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A Saponin Complex, KPRG-C, and Its Sapogenin Complex, KPRG-D, Reduce Nociception and Inflammation in Animals

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Abstract - To develop a clinically available saponin- or sapogenin complex from Oriental medicines, the EtOH extract (KPRG-A) was obtained by extracting from the four crude drugs, Kalopanacis Cortex, Platycodi Radix, Rubi Fructus and Glycyrrhizae Radix. The BuOH fraction (KPRG-B), a crude saponin complex, was prepared by fractionating KPRG-A, which were further completely hydrolyzed to afford the sapogenin complex (KPRG-D). In an attempt to find the antinoicpetive effects of the saponin complex and sapogenin complex, KPRG-C, and -D, were assayed by writhing-, hot plate-, and tail-flick tests using mice or rats. The three samples were also subjected to antiiflammatory tests using serotonin-induced and carrageenan-induced hind paw edema mice and rats, respectively. The three samples significantly reduced inflammations and pains of the experimental animal. The potency were found in the order of KPRG-D> KPRG-C> KPRG-B. The most active sample, KPRG-D, caused no death, no body increase or no anatomical pathologic change even at 2,000 mg/kg dose. These results suggest that a sapogenin complex, KPRG-D, which was found to contain mainly hederagenin, platycodigenin, polygalacic acid, 23-hydroxytormentic acid, glycyrrhetic acid together with minor triterpene acids, could be a potential candidate for antiinflammatory therapeutics.

Key words - Saponin complex, Sapogenin complex, Kalopanacis Cortex, Platycodi Radix, Rubi Fructus and Glycyrrhizae Radix, Antinociceptive, Antiinflammatory

Introduction

We previously studied on the bioactive saponin components of each Chinese traditional medicine, Kalopanacis Cortex (Choi *et al.*, 2002a; Choi *et al.*, 2002b), Rubi Fructus (Choi et al., 2003; Nam *et al.*, 2003), Kochiae Fructus (Choi *et al.*, 2003; Lee *et al.*, 2002), and Akebiae Caulis (Jung *et al.*, 2004). Oral administration of rats with those bioactive saponins exhibited marked antinociceptive and anti-inflammatory activities mainly due to the sapogenin moiety (Jung *et al.*, 2005). This suggests that the saponin itself cannot be easily absorbed from the animal' s gastrointestinal tract. Nevertheless, the saponin-contaning oriental herb medicines have been very frequently used to treat neurotic pain, arthritic disease, diabetic syndrome. We also previously reported that saponins produce sapogenins or prosapogenins as metabolites by human intestinal bacteria (Kim *et al.*, 1998; Kim *et al.*, 2002).

We also previously reported the inhibitory effect of the triterpenoid, niga-ichigoside F_1 and 23-hydroxtormentic acid isolated from *R. coreanus*, on the rat' s gastropathy caused by sodium salicylate + EtOH (Nam *et al.*, 2003). These results support the saponin or sapogenin can substitute non-steroidal- or steroidal anti-inflammatory drugs (NSAIDs or SAIDs) which provoke gastropathy by long term-oral administration of them. Therefore, we thought that the natural saponins or sapogenins should be clinically or commercially used.

Oriental herb medicines are more frequently used as the prescribed medicines composed of several individual herbal drugs than as a single herb. We have reported that the extract complex is more suitable for treatment of aging disease. In this research, we prepared the complex extract, complex saponin fraction and sapogenin fraction by use of a more safe procedure to be a dosage form for human from the three Chinese drugs, Kalopanacis Cortex, Platycodi Radix, Rubi Fructus and Glycyrrhizae Radix to test antinociceptive anti-inflammatory activity in rats and mice. We assume that the complex extract or fraction obtained from several Chinese herbs may show at least the average activity potency of the individual herb in addition to reducing toxic action. Actually, the herbal drug complexes are now commercially available because many Chinese herb medicines are reveled to be active against aging disease.

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We and other researchers have elucidated antiinflammatory effect of the individual drugs, Kalopanacis Cortex, Platycodi Radix, Rubi Fructus and Glycyrrhizae Radix. Each herbal drugs contain the saponin constituents as follows: Kalopanacis Cortex (kalopanaxsaponins) (Sano *et al.*, 1991), Platycodi Radix (platycodins and polygalacins) (Kim *et al.*, 2005), Rubi Fructus (19 α-hydroxyursane-type triterpenoids) (Kim *et al.*, 1993), and Glycyrrhizae Radix (glycyrrhizin) (Ram *et al.*, 2006). Glycyrrhizae Radix is very often prescribed in the oriental medicines for the purpose of detoxification or a sweet ingredient because glycyrrhizin of the sweet ingredient has anti-allergic and anti-inflammatory effects (Cherng *et al.*, 2006; Kawashima *et al.*, 2006).

In this experiment, we extracted from the four Chinese medicinal drugs to give the EtOH extract (KPRG-A) and then prepared the BuOH fraction (KPRG-B), which is so-called saponin fraction by fractionating KPRG-A. The crude saponin complex of KPRG-B was passed through charcoal and Diaion HP-20 column to afford more refined saponin complex (KPRG-C). KPRG-C was completely hydrolyzed under acid condition to produce the sapogenin complex (KPRG-D). Antinociceptive activity was measured in mice and rats using writhing-, hot plate- and tail-flick assays. Serotonin- and carrageenan-induced hind paw edema of rats were used to test the antiiflammatory activity. The inhibitory effect on acetic acid-induced vascular permeability was measured to confirm the antiiflammatory effect. To find the toxic action of KPRG-D, which were found to have the most potent activity in this research, body weight increase, anatomical investigation, and death ratio were checked after each single administration of KPRG-D with 500, 1000, 2000mg/kg.

Materials and Methods

Plant material

After the four Chinese medicinal drugs, Kalopanacis Cortex, Platycodi Radix, Rubi Fructus and Glycyrrhizae Radix, were purchased from Chun-Il herbal drug store in Wonju, Korea, they were identified to be the stem bark of *Kalopanax pictus*, the roots of *Platycodon grandiflorum*, the unripe fruits of *Rubus coreanus*, the roots of *Glycyrrhiza uralensis*, respectively, by Prof. Sang-Cheol Lim, Department of Botanical Resources, Sangji University, Korea.

Preparation of KPRG-A, -B, -C and -D

Each 300g of Kaolopacis Cortex, Platycodi Radix, Rubi Fructus and 150g of Glycyrrhizae Radix, were put in the round flask and then extracted three times with EtOH for 5h under reflux. The extracted solution was filtered, evaporated under reduced pressure on a rotatory evaporator and then lyophilized to give solid extract (KPRG-A, 156g). KPRG-A (150g) was suspended in 1L distilled water and partitioned with 0.8L diethyl ether three times. Aqueous layer was also partitioned with 0.50L BuOH three times and BuOH layer was concentrated to dryness to give BuOH fraction (KPRG-B, 33g). Twenty eight g of KPRG-B was suspended in distilled water and then passed to charcoal (70g) column to remove phenolic substances or pigments. The eluted solution was again subjected to diaion HP-20 column by 2.0L distilled water to remove sugars or salts and then successively eluted with 2.0L EtOH. The eluted EtOH solution was evaporated *in vacuo* and finally lyophilized to give the saponin fraction (KPRG-C, 22g). On thin layer chromatography (TLC) sprayed by 10% sulfuric acid, KPRG-C showed only reddish or indigo spots indicating the characteristic color of triterpenoids or saponins.

Preparation of KPRG-D

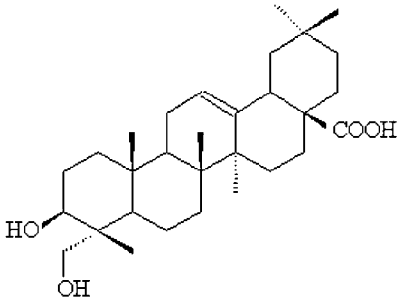
To prepare the sapogenin complex, 15g of KRPR-C was refluxed in 5%-sulfuric acid (1L) in MeOH-H2O (2:8) for 8 h. The reaction mixture was cooled and then partitioned with 0.8 L EtOAc three times. The EtOAc fraction was washed with distilled water two times, concentrated to dryness in vacuo and lyophilized to yield a solid sapogenin complex (KPRG-D, 20g). By comparison of TLC of KPRG-D with authentic specimens, it was found that KPRG-D had hederagenin, polygalacic acid, platycodigenin, 23-hydroxytormentic acid, euscaphic acid, tormentic acid, and glycyrrhetic acid and other minor triterpenoids. The structure of these sapogenins was shown in Fig. 1.

Animals

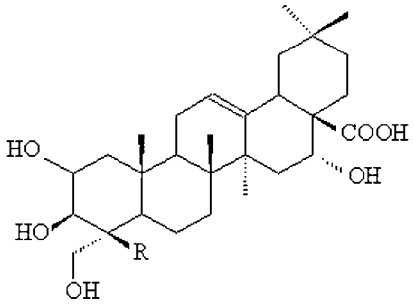
Both ICR male mice with 20-50g body weight and Sprague-Dawley male rats with 130-150g body weight were purchased from Dae-Han BioLink Co., and adapted them in a constant condition (temperature: 20±2℃, dampness: 40-60%, light/dark cycle: 12hr) for two weeks or more. Twenty-four hours before the experiment, only water was offered to the animals. Considering the variation of enzyme activity during one day, the animals were sacrificed at fixed time (10:00 A.M.-12:00 A.M.).

Preparation of sample solutions

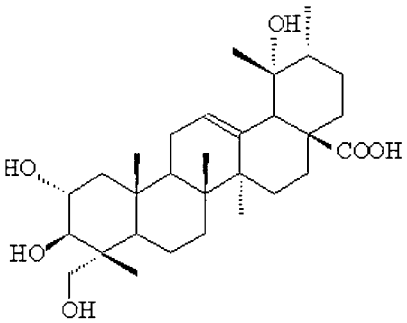
The complex crude saponin fraction (KPRG-B), the complex



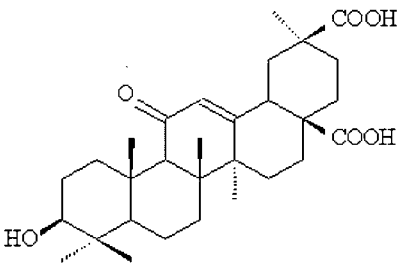
Hederagenin



Platycodigenin (R=CH_2OH)
Polygalacic acid (R=CH_3)



23-Hydroxytormentic acid



Glycyrrhetic acid

Fig. 1. Structure of five main sapogenins contained in KPRG-D.

saponin fraction (KPRG-C) and the complex sapogenin (KPRG-D) obtained from the four oriental herb medicines were dissolved in 10% Tween 80 and used being diluted with saline. The test solution was orally administered *ad libitum* for 2 weeks once a day with 100, 200mg/kg dose. The same volume of vehicle was administered to the control group.

Induction of hind paw edema of rats and mice

The initial hind paw volume of the Sprague-Dawley strain rats or ICR mice was determeined volumetrically using plethysmometer (Yesilda *et al.*, 1988). A 0.1ml/rat of 1% carrageenan (Sigma Co.) was injected subcutaneously into the rat' s right hind paws 1 h after the last administration of the test solution to induce edema. Ibuprofen (100mg/kg), an inflammatory drug, was used as a positive control in carrageenan-induced edema model. A 1% solution of serotonin in saline (0.5μg/rat) was injected subcutaneously into right hind paws of mice as with carrageenan-induced edema model. Indomethacin was used in serotonin-induced edema model as a positive control.

Acetic acid-induced permeability in mice

The effects of the test materials on the increased vascular permeability induced by acetic acid in mice were determined according to the method described by Eddy and Leimback (1953), with some modifications. The mice were dosed orally with the test substances suspended in 0.2% carboxymethyl cellulose (CMC) solution 30 min before the injection of acetic acid-saline solutions. Thirty min after the injection (I,p, 0.7% acetic acid-saline 0.1ml/10 g), 4% Evans blue-saline (10ml/kg) was also injected to the tail vein. After 20 min, the mice were sacrificed and viscera were exposed and irrigated with 10ml of distilled water. This washed solution was then centrifuged (3000 rpm, 10 min) and the absorbance of the supernatants was measured at 590nm wavelength using a UV/VIS spectrophotometer. The vascular permeability effects are expressed in terms of dye per ml which leaked into the intraperitoneal cavity. Indomethacin was used as the standard.

Writhing test in mice

The acetic acid-induced abdominal constriction testing was performed as described by Whittle (1949). Vehicle, aspirin (100 mg/kg), and the test solution (100 and 200mg/kg) were orally administered 30 min before the experiment, and 0.1ml/10g of 0.7% acetic acid-saline was then injected i.p. 10 min later, the frequency of

writhing in mice was counted over the following 20 min.

Hot plate test

The hot plate test was used to measure the response latencies according to the method described previously by Eddy and Leimback, with minor modifications. In these experiments, the hot plate (Ugo Basile, model-DS 37) was maintained at 56±1℃. The reaction time was noted by observing either the licking of the hind paws or the jumping movements before and after drug administration. The cut-off time was 20 sec and morphine sulphate 4.0mg/kg (Kuju Pharmaceutical CO.), administered intraperitoneally, was used as a reference drug.

Tail-flick test

The antinoceptive responses were determined by measuring the time required to respond to a radiating thermal stimulus. The rat was restrained so that the radiant heat source was focused onto the base of the tail. An automatized tail-flick analgesymeter (Ugo Basile) was used, and the cutoff time was set at 15 seconds. For each rat, three determinations were carried out before material administration (control latency). The tail-flick latency responses are expressed as a percentage of analgesia calculated. The intensity of the thermal stimulus was adjusted to obtain control latency between 4 and 6 seconds.

Short-term toxicity test

For the short-term toxicity test, the body weight of male and female rats was over 192.2-216.2g and over 146.8-165.6g, respectively. A dose of 2,000mg/kg and a dose of 500mg/kg were chosen as the highest dose group and the lowest dose group, respectively, in the rat. For vehicle only treatment, 10% Tween 80 in saline was offered to rats. Clinical signs were observed for 6h following treatment of KPRG-D on the day of administration and once everyday thereafter for 14 days. Body weight was measured before dosing and 1, 3, 7, and 14 days after dosing of KPRG-D and several days after the treatment. Following the observation period, all animals were anesthetized with carbon dioxide gas. Autopsy was conducted on every animal and all major organs were examined for gross lesions.

Results

Antinociceptive effects - The antinociceptive effects of KPRG-B, -C, and -D were shown in Table 1 and Fig. 2, which were resulted from acetic acid-induced writhing test, hot plate test and tail-flick test. All three antinociceptive effects were shown in the order of potency, KPRG-D> KPRG-C> KPRG-B. Aspirin and morphine was used in the positive control where the former one was used to test peripheral analgesic activities in writhing test, and the latter was used to compare central analgesic properties in hot plate- and tail-flick tests. KPRG-D exhibited significant analgesic activities in dose dependent manner, though the activity was less than the positive control. It was also observed that KPRG-D was more active in peripherally mediated antinociceptive activity than in

Table 1. Antinociceptive effect of KPRG-B, C and D on hot plate- and tail-flick reactions

Treatment	Dose (mg/kg)	Reaction time (sec)	Intensity response (sec)
Normal	0	9.36 ± 0.41^{f}	2.43 ± 0.39^{c}
KPRG-B	50	9.54 ± 0.39^{f}	2.50 ± 0.27^{c}
	100	$9.89 \pm 0.27^{e,f}$	2.55 ± 0.30^{c}
	200	$10.2 \pm 0.25^{d,e}$	$2.61 \pm 0.24^{b,c}$
KPRG-C	50	$9.77 \pm 0.20^{e,f}$	2.48 ± 0.22^{c}
	100	10.6 ± 0.27^{d}	$2.60 \pm 0.30^{b,c}$
	200	11.7 ± 0.19^{c}	$2.77 \pm 0.31^{b,c}$
KPRG-D	50	$10.2 \pm 0.23^{d,e}$	$2.59 \pm 0.19^{b,c}$
	100	11.6 ± 0.25^{c}	$2.82 \pm 0.23^{b,c}$
	200	14.8 ± 0.17^{b}	3.06 ± 0.25^{b}
Morphine	10	28.7 ± 0.63^{a}	4.29 ± 0.39^{a}

Value represents mean ± S.D. (n = 6). Values sharing the same superscript letter are not significantly different each other (p = 0.05) by Duncan' s multiple range test.

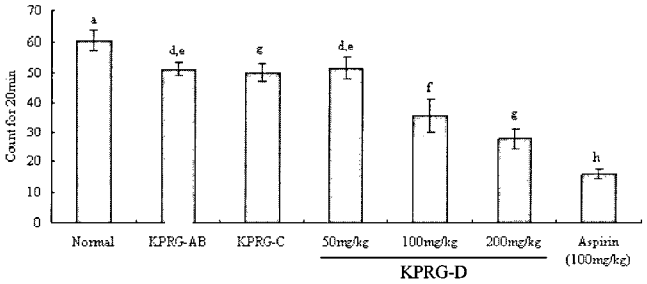


Fig. 2. Antinociceptive effects of KPRG-B, C, and D on acetic acid-induced writhing in mice.
Values represent means ± S.D. (n = 10). Values sharing the same superscript letters are not significantly different each other (P<0.05) in Dunca' s multuple-range test.

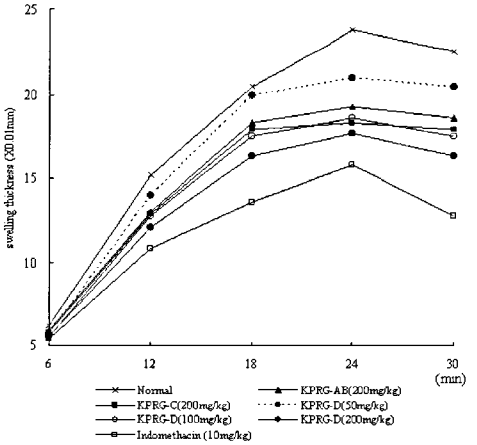


Fig. 3. Inhibitory effect of KPRG-B, C, and D pretreatment on serotonin-induced paw edema.
Values represent mean ± S.D (n = 10).

centrally mediated one.

Effect on serotonin-induced edema in mice - Hind paw edema in mice was measured from 6 min to 30 min after the injection of serotonin, which results are shown in Fig. 3. Injection of serotonin increased the paw edema up to 24 min and then reduced after 30 min. As shown in Fig. 3, all the samples, KPRG-B, -C, and -D decreased serotonin-induced edema, though it was shown in the order of potency: KPRG-D> KPRG-C> KPRG-B. The data of KPRG-B and C measured at 100mg/kg are not shown. Test samples were less active than indomethacin.

Effect on carageenan-induced edema in mice - Inhibitory effect of KPRG-B, -C, and -D on carrageenan-induced edema in rats are shown in Fig. 4. Hind paw edema of rats induced by carrageenan was measured every 1h from 1h to 5h after carrageenan injection. Maximal edema was observed at 3 h after carrageenan injection, and thereafter it was reduced. KPRG-B, -C, and -D exhibited dose-dependent anti-inflammatory effects, which were less active than ibuprofen. The data of KPRG-B and C measured at 100mg/kg are not shown. The potency of the three samples were shown in the order of KPRG-D> KPRG-C> KPRG-B. In particular, KPRG-B, the complex sapogenin, was active even at 50mg/kg dose.

Effect on acetic acid-induced vascular permeability - As shown in Table 2, induction of vacular permeability with acetic acid caused the increase of evans blue concentration by 8.32-0.53μg/ml whereas treatment of rats with indomethacin reduced it to 3.26-0.33μg/ml.

Table 2. Effect of KPRG-B, C, D on vascular permeability induced by acetic acid in mice

Treatment	Dose (mg/kg)	Evans blue concentration μg/ml
Normal		8.32 ± 0.53^{a}
KPRG-B	50	$8.13 \pm 0.46^{a,b,c}$
	100	$7.96 \pm 0.55^{a,b,c}$
	200	$7.42 \pm 0.38^{c,d}$
KPRG-C	50	$8.20 \pm 0.29^{a,b}$
	100	$7.52 \pm 0.50^{b,c,d}$
	200	7.03 ± 0.46^{d}
KPRG-D	50	$7.98 \pm 0.39^{a,b,c}$
	100	7.18 ± 0.28^{d}
	200	6.23 ± 0.40^{e}
Indomethacin	10	3.26 ± 0.33^{f}

Value represents mean ± S.D. (n = 6). Values sharing the same superscript letter are not significantly different each other (p = 0.05) by Duncan' s multiple range test.

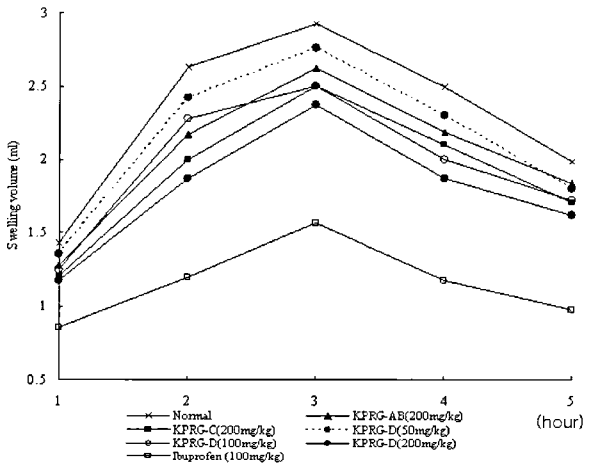


Fig. 4. Inhibitory effect of KPRG-B, C, and D pretreatment on carrageenan-induced paw edema.
Values represent mean ± S.D (n = 10).

These results suggest that indomethacin inhibited vascular permeability. KPRG-B, -C and -D also decreased vascular permeability caused by acetic acid, which results were weaker than indomethacin.

Acute toxicity - No particular clinical symptoms were observed at any treated groups of male and female mice with 0, 500, 1000, and 2000mg/kg dose. No dead mouse was shown through the period of acute toxicity test (data not shown). Significant change of body weight was not also observed through the period in male and female group orally administered KPRG-D, as shown in Fig. 5. Anatomical investigation also indicated no signs of pathological change in the animal alive.

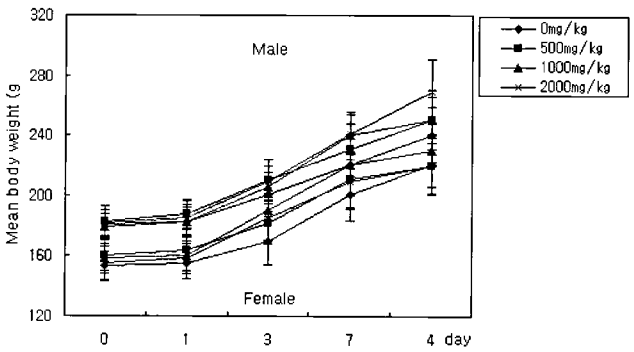


Fig. 5. Body weight changes in rats treated orally once treated with KPRG-D

Discussion

The oriental herb medicines, Kalopanacis Cortex, Platycodi Radix, Rubi Fructus and Glycyrrhizae Radix, used in this experiment mainly contain the saponin constituents: Kalopanacis Cortex (kalopanaxsaponin A-K) (Sano *et al.*, 1991), Platycodi Radix (more than 10 saponins belonging to platycodins or polygalacins) (Kim *et al.*, 2005), Rubi Frctus (glycosides of 19α-hydroxytursane-type triterpenoids) (Kim *et al.*, 1993), and Glycyrrhizae Radix (glycyrrhizin) (Ram *et al.*, 2006). Although BuOH fraction is said as so-called saponin fraction, this fraction is not pure saponin mixture but crude saponin mixture. Therefore, the BuOH fraction, KPRG-B, was passed through charcoal and Diaion HP-20 column to afford the saponin complex, KPRG-C, to remove phenolic or non-saponin fraction. It was shown on TLC that KPRG-C was composed of saponins. The complex sapogenin, KPRG-D, obtained from the hydrolysis of KPRG-C mainly included hederagenin, platycodigenin, polygalacic acid, euscaphic acid, tormentic acid, 23-hydroxytormentic acid, and glycyrrhetic acid, as shown in Fig. 1, and other minor sapogenins, which were found compared with authentic specimens on TLC.

This research was attempted to strengthen the clinical availability of the saponin complex or sapogenin complex, because the prescribed medicinal drugs with oriental medicine complex are now commercially available. It was shown that KPRG-C, more refined saponin complex, had more active in antinociceptive and anti-inflammatory tests than KPRG-B, indicating that the saponin complex is responsible for the activities rather than non-saponins. Since it is accepted that saponins are hardly absorbed from the gastrointestinal tract, the sapogenin complex was prepared and then pharmacologically tested.

While investigating the inflammatory and antinociceptive effects of KPRG-B, -C, and -D *in vivo*, we found that they significantly reduced the edema induced by serotonin or carrageenan (Fig. 2 and 3), where peak edema characterized by the presence of prostaglandins (Yang *et al.*, 1996). Increased vascular permeability swelling are common during the early stage of many type of inflammation, which is principally associated with serotonin increase. Carrageenan-induced inflammation in the rat paw is the classical edema model and hyperalgesia that has been extensively used in the development of NSAIDs. Antinoceiceptive effects of test samples were assayed using three different models: acetic acid-induced writhing test, hot plate test and tail-flick test, where the former assay is used to find peripherally mediated analgesic properties and latter two assays were to find centrally mediated one. KPRG-B, -C and -D are more active in writhing test rather than in

other two antinociceptive assays, which results represent that they mainly have peripherally mediated analgesic properties. Significant antinociceptive anti-inflammatory effects of KPRG-D suggest that KPRG-D could be a potential candidate for natural medicines.

Although the triterpene acid complex is not easily prepared from the natural sources, saponin complex will be prepared in a commercially available amount. Therefore, large amount of complex sapogenins is also possibly prepared by complete hydrolysis of the saponin complex. It has been reported that a commercial medicinal drug, SKI306X (SK Chemical Co.) is the fraction of the roots of *Trichosanthes kirilowii* (Trichosanthis Radix), the herbs of *Prunella vulgaris* (Prunellae Herba) and the *Clematis mandshurica* (Clematidis Radix) (Kim *et al.,* 2005a; Kim *et al.,* 2005b). Our results on antinociceptive and antinflammatory effects of KPRG-D are comparable to the bioactivity of SKI306X by WHO *et al.* Kawashima *et al* (2006) reported that the mixture of glycyrrhizin and geinsenosides and etc. inhibited the gastropathy. Since KPRG-C and D are the saponin complex and sapogenin complex, respectively, the efficacy will be predictable and the toxicity is weak. Together with this, KPRG-D showed no signs of toxicity in the acute toxicity test.

Therefore, it is suggested that KPRG-D could be a potential candidate for treatment of inflammatory disease like rheumatoid arthritis and that it could substitute NSAIDs or SAIDs causing side effects. KPRB-C can be also a candidate for treatment of chronic inflammatory disease as a saponin complex.

Acknowledgements

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