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Evaluation of Phthalate in Breast Milk and Urine of Lactating Women in Egypt

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Phthalates are widely used in softening plastics especially it is cheap. But due to its harmful effect on health in many countries non phthalate plasticizers they are used instead in many products. As Phthalates rapidly metabolized and excreted in urine and feces.in the form of Monoesters (mono butyl phthalate) which can be measured in urine and breast milk using High Performance Liquid Chromatography (HPLC), So aim of our study to detect and counting of Mono-n-Butyl phthalate (MnBp) in the breast milk and urine of 20, randomly selected, lactating females house wife's with mean age of mothers from 17-39 years from Helwan Primary Health Center – Cairo – Egypt after their consent. . with detailed history regarding Sample analysis was conducted in The Micro Analytical Center – Faculty of Science – Cairo University using HPLC machine . As high non-occupational human exposures due to wide uses of phthalates is reported, so in our study we assess phthalate level in breast milk and urine in correlation with uses of personal care products(Cosmetics),Vinyl use in walls and in home flooring , drinking and eating in plastics containers and in relation to smoking. Our study shows a high statistically significant relation between phthalate level in mothers' urine and in breast milk with PVC or Vinyl use in walls and in home flooring, while its level in mothers' urine was highly significant in cases of drinking in plastic bottles and in eating in plastic containers. shows a high statistically significant relation between phthalate level in mothers' urine and in breast milk with PVC or Vinyl use in walls and in home flooring, while its level in mothers' urine was highly significant in cases of drinking in plastic bottles and in eating in plastic containers.

Keywords: Mono-N- Butyl Phthalate; HPLC; Breast Milk; Urine.

Phthalates[1], or phthalate esters are added to plastics making them more flexible, durable, transparent and long lived; therefore they are used in softening polyvinyl chloride (PVC). Due to low cost, di(2-ethylhexyl) phthalate (DEHP) was the main plasticizer and benzylbutylphthalate (BBP) is used in the manufacture of flooring material. In the US, Canada and European Union, they are replaced by many products due to health concerns where nonphthalate plasticizers are used instead[2]

They are also found in modern electronics, catheters, enteric coatings of pharmaceutical pills, blood transfusion devices as well as nutritional supplements and personal-care products (perfumes, eye shadows, moisturizers, nail polish, liquid soap, hair spray).[3] This leads to high non-occupational

human exposure (ingestion, dermal contactand[4], parenteral exposure).[5]

They are rapidly metabolized and excreted in urine and feces.[4] Monoesters (mono butyl phthalate) are excreted unchanged in urine and feces or may undergo biotransformation to produce more water soluble glucuronide conjugates increasing urinary excretion.[4,6], Monoesters can be measured in urine, breast milk, serum, saliva, seminal plasma and amniotic fluid using High Performance Liquid Chromatography (HPLC).[7,8,9].

During gestation, phthalates may cause male infertility where semen quality and volume are decreased and sperms show increased damaged DNA and decreased motility.[10,11,12,13,14] Exposure may be also associated with diabetes[15,16], breast cancer, obesity, metabolic disorders, and immune function. [17] It may also develop ADHD, autistic behaviors in children.[18,19].

So in our study, detection of Monoesters(mono butyl phthalate) by HPLC is very important to be detected in breast milk and urine of lactating women is important to avoid potential risk of phthalate toxicity to mother as well as her newborn infant.

Aim of the study

Since phthalates are proven harmful to the health of human beings and newborn infants (especially neurodevelopment), so our study is concerned about assessing and detecting phthalates in milk of lactating women in different stages of lactation as milk is the main nutritional source for newborns and infants compared to urinary detection of exposure level of mothers.

METHODOLOGY

Mono-n-Butyl phthalate (MnBp), a metabolite of di-n-butyl phthalate was detected in the breast milk and urine of 20, randomly selected, lactating females house wife's (to exclude occupational exposures) fromHelwan Primary Health Center – Cairo – Egypt.

Written informed consents from all participant in the study with no disclosure for names or any data of patients as we use serial number in all our samples and the consent include acceptance for publication of the data as well as the approval of Human Research Ethical Committee were done. Sample analysis was conducted in The Micro Analytical Center – Faculty of Science – Cairo University.

History

• Personal history à name, age, marital status, special habits of medical importance.

• Medical history à hypertension, diabetes, kidney or liver disease, any neurological symptoms as well as history of blood transfusion or intravenous drug intake.

• Exposure history à smoking (active or passive) and duration of exposure, cosmetic use, drinking in plastic bottles and eating in plastic containers.

• Surgical history.

Laboratory investigations

Comparative assessment ofMnBP in breast milk and urine obtained from 20 lactating females.

• MnBp (>99.9%) à from FLUKA, Inc.

• Methanol, acetonitrile, ethyl acetate and dichloromethane à from Carlo Erba group, Inc. All solvents are HPLC grade.

• â-glucuronidase (*Escherichia coli*-K_{12}) à from Roche Biomedical.

• Water was purified using a direct-Q gradient 8 UV system (Millipore).

Sample collection

Urine Specimens

• Urine specimenswerelabeled with the subject identification number.

• Turbid samples or those containing blood were excluded.

• They were then analyzed by HPLC in the laboratory of Faculty of Science – Cairo University.

• Collected urine samples (pooled from individuals) were stored at -40 °C.

• In this study, plastic equipment's were not used to avoid contamination; all glass apparatuses were washed with chromic acid solution and rinsed with deionized water and methanol before drying.

Breast Milk Specimens

• A single sample was collected from each lactating female.

• To assess average exposure, samples consisted of many small aliquots collected over successive infant feeds up to a volume of 50ml.

• Samples were collected and stored in 250-mL Pyrex glass bottles with Teflon-coated caps.

• Oral and written instructions were given to mothers to feed their baby first before milk aliquot collection (hind milk).

Table 1. English translated questionnaire

Name:	
Age:	
Occupation:	
Present history:	Complaint
System related to complaint	
Past history	Similar problems: • Previous illness (DM, HTN, bronchial asthma, others) • Operations • Blood transfusion • Hospital admissions
Drug history	• Medications or over the counter drugs à regular or irregular intake and duration of intake. • Illicit drug use.
Family history	• Similar problems. • DM, HTN, bronchial asthma.
Social history	• Daily activity. • Smoking, alcohol. • Family.

Potential Sources of Exposure to Phthalates

Occupational Exposure

• PVC plastic factories
• Perfume industry
• Cosmetic industry

Dietary Exposure

	Container type	Frequency/week
• Milk		
• Other dairy products		
• Beverages		
• Fish		
• Fruits/ vegetables		
• Meat and meat products		
• Oils types		

Consumer Products

• Household cleaners 2hours/ week
• Perfumes
• Soaps , shampoos

Material Used in Building

• Home built time period
1969 or earlier
1970-1979
1980-1989
1990-1999
2000-2010
In last year

Flooring material

• Poly vinyl flooring
• Wood

Wall materials

• Wall paper
• Vinyl
• Wood
• Textile

Ventilation system

• Natural
• Mechanical exhaust
• Mechanical exhaust and supply

Medical Exposure

• Chronic need for blood transfusion
• Chronic use of any IV preparations

Do you agree to share in research about phthalate exposure NO YES

• This collection method was chosen to ensure that breastfeeding had been well established beforehand.
• Each sample was frozen consecutively in a household freezer at -20°C in a single glass bottle as additive aliquots and delivered frozen for analysis.
• Breast milk samples with a volume > 50 mL were included in the analyses.[20]

HPLC apparatus

• A high-pressure isocratic system was used, consisting of a DionexUlti Mate 3000 UHPLC; RS pump, auto sampler, column compartment, and Diode Array detector (2012).
• Column C8
• Mobile phase acetonitrile + 0.1% acetic acid = 100% UV/VIS detector
• WL 254nm
• Flow rate 1ml/minute
• Injection volume 20 UL
• Chromatographic column reversed phase 150 mm × 4.6 mm Hypersil BDS, C_{18} particle size 5U.

Solid Phase Extraction

• Hypersep glasses block 16 port vacuum manifolds and vacuum pump ROCKER 400 Thermoscientific.
• SPE columns were purchased from THERMO SCIENTIFIC. HYPERSEP C8 500MG/3ML/50PKG.
• Dimension RxL Max analyzer (Siemens Healthcare GmbH-HenKestr. 127, 91052 Erlangen, Germany) by colorimetric techniques.

Stock Solution

• Stock solution was prepared using 10mg analytical standard added to 10ml of acetonitril.
• Each 1ml of the solution contains 1mg of monobutylphthalate.

Fig. 1. Pump

Fig. 2. Auto Sampler

Fig. 4. Diode Array Detector

Fig. 3. Column Compartment

Fig. 5. BDS Hypersil Column

Sample Pretreatment (Solid Phase Extraction)

• Urine samples were thawed and vortexed homogeneously.

• Each 950-µL urine sample was transferred into a glass tube. Then, 5 µL of â-glucuronidase (200 U/mL) and 245 µL ammonium acetate buffer (1 M, pH 6.5) were added to the tube and vortexed in turn.

• Samples were then incubated at 37 °C for 90 min.[21]

Solid Phase Extraction

• Conditioning àwith 1mL methanol, 1mL acetonitrile, and 1mL phosphate buffer solution (pH 2.0) were added successively.

• Loading à 1mL urine sample was diluted with 1mL phosphate buffer solution (pH 2.0) and added to the SPE column.

• Wash à cartridges were then washed with 2mL formic acid solution (0.1M) and 1mL water. Cartridges were dried under negative pressure. The target analytes were?

• Elution à sequentially with 1mL acetonitrile and 1mL ethyl acetate, the eluent was collected together, concentrated, and evaporated.

• The dry residue was reconstituted with 200 µL of 1:9 (v/v) acetonitrile–water.[22]

Chromatographic conditions

Mobile Phase (0.1% acetic acid in acetonitrile).

• To make 1L, 1.0 mL of acetic acid is added to 1000 mL HPLC grade acetonitrile.

• This solution is stored at room temperature in an amber bottle.

• Column temperature was set at 40 °C.

• Sample injection volume was 20µL.

• Flow rate was 0.3 mL/min.

• UV 254.[23]

Method Validation

The analytical method was validated to demonstrate:

• Linearity

Fig. 6. Vacuum Manifold and Vacuum Pump

Fig. 7. Solid Phase Extraction Column

Fig. 8. Balance

Fig. 9. Milli-Q Water Purification System

• LOD & LOQ
• Accuracy
• Precision

Statistical analysis

• Recorded data was analyzed using the statistical package for social sciences, version 20.0 (SPSS Inc., Chicago, Illinois, USA).
• Quantitative data was expressed as mean ± standard deviation (SD).
• Qualitative data was expressed as frequency and percentage.

The following tests were done:

• Independent-samples t-test of significance was used when comparing between two means.

Distribution of all examined parameters of the study group.

	Total number (n) = 20
Age of mother (years)	17-39 yrs. [27.80±5.70]
Age of infant (months)	2-24 months [9.75±6.89]
PVC, Vinyl use in walls and in home flooring	
Yes	4 (20.0%)
No	16 (80.0%)
Active and passive smoking	
Yes	15 (75.0%)
No	5 (25.0%)
Cosmetic use	
Yes	4 (20.0%)
No	16 (80.0%)
Drinking in plastic bottles	
Yes	14 (70.0%)
No	6 (30.0%)
Eating in plastic containers	
Yes	14 (70.0%)
No	6 (30.0%)
Phthalate level in mothers' urine (mg/L)	0-24.22[12.52±6.65]
Phthalate level in breast milk (mg/L)	0.02-8.80[2.51±2.92]

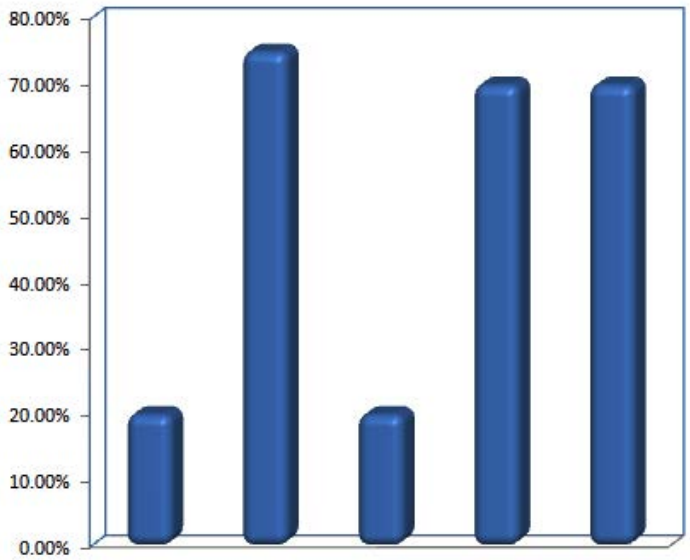

Fig. 10. Distribution of the study groups. A →PVC or Vinyl use in walls and in home flooring; B → active and passive smoking; C →cosmetic use; D →drinking in plastic bottles; E →eating in plastic containers.

• Pearson's correlation coefficient (r) test was used to assess the degree of association between two sets of variables
• The confidence interval was set to 95% and the margin of error accepted was set to 5%. So, the p-value was considered significant as the following (probability: P-value):
• $P \leq 0.05$ à significant.
• $P \leq 0.001$ à highly significant.
• $P > 0.05$ à insignificant.

RESULT

As observed in table (2), the mean age of mothers (years) was 17-39 years [27.80±5.70], the mean age of infants in months was 2-24 months [9.75±6.89], number of those using PVC and/or Vinyl in home walls and flooring are 4 (20.0%), number of active and passive smokers was 15 (75.0%), those using cosmetics were 4 (20.0%), mothers drinking in plastic bottles were 14 (70.0%) while those eat in plastic containers were 14 (70.0%). Mean phthalate level in mothers' urine was 0-24.22µg/l [12.52±6.65] and in breast milk 0.02-8.80µg/l [2.51±2.92].

As seen in table (3), there was a positive correlation and a statistical significance between phthalate level in mothers' urine in relation to their age and a significant correlation was detected between phthalate level in breast milk and in mothers' urine (µg/L).

Table (4) shows a high statistically significant relation ($p<0.001$) between phthalate level in mothers' urine and in breast milk with PVC

Table 3. Correlation between phthalate level in mothers' urine (µg/L) and in breast milk (µg/L) with all parameters, using Pearson Correlation Coefficient of the study group

		Phthalate level in mother urine (µg/L)	Phthalate level in breast milk (µg/L)
Age of mother (years)	r	0.381	0.518
	p-value	0.098	0.019*
Age of infant (months)	r	0.334	0.244
	p-value	0.150	0.299
Phthalate level in mother urine (µg/L)	r	—	0.459
	p-value	—	0.028*

r-Pearson Correlation Coefficient;
p-value>0.05 NS; *p-value <0.05 S; **p-value <0.001 HS

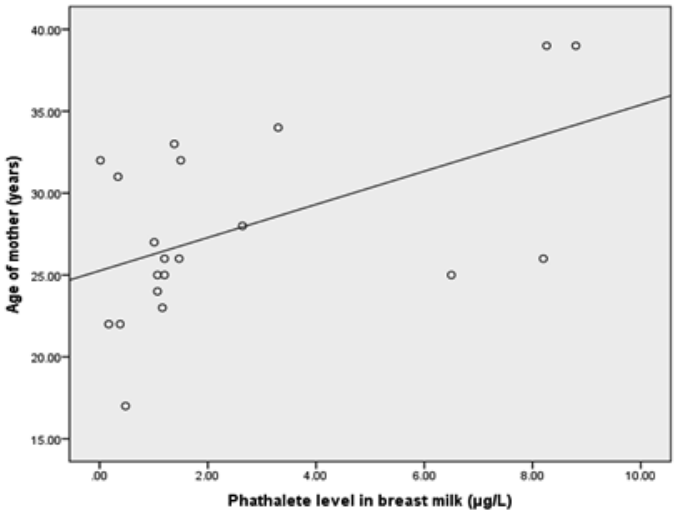

Fig. 11. Scatter plot between Phthalate level in breast milk and age of mothers

Table 4. Relation between phthalate level in mothers' urine (mg/L) and in breast milk (mg/L) with PVC or Vinyl use in walls and in home flooring, active and passive smoking, cosmetic use, drinking in plastic bottles and eating in plastic containers of the study group

Parameters		Phthalate level in mother urine (µg/L) Mean	±SD	Phthalate level in breast milk (µg/L) Mean	±SD
PVC or Vinyl use in walls	Yes	22.25	3.00	6.69	3.47
and in home flooring	No	10.09	4.77	1.46	1.59
Independent Sample t-test	t-test	23.089	21.225		
	p-value	<0.001**	<0.001**		
Active and Passive smoker	Yes	13.25	6.70	2.58	3.11
	No	10.35	6.72	2.29	2.53
Independent Sample t-test	t-test	0.699	0.037		
	p-value	0.414	0.850		
Cosmetic use	Yes	13.71	8.48	1.29	0.19
	No	12.23	6.42	2.81	3.21
Independent Sample t-test	t-test	0.152	0.868		
	p-value	0.702	0.364		
Drink in plastic bottles	Yes	15.49	5.27	2.81	3.16
	No	5.60	3.70	1.81	2.36
Independent Sample t-test	t-test	17.213	0.474		
	p-value	<0.001**	0.500		
Eat in plastic containers	Yes	15.49	5.27	2.81	3.16
	No	5.60	3.70	1.81	2.36
Independent Sample t-test		17.213	0.474		
		*<0.001***	*0.500*		

p-value>0.05 NS; *p-value <0.05 S; **p-value <0.001 HS

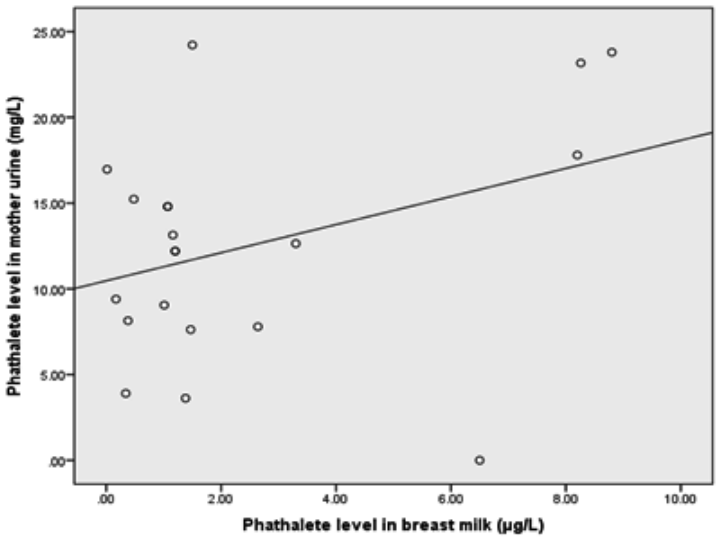

Fig. 12. Scatter plot between phthalate level in breast milk and in mothers' urine (µg/L)

or Vinyl use in walls and in home flooring, while its level in mothers' urine was highly significant ($p<0.05$) in cases of drinking in plastic bottles and in eating in plastic containers.

DISCUSSION

In this study, 20 lactating females were randomly selected from Helwan – Cairo – Egypt, with mean age of mother 17-39 years [27.80±5.70] and mean age of infants 2-24 months [9.75±6.89]. Mothers having PVC and/or Vinyl in walls and in home flooring were 4 (20.0%), active and passive smokers were 15 (75.0%), those using cosmetics were 4 (20.0%), mothers drinking in plastic bottles were 14 (70.0%) while those eating in plastic containers were 14 (70.0%).

Mean phthalate level in mothers urine was 0-24.22 µg/l [12.52±6.65] and in breast milk was 0.02-8.80 µg/l [2.51±2.92].

There was a positive correlation and a statistical significance between phthalate level in mothers' urine in relation to their age and a significant correlation was detected between

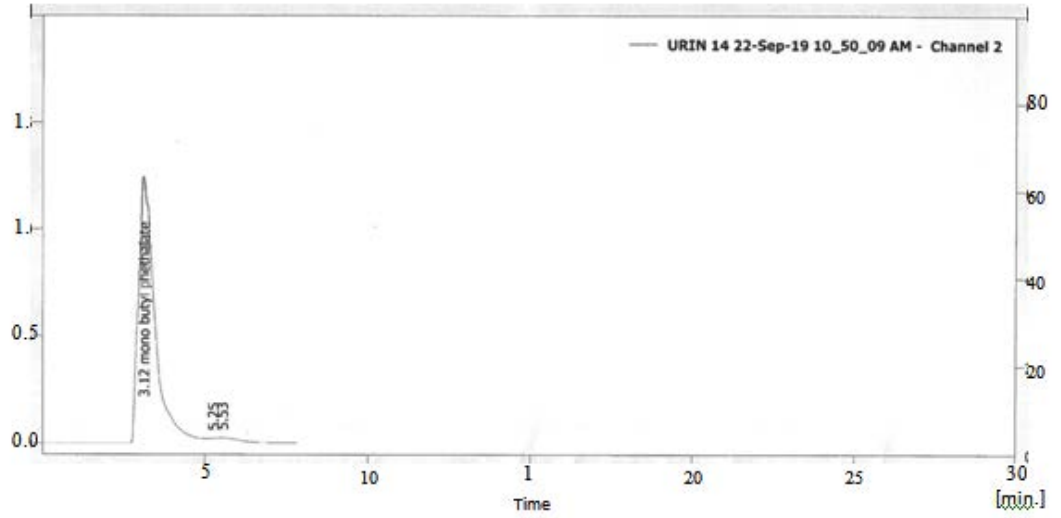

Fig. 13. A curve showing an example for HPLC result of mono-n-butyl phthalate level in urine in sample number 14

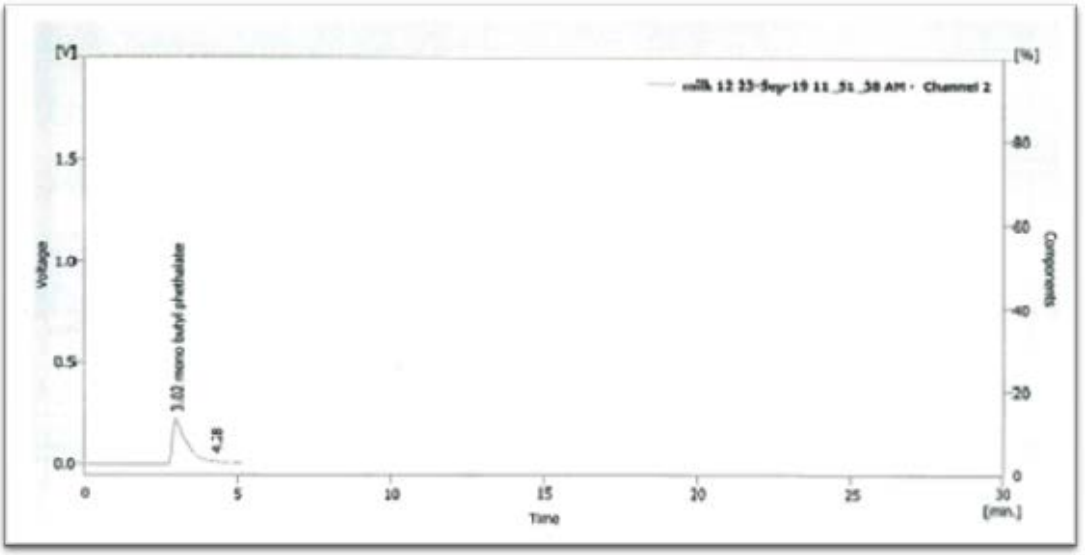

Fig. 14. A curve showing an example for HPLC result of mono-n-butyl phthalate level in milk in sample number 12

phthalate level in breast milk and in mothers' urine (μg/L).

The current study shows a high statistically significant relation between phthalate level in mothers' urine as compared to its level in breast milk using PVC or Vinyl in the walls and home flooring. This goes with Allan C. Just *et al*.,(2015) which showed an increase in urinary metabolites of phthalate in Vinyl flooring in homes with an increased risk of bronchial asthma.[24]

Phthalate levels were highly significant in the urine of mothers drinking in plastic bottles and eating in plastic containers which was consistent with Dong Rui Hua et., al (2017) which showed that diet was a major exposure source for phthalates and that it is likely that plastic containers contributed to phthalate contamination of foods.[25]

On the other hand, results were not consistent withRose O. Sulentic *et al*., (2018) who did not identify water consumption or consumer product use as major sources contributing to phthalate exposure.[26]

No significant correlation was observed between active and passive smoking and cosmetic use with mono-butyl-phthalate level in mothers' urine and in breast milk samples; on the contrary, Lauren E. Parlett and colleagues (2013) showed that personal care products (PCPs) use was widespread in this group of recently pregnant females. Female's use of PCPs, particularly perfumes and fragrance products, was positively associated with urinary concentration of multiple phthalate metabolites[27], that mostly explained that in our study random sample of lactating females where only 20% were using cosmetics like shampoos, perfumes in a sporadic manner in a rate of once per week or less.

CONCLUSION

Inour study which was conducted on 20 lactating mothers in Egypt for assessment of mono-butyl-phthalate in breast milk and urine, phthalate level in mothers' urine and in breast milk showed a highly significant increase with PVC or Vinyl use in walls and in home flooring, while the level in mothers' urine was highly significant in mothers drinking in plastic bottles and eating in plastic containers. For further research for better health of lactating mothers and their fetuses, especially that phthalate is highly toxic material to both mother and fetus.

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