

Assessment of anti-coagulant activity of *Nelumbo nucifera* fruit

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Abstract: The herbal agents rich in flavonoids are progressively becoming popular these days as they are considered to have anti-oxidant effects and also lack bothersome effects. That's why existing research was encircled around the anti-coagulant effect of *Nelumbo nucifera* fruit (NNF) as it is also a rich source of flavonoids and cultivated in abundance especially in tropical regions of Asia but its usefulness as anti-coagulant agent was never determined pharmacologically. Anticoagulant assessment was done in thirty five male Wister rats which were separated equally in 5 groups. Results of the current study revealed that NNF 200mg/kg significantly prolonged prothrombin time and thrombin time, whereas fibrinogen level was highly significantly reduced as compared to control. Fibrinogen level was also reduced highly significantly with NNF 100 mg/kg as compared to control without affecting other parameters of coagulation i.e. activated partial thromboplastin time, prothrombin time and thrombin time. NNF exhibited strong anti-coagulant activity which may be due to the inhibitory effects on platelet activation, adhesion and aggregation along with inhibitory effects on thromboxane A₂ formation. Presence of alkaloid i.e. neferine and flavonoids in it may be a reason of its anti-coagulant activity but more pre-clinical and clinical evaluation needs to be conducted to establish these findings.

Keywords: *Nelumbo nucifera*, anticoagulant, neferine, flavonoids, thromboxane A₂

INTRODUCTION

The uses of herbal drugs are becoming progressively more popular as they are supposed to be natural, advantageous and lack unwanted effects (Leonardo *et al.*, 2000). Mostly the plant derived drugs are taken randomly by local population for the treatment of various diseases without having adequate information regarding its usefulness. Hence for proper guidance of the general population, especially users of natural products, there is a need to scientifically prove the effectiveness of these medicinal plants (Agbaje *et al.*, 2009).

Nelumbo nucifera, a *Nymphaeaceae* family plant is commonly cultivated in the hot and humid climatic zones of Thailand, Pakistan, India and China (Mukherjee *et al.*, 2009). Its fruit contains seeds plus pods (lotus bulbs). The green colored pods offer add-on to the seeds, which are black, firm and egg shaped to round. They are organized in spirals (Sridhar and Bhat, 2007) and edible portion of seeds have to be peeled separately before they are eaten (Carlo *et al.*, 2013).

Seeds are wonderful source of protein, starch, fat, unsaturated fatty acids and asparagines. The key active principles in seeds are flavonoids, alkaloids, principally liensinine, lotusine, isoliensinine, dauricine, proniciferine, nuciferine, roemerine, procyanidin,

neferine plus armepavine. The seeds also contain ample amount of various minerals for instance potassium, magnesium, calcium, sodium, iron, chromium, manganese, copper and zinc (Indrayan *et al.*, 2005; Pal *et al.*, 2015). They also contain gallic acid and isoquininolinol (Mukherjee *et al.*, 2009).

Recently conducted study on NNF pods has shown the existence of numerous active bioactive principles for instance tannins, saponins, terpenoids flavonoids and alkaloids (Rajput and Khan, 2017). Alkaloid procyanidin was also squeezed from NNF pods (Mukherjee *et al.*, 2009).

The fruits are commonly used up as a healthy component of Asian cuisine and also as a traditional cure of various ailments e.g. chronic diarrhea, hypertension, palpitation, arrhythmia, fever, pain, inflammation, sleep disorders, menorrhagia, spermatorrhea, leucorrhoea, bad breath and leprosy (Chopra *et al.*, 1956; Vershney and Rzoska, 1976). Current research work revealed LD₅₀ value of NNF higher than 5g/kg whereas its Neuropharmacological role was also established as an anxiolytic, antidepressant and antiepileptic agent (Rajput and Khan, 2017; Rajput *et al.*, 2017) however very inadequate literature is obtainable concerning its effects on coagulation parameters, therefore existing study was centered towards the evaluation of coagulation parameters in order to determine its use in various thrombotic and atherosclerotic conditions.

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MATERIALS AND METHODS

Experimental design

Research work was executed utilizing the laboratory facilities of Pharmacology Department and the Research Institute of Pharmaceutical Sciences, University of Karachi, following approval from the Board of Advance Studies and Research reference number BASR/02149/Pharm. Both standard drug and NNF were administered for 15 days and blood samples were collected on 15th day forty minutes after administration of last dose.

Selection of animals

The anticoagulant activity was assessed in male Wister rats divided in to 5 groups i.e. control group, reference group and 3 test groups each having 7 animals. The weight of the rats ranges from 180-230g. The departmental research committee of Pharmacology, Karachi University allowed the use of animals in experiments according to the guidelines provided by NAELAR for Use of Laboratory Animals (National Advisory Committee for Laboratory Animal Research, 2004) and NIH (The National Institute of Health, 2010).

Animal housing

The rats were kept in flexible polypropylene cages with proper maintenance of temperature kept close to 25°C and moistness 50 to 60% in an alternating twelve hour light and dark cycle. Each mouse was provided access to normal diet and water. The rats were carried to the research laboratory about an hour prior to the experiments. Physical fitness of each rat was evaluated for 7 days prior to administration of drugs, during adaptation phase chiefly noticing absence of movements, edema, diarrhea and ulceration.

Preparation of fruit extract

After obtaining fruits from domestic fruit bazaar of Hyderabad, Pakistan in August 2016, they were initially presented to department of Pharmacognosy, University of Karachi for identification and authentication and afterwards receipt no NNF-03 was deposited in the same department.

Crude extract was prepared through cold extraction procedure (Hossain *et al.*, 2010; Assad *et al.* 2018). Six kg fruits were initially rinsed with tap water and the seeds were separated from the pods manually. The seeds have high contents of water that's why they need to be chopped first then left for 06 days for drying out in shade. The dried material obtained was thick so again needs to be ground in to fine powder. In contrast pods were chopped once only and were allowed to dry in shade for 03 days. The dried pod material takes a coarse powder form. So for better separation and collection of NNF constituents (secondary metabolites) they need to be chopped and dried separately before soaking up together in ethanol (98%) for thirty days with occasional shaking.

Afterwards it was sieved using separator (Whatman No. 1). Later it was evaporated using rotary machine under condensed pressure at 40°C to 45°C. The condensed material was freeze dried in a freeze dryer at -30°C. The amount of material so gained was preserved at -20°C until further use in doses of 50, 100 and 200mg/kg orally (Rajput and Khan, 2017). The ultimate amount of the extract acquired was 400 g of dry weight.

Grounding of drugs

Tragacanth gum was acquired from Merck whereas warfarin sodium tablets (5 mg) were obtained from one of the well-known pharmacy shops at Karachi. The three doses of NNF i.e. 50, 100 & 200 mg/kg were given as 2% tragacanth gum suspension (Danamurthy *et al.*, 2018) acquired from Merck. Control group was given only 2% gum tragacanth suspension in the dose of 10ml/kg orally. 100ml of warm distilled water was added in 2 g tragacanth gum to make the suspension, which was prepared freshly when required (Madhu *et al.*, 2009; Rajput *et al.*, 2013). Warfarin Sodium 5mg tablets were pulverized and then diluted in distilled water and was given to rats in 0.54mg/kg PO dose (Zacchigna *et al.*, 2004).

Effect of NNF on coagulation parameters

The anticoagulant activity was assessed by the method as described by Rajput *et al.*, 2012. A population of thirty five male Wister rats were equally placed in 5 groups; 3 test groups, one control, and one reference group. Three test groups were treated with 50, 100 and 200 mg/kg NNF respectively, control group was given gum tragacanth in a dose of 10ml/kg and reference group was treated with warfarin at a dose of 0.54mg/kg. All drugs were given by orogastric tube.

Blood sample (3ml) was collected in coagulation tubes; plasma was separated by centrifugation at 3000 rpm for 15 minutes by 14k Humax centrifuge. Prothrombin time (PT), Activated Partial Thromboplastin time (aPTT), Thrombin time (TT) and Fibrinogen (Fg) were evaluated through Humaclot duo Germany, utilizing standard reagent kits obtained from Human (Chan *et al.*, 2007).

STATISTICAL ANALYSIS

The figures obtained in experiment were used to take average and standard error to the average using two sample student T- test. The values of P less than 0.05 were reflected as significant and P less than 0.005 as extremely significant. All numerical methods were accomplished with SPSS software version 20.

RESULTS

Effect of NNF on coagulation parameters

Table-1 reveals the comparison of NNF and warfarin on coagulation parameters. NNF extract at dose of 200mg/kg

Table 1: Outcomes of NNF and warfarin on coagulation parameters

Parameters	Control 10ml/kg	NNF 50 mg/kg	NNF 100 mg/kg	NNF 200 mg/kg	Warfarin 0.54 mg/kg
aPTT (sec)	8.4±0.2	8.8±0.3	9.1±0.2	8.8±0.3	9.8±0.3*
PT (sec)	5.5±0.7	5.4±0.3	6.1±0.4	6.7±0.3*	8.5±0.3**
TT (sec)	9.3±0.3	9.0±0.3	9.7±0.3	10.5±0.3*	12.3±0.3**
Fibrinogen (mg/dl)	429.4±1.9	428.5±2.8	409.0±2.1**	356.5±1.5**	431.1±0.9

n=7

The expressions were calculated by taking mean ± standard error to the mean

*p value less than 0.05 was counted as significant in comparison to control

**p value less than 0.005 was counted as extremely significant in comparison to control

considerably prolonged PT and TT, whereas fibrinogen level was highly significantly reduced as compare to control. Fibrinogen level was also reduced highly significantly with NNF extract dose of 100 mg/kg in comparison to control without affecting other parameters of coagulation i.e. aPTT, PT and TT. Conversely warfarin 0.54 mg/kg highly significantly prolonged PT and TT and significantly prolonged aPTT as matched with control, whereas fibrinogen level didn't affected considerably with warfarin in contrast to control.

DISCUSSION

Abnormalities of coagulation are frequently present in seriously sick patients and usually result in disability and death hence requires prompt diagnosis and treatment (Marcel and Steven, 2006). Acute platelet thrombus formation leads to the development of atherosclerosis, followed by embolization of stenosed vessels (Lou *et al.*, 1989). Platelets and thrombin are interdependent; since thrombin induced activation of platelets is as important as platelets availability for thrombus formation (Riaz *et al.*, 2009).

Results of the current study also revealed that NNF extract at dose of 200mg/kg considerably prolonged PT and TT, whereas there was highly significant decrease in fibrinogen level as compare to control. There was also highly significant decrease in fibrinogen level with NNF extract at the dose of 100 mg/kg as compared to control without affecting other parameters of coagulation i.e. aPTT, PT and TT.

Neferine, an alkaloid and one of the constituents of *N. nucifera* seed has exhibited antithrombotic effects by inhibiting platelet activation, adhesion and aggregation (Zhou *et al.*, 2013). Similarly liensinine which is also an isoquinoline alkaloid like neferine has considerably inhibited platelet aggregation and prolonged PT, aPTT and TT and thus exhibited strong effects against thrombus formation (Wang *et al.*, 2010).

Previous studies have demonstrated, powerful antithrombotic effects of flavonoids and is thought to be through its inhibitory effect on thromboxane A₂

formation. Thromboxane A₂ increases the expression of glycoprotein 11b/111a receptor complex on the membranes of the platelets and thus stimulates platelet aggregation. Circulating fibrinogen sticks to these platelets and give further strength to the clot (Tzeng *et al.*, 1991). Since flavonoids and alkaloids (neferine & liensinine) are significant constituents present in NNF, so it can be stated that antithrombotic effects of NNF may be due to the presence of these constituents in it.

CONCLUSION

NNF has exhibited strong anti-coagulant effects which may be useful in treating various thrombotic and atherosclerotic conditions but further studies needs to be conducted pre-clinically and clinically to ratify these outcomes.

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