ISSN Print: 2664-844X
ISSN Online: 2664-8458
NAAS Rating (2025): 4.97
IJAFS 2025; 7(8): 1283-1286
www.agriculturaljournals.com
Received: 26-06-2025
Accepted: 30-07-2025

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Methods for extraction of Acetogenins from the seeds of Annona squamosa: A review

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DOI: https://www.doi.org/10.33545/2664844X.2025.v7.i8l.703

Abstract
Annona squamosa L., commonly known as custard apple or sugar apple, is a tropical fruit tree of the Annonaceae family, renowned for its seeds' rich content of annonaceous acetogenins (ACGs). These lipophilic polyketides exhibit potent bioactivities, including anticancer, antiparasitic, and pesticidal properties, making them promising candidates for pharmaceutical and agricultural applications. Efficient isolation and purification methods are critical to harnessing these compounds due to their complex chemical structures and low natural abundance. This review comprehensively examines recent advancements in the extraction, isolation, and purification of ACGs from *A. squamosa* seeds, focusing on techniques such as solvent extraction, ultrasound-assisted extraction (UAE), thermosonication-assisted extraction (TSAE), supercritical fluid extraction (SFE), and chromatographic methods. The review discusses the advantages, limitations, and optimization strategies of these methods, alongside their impact on yield, purity, and bioactivity. Challenges such as toxicity concerns and scalability are addressed, and future directions for improving extraction efficiency and sustainability are proposed.

Keywords: Annona squamosal, Aannonaceous acetogenins, custard apple, spectroscopic, Extraction

Introduction
Annona squamosa L., a member of the Annonaceae family, is widely cultivated in tropical and subtropical regions for its edible fruit and traditional medicinal uses. The seeds of *A. squamosa* have been used in folk medicine in South China to treat "malignant sores" (cancer) and as an insecticide (Chen *et al*., 2011) [8]. The primary bioactive constituents responsible for these properties are annonaceous acetogenins (ACGs), a class of C35/C37 polyketides derived from the polyketide pathway, characterized by a long fatty acid chain, tetrahydrofuran (THF) rings, and a γ-lactone moiety (Bermejo *et al*., 2005) [4]. Over 164 bis-THF and 224 mono-THF ACGs have been isolated from *A. squamosa* seeds, including squamocin, bullatacin, and annosquacins A–D, which exhibit potent cytotoxicity against various cancer cell lines (Chen *et al*., 2011; Miao *et al*., 2016) [8, 12].
The isolation and purification of ACGs are challenging due to their lipophilic nature, structural complexity, and low concentrations in plant material (typically 0.00019–0.003% yield) (Durán *et al*., 2021) [10]. Traditional extraction methods like Soxhlet and maceration are time-consuming and solvent-intensive, prompting the development of advanced techniques to improve yield, purity, and environmental sustainability. This review synthesizes recent research on the development of isolation and purification methods for ACGs from *A. squamosa* seeds, evaluating their efficacy, scalability, and potential for therapeutic applications.

Materials and Methods
Chemical Characteristics of *Annonaceous Acetogenins*
ACGs are secondary metabolites unique to the Annonaceae family, comprising a long aliphatic chain (C32–C34) with one to three THF rings, hydroxyl groups, and a terminal γ-lactone, often methylated and α,β-unsaturated (Alali *et al*., 1999) [2]. These structural features contribute to their bioactivity, particularly their ability to inhibit mitochondrial complex I, leading to cytotoxicity in cancer cells (Bermejo *et al*., 2005) [4]. Key ACGs from *A. squamosa* seeds include:

- **Squamocin:** A bis-THF ACG with potent cytotoxicity against leukemia (HL-60) and other cancer cell lines (Chen *et al*., 2012) [9].
- **Bullatacin**: Known for its high antitumor activity against MCF-7 and HepG2 cells, with minimal toxicity to normal cells at low doses (Chen *et al*., 2012) [9].
- **Annosquacins A–D:** Novel bis-THF ACGs with enhanced cytotoxicity against lung (A-549) and breast (MCF-7) cancer cell lines (Miao *et al*., 2016) [12].

The structural elucidation of ACGs relies on advanced spectroscopic techniques such as nuclear magnetic resonance (NMR), high-performance liquid chromatography (HPLC), and mass spectrometry (MS), which are critical for their identification and purification (Yang *et al*., 2009b) [15].

Extraction Methods for Acetogenins from *Annona squamosa* Seeds

- Soxhlet Extraction: This method involves continuous solvent extraction using organic solvents like ethanol, methanol, or ethyl acetate. Soxhlet extraction has been widely used for ACG extraction due to its simplicity and ability to extract lipophilic compounds. For instance, used 95% ethanol in a Soxhlet apparatus to isolate six new ACGs (annosquacins A–D, annosquatin A, and B) from *A. squamosa* seeds, achieving high purity (>98%) via subsequent chromatography. However, Soxhlet extraction is time-consuming (6–8 hours), requires large solvent volumes, and may degrade thermolabile ACGs due to prolonged heating.
- Maceration: Maceration involves soaking seed powder in solvents like hexane, chloroform, or methanol. Yang *et al*. (2009a) [15] reported maceration with ethanol to extract ACGs, followed by partitioning with ethyl acetate–water to isolate compounds like squamostatin-A. Maceration is less equipment-intensive but yields lower ACG content (e.g., 1–2% dry weight) compared to advanced methods and requires extended extraction times (up to 72 hours).
- Ultrasound-Assisted Extraction (UAE): UAE uses ultrasonic waves to disrupt plant cell walls, enhancing solvent penetration and compound release. León-Fernández *et al*. (2020) [11] optimized UAE for *A. muricata* seeds, achieving a total acetogenin content (TAC) of 13.01 mg/g dry weight (DW) in *A. squamosa* seeds using ethanol at 100% amplitude, 5–15 min, and 0.4–1 s pulse-cycle. Compared to maceration, UAE increased TAC by 993% for seeds, demonstrating its efficiency. However, UAE's effectiveness depends on parameters like amplitude, time, and solvent polarity, and it may require optimization via response surface methodology (RSM) to maximize yield.
- Thermosonication-Assisted Extraction (TSAE): TSAE combines ultrasound with controlled heating to enhance extraction efficiency. A 2022 study by Aguilar-Hernández *et al*. optimized TSAE for *A. muricata* seeds, achieving a TAC of 35.89 mg/g DW at 50 °C, 100% amplitude, and 0.5 s pulse-cycle, outperforming Soxhlet (15.60-fold higher TAC) and UAE at 25 °C (2.17-fold higher). TSAE's ability to extract ACGs like pseudoannonacin and annonacin from *A. squamosa* seeds suggests its potential for *A. squamosa*, though specific studies are limited. TSAE reduces extraction time and solvent use but requires precise temperature control to avoid ACG degradation.
- Supercritical Fluid Extraction (SFE): SFE using CO_2 is an eco-friendly method that minimizes solvent residues. Yang *et al*. (2009a) [15] applied SFE to extract eight ACGs from *A. squamosa* seeds, achieving high purity with minimal environmental impact. SFE is selective for non-polar ACGs but requires high-pressure equipment, limiting its scalability for small-scale laboratories.
- Solvent Selection and Optimization**:** Solvent polarity significantly affects ACG extraction. Non-polar solvents (e.g., hexane, chloroform) extract less polar ACGs, while polar solvents (e.g., ethanol, methanol) are effective for hydroxylated ACGs like squamocin. Nik Mat Daud *et al*. (2016) [13] used a sequential polarity-guided extraction (hexane, chloroform, methanol) to isolate bullatacin and squamocin, identifying fractions rich in ACGs using the Kedde reagent, which forms a dark green complex with the γ-lactone group. RSM has been employed to optimize solvent-to-solid ratios, extraction time, and temperature, with ethanol at 1:6 (solvent:seed) and 4–8 hours yielding optimal TAC for *A. squamosa* (Nik Mat Daud *et al*., 2016) [13].

Purification Methods for Acetogenins

Chromatographic Techniques

- **Silica Gel Chromatography:** This is the most common method for ACG purification due to its ability to separate compounds based on polarity. Chen *et al*. (2011) [8] used silica gel column chromatography with ethyl acetate–hexane gradients to isolate annosquacins A–D from *A. squamosa* seed extracts, achieving >98% purity confirmed by HPLC and NMR. The method is cost-effective but requires large solvent volumes and multiple steps for complex mixtures.
- **High-Performance Liquid Chromatography (HPLC):** HPLC, often coupled with diode-array detection (HPLC-DAD) or mass spectrometry (HPLC-MS), is used for precise separation and quantification of ACGs. Yang *et al*. (2009b) [15] employed HPLC-DAD to quantify 12,15-cis-squamostatin-A and bullatacin, achieving detection limits of 0.1–0.5 µg/mL. Reverse-phase HPLC with C18 columns is preferred for ACGs due to their lipophilic nature, but high costs and specialized equipment are limitations.
- **Thin-Layer Chromatography (TLC):** TLC is used for preliminary screening and fraction identification. Al Kazman *et al*. used TLC with Kedde reagent to detect ACGs in *A. squamosa* seed extracts, observing pink bands indicative of γ-lactone groups. TLC is simple and rapid but lacks the resolution for final purification.

Spectroscopic Confirmation

Post-purification, ACGs are characterized using:

- **NMR Spectroscopy***:* 1H and 13C NMR, along with 2D techniques (COSY, HMBC), confirm THF rings and γ-lactone moieties. For example, Miao *et al*. (2016) [12] used 600 MHz 1H NMR to elucidate annosquacin structures.
- **Mass Spectrometry (MS):** High-resolution electrospray ionization MS (HRESI-MS) determines molecular formulas, as seen with dieposabadelin

(C35H62O4, m/z 569.4560) (Bajin ba Ndob *et al*., 2009).

- **Fourier Transform Infrared Spectroscopy (FT-IR):** FT-IR identifies functional groups, with peaks at 1748–1752 cm^{-1} (carbonyl) and 3418 cm^{-1} (hydroxyl) confirming ACG structures (Chen *et al*., 2012) [9].

Bioactivity and Applications of Acetogenins

ACGs from *A. squamosa* seeds exhibit diverse bioactivities:

Anticancer Activity: Annosquacins A–D and bullatacin inhibit tumor cell lines (e.g., MCF-7, A-549) by targeting mitochondrial complex I, with IC50 values as low as 7.8 nM (Chen *et al*., 2011; Miao *et al*., 2016) [8, 12].

Anthelmintic Activity: A C37 trihydroxy bis-THF ACG inhibited *Haemonchus contortus* egg hatching at 25 mg/mL, supporting its use in veterinary medicine (Castañeda-Ramírez *et al*., 2019) [6].

Pesticidal Activity: Squamocin damages insect midgut and inhibits NADH ubiquinone oxidoreductase, making it a natural insecticide (Bonneau *et al*., 2017) [5].

Challenges and Limitations

- **Low Yield and Scalability:** ACG yields are low (0.00019–0.003%), requiring large quantities of seeds and solvents, which hinders commercial production (Durán *et al*., 2021) [10].
- **Toxicity Concerns:** High doses of ACGs, particularly bullatacin, cause liver and kidney toxicity in rats via increased reactive oxygen species and Bax expression (Chen *et al*., 2012) [8]. Chronic consumption may lead to neurodegenerative tauopathies, necessitating cautious use (Champy *et al*., 2011) [7].
- **Environmental Impact:** Conventional methods like Soxhlet use large solvent volumes, posing environmental risks. Green methods like SFE and TSAE are more sustainable but require costly equipment (Aguilar-Hernández *et al*., 2022) [1].
- **Structural Complexity:** The presence of multiple ACGs in extracts complicates purification, requiring multi-step chromatography (Nik Mat Daud *et al*., 2016) [13].

Future Directions

- **Green Extraction Technologies:** Expanding TSAE and SFE applications for *A. squamosa* seeds could reduce solvent use and environmental impact. Pilot-scale TSAE studies are needed to assess industrial feasibility.
- **Optimization with RSM:** Further RSM studies can optimize UAE and TSAE parameters (e.g., temperature, amplitude, solvent type) to maximize TAC and minimize degradation.
- **Synthetic Mimics:** Developing ACG mimics like AA005, which are easier to synthesize, could overcome low natural yields (Durán *et al*., 2021) [10].
- **Toxicity Mitigation:** Encapsulation techniques for single ACGs (e.g., bullatacin) could reduce toxicity and enhance targeted delivery for cancer therapy (Nik Mat Daud *et al*., 2016) [13].
- **Bioactivity-Guided Fractionation:** Integrating bioassays (e.g., MTT, brine shrimp lethality) during purification can prioritize fractions with high anticancer or antiparasitic activity (Chen *et al*., 2011) [8].

Conclusion

The isolation and purification of acetogenins from *Annona squamosa* seeds have advanced significantly with the development of UAE, TSAE, and SFE, which offer higher yields and sustainability compared to conventional Soxhlet and maceration methods. Chromatographic techniques, particularly silica gel and HPLC, remain essential for achieving high-purity ACGs, while NMR, MS, and FT-IR ensure accurate structural elucidation. Despite challenges like low yields and potential toxicity, the potent bioactivities of ACGs, including anticancer and anthelmintic properties, underscore their therapeutic potential. Future research should focus on scalable, eco-friendly extraction methods and toxicity mitigation strategies to facilitate the clinical and industrial application of *A. squamosa* ACGs.

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