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Original Article

HPLC OF PHENOLIC COMPOUNDS, ANTIOXIDANT AND ANTIMICROBIAL ACTIVITY OF BULBS FROM THREE *ORNITHOGALUM* SPECIES AVAILABLE IN INDIA

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ABSTRACT

Objective: The aim of the study was to analyze phenolic compounds of three species of *Ornithogalum* viz *Ornithogalum virens*, *Ornithogalum thyrsoides, Ornithogalum dubium* and to assess their bioactivity in terms of antimicrobial and antioxidant potential.

Methods: Extracts were prepared in 20% aqueous methanol. Each extract was subjected to phenolic and flavonoid estimation. Antioxidant activity was tested using DPPH method, and their antimicrobial activity was tested on six pathogenic strains namely *Enterobacter cloacae, Serratia marcescens, Escherichia coli*, *Shigella dysenteriae*, *Staphylococcus aureus* and *Pseudomonas aeruginosa.* The extracts were subjected to HPLC analysis with different standards namely gallic acid, caffeic acid, p-coumaric acid, syringic acid, sinapic acid, ferulic acid, methyl gallate, catechin, rutin, apigenin, quercetin, myricetin, and kaempherol.

Results: *O. dubium* was found to have highest antioxidant activity (IC_{50} 311 µg/g extract). Inhibition zone was minimum in *S. marcescens* and *E. coli* on the application of extracts of *O. virens,* and the consequent MIC was 670 µg and 650µg/g dry weight respectively. None of the three extracts was found to have any effect either on *S. aureus* or *P. aeruginosa*. HPLC analyses have shown that myricetin was the primary flavonoid constituent of the extract of *O. dubium* and gallic acid of *O. virens*.

Conclusion: *O. dubium* shows maximum antioxidant and antimicrobial activity. Extracts of O. virens also shows maximum polyphenol content. From the HPLC results, it is evident that the flavonoids present in *O. dubium* are myricetin, rutin, p-coumaric acid along with some phenolic compounds, which confers bioactivity to the extract.

Keywords: Ornithogalum, Phenolic acid, Flavonoids, HPLC, Antioxidant activity, Antimicrobial activity

INTRODUCTION

The genus *Ornithogalum*, commonly called stars of Bethlehem is a native to temperate regions of Europe, Asia and Africa. The genus comprised of approximately 200 species [1]. Since distant past, its beautiful flowers was always given importance for the purpose of horticulture and gardening. The cut flower of *Ornithogalum thyrsoides* and *Ornithogalum arabicum* are considered most important in Kenya for its export potential [2]. In India, some species such as *Ornithogalum virens*, *Ornithogalum thyrsoides, Ornithogalum dubium,* are cultivated as ornamental garden plants in the sub-Himalayan temperate regions.

Apart from the horticultural importance and aesthetic values, *Ornithogalum* has been used in traditional medicine [3]. *Ornithogalum umbellatum* is highly valued in homeopathy medicines [4]. Even the extracts are useful in treatments of stomach upsets like gastric ulcers, peptic ulcers, duodenal ulcers, acidity, etc. It has been reported that this plant showed bioactivity related to anticancer, antimicrobial, cytotoxic and antioxidant properties [5]. A number of phytochemicals e. g. steroidal glycosides [6], monoterpene lactone [7] and homo isoflavone [8] have been isolated to corroborate bioactivity. Acylated cholestane glycoside has been extracted from the bulbs extract of *Ornithogalum saundersiae* [9]. These compounds are potentially bioactive especially cytotoxic against tumor cells. Two cholestane tri glycosides from bulb extract of *Ornithogalum saundersiae* with potent cytotoxic activity have also been reported [8-10].

A variety of human diseases like cancer, diabetes, hypertension, and aging are thought to be due to the generation of excessive reactive oxygen species (ROS). A plethora of medicinal plants has been investigated for potential antioxidant activity to inhibit the progression of these diseases. Eventually, some *Ornithogalum* species have also been reported to have strong antioxidant activity. Antioxidant activity has been reported in species like *O. sintenisii* [11], *O. umbellatum* [12], *O. alpeginum* [13]. It has been shown that the salient compounds responsible for antioxidant activity are phenolic constituents of plant extracts. Scientific reports are also plenty where bactericidal, and bacteriostatic activity was clearly correlated to the antioxidant property of plant extracts. In this context, it is worth to notice that *O. cuspidatum* [14] and *O. sintenisii* [9] have been shown to exhibit promising antimicrobial activity.

The main objective of this study was to investigate antioxidant and antimicrobial activity of extracts from three Indian species of *Ornithogalum* namely, *O. virens, O. thyrsoides* and *O. dubium*. Each bulb extract of *Ornithogalum* species was investigated by high-performance liquid chromatography (HPLC) to delineate the role of phenolic acids and flavonoids present.

MATERIALS AND METHODS

Collection of plant materials

The bulbs of the three *Ornithogalum* species, *Ornithogalum dubium* Houtt., *Ornithogalum thyrsoides* Jacq. and *Ornithogalum virens* Lindl. were collected from Darjeeling (27.0500 ° N, 88.2667 ° E and 2,134 meters above mean sea level) in winter. The voucher specimens (voucher specimen no. PU/16, PU/17 and PU/18, deposited to the Presidency University Herbarium) were identified by Taxonomist Dr Subhasis Panda (Ph. D. FIAT, FEHT), Angiosperm Taxonomy & Ecology Lab, Post-Graduate Department of Botany, Darjeeling Govt. College (University of North Bengal).

Equipment and chemicals

The instruments used were Rotavapor (SB1100, Eyela, Japan), Spectrophotometer (V-530, Jasco, Japan) and HPLC (Dionex Ultimate 3000, Thermo, USA). The standard phenolic compounds for HPLC analysis were purchased from Sigma-Aldrich, USA such as Gallic

acid, Caffeic acid, p-Coumaric acid, Syringic acid, Sinapic acid, Ferulic acid, Methyl gallate, Catechin, Rutin, Apigenin, Quercetin, Myricetin, Kaempherol. Other chemicals like 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Sigma, USA), Aluminium chloride (Sigma, USA), Folin-Ciocalteu (Merck, Germany), Potassium acetate (SRL, India), Sodium carbonate (Merck, Germany), Mueller-Hinton agar (Hi-media, India), and Acetonitrile (SRL, India) were procured for experiment.

Extraction

The bulbs of the three species were thoroughly washed, soaked in blotting paper and then cut into 5 cm small pieces and dried on the shade for 15 d. The dried material was powdered in a pulverizer and passed through a mesh (mesh size 80). 2 g sample of each species was extracted with 20 ml of aqueous methanol (methanol-water, 80:20, v/v)[15] for overnight in a shaker at 180 rpm. After 24 h the extract was filtered (Whatman no.1 filter paper) and the residue was subjected to further extraction twice sequentially. The total filtrate was pooled and concentrated in reduced pressure using rotary evaporator. The residue was dissolved in 2 ml methanol for further experiments [11].

Total phenolic content

Total phenolic content was estimated for each extract following the method of Singleton and Rossi [16]. Briefly, 200 µl sample was taken in a test tube, 1 ml Folin-Ciocalteu reagent and 0.8 ml of sodium carbonate (7.5%) were added. After thorough mixing, it was incubated for 30 min. Absorption was measured at 765 nm in a spectrophotometer. Total phenolic content was measured as gallic acid equivalent (GAE) using gallic acid as standard.

Total flavonoid content

A spectrophotometric assay [17] was adopted for total flavonoid content in each extract. Extracts (200 µl) were mixed with 0.1 ml of 10% aluminum chloride and 0.1 ml of 1M potassium acetate. The mixture was incubated for 30 min. and OD was measured at 415 nm. Quercetin was used for calibration to estimate flavonoid content as quercetin equivalent (QE) for each extract.

DPPH radical scavenging activity

A modified method [15] for radical scavenging activity was followed using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. Aliquots of the sample (20-100 µl) were mixed with 3.9 ml of DPPH solution (25 mg/l methanol) and kept in dark for 30 min. Absorption was measured at 517 nm for blank and sample at different concentrations. DPPH radical scavenging activity was calculated by using the formula $[(A_0-A_t)/A_0] \times 100$ where, A_0, A_t are absorptions of blank and sample respectively. The 50% radical scavenging activity denoted by IC_{50} was calculated from regression equations. The regression equations for *O. virens, O. thyrsoides* and *O. dubium* were $y=23.65x+39.05$, $r^2=0.996$, $y=37.25x+23.18$, $r^2=0.989$ and $y=50.05x+34.45$, $r^2 = 0.997$ respectively.

Antimicrobial activity

Antimicrobial activity was carried out on microorganisms *E. coli* (ATCC25922), *P. aeruginosa* (ATCC27853), *S. marcescens* (ATCC13880), *S. dysenteriae* (ATCC9361), *S. aureus* (ATCC25923) and *E. cloacae* (ATCC13407) by disc diffusion method [18]. The strains were cultivated in nutrient broth for 24 h at 37 ^{0}C in shakers (180rpm). Inoculums (50 µl of each strain) were spread on Mueller-Hinton agar medium in 90 mm plates. Small paper discs of 5 mm diameter were saturated with 10µl of extracts of *O. virens*, *O. thyrsoides* and *O. dubium*. The discs were then aseptically placed on the plates of each strain and were incubated at 37 ^{0}C for 24 h. Streptomycin (10 mg/ml) was used as a positive control and methanol as a negative control. Minimum inhibition zone (MIC) was determined by serial dilution of the each of the extracts in 1:10 ratio in 100 µl aliquots.

HPLC analysis of phenolic compounds

HPLC analysis was performed using Dionex Ultimate 3000 (Germany) HPLC instrument with the quaternary solvent delivery system (LPG 3400 SD) including a diode array detector (DAD 3000), manual sample injection valve equipped with a 20 µl loop and Chromeleon 6.8 software was used to process data. The separation was achieved by a gradient elution of a mobile phase containing 1% aq. acetic acid (Solvent A) and acetonitrile (Solvent B), the flow rate was adjusted to 0.7 ml/min and a reversed-phase column, Acclaim ™ 120 C18 (25 cm x 4.6 mm, 5 µm) has been used for analysis. The elution program was set (from 10-40% B in 28 min; 40-60 % B in 39 min; 60-90 % B in 50 min) to get adequate resolution for standard mix comprised of 13 components. Detection and quantitation of each phenolic compound in each extract of *Ornithogalum* species was performed by following a validated method [19].

Statistical evaluation

Each experiment was done in triplicates. The mean and standard deviation were calculated by employing statistical software SPSS, version 17.0.

RESULTS

The amount of total phenolics

Three species *O. thyrsoides, O. dubium* and *O. virens* have been studied for antioxidant activity. Total phenolic content (TPC) was estimated as Gallic acid equivalent (GAE) where gallic acid was used as standard phenolic acid ($y=0.002x-0.029$, regression coefficient $r^2 = 0.998$). It is shown in Table-1 that *O. virens* have highest TPC 78.13µg/g GAE, then *O. dubium* (68.35µg/g) and the least found in *O. thyrsoides* (41.18µg/g). The flavonoids have been estimated and expressed as Quercetin equivalent (QE), ($y=0.006x+0.061$, regression coefficient $r^2 = 0.995$), considering quercetin as standard. A similar trend of QE was observed for *O. virens* (42.28µg/g), *O. dubium* (27.05µg/g) and *O. thyrsoides* (17.52µg/g) (Table-1).

DPPH radical scavenging activity

Antioxidant activity was measured by DPPH radical scavenging method. The crude extract of *O. virens, O. thyrsoides* and *O. dubium* showed 89.85%, 88.83% and 65.06% inhibition respectively (table 1). IC_{50} was lowest in *O. dubium* (311µg/ml) and maximum in *O. thyrsoides* (699µg/ml) whereas for *O. virens* it was 457µg/ml.

Table 1: Radical scavenging activity, total phenolic content, flavonoid content and extractive value of three *Ornithogalum* species

Sample	QE[a] (mg/g) mean±SEM	GAE[b] (mg/g) mean±SEM	DPPH inhibition (%) mean±SEM	IC_{50} value (µg/ml)[c] mean±SEM	Extractive value (mg/g) mean±SEM
O. virens	42.28±2.90	78.13±5.53	88.83±2.12	457±30.62	201.08±5.09
O. thyrsoides	17.52±1.08	41.18±3.97	65.06±0.37	699±14.14	115.49±8.65
O. dubium	27.05±2.14	68.35±5.34	89.85±1.85	311±49.14	170.66±11.63

Data presented as mean±SEM. All the experiments were performed in triplicate (n=3), [a]QE means mg of quercetin equivalent per g of dry material, ($y=0.006x+0.061$, $r^2 = 0.995$), [b]GAE means mg of gallic acid equivalent per g of dry material, ($y=0.002x-0.029$, $r^2 = 0.998$), [c]µg/ml of crude extract, Ascorbic acid was used as positive control, ($y=0.008x+0.013$ $r^2 = 0.997$)

Antimicrobial activity

The aqueous methanolic extracts of the three species of *Ornithogalum* showed potential antimicrobial activity (Table2). The inhibition zone and minimum inhibitory concentration (MIC) for each extract were evaluated against six pathogenic strains viz. *Enterobacter cloacaea* lower respiratory tract pathogen, *Serratia marcescens* a urinary tract pathogen, *Shigella dysenteriae* that causes

severe dysentery, *Escherichia coli* that cause diarrhea, *Staphylococcus aureus* a respiratory tract pathogen and *Pseudomonas aeruginosa* an eye pathogen. Maximum activity was found in *O. dubium* and minimum in *O. thyrsoides* (table 2). MIC was found to be minimum for *S. dysenteriae* and it has shown the largest inhibition zone on the application of the concentrated extract of *O. dubium* (23.33 mm). Inhibition zone was minimum in *Serratia* and *E. coli* on the application of extracts of *O. virens* and consequent MIC was 670.33µg and 648.33µg respectively. None of the three extracts have any effect either on *S. aureus* or *P. aeruginosa*. *O. dubium* extract was found to be most effective against microorganisms tested (table 2) compared to other species of *Ornithogalum*.

Table 2: Antimicrobial activity of three *Ornithogalum* species

Name of the Organism	*O. thyrsoides*		*O. virens*		*O. dubium*		Positive Control (Streptomycin)	Negative Control (Methanol)
	Zone (mm)	MIC µg/ml	Zone (mm)	MIC µg/ml	Zone (mm)	MIC µg/ml		
Enterobacter cloacae (ATCC 13047)	8.33±1.08	526.00±3.93	15.33±2.48	255.00±6.28	19.00±1.41	255.66±6.0	11.66±0.4	ND[a]
Serratia marcescens (ATCC 13880)	9.00±1.41	670.33±5.30	13.66±1.77	510.66.00±6.79	18.66±2.40	258.33±7.4	13.00±1.4	ND[a]
Escherichia coli(ATCC 25922)	8.00±1.87	648.33±4.70	20.33±2.27	355.66±7.11	21.00±2.54	250.66±7.90	16.66±1.08	ND[a]
Shigella dysenteriae (ATCC 9361)	12.33±1.88	429.00±6.74	21.66±2.51	200.33±6.79	23.33±1.47	125.00±6.16	ND[a]	ND[a]
Staphylococcus aureus (ATCC 25923)	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]	10.66±1.08	ND[a]
Pseudomonas aeruginosa (ATCC 27853)	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]	6.66±0.81	ND[a]

*MIC presented in µg of extract per ml, [a]ND means no inhibition zone detected, Data presented as mean±SEM. All the experiments were performed in triplicate (n=3)

HPLC analysis

The phenolic compounds present in the extracts of *O. virens, O. thyrsoides* and *O. dubium* were analyzed by HPLC and chromatograms generated are given in fig. 1, fig. 2, and fig. 3 respectively. Identification of the constituents was done using 13 phenolic acid and flavonoid standards as depicted in table 3. apigenin, Methyl gallate, and Ferulic acid was found absent in all three extracts, on the other hand, catechin, caffeic acid and kaempferol were present. *O. thyrsoides* was found to contain least components in comparison to other two species.

The major difference between *O. virens* and *O. dubium* was noteworthy on the basis of structural variation phenolic compounds. *O. virens* was rich in phenolic acids compared to *O. dubium* which in turn is rich in flavonoids (table 3). The flavonoid myricetin was found maximum (357.92 µg/g) in *O. dubium* whereas gallic acid was found to be significant (162.81 µg/g) in *O. virens*.

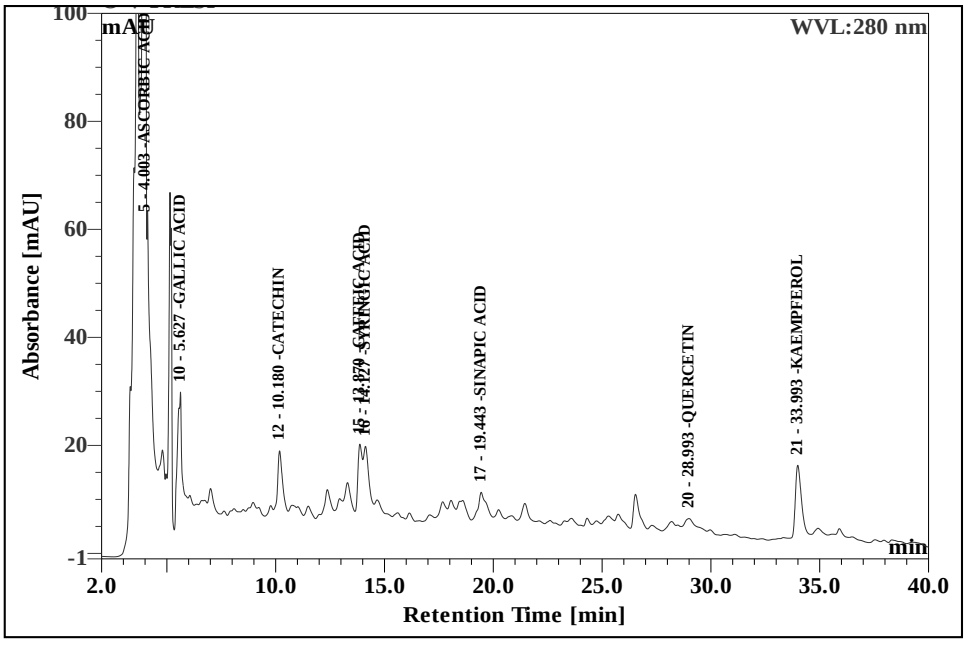

Fig. 1: HPLC chromatogram of *O. virens*

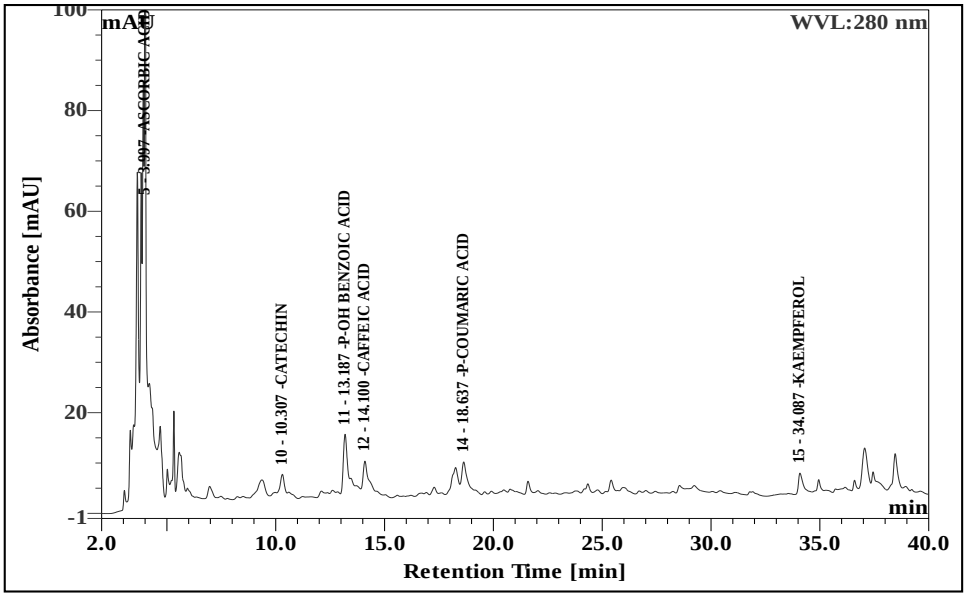

Fig. 2: HPLC chromatogram of O. thyrsoides

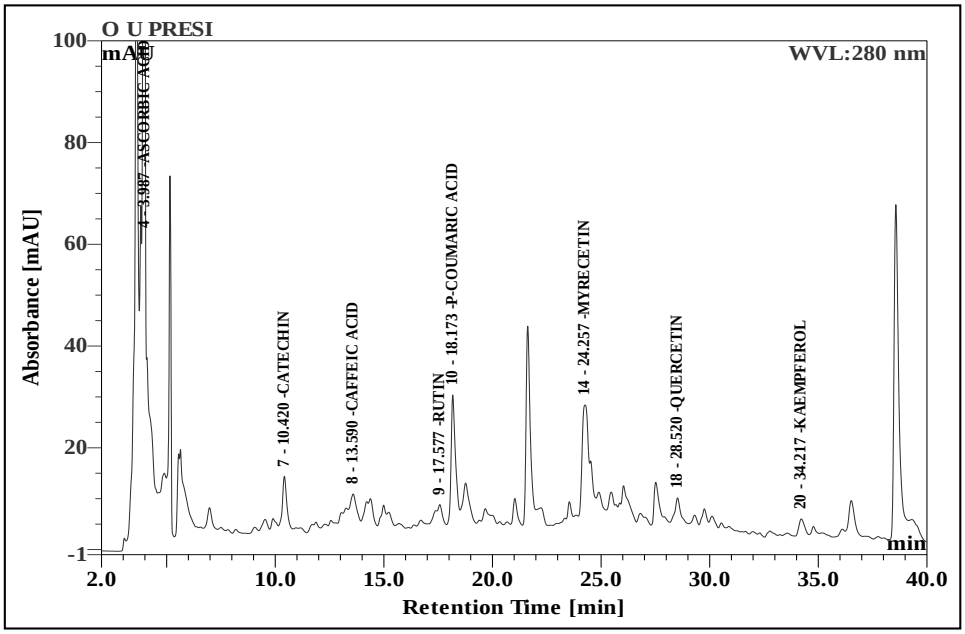

Fig. 3: HPLC chromatogram of *O. dubium*

Table 3: HPLC analysis of phenolic compounds of three *Ornithogalum* extracts

S. No.	Name of components	*O. thyrsoides* (µg/g)*	*O. dubium* (µg/g)*	*O. virens* (µg/g)*
1	Catechin	76.20±2.71	244.22±2.35	306.34±3.17
2	Gallic acid	-	-	162.81±5.06
3	Caffeic acid	25.63±2.96	23.28±1.72	50.30±3.42
4	p-Coumaric acid	30.64±2.11	92.71±1.44	-
5	Syringic acid	-	-	62.93±1.82
6	Sinapic acid	-	-	105.64±3.70
7	Rutin	-	109.71±±3.61	-
8	Myricetin	-	357.92±6.03	-
9	Quercetin	-	47.75±7.25	24.48±2.59
10	Kaempherol	36.62±2.13	31.92±1.96	153.96±7.83
11	Ferulic acid	-	-	-
12	Methyl gallate	-	-	-
13	Apigenin	-	-	-

*per gram of dry weight of the material, Data presented as mean±SEM. All the experiments were performed in triplicate (n=3)

DISCUSSION

Ornithogalum species is exotic to India and available in a particular climatic zone. Although the flowers are widely known for its aesthetic value and commercially exploited from nursery cultivation, the bulbs have different usage particularly in folk medicine [3, 4]. The phytochemical studies have revealed the presence of steroidal glycosides having potential anticancer activity [20]. It has been reported in several scientific reports that the polyphenolic compounds like phenolic acids and flavonoids are common in plants which are consumed as food or used for medicinal purposes and these compounds have strong antioxidant properties [21]. In plants, the polyphenols prevent pathogen aggression and protects from UV radiation [22]. Dietary consumption of polyphenols on a regular basis provides protection from neurodegenerative diseases, cardiovascular diseases, diabetes, osteoporosis and even cancer development [23, 24].

There are few reports available on the antioxidant potential of *Ornithogalum* species such as *O. sintenisii* [12] although the salient phenolic compounds are not identified or quantitated. It was reported that the phenolic content in extracts of *O. sintenisii* [11] and *O. umbellatum* [12] bulbs was 8.4±0.3 mg/g and 2.1±0.9 mg/g respectively which is much lower than *Ornithogalum* species investigated in this study. Flavonoids are also a group of polyphenolic compounds which are of common occurrence among plants. They have anti-inflammatory, anti-platelet, cytotoxic, antioxidant, anti-allergic properties and also cardio and neuroprotective [25]. In *O. virens* flavonoid content is much better than both *O. sintenisii* (23.5±1.3 mg/g) [11]), and *O. umbellatum* (0.2±0.2 mg/g) [12]. The result in Table-1 shows a significant variation that might be due to different species. *O. virens* is a more potential source of polyphenolics than *O. dubium* and*O. thyrsoides*.

Antiradical activity is often a good marker for the antioxidant activity of plant extracts. The polyphenolics play a major role for radical scavenging and to evaluate the potential DPPH, is commonly used as a radical generator *in vitro*. Maximum inhibition percentage (89.95%) was shown by *O. dubium* and minimum by *O. thyrsoides* (65.06%). The IC_{50} values shown in Table-1 corroborated radical scavenging was maximum for *O. thyrsoides* (699 µg/ml) and minimum for *O. dubium* (311 µg/ml). Antioxidant activity helps to scavenge free radicals and thus provides protection from different pathogens as well as chronic diseases [26]. The change in the structural conformation due to giving up of a hydrogen atom is responsible for free radical scavenging activity of the antioxidants [27]. Ebrahimzadeh (2010) has reported IC_{50} of bulb extracts of *O. sintenisii* as 669±25 µg/ml. DPPH dependent antioxidant activity assay by Makasci *et al.* (2010) have shown 90.38% of inhibition in the methanolic extraction of bulbs of *O. alpigenum* [13, 28]. The overall antioxidant potential with respect to IC_{50} of the three species of *Ornithogalum* appears as *O. dubium* (311 µg/ml)>*O. virens* (457 µg/ml)>*O. thyrsoides* (699 µg/ml).

The secondary metabolites especially polyphenolic compounds of many plants are often found to have potent antimicrobial activity. Plant extracts serve as an important source of some antimicrobial agents [29] and their use in human welfare dates back to early civilizations. These drugs are preferred more than the synthetic ones because of the fact that they are more often far less deleterious on human health than that of the synthetic drugs. Literatures are available regarding antimicrobial properties of different species of *Ornithogalum*. Makasci *et al.* (2010) have reported that maximum activity was shown by the extracts of O. *alpigenum* against *Bacillus subtilis* and *B. cereus* [13]. As per our findings, O. *dubium* has maximum potential as an antimicrobial agent and it has lowest MIC for *Shigella dysenteriae* (125µg). Antimicrobial activity has also been reported in *O. cuspidatum*, maximum activity is being found against *B. cereus* with an MIC of 7.5 mg/ml [30].

In our experiments, *O. dubium* has been found to have maximum bioactivity and its HPLC analysis reveals that the myricetin (357.92µg/g dry weight) is the chief flavonoid present in the extract. Moreover, catechin and rutin are also present in significant concentration. Flavonoids like myricetin, in particular, have been shown to inhibit bacterial DNA polymerase, RNA polymerase, Reverse Transcriptase and Telomerase [31]. Griep *et al.* (2007) reported that myricetin is capable of blocking DnaB helicase of *E. coli*. Extracts of *O. dubium* has been shown maximum antioxidant activity (table 1) and potent antimicrobial activity (table 2) compared to other species, and that might be related to the presence of myricetin in considerable quantity. Moreover, it has been investigated that rutin, a flavonoid glycoside, enhance the antimicrobial efficacy of flavonoids like myricetin, morin, quercetin, kaempherol [32]. Hence, the presence of myricetin and rutin in *O. dubium* extract was found to be more effective than *O. virens, O. thyrsoides*. *p*-coumaric acid has been shown to have potent antioxidant activity and consequently has been found to be a potential antimicrobial agent [33]. This study indicated that *O. dubium* has antioxidant potential, prominent antimicrobial activity, and these properties have further been consolidated by phenolic compounds analyzed by HPLC.

CONCLUSION

It has been shown that *O. dubium* bulb extract has significant radical (DPPH) scavenging activity as well as potential antimicrobial activity compared to other species *O. virens,* and *O. thyrsoides*. The extracts are rich in phenolic constituents as evident from GAE and QE estimation. Identification of phenolic components was further confirmed by HPLC analyses. The quantity of each phenolic acid and flavonoid constituents was determined using external standards and validated method. *O. dubium* was found to contain myricetin, a flavonoid and *O. virens* contain gallic acid, a phenolic acid in a significant amount. To the best of our knowledge, this phytochemical and bioactivity study is the first report on species like *O. dubium and O. virens*.

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CONFLICT OF INTERESTS

Declared none

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