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EVALUATION OF THE BIOACTIVE ROLE OF INDIGENOUS *VIOLA ODORATA* LEAF EXTRACTS

TAHREEM SIKANDER, AQSA ANUM BHATTI, ZAHID MUSHTAQ*, MUHAMMAD SHAHID, FARZANA NIGHAT AND SIDRA ANWAR

Bioactive Molecules Research Lab (BMRL), Dept of Biochemistry, University of Agriculture, Faisalabad. 38040, Pakistan
**Corresponding author's email: zahidmushtaquaf@uaf.edu.pk*

Abstract

Medicinal plants are rich source of bioactive compounds and other secondary metabolites and are used to prepare drugs for preventing infectious diseases because natural sources like plants have least or no side effects and less expensive as compared to other synthetic drugs. In this research bioactive extracts were prepared and fractionated from *Viola odorata* leaves using organic solvents (in increasing polarity order) like methanol (CME) for first crude extract and fractionated it further in *n*-hexane (*n*-HF), chloroform (CF), ethyl acetate (EAF) and water. The residue left from CME was solubilized in methanol (MSF) and water (WSF). Total phenolic (TPC) and Total flavonoid compounds (TFC) were also estimated. The bioactive potential of extracts/fractions was checked by using different assays for evaluating antimicrobial, anti-oxidant, thrombolytic, cytotoxic, α-amylase inhibition & lipase inhibition potential. The maximum antibacterial (ZOI =14 mm) and antifungal activity (30.33 mm) was shown by MSF (methanol soluble fraction) against *E. coli* and *n*-HF against *Fusarium solani* respectively. Maximum TPC was shown by MSF 3.4 mg/g and maximum TFC was shown in *n*-HF 0.21 mg/g. MSF also exhibited maximum α-amylase inhibition % age 42.163±2.74 and CME exhibited maximum lipase inhibition (95±0.89). EAF showed maximum antioxidant activity that is 62.13±4.26 by using DPPH assay. CF showed maximum cytotoxicity (76.31±0.252). Maximum clot lysis was shown by MSF 26.44±0.485. Moreover HPLC results showed high amount of phenolics in CME and *n*-HF that could be related to their bioactivities. Hence *V. odorata* proved an excellent source of bioactive compounds that can be analyzed individually in future.

Key words: *Viola odorata*, Antimicrobial activity, Cytotoxicity, Enzyme inhibition, Extracts/fractions.

Introduction

New evolving contagious diseases have been appeared as the cause of major deaths in animals worldwide (Daszak *et al.*, 2000). Hepatitis, AIDS, cancer, diarrhea, tuberculosis and some other contagious diseases are intimidations for a healthy life (Ferber, 2010). These diseases have to be controlled by using some alternate sources of treatment against these pathogens (Delahaye *et al*., 2009). Cardiovascular diseases are non-contagious and caused by intravascular thrombosis; main cause of deaths (Holden, 1990). Antibiotics consumption has controlled the bacterial diseases to an extent but the other antimicrobials have to be promoted because the unnecessary use of these antibiotics has developed the resistance in the microorganisms (Arora & Kaur, 2007). This alternative source could be the use of bioactive compounds in drug development. Bioactive compounds are known as important therapeutic compounds as they possess a variety of bioactivities including anti-inflammatory, antibiotic, antioxidant, antifungal, thrombolytic and anticoagulant (Long *et al.,* 2016).

Most of the diseases are due to oxidation in the body (Ahmad *et al*., 2000). ROS produced at high levels due to environmental stress, this higher oxidative stress can damage proteins and DNA (Devasagayam & Kesavan, 2004). Antioxidants containing flavonoids, phenolic acids and polyphenols can give an effective outcome by eliminating free radicals, inhibiting the formation of reactive oxygen species (ROS) or triggering the detoxifying protein (Halliwell *et al.,* 1999).

Many beneficial bioactivities are described by natural products like anticancer, antimicrobial, antioxidant, antidiarrheal, analgesic and wound healing activities. People accepted the advantages of natural products in the past as well as present. Primarily plants and other organisms like algae, fungi and bacteria are also rich in bioactive compounds (Debbab *et al.*, 2010). Violaceae family is well known in the field of pharmaceuticals. *Viola odorata* used in current project belongs to this family and was first considered to be a medicinal plant and was used for anti-tumorous role by Lindholm *et al*., (2002). The history of *V. odorata* described its used as a medicinal herb to relief pain (Kapoor, 1990). In past, it has been used as remedy for cough, fever, anxiety, lower blood pressure, bronchitis, rheumatism, sneezing, kidney and liver disorders (Qadir *et al*., 2014). *V. odorata* is also effectively good in antipyretic, antihypertensive and antidyslipidemic activity. Violaceae family especially *Viola odorata* is successfully used to treat the respiratory diseases and as anti-inflammatory agent (Ebrahemzadeh *et al.*, 2010 & Elhassaneen *et al*., 2013). Thus, drugs derived from natural sources like plants play a significant role in the prevention and treatment of human diseases (Bahlodia & Shukkla, 2011).

This project was designed to evaluate the bioactive potential of the *V. odorata* leaf's methonolic crude extract (CME) and its fractions which were obtained by using organic solvents in increasing polarity manner. Different bioactivities like antimicrobial, thrombolytic, antioxidant, enzyme inhibition, TPC/TFC and cell cytotoxicity were performed to evaluate the said potential of indigenous plant.

Materials and Methods

Collection of sample: *Viola odorata* was obtained from the botanical garden established by the Department of Botany in University of Agriculture, Faisalabad.

Extraction and fractionation through different polar and non-polar solvents: Fresh plant leaves were separated and washed with sterile distilled water, dried at room temperature and ground to powder. Plant crude extract was obtained in methanol (CME) after 2 days of maceration and then this crude extract was fractionated using a series of immiscible solvents with increasing polarity to attain the fractions of *n*-hexane (*n*-HF), chloroform (CF) and ethyl acetate (EAF). Methanol soluble fraction (MSF) and water soluble fraction (WSF) were also obtained from the remaining residue of CME. Each extract and fraction was evaporated, weighed and dissolved in DMSO and was further used to perform bioassays (Muhit *et al*., 2010).

Phytochemical screening

Total phenolic content (TPC): Content of total phenolics in polar and nonpolar extract or fractions of *Viola odorata* leaves was determined by Folin Ciocalteau's reagent (FCR) method (Vivek *et al*., 2013). Standard curve was plotted by using different concentration of gallic acid (standard, 50-250 µg/mL) and TPC of extract was expressed as µg gallic acid equivalents from the graph.

Total phenolic content = µg of Gallic acid Equivalents per gram dried extract

Following formula was used to calculate Total Phenol:

Concentration (µg/g) = Absorbance in nm – (value of y- intercept) value of slope of standard curve

Total flavonoid content (TFC): For the determination of total flavonoids Aluminum chloride based colorimetric method was used (Vivek *et al*., 2013). Standard curve was plotted by using different concentrations of quercetin (as standard, 50-250 µg/mL) and TFC of extract was expressed as µg quercetin equivalents from the graph.

Total flavonoid content = µg of Quercetin Equivalents per gram dried extract

Following extract was used to calculate Total Flavonoid:

Concentration (µg) = Absorbance in nm – (value of y-intersept) Value of slope of standard curve.

DPPH free radical-scavenging activity: The DPPH assay was followed as described by Roopa *et al*., 2015 according to which 5 µL of leaf fraction/extract was added in 95 µL of methanolic solution of DPPH. After 30 minutes incubation in darkness at room temperature the absorbance was noted at 517 nm. Ascorbic acid was used as standard control. Inhibition of free radical by DPPH was calculated in the following way:

$$I\ (\%) = \{(\text{Abs. blank} - \text{Abs. sample}) / \text{Abs. blank}\} \times 100$$

where, **I (%)** = percentage inhibition of DPPH free radical, Abs. blank = absorbance of the control reaction mixture excluding the test compounds and Abs. sample= absorbance of the test compounds.

Antimicrobial activity: Antimicrobial activity of *Viola odorata* leave extract and fractions was determined by using different bacterial strains like *Bacillus subtilis, Staphylococcus aureus, E. coli, Pasteurella multocida* and fungal strains like *Fusarium solani* and *Aspergillus niger.* The activity was determined by well diffusion method according to Candan *et al.*, 2003. Nutrient agar for antibacterial and potato dextrose agar (PDA) for antifungal activities were dissolved in distilled water and adjusted the respective pH of each media with 0.1 N HCl/NaOH. The medium was sterilized by autoclaving at 121ºC for 30 minutes. 100 µL of inoculum was added from liquid media to each plate and mixed well with the agar media. In the solidified agar, the wells of 4 mm were cut with the help of sterilized borer. 50-70 µL of sample was poured into the wells. Ciprofloxacin and fluconazole were used as positive controls for antibacterial and antifungal activities respectively (Nighat & Zahid, 2019). The Petri plates were incubated at 37ºC or at each strain's specific temperature for 24 hours (Zaidan *et al*., 2005; Tepea *et al*., 2004).

***α-amylase* inhibitory assay:** α-amylase inhibitory assay was determined by using method of Kazeem *et al.,* 2013. 250 µL of extract was added in a tube and then 250 µL of 0.02 M sodium phosphate buffer with pH 6.9 and α-amylase solution (0.5 mg/mL) was added in each test tube. The solution was incubated for 10 min at 25ºC, after this incubation 250 µL of 1% starch solution as substrate (prepared in 0.02 M sodium phosphate buffer with pH 6.9) was added in test tubes and then further incubated for 10 min at 37ºC. By adding 500 µL of dinitrosalicylic acid (DNS) reagent the reaction was finally terminated. At the end the whole reaction, each mixture was diluted with 5 mL distilled water and then the absorbance was measured at 540 nm by using a spectrophotometer. The control consisted of DMSO i.e. without any sample. The α-amylase inhibitory activity was calculated as percentage inhibition:

$$\%\ \text{Inhibition} = [(\text{Absorbance of Control} - \text{Absorbance of Extracts}) / \text{Absorbance of Control}] \times 100$$

Lipase inhibition assay: To check the porcine pancreatic lipase (PPL) inhibition by the leaves extracts of *Viola odorata* chromogenic olive oil plate assay was performed as described by Patil *et al*., 2017. The assay is based on change in pH from neutral to acidic after lipase activity which changes color of the medium. Plates were prepared by using the medium which is composed of 2% agar (w/v) along with 2.5% (v/v) olive oil and 1% (w/v) phenol red as an indicator. Lipase was prepared in 100 mM Tris-HCl buffer (30 mg/mL). After this 60 µL of the master mix was prepared using 30 µL of porcine PL and 30 µL of extract (20 mg/mL) and poured into wells. The control well contained 30 µL of porcine PL and buffer. The plates were incubated for 24 h at 37°C. After incubation, the change in color in control (a yellow zone around well of red background due to acidic pH) which indicated the activity of PPL. Lipase inhibitory activity was calculated as percentage inhibition:

$$\%\ \text{Inhibition} = [(\text{Zone of control} - \text{zone of sample}) / \text{Zone of control}] \times 100$$

Thrombolytic activity: Thrombolytic activity of plant extract was estimated by following the procedure given by Prasad *et al*., (2006). The blood of volume 0.5 mL in microcentrifuge tube was incubated for 45 minutes at room temperature and centrifuged to remove the serum after the blood clot had formed. After removing the serum weighed the tubes again. We will get weight (Wt) of clot as;

Wt. of clot (before lysis) W1 = Wt. of tube + clot - Wt. of empty tube

Test samples of 100 µL were added and incubated for 90 minutes and centrifuged, weighed the clot lysis by the sample as;

Wt. of clot (after lysis) = Wt. of tube + clot (after lysis) – Wt. of empty tube
W2 = W1 –W3

Diff. (amount of lysis) = Wt. of clot (before lysis) – Wt. of clot (after lysis)
W = W1 – W2

% age clot lysis = Diff. (amount of lysis) / Wt. of clot ×100
% age lysis = W /W1×100

Cytotoxicity activity by hemolytic assay: The cytotoxic activity of extract and fractions was studied by hemolytic activity following the procedure of Powell *et al*., 2000. The 20 µL of each sample was taken in 2 mL microcentrifuge tubes, then added 180 µL RBCs suspension. For each test, 0.1% Triton-X100 was taken as a positive control and phosphate buffer saline (PBS) was taken as a negative control and absorbance was noted at 576 nm at ELISA reader (Powell *et al*., 2000).

Lysis of RBCs (%) = {(Abs. sample–Abs. Negative control)/Abs. Positive control} × 100

where Abs. = Absorbance and Hemolytic %age is also called lysis of RBCs.

High performance liquid chromatography (HPLC) analysis: For the quantification analysis of individual phenolic and flavonoid compounds from organic and aqueous extracts we used a reverse phase HPLC system according to given method described by Hakkim *et al*., 2008.

Results and Discussions

Extraction and fractionation from *Viola odorata* leaves: An extraction based on solvents described by Nisa, 2011 & Muhit *et al.*, 2010 with little modifications was performed using macerations and mixing of immiscible solvents to franctionate CME. The concentration and % age yield of CME extract and its fractions (*n*-HF, CF, EAF, MSF, WSF i.e. from non-polar to polar) are listed below in table 1. Each sample was dissolved in DMSO for bioactivities.

Total phenolic and flavonoid contents (TPC/TFC): In the *V. odorata* leave extract total phenolic and total flavonoid contents were measured and each concentration is listed in Table 3.

TPC have been expressed in terms of Gallic acid equavilants mg/g plants. In *V. odorata* leave extract the highest TPC recorded was in MSF (3.44 mg/g) and the least TPC was in WSF (1.82 mg/g). Similarly TFC have been expressed in terms of quercetin equivalents mg/g plants. Highest TFC was recorded in *n*-HF (0.21mg/g) and the least in WSF (0.09mg/g).

Different CME, WSE of different plants like *Bryophyllum pinnatum* (18.4 mg/g TPC, 8.4 mg/g TFC), *Ipomea aquatica* (18.8 mg/g TPC, 37.6 mg/g TFC), *Oldenlandia corymbose* (11.6 mg/g TPC, 4.4 mg/g TFC) also showed different total phenolic and total flavonoid content (Yadav & Agarwala, 2011). This usually varies due to number of reseaons of either expression, plant part, plant type, extraction procedures etc. but however these contents are responsible for a number of plant extract's activitites (Mushtaq *et al*., 2017).

Antioxidant studies obtained by DPPH assay: All the fractions showed significant results ranging from 6.1467 % ± 1.11 to 62.133±4.26 but are mostly active in scavenging radicals. For example, EAF showed highest antioxidant activity (62.133±4.26) DPPH free radical scavenging activity. EAF in literature also showed significant antioxidant activity (61.35%) as described by Siddiqui *et al.,* 2013 for *Holarrhena pubescens* which showed 70% antioxidant activity by same method.

Antibacterial activity: Against four bacterial strains the antibacterial activity of each sample of *V. odorata* leaf was carried out using *B. subtilis, E. coli* and *S. aureus* as Gram positive and *P. multocida* as Gram negative. Mean ZOI (zone of inhibition) of different bacteria are shown in Table 3.

ZOI was shown by CME against *B. subtilis, S. aureus, E.coli* and *P. multocida* is (8 mm). MSE showed maximum zone of inhibition against *S. aureus* (9 mm), *E. coli* (14 mm), *B. subtilis* (11 mm) and *P. multocida* (10 mm). CF also showed significant ZOI against *S. aureus* and *E.coli* (8 mm) and against *B. subtilis* and *P. multocida* (10 mm). All these activities are shown in figures as given below (Figs. 1-4). Ciprofloxacin as positive control was used in each experiment of antibacterial studies. Negative control of DMSO was used in each experiment too in which samples were prepared. HPLC results (Table 2) of extract showed that the most active phenolic compound in MSE is Ferulic acid (12.85 ppm) and in EAF Caffeic acid (30.3 ppm) which may be responsible for antibacterial activity. Literature showed that in *Debregeasia salicifolia,* the EAF, MSF and CME showed antibacterial potential against the different bacteria like *Bacillus subtilis, Staphylococcus aureus* and *Escherichia coli* i.e 13.9 mm, 12.3 mm and 16.2 mm respectively (Nisa, 2011 & Gautam *et al*., 2012).

Table 1. *V. odorata* leaf extracts with different bioactive fractions in crude form.

S. No.	Organic & Aqueous fractions	Fractions conc. (mg/mL)	% Age yield of CME fractionated
1.	Crude Methanol Extract (CME)	144	16.25*
2.	n- Hexane Fraction (*n*-HF)	770	25.01**
3.	Chloroform Fraction (CF)	130	2.36**
4.	Ethyl acetate Fraction (EAF)	120	1.10**
5.	Methanol Soluble Fraction (MSF)	2200	55.43**
6.	Water Soluble Fraction (WSF)	63	0.70**

* % age of total plant mass, ** % age of total CME fractionated

Table 2. HPLC based fingerprints of phenolics and flavonids in leaves samples of *V. odorata.*

Sample name	Identified phenolic compound	Retention time (Rt)	Peak area (m V.s)	Concenration (ppm)
CME	Quercetin	3.287	40.142	2.12
	Gallic acid	4.953	105.322	3.79
	Caffeic acid	12.960	69.624	3.21
	Benzoic acid	14.853	36.844	3.91
	Chlorogenic acid	15.720	93.870	6.75
	Ferulic acid	22.340	178.555	12.85
n-HF	Quercetin	2.833	3.211	0.16
	Gallic acid	4.980	23.649	0.84
	Caffeic acid	12.233	38.551	1.77
	Vanillic acid	13.147	88.999	55.17
	Chlorogenic acid	15.260	67.637	5.27
	p- coumeric acid	17.833	212.252	2.75
CF	Quercetin	3.240	164.466	8.71
	Gallic acid	4.933	82.331	2.95
	Caffic acid	12.260	40.381	1.85
	Vanillic acid	13.273	174.542	10.78
	Ferulic acid	21.867	164.952	11.18
	Cinamic acid	25.013	155.870	5.48
EAF	Quercetin	3.347	4.400	0.23
	Gallic acid	4.960	4.874	0.17
	Caffic acid	12.707	659.477	30.31
	Cinamic acid	25.020	525.394	18.37

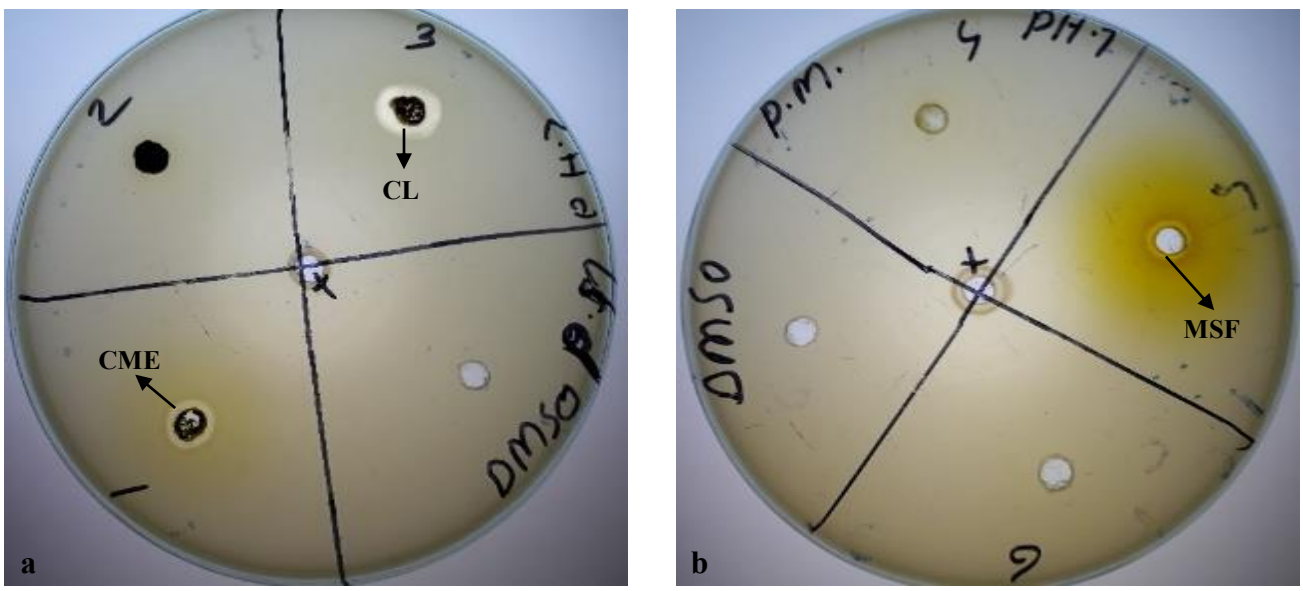

Fig. 1. Antibacterial potential against *P. multocida.* (a and b) CME, CF and MSF showing activity against *P. multocida*.

Table 3. Statistical evaluation of bioactivities showed by each organic extract or fraction of *V. odorata* leaves.

Sample name	TPC (GAE mg/g)	TFC (QE mg/g)	Anti-microbial assay (Mean ZOI (mm) ± S.E)					% α-amylase inhibition	% of free radical scavenging	% Lipase inhibition	Thrombolytic	Cytotoxicity
			B. subtills	*E. coli*	*P. multocida*	*S. aureus*	*F. solani*					
CME	$2.11^{C} \pm 0.09$	$0.17^{B} \pm 0.08$	$8^{B} \pm 0.513$	$8^{B} \pm 0.31$	$8^{B} \pm 0.40$	$8^{A} \pm 0.20$	$22^{BC} \pm 0.58$	$3.4300^{D} \pm 1.00$	$49^{AB} \pm 5.9$	$95^{A} \pm 0.89$	$18^{D} \pm 0.51$	$40^{C} \pm 0.33$
n-HF	$2.04^{C} \pm 0.02$	$0.21^{A} \pm 0.064$	Nil	Nil	Nil	$7^{B} \pm 0.42$	$33^{A} \pm 0.75$	$13.770^{C} \pm 2.30$	$44^{AB} \pm 2.1$	$34^{B} \pm 1.24$	$8^{F} \pm 0.59$	$37^{D} \pm 0.66$
CF	$2.40^{B} \pm 0.03$	$0.150^{B} \pm 0.03$	$10^{A} \pm 0.40$	$8^{B} \pm 0.40$	$10^{A} \pm 0.40$	$8^{A} \pm 0.20$	$26^{B} \pm 0.38$	$28.177^{B} \pm 2.96$	$32^{B} \pm 10$	$22^{C} \pm 1.43$	$11^{E} \pm 0.35$	$76^{A} \pm 0.25$
EAF	$2.07^{CD} \pm 0.15$	$0.13^{AB} \pm 0.02$	Nil	$5^{C} \pm 0.20$	Nil	$6^{C} \pm 0.31$	Nil	$17.487^{C} \pm 0.93$	$62^{A} \pm 4.26$	$6^{D} \pm 1.28$	$22^{C} \pm 0.40$	$15^{E} \pm 0.50$
MSF	$3.44^{A} \pm 0.018$	$0.12^{AB} \pm 0.015$	$11^{A} \pm 0.40$	$14^{A} \pm 0.31$	$10^{A} \pm 0.40$	$9^{A} \pm 0.40$	Nil	$42.163^{A} \pm 2.74$	$43^{B} \pm 17.68$	$19^{C} \pm 1.43$	$26^{A} \pm 0.48$	$45^{B} \pm 0.33$
WSF	$1.82^{D} \pm 0.02$	$0.09^{B} \pm 0.015$	Nil	Nil	Nil	Nil	$19^{D} \pm 0.63$	$27.193^{B} \pm 2.57$	$6^{C} \pm 1.11$	$29^{B} \pm 2.31$	$24^{B} \pm 0.43$	$7^{F} \pm 0.33$

*Data are Means ± SD of 3 replications. Means sharing same alphabets are statistically non-significant by Duncan's test (p>0.05). Different alphabets showed that the results shown by bioactive fractions of *V. odorata* are significant

Antifungal activity of *V. odorata* leaves: In antifungal activity against *Fusarium solani* was performed and *n*-HF showed maximum ZOI (33 mm) and minimum ZOI was given by MSF (19 mm). Fluconazole as a positive control and DMSO as negative control was used in each experiment (Jamil *et al*., 2007). Mean ZOI of *F. solani* is shown in Table 3. The activity shown by each sample is given below in figure 5a and 5b.

In table 2 HPLC results are showing that maximum concentration of vanillic acid (55.17 ppm) in *n*- HF that could be responsible for showing the maximum activity.

α-Amylase Inhibition: The inhibitory activity of the leaf extract and fractions of *V. odorata* was studied using α-amylase. % α-amylase inhibition was calculated and represented in Table 3. In which MSF showed highest inhibition %age (42.163±2.74) and CME showed minimum inhibition 3.43±1.00. MSF of *Carica papaya* leaves also showed 25% α-amylase inhibition on literature survey which supports our findings (Nickavar & Yousefian, 2009 & Ogundele *et al*., 2017).

Lipase inhibition assay: The lipase inhibition activity of each sample was studied using phenol red stained plate method. % lipase inhibition is presented in Table 3. Our findings showed that *n*-HF possessed maximum lipase inhibition in % age 34±0.89 and ethyl acetate (EAF) fraction showed minimum inhibition of 6%±1.28. HPLC results showed (Table 2) that most active compound in *n*-HF is Vanillic acid (55.17 ppm) which may be responsible for lipase inhibition. CME of *Rubi Fructus*, *Corni Fructus*, *Salicis Radicis Cortex* and *Geranium nepalense* showed 32%, 34%, 38% and 31% of PPL inhibition (Changhyun & Jung, 2012). Figure 6 (a & b) shows the plate test results, in each plate the positive control is with enzyme only (no sample) and negative control was only DMSO.

Evaluation of *In vitro* thrombolytic activity: The thrombolytic activity of leaf samples of *V. odorata* was checked and results showed that WSF showed 26.44%±0.48 of thrombolytic activity which was maximum of clot lysis among all samples and minimum activity was shown by *n*-HF (8.860±0.591). Thrombolytic compounds have been isolated from most species of plant family Violaceae which shows that bioactive compounds obtained from plants are gaining much more importance not only in producing functional food additives but also medicines to treat serious diseases like thrombosis (Peng *et al*., 2005). All results are shown in table 3 and for this study SK enzyme was used as a positive control and DMSO as negative control.

Evaluation of hemolytic activity: Bioactive CME and its fractions of *V. odorata* leaves were checked for cytotoxicity by *In vitro* hemolytic assay. Maximum % hemolysis was shown by CF (76.317±0.252) and minimum hemolysis by WSF (7.8847±0.33). These values showed assessment of safe use of each leaf extract and fraction. Reported agent Triton X-100 was used as positive control which gave 87.29% hemolysis and most samples gave less range of hemolysis (Ashraf *et al*., 2020). The extracts rich in phytochemicals from Lepidiumaucheri Boiss exhibited hemolytic activity (48.09%) which shows that medicinal plants extracts also possess hemolytic role (AL-Khuzaie, 2014).

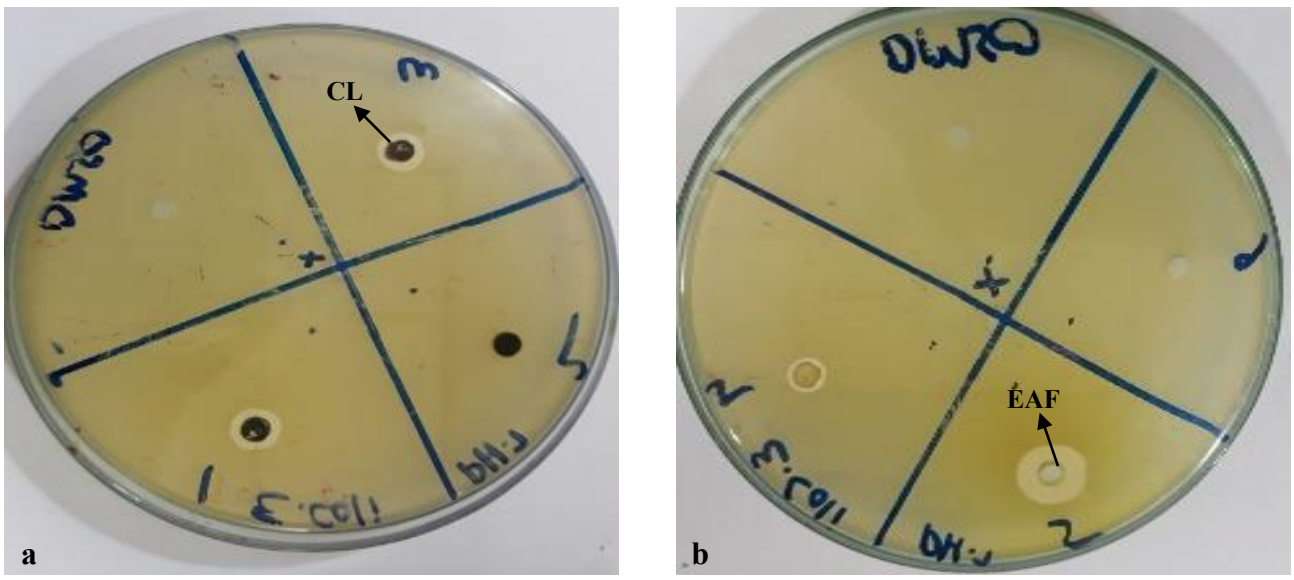

Fig. 2. Antibacterial potential against *E. coli.* (a and b) C F and EAF showing activity against *E. coli.*

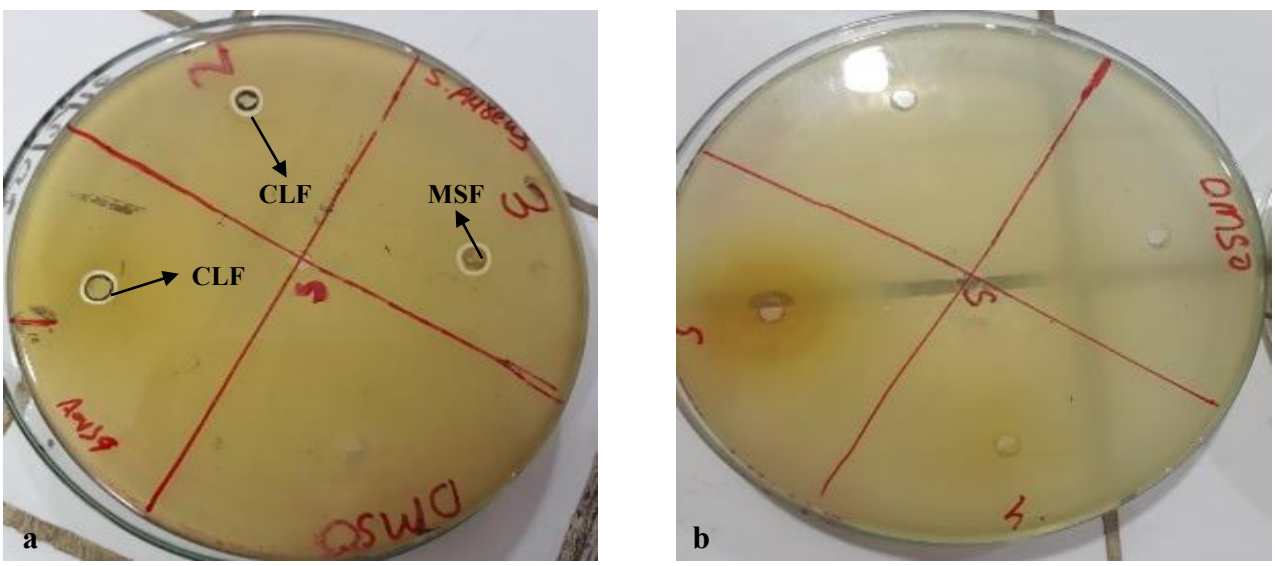

Fig. 3. Antibacterial potential against *S. aureus.* (a and b) CME, CF and MSF showing activity against *S. aureus*

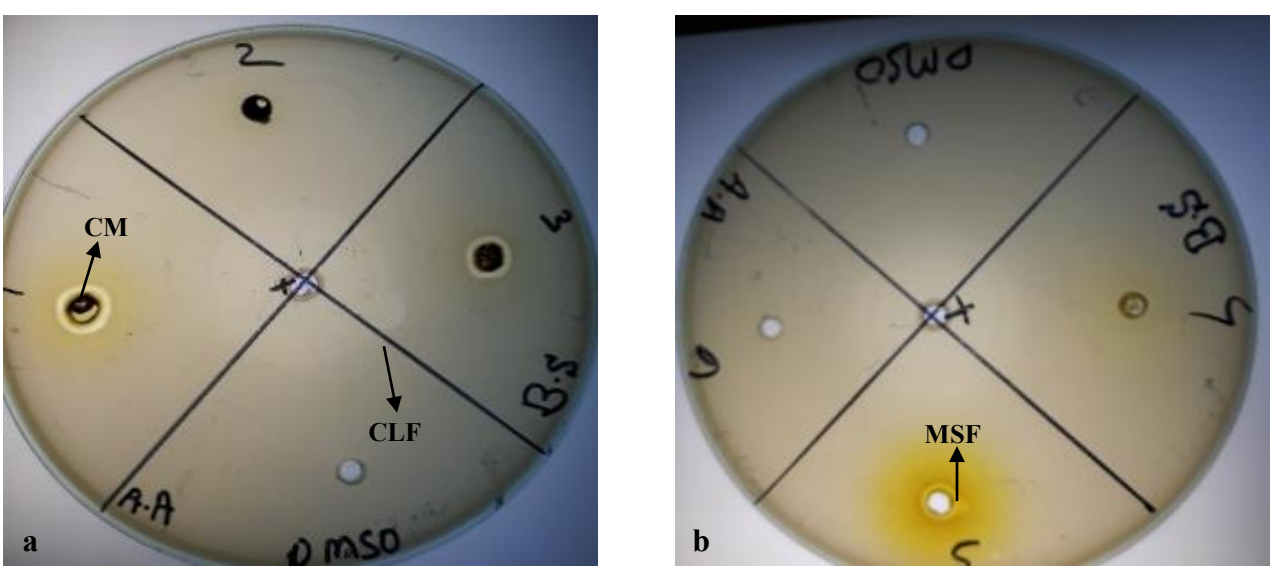

Fig. 4. Antibacterial potential against *B.subtilis.* (a and b) CME, CF and MSF showing activity against *B. subtilis.*

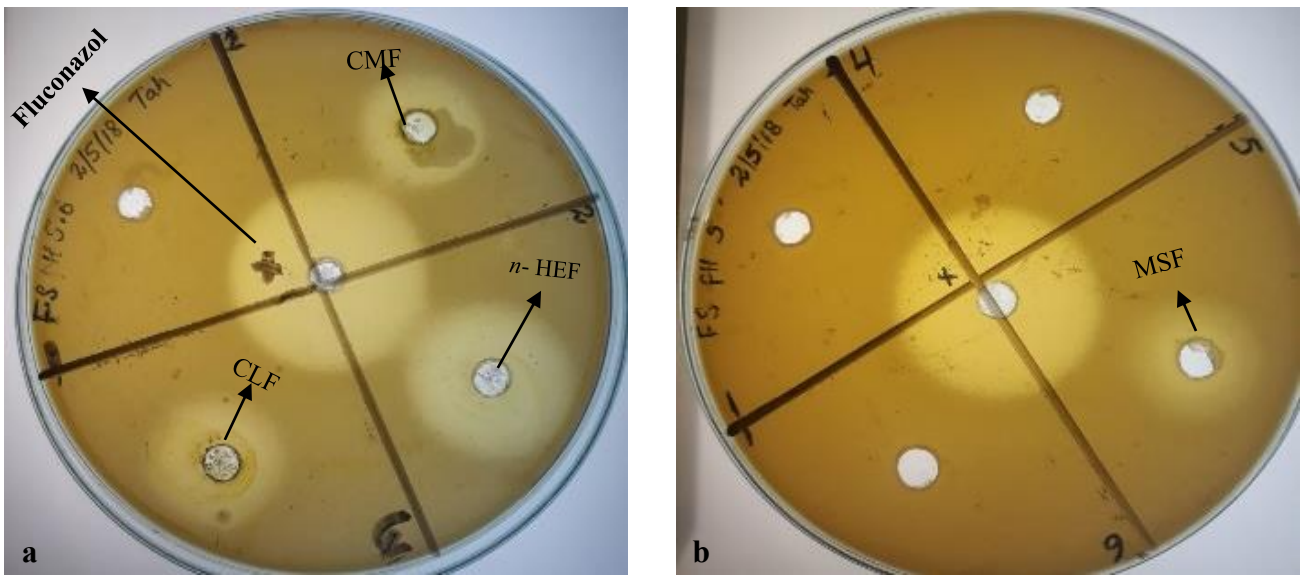

Fig. 5. Antifungal activity of *V. Odorata* samples: a. Showing the antifungal behavior of CME, *n*- HF and CF while fluconazole is used as positive control. b. showing sample MSF as antifungal fraction.

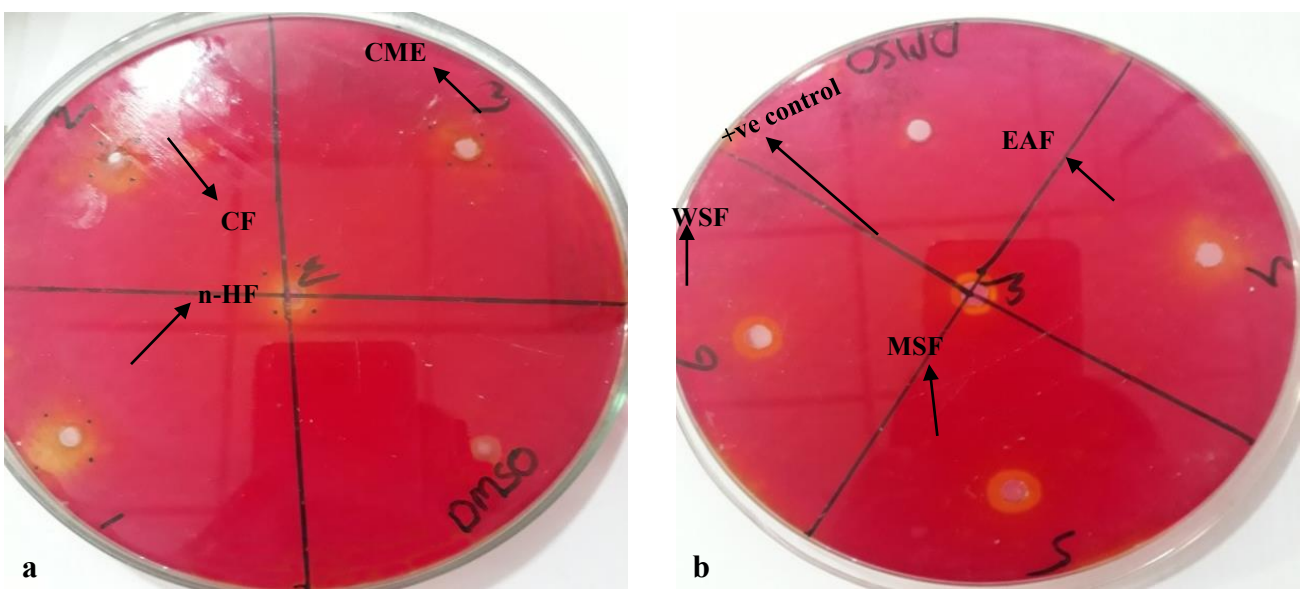

Fig. 6. Lipase Inhibition Assay using Phenol red plate method. a) Shows inhibition zones by CF, n-HF, CME samples and only lipase was used as +ve control b) shows MSF and EAF results of inhibition.

Estimation of phenolics and flavonoids by high performance liquid chromatography (HPLC): HPLC results obtained from the Central HiTech labs of University of Agriculture Faisalabad indicated that leaves extract and fractions of *V. odorata* contains phenolics and flavonoids compounds. In CME, Ferulic acid is more active phenolic compound with retention time (22.34) and concentration =12.85 ppm. The least available compound in CME was Caffeic acid (3.21 ppm). In *n*- HF, Vanillic acid with high concentration (55.17 ppm) was present. Similarly, in CF the most active phenolic compound was Ferulic acid with a concentration of 11.18 ppm. Whereas, in EAF Caffeic acid with 30.3 ppm concentration was present. Gallic acid, quercetin and caffeic acid was detected in almost all samples. Whereas other detected phytochemicals in samples were benzoic acid, chlorogenic acid, ferulic acid, vanillic acid and cinamic acid which were different in concentrations in different samples that could be due to their solubilities in respective solvents and these components are the possible agents that showed bioactivities of samples tested (Kabera *et al*., 2014 & Mustafa *et al*., 2016). HPLC results listed in Table 2.

Conclusion

The present study was an attempt to identify the phenolics and flavonoid compounds from the leaves methanolic extract and its fractions which comprised different bioactive phytochemicals. The samples displayed antimicrobial activities against a variety of bacterial & one fungal strain. Other bioactivities evaluated were antioxidant, enzyme inhibition (amylase + lipase), thrombolytic and hemolytic. Our results indicate that the CME and its fractions are able to combat most

microorganisms that could have developed resistance to existing synthetic antibiotics. So, *Viola odorata* of our indigenous origin could a very potential medicinal plant & different extraction method can be helpful in obtaining different bioactive compounds that could be reason of different bioactivities. Further investigation is needed in order to identify, determine the structure of active compounds and to scale up the production of bioactives and *In vivo* study must be accomplished to evaluate the better results for better understanding towards drug development.

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