ANTI-HEAD LICE EFFECT OF *ANNONA SQUAMOSA* SEEDS

Junya Intaranongpai[1], Warinthorn Chavasiri[2] and Wandee Gritsanapan[1]

[1]Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University, Bangkok;
[2]Department of Chemistry, Faculty of Science, Chulalongkorn University, Bangkok, Thailand

Abstract. The present study focused on the separation and identification of the active compounds against head lice from the hexane extract of *Annona squamosa* L seed. Chromatographic and spectroscopic techniques revealed that two major compounds of the hexane seed extract were oleic acid and triglyceride with one oleate ester. The yields of these compounds were 13.25% and 7.74% dry weight, respectively. The compounds were tested *in vitro* against head lice, comparing to the crude hexane extract of the seed. The triglyceride with one oleate ester and the crude hexane extract diluted with coconut oil 1:1. These compounds were found to kill all tested head lice in 49, 11 and 30 minutes, respectively. The triglyceride ester can be used as a marker for quantitative analysis of the active compound for quality control of the raw material *A. squamosa* seed and its extract. This first finding will be useful for quality assessment and the chemical stability of the anti-head lice preparation from this plant.

INTRODUCTION

Annona squamosa L. (Custard apple) is a plant belonging to the family Annonaceae. It is popularly cultivated in all parts of Thailand, especially in the northeast, as a sweet fruit. The seed of this plant is well known for killing head lice in many countries (Boonyaprapasara, 1998).

The human head louse (*Pediculus humanus capitis*) is a small insect causing a public health problem, especially in poor sanitary conditions. In Thailand, research has shown the anti-head lice activity of *A. squamosa*. Puapatanakul (1980) reported that the extract of custard apple seeds in coconut oil at the ratio of 1:2 can kill 98% of head lice within two hours, while the leaf extract shows less potency. Gritsanapan *et al* (1996) found that the petroleum ether extract of the leaves and seeds dissolved in coconut oil at a ratio of 1:1, kill 90% of head lice *in vitro* by 53 and 26 minutes, respectively. A 20% cream (oil/water) preparation of petroleum ether extract of custard apple seeds can kill 93% of head lice within 3 hours (Areekul and Chaikledkaew, 1994, personal communication). Gritsanapan *et al* (1996), reported that 20 g of 20% freshly prepared cream can kill 94.5±9.1% of head lice within 3 hours of application to school girls' hair. Tiangda *et al* (2000) found the cream preparation of custard apple seed is biologically stable for at least 12 months. However, it is easier to control the quality and stability of the preparation by quantitative analysis of the active chemical components. The active compounds of *A. squamosa* seed extract have not been reported elsewhere. The present study, therefore, is focused on the isolation and identification of the anti-head lice components in the seeds of *A. squamosa.*

Correspondence: Wandee Gritsanapan, Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University, 447 Si Ayutthaya Road, Ratchathewi, Bangkok 10400, Thailand.
Tel: +66 (0) 2644-8677 ext 1500, 5530; Fax: +66 (0) 2644-8701
E-mail: pywgs@mahidol.ac.th

MATERIALS AND METHODS

Preparation of plant extracts

A. squamosa seeds were purchased from Pak Chong District, Nakhon Ratchasima Province, Thailand in October 2004. The samples were identified by comparison with the herbarium at Forest Herbarium, Department of National Parks, Wild-life and Plant Conservation, Ministry of Natural Resources and Environment, Bangkok. The voucher specimen (WAS 0704) has been deposited at the Department of Pharmacognosy, Faculty of Pharmacy, Mahidol Uni-

versity, Bangkok, Thailand. The seeds were washed and dried in a hot air oven at 55ºC for 24 hours. The dried seeds were ground in an electric mill.

Extraction and separation of major compounds

The powdered seeds of *A. squamosa* (1.1 kg) were macerated with hexane (2x3 l) for three days at room temperature. The mixture was filtered and the filtrate was concentrated by a rotary evaporator and evaporated in a hot water bath until a constant weight (282.4 g) was obtained. The extract (75 g) was separated using silica gel column chromatography (400 g silica). Hexane (CH_2Cl_2 (1:1) - 100% CH_2Cl_2 : CH_2Cl_2 : MeOH (1:1)) was used as an eluent. Fifty milliliter fractions were collected and the fractions with the same TLC pattern ($SiGF_{254}$ hexane: ethyl acetate 10:3) were combined. The fractions containing two major spots (R_f 0.20 and 0.72) were eluted in 100% CH_2Cl_2 fractions.

To isolate pure compounds, the fractions containing major compounds were combined and concentrated. The mixture was further fractionated using silica gel column chromatography (200 g). Isocratic elution by hexane: ethyl acetate (10:3) was performed (approximately 25 ml per fraction). The fractions with the same TLC pattern were combined to yield five fractions. The second and fourth fractions gave compound AS1 (38.7 g) and compound AS2 (22.6 g), respectively. Compounds AS1 and AS2 were purified to give pure compounds.

Testing for anti-head lice activity of pure compounds and crude extract

The hexane extract and the two major pure compounds were tested for anti-head lice activity according to the method of McCage (2002). The extract and pure compounds were separately dissolved in coconut oil at dilutions of 1:1 to 1:8 w:w. The same amount of each solution (0.05 ml) was put in a Petri dish and spread in a thin layer over a 2 cm^2 area. Seven equal sized head lice collected from school girls' hair were placed in the Petri dish containing solutions of the extract and the two major pure compounds. Non-moving head lice, which were determined as dead lice, were counted every 5 minutes until all the lice were dead. A commercial anti-head lice cream, Hexin™, which is gamma benzene hexachloride (1% w/w) and coconut oil were used as a positive and negative controls, respectively.

RESULTS

Compound AS1 was a pale yellow oil, yielded 13.25% w/w of dried seeds. TLC ($SiGF_{254}$, hexane:ethyl acetate 10:3) had an R_f value of 0.20 (Fig 1). The EI mass spectrum had a molecular ion peak at *m/z* 283.2 [M+1] and a prominent peak at *m/z* 264.3.

The IR spectrum of compound AS1 revealed absorption peaks at 3000-2930 (O-H stretch), 2850 (C-H stretch), and 1700 (C=O stretch, carboxylic) cm^{-1}.

The ^1H NMR spectrum of compound AS1 indicated the presence of one methyl proton at δ 0.90 (3H, t, H-18); a methylene proton group at δ 1.26 (20H, m, H-4-7 and H-12-17); two methylene proton groups at δ1.65 (2H, m, H-3); four methylene proton groups at δ 2.00 (4H, m, H-8, 11); two methylene proton groups at δ 2.35 (2H, t, H-2); two olefinic methane proton groups at δ 5.35 (2H, m, H-9,10) and the broad peak of a hydroxyl proton at δ 10.15 (1H).

The ^{13}C NMR spectrum and Distortionless Enhancement by Polarization Transfer (DEPT) exhibited 16 carbon resonances, revealing the presence of thirteen methylene carbons, one methyl carbon, two olefinic methine carbons and one carbonyl carbon.

These spectral data suggested that compound AS1 was a fatty acid. Comparing the NMR spectra of compound AS1 with Aldrich Library (1993) of ^{13}C and ^1H FT NMR spectra, confirmed the molecular structure of AS1 was an oleic acid (Fig 2).

Compound AS2 was also pale yellow oil, yielded 7.74% w/w of dried seeds. The R_f value ($SiGF_{254}$, hexane: ethyl acetate 10:3) was 0.72 (Fig 1).The ^1H NMR spectrum looked similer to the AS1 spectrum, with additional signals at δ 4.15 and 4.30.

The IR spectrum of compound AS2 showed bands at 2925 (C-H stretch) and 1746 (C=O stretch, ester) cm^{-1}.

Table 1
Head lice killing time of the crude extract and pure compounds from *Annona squamosa* seeds (n=3).

Test sample	Dilution (w:w)	Killing time (min)
Hexane crude extract	1:1	30.67±4.04
	1:2	34.33±4.04
	1:4	41.00±3.61
	1:8	55.00±5.00
Oleic acid (AS1)	1:1	49.33±3.06
	1:2	54.67±5.51
	1:4	59.00±6.56
	1:8	61.33±4.16
Triglyceride with one oleate ester (AS2)		
	1:1	11.00±1.00
	1:2	12.00±2.00
	1:4	16.00±1.00
	1:8	22.33±2.52
Coconut oil (-ve control)	Not diluted	>180
Hexin™ (+ve control)	Not diluted	>180

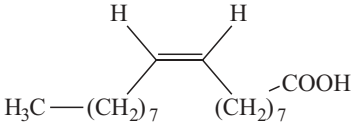
After comparing the NMR spectra of compound AS2 with Aldrich Library (1913) of ^{13}C and ^{1}H FT NMR spectra, compound AS2 was felt to be a triglyceride with one oleate ester (Fig 2).

The hexane crude extract of compound AS1 (oleic acid) and compound AS2 (triglyceride with one oleate ester) from *Annona squamosa* seeds showed *in vitro* anti-head lice activity as summarized in the Table 1.

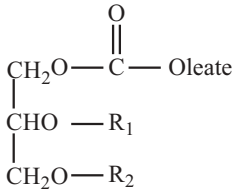
The data show that the triglyceride with one oleate ester was the most active compound against head lice. It killed all tested head lice within 11 minutes when diluted with coconut oil to a ratio of 1:1. Both the Hexin™ and the coconut oil killed all the head lice within a period of 180 minutes.

1 = AS1, 2 = AS2, 3 = hexane crude extract.

Fig 1–TLC chromatogram of AS1, AS2 and hexane crude extract.



Oleic acid



Triglyceride with one oleate ester
R_1, R_2 = H/ other fatty acid

Fig 2–Structure of separated compounds from *Annona squamosa* seed.

DISCUSSION

The triglyceride with one oleate ester in coconut oil (1:1) was significantly more active against head lice than gamma benzene hexachloride 1% cream and the hexane crude extract. These data are supported by previous reports (Gritsanapan *et al*, 1996; Tiangda *et al*. 2000). This result is useful for the standardization of *Annona squamosa* seed and its extract. The active compound may be used for the qualitative assessment of the chemical stability of the custard apple cream preparation. This is the a first report of the active anti-head lice components from *A. squamosa* seeds.

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