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Original Research Article

Effects of Aqueous and Butanol Leaf Fractions of *Olax subscorpioidea* Oliv. on Inflammatory Cytokines in Wistar Rats

Saidi Odoma[1,2]*, Abdulkadir U. Zezi[3], Muhammed N. Danjuma[3], Ahmed Abubakar[4], Garba M. Magaji[3]

[1]*Department of Pharmacology and Therapeutics, Kogi State University, Anyigba, Nigeria*
[2]*Department of Pharmacology and Toxicology, Kampala International University, Uganda*
[3]*Department of Pharmacology and Therapeutics, Ahmadu Bello University, Zaria, Nigeria*
[4]*Department of Pharmacognosy and Drug Development, Ahmadu Bello University, Zaria. Nigeria*

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ABSTRACT

Olax subscorpioidea leaf is used traditionally in the management of yellow fever, painful swellings, and venereal diseases. Its anti-inflammatory activity has been reported; furthermore, an anti-inflammatory guided fraction of its leaf extract revealed that the aqueous and butanol fractions were the most active. Considering the involvement of cytokines in inflammation, we investigated the effects of the anti-inflammatory activities of the aqueous and butanol leaf fractions on inflammatory cytokines in Wistar rats.

Rats were divided into four groups (n = 6) and were orally administered distilled water (1 mL), aqueous/butanol fraction (1,000 mg/kg), and acetylsalicylic acid (ASA, 300 mg/kg). One hour post-treatment, carrageenan (0.1 mL, 1% w/v) was intraperitoneally injected into the right hind paws of the rats. Four hours post inflammation induction, the rats were anesthetized and the hind paws cut-off, homogenized, and centrifuged before collecting the paw exudates. The concentrations of inflammatory cytokines in the exudates were measured.

Both fractions significantly ($p < 0.05$ and $p < 0.01$) reduced the concentrations of pro-inflammatory cytokines, such as interleukin-1 (IL-1), vascular endothelial growth factor (VEGF), and epidermal growth factor (EGF); and increased the concentrations of anti-inflammatory cytokines such as IL-5, IL-6, and interferon-γ (IFN-γ).

The result of the study suggests that the aqueous and butanol leaf fractions of *Olax subscorpioidea* may be exhibiting their anti-inflammatory activities via the inhibition of pro-inflammatory cytokines such as IL-1, VEGF, and EGF and/or stimulation of the synthesis of anti-inflammatory cytokines such as IL-5, IL-6, and IFN-γ.

Keywords: Cytokines, inflammation, inflammatory mediators, interferon-γ, interleukins, Olax subscorpioidea.

Introduction

Inflammation is regarded as the primary physiologic defense mechanism that helps protect the body against allergens, infection, toxic chemicals, burns, or other noxious stimuli.[1] It has been demonstrated as the root cause of almost all chronic diseases, such as cardiovascular diseases, cancer, inflammatory bowel syndrome, atherosclerosis, arthritis, and autoimmune diseases.[2,3] Likewise, inflammatory and oxidative processes have been implicated in the pathological features associated with the central nervous system in Alzheimer's disease.[4]

Inflammation is caused by a variety of soluble factors; which include lipoxins, platelet activating factor (PAF), leukotrienes, prostaglandins, and cytokines.[5] Cytokines are the major determinants of the systemic responses to inflammation.[5] They are small proteins that are secreted and released by cells and they have a specific effect on the communications and interactions between cells.[6] They are divided into two; the pro-inflammatory cytokines (which worsen disease) and the anti-inflammatory cytokines (which reduce inflammation and promote healing). Due to the critical role cytokines play in the origination and development of inflammation, a variety of drugs designed to treat inflammatory diseases are focused on the inhibition of pro-inflammatory cytokines or potentiation of anti-inflammatory cytokines.[2]

Non-steroidal anti-inflammatory drugs (NSAIDs) like acetylsalicylic acid (aspirin), ibuprofen, and so on are among the most commonly prescribed anti-inflammatory agents;[7] but, they are prone to evoking serious adverse reactions.[8] Their use has been limited as a result of side effects such as blood disorders, gastrointestinal tract (GIT) irritation, renal damage, liver damage, hypersensitivity reactions, tinnitus, and so on.[9] Thus, there is an urgent need for new anti-inflammatory substances from the vast available natural products; as most of the medicinal plants are moderately toxic compared to the orthodox medicines.[8]

Ever since ancient times, medicinal plant products have been used for the management of different kinds of inflammatory diseases;[10] one of such medicinal plants is *Olax subscorpioidea* Oliv.[11] *Olax subscorpioidea* is a member of the Olacaceae family, it is widely distributed in Africa tropics, and it is used in the management of several diseases which include arrhythmia, rheumatism, and microbial diseases.[12,13] The presence of phytochemicals such as tannins, carbohydrates, flavonoids, saponins, alkaloids, cardiac glycosides, steroid, and triterpenoid have been reported in the leave extract of *Olax subscorpioidea*.[11,14]

*Corresponding author. E mail: odoma.s@ksu.edu.ng
Tel: +2348027547778

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The anti-inflammatory activities of the crude methanol leaf extract of *Olax subscorpioidea* have been previously reported by Odoma *et al*.[11] We have also shown that the butanol and aqueous fractions of its crude methanol extract have more anti-inflammatory activities than the other fractions.[15] However, whether cytokines were affected or not in our reported anti-inflammatory effect of *Olax subscorpioidea* has not been reported. This study investigated the effects of the butanol and aqueous leaf fractions of *Olax subscorpioidea* on inflammatory cytokines; using the dose with the best anti-inflammatory response (1,000 mg/kg) from our previous study where each fraction was tested in graded doses (250, 500 and 1,000 mg/kg).[15]

Materials and Methods

Plant collection and identification

Olax subscorpioidea leaf was collected in March 2013, from a farm in Anyigba, Kogi State, North Central Nigeria. The identification was done by a taxonomist, Dr. Emmanuel I. Aigbokhan, of the Department of Biological Sciences, Faculty of Natural Sciences, Kogi State University, Anyigba, Kogi State, Nigeria; and a voucher specimen number (KSUH-277-2013-01) was deposited for future references.

Plant extraction and fractionation

The extraction and fractionation of the plant material have also been reported.[11,15] The plant material was shade dried for several days until crimpy, it was reduced into a fine powder with the aid of a mortar and pestle. About 1kg of the powdered material was extracted exhaustively with 2.5 L of aqueous-methanol (20-80%$^{v}/_{v}$), using continuous soxhlet apparatus. The extract was concentrated under reduced pressure to yield a dark brown mass (methanol extract). The fractionation was achieved by suspending 100 g of the methanol extract in 500 mL of water and successively partitioned with hexane (5 x 500 mL), ethyl acetate (5 x 500 mL), and butanol (5 x 500 mL) to afford the corresponding fractions. The butanol and residual aqueous fractions were concentrated using a water bath set at 50°C. Each of the fractions was placed in a bottle container and stored in a desiccator before use. Solutions of the fractions were prepared freshly with distilled water for each study.

Laboratory animals

Adult Wistar Rats (180-220 g) of either sex were obtained from the Animal House Facility of the Department of Pharmacology, Therapeutics, and Toxicology, Faculty of Basic Medical Sciences, College of Medicine, University of Lagos, Idi-Araba Lagos, Nigeria. The animals were housed in standard cages at room temperature under standard environmental conditions of humidity and illumination cycle; and were fed with a standard rodent pellet diet (Vital feeds, Jos, Nigeria) and water *ad libitum*. The approval for the experiments was given by the Ethical Committee of the Department of Pharmacology and Therapeutics, ABU, Nigeria (protocol number: DAC/IW-OT/137/14) and was carried out following the criteria outlined in the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health (Publication No. 80-23, revised 1996).

Inflammatory stimulus

Four randomly-selected groups of rats (n = 6) were orally administered distilled water (1 mL), aqueous fraction, butanol fraction (1,000 mg/kg) and acetylsalicylic acid (ASA, 300 mg/kg). Sixty minutes post-treatment, inflammation was induced by injecting each rat with 0.1 mL of 1% carrageenan into the plantar surface of the rat's right hind paw.[16]

Determination of inflammatory cytokines' concentration

Four hours post inflammation induction, rats were anesthetized using chloroform, and the hind paws were removed at the level of the calcaneus bone. The paws were homogenized using phosphate-buffered saline (PBS) and centrifuged at 10,000 g for 10 minutes at 4°C before collecting the exudates (edema fluid). The concentrations of inflammatory cytokines (in the fluid) were measured using rat cytokine 27-plex discovery assay by Eve Technologies (Calgary, Alberta, Canada).

Statistical analysis

All values were expressed as Mean ± Standard Error of the Mean (SEM). The data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test using the Graph Pad Prism (statistical) software, version 8. The differences between means were considered significant when $p < 0.05$.

Results and Discussion

The anti-inflammatory and analgesic activities of the crude methanol leaf extract of *Olax subscorpioidea* have been previously reported by Odoma *et al*.[11] We have also shown that the butanol and aqueous fractions of its crude methanol extract have more anti-inflammatory and analgesic activities than the other fractions.[15] Furthermore, we elucidated the mechanism of the analgesic action of its butanol fraction; which revealed the involvement of serotonergic, opioidergic, and nitric oxide-l-arginine pathways in the analgesic effect of the butanol leaf fraction.[17] This current study investigated the effects of the butanol and aqueous leaf fractions of *Olax subscorpioidea* on inflammatory cytokines, using the carrageenan-induced inflammation model; using the dose with the best anti-inflammatory response (1,000 mg/kg) from our previous study.[15]

Carrageenan-induced inflammation is a well-established method of inducing hind paw inflammation in rats and mice.[16] After the carrageenan injection, agents such as bradykinin, histamine, serotonin and are released, these agents stimulate the release of tumor necrosis factor-α (TNF-α), which in turn stimulates the release of other pro-inflammatory cytokines such as interleukin-1 beta (IL-1β). The pro-inflammatory cytokines promote the release of the cycloxygenase enzymes, which convert arachidonic acid to prostaglandins. [18,19]

Cytokines are divided into pro-inflammatory and anti-inflammatory cytokines. The pro-inflammatory cytokines are characterized due to their ability to cause inflammation; whereas, the anti-inflammatory cytokines are characterized because of their ability to inhibit the synthesis of major pro-inflammatory cytokines;[20] and/or stimulate the synthesis of pro-inflammatory cytokines' receptor antagonists.

Effect of aqueous and butanol leaf fractions of Olax subscorpioidea on pro-inflammatory cytokines

The concentrations of epidermal growth factor (EGF), interleukin-1α (Il-1α), and vascular endothelial growth factor (VEGF) were significantly ($p < 0.05$ and $p < 0.01$) decreased by the aqueous and butanol fractions of *Olax subscorpioidea;* while the concentrations of IL-1β was significantly ($p < 0.05$) increased by both fractions when compared with the control (Figure 1). The concentrations of tumor necrosis factor-α (TNF-α) and IL-2 were not significantly affected by either of the fractions.

IL-1 causes fever by enhancing the synthesis of prostaglandin E_2 (PGE_2) by the vascular endothelium of the hypothalamus and it can also stimulate T cell proliferation. IL-1 also stimulates the release of histamine from mast cells at the inflammation site. The released histamine initiates early vasodilation and thus increases vascular permeability.[20] Il-1 is subdivided into Il-1α and Il-1β which are equally potent pro-inflammatory cytokines. They activate inflammatory processes and cause devastating diseases manifested by severe acute or chronic inflammation.[21] The biogenesis and the distinctive role of Il-1α in inflammation are poorly understood.[21] From our studies, it was observed that the concentration of Il-1α was significantly reduced by both fractions especially BFOS. Il-1β is produced and secreted by immune cells such as macrophages, microglia, and monocytes; and is secreted under conditions of stress.[22] An increase in the concentration of IL-1β has been implicated in various diseases which include neuropathic pain due to diabetes, rheumatoid arthritis, type 2 diabetes, inflammatory bowel disease, leprosy neuropathy, and other inflammatory diseases.[22,23] It was observed from our studies that the concentration of IL-1β was

increased in the fractions treated groups; the observed increase may be due to direct stimulation of IL-1 receptor antagonist (IL-1ra) by the fractions or indirectly via the IL-6 (and/or other anti-inflammatory cytokines). IL-1ra is a specific IL-1 receptor antagonist that competitively binds to the same receptor as IL-1*β*, consequently blocking IL-1*β*-mediated cellular changes leading to the increase of IL-1*β* concentration.[6,24] Vascular Endothelial Growth Factor (VEGF) is a member of the growth factors sub-family and an angiogenic cytokine.[25] It is a key factor implicated in nearly all human tumors.[26] It regulates vascular permeability thus causing tissue edema.[27] It also stimulates angiogenesis in adults. Angiogenesis is a prerequisite for the growth of tumors from a benign to a malignant phenotype and metastasis, consequently making VEGF a key player in tumorigenesis.[26] Epidermal Growth Factor (EGF) is also a member of the growth factors sub-family. It mediates the activation of NF-kB and inflammation *in vivo* and *in vitro*. The mutations, amplifications, or misregulations of EGF receptors have been implicated in about 30% of all epithelial cancers. It has also been reported to play an important role in the pathogenesis of asthma.[28] The plant fractions were able to significantly reduce the concentrations of the growth factors sub-families (VEGF and EGF).

Effect of aqueous and butanol leaf fractions of Olax subscorpioidea on anti-inflammatory cytokines

The concentrations of IL-6, IL-5, and interferon-γ (IFN-γ) were significantly ($p < 0.05$ and $p < 0.01$) increased by the aqueous and butanol fractions of *Olax subscorpioidea* when compared with the control (Figure 2). The concentrations of IL-13, IL-10, and IL-4 were not significantly affected by either of the fractions.

IL-5 is produced by various cell types such as type 2 T helper cells (Th2) and mast cells. Its essential activity is eosinophil growth and function.[6] IL-5 is involved in the activation and differentiation of eosinophil; and also stimulate the switching of immunoglobulin class to IgA;[20,23] it helps in the regulation of chronic inflammation and disease control. The potential roles of IL-5 are in immune responses, allergy, and autoimmunity.[29] It has also been reported to enhance the cytotoxicity of T cells and increase activation of B cell proliferation.[20] From the studies, the concentration of IL-5 was significantly increased by the fractions especially AFOS. IL-6 is a multifunctional pleiotropic cytokine. It regulates, hematopoiesis, immune response, acute-phase response, and inflammation.[30] It possesses the characteristics of both pro-inflammatory and anti-inflammatory cytokine properties but, it possesses more of the anti-inflammatory cytokine properties.[20,31,32] In models of chronic inflammatory diseases, such as murine colitis, collagen-induced arthritis, or experimental autoimmune encephalomyelitis, IL-6 is pro-inflammatory, whereas, in models of acute inflammation such as carrageenan-induced inflammation, IL-6 exhibits an anti-inflammatory profile.[33] IL-6 is generated in an infectious lesion and sends out a warning signal to the entire body. Thus, it serves as a mediator for notification of the occurrence of some emergent event as it issues a warning signal in the event of tissue damage.[30,34] IL-6 inhibits the production of pro-inflammatory cytokines such as TNF and IL-1. Thus, it limits the acute inflammatory response via a negative feedback mechanism.[20] In this study, the concentration of IL-6 was significantly increased especially by AFOS. Interferons (IFN) are produced and secreted in response to viral infections. It possesses both anti-proliferative and anti-viral properties and it is classified as type I and type II IFN. Type I IFN such as IFN-α and IFN-*β* induces anti-viral and anti-proliferative activity; but, type II IFN e.g. IFN-γ possesses potent immunomodulatory activity with a weaker anti-viral activity.[24] IFN-γ stimulates the activation of phagocytes and macrophages; the macrophages in turn kill intracellular pathogens.[20,24] IFN-γ also activates and increases the anti-microbial and tumoricidal activity of neutrophils, macrophages, monocytes, and natural killer (NK) cells.[23] The concentration of IFN-γ was significantly increased by the plant fractions as observed from the results.

From this study, it was observed that the concentrations of IL-1α, VEGF, and EGF were reduced in the fractions treated groups; the observed reductions may be due to direct inhibition by the fractions or by IL-6 and/or other anti-inflammatory cytokines. IL-13, IL-10, IL-6, and IL-4 were all reported to inhibit the synthesis of IL-1, simultaneously they stimulate the synthesis of IL-1ra.[20] It was also observed from the study that the plant fractions were able to stimulate the production of IL-6; this is evidenced by the increase in the concentration of IL-6; consequently, it inhibited the production of pro-inflammatory cytokines and/or stimulating the production of pro-inflammatory cytokines receptor antagonists. Reductions in the concentration of the growth factors sub-families (VEGF and EGF) were also observed. The ability of the leaf fractions to decrease the concentrations of VEGF and EGF may also justify the use of the plant as an anti-tumor agent. Oloyede *et al*[35] reported the utilization of *Olax subscorpioidea* leaf as one of the seven therapeutic plants combined to form a local decoction (Joloo) traditionally used in Southwestern Nigeria for the management of breast cancer. These leaf fractions may also serve as a prophylactic agent for malignancies; as inflammation, supported by pro-inflammatory cytokines; precede tumor appearance.[36]

In this study, the concentrations of a potent pro-inflammatory cytokine, TNF-α, and that of a potent anti-inflammatory cytokine, IL-10, were not affected by the leaf fractions. The reasons may be due to the complexity in regulatory networks that govern inflammatory cytokine secretion upon specific stimulation, which might contribute to the different effects of the various cytokines tested.[36] These also suggest that measuring just one inflammatory cytokine may be misleading and may also give an unreliable result.

Phytochemicals have been shown to modulate various points in inflammatory processes by disconnecting the amplification of inflammatory processes and thereby reducing subsequent diseases risk.[37] Different medicinal plants e.g. *Rauwolfia serpentine* root, *Curcuma longa* rhizome, *Zingiber officinale* rhizome, *Allium sativum* bulb, have been reported to modulate cytokine secretion by their phytochemical constituents.[38] Moreover, wide ranges of phytoconstituents including flavonoids, saponins, alkaloids, and tannins have been reported to inhibit pro-inflammatory cytokines.[39] The presence of phytochemicals such as tannins, flavonoids, saponins, alkaloids, cardiac glycosides, carbohydrates, steroids, and triterpenoids have been previously reported in the aqueous and butanol fractions of the crude methanol leave extract of *Olax subscorpioidea*.[14] The presence of these phytochemicals in both fractions of *Olax subscorpioidea* used in this study might have contributed to the modulation of the cytokines, which could have been responsible for the observed anti-inflammation.

Conclusion

In conclusion, the results of the experiment suggest that the aqueous and butanol leaf fractions of *Olax subscorpioidea* may be exhibiting their anti-inflammatory activities via the inhibition of pro-inflammatory cytokines such as IL-1, VEGF, and EGF and/or stimulation of the synthesis of anti-inflammatory cytokines such as IL-5, IL-6, and IFN-γ.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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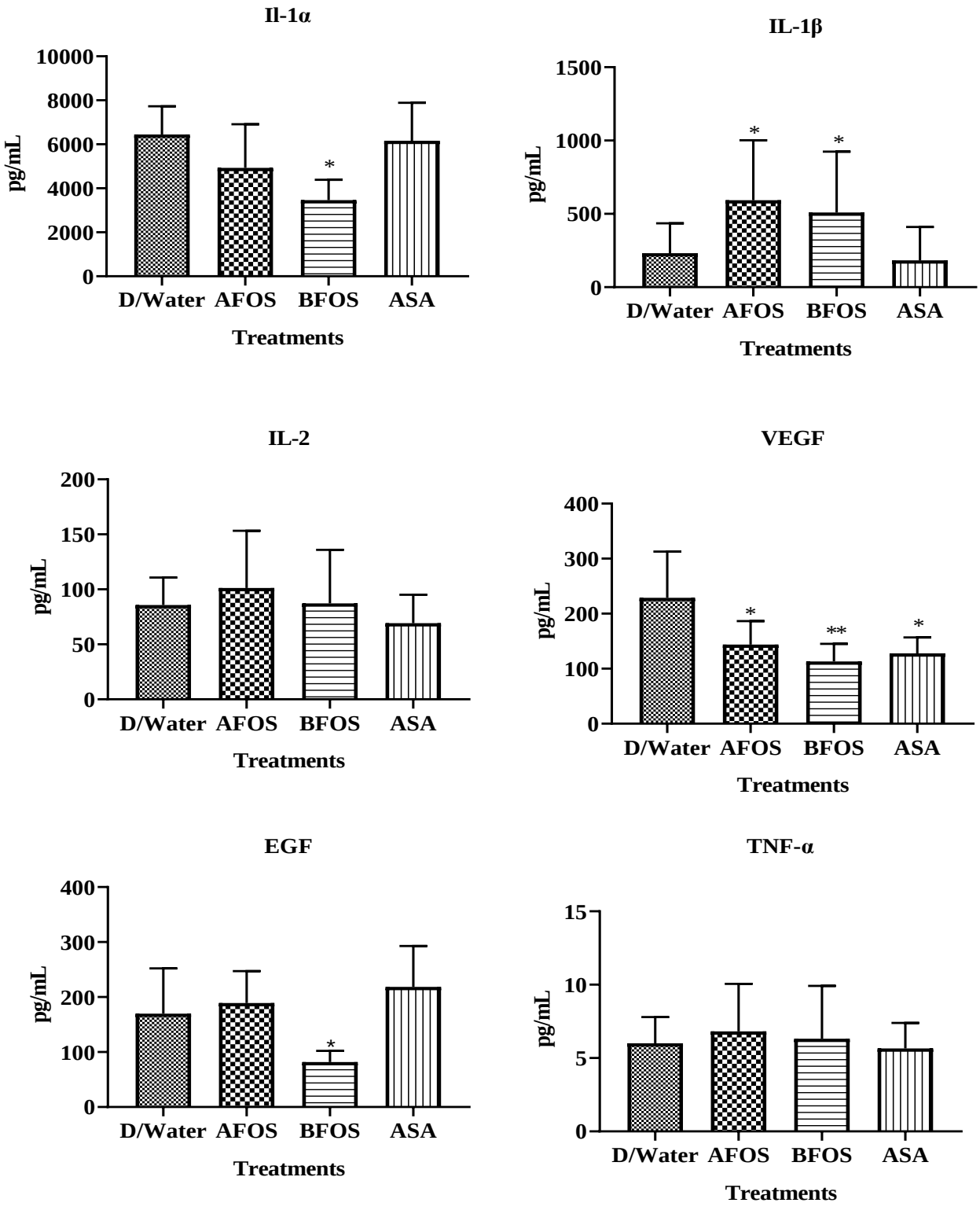

Figure 1: Effect of aqueous and butanol fractions of *Olax subscorpioidea* on Pro-inflammatory Cytokines.
*$p < 0.05$, **$p < 0.01$ versus control (D/Water). AFOS = aqueous fraction, BFOS = butanol fraction, ASA-acetylsalicylic acid, IL = Interleukin, TNF = tumor necrosis factor, VEGF = Vascular Endothelial Growth Factor, EGF = Epidermal Growth Factor, n = 6.

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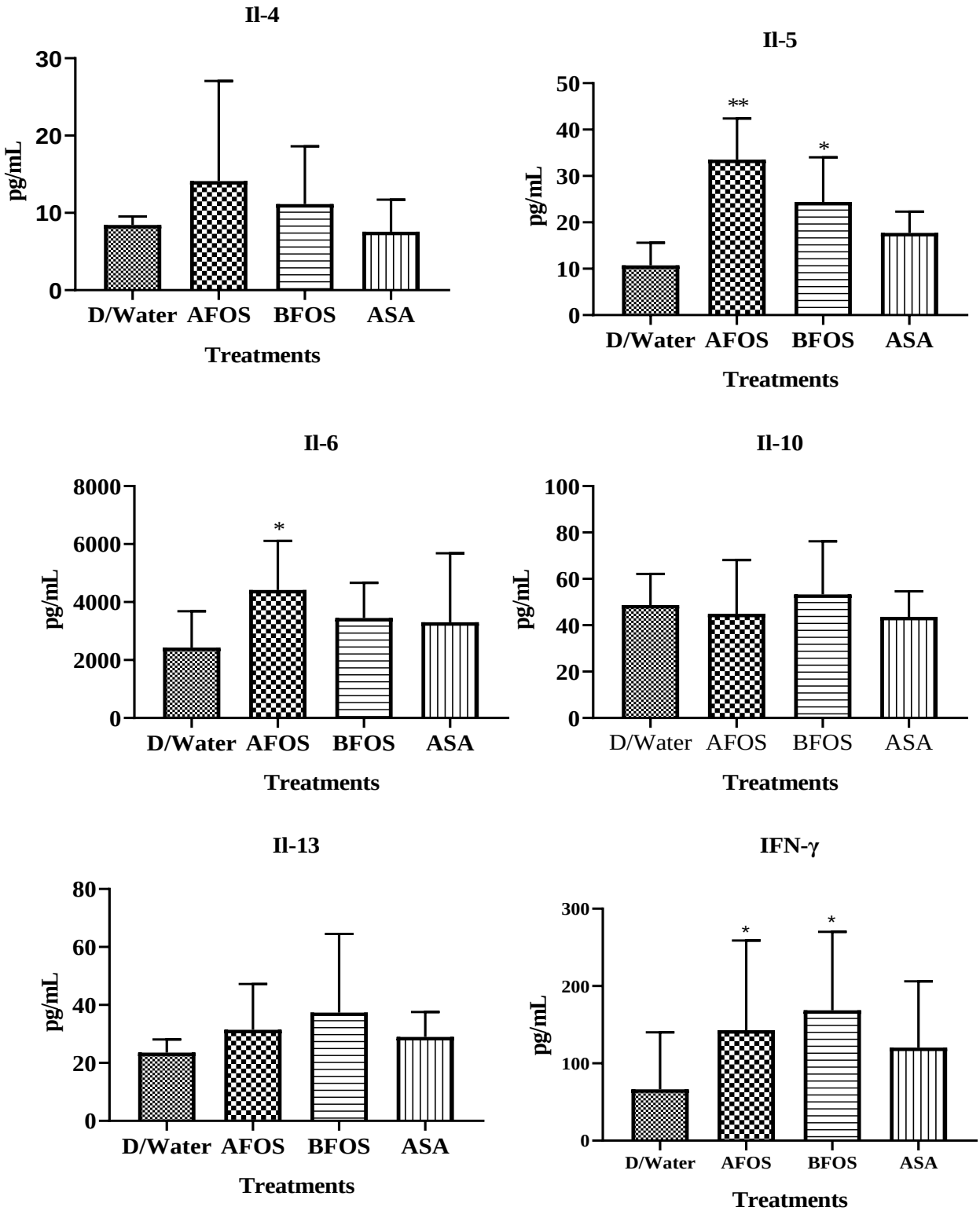

Figure 2: Effect of Residual Aqueous and Butanol Fractions of *Olax subscorpioidea* on Anti-inflammatory Cytokines.
*$p < 0.05$, **$p < 0.01$ versus control (D/Water). AFOS = aqueous fraction, BFOS = butanol fraction, ASA = acetylsalicylic acid, IL = Interleukin, IFN = Interferon, n = 6.

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