

# Effect of 3 $\beta$ , hydroxy-lup-20(29)-en-28-oic acid on 7,12-Dimethylbenz(a)anthracene impaired cellular homeostasis in extrahepatic organs of Sprague Dawley rats

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## Abstract

3 $\beta$ , hydroxy-lup-20(29)-en-28-oic acid (Betulinic acid), a pentacyclic lupane-type triterpene has diverse pharmacological functions both *in vitro* and *in vivo*. The present study focuses its protective effect on polycyclic aromatic hydrocarbon (7,12-Dimethylbenz(a)anthracene or DMBA) induced alterations in membrane bound ATPases, detoxification enzymes and antioxidant enzymes in stomach and lungs of female Sprague Dawley rats. Healthy female Sprague Dawley rats were randomly assorted into six groups and the treatments were given orally for 7 weeks on alternate days. It was observed that betulinic acid facilitated the downregulation of elevated membrane bound ATPases (Na<sup>+</sup>/K<sup>+</sup>-ATPase, Ca<sup>2+</sup>-ATPase and Mg<sup>2+</sup>-ATPase) in DMBA administered rats. Likewise, the detoxification enzymes as well as antioxidant enzymes were modulated to normalcy in rats. Overall, betulinic acid was seen to be effective modulator of DMBA induced alterations in biochemical parameters.

## Introduction

Xenobiotics are responsible for a varied degree of stress in humans and animals. The exposure to xenobiotics may occur deliberately (food additives), accidentally (food contaminants and pesticides) or coincidentally (industrial chemicals and environmental pollutants/mutagens) which lead to oxidative stress triggering spread of various diseases and cancer.<sup>1,2</sup> Thus, the myriad of enzyme systems have evolved to assist the disposition of xenobiotics and to retain cellular homeostasis. Xenobiotic metabolizing enzymes in the extrahepatic tissues of ani-

mals serve the additional purpose of detoxification defence against toxins that mainly act on these organs. In addition, liver diseases (cirrhosis, infection, necrosis *etc.*) or the damage of organ compromise the role of liver by shifting the dependence for drug disposition to extrahepatic tissues.<sup>3</sup> The gastrointestinal tract (GIT) is the second major site in the body for detoxification. It is the main portal of entry for a wide array of compounds including xenobiotics as most of the contaminants and pollutants are consumed orally. During the entire life span, gastrointestinal tract processes more than 25 tons of food which correspond to the largest load of xenobiotics confronting the body parts.<sup>4</sup> Nearly 75% of the blood reaching the liver arrives directly from gastrointestinal organs and then spleen *via* hepatic portal vein.<sup>5</sup> The lungs can absorb, modify and excrete out the foreign substances and drugs.<sup>6</sup> They are situated at the air/blood interface and play a noteworthy role in the elimination of variety of compounds.<sup>7</sup> The complex architecture of lungs reflects over 40 different cell types with remarkable diversity in cell functionality and is capable of extracting drugs from the blood circulation because all the cardiac output goes through it.<sup>8,9</sup>

7,12-Dimethylbenz(a)anthracene (DMBA) is a class of polycyclic aromatic hydrocarbon (PAH). It is absorbed mainly through the skin, respiratory, intestinal tracts; by ingestion and inhalation.<sup>10</sup> The induction of gastric cancer in rats by injecting DMBA in the glandular stomach has been reported in literature.<sup>11</sup> Previous reports have also shown the DMBA induced tumor formation in forestomach of mouse.<sup>12</sup> It was also found that lungs showed occasional tumors after intraperitoneal injection of DMBA.<sup>13</sup> In other reports, DMBA has been found to induce neoplasms in the mammary gland, liver, heart and lungs after being metabolically activated.<sup>14</sup>

3 $\beta$ , hydroxy-lup-20(29)-en-28-oic acid (Betulinic acid), a pentacyclic lupane-type triterpene is a phytochemical agent and has various botanical sources like *Betula alba*, *Tryphyllum peltatum*, *Ancistrocladus heyneanus*, *Diospyros leucomelas*, *Tetracera boliviana*, *Zizyphus joazeiro*, *Syzygium formosanum* *etc.* Various fruits and vegetables are also a good source of this compound.<sup>15-17</sup> Betulinic acid is known for various biological activities including anti-inflammatory, anti-viral, anti-neoplastic, anti-tumor, and anti-HIV.<sup>18-22</sup> Its anti-tumor activity has also been reported in human neoplastic cell lines including lung, ovarian, cervical, colorectal, breast and prostate.<sup>23</sup> Induction of apoptosis *via* direct mitochondrial alterations, formation of reactive oxygen

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Contributions: PK carried out the biochemical alteration study in stomach and lung of female Sprague Dawley rats, performed the statistical analysis and drafted the manuscript. RK conceived of the study, participated in its design and helped to draft the manuscript. RA analyzed the data and helped to draft the manuscript. SA contributed reagents/materials/analysis tools, design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Conflict of interest: the authors declare no conflict of interest.

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species, modulation of BCL-2 and BAX levels and topoisomerases Ia and IIa have been suggested as its possible mechanism of action.<sup>24,25</sup>

A number of *in vivo* studies have shown betulinic acid to be effective against carcinogen induced hepatotoxicity with fewer studies probing its effect in DMBA evoked extrahepatic damage.<sup>26</sup> The available literature on detoxification highlights the liver as the chief site of detoxification activity for both endogenous and exogenous toxins. The focus on biotransformation of chemical assaults (xenobiotics) in extrahepatic organs is limited. Keeping in mind the aforementioned facts, the present study

extends this observation by examining the intervention of betulinic acid with DMBA induced alterations in membrane bound ATPases, detoxification enzymes and antioxidant enzymes in stomach and lungs of female Sprague Dawley rats.

### Ethical approval for animal research

The animals were sheltered in the central animal house of Guru Nanak Dev University, Amritsar as per strict compliance with the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Government of India. The experimental protocol was duly reviewed and approved by the Institutional Animal Ethics Committee, Guru Nanak Dev University, Amritsar, India (671/BT dated May 20, 2011).

### Materials and Methods

Studies were carried out using healthy female Sprague Dawley rats (8-12 weeks old), weighing 140-200 g, procured from National Institute of Pharmaceutical Education and Research (NIPER), Mohali, Punjab (India). The animals were randomly assorted into 6 groups (n=6 for each group) and then treated with DMBA, test compound betulinic acid and standard compound indole-3-carbinol (I3C). This polycyclic aromatic hydrocarbon (DMBA) is an

important chemical in inducing mutagenesis and carcinogenesis in animal models.<sup>27</sup> The different concentrations of betulinic acid, DMBA and I3C were made using peanut oil (used as control vehicle) administered as per body weight (bw) and the compounds were given to animals *via* oral route. The different groups are described in Table 1. The treatments were made for seven weeks on alternate days and the experiment was terminated at the end of 14<sup>th</sup> week. Animals were sacrificed by cervical dislocation following overnight fasting.

### Preparation of homogenate

The entire stomach and lung of animals were isolated, perfused immediately with cold 0.9% NaCl, cleaned of adhering fat and connective tissues and thereafter rinsed in chilled 0.15 M Tris-KCl buffer (0.15 M KCl + 10 mM Tris-HCl, pH 7.4). The organs were then blotted dry, weighed quickly and homogenized in ice cold 0.15 M Tris-KCl buffer to yield 10% (w/v) homogenate. Homogenate (0.5 ml) was then precipitated with 5% trichloroacetic acid, centrifuged at 2000 rpm and the supernatant was used for the estimation of reduced glutathione content. The homogenate, after discarding lipid layer was used for the estimation of various biochemical parameters.

### Biochemical analysis

#### Estimation of membrane bound ATPases

Na<sup>+</sup>/K<sup>+</sup>-ATPase was determined according to the method of Bonting.<sup>28</sup> Ca<sup>2+</sup>-

ATPase was assayed as described by Hjerten and Pan.<sup>29</sup> Mg<sup>2+</sup>-ATPase was determined by the method of Ohnishi *et al.*<sup>30</sup> The supernatant was used for the estimation of phosphorous content as per the method described by Fiske and Subbarow.<sup>31</sup>

#### Estimation of detoxification enzymes

Glutathione-S-transferase (EC 2.5.1.18) activity was determined spectrophotometrically at 37 °C as described by Habig *et al.*<sup>32</sup> NAD(P)H:quinone oxidoreductase (EC 1.6.99.2) also known as DT-diaphorase was measured as described by Ernster.<sup>33</sup>

#### Estimation of antioxidant enzymes

The activity of superoxide dismutase (EC 1.15.1.1) was assayed following the method given by Kono with slight modifications.<sup>34</sup> Catalase (EC 1.11.1.6) activity was estimated following the method given by Aebi.<sup>35</sup> Activity of Ascorbate peroxidase (EC 1.11.1.11) was determined using method given by Asada.<sup>36</sup> Guaiacol peroxidase (EC 1.11.1.7) was assayed by the procedure described by Putter with slight modifications.<sup>37</sup> Glutathione reductase (EC 1.8.1.7) was determined by the procedure described by Carlberg and Mannervik.<sup>38</sup> Lactate dehydrogenase (EC 1.1.1.27, L-lactate: NAD<sup>+</sup> oxidoreductase) activity was determined following the method of Kuznetsov and Gnaiger with slight modifications.<sup>39</sup>

#### Estimation of oxidative stress parameters

Lipid Peroxidation (LPO) was estimated as evidenced by the formation of

**Table 1. The treatment protocol of Betulinic acid, DMBA and I3C among the Sprague Dawley female rats assorted in 6 groups (n=6 for each group).**

Treatment Protocol			
Groups	Compound	Concentration	Treatment type
I	Standard pellet diet and tap water <i>ad libitum</i>	-	Untreated Control
II	DMBA	10 mg/kg bw	Mutagen
III	DMBA+ Betulinic acid	10 mg/kg bw + 2 mg/kg bw	Treatment groups to check the inhibitory effect of test compound
IV	DMBA+ Betulinic acid	10 mg/kg bw + 5 mg/kg bw	
V	DMBA+ Betulinic acid	10 mg/kg bw + 10 mg/kg bw	
VI	DMBA+ Indole-3-carbinol	10 mg/kg bw + 10 mg/kg bw	

Thiobarbituric Acid Reactive Species (TBARS) and lipid hydroperoxides following the method of Devasagayam *et al.* and Jiang *et al.* respectively with slight modifications.<sup>40,41</sup> The amount of reduced glutathione (GSH) was determined using method described by Anderson.<sup>42</sup>

#### Determination of protein content

The protein content of various samples was determined using the method given by Smith *et al.* using BSA as a standard.<sup>43</sup>

#### Statistical analysis

The experimental data were expressed as mean  $\pm$  SE. One-way analysis of variance (ANOVA) and HSD value were carried out to determine significant differences between the means at  $P \leq 0.05$ .

## Results

### Betulinic acid modulates DMBA induced impairment of membrane bound ATPases

ATPases are lipid dependent membrane

bound enzymes responsible for cellular viability by controlling several crucial cellular functions. Transmembrane ATPases import many of the metabolites necessary for cell metabolism and export toxins, wastes, and solutes that can hinder cellular processes. Changes in the activities of these enzymes either deteriorate or accelerate the different pathological conditions.<sup>44</sup> In stomach, DMBA treatment (Group II) resulted in the higher amount of phosphorous liberation as compared to the untreated control (Table 2). The oral supplementation of betulinic acid and I3C (known anticarcinogen) in combination with DMBA at all the tested doses showed the decrease in  $\text{Na}^+/\text{K}^+$ -ATPase activity in both stomach and lung homogenate as compared to Group II animals. Similar results were obtained in the activity of  $\text{Ca}^{2+}$ -ATPase in stomach as well as lungs (Group III, IV, V and VI). In the case of  $\text{Mg}^{2+}$ -ATPase, group III, V and VI witnessed significant reduction of its activity in stomach compared to DMBA group. Similar results were observed in lung homogenate of betulinic acid treatment groups III and IV.

### Betulinic acid modulates DMBA induced impairment of detoxification enzymes

Exposure of rats to DMBA resulted in the decreased activity of GST as compared to the control group in stomach. While, GST increased activity was noted in case of the lung. The treatment of I3C and three tested doses of betulinic acid lowered the GST activity as compared to DMBA group in both stomach and lung. Extrahepatic DT-diaphorase enzyme activity in case of DMBA treated rats showed increased activity in comparison to untreated control. The specific activity was found to be lowered on betulinic acid administration in both organs except at betulinic acid 5 mg/kg bw in the stomach (Table 3).

### Betulinic acid modulates DMBA induced impairment of antioxidant enzymes

Table 4 depicts the activities of various antioxidant enzymes among different treatment groups. DMBA treated group elicited increased SOD activity as compared to control in both the extrahepatic organs (stom-

**Table 2. Modulation of DMBA induced impairment of membrane bound ATPases by betulinic acid among different treatment groups of female Sprague Dawley rats.**

Parameter	Group I	Group II	Group III	Group IV	Group V	Group VI	F-ratio	HSD value
<b><math>\text{Na}^+/\text{K}^+</math>-ATPase</b> ( $\mu\text{moles}$ of phosphate liberated/min/mg protein)								
Stomach	67.120 <sup>a</sup> $\pm$ 0.329	185.821 <sup>b</sup> $\pm$ 0.616	26.890 <sup>c</sup> $\pm$ 0.0587	102.770 <sup>d</sup> $\pm$ 1.017	32.830 <sup>e</sup> $\pm$ 0.386	157.250 <sup>f</sup> $\pm$ 0.673	5.064*	0.1528
Lung	29.698 <sup>a</sup> $\pm$ 0.587	60.836 <sup>b</sup> $\pm$ 0.210	31.815 <sup>c</sup> $\pm$ 0.645	31.388 <sup>d</sup> $\pm$ 0.732	40.535 <sup>e</sup> $\pm$ 0.711	39.493 <sup>f</sup> $\pm$ 0.268	27.270*	0.0546
<b><math>\text{Ca}^{2+}</math>-ATPase</b> ( $\mu\text{moles}$ of phosphate liberated/min/mg protein)								
Stomach	111.285 $\pm$ 0.501	254.410 $\pm$ 0.386	61.863 $\pm$ 0.243	105.80 $\pm$ 0.558	31.913 $\pm$ 0.558	136.503 $\pm$ 0.616	1.731	-
Lung	50.231 $\pm$ 0.036	57.348 $\pm$ 0.674	104.054 $\pm$ 0.674	54.424 $\pm$ 0.471	91.757 $\pm$ 0.384	62.890 $\pm$ 0.645	1.043	-
<b><math>\text{Mg}^{2+}</math>-ATPase</b> ( $\mu\text{moles}$ of phosphate liberated/min/mg protein)								
Stomach	71.060 <sup>a</sup> $\pm$ 1.959	137.650 <sup>b</sup> $\pm$ 1.80	97.80 <sup>c</sup> $\pm$ 2.458	141.190 <sup>d</sup> $\pm$ 0.2358	40.940 <sup>e</sup> $\pm$ 2.344	135.490 <sup>f</sup> $\pm$ 1.732	3.458*	0.2830
Lung	44.360 <sup>a</sup> $\pm$ 0.442	60.950 <sup>b</sup> $\pm$ 0.645	55.460 <sup>c</sup> $\pm$ 0.181	56.060 <sup>d</sup> $\pm$ 0.529	147.95 <sup>e</sup> $\pm$ 0.7011	52.750 <sup>f</sup> $\pm$ 0.501	12.063*	0.1482

\*Represents significance at  $P \leq 0.05$ . Superscripts a-f represents comparison of group I, II, III, IV, V and VI means using HSD value.

**Table 3. Modulation of DMBA induced impairment of detoxification enzymes by betulinic acid among different treatment groups of female Sprague Dawley rats.**

Parameter	Group I	Group II	Group III	Group IV	Group V	Group VI	F-ratio	HSD value
<b>Glutathione-S-transferase</b> (milli IU/mg protein)								
Stomach	8.010 <sup>a</sup> $\pm$ 0.003	5.870 <sup>b</sup> $\pm$ 0.004	2.320 <sup>c</sup> $\pm$ 0.002	2.050 <sup>d</sup> $\pm$ 0.001	1.220 <sup>e</sup> $\pm$ 0.001	5.140 <sup>f</sup> $\pm$ 0.0028	89.988*	0.00407
Lung	3.911 <sup>a</sup> $\pm$ 0.006	9.665 <sup>b</sup> $\pm$ 0.0018	2.346 <sup>c</sup> $\pm$ 0.0063	2.849 <sup>d</sup> $\pm$ 0.007	7.991 <sup>e</sup> $\pm$ 0.005	2.123 <sup>f</sup> $\pm$ 0.0018	14.349*	0.00839
<b>DT-diaphorase</b> ( $\mu\text{mol}$ IU/mg protein)								
Stomach	106.70 <sup>a</sup> $\pm$ 0.085	407.10 <sup>b</sup> $\pm$ 0.085	361.40 <sup>c</sup> $\pm$ 0.086	541.50 <sup>d</sup> $\pm$ 0.286	330.10 <sup>e</sup> $\pm$ 0.171	679.20 <sup>f</sup> $\pm$ 0.085	10.537*	0.006924
Lung	319.160 $\pm$ 1.714	431.040 $\pm$ 0.100	408.730 $\pm$ 0.571	268.340 $\pm$ 0.571	254.40 $\pm$ 0.541	278.933 $\pm$ 1.142	2.944	-

\*Represents significance at  $P \leq 0.05$ . Superscripts a-f represents comparison of group I, II, III, IV, V and VI means using HSD value.



ach and lung). On the other hand, betulinic acid and I3C treated groups in combination with DMBA were found to have lower SOD activity than DMBA administered rats. The enzymatic activity of CAT in the case of stomach homogenate of DMBA treated group was found to be lowered in contrast to control group. However, co-treatment of betulinic acid with DMBA resulted in the reduced activity of CAT. No significant change was observed in the status of CAT activity in lung among various groups employed in the present study. The specific activity of APOX enzyme was significantly enhanced by DMBA in comparison to normal rats in both the organs. The activity was observed to be lower in all the other treatment groups (III, IV and V of stomach and III of lung). An increase in the level of enzymatic activity of GPOX was observed in stomach and lung on administration of DMBA as compared to untreated group. All the other tested doses of betulinic acid and I3C in combination with DMBA employed in the present study showed reduced enzymatic level. Both the extrahepatic organs showed reduced GR activity in DMBA treated group in comparison to control group. Betulinic acid treatment groups resulted in a significant increase in the specific activity as compared to DMBA group.

LDH activity was found to be lowered in DMBA group as compared to the control group. The treatment with betulinic acid resulted in an increase in the specific activity in both organs.

### Betulinic acid modulates DMBA induced oxidative stress

As depicted in Table 5, a significant increase in lipid peroxidation was observed in DMBA group when compared with the normal control in both the extrahepatic organs. DMBA induced increment in lipid peroxidation was observed to be lowered on co-treatment with betulinic acid and I3C. The amount of lipid hydroperoxides was found to be very low in DMBA treated rats among different treatment groups in stomach and lung. Betulinic acid co-administration elevated the lipid hydroperoxide content at all the given tested doses. A marked decrease in the extrahepatic GSH level was observed in DMBA recipient animals than the normal control. The treatment with betulinic acid and I3C afforded protection by elevating the GSH level in comparison to DMBA treatment group at all the tested doses.

### Protein content analysis

Group II animals that received DMBA

showed decreased protein content as compared to untreated control. Administration of betulinic acid and I3C in combination with DMBA resulted in higher protein content in the stomach and lungs of rats (gp IV, V and VI).

## Discussion

Although, liver is an organ of vital significance due to its crucial role in maintaining the biological equilibrium of vertebrates but the drug metabolizing enzymes are also present at other sites. However, diverse types of hepatic dysfunctions and varied sites of action of different drugs may result in increased drug action and reduced drug elimination. In such cases, the extrahepatic organs and tissues supplement the metabolism of toxic chemical insults using xenobiotic metabolizing enzymes to a specific extent.<sup>3</sup> The activities of the drug metabolizing enzymes representing major classes of enzymatic machinery (oxidation, reduction, hydrolysis and conjugation) are known to be present in the subcellular fractions of human renal cortex, lung, gastric mucosa, colonic mucosa, spleen and prostate to certain degrees.<sup>45</sup> The first pass

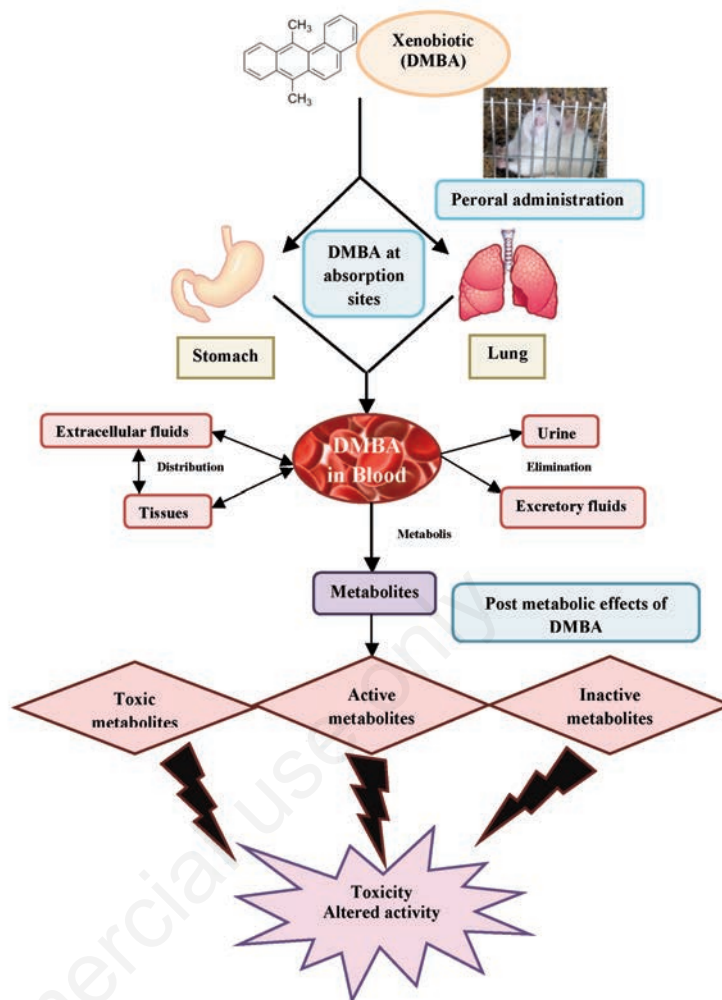
**Table 4. Modulation of DMBA induced impairment of antioxidant enzymes by betulinic acid among different treatment groups of female Sprague Dawley rats.**

Parameter	Group I	Group II	Group III	Group IV	Group V	Group VI	F-ratio	HSD value
<b>Superoxide Dismutase (IU/mg protein)</b>								
Stomach	16.085 <sup>a</sup> ±0.0001	51.090 <sup>b</sup> ±0.009	12.280 <sup>c</sup> ±0.008	17.003 <sup>d</sup> ±0.001	18.777 <sup>e</sup> ±0.016	29.237 <sup>f</sup> ±0.008	7.668*	0.00016
Lung	13.640 <sup>a</sup> ±0.270	17.692 <sup>b</sup> ±0.0003	14.294 <sup>c</sup> ±0.022	13.964 <sup>d</sup> ±0.009	17.464 <sup>e</sup> ±0.014	11.252 <sup>f</sup> ±0.009	6.932*	0.0745
<b>Catalase (IU/mg protein)</b>								
Stomach	39.364 <sup>a</sup> ±4.329	37.514 <sup>b</sup> ±3.896	10.021 <sup>c</sup> ±2.597	13.727 <sup>d</sup> ±4.218	10.259 <sup>e</sup> ±4.117	51.460 <sup>f</sup> ±4.210	17.516*	0.0058
Lung	22.012 <sup>a</sup> ±12.987	8.509 <sup>b</sup> ±3.812	33.365 <sup>c</sup> ±12.884	15.597 <sup>d</sup> ±4.329	29.803 <sup>e</sup> ±4.310	24.3908 <sup>f</sup> ±5.316	45.584*	0.0122
<b>Ascorbate Peroxidase (milli IU/mg protein)</b>								
Stomach	56.700 <sup>a</sup> ±0.021	73.30 <sup>b</sup> ±0.010	12.360 <sup>c</sup> ±0.024	28.210 <sup>d</sup> ±0.045	21.620 <sup>e</sup> ±0.044	53.90 <sup>f</sup> ±0.023	4.282*	0.0475
Lung	21.370 <sup>a</sup> ±0.022	35.10 <sup>b</sup> ±0.021	26.630 <sup>c</sup> ±0.043	39.090 <sup>d</sup> ±0.047	45.040 <sup>e</sup> ±0.032	18.80 <sup>f</sup> ±0.020	11.870*	0.0136
<b>Guaiacol Peroxidase (milli IU/mg protein)</b>								
Stomach	5.140 <sup>a</sup> ±0.001	17.760 <sup>b</sup> ±0.001	3.360 <sup>c</sup> ±0.003	3.950 <sup>d</sup> ±0.005	3.150 <sup>e</sup> ±0.004	4.410 <sup>f</sup> ±0.003	43.392*	0.0120
Lung	1.776 <sup>a</sup> ±0.008	5.224 <sup>b</sup> ±0.003	2.529 <sup>c</sup> ±0.0008	8.446 <sup>d</sup> ±0.011	1.391 <sup>e</sup> ±0.009	2.387 <sup>f</sup> ±0.004	16.957*	0.00729
<b>Glutathione Reductase (milli IU/mg protein)</b>								
Stomach	10.60 <sup>a</sup> ±0.006	4.800 <sup>b</sup> ±0.009	8.701 <sup>c</sup> ±0.013	7.001 <sup>d</sup> ±0.006	1.20 <sup>e</sup> ±0.005	10.90 <sup>f</sup> ±0.042	5.0952*	0.06612
Lung	2.179 <sup>a</sup> ±0.002	0.936 <sup>b</sup> ±0.001	6.039 <sup>c</sup> ±0.003	3.4705 <sup>d</sup> ±0.008	2.873 <sup>e</sup> ±0.006	1.5752 <sup>f</sup> ±0.008	10.6006*	0.01417
<b>Lactate Dehydrogenase (milli IU/mg protein)</b>								
Stomach	36.040 <sup>a</sup> ±0.058	17.180 <sup>b</sup> ±0.001	55.640 <sup>c</sup> ±0.005	17.730 <sup>d</sup> ±0.014	10.947 <sup>e</sup> ±0.019	32.630 <sup>f</sup> ±0.024	11.229*	0.0256
Lung	13.016 <sup>a</sup> ±0.005	10.744 <sup>b</sup> ±0.005	13.19 <sup>c</sup> ±0.005	10.997 <sup>d</sup> ±0.019	11.166 <sup>e</sup> ±0.020	10.011 <sup>f</sup> ±0.010	8.186*	0.0127

\*Represents significance at P≤0.05. Superscripts a-f represents comparison of group I, II, III, IV, V and VI means using HSD value.

metabolism in gastrointestinal tract (GIT) may also establish the equilibrium between bioactivation/deactivation for orally ingested xenobiotics.<sup>46</sup> Both rodent and human GIT contain enzymes capable of metabolizing a broad range of xenobiotics.<sup>47</sup> The presence of food in the rat stomach influences the bioavailability and pharmacokinetics of these chemicals.<sup>48</sup> The interaction of pesticides with P-glycoproteins was reported to be upregulated in rat stomach, jejunum and liver.<sup>49</sup> In another report, presence of benzo(a)pyrene hydroxylase in GIT, lungs and skin suggests that these tissues act as portals of entry and site of metabolism in the body and metabolize the noxious agents.<sup>50</sup>

In the present study, a polycyclic aromatic hydrocarbon (DMBA) was chosen for its ability to imbalance the different biochemical parameters with the consideration that this compound is readily metabolized into reactive carcinogens to form DNA adduct.<sup>51</sup> Figure 1 shows the possible fate of DMBA after administration into the body. Betulinic acid has received a lot of attention because of its enthusiastic biochemical modulatory influences both in *in vitro* and *in vivo* system. The current study employed the use of female Sprague Dawley rats as a number of studies in literature reveals that a high incidence of spontaneous tumors (57%) occur in normal female rats extended over their entire adult life. The male rats of same strain were found to be less susceptible to tumor formation than females.<sup>52,53</sup> The protocol was followed for 7 weeks and was terminated at the end 14<sup>th</sup> week in order



**Figure 1.** Schematic representation of possible fate of DMBA after administration into the body.

**Table 5. Modulation of DMBA induced impairment of oxidative stress parameters by betulinic acid among different treatment groups of female Sprague Dawley rats.**

Parameter	Group I	Group II	Group III	Group IV	Group V	Group VI	F-ratio	HSD value
<b>Lipid Peroxidation (TBARS)</b> ( $\mu$ moles MDA equivalent/g of tissue)								
Stomach	117.913 <sup>a</sup> $\pm$ 0.003	119.632 <sup>b</sup> $\pm$ 0.002	139.983 <sup>c</sup> $\pm$ 0.006	65.728 <sup>d</sup> $\pm$ 0.009	110.762 <sup>e</sup> $\pm$ 0.008	121.213 <sup>f</sup> $\pm$ 0.01	20.021*	0.0384
Lung	19.594 <sup>a</sup> $\pm$ 0.001	44.483 <sup>b</sup> $\pm$ 0.017	29.564 <sup>c</sup> $\pm$ 0.007	42.971 <sup>d</sup> $\pm$ 0.003	15.675 <sup>e</sup> $\pm$ 0.006	43.315 <sup>f</sup> $\pm$ 0.004	3.113*	0.0503
<b>Lipid Hydroperoxides</b> (mM H <sub>2</sub> O <sub>2</sub> equivalents/g of tissue)								
Stomach	1.582 $\pm$ 0.004	0.730 $\pm$ 0.01	4.077 $\pm$ 0.012	1.345 $\pm$ 0.013	1.906 $\pm$ 0.017	2.190 $\pm$ 0.009	0.1445	-
Lung	2.522 <sup>a</sup> $\pm$ 0.006	0.983 <sup>b</sup> $\pm$ 0.004	2.924 <sup>c</sup> $\pm$ 0.006	1.367 <sup>d</sup> $\pm$ 0.008	1.767 <sup>e</sup> $\pm$ 0.002	1.401 <sup>d</sup> $\pm$ 0.021	8.017*	0.0558
<b>Reduced Glutathione</b> ( $\mu$ M of SH content/g of tissue)								
Stomach	764.130 $\pm$ 0.001	523.460 <sup>b</sup> $\pm$ 0.002	1895.310 <sup>c</sup> $\pm$ 0.010	1744.88 <sup>d</sup> $\pm$ 0.003	625.750 <sup>e</sup> $\pm$ 0.001	1143.20 <sup>f</sup> $\pm$ 0.002	4.659*	0.0216
Lung	1186.610 $\pm$ 0.008	983.770 $\pm$ 0.002	2216.02 $\pm$ 0.008	2190.0 $\pm$ 0.002	1222.110 $\pm$ 0.002	1566.930 $\pm$ 0.003	0.6193	-
<b>Protein Content</b> (mg/g of tissue)								
Stomach	40.140 <sup>a</sup> $\pm$ 0.043	17.540 <sup>b</sup> $\pm$ 0.02	67.180 <sup>c</sup> $\pm$ 0.02	39.420 <sup>d</sup> $\pm$ 0.007	135.020 <sup>e</sup> $\pm$ 0.033	27.340 <sup>f</sup> $\pm$ 0.006	71.401*	0.1194
Lung	63.916 <sup>a</sup> $\pm$ 0.005	57.233 <sup>b</sup> $\pm$ 0.004	30.166 <sup>c</sup> $\pm$ 0.011	54.816 <sup>d</sup> $\pm$ 0.002	41.033 <sup>e</sup> $\pm$ 0.001	61.233 <sup>f</sup> $\pm$ 0.031	20.114*	0.0832

\*Represents significance at  $P \leq 0.05$ . Superscripts a-f represents comparison of group I, II, III, IV, V and VI means using HSD value.

to develop the toxic effects of DMBA on the extrahepatic organs and to observe the impact of betulinic acid on DMBA evoked damage.

The level of lipid peroxides (LPO) is the diagnostic index for evaluating the extent of membrane damage as it causes polymerization, cross-linking and/or fragmentation of the membrane components leading to altered membrane permeability and destructing cellular constituents like lipids, proteins and nucleic acids.<sup>54</sup>  $\text{Na}^+/\text{K}^+$ -ATPase, a membrane-bound enzyme, is essential to maintain the  $\text{Na}^+$  and  $\text{K}^+$  gradients between the cells and their environment.<sup>55</sup>  $\text{Ca}^{2+}$ -ATPase is of primary importance in control of hydration, volume, nutrient uptake, contractility and excitability of muscles.<sup>56,57</sup>  $\text{Mg}^{2+}$ -ATPase regulates the rate of protein synthesis and cell growth.<sup>58</sup> In the present investigation, administration of DMBA increased the activities of physiologically important membrane bound ATPases namely,  $\text{Na}^+/\text{K}^+$ -ATPase,  $\text{Ca}^{2+}$ -ATPase and  $\text{Mg}^{2+}$ -ATPase. Disturbances in the ionic equilibrium of ATPases might be due to the failure of optimal interaction with the membrane components, as an outcome of increased LPO. Co-treatment of betulinic acid caused the modification in the altered membrane fluidity and thereby improved the cell membrane integrity as a result of enhancement of GSH and protection against LPO provided by the treatment.

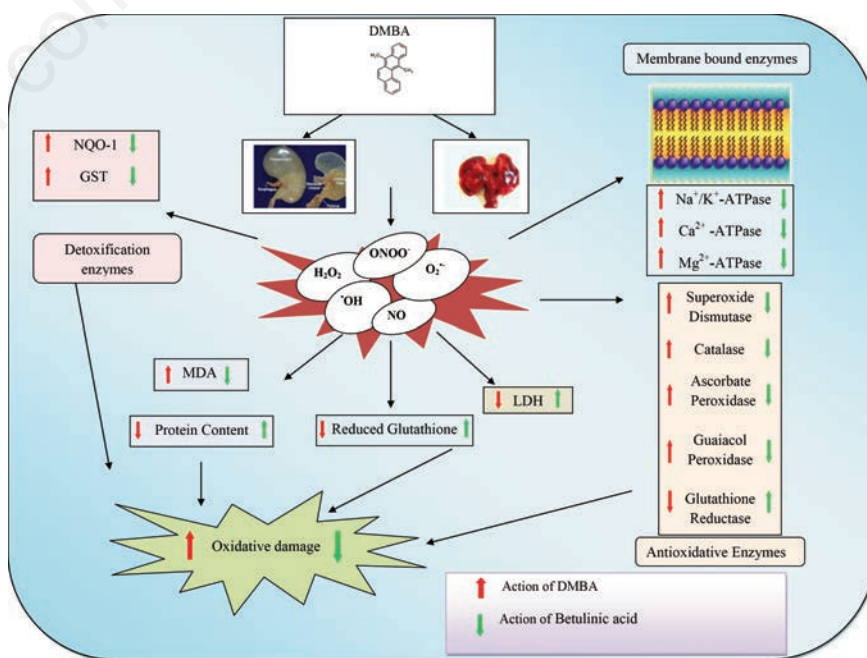
The disposition of DMBA and thereby defense against oxidative injury occurs in the presence of several detoxification enzymes. One of the important pathways is GSH conjugation catalyzed by GST that is concerned with the elimination of the proximate and ultimate carcinogens through formation of more water-soluble and non-electrophilic detoxification products.<sup>59</sup> DT-diaphorase acts as a chemical detoxifier, superoxide scavenger and maintains endogenous antioxidant vitamins.<sup>60</sup> Elevated expressions of GST and DT-diaphorase cited in several previous reports are well in line with our findings.<sup>61,62</sup> This might be due to the reason that PAHs activate transcription of GST and DT-diaphorase by binding to the xenobiotic response element present in the promoter region of genes.<sup>63</sup>

The antioxidant enzyme activity varies from tissue to tissue and also depends on the continuance and rigor of the toxin. SOD and CAT acts as mutually supportive antioxidants and provide protective defence against reactive oxygen species (ROS). The enhancement of the various antioxidant enzymes *viz.*, SOD, CAT, APOX and GPOX indicates the presence of more oxygen free radicals in the system. This

increase might be due to the activation of genes that code for antioxidant enzymes by oxidants.<sup>64</sup> The reduction in the enzyme status of almost all the antioxidant related enzymes by betulinic acid supplementation in comparison to DMBA recipient rats explains its significance in eliminating free radical-mediated consequences that may affect the normal functioning of cells. GR catalyzes the reduction of glutathione disulfide by NADPH.<sup>65</sup> The diminution in the GR activity in DMBA administered rats might be ascribable to the decline in the production and availability of reduced glutathione to overcome  $\text{H}_2\text{O}_2$  as GR is responsible for regeneration of GSH. On the contrary, the expression of GR was increased on treatment with betulinic acid suggesting GSH-dependent detoxification of free radicals as GSH is one of the guarding factors against oxidative stress. LDH, a cytoplasmic marker enzyme catalyses the readily reversible reaction involving oxidation of lactate to pyruvate and serves as a biochemical indicator of organ dysfunction, differentiates normal tissues from their damaged counterparts and spot various stages of tissue development.<sup>66,67</sup> In the present investigation, damage to stomach and lung tissues by DMBA is reflected by a substantial decrease in the activity of LDH showing suppressed lactate metabolism. Furthermore, LDH is believed to vary

according to the metabolic necessity of each tissue and alteration in LDH levels have been observed during development under changing biological conditions and in response to pathological processes.<sup>68</sup>

Oxidative damage is an indicator of the diseases caused by disruption in tissue functions. The process of lipid peroxidation is under the control of efficient endogenous cellular defense systems that ensures optimum metabolic functions. In this study, an increase in lipid peroxidation was observed in DMBA group of both extrahepatic organs due to the production of excessive, toxic and diffusible ROS during the metabolism of DMBA indicating the damage to cell membranes and cellular dysfunction. Treatment with betulinic acid reduced the level of MDA, which could be associated with its protective role as a free radical scavenger. GSH, a co-factor for several detoxifying enzymes has a putative role in regulation of cell proliferation, reduction of peroxides and maintenance of protein thiols in the reduced state. The present investigation showed a declined level of reduced glutathione in DMBA administered rats. The reduction in GSH content is associated with disproportionate synthesis of ROS coupled with the lessened activity of gluco-6-phosphate dehydrogenase, a GSH-synthesizing enzyme.<sup>69,70</sup> Co-administration of betulinic acid credited its biological significance by



**Figure 2. Overall schematic representation of Betulinic acid mediated modulation of DMBA evoked extrahepatic oxidative injury.**



elevating the level of GSH and thus, protecting cellular proteins against oxidative stress.

Variations in the protein content among different groups is related to the oxidative stress that cause DNA, protein and lipid damage leading to changes in chromosome instability, genetic mutations or modulations in cell growth.<sup>71</sup>

## Conclusions

In nutshell, we conclude that the normal cellular protective response is altered by tissue damage caused by a xenobiotic. This damage depends on the bioavailability of xenobiotic, its metabolism and various other factors. The damaging effect of DMBA is realized through the formation of free radicals or ROS, activation of membrane lipid peroxidation, their release in circulation, disturbances in energy homeostasis, altered enzymatic machinery and thus, failure of adaptive potential of body to conditions of increased oxidative stress. Betulinic acid controlled the formation and release of free radical cascades thereby reducing toxic side-effects in animals (Figure 2).

## Research highlights

- Extrahepatic organs cope up with scores of xenobiotics by acting as first line detoxification defense.
- Betulinic acid neutralizes the oxidant radicals evoked by such xenobiotics (DMBA) in extrahepatic sites maintaining electrophysiological energetic and cellular homeostasis.
- Thus, betulinic acid offers protection against tissue damage induced by DMBA.

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