Full Length Research Paper

Immunoregulatory activity of root bark of Cassia sieberiana D.C. in a modified adjuvant-induced arthritis in rat

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The present study seeks to evaluate the immunoregulatory effects of extracts of the root bark of Cassia sieberiana, a plant used in Ghana for various painful inflammatory conditions, in a modified adjuvant arthritis model induced by administration of very low dose of Mycobacterium tuberculosis (MT) – carrageenan mixture in the rat. A volume of 0.1 mg kg⁻¹ heat killed MT in paraffin oil was mixed with equal volume of 0.05% (w/v, normal saline) carrageenan. A single intraplantar dose of 0.1 ml of the MT-carrageenan mixture was administered to experimental animals. Groups were administered extracts (20 to 200 mg kg⁻¹, p.o.), dexamethasone (0.3 mg kg⁻¹, p.o.) or vehicle an hour prior to the test and daily from test day till the 6th day. Paw volume (ml) of the injected hind limbs were measured using a plethysmometer, while paw withdrawal thresholds were determined using an analgesy meter. Serum levels of IL-1α, IL-6, IL-10 and TNF-α were determined via enzyme linked immunosorbent assay (ELISA). Results showed that the extracts attenuated the inflammation and hyperalgesia caused by the intraplantar injection of MT-carrageenan mixture in the rats in a dose-dependent fashion. Similarly, the extracts reduced the serum levels of IL-1α, IL-6 and TNF-α while increasing the levels of IL-10. It can be concluded that the anti-inflammatory activity of extracts of root bark of C. sieberiana may be attributable to their immunomodulatory effects via suppression of pro-inflammatory cytokines, TNF-α, IL-1α and IL-6; and elevation of the anti-inflammatory cytokine, IL-10 levels, in serum.

Key words: Immunoregulatory, pro-inflammatory cytokines, TNF-α, adjuvant, C. sieberiana, IL-10.

INTRODUCTION

Cassia sieberiana is a woody shrub which grows well in tree or shrub savanna with less than 800 mm annual rainfall (Von Maydell, 1990), and it is a tropical plant which is native to Africa. The plant has many uses including food, medicine and other unspecific uses (Freedman, 2012). It has many folkloric uses including

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the root bark being used for treating swelling, gout and dropsy, and leaves used to treat rheumatism and arthritis (Obidah et al., 2009; Madusolumuo et al., 1999). Earlier, evaluations on the anti-inflammatory effects of the aqueous and ethyl acetate fractions of ethanolic extract of the root barks of C. sieberiana in the carrageenan-induced edema and formalin tests have been conducted (Donkor et al., 2013). In this follow up study, its immunoregulatory effect is studied as a possible mechanism responsible for its anti-inflammatory activity.

Inflammation is a localized protective reaction of cells/ tissues of the body to allergic or chemical irritation, injury and/or infections. It is characterized by pain, heat, redness, swelling and loss of function that result from dilation of the blood vessels leading to an increased blood supply and increased intercellular spaces resulting in the movement of leukocytes, protein and fluids into the injured regions (Parham, 2000). It is evident that the immune system is intricately linked to the etiologic and pathophysiologic mechanisms of inflammation (Chun et al., 2016). It is known that some cytokines (IL-3, IL-4, IL-10, -13) released during inflammation are beneficial by acting as anti-inflammatory mediator within the cells while pro-inflammatory mediators present pathways through which disorders in the body may be eliminated (Esch and Stefano, 2002).

Indeed studies suggest that pro-inflammatory cytokines have to be taken care of in order to completely overcome the effect of inflammatory responses (Meshram et al., 2016). Medicinal plants are rich sources of substances which are claimed to induce non-specific immunomodulatory effects (Sharififar et al., 2009).

Complete Freund’s Adjuvant (CFA)-induced arthritis is the commonest chronic arthropitc animal model. The main challenge associated with this assay is that the heat killed Mycobacterium tuberculosis used is quite expensive thus many resource limited labs are unable to acquire it. Therefore, the present study seeks to modify it with the aim of reducing the cost of carrying it out by administration of very low dose of Mycobacterium tuberculosis – carrageenan mixture in rat.

**METHODOLOGY**

**Plant collection**

The roots of C. sieberiana was obtained from the Arboretum of the Centre for Plant Medicine Research (CPMR), Mampong-Akwapim, Ghana. The barks of the root was peeled and air-dried. The plant was authenticated by the Plant Development Department (PDD) of the CPMR and a voucher specimen (CSRPM No. 315) kept in the Herbarium of the PDD of the CPMR, Mampong-Akwapim, Ghana.

**Extract preparation**

The dried root barks were crushed in a mortar, and 2.0 kg was soaked in 4 L of absolute ethanol for 3 days. The mixture was filtered, and the filtrate dried at 65°C under pressure using rotary evaporator (Eyela, N-1100, Rikikigak Co. Ltd, Tokyo, Japan). The extract was defatted using 350 ml of petroleum ether. The filtrate was mixed with 350 ml of 90% ethanol and the ethanol was evaporated under pressure at 65°C. The resultant aqueous filtrate was mixed with 250 ml distilled water. A volume of 250 ml of ethyl acetate was used to partition the residue three times, and a freeze dryer used to lyophilize the aqueous layer to dry powder; the aqueous extract was labeled as CS-Aq. The ethyl acetate was evaporated under pressure as previously described to a paste, lyophilized thereafter to dryness and labeled as ethyl acetate extract (CS-Ea).

**Animals**

Male Sprague-Dawley rats (180 to 200 g) were obtained from the Animal Unit, CPMR, Mampong-Akwapem, in the Eastern Region of Ghana. The animals were fed on feed obtained from Ghana Agro Food Company (GAFCO), Tema, Ghana. They were also allowed free access to clean water. The research was conducted in accordance with the internationally accepted principles for laboratory animal use and care (NIH, No.85 to 23, revised 1985). All protocol were approved by the Pharmacology/Toxicology Department of the CPMR with animal use authorization number (CPMR No. 12/15) kept at the repository of the department. The paper was written using the ARRIVE guidelines.

**Chemicals and drugs**

Dexamethasone and heat killed M. tuberculosis were obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA). TNF-α, IL-1α, IL-6 and IL-10 rat serum ELISA kits were obtained from Abcam Company Ltd (Cambridge, USA). All other chemicals were purchased in their purest form available from British Drug House (BDH) Ltd (Poole, UK).

**Induction of FA-carrageenan-induced arthritis**

In adjuvant-induced arthritis, heat killed M. tuberculosis in paraffin oil is normally administered at 4 to 10 mg kg⁻¹ while carrageenan is usually administered (0.1-1%, w/v) in carrageenan-induced edema assay (Pearson, 1956). In an attempt to modify CFA-induced arthritis with the aim of reducing the cost involved, a volume of 0.1 mg kg⁻¹ heat killed M. tuberculosis in paraffin oil was mixed with equal volume of 0.05% (w/v) normal saline (carrageenan). Right hind paw of rats were injected with a single intraplantar dose of 0.1 ml of the M. tuberculosis (MT) - carrageenan mixture. Groups were administered extracts (20, 100 and 200 mg kg⁻¹, p.o.), dexamethasone (0.3 mg kg⁻¹, p.o.) or vehicle an hour prior to the test, and daily from test day till the 6th day. On the day of test, paw volume (ml) of the right hind limbs were measured using a plethysmometer (7150, Ugo basile, Comerio-Varese, Italy), prior to the induction of arthritis (baseline) and thereafter readings were taken daily until the 6th day. The anti-inflammatory activity was calculated as the degree of paw edema (e) using the formula:

\[
e = \frac{E_t - E_o}{E_o} \times 100\%
\]

where, \(E_t\) and \(E_o\) are paw volume at baseline, and at a given reading day of the right hind paw.
Figure 1. (a) Effect of EA-F (20-200 mg kg\(^{-1}\), p.o.), (c) AQ-F (20-200 mg kg\(^{-1}\), p.o.) and dexamethasone, DXT (0.3 mg kg\(^{-1}\), p.o.) on the time course of MT-Carrageenan-induced arthritis in rats; (b) and (d) are AUCs determined from (a) and (c) respectively. Each point/column represents Mean±SEM (n = 5) (**p<0.01 and ***p<0.001 are compared to untreated controls).

Hyperalgesia determination

Paw withdrawal thresholds (PWTs) were determined using an analgesy-meter (7200, Ugo Basile, Comerio-Varese, Italy). The PWTs were measured prior to the induction of arthritis (baseline) and thereafter readings taken daily until the 6th day. The analgesic activity of the extracts/drug was calculated as analgesic coefficient (k) using the formula:

\[ k = \frac{a + b + c + d + e + f}{y \times 6} \times 100\% \]

Where a to f are daily PWTs up to day 6, and y is baseline PWTs.

Determination of serum levels of TNF-α, IL-α, IL-6 and IL-10

On day 6 (termination of treatment), blood samples were collected via tail bleeding, and serum prepared and stored at appropriate temperature for determination of TNF-α, IL-α, IL-6 and IL-10 levels via ELISA following the procedure of the manufacturers.

Statistical analysis

One way analysis of variance (ANOVA) and Bonferroni post-hoc tests were conducted between control and tests to determine statistical significance. Further comparisons between vehicle- and drug-treated groups were performed using the Newman - Keuls' Test. GraphPad Prism for Windows Version 5.00 (GraphPad Software, San Diego, CA, USA) was used for all graphics and statistical analyses. The 5% level of probability was used as criterion of significance in all instances.

RESULTS

FA-carrageenan-induced arthritis

The effects of the extracts and dexamethasone on MT-carrageenan-induced arthritis are represented in Figure 1. The sub-plantar injection of MT-carrageenan mixture caused an increase in paw volume of all experimental rats
with controls experiencing a sustained increase in paw volume over the 6 day study period which peaked after a day post-arthritis induction. Pretreatment with dexamethasone (0.3 mg kg\(^{-1}\), p.o.) completely reversed the edema after day 2 post-arthritis induction. Both extracts caused significant (p < 0.001) dose-dependent reduction in the percentage edema formation compared to the controls. The degree of edema inhibition over the treatment period calculated as area under the curve (AUC) for the ethanolic and aqueous fractions of the ethanolic extract (38.2 to 76.1%) was lower than dexamethasone (95.4%).

Mechanical hyperalgesia

Figure 2 shows the effects of the extract and dexamethasone on the MT-carrageenan-induced mechanical hyperalgesia following the sub-plantar injection of MT-carrageenan mixture. The ipsilateral paw showed marked hyperalgesia in all experimental rats after a day of injection of the MT-carrageenan which was reduced significantly in both extracts and reference drug (p < 0.0001) in a dose-dependent fashion compared to controls.

Involvement of TNF-\(\alpha\)

The effects of the extracts (20 to 200 mg kg\(^{-1}\), p.o) and dexamethasone (0.3 mg kg\(^{-1}\), p.o) on the serum levels of TNF-\(\alpha\) in MT-carrageenan-induced arthritis in the rat after 6 days are presented in Figure 3. Intraplantar injection of adjuvant caused an elevation in the serum TNF-\(\alpha\) levels of rats which was attenuated by extracts and dexamethasone. Pretreatment with extracts significantly reduced serum TNF-\(\alpha\) levels in both extracts-treated animals (p<0.0001) in a dose-related manner compared to controls.

Involvement of IL-1\(\alpha\) and IL-6

The effects of the extracts (20 to 200 mg kg\(^{-1}\), p.o) and dexamethasone (0.3 mg kg\(^{-1}\), p.o) on the serum levels of IL-1\(\alpha\) and IL-6 in MT-carrageenan-induced arthritis in the rat after 6 days are presented in Figures 4 and 5. Intraplantar injection of adjuvant caused an elevation in the serum IL-1\(\alpha\) and IL-6 levels of rats which were attenuated by extracts and dexamethasone. Pretreatment with extracts significantly reduced serum IL-1\(\alpha\) levels in both extracts (p < 0.0001) in a dose-dependent manner compared to controls. Similarly, serum IL-6 levels were gaudily reduced in both extracts-treatment (p < 0.0001) rats in a dose-related manner compared to controls.

Involvement of IL-10

The effects of the extracts (20 to 200 mg kg\(^{-1}\), p.o) and dexamethasone (0.3 mg kg\(^{-1}\), p.o) on the serum levels of IL-10 in MT-carrageenan-induced arthritis in the rat after 6 days are presented in Figure 6. Intraplantar injection of adjuvant caused a reduction in the serum IL-10 levels of rats. Pretreatment with extracts and dexamethasone significantly increased serum IL-10 levels in both extracts (p < 0.0001), in a dose-dependent manner compared to controls.

Figure 2. (a) Effect of Ea-F (20-200 mg kg\(^{-1}\), p.o.), (b) AQ-F (20-200 mg kg\(^{-1}\), p.o.) and dexamethasone, DXT (0.3 mg kg\(^{-1}\), p.o.) on M. tuberculosis carrageenan-induced hyperalgesia in rats. Values are Mean±SEM (n = 5) (**p<0.01 and ***p<0.001 compared to untreated controls).
DISCUSSION

It has been suggested that all pain, whether acute or chronic, peripheral or central, originates from inflammation and the inflammatory responses (Omoigui, 2007). Thus, the treatment of inflammation is a major clinical concern. We had earlier on established the anti-inflammatory effects of the root bark of C. sieberiana in both carrageenan-induced edema and formalin tests (Donkor et al., 2013).

A preliminary fractionation process of the crude ethanolic extract of root bark of the plant revealed that about 70% of the extract was in the ethyl acetate fraction. The root bark of the plant is traditionally boiled in water and drank. Thus, the present study sought to evaluate the immunoregulatory activity of ethyl acetate and aqueous fractions of ethanolic extract of the root bark of the plant as a possible mechanism for its anti-inflammatory properties. In this study, we have successfully induced arthritis using a single administration of very low dose mixture of Freud’s adjuvant and carrageenan. The edema and hyperalgesia caused by the induction of arthritis was attenuated by both fractions of the extract. The extracts also significantly reduced serum levels of pro-inflammatory cytokines, TNF-α, IL-1α and IL-6, while elevating levels of the anti-inflammatory cytokine IL-10.

A single intraplantar injection of 0.1 ml of M.

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**Figure 3.** (a) Effect of EA-F (20-200 mg kg⁻¹, p.o.), (b) AQ-F (20-200 mg kg⁻¹, p.o.) and dexamethasone, DXT (0.3 mg kg⁻¹, p.o.) on serum TNF-α levels in MT-Carrageenan-induced arthritis in the rats after 6 days of treatment. Values are Mean±SEM (n = 5). *p<0.05, **p<0.01, and ***p<0.001 compared to untreated controls.

**Figure 4.** (a) Effect of EA-F (20-200 mg kg⁻¹, p.o.), (b) AQ-F (20-200 mg kg⁻¹, p.o.) and dexamethasone, DXT (0.3 mg kg⁻¹, p.o.) on serum IL-1α levels in MT-carrageenan-induced arthritis in the rats after 6 days of treatment. Values are Mean±SEM (n = 5). **p<0.01, and ***p<0.001 are compared to untreated controls.
tuberculosis - carrageenan mixture induced inflammation in the rat which is identical in appearance to the arthritis induced by CFA. Like the arthritis induced by CFA, this one is also irreversible naturally saved by pharmacological intervention. Carrageenan-induced edema and the arthritis induced by Freud’s adjuvant are known to be mediated by histamine, serotonin and kinin at the early phases, and they are sustained by prostaglandins and cytokines released from infiltrated leukocytes (Phillippe et al., 1997; Maleki et al., 2001; Okada et al., 2014).

Though the chemical nature of the current model of chronic inflammation is not yet characterized, it is strongly believed that the inflammatory mediators would be similar to that of carrageenan-induced inflammation and CFA-induced arthritis. The advantage of this model is that the reagents used in inducing the inflammation are low in concentration thus more tolerable and humane to the subjects. It would also be useful in a resource limited facility in terms of cost reduction.

It has been reported that infiltrated neutrophils-released prostaglandin E2 directly sensitizes mechanical nociceptors to produce hypernociception in carrageenan assay (Furst and Emery, 2014). It has also been shown that the inflammation causes a lowering of the thresholds of various mechanoreceptors and mechanotransduction pathways (Park et al., 2008; Liu et al., 2014).

Similarly, the production of pro-inflammatory cell-mediated cytokines such as IL-1α, IL-6 and TNF-α are considered as the main reason for hyperalgesia and
alldynia induction during acute and chronic inflammation situations (Lipsky, 2006; Chun et al., 2016). The ethyl acetate and aqueous fractions of the ethanolic extract together with dexamethasone significantly reduced the hyperalgesia caused by the injection of the *M. tuberculosis*-carrageenan mixture suggesting interference in the production and/or release of the associated inflammation mediators peripherally.

In the mechanical hyperalgesia test, it has been suggested that the stimulus applied is likely to activate slowly-adapting mechanoreceptors with decreased thresholds, which are predominantly C-fiber located in the cutaneous and subcutaneous structures that would have required greater stimulus intensities for activation (Birder and Perl, 1994; Lewin and Moshourab, 2004; Abdelwahab et al., 2013). Dexamethasone is also known to attenuate hyperalgesia by decreasing transmission of impulses in C-fibers (Shariffar et al., 2009). Thus, the extracts may have acted via a similar mechanism.

Daily administration of the ethyl acetate and aqueous fractions of the ethanolic extract and dexamethasone for 6 days attenuated the inflammation induced in the assay with dexamethasone completely reversing the inflammation within three days. Dexamethasone is known to inhibit leukocyte infiltration at the site of inflammation resulting in decreased release of bradykinin, TNF-α, IL-1, IL-2 and IL-6 (Zhang et al., 2014). Its action is also thought to involve phospholipase A2 inhibitory proteins, lipocortins, which control the biosynthesis of potent mediators of inflammation such as prostaglandins and leukotriene (Tsurufuji et al., 1984; Huang et al., 2013). Thus, like dexamethasone, the anti-inflammatory activity of the extracts may be as a result of decrease in the production of these mediators. This assertion was investigated by measuring serum levels of TNF-α, IL-1, IL-6 and IL-10 in the current assay at termination of treatment.

IL-1α, which activates the innate and adaptive immune responses, stimulates the production of INFγ by T lymphocytes (Arend, 2002; Chun et al., 2016). INFγ can induce IL-1α expression and enhance the cytotoxic action of TNF-α (Banno et al., 2004; Chun et al., 2016). TNF-α is an important cytokine involved in systemic inflammation, such as the one induced via adjuvant and acute phase response (Billiau, 1996; Jubri et al., 2013). It is released by white blood cells, endothelium and several other tissues in the course of damage and its release is stimulated by several mediators including IL-1α (Locksley et al., 2001).

IL-6 is a pro-inflammatory cytokine secreted by T cells and macrophages to stimulate the immune response to trauma and other tissue damage leading to inflammation (Hennes et al., 1996; Liu et al., 2014). Deregulation of IL-6 expression results in the synthesis and release of many inflammatory mediators which cause pain and edema, and it is a main factor for nociceptor excitation (Al-Hindawi et al., 1989; Yoshimura et al., 2009; Zhang et al., 2014). It has been shown that stimulation of IL-6 receptors on the afferent fibers of nociceptors can cause hyperalgesia during inflammation.

Elevation in serum levels of IL-6 increases the amount of secretion of some neurotransmitters such as substance P and calcitonin gene-related peptide through making an effect on β subunit of its receptors in nociceptors (Ruzek et al., 1997; Herder et al., 2013). The extracts of *C. sieberiana* decreased serum IL-6 as well as TNF-α and IL-1α during which was aligned with hyperalgesia and edema reduction during the period of the study suggesting that the anti-inflammatory activity of the extracts may be due to their immunosuppressive effects via reduction in the synthesis of these pro-inflammatory cytokines.

IL-10, also known as human cytokine synthesis inhibitory factor, is an anti-inflammatory cytokine. Previous studies have suggested the function of IL-10 as an essential immunoregulator in the intestinal tract and, indeed, patients with Crohn's disease react favorably towards treatment with recombinant interleukin-10 - producing bacteria, demonstrating the importance of IL-10 for counteracting the hyperactive immune response (Braat et al., 2006). IL-10 is capable of inhibiting synthesis of pro-inflammatory cytokines such as IFN-γ, IL-2, IL-3, TNF-α and GM-CSF made by cells such as macrophages and regulatory T-cells (Chun et al., 2016). The extracts elevated serum IL-10 confirming their immunoregulatory effects.

In all the assays carried out in this study, the ethyl acetate fraction had a slightly higher activity save in the TNF-α but this is not statistically significant. In our earlier study, we showed that both fractions have the same phytochemical constituents, namely saponins, flavonoids, anthraquinones and phenolic compounds (Donkor et al., 2013). Thus, it is not surprising that there were no significant difference in the activity of both fraction, and it is possible that these secondary metabolites found in the fractions may be implicated in the immunoregulatory activity of the extract.

**Conclusion**

Intraplantar injection of a mixture of low concentrations of heat killed *M. tuberculosis* and carrageenan successfully induce arthritis in the rat which is reversible via pharmacological intervention but not naturally. The anti-inflammatory activity of extracts of root bark of *C. sieberiana* may be attributable to their immunoregulatory effects via suppression of pro-inflammatory cytokines, TNF-α, IL-1α and IL-6; and elevation of the anti-inflammatory cytokine, IL-10 levels in serum.

**CONFLICT OF INTERESTS**

The authors have not declared any conflict of interests.
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