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Anti-Inflammatory and Antioxidant Activities of Alkaloid Extract of *Citrus aurantium* Leaves

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Abstract: **Inflammation is a complex mechanism used by the body to promote healing and restore normal function after an injury. The crude alkaloid extract of *Citrus aurantium* leaves was tested for its anti-inflammatory and antioxidant activities. The extract's anti-inflammatory activity was assessed using cyclooxygenase (COX-1 and COX-2) and 5-lipoxygenase (5-LOX) inhibition assays. The antioxidant activity of the extract was assessed using DPPH (2, 2-diphenyl-1-picrylhydrazyl) and ABTS (2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) assays. *The* result showed that inhibition of COX-1 (IC_{50} = 57.27 ± 0.36 µg/mL) and COX-2 (IC_{50} = 54.35 ± 0.54 µg/mL) was within the range of 23.31% to 60.12% and 15.80 to 43.45 % respectively. *The* inhibition of LOX (IC_{50} = 61.20 ± 0.34 µg/mL) was within the range from 13.21% to 47.79% at concentrations ranging from 20 to 100 µg/mL. *The* extract demonstrated good antioxidant activity by scavenging DPPH (IC_{50} = 42.33 ± 0.42µg/mL) and ABTS (IC_{50} = 52.31± 0.22µg/mL) radicals in a concentration - dependent manner. The extract's IC_{50} values were comparable to those of standard drugs (Ibuprofen, Celecoxid and BHT). The alkaloid extract of *C. aurantium* leaves had good anti-inflammatory and antioxidant activities. These findings suggest that *C. aurantium* leaves can be a promising natural remedy for treating inflammation and oxidative stress related conditions.**

Keywords: ***Citrus aurantium*****, Alkaloid extract, Inflammation, Antioxidant, Cyclooxygenase, 5-Lipoxygenase, Radicals.**

I. INTRODUCTION

Inflammation is an immune response that is initiated by different stimuli, including chemical, physical and biological processes [1]. Inflammation is triggered by the recognition of a harmful agent or activation and amplification of the immune response, thus, resulting in the release of various mediators responsible for the inflammatory response [2]. Inflammatory mediators (TNF-cytokines IL-1, IL-6, IL-12) are responsible for inducing the expression of adhesion molecules and leukocytes segregation from the bloodstream towards the affected site [3]. As a result, uncontrolled inflammation can result in severe tissue damage.[4].The reactive oxygen species (ROS) creates oxidative stress in the cells leading to inflammatory and infectious conditions. ROS deregulates the cellular functions causing cellular and tissue damage, which aggravates inflammation [5,6,7]. Non-steroidal anti-inflammatory drugs (NSAIDs) are clinically used to combat inflammatory processes. Scientific reports have indicated that about 30 million of the world population use NSAIDs to treat inflammation and related disorders [8,9]. However, the prolonged use of non-steroidal anti-inflammatory drugs has been reported to pose serious side effects such as gastrointestinal and cardiovascular complications [10]**.** Natural plant products are reliable alternative therapeutic options to formulate anti-inflammatory drugs as they are cheap, abundantly available, and relatively less toxic [11].

Citrus aurantium L. (*Rutaceae*), popularly known as bitter orange, is a subtropical plant native to Africa, Guinea, West Indies, Brazil, California and Florida [12]. The height of the bitter orange tree ranges from 2 to 9 m and has a more compact pinnacle than that of the sweet orange; it has smooth, brown bark, green twigs and is angular and flexible. The plant produces

leaves all through seasons of the year [13]. The plant is commonly used as a flavoring agent, an acidifying agent in food and a source of flavonoid-type compounds with various biological effects [14-15]. Due to the abundance of health-giving secondary metabolites, *C. aurantium* is also used for the treatment of several ailments such as anxiety [16], lung and prostate cancers [17], and gastrointestinal disorders and obesity [18]. *C. aurantium* it has found an important place as a preferred agent to replace ephedra, as it contains *p*-synephrine, a phenylethanolamine type alkaloid, which is chemically similar to adrenergic agents, as appetite suppressants [18–19]. *C. aurantium's* chemical composition is responsible for its health-promoting properties. Vitamins, minerals, phenolic compounds, and terpenoids are among the chemical components [20-21]. *C. aurantium* is widely used in folklore medicine for various purposes. Only a few studies have reported the pharmacological properties of the plant. This work aims to investigate the anti-inflammatory and antioxidant effects of crude alkaloids extracted from the leaves of the plant.

II. MATERIALS AND METHODS

Plant material collection and identification

The leaves of *C. aurantium* were collected from a farm in Odo Ayedun Ekiti, South-Western Nigeria. Mr. Omotayo (Herbarium curator) at the Department of Plant Science and Biotechnology, Ekiti State University, Ado Ekiti, Nigeria, authenticated the plant and voucher specimen was deposited in the Herbarium

Chemicals

All solvents and reagents used were of analytical grade. 5-lipoxygenase (5-LOX), trichloroacetic acid (TCA),

Cyclooxygenase 1 and Cyclooxygenase 2 (COX-1 and COX-2 were purchased from Sigma Aldrich (USA).

Preparation of alkaloid extract of the leaves

The alkaloid extract of *C. aurantium* leaves was prepared using the Oboh *et al.* [22] method. The pulverized samples were defatted in n-hexane at room temperature for 24 hours. Following that, 10 g of defatted samples were weighed into a 250 mL beaker and 100 mL of 10% acetic acid in ethanol were added and covered. This was vigorously shaken, venting the built-up pressure, and left to stand for 24 hours to allow for adequate extraction. Thereafter, the mixtures were filtered using Muslin cloth and then filter paper (Whatman no. 1) to obtain a clear filtrate that was concentrated under vacuum using a rotary evaporator set to 45 °C. Concentrated ammonium hydroxide was added to the concentrated filtrate drop by drop until precipitation was complete. To obtain the alkaloid extracts, the entire solution was allowed to settle; the precipitate was collected and rinsed with dilute ammonium hydroxide. The extracts were collected and refrigerated at 4^{o}C for further analysis.

In-Vitro Cyclooxygenase (COX−1 and COX−2) Assay

The alkaloid extract's inhibitory potential against COX1 and COX2 enzymes was determined using the standard method of Ilic *et al.* [23] with minor modifications. A 300 U/ml concentration of COX enzyme solution was prepared. This solution (10 µL) was activated by placing it on ice for 5-10 minutes. Furthermore, a 50 µL concentration of cofactor solution containing 0.9 mM glutathione, 1 mM hematin, and 0.24 mM N,N,N,N-tetramethyl-p-phenylenediamine dihydrochloride (TMPD) in 0.1 Mole Tris HCl buffer with 8.0 pH was added to this enzymatic solution. Finally, 20 µL of the extract in various concentrations ranging from 20 to 100 g/ml and enzyme solution (60 µL) were left at 25 °C for five minutes. Similarly, the reaction was started by adding 30 mM arachidonic acid in a volume of 20 µL. This solution mixture was then incubated for about 4-5 minutes. After the incubation period, absorbance was measured at 570 nm using a UV-visible spectrophotometer. The absorbance value per unit time was used to calculate the inhibitory activity of the enzyme (COX1 or COX2) solution. The percentage inhibition was calculated using the following formula:

Percentage Inhibition = Control Abs − Sample Abs/Control Abs × 100

Lipoxygenase Inhibition Assay

The extract's lipoxygenase inhibition activity was determined using the method described by Gunathilake *et al.* [24]. In a 1 mL cuvette, a mixture of sodium borate buffer (1 mL, 0.1 M, pH 8.8) and lipoxygenase (10 µL, final concentration 8000 U/mL) was incubated with 10 mL leaf extract for 5 minutes at room temperature (30 ± 2 °C). The reaction was started by adding 10 L of linoleic acid substrate (10 mmol). A UV/VIS spectrometer was used to measure the absorbance of the

reaction solution at 234 nm. The control solution was phosphate buffer solution, and the percentage inhibition of lipoxygenase was calculated using the following equation:

% inhibition = 100 × (absorbance of the control − absorbance of the sample)/absorbance of the control

In vitro antioxidant assays

DPPH radical scavenging assay

The scavenging ability of the extract against DPPH radical was determined using the method of [25]. One millilitre of 0.135mM of DPPH in methanol was mixed with 1 ml of different concentrations (20 – 100 µg) of the alkaloid extract. The mixture was kept in a dark cupboard for 30 minutes. The absorbance of the resulting solution was measured spectrophotometrically at 517 nm and the scavenging ability of the extract was calculated as:

% inhibition = 100 × (absorbance of the control − absorbance of the sample)/absorbance of the control

ABTS Assay

The antioxidant ability of the crude alkaloid extract was also evaluated using the 2,2azinobis [3ethylbenzthiazoline]-6-sulfonic acid (ABTS) protocol. A solution of (ABTS) 7 mM and (K2S2O4) 2.45 mM was prepared. This mixture was darkened for nearly 12-16 hours to obtain a dark-colored solution containing ABTS+ cation. For activity, a 0.01 M phosphate buffer with a pH of 7.4 was used to dilute the ABTS+ solution and regulate absorbance at 734nm. The antioxidant effect of the extract was estimated by preparing a solution of extract (20-100 µg) in a concentration of 300 µL with an ABTS solution of 3.0 ml. The solution was vortexed for one minute in order to measure the reduction in absorbance spectrophotometrically [26]. The above protocols were repeated three times and inhibition percent was estimated by the following formula;

% scavenging effect = (Control absorbance − Sample absorbance/Control absorbance) × 100

III. RESULTS AND DISCUSSION

Herbal remedies have long been used to maintain health and treat diseases in many parts of the world. However, they are not officially recognized internationally because there is insufficient qualitative and quantitative evidence on their safety and effectiveness [27]. Herbal medications used in traditional folk medicine have long been used in rural and urban health care in various countries [27, 28]. The current study shows that alkaloids extracted from *C. aurantium* leaves have anti-inflammatory properties in vitro in several models, including COX-1, COX-2, and LOX inhibition. Inflammation is a complex process that involves the production of free radicals derived from neutrophils, NO, ROS, cytokines, and prostaglandins [29]. The inflammation mechanism consists of a series of events in which arachidonic acid metabolism plays an important role. Prostaglandin I2, thromboxane A2, prostaglandin E2, arachidonic acid, and leukotrienes are overproduced during the pathogenesis of inflammatory diseases via two metabolic pathways, the cyclooxygenase (COX) pathway and the 5-lipoxygenase (5-LOX) pathway [30]. COX and 5-LOX are the primary enzymes involved in the synthesis of prostanoids and eicosanoids from polyunsaturated fatty acids in a variety of inflammatory and allergic disorders. The dual inhibition of LOX and COX is important for the effective reduction of chronic inflammatory conditions [24]. Substances that can inhibit both COX and 5-LOX, resulting in a significant reduction in leukotriene and prostaglandin production, produce a broad spectrum of anti-inflammatory activity and have an excellent pharmacological safety profile in clinical practice [27]. The anti-inflammatory activity of alkaloids extracted from *C. aurantium* leaves was assessed using two methods: COX-2 and lipoxygenase (LOX) assays. Alkaloids extracted from *C. aurantium* leaves demonstrated excellent concentration - dependent anti-inflammatory activity against 5-lipoxygenase and cyclooxygenase 2 enzymes, both of which are active mediators of inflammation in this study. At concentrations of 20, 40, 60, 80, and 100 µg/ml, the crude extract of *C. aurantium* leaves inhibited COX-1 enzyme by 23.31, 36.20, 41.72, 47.85, and 60.12%, respectively (Figure 1). Furthermore, at concentrations of 20, 40, 60, 80, and 100 µg/ml, it inhibited COX-2 enzyme with 15.80, 25.05, 35.88, 35.94, and 43.45 %, respectively (Figure 2). The crude alkaloid extract inhibited the 5-LOX enzyme effectively. It showed inhibition of 13.21, 18.50, 28.50, 32.49 and 47.79% at concentrations of 20, 40, 60, 80 and 100 µg/mL respectively (Figure 3). The extract's inhibitory activity against the cyclooxygenases and lipoxygenase pathways indicates that it is capable of significantly inhibiting the production of prostaglandins and leukotrienes, indicating its anti-inflammatory activity. The IC_{50} values (Table I) of the extract was comparable with those of the standard drugs (Ibuprofen, Celecoxid and BHT).

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Table I: IC_{50} values of extract and standard drugs

	IC_{50} values (µg/mL)				
Sample	COX - 1	COX - 2	5-LOX	DPPH radical	ABTS radical
Extract	57.27 ± 0.36	54.35 ± 0.54	61.20 ± 0.34	42.33 ± 0.42	52.31± 0.22
BHT	-	-	-	46.50 ± 0.67	46.01 ± 0.58
Celecoxid	-	44.55 ± 0.13	-	-	-
Ibuprofen	52.45 ± 1.13	-	53.08 ± 1.22	-	-

Each value is expressed as mean ± SD (*n* = 3).

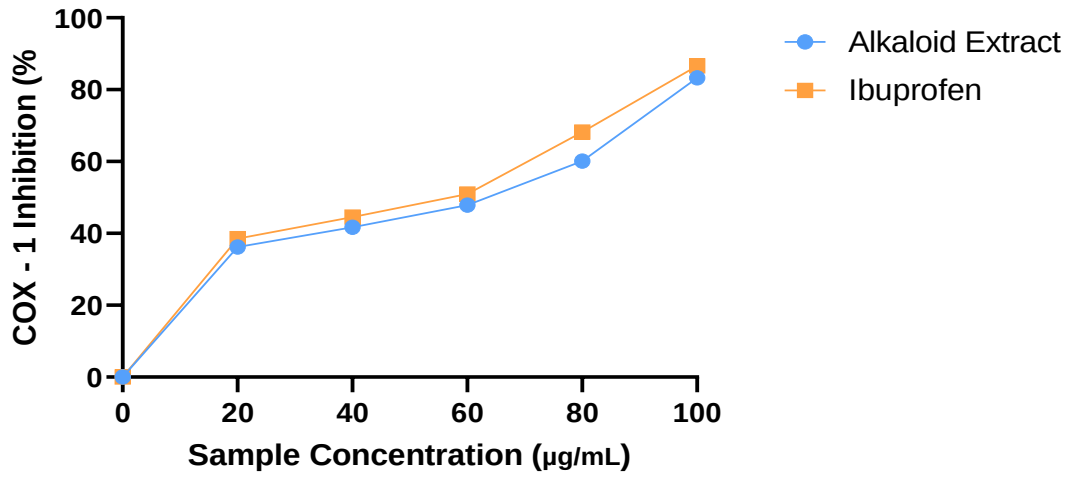

Figure 1: COX - 1 inhibitory activity of alkaloid extract from *C aurantium* leaves. Data expressed as means± SD (n = 3).

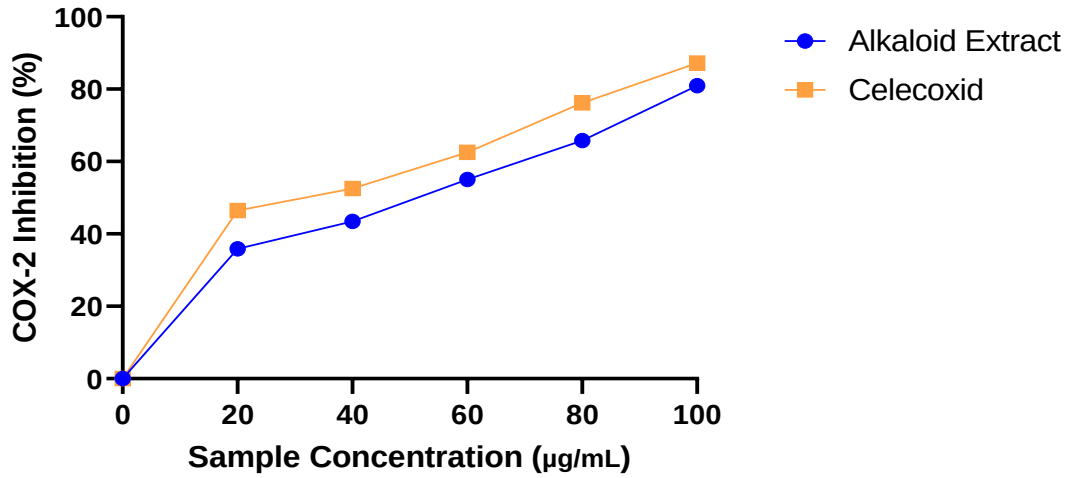

Figure 2: COX - 2 inhibitory activity of alkaloid extract from *C aurantium* leaves. Data expressed as means± SD (n = 3).

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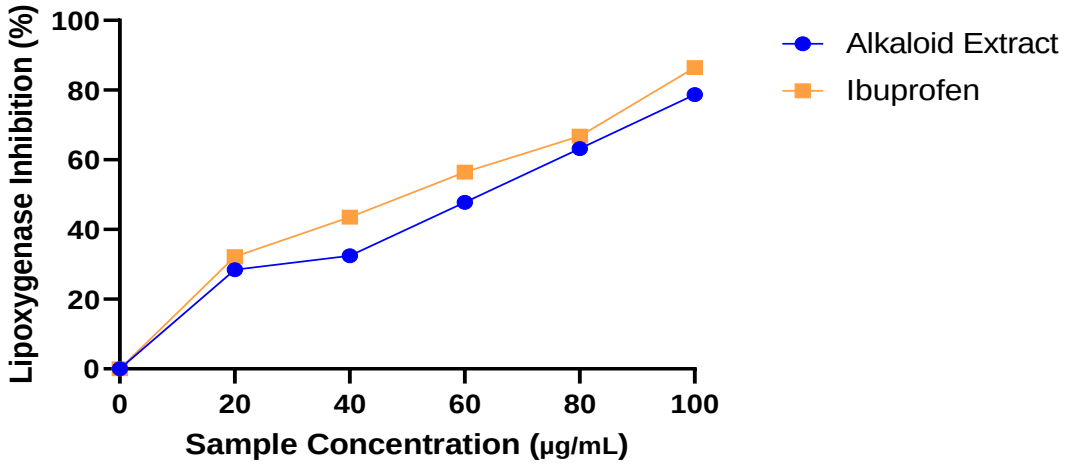

Figure 3: Lipoxygenase inhibitory activity of alkaloid extract from *C aurantium* leaves. Data expressed as means± SD (n = 3).

Reactive oxygen species (ROS) play an important role in human pathological and physiological processes [31]. In most cases, there appears to be a balance between free radical production and endogenous antioxidant defense mechanisms. If there is a discrepancy, oxidative stress can occur. This level of oxidative stress can cause cell death by causing damage to all critical cellular constituents such as proteins, DNA, and membrane lipids [32, 33]. When the body is under extreme stress, the formation of reactive oxygen species (e.g., hydroxyl radicals, hydrogen peroxide, etc.) increases. Endogenous enzymatic and non-enzymatic antioxidant molecules are incapable of dealing with ROS overload, resulting in metabolic imbalances, cell damage, and health problems [34]. Secondary complications can include cardiovascular disease, diabetes, inflammation, degenerative diseases, cancer, anemia, and ischemia [32]. Natural compound-based antioxidants prevent free radical formation, making them one of the most effective therapeutic substances for reducing illnesses caused by oxidative stress in the body. **An accumulation of free radicals damages cells and is thought to be the root cause of a wide range of diseases. Antioxidant analysis revealed that alkaloids extracted from *C. aurantium* leaves had significant antioxidant potential. The hydrogen-donating capability of the alkaloid extract may be responsible for the effect the extract had on DPPH and ABTS radicals (Figures 4 and 5). (Figures 4 and 5).**

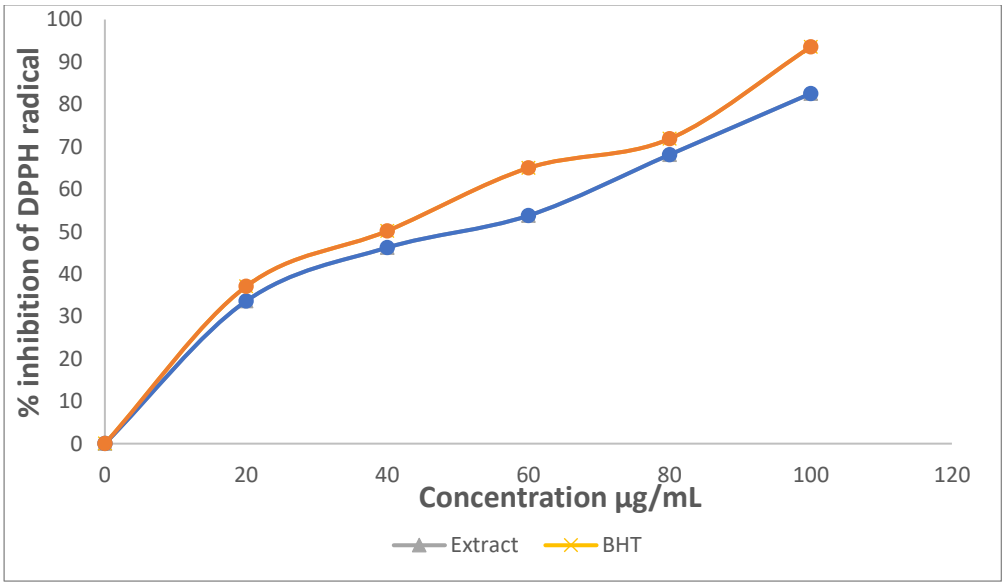

Figure 4: DPPH radical scavenging ability of alkaloid extract from *C aurantium* leaves. Data expressed as means± SD (n = 3).

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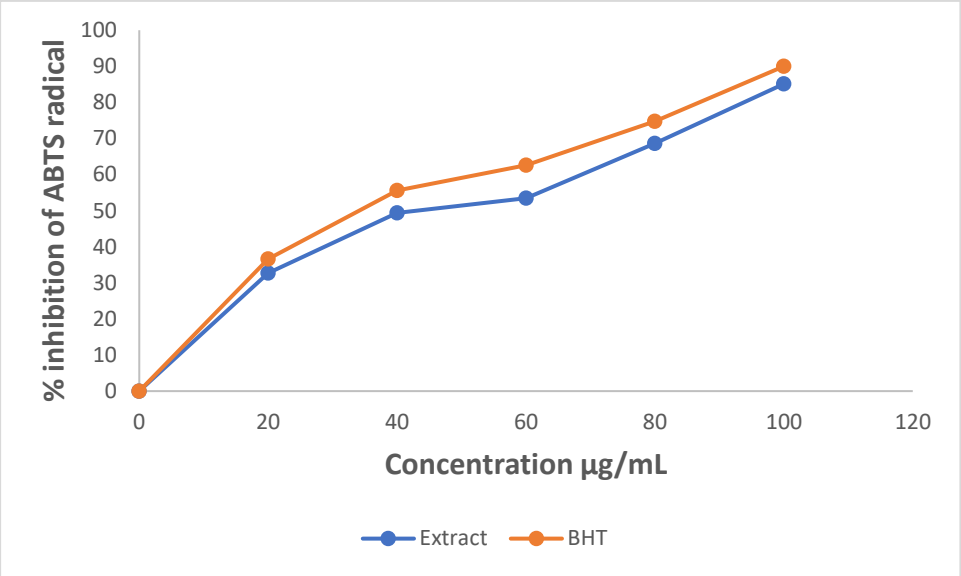

Figure 5: ABTS radical scavenging ability of alkaloid extract from *C aurantium* leaves. Data expressed as means± SD (n = 3).

IV. CONCLUSION

These studies provide evidence that the alkaloids extracted from C. *aurantium* leaves exert anti-inflammatory effect by inhibiting both COX and 5-LOX activities in an *in-vitro* model.

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