ORIGINAL ARTICLE

In vivo and *in vitro* Pharmacological Evaluation of *Gymnosporia* Royleana

H Khan[2], AA Shad[1], I Khan[2], A Aziz[1], G Ali[2], SM Hizbullah[2], I Jan[1]

ABSTRACT

Objective: Gymnosporia royleana (G royleana) *Wall ex MA Lawson, locally known as (Sur Azghee), is traditionally used for the management of various diseases. In the current investigation, we made an effort to scientifically validate its traditional use in various pathological conditions, such as microbial infections and cancer, and to explore its additional pharmacological activities* via *random screening against locally accessible pharmacological methods, irrespective of its traditional uses like antidiabetic, haemagglutination and antioxidant assays.* ***Methods:*** *Extraction was carried out using a cold maceration methodology. Dilution method was used for antimicrobial susceptibility testing using different concentrations. Streptozocin (STZ) induced protocol was used to assess antidiabetic activity at a dose level of 200, 400 mg/kg. Antioxidant activity, haemagglutination activity, and anticancer activities against HepG-2 and MCF-7 cell lines were determined as per established protocols. Similarly, the maximum amount of phenolic content (12.02 mg 100 g) was determined by using Folin Ciocalteu assay.* ***Results:*** *Promising antimicrobial activities in terms of minimum inhibitory concentration (MIC) were noted for crude extract (25–200 µg/mL), n-hexane (100–400 µg/mL), ethyl acetate (50–200 µg/mL) and aqueous (100–400 µg/mL). Antidiabetic potential was significant at a dose level of 200–400 mg/kg bodyweight by reducing the blood glucose level at days 10 and 15. The percentage of 2,2-diphenyl-1-picrylhydrazyl (DPPH) values increase by increasing the concentration of the plant extract (10–100 µg/mL). The methanol extract was found to possess high agglutination activity.*

Conclusion: *It was concluded that this plant species possess significant antimicrobial, antidiabetic, antioxidant, anticancer and haemagglutination activities, which could be attributed to the phenolic content of the extract.*

Keywords: Antidiabetic, antimicrobial, DPPH, *Gymnosporia Royleana*, MTT

Evaluación Farmacológica de *Gymnosporia Royleana in vivo* e *in vitro*

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RESUMEN

Objetivo: *Gymnosporia royleana (G royleana) Wall ex MA Lawson, localmente conocida como "Sur Azghee", se utiliza tradicionalmente para el tratamiento de diversas enfermedades. En la investigación presente, tratamos de validar científicamente su uso tradicional en varias condiciones, tales como las infecciones microbianas y el cáncer, así como explorar sus actividades farmacológicas adicionales mediante el tamizado aleatorio frente a los métodos*

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farmacológicos localmente accesibles, independientemente de sus usos tradicionales como ensayos antidiabéticos, hemaglutinantes, y antioxidantes.
Métodos: *La extracción se realizó mediante una metodología de maceración en frío. Un método de dilución se utilizó para la prueba de susceptibilidad antimicrobiana utilizando diferentes concentraciones. Se utilizó el protocolo inducido por estreptozotocina (STZ) para evaluar la actividad antidiabética a un nivel de dosis de 200, 400 mg/kg. La actividad antioxidante, la actividad de hemaglutinación, y las actividades anticancerígenas contra las líneas celulares HepG-2 y MCF-7, se determinaron según los protocolos establecidos. De modo similar. la cantidad máxima de contenido fenólico (12.02 mg 100 g) se determinó mediante el uso del ensayo Folin-Ciocalteu.*
Resultados: *Se observaron actividades antimicrobianas prometedoras en términos de la concentración inhibitoria mínima (CIM) para el extracto crudo (25–200 μg/mL), el n-hexano (100–400 μg/mL), el acetato de etilo (50–200 μg/mL), y el extracto acuoso (100–400 μg/mL). El potencial antidiabético fue significativo a un nivel de dosis de 200–400 mg/kg de peso corporal mediante la reducción del nivel de glucosa en sangre a los 10 y 15 días. El porcentaje de los valores de 2,2-difenil-1-picrilhidracilo (DPPH) se incrementa al aumentar la concentración del extracto de la planta (10–100 μg/mL). Se halló que el extracto de metanol posee una alta actividad de aglutinación.*
Conclusión: *Se concluyó que esta especie de planta posee importantes actividades antimicrobianas, antidiabéticas, antioxidantes, anticancerígenas y hemaglutinantes, que podrían atribuirse al contenido fenólico del extracto.*

Palabras clave: Antidiabética, antimicrobiana, DPPH, *Gymnosporia royleana*, MTT

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INTRODUCTION

Natural products-based drug discovery is still a challenging field for scientists across the globe. New yet effective therapeutic agents from natural sources, especially plants, are an ever expanding area (1–6). *Gymnosporia royleana* (*G royleana*) Wall ex MA Lawson (Sur Azghee), is a herbaceous medicinal plant, which belongs to the family Celastraceae (Fig. 1).

This family comprises around 900–1000 different genera and nearly 1300 species (7). The geographical study reveals that the plant is cosmopolitan in the Northern region of Pakistan and locally known as "Sur Azghee". The plant is traditionally used for the treatment and management of stomach disorders, gastritis, rheumatism, influenza and cancer (8–10). Literature survey shows that the plant is used in folk medicine for analgesic, gastro-protective and anticancer properties and for toothaches (11).

An *in vitro* bioassay has reported that the root of *G royleana* possesses phytotoxic, cytotoxic and antibacterial activity (12). A large number of bioactive constituents like cytotoxic maytensine alkaloids, triterpenes, namely salaspermic acid and orthosphenic acid have been isolated from different *Gymnosporia* species (12–14). In the current investigation, we have made an effort to scientifically validate some of its traditional uses in various pathological conditions, assessing its antimicrobial and anticancer activities. We were also

Fig. 1: Photographs of *Gymnosporia royleana* (Wall ex MA Lawson).

interested in exploring its additional pharmacological activities *via* random screening against locally accessible pharmacological methods irrespective of its traditional uses like antidiabetic, haemagglutination and antioxidant assays. As polyphenolic compounds are generally associated with antioxidant, antidiabetic, antimicrobial and anti-cancer activities (15–16), we also decided to carry out quantitative anaylsis of the total phenolic content with the aim of correlation with the pharmacological activities of *G royleana*.

Ethical approval

The current study "*In vivo* and *in vitro* pharmacological evaluation of *G royleana*" was approved under a project entitled "Investigation of secondary metabolites of *G royleana*: Phytochemical and pharmacological studies" by the Departmental Research Ethical Committee of the Pharmacy, University of Peshawar, Peshawar, Khyber Pakhtunkhwa (KP), Pakistan, who granted an approval certificate number 05/EC-15/Pharm.

MATERIAL AND METHODS

Plant collection and identification

Gymnosporia royleana was collected from Swat Valley, Khyber Pakhtunkhwa, Pakistan. The plant was collected from its natural habitat and was identified by Mahboob Ur Rehman, plant taxonomist, (Assistant professor, Government Post-Graduate Jehanzeb College, Saidu Sharif, Swat, Khyber Pakhtunkhwa, Pakistan) and a voucher specimen (RG-12) was deposited in the herbarium of cited college and Pakistan Council of Scientific and Industrial Research (PCSIR), Peshawar, Khyber Pakhtunkhwa, Pakistan.

Sampling and sample preparation

In our study, the aerial parts of *G royleana* were used for pharmacological investigation. The respective parts were washed with tap water to remove dust, filth and adherent materials. The plant material was then dried in shade for complete dryness, which was achieved within seven days. The dry sample was then crushed and chopped with the help of an electrical grinder (Yigan, Model WF 130). The powder sample was packed and labelled in clean plastic bags and was refrigerated below 4 °C for analysis.

Extraction and Fractionation

Extraction was done using a cold maceration method. Firstly, powdered plant material, about 1.5 kg, was suspended in approximately 2 L methanol at room temperature for seven days (17–18). The extracts were then filtered through Whatman filter paper No.1. Methanol was then evaporated from the filtrate using a rotary evaporator (model, Buchi Rotavapor R-200) under vacuum at 45 °C. Fractionation of the extract was carried out by suspending the crude extract in 150 mL water, and then partitioning with different organic solvents (hexane and ethyl acetate), in order to increase polarity by using a separating funnel.

All the fractions were dried by evaporating respective solvent using a rotary evaporator. Dried extracts were stored at 4 °C in a refrigerator until analysed. We were interested in screening for the biological effects of the fractions of crude extract in some assays, based on the limited choice of available organic solvents (*n*-hexane and ethyl acetate). *N*-hexane is used to separate less polar constituents of the crude extract while ethyl acetate is used to separate polar constituents in assessing, their comparative biological effects.

Antimicrobial activity

Determination of the minimum inhibitory concentration (MIC, mg/mL) by the dilution method was carried out as reported previously (18–19), using a cell suspension of about 1.7×10^6 CFU/mL, which was obtained following Macfarland and Turbidity Standard No. 0.5. Concentration of the suspension was standardized by adjusting the optical density to 0.1 at 600 nm (Shimadzu, UV-VIS Spectrophotometer). Extracts and fractions were dissolved in dimethylsulfoxide (DMSO) and serially diluted with sterile water in microplates, in a laminar flow cabinet. The same volume of an actively growing culture of the test bacteria was added to the different wells and cultures were grown overnight in 100% relative humidity (at 37 °C). The next morning, tetrazolium violet was added to all the wells. Growth was indicated by a violet colour in the culture. The lowest concentration of the test solution that led to inhibition of growth was taken as the MIC. Streptomycin, miconazole and amphotericin B were used as controls for comparison.

Test micro-organisms

Tests were performed using well-known bacterial and fungal strains. Bacterial strains were *Escherichia* Coli (*E coli*) [ATCC 25922], Bacillus subtilis (*B subtilis*) [ATCC 6633], *Staphylococcus* aureus (*S aureus*) [ATCC 25923], *Pseudomonas aeruginosa* (*P aeruginosa*) [ATCC 27853] and *Salmonella* typhi (*S typhi*) [ATCC 19430]. Fungal strains included *Candida* albicans (*C albicans*) [ATCC 2091] and Aspergillus flavus (A flavus) [ATCC 32611], which were maintained on an agar slant at 4 °C.

Bacteria and fungi were activated at 37 °C for 24 hours on nutrient agar or Sabouraud glucose agar, respectively, prior to any screening.

Antidiabetic activity

In-vivo antidiabetic activity was performed using laboratory mice. All animals were kept according to standard laboratory protocols and guidelines (2). These experiments were conducted according to well-established protocols, published and accepted internationally. Methanolic extract of *G royleana* was evaluated for antidiabetic activity by utilizing the standard protocol of Chen et al (20). Diabetes mellitus was induced in mice by injecting freshly prepared STZ (Sigma) solution in citrate buffer (0.01 Mol L^{-1}, pH 4.5), at a dose level of 35 mg kg^{-1} bodyweight. Prior to induction of diabetes, all animals were fed with a high-fat diet. All diabetic animals, meeting the criterion of diabetes were included in the study. All animals were divided into five groups (n = 6). Group 1 included a normal control (healthy animals). Group 2 included animals with induced diabetes as a disease-control group. Groups 3 and 4 included diabetic animals treated with *G royleana* 200 mg/kg and *G royleana* 400 mg/kg, respectively. Finally, Group 5 was based on diabetic animals treated with glibenclamide, 10 mg/kg, as a positive control group.

Determination of antioxidant activity

Antioxidant activity of methanol extract of *G royleana* was evaluated, using the standard protocol of Blois (21). Plant extract (25 mg) was dissolved in 2 mL methanol, to make different concentrations by the serial dilution method (22). Then, 4 mL of solution in the test tube was taken, to which 1 mL of 0.001 M of DPPH (2,2-diphenyl-1-picrylhydrazyl) was added. For control solution, 1 mL DPPH solution and 5 mL methanol were mixed and were incubated in the dark for 30 minutes. The antioxidant potential of the methanolic fraction was measured spectrophotometrically by the change in absorbance of DPPH at 517 nm employing the following formula:

Percentage of DPPH activity = Control – test Absorbance / Control × 100

Haemagglutination activity

In order to make the red blood cells (RBC) suspension, the fresh human blood was washed thoroughly with phosphate buffered saline (PBS) and centrifuged for 10 minutes at 2900 revolutions per minute (RPM). Phosphate buffered saline was used to wash the suspension repeatedly by adding trypsin solution (0.25%). The routine protocol was used for making 1% suspension of RBC. Afterwards, 100 µL of the RBC suspension were transferred to each well in 96-well plates, 100 µL of untreated PBS, phytohaemagglutinin (PHA) and the fraction were added, in triplicates. After 20, 40, 60 and 80 minutes, four sets of plates were allowed to react individually. Supernatants were analysed using ELISA reader at a wavelength of 405 nm and a different wave-length of 650 nm, 100 µL of 0.1% solution of Triton × 100 was added to each well and homogenized for 10 minutes on an MTP shaker at room temperature. Finally the plates were again analysed on an ELISA reader by using the same wavelength (23).

Determination of polyphenol content and preparation of extract

The total phenolic content (TPC) in *G Royleana* was determined by follin ciocalteu assay as described by Grubesic *et al* (24). About 2 g of the powdered sample was dissolved in 160 mL methanol (30%) by heating it at 70 °C for 15 minutes in a water bath. The extract was cooled and then filtered. Afterwards, the basic sample solution was prepared by adding 30% methanol in the cooled extract and the volume of each extract was made up to 200 mL. Furthermore, 2 mL of the basic sample solution was dissolved in 8 mL of water and 10 mL of acetate buffer (solution 1, S1). Then, 10 mL of S1 was mixed with casein and shook for 45 minutes and then filtered (solution 2, S2). The absorbance was measured at 720 nm. The blank solution was prepared by using 10 mL S1 and 50 mg casein with normal shaking for 45 minutes and then filtered. The total polyphenol concentration was measured by taking three replicates independently. Concentration of phenols in the sample was determined *via* absorbance data. Total phenolic content was expressed as a percentage of the dry mass.

Anticancer activities against HepG-2 and MCF-7 cell lines

Cell cultures

HepG-2 (hepatic cancer) and MCF-7 (breast cancer) human cell lines were maintained in DMEM complete media (L-glutamine supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin) in addition to 10 mM non-essential

amino acids. Cells were grown at 37 °C in a humidified atmosphere of 5% CO_2. All experiments were performed with cells in the logarithmic growth phase.

Cytotoxicity assay

Sensitivity of the cancer cells to drugs was determined in triplicate using the 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT) cell viability assay (25). The extracts and individual substances were dissolved in dimethylsulfoxide (DMSO) and further serially diluted with the medium in two-fold fashion into ten different concentrations so as to attain final concentrations ranging from 0.977 to 500 µg/mL for extracts, and from 0.977 to 500 µM for isolated substances, in 96-well plates; each well contained 100 µL medium. Dimethylsulfoxide was used in a concentration below 0.05% in the medium. Wells containing the solvent and wells without the solvent were included in the experiment. Exponentially, growing cells were seeded in a 96-well plate (2×10^4 cells/well), the cells were cultivated for 24 hours and then incubated with various concentrations of the serially diluted tested samples at 37 °C for 24 hours and then with 0.5 mg/mL MTT for four hours. The formed formazan crystals were dissolved in 100 µL DMSO. The absorbance was detected at 570 nm, using a microplate reader.

In-vivo acute toxicity

The LD_{50} in mice was determined according to the method described previously (26). In brief, nine mice were divided into three groups of three mice each, representing three geometrically increasing dose points of 10, 100 and 1000 mg/kg of the extract, which were administered intraperitoneally. Animals were observed for mortality within each group, over a 24-hours period. The LD_{50} was calculated as the geometric mean of the highest non-lethal dose and the lowest lethal dose.

The use of a limited number of mice, three per group, for the LD_{50} toxicity studies is already reported in the literature (27–28). Secondly, due to a shortage of healthy and suitable laboratory mice, we had no other way to conduct experiments but on three animals per group.

Statistical analysis

GraphPad Prism version 5.0, Inc. Version, California, United States of America was used for analysis (http://www.graphpad.com). Analysis of variance (ANOVA) followed by appropriate *post-hoc* analysis was applied to data for statistical significance. Values of $p < 0.05$ were considered significant in all cases.

RESULTS AND DISCUSSION

This study revealed promising antimicrobial activities in terms of minimum inhibitory concentration (MIC, µg/mL). Results of the antimicrobial activity of standard, control, crude, *n*-hexane, ethyl acetate and aqueous fractions of *G royleana* are showen in Table 1.

The most susceptible microorganism among the bacterial and fungal strains were *E coli*, *B subtilis*, *S aureus*, *P aeruginosa*, *S typhi*, *C albicans* and *A flavus*. Results revealed that the MIC values of methanol extract, *n*-hexane, ethyl acetate and aqueous fraction against *E coli*, *B subtilis*, *S aureus*, *P aeruginosa*, *S typhi*, *C albicans* and *A flavus* were (100, 50, 50, 100, 25, 100, 200 MIC, µg/mL), (400, 100, 100, 400,100, 200, 400 MIC, µg/mL), (100, 50, 50, 100, 50, 100, 200 MIC, µg/mL) and (200, 100, 100, 200, 100, 200, 400 MIC, µg/mL), respectively. These data revealed more potent activity against *S typhi* for methanol extract (25 MIC, µg/mL), *B subtilis*, *S aureus*, *S typhi* for *n*-hexane (100 MIC, µg/mL), *B subtilis*, *S aureus*, *S typhi* for ethyl acetate (50 MIC, µg/mL) and *B subtilis, S aureus, S typhi* for aqueous fraction (100 MIC, µg/mL). In case of positive control standard antibiotic streptomycin, miconazole and amphotericin B

Table 1: Antimicrobial activity of methanol extract and fractions of *Gymnosporia royleana* represented as minimum inhibitory concentration (MIC, µg /mL).

Microorganism	Minimum Inhibitory Concentration (MIC, µg/mL)					
	Standard	Control	Crude	*n*-Hexane	Ethyl acetate	Aqueous
E coli	10[1]	800	100	400	100	200
B subtilis	8[1]	800	50	100	50	100
S aureus	10[1]	800	50	100	50	100
P aeruginosa	9[1]	800	100	400	100	200
S typhi	10[1]	800	25	100	50	100
C albicans	1.7[2]	800	100	200	100	200
A flavus	2.4[3]	800	200	400	200	400

[1]Streptomycin, [2]Miconazole, [3]Amphotericin B

exhibited potent activity against bacteria *E coli*, *B subtilis*, *S aureus*, *P aeruginosa*, *S typhi* (MIC; 8–10 µg/mL) and fungal strain *C albicans*, *A flavus* (MIC; 1.7, 2.4 µg/mL). The negative control, that is, DMSO had no influence on the growth, even at the highest concentration used.

Results of Table 2 clearly indicate that both doses (200, 400 mg kg^{-1} bodyweight) of methanolic extract of *G royleana* were effective in reducing the blood glucose level in diabetic mice at day 10 and day 15 ($p < 0.05$). The results were comparable with that of standard drug. However, both doses failed to reduce the blood glucose level at day five of experimental protocol.

Results of agglutination values of methanolic extract of *G royleana* are shown in Table 3. The haemagglutination inhibition activity was investigated by taking various concentrations of extracts (0.0001–1 mg mL^{-1}) against different types of human blood groups. Analysis of variance and Dunnet *post-hoc* tests have revealed that there was a significant difference between the agglutination values observed with methanolic extract of *G royleana* and PHA ($p < 0.05$).

The effects of the antioxidant potential methanolic extract of *G royleana* are depicted in (Fig. 2).

Methanolic extract of the plant proved to be a good source of free radical scavenging activity with a percentage of DPPH value ranging from 7.43–77.31% at varying concentrations. The results of total phenolic content of *G royleana* are demonstrated in (Fig. 3) and were expressed as tannic acid equivalent (TAE) in mg 100 g^{-1}. The subject plant was found to possess significant phenolic content of 12.02 mg 100 g^{-1}.

Cancer is a major cause of morbidity and mortalilty worldwide. Emergence of cancer resistance to available drugs is another dilemma, which necessitates the prompt and effective discovery of new anticancer agents with promising safety profiles. HepG-2 cells are suitable models for investigation of new agents against hepatocellular carcinoma. Owing to their high degree of morphological and functional differentiation *in vitro*, HepG2 cells are a suitable model to study the intracellular trafficking and dynamics of bile canalicular and sinusoidal membrane proteins and lipids in human hepatocytes *in vitro*. A MCF-7 cell belongs to non-invasive breast cancer cells. This cell line retained several characteristics of differentiated mammary epithelium, including the ability to process oestradiol *via* cytoplasmic oestrogen receptors and the capability of forming domes. Natural products have proved to be rich sources of bio-active compounds potentially useful for better management of cancer and other diseases. In the current investigation, two of three test samples showed reasonable activity against the tested cancer cell lines, showing considerably significant activity against HepG-2 and MCF-7 cells Table 4.

This preliminary study data supports the presence of various classes of bioactive compounds, which might potentially provide significant anticancer activity. New compounds can be isolated and identified using bioactivity-guided isolation against various cancer cell lines. Extensive phytochemical and pharmacological investigations of *G royleana* are recommended for ongoing anticancer-based drug research. *In-vivo* toxicity revealed that the LD50 of the extract in mice was found to be 1250 mg/kg *via* intraperitoneal route.

CONCLUSION

From the current study, it is concluded that the various fractions of the aerial parts of *G royleana* showed promising pharmacological activities due to the presence of polyphenolic bioactive compounds. Therefore, further work is required to isolate these compounds by applying advanced analytical techniques.

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AUTHOR'S NOTE

The authors declare that we have no conflict of interest.

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Table 2: Anti-diabetic potential of methanol extract of *Gymnosporia Royleana.*

Groups and doses	Initial Day Mean ± SD	Day 5 Mean ± SD	Day 10 Mean ± SD	Day 15 Mean ± SD
NC (Normal control)	70.81 ± 0.46	72.69 ± 0.22	72.03 ± 0.38	72.06 ± 0.11015
DC (Diabetic control)	184.36 ± 0.37	185.78 ± 0.41	187.92 ± 0.46	192.53 ± 0.23
GR (200 mg/kg)	183.21 ± 0.57	157.28 ± 0.63	116.17 ± 0.43*	97.91 ± 0.51*
GR (400 mg/kg)	183.87 ± 0.33	141.82 ± 0.57	102.77 ± 0.59*	86.45 ± 0.29*
Glibenclamide (10 mg/kg)	193.37 ± 0.52	154.93 ± 0.48	109.27 ± 0.31*	75.78 ± 0.31*

Values are presented as mean ± SD. ANOVA followed by *Post-hoc* Dunnett's test. Asterisks indicated statistically significant results. *$p < 0.05$. NC: Normal control; DC: Diabetic control; GR: *Gymnosporia Royleana*.

Table 3: Haemagglutination potential of methanolic extract of *Gymnosporia Royleana*.

Concentrations	PHA	*Gymnosporia Royleana*
1	3.71 ± 0.223	4.671 ± 0.357*
0.1	3.89 ± 0.215	4.329 ± 0.312*
0.01	3.71 ± 0.298	4.211 ± 0.287*
0.001	3.70 ± 0.301	4.248 ± 0.419*
0.0001	3.70 ± 0.271	4.252 ± 0.554*

Each point is the mean ± SEM (ANOVA and Dunnet's *post-hoc* test: *$p < 0.05$, v = 6 against PHA (phytohaemagglutinin) at each separate concentration).

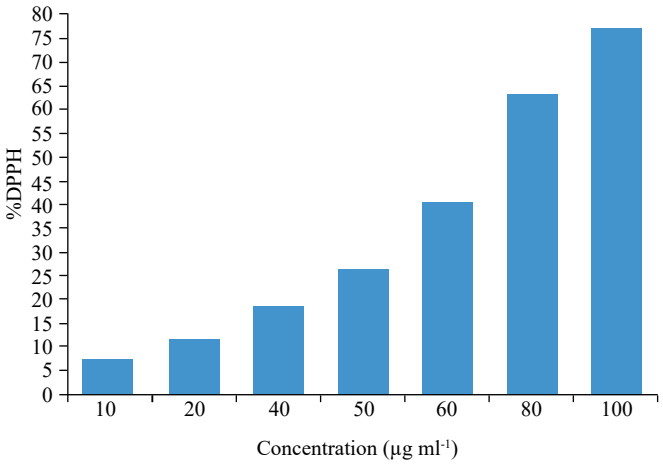

Fig. 2: Antioxidant activity of methanol extract of *Gymnopsporia Royleana.* The effects of the antioxidant potential of *Gymnopsporia Royleana* are depicted in figure above. The percentage of DPPH (2,2-diphenyl-1-picrylhydrazyl) values increase by increasing the concentration of the plant extract (10-100 µg/mL). Methanolic extract of the plant proved to be a good source of free radical scavenging activity with a percent DPPH value ranged from 7.43–77.31% at varying concentrations.

Table 4: Anticancer activity results of *Gymnosporia Royleana*

Sample	IC_{50} (µg/mL) HepG-2	MCF-7
Gymnopsporia Royleana (GR)	162.1± 0.02	241.7± 0.08
Doxorubicin (Standard)	0.39 ± 0.03	0.28 ± 0.02

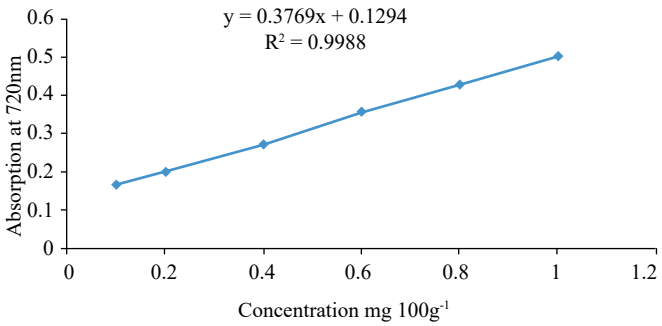

Fig. 3: Total phenolic content (TPC) of *Gymnosporia Royleana*. The outcome of total phenolic content of *G Royleana* have demonstrated in figure above and were expressed as tannic acid equivalent (TAE) in mg 100 g^{-1}. The subject plant was found to possess significant phenolic content of 12.02 mg 100 g^{-1}.

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