PHYTOMODULATORY EFFECTS OF MURRAYA KOENIGII IN DMBA/TPA INDUCED ANGIogenesis, Hepatotoxicity and Renal Toxicity During Skin Carcinogenesis In Mice

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ABSTRACT

The present study was aimed to evaluate the protective effects of Hydroethanolic Murraya koenigii leaves extract (HEMKLE) against DMBA/TPA-induced angiogenesis, hepatotoxicity and renal toxicity during skin carcinogenesis in mice. For the study, male LACA mice were divided into four groups: Group I (C), Group II (DMBA/TPA), Group III (HEMKLE), and Group IV (HEMKLE+DMBA/TPA). 7, 12-dimethylbenz(a)anthracene (DMBA), and 12-O-tetradecanoylphorbol-13-acetate (TPA) were applied on the depilated skin of the mice to raise skin tumors in Group II and Group IV. The chemopreventive response of HEMKLE was evident by histoarchitectural and morphometric analysis of skin/tumors in Group IV (HEMKLE+DMBA/TPA). In addition, HEMKLE administration also decreased the mRNA and protein expression of HIF-1α, HMOX-1, VEGF, bFGF, and ANGPT-2 in Group IV (HEMKLE+DMBA/TPA) when compared to Group II (DMBA/TPA) that suggest its anti-angiogenic effect. Moreover, HEMKLE administration protected the liver and kidney tissues from damages incurred during skin carcinogenesis as evident through histological analysis, assessment of ROS and LPO levels, assessment of liver and kidney function markers (viz., SGOT, SGPT, D-bilirubin, T-bilirubin, ALP, urea, creatinine and BUN) and activities of the antioxidant enzymes in Group IV (HEMKLE+DMBA/TPA) when compared to Group II (DMBA/TPA). The outcome of the present study showed that HEMKLE administration markedly alleviated the angiogenesis and also showed the protective effects against damages incurred in liver and kidney tissues during skin carcinogenesis. However, further extensive studies are needed to explore the efficacy of HEMKLE on metastasis before going to human trials.

Keywords: Skin tumor, Hepatotoxicity, Renal toxicity, Angiogenesis, Murraya koenigii.

1. INTRODUCTION

Skin is the largest body organ that serves as a virtual environmental interface and provides a protective envelope[1]. However, the accidental or occupational exposure of skin to several chemicals or carcinogens leads to skin cancer [2]. Skin cancer is broadly categorized as Melanoma skin cancer (MSC) and Non-melanoma skin cancer (NMSC). The incidence of NMSC is continually rising, and according to GLOBOCAN 2020, there were 1,198,073 new cases of NMSC (excluding BCC) and 63,731 deaths in total from NMSC [3]. NMSC is further sub-categorized as Basal Cell Carcinoma (BCC) (which occurs in basal cells of the epidermis) and Squamous Cell Carcinoma (SCC) (which occurs in squamous cells of the epidermis). SCC is the most aggressive form of skin cancer since it can spread to various vital organs of the body [4]. Mutated cells of the skin demand to attain distinct abilities such as hyper-proliferation, angiogenesis, tissue invasion, and metastasis to prosper. Out of these, angiogenesis is a decisive event for the further progression of solid tumors, which is regulated by many factors. The imbalance between these angiogenic factors commences to metastasis (by forming new capillaries, required for proper blood and nutrient supply) [5]. Thus, targeting angiogenesis is the most promising strategy for cancer suppression and treatment [6].

7, 12, dimethylbenz[a]anthracene (DMBA), a polycyclic aromatic hydrocarbon (PAH) (mostly found in
environment), is commonly used to generate skin carcinogenesis in laboratories. DMBA can further metabolize in liver and kidney tissues, produces excess reactive oxygen species (ROS) [7]. Further, lipid peroxidation (LPO) from ROS generation may initiate many pathologies [8]. Excess ROS molecules also triggers oxidative stress and can further initiate apoptosis, necrosis, and autophagy [9]. Thus emphasis on controlling ROS during carcinogenesis is the need of the hour to retard further damages to other organs.

Due to adverse side-effects and the economic burden of the modern drug, the interest of the scientific community, as well as the general public, is growing exponentially towards traditional medicines (as they are easily available, safe, cost-effective and multi-targeted). Several plants have already shown promising antioxidant characteristics in various in vitro and in vivo studies [10].

One such plant of significant importance is Murraya koenigii (Linn.) Spreng, which is an inhabitant of Asian countries and relates to the Rutaceae family. It is well-reputed in the Ayurveda and Siddha system of medicine from ancient times (as a traditional medicinal plant) to tackle ailments viz., obesity, hypertension, mental disorders and diarrhoea [11-12]. Also, Murraya koenigii is found competent to tackle various extrinsic skin problems such as pigmentation, bruises, sunburns, skin eruptions, allergies, cuts, and wounds. Moreover, it also guarded the skin from oxidative stress, bacterial and fungal infections [12]. Despite many skin-protecting properties, reports on its chemoprotective effect on skin cancer are scanty. In a previous study from our laboratory, Hydroethanolic Murraya koenigii leaves extract (HEMKLE) showed suppressive effect against chemically induced skin carcinogenesis in mice as evident through assessment of tumor statistics, oxidative stress markers, antioxidant enzymes activities, and mRNA and protein expressions of apoptosis associated markers [13]. So, by further extending our previous study, the present study was aimed (i) to assess the antiangiogenic potential of HEMKLE on chemically induced skin carcinogenesis in mice, (ii) to assess the protective effect of HEMKLE against damages incurred in liver and kidney tissues during skin carcinogenesis.

2. MATERIAL AND METHODS

2.1. Chemicals

7,12-Dimethylbenz(a)anthracene (DMBA), phorbol 12-myristate 13-acetate (TPA), bovine serum albumin (BSA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonate (ABTS), butylated hydroxytoluene (BHT), ethidium bromide (EtBr) were bought from Sigma Chemical Co. (St Louis, MO, USA). Molecular biology grade chemicals for RNA isolation viz., chloroform, isopropanol, ethanol, and formaldehyde were procured from Amresco, Ohio (USA). Primary antibodies and peroxydase-conjugated anti-rabbit secondary antibodies were purchased from Santa Cruz Biotechnology (California, USA). Enzymatic kits for serum glutamic pyruvic transaminase (SGPT), serum glutamic oxaloacetic transaminase (SGOT), alkaline phosphatase (ALP), direct bilirubin (D-Bilirubin), total bilirubin (T-Bilirubin), urea, creatinine, and blood urea nitrogen (BUN) were obtained from Reckon Diagnostic Pvt. Ltd (ENZOPAK, India). All other chemicals used in this study were of analytical grade and purchased from reputed Indian firms (Sisco Research Laboratories (SRL), India; Central Drug House (CDH), India).

2.2. Preparation of Hydroethanolic Murraya koenigii leaves extract (HEMKLE)

For the study, fresh Murraya koenigii leaves were obtained from the Botanical garden of Panjab University, Chandigarh (India). Further, HEMKLE was prepared in our laboratory as described previously [13]. First of all, the leaves were rinsed with water, shade dried, and powdered. 40 g of dried powder was added to 250 mL of ethanol: water (1:1), and this was kept on a shaker (for 3 days). After which, that liquid was centrifuged, and the supernatant so obtained, was filtered, lyophilized, and stored at 4 degree Celsius in a dark container for further use.

2.3. In vitro Antioxidant assay

In vitro, the antioxidant activity of HEMKLE was determined by DPPH and ABTS radical scavenging assays by following the methods of Mishra [14], and Re et al. [15], respectively.

2.4. Animal procurement

The experimental protocols requiring animals were approved by the Institutional Animal Ethics Committee (IAEC) (Ethical approval no: PU/4S/99/CRSEA/IAEC/2019/295) by following the Indian National Science Academy guidelines. Male LACA mice (20-25 g) (of age 4 weeks), were obtained from the University Central Animal House and were housed in well-aerated rooms having ambient temperature (21±2°C) during the treatment period. Animals were placed in
polypropylene cages bedded with rice husk and provided with a standard laboratory pellet diet and water ad libitum. Before initiating experiments, animals were acclimated to laboratory conditions for one week.

2.5. Two-stage model of skin carcinogenesis and its chemoprevention by HEMKLE

A twenty week study was conducted for the present experimental work. Male LACA mice were divided into four different groups having ten animals in each group. Group I (C): mice received a topical application of acetone (100µL/mouse) (vehicle treatment) twice a week for twenty weeks.

Group II (DMBA/TPA): mice received a topical application of DMBA (500nmol/100µL of acetone) for two weeks (twice), followed by TPA (1.7nmol/100µL of acetone) twice a week till 20th week.

Group III (HEMKLE): mice were administered with HEMKLE (200mg/kg b.w.; through oral gavage) thrice a week till the 20th week.

Group IV (HEMKLE+DMBA/TPA): mice received DMBA/TPA treatment as per Group II and were administered with HEMKLE as per Group III.

The first dose of DMBA was given to the animals after 2 weeks of pre-treatment with HEMKLE.

2.6. Collection of Blood and Preparation of Serum

At the end of the treatment period, blood samples were collected by retro-orbital puncture with the help of a thin capillary tube, after which, blood was collected into a vial. Blood was then centrifuged at 2200-2500 rpm for 15 minutes within one hour of collection to prepare the serum. Then, the mice were sacrificed through cervical dislocation after subjecting them to mild ether anaesthesia.

2.7. Histological analysis

Histopathological analysis of tissues was conducted using Hematoxylin and Eosin (H&E) staining as described by Humanson [16]. Skin/tumors, liver and kidney tissues were removed and immediately transferred to neutral formalin and allowed to fix for 12 h. Next, the tissues were dehydrated gradually in ascending series of ethanol. For embedding, the dehydrated samples were placed in benzene, then sequentially in 1:1 benzene:paraffin wax and two changes in pure melted wax, before finally embedding in paraffin wax. Thin sections (5 µm) were obtained using a manual hand-driven microtome and transferred to the glass slides. These were dewaxed in xylene, rehydrated in descending series of ethanol, and stained with H&E. The stained slides were then cleared in xylene and mounted in distyrene plasticizer xylene (DPX). The final sections were viewed and photographed under a light microscope (LEICA DM 3000).

Further, H&E stained sections of skin/tumor were utilized for morphometry (Epidermal thickness and cell count determination). Epidermal thickness was measured manually with the help of a calibrated ocular micrometre scale [17]. Epidermal cell counting was performed in an area approximately equal to 2,500µm² using PICASA software.

2.8. Hepatic and renal function markers

Enzymatic kits obtained from Reckon Diagnostic (ENZOPAK, India) were used for the determination of SGPT, SGOT, ALP, D-Bilirubin, T-Bilirubin, urea, creatinine, and BUN in blood serum.

2.9. Biochemical estimations in hepatic and renal tissues

The level of oxidative stress and antioxidants enzyme activities in the liver and kidney tissues were assessed by Reactive oxygen species (ROS) [18], Lipid peroxidation (LPO) [19], Reduced glutathione (GSH) [20], Glutathione-S-transferase (GST) [21], Glutathione peroxidase (GSH-Px) [22], Superoxide Dismutase (SOD) [23], and Catalase (CAT) [24] estimations. The total protein content of liver/kidney tissues was determined by following the method of Lowry et al. [25].

2.10. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA isolated from skin tissue/skin tumors after twenty weeks of treatment was subjected to reverse transcriptase-polymerase chain reaction (RT-PCR) using a thermocycler PCR machine (G-STORM, Akribis Scientific Limited, UK). The PCR reaction mixture was prepared according to kit instructions (Invitrogen, Thermo Fisher Scientific, USA). The final PCR products were analysed using horizontal agarose gel (1.2%) electrophoresis. The densitometric analysis of the bands was done by using ImageJ software (NIH, USA). β-actin was used as a loading control. The following primer pairs were used for RT-PCR; β-actin: forward primer: 5′-ATCCGTAAGACCTCTATGC-3′, reverse primer: 5′-AACGCACTTGTAACAGTC-3′, HIF-1α: forward primer:5′-CATAAAGTCTGCAACATG GAAGGT-3′ reverse primer:5′-ATTGTGGTGATGGTTGAGGT3′, HMOX-1: forward primer: 5′-CTTGGCACAAGGA
GTGTTAAC-3', reverse primer: 5'-CATCCTGCTT
GTCTCTCACC-3', VEGF: forward primer: 5'-
TCTTCAAGCATCCTGTG-3', reverse primer:
5'-ATCCGCATAATCTGCACT-3', bFGF: forward
primer: 5'-AGCGGCTCTACTGCAAGA-3', reverse
primer:5'-TATGCCCTCTGTCCAGGT-3', ANGPT-
2:forward primer: 5'-GGGGAGAAGAGAAGAGA-
G-3', reverse primer: 5'-CAGGGCATGGAAGAGAG-
G-3').

2.11. Enzyme-Linked Immunosorbent Assay
(ELISA)
Cytoplasmic (for VEGF, bFGF, and ANGPT-2) and
nuclear (for HIF-1α and HMOX-1) extracts of skin
tissues/tumors were used to analyze protein expression
employing the standard sandwich ELISA method.
Briefly, 5 μg of protein sample was loaded onto ELISA
strip wells in carbonate buffer (100 μL) and kept
overnight at 4°C. The wells were then blocked with 1%
BSA for 1 hour at 37°C. After washings with PBS,
PBS-Tween-20 (0.05%), and PBS for 5 minutes, the wells
were incubated with respective primary antibodies
(1:200) for 2 hours at 37°C. After washing with PBS,
PBS-Tween-20 (0.05%), and PBS, the wells were
incubated with peroxidase-labelled secondary antibody
(1:5000) for 2 hours at 37°C. Then the substrate, 2, 2'-
azino-bis (3-ethylbenzothiazine-6-sulphonic acid)
(ABTS), was added with H2O2 and kept in the dark for
20 minutes. The color obtained was quantified by
determining the absorbance at 405 nm using a
microstrip reader (STAT FAX 325+, Awareness
Technology Inc., USA).

2.12. Statistical Analysis
Data are expressed as mean ± standard deviation (SD),
and the Statistical Package for the Social Sciences (SPSS)
software (version 22.0) for one-way analysis of variance
(ANOVA) followed by Tukey's post hoc was utilised for
the analysis. p≤0.05 was considered as statistically
significant.

3. RESULTS
3.1. In vitro antioxidant activity
DPPH assay revealed an increase in the percentage free
radical scavenging activity of HEMKLE (78.85-96.14%)
in comparison to the standard ascorbic acid (30-
69.28%) against DPPH free radicals for an increase in
concentration (0.01-0.05 mg/mL), as shown in (as
shown in Fig. 1a).
ABTS assay revealed a marked increase in percentage
free radical scavenging activity of HEMKLE (16.57-
89.12%) in comparison to the standard ascorbic acid
(6.42-50.57%) against ABTS free radicals for an
increase in concentration (0.001-0.01 mg/mL), as
shown in (as shown in Fig. 1b).

Fig. 1: In vitro antioxidant activity of HEMKLE (a) DPPH assay (b) ABTS assay

3.2. Histoarchitectural analysis
The skin tissues from Group I (C) and Group III
(HEMKLE) revealed normal histoarchitecture with
the uniformly arranged epidermis, dermis, and
subcutaneous tissue. After ten weeks of DMBA/TPA
treatment, hyperplasia, and hyper-proliferation of
epidermis was observed in the skin sections of Group II
(DMBA/TPA). However, HEMKLE administration in
Group IV (HEMKLE+DMBA/TPA) resulted in
inhibition of hyperplasia and hyper-proliferation of
epidermis. After twenty weeks of DMBA/TPA
 treatment, hyper-keratinization, and hyper-proliferation
of epidermis was observed in tumor sections obtained
from Group II (DMBA/TPA). However, in Group IV
(HEMKLE+DMBA/TPA), a marked decrease in hyper-
kiratinization, and hyper-proliferation was observed.
The tumors in both groups were histologically identified as SCC (as shown in Fig. 2). Further, morphometric analysis of skin tissues/tumors showed a significant ($p\leq0.05$) increase in epidermal thickness as well as epidermal cell count at both the 10th and 20th week in Group II (DMBA/TPA) when compared to Group I (C) and Group III (HEMKLE). Interestingly, HEMKLE administration significantly ($p\leq0.05$) reduced the epidermal thickness and epidermal cell count at both the 10th and 20th week in Group IV (HEMKLE+DMBA/TPA) when compared to Group II (DMBA/TPA) (as shown in Fig. 2).

**K-Keratinocytes, E-Epidermis, D-Dermis, SC-Subcutaneous layer, M-Muscle, HK-Hyper-keratinization, HE-Hyper-proliferation.**

Group I is Control, Group II is DMBA/TPA, Group III is HEMKLE, and Group IV is HEMKLE+DMBA/TPA. Data is expressed as Mean ± SD ($n=5$). Data is analysed by one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test at Statistical significance: $^a p \leq .05$ significant with respect to Group I (C); $^b p \leq .05$ significant with respect to Group II (DMBA/TPA); $^c p \leq .05$ significant with respect to Group III (HEMKLE). “a” represents significance with respect to Group I (C); “b” represents significance with respect to Group II (DMBA/TPA); “c” represents significance with respect to Group III (HEMKLE).

**Fig. 2: Effect of DMBA/TPA or HEMKLE on histology/morphometry in haematoxylin and eosin stained skin/tumor sections from (a,e) Group I (C), (b,f) Group II (DMBA/TPA), (c,g) Group III (HEMKLE) and (d, h) Group IV (HEMKLE+DMBA/TPA), (i, k) Quantitative determination of epidermal thickness after 10 and 20 weeks, (j, l) Epidermal cell count after 10 and 20 weeks.**

Histopathological analysis of the liver tissues from Group I (C) and Group III (HEMKLE) revealed normal histoarchitecture. DMBA/TPA treatment to Group II (DMBA/TPA) caused several histological alterations such as the distorted structure of hepatocytes, as evident from widened and dilated central vein with ruptured endothelial lining of cells associated with congested plates of vascular sinusoidal spaces along with vascular channels radiating out from the central veins. This might be due to the shrinkage and necroses of hepatocytes. However, HEMKLE administration in Group IV (HEMKLE+DMBA/TPA), reduced the cytoplasmic vacuolization and sinusoidal expansions when compared to Group II (DMBA/TPA), although most areas were still distorted. This indicated a marked reduction in the damages (incurred due to DMBA/TPA) upon HEMKLE administration (as shown in Fig. 3).
Histological analysis of the kidneys from Group I (C) and Group III (HEMKLE) animals showed normal morphology with well-preserved glomerulus and no loss of tubular epithelial cells. Group II (DMBA/TPA) showed mild tubulointerstitial injury, along with extensive loss of tubular epithelial cells, tubular dilation, intratubular debris, tubular cell atrophy, inflammatory cell infiltration, and increased interstitial areas. However, in Group IV (HEMKLE+DMBA/TPA), marked improvement was evident, which indicated the protective effect of HEMKLE (as shown in Fig. 4).

![Histological analysis of the kidneys](image)

**Fig. 3: H & E-stained sections of hepatic tissue at 10X**

(a) Group I (Control) (b) Group II (DMBA/TPA) (c) Group III (HEMKLE) (d) Group IV (HEMKLE+DMBA/TPA) [CV: Central vein; S: Sinusoids; H: Hepatocyte; V: Vacuolization of cytoplasm; E: Congested sinusoidal spaces]

3.3. Liver and kidney injury markers

DMBA/TPA treatment caused a significant (p≤0.05) increase in SGOT, SGPT, ALP, D-Bilirubin, and T-Bilirubin marker enzymes in Group II (DMBA/TPA) when compared to Group I (C) and Group III (HEMKLE). However, HEMKLE administration significantly (p≤0.05) attenuated their levels/activities in Group IV (HEMKLE+DMBA/TPA) when compared to Group II (DMBA/TPA). No significant change was observed in Group III (HEMKLE) when compared to
DMBA/TPA treatment caused a significant (p≤0.05) increase in Urea, Creatinine, and BUN marker enzymes in Group II (DMBA/TPA) when compared to Group I (C) and Group III (HEMKLE). However, HEMKLE pre-treatment significantly (p≤0.05) attenuated their levels/activities in Group IV (HEMKLE+DMBA/TPA) when compared to Group II (DMBA/TPA). No significant change was observed in Group III (HEMKLE) when compared to Group I (C) (as shown in Figs. 6 a-c).
3.4. Oxidative stress markers & antioxidant enzymes

DMBA/TPA treatment caused a significant (p≤0.05) increase in ROS, LPO, and GSH-Px levels/activities in both liver and kidney tissues of Group II (DMBA/TPA) when compared to Group I (C) and Group III (HEMKLE). However, HEMKLE administration significantly (p≤0.05) attenuated their levels/activities in Group IV (HEMKLE+DMBA/TPA) when compared to Group II (DMBA/TPA). No significant change was observed in Group III (HEMKLE) when compared to Group I (C) (as shown in Table 1 & 2).

Table 1: Modulatory effect of DMBA/TPA and/or HEMKLE on oxidative stress and antioxidant defense system in liver tissues

<table>
<thead>
<tr>
<th></th>
<th>Group I (C)</th>
<th>Group II (DMBA/TPA)</th>
<th>Group III (HEMKLE)</th>
<th>Group IV (HEMKLE +DMBA/TPA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROS (relative intensity of DCF)</td>
<td>6.31±1.62</td>
<td>13.39±1.61*</td>
<td>7.35±0.83#</td>
<td>9.72±0.56**#</td>
</tr>
<tr>
<td>LPO (nmole MDA-TBA chromophore formed/min/mg protein)</td>
<td>9.53±1.75</td>
<td>49.36±3.49*</td>
<td>9.72±1.25#</td>
<td>22.16±2.16**#</td>
</tr>
<tr>
<td>GSH (μmoles GSH/mg protein)</td>
<td>11.13±1.61</td>
<td>5.40±0.84*</td>
<td>13.50±4.14#</td>
<td>7.67±1.36**#</td>
</tr>
<tr>
<td>GST (μmoles GSH-CDNB conjugates formed/mg of protein)</td>
<td>31.74±2.99</td>
<td>5.18±1.00*</td>
<td>30.49±4.32#</td>
<td>9.86±0.41**#</td>
</tr>
<tr>
<td>GSH-Px (μmoles NADPH oxidized/min/mg protein)</td>
<td>10.65±1.44</td>
<td>39.53±1.00*</td>
<td>6.19±1.48#</td>
<td>21.03±4.04**#</td>
</tr>
<tr>
<td>SOD (μmoles/min/mg protein)</td>
<td>2.19±0.18</td>
<td>1.75±0.05*</td>
<td>2.19±0.12#</td>
<td>1.94±0.09**#</td>
</tr>
<tr>
<td>CAT (μmoles H₂O₂ decomposed/min/mg protein)</td>
<td>2.45±0.72</td>
<td>0.51±0.07*</td>
<td>2.054±0.56#</td>
<td>1.03±0.17**#</td>
</tr>
</tbody>
</table>

*p ≤ .05 significant with respect to Group I (C).
# p ≤ .05 significant with respect to Group II (DMBA/TPA).
** p ≤ .05 significant with respect to Group III (HEMKLE). Data is expressed as Mean ± SD (n = 5). Data is analysed by one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test at Statistical significance. “a” represents significance with respect to Group I (C); “b” represents significance with respect to Group II (DMBA/TPA); “c” represents significance with respect to Group III (HEMKLE).

Table 2: Modulatory effect of DMBA/TPA and/or HEMKLE on oxidative stress and antioxidant defense system in kidney tissues

<table>
<thead>
<tr>
<th></th>
<th>Group I (C)</th>
<th>Group II (DMBA/TPA)</th>
<th>Group III (HEMKLE)</th>
<th>Group IV (HEMKLE +DMBA/TPA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROS (relative intensity of DCF)</td>
<td>32.94±2.58</td>
<td>75.8±1.48*</td>
<td>36.61±1.37#</td>
<td>49.32±2.42**#</td>
</tr>
<tr>
<td>LPO (nmole MDA-TBA chromophore formed/min/mg protein)</td>
<td>8.66±1.22</td>
<td>51.19±5.12*</td>
<td>10.36±0.54#</td>
<td>33.53±2.36**#</td>
</tr>
<tr>
<td>GSH (μmoles GSH/mg protein)</td>
<td>2.75±0.39</td>
<td>0.52±0.21*</td>
<td>2.49±0.74#</td>
<td>0.98±0.20**#</td>
</tr>
<tr>
<td>GST (μmoles GSH-CDNB conjugates formed/mg α-protein)</td>
<td>3.91±0.47</td>
<td>0.69±0.09*</td>
<td>3.15±0.83#</td>
<td>1.74±0.17**#</td>
</tr>
<tr>
<td>GSH-Px (μmoles NADPH oxidized/min/mg protein)</td>
<td>0.22±0.05</td>
<td>5.39±0.81*</td>
<td>0.199±0.043**#</td>
<td>3.24±0.90**#</td>
</tr>
<tr>
<td>SOD (μmoles/min/mg protein)</td>
<td>2.03±0.12</td>
<td>1.61±0.11*</td>
<td>2.13±0.34#</td>
<td>1.91±0.14**#</td>
</tr>
<tr>
<td>CAT (μmoles H₂O₂ decomposed/min/mg protein)</td>
<td>0.90±0.22</td>
<td>0.02±0.002*</td>
<td>0.85±0.15#</td>
<td>0.39±0.25**#</td>
</tr>
</tbody>
</table>

*p ≤ .05 significant with respect to Group I (C). ** p ≤ .05 significant with respect to Group II (DMBA/TPA). ** p ≤ .05 significant with respect to Group III (HEMKLE). Data is expressed as Mean ± SD (n = 5). Data is analysed by one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test at Statistical significance. “a” represents significant with respect to Group I (C); “b” represents significance with respect to Group II (DMBA/TPA); “c” represents significance with respect to Group III (HEMKLE).
Also, DMBA/TPA treatment caused a significant (p≤0.05) decrease in GSH, GST, SOD, and CAT levels/activities in both liver and kidney tissues of Group II (DMBA/TPA) when compared to Group I (C) and Group III (HEMKLE). However, HEMKLE administration significantly (p≤0.05) enhanced their levels/activities in Group IV (HEMKLE+DMBA/TPA) when compared to Group II (DMBA/TPA). No significant change was observed in Group III (HEMKLE) when compared to Group I (C) (as shown in Table 1 & 2).

3.5. Expressions of Hypoxia and angiogenesis markers

A significant (p≤0.05) increase in mRNA and protein expressions of HIF-1α, HMOX-1, VEGF, bFGF, and ANGPT-2 was observed in Group II (DMBA/TPA) when compared to Group I (C) and Group III (HEMKLE). However, HEMKLE supplementation significantly (p≤0.05) decreased the mRNA and protein expressions of these in Group IV (HEMKLE+DMBA/TPA) when compared to Group II (DMBA/TPA). No significant change was observed in Group III (IKLE) when compared to Group I (C) (as shown in Figs. 7 & 8).

Fig. 7: Modulatory effect of HEMKLE on mRNA expressions of hypoxia and angiogenesis associated markers in skin/tumors

Fig. 8: Modulatory effect of HEMKLE on protein expressions of hypoxia and angiogenesis associated markers in skin/tumors
4. DISCUSSION
The imbalance between the generation of free radicals and their scavenging leads to oxidative stress, which is considered a driving factor behind carcinogenesis. It is considered that phytochemicals are potentially helpful in maintaining this balance, thus impeding carcinogenesis [26]. In addition, cancer cells evolve multiple pathways to survive, such as evading apoptosis, angiogenesis, and metastasis. So, targeted regulation of any of the varied pathways could be of great potential in preventing cancer [27]. Our previously published study showed the pro-apoptotic effect of HEMKLE against DMBA/TPA-induced skin carcinogenesis in mice, as evident through assessment of tumor statistics, histology of skin/tumors, oxidative stress, activities of antioxidant enzymes, mRNA and protein expressions of apoptosis-associated genes [15]. Further extending our previous study, the present study was aimed to evaluate the role of HEMKLE upon hypoxia and angiogenesis during skin carcinogenesis. Also, we examined the modulatory effects of HEMKLE upon damages incurred in liver and kidney tissues during skin carcinogenesis in mice.

The ABTS and DPPH assays are standard methods for evaluating a compound's antioxidant potential. HEMKLE showed significant radical scavenging and antioxidant activity when tested using the ABTS and DPPH assays in corroboration to the previous study [28]. These findings suggest that HEMKLE act as a potential antioxidant agent, and could be further used in drug development.

Hyperproliferation is a characteristic feature of most cancers, and anticancer agents do targeted inhibition of excessive cell proliferation [29]. In the present study, histopathological investigation revealed that HEMKLE supplementation markedly decreased the extent of hyper-keratinization, hyper-proliferation, epidermal thickness and cell count in HEMKLE+DMBA/TPA co-treated group when compared to DMBA/TPA alone treated group, which indicated the potential of HEMKLE to inhibit aberrant cell proliferation during skin carcinogenesis. Our previous study supports this observation. This is in corroboration with our previous study, in which we observed that HEMKLE supplementation markedly improved the histarchitecture of skin/tumors in HEMKLE+DMBA/TPA co-treated group [15].

Toxicity profiling during skin carcinogenesis was studied by assessing the liver and kidney function markers viz., SGOT, SGPT, ALP, BUN, Bilirubin, Urea, and Creatinine. Increase levels of SGOT, SGPT, and ALP in serum indicate liver damage. Likewise, elevated levels of BUN, Bilirubin, Urea and Creatinine in serum are the indicators of kidney disease, and kidney failure [30-31]. In the present study, DMBA/TPA exposure increased the SGPT, SGOT, ALP, bilirubin, urea, creatinine, and BUN levels in Group II (DMBA/TPA) mice, which suggests cellular damage. However, HEMKLE administration markedly restored their levels in Group IV (HEMKLE + DMBA/TPA). HEMKLE was prepared following the method of Ningappa et al. [32], who reported that Hydroethanolic Murraya koenigii leaf extract contain various carbazole alkaloids viz., mahanine, mahanimbine, koenimbine, girtinimbine that possess high antioxidant and radical scavenging activities against ROS. Similar to our observation, Kesari et al. [33] also reported that oral administration of Murraya koenigii leaves extract for one month caused a marked decrease in the SGOT, SGPT, ALP, and creatinine levels. Another study by Joel Enoch, [34] also reported that administration of Murraya koenigii leaf extract caused a significant decrement in creatinine, protein, and urea levels in diabetic rats.

DMBA is an exogenous hepatotoxin, which is well known for modulating phase I and II xenobiotic metabolizing enzymes of the liver. ROS formed during the metabolism of DMBA leads to oxidative stress, and chromosomal damage [35]. ROS generated by DMBA is potent and plays an essential role in pathogenesis by damaging cellular macromolecules in the cells, including lipids (forms lipid peroxides), proteins, and nucleic acids [36]. The high oxidative stress generated during DMBA metabolism causes damages to several body organs viz., liver, kidney, testis etc [37]. On the contrary, a variety of antioxidant enzymes present in the cell alleviates excessive free radicals and can protect itself from ROS-mediated insult [38]. GSH scavenges free radicals, oxidizes itself to form oxidized glutathione (GSSG). Further, GST helps in eliminating DMBA metabolites out of the cell and thus protects against oxidative stress. SOD converts the superoxide radicals to molecular oxygen and hydrogen peroxides, whereas, GSH-Px catalyzes the reduction of hydrogen peroxide to water and molecular oxygen. CAT catalyzes the degradation of hydrogen peroxide to harmless water and molecular oxygen, consequently completing the detoxification process initiated by SOD [39]. In the present study, DMBA/TPA exposure caused a significant increase in ROS, LPO, and GSH-Px and a marked decrease in GSH, GST, SOD, and CAT in liver and kidney tissues, which reflect cellular damage to these organs. Cellular damage due to DMBA/TPA exposure was further confirmed by histological analysis.
of these tissues, where distorted histoarchitecture was observed. However, HEMKLE administration in HEMKLE+DMBA/TPA co-treated group profoundly decreased the cellular damages as evident from the reduced ROS, and LPO levels, increase in the activities of antioxidant enzymes, and restoration of the histoarchitecture. Various carbazole alkaloids, flavonols, and terpenoids found in MK leaf exhibit free radical-scavenging activities. A study by Desai et al. [40] reported that administration of Murraya koenigii leaves extract significantly increased the hepatic SOD, CAT, GSH, and decreased the LPO in carbon tetrachloride (CCl₄) treated animals. Our previously published report also stated that HEMKLE administration caused a significant decline in oxidative stress (ROS, and LPO), and restored the antioxidant enzyme activities in skin /tumors HEMKLE+DMBA/TPA co-treated group [15]. Chronic oxidative stress and hypoxia are the most common characteristics of fast-growing solid tumors [41]. The formation of tumor mass creates a hypoxic environment that activates HIF-1α, a transcription factor and a decisive factor for angiogenesis or apoptosis. Earlier studies have reported that cancer cells become adaptive to hypoxia and resist their apoptosis in a nutrient-deprived hypoxic environment [42]. This is in corroboration to our previous study, in which DMBA/TPA treatment caused a marked decrease in apoptosis assessed through mRNA and protein expressions of Bcl-2, caspase-9, and caspase-3 [15]. In the present study, DMBA/TPA exposure caused a significant increase in mRNA and protein expression of HIF-1α, evidenced that DMBA treatment created hypoxic conditions, which further facilitated the angiogenesis. However, HEMKLE supplementation attenuated the expression of HIF-1α in HEMKLE+DMBA/TPA co-treated group, which represents that HEMKLE restored the normal levels of HIF-1α within tissues.

Heme oxygenase-1 (HMOX-1) is a stress-inducible enzyme known to be involved in angiogenesis. HIF-1α is known to induce the expression of HMOX that facilitates the release of iron, biliverdin, and carbon monoxide from heme molecule during hypoxia [43]. In the present study, DMBA/TPA exposure caused a significant increase in HMOX-1 mRNA and protein expression, which provided an evidence that DMBA treatment increased HMOX-1 expression in cancer conditions further facilitated angiogenesis. The increase in the expression of HMOX-1 could be due to hypoxic conditions in the tumors. However, HEMKLE supplementation attenuated the expression of HMOX-1 in HEMKLE+DMBA/TPA co-treated group, which represents that HEMKLE restored the normal levels of HMOX-1 within tissues. HIF-1α also acts as a trigger/switch for activating most of the angiogenic markers, viz., vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and Angiopoietin-2 (ANGPT-2). VEGF, the most critical gene required for angiogenesis, is mainly governed by HIF-1α [44]. VEGF is considered a master switch of angiogenesis, and VEGF, bFGF, and ANGPT-2 promote angiogenesis and neovascularization. ANGPT-2 is chiefly crucial for disrupting the host vasculature and sensitizing the endothelial cells towards angiogenic signals [45]. A noteworthy enhanced VEGF, bFGF, and ANGPT-2 expressions upon DMBA/TPA treatment advocate enormous tumor growth through angiogenesis. In contrast, HEMKLE supplementation led to a significant down-regulation in mRNA and protein expression of VEGF, bFGF, and ANGPT-2 in HEMKLE+DMBA/TPA co-treated group, which showed angiogenesis inhibiting effect of HEMKLE. It is well-reported that the targeted regulation of angiogenesis is among the best chemotherapeutics and chemo preventive approaches [46]. Girinimbine, a carbazole alkaloid, also an active component of MK leaf, showed in vitro inhibition of HUVECs (human umbilical vein endothelial cells) cell proliferation, endothelial cell invasion, migration, tube formation, and wound healing. Girinimbine administration also revealed in vivo inhibition of blood vessel formation in zebrafish embryos, indicating anti-angiogenic activity by balancing the expression of VEGF and bFGF proteins [47].

5. STUDY LIMITATIONS

Further extensive studies are obligatory to explore the underlying mechanism of action and pharmacokinetics of HEMKLE.

6. CONCLUSION

The observations of this study indicate the positive effects of HEMKLE in inhibiting chemically induced skin cancer in mice, and associated toxicity incurred in liver and kidney tissues. The inhibition in hypoxia and angiogenesis, reveals its potential in impeding skin carcinogenesis. HEMKLE also showed protective effect against damages induced in liver and kidney tissues during skin tumorigenesis. We believe that the successful outcomes of the current study may go further and may lay down the foundation of further/future research.
REFERENCES