Research Article **ISSN: 2347–8330**

Journal of Pharmaceutical and Biological Research
Online at www.pharmaresearchlibrary.com/jpbr
JPBR, 2014, Vol.2(2): 168-173

A Quest for Potent Plant Based Thrombolytics

Shalini H Kumar* and Pushpa.A

Department of Biochemistry, Biotechnology and Bioinformatics,
Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, India

NCBC'14, 27 August 2014, Organized by PG & Research Department of Biotechnology,
Sree Narayana Guru College, K.G. Chavadi, Coimbatore, India-641105.

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***Corresponding author**
Shalini H Kumar
Department of Biochemistry,
Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, India
Manuscript ID: NCBC2014-JPBR2274

PAPER QR-CODE

Abstract

To combat the shortcomings of the commercially available thrombolytic agents, thirteen different plant extracts namely leaves and fruits of *Phyllanthus emblica*, seeds of *Sesamum indicum* and *Brassica juncea*, leaves of *Commiphora molmol*, water and endosperm of *Cocos nucifera*, leaves and pulp of *Carica papaya*, aril and rind of *Punica granatum* and pulp of *Ananas comosus* were screened for thrombolytic activity in terms of *in vitro* clot lysis. *Punica granatum* exerted a higher thrombolytic activity and accordingly it was chosen for further studies. Streptokinase was used as a positive control and water was used as a negative control. The minimum effective concentration for thrombolysis of aqueous and ethanolic extracts of aril and rind of *Punica granatum* were analysed. The cytotoxic property was assessed by lethality assay using *Artemia salina* and total flavonoid and phenolic content were also determined. It was found that *Punica granatum* had high phenolic and flavonoid content which could be endorsed for the significant thrombolytic activity.

Keywords: *Punica granatum*, thrombolysis, total phenol, cytotoxic

1. Introduction

Myocardial infarction, commonly known as a heart attack, is the irreversible necrosis of heart muscle resulting from a period of prolonged ischemia. This usually results from an imbalance in oxygen supply and demand, which is most often caused by plaque rupture with thrombus formation in a coronary vessel, resulting in an acute reduction of blood supply to a portion of the myocardium. Thrombolytics offer a dramatic improvement in the treatment of myocardial infarction. Thrombolytic drugs like tissue plasminogen activator (t-PA), urokinase, streptokinase etc.

play a crucial role in the management of patients with cerebral venous sinus thrombosis (Chowdhury *et al*., 2011). All available thrombolytic agents have significant shortcomings, including limited specificity and efficiency in low dose and need for large doses to be maximally effective whose safety remains to be confirmed and bleeding tendency at times leading to death (Elumalai *et al*., 2012).

Drugs based on herbs have become a common form of therapy because they are often perceived as being natural and therefore harmless. Today, they are one of the hottest trends and most sought after in the field of nutrition or herbal therapeutics (Singh *et al*., 2012). Fruits in general have greater antioxidant capacity, and are enriched with fibre, and contain a certain percentage of vitamins and mineral salts. Small quantities of protein and fat are also contained in fruits. The minerals commonly found in fruits are iron, lime, sodium, magnesium, potash and phosphorus.

Along with the nutritional value, plants contribute in the protection from free radical deterioration by hindrance of lipid peroxidation via numerous mechanisms including scavenging free radicals, inducing antioxidant enzymes, modulating protein kinase, and lipid kinase signalling pathway. Oxidative damages done by free radicals causes pathogenesis of many deadly diseases like cancer, Alzheimer's and diabetes (Islam *et al*., 2013). Though all the selected samples are exploited for their antioxidant potential, very little emphasis has been laid for their thrombolytic potential. Hence this study is focussed on evaluating the thrombolytic potential in the selected fruits to provide new insights and promote progress towards the development of the ideal thrombolytic therapy.

2. Materials and Methods

Sample Collection:

All samples were collected from Coimbatore city and they were first cleaned and washed with water to remove any dust or soil particles. Samples were weighed separately (10 g each) and extracted with 20 ml of water. They were then filtered through a fine cheese cloth and finally with Whatman No.1 filter paper. Similarly, for organic solvent extracts, the samples were extracted with ethanol.

Thrombolytic activity:

The thrombolytic activity of all extracts was evaluated by the method developed by Daginawala (2006). Whole blood was drawn from healthy human volunteers without a history of anticoagulant therapy. The blood was distributed in different pre-weighed sterile microcentrifuge tube (1 ml/tube) and incubated at 37°C for 45 minutes. After clot formation, the serum was completely removed without disturbing the clot and each tube containing the clot was again weighed to determine the clot weight (clot weight = weight of clot containing tube – weight of tube alone).

To each microcentrifuge tube with the pre-weighed clot, 200 µl of the extract of different samples was added separately. All the tubes were then incubated at 37 °C for 90 minutes and observed for clot lysis. After incubation, the released fluid was removed and the 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 as shown below:

% of clot lysis = (wt of released clot /clot wt) × 100

Streptokinase (SK):

Commercially available lyophilized Streptokinase vial of 10,000 kU was collected and 5 ml sterile distilled water was added and mixed properly. This suspension was used as a stock from which 100 µl was used for *in vitro* thrombolysis.

Brine shrimp lethality bioassay:

Brine shrimp lethality bioassay was performed (Meyer *et al*., 1982 and McLaughlin *et al*., 1998) to investigate the cytotoxicity of aqueous and ethanolic extracts of *Punica granatum*. Brine shrimp (*Artemia salina*) eggs were hatched in sterile artificial seawater (Nacl 38.0 g/L; adjusted pH 8.5). They were kept for 48 hours with continuous aeration. After hatching, active nauplii free from egg shells, were collected from brighter portion of the hatching chamber. The extracts were mixed with the sea water and each test tube was introduced with ten brine shrimp larvae (10 nauplii). All test tubes were maintained at room temperature for 24 hours. The numbers of surviving and dead shrimps were counted and percentage mortality was determined.

Total phenolics analysis:

Total phenolic content of the selected fruit extracts were measured by the method (Skerget *et al*., 2005). In brief, Folin-Ciocalteu reagent was used as an oxidizing agent and gallic acid as a standard. 0.5 ml of extract solution, 2.5 ml of Folin-Ciocalteu reagent (diluted 10 times with water) and 2.0 ml of sodium carbonate (7.5 % w/v) solution were added. After 20 minutes of incubation at room temperature the absorbance was measured at 760 nm using a UV-visible spectrophotometer. Total phenolics were quantified by calibration curve obtained from measuring the known concentrations of gallic acid (0-100 µg/ml) and were expressed as mg of GAE (gallic acid equivalent) / gm of the dried extract.

3. Results and Discussion

Percent clot lysis of different plants

Percent clot lysis of different samples were investigated for determining the thrombolytic efficiency of the samples with a positive and negative control and the results are as in table 1

Table 1: Percent Clot Lysis of Various Samples

S.No	Sample	% Clot Lysis
1	*Phyllanthus emblica* leaves (S1)	2.28
2	*Sesamum indicum* (S2)	2.35
3	*Commiphora molmol* leaves (S3)	2.44
4	*Cocos nucifera* endosperm (S4)	2.91
5	*Brassica juncea* (S5)	3.26
6	*Cocos nucifera* water (S6)	3.45
7	*Carica papaya* leaves (S7)	4.15
8	*Zingiber officinale* (S8)	7.58
9	*Phyllanthus emblica* fruit (S9)	9.37
10	*Carica papaya* pulp (S10)	10.18
11	*Ananas comosus* pulp (S11)	17.1
12	*Punica granatum* rind (S13)	27.62
13	*Punica granatum* aril (S14)	30.36
14	Streptokinase	58.38
15	Water	4.49

Streptokinase that served as a positive control showed 58.38% and water which was treated as a negative control exhibited 4.49% clot lysing ability. The thrombolytic activity of extracts was confirmed by comparing all the extracts with positive control and negative control. Leaves of *Phyllanthus emblica* exhibited the least activity of 2.28% and the pulp of *Punica granatum* showed the highest thrombolytic activity of 30.36%. It was noted unambiguously that among the samples analyzed for the thrombolytic activity, fruits exerted a better activity than other samples. This could be attributed to the presence of high antioxidant capacity in fruits.

A wide variation in the degree of thrombolysis was seen among the different aerial parts of the same plant. For instance, *Phyllanthus emblica* leaves revealed 2.28% activity while the fruit showed 9.37%. Similar trend for thrombolysis was observed between *Carica papaya* leaves and fruit also. Concurrent findings were reported by Islam *et al*. (2013), who reported that the chloroform fruit extract of *Spondias dulcis* had more clot lysing ability (25.3%) than the leaf extract (6.48%) of the same solvent. Fruit pulp yielded good thrombolytic activity and this is in agreement with the findings of Hossen *et al*., 2012, who have reported that, the fruit pulp of *Tamarindus indica* has excellent therapeutic potential.

Minimum Effective Concentration:

Aqueous and ethanolic extract of aril and rind of *Punica granatum* were assessed for thrombolytic activity at different concentrations using human blood and the results are as depicted in figure 1.

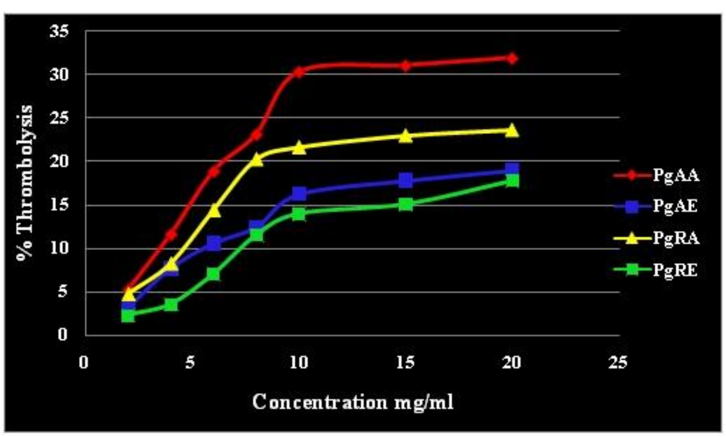

Figure 1: Percent Clot Lysis of Various Samples

PgAA – *Punica granatum* aril aqueous extract, PgAE - *Punica granatum* aril ethanolic extract, PgRA - *Punica granatum* rind aqueous extract, PgRE-*Punica granatum* rind ethanolic extract.

A marked increase in thrombolysis was observed as the concentration of the extract also increased. The thrombolytic action of the plant extract was dose dependant. Ratnasooriya *et al*., 2013 stated that the Sri Lankan low gown orthodox O.P. grade black tea possesses marked *in vitro* thrombolytic activity and found that to be dose dependant. The author claimed the effect to be genuine, intrinsic, causal, specific and possibly receptor mediated. The same could be extrapolated for the present study also. The fact that the aqueous extract was more powerful than the ethanolic extract is a noteworthy observation that was made. In addition, the aril of the fruit revealed a better clot lysing potential than the rind. Sarker *et al*., 2012 in the study of thrombolytic activity and preliminary cytotoxicity of five different fractions of methanol extract of *Allamanda cathartica* leaf found that the aqueous extract was better than the methanolic extract in thrombolysis.

Cytotoxicity of *Punica granatum* extracts:

Bioassay screening was performed for the *Punica granatum* rind and aril extracts to assess the preliminary toxicity against *Artemia salina* and the results depicted the lethality of the shrimp at 6 different concentrations. Potassium dichromate was used as a positive control for the bioassay screening and the results are as in figure 2 and 3.

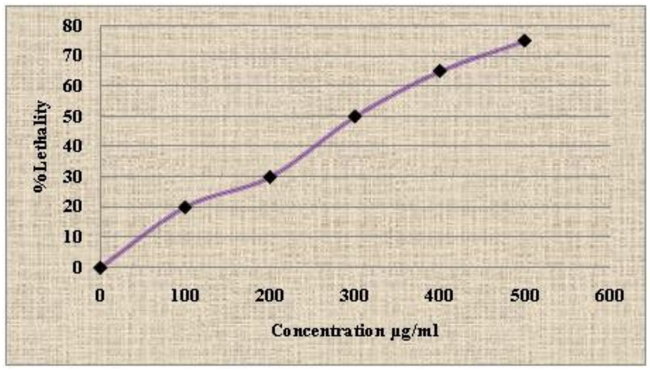

Figure 2: Percent Lethality of Potassium Dichromate against Brine Shrimp Nauplii

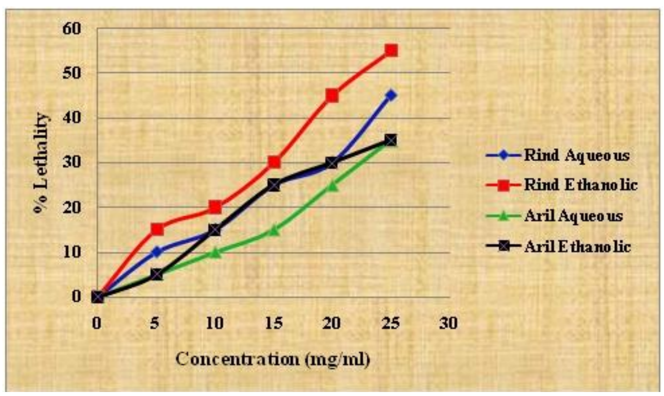

Figure 3: Percent Lethality of *Punica Granatum* Extracts Against Brine Shrimp Nauplii

A significant difference in lethality to *Artemia salina* was observed with exposure to different dose levels of all the extracts. There was an increase in lethality as the concentration of the extract increased. Potassium dichromate that was used as a positive control showed an LC_{50} value of 310 which was very low when compared to the extracts, proving the non toxic nature of the extracts. Maximum mortality was seen at a concentration of 25 mg/ml for all the extracts. The order of lethality among the different extracts was found to be PgAA < PgAE < PgRA < PgRE with their LC_{50} values as 45.14 > 36.47 > 29.89 > 22.32 respectively. Among the four extracts, the ethanolic extracts of both aril and rind manifested a slight increase in cytotoxicity than the aqueous extract.

Thus the results of the present study portray that the fruit is not toxic even at a concentration of 25 mg/ml. Concurrent findings were reported by Arulvasu *et al*., 2014, who have observed an increase in mortality in brine shrimp with the increase in concentration of silver nano particles. Syahmi and others in 2010 have demonstrated a proportional relationship between the concentration of the methanolic extracts of *Elaeis guineensis* and the degree of lethality of brine shrimp exhibiting a linear correlation.

Total phenols and flavonoids:

The rind and aril of *Punica granatum* were assessed for total phenols and flavonoids and the results are as shown in figure 4.

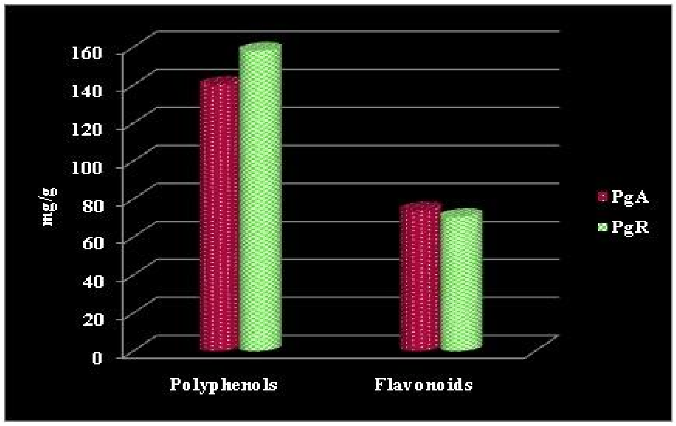

Figure 4: Level of Phenols and Flavonoids in *Punica granatum*

Polyphenols were found to be higher in rind than in aril while the vice versa was observed for the level of flavonoids. Shabbir *et al*. (2013) have suggested that high concentration of phenolics, flavonoids, tannins and terpenoids is the cause for the protective potential of *Maytenus royleanus* leaf extract. Oxidative stress is peaked during ischemia and reperfusion and translational research of the combination of antioxidants and thrombolytic agents seems vindicated. Pomegranate is a potent antioxidant, superior to red wine and equal to or better than green tea and thus it activity could serve as an excellent candidate for thrombolysis also for the reason that they can quench the oxidative stress formed during ischemia and reperfusion.

4. Conclusion

Lifestyle disease like diabetes, cardiovascular diseases, hypertension etc is very widespread in present day's world. From the results of the present study and with reference to the available literatures, the fruit *Punica granatum* gain significance as a potent antioxidant and a thrombolytic agent. Further research is required in *Punica granatum* to understand the molecular mechanisms and principle compounds involved in thrombolysis to be included for pharmaceutical preparations.

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