Cudrania tricuspidata Extract Protects against Reflux Esophagitis by Blocking H2 Histamine Receptors.

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ABSTRACT: Cudrania tricuspidata has been used in East Asia as a folk medicine for symptoms such as inflammation, allergy, and gastritis. Administration of C. tricuspidata extracts to pylori-ligated rat stomachs reduces gastric acid secretion and alleviates esophagus damage caused by gastric reflux. Therefore, in this study we aimed to investigate whether C. tricuspidata extracts inhibit reflux esophagitis by blocking H2 histamine receptor (H2R). Dimaprit, a H2R specific agonist, induced intracellular cyclic adenosine monophosphate (cAMP) production in U937 cells. Pretreatment with C. tricuspidata extracts significantly blocked dimaprit-induced cAMP production in a concentration-dependent manner. To extracted C. tricuspidata with different ethanol concentrations to determine the optimum method. We found that the 70% ethanol extract showed the most potent H2R antagonistic effect against dimaprit-induced cAMP production. However, water extract did not show any H2R blocking effect. These findings suggest that C. tricuspidata extracts extracted using ethanol specifically inhibits gastric acid secretion and reduces esophageal injury by blocking H2R in a competitive manner. Therefore, C. tricuspidata extracts may be used in food or medicine to prevent H2R-related diseases, such as gastric hyperacidity and reflux esophagitis.

Keywords: Cudrania tricuspidata, H2 histamine receptor, reflux esophagitis, gastric juice secretion, gastric hyperacidity

INTRODUCTION

Gastroesophageal reflux disease (GERD), which includes reflux esophagitis, is a disease during which stomach contents (mainly acid and pepsin) are refluxed into the esophagus, resulting in various clinical symptoms and mucosal changes. Reflux esophagitis is a chronic condition characterized by severe lesions caused by prolonged exposure to acid. In general, strong acidic gastric juice stimulates the esophagus to induce refluxing accompanied by a burning sensation from the chest to the throat, leading to chest tightness, breathing difficulty, and, in severe cases, heartburn. Frequent coughing and reflux secrections lead to bitter tastes in the mouth from which aspiration pneumonia may develop as a complication (Rieder et al., 2010; Moore et al., 2016).

The main cause of reflux esophagitis is eating habits. Intakes too high in fatty, fried, or spicy food can increase gastric acid secretion, while overeating and rapid food intake increase gastrointestinal pressure which makes it difficult to neutralize stomach acid. Another cause of reflux esophagitis is the habit of lying down straight after meals. Lying down after overeating is often linked to reflux esophagitis. Moreover, cigarettes, caffeine, soft drinks, chocolate, peppermint, and orange juice can weaken the squeezing ability of the sphincter, causing reflux esophagitis (Eslick and Talley, 2009; Hsu et al., 2013; Henry, 2014). The most commonly used drugs for treatment of reflux esophagitis are proton pump inhibitors and histamine H2 receptor (H2R) blockers. These drugs lower the acidity of gastric juice and reduce irritations when gastric reflux occurs (Vakil et al., 2006; Abdul-Hussein et al., 2015).

The genes encoding four histamine receptor subtypes (H1, H2, H3, and H4) have been cloned, and the receptors and their downstream signaling pathways have been pharmacologically characterized (Repka-Ramirez, 2003). H2R is a G protein-coupled receptor that couples with adenyl cyclase, which produces the intracellular second messenger cyclic adenosine monophosphate (cAMP) (Klinker et al., 1996a; Klinker et al., 1996b). In general, H2R is present in gastric parietal cells and regulates secretion signals in the stomach. Activation of H2R in gastric parietal cells increases production of cAMP and induces release of hydrogen ions (H+) in the gastric juice by proton pump, leading to increased gastric juice acidity. There-
fore, H₂R blockers may be useful for treating diseases that involve gastric acid hypersecretion, such as gastric ulcers and reflux esophagitis (Kowalsky et al., 1991; Onodera et al., 1999; Weiser et al., 1983).

*Cudrania tricuspidata* is a deciduous tree that has been used in traditional medicine to treat eczema, mumps, pulmonary tuberculosis, allergy, and acute arthritis (Xin et al., 2017). It has been reported that different parts of the tree (fruit, root, stem, and leaf) may exert beneficial anti-inflammatory, anti-obesity, and antioxidant effects (Park et al., 2006; Lee et al., 2006; Kim et al., 2016; Jo et al., 2017). Although there is increasing evidence for the different physiological activities induced by *C. tricuspidata*, the molecular mechanisms of action of *C. tricuspidata* on GERD and reflux esophagitis have not yet been elucidated. In the present study, we used the U937 monocyte cell line and pylorus-ligated rat models to evaluate the potential activity of *C. tricuspidata* as an H₂R antagonist and reflux esophagitis agent. The aim of this study is to provide evidence that ethanol extracts of *C. tricuspidata* leaves negatively regulate H₂R activity and apparently act as specific and competitive H₂R antagonists in terms of the secretion blockage of gastric acid similar to that of ranitidine.

**MATERIALS AND METHODS**

**Preparation of C. tricuspidata extract**

Dried *C. tricuspidata* leaves were pulverized to an appropriate size and placed in an extraction vessel. Ethanol (0% to 70%, corresponding to 10 times the weight of the leaves) was added to the extraction vessels, refluxed, and stirred at 50°C for 6 h. *C. tricuspidata* extracts were adsorbed and filtered by perlite to remove insoluble impurities. The filtered juice extract was concentrated using a rotary vacuum evaporator (EYELA, Tokyo, Japan), lyophilized, pulverized, and powdered. The dried materials were resuspended in 0.5% methyl cellulose (MC) for animal experiments and in dimethyl sulfoxide (DMSO) for cellular experiments.

**Animals**

Six weeks old male Sprague-Dawley rats were maintained at a temperature of 23±3°C, a relative humidity of 55±15%, a ventilation frequency of 10~20 times/h, and with 12 h of light (8:00 am to 8:00 pm off). Temperature and relative humidity were measured every hour using a computer system, and the frequency of ventilation and illumination was measured periodically. There were no abnormalities that could affect the test results during the experiments. All studies were approved by the Institutional Animal Care and Use Committee of Gyeonggi Bio Research Center (approval no. 2016-10-0011, 2017-05-0005).

**Gastric secretions and gastric acidity measurements**

Animals were fasted for 24 h before administration of test substances. Test substances (*C. tricuspidata* 70% ethanol extract or ranitidine) were suspended in 0.5% MC before administration. In the vehicle group, only 5% MC was administered. One hour after oral administration of test substances, animals were anesthetized with isoflurane, and the pylorus was ligated. After 8 h of pyloric ligation, animals were sacrificed with CO₂ gas and gastric juice was collected from the stomach using a 10-mL syringe. The gastric juice was centrifuged at 3,000 rpm for 10 min, the supernatant removed, and the gastric fluid amount (mL), pH, and acidity were measured. To determine gastric acidity, 1 mL of centrifuged gastric juice was dispensed into a tube and titrated to pH 7.0 with 0.1 N NaOH; the amount of 0.1 N NaOH used in the titration was measured. Total acidity was calculated using the following formula:

Total acidity=\(\frac{\text{amount of titrated NaOH (mL)×total}\text{ gastric volume (mL)×0.1 N (correction of NaOH) ×50 (acidity factor)}}{\text{ligation time (h)}}\)

**Induction of reflux esophagitis and esophageal lesion measurement**

All animals were fasted for 36 h before test substances were administered. One hour after oral administration of test substance (*C. tricuspidata* 70% ethanol extract or ranitidine), animals were anesthetized with tiletamine/zolazepam (10 mg/kg; Zoletil 50, Virbac, Carros, France) and 2% xylazine hydrochloride (2 mg/kg, Rumpun, Byer Co., Seoul, Korea). The abdomen of the anesthetized rats was shaved and disinfected with povidone, followed by incision along the midline at 4 to 5 cm. The exposed pylorus and the limiting ridge were ligated with silk (3-0, B. Braun Surgical S.A., Barcelona, Spain). The peritoneum was closed with 3/0 absorbable suture (3/0 Surgisorb, Samyang, Seoul, Korea), and the skin was ligated with silk (3-0, B. Braun Surgical S.A.). Eight hours following the operations, animals were sacrificed with CO₂ gas, and the stomachs and esophagi were collected. Stomachs and esophagi were cut longitudinally using surgical scissors, and the blood was washed with phosphate buffered saline. The dissected stomachs and esophagi were spread on clean paper and photographed with a digital camera (Coolpix P5100, Nikon, Tokyo, Japan) under 200 to 300 lux illumination at the designated site. The distance between the camera and the specimen was measured with object markers and scale bars (mm unit) at the time of photographing. The lesion area of injured esophagus mucosa was measured using Image J (Wayne Rasband; National Institutes of Health, Bethesda, MD, USA).
**RESULTS**

**Effect of *C. tricuspidata* on gastric acid secretion**

The pylori-ligated rat model (Satyanarayana et al., 1989) was used to investigate the effects of *C. tricuspidata* extracts on total gastric volume and total acidity of gastric juice. Analysis of gastric contents showed that *C. tricuspidata* extracts decreased the amount and total acidity of gastric juices in a concentration-dependent manner. Specifically, 20 mg/kg of *C. tricuspidata* extract showed an inhibitory effect on gastric acid secretion almost comparable to the H2R inhibitor ranitidine (Fig. 1). The *C. tricuspidata* extract showed an inhibitory effect on gastric acid secretion almost comparable to the H2R inhibitor ranitidine (Fig. 1). The *C. tricuspidata* extract treated group did not show dose-dependent inhibition of esophageal mucosal damage, but the mean level of damage was comparable to that of the ranitidine-treated group. The high-dose *C. tricuspidata* extract-treated group (50 mg/kg) showed the greatest reduction of esophageal mucosal damage.

**Effect of *C. tricuspidata* on reflux esophagitis**

In the vehicle group, most of the esophageal mucosa were damaged by stomach acid, resulting in severe redness. In contrast, rats treated with ranitidine and *C. tricuspidata* extracts showed a decrease in redness due to gastric acid secretion (Fig. 2A). The esophageal injury ratio of the vehicle group was 57.72±5.16%. *C. tricuspidata* extracts at concentrations of 20 and 50 mg/kg attenuated the esophageal injury ratio to 29.41±7.84% and 25.18±7.57%, respectively. Ranitidine at 7.7 mg/kg also attenuated the lesion score to 29.90±6.3% (Fig. 2B, Table 1). The *C. tricuspidata* extract-treated group did not show dose-dependent inhibition of esophageal mucosal damage, but the mean level of damage was comparable to that of the ranitidine-treated group. The high-dose *C. tricuspidata* extract-treated group (50 mg/kg) showed the greatest reduction of esophageal mucosal damage.
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Fig. 3. Inhibition of H2 histamine receptor (H2R)-mediated cyclic adenosine monophosphate (cAMP) production by Cudrania tricuspidata extract in U937 cells. (A) C. tricuspidata ethanol extracts inhibit cAMP production in a dose-dependent manner. (B) H2R inhibitory activity of C. tricuspidata extracted with various concentrations of ethanol. U937 cells were pretreated with 10 μM of ranitidine or C. tricuspidata extracts for 5 min. Next, cells were stimulated with 10 μM of dimaprit. The control group was treated with the same amount of DMSO instead of ranitidine or C. tricuspidata extracts. Values are mean±SE. Significantly different from Veh group at **P<0.01.

Table 1. Gross esophageal damage in rats with reflux esophagitis

| Groups | Total area (mm²) | Lesion area (mm²) | Injury ratio (%) |
|--------|-----------------|------------------|-----------------|
| G1     | 448.18±21.71    | 261.75±30.22     | 57.72±5.16      |
| G2     | 434.04±26.08    | 136.05±33.67     | 29.90±6.30      |
| G3     | 398.89±20.90    | 116.54±31.23     | 29.41±7.84      |
| G4     | 408.04±12.68    | 106.60±32.60     | 25.18±7.57      |

Data were expressed as mean±SE. The results were statistically analyzed by one-way ANOVA. **Significantly different from G1 at P<0.01.

C. tricuspidata inhibits H2R-mediated cAMP production in U937 cells.

H2R is a major target of anti-ulcer drugs (Kowalsky et al., 1991). Inhibitory effects of H2R blockers on gastric acid secretion have been demonstrated in many animal model systems (Konturek et al., 1980; Ohsawa et al., 2002; Kim et al., 2005). H2R is expressed in immune cells, such as the U937 cell line, which is widely used as a model system for H2R activation and GI tract cells (Kim et al., 2005; Jutel et al., 2001; Delgado et al., 2002). Therefore, we investigated whether C. tricuspidata extracts inhibited cAMP production by dimaprit, a selective H2R agonist, in U937 cells. Treatment of U937 cells with dimaprit resulted in a significant increase in cAMP, which was
inhibited by *C. tricuspidata* extract in a dose-dependent manner (Fig. 3A).

In the above animal experiments, we found that the *C. tricuspidata* 70% ethanol extract effectively inhibited gastric acid secretion and reflux esophagitis. Therefore, we investigated the effects of various concentrations of *C. tricuspidata* ethanol extractions on H2R inhibition. Higher ethanol concentration induced greater H2R inhibition. The 0% ethanol extract (hot water extract) did not affect H2R inhibition (Fig. 3B). Ranitidine, a H2R-specific antagonist, almost completely inhibited cAMP production by H2R at a concentration of 10 µM (Fig. 3B). When ethanol was used as the solvent at concentrations over 70%, only a very small amount of extract was obtained.

There was no cellular damage in U937 cells treated with ranitidine or *C. tricuspidata* extracts (data not shown), suggesting that the inhibitory effects of H2R by *C. tricuspidata* extracts was not due to inducing cell death. We also measured the H2R inhibitory effect of *C. tricuspidata* fruit and stem extracts; the fruit extract did not inhibit H2R, and the stem extract only showed a small inhibitory effect (data not shown).

**DISCUSSION**

*C. tricuspidata* has long been used for treatment of gastrointestinal diseases in folk medicine. Although many studies have reported the effects of *C. tricuspidata* on allergy, inflammation, diabetes, and obesity (Kim et al., 2016; Jo et al., 2017; Lee et al., 2012; Kim et al., 2015; Jo et al., 2014; You et al., 2017), the mechanism of action is unclear for gastrointestinal diseases such as gastritis and reflux esophagitis. In this study, we suggest that *C. tricuspidata* ethanol extracts may block H2R and prevent gastric hyperacidity and reflux esophagitis.

A single oral administration of *C. tricuspidata* ethanol extract reduced gastric acid secretion in pyloric-ligated animals, similar to the effects of ranitidine (Fig. 1), and effectively suppressed esophageal damage in the reflux esophagitis model (Fig. 2). These results suggest that *C. tricuspidata* ethanol extracts inhibit signals related to gastric acid secretion. Thus, we determined whether *C. tricuspidata* inhibits H2R-mediated cAMP production in U937 cells. We found that the ethanol extract of *C. tricuspidata* inhibited H2R activity in a concentration-dependent manner (Fig. 3A). Inhibition of cAMP signaling by *C. tricuspidata* ethanol extracts was due to blockade of histamine binding to H2R, rather than accelerating degradation, based on inhibition observed in the presence of the phosphodiesterase inhibitor RO-20-1724. In addition, when *C. tricuspidata* was extracted with high concentration of ethanol, higher H2R inhibitory activity was observed compared with extraction with low concentrations of ethanol or water. These results suggest that the active component of *C. tricuspidata* that inhibits H2R is a hydrophobic, and not hydrophilic material. Therefore, we are conducting a follow-up study to identify a single component that specifically inhibits H2R in *C. tricuspidata* by fractionating the *C. tricuspidata* extract with hydrophobic solvents such as ethyl acetate.

Antagonism of H2R has been the cornerstone of pharmacological treatment of gastrointestinal tract acid disorders, such as gastric hyperplasia and gastritis (Kowalsky et al., 1991). Selective H2R inhibition and gastric acid secretion by *C. tricuspidata* indicates that *C. tricuspidata* extracts or its active ingredients may be a promising candidate for the treatment of gastric ulcers and other H2R-related diseases. Therefore, further studies are needed to demonstrate the effect of *C. tricuspidata* on gastrointestinal dysfunction, such as hypergastric acidity and reflux esophagitis, in humans, and to identify individual *C. tricuspidata* components that inhibit H2R.

In conclusion, our data show that *C. tricuspidata* extract can inhibit H2R and may effectively prevent diseases such as gastric hyperacidity and reflux esophagitis associated with H2R-mediated gastric secretion.

**AUTHOR DISCLOSURE STATEMENT**

The authors declare no conflict of interest.

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