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Full Length Research Paper

Evaluation of anticandidal potential of *Quercus baloot* Griff. using contact bioautography technique

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The activities of fractions derived from hydroalcoholic extract of *Quercus baloot* leaves against a dimorphoic pathogenic yeast, *Candida albicans (*Cl. I. 4043) was evaluated. The hydroalcoholic extract was sequentially fractionated to give n-hexane, dichloromethane, ethylacetate, and n-butanol fractions, which were also subjected to qualitative phytochemical analyses. Preliminary anticandidal screening was carried out using disk diffusion assay with clotrimazole and chloroform serving as positive and negative controls, respectively. Using optimized solvent systems for each fraction, thin layer chromatography was done to separate the secondary metabolites that were evaluated for bioactivity using contact bioautography technique. Minimum inhibitory concentrations of the individual fractions were found using broth microdilution method. Preliminary screening showed all the fractions, apart from aqueous fraction, to have anticandidal potential (zone of inhibition ≥ 10 mm) against test yeast. However, contact bioautography revealed that only dichloromethane fraction was bioactive with inhibition zones at R_f = 0.44 and 0.50, which showed location of inhibitory compound. The MIC of 125 µg/ml also support the presence of anticandidal moieties in this fraction. Phytochemical analyses indicated flavonoids, terpenoids, and steroids in the fractions.

Key words: *Quercus baloot,* Fagaceae, anticandidal activity, contact bioautography.

INTRODUCTION

Quercus baloot Griff. belong to oaks family. The wood of this plant is used as timber, fire-wood and its bark is a source of tannins. Traditional uses of various species of *Quercus* include utilization in problems of skin and wounds, locomotor organs, and gastrointestinal tract (Viegi et al., 2003). They have been reported for a number of pharmacological activities that include as anti-hyperlipidemic agent (Pieroni et al., 2005), in problems of skin and wounds, ailments of locomotor organs (Viegi et al., 2003), anti-inflammatory, mild antiseptic, anti-hemorrhagic (Leporatti and Ivancheva, 2003), as astringent (Aburjai et al., 2007; Leporatti and Ivancheva, 2003), gastrointestinal ailments (Viegi et al., 2003; Lentz et al., 1998; Aburjai et al., 2007), stomach cancer, gastric and duodenal ulcers, and diarrhea (Castillo-Juárez et al., 2009), as febrifuge, and an antiseptic (Adonizio et al., 2006).

The isolated phytochemicals are predominantly phenolics that have been historically associated with *Quercus* genus (Martens and Mithöfer, 2005). The

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presence of flavonoids (Harborne and Williams, 2000) especially quercetin 3-*O*-*α*-L-arabinopyranoside, quercetin 3-*O*-*α*-Dgalactopyranoside and tannins isolated as peracetates of (+)-catechin, (+)-gallocatechin, a procyanidin epicatechin-(4*β*→8)-catechin, isolated as a peracetate (Sakar et al. 2005), 4,5-di-*O*-galloyl (+)-protquercitol and 3,5-di-*O*-galloyl protquercitol and Gallic acid (Serit et al., 1991) have been documented and these metabolites can be linked to various antimicrobial activities. However, further scientific screenings are required to evaluate the pharmacological potentials of this genus.

To our best knowledge, *Q. baloot* has not yet been studied for bioactivities, therefore, keeping in view the potentials of various species of *Quercus* genus, it can be hypothesized that *Q. baloot* may contain potential antimicrobial entities. The objective of the current study was to explore the anticandidal potential through activity guided bioassay technique.

MATERIALS AND METHODS

Chemicals, media and equipment

All of the organic solvents were obtained from Merck, Darmstadt, Germany, except dichloromethane that was acquired from LabScan, Dublin, Ireland. Sabourad dextrose agar (SDA) and sabourad dextrose broth (SDB) were of Oxoid, Hampshire, UK. Clotrimazole, triphenyl tetrazolium chloride (TTC) were from Sigma-Aldrich, Steinheim, Germany. The pre-coated glass TLC plates (Kieselgel 60, F_{254}, layer thickness 0.25 mm) and 96-well micro-titer plates were purchased from Sterlin, Aberbargoed, UK.

Plant material

Q. baloot Griff. belong to the Fagaceae family. The leaves of the plant were collected from Swat (Latitude 35° 0' 0" and Longitude 72° 30' 0"), Khyber Pukhtoon Khwa, Pakistan, in October 2007. A specimen was matched for confirmation of identity with the reference voucher number 379, preserved in the Herbarium of Pakistan (Quaid-i-Azam University, Islamabad, Pakistan), and was confirmed by Associate Professor Rizwana A. Qureshi, plant taxonomist, Department of Plant Sciences, Quaid-i-Azam University, Islamabad, Pakistan.

Preparation of extract

The shade dried leaves (weighing 15 kg) of *Q. baloot* were pulverized to a fine powder. Methanol (80% *v/v*) was used to soak the pulverized material in a percolator at room temperature. The crude bulk hydroalcoholic extract *Q. baloot* was first deprived off the alcohol, and then fractionated using *n*-hexane, dichloromethane, ethyl acetate, and n-butanol. All of the fractions so obtained were filtered twice using Whatman No. 1 filter paper sheets (Whatman Biometra, Göttingen, Germany) and concentrated under reduced pressure using Rotavapor R-200 (Büchi, Flawil, Switzerland) at 30 ± 5°C. The collections obtained were stored at 4°C.

Preliminary phytochemical screening

The plant underwent phytochemical screening for alkaloids, steroids, tannins, triterpenoids, flavonoids, cyanogenic and coumarin glycosides (Edeoga et al., 2005).

Preliminary anticandidal screening

Candida albicans (Cl. I. 4043) was maintained on SDA slants and refreshed fortnightly. The slants were stored at 4°C. Upon each subculture, the microorganism was Gram stained for morphology and further characterized by simple germ tube test (Cheesbrough, 1985). One hundred microliter of inoculum (ca. 5×10^5 CFU/ml) from an optimized culture was evenly spread, with the help of a sterile glass spreader over the entire surface area of 15 cm diameter Petri plates containing SDA. The seeded plates were allowed to dry under laminar flow. The disks containing the plant fractions, ethanol (negative control for fractions), 0.33% (*w/v*) Clotrimazole in $CHCl_3$ that served as positive control, were prepared and applied using sterile forceps. The plates were placed in inverted position in refrigerator for a period of two hours in order to allow the materials to diffuse around the disks. The inhibition zones were measured after 48 h of incubation at 37°C in inverted position. The tests were run in triplicate.

Thin layer chromatography and contact bioautography

TLC chromatograms were developed using optimized solvent systems as shown in Table 1, and were examined under UV light of 254 and 365 nm wave lengths and metabolites were marked. Previously reported approach (Khurram et al., 2009) was undertaken to carry out contact bioautography experiments with slight modification. The sterilized developed chromatograms were placed on the seeded plates (prepared as mentioned above) aseptically and were left for a period of two hours, in order to allow the materials from them to diffuse on to the seeded plates. Clotrimazole disks (mentioned above) were used as positive control and the area, submerged in the mobile phase in case of each fraction, of TLC chromatogram served as negative control. Thereafter, TLC plates were removed from the surface with the help of sterile forceps and the plates were incubated in inverted position for 48 h at 37°C. The areas of inhibition were marked and relevant R_f (retardation factor) values were recorded by comparing them with the TLC chromatograms. All the tests were run in triplicate.

Susceptibility assay

A broth microdilution method (Johann et al., 2007) using sterile flat-bottom 96-well micro-titer plates was applied to carry out MIC assays. Briefly, fractions were tested at eight concentrations that varied from 1000 to 7.8 µg/ml while positive control clotrimazole concentrations varied from 25 to 0.195 µg/ml. Inoculum was prepared as described earlier and was transferred in 100 µl volume in each of the test wells and controls. The plates were incubated at 37°C for 48 h. Activity in test wells was detected by adding 20 µl of 1% (*w/v*) TTC aqueous solution. MIC was defined as the lowest dilution of respective fraction that inhibited visible growth of test yeast, as indicated by the TTC color change from colorless to red after 3 h incubation at 37°C. Tests were run in triplicate.

Statistical analysis

Results were analyzed using two sample t-test at 95% level of

Table 1. Optimized solvent systems for *Q. baloot* fractions.

Fraction	Solvent system/(s)
QHX	n-Hexane:$CHCl_3$ (1:3)
QDM†	(i) CH_3OH:CH_2Cl_2 (1:10) basify with sufficient NH_4OH so that pH of the solution becomes 9 (i) CH_2Cl_2:CH_3OH (1:2)
QEA	n-Butanol: Acetic acid: Water (20:3:2)
QBN	n-Butanol: Acetic acid: Water (12:3:5)

†TLC plates loaded with the dichloromethane fraction were first developed to a maximum with system (i) and then half developed with system; (ii) QHX = *Q. baloot*; n-hexane fraction; QDM = *Q. baloot* dichloromethane fraction; QEA = Q. baloot ethyl acetate fraction; QBN = *Q. baloot* n-butanol fraction.

Table 2. The susceptibility (expressed in mm mean ± SEM) of *C. albicans* (Cl. I. 4043) and minimum inhibitory concentrations (MIC) of *Q. baloot* fractions.

Fraction	Zone of inhibition (in mm) (n=3)	MIC (in µg/ml) (n=3)	Control	Zone of inhibition (in mm) (n=3)	MIC (in µg/ml) (n=3)
QHX	10.0 ± 0.29	>1000	CLOT†	28.67 ± 0.44	0.39
QDM	12.0 ± 0.12	125	CHCl3‡	0	NT‡
QEA	10.0 ± 0.29	500	Ethanol‡	0	NT‡
QBN	11.7 ± 0.44	250			
QAQ	0	NT‡			

‡ = negative controls; † = positive control; CHCl3, chloroform; CLOT, clotrimazole.

significance (α = 0.05). Analysis was done using Statistical package Minitab v14 (Minitab Inc., Pennsylvania, USA).

RESULTS

Extraction of 15 kg plant material was done using 80% (*v/v*) methanol in water and it gave 3.2 L of crude extract after evaporation under reduced pressure. The liquid-liquid fractionation of the crude extract of *Q. baloot* gave 20 g of a soft mass like n-hexane fraction (QHX), 140 g of sticky gum like dichloromethane fraction (QDM), 102 g of highly viscous ethyl acetate fraction (QEA), 75 g of viscous n-butanol fraction (QBN) that slowly solidified in to a brittle mass and 135 g of thick aqueous fraction (QAQ). Phytochemical screening indicated the presence of tannins, flavonoids and alkaloids.

The bioactivity of *Q. baloot* fractions against the yeast *C. albicans* showed good inhibitory potentials (zone of inhibition ≥ 10 mm). The results, given in Table 2, indicated that all of the fractions were having activity against the test strain except for QAQ fraction. The highest bioactivities were observed in case of QDM and QBN fractions, with zone of inhibitions of 12.0 ± 0.5 and 11.7 ± 0.29 mm, respectively, while QHX fraction expressed lowest inhibitory effect of 10.0 ± 0.15 mm. It was observed that QDM was more effective then QHX ($p = 0.019$) but had a statistically equal effect in comparison to QBN ($p = 0.113$) and QEA ($p = 0.170$). QHX had a statistically insignificant ($p = 0.974$) impact upon the control of the test yeast when compared to QBN. Moreover, there was an insignificant difference between QBN and QEA ($p = 0.012$) for the control of *C. albicans.* QAQ fraction and negative controls (pure chloroform and ethanol that were used to solubilise clotrimazole and test fractions, respectively), were found to pose no negative effect on the growth of the test yeast ($p = 0.50$). Clotrimazole, used as positive control, was effective against the test strain with zone of inhibitions of 28.7 ± 0.60 mm, and have had very significant impact in term of control of the test yeast, in comparison to all of the test fractions and negative controls ($p < 0.05$). The MIC data presented in Table 2 indicates QDM fraction to have highest inhibitory potential (MIC = 125 µg/ml). MIC of clotrimazole, used as positive control, shows that the test strains is sensitive to it.

Based upon the preliminary anticandidal screening results of the fractions, contact bioautography was employed to test if a single compound or multiple compounds were responsible for the anticandidal effects of the separated fractions. Solvent systems used for the development of thin layer chromatograms and contact bioautography results for the respective fractions of plant are presented in Table 3. In the chromatograms, eight R_f points were observed for QHX fraction, fourteen for QDM, and four each for QEA and QBN fractions. These analyses not only indicated presence of separated metabolites but also illustrated the diversity in these

Table 3. Rf values of metabolites and corresponding inhibition zones for *Q. baloot* fractions against *C. albicans* (Cl. I. 4043).

Fraction	TLC (Metabolites observed at Rf)	Contact bioautography (Inhibitions observed at Rf)
QHX	0.04± 0.01 , 0.08 ± 0.01, 0.30 ± 0.01, 0.40 ± 0.01, 0.55 ± 0.02, 0.58 ± 0.02, 0.75 ± 0.01, 0.85 ± 0.01	-*
QDM	0.08 ± 0.02, 0.20 ± 0.02, 0.25 ± 0.01, 0.30 ± 0.01, 0.34 ± 0.01, 0.42 ± 0.01, 0.44 ± 0.01, 0.50 ± 0.02, 0.65 ± 0.01, 0.70 ± 0.02, 0.80 ± 0.01, 0.84 ± 0.01, 0.90 ± 0.02, 0.95 ± 0.01	0.44, 0.50
QEA	0.48 ± 0.01, 0.80 ± 0.01, 0.84 ± 0.01, 0.86 ± 0.01	-
QBN	0.30 ± 0.01, 0.52 ± 0.01, 0.74 ± 0.01, 0.86 ± 0.02	-

*- = No inhibition.

fractions. However, the results of contact bioautography revealed anticandidal activities only in QDM fraction at R_f = 0.44 and 0.50. No inhibition was observed in case of any mobile phases used for separation of respective fractions. The contact bioautography and MIC data of QDM suggest presence of some potential moieties having activity against the test microorganism.

DISCUSSION

Quercus spp. have been used in the problems of skin and wounds (Viegi et al., 2003), against microorganisms as antiseptics (Leporatti and Ivancheva, 2003; Adonizio et al., 2006) that indicate their possible antimicrobial potential. *Candida* spp. may cause severe nosocomial opportunistic infections. The resistance against various drugs used against *Candida* spp. is variable around the globe, yet it is increasing especially against azoles that serve as main drugs used for the candidiasis (Wroblewska et al., 2002).

In the present study, anticandidal activity was seen in all of the test fractions with the exception of aqueous fraction, with highest activity seen in the QDM fraction. This shows that fractionation resulted in the separation of the components to some extent that is obvious in the cases of complex mixtures of natural origin, especially obtained from the extraction of plant materials.

Since plants contain an enormous number of metabolites (Dixon, 2001) and, though separation can be achieved by liquid – liquid partitioning; the method adopted in this study at the start, yet it is impossible to obtain one single molecule from the fractions, mainly due to the close association of the molecules in the extracts and complex nature of plant metabolites, thereby, necessitating further extensive separation. Therefore, TLC was carried out, that resulted in further separation of metabolites. TLC is an easy and cost-efficient technique used in the separation of components of complex mixtures, commonly used for natural products. Suitable solvent systems can resolve effectively the compounds present in the test materials. The main benefits of this technique include low cost analysis, high-throughput screening of samples, and minimal sample preparation (Wen et al., 2004). An additional benefit is that the chromatograms can be screened for antimicrobial activity. This separation technique can be hyphenated to microbiological assays like bioautography.

In the present study, the combination of TLC – contact bioautography resulted in rapid identification of metabolites having anticandidal activity. This spotting of potential metabolites was further supported by the results obtained from MIC assays. Therefore, this approach can be adopted in the preliminary screenings of extracts, and fractions thereof to evaluate anticandidal potentials at very rapid pace. Although the fractions were active against test strain, as implicated in the disk diffusion assay, but when they were subjected to more extensive separation through TLC, the activity appeared to vanish. This indicates that it was probably due to the synergestic or potentiating effects of the secondary metabolites that was lost when they got separated during TLC. However, activity was present in only one fraction at two different R_f points, which signifies their potential. Further work is underway to isolate the molecules indicating this activity.

The results of the studies confirm the validity of the methods used for the separation of compound, thus paving a way for further studies like purification and characterization of the compound, isolation of further potential metabolites having antimicrobial potential, study on various parts of the plant to spot the area having highest amount of isolated agent, identification of geographical location impact on the active secondary metabolites presence and yield. This approach can be further capitalized to rapidly screen plants used in traditional medicines for ailments resulting from microorganism as well as in the extraction of potential molecules that can be inserted in to future therapeutics.

Conclusion

Based on general screening of the fractions and following TLC – Bioautography, and MIC assays it is evident that dichloromethane fraction contains some useful potential metabolites that have good inhibitory potential against a notable pathogen *C. albicans*. The experimental approaches are valid for rapid screening and identification of metabolites against this and such microorganisms that can be grown on solid media.

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