ORIGINAL ARTICLE

Antibacterial and Antifungal Activities of Compounds Isolated from *Gymnosporia Royleana* (*Celastraceae*)

H Khan[1], I Khan[1], MR Shah[2], A Shahidullah[1], NK Khalil[1], A Aziz[3], AW Khalil[4], H Jan[5], IU Rahman[6]

ABSTRACT

Objective: *In the present study, five new source compounds isolated from aerial parts of* Gymnosporia royleana (G royleana) *were screened for antibacterial and antifungal activities.*

Methods: *Extraction from plant material was carried out using cold maceration technique. Isolation of pure compounds was accomplished through repeated column chromatography of different fractions obtained from crude extract and using silica gel as stationary phase. Their structures were established* via *advanced spectroscopic techniques along with the spectral data previously reported for these compounds. Dilution method was used for the evaluation of antimicrobial potential of the compounds against various microbial strains.*

Results: *Among the tested compounds, Gymnosporin B displayed moderate antimicrobial activity against* Escherichia *Coli* (E coli), Staphylococcus *aureus* (S aureus), Candida *albicans* (C albicans) *and* Aspergillus *flavus* (A flavus) *[minimum inhibitory concentration (MIC) range; 32–64 µg/mL]. Similarly, Gymnosporin C also showed moderate activity against* E coli *and* S aureus *(MIC; 32 µg/mL each) as well as weak activity against* C albicans *and* A flavus *(MIC; 64 µg/mL each). However, Royaflavone showed moderate antibacterial activity against* S aureus *only (MIC; 32 µg/mL). Antimicrobial activity of the rest of the compounds was weak and negligible.*

Conclusion: *The present study has provided fascinating results of antimicrobial activities of the isolated compounds. However, the broad antimicrobial spectrum of Gymnosporin B and Gymnosporin C demands for further exploration of these triterpenes, both on the basis of mechanism and quantitative structure-activity relationship.*

Keywords: Antimicrobial, *Celastraceae*, *Gymnosporia royleana*, minimum inhibitory concentration, triterpenes

Actividades antibacterianas y antifúngicas de compuestos aislados de *Gymnosporia Royleana (Celastraceae)*

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RESUMEN

Objetivo: *En el presente estudio, cinco nuevos compuestos de origen aislados de partes aéreas de* Gymnosporia royleana (G royleana) *fueron tamizados en sus actividades antibacterianas y antifúngicas.*

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Métodos: *La extracción de material vegetal se realizó mediante la técnica de maceración en frío. El aislamiento de compuestos puros se logró a través de la cromatografía en columna repetida de diversas fracciones obtenidas del extracto crudo y usando gel de silicona como fase estacionaria. Sus estructuras fueron establecidas mediante técnicas espectroscópicas avanzadas junto con los datos espectrales previamente reportados para estos compuestos. El método de dilución fue usado para evaluar el potencial antimicrobiano de los compuestos contra diversas cepas microbianas.*

Resultados: *Entre los compuestos sometidos a prueba, Gymnosporina B mostró una actividad antimicrobiana moderada contra* Escherichia *Coli (*E coli*),* Staphylococcus *aureus (*S aureus*),* Candida *Albicans (*C albicans*) y* Aspergillus *flavus (*A flavus*) [rango de concentración inhibitoria mínima (CIM); 32 – 64 μg/mL]. De manera similar, Gymnosporina C también mostró actividad moderada contra* E coli *y* S aureus *(CIM; 32 μg/mL cada uno) así como débil actividad frente a* C albicans *y* A flavus *(CIM; 64 μg/mL cada uno). Sin embargo, Royaflavone mostró actividad antibacteriana moderada sólo frente a* S aureus *(CIM; 32 μg/mL). La actividad antimicrobiana del resto de los compuestos fue débil e insignificante.*

Conclusión: *El presente estudio ha proporcionado resultados interesantes acerca de las actividades antimicrobianas de los compuestos aislados. Sin embargo, el amplio espectro antimicrobiano de la Gymnosporina B y la Gymnosporina C exige una mayor exploración de estos triterpenos, tanto sobre la base del mecanismo como a partir de la relación cuantitativa estructura-actividad.*

Palabras clave: Antimicrobiano, *Celastraceae*, *Gymnosporia royleana*, concentración inhibitoria mínima, triterpenos

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INTRODUCTION

For centuries, the survival of the human race has been challenged by infectious agents causing morbidities and mortalities worldwide. In 2011 around 14 million lives were lost globally due to infectious diseases (1). A recent survey showed that a handful of 20 species out of nearly 1400 known human pathogens were responsible for mortalities in 2010 (2). Despite new discoveries in antimicrobial agents, newer infections along with emerging bacterial resistance remain a concern today.

Additionally, invasive aspergillosis and other fungal opportunistic infections are also emerging particularly in subjects treated with immune-modulating agents (3). The control of these fungal infections is difficult and challenging for healthcare professionals. The arsenal against fungal infections consists of several agents but still there is a great deficiency of antifungal drugs having broad spectrum and ideal safety. The majority of the present day antifungal agents have pronounced adverse effects or toxicity (amphotericin B), have potential for drug interactions (azoles), or are prone to development of resistance (4). Although the use of combination therapy has been somewhat successful, a need definitely exists for novel antifungal agents with broad spectrum coverage and ideal safety profiles.

In the present scenario re-emerging, multidrug resistance as well as novel microbial infections have outpaced the discovery of new anti-infective agents and presently, a serious need for novel antimicrobial agents exists. Historically natural resources have provided outstanding antimicrobial drugs. As the natural product resources especially the plants have only partially been explored, there is an immense potential in these unexplored reservoirs for producing novel antimicrobial leads.

Gymnosporia Royleana (*G royleana*) belongs to the genus *Gymnosporia*, an important genus of *Celastraceae* family. The plant is a thorny shrub with stiff branches and flowers in the month of March and April. Flowers are white in colour and after maturation forms 3-gonous capsule bearing black seeds (5, 6). It is widely distributed in northern areas of Pakistan, especially in Kaghan, Kashmir, Swat and Buner (5, 6). The plant is widely used in local traditional systems as antimicrobial, anticancer, analgesic, anti-diarrheal, anti-dysentric, antispasmodic, gastro-protective, abortifacient and as insecticidal agent

(7–10). Furthermore, the root extracts of *G Royleana* have shown potential antimicrobial, phytotoxic and anticancer properties (7). Similarly the leaf extracts of the plant have also demonstrated potential anti-haemolytic, anti-lipid peroxidation and antioxidant properties (6).

In the current investigation, we have made an attempt to identify and screen some of the isolated compounds of *G royleana* for antibacterial and antifungal properties.

MATERIAL AND METHODS

Plant collection

The aerial parts of *G royleana* were collected from Mata, in the district of Swat Valley, Khyber Pakhtunkhwa, Pakistan and identified by taxonomist and a voucher specimen (Bot. 20044/pup) was submitted in Department of botany, University of Peshawar.

After garbling and washing with distilled water, the material was placed under shade at room temperature for drying. Then the material was chopped and finely powdered by using mechanical grinder (Yigan, Model WF 130).

Extraction and isolation

Extraction of chemical constituents from *G royleana* was accomplished using cold maceration technique. Powdered plant material (9.7 kg) was macerated repeatedly in methanol at room temperature with intermittent shaking. The combined filtrate was evaporated using a rotary evaporator (Bucchi Rotavapor R 200), that finally provided crude extract (580 g). Methanolic extract was suspended in distilled water and was shaken in a separating funnel with *n*-hexane (3 x 1.5 L), dichloromethane (3 x 1.5 L), ethyl acetate (3 x 1.5 L) and *n*-butanol (3 x 1.5 L), which resulted in respective fractions.

The dichloromethane fraction (20 g) was loaded to a column packed with silica gel, and the sample was eluted with mobile phases ranging from *n*-hexane-DCM (1:1) to DCM-methanol (98:2) gradient. This separation process provided nine sub-fractions (GRD1-GRD9). The sub-fraction GRD-5 (113 mg) was also loaded on Silica gel and eluted with *n*-hexane-DCM (4:6), which lead to isolation of compound **4** (Gymnosterol; 18 mg). The sub-fraction GRD-7 (649 mg) was rechromatographed over silica gel, using *n*-hexane-DCM (7:3), which provided compound **1** (Gymnosporin A; 21 mg).

Ethyl acetate fraction (43 g) was also loaded to a glass column packed with Silica gel. At the beginning, the sample was eluted with hexane-DCM (9:1) gradient which eventually lead to DCM (100%) gradient. Furthermore, the same sample was eluted with DCM-methanol (99:1, 98:2, 96:4, 94:6, 9:1, 8.5:1.5 and 8:2) gradients. This process resulted in various sub-fractions, which were combined according to TLC profiles. Total 12 sub-fractions (GRE1-GRE12) were obtained. The sub-fraction GRE-4 (322 mg) was subjected to chromatography using silica gel as stationary phase, and eluted with DCM-methanol (98:2) gradient that ultimately provided compound **2** (Gymnosporin B; 18 mg) and compound **3** (Gymnosporin C; 32 mg). The sub-fraction GRE-7 (172 mg) was eluted with DCM-methanol (93:7) gradient and provided compound **5** (Royaflavone; 37 mg) using silica gel based column chromatography.

Chemical structures of isolated compounds were identified by comparing nuclear magnetic resonance (NMR) and mass spectrometry (MS) data, with the data already reported in literature (11–14).

Antimicrobial assays

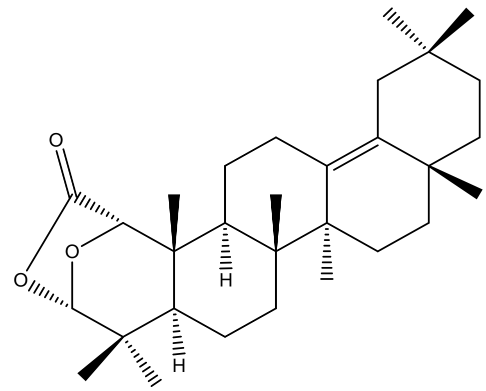
Antifungal and antibacterial activities of the isolated compounds from aerial parts of *G royleana* were determined using dilution method.

Bacterial and fungal cultures

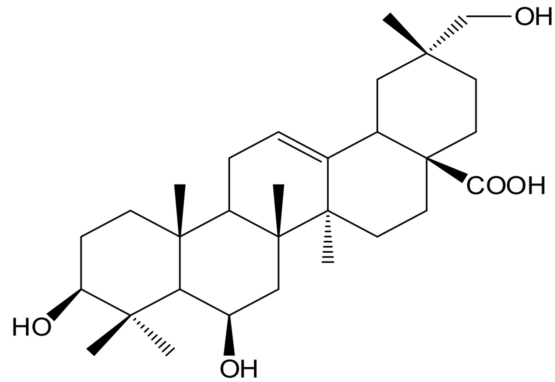
Antimicrobial bioassay of isolated compounds were executed using four bacterial and three fungal strains. The selected bacterial strains for the bioassay were *E coli* (ATCC 25922), *S aureus* (ATCC 25923), *P aeruginosa* (ATCC 27853) and *S typhi* (ATCC 19430). Whereas, *C albicans* (ATCC 2091), *A flavus* (ATCC 32611), *T longifusus* (clinical isolate) were the chosen fungal strains for the study. These microbial strains were activated, when required on nutrient agar (bacteria) or Sabouraud glucose agar (fungi) at 37 °C, for 24 hours before screening.

Determination of minimum inhibitory concentration by macrodilution method

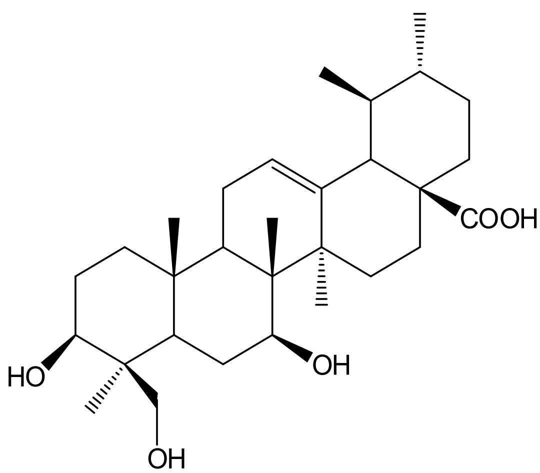
Antimicrobial activity (in terms of minimum inhibitory concentration) of compounds isolated from *G royleana* was evaluated according to reported protocols (15, 16). Samples were solubilized in dimethylsulfoxide and diluted serially in micro-plates with sterile water, using a laminar air flow hood. Similar volumes of actively growing microbial test cultures (approximately 1.5×10^6 CFU/mL), after adding to different wells, were incubated overnight at 37 °C. On the next morning, each well was treated with tetrazolium violet. Microbial growth, if any, was reflected by violet colouration of the culture medium. Based on visual observation, the lower-most concentration of the test sample causing



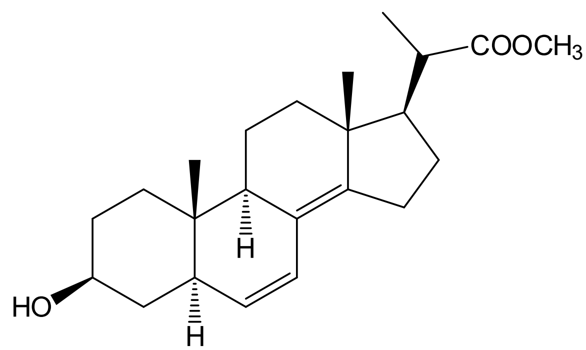
Gymnosporin A



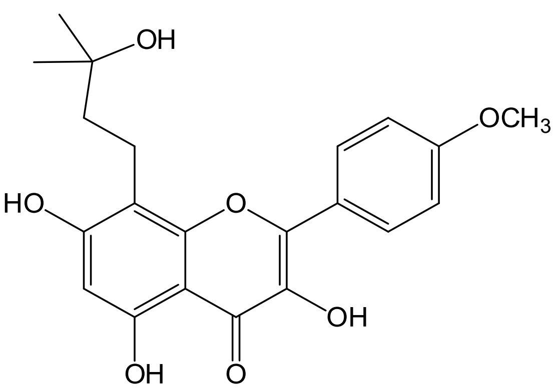
Gymnosporin B



Gymnosporin C



Gymnosterol



Royaflavone

Figure: Chemical structures of isolated compounds.

inhibition of microbial growth was considered as the MIC. The inertness of dimethylsulfoxide (negative control) towards inhibiting microbial growth was verified even at the highest concentration used. Miconazole, Streptomycin and Amphotericin B were employed as standard drugs.

RESULTS

Results presented in the Table clearly show the antimicrobial potential of some of the isolated compounds, to varying degrees, depending upon the microbial strain. The isolated compounds were active against Gram-positive and Gram-negative bacteria as well as fungi. Among the tested microbial cultures, *S aureus* (MIC; 32–256 µg/mL) and *E coli* (MIC; 32–512 µg/mL) were the most susceptible bacteria, whereas, *C albicans* (MIC; 32–512 µg/mL) and *A flavus* (MIC; 32–512 µg/mL) were the most sensitive fungal strains. Alternatively, compound **2** (MIC; 32–512 µg/mL) and **3** (MIC; 32–512 µg/mL) were the most active compounds towards inhibiting the microbial strains.

Table: Antibacterial and antifungal activity of compounds isolated from aerial parts of *Gymnosporia royleana*.

Microorganism	Minimum Inhibitory Concentration (MIC, µg/mL)					
	Standard	1	2	3	4	5
E coli	10[1]	128	32	32	512	256
S aureus	10[1]	256	32	64	256	32
P aeruginosa	9[1]	≥ 1024	512	512	≥ 1024	256
S typhi	10[1]	≥ 1024	256	512	≥ 1024	≥ 1024
C albicans	1.8[2]	512	64	128	512	256
A flavus	2.5[3]	512	64	128	≥ 1024	512
Trichophyton longifusus	1.4[2]	≥ 1024	≥ 1024	≥ 1024	≥ 1024	512

[1]Streptomycin, [2]Miconazole, [3]Amphotericin B
Gymnosporin A = (**1**)
Gymnosporin B = (**2**)
Gymnosporin C = (**3**)
Gymnosterol = (**4**)
Royaflavone = (**5**)

DISCUSSION

As a whole, the result of this study could be regarded as very promising if viewed in the context of a novel drug discovery from plant origin and the clinical significance of the test micro-organisms. *Staphylococcus* aureus, a Gram-positive pathogen, is a main causative agent of community and nosocomial infections with 7–10% estimated mortality rate. Furthermore, some 0.5 million patients clinics in the United States are diagnosed with *staphylococcal* infection each year. *Escherichia* Coli, a Gram-negative organism is responsible for food poisoning, gastroenteritis, neonatal meningitis and urinary tract infection (UTIs). *Salmonella* typhi is well-known for typhoid fever, a serious health problem in the developing world. According to The World Health Organization (WHO), the annual reported typhoid cases go beyond 21 million claiming more than 200 000 lives (17). *Pseudomonas aeruginosa* is commonly responsible for respiratory tract infections, UTI's, burn and wound infections, otitis externa as well as other systemic infections. Similarly, nearly 80% of deaths of immune-deficient individuals is attributed to mycotic infections, including *Candidiasis* and others. Furthermore, three out of every four females develop candidial vulvo-vaginitis, at least once during their lives and is considered to be the most common cause of vaginitis after bacterial vaginosis. *Aspergillus* flavus is one of the leading causes of invasive and non-invasive aspergillosis (18).

The results clearly support the antimicrobial use of the plant in folk medicine including antidiarrheal use and support our previous results (19). Furthermore, the probable mode of antimicrobial action of these compounds could be explained on the basis of the chemical classes to which these compounds belong. Hence, the likely mode of action of Gymnosporin A, Gymnosporin B and Gymnosporin C (triterpenes) as well as Gymnosterol (sterol) is *via* disruption of microbial membrane. Similarly, the antimicrobial activity of Royaflavone might be attributed to its complexation with microbial cell wall, thus, preventing the microbial growth (18, 20).

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AUTHOR'S NOTE

HK, MRS and IK designed the study, HK, AS and AW performed the experiments, NK and HJ wrote the paper. Whereas, MRS, IK and IR supervised the experiment and analysed the final draft.
Authors declare no conflict of interest.

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