Journal of
Medicinal Plant Research
Volume 11 Number 45, 3 December, 2017
ISSN 1996-0875

Academic Journals

ABOUT JMPR

The Journal of Medicinal Plant Research is published weekly (one volume per year) by Academic Journals.

The Journal of Medicinal Plants Research (JMPR) is an open access journal that provides rapid publication (weekly) of articles in all areas of Medicinal Plants research, Ethnopharmacology, Fitoterapia, Phytomedicine etc. The Journal welcomes the submission of manuscripts that meet the general criteria of significance and scientific excellence. Papers will be published shortly after acceptance. All articles published in JMPR are peer reviewed. Electronic submission of manuscripts is strongly encouraged, provided that the text, tables, and figures are included in a single Microsoft Word file (preferably in Arial font).

Contact Us

Editorial Office: jmpr@academicjournals.org

Help Desk: helpdesk@academicjournals.org

Website: http://www.academicjournals.org/journal/JMPR

Submit manuscript online http://ms.academicjournals.me/

Editorial Board

Journal of Medicinal Plants Research

Table of Contents: Volume 11 Number 45 3 December, 2017

ARTICLE

Phenolic profile and antioxidant capacity of *Cnidoscolus chayamansa* and *Cnidoscolus aconitifolius*: A review 713
A. Kuri-García, J. L. Chávez-Servín and S. H. Guzmán-Maldonado

Bioactivity of bizzy nut extract in prostate cancer cells 728
Orville Phillip, Caroline Telles and Wesley Gray

academicJournals

Vol. 11(45), pp. 713-727, 3 December, 2017
DOI: 10.5897/JMPR2017.6512
Article Number: 4A3FF2F66877
ISSN 1996-0875

http://www.academicjournals.org/JMPR

Journal of Medicinal Plants Research

Review

Phenolic profile and antioxidant capacity of *Cnidoscolus chayamansa* and *Cnidoscolus aconitifolius*: A review

A. Kuri-García[1], J. L. Chávez-Servín[1*] and S. H. Guzmán-Maldonado[2]

[1]Laboratory of Cellular and Molecular Biology, Facultad de Ciencias Naturales, Campus Juriquilla, Universidad Autónoma de Querétaro, Av. de las Ciencias S/N, Juriquilla, Querétaro, Qro. CP 76230, México.
[2]Laboratorio de Alimentos-Campo Experimental Bajío (INIFAP), Km 6.5 Carretera Celaya-San Miguel de Allende, C. P. 38110 Celaya, Gto., México.

Received 28 September, 2017; Accepted 16 November, 2017

Research into ancient cultures has yielded a large body of evidence on the use of medicinal plants for preventive and/or therapeutic purposes. Such plants may have many metabolic activities and functions in the body-antioxidant, anti-inflammatory, platelet aggregation inhibitory and immunological and they can act at different molecular levels. This work offers a comprehensive review of research into the phenolic profile and antioxidant capacity of a plant used since the pre-Columbian era, native to southeast Mexico, commonly known as "chaya". The most prevalent phytochemicals in this plant are its phenolic compounds, and their antioxidant capacity is responsible for many of its health benefits, specifically in controlling chronic diseases. In the chaya leaf, there is a general trend toward the presence of different phenolic groups, such as coumarin, flavonoids, phenols, tannins, anthraquinones and flobotanins in aqueous and alcoholic extracts. Aside from environmental factors, there are differences in the ways samples are treated before the extraction process, such as the treatment type and the drying conditions. There are also differences in the solvents used and in the methods of extraction and concentration of compounds. Finally, a diversity of techniques is used, and even the data are quantified and expressed differently. Chaya has great potential for production as food and as a medicinal plant, but much more research is needed on the composition of its leaf and the biological effects of its components.

Key words: Chaya, *Cnidoscolus aconitifolius*, *Cnidoscolus chayamansa*, phenolic compounds, antioxidant capacity.

INTRODUCTION

The use of plants in medicine goes back to the beginnings of human civilization. Substantial evidence has been found on the use of plants for preventive and/or therapeutic purposes in ancient cultures (Mwine and Van

*Corresponding author. E-mail: jorge.chavez@uaq.mx.

Damme, 2011). According to the World Health Organization (WHO), a medicinal plant is one that contains substances that can be used for therapeutic purposes and/or can serve as active ingredients for the synthesis of new drugs (WHO, 2005). The use of traditional medicines and medicinal plants has been widely observed in most developing countries, where they are seen as therapeutic agents for the maintenance of good health (Soetan and Aiyelaagbe, 2009).

For several decades, various lines of research have been pursued into medicinal plants and their components. One of them focuses on the study of the composition of minority compounds, mainly phenolic compounds, given their various benefits in battling chronic disease, including cardiovascular disease, neurodegeneration, and cancer (Del Rio et al., 2013). They cover a wide range of metabolic activities and have many functions in the body: antioxidant, anti-inflammatory, platelet aggregation inhibitory and immunological; and they can act at different molecular levels. Thus, the consumption of phenolic compounds is associated with health benefits (Rangel-Huerta et al., 2015). Also, several studies in plants report on their antioxidant capacity. There are a large number of publications on different plants, applying a variety of methods for extracting and measuring phenolic compounds and antioxidant capacity (Gutiérrez-Grijalva et al., 2016). These publications differ considerably in the types of processing used for the raw material, and also the solvents used (for example, aqueous, alcoholic and non-polar, as well as different mixtures thereof), as well as the times, temperatures, concentration, and other factors. Finally, there are diverse ways of expressing the content of phenolic compounds and antioxidant capacity. This work offers a comprehensive review of the literature on the phenolic profile and antioxidant capacity of a plant that has been in use since the pre-Columbian era, native to southeast Mexico, commonly known as "chaya".

CHAYA (*CNIDOSCOLUS* SPECIES)

Chaya refers to any group of plants of the genus *Cnidoscolus*, which is a part of the family Euphorbiaceae (Cifuentes et al., 2010). This genus is composed of 50 species, 20 of which are endemic to Mexico. They are distributed in tropical and subtropical areas, mainly in regions of low deciduous forest and xerophilous scrub of Mexico (Kolterman et al., 1984). Some species of *Cnidoscolus* are of interest for their nutritional potential, particularly the most commonly used for both consumption and traditional uses such as medicinal and ornamental plants, *Cnidoscolus aconitifolius* and *Cnidoscolus chayamansa* (Kolterman et al., 1984). *C. aconitifolius* has pentalobulated leaves, with lobed, serrated edges, with a long petiole length, without pubescences, with sagittate base, with the presence of glands and with white flowers (Adebiyi et al., 2012). In contrast, *C. chayamansa* has three-lobed leaves, with smooth lobed edges, with short petiole length, without pubescence, and similarly with sagittate base, with the presence of glands and with white flowers (Standley and Steyerman, 1949) (Figure 1). These two species originated in the Yucatán region of southern Mexico and are fast-growing perennial shrubs (Grubben and Denton, 2004).

The chaya plant is a domesticated shrub, highly valued by people in rural communities of central and southern Mexico as food, as a medicinal plant and as an ornamental. Chaya has been used as food since pre-Columbian times and is currently consumed regularly in some populations (Ross-Ibarra and Molina-Cruz, 2002). In addition, chaya leaves have been found to be an important source of protein, β-carotene, vitamins, ascorbic acid, calcium, potassium, and iron (Jiménez-Arellanes et al., 2014; Kuti and Kuti, 1999).

Chaya is consumed in a manner similar to spinach, which is why it is also called “Mayan spinach” (Ross-Ibarra, 2003). But its nutrient content is far superior to spinach: 78% more proteins, 111% more fiber, 100% more iron and 242% more vitamin C (Kuti and Torres, 1996) (Table 1).

Chaya leaves contain a cyanogenic glycoside called Linamarin. Linamarin is a glucoside conjugate of an acetone and a cyanide (Kuti and Konuru, 2006). It is a secondary metabolite of plants that performs defense functions, since when it is hydrolyzed by enzymes it releases hydrogen cyanide, a process called cyanogenesis. The content of cyanogenic glycosides according to Gonzalez-Laredo et al. (2003) is 2.37 to 4.25 mg/100 g dry matter (DM). These authors tested various thermal treatments to remove this compound from the leaves and reported that 5 min in boiling water is sufficient to remove any residue of cyanide (Figure 2).

The use of chaya leaves has been reported in traditional medicine for various pathologies, where it is believed to have antidiabetic, antioxidant, hepatoprotective, and hormone-related properties on the pituitary-gonadal axis (García-Rodríguez et al., 2014; Hitchcock et al., 1997; Jiménez-Arellanes et al., 2014; Kulathuran et al., 2012; Kuti and Konuru, 2006; Kuti and Torres 1996; Loarca-Piña et al., 2010; Lucky and Festus, 2014; Miranda-Velasquez et al., 2010).

These plants can grow up to 6 m high, with lobed leaves, milky sap and small dichotomous white flowers at the tip of the branches. It is propagated by planting stem cuttings or woody stem cuttings. Within the chaya subspecies there is a considerable morphological and phenological variation. In research carried out by Ross-Ibarra and Molina-Cruz (2002), four cultivated varieties of chaya were identified, with easily separable and quite consistent morphological differences, but their taxonomy is not yet assigned. These are classified as star, beaked, chayamansa and round. Seeds and ripe fruit are rare and unknown (McVaugh, 1994). Given the ease of its

Table 1. Comparison of nutritional compositions of chaya leaves (*Cnidoscolus chayamansa* McVaughn) and spinach (*Spinacia oleraceae* L.) per 100 g fresh weight.

Component	Chaya	Spinach	Δ (%)
Water (%)	85.3	90.7	-6
Protein (%)	5.7	3.2	78
Lipid (%)	0.4	0.3	33
Fiber (%)	1.9	0.9	111
Calcium (mg/100 g)	199.4	101.3	96
Phosphorus (mg/100 g)	39.0	30.0	30
Potassium (mg/100 g)	217.2	146.5	48
Iron (mg/100 g)	11.4	5.7	100
Ascorbic acid (mg/100 g)	164.7	48.1	242

Δ (delta) represents the change (increase or decrease) of the value of a variable, using as reference the values of spinach. Adapted from (Kuti and Torres, 1996).

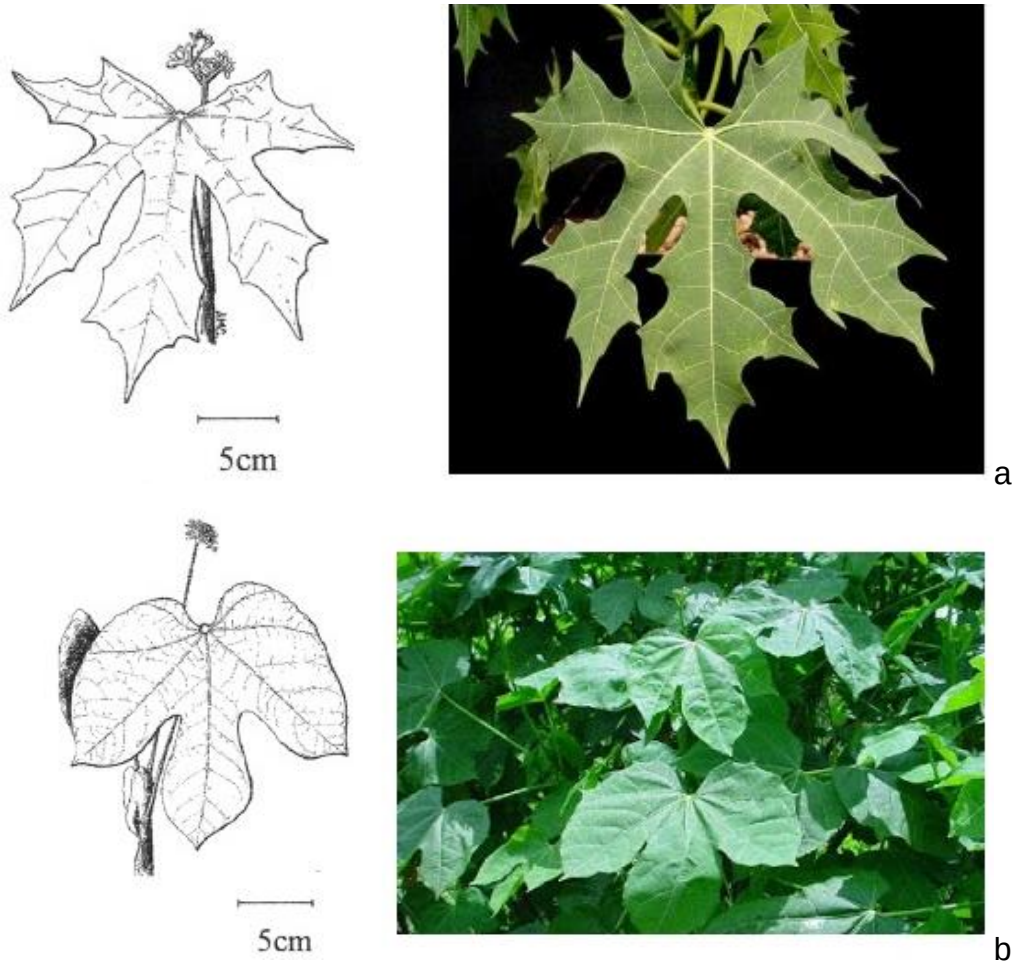


Figure 1. Images of (a) *C. aconitifolius* and (b) *C. chayamansa*, known locally as chaya. Source: Adebiyi et al. (2012); Cifuentes et al. (2010).

cultivation, its potential productivity, and above all its high nutritional value, chaya has been proposed as a potential crop for regions outside of Mesoamerica (Kuti and Torres, 1996; Molina-Cruz et al., 1997; Ross-Ibarra and

Molina-Cruz, 2002).

IMPORTANCE OF PHENOLIC COMPOUNDS AND ANTIOXIDANT CAPACITY

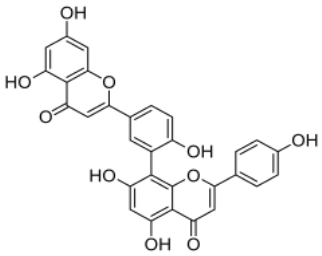
Atoms or molecules containing one or more unpaired electrons are called free radicals. Free radicals are responsible for tissue degeneration through damage to DNA, proteins and lipid peroxidation through oxidative stress, which has been implicated in the pathophysiology of different diseases. Some authors have found that the degree of damage caused by free radicals can be mitigated by supplementation with one or more antioxidants (Marchioli et al., 2001). Several compounds with differential antioxidant properties are found in plants and these plants are considered to have high biological potential in the context of the prevention and treatment of damage caused by free radicals. Several medicinal plants have been examined and evaluated for their properties in antagonism toward free radicals induced by oxidative stress (Esparza-Martínez et al., 2016; Vinson et al., 2001).

Some of these plants' medicinal properties are attributed to their phytochemical composition, specifically a variety of minority compounds derived from the secondary metabolism of plants, which have attracted interest recently for their bioactive effects. Phenolic compounds are among these, and are ubiquitous in foods of plant origin. The main functions of phenolic compounds in plants have to do with pigmentation and protection against pathogens and predators. They are chemical compounds having at least one aromatic ring to which one or more hydroxyl groups are attached to aromatic or aliphatic structures (Bravo, 1998). There are over 10,000 different phenolic compounds, ranging from the simplest to the most complex, and their wide diversity in nature is evident upon analysis of their characteristics (Gutiérrez-Grijalva et al., 2016; Neveu et al., 2010; Rothwell et al., 2014; Zare et al., 2014). Many constituents of these plants can contribute to their protective properties, including: vitamins C and E; selenium and other mineral micronutrients; carotenoids; phytoestrogens; glucosinolates and indoles; ditholthiones; isothiocyanates; protease inhibitors; fiber; and folic acid. These compounds may act alone or in combination, as anticarcinogenic or cardioprotective agents, through a variety of mechanisms. One of these protective mechanisms, attributed to vitamins C and E and to carotenoids, is antioxidant activity (radical barrier) (Rice-Evans et al., 1997).

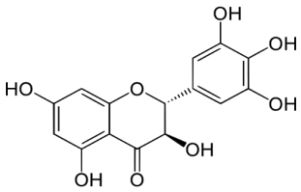
There are several classes of flavonoids, which differ in the level of oxidation and saturation of ring C, and individual compounds within each class differ in the substitution pattern of rings A and B (Wojdyło et al., 2007). Researchers have been looking into the antioxidant properties of many plant species for at least 50 years. There is currently a great deal of interest in the commercial production of plants as sources of antioxidants that can enhance the properties of food, both for nutritional and medicinal purposes. Numerous epidemiological studies have shown an inverse relationship between consumption of fruits, vegetables and cereals and the incidence of coronary heart disease and certain cancers (Gunjan et al., 2011). The plant kingdom is vast, with thousands of species and varieties that demand study. The phenolic composition and antioxidant activity of plants, both wild and cultivated traditionally, are a particularly rich area for future research. The antioxidant capacity of various plants is generally studied with respect to the content of total phenolic compounds using traditional methods, and only one test is used to determine free radical scavenging ability. Although extensive studies of bioactive compounds and their content of total phenolic compounds have been carried out in many species, the phenolic identification data are still insufficient and incomplete. In particular, quantitative data on specific phenolic compounds in plant species remains a pending task. There are also few comparisons of the phenolic constituents identified in several species of different plant families. Further research is required into the structure-activity relationships of phenolic compounds present in plant species (Czapecka et al., 2005; Ivanova et al., 2005). The objective of this work is to review the literature on phenolic composition and the antioxidant capacity of different extracts derived from the leaves of *C. aconitifolius* and *C. chayamansa*. A comprehensive search was performed using the terms "*Cnidoscolus chayamansa*" and "*Cnidoscolus aconitifolius*" without reducing or limiting the search elements. A total of 57 publications were consulted on the main scientific portals (Scopus, PubMed, Science Direct, Springer-Link, Wiley, Redalyc, Google Scholar, and Web of Science). The information was subsequently analyzed and classified as described subsequently.

PREPARATION FOR PHENOLICS EXTRACTIONS

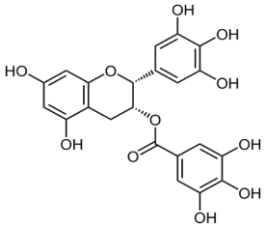
Plant extracts are a complex mixture, with a multitude of chemical compounds obtained by physical and chemical processes from a natural source and usable in almost any technological field. The WHO estimates that 80% of developing country populations rely on traditional medicines, mostly plant drugs, for their primary health care needs (Soetan and Aiyelaagbe, 2009). Plant extracts have been used since the beginning of civilization because they increase the useful life of the compound. There are few synthetic chemicals that can be used without toxicity or side effects, but nature is a potential source for discovering new structures that may have therapeutic qualities. Various phenolic compounds such as flavonoids can be extracted from fresh or dry



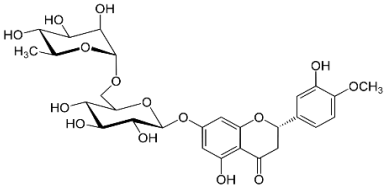
Amentoflavone (Figueroa-Valverde et al., 2004; González-Laredo et al., 2003)



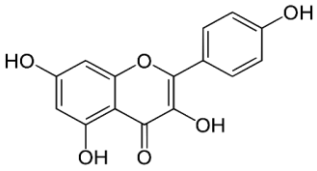
Dihydromyricetine (Figueroa-Valverde et al., 2004; González-Laredo et al., 2003)



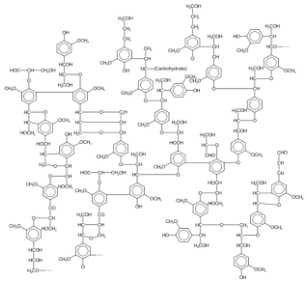
Epigallocatech gallato (Numa et al., 2015; Ramos-Gómez et al., 2016)



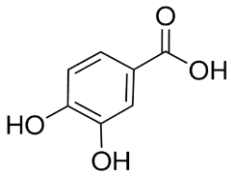
Hesperidin (Figueroa-Valverde et al., 2004; Ramos-Gómez et al., 2016)



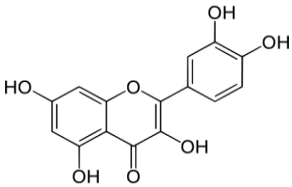
Kaempferol (Kolterman et al., 1984; Kuti and Konuru, 2004)



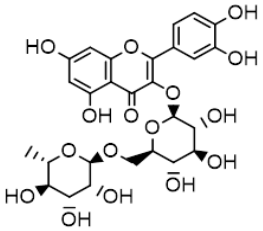
Lignin (Numa et al., 2015; Sarmiento-Franco et al., 2003)



Protocatechic acid (Loarca-Piña et al., 2010; Ramos-Gómez et al., 2016).



Quercetin (Figueroa-Valverde et al., 2004; Kolterman et al., 1984; Kuti and Konuru, 2004; Ramos-Gómez et al., 2016; Valenzuela-Soto et al., 2015)



Rutine (Figueroa-Valverde et al., 2004; Loarca-Piña et al., 2010; Ramos-Gómez et al., 2016; Valenzuela-Soto et al., 2015)

Figure 2. Structures of the most reported phenolic compounds in chaya leaves.

material, as long as proper methods and care are used to avoid significant alteration of their contents and composition. Nonpolar or slightly polar solvents are initially used to separate chlorophylls, gums and aglycones from highly methoxylated flavonoids. Flavonoids, which have many unsubstituted hydroxyl groups or sugars, are considered polar, so they are slightly soluble in polar solvents such as methanol, ethanol, acetone, dimethyl sulfoxide (DMSO) or water. The final filtrate is usually concentrated and the solvent is removed (Sarker and Nahar, 2012; Skerget et al., 2005). Most phenolic compounds are found within plant cells in aglyconated or in glycosylated form. This protects them from degradation, diminishes their toxic effects and at the same time aids transport through membranes, increasing their aqueous solubility. These compounds, in any of their forms, are already aglyconated or glycosylated, are in the vacuoles of plant cells and are in a soluble polar fraction.

Therefore, these aglyconated and glycosylated compounds can be extracted relatively easily using polar solvents (Jones and Vogt, 2001).

EXTRACTIONS FROM THE CHAYA LEAF WITH DIFFERENT SOLVENTS

Table 2 shows different forms of extraction of chaya leaf compounds reported in the literature.

Water extraction

Awoyinka et al. (2007) report that the aqueous extraction was performed from the dry leaves of *C. aconitifolius* that had been processed with a mortar and pestle. At the end, the substance was heated in an oven at 45°C until it reached a constant weight, although the proportion of the extraction is not specified. Musa et al. (2008) allowed the leaves of *C. aconitifolius* to dry at 40°C for 48 h. The reported extraction rate was 20 g of dried ground leaf to 1 L of cold distilled water, mixing for 48 h at a constant

Table 2. Studies that have reported on analysis of chaya leaf composition.

Solvent species	Plant samples	Extraction process	Elimination of cyanogenic glucoside	Reference
		Water extraction		
C. aconitifolius	Leaves dried at 45°C	NR	NR	Awoyinka et al. (2007)
C. aconitifolius	Leaves dried at 40°C for 48 h	20 g of DM in 1000 ml of distilled water	NR	Musa Toyin Yakubu et al. (2008)
C. aconitifolius	Sun-dried leaves	218 g of DM in 500 ml of distilled water	NR	Mordi and Akanji (2012)
C. aconitifolius	Leaves air dried at 28°C for 28 days	NR	NR	Obichi et al. (2015)
C. chayamansa	Leaves air dried for 15 days	5 g of DM in 100 ml of distilled water at 19°C for 10 min.	10 min at 90°C	Valenzuela et al. (2015)
C. aconitifolius	Leaves divided into 5 groups: fresh, bleached, boiled, extract and extract residue	Bleached leaves, 65°C for 10 min; leaves boiled, 100°C for 15 min. Juice was extracted from the leaf and juice residue	15 min at 100°C	Babalola and Alabi (2015)
C. chayamansa	Ethno-botanical information available (without quotation)	20 g of DM in 1000 ml of boiling water for 20 min	Boiling water during 20 min	Ramos-Gomez et al. (2016)
		Extraction using ethanol and mix polar solvents		
C. aconitifolius	NR	5 g DM in 20 ml ethanol/acetone/water/acetic acid (40:40:20:0.1 v/v)	heating in a microwave oven for 2 min	Kuti and Konuru (2004)
C. aconitifolius	Dried leaves ground in a mortar	96% ethanol for 3 h rotaevaporated at 30°C for 25 min and dried in an oven at 45°C	NR	Awoyinka et al. (2007)
C. aconitifolius	NR	5 g DM in 40 ml in ethanol/acetone/water/acetic acid (40:40:20:0.1 v/v) dried in a 60°C water bath for 1 h	NR	Johnson et al. (2008)
C. aconitifolius	Leaves air-dried at room temperature	1000 g of mature leaves in 70% ethanol, reduced by evaporation at 50°C and defatted with *n*-hexane	NR	Mordi and Akanji (2012)
C. chayamansa	NR	135 g of DM using 9.44 g of ethanol, the solvent was rotaevaporated and allowed to dry at 25°C in an oven	NR	García-Rodríguez et al. (2014)
C. aconitifolius	Dried and macerated leaves	7 days in 96% ethanol with solvent change daily	NR	Numa et al. (2015)
		Extraction using methanol and mix polar solvents		
C. aconitifolius	Ground dried leaves	70% methanol and 30% water	NR	Kolterman et al. (1984)
C. chayamansa	Leaves dried at 60°C for 6 h	It was extracted with methanol (x2) and rotaevaporated (<40°C), finally separated with hexane, ethyl ether and ethyl acetate (x2)	Boiled in water for 1, 5, 10, and 15 min, soaked in water at 20°C, 60 min; 70°C, 30 min, and sun-dried for 4 days	Gonzalez-Laredo et al. (2003)

Table 2. Contd.

C. chayamansa	Leaves previously dried	20 g in 250 ml of 80% methanol for 8 h	NR	Figueroa-Valverde et al. (2009)
C. chayamansa	Dried and macerated leaves.	500 g DM in 1000 ml using hexane-acetone (1:1 v/v) at room temperature for 5 days 2 times a day. The material was extracted with 100% methanol under the same conditions	NR	Loarca-Piña et al. (2010)
C. aconitifolius	Dry leaves (3 kg) in an extractor at 30°C	Methanol for 5 h, rotaevaporated at 35°C for 30 min.	NR	Adaramoye et al. (2011)
C. aconitifolius	Air dried in the laboratory for 5 days at room temperature followed by oven drying at 40°C followed by grinding to powder form using an electric mill.	1000 g DM in 2500 ml methanol. Rotaevaporated at 40°C	NR	Ikpefan et al. (2013)
		Other solvents		
Ethyl acetate *C. aconitifolius*	NR	135 g of DM was treated with 5.27 g of ethyl acetate. It was rotaevaporated to dryness and dried in an oven at 25°C	NR	García-Rodríguez et al. (2014)
Dichloromethane *C. aconitifolius*	20 freshly cut leaves	1000 ml of methylene chloride for 20 secs and evaporated to obtain 5333.3 mg of residue	NR	Escalante-Erosa et al. (2004)
Hexane *C. aconitifolius*	NR	135 g of DM treated with 5.68 g of hexane. It was rotaevaporated to dryness and oven dried at 25°C	NR	García-Rodríguez et al. (2014)
		Studies that do not report extraction		
C. aconitifolius fresh matter	The leaves from each plant were stored in plastic bags and frozen at −10 ◦C until analysis.	NR	NR	Sarmiento-Franco et al. (2003)
C. aconitifolius dry matter	Leaves cooked at 80 and 90°C for 19 min and allowed to dry	NR	Cooked 10 min at 90°C	Aye (2012)
C. aconitifolius dry matter	Leaves dried in oven at 40°C	NR	Boiled for 20 min.	Akachukwu et al. (2014)
C. aconitifolius dry matter	Leaves dried in oven at 70°C for 3 days to constant weight	NR Fresh or dry matter is related with preparation of leaf	NR	Jiménez-Aguilar and Grusak (2015)
		Studies that do not report extraction		
C. aconitifolius fresh matter	The leaves from each plant were stored in plastic bags and frozen at −10 ◦C until analysis.	NR	NR	Sarmiento-Franco et al. (2003)

Table 2. Contd.

C. aconitifolius dry matter	Leaves cooked at 80 and 90°C for 19 min and allowed to dry	NR	Cooked 10 min at 90°C	Aye (2012)
C. aconitifolius dry matter	Leaves dried in oven at 40°C	NR	Boiled for 20 min.	Akachukwu et al. (2014)
C. aconitifolius dry matter	Leaves dried in oven at 70°C for 3 days to constant weight	NR Fresh or dry matter is related with preparation of leaf	NR	Jiménez-Aguilar and Grusak (2015)

Only the information available in each of the references is mentioned. NR: Not reported.

temperature. The mixture was then filtered and concentrated in a steam bath until 4.88 g of residue remained. Mordi and Akanji (2012) dried the *C. aconitifolius* leaves in the sun, and then macerated them. The proportion was 218 g of dry matter to 500 ml of distilled water using a rotoevaporator at 50°C. This residue was then lyophilized. Obichi et al. (2015) mentioned that only *C. aconitifolius* leaves were harvested, cleaned and air dried at 28°C for 28 days before use. Valenzuela et al. (2015) reported drying the *C. chayamansa* leaf for 15 days at room temperature in a closed and ventilated area, where the sample was then ground with a mortar and stored at room temperature. The sample was prepared by mixing 5 g of dry matter into 100 ml of distilled water at 90°C for 10 min. This was then filtered with Whatman paper (No. 4, 110 mm) and the extract was stored at 5°C for analysis. Babalola and Alabi (2015) reported four different processes: in the first group, the leaves of *C. aconitifolius* were bleached at 65°C for 10 min, in the second they were boiled at 100°C for 15 min, in the third the juice was extracted from the leaf, and in the fourth the residue of the juice was collected after extraction. Ramos-Gomez et al. (2016) used a technique gathered from available ethno-botanical information for *C. chayamansa*, which was to boil 20 g in 1 L of drinking water for 20 min, then to pass this mixture through a 0.5-mm pore size filter.

Extraction using ethanol and mix polar solvents

Kuti and Konuru (2004) mention that the extraction of *C. aconitifolius* was 5 g DM in 20 ml of ethanol/acetone/water/acetic acid (40:40:20:0.1 v/v). This is the only study that reports using a microwave oven (1.3 cu ft Panasonic microwave 1000-W), in which the sample was heated for 2 min, to remove the cyanogenic glycoside from the plant. Awoyinka et al. (2007) mention that *C. aconitifolius* dried leaves were ground in a mortar and that the extraction was carried out with 96% ethanol for 3 h. The resulting solution was placed in a rotoevaporator at 30°C for 25 min, then placed in a drying oven at 45°C until a constant weight was reached. Johnson et al. (2008) reported placing a mixture of 5 g of *C. aconitifolius* dried leaf in 40 ml of an ethanol/acetone/water/acetic acid solution (40:40:20:0.1 v/v), in a water bath at 60°C for 1 h. Mordi and Akanji (2012) mention that the air-dried powder from *C. aconitifolius* leaves (1 kg) of fresh matured *C. aconitifolius* were extracted by percolation at room temperature with 70% ethanol (EtOH). A leaf extract from *C. aconitifolius* was concentrated under reduced pressure (bath temperature 50°C) and finally defatted with n-hexane. The extract was evaporated to dryness. This yielded 69.9 g of dried mass. García-Rodríguez et al. (2014) mentioned that approximately 135 g of *C. aconitifolius* dried leaves were extracted by maceration using ethanol (9.44 g) at room temperature (25°C). The samples were kept in the dark at room temperature for successive testing during the course of the research reported. The solvent was removed by rotary evaporation to dryness and the resulting material dried completely in an oven at 25°C. Numa et al. (2015) mention that to prepare the soluble extract in ethanol, the *C. aconitifolius* leaf was dried, ground and macerated for 7 days in a solution of 96% ethanol, changing the solvent daily.

Extraction using methanol and mix polar solvents

The first report was by Kolterman et al. (1984), in which the dry matter of *C. aconitifolius* was extracted in 70% methanol/30% water. Later, González-Laredo et al. (2003) reported drying the leaves of *C. chayamansa* at 60°C for 6 h. These

authors also performed a duplicate extraction with methanol and rotary evaporation at <40°C. Subsequently a separation was performed in duplicate using hexane, ethyl ether and ethyl acetate. Figueroa-Valverde et al. (2009) performed their extraction by placing 20 g of previously dried leaves of *C. chayamansa* in 250 ml of 80% methanol for 8 h, then performing a rotary evaporation of the mixture. They then added a chloroform: water solvent mixture (4:1 v/v) to remove the organic phase from the aqueous. The volume of the organic phase was reduced to dryness and the obtained mixture was reconstituted with 70% ethanol to be used as stock solution. Loarca-Piña et al. (2010) reported drying and macerating the *C. chayamansa* leaves, then performing the extraction by placing 500 g of dry leaf in 1000 ml of solvent (hexane-acetone, 1:1) at room temperature for 5 days, twice daily. Subsequently, the material was extracted with methanol under the same conditions. It was then dried in a rotoevaporator and stored at 4°C. In Adaramoye et al. (2011), approximately 3 kg of dry *C. aconitifolius* leaves were placed in an extractor at 30°C using methanol for 5 h and the extract was concentrated in a rotary evaporator at 35°C for 30 min. In Ikpefan et al. (2013), *C. aconitifolius* leaves were air dried for 5 days in a laboratory at room temperature. Oven drying was then carried out at 40°C, followed by milling in powder form, using an electric mill. 1 kg of the dry matter was extracted in 2.5 L of methanol. The extracted liquid obtained was concentrated using a rotoevaporator at a steady temperature of 40°C then kept in refrigeration afterwards.

Other solvents

García-Rodríguez et al. (2014) reported extraction from approximately 135 g of dried leaves of *C. aconitifolius* by maceration, using 5.27 g of ethyl acetate at room temperature (25°C). The solvent was removed by rotary evaporation to dryness and completely dried in an oven at 25°C. Escalante-Erosa et al. (2004) reported that they used 20 freshly cut *C. aconitifolius* leaves. Subsequently, they added 1 L of methylene chloride for 20 s. Afterwards, the mixture was subjected to rotary evaporation to produce 533.3 mg of wax. García-Rodríguez et al. (2014) reported that approximately 135 g of dried leaves were extracted by maceration using 5.68 g of hexane at room temperature (25°C). The solvent was removed by rotary evaporation to dryness and dried completely in an oven at 25°C.

Studies that do not report extraction

Sarmiento-Franco et al. (2003) mentioned only the use of ground dry matter from *C. aconitifolius*. Unlike Oyagbemi et al. (2011), they mentioned that the leaves of *C. aconitifolius* were collected, cleaned and air dried at room temperature. Aye (2012) reported that the preparation of *C. aconitifolius* leaves was washed, weighed and cooked in batches of 80 and 90°C for 10 min, and then allowed to dry. Akachukwu et al. (2014) mentioned only that the leaves of *C. aconitifolius* were dried in an oven at 40°C and subsequently ground. Jiménez-Aguilar and Grusak (2015) reported that *C. aconitifolius* leaves were dried in an oven at 70°C for 3 days to maintain a constant weight.

Phenolic compounds detected in chaya leaf

In an aqueous extraction, Musa et al. (2008) found different phenolic compounds in different concentrations: 1.86% phenols, 0.93% tannins, 0.30% flavonoids, 0.072% anthraquinones, and 0.065 % flobotannins (Table 3). Mordi and Akanji (2012), also using an aqueous extraction, found a moderate presence of phenols (++), a low presence of tannins (+), and a high presence of flobotannins (+++). In an aqueous extraction of chaya leaf, Obichi et al. (2015) found 5.7% of tannins and 23.7% of flavonoids. Babalola and Alabi (2015) also tested an aqueous extraction of chaya leaf and found 15.17 gallic acid equivalents (GAE)/100 g fresh matter (FM) of total phenolic compounds, and 243.33 mg/100 g FM of flavonoids. Valenzuela et al. (2015) performed a chaya leaf infusion and reported a total phenolic compound concentration of 6.34 mg GAE/ml. Kuti and Konuru (2004) performed leaf extractions of chaya using ethanol as solvent, reporting on the concentration of total phenolic compounds in raw and cooked leaves, finding values of 2906.2 and 2241.4 mg chlorogenic acid equivalents (CAE/kg) FM, in raw and cooked leaves, respectively. Various researchers analyzed ethanolic extracts: Awoyinka et al. (2007) reported a mean presence of tannins; Johnson et al. (2008) reported a total phenolic compound concentration of 5.6 mg GAE/g DM; Mordi and Akanji (2012) in an ethanolic extract reported a high presence of phenols and tannins and a low presence of flobotanins and flavonoids; García-Rodríguez et al. (2014) reported a low presence of coumarin and flavonoids, and a total phenolic compound concentration of 35.7 mg GAE/g DM. These authors also tested a hexanoic extract, in which reported a total phenolic compound concentration of 22.3 mg GAE/g DM. On the other hand, Loarca-Piña et al. (2010) tested a methanolic extract, and reported a concentration of phenolic compounds of 71.3 mg GAE/g extract, and a total flavonoid concentration of 42.7 mg catequin equivalents (CE)/g extract. Among other authors who tested a methanolic extract, Oyagbemi et al. (2011) reported a high presence of flavonoids and a low presence of tannins. Adaramoye et al. (2011), reported a high presence of flavonoids and a moderate presence of tannins. Aye (2012) reported a total phenolic compound concentration of 3.78% TE. Akachukwu et al. (2014) reported a tannin concentration of 0.14%, a phenol of 0.19% and a flavonoid of 2.36%. Jimenez-Aguilar and

Table 3. Phenolic compounds reported in chaya leaves.

Solvent system used/species	Phenolic compounds reported	Reference
Aqueous		
C. aconitifolius	Phenols: 1.86%, Tannins: 0.93%, Flavonoids: 0.30%, Anthraquinones: 0.072%, and Flobotanins: 0.065%	Musa et al. (2008)
C. aconitifolius	Phenols (++) Tannins (+) Flobotanins (+++)	Mordi and Akanji (2012)
C. aconitifolius	Tannins 5.7% and Flavonoids 23.7%	Obichi et al. (2015)
C. aconitifolius	TFC 15.17 GAE/100 g DM and Flavonoids 183.33 mg/100 g DM	Babalola and Alabi (2015)
C. chayamansa	TFC 6.34 mg GAE/ml infusion	Valenzuela-Soto et al. (2015)
Ethanolic		
C. aconitifolius	TFC Crude: 2906.2 and Cooked: 2241.4 mg CAE/kg FM	Kuti and Konuru (2004)
C. aconitifolius	Tannins (++)	Awoyinka et al. (2007)
C. aconitifolius	TFC 5.6 mg GAE/g DM	Johnson et al. (2008)
C. aconitifolius	Phenols (+++), Tannins (+++), Flobotanino (+), Flavonoids (+)	Mordi and Akanji (2012)
C. aconitifolius	Coumarin (+), Flavonoids (+), TFC : 35.7 GAE/g DM	García-Rodríguez et al. (2014)
Methanolic		
C. chayamansa	TFC 71.3 mg GAE/g extract; Total flavonoids 42.7 mg CE/g extract	Loarca-Piña et al. (2010)
C. aconitifolius	Flavonoids (+++), Tannins (+)	Oyagbemi et al. (2011)
C. aconitifolius	Flavonoids (+++) and Tannins (++)	Adaramoye et al. (2011)
C. aconitifolius	TFC 3.78% TE (average)	Aye (2012)
C. aconitifolius	Flavonoids (+++) and Tannins (+++)	Ikpefan et al. (2013)
C. aconitifolius	Tannins: 0.14%, Phenols: 0.19% and Flavonoids: 2.36%	Akachukwu et al. (2014)
C. aconitifolius	TFC 5.66 mg GAE/g FM; Total flavonoids 332.8 µg CE/g FM	Jiménez-Aguilar and Grusak (2015)
Other solvents		
Ethyl acetate *C. aconitifolius*	Coumarin (+), Flavonoids (+), TFC 13.2 GAE/g DM.	García-Rodríguez et al. (2014)
Hexanoic *C. aconitifolius*	TFC 22.3 GAE/g DM	García-Rodríguez et al. (2014)

Results presented as reported by the authors.

Grusak (2015) reported total phenolic compounds content of 5.66 mg GAE/g FM, and a total flavonoid content of 332.8 µg CE/g FM. Finally, using an ethyl acetate extractant, García-Rodríguez et al. (2014) found a weak presence of coumarin and flavonoids. They also found a concentration of 13.2 mg GAE/g DM of total phenolic compounds in a hexanoic extract.

Determination of individual compounds

Figure 2 presents the structures of the most reported phenolic compounds in chaya leaves. Valenzuela et al. (2015) reported the presence of quercetin and rutine in an aqueous extraction of *C. chayamansa* leaf (Table 4). Ramos-Gómez et al. (2016) performed an aqueous extraction of *C. chayamansa* leaf and analyzed by high performance liquid chromatography with a diode-array detector (HPLC-DAD)/mass spectrometer

Table 4. Identification of specific phenolic compounds in chaya leaves.

Solvent system used/species	Technique used	Phenolic compounds identified	Reference
Aqueous/ *C. chayamansa*	Only one chromatogram is shown	Quercetin and Rutine	Valenzuela-Soto et al., 2015
Aqueous/ *C. chayamansa*	HPLC-DAD/MSD	Epigallocatech gallato 27.4 mg/g FM Rosmarinic Acid 26.8 mg/g FM Hesperidin 16.2 mg/g FM Vanillin 11.3 mg/g FM Rutine 10.6 mg/g FM Chlorogenic Acid 8.6 mg/g FM 4-Hydroxybenzoic acid 8.1 mg/g FM Coffeic Acid 5.4 mg/g FM Ferulic Acid 4.7 mg/g FM Catechin 4.3 mg/g FM Protocatechic acid 4.2 mg/g FM P-coumaric acid 3.0 mg/g FM Naringenin 2.7 mg/g FM Synaptic Acid 1.7 mg/g FM Quercetin 1.4 mg/g FM Ellagic Acid 0.8 mg/g FM Galocatequin gallate 0.5 mg/g FM	Ramos-Gómez et al. (2016)
Ethanol/*C. aconitifolius*	HPLC-DAD	Crude: Kaempferol 58.2, Quercetin 16.9 and Cooked: Kaempferol 50.0, Quercetin 12.6 µg/g FM	Kuti and Konuru (2004)
Ethanol/*C. aconitifolius*	HPLC–DM	Hispidulin Sulfate and Eucalyptine, Epigalocatechin di-O-gallate, Epicatequin di-O-gallate, Acutifoline D and Tiegusanin F Lignin and Coumarin Fraxetin.	Numa et al. (2015)
Fresh matter/*C. aconitifolius*	AOAC Method	Lignin 39.6 g/kg FM	Sarmiento-Franco et al. (2003)
Methanol/*C. aconitifolius*	Gas-liquid Comatography	Glucosidated flavonols present are Galactosidized, Glucosidized, Ramnosididated and Ramnosylglucosidates of Quercetin and Kaempferol, and two triglycosides of Quercetin	Kolterman et al. (1984)
Methanol/*C. chayamansa*	Nuclear magnetic resonance	Dihydromyricetine was observed in the stem, and in the leaves, the biflavonoid (3 '→ 8) - Diapigenin (Amentoflavone) and the Kaempferol-3-O-glucoside (Astragalin) and Kaempferol-3-O-rutinoside glycoside	González-Laredo et al. (2003)
Methanol/*C. chayamansa*	Ultraviolet spectrophotometric analysis	Dihydromyricetine, Amentoflavone, Rutin, Quercetin, Naringin, Hesperidin, Nobiletine	Figueroa-Valverde et al. (2009)
Methanol/*C. chayamansa*	HPLC-DAD	Protocatecuic Acid 0.24 mg/g and Rutine 2.00 mg/g freeze-dried.	Loarca-Piña et al. (2010)

Results presented as reported by the authors.

(MSD). They reported the concentration of different phenolic compounds: epigallocatequin gallate 27.4 mg/g FM, rosmarinic acid 26.8 mg/g FM, hesperidin 16.2 mg/g FM, vanillin 11.3 mg/g FM, rutin 10.6 mg/g FM, chlorogenic acid 8.6 mg/g FM, 4-hydroxybenzoic acid 8.1 mg/g FM, caffeic acid 5.4 mg/g FM, ferulic acid 4.7 mg/g FM, catechin 4.3 mg/g FM, protocatechic acid 4.2 mg/g FM, p-coumaric acid 3.0 mg/g FM, naringenin 2.7 mg/g FM, synapic acid 1.7 mg/g FM, quercetin 1.4 mg/g FM, ellagic acid 0.8 mg/g FM, and galocatequin gallate 0.5 mg/g FM. Kuti and Konuru (2004) analyzed raw and boiled chaya leaf extracts and analyzed them by HPLC-DAD. They reported kaempferol 58.2 µg/g FM and 50.0 µg/g FM, quercetin 16.9 µg/g FM and 12.6 µg/g FM in the raw and boiled extracts, respectively. Numa et al. (2015) analyzed an ethanolic extract of *C. aconitifolius* leaf by HPLC-DM, finding the presence of hispidulin sulphate, eucalyptine, epigallocatechyne di-O-gallate, epicatequin di-O-gallate, acutifolin D, lignin Tiegusanin F, and coumarin fraxetin. Sarmiento-Franco et al. (2003) harvested *C. aconitifolius* by cutting off all the leaves first, and then allowing the young stems to reach approximately 1 m in height. The leaves from each plant were stored in plastic bags and frozen at -10°C until analysis. By this process, they determined the presence of lignin: 39.6 g/kg FM carried out according to AOAC methods (AOAC, 1980). Kolterman et al. (1984) performed a methanolic extraction of *C. aconitifolius* leaf and analyzed it by gas-liquid chromatography, identifying glucosidic flavonols, such as galactosidated, glucosidized, rhamnosididated and rhamnosylglucosidates of quercetin and kaempferol, and two quercetin triglycosides. Gonzalez-Laredo et al. (2003) performed methanolic extractions from the stem and leaf of *C. chayamansa* and analyzed them by nuclear magnetic resonance. They reported dihydromyricetin in the stem, and in the leaves, biflavonoid (3 '→ 8) - diapigenin (amentoflavone), glycoside kaempferol-3-o-glucoside (astragalin) and kaempferol-3-o-rutinoside. Figueroa-Valverde et al. (2009) examined a methanolic extract of *C. chayamansa* leaf using ultraviolet spectrophotometric analysis, and found the presence of dihydromyricetine, amentoflavone, rutin, quercetin, naringin, hesperidin, and nobiletin. Loarca-Piña et al. (2010) found a protocatecuic acid (0.242 ± 0.001 mg/g of extract) and rutin (2.00 ± 0.097 mg/g) in a methane extract from *C. chayamansa* leaf, analyzed by HPLC-DAD.

Antioxidant capacity of chaya leaf

García-Rodríguez et al. (2014) performed a non-polar extraction using ethyl acetate as solvent and analyzed antioxidant capacity using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reduction activity potential (FRAP) techniques (Table 5). They reported an 11.6% inhibition by DPPH and 387.1 µmol Fe/L by the FRAP technique. Valenzuela et al. (2015) performed an infusion with chaya leaf and found an antioxidant capacity of 5.9 mM Trolox equivalents/ml infusion. In an aqueous extract, Ramos-Gómez et al. (2016) reported an antioxidant capacity of 25.5 µg/ml by DPPH, of 44.3 µg/ml by 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), and 38.5 µg/ml by NO. Kuti and Konuru (2004) performed ethanolic extractions on raw and boiled chaya leaves, testing for antioxidant capacity using the oxygen radical absorbance capacity (ORAC) technique, which yielded values of 15.3 µmol Trolox equivalents/g FM in the extract of raw leaves and 11.8 µmol Trolox equivalents/g FM in the extract of cooked leaves. García-Rodríguez et al. (2014) reported the antioxidant capacity in an ethanolic extract of chaya leaves using the DPPH and FRAP techniques. These authors reported a 10.6% inhibition by DPPH, and 245.0 µmol Fe/L with the FRAP technique. Also, in a hexanoic extract, they reported 0.5% inhibition by DPPH and 239.4 µmol Fe/L by FRAP. Loarca-Piña et al. (2010) analyzed the antioxidant capacity of the chaya leaf, reporting a 45.5% inhibition by DPPH, and a 95% inhibition by ABTS. They reported IC50 of 1693 µg/ml. Finally, Jiménez-Aguilar and Grusak (2015) analyzed the antioxidant capacity of a chaya leaf methanolic extract, reporting 34.38 µmol Trolox equivalents/g FM.

The most commonly used methods for analyzing antioxidant capacity are ABTS+, DPPH, ORAC and FRAP. These are highly reproducible under certain assay conditions, but also show significant differences in their response to antioxidants. The free radical DPPH (DPPH) does not require any special preparation, whereas the radical cation ABTS (ABTS+) must be generated by enzymes or chemical reactions (Arnao, 2000). Another significant difference is that ABTS+ can be dissolved in aqueous and organic media, in which antioxidant activity can be measured, given the hydrophilic and lipophilic nature of the compounds in the samples. In contrast, DPPH can only be dissolved in organic media, especially in ethanol, which is a significant limitation in interpreting the role of hydrophilic antioxidants. In both radicals, however, reductive capacity does not necessarily reflect antioxidant activity, as suggested by Wong et al. (2006), Katalinic et al. (2006) and Wojdyłol et al. (2007). From a scientific standpoint, the best approach is to conduct a variety of tests to evaluate antioxidant capacity, since this yields a more complete and ultimately more accurate analysis.

The content of the phenolic compounds and their antioxidant capacity varies from one extract to another, not only in the environmental factors, but also by the way in which the data are expressed, either in different units or in different states of the sample, for example, lyophilized, dried or fresh matter. The results also vary in that the distinct types of extractions are not usually 100% of a single solvent, but instead use different mixtures

Table 5. Antioxidant capacity reported in chaya leaves.

Solvent system used/species	Antioxidant capacity	Reference
Aqueous		
C. chayamansa	5.9 mM Trolox Eq/ml of infusion	Valenzuela-Soto et al. (2015)
C. chayamansa	DPPH 25.5, ABTS 44.3 and NO 38.5 (IC_{50} µg/mL)	Ramos-Gómez et al. (2016)
Ethanolic		
C. aconitifolius	ORAC raw leaf: 15.3, cooked leaf: 11.8 µmol Trolox Eq/g FM	Kuti y Konuru (2004)
C. aconitifolius	DPPH: 10.6% inhibition and in FRAP: 245.0 µmol Fe/L	García-Rodríguez et al. (2014)
Methanolic		
C. chayamansa	DPPH 45.5% inhibition and ABTS 95% inhibition and 1693 (IC_{50}) µg/mL	Loarca-Piña et al. (2010)
C. aconitifolius	ORAC - APPH 34.38 µmol Trolox Eq/g FM	Jiménez-Aguilar y Grusak (2015)
Other solvents		
Ethyl acetate/*C. aconitifolius*	DPPH 11.6% inhibition and in FRAP 387.1 µmol Fe/L	García-Rodríguez et al. (2014)
Hexanoic/*C. aconitifolius*	DPPH: 10.5% inhibition and in FRAP: 239.4 µmol Fe/L	García-Rodríguez et al. (2014)

Results presented as reported by the authors.

and proportions, in addition to various extraction conditions and various determination methodologies.

Future perspectives

The studies presented in this review do not enable us to clearly determine which is the best extraction method for the phenolic compounds of the chaya leaf. This is because of the highly diverse processes mentioned by the different authors, as can be seen in Tables 2 to 4. Apart from the environmental factors, there are differences in the treatment of the sample before the extraction process, such as the type and the drying conditions. There are also differences in the solvents used and in the methods of extraction and concentration of compounds. Finally, a diversity of techniques are used, and even the data themselves are quantified and expressed differently. Even so, it can be said that the greatest amount and variety of phenolic compounds was obtained with different mixtures of hydroalcoholic proportions. Common knowledge tells us that the best drying method is one in which the conditions used to remove the water are not very aggressive with the biological material, for example, temperatures no higher than 40°C and a short drying time to avoid the degradation of the compounds of interest.

Specific further study is needed to evaluate different types of solvents and mixtures of them for the extraction of phenolic compounds, where the same methodology is used for sample handling, from the harvesting of chaya leaves, the method of drying, grinding and extraction conditions, through the analysis of the compounds to create a phenolic profile. This would enable researchers to determine the best solvent for extracting certain type of phenolic compounds in chaya leaves. It would also be useful to perform the extractions from both raw and boiled leaves since it is known that the raw leaves have a cyanogenic glycoside that is eliminated by boiling the leaves in water, and this heat treatment could affect the phenolic profile.

CONCLUSIONS

In the chaya leaf, there is a general trend toward the presence of different phenolic groups, such as coumarin, flavonoids, phenols, tannins, anthraquinones, and flobotanins in aqueous and alcoholic extracts. The chaya plant has potential for production as food and as a medicinal plant, but the task of comparing the results obtained from the different research articles is complicated by the different processes used by each of the researchers to report the phenolic compounds and the antioxidant capacity of this plant. Apart from the analysis of different extraction methods, solvents and forms of preparation, as well as the diversity of extracted compounds, further research is also important and necessary through *in vitro* and *in vivo* studies of each type of extract in order to evaluate their biological effects on health, for example, in reducing glucose levels, or as a possible chemopreventive or chemoprotector agent against colon cancer.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

Abbreviations

GAE, Gallic acid equivalents; **CAE,** chlorogenic acid equivalents; **CE,** catequin equivalents; **TE,** tannin equivalents; **FM,** fresh matter; **DM,** dry matter.

ACKNOWLEDGEMENT

Publication partially funded with PFCE-2016 resources.

REFERENCES

Adaramoye OA, Aluko A, Oyagbemi AA (2011). *Cnidoscolus aconitifolius* leaf extract protects against hepatic damage induced by chronic ethanol administration in Wistar rats. Alcohol 46(4):451-458.

Adebiyi OA, Adebiyi OO, Ilesanmi OR, Raji Y (2012). Sedative effect of hydroalcholic leaf extracts of *Cnidoscolous acontifolius*. Int. J. Appl. Res. Nat. Prod. 5(1):1-6.

Akachukwu D, Okafor PN, Ibegbulem CO (2014). Phytochemical content of *Cnidoscolus aconitifolius* and toxicological effect of its aqueous leaf extract in Wistar rats. J. Investig. Biochem. 1(1):26-31.

Arnao MB (2000). Some methodological problems in the determination of antioxidant activity using chromogen radicals: A practical case. Trends Food Sci. Technol. 11:419-421.

Association of Official Analytical Chemists (AOAC) (1980). Official Methods of Analysis, 13th ed. Washington, DC.

Awoyinka OA, Balogun IO, Ogunnowo AA (2007). Phytochemical screening and in vitro bioactivity of *Cnidoscolus aconitifolius* (Euphorbiaceae). J. Med. Plants Res. 1(3):63-65.

Aye PA (2012). Effect of processing on the nutritive characteristics, anti-nutritional factors and functional properties of *Cnidoscolus aconitifolius* leaves. Am. J. Food Nutr. 2(4):89-95.

Babalola JO, Alabi OO (2015). Effect of processing methods on nutritional composition, phytochemicals, and anti-nutrient properties of chaya leaf (*Cnidoscolus aconitifolius*). Afr. J. Food Sci. 9(12):560-565.

Bravo L (1998). Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. Nutr. Rev. 56(11):317-333.

Cifuentes R, Poll E, Bresanni R, Yurrita R (2010). Caracterización botánica, molecular, agronómica y química de los cultivares de Chaya (*Cnidoscolus aconitifolius*) de Guatemala. Rev. Valle Guatemala 21:34-49.

Czapecka E, Mareczek A, Leja M (2005). Antioxiadant activity of fresh and dry herbs of some Lamiaceae species. Food Chem. 93:223-226.

Del Rio D, Rodriguez-Mateos A, Spencer J, Tognolini M, Borges G, Crozier A (2013). Dietary (poly)phenolics in human health: structures, bioavailability, and evidence of protective effects against chronic diseases. Antioxid. Redox Signal. 18(14):1818-1892.

Escalante-Erosa, Ortegón-Campos I, Parra-Tabla V, Peña-Rodríguez LM (2004). Chemical Composition of the Epicuticular Wax of *Cnidoscolus aconitifolius*. Rev. Soc. Quím. Méx. 48:24-25.

Esparza-Martínez FJ, Miranda-López R, Guzmán-Maldonado SH (2016). Effect of air-drying temperature on extractable and non-extractable phenolics and antioxidant capacity of lime wastes. Ind. Crops Prod. 84:1-6.

Figueroa-Valverde, Díaz-Cedillo F, Camacho-Luis A, López-Ramos M (2009). Efectos inducidos por *Ruta graveolens* L., *Cnidoscolus chayamansa* McVaugh y Citrus aurantium L. sobre los niveles de glucosa, colesterol y triacilglicéridos en un modelo de rata diabética. Braz. J. Pharmacogn. 19(4):898-907.

García-Rodríguez RV, Gutiérrez-Rebolledo GA, Méndez-Bolaina E, Sánchez-Medina A, Maldonado-Saavedra O, Domínguez-Ortiz MA, Vázquez-Hernández M, Muñoz-Muñiz OD, Cruz-Sánchez JS (2014). *Cnidoscolus chayamansa* Mc Vaugh, an important antioxidant, anti-inflammatory and cardio protective plant used in Mexico. J. Ethnopharmacol. 151:937-943.

González-Laredo RF, Flores ME, Quintero-Ramos MJ, Karchesy JJ (2003). Flavonoid and Cyanogenic Contents of chaya (Spinach Tree). Plant Foods Hum. Nutr. 58:1-8.

Grubben GJ, Denton OA (2004). Plant resources of Tropical Africa 2. Nord. J. Bot. 23(3):298.

Gunjan V, Rajkumar R, Ashok K, Lazar M (2011). Therapeutic potential of polar and non-polar extracts of *Cyanthillium cinereum* in vitro. J. Evid. Based Complement. Altern. Med. 2011:1-10.

Gutiérrez-Grijalva EP, Ambriz-Pérez DL, Leyva-López N, Castillo-López RI, Heredia JB (2016). Review: Dietary phenolic compounds, health benefits and bioaccessibility. Arch. Latinoam. Nutr. 66(2):87-100.

Hitchcock P, Pugh J, Larme A, Marsh G (1997). The use of traditional plant medicines for non-insulin dependent diabetes mellitus in south Texas. Phytother. Res. 11:512-517.

Ikpefan EO, Ayinde BA, Gita TD (2013). In vitro comparative cytotoxic and growth inhibitory effects of the methanol extracts of the leaf, stem and root barks of *Cnidoscolus acontifolius* (Mill.) Johnst (Euphorbiaceae). Int. J. Bioassays 2(2):445-449.

Ivanova D, Gerova D, Chervenkov T, Yankova T (2005). Polyphenols and antioxidant capacity of Bulgarian medicinal plants. J. Ethnopharmacol. 96:145-150.

Jiménez-Aguilar DM, Grusak MA (2015). Evaluation of minerals, phytochemical compounds and antioxidant activity of Mexican, central American, and African green leafy vegetables. Plant Foods Hum. Nutr. 70:357-364.

Jiménez-Arellanes MA, García-Martínez I, Rojas-Tomé S (2014). Potencial Biológico de Especies Medicinales del género *Cnidoscolus* (Euphorbiaceae). Rev. Mex. Cienc. Farm. 45(4):1-6.

Johnson CE, Oladeinde FO, Kinyua AM, Michelin R, Makinde JM, Jaiyesimi AA, Mbiti WN, Kamau GN, Kofi-Tsekpo WM, Pramanik S, Williams A, Kennedy A, Bronner Y, Clarke K, Fofonoff P, Nemerson D (2008). Comparative assessment of total phenolic content in selected medicinal plants. Niger. J. Nat. Prod. Med. 12:40.

Jones P, Vogt T (2001). Glycosyltransferases in secondary plant metabolism: tranquilizers and stimulant controllers. Planta 213(2):164-174.

Katalinic V, Milos M, Jukic M (2006). Screening of 70 medicinal plant extracts for antioxidant capacity and total phenols. Food Chem. 94:550-557.

Kolterman DA, Breckon GJ, Kowal RR (1984). Chemotaxonomic Studies in *Cnidoscolus* (Euphorbiaceae). II. Flavonoids of *C. aconitifolius, C. souzae, and C. spinosus*. Syst. Bot. 9(1):22-32.

Kulathuran PK, Narayanan N, Chidambaranathan N, Halith MM, Jayaprakash S (2012). Antitumor activity of ethanolic extract of *Cnidoscolus chayamansa* Mc. Vaugh against Dalton's ascitic lymphoma in mice. Int. J. Pharm. Sci. 4(4):647-652.

Kuti JO and Konoru HB (2006). Cyanogenic glycosides content in two edible leaves of tree spinach (*Cnidoscolus* spp). J. Food Comp. Anal. 19(6-7):556-561.

Kuti JO, Konuru HB (2004). Antioxidant capacity and phenolic content in leaf extracts of tree spinach (*Cnidoscolus* spp.). J. Agric. Food Chem. 52(1):117-121.

Kuti JO, Kuti HO (1999). Proximate composition and mineral content of two edible species of *Cnidoscolus* (tree spinach). Plant Foods Hum. Nutr. 53(4):275-283.

Kuti JO, Torres ES (1996). Potential nutritional and health benefits of tree spinach. J. Janick (Eds). Progress in new crops. ASHS Press Arlington, VA. pp. 516-520.

Loarca-Piña MG, Mendoza S, Ramos-Gómez M, Reynoso R (2010). Antioxidant, antimutagenic, and antidiabetic activities of edible leaves from *Cnidoscolus chayamansa* Mc. Vaugh. J. Food Sci. 75(2):H68-H72.

Lucky SC, Festus OA (2014). Effects of aqueous leaf extract of chaya (*Cnidoscolus aconitifolius*) on pituitary-gonadal axis hormones of male Wistar rats. J. Exp. Clin. Anat. 13(2):34-39.

Marchioli R, Schweiger C, Levantesi G, Tavazzi L, Valagussa F (2001). Antioxidant vitamins and prevention of cardiovascular disease: Epidemiological and clinical trial data. J. Lipids 36:S53-S63.

McVaugh R (1944). The Genus *Cnidoscolus*: Generic Limits and Intrageneric Groups. Bull. Torrey Bot. Club 71(5):457-474.

Miranda VL, Oranday-Cardenas A, Lozano-Garza H, Rivas-Morales C, Chamorro-Cevallos G, Cruz-Vega DE (2010). Hypocholesterolemic

activity from the leaf extracts of *Cnidoscolus chayamansa*. Plant Foods Hum. Nutr. 65(4):392-395.
Molina-Cruz A, Curley LM, Bressani R (1997). Redescubriendo el valor nutritivo de las hojas de chaya (*Cnidoscolus aconitifolius*; Euphorbiaceae). Cienc. Acción Universidad Valle Guatemala 3:1-4.
Mordi JC, Akanji MA (2012). Phytochemical screening of the dried leaf extract of *Cnidoscolus aconitifolius* and associated changes in liver enzymes induced by its administration in Wistar rats. Curr. Res. J. Biol. Sci. 4(2):153-158.
Musa TY, Musbau AA, Adenike TO, Abdul WO, Olatinwo, Abdulfatai AA, Monsurat OY, Bamidele VO, Taofik OS, Moyosore SA (2008). Effect of Cnidoscolous aconitifolius (Miller) I.M. Johnston leaf extract on reproductive hormones of female rats. Iran J. Reprod. Med. 6(3):149-155.
Mwine JT, Van Damme P (2011). Why do Euphorbiaceae tick as medicinal plants? A review of Euphorbiaceae family and its medicinal features. J. Med. Plants Res. 5(5):652-662.
Neveu V, Perez-Jimenez J, Vos F, Crespy V, du Chaffaut L, Mennen L, Knox C, Eisner R, Cruz J, Wishart D, Scalbert A (2010). Phenol-Explorer: An online comprehensive database on polyphenol contents in foods." Database 2010 (2010): bap024. Available at: https://academic.oup.com/database/article/doi/10.1093/database/bap024/401207
Numa S, Rodríguez L, Rodríguez D, Coy-Barrera E (2015). Susceptibility of *Tetranychus urticae* Koch to an ethanol extract of *Cnidoscolus aconitifolius* leaves under laboratory conditions. Springerplus 4:338.
Obichi EA, Monago CC, Belonwu DC (2015). Effect of *Cnidoscolus aconitifolius* (family Euphorbiaceae) aqueous leaf extract on some antioxidant enzymes and haematological susceptibility of *Tetranychus urticae* koch to an ethanol extract of *Cnidoscolus aconitifolius* leaves under laboratory conditions parameters of high fat diet and streptozotocin induced diabetic Wistar albino rats. J. Appl. Sci. Environ. Manag. 19(2):201-209.
Oyagbemi AA, Odetola AA, Azeez OI (2011). Phytochemical investigation and proximate analysis on the leaves of *Cnidoscolus aconitifolius*. J. Med. Food 14(3):322-324.
Ramos-Gómez M, Figueroa-Pérez M, Guzmán-Maldonado H, Loarca-Pina G, Mendoza S, Quezada-Tristan T, Reynoso-Camacho R (2017). Phytochemical profile, antioxidant properties and hypoglycemic effect of chaya (*Cnidoscolus chayamansa*) in stz-induced diabetic rats. J. Food Biochem. 41:e12281.
Rangel-Huerta OD, Aguilera CM, Martin MV, Soto MJ, Rico MC, Vallejo F, Tomas-Barberan F, Pérez-de-la-Cruz AJ, Gil A, Mesa MD (2015). Normal or high polyphenol concentration in orange juice affects antioxidant activity, blood pressure, and body weight in obese or overweight adults. J. Nutr. 145(8):1808-1816.
Rice-Evans C, Miller N, Paganga G (1997). Antioxidant properties of phenolic compounds. Trends Plant Sci. 2:152-159.
Ross-Ibarra J (2003). Origen y domesticación de la chaya (*Cnidoscolus aconitifolius* Mill I. M. Johnst): La espinaca Maya. Mex. Studies/Estudios Mex. 19(2):287-302.
Ross-Ibarra J and Molina-Cruz A (2002). The ethnobotany of chaya. Econ. Bot. 56(4):350-365.
Rothwell JA, Pérez-Jiménez J, Neveu VM, M'Hiri NG, Manach C, Eisner R, Wishart D, Scalbert A (2013). Phenol-Explorer 3.0: A Major Update of the Phenol-Explorer Database to Incorporate Data on the Effects of Food Processing on Polyphenol Content. Available at: https://academic.oup.com/database/article/doi/10.1093/database/bat070/342410
Sarmiento-Franco L, Sandoval-Castro C, McNab J, Quijano-Cervera R, Reyes-Ramirez R (2003). Effect of age of regrowth on chemical composition of chaya (*Cnidoscolus aconitifolius*) leaves. J. Sci. Food Agric. 83:609-612.
Sarker SD, Nahar L (2012). An Introduction to Natural Products Isolation. In: Sarker S, Nahar L (eds.), Natural Products Isolation. Methods in Molecular Biology (Methods and Protocols), Humana Press 864:1-25. Available at: https://link.springer.com/protocol/10.1007/978-1-61779-624-1_1#citeas
Skerget M, Kotnik P, Hadolin M, Hraš AR, Simonič M, Knez Ž (2005). Phenols, proanthocyanidins, flavones and flavonols in some plant materials and their antioxidant activities. Food Chem. 89(2):191-198.
Soetan KO, Aiyelaagbe OO (2009). The need for bioactivity-safety evaluation and conservation of medicinal plants - A review. J. Med. Plants Res. 3(5):324-328.
Standley PC, Steyermark JA (1949). Flora of Guatemala Vol-24.
Valenzuela R, Morales ME, Verde MJ, Oranday A, Preciado-Rangel P, González JA, Esparza-River JR (2015). *Cnidoscolus chayamansa* organic hydroponic and its hypoglycemic capacity, nutraceutical quality and toxicity. Rev. Mex. Cienc. Agric. 6(4):815-825.
Vinson JA, Su X, Zubik L, Bose P (2001). Phenol antioxidant quantity and quality in foods: fruits. J. Agric. Food Chem. 49(11):5315–5321.
Wojdyło A, Oszmianski J, Czemerys R (2007). Antioxidant activity and phenolic compounds in 32 selected herbs. Food Chem. 105:940-949.
Wong CC, Li HB, Cheng KW, Chen F (2006). A systematic survey of antioxidant activity of 30 Chinese medicinal plants using the ferric reducing antioxidant power assay. Food Chem. 97:705-711.
World Health Organization (WHO) (2005). WHO monographs on selected medicinal plants Volume 4. Available at: http://apps.who.int/iris/bitstream/10665/42052/4/9789241547055_eng.pdf
Zare K, Movafeghi A, Mohammadi SA, Asnaashari S, Nazemiyeh H (2014). New phenolics from *Linum mucronatum* subsp. orientale. BioImpacts BI. 4(3):117-122.

academicJournals

Vol. 11(45), pp. 728-741, 3 December, 2017
DOI: 10.5897/JMPR2017.6494
Article Number: 6EC6B9E66879
ISSN 1996-0875

http://www.academicjournals.org/JMPR

Journal of Medicinal Plants Research

Full Length Research Paper

Bioactivity of bizzy nut extract in prostate cancer cells

Orville Phillip[1], Caroline Telles[2] and Wesley Gray[1]*

[1]Department of Environmental Toxicology, Southern University, Baton Rouge, LA 70813, United States.
[2]Department of Biological Sciences, Southern University, Baton Rouge, LA 70813, United States.

Received 11 September, 2017; Accepted 31 October, 2017

Cola acuminate **is one of the three edible forms of bizzy nut that has been used for medicinal and therapeutic purposes. However, the precise compound responsible for its biological effects has not been completely identified. Using sequential solid-liquid extraction of bizzy nut coupled with bioactive screening, ether extract was obtained (Biz-2) and it possesses tumor inhibitory activity specific for prostate cancer cells. Enrichment of this tumor inhibitory activity (Biz-2Fr.3) resulted in the elimination of caffeine and tannin, suggesting that Biz-2-Fr.3 activity is due to a unique set of compounds. Biz-2-Fr.3 contains three to four unique compounds with a molecular mass ranging from 120 to 440 amu as evident by high performance liquid chromatography (HPLC), UV-Vis spectroscopy and LC/MS. Biz-2-Fr.3 was found to exhibit growth inhibition and cytotoxicity against the hormone-independent (DU-145) and hormone-dependent (LNCaP) prostate cancer cell lines via microculture tetrazolium (MTT) assay. In the DU145 cell, Biz-2Fr.3 induces a growth-inhibition with a GI_{50} of 120 ppm with no apparent toxicity in normal transformed prostate cells. The inhibition of DU145 cell proliferation by Biz-2Fr.3 was as a result of retardation of the cell cycle occurring mainly in the G1 phases of the cycle. This cell cycle arrest was associated with the decrease in cyclin D protein levels following Biz-2Fr.3 treatment. It was observed that Biz-2Fr.3 did not elicit any toxicity as evidenced by biochemical markers of liver injury which caused decrease in body weight or serum protein profiles. These results suggest that *C. acuminate* possesses an anti-cancer activity that is distant from its previously reported biological effects.**

Key words: *Cola acuminate*, bizzy nut, prostate, toxicity.

INTRODUCTION

Prostate cancer (PCa) is a major public health problem worldwide. Epidemiological studies have illustrated a wide variation in PCa incidence among different ethnic populations. The incidence of PCa is lower in Asia and China as compared to Western countries such as Europe and the Americas (American Cancer Society, 2013, 2014; Torre et al., 2016). It is estimated that, in the United States, 241,740 men will be diagnosed, and 28,170 men will die of cancer of the prostate in 2016 (Howlader et al., 2017). At the same time, the incidence rate of the diseases in African American men is one-half to three times higher as compared to other ethnic groups (American Cancer Society, 2016). Interestingly, a thirty-year retrospective study on PCa in Black men

*Corresponding author. E-mail: Wesley_Gray@subr.edu.

men from the Caribbean island of Jamaica indicated a lower PCa incidence as compared to black men from the USA and the United Kingdom (Gibson et al., 2011). This disparity between prostate cancer among blacks is presumable due to the difference in life-style and diet.

Studies over the past ten years have provided convincing evidence that natural products can suppress cancer cell growth in cultures and in some animal models. Although the mechanisms of cancer inhibition by natural products vary, they all appear to exert their effect at one or more stages in the cell cycle. For example, Berberine, a naturally occurring isoquinoline alkaloid present in the roots, rhizome, mediates its potential effectiveness in prostate cancer cells through interference with cell cycle progression and induction of apoptosis (Mantena et al., 2006). Andrographolide (AGP), a natural compound isolated from *Andrographis paniculata,* displayed selective inhibitory properties in androgen resistant prostate cells through induction of the G2/M phase cell cycle arrest leading to apoptosis (Varma et al., 2011; Yan et al., 2012; Wong et al., 2011). CKBM, a product contained in the water extracts of wu wei zi (*Schisandra chinensis*), ginseng (*Panax ginseng*), hawthorn (*Fructus Crataegi*), jujube (*Ziziphus jujube*), soybean (*Glycine Max*) and baker's yeast (*Saccharomyces cerevisiae*) inhibits cell proliferation through the induction of the G2/M cell cycle arrest and dose dependent apoptotic effect (Luk et al., 2005).

Chemopreventive agents, isolated from natural products including silibinin, selenium, inositol hexaphosphate, decursin, apigenin, acacetin, curcumin, and epigallocatechin-3 gallate have been identified in laboratory studies as potential useful agents in managing PCa (Singh and Agarwal, 2006; Deep et al., 2016; Tilley et al., 2016). In a clinical trial, lycopene was found to be associated with a decreased risk of PCa development and to inhibit the growth of PCa cells (Dahan et al., 2008; Kumar et al., 2008; Maru et al., 2016). Epigallocatechin-3 gallate (EGCG), a compound found in green tea has been shown to inhibit both androgen dependent and androgen-independent prostate cancer growth (Kallifatidis et al., 2016; Kumar et al., 2016).

EGCG appears to target prostate cell proliferation by inhibiting the production of androgens thus inhibiting 5-alpha reductase and depleting the level of polyamines, both of which are involved in prostate cell proliferation. Other sources of dietary-based natural products that have been examined as a possible treatment and prevention regime for prostate cancer are garlic, grape seed extract, green tea, bitter almonds, and tomato based products. Although several of the natural product remedies are being used clinically and as over the counter medication for treatment of prostate disease but none of them have emerged as a gold standard for prevention or treatment of the disease.

In the laboratory, interest is placed on natural products that contain phytoestrogens, phyto-androgens, or compounds that are anti-androgenic in nature. Recently, it was reported that the common Jamaican herbal medicine, (Bizzy), contains a putative non-steroidal compound with bioactivity in both breast and prostate cancer cells (Fontenot et al., 2007; Solipuram et al., 2009). *C. acuminate*, also known as obi or bizzy nut to the Ettu people of Jamaica, is a "cure-all" herbal medicine. It reportedly affects many biological processes, which are directly, or indirectly, modulated by hormones. Available ethnobotanical information suggests that *C. acuminate* may contain bioactive chemicals that possess estrogenic and androgenic properties (Kamatenesi-Mugish and Oryem-Origa, 2005; Endrini et al., 2011; Osterburg et al., 2009; Lowe et al., 2014).

To begin to elucidate the biology of bizzy nut, several of its hormonally bioactive extracts that were capable of inhibiting the growth of different cancer cell lines were identified (Fontenot et al., 2007). The putative androgenic effects of a Biz-2Fr.3 extract (acetone extract of bizzy Nut) on pathways mediated by an androgen receptor (AR) in LNCaP cells was demonstrated. This bioactive fraction of Bizzy nut is able to induce apoptosis in a prostate cell and modulate AR-dependent gene expression (Solipuram et al., 2009). Furthermore, it was demonstrated that Biz-2Fr.3 induces apoptotic cell death and the cytostatic effect was twice as potent in AR positive LNCaP cells line as compared to the AR negative DU145 cells. However, the biochemical mechanism by which Biz-2Fr.3 induces toxicity in prostate cancer and its effects on prostate cell function is undefined. According to recent studies, many chemo-preventive and/or chemotherapeutic agents can cause cell death via the induction of apoptosis. Therefore, the induction of apoptotic cell death is an important mechanism in the anticancer properties of many drugs. In this study, the anti-cancer effects of Biz-2Fr.3 in the two main stages of prostate cancer, namely, androgen-sensitive and androgen-insensitive tumors is reported. It is shown that a HPLC enriched fraction of bizzy nut is able to inhibit prostate cancer cell growth via induction of the G1--cell cycle blockage. Under the conditions used in this study, bizzy nut did not affect the viability of non-neoplastic human prostate epithelial cells and showed no signs of toxicity in mice. Taken together, a potent anticancer bioactive fraction from bizzy nut which is apparently safe in mice has been identified.

MATERIALS AND METHODS

Cell culture

All cell lines used in this study were obtained from the American type culture collection (ATCC; Rockville, MD, USA). The human prostate cancer cell lines DU145, LNCaP, and PC-3 were cultured in RPMI-1640 (Invitrogen, Grand Island, NY, USA) with 4 mM L-glutamine, and no-phenol red and adjusted to contain 10% fetal bovine serum and 100 U/mL penicillin-streptomycin. The human

breast cancer cell lines MCF-7 and MCF-10A were cultured in DMEM-F12 with 4 mM L-glutamine and adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose supplemented with 0.005 mg ml/mL bovine insulin and 10 nM dehydroisoandrosterone, 10%; fetal bovine serum (BD Biosciences, San Jose, CA, USA). The non-neoplastic immortalized adult human prostatic epithelium cell line RWPE-1 was cultured in K-SFM supplemented with recombinant human epidermal growth factor (rhEGF) and bovine pituitary extract (BPE). The cells were incubated at 37°C in 95% air, 5% CO_2 atmosphere until they approached 80% confluence.

Cell viabilityanalysis

For experiments involving cell growth and gene induction studies, cells were grown for 24 h in appropriate medium containing 5% fasting blood sugar (FBS) that was stripped three times with dextran-coated charcoal or in corning™ serum free medium, with L-glutamine and without phenol red. Cells were plated in 96-well plates (2 x 103 cells/well) and allowed to attach overnight. Bizzy nut extract was added at five to 10-fold dilutions of a 1E4 ppm stock Biz-2Fr.3 solution in 0.5% dimethyl sulfoxide (DMSO) to a 96-well plate. As a control and reference, 10-8 M DHT and 100 ng/ml TNF-a were added to separate wells of each plate and each treatment and time point had four replicates. In each treatment, the final concentration of vehicle solvent (DMSO) did not exceed 0.1% v/v in the medium. After 24 h exposure to the test compounds, the effect on cell viability and gene expression was determined. Cytotoxicity was determined by the CellTiter 96® aqueous one solution cell proliferation assay (Promega, Madison, WI) or sulforhodamine B assay according to the manufacturer's instructions. After incubation with 3-(4,5-dimethyl-2-yl)-5-(3--carboxymethoxyphenyl)--2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS), absorbance at 490 nm was measured using a ELX800UV universal microplate reader (Bio-Tek, Inc.). The absorbance data from the sulforhodamine B assay and the CellTiter 96® aqueous one assay was analyzed using Prism 5.

Analysis of cell cycle progression

Cells were seeded in a 75 cm2 flask at a density of 5 x 106 cells/flask. Cells were treated with Biz--2Fr.3 (five to 10-fold dilutions of 1E4 ppm stock), and incubated for 0, 6, 12 or 24 h. Cells were trypsinized, harvested, and fixed in 1 ml of 80% cold ethanol in test tubes and incubated at 4°C for 15 min. After incubation, cells were centrifuged at 1,500 rpm for 5 min and the cell pellets were suspended in 500-µl propidium iodine (10 µg/ml) containing 300 µg/ml RNase (Sigma, MO, USA). Then, the cells were incubated on ice for 30 min and filtered with 53 µm nylon mesh. The cell cycle distribution was determined from 20,000 cells using the Beckman Coulter Cytoflex flow cytometer. The stained cells were analyzed by ModFit software for cell cycle distribution, including sub-G1, G0/G1, S, and G2/M phases.

Western blot analysis

Immunobloting was performed as previously described (Stahl et al., 1998; Washington et al., 2001). Cells at 80% confluence were treated with Biz-2Fr.3 (5 to10-fold dilution of 1 E4 ppm) for 24 h. Treated cells were trypsinized, washed in PBS, and then pelleted by centrifugation at 100 *g* for 5 min. The cell pellet was then suspended in lysis buffer [(20 mM Tris-HCl pH 7.4, 2 mM EDTA pH 7.4, 2 mM EGTA pH 7.4, 6 mM b-mercaptoethanol, 10 $mgmL^{-1}$ of leupeptin, 2 $mgmL^{-1}$ of aprotinin and 1% Nonidet (NP-40)] and sonicated (Soniprep 150, MSE, USA) at 26 amplitude microns on ice. The cell lysate was centrifuged at 140,000 *g* for 15 min at 4°C and the supernatant was collected and stored at 70°C. The concentration of protein was determined using a BCA protein assay reagent, according to the manufacturer's instructions. An equal amount of protein (25 ug) was separated by 10% SDS-PAGE. After electrophoresis, the proteins were transferred to a PVDF membrane, blocked overnight with 1% skimmed milk in TBS at 4°C then reacted with the following antibodies: anti-BCl2 0.5 ug/ml and anti-Bax 0.5 ug/ml (Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-AR 1.5 ug/ml, anti-PARP 0.5 ug/ml and anti-GDPH 0.5 ug/ml (Cell Signaling, Danvers, MA), mouse monoclonal antibody against CDK1, CDK4 or Actin (Ab-1) (Santa Cruz Biotechnology). After reaction with horseradish peroxidase-conjugated goat anti-mouse antibody, the immune complexes were visualized by using the ECL-detection reagents following the manufacturer's procedure. The immunoblot signal was captured using an AlphaInnotech Fluorochem HD 9900 (Alpha Innotech, San Leandro, CA) equipped with a CDD camera and curves and graphs were fitted with GraphPad Prism 5.0 software (GraphPad, San Diego CA)

Immunofluorescence staining

Cells were grown on microscope slides and induced with Biz-2Fr.3 for 24 h. Cells were fixed with 100% methanol (-20°C for 10 min) and cross linked with 4% paraformaldehyde at room temperature for 10 min. The slides were blocked with 1% rabbit serum solution at room temperature for 1 h. Slides were probed with anti-AR at a 1:100 dilution for 1 h at room temperature, washed and then incubated with fluorescence-labeled anti-rabbit IgG (1:5000 dilution) for an additional hour at room temperature. For dual antibody staining, slides were washed with TBS-T and blocked in 10% sheep serum for 1 h and probed with anti---tubulin (1:200) for 1 h. The tubulin signal was developed by addition of Cy3---labeled anti-mouse IgG for 1 h. Slides were washed with TBS and stained with prolong gold anti-fade reagent containing DAPI (4,6-diamidino-2-phenylindole). Slides were visualized using a Nikon Optiphot fluorescent micro--- scope with green fluorescent (525 nm) and red fluorescent (620 nm) filters.

Solid–liquid extraction of bizzy nut

C. acuminate, commonly known as bizzy nut is from the west and central African genus of the family *Sterculiaceae*. Ripened bizzy nut was obtained from Lambs River, Jamaica in August and the dark brown nut was blended to a fine powder before use. A 1.2 kg sample of finely ground nut was sequentially extracted in a Soxhlet apparatus (120 cm X 500 cm) using 100% hexane, ether, acetone, methane, and water to produce five independent extracts with compounds of unique polarity. The extraction mixture was refluxed for seven days at temperatures corresponding to the boiling point of the respective solvent and the extraction monitored by HPLC chromatography. Following extraction, particulate matter was removed by filtering the samples through a 0.45 µm glass–fritted filter, and the extracts evaporated to dryness using a combination of simple distillation and rotary evaporation. All extracts were dissolved in 50% DMSO and represent the starting point for characterizing the bioactivity of bizzy nut.

HPLC with diode array detection (HPLC-DAD) analysis

HPLC purification of Biz-2Fr.3 was performed on an Agilent 1200 Series system (Agilent, USA), equipped with diode array UV/VIS detector, a quaternary LC- pump, a degassing unit auto injector column oven, a fraction collector and chem station data system.

Chromatographic separation was performed on a 10 micron, 4.6 mm, 250 mm x 4.6 mm. i.d. semi preparative Phenomenex C18 reversed phase column, (Agilent, USA) at 25°C with a guard column (4.6Å~12.5 mm, 5 µm, Zorbax eclipse plus). The mobile phase consists of water (Solvent A was water/formic acid 100:0.1:(v/v)) and acetonitrile/isopropanol (solvent B, acetonitrile/isopropanol 70:30 (v/v).) which was used for gradient elution. The gradient starts linear at 20% B up to 70% B at 15 min, 50% B at 30 min, 60% B at 35 min and then down to 6% B at 41 min. Sample volume was 1200 uL, at a flow rate of 5 mL min-1 and peak detection at 250 nm and 280 nm.

HPLC with electrospray ionization mass spectrometry (HPLC-ESI-MS)

LC-MS identification of Biz-2Fr.3 analytes were performed on the Agilent Technologies Agilent 6210 Quadrupole LC-MS equipped with a mass analyzer and an electrospray ionization interface. An octadecyl silica (C18) column, 10 cm × 2.1 mm i.d., 100 Å pore C18 Ace® analytical column and guard column, from Mac Mod Analytical, Inc (Chadds Ford, PA) was used for analysis. The MA analysis parameters were as follows: Q1 MS; polarity positive, ion source, turbo spray (ESI); declustering potential (DP), 80 V; entrance potential (EP), 10 V; curtain gas, 20 psi; ion spray voltage (IS), 5500 V; temperature, 550C; ion source gas 150 psi; ion source gas 250 psi; interface. The LC mobile phase consisted of gradient water: methanol: ammonium acetate (v/v/w). The mobile phase started with 100% A for the first min, followed by a linear increase to 100% B from 1 to 16 min. This was followed by 100% B from 16 to 31 min, then a linear decrease to 100% A from 31 to 40 min. The injection volume was 20 µl and the eluent flow rate was 0.25 ml/min.

NMR and FTIR Analysis of Biz-2Fr.3

^{1}H NMR and ^{1}H-decoupled 13C-NMR spectra of Biz-2Fr.3 in DMSO-d6 analysis were obtained using a Bruker ARX 500 MHz NMR. A 30° pulse width was used for the 1H NMR, with a 1 s pulse delay. A 30° pulse width was used for the 13C-NMR spectra, with a 2 s pulse delay. The hydrogen and carbon chemical shifts were referenced to the DMSO peaks, which were set to 2.50 ppm for hydrogen and 39.50 ppm for carbon, respectively. The Attached Proton Test (APT) was used to distinguish between two groups of signals, namely, methyl/methane and methylene/quaternary.

Animal study

C57BL/6 male mice of age 4 to 6 weeks were used for the subacute toxicity profiling. They were fed *ad libitum* with standard feed, and had free access to water. They were also maintained under standard conditions of humidity, temperature, and 12 h light/dark cycle. The animals were acclimatized for a week before the commencement of the study. A standard protocol was drawn up in accordance with current guidelines for the care of laboratory animals and ethical guidelines for investigations of experiments in conscious animals

Dosing

C57BL6 mice of average weight between 22 and 24 g were selected by stratified randomization and then divided into four groups of five mice. Group II, Group III, and Group IV were given 10, 100 and 200 mg/kg body weight, respectively, of Biz-2 or Biz-2Fr3 fraction orally every 24 h for 21 days. Biz--2, is a crude ether extract of the C acuminate nut and Biz-2Fr3 is a partial HPLC purified fraction of Biz-2 containing both active and inactive ingredients. Group I served as the control group and received DMSO in PBS. The body weight of each mouse was expressed using a sensitive balance during the acclimatization period, once before commencement of dosing, once daily during the experimental period and on the day of sacrifice. The first day of dosing was taken as Day 0 and blood was collected on Day 21 and used for biochemical analysis.

Determination of biochemical parameters

Blood was collected by the cardiac puncture technique. The clear serum supernatant was prepared and stored in a clean sample bottle for the biochemical tests. A mini blood chemistry panel was performed which included measurement for blood glucose, total serum protein, serum albumin, serum cholesterol, serum alanine aminotransferase (ALT), serum aspartate aminotransferase (AST), serum alkaline phosphatase (ALP), serum urea, and serum creatinine. All hematological parameters were determined at room temperature following standard laboratory procedures.

Organ weight

The liver, kidney, brain, prostate, and stomach of mice in the various groups were excised on the Day 21 immediately after blood collection. Following excision, the organs were trimmed of extraneous tissues, placed on a saline soaked gauze pad to retard desiccation and were immediately weighed (paired organs were weighed together) to one decimal place and calculated for organ weight ratio.

Statistical analysis

All numerical data were expressed as mean ±SEM. In each assay, three or four measurements were made. Means for the treatment groups were compared using analysis of variance and Duncan's multiple range test ($P< 0.05$). To analyze the absorbance density from Western blot data, a two-tailed t test ($P< 0.05$) was used to compare the mean (n=4) for each treatment group with the mean for the untreated control group. The GraphPad Prism 5.0 software program (GraphPad, San Diego CA) was used for the statistical analysis.

RESULTS

Enriching the anti-tumor bioactivity in bizzy nut

Previously we reported that a crude ether extract of bizzy Nut (Biz-2) contains bioactive compounds that elicited an anti-proliferative effect in both hormone---responsive (LNCaP cell) and hormone-resistant (DU145 cell) prostate cancer cells suggesting that Biz-2 may be a potential agent for managing PCa. To generate an enriched fraction of the bioactive compounds present in Biz-2, a reverse phase HPLC chromatographic separation method was developed using a 2.6 µm C18 250b × 4.6 ID phenomenex column and the Agilent 1200 Series HPLC equipped with diode array detector, a quaternary pump, and a standard fraction collector. The analytes present in Biz-2 were separated based on retention time and a peak threshold corresponding to 5 nng of analytes at 250 nm absorbance. Optimizations of

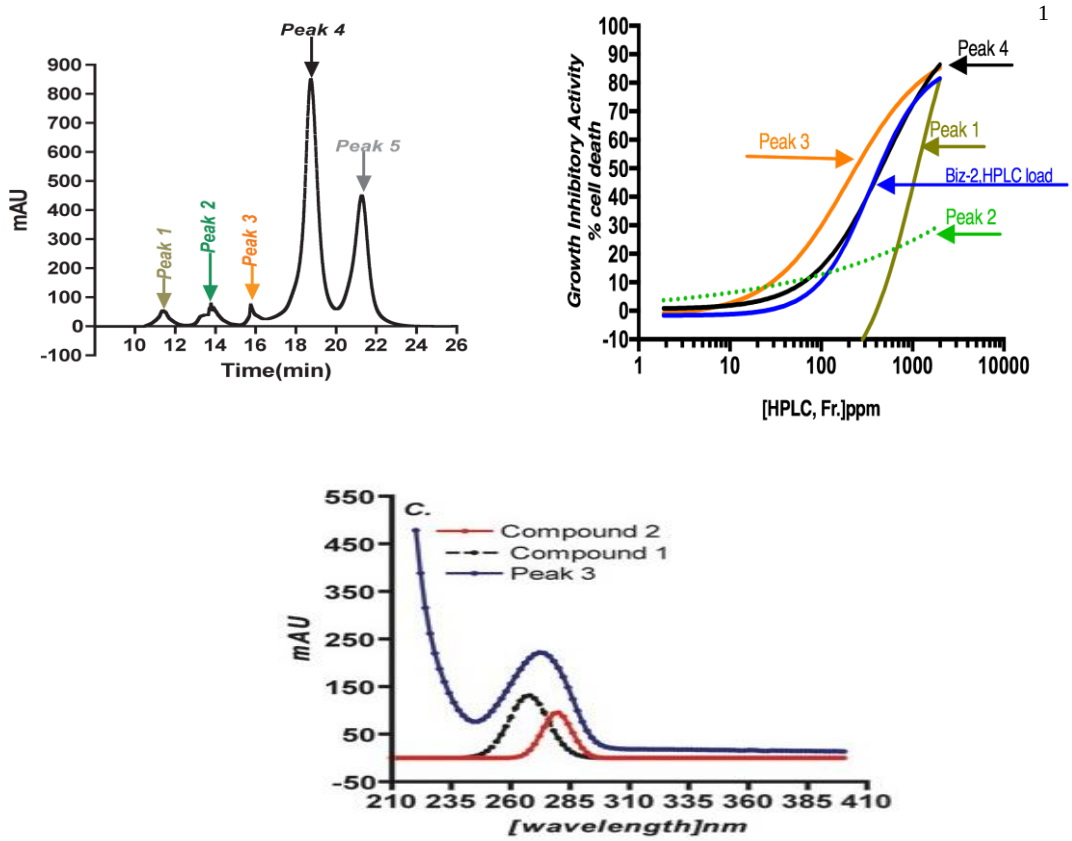


Figure 1. HPLC enrichment of the bioactive compounds in Bizzy nut.

the Biz-2 enrichment conditions were performed on an analytical size column and include chromatography column selection, mobile phase buffer optimization and detection wavelength. In several HPLC runs, a mobile phase of 70%/acetonitrile/30% isoproponal was able to identify five peaks having an absorbance response greater than 5 ng using our separation conditions. The HPLC conditions that were developed resulted in excellent separation with each peak having a resolution factor (K') of 2.1 to 2.4. Fractionation of the Biz-2 extract by HPLC revealed the presence of five distinct peaks (Fr.1-5), all eluting within the first 25 min of the HPLC run (Figure 1A). The resulting peak areas of the five-distinct fraction were used as a marker for evaluation of Biz-2 extraction efficiency. There were no significant differences in the peak areas of the five fractions among different batches of extractions and HPLC separation. Therefore, the extraction method for identifying the bioactivity in bizzy nut was selected as extraction with 100% hexane 70°C for 24 h, followed by 100% diethyl ether at 50°C for 48 h.

Identification of bioactivity in HPLC Fraction

To determine which of the HPLC fraction contains the observed bizzy nut bioactivity, each HPLC peak was concentrated to remove the HPLC solvent, dissolved in 10% DMSO, and were subjected to growth-inhibitory screens in LNCaP prostate cancer cells using the MTS viability assay (Figure 1B). The percent growth-Inhibition was determined and the resulting inhibition data analyzed using Sigmoidal Dose-response with a variable slope was used to determine which fraction contained the bioactivity. Analysis of the GI50 in DU145 cells indicated that most of the activity eluted in peak 3. The GI50 associated with peak 3 was between 150 to 210 ppm as compared to the other HPLC fractions which has a GI50 greater than 500 pm. The strongest correlation between the inhibitory activity of the HPLC fractions was observed in peak 3 of the HPLC chromatograph. The GI_{50} for peak 3 (referred to as Biz-2Fr.3) in DU145 cells was 100±1.3 ppm; 2.5 times lower than that of the crude extract (Figure 1B)

Chemical characterization of Biz-2Fr.3

Next, we examined the purity and determined the number of compounds present in peak 3 (Biz-2Fr.3Fr.3) using U-visible and LC-MS spectroscopy. A spectrum scan of peak 3 from 210 to 700 nm detected the presence of at

least two to three individual compounds with maximum absorbance at 278 nm and 284 nm (Figure 2A). To provide clues as to the chemical identity of Biz-2Fr.3, LC-MS spectroscopy analysis was subjected peak 3. LC-MS because of its sensitivity, reproducibility, speed and versatility was applied. An Agilent 6210 Quadrupole LC-MS with an Agilent Ultra-2 fused silica capillary MS column both positive and in the negative ion mode was used to analyzed Biz-2Fr.3 because it provides more information about chemical structure. LC-MS chromatography analysis resulted in the identification of various compounds present in Biz-2Fr.3. The LC-MS resulted in three major peaks with retention times of 1.1, 5.8 and 7.5 min (Figure 2B). Characterization of the molecular ions at 1.1 min revealed a range of analytes with m/z ranging from 120 to 445 amu. The MS peaks at 5.8 and 7.5 min contained a single molecular ion with amu of 181 (Figure 2B.)

In vitro growth Inhibitory properties of Biz-2Fr.3 in normal and prostate cancer cells

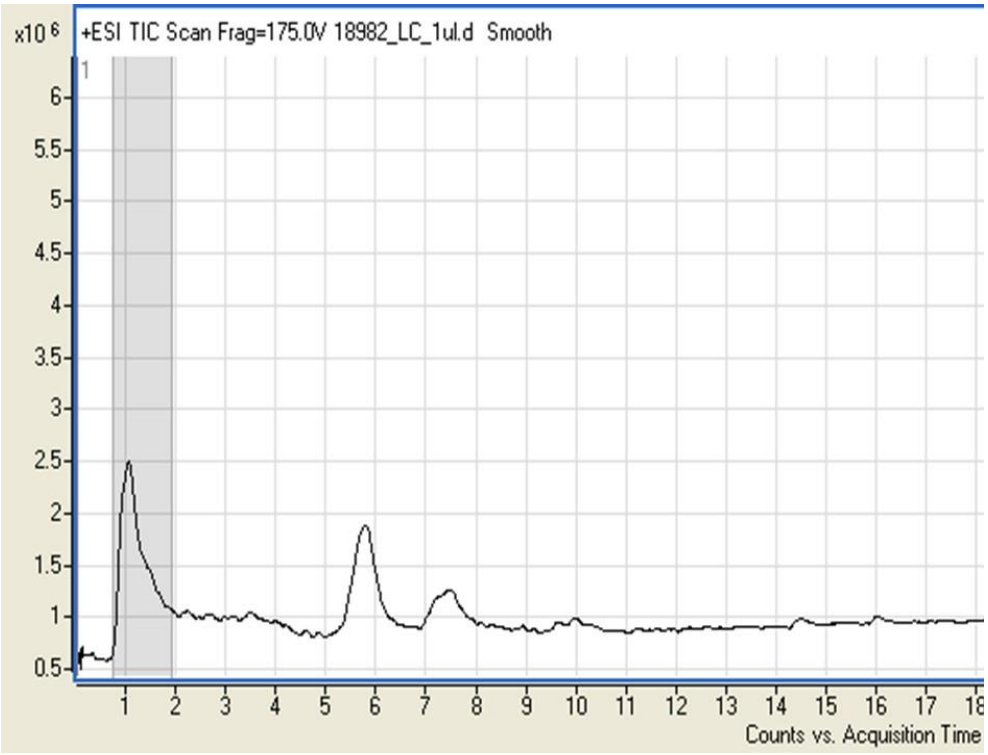
To determine if there were any selectivity of anti-proliferative effects of Biz-2Fr.3 in towards prostate cancer, a panel of cell lines representing functional disease states was tested. The panel contains four androgen receptor-selected lines representing hormone-responsive (LNCaP cell), hormone-resistant (DU145), hormone refractory (PC-3) cell and normal transformed (RWPE-1) prostate cells. Cells (1E4) were grown in 96-well plates, induced with varying concentration of Biz-2Fr.3 in for 24 h and the degree of the cytotoxicity measured using the MTT assay. Biz-2Fr.3 was found to inhibit the proliferation of all tested cell lines in a concentration-dependent and time-dependent manner (Figure 3). The growth inhibitory concentration (GI_{50}) of Biz-2Fr.3, calculated by the four-parameter logistic method, shows higher values in normal transformed prostate cells than in prostate cancer cell lines ($p>0.001$) implying Biz-2 possesses relatively selective cytotoxicity towards cancer cells (Figure 3). Biz-2Fr.3 was found to be 5 times more potent towards the androgen insensitive cell DU145 line as compared to the normal transform RWP-1 (GI_{50} of 126 ppm verses 650 ppm). The androgen responsive cell line showed a marginal sensitivity (260 ppm LNCaP verses 126 ppm DU145), whereas the hormone refractive PC-3 was unaffected by Biz-2Fr.3. To corroborate the cytotoxicity of Biz-2Fr.3, toward prostate cancer cells, its growth inhibitory activity in MCF-7 was examined which possesses a functional AR receptor. Growth of MCF-7 cells in the presence of Biz-2Fr.3, resulted in little to no toxicity. The GI_{50} after 24 and 48 h was 600ppm, similar to that observed in the normal untransformed prostate cells. Thus, Biz-2Fr.3 produces a significant increase in its cell inhibitory activity in DU145 prostate cells implicating that Biz-2Fr.3 possesses relatively selective cytotoxicity towards cancer cells.

Inhibition of cell cycle progression by Biz-2Fr.3

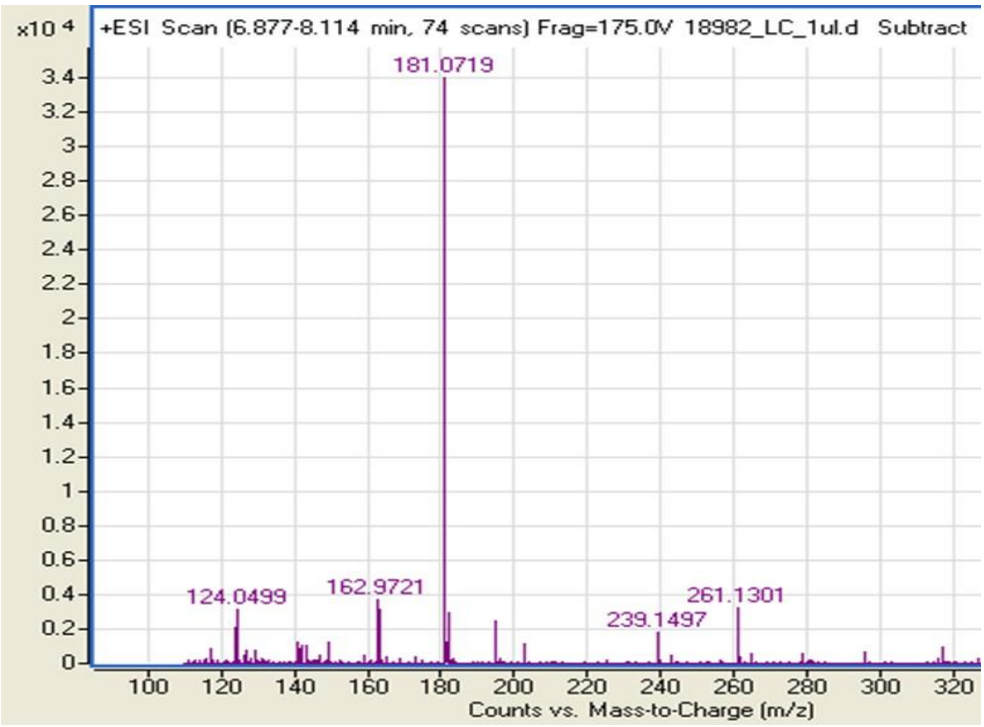
Observing that Biz-2Fr.3 elicited a greater anti-proliferative effect in DU-145, it was used as a model to examine the mechanism of Biz-2Fr.3 anti-proliferative activity. PI staining coupled to flow cytometry was used to determine where in the cell-cycle Biz-2Fr.3 induced proliferation blockage. Analysis of untreated, synchronized DU-145 cells by flow cytometry showed that 40% of the cells were in G1/S, and 17% in the G2/M phase of the cell cycle. Upon treatment with Biz-2Fr.3, there was a significant increase in G1 cells, which was accompanied by a decrease of cells in the S phase after 24 and 36 h. (Figure 4). As summarized in Figure 4, treatment of DU145 cells with Biz-2Fr.3 for 48 h resulted in a significantly higher number of cells in the G1 phase at the concentrations used, 250 ppm (72%), compared with non Biz-2Fr.3 treated control (41%). These experiments suggested that Biz-2Fr.3 induces G1-phase cell cycle arrest in DU145 cells. When these experiments were performed in LNCaP, we observed an accumulation of cells in the G2/M phase of the cell cycle (data not shown). Next, Biz-2Fr.3 inhibition of cell cycle regulatory proteins in prostate cancer cells was examined. Considering the essential role of cycling D/p27 in the G1/S transition, it was investigated whether the Biz-2Fr.3 induced accumulation of cells in G1 phase in the DU-145 was a direct modulation of cyclin D expression. To test this hypothesis, the protein expression levels of cycling D1 in DU145 prostate cancer cell lines following exposure to 250 ppm Biz-2Fr.3 for 36 h was measured. As observed low levels of cyclin D1 in DU145, was unaffected by Biz-2Fr.3 treatment. There was an increase in cycling D1 levels in LNCaP and a decrease in its level in PC-3 cells following Biz-2Fr.3 treatment (Figure 5). Since the AR functions as a driver of G1 progression through cross-communication with the cell cycle machinery and regulation of transcription of genes that control the G1-S transition the effect of Biz-2Fr.3 on AR levels was examined. Analysis of AR levels suggested that Biz-2Fr.3 regulation of cycling D1 levels might be dependent on AR levels since there was an apparent increase in its protein levels following Biz-2Fr.3 exposure (Figure 5). In DU-145 cells (which contains a hormone insensitive AR) the levels of AR was inhibited in the presence of Biz-2Fr.3. Taken together, these results suggest that AR is involved in Biz-2Fr.3 inhibition of prostate cancer cells.

Body weight changes in mice fed Biz-2Fr.3 for 21-days

Given that Biz-2Fr.3 has the potential to inhibit prostate



A



B

Figure 2. LC-MS Chromatogram of Biz-2Fr.3. Biz-2Fr.3 was analyzed using an Agilent 6210 Quadrupole LC-MS with an Agilent Ultra-2 fused silica capillary MS column (A) and the M/Z for each of the resulting peak was determined (B).

cancer cell growth, the pharmacological potential of the extract and its safety in C57BL/6 mice was evaluated. The effect of Biz-2Fr.3 on the hematology, kidney and liver profile of C57BL/6 mice was investigated. Acute

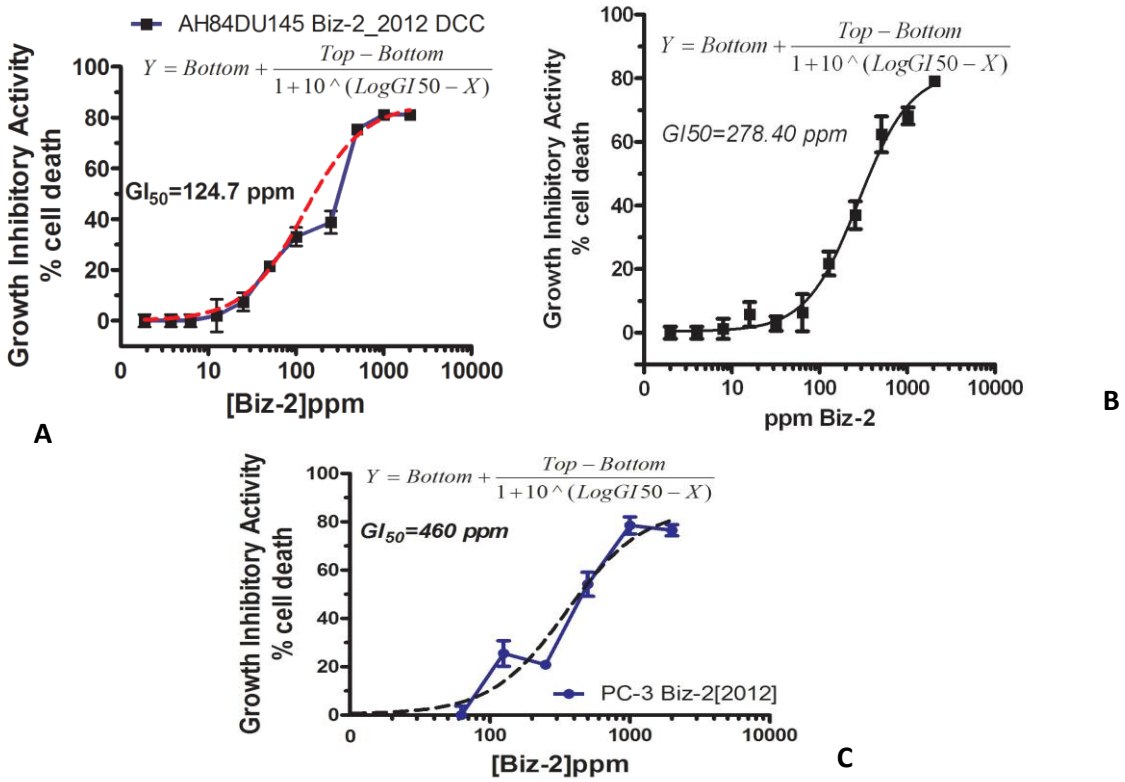


Figure 3. Growth inhibitory properties of Biz-2Fr.3 in prostate cancer cells. Prostate cancer cell lines were seeded at $1E^3$ or $1E^4$ cell /well in 96-well plates and then treated with increasing concentrations of Biz-2Fr.3. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described in materials and methods and growth inhibition assessed using the MTT assay. **A.** DU-145 cells; **B.** LNCaP cell; **C.** PC-3 cells.

toxicity study (LD50) of Biz-2Fr.3 was performed in 8 week old B57BL/6 mice using doses as high as 500 mg/kg. Animals were gavaged daily with 0, 10, 100 or 200 mg/kg body weight and changes in body weight, signs of toxicity, mortality, and general behaviors were observed. As observed no signs of toxicity or mortality up to the dose of 500 mg/kg was seen. However, there were partial decreases in activity and increase in respiratory rates in mice receiving 200 and 500 mg/kg of Biz-2Fr.3. The changes in body weight of mice over 21 days following oral gavage of Biz-2Fr.3 are shown in Figure 6. There was a significant increase in body weight in the treatment groups as compared to the control (Figure 6 (control, 22.22±0.117 g versus 200 ppm Biz-2Fr.3, 24.24±0.20 g; p<0.0001). The increase in overall body weight was more pronounced in the low dose group. The effect of Biz-2Fr.3 on organ reproductive and detoxification tissues were examined in mice after a 21-day treatment as shown in Table 1. We observed no significant (P>0.05) difference in the weight of the liver, kidney or brain of mice after Day 21. However, there were a significant (P<0.05) increase in wet weight of urogenital sinuous (control, 135.11 ug ± 5.12 g versus Biz-2Fr.3, 176.09 ± 25.20 g ug) after correcting for changes in body weight in the treatment group.

Clinical biochemistry of mice exposed to Biz-2Fr.3 for 21-days

There were no consistent significant differences in serum profiles between treated and control animals following Biz-2Fr.3 exposure. Table 2 shows the effect of Biz-2Fr.3 on hematological parameters in mice. After Day 21, there was a significant (P<0.01) effect on the total WBC in the treated group compared to the control group. There was a two-fold increase in the WBC (2.12.1±0.4 control versus 6.20±2.10) at the high doses of Biz-2Fr.3 throughout the study. There was also no significant (P>0.05) effect on mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), or mean corpuscular haemoglobin concentration (MCHC) in the treated groups as compared with the control group. However, there was a significance decrease in the platelet levels between the control group and treatment group. Administration of 100 mg/kg Biz-2Fr.3 for 21 days resulted in a 29% decrease in platelets (Control 1354 103 uL^{-1} versus 953 103 uL^{-1} in treatment group).

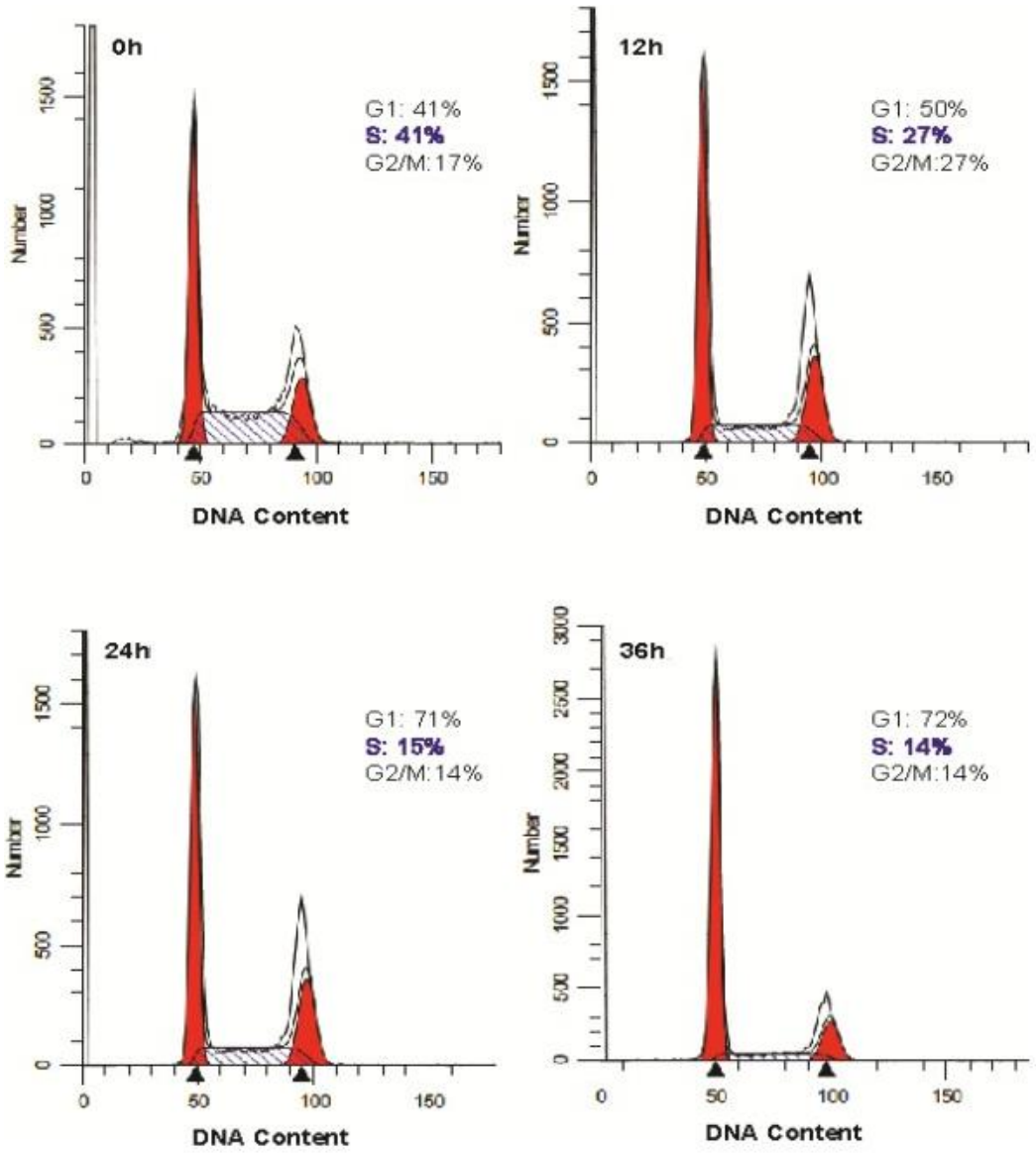


Figure 4. Time dependent effect of Biz-2fr.3 on cell cycle progression of du-145 human prostate cancer cells. Cells were cultured in complete medium and treated either with vehicle (0.5% DMSO in medium) or 250 ppm of Biz-2Fr3. After the indicated time of treatment, cells were harvested, washed with cold PBS buffer, and digested with RNase. Cellular DNA was stained with propidium iodide and flow cytometric analysis was done to determine the cell cycle distribution as described in the materials and methods.

Biochemical profile of kidney and liver of mice exposed to Biz-2Fr.3

The effects of Biz-2Fr.3 on kidney and liver functions are illustrated in Figure 7 after day 21 of daily gavage of Biz-2Fr.3 liver AST was significantly increased ($P<0.05$) in the treatment group as compared to the control (Group IV, 101.00±16.00 versus control, 47.33±1.86). There were also a significant ($P<0.05$) increase in ALT in Group II and IV with no significant change in the middle dose group (Group III). Alkaline phosphatase was markedly decreased in the entire treated group but not significantly ($P>0.05$) different from the control. There was no statistical significant ($P>0.05$) difference in glucose, urea, or creatinine levels.

DISCUSSION

Identification of prostate specific bioactivity

Bizzy Nut is generally recognized for its enriched caffeine

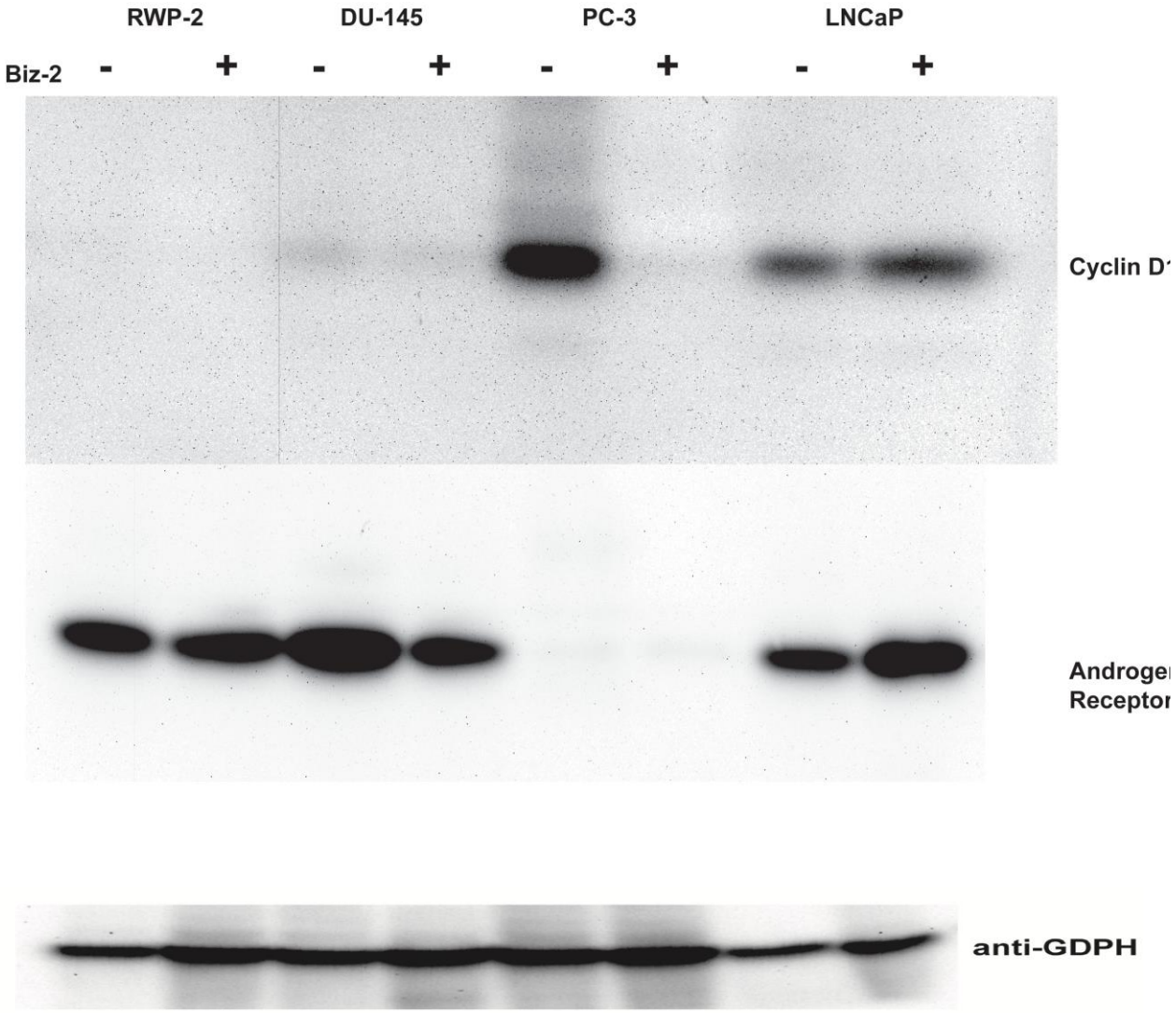


Figure 5. Regulation of G1 cell cycle regulatory proteins by Biz-2Fr.3 in DU145 Cells. The DU145 cells were cultured in complete medium and treated either with vehicle or 250 ppm Biz-2Fr.3 for 24 h, then subjected to SDS-PAGE followed by Western blot analysis, as described in materials and methods. Blot were probed with antibodies for cyclin D1, anti-AR and b-Actin.

constituents which are attributed to its reported bioactivity and therapeutic effects. In this study, solid-liquid extraction, using solvents of increasing polarity to identify and characterize medicinally relevant, putative anti-prostatic or anti--tumor compounds present in bizzy nut using a cancer cell inhibition assay screen was performed. The bizzy nut was sequentially extracted with 100% hexane, Biz-1; ether, Biz-2; acetone, Biz-3; methane, Biz-4 or water, Biz-5 using solid-liquid extraction. Our bioactivity drive isolation scheme used to isolate the tumor inhibitory activity (ether extract, Biz-2) resulted in the elimination of the major constituents of bizzy nut such as caffeine and tannin suggesting our tumor inhibitory activity is due to a unique compound. To characterize this new bioactivity, a liquid chromatography (LC) method was developed for fingerprinting and quantifying the anti-prostatic activity existing in Biz-2. Using this method, the chemical fingerprint of Biz-2Fr.3 was established, in which the separation of more than five analytes was accomplished in about 30 min, and 6 to 8 distinctive peaks were identified by LC/MS analysis. Characterization of the molecular ions re-showed the presence of the analyte, with m/z ranging from 120 to 225 m/z. Peaks two and three, on the other hand, contained a

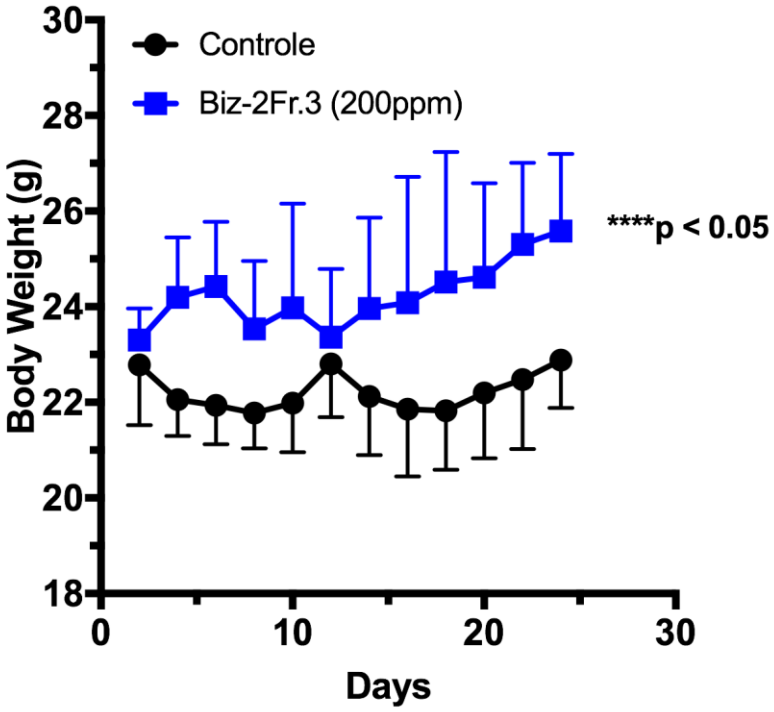


Figure 6. Change in body weight following chromic exposure of mice to Biz-2Fr.3. Mice were given 200 mg/kg body weight Biz-2Fr.3 orally every 24 h for 21 days. Changes in body weight were determined every 24 h and the results are presented as mean ± SEM of quintuple values. Two-tail-t-tests reveals significant difference at p<0.001 compared to the control after 21-days.

single molecular ion with m/z of 181. The LC-MS data suggested that we have three groups of compounds in the Biz-2Fr.3.

Mechanism of anticancer activity

In order to systematically evaluate the toxicity of the enriched fraction of Biz-2, (Biz-2Fr.3) towards target prostate cancer cells, a cell line panel representative of different stages of prostate disease was compiled. The data presented here suggests that the mechanism of toxicity induced by Biz--2Fr.3 is dependent on the cell phenotype. Also, it was observed that prostate cancer cells were more sensitive to Biz-2Fr.3 induced toxicity as compared to breast or neuronal cells lines (data not shown). The order of sensitivity of prostate cells toward Biz-2Fr.3 was DU-145, LNCaP, PC-3 and normal prostate cells. This differential sensitivity of prostate cancer cell lines to Biz-2Fr.3 could be explained in part due to the difference in molecular characteristics of the three-different prostate cancer cell lines tested. LNCaP cells with hormone -sensitive and p53-wild type confer sensitivity to Biz-2Fr.3. DU145 is resistance to Biz-2Fr.3 and is hormone-insensitive and has a p53-mutant. It will be investigated whether these phenotypes and other factors contribute to the mechanism of action of Biz-2Fr.3 in prostate cancer cells. Prostate cancer development is dependent on androgens, and majority of patients respond to androgen ablation. However, virtually every patient will develop hormone---resistant prostate cancer and can no longer respond to androgen deprivation therapy (ADT). Therefore, the need to identify chemo preventative compounds that does not result in androgen resistance is urgently needed. In this study, it was demonstrated that our Biz-2Fr.3 bioactive extract is more potent to androgen insensitive DU145, suggesting that this extract may be developed as a treatment hormone-resistant prostate cancer. To begin to understand the mechanism of Biz-2Fr.3 anti-proliferative activity in DU-145 cell, flow cytometry was used to determine where in the cell-cycle Biz-2Fr.3 induces proliferation blockage. Biz-2Fr.3 was able to induce a G1 blockage in a dose and time dependent manner. Control of cell cycle progression in PCa and other cancer cells is considered to be a potentially effective strategy for the control of tumor growth (Endrini et al., 2011; Osterburg et al., 2009). Molecular analysis of human cancers has revealed that cell cycle regulators are frequently mutated in most common malignancies and natural product

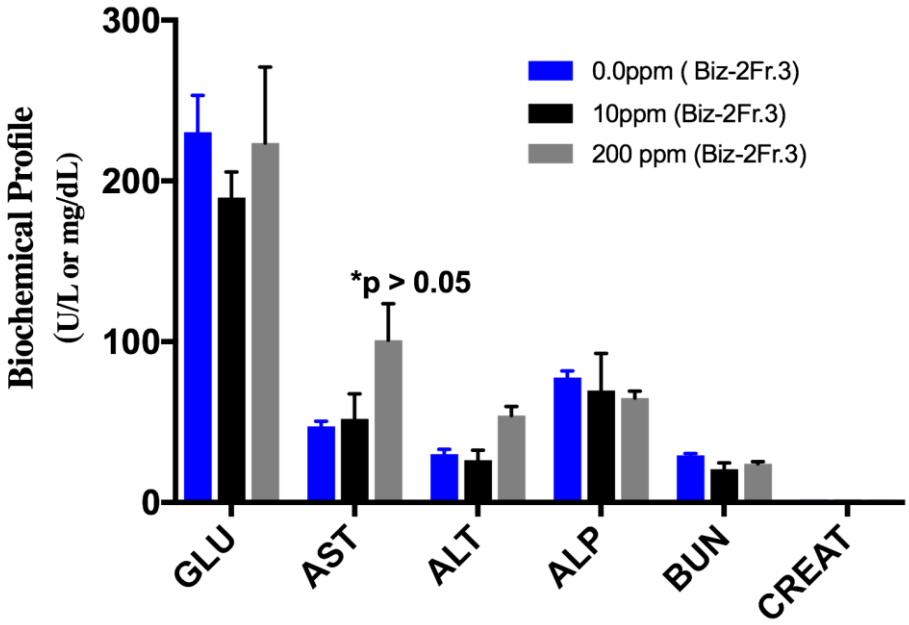


Figure 7. Biochemical profile of mice exposed to chromic consumption of Biz-2Fr.3. Mice were dosed orally with 0, 10, 200 ppm Biz-2Fr.3 daily for 21 days. At the end of the treatment period, blood was collected by cardiac puncture and used to prepare serum for the biochemical tests. The values of blood glucose (GLU), serum alanine aminotransferase (ALT), serum aspartate aminotransferase (AST), serum alkaline phosphatase (ALP), serum urea and serum creatinine were determined following standard laboratory procedures. * Significantly different at $p<0.05$ compared to the control.

tended to target the cell cycle pathway (Gioti and Tenta, 2015; Gunn et al., 2011; Rafi et al., 2002). The evaluation of ancient herbal medicines such as bizzy nut may indicate novel strategies for the treatment of prostate cancer, which remains the leading cause of cancer-¬-related deaths in American men. In our present investigation, it shown that a naturally occurring specific bioactive fraction of bizzy nut significantly inhibits the proliferation and reduces the viability of DU145 as well as LNCaP cells (Figure 1), which suggests that Biz-2Fr.3 may be an effective chemotherapeutic agent against both androgen-sensitive and androgen-insensitive prostate cancer cells. There is significant global exposure of humans to bizzy nut (usually an ethanolic extract of the nut) in the form of a flavoring ingredient and food coloring (Burdock et al., 2009; Agency's, 2011). In addition, oral exposure can be dated back to the late 19th century with no documented adverse side effects. Studies in animals on the effects of chronic (28 days) consumption of Bizzy nut and its active major constituent caffeine have been investigated in mice. These studies report that the chronic consumption of Bizzy nut and caffeine diets caused an apparent toxicity as side effects from a decrease in food intake and body weight and the observed effects are largely due to the high caffeine content (Umoren et al., 2009; Salahdeen et al., 2015; Moradi et al., 2016). Humans have consumed plant phytochemicals for an extensive period and so they are perceived to be reasonably safe. However, our phytochemical enrichment scheme may have concentrated unwanted chemical that may pose safety issues. Furthermore, Bizzy has been consumed in Jamaica for century without any reported side effect. Given that bizzy nut may have multi-¬-targeting properties along with relatively lower systemic toxicity, the compounds in Biz-2Fr.3 can offer significant therapeutic advantages for prevention and treatment of PCa in Jamaica (Lowe et al., 2014; Jamaica, 2006; Mitchell and Ahmad, 2006). Since that Biz-2Fr.3 has the potential to inhibit prostate cancer cell growth, the pharmacological potential of the extract and evaluate its safety in mice was evaluated. To examine whether Biz-2Fr.3 administration induces toxicity, for 5-week C56B mice with Biz-2Fr.3 for 21 days was gavage-fed. Biz-2Fr.3 administration (at 10 or 200 mg/kg body weight) produces a slight increase in body weight (Figure 6). At the time of sacrifice (at 8 weeks of age), there was no considerable difference in organ (liver, lung, kidney, and spleen) weights between Biz-2Fr.3 and control groups (Table 1). Additionally, the levels of creatinine ALP and alanine aminotransferase (ALT) in the sera were not increased by Biz-2Fr.3 administration (Figure 7). However, the activity of aspartate aminotransferase (AST) was significantly increased relative to the control.

Table 1. Relative organ weight of mice in the presence of Biz-2Fr.3Fr.3 over a 21 day period.

Organ	Group I (0.5%DMSO)	Group II (Biz-2Fr.3 10 mg/kg)	Group III (Biz-2Fr.3 100 mg/kg)	Group IV (Biz-2Fr.3 200 mg/kg)
Brain	378.33±13.31	372.75±51.142	450.25±34.55	344.25±57.55
Liver	1185.75±74.90	1364.14±100.94	1181.25±93.75	1312.16±72.01
Kidney	275.11±9.64	321.25±36.62	304.75±29.15	305.28±26.52
Prostate	135.33±5.50	204.25±5.37a	199.75±7.32a	176.09±25.62a
Stomach	366.51±43.13	294.25±25.78	316.33±70.46	335.52±14.15

Values are represented as mean ±SD of quintuple expressed in mg. Superscript letters differ significantly (aP<0.05) from the control.

Table 2. Hematological analysis of mice exposure to Biz-2Fr.3.

Parameters	Group I (Control, 0.2%DMSO)	Group II (Biz-2Fr.3 10 mg/kg)	Group III (Biz-2Fr.3 100 mg/kg)	Group IV (Biz-2Fr.3 200 mg/kg)
WBC (103/uL)	2.1±0.4	5.4	5.06±0.71	6.20±2.10
RBC (106/uL)	7.545±0.29	8.51±0.48	8.36±0.41	6.77±0.93
HGB (g/dL)	11.10±0.50	12.66±0.73	9.2±3.8	10.15±4.30
HCT (%)	38.80±1.30	42.13±2.20	41.76±1.58	35.30±0.85
MCV (fL)	51.40±0.30	49.50±0.25	49.96±0.61	52.25±0.30
MCH (pg)	14.75±0.05	14.86±0.08	15.00±0.15	15.00±0.30
MCHC (g/dL)	28.65±0.35	30.00±0.20	30.06±0.27	28.75±0.05
CHCM (g/dL)	26.05±0.25	27.33±0.08	27.10±0.27	25.95±0.35
RDW (%)	13.25±0.35	12.70±0.15	14.00±0.23	14.00±0.20
PLT (103/uL	1354.00±211	1145.00±10.69a	953.00±68.87b	1125.50±203a
MPV (fL)	6.00±0.20	6.10±0.25	5.86±0.12	7.45±1.35

Values are represented as mean ±SEM of triplicates. Values on the same row followed by superscript letters differ significantly (a, P<0.01; b, P<0.001) from the control. WBC, differential leukocyte count; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume, MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, Red cell distribution width; PLT, Platelets; MPV, Mean platelet volume.

As AST is not a liver specific enzyme a high level of this enzyme can also be released from skeletal and cardiac muscle or red blood cells (Etuk and Muhammad, 2010). These results indicate that the chronic administration of Biz-2Fr.3 (10 or 200 mg/kg/day) was not toxic to the kidney or liver in mice. In the present study, the inhibition of prostate cancer cell growth and cell cycle progression are observed at 120 ppm Biz-2Fr.3 concentrations. It is hard to predict whether such concentration of Biz-2Fr.3 was achievable in the in vivo studies in the absence of pharmacokinetic data. Nonetheless, the Biz--2 concentrations used in the present study are within the range employed in previous studies to document cellular effects of this natural product (Salahdeen et al., 2015). In summary, the present study identified a bioactive component of bizzy nut that suppresses the growth of androgen--responsive (LNCaP) as well as androgen-independent (DU145) human prostate cancer cells in association with G1 phase cell cycle arrest. The Biz-2Fr.3-mediated cell anticancer activity is not associated with any sign of toxicity in normal prostate cells or in mice. Collectively, these results suggest that the Biz-2Fr.3 fraction of bizzy nut should be seriously considered for further investigation to determine its possible chemopreventive and/or therapeutic efficacy against prostate cancer in humans. A sample of finely ground bizzy root was sequentially extracted with solvents of increasing polarity and the resulting ether extract (Biz-2) was fractionated on a reversed phase high performance liquid chromatography (RP-HPLC) (A). Growth-inhibitory activity (GI50) of each fraction were determined (B) and spectra of each peak was monitored from 190 to 400 nm to identify the number of putative bioactive compounds in each peak (C).

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Agency's EM (2011). Committee on Herbal Medicinal Products (HMPC) Assessment report on Cola nitida (Vent.) Schott et Endl.

and its varieties and Cola acuminata (P. Beauv.) Schott et Endl., semen" 2011. Available at: http://docplayer.net/41584067-Assessment-report-on-cola-nitida-vent-schott-et-endl-and-its-varieties-and-cola-acuminata-p-beauv-schott-et-endl-semen.html

American Cancer Society (ACS) (2016). Cancer Facts & Figures 2016. Atlanta, Ga. American Cancer Society, 2016.

American Cancer Society (ACS) (2013). Cancer Facts & Figures for African Americans 2013-2014. American Cancer Society, 2016. Available at: https://www.cancer.org/content/dam/cancer-org/research/cancer-facts-and-statistics/cancer-facts-and-figures-for-african-americans/cancer-facts-and-figures-for-african-americans-2013-2014.pdf

Burdock GA, Carabin IG, Crincoli CM (2009). Safety assessment of kola nut extract as a food ingredient. Food Chem. Toxicol. 47(8):1725-1732.

Dahan K, Fennal M, Kumar NB (2008). Lycopene in the prevention of prostate cancer. J. Soc. Integr. Oncol. 6(1):29-36.

Deep G, Kumar R, Jain AK, Dhar D, Panigrahi GK, Hussain A, Agarwal C, El-Elimat T, Sica VP, Oberlies NH, Agarwal R (2016). Graviola inhibits hypoxia-induced NADPH oxidase activity in prostate cancer cells reducing their proliferation and clonogenicity. Sci. Rep. 6:23135.

Endrini S, Jaksa S, Marsiati H, Othman F, Rahmat A (2011). Effects of cola nut (Cola nitida) on the apoptotic cell of human breast carcinoma cell lines. J. Med. Plants Res. 5(11):2393-2397.

Etuk E, Muhammad A (2010). Safety evaluations of aqueous stem bark extract of Lophira lanceolata in Sprague dawley rats. Int. J. Res. Pharm. Sci. 1(1):28-33.

Fontenot K, Naragoni S, Claville M, Gray W (2007). Characterization of Bizzy Nut extracts in estrogen-responsive MCF-7 breast cancer cells. Toxicol. Appl. Pharmacol. 220(1):25-32.

Gibson TN, Hanchard B, Waugh N, McNaughton D (2011). Thirty-year trends in incidence and age-distribution of prostate cancer in Kingston and St Andrew, Jamaica, 1978-2007. West Indian Med. J. 60(1):9-12.

Gioti K, Tenta R (2015). Bioactive natural products against prostate cancer: mechanism of action and autophagic/apoptotic molecular pathways. Planta Med. 81(7):543-62.

Gunn EJ, Williams JT, Huynh DT, Iannotti MJ, Han C, Barrios FJ, Kendall S, Glackin CA, Colby DA, Kirshner J (2011). The natural products parthenolide and andrographolide exhibit anti-cancer stem cell activity in multiple myeloma. Leuk. Lymphoma 52(6):1085-1097.

Howlader NNA, Krapcho M, Miller D, Bishop K, Kosary CL, Yu M, Ruhl J, Tatalovich Z, Mariotto A, Lewis DR, Chen HS, Feuer EJ, Cronin KA (2017). SEER Cancer Statistics Review, 1975-2014. Available at: https://seer.cancer.gov/csr/1975_2014/

Kallifatidis G, Hoy JJ, Lokeshwar BL (2016). Bioactive natural products for chemoprevention and treatment of castration-resistant prostate cancer. Cancer Biol. 40:160-169.

Kamatenesi-Mugisha M, Oryem-Origa H (2005). Traditional herbal remedies used in the management of sexual impotence and erectile dysfunction in western Uganda. Afr. Health Sci. 5(1):40-49.

Kumar NB, Pow-Sang J, Spiess PE, Park J, Salup R, Williams CR, Parnes H, Schell MJ (2016). Randomized, placebo-controlled trial evaluating the safety of one-year administration of green tea catechins. Oncotarget 7(43):70794-70802.

Kumar NB, Besterman-Dahan K, Kang L, Pow-Sang J, Xu P, Allen K, Riccardi D, Krischer JP (2008). Results of a Randomized Clinical Trial of the Action of Several Doses of Lycopene in Localized Prostate Cancer: Administration Prior to Radical Prostatectomy. Clin. Med. Urol. 1:1-14.

Lowe HI, Watson CT, Badal S, Peart P, Toyang NJ, Bryant J (2014). Promising Efficacy of the *Cola acuminata* Plant: A Mini Review. Adv. Biol. Chem. 4(4):6.

Luk SC, Siu SW, Lai CK, Wu YJ, Pang SF (2005). Cell Cycle Arrest by a Natural Product via G2/M Checkpoint. Int. J. Med. Sci. 2(2):64-69.

Mantena SK, Sharma SD, Katiyar SK (2006). Berberine, a natural product, induces G1-phase cell cycle arrest and caspase-3-dependent apoptosis in human prostate carcinoma cells. Mol. Cancer Ther. 5(2):296-308.

Maru GB, Hudlikar RR, Kumar G, Gandhi K, Mahimkar MB (2016). Understanding the molecular mechanisms of cancer prevention by dietary phytochemicals: From experimental models to clinical trials. World J. Biol. Chem. 7(1):88-99.

Mitchell S, Ahmad M (2006). A Review of Medicinal Plant Research at the University of the West Indies, Jamaica, 1948-2001. West Indies Med. J. 55:243-269.

Moradi MT, Karimi A, Alidadi S (2016). *In vitro* antiproliferative and apoptosis-inducing activities of crude ethyle alcohole extract of *Quercus brantii L.* acorn and subsequent fractions. Chin. J. Nat. Med. 14(3):196-202.

Osterburg A, Gardner J, Hyon SH, Neely A, Babcock G (2009). Highly antibiotic-resistant *Acinetobacter baumannii* clinical isolates are killed by the green tea polyphenol (-)-epigallocatechin-3-gallate (EGCG). Clin. Microbiol. Infect. 15(4):341-346.

Rafi MM, Vastano BC, Zhu N, Ho CT, Ghai G, Rosen RT, Gallo MA, DiPaola RS (2002). Novel polyphenol molecule isolated from licorice root *(Glycrrhiza glabra)* induces apoptosis, G2/M cell cycle arrest, and Bcl-2 phosphorylation in tumor cell lines. J. Agric. Food Chem. 50(4):677-684.

Salahdeen HM, Omoaghe AO, Isehunwa GO, Murtala BA, Alada AR (2015). Gas chromatography mass spectrometry (GC-MS) analysis of ethanolic extracts of kolanut (*Cola nitida*) (vent) and its toxicity studies in rats. J. Med. Plants Res. 9(3):56-70.

Singh RP, Agarwal R (2006). Mechanisms of action of novel agents for prostate cancer chemoprevention. Endocr. Relative Cancer 13(3):751-778.

Solipuram R, Koppula S, Hurst A, Harris K, Naragoni S, Fontenot K, Gray W (2009). Molecular and biochemical effects of a kola nut extract on androgen receptor-mediated pathways. J. Toxicol. 2009:530279.

Stahl S, Chun TY, Gray WG (1998). Phytoestrogens act as estrogen agonists in an estrogen-responsive pituitary cell line. Toxicol. Appl. Pharmacol. 152(1):41-48.

Tilley C, Deep G, Agarwal C, Wempe MF, Biedermann D, Valentová K, Kren V, Agarwal R (2016). Silibinin and its 2,3-dehydro-derivative inhibit basal cell carcinoma growth via suppression of mitogenic signaling and transcription factors activation. Mol. Carcinogen. 55(1):3-14.

Torre LA, Siegel RL, Ward EM, Jemal A (2016). Global Cancer Incidence and Mortality Rates and Trends--An Update. Cancer Epidemiol. Biomark. Prev. 25(1):16-27.

Umoren EB, Osim EE, Udoh PB (2009). The comparative effects of chronic consumption of kola nut (Cola nitida) and caffeine diets on locomotor behaviour and body weights in mice. Niger. J. Physiol. Sci. 24(1):73-78.

Varma A, Padh H, Shrivastava N (2011). *Andrographolide:* a new plant-derived antineoplastic entity on horizon. Evid. Based Complement. Altern. Med. 2011:815390.

Washington W, Hubert L, Jones D, Gray WG (2001). *Bisphenol a binds to the low-affinity estrogen binding site.* In vitro Mol. Toxicol. 14(1):43-51.

Wong HC, Sagineedu SR, Lajis NH, Loke SC, Stanslas J (2011). *Andrographolide induces cell cycle arrest and apoptosis in PC-3 prostate cancer cells.* Afr. J. Pharm. Pharmacol. 5(2):225-231.

Yan J, Chen Y, He C, Yang ZZ, Lü C, Chen XS (2012). Andrographolide induces cell cycle arrest and apoptosis in human rheumatoid arthritis fibroblast-like synoviocytes. Cell Biol. Toxicol. 28(1):47-56.

Journal of Medicinal Plant Research

Related Journals Published by Academic Journals

- *African Journal of Pharmacy and Pharmacology*
- *Journal of Dentistry and Oral Hygiene*
- *International Journal of Nursing and Midwifery*
- *Journal of Parasitology and Vector Biology*
- *Journal of Pharmacognosy and Phytotherapy*
- *Journal of Toxicology and Environmental Health Sciences*

academicJournals