



EVALUATION OF THROMBOLYTIC ACTIVITY OF *Murraya koenigii* AND *Spinacia oleracea* IN VITRO AND IN VIVO

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Abstract:

Green leafy vegetable consumption has been associated with decreased risk of persistent metabolic diseases. The *in vitro* thrombolytic property of curry leaves (*Murraya koenigii*) and palak (*Spinacia oleracea*) was determined by using human blood. Aqueous extract of *Murraya koenigii* exhibited maximum clot lysis at a concentration of 10mg/ml contributing to 22.14%. *Spinacia oleracea* revealed maximum clot lysis of 40.9% at 20mg/ml concentration. The plant extracts, when given in combination showed clot lysis of 33.22% at 20mg/ml. The thrombolytic activity was correlated with serum cholesterol and the findings suggested that the plant extract could possibly lyse a clot regardless of the level of cholesterol. Membrane stabilizing potential of the selected plants was analysed and both the plant extracts moderately protected the human RBC membrane which is an essential quality for thrombosis. To ensure the thrombolytic efficacy of *Murraya koenigii* and *Spinacia oleracea*, *in vivo* models of FeCl₃-induced thrombosis was developed. Haematological and biochemical parameters were determined in experimental animals. Histopathological examination was also carried out after the experimental period. This investigation can help in developing cost effective novel thrombolytic agents from common plant sources, which are advantageous over synthetic drugs.

Keywords: Thrombolysis; *Murraya koenigii*; *Spinacia oleracea*.

Introduction

Many of the modern day's vital drugs and processed medicines are of plant origin. There are 45,000 species of wild plant out of which 9,500 species are ethnobotanically important species. Of these, 7,500 species are in medicinal use for indigenous health practices. About 3,900 plant species are used by tribals as food out of which 521 species of leafy vegetables (Kamble and Jadhav, 2013). Thrombosis is one of the major causes of the death Worldwide. It occurs due to imbalance between prothrombotic and fibrinolytic pathway (<http://departments.edu/chpweb/hemophilia/mechanis>

[m_of_blood_coagulation.htm](#)). Thrombolytic agents lyse pre-existing thrombus, either by potentiating the body's own fibrinolytic pathway or by mimicking natural thrombolytic molecule (Prakash, and Manavalan, 2011). Commonly used thrombolytic agents are alteplase, anistreplase, streptokinase, urokinase and tissue plasminogen activator (t-PA) to dissolve clots (Anwar *et al.*, 2011). However, the relatively weak specificity of first generation agents (streptokinase and urokinase) can result in a state of systemic fibrinolysis and associated bleeding complications (Khan *et al.*, 2011). Hence there is great interest in the use of plants with cytotoxic and clot lysis potential as source of novel antitumor agents and thrombolytic drugs (Hossain *et al.*, 2013). Epidemiologic studies have provided evidence that foods with experimentally proved antithrombotic effect could reduce risk of thrombosis. Some plants or plant parts showing thrombolytic activity have also been reported (Rahman *et al.*, 2013). However, there is no reports relating to the thrombolytic properties of green leafy vegetables.

In the present study, six different green leafy vegetables were screened for *in vitro* thrombolytic property. The two plants which showed higher percentage of clot lysis were selected for further investigation.

2. Materials and Methods

Screening of the Plants for Thrombolytic Potential

Six different plants namely *Murraya koenigii*, *Spinacia oleracea*, *Basella alba*, *Talinum portulacifolium*, *Trigonella foenum-graecum* and *Mentha piperita* were collected from Coimbatore District, Tamilnadu and screened for thrombolytic activity. The two plants that showed the maximum effect namely *Murraya koenigii* and *Spinacia oleracea* were selected for the present investigation.

Plant Material

Murraya koenigii and *Spinacia oleracea* were collected from Coimbatore District and the plants were identified and authenticated by Botanical Survey of India, Coimbatore, Tamilnadu. Fresh leaves of the plants were collected, washed and

homogenized using distilled water for the preparation of aqueous extract.

Determination of Thrombolytic and Membrane Stabilizing Activity of the Selected Plants.

Thrombolytic activity of *Murraya koenigii* and *Spinacia oleracea* was determined using human blood by the method of Prasad *et al.*(2006). All the experimental procedures were initiated after reviewed by Institutional Human Ethics Committee (AUW/IHEC-13-14/XPD-11). Venous blood drawn from healthy volunteers (n=20) was transferred into different pre weighed sterile micro centrifuge tube (500µl/tube) and incubated at 37°C for 45 minutes. After clot formation, serum was completely aspirated out without disturbing the clot formed. Each tube having clot was again weighed to determine the clot weight (clot weight=weight of clot containing tube-weight of tube alone). Each micro centrifuge tube containing clot was properly labeled and 100µl of streptokinase (30,000 IU) along with various dilution in aqueous plant extracts (5-25mg/ml) was added to the tubes. Water was also added to one of the tubes containing clot and this served as a negative thrombolytic control. All the tubes were again weighed to observe the difference in weight after clot disruption. Differences obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis. This test was repeated twenty times with different concentrations of the plant extracts in blood samples of twenty different healthy volunteers.

Total cholesterol was estimated in serum obtained from human blood used for the determination of thrombolytic activity by Allian *et al.*(1974).The membrane stabilizing activity of the extracts was assessed by the method of Shinde *et al.*(1999) by using hypotonic solution – induced and heat-induced human RBC haemolysis.

Determination of Thrombolytic Potential *in vivo*

Acute oral toxicity was performed as per Organization for Economic Co-operation for Development (OECD) guideline 423 method to find out the LD₅₀. All the experimental procedures were initiated after getting approval from the Institutional Animal Ethics Committee (KMCRET/PhD/5/2013-14). Male Wistar rats 8-10 weeks old, weighing 200-250g were used for the FeCl₃ (35% w/v) induced thrombus in tail injury model. Animals were housed in polyethylene cages in an animal room with a controlled temperature at 24±2 C, a constant humidity of 50±10% and a 12 hours light-dark cycle. Animals had free access to a standard diet and tap water ad libitum.

The animals were divided into 6 main groups each group containing six animals.

Group I - Control

Group II - FeCl₃

Group III - FeCl₃ + Streptokinase

Group IV - FeCl₃ + *M.k* extract (250mg/kg)

Group V - FeCl₃ + *S.o* extract (250mg/kg)

Group VI -FeCl₃+*M.k*(250mg/kg)+*S.o* (250mg/kg)

Experimental rats were anaesthetized with i.p injection of ketamine hydrochloride (100 mg/kg). Thrombus was induced by applying a Whatmann filter paper saturated with 35% FeCl₃ on top of the tail vein for two minutes.

Thrombus formation was monitored to confirm the development of thrombus. After formation of thrombus the animals were treated with respective extracts and standard drugs, once a day orally for thirty days. After the experimental period animals were anaesthetized using intra peritoneal injection of ketamine hydrochloride (100mg/kg). Blood was collected from retro-orbital sinus by using capillary into a centrifugation tube which contains EDTA for haematological parameters and without EDTA for biochemical parameters.

Haematological parameters such as WBC count, RBC count, platelet count, haemoglobin, bleeding time and activated partial thromboplastin time (APTT) were analysed in the control and experimental animals. Biochemical parameters namely fibrinogen, D-dimer and creatine phosphokinase were analysed in the serum sample of experimental animals. Total protein, SOD and catalase were assessed in the liver sample of experimental animals. Histopathological examination was carried out to study the effect of plant extracts on heart, liver and kidney.

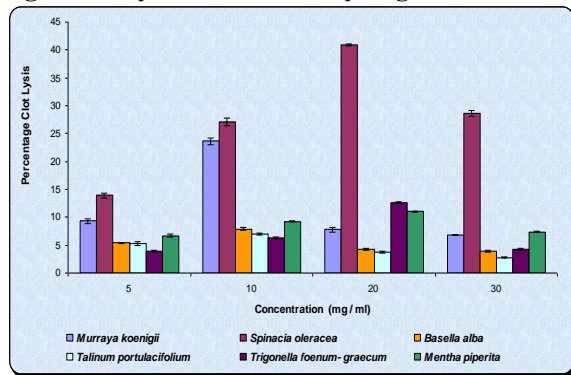
Statistical Analysis

The data of various observations were subjected to statistical analysis to find the significant difference in the parameters studied

Results and Discussion

Screening of Plants for Thrombolytic Activity The thrombolytic activity of the aqueous extracts of plants namely *Murraya koenigii*, *Spinacia oleracea*, *Basella alba*, *Talinum portulacifolium*, *Trigonella foenum-graecum* and *Mentha piperita* was determined *in vitro* using human blood. The percentage clot lysis is shown in Figure I.

Fig 1 Clot Lysis of Green Leafy Vegetables



Thrombolytic activity of all the plants tested did not follow a dose-dependent increase. On the other hand, the extent of clot lysis peaked at a particular optimum concentration, on either side of which, a lower activity was observed. This peak activity was attained at 10mg/ml concentration for *Murraya koenigii*, *Basella alba* and *Talinum portulacifolium*, while it was at 20mg/ml for *Spinacia oleracea*, *Trigonella foenum-graecum* and *Mentha piperita*. Such an optimal dose effect was also reported by Islam *et al.* (2013) for *Tinospora crispa*. The comparison of the thrombolytic activity of the six different plants indicated that *Murraya koenigii*

and *Spinacia oleracea* exhibited a higher extent of clot lysis than the other plants. Hence further analysis was carried out with these two plants.

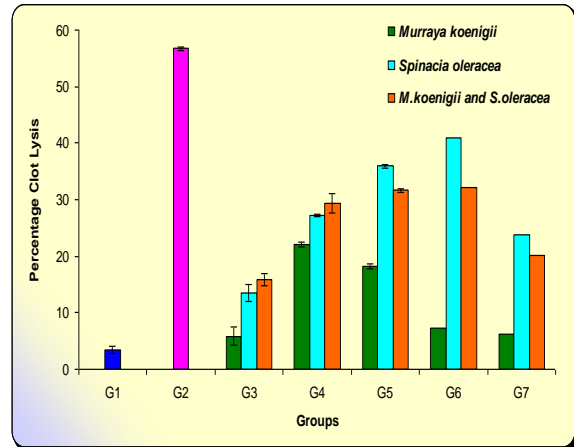
Thrombolytic Activity of *Murraya koenigii* and *Spinacia oleracea*

Figure 2 represents the thrombolytic activity of various concentrations (5-25mg) of aqueous extracts of *Murraya koenigii*, *Spinacia oleracea* and combination of both the plants. All the five different concentrations of the plant extracts induced significant clot lysis when compared to negative control. The positive control streptokinase (30,000 IU) evoked a huge and significant clot lysis. *Murraya koenigii* exhibited maximum clot lysis at a concentration of 10mg/ml while *Spinacia oleracea* showed the maximal value at a concentration of 20mg/ml.

It is evident from the Figure that percentage clot lysis of the extracts at the highest dose was lesser than that produced by the penultimate dose. Similar findings have been reported by Ratnasooriya *et al.* (2008) who have indicated that the thrombolytic activity of the highest dose (20mg/ml) was lower than that produced by the penultimate dose (10mg/ml). All the six plants tested showed clot-lysing abilities, albeit to varying extent. An another

striking observation that can be made from our results is that When the two different plants *Murraya koenigii* and *Spinacia oleracea* were used individually, *Spinacia oleracea* registered greater thrombolytic activity (40.9%) than *Murraya koenigii* at a concentration of 20mg/ml. When they were used in combination maximum clot lysis (32.22%) was

Fig 2 Thrombolytic Activity of the Selected Plants



G1 Negative Control G2 Positive Control observed at the same concentration (20mg/ml) but percentage was lesser than the value recorded by *Spinacia oleracea* when used individually indicating that some compounds in *Murraya koenigii* would have interfered in lysing the clot. Thus the results revealed that *Murraya koenigii* and *Spinacia oleracea* exhibited considerable percentage of clot lysis whether used individually or in combination suggesting that both plants can be exploited for thrombolytic therapy.

Comparison of Serum Cholesterol Level and Thrombolytic Activity of Selected Plants

Total cholesterol was estimated in the serum obtained from an aliquot of the blood samples used for clot lysis. A comparison was made between the serum cholesterol level and percent clot lysis. The values are indicated in Figures 3a,3b and 3c. The scattergram of paired values of cholesterol levels and the corresponding clot lysis values in each sample varied widely. While a mild positive correlation observed with *Murraya koenigii* and the mixture of two leaf extracts, there was a slight negative correlation in the samples treated with *Spinacia oleracea*. This lack of correlation indicates that both the leaves can render good protection against clot induced blocks in blood vessels, irrespective of the cholesterol levels of the individual. It also suggests that both the leaves can act as sources of potential

thrombolytic agents, irrespective of the cause of clot formation.

Fig 3a Correlation between Serum Cholesterol and Thrombolytic Activity of *Murraya koenigii*

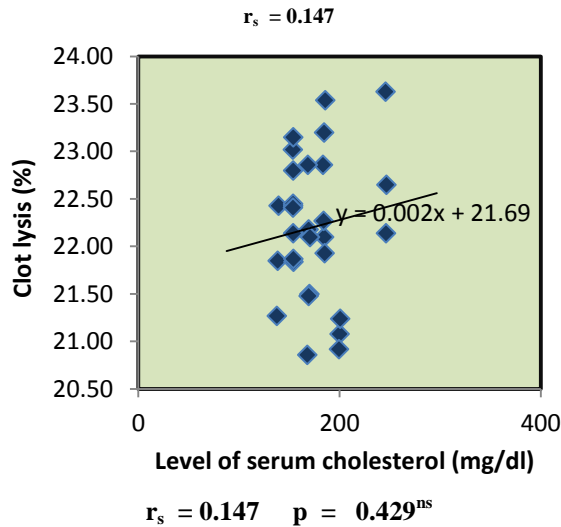
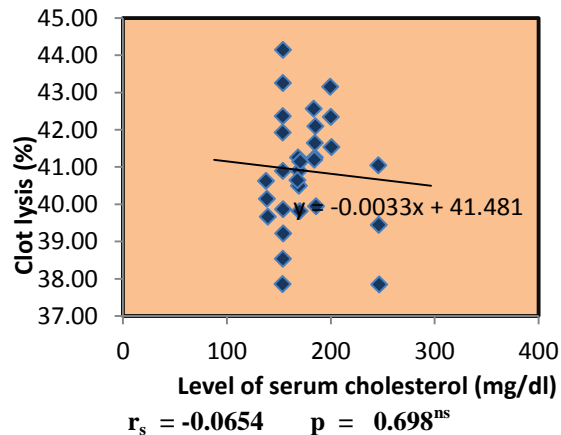


Fig 3b Correlation between Serum Cholesterol and Thrombolytic Activity of *Spinacia oleracea*



Determination of Membrane Stabilizing Activity of the Selected Plants

Figure 4 depicts the membrane stability of the selected plants. The results suggest that the standard exhibited 59.36% and 64.75% inhibition for heat induced and hypotonic solution induced haemolysis respectively. Both the plants namely *Spinacia oleracea* and *Murraya koenigii* also showed inhibiting potential. However, the magnitude of inhibition for *Murraya* was comparatively low (46.17% for heat induced and 40.30% for hypotonic solution induced) when compared to *Spinacia*

oleracea which showed 57.82% for heat induced and 74.82% for hypotonic solution induced haemolysis. The percentage inhibition of the latter was significant when compared to the standard. Shahriar *et al.* (2012) showed that in heat induced and hypotonic conditions the methanol extract of *Withania somnifera* inhibited 45.91% and 63.95% of haemolysis of RBCs respectively as compared to 42.12% and 72.9% inhibition by acetyl salicylic acid respectively (0.1 mg/ml). The findings of the study are in accordance with the present report. Similar results were also reported by Kawsar *et al.* (2011) and Khan *et al.* (2013). Both the plant extracts moderately protected the human RBC membrane which is an essential quality for thrombolysis. This significant inhibition of haemolysis *in vitro* could have a potential therapeutic effect on the disease process causing destabilization of biological membrane.

Fig 3c Correlation between Serum Cholesterol and Thrombolytic Activity of *Murraya koenigii* and *Spinacia oleracea*

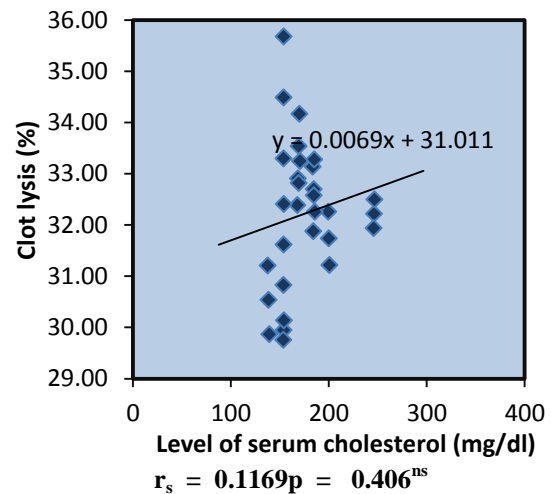
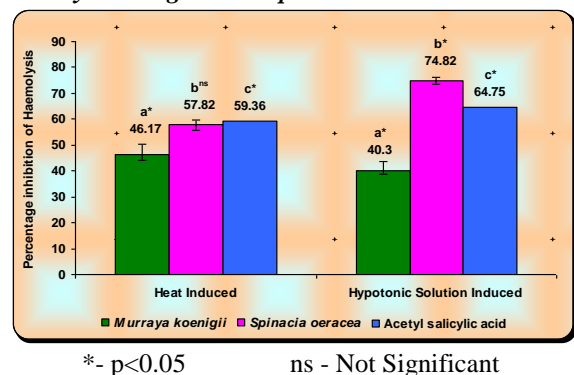


Fig 4 Percentage Inhibition of Haemolysis by *Murraya koenigii* and *Spinacia oleracea*



Thrombolytic Property of Selected Plants *in vivo*

This study was designed to explore the effect of selected plant extracts on ferric chloride (FeCl₃) - induced thrombus in experimental rats. An acute toxicity study was carried out to find out the effective dose of plant extracts to be administered. Appearance of thrombus in the tail region of experimental animals is shown in Plate 1.

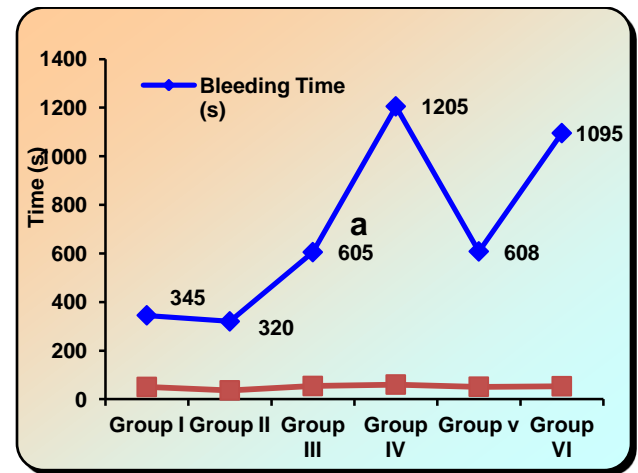
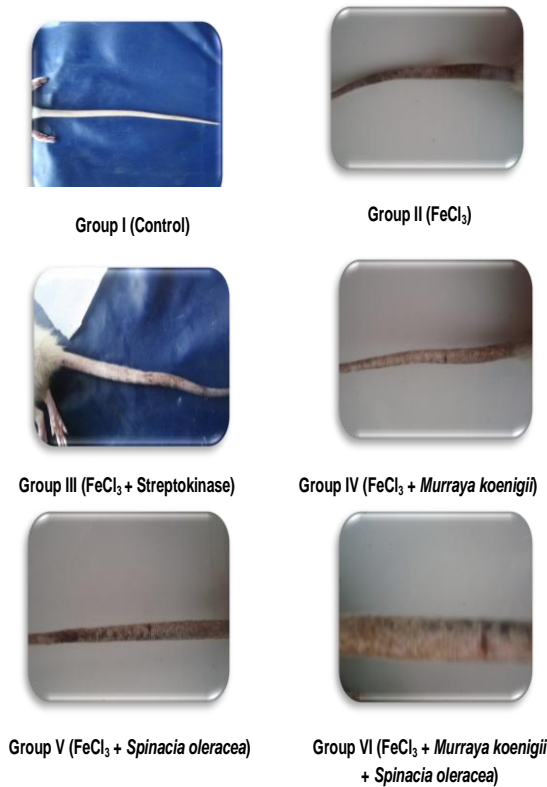
Haematological Parameters in Experimental Animals

WBC, RBC, Haemoglobin level and Platelet count and in experimental rats are depicted in Table 1. The control groups showed 7.73×10⁶ cells/μl of RBC. When a thrombus was induced, RBC count increased to 9.06×10⁶ cells/μl, which is similar to the findings of Cadroy and Stephen (1990) who have described that thrombus formation increases when RBC count increased. On the treatment of the thrombus with streptokinase, the levels decreased but were well within the control range, featuring the initiation of lysis.

extracellular haemoglobin may also contribute to platelet activation and thrombosis. The platelet count in the control group which decreased when a thrombus was formed. Similar finding was observed by Monreal *et al.* (1991) who have described that patients with thromboembolism may have lower platelet count. The number of cells increased to 707×10³ cells/μl, when treated with streptokinase, posing a possible reocclusion that remains a drawback for thrombolytic therapies as described by Montrucchio *et al.* (1993). In contrast, when the plant extracts were administered, platelets count decreased to 649×10³ cells /μl for *Murraya koenigii* and 660×10³ cells /μl for *Spinacia oleracea*. The plant extracts when given in combination the count was decreased to 610×10³ cells/μl which was still lower than when given individually. This effect could possibly complement the negative effect of streptokinase in thrombolytic therapies. Figures 6 represents the bleeding and activated partial thromboplastin time in experimental rats.

Figure6 : Bleeding Time and Activated Partial Thromboplastin Time in Experimental Animals.

Plate 1
Appearance of Thrombus in the Tail of Experimental Animals



Bleeding time and Activated Partial Thromboplastin Time (APTT), the key influential factors of thrombolysis were reduced on initiation of a clot. Lysis of the clot with extracts led to an increase in BT and APTT. A similar trend was seen in plant extracts also, with *Spinacia oleracea* recording the least value of 608 s for bleeding time and 51 s for APTT, which was comparable with the values of streptokinase. Though the BT and APTT have increased after treatment, they lie within the normal range depicting that there is a minimal chance of bleeding, a major complication in thrombolysis treatment. Prezoto *et al.* (2002) evaluated the possible antithrombotic and thrombolytic activities of *Lonomia obliqua* caterpillar bristle extract (LOCB) in

The results are similar to the findings of Zhou *et al.* (2011), who have suggested that the excessive

the experimental animals and reported that significant increase in bleeding time seen in LOCBE treated rats might be induced by the antithrombotic components or by other substances present in the crude extract.

Table 2 represent the level of fibrinogen, D-dimer and Creatine phosphokinase in experimental rats

Table 2: Fibrinogen, D-dimer and Creatine phosphokinase Levels in Experimental Animals
Column means followed by common superscript are not significant at 5% by DMRT

Groups	Fibrinogen mg/dl	D-dimer ng/ml	Creatine phosphokinase U/L
G1	700.0± 6.78 ^a	349.0±2.67 ^f	129.00±1.94 ^d
G2	650.0±6.18 ^b	372±4.56 ^e	84.00±1.45 ^e
G3	425.0±5.39 ^e	629.00±4.82 ^a	164.00±1.23 ^a
G4	569.0±4.89 ^c	549.00±4.13 ^c	146.00±1.55 ^b
G5	465.0±6.15 ^c	512.00±3.98 ^d	146.00±2.05 ^b
G6	529.0±5.71 ^d	592.004.52 ^b	141.00±1.79 ^c
CD (p<0.05)	8.237	6.452	2.489

The concentrations of fibrinogen and D-dimer are inversely proportional to each other, i.e., when a thrombus is formed, fibrinogen is converted to fibrin, which is then degraded to D-dimer on thrombolysis. The same pattern was observed in the present study, where the levels of fibrinogen decreased on the incidence of clot, and on clot lysis, the level of fibrinogen decreased further, while the D-dimer concentration increased. The D-dimer concentration did not differ significantly when compared to control and FeCl₃ induced group. But when streptokinase was administered, the D-dimer level increased to 629 ng/ml confirming clot lysis. Similarly, when the plant extracts were given individually and in combination the D-dimer level was increased. These results collectively suggest that the plant extracts have clot lysing ability which is comparable to that of streptokinase. Creatine phosphokinase followed a similar pattern like fibrinogen, where the activity was reduced when a thrombus was formed. Subsequently, when the clot was lysed either with streptokinase or with plant extracts, the activity of creatine phosphokinase increased significantly

Catalase, Superoxide dismutase and Total Protein Content in the Liver of Experimental Animals

Table 3 represents the level of catalase, superoxide dismutase (SOD) and total protein in experimental rats.

Groups	Catalase (U/g)	Superoxide dismutase (U/g)	Total Protein (U/g)
G1	0.493 ± 0.005 ^b	0.133 ± 0.004 ^d	1.39 ± 0.17 ^b
G2	0.276 ± 0.004 ^f	0.066 ± 0.004 ^e	0.62 ± 0.72 ^c
G3	0.413 ± 0.003 ^e	0.127 ± 0.005 ^c	0.78 ± 0.71 ^c
G4	0.424 ± 0.004 ^d	0.124 ± 0.004 ^c	1.51 ± 0.21 ^b
G5	0.483 ± 0.003 ^c	0.191 ± 0.007 ^b	1.53 ± 0.13 ^b
G6	0.528 ± 0.003 ^a	0.213 ± 0.006 ^a	2.11 ± 0.01 ^a
CD (p<0.05)	0.0062	0.0091	0.578

Column means followed by common superscript are not significant at 5% by DMRT

Catalase: Amount of enzyme required to decrease the optical density by 0.05 units, SOD: The amount that causes 50% reduction in the extent of NBT oxidation

It can be seen from the values in the table, that the administration of FeCl₃ to the experimental animals decreased the activity of catalase, superoxide dismutase and the concentration of total protein in liver. After thrombus formation, when the animals were treated with the standard drug streptokinase and the plant extracts, the enzymatic activity and the protein concentration were found to be enhanced. Among the three groups which received the plant extracts individually and in combination, the combined administration showed significantly higher activity of catalase and SOD than the groups which received individual plant extract. In the case of protein combined administration showed higher protein content than individual administration of plant extract. Studies conducted by Mitra *et al.* (2012) indicated that the aqueous extracts of *Murraya koenigii* leaf conferred significant protection to rat cardiac tissue against cadmium-induced oxidative stress, probably due to its antioxidant activity.

Histopathological Examination of Experimental Animals

The cellular changes in liver, heart and kidney are indicated in plates 2, 3 and 4 respectively and the findings of histopathological examination are discussed below.

The histology of the liver sections of control animals showed intact hepatocytes and there was no inflammation, fibrosis and toxic change. Liver sections of FeCl₃ treated animals revealed mild lobular inflammation. The sinusoids showed dilatation and central vein showed congestion. Portal

traiditis was noticed in the portal tract. In the case of animals treated with streptokinase showed normal hepatocytes without any inflammation. Liver section of animals treated with *Murraya koenigii*, *Spinacia oleracea* and combined extracts of *Murraya koenigii* and *Spinacia oleracea* revealed moderate periportal inflammation.

Heart section of control animals showed normal myocardium and mild congestion was observed in blood vessels. There was no inflammation, edema or necrosis. Heart sections of FeCl₃ treated animals revealed mild scattered lymphocytic infiltrates in the myocardium. Myocytes showed mild degeneration and areas of congested vessels and haemorrhage were also observed.

In the case of streptokinase treated animal showed normal cardiac muscle and the blood vessels showed mild congestion. No inflammation, edema or necrosis was observed. Heart sections of animals treated with individual and combined administration showed mild congestion.

The histopathological examination of the kidney sections of control animals showed normal cortex, medulla and pelvicalyceal system. The blood vessels showed mild congestion and thickening. There was no inflammation or tubular necrosis. Kidney sections of FeCl₃ treated animals revealed focal lymphocytic infiltration. Tubules showed focal nuclear loss. The blood vessels showed thickening and congestion. The kidney sections of streptokinase treated animals showed normal cortex and medulla without any inflammation. The animals treated with *Murraya koenigii* indicated normal tubules. Treatment with *Spinacia oleracea* animals showed nuclear loss in the tubules. Kidney sections of animals treated with combined extracts revealed normal glomeruli and tubules.

Jadhav *et al.* (2013) have indicated that the altered histoarchitecture of heart and kidney tissue due to doxorubicin treatment was improved with *Luffa acutangula* Roxb. Potential cardioprotective effect of *Saraca Indica* supported by histopathological examination was demonstrated by Swamy *et al.* (2013).

Plate 2 Histopathological observations of Liver of Control and Experimental Animals

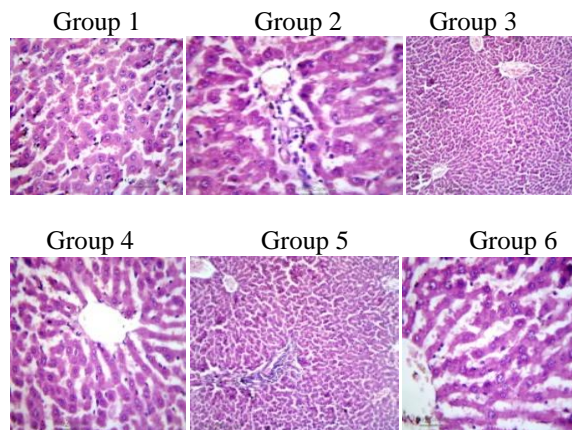


Plate 3 Histopathological observations of Heart of Control and Experimental Animals

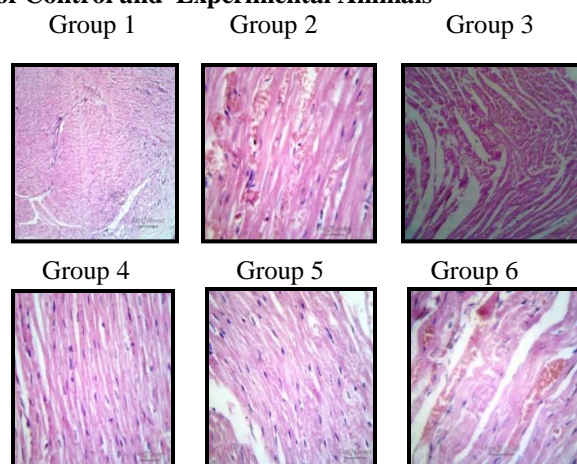
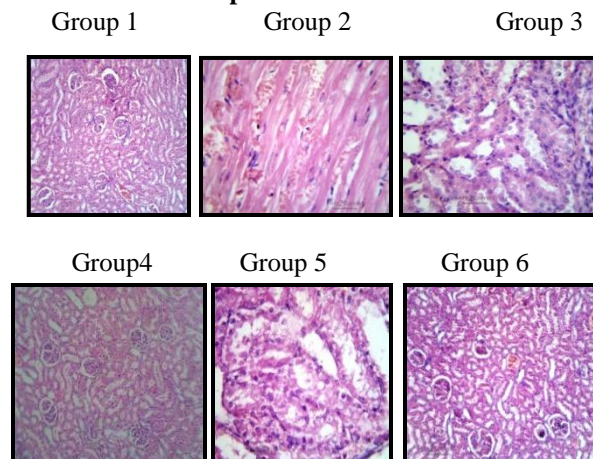


Plate 4 Histopathological observations of Kidney of Control and Experimental Animals



The results of histopathological examination showed that the changes observed in the histoarchitecture of heart, liver and kidney due to thrombus formation were improved by administration of the standard drug and the plant extracts.

Conclusion

Assessment of thrombolytic properties of *Murraya koenigii* and *Spinacia oleracea* revealed that these plants can be considered as a valuable source of therapeutic agents for human health as thrombolytic agent. The present study might have important implications in cardiovascular health in developing countries where cheap and effective medicines are the need of the hour.

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