

## ISOLATION FROM *CANNABIS SATIVA* L. OF CANNFLAVIN—A NOVEL INHIBITOR OF PROSTAGLANDIN PRODUCTION

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**Abstract**—The isolation from *Cannabis sativa* L. of an inhibitor of prostaglandin (PG) E<sub>2</sub> production by cultured rheumatoid synovial cells is described. This agent, for which the name Cannflavin has been coined, is distinct from cannabinoids on the basis of isolation procedure, preliminary structural analysis and biological properties. The activity of Cannflavin has been compared with several established anti-inflammatory drugs and the major cannabinoids.

Both analgesic and anti-inflammatory properties have been attributed to extracts of the cannabis herb [1]. However, testing these activities in animal models using pure tetrahydrocannabinol (THC) has given equivocal results [2]. Several investigators have attempted to link the mode of action of THC in producing central effects with modulation of prostaglandin levels [3]. Prostaglandins are also believed to be important mediators of vasodilatation, oedema and hyperalgesia associated with inflammation [4]. Therefore it is possible that the reported analgesic and anti-inflammatory effects of cannabis constituents may be exerted via inhibition of prostaglandin biosynthesis. In a preliminary report, we showed that a THC-free extract of cannabis inhibited PGE<sub>2</sub> production by cultured human rheumatoid synovial cells in response to the proinflammatory agent 12-O-tetradecanoylphorbol-13-acetate (TPA) [5]. This work followed from the demonstration by Fairbairn and Pickens that cannabinoid-free extracts of cannabis and cyclo-oxygenase inhibitors could reduce the cataleptic effect of THC on mice [6]. The effect of cannabinoids on prostaglandin production *in vitro* has been studied by Burstein and coworkers who found both stimulation of prostaglandin release from various types of cultured cells and inhibition of prostaglandin biosynthesis by seminal microsomal enzymes [7].

In this communication we describe the isolation of a previously undescribed component of the cannabis herb, its separation from the major cannabinoids and a comparison of its activity with both the cannabinoids and established anti-inflammatory drugs.

### MATERIALS AND METHODS

**Synovial cell assay.** Plastic-adherent synovial cells were isolated by proteolytic dispersal essentially as described by Dayer *et al.* [8]. Synovial tissue was

obtained from rheumatoid arthritis patients undergoing synovectomy. Within 3 hr of surgery the tissue was immersed in Dulbecco's modified Eagle's medium (DMEM) containing 20 mM Hepes buffer, pH 7.3, dissected to remove fat and cartilage, diced and placed in 10 ml collagenase, 5 mg/ml (Type I from *Clostridium histolyticum*, Sigma Chemical Co., St. Louis, MO). The tissue was digested for 3 hr at 37° with mixing every ½ hr before 10 ml of trypsin-EDTA (0.5 g trypsin, 1:250 and 2.0 g of EDTA/titre modified Puck's saline A) was added for a final hour of incubation with mixing every ½ hr. The isolated cells were washed several times by centrifuging at 700g for 15 min at room temperature, before culturing in DMEM-containing antibiotics (penicillin 100 units/ml and streptomycin 100 µg/ml) and supplemented with 10% v/v heat-inactivated (56° for 30 min) foetal calf serum. The cells, grown to confluence in 75 cm<sup>2</sup> tissue culture flasks (Falcon) were subcultured by incubating for 10–15 min in trypsin-EDTA and washed as above. In the second and subsequent subcultures the spontaneous PGE<sub>2</sub> production of the cells was low and these cells were grown to confluence in 24-well (2 cm<sup>2</sup>) Linbro plates. Aliquots of drugs either dissolved in media or alcohol (final concentration 2% by volume, which had no effect on cell viability or PGE<sub>2</sub> production) were added to the wells maintaining a final volume of 0.5 ml. For inhibition assays, the cells were stimulated to release PGE<sub>2</sub> with TPA, 10 ng/ml. Following incubations at 37° in 5% CO<sub>2</sub>, 95% air-humidified atmosphere for 48 hr, the PGE<sub>2</sub> content of supernatant fluids was measured by direct radioimmune assay [9]. Crossreactivity of the antisera has been previously reported by Jose *et al.* [9]. The results of inhibition assays were quoted as IC<sub>50</sub> values, i.e. the drug concentration required for 50% inhibition of PGE<sub>2</sub> release.

**Plants.** *Cannabis sativa* L. seeds originating from South Africa (UNC 335) and Turkey (UNC 354) were supplied by the United Nations Council for Drug Abuse, Geneva. They were germinated in a

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greenhouse using standard horticultural techniques and planted out in local botanical gardens during May of years 1980, 1981 and 1982. Plants were harvested at maturity in the autumn, air-dried and stored in the dark. Leaf material was separated from the large stems and crushed by hand before passing through a No. 10 metal sieve to remove small stems and seeds.

**Extraction of cannabis herb.** The powdered plant material was repeatedly macerated with petroleum spirit (40–60°) until the solvent remained colourless and tested negative with Fast Blue Salt B. The resultant marc was air dried and then repeatedly macerated in 100% ethanol until the solvent remained colourless. The ethanolic tincture was concentrated using a rotary evaporator at 40° and tested for the presence of cannabinoids by analytical TLC on silica gel G developed with toluene and visualized with Fast Blue Salt B. The extract was assayed on synovial cells in serial 10-fold dilutions in ethanol (2% v/v).

**Isolation.** The biologically active component of the extract was isolated using standard preparative TLC techniques on silica gel G. Extracts dissolved in a minimum of ethanol were applied as a streak to the base of the 0.5 mm, 20 × 20 cm or 20 × 40 cm preparative plates and developed in a solvent mixture (1 below) over a distance of 15 cm. The plates were then divided into 1-cm horizontal bands and the silica in each band eluted separately with ethanol. The fractions were evaporated to dryness, weighed and tested at 2 or 3 serial 10-fold dilutions for inhibition of PGE<sub>2</sub> release. Eluates demonstrating maximum activity were pooled and further purified by preparative TLC. Four consecutive solvent systems were used in the following order: (1) Chloroform: ethanol 100:15; (2) cyclohexane: butanone: ethanol 70:25:5; (3) heptane: chloroform: ethanol 50:50:15; and (4) chloroform: toluene: ethyl acetate 35:35:30.

**Cell viability—lactate dehydrogenase assay.** The presence of lactate dehydrogenase in the cells and the surrounding supernatant was tested with and according to the procedure of Sigma diagnostic kit No. 340-UV. Supernatants were removed for assay as for PGs and cells were frozen in 1% Triton X-100, then thawed, centrifuged and the supernatant assayed.

**Materials.** All cell culture media was obtained from Gibco, Grand Island, NY. Tetrahydrocannabinol (THC), Cannabinol (CBN), Cannabidiol (CBD), Cannabigerol (CBG) and Olivetol, all from Makor Chemicals, Israel, were dissolved in ethanol which was added directly to the cell supernatants. Indomethacin (Merck, Sharp and Dohme, West Point, PA) and aspirin (BDH) were initially dissolved in ethanol before further dilution in DMEM. Dexamethasone sodium phosphate (Merck, Sharp and Dohme) was dissolved directly in DMEM and sterilized by membrane filtration (0.2 µm Millex) prior to further dilution in DMEM. 12-O-Tetradecanoylphorbol-13-acetate (TPA) (Sigma Chemical Co., St. Louis, MO) was initially dissolved in acetone before further dilution in DMEM.

All chromatographic solvents were obtained from Koch-Light (Colnbrook, U.K.) and redistilled in glass before use. Thin layer chromatography plates were prepared using a Jobling Laboratory Division moving Spreader Apparatus with silica gel G from BDH Chemicals, Poole, U.K. Fast Blue Salt B (Koch-Light, Colnbrook, U.K.) was freshly prepared as a 0.5% aqueous solution in 0.01 N potassium hydroxide.

## RESULTS

### Crude extracts

Figure 1 illustrates inhibition of TPA-induced PGE<sub>2</sub> release from synovial cells by two crude THC-

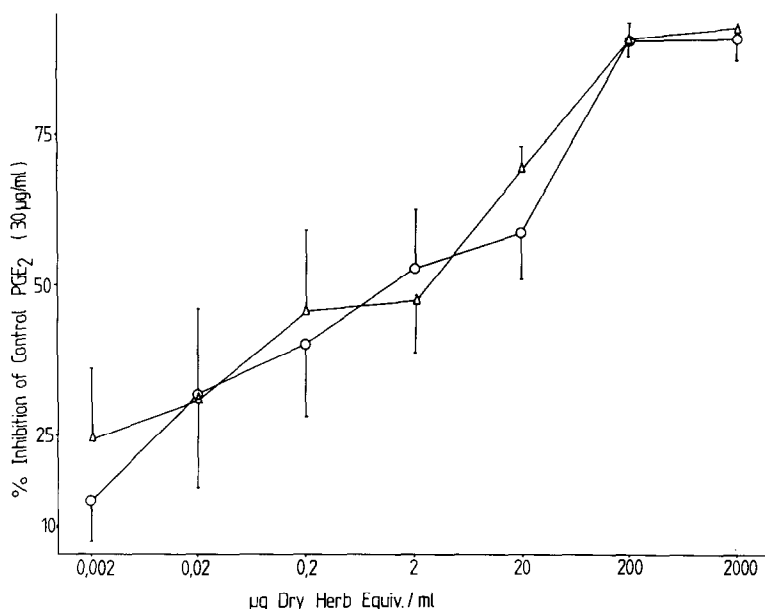


Fig. 1. Inhibition of TPA-induced PGE<sub>2</sub> release from cultured human rheumatoid synovial cells by "cannabinoid-free" extracts of Cannabis herb (variety UNC 335, crops 1980 (○) and 1981 (△)).

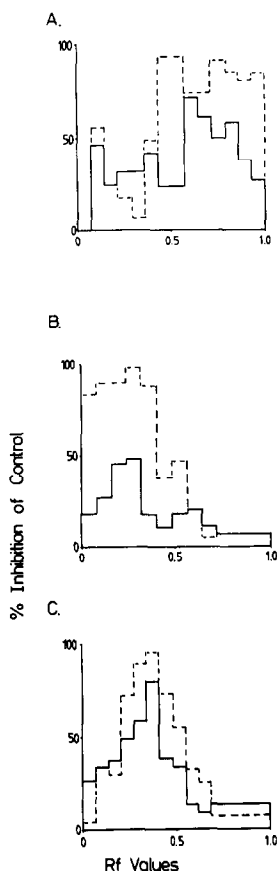


Fig. 2. Inhibition of PGE<sub>2</sub> release by eluates of TLC bands from the fractionated ethanolic extract. Developmental solvent systems were used sequentially as follows: (A) chloroform:ethanol (100:15), (B) cyclohexane:butanone:ethanol (70:25:5) and (C) heptane:chloroform:ethanol (50:50:15). Activity of top concentration of fractions (approx 20 µg/ml) indicated by broken lines and 10-fold dilutions indicated by solid lines.

free ethanolic extracts of *Cannabis sativa*. In five batches of plants extracted and tested, the IC<sub>50</sub> ranged from 1.2 to 3.8 µg dry wt equivalent of herb/ml. Four of these plant batches were produced by seed UNC 335 from South Africa and contained THC as the major cannabinoid. The activities of extracts of 1980 and 1981 crops of UNC 335 are depicted in Fig. 1. One of the five plant batches tested was produced by seed UNC 354 from Turkey and contained CBD as the major cannabinoid. The IC<sub>50</sub> of this batch (2.6 µg herb equivalent/ml) was within the range of the others. This suggested that

the presence of the inhibitor in the cannabis herb was independent of chemotaxonomic race based upon cannabinoid content.

### Isolation

Isolation of the inhibitory component of the ethanolic extract has been described in the Materials and Methods. Figure 2 depicts activity of eluates from 1-cm bands of the prep-TLC plates. The activity of the top concentration of the fractions (approx 20 µg/ml) and 10-fold dilution are illustrated as overlaying histograms. In the first separation with solvent system 1 of chloroform:ethanol 100:15, fractions with *R<sub>f</sub>* values from 0.57 to 0.80 contained the most activity and these were pooled and developed in the second system of cyclohexane:butanone:ethanol 70:25:5. Maximum inhibitory activity was found in between *R<sub>f</sub>* values of 0.16 and 0.32. These fractions were run in the third system of heptane:chloroform:ethanol 50:50:15 and the most active fraction had an *R<sub>f</sub>* value of 0.34 to 0.40. In the last chromatographic system of chloroform:toluene:ethyl acetate 35:35:30 (not illustrated), the component with maximum activity had an *R<sub>f</sub>* value of 0.26. Table 1 lists the *R<sub>f</sub>* values of the resulting isolated compound which appeared as one yellow spot when run in all four TLC systems and visualized with 60% H<sub>2</sub>SO<sub>4</sub>.

The compound produced was a yellow resin soluble in ethanol and acetone but which failed to crystallize.

The yield from 130 g herb was 18 mg or 0.014%. In the high resolution electron-impact mass spectrum (70 eV, 195°, direct inlet), a molecular ion of *m/e* 436.185454 was detected. Major fragment ions at *m/e* 367 (base peak, intensity 100%), 313 (81%), 107 (82%) and 69 (97%) were also present. Elemental analysis showed no nitrogen but 69.32% carbon, 6.68% hydrogen and 24.0% remainder. A molecular formula of C<sub>26</sub>H<sub>28</sub>O<sub>6</sub> satisfies both the molecular ion and the elemental analysis. When reacted separately with aqueous NaOH, concentrated H<sub>2</sub>SO<sub>4</sub> and Mg-HCl, the compound produced a yellow colour characteristic of the flavone class of flavonoids. The u.v. spectrum of the compound in methanol gave the following absorbance maxima, 220 nm ( $\epsilon$  38,000), 280 ( $\epsilon$  19,000) and 346 ( $\epsilon$  26,000). These maxima and their shifts in the presence of diagnostic reagents [10] as displayed in Table 2 were characteristic of a flavone. A fuller description of the elucidation of the chemical structure of this compound, which was named Cannflavin, will be given elsewhere.

### Comparison with the effects of cannabinoids

Cannabinoids THC, CBN, CBD and CBG caused

Table 1. *R<sub>f</sub>* Values of Cannflavin in four TLC systems on silica gel

TLC solvent system	<i>R<sub>f</sub></i> value
Chloroform:ethanol (100:15)	0.59
Cyclohexane:butanone:ethanol (70:25:5)	0.24
Heptane:chloroform:ethanol (50:50:15)	0.35
Toluene:chloroform:ethyl acetate (35:35:30)	0.28

Table 2. Ultraviolet absorption maxima of Cannflavin upon the addition of shift reagents

Reagent	Absorption maxima (nm)	$\Delta$ Band 2 (240–285 nm)	$\Delta$ Band 1 (300–400 nm)
MeOH	280, 346		
+NaOMe	283, 343, 408	+3	+62
+AlCl <sub>3</sub>	264(sh), 290, 370	+10	+24
+HCl	264(sh), 292, 364	+12	+18
+NaOAc	278, 400, 522	–2	+54
+H <sub>3</sub> BO <sub>3</sub>	277, 347, 522	–3	+54

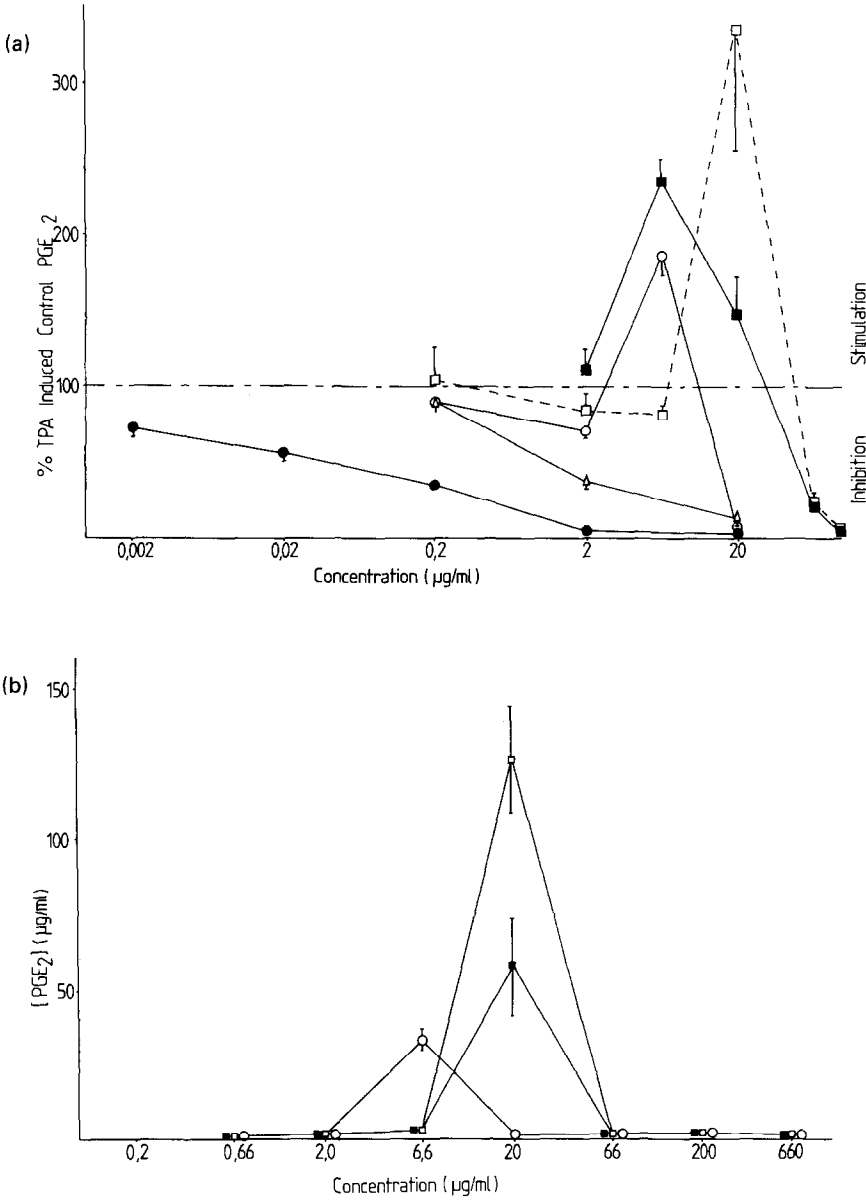


Fig. 3(a). Effects of Cannabinoids THC (□), CBN (■), CBD (○), CBG (△) and Olivetol (●) on TPA-induced PGE<sub>2</sub> release. (b) Stimulation of PGE<sub>2</sub> release by THC (□), CBN (■) and CBD (○) alone on synovial cells.

90% inhibition of TPA-induced PGE<sub>2</sub> release at concentrations of 20–200 µg/ml as depicted in Fig. 3(a). With the exception of CBG, the cannabinoids caused further stimulation of PGE<sub>2</sub> release at slightly lower concentrations of 6.6–20 µg/ml. This stimulatory effect was also seen when THC, CBN and CBD were administered alone to the synovial cells. As illustrated in Fig. 3(b), stimulation took the form of a bell shaped dose–response curve with THC and CBN effective at 20 µg/ml and CBD at 6.6 µg/ml.

Olivetol, a biosynthetic precursor of the cannabinoids which did not cause stimulation, was 25-fold more potent than CBG in inhibiting TPA-induced PGE<sub>2</sub> release.

The sharp transition between additional stimulation of TPA-induced PGE release by the major cannabinoids and 90% inhibition with a 3-fold increase in concentration raised the possibility that the inhibition may be due to toxicity. When the cells subjected to inhibitory concentrations of cannabinoids were examined microscopically, some loss of adherence was observed. Release of the cytoplasmic enzyme lactate dehydrogenase, an index of loss of cell viability, was found to increase by 25–38% over the control of 6% at concentrations of 20 µg/ml (CBD, CBG, Olivetol) or 200 µg/ml (CBN).

#### *Distinction of Cannflavin from Cannabinoids*

The isolated compound was distinct from the major cannabinoids on the basis of the extraction procedure. The crude ethanolic extract, when examined by TLC, was shown to be devoid of THC, CBN, CBD or CBG. When cochromatographed with the cannabinoids, cannflavin had a distinct *R<sub>f</sub>* value and was the only compound visualized with stannic chloride. Neither the crude ethanolic extract nor Cannflavin stimulated PG release from the synovial cells in contrast to most of the cannabinoids. The inhibitory activity of Cannflavin by weight is similar to that of Olivetol, however the two compounds differ substantially in molecular weight and therefore the activity of Cannflavin is 2.4-fold that of Olivetol on a molar basis.

#### *Comparison with common established anti-inflammatory drugs*

Established anti-inflammatory drugs also inhibited TPA-induced PGE<sub>2</sub> release from synovial cells. Cannflavin with a mean IC 50 of 31 ng/ml was more active than aspirin, (IC 50, 840 ng/ml) but less active than indomethacin (IC 50, 1.7 ng/ml) and dexamethasone (IC 50, 0.27 ng/ml). The relative inhibitory potencies listed in Table 3 remained consistent

whether the synovial cells were spontaneous producers of PGE<sub>2</sub> or induced by TPA or the monokine interleukin 1 (IL-1).

#### DISCUSSION

These studies revealed the presence of a novel, potent inhibitor of prostaglandin production in *Cannabis sativa* L. herb. The active component was isolated by chromatographic separation of an ethanolic extract of herb previously exhausted with petroleum ether. Structural analysis indicated that this compound was flavonoid in nature and has therefore been named Cannflavin. It appears to be present in the plant regardless of chemical race as extracts of “drug plants” (THC + CBN content > CBD content) and “fibre plants” (CBD content > THC + CBN content) had similar activity.

The Cannabinoids, which were removed from the herb by extraction with petroleum ether, also affect prostaglandin production. Tetrahydrocannabinol, CBN, and CBD at concentration of 6–20 µg/ml stimulated PGE<sub>2</sub> production by synovial cells, possibly through activation of phospholipase A<sub>2</sub> [11]. However, at higher concentrations in conjunction with the stimulant TPA, inhibition of PGE<sub>2</sub> production was observed. In contrast to the other cannabinoids, CBG and Olivetol, a biosynthetic precursor of the cannabinoids, were found only to inhibit PG formation and of the two, Olivetol was 25-fold more potent. The ability of the cannabinoids to stimulate and inhibit prostaglandin production *in vitro* has been reviewed by Burstein and Hunter [7]. Stimulation of arachidonic acid and prostaglandin release was noted in mouse mammary tumour epithelial cells, HeLa cells and WI-38 human lung fibroblast cultures. Inhibition of prostaglandin biosynthesis was reported in microsomal vesicles and Olivetol, which represents a portion of the cannabinoid structure, was more potent than the cannabinoids and was proposed as the active component of the cannabinoid molecule [12]. Olivetol was also much more potent than the cannabinoids in inhibiting PGE<sub>2</sub> release in this study and its action is likely to be due to inhibition of cyclo-oxygenase. However, at the high concentrations of Cannabinoids necessary for inhibition of PGE<sub>2</sub> release, cytopathic effects indicated by cell detachment and LDH release were apparent. Burstein and coworkers [13] proposed that cannabinoids interact with sterol-binding sites in membranes where they may effect changes in the activities of membrane bound enzymes such as phospholipase A<sub>2</sub> and cyclo-oxygenase. It may be that at high

Table 3. Comparison of Cannflavin with established anti-inflammatory drugs in inhibiting TPA-induced PGE<sub>2</sub> release from cultured synovial cells

Drug	IC 50 (ng/ml) mean (range)	Relative potency
Cannflavin	31 (4.4–58)	1
Aspirin	840 (460–1500)	0.037
Indomethacin	1.7 (0.3–4.0)	18
Dexamethasone	0.27 (0.036–0.5)	115

The IC 50 values are the means of four assays of each drug; the range of values observed in the assays is also given. Cannflavin was arbitrarily assigned a potency of 1 for comparison of potency with other drugs.

concentrations they also tend to disrupt these membranes and thus display toxicity.

Cannflavin and Olivetol were distinct in that they both caused over 90% inhibition of PG release without displaying toxicity, however, with a 10-fold increase in concentration, Olivetol proved the more toxic of the two. The ability of Cannflavin to inhibit PGE release from synovial cells was compared with established anti-inflammatory drugs. Synovial cells from patients with rheumatoid arthritis spontaneously release PGE<sub>2</sub> during primary culture and in subcultures following stimulation by TPA or IL-I, [14]. PGE<sub>2</sub> is believed to be an important mediator of inflammatory erythema, oedema and pain. Its biosynthesis is inhibited by both anti-inflammatory glucocorticoids, e.g. dexamethasone, and non-steroidal anti-inflammatory agents, i.e. aspirin and indomethacin. Aspirin and indomethacin act directly through inhibition of cyclo-oxygenase and dexamethasone acts indirectly through inhibition of arachidonic release from phospholipids. Cannflavin was intermediate in potency between aspirin on the one hand and indomethacin and dexamethasone on the other hand; however, its mode of action has yet to be determined. Possible indication of its anti-inflammatory potential may be drawn from early accounts of analgesic and anti-inflammatory properties of cannabis herb. Although Cannflavin has yet to be tested *in vivo*, the ethanolic extract of Cannabis did exhibit appropriate activity in the mouse phenylbenzoquinone-writhing test which identifies analgesic non-steroidal anti-inflammatory drugs (Pickens, Barrett and Fairbairn, unpublished observations). Anti-inflammatory activity in animal models has been demonstrated by flavonoids [15] which, depending upon their structure, may inhibit either one or both arachidonic acid metabolising enzymes, cyclo-oxygenase and lipoxygenase [16]. Lipoxygenase products include leukotriene B<sub>4</sub> (LTB<sub>4</sub>) which has been implicated in inflammatory processes [17]. Further studies are required to determine whether Cannflavin shares such inhibitory properties.

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