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ETHNOPHARMACOLOGICAL AND PHYTOCHEMICAL EVALUATIONS OF DESERT PLANT *CALLIGONUM POLYGONOIDES* –POLYGONACEAE

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Abstract

The use of medicinal plants for the treatment of various ailments has significantly expanded due to the high cost and adverse effects of allopathic medicines. In the current investigation stem, root, and flower of *C. polygonoides* were used to make Aqueous (Aq), MtOH, EtOAc, EtOH, and *n*-hexane extracts. Qualitative phytochemical analysis has revealed the presence of carbohydrates, proteins, glycosides, and other phytochemicals. According to HPLC-PDA analysis results different phenolic compounds were present in significant quantities. In antibacterial activity, EtOAc extracts of stem and root whereas EtOH extract of the flower was most effective though all the other extracts were positive in varying order. In the antibiofilm assay, EtOH extract of stem and root whereas multiple extracts of the flower have shown high antibiofilm potential. Antioxidant potentials observed by DPPH assay revealed that MtOH extract of the stem, EtOH extract of roots, and EtOH extract of flowers showed max scavenging potential. Similarly, in FRAP assay the EtOAc extract of the stem, MtOH extract of root, and n-hex extract of the flower have shown the highest antioxidant potentials. In case of α-glucosidase inhibition assay, Aq extracts of stem and root have shown max activity, though, all extracts of flowers have shown excellent inhibition potential. According to antiviral activity, Aq extracts of different parts were most active against $AIVH_9N_2$ whereas, in the case of IBV different extracts were active in varying order. It can be positively stated that *C. polygonoides* possesses great pharmacological importance and is a rich source of multiple compounds with biological activity.

Key words: Medicinal plants, Antibiofilm, Phytochemical, Antiviral, Phenolic content.

Introduction

Use of medicinal plant is as old as the huminty is (Yuan *et al*., 2016). Recently use of herbal product(s) is increased all over the world. According to WHO, 75% of people around the world rely on herbs for their fundamental healthcare requirements. More than, 53,000 plant species are being used as herbal medicines (Pan *et al*., 2014). One of the most important reasons for relying on herbal treatment is their cost-effectiveness, no/limited side effects, and ease of availability. Additionally, herbal medicines are considered as naturally balanced and moderate approach of therapy. The medicinal properties of plants are mainly associated with secondary metabolites which are principal active agent to confer pharmacological activity to plant. Risks of degenerative diseases like cardiovascular disorders, diabetes, and cancer, can be substantially reduced by the consumption of phytochemicals (Björkman *et al*., 2011). Phytochemicals, unlike pharmaceutical drugs, may be termed as "human-friendly medicines" since they heal without causing side effects.

The excessive and improper use of antibiotics has created the problem of antibiotic resistance in bacteria which are causing adverse effects on human health. Recent studies have reported that medicinal plants are potent sources of antibacterial agents. The use of natural products for bacterial infections is not only cost-effective but also address the issues related to side effects of antibiotics (María *et al*., 2018). Many bacterial strains are capable of developing biofilm on different surfaces. According to the National Institutes of Health, biofilms formation is the reason for up to 80% of human bacterial infections. Bacteria including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli* are major causes of biofilm-related infections in the world today (Römling *et al*., 2012). For dissociation of biofilms or inhibition of biofilm development, studies have been focusing on medicinal plants and various physiologically active plant-derived pharmaceutical substances. Natural products are not only effective in dealing biofilm-based infections but also have less toxic and fewer side effects compared to synthetic compounds (Quave *et al*., 2008). To the best of our knowledge, the antibiofilm potential of *C. polygonoides* has not been reported yet so the current investigation was done to examine its antibiofilm potential from different parts of plant.

A free radicals are highly reactive and unstable species causes many adverse effects on human health. Free radicals damage proteins by causing a loss in enzymatic activity. Free radicals also induce DNA damage, which can result in cancer (Lobo *et al*., 2010). Therefore, the use of antioxidants especially natural antioxidants is gaining interest day by day. Plants are rich source of natural antioxidants including phenolic compounds which are the most common secondary metabolites. The presence of phenolic acids, flavonoids, and phenolic diterpenes contributes to the antioxidant potential of a plant (Noreen *et al*., 2017; Akhtar *et al*., 2018). The use of medicinal plants as antidiabetic agents has tremendously increased across the world. Plants mediate the antihyperglycemic effects either via raising insulin production or decreasing glucose retention in the intestine (Salehi *et al*., 2019). This study is designed to evaluate the pharmacological potential of *C. polygonoides*. The genus Calligonum of the family

Polygonaceae consists of two Greek words Kalli "beautiful" and gony "knee joint". *C. Polygonoides* is locally known as "phog". It is a perennial shrub but sometimes it may develop into a small tree. The flowers of this plant are used as a vegetable (Ahmed *et al*., 2016).

Viral infections in poultry birds have been a significant cause of economic losses in poultry flocks especially in developing countries, like Pakistan. In this study, the antiviral potential of *C. polygonoides* against two common poultry viruses, the Infectious bronchitis virus (IBV) and Avian influenza virus (AIV-H9N2), was evaluated. Studies in the field of phytoantivirals have led to the discovery of novel antiviral agents such as polyphenols, flavonoids, terpenoids, and saponins. These metabolites are very effective antiviral agents Mukhtar *et al*., (2008). To the best of our knowledge antiviral properties of *C. polygonoides* have not yet been explored. In short, different parts of this plant have shown different levels of antibacterial, antibiofilm, antioxidant, antiviral, and α-glucosidase inhibitory activities.

Material and Methods

Collection of plant: *C. polygonoides* was collected from the Cholistan desert near Bahawalpur, Pakistan. Plant identification was done by a taxonomist at the Department of Botany, The Islamia University of Bahawalpur (voucher no. 560). The copy of voucher was save at Department of Botany, The Islamia University of Bahawalpur.

Sample preparation: The root, stem, and flower of the clean fresh plant, were dried under shade for 20-30 days and pulverized to a fine powder. 10g powder of each part of plant was mixed in 200ml of respective solvents including Aq, MtOH, EtOAc, EtOH, and *n*-hex. The solutions were kept in air-tight containers for 72 hrs at RT under constant shaking. All the extracts were filtered and the filtrates were dried in a rotary evaporator at 40-45ºC. Finally, the extract was dissolved in the same solvent, and solutions of different concentrations were made for different biological assays.

Qualitative phytochemical analysis: Preliminary phytochemicals analysis was performed to confirm the presence of certain secondary metabolites qualitatively. All the tests were performed with the standard methods given by (Santhi *et al*., 2016).

HPLC-PDA determination and chemical fingerprint of phenolic content: The active metabolites were quantified by the HPLC-PDA at the Department of Pharmacy, Via dei Vestini, Chieti, Italy. The analysis was carried out on Waters liquid chromatograph equipped with a model 600 solvent pump and 2996 photodiode array detector (PDA). A C_{18} reversed-phase packing column (4.6 × 150 mm, 5 µm) was used for the separation and the column was thermostated at 30 ± 1°C. The Empower *v.2* Software was used for the acquisition of data and UV/Vis acquisition wavelength was adjusted to 200–500 nm. Briefly, the weighed extracts were solubilized in mobile phase A (milliQ water + acetic acid): B (acetonitrile + acetic acid) (93:7 *v:v*), adding 20% dimethyl sulfoxide (DMSO). The 20 µL extract (1 mg/250 µL) was injected in the HPLC system waith gradient elution flow rate of 1 mL/min. Total of 22 polyphenols was used as standard. The retention times of 4.99, 13.36, 14.29, 14.71, 17.31, 18.30, 18.50, 19.41, 22.08, 22.65, 25.38, 26.18, 27.75, 29.78, 30.36, 31.20, 34.81, 40.57, 45.49, 45.87, 46.74, 49.95 min belonged to corresponding compounds gallic acid, catechin, chlorogenic acid, 4-hydroxybenzoic acid, vanillic acid, epicatechin, syringic acid, 3-hydroxybenzoic acid, 3-hydroxy-4-methoxybenzaldehyde, *p*-coumaric acid, rutin, sinapinic acid, *t*-ferulic acid, naringin, 2,3-dimethoxybenzoic acid, benzoic acid, *o*-coumaric acid, quercetin, harpagoside, *t*-cinnamic acid, naringenin, and carvacrol respectively. By comparing the standard peak and UV/VIS spectrum, the presence of polyphenols in extracts was determined. In addition, the concentration of each target compound was calculated using the calibration curve.

Antibacterial assay

Disc diffusion method: Bacterial strains selected for this assay include *E. coli*, *K. Pneumoniae*, *P. Aeruginosa*, *P. Aeruginosa* MDR, *P. Vulgaris*, *Staph aureus,* and *Staph aureus* MDR. The antibacterial potential of the plant extracts was assessed by the disc diffusion method. The pre-soaked GF-1 grade filter paper discs were used @ 200µg of extract/disc were used. The bacteria were inoculated on agar plates, incubated for 40 min at 37°C, and then discs were applied. The culture was again incubated at 37°C overnight and the zone of inhibition (ZoI) was recorded as per standard guidelines provided by CLSI. Pure solvents were used as the negative control. Ampicillin and Moxifloxacin were used as a positive control for normal and drug-resistant bacteria respectively (Siddique *et al*., 2018).

MIC (minimum inhibitory concentration assay): Minimum inhibitory concentration assay was performed by the method given by (Eloff, 1998).

Antibiofilm assay: Antibiofilm assay was performed with slight modifications to the method provided by (Sandasi *et al*., 2010). OD_{630nm} was taken through ELISA reader and % inhibition was calculated by the formula: % Inhibition = $[(A_0\text{-As})/A_0]$ *100, where A_0 is the absorbance of negative control and As is the absorbance of the sample.

Antioxidant assays

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity: The assay was performed with method described by (Molyneux, 2004) with few modifications. Following formula was used to calculate radical scavenging activity (% RSA= $[(A_0\text{-}A_X)/A_0]$ *100) where A_0 is the absorbance of negative control and A_X is the absorbance of the sample.

FRAP ferric reducing antioxidant power: FRAP assay was performed as the method given by (Puangkam *et al*., 2017). The assay was performed in triplicates and OD_{700nm} was taken through an ELISA reader.

α-glucosidase inhibition assay: *In vitro* assay was performed, according to the method reported by (de Souza *et al*., (2012). The absorbance of *p*-nitrophenol was measured at 405nm. The activity was measured in the form of % inhibition by formula:

$$\% \text{ Inhibition} = \frac{OD_0 - OD_S}{OD_0} \times 100$$

where OD_0 is the absorbance of negative control and ODs is the absorbance of the sample.

Antiviral activity

Inoculation of poultry viruses: Specific pathogen-free 9-11 days old chicken embryonated eggs were taken and their viability was confirmed through candling. The viral strains were inoculated through the Chorio-allatonic route under sterile conditions. Eggs were incubated at 37ºC for 48 hrs. The eggs were harvested and allantoic fluids were collected. The titer of each virus was confirmed through the HA test.

Hemagglutination (HA) test: HA test was performed by the method given by Hirst *et al*., (1944).

Statistical analysis

The data is represented as means ± standard error of mean (n = 3). The results are analyzed by using two- way ANOVA, Dunnett's multiple comparisons Test. For the data analysis and IC_{50} calculation GraphPad Prism version 8.0 for Windows was used.

Results

Qualitative phytochemical analysis: The results confirmed the presence of active phytochemicals from different parts of plant (Table 1). All the floral extracts were positive for carbohydrates whereas few extracts of stem and root also confirm the presence of carbohydrates. The *n*- hex extracts of stem and root were negative for glycosides but the floral *n*-hex extract was positive for it. All extracts of all parts of plant were found negative for Ninhydrin test but when tested for soluble proteins through Millon's test extracts of stem, root, and flower, showed positive results (Saad *et al*., 2014).

HPLC-PDA determination and chemical fingerprint of phenolic content: Results of the current investigation revealed that different parts of this plant are the richest source of different phenolic compounds. The phenolic compound present in the highest quantity was 2,3-diMeO benzoic acid and was found in EtOAc extract of root (55.51µg/mg). The lowest phenolic content was *n*-hex in extract of root, whereas *n*-hex extracts of stem and flower showed significantly high level of phenolic content(s). Gallic acid was present in considerable amounts in all extracts of stem and flower except Aq extracts (Table 2). Overall the EtOH, EtOAc, and MtOH extracts were found best in possessing different phenolic compounds compared to Aq and *n*-hex extracts.

Antibacterial Activity: According to antibacterial activity, the EtOAc extract of the stem showed max activity against most of selected bacterial strains. In the case of *S. aureus* MDR EtOAc extract of the stem exhibited 17.5mm ZoI which was greater than ZoI of Moxifloxacin i.e., 16.5mm (Arulmozhi *et al*., 2018). When tested against *P. aeruginosa* MDR, EtOAc extract of root (12.5 mm ZoI) again better than Moxifloxacin (10.5 mm ZoI). In case of *P.vulgaris* EtOAc extract of root was highly effective and showed 14.5mm ZoI even better than Ampicillin i.e 13.5mm ZoI (Khan *et al*., 2013). In the case of the flower, EtOH and MtOH extracts were more active against selected bacterial strains. Against *S. aureus* MDR, EtOH extract showcased high antibacterial potential with 15.5mm ZoI. In case of *P. vulgaris* MtOH extract of flower and Ampicillin were equally effective with 13.5mm ZoI (Renisheya *et al*., 2011) (Table 3).

Table 1. Qualitative phytochemical analysis of *C. polygonoides*.

Standard test	Phytochemical	Stem					Root					Flower				
		Aq	MtOH	EtOAc	EtOH	*n*-hex	Aq	MtOH	EtOAc	EtOH	*n*-hex	Aq	MtOH	EtOAc	EtOH	*n*-hex
Molish test	Carbohydrates	+	--	--	+	--	--	+	--	+	--	+	+	+	+	+
Benedict test	Reducing sugars	+	+	--	+	--	+	+	+	--	--	--	+	--	+	--
Ninhydrin test	Amino acid	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Biuret test	Peptide bond	±	±	+	+	+	--	+	+	--	+	--	±	+	±	+
Millon's test	Soluble Protein	+	+	--	+	+	+	+	--	+	+	+	+	--	+	+
Terpenoids test	Terpenoids	--	--	+	--	+	--	--	+	+	+	+	+	+	+	+
Wagner's test	Alkaloids	--	±	--	--	--	--	--	--	±	--	--	--	±	--	--
Salkowaski's test	Glycosides	+	+	+	+	--	+	+	+	+	--	+	+	+	+	+
Libermann's test	Sterols	--	+	+	+	--	--	+	--	+	--	+	+	--	+	+
Froth test	Saponins	+	+	--	--	--	+	+	--	--	--	+	+	±	+	--
Alkaline reagent test	Flavonoids	+	--	+	--	--	+	+	+	--	--	±	±	+	±	+
Phenols test	Phenols	--	+	--	+	--	--	+	--	+	--	+	+	--	+	+

+ = Detected -- = Not detected ± = Detected in low quantity

Table 2. HPLC-PDA determination and chemical fingerprint of phenolic content of *C. polygonoides*.

Analytes	Stem					Root					Flower				
	Aqueous	**MtOH**	**EtOAc**	**EtOH**	***n*-hex**	**Aqueous**	**MtOH**	**EtOAc**	**EtOH**	***n*-hex**	**Aqueous**	**MtOH**	**EtOAc**	**EtOH**	***n*-hex**
Gallic acid		3.95 (± 0.20)	2.45 (± 0.12)	3.37 (± 0.17)	1.87 (± 0.09)	2.59 (± 0.13)	2.77 (± 0.14)	2.31 (± 0.12)	4.12 (± 0.25)	BLQ		8.32 (± 0.42)	15.21 (± 0.91)	7.02 (± 0.42)	3.23 (± 0.16)
Catechin	2.84 (± 0.14)	2.34 (±0.12)	0.96 (± 0.06)	2.37 (± 0.12)	6.15 (± 0.31)		14.24 (± 0.71)	21.26 (± 1.49)	17.56 (± 1.23)						
Chlorogenic acid		BLQ										26.59 (± 1.33)	8.39 (± 0.50)	21.87 (± 1.75)	7.29 (± 0.44)
p-OH benzoic acid			BLQ (>LOD)			BLQ					BLQ	BLQ (>LOD)	BLQ	0.27 (± 0.02)	0.26 (± 0.01)
Vanillic acid													1.13 (± 0.08)		0.32 (± 0.02)
Epicatechin	8.4 (± 0.42)			1.76 (± 0.11)				12.48 (± 1.00)	6.82 (± 0.41)						
Syringic Acid		0.3 (± 0.02)		BLQ (>LOD)						BLQ (>LOD)					
3-OH benzoic acid											BLQ (>LOD)				
3-OH-4-MeO benzaldehide											BLQ				
p-coumaric acid			0.33 (± 0.02)									0.77 (± 0.04)	3.41 (± 0.27)	0.78 (± 0.05)	0.88 (± 0.04)
Rutin	1.65 (± 0.08)	6.78 (± 0.34)	4.18 (± 0.29)	3.56 (± 0.25)	0.38 (± 0.02)	0.2 (± 0.01)					BLQ	3.18 (± 0.16)	2.04 (± 0.14)	7.68 (± 0.69)	
Sinapinic acid															BLQ
t-ferulic acid							BLQ		0.29						
Naringin		0.51 (± 0.02)					(>LOD)		(± 0.01)						
2,3-diMeO benzoic acid		5.24 (± 0.26)	22.16 (± 1.33)	2.29 (± 0.14)	18.98 (± 1.52)	3.55 (± 0.21)	38.21 (± 1.91)	55.51 (± 2.78)	48.64 (± 2.92)	0.64 (± 0.03)					
Benzoic acid											BLQ (>LOD)				
o-coumaric acid															
Quercetin dihydrate		BLQ	0.69 (±0.03)	1.27 (± 0.08)	0.84 (± 0.04)	BLQ	1.58 (± 0.08)	1.13 (± 0.07)	1.8 (± 0.11)						0.2 (± 0.01)
Harpagoside		BLQ													BLQ
Trans-cinnamic acid															
Naringenin															BLQ
Carvacrol															

BLQ: below Limit of Quantification; >LOD: Over Limit of Detection

Table 3 Antibacterial activity of different extracts of *C. polygonoides*.

Bacterial strains	Extract	Stem		Root		Flower	
		ZoI(mm) ± SEM	MIC (µg/µl)	ZoI(mm) ± SEM	MIC (µg/µl)	ZoI(mm) ± SEM	MIC (µg/µl)
S. aureus	Aq	8 ± 0	-	10 ± 0	125	7 ± 0	1562.5
	MtOH	7 ± 0	-	6 ± 0	-	7 ± 0	-
	EtOAc	7 ± 0	6250	6.5 ± 0.5	1562.5	7 ± 0	-
	EtOH	6 ± 0	-	6 ± 0	-	7 ± 0	3125
	n-hex	8 ± 1	12500	7.75 ± 0.25	1562.5	7.25 ± 0.25	-
	Ampicillin	13.5 ± 0.5	6250	13.5 ± 0.5	125	13.5 ± 0.5	6250
S. aureus MDR	Aq	9 ± 0	3125	10 ± 1	6250	8 ± 0	1562.5
	MtOH	8.75 ± 0.2	-	9.5 ± 0.5	-	12 ± 0	3125
	EtOAc	17.5 ± 0.5	3125	11.5 ± 0.25	781.25	9 ± 0.5	6250
	EtOH	8 ± 0	-	10 ± 0	12500	15.5 ± 0	781.25
	n-hex	14 ± 0.2	6250	12 ± 2	3125	10 ± 0	390.62
	Moxifloxacin	16.5 ± 1.5	390.6	16.5 ± 1.5	390.6	16.5 ± 1.5	390.62
K. pneumoniae	Aq	9 ± 0	-	9.5 ± 0.5	-	11.5 ± 0.5	62500
	MtOH	6 ± 0	-	8 ± 0	-	8.5 ± 0.5	12500
	EtOAc	14 ± 1	390.62	14 ± 0	390.62	10.5 ± 0.5	12500
	EtOH	8 ± 0	-	8 ± 0	-	8.5 ± 0.5	12500
	n-hex	8 ± 0	-	12.75 ± 0.75	1562.5	8.5 ± 0.5	-
	Ampicillin	16 ± 1	195.3	16 ± 1	195.3	16 ± 1	
P. aeruginosa	Aq	10 ± 1	-	9 ± 0	12500	9 ± 0	-
	MtOH	7.5 ± 0.5	-	9.5 ± 0.5	781.25	9.5 ± 1	-
	EtOAc	11.5 ± 0.5	-	11.5 ± 0.5	1562.5	8.75 ± 0.5	-
	EtOH	7.5 ± 0.5	-	7 ± 0	-	12.5 ± 0.5	6250
	n-hex	14.5 ± 0.5	12500	12 ± 0	1562.5	8.5 ± 0.5	6250
	Ampicillin	15 ± 0	12500	15 ± 0	12500	15 ± 0	12500
P. aeruginosa MDR	Aq	8.5 ± 0.5	-	9 ± 0	-	7 ± 0	-
	MtOH	8 ± 0	1562.5	9 ± 0	390.62	8 ± 0	-
	EtOAc	8.5 ± 0.5	3125	12. 5 ± 0.5	781.25	8 ± 0	-
	EtOH	8 ± 0	-	7.75 ± 0.5	781.25	9 ± 0	-
	n-hex	10 ± 0	195.31	7.75 ± 0.25	-	8.5 ± 0.5	-
	Moxifloxacin	10.5 ± 0.5	1562.5	10.5 ± 0.5	1562.5	10.5 ± 0.5	1562.5
E. coli	Aq	10.5 ± 1.5	3125	9.5 ± 0.5		8.5 ± 0.5	-
	MtOH	8 ± 0	6250	8 ± 0	3125	9 ± 1	6250
	EtOAc	12 ± 0	781.25	12.5 ± 0.25	1562.5	8.5 ± 0.5	-
	EtOH	7.5 ± 0.5	-	10 ± 0	1562.5	10.5 ± 0.5	-
	n-hex	12 ± 1	781.25	11.5 ± 0.5	3125	10 ± 0	-
	Ampicillin	17.5 ± 2.5	12500	17.5 ± 2.5	12500	17.5 ± 2.5	12500
P. vulgaris	Aq	9.5 ± 0.5	-	9 ± 0	6250	9.5 ± 0.5	3125
	MtOH	8 ± 0	-	9.5 ± 0.5	-	13.5 ± 0.5	3125
	EtOAc	9.5 ± 0.5	-	14.5 ± 0.5	1562.5	9.5 ± 0.5	-
	EtOH	8 ± 0	-	10.5 ± 1.5	-	10 ± 0	12500
	n-hex	9.5 ± 0.5	-	11 ± 0	6250	8.5 ± 0.5	-
	Ampicillin	13.5 ± 1.5	195.3	13.5 ± 1.5	195.3	13.5 ± 1.5	195.312

Antibiofilm activity: EtOH extract of the stem showed max antibiofilm potential. However, in the case of root and flower, EtOAc and MtOH extracts were most active against most of the selected strains. It was observed that Aq extract of root was significantly active followed by Aq extract of stem and flower. The *n*-hex extract of the flower was more active than *n*-hex extracts of stem and root. Among all bacterial strains, best antibiofilm activity was seen against *K. pneumoniae* and *P. vulgaris*, where all extracts of stem and root exhibited up to 80% inhibition except *n*-hex. When tested against *P. aeruginosa* MDR different extracts of flower showed more than 60% inhibition of biofilm. Different floral extracts exhibited good potential against *K. pneumoniae* (Fig. 1). On the basis of results of this experiment, extracts of *C.polygonoides* are found more potent against Gram-ve bacteria as compared to Gram +ve bacteria. (Nikaido *et al*., 1985).

Antioxidant activity

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity: According to results of this experiment, all the extracts of the stem have exhibited good radical scavenging potential except Aq and *n*-hex. MtOH extract was very active as it exhibited lowest IC_{50} (266.16µg/ml) (Fig. 2). As compared to Aq extract of the stem, Aq extract of root showed significant % RSA with IC_{50} (474.25µg/ml). EtOH extract was most active in the case of root with IC_{50} (239.71µg/ml) whereas *n*-hex was the least active extract (Fig. 3). EtOH extract of the flower was the most active of all extracts and showed IC_{50} (460.72µg/ml). The *n*-hex extracts of stem and root were the least active but surprisingly the *n*-hex extract of the flower has shown significant radical scavenging activity with IC_{50} (451.21µg/ml) (Fig. 4).

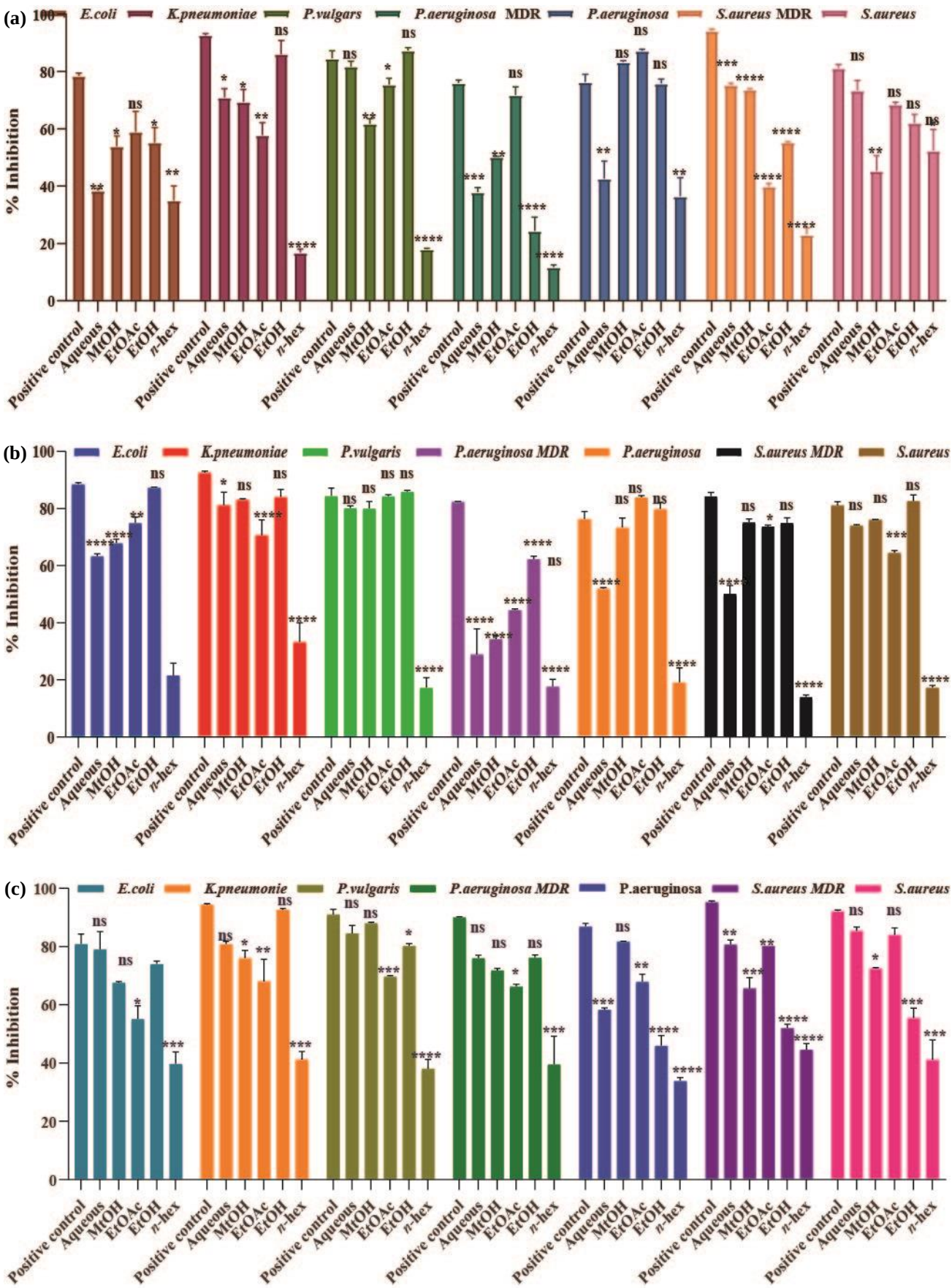


Fig. 1. Antibiofilm activity of different parts of *C. polygonoides* against selected bacterial strains. (a) stems (b) roots (c) flowers. Data is presented as % inhibition± standard error. The results are analyzed using two- way ANOVA, Dunnett's multiple comparisons Test. The comparisons are made with positive control and results are considered significant (*) if P<0.05, more significant (**) if P< 0.01, very significant (***) if P<0.001, highly (****) if P<0.0001 and ns if P >0.05.

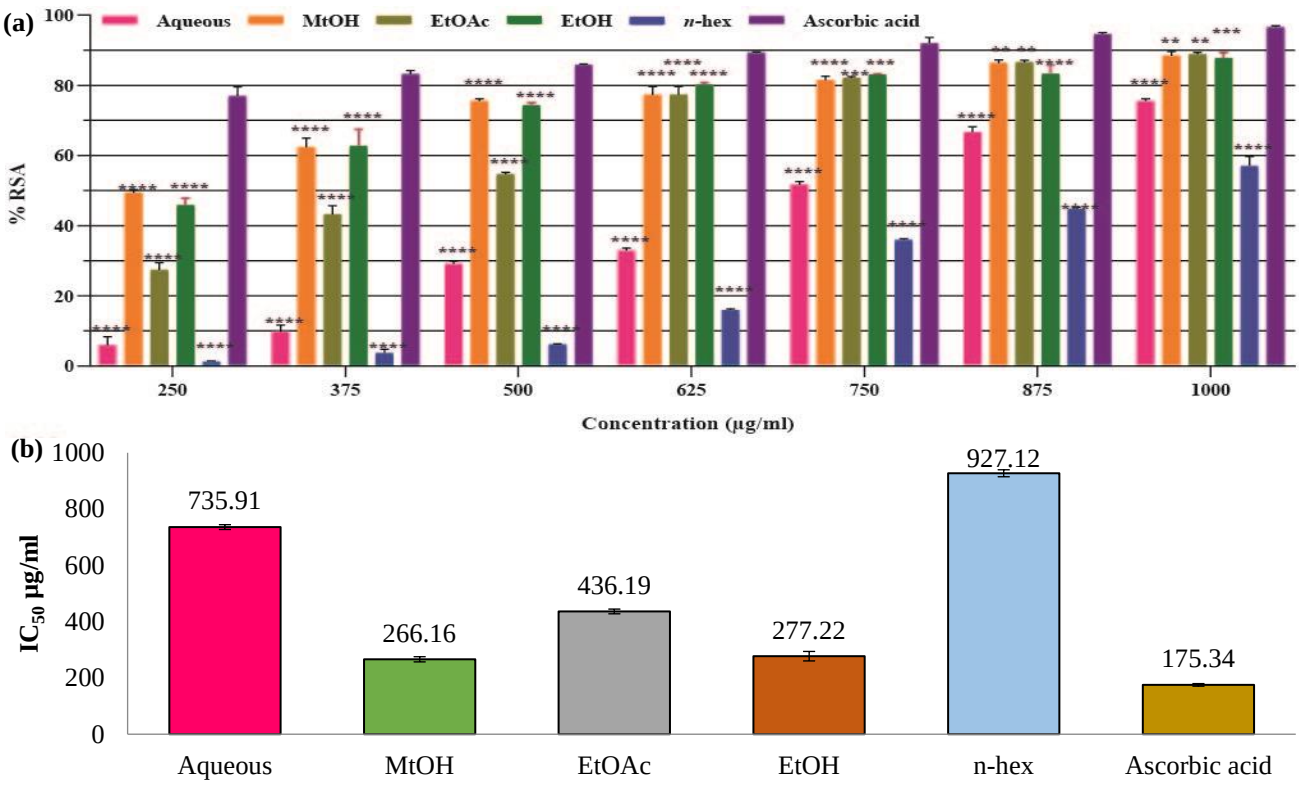


Fig. 2. (a) %RSA of different extracts of the stem. Values are presented as %RSA± standard error. The results are analyzed using two-way ANOVA, Dunnett's multiple comparisons Test. The comparisons are made with positive control and results are considered significant (*) if $p<0.05$, more significant (**) if $p< 0.01$, very significant (***) if $p<0.001$, highly (****) if $p<0.0001$ and ns if $p>0.05$ (b) IC_{50} of different extracts of the stem was calculated by using graph pad prism version 8.0.

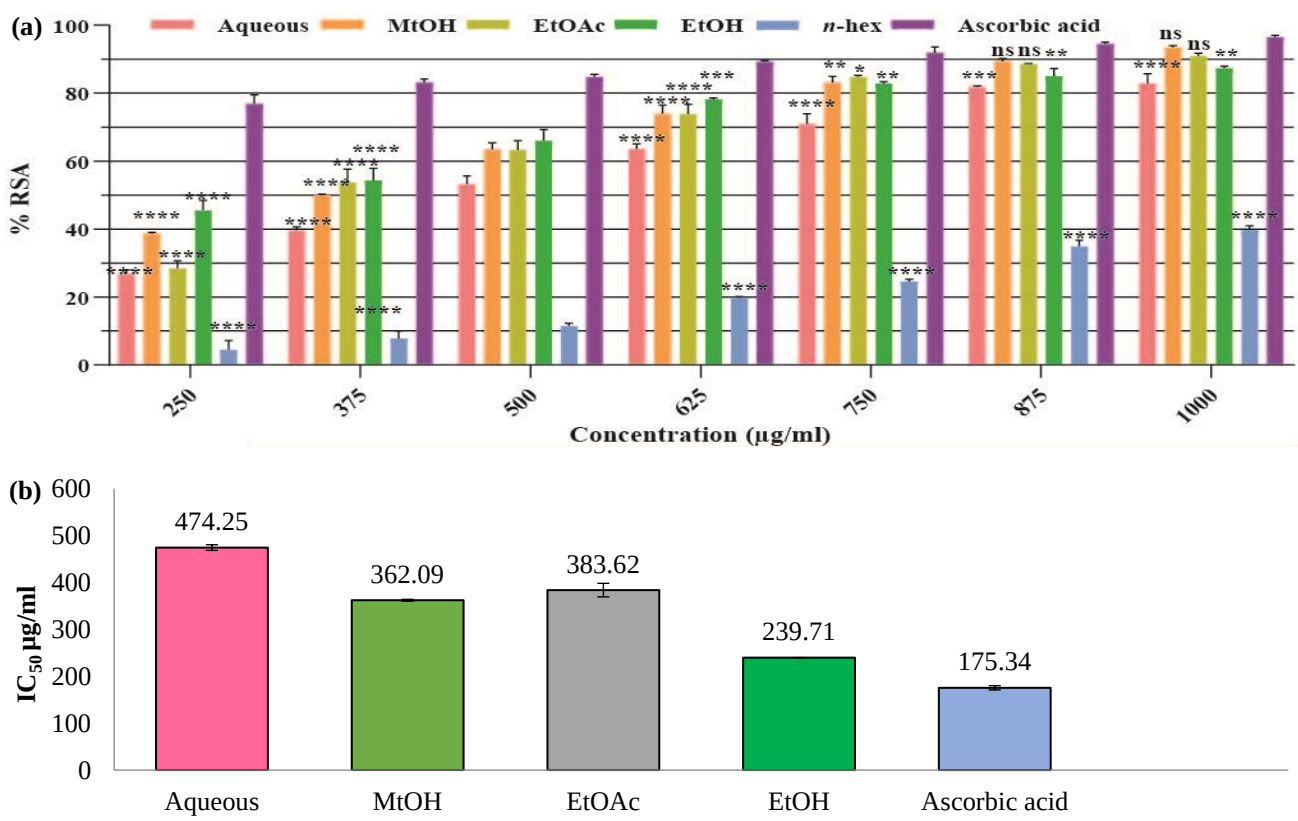


Fig. 3. (a) %RSA of different extracts of root. Values are presented as %RSA± standard error. The results are analyzed using two- way ANOVA, Dunnett's multiple comparisons Test. The comparisons are made with positive control and results are considered significant (*) if $p<0.05$, more significant (**) if $p< 0.01$, very significant (***) if $p<0.001$, highly (****) if $p<0.0001$ and ns if $p>0.05$ (b) The IC_{50} of different extracts of the root was calculated by using graph pad prism version 8.0.

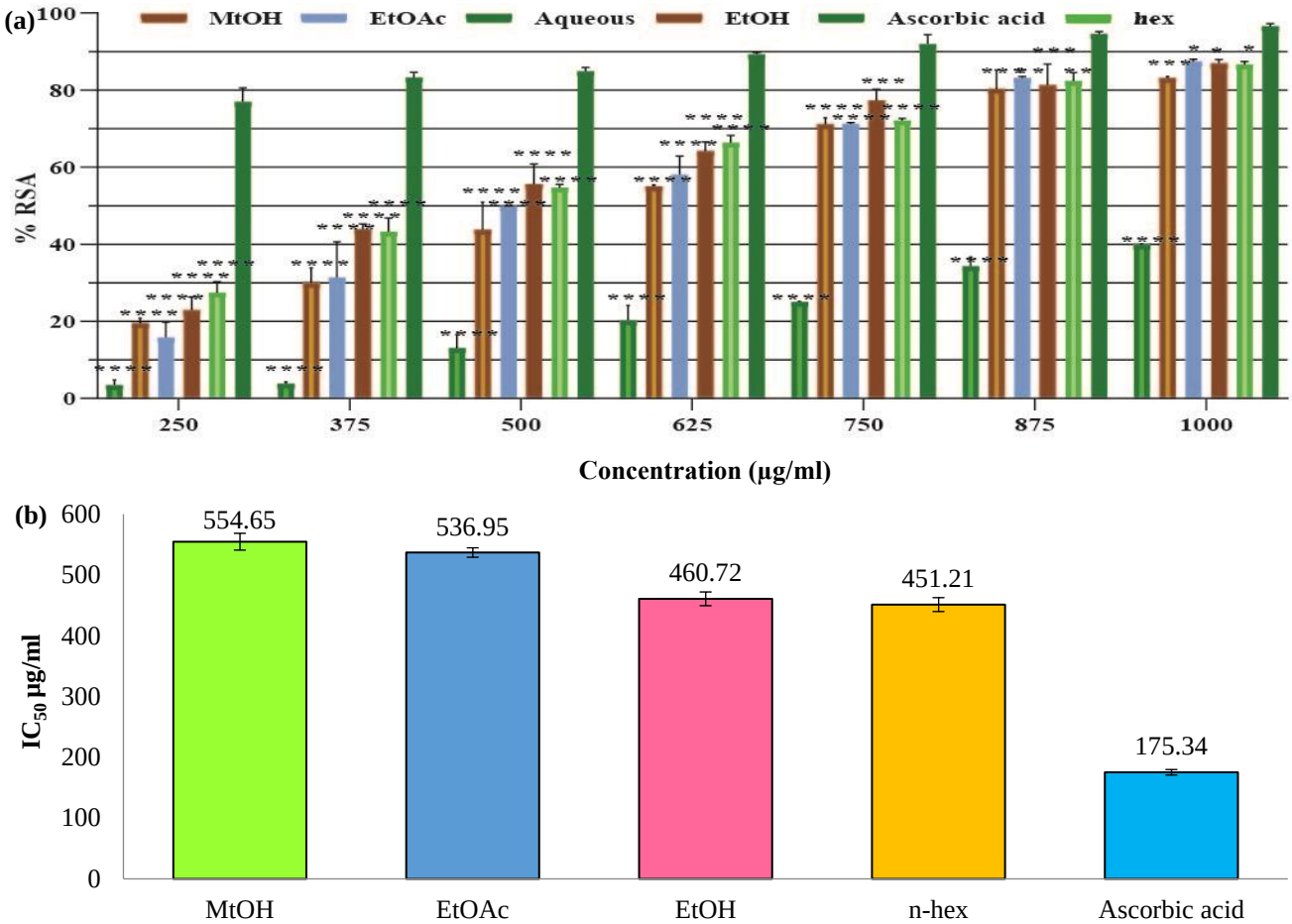


Fig. 4 (a). %RSA of different extracts of the flower. Data is presented as %RSA± standard error. The results are analyzed using two-way ANOVA, Dunnett's multiple comparisons Test. The comparisons are made with positive control and results are considered significant (*) if $p<0.05$, more significant (**) if $p< 0.01$, very significant (***) if $p<0.001$, highly (****) if $p<0.0001$ and ns if $p>0.05$ (b) IC_{50} of different extracts of the flower was calculated by graph pad prism version 8.0.

FRAP assay: According to the results of this assay EtOH, MtOH, and EtOAc extracts of stem and root were very active and indicated high reduction potential at different conc. The *n*-hex extracts of stem and root were less active at lower conc, but with the increase in conc reduction potential was also increased. Aq extract of roots was slightly more active than Aq extract of the stem. MtOH, EtOH and EtOAc extracts of the flower were among the most active extracts, at various conc. The *n*-hex extract of the flower exhibited good antioxidant potential as compared to *n*-hex extracts of stem and root (Fig. 5).

α-glucosidase Inhibitory assay: In case of stem and root, the Aq extract has shown max inhibition of α-glucosidase whereas in the case of flower Aq extract was least active (Fig. 6).

Antiviral activity: Results of this study reveiled that Aq extract of all parts of plant has showcased highest antiviral potential against HIVH9N2. EtOAc extracts of stem and flower were next in order. Against IBV, MtOH extract of root and flower and EtOH extract of stem were active (Tables 4 & 5).

Discussion

Secondary metabolites found in plants are the real source of medicinal potentials and can be exploited in different ways to treat a variety of medical conditions (Arulmozhi *et al*., 2018). In accordance to results of this study, many previous studies have reported that the EtOAc extracts of different parts of *C. polygonoides* is a potential source of flavonoids (Ahmed *et al*., 2016). The study conducted by (Samejo *et al*., 2011) was comparable to the current investigation and has reported the presence of flavonoids, tannins, phenols, terpenoids, and carbohydrates in different parts of *C. polygonoides*. Many oxidative stress-related disorders may be prevented and treated with phenolic substances (Cai *et al*., 2004). The variation in phenolic content of different extracts is due to solvent polarity. Results indicated that EtOH, EtOAc, and MtOH extracts were the best solvents for the extraction of different phenolic compounds (Marinova *et al*., 1997). In a similar study, it was observed that a complex mixture of terpenoids, acid derivatives, and hydrocarbons was present in different parts of *C. polygonoides* (Samejo *et al*., 2013). Gomes *et al*., (2015) had reported that floral extracts of this plant were rich in aromatic substances. Studies conducted on a different plant of the same genus i.e., *Calligonum azel* Maire have unveiled the presence of, quercetine, hordenine, and vanilline in different extracts (Bannour *et al*., 2016). The results of the current study are comparison with that of Pervaiz *et al*., (2020) which reported the prevalance of gallic acid in different extracts of *C. polygonoides*.

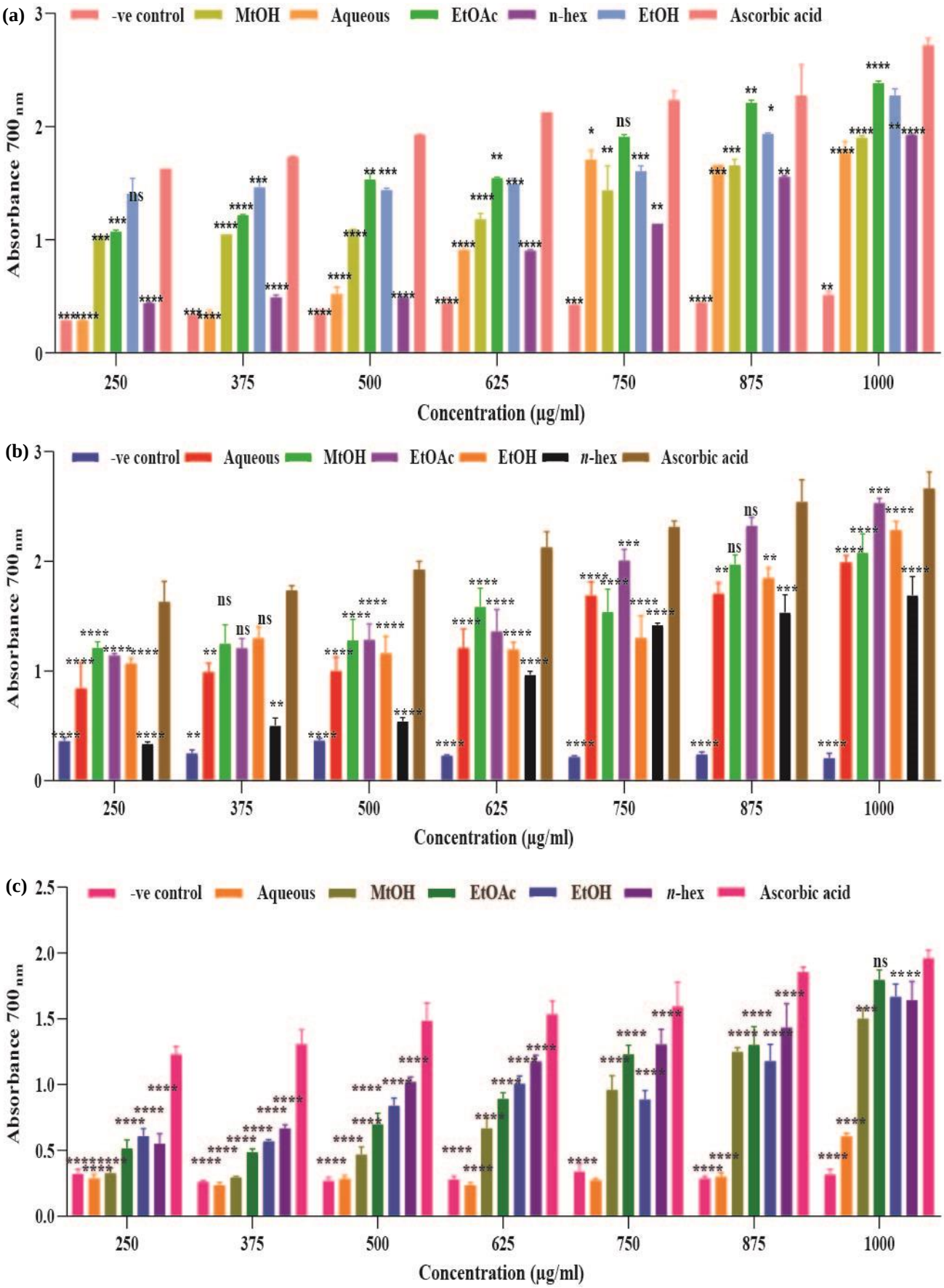


Fig. 5. FRAP assay of different parts of *C. polygonoides*. (a) stems (b) roots (c) flowers. Data is presented as mean± standard error. The results are analyzed using two- way ANOVA, Dunnett's multiple comparisons Test. The comparisons are made with positive control and results are considered significant (*) if $p<0.05$, more significant (**) if $p< 0.01$, very significant (***) if $p<0.001$, highly (****) if $p<0.0001$ and ns if $p>0.05$.

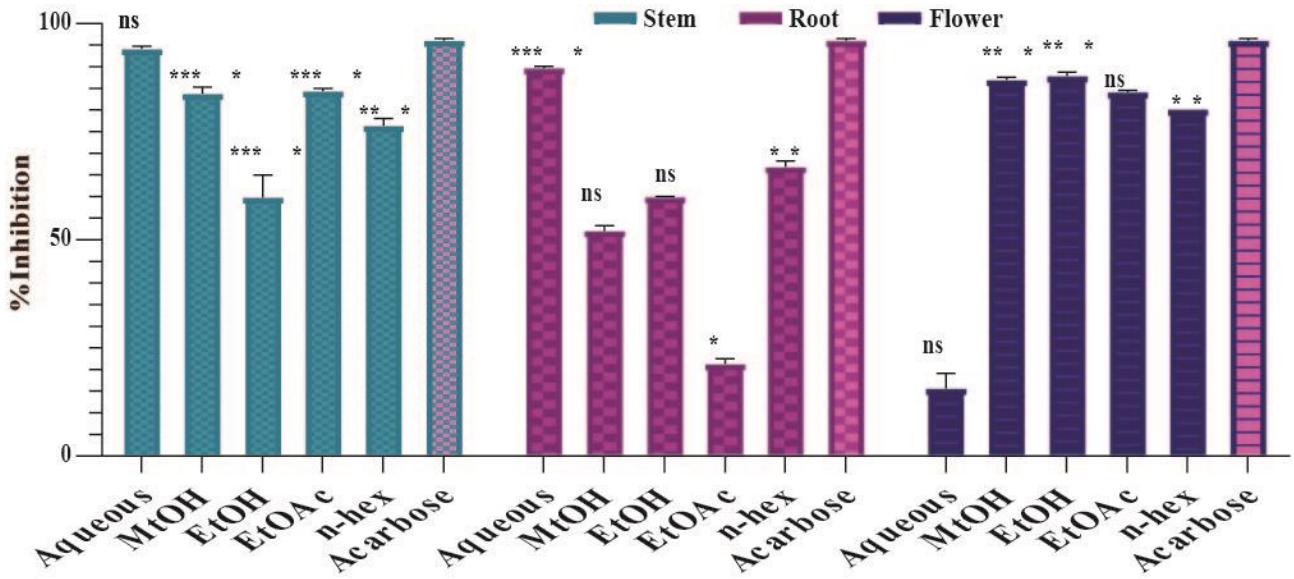


Fig. 6. α-glucosidase Inhibitory assay of different extracts of stem, root, and flower. Values are presented as mean ± standard error. The results are analyzed using two- way ANOVA, Dunnett's multiple comparisons Test. The comparisons are made with positive control and results are considered significant (*) if $p<0.05$, more significant (**) if $p< 0.01$, very significant (***) if $p<0.001$, highly (****) if $p<0.0001$ and ns if $p>0.05$.

Table 4. Antiviral activity of different extracts of *C. polygonoides* against HIVH9N2.

HIVH9N2									
Extract type	**Stem**			**Root**			**Flower**		
	HA Titer	**Log Reduction**	**IC_{50}**	**HA Titer**	**Log Reduction**	**IC_{50}**	**HA Titer**	**Log Reduction**	**IC_{50}**
Aq	0	<log2	12.5	0	<log2	12.5	0	<log2	12.5
MtOH	2	9	25	16	6	25	2	9	25
EtOAc	0	<log2	3.125	64	4		0	<log2	3.125
EtOH	8	7	50	0	<log2	12.5	16	6	25
n-hex	2	8	25	4	8	25	4	8	25
Control	1024	0		1024	0		1024	0	

*Log reduction= [log_2 (control=10)-log_2 Sample]

Table 5. Antiviral potential of different extracts of C. polygonoides parts against IBV.

IBV									
Extract type	**Stem**			**Root**			**Flower**		
	HA Titer	**Log Reduction**	**IC_{50}**	**HA Titer**	**Log Reduction**	**IC_{50}**	**HA Titer**	**Log Reduction**	**IC_{50}**
Aq	8	7	50	16	6	25	4	8	25
MtOH	2	9	25	0	<log2	3.125	0	<log2	12.5
EtOAc	2	9	25	4	8	25	0	<log2	3.125
EtOH	0	<log2	12.5	2	9	12.5	8	7	50
n-hex	8	7	50	4	8	25	0	<log2	12.5
Control	1024	0		1024	0		1024	0	

*Log reduction= [log_2 (control=10)-log_2 Sample]

Plant based antibacterial compounds are usually found very effective against variety of microorganisms. According to multiple studies the difference in antimicrobial effects of extracts is due to the presence of different phenolic compounds (Mahboubi *et al*., 2014; María *et al*., 2018). Gallic acid is one of those phenolic compounds which are responsible for antibacterial activity. It was found to be present in significant quantities in active extracts like EtOH and EtOAc (Tanase *et al*., 2019). Bacterial biofilms have become a worldwide health hazard due to higher level of resistance and the tendencies to worsen the nosocomial infections. Therefore seeking new and more effective substances to combat the issue is a mater of highest priority (Nostro *et al*., 2016). The difference in biofilm inhibition potential of different extracts is based on presence or absence of different phenolic compounds in varying order (Daglia, 2012; Wijesundara *et al*., 2019). Active extracts have shown the presence of gallic acid, quercetin, and catechin which acts as biofilm inhibitors (Slobodníková *et al*., 2016).

The use of medicinal plants as natural antioxidant agents and their value in the treatment of a variety of oxidative stress-related disorders are well established (Carocho *et al*., 2013). This could be due to the presence of chlorogenic acid which was a widely reported antioxidant and was found exclusively in the *n*-hex extract of flowers. According to earlier reports a positive correlation exist between phenolic content and antioxidant potential (Takao *et al*., 2015). Phenolic compounds like quercetin, catechin, and gallic acid were also responsible for antioxidant potential and also found in significant quantities in active extracts (Song *et al*., 2010). Apak *et al*., (2007) suggested that antioxidant potential should be studied with more than one assay, so the FRAP assay was also used to strengthen the current investigation. This could be due to the presence of *p*-coumaric acid which is a strong antioxidant and occurs exclusively in the *n*-hex extract of flower (Santhi *et al*., 2016). Studies have reported that different flavonoid derivatives like quercetin, and rutin, are responsible for α-glucosidase inhibition (Lin *et al*., 2016). These phenolic compounds were present in significant quantities in active extracts but the Aq extract of flower contained phenolic compounds in very little quantities. That's why floral Aq extract was less active compared to Aq extract of root and stem. Viral infections in poultry birds have been a major cause of economic losses to poultry flocks. Medicinal plants are a potential source of a variety of secondary metabolites that can act as antiviral agents (Mukhtar *et al*., 2008). The antiviral potential of Cholistani plants has been widely evaluated by (Shahzad M. *et al*., 2020; Shahzad *et al*., 2019). The presence of natural components such as polyphenols and tannins in different extracts of plants attribute to the antiviral properties of these plants (Jassim *et al*., 2003).

Supplementary Material

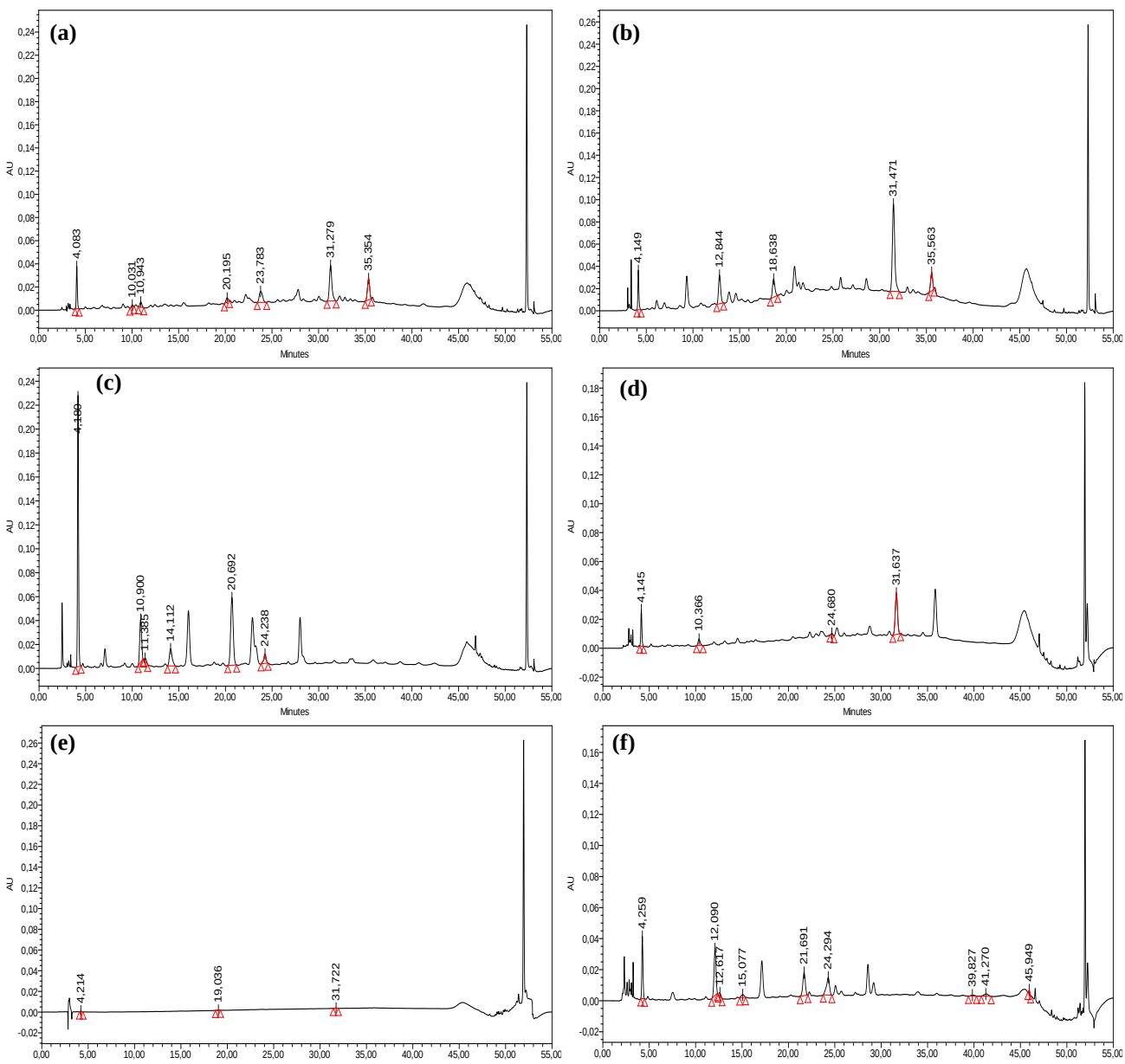
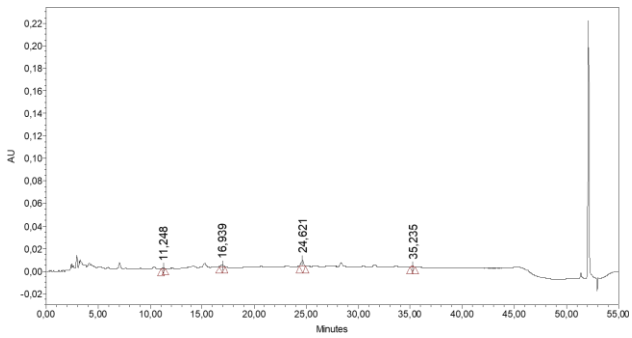
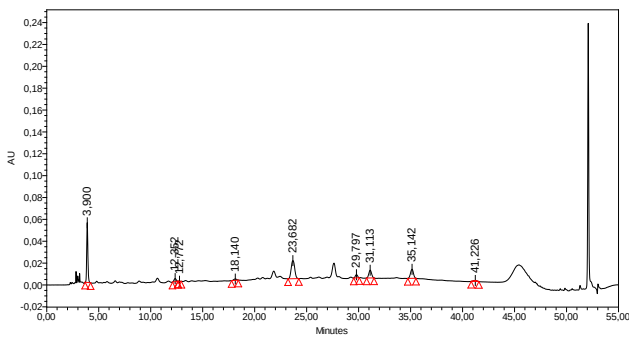


Fig. 1. HPLC-PDA determination and chemical fingerprint of the phenolic content of *C. polygonoides*. (a) EtOAc extract of stem (b) EtOAc extract of root (c) EtOAc extract of flower (d) *n*-hex extract of stems (e) *n*-hex extract of roots (f) *n*-hex extract of flower.





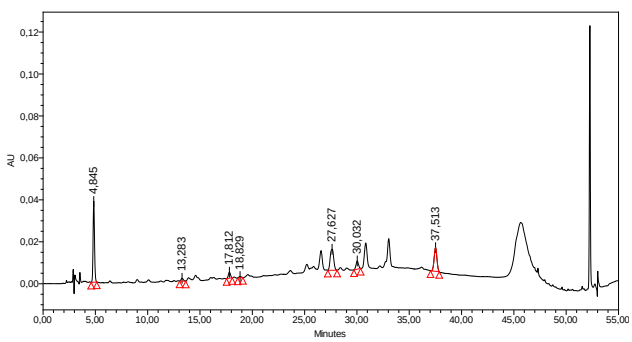
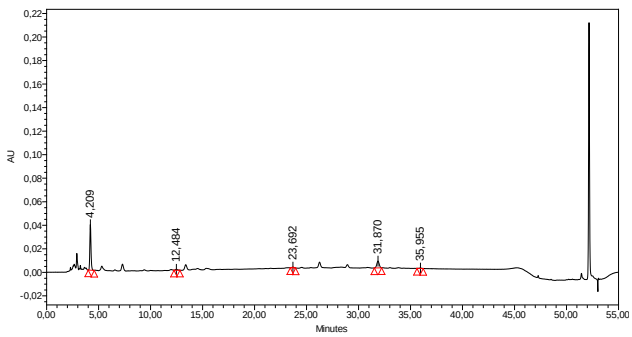
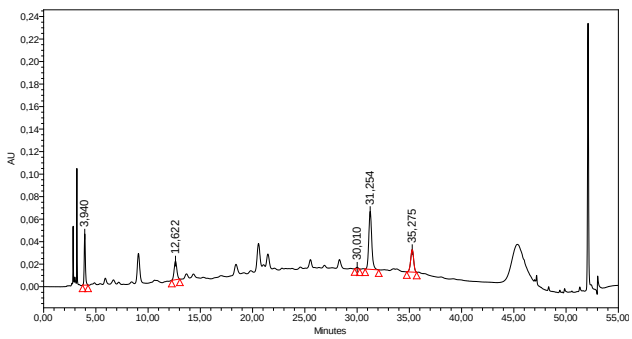


Fig. 2. HPLC-PDA determination and chemical fingerprint of the phenolic content of different extracts of *C. polygonoides* stem (a) Aqueous (b) MtOH (c) EtOH.





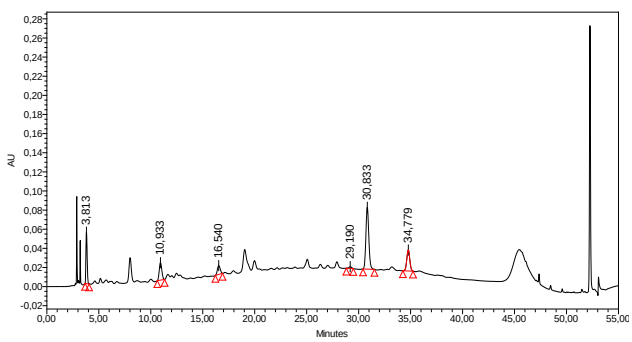
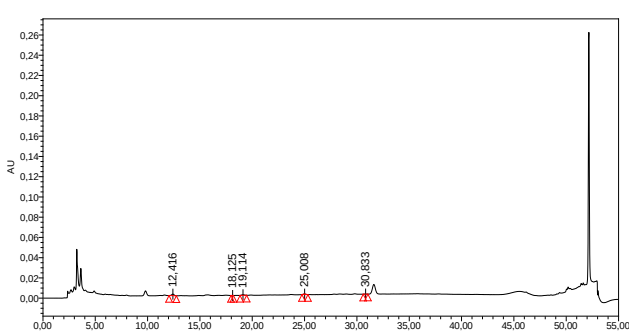
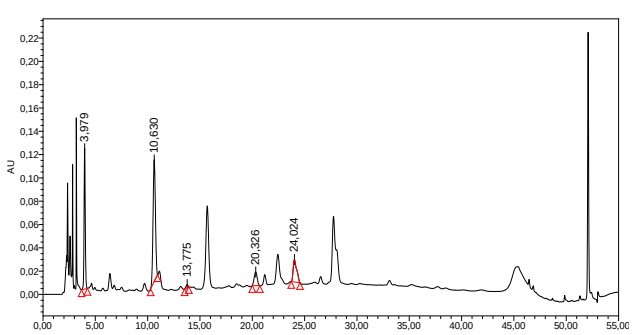


Fig. 3. HPLC-PDA determination and chemical fingerprint of the phenolic content of different extracts of *C. polygonoides* root (a) Aqueous (b) MtOH (c) EtOH.





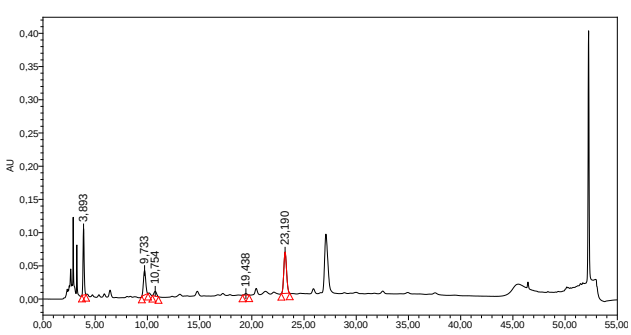


Fig. 4. HPLC-PDA determination and chemical fingerprint of the phenolic content of different extracts of *C. polygonoides* flower (a) Aqueous (b) MtOH (c) EtOH.

Conclusion

This study concludes that different parts of *C. polygonoides* contain a variety of phytochemicals that confer antimicrobial, antioxidant antiviral, and antidiabetic activities through different extracts. Quantitative analysis of phenolic content revealed that stem extracts are rich in phenolic compounds and therefore exhibit strong antioxidant activities compared to root and floral extracts. In terms of antibacterial and antibiofilm potential different extracts of all parts exhibited varying potentials against selected bacterial strains. Furterh, the study proved that *C. polygonoides* also possesses good antidiabetic potential, especially in stem extracts. All the extracts of different parts of *C. polygonoides* showed good antiviral potential against AIVH9N2 and IBV. The study proved that *C. polygonoides* is a rich source of pharmacologically active agents.

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