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Original Article

In vitro anticancer assessments of *Annona muricata* fractions and *in vitro* antioxidant profile of fractions and isolated acetogenin (15-acetyl guanacone)

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ABSTRACT

Annona muricata has been attributed with numerous health benefits – including anticancer properties. The aim of this study was to carry out anticancer investigations of fractions of *Annona muricata*, by isolating a key compound and assessing antioxidant properties. The anticancer properties of fractions of *Annona muricata* were assessed using cell lines, and the potent fractions were subjected to isolation procedures. The isolated compound was examined using standard spectroscopies and further assayed with fractions for antioxidant properties. The cell viability of the most potent fractions revealed that ethyl acetate fruit (EAF, 10.62%) and ethyl acetate leaf (EAL, 10.83%) had the highest anticancer abilities, followed by ethyl acetate root-bark (11.44%), crude methanol leaf extract (16.21%) and crude methanol fruit extract (53.50%). The bi-phasic effect was observed for ethyl acetate fruit. The "*reverse cycle S-G2 phase disproportionation*" pattern was also observed with EAF and EAL. EAF caused a significant reduction of integrins leading to a decline in cell – cell interactions and increased cell death. The isolated compound demonstrated the presence of α, β-unsaturated γ-lactone and hydroxyl functional groups and molecular weight, 662.4 arriving at a molecular formula of $C_{39}H_{66}O_8$ and elucidated as 15-acetyl guanacone. The observations from antioxidant assays suggested that 15-acetyl guanacone possessed a better antioxidant potential compared to fractions. The superior antioxidant properties of EAF compared to EAL correlated significantly with 15-acetyl guanacone ($r = 0.563$ and $r = 0.682$, respectively). Thus, the possible *in vitro* anticancer mechanisms of *Annona muricata* could be attributed to pro-apoptotic, bi-phasic and karyokinesis effects, "*reverse-cycle S-G2 phase disproportionation*", and inhibition of integrin and antioxidant capacity.

1. Introduction

The *Annonaceae* is comprised of some 120 genera and includes over 2100 species. *Asimina triloba* (pawpaw) is the major temperate specie, while the rest of the family is tropical or subtropical, *e.g. Annona muricata*. Using cell lines, activity-guided fractionation of extracts of the *Annonaceae* family have led to the isolation and molecular characterization of unique annonaceous acetogenins.[1,2] These annonaceous acetogenins are derivatives of long chain (C32 or C34) fatty acids.[1,3] Their mechanism of cytotoxicity includes being potent inhibitors of mitochondrial (complex I) as well as a cytoplasmic (anaerobic) production of adenosine triphosphate (ATP) and the related nucleotides.[4] According to Gupta et al.,[1] they inhibit complex I (NADH: ubiquinone oxidoreductase) of mitochondrial electron transport systems (ETS). It is believed that they caused tumor cell inhibition by blocking oxidative phosphorylation, limiting the level of ATP, and, therefore, inducing a type of suffocation (ATP deprivation) at the cellular level. The acetogenins are also inhibitors of the NADH oxidase which is prevalent in the plasma membranes of cancer cells.[2] Agu et al.[5] also suggested that these compounds target and bind to vascular endothelial growth

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factor 2, *VEGF2* and α1 β2 integrin. These modes of action deplete ATP (adenosine triphosphate) and nutrient supply ultimately inducing programmed cell death (apoptosis).[4] Cancer cells are better targets for the acetogenins than normal cells because they have elevated levels of NADH oxidase accompanied by higher ATP demand.[1,2] The NADH oxidase inhibition lowers intracellular ATP levels by blocking NAD regeneration and, thus, inhibiting glycolytic (substrate level) phosphorylation in the cytosol; these combined modes of action likely lead to apoptosis (programmed cell death).[6] The selectivity of the acetogenins for tumor cells could then be explained both by the higher activities of the NADH oxidase that is peculiar to tumor cells, as well as the increased ATP demands that are inherent due to their uncontrolled growth.[7] Acetogenins help to modulate or reduce the growth of blood vessels near the cancerous cells.[5] Since the cancer cells need more ATP, they need more glucose to convert into it. Thus, the body tends to grow new blood vessels to deliver the glucose. Actually, the new blood vessel growth needs ATP itself, so the ATP reduction helps reduce that growth that is necessary for cancer cell survival.[3] One of the ways that cancer cells develop resistance to chemotherapy drugs is by creating an intercellular efflux pump called a P-glycoprotein mediated pump.[8] These types of pumps are capable of pushing anticancer agents out of the cell before they can kill it. On average, only about 2% of the cancer cells in any given person might develop this pump, but they are the 2% that can eventually grow and expand to create multi-drug-resistant tumors (MDRs). Collnot et al.[9] proposed competitive inhibition of substrate binding, alteration of membrane fluidity, and inhibition of efflux pump ATPase as possible mechanisms by which the effectiveness of these intracellular pumps are reduced in these MDRs.[10–12] Xia et al.[13] also proposed a possible down-regulation of gene expression of this pump in these MDRs. However, Qian et al.[14] reported that MDR proteins such as *MDR1, MRP1, Topo-IIα, GST-π, cyclin D1, Survivin* and *bcl-2* are down-regulated, while intracellular *Rh-123* and *caspase-3/8* are up-regulated by annonaceous acetogenins. They also reported decreased activities of *NF-κB* and *Akt* by annonaceous acetogenins, and that *Akt/NF-κB* pathway is involved in annonaceous acetogenins reversal of MDRs.

Hence, when the acetogenins, as potent complex 1 and NADH oxidase inhibitors, decrease intracellular ATP levels, they, therefore, decrease the effectiveness of the P-glycoprotein efflux pump. Some of the latest research on acetogenins reported that they were capable of shutting down these intercellular pumps. Acetogenins preferentially kill MDRs by blocking the transfer of ATP into them. A tumor cell needs the energy to grow and reproduce, and a great deal more to run its pump and expel attacking agents. By inhibiting energy to the cell, it can no longer run its pump. When acetogenins block ATP to the tumor cell over time, the cell no longer has sufficient energy to operate sustaining processes and it dies.[15] Normal cells seldom develop such a pump; therefore, they don't require large amounts of energy to run a pump, and generally are not adversely affected by ATP inhibition.

Anoikis is a form of programmed cell death that is induced by anchorage-dependent cells detaching from the surrounding extracellular matrix (ECM).[16] Usually, cells stay close to the tissue to which they belong since the communication between proximal cells as well as between cells and ECM provide essential signals for growth or survival. When cells are detached from the ECM, there is a loss of normal cell–matrix interactions, and they may undergo *anoikis*. However, metastatic tumor cells may escape from *anoikis* and invade other organs.

According to Potapova et al.,[17] cells exiting mitosis are able to undo this process and return to the metaphase if cyclin B is preserved. The destruction of *cyclin B* at anaphase, and the resultant inactivation of *Cdk1* results in mitotic exit and cytokinesis. By altering this process through mitotic regulators, Gorbsky's research group was able to reverse mitotic exit in vertebrate cells. They inhibited the ubiquitin proteasome activity, thus preserving *cyclin B* at anaphase. They then forced these cells into cytokinesis through the inhibition of *Cdk1* activity. When they withdrew the *Cdk1* inhibitor, the cells reverted back into mitosis. The cleavage furrow opened, the nuclear envelope dissolved, the chromatin recondensed, and the mitotic spindles reformed and recaptured the chromosomes.

In this research, we subjected the vacuum-liquid chromatography fractions of *Annona muricata* to *in vitro* anticancer studies using some selected cancer cell lines. We also attempted to put into focus the argument that Potapova et al.[17] put forward: namely, that the cell cycle reverse could be a mode of action of some antineoplastic agents. We also isolated and elucidated an acetogenin, and carried out *in vitro* antioxidant studies of the fractions and isolated compound.

2. Materials and methods

Plant Materials: The various parts of *Annona muricata* (Soursop), *i.e.*, matured unripe fruit, leaf, stem-bark and root-bark, were collected from the different location around the University of Benin and its environs, and was authenticated in the school's botany department and deposited in the herbarium with voucher number, UBHa 0205.

Preparation of plant extracts: The powdered plant samples (500 g) were each extracted by maceration with methanol (3 L) at room temperature and concentrated to dryness using a rotary evaporator *in vacuo*. The percentage yield of the crude extracts, CE (119, 323, 147, and 131, for fruit, leaf, stem-bark and root-bark) respectively, were obtained. Thereafter, dried samples were stored at 4 °C until further use.

Preparation of fractions used for cytotoxicity assay: The various methanolic extracts were further subjected to VLC fractionation using a vacuum pump, sintered funnel and silica gel. From 0.44 g to 0.93 g of the crude extracts were used for forming a uniform slurry with silica gel using a mortar and pestle. The solvent used included hexane (Hx; 100%), hexane-ethyl acetate (Hx-EA; 50:50%), ethyl acetate (EA; 100%), ethyl acetate-methanol (EA-MeOH; 50:50%), methanol (MeOH; 100%) and methanol-water (MeOH-water; 90:10%). Ultimately, each of the VLC fractions were dissolved in dimethyl sulfoxide (DMSO) to generate the desired stock solution of 50 mg/ml which was aliquoted and stored at −80 °C.

Proton Nuclear Magnetic Resonance (H^1NMR) spectroscopy: The sample was dissolved in $CDCl_3$. The different functional groups based on proton association were identified using NMR (JNMPMX60SI) at 500 MHz.

Infrared (IR) Spectroscopy: Using a Perkin-Elmer 1430 infrared spectrophotometer, the functional groups of the compound were identified. The measurements were carried out at an infra-red spectra of between 400 and 4000 nm.

Electron Impact$^+$ Mass Spectra (MS): A mass spectrophotometer (MS-600H-1) was used. The product was subjected to a stream of high energy electrons at elevated temperature of up to 100 °C (vaporization, fragmentation and ionization). The cleavage fragments yielded were characterized by mass/charge from mass spectra data.

The Culture of Cell Lines. The cell lines were obtained from the American Type Culture Collection, (Manassas VA, USA). Cells were maintained at 37° C and in a 5% CO_2 atmosphere in a monolayer in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Germany) with 10% fetal bovine serum (PAA Laboratories, GmbH, Germany) and antibiotic-antimycotic (1% penicillin G/streptomycin and 0.5% amphoteric B) solution (Ratiopharm, Germany). Confluent cells

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