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Original Research Article

Anti-thrombotic effect of *Zingiber officinale* (ginger) in sprague dawley rats

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ABSTRACT

Background: The prevalence of thrombotic diseases is rising globally. Presently, stroke and ischemic heart disease account for 25% of all deaths. Use of anti-thrombotic drugs have proven effective in prevention of these ailments but might not be affordable especially in developing countries. They are also associated with undesirable side effects. This study sought to determine the anti-thrombotic effect of ginger since it is affordable, accessible and is widely used as a food enhancer and a medicinal herb.
Methods: The current study employed an *in-vivo* experimental study design. Three groups Sprague dawley rats (N=5) were given different doses of methanolic extract of ginger for 30 days. Two other groups (N=5) which served as controls received 5% dimethyl sulfoxide and aspirin for the same duration. Measurement of bleeding time, platelet count, prothrombin time, activated partial thromboplastin time and thrombin time was done to assess the anti-thrombotic property.
Results: There was a statistically significant difference in bleeding time (P=0.03) across the groups investigated. There was however no significant difference across the groups in platelet count, prothrombin time, activated partial thromboplastin time and thrombin time (P=>0.05).
Conclusion: This study demonstrates that methanolic extract of ginger possesses an anti-thrombotic property probably through inhibition of platelet function. Regular consumption of ginger may therefore confer protection against thrombotic diseases.

Keywords: Coagulation, Ginger, Thrombosis, *Zingiber officinale*

INTRODUCTION

Zingiber officinale (ginger) is a popular plant that is widely used as a food spice and as a medicinal herb.[1] It is used in management of gastroenteritis, asthma, diabetes, pain, hyperlipidemia among other ailments.[2] In addition, recent studies have elucidated that it could as well be having anti-thrombotic properties.[3] Though data exists on its medicinal values in management of many conditions, there is paucity of data on its efficacy as an anti-thrombotic agent, owing to the inconsistency of the few studies that investigate it's anti-thrombotic effect.[4] Ginger is a grass like crop that grows two to four feet tall from the ground. It is its root (rhizome) that is widely used for dietary and medicinal purposes. This crop is native to India and China but is now grown across the world including Africa.[1]

Hemostasis is the process in which the body prevents blood loss following damage of blood vessel and it involves vasoconstriction, platelet plug formation and activation of the coagulation cascade.[5] The processes involved in hemostasis are desired when there is breach of vascular integrity as they help to curb blood loss. However thrombosis which involves intravascular clot formation without breach of vessel wall continuity is undesired as this might occlude the vessel and compromise perfusion of distal tissue. Thrombosis is implicated as the main pathology that underlies ischemic heart disease and ischemic stroke.[6] Presently, thrombotic diseases are among the leading causes of mortality and morbidity. It is estimated that ischemic heart disease and stroke are responsible for one in four deaths around the globe.[6]

Anti-platelets and anti-coagulants drugs like aspirin and warfarin respectively have been utilized for prophylaxis and treatment of thrombotic disorders.[7] However, the cost and the undesired side effects of these conventional drugs pose a challenge. For instance, prolonged use of aspirin may cause stomach and duodenal ulceration while warfarin have been documented to cause dermatonecrosis and teratogenic effects.[8] Clopidogrel, another antiplatelet drug has been documented to be more effective than aspirin but this drug is costly and has a wider profile of adverse effects like neutropenia and thrombocytopenia.[9]

Some studies have suggested that ginger inhibits platelet function. Most of these studies assess *in vitro* outcomes of various extracts of ginger. However, other studies have reported that ginger has no effect on platelet function. A systematic review by Marx and colleagues reported different results in eight randomized control trials. Four of these studies reported that ginger possessed anti-platelet function while four reported no effect.[4] It is these conflicting results in studies in addition to their limited number that prompted the need to carry out the current study. It sought to determine the anti-thrombotic effect of *Zingiber officinale* (Ginger) in Sprague dawley rats.

METHODS

Preparation of ginger extract and aspirin

Fresh *Zingiber officinale* rhizomes were obtained from a vegetable vendor. Botanical confirmation was done at the department of botany of Jomo Kenyatta University of Agriculture and Technology. They were then washed with water, peeled and cut into small pieces. Extraction of *Zingiber officinale* was done using methanol over 48 hours and later the solvent was left to evaporate for another 48 hours using rotatory evaporator. The extract was then weighed and dissolved in 5% dimethyl sulfoxide (DMSO). This ginger solution was then refrigerated at 4^0C for use in the experiment. Aspirin (acetyl salicylic acid) was used as a positive control. Pure molecules were obtained and dissolved in 5% DMSO to make aspirin solution. The prepared solution was then refrigerated for use in the experiment.

Animals

Twenty five Sprague dawley rats were procured from and kept in the animal facility of university of Nairobi. They were kept in appropriate rodent cages and allowed access to approved food pellets and tap water ad libitum. They were handled humanely and the rules and regulations of the animal house were adhered to. FELASA guidelines were adhered to at all times during the course of the study as the principles of replacement, reduction and refinement were observed.[10] Twenty five animals were randomized into five groups (N=5). Administration of treatment agents was then done through oral gavage every morning as indicated below:

- **Group 1:** This served as negative control group and received 2ml of 5% dimethyl sulfoxide (DMSO) for 30 days.
- **Group 2:** This served as a positive control which received 10mg/kg of aspirin (low dose) for 30 days.
- **Group 3:** This served as the first treatment group which received 50mg/kg (low dose) of ginger for 30 days.
- **Group 4:** This was the second treatment group which received 250mg/kg (medium dose) of ginger for 30 days.
- **Group 5:** This was the third treatment group which received 500 mg/kg (high dose) of ginger for 30 days.

Assessment of antithrombotic effects

All measurements were done 24 hours after administration of the last dose. Animals were sedated using intra-peritoneal injection of ketamine (100mg/kg) before performing bleeding time and collecting blood samples. Bleeding time was assessed by cutting the tail at one centimeter from the distal end. The time interval from appearance of blood and cessation of bleeding was noted by making drops of blood on a piece of paper. This indicated the bleeding time and was recorded in seconds. Blood was collected from retro orbital plexus into citrated vacutainers, for prothrombin time (PT), activated partial thromboplastin time (APTT), and thrombin time (TT), and into EDTA containing vacutainers, for platelet count. Citrated blood was then centrifuged at 2000r/min for 10 minutes to obtain plasma which was used to perform PT, APTT and TT. Whole blood was used to perform platelet count. Platelet count was done using Yuesen Med automated hemocytometer. Prothrombin time was done by adding PT reagent (tissue thromboplastin) to the plasma sample and incubating it at 37^0 C. The time it took for the clot to appear after addition of the reagent was taken as the prothrombin time and was recorded in seconds. APTT was done by adding APTT reagent (kaolin and phospholipid) to plasma sample and then incubating it at 37^0C. The time it took for clot to form after addition of reagent was noted and taken as the

APTT and recorded in seconds. Thrombin time was then assessed by adding TT reagent (thrombin) to the plasma sample and incubating it at 37^0 C. The time it took for a clot to appear was recorded in seconds as thrombin time.

Data analysis and presentation

Data was analyzed using Scientific Package for Social Sciences (SPSS) version 21 (SPSS Inc. Chicago, IL). Means were compared using one way analysis of variance (ANOVA) and $p<0.05$ was considered significant. Post hoc test (Tukey) was performed to identify the group (s) with significant difference. Results are presented in tables as means±standard error.

RESULTS

Bleeding time

The current study investigated the effect of ginger extract on bleeding time to determine its effect on platelet function. There was a statistically significant difference across groups investigated for bleeding time (p=0.03). High dose group (HDG) had a mean of 139.2 while aspirin (positive control) had a mean of 141. 4. These 2 groups were significantly different from the other groups. Despite the lack of statistically significant difference across low dose (LD), medium dose (MD) and 5%DMSO, bleeding time increased in a dose dependent manner with MD and 5% DMSO registering the highest (130.6) and the lowest (127.2) readings respectively. The results for bleeding time are shown in Table 1.

Table 1: Bleeding time.

Group	N	Mean	Std. Error
5% DMSO	5	123.2	3.54
High dose ginger	5	139.2	2.95
Medium dose ginger	5	130.6	5.37
Low dose ginger	5	127.2	1.95
Aspirin	5	141.4	5.73

p=0.03

Platelet count

Authors sought to determine whether the ginger extract exert antiplatelet effect through inhibition of thrombopoiesis by conducting platelet count. There was no statistically significant difference in platelet count across all groups (p=0.08). 5% DMSO group registered the highest platelet count whereas MD had the lowest. The results for platelet count are shown in Table 2.

Thrombin time (TT)

This study sought to examine the effect of ginger extract on fibrinogen function by measuring thrombin time across all groups. There was no statistically significant difference between groups studied for TT (p=0.43). However, TT was higher in ginger groups compared to negative control. The results for TT are shown in Table 3.

Table 2: Platelet count.

Group	N	Mean	Std. Error
5% DMSO	5	744.40	38.32
High dose ginger	5	648.80	21.63
Medium dose ginger	5	615.60	48.04
Low dose ginger	5	663.80	16.28

Table 3: Thrombin time.

Group	N	Mean	Std. Error
5% DMSO	5	19.07	1.29
High dose ginger	5	20.04	1.41
Medium dose ginger	5	22.03	2.39
Low dose ginger	5	19.83	1.28

Prothrombin time (PT)

To determine the effect of the extract on the extrinsic pathway of coagulation, we performed a prothrombin time. There was no statistically significant difference between groups studied for PT (p = 0.64). PT was highest in HDG. Results for prothrombin time are presented in Table 4.

Table 4: Prothrombin time.

Group	N	Mean	Std. Error
5% DMSO	5	30.28	3.89
High dose ginger	5	31.89	3.28
Medium dose ginger	5	26.67	1.52
Low dose ginger	5	28.05	1.35

Activated partial thromboplastin time (APTT)

APTT was performed to assess whether the ginger extract affect the intrinsic pathway of coagulation There was no statistically significant difference between groups studied for APTT (p=0.99). However, the highest APTT was seen in HDG. Results for APTT are presented in Table 5.

Table 5: Activated partial thromboplastin time.

Group	N	Mean	Std. Error
5% DMSO	5	38.34	4.16
High dose ginger	5	39.17	3.87
Medium dose ginger	5	38.50	3.08
Low dose ginger	5	36.38	5.70

DISCUSSION

Platelet function

The current study has shown that methanolic extract of *Zingiber officinale* possesses an antiplatelet activity. High dose of *Zingiber officinale* significantly prolonged

bleeding time in rats. This study has also depicted that the extract has no statistically significant effect on platelet count. Absence of the latter effect hence implies that *Zingiber officinale* inhibits function rather than formation of platelets.

Platelets are small nucleus-free cells fragments of blood. They are discoid in shape and are involved in the process of hemostasis following vascular injury. They are products of megakaryocytes produced in the bone marrow. They amount 150-450 billion/liter of blood in normal humans and have a life span of 8 to 10 days in circulation. They contain special organelles, alpha and dense granules, which stores and release vital molecules that mediate hemostasis.[11,12]

Following vascular injury, exposed collagen binds to platelets via glycoprotein VI (GPVI) receptors leading to adherence of platelets to the injured endothelium. Von Willie brands factor (VWF) which is a product of endothelial cells and megakaryocytes also binds to platelets via GPIb-IX-V receptor and anchors to the injured endothelium.[13] Binding of these ligands to platelets, aside causing adherence of platelets to site of injury, also initiates signal transduction pathways that lead to platelets activation.

The main pathway activated by the ligands involves the activation of membrane bound phospholipase C which in turn generates inositol triphosphate (IP3) from 4' 5' phosphatidyl-inositol-diphosphate. IP3 activates calcium channels from the endoplasmic reticulum which in turn leads to entry of calcium into the cytosol from the intracellular stores [14]. Presence of calcium in the cytosol facilitates influx of additional calcium from the exterior of the cell. Both processes lead to rise in the cytosolic calcium which in turn initiates calcium dependent exocytosis of the contents of alpha and dense granules. Platelet activation is also called release reaction and involves secretion of adenosine diphosphate (ADP), serotonin and fibrinogen [11]. Stimulated platelets also utilize phospholipase A_2 to release the arachidonic acid (AA) from the inner part of the cell membrane phospholipids. The cell then uses cyclooxygenase to generate thromboxane A_2 (TXA_2) from the liberated AA. This eicosanoid (TXA_2) is crucial stimulator of platelet aggregation.[14]

ADP and TXA_2 bind to and activate additional platelets. ADP binding is through any of its receptors i.e. P_2Y_{12}, P_1Y_1, or P_1X_1 while TXA_2 binds to TP receptor. Both receptors initiate the signal transduction cascades that lead to more platelets activation hence amplifying the platelet response.[15] During this phase, platelets also express glycoprotein IIb/IIIa (GPIIb/IIIa) on the outer side of their altered irregular membrane. The irregularity of the cell membrane is also a consequence of platelet activation and involves transformation of a discoid shaped platelet to an irregular shaped platelet with finger like projections.[13] This change in shape serves to increase the surface area for platelet to platelet interaction. Numerous platelets that have been activated are linked together by fibrinogen and von Willie brand factor. This linkage is facilitated by binding of fibrinogen and vWF to the expressed GPIIb/IIIa.[14] These ligands can bind to more than one platelet or to platelet and endothelium at once. The consequence is that many platelets are trapped within the injured site; a process called platelet aggregation, and will temporarily seal the discontinuity of the vessel preventing blood loss.[16,11] Activated platelet also provides a surface for activation of thrombin. Conversion of prothrombin to thrombin occurs on the outer surface of activated platelet's membrane. Once activated, thrombin also stimulates additional platelets.[17]

The methanolic extract of *Zingiber officinale* could be exerting its antiplatelet property by inhibiting any of the steps of platelet adhesion, activation and aggregation. Possible mechanisms of actions include inhibition of cyclooxygenase (COX) necessary to generate TXA_2 or inhibition of any of the numerous platelet receptors e.g. GP IIb/IIIa, TP, P_2Y_{12}, GPVI or GPIb-IX-V.

Vascular injury that initiates these processes of platelet adhesion, activation and aggregation can be due to trauma leading to severing of the vessel or endothelial damage following rupture of atherosclerotic plaque.[11] The former is a desired mechanism because it leads to cessation of blood loss. However the latter is undesired because it occurs in an intact vessel. It is the main mechanism involved in cardiovascular events like myocardial infarction and stroke because the ensuing platelet aggregation leads to occlusion of a vessel leading to reduction or loss of blood supply to the myocardium and the brain respectively.[18]

Ginger significantly prolonged bleeding time in rats compared to placebo in a past study. Further, it also did not significantly affect platelet levels.[19] The results also agree with those which suggested that *Zingiber officinale* combined with nifedipine possesses synergistic antiplatelet function among normal persons and hypertensive patients. Nifedipine is a calcium channel antagonists that inhibits entry of calcium into the cell hence inhibiting platelet release reaction.[20]

The findings in this study contrast those by Jiang et al, where ginger registered no antiplatelet aggregation activity. Different outcome seen in this study could be attributed to the study design used and the subjects utilized. Jiang and colleagues used crossover experimental design to assess platelet aggregation in humans pre-treated with both ginger and warfarin.[21]

Coagulation

This study has also shown that methanolic extract of *Zingiber officinale* has no anticoagulant activity in rats. There was no significant difference among the means of

different groups studied for prothrombin time, activated partial thromboplastin time and thrombin time.

Coagulation is conventionally described as the function of extrinsic and intrinsic pathways and together with platelet aggregation serve to prevent blood loss. Extrinsic pathway begins with binding of tissue factor and clotting factor VII. Tissue factor is a trans- membrane protein which is expressed by cells that are not in constant contact with flowing blood. Such tissues include sub-endothelial matrix. Following endothelial damage, tissue factor is exposed. It then combines with and activates factor VII. Factor VII-tissue factor complex activates additional factor VII. Subsequently, factor VIIa, tissue factor and calcium form a tri-molecular complex that activates factor X. Activated factor X together with activated co factor V (activated by thrombin) and calcium form a tri-molecular complex called prothrombinase. Prothrombinase then proteolytically cleaves prothrombin to thrombin which in turn converts fibrinogen to fibrin monomers. Fibrin monomers undergo automatic polymerization to form a fibrin mesh which seals off the injured vessel preventing blood loss. Extrinsic pathway ends at the generation of activated factor X with the subsequent steps being referred to as a common pathway .[22,23] Intrinsic pathway begins when factor XII comes in contact with negatively charged surfaces like cell membrane of activated platelets or other subendothelial proteins. Activated factor XII together with co factors kallikrein and high molecular weight kininogen activates factor XI. Activated factor XI generates factor IXa (active) from IX (inactive). Activated factor IX with the help of thrombin and factor V activates factor VIII. Finally, factor VIIIa, IXa and calcium form a tri-molecular complex which converts factor X to its active form. From here the cascade follows the common pathway as described initially.[22,23]

This process of coagulation pathway is regulated by naturally occurring anticoagulants in plasma to ensure that it occurs only when it is needed e.g. following vascular trauma. Tissue factor pathway inhibitor (TFPI) inactivates the tissue factor- factor VII-calcium tri-molecular complex, thus inhibiting conversion of factor FX to FXa.[23] Anti-thrombin III with the help of heparin and heparan sulphate binds to and inactivates factor X and thrombin.[24] Protein C binds to thrombin-thrombomodulin complex and is activated to inactivate factors VIII and V. Protein S is required as a cofactor in this protein C mediated inhibition.[25] Thrombomodulin which is a product of endothelial cells, acting independently, binds to and removes thrombin from circulation.[26] These anticoagulants ensure that blood remain in a fluid state that can easily be transported inside the blood vessels. As already noted, calcium serves as a co factor in numerous steps of the coagulation pathway besides being involved in release reaction of platelets during platelet activation. States of calcium deficiency have been associated with coagulation dysfunctions.[27,28]

Prothrombin time which is the time taken for a clot to form after anti-coagulated plasma is mixed with tissue thromboplastin at physiologic temperature is a measure of the function of the extrinsic pathway. Prolongation is seen in defects that lead to both qualitative and quantitative dysfunctions of coagulation factors of the extrinsic and common pathways.[29] Activated partial thromboplastin time involves measurement of time taken for blood to clot after addition of kaolin and phospholipid (activated partial thromboplastin). Prolongation is seen in states of deficiency or inhibition of clotting factors in the intrinsic and common pathways.[29] Thrombin time assesses both qualitative and quantitative dysfunction of fibrinogen and refers to the time it takes for clot to appear after blood is mixed with thrombin.[29]

The insignificant results reported in this study on anticoagulant effects of *Zingiber officinale* are similar to those reported by prassad and colleagues. In that study, fresh ginger juice did not alter clotting time, prothrombin time, activated partial thromboplastin time and thrombin time [19]. However, these results contrasts those of Taj et al, which suggested that aqueous extract of *Zingiber officinale* has an anti-coagulant activity as demonstrated by prolongation of prothrombin time. This variation in outcome seen in between this study could be attributed to the different methods employed. While the setting of the current study was in-vivo using rats, Taj and colleagues assessed in-vitro outcomes using human samples.[30] Some components of ginger could undergo metabolism in the body systems leading to suppression of anticoagulant activity *in vivo*. They also contrast those of Ajala et al, which suggested that methanolic extract of *Zingiber officinale* prolongs prothrombin time, thrombin time and partial thromboplastin time. The difference between these two studies is that dried ginger rhizome was used in the past study while fresh ginger rhizome was used in this study.[31] There is slight variation in the phytochemical contents of *Zingiber officinale* between fresh and dry rhizome and this could contribute to the discrepancy in between the two studies.[32]

CONCLUSION

The current study has shown that methanolic extract of *Zingiber officinale* has anti-thrombotic activity in rats as evidenced by inhibition of platelet function. Suggestion can be made that regular consumption of *Zingiber officinale* may lower the risk of developing cardiovascular events like myocardial infarction and stroke by reducing the risk of thrombosis. Recommendations are made for further investigations to identify the phytochemical component (s) of ginger with this property.

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