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Enzyme inhibition studies of ethyl acetate extract of *Pinus wallichiana* needles from Azad Kashmir, Pakistan

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Abstract

Ancient people preferred plants as a medicinal source for the treatment of many diseases due to different specific properties such as natural source, easy and cheap availability, less toxicity, and side effects. Synthetically designed drugs are sometimes toxic and have many side effects. Dietary disorders are the main routes of other diseases like obesity, imbalance of blood glucose, gastric ulcers, and many others due to imbalance and not proper functioning of these basic enzymes including amylase, pepsin and lipase in digestion. Plants have many bioactive compounds with potentials for enzyme inhibition and many other properties. *Pinus* family plants such as *Pinus wallichiana* also have all these specific properties and these plants are found mostly in mountain areas in all over the world including Pakistan. Therefore in present work, the *P. wallichiana* plant needle's ethyl acetate macerated (E) and soxhlet (S) extracts were used to estimate phytochemicals to test enzyme inhibition potential of each sample for α-amylase, pepsin and lipase enzymes. Results showed that Soxhlet (i.e. 24.75% extract yield) extracted more crude sample as compared to maceration (2.35%). It was observed that all inhibition bioactivities are also concentration dependent because they show gradually increased by increase in concentrations. TPC & TFC were more in soxhlet extract and total protein was more in macerated extract. HPLC analysis of the extracts showed the presence of bioactive compounds like gallic acid, vanillic acid, sanapic acid and caffeic acids which could be responsible for different bioactivities. Diluted 5 mg/mL Se sample showed maximum amylase inhibition (27.23%), maximum lipase inhibition was shown by 5 mg/mL Ee and maximum pepsin inhibition (54.9%) was shown by Se. Further study is needed on potential enzyme inhibitor's identification and purification that were responsible for activities. *P. wallichiana* needles ethyl acetate extracts from Kashmir proved to be a potential source of enzyme inhibitors that could be used in the field of medicines important for the treatment of digestive disorders.

Keywords: *Pinus wallichiana*, Ethyl acetate, enzyme inhibition, Phenolics, Flavonoids, HPLC

Full length article **Corresponding Author*, e-mail: zahidmushtaquaf@uaf.edu.pk

1. Introduction

Medicinal plants are known as major source of therapeutics to fight illness and maintain health because these are natural sources and have lesser side effects as compared to chemically formulated drugs specially for dietary disorders (e.g. Xenical, saxenda and belviq) [1]. *Pinus wallichiana* is a medicinal plant known as blue pine that is widely spread in various regions of world including Pakistan and Kashmir having different biologically active compounds which possess different bioactivities like antioxidant, antibacterial and enzyme inhibition potential [2-4]. The needles of *Pinus* plants are so much important as compared to other parts due to rich in phenolics, flavonoids, tannins and other compounds as a drug candidate to treat oxidative inflammatory and microbial diseases [5].

Imbalance in energy intake and expenditure causes the weight increase. Fat absorption pharmaceutical drugs have side effects like steatorrhea, oily stool, diarrhea and cholelithiasis so medicinal plants are preferable as a source for weight control. Lipase inhibition activity was used to discover the anti-obesity compounds for weight lowering activity in different plants namely *Carica papaya, Corcorus olitorius, Chrysophyllum cainito, Cymbopogon citrates,* and *Syzygium cumini.* Alcoholic extract of *C. cainito* shows highest lipase inhibition of 74.91% while *S. cumini, C. citratus, C. olitorius* and *C. papaya* show less than 50%

average inhibition. Further hexane and ethyl acetate extract of *C. cainito* show 92.11% inhibition and 21.9% inhibition. So hexane extract have high anti-lipase constituents. High amount of endothelium bound lipoprotein lipase lower the triglyceride level and lead to beta-oxidation activation in the liver. Plants have lipase inhibition activity because of alkaloids and phenolic compounds [6]. Antioxidant and enzyme inhibition activity of unripen apple polyphenols with their five different varieties was checked by fluorescence quenching and differential scanning calorimeter which shows strong antioxidant activity and inhibition activity on porcine pancreatic α-amylase. The highest inhibition is suggested by the lowest value of IC50. Phenolic extract show inhibition against α-amylase with the lowest IC50 value then acarbose such as tea extracted polyphenols. [7]. The fermented extract of endophytic fungus *D. arengae* also showed porcine pancreatic lipase inhibitory potential which has the capacity as drug potential [8].

The flowers leave and stem of traditional medicinal plant namely *O. angustissima* were extracted with distilled water, aqueous methanol, n-butanol and ethyl acetate which show highest inhibition activity on α-glucosidase enzyme as compared to α-amylase [9]. Plants contain different chemical compounds which show α-amylase inhibitory potential which can be used therapeutically for carbohydrate hydrolyzation and maintaining blood glucose level in diabetes patients [10].

Obesity is a root cause of diabetes mellitus and cardiovascular disorders and also related with the growth of breast, liver, ovaries, prostate, bladder, colon and kidney malignancies. Lipase inhibitors show alternative effect for weight control. Chalcone and xanthine derivatives have anti-lipase activity. From 19 chalcones only isoliquiritigenin and licuroside established an effect on preventing weight gain and increase in total cholesterol and triglycerides from their high lipase inhibition activity, obtained from plants [11].

Orange peel oil has major compound d-limonine while propolis contains phenolic, flavonoids and terpenes which show anti-diabatic activity. 70% ethanol is best solvent for propolis extraction which have higher α-amylase inhibitory activity [12]. Hypertension increase the blood pressure which is the main cause of severe diseases including cardiovascular disease, cerebrovascular disease, diabetes and renal failure in 20% to 45% of worldwide population. Oil extracted Chia meal waste is a main source of protein which is hydrolyzed by using different enzymes such as pepsin, trypsin and *α*- chymotrypsin while pepsin exhibited the highest antihypertensive property by showing highest ACE inhibition potential by interacting with catalytic site through H-bond and hydrophobic interactions [13].
Phenolics, flavonoids, proteins and carotenoids are plant extracted natural compounds with applications on industrial scale specially in pharmaceuticals [14]. Bioactive peptides of protein containing foods are used for treating chronic diseases as natural substitutes for most expensive chemical medicines that are usually used for chronic diseases [15].

α-amylase is a carbohydrate hydrolyzing enzyme and play vital role in blood glucose level in patients of type II diabetes. Many plants have α-amylase inhibition potential like *Raphanus raphanistrum* show highest inhibition activity by using sodium phosphate as a best extraction buffer [16]. The peptide fractions of Pea protein had highest α -amylase inhibitory potential as compared to α -glucosidase due to competitive and non-competitive inhibitors and also possessed pancreatic lipase inhibition activities [17, 18, 19].. Pistachio green hull extract with tannin fraction had higher lipase inhibition activity as compared to non-tannin fraction due to presence of polyphenolic compounds [20].Therefore to the best of our knowledge, we for the first time are trying to study the enzyme inhibition potential of α-amylase, pepsin and lipase of ethyl acetate extract by using maceration and Soxhlet extraction technique and correlating it with different phytochemicals analysis also.

2. Materials and methods

2.1. Chemicals

All the chemicals that were used in our project were of high quality and were from recognized chemical companies like Sigma-Aldrich, Merck etc. The name of the used chemicals were DNS, ethyl acetate, DMSO, Tris HCl, Bradford reagent, phenol red, lipase, alpha amylase, pepsin, catechin and FC reagent.

2.2. Plant collection

The needles of this plant were collected from Muzaffarabad, Azad Kashmir, Pakistan and taxonomic identification was done by Department of Botany, University of agriculture Faisalabad and a voucher number 518-12-2018 was issued. The entire research work was completed in Bioactive Molecules Research Lab (BMRL), Department of Biochemistry, University of Agriculture Faisalabad, Pakistan.

2.3. Extraction

Bioactive compounds were extracted by using Soxhlet and maceration extraction techniques with ethyl acetate as organic solvent following the procedure of Palmieri *et al*., [21] with slight modifications. Soxhlet extraction was done by the ratio of 1:10 (g/mL) at 63 ºC for four days. Maceration was also done by the ratio of 1:10 at 250 rpm for 4 days and got extract after filtration by using filter paper (Whatman No. 1). 0.47 g gel like viscous end product was obtained from maceration and 4.95 g from Soxhlet extraction from 20 g each plant powder followed by evaporation at 60 ºC on dry bath.

2.4. Preparation of samples

0.2 g extract of Soxhlet and maceration was dissolved in 2 mL DMSO and labeled these samples S1 and E1. Then further dilution of sample S1 and E1was done by 1:9 ratio and named them S2 and E2. Ten samples namely Sa, Sb, Sc, Sd, Se and Ea, Eb, Ec, Ed, Ee of different concentrations including 1mg/mL, 2 mg/mL, 3 mg/mL, 4 mg/mL and 5 mg/mL were prepared from both S2 and E2 respectively.

2.5. Lipase inhibitory assay by plate method

Evaluation of lipase inhibition was done by chromogenic olive oil plate test using the method of Patel *et*

al., with slight modifications [22, 35]. Plates medium was prepared by using 2% (w/v) agar with 2.5% (v/v) olive oil and 5% (v/v) phenol red as an indicator and its pH was maintained at 7. Then, lipase solution of 30 mg/mL concentration was prepared by dissolving it into 100 mM Tris-HCl buffer. After this, agar medium was poured in plates and wells were made after gel like solidification. Then 30 uL sample and 30 uL enzyme poured in each well while enzyme itself was used as positive control and DMSO as a negative control. The plates were incubated overnight at 37 °C. After incubation, the color was changed from red to yellow around each well because of pH change from neutral to acidic. The test was performed in triplicates and their mean and SD were calculated by measuring inhibition zone with Vernier caliper.
Inhibition (%) = [(Zone of +ve control-zone of extract)/zone of +ve control] ×100

2.6. α-amylase inhibition by DNS chromogenic method

α-amylase inhibition potential was tested by following the chromogenic method of Akanji *et al.*, with slight modifications [23, 34]. Briefly, starch solution was prepared by dissolving 0.01 g/mL into dH_2O. Then α-amylase solution (0.0024 g/mL) was also prepared by dissolving in $d.H_2O$. After that, 250 uL sample along with 250 uL starch solution was added in each test tube to start the reaction and incubated for 30 min at 37 °C. Then 250 uL enzyme solution was added while negative control was replaced by DMSO and also incubated it at 37 ^{0}C for 30 min. After that, 500 uL DNS was added in each test tube and boiled for 5 min to stop the reaction which change the color from light reddish brown to dark brown. Positive control was much darker than others. After cooling at room temperature 5 mL $d.H_2O$ was added and took absorbance at 540 nm from spectrophotometer. Percentage inhibition was calculated by following formula;

Inhibition (%) = [(Abs control – Abs samples)/ Abs control] × 100

2.7. Pepsin inhibition potential

Pepsin inhibition potential was examined by following the method of Rege *et al.*, with slight modifications [24]. Test solution was prepared in each test tube with the composition of 100 uL plant extract, 100 uL pepsin solution (5 mg/0.01N HCL) which was incubated for 30 min at 37 °C and 200 uL albumin of 5 mg/mL in H_2O as a substrate which was also incubated for 20 min at 37 ^{0}C while DMSO was used as negative control. After that, 1600 µL biuret reagent was added and absorbance was taken at 540 nm with spectrophotometer. Percentage inhibition was calculated by following formula.
Inhibition (%) = [(Abs control – Abs test sample)/ Abs control] × 100

2.8. Determination of total phenolic contents

To estimate the total phenolic content of macerated and Soxhlet extracts Folin–Ciocalteu reagent was used by applying the procedure of Ghafar *et al.*, with slight modifications [25]. For obtaining standard curve from standard solution of gallic acid same procedure was applied. The mean value is reported in gallic acid equivalents (GAE) using units of µg/mg of extract by using formula y=mx + c where m is the slope of calibration curve, y is absorbance, c represents intercept and x concentration from the calibration curve. TPC was calculated as follows;

$$TPC = cV/m$$

Here c is the concentration from calibration curve, V volume of the extract used, and m represents the mass of the extract used.

2.9. Determination of total flavonoid content

Total flavonoid contents from ethyl acetate extract were examined by aluminium chloride colorimetric method following the protocol of Babashpour *et al*., and catechin was used as standard [26]. Then absorbance was taken at 510 nm. Same protocol was performed by for the standard curve of catechin standard solution. The results were evaluated from the calibration curve (y=mx+c) of catechin and reported in catechin equivalent using µg/mg of extract.

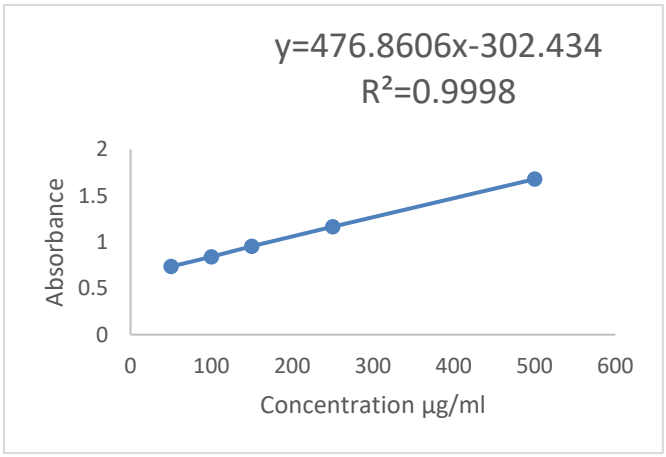

Fig. 1: Standard curve of gallic acid as standard solution for TPC evaluation.

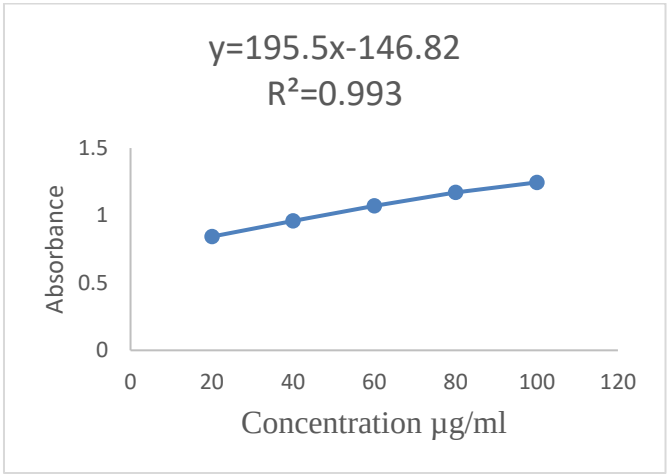

Fig. 2: Standard curve of catechin for TFC evaluation.

2.10. Determination of total protein content

Bradford protein assay was utilized to evaluate total protein content from extracted samples by following the procedure of Jones *et al*., [27].

2.11. Analysis by HPLC

Phenolic compounds analysis was performed by following the procedure of Naeem *et al*., with slight modifications using HPLC system consisting of pump (1500 series), UV spectrophotometer detector (SPD) and column of C18, (250 × 4.6 mm, 5 mm particle sizes). 20 uL sample of 25 ppm was observed at 280 nm and 320 nm by using SPD [28]. Phenolic compounds are evaluated by comparing retention time of standard and sample.

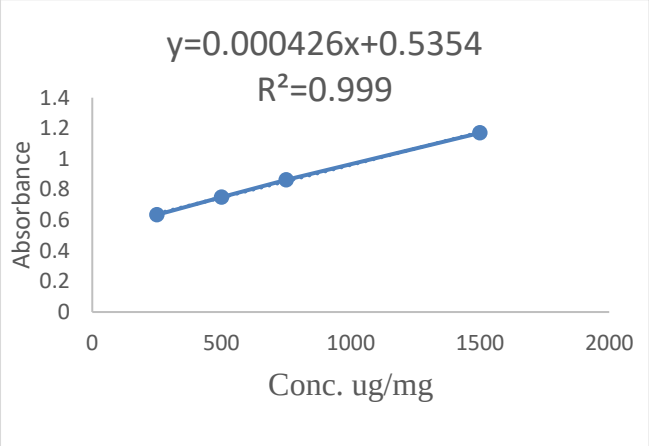

Fig. 3: Standard curve of albumin as standard solution for total protein.

3. Results and Discussion

From last many years research has been increasing for natural products having bioactive roles with low toxicity, high bioactive potential and possess ability to oxidize fats, control appetite, regulate levels of hormones related to obesity and inhibit digestive enzymes involved in the absorption of carbohydrates, protein and lipids. Plants possess all these properties due to which scientists prefer their use abundantly as compared to synthetic compounds [29]. The present project is also planned in following segments to search in *P. wallichiana* from Azad Kashmir.

3.1. α amylase inhibition assay

α amylase inhibition assays was done to check the potential of this plant for drug designing and as an alternative free of side effect. α amylase inhibition potential shows the gradually increase in the inhibition activity by increasing the concentration of the samples. Soxhlet extracted sample shows highest activity as compared to macerated samples (Figure 4). Research shows that the inhibition is affected by changing extraction technique, organic solvents and concentration of sample while inhibition caused by bonding interactions between them. Overall, ethyl acetate extract of *P. wallichiana* needles showed moderate inhibition activity. Maximum activity was in soxhlet extracts with more TPC and TFC (Figure 8, 9) Extract bioactive compounds compete with the substrate for binding to the active site of the enzyme by preventing the breakdown of oligosaccharides to disaccharides. This inhibitory activity is possibly due to the presence of several phytochemicals such as flavonoids, phenolic acid and tannins in it [30]. Inhibition of amylases will slow down the degradation of starch in the gastrointestinal tract by preventing hyperglycemia in an uncompetitive manner. This inhibition leads to reduction in both substrates Km and Vmax and is useful in the design of *α*-amylase inhibitors for the treatment of diabetes mellitus [23].

3.2. Lipase inhibition activity

Lipase enzyme assays also showed concentration dependent activity while the macerated sample showed more inhibition as compared to Soxhlet which shows moderate activity overall (figure 5, 6). Maximum activity was shown by Ee sample with 5 mg/mL concentration i.e. approximately 15% only. The plants show such activities due to presence of phytochemicals such as saponins and polyphenols that can inhibit pancreatic lipase and lighten weight gain in high fat diets [29]. Phenolic acid, stilbenes, anthocyanidins and lignans are also linked in lowering of pancreatic lipase activity by eliciting their inhibitory activity by non-specific binding to enzyme. There are many parameters such as pH, incubation, temperature and concentration which are reasons of optimum inhibition activity [31, 34].

3.3. Pepsin inhibition assay

In pepsin inhibition every sample showed the inhibition in increasing order with the gradually increase in concentrations. Soxhlet extracted sample (Sa) with minimum concentration do not show the inhibition. Overall inhibition study describes that pepsin was maximum inhibited as compared to lipase and amylase. Pepsin has a close resemblance with HIV-protease in proteolytic activity because both of them belong to same aspartate enzyme family so pepsin study is useful for HIV-protease. A HIV protease inhibitor is important due to structural and functional similarities with pepsin [24]. Inhibition of HIV protease may be due to presence of phenolics including tannins, simple phenols and phenolic acids, quinones, flavonoids, flavones and flavanols [32, 33] which are also reported by our research work (Figure 11,12).

3.4. Estimation of total phenolic contents

Soxhlet extracted samples have more TPC as compared to macerated sample (Figure 8). Overall study from research papers describes that *pinus* needles have high amount of phenolics, flavonoids and tennins. Bioactive compounds such as phenolic compounds are the main agents that can donate hydrogen to free radicals and thus break the chain reaction of lipid oxidation at the first initiation step so these compounds are responsible for high bioactive potential [26].

3.5. Evaluation of total flavonoid contents

By comparison it was observed that Soxhlet extract possess (figure 9) more flavonoids content then macerated extract which suggests the use of Soxhlet extraction for medicinal purpose. The presence of flavonoids is responsible for such bioactivities [26].

3.6. Estimation of total protein by Bradford method

In total protein analysis it is estimated that the macerated extract contains much amount of proteins as compared to Soxhlet (Figure 10). So, this concentration of proteins may be reason of pepsin inhibition activity. The presence of phenolic acid in plant tissues is also reason of this inhibition e.g. due to polyquinine complex with protein because these compounds react with protein [27].

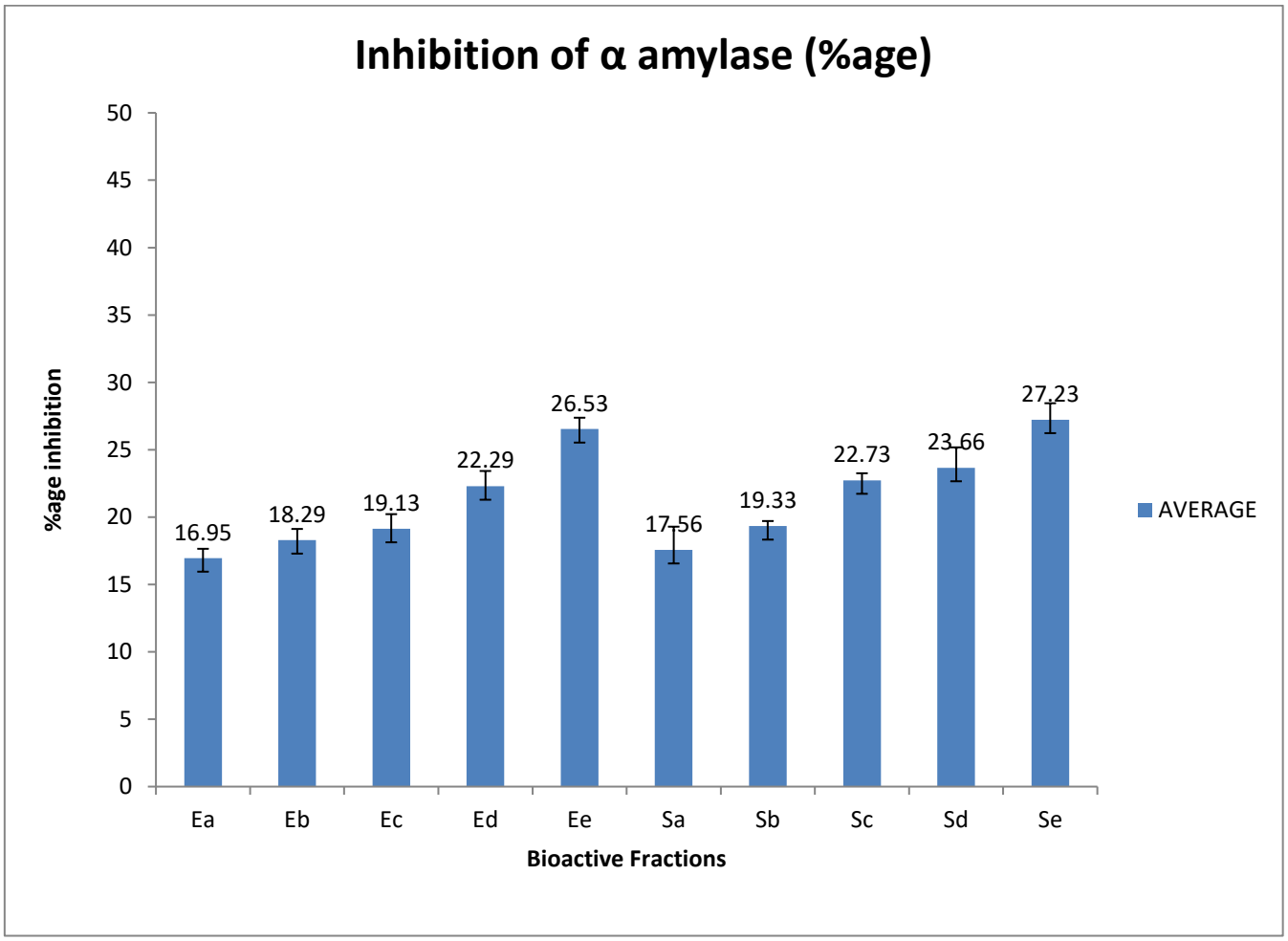

Fig. 4: α-Amylase inhibition of ethyl acetate extracts of different concentrations. Gradual increase in inhibition activity was seen by gradually increasing sample concentrations. Each test was performed in triplicate.

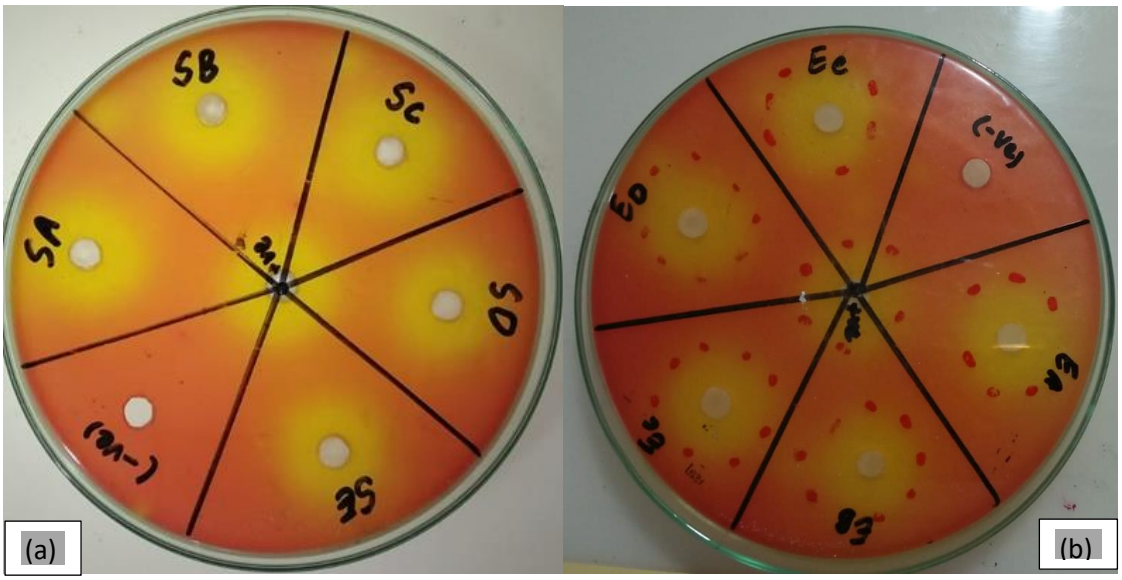

Fig. 5: Lipase inhibition plate method (Phenol red) using Soxhlet (a) and macerated (b) extracts.

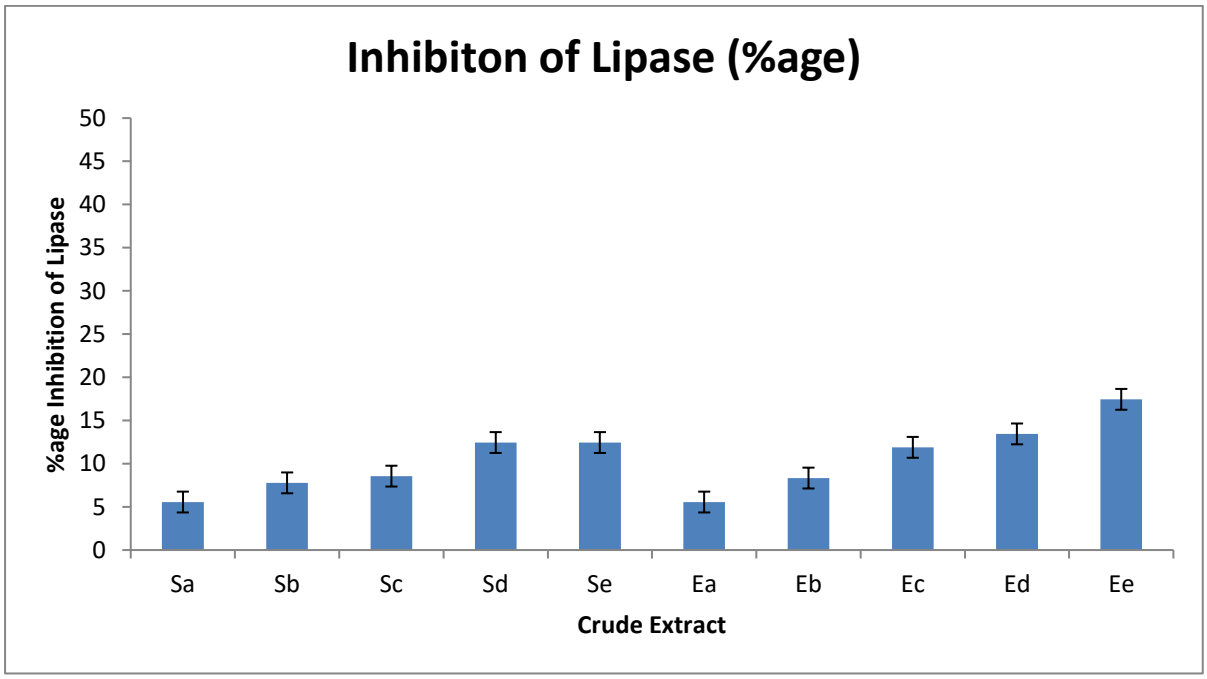

Fig. 6: Lipase inhibition potential of macerated samples at different concentrations.

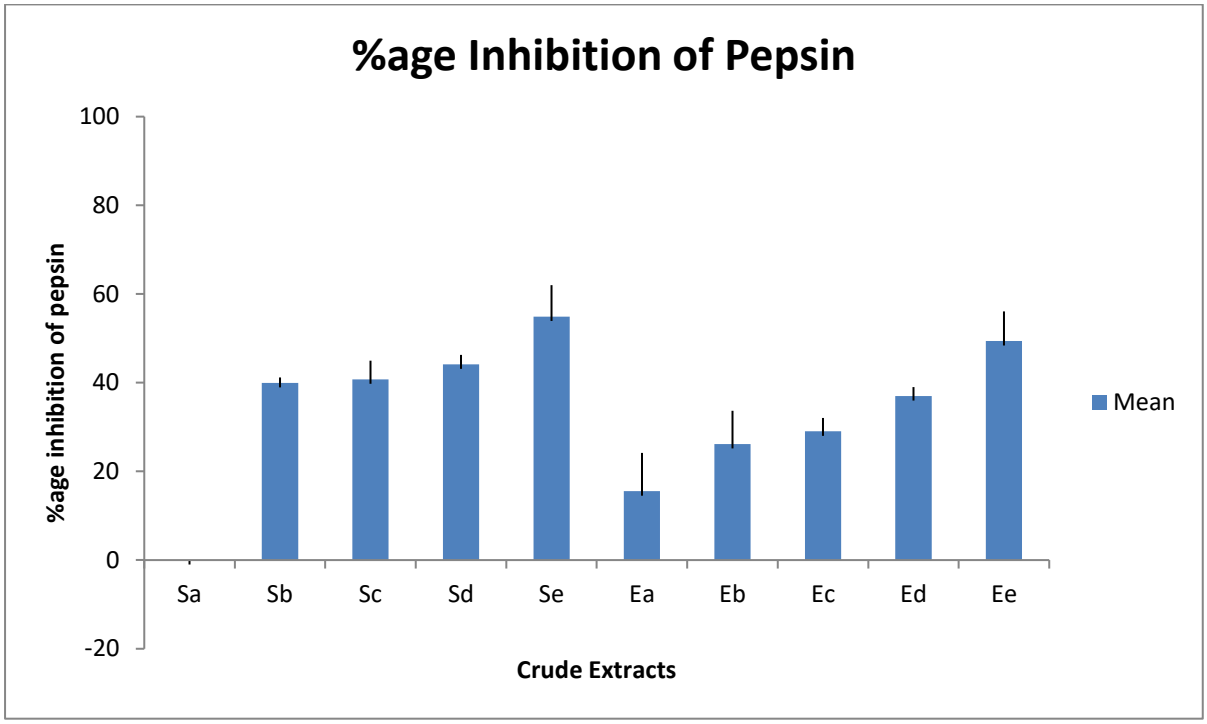

Fig. 7: Pepsin inhibition potential at different Concentrations of macerated and Soxhlet extracts.

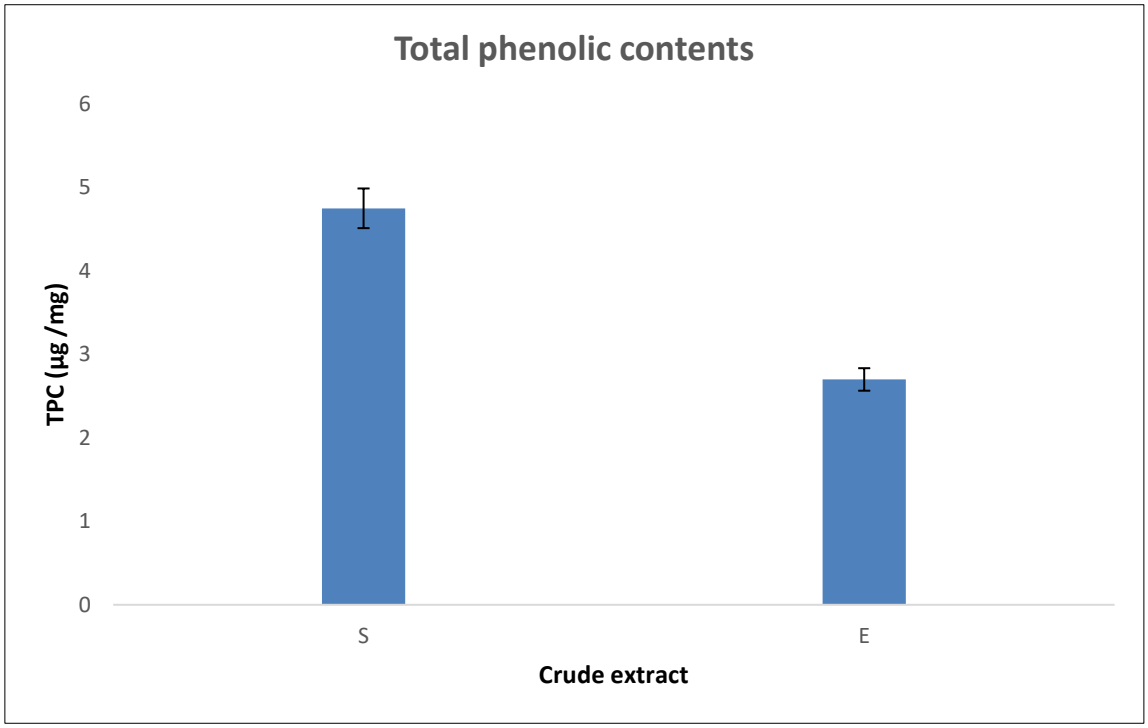

Fig. 8: TPC of Soxhlet and macerated extracts of *P. wallichiana* needles.

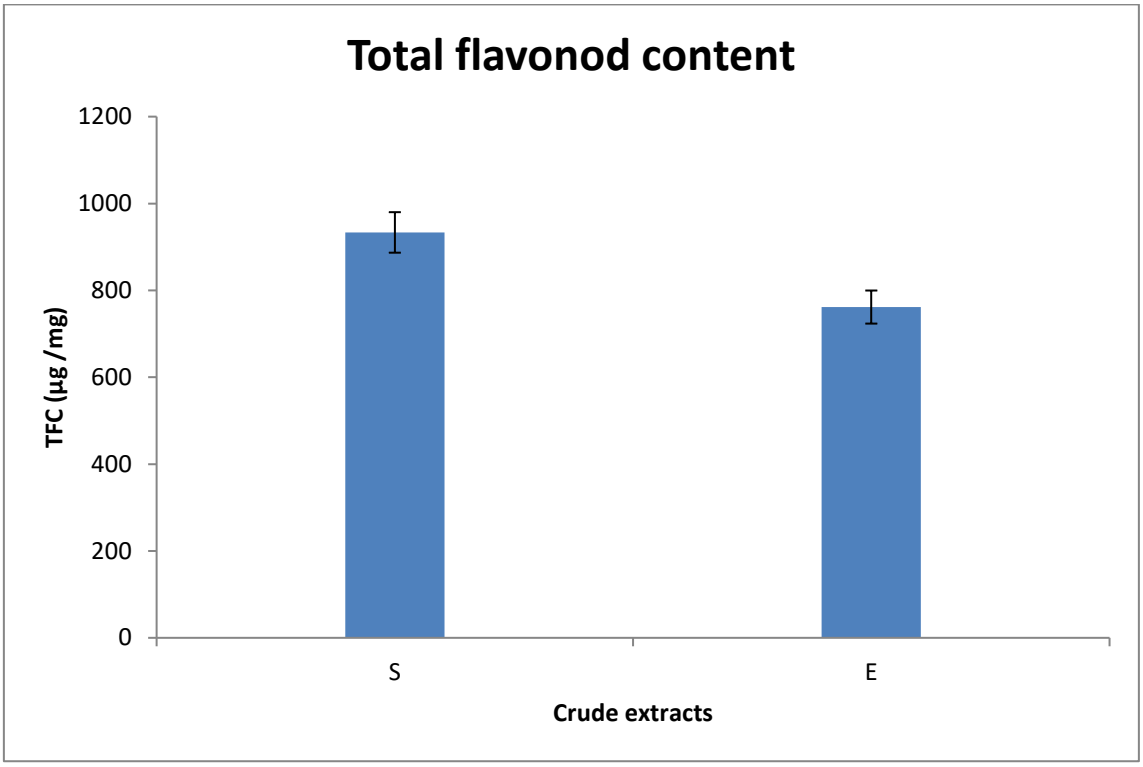

Fig. 9: TFC of Soxhlet and macerated extracts of *P. wallichiana* needles.

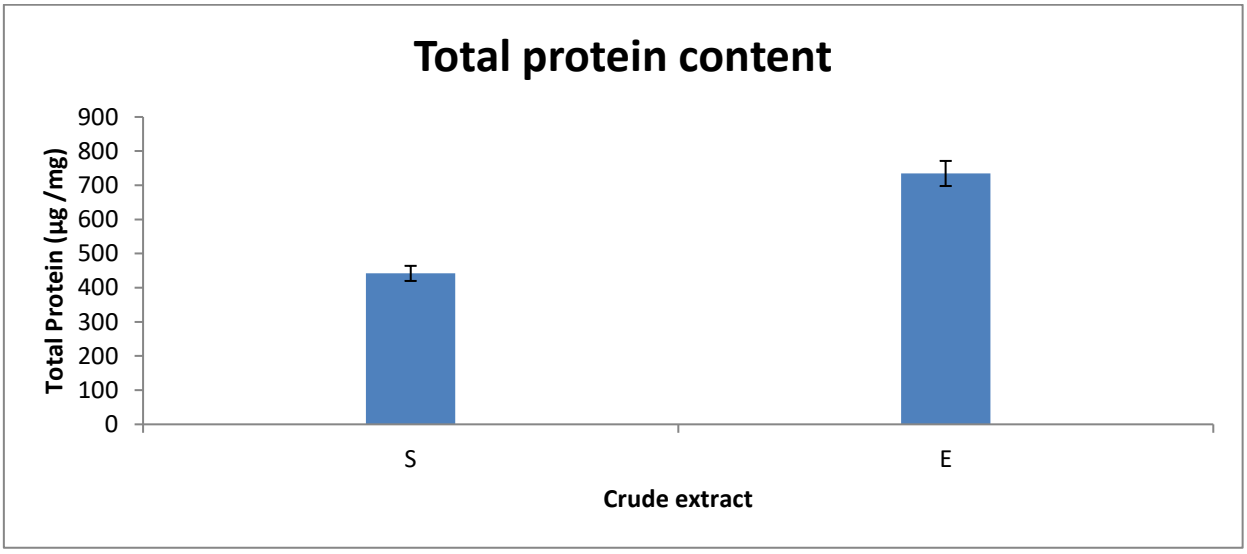

Fig. 10: Total Proteins of Soxhlet and macerated extracts of *P. wallichiana* needles

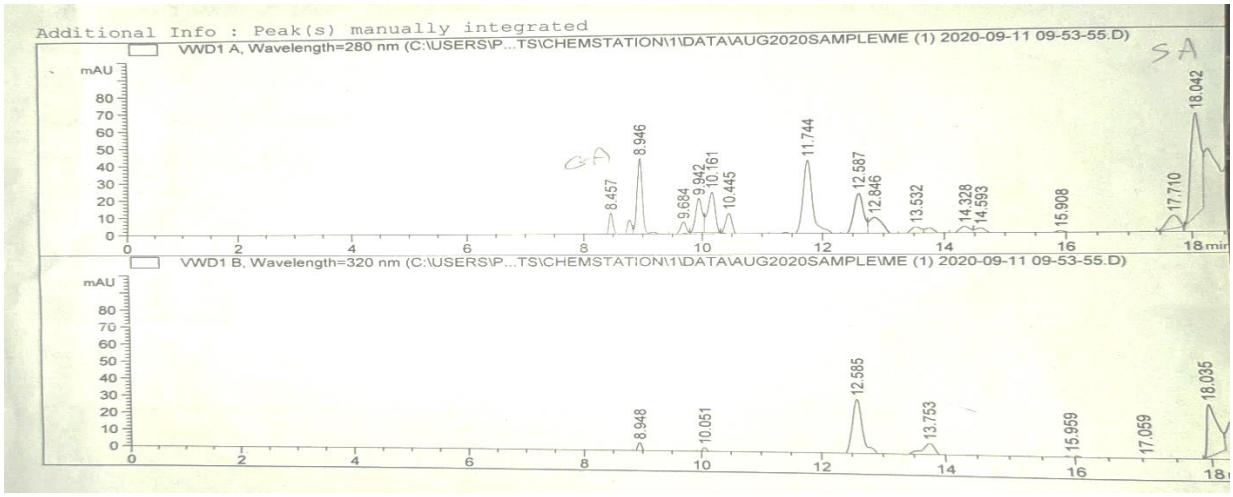

Fig. 11: HPLC Chromatograms of *P. wallichiana* needles macerated extracts.

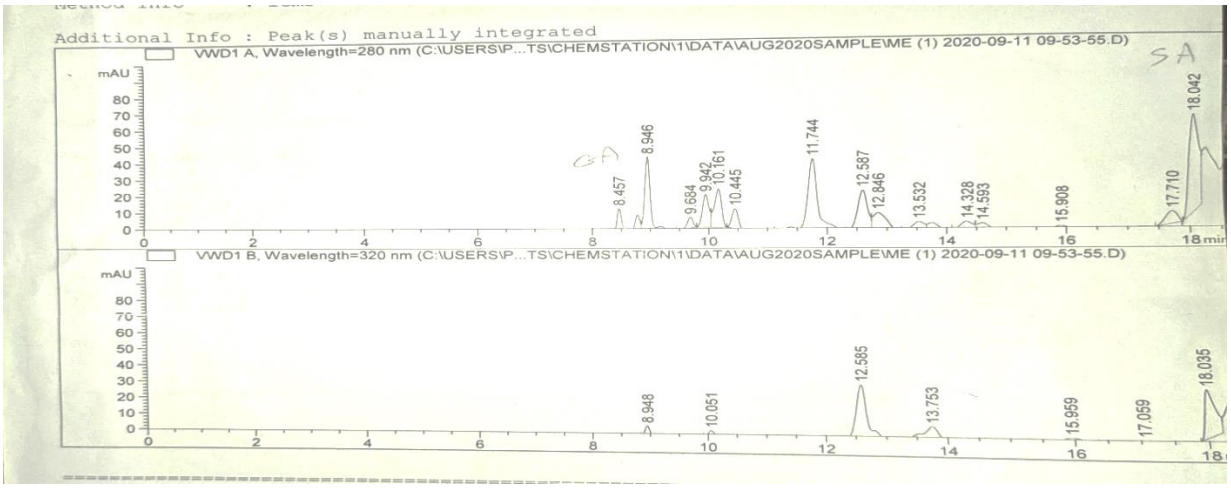

Fig. 12: HPLC Chromatograms of *P. wallichiana* needles Soxhlet extract.

3.7. HPLC analysis of P. wallichiana needles extract

HPLC chromatogram showed the presence of many bioactive compounds. Ethyl acetate extract of *P. wallichiana* needles possesses less bioactive components as compared to extracts of other organic solvents [28]. Soxhlet extracted sample had better inhibition and bioactivities results then the macerated one. At 280 nm (SPD) showed more bioactive phytochemicals as compared to 320 nm shown in (Fig. 11, 12). Overall, the main detected bioactive compounds are phenolics and flavonoids like gallic acid, vanillic acid, caffiec acid and sanapic acid detected by comparing their retention times with standards, these are responsible for the bioactive potential of this plant extracts.

3.8. Statistical analysis

These experiments were conducted in completely randomized design (CRD) with three replications. Statistical analyses were done using the SAS 9.1 program. Data was subjected to analysis of variance and means were separated using LSD test at P< 0.01 significance level.

4. Conclusions

Overall our research describes that *P. wallichiana* needles extract have moderate inhibition activities while concentration dependent behavior was observed. Soxhlet extraction yielded more crude extract in amounts then the maceration method. According to overall research review study ethyl acetate have minimum or moderate inhibition activity in other plants too. In some cases enzymes inhibition assays the Ee or Se was more active due to the differences in their constituents which we studied like TFC, TPC and Total proteins. HPLC analysis although detected less number of compounds but yet showed presence of famous phytochemicals which already have reported same activities. This is perhaps first reported data by our findings for *P. wallichoiana* needle from Azad Kashmir and with ethylacetate solvent showing the potential presence of inhibitors of different digestive enzymes thus these smples can play a vital role in future as a medicinal use specially for digestive system and will also be helpful to control overweight and diabetes.

References

[1] A. Sharma, L. Sharma and R. Goyal. (2018). A review on himalayan pine species: Ethnopharmacological, phytochemical and pharmacological aspects. Pharmacognosy Journal. 10: 611-619. DOI:10.5530/pj.2018.4.100

[2] T.U. Rahman, G. Uddin, K.F. Khattak, W. Liaqat and M.I. Choudhary. (2016). Antibacterial, antifungal, insecticidal and phytotoxic activities of leaves of *Pinus wallichiana*. Journal of Chemical and Pharmaceutical Research. 8: 420-424.

[3] I.K. Nazish, K. Khan, S. Azam and I.A.a.I. Ullah. (2018). An insight into in vitro and in vivo pharmacological activities of pinus wallichiana. Pak. J. Bot. 50: 1487-1497.

[4] N. Khan, I. Khan, A. Nadhman, S. Azam, I. Ullah, F. Ahmad, H.A. Khan and Reviews. (2020). *Pinus wallichiana*-synthesized silver nanoparticles as biomedical agents: in-vitro and in-vivo approach. Green Chemistry Letters. 13: 69-82.

[5] L. Singh, P. Dixit, R.P. Srivastava, S. Pandey, P.C. Verma and G. Saxena. (2019). Ethnobotany and Pharmacology of Pinus Species Growing Naturally in Indian Himalayas: A Plant Review. Current Pharmaceutical Biotechnology. 20: 1281-1287.

[6] A. Briones and C. Chichioco-Hernandez. (2017). Lipase inhibitory activity of *Carica papaya*, *Chrysophyllum cainito*, *Corcorus olitorius*, *Cympogon citrates* and *Syzygium cumini* extracts. Food Research. 2: 51-55.

[7] T. Zhao, L. Sun, Z. Wang, T. Nisar, T. Gong, D. Li, P. Niu and Y. Guo. (2019). The antioxidant property and α-amylase inhibition activity of young apple polyphenols are related with apple varieties. LWT. 111: 252-259.

[8] M.P. Patil and R.H. Patil. (2019). Data on the inhibitory effect of endophytic fungi of traditional medicinal plants against pancreatic lipase (PL). Data in brief. 27: 104797. Doi: org/10.1016/j.dib.2019.104797

[9] H. Laoufi, N. Benariba, S. Adjdir and R. Djaziri. (2017). *In vitro* α-amylase and α-glucosidase inhibitory activity of Ononis angustissima extracts. J. Appli. Pharm. Sci. 7: 191-198.

[10] S. Hussein, A. Kaluf, Y. Ahmed, B. Ahmed and Iyad. (2020). Determination of inhibition activity of α-amylase enzyme, antioxidant activity, antibacterial activity and phenolic compounds by using some medical plants. The Iraqi Journal of Agricultural Science. 51: 411-421.

[11] D.N.H. Tam, E.M. Mostafa, V.L. Tu, A.I. Rashidy, E. Matenoglou, M. Kassem, D.T. Soa, A. Bayumi, H.E.S. Emam and L. Tran. (2020). Efficacy of chalcone and xanthine derivatives on lipase inhibition: A systematic review. Chemical Biology Drug Design. 95: 205-214.

[12] Ş. Keskin. (2020). Orange peel volatile oil: A green solvent for propolis extraction, enhanced α-amylase inhibition activity. Flavour Fragrance Journal. 35: 411-416.

[13] B. San Pablo-Osorio, L. Mojica and J.E. Urías-Silvas. (2019). Chia Seed (*Salvia hispanica L.*) Pepsin Hydrolysates Inhibit Angiotensin-Converting Enzyme by Interacting with its Catalytic Site. Journal of food science. 84: 1170-1179.

[14] P. Ferreira-Santos, Z. Genisheva, C. Botelho, J. Santos, C. Ramos, J.A. Teixeira and C.M. Rocha. (2020). Unravelling the Biological Potential of *Pinus* pinaster Bark Extracts. Antioxidants. 9: 334. Doi: 10.3390/antiox9040334

[15] S.N. Mazloomi-Kiyapey, A. Sadeghi-Mahoonak, E. Ranjbar-Nedamani and E. Nourmohammadi. (2019). Production of antioxidant peptides through hydrolysis of medicinal pumpkin seed protein using pepsin enzyme and the evaluation of their functional and nutritional properties. Arya Atherosclerosis. 15: 218–227. Doi: 10.22122/arya.v15i5.1755

[16] S. Hussein, A. Kaluf, Y. Ahmed, B. Ahmed and A. Iyad. (2020). Determination of inhibition activity of α-amylase enzyme, antioxidant activity, antibacterial activity and phenolic compounds by using some medical plants. Journal of Agricultural Science. 51: 411-421.

[17] T.O. Awosika, R.E. Aluko and Technology. (2019). Inhibition of the in vitro activities of α-amylase, α-glucosidase and pancreatic lipase by yellow field pea (*Pisum sativum L.*) protein hydrolysates. International Journal of Food Science. 54: 2021-2034.

[18] A.B. Shori. (2020). Inclusion of phenolic compounds from different medicinal plants to increase α-amylase inhibition activity and antioxidants in yogurt. Journal of Taibah University for Science. 14: 1000-1008.

[19] G. Izvekova and T. Frolova. (2017). Proteolytic enzymes and their inhibitors in cestodes. Biology Bulletin Reviews. 7: 150-159.

[20] Z. Noorolahi, M.A. Sahari, M. Barzegar and H. Ahmadi Gavlighi. (2020). Tannin fraction of pistachio green hull extract with pancreatic lipase inhibitory and antioxidant activity. Journal of Food Biochemistry. 44: Doi: 10.1111/jfbc.13208. e13208.

[21] S. Palmieri, M. Pellegrini, A. Ricci, D. Compagnone and C. Lo Sterzo. (2020). Chemical Composition and Antioxidant Activity of *Thyme*, *Hemp* and *Coriander* Extracts: A Comparison Study of Maceration, Soxhlet, UAE and RSLDE Techniques. Foods. 9: 1221. Doi: 10.3390/foods9091221.

[22] M. Patil, R. Patil, B. Bhadane, S. Mohammad, V. Maheshwari and a. biotechnology. (2017). Pancreatic lipase inhibitory activity of phenolic inhibitor from endophytic Diaporthe arengae. Biocatalysis. 10: 234-238.

[23] M.A. Akanji, S.O. Olukolu, M.I. Kazeem and C. Longevity. (2018). Leaf extracts of aerva lanata inhibit the activities of type 2 diabetes-related enzymes and possess antioxidant properties. Oxidative Medicine and Cellular Longevity. 2018: Doi: 10.1155/2018/3439048

[24] A.A. Rege, Ramkrishna Y Chowdhary, Abhay S. (2014). Effect of Costus Pictus D. Don. on pepsin enzyme. Internationnal Journal of Pharmacy and Pharmaceutical Sciences. 6 (5):178-180.

[25] F. Ghafar, T. Nazrin, M. Salleh, N.N. Hadi, N. Ahmad, A.A. Hamzah, M. Yusof and I.N. Azman. (2017). Total phenolic content and total flavonoid content in *Moringa oleifera* seed. Galeri Warisan Sains. 1: 23-25.

[26] M. Babashpour-Asl, M. Piryaei and Technology. (2021). Free Radical Scavengering and Phenolic Compounds of Peel and Pulp of *Quince*. International Journal of Horticultural Science. 8: 91-101.

[27] C.G. Jones, J.D. Hare and S.J. Compton. (1989). Measuring plant protein with the Bradford assay. Journal of chemical ecology. 15: 979-992.

[28] I. Naeem, A. Taskeen, H. Mubeen and A. Maimoona. (2010). Characterization of flavonols present in barks and needles of *Pinus wallichiana* and *Pinus roxburghii*. Asian Journal of Chemistry. 22: 41-44.

[29] J.O. Unuofin, G.A. Otunola and A.J. Afolayan. (2018). In vitro α-amylase, α-glucosidase, lipase inhibitory and cytotoxic activities of tuber extracts of Kedrostis africana (L.) Cogn. Heliyon. 4: Doi: 10.1016/j.heliyon.2018. e00810.

[30] M. Kazeem, J. Adamson and I. Ogunwande. (2013). Modes of inhibition of α-amylase and α-glucosidase by aqueous extract of *Morinda lucida* Benth leaf. BioMed research international. 2013: 6. Doi: 10.1155/2013/527570

[31] J.O. Unuofin, G.A. Otunola and A.J. Afolayan. (2019). Inhibition of Key Enzymes Linked to Obesity and Cytotoxic Activities of Whole Plant Extracts of Vernonia mesplilfolia Less. Processes. 7: 841. Doi: 10.3390/pr7110841

[32] A. Rege, A.S.S.R. Chowdhary and Research. (2014). Evaluation of *Ocimum sanctum* and *Tinospora cordifolia* as probable HIV protease inhibitors. Internationnal Journal of Pharmaceutical. 25: 315-318.

[33] K. Singh1â and B. Upadhyay. (2010). Screening of *Adhatoda vasica Nees* as a putative HIV-protease inhibitor. Journal of Phytology. 2: 78–82.

[34] T. Sikander, A.A. Bhatti, Z. Mushtaq, M. Shahid, F. Nighat and S. Anwar. (2021). Evaluation of the bioactive role of indigenous *Viola odorata* leaf extracts. Pakistan Journal of Botany. 53(2): 541-549.

[35] F. Nighat, Z. Mushtaq, M. Maqsood, M. Shahid, M.A. Hanif, and A. Jamil. 2020. Cytotoxic, a-amylase inhibitory and thrombolytic activities of organic and aqueous extracts of *Bacillus clausii* KP10. Pakistan Journal of Pharmaceutical Sciences, 33(1):135-139.