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EXTRACTION, PHYTOCHEMICAL POTENTIAL AND IN VITRO BIOLOGICAL ACTIVITIES OF *AMYGDALUS BRAHUICA* ROOTS

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ABSTRACT

The major aim of this research work was to explore the phytochemical and biological potential of *Amygdalus brahuica*, a traditional medicinal plant which has not been extensively investigated in literature archives. The bioactive compounds from roots of this plant were extracted by using methanol and further fractionated into methanol, dimethyl sulfoxide (DMSO), ethyl acetate and hexane. The antioxidant potential was determined by DPPH and H_2O_2 scavenging assays while anti-inflammatory response was evaluated through protein denaturation method. The cytotoxic potential of these fractions was evaluated by using HeLa cervical cancer cell line. Antibacterial potential of various fractions was measured by zone of inhibition developed against exposure to different gram +ve and -ve bacterial strains. HPLC analysis revealed that methanolic fraction possess maximum concentration of p-coumaric acid (200.10 µg/g) along with other four phenolic compounds. Moreover, results of total phenolic content showed maximum concentration in methanolic fraction (56.8±0.79 mg GAE/g) while maximum total flavonoid contents in DMSO fraction (46.16±0.74 mg QE/g). Maximum DPPH scavenging potential with lowest IC_{50} (17.01 µg/mL) was showed by methanolic fraction while ethyl acetate fraction showed maximum H_2O_2 inhibition with lowest IC_{50} (13.71 µg/mL) as compared to all other fractions. Moreover, maximum anti-inflammatory response was observed in ethyl acetate fraction (79.29 ± 0.30%). The ethyl acetate fraction exhibited maximum antibacterial potential as compared to other fractions while, methanolic fraction showed maximum antiproliferative potential 44.4% against HeLa cells.

Keywords; A*mygdalus brahuica*, DPPH, phytochemicals, MTT assay, antibacterial, anti-inflammatory

INTRODUCTION

Medicinal plants are diverse sources of new and potential compounds that may support healthcare by providing new therapeutic agents with various pharmacological properties (Aumeeruddy & Mahomoodally, 2019). In the modern age, around 25% of the pharmaceutical products are derived and developed from plants (Aumeeruddy-Elalfi et al., 2018). World health organization (WHO) reported that almost 80% population in the world use herbs as medicinal plants to get cure from aliments (Veiga, Costa, Silva, & Pintado, 2020). Antioxidant, antibacterial, antifungal, antiviral, anti-inflammatory, anti-aging and insect repellant activities rely on many kinds of secondary metabolites such as lycopene, omega-3 fatty acids, glucosinolates, anthocyanin's, phytoestrogens

and polyphenols isolated from various plant sources by high-throughput instruments. With these features, approximately 20,000 types of plants have been studied extensively for their medicinal properties (Bursal, Aras, & Kılıç, 2019).

"Reactive oxygen species (ROS) are produced both during cellular metabolism and by external sources such as chemical intoxicants, smoking, UV radiation, and environmental stress (Salehi et al., 2019). Such risk leads to accumulation of highly reactive agents in the living body that damage many vital biomolecules of DNA, proteins, fats and sugars (Bursal, Taslimi, Gören, & Gülçin, 2020). It has been showed that ROS are linked to certain illnesses like diabetes, insulin resistance, heart diseases, respiratory distress and induction of cancer (Hassan et al., 2017). Moreover, plant derived bioactives have less side effects and cost effectiveness therefore, emerge as an alternative to existing therapies (Ige & Liu, 2020). For the healthcare and food industry, there is continuous search for antioxidant materials. Antioxidants are essential to sustain the normal healthy status by scavenging and neutralizing the free radicals and ROS (Chen, Wang, Zhu, Xiao, & Zhang, 2018).

Almonds have been used traditionally to treat many diseases due to its nutritious, calming and nerve-strengthening properties. It is also used as diuretic, emollient and laxative (Siddiqui & Begum, 2023). The use of almonds is associated with different health benefits like regulation of body weight glucose levels and it may act against various diseases like diabetes and other cardiovascular disease (Barreca et al., 2020). Almond is also hepato-protective, anti-aging, antidepressant, memory enhancer and antioxidant (Arangia et al., 2023).

Almond contains oil contents which possess oleic acid, linoleic acid, palmitic acid and stearic acid, α-tocopherol as well as protein content in its kernels (21.4-27.7%) (Kodad, Estopañán, Fagroud, Juan, & Socias i Company, 2013). Moreover, antioxidant potential and antimicrobial activities of the almond gum extracts revealed that it is rich source of polyphenols (Bouaziz et al., 2017). Thus, the objective of this work was to assess the phytochemical properties and in vitro biological effects (including antioxidant, antibacterial, anti-inflammatory, and anticancer activities) of various fractions obtained from the roots of Amygdalus brahuica, native to the Balochistan province of Pakistan.

MATERIALS AND METHODS

Plant material collection and storage

Amygdalus brahuica as whole plant was collected from Baluchistan, Pakistan. Roots were isolated and cleaned under fresh water to remove dirt and unnecessary material. Roots were dried under the shade, powdered and stored in appropriate container at room temperature for further use.

Preparation of extracts and fractionation

Amygdalus brahuica roots (500g) were air dried and grinded to coarse powder that was subjected to maceration in methanol (Truong et al., 2019). After maceration methanolic crude extract was dried and saved for further fractionation. Finally, the 2 g of saved extract was taken and fractionated using a separating funnel with four solvents including methanol, DMSO, ethyl acetate and n-hexane. The fractions obtained were labelled as Methanol (ABRMF), DMSO (ABRDF), ethyl acetate (ABREF) and n-hexane (ABRHF). The fractions were air dried and stored for further analysis.

Estimation of total phenolic (TPC) and flavonoids contents (TFC)

The Folin-Ciocalteu (FC) protocol, as described earlier (Aryal et al., 2019) was adopted with slight modifications to measure the total phenolic contents in these fractions of *Amygdalus brahuica* root extract. The solution of dried fractions was prepared to get final concentration of 1 mg/mL that was mixed with FC reagent. After vertexing, 0.8 mL sodium carbonate solution was added and incubated at room temperature for a period of 30 min. A volume of 200 µL from final solution was shifted to 96 well microplate and measured the absorbance at wavelength of 765 nm employing micro plate reader BioTek Synergy HTX (Winooski, VT, USA). TPC was expressed in mg of gallic acid equivalents per gram of dry extract (mg GAE/g extract).

Estimation of Total Flavonoid Contents (TFC)

The total flavonoid contents of different fractions of *Amygdalus brahuica* root extract were measured by $AlCl_3$ assay as previously described by (Aryal et al., 2019) with slight alterations. The solution of fractions was prepared to get final concentration of 1 mg/mL. Briefly, sample solution (1 mL), deionized water (4 mL), 10% aluminum chloride (300 µL) and 5% $NaNO_2$ (300 µL) were mixed together along with 2 mL of 1M NaOH. The final volume incubated for 6 minutes at room temperature. A volume of 200 µL from final solution was shifted to 96 well microplate and measured the absorbance at wavelength of 415 nm by micro plate reader BioTek Synergy HTX (Winooski, VT, USA). The results of TFC were expressed as (mg QE/g) mg of quercetin equivalent per gram of dry extract.

DPPH (1,1-diphenyl-2-picrylhydrazyl)

DPPH scavenging activity was measured by previously described protocol with some modifications (Al Rashdi, Hossain, & Al Touby, 2021). A volume of 1 mL of sample solution of fractions of *Amygdalus brahuica* extract was mixed with 1 mL of DPPH solution. From final solution volume of 200 µL was added in microplate and incubated for 30 minutes at room temperature while keeping in dark ambience. Absorbance was estimated at 517 nm micro plate reader BioTek Synergy HTX (Winooski, VT, USA).

Hydrogen peroxide (H_2O_2) Scavenging assay

The (H_2O_2) scavenging effect of fractions of *Amygdalus brahuica* root extract was determined by the previously described procedure (Mohammed & Niamah, 2022). The absorbance of final solution was measured at wavelength of 230 nm by microplate reader BioTek Synergy HTX (Winooski, VT, USA).

Phenolic acid profiling by HPLC

Individual phenolic contents in methanolic fractions of *Amygdalus brahuica* root extract were determined using by already reported method which based upon high performance liquid chromatography method (Zulfiqar, Hussain, Ali, Rathore, & Ahmed, 2024). HPLC system (Flexer Chromera, Perkin Elmer, USA) was consisted of a C18 column which had dimensions of 250 × 4.6 mm and particle size of 5 µm and having binary LC pump and a UV–visible LC detector. The analysis was conducted by employing a gradient system which comprise of two solvent systems [solvent A; mixture of acetonitrile and methanol (70:30) and solvent B; 0.5 % glacial acetic acid in double distilled water].

MTT Cytotoxicity Assay

MTT assay was conducted to evaluate the cytotoxic response of different fractions of *Amygdalus brahuica* roots on HeLa cells as described previously (Achakzai et al., 2020). The quantification of MTT reduction to formazan within cells was determined by measuring the absorbance at 570 nm, utilizing a microplate reader (Spectra Max plus, Molecular Devices, CA, USA).

Anti-inflammatory activity

Anti-inflammatory activity of different fractions of *Amygdalus brahuica* roots was evaluated in-vitro by the inhibition of protein denaturation protocol described previously (Nahari et al., 2022). Denaturation of protein was observed in water bath at 70 ℃ for 10 min. Final solution was cooled down at room temperature and absorption was measured at 660 nm by microplate reader BioTek Synergy HTX (Winooski, VT, USA).

Inhibition (%) = (ABS) of control – (ABS) of sample / (ABS) of control Whereas (ABS) = Absorbance

Antibacterial Activity

The antibacterial potential of fractions of methanolic extract of *Amygdalus brahuica* roots was evaluated against *Bacillus subtilis (ATCC23857)*, *Staphylococcus aureus (ATCC25923)* and

Escherichia coli (ATCC25922). Antibacterial activity of different fractions of *Amygdalus brahuica* roots was determined by employing well diffusion as described by (Iqbal, Chatha, Chauhdary, Ijaz Hussain, & Khan, 2023). The negative control well for hexane fraction contained hexane solvent whereas, (methanol, water 50:50) was used for other all fractions. The positive control (ciprofloxacin, 10 µg/Disc) were also placed. The zone of inhibition was calculated after incubating the petri plates at 37 ℃ for 24 hours.

Statistical analysis

The experiment was conducted following a complete randomized design repeating three times (n = 3). Collected data was presented as mean ± SEM and analyzed by employing analysis of variance and Dunacan's Multiple Range (DMR). GraphPad prism version 9.2.0 software (225 Franklin Street. Fl. 26 Boston, MA 02110) was used to analyze the data.

RESULTS

Extract/Fraction Yield (%)

The yield of crude methanolic extract of root of *Amygdalus brahuica* was about 8.65%. The methanolic extract (2 g) was used for fractionation in methanol (ABRMF), DMSO (ABRDF), ethyl acetate (ABREF) and n-hexane (ABRHF) which have yield of 27.5%, 25%, 40% and 7.5% respectively.

Total phenolic content (mg GAE/g)

The total phenolic contents (TPC) estimated in different fractions of methanolic crude extract of root of *Amygdalus brahuica* were within the range of (56.8±0.79-29.42±0.70 mg GAE/g of dry extract). However, maximum TPC were found in ABRMF; methanolic fraction (56.8±0.79 mg GAE/g) while ABRHF; n-Hexane fraction showed minimum phenolic contents (29.42±0.70 mg GAE/g of dry extract) as shown in Table 1, Figure 1.

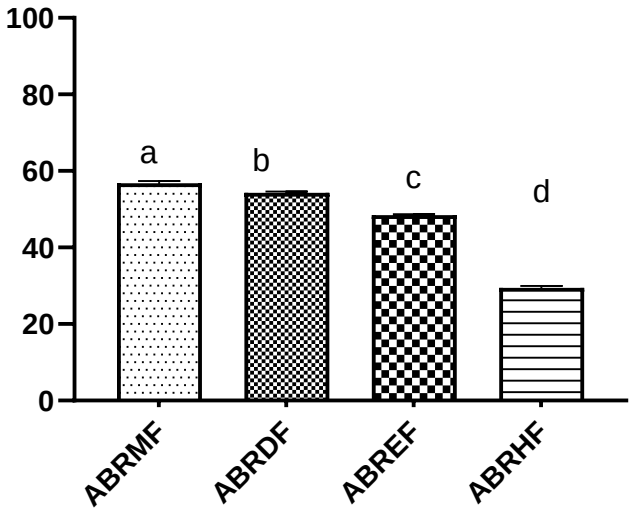

Figure 1. Total phenolic contents (mean ± SEM) mg of GAE/g in ABRMF, ABRDF, ABREF and ABRHF fractions of methanolic crude extract of root of *Amygdalus brahuica*. Values showing different superscripts (a, b, c and d) significantly differ from each other as $p \leq 0.05$. ABRMF = methanolic fraction, ABRDF = DMSO fraction, ABREF = ethyl acetate fraction and ABRHF = n-hexane fraction.

Total flavonoid content (mg QE/g)

The total flavonoid contents (TFC) estimated in different fractions of methanolic crude extract of root of *Amygdalus brahuica* were within the range of (46.16±0.74-35.92±0.79 mg QE/g). However, maximum TFC were found in ABRDF; DMSO fraction (46.16±0.74 mg QE/g) while ABRHF; n-

hexane fraction showed minimum contents (35.92±0.79 mg QE/g of dry extract) as shown in Table 1, Figure 2.

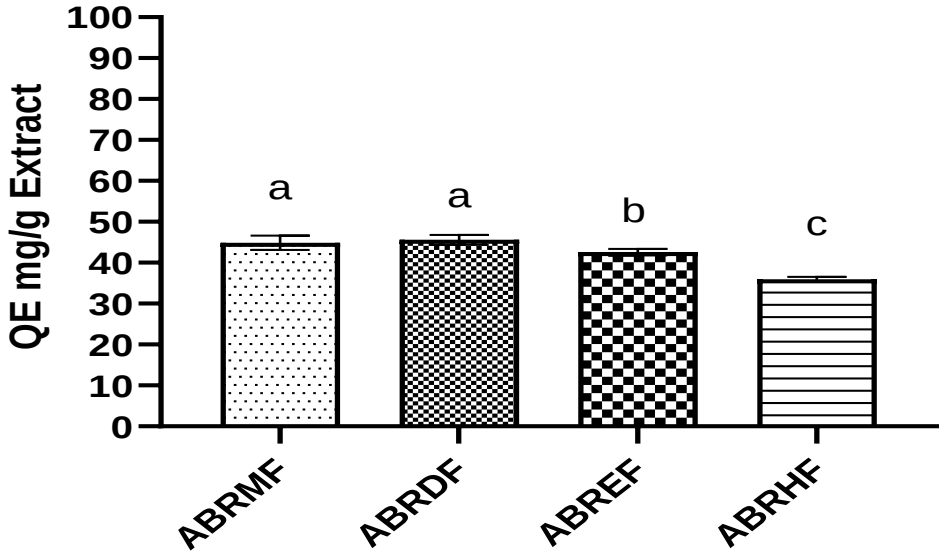

Figure 2. Total flavonoid contents (mean ± SEM) mg QE/g in ABRMF, ABRDF, ABREF and ABRHF fractions of methanolic crude extract of root of *Amygdalus brahuica*. Values showing different superscripts (a, b, c and d) significantly differ from each other as p≤0.05. ABRMF = Methanolic fraction, ABRDF =DMSO fraction, ABREF = ethyl acetate fraction and ABRHF = n-hexane fraction.

Table 1: TPC, TFC present in different fractions ABRMF = methanolic fraction, ABRDF = DMSO fraction, ABREF = ethyl acetate fraction and ABRHF = n-hexane fraction of methanolic crude extract of roots of *Amygdalus brahuica*.

No.	Samples	Total phenolic content (mg GAE/g)	Total flavonoid content (mg QE/g)
1	ABRMF	56.8 ± 0.79^{d}	44.86 ± 1.76^{a}
2	ABRDF	54.29 ± 0.42^{b}	46.16 ± 0.74^{a}
3	ABREF	48.44 ± 0.34^{a}	42.60 ± 1.12^{b}
4	ABRHF	29.42 ± 0.70^{c}	35.92 ± 0.79^{c}

Results are presented as mean ± SEM of three replicates (n = 3). Different superscript a, b, c and d linked values show significant differences among different fractions while setting $p \leq 0.05$.

DPPH activity

The free radical scavenging potential of different fractions ABRMF, ABRDF, ABREF and ABRHF of crude methanolic extract of roots of *Amygdalus brahuica* was detrmined by DPPH scavanging assay. The results revealed that antioxidant potential of all fractions was concentration dependent (0.025-0.75mg/mL) at p≤0.05 as shown in Figure 3. IC_{50} values of various fractions varied significantly (p≤0.05) from each other in the range of 33.92-17.01 µg/mL. Overall maximum radical scavenging potential was shown by methanolic fraction (ABRMF) with lowest IC_{50} (17.01 µg/mL). This showed that methanolic fraction possess maximum antioxidant potential as compared to other fractions.

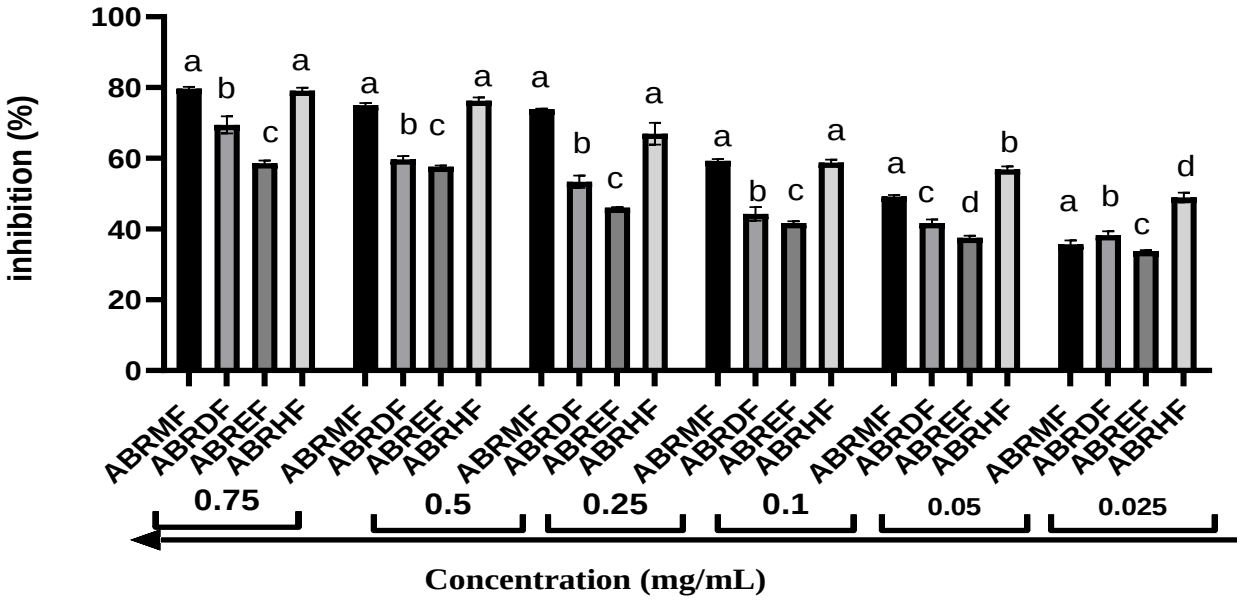

Figure 3. DPPH Percentage inhibition (%) potential of different fractions (ABRMF, ABRDF, ABREF and ABRHF) of *A. brahuica* roots methanolic crude extract. Columns indicating superscript labelled as a, b, c and d differ significantly from each other while setting P value ≤0.05. The abbreviations ABRMF, ABRDF, ABREF and ABRHF indicate methanolic, DMSO, ethyl acetate, and n-hexane fraction respectively.

Hydrogen peroxide (H_2O_2) inhibition assay

Scavenging potential of different fractions ABRMF, ABRDF, ABREF and ABRHF of methanolic crude extract of root of *A. brahuica* against hydrogen peroxide was determined which was also concentration dependent (0.025-0.75mg/mL) as shown in Figure 4. Ethyl acetate fraction displayed the strongest hydrogen peroxide scavenging activity with IC_{50} 13.71 µg/mL as compared to all other fractions. .

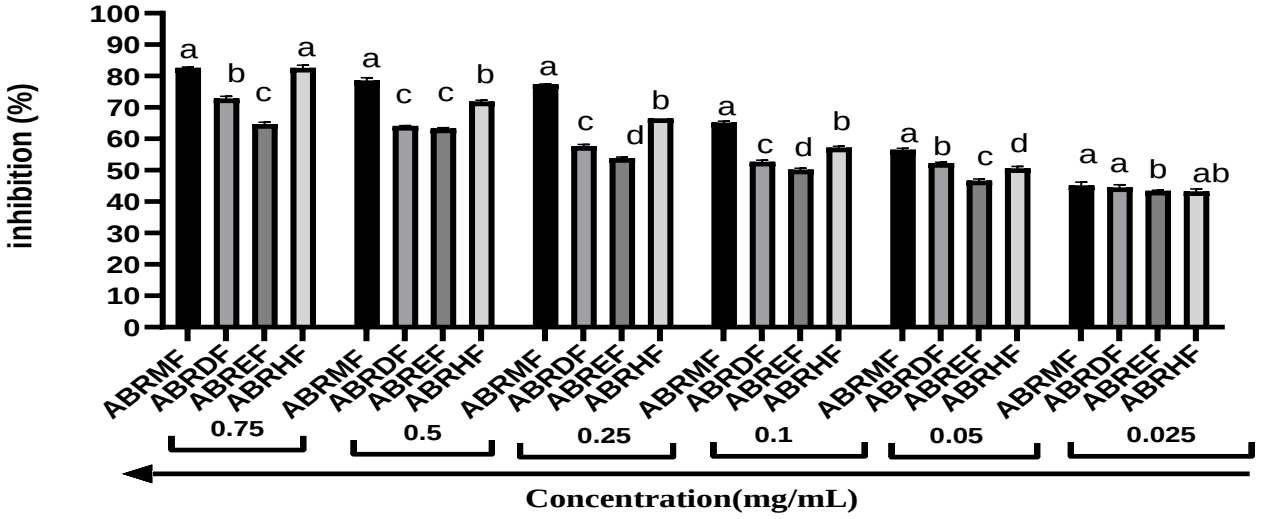

Figure 4. Hydrogen peroxide H_2O_2 inhibition (%) potential of various fractions (ABRMF, ABRDF, ABREF and ABRHF) of *A. brahuica* roots methanolic crude extract. Columns indicating superscript labelled as a, b, c and d differ significantly from each other while setting P value ≤0.05. Abbreviations of ABRMF, ABRDF, ABREF and ABRHF indicate methanolic fraction, DMSO fraction, ethyl acetate fraction and n-hexane fraction respectively.

Identification of Phytochemicals by RP-HPLC-UV

Maximum bioactive phenolic compounds were detected in methanolic fraction (ABRMF) while minimum in n-hexane fraction (ABRHF) of methanolic crude extract of root of *Amygdalus brahuica* as shown in Table 2, Figure 5. In ABRMF fraction p-coumaric acid concentration (200.10 µg/g) existed maximum while vanillic acid concentration (13.66 µg/g) was lowest among four phenolic compounds which also included gallic acid (89.88 µg/g) and salicylic acid (38.97µg/g). DMSO fraction (ABRDF) depicted three phenolic compounds having maximum concentraion of quercetin 572.49 µg/g followed by the concentration of gallic acid (144.98µg/g) and caffeic acid (23.96 µg/g). Ethyl acetate fraction (ABREF) revealed two phenolic compounds including quercetin (1010.7 µg/g) having higher concentration and coumarin (396.24 µg/g. However, n-hexane fraction showed only chlorogenic acid 45.48 µg/g during HPLC analysis.

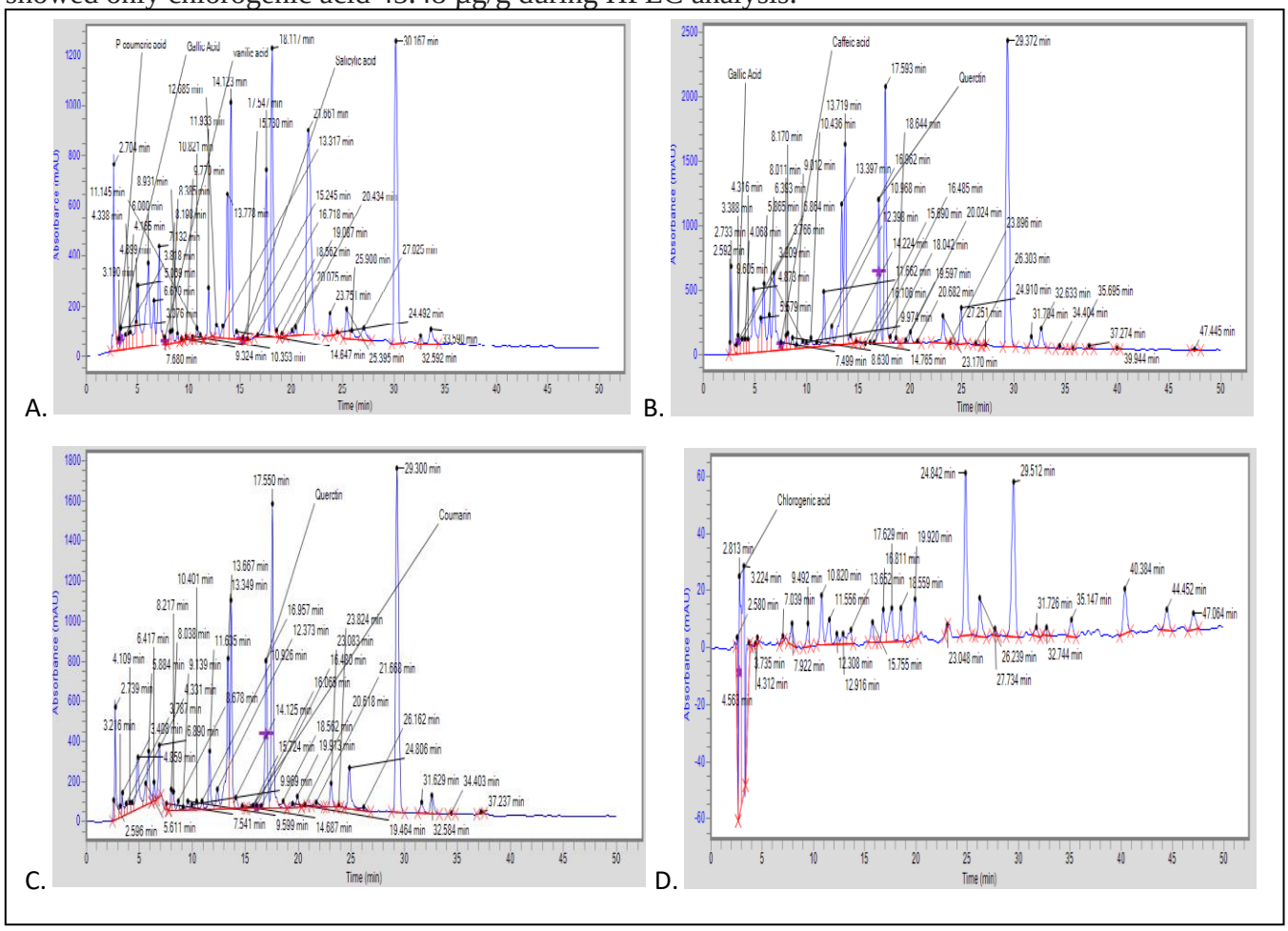

Figure 5. Chromatograms of methanol fraction (A), DMSO fraction (B), ethyl acetate fraction (C) and n-hexane fraction (D) of extract of root of *Amygdalus brahuica*.

Table 2: Quantification of various polyphenols found in different fractions of methanolic crude extract of root of *Amygdalus brahuica* by HPLC-UV gradient elution.

No	Fraction	Compound	M. F	Peak Area	K factor	µg/g of dry extract
1	ABRMF	P-coumaric acid	$C_9H_8O_3$	667,017.5	0.0003	200.10
		Gallic acid	$C_7H_6O_5$	1,137,736.8	0.000079	89.88
		Vanillic acid	$C_8H_8O_4$	310,433.6	0.000044	13.66
		Salicylic acid	HOC_6H_4COOH	103,372.8	0.000377	38.97
2	ABRDF	Gallic acid	$C_7H_6O_5$	1,832,302.9	0.000079	144.98
		Caffeic acid	HOC_6H_4COOH	737,232.7	0.0000325	23.96
		Quercetin	$C_{15}H_{10}O_7$	6,026,215.7	0.000095	572.49
3	ABREF	Coumarin	$C_9H_6O_2$	330,205.2	0.0012	396.24
		Quercetin	$C_{15}H_{10}O_7$	10,639,305.0	0.000095	1010.7
4	ABRHF	Chlorogenic acid	$C_{16}H_{18}O_9$	,349,872.2	0.00013	45.48

Inhibition of protein denaturation

The different concentrations (0.025-0.75 mg/ml) of methanolic (ABRMF), DMSO (ABRDF), ethyl acetate (ABREF) and n-hexane (ABRHF) fractions of methanolic extract of root of *Amygdalus brahuica* showed inhibitory effect on protein denaturation in dose dependent manner showing maximum inhibition (%) by ABRDF (74.79 ± 1.01%) at 0.025 mg/ml significantly higher $p \leq 0.05$ as compared to all other fractions but significantly $p \leq 0.05$ lower than diclofenac used as reference standard. However, at maximum concentration of 0.75 mg/ml protein inhibition by ABREF (79.29 ± 0.30%) and ABRHF (78.45 ± 0.41%) was non-significant $p \geq 0.05$ with each other but significantly $p \leq 0.05$ higher than ABRMF (77.42 ± 0.23%) and ABRDF (74.79 ± 1.01%) and significantly $p \leq 0.05$ lower than Diclofenac (89.43 ± 0.21%) showing maximum inflammatory response as shown in Figure 6.

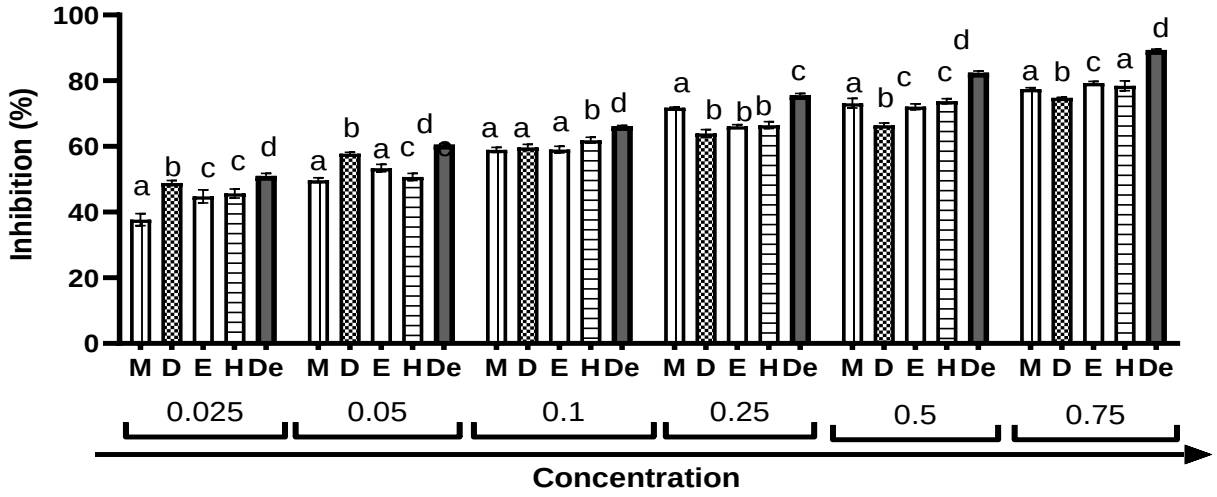

Figure 6. Protein denaturation percentage inhibition (mean %± SEM) mg of M = ABRMF, D = ABRDF, E = ABREF H = ABRHF methanolic, DMSO, ethyl acetate and n-Hexane fraction of methanolic crude extract of roots of *Amygdalus brahuica respectively*. whereas De = Diclofenac was used as control.

Anticancer activity (MTT assay)

Cytotoxic effect of methanolic (ABRMF), DMSO (ABRDF), ethyl acetate (ABREF) and n-hexane (ABRHF) fractions of crude methanolic extract of *Amygdalus brahuica* on the growth of HeLa cancer cell lines was evaluated at 30 µg/mL concentration. Doxorubicin was used as reference standard showing 100% inhibitory effect on HeLa cells at dose of 30 µg/mL depicted IC_{50} (0.9 ± 0.14 µg/mL) see Table 3. The impact of all fractions prepared using different solvents on cell viability was investigated by using MTT assay. Out of four fractions, ABRMF showed significantly higher ($p \leq 0.05$) potential against HeLa cell lines as compared to all other fractions.

Table 3. Cytotoxic effect of methanolic (ABRMF), DMSO (ABRDF), ethyl acetate (ABREF) and n-hexane (ABRHF) fractions of methanolic crude extract of root of *Amygdalus brahuica* on the growth of HeLa cancer cell lines.

Sample code	Conc.	% Inhibitions	IC_{50} µg/mL
Doxorubicin	30 µg/mL	100	0.9 ± 0.14
ABRME	30 µg/mL	44.4	-
ABRDF	30 µg/mL	37.4	-
ABREF	30 µg/mL	35.8	-
ABRHF	30 µg/mL	30.2	-

Results are presented as mean ± SEM of three replicates (N=3). Different superscripts a, b, c and d linked values show significant differences among different fractions while setting P≤0.05.

Antibacterial activity

The well diffusion method was used to evaluate antibacterial potential of methanolic (ABRMF), DMSO (ABRDF), ethyl acetate (ABREF) and n-hexane (ABRHF) fractions of *Amygdalus brahuica* roots at a dose of 40 mg/mL. The ethyl acetate fraction (ABREF) showed the most significant results with zone of inhibition (25 mm) against *Escherichia coli* at a dosage of 40 mg/mL. While the lowest activity (21 mm) was found against Staphylococcus aureus for (ABREF). The methanolic fraction (ABRMF) exhibited the highest zone of inhibition (23 mm) against *Escherichia coli*, while displaying the lowest activity (20 mm) against both *Staphylococcus aureus* and *Bacillus subtilis*. On the other hand, the DMSO fraction (ABRDF) exhibited the highest inhibition zone (18 mm) against *Escherichia coli* and *B. subtilis*, whilst the lowest activity (16 mm) was observed against *Staphylococcus aureus*. The (ABRHF) n-hexane fraction at a concentration of 40 mg/mL exhibited the lowest activity among all other samples, with ZI against *Staphylococcus aureus*, 13 mm while minimal activity of (ABRHF) was observed against *E. coli* (9 mm), and *B. subtilis* (12 mm) respectively as shown in Figure 7 and Table 4.

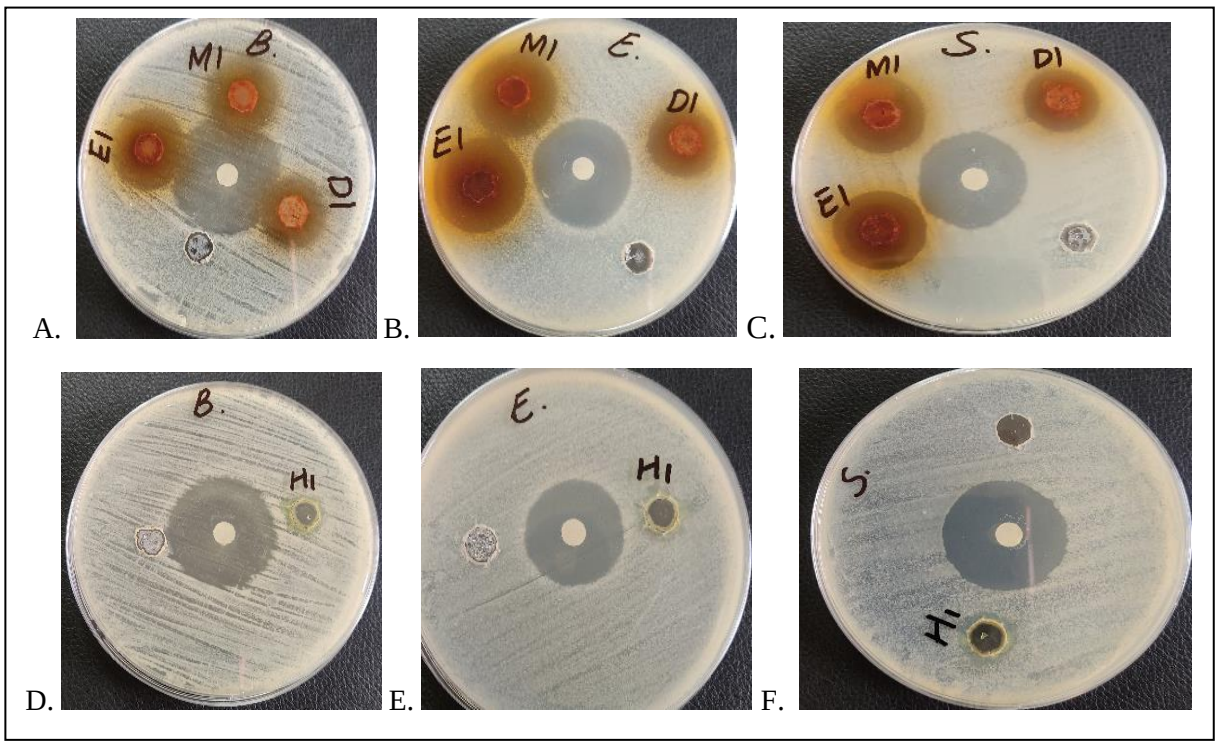

Figure 7. Zone of inhibitions in (A, B, C) represent methanolic (ABRMF), DMSO (ABRDF), ethyl acetate (ABREF) fractions whereas, (D, E, F) show n-hexane (ABRHF) fraction of root of *Amygdalus brahuica*.

Table 4: Antibacterial activity of methanolic (ABRMF), DMSO (ABRDF), ethyl acetate (ABREF) and n-hexane (ABRHF) fractions of methanolic crude extract of roots of *Amygdalus brahuica* at a dose of 40 mg/mL.

Fraction	**Strain**	**ZI (mm) of sample**	**ZI (mm) of negative control**	**ZI (mm) of (ciprofloxacin) (Conc. 10 µg/mL)**
ABRMF	*Bacillus subtilis*	**20**	**-**	**28**
	Escherichia coli	**23**	**-**	**29**
	Staphylococcus aureus	**20**	**8**	**30**
ABRDF	*Bacillus subtilis*	**18**	**-**	**28**
	Escherichia coli	**18**	**-**	**29**

	Staphylococcus aureus	**16**	**8**	**30**
ABREF	*Bacillus subtilis*	**22**	**-**	**28**
	Escherichia coli	**25**	**-**	**29**
	Staphylococcus aureus	**21**	**8**	**30**
ABRHF	*Bacillus subtilis*	**12**	**-**	**28**
	Escherichia coli	**9**	**-**	**29**
	Staphylococcus aureus	**13**	**8**	**30**

DISCUSSION

The present study revealed that fractions of *Amygdalus brahuica* root methanolic extract are rich source of phytochemicals. Major constituents found such as phenolic acids and flavonoids are responsible for anti-oxidant, analgesic, antimicrobial and anti-cancer activities (Atanassova, Georgieva, & Ivancheva, 2011). Previous studies of *Amygdalus* genus suggest the presence of different phenolic and flavonoid bioactive compounds (Sajjadi, Oskoueian, Karimi, & Ebrahimi, 2021). The methanolic fraction showed maximum phenolic contents (56.8±0.79 mg GAE/g) while (ABRHF) showed minimum phenolic contents (29.42±0.70 mg GAE/g). These higher quantities of poly phenolic bioactive compounds may be attributed to root part than leaves of *Amygdalus brahuica* used in current study. The study reported that methanolic extract of *Dracaena reflexa* TPC (88.16 mg GAE/g) whereas n-butanol fraction contained (92.72 mg GAE/g) slightly higher in *Dracaena reflexa* but the n-Hexane fraction contained the least among all fractions (Ghalloo et al., 2022).

Maximum total flavonoid contents (TFC) were found in DMSO fraction (46.16 ± 0.74 mg QE/g) while minimum contents (35.92±0.79 mg QE/g of dry extract) in n-hexane fraction of root extract of *Amygdalus brahuica.* The literature review on *Amygdalus brahuica* from Balochistan, Pakistan showed that up to author(s) best knowledge no study has been conducted on TPC and TFC of any part of the plant yet. In current study, the different fractions of *Amygdalus brahuica* depicted that it has huge potential to be a potent source of bioactive compounds. Polyphenol found in plants are biologically active compounds that show their response as antibacterial, antiviral, antioxidant, anticancer and anti-diabetic (Marín, Miguélez, Villar, & Lombó, 2015). Flavonoids show anti-inflammatory, anti-allergy, antioxidant and anticancer activities as biological response (Karak, 2019). Maximum DPPH potential of methanolic fraction with lowest IC_{50} 17.01 µg/mL showed maximum antioxidant capacity while ethyl acetate (ABREF) fraction showed maximum H_2O_2 inhibition with lowest IC_{50} 13.71 µg/mL as compared to all other fractions.

Free radicals are reactive oxygen species (ROS) frequently generated during metabolic processes and their accumulation leads to adverse change in the fate of cell membrane, proteins, DNA, fatty acids and other biomolecule thereby inducing inflammation and injury in different tissues in the body. In order to neutralize and detoxify the free radicals, antioxidants are capable of scavenging the reactive oxygen species. However, synthetic antioxidants due to high cost and possible adverse effects are less favorable over natural antioxidants. Naturally, plants are potential source of wide range of antioxidants which can be used for different biological activities (Aras et al., 2021).

Maximum anti-inflammatory response was observed with ethyl acetate fraction (79.29 ± 0.30%). Denaturation of protein is an unpredictable mechanism majorly involves hydrophobic interactions, disulfide bonding, H-bonding and electrostatic interactions (Das & Mukhopadhyay, 2009; Sen et al., 2015). The process of denaturation of protein produces autoantigen during rheumatic arthritis. So, by inhibiting denaturation of protein inflammatory response can be inhibited (Sangeetha & Vidhya, 2016). In current study diclofenac (NSAIDs) was used as reference drug. NSAIDs inhibit cyclooxygenases to prevent inflammation, however such drugs cause hemorrhage, ulcer and perforation therefore, there is need to develop plant based anti-inflammatory drug on commercial scale (Panchal & Sabina, 2023; Sostres, Gargallo, Arroyo, & Lanas, 2010). None of the studies have been conducted before to show anti-inflammatory activity of root fraction of *Amygdalus brahuica.*

Chemotherapy has recently emerged as a viable cancer treatment option. However, the use of natural products in disease treatment has received a lot of interest because of their potential

biological properties. In contrast to synthetic compounds, which can produce serious adverse responses, natural products provide a safer alternative with fewer side effects (Amin, Kucuk, Khuri, & Shin, 2009). Therefore, the current study aimed to explore the antiproliferative activity of different fractions of Amygdalus roots against HeLa cell line, results show that methanolic fraction showed maximum inhibition potential 44.4% inhibition of HeLa cells.

The ethyl acetate fraction exhibited maximum antibacterial potential due to presence of highest content of quercetin 1010.7 µg/g as compared to all other fractions. The ethyl acetate fraction (ABREF) at 40 mg/mL showed ZI (25 mm) maximum, against *Escherichia coli* and minimum (21 mm) was observed for *Staphylococcus aureus*. Antibacterial activity of quercetin was also previously estimated against eleven microbes including *Streptococcus species* and *Lactobacillus acidophilus* (Shu et al., 2011), *Staphylococcus aureus* (Jaisinghani, 2017) and its resistant strains Methicillin-resistant *Staphylococcus aureus* (MRSA) and Methicillin-sensitive *S. aureus*. Quercetin disrupts the bacterial quorum sensing pathways to prevent bacterial adhesion (Yang, Wang, Long, & Li, 2020).

While methanolic fraction (ABRMF) exhibited maximum ZI (23 mm) against *Escherichia coli* and minimum activity (20 mm) against both *Staphylococcus aureus* and *Bacillus subtilis*. Methanolic fraction (ABRMF) contained p-coumaric acid concentration 200.10 µg/g. Pre-clinical and clinical trials on p-coumaric acid showed efficacy as antimicrobial, antioxidant, anti-inflammatory and anti-tumor response exhibiting its potential in health care, pharmaceutical and food industry (Li et al., 2022).

CONCLUSION

Current study explored the phytochemical potential and biological activities of methanol, DMSO, ethyl acetate and n-hexane fractions of methanolic crude extract of root of *Amygdalus brahuica*. HPLC analysis depicted that methanolic fraction showed maximum concentration of p-coumaric acid while DMSO fraction and ethyl acetate fraction contained quercetin and n-hexane fraction showed chlorogenic acid. The ethyl acetate fraction showed maximum anti-inflammatory response while methanolic fraction revealed maximum antiproliferative potential against HeLa cell line (cervical cancer). The Ethyl acetate fraction exhibited maximum antibacterial potential as compared to other fractions. In conclusion, current study suggests potential pharmaceutical significance of different fractions of root extract of *Amygdalus brahuica* that it should be further explored for exploration bioactive compounds for versatile medical applications. Further research on *Amygdalus brahuica is* needed for identification and isolation of biologically active compounds for developing novel biobased products against emerging infections as well as chronic diseases.

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