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ANTIOXIDANT AND THROMBOLYTIC ACTIVITY OF SOUTH INDIAN DATEPALM- *PHOENIX PUSILLA*

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ABSTRACT

The present investigation was carried out to evaluate the antioxidant, free radical scavenging and thrombolytic potential of fruit and seed methanolic extract of *Phoenix pusilla.* The enzymatic and non-enzymatic antioxidant assays, Thrombolytic and fibrinolytic activity were also determined using *in vitro* methods. Inhibitions of lipid peroxidation in the egg of hen were determined using a modified method thiobarbituric acid- reactive species (TBARS) assay. The fruit pulp showed higher antioxidant activity than the seed extract which is due to the difference in the presence of phytoconstituents in them. The free radical scavenging activities of the methanol extracts of both the samples were dose dependent with all the radicals. The lysis of clot increases exponentially with time. The percentage of clot lysis produced by pulp extract is higher than the seed extract. Thus, the extracts possess considerable thrombolytic and anticoagulant activity. Such a discovery is crucial as it provides clues for potential treatment of blood coagulant disorders.

KEYWORDS: Antioxidant, *Phoenix pusilla*, thrombolytic, free radicals, lipid peroxidation.

INTRODUCTION

Living cells may generate free radicals and other reactive oxygen species by-products as a results of physiological and biochemical processes. Free radicals can cause oxidative damage to lipids, proteins and DNA, eventually leading to many chronic diseases, such as cancer, diabetes, aging, and other degenerative diseases in humans.[1] The ingestion of natural antioxidants has been associated with reduced risks of cancer, cardiovascular disease, diabetes, and other diseases associated with ageing[2] and in recent years, there has been a worldwide trend toward the use of natural phytochemicals present in berry crops, teas, herbs, oil-seeds, beans, fruits and vegetables.[3] Recently, the demand for fruit and fruit products has increased considerably, as fruits are well-known storehouse of polyphenols and phytonutrients which possess antioxidant activities.[4] Phytochemical screening of various plants has been reported by many workers. These studies have revealed the presence of numerous chemicals, including alkaloids, flavonoids, steroids, phenols, glycosides, and saponins. The phenolic compounds are one of the largest and most ubiquitous groups of plant metabolites. A number of studies have focused on the biological activities of phenolic compounds, which are antioxidants and free radical scavengers. The crude extracts of herbs, spices and other plant materials rich in phenolics and flavonoids are of increasing interest in the food industry because they retard oxidative degradation of lipids and thereby improve the quality and nutritional value of food.[5]

Since ancient times, herbal preparations have been used for the treatment of several diseases. The leaves and or twigs, stem, bark and underground parts of plants are most often used for 27 traditional medicines.[6] Considerable efforts have been directed towards the discovery and development of natural products from various plant and animals which have anti-platelet, anticoagulant antithrombotic, and thrombolytic activity. This method was done to investigate whether extract from plants possess thrombolytic activity or not. One of the major causes of blood circulation problem is the formation of blood clots. Thrombo embolic disorders such as pulmonary emboli, deep vein thrombosis, strokes and heart attacks are the main causes of morbidity and mortality in developed countries. Thrombi can lodge in a blood vessel and block the flow of blood in that location depriving tissues of normal blood flow and oxygen. This can result in damage, destruction or even death of the tissues (necrosis) in that area. Atherothrombotic diseases such as myocardial or cerebral infarction are also serious consequences of the thrombus formed in blood vessels.[7] Various thrombolytic agents are used to dissolve the

clots that have already formed in the blood vessels. One of the major causes of blood circulation problem is the formation of blood clots. Thrombi or emboli can lodge in a blood vessel and block the flow of blood in that location depriving tissues of normal blood flow and oxygen. Commonly used thrombolytic agents are alterplase, anistreplase, streptokinase, urokinase and tissue plasminogen activator (tPA) to dissolve clots.[8] Streptokinase is an antigenic thrombolytic agent used for the treatment of acute myocardial infarction. It reduces mortality as effectively as the nonantigenic altreplase in most infarct patients while having the advantages of being much less expensive. Tissue-type Plasminogen activator (tPA) is generally preferred as being effective and safer than either urokinase or streptokinase type activators.[9] Streptokinase forms a complex with plasminogen which then converts plasminogen to plasmin. Plasmin breaks down clots as well as fibrinogen and other plasma proteins. [10]

Phoenix pusilla Gaertn., (Family: Arecaceae) a multipurpose palm species closely related to the date palm, is commonly known as the small date palm in India, as it only grows to 100 cm tall.[11] It is a beautiful shrubby suckering palm with a very short stem enveloped in persistent leaf sheaths. A crown of about 15-17 leaves is produced every year. Just like the true date palm, Phoenix dactylifera it is dioecious, producing male and female flowers on separate trees. It grows wild in dry areas in India at low elevations. Its flowering season starts in November and runs through January. Clusters of edible orange-red fruits turn into black drupes in the months of July and August.[12] The pulp of the fruit is fleshy, sweet and mealy. The tender part of the palm is often eaten by the poorer people as a meal called kanji. The leaflets are woven into mats and the split petioles into baskets. Brooms were also made out of the leaves of this palm. Its fruit is used in herbal medicines, as it is sweet, sour, cooling and laxative, cardiotonic, aphrodisiac, carminative and roboranto. The fruit is also used for hyperdipsia, burning sensation, fevers, consumption, cardiac debility, seminal weakness, gasteropathy and general debility. Various ethanobotanical surveys give information that it commonly existing in Srilanka and South India.[13] Nucleated succession study of *P.pusilla* reveals that it cools the soil as well as found to decrease the radiation and so it was identified as better nurse plant to improve the biodiversity conservation.[14]

Heparin and Aspirin are just decently efficient for acceleration of lysis and prevention of reocclusion, however are safe. More particular thrombin inhibitors and antiplatelet agents are more powerful, yet their safety stays to be affirmed. Proceeded investigation in this area will provide new insights and advance advancement toward the improvement of the perfect thrombolytic treatment, characterized by maximized stable coronary arterial thrombolysis with minimal bleeding. Hence, the point of the present research is to find out antioxidant and thrombolytic activity of methanolic extract of fruit pulp and seed from *Phoenix pusilla*.

MATERIALS AND METHODS

Preparation of the Sample

The fruit pulp and seed of *Phoenix pusilla* were separated after drying the whole fruit. They were soaked in methanol separately [25g in 100ml] and left undisturbed for 24hrs. Then the solution was filtered and to the filtrate methanol was added repeatedly till 72hrs for every 24 Hrs. The filtered solution were pooled and dried for the evaporation of the solvent. Then they were dissolved in methanol at concentration of 1mg/ml and used for further analysis.

Total antioxidant activity and Free radical Scavenging Assays

The total antioxidant and radical scavenging activity of the methanolic extract of fruit pulp and seed of *Phoenix pusilla* was determined by standard protocol. *In vitro* assays such as DPPH assay, Hydrogen peroxide activity, Nitric oxide scavenging assay, Ferric reducing power assay, Deoxyribose non-site specific hydroxyl radical scavenging activity, Superoxide radical, ABTS assay, estimation of lipid peroxidation using egg yolk and β carotene linoleic acid assay was carried out based on the protocol followed by [42].

Enzymatic and Nonenzymatic Antioxidant Assays

Enzymatic and non- enzymatic antioxidant assay like Catalase activity (Sinha A K, 1972), Superoxide dismutase [15], Determination of Vitamin E [16] and Vitamin C [17] was carried out for both methanolic extract of fruit pulp and seed of *Phoenix pusilla*.

Thrombolytic activity

2ml of venous blood (n= 4) were drawn from healthy human volunteers without a history of oral contraceptive or anticoagulant therapy which were taken in to different pre-weighed sterile microfuge tube. The microfuge tubes were incubated at 37°C for 45 minutes. After clot formation, serum was completely removed without disturbing the clot and each tube having clot was again weighed to determine the clot weight (clot weight = weight of clot containing tube – weight of tube alone). Then to each of the microfuge tube containing clot, 100 µl of fruit pulp and seed extract, 100 µl of streptokinase (positive control) and 100 µl of distilled water (negative control) were separately added. All the tubes were then incubated at 37°C for 90 minutes and observed for clot lysis. After incubation, the released fluid was discarded and tubes were again weighed to observe the difference in weight after clot disruption.[18]

Finally percentage of clot lysis was determined as % of clot lysis = (wt of released clot /clot wt) × 100.

Evaluation of Fibrinolytic Activity

For determination of Fibrinolytic activity, 4 ml venous blood drawn from healthy volunteers was distributed in four different pre-weighed sterile micro centrifuge tube

(0.5 ml/tube) and incubated at 37 °C for 45 minutes. After clot formation, serum was completely removed without disturbing the clot and each tube having clot was again weighed to determine the clot weight (clot weight = weight of clot containing tube-weight of tube alone). To one micro centrifuge tube containing pre-weighed clot, 100 µl of each extract of fruit and pulp were added. As a positive control, 100 µl of SK and as a negative non fibrinolytic control, 100 µl of distilled water were separately added to the control tubes numbered. All the tubes were then incubated at 37 °C for 90 minutes and observed for clot lysis. After incubation, fluid released was removed and tubes were again weighed to observe the difference in weight after clot disruption. Difference obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis. The experiment was repeated three times with the blood samples of 20 volunteers. The significance between % clot lysis by Streptokinase, fruit pulp and seed extract, water by means of weight difference was determined.[19]

Anticoagulant activity

Collection of blood samples

The blood samples were obtained from normal individuals by using sterile syringes, withdrawn from vein of right arm of each individual and placed separately in containers containing tri-sodium citrate to prevent the clotting process. Centrifugation (15 minutes at rate 3000 rpm) was carried out to separate the blood cells from plasma in order to obtain pure platelet plasma (ppp) for prothrombin time test. The obtained plasma sample of each individual were poured separately in plane containers using automatic pipette and stored at room temperature.

Collection of blood and Plasma re-calcification

0.2 ml plasma, 0.1 ml of fruit pulp and seed extract of different concentration and different volume of CaCl2 (25 *m*M) were added together in a clean fusion tube and incubated at 37°C in water bath. For control experiment extract solution was replaced by same volume of 0.9% saline water. The clotting time was recorded with stopwatch by tilting the test tubes every 5 seconds. This time is called the prothrombin time.[20]

RESULTS AND DISCUSSION

Total antioxidant activity

Table 1: Total Antioxidant Activity of Methanolic Extract of Fruit Pulp and Seed.

S.No	Concentration µl	Standard OD	Fruit pulp OD	Seed OD
1	100	0.002	0.004	0.006
2	200	0.006	0.009	0.014
3	300	0.008	0.012	0.021
4	400	0.012	0.017	0.029
5	500	0.017	0.021	0.034

The OD Values are Mean of Triplicates

An antioxidant has been defined as any substance that when present in low concentrations compared with that of an anti-oxidisable substrate, significantly delays or inhibits the oxidation of that substrate. Recently, the demand for fruit and fruit products has increased considerably, as fruits are well-known storehouse of polyphenols and phytonutrients which possess antioxidant activities. The total antioxidant activity of standard ascorbic acid and test samples were taken in the concentration of 100 – 500 µg/ml and the OD values were noted in the **Table 1**. The fruit pulp showed higher antioxidant activity than the seed extract which is due to the difference in the presence of phytoconstituents in them. This supports earlier reports, correlating the presence of phenolic compounds to antioxidative actions.[21,22] Variation observed in antioxidant activities solely depends on varieties, location and growth conditions.[23]

Free Radical Scavenging Assays of methanolic extract of *Phoenix pusilla* (Fruit pulp and seed)

DPPH radical scavenging assay

In DPPH scavenging assay, the antioxidant activity was measured by the decrease in absorbance as the DPPH radical received an electron or hydrogen radical from an antioxidant compound to become a stable diamagnetic molecule.[24] In the present study, the methanolic extract of *P pusilla* showed a good antiradical activity by scavenging DPPH radical. The methanolic extract of both fruit pulp and seed was found to exhibit better inhibition of DPPH radical with an IC50 value of 337.473µg/ml and 323.100 µg/ml respectively **(Table 2)**.

Table 2: DPPH Radical Scavenging Activity of Methanolic Extract of Fruit Pulp and Seed.

S.No	Concentration µl	Standard OD	Standard %	Fruit pulp OD	Fruit pulp %	Seed OD	Seed %	Control OD
1	100	0.956	13.72	0.922	16.79	0.912	17.69	1.108
2	200	0.821	25.90	0.812	26.71	0.798	27.98	
3	300	0.685	38.17	0.599	45.94	0.583	47.38	
4	400	0.612	44.77	0.448	59.57	0.421	62.00	
5	500	0.391	64.71	0.293	73.56	0.252	77.26	
IC 50 Values		-	403.815	-	337.473	-	323.100	

The OD Values are Mean of Triplicates

Hydrogen peroxide scavenging activity

Table 3: H_2O_2 Radical Scavenging Activity of Methanolic Extract of Fruit Pulp and Seed.

S.No	Concentration µl	Standard OD	Standard %	Fruit pulp OD	Fruit pulp %	Seed OD	Seed %	Control OD
1	100	1.615	23.68	1.589	24.91	1.563	26.13	2.116
2	200	1.259	40.50	1.198	43.38	1.175	44.47	
3	300	0.954	54.91	0.817	61.39	0.796	62.38	
4	400	0.612	71.08	0.565	73.30	0.533	74.81	
5	500	0.333	84.26	0.193	90.88	0.179	91.54	
IC 50 Values		-	267.800	-	245.805		238.781	

The OD Values are Mean of Triplicates

H_2O_2 is rapidly decomposed into oxygen and water and this may produce hydroxyl radicals ($OH^{\cdot}$) that can initiate lipid peroxidation and cause DNA damage in the body[25] The H_2O_2 radical scavenging potential of fruit pulp and seed was determined and the values were recorded. The percentage scavenging ability and IC 50 values of each samples were also calculated (**Table 3**). The hydroxyl radical scavenging activities of the methanol extracts of both the samples were dose dependent. The highest activity among the samples was expressed by the fruit pulp with IC50=245.805 µg/ ml. This was followed by the seed with IC50=238.78 µg/ ml.

Nitric oxide scavenging activity

Nitric oxide is generated when sodium nitroprusside reacts with oxygen to form nitrite. Some samples inhibit nitrite formation by competing with oxygen to react with nitric oxide directly. These compounds alter the structure and function of many cellular components. Any compound, natural or synthetic, with antioxidant properties might contribute towards the partial or total alleviation of this damage.[26] The NO radical scavenging ability and IC 50 values of each samples were also calculated **(Table 4)**. The nitric oxide radical scavenging activities of the methanol extracts of both the samples were dose dependent. The highest activity among the samples was expressed by the fruit pulp with IC50=325.477µg/ ml. This was followed by the seed with IC50=289.891µg/ ml.

Table 4: NO Radical Scavenging.

S.No	Concentration µl	Standard OD	Standard %	Fruit pulp OD	Fruit pulp %	Seed OD	Seed %	Control OD
1	100	1.653	14.53	1.678	13.24	1.611	16.70	1.934
2	200	1.291	33.25	1.321	31.70	1.245	35.63	
3	300	0.889	54.03	0.997	48.45	0.843	56.41	
4	400	0.632	67.32	0.703	63.65	0.598	69.08	
5	500	0.411	78.75	0.512	73.53	0.381	80.30	
IC 50 Values		-	302.609	-	325.477	-	289.891	

Activity of Methanolic Extract of Fruit Pulp and Seed.

The OD Values are Mean of Triplicates

Ferric reducing antioxidant Power (FRAP)

Table 5: FRAP Radical Scavenging Activity of Methanolic Extract of Fruit Pulp and Seed.

S.No	Concentration µl	Standard OD	Fruit pulp OD	Seed OD
1	100	0.012	0.008	0.005
2	200	0.026	0.018	0.013
3	300	0.039	0.026	0.017
4	400	0.051	0.036	0.022
5	500	0.062	0.042	0.037

The OD Values are Mean of Triplicates

The Ferric Reducing Antioxidant Power assay takes advantage of electron-transfer reactions. Here in a ferric salt, Fe (III) (TPTZ) 2 Cl3 (TPTZ) 2, 4, 6-tripyridyls-triazine), is used as an oxidant. The change in absorbance is therefore, directly related to the combined or "total" reducing power of the electron donating antioxidants present in the reaction mixture. Increase in absorbance of the reaction mixture indicates the reducing power of the samples.[27] Ferric reducing antioxidant power was performed for the test samples along with the standard ascorbic acid. The values obtained were recorded in **Table 5**. Each sample was tested with different concentration in the range of 100 –500 µg/ml. The transition metal ion Fe2+ of iron has been found to cause the production of oxyradicals and lipid peroxidation. Therefore, chelation of these ions affords protection against oxidative damage.

Deoxyribose Radical Scavenging Activity

In this assay, the antioxidant activity was determined based on the ability of the antioxidant components in the samples to inhibit deoxyribose oxidation by reactive OH^- generated from Fenton's type reaction. **Table 6** indicates the Deoxyribose radical scavenging assay of the test samples and of the standard Ascorbic acid. From each OD, the percentage of inhibition of both the samples at different concentration was calculated based on control values. It is seen that the standard (23.00% to 82.45%) showed less activity when compared with the test samples with fruit pulp showing 25.77% to 85.50% and seed 24.55 % to 84.57% respectively. The IC 50 values of the test samples were comparatively similar to the standard.

Table 6: Deoxy Ribose Radical Scavenging Activity of Methanolic Extract of Fruit Pulp and Seed.

S.No	Concentration µl	Standard OD	Standard %	Fruit pulp OD	Fruit pulp %	Seed OD	Seed %	Control OD
1	**100**	1.891	23.00	1.823	25.77	1.853	24.55	2.456
2	**200**	1.559	36.52	1.534	37.54	1.541	37.26	
3	**300**	1.111	54.76	1.075	56.23	1.098	55.29	
4	**400**	0.759	69.10	0.694	71.74	0.712	71.01	
5	**500**	0.431	82.45	0.356	85.50	0.379	84.57	
IC 50 Values		-	279.10	-	265.144		270.505	

The OD Values are Mean of Triplicates

ABTS [2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] Radical Cation Scavenging Assay

ABTS assay is a simple indirect method for determining the activity of natural antioxidants. In the absence of phenolic, ABTS radical is rather stable, but it reacts energetically with an H^- atom donor such as phenolic, being converted into a non-colored form of ABTS.[28]

In present study, the ABTS radical cation-scavenging assay was performed and the results showed that the free radical scavenging activity increases with increase in the concentration of the samples **(Table 7)**. Percentage and IC 50 values are calculated from OD values obtained for standard Gallic acid and test samples based on control values. The IC50 values of ABTS+ radical scavenging activity of standard was 260.203µg/ml and its IC50 values were higher than that of fruit pulp (238.620µg/ml) but lower than that of seed (282.720µg/ml). The results of the present study indicate that the methanolic seed extract exhibited higher free radical scavenging activity.

Table 7: ABTS Radical Scavenging Activity of Methanolic Extract of Fruit Pulp and Seed.

S.No	Concentration µl	Standard OD	Standard %	Fruit pulp OD	Fruit pulp %	Seed OD	Seed %	Control OD
1	100	0.512	29.96	0.493	32.56	0.522	28.59	0.731
2	200	0.432	40.90	0.417	42.95	0.446	38.99	
3	300	0.310	57.59	0.295	59.73	0.347	52.53	
4	400	0.237	67.58	0.207	71.68	0.258	64.71	
5	500	0.156	78.66	0.127	82.63	0.179	75.51	
IC 50 Values		-	260.203	-	238.620	-	282.720	

The OD Values are Mean of Triplicates

Superoxide radical scavenging activity

Superoxide radical is known to be a very harmful species to cellular components as a precursor of more reactive oxygen species (ROS). Superoxide is produced from molecular oxygen due to oxidative enzymes of body as well as via non-enzymatic reaction such as autoxidation by catecholamines.[29] Superoxide anion is capable of generating singlet oxygen and hydroxyl radicals which may lead to redox imbalance and harmful physiological consequences. The samples showed the scavenging activity in a dose-dependent manner, by donating their electrons to the superoxide and preventing their interaction with NBT **(Table 8)**. Fruit pulp methanolic extract (IC50=219.551 µg/ ml) revealed the maximum

scavenging potential than seed extract (IC50=208.071 µg/ ml) as represented in Figure 9. IC50 value of the standard BHT was 230.790 µg/ ml.

Table 8: SO- Radical Scavenging Activity of Methanolic Extract of Fruit Pulp and Seed.

S.No	Concentration µl	Standard OD	Standard %	Fruit pulp OD	Fruit pulp %	Seed OD	Seed %	Control OD
1	100	0.715	29.14	0.698	30.82	0.672	33.40	1.009
2	200	0.579	42.62	0.561	44.40	0.547	45.79	
3	300	0.342	66.11	0.327	67.59	0.319	68.38	
4	400	0.239	76.31	0.218	78.40	0.201	80.08	
5	500	0.114	88.70	0.097	90.39	0.083	91.77	
IC 50 Values		-	230.790	-	219.551		208.071	

The OD Values are Mean of Triplicates

Estimation of lipid peroxidation using egg yolks

LPO is known to be very harmful to cellular components as a precursor of more reactive oxygen species. Unsaturated fatty acids in membrane lipids are predominantly susceptible to oxidative processes. Particularly, linoleic acid and arachidonic acid are the usual targets of lipid peroxidation. FTC method is used to measure the peroxide level during the initial stage of lipid oxidation. Low absorbance values compared to the control indicated high levels of antioxidant activity.

Table 9: Lipid Peroxidation Activity of Methanolic Extract of Fruit Pulp and Seed.

S.No	Concentration µl	Standard OD	Standard %	Fruit pulp OD	Fruit pulp %	Seed OD	Seed %	Control OD
1	100	1.018	17.70	0.993	19.73	0.981	20.70	1.237
2	200	0.837	32.34	0.816	34.03	0.804	35.00	
3	300	0.671	45.76	0.658	46.81	0.642	48.10	
4	400	0.419	66.13	0.401	67.58	0.387	68.71	
5	500	0.272	78.01	0.253	79.55	0.242	80.44	
IC 50 Values		-	313.030	-	303.003	-	296.149	

The OD Values are Mean of Triplicates

Lipid peroxidase activity for fruit pulp and seed methanolic extracts along with standard BHT was performed and values are tabulated **(Table 9)**. The IC values and percentage of inhibition were also calculated from the obtained OD values. Percentage of inhibition by standard BHT was founded to be maximum of 78.01% at a concentration of 500 µg/ml which is slightly lesser than the test samples. Among the pulp and seed samples, the seed methanolic extract showed better activity with IC 50 value of 296.149 µg/ ml. Inhibition of lipid peroxidation by these antioxidants might be due to their free radical scavenging activities. In general, the antioxidant activity by TBA method was higher than that of FTC method. This might suggest the greater stability of the secondary product and thus the amount of peroxide in the secondary stage of lipid per oxidation was more than that in the primary stage.

β carotene linoleic acid assay

Table 10: β carotene Scavenging Activity of Methanolic Extract of Fruit Pulp and Seed.

S.No	Concentration µl	Standard OD	Standard %	Fruit pulp OD	Fruit pulp %	Seed OD	Seed %	Control OD
1	100	0.472	18.48	0.491	15.20	0.483	16.58	0.579
2	200	0.345	40.41	0.378	34.72	0.394	31.95	
3	300	0.231	60.10	0.263	54.58	0.280	51.64	
4	400	0.146	74.78	0.169	70.81	0.185	68.05	
5	500	0.071	87.74	0.098	83.07	0.117	79.79	
IC 50 Values		-	263.549	-	290.246	-	302.449	

The OD Values are Mean of Triplicates

This is one of the rapid method to screen antioxidants, which is mainly based on the principle that linoleic acid, which is an unsaturated fatty acid, gets oxidized by ‘‘Reactive Oxygen Species’’ (ROS) produced by oxygenated water. The products formed will initiate the β-carotene oxidation, which will lead to discoloration.[30] Percentage of inhibition for standard BHT showed 18.48% at 100 µg/ml concentration and 87.74% at 500 µg/ml. Among the pulp and seed samples, the seed

methanolic extract showed better activity with IC 50 value of 302.449µg/ ml **(Table 10)**.

Enzymatic and Non-enzymatic activity

Vitamin C and vitamin E are among the widely studied dietary antioxidants. Vitamin C (ascorbic acid) is considered the most important water soluble antioxidant in extracellular fluids. It is capable of neutralizing reactive oxygen species in the aqueous phase before LPO is initiated. Vitamin E, a major lipid-soluble antioxidant, is most effective chain-breaking antioxidant within the cell membrane where it protects membrane fatty acids from LPO. Vitamin C has been cited as being capable of regenerating vitamin E.[31]

Table 11: Enzymatic and Non-enzymatic Antioxidant Activity of Methanolic Extract of Fruit Pulp and Seed.

S.NO	Composition	Fruit pulp Content mg/g of extract	Seed Content mg/g of extract
1	Catalase	49.67 ± 0.57	58.51 ± 0.65
2	SOD	21.23 ± 0.13	33.46 ± 0.54
3	Vitamin C	113.21 ± 0.58	151.32 ± 0.62
4	Vitamin E	111.57 ± 0.21	122.48 ± 0.41

The Values are Mean±S.D of Triplicates

The level of enzymatic antioxidants such as catalase and SOD was found to be 49.67 ± 0.57 units/mg of extract and 21.23 ± 0.13 units/mg of extract respectively for fruit pulp. Similarly for seed extract it is found to be 58.51 ± 0.65 units/mg of extract and 33.46 ± 0.54 units/mg of extract respectively. The activity of Vitamin C and vitamin E was found to be 113.21 ± 0.58units/mg of extract and 111.57 ± 0.21 units/mg of extract respectively for fruit pulp. Similarly for seed extract it is found to be 151.32 ± 0.62 units/mg of extract and 122.48 ± 0.41 units/mg of extract respectively **(Table 11)**.

The formation of ROS is prevented by an antioxidant system: low molecular mass antioxidants (ascorbic acid, glutathione, and tocopherols), enzymes regenerating the reduced forms of antioxidants, and ROS-interacting enzymes such as SOD, peroxidases and catalases.[32] The SOD enzyme destroys the superoxide radical; however, as a result of that it creates hydrogen peroxide, which also has high toxic properties.[33] It has been reported as one of the most important antioxidant defense enzyme that scavenge superoxide anion by converting to hydrogen peroxide thus diminish the toxic effect caused by this radical.[34] Catalase is a tetrahedrical protein, constituted by four heme groups which catalyze the dismutation of hydrogen peroxide in water and oxygen.[35] Reduced thiols have long been reported to be essential for recycling of antioxidants like vitamin E and vitamin C.[36]

Thrombolytic activity

The fundamental task of thrombolytic or fibrinoytic therapy is the degradation of fibrin by plasmin, which can be activated by plasminogen.[37] The percentage of clot lysis of positive control was 22.72%. The sample methanolic extract of seed extract of P. *pusilla* shows 40% of clot lysis and the pulp showed comparatively high percent of clot lysis of 42.86% **(Table 12)**. The lysis of clot increases exponentially with time. Thus, the extracts possess considerable thrombolytic activity.

Table 12: Thrombolytic activity of Methanolic Fruit Pulp and Seed Extracts of *P. pusilla.*
Weight of the tube: 1 gram
Weight of clot after serum separation: 2.5 gram

	100 ml of fruit pulp extract	100 ml of seed extract	100 ml of Distilled water	100 ml of the drug
24 hr Wt of clot after fluid release	1.4	0.5	2.2	0.8
Wt of released fluid	0.6	0.2	0.5	0.4
% of clot lysis	**42.86**	**40.00**	**22.72**	**50.00**
48 hr Wt of clot after fluid release	0.9	1.8	1.9	1.6
Wt of released fluid	0.4	0.8	0.5	0.9
% of clot lysis	**44.44**	**44.44**	**26.32**	**56.25**
72 hr Wt of clot after fluid release	1.2	1.5	1.2	1.4
Wt of released fluid	0.7	0.9	0.7	0.8
% of clot lysis	**58.33**	**60.00**	**58.33**	**57.14**

Fibrinolytic Activity

Table 13: Fibrinolytic activity of Methanolic

S.NO	Treatment	% of Clot Lysis
1	Streptokinase	63.92 ± 0.57
2	Distilled Water	21.23 ± 0.13
3	Methanolic Pulp Extract	43.21 ± 0.58
4	Methanolic Seed Extract	31.57 ± 0.21

Fruit Pulp and Seed Extracts of *P.pusilla.*
The Values are Mean ± S.D of Triplicates

Fibrin is the more important component of clots that form in veins but platelets are the major component of clots that form in arteries where they can cause heart attacks and strokes by blocking the flow of blood in the heart and brain, respectively.[38] The fibrinolytic enzyme prevents the formation of fibrin clot in the circulatory system. Some medicines like urokinase and streptokinase are widely used to inhibit hemostatic disorders, particularly thromboembolism.[39] The percentage of clot lysis produced by pulp extract is higher than the seed extract **(Table 13).**

Anticoagulant activity

The prothrombin time test (also known as the pro test or PT test) is a useful screening procedure for the extrinsic coagulation mechanism including the common pathway. Anti-coagulant activities of fruit pulp and seed extract of *P.pusilla* were carried out. From the present study it is proved that both the extract have remarkable anti-coagulant activity than the control solution **(Table 14)**. The phytoconstituents were suggested to have played a role in the anticoagulant activity, especially prolonging blood coagulation in the intrinsic pathway.

Table 14: Anticoagulant activity of Methanolic Fruit Pulp and Seed Extracts of *P.pusilla.*

Plant species	CONC (in ppm)	Prothrombin time (mins)
Fruit pulp	500	12:21
	1000	32:02
	1500	46:25
Seed	500	14:23
	1000	35:04
	1500	48:31
Isosaline (control)	500	05:21
	1000	02:20
	1500	02:42

The mechanism by which the extract exhibited anticoagulant activity is not understood but chelating agents, warfarin and vitamin K antagonists are known to interfere with blood clotting processes. Warfarin can inhibit clotting factor IXa, XIa and thrombin but its action on factor Xa accounts for its potency as an anticoagulant.[40] Heparin can inhibit both the generation of thrombin and also the formed thrombin. The synthesis of clotting factors II, VII, IX and X in the liver depends on adequate amounts of vitamin K. Coumarin and inadedione group of oral anticoagulant drugs antagonizes the synthesis of non-functional forms of coagulant proteins and thereby prevents blood clot formation.[41]

CONCLUSION

Many compounds present in diets may affect coagulation processes. Many of them are bioactive compounds, such as phenolic and polyphenolic compounds, vitamins and carotenoids, found in vegetables and fruits. Studies have shown these compounds have inhibitory effects on platelet function, thus having beneficial effects on heart disease and atherosclerosis. The present study reveals that methanolic extract of fruit pulp and seed extract of *P.pusilla* is a rich source of natural antioxidants which could be extracted efficiently with methanol. The methanolic extract of fruit pulp and seed extract of *P.pusilla* extracts showed a higher potency in scavenging of DPPH free radical. This may be related to the high amount of phenolic compounds and flavonoids in this plant extract. The data clearly indicated that the extracts showed good antioxidant, radical scavenging and thrombolytic activity. The results of the present study also demonstrated that the methanolic extract of fruit pulp and seed extract of *P.pusilla* possesses pharmacologically active anticoagulant principles that could be isolated and evaluated for clinical or physiological purposes. Thus, the extracts possess considerable thrombolytic activity. Such a discovery is crucial as it provides clues for potential treatment of blood coagulant disorders.

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