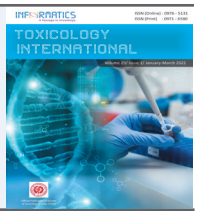
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Cleistopholis patens Extracts Attenuate Inflammatory Cytokine and Oxidative Stress Status in Complete Freund's Adjuvant-induced Rheumatoid Arthritis in Wistar Albino Rats

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Abstract

Rheumatoid Arthritis (RA) is an autoimmune inflammatory disease. Accumulating evidences show that natural products use for RA treatment is gaining importance in the medical world due to negative effects associated with orthodox medicines. This study investigated the anti-inflammatory and antioxidant potency of Stem Bark of *Cleistopholis Patens* (SBCP) on Chicken Type II collagen in Complete Freund's Adjuvant (CFA) induced RA in rats. Experimental rats were divided into nine groups (n= 15). RA was established in the rats by a single intradermal injection of 0.1ml Chicken Type II collagen in CFA through the left hind paw. The serum concentrations of Inter-interleukin-1 beta (IL-1β), interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), malondialdehyde (MDA), nitric oxide (NO), superoxide dismutase (SOD) and catalase (CAT) were evaluated. Unlike the untreated arthritic group, the test groups (4-9) orally administered with graded doses (400, 600 and 800 mg/kg) of SBCP extracts for twenty-one days showed significant reduction ($P < 0.05$) in concentrations of IL-1β, IL-6, TNF-α, MDA and NO while the activities of SOD and CAT increased significantly ($P < 0.05$) when compared to the negative control. The anti-inflammatory and anti-oxidative effects of SBCP extracts were comparable to that of the reference drug, indomethacin. This study indicates that SBCP ethanol and aqueous extracts could suppress proinflammatory cytokines and reduce oxidative stress in rheumatoid arthritic rats.

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1. Introduction

Rheumatoid Arthritis (RA) is an inflammatory and autoimmune ailment with a non-specific defined etiology affecting the joints, causing pain, swelling, joint destruction and malformation[1]. The prevalent rate is more in women than men, affecting mostly the elderly of 50 and 60 years[2]. Free radicals induced oxidative stress and compromised antioxidant scavenging systems give rise to an imbalance in pro-oxidant/antioxidant and cause damage to joints in RA[3]. Reports showed that IL-1β and TNF-α were implicated in the emergence of deleterious peroxynitrite by boosting the activity of nitric oxide synthase (NOS) enzyme, thus, reflecting powerful inflammatory mediator[3].

Therapeutic agents such as non-steroidal anti-inflammatory drugs (NSAIDs) and disease-modifying anti-rheumatic drugs (DMARDs) have been used over time for managing rheumatoid arthritis[4]. At the cellular level, NSAIDs prevent the production of prostaglandins through its inhibitory actions on cyclooxygenase 1 and 2 (COX-1 and COX-2) leading to anti-arthritis and anti-inflammation (3,4). The uses of NSAIDS and DMARDS for the management of rheumatoid arthritis are limited due to their adverse effects such as gastrointestinal distress, peptic ulcer, other potential toxicities and high cost among others[5,6]. These adverse effects call for new therapeutic approach that is safer and more affordable for the management of rheumatoid arthritis.

The tree, *Cleistopholis patens* (Benth) belongs to the Annonaceae family and grows up to 30m high. It is mostly found in Uganda, Zaire and eastwards of Sierra Leone[7]. It is called different names in Nigeria, including 'ojo' by the Igbos and 'apako' by the Yorubas[8]. Reports have shown that various parts of *C. patens* contain potent phytochemicals such as cardiac glycosides, steroids, phenols, tannins, saponins, flavonoids, etc. and high level of Zn among other minerals[9]. Documented ethnomedicinal usage included treatment of infectious diseases caused by *Staphylococcus aureus* among others[10]. Robust evidence had shown that phytochemicals such as phenols, flavonoids, tannins, terpenoids, amino acids, ascorbic acid, tocopherols, carotenoids, alkaloids and phytates seen in ample quantities in many plants are antioxidants that help to mop up free radicals[11]. Traditionally, the indigenous people of Ugwulangwu in Ohaozara, Ebonyi state, Nigeria employ *C. patens* extracts in the control and treatment of rheumatoid arthritis. This practice has no scientific backing. Hence, this study sought to verify the effect of SBCP extracts on proinflammatory cytokines and oxidative stress in rheumatoid arthritic rats in order to validate its potency, possible mechanism, safety and wholesomeness or otherwise.

2. Materials and Methods

2.1 Chemicals and Reagents

Analytical graded chemicals, chicken type 11 collagen, Complete Freund's Adjuvant (CFA) among others used for the experiment were purchased from Sigma Aldrich, Saint Louis, U.S.A.

2.2 Experimental Animals

One hundred and thirty-five (135) albino Wistar rats weighing 155 – 185g were obtained from the University of Nigeria Nsukka (Faculty of Veterinary Medicine animal house facility). The rats were moved to Ebonyi State University Abakaliki (Department of Biochemistry animal house facility). The Wistar albino rats were allowed to acclimatize under standard laboratory conditions for two weeks and fed using growers' mash and water *ad libitum*. Ethical approval for use of animals for this study was granted by Ebonyi State University, Abakaliki, Faculty of Medicine, Pre-Clinical Research and Ethics Committee with approval reference no: EBSU/REC/FBMS/1808/02/002. The guidelines for care and use of laboratory animals published by NIH were strictly observed in handling the albino Wistar rats to ensure humane treatment.

2.3 Plant Material and Preparation of Extracts

The Stem Bark of *Cleistopholis Patens* (SBCP) was obtained from farmlands situated at Umuigboke clan in Ugwulangwu autonomous community in Ohaozara L.G.A. Ebonyi State Nigeria. The plant was authenticated at the International Centre for Ethnomedicine and Drug Development Nsukka, Enugu State Nigeria by a taxonomist, Mr. Alfred Ozioko and it was deposited in the herbarium with a voucher specimen number: Inter

CEDD/505-cleitopholis. The SBCP was cut into pieces, shade dried and pulverized into fine powder. The method outlined by Oluduro and Aderiye[12] was followed to prepare the SBCP extracts from its processed powdered form.

2.4 Acute Toxicity

The acute toxicity test was done to determine the median Lethal Dose (LD_{50}) of *C. patens* ethanol and aqueous extracts and to choose the safe doses for the extracts. The acute toxicity study method described by Lorke[13] with slight modifications was followed in the acute toxicity study of SBCP extracts on sixty-eight (68) albino Wistar rats. The weights of the rats were recorded, assigned to different treatment groups: A, B and C and fasted overnight before the experiment. Each group had four albino Wistar rats. Group A (normal control) received normal saline via oral intubation. Group B were divided into eight sub-groups of four rats each and each group received aqueous extracts of SBCP at a dosage of 200, 400, 800, 1200, 1800, 2000, 3000 and 5000 mg/kg bw respectively. Group C were also divided into eight sub-groups of four rats each and each group received ethanol extracts of SBCP at a dosage of 200, 400, 800, 1200, 1800, 2000, 3000 and 5000 mg/kg bw respectively. The treated albino Wistar rats were monitored at 2 h intervals for any behavioral changes and mortality for 24 h.

2.5 Experimental Design

2.5.1 Induction of Arthritis

The method described by Pearson[14] was used to induce RA in the albino Wistar rats. Using sterile syringe and needle, 0.1ml chicken type II collagen in CFA (heat-killed *Mycobacterium tuberculosis* + 10 mg/ml sterile paraffin oil) were administered (taking the body weight of the rats into cognizance) via intradermal injection through the hind paws in groups 2 to 9. The treatment with SBCP extracts commenced on the 10th day after arthritis induction and continued for the next 21 days.

2.5.2 Experimental Groups

One hundred and thirty-five albino Wistar rats used for the experiment were divided into nine randomized groups of fifteen each.

Group 1: Albino Wistar rats received 5 ml/kg normal saline (normal control)
Group 2: Arthritic albino Wistar rats received 5 ml/kg normal saline (negative control)
Group 3: Arthritic albino Wistar rats received 10 mg/kg bw indomethacin standard drug (positive control)
Group 4: Arthritic albino Wistar rats + 400 mg/kg bw SBCPEE
Group 5: Arthritic albino Wistar rats + 600 mg/kg SBCPEE
Group 6: Arthritic albino Wistar rats + 800 mg/kg SBCPEE
Group 7: Arthritic albino Wistar rats + 400 mg/kg stem SBCPAE
Group 8: Arthritic albino Wistar rats + 600 mg/kg SBCPAE
Group 9: Arthritic albino Wistar rats +800 mg/kg SBCPAE

2.5.3 Determination of Pro-inflammatory Cytokines

Serum concentrations of proinflammatory cytokines (interleukin-1 beta (IL-1β), interleukin 6 (IL-6) and tumor necrosis factor-alpha (TNF-α)) were determined in a method outlined by Krakauer[15] and Rysz *et al.*[16]. Employing enzyme-linked immunosorbent assay (ELISA) test kits (Sigma Aldrich, Saint Louis, U.S.A.) with manufacturer's instruction strictly followed.

2.5.4 Assessment of Oxidative Stress Markers

Malondialdehyde (MDA) in serum was determined spectrophotometrically by measuring thiobarbituric acid reactive substance (TBARS) as described by Buege and Aust[17] while nitric oxide (NO) was determined according to the method described by Titheradge[18]. Serum superoxide dismutase (SOD) activity was assayed by the method described by Fridovich and Mc-Cord[19]. CAT activity was assayed by the method described by Sinha[20].

2.6 Statistical Analysis

The data were expressed as mean ± standard deviation. The mean results were analyzed with SPSS software version 20 using one-way analysis of variance (ANOVA) and compared for significant variations using Duncan multiple range test. Statistical differences were established at $p < 0.05$.

3. Results

3.1 Acute Toxicity Study

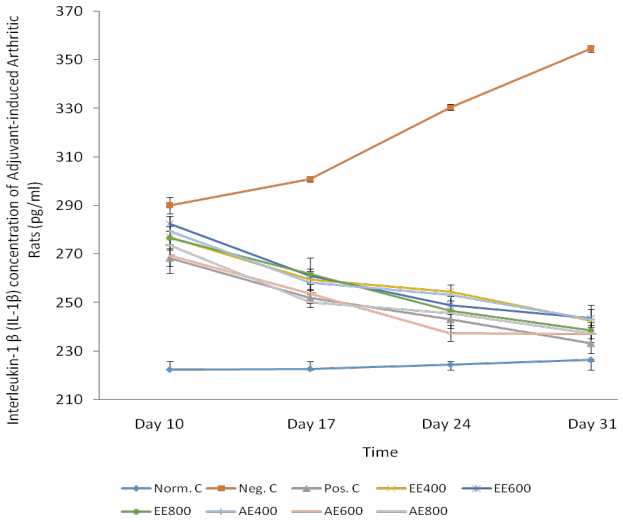
The SBCPEE and SBCPAE caused no death at the maximum dosage of 5000 mg/kg administered. Hence, 400, 600 and 800 mg/kg were selected for this study.

3.2 Effect of SBCPEE on Cytokine Levels of Adjuvant-induced Arthritic Rats

The results of the effect of SBCPEE and SBCPAE on interleukin-1 beta (IL-1β), interleukin 6 (IL-6) and tumor necrosis factor-alpha (TNF-α) levels in adjuvant-induced arthritic rats are presented in Figures 1, 2 and 3 respectively. These results showed that rats injected with the adjuvant exhibited a significant increase ($p < 0.05$) in the levels of IL-1β, IL-6 and TNF-α in comparison to the normal control. The results indicated dose and time-dependent significant diminution ($p < 0.05$) in the levels of IL-1 β, IL-6 and TNF- α after 21 days of oral administration of SBCPEE and SBCPAE in a similar way with that of indomethacin (reference drug) as compared to untreated arthritic albino Wistar rats.

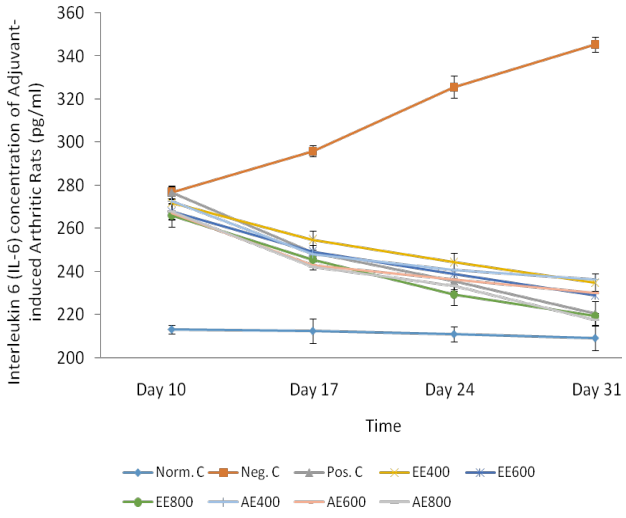
3.3 Effect of SBCPEE on MDA, NO, SOD and CAT in RA Rats

The results of the effect of SBCPEE and SBCPAE on MDA (Figure 4) and NO levels (Figure 5), SOD (Figure 6) and CAT (Figure 7) activities of adjuvant-induced arthritic albino Wistar rats indicated that exposure of the rats to the Complete Freund's adjuvant produced significant ($p< 0.05$) elevation in serum concentrations of MDA and NO while there was a significant reduction ($p< 0.05$) in the activities of SOD and CAT relative to the normal control. However, treatment with 400, 600 and 800 mg/kg of SBCPEE and SBCPAE significantly decreased ($P < 0.05$) the concentration of MDA and NO and significantly



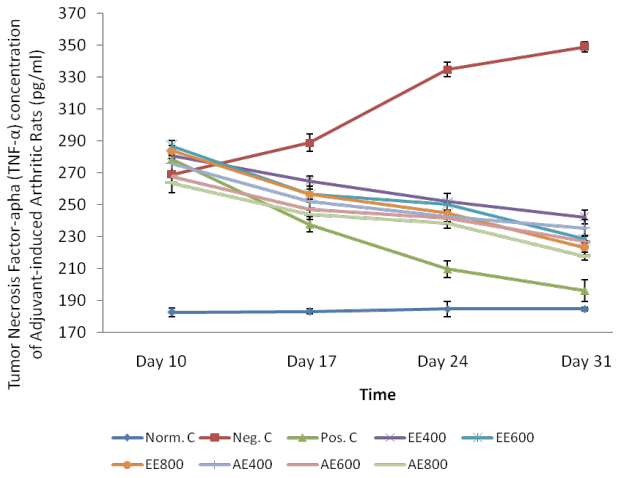
Norm. C= normal control, Neg. C= negative control, Pos. C= positive control, EE= ethanol extract, AE= aqueous extract. SBCPEE= Stem bark of *Cleistopholis patens* ethanol extract; SBCPAE= Stem bark of *Cleistopholis patens* aqueous extract; Norm.C= Normal control (rats that were not induced), Neg.C= Negative control (untreated arthritic rats); Pos. C= Positive control (arthritic rats treated with 10 mg/kg indomethacin); EE400= 400 mg/kg SBCPEE; EE600= 600 mg/kg SBCPEE; EE800= 800 mg/kg SBCPEE; AE400= 400 mg/kg SBCPAE; AE600= 600 mg/kg SBCPAE; AE800= 800 mg/kg SBCPAE.

Figure 1. Effect of SBCPEE and SBCPAE on IL-1 β concentration in adjuvant-induced arthritic rats.



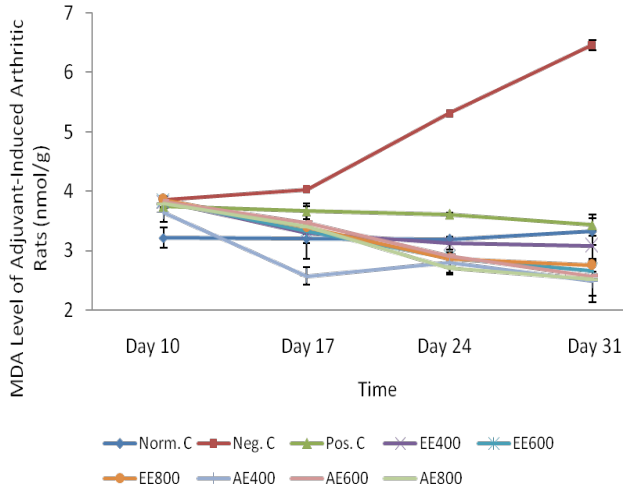
Norm.C= normal control, Neg.C= negative control, Pos. C= positive control, EE= ethanol extract, AE= aqueous extract. SBCPEE= Stem bark of *Cleistopholis patens* ethanol extract; SBCPAE= Stem bark of *Cleistopholis patens* aqueous extract; Norm.C= Normal control (rats that were not induced), Neg.C= Negative control (untreated arthritic rats); Pos. C= Positive control (arthritic rats treated with 10 mg/kg indomethacin); EE400= 400 mg/kg SBCPEE; EE600= 600 mg/kg SBCPEE; EE800= 800 mg/kg SBCPEE; AE400= 400 mg/kg SBCPAE; AE600= 600 mg/kg SBCPAE; AE800= 800 mg/kg SBCPAE.

Figure 2. Effect of SBCPEE and SBCPAE on IL-6 concentration of adjuvant-induced arthritic rats.



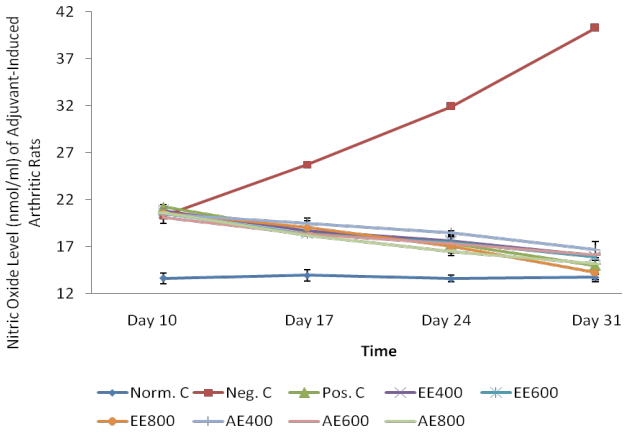
Norm.C= normal control, Neg.C= negative control, Pos. C= positive control, EE= ethanol extract, AE= aqueous extract. SBCPEE= Stem bark of *Cleistopholis patens* ethanol extract; SBCPAE= Stem bark of *Cleistopholis patens* aqueous extract; Norm. C= Normal control (rats that were not induced), Neg.C= Negative control (untreated arthritic rats); Pos.C= Positive control (arthritic rats treated with 10 mg/kg indomethacin); EE400= 400 mg/kg SBCPEE; EE600= 600 mg/kg SBCPEE; EE800= 800 mg/kg SBCPEE; AE400= 400 mg/kg SBCPAE; AE600= 600 mg/kg SBCPAE; AE800= 800 mg/kg SBCPAE.

Figure 3. Effect of SBCPEE and SBCPAE on TNF-α concentration of adjuvant-induced arthritic rats.



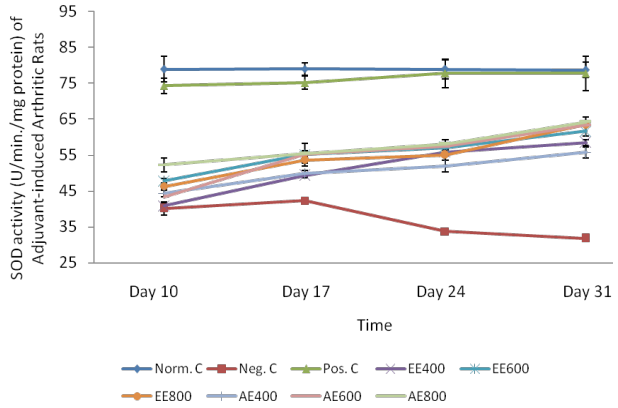
Norm.C= normal control, Neg.C= negative control, Pos. C= positive control, EE= ethanol extract, AE= aqueous extract. SBCPEE= Stem bark of *Cleistopholis patens* ethanol extract; SBCPAE= Stem bark of *Cleistopholis patens* aqueous extract; Norm. C= Normal control (rats that were not induced), Neg.C= Negative control (untreated arthritic rats); Pos.C= Positive control (arthritic rats treated with 10 mg/kg indomethacin); EE400= 400 mg/kg SBCPEE; EE600= 600 mg/kg SBCPEE; EE800= 800 mg/kg SBCPEE; AE400= 400 mg/kg SBCPAE; AE600= 600 mg/kg SBCPAE; AE800= 800 mg/kg SBCPAE.

Figure 4. Effect of SBCP Extracts on MDA Level of Arthritic Albino Wistar Rats induced with adjuvant.



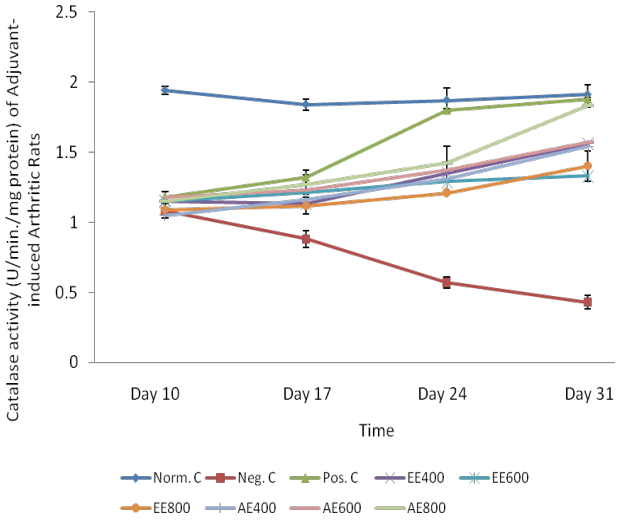
Norm.C= normal control, Neg.C= negative control, Pos. C= positive control, EE= ethanol extract, AE= aqueous extract. SBCPEE= Stem bark of *Cleistopholis patens* ethanol extract; SBCPAE= Stem bark of *Cleistopholis patens* aqueous extract; Norm.C= Normal control (rats that were not induced), Neg.C= Negative control (untreated arthritic rats); Pos. C= Positive control (arthritic rats treated with 10 mg/kg indomethacin); EE400= 400 mg/kg SBCPEE; EE600= 600 mg/kg SBCPEE; EE800= 800 mg/kg SBCPEE; AE400= 400 mg/kg SBCPAE; AE600= 600 mg/kg SBCPAE; AE800= 800 mg/kg SBCPAE.

Figure 5. Effect of SBCP extracts on NO Level of Arthritic Albino Wistar rats induced with adjuvant.



Norm.C= normal control, Neg.C= negative control, Pos. C= positive control, EE= ethanol extract, AE= aqueous extract. SBCPEE= Stem bark of *Cleistopholis patens* ethanol extract; SBCPAE= Stem bark of *Cleistopholis patens* aqueous extract; Norm.C= Normal control (rats that were not induced), Neg.C= Negative control (untreated arthritic rats); Pos. C= Positive control (arthritic rats treated with 10 mg/kg indomethacin); EE400= 400 mg/kg SBCPEE; EE600= 600 mg/kg SBCPEE; EE800= 800 mg/kg SBCPEE; AE400= 400 mg/kg SBCPAE; AE600= 600 mg/kg SBCPAE; AE800= 800 mg/kg SBCPAE.

Figure 6. Effect of SBCP extracts on SOD Activity of Arthritic Albino Wistar Rats induced with adjuvant.



Norm.C= normal control, Neg.C= negative control, Pos. C= positive control, EE= ethanol extract, AE= aqueous extract. SBCPEE= Stem bark of *Cleistopholis patens* ethanol extract; SBCPAE= Stem bark of *Cleistopholis patens* aqueous extract; Norm.C= Normal control (rats that were not induced), Neg.C= Negative control (untreated arthritic rats); Pos. C= Positive control (arthritic rats treated with 10 mg/kg indomethacin); EE400= 400 mg/kg SBCPEE; EE600= 600 mg/kg SBCPEE; EE800= 800 mg/kg SBCPEE; AE400= 400 mg/kg SBCPAE; AE600= 600 mg/kg SBCPAE; AE800= 800 mg/kg SBCPAE.

Figure 7. Effect of SBCP extracts on CAT Activity of Arthritic Albino Wistar Rats induced with adjuvant.

increased ($p < 0.05$) SOD and CAT activities. This effect was analogous to that of indomethacin, a reference drug when compared with that of the untreated arthritic rats.

4. Discussion

The results showed that SBCPEE and SBCPAE caused no death at a maximum dosage of 5000 mg/kg administered and therefore considered to be relatively safe especially for the maximum dosage used. This study equally revealed elevation of proinflammataory cytokines IL-1β, IL-6 and TNF-α in the adjuvant-induced arthritic rats compared to the normal control group. These observations could be ascribed to the stimulation of the immune system by the adjuvant as previously reported[21,22]. The study also showed that treatment with graded doses of SBCPEE and SBCPAE decreased the level of these cytokines (IL-1β, IL-6 and TNF-α) in arthritic albino Wistar rats. Flavonoid a phytochemical constituent of the plant extracts might have caused the decrease in the level of the cytokines due to its antioxidative properties[9]. Flavonoids such as rutin and quercetin have been reported to inhibit the liberation of NO, TNF-α, IL-1β, IL-6 as well as T-cells proliferation in sub chronic and chronic process of adjuvant arthritis and decrease the level of IL-1β in rat adjuvant arthritis[23,24].

Research evidence showed negative correlation in the levels of IL-1β, TNF-α, and the levels of zinc in rheumatoid arthritis patient's serum. This negative correlation has been attributed to be due to the increased concentration of zinc-containing proteins in the liver and inflamed joints prevalent in RA[25]. Osuntokun[9] reported high level of zinc in the stem bark of *C. patens*. Thus, the presence of zinc in the stem bark could have contributed to lowering the concentrations of IL-1β and TNF- α in treated RA rats. This immune-suppressant activity caused by antioxidative phytochemicals and minerals has been previously reported[26].

Oxidative stress contributes substantially to the pathologic process of rheumatoid arthritis. Uncontrolled amounts of reactive oxygen (ROS) and nitrogen (RNS) species produced by neutrophils and macrophages cause injury to joints mainly through up-regulation of matrix metalloproteinases and activation of the activities of osteoclast[27]. The release of free radicals especially reactive oxygen species and compromise in antioxidant scavenging potentials are implicated in the pathologic process of RA which essentially perpetuates tissue destruction as seen in RA[28].

Elevated MDA levels in RA albino Wistar rats observed in this study indicated raised MDA concentrations in CFA-induced oxidative stressed rats as a result of lipid peroxidation of membranes similarly reported by Rasoola and Varalakshmi[29]. This lipid peroxidation is probably caused by a disparity in the antioxidants (oxidative scavengers) and the free radical generation processes[30]. The SBCP extracts decreased the levels of MDA in RA rats and as such restored the morphology of the cell membrane probably due to its antiperoxidative effects which may also be due to the raised levels of the activities of SOD and CAT.

The elevated level of NO in RA rats in this study might be attributed to enhanced inducible nitric oxide synthase (iNOS) expression induced in the inflamed

joints as implicated in the pathologic process leading to inflammatory arthritis via increment in blood flow to the synovial joint and regulation of cellular activities within the synovial membrane and cartilage[31]. Treatment with ethanol and aqueous extracts of SBCP reduced NO levels in RA rats and this could be due to inhibition of expression of iNOS[32]. TNF-α reportedly is involved in the regulation of induction of iNOS[33] and as such, the observed effects of the extracts on TNF-α is in tandem with NO level.

Tissue injury is the aftermath of compromise in the antioxidant system which culminates in an unrestricted build up of ROS and the related metabolites[34,35]. The results showed a decrease in the activities of CAT and SOD in arthritic albino Wistar rats. The decrease in CAT and SOD activities may be linked to their ROS mediated degradation during the detoxifying process or due to the fact that they are highly utilized in scavenging free radicals[36]. Oxidative stress and RA have been reported to be associated with an increase in serum lipid peroxidation maker levels, decrease in the activities of SOD and dysfunctional antioxidant enzymes[37]. The results showed that different doses of the extract increased CAT and SOD activities in the RA-treated rats. These antioxidative and free radical scavenging potentials of the extracts may be due to the presence the phytochemical and minerals contents in the extracts.

5. Conclusion

This study indicated that SBCPEE and SBCPAE are safe at a maximum dosage not exceeding 5000 mg/kg. The study proposed that the extracts could suppress proinflammatory cytokines and reduce stress due to reactive oxygen species associated with rheumatoid arthritis. The anti-arthritic effects of these extracts were proposed to be mediated via multiple mechanisms including activities such as anti-inflammation of the joints and anti-oxidation of reactive oxygen species.

6. Acknowledgement

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8. Conflict of Interest

The authors declared no conflict of interest

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