Chemistry and Pharmacology of Flavone-C-Glycoside from Zizyphus Seeds

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Introduction

Zizyphus jujuba alias *Zizyphus vulgaris* var. *spinosus* is a deciduous shrub and its seeds have long been used for treatment of insomnia and nervous breakdown in Korea and other parts of Asia as well.

This chinese drug is known to be mild in effect and not to induce any drug dependence and tolerance on chronic administration.

The chemical investigations so far on this plant seeds as far as we know resulted in isolation of triterpenoids[1], betulin (1) and betulinic acid (2) and saponins[2] named jujubosides A (3) and B (4). Some pharmacological works have also been done[3,4], however, active components have not been identified.

Therefore, we set to work on sedative components of this plant and firstly found that saponin fraction as well as flavonoid fraction possessed some mild sedative activity through preliminary experiment. This communication deals with isolation of C-glycosylflavones, one known compound (5) and four new flavonoids (6-9) and their sedative activity.

Isolation

Scheme 1 shows the procedure of extraction and fractionation. The seeds were defatted with petroleum ether and then extracted with methanol. The methanol extract was fractionated as shown and each fraction was investigated to determine which fractions possess sedative activity.

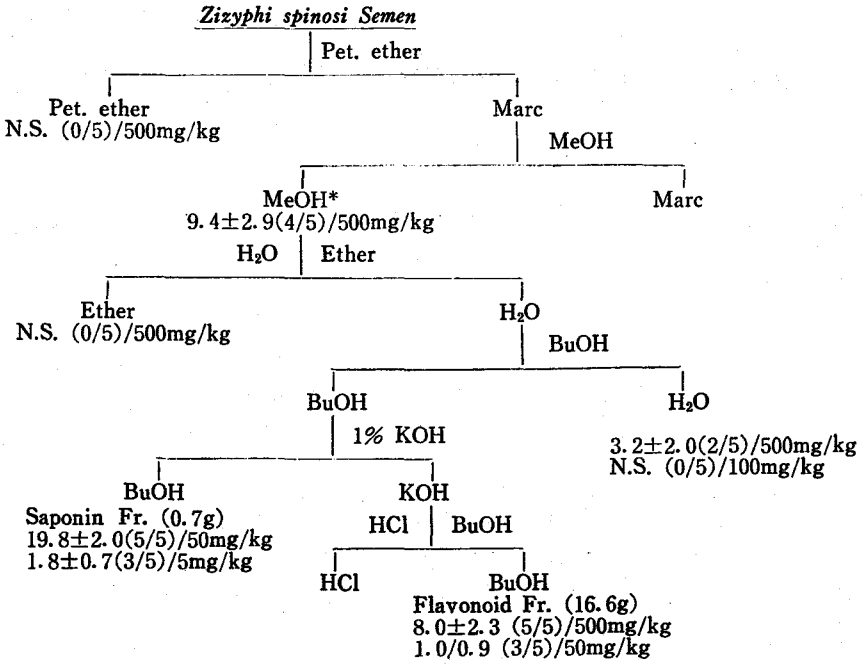
As a result, it was found that saponin and flavonoid fractions were active.

In animal experiment, male mice were employed. Each fraction was given intraperitoneally and then 10 min later 38mg/kg of hexobarbital sodium was injected, and the sedative activity was determined by measuring the duration of sleep which was induced by hexobarbital sodium administration. At this dose of hexobarbital sodium, the mice in the control group got into a state of ataxia, but they were not asleep.

Administration of saponin fraction induced sleep for 2 min at a dose of 5mg/kg and for 20min at a dose of 50mg/kg. In case of flavonoid fraction, sleep was induced for 1 min at 50mg/kg dose and for 8 min at 500mg/kg dose. The denominators in parentheses represent the number of mice used in the experiment and numerators, the number of mice which fell asleep.

So at 5 mg/kg dose of saponin fraction 3 mice out of 5 mice got to sleep and all 5 mice became asleep at a dose of 50 mg/kg.

From this result it can be estimated that hexobarbital induced sleeping time potentiation activity of saponins is about 10 times stronger than that of flavonoids. However yield of flavonoids was 16g, which was 24 times larger than that of saponins. Therefore the flavonoids



* Sleeping time±S.E./dose
(No. Anim. slept/No. Anim. used)
38mg/kg Hexob.: ataxia; no sleep (N.S.)

Scheme 1. Fractionation of flavonoids

were considered to play a main role in pharmacological action of *Zizyphus* seeds.

Repeated chromatography of flavonoid fraction on silica gel ($CHCl_3$-MeOH-H_2O=13:7:2) gave swertisin, a known compound and a new compound named spinosin and acylated spinosin mixture which was further chromatographed using another eluent (EtOAc-MeOH-H_2O=6:1:0.7) to yield three compounds called A,B and C in order of decreasing polarity.

Chemistry

Swertisin: $C_{22}H_{22}O_{10}$, mp 242-4°, $[\alpha]_D^{24}$+40.7°, which was first isolated by Nakaoki[5] from *Swertia japonica* (Gentianaceae) and structure of which was elucidated by Komatsu, *et al.*,[6] was identified by mmp, UV, NMR and co-TLC with an authentic sample.

Spinosin: $C_{28}H_{32}O_{15}$ .H_2O, mp 255-6°, $[\alpha]_D^{24}$ +16.5° was elucidated to be 2″-O-β-glucosylswertisin. In order to prove the fact that the second glucose is linked to 2″-position of swertisin, spinosin was methylated to dimethylether by treatment with diazomethane. The UV spectrum of this compound was not changed by shift reagents. And acid hydrolysis of this compound gave trimethylisovitexin. Moreover treatment of spinosin with acetone gave diacetonide. These results indicate that second glucose moiety could exist at the position of 2″- or 3″ rather than 4″, 6″ or phenols. In NMR spectrum (DMSO-d_6) of spinosin, oxymethyl signals appeared at δ3.93, two anomer proton signals appeared at δ4.23 and 4.75, respectively, as a doublet. The peak at δ 4.23 is due to O-glucoside anomer proton and J value of 7Hz indicates β-orientation of O-glucosidic linkage. The singlet equivalent to two protons at δ6.8 is assigned to the hydrogens at 3-and

8-positions. Two pairs of *ortho* coupled doublets due to aromatic protons on B ring appeared at δ6.97 and 7.97, andaphenolic proton signal at 5-position appeared at δ12.4.

β-orientation of O-glucosidic linkage was proved by not only NMR data but also molecular rotation difference between spinosin and swertisin[7]. The value of difference ($-81°$) is very similar to the molecular rotation ($-61°$) of β-methyl-glucoside.

NMR spectrum ($CDCl_3$) of spinosin peracetate showed acetyl signals in the high field region and one of them appeared at unusally high field position, δ1.83. NMR ($CDCl_3$) of spinosin octaacetate prepared under milder conditions showed a signal at δ1.78. Such shift values are within a typical range for 2″-acetate of 6-C-glucosylflavone[8]. This observation made us infer the presence of acetyl group at 2″-position. Therefore 1-3 glucosidic linkage in the spinosin structure was once suggested.

However the mass spectrum of permethylated spinosin shows the base peak at m/e 499, which is derived from molecular ion having 734 mass units by the loss of O-glucoside moiety and an intense peak at m/e 515 derived by the loss of glucoside, but the peaks for fragmentions, corresponding to the loss of methyl and loss of methoxy from molecular ion can hardly be recognized.

Recently, Chopin and co-workers[9,10] reported the mass data of flavone-C-O-glucosides and found that the 2″-substituted permethylated 6-C-glucosylflavones could easily be distinguished from 3″, 4″ or 6″ isomer, by the absence of M-15 and M-31 peaks. Base peak for these compounds was M−sugar peak and M−sugar without oxygen peak was abundant.

According to their finding these mass fragmentation patterns strongly suggest the presence of 1-2 glucosidic linkage in spinosin structure.

In order to clarify such an ambiguity, we conducted the decoupling experiment on hexamethylisovitexin monoacetate which was prepared from permethylated spinosin by hydrolysis and then acetylation. NMR spectrum of this substance showed an anomer proton signal at δ4.88 as a doublet with J value of 10Hz and acetylated oxymethine proton signal at δ5.82 as a multiplet. In this case acetyl methyl signal appeared at δ1.81. When the proton at δ5.82 was irradiated, the anomer proton signal collapsed to a singlet. Moreover when the anomer proton was irradiated, the multiplet at δ5.82 was converted into a doublet. These observations indicate that the acetylated oxymethine proton must be on 2″-C. Therefore the point of attachment of second glucose moiety to swertisin was settled to be 2″-position of swertisin.

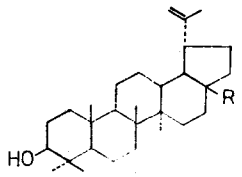
The ^{13}C NMR spectrum (DMSO-d_6) of spinosin was also in agreement with this structure. Each of the values of the flavone moiety, C-glucoside and O-glucoside moieties was compared with each of reported values of genkwanin[11], and C-glucoside portion of isoorientin[12] and methylglucoside[13]. The chemical shifts observed were almost the same as those reported with the exception of those for 6-C and 2″-C which were displaced downfield by +10.5 and +9.9 ppm, respectively. This evidence also revealed that spinosin must be represented by 2″-O- glucosylswertisin.

Recently, Wagner, *et al.*[14] isolated two new 2″-O-glycosylated flavone-C-glycosides, 2″-O-glucosylisovitexin-7-O-galactoside and 2″-O-rhamnosy isovitexin-7-O-galactoside, from *Melandrium album* and encountered upfield acetyl signals at δ1.78 and 1.71, respectively, in the NMR of peracetates.

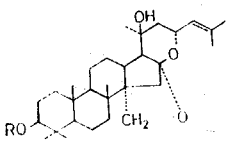
Therefore, we would like to point out that there exists an exception in certain compounds in deducing presence of acetyl group at the

2″-position of flavone-C-glucoside from proton NMR data.

Acylated spinosin A(7): $C_{39}H_{42}O_{19}\cdot 2H_2O$, mp 198-204°, $[\alpha]_D^{20}$ -40.5°; **B(8)**, $C_{38}H_{40}O_{18}\cdot 2H_2O$, mp 194-7°, $[\alpha]_D^{20}$, -45.2°, **C(9)**, $C_{37}H_{38}O_{17}\cdot 2H_2O$ proved to be 6‴-sinapoyl-, 6‴-feruloyl-, and 6‴-*p*-coumaroyl spinosin, respectively. Acid hydrolysis gave swertisin, glucose and corresponding cinnamic acid derivatives whereas spinosin was obtained on mild alkaline hydrolysis. Acetone treatment yielded monoisopropylidene derivatives. Permethylated compounds and permethylated isopropylidene derivatives showed in their mass spectra the intense peaks at m/e 499 and 515 and m/e 511 and

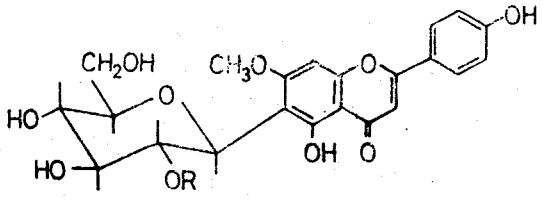


1. Betulin R=CH_2OH
2. Betulinic acid R=COOH

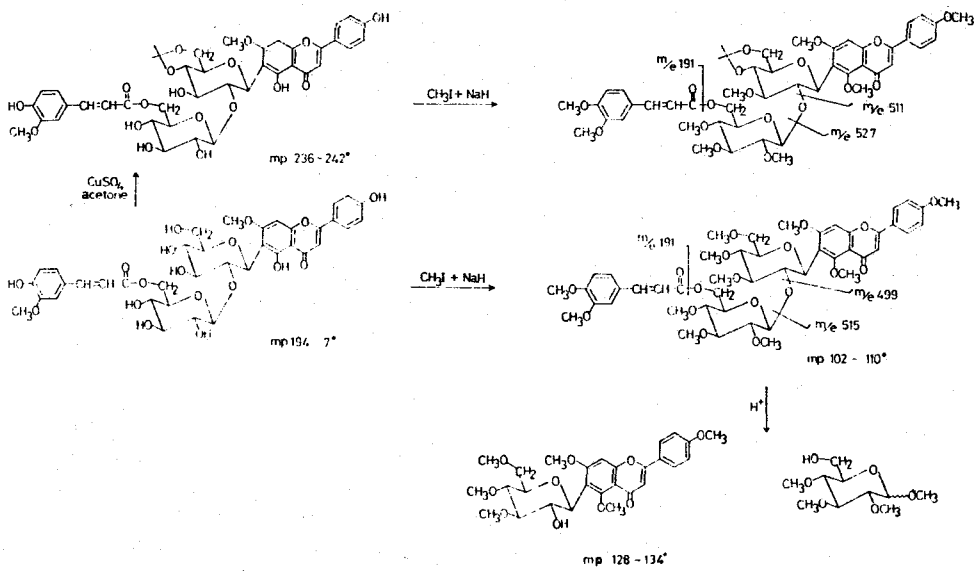


Jujuboside

3. A : R=$\begin{matrix}\text{D-Glc}(\beta1\to6)\\ \text{D-Xyl}(\beta1\to2)\end{matrix}$ > $\begin{matrix}\text{L-Rha}(\alpha1\to2)\\ \text{D-Glc}(\beta1\to3)\end{matrix}$ > L-Ara
4. B : R=D-Xyl(β1→2) $\begin{matrix}\text{L-Rha}(\alpha1\to2)\\ \text{D-Glc}(\beta1\to3)\end{matrix}$ > L-Ara



5. Swertisin R=H
6. Spinosin R=Glc
7. Acylspinosin A R=6‴-Sinapoyl-Glc
8. Acylspinosin B R=6‴-Feruloyl-Glc
9. Acylspinosin C R=6‴-p-Coumaroyl-Glc



Scheme 2. Derivatization of acylspinosin B

527, corresponding to the loss of permethylated acyl glucosyl moiety with and without oxygen from respective molecular ions (Scheme 2).

These results demonstrate that acyl residues must be on the 4‴ or 6‴-position. Methanolysis of each of permethylated compounds gave 2″-OH free permethylated isovitexin and methyl-2, 3, 4-tri-O-methylglucoside. Therefore the point of attachment of acyl residue proved to be 6‴-position.

^{13}C-NMR chemical shifts of compound B were in agreement with the formulation of this compound as 6‴-feruloylspinosin.

The signals for the sugar carbon atoms appeared in the region from 81.4 ppm to 61.3 ppm. Compared with the corresponding carbon resonances in the spectrum of spinosin, the 6‴-carbon signal in the compound was 2.1 ppm downfield and 5‴-carbon signal was 2.3 ppm upfield. Such changes in the chemical shifts of 6‴-carbon and 5‴- carbon can only be explained if the primary hydroxyl group at 6‴- position is esterified with ferulic acid.

Pharmacology

In this section we will discuss the pharmacology of *Zizyphus* flavonoids. The pharmacology of jujuboside B which is main saponin of *Zizyphus* seeds was also included for comparison.

Toxicology: Intraperitoneal injection of each compound did not bring about death to mice even at the dose of 10 g/kg.

At this dose, these flavonoids did not put the animals to sleep but decreased spontaneous movement. Any other distinct symptom could not be observed except passivity and ptosis. This finding indicates that *Zizyphus* flavonoids are physiologically inocuous.

LD_{50} value of jujuboside B, was measured to be 110 mg/kg and haemolytic index was 2800. So the saponin is rather toxic compared with the flavonoids.

Potentiation of hexobarbital: Table I shows the effect on hexobarbital induced sleep in mice. Experimental conditions were the same as mentioned before.

Table I. Effect of flavonoids and saponins on hexobarbital induced hypnosis in mice.

Treatment	Dose (mg/kg, i.p.)	No. of mice	Potentiation of sleeping time (min)
Control	—	5(0)	N. S.
Swertisin	50	5(5)	5.0±1.3
	100	5(5)	7.6±2.6
	500	5(5)	14.2±0.4
Spinosin	50	5(3)	3.2±2.5
	100	5(4)	4.6+1.9
	500	5(5)	14.0±1.0
Acylspinosin*	50	5(1)	1.4±1.4
	100	5(2)	2.8±2.0
	500	5(4)	10.0±3.4
Saponin**	5	5(3)	1.8+0.7
	10	5(4)	6.6±2.2
	50	5(5)	19.8±2.0
Chlorpromazine	1	5(2)	9.2±6.7
HCl	2	5(5)	19.8±4.5
	3	5(5)	48.8±6.9

Test materials were administered 10 min prior to the injection of hexobarbital sodium (38mg/kg, i.p.). Hexobarbital caused ataxia and no sleep. N.S.=no sleep.

Figures in parentheses represent number of mice which slept.

* rich in compound B

** rich in jujuboside B

Figures in parentheses represent the number of mice which fell asleep. Chlorpromazine, one of the potent tranquilizing agents, was used as a positive control substance. Chlorpromazine exhibited a profound hypnotic effect. The potency of flavonoids is relatively low as compared with that of chlorpromazine but it is obvious that flavonoids have the synergistic effect on

barbiturate.

Among them swertisin showed the most potent activity. Acylspinosin rich in compound B showed slightly weak activity. Activity of saponin rich in jujuboside B was about 10 times stronger than that of flavonoids.

Activity in ladder-climbing test: Table II shows the effect on ladder-climbing activity in mice. One hr. after injection of test compounds animals were put into the cage supplied with a ladder in it, and the number of animals which did not climb the ladder within 10 minutes was counted.

Table II. Effect of flavonoids and saponins on ladder-climbing activity in mice.

Treatment	Dose (mg/kg, i.p.)	No. of mice uncli-mbed*	Inhibi-tion (%)	CD50 (mg/kg)
Control	—	0(24)	0	
Swertisin	50	12(24)	50	
	100	16(24)	67	46
	200	18(24)	75	
Spinosin	100	6(20)	30	
	200	8(20)	40	247
	500	14(20)	70	
Acylspinosin **	200	2(10)	20	
	500	6(10)	50	421
	1000	8(10)	80	
Saponin***	10	3(10)	30	
	20	5(10)	50	17.5
	50	9(10)	90	

* The number of animals which did not climb the ladder during 10 min periods at 1 hr after dosing. Fugures in parentheses are the number of mice used.
** rich in compound B
*** rich in jujuboside B

Figures in parentheses represent the number of animal used.

CD_{50} value, that is, the dose necessary to reduce the number of climbing mice to 50% was calculated to be 46 mg for swertisin but 247 mg for spinosin. Activity of acylspinosin was weak compared with that of spinosin. Activity of saponin was very stronger than those of flavonoids.

Activity in hole-cross test: Table III shows the effect on caffeine-induced hyperactivity in mice. Test compounds were given immediately after the injection of 20 mg/kg of caffeine. And then animals were put into the cage divided into two rooms with sponge sheet having a small hole 4 cm of diameter in its centre. The frequency of hole crossing was counted during first 1 hr after drug administration.

Table III. Effect of flavonoids and saponins on caffeine induced hyperactivity in mice (Hole-Cross Test).

Treatment	No. of mice	Dose (mg/kg, i.p.)	Freq. of Crossing for 1 hr (Mean± S.E.)	Inhibi-tion (%
Control				
Caffeine untreated	5	—	30.8±9.4	
Caffeine treated	5	—	56.2±8.2	
Spinosin	5	100	10.6±4.7	81.1
	5	200	4.8±2.6	91.5
	5	500	2.0±0.5	96.4
Acylspinosin*	5	100	23.8±4.9	57.7
	5	200	20.8±2.6	63.0
	5	500	11.2±6.4	80.1
Saponin**	5	10	14.0±4.1	75.1
	5	50	1.4±0.8	97.5
Chlorpromazine HCl	5	3	10.8±8.0	80.9
	5	6	4.4±4.3	92.2

Test compounds were administered i.p. immediately after s.c. injection of caffeine (20mg/kg).
The hole crossing was checked for 1 hr after dosing.
* rich in compound B
** rich in jujuboside B

Normal mouse crossed the hole 30 times for 1 hr on the average.

When 20 mg/kg of caffeine was injected, the frequency of the hole crossing was increased to 56 times. All compounds tested significantly decreased the frequency of the hole crossing, and the activity of saponin was 10 times stronger

than that of spinosin.

Activity in rotarod test: Table IV shows the results in rotarod test. Test compounds were given 30 minutes before testing and animals were placed on rotating rod at a rate of 10 revolutions per min. And the number of mice which fell off the rod within 1 min was counted.

Table IV. Activity of flavonoids and saponins in the rotarod test

Treatment	Dose (mg/kg,i.p.)	No. of mice dropped*
Spinosin	200	1(6)
	500	2(6)
	1000	3(6)
Acylspinosin**	200	0(6)
	500	0(6)
	1000	0(6)
Saponin***	20	0(6)
	50	2(6)
	100	4(6)
Chlorpromazine HCl	5	1(6)
	10	5(6)

The dose was given 30 min before testing.

* The number of mice fell off the rod (10 revolutions/min) within 1 min. Figures in parentheses are the number of mice used.

** rich in compound B

*** rich in jujuboside B

Figures in parentheses represent the number of mice used. All compounds showed weak activity or inactivity in rotarod test. It means *Zizyphus* seeds did not cause neurological deficit.

Conclusion

In conclusion five flavone-C-glycosides isolated from *Zizyphus* seeds were found to have mild sedative activity through preliminary animal experiments using four typical models for the test of CNS-depressant activity.

Although saponins showed potent activity, flavonoids seem to play a leading role in pharmacological action of *Zizyphus* seeds considering the high content of them.

Among the flavonoids swertisin showed the highest sedative activity. However it existed in trace amounts.

Consequently it is postulated that main pharmacologically active principles of *Zizyphus* seeds might be spinosin and its acylated derivatives.

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