BIOLOGICALLY ACTIVE PRINCIPLES OF CRUDE DRUGS. PHARMACOLOGICAL EVALUATION OF CHOLAGOGUE SUBSTANCES IN CLOVE AND ITS PROPERTIES

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The present study was carried out to elucidate the effect of clove and its active principles in view of cholagogue. The cholagogue effect was observed in acetone extract of clove and eugenol. Acetyl-eugenol also possessed cholagogue property.

Keywords—biologically evaluation of crude drug; clove; cholagogue effect; acetone extract of clove; eugenol; acetyl-eugenol

Clove is a crude drug used as a material for home medicine, aromatic stomachic agent and spice. It is also formulated in Kanpo. β-Caryophyllen, eugenol and acetyl-eugenol are the known substances present in clove. Though clove has traditionally been used as aromatic stomachic, its pharmacological study is meager.1–3 We have been studying effects of various natural drugs and their active principles, together with the studies on the effects of stomachic crude drugs which have mostly been categorized as the pharmacological effects of crude drugs in a series of our studies. Since the bile facilitates absorption and digestion of diet, a cholagogue effect is considered to improve part of the characteristic of stomachic agent. The present study was designed to elucidate the cholagogue effect of clove and its active principles.

MATERIALS and METHODS

Materials

Clove purchased from the market in Osaka was cut coarsely and macerated in about 5-fold volume of acetone for 3 d. After filtration the filtrate was concentrated below 50°C under reduced pressure to prepare acetone dry extract as a sample. To obtain the active principles, the dry extract was fractionated by the column chromatography using silica gel as absorbent (fraction 1, 2, 3). Fraction 1 was further subfractionated by the same method to isolate β-

<table>
<thead>
<tr>
<th>Caryophylli flos acetone ext. (50 g)</th>
<th>SiO2 column chromatogr. elute: n-hexane: acetone (30:1 → 1:1).</th>
</tr>
</thead>
<tbody>
<tr>
<td>fraction I (24.2 g)</td>
<td>fraction II (9.9 g)</td>
</tr>
<tr>
<td>fraction III (12.0 g)</td>
<td></td>
</tr>
</tbody>
</table>

TLC patterns of fraction

<table>
<thead>
<tr>
<th>C. flos acetone ext.</th>
<th>fraction I</th>
<th>fraction II</th>
<th>fraction III</th>
<th>eugenol</th>
<th>acetyl-eugenol</th>
<th>caryophyllene</th>
</tr>
</thead>
<tbody>
<tr>
<td>solvent: benzene: acetone (8:1)</td>
<td>plate: Kieselgel 60 F254</td>
<td>spray: anise aldehyde-H2SO4</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
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CHART 1. Flow Diagram of Fractionation of Caryophylli flos Acetone Ext.
caryophyllene, eugenol and acetyl-eugenol. These compounds were identified with each reference standard in thin layer chromatography (TLC), infrared (IR) and nuclear magnetic resonance (NMR) values. Their TLC patterns and yield of each fraction are illustrated in Chart 1. The amount of eugenol and acetyl-eugenol in fraction 1 determined by the gas chromatography (column: stainless column of 4 mm × 1 m; column packing: SE-30; temperature: 130°C hold; injection-detection: 180°C; carrier gas: N₂ 1 kg/cm², 20 ml/min; H₂: 1 kg/cm²; air: 1.5 kg/cm²) was found to be about 74% and about 86%, respectively.

Methods

1. Cholagogue Action — (a) Measurement of Bile: Groups of 6–7 male Wistar rats weighing about 300 g were fasted for 6 h before operation. After abdominal opening under simultaneous use of urethane (700 mg/kg, i.p.)–ether anaesthesia a polyethylene cannula (Hibiki) was inserted into the common bile duct. The animals were kept for 1 h to obtain a steady state. Each test drug suspended in 5% acacia solution was administered intraduodenally 30 min later to measure the amount of bile at 0.5, 1, 1.5, 2, 3, 4 and 5 h after injection. The bile outflux rate (%) measured at each time was calculated as the change in the amount of the bile taking the amount of a 30-min bile before administration of each drug as 100% (about 0.3 ml). The dose of each fraction administered was established according to the corresponding yield of fractions from the extract.

(b) Amount of Solid Constituents of Bile: The bile samples were collected at each scheduled time from the animals of all group, freeze-dried and weighed. The percent weight of solid constituents obtained from the bile at each time was calculated as the change in the amount of solid constituents taking the weight of a 30-min bile before intraduodenal injection of the test drugs as 100%.

(c) Amount of Bile Acids in Bile: A portion of 30-min and 1-h bile collected after administration of the drug that exerted the most potent

![FIG. 1. Effects of Caryophylli flos Acetone Ext. on Bile Secretion in Rats](image1)

--- ▲ —: control, —△ —: caryophylli flos Acetone ext. 500 mg/kg.
Each value is the mean with standard error from 7 rats.
Significant difference: a) p< 0.01.

![FIG. 2. Effects of Fractions of Caryophylli flos Acetone Ext. on Bile Secretion in Rats](image2)

— ▲ —: control, —△ —: fraction I 300 mg/kg, —□ —: fraction II 50 mg/kg, —○ —: fraction III 150 mg/kg.
Each value is the mean with standard error from 7 rats.
Significant difference: a) p< 0.05, b) p< 0.01.
cholagogue action by comparing to the volume of the bile collected 30 min before dosing of test drugs was dissolved in methanol. The amount of bile acids in the filtrate was determined by the high performance liquid chromatography (HPLC) bile acids analysis system (Japan Spectroscopic Co., Ltd.). Condition for HPLC:

Chromatograph: TWINCLE, Solvent programmer: GP-A30, Detector: FP-110C, Ex=365 nm, Ex=470 nm, Reagent pump: SP-024-1, Column: Jasco Bilepac, Column temperature: 25°C, Mobile phase: A: acetonitrile-phosphate buffer =40/60, B: acetonitrile-phosphate buffer =20/80, A/B (0/100) → A/B(99/1) 64 min: concave gradient, Flow rate: 1.0 ml/min, Immobilized enzyme column: Jasco Enzymepak-HSD, Column temperature: 25°C, Reagent: 0.3 mM NAD, 10 mM phosphate-buffer pH 7.00, 1 mM EDTA 0.05% 2-mercaptoethanol, Reagent flow rate: 0.5 ml/min.

Mixture of various standard bile acids, cholic acid, deoxycholic acid, ursodeoxycholic acid, chenoxycholic acid, free lithocholic acid, glycine and taurine conjugates (bile acids, HPLC standard kit 15, Technochemical CO., Ltd.) were determined by HPLC to quantify bile acid. Thereby the calibration curve was drawn to calculate the absolute amount of various bile acids. The measured amount of each bile acid was added and expressed as the total amount of bile acids. Assuming the amount of various bile acids and total amount of bile acids measured for 30-min before administration of test drugs to be 100%, their percentage at each time interval was calculated and expressed as the change in the amount of bile acids.

**FIG. 3. Effect of Eugenol, Acetyl-eugenol and Sodium Dehydrocholate on Bile Secretion in Rats**

- ▲ — : control, —△— : eugenol 100mg/kg,
- ○ — : acetyl-eugenol 100mg/kg, —●— : sodium dehydrocholate 100mg/kg.
Each value is the mean with standard error from 6 rats.
Significant difference: a) p<0.05, b) p<0.01.

**FIG. 4. Effects of Eugenol, Acetyl-eugenol and Sodium Dehydrocholate on Solid Weight of Bile in Rats**

- ▲ — : control, —△— : eugenol 100mg/kg,
- ○ — : acetyl-eugenol 100mg/kg, —●— : sodium dehydrocholate 100mg/kg.

Drugs were administered i.d. at 0 h.
Significant difference: a) p<0.05, b) p<0.01.
Each value is the mean with standard error from 6 rats.
(d) Measurement of Cholesterol and Phospholipids: An aliquot of 30-min and 1-h bile obtained after administration of the test drug showing the most potent chologogue action was used for measurement of cholesterol and phospholipids by the COD-\textit{p}-chlorophenol colorimetry (Cholesterol CII-Test Wako, Wako Pure Chem. Ind., Ltd.), and enzymic method (Phospholipids-B-Test Wako, Wako Pure Chem. Ind., Ltd.), respectively. Changes in values of cholesterol and phospholipids at each fixed time were determined by regarding both 30-min values obtained before administration of test drugs as 100.

RESULTS

1. Chologogue Action

(a) Amount of Bile — Acetone clove extract and fraction 1 exerted a potent lasting chologogue effect (Figs. 1–3). \( \beta \)-Caryophyllene obtained from fraction 1 did not reveal any marked chologogue effect, but eugenol and acetyl-eugenol from fraction 1 increased the flow of bile significantly compared to the control. The chologogue activity of eugenol was more potent than that of acetyl-eugenol. The activities of eugenol and acetyl-eugenol were stronger than that of dehydrocholic acid sodium (DC-Na) used as the control.

(b) Amount of Solid Constituents of Bile — Eugenol, acetyl-eugenol and control drug, DC-Na, increased the amount of solid constituents along with the increase in the rate of bile flow (Fig. 4).

(c) Amount of Bile Acids — Eugenol increased significantly the amount of total bile acids 30 min after administration, in comparison with the control group. However, the rate of increase was less than that of bile (about 240\% at 30 min after administration) (Fig. 5). The amount of bile acids increased slightly 30 min after administration of DC-Na, and the increase in the rate of DC-Na group was significantly smaller than that in eugenol group.

While various bile acids decreased in the control group (Figs. 6–9), ursodeoxycholic acid increased significantly between 30 min and 1 h.

\[ \text{FIG. 5. Effects of Eugenol, Acetyl-eugenol, Sodium Dehydrocholate on the Amount of Total Bile Acids in Bile} \]

\(-\Delta-\): control, \(-\bigcirc-\): eugenol, \(-\bullet-\): acetyl-eugenol, \(-\bullet-\): sodium dehydrocholate.

Each value is the mean with standard error from 6 rats.

\[ \text{FIG. 6. Change of Bile Acid in Bile in Untreated Rats} \]

\[ \text{free type, } \square \text{ glycine conjugation type, } \bigcirc \text{ taurine conjugation type.} \]
after administration of eugenol, in addition to a slight increase in cholic acid 30 min later. The profile of the action of acetyl-eugenol was similar to that of eugenol, except that the action of the former was weaker than that of the latter.

(d) Cholesterol and Phospholipids — Both cholesterol and phospholipids decreased with the lapse of time in the control and eugenol groups (Figs. 10, 11). The decreasing rate was higher in eugenol group than in the control group. The pattern of action of acetyl-eugenol was similar to that of eugenol on cholesterol and phospholipids, but the former was weaker than the latter. Cholesterol somewhat increased at 30 min in DC-Na group, but phospholipids increased significantly.

DISCUSSION and CONCLUSION

A considerable number of crude drugs can be brought under stomachic category. Stomachics are classified by bitter, pungent and aromatic ones depending upon the characteristic principles in crude drugs. However, their effects have never

FIG. 7. Change of Bile Acid in Bile after Intraduodenal Administration of Eugenol
- free type, [] glycine conjugation type, [ ] taurine conjugation type.

FIG. 8. Change of Bile Acid in Bile after Intraduodenal Administration of Acetyl-eugenol
- free type, [] glycine conjugation type, [ ] taurine conjugation type.

FIG. 9. Change of Bile Acid in Bile after Intraduodenal Administration of Sodium Dehydrocholate
- free type, [] glycine conjugation type, [ ] taurine conjugation type.
FIG. 10. **Effects of Eugenol, Acetyl-eugenol and Sodium Dehydrocholate on the Amount of Phospholipid in Bile**

- ▲—: control, - △—: eugenol, - ○—: acetyl-eugenol, - ●—: sodium dehydrocholate.

Significant difference: a) \( p < 0.05 \), b) \( p < 0.01 \).

Each value is the mean with standard error from 6 rats.

FIG. 11. **Effects of Eugenol, Acetyl-eugenol and Sodium Dehydrocholate on the Amount of Cholesterol in Bile**

- ▲—: control, - △—: eugenol, - ○—: acetyl-eugenol, - ●—: sodium dehydrocholate.

Each value is the mean with standard error from 6 rats.

been justified objectively. It is, therefore, necessary to clarify their effects from various pharmacological points of view.

The chologogue property of clove and eugenol, which could be verified in this study, differed obviously from that of the control drug DC-Na; the chologogue effect of clove and eugenol was long-acting and increased the solid constituents of the bile together with the increase in bile acids. Moreover, the result of HPLC for constituents of bile acids proved that eugenol induces the increase in ursodeoxycholic acid being reported empirically to have a cholelitholytic action. The analysis of bile lipids also demonstrated the decrease in cholesterol and phospholipids after administration of eugenol. These results suggest that clove and eugenol is possibly applicable as a cholelitholytic agent in future. We are in consideration to study this point.

It is reasonable to measure the contents of eugenol and acetyl-eugenol which are present in about 30—60% in clove for quality evaluation of this crude drug. It is also conceivable that clove is a useful crude drug as aromatic stomachic.

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**REFERENCES**

