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AN EASY SCREENING THROUGH*IN SILICO* STUDY FOR PREDICTIVE TOXICITY MECHANISMS OF DIFFERENT PHTHALATE COMPOUNDS BY USING ONLINE TOOL (PROTOX-II WEBSERVER)

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ABSTRACT

The phthalate compounds (PCs) are well-known plasticizers and easily exposed through environment. The present objective was an *in silico* study to detect toxicity mechanisms of common phthalates by using ProTox-II webserver. Different types of common PCs were selected as per recent literatures study. Total 14 nos. of PCs were selected for present predictive study. These PCs such as DEHP, DINP, DIDP, DPHP, DMP, DEP, BBP, MBP, PA, DNPP, DCHP, DAP, DNHP and DHP were studied. The prediction of different toxicity mechanisms was done by using ProTox-II webserver. The mechanism of toxicity of PCs indicated 10 compounds were obtained between the class of IV and V while 4 compounds were found class VI. The hepatotoxicity and immunotoxicity results were observed inactive for all compounds. All the compounds were found cytotoxic and mutagenic inactive, but 8 compounds obtained carcinogenic active.TheTox21-nuclear receptor signalling pathways revealed AhR, AR, AR-LBD, Aro, ER, ER-LBD, PPAR-Gamma were inactive except 1 compound active for ER and ER-LBD. For Tox21-stress response pathways, it was observed that 2 compounds were active for nrf2/ARE and HSE. The parameter MMP was active only for 1 compound. Other two parameters viz. p53 and ATAD5 obtained all the compounds were inactive. In conclusion, the present predictive results indicated that few PCs are harmful to animals and scattered information on toxicity mechanisms by few compounds found for human studies. This prediction may be suitable for further *in vitro* and *in vivo* research works in future to validate the present prediction.

Keywords: *In silico* study, Phthalate compounds, Plasticizers, Predictive toxicology, Mechanism of toxicity, Nuclear receptor signaling, Stress response pathways

1. INTRODUCTION

Phthalate derivatives are used for the manufacturing of plastic materials. On the other hand, in present days, plastics are used to make toys, container for blood and several liquid medicines, potable water, raw and cooked food materials, etc. [1-5]. According to the researchers, phthalates are not covalently bound to plastics and it has tendency to leach into the medium [4, 6-10].

It has been well-established that these phthalates cause several types of cancer, endocrine disruption, teratogenicity, etc. [5, 10-12]. An informative research work revealed that the higher energy intake in the overweight and obese due to higher di-2-ethylhexyl phthalate (DEHP) exposure, which indicated close relationship between body mass index and DEHP[13].In another study it was observed positive correlations between serum dibutyl phthalate (DBP) or mono(2-ethylhexyl) phthalate (MEHP), and serum estradiol (E2) and/or luteinizing hormone (LH) in prepubescent children while serum monobutyl phthalate (MBP) levels were found to be negatively correlated with serum triiodothyronine (T3) or thyroxine (T4) in male participants, and serum DEHP levels with serum thyroid stimulating hormone (TSH) in female adolescents. Low-density lipoprotein (LDL) levels were positively correlated with serum phthalic acid (PA) levels in children and adolescents. DEHP, DBP or its metabolites may be associated with altered hormone levels in Korean children and adolescents [14].

In earlier research work, researchers have been studied individual phthalate or multiple phthalates to determine health impact in relation to particular parameter such as toxicity, carcinogenicity especially particular cancer type, teratogenicity, endocrine disruption, etc. on human and/or mammals, which was observed long duration, financial burden as well as animal harming, etc.

But *in silico* study through computational simulation by using online tool is an achievement for faster screening of several compounds within an hour with many parameters of toxicological mechanisms to obtain narrow range of toxic compounds and also fulfil the prioritization of regulatory agencies [15-17].

Present *in silico* study was to predict rat oral acute toxicity, hepatoxicity, immunotoxicity, genetic toxicity endpoints, nuclear receptor signalling, and stress response pathways of different phthalates by using ProTox-II webserver.

2. MATERIAL AND METHODS

2.1. Selection of compounds

Different types of phthalate compounds (PCs) were selected as per recent literatures [4-5, 10]. Total 14 nos. of PC were selected for present predictive study. These PCs such as Di(2-ethylhexyl) phthalate (DEHP), Diisononyl phthalate (DINP), Diisodecylphthalate (DIDP), Di(2-propylheptyl) phthalate (DPHP), Dimethyl phthalate (DMP), Diethyl phthalate (DEP), Butylbenzyl phthalate (BBP), Monobutyl phthalate (MBP), Phthalic acid (PA), Di-n-pentyl phthalate (DNPP), Dicyclohexyl phthalate (DCHP), Diallyl phthalate (DAP),Di-n-hexyl phthalate (DNHP) and Diheptyl phthalate (DHP) were studied. The structure of all the compounds are depicted in Fig. 1 (obtained from ProTox-II webserver).

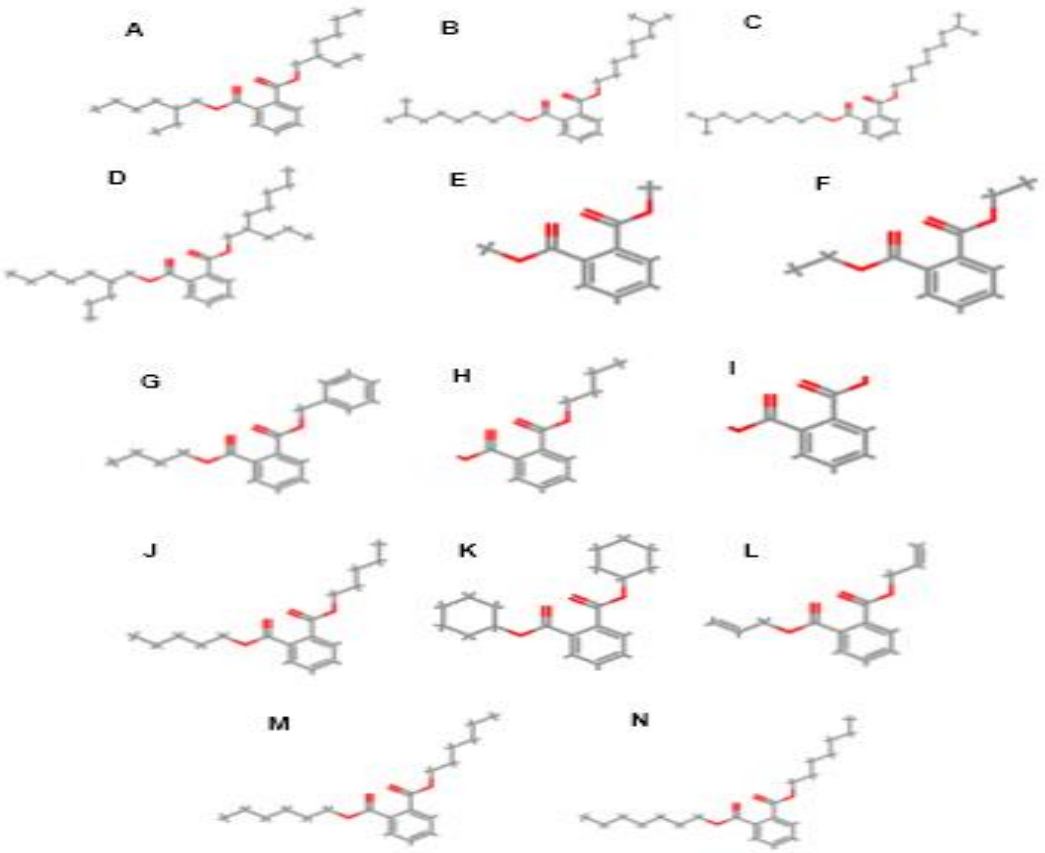

Fig. 1: Structure of studied compounds (A =DEHP; B = DINP; C = DIDP; D = DPHP; E = DMP; F = DEP; G = BBP; H = MBP; I = PA; J = DNPP; K = DCHP; L = DAP; M = DNHP and N = DHP)

2.2. *In silico* study for toxicity mechanisms of PCs

According to Banerjee et al. [18], the ProTox-II platform is classified into a five different steps such as(1) oral acute toxicity prediction models per six different toxicity classes mentioned by Drwal et al. [19]; (2) organ toxicity model for hepatotoxicity prediction;(3) immunotoxicity model and geno-toxicological (cytotoxicity, mutagenicity and carcinogenicity model) endpoints; (4) toxicological pathways such as nuclear receptor signalling pathways is classified seven target-pathway based models viz. aryl hydrogen receptor (AhR), androgen receptor (AR), androgen receptor ligand binding domain(AR-LBD), aromatase, estrogen receptor alpha (ER), estrogen

receptor ligand binding domain (ER-LBD), and peroxisome proliferator activated receptor gamma (PPARGamma) as well as stress response pathways is classified five target-pathway based models such as nuclear factor(erythroid-derived 2)-like 2/antioxidant responsive element(ARE), heat shock factor response element (HSE), mitochondrial membrane potential (MMP), phosphoprotein tumor suppressor (p53), and ATPase family AAAdomain-containing protein 5 (ATAD5) and toxicity targets model of 14 nos. All the predictive models for toxicology pathways have been implemented as toxicology in the 21st Century (Tox21), which is a federal collaboration among United States Environmental Protection Agency (EPA), National Institute of Health (NIH), including National Center for Advancing Translational Sciences, and the National Toxicology Program at the National Institute of Environmental Health Sciences, and the Food and Drug Administration [20].

3. RESULTS

The results of different derivatives of phthalates obtained the predictive rat oral acute toxicity (LD_{50}) values (mg/Kg) along with activity or inactivity on liver toxicity, immunotoxicity, genetic toxicity end points viz. cytotoxicity, mutagenicity and carcinogenicity as well as toxicological pathways (nuclear receptor signalling and stress response).

Table 1: Prediction of oral acute toxicity, class and accuracy of different PCs

Compounds name	Oral LD_{50} value (mg/Kg)	Predicted toxicity class	Prediction accuracy (%)
DEHP	1340.0	IV	100
DINP	1340.0	IV	100
DIDP	1340.0	IV	100
DPHP	1340.0	IV	100
DMP	1850.0	IV	100
DEP	6172.0	VI	100
BBP	2330.0	V	100
MBP	3474.0	V	100
PA	2530.0	V	100
DNPP	26000.0	VI	72.9
DCHP	10000.0	VI	72.9
DAP	656.0	IV	100
DNHP	10000.0	VI	100
DHP	1340.0	IV	100

Class IV: harmful if swallowed ($300 < LD_{50} \leq 2000$); Class V: may be harmful if swallowed ($2000 < LD_{50} \leq 5000$) and Class VI: non-toxic ($LD_{50} > 5000$)

In Table 1, the rat oral acute toxicity (LD_{50}) as mg/Kg, predicted different toxicity classes (IV–VI) and prediction accuracy in % for studied compounds. The (LD_{50}) values (mg/Kg) for all studied compounds were obtained 1340 for DEHP, DINP, DIDP, DPHP and DHP; 1850 for DMP and 656 for DAP as class IV ($300<LD_{50}\leq2000$, prescribed harmful if swallowed) while 2330 for BBP; 3474 for MBP and 2530 for PA as class V ($2000<LD_{50}\leq5000$, prescribed may be harmful if swallowed) but 6172 for DEP, 10000 for DCHP and DNHP and 26000 for DNPP as class VI ($LD_{50}>5000$ prescribed non-toxic). All the studied compounds were obtained 100% prediction accuracy except two compounds viz. DNPP and DCHP showed 72.9% prediction accuracy.

The prediction of hepatotoxicity and immunotoxicity results were observed inactive for all 14 compounds. The hepatotoxicity probability score (%) for DNHP and DHP (84); DEHP and DPHP (82); DINP, DIDP and DNPP (79); DEP (77); MBB (69); BBP (68); DAP (66); PA (65); DMP (64) and DCHP (0.59) respectively were obtained. In case of immunotoxicity end points,all of these studied compounds were obtained probability score (%) for DMP, DEP, MBP, PA and DAP (99); BBP and DCHP (98); DEHP and DPHP (97); DINP and DIDP (95); DNPP (81) and DNHP and DHP (69) respectively (Table 2).

Table 2: Prediction of hepatotoxicity and immunotoxicity end points of different PCs

Compounds name	H	P	I	P
DEHP	I	0.82	I	0.97
DINP	I	0.79	I	0.95
DIDP	I	0.79	I	0.95
DPHP	I	0.82	I	0.97
DMP	I	0.64	I	0.99
DEP	I	0.77	I	0.99
BBP	I	0.68	I	0.98
MBP	I	0.69	I	0.99
PA	I	0.65	I	0.99
DNPP	I	0.79	I	0.81
DCHP	I	0.59	I	0.98
DAP	I	0.66	I	0.99
DNHP	I	0.84	I	0.69
DHP	I	0.84	I	0.69

H = Hepatotoxicity; I = Immunotoxicity; I = Inactive; A = Active and P = Probability

Table 3: Prediction of genetic toxicity end points of different PCs

Compounds name	Cy	P	Mug	P	Ca	P
DEHP	I	0.87	I	0.99	A	0.86
DINP	I	0.86	I	0.99	A	0.85
DIDP	I	0.86	I	0.99	A	0.85
DPHP	I	0.87	I	0.99	A	0.86
DMP	I	0.93	I	0.89	I	0.81
DEP	I	0.92	I	0.87	I	0.65
BBP	I	0.91	I	0.92	A	0.52
MBP	I	0.89	I	0.89	I	0.60
PA	I	0.90	I	0.99	I	0.69
DNPP	I	0.89	I	0.95	A	0.72
DCHP	I	0.84	I	0.93	I	0.69
DAP	I	0.92	I	0.91	I	0.80
DNHP	I	0.89	I	0.99	A	0.75
DHP	I	0.89	I	0.99	A	0.75

Cy = Cytotoxicity; Mug = Mutagenicity; Ca =Carcinogenicity; I = Inactive; A = Active and P = Probability

The prediction of genotoxicity endpoints especially cytotoxicity, mutagenicity and carcinogenicity were studied. All the studied 14 compounds were found cytotoxic and mutagenic inactive. The cytotoxic probability scores (%) for DMP (93); DEP and DAP (92); BBP (91); PA (90); MBP, DNPP, DNHP and DHP (89); DPHP and DEHP (87); DINP and DIDP (86) and DCHP (84) respectively and the mutagenic probability scores (%) for DEHP,DINP, DIDP, DPHP, PA, DNHP and DHP (99); DNPP (95); DCHP (93); BBP (92); DAP (91); DMP and MBP (89) and DEP (87) respectively were obtained. For carcinogenicity prediction, few compounds such as DEHP, DPHP, DINP, DIDP, DNHP, DHP, DNPP and BBP were obtained active with probability score (%) 86; 85; 75; 72 and 52 while rest compounds such as DMP, DAP, PA and DCHP; DEP and MBP were obtained inactive with probability score (%) 81; 80; 69; 65 and 60 respectively (Table 3).

For Tox21-nuclear receptor signalling pathways, several parameters such as AhR, AR, AR-LBD, Aro, ER, ER-LBD and PPAR-Gamma were predicted for 14 phthalates (Table 4). All the studied 14 compounds such as DINP, DIDP, DNHP and DHP; DEHP, DPHP, DEP, PA, DNPP and DAP; MBP; DMP; BBP and DCHP were observed Ahr inactive with probability scores (%) 100; 99; 98; 97; 96 respectively. All the studied 14 compounds such as DINP, DIDP, DEP, DMP, MBP, DNPP, DNHP and DHP; DEHP, DPHP, PA, BBP and DAP and DCHP were observed AR inactive with probability scores (%) 100; 99; 98 and 97 respectively. For AR-LBD prediction, all compounds were found inactive with probability scores (%) for DEHP, DINP, DIDP, DPHP, DEP, MBP, DNPP, DNHP and DHP (100); DMP, BBP, PA and DAP (99) and DCHP (98) respectively. Another parameter Aro prediction, all compounds were also observed inactive with probability score (%) 100 for all 10 compounds but 99 for 2 compounds, 98 and 96 for BBP and DCHP compound. For ER, all compounds were inactive except BBP and probability score (%) 100 for BBP, DNHP and DHP; 99 for DEHP, DINP, DIDP, DPHP, DMP, PA and DNPP; 97 for DAP and 92 for DCHP respectively. For ER-LBD, all compounds were inactive except BBP and probability score (%) 100 for DEHP, DINP, DIDP, DPHP, DMP, PA, DNHP and DHP; 99 for DEP, DNPP and DAP; 96 for DCHP and 94 for MBP respectively. For PPAR-Gamma prediction, all compounds were inactive and probability score (%) 100 for DINP, DIDP, DMP, PA, DNHP and DHP; 99 for DEP and DNPP; 98 for DEHP and DPHP; 95 for BBP; 92 for MBP; 91 for DAP and 83 for DCHP respectively.

Table 4: Prediction of Tox21-nuclear receptor signalling pathways of different PCs

Compounds name	Tox21-Nuclear receptor signalling pathways													
	Ahr	P	AR	P	AR-LBD	P	Aro	P	ER	P	ER-LBD	P	PPAR-Gamma	P
DEHP	I	0.99	I	0.99	I	1.0	I	1.0	I	0.99	I	1.0	I	0.98
DINP	I	1.0	I	1.0	I	1.0	I	1.0	I	0.99	I	1.0	I	1.0
DIDP	I	1.0	I	1.0	I	1.0	I	1.0	I	0.99	I	1.0	I	1.0
DPHP	I	0.99	I	0.99	I	1.0	I	1.0	I	0.99	I	1.0	I	0.98
DMP	I	0.97	I	1.0	I	0.99	I	1.0	I	0.99	I	1.0	I	1.0
DEP	I	0.99	I	1.0	I	1.0	I	1.0	I	0.92	I	0.99	I	0.99
BBP	I	0.97	I	0.99	I	0.99	I	0.98	A	1.0	A	1.0	I	0.95
MBP	I	0.98	I	1.0	I	1.0	I	0.99	I	0.89	I	0.94	I	0.92
PA	I	0.99	I	0.99	I	0.99	I	1.0	I	0.99	I	1.0	I	1.0
DNPP	I	0.99	I	1.0	I	1.0	I	1.0	I	0.99	I	0.99	I	0.99
DCHP	I	0.96	I	0.98	I	0.98	I	0.96	I	0.92	I	0.96	I	0.83
DAP	I	0.99	I	0.99	I	0.99	I	0.99	I	0.97	I	0.99	I	0.91
DNHP	I	1.0	I	1.0	I	1.0	I	1.0	I	1.00	I	1.00	I	1.0
DHP	I	1.0	I	1.0	I	1.0	I	1.0	I	1.00	I	1.00	I	1.0

AhR = Aryl hydrocarbon Receptor; AR = Androgen receptor; AR-LBD = Androgen Receptor Ligand Binding Domain; Aro = Aromatase; ER = Estrogen Receptor Alpha; ER-LBD = Estrogen Receptor Ligand Binding Domain; PPAR-Gamma = Peroxisome Proliferator Activated Receptor Gamma; I = Inactive; A = Active and P = Probability

Table 5: Prediction of Tox21-stress response pathways of different phthalates

Compounds name	Tox21-Stress response pathways									
	nrf2/ARE	P	HSE	P	MMP	P	p53	P	ATAD5	P
DEHP	I	0.99	I	0.99	I	0.99	I	1.0	I	1.0
DINP	A	0.84	A	0.84	I	1.0	I	1.0	I	1.0
DIDP	A	0.84	A	0.84	I	1.0	I	1.0	I	1.0
DPHP	I	0.99	I	0.99	I	0.99	I	1.0	I	1.0
DMP	I	1.0	I	1.0	I	0.99	I	0.99	I	0.99
DEP	I	0.99	I	0.99	I	1.0	I	0.99	I	0.99
BBP	I	0.94	I	0.94	I	0.97	I	0.98	I	0.97
MBP	I	0.99	I	0.99	I	0.96	I	0.99	I	0.97
PA	I	1.0	I	1.0	I	0.99	I	1.0	I	1.0
DNPP	I	0.99	I	0.99	I	0.99	I	1.0	I	0.99
DCHP	I	0.95	I	0.95	A	0.94	I	0.97	I	0.96
DAP	I	0.96	I	0.96	I	0.99	I	0.98	I	0.96
DNHP	I	0.99	I	0.99	I	1.0	I	1.0	I	1.0
DHP	I	0.99	I	0.99	I	1.0	I	1.0	I	1.0

nrf2/ARE = Nuclear factor (erythroid-derived 2)-like 2/antioxidant responsive element; HSE = Heat shock factor response element; MMP = Mitochondrial Membrane Potential; p53 = Phosphoprotein (tumour supressor); ATAD5 = ATPase family AAA domain-containing protein 5; I = Inactive; A = Active and P = Probability

The Tox21-stress response pathways such as nrf2/ARE, HSE, MMP, p53 and ATAD5 were predicted for all compounds (Table 5). For nrf2/ARE and HSE, 2 compounds viz. DINP and DIDP were active and probability score (%) was obtained 84 while other compounds were inactive and probability score (%) 100 for DMP and PA; 99 for DEHP, DPHP, DEP, MBP, DNPP, DNHP and DPP; 96 for DAP; 95 for DCHP and 94 for BBP respectively. For MMP, 1 compound namely DCHP was active and probability score (%) obtained 94

while rest compounds were inactive and probability score (%) 100 for DINP, DIDP, DEP, DNHP and DHP; 99 for DEHP, DPHP, DMP, PA, DNPP and DAP; 97 for BBP; 96 for MBP and 94 for DCHP respectively. For p53 and ATAD5, all studied compounds were inactive and probability score (%) 100 for DEHP, DINP, DIDP, DPHP, PA, DNHP and DHP; 99 for DMP, DEP but 98 and 97 for BBP, 99 and 97 for MBP, 100 and 99 for DNPP, 97 and 96 for DCHP and 98 and 96 for DAP respectively.

4. DISCUSSION

The mechanism of toxicity of phthalates indicated 10 compounds were obtained between the class of IV and V (harmful and may be harmful if swallowed) while 4 compounds were found class VI (non-toxic). These toxicity classes have been prescribed by Drwal et al. [19] in ProTox-II webserver. It has been reported that few phthalates showed lower value of toxicity in rodents [21-25].Interestingly, all compounds were predicted non-hepatotoxic or hepatotoxic inactive but chronic exposure through oral route by DEHP in rodents may cause hepatomegaly due to hyperplasia and hypertrophy of liver parenchymal cells [25-26].

The toxicity mechanism of DEHP in hepatocytes revealed several actions such as activation of PPARα, induction of peroxisomal proteins and proliferation of peroxisomes, nonperoxisomal metabolism proteins, cell proliferation, suppression of apoptosis, formation of reactive oxygen species, oxidative DNA damage, and inhibition of gap junctional intercellular communication [25, 27]. In present prediction, all the compounds were obtained non-immunotoxic or immunotoxic inactive, which has close similarities in other reports [28-29].

The prediction of genotoxicity endpoints especially cytotoxicity, mutagenicity and carcinogenicity were revealed all the compounds were cytotoxic and mutagenic inactive but 8 compounds such as DEHP, DPHP, DINP, DIDP, DNHP, DHP, DNPP and BBP were obtained carcinogenic active. According to López-Carrillo et al. [12], it was found that exposure to DEP and its metabolite as urinary MEP concentrations could be associated with breast cancer risk (BCR) while the exposure to other phthalates and metabolite in the urinary concentrations as MBP and BBP might be negatively associated with BCR. On the other hand, Hardell et al. [30] reported exposure to DEHP causes different types of cancer and they observed testicular cancer. Phthalates such as DEHP and BBP were reported carcinogenic as per experimentation in animals and possibilities as human carcinogens [5].

Among all studied compounds, only BBP was observed ER and ER-LBD active. This prediction is supported by Chen et al. [10].Their findings revealed that lower concentrations of phthalates interfere with the effects of 17β-oestradiolon the growth of MCF-7 breast cancer cells and BBP, DBP and DEHP significantly enhanced the cell proliferation. Beside these, phthalates are well-established an endocrine disrupter due to their estrogenicand antiandrogenic activities [10]. It was also considered that phthalates may activate steroid hormone dependent cancer viz. breast cancer, etc.[12].

In present prediction, DINP and DIDP were obtained active for nuclear factor (erythroid-derived-2)-like2antioxidant signaling (nrf2/ARE) and heat shock transcription factor responsive element (HSE) as disruptor of antioxidant capacity lead to oxidative stress [31-34] as well as expression of a variety of genes, which involved in cell survival, protein chaperones, the protein degradation machinery, anti-apoptotic proteins, and transcription factors. Ultimately, lead to neurodegenerative disease and cancer [33, 35-38]. Another stress response pathway i.e. mitochondrial membrane potential (MMP), 1 compound namely DCHP was obtained active. Mitochondria provides the energy to the cell due to the presence of double membrane, which provides oxidative phosphorylation and prevent apoptosis [39]. Pariskh et al. [40] stated that yeast mitochondria adapt a mitochondria-to-nucleus signal transduction pathway termed the retrograde response to induce the transcription of nuclear-encoded mitochondrial genes, which prevent mitochondrial stress. It was known that mitochondrial stress by toxins may lead to various diseases [41]. Recently, Richter et al. [42] reported that toxins inhibit the mitochondrial protein synthesis and block with the stress response. Other two stress response parameters viz.p53 or Phosphoprotein (tumour suppressor) and ATPase family AAA domain-containing protein 5 (ATAD5) observed inactive for all the studied compounds. The p53 gene controls the cell cycle arrest, carcinogenesis, DNA damage, apoptosis, etc. but in the present prediction inactivity for both parameters contradicted the results of carcinogenesis of 8 compounds obtained in Table 3.

5. CONCLUSION

It is concluded from the present predictive results that few phthalates are harmful to animals and scattered

information on toxicity mechanisms by few compounds found for human studies. Moreover, the study of predictive toxicity mechanisms by using ProTox-II online server has been used by many researchers to screen easily several organic compounds within short duration [18, 20, 43-44]. This easy screening and prediction of PCs may be suitable for further *in vitro* and *in vivo* research works in future. This web server helps faster screening of large number of compounds and per compound within a minute, without financial burden and animal testing. This study is suggested further experimental analysis to validate the present prediction.

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Conflict of Interest:

No conflict of interest in the present study.

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