Screening of Bioactive Compounds in Oriental Medicinal Drugs

Ushio Sankawa
Faculty of Pharmaceutical Sciences, University of Tokyo
Tokyo, Japan

Introduction

Medicinal drugs based on traditional medicine have been widely used in Southeast Asian countries. In Japan, Chinese medicinal drugs consisting of various medicinal plants are still in clinical use. Recently, about 100 kinds of Chinese drug recipes are officially approved as the drugs covered by social health insurance. The real actions and active principles of the drugs prescribed by the systematic theory of Chinese medicine are very interesting and challenging subject of study. Although a large number of compounds was isolated from Chinese medicinal drugs, only a few is recognized as active principles. Ephedrine, an alkaoids of *Ephedra* herb, is one of the limited examples whose pharmacological activity and function in the prescriptions has been firmly established by scientific investigations. Recently, pharmacological studies on Chinese medicinal drugs have revealed that constituents isolated in phytochemical studies actually possess various biological actions. Takagi and Saito reported that Ginseng saponins exhibited both stimulative and depressive actions upon central nervous system (CNS), antifatigue activity and stimulation for discrimination learning.[1] In biochemical study, Ginseng saponins were shown to stimulate RNA and protein syntheses in rat liver cells,[2] and to potentiate the effect of nerve growth factor (NGF) in organ cultures of chick embrionic dorsal root ganglia.[4] Saponins of *Bupleurum* root and *Platycodon* root exhibited antiinflammatory and expectorant actions as expected from their use in Chinese medicine.[5]

Recently, two Japanese groups have demonstrated the effectiveness of the work in which isolation of bioactive compounds contained in Chinese medicinal drugs is carried out in parallel with bioassay test. They isolated cardioactive substances, demethylcoclaurine and coryneine, from *Aconitum* root, which is applied to patient who requires general potentiation.[6] A hypotensive alkaloid was isolated from *Ephedra* root by monitoring hypotensive activity in isolation process.[7]

In most of Chinese medicinal drugs, their actions have not been fully proved with biological tests. It is generally recognized that the action of Chinese medicinal drug is very weak and the detection of the action with pharmacological screening, such as behavior test, was very difficult. Another difficult problem in studying active principles of Chinese medicinal drugs is to choose bioassay tests best fit to their use in Chinese medicine. Description in the books of Chinese medicine is quite different from that of modern medicine and terms to indicate biological actions are very difficult to be interpreted by modern pharmacology. To overcome these problems we have applied random screening method using *in vitro* bioassay tests to the investigation of bioactive

compounds in Chinese medicinal drugs. *In vitro* bioassay tests are provided with high sensitivity and efficiency which are prerequisite to our investigation. This method certainly has advantages over *in vivo* pharmacological tests, however lack of direct correlation to *in vivo* activity would be a main disadvantage. In ancient time, Chinese medicinal drugs were selected from numerous plants by the repetition of "trial and error". It was the first screening of Chinese drugs and what we want to do is the secondary screening. If bioassay test is well suited for the biological activity of Chinese medicinal drugs, they will show the activity in much higher probability.

Preparation of Extracts

Since Chinese medicinal drugs are mainly taken as decoction, their extracts for the screening were prepared from hot water extracts by frozen-dried procedure. About 250 kinds of drugs available in Japanese and Taiwan markets were submitted for the tests.

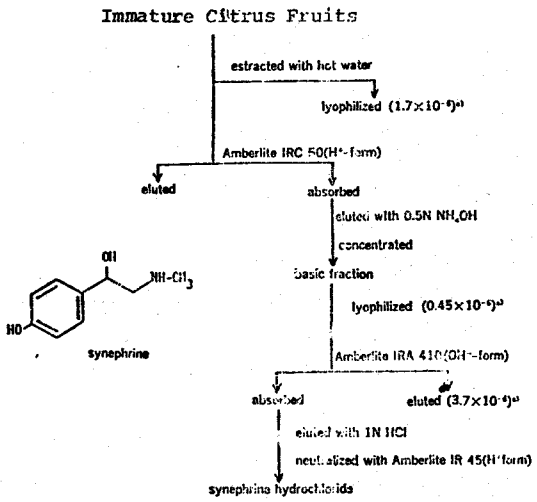
Active substances for smooth muscle preparation

Two kinds of smooth muscle preparations were used in bioassay. In an experiment using uterus from estrogen treated rat nine samples out of 150 showed inhibition against contraction induced by serotonin. *Ephedra* herb played a role of positive control in this screening, because ephedrine is β-adrenergic substance and possesses competitive action against the contraction induced by serotonin. Of nine active extracts four originate from plants belonging to *Citrus* species and active substance was expected to be the same. Immature *Citrus* fruits were extracted with hot water and fractionated as illustrated in Chart 1.

Table 1. Inhibitory action of Chinese medicinal drugs against rat uterine contraction induced by serotonin and the contents of synephrine in the drugs

Drugs	ID_{50} g/ml	Synephrine%
Immature Citrus peel	1.6×10^{-7}	0.26
Citrus peel	2.7×10^{-7}	0.22
Ephedra herb	1.4×10^{-6}	—
Imature Citrus fruit	1.7×10^{-6}	0.21
Citrus fruit	1.8×10^{-6}	0.13
Sinomenium stem	1.5×10^{-6}	—
Magnolia flower	4.0×10^{-6}	—
Zanthoxylum fruit	3.0×10^{-6}	—
Evodia fruit	1.0×10^{-5}	0.16

Chart 1. Extraction and purification of synephrine from Immature *Citrus* Fruits



Active substance was absorbed by both cation and anion exchanging resins. Finally the active compound was obtained as hydrochloride and identified as synephrine hydrochloride by spectral investigation. Synephrine hydrochloride showed $[\alpha]_D$-22.6°, whereas authentic value is reported to be -55.6°. Thus, sample obtained from immature *Citrus* fruits is a quassi racemate. Synephrine is a synthetic sympathomimetic drug developed for oral administration[8]. Stewart *et al.* isolated synephrine from juice of tangerine

as a compound showing hypertensive action.[9] The presence of synephrine in immature *Citrus* fruits is well in accord with the observation that the aqueous extracts suppress uterine movement and caused hypertension. Synephrine contents in the drugs listed in Table 1 were determined by TLC densitometry using Shimadzu Double Wavelength Chromatoscanner CS 900. Basic fractions were developed on TLC and the spot of synephrine was detected by ninhydrin colouration. The drugs of *Citrus* origin contain 0.13-0.26% of synephrine.[10] The results indicate that synephrine is a main active principle in these drugs. In our separate work on minor basic constituents of *Evodia* fruit, synephrine was isolated along with 6-methoxy-N, N-dimethyltryptamine.[11] Although synephrine content in *Evodia* fruits was 0.19%, ID_{50}(50% inhibition concentration) of the extract was lower than those of the drugs of *Citrus* origin. This observation was reasonably explained by assuming the presence of a substance contracting rat uterus. The basic fraction from which synephrine had been removed by passing anion exchanging resin contracted rat uterus and guinea pig illeum. Characterization of this bioactive substance is now under investigation. Old Chinese books on medicinal plants describes that immature *Evodia* fruits should be used for drug. We examined time course variation of synephrine content in *Evodia* fruits. As appears in Figure 1, synephrine content in the fruits was highest just after fruit formation and then decreased rapidly. It would be the reason why immature fruits should be used for the drug.[11]

Fig. 1. Changes of the contents of synephrine in some specimens of fruits of *Evodia rutaecarpa*, harvested at intervals of 1-2 weeks from full bloom.

Phosphodiestrase inhibitors

Since Sutherland found cyclic AMP as a messenger inside cells,[12] many observations regarding synchronized changes of cAMP level and physiological conditions have been reported.[13] cAMP triggers successive reactions leading to the induction of enzymes that play definitive roles in various physiological phenomena.[14] The level of cAMP concentration is controlled by two enzymes, adenyl cyclase mediating reaction to form cAMP from ATP and phosphodiesterase (PDE) catalyzing the hydrolysis of cAMP to give AMP. Compound inhibits PDE would act to increase the concentration of cAMP, resulting in the induction of physiological changes. Weinryb *et al.* reported PDE inhibition by drugs used clinically.[15] In addition to well known PDE inhibitors, such as papaverine and theophylline, chloramphenicol, testosterone, ethinylestradiol, diazepam and oxytocin were found to be potent inhibitors of PDE. Therefore, we can expect a variety of actions for PDE inhibitors obtained from Chinese medicinal drugs. Series of investigations on enzyme inhibitors of microbial origin have been reported by Umezawa's group. They isolated reticurol,[16] PDE-I, PDE-II,[16] isoflavones and dehydrodicaffeic acid dilactone[17] as PDE inhibi-

Table 2. PDE Inhibition activity of Chinese medicinal drugs

Samples	Inhibition ratio (%) 10^{-4}g/ml			
	H_2O ext.		CH_3Cl frac.	H_2O frac.
	I	II		
Iris root	44.3	46.6	77.3	—
Polygala root	52.5	43.4	84.4	36.9
Glycyrrhiza root	45.5	72.1	72.4	34.8
Nepeta herb	31.6	65.1	67.0	—
Cassia seed	38.2	61.7	88.1	—
Daphne flower	51.0	66.9	70.4	44.2
Carthamus flower	30.7	39.0	11.7	—
Bupleurum root	38.1	43.1	74.1	—
Asiasarum root	34.4	37.5	63.3	—
Zanthoxylum fruit	36.2	51.5	—	—
Fraxinus bark	54.4	60.6	26.6	0.07
Immature Citrus peel	43.3	40.9	81.4	—
Nuphar root	51.6	57.9	60.1	40.7
Inula flower	84.9	38.2	11.5	0.1
Amomum fruit	42.7	42.3	67.0	0.03
Perilla leaf	30.4	42.3	62.6	0.07
Areca peel	33.8	31.8	78.6	26.8
Aralia root	39.2	47.9	67.4	—
Bamboo stem	61.7	47.9	78.4	47.7
Anemarrhena root	48.0	49.1	94.5	—
Caesalpinia stem	68.8	59.2	85.7	61.1
Forsythia fruit	44.7	34.6	56.3	37.3

tors. These PDE inhibitors exhibited hypotensive action in spontaneous hypertensive rats (SHR).

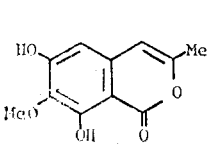
About 250 frozen-dried aqueous extracts of Chinese medicinal drugs were tested for PDE inhibition. Assay method employed in our work is based on Brooker's procedure with some modification,[18] and commercially available PDE was used for the bioassay. Screening was carried out with a final concentration of 100μg/ml and those showed more than 30% inhibition twice in two successive tests were submitted to further investigation.

Thirty samples out of 250 extracts tested showed reproducible activity. Several drugs inhibited all enzyme reactions so far we have tested and their inhibition were regarded as nonspecific inhibition caused by polyphenolic compounds like tannins. As it appears in Table 2, most of the active compounds were soluble in chloroform, but in some cases both chloroform and water soluble fractions were active. It was not expected before that hot water extracts contain a large amount of lipophylic compounds and they are the real active principles in PDE inhibition. Some examples of PDE inhibitors identified in our work will be discussed.

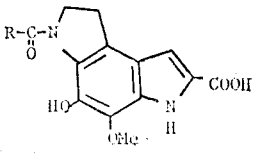
Polygala root: Polygala root is a Chinese medicinal drug derived from *Polygala tenuifolia* (Polygalaceae). It is used as tonic and sedative agent in recipes. The extract of Polygala roots inhibited PDE reaction about 50% at a dose of 100μg/ml. Fractionation and bioassay revealed that two kinds of active substances were present in the extract, one is soluble in chloroform and the other is insoluble in chloform and soluble in butanol. The chloroform fraction was further fractionated by column chromatography and preparative TLC, and an active compound was finally identified as oleic acid. Following to this finding various fatty acids were tested for PDE inhibition, and C_{16} and C_{18} unsaturated fatty acids were found to be most active. At this moment, it is not clear, if unsaturated fatty acids can function as PDE inhibitors *in vivo*.

Butanol soluble PDE inhibitors showed characteristic behavior of saponin on TLC and the samples of saponins, onjisaponin A-F, isolated from *Polygala* roots by Shoji *et al.*[19] were examined for PDE inhibition. The polar saponin, onjisaponin F, showed the highest activity and its IC_{50} value (2.9×10^{-5}M) was about one third that of papaverine (1.1×10^{-5}M). Kinetic experiment revealed the mode of inhibition was uncompetitive to cAMP, the same as

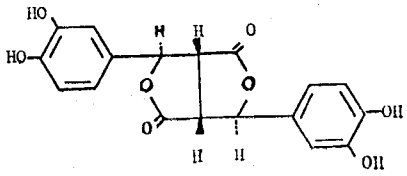
papaverine. Then bioactive saponins were subjected to pharmacological investigation. Since *Polygala* root is mainly used as a sedative agent in Chinese medicine, potentiation of onjisaponins on the action of hexobarbital was tested. Onjisaponin F caused prolongation of sleeping time induced by hexobarbital at a dose of 5mg/kg and saponin mixture was also active. The results so far obtained clearly demonstrates that onjisaponins are the active principles in *Polygala* root. The study of *Polygala* root was somewhat ideal, because biological activity was detected by highly sensitive PDE inhibition test and bioactive saponins identified were proved to possess sedative action in pharmacological test.



Reticulol



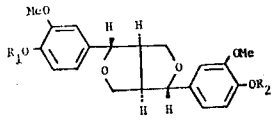
PDE I: R=NH_2
PDE II: R=Me



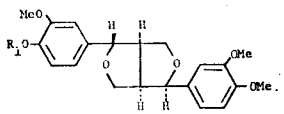
Dehydrodicaffeic acid dilactone

Phosphodiesterase (PDE) inhibitors from microbial cultures

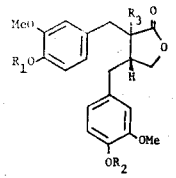
Forsythia fruit: *Forsythia* fruit is derived from several *Forsythia* plants and used as an antiinflammatory, diuretic and detoxication agent. Preliminary investigation revealed both chloroform and ethyl acetate fractions obtained by the extraction of aqueous extracts were active. TLC showed the fractions contained lignans and lignan glycosides, which had been isolated by Nishibe *et al.* in their study on the constituents of *Forsythia* fruits.[20] Pinoresinol, pinoresinol glucoside and pinoresinol diglucoside, contained in *Forsythia suspensa*, showed considerable inhibition against PDE reaction, whereas phylligenin and phyllylin, contained in the same plant, were inactive. Matairesinol and arctigenin, constituents of *Forsythia viridissima*, were active while their glucosides were inactive. Systematic investigation on inhibitory action of lignans were carried out to clarify structure activity relationship of lignans. So far we have tested the most active class of lignans were trachelogenin and nortrachelogenin. Recently, (+)nortrachelogenin was isolated from a



$R_1=R_2=H$ Pinoresinol (7.5)*
$R_1=H$ $R_2=glc$ Pinoresinolglucoside (14.2)*
$R_1=R_2=glc$ Pinoresinoldiglucoside (15.4)*



R=H Phyllygenin (>50)*
R=glc Phyllylin (>50)*



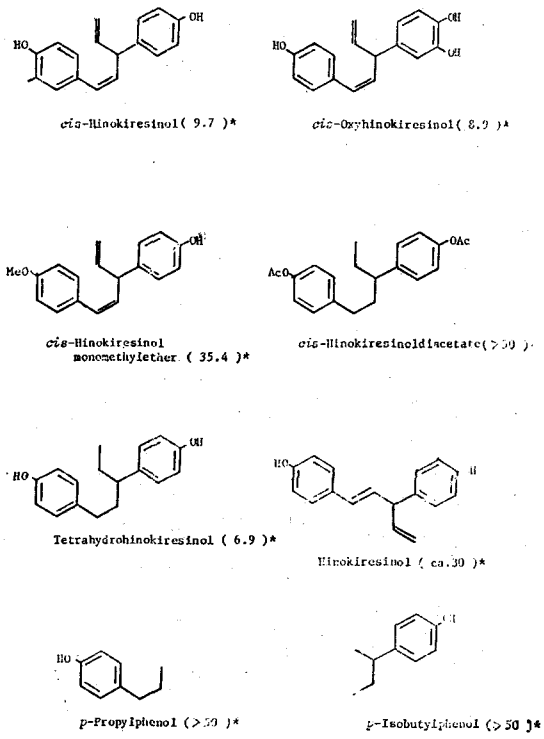
$R_1=R_2=R_3=H$ Matairesinol (9.8)*
$R_1=R_3=H$ $R_2=Me$ Arctigenin (13.9)*
$R_1=R_2=H$ $R_3=OH$ Nortrachelogenin (2.0)*
$R_1=H$ $R_2=Me$ $R_3=OH$ Trachelogenin (2.3)*

*($IC_{50}\times10^{-5}M$)

PDE Inhibitors from *Forsythia* fruits

folklore medicinal plant of Taiwan, ***Wiksotroemia indica***, and shown to possess sedative activity.[21] On the other hand, pinoresinol diglucoside was isolated from the barks of ***Eucomia ulmoides***, which has been used as a Chinese drug to treat hypertension, and its hypotensive activity was confirmed by pharmacological experiment using SHR.[22] At this moment it is not fully clear that PDE inhibitions are directly related to hypotensive action. It is worth to mention here that pinoresinol has very similar structure to that of dehydrodicaffeic acid dilactone isolated from microbial culture as PDE inhibitor and shown to possess hypotensive action.

Anemarrhena root: *Anemarrhena* root is derived from ***Anemarrhena asphodeloides*** and used as sedative and antipyretic agent. In PDE inhibition study, active substances were shown to be chloroform soluble (Table 1). The main constituents of chloroform fraction were norlignans, *cis*-hinokiresinol and *cis*-oxyhinokiresinol,[23] which were shown to be strong inhibitors of PDE in preliminary experiment using preparative TLC. Detailed investigation revealed that norlignans having two *p*-hydroxyl groups are most active. The activity of monomethyl derivative was less and the diacetate completely lost original activity. Hinokiresinol is less active than *cis* isomer, but reduction of double bond caused no change in the activity. The results so far obtained indicates that the presence of two *p*-hydroxyphenyl groups are essential for the activity. This structure activity relationship is the same that observed in lignans, since lignans possessing two *p*-hydroxyphenyl groups are most active. Moreover, the structure of *cis*-hinokiresinol is somewhat similar to that of ethinylestradiol, which has been reported to be a PDE inhibitor.[15] Hormonal activity of *cis*-hinokiresinol has not yet been tested, but it exhibited a weak sedative activity in pharmacological test.



*($IC_{50} \times 10^{-5}$M)

PDE inhibitors from *Anemarrhena* Roots

The result so far obtained in the study of PDE inhibition suggests that the inhibitors contained in Chinese medicinal drugs act to depress central nervous system. In preliminary experiments on the rest of drugs shown in Table 2, isoflavonoids, anthraquinones, coumarins and saponins were identified as active compounds and further study is now in progress.

Conclusion and Remarks

The screening method using ***in vitro*** bioassay tests has been shown to be very effective for the investigation of the active principles of Chinese medicinal drugs. Highly sensitive bioassay tests play a role to detect the presence of bioactive substances in the extracts of

drugs, and they are also used as monitors in successive separation works. Some bio-active compounds obtained in this study showed the same pharmacological actions as expected in traditional medicine. Although only a limited number of active principles have been clarified so far we have done in these couple of years, it is, however, just a beginning and further introduction of new sensitive bioassay test would lead to the elucidation of active principles.

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