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Assessment of phthalate esters (PAEs) contamination in *Urtica dioica* L.

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Abstract

Phthalate esters are a group of chemical compounds of ubiquitous nature which nowadays have become a colossal threat to the environment, human -animal and plant health, because of its higher potential of accumulation in soil and aquatic habitat leading to environmental contamination due to its widespread industrial and agricultural usage. The present research aims to analyze the phthalate esters accumulation in *Urtica dioica* L. For this study, the *Urtica dioica* L. is tested for the presence of phthalates by using Gas Chromatography-Mass spectrometry. The Gas Chromatography-Mass spectrometry observations show the presence of 11 phthalate esters, among which diethyl phthalate (DEP) and bis(2-ethylhexyl) phthalate (BEHP), dibutyl phthalate (DBP), and diisobutyl phthalate (DIBP) were found to be in significantly higher amount. The sum concentrations of the phthalate ester in different extracts of plant range from 16.25% to 84.07%. The % composition of diethyl phthalate is found to be comparatively higher than other phthalate esters in methanolic extract of *Urtica dioica* while diisobutyl phthalate and bis (2-ethylhexyl) phthalate accumulation is found relatively higher in the ethyl acetate and diethyl ether fractions. The observations show the contamination of the *Urtica dioica* plant with phthalate esters and also indicate the phthalate accumulating potential of the plant.

Keywords

Phthalic acid esters, *Urtica dioica*, Gas Chromatography-Mass spectrometry, Diethyl phthalate, Bis (2-ethylhexyl) phthalate, Diisobutyl phthalate, Dibutyl phthalate

Introduction

Phthalic acid esters (PAEs) commonly called phthalates are a group of synthetic chemical compounds which are known as 1, 2-benzene dicarboxylate esters or dialkyl or alkyl aryl esters of *o*-phthalic acid (1, 2). PAEs have become a serious concern as they possess potential hazardous effects as an environmental contaminant due to their widespread usage with a total global production of 8M tonnes in 2011 (3). PAEs are used as a common plasticizer that is added to plastic polymers for increasing the strength, flexibility, transparency and durability of plastic (1, 4). The most common usage is found in toys, cosmetics, food and pesticides packaging, medical instruments and many other agricultural and household products (5-7). The PAEs remain free of any covalent bond binding it to the surrounding matrix, so it is readily accessible for leaching into the environment released from the higher molecular mass carbon chains (8-10) and spread itself ubiquitously in water, air and soil (11, 12). PAEs are considered among the priority pollu-

tants as it has been reported to cause oxidative stress, apoptosis and cytotoxicity via DNA damage (13-16). They are often grouped as compounds with endocrine disruptive potential, interfering with the normal process of synthesis, secretion and metabolism of hormones involved in sexual development, thus causing an adverse effect on reproductive health (17, 18). Other health issues attributed to PAEs are hyperactivity disorders, allergy, asthma, thyroid cancer and hypertension (19-22).

The plant chosen is collected from the roadsides, drainage areas (adjacent to the agricultural fields) from the Palampur district of Himachal Pradesh. Major sources of phthalate esters pollutant to be reaching the soil in Himalaya region of Himachal Pradesh is the increased protective cultural practices in the hill slopes, promoting the use of greenhouse/polyhouse farming practices. These polyhouses are made of cheaper polythene or plastic instead of glass on the greenhouse roof (23). Other major sources of PAEs pollutants are wastewater irrigation (24), use of agricultural chemicals and fertilizers (25, 26), and emission of industrial waste (27, 28). From the contaminated soil, the PAEs further get distributed in the environment through biogeochemical cycling (Fig. 1).

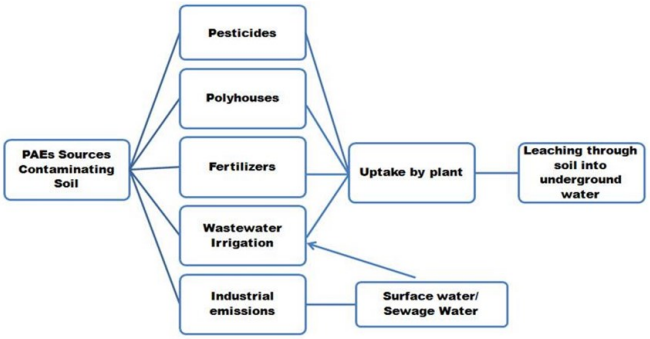


Fig. 1. Soil Contamination sources of PAEs.

The past few decades have witnessed a massive surge of inclination in the ideology of people towards the use of medicinal plants due to their easy availability, effectiveness with minimalistic side effects, less toxicity and long-term beneficial potentials (29-33). *Urtica dioica* L., a notorious weed of invasive nature comes under the least concern group of IUCN list, is a plant that has long been used for fiber, food and traditional medicine for its potential property in acting against arthritic conditions, musculoskeletal pain, inflammations and for its anti-diabetic, anti-hypertensive and cardiovascular potential (34-37). Although known for its traditional use in Urtication therapy for the treatment of numb joints in case of paralysis and rheumatoid arthritis (38, 39); the plant could not overcome its reputation and still found growing as a neglected weed species in barren lands, waste places, roadsides, neglected yards, stream banks and ditches. The plant grows best in moist soils that are rich in nutrients (40).

U. dioica is found widespread throughout the cooler, temperate climate of Europe, also occurs in North America, North Africa and parts of Asia (41). The species is although invasive in nature, nowadays growing stinging nettle has been encouraged by conservation groups of UK wildlife as it supports more than 40 species of insects including tortoise shell, peacock butterflies and various kinds of caterpillars. Nettles serve as a food source for butterflies and moth larvae. The seeds produced from the plant make food source for the native birds. The plant is usually insect-prone and is useful as an insect feed for many types of caterpillars and thus has been deliberately planted (42).

Taxonomic classification of the plant (43, 44) is as follows:

Kingdom	:	Plantae
Sub-Kingdom	:	Tracheophyta
Superdivision	:	Spermatophyta
Division	:	Magnoliophyta
Class	:	Magnoliopsida
Subclass:	:	Hemamelididae
Order	:	Urticales
Family	:	Urticaceae
Genus	:	*Urtica*
Species	:	*dioica*

The ever-increasing contamination of agronomically important soil is becoming a serious problem as the presence of contaminants disrupts the normal functioning of biogeochemical balance in the environment. Physicochemical remediation technology has various limitations (45), hence an alternative of phytoremediation has been put forth using plants for removal of pollutants from the environment (46). Various plant species possess an inbuilt mechanism for detoxification of the xenobiotic compound. These plants have high toleration capacity and the potential to hyper accumulate toxic chemicals almost up to 1% of their weight (47). *U. dioica* is also one such hyperaccumulating plant that has already been evaluated for its phytoremediation potential in eradicating the Cr metal stress (48) and soils contaminated with polychlorinated biphenyls (PCBs) (47). The main objective of the present study is determining the contamination in the phytoconstituent composition of *U. dioica* plant with phthalate esters and evaluation of type PAEs accumulated in the plant through GC-MS analysis.

Materials and Methods

Collection and Preparation of Plant extracts

The whole plant of *U. dioica* L. was collected from the roadsides and drainage areas (adjacent to the agricultural fields) of Palampur District of Himachal Pradesh, India. These areas are chosen deliberately to test the impact of employed agricultural practices on the phytochemical composition of the plants growing in the vicinity. A dried plant completely pressed in blotting papers was submitted for identification of the plant species and specimen no. PLP-18301 has been given as a herbarium specimen to IHBT Palampur. The collected plant material except the one to be provided for herbarium is at first rinsed in run-

ning tap water to remove the surface impurity and blotted dry to remove any type of excess moisture. The whole plant was then dissected into root, stem and leaves which were then allowed to dry in shade for 7 days, occasionally mixed and cut into smaller pieces. The dried stock samples were then powdered using a grinder and the powdered material was kept stored in an airtight container at 4 °C for future extraction. About 25 g of powdered sample was weighed and extracted with 200 ml methanol using Soxhlet extraction for methanolic (ME) extract until the plant material is completely exhausted and for the diethyl ether (DEM) and ethyl acetate (EAM) fraction of methanol extract, about 25 g of powdered sample is soxhlet extracted for 24 hrs in 80% methanol. Soxhlet extraction is carried out at a temperature of 64 °C for both sets. The solvent of the plant extracts was then evaporated in a hot air oven at 40 °C to obtain a viscous liquid. For the DEM and EAM extract 80% methanol concentrated dried extract was taken and re-extracted with pet ether, diethyl ether and ethyl acetate sequentially. The plant residue collected was discarded and the concentrated solvent extracts were transferred to vials of 15 ml sizes and the remaining solvent is further allowed to evaporate in the laboratory at room temperature. The remaining completely dried extract was weighed to evaluate the yield of the extract and then it is stored in a refrigerator at 4 °C until further analysis.

GC-MS Analysis

Gas Chromatography-Mass Spectrometry analysis of the methanolic plant extract was carried out on a Shimadzu QP-2010 Plus equipped with a Thermal desorption system TD 20, helium carrier gas, Rxi-5Sil MS column (30 m length, 0.25 mm ID, 0.25 µm thickness), Column Oven temperature of 60 °C for ME extract and 100 °C for DEM and EAM extracts, injector temperature was maintained at 260 °C, pressure 81.7 kPa for ME and 90.5 kPa for DEM and EAM extracts, total flow 16.3 ml/min, column flow 1.21 ml/ min, linear velocity 40.1 cm/ sec, purge flow 3.0 ml/ min, the split ratio was 10.0, ion source temperature : 230 °C, Interface temperature: 270 °C, Oven temperature program: 60 °C (hold for 5 min) for ME and 100 °C for DEM and EAM with a hold time of 2 min. raised to 300 °C at the rate of 10 °C/ min: ending with an isothermal temperature of 300 °C for 19 minutes, run time: 44.98 min for ME and 40 min for DEM and EAM extracts. The instrument was operated using GC-MS solutions software. Scan range was 40-650 m/z. For GC-MS analysis crude extract was re-dissolved in methanol to make a stock solution. 1 µl stock solution was prepared for GC-MS analysis. The relative abundance of the compound in the extract was calculated by comparing the area of average peak to the total area, software adopted to handle mass spectra and the chromatogram was turbo mass. The relative percentage of every constituent present in the extract was expressed as a percentage with their respective peak area. The phytoconstituents in the extracts were determined by the comparison of their retention time and mass weight with recorded authentic samples obtained by GC as well as from the mass spectra of NIST (National Institute of Standards and Technology, US) a database having 62000 patterns and Wiley pesticide library 3 rd edition, for estimating the presence of probable compounds in the extract, which is further cross-checked using PubChem online database and other research articles for the activities and structure of the detected compound.

Results and Discussion

The main aim of the present study is the extraction and evaluation of methanolic, ethyl acetate, and diethyl ether extract of root, stem and leaves of *U. dioica* for the presence and the extent of accumulation percentage of PAEs. The solvents used for the sample injection in GC-MS were pure with no phthalate contamination and as a precautionary measure, the solvents were put in the dried extract immediately before putting the sample into injection. So, the chance of introduction of any PAEs peak in the GC-MS chromatogram originating from the laboratory contamination is kept minimal.

The completely dried plant methanolic soxhlet extractions of root, leaves and stem of the plant were weighed for the total yielding capacity. The yield of the extract is measured by comparing the weight of the solvent extract with the sample weight of the powdered extract of the plant. The yield is measured in the amount of extract given by 100 g of the sample plant part weight (g/100 g dry weight). The result of the extraction yield obtained from each extract is compiled in Table 1. The methanolic extraction yield is found to be the highest in leaves among the tested plant parts and the root seems to be the one with the lowest yield. To make sure that the phthalate compounds source might not be from any sort of laboratory plastic-ware, all the steps and processes of extraction, storage and analysis were monitored for avoiding the use of any type of plastic container in the laboratory.

Table 1. Extraction Yield of different plant parts of *U. dioica*

Plant Part	Yield (g/100g dry weight)		
	ME	**DEE**	**EAE**
Root	6.086±0.0728	0.251±0.0049	3.74±0.001
Stem	7.612±0.0538	0.454±0.107	1.034±0.011
Leaves	10.098±0.109	0.13±0.017	2.34±0.006

ME: Methanol Extract, **DEE:** Diethyl ether Extract, **EAE:** Ethyl Acetate extract
**Each value is expressed as mean±standard deviation (n=3)*

GC-MS chromatogram results of the different extracts of *U. dioica* exhibited the presence of about 11 PAEs compounds which are diisobutyl Phthalate, dibutyl phthalate, bis(2-ethylhexyl) phthalate, diethyl phthalate, dimethyl phthalate, diamyl phthalate, butyloctyl phthalate, 5-methylhex-2-yl isobutyl phthalate, 5-methylhex-2-yl butyl phthalate, dioctyl terephthalate, dimethoxyethyl phthalate. The phthalates compounds with major contamination in the *U. dioica* extract are illustrated in Fig. 2 with their molecular structure and their respective mass spectra. The GC-MS chromatograms of the extracts are provided in Fig. 3: Root, Fig. 4: Stem and Fig. 5: Leaves.

Leaves of *U. dioica* show the most considerable amount of contamination both in terms of the accumula-

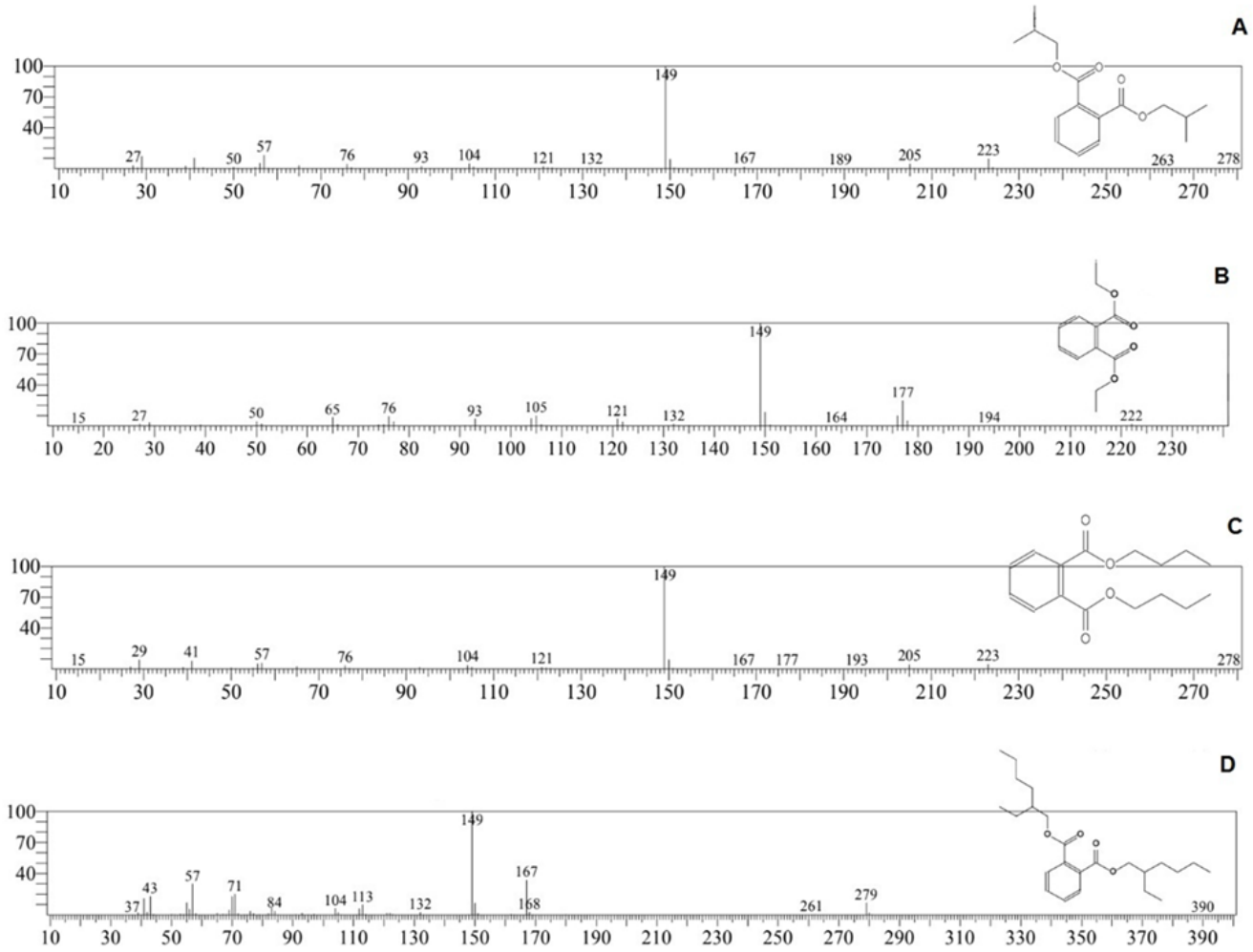
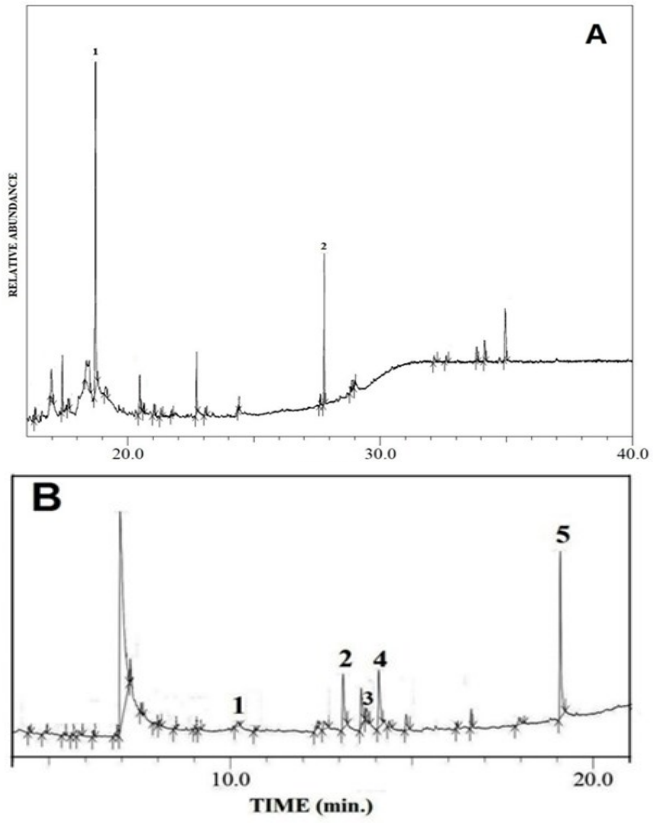


Fig. 2. GC-MS mass spectra of four major phthalate contaminants in *U. dioica* **A**. Diisobutyl phthalate (DIBP), **B**. Diethyl phthalate (DEP), **C**. Dibutyl phthalate (DBP), **D**. Bis (2-ethylhexyl) phthalate (BEHP).



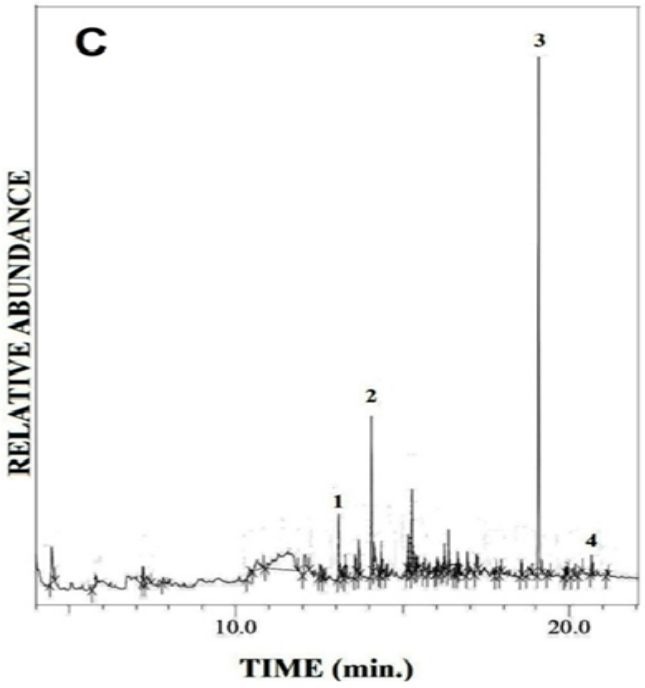


Fig. 3. Mass Chromatograms of *U. dioica* Root **A.** Methanol Extract. 1.DEP, 2. BEHP; **B.** Ethyl Acetate Extract **1**. DEP, **2**. DIBP, **3**. BOP, **4**. DBP, **5**.BEHP; **C.** Diethyl ether Extract**, 1**. DIBP, **2**. DBP, **3**. BEHP, **4**. DOTP.

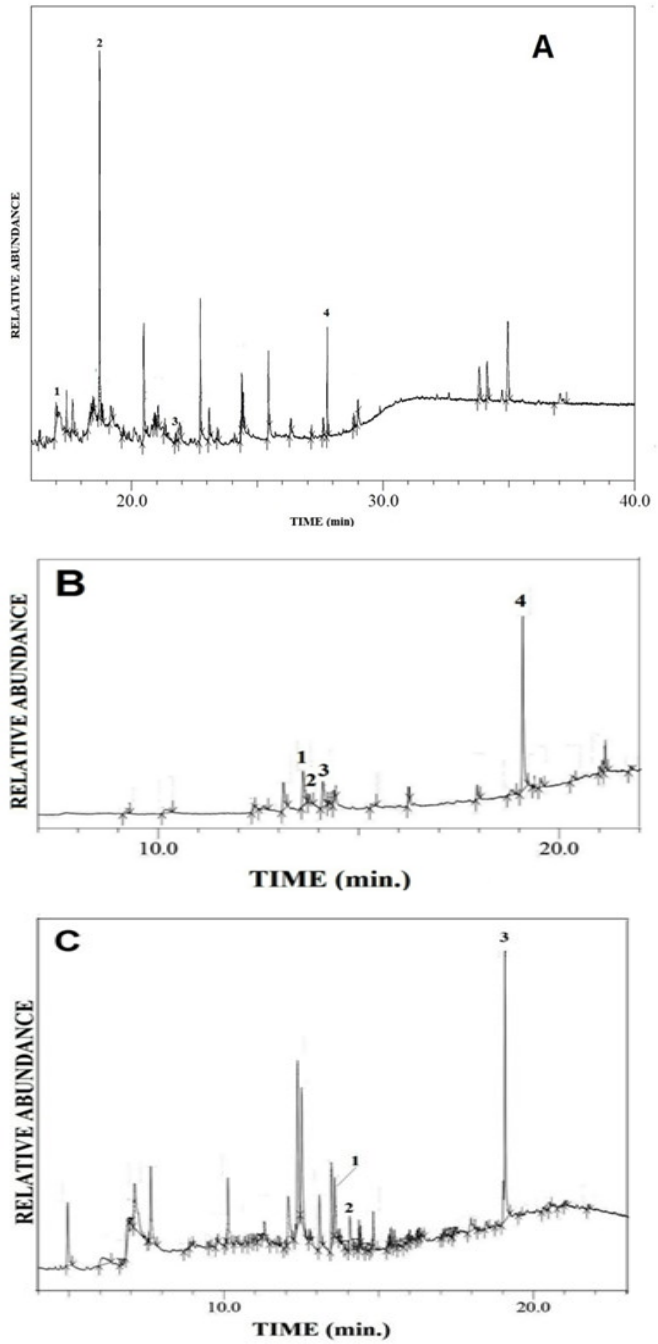
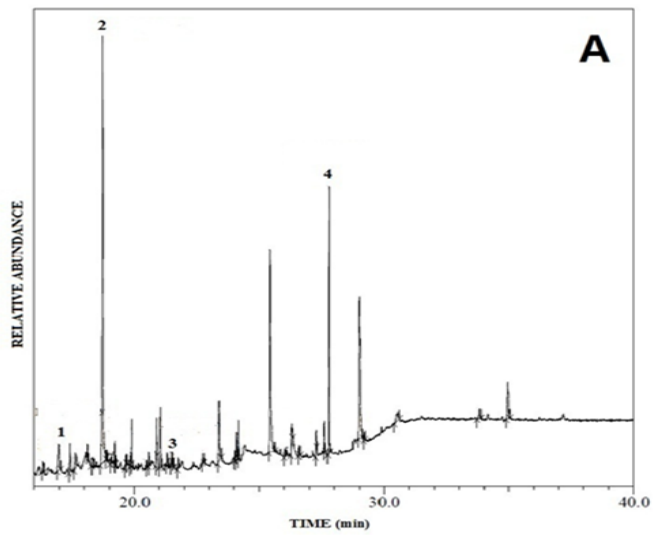


Fig. 4. Mass Chromatograms of *U. dioica* Stem **A.** Methanol Extract**,** **1**.DMP, **2**. DEP, **3**. BOP, BEHP; **B.** Ethyl Acetate Extract **1**. DIBP, **2**. DBP, **3**. BOP, **4**. BEHP; **C.** Diethyl ether Extract**,** **1**. DIBP, **2**. DBP, **3**. BEHP.



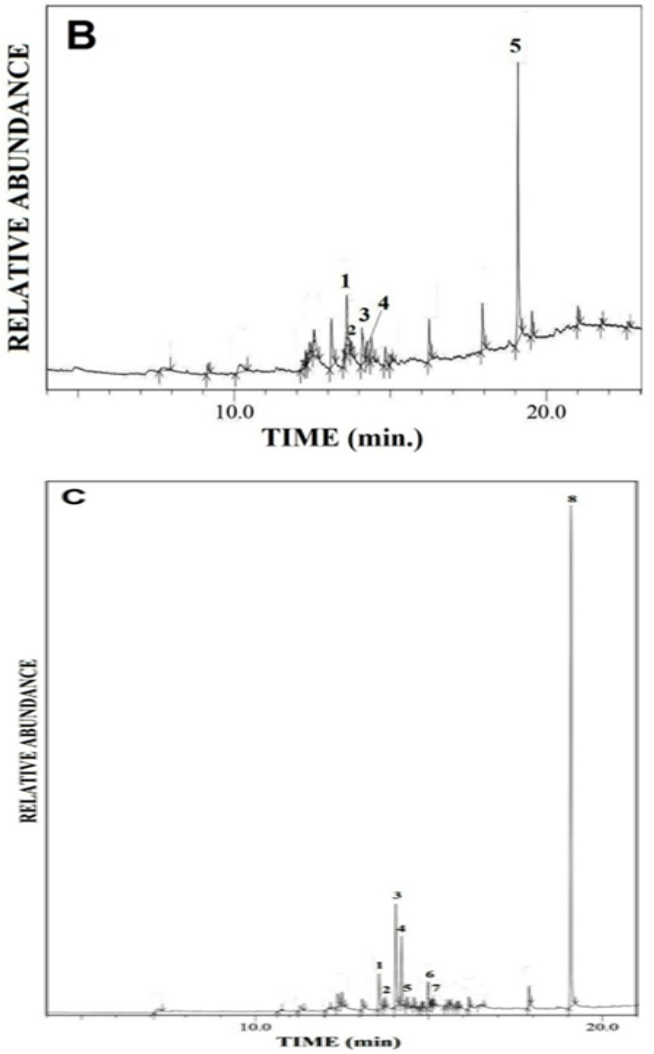


Fig. 5. Mass Chromatograms of *U. dioica* Leaves **A.** Methanol Extract**,** **1**. DMP, **2**. DEP, **3**. DBP, **4**. BEHP; **B.** Ethyl Acetate Extract**,** **1**. DIBP, **2**. MHIBP, **3**. BOP, **4**. DMEP, **5**. BEHP; **C.** Diethyl ether Extract**,** **1**. DIBP, **2**. MHIBP, **3**. DBP, **4.** MHBP, **5**. DAP, **6**. BOP, **7**. PPP, **8**. BEHP; **1**. DIBP, **2**. DBP, **3**. BEHP.

tion % as well as in terms of various types of phthalate ester observed from the GC-MS results obtained. The diethyl ether extract of the leaves exhibit the highest accumulation of phthalate compounds taking up about 84.07% peak area of the resultant chromatogram. DEP presence is observed only in the methanolic fraction of all plant parts in significantly higher amounts and only in trace amounts from the ethyl acetate fraction of the root. The methanolic leaves extract exhibits the highest % of peak area covered up by DEP.

The comparative phthalate ester contamination in various extracts of *U. dioica* is illustrated in Fig. 6. A relatively higher concentration of DBP and DIBP is found in the diethyl ether and ethyl acetate extract; while BEHP shows its accumulation in all the extracts in significant quantities.

Previous GC-MS analysis conducted on the hexane and aqueous extract of *U. dioica* leaves reported the presence of dibutyl phthalate (DBP) and diisooctyl phthalate esters and were observed to be responsible for imparting antimicrobial properties to the plant (49-52). There is no further evidence and not much research has been conducted on testing the phthalate esters being an indigenous plant metabolite in plant *U. dioica*.

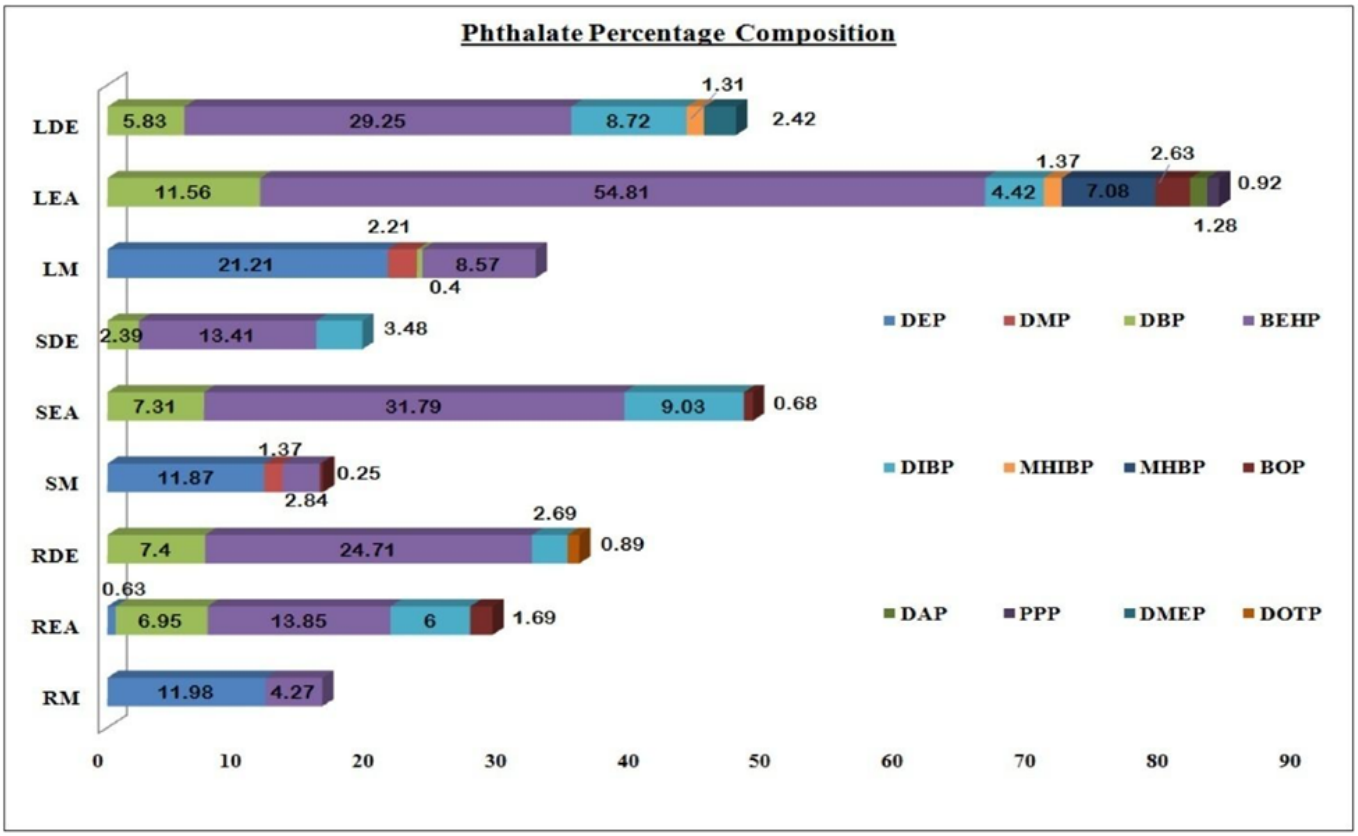


Fig. 6. Percentage Contamination of the extracts of *U. dioica* by phthalate esters. [LDE: Leaf diethyl ether extract; LEA: Leaf ethyl acetate; LM: Leaf Methanol;SDE: Stem diethyl ether extract; SEA: Stem ethyl acetate;SM: Stem Methanol; RDE: Root diethyl ether extract; REA: Root ethyl acetate; RM: Root Methanol; Diethyl phthalate (DEP), Dimethyl phthalate (DMP), DBP: Dibutyl phthalate, BEHP: Bis (2-ethylhexyl) phthalate, Diisobutyl phthalate, MHiBP: 5-Methyl hex-2-yl isobutyl phthalate, MHBP: 5-methylhex-2-yl butyl phthalate, BOP: Bis-octyl phthalate, DAP: Diallyl phthalate, PPP: Poly-propylene phthalate, DMPP: Di-(methoxyethyl) phthalate, DOTP:Dioctyl terephthalate].

The detection of mentioned PAE compounds revealed the ability of the plant to absorb these contaminants either from the contaminated soil or the polluted water source, keeping in mind that the plasticizer contamination while handling, storage and extraction is kept minimal. The discussed plant grows as a common weed in the mountain region situated near floating river beds with rich soil. The major source of such contaminations in the soil or the draining water is the absence of proper waste plastic disposal or recycles and the increasing plastic warehouse cultivation practices and use of mulching films in agriculture also increases the chances of leaching of PAEs into the soil and further to the groundwater sources (53, 54). Among the commonly used phthalate esters, DMP shows the highest water solubility followed by DEP (55). BEHP is also reported to be slightly water-soluble in nature making the phthalate ester capable of easily leaching and contaminating the water sources (56-59). The prime cause of phthalate pollution in the environment remains the neglect of the proper waste disposal resulting in a manifold increase in phthalate pollutant percentage in the environment (60).

Phthalates constant release into the environment has raised concern about the deteriorating effect of phthalates on human and animal health (61-65). Toxicological study on PAEs reported DEHP with endocrine disruption effect as well as cytotoxicity in various types of cells while DBP is reported to significantly reduce the cell viability. Phthalates that are reported as reproductive as well as developmental toxicants are DiBP (66-68), DEHP (69-71), DiNP (66, 69), BBzP (72, 73). Phthalates temper with the normal production and functioning of follicle-stimulating hormone, testicular testosterone (fetal) (74). Major critical ill effects can be seen as the structural and functional disruption of male reproduction (75, 76). Phthalate toxicity also shows effects like retention of nipples/areola in male rodents and a reduced anogenital distance; this marks the first sign of feminization and demasculinization (49, 73). This group of symptoms in animals is known as ‘‘phthalate syndrome’’ (75, 77).

Previous research reported phthalate esters to cause adverse effects on the reproductive health of humans have recently been surveyed which concluded women to have relatively broader exposure risk in day-to-day life compared to men as they are more exposed to use cosmetics and personal care products that uses phthalate in their packaging and storage (78). Although phthalate pollution poses a greater threat upon humans and animals' health, the phyto composition of food crops and medicinal plants are also getting greatly affected due to phthalate pollution in the environment. There have been reports on the higher accumulation of PAEs in three staple food crops *Triticum aestivum, Brassica napus, Zea mays* (48). In some practical experiments, the researchers used the PAEs accumulation potential of the plant *Benincasa hispida* to eradicate and reduce the risk of absorption of DEHP by the planted vegetable crop, thus adapting a new way for eradicating the PAEs removal from the agriculture fields (79).

Conclusion

The present study reported the phthalate esters accumulation in *Urtica dioica* L.. The toxic compound that has been adapted for use as a plasticizer in agriculture, pharmaceutical, cosmetic, personal care product, industrial uses has an easy pathway to get leach from the sources and getting mixed into soil and water sources contaminating them. From here these pollutants get absorbed by the plants through roots. Pollution of medicinal and food crop plants has become a serious problem in the developing countries which requires special attention in standardization and restriction of PAEs plasticizers and increase in the capacity of production as well as promoting and making people aware about using phthalate-free plasticizers. Proper toxicological evaluation and examination of medicinal plants are recommended to determine the extent of phthalate accumulation in the plant before taking into consideration for use as an herbal drug to avoid creating an adverse effect. Although the restriction in usage of PAEs remains the first step, but finding new ways for the eradication of such toxic compounds from the soil and water sources may also have benefits.

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Authors contributions

All the authors have read and approved the final version of the article. The research conducted and the analysis done was solely done by PR under the guidance of RAS.

Compliance with ethical standards

Conflict of interest: The authors declare no conflict of interest to the presented research.

Ethical issues: None

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