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Research Article

Evaluation of Anti-Platelet Activity of Ethanolic Extract of *Lagenaria Sicereria* Fruit: *In Vitro* Study in Healthy Human Volunteers

Bhide SS, Bhojne SK, Maurya MK*, Raut SB, Uchil DA, Athavale CA and Kale JK

Department of Pharmacology and Therapeutics, Seth GS, Medical College and KEM Hospital, Mumbai, Maharashtra, India

***Corresponding author:** Mitesh Kumar Maurya, Department of Pharmacology and Therapeutics, Seth GS, Medical College and KEM Hospital, Mumbai, Maharashtra, India

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Abstract

Purpose: To evaluate the anti-platelet activity of Ethanolic Extract of *Lagenaria Siceraria* (EELS) fruit in healthy human volunteers. EELS has been shown to possess fibrinolytic and antiplatelet activity in animal models and it was interesting to find if it had a similar effect on human platelets.

Material and Methods: Blood was collected from 12 healthy human volunteers. Platelet viability using Lactate Dehydrogenase (LDH) assay was determined. Maximum platelet aggregation was found by an optical method on platelet aggregometer. Platelet aggregation was induced by ADP.

Results: The LDH estimated in all the groups was comparable and was not raised significantly suggesting platelet viability. All the study groups of Ethanolic Extract of fruit of *Lagenaria Sicereria* (EELS) 75, 100, 150 and 200 µg/ml significantly decreased the Mean Platelet Aggregation (MPA) as compared to vehicle control. The Percentage Inhibition (PI) of platelet aggregation in all doses of EELS was comparable to that produced by Aspirin.

Conclusion: Ethanolic extract of LS has antiplatelet effect in doses of 75, 100, 150 and 200 µg/ml in ADP induced platelet aggregation *in vitro* in human volunteers. The antiplatelet effect of EELS is not dose dependent.

Keywords: ADP; Platelet aggregation; Aspirin; Ethanolic extract; Lagenaria siceraria

Abbreviations

EELS: Ethanolic Extract of *Lagenaria Sicereria*; ADP: Adenosine Di-Phosphate; LDH: lactate Dehydrogenase; MPA: maximum platelet aggregation

Introduction

Hemostasis is a finely regulated dynamic process of repairing vascular injury and limiting blood loss while avoiding vessel occlusion (thrombosis) and inadequate perfusion of vital organs. In normal hemostasis, the activation of platelets followed by coagulation prevents hemorrhage after injury and thereby preserves vascular integrity [1]. Platelet activation and coagulation normally do not occur in an intact blood vessel [2]. However, platelets play a key pathophysiological role in the formation of a thrombus when a vascular injury such as rupture of an atherosclerotic plaque occurs. This may lead to vascular occlusion with resultant hypoxia and infarction of distal tissues [3]. Therefore, inhibition of platelet function is a useful prophylactic and therapeutic strategy against myocardial infarction and stroke caused by thrombosis in coronary and cerebral arteries respectively.

The classes of anti- platelet agents in current clinical use include- the cyclo-oxygenase (COX) inhibitors (e.g. aspirin), phosphodiesterase inhibitors (e.g. dipyridamole), ADP receptor pathway inhibitors (e.g. ticlopidine and clopidogrel) and platelet glycoprotein IIb/IIIa receptor antagonists (e.g. abciximab, tirofiban, and eptifibatide) [4]. They have an established role in the treatment and prevention of thrombotic vascular diseases but they have certain limitations. These include- weak and incomplete inhibition of platelet function (aspirin), slow onset of action (clopidogrel) and interpatient variability in anti- platelet response, inability to transform the success of intravenous GPIIb-GPIIIa antagonist therapy into successful oral therapy etc [5]. Also, there is an inability to completely prevent bleeding as demonstrated in CHARISMA trial [6].

Lagenaria siceraria is a common dietary ingredient (vegetable) in Indian food. It has been proved to have multiple therapeutic effects in chronic diseases like Diabetes, Hypertension, Constipation, skin diseases & bronchial disorders [7]. Additionally it has been reported to have antitumor, antiviral, antiproliferative, anti-hyperlipidemic and anti-atherosclerotic activities [8-10]. Ethanolic extract of Lagenaria has been shown to possess fibrinolytic [11] and antiplatelet [12] activity in animal models. With this background, it was interesting to find if it had a similar antiplatelet effect on human platelets. Hence the present study was undertaken with following objectives.

Objectives

To evaluate the concentration-dependent inhibition of platelet aggregation induced by ADP using an Ethanolic Extract of the fruit of *Lagenaria sicereria* (EELS) in normal healthy human volunteers with 4 doses 75,100,150 and 200 µg/ml.

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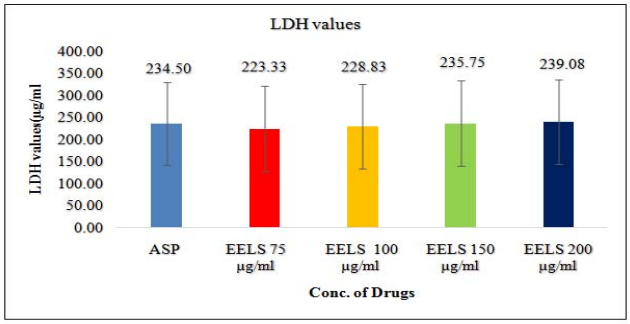

Figure 1: LDH values.
Mean LDH values in various groups. (n=12) EELS: Ethanolic Extract of *Lagenaria Sicereria.*

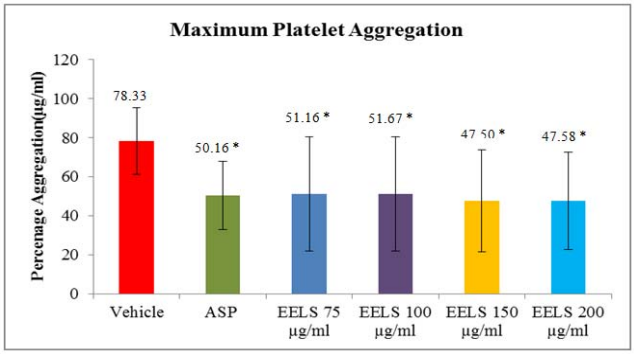

Figure 2: Maximum platelet aggregation.
Effect of 75µg, 100µg, and 150µg, 200µg of EELS and Aspirin on maximum platelet Aggregation. (n=12) Values are expressed in mean + SD, *Values Significant versus Vehicle group, P value <0.05 Using One way ANOVA followed by post-hoc Tukeys test.
EELS: Ethanolic Extract of *Lagenaria Sicereria;* ASP: Aspirin

Materials and Methods

Prior to the commencement of the study, an approval from the Institutional Ethics Committee of Seth G.S. Medical College and K.E.M. Hospital was sought (EC/OA-78/2016).

Study drugs

Test Drug: Ethanolic Extract of the fruit of *Lagenaria sicereria* (EELS) was procured from M/s. Konark Herbals and Healthcare, Mumbai in a powder form. It has undergone all qualitative analysis and the extract which obtained was up to the mark. The extract was stored at –20°C and dissolved in distilled water and used for the study at the working concentrations of 1.5, 2.0, 3.0 and 4.0 mg/ml. which gave a final concentration of 75, 100,150 and 200 µg/ml respectively [13]. The extractive value of EELS was 85.45%.

Standard Drug: Acetylsalicylic acid (Aspirin) procured from Sigma Chemical Co., St. Loius, Mi, USA was selected to compare the effect of EELS on platelet aggregation. Aspirin was obtained in a powder form and stored at room-temperature. Freshly prepared solution of Aspirin in distilled water was taken at a working concentration of 0.5mg/ml to get a final concentration of 0.025mg/ml. As Aspirin was sparingly soluble in distilled water, the solution was warmed at 80°C before use.

Vehicle: Distilled water which was used to dissolve EELS and aspirin served as Vehicle control in the study.

Platelet aggregating agents

ADP (Sigma Chemical Co., St. Loius, Mi, USA) procured in powder form was dissolved in normal saline to prepare a working solution of 50µM that gave a final concentration of 2.5µM were used as inducers of platelet aggregation. These concentrations are found to cause maximum aggregation of Platelet-Rich Plasma (PRP) (with platelet count adjusted to 2.5 × 105/µL) and have been standardized in our laboratory. The working solutions of ADP were stored at –20°C. Required dilutions of all study drugs were prepared freshly before use.

Sample size and Study population

As there were no studies in literature studying the anti-platelet effect of *Lagenaria sicereria* in healthy volunteers, the present study was done as a proof of concept study and therefore 12 volunteers were recruited for the study. After administering written informed consent, the study was carried out on *in vitro* in blood obtained from 12 healthy volunteers if they met eligibility criteria. The evaluation of the antiplatelet effect of all the four doses was done on samples from all the 12 volunteers; that are all the doses of EELS were tested on blood sample from each volunteer. Volunteers were recruited by mouth to mouth publicity.

Various study groups have shown in figure 1.

Study participants

Inclusion criteria:

1. Healthy volunteers in the age group of 18- 55 years.
2. Subjects ready to give written informed consent.

Exclusion criteria:

1. Patient taking medication known to affect platelet function in the past 30 days like Aspirin, NSAIDS, inhibitors of platelet receptors (abciximab, tirofiban, eptifibatide, ticlopidine, clopidogrel, prasugrel, cangrelor, ticagrelor), PDE inhibitors, anticoagulants, antimicrobials (penicillins, cephalosporins, amphotericin, hydroxychloroquine), Tricyclic anti-depressants, Selective Serotonin Reuptake Inhibitors etc.
2. History of diabetes mellitus or hypertension or gastrointestinal disorders.
3. History of smoking and alcohol intake within 24 hours of baseline.
4. History of known or suspected dysfunction of kidney, liver or bone marrow
5. History of any bleeding disorder or a family history of bleeding disorder
6. History of intake of any medication from allopathic or any alternative systems of medicine.
7. History of any medical or surgical illness that precludes participation in the study.
8. Pregnancy and lactation

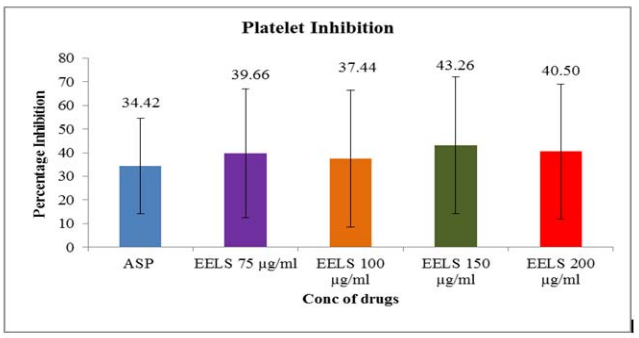

Figure 3: Percentage inhibition.
Comparison of percentage inhibition with Aspirin, 75µg, 100µg, 150µg and 200µg of EELS. (n=12)
Values are expressed in mean + SD, Kruskal Wallis test followed by post Hoc- Dunn's test.

The study was conducted on platelets harvested from eligible healthy volunteers. Presence of any disease or condition precluding participation in the study was ascertained on history, clinical examination and laboratory evaluation. Twelve volunteers who were non- smokers, non- alcoholics, with no history/ family history of bleeding diathesis, not consuming drugs known to affect platelet function in the past 30 days, and having their hemogram, liver function tests, renal function tests and lipids within normal laboratory range were included in the study. Volunteers were asked to take light diet on a preceding night and to avoid lipid-rich food.

Procedure for harvesting platelets

Volunteers were asked to report to the laboratory after overnight fasting. Twenty- five milliliters of venous blood was collected from these volunteers using aseptic precautions, in a citrated plastic tube (1ml of 3.8% trisodium citrate for 9ml of whole blood). The tubes were centrifuged at 800 Rotations Per Minute (rpm) for 8 minutes to obtain Platelet-Rich Plasma (PRP). The PRP was carefully removed, placed in stoppered plastic tubes and kept at room temperature. The remaining blood sample was recentrifuged at 4000rpm for 15 minutes to obtain PPP (platelet poor plasma). Platelets from platelet-rich plasma were counted using Abacus automated hematology analyzer. The platelet count was adjusted to 2.5 X 105 per µL using autologous PPP. The plasma with the adjusted platelet count is called as WPRP (working platelet rich plasma). WPRP was used for the various parts of the study.

Study procedures

The study was conducted in two parts as follows:

Part 1: Platelet viability [14]

Platelet viability using Lactate Dehydrogenase (LDH) assay was tested in volunteers for all concentrations of EELS (75,100,150 & 200 µg/ml) as well as aspirin and distilled water. Platelets contain LDH which is released when the platelets lose their membrane Permeability. This change in permeability occurs following platelet death. A significant rise in LDH release after incubation of WPRP with the study drugs for 3 minutes was indicative of loss of platelet viability. It was decided that any concentration of the study-drugs demonstrating a significant rise in LDH release would be excluded from evaluation in further part of the study. The LDH levels were measured using commercial LDH kits, on a semi-automated Biochemistry Analyzer at 340nm.

Table 1: Various study groups.

Groups ((N = 12)	Drug added in vitro to blood collected from healthy volunteers
Vehicle control	Distilled water
Positive control	Aspirin (0.025 mg/ml)
Group 1	75 µg/ml of EELS
Group 2	100 µg/ml of EELS
Group 3	150 µg/ml of EELS
Group 4	200 µg/ml of EELS

EELS: Ethanolic Extract of *Lagenaria Sicereria*

Part 2: Effect of EELS and Aspirin on ADP induced platelet aggregation [15]

Platelet aggregation was studied using the optical method described by Born in 1962 on a platelet aggregometer (Chrono-log Model 700 Whole blood/ Optical Lumi aggregometer, Havertown, PA, USA). The WPRP (440µL) was pre-warmed at 370 C for 5 minutes in siliconized glass cuvettes (provided by Chronolog Corporation, USA). Ten µL of either distilled water, different doses of EELS [75,100,150 & 200 µg/ml] or aspirin [0.025 mg/ml] were added to these WPRP cuvettes and incubated for 3 minutes. Then, the WPRP was transferred to the reading wells. The autologous PPP (platelet poor plasma) served to adjust the transmission to 100%. Platelet aggregation inducers i.e. ADP was added to the WPRP cuvettes in a volume of 50µL with continuous stirring using Teflon-coated stir bars to get a final concentration of 2.5 µM. The optical curves were allowed to run for 7 minutes. The aggregation pattern was recorded as percent aggregation versus time. The Maximum Platelet Aggregation (MPA) was recorded as an optical curve on the aggregometer (AGGRO/ LINK-8 and Vw Cofactor Software packages). The Percentage Inhibition (PI) shown by aspirin and EELS was calculated from the values of MPA using the formula,

$$\text{Percentage Inhibition}(\%) = \frac{1 - \text{MPA of } \textit{Lagenaria sicereria} \text{ or aspirin}}{\text{MPA of vehicle}} \times 100$$

The percent inhibition of mean platelet aggregation of the volunteers was used to obtain a mean value of percent inhibition.

Statistical analysis

The results were expressed as mean ± SD. The data was checked for normality. The groups were compared with one way ANOVA followed by Tukey's test or Kruskal Wallis followed by post Hoc-Dunn's test for parametric and non-parametric data respectively. The p-value of <0.05 was considered significant.

Results

Part 1: Platelet viability

LDH Values were compared using Kruskal Wallis test followed by post Hoc- Dunn's test since the data was not normally distributed. The LDH values of platelets treated with EELS 75µg/ml was 223.33± 96.6 IU/l, with100 µg/ml it was 228.83±96.5 IU/l, with 150µg/ml it was 235.75±96.49 IU/l and with 200 µg/ml 239.08± 95.56 IU/L. LDH value of aspirin treated platelets was 234.50±94.02IU/l (Figure 1).

Part 2: Maximum platelet aggregation

Maximum platelet aggregation in groups treated with 75µg/ml EELS was 51.16±29.3% , with 100µg/ml EELS it was 51.67± 29.4%, with150µg/ml EELS 47.50±26.06 %and with 200µg/ml EELS it was 47.58±24.08%. The MPA of EELS treated groups were comparable to positive control aspirin (50.16±17.49) and also values didn't differ significantly between all the test groups (Figure 2).

Percentage Inhibition

Percent inhibition of platelet aggregation with EELS 75µg/ml was 39.6±27.2%, 100µg/ml EELS was 37.4±28.8%, 150µg/ml EELS was 43.2±28.9% and with 200µg/ml it was 40.5±28.4%.The percentage inhibition produced by all the doses of EELS were comparable with Aspirin ($p>0.05$) (Figure 3).

Discussion

Platelets play a central role in the pathophysiology of Atherothrombosis. A vascular injury followed by platelet activation results in to acute ischemic complications like acute myocardial infarction, ischemic stroke [3]. Currently, the main classes of antiplatelet agents approved for the use in such complications are Thromboxane A2 synthesis inhibitors (aspirin), ADP receptor inhibitors (clopidogrel), Platelet phosphodiesterase inhibitors (dipyridamol) and Glycoprotein IIb/IIIa receptor inhibitors (Abciximab). But still current therapy has significant limitations such as weak and incomplete platelet inhibition, poor compliance, increased bleeding risk which occuring due to aspirin therapy [16]. There is inter-patient variability in platelet inhibition occurs due to Clopidogrel- Aspirin dual therapy. This variabllity of response may be due to genetic factors like polymorphism of CYP & P2Y12 receptors present on platelets. On the other side cellular factors like increased ADP exposure, upregulation of P2Y1, P2Y12 receptor pathway also causes inadequate platelet inhibition [17]. Delayed onset of action is also one of the drawback of anti-platelet therapy [18].

In the present study, we observed that of EELS has antiplatelet effect in ADP induced platelet aggregation in human platelets in the doses of 75, 100, 150 and 200 µg/ml *in vitro* which was comparable to Aspirin. Our results are in accordance with Rajput et al. who observed that ethanolic extract of the fruit of LS significantly increased bleeding time in mice by inhibition of ADP-mediated platelet aggregation [11]. A clinical study conducted by C. Katare also showed improvement in Cardiac risk ratio, atherogenic coefficient, and atherogenicity index of plasma when 200ml freshly prepared fruit extract was administered daily for 90 days [19]. In addition to anti-atherosclerotic effect, inhibition of Platelet aggregation would enhance the cardiovascular beneficial effect of LS. This study was conceptualized based on these reports and hence it was interesting to evaluate the anti-platelet action of EELS in human platelets *in vitro*.

Role of raised oxidative stress during platelet aggregation is well established, Superoxide at high levels also stimulate aggregation. NADPH through ADP increases the availability of ADP and increases the recruitment of platelets in the process of aggregation [20]. The antiplatelet activity of Ethanolic extract of *Lagenaria siceraria* may be due to its free radical scavenging and antioxidant properties though in our study we did not ascertain the mechanism of action of EELS [21,22]. Rajput et. al has proposed a non-cellular mechanism of EELS which plays important role in preventing ADP induced thromboembolism [12]. Further studies are needed to support the mechanism of action and to evaluate its anti-platelet effect in vivo. It will also be desirable to study the effect of EELS on various inflammatory markers involved in the process of coagulation [23].

Conclusion

Ethanolic extract of *Lagenaria sicereria* has antiplatelet effect in ADP induced platelet aggregation in human platelets in the doses of 75, 100, 150 and 200 µg/ml *in vitro* which was comparable to Aspirin & there was no dose dependent effect seen.

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