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# Screening of *Rhamnus Purpurea* (Edgew.) Leaves for Antimicrobial, Antioxidant, and Cytotoxic Potential

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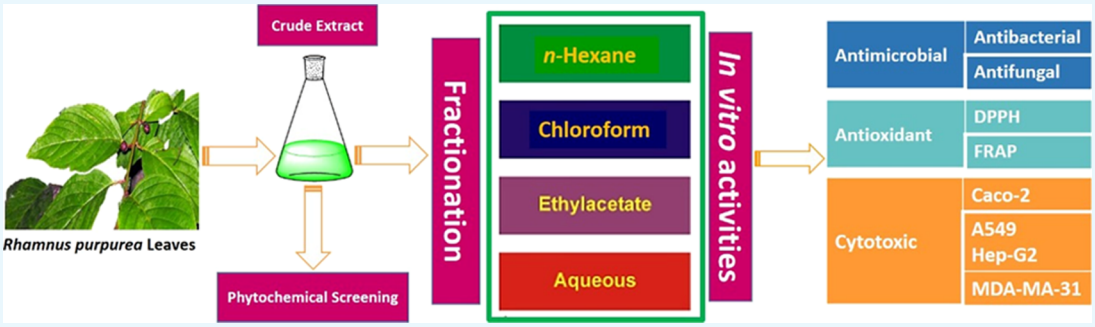


**ABSTRACT:** Exploring new antimicrobial and cytotoxic drugs has been one of the most active areas of research. *Rhamnus purpurea* (Edgew.) buckthorn (*Rhamnaceae*) is a wild shrub traditionally used in Pakistan for the treatment of various ailments including cancer and infectious diseases. The aim of this study is to find novel antimicrobial and cytotoxic agents of plant origin. The crude methanol extract and full range of fractions of *R. purpurea* leaves were screened for the said activities using *in vitro* antimicrobial, antioxidant, and cytotoxic models following standard protocols. The antimicrobial activity was evaluated using the agar well diffusion method, while the antioxidant activity was assessed with 1,1-diphenyl-2-picryl hydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) assays. The cytotoxic effect was investigated against the human cancer cell lines i.e. Caco-2 (gut), A549 (lung), HepG2 (liver), and MDA-MB-231 (breast) by MTS assay. In addition, toxicity studies were conducted on renal and alveolar primary epithelial cells (HRPTEpiC and HPAEpiC, respectively). Phytochemical investigation showed the presence of secondary metabolites such as alkaloids, saponins, tannins, glycosides, phenols, carbohydrates, proteins, and flavonoids. The *n*-hexane and chloroform fractions showed significant activity against *Staphylococcus aureus* (MIC 0.60 and 0.68 mg/mL, respectively), *Salmonella typhi* (MIC 0.48 and 0.45 mg/mL, respectively), and *Bacillus subtilis* (MIC 0.54 and 0.76 mg/mL, respectively). Among fungal strains, crude methanol and chloroform fractions exhibited significant activity against *Fusarium solani* (MIC 0.53 and 0.44 mg/mL, respectively) and *Aspergillus niger* (MIC 0.47 and 0.42 mg/mL, respectively). The crude methanol, *n*-hexane and chloroform fractions revealed the highest antioxidant activity at 1000 $\mu$g/mL, compared to that of ascorbic acid. The *n*-hexane fraction showed a significant cytotoxic effect against Caco-2, A549, and HepG2 cell lines with $IC_{50}$ values of 5.65 ± 0.88, 5.50 ± 0.90, and 4.95 ± 1.0 $\mu$g/mL, respectively. Similarly, the chloroform fraction depicted significant activity against Caco-2, A549, and HepG2 cell lines with $IC_{50}$ values of 4.55 ± 1.25, 4.65 ± 1.55, and 2.85 ± 0.98 $\mu$g/mL, respectively. The crude methanol extract and almost all fractions exhibited the highest selectivity index (>2.0) for Caco-2, A549, and HepG2 cancer cell lines, providing safety data for this study. The results showed that *R. purpurea* leaves have excellent antimicrobial, antioxidant, and cytotoxic potential and warrant further studies to search for novel compounds for the said activities.

## 1. INTRODUCTION

Antimicrobial resistance (AMR) is a serious issue across the globe, particularly in developing countries such as those of South Asia.[1] The major reasons include its availability over the counter that has caused approximately 5.3 million deaths worldwide annually. In Pakistan, both extensively drug-resistant (XDR) and multidrug-resistant (MDR) bacteria have been identified in recent years.[2,3] It has been reported that *Enterobacteriaceae* has developed a significant resistance against quinolones. Similarly, *Salmonella* species has shown almost 100% resistance against fluoroquinolones.[4] Furthermore, in the US (1999−2012), 68.4% *Staphylococcus epidermidis*, 47.9% *Acinetobacter bauman-*

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*nii*, and 13.7% *Escherichia coli* were ciprofloxacin-, carbapenem-, and β-lactam-resistant, respectively.[5,6] A similar resistance has emerged in Canada (2007−2011), where 27% *E. coli*, 19.3% *Staphylococcus aureus*, and 16.8% *Streptococcus pneumoniae* were ciprofloxacin-, methicillin-, and penicillin-resistant, respectively.[7]

Likewise, cancer is one of the leading causes of mortality around the globe. According to the World Health Organization (WHO), approximately 13 million deaths are expected from this fatal disease by the end of 2030.[8,9] Among these, breast cancer is the most common malignancy worldwide. It is noteworthy that Pakistan has the highest rate of breast cancer in Asia with more than 40,000 deaths and 90,000 cases reported annually.[10] It has 25% more incidence in women; however, men are also susceptible, but fortunately, the mortality rate is very low in the latter (1:1000).[9] Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide, with approximately 5.7% new cases annually. Globally, it remains the third leading cause of cancer-related deaths with an overall survival rate of only 3−5%. It is more prevalent in South Asia, with 55% cases in China only. Hepatitis B and C are the well-known factors for HCC.[11,12] Lung cancer accounts for 470,000 cases in Europe each year. In Pakistan, its incidence, prevalence, and mortality are not known due to the absence of population-based cancer registry. According to regional cancer registries, this is the second most common cancer in men and third most common cancer in both sexes.[13] Colorectal cancer is the third most common type of cancer worldwide. This type of cancer is closely associated to the age, as it is more common in people over the age of 65 years and accounts for approximately 70% of all cancer cases.[14,15] It is more prevalent in developed countries; however, its prevalence is on rise in developing countries such as Pakistan and India. Various chemotherapeutic drugs are used for the treatment of these cancers. However, they are associated with resistance and severe side effects such as nausea, vomiting, hair loss, and fatigue. Therefore, there is a need to search for safe and more selective/specific cytotoxic agents.

Production of free radicals is among the several factors responsible for causing a variety of cancers. According to the literature, oxidation is one the important processes in the production of free radicals, i.e., hydroxyl and perhydroxyl.[16,17] Synthetic antioxidants are frequently used to prevent the generation of free radicals and subsequently stop the oxidation process. However, they are usually associated with several side effects. Butylated hydroxy toluene and hydroxy anisole are frequently linked to liver cirrhosis and cancer.[18] Therefore, there is a need to search for more safe and effective antioxidants especially of natural origin.

*Rhamnus* is a genus of approximately 110 species, commonly known as buckthorns. Some of these species are *Rhamnus alaternus*, *Rhamnus caroliniana*, *Rhamnus cathartica*, *Rhamnus diffusus*, *Rhamnus globosa*, *Rhamnus japonica*, and *Rhamnus saxatilis*. *Rhamnus purpurea* (Figure 1), commonly known as *buckthorns*, is a wild shrub, widely distributed in East Asia and North America with species ranging in height from 1 to 10 meter. Several phytochemicals have been isolated from the *Rhamnus* genus including flavonoids, alkaloids, glycosides, phenols, and anthraquinones. To date, no pharmacological activity of this plant has been reported. However, in folk medicine, it has been widely used in the treatment of cancer, wound and throat infections, inflammation, and other health problems.[19−21] Due to its widespread traditional use, the current study scientifically investigates different fractions of the said plant for antimicrobial, antioxidant, and cytotoxic potential.

**Figure 1.** *Rhamnus purpurea* Edgew. (photograph courtesy of Dr. Atif Ali Khan Khalil; the photo is from a free domain).

## 2. MATERIALS AND METHODS

**2.1. Chemicals.** Imipenem and Miconazole were donated by Ferozsons Laboratories Limited, Nowshera, Pakistan, and 1,1-diphenyl-2-picryl hydrazyl (DPPH), ferric reducing antioxidant power (FRAP) reagents, acetate buffer, HCl, methanol, chloroform, *n*-hexane, and ethyl acetate were purchased from Sigma-Aldrich, Germany.

**2.2. Plant Materials.** The plant was collected (July 2018) from Dir and Swat valleys (latitude 35° 3′ 11″ N; longitude 72° 33′ 42″ E), Khyber Pakhtunkhwa (KPK), Pakistan, and was identified by Dr. Fazal Hadi, Department of Botany, University of Peshawar. (Voucher no. F.Khuda-32522).

*2.2.1. Preparation of the Extract.* The leaves were thoroughly washed, dried under shade, and subsequently powdered. The powdered material (1000 g) was macerated for at least 3 days in methanol and stirred occasionally. The macerate was filtered and concentrated to dryness under vacuum (40 °C) using a rotary evaporator.

*2.2.2. Fractionation.* The dark-green extract was dissolved in distilled water and subsequently partitioned with different solvents to obtain chloroform, n-hexane, ethyl acetate, and aqueous fractions. The crude methanol extract and its fractions were then screened for the following biological activities.

**2.3. Phytochemical Screening.** The extract was screened for the presence of the following phytochemicals: alkaloids, saponins, tannins, glycosides, phenols, carbohydrates, proteins, and flavonoids. The method of Farzana et al. was used for conducting the following assays.[22]

*2.3.1. Tests for Alkaloids. 2.3.1.1. Mayer's Reagent Test.* In this test, 2 mL each of methanol crude extract and conc. hydrochloric acid was mixed, followed by the dropwise addition of Mayer's reagent. The appearance of the green color or white precipitate confirmed the presence of alkaloids.

*2.3.1.2. Hager's Test.* In this test, 2−3 drops of Hager's reagent (picric acid) were added to the plant extract (2 mL). The appearance of a yellow precipitate revealed the presence of alkaloids.

*2.3.2. Test for Saponins.* In a test tube, 2 mL each of the plant extract and distilled water was mixed and shaked vigorously for 5 min. The formation of a thick foam at the top of the tube indicted the presence of saponins.

*2.3.3. Test for Flavonoids.* This test was performed by the addition of a few drops of ferric chloride to about 2 mL of the plant extract. The appearance of blackish-red precipitates confirmed the presence of flavonoids.

*2.3.4. Test for Tannins. 2.3.4.1. Alkaline Reagent Test.* In this test, 2 mL each of the extract and sodium hydroxide (1N) was added and mixed thoroughly. The formation of red or yellow precipitates showed the presence of tannins.



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*2.3.5. Test for Glycosides. 2.3.5.1. Keller Killiani Test.* This test was performed by the addition of 1 mL each of the extract and glacial acetic acid, followed by cooling and adding 2−3 drops of ferric chloride. Finally, sulfuric acid (0.5 mL) was added along the sides of the test tube. The formation of a reddish-brown ring at the junction of the two layers confirmed the presence of glycosides.

*2.3.6. Test for Phenols. 2.3.6.1. Ellagic Acid Test.* To 1 mL of the plant extract, few drops of glacial acetic acid (5%) were added and mixed, followed by the addition of few drops of sodium nitrite (5%). The appearance of muddy brown color indicated the presence of phenols.

*2.3.7. Test for Carbohydrates. 2.3.7.1. Benedict's Test.* A few drops of Benedict's reagent were mixed with the plant extract, followed by boiling. The formation of reddish-brown precipitates showed the presence of carbohydrates.

*2.3.8. Test for Proteins. 2.3.8.1. Xanthoproteic Test.* This test was conducted by the addition of few drops of conc. nitric acid to about 1 mL of the extract. The appearance of yellow color revealed the presence of proteins in the extract.

**2.4. Biological Activities.** *2.4.1. Antimicrobial Assay. 2.4.1.1. Microorganisms.* The following reference strains of bacteria were tested: *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *S. aureus* (ATCC 25923), *Salmonella typhi* (ATCC 19430), and *Bacillus subtilis* (ATCC 6633). Fungal strains include the following: *Fusarium solani* (ATCC 11712), *Aspergillus terreus* (ATCC 20542), *Aspergillus flavus* (ATCC 32611), and *Aspergillus niger* (ATCC10549).

*2.4.1.2. Antibacterial Activity.* The agar well diffusion method was used for conducting the assay.[23] The stated bacterial strains were cultured on nutrient broth media at 37 °C for 24 h. After incubation, the bacterial suspension (100 $\mu$L) was spread on sterilized medium plates. Wells were made using a sterilized cork borer for both the test samples and control. Test samples were prepared by dissolving 5 mg of the crude methanol extract and its fractions in dimethyl sulfoxide (DMSO). These samples (100 $\mu$L) were then added to the respective wells along with negative (DMSO) and positive (Imipenem) controls. The plates were incubated for 48 h at 37 °C. The percent inhibition growth was calculated by measuring the diameter around cleared zones created by the sample and positive control. The experiment was conducted in triplicate, and the mean values were recorded.

*2.4.1.3. Antifungal Activity.* This activity was conducted using the agar slanting method.[24] The cultured medium was poured into sterilized test tubes. Test samples were prepared by dissolving 25 mg of the crude methanol extract and its fractions in DMSO (3%). About 100 $\mu$l of each sample was added into each test tube containing Sabouraud dextrose agar media (SDA) and allowed to solidify in the tilted position. Subsequently, it was incubated at 30 °C for at least 1 week. DMSO and miconazole were used as negative and positive controls, respectively. Finally, the test tubes were measured for linear growth inhibition, and % inhibition was determined using eq 1.

$$\%\ \text{inhibition} = 100 - \frac{\text{linear growth (test)}}{\text{linear growth (control)}} \times 100 \quad (1)$$

*2.4.1.4. Determination of the Minimum Inhibitory Concentration (MIC).* The MIC of different fractions of the methanolic extract was determined by the microdilution method, using 96-well microtiter plates.[25] The extract and fractions were dissolved in DMSO and serially diluted with the respective broth to obtain different dilutions (0.1−30 mg/mL). Susceptible strains of bacterial and fungal cultures were then added to each well and incubated at 37 °C for 24 and 72 h, respectively. Tetrazolium salt was added to each well as an indicator; the appearance of violet color in culture media indicates the growth of microorganisms. Imipenem and miconazole serve as positive controls, while DMSO serves as a negative control.

**2.5. Antioxidant Assay.** *2.5.1. 1,1-Diphenyl-2-Picryl Hydrazyl (DPPH) Assay.* This assay was performed with DPPH following a well-established procedure with slight modifications.[26] The reagent solution was prepared (0.01 mM) by dissolving it in methyl alcohol (95 mL). Similarly, stock solutions of the crude extract and its fractions were prepared in methyl alcohol (1 mg/mL) and subsequently diluted to get working solutions of different concentrations (62.5, 125, 250, 500, and 100 $\mu$g/mL). From each working solution, 100 $\mu$l was mixed with 3 mL of DPPH solution and allowed to incubate for 15 min at 23 °C. Finally, the maximum absorbance was measured using a spectrophotometer (517 nm), and % radical scavenging activity of the extract and its fractions was calculated using eq 2.

$$\%\ \text{radical scavenging potential} = \frac{C_{\text{Abs}} - S_{\text{Abs}}}{C_{\text{Abs}}} \times 100 \quad (2)$$

where $C_{\text{Abs}}$ and $S_{\text{Abs}}$ are the absorbance of the control and test samples/standard, respectively.

*2.5.2. Ferric Reducing Antioxidant Power (FRAP) Assay.* The method of Iris and Strain was adopted for the conduction of this assay.[27] The assay is used to determine the reducing power of the tested compound. Compounds with antioxidant activity reduces the ferric ion ($Fe^{3+}$) to the ferrous ion ($Fe^{2+}$); the latter form a blue complex ($Fe^{2+}$/TPTZ) that significantly increases the total absorption at 593 nm. The assay mixture comprised TPTZ solution (0.25 mL; 10 mM) in HCl (40 mM), acetate buffer (2.5 mL; 300 mM, pH 3.6), $FeCl_3$ (0.25 mL; 20 mM), and crude methanol extract along with its fractions at various concentrations (62.5, 125, 250, 500, and 100 $\mu$g/mL). The FRAP reagent (4 $\mu$L) and sample solutions (100 $\mu$L) were added to each well and mixed thoroughly. Following incubation for 30 min, the absorbance was measured at 593 nm, and % radical scavenging activity of the extract and its fractions was calculated. The assay was performed in triplicate.

**2.6. Cytotoxicity Assay.** The cytotoxic potential against four human cancer cell lines, i.e., hepatocellular carcinoma (HepG2), lung adenocarcinoma (A549), intestinal epithelial (Caco-2), and breast adenocarcinoma cells (MDA-MB-321) (Korea cell line bank, Seoul, Korea), and two normal cell lines, i.e., primary epithelial cells (HPAEpiC and HRPTEpiC), was investigated by the MTS assay with slight modifications.[28] The cells were cultured in RPMI-1640 media containing streptomycin/penicillin solutions (1% v/v) and fetal bovine serum (10%) (FBS) and subsequently incubated at 37 °C with 5% $CO_2$. Before seeding, cell lines with 80−90% confluency were expanded up to three passages. Prior to the addition of fresh media, Dulbecco's phosphate buffer saline (DPBS) was used to wash the cells to remove cell debris or any metabolites. Cells showing 90% confluency were trypsinized (0.05% trypsin-EDTA solution) and suspended in RPMI-1640 media containing FBS. About 24 h prior to the treatment, cell lines were seeded in 96-well microplates having 2000 cells/well.


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Following an overnight incubation, the cells were treated with various concentrations of the crude methanol extract and its fractions. Wells containing media, cells, and tested drugs served as positive controls, while those containing only cells and media served as the negative control. After incubation for 48 h, the MTS reagent (25 $\mu$L) was added to each well and allowed to incubate for 30 min. Using a multimode microplate reader (Varioskan Flash, Thermo Scientific, Waltham, MA), the absorbance was measured at 490 nm. Finally, the percent cell viability for the extract and fractions was determined using eq 3. The concentration required to kill 50% of the cell population or $IC_{50}$ was computed using linear regression of the graph of absorbance against concentration. To find out the specificity of the extract and fractions to cancer cells, the data were analyzed to calculate the selectivity index (SI). It was calculated by dividing the $IC_{50}$ values for the noncancer (HPAEpiC and HRPTEpiC) cell lines by the values of $IC_{50}$ for cancer (Caco-2, A549, HepG2 and MDA-MB-231) cell lines. A value of 2 or more indicated high specificity.

$$\%\ \text{viable cells} = \left(\frac{\text{abs}_{\text{sample}} - \text{abs}_{\text{blank}}}{\text{abs}_{\text{control}} - \text{abs}_{\text{blank}}}\right) \times 100 \quad (3)$$

**2.7. Statistical Analysis.** One-way ANOVA (GraphPad Software) was used for data analysis, and it was presented as the mean ± standard error of the mean (SEM) ($n$ = 5).

## 3. RESULTS

**3.1. Phytochemical Screening.** The results of phytochemical screening are summarized in Table 1. Based on phytochemical analysis, the *R. purpurea* crude methanol extract contains alkaloids, glycosides, saponins, phenols, and flavonoids as the major secondary metabolites. Ellagic acid and Xanthoproteic tests were unable to confirm the presence of carbohydrates and proteins, respectively.

**Table 1. Phytochemical Screening of the Crude Extract of *R. Purpurea*[a]**

| phytochemicals | chemical tests | results |
|---|---|---|
| alkaloids | Mayer's reagent test | + |
| | Hager's test | + |
| saponins | Foam test | + |
| tannins | alkaline reagent test | + |
| glycosides | Keller Killiani test | + |
| phenols | Ellagic acid test | + |
| carbohydrates | Benedict's test | N |
| proteins | Xanthoproteic test | N |
| flavonoids | general test | + |

[a]Abbreviations: + sign, present; N, not indicated.

**3.2. Antimicrobial Assays.** The antibacterial and antifungal activities were characterized using agar well diffusion and agar slanting methods, respectively.

*3.2.1. Antibacterial Activity.* The antibacterial activity of the *R. purpurea* leaf extract and fractions was investigated according to their inhibition zones and MIC values against the selected bacterial strains (Table 2). The methanol extract, chloroform, and n-hexane fractions showed significant inhibitory zones against *P. aeruginosa*, *S. aureus*, *S. typhi*, and *B. subtilis*. Among the tested strains, *S. typhi* remained the most sensitive organism against chloroform and *n*-hexane fractions (% IZ: 71 and 76%, respectively) with MIC values of 0.48 and 0.45 mg/mL, respectively, as compared to the standard. Likewise, the said fractions showed significant activity against *B. subtilis* (% IZ: 64 and 56%, respectively) with MIC values of 0.54 and 0.76 mg/mL, respectively. The crude methanol extract and *n*-hexane and chloroform fractions also depicted significant activity against *S. aureus* (% IZ: 52, 63, and 55%, respectively) with MIC values of 0.77, 0.60, and 0.68 mg/mL, respectively. Results of antibacterial activity suggest that *E. coli* remained the most resistant strain to the crude methanol extract and all of its fractions. On the other hand, ethyl acetate and aqueous fractions did not show considerable activity against any of the tested bacteria.

*3.2.2. Antifungal Activity.* The zones of inhibitions exhibited by the crude methanol extract, its fractions, and standard against different fungal strains are shown in Table 3. The crude methanol extract and chloroform fractions exhibited significant activity against *F. solani* and *A. niger* (% I: 48, 58 and 55, 62, respectively) with MIC values of 0.53 and 0.47 mg/mL for the former and 0.44 and 0.42 mg/mL for the latter. Among the fungal strains, *A. terreus* remained the most resistant strain and did not show considerable sensitivity against the crude methanol extract and any of its fractions. The *n*-hexane, ethyl acetate, and aqueous fractions did not show any considerable activity against the tested fungal strains.

**3.3. Antioxidant Activity.** The antioxidant activity values of the extract, its fractions, and standard are shown in Tables 4 and 5. The DPPH radical scavenging assay demonstrated dose-dependent activity for the extract and fractions, i.e., *n*-hexane, chloroform, ethyl acetate, and aqueous with $IC_{50}$ values of 22.35, 25.20, 19.52, 25.78, and 28.70 $\mu$g/mL, respectively, compared to that of the standard (14.55 $\mu$g/mL). Among various fractions, the chloroform fraction showed significant antioxidant activity particularly at higher concentrations (65% at 1000 $\mu$g/mL with an $IC_{50}$ of 19.52) followed by the crude methanol extract, which depicted 47% inhibition ($IC_{50}$ 22.35) at the same concentration. In the present study, the aqueous fraction demonstrated the lowest free radical scavenging activity at all concentrations (Table 4). Similarly, the FRAP assay demonstrated a dose-dependent activity for the crude methanol extract and all of its fractions (Table 5). The *n*-hexane and chloroform fractions

**Table 2. Antibacterial Activity of the Crude Extract and Its Fractions against Selected Bacterial Strains[a]**

| | crude extract | | | *n*-hexane | | | Chloroform | | | ethyl acetate | | | aqueous | | | std. | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| test microorganisms | IZ | % I | MIC | IZ | %I | MIC | IZ | %I | MIC | IZ | % I | MIC | IZ | % I | MIC | IZ | MIC |
| *E. coli* | 13 | 38 | 0.92 | 11 | 32 | 0.68 | 10 | 29 | 0.72 | 8 | 23 | 1.88 | 13 | 30 | 1.87 | 34 | 0.0020 |
| *P. aeruginosa* | 18 | 51 | 0.77 | 22 | 62 | 0.55 | 20 | 57 | 0.69 | 11 | 31 | 1.76 | | | | 35 | 0.0019 |
| *S. aureus* | 14 | 52 | 0.77 | 17 | 63 | 0.60 | 15 | 55 | 0.68 | | | | 5 | 18 | 1.90 | 27 | 0.0014 |
| *S. typhi* | 19 | 50 | 0.79 | 27 | 71 | 0.48 | 29 | 76 | 0.45 | 14 | 36 | 1.0 | 8 | 21 | 1.92 | 38 | 0.0018 |
| *B. subtilis* | 10 | 40 | 0.90 | 16 | 64 | 0.54 | 14 | 56 | 0.76 | 7 | 28 | 1.25 | 14 | 56 | 0.68 | 25 | 0.0022 |

[a]IZ, inhibition zone (mm); % I, percent inhibition; MIC, minimum inhibitory concentration (mg/mL); Std, imipenem.



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**Table 3. Antifungal Activity of the Crude Extract and Its Fractions against the Selected Fungal Strains[a]**

| | crude extract | | *n*-hexane | | chloroform | | ethyl acetate | | aqueous | | std. | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Test microorganisms | % I | MIC | % I | MIC | % I | MIC | % I | MIC | % I | MIC | % I | MIC |
| *F. solani* | 48 | 0.53 | 17 | 1.55 | 58 | 0.44 | 32 | 1.15 | 18 | 1.50 | 72 | 0.0004 |
| *A. terreus* | 23 | 1.78 | 18 | 1.88 | 16 | 1.70 | 10 | 1.88 | | | 58 | 0.0018 |
| *A. flavus* | 38 | 0.84 | 44 | 0.64 | 40 | 0.68 | 28 | 1.0 | | | 66 | 0.0012 |
| *A. niger* | 55 | 0.47 | 21 | 1.45 | 62 | 0.42 | 33 | 0.89 | 11 | 1.98 | 75 | 0.0005 |

[a]% I, percent inhibition; MIC, minimum inhibitory concentration (mg/mL); Std, miconazole.

**Table 4. DPPH Free Radical Scavenging Activity**

| | DPPH scavenging activity (%) | | | | | |
|---|---|---|---|---|---|---|
| concentration ($\mu$g/mL) | extract | *n*-hexane | chloroform | ethyl acetate | aqueous | ascorbic acid |
| 62.5 | 7.20 ± 2.0 | 3.12 ± 0.80 | 11.56 ± 1.55 | 4.25 ± 0.56 | | 22.32 ± 4.67 |
| 125 | 11.65 ± 2.0 | 8.45 ± 1.65 | 23.65 ± 3.10 | 11.34 ± 1.20 | 4.45 ± 0.65 | 48.34 ± 5.20 |
| 250 | 22.55 ± 2.53 | 18.50 ± 2.20 | 41.80 ± 5.55 | 17.87 ± 1.88 | 11.23 ± 1.21 | 63.30 ± 2.52 |
| 500 | 39.70 ± 4.0 | 31.78 ± 3.89 | 56.70 ± 7.28 | 30.38 ± 3.43 | 21.50 ± 2.39 | 73.80 ± 4.55 |
| 1000 | 47.0 ± 2.56 | 39.90 ± 5.32 | 65.98 ± 7.10 | 37.72 ± 4.45 | 30.88 ± 3.10 | 85 ± 5.87 |
| IC50 | 22.35 | 25.20 | 19.52 | 25.78 | 28.70 | 14.55 |

**Table 5. FRAP Scavenging Activity**

| | FRAP scavenging activity (%) | | | | | |
|---|---|---|---|---|---|---|
| concentration ($\mu$g/mL) | extract | *n*-hexane | chloroform | ethyl acetate | aqueous | ascorbic acid |
| 62.5 | 9.12 ± 1.10 | 11.55 ± 1.10 | 19.45 ± 0.88 | | 2.11 ± 0.45 | 29.45 ± 3.23 |
| 125 | 17.23 ± 2.34 | 19.15 ± 1.70 | 33.18 ± 1.0 | 9.18 ± 0.88 | 9.88 ± 0.89 | 47.52 ± 4.20 |
| 250 | 29.80 ±1.55 | 38.22 ± 3.52 | 47.90 ± 1.80 | 14.54 ± 1.90 | 15.20 ± 1.0 | 69.90 ± 7.89 |
| 500 | 44.65 ± 3.40 | 56.60 ± 5.10 | 66.38 ± 4.24 | 23.52 ± 1.88 | 21.52 ± 1.89 | 81.35 ± 7.38 |
| 1000 | 52.0 ± 6.75 | 68.48 ± 7.75 | 72.70 ± 5.76 | 31.60 ± 4.12 | 29.98 ± 2.20 | 92.78 ± 8.80 |
| IC50 | 20.45 | 17.68 | 15.34 | 24.48 | 27.34 | 9.25 |

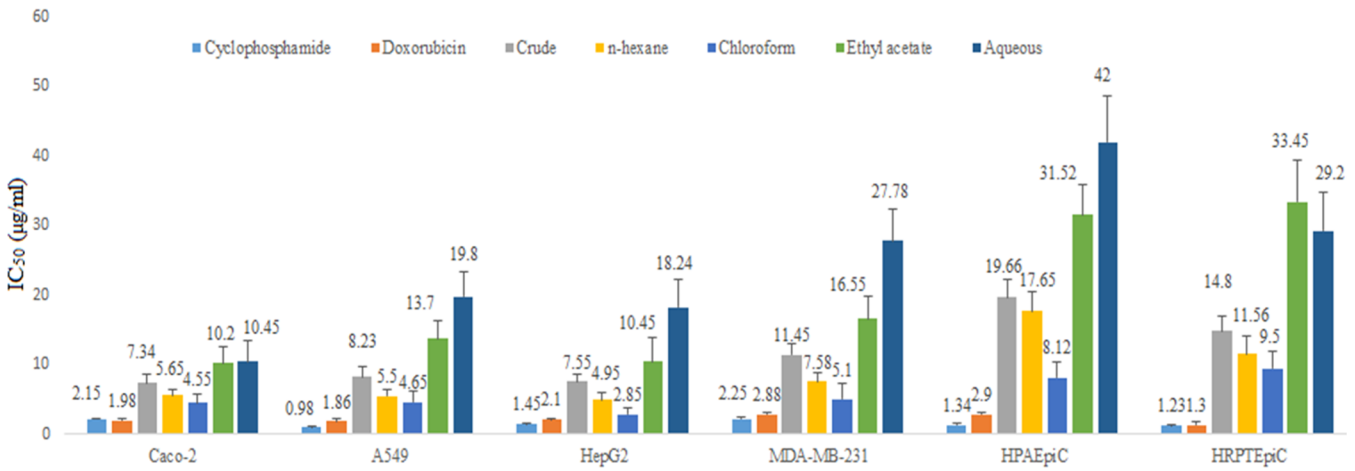


**Figure 2.** $IC_{50}$ values of the plant extract and fractions against different cell lines using cyclophosphamide and doxorubicin as positive controls and DMSO as a negative control.

showed significant activity (68 and 72% inhibition at 1000 $\mu$g/mL, respectively) with $IC_{50}$ values of 17.68 and 15.34 $\mu$g/mL, respectively. The ethyl acetate ($IC_{50}$; 24.48 $\mu$g/mL) and aqueous ($IC_{50}$; 27.34 $\mu$g/mL) fractions did not show any considerable activity compared to that of the standard ($IC_{50}$; 9.25 $\mu$g/mL). Likewise, the aqueous fraction remained the most inactive at all tested concentrations. Results of both assays depicted that the crude methanol extract and *n*-hexane and chloroform fractions had comparable free radical scavenging activities, as evident from the $IC_{50}$ values. On the other hand, ethyl acetate and aqueous fractions did not show any considerable antioxidant activities.

**3.4. Cytotoxicity Assay.** In the present study, the crude methanol extract and different fractions of *R. purpurea* were investigated against various cancer cell lines including Caco-2, A549, HepG2, and MDA-MB-321 and two normal cell lines, i.e., primary epithelial cells (HPAEpiC and HRPTEpiC) using the MTS assay. The $IC_{50}$ values are depicted in Figure 2.

The chloroform fractions revealed significant activity against Caco-2, A549, and HepG2 cell lines with $IC_{50}$ values of 4.55, 4.65, and 2.85 $\mu$g/mL, respectively, as compared to standard


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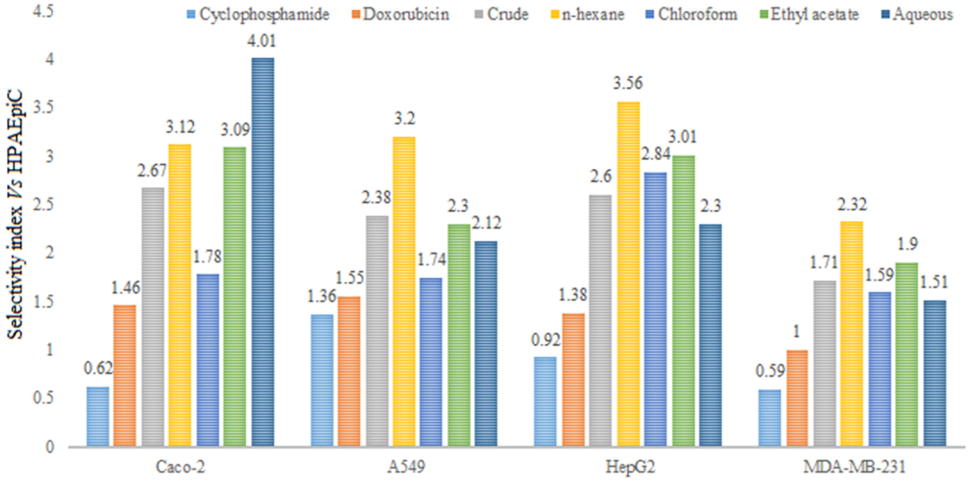


**Figure 3.** Selectivity indices of the standards, extract, and fractions against HPAEpiC.

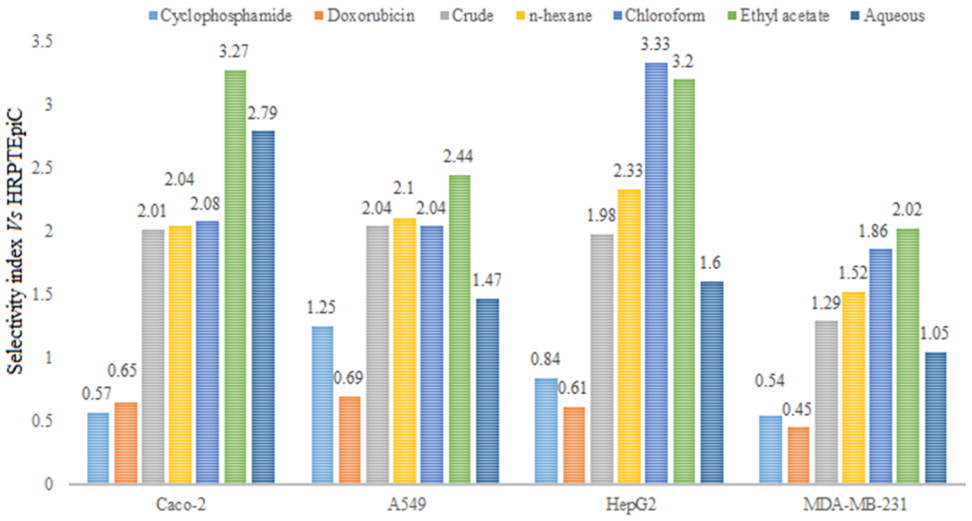


**Figure 4.** Selectivity indices of the standards, extract, and fractions against HRPTEpiC.

drugs, i.e, cyclophosphamide and doxorubicin ($IC_{50}$ values of 2.15 and 1.98; 0.98 and 1.86; and 1.45 and 2.10 $\mu$g/mL against the mentioned cell lines, respectively). Similarly, the *n*-hexane fraction showed significant activity against the same cell lines with $IC_{50}$ values of 5.65, 5.50, and 4.95 $\mu$g/mL, respectively. The corresponding percent cell viability values are depicted in Figures S1−S4. A lower percent cell viability value shows a greater potential for cytotoxic activity. In terms of percent cell viability, the *n*-hexane fraction showed significant cytotoxic activity (at 10 mg/mL dose) against Caco-2 (11%), A549 (17%), and HepG2 (13%) cell lines. Similarly, the chloroform fraction depicted significant activity against Caco-2 (9%), A549 (18%), and HepG2 (22%) cell lines at the same concentration. The ethyl acetate and aqueous fractions did not show any considerable cytotoxic activity against the studied cell lines. It is pertinent to mention that the crude methanol extract and fractions showed a dose-dependent activity. Compounds that exhibit potent cytotoxicity should be highly selective against cancer cells and remain safe or noncytotoxic against normal human cell lines. To investigate the safety profile or selectivity of the extract and fractions, they were tested against the primary epithelial cells HPAEpiC and HRPTEpiC (Figures S5 and S6). The percentage cell viability of all fractions and the crude methanol extract remained high compared to that of the standard drugs, i.e., cyclophosphamide and doxorubicin, when tested against HPAEpiC and HRPTEpiC cells. To assess and elaborate on the selectivity of the tested samples for cancer cell lines, the selectivity index was calculated (Figures 3 and 4). In the present study, the crude methanol extract and all fractions except chloroform exhibited high selectivity index values for Caco-2, A549, and HepG2 cancer cell lines, compared to those of standard drugs.

## 4. DISCUSSION

The literature regarding *R. purpurea* is scarce; therefore, this study may validate the traditional use of this plant as antimicrobial and cytotoxic agents. In the present study, the crude methanol extract and *n*-hexane and chloroform fractions revealed significant activity against *P. aeruginosa*, *S. aureus*, *S. typhi*, and *B. subtilis*. It has been documented that typhoid fever, throat, skin, and other frequent infections are caused by these pathogens. *S. aureus* is a well-known bacteria that causes infections in wounds and throat.[29] Overall, the results suggest that the *R. purpurea* extract may be used to mitigate infections caused by the mentioned pathogens. In addition, the traditional use to treat several infections, particularly throat and skin, supports the antibacterial findings of this study. Phytochemical analysis revealed the presence of phenolic compounds in the


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extract, which may be possibly involved in the antibacterial activity observed in the present study. The literature review showed that phenolics are the major plant constituents exhibiting antibacterial activity.[30] The bactericidal effect depicted by these substances may involve several mechanisms such as pore formation, inhibition of DNA gyrase and nucleic acid synthesis, and alteration in the physicochemical properties of bacterial membrane.[31] However, further studies are needed to screen *n*-hexane and chloroform fractions and to isolate the specific compounds responsible for the said activity and to elucidate the specific mechanism for the same.

Among the fungal strains, the crude methanol extract and chloroform fractions exhibited significant activity against *F. solani* and *A. niger* as compared to the standard. The same fractions showed considerable activity against *A. flavus*. The literature review showed that about half of the human diseases involving *Fusarium* are caused by *F. solani*.[32] These infections include, but not limited to, keratitis and skin infections. Similarly, the *Aspergillus* species are the common opportunistic filamentous fungi and are highly resistant to most of the available antifungal drugs.[33] In this study, the *R. purpurea* extract showed significant activity against the mentioned fungal strains. In addition, this plant is famous for the treatment of skin-related disorders; therefore, the extract and/or fractions derived from the said plant may be considered as an alternative effective therapy for the mitigation of the above-mentioned disorders. Possible mechanisms for antifungal drugs may include, but not limited to, the inhibition of cell wall synthesis and pore formation; however, further studies are required to elucidate the exact mechanism for the same.

According to the WHO, most of the world population (approx. 80%) rely on herbal medicines for their primary health care needs. Under stress conditions, the human body produces more reactive oxygen species such as hydroxyl and superoxide anion radicals, compared to enzymatic antioxidants. At high concentrations, these radicals are very much hazardous to the body and damage most of the cell components including DNA, cell proteins, and membranes, which eventually leads to the development of cancer and other health conditions.[34] Synthetic antioxidants are widely used in the food industry; however, they are associated with several side effects including carcinogenesis and liver cirrhosis. Therefore, in recent decades, the use of natural antioxidants has got much attention. The DPPH and FRAP assays showed that *R. purpurea* contains a considerable amount of antioxidants that can be useful in the treatment of several diseases including cancer. Furthermore, as chloroform and *n*-hexane fractions depicted significant antioxidant activity, the same fractions may be further investigated for the isolation of specific antioxidant compounds.

Despite advances in cancer research, it remains one of the most devastating diseases, being a major public health problem and one of the foremost causes of death worldwide.[35] To date, a number of anticancer drugs have been developed; however, the emergence of resistance and serious side effects associated with the existing drugs demands for the search of safe and effective cytotoxic compounds. Since ancient times, the plant kingdom has provided a number of diverse secondary metabolites for the treatment of several human aliments including different cancers. According to Cao et al., flavonoids isolated from different plants showed significant cytotoxic activity against human cancer cell lines such as breast (MCF-7) and liver (HepG2) cancers.[36] Alkaloids isolated from *Catharanthus roseus* (vinblastine, vincristine), *Solanum mauritianum* (solasodine), *Camptotheca acuminata* (Campothecin), etc. were effectively used in the treatment of ovarian and colorectal cancers.[37] Several saponins obtained from *P. japonocus*, *Panax ginseng*, and *P. quiquefolius* showed excellent cytotoxic properties against oral squamous carcinoma and other related cancers.[38] Similarly, other secondary metabolites such as polyphenols, tannins, brassinosteroids, anthocyanin, podophyllotoxin derivatives, terpenoids, and essential oils exhibited significant cytotoxic activity against various cancer cell lines.[39]

Fortunately, Pakistan has the world's richest natural flora. There are almost 5700 species of different plants, of which around 2000 species have medicinal values.[40] Exploring the medicinal values of these plants may not only contribute to drug discovery for various aliments but also play a vital role in the revenue generation. The present study was therefore designed to screen the *R. purpurea* extract, in search for new lead compounds with potential cytotoxic properties. Different fractions of the said plant showed significant activities against various malignancies. The crude methanol extract and chloroform and *n*-hexane fractions showed almost similar significant activities against gut, liver, and lung cancers. The results suggest further studies to explore new chemical entities that will help discover new therapeutic agents to treat a variety of cancers and infectious diseases.

## 5. CONCLUSIONS

To the best of our knowledge, this is the first detailed study regarding the antimicrobial, antioxidant, and cytotoxic potential of *R. purpurea*. The crude methanol extract and *n*-hexane and chloroform fractions revealed significant activity against the well-known pathogens of bacterial and fungal origin including *P. aeruginosa*, *S. aureus*, *S. typhi*, *F. solani*, *A. flavus*, and *A. niger*. The same fractions showed considerable antioxidant activity particularly at high concentrations (1000 $\mu$g/mL). In addition, the *n*-hexane and chloroform fractions demonstrated significant cytotoxic activity against Caco-2, A549, and HepG2 cell lines. Furthermore, the same fractions exhibited high selectivity against the mentioned cell lines, which confirms their safety for normal human cells. Based on the results, *R. purpurea* warrants further studies to isolate new leads for the effective treatment of a variety of cancers and infectious diseases. This study also suggests evaluating the *in vivo* cytotoxic potential of *R. purpurea* when used alone or in combination with cyclophosphamide or doxorubicin to lessen the side effects of the latter.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c03094.

(PDF)

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### Notes

The authors declare no competing financial interest.

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