.82	18.00±0.82	18.67±0.47	0.3739

[†]Mean values are of three determinations; each from different plates, [*]Student *t*-test comparing zone sizes between methanol and aqueous extracts. ATCC: American Type Culture Collection, CLIS: Clinical isolates, *Q. infectoria: Quercus infectoria, C. albicans: Candida albicans, C. glabrata: Candida glabrata, C. krusei: Candida krusei, C. tropicalis: Candida tropicalis, C. parapsilosis: Candida parapsilosis,* SEM: Standard error of mean

Anti-candida activity of *Q. infectoria* gall extracts against *Candida* species by disc diffusion test

Table 2

Yeast	Methanol extract			Aqueous extract		
	MIC	MFC	MFC/MIC ratio	MIC	MFC	MFC/MIC ratio
C. albicans ATCC	2.00	8.00	4	1.00	4.00	4
C. albicans CLIS	2.00	8.00	4	1.00	2.00	2
C. glabrata ATCC	0.50	1.00	2	0.25	1.00	4
C. glabrata CLIS	0.50	2.00	4	0.25	2.00	8
C. krusei ATCC	0.06	0.06	1	0.06	0.25	4
C. krusei CLIS	0.06	0.25	4	0.06	0.33	5
C. tropicalis ATCC	1.00	2.00	2	2.00	8.00	4
C. tropicalis CLIS	1.00	2.00	2	2.00	8.00	4
C. parapsilosis ATCC	0.25	1.00	4	0.50	2.00	4
C. parapsilosis CLIS	0.25	1.00	4	0.50	4.00	8

ATCC: American type culture Collection, CLIS: Clinical isolates, *Q. infectoria: Quercus infectoria*, MIC: Minimal inhibitory concentration, MFC: Minimal fungicidal concentration, *Q. infectoria: Quercus infectoria*, *C. albicans: Candida albicans*, *C. glabrata: Candida glabrata*, *C. krusei: Candida krusei*, *C. tropicalis: Candida tropicalis*, *C. parapsilosis: Candida parapsilosis*

MIC and MFC values in mg/ml of *Q. infectoria* gall extracts against *Candida* species

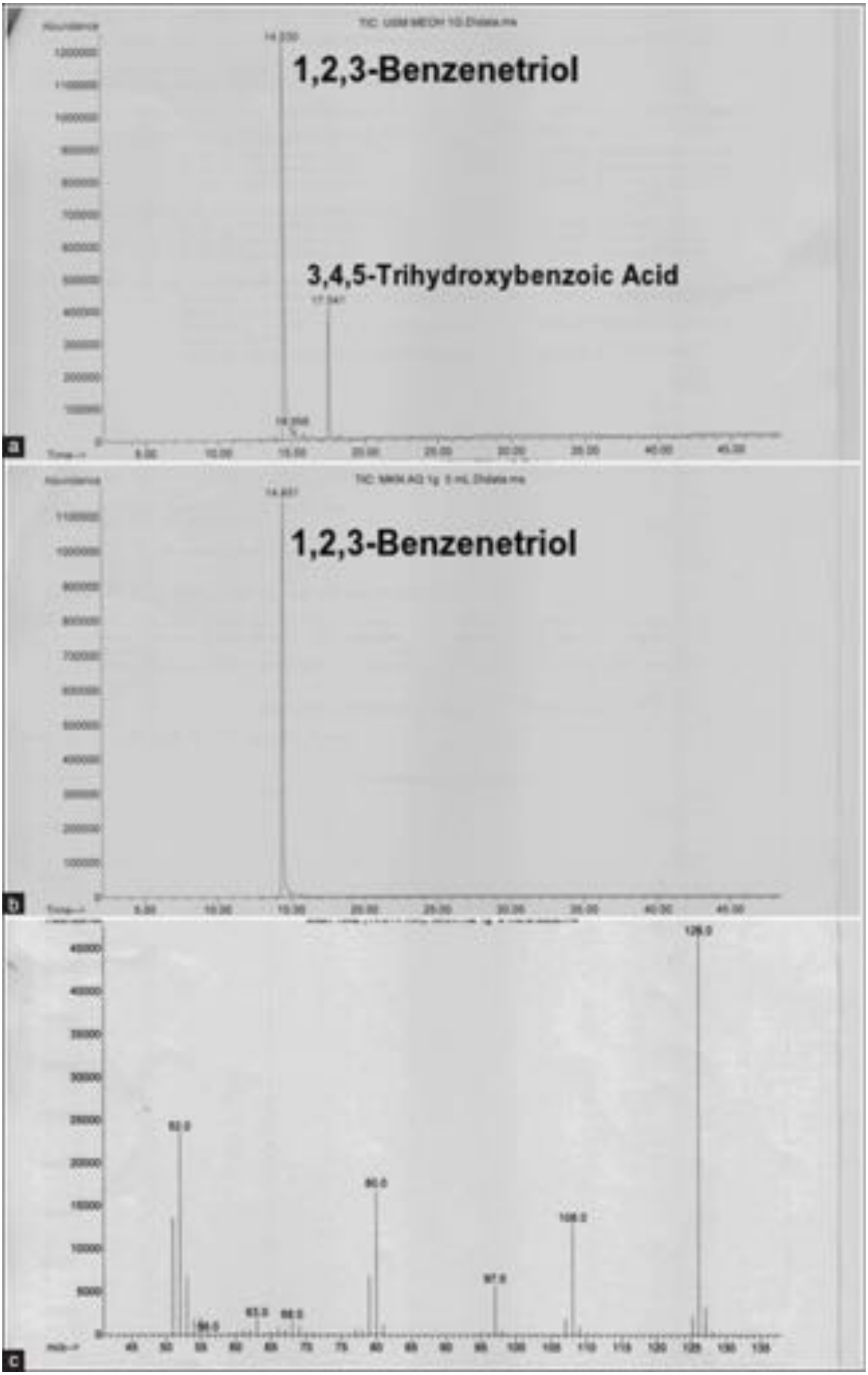
Table 3

Compound	Methanol extract	Aqueous extract
Tannins	+	+
Phenol	+	+
Flavonoid	-	-
Saponin	-	-
Alkaloid	-	-

+: detected, -: not detected, *Q. infectoria: Quercus infectoria*

Phytochemical screening of *Q. infectoria* gall extracts

Figure 2



Chromatogram of methanol extract (a) and aquoeus extract (b) of *Quercus infectoria* gall by gas chromatography-mass

spectrometry (GC-MS) Analysis. The GC-MS spectrum at retention time 14.50 min of major compound of the 1,2,3-Benzenetriol (pyrogallol) with molecular formula of $C_6H_6O_3$ and molecular weight of 126.0 g/mol (c)

Figure 3

Chemical structure of pyrogallol (1, 2, 3 – Benzenetriol)[37]