

Full Length Research Paper

Antibacterial potentials of *Quercus baloot* Griff.

Muhammad Khurram^{1,2*}, Abdul Hameed², Murad Ali Khan³, Muhammad Usman Amin⁴,
Muhammad Hassan⁵, Naseem Ullah¹, Wajeeha Manzoor⁶, Abdul Qayum⁷, Muhammad Bilal⁸,
Umberin Najeeb⁹ and Farhat Ali Khan¹

¹Department of Pharmacy, Faculty of Life Sciences, Sarhad University of Science and Information Technology, Peshawar, Pakistan.

²Department of Microbiology, Quaid-i-Azam University, Islamabad, Pakistan.

³Department of Chemistry, Kohat University of Science and Technology, Kohat, Pakistan.

⁴Department of Pharmacology, Kohat Institute of Medical Sciences, Kohat, Pakistan.

⁵Gandhara College of Pharmacy, Gandhara University, Peshawar, Pakistan.

⁶Frontier College for Women, Peshawar, Pakistan.

⁷Department of Pharmacology, Peshawar Medical College, Peshawar, Pakistan.

⁸Department of Plastic surgery, Khyber Teaching Hospital, Peshawar, Pakistan.

⁹Department of Medicine, University of Toronto, Toronto, Canada.

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Quercus baloot fractions were subjected to antibacterial susceptibility evaluation that illustrated inhibition zones against *Staphylococcus aureus*, *Micrococcus luteus*, *Bacillus subtilis*, *Escherichia coli*, and *Pseudomonas aeruginosa*. The active fractions were chromatographed by thin layer chromatography (TLC) and then contact bioautography was utilized as hyphenated bioassay, which showed inhibition zones at different R_f values against *B. subtilis*, *M. luteus*, *E. coli*, and *S. aureus*. The minimum inhibitory concentrations (MICs) of active fractions were found using a 96-well micro-titer plate method and the non-viability of the organisms was ascertained by determining the minimum bactericidal concentration (MBC) of the fractions. This is the first report of antibacterial activities for *Q. baloot*.

Key words: *Quercus baloot*, contact bioautography, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC).

INTRODUCTION

Quercus baloot Griff. belongs to family Fagaceae, commonly known as Oak, Holy oak, Holm oak, Shah baloot (Urdu) locally as Tor banj (Pushto). It is an evergreen small tree or shrub that may grow to a height of 2.5 to 8.0 m. It is present in Afghanistan, Pakistan, and Kashmir at altitude range of 1800 to 3000 m. Viegi et al. (2003) in his review of plants used as veterinary folk medicines in Italy, described *Quercus cerris* L., *Quercus pubescens* Willd., *Quercus robur* L., and *Quercus suber* L. that are generally used for the problems of skin and wounds, ailments of locomotor organs, and gastrointestinal ailments. The bark decoctions of *Quercus*

petraea and *Q. robur* have been utilized for their astringent, anti-inflammatory, mild antiseptic and anti-hemorrhagic potentials in Italy and Bulgaria (Leporatti and Ivancheva, 2003). *Quercus virginiana* Mill. leaves have been described for their use as febrifuge, in diarrhea, and as an antiseptic (Adonizio et al., 2006).

Antibacterial activity of crude ethanolic extracts from wood and bark of *Quercus rubra* were tested against 8 bacteria including Gram-positive and Gram-negative organisms as well 6 fungal strains. The results showed that the bark extract was active against Methicillin sensitive *Staphylococcus aureus* (MSSA), *Bacillus subtilis*, *Mycobacterium phlei* but was ineffective against the Gram-negative bacteria. The results obtained from the wood extract showed its activities against MSSA and *B. subtilis* but was inactive against the Gram-negative bacteria and the test fungi (Omar et al., 2000). The crude

*Corresponding author. E-mail: pharmacistkhurram@hotmail.com. Tel: +92-91-5230931-33. Fax: +92-91-5230930

ethanolic extract of *Quercus infectoria* was found to have very good activity against enterohaemorrhagic *Escherichia coli* strains (Voravuthikunchai et al., 2004).

Sakar et al. (2005) reported the isolation of 2 flavonoids (quercetin 3-*O*- α -L-arabinopyranoside, quercetin 3-*O*- α -Dgalactopyranoside), 2 tannin precursors [isolated as peracetates of (+)-catechin and (+)-gallocatechin] and a procyanidin epicatechin-(4 β →8)-catechin (isolated also as a peracetate). The crude extracts of various *Quercus* spp. are under scientific investigations but isolation of compounds responsible for the antimicrobial activities has not been undertaken except for the study carried out by Serit et al. (1991) who reported two active compounds isolated from *Quercus acuta* Thunb. exhibiting antibacterial activity against Gram-positive and Gram-negative bacteria that is, 4,5-di-*O*-galloyl (+)-protquercitol was found to be more active against *E. coli* (MIC = 12.5 μ g/ml) while against 3 *Bacillus* strains it had MIC's = 50 to 75 μ g/ml.

To our best knowledge *Q. baloot* has not yet been studied for any such or other pharmacological and phytochemical studies. Keeping in view the potentials and phytochemical contents of the various species of *Quercus* spp. it can be hypothesized that *Q. baloot* may contain potential antibacterial entities. The objective of the current study is to explore the antibacterial potentials of *Q. baloot* and to mark the class of compounds that are responsible for the antibacterial activities.

MATERIALS AND METHODS

Chemicals, media, and equipment

All of the organic solvents and pre-coated glass TLC plates (Kieselgel 60, F₂₅₄, layer thickness 0.25 mm) were obtained from Merck, Darmstadt, Germany, while dichloromethane was obtained from LabScan, Dublin, Ireland. Nutrient agar, nutrient borth, Muller Hinton agar, Muller Hinton broth were of Oxoid, Hampshire, UK. Ciprofloxacin and triphenyl tetrazolium chloride (TTC) were from Sigma-Aldrich, Steinheim, Germany and 96-well micro-titer plates were purchased from Sterlin, Aberargoed, UK.

Plant material

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

Bacterial strains

Test bacteria included; *Staphylococcus aureus* (ATCC 6538), *E. coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 9721), *Micrococcus luteus* (ATCC 10240), *B. subtilis* (Wild type), *Bacillus cereus* (Clinical isolate), and *Salmonella typhi* (Clinical isolate).

Extraction and fractionation

Pulverized shade dried leaves (weighing 15 kg) of *Q. baloot* were extracted using methanol (80% v/v) in a percolator at room temperature. After complete removal of alcohol under reduced pressure, extract was subjected to fractionation using *n*-hexane, dichloromethane, ethyl acetate, and *n*-butanol. All of the fractions were concentrated under reduced pressure using Rotavapor R-200 (Büchi, Flawil, Switzerland) at 45° ± 5°C. The collections obtained were kept 4°C.

Phytochemical screening

Phytochemical screenings of plant extract and fractions thereof were carried to the methods adopted by Edeoga et al. (2005) to mark the presence of alkaloids, steroids, tannins, triterpenoids, flavonoids, cyanogenic and coumarin glycosides.

Preliminary antibacterial screening

The crude extract fractions were dissolved in pure methanol to make the concentration equal to 16.7% (w/v). Blank disks of diameter 6 mm were punched from Whatman No. 1 filter paper sheets and sterilized prior to application of 6 μ l volume from each of the prepared methanolic crude extract using Millex, 33 mm filter units having pore size 0.22 μ m (Millipore, Cork, Ireland). The disks were allowed to dry in the laminar flow. The negative control disks and positive control disks were prepared by applying same volume of pure methanol and 0.84% (w/v) Ciprofloxacin in 0.1 N HCl, respectively. The optimized bacterial cultures, equivalent to 0.5 McFarland turbidity standard, were aseptically spread on the entire area of Petri plates containing Muller Hinton agar, and were then allowed to dry. The disks containing the plant crude extracts, negative control disks (methanol) and positive control (standard antibiotics) were aseptically placed on the seeded plates. 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 area. The zones of inhibition were measured after 24 h incubation at 37°C, in case of all the organisms, except for *M. luteus*, which was incubated for a period of 48 h, in an inverted position. All the tests were run in triplicate.

Thin layer chromatography and contact bioautography

Thin layer chromatography (TLC) chromatograms were developed as done by Khurram et al. (2011), using optimized solvent systems, followed by contact bioautography according to the method adopted by Khurram et al. (2009). Briefly, seeded bacterial plates were prepared, as mentioned earlier and then sterilized chromatograms were aseptically placed upon the bacterial lawn and left in refrigerator for a period of two hours, in order to allow the materials from them to diffuse on to the seeded plates. Ciprofloxacin disks were used as positive controls whereas, area below the line of application of the test sample on the TLC chromatograms served as negative control. Thereafter, TLC plates were aseptically removed from the surface of the plates that were incubated in inverted position for 24 h, in case of all the organisms except *M. luteus* that was incubated for 48 h. The areas of inhibition were marked and relevant *R_f* values were recorded by comparing with the TLC chromatograms. All the tests were run in triplicate.

Minimum inhibitory concentration (MIC) assays

A broth microdilution method (Grare et al., 2008) with slight modification was applied to carry out MIC assays. Briefly, fractions

Table 1. Phytochemical analysis of *Quercus baloot* fractions.

Fraction	A	S	Ta	Tp	F	Cy	Co
n-Hexane	-	-	+	-	+	-	-
Dichloromethane	-	-	+	+	+	-	-
Ethyl acetate	-	-	+	+	+	-	-
n-Butanol	+	-	+	-	+	-	-

A = alkaloids, S = steroids, Ta = tannins, Tp = triterpenoids, F = flavonoids, Cy = cyanogenic glycoside, Co = coumarin glycosides, + = Present, - = No reaction.

were tested at nine concentrations that varied from 2000 to 7.8 µg/ml while positive controls ciprofloxacin concentrations varied from 12.5 to 0.05 µg/ml. Inocula were prepared as described earlier and transferred in 100 µl volume in each of the test wells and controls. The plates were incubated at 37°C for 48 h. Any bacterial 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 bacteria, as indicated by the TTC color change from colorless to red after 3 h incubation at 37°C. Tests were run in triplicate.

Minimum bactericidal concentration (MBC) assays

It is defined as the concentration of the antimicrobial that results in a 99.9% reduction in CFU/ml compared with the organism concentration in the original inoculum. The micro-titer plates were prepared in the same manner as discussed earlier for MIC studies. After 24 h incubation for all test bacteria and 48 h incubation in case of *M. luteus*, from each of the visibly clear wells containing plant test materials, where TTC colour remained unchanged, 100 µl aliquots were taken and aseptically spread evenly on the entire surface of nutrient agar plates. The plates were allowed to dry and then kept in inverted position in incubator at 37°C for 24 h for all of the test organisms except the *M. luteus* that was incubated for 48 h at 37°C. After the incubation period, the colonies were counted and compared with negative control. The concentration of the plant extract based fraction that completely inhibited the growth of respective test organisms (99.9% reduction in CFU/ml) was taken as the MBC. The tests were run in triplicate.

RESULTS

Extraction, fractionation and phytochemical screening

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 results are given in

Table 1.

Bacterial susceptibility evaluations

The crude fractions of *Q. baloot* were tested against Gram-positive and Gram-negative bacteria. The results (Table 2) indicate the effectiveness of QDM that was able to cause inhibition against *M. luteus*, *B. subtilis*, and *S. aureus*. QBN was inhibitory to *M. luteus* and *B. subtilis*, while QEA only gave inhibition against *M. luteus*. QAQ and QHX fractions had no activity against any of the test bacteria. QDM fraction showed good activity against the test Gram-negative bacteria. It caused inhibition of *P. aeruginosa* and *E. coli*. The QEA fraction inhibited *E. coli* and QBN gave a slight activity against *P. aeruginosa*. QAQ and QHX fractions had no activity against any of the test bacteria. *P. aeruginosa* and *E. coli* were main susceptible bacteria as they got restricted by two of the five fractions. *S. typhi* was resistant against all of the test fractions. The susceptibility assay results got quantified through MIC's and MBC's assays. With QDM fraction showing effectiveness against Gram-negative bacteria *E. coli* and *P. aeruginosa* having MIC's between 125 to 250 µg/ml range and MBC's 250 to 500 µg/ml, respectively. In case of Gram-positive bacteria were concerned *M. luteus* and *B. subtilis* were having good MIC's of 125 µg/ml and MBC's of 250 µg/ml. *S. aureus* posed a tougher challenge with MIC of 500 µg/ml and MBC of 1000 µg/ml. In case of QBN fraction MIC of 500 µg/ml and MBC 1000 µg/ml were observed for *P. aeruginosa* that was the only Gram-negative bacterium responding to it and in case of Gram-positive bacteria *M. luteus* and *B. subtilis* showed moderate MIC's of 250 µg/ml and MBC's of 500 µg/ml were seen. The QEA gave MIC of 500 µg/ml and MBC 1000 µg/ml against *E. coli* and in case of Gram-positive bacteria only *M. luteus* responded to it with a fair MIC's of 250 µg/ml and MBC's of 500 µg/ml. Since *S. typhi* and *B. cereus* did not show any activity in the susceptibility assays, therefore, they were not subjected to assays pertaining to MIC's and MBC's of fractions. The strains showed excellent sensitivities to positive control (ciprofloxacin) with Gram-positive bacteria having MIC's (0.097 to 0.39 µg/ml) and MBC's (0.195 to 0.78 µg/ml) range and also the Gram-negative bacteria having MIC's

Table 2. Susceptibilities of Gram-positive and Gram-negative bacteria against *Q. baloot* fractions (n = 3).

Bacteria	QHX	QDM	QAE	QBN	QAQ	PC†
<i>S. typhi</i>	0.0 ^a	0.0	0.0	0.0	0.0	31.16 ± 0.76
	- ^b	-	-	-	-	0.39 ^c
	-	-	-	-	-	0.78 ^d
<i>P. aeruginosa</i>	0.0	11.17 ± 0.76	0.0	8.67 ± 0.29	0.0	31.2 ± 0.8
	-	500	-	500	-	0.195
	-	250	-	1000	-	0.39
<i>E. coli</i>	0.0	12.0 ± 0.78	9.17 ± 0.29	0.0	0.0	31.0 ± 0.7
	-	250	500	-	-	0.05
	-	125	1000	-	-	0.097
<i>M. luteus</i>	0.0	13.2 ± 0.36	10.76 ± 0.25	12.83 ± 0.29	0.0	30.0 ± 0.5
	-	250	250	250	-	0.39
	-	125	500	500	-	0.78
<i>B. subtilis</i>	0.0	11.76 ± 0.25	0.0	10.9 ± 0.36	0.0	32.76 ± 0.75
	-	250	-	250	-	0.097
	-	125	-	500	-	0.195
<i>S. aureus</i>	0.0	12.77 ± 0.26	0.0	0.0	0.0	29.03 ± 0.25
	-	500	-	-	-	0.195
	-	1000	-	-	-	0.39
<i>B. cereus</i>	0.0	0.0	0.0	0.0	0.0	25.67 ± 0.29
	-	-	-	-	-	0.39
	-	-	-	-	-	0.78

^a Zone of inhibition (mm ± SD); ^b - Not tested; ^c MIC (µg/ml); ^d MBC (µg/ml); † PC (Positive Control) = Ciprofloxacin.

(0.05 to 0.39 µg/ml) and MBC's (0.097 to 0.78 µg/ml) range.

Thin layer chromatography – Contact bioautography

TLC was performed upon QHX, QDM, QEA, and QBN fractions with the separations achieved that are enlisted in Table 3, which were spotted under UV light. The contact bioautography was carried upon the developed chromatograms with multiple inhibition zones. QDM gave maximum of six inhibition zones with two each for *B. subtilis* and *M. luteus*. *S. aureus* was inhibited at a single point and so does *E. coli*. QBN showed a single point where *M. luteus* and *B. subtilis* got inhibited while QEA was only able to give lone inhibition against *M. luteus*. There were no zones of inhibition as far as QHX fraction was concerned. *P. aeruginosa*, *S. typhi* and *B. cereus* remained unaffected by any of the test fractions.

DISCUSSION

Quercus spp. have been used for dermatological problems (Viegi et al., 2003), against microorganisms as antiseptics (Leporatti and Ivancheva, 2003; Adonizio et

al., 2006), and in cases of diarrhea (Castillo-Juárez et al., 2009) that show their possible antimicrobial potential. Antibacterial susceptibilities of *Q. baloot* fractions, indicated QDM fraction to have inhibitory potential against Gram-positive bacteria; *M. luteus*, *B. subtilis*, *S. aureus* and for Gram-negatives *P. aeruginosa* and *E. coli*. Rather feeble activities were observed for QEA and QBN fractions that show that fractionation results in the separation of the components obtained from the extraction of plant materials.

Ease of application and cost effectiveness of TLC is well known (Wen et al., 2004); therefore, further separation of the components was undertaken through it. Contact bioautography was used as hyphenated bioassay with the TLC to locate antimicrobial components. The results showed inhibition zones at different R_f values against *B. subtilis*, *M. luteus*, *E. coli*, and *S. aureus* indicating the presence of antibacterial components in the QDM, QEA and QBN fractions with QDM fractions showing very promising results.

The antibacterial evaluations of various extracts obtained from *Quercus spp.* indicate potential against bacteria (Omar et al., 2000; Sakar et al., 2005; Berahou et al., 2007; Hayouni et al., 2007; Castillo-Juárez et al., 2009). The results from the preliminary screening

Table 3. R_f values of metabolites and corresponding inhibition zones for *Q. baloot* fractions against bacteria.

Fraction	R_f of the compounds present in each examined fraction	Contact bioautography (Inhibitions observed at R_f)						
		ST	PA	EC	ML	BS	SA	BC
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	–	–	0.65	0.42, 0.70	0.42, 0.70	0.70	–
QEA	0.48 ± 0.01, 0.80 ± 0.01, 0.84 ± 0.01, 0.86 ± 0.01	–	–	–	0.80	–	–	–
QBN	0.30 ± 0.01, 0.52 ± 0.01, 0.74 ± 0.01, 0.86 ± 0.02	–	–	–	0.30	0.30	–	–

ST = *S. typhi*; PA = *P. aeruginosa*; EC = *E. coli*; ML = *M. luteus*; BS = *B. subtilis*; SA = *S. aureus*; BC = *B. cereus*; *– = No inhibition.

and contact bioautography indicated dichloromethane fraction to have good potential in terms of antibacterial activities followed by n-butanol and ethyl acetate fractions. This was further supported in the phytochemical analyses of fractions in which tannins and flavonoids were indicated in the DCM, DEA, and DBN fractions. Since these classes of metabolites have proven antimicrobial activities, which confirm the obtained results and further validated in the MIC and MBC assays. As far as *Q. baloot* is concerned, this is the first such study carried on it, which confirm the potentials of *Quercus* genus in general and *Q. baloot* in particular.

Bacterial resistance is a global issue and an ever growing threat. The important resistances in various groups include; resistance in Gram-positive cocci including MRSA and VRE, resistance in ESBL-producing Gram-negative bacilli especially enterobacteriaceae, fluoroquinolone resistance in Gram-negative bacilli, drug-resistant *P. aeruginosa*, metallo- β -lactamase producing Gram-negative bacilli, and extreme drug resistance among Gram-negative bacilli.

Since the first report of MRSA in 1960, its incidence and prevalence has increased around the globe (Istruiz, 2008). Antibiotic resistance appears as an inevitable consequence and we are running out of our armory against vital pathogens at a brisk pace. It requires prompt actions that include exploration of new therapeutic strategies, preservation of the activity of available antibiotics, and stern efforts in order to develop new antimicrobial agents (Falagas et al., 2008).

The results indicate potential moieties especially in the dichloromethane fraction. The adopted methodology not only highlighted a path in the exploration of antibacterial potentials of this plant but also gave an easy working protocol that can be adopted in such kind of studies.

Conclusion

The results of the studies confirm the validity of the methods used for the identification of potential fractions that can now be further worked upon in order to perform further studies like isolation,

purification and chemical characterization of active compounds, thus yielding molecules that can be taken up in therapeutics after mandatory pharmacological, toxicological studies.

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