Characterization of Polyphenolic Content and Antioxidation Activity of Pawpaw (*Asimina triloba*) and Its Anti-cancer Effects in Hepatocellular Carcinoma Cell Lines-Derived From Racially Diverse Patients

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Abstract

This study was focused on assessing the phenolic content and antioxidant properties of three distinct pawpaw species. Moreover, it explored the impact of pawpaw treatment on the cell viability of hepatocellular carcinoma cells-derived from racially diverse patients. The findings revealed that the Atwood sample displayed the highest phenolic content, which was determined using Folin&Ciocalteu’s (FC) reagent, while the Sunflower sample exhibited the most significant antioxidant activity, as indicated by both the DPPH and ABTS assays. Additionally, the Atwood sample demonstrated the most potent inhibition of cell proliferation based on the WST-1 assay. Collectively, these experimental results lend support to the notion that pawpaw may possess anticancer effects on liver cancer cell lines; however, the effect was not significantly different between HCC derived from Caucasian and Africa American patients.

Keywords: HepG2; O/20-HCC; Cell viability; Antioxidation; Polyphenols

Introduction

Hepatocellular carcinoma (HCC) represents the predominant subtype of primary liver cancer and ranks as the fifth leading cause of cancer mortality in the United States [1]. Given the considerable toxicity associated with current cancer treatments, there is a distinct need for the emergence of alternative therapies that offer potent efficacy while minimizing side effects for liver cancer treatment. Henceforth, researchers are consistently exploring innovative therapeutic agents to treat various kinds of cancers.

The integration of dietary compounds has emerged as a potential strategy to attenuate various malignancies and tumors. Certain natural dietary components have demonstrated a synergistic potential when combined with drug treatments, leading to an inhibitory impact on the proliferation of tumor cells [2]. Furthermore, combined therapeutic approaches have proven adept at bolstering immune function and orchestrating the regulation of various signaling pathways implicated in the onset and progression of cancer [3]. Fruits are rich in vitamins, minerals, and phytochemicals that have been reported to exert beneficial biological effects including anti-cancer, antioxidant and anti-inflammatory activities [4].

The Pawpaw, scientifically known as *Asimina triloba*, is an edible fruit that is native to the United States; however, it is now grown in South Korea, Japan, Italy, China, Israel, Romania, Portugal, Nigeria, and Belgium [5]. Belonging to the Annonaceae family, and commonly referred to as custard apples, is the only member of the family that is a temperate species. It can be found in 25 states ranging from northern Florida to southern Ontario and extending as far west as Nebraska [5]. According to a historical review by Hormaza, the utilization of this fruit can be traced back to the era of Native Americans and the early settlers[6]. Pawpaw played a significant role in the Native American diet, with the Shawnee tribe even dedicating a month to celebrate this fruit. The first recorded mention of Pawpaw by a European nation dates to 1540 when a traveler during Hernando de Soto’s Mississippi expedition observed Native Americans cultivating this fruit. Journal entries from the 1800s reveal that the pawpaw was a crucial food source that sustained the Lewis & Clark expedition.

Pawpaw possesses elevated levels of glutamic acid, oleic acid,
linoleic acid, potassium, phosphorus, calcium, and sulfur [7]. The medicinal properties of the bark, leaves, and twigs of pawpaw are proven to have cancer-fighting properties [8]. These three components of the pawpaw tree contain a specific phytochemical known as acetogenin, which has the ability to reduce ATP production within cells [9]. This inhibition of ATP production is crucial in preventing the growth of tumors, particularly in cases where they have already formed. The essential oils of pawpaw were found to display anticancer effects on the human lung carcinoma cell line A549 and the human breast carcinoma cell line MDA-MB231[10]. Unripe pawpaw exhibits a greater ability to prevent the growth of cancer cells compared to ripe pawpaw [8]. This characteristic makes pawpaw a potential option for cancer treatment, with the advantage of the fruit being less toxic than chemotherapy drugs.

We hypothesized that because of its anti-inflammatory properties, pawpaw might be more effective in HCC cells. We investigated the effects of pawpaw extract on the proliferation of HepG2 (from Caucasian patient) and O/20 (from African American patient) HCC cell lines. The aim of this study was to determine the phytochemical and antioxidant profile of three pawpaw species for their in vitro anticancer potential in HCC. To our knowledge, the anticancer potential of pawpaw fruits in mitigating HCC is not known. Our findings might provide insights into unraveling biological activities and the inclusion of pawpaw as an adjuvant therapeutic agent in the HCC.

Materials and Methods

Ethanolic Extraction of Pawpaw Samples

The samples of three Asimina triloba species namely, Atwood, Susquehanna and Sunflower, were acquired from University of Kentucky, each originating from separate trees. The pulp of these fruits were freeze-dried, and then ground using a Scienceware Bel ArtMi-cromill (Pequannock, NJ). The powder was extracted with 80% ethanol (50 mg/ml) for 18 h at ambient temperature with 250 rpm on a Scilogex SK-0330-PRO orbital shaker (SciLogic, CT) followed by centrifugation at 2,500 × g at 20°C for 40 min. The ethanolic extract was evaporated in a nitrogen evaporator (Organamation Associates, Inc.) followed by freeze-drying overnight to remove the traces of ethanol/water residues. The dried residues were dissolved in DMSO. All extracts were kept under nitrogen and stored at −20°C until further analyzed.

Total Phenolic Content (TPC)

The TPC was measured using Folin&Ciocalteu’s (FC) reagent with slight modification to a adopt a 96-well microplate version[11]. Briefly, the reaction was conducted in a mixture containing 80 µl pure water, 20 µl pawpaw samples (25 mg/ml), 20 µl FC reagent, and 160 µl 7% sodium carbonate. The reaction was kept in the dark for 2 h at ambient temperature. Absorbance was measured at 765 nm using a microplate reader SpectraMax M5 (Molecular Devices, LLC). Total phenolic content was determined using gallic acid as a standard curve. The analysis was performed in triplicate, and data were expressed as milligrams of gallic acid equivalents (GAE)/g of dried sample.

Antioxidants assay

DPPH Assay

The oxygen-scavenging capacities of the pawpaw extracts were determined using a DPPH assay as previously described [11]. A 100 µl of freshly prepared DPPH+ methanol solution was mixed with 100 µl of pawpaw samples (50 µg/ml) in a 96-well microplate. Absorbance readings were measured at 517 nm after a 30-min reaction. Equal amounts of 80% methanol was used as a blank. Trolox was used to generate a calibration curve (0–70µM). DPPH+ scavenging capacity of pawpaw was calculated by plotting against Trolox antioxidant standard curve. The experiment was conducted in triplicate, and data were expressed as µM of Trolox equivalents (TE)/g of dried sample.

ABTS Assay

The antioxidation activity of pawpaw was determined using 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS•+) ) assay following a previously described procedure with slight modification [11]. Initially, 5 mM highly oxidized ABTS + cation radical was generated by thoroughly mixing ABTS and manganese dioxide at ambient temperature for 18 h. Extra manganese dioxide was removed after reaction by filtration through Whitman paper. The ABTS•+ solution was then diluted with 0.1 M phosphate buffer saline (pH 7.4) to reach an absorbance of around 0.5 at 734 nm. For ABTS assay, 230 µl ABTS•+ solution was added to a 96-well microplate and absorbance was recorded at 734 nm. 20 µl of standard or pawpaw extracts (1 mg/ml) was then added and followed for 30 s by intermittent shaking at room temperature. The absorbance was measured again at 734 nm. 80% methanol was used as the solvent blank. Differences between ABTS reading before and after adding standard or sample were calculated and ABTS•-reducing activity in pawpaw extracts was determined against a Trolox standard curve. The assays were performed in triplicate. Trolox equivalents (TE) were calculated by expressing in µM TE/g dried sample.

Cell Viability Assay

The HepG2 cell line-derived from a Caucasian patient was purchased from ATCC (Manassas, VA) whereas 0/20 cell-derived from an African American patient was obtained from Dr. Devanand Sarkar’s laboratory at VCU [12]. Liver cancer cell lines were cultured in Eagle’s Minimum Essential Medium (EMEM) supplemented with 1% antibiotic solution and 10% fetal bovine serum (FBS) in separate flasks at 37°C. For cell viability assay, 10,000 cells were seeded in a 96-well plate. After overnight incubations, cells were treated with varying concentrations of pawpaw extracts (0 µg/mL, 10 µg/mL, 25 µg/mL, 50 µg/mL, 75 µg/mL, 100 µg/mL, 125 µg/mL, and 250 µg/mL) in a serum-free EMEM. Control wells were treated with a corresponding concentration of DMSO for each pawpaw dilution. The cells were then incubated overnight. Next day, the cells were treated by...
adding 10 μl WST-1 and incubated at 37°C for an additional 2 hours. The absorbance was measured using a microplate reader at 440 nm.

**Results**

**Total Phenolic Content in Pawpaw**

As shown in Figure 1, the phenolic content was the highest in Atwood pawpaw samples, followed by Sunflower and Susquehanna samples. Although statistically not significantly different, the Atwood sample had the highest amount of TPC (6.08 mg GAE/g Powder) as compared to the Susquehanna (4.71 mg GAE/g Powder) and the Sunflower (5.11 mg GAE/g Powder) samples.

**Anti-oxidation Activity of Pawpaw**

As presented in Figures 2a and 2b, the results reveal that the Sunflower sample exhibited the highest antioxidant activity, followed by Atwood and Susquehanna. Interestingly, the patterns of DPPH and ABTS activities differed from their TPC. The Atwood sample showed the highest TPC, in contrast to the DPPH and ABTS assays where the Sunflower sample displayed the greatest antioxidant activity. Specifically, the DPPH free radical scavenging capacity of the sunflower sample measured 15.06 TE μM/g dry powder, significantly differing from the values of the Atwood sample (12.23 TE μM/g dry powder) and the Susquehanna sample (11.94 TE μM/g dry powder). Similarly, the ABTS scavenging activity showed the highest value in the sunflower sample (20.59 TE μM/g dry sample), followed by Atwood (14.65 TE μM/g dry powder) and Susquehanna (12.71 TE μM/g dry powder).

**Effect of Pawpaw on Hepatocellular Carcinoma Cell Viability**

To determine the effect of pawpaw samples on HepG2 and O/20 cell lines, a cell viability assay was performed using WST-1. Treatment with Atwood, Susquehanna and Sunflower (10, 25, 50, 75, 100, 125, 250 μg/mL) on HCC cells induced antiproliferative effect in a dose-dependent manner. Both cell lines were more sensitive to Atwood treatment, followed by Susquehanna and Sunflower samples [Figure 3]. The calculated IC50 of the Atwood was 97.4 μg/ml, and 128 μg/ml for the Susquehanna samples in HepG2 cells. Similar findings were observed in O/20 cells. Atwood (90 μg/ml), and Susquehanna (110 μg/ml) treatment reduced the cell viability by 50%, whereas the sunflower samples exhibited a weak effect in both cell lines.

**Discussion**

The quest for identifying novel dietary sources rich in biologically active compounds has emerged as a pressing area of research in modern biological sciences, constituting one of its most pivotal and urgent facets. Researchers are increasingly directing their efforts towards uncovering new plant species and fruits with a primary focus on those harboring biologically active compounds known to confer health benefits. A substantial body of research has highlighted the presence of diverse biological
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Figure 2: Antioxidant properties of pawpaw. The anti-oxidation activity in three different pawpaw samples were determined using a) DPPH and b) ABTS. The data is expressed as mean ± SD for 8 different samples. In both experiments, the sunflower sample has shown the highest activity, followed by Atwood and Susquehanna.

activities, including antioxidative, anti-inflammatory, and anticancer properties, within various plants and fruit species [4, 13]. In line with these previous findings, our investigation was focused on elucidating the potential anticancer effects of different varieties of pawpaw extracts on hepatocellular carcinoma (HCC) cell lines.

Our study findings demonstrate that pawpaw treatment, particularly from the Atwood and Susquehanna varieties, substantially inhibits the proliferation of HepG2 and O/20 cells. These results align with similar observations of antiproliferative effects induced by extracts from other fruits on various cancer cell lines [14]. Fruits and vegetables represent rich sources of antioxidants and phytochemicals that exert anti-tumorigenic effects by mitigating oxidative stress [13, 14]. Annona fruits, in particular, are recognized for their potent antioxidative activity and their potential to combat cancer due to their polyphenolic content [15, 16]. Notably, our study identifies Atwood as the most potent anti-cancer fruit among the three pawpaw varieties studied. Atwood extract exhibits the highest total phenolic content, while Sunflower and Susquehanna samples display lower phenolic content. However, Sunflower stands out in terms of antioxidant activity, as demonstrated by DPPH and ABTS assays.

In our previous studies, we have investigated anti-cancer activities in several fruits. We investigated the effect of ginger for its anticancer properties in HepG2 cells. The phenolic content and DPPH and ABTS scavenging activities in ginger were notably higher compared to the pawpaw samples. The highest phenolic content observed in ginger was 16.9 mg GAE/g dry powder, surpassing the values obtained from all three pawpaw samples by over 10 mg GAE/g dry powder [11]. The DPPH free radical scavenging capacity and ABTS value were also remarkably higher in ginger, measuring 135.5 TE μM/g dry sample and 255.6 TE μM/g dry sample, respectively, around 10 times greater than those found in the pawpaw samples [11]. Despite the significant differences in antioxidant activity favoring ginger, the pawpaw extracts exhibited greater cytotoxicity in HepG2 cells [11]. The IC50 value of the ginger extract was 186 μg/ml, while the Atwood and Susquehanna samples had IC50 values of 97.4 μg/ml and 125 μg/ml, respectively. These results suggest that pawpaw inhibited cell proliferation to a greater extent compared to the ginger extract, even though the antioxidant activity was found to be higher in ginger samples.

In our previous study, the antioxidant activity of green papaya was investigated in breast cancer cell lines [17]. The fruit was fractionated into distinct parts, including leaves, skin, pulp, and seeds, each being individually tested for their effects. The results from the TPC test demonstrated that the seeds of green papaya possessed the highest Gallic acid Eq., with a phenolic value of approximately 14 mg GAE/g dry extract, slightly surpassing the TPC of the pawpaw extract. However, the TPC of the leaves, skin, and pulp were notably lower in comparison to that of the seeds. Surprisingly, none of the papaya components exhibited any significant effects on the breast cell lines tested. Moreover,
Annona, another fruit, underwent testing to assess its antioxidant activity and anticancer effects. Similar to papaya, the Annona fruit was divided into three parts - the skin, pulp, and seeds - with each part tested independently. Unlike papayas, it was discovered that the skin of Annona had the most remarkable antioxidant effect. In the methanol extract, the DPPH assay yielded high value of almost 8000 TE μM/g dry weight, approximately 530 times higher than the value obtained for the pawpaw fruit [18]. These data suggest that total polyphenolic content and antioxidation activities play a role in exerting anticancer activity; however, the types and

Figure 3: Antiproliferative activity of pawpaw samples in HepG2 and O/20 cell lines. The cells were treated with varying concentrations of pawpaw extract (0, 10, 25, 50, 75, 100, 125, 250 μg/ml). Cell viability was determined using a WST-1 assay. The data is expressed as mean ± SD for 3 different samples.
quantities of specific polyphenol in pawpaw versus other fruits may have played an important role towards the potency of their anticancer effects.

**Conclusion**

In conclusion, our data suggested that pawpaw extracts equally exhibited anticancer activities in HCC cell lines-derived from Caucasian or African American patients. Nonetheless, further research is required to identify and characterize the active compounds from the pawpaw fruit for potential therapeutic use and for a better understanding of the underlying mechanism. One promising avenue is conducting cell viability tests on various cell lines to ascertain if pawpaws exhibit heightened cytotoxicity in other cancer cell types. Moreover, the present study is focused only on in-vitro experiments, a comprehensive in-vivo (animal) study using the active compound of Asimina trifolia might help to unravel the underlying mechanism of anti-cancer effects in liver cancer and provide a deeper insight.

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**References**


