doi.org/10.36721/PJPS.2020.33.1.REG.135-139.1

Cytotoxic, α-amylase inhibitory and thrombolytic activities of organic and aqueous extracts of *Bacillus clausii* KP10

Farzana Nighat[1], Zahid Mushtaq[1,2*], Munaza Maqsood[1], Muhammad Shahid[3], Muhammad Asif Hanif[4] and Amer Jamil[2]
[1]Bioactive Molecules Research Lab (BMRL), Department of Biochemistry, University of Agriculture, Faisalabad, Pakistan
[2]Molecular Biochemistry Lab, Department of Biochemistry, University of Agriculture, Faisalabad, Pakistan
[3]Medicinal Biochemistry Research Laboratory, University of Agriculture, Faisalabad, Pakistan
[4]Department of Chemistry, University of Agriculture, Faisalabad, Pakistan

Abstract: Humans are experiencing serious health issues like myocardial infarction and diabetes. Thrombosis is the reason of myocardial infarction that may cause death. Bioactive compounds or enzymes can be used to dissolve the clot. Whereas diabetes is a disorder of metabolism in which the level of glucose in blood becomes high. It can be controlled by inhibiting α-amylase enzyme. The current project was, therefore planned to investigate the thrombolytic, α-amylase inhibitory and cytotoxic (to access drug safety) potentials of the organic and aqueous bioactive fractions of *Bacillus clausii* KP10. The cytotoxicity was assessed with hemolytic assay, α-amylase inhibition assay was done by using DNS and *in-vitro* thrombolytic effect was checked with human blood. In our experiments, the maximum hemolytic activity was shown by ethyl acetate fraction (12.64%). Results were compared with standard Triton X-100 which showed 91.61% hemolytic activity whereas all other fractions showed least cytotoxic activity. The extracts were also evaluated as thrombolytic agents as correlated to streptokinase (73.83%). All the extracts showed clot lysis activity, among which water soluble fraction exhibited maximum (35.16%) clot lysis activity. In our experiment methanol soluble fraction of *B. clausii* KP10 showed maximum 26.49% α-amylase inhibitory activity. Results were analyzed statistically through analysis of variance (ANOVA).

Keywords: Thrombolytic, cytotoxic, clot lysis, α-amylase, *Bacillus clausii* KP10.

INTRODUCTION

When accumulation of blood occurs inside a blood vessel it causes thrombosis. It obstructs the blood circulation in body. Platelets and fibrins are used by the body to form blood clot after injury to avoid loss of blood. Blood clots can be formed even when a blood vessel is not injured (Ali *at el.*, 2013). Thrombosis is a main reason for complications that may cause death. Streptokinase is a fibrinolytic enzyme that is widely used to dissolve the clot (Basta *et al.,* 2004). Streptokinase can break the unbound plasminogen to form plasmin. It is used as a useful and low-priced thrombolytic medicine for the treatment of myocardial infarction (Nikhil and Amit, 2007) and pulmonary embolism (Ali *at el.*, 2013). According to a report given by WHO 17 million people die due to intravascular thrombosis every year. Thrombolytic agents have been used to treat the blood clot over the years. The *Bacillus* species produce fibrinolytic enzymes. They are capable of treating the thrombus (Yuan *et al*., 2012). Nattokinase is produced by *Bacillus subtilis.* Many other microorganisms also produce enzymes that have significant thrombolytic potential e.g staphylokinase produced by *Staphylococcus aureus* and Streptokinase produced by *Streptococcus hemolyticus* (Peng *et al.,* 2005).

**Corresponding author:* e-mail: zahidmushtaquaf@uaf.edu.pk

Diabetes mellitus is a disorder of glucose metabolism. It increases the glucose level in blood that leads to hyperglycemia. Treating hyperglycemia is a major challenge which may reduce by the use of oral hypoglycemic medicines such as biguanides, insulin secretagogues and α-amylase inhibitors (Kazeem *et al.,* 2013). All these drugs have good effects and control the disease but they also induce a gastrointestinal side effect that limits their use in a preventive approach. In small intestine inhibitors of α-amylase hold up the carbohydrate break down and reduce the blood glucose excretion after meal (Kwon *et al.,* 2007). α-Amylase is an important enzyme that is present in the pancreatic juice and saliva. It breaks the big insoluble starch particles into small soluble particles (Afifi *et al.,* 2008). The inhibition of α-amylase can be an important approach to lower the blood glucose level. There is need to find enzyme inhibitors from natural sources because synthetic inhibitors are associated with numerous gastrointestinal side effects (Bhutkar and Bhise, 2012). It is well known that in infectious diseases too, hemolysis occurs as a side effect of medicines (Sharma and Sharma, 2001). The bioactive fractions obtained from microbes may prevent the hemolysis. The membrane of erythrocytes can be affected by utilization of bioactive compounds from microbes and plants. So there is need to analyze the hemolytic potential of the bioactive microbial and phytocompounds (Zohra and Fawzia, 2014).

The above contention encourages the alternative techniques for screening of bioactive compounds from microorganisms to overcome the serious health problems like thrombosis and diabetes. Hence, the main objective of the research is to screen the potential of bioactive fractions extracted in organic and aqueous solvents of *B. clausii* KP10 by different thrombolytic, cytotoxic and α-amylase inhibitory assays.

MATERIALS AND METHODS

Selection of bacterial species

Locally isolated and identified strain of alkalophillic *Bacillus clausii* KP10 by our research group was used in the research as a source of microbial biomass from where bioactive fractions were extracted and evaluated through bioassays (Erum *et al*., 2017).

Media preparation and bacterial culturing

Nutrient agar and nutrient broth (MERCK) was used to grow bacterial cultures (Muller *et al.,* 2016). Then the bacterial cell mass was obtained when centrifuged at 10,000 rpm for 10 minutes at 4°C to obtain extracts of bioactive fractions. Cell mass was dried, weighed and used for extraction of biologically active agents.

Extraction of bioactive compounds

a) *Soxhlet extraction*

The Soxhlet extraction was done by using 5 g of dry cell mass of *B. clausii* KP10 on soxhlet apparatus. 12 hours of extraction process at 78°C, followed by 30 minutes solvent rinse and 30 min solvent evaporation. Absolute ethanol was used for extraction. The extract was dried and weighed. Then dissolved it with DMSO and used for further bioactive potential (Li *et al*., 2014).

b) Isoprenoid quinones and polar lipids extraction

Chloroform and methanol mixture was shown to provide efficient extraction of isoprenoid quinone and polar lipids, and the procedure described by Minnikin and others was used for the extraction of bacterial polar lipids and isoprenoid quinones (Minnikin *et al.,* 1984).

c) Protein extraction

2 g organism dry cell mass and 4 mL protein extraction buffer (PEB) (10 mM Na_2HPO_4, 15mM NaH_2PO_4, 100 mM KCl, 2 mM EDTA, 1.5% polyvinylpoly pyrrolidone (PVPP), 1 mM phenyl methyl sulfonylfloride (PMSF) and 2 mM thiourea) were mixed carefully. The cell suspension was sonicated for 5 min. The supernatant was filtered after centrifugation (12000 rpm) at 4°C for 20 minutes, to remove debris present in it. The filtrates were placed at -4°C in sterilized tube. By using BSA (bovine serum albumin) as standard curve the protein contents of *Bacillus clausii* KP10 were estimated spectrophotometrically by Bradford method (Mushtaq *et al.,* 2017).

d) Extraction and fractionation using different organic and aqueous solvents

Method for solvent extraction was described by Nisa (2011). Accordingly 5 g dry cell mass of *Bacillus clausii* KP10 was used for extraction. Extraction was done by mixing the dry cell mass of *Bacillus clausii* KP10 in methanol followed by continuous stirring for overnight and centrifuged. Further, the crude methanol extract was partitioned with different aqueous (water) and organic solvents (*n*-hexane, chloroform, ethyl acetate and methanol) depending upon the increasing order of polarity of solvents. Carefully separated the layers, dried and weighed them. Then dissolved in DMSO and stored at 4 °C for further bioactivity assays.

α -Amylase inhibition assay

A 250 µL volume of extracts of *B. clausii* KP10 (0.5 mg/mL) and 250 µL of α-amylase solution (0.5 mg/mL) (prepared in 0.02 M sodium phosphate buffer of pH 6.9) was placed in a tube. The mixture of extracts and α-amylase solution was incubated at 25˚C for 10 minutes. Then, 250 µL of 1% starch solution (prepared in 0.02 M sodium phosphate buffer pH 6.9) was added. 500 µL of DNS (dinitrosalicylic acid) was added to discontinue the reaction after incubation at 25˚C for 10 minutes. The tubes were placed in boiling water for further incuabtion for 5 minute. After incubation in boiling water, tubes were cooled down to room temperature. Then, 5 mL of distilled water was added in tubes to dilute the reaction mixture. The absorbance was recorded at OD 540 nm (using spectrophotometer). Distilled water (as a control) was used instead of extract. The α-amylase inhibitory activity was estimated as percentage inhibition:

$\%\text{Inhibition} = [(\text{Abs}_{\text{control}} - \text{Abs}_{\text{extracts}})/ \text{Abs}_{\text{control}}] \times 100$ (Kazeem *et al.,* 2013).

Hemolytic activity

The cytotoxic studies of the *Bacillus clausii* KP10 extracts were carried out by hemolytic activity (Powell *et al.,* 2000). A 3 mL volume of blood (freshly obtained) was added in heparinized tubes to avoid coagulation. Mixed lightly and placed into a sterilized centifuge tube. Centrifuged for 5 minutes at 13000 rpm. The supernatant was discarded and 5 mL of chilled (4˚C) sterile isotonic PBS (phosphate buffer saline, pH 7.4) solution was used for the washing of Red Blood Cells. The 20 µL (0.5 mg/mL) of *B. clausii* KP10 extracts and fractions were placed in 2 mL microcentrifuge tubes and 180 µL of diluted Red Blood Cells (RBCs) suspension was added. After incubation (for 35 minutes at 37 ˚C) and agitation (10 minutes) the tubes were placed on ice for 5 minutes. Then centrifuged the tubes for 5 minutes at 13000 rpm and 100 µL supernatant (taken after centrifugation) was placed in microcentrifuge tubes and diluted with 900µL of phosphate buffer saline. All microcentrifuge tubes were kept on ice. Then 200 µL mixture from each microcentrifuge tubes was added into ELISA plates.

Triton X-100 (0.1%) was used as a positive control and PBS as a negative control. The absorbance (A) was checked at 576 nm on ELISA reader (Powell *et al*., 2000). Hemolytic activity was calculated with the help of following formula:

Lysis of RBCs (%) = $\{(A_{sample} - A_{Negative\ control})/A_{Positive\ control}\} \times 100$

Thrombolytic activity

Thrombolytic activity was performed with little modifications by following the method described by Prasad *et al.,* (2006). Blood samples were collected from different blood banks of Government hospitals of Faisalabad. Blood was drawn from veins of healthy human volunteers. Took 500 µL blood into pre-weighed microcentrifuge tubes. Each pre-weighed tube was labeled and 100 µL (0.5 mg/mL) of *B. clausii* KP10 extracts were added to the tubes. Streptokinase (100 µL) was used as a positive control and DMSO (100 µL) was used as a negative control. All microcentrifuge tubes were incubated overnight at 37°C and observed for clot lysis and following calculations were done;

Percentage of clot lysis = [{Difference (amount of lysis)}/weight of whole clot] ×100

%age clotlysis = W /W1×100

STATISTICAL ANALYSIS

The data was analysed statistically through analysis of variance (ANOVA) (Oliveira, 2015). One way ANOVA was performed on all main effect means from hemolysis data, α-amylase inhibitory data and thrombolytic data. Data were then compared with pair wise comparison statistically (Powell *et al.,* 2000). The software Statistix 9 was used for one way ANOVA.

RESULTS

The bioactive fractions were obtained from *B. clausii* KP10 includes Isoprenoid quinone, polar lipids and methanol and water soluble fraction and yields were 566, 30 and 90 mg/mL of DMSO respectively. 140 mg/mL crude ethanolic extract was obtained from Soxhlet extraction that was dissolved in DMSO. Crude methanolic extract, *n*-hexane extract, chloroform extract, ethyl acetate extract, methanol soluble fraction and water soluble fraction with yields of 580, 100, 80, 60, 150 and 30 mg/mL of DMSO respectively were obtained. 218 µg/mL of total crude protein was estimated. 0.5 mg/mL of each sample was used for thrombolytic, hemolytic and α-amylase inhibition assay. Streptokinase was used as a positive control for comparison in thrombolytic activity that showed 73.83% clot lysis. When clots were treated with DMSO as negative control, it showed negligible clot lysis. The *in vitro* thrombolytic activity study exposed that water soluble fraction exhibited 35.16% clot lysis. The thrombolytic activity showed by the bioactive fractions of *B. clausii* KP10 is shown in table 1. In our project chloroform extract of *B. clausii* KP10 exhibited 18.88% clot lysis activity. The percentage of weight loss of clot after use of extracts obtained from *B. clausii* KP10 was taken as the prospective indication for thrombolytic activity. It can be used for the treatment of myocardial infarction. Maximum hemolytic activity was shown by Ethyl acetate fraction (12.64%). Results of all samples were compared with standard Triton X-100 only (as a positive control) which showed 91.61% hemolytic activity. The hemolytic activity shown by the bioactive fractions of *B. clausii* KP10 is shown in table 2. It showed that all the bioactive fractions of *Bacillus clausii* KP10 are safe for medicinal use because they do not damage the red blood cells. Methanol soluble fraction of *B. clausii* KP10 showed 26.49% α-amylase inhibitory activity. It can be used for the control of diabetes. It will help to lower the blood glucose level. The α-amylase inhibition shown by the bioactive fractions of *B. clausii* KP10 is shown in table 3. Ethanolic extract exhibited 24.52% α-amylase inhibition. In our experiment the methanol extract of *B. clausii* KP10 showed 19.26% α-amylase inhibitory activity. Water soluble fraction showed 23.49% α-amylase inhibitory activity. All explained results showed that *B. clausii* KP10 extracts may be potential candidates for the treatment of thrombus and diabetes.

Table 1: Percentage clot lysis activity by bioactive fractions of *B. clausii* KP10

Sr. No.	Sample Name	%age clot lysis ± S.D
1	Ethanolic Extract	16.83^{I}±0.14
2	Methanolic Extract	23.36^{D}±0.30
3	n-Hexane Soluble Fraction	20.40^{F}±0.43
4	Chloroform Soluble Fraction	18.88^{G}±0.12
5	Ethyl acetate Fraction	27.58^{C}±0.31
6	Methanol Soluble fraction	13.77^{K}±0.13
7	Water Soluble Fraction	35.16^{B}±0.11
8	Isoprenoid Quinone	22.77^{E}±0.11
9	Polar Lipids	17.62^{H}±0.28
10	Methanol & Water Soluble Fraction	9.73^{L}±0.19
11	Protein Extract	15.33^{J}±0.40
12	Streptokinase (+ve)	73.83^{A}±0.15

%age hemolysis= Mean, S.D= Standard deviation. Means having similar alphabets are statistically non-significant (P>0.05).

DISCUSSIONS

Microbes play a vital role for the benefit of mankind, they cover the basic health needs in developing countries. Moreover, microbes may offer a new source for thrombolytic compounds, α-amylase inhibitors and may prevent the cytotoxicity. Therefore, the thrombolytic, cytotoxicity and α-amylase inhibition activities of bioactive fractions of *B. clausii* KP10 were evaluated. The bioactive fractions were obtained from *B. clausii* KP10 includes Isoprenoid quinone, polar lipids and methanol and water soluble fraction. The *in vitro* thrombolytic activity study exposed that water soluble fraction exhibited maximum clot lysis. In a study conducted by Sayeed *et al.* (2014), the thrombolytic potential of *Trevesia palmate* methanolic fractions (*n*-hexane, chloroform, ethyl acetate, methanol soluble, and aqueous soluble fractions) were studied. Ethyl acetate fraction exhibited maximum clot lysis activity (44.30%). In research work ethyl acetate fraction exhibited maximum (27.58%) thrombolytic activity. Chloroform extracts of fruits and leaf extracts *S. dulcis* showed maximum thrombolytic activity (25.30%) (Islam *et al.,* 2013). In our project chloroform extract of *B. clausii* KP10 also exhibited clot lysis activity (18.88%). The triterpenoids saponins from *Lepidium aucheriboiss* showed hemolytic activity (48.09%) at 1 mg/mL concentration (AL-Khuzaie, 2014). The microbial peptide ESF1B exhibited 73% hemolytic activity (Powell *et al.,* 2000). In our work ethyl acetate fraction exhibited maximum hemolytic activity. But the results showed that the fractions are safe for medicinal purpose, as they will not damage the RBCs. Methanol soluble fraction of *B. clausii* KP10 showed maximum α-amylase inhibitory activity (26.49%). It will help to reduce the blood glucose level. The ethanolic extract prepared from the leaf of *Croton bonplandianum* showed significant α-amylase inhibitory activity with an IC_{50} value of 17.22±0.05 (Keerthana *et al.,* 2013). An extract was prepared from *Andrographis paniculata* by using ethanol; it exhibited 11.3% α-amylase inhibitory activity (Subramanian *et al.,* 2008). In our project ethanolic extract also exhibited α-amylase inhibition (24.52%). The methanolic extract of *S. obtusifolia* that showed 16.67% α-amylase inhibitory activity (Anisuzzaman *et al.,* 2014). In our experiment the methanol extract of *B. clausii* KP10 showed α-amylase inhibitory activity (19.26%) and water soluble fraction showed activity (23.49%). Water soluble extract of *Morinda lucida* leaf showed maximum activity 9.99% (Kazeem *et al*., 2013). All above discussions showed that *B. clausii* KP10 extracts may be potential candidates for the treatment of thrombus that may lead to myocardial infarction and high glucose level in blood that causes diabetes.

Table 2: Percentage hemolytic activity by bioactive fractions of *B. clausii* KP10

Sr. No.	Sample Name	%age hemolysis ± S.D
1	Ethanolic Extract	$9.38^{D}\pm0.06$
2	Methanolic Extract	$5.74^{G}\pm0.14$
3	n-Hexane Soluble Fraction	$8.04^{F}\pm0.06$
4	Chloroform Soluble Fraction	$6.33^{E}\pm0.30$
5	Ethyl acetate Fraction	$12.64^{B}\pm0.13$
6	Methanol Soluble Fraction	$6.47^{F}\pm0.28$
7	Water Soluble Fraction	$4.16^{I}\pm0.08$
8	Isoprenoid Quinone	$4.76^{H}\pm0.12$
9	Polar Lipids	$3.84^{I}\pm0.14$
10	Methanol & Water Soluble Fraction	$4.64^{H}\pm0.13$
11	Protein Extract	$9.81^{C}\pm0.59$
12	Triton X-100	$91.61^{A}\pm0.35$

%age hemolysis= Mean, S.D= Standard deviation. Means having similar alphabets are statistically non-significant (P>0.05).

Table 3: Percentage α-amylase inhibition by bioactive fractions of *B. clausii* KP10

Sr. No.	Sample Name	%age inhibition ± S.D
1	Ethanolic Extract	$24.52^{C}\pm0.28$
2	Methanolic Extract	$19.26^{E}\pm0.20$
3	n-Hexane Soluble Fraction	$15.63^{G}\pm0.27$
4	Chloroform Soluble Fraction	$18.39^{F}\pm0.17$
5	Ethyl acetate Fraction	$25.57^{B}\pm0.44$
6	Methanol Soluble Fraction	$26.49^{A}\pm0.38$
7	Water Soluble Fraction	$23.49^{D}\pm0.42$
8	Isoprenoid Quinone	$14.54^{H}\pm0.42$
9	Polar Lipids	$26.45^{A}\pm0.42$
10	Methanol & Water Soluble Fraction	$10.49^{I}\pm0.20$
11	Protein extract	$18.39^{F}\pm0.17$
12	DMSO	$4.62^{J}\pm0.28$

%age inhibition= Mean, S.D= Standard deviation. Means having similar alphabets are statistically non-significant (P>0.05).

CONCLUSIONS

The organic and aqueous extracts of *Bacillus clausii* KP10 showed thrombolytic, α-amylase inhibitory and cytotoxic activities. Based on this primary research, more investigations are needed to examine the bioactive agents which are responsible for the extract's activities as well as their mechanisms of action.

ACKNOWLEDGEMENTS

We acknowledge the support of Molecular biochemistry lab and Medicinal biochemistry research lab for providing the facilities of thrombolytic, hemolytic and α-amylase inhibitory assays based studies.

REFERENCES

Afifi AF, Kamel EM, Khalil AA, Foaad MA, Fawziand EM and Houseny M (2008). Purification and characterization of α-amylasefrom *Penicillium olsonii* under the effect of some antioxidant vitamins. *GJBB*, **3**(1): 14-12.

Ali MS, Faruq KU, Islam A, Nurullah AMM, Chowdhury KAA and Sayeed MA (2013). Thrombolytic and cytotoxic activities of *Terminalia bellerica* Roxb. *BPJ,* **16**(2): 131-135.

AL-Khuzaie MQS (2014). Extraction, identification and bioactivities of the triterpenoids saponins from *Lepidium aucheri boiss.* M.Sc. Thesis. The College of Science. Univ. Thi-Qar. Republic of Iraq.

Anisuzzaman M, Ahsan MQ, Kuddus MR and Rashid MA (2014). Pharmacological Activities of *Senna obtusifolia* Linn.: A Medicinal Plant of Bangladesh. *BPJ.,* **17**(2): 182-186.

Basta G, Lupi C, Lazzerini G, Chiarelli P, LAbbate A and Rovai D (2004). An *in vitro* study on blood of normal subjects and patients with coronary artery disease. *Thromb. Haemost*., **91**(6): 1078-1083.

Bhutkar MA and Bhise SB (2012). *In vitro* assay of α-amylase inhibitory activity of some indigenous plants. *Int. J. Chem. Sci.,* **10**(1): 457-462.

Emran BT, Rahman MA, Uddin MMN, Rahman MM, Uddin MZ, Dash R and Layzu C (2015). Effects of organic extracts and their different fractions of five Bangladeshi plants on *in vitro* thrombolysis. *J. Altern. Complement. Med.,* **15**(128): 2-8.

Erum N, Mushtaq Z and Jamil A (2017). Isolation and identification of a catalase producing thermoduric alkalotolerant *Bacillus* sp. Strain kp10 from hot springs of Tatta Pani, Azad Kashmir. *The J. Anim. Plant Sci*., **27**(6): 2056-2062.

Islam A, Ali MS, Faruq KU, Nurullah AMM, Chowdhury KAA and Sayeed MA (2013). Thrombolytic and cytotoxic activities of *Terminalia bellerica* Roxb. *BPJ,* **16**(2): 131-135.

Kazeem IM, Adamson JO and Ogunwande IA (2013). Modes of inhibition of α-amylase and α-*glucosidase* by aqueous extract of *Morindalucida* benth leaf. *BioMed. Res. Int.,* **11**(527570): 1-6.

Keerthana G, Kalaivani MK and Sumathy A (2013). *In vitro alpha amylase* inhibitory and antioxidant activities of ethanolic leaf extract of *Croton bonplandianum. Asian J. Pharm. Clin. Res.,* **6**(4): 32-36.

Kwon YI, Apostolidis E and Shetty K (2007). Evaluation of pepper (*Capsicum annuum*) for management of diabetes and hypertension. *J. Food Biochem.,* **31**(3): 370-385.

Li Y, Naghdi FG, Garg S, Adarme-Vega TC, Thurecht KJ, Ghafor WA, Tannock S and Schenk PM (2014). A comparative study: the impact of different lipid extraction methods on current microalgal lipid research. *Microbial. Cell Factories.,* **13**(14): 2-9.

Minnikin DE, Donnell AG, Goodfellow M, Alderson G, Athalye M, Schaal A and Parlett JH (1984). An integrated procedure for the extraction of bacterial isoprenoid quinines and polar lipids. *J. Microbiol. Meth.,* **2**(5): 233-241.

Muller A, Behsnilian D, Walz E, Graf V, Hogekamp L and Greiner R (2016). Effect of culture medium on the extracellular synthesis of silver nanoparticles using *Klebsiella pneumoniae*, *Escherichia coli* and *Pseudomonas jessinii*. *Biocatal. Agric. Biotechnol.,* **6**: 107-115.

Mushtaq Z, Iqbal T, Ahmed N and Jamil A (2017). Antioxidants from selected indigenous plants possessing cyclotides. *Oxid. Commun.,* **40**(1-I): 102-119.

Nikhil S and Amit B (2007). A history of streptokinase use in acute myocardial infarction. *Tex. Heart Inst. J.,* **34**(3): 318-327.

Nisa S (2011). Bioactivities and phytochemical analysis of four plants of medicinal importance. Ph.D. thesis. Dept of Microbiol. Quaid-i-Azam University, Islamabad, Pakistan.

Oliveira CCD, Calado VMDA, Ares G and Granato D (2015). Statistical approaches to assess the association between phenolic compounds and the in vitro antioxidant activity of *Camellia sinensis* and *Ilex paraguariensis* Teas. *Crit. Rev. Food Sci. Nut.,* **55**(10): 1456-1473.

Peng Y, Yang X and Zhang Y (2005). Microbial fibrinolytic enzymes: An overview of source, production, properties, and thrombolytic activity *in vivo*. *Appl. Microbiol. Biotechnol.,* **69**(2): 126-132.

Powell WA, Catranis CM and Maynard CA (2000). Design of self-processing antimicrobialpeptides for plant protection. *Appl. Microbiol.,* **31**(2): 163-168.

Prasad S, Kashyap RS, Deopujari JY, Purohit HJ, Taori GM and Daginawala HF (2006). Development of an in vitro model to study clot lysis activity of thrombolytic drugs. *Thrombo. J.*, **4**(14): 1-4.

Sayeed MA, Faruk M, Chowdhury AI, Faisal AR, Mamun AA and Islam M (2014). Thrombolytic and Anti-arthritic Activities of Methanolic Extract of *Trevesia palmate*. *J. Sci. Technol*., **5**(9): 119-124.

Sharma P and Sharma JD (2001). *In vitro* hemolysis of human erythrocytes by plant extracts with antiplasmodial activity. *J. Ethnopharmacol*., **74**(3): 239-243.

Subramanian R, Asmawi MA and Sadikun A (2008). *In vitro α-glucosidase* and *α-amylase* enzyme inhibitory effects of *Andrographis paniculata* extract and andrographolide. *Acta. Biochemica. Polonica.,* **55**(2): 391-398.

Yuan J, Zhuang Z, Yang Y, Lin L and Wang S (2012). Thrombolytic effects of Douchi Fibrinolytic enzyme from *Bacillus subtilis* LD-8547 *in vitro* and *in vivo*. *BMC Biotechnol.,* **12** (36): 1-9.

Zohra M and Fawzia A (2014). Hemolytic activity of different herbal extracts used in Algeria. *IJPSR,* **5**(8): 495-500.