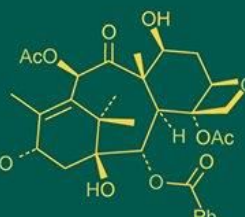
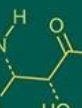
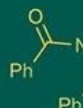


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Development of isolation and purification method for extraction of acetogenin from the seeds of custard apple

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Abstract

Acetogenins are bioactive compounds predominantly found in the seeds of *Annona squamosa* (custard apple) and have demonstrated significant pesticidal, anticancer, and antimicrobial properties. However, their practical application is often limited by the lack of standardized and efficient extraction methods that ensure high purity and yield. This study focuses on the development of an optimized protocol for the isolation and purification of acetogenin from custard apple seeds. The procedure integrates seed preparation, solvent extraction, and chromatographic purification to maximize recovery while minimizing degradation of the target compound. The extraction efficiency was evaluated using different solvent systems, and purification was achieved through column chromatography with gradient elution. The yield and purity of acetogenin were assessed using spectroscopic and chromatographic analyses (UV-Vis, TLC, and HPLC). The optimized method yielded acetogenin of high purity, offering a reproducible and scalable approach suitable for further pharmacological studies and industrial applications. This work provides a foundation for cost-effective large-scale production and potential exploitation of acetogenin in pharmaceutical and agrochemical sectors.

Keywords: Annona squamosa, acetogenins, extraction of acetogenin, column chromatography

1. Introduction

Acetogenins are a class of natural compounds primarily found in the Annonaceae family, notably in species like *Annona squamosa* (sugar apple or sweetsop), *Annona muricata* (soursop), and *Annona cherimola* (cherimoya). These polyketide-derived molecules, characterized by a long aliphatic chain with a terminal lactone ring, are predominantly located in the seeds, leaves, and bark of these plants. Acetogenins exhibit a range of biological activities, including anticancer, antiparasitic, insect-repellent, and neurotoxic properties, making them a subject of significant pharmacological and agricultural interest. Their primary mode of action involves inhibiting mitochondrial complex I (NADH: ubiquinone oxidoreductase), disrupting ATP production, which selectively targets energy-demanding cells like cancer cells or parasites (Alali *et al.*, 1999) [1]. Additionally, acetogenins inhibit multidrug resistance proteins, enhancing their potential in overcoming chemotherapy resistance (Oberlies *et al.*, 1997) [7].

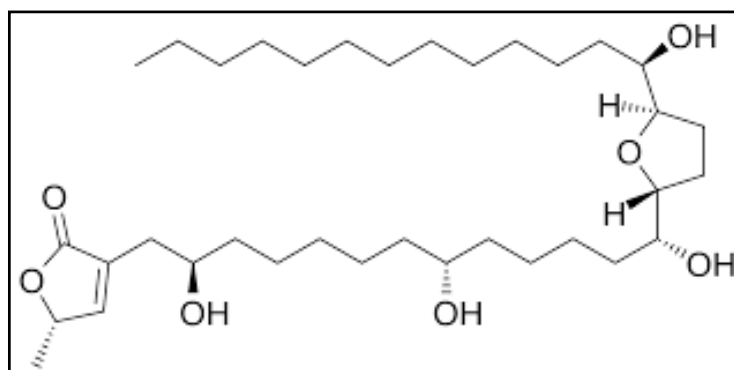


Fig 1: Structure of acetogenin.

The chemical structure of acetogenins typically includes a 35-37 carbon backbone with oxygenated functional groups, such as hydroxyls, ketones, or epoxides, and one to three tetrahydrofuran (THF) rings, contributing to their bioactivity. Over 400 acetogenins have been identified, with notable examples like annonacin, squamocin, and bullatacin, each varying in potency and specificity. For instance, annonacin, abundant in *Annona muricata*, has shown cytotoxicity against various cancer cell lines, including breast, lung, and colon cancers, with IC₅₀ values often in the nanomolar range (Liaw *et al.*, 2010) [6]. However, their neurotoxicity, particularly linked to annonacin, raises concerns, as chronic consumption of annonaceous fruits or teas has been epidemiologically associated with atypical parkinsonism in regions like Guadeloupe, likely due to mitochondrial dysfunction in neurons (Höllerhage *et al.*, 2009) [3].

In agriculture, acetogenins serve as natural insecticides and antifeedants. Powdered seeds of *Annona squamosa*, rich in squamocin, are traditionally used to control lice and pests, leveraging their toxicity to disrupt insect mitochondrial metabolism (Leatemia & Isman, 2004) [5]. Despite their therapeutic potential, clinical applications are limited by challenges in solubility, bioavailability, and toxicity. Acetogenins are poorly water-soluble, requiring advanced delivery systems like liposomes or nanoparticles to enhance efficacy. Moreover, their non-selective toxicity necessitates careful dose optimization to avoid adverse effects on healthy cells. Current research focuses on semi-synthetic derivatives to improve specificity and reduce neurotoxic risks, alongside exploring their antimalarial and antiviral properties (Bermejo *et al.*, 2005) [2].

The extraction of acetogenins from plants in the Annonaceae family, such as *Annona squamosa* (sugar apple) and *Annona muricata* (soursop), is a critical process for studying their potent bioactivities, including anticancer, antiparasitic, and insect-repellent properties. Acetogenins, polyketide-derived compounds with a long aliphatic chain and a terminal lactone ring, are primarily concentrated in seeds, leaves, and bark, requiring careful extraction to preserve their structural integrity and bioactivity.

Further purification is achieved through column chromatography, often employing silica gel with a gradient of hexane and ethyl acetate, which allows for the isolation of individual acetogenins based on their differential interactions with the stationary phase (Bermejo *et al.*, 2005) [2]. High-performance liquid chromatography (HPLC) is frequently used for final purification, with reversed-phase C18 columns and methanol-water or acetonitrile-water mobile phases to achieve high-purity acetogenin fractions. Detection is typically performed using UV-Vis spectroscopy at 210-220 nm, as acetogenins exhibit weak UV absorption due to their lactone moiety (Liaw *et al.*, 2010) [6]. For acetogenins, which are lipophilic with varying degrees of polarity due to hydroxyl and tetrahydrofuran groups, silica gel column chromatography effectively separates them from less polar lipids and more polar phenolics. Fractions are collected in small volumes, monitored by thin-layer chromatography (TLC) with visualization under UV light or staining with reagents like vanillin-sulfuric acid, which detects acetogenins' lactone moieties (Alali *et al.*, 1999) [1]. The elution gradient is tailored to the target compounds; for example, a hexane:ethyl acetate ratio starting at 9:1 and gradually increasing to 1:1 is common for isolating

acetogenins like squamocin or annonacin from *Annona squamosa* seed extracts. The technique's versatility allows for scale-up in preparative chromatography, where larger columns and automated fraction collectors enhance throughput for industrial applications (Snyder *et al.*, 2012) [8].

2. TLC for Isolation of Actogenins

The choice of mobile phase is critical, with typical ratios like hexane:ethyl acetate (7:3 or 8:2) effectively separating acetogenins due to their intermediate polarity, influenced by hydroxyl and tetrahydrofuran moieties. After development, acetogenins are visualized under UV light at 254 nm, where their lactone rings exhibit weak absorption, or by spraying with reagents like vanillin-sulphuric acid, which produces colored spots (e.g., pink or purple) specific to acetogenins, facilitating their identification (Bermejo *et al.*, 2005) [2]. Retention factor (R_f) values, calculated as the ratio of the distance traveled by the compound to that of the solvent front, are used to characterize acetogenins, with R_f values typically ranging from 0.3 to 0.6 in hexane:ethyl acetate systems for compounds like annonacin (Liaw *et al.*, 2010) [6]. Preparative TLC, using thicker silica gel plates (e.g., 1-2 mm), allows for larger sample loads to isolate milligram quantities of acetogenins, where bands are scraped off, eluted with methanol or chloroform, and further purified, often by high-performance liquid chromatography (HPLC) for higher resolution (Hostettmann *et al.*, 1998) [4]. TLC's advantages include its rapid setup, low cost, and ability to process multiple samples simultaneously, making it ideal for screening crude extracts and monitoring column chromatography fractions. However, limitations include lower resolution compared to HPLC, potential overlap of structurally similar acetogenins, and sensitivity to environmental factors like humidity, which can affect R_f values. To enhance separation, two-dimensional TLC or high-performance TLC (HPTLC) with finer particle size stationary phases can be employed, offering improved resolution and reproducibility (Snyder *et al.*, 2012) [8]. For acetogenin isolation, TLC is often a complementary step, guiding the purification process by identifying fractions rich in target compounds before final purification. Its role in acetogenin research underscores its importance in natural product chemistry, balancing accessibility with sufficient sensitivity for preliminary isolation tasks.

30 cm column was used to fractionate the sample. F4 bit was transferred to an open column chromatography with silica gel 60 as the stationary phase. Detergents used as eluents were hexane, hexane-chloroform (82), hexane-chloroform (11) and ethyl acetate. Pink colour spot on TLC distance after dipping in kedde's reagent confirm the presence of acetogenin in CC bit.



Fig 2: Setting up the column for chromatography.

3. Materials and Methods

Acetogenins are bioactive polyketides found in plants of the Annonaceae family, known for their cytotoxic, antitumor, and pesticidal properties. The extraction, isolation, and identification of acetogenins involve a series of phytochemical techniques aimed at obtaining pure compounds for further analysis or application.



Fig 3: Seeds of Custard Apple

3.1 Sample Extraction

The process begins with grinding seeds to increase surface area, followed by sequential extraction using solvents of increasing polarity (n-hexane, chloroform, methanol) to isolate acetogenins, which are polar compounds. Non-polar solvents remove lipids, while methanol extracts polar acetogenins. The methanol extract is partitioned with ethyl acetate to concentrate acetogenins in the organic phase. This method leverages the principle of differential solubility to separate compounds based on polarity (Harborne, 1998) [4].

3.2 Column Chromatography for Isolation

Column chromatography uses silica gel (SiO_2) as a stationary phase and a gradient of n-hexane: chloroform as the mobile phase to separate acetogenins based on their affinity for the stationary phase. The gradient elution ensures that compounds with varying polarities are eluted sequentially, allowing for the isolation of acetogenins in specific fractions (Liaw *et al.*, 2010) [6].



Fig 4: TLC chamber of ethyl acetate extract

3.3 Thin-Layer Chromatography (TLC) for Identification

TLC confirms the presence of acetogenins using silica gel 60 as the stationary phase and chloroform: methanol (9:1) as the mobile phase. Visualization with Kedde's reagent,

which reacts with the lactone ring of acetogenins to produce a pinkish color, confirms their presence. The R_f value aids in identification by comparing migration distances (Stahl, 1969) [9].

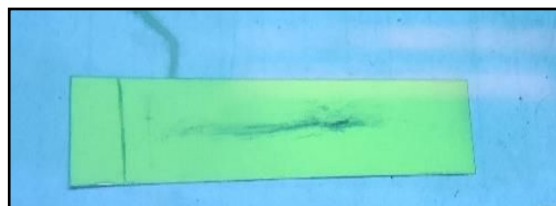


Fig 5: TLC appearance of purple colour on the ethyl acetate plate in UV chamber

4. Identification of acetogenin by using kedde reagent

All the fragments (F1, F2, F3 and F4) were carried out on logical TLC distance with silica gel 60 as stationary phase and chloroform-methanol (9:1) as mobile phase. F4 bit showed the conformation of Pinkish purple colour spot on TLC distance after dipping in kedde's reagent which indicates the presence of acetogenins and this spot disappears within a nanosecond. There's no confirmation of pink spot on all other fragments (F1, F2 and F3) which indicates the absence of acetogenins.

All the chemicals and detergents were carried as a logical grade. Thin level Chromatography (TLC) analysis used by TLC on pre-coated aluminium wastes, silica gel GF254/366 (20 cm x 20 cm, 0, 15 mm consistence, Merck) and emulsion were detected under UV light (254 & 366 nm) and scattering with $\text{Ce}_2(\text{SO}_4)_3/\text{H}_2\text{SO}_4$ with 5 methanol followed by heating the plates at 110°C for 5 minutes.

Analytical TLC was carried out on silica gel 60 as stationary phase and chloroform-methanol (9:1) as mobile phase. Acetogenin imaged by scattering kedde reagent on TLC plate. Kedde's reagent which consists of equal volumes of 2 result of 3, 5-dinitrobenzoic acid in ethanol and 5.7% result of KOH in ethanol. It forms pinkish purple colour spot in the presence of acetogenin.

5. Conclusion

Annona squamosa seeds contain powerful bioactive compounds, especially acetogenins, which exhibit strong cytotoxic and anti-proliferative effects against a wide variety of cancer cell lines. These effects are achieved through mechanisms such as mitochondrial inhibition, apoptosis induction, ROS generation, and cell cycle arrest. The cytotoxic activity has been proven in both *in vitro* and *in vivo* models, showing potential for clinical applications. However, care must be taken due to potential toxicity, and future work should focus on purification, formulation, and human testing. Thus, with proper scientific validation and formulation, *Annona squamosa* seed extract may serve as a promising natural source for anticancer therapies.

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