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Phthalate derivatives are naturally occurring in *Arum Palaestinum*

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KEYWORDS

Arum palaestinum, phthalates, HPLC, environmental

A B S T R A C T

Arum palaestinum Boiss, a wild edible plant in Palestine, is one of about 26 species of the arum genus which are flowering plants belonging to Araceae. The plant is widely used in Traditional Arabic Palestinian Herbal Medicine (TAPHM) in the treatment of several human diseases mainly cancer. This investigation reports the separation and identification of phthalate compounds such as dipropyl phthalate, diisobutyl phthalate and di-n-octyl phthalate for the first time from the extract of *Arum palaestinum*, using HPLC and GCMS techniques. In addition to these finding the amounts of phenolics, flavonoids and phthalate contents were evaluated in the whole plant. Total phenolics were found to be 3.9µg/mg pyrocatechol equivalent, and total flavonoids were found to be 270µg/mg as quercetin equivalent. One of the interesting results of this study also was the evaluation of phthalates in different parts of the plant. Average values of phthalates in the fresh plant parts were (36.3, 26.1, 5.6, 7.0µg/g) in leaves, inflorescence, stem and inflorescence base respectively. In addition, the average values of phthalates in different areas of Nablus region for whole plant were 48.3, 53.0, 50.9 µg/g. The findings of this study is discussed with relation to medicinal and food uses of the plant in Palestinian communities.

Introduction

Phthalates are high-production-volume synthetic chemicals with ubiquitous human exposures because of their use in plastics and other common consumer products. Globally, more than 18 billion pounds of phthalates and phthalate ester (PE), including di-(n-butyl)-phthalate (DBP) and di-(2-ethylhexyl)-phthalate (DEHP) are used each year, primarily as plasticizers in flexible polyvinyl chloride (PVC) products (Blount et al., 2000a).

Although phthalate derivatives are harmful chemicals which are regarded as environmental health hazards and is considered to be a potential carcinogen, teratogen, and mutagen due to their toxicity (Heudorf et al., 2007; Fushiwaki et al., 2003; Fatoki et al., 2010), they are used in a wide variety of products such as paints, adhesives, inks and cosmetics (Ling et al., 2007; Huang et al., 2008). As a result synthetic phthalate esters are widely become

distributed and detected in the environments, such as sediments, natural waters, soils, plants, and aquatic organisms (Sultan et al., 2010; Staple et al., 1997; Yuan et al., 2008). Different studies showed that phthalate ester (PE) has been detected in water samples from various aquatic environments (Hashizume et al., 2002), as well as in bacteria, plants and the fatty acid fractions of certain species of marine macro-algae (Namikoshi et al., 2006). However, there are reports that phthalate esters are naturally produced extracellularly by microorganisms such as bacteria, fungi and yeasts (Mahmoud et al., 2006). One of the previous studies showed that certain cyanobacteria were capable of intracellular degradation of PE (Babu and Wu, 2010).

Arum palaestinum is one of about 26 species of the arum genus which are flowering plants belonging to Araceae. Previous studies on the characteristics of secondary metabolites of the Araceae family showed that it has a simple profile of polyphenols and alkaloids with flavone c-glycosides, flavanols, flavones, proanthocyanidins and polyhydroxy alkaloids as main classes (Kite et al., 1997). Few phytochemical and biological investigations have been reported on this plant (Saad et al., 2005). One of the studies was attempted to analyze the ethyl acetate fraction of the plant and led to the isolation and identification of a new polyhydroxy alkaloid compound $C_4H_5NO_4$ (EL-Desouky et al., 2007a, 2007b). Moreover, ethanolic extract of this plant was examined and found to possess antioxidant activity via evaluation of its scavenging of DPPH free radicals (IC_{50}=368µg/ml), in addition to its anti-cancer activity against breast carcinoma cells MCF-7, (IC_{50}=500-600 µg/ml) using the 3-[4,5-dimethyl-2-thiazolyl)-2,5- diphenyl-2Htetrazolium bromide (MTT) cytotoxicity assay (Husein et al., 2014). In continuation of our research for identification of plant constituents, we reported three phthalate derivatives, dibutyl phthalate, dioctyl phthalate, and diisobutyl phthalate.In addition to those finding, the ammounts of plthalate in defferent locations in Palestine have been evaluated for different parts of the plant.

Experimental

Chemicals

Benzoic acid, salicylic acid, cinnamic acid, diisobutyl phthalate, ascorbic acid, resorcinol, thymol, phthalic acid, sorbic acid, n-diprpyl phthalate, di-n-octyl phthalate, pyrocatechol, quercetin, Tween-20, Folin-ciocalteu's reagent (FCR), Sodium sodium carbonate were purchased from Sigma,(Sigma, Aldrich GmbH, Sternheim, Germany,). While chloroform, ethanol, methanol were purchased from Merck (Darmstat, Germany),Sigma,(Sigma, Aldrich GmbH, Sternheim, Germany). and all other chemicals and reagents were of analytical grade. All chemicals and reagents were kept in glass bottles and vials to avoid any contamination of phthalates.

Plant collection

The plant samples screened in this study were collected in April – June 2013 from three different places natural, unditurbed locations (2 in Tulkarm District, and one in Nablus District) south Beit Leed town , north Beit Leed town and Til village) and were identified by Prof. M. S. Ali-Shtayeh in the Biodiversity and Environmental Research Center (BERC), Til / Nablus. The plant material was collected and kept in glass containers to avoid any contamination of phthalates from the envionment. Voucher specimens (BERC-BX-C-0064) are deposited in the Herbarium of BERC.

Extracts preparation

The fresh parts of the plant were grinded using a stainless steel Molenix (Mooele Depose type 241) for a minute. 50g from each sample (leaves, stems, inflorecence and inflorecence base) were extracted by continuous stirring with 200 ml ethanol at 24°C for 72 h and filtering through Whatman No. 4 filter paper. The residue was then washed with additional 5 ml ethanol. The combined ethanolic extracts were dried using freeze drying and stored at -20 °C for future use.

Apparatus and conditions

The device "Perkin Elmer clarus 500 Gas chromatography" and "Perkin Elmer clarus 560D mass spectrometer" were used in the analysis.
He gas, temperature of the injector = 280 ºC, carrier was 1ml/ min and total flow was 60ml/min. Column was Elite – 5MS, 30 meter 0.25 µm df. Max projection at temperature 360 ºC, and minimum bleed at 320 ºC.

The experiment was done as follows:

Temperature (ºC)	Time (min.)	Rate (10/min.)
60 (beginning)	15	10
110	5	10
200	5	10
280	5	

Sampling

Diluted samples (10 µl) of the ethanolic extract of the plant were injected in the GC-MS spectrometer.
A few compounds were identified by GC-MS such as [diisobutyl phthalate, Di-n-octyl phthalate (figures 1, 2) , Methyl 3-hydroxy benzoate and 4H-pyran-4-one 2,3 dihydro-3,5-dihydroxy -6-methyl] depending on the total ionic concentration values (TIC) and mass spectrum for *A. palaestinum*. In addition to those, many compounds were detected but not identified Figures 1&2.

Identification of compounds in the plant extract using HPLC analysis

HPLC methodologies represent, to date, the most widely used approach to the analysis of phenolics (Martinez et al., 2002). Reversed-phase chromatography has been extensively employed for the separation of flavonoids on C18 columns (Penazzi et al., 1995; Molyneux et al., 2002) with polar mobile phases, such as methanol, acetonitrile, tetrahydrofuran or acid solutions (Merken and Beecher, 2000). Under normal reversed-phase conditions, the more polar compounds are generally eluted first. The classes of flavonoids that characterize citrus species (flavanones, flavones, and, to a lesser extent flavonols/flavanols) have their maximum absorption at specific wavelength ranges: flavanones (280- 290 nm), flavones (304-350 nm) and flavonols (352-385 nm). Moreover, at 325 nm flavones show an absorption peak, which is similar to the corresponding peak at 280 nm Figure 3.

Ethanol extract of the plant is tested for the presence of phenolic compounds and flavonoids according to the retention time of calibration curve standards Table 1.

Apparatus and reagents

Equipped with suitable injection device (Shimadzu SPD-M 10AVP HPLC), solvent delivery system, UV detector, electronic integrator and µBondapak C_{18} column, 300x3.9 (id) mm waters/Millipore. Flow rate 2mL/min, injection volume 10 µL, detector

wavelength 254 nm, temperature ambient. Mobile phase: 20% aqueous CH_3COOH (volume/ volume) buffered to pH 3.0 with saturated sodium acetate solution.

Working Standard Solutions for Calibration

Dilute 50 µL of the Mixture of three phthalates standard solution (1 mg /mL for each component) to 1 mL with 950 µL of methanol/water solution (3:1, v/v). Prepare five working standard solutions for the calibration with 10, 20, 30, 40 and 50 µg/ml concentrations by adding the proper amounts of stock standard of phthalate Mixtures.

Calibration linearity for UV detection of the phthalates was investegated by making five consecutive meaurements of a mixed standard solution prepared at five different concentrations. Best linearity was observed from 1.0 to 40.0 µg/ml when plotting the concentration versus the peack area and the coefficeits of determination were ≥ 0.99 for all analytes Table 2.

Standard solutions

Fifty mg of each standard was dissolved in 5ml ethanol and diluted to 0.1mg/mL. The final solution was filtered through a 0.45 membrane filter.

Sample preparation

10 µL /ml of each part of the plant extract from different areas were diluted to 100 µL /ml with methanol/water solution (3:1, v/v), and filtered through a 0.45 µm filter prior to measurement

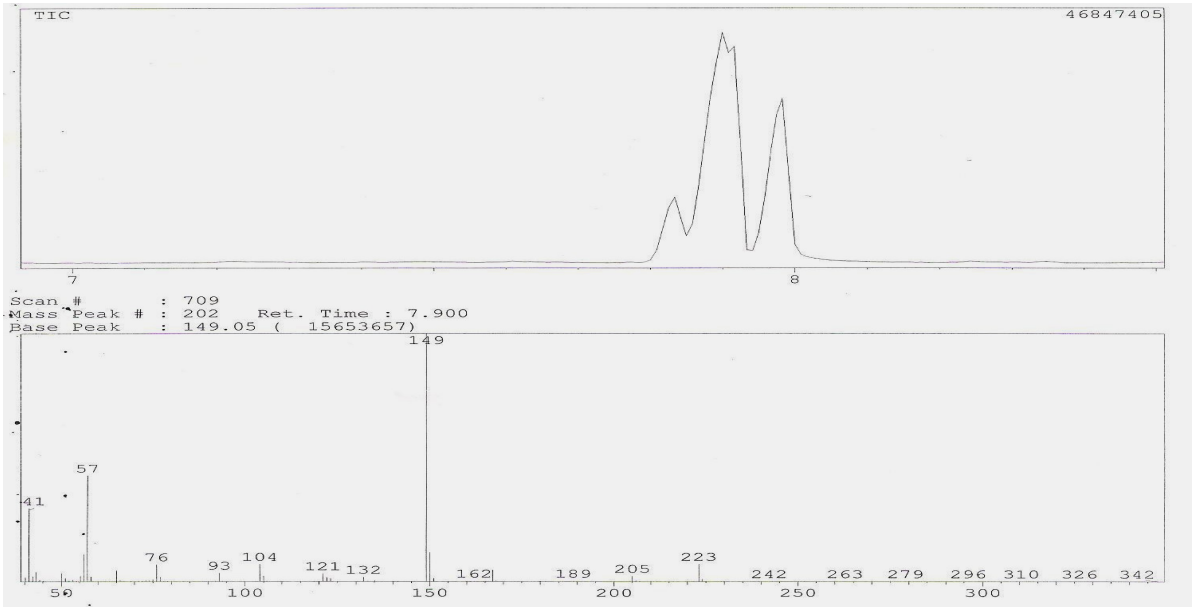

Figure.1 Values of TIC and MS spectra for diisobutyl phthalate

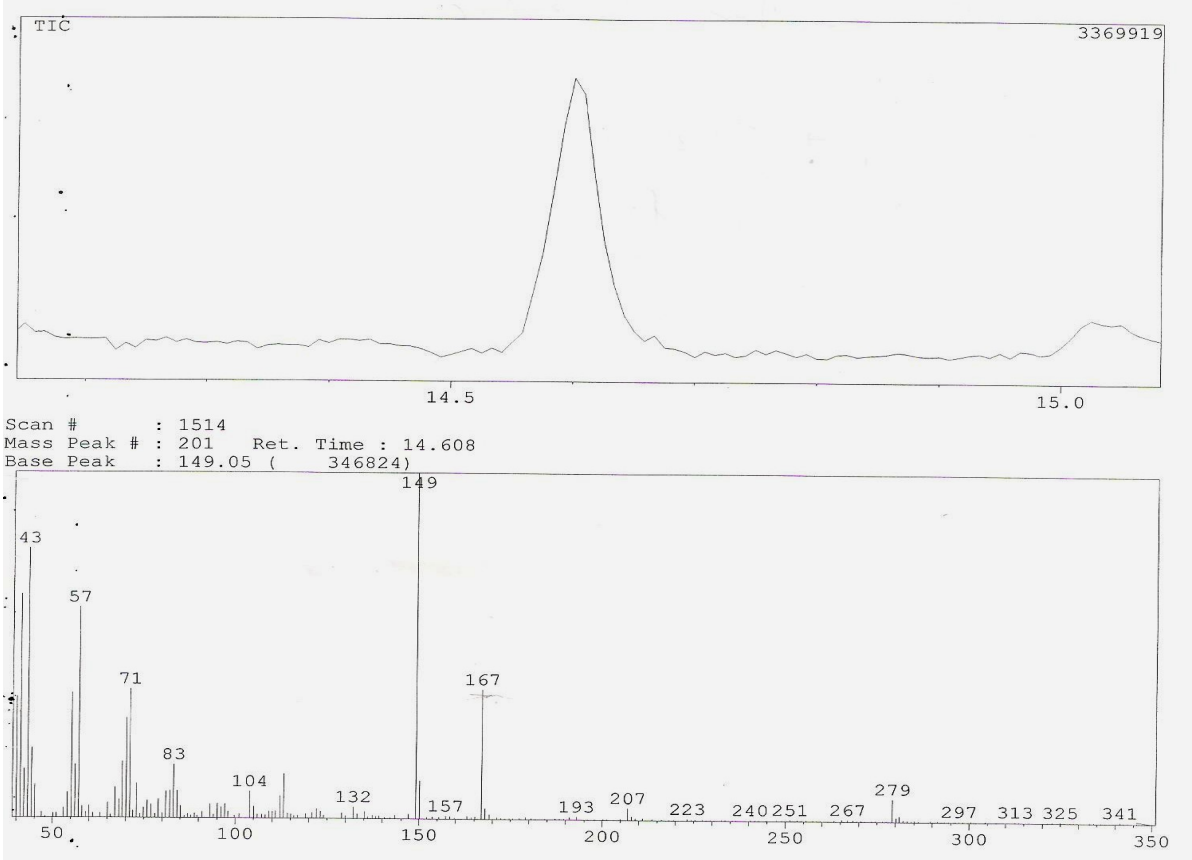

Figure.2 Values of TIC and MS for di-n-octyl phthalate

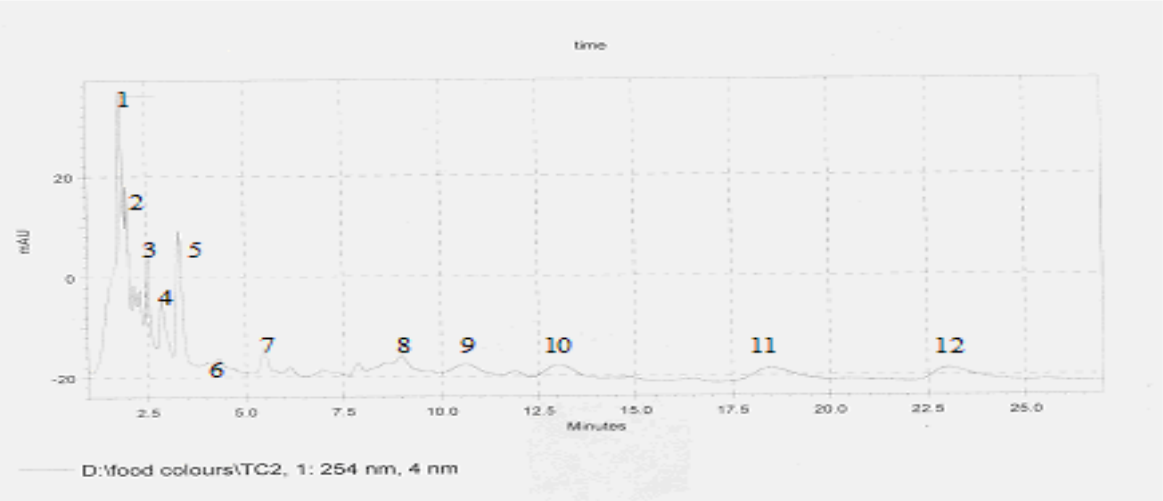

Figure.3 Chromatogram of A. palaestinum

Table.1 Compounds detected in *A. palaestinum* extract by HPLC according to the retention times

No.	Standards	Ret. Time (min)	*A. palaestinum* extract
1	Benzoic acid	10.5	Present
2	Salicylic acid	2.9	Present
3	Cinnamic acid	18.8	Present
4	Quercetin	22.8	
5	Pyrocatechol	4.4	
6	Diisobutyl phthalate	7.9	Present
7	Ascorbic acid	*N.D	
8	Resorcinol	3.1	
9	Thymol	9.8	
10	Phthalic acid	3.3	
11	Sorbic acid	1.8	
12	2-Phenoxy ethanol	21.1	
13	Methyl 3-hydroxy benzoate	19	
14	n-diprpyl phthalate	3.5	Present
15	Di-n-octyl phthalate	14.6	Present

* N.D : Not deternined

Table.2 Calibration curves for phthalates.

Analyte	Equation	R^2	Range
Diisobutyl phthalate	Y=3.417+0.324	0.999	
n-diprpyl phthalate	Y= 3.073+0.231	0.999	1.0-40.0
Di-n-octyl phthalate	Y= 2.366+0.188	0.999	µg/ml

Result and Discussion

One of the interesting results from the present study was the separation and identification of phthalate compounds such as diisobutyl phthalate and di-n- octyl phthalate for the first time from the extract of *A. palaestinum*, using HPLC and GCMS techniques. To confirm these results *A. palaestinum* is cultivated from different natural sites in Nablus and Tulkarm areas and kept in glass containers. All experiments were carried out in glass equipments to avoid any contamination of phthalates from the environment. Table 3 showed the values of phthalates (µg/g of fresh plant) in different parts of the plant collected from different locations. The highest values were leaves (average= 36.29 ±2.7 µg/g), then the inflorescence part (average= 26.1 ± 2.8 µg/g). The least values were in stems (average= 5.6 ± 2.2 µg/g) and inflorescence base (average= 7.0 ± 0.9 µg/g) for fresh plants. These results are consistent with the study done by Saleem et al. (2009), who showed that some species of the genus *Phyllanthus*, the famous medicinal plants, produce phthalates (bis (2-ethyloctyl) phthalate and bis (2-ethylicosyl) phthalate), which most often exhibited antimicrobial activities. In addition, the leaves of *Pongamia pinnata*, an Indian medicinal plant, have been reported to consist of bis (2-methylheptyl) phthalate and the mentioned compound exhibited inhibitory activity against White Spot Syndrome Virus (WSSV) (Rameshthangam and Ramasamy, 2007).

Interestingly, plants receive both nutrients and toxic substances through the roots as well as above-ground green parts. High

accumulation of phthalates in stems of some types of crops has been reported including *Triticum aestivum, Brassica napus, Zea mays* and Raphanus salivas (Yin et al., 2003; Zorníkova et al, 2011; Virgin, 1988). The results of this study confirm the production of phthalates in the plant because phthalates are found in different parts with different concentrations for all places.

Additionally, it was proved that brown algae like (*Sargassum*) can synthesize phthalate esters, but their production process and physiological role have not been clear so far (Chen, 2004). The challenge will be raised when many of these plants and marine algae are consumed as food or medicinal resources. Accumulation of phthalates can occur in some medicinal plants *e.g Lythrum*, that usually grows in water flow in rivers and canals **(**Saeidnia and Abdollahi, 2013**).** In such cases, wastewater might be the origin of pollution and phthalate exposure to these plants. Sometimes, high exposure to phthalates resulted in about half part of essential oil extraction, which can cause worries to consume such medicinal plants, crops or vegetables.

Phthalates are able to possess remarkable toxic variations depending on their structures. So far, DEHP and DBP have been found to cause reproductive and developmental toxicities. The U.S. Environmental Protection Agency (EPA) classified DEHP as probable human carcinogen. To the best of our knowledge, phthalates showed diverse toxicity profiles according to their structures in the liver, kidneys, thyroid, and testes, which are involved in general toxicity. Furthermore, they are introduced as hormonally-active agents, because they can interfere with the endocrine system in human (Herr et al., 2009).

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