Plant Archives Vol. 20, Supplement 1, 2020 pp. 3285-3291 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

BIOASSAY-GUIDED APPROACH EMPLOYED TO ISOLATE AND IDENTIFY ANTICANCER COMPOUNDS FROM *PHYSALIS PERUVIANA* CALYCES

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Abstract

This work aimed to maximize the knowledge on the use of natural bioactive compounds, isolated from fruit wastes, in human therapy. Fruit wastes have been long scrutinized in the quest of cancer treatment since they are cheap, safe, active, and possess no side effects. *Physalis peruviana* fruit is highly appreciated for its therapeutic value. Extensive research has been carried on the fruit of *P. peruviana* with few studies focusing on its by-products (calyces). In the present work, *P. peruviana* calyces were subjected to phytochemical screening, including; total phenols, total flavonoids and HPLC analyses for phenolic compounds. Besides, the anticancer activity of *P. peruviana* calyces extracts against hepatocellular carcinoma cell line (HepG2) was evaluated. Results showed that ethyl acetate (EtOAc) extract of *P. peruviana* exhibited a potent anticancer effect against HepG2. The EtOAc fraction was subjected to bioactivity guided fractionation on sephadex column LH-20 to five subfractions. Among these fractions, F5 was promising as an anticancer agent, two compounds (rutin and oleanolic acid) were isolated and their structure was elucidated by spectral analysis. Oleanolic acid was obtained from this plant for the first time from this species. The present investigation suggests the possibility of extraction of bioactive molecules from *P. peruviana* fruit waste that are promising in the management of liver cancer.

Key words : P peruviana; calyces; cytotoxicity; HepG2; caspases.

Introduction

Plants and herbs represented a crucial source for preparation of new drugs for the sake of treatment of various ailments from the ancient time. Recently there is a growing interest in using plant byproducts for therapeutic purposes due to their potentiality, availability and low cost in comparison to modern therapeutic drugs. Plant byproducts are gaining greater importance as they are used for the treatment of dangerous diseases such as cancer and Alzheimer (Mossa *et al.*, 2015; El Gengaihi *et al.*, 2016a,b; Salem *et al.*, 2016).

In this sense, Physalis species is considered as an important therapeutic plant that is popular worldwide. Physalis species that lacks scientific scrutiny is *P peruviana*, commonly known as Cape gooseberry, and its fruit belongs to the Solanaceae family. Recent research has shown that *P peruviana* is a promising source of phytochemicals. Different parts of the plant show antioxidant (Wu *et al.*, 2004, Puente *et al.*, 2010, Ramadan 2012, Valdenegro *et al.*, 2012,), anti-inflammatory (Wu *et al.*, 2006; Chang *et al.*, 2008; Arun and Asha 2007; Gastro *et al.*, 2015) and antiproliferative effects on hepatoma cells (Wu *et al.*, 2005). In addition, the fruit has excellent potential as a food-based strategy for anti-diabetic and anti-hypertensive products (Pinto *et al.*, 2009). *P. peruviana*, possesses anticancer (Yen *et al.*, 2010), antibacterial, antipyretic, and immunomodulatory effects, and has been used for the treatment of malaria, asthma, hepatitis, dermatitis, and rheumatism (Pietro *et al.*, 2000; Soares *et al.*, 2003).

Chemical studies on *P. peruviana*, showed the presence of withanolides, steroids, alkaloids, and glycosides (Wu *et al.*, 2006). Many chemical compounds;

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viz. 28-hydroxywithanolide, withanolides, phygrine, kaempferol, and quercetin di- and tri-glycosides are reported to be present in *P. peruviana* (Keith *et al*., 1992). Phytochemical studies made on *P. peruviana* calyces reported the presence of flavonoids, steroids, triterpenes, and lactones. The oleaginous fruit by-products may become one of the important oil sources. The oil is rich in essential fatty acids, natural antioxidants and phytosterols (Ramadan and Moersel, 2003).

The experimental studies of *P. peruviana* have been focused on the chemical and biological properties of the whole plant, leaves, and fruits, with only a few reports on calyces. Hence, the present work focused on physalis calyces in order to maximize its value and to test its potentials in the management of liver cancer. The aim of the present study could be considered the first trial targeting the study of anticancer activity of calyces.

Materials and Methods

Phytochemical screening

The presence of carbohydrates, tannins, alkaloids, flavonoids, phenols, saponins, triterpenoid and coumarins in the ethanolic extract has been reported by Harborne (1973).

Quantification of flavonoids and phenols

The total phenolic and Flavonoid content were determined according to Zilic *et al.,* (2012).

Identification of Phenolic and Flavonoid Compounds

The phenolic and flavonoid compounds of *P. peruviana* were extracted according to the method described by Hakkinen *et al.,* (1998) and Mattila *et al.* (2000). HPLC analysis was carried out according to Kim *et al.,* (2006) with slight modifications using an Agilent Technologies 1100 series liquid chromatograph equipped with an auto sampler and a diode-array detector. The analytical column was Agilent Eclipse XDB C18 (150 x4.6 µm; 5 µm) with a C18 guard column. The analytical column was ZORBAX Eclipse XDB C18 column (15 cm x 4.6 mm I.D., 5 µm, USA).

Extraction, fractionation, isolation and identification

Air-dried and powdered Calyces were extracted successively with hexane, dichloromethane, ethyl acetate, and methanol with gradual increase in the polarity by percolation at room temperature until exhaustion of the material. All extracts were evaporated under vacuum and concentrated in a rotary evaporator.

The ethyl acetate part which exhibited powerful anticancer activity against HepG2, was evaluated using HPLC and chromatographed over sephadex LH-20 column by elution with 50 to 100 % methanol. On the basis of TLC behavior, the appropriate fractions were combined to give five main fractions which were tested for inhibitory activity against Hep-G2. The best fraction was subjected to sephadex LH-20 column by elution with 50 to 100 % methanol to obtain two major compounds.

General Experimental Procedures

NMR spectra were acquired on a Varian Mercury 400 MHz spectrometer at 400 (^{1}H) and 100 (^{13}C) MHz in $CDCl_3$ and DMSO using the residual solvent as an internal standard. Multiplicity determinations (DEPT) and 2D-NMR spectra (HMQC, HMBC, NOESY) were obtained using standard Bruker pulse programs.

Anticancer activity

Cytotoxicicity test

Hepatocellular carcinoma cells (HepG2) was supplied by Naval American Research Unit-Egypt (NAmRU). Cytotoxicity against cancer cells was assessed by MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay (Mosmann 1983). The percentage of change in viability was calculated according to the formula:

[(Reading of extract / Reading of negative control) - 1)] x 100

Caspase-3/8/9 activity

Caspase activity was determined using Caspase-3/ 8/9 Colorimetric Assay Kit as per manufacturer's protocol (GenScript, USA). Seeding of HepG2 cells was done at a density of 4 x 106 cells/mL and followed by treatment with DTN (IC_{50}c for 12h, 24h, 48h and 72 h). At the same time, negative control was set. Resuspension of the cells was done in cold lysis buffer followed by incubation on ice for 1 h. After centrifugation, 10 ìL of supernatant was used to assay the protein concentration by Bradford method. Equal amounts of protein were mixed with 2X reaction buffer and 200 ìM DEVD-pNA substrate (caspase-3)/ IETD-pNA substrate (caspase-8)/ LEHD-pNA substrate (caspase-9) and further incubated at 37°C for 4h, away from light. The plates were read at 405 nm.

Statistics

All results were expressed as mean value of three replicates. Data were statistically analyzed through analysis of variance (ANOVA) and Duncans test using Co-Stat Statistics Software, where unshared letters were considered significant at $P \leq 0.05$.

Result and discussion

Phytochemical screening

The qualitative phytochemical analysis of *P. peruviana* L. calyces extract showed the presence of biologically active phytoingredients such as tannins, flavonoids, glycosides, sterols, saponins, coumarin and alkaloids (Table 1). The medicinal value of this plant mainly depended on phytocompounds that produced definite biological actions on human and these constituents were produced to defend the plant from the environment.

Flavonoids and phenol Content

In addition, the phenolic and flavonoid contents in the calyces extract were found to be 120±0.66 mg/g and 254±1.45 mg/g, respectively (Table 2). The extraction yield of total phenolic content relied on solvent type and polarity (Xu *et al.,* 2014), harvesting time, ripening stage (Valdenegro *et al.,* 2012), organ, growth stage when harvested, storage after harvesting, geographic place of production and the ripening stage of the fruit (Bravo *et al.,* 2015), as well as after defense mechanisms against stress factors and pathogens (Acosta-Estrada *et al.,* 2014; Allam *et al.,* 2018). According to other findings, phenolic content of *P. peruviana* fruits had been studied at different harvesting time, showing a content range from 0.77 to 0.59 g GAE/ kg (Li *et al.,* 2014).

Good amounts of phenolic compounds were estimated in *P. peruviana* juices, wherein the levels of total phenols varied from 6.09 to 6.30 mg/100 g juice as caffeic acid equivalents, highest content in total flavonoids (226.19 ± 4.15 mg/g) and phenols (100.82 ± 6.25 mg/g), quercetin is the main phenolic compound, followed by myricetin and kaempferol (Hδkkinen *et al.,* 1999). Cardona *et al.,* (2017) evaluated the effect of different extraction processes of *P. peruviana* calyces extract on rutin content and total phenolic content, where the results showed that the optimal conditions were the use of ethanol 70%, and a percolation time of 72 hours.

Table 1: Phytochemical screening of *P. peruviana* calyces alcoholic extract.

	Alcoholic extract
Tannins	++
Flavonoids	+++
Glycosides	+
Alkaloids	+++
Saponin	++
Sterols and triterpenes	+
Coumarin	+++

Table 2: Total Flavonoids and phenol content in alcoholic extract of *P. peruviana*calyces.

	Alcoholic extract
Total Flavonoids	254±1.45 mg/g
Total phenolic	120±0.66 mg/g

Analysis of phenolic compounds using HPLC

The main flavonoids found in *P. peruviana* calyces were Hespirdin (32760.00 ppm) followed by rutin (18412.17 ppm**),** while the main phenolic acids were vanillic acid followed by ellagic acid (30790.35 ppm and 19748.74 ppm, respectively) (Table 3). In *P. peruviana* fruits, quercetin was the main phenolic compound, followed by myricetin and kaempferol (Hδkkinen *et al.,* 1999). Previous reports indicated the presence of kaempferol, rutin, quercetin, myricetin, Apg 6 glucose 8 rhamnose, Luteo 7 glucose, Narengin, Apig 7 O neohespiroside, Kamp3-7 di-rhamnoside and Kamp3(2-p-manryl) glucose (747.27, 913.95, 3023.39, 1260.17, 468.32 and 4742.23, respectively). For further investigations, different extraction methods and types of solvents could be studied for *P. peruviana* calyces. The extraction efficiency may vary depending on extraction techniques resulting in different patterns of the bioactive ingredients that may affect the capacity of the pharmacological properties.

Cytotoxic activity

The effect of the *P. peruviana* calyx's fractions and EtOAc sub-fractions on HepG2 cells were evaluated using MTT assay. The cells were treated for 24 h with different concentrations (10- 100 µg/mL). Data were presented in Figure 1. Ethyl acetate fraction showed a high cytotoxic activity where the IC_{50} was 25µg/mL, while the methanol and chloroform fractions exhibited weak cytotoxic activity with IC_{50} > 100 µg/ mL. Hexane fraction was non-cytotoxic where the IC_{50} was 200 µg/ mL. The cytotoxic EtOAc fraction was fractionated into five sub-fractions and the cytotoxic activity was tested, then the caspase activity of the treated HepG2 cells was determined (Figure 2). All sub-fractions induced caspase activity that was supposed to induce apoptosis, except f2, in a percentage exceeding that of the ethyl acetate itself. Sub-fraction F1 induced caspase activity in a percentage of 257 while, F3, F4, and F5 increased the activity by approximately 100 %. Therefore, a bioassay-guided approach was employed to isolate the bioactive compounds responsible for the activity from F1 (rutin and oleanolic acid, Figure 3).

Cytotoxic effects of *P. peruviana* whole plant, leaves, and stems on different cell lines such as leukemia, breast, colon, lung, and liver cancers were also investigated (Wu *et al.,* 2009). Cakir *et al.,* (2014) suggested that *P.*

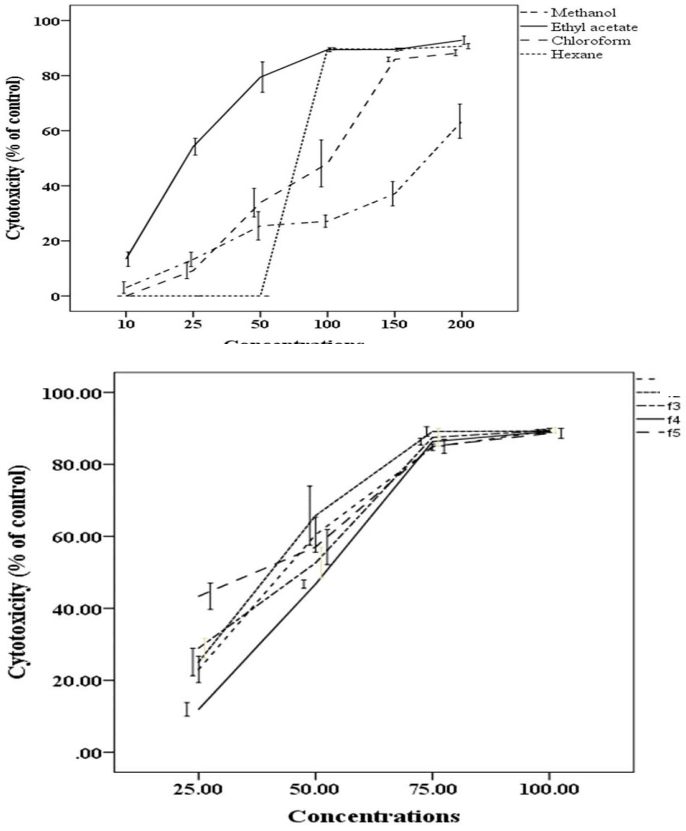

Fig. 1: Cytotoxic activity of *P. peruviana* fraction (A) and subfraction (B) against HepG2 treated for 24h at various concentrations of Data were expressed as percent of control ± SE (n=3).

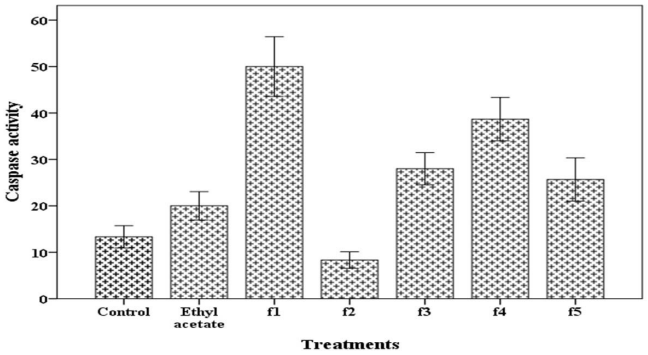

Fig. 2: The induction of Caspases 3, 8, 9 of HepG2 cells treated with EtOAc subfractions.

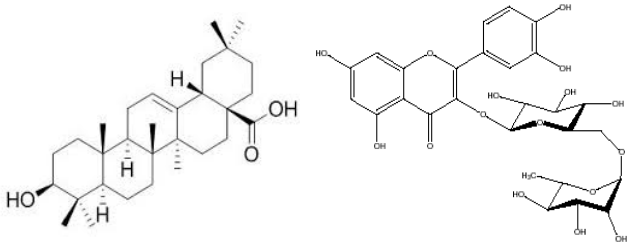

Fig. 3: Isolated compounds from the most active subfraction a) Rutin b) Oleanolic acid.

peruviana ethanolic extract of shoot and leaf can induce apoptotic cell death against HeLa cells when applied by100 µg/mL. Studies performed on fruits (Mehmet and Hameaz-Koocabaa 2014) observed that IC_{50} values of *P. peruviana* fruit for HT-29, Hep3B, SaOS-2 and SH SY5Y cell lines were evaluated as 40.79, 24.92, 15.44, and 44.24 µg/ml, respectively. Another study on *P. peruviana* fruit showed high cytotoxic potential in L929 and HeLa through cell inhibition and blocking cytokines (Giraldo, *et al.*, 2017). Wu *et al*., (2004) observed that *P. peruviana* fruits' crude extract was more cytotoxic than the extract from the whole plant.

Identification of isolated compounds from F1

For compound (1), the 1D and 2D NMR data showed the presence of 27 carbons and 2 carbohydrate units, where their anomeric protons were at δ 4.4 and 5.4 ppm, while their carbons showed up at δ 101, and 102 ppm. The DEPT 135 showed the presence of 5 doublet aromatic carbons at δ 93, 99, 115, 116, and 121. The carbon showed the presence of one carbonyl carbon at δ 177 and 7 oxygenated sp2 carbons at δ 133, 144, 148, 156, 157, 161, and 164. This data suggested the presence of flavonoid glycosides similar moiety. Searching the literature for the previously reported compounds from this species showed that compound (1) was quercetin-3-*O*-rutinoside, also known as rutin and rutoside, where the ^{1}H and ^{13}C NMR showed agreement with results of Toro *et al.,* (2014).

Concerning compound (2), the 1D and 2D NMR data showed the presence of 31 carbons. The carbon and DEPT 135 showed the presence of one carbonyl carbon at δ 183 and 2 sp2 carbons at δ 123 and 144, respectively, and one oxygenated sp3 carbon at δ 79, and 2 sets of germinal methyl carbons, where the first set showed up at δ 16 and 24, while the second set showed up at δ 23 and 33. This data suggested the presence of triterpenes similar moiety. Searching the literature for the previously reported compounds from this species and genus, it was found that compound (2) was Oleanolic acid, which has been previously identified from the genus but not from the species. The ^{1}H and ^{13}C NMR showed agreement with that reported by Seebacher *et al*. (2003). This was the first time to isolate oleanolic from *P. peruviana* that was previously isolated from another physalis species (Shim *et al.*, 2002; Li *et al.*, 2012).

It was found that phenolic compounds such as flavonoids had variety of medicinal activities because of their structure. They showed to be very efficient scavengers of free radicals (ElGengaihi *et al*., 2014; 2015) with hepato-protective properties (ElGengaihi *et al.*, 2016a;b). They also showed to decrease adverse effects

Table 3: Polyphenolic content in *P. Peruviana* calyces using HPLC.

Phenolic compound	µg/g	Flavonoids	µg/g
Pyrogallol	6712.11	Luteo 6 arbinose 8 glucose	13726.14
Gallic	171.02	Luteo 6 glucose 8 arabinose	677.58
4-aminobenzoic	111.11	Apg 6 arabinose 8 glucose	1310.53
Protocatchuic	1071.93	Apg 6 rhamnose 8 glucose	1179.99
Catechin	483.68	Apg 6 glucose 8 rhamnose	747.27
Catechol	2266.22	Luteo 7 glucose	913.95
Epicateachin	1099.38	Narengin	3023.39
P-OH benzoic	614.35	Hespirdin	32760.00
Caffeine	1558.67	Rutin	18412.17
Chlorogenic	1394.35	Apig 7 O neohespiroside	1260.17
Vanillic	874.53	Kamp3-7 di-rhamnoside	468.32
Caffeic	206.79	Quercetrin	1381.67
P-coumaric	255.03	Rosmaniric	245.26
Ferulic	652.43	Quercetin	202.45
Iso- ferulic	35.15	Narengenin	276.46
E-vanillic	30790.35	Kamp3(2-p-manryl)glucose	4742.23
Alpha-coumaric	187.84	Hespertin	945.52
Ellagic	19748.74	Kampferol	83.33
3,4,5 methoxy cinnamic	2757.11	Rhamnetin	153.08
Coumarin	221.6	Apigenin	114.71
Cinnamic	50.15	Apigenin 7 glucose	165.06
Salycilic	2259.3	Acacetin	1348.86

of pesticides (Mossa *et al.,* 2015), and to acquire antihypertensive (Salam *et al.,* 2016), antiinflammatory (Ibrahim *et al.,* 2016), antibacterial (Abou Baker *et al.,* 2020) and cholinesterase inhibition (El Gengaihi *et al.,* 2017) activities.

In agreement with results obtained from the present study, it has been reported by literature that rutin and oleanolic acid showed anticancer activities and activated caspase activities which may lead to the induction of apoptosis in different cancer cell lines (Tsai and Yin, 2008; Ramos *et al.,* 2008). Such effects could be due to the reduced oxidative stress that is known to play an important role in inflammatory carcinogenesis (Ibrahim *et al.,* 2016).

Conclusion

In conclusion, limited number of reports, over the past decades, has been done for the evaluation of *P. peruviana* calyces, their bio-active constituents and their mechanisms of action. The Results introduced by the present study could be encouraging for further investigation regarding the bioactive constituents of *P. peruviana* calyces as an anticancer plant byproduct in particular.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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