Antinociceptive and anti-inflammatory effects of roots extracts from *Actinidia arguta* (Sieb.et Zucc.) Planch

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ABSTRACT

Aims: The roots of *Actinidia arguta* (Sieb. et Zucc.) Planch (also named *tengligen*) have medicinal uses as anti-tumour, antinociceptive, and anti-inflammatory agents. In this study, we evaluated the antinociceptive and anti-inflammatory effects of 95% ethanol extract and different fractions of the roots of *A. arguta* (Sieb.et Zucc.) Planch *(Tengligen)*. ***Methods:*** Three conventional methods were used to carry out the antinociceptive effect: *acetic acid*-induced abdominal writhing, formalin induced hind paw licking, and hot plate test. In addition, the anti-inflammatory effect was investigated by carrageenan-induced paw edema in rats. ***Conclusion:*** From the obtained results, we found that the total ethanol extract, ethyl acetate fraction, and n-butanol fraction all significantly inhibited acetic acid-induced writhing and both phases of the formalin induced pain response, increased the time of response to thermal stimulation in hot plate test, and exhibited significant dose-related inhibition of carrageenan induced paw edema volumes when compared with the control group. Based on our findings, we conclude that the flavonoid and saponin contents of *tengligen* are responsible for the antinociception and anti-inflammatory effects of *Actinidia arguta* (Sieb. et Zucc.) Planch, respectively.

Keywords: *Actinidia arguta* (Sieb.et Zucc.) Planch; antinociceptive; anti-inflammatory.

INTRODUCTION

Inflammation and pain are two kinds of defense reactions of living systems in reply to any invasive factor. Considering the frequent occurrence of adverse side effects of current drugs, the screening and development of new agents with more powerful analgesic and anti-inflammatory effects and with lesser side effects is still in progress.

Actinidia arguta (Sieb.et Zucc.) Planch belongs to Actinidia genus in the family Actinidiaceae, which is a family composed of large, deciduous vines. The genus *Actinidia* contains 54 species. Most species in this genus are found in the mountains of South China, but some species are also found in Siberia, Japan, Indochina, Malaysia, Indonesia, and New Zealand.[1–3]

The fruits of *A. arguta* (Sieb.et Zucc.) Planch (kiwi fruit) are edible, while the roots of the plant, which are called *Tengligen*. *Tengligen* is normally used as an ingredient in folk medicine, different preparations of this plant such as, decoctions, infusions and powders, are used in traditional Chinese medicine to treat several diseases such as inflammatory, ache, tumors, diabetes, hyperlipidemia, hepatitis, and so on. Especially, *Tengligen* has been used to treat alimentary canal tumors, such as those characterizing gastric, esophagus, and liver cancer.[4–6] Phytochemical investigation previous resulted in the isolation and identification of many metabolites including flavonoids, saponins, terpenoids, polysaccharide, and essential oils from *Tengligen*.[7]

To the best of our knowledge, there is no study to date evaluating the antinociceptive and anti-inflammatory effects of the major fractions of *Tengligen*, despite the prevalent historical use of these plant parts in traditional

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Chinese medicine for more than 2000 years. Here, we filled this gap in knowledge by evaluating the antinociceptive and anti-inflammatory effects of 95% ethanol extract and different fractions of the roots of *A. arguta* (Sieb.et Zucc.) Planch *(Tengligen)*. The antinociceptive effect was examined on chemically and thermally induced nociceptive pain in mice through *acetic acid*-induced abdominal writhing, formalin induced hind paw licking, and hot plate test. In addition, the anti-inflammatory effect was investigated by carrageenan-induced paw edema in rats.

MATERIALS AND METHODS

The roots of *A. arguta* (Sieb.et Zucc.) Planch were collected in the Changbai Mountains, Jilin Province, China, in March of 2012, and identified by Dr. Yue-Chun Sun. A voucher specimen was deposited at Life Science and Technology College, Heilongjiang Bayi Agricultural University (No120326).

Phytochemical screening

Approxmiately 5kg of dried *Tengligen* was extracted with 95% ethanol(2×10L) under heat reflux for 2h in duplicate, and the solvent was removed under reduced pressure. The residue of the ethanol extract (525.3g) was suspended in 1000mL of distilled water and was partitioned sequentially with cyclohexane, chloroform, ethyl acetate, and then n-butanol at room temperature. In total, 5 major fractions were collected and concentrated under reduced pressure until all the solvent had been removed to give an extract sample. The chemical constituents of the extract were analyzed qualitatively and screened to detect saponins, flavonoids, alkaloids, and terpenoids by thin layer chromatography.[8,9] The extract was stored at –20ºC until pharmacological tests.

Animals

Wistar rats (aged 8–12 weeks; weight, 180–200g) and ICR(Institute of Cancer Research,USA) mice (aged 2–3 weeks; weight, 18–22g) of either sex were used for experiments described here. Animals were bought from the Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and used once each. All animal treatments were strictly in accordance with international ethical guidelines concerning the care and use of laboratory animals, and all the experiments were carried out under the approval of the Committee of Experimental Animal Administration of the University (number CEAAU-189). All animals were kept in standard laboratory conditions (relative humidity 55–60%, room temperature 25±2ºC, 12h light/dark cycle), and had free access to standard diet and water *ad libitum* during the duration of the experiment. All animals were acclimated to the laboratory environment for a period of 7 days prior to performing the experiments.

Writhing test

Writhing test was carried out as described by Pinheiro et al.,(2010) and Mariana et al., (2012).[10,11] In brief, the mice were pre-treated with positive drug and test drug for 3 days; approximately a half hour after the final administration on the third day, 0.6% acetic acid (0.1mL/10g) was given by intraperitoneal injection. The number of writhing movements during the next 15 minutes was recorded. The number of writhes in each treated group was compared with the number observed in the control group, which received only a saline injection instead of acetic acid, but was otherwise treated identically. The inhibition rate of writhes $[(\bar{x}$ control-$\bar{x}$ test$)/\bar{x}$ control$] \times 100$, was then calculated.

In the writhing test, mice were randomly divided into 8 groups. These groups were (1) control group with intragastrical administration (i.g.) isometrical physiological saline, the test drug groups at dosages of (2–6) 50mg/kg of 5 major fractions, (7) total ethanol extract (TEE, 200mg/kg), (8) a positive drug control group (i.g. ibuprofen [50mg/kg]).

Formalin induced nociception

The procedure we followed for formalin-induced nociception was similar to the method described by Hunskaar and Hole (1987),[12] but with modifications by Gomes et al., (2007).[13] In short, animals were divided into 7 groups of 10 mice each. Each mouse was pre-treated with an oral dose of 1 of the 5 major fractions (50mg/kg, respectively), TEE (200mg/kg), or ibuprofen (50mg/kg) (positive drug). The mice were pre-treated with the positive drug and the test drugs for 3 days; a half hour after the final administration on the third day, 20µl of 5% v/v formalin was injected subcutaneously into the right hind paw of the mouse. The time that the animal spent on licking or biting of the injected paw was recorded as the animal's pain response. Based on previous studies of this response pattern,[11] we measured responses up until 5 min after formalin injection (early phase, neurogenic pain response), and from 15–30 min after formalin injection (later phase, inflammatory pain response).

Hot plate test

Hot plate test was conducted as described by Eddy and Leimbach (1953).[14] In this test, female mice were placed in a 24cm diameter glass cylinder on a heated metal plate that was maintained at 55±1ºC. Each animal was habituated twice to the hot plate prior to the experiment. The response was defined as licking or biting of a paw, or jumping. The time in seconds between the placing of the animal on the platform and the first reaction observed was recorded as the response latency time. The mice exhibiting latency times greater than 30s or less than 5 s was excluded from analyses. Animals were divided into 8 groups of 10 mice each and pretreated with 1 of the 8 treatments described above. Mice were tested at 30 min, 60 min, 90 min, and 120 min intervals after oral administration of the extracts or ibuprofen.

Carrageenan induced paw edema

Carrageenan induced hind paw edema model was used to determine the anti-inflammatory effect.[15] Rats were orally treated with 1 of the 7 previously described treatments approximately 30 min prior to injection of 1% carrageenan (0.1mL) in the right hind paw (sub-plantar region) of each rat. Hind paw edema volumes were measured using the plethysmometer at 0.5h, 1h, 2h, and 3h intervals post injection. The percentage inhibition was calculated according to the hind paw volume.

Statistical analysis

The experimental data was expressed as mean±standard error of the mean (SEM). The statistical analysis was carried out using a one way analysis of variance (ANOVA) followed by Tukey's t-test. The differences with $p<0.05$ were considered statistically significant, $p<0.01$ were considered highly significant.

RESULTS

Fractionation and phytochemical screening

Extraction yield was measured and comprised about 42.7% of the final product, from which 5 major fractions were separated. As shown in Table 1, during phytochemical screening, the fractions of ethyl acetate and n-butanol exhibited strong positive reactions for flavonoids and saponins, respectively.

Writhing test

In the acetic-acid-induced writhing test (shown in Table 2), the TEE, EAF (ethyl acetate fraction), and NBF (n-butanol fraction) exhibited significant analgesic effects after oral administration in mice that were subjected to acetic acid-induced writhing compared with the control ($p<0.01$). In addition, the highest analgesic activity was observed with the TEE (200mg/kg), which was lower than the analgesic activity observed with 50mg/kg ibuprofen alone. The maximum inhibition of the nociceptive response (81.37%) was achieved with the 50mg/kg ibuprofen dose (alone). However, no significant analgesic effects were observed for any of the doses of CYF (Cyclohexane fraction) group, CHY (Chloroform fraction) group or the aqueous fraction group.

Formalin induced pain

Treatment with TEE (200mg/kg), EAF (50mg/kg), NBF (50mg/kg), and ibuprofen alone (50mg/kg) each caused significant increases in the percentage pain inhibition in both the early and later phases of the formalin test (both $p<0.01$) (Figure 1). Moreover, in both early and late phases of the experiment, the percentage of pain inhibition observed during treatment with TEE, EAF, and NBF was as good as with ibuprofen alone. However the

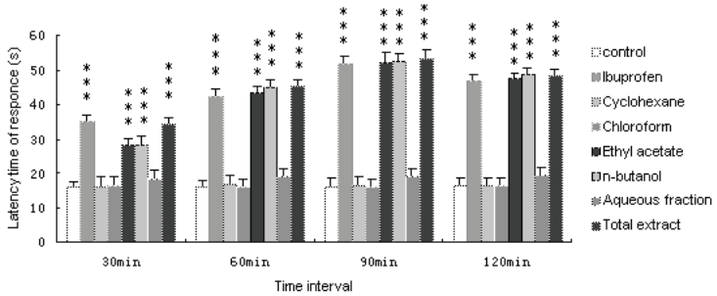

Figure 1. The antinociceptive effect of total ethanol extract and major fractions separated from Tengligen in hot plate test. The effect has been calculated on the basis of latency time of response. Each point is the mean±SEM of ten animals.*** $p < 0.01$ in comparison to normal saline group.

percentage pain inhibition observed during treatment with TEE, EAF, NBF, and ibuprofen alone was greater in the later phase than in the early phase.

Hot plate test in mice

In the hot-plate test, the TEE, EAF, NBF, and ibuprofen treatments produced a significant increase in the response time from 30 min to 120 min (both $p<0.01$) (Figure 2). At the same time, the antinociceptive response observed with the TEE, EAF, and NBF were considerably more pronounced than that obtained with ibuprofen at all the tested doses from 60 min to 120 min. Lastly, the analgesic activity observed with the TEE, EAF, and NBF was lower than the analgesic activity of ibuprofen alone group from 0 min to 30 min.

Carrageenan-induced paw edema

In the carrageenan-induced hind-paw edema experiment we conducted the treatments we evaluated all demonstrated significant anti-inflammatory activity at all tested doses in comparison with the negative control 3h after carrageenan administration ($p<0.01$) (Table 3). Amongst the

Table 1. The result of phytochemical screening of total ethanol extract and separated fractions from Tengligen.

Sample	color	Saponin	Flavonoid	Alkaloid	Terpenoids
TEE	yellowish	+++	+++	–	++
CYF	whitish	–	–	–	++
CHF	whitish	–	–	–	–
EAF	yellowish	+	+++	–	–
NBF	yellowish	+++	+	–	–
AQF	slight yellowish	+	+	–	–

TEE (total ethanol extract); CYF(cyclohexane fraction); CHF(chloroform fraction); EAF(ethyl acetate fraction); NBF(n-butanol fraction); AQF(aqueous fraction).
+++: high content; ++: medium content; +: low content; -: no content (content was evaluated as the sediment or the intensity of color by thin layer chromatography).

Table 2. The antinociceptive effect of total ethanol extract and major fractions separated from Tengligen in acetic acid-induced nociception (per 15 min).

Group	Dose (mg/kg)	Number of writhing	Inhibition(%)
Control	–	56.27 ± 2.03	–
Ibuprofen	50	10.48 ± 2.46***	81.37***
CYF	50	54.12 ± 2.49	3.82
CHF	50	52.27 ± 2.12	7.11
EAF	50	12.59 ± 2.26***	77.63***
NBF	50	16.41 ± 2.57***	70.84***
AQF	50	53.43 ± 2.64	5.47
TEE	200	11.73 ± 2.87***	79.15***

CYF(cyclohexane fraction); CHF(chloroform fraction); EAF(ethyl acetate fraction); NBF(n-butanol fraction); AQF(aqueous fraction) ;TEE (total ethanol extract).
*** $p < 0.01$ significantly different from control.

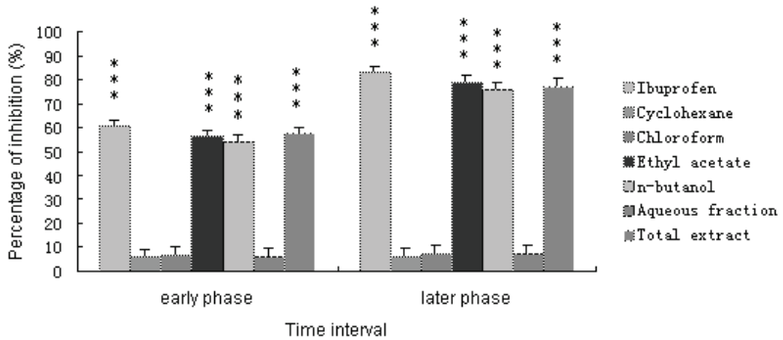

Figure 2. The antinociceptive effect of total ethanol extract and major fractions separated from Tengligen in formalin-induced nociception. The effect has been calculated on the basis of percentage of pain inhibition. Each point is the mean±SEM of ten animals.*** $p < 0.01$ in comparison to normal saline group.

Table 3. The antinociceptive effect of total ethanol extract and major fractions separated from Tengligen in carrageenan-induced hind paw edema.

Group	Percent of inhibition (%)			
	0.5h	1h	2h	3h
Ibuprofen (50mg/kg)	8.21 ± 1.68	43.73 ± 2.51**	71.79 ± 2.49**	96.61 ± 2.16***
CYF (50mg/kg)	3.15 ± 2.62	19.82 ± 2.85	36.82 ± 2.57	45.07 ± 3.93**
CHF (50mg/kg)	4.41 ± 2.31	16.58 ± 2.03	45.73 ± 2.71**	49.79 ± 2.17**
EAF (50mg/kg)	16.57 ± 1.87	46.45 ± 1.73**	78.96 ± 2.63**	95.31 ± 2.82***
NBF (50mg/kg)	17.15 ± 2.69	44.82 ± 2.36**	74.898 ± 2.27**	93.63 ± 2.93***
AQF (50mg/kg)	11.47 ± 2.52	19.87 ± 2.93	45.25 ± 3.09**	54.46 ± 2.97**
TEE (200mg/kg)	15.36 ± 1.82	42.76 ± 2.09**	79.74 ± 2.94**	97.62 ± 1.88***

CYF(cyclohexane fraction); CHF(chloroform fraction); EAF(ethyl acetate fraction); NBF(n-butanol fraction); AQF(aqueous fraction) ;TEE (total ethanol extract).
** $p < 0.05$ significantly different from control
*** $p < 0.01$ significantly different from control

treatments we examined, the TEE (200mg/kg), EAF (50 mg/kg), NBF (50mg/kg), and ibuprofen alone (50mg/kg) all significantly reduced paw edema volume from 1h to 3h of the experiment ($p<0.05$ and $p<0.01$).The TEE resulted in the highest inhibition of paw edema volume at 3h in comparison with the negative control group ($p< 0.01$).

DISCUSSION

Pain is the most common motivating factor to seek medical attention. Although adequate pain relief is achieved with the currently available analgesic agents like opioids or NSAIDs, some of their serious side effects are major limitations to their routine use in therapy. Recently, many natural medicines derived from plants, soil microbes, marine organisms,etc, were considered as the effective and safer for the treatment of various diseaces including inflammation and pain.

The roots of *Actinidia arguta* (Sieb. et Zucc.) Planch (*Tengligen*) is a traditional herb, which has been used for treatment of several diseases such as inflammatory, ache, tumors, diabetes, hyperlipidemia, hepatitis, and so on. In this study, we evaluated the antinociceptive and anti-inflammatory effects of 95% ethanol extract and different fractions of the roots of *A. arguta* (Sieb.et Zucc.) Planch (*Tengligen*).

Here, we investigated the antinociceptive and anti-inflammatory effects of *Tengligen* in three analgesic models: the acetic acid-induced writhing model, the formalin induced licking model, and the hot-plate test. All of these were employed with the goal of assessing the antinociceptive effects of the total ethanol extract and its sub-fractions. In addition, the carrageenan-induced hind paw edema model was used to assess anti-inflammatory properties of the total ethanol extract and its sub-fractions.

The acetic acid-induced writhing reaction in mice has long been used as a screening tool for the assessment of analgesic or anti-inflammatory properties of new agents, and is described as a typical peripheral analgesic model for visceral inflammatory pain.[16] The hot-plate test, which uses thermal stimulus to induce pain, is frequently used to evaluate the centrally mediated antinociceptive effect.[17] The acetic acid-induced writhing test and hot plate test are both regarded as gold standards for determining the peripheral- and central-acting analgesic effects of drugs, respectively.[12,18] The peripheral analgesic effect may be mediated via the inhibition of cyclooxygense and/or lipoxygenases(and/or inflammatory mediators), while the central analgesic action may be mediated via inhibition of central mediators. For this reason, we selected the acetic acid-induced writhing test and the hot plate test to test analgesic potential. In order to explore the analgesic activity pathway of 95% ethanol extract and different fractions of *Tengligen*, ibuprofen was selected as the reference drug. Ibuprofen is a nonselective COX inhibitor, in that it inhibits two isoforms of cyclooxygenase, COX-1 and COX-2. The analgesic, antipyretic, and anti-inflammatory activity of NSAIDs appears to operate mainly through inhibition of COX-2.

Our results indicated that the TEE, EAF, and NBF all exhibited significant analgesic properties, apparent by their statistically significant inhibition of writing, and the increased latency of response to chemical and thermal stimulation, respectively, in comparison with control groups (Table 2 and Figure 1). Overall, the results of our study show that at all dose levels examined, the TEE, EAF, and NBF treatments significantly reduced the number of acetic acid-induced writhes, which suggests that the analgesic effects of TEE and some fractions may be mediated via peripheral pathways of pain perception. The increase in reaction time, as evidenced by the latency measured in the hot plate test, indicates

that the TEE, EAF, and NBF may also possess a central analgesic effect.

Formalin induced nociception is a well-described model for evaluating the mechanism of pain and analgesia.[13] The nociceptive behavior after formalin injection was distinctly recorded in two phases. The early phase of paw licking/biting response starts immediately after injection and is considered probably due to direct stimulation of nociceptors.[19] The later phase which appears little later is considered to be due to a combination of an inflammatory reaction in the peripheral tissue and changes in central processing.[20] In formalin test, the central antinociceptive agents can inhibit both phases of formalin-induced pain while peripherally active ones inhibit the later period of pain.[21]

Our study here demonstrates that TEE, EAF, and NBF of *Tengligen* can inhibit both phases of formalin induced pain with a more potent effect on the later phase than the early phase (Figure 2). Based on the results we observed, we tentatively suggest that the analgesic activity of the extract is dependent upon both central and peripheral sites of action. This conclusion is congruent with that reported by Shibata et al in 1989.[22] Taken together, our data suggest that the ability of the TEE, EAF, and NBF of *Tengligen* to suppress pain perception may be mediated via both the peripheral and central pathways of pain perception.

The carrageenan test is highly sensitive to non-steroidal anti-inflammatory drugs and has long been used as a phlogistic tool for evaluating new anti-inflammatory drugs. Carrageenan-induced inflammation has been used previously to detect orally active anti-inflammatory agents, and therefore has a significant predictive value for anti-inflammatory agents that act by inhibiting the mediators of acute inflammation.[23] The results we obtained here show that the *Tengligen* extract does indeed possess anti-inflammatory activity (Table 3).

Previous investigations have reported that saponins, flavonoids, phenylpropanoids, quinines, and steroid compounds have all been separated and structurally identified from *Tengligen*. Flavonoids, saponins, tannins, phenolic compounds, and glycosides have all been associated with various degrees of anti-inflammatory and analgesic activities.[24–28] Our results indicate that the mechanism of antinociceptive and anti-inflammatory effects of *Tengligen* extracts may be related to flavonoids and saponins. Flavonoids inhibit cyclooxygenase and lipoxygenase which are involved in initiation stage of inflammation reactions, but the precise mechanism of flavonoids in inhibition of these enzymes is not known. In addition, flavonoids are putative antioxidant with high activity of free radical scavenging. Free radicals can attract various inflammatory mediators, contributing to a general inflammatory response.[29]

In the past, pharmacological studies have focused mainly on crude extracts, and many of the constituents responsible for different pharmacological activities remain unknown. The antinociceptive and anti-inflammatory effects observed in this study may be in part due to the activity(s) of one or a combination of some of the classes of compounds we identified and examined here. However, more studies are needed to firmly establish the clinical efficacy of the TEE and fractions of *Tengligen* we examined, as well as to reveal the exact mechanism of action that characterizes the response to these substances.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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