Magna Scientia Advanced Research and Reviews

eISSN: 2582-9394

Cross Ref DOI: 10.30574/msarr

Journal homepage: https://magnascientiapub.com/journals/msarr/

(RESEARCH ARTICLE)

Investigation into the effect of ethanol leaf extract and fractions of *Justicia Secunda* on platelets and WBC counts in mice

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Magna Scientia Advanced Research and Reviews, 2022, 06(01), 001–007

Publication history: Received on 17 July 2022; revised on 27 August 2022; accepted on 29 August 2022

Article DOI: https://doi.org/10.30574/msarr.2022.6.1.0059

Abstract

Aim: This study investigated the effect of the ethanol leaf extract and fractions of *Justicia secunda* on platelets and WBC counts in mice.

Methods: Phytochemical analysis were carried out on the ethanol leaf extract of *justicia secunda*. A total of 96 apparently healthy mice of different sex were used in this study**.** Fourteen treated and two control groups of six mice each were used to investigate the effect of leaf extract and fractions of *justicia secunda* on platelets and white blood cell counts in mice (Groups 1-12) using 20mg/kg/i.p. phenyl-hydrazine (PHZ) daily for 2 days, and then intervention with ethanol extract and 3 fractions (n-hexane, ethylacetate and n-butanol) for 6 days. The positive control group received 20 mg/kg PHZ i.p. only, while the negative control group received food and water only. Blood samples were collected from the retro-orbital plexuses of the mice into EDTA-containing specimen bottles on the 7th day and analyzed for white blood cell (WBC) and platelet (PLT) count.

Results: The ethanol leaf extract significantly decreased the platelets and white blood cell count compared to the negative control groups ($p< 0.05$), they were almost brought back to normal(baseline).The fractions (n-hexane MD, ethyl acetate HD and n-butanol HD) had statistical significant effect on WBC ($p< 0.009$) in a dose dependent manner. Some of the fractions of *J. secunda* however had no significant effect on platelets counts ($p > 0.146$) compared with the control.

Conclusion: n-butanol fraction of *J. secunda* leaf possessed better significant effect on WBC when compared with other fractions, thus, perhaps eliciting immunosuppresant action in the mice.

Keywords: Platelet aggregation; White blood cell; *Justicia secunda*; Phenylhydrazine; Fractions

1. Introduction

Platelets, the smallest blood element in the circulation, play the important roles in normally hemostatic processes including adhesive and cohesive functions in the thrombus formation and the activation. Blood is a vital tissue of body that transports oxygen and nutrients to every cell of the body and eliminates waste products from tissues. Platelets are cell fragments that prevent excessive bleeding by forming a clot [1]. Blood clot is a bulk of blood cells and blood constituents which is produced to stop bleeding resulting from blood vessel injuries. During this process, platelets in the blood become sticky and clump together at the site of the injury. Clotting is the body's normal response to prevent a person from bleeding to death [2]. However, blood clot formation can be dangerous if it occur within healthy blood

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vessels, or if not degraded after due time. Many diseases like heart attack, stroke and pulmonary embolism are associated with inappropriate blood clot formation [3].

White blood cells (WBC) are a heterogeneous group of nucleated cells that can be found in circulation for at least a period of their life. Their normal concentration in blood varies between 4000 and 10,000 per microliter. They play the most important role in phagocytosis and immunity and therefore in defense against infection. WBC is classified into granulocytes, lymphocytes, and monocytes. Granulocytes owe their name to the presence of distinct cytoplasmic granulation. Three varieties are recognized: neutrophils (or polymorphonuclear granulocytes), eosinophils, and basophils.

The main function of neutrophilic granulocytes is phagocytosis of bacteria. This is a complex multistage process that includes engulfment of the organism, incorporation into the cytoplasm, and fusion with a lysosome where enzymes are liberated that will destroy the bacterium while a burst of energy is generated. Eosinophils and basophils have a similar development. After release from the bone marrow, eosinophils promptly abandon the intravascular compartment (where they constitute up to 5% of WBC), entering the tissues, they are not able to reenter the blood. Basophils constitute about 1 to 2% of circulating leukocytes. Their physiologic role is also not known with precision. In their granules they carry heparin and histamine. IgE can be found bound to their surface. Macrophages and lymphocytes are known collectively as mononuclear leukocytes. Both play important roles in cellular and humoral immunity. These cells are able to exit and reenter circulation, retaining their function [4].

Monocyte–macrophages phagocytose bacteria and particulate material, play a role in the inflammatory reaction, and are important in the immune apparatus where they process antigenic material and "communicate" with T lymphocytes through a cell–cell interaction process. Monocytes are able to secrete interleukin, a substance that potentiates B and T lymphocytes. They participate in fibrinolysis by secreting plasminogen activators. Lymphocytes are immune cells fundamental in cellular and humoral immunity. In the blood they represent 20 to 45% of WBC. They belong to the B (*b*ursa or *b*one marrow) or T (*t*hymus) systems. Both cells are morphologically indistinguishable, the B system is responsible for synthesis of antibodies. The T system constitutes the cellular immune system and regulates the whole immune apparatus. Several subsets of T cells can be identified with monoclonal antibodies specific against different membrane antigens. For instance, helper T cells favor the function of B cells, whereas suppressor T cells inhibit them. Some T cells are responsible for cell-mediated cytotoxicity; natural killer (NK) lymphocytes are responsible for nonspecific lysis of certain cells. In the peripheral blood, approximately 15 to 25% of lymphocytes are B cells and 40 to 75% are T cells [4].

However, abnormalities of WBC can be quantitative or qualitative; neoplasia, metabolic and collagen diseases, hypersensitivity reactions; hemorrhage, hemolysis, and stress. Neutropenia, May Hegglin, pelger-Huët anomaly, monocytosis, eosinophilia, basophilia, lymphocytopenia, leukemias; Acute leukemia, granulocytopenia, thrombocytopenia, lymphadenopathy and hepatosplenomegaly, chloromas, and, in monocytic leukemia, gum hypertrophy. Platelets are decreased, chronic myelogenous leukemia (CML), leukostasis, chronic lymphocytic leukemia (CLL)**,** Splenomegaly is common, and Multiple myeloma, the hyper-globulinemia, and all other immunoglobulins are decreased. Hypercalcemia and lytic bone lesions [4]. Although, some synthetic blood thinners such as aspirin and heparin are available in market, these have side effects like cancer [5]. Thus, anti-platelet substances with minimal side effects from natural origins are primary targets for drug discovery. Several classes of natural products have been investigated for the anti-platelet activity, including flavonoids, stilbenoids, coumarins, and indole alkaloids. So now scientists are in search for natural substance that decrease coagulation (anti-coagulants) and WBC count from plant sources that are safe, cost effective and available from indigenous resources. The knowledge and practices of herbal medicinal system have been handed down through the ages, and for many years, medicinal plant were the only sources of treatment for disease in humans. Indeed, many of today's drugs have been isolated from medicinal plants. The world health organisation (WHO) has reported that up to 80% of the population in Africa depend on traditional medicine to meet their health care need in Nigeria, it is common knowledge that many of the local populace use herbal medicines for treatment of diseases, this is either because the medicines are cheap or of the erroneous belief that herbal medicines are safe because they are natural.

Justicia seconda is an evergreen perennial plant with stems that sometimes become more or less woody, it can grow from 90-200cm tall. The plant is harvested from the wild for local use as medicine, it belongs to the Acanthaceae family, order Scrophulariales, super order Lamiiflorae (sensu Dahlgren), comprises almost 250 genera with 2,500 species. *J. secunda* species are widespread in tropical regions of the world [6] and are poorly represented in temperate regions [7]. The folkloric uses of the plant include wound healing, anemia and abdominal pain [8,9]. In South-East Nigeria, Congo and South Coˆte-d'Ivoire, the leaf decoction is used as a treatment of anaemia in Congo by Jehovah's Witnesses, well

known for their refusal of blood transfusions [8]. The anti-sickling, haematinic, antimicrobial and anti-hypertensive activities of *J. secunda* have been reported [10].

The aim of this study is to investigate the effect of the ethanol leaf extract and fractions of *justicia secunda* on platelet and WBC counts in mice.

2. Methods

2.1. Experimental animals/Study site

96 Apparently healthy albino mice of different sex weighing 28-30g were purchased from the Animal House Unit, Faculty of Pharmaceutcal Sciences, Nnamdi Azikiwe University, Agulu Campus was used throughout the study. The animals were housed in cages and fed with palletized commercial rat feed (vital fed) and tap water. Prior to administration of the ethanol leaf extract, the animals were kept for two weeks to acclimatize. The animals were allowed free access to feed and water *ad libitum.* The study protocol (NAUTH/CS/66/VOL.11/187/2018/122 was approved by the institutional ethics committee Nnamdi Azikiwe University Teaching Hospital, Nnewi, Anambra.

2.2. Collection and Identification of plant material

Justicia secunda plant (leaves) were collected at Abakiliki, Ebonyi State, Nigeria, and was identified by a Plant Taxonomist; Mr. A. Ozioko, formerly of the Herbarium Section, Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka. Currently at 110, Aku Road, Nsukka, Enugu state, Nigeria. Voucher number *justiciasecunda*-intercedd/29648 was deposited at Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka.

2.3. Extraction

Agitation extraction was used which involved successive extraction with solvents of increasing polarity from a non-polar n-hexane to a more polar solvent like ethanol to ensure that a wide polarity range of compound is extracted as well as choosing solvent that will give a higher percentage yield. This was carried out by weighing 1000grams of the pulverized sample and kept in Erlenmeyer flask containing 80% ethanol in water for 72h, using solid/liquid ratio of 1:10 [11, 12]. With intermittent shaking every 2min in an orbital shaker, this was thereafter filtered with Whatman No.1 filter paper. The filtrates was concentrated using a rotary evaporator at 40^0 C. The paste weighed 85.4g which was stored in a refrigerator.

2.4. Phytochemical analysis (qualitative and quantitative)

This was carried out on the plant extract by the methods of Trease and Evans [13]. All measurements were done in triplicate.

2.5. Acute toxicity test (Median lethal dose)

This study was carried out using the up and down method of acute toxicity in accordance with Guideline No. 425 of the Organization for Economic Cooperation and Development (OECD) [14].

2.6. Animal study

Apparently healthy albino mice numbering 96 of different sex weighing 28-30g were divided into 16groups 0f 6 mice per group, Animals of groups 1-12 were treated with 20mg/kg(i.p.) Phenyylhydrazine (PHZ) for 2 days. and from the 3rdday onwards the animals administered 2.7 mg/kg(p.o.) (low dose, LD), 8.3 mg/kg (medium dose, MD) and 24.9 mg/kg (high dose, HD) of ethanol extract, n-hexane, ethyl acetate and n- butanol fractions of *J.secunda* for 6 days. The positive control (Group 13) received 20 mg/kg (i.p.) PHZ only, while the negative control (untreated) (Group 14) received only feed and water. All the animals in the positive control (Group 13) died on the 3rd day of treatment.

2.7. Haematological assays

On the 7th day 1ml blood samples were be collected from the retro-orbital plexus in vials containing ethylene diamine tetra acetic acid (EDTA) as the anti-coagulant. The samples were evaluated for Full blood count using hematology automated analyser machine (Mindrays, Model BC-2800Vet, China). The haematolgical parameters analyzed for white blood cell, and platelet counts.

2.8. Statistical Analysis

Data were presented as mean ± standard deviation (SD). Data analysis was performed using GraphPad Instant software (GraphPad Prism®, Standard version 7.0). And analyzed by using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test (post-test); $P \leq 0.05$ were considered as statistically significant in all analyses.

3. Results

Table 1 Phytochemical analysis of *J. secunda* ethanol leaf extract

Phytochemical	Qualitative	Qualitative (%)
Saponins	++	9.2
Tannins	++	9.0
Flavonoids	+	7.0
Alkaloids	+	2.4
Terpenoids	+	-
Carbohydrates	+	-
Reducing Sugar	+	-
Steroids	-	-
Glycosides	-	-
Protein	-	-

Key: + = Trace; ++ =Moderate; – = Not detected

Table 2 Effect of *J. secunda* leaf extract and fractions on white blood cell count (10^9/L)

Group	Baseline	Induction	Treatment	F	*P-value*
Ethanol extract (mg/kg)					
LD	4.54 ± 0.27	3.05 ± 0.39*	3.87 ± 0.59#	23.41	0.013
MD	3.46 ± 0.50	4.90 ± 2.10	4.45 ± 0.40	1.68	0.271
HD	4.30 ± 0.53	3.10 ± 0.81	4.00 ± 0.67	4.7	0.165
n-Hexane fraction (mg/kg)					
LD	4.28 ± 0.30	3.65 ± 1.30	3.57 ± 0.57	1.17	0.326
MD	3.58 ± 0.54	2.25 ± 0.72*	4.18 ± 0.59#	11.33	0.028
HD	3.52 ± 0.68	3.28 ± 0.98	3.00 ± 0.42	0.450	0.340
Ethyl acetate fraction (mg/kg)					
LD	3.30 ± 1.32	4.10 ± 1.54	3.00 ± 0.44	2.55	0.182
MD	4.10 ± 0.88	2.80 ± 0.66	2.70 ± 0.44	23.19	0.079
HD	4.00 ± 0.88	2.80 ± 0.66*	3.90 ± 1.32#	15.86	0.009
n-butanol fraction(mg/kg)					
LD	3.75 ± 0.28	3.84 ± 0.98	2.86 ± 0.50	2.821	0.206
MD	3.78 ± 0.73	3.15 ± 0.57	3.60 ± 0.49	1.22	0.329
HD	2.70 ± 0.44	2.70 ± 0.44	4.50 ± 0.00#	94.66	0.0002
Negative control	4.34±0.48	2.98±0.64*	4.33±0.35#	11.06	0.003

LD = low dose; MD = median dose; HD = high dose; *significantly lower than baseline value ($p < 0.05$); #significantly higher than the induction value ($p < 0.05$)

The qualitative phytochemical analysis showed that *J. secunda* ethanol leaf extract contains flavonoids, carbohydrate saponins, tannins, terpenoids, reducing sugars and alkaloids. Saponin were more present than all the other phytochemicals in the extract (Table 1).The quantitative phytochemical analysis of the extract showed that saponins had the highest concentration of 9.2%,followed by tannins and flavonoids (9.0 and 7.0 % respectively), while alkaloids had the least concentration (2.4%).

Administration of 2000mg/kg (p.o.) of the extract produced no death or any signs of toxicity, so the LD_{50} was taken as >2000 mg/kg, following the up and down procedure of LD_{50} determination.

The LD ethanol extract, MD n-hexane and HD n-ethyl acetate and HD n-butanol fractions all had significant increase in the WBC counts of the animals in comparison with those of the animals induced platelet aggregation and leukocytosis (Table 2). Platelet counts of all the animals in the baseline, induction and treatment groups of ethanol extract and all the fractions did not differ statistically from one another, except in the LD and MD ethanol extract, which caused significant decrease ($p< 0.05$) in comparison with the baseline value. The LD ethanol extract treatment also caused a significant increase ($p = 0.014$) in the platelet count (1334.0 ± 348.90) in comparison with the phenylhydrazine induced mice (1039.00 ± 220.20) (Table 3).

Table 3 Effect of *J. secunda* leaf extract and fractions on platelet counts (10^9/L) in mice

Group	Baseline	Induction	Treatment	F	*P-value*
Ethanol extract (mg/kg)					
LD	1850.00±140.60	1039.00±220.20*	1334.0±348.90*#	14.55	0.014
MD	1622.00±207.20	1041.00±159.20*	1100.0±118.10*	21.19	0.000
HD	1174.00±81.85	983.70±69.10	1334.0±214.60*	5.74	0.054
n-Hexane fraction (mg/kg)					
LD	1174.00±140.90	853.00±490.80	957.0±106.90	1.57	0.265
MD	1025.00±121.50	1063.00±101.80	1179.0±153.90	1.83	0.229
HD	1135.00±451.80	1158.00±237.40	1031.0±190.10	0.12	0.767
Ethyl acetate fraction (mg/kg)					
LD	1408.00±410.40	1011.00±41.36	1169.0±152.70	3.24	0.146
MD	977.30±75.29	1113.00±139.80	989.30±61.45	2.75	0.148
HD	1046.00±85.02	1276.00±177.80	1115.0±44.23	4.41	0.106
n-butanol fraction(mg/kg)					
LD	973.70±157.50	1020.00±364.60	987.0±65.02	0.10	0.782
MD	1025.00±112.60	1191.00±181.70	1129.0±150.50	1.67	0.237
HD	986.00±65.22	1034.00±71.65	976.0±166.60	0.39	0.561
Negative control	1136.00±125.90	1225.00±203.00	1156.0±154.70	0.42	0.561

LD = low dose; MD = median dose; HD = high dose*significantly lower than baseline value ($p < 0.05$)#significantly higher than the induction value ($p < 0.05$)

4. Discussion

This study investigated the effect of the ethanol leaf extract and fractions of *Justicia secunda* on platelets and WBC counts in mice, it was found that *j.secunda* contained a number of phytochemicals; saponins, tannins, flavonoids and alkaloids, they are potent inhibitors of hydrolytic enzymes, and this may account for the plant's antiplatelet and antileucopenic properties. The presence of phytochemicals in *j.secunda* was in agreement with the study carried out by Krishnaraju et al [15]. Which also found that *J.secunda* contained the presence of Tannins, saponins, protein, reducing sugar, flavonoids and quinines. Our findings did not reveal the presence of glycosides and steroids which were reported by

Krishnaraju *et al* [15] and his team, the differences in the phytochemical contents could be due to environmental factors, viz; pH, water supply climate, soil etc in which the plant was grown.

The acute toxicity (LD_{50}) test revealed that the extract, at a dose of 2000mg/kg were not able to cause death or any sign of toxicity after 14 days of exposure. This is an indication that the extract is practically non-toxic.

With the economic harsh realities in the society, some patients are unable to afford the standard drugs used in the management of platelet aggregation like Ticlopidine etc, coupled with their adverse effect profile, however, herbal medicines used in therapy are comparatively cheap, readily available with supposed minimal side effect compared with standard agents. Moreover, the use of herbal remedy without safety evaluation could be noxious. Our study showed that phenylhydrazine (20 mg/kg, i.p.) caused significant decrease in platelets and WBC after 2 days administration. This was in agreement with the work of Gheith and El- Mahmoudy [16] which reported induction of platelet aggregation and leukocytosis with PHZ 2 days post-treatment.

The antithrombotic potentials of the extract were demonstrated by the restoration of platelets count to even normal levels after treatment with the various doses of *J.secunda* ethanol leaf extract as short as 6 days. Essentially, treatment with low dose ethanol extract decreased the WBC and Platelet levels of the animals significantly in comparison with both the induction and baseline values, This effect may be due to the presence of the phytochemicals in the plant extracts. These bio-active compounds present in extract might have prevented the adhesion and aggregation of platelets, besides the release of cytoplasmic calcium that stimulates the release of ADP and 5-HT. This is in consonance with the study conducted by Imran *et al*[17] which opined that *Acacia leucophloea* extract may have beneficial effects in primary prevention of cardiovascular disease by reducing platelet activation, which may contribute to a reduction in thrombotic events. Bigoniya *et al* [18] Reported good anti-thrombotic activities in *Wrightia tinctoria* bark methanolic extract, which is rich in flavonoid and polyphenolic compounds. This may be responsible for the platelets aggregation inhibitory properties of *J. secunda*. The exact mechanisms by which the extracts exhibited the reported effect need further mechanistic investigations.

There were significant changes(decrease) in the white blood cell in the fractions with varying dose, however, there were no significant changes in the platelet counts of the treatment and baseline groups in all the fractions used except in ethanol plant extract. This is in consonance with the result of the study of Anacletus *et al* [19], which reported that Aqueous Extract of *Limonia acidissima* leave had effect on WBC. The study of Gheith I and El-Mahmoudy [17], reported however that *Beta vulgaris* leaf and stalk extract had no significant haemopoeitic activity on WBC and platelet count.It may be plausible that the fractions of *J. secunda* do not exert any significant effects on thrombocytes.

5. Conclusion

The results of this study confirm the hypothesis that *Justicia secunda* ethanol leaf extract and its fractions (n-hexane, ethyl acetate and n-butanol) (with the ethanol plant extract producing better therapeutic effect) decreased platelets and WBC count in mice and might produce an immunosuppressant effect along with reduced susceptibility to atherothrombotic events.

Compliance with ethical standards

Acknowledgments

I express sincere thanks to my co-author; associate provost College of Health Science Nnamdi Azikiwe University (UNIZIK) involved in study and others who directly or indirectly helped me to make this study a success.

Disclosure of conflict of interest

No conflict of interest.

Statement of ethical approval

The study was conducted according to the Animal Ethical Committee Guidelines of Nnamdi Azikiwe University Teaching Hospital, Nnewi Campus, Anambra State (*(NAUTH/CS/66/VOL.11/187/2018/122*) and every effort was made to minimize animal suffering.

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