Study on antiplatelets activity of Gum resins of selected Indian Medicinal Plants

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Abstract- Ayurveda, the ancient Indian medicine system is renowned as one of the major systems of alternative and complimentary medicine. As other herbal systems, greater parts of its medicaments are based on indigenous herbals. In the past few decades, the interest in medicinal plants has increased in a great deal because of high efficacy and low side effects. The fact that the world's most common antiplatelet drug –Aspirin stems from a plant source *Spirea alba* there is a scope of developing many more drugs from plant origin producing similar effects without side effects. In the present study, we have tried to evaluate the antiplatelet properties of two most commonly used Ayurvedic plants viz. *Ferula asafoetida*-Hing, and *Aloe vera*-Kumari. Since there were many difficulties in making a clear and whole drug solution for the assay, only a part of the oleogum resin could be used for the antiplatelet study, i.e. ethanol soluble extract of *Ferula asfoetida* resin and water soluble extract of *Aloe vera* resin. However, *Shorea robusta* could not be studied for its antiplatelet activities as a suitable solvent was not available to dissolve the oleogum resin. The present study provides a strong basis that the oleogum resin of the two Indian Medicinal plant possess antiplatelet effects comparable to that of Aspirin and thus also provide a scope for further investigations in the related field for developing an effective yet safer antiplatelet medicine.

Key words: - Ayurveda, antiplatelet, Aspirin, *Spirea alba and Ferula asfoetida*

Introduction

Platelets have long been recognized to be of central importance in haemostasis preventing uncontrolled hemorrhage; but their participation in pathological conditions such as thrombosis, atherosclerosis is also well established[1]. They play a major role in arterial thrombosis, which is the final event complicating cardiovascular diseases as well as peripheral vascular diseases [2]; abnormal platelet reactivity has been linked to unstable angina, myocardial infarction, post angioplasty stenosis, cerebral ischemia, thrombotic stroke and a variety of inflammatory vascular disorders[3]. Approximately 71.3 million Americans have at least one of these cardiovascular diseases. An estimated 2,500 die of cardiovascular disease each day, an average of 1 death every 35 seconds[4]. Since platelets play a key role in the development of arterial thrombosis, antiplatelet drugs serve as a cornerstone in the prevention and the treatment of these conditions[5]. The most commonly prescribed anti-platelet drugs are Aspirin, Clopidogrel known as Plavix®, Cilostazol, Ticlid (ticlopidine hydrochloride)), dipyridamole (Persantine), etc. Acetylsalicylic acid or aspirin, synthesized by Hoffmann in 1898 is the most widely used drug in the world and is the drug of choice for cardiovascular diseases due to its good cost effectiveness profile. Although already used in the ancient world as an anti-inflammatory agent, aspirin was adopted for antithrombotic therapy only in the 1960s[6]. It inhibits platelets by irreversibly inactivating cyclooxygenase-1, thereby blocking the generation of thromboxane A_2, a potent vasoconstrictor

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and platelet agonist[7] thus reducing thrombus formation on the surface of the damaged arterial wall. Since the demonstration that aspirin is effective in the primary prevention of myocardial infarction, the prophylactic use of aspirin for thrombotic disorders has increased enormously. It has also become a standard treatment for patients with both cardiovascular and cerebrovascular diseases[8]. Studies have shown aspirin to reduce the occurrence of major cardiovascular events such as death, recurrent myocardial infarction, recurrent angina, nonfatal stroke, or progression to severe angina[4].
Despite of its great success, benefits of aspirin need to be weighed against its principle adverse effects such as gastrointestinal bleeding and haemorrhagic stroke, also recurrent thromboembolic vascular event. In children and adolescents, aspirin is no longer used to control flu-like symptoms or other viral illnesses, because of the risk of Reye's syndrome[9, 10]. Hence there is a need for potent yet safer antiplatelet agents to be developed as adjuncts to or substitutes for aspirin.
Medicinal plants have been in common use for centuries and are known to provide innumerous benefits without any side-effects. The traditional Indian medicinal system —Ayurveda remains one of the most ancient and yet alive tradition practiced widely in India, Sri Lanka and other countries. It describes various plants with antiplatelet properties that pose minimal side effects with higher efficacy. The most widely used cardiovascular drug - Aspirin also originated from a plant *Spiraea alba* that contains salicylate and therefore there lies a scope for developing more cardiovascular drugs from medicinal plants.
Recent studies have shown that the oleogum resins of many medicinal plants possess potent anti-platelet activities. Hence the current project was planned to further screen the oleogum resins of three other plants from Ayurveda *viz. Ferula asafoetida, Aloev vera* and *Shorea robusta* for their anti-platelet activities.

Ferula asafoetida is used as an antidote for flatulence[11]and for respiratory conditions like asthma, bronchitis, and whooping cough[12]. The gum resin of *Asafoetida* is used as a flavoring, food preservative and fragrance. Its oleo-gum-resin has been reported to be antiepileptic in classical Unani as well as ethnobotanical literature[13]. *Aloe vera* is a common herb used for its anti-inflammatory and anti-arthritic activity[14], as well as antibacterial[15], hypoglycemic[16, 17] and lipid-lowering effects[18]. Compounds contained within *Aloe Vera* can cause a reduction in prostaglandin synthesis, which may inhibit secondary aggregation of platelets[19.] The resin of *Shorea Robusta* is used in the indigenous systems of medicine as an astringent and detergent[20]. It is also used as an ingredient of ointments for skin diseases and in ear troubles.

In Ayurveda, *Shorea robusta* is used in treatment of arthritis, infection, wounds, ulcers, burns, pruritus, fracture, fever, hemorrhoid, menorrhagia, jaundice, splenomegaly, obesity, headache , and skin diseases whereas *Aloe vera* is used as a laxative, as a liver tonic, as a wound-healing agent, as an adaptogen and also widely for beauti care. Ferula asafoetida is used in treatment of digestive problems, respiratory conditions like asthma, bronchitis, fertility related problems, etc.

MATERIALS AND METHOD

The details of the materials and methods used to achieve the objectives of the study are described as under:

PART I

(a) Procurement and processing of plant drugs: Oleogum resins of the selected plants *Ferula asafetida, Aloe vera* and *Shorea robusta* were procured from a single standard source i.e. Dadar Pharmacy, Dadar. The drugs were crushed to fine powder using pestle and mortar then in a mixer grinder. The pulverized drugs were stored in dark brown air tight glass bottles at room temperature.

(b) Standardization of the crude drugs (Physico-Chemical Analysis) : As per WHO guidelines, the quality of the plant materials to be incorporated in the study should be determined and ensured prior to the commencement of the actual study. The quality of the plant drugs was ensured by carrying out a number of Physico-Chemical tests. The results; n=3 for each test were

compared to standard established values. The following test procedures were performed to assess the quality of the plant drugs:

Determination of Extractable Matter/Percentage solubility of the plant drugs: This method determines the amount of active constituents extracted with solvents from a given amount of medicinal plant material. It is employed for materials for which as yet no suitable chemical or biological assay exists.

Hot extraction: Solvent Used: Water: About 4.0 g of coarsely powdered air-dried material, accurately weighed was placed in a clean and dry conical flask. Take 100 ml of double distilled water at 80°C was added to the flask. The flask was allowed to stand for 1 hour at Room Temperature with intermittent shaking. The flask was then placed on a water bath at 100°C for 1 hour and allowed to cool. The contents of the flask were shaken well and filtered rapidly through Whatman's Filter Paper No. 1. About 25 ml of the filtrate was transferred to a pre-weighed petri-plate and further evaporated to dryness on water bath at 100^{0}C.It was further allowed to dry at 105^{0}C until the weight became constant. The weight of the residue was calculated as the difference in the weight of the empty petri-plate and the weight of petri-plate plus the dried residue. The percent extractable matter was calculated using the following relationship:

% Extractable Matter/ % water solubility = Weight of the Residue X 100/Weight of the Sample X 100/20

Cold maceration: About 4.0 g of coarsely powdered air-dried material, accurately weighed was placed in a clean and dry conical flask. Take 100 ml of double distilled water was added to the flask .The flask was allowed to stand for 1 hour at Room Temperature with intermittent shaking. The contents of the flask were then filtered rapidly through a Whatman's Filter Paper No.1.25 ml of the filtrate was transferred to a pre-weighed petri-plate and further evaporated to dryness on water bath 100 ^{0} C. It was further allowed to dry at 105^{0}C until the weight became constant. The weight of the residue was calculated as the difference in the weight of the empty petri-plate and the weight of petri-plate plus the dried residue. The percent extractable matter was calculated using the following relationship:

% Extractable Matter/ % water solubility = Weight of the Residue X 100/Weight of the Sample X 100/20

Solvent used: 70% Ethanol: About 4.0 g of coarsely powdered air-dried material, accurately weighed was placed in a clean and dry conical flask.100 ml of the 70% Ethanol was added to the flask and the total weight (powder+ethanol+flask) was calculated. The flask was allowed to stand for 18 hours at Room Temperature. The contents of the flask were then filtered rapidly through a Whatman's Filter Paper No.1. About 25 ml of the filtrate was transferred to a pre-weighed petri-plate and further evaporated to dryness on a water bath at 100°C. It was further allowed to dry at 105^{0}C until the weight became constant. The percent extractable matter was calculated using the following relationship:

% Extractable Matter/ % alcohol solubility = Weight of the Residue X 100/Weight of the Sample X 100/20

Determination of Ash: The ash remaining following ignition of medicinal plant materials is determined by different methods of which two methods *viz*. total ash and acid insoluble ash were performed as a part of Quality Control Studies of the plant drugs. The total ash method is designed to measure the total amount of material remaining after ignition. This includes both 'physiological ash', which is derived from plant tissue itself, and 'non-physiological ash' which is

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the residue of the extraneous matter (e.g. sand and soil) adhering to plant surface. Acid-insoluble ash is the residue obtained after boiling the total ash with dilute hydrochloric acid, and igniting the remaining insoluble matter. This measures the amount of silica present, especially sand and siliceous earth.

Total Ash: Take 1 g of the ground air-dried material, accurately weighed was placed in a tared, pre weighed crucible. The crucible was weighed along with the sample and the weight noted. The material was evenly spread in the crucible and ignited in a muffle furnace at 700^0C, until it turned white, indicating the absence of carbon. The crucible was allowed to cool in a desiccator and weighed. The total ash value was then calculated as the difference between weight of the crucible plus the weight of the sample before and after ignition.

Acid-Insoluble Ash: To the crucible containing total ash, 25 ml of 35% Hydrochloric acid was added. The crucible was covered with a watch glass and boiled gently for 5 minutes. The watch glass was rinsed with 5 ml of hot water and this liquid was added to the crucible. The contents of the crucible were then filtered through ash less filter paper and the residue was washed with hot water twice or thrice until the filtrate was neutral. The filter paper containing the insoluble matter was transferred to the original crucible and ignited in muffle furnace at 700^0C.The crucible was allowed to cool in the desiccator for 30 minutes and percent acid-insoluble ash in dried plant materials was calculated.

Determination of Microbial Load: Medicinal plant materials normally contain a great number of bacteria and moulds often originating in soil. The conditions for the test of microbial contamination are designed to minimize accidental contamination of the material being examined.

In order to determine the microbial load, a series of six tubes containing 10 ml (in the first tube) and 9 ml of sterile saline in the subsequent tubes were prepared. To the first tube 1 g of the powdered plant material was added. To the next five tubes 1 ml of aliquot from the previous dilution was added. The last four tubes of dilutions (10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}) were then used for determination of microbial load. This examination was carried out on sterile Nutrient Agar and sterile Sabouraud's agar plates by spread plate method.

Study protocol for anti-aggregatory studies for Ethics Committee approval.: Before the actual study commenced, an approval for the study from Ethics Committee was taken as it involved human volunteers. Hence a detailed protocol of the entire study plan was drafted based on referencing from relevant sources. Informed Consent Documents indicating the free will of the volunteers to participate in the study were also drafted in three different languages *viz.* English, Hindi and Marathi. In addition, Case Record Forms for recording the health status of the volunteer during the study process were also drafted. All the documents were submitted to the Ethics Committee for approval prior to the commencement of the study.

Standardization of Platelet-Aggregation Methodology: Platelet aggregation methodology described by GVR Born (1962) and modified by Chanarin (1989) was standardized in the laboratory

Sample Size : ***12 normal healthy volunteers (6 males and 6 females) were recruited for the study (n=6).***

Study Population: The study included individuals satisfying the following criteria:

Inclusion Criteria: Normal, healthy volunteers of either sex in the age group 18-30 years. Volunteers ready to give written informed consent to the study procedures.

Exclusion Criteria: Volunteers with smoking habits. Volunteers with history of cardiovascular disease, metabolic or systemic disease. Volunteers who have taken any NSAIDs during 2 weeks prior to enrollment. Volunteers with history of receiving treatment for any condition during 2 weeks prior to enrollment. Volunteers who have participated in any investigational study 2 weeks prior to enrollment.

Standard Drugs

(a) Anti-platelet Drug: Aspirin (5 µM, 2.5 µM).

(b) Platelet Aggregating Agent: ADP (Adenosine Diphosphate, 5 µM).

Materials for platelet aggregation study: About 3.8 % Trisodium Citrate Platelet Aggregometer: (Four channel Automatic Optical Aggregation System, Chronolog Corporation, USA)**.** Siliconised glass cuvettes: washed cuvettes dipped in 0.5% silicone oil emulsion and dried in hot air oven at 120^0C for 1 hour. Teflon coated stir bars. Aggregating Agent: 50µl of the aggregating agent *viz*. 5µM Adenosine Diphosphate (ADP) was used for *in vitro* analysis. The details of the preparation of aggregation agents have been described in the subsequent paragraphs.

Preparation of Reagents

Adenosine Diphosphate (ADP): ADP was weighed and dissolved in distilled water to a concentration of 1 mg/ml. This was the stock solution. From this 40µl of stock solution was added to 860µl of normal saline. This is the working solution of 100µM ADP, which gives a final concentration of 10 µM in the working test volume. Further dilutions were prepared to obtain a concentration of 5 µM. Stock solutions of ADP were kept refrigerated at 4^oC.1:1 dilution of 5 µM ADP gave 2.5 µM ADP.

Aspirin*:* 10 mg of Aspirin was dissolved in 10 ml of Distilled Water and kept in a hot water bath for 10-15 min at 60^oC. This gave 20 µg/ml of Aspirin.

Trisodium citrate (3.8%): 3.8 g of trisodium citrate was dissolved in 100 ml distilled water.

Methodology of Platelet aggregation; The study was initiated only after obtaining an approval from the Ethics Committee of the Institute. On the day of the study, 9 ml of venous blood was collected in a fasting state atraumatically from the volunteers who gave written informed consent for the study. Blood was collected in a polystyrene tube containing 3.8% Trisodium Citrate (1:9 blood: Trisodium Citrate) and centrifuged at 1000 rpm for 10 min at 25^OC to obtain Platelet Rich Plasma (PRP). It was again centrifuged at a higher speed of 4000 rpm for 15 min at 25^OC to obtain Platelet Poor Plasma (PPP). Platelets from PRP were counted using cell counter and adjusted to $2X10^5$ platelets /ml using autologous platelet poor plasma. The adjusted plasma i.e. the Working Platelet Rich Plasma (WPRP) was used for studying platelet aggregation.

Platelet aggregation study was carried out as follows

Determination of Platelet Aggregation: Aggregometer was switched “On” and allowed to warm up to 37^0C. The stirrer speed was set to 1000 rpm. The PPP sample (450 µl) and the WPRP samples (440 µl) were incubated in incubation wells for 5 minutes. 10 µl of distilled water (vehicle), Aspirin (5 µM and 2.5µM) at a time, were added to the WPRP cuvettes and incubated at 37^0C in the incubation wells for 3 minutes. After incubation, the cuvettes were transferred to their respective wells on the aggrecorder; PPP cuvette containing Platelet Poor Plasma to the PPP well and WPRP cuvettes to the three different WPRP wells. A magnetic stirrer was added to each cuvette. The ‘Set baseline’ button on the aggregometer was pressed to set the baselines. The tracings on the computer screen were moved to the 100% line on the screen automatically setting the 100% (PPP) baseline. The ‘set baseline’ buttons were released. The tracings on the computer screen moved to 0% baseline automatically setting the 0% baseline. All the stirrers were set on and 50µl of the aggregating agent (ADP-5µM) was added slowly to the WPRP samples with continuous stirring. Optical Curve was allowed to run for the desired time interval (7-8 minutes) The procedure was then terminated by stopping the test after 7 minutes incase of ADP. The time for starting the aggregation after adding the aggregating agent and the time for stopping the procedure was set. The amplitude and the slope were then calculated by the software. The aggregation pattern was recorded as percent aggregation versus time for a period

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of 7 minutes in presence of ADP. The percentage inhibition shown by Aspirin at two concentrations *viz*. 5 µM and 2.5µM was then calculated by the formula:

$$\% \text{ Inhibition} = 1 - \frac{\text{MPA of test}}{\text{MPA of Vehicle}} \times 100$$

For each volunteer, the aggregation pattern of Aspirin and the vehicle control (Distilled Water) was tested using Adenosine Diphosphate (ADP, 5µM). The different channels of the aggregometer were used simultaneously to test the study Drugs at different concentrations.

Precautions to be taken while performing Platelet Aggregation Studies: Several precautions should be taken while carrying out platelet studies, some of which are described as under:

Blood Collection: Blood should be drawn from fasting donors since several lipemia may interfere with platelet aggregatory response. Blood should be collected in polystyrene tubes only, since platelets tend to adhere to the walls of the glass tube. It is preferable to maintain the platelets at either room temperature or at 37°C during all manipulations.In general, the blood products should be considered biohazardous and should be handled according to individual institute's regulations.Blood collection should be smooth to avoid haemolysis and premature activation of platelets. Blood should be collected from a forearm vein and should be drawn into syringes in an even manner. Haemolysed blood samples should not be used since the red cells contain ADP

Anti-coagulants*:* A number of anti-coagulants are available. The choice depends ultimately on what aspect of platelet function is ultimately to be examined. Heparin, if used as an anti-coagulant is known to induce platelet aggregation while EDTA, has been used but is not considered ideal for platelet studies since it affects cell surface receptors and may delete intracellular calcium stores. Any one of the three citrate based anti-coagulants may be used:Tri Sodium Citrate, Citrate-citric acid-Dextrose (CCD), Acid-citric acid-Dextrose (ACD)

Platelet Count: Slow and weak aggregation is observed with platelet count is below 150 $x10^5$/µl or over 400 $x10^5$/l. Hence, the platelet counts are adjusted to $2X10^5$ platelets/µl.

Sample Aspiration: PPP or PRP samples should be slowly aspirated with autopippetes without disturbing the RBC layer after centrifugation and slowly dispensed into plastic tubes with tight fitting caps. Any jerk to the PPP or PRP might cause premature platelet activation.

pH : All the samples (PRP and PPP) should be capped properly before dispensing them into the cuvettes to prevent CO_2 loss, which helps to maintain pH of the samples. As pH less than 7.7 inhibits platelet aggregation and pH greater than 8.0 enhances aggregation. pH of 6.5 to 8.5 is the most accurate for aggregation studies (Han and Ardlie,1974).

Temperature: Temperature greater than 35°C causes decrease in platelet aggregation, whereas cooling inhibits platelet aggregation. Hence the temperature should be maintained at 37^0C during the aggregation procedure.

Stirring Speed: Stirring is necessary to bring the platelets in close contact with each other to allow aggregation to occur. Hence the mixing speed should be at 1000 rpm and not below 800 rpm and above 1200 rpm.

Cuvettes; Clean siliconised cuvettes should be used, as dirty cuvettes may slow down or enhance the aggregation and stained cuvettes may obstruct light transmission. Before placing the cuvettes into the wells, the outer surface of the cuvettes should be properly wiped with tissue paper to remove any outer stains.

PART II

Preparation of Extracts

Solubility of the plant drugs:

Solubility tests were carried out using different solvents *viz*. cold water, hot water, DMSO, ethanol, acetone and methanol. Since the drug sample comprised of oleogum resins (mixture of resins) they were incompletely soluble in all of the above mentioned solvents giving turbid solutions. *Ferula asfoetida* became turbid white on addition of water whereas *Shorea robusta* became gummy and dissolved to a minimum, *Aloe vera* on the other side was less turbid in water but not completely soluble. Therefore, In search of a clear, non opaque solution of drug that affects the light transmission (in aggregometer) to a minimum extent and dissolves the drug completely, different organic solvents such as Propanol, Ethyl acetate, Chloroform, Benzene, Petroleum ether, and different combinations of Ethanol-Methanol were also used for the solubility of the drugs. However these organic solvents were not suitable to prepare the drug solution as they are known to affect the viability of the platelets which are extremely sensitive to *in vitro* conditions **(Table 1, 2 &3).** Ethanol was selected for *Ferula asafoetida* as it gives clearer solution then water and the percentage of Ethanol selected was 30% as decreasing below this amount, increases turbity of the solution and increasing affects viability of the platelets. *Aloe vera* was comparatively more soluble and clear in water then in Ethanol. Ethanol was selected for *Shorea robusta* though it was clearly soluble in Chloroform and partially soluble in Propanol as both are known to affect viability of platelets.

Hot Water Extraction: 20 ml of distilled water at 80°C was added to1 g of accurately weighed, dried, powdered form of *Aloe vera* placed in a clean conical flask. The flask were shaken vigorously and allowed to stand at Room Temperature for 10-15 minutes. The flask was then placed in boiling water bath at 100°C for1 hour. The contents of each flask were well shaken and then filtered through Whatman Filter Paper No.1 with continuous and gentle shaking. 4 ml of filtrate was added to a pre-weighed petri-plate and the plate was then placed on water bath until properly dried. The petri-plate was then placed in the oven (105°C) until its weight became constant. This was re-weighed and the final weight i.e. the weight of petri plate plus the residue was subtracted from the weight of the empty petri-plate initially measured. This gave the weight of the residue. The extractive value for each drug was calculated using the following formula:

%Water Soluble Extract = Weight of the residue x 20/weight of the sample x 100/4

Ethanol extraction 30% : About 1.0 g of coarsely powdered, accurately weighed form of *Ferula asafetida* and *Shorea robusta* were placed in clean and dry conical flasks. 20 ml of the 30% Ethanol was added to each flask and the total weight (powder+ethanol+flask) was calculated for each drug. The flasks were allowed to stand for 18 hours at Room Temperature. The contents of the flasks were then filtered rapidly through a Whatman's Filter Paper No.1. 8 ml of the filtrate was transferred to 2 pre-weighed petri-plates and further evaporated to dryness on a water bath at 100°C. These were further allowed to dry at 105^{0}C until the weight became constant. The percent extractable matter was calculated for both the drugs using the following relationship:

% Extractable Matter/ % alcohol solubility = Weight of the Residue X 20/Weight of the Sample X 100/4

The percentage extraction for each drug was calculated (n =3) using the above relation and the average value obtained from the three readings for each drug was considered as the extractive value of the drug. After obtaining the extractive values of plant drugs it was decided to use water soluble portion of Aloe vera and alcohol soluble portion of *Ferula asafoetida* and *Shorea robusta* to be dropped for further assay due to less then 30% alcohol extractive value and lack of any other suitable solvent for platelet aggregation studies. The extracts obtained were dried to a powder form and dissolved in respective solvents to prepare stock solutions of the plant drugs for further assay **(Table 4).**

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Preparation of Stock Solution

It was decided to prepare a stock solution of 5mg/ml for each drug. Stock solution was prepared using the following relationship

Platelet Function Studies

Platelet Viability Studies: Before studying the effect of drugs on platelet aggregation, it was decided to check the effects of selected concentrations of plant drugs on viability of platelets. Hence first viability studies were done. In these experiments, platelets were incubated with plant drugs for three minutes. Distilled water, the vehicle used to prepare drug solutions, served as Vehicle control and Aspirin (5µM) as the positive control. Release of LDH from platelets served as the measure of viability.LDH was estimated using Erba autoanalyser chem7.

Materials for Platelet Viability Study : Cell Counter- MicroDiff 18 Coulter Counter, Coulter Electronics Ltd., England. LDH-P Kit (Erba Kits, LDH-P, single step, kinetic); Kits were procured from Noble Kinetics, Mumbai. Erba Autoanalyser (Autoanayser Erba Chem-5); Transasia Bio-medicals Ltd., Mumbai. Siliconised Glass Cuvettes (washed cuvettes dipped in 0.5 % silicone oil emulsion and dried in hot air oven at 120 ^{o} C for 1 hour). Autopippetes- Volume Range 5-10, 10-100,100-1000 µl.

Methodology of testing effect of plant drugs on Platelet Viability (Snyder et al, 1981)

After taking an informed written consent from normal volunteers (n=3), they were asked to report to the laboratory after overnight fasting. 9 ml of venous blood was collected atraumatically from these volunteers in a tube containing 1 ml of 3.8% Trisodium Citrate and centrifuged at 1000 rpm for 10 min to obtain platelet rich plasma (PRP). It was again centrifuged at a higher speed of 4000 rpm for 15 min to obtain Platelet Poor Plasma (PPP). Platelets from PRP were counted using cell counter and adjusted to $2X10^{5}$ platelets /ml using autologous platelet poor plasma. The adjusted plasma i.e. the Working Platelet Rich Plasma (WPRP) was used for studying platelet viability. Aliquots of 220 µl of WPRP were incubated with 5 µl of the test drugs of concentrations 5-100 µg/ml, Aspirin (positive control, and vehicle control (D/W) for 3 min 37°C. From the mixture, 10µl was pipetted out into 500 µl of reconstituted LDH reagent from Erba kits. The mixture was well shaken and then assayed for LDH using an autoanalyser. The absorption in kinetic mode was measured at 340 nm. Basal LDH levels were measured after addition of 5µl of the test drug to 220 µl of WPRP without incubation. A statistical significant change in the indicators is looked for, while evaluating the effect on platelet viability. The post-incubation values of released LDH for each concentration of the drug were compared to the post-incubation values of released LDH for Vehicle control. All those concentrations which showed no significant difference as compared to Vehicle control were selected for the study.

Platelet Aggregation Studies: The study was initiated after obtaining permission from Institutional Ethics Committee. Healthy volunteers in the age group 18-30 years willing to abide by the study procedures and given written informed consent were recruited in the study. The volunteers were initially screened (Visit1) for their health status by taking a detailed history and performing physical examination. Blood (8 ml) was collected in a fasting state for various laboratory investigations *viz.* Hemoglobin, total and differential leukocyte count, ESR, and biochemical function tests viz. serum bilirubin, alanine, amino transferase, aspartate amino transferase, alkaline phosphatase, total protein and serum albumin, renal function tests viz. Blood-urea nitrogen, serum creatinine, fasting sugar and for the presence of HIV & Hepatitis B antigen. Once the healthy status of a volunteer was confirmed, he/she was recruited in the study. The volunteer was requested to come for a second visit (Visit 2) after a period of 8 days to the Clinical Pharmacology ward. Each volunteer was assigned a specific study number. 18 ml blood was collected from the volunteers in a fasting state in two polystyrene tubes (9 ml in each tube) containing 1ml of 3.8% Trisodium citrate in each tube for further processing.

Methodology of Platelet aggregation: This was performed using turbidimetric method of Born GV standardized in our laboratory. The sample size was chosen to be 12 (6 males and 6

females).Platelet rich plasma (PRP) was obtained from the collected blood as a supernatant by centrifugation (1000rpm for 10 min at 25^0 C) and the remaining blood was centrifuged (4000 rpm for 15 min at 25^0 C) to obtain platelet poor plasma (PPP). Platelets in the PRP were counted using a platelet counter and the count was adjusted to $2x10^5$ / ml using autologous PPP for dilution (Working plasma). Initially the viability of platelets was assessed using LDH (Lactate Dehydrogenase) kit. Working plasma (440µl) was incubated with 10µl of distilled water (vehicle), standard drug i.e. positive control (Aspirin; 5µM) and the test drugs in different concentrations for 3 mins at 37°C. Adenosine Diphosphate (ADP, known platelet aggregating agent, 5µM) was then added and platelet aggregation was studied using Chronolog platelet aggregocorder.

Platelet Aggregation Kinetics Studies

Calculation of time required for each concentration of the drug for initiation of platelet aggregation.

The time required for initiation of action of the drug was calculated as the time interval between the peak on the curve and the starting point of the secondary wave. Secondary wave results due to the release of platelet aggregation inducing granules from the platelets. Thus the time period between the binding of ADP to receptors on platelets (indicated by the peak) through the primary wave (representing change of platelet shape) and up to the release of granules from platelets (point of initiation of secondary wave) is taken as the time required for initiation of drug action.

Statistical Analysis

The results were analyzed by ANOVA for inter-group analysis and Student's t-test for intra-group analysis. **(Figure 1, 2 &3)**

RESULT AND DISCUSSION

Physico-chemical Analysis

The oleogum resins of the selected plant drugs were procured from a single standard source i.e.Dadar Pharmacy, Dadar. Physicochemical tests of the crude drugs were performed as per WHO guidelines for standardization of the plant drugs; the results of which are as detailed in Tables 7, 8 and 9. All the tests for both the plant drugs provided values within the limits stated by standard sources **(Table 5, 6 & 7).**

Standardization of Platelet Aggregation Methodology (n=6)

Platelet aggregation methodology described by GVR Born (1962) and modified by Chanarin (1989) was standardized using ADP (5 µM) as the aggregating agent and10 µg/ml and 20 µg/ml Aspirin. The results are summarized in **Table 8**.

Platelet Function Studies

Platelet Viability Studies (n=3)

The effects of four different concentrations (5-100 µg/ml) of the aqueous extracts of the selected plant drugs on platelet viability were assessed by measuring the release of LDH by platelets upon treatment with the selected concentrations of the Test Drugs, Positive Control (Aspirin) and Vehicle Control (Distilled Water).

No significant difference between the post-incubation values of LDH released was observed between the Vehicle control group and drug-treated group indicating that platelet viability was not affected by the selected concentrations of the drugs. Hence all the concentrations in the range of 5-100 µg/ml were decided to be employed for the study.

The results of Platelet Viability studies have been summarized in **Table 9**.

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Percent aggregation and Inhibition (n=8)

The effect of five different concentrations of the test drugs i.e. Ethanol (30%) soluble content of *Ferula asafoetida* and Water soluble content of *Aloe vera* oleogum resins on inhibition of platelet aggregation was studied by platelet aggregation methodology standardized in the laboratory. Both the drugs showed a concentration -dependent effect on platelet aggregation.

Ferula asafoetida exhibited significantly more percent inhibition than *Aloe vera* at higher concentrations *viz*. 50 µg/ml ($p<0.05$) and 100 µg/ml ($p<0.01$).

The activity of *Ferula asafoetida* was significantly more than Aspirin at concentration of 100µg/ml ($p<0.001$) while *Aloe vera* showed significantly lower inhibition as compared to Aspirin at all concentrations ($p<0.001$).

The results of Platelet aggregation and Inhibition are summarized in **Table.10, 11& 12** and **Figure 4, 5 and 6**

Platelet Aggregation: Kinetics Study

Calculation of time required for each concentration of the drug for initiation of platelet aggregation. The time of initiation of action of the drug is calculated as the time interval between the peak on the curve and the starting point of the secondary wave. Secondary wave results due to the release of platelet aggregation inducing granules from the platelets. Thus the time period between the binding of ADP to receptors on platelets (indicated by the peak) through the primary wave (representing change of platelet shape) and upto the release of granules from platelets (point of initiation of secondary wave) is taken as the time required for initiation of drug action

It was observed that both the plant drugs showed a decrease in the time required for initiation of platelet aggregation as their concentrations increased. A dose-dependant response was thus observed. Also the time required for initiation of platelet aggregation by *ferula asafoetida* at a particular concentration was lesser as compared to the time required for initiation of platelet aggregation by *Aloe vera* at the same concentration.

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Table 1- Solubility details of Ferula asafetida

Observation	D/W	30%Ethanol	70% Ethanol	Propanol	Chloroform	Ethyl acetate	Petroleum Ether	Methanol
Appearance	Dissolves, Opaque,	Dissolves, turbid,	Dissolves, turbid,	Dissolves, turbid,	Dissolves, Clear,	Dissolves	Doesn't Dissolves	Dissolves minimally
Color	Milky white.	Milky white.	Milky white.	Milky white.	Transparent white	Milky white.	Milky white.	Milky white.
Solubility	++	++++	+++	++	++++	++	-	+

Table 2- Solubility details of Aloe vera

Observation	D/W	30%Ethanol	70% Ethanol	Propanol	Chloroform	Ethyl acetate	Petroleum Ether	Methanol
Appearance	Dissolves, Clear,	Dissolves, Clear	Dissolves, Transparent,	Doesn't dissolve, Powder remains as it is.	Doesn't dissolve, Powder remains as lumps	Doesn't dissolve	Dissolves very little	Dissolves minimally
Color	Dark brown	Yellowish brown	Light yellowish brown	Brown	Dark brown	Dark brown	Dark brown	Dark brown
Solubility	++++	+++	++	-	-	-	-	-

Table 3-Solubility details of Shorea robusta

Observation	D/W	30%Ethanol	70% Ethanol	Propanol	Chloroform	Ethyl acetate	Petroleum Ether	Methanol
Appearance	Doesn't Dissolve, clear with gummy resin.	Dissolves, Powder remains	Dissolves, Transparent, with gummy resin.	Dissolves, Clear solution with foams.	Dissolves completely, Clear solution	Doesn't dissolve.	Dissolves very little.	Dissolves Minimally,
Color	Reddish brown	Clear with gummy red powder	Clear watery solution	reddish brown	Reddish tinge	Clear solution	Reddish brown remains	Red tinge
Solubility	-	++	+++	+++	++++	++	+	+

Table 4- Dilution chart

Concentration (µg/ml)	Volume of Stock (µl)	Volume of Diluent	Total volume
		(µl)	(µl)
5	5	95	100
25	25	75	100
50	50	50	100
100	100	-	100

Table 5- Standardization of oleogum resin of Ferula asafoetida (n=3)

Sr. No	Test	Results	Limits
1.	Description	yellowish or reddish-brown with shining surface, becomes red on exposure	
2.	Moisture (% w/w)	6±0.00	< 5.0 %
3.	Alcohol Solubility (%)	44.33±4.72	Not less than 60%
4.	Total Ash value (%w/w)	1.03±0.00	Not more than 15%
5.	Acid Insoluble Ash (%w/w)	1.6±2.05	Not more than 8%
6.	Water Solubility	52.46±4.23	Not less than 28%
7.	Fungal Growth	$6.25 \pm 0.7 \times 10^2$ cfu/g	$< 10^3$ cfu/ g
8.	Microbial Load	$1.73 \pm 0.5 \times 10^2$ cfu/g	$< 10^4$ cfu/g

Results expressed as Mean ± SD

Table 6- Standardization of oleogum resin of Aloe vera

Sr. No	**Test**	**Results**	**Limits**
1.	Description	Pale yellowish to brown yellow coloured masses ,reddish tinge, covered with dull white dust	
2.	Moisture (% w/w)	3.0%	Not more than 12%
3.	Alcohol Solubility (%)	20.57±5.25	Not less than 45%
4.	Total Ash value (%w/w)	1.05±0	Not more than 3%
5.	Acid Insoluble Ash (%w/w)	4±0.00	Not more than 8%
6.	Water Solubility	81.10±3.64	Not less than 50%
7.	Fungal Growth	$8.4 \pm 0.9 \times 10^2$ cfu/g	$< 10^3$ cfu/ g
8.	Microbial Load	$6.10 \pm 0.5 \times 10^3$ cfu/g	$< 10^4$ cfu/g

Results expressed as Mean ± SD

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Table 7- Standardization of oleogum resin of Shorea robusta (n=3)

Sr. No	Test	Results	Limits
1	Description	Cream to reddish Brown with shiny and sticky surface	
2.	Moisture (% w/w)	1±0.00	< 10.0
3.	Alcohol Solubility (%)	32.33±5.64	Not less than 21%
4.	Total Ash value (%w/w)	1.00±0.0	< 2.8 %,
5.	Acid Insoluble Ash (%w/w)	0.00±0.00	< 1.0%
6.	Water Solubility	0.43±0.03	Not less than 37%
7.	Fungal Growth	6.3±0.7 x 10^3 cfu/g	< 10^4cfu/ g
8.	Microbial Load	5.14±0.5 x 10^3 cfu/g	< 10^4 cfu/g

Results expressed as Mean ± SD

Table 8- Standardization of platelet aggregation methodology (n=6)

Drug	% Aggregation	% Inhibition
Vehicle Control (D/W)	88.25 ±8.54	-
Aspirin (10 µg/ml)	49.75±16.46	43.30±18.98
Aspirin (20 µg/ml)	64.75±10.53	24.49±19.58

Results expressed as Mean ± SD

Table 9-Platelet Viability Studies (n =3)

Groups	**LDH Activity (IU/L)** **Post-Incubation**
Vehicle (D/w)	216.6 ± 32.73
Aspirin (20 g/ml)	210.6 ± 34.75
30% Ethanol	281.43±12.44
Ferula asafoetida	
5 □g/ml	285.3±10.73
10□g/ml	301.4±11.89
25□g/ml	302.73±10.18
50 □g/ml	315.17±14.35
100 □g/ml	363.7±28.42
Aloe vera	
5 □g/ml	218.6±79.35
10□g/ml	264.03±56.52
25□g/ml	286.63±54.52
50 □g/ml	310.7±62.82
100 □g/ml	382.77±69.72

Results expressed as Mean ± SD

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Table 10-Effect of the two plant drugs on platelet aggregation in response to ADP (n =8)

Groups	**% Aggregation**
Vehicle (D/w)	70.33±15.62
Aspirin (20 g/ml)	36.83 ± 16.02
Ferula asafoetida	
5 g/ml	42.5±17.03
10g/ml	39.83±17.63
25 g/ml	32.33±23.02
50 g/ml	28.67±17.44
100 g/ml	25±14.24
Aloe vera	
5 g/ml	58.33±20.08
10 g/ml	54.17±21.97
25 g/ml	50±25.62
50 g/ml	48.8±20.9
100 g/ml	44.8±20.66

Results expressed as Mean ± SD

Table 11- Effect of the two plant drugs on inhibition of platelet aggregation (n =8)

Groups	**% Inhibition**
Aspirin (20 g/ml)	43.42 ± 5.14
Ferula asafoetida	
5 □g/ml	39.47±15.57
10 □g/ml	44.495±17.97
25 □g/ml	54.93±24.51
50 □g/ml	57.8±12.64
100 □g/ml	61.05±10.65***
Aloe vera	
5 □g/ml	21.5± 12.39
10 □g/ml	27.71± 11.80*
25□g/ml	25.36± 20.34
50 □g/ml	32.76± 25.77
100□g/ml	30.05± 20.07

Results expressed as Mean ± SD

*p<0.05 and ***p<0.001 as compared to Aspirin using ANOVA followed by Tukey’s post test

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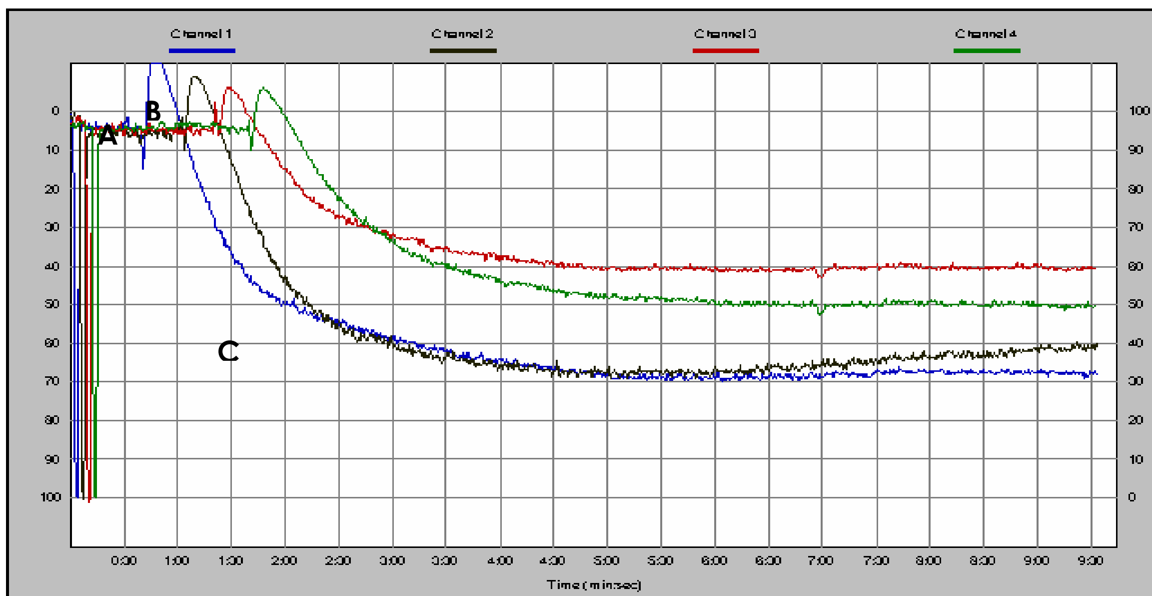
Table 12- Platelet aggregation Kinetics: Time

Concentration (µg/ml)	Time required for initiation of drug action (sec)	
	Aloe vera	***Ferula asafoetida***
5	175 ± 50.24	157 ± 17.67
25	139 ± 93.40	126 ± 41.6
50	113 ± 58.48	89.8 ± 55.6
100	107 ± 64.17	80.33 ± 49.9

Results expressed as Mean ± SD



Key:

Channel 1: Vehicle control (Distilled water)

Channel 2: Positive control (Aspirin)

Channel 3: *Ferula asafoetida* (25 µg/ml)

Channel 4: *Aloe vera* (25 µg/ml)

A single curve of platelet aggregation graph typically shows the following:

A: Representing addition of aggregating agent

B: Peak, representating binding of ADP to receptors on the platelets

From B onwards to C: Primary wave, representing change in platelet shape

C onwards: Secondary wave, release of platelet granules

Fig. 1- Aggregation Pattern as recorded on the Aggregocorder.

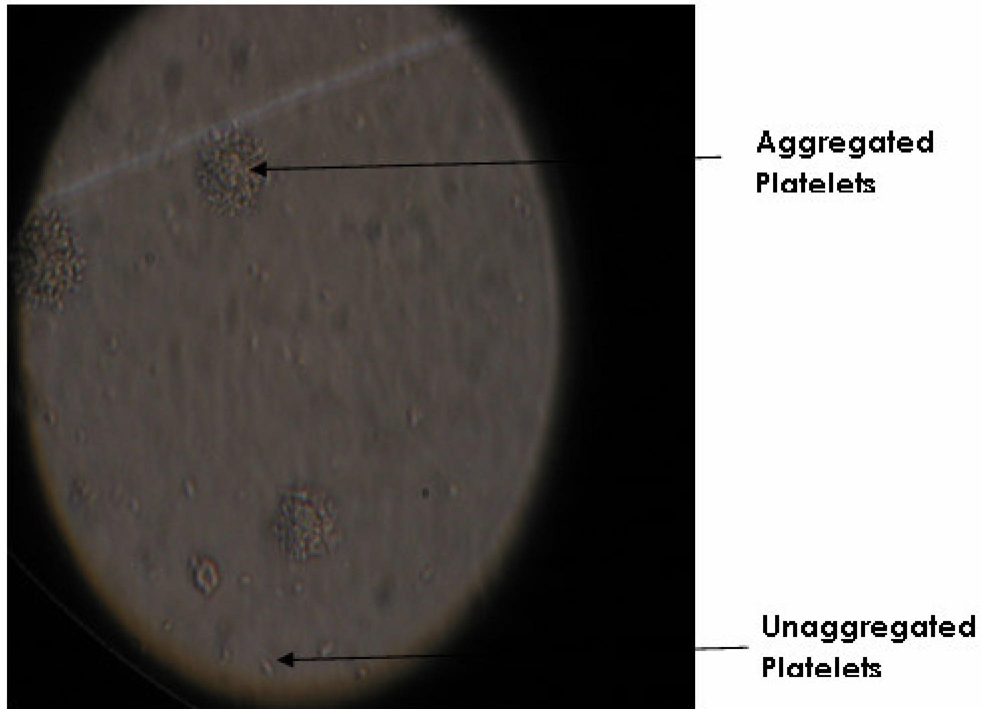


Fig. 2- ADP induced Platelet aggregation (observed under 45X)

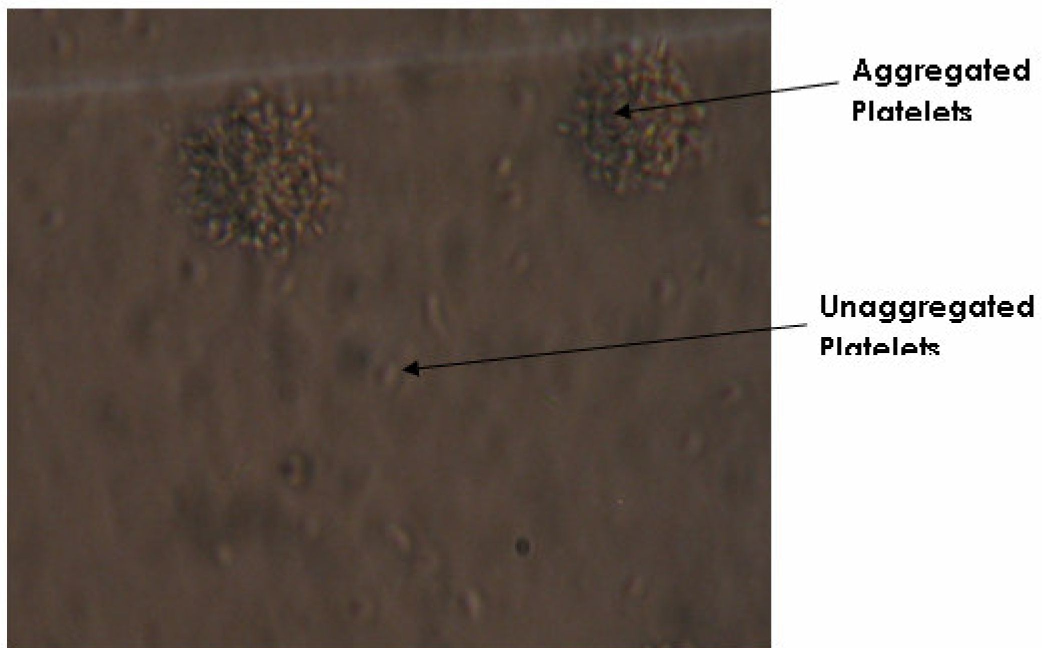


Fig. 3- ADP induced Platelet aggregation (observed under 10X)

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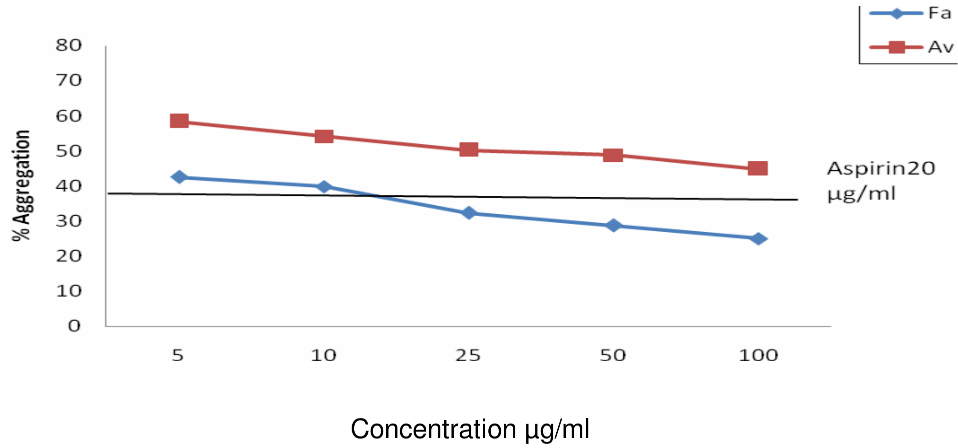
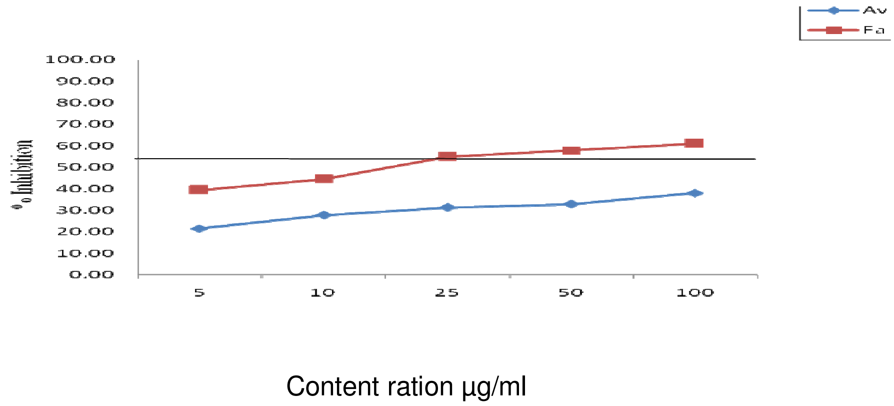


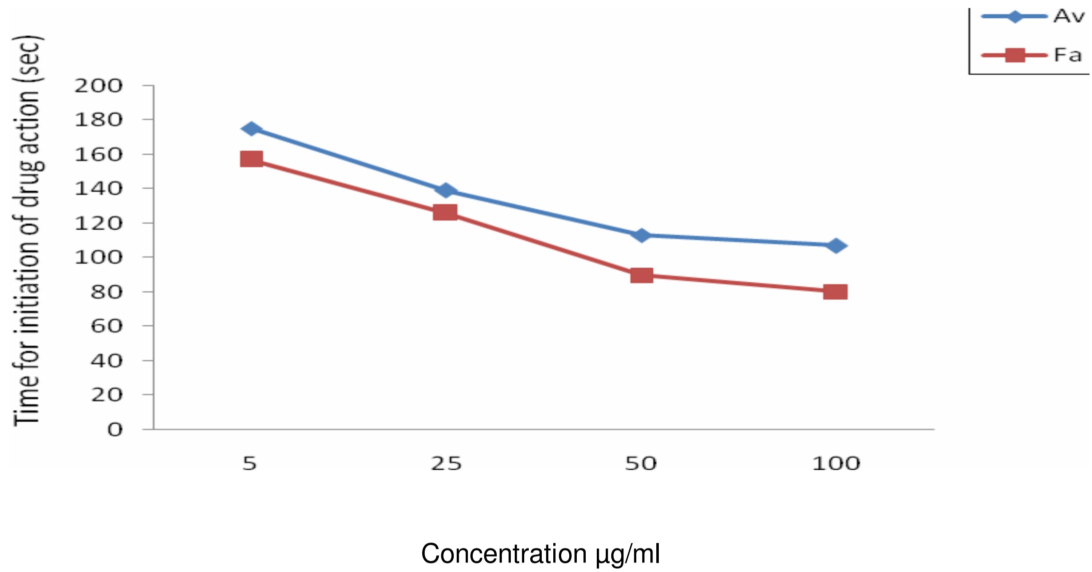
Concentration µg/ml

Fig. 4- Effect of the two plant drugs on platelet aggregation in response to 5µM ADP (n =8)



Content ration µg/ml

Fig. 5- Effect of the two plant drugs on platelet inhibition (n =8)



Concentration µg/ml

Fig. 6- Time for initiation of drug action