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Sedative Action of Flavonoids and Saponin from the Seeds of *Zizyphus vulgaris* var. *spinosus* Bunge

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산조인의 플라보노이드 및 사포닌의 진정작용

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The acute toxicity and sedative activity of flavonoids and saponin isolated from *Zizyphus* seeds have been evaluated in mice. All the compounds elicited potentiation of hexobarbital-induced hypnosis, inhibition of ladder-climbing and caffeine-induced hyperactivity. Swertisin was the most potent of all flavonoids tested. The potency of saponin in these tests was found to be higher than that of swertisin. The activities in rotarod test and electroshock seizure test, on the other hand, were relatively weak or nil. From these findings it was suggested that flavonoids and saponin from *Zizyphus* seeds have neuroleptic activity without anticonvulsant or muscle relaxant activity accompanied by neurological deficit.

Zizyphus vulgaris var. *spinosus* Bunge (Rhamnaceae) is a deciduous shrub and its seeds have long been reputed for treatment of insomnia and nervous breakdown in indigenous medicine.

Although the tranquilizing and sedative activities of fractions obtained from the seeds were demonstrated[1~3] and several compounds were isolated,[4~7] pharmacologically active principles have not been distinguished.

The present report deals with comparative investigation for sedative activity of flavonoids and saponin isolated from the butanol soluble fraction of methanol extract, which exhibited sedative activity. The preliminary pharmacological data on spinosin was reported in a previous communication.[8]

Materials and Methods

Animals: Male albino mice weighing 20±3g were used and fed lab. chows and tap water *ad. lib.* maintaining in a constant temperature environment throughout the experiments. Male rabbits of New Zealand White strain weighing 2.5kg were also used.

Materials: Flavonoids and saponin were isolated from the seeds of *Zizyphus vulgaris* var. *spinosus*. The plant material (40g) was repeatedly extracted (three times) with *n*-hexane, followed by methanol (five times) and the methanol extract (4.05g) was partitioned between $CHCl_3$ and water. The aqueous layer was then extracted with *n*-butanol. The *n*-butanol extract was concentrated to dryness (0.184g) and fractioned

by gel filtration with Sephadex LH-20 using methanol as an eluent to give fractions containing saponin(0.0175g, rich in jujuboside B) and flavonoids(0.132g). Rechromatography of flavonoids fraction on silica gel($CHCl_3$:MeOH:H_2O=13:7:2) allowed the separation of spinosin and subsquently mixture of acylspinosin(rich in feruloylspinosin). Recrystallization of crude saponin from methanol gave yellowish needles, mp 255~6°. Swertsin was obtained by acid hydrolysis of spinosin. Crude swertisin thus obtained was recrystallized from methanol to give yellowish crystals, mp 242~4°.

Flavonoids were suspended in a 0.5% aqueous solution of sodium carboxymethylcellulose to give *i.p.* or *p.o.* to mice in a volume of 10~20ml/kg. For *i.v.* administration, sodium salt of each corresponding flavonoids was prepared and dissolved in 0.9% NaCl solution.

Hexobarbital sodium was prepared by reacting equimolar quantity of metalic sodium with hexobarbital base(Cyclopan, TRK) in absolute methanol. Caffeine and chlorpromazine HCl were obtained commercially. Saponin, hexobarbital sodium, caffeine and chlorpromazine HCl were dissolved in 0.9% NaCl solution and administered in the same volume as described above.

Acute toxicity

The animals were administered with the test compounds to observe for any change in behavior and 72 hrs later to assess mortality. LD_{50} was calculated by up and down method.

Hemolysis test

The method of Fujita *et al.*[9] was employed. In brief, two percent blood suspension was made by mixing and then diluting one ml aliquot of rabbit blood with 3.6% citrate buffer and subsequently with isotonic phosphate buffer(pH 7.4). To one ml of this blood suspension, one ml each of the diluted saponin solution of various concentration was added, thoroughly mixed and stood at room temperature for five hrs. The hemolytic potency was determined by the dilution ratio of the saponin in the reaction mixture which caused a complete hemolysis.

Hexobarbital-hypnosis potentiation test

Five animals in each group were injected with 38mg/kg of hexobarbital sodium(a subhypnotic dose) 10min after the intravenous and intraperitoneal, or 30 min after the oral administration of graded doses of the sample. When a loss of righting reflex for more than one min was observed hypnosis potentiation was considered to be positive. HP ED_{50}, the dose which potentiated the hexobarbital-induced hypnosis in 50% of the animals was determined by probit method.

Ladder climbing test

The method described by Sandberg[10] was employed. Animals were put in a rectangular cage(36×27×27cm) supplied with a ladder, and the animals climbed the ladder within 10min were selected. Groups of 24 animals were intraperitoneally injected with graded doses of samples and put into the cage. Thirty min after the administration of the samples the number of animal which did not climb the ladder within 10 min were counted. LC ED_{50}, the dose which caused a half of the animals unable to climb the ladder was determined by probit method.

Hole-cross test

The method described by Shibata, *et al.*[3] was employed with a slight modification. Animals were put in one side of rooms of a black cage (36×27×27cm) which was devided into two rooms with a rectangular sponge sheet having a small hole 4 cm in diameter in its center and the animals which crossed the hole within 10 min were selected and used for the experiment. Immediately after the subcutaneous injection of caffeine(20mg/kg), graded doses of the test compounds were administered intraperitoneally. The frequency of crossing the hole was counted

during the first one hr after dosing. HC ED_{50}, the dose which reduced the total frequency of the caffeine-treated control by 50% was determined by probit method.

Rotarod test

A method of Dunham and Miya[11] was used. Animals which stayed for at least one min on plastic rod (4 cm in diameter) rotating at 10 revolution/min were selected and used for the test. Animals were pretreated with samples 30 min prior to the test and the number of animals dropped within one min was recorded. RR ED_{50}, the dose which caused a half of animals unable to stay on the rod during one min was determined by probit method.

Maximal electroshock seizure test

The electroshock apparatus and the method were employed according to the description by Woodbury and Davenport.[12] Animals were administered *i.p.* with the test compounds 30 min prior to the electroshock induced on the surface of the cornea through the bipolar electrode. Maximal electroshock seizure induced by supramaximal alternating current (60 cycle, 50mA) for 0.2 sec. The typical seizure (initial tonicflexion and then tonic extension) lasted approximately 22 sec. Failure to extend the hind limbs to an angle with the trunk greated than 90° was defined as protection (Swinyard, *et al.*[13]).

Results

Acute toxicity and hemolytic index

Intraperitoneal injection of each flavonoids did not bring about death to mice even at the dose of 10g/kg. At this dose, these flavonoids did not cause the animals to sleep but decreased the spontaneous movement. Any other distinct symptom could not be observed except passivity and ptosis. LD_{50} value of saponin was measured to be 110mg/kg *i.p.* and 69.6mg/kg *i.v.* No death was observed in mice which were given orally upto 1g/kg of saponin. Its hemolytic index was calculated to be 2,857 (that of reference saponin was 40,000).

Effect on Hexobarbital-induced hypnosis

The effect of flavonoids and saponin on hexobarbital-induced sleep was tested and the results were summarized in Table I. For comparison in detail of the potency between test compounds with respect to the route of administration, the samples were treated *i.p.*, *i.v.* as well as *p.o.* Regardless of the route of administration, the potency of the flavonoids and saponin was found to be relatively low as compared with that of chlorpromazine, but it is obvious that they showed significant potentiation of hexobarbital-induced hypnosis and thus have the synergistic effect on barbiturate.

Table I. Effect of flavonoids and saponin on hexobarbital-induced hyponsis

Treatment / Compounds	HP ED_{50} (mg/kg)		
	p.o.	*i.p.*	*i.v.*
Spinosin	72.2	35.2	20.8
Swersisin	58.2	24.8	13.3
Acylspinosin	332.9	148.1	68.6
Saponin	54.9	13.4	10.0
Chlorpromazine-HCl	3.3	2.0	2.0

It was observed that swertisin and spinosin, when administered orally, have similar ED_{50} values which were determined to be 58.19 and 72.16mg/kg, respectively. Acylspinosin, however, showed a weak activity (about 1/4 of that of spinosin) compared to the other compounds tested. The patency of saponin was approximately equal to that of spinosin.

In case of *i.p.* and *i.v.* administration of the test compounds, the potency of swertisin was revealed to be the strongest among flavonoids. Swertisin was approximately equipotent to saponin in potentiating effect, when it was admin-

istered intravenously. Acylspinosin, when treated intraperinoneally, exhibited the least activity, being 1/4 times as potent as spinosin. The potency of acylspinosin, with *i.v.* administration, was also shown to be 1/3 times as potent as spinosin, which represents about a five fold increase over that of oral administration of it. The degree of the potentiating activity of saponin, when administered *p.o.*, was approximately 1/5 times as strong as those of intraperitoneal and intravenous administration of it.

Effect on ladder-climbing activity

The inhibitory effect of the test compounds on ladder-climbing activity in mice was evaluated and summarized in Table II. Swertisin exhibited the most potent inhibitory activity among flavonoids. The potency was five times stronger than that of spinosin. The activity of acylspinosin was rather weak compared with that of spinosin. The activity of saponin in suppressing ladder-climbing was stronger than that of flavonoids and approximately 2.6, 14 and 24 times as potent as swertisin, spinosin and acylspinosin, respectively.

Effects on caffeine-induced hyperactivity

The inhibitory effect of the test compounds on caffe-ineinduced hyperactivity was evaluated and shown in Table II. Swertisin was approximately equipotent to spinosin and acylspinosin had the least activity among the flavonoids, being about 1/2 times as potent as the other two flavonoids. The potency of saponin was about seven times as strong as spinosin.

Effects on rotarod performance

The results on rotarod test were summarized in Table II. Not only flavonoids but also saponin showed a weak activity in rotarod performance. Acylspinosin showed no inhibitory effect even at a dose of 1g/kg *i.p.*

Effect on electroshock seizure

The inhibitory effects of flavonoids and saponin on maximal electroshock seizure were tested (Table II), however, it was revealed that neither flavonoids nor saponin gave any influence on the seizure even at the highest dose used. Saponin showed no protective effect even at a dose of 100mg/kg *i.p.* which is near its LD_{50} value.

Discussion

The neuropharmacological properties of flavonoids and saponin isolated from *Zizyphus* seeds were tested and compared utilizing several experimental models for evaluation of CNS-depressant activity.

As a result in general, it was demonstrated that flavonoids and saponin exhibited a significant activity in tests such as hexobarbital-induced hypnosis potentiation, ladder-climbing and hole-cross but were inactive in electroshock seizure test. It was also shown that the test

Table II. The evaluation of sedative activity of flavonoids and saponin in ladder-climbing test, rotarod test and maximal electroshock seizure test*

Compounds	Treatment	LC ED_{50} (mg/kg)	HC ED_{50} (mg/kg)	RR ED_{50} (mg/kg)	MES ED_{50} (mg/kg)
Spinosin		247.0	49.2	995.0	>1,000
Swertisin		46.0	44.5	950.0	>1,000
Acylspinosin		421.0	99.8	>1,000	>1,000
Saponin		17.5	7.0	71.5	>100
Chlorpromazine-HCl		1.0	1.8	7.1	—

*Samples were administered *i.p.*

compounds possess only weak activity even at the highest dose in the rotarod test. These results strongly suggest that both flavonoids and saponin have a significant neuroleptic activity but are devoid of anticonvulsant or muscle relaxant activity accompanied by neurological deficit. Swertisin, by parenteral administration, was found to exhibit the most potent activity of the flavonoids tested. In the experiment for measurement of barbiturate-induced hypnosis, swertisin was demonstrated to exhibit about a four fold stronger activity by intravenous administration than by oral administration. Its potency by *i.v.* was almost equal to saponin. In case of acylspinosin, the potency was shown to increase about five fold by *i.v.* administration. The difference of potency between *i.v.* and *p.o.* of spinosin was relatively small. From these results, it can be postulated that spinosin and its acylated derivatives might be absorbed in the gastrointestinal tract and biotransformed into swertisin to elicit their sedative activities. Flavonoids caused no death to mice treated with them even at a dose of 10g/kg *i.p.* This indicates that flavonoids are physiologically innocuous. Saponin, however, is considered to be rather toxic compared to flavonoids judging from their acute toxicities and hemolytic index of saponin.

In the schematic fractionation of *Zizyphus* seeds, it was demonstrated that the yield of flavonoids was about 8 times larger than that of saponin. And furthermore, swertisin was evidenced to exist in trace amount in the seeds, although it showed the highest sedative activity among the flavonoids. Consequently, spinosin and saponin seem to play a leading role in pharmacological action of *Zizyphus* seeds.

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