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Antimicrobial potential of *Viola canescens* Wall

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Key words: *Viola canescens, Escherichia coli, Staphylococcus aureus, Clavicep purpurea, Penicillium italicum.*

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Abstract

Viola canescens Wall. is an important ethnomedicinal plant, widely used in traditional system of medicines for treating various ailments. The present study was therefore conducted to evaluate the antibacterial and antifungal activity of this ethnomedicinally important plant.The antibacterial and antifungal potentials of *V. canescens* was investigated through methanolic extract of the whole plant (CME) and its various organic solvent fractions i.e. ethyl acetate fraction (EAF), Chloroform fraction (ChF), uncharacterized crystalline compound obtained from ethyl acetate fraction (UCC), n-hexane fraction (NHF), total phenol content (CTP) and total flavonoid content (CTF) using various plant and human/animal pathogens. The antibacterial potential of the using agar well diffusion assay while the antifungal activity was determined using disc diffusion assay.In antibacterial assay except the NHF, CTP, and CTF all the other extract/fractions exhibited significant activities against most of the test bacteria. EAF was the most effective fraction in comparison with the other. The highest activity of EAF was recorded against *X. campestris*, followed by *E. coli*, *P. vulgaris, P. auriginosa, A. tumefecian, S. aureus, B. subtilis*and *S. pneumoniae* with LD_{50} values equal to 6.28, 9.07, 13,26, 15.64, 16.28, 16.89, 20.57 and 20.99 respectively. In antifungal assay, all the extract/fractions exhibited significant activity against most of the tested fungal strains.In antibacterial assay, the CME was found more effective against the test fungi. CME showed maximum activity against *A. niger* (LD_{50} = 7.87). Least activity of CME was observed against *C. purpurea* (LD_{50} = 58.77) and *R. stolonifera* (LD_{50} = 154.83).

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Introduction

Harmful bacteria are the major cause of several diseases and lethality of humans from the start of human civilization. A number of antibiotics have been produced by various pharmaceutical companies to combat the organisms time to time. In the recent years, the major problems that pharmaceutical companies have is the development of resistance of bacterial strains towards the conventional antibiotics. The development of drug resistance has significantly affected the success rate of treatments using various drugs. This situation demand the search for novel potent substances and the most important source for such compounds are the plants that contain various biologically active compounds (Djeussi *et al.*, 2013). The infectious diseases are the major health issues worldwide and accounts for about 43 percent of health problems. The main reason for this the failure of synthetic drugs used to treat these diseases. The pharmaceutical companies are continuously introducing new synthetic drugs which produce various severe side effects. The effective way to deal the situation to obtain natural potent substances which may be more effective and safe as compared to the synthetic drugs (Al-Mariri and Safi, 2014; Ishaq *et al.*, 2014).

Fungus attack is one of the major cause in decreasing agricultural production. Many genera of fungi like *Aspergillus, Fusarium* cause spoilage of many fruits and other food products. The spoilage is due the production of various toxins secreted by fungi. Fungi also attack economically important crops like corn, cotton, wheat, barley and even trees. The fungal attack affects both the quality and quantity of different crops. The toxins produced by fungi not only affect the plants but are also toxic to animals. To control the adverse effect of fungi different synthetic fungicides are used. Normally the synthetic fungicides are not environment friendly and produce severe effects, and many of these are toxic to humans. Best alternative to the synthetic fungicides is biological control by obtaining antifungal substances of natural origin. Substances of natural source are safe, environment friendly. The other advantage of these substances is their efficacy because many of the fungi has developed drug resistance against the conventional synthetic drugs (Mahlo *et al.*, 2010).

Viola canescens is an important medicinal plant, used in traditional system of medicine in different regions of the Pakistan, especially in rural areas. In Malakand tribal region the plant is locally called Banafsha and is used to treat cancer, as a purgative and antipyretic agents and to increase perspiration (Murad *et al.*, 2013). In other parts of Khyber Pakhtunkhwa, Pakistan, the plant is used to treat cough, cold and treating liver problems (Haq and Ullah, 2011). The present study was conducted to evaluate the antibacterial and antifungal potential of the plant.

Materials and methods

Antibacterial assay

Antibacterial potential of *V. canescens* Was evaluated through the whole plant crude methanolic extract (CME) and its subsequent organic solvent fractions i.e. ethyl acetate fraction (EAF), Chloroform fraction (ChF), uncharacterized crystalline compound obtained from ethyl acetate fraction (UCC), n-hexane fraction (NHF), total phenol content (CTP) and total flavonoid content (CTF) using agar well diffusion method following Carron *et al.* (1987) with minor modification, against both Gram positive and Gram negative bacteria. The tested bacterial strains include both plant and animal/human pathogenic bacteria i.e. *Escherichia coli, Bacillus subtillis, Pseudomonas auriginosa, Staphylococcus aureus, Proteus vulgaris, Streptococcus pneumonia, Xanthomonas campestris* and *Agrobacterium tumefaciens.*

Different concentrations (20, 40,60,80,100 µg/ml) of each extract/fraction were prepared by dissolving the extract/fractions in DMSO. Agar plates were prepared by pouring about 30 ml of sterilized molten agar in petri plates. The plates were kept for some time in the laminar flow hood to became hard. When the surface of the agar plates was hardened enough, about 50 µl of fresh bacterial cultures were spread uniformly on the surface of agar plates. For each culture, the Petri plates were taken in replicates. Wells of about 6mm

diameter equally spaced were made in the plates, with the help of sterilized metallic borer. Finally, the 100 µl of each concentration were poured in the wells with the help of micropipettes. The plates were then transferred from laminar flow to the incubator and kept for 72 hours at 37°C. After three days, zones of inhibitions were calculated for each concentration. In the assay DMSO and standard antibiotic drugs were used as negative and positive controls respectively. Percent inhibition for each concentration of extract/fraction, against each bacterial strain was calculated with reference to positive control. Based on the inhibitions of different concentrations of extract/fractions LD_{50} values were calculated for each extract/fraction against tested bacterial strains by Probit analysis using SPSS (Version 20).

Antifungal potential of *V. canescens* was evaluated using methanolic extract and various organic solvent fractions, crude phenols and crude flavonoids of the plant using disc diffusion method following Atta Ur Rehman*et al.* (2001) with modification. Sterilized molten sabouraud dextrose agar were poured in to petri plates. Petri plates were left for some time in the laminar flow hood, until the agar surface became hard enough.

Fresh cultures of test fungal strains (*Clavicep purpurea, Aspergillus niger, Rhizopus stolonifera, Penicillium italicum, Candida albicans* and *Alternaria solani*) were used in the experiment. 100 ml of inoculum of each fungal strain were uniformly spread on the surface of separately on the agar plates. Different dilutions (20,40,60,80,100µg) of crude methanolic extract and other fractions were loaded on sterile paper disc of about 8mm diameter. The dried loaded discs were then placed on the surface of plates. The petri plates were then transferred to incubator and incubated at 30°C for 72 hours. Disc loaded with DMSO and standard drugs were used as negative and positive control respectively. After 72 hours of incubation zone of inhibitions around the discs were measured. Percent inhibition was calculated for each concentration of various extract and fractions used. On the basis of inhibitions caused by various concentrations, LD_{50} values were calculated for crude methanolic extract and other fractions, by Probit analysis using SPSS (Version 20).

Results

Antibacterial activity

Except the NHF, CTP, and CTF all the other extract/fractions exhibited significant activities against most of the test bacteria.EAF was found to the most effective fraction used in the assay. The highest activity of EAF was recorded against *X. campestris*, followed by *E. coli*, *P. vulgaris, P. auriginosa, A. tumefecian, S. aureus, B. subtilis* and *S. pneumoniae* with LD_{50} values equal to 6.28, 9.07, 13,26, 15.64, 16.28, 16.89, 20.57 and 20.99 respectively.CME, AqF, ChF, and UCC showed moderate activities against the tested fungal strains. The least activities was exhibited by CTP and CTF against most of the tested bacterial strains (Table 1).

Table 1. Antibacterial potential of *V. canecens*.

Treatments	*E. coli*	*B. subtillis*	*P. auriginosa*	*S. aureus*	*P. vulgaris*	*S. pneumonia*	*X. campestris*	*A. tumefaciens*
CME	20.03	34.74	59.81	59.81	34.80	37.56	9.54	19.60
EAF	09.07	20.57	15.64	16.98	13.26	20.99	6.28	16.28
AqF	242.36	231.47	562.49	1323.42	41.63	667.56	321.18	203.60
NHF	1422.31	400.52	371.87	1311.03	255.44	115.60	55.85	2021.88
ChF	14.90	05.84	22.14	13.11	09.01	16.23	05.07	53.33
UCC	26.91	22.81	50.57	73.50	50.60	44.64	27.98	55.72
CTP	949.35	364.45	306.92	6344.96	459.98	44.64	27.98	55.72
CTF	310.23	451.37	482.92	604.92	295.51	1324.36	303.69	1070.16

Each value indicates the LD_{50} calculated from percent inhibitions recorded at 20, 40, 60, 80 and 100 µg/ml.

The activities of the extract/fractions were positively correlated with the concentration of the dose i.e. showed maximum activities at highest concentration (100µg/ml). Against the *E. coli* maxim inhibition at highest concentration was exhibited by EAF (90.63%), while least activity was exhibited by NHF (28.75%), and CTP (26.97%). (Fig.1). Against *B. subtilis* the maximum inhibition at highest concentration was recorded for ChF (91.23%), while least was recorded for NHF (34.10%) and CTF (32.57%) (Fig. 2).

Against *P. auriginosa* the maximum inhibition was caused by EAF (96.68%), followed by ChF (86.89%). NHF was found least effective against the *P. auriginosa* (Fig. 3). Against *S. aureus*and *P. vulgaris* the maximum activities were recorded for EAF (85.93%)and (84.44%) respectively, while least activities were recorded for CTP (19.25%) and (36.93%) respectively. EAF also exhibited maximum activity (91.82%) and (79.55%) against *X. campestris* and *A. tumefaciens* respectively (Fig. 4,5,6,8).

Table 2. Antifungal potential of *V. canecens*.

Treatments	*C. purpurea*	*A. niger*	*R. stolonifera*	*P. italicum*	*C. albicans*	*A. solani*
CME	58.77	07.87	154.83	36.93	17.50	12.16
EAF	73.27	65.45	171.42	21.44	17.89	15.13
AqF	233.32	62.29	13.91	36.93	35.96	68.47
NHF	564.85	140.13	125.84	82.66	03.31	11.05
ChF	68.34	04.02	12.36	24.06	15.77	41.78
UCC	356.24	21.40	75.49	75.71	50.41	73.75
CTP	235.76	49.00	95.64	119.82	49.54	78.93
CTF	383.12	59.91	60.50	96.70	73.56	40.64

Each value indicates the LD_{50} calculated from percent inhibitions recorded at 20, 40, 60, 80 and 100 µg/ml.

Against *S. pneumoniae* the maximum activity at highest concentration was recorded for ChF (82.07%) and EAF (81.97%), while leas was recorded for CTF (28.38%) (Fig. 7).

Antifungal activity

All the extract/fractions exhibited significant activity against most of the test fungal strains. Though the effectiveness of different treatments varied against different strains used, but in general EAF, ChF and CME were found more effective against the test fungi. CME showed maximum activity against *A. niger* (LD_{50} = 7.87) while found leas effective against *R. stolonifera* (LD_{50} = 154.83). EAF exhibited maximum activity against *A. solani* (LD_{50} = 15.13). Least activity of EAF was recorded against *R. stolonifer* (LD_{50} = 171.42). AqF was most effective against *R. stolonifera* (LD_{50} = 13.91).

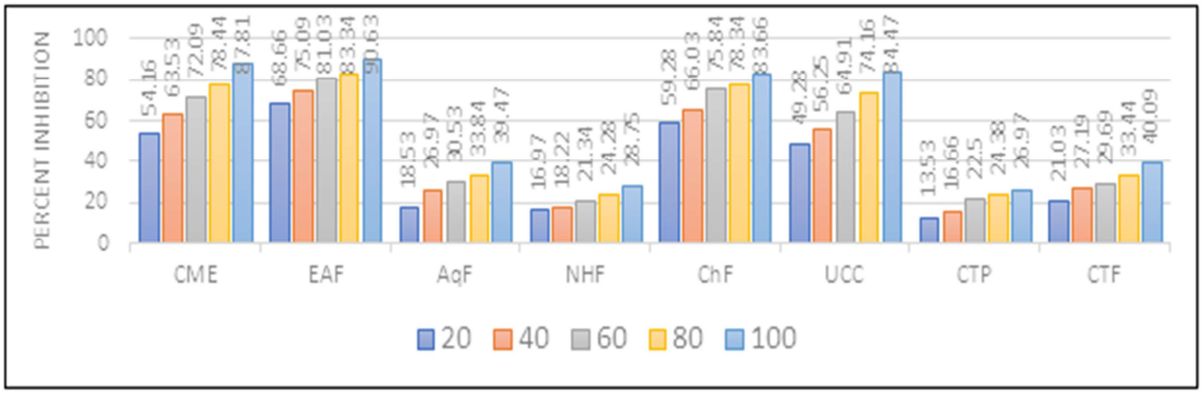

Fig. 1. Antibacterial activity of *V. canescens* against *Escherichia coli.*

The fraction was found least effective against *A. Solani, A. niger* and *C. purpurea* with LD50 equal to 68.47, 62.29 and 233.32 respectively. NHF showed maximum potential against *C. albicans* (LD_{50} = 3.31). Maximum activity of ChF was recorded against *A. niger* (LD_{50} = 4.02), *R. stolonifera* (LD_{50} = 12.36) and *C. albicans* (LD_{50} = 15.77). For UCC maximum activity was recorded against *A. niger* (LD_{50} = 21.40). CTP exhibited maximum activity against *A. niger* (LD_{50} = 49.00) and *C. albicans* (LD_{50} = 49.54) respectively. CTF showed maximum activity against *A. solani* (LD_{50} = 40.64) (Table 2).

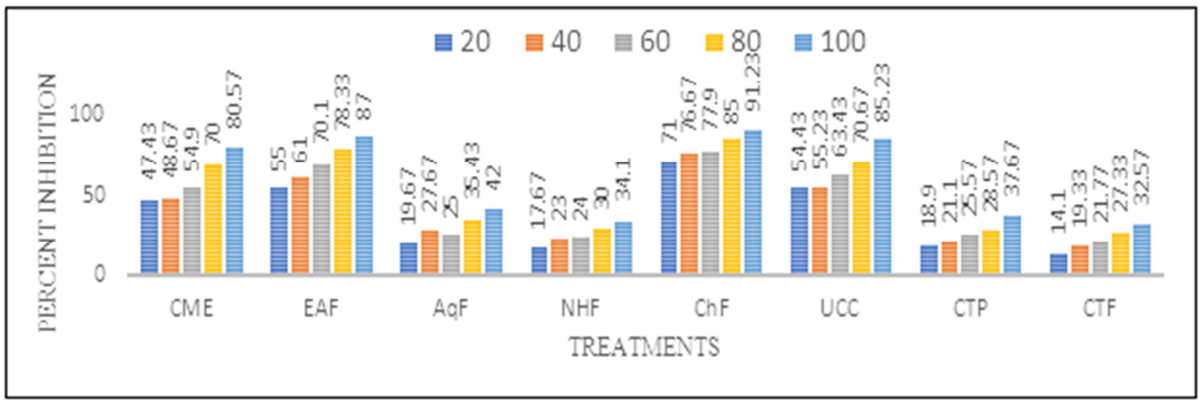

Fig. 2. Antibacterial activity of *V.canescens* against *Bacillus subtillis*.

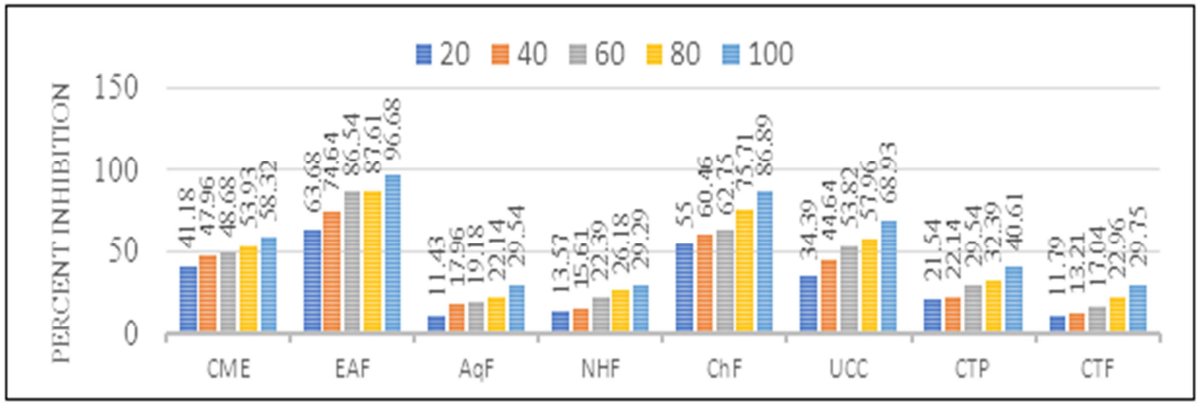

Fig. 3. Antibacterial activity of *V. canescens* against *Pseudomonas auriginosa*.

The results revealed that *C. abicans* and A. niger were comparatively more susceptible to most of the treatments, while *C. purpurea* was least affected by the various treatments.

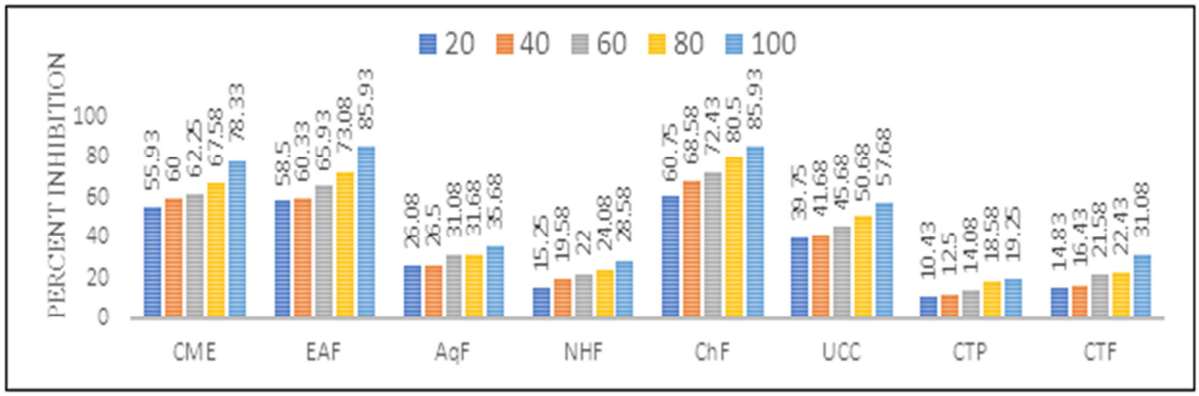

Fig. 4. Antibacterial activity of *V. canescens* against *Staphylococcus aureus*.

Furthermore, the effectiveness of the treatments was strongly positively correlated with their concentrations i.e. maximum activity for all extract/fractions was recorded at highest concentration (100µg/ml) In case of *C. purpurea* the maximum inhibition at higher concentration (100µg/ml) was recorded for EAF (58.71%) while least affected by NHF (37.10%) (Fig. 9).

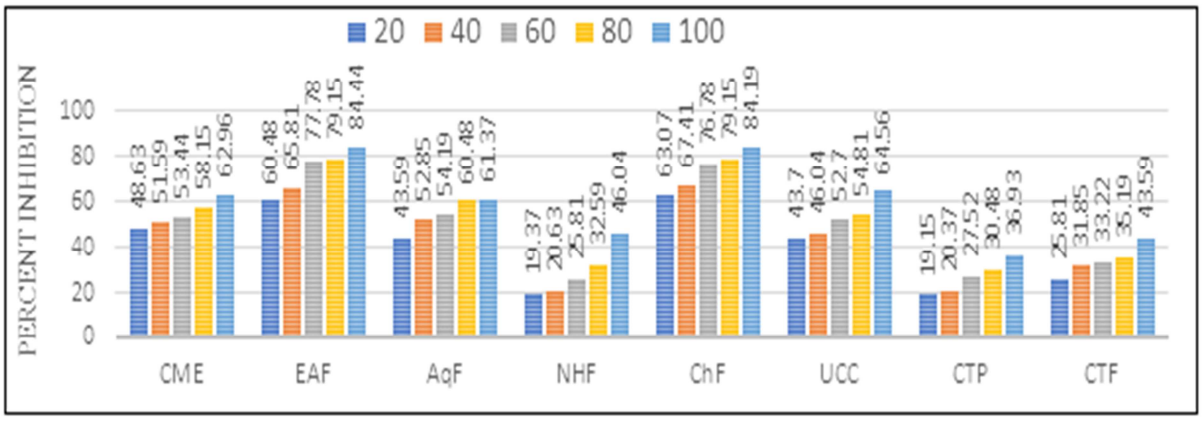

Fig. 5. Antibacterial activity of *V. canescens* against *Proteus vulgaris.*

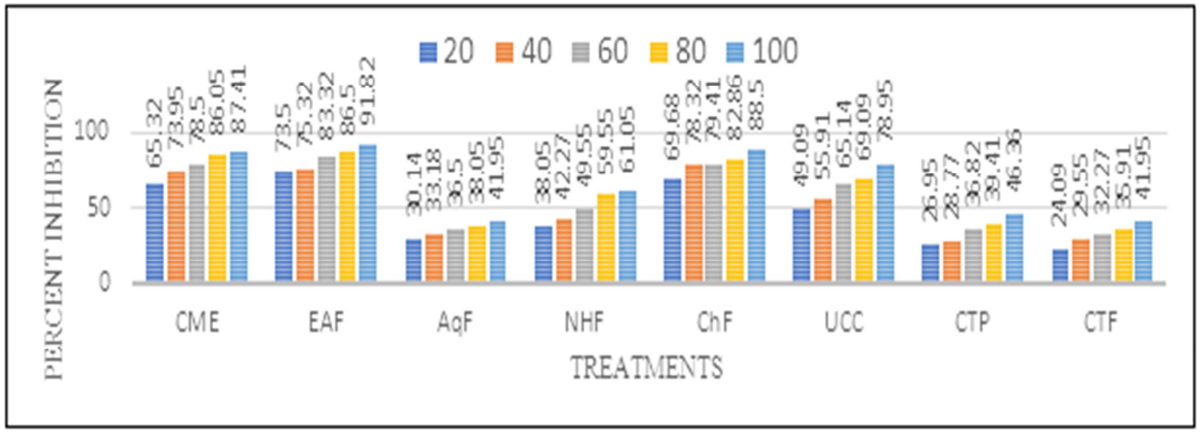

Fig. 6. Antibacterial activity of *V. canescens* against *Xanthomonas campestris.*

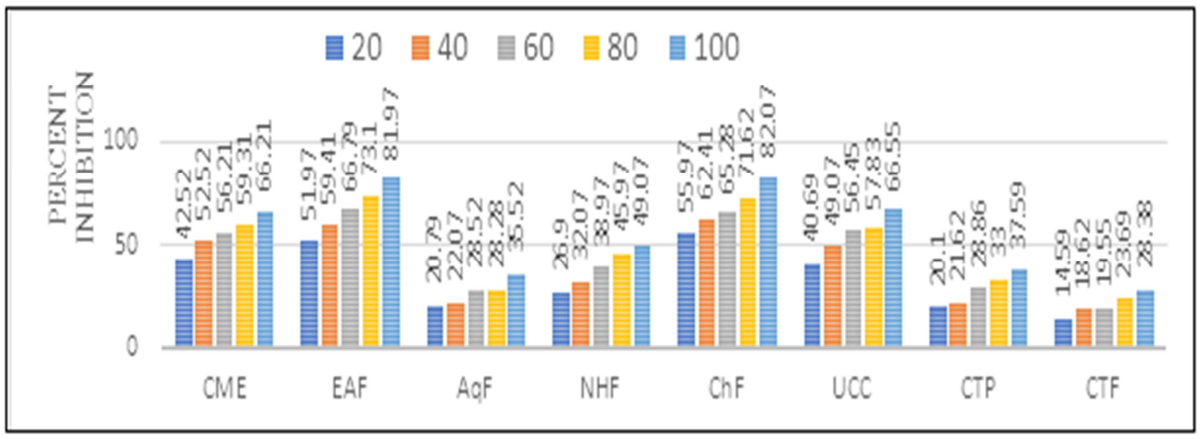

Fig.7. Antibacterial activity of *V. canescens* against *Streptococcus pneumoniae.*

In case of *A. niger* maximum inhibition at highest concentration was recorded for UCC (92.68%) while least was recorded for NHF (50.68%) (Fig. 10) For *R. stolonifer* the maximum inhibition at highest concentration was recorded for AqF (91.29) and least for CME (49.19%) (Fig.11). Against *P. italicum*

maximum inhibition was observed for ChF (78.46%) while leas was recorded for CTP (52.79%). Least inhibition against *P. italicum* (51.67%) was observed for CTF (Fig. 12). In case of *C. albicans* the maximum inhibition at highest concentration was caused by NHF (87.68%), while lowest (64.41%) was recorded for CTF (Fig. 13). Against *A. solani* the maximum inhibition at highest concentration was recorded for CME (87.70%) while the lowest was recorded for CTP (Fig. 14).

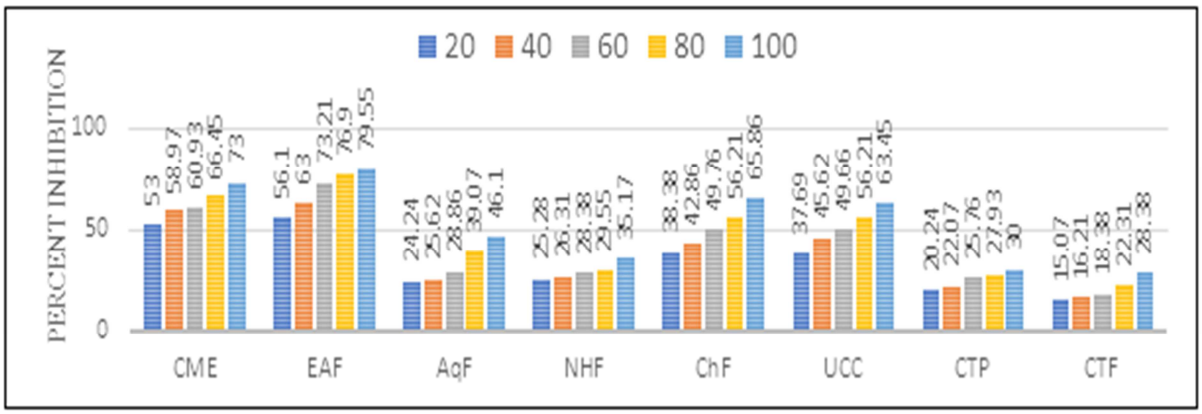

Fig. 8. Antibacterial activity of *V. canescens* against *Agrobacterium tumefaciens*.

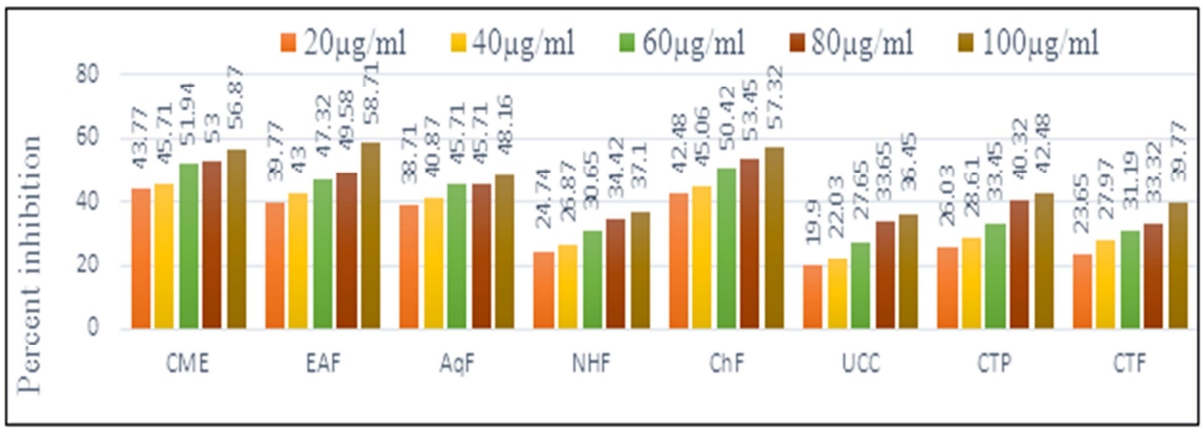

Fig. 9. Antifungal activity of *V. canescens* against *C. purpurea*.

Discussion

The Genus *viola* has significant antimicrobial potential and other species of the genus have also been reported to possess significant antimicrobial potentials.

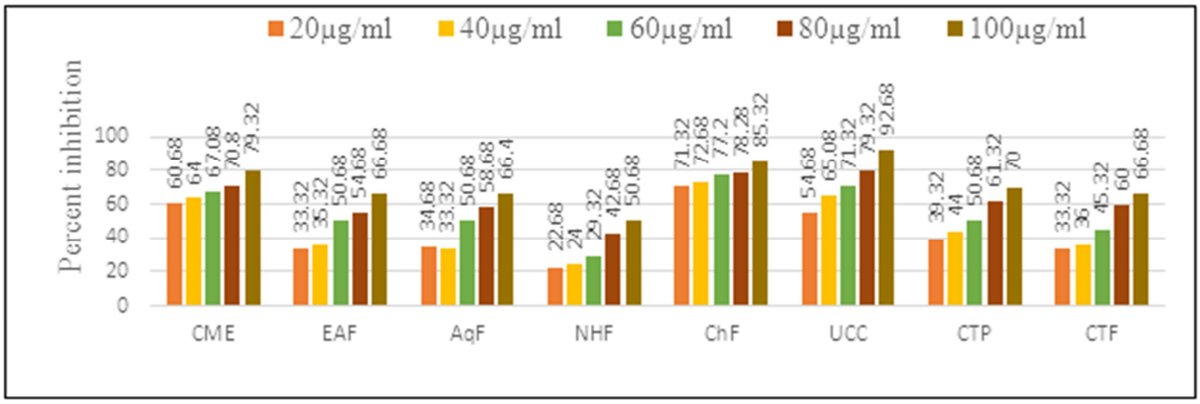

Fig.10. Antifungal activity of *V. canescens* against *A. niger*.

The results of the present study revealed that EAF has the highest antibacterial potential followed by ChE, UCC and CME, while NHF, CTP, CTF and AqF were found comparatively least effective against most of the bacterial strains. Similarly, in case of antifungal activity all the extract/fractions exhibited significant activity against most of the test fungal strains. Though the effectiveness of different treatments varied against different strains used, but in general EAF, ChF and CME were found more effective against the test fungi.

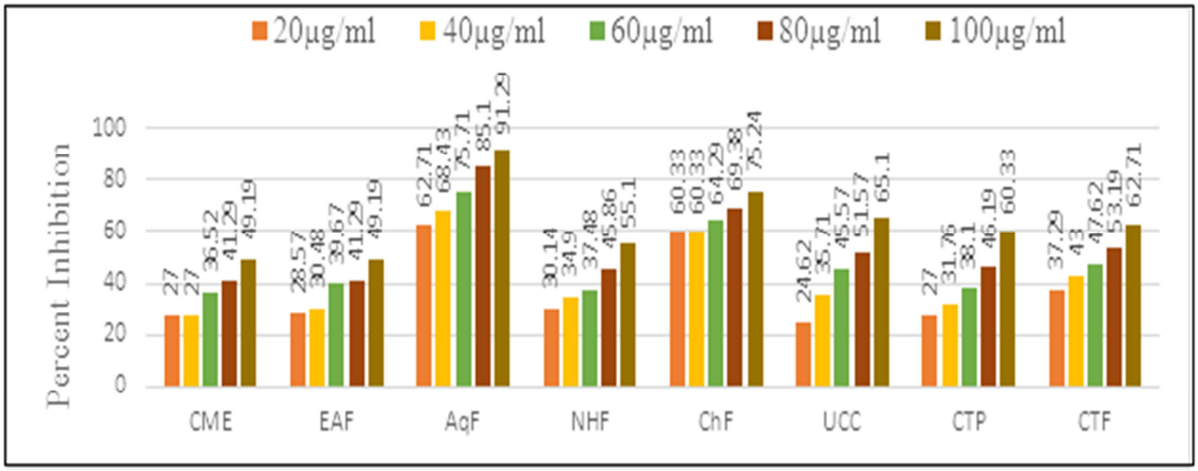

Fig. 11. Antifungal activity of *V. canescens* against *R. stolonifer*.

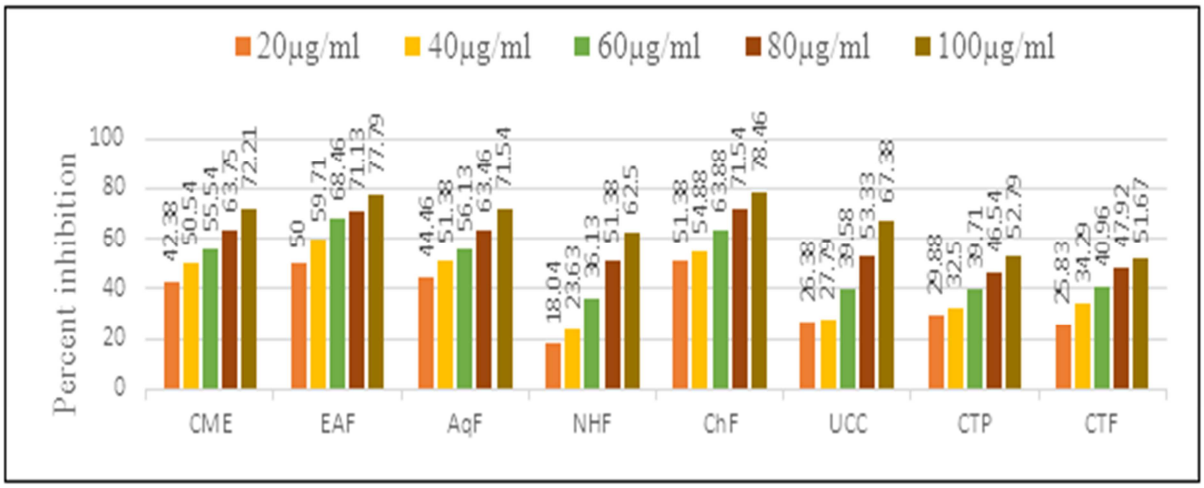

Fig. 12. Antifungal activity of *V. canescens* against *P. italicum*.

The present results are in agreement with in some aspects with other works conducted before on the other species of *Viola* (genus).

The methanolic extract and its various organic solvent fractions of *V. betonicifolia* tested by Muhammad *et al.* (2013) also exhibited significant dose dependent activity against human pathogens including *E. coli*, *Staphylococcus aureus* and *Aspergilis niger*, similarly the decoction and Alcoholic extract of *V. tricolor* exhibited significant dose dependent activities against tested microorganisms (Witkowska-Banaszczak, 2003). *V. odorata* also exhibited significant activities against human pathogenic bacteria including those causing respiratory tract infections (Arora and Kaur, 2007; Gautam *et al.* 2012).

The genus Viola contain alkaloids, saponins, flavonoids, tannins (Zhu *et al.,* 2016) and variety of cyclotides (Zarrabi *et al.,* 2013) which alone are their synergetic effect may be responsible for the exhibited antibacterial and antifungal potentials of the genus.

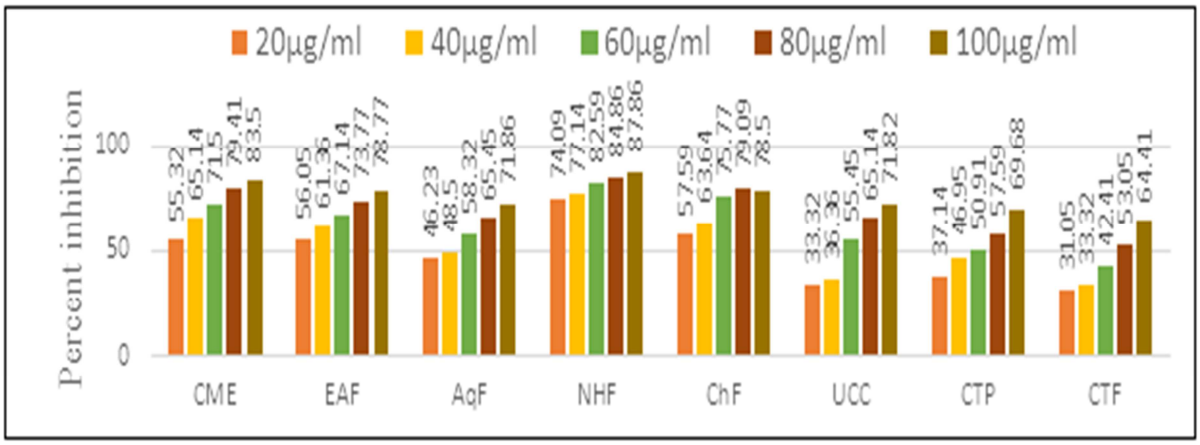

Fig.13. Antifungal activity of *V. canescens* against *C. albicans*.

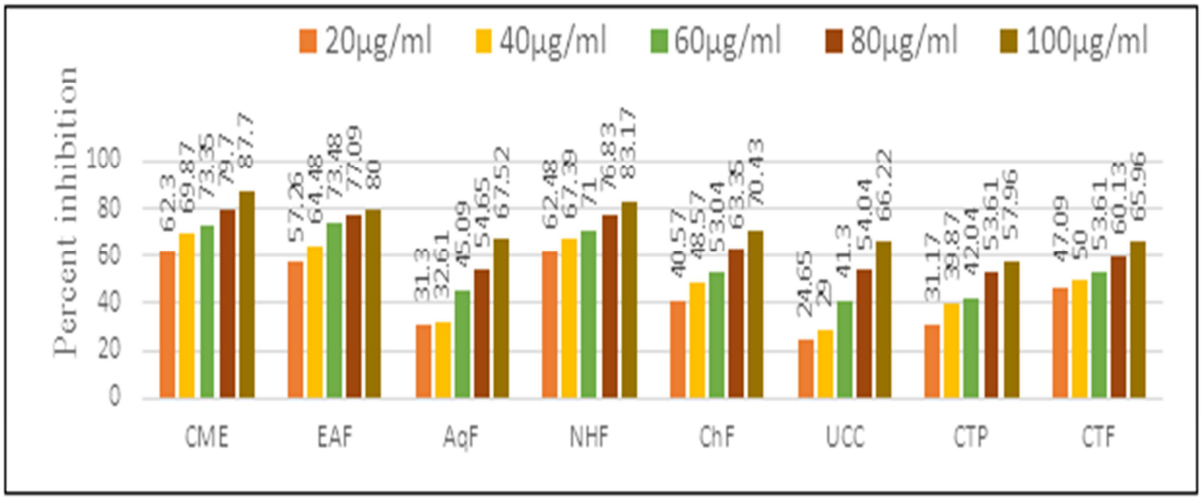

Fig.14. Antifungal activity of *V. canescens* against *A. solani*.

Conclusion

The results confirmed that *V. canecens* possesses significant antibacterial and antifungal potential and thus can be a prominent source of novel antibiotics and fungicides. The results also confirmed the traditional use of this valuable plant for the treatments of various microbial infections. The study further suggest that further work is required to isolate the active constituents of the plant, especially the fractions having greater potentials.

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