

# Cannabidiolic Acid as a Selective Cyclooxygenase-2 Inhibitory Component in Cannabis

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## ABSTRACT:

In the present study it was revealed that cannabidiolic acid (CBDA) selectively inhibited cyclooxygenase (COX)-2 activity with an  $IC_{50}$  value (50% inhibition concentration) around 2  $\mu$ M, having 9-fold higher selectivity than COX-1 inhibition. In contrast,  $\Delta^9$ -tetrahydrocannabinolic acid ( $\Delta^9$ -THCA) was a much less potent inhibitor of COX-2 ( $IC_{50} > 100 \mu$ M). Nonsteroidal anti-inflammatory drugs containing a carboxyl group in their chemical structures such as salicylic acid are known to inhibit nonselectively both COX-1 and COX-2. CBDA and  $\Delta^9$ -THCA have a salicylic acid moiety in their structures. Thus, the structural requirements for the CBDA-mediated COX-2 inhibition were next studied. There is a structural difference between CBDA and  $\Delta^9$ -THCA; phenolic hydroxyl groups

of CBDA are freed from the ring formation with the terpene moiety, although  $\Delta^9$ -THCA has dibenzopyran ring structure. It was assumed that the whole structure of CBDA is important for COX-2 selective inhibition because  $\beta$ -resorcylic acid itself did not inhibit COX-2 activity. Methylation of the carboxylic acid moiety of CBDA led to disappearance of COX-2 selectivity. Thus, it was suggested that the carboxylic acid moiety in CBDA is a key determinant for the inhibition. Furthermore, the crude extract of cannabis containing mainly CBDA was shown to have a selective inhibitory effect on COX-2. Taken together, these lines of evidence in this study suggest that naturally occurring CBDA in cannabis is a selective inhibitor for COX-2.

Cannabis is one of the oldest known medicinal plants and produces pharmacologically important substances. Among them, most important are the cannabinoids that are unique components in the cannabis plant.  $\Delta^9$ -Tetrahydrocannabinol ( $\Delta^9$ -THC) and cannabidiol (CBD) are known to be major cannabinoids in the plant.  $\Delta^9$ -THC is known to have pharmacological effects such as psychoactivity and hallucination (Dewey, 1986; Howlett et al., 2002). Cannabinoids (i.e.,  $\Delta^9$ -THC and CBD) are also being used as a rheumatoid arthritis agent in clinical settings (Klein and Newton, 2007) because of their anti-inflammatory effects (Formukong et al., 1988). In fresh plant materials, most of  $\Delta^9$ -THC and CBD exist as their respective acid forms,  $\Delta^9$ -tetrahydrocannabinolic acid ( $\Delta^9$ -THCA) and cannabidiolic acid (CBDA) (Yamauchi et al., 1967; Turner et al., 1980; Taura et al., 2007). The specific use of acidic cannabinoids including  $\Delta^9$ -THCA and CBDA as the active pharmaceutical ingredients is not disclosed to date because these acid forms of cannabinoids are recognized as pharmacologically

inactive forms (Yamauchi et al., 1967; Razdan, 1986; Burstein, 1999). By focusing on the structures between  $\Delta^9$ -THCA and CBDA, it was revealed that both acidic cannabinoids have a salicylic acid moiety in their structures (Fig. 1). Salicylic acid is known to be an inhibitor of cyclooxygenases (COXs, also referred as prostaglandin H synthases). Most of the conventional COX-1 and/or nonselective inhibitors contain a carboxylic acid group in their structures, and the COX-2 selective inhibitors reported lack the acid group but contain a sulfonyl-like group.

COX, which exists in at least two isoforms, catalyzes the first key steps in the synthesis of all the prostaglandins (PGs) by converting arachidonic acid (AA) into  $PGH_2$ . Thus, COX is a bifunctional enzyme exhibiting both cyclooxygenase (from AA to  $PGG_2$ ) and peroxidase (from  $PGG_2$  to  $PGH_2$ ) activities (DeWitt, 1999; Hinz and Brune, 2002). COX-1 is constitutively expressed as a housekeeping enzyme in nearly all the tissues and mediates physiological responses (e.g., cytoprotection of the stomach, platelet aggregation). On the other hand, COX-2 is expressed by cells that are involved in inflammation and has emerged as the isoform primarily responsible for the synthesis of prostanoids involved in acute and chronic inflammatory states of pathological processes (DeWitt, 1999; Hinz and Brune, 2002). Classical nonsteroidal anti-inflammatory drugs (NSAIDs) such as acetylsalicylic acid (aspirin) and diflunisal, which are grouped into the salicylate derivatives of NSAIDs, were shown to inhibit both COX-1 and COX-2 activities (DeWitt, 1999; Warner et al., 1999).

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**ABBREVIATIONS:**  $\Delta^9$ -THC,  $\Delta^9$ -tetrahydrocannabinol; CBD, cannabidiol;  $\Delta^9$ -THCA,  $\Delta^9$ -tetrahydrocannabinolic acid; CBDA, cannabidiolic acid; COX, cyclooxygenase; PG, prostaglandin; AA, arachidonic acid; NSAID, nonsteroidal anti-inflammatory drug; GC, gas chromatography; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

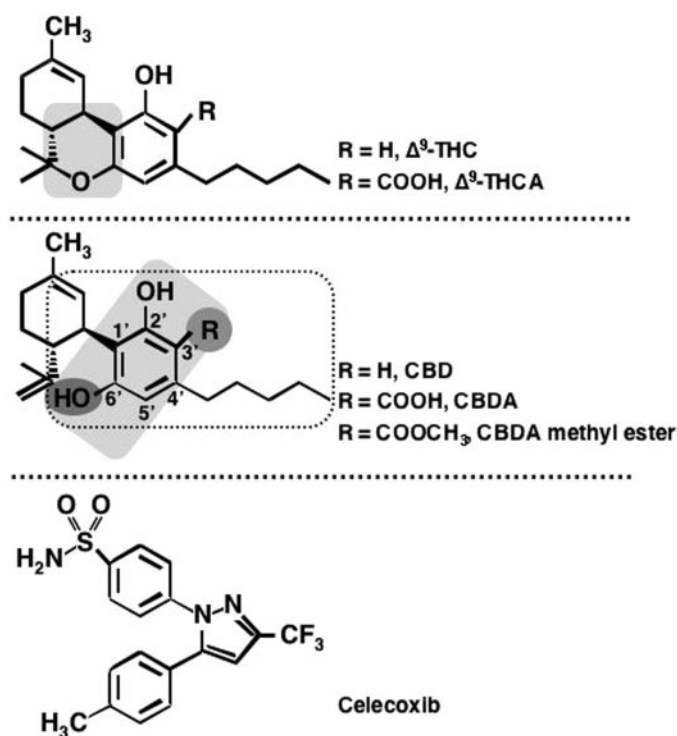


FIG. 1. Structures of cannabinoids tested and celecoxib.

None of the COX-2 selective inhibitors belonging to salicylates, which show selectivity for COX-2 inhibition with low concentrations, are reported to date (DeWitt, 1999; Warner et al., 1999). Inhibition of COX-2-dependent PG synthesis accounts for the anti-inflammatory and analgesic effects of NSAIDs, whereas suppression of COX-1 can lead to many unwanted side effects (e.g., gastrointestinal ulceration and bleeding, platelet dysfunctions). Thus, it has been thought that specific inhibitors for the COX-2-mediated reaction might have ideal therapeutic actions similar to those of classical NSAIDs without causing adverse effects. Burstein et al. (1973) have reported that some of natural cannabinoids inhibited PGE synthesis in bull seminal vesicles. However, there is no report whether any cannabinoid(s) selectively inhibit the COX-2 isoform.

The present report describes that CBDA is a selective COX-2 inhibitor in cannabis. The mechanism of selective COX-2 inhibition by CBDA is discussed.

#### Materials and Methods

**Cannabinoids and Chemicals.** Cannabis leaves were harvested from *Cannabis sativa* L. of  $\Delta^9$ -THCA (Mexican) and CBDA strains grown in the botanical garden of Hokuriku University.  $\Delta^9$ -THC,  $\Delta^9$ -THCA, CBD, and CBDA were isolated and purified from the cannabis leaves according to the methods described elsewhere (Aramaki et al., 1968). Purities of these cannabinoids were checked to be at least 95 to 98% by gas chromatography (GC) (Watanabe et al., 2005). The crude extract from CBDA strain was prepared by the methods described previously (Watanabe et al., 2005) except that the crude extract was not treated with heating to decompose the acid forms (i.e., decarboxylation). In fresh plant material, most of CBD has been reported to exist as its acid form (Turner et al., 1980). The relative contents of CBDA (77%) and CBD (23%) were determined by thin-layer chromatography analysis using Fast Blue BB salt as a coloring reagent (Watanabe et al., 2005). To obtain more information GC analysis was used. In GC analysis, because the applied CBDA in cannabis is subject to heating conditions causing its decomposition into CBD (~100%), the apparent content of CBDA in the strain was determined as CBD. The extract from CBDA strain contained 0.22 mg/ml of CBD. The content of  $\Delta^9$ -THC in the extract of CBDA strain was not deter-

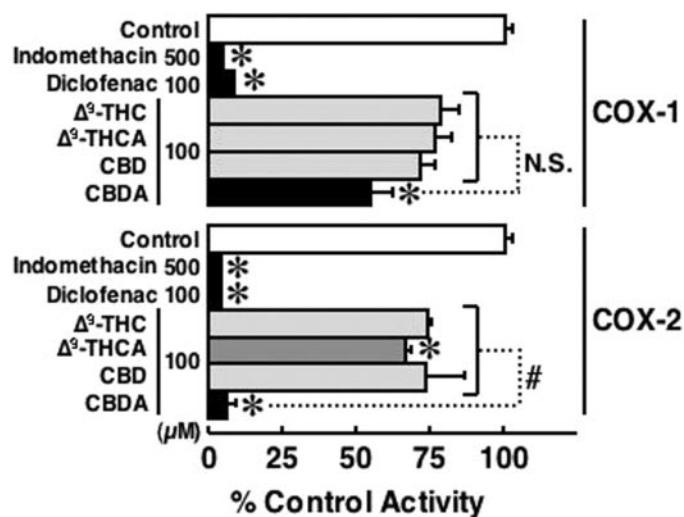


FIG. 2. Effects of cannabinoids on COX activity. CBDA was a potent inhibitor for COX-2. Reactions were initiated with AA, and TMPD oxidation was monitored at 590 nm. Details of the assay conditions are described under *Materials and Methods*. Each bar represents the mean  $\pm$  S.D. (triplicate determinations) of the relative activity to the control. \*, significantly different ( $p < 0.05$ ) from control; #, significantly different ( $p < 0.05$ ) from  $\Delta^9$ -THC-,  $\Delta^9$ -THCA-, and CBD-treated groups. N.S., not significant.

mined because  $\Delta^9$ -THC concentration was less than the detection limit ( $\leq 0.01$  mg/ml). CBDA methyl ester was prepared by the methylation of CBDA with diazomethane (Watanabe et al., 1988). 2,4-Dihydroxybenzoic acid ( $\beta$ -resorcylic acid), indomethacin, and resorcinol were purchased from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). Diclofenac was purchased from Sigma (St. Louis, MO). All the other reagents were of analytical grade.

**Enzyme Sources.** Assay of recombinant COX-1 and COX-2 activity was performed by using a commercially available colorimetric COX (ovine) inhibitor screening assay kit (Cayman Chemical Company, Ann Arbor, MI; lot 184104). All the inhibitors added to the reaction system were dissolved in ethanol and prepared just before use. In this assay, the COX activity was measured by using *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) as a cosubstrate with AA (reduction of PGG<sub>2</sub> to PGH<sub>2</sub>). TMPD oxidation was monitored spectrophotometrically with a 96-well plate reader at 590 nm. No colorimetric change was observed in control incubations that were performed by omitting enzymes or with heat-denatured enzymes and inhibitors in combination with TMPD.

**Data Analysis.** The concentration of the inhibitor that is required to produce 50% inhibition of the enzymatic activity (IC<sub>50</sub>) was determined from the curves plotting enzymatic activity versus inhibitor concentrations using Origin 7.5J software (OriginLab Corp., Northampton, MA). The details of the calculations were described in our previous articles (Takeda et al., 2006; Yamaori et al., 2007). Differences were considered significant when the  $p$  value was calculated to be less than 0.05. All the statistical analyses were performed by Scheffé's F test, which is a type of post hoc test for analyzing results of analysis of variance testing. These calculations were done using StatView 5.0J software (SAS Institute Inc., Cary, NC).

#### Results

**Effects of Cannabinoids on COX Activity.** The inhibitory effects of  $\Delta^9$ -THC and CBD and their respective acid forms  $\Delta^9$ -THCA and CBDA on COX-mediated TMPD oxidation activity were examined using purified COX as enzyme sources. Although COX-1 activity was not significantly inhibited by the addition of 100  $\mu$ M  $\Delta^9$ -THC,  $\Delta^9$ -THCA, or CBD except for CBDA, COX-2 activity was strongly inhibited by CBDA treatment compared with  $\Delta^9$ -THCA (around 10%) (Fig. 2). NSAIDs used in this study (indomethacin and diclofenac) nonselectively inhibited COX-1/-2 (see also Table 1). Furthermore, it should be noted that although both  $\Delta^9$ -THCA and CBDA have the

TABLE 1

Comparison of  $IC_{50}$  values ( $\mu M$ ) of various COX inhibitors

The  $IC_{50}$  values for each of the inhibitors are taken from the published data performed by using ovine COX-1 and COX-2 as enzyme sources (see *Materials and Methods*).

Inhibitors	COX-1	COX-2	COX-1/COX-2 Ratio <sup>a</sup>	Reference
CBDA	20	2.20	9.1	This work
Celecoxib	26.61	0.44	60.48	Tsai et al., 2006
Diclofenac	0.06	0.22	0.27	Johnson et al., 1995
Indomethacin	0.004	0.34	0.01	Tsai et al., 2006

<sup>a</sup> Ratio of the  $IC_{50}$  values for COX-1 and COX-2 can be used as an indication of the COX-2 selectivity of inhibitors. A COX-1/COX-2 ratio of more than 1 indicates preferential COX-2 selectivity.

same structural moiety, namely, salicylic acid (Fig. 1), the inhibition potency between these two acids was quite different. Based on the results obtained in Fig. 2, the following experiments focused on the inhibition potential of CBDA for COX-2 activity. To obtain a selectivity index (COX-1/COX-2 ratio of  $IC_{50}$  values), we next determined  $IC_{50}$  values for the inhibition of the two COX isoforms by CBDA. Although CBDA inhibited both COX-1 and COX-2, with apparent  $IC_{50}$  values of  $20 \pm 1.5$  and  $2.2 \pm 0.3$ , respectively (Fig. 3, A and B), it was revealed that CBDA was a selective inhibitor for COX-2 based on its selectivity index of 9.1 (i.e.,  $>1$ ) (Fig. 3; Table 1).

**Structural Requirement for Inhibitory Effect of CBDA on COX Activity.** To determine key structural determinants of CBDA-mediated COX-2 selective inhibition, we performed structure-activity relationship analysis. Interestingly,  $\beta$ -resorcylic acid only significantly inhibited COX-1 activity, although resorcinol equipotently inhibited COX enzymes (Fig. 4A). These structural components of CBDA were added to the reaction system at  $2 \mu M$ , as well as CBDA based on the  $IC_{50}$  value for COX-2 inhibition of CBDA (Fig. 3B). Thus, we hypothesized that  $\beta$ -resorcylic acid moiety of CBDA is one of the key structures of CBDA-mediated inhibition of COXs (COX-2), although  $\beta$ -resorcylic acid itself is insufficient for selective inhibition of COX-2. We discussed this point under *Discussion*. We next studied the effect of the methyl ester form of CBDA on COX-2 activity (Fig. 4B). The result indicated that the free carboxylic acid portion of CBDA is important to express its full inhibitory activity. Collectively, it was revealed that CBDA is able to inhibit COX-2 activity, which relies on the  $\beta$ -resorcylic acid moiety whose 6'-hydroxyl residue has to be freed from ring formation with the terpene moiety (see the structure of  $\Delta^9$ -THCA in Fig. 1).

**Effect of the Crude Extract from CBDA Strain Cannabis on COX Activity.** This study was performed to investigate the possibility that CBDA is a COX-2 specific inhibitory component in cannabis; the inhibition by CBDA would be also seen even in the presence of other constitutive components in cannabis. The extract from CBDA strain, which contains CBDA as a major cannabinoid, inhibited both COX-1 and COX-2 activities at a concentration of  $37.5 \mu g/ml$  ( $25 \mu M$  in terms of CBDA concentration) (Fig. 5), although this inhibitory effect was not observed at a concentration of  $7.5 \mu g/ml$  ( $5 \mu M$  in terms of CBDA concentration) (Fig. 5). The inhibitory magnitude of COX-2 by the extract from CBDA strain cannabis was clearly higher than that of COX-1. Therefore, CBDA itself is suggested to be a selective COX-2 inhibitory component in cannabis. However, it was also revealed that the inhibitory potency of CBDA in the extract might be much weaker than that of pure CBDA (Figs. 3 and 5). We discussed this inconsistency under *Discussion*.

### Discussion

Although it was considered that cannabinoid acids in cannabis plant were inactive cannabinoids, in the present study it was revealed that

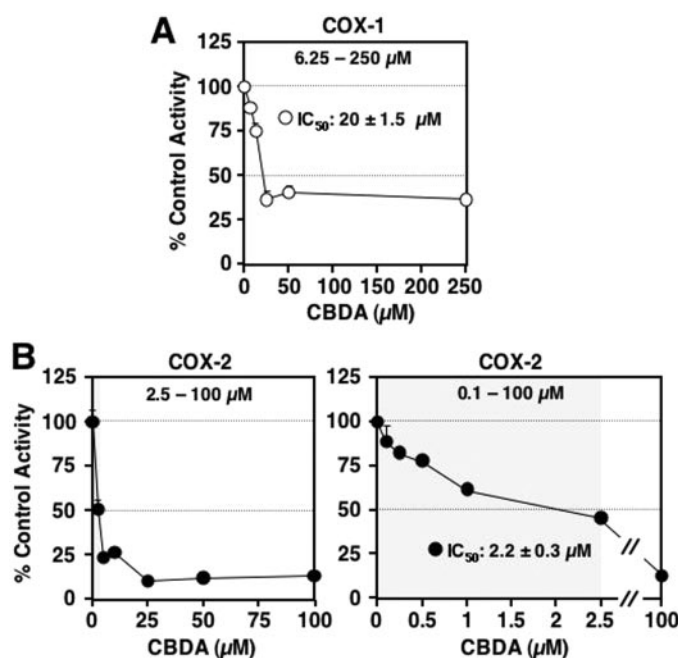


Fig. 3. Dose-dependent inhibition by CBDA on COX activity. A and B, TMPD oxidation by two isoforms of COX enzyme (A, COX-1; B, COX-2) was examined in the presence of indicated concentrations of CBDA. B, is composed of two parts; left is high concentration range (2.5–100  $\mu M$ ), right is low concentration range (0.1–100  $\mu M$ ). Reactions were initiated with AA, and TMPD oxidation was monitored at 590 nm. Details of the assay conditions are described under *Materials and Methods*. Each plot represents the mean  $\pm$  S.D. (triplicate determinations) of the relative activity to the control.

CBDA is a selective COX-2 inhibitor in vitro (selectivity index = 9.1; Table 1). Burstein et al. (1973) reported that several cannabinoids were able to suppress the biosynthesis of PGE in bull seminal vesicles, with large  $IC_{50}$  values ranging from 70 to 300  $\mu M$ . Because COX-2 is basically inducible by stimulations (DeWitt, 1999; Hinz and Brune, 2002), it is reasonable to understand that they only focused on the relationship between COX-1 and cannabinoids investigated. In agreement with their report,  $\Delta^9$ -THC was also a very weak inhibitor for COX-1 in our experiments ( $IC_{50}$  value;  $>100 \mu M$ ) (Fig. 2). After the discovery of COX-2 (Kujubu et al., 1991; O'Banion et al., 1991; Xie et al., 1991), it became a therapeutic target to avoid side effects by nonselective COX inhibitors. Thus, we set out to discover constituent(s) that have COX-2 selectivity in cannabis, and then CBDA was found to be an inhibitor for COX-2 (Figs. 2 and 3). Although  $\Delta^9$ -THC and CBD have been reported to have potential use as an analgesic for patients with rheumatoid arthritis (Klein and Newton, 2007), it has been suggested that the anti-inflammatory effect of  $\Delta^9$ -THC and CBD is not mediated by COX enzyme inhibition (Russo and Guy, 2006). Thus, it is assumed that the inhibition mechanism between  $\Delta^9$ -THC/CBD and CBDA in anti-inflammation is different. However, including this possibility, we are left with further questions that we were not able to address in these studies, such as does CBDA have potential to inhibit the COX-2-mediated PG production, which is able to lead to an anti-inflammatory action in vivo? A study about this possibility is under investigation.

It is well known that NSAIDs (salicylates) with an acidic carboxylic acid moiety, such as diflunisal and salicylic acid, inhibit COX activity via forming a salt bridge with Arg120 in COX enzymes (Picot et al., 1994; Mancini et al., 1995; Kurumbail et al., 1996; Luong et al., 1996). Thus, cannabinoids containing a carboxylic acid residue in their structures (i.e., both  $\Delta^9$ -THCA and CBDA) were expected to be effective COX inhibitors (Fig. 1). However, this does not seem to be

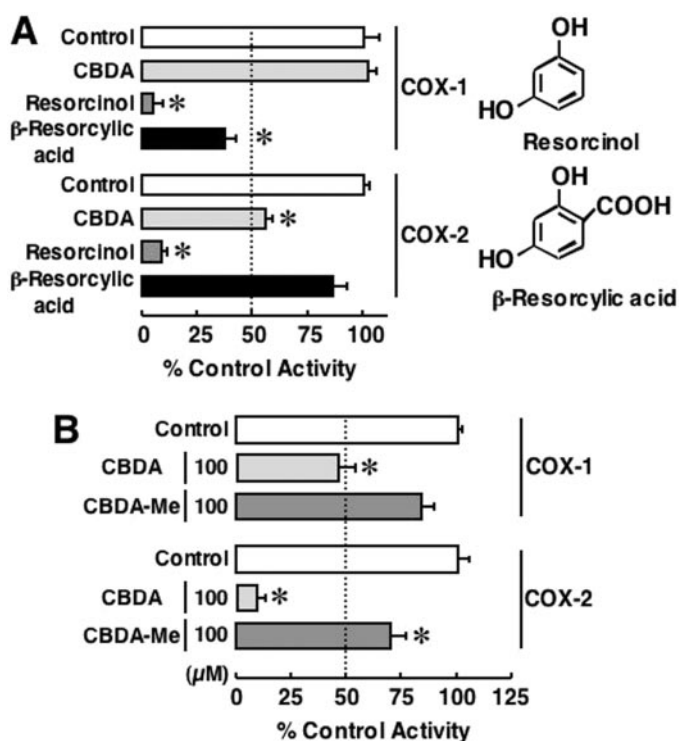


FIG. 4. Structural requirement of CBDA-mediated COX-2 selective inhibition. A, effects of structural moieties of CBDA (resorcinol and  $\beta$ -resorcylic acid) on the COX activities. These were added to the reaction mixture at  $2 \mu\text{M}$  (determined based on  $\text{IC}_{50}$  value for the COX-2 inhibition of CBDA; see Fig. 3B), and their structures are shown. B, effect of CBDA methyl ester (CBDA-Me) on the COX-mediated TMPD oxidation. Reactions were initiated with AA, and TMPD oxidation was monitored at 590 nm. Details of the assay conditions are described under *Materials and Methods*. Each bar represents the mean  $\pm$  S.D. (triplicate determinations) of the relative activity to the control. \*, significantly different compared with the control group ( $p < 0.05$ ).

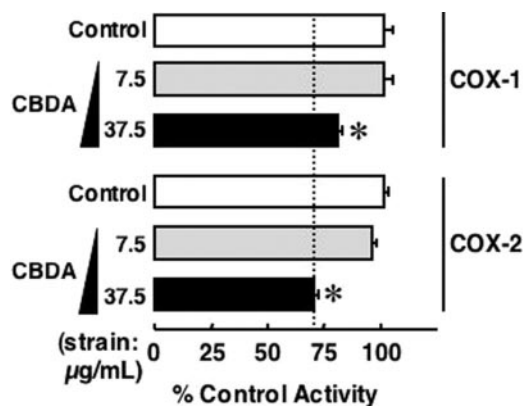


FIG. 5. Effect of the crude extract from CBDA strain on COX activity. TMPD oxidation by two isoforms of COX enzyme (COX-1 and COX-2) was examined in the presence of indicated concentrations of the crude extract. Reactions were initiated with AA, and TMPD oxidation was monitored at 590 nm. Details of the assay conditions are described under *Materials and Methods*. Each plot represents the mean  $\pm$  S.D. (triplicate determinations) of the relative activity to the control. \*, significantly different compared with the control group ( $p < 0.05$ ).

the case with  $\Delta^9$ -THCA (Fig. 2). There is only one structural difference between these, namely, the 6'-hydroxyl group of CBDA is freed from the ring formation with the terpene moiety. Based on this information, to obtain further experimental evidence we studied the effects of the structural moieties of CBDA, resorcinol and  $\beta$ -resorcylic acid, on COX activity. As expected, both COX-1 and COX-2

activities were inhibited by the reducing agent (i.e., antioxidant) resorcinol because it has been reported that the COX activity is sensitive to a large number of reducing agents that act as reducing cosubstrate for peroxidase reaction of COX enzymes (Markey et al., 1987). On the other hand, COX-1 but not COX-2 was significantly inhibited by  $\beta$ -resorcylic acid, a nonreducing agent (Seeram et al., 2001) (Fig. 4A). It should also be noted here that COX-2 activity was inhibited by CBDA itself, but COX-1 was not inhibited (Fig. 4A). It appears that the inhibitory effects of pure CBDA and the crude extract are different (Figs. 3 and 5). The reason for this discrepancy is not clear at present, although there is a possibility that CBDA crude extract contains other interfering component(s) for attenuating CBDA-mediated COX-2 inhibition. The X-ray crystal structures of the COX-1 and COX-2 enzymes have presented insight into how COX-2 specificity is achieved. Within the hydrophobic channel of the COX proteins, a single amino acid difference in position 523 (isoleucine in COX-1, valine in COX-2) has been shown to be critical for the COX-2 selectivity (Hood et al., 2003). Thus, the total NSAID binding site is around 17% larger in the COX-2 isoform (Luong et al., 1996), which allows COX-2 to bind bulky inhibitors more readily than COX-1 (Kurumbail et al., 1996). Although the results obtained here (Fig. 4A) are complex, at least two possibilities might be that 1)  $\beta$ -resorcylic acid itself can enter the catalytic site of both COX enzymes because of its smaller molecular size compared with that of CBDA, and 2) the whole molecule of CBDA is fitted by ideal configuration(s) with COX-2, which leads to COX-2 selective inhibition via its carboxylic acid moiety (see also Fig. 4B). Because there are no structural similarities between CBDA and celecoxib, a highly selective COX-2 inhibitor (selectivity index = 60.48; Table 1; see also Fig. 1), we propose the possibility that CBDA will be a useful "prototype" for producing COX-2 selective inhibitors different from celecoxib.

Taking all these findings into consideration, we have shown that CBDA in cannabis is a potent and selective inhibitor for COX-2 in vitro. Further studies are necessary to obtain information about molecular mechanism of the inhibition.

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