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Effect of High Dose of Formononetin and Biochanin A on Blood Clotting Parameters in Sprague Dawley Rats

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Abstract: ***Raloxifene is a synthetic estrogenic receptor modulator which is currently used in the therapy osteoporosis. One of the side effects associated with raloxifene therapy is the increased incidence of thromboembolic effects. Phytoestrogens are plant derived compounds that have functional similarity with estrogenic. One of the interesting properties of phytoestrogens is that many of them possess tissue selective estrogenic receptor agonistic or antagonistic actions enabling them to function as natural Selective Oestrogen Receptor Modulators (SERMs). Is flavones Formononetin and Biochanin A are phytoestrogens derived from the plant Trifolium pratense belonging to the family Leguminosae. Formononetin and Biochanin A are believed to selectively modulate the estrogen receptors depending on the target tissue making them potential ant osteoporotic candidates. Trifolium pratense has been reported to induce haemorrhage due to the presence of coumestans and is contraindicated in patients with known episodes of haemorrhage. To understand whether the is flavones Formononetin and Biochanin A derived from Trifolium pratense possess procoagulatory or anticoagulatory effects on the blood, few parameters which are of considerable importance in the clotting cascade was evaluated in Sprague Dawley rats. Results of the study showed that sub chronic administration of Formononetin and Biochanin A at a dose of 100mg/kg orally for 30 days did not induce any significant alterations in parameters like Prothrombin time, Partial thromboplastic time, bleeding time and clotting time thereby showing that although the is flavones are derived from Trifolium pratense, they did not exhibit any adverse effects on the clotting cascade.***

Keywords: SERM, Raloxifene, Formononetin, Biochanin A, PTT

I. INTRODUCTION

Trifolium pratense commonly known as red clover belonging to the family leguminosae is widely cultivated in Europe, America and Asia. The plant is known as Tripura in Hindi which refers to the clover shaped trifoliate leaves characteristic to the plant (Jan., *et al.*, 2014). The plant is widely used in many systems of alternative medicine including Traditional Chinese Medicine (TCM) for the treatment of various ailments. The plant is proven to be beneficial to counteract postmenopausal symptoms in women including management of hot flashes and is reported to possess osteoprotective properties (Chen *et al.*, 2015). It is also said to be beneficial in the treatment of asthma, psoriasis and eczema thereby implicating the anti-inflammatory effects of the plant. The biological health benefits of the plant is mainly due to the presence of phytoestrogen is flavones Formononetin, Biochanin A, Einstein and Diadem. Among these is flavones, much has been studied about the biological effects of the is flavones Feinstein and Daidzein, but very less has been studied with respect to the other two isoflavones Formononetin and Biochanin A which are the major isoflavones in the plant. Formononetin and Biochanin A are selective estrogenic receptor modulators that could be beneficial in the therapy of postmenopausal osteoporosis. (Genera *et al.*, 2007) Because of their dual role as an estrogenic receptor agonist and antagonist based on the target tissue, they have lot of potential to be exploited in the treatment of many conditions wherein there is an over expression of estrogen (breast cancer) or deficiency of estrogenic (post-menopausal osteoporosis).

Raloxifene, the only synthetic Selective Oestrogen Receptor Modulator (SERM) approved by the FDA for the therapy of osteoporosis has the side effect that it has an increased risk of thromboembolic events in the patients (Maximum *et al.*, 2013) Trifolium pratense is reported to possess a class of phytoestrogen compounds called coumestans which are known to possess anticoagulatory blood thinning effects. As Formononetin and Biochanin A are also derived from Trifolium pratense, and they are phytoestrogen SERMs, it will be desirable to understand whether it has procoagulatory effects like raloxifene or it has anticoagulatory effects like coumestans present in their source plant Trifolium pratense. Hence the aim of the current study is to understand whether subchronic administration with high dose of Formononetin and Biochanin A has any adverse influence on the blood clotting parameters in Sprague Dawley rats.

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II. MATERIALS AND METHODS

A. Chemicals and Reagents

Formononetin, Biochanin A were purchased from M/S Sigma Aldrich Co. (St louis, USA).

B. Experimental animals

Adult Sprague-Dawley rats weighing between 100-120g obtained from the approved source of Bangalore University were used for the study. The animals were kept in quarantine for a period of two weeks after procurement. After the quarantine period, they were acclimatized to animal house conditions and were fed on commercial pelleted rat chow (M/s Hindustan Lever Limited, Bangalore, India) and water *ad libitum*. For the *in vivo* experiments, test compounds were administered orally by intra gastric intubation (Feedy intubation tube, India, size 05, Diameter 1.70 mm).

The animals were segregated into three groups. Two groups of animals were administered with different dosages of Formononetin and Biochanin A *(*100mg/kg body weight/day orally). The untreated group served as the control. After the experimental period, the animals were subjected to mild CO_2 asphyxiation. Blood samples collected from different groups of animals were used for various the blood clotting parameters after the separation of serum or plasma.

C. Bleeding time

The bleeding time is the most basic test to evaluate thrombocyte function. A sterile disposable needle or a special lancet is used for pricking the tail tip. The tail blood was wiped filter paper or paper towel and the duration of bleeding time recorded to the nearest 30 seconds till the bleeding is stopped. (Deana *et al., 1982)*

D. Clotting time

Approximately 5-6 ml of venous blood is collected. The blood is immediately distributed in 3 test tubes that were prewarmed in a 37 ºC water bath. Blood clotting is tested by tipping the tube back and forth every 30 seconds. The clotting time is measured when the blood does not flow out of the test tubes when tilted horizontally. The clotting time is calculated by averaging the results obtained with the 3 test tubes. The normal value is 5-8 min. (Lewis *et al.*, 1985)

E. Prothrombin Time

Prothrombin time test determines the amount of prothrombin in the sample. The speed of coagulation depends on the concentration of prothrombin. 4.5 ml of venous blood is drawn into a sterile syringe already containing 0.5 ml of 3.8% Na-citrate. The citrated blood is centrifuged as soon as possible, max. 2 hours after the blood collection, for 10 min at 1000 g. Then 0.1 ml plasma is pipetted onto a silicon/ wax covered watch glass and placed in a 37 ºC water bath for 3 min. The liquiplastin is also prewarmed in a test tube at 37 ºC. 0.2 ml of prewarmed liquiplastin is added to the 0.1 ml of plasma in the watch glass and the timer is started. Approximately every second, the tip of an injection needle is pulled through the plasma. The prothrombin time is recorded when the first fibrin fibre appears at the tip of the needle (Salami *et al.*, 1994)

F. Partial thromboplastic time (PTT, theoretically only)

The activity of plasma clotting factors is measured in the presence of calcium and a phospholipid reagent substituting thrombocyte factor 3. Na-citrate is added to the blood sample in a ratio of Na-citrate : blood = 1 : 9, and the mixture is centrifuged. The time elapsed until the appearance of the first fibrin thread is measured after the addition of 0.2 mmol/L calcium chloride 0.1ml and a cephaloplastin reagent of 0.1 ml time is noted (Goya *et al.*, 2015)

III. RESULTS

A. Effect of Formononetin and Biochanin A on Food intake, Water Consumption and Body Weight Measurement (Fig 1, Fig 2)

The body weight, food and water of rats was recorded ever week until the end of experimental period. It was observed that there were no significant ($p > 0.001$) changes in body weight, food and water consumption between the control and treated rats**.**

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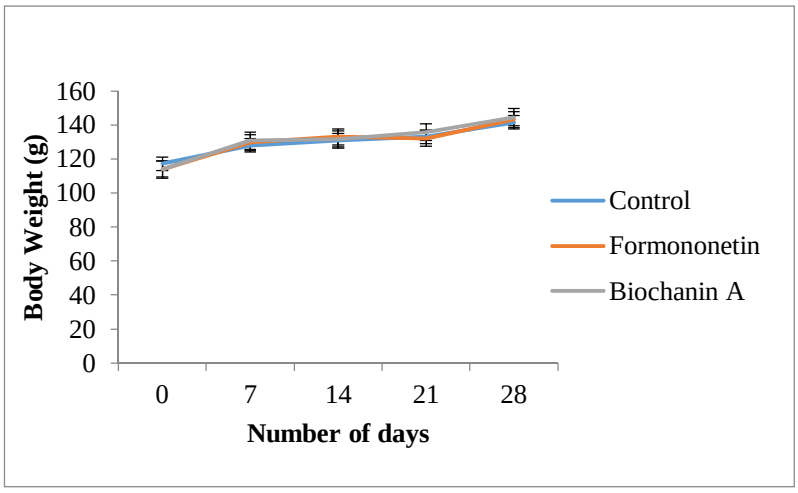

Fig. 1: Body weight of control and treated rats in the toxicity study. Data indicate mean ± SD, n = 6. There are no significant differences between the control and treated rats in their body weight.

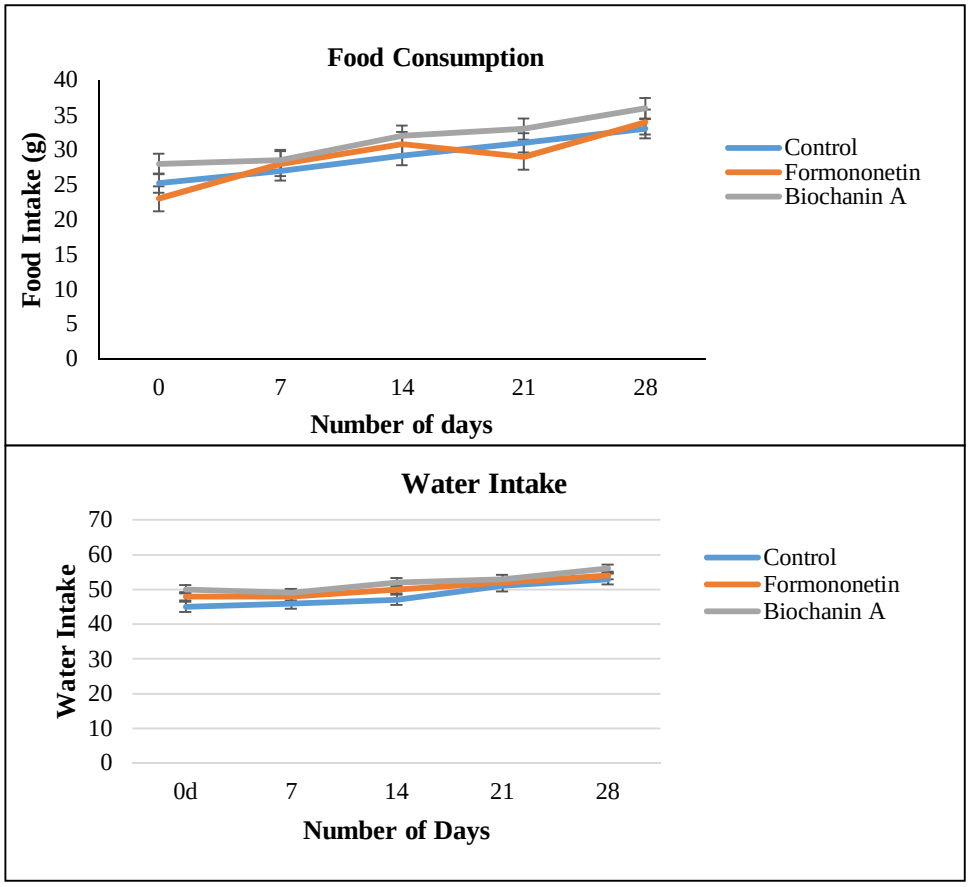

Fig. 2: Consumption of food and water by the control and treated rats. Data indicate mean ± SD, n = 6. There are no significant differences between the control and treated rats in their food and water intake.

B. Effect of Formononetin and Biochanin A on Bleeding Time, Clotting time, Prothrombin Time, Partial thromboplastin (Blood clotting parameters-Table 1 and Fig 3) The bleeding time, clotting time, prothrombin time, partial thromboplastin of the rats administered with higher doses of Formononetin and Biochanin A did not show any significant changes in blood clotting parameters between control and treated rats indicating no prothrombotic effect of test compounds at higher doses.

Parameters	Control	Formononetin	Biochanin A
Prothrombin time (sec)	14.1± 1.28	15.3 ± 0.96 NS	13.8 ± 1.35 NS
Partial thromboplastin (sec)	36.2 ± 2.1	35.5 ± 2.2 NS	34.3 ± 2.6 NS
Bleeding time (sec)	150.3 ± 1.5	149.6 ± 4 NS	148.1 ± 1.5 NS
Clotting time (min)	7 ± 1.1	6.7 ± 1.10 NS	6.8 ± 1.01 NS

Table I: Results of blood clotting parameters in control and treated rats given a dosage of 100 mg/kg body wt. Data represent mean± SD of 6 replicates. Student's t-test. Comparisons were made between treated group Vs untreated control. NS – non significant.

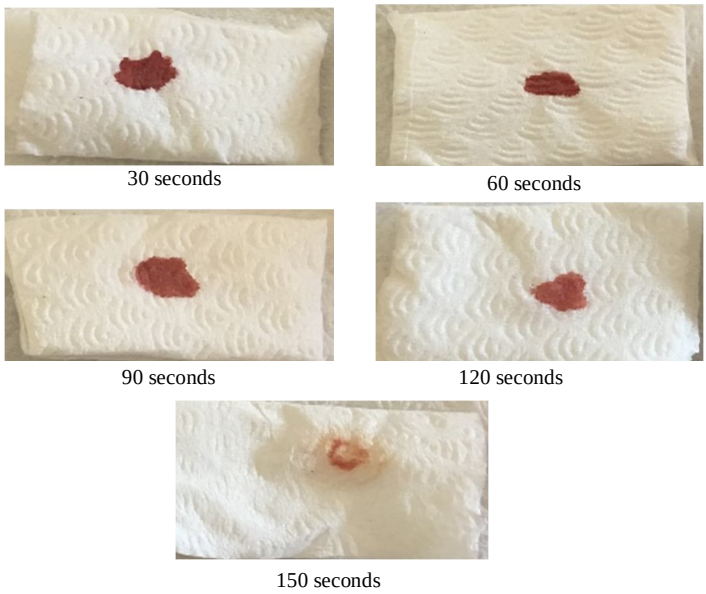

Figure 3: Results of bleeding time that was recorded every 30 seconds until the bleeding stops

IV. DISCUSSION

The increased risk of thromboembolic events in patients undergoing therapy with SERM raloxifene limits its usage in elderly patients who are already prone to develop thrombotic effects due to advancing age. This indicates the necessity to look for other natural SERMs which will possess the osteoprotective functions of raloxifene but is devoid of its side effects. Trifolium pratense also called as red clover is a plant rich in phytoestrogens Formononetin and Biochanin A which are reported to possess osteoprotective properties and tissue specific estrogen receptor modulating properties (Suita and Pattanayak. 2011). Formononetin and Biochanin A could be potential alternatives to raloxifene in the management of osteoporosis but the fact that they are derived from Trifolium pratense necessitates investigations pertaining to their effects on blood clotting because of the increased thinning tendency of the blood in patients administered Trifolium pratense which is contributed to the presence of a class of anticoagulatory compound referred to as coumestans (Osoki.,m 2003).Prothrombin time, partial thromboplastic time, bleeding time and clotting time are simple parameters which will provide an idea about the effect of test compounds on the clotting cascade (Arber., 1990). Prothrombin is a serine protease which is the inactive precursor of the protein thrombin. Thrombin is essential for the final step of the clotting cascade- the conversion of fibrinogen to fibrin. Thromboplastic on the other hand is a protein which is required for the conversion of inactive prothrombin to active thrombin. Partial thromboplastic time implies the time required for the conversion of prothrombin to thrombin and consequently the time required to form the fibrin clot. Measurement of Bleeding time gives an idea about the time till which the blood can remain in a liquid state when it is made to ooze from a source (injured blood capillary/vessel or a deliberate prick) and measurement of clotting time provides an idea about the time required for the liquid blood to form the insoluble fibrin clot. All these parameters together provide an idea about the influence of a test compound as a procoagulatory or anticoagulatory agent.

In the study sub chronic administration of high dose (100mg/kg bodt. wt/ day for 30 days) of Formononetin and Biochanin A treated rats at each dosage group continued to gain weight throughout the experimental periods. This suggests that administration of the Formononetin and Biochanin A did not affect the body weight of the rats. In addition, no significant changes in the food and water consumption of the treated rats compared to the control rats in toxicity studies. Utilization of food and water exhibited normal metabolism in the animals and this suggests that the administration of the Formononetin and Biochanin A did not retard the growth of the rats. Animals treated with Formononetin and Biochanin A did not cause significant changes in any of the clotting parameters studied as compared to the untreated control. This indicates that both the compounds did not exhibit prothrombotic effects like raloxifene or antithrombotic effects like Trifolium pratense. The findings of the study are important as this is the first scientific report elaborating the role of Formononetin and Biochanin A on hemostasis. As maintenance of hemostasis is required in maintaining the homeostatic conditions operating in the body, avenues are wide open for Formononetin and Biochanin A to be explored as hemocompatible, natural phytoestrogenic SERMs with potential osteoprotective properties.

V. ACKNOWLEDGEMENTS

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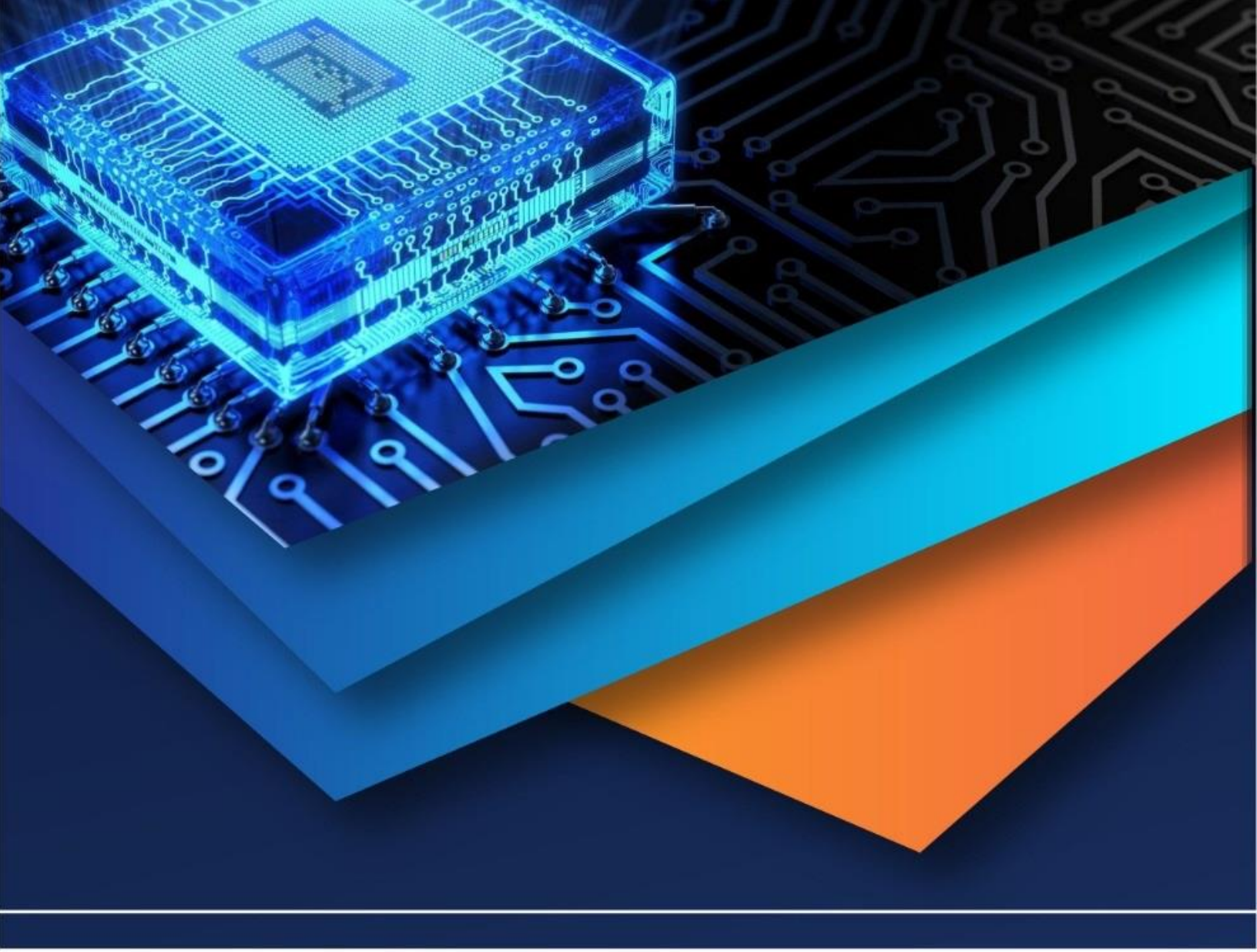

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