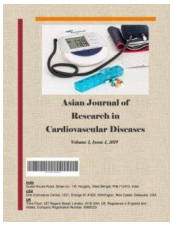

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Effects of Aqueous Leaf Extract of *Thymus schimperi*on Hematologic Profiles of Animal Models of Pre-eclampsia

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Authors' contributions

This work was carried out in collaboration among all authors. Author KM designed the study, performed the experimental procedures, statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors YAM, TT, EM and SG wrote the 2nd draft of the manuscript and supervised lab work. Author YB involved in lab work. Authors AA, KG, AT and AD organized data, managed the literature searches, assisted plant material preparation. All authors read and approved the final manuscript.

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ABSTRACT

Background: Hypertensive disorders of pregnancy such as pre-eclampsia (PE) complicate pregnancy outcomes among women worldwide and are responsible for a high incidence of maternal and fetal mortalities. Complete blood count tests (CBC), including hematocrit (HCT) and red cell distribution width (RDW), are easy, inexpensive, routinely reported investigations, which might

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allowthe acquisition of significant diagnostic and prognostic information in patients with PE. They also help to evaluate the severity of the disorder.Low platelet count (Thrombocytopenia) is well recognized when PE is complicated by hemolysis, elevated liver enzymes and low platelet count (HELLP) syndrome.

Objective: The aim of this study wasto determine the effects of aqueous leaf extract of *Thymus schimperi*on the levels of some hematologic parameters among PE rat models induced by N (ω)-nitro-L-arginine methyl ester (L-NAME).

Methods: A case-control experimental study was employed to evaluate hematologic variables. The red blood cell (RBC) indices were determined using hematology analyzer after blood sample obtained by cardiac puncture at gestation day 20.

Results: There was a significant difference in RBC indices particularly hemoglobin (Hb), HCT, mean corpuscular hemoglobin (MCH) and RDW of PE animal models compared to the normal pregnant controls. However, there was no significant difference in RBC count, mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) among PE and normotensive pregnant rats. Oral administration of calculated doses of *Thymus* leaf extract to PE rat models have shown normalized levels of RBC indices compared to untreated PE cases. All PE rat models in the present study had lower count of platelets and higher count of leukocytes. The total leukocytes and platelets counts were regulated in those PE groups which received *Thymus* extract.

Conclusion: Administrationof*Thymus* leaf extract to PE rat models might have regulating effects on hematologic parameters which are usually deranged in PE condition; hence plant extract might have potential therapeutic effects against PE.

Keywords: Pre-eclamsia; RBC indices; platelets; leukocytes; Thymus schimperi; therapeutic.

ACRONYMS

L-NAME : N (ω)-nitro-L-arginine methyl ester
Hb : Hemoglobin
HCT : Hematocrit
MCH : Mean Corpuscular Hemoglobin,
RDW : Red Cell Width,
MCHC : Mean Corpuscular Hemoglobin Concentration
HELLP : Hemolysis, Elevated Liver Enzymes and Low Platelet Count Syndrome
PE : Pre-Eclampsia
CBC : Complete Blood Count
EDTA : Ethylene-Diamine Tetra-Acetic Acid

1. INTRODUCTION

Pre-eclampsia (PE) is a pregnancy-specific disorder characterized by hypertension and excess protein excretion in the urine. It is an important cause of maternal and fetal morbidity and mortality worldwide [1,2]. The disease is almost exclusive to humans and delivery of the pregnancy continues to be the only effective treatment. The disorder is probably multifactorial, although most cases of PE are characterized by abnormal maternal uterine vascular remodeling by fetally derived placental trophoblast cells [3]. Numerous *in vitro* and animal models have been used to study aspects of PE, the most common being models of placental oxygen dysregulation, abnormal trophoblast invasion, inappropriate maternal vascular damage and anomalous maternal-fetal immune interactions [4].

Investigations into the pathophysiology and treatment of PE continue to move the field forward, albeit at a frustratingly slow pace. There remains a pressing need for novel approaches, new disease models and innovative investigations to effectively tackle this complex and devastating disorder [5].

PE is the most common hypertensive disease of pregnancy, affecting 5-8% of pregnancies [6] and accounting for nearly 18% of maternal deaths in the United States.

PE is also associated with adverse fetal outcomes, including intrauterine growth retardation (IUGR), placental abruption, oligohy dramnios and non-reassuring fetal surveillance [7]. It is clinically defined as hypertension and proteinuria with onset following the 20th week of pregnancy [8]. PE can be further differentiated into mild and severe forms. Mild PE is defined by a systolic blood pressure of >140 mmHg or a diastolic blood pressure >90 mmHg in combination with 300 mg of proteinuria over 24 hours [9].

Complete blood count test, especially red cell distribution width (RDW), is an easy, inexpensive, routinely reported investigation, which might allow the acquisition of

significantdiagnostic and prognostic information in patients with PE and to determine the severity of PE [10].

Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), and red blood cell (RBC) are known as the red blood cell indices. MCV defines the size of the red blood cells. MCH determines the amount of hemoglobin per red blood cell. MCHC indicates the amount of hemoglobin per unit volume. RDW is an indicator of red cell size variation [11]. It reflects early changes in red blood cells. All these parameters are important for detecting and investigating anemia. They are usually evaluated in a fully automated hematology analyzer, as part of the complete blood count. In many literatures, it has been shown that RDW was higher in prehypertension. Prehypertension was described as slightly elevated blood pressure which would likely turn into high blood pressure (hypertension) in non-pregnant people if lifestyle changes like eating healthier and starting to exercise were not done [12].

Furthermore, the association between RDW values and non-cardiac and cardiac mortality in patients with cardiovascular and thrombotic disorders, diabetic ketoacidosis, acute and chronic heart failure, coronary artery disease, and stroke has been investigated [13]. However in the literature, there have been limited data on the relationship between red blood cell indices, including RDW, and PE [14]. Hematological parameters play an important role in severe PE, especially at microcirculatory regions with high shear stress such as inter-villous space of placenta [15]. High levels of Hb and Hct could be used as a prediction factor for early PE diagnosis [16]. Women with high hemoglobin concentration in the first trimester carry an increased risk of pregnancy induced hypertension [17]. Thrombocytopenia is well recognized when PE is complicated by hemolysis, elevated liver tests and low platelet count (HELLP) syndrome [18,19]. Hematological changes associated with the red blood and white blood cells, platelets and hemostatic profile could occur during pregnancy.

Women with PE have raised leukocyte count and low platelets [20]. Lymphocyte count increases during the third trimester. There is an absolute monocytosis during pregnancy, especially in the first trimester, but decreases as gestation advances. Monocytes help in preventing fetal allograft rejection by infiltrating the decidualtissue (7^{th} –20^{th} week of gestation) possibly, through PGE2 mediated immunosuppression. The monocyte to lymphocyte ratio is markedly increased in pregnancy. Eosinophil and basophil count, however, do not change significantly during pregnancy [21]. *Thymus* is a plant largely distributed in temperate zones and is uncommon in the African tropics. Ethiopia however has two species of Thyme called *Thymus schimperi* and Thymus *serrulatus*. *Thymus schimperi* locally called 'Tosign' is wild growing species of *Thymus* and comparatively well-known in Central, Eastern and Northern Ethiopia [22]. Aqueous leaf extract of *Thymus serrulatus* (another endemic species) has vasodilatory activity which might bring anti-hypertensive effects that might support the traditional claim of the plant as anti- hypertensive agent [23]. In Ethiopia, selected parts of *Thymus* are being used for the treatment of hypertension. The result of the present study is thus expected to provide the prophylactic and/or therapeutic effects of *Thymus schimperi* against PE. This plant is claimed to help maintain normal hematological parameters which are deranged in women with PE. The aim of the present study is, therefore, to confirm this claim through investigating the regulatory effects of *Thymus* leaf extract on some hematologic parameters of PE rat models.

2. MATERIALS AND METHODS

2.1 Plant Material Extraction

Enough amount of leaves of the plant was collected. Then the plant samples were identified by a taxonomist and a voucher number (01/2016Tc) was given and deposited at the National Herbarium of Addis Ababa University. The fresh leaves were cleaned and dried under shade at room temperature of 20-25°C. Then, dried leaves were reduced by manual crusher to obtain powder. One kilogram of powdered leaves was macerated with distilled water for 2 hours with intermittent agitation by an orbital shaker. Then, the supernatant part of agitated materials were decanted and filtered with What man's filter paper. The filtrates were freeze-dried at -20°C and reduced pressure, and then lyophilized to obtain aqueous crude extract.

2.2 Experimental Animals

Virgin female Wistar rats weighing 200-250gms with age of 3-6months were used to conduct the

present experimental study. The animals were housed at room temperature between 20°C and 25°C with relative humidity of 30% to 70%. Dark and light cycle each of 12 hours were followed. The animals were acclimatized to laboratory condition for one week before commencement of experiment and dosing.

The female rats which were in estrous phase (identified by microscopic demonstration of typical epithelial cells on vaginal smear) were housed and co-habited with fertile male rats in a ratio of 2:1. (F: M). The gestation day one was depicted as the day that copulation occurred as demonstrated by the presence of vaginal plugs and sperm cells on vaginal plug hence pregnancy, the male rats were separated from the female rats. N (ω)-nitro-L-arginine methyl ester (L-NAME), an L-arginine analogue widely used inhibitor of nitric oxide synthase (NOS) activity both *in vitro* and *in vivo*, was used to induce PE in rats with oral doses of 50 mg/kg/d at gestation day 11 (GD11). Nifedipine was used as a standard anti-hypertensive agent in positive control group.

The animals were randomly grouped as follows: G5-untreated PE rats (negative controls of PE), G6-PE rats treated with Nifedipine (20 mg/kg/d) from GD12 to GD20. G7-PE rats treated with 250 mg/kg/d of *Thymus* extract, G8A-PE rats treated with 500 mg/kg/d of *Thymus* extract, G8B-PE rats treated with 1000 mg/kg/d of *Thymus* extract from GD12 to GD20. G11- normotensive pregnant controls.

2.3 Blood Sampling and Analysis

Two milliliters (2ml) of blood was sampled from each animal by cardiac puncture at gestation day 20. Then, the blood was kept in a test tube with anti-coagulant (EDTA) and gently shacked to mix thoroughly before analysis. Hemoglobin, hematocrit, MCV, MCH, MCHC, platelets, RBC and WBC counts were analyzed using a hematology analyzer (Hematological Analyzer, SYSMEX XT-1800i; SYSMEX CORPORATION, Japan) at Ethiopian Public Health Institute national laboratory. RBC, Hb, HCT, MCV, MCH, MCHC, PLT were analyzed using Impedance and Fluorescent Optical methods while, WBC with differentials were evaluated using Fluorescent Flow Cytometry.

2.4 Statistical Analysis

All experimental data were expressed as mean values ± S.E.M and were subjected to bio-statistical interpretation by SPSS windows version 21 statistical packages all the way through a one-way ANOVA followed by post-hoc test (Tukey Test) for multiple comparisons of the mean differences and responses of drugs and extract. $P < 0.05$ was considered as statistically significant.

3. RESULTS

Therapeutic effects of *Thymus* leaf extract were evaluated at different standard doses on PE case groups (G7, G8A & G8B) and compared with untreated controls (G5), positive controls of PE (G6) and normal controls (G11). The results indicated that PE rats that received *Thymus* leaf extract had RBC counts in near to that of the normal pregnant controls (G11) in a dose dependent manner which were comparable to that positive controls of PE (G6) that were treated with a standard antihypertensive agent, nifedipine. The levels of Hb and HCT were reduced in a dose dependent manner while the other parameters were unaffected in dose-dependent fashion. Generally *Thymus* leaf extract treated PE groups (G7, G8A & G8B) showed RBC indices within their normal ranges; while the values were deranged in untreated PE cases (G5) (Table 1). There was a dose-dependent increase in platelets, total leukocyte count including lymphocyte and monocyte count in PE rat models treated with *Thymus* leaf extract compared to untreated cases (G5). However, significant increase in these parameters was seen with highest dose of the extract (1000 mg/kg) (Table 2).

4. DISCUSSION

All PE rat models had significantly elevated levels of Hb and HCT. These findings are in line with previous studies [24,25]. According to the current study, administration of *Thymus* leaf extract could significantly decrease RBC count, Hb, MCHC and RDW-CV%; increase MCV, MCH and MCHC in a dose dependent manner but had no significant effects on MCV and MCH values. These findings are consistent with previous studies conducted on RBC indices in PE cases [26-28].

In the present study, all pregnant rats had significantly higher total leukocyte count compared to non-pregnant normal controls which could be explained by physiological adaptation for pregnancy as the stress of pregnancy and delivery leads to brisk leukocytosis which is in line with previous studies [29].

Table 1. Effects of *Thymus* leaf extract on RBC indices of PE rat models

CBC	G7 (250 mg/kg)	G8A (500 mg/kg)	G8B (1 gm/kg)	G6 Nifedipine treated PE	G5 Untreated PE	Group 11 (normal control)
RBC (10^6/uL)	7.1±0.1	6.8±0.09^{b**}	6.3±0.02^{b**c*}	7.2±0.4	7.7±0.1^{a*}	7.1±0.2
Hb (g/dL)	14.4±0.1^{b**}	14.2±0.4^{b**}	13.8±0.2^{b***}	14.2±0.2^{b***}	16.7±0.2^{a***}	13.6±0.5
HCT (%)	43.5±0.1^{b*}	42.9±1.4^{b*}	41±0.9^{b**}	42.9±1	45.3±0.7^{a*}	41.1±2.1
MCV (fL)	61.1±1.1^{b*}	63.1±1.8^{b*}	66.9±1.3^{a**b**c**}	57.8±1.8	58.7±1.6^{a*}	57.4±1.2
MCH (pg)	20.2±0.3^{b*c*}	20.9±0.4^{a**c**}	21.8±0.3^{a***c***}	18.8±0.1^{b***}	21.7±0.5^{a***}	18.9±0.1
MCHC (g/dL)	32.9±0.1^{b**}	33.2±0.6^{b*}	33.6±0.3^{b*}	33.6±0.6^{b*}	36.3±0.7^{a**c*}	33.1±0.5
RDW-CV (%)	20.1±0.3^{a*b**}	19.1±1^{b**}	16.7±0.1^{b**}	17.2±1^{b**}	25.8±1^{a***}	16±0.8

Values are, mean ±SEM; $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; ^{a}p-compared to normal controls, ^{b}p-compared to negative controls; ^{c}p-compared with positive controls, RBC-red blood cells, Hb-hemoglobin, HCT- hematocrit, MCV-mean cell volume, MCH-mean corpuscular hemoglobin, MCHC-mean cell hemoglobin concentration, RDW-red cell width, n=6

Table 2. Effects of *Thymus* leaf extract on platelets and leukocyte counts in PE rat models

Group	Platelets ($x10^3$/µL)	WBC ($x10^3$/µL)	Neutrophil ($x10^3$/µL)	Lympho. ($x10^3$/µL)	Monocyte ($x10^3$/µL)
G7	769±46^{c*}	7.8±1	1.41±03	5.1±1	0.27±0.08
G8A	873.2±78^{b*}	8±1.1	0.96±0.2	5.2±0.1	0.46±0.1
G8B	959±35^{a*b***}	7.6±0.05	1.38±0.06	6.05±0.1	0.53±0.1
G6	889±27^{b**}	7.3±0.5^{b*}	1.1±0.3	5.25±0.5	0.3±0.08
G5	738±12^{a*c**}	8.5±0.1	1.46±0.3	6.49±0.1	0.45±0.07
G11	827±20	8.2±0.2	2.14±0.3	5.68±1	0.5±0.09

Values are, mean ± SEM; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ^{a}p-compared to normal controls, ^{b}p-compared to negative controls; ^{c}p-compared with positive controls, G7-PE rats treated with 250 mg/kg of Thymus leaf extract, G8A-PE rats treated with 500 mg/kg of Thymus leaf extract, G8B-PE rats treated with 1000mg/kg of Thymus leaf extract, G6-nifedipine treated PE cases, G5-untreated PE cases, n=6

All PE rat models, in the present study, had significantly lower platelets count (thrombocytopenia) and higher leukocyte count (leukocytosis) attributed to the course of HELLP syndrome that supports the hypothesis which may represent an inflammatory process in PE. Results were in line with previous reports [30,31]. Therefore, platelet count can be used as a simple and cost effective tool to monitor the progression of PE, thereby helping to prevent life threatening complications. Due to increased platelet destruction and turn-over, low platelet count may play a role in predicting PE. Platelet indices are therefore, simple, cheap and practical tools in predicting severity of PE [32].

In the current study, there is relatively higher lymphocyte count among PE than neutrophil and monocyte count; this might be due to the fact that blood sample was taken at the end of the gestation period which is in line with previous report on lymphocytosis in the 3rd trimester and monocytosis in the 1st and 2nd trimester [33]. There was no significant difference in monocyte and basophil count in PE and normal pregnant controls; which is consistent with previous reports [34]. However, the results of the present study were inconsistent with previous studies that reported the total neutrophil count further increased 48 hours after delivery in the group with severe PE and the increased neutrophil count contributed for the leukocytosis in women with PE [35]. The difference might be due to sampling time and other confounders such as bacterial infections that might cause neutrophilia. Generally, administration of *Thymus* leaf extract might have dose dependent significant effects by increasing platelet and decreasing leukocyte count in PE rat models. The results show that the extract might have therapeutic effects against PE.

5. CONCLUSION

Thymus leaf extract might have significant regulating effects on hematologic parameters

which are deranged as a result of pathogenesis of PE; hence the extract might have potential therapeutic benefits against PE syndrome.

CONSENT

It is not applicable.

ETHICAL APPROVAL

Ethical clearance was obtained from Institutional Review Board of College of Health Sciences of Addis Ababa University with a protocol number of 029/16/032/16/ Physio before commencement of the animal experimentation.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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