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The Flowers of *Carthamus tinctorius* : Potential Agent for Postmenopausal Disorder

Moon Young HEO*, Cheon Ho KIM[1], Jae Sung KANG[2], Kyung Nam UR[2] and Hyun Pyo KIM

College of Pharmacy and [1]Dept. Vet. Med., Kangwon National University, Chunchon 200-701, and [2]Samchully Pharm. Co., Ltd., 947-7 Daechi-dong Kangnam-ku, Seoul 135-280, Korea

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Abstract – In this study, 75% ethanol extract from the flowers of *Carthamus tinctorius* was prepared and biological activities were examined. The extract showed the inhibitory activity of vascular smooth muscle contraction and antithrombotic activity judged by bleeding time measurement. It also showed anti-inflammatory and potent analgesic activities *in vivo*. By oral administration of the extract, no acute toxicity was observed up to 5 g/kg in mice and rats. All these results strongly suggest that this extract may be beneficial for postmenopausal disorder by enhancing blood circulation.

Keywords □*Carthamus tinctorius* L. (Compositae), postmenopausal disorder, vascular contraction, anti-inflammatory activity, analgesic activity, kaempferol-3-O-rutinoside

The flowers of *Carthamus tinctorius* L. (Compositae) has been widely used for enhancing blood circulation and postmenopausal disorder of women (Korean translation Committee of Dong Eui Bo Gam, 1966a; Shanghai Science Technology Publishing Co., 1985). Menopause is defined as the permanent cessation resulting from the loss of ovarian follicular activity. The relative estrogen deprivation in postmenopausal women is associated with physiological changes and increased risks of several diseases, including cardiovascular disorder, atherosclerosis, osteoporosis and chronic pain etc.

Various constituents were previously reported from the flowers of *C. tinctorius*. They include fatty acids, lignans, steroids and flavonoids (Namba, 1993), among which several flavonols such as kaempferol and quercetin glycosides are the major constituents (Kim *et al.*, 1992; Masao *et al.*, 1992). Many investigations so far have shown that water or ethanol extract of Carthamus flos possessed biological activities such as antithrombosis, inhibition of platelet aggregation, anti-inflammation, etc. (Commission of Japanese Pharmacopeia, 1996; Jung *et al.*, 1999). These literatural backgrounds and many biological activities verified prompted us to study the biological activities of the flowers of *C. tinctorius* for plant-based drug development. In this study, kaempferol-3-O-rutinoside as an active compound was isolated from the flowers of *C. tinctorius*, and biological activities of this compound and the ethanol extracts were compared. The original preparing method used for treatment of various disorders of women is boiling the flowers of *C. tinctorius* in alcoholic beverage according to the ancient literature (Korean translation Committee of Dong Eui Bo Gam, 1966b). To modernize this procedure, in this study, standard ethanol extract from the flowers of *C. tinctorius* was prepared after comparing biological activities and production yields of the preparations using various extraction procedures. From this extract, four flavonoid derivatives were successfully isolated and their contents were determined. And the biological activities focused on improvement of blood circulation, anti-inflammatory and analgesic activity in postmenopausal disorder were evaluated.

*To whom correspondence should be addressed.

MATERIALS AND METHODS

Apparatus and animals

Melting point was determined by Fisher-Johns melting point apparatus and uncorrected. ^{1}H- and ^{13}C-NMR spectrum was recorded using TMS as an internal standard with Varian 200 MHz NMR. Purity of the isolated compounds was determined using TLC with at least two different solvent systems. ICR mice and Sprague-Dawley (SD) rats were purchased from Chales River (Japan) and acclimatized in our SPF animal facility at least for 7 days before use.

Extraction and isolation of flavonoids

The flowers of *C. tinctorius* were purchased from local market (Chunchon, Korea) and brochure specimen was deposited in College of Pharmacy (KNU). The flowers were put into various concentrations of ethanolic solutions for 1-7 days at 4°C-37°C. After appropriate time of extraction, the extracts were filtered and dried under vacuo in order to compare the production yields as well as their biological activities. For isolation of flavonoid constituents, the flowers of *C. tintorius* (1 kg) were extracted in 75% ethanol (10 L) for 5 days at room temperature. After filtration, the filtrate was dried under vacuo to yield 256 g dried residue. The residue was mixed in distilled water and partitioned with n-hexane, ethyl acetate and n-butanol. After each fraction was dried, ethyl acetate fraction was dissolved in small amount of methanol and poured into silica gel column chromatography. Using chloroform:methanol:water (10:3.5:0.5) as a mobile phase, compound I and II were isolated. From n-butanol fraction, silica gel column chromatography using ethyl acetate:methanol:water (100:13.5:10) as a mobile phase gave compound III and IV.

Compound I; Astragalin (kaempferol-3-O-β-glucoside), Recrystallized from acetone, yellow needles, m.p.=176-178, ^{1}H-NMR (DMSO-d_6) δ 5.35-5.50 (1H, m, H-1"), 6.22 (1H, d, J =2 Hz, H-6), 6.45 (1H, d, J=2 Hz, H-8), 6.90 (2H, d, J=8.8 Hz, H-3' and H-4'), 8.06 (2H, d, J=8.8 Hz, H-2' and H-6'), 10.20, 10.88 (2H, 2s, 2OH), 12.64 (1H, s, 5-OH)., ^{13}C-NMR (DMSO-d_6) δ 177.4 (C-4), 164.1 (C-7), 161.2 (C-5), 159.9 (C-4'), 156.3 (C-2 or C-9), 156.2 (C-2 or C-9), 133.1 (C-3), 130.8 (C-2' and C-6'), 120.8 (C-1'), 115.0 (C-3' and C-5'), 103.9 (C-10), 100.7 (C-1"), 98.6 (C-6), 93.5 (C-8), 77.4 (C-3"), 76.3 (C-5"), 74.1 (C-2"), 69.7 (C-4"), 60.7 (C-6")., Acid hydrolysis products: kaempferol and glucose

Compound II: Isoquercitrin, Structurally identified by direct comparison with an authentic sample from Aldrich Chem. Co.

Compound III: Kaempferol-3-O-rutinoside, Recrystallized from acetone, yellow powder, m.p.=173-176°C, ^{1}H-NMR (DMSO-d_6) δ 0.99 (3H, d, J=6 Hz, Rham-6), 5.10 (1H, m, H-1", Rham-1), 5.38 (1H, m, H-1", Glu-1), 6.21 (1H, d, J=2 Hz, H-6), 6.42 (1H, d, J=2 Hz, H-8), 6.89 (2H, d, J=9.0 Hz, H-3' and H-5'), 8.0 (2H, d, J = 9.0 Hz, H-2' and H-6'), 12.58 (1H, s, 5-OH)., ^{13}C-NMR (DMSO-d_6) δ 177.37 (C-4), 164.10 (C-7), 161.16 (C-5), 159.84 (C-4'), 156.79 (C-9), 156.44 (C-2), 133.13 (C-3), 130.80 (C-2' and C-6'), 120.79 (C-1'), 114.98 (C-3' and C-5'), 103.86 (C-10), 101.20 (C-1-glu), 100.64 (C-1-rham), 98.59 (C-6), 93.60 (C-8), 76.18 (C-3-glu), 75.57 (C-5-glu), 74.01 (C-2-glu), 71.63 (C-4-rham), 70.41 (C-3-rham), 70.32 (C-2-rham), 69.75 (C-4-glu), 68.08 (C-5-rham), 66.71 (C-6-glu), 17.49 (C-6-rham)., Acid hydrolysis products: kaempferol, rhamnose and glucose.

Compound IV: Rutin, structurally identified by direct comparison with an authentic sample from Aldrich Chem. Co.

HPLC analysis of the extract

For HPLC analysis, HPLC system equipped with Shimpak-C_{18} column (Shimadzu) was used. The extract of the flowers of *C. tinctorius* was diluted with acetonitrile:water (1:1) and injected to HPLC (Shimadzu 9A). For a mobile phase, acetonitrile:water (19:81) was used. Peaks were detected at UV 360 nm. The standard compounds including each 10 ug/ml of rutin, isoquercitrin, kaempferol-3-rutinoside and astragalin were injected and retention times were compared.

Inhibition of vascular smooth muscle contraction

In all following biological tests, 75% ethanolic standard extract was used. For a standard compound, kaempferol-3-O-rutinoside was also used. The inhibitory effect of the extract on KCl or norepinephrine-induced vessel contraction was tested with the isolated aorta from female SD rats (200-250 g) in organ bath. Polygraph (Grass, USA) was used according to the previously described method (Abebe and Agrawal, 1995).

Inhibition of blood coagulation (Total bleeding time)

For examination of anticoagulating effect, total bleeding time was measured according to the reported procedure (Han *et al.*, 1987). Female ICR mouse (20-25 g) were anethesized with i.p. injection of sod. pentobarbital (400 mg/kg). The tail was transsected at 5 mm from the tip, and the distal 5 cm of the tail was immersed vertically in saline solution at 37.5°C. Bleeding time was counted until breed stops by the formation of loose and temporary platelet plugs.

Antioxidative and radical scavenging activity

Using Fenton's reagent, TBA method was employed at 535 nm for measuring antioxidative activity according to the previous described procedure (Ohkawa *et al.*, 1979). Free radical scavenging activity was tested using 60 uM DPPH (1,1-diphenyl-2-picrylhydrazyl). Absorbance was measured at 520 nm after incubating at 37°C for 30 min (Fugita *et al.*, 1988).

Effects on plasma cholesterol and triglyceride levels

The standard ethanol extract was administered orally to female ICR mice once a day for 7 days. Serum cholesterol and triglyceride levels were measured using standard assay kits (Asan, Korea). For elevating levels of cholesterol and triglyceride, the diet containing 1% cholesterol and 0.5% cholic acid was fed to mice for 7 days. The extract was orally

administered for 5 days and levels of cholesterol and triglyceride were also checked.

Anti-inflammatory activity

For an acute inflammatory animal model, mouse ear edema bioassay was employed (Kim *et al.*, 1993). And rat adjuvant induced arthritis was carried out to measure anti-inflammatory activity against chronic inflammation according to the modified procedure (Kim *et al.*, 1997).

Analgesic activity

For evaluation of analgesic activity, acetic acid induced writhing test was carried out using mice according to the previously described procedure (Bentley *et al.*, 1983).

Evaluation of acute toxicity

Following a guideline from KFDA (1996), acute toxicity test was carried out with male and female ICR mice and rats.

Statistics

Data were presented as arithmetic mean±SD. Statistical significance was compared using Student's t-test.

RESULTS

Using various extraction procedures, the optimal conditions of extraction were established mainly based on the production yields and their biological activities (data not shown).

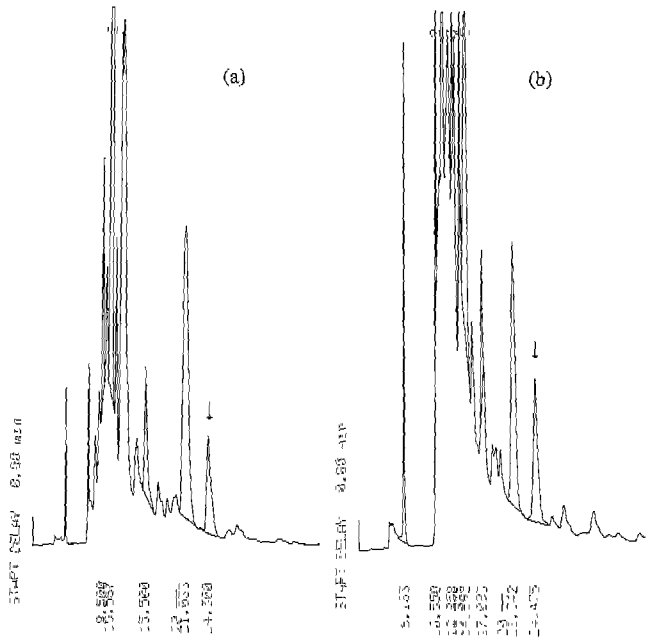

Fig. 1. HPLC chromatogram of the extract.
(a) The 75% ethanol extract, (b) The extract based on the original procedure described in ancient literature. Kaempferol-3-O-rutinoside (←).

The selected method is an extraction of the flowers of *C. tinctorius* with 75% ethanol solution at room temperature for 5 days. From the extract, four flavonoid derivatives including astragalin, isoquercitrin, kaempferol-3-O-rutinoside and rutin were isolated and structurally identified according to the previous results (Kim *et al.*, 1992; Masao *et al.*, 1992). Among these derivatives, kaempferol-3-O-rutinoside was selected as a standard compound since the content and activities of this compound were higher than other flavonoids isolated. Its content was measured with HPLC, in which the extract prepared showed almost same HPLC chromatogram compared with the extract prepared based on ancient literature (Fig. 1). The usual contents of kaempherol-3-O-rutinoside were revealed to be approximately 0.41±0.02 %, w/w (n=3).

When the extract was tested in KCl-induced vasocontraction, it inhibited vascular smooth muscle contraction dose-dependently (Fig. 2a). IC_{50} values for the extract and the ethyl acetate fraction of *Ginkgo biloba* leaves were

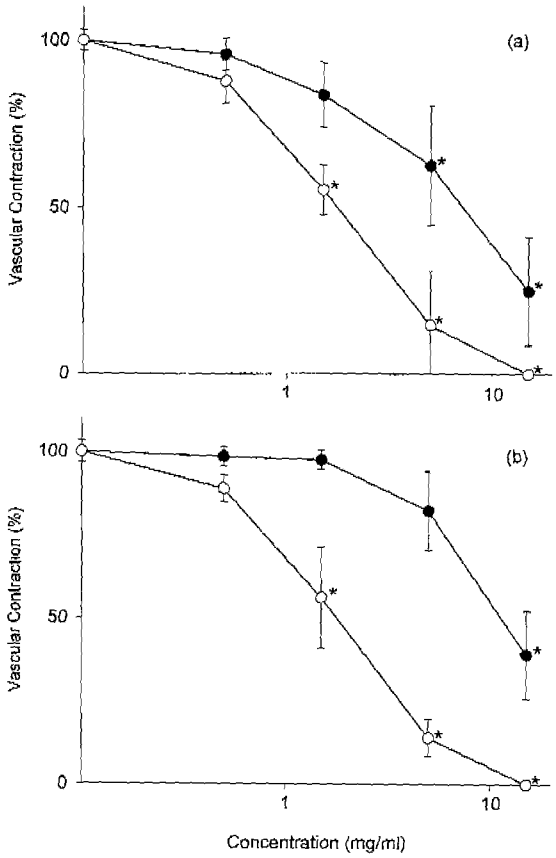

Fig. 2. Effects on vascular smooth muscle contraction by *C. tinctorius* extract.
(a) KCl(72.7 mM)-induced contraction, (b) Norepinephrine(3×10^{-7} M)-induced contraction. Gingko extract (○), *C. tinctorius* extract (●), *P<0.01, significantly different from control.

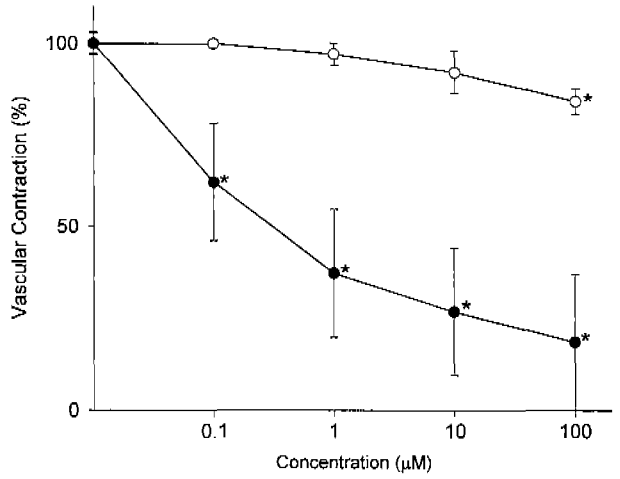

Fig. 3. Effects of kaempferol-3-O-rutinoside on vascular smooth muscle contraction KCl (72.7 mM)-induced contraction (○), Norepinephrine(3×10^{-7} M)-induced contraction (●), *P<0.01, significantly different from control.

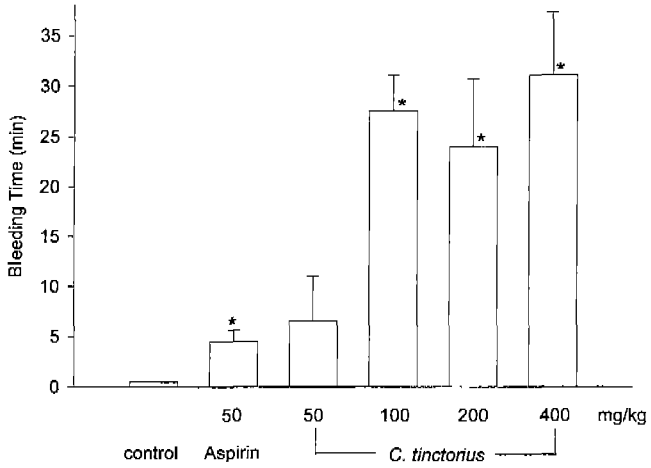

Fig. 4. Effects of *C. tinctorius* extract on bleeding time in mice. *P<0.05, significantly different from control.

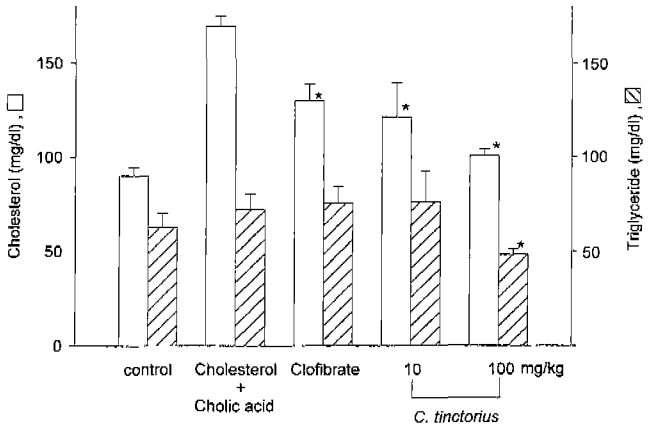

Fig. 5. Effects of *C. tinctorius* extract on serum cholesterol and triglyceride level. *P<0.05, significantly different from cholesterol+cholic acid treated group.

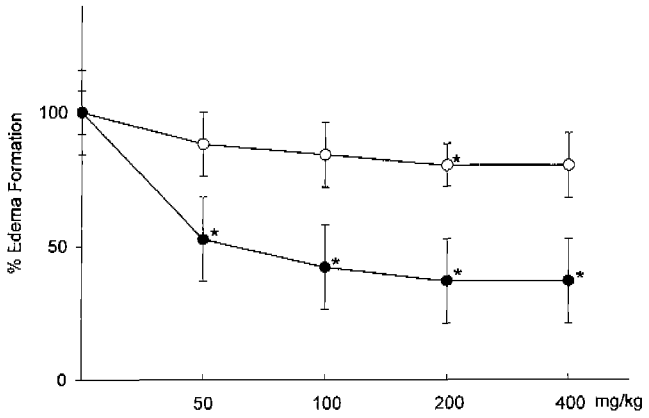

Fig. 6. Anti-inflammatory activity of *C. tinctorius* extract in mice ear edema Croton oil-induced ear edema (○), Arachidonic acid-induced ear edema (●), *P<0.05, significantly different from control.

approximately 7.9 and 1.8 mg/ml, respectively. Fig. 2b demonstrated the inhibitory activity of the extract against norepinephrine-induced vascular smooth muscle contraction. IC_{50} values for the extract and the ginkgo extract were found to be 15.4 and 1.8 mg/ml, respectively. When the same experimental conditions were used, kaempferol-3-O-rutinoside was also found to possess significant inhibitory activity against KCl- as well as norepinephrine-induced vascular smooth muscle contraction (Fig. 3). Fig. 4 showed antithrombotic activity of the extract. By oral administration, the extract clearly delayed bleeding time more than 10 times at 50 mg/kg. Aspirin used as a reference drug also showed approximately 10 times delay of bleeding time at 50 mg/kg. In order to check the antioxidative activity, antioxidative and free radical scavenging activities of the extract were examined. The extract and its standard compound, kaempferol-3-O-rutinoside, showed the significant antioxidative as well as free radical scavenging activities (data not shown). And this activity is thought to involve the lowering effects on serum cholesterol and triglyceride levels, at least in partly (Fig. 5). When the anti-inflammatory activity was examined, the extract actually showed the inhibitory activity against acute inflammation (Fig. 6), but not against chronic inflammatory animal model (data not shown). Especially, the extract strongly exhibited analgesic activity using mouse acetic acid induced writhing test (Fig. 7). IC_{50} value of the extract was found to be approximately 100 mg/kg orally. When body weight changes were examined in mice and rats for acute

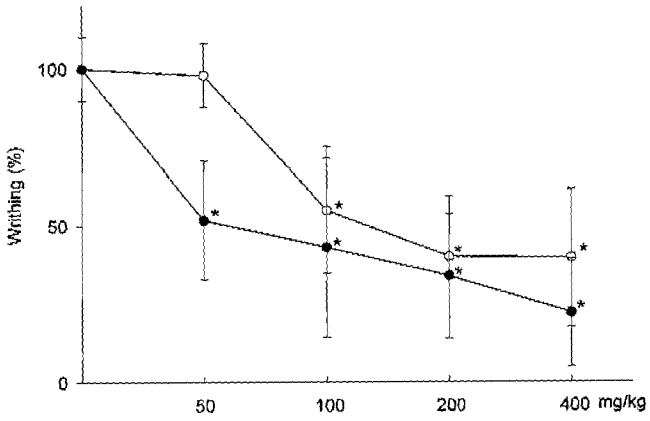

Fig. 7. Analgesic activity of *C. tinctorius* extract in acetic acid induced writhing. Aspirin (○), *C. tinctorius* (●), *P<0.01, significantly different from control.

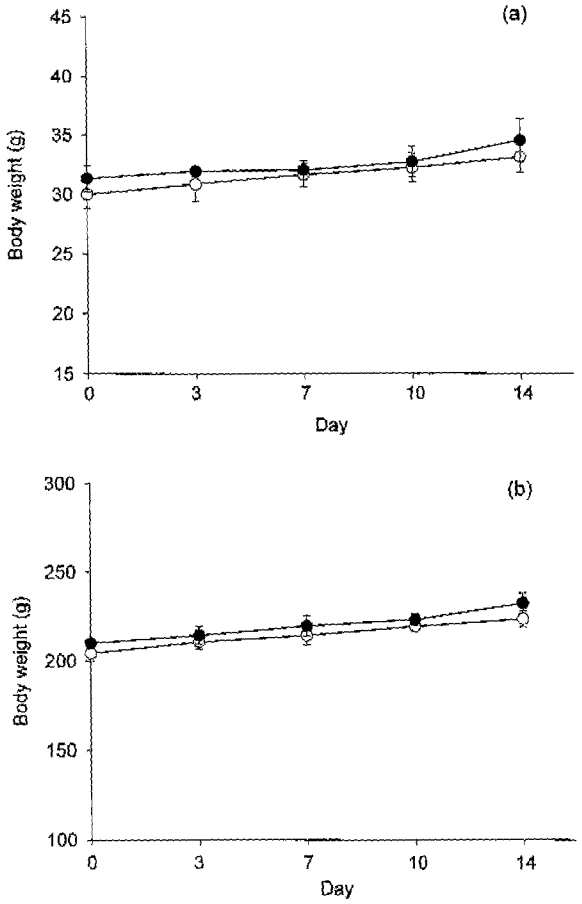

Fig. 8. Changes of body weight of mice (a) and rats (b) after single oral administration of *C. tinctorius* extract. Acute toxicity test using male and female mice and rats at 0-5 g/kg were carried out. Here represented only the data for female mice and female rats at 5 g/kg (n = 5). Vehicle (○), *C. tinctorius* (●).

toxicity test, this extract did not show any significant change for two weeks period up to the bollus dose of 5 g/kg (Fig. 8a and 8b). It was also observed that there was no dead animal and no morphological change of each organ after two weeks.

DISCUSSION

The postmenopausal disorder of elderly women is induced by the loss of ovarian follicular activity and the decreased level of estrogen (Barrett-Connor and Stuenkel, 1999). Accordingly, hormone replacement therapy is widely used and gives favorable results in most cases. However, a higher incidence of breast cancer is observed (Colditz, 1999). Therefore, there is a definite need for new agent without side effect. One of the potential candidates is plant extract therapy. Since the flowers of *C. tinctorius* have been widely used in Asia especially for postmenopausal disorder, the standard ethanol extract from the flowers of *C. tinctorius* was prepared in this study for new drug development. And biological activities focused on the treatment of postmenopausal disorder were evaluated to verify the pharmacological activities of the extract prepared because there is yet no developed animal model directly for postmenopausal disorder. They included inhibition of vascular smooth muscle contraction, anti-inflammatory activity, etc. The extract inhibited vascular smooth muscle contraction induced by KCl or norepinephrine. In addition, it possessed antithrombotic activity and lowering effects on serum levels of cholesterol and triglyceride. These results may lead to enhanced blood circulation, thereby reducing deleterious effects of postmenopausal disorder. This favorable effect of the extract was also supported by its anti-inflammatory and potent analgesic activity. Several reports demonstrated that dietary flavonoid intake reduced the risk of cardiovascular disease (Yochum *et al.*, 1999) and the bone density changes in postmenopausal women (Aloysio *et al.*, 1997). Therefore, it is suggested that the flavonoid compounds including kaempferol-3-O-rutinoside from *C. tinctorius* may be responsible for improvement of postmenopausal disorder at least in part.

From this investigation, it could be concluded that the 75% ethanol extract of the flowers of *C. tinctorius* may be useful for postmenopausal disorder by enhancing blood circulation, anti-inflammatory and analgesic activities. Kaempferol-3-O-rutinoside was found to be one of the active principles.

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