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BRIEF REPORT

In vivo anti-inflammatory activity of β-caryophyllene, evaluated by molecular imaging

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We have recently reported that β-caryophyllene (BCP) can suppress metastasis of colon cancer, induce apoptosis, and inhibit a broad spectrum of microorganisms. Here, we report anti-inflammatory effect of BCP in the carrageenan-induced edema paw model, supported by molecular imaging using fluorescence molecular tomography (FMT). A significant ($p<0.01$) reduction in paw volumes, and low intensity of fluorescent signal was noted in experimental animals when compared with negative control. Noteworthy, the low toxicity of BCP and its high ability of skin penetration attributed to the strong anti-inflammatory and analgesic properties. Intriguingly, BCP is a natural component of many volatile plants, spices,foods and major constituent of *Cannabis*, which is recognized as natural selective agonist to the CB2 receptor. Collectively, the result of present study illustrate that the anti-inflammatory properties of BCP could be beneficial for prevention and management inflammation-related diseases.

Keywords: β-caryophyllene; FMT; anti-inflammatory

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Introduction

From the early stage of civilization, natural products have been the major source of most biologically active molecules. The first reference of natural product based- plant use date back to 4000 years ago, depicted on a Sumerian clay table with list of most valuable plants for various diseases [1]. Recently, the Dictionary of Natural Product has listed about 200,000 plant secondary metabolites with a broad spectrum of biodiversity and high degree of stereochemistry [2]. However, it is estimated that only 0.5% of medicinal plant have been chemically investigated thus far, leaving an abundant source for further examination [3]. The genus of Aquilaria (Thymelaeceae) particularly species of *Aquilaria crassna* are identified as a convenient and useful source of bioactive components such as phenolic, flavonoids and benzophenones, xanthones and Sesquiterpenes [4, 5]. Earlier communications from this laboratory reported the extraction and purification of β-caryophyllene from the essential oils of *Aquilaria crassna*. The biological studies on *A.crassna* and its active principle possess potent anticancer activity against colorectal carcinoma cells (HCT 116) and pancreatic cancer cells (MIA PaCa-2) which mediated via apoptotic mechanism [6-8]. It appears that the Privileged structure and wide range of biological activities of some natural products has increased the interest in drug discovery campaigns. In general, β-caryophyllene (BCP) is a common sesquiterpene component, which present in enormous essential oils plant, vegetable species, fruits and medicinal herbs, and has been used as food flavoring additive [9]. Several studies have been ascribed the biological activities of BCP. In particular, it seems to possess antioxidant capacity [9], antileishmanial

activity [10]. Induce apoptosis in tumor cells [11], displayed cytotoxicity effect against cancer cells [12]. Furthermore, BCP was identified as a major constituent (35%) in the essential oil of *Cannabis sativa* L, which makes BCP an important dietary cannabinoid with high selectivity binding to cannabinoid receptor2(CB2) [13]. Recent studies have suggested that CB2 receptor play key roles in the modulation of inflammatory and neuropathic pain responses [15]. Consequently, oral administration of BCP significantly reduced spinal neuroinflammation in animal models [14], and improves colitis via activation of CB2 receptor [15].

It is now becoming clear that cancer and inflammation are closely integrated processes. In some type of cancer, inflammation can promote initiation step of cancer cells , and cancer can trigger inflammation by several mediator and inhibitor factors including vascular endothelial growth factor (VEGF), matrix metalloproteinases (MMPs), tumor necrosis factor-a (TNF-a) and cyclo-oxygenase-2 (COX-2). These molecular events are strongly implicated in the regulatory network process of tumor microenvironment and inflammation, targeting these pathways with new target molecules may lead to improved diagnosis and treatment [16, 17].

In view of multi-functional properties of β-caryophyllene, the present study was design to screen the anti-inflammatory activity of BCP on acute inflammation using *in vivo* molecular imaging fluorescence molecular tomography (FMT).

Materials and Methods

Chemicals and reagents

Carrageenan and diclofenac sodium were procured from Sigma Aldrich (St. Gallen, Switzerland). MMPSense 680 Fluorescent Imaging Agent was obtained from perkin elmer (USA). Chloroform, n-hexane and methanol were obtained from R & M Marketing (Essex, UK).

Isolation and characterization of β-caryophyllene

As described in our previous study [7]. β-caryophyllene was purified from *Aquilaria crassna* essential oils as a brown-colored crystalline compound which was washed with *n*-hexane and recrystallized from hot methanol to produce colorless β-caryophyllene (0.4 g, 0.2%)[7].. The structure of (BCP) was elucidated using FTIR, 1H- and 13C-NMR and GC-MS spectral studies.

Experimental Animals

Swiss mice (25-30 g) and Sprague Dawley(SD) rats of either sex weighing (150-200 g) were collected from the animal unit facility of University Science Malaysia (USM). All the experimental procedures were performed in in accordance with the guidelines of USM Animal Ethical committee with approval number (USM/PPSF/50 (084) Jld.2). The animals were provided free access to water and food in well ventilated cages at 12 h dark/light cycle

In vivo acute anti-inflammatory assay

The acute anti-inflammatory effect of BCP was measured by carrageenan-induced rat hind paw edema model [18, 19] with minor modifications. Animals were divided into five groups, each group contains six rats o (n = 6). First group received 0.1 ml of distilled water as negative control; second group received 5 mg/kg of the reference drug indomethacin as positive control. Whereas, the treated groups(Third, fourth, and fifth) received oral treatment with 200, 100, and 50 mg/kg body weight of BCP, respectively. One hour after administration of the regimens by oral gavage, 0.1 mL of 1% freshly prepared carrageenan in normal saline was injected into the sub-plantar region of the right hind paw. plethysmometer (Ugo-Italy) was used to measure the thickness of the right hind paw of rats at times 0, 1, 2, 3, 4 and 5 h after carrageenan administration. The percentage of inflammation was calculated using following formula: % inflammation = $(A - B) / B \times 100$

Where A is paw thickness 3 h after carrageenan-induced edema and B is paw thickness before carrageenan-induced edema.

Fluorescence molecular tomography

Acute in vivo inflammation model using carrageenan-induced mice was used to detect and quantify fluorescent agents in BCP-treated and non-treated groups of mice. The animals were injected subcutaneously with 0.03 ml of carrageenan into the shaved region of the dorsal side. Diclofenac sodium (5 mg/kg) was used as the positive control BCP at 200 and 100 mg/kg were administered one hour before carrageenan injection. After 4 h, 100 µL of MMPSenes Perkin Elmer fluorescent imaging probe were injected into the tail vein of each mouse. After 24 h, the animals were anaesthetized using Phenobarbital. Each mouse was placed in the portable animal imaging cassette of the FMT and imaged using a Molecular Light Imager (Berthold Technologies) for 10 ms using a HQ 470 excitation filter (Chroma), HQ 525 emission filter (Chroma), and the WinLight32 software supplied with the instrument [20]. Imaging was then performed using a two-step process and the WinLight32 software. First, a black and white photographic image was acquired using a 15 ms exposure.

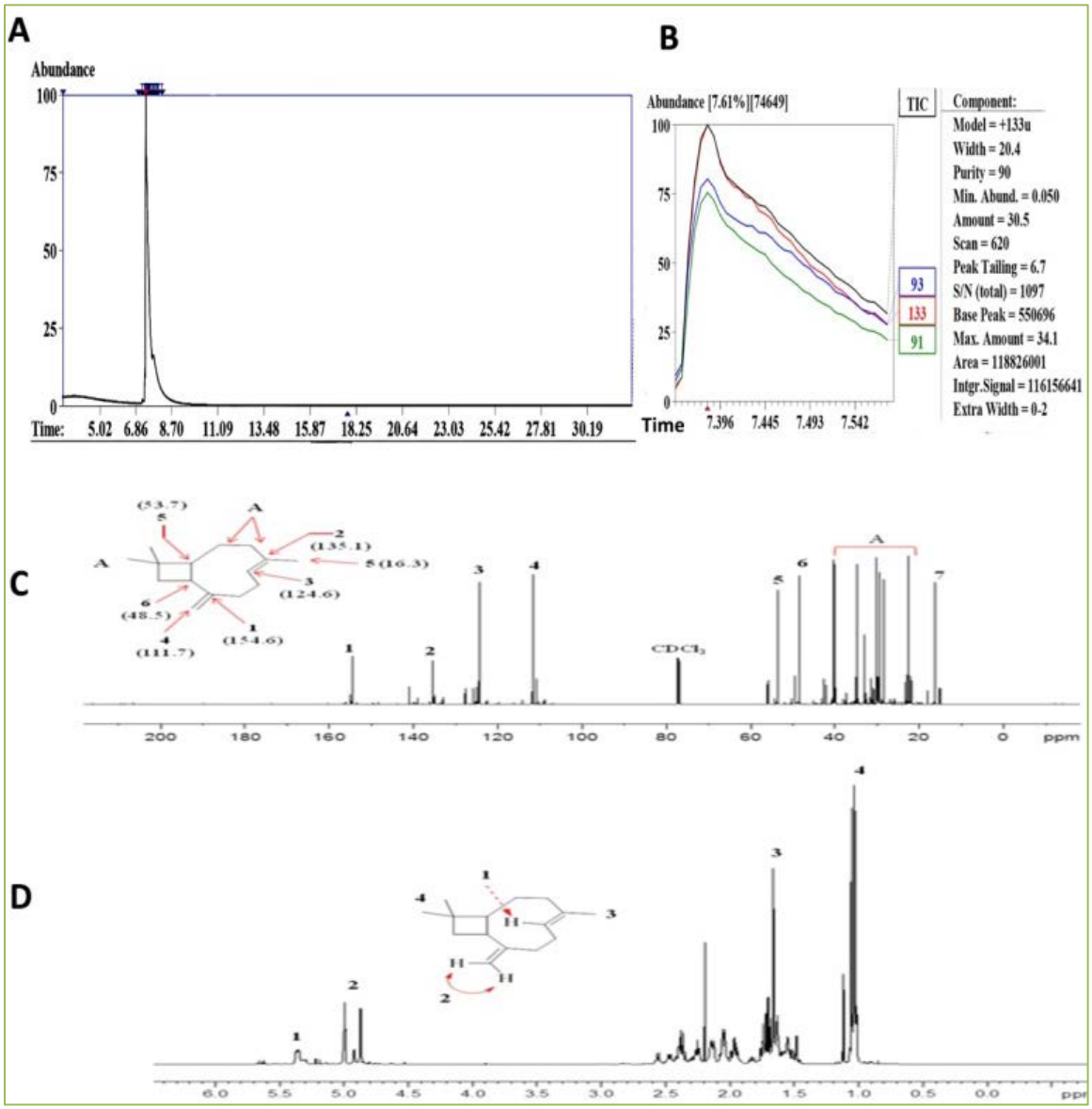

Figure 1. Fingerprints of β-caryophyllene. (A) The main peak corresponds to the isolated component identified as BCP, purified from the essential oil of Agarwood.(B) TLC fingerprint of BCP. (C) Chemical structure of β-caryophyllene illustrating the ^{13}C assignments obtained fro^{13}C-NMR spectrum. (D) Chemical structure of BCP depicting the ^{1}H-NMR assignments.

Next, the fluorescent image was acquired using a 5 min photon integration period with background subtraction. The fluorescent image was processed to colorize the fluorescence intensity, and then it was overlaid onto the black and white image for presentation. Quantification of the images was conducted, and the data were used to characterize the extent of the anti-inflammatory effect and protection provided by BCP against the carrageenan-induced mice [20].

Statistical analysis

The data obtained was statistically analyzed using one-way ANOVA. This was followed by Dunnett's or Tukey's posthoc tests when the ANOVA produced significant results. All data were expressed as the mean±SEM. Statistical differences were considered to be significant at $p<0.0$ Statistical differences were considered to be significant at $p<0.05$

Result

Characterization of β-caryophyllene

As shown in Figure 1. The chemical structure β-caryophyllene was elucidated using spectroscopic data. Analysis in TLC indicates BCP as the main constituents of

Table 1. Effect of BCP on carrageenan induced paw edema in rats

Groups mg/kg	Changing in paw edema(ml)									
	1h		2 h		3 h		4 h		5 h	
	PV	%IPV	PV	%IPV	PV	%IPV	PV	%IPV	PV	%IPV
Vehicle DW	0.621±0.05	-	0.723±0.04	-	0.722±0.02	-	0.711±00.4	-	0.615±0.03	-
BCP 200	0.60±0.02	-	0.792±0.02	-	0.592±0.03	25.3	0.300±0.02	53.2*	0.184±0.02	87.6**
BCP 100	0.622±0.03	-	0.701±0.02	-	0.512±0.04	17.6	0.485±0.03	41.5*	0.211±0.02	71.4**
BCP50	0.614±0.03	-	0.70±0.03	-	0.600±0.03	12.8	0.467±0.02	30.9*	0.222±0.03	56.2**
Indomethacin 10	0.618±0.02	-	0.7 20±0.01	-	0.516±0.01	23.4	0.400±0.02	45.5*	0.224±0.02	75.5**

Values are presented in Mean ± S.E.M (n=6); *p<0.05 and **p<0.01 when compared with Control using One way ANOVA. DW= Distilled Water PV= Paw Volume %IPV= Percentage Inhibition Paw Volume

Aquilaria crassna essential oils with retention time 7.38 min, peak aria 8.111%, molecular weight 204, molecular formula $C_{15}H_{24}$ and 90% purity. Further analyzed for BCP was carried out using ^{1}H- and ^{13}C-NMRspectroscopic technique (Fig 1 C-D).

Inhibitory effect of BCP on carrageenan-induced paw edema in rats

The carrageenan-induced rat hind paw edema model was used to assess the anti-inflammatory effect of BCP in comparison with the standard reference drug indomethacin as given in Table 1. Sub plantar injection of carrageenan results has revealed continuous increasing in paw edema in vehicle group which received distil water only, whereas Inflammation was drastically reduced in the treated groups. The percent inflammation values of treatment with BCP (200, 100 and 50 mg/kg) were 87.6 %, 71.4% and 56.2%, respectively. Indomethacin treatment resulted in 75.5% % inhibitions at 10 mg/kg.

FMT

Carrageenan is a sulphated polysaccharide which has been used wildly to induce inflammation in animal model. In the present study, FMT system was used to quantify the carrageenan which is the specific aspects of acute inflammation. The FMT scanning analysis showed significantly high intensity of fluorescent signal in the negative control animals bearing acute inflammation induced by the carrageenan. Whereas, treatment with BCP showed a dose-dependent reduction of the fluorescence intensity. These results can be compared with the standard reference drug, diclofenac sodium (Figure 2).

Discussion

Inflammation process is the cornerstone of many physiological and pathological diseases such as atherosclerosis, allergy, rheumatoid arthritis, diabetes, neurodegeneration and cancer [21]. Natural products are widely used as a folk remedy to treat inflammation; several *in vitro* and *in vivo* studies have reported the anti-inflammatory properties of curcumin, cucurbitacins, parthenolide and cineole as bioactive molecules isolated from different plants [22]. Although, an increasing interest among the scientific community have supported the notion of multi-target natural products for developing anti-inflammatory multifaceted approach [23]. It is also important to understand that the Inflammation is a complex biological process, which consists a broad spectrum of mediators, regulated by multiple signaling pathways and connected with a wide range of cellular responses [23].

β-caryophyllene is a natural volatile bi-cyclic sesquiterpene that occurs in essential oils from several plants such as cinnamon, clove oil, black pepper, oregano and copaiba balsam, which have been utilized for different medicinal purposes [9, 13]. Due to the spicy odour and low toxicity of BCP, it has been approved by the US Food and Drug Administration as food additive and flavouring agent [24]. Although, BCP is the first natural CB2 receptor agonist, which could orally attenuated thermal hyperalgesia, mechanical allodynia, and reduced inflammatory responses in different animal models [14]. Beside many pharmacological properties, BCP has shown to have skin penetration enhancing activities [25].

Thus, on the base of multi-target properties of BCP, we evaluated the anti-inflammatory effect of BCP by *in vivo* experimental system using carrageenan-induced rat paw edema model and molecular imaging using FMT. It is well established that the inflammatory model of rat paw edema induced by carrageenan have been extensively used as a suitable and accessible method to screen and develop anti-inflammatory agents [26]. In this study, the degree of swelling of carrageenan-injected rat paws was reached the maximum at the 2nd h after injection. BCP significantly inhibited the development of edema at the 4th and the 5th h after treatment ($P < 0.01$). Furthermore, in order to gain close insight into the anti-inflammatory effect of BCP, FMT has been used to assess the MMP fluorescent imaging agent in carrageenan induced mice. This technique offers non-invasive, complete body, deep tissue imaging in small animal models, and in this study it generated three-dimensional information-rich results to evaluate the efficacy of BCP against acute inflammation

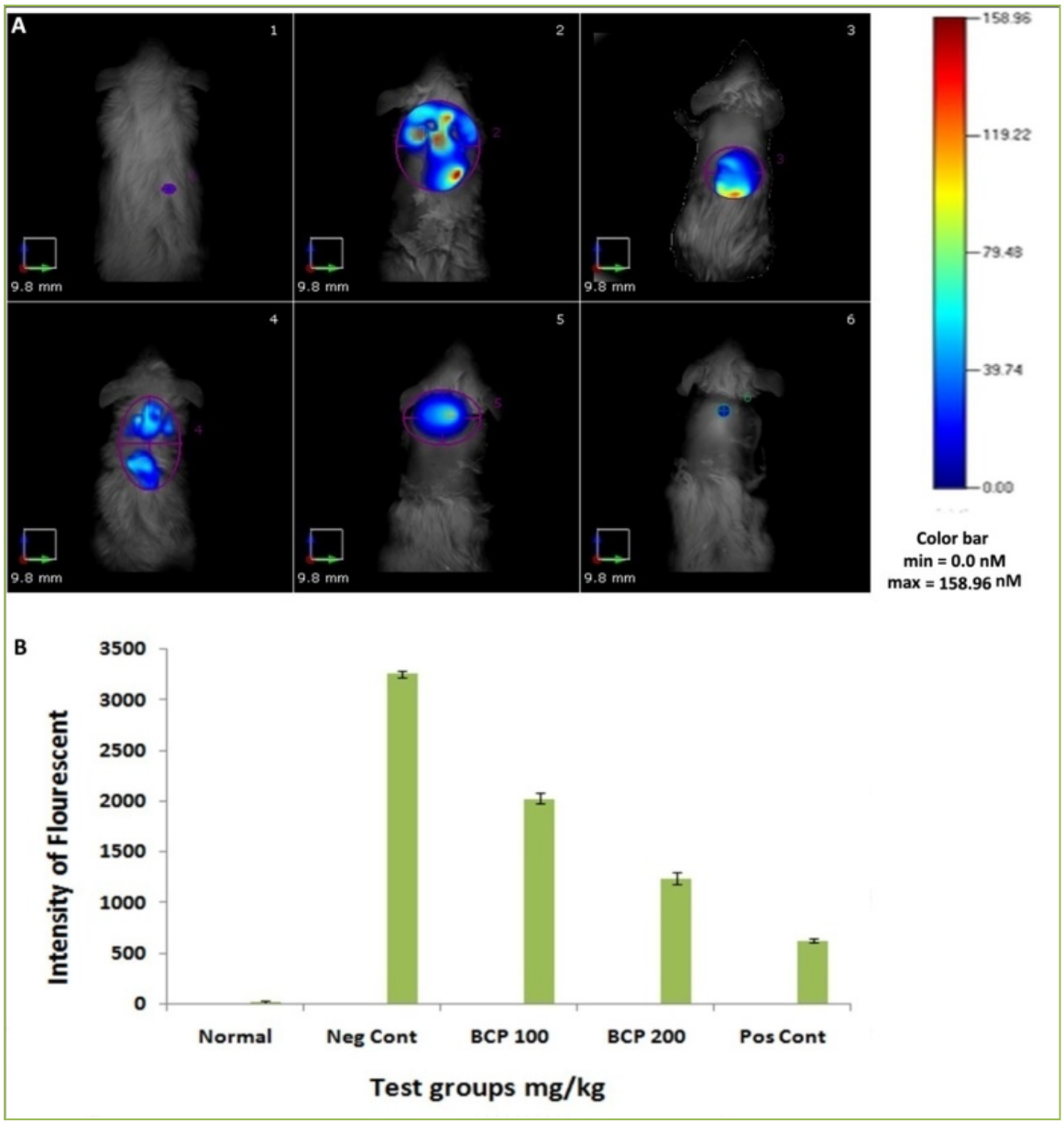

Figure 2: FMT tomographic imaging to assess acute inflammation disease using MMTSense fluorescent imaging agent. (A) (1 & 6) Tomographic images of normal control without carrageenan treatment. (2) The fluorescent signal of the negative control is represented as a maximum intensity projection. (3) Result for the group treated with the low concentration (100 mg/kg) of BCP. (4) Results for the group treated with the high concentration (200 mg/kg) of BCP. (5) Result for the group treated with the positive control, diclofenac sodium (10 mg/kg). (6) Normal control without carrageenan treatment. (B) Quantifications of signal fluorescence for each of the two BCP doses and the positive and negative controls.

[27]. The advantage of using FMT over other method, it provides a deep understanding of the mechanism and progression of diseases and it supports results from other models. It produced accurate readouts regarding the promotion and progression of the disease, and that it may prove possible to apply FMT data to indicate disease development. In the present study, MMPSenes is activated by the key disease associated proteases, FMT system was applied to quantify the carrageenan to study disease progression and therapeutic response in animal models of acute inflammation. FMT results were consistent with carrageenan-induced rat paw edema results, where BCP inhibited acute inflammation in a dose-dependent manner.

Conclusion

The results of the present study reported that BCP is a potent natural anti-inflammatory agent. Since BCP is available naturally in severals plants, spices and foods, it could be one of richest source of biologically-active

molecules with potential impact on human health. Further studies are required to consolidate the multi-functional activities of BCP as anticancer and anti-inflammatory agents.

Conflicting Interests

The authors declare that they have no conflicting interests.

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