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# Antioxidant and Antibacterial Potential of *Achillea millefolium*, *Sisymbrium irio* and *Viola canescens* from Azad Jammu Kashmir, Pakistan

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**Abstract.** Utilization of plants to cure different ailments has been in practice from centuries. In current study, evaluation of phytochemical constituents, antioxidant and antibacterial activity of *Achillea millefolium*, *Sisymbrium irio* and *Viola canescens* collected from Azad Jammu and Kashmir was performed. Presence of various phytochemicals was detected in ethanolic extracts of the selected plants. *V. canescens* extract exhibited maximum antioxidant activity (86%) tested by DPPH (1, 1-diphenyl-2-picryl-hydrazyl) assay phosphomolybdate (526µg/mL) and antibacterial activity against *Bacillus* (12mm) and *Pseudomonas* (3mm) strains. Minimum inhibitory concentration (MIC) of *V. canescens* was found to be 6.25mg/mL. Thin Layer Chromatography (TLC) of *V. canescens* showed 15 spots and out of these only three spots gave antibacterial activity against *Bacillus subtilis* strain. GC-MS analysis revealed 2H-1,4-benzodiazepin-2-one, 7-chloro-1,3-dihydro-5-phenyl-1- (trimethylsilyl)-oxy, cyclohexasiloxane, dodecamethyl and octasiloxane hexadecamethyl as bioactive components of *Viola canescens*. These compounds can be exploited in pharmaceutical sector in place of the synthetic drugs.

**Keywords:** *Viola canescens*, antioxidant activity, antibacterial activity, GC-MS analysis

## Introduction

Man has been searching for drugs in plants from centuries. In developing countries, major population relies on herbal medication for the management of numerous infections. Plant based medicines minimize many side effects like allergies, hypersensitivity and immune suppression that are often associated with the use of synthetic medicines. The indiscriminate use of synthetically prepared antimicrobials has resulted in the origin of multiple drug resistant bacteria (MDR), which are posing a great threat to medical sector. Hence, modern pharmacopoeia is currently investing a lot on the formulation of plant based medicines (Khanam *et al*., 2015). A diverse range of bioactive molecules are found in plants as secondary metabolites. Mostly, these compounds are phenols, flavonoids, alkaloids steroids, resins, fatty acids and tannins (Shinde *et al*., 2015).

In Pakistan, as one of the developing countries plant based medication is a vital part of our cultural heritage. The herbal medicine sector is playing a major role in providing healthcare to a great number of people. There are about 600 to 700 species of plants that are used for medicinal purposes (Shinwari and Qaiser, 2011). Tatta Pani Region of Azad Jammu Kashmir (AJK) is least investigated area from ethno-medicinal point of view. The vegetation of the area is largely influenced by monsoon rainfall and climate of the area varies from humid to temperate. Many plants of medicinal importance are native of AJK. The local communities of the region are aware of the traditional uses of the local plants. These plants are utilized to cure nearly every kind of disease leading from headache to stomachache and even to heal small cuts and wound (Mahmood *et al*., 2011).

In present study, *Achillea millefolium* (Kangi), *Sisymbrium irio* (Khoob Kala) and *Viola canescens* (Banafsha) were collected from Tatta pani region of AJK. *Achillea millefolium* is popularly known as “Yarrow” and “Kangi”. This flowering plant belongs to family Asteraceae. and *A. millefolium* is an herbaceous, erect and perennial herb that can have one to numerous stems 0.2 to 1 m in height with flowers which range from white to pink. The plant has a strong, sweet scent. *A. millefolium* is known to be utilized for the curing of hemorrhage, pneumonia, wounds healing and rheumatic pain. *A. millefolium* tea also improves blood circulation. It is used in cosmetics and fragrances (Khan *et al*., 2012).

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*Sisymbrium irio*, also known as "Khoob Kala" belongs to family Brassicaceae. The edible plant *S. irio* is an annual herb. The plants are 3 or more than 3 feet tall with open thin stem branches. It has small flowers having 4 yellow petals (Ullah Jan *et al*., 2012). The fruit is narrow, long, cylindrical and sleek which retains its green colour after ripening. The fruit contains tiny reddish-brown oblong seeds (Al-Massarani *et al*., 2017). Seeds of *S. irio* are used to detoxify liver and spleen, treat rheumatism, piles, dysentery, diarrhea, bronchitis, pneumonia, asthma, cough and reduce swelling(Shah *et al*., 2014). *Viola canescens* which is commonly known as "Banafsha" or as "Himalayan White Violet" belongs to family Violaceae. It is present in the Himalayan regions of Pakistan, Bhutan, Nepal and India (Muhammad *et al*., 2012). This plant is a perennial herb. Herbal practitioners utilize plant extract against epilepsy, eczema, stomach acidity and rheumatism and as a therapy for respiratory problems(Masood *et al*., 2014). Paste of the plant is externally applied on wounds, cuts and boils as antiseptic (Rana *et al*., 2014). The goal of the current study was to screen the phytochemical constituents which are responsible for imparting antioxidant and antibacterial activity to *Achillea millefolium*, *Sisymbrium irio* and *Viola canescens* collected from Tatta Pani Region of Azad Jammu and Kashmir. So, these identified phytochemicals can then be exploited in pharmaceutical sector to get cheaper herbal medicines in place of the synthetic drugs.

## Materials and Methods

**Plant collection and processing.** *Achillea millefolium* (whole plant), *Sisymbrium irio* (seeds) and *Viola canescens* (leaves and flowers) about 1000g each which were collected from Azad Jammu and Kashmir, Tatta pani region (31.2488 N̊, 77.0895 E̊) in paper bags. *A. millefolium* and *V. canescens* were rinsed with distilled water to dispose of dust particles and shade dried for 2 to 3 days. *S. irio* seeds were cleaned by threshing. Plants were then crushed by mortar and pestle and stored in clean and dry glass jars.

**Plant extract preparation.** Ten grams of powdered plant material was added in 100 mL absolute ethanol and placed in dark place at room temperature. After 72 h, the filtrate was collected by filtration of sample and stored in dark place.

**Phytochemical analysis.** In order to find out bioactive compounds present in the selected plants different phytochemical tests were performed.Solvent, in which extracts were made, was taken as a negative control while change in colour was interpreted as a positive result.

**Alkaloids.** Picric acid (few drops) was supplemented in 3ml of plant extract, production of yellow precipitates upon reaction, confirmed the presence of alkaloids (Bhandary *et al*., 2012).

**Carbohydrates.** In 3mL of plant extract few drops of Benedict's reagent were mixed and the mixture was boiled. After boiling, appearance of red precipitates showed the carbohydrates presence (Dilshad *et al*., 2018).

**Coumarins.** Plant extract (2mL) was added to 1mL of chloroform and 1mL of 10% NaOH. Development of yellowish colour confirmed the coumarins (Abdirahman *et al*., 2016).

**Flavonoids.** Flavonoids were confirmed by mixing 2mL of plant extract with 2mL of 10%NaOH, increase in intensity of yellow colour or appearance of yellow colour showed that flavonoids are present and this colour faded away when few drops of dilute HCl were added (Khanam *et al*., 2015).

**Phenols.** Few drops of 5% ferric chloride ($FeCl_3$) solution were mixed with 3mL of plant extract. Formation of red, green or purple colour indicated the phenols (Khanam *et al*., 2015).

**Phlobatanins.** For detection of phlobatanins, 3mL of plant extract was boiled with 1ml of 2% HCl, formation of red colour confirmed the presence of phlobatanins (Abdirahman *et al*., 2016).

**Proteins.** In 2mL of plant extract 2mL of 10% NaOH was added and 2 drops of 1% copper sulphate were mixed. Appearance of violet pink colour revealed the existence of proteins in the solution (Khanam *et al*., 2015).

**Quinones.** In 1mL of plant extract, 1mL 10% sodium hydroxide solution was added. Appearance of blue green or red colour showed that quinones are present in the sample (Abdirahman *et al*., 2016).

**Saponins.** In 2mL plant extract, 2mL distilled water was added. After shaking vigorously, test tube was left for 10-15 mins. Occurrence of persistent foam at the surface of the extract revealed the presence of saponins (Pokhrel *et al*., 2015).

**Antibacterial activity screening.** Antibacterial property of plant extracts was checked by agar well diffusion assay and disc diffusion assay. In present study, two ampicillin resistant bacterial strains were used, *Bacillus subtilis,* (KC 881030) as gram positive bacterial strain and *Pseudomonas aeroginosa* (KC 881031) as gram negative bacterium. In agar well diffusion assay, 24 h old culture was swabbed on 90 mm sterile Muller Hinton (MH) agar plate and 3 wells were made in each plate with the aid of sterile Pasteur pipette. In each well, 50µL of plant extract, absolute ethanol (as negative control) and ampicillin i.e. 10µg/mL (as standard) were added and incubated at 28 ºC for 24 h. After 24 h, the inhibition zones (mm) were measured (Sheikh *et al*., 2012). For the purpose of disc diffusion assay, 6mm discs were made from Whatman filter paper no. 1 and autoclaved. After autoclaving, discs of filter paper were kept in hot air oven at 60ºC until properly dried. These discs were then soaked in each plant extract, absolute ethanol and ampicillin under aseptic conditions and allowed to dry. These discs were placed on MH agar plates which were already swabbed with bacterial culture. Plates were then put in incubator at 28 ºC for 24 h. After 24 h, the bacterial growth inhibition (mm) was measured (Van Dijk *et al*., 2014). This experiment was conducted in triplicates and mean value was noted.

**Estimation of antioxidant activity of medicinal plant extracts by DPPH assay.** DPPH (1, 1-diphenyl-2-picryl-hydrazyl) is a stable free radical which has an unpaired valence electron at one atom of nitrogen bridge. In DPPH antioxidant assay, scavenging of DPPH radical take place. DPPH has dark violet colour but on reacting with antioxidants it changes colour to yellow. DPPH is also light, oxygen and pH sensitive. So, the reaction tubes were wrapped in aluminum foil and incubated in dark at 30°C for half an hour. DPPH working solution was prepared from stock (24 mg/100mL) by adjusting the absorbance of stock to 0.98±0.02 at 517nm. Three ml of DPPH working solution was added to 100µL of plant extract and left for 30 min in the dark. Same was done in case of negative control in which 100µL of absolute ethanol was used. The procedure was performed in triplicates and O.D was measured at 517 nm (Baba *et al*., 2015).

**Phosphomolybdate assay.** Total antioxidant potential of the plant was estimated by phosphomolybdate assay. A reagent containing 0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate solution was prepared and 3 mL of this reagent was mixed in 300µL of the plant extract. The capped tubes were incubated at 95ºC for one and half hour. The sample was then cooled down to room temperature and the optical density was determined at 765 nm against the blank by UV spectrophotometer. The total antioxidant capacity was described as µg of ascorbic acid equivalents per mL by using the standard Curve of ascorbic acid graph (Ahmed *et al*., 2015).

**Reducing power assay.** Reducing power assay was done for the determination of antioxidant capacity. For this purpose, plant extract i.e. 2.5mL was combined with same volume of each of 0.2 M sodium phosphate buffer and 1% potassium ferricyanide. The incubation was done in a water bath for 20 min at 50°C. The sample was then cooled to room temperature and 10% trichloroacetic acid (2.5mL) was supplemented and centrifuged at 650 rpm for 10 min. To the supernatant, 5 mL distilled water and 1 mL of 0.1% ferric chloride solution was added. Absorbance was taken at 700 nm by UV spectrophotometer. A blank (negative control) was also run using the same procedure (Ahmed *et al*., 2015).

**Minimum inhibitory concentration (MIC) of *Viola canescens*.** Broth micro-dilution test was done to estimate the MIC of *Viola canescens* against *Bacillus subtilis* strain. For this, 96 well tissue culture microtiter plate was used and 50µL Nutrient broth was poured in each well under aseptic conditions. In first and second well each, 50µL plant extract was added and serially diluted up to 10th well. In second row, absolute ethanol was added, while in third to eighth row antibiotics, ampicillin, penicillin, erythromycin, tetracycline, chloramphenicol and streptomycin (2µg/mL each) were added respectively. Then, 50 µL of 24 h *Bacillus* culture with 0.5 absorbance at 600nm was added in each well except column 12 (*i.e.*, negative control) and plate was incubated at 37ºC for 24 h. After 24 h, 10µL of 0.1% tetrazolium salt was added in each well and kept in dark for thirty minutes. Later, the development of pink colour showed the presence of bacteria (Klanènik *et al*., 2010).

**Thin layer chromatography.** In this technique, *Viola canescens* extract was applied in the form of spot on TLC plate coated with silica gel. When the spot had dried, the plate was put in chromatographic tank containing ethyl acetate, chloroform 1:1 with one drop of water (i.e. mobile phase). As mobile phase traveled, upwards the components of sample got separated. When solvent front had travelled to the top and just 1 inch

was left. the plate was taken out and solvent front was marked. The plate was allowed to dry and observed under short and long UV in TLC plate viewer. The spots which got separated were marked with the help of lead pencil. This TLC plate was then developed in iodine jar to check iodine active compounds (Abdirahman *et al*., 2016).

**Antibacterial activity of TLC plate spots.** The marked spots were scratched from TLC plate and collected in extraction bottles. Two mL of absolute ethanol was poured in them. After 24 h, the extracts were filtered, allowed to evapourate and antibacterial activity of these fractions was estimated by measuring the zone of inhibition in millimeters (mm).

**GC-MS.** Gas chromatography and mass spectrometry (GC-MS) analysis of spot number 1 was done as it gave maximum inhibition zone among all other spots. Length of the column of spectrophotometer was 30 m. Carrier gas in this experiment was helium. Temperature of the Injection port was 230 ºC. One µL of sample was added and flow rate was set at 1mL/min. Temperature change rate was maintained at 5ºC/min, where initial temperature was 100 ºC and final temperature was 270 ºC. The column was run for 35 min and peaks were observed (Gupta and Kumar, 2017).

## Results and Discussion

Quinones were not detected in *Viola canescens* and *Sisymbrium irio*, while alkaloids were detected in *Achillea millefolium* and *Sisymbrium irio* only.Proteins were absent in all extracts when biuret test was performed (Table 1). These results are correlated with previous studies that carbohydrates, coumarins, flavonoids, phenols, phlobatanins and saponins are present in *A. millefolium*, *V. canescens* and *S. irio* (Masood *et al*., 2014; Al-Jaber, 2011). Flavonoids and phenols are reported as most important set of compounds acting as primary antioxidants or free radical scavengers (Sarker and Oba, 2020).

All plant extracts showed antibacterial activity against the selected *Bacillus* (8-12 mm) and *Pseudomonas* (2-3 mm) strains. *Viola canescens* (Banafsha) demonstrated the highest antibacterial potential. *V. canescens* showed 12.1mm zone of inhibition against *Bacillus* and 3mm against *Pseudomonas* (Fig. 1 and Table 1). Antibacterial activity of plant extracts depends upon the solvent in which extraction was done as it will determine the solubility of bioactive compounds (Adnan *et al*., 2020). Plant extracts were made in ethanol because it has less polarity than water hence plants bioactive components can easily dissolve in it. Ethanol is more potent to dissolve bioactive compounds as compared to other organic solvents (Hazli *et al*., 2019). Antibacterial activity of plants against *Bacillus* was recorded to be as compared to more *Pseudomonas.* As gram negative bacteria have an additional lipo-polysaccharide layer which makes it difficult for antibacterial compounds to penetrate in bacterial cell and inhibit its growth (Mouhcine *et al*., 2019).

MIC for *V. canescens* was analyzed by broth micro-dilution method. It was found that minimum concentration of extract required to inhibit tested bacterial strain is 6.25mg/mL, while antibiotic standards were used to compare the effect of plant extract to them. It was observed that antibiotics were more efficient to kill bacterial cells than plant extract (Fig. 2). It is most suitable for a quick quantitative determination of the antimicrobial activity of plant extracts (Klanènik *et al*., 2010).

DPPH assay revealed that all of the selected plants were found to have antioxidant activity (62-86%). *Viola canescens* antioxidant activity was maximum (86%). Whereas, *Sisymbrium irio* extract had antioxidant potential greater than *Achillea millefolium* (Table 2). The genus *Viola* has already been reported to have excellent antioxidant ability due to phenolic compounds

**Table 1**. Phytochemical analysis and antibacterial activity of *A. millefolium*, *S. irio* and *V. canescens*.

| Extracts | Phytochemical tests | | | | | | | | Antibacterial activity (mm) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Alkaloids | Carbohydrates | Coumarine | Flavonoids | Phenols | Phlobatanins | Protein | Quinone | Saponins s | *Bacillus ubtilis* | *Pseudomonas aeroginosa* |
| *A. millefolium* | + | + | + | + | + | + | - | + | + | 8±0.05 | 2±0.02 |
| *S. irio* | + | + | + | + | + | + | - | - | + | 10±.05 | 2±0.01 |
| *V. canescens* | - | + | + | + | + | + | - | - | + | 12.1±0.5 | 3±0.02 |

Results are given as mean of three replicates ± Standard error of mean

presence (Muhammad *et al*., 2012). The trend of antioxidant activity was:

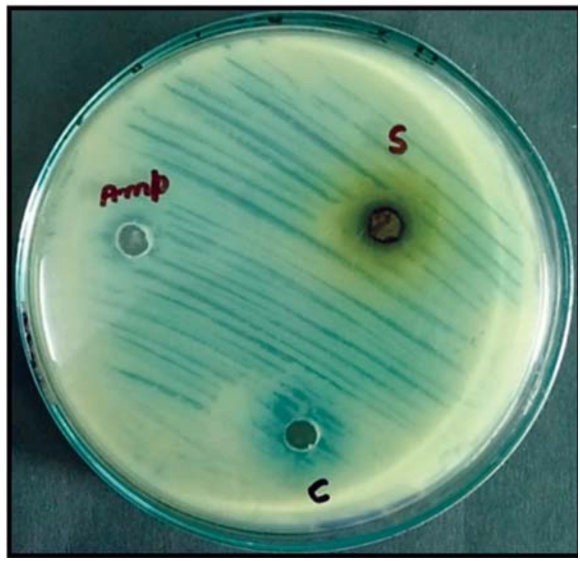


**Fig. 1.** Antibacterial Activity of *V.canescens* by agar well diffusion assay against (**a**) gram positive (**b**) gram negative bacteria.

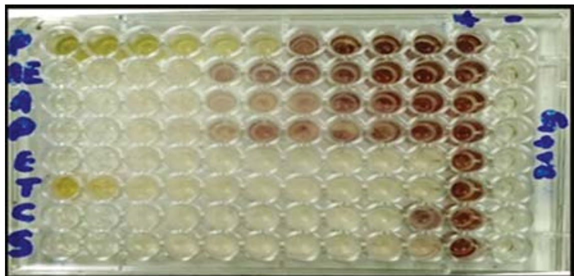

**Fig. 2.** Microtiter plate showing MIC of *V. canescens* against *Bacillus*. 1st row: *V.canescens* extract; 2nd row: Absolute ethanol; 3rd row: *Ampicillin*; 4th row: *Penicillin*; 5th row: *Erythromycin*; 6th row: *Tetracycline*; 7th row: *Chloramphenicol* and 8th row: *Streptomycin*. Pink colour is indicating the presence of bacterial growth i.e. bacterial strain could not be inhibited by this concentration of plant extract. Light pink colour is showing the decreased growth of bacterial strain while yellow and white colour are representing absence of bacterial growth due to inhibitory potential of plant extract.

***Viola canescens>Sisymbrium irio>Achillea millefolium.*** The total antioxidant capacity (TAC) of the ethanolic extracts of *A. millefolium*, *S. irio* and *V. canescens* was determined using phosphomolybdate assay. *V. canescens* antioxidant capacity was found to be maximum (526.57µg/mL) whereas antioxidant activity of *A. millefolium* and *S. irio* was calculated as 524.98 and 524.80µg/mL respectively. Ethanol was taken as control and its antioxidant capacity was 25.44 AAE µg/mL (Table 2). *V. canescens* showed maximum TAC, it means that it has more amount of phenols and flavonoids and they are associated with antioxidation capacity of extract. Reducing power assay showed that *Viola canescens* antioxidant activity was best amongst the selected plants. The extract with greater optical density (O.D.) at 700 nm has more antioxidant activity than the others (Table 2). Greater O.D. indicates greater reduction of

**Table 2.** Antioxidant activity analysis of selected medicinal plants

| Extracts | DPPHAssay | | Phosphomolybdate assay | | Reducing power assay |
|---|---|---|---|---|---|
| | O.D. at 517 nm | Antioxidant activity % | O.D. at 765 nm | Antioxidant capacityAAE µg/mL | O.D. at 700 nm |
| *Achillea millefolium* | 0.395±0.5 | 62% | 3.024±0.14 | 524.80 | 1.065±0.12 |
| *Sisymbrium irio* | 0.331±0.6 | 71% | 3.023±0.27 | 524.98 | 1.481±0.32 |
| *Viola canescens* | 0.502±0.8 | 86% | 3.033±0.55 | 526.57 | 1.878±0.45 |

Mean of three replicates ± standard error of mean

ferricyanide complex into ferrous and hence, more antioxidant potential (Ahmed *et al*., 2015).

For the determination of bioactive fractions, *V. canescens* extract's TLC was performed. The chromatogram showed 15 spots under short and long U.V light. When this plate was stained with iodine, it gave no brown spots which indicated the absence of iodine sensitive compounds. The antibacterial activity of the spots scratched from the TLC plate was performed by disc diffusion assay. Three out of the 15 spots showed zone of inhibition against *Bacillus* (Fig. 3). Maximum zone was exhibited by spot 1 which was chosen for Gas chromatography and Mass spectrometry analysis.

Identification of the biologically active components of the extract was done by GSMS analysis. 2H-1,4-benzodiazepin-2-one, 7-chloro-1,3- dihydro-5-phenyl-1-(trimethylsilyl)-oxy, cyclohexasiloxane, dodecamethyl and octasiloxane,1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl were detected in extract of *V. canescens* (Table 3). Octasiloxane,1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl is a volatile organic compound that is known to have antibacterial potential against *Salmonella typhi, Escherichia coli* and *Shigella dysentriae*. 2H-1,4-benzodiazepin-2-one, 7-chloro-1,3- dihydro-5-phenyl-1-(trimethylsilyl)-oxy is a derivative of oxazepam that originates from a class of benzodiazepines which is known to relieve insomnia and anxiety. It has been stated to possess antioxidant activity and also have antibacterial potential against *Staphylococcus aureus*, *Klebsiella pneumonia* and *Aspergillus niger* (Sherazi *et al*., 2016). Cyclohexasiloxane, dodecamethyl belongs to organosilicon family and is known to be active against *Proteus vulgaris*, *Klebsiella pneumoniae*, *Shigella flexneri*, *Pseudomonas aeruginosa* (Wadkar *et al*., 2017). In this research, these compounds showed antibacterial activity against *Bacillus.* These compounds have been stated to have anticancer, antioxidant, antimicrobial, cytotoxic and anti-inflammatory activities as shown in Table 3 (Atolani and Olatunji, 2016; Sherazi *et al*., 2016; Mohansrinivasan *et al*., 2015).

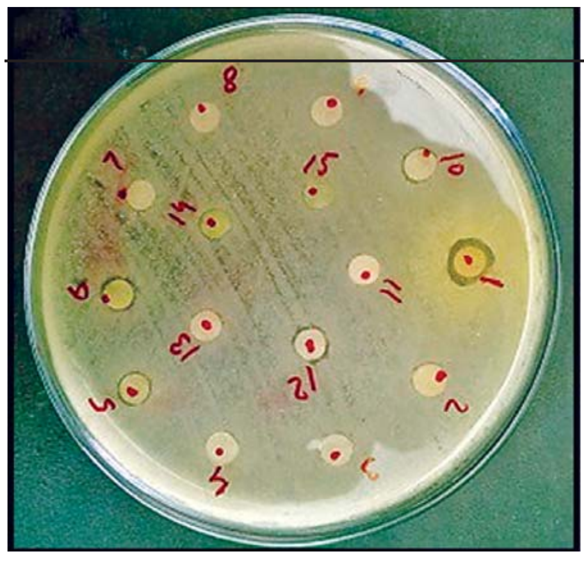

**Fig. 3.** Antibacterial activity shown by TLC spots.

## Conclusion

Findings of the current study revealed that, due to considerable antioxidant and antibacterial activity of *V. canescens,* it can be a good substitute for the synthetic drugs. Future research should be focused on the analysis of *V. canescens* for its anticancer potential and synthesis of new plant based medicinal drugs.

**Table 3.** Bioactive compounds obtained from GC-MS analysis of partially purified *V. canescens* extract

| Compound name | Molecular formula | Molecular weight | Retention time | Structure | Reported activity |
|---|---|---|---|---|---|
| 2H-1,4-Benzodiazepin-2-one, 7-chloro-1,3- dihydro-5-phenyl-1-(trimethylsilyl)-oxy | $C_{18}H_{19}ClN_2OSi$ | 430 | 25.842 | | Antimicrobial and Antioxidant activity (Sherazi, Jabeen *et al*., 2016) |
| Cyclohexasiloxan, dodecamethyl | $C_{12}H_{36}O_6Si_6$ | 444 | 29.708 | | Antibacterial and Antioxidant activity (Gupta and Kumar, 2017) |
| Octasiloxane,1,1,3,3,5, 5,7,7,9,9,11,11,13,13, 15,15-hexadecamethyl | $C_{16}H_{50}O_7Si_8$ | 578 | 31.708 | | Antimicrobial Activity (Atolani and Olatunji, 2016) |

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**Conflict of Interest.** The authors declare they have no conflict of interest.

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