Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
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ORIGINAL ARTICLE

Hemidesmus indicus (L)-Derived 2-Hydroxy-4-Methoxy Benzoic Acid Attenuates DNA Damage and Autophagy in SK-MEL28 Cells via p-ERK Pathway

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Submitted: 29-Oct-2020 Revised: 04-Mar-2021 Accepted: 28-May-2021 Published: 24-Jan-2022

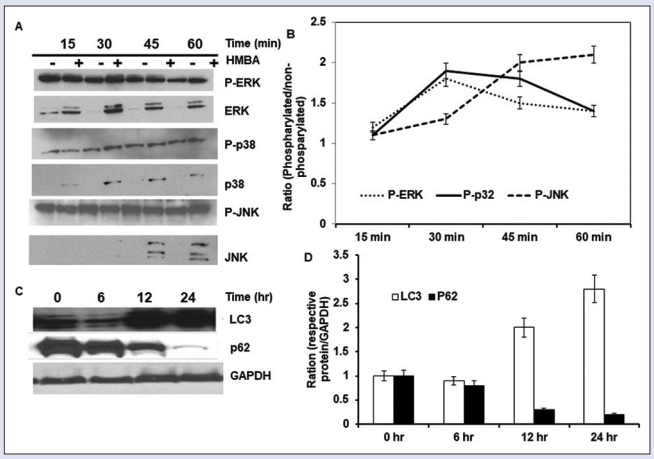
ABSTRACT

Background: Melanoma, a type of skin cancer, is a leading cause of death worldwide. Currently available therapy shows numerous side effects; therefore, there is an urgent need to develop safe drugs to treat this malignancy. Recently, 2-hydroxy-4-methoxy benzoic acid (HMBA), a bioactive plant component, has been reported to demonstrate various biological functions. **Objectives:** In this study, the anticancer effect of HMBA was assessed against SK-MEL-28 cells. **Materials and Methods:** DNA damage was assessed using DNA strand break assay (comet assay), apoptosis-mediated cell death by Annexin-V, and Terminal deoxynucleotide transferase dUTP Nick End Labeling experiments. Western blot analysis was performed to assess the phosphorylation of ERK, p38, and JNK. **Results:** Increasing the time of exposure decreases the IC_{25}, IC_{50}, and IC_{75} value of HMBA. Activity of lactate dehydrogenase in the culture medium of control and HMBA-exposed cells directly correlate its cytotoxic property. HMBA caused dose-dependent DNA damage and induced apoptosis in SK-MEL-28 cells. AO-staining showed autophagy in HMBA-treated cells, whereas Western blot analysis revealed increase in the phosphorylation of ERK, p38, and JNK. Furthermore, our results show that ERK phosphorylation is responsible for the activation of autophagy protein such as LC3 and p62. These observations reveal that the activation of caspase-3 and commencement of autophagy is mediated through activation of ERK phosphorylation. **Conclusion:** Therefore, HMBA inhibits propagation of SK-MEL-28 cells via stimulation of apoptosis and autophagy. In addition, HMBA promotes apoptosis and autophagy by phosphorylation of vital signaling protein such as ERK, however other vital signaling such as p38 and JNK also phosphorylate. Thus, HMBA may be of therapeutic importance for the treatment of melanoma.

Key words: 2-hydroxy-4-methoxy benzoic acid, apoptosis, autophagy, melanoma, p-ERK

SUMMARY

- This study demonstrates the anticancer, apoptotic, and autophagy activation property of 2-hydroxy-4-methoxy benzoic acid (HMBA) in SK-MEL-28 human malignant melanoma cells. ERK phosphorylation and autophagy activation were initiated by HMBA leading to apoptosis-mediated cell death. Furthermore, as evidenced by the annexin V-cy3 and 7-AAD staining, HMBA did not cause any necrosis-mediated cell death. Thus, HMBA could be recognized as a significant escort molecule.



Abbreviations used: HMBA: 2-hydroxy-4-methoxy benzoic acid; NF1: Neurofibromin 1; MAPK: mitogen-activated protein kinase; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMEM: Dulbecco's modified Eagle's medium; FBS: fetal bovine serum; TUNEL: Terminal deoxynucleotide transferase dUTP Nick End Labeling; AVO: Acidic vesicular organelles.

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DOI: 10.4103/pm.pm_489_20

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INTRODUCTION

The current development in the treatment of different types of cancer has greatly improved; however, there is a significant amount of information needed in the area of skin cancer. Melanoma, in skin has low occurrence, however, it is accountable for ~80% of the skin cancer-related deaths.[1,2] Furthermore, it is known that skin cancer has a higher tendency to metastasize. In addition, it weakly responds to the majority of the currently available drugs such as carboplatin and paclitaxel.[3] Moreover, these medications can be lethal to the patients not because of their adverse effects but because they weaken the immune system of the patients, thereby making the patients more susceptible to other infectious diseases.[4,5] Therefore, researchers are searching for new compounds that can have better anticancer effect and less adverse effects. Natural

Cite this article as: Miao Y, Chen Z, Wang Z, Hu C, Chen L, Sun H. *Hemidesmus indicus* (L)-derived 2-hydroxy-4-methoxy benzoic acid attenuates DNA damage and autophagy in SK-MEL28 cells via p-ERK pathway. Phcog Mag 2021;17:819-27.

sources such as plants provide safe anticancer drugs that can be utilized in the treatment of cancer, for example, vincristine, taxol, vinblastine, irinotecan, topotecan, podophyllotoxin, and camptothecin.[6,7] These aforementioned compounds also serve as lead molecules in the development of new anticancer drugs.[8] It is noteworthy that not all the plant-based products are non-toxic to humans; therefore, it is important to validate such compounds for minimal toxicity and maximum efficacy.

Hemidesmus indicus (L) belongs to the family Asclepiadaceae. The plant has been used in ancient medication to treat inflammation and various blood disorders.[9-12] One of its active ingredients is 2-hydroxy-4-methoxy benzoic acid (HMBA), which exhibits anti-inflammatory, antioxidant, and antidiabetic properties.[11-16] Recent studies on HMBA encouraged us to HMBA could be an effective candidate against blood cancer such as malignant melanoma.

Mutated oncogene is responsible for most of the cancers, for example, v-Raf murine sarcoma viral oncogene homolog B (*BRAF*), among all mutated melanomas, cause 80% of the *BRAF*-mutated tumors.[17-19] Another significant mutated oncogene is the neuroblastoma RAS viral oncogene homolog (*NRAS*), which is mutated in ~30% of all melanomas.[17-19] Neurofibromin 1 (*NF1*), a recently identified mutated oncogene, accounts for 10% of all melanomas.[17] All these three mutations act through mitogen-activated protein kinase (MAPK) signaling pathway. MAPK is the crucial signaling pathway in the development of cancer. This encouraged us to identify a novel therapeutic compound for use against melanoma. Therefore, in this study, we scrutinized the possible mechanism of HMBA in melanoma cancer cells.

MATERIALS AND METHODS

Chemicals and reagents

In this study, 2-hydroxy-4-methoxy-benzoic acid (HMBA; Catalog No: 2237-36-7) (95% purity by HPLC); dimethyl sulfoxide (DMSO); streptomycin; penicillin; and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were procured from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) (10% heat-inactivated), and 3.5% trypsin were acquired from Gibco (Carlsbad, CA, United States). From Cell Institute, Chinese Academy of Sciences (Shanghai, China), the human malignant melanoma cell line (SK-MEL-28) was obtained. The cells were grown in DMEM containing 5% heat-inactivated FBS, 100 µg/mL streptomycin, and 100 U/mL penicillin with 5% CO_2 at 37°C.

Assessment of cell cytotoxicity

Before beginning the actual experiment, SK-MEL-28 cells (1×10^5 cells/mL) were grown in a 96-well cell culture plate (10,000 cells/chamber) for 24 h. After 24 h, the cells were then exposed to 0, 6.25, 12.5, 25, 50, 100, 200, and 400 µM of HMBA dissolved in DMSO (0.1%) for 24 and 48 h. Cells exposed to 0 µM concentration of HMBA were kept as control. In order to avoid the effect of solvent in control and experiment cells, equal volume of DMSO (10 µL of 0.1% DMSO/mL of medium) was supplemented to per mL of growth medium to reach the required dose of HMBA, including 0 µM of HMBA. After the experimental period, a fresh medium containing MTT was added to the control and experimental cells and then incubated for another 4 h. The formazan crystals formed were dissolved. The optical density was read on a programmed microplate reader (BioTek Instruments, Inc., Winooski, VT, USA) at 570 nm. The inhibition of cell proliferation was measured based on the percentage difference as compared with normal cells (0 µM of HMBA). The cytotoxicity was calculated as the dose of HMBA preventing cell propagation by 25% (IC_{25} concentration), 50% (IC_{50} concentration), and 75% (IC_{75} concentration).

Estimation of lactate dehydrogenase activity

The activity of lactate dehydrogenase (LDH) was estimated by kit obtained from Stanbio Laboratory (Boerne, Texas, USA; Cat. No. G428-86).

Assay of DNA damage (strand breaks) by single-cell electrophoresis (comet assay)

Comet assay is a sensitive technique, which is frequently used by various investigators to estimate DNA damage within a cell. In this study, cells were grown after exposing them to different concentrations of HMBA. Then, the cells were harvested through trypsin digestion. Excess trypsin was removed by washing the cells in phosphate-buffered saline (PBS) following centrifugation at 80 ×*g* for 2 min. Then, the comet assay was performed and data were processed as mentioned previously.[20,21] Briefly, the slides were precoated by using 1% normal melting point agarose (NMPA). After the cells were grown, they were harvested by enzymatic digestion and counted (1×10^6 cells/mL). The cells were suspended in 0.5% low (L) NMPA (1:1000) and were placed (50 µL) on the slide, which were precoated with NMPA. Next, the cell layer was covered with 1% NMPA (40 µL). The experimental slides were chilled down on ice. The cells were lysed by keeping the slides in lysis buffer (2.5 M NaCl, 10 mM Tris HCl, pH 10 with 100 mM ethylenediaminetetraacetic acid [EDTA], detergents, and 10% DMSO) for 1 h and subsequently neutralized with 0.4 M Tris-HCl (pH 7.5). Then, we performed electrophoresis in a dark room at 40 mA for 25 min with alkaline buffer (pH 13). After electrophoresis, the cells in slides were stained with ethidium bromide (10 mg/mL concentration), and the cells were observed using a fluorescent microscope at 40× objective. The experimental cell images were obtained through microscope and processed using Tritek Comet Score freeware™ v1.5.

Assay of DNA adducts

To quantify the DNA adducts, before treating the cells with HMBA, they were cultured in glass coverslips. Subsequent to the experiment, cells were fixed in 70% ethanol. Then, the processed cells were exposed to RNase A (100 ng/mL) for 1 h at 37°C. The cells were exposed to 4 mol/L HCl for 7 min, and the DNA was hydrolyzed and neutralized in 50 mmol/L Tris base for 2 min. The nonspecific sites were masked in 10% goat serum. The processed cells were exposed to anti 8-oxo-dG (1:1000; GTX41980; Gene Tex, Irvine, CA) antibody for 10 ± 12 h at 25°C. Then, the cells were quenched with 3% H_2O_2 in methanol for 30 min and incubated with peroxidase-conjugated goat anti-mouse IgG (A3682; Sigma-Aldrich) for 1 h at 25°C. After washing, the color was generated by chromogenic enzyme reaction with diaminobenzidine. The nuclei were counter-stained with toluidine blue. We used 10 diverse grounds under the microscope at ×400 to calculate the 8-oxo-dG-positive cells, which were counted through ImageJ software (NIH).

Flow cytometric analysis to detect apoptosis

Cells were exposed to three different concentrations of HMBA (IC_{25}, IC_{50}, and IC_{75}). After exposing the cells with HMBA, they were washed with ice-cold PBS. Then, the cells were harvested via trypsin-EDTA digestion. The control and experimental cells were treated with 7-aminoactinomycin D (7-AAD) and annexin V-cy3 dyes. Furthermore, 7-AAD and annexin V-cy3-stained cells were processed as described by Thirunavukkarasu *et al.*[22]

Terminal deoxynucleotide transferase dUTP Nick End Labeling assay

Terminal deoxynucleotide transferase dUTP Nick End Labeling (TUNEL) assay was performed by using TACS 2 TdT-Fluor *in situ* apoptosis detection kit (Trevigen Inc, MD, USA; Cat No. 3500-096-01). For each condition, 10 different fields were selected and quantified through fluorescence microscope. The experiment was repeated thrice with second copy in every instance.

Detection of acidic vesicular organelles

Approximately 1 × 10^5 cells were grown on coverslips in 24-well cell culture plates. The cells were exposed to different concentrations of HMBA (IC_{25}, IC_{50}, and IC_{75}) and DMSO (control). After the experimental duration, the cells were exposed to staining dye such as acridine orange (AO) (1 μg/mL in PBS) for 15 min. After staining the cells, excess AO was removed through washing the cells with PBS. The stained cells were observed through Olympus fluorescence microscope at ×60.

Assay of caspase-3 activity

After the experimental duration, the cells were harvested as explained in the previous section (flow cytometric analysis to assay apoptosis). The cells were lysed by using 50 μL of ice-cold cell lysis buffer. The cells were maintained on ice for another 10 min to complete the lysis. After this, the supernatant (cytosolic fraction) was obtained via centrifugation at 10,000 ×*g* for 1 min. The caspase 3 activity was estimated from cytosolic fraction. The activity was assayed through commercially obtainable fluorescent caspase 3 assay kit (BD Biosciences-Clontech; Catalog No. 556485). The experiment was conducted based on manufacturer's protocol to avoid any background reading.

Quantification of signaling proteins by Western blot analysis

Protein was extracted from the cells exposed with IC_{50} concentration of HMBA and untreated cells for indicated timepoints. From the control and experimental cells, 30 μg of protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After the proteins were separated, the protein bands were reloaded onto Immobilon-P membrane (Millipore, Bedford, MA). Protein-transferred Immobilon-P membrane was masked for 1 h with 1.0% bovine serum albumin (BSA) in TBS. A Immobilon-P membrane carrying different experimental proteins and nonspecific site blocked with 1.0% BSA was incubated with anti-P-ERK (Cell signaling; #9101), anti-ERK (Cell signaling; #9102), anti-P-p38 (Cell signaling; #9211), anti-p38 (Cell signaling; #9212), anti-P-JNK (Cell signaling; #4668), anti-JNK (Cell signaling; #4664), anti-LC-3 (Cell signaling; #2775), and anti-p62 (Cell signaling; #5114) antibodies (all at 1:1000 dilution) for 10 ± 12 h at 4°C. After incubating the membrane with respective primary antibody, the membrane was washed with TBST buffer (×4). The washed membranes were then incubated with respective host-specific peroxidase-linked secondary antibodies (1:25,000) for 1 h at 25°C. After washing the membrane with TBST buffer (×4), the specific immunoreactive proteins were found through an improved chemiluminescence (ECL) kit (Amersham-Pharmacia). To ensure identical protein loading, GAPDH (Cell signaling; 14C10) immunoreactive protein was calculated in control and experimental samples. Using ImageJ software (NIH, Bethesda, USA), the immunoreactive protein band density was calculated.

Statistical analysis

The results are shown as mean ± standard deviation. The differences between mean of control and HMBA-exposed cells were analyzed through one-way analysis of variance Tukey's *post hoc* test. Every assay was conducted thrice, if not otherwise mentioned. $P < 0.05$ was considered statistically significant.

RESULTS

Cytotoxicity of 2-hydroxy-4-methoxy benzoic acid

MTT assay was used to evaluate the cytotoxicity of HMBA. The cells were exposed to 6.25, 12.5, 25, 50, 100, 200, and 400 μM of HMBA. According to the results, HMBA is a very powerful cytotoxic agent, and it demonstrated both dose- and time-dependent inhibition of cancer cell propagation [Figure 1a]. HMBA at 50 and 25 μM concentration showed significant cytotoxic effect after 24 and 48 h respectively. The IC_{25}, IC_{50}, and IC_{75} values for HMBA were respectively 46, 105, and 189 μM at 24 h and 34, 65, and 113 μM at 48 h. These results show that the concentration of HMBA decreased to accomplish comparable efficacy with a higher treatment period.

After determining the cytotoxicity of HMBA, we estimated the LDH activity in the medium of control and HMBA-exposed cells [Figure 1b]. This assay was performed to know that reduced MTT output is due to plasma membrane rupture. LDH is mainly present in the cytosol of the cell, which is released into the medium after the rupture of the plasma membrane. Therefore, the level of LDH activity in the cell culture medium directly corresponds to the cytotoxic activity of the drug candidate. Similar to the MTT assay, LDH activity in the medium of HMBA-treated cells was increased when compared to control cells. Furthermore, we observed that the concentration of HMBA is directly proportional to the activity of LDH in the culture medium. Moreover, 50 and 25 μM of HMBA demonstrated significant LDH activity ($P < 0.05$)

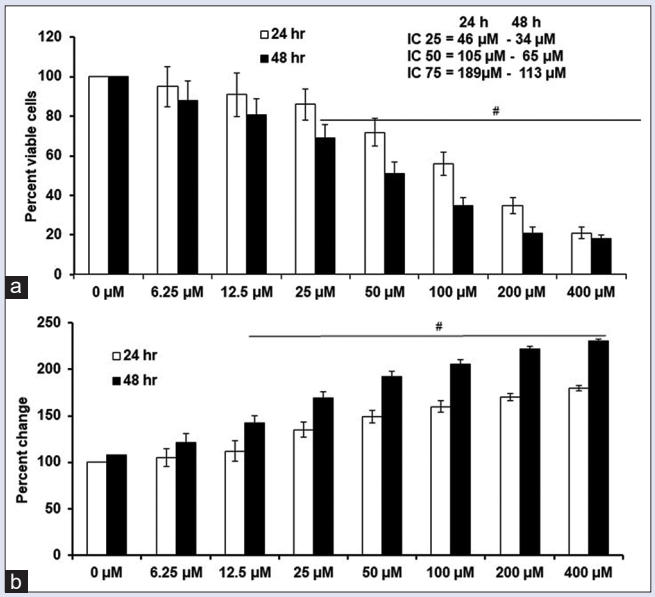

Figure 1: 2-hydroxy-4-methoxy benzoic acid-induced dose-dependent cytotoxicity in SK-MEL-28 cells. The cells were treated with 6.25, 12.5, 25, 50, 100, 200, and 400 μM 2-hydroxy-4-methoxy benzoic acid and then incubated for 24 and 48 h. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (a) and lactate dehydrogenase activity in the medium (b) were performed in control and experimental cells to assess the 2-hydroxy-4-methoxy benzoic acid -induced cytotoxicity. Data are presented as the mean ± standard deviation of four different experimentation. One-way analysis of variance Tukey's *post hoc* test was employed to analyze the statistical differences. $^*P < 0.05$, $^@P < 0.01$, $^\#P < 0.001$ versus 0 μM (control)

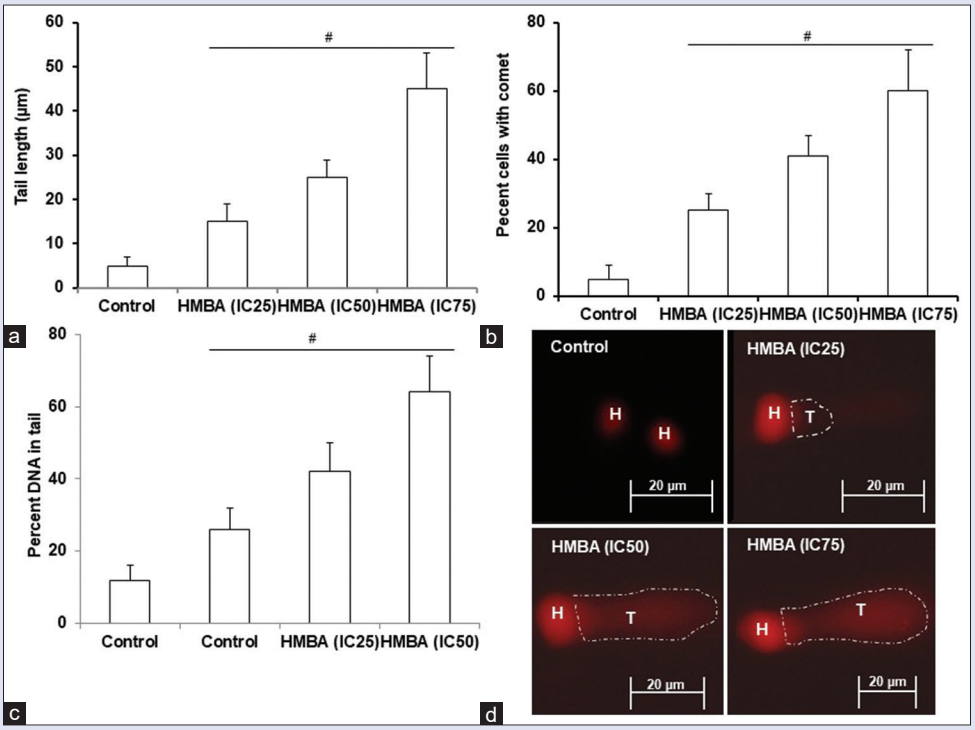

Figure 2: Impact of 2-hydroxy-4-methoxy benzoic acid on DNA damage. Cells were exposed to IC_{25}, IC_{50}, and IC_{75} concentrations of 2-hydroxy-4-methoxy benzoic acid for 24 h. (a) Tail length, (b) percent cells with a comet, (c) percent DNA in tail, and (d) representative photograph of normal and 2-hydroxy-4-methoxy benzoic acid-exposed cells. Data are presented as the mean ± standard error of mean. Experiments were repeated thrice with a copy each time. One-way analysis of variance Tukey's *post hoc* test was employed to test the statistical differences. $P < 0.05$ was considered as significant. $^{\#}P < 0.001$ versus control

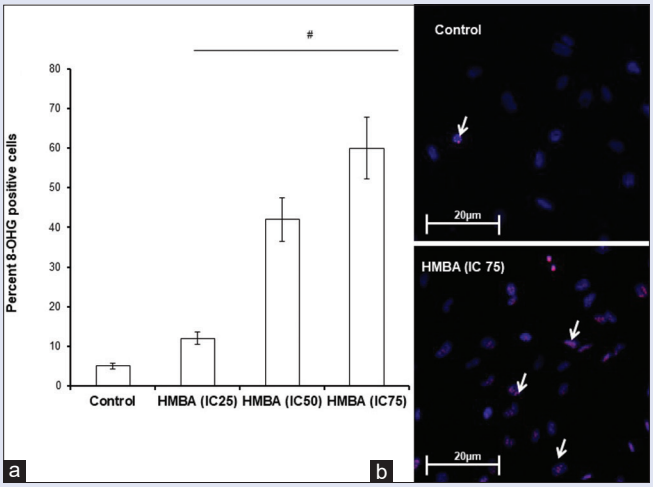

Figure 3: DNA damage by 2-hydroxy-4-methoxy benzoic acid in control and experimental cells. Cells were treated with IC_{25}, IC_{50}, and IC_{75} concentration of 2-hydroxy-4-methoxy benzoic acid for 24 h. (a) Percent cells with 8-hydroxydeoxyguanosine-positive and (b) representative picture of control and 2-hydroxy-4-methoxy benzoic acid -exposed cells. Data are presented as the mean ± standard deviation of four independent experiments. Experiments were repeated thrice with a copy each time. One-way analysis of variance Tukey's *post hoc* test is employed to test the statistical differences. $^{*}P < 0.05$, $^{@}P < 0.01$, $^{\#}P < 0.001$ versus 0 μM (control)

at 24 and 48 h, respectively. Furthermore, lower concentration of HMBA shows a significant level of cytotoxicity upon increasing the duration of the treatment. Therefore, we tested the cells with IC_{25}, IC_{50} and IC_{75} concentration of HMBA for 24 h only.

2-hydroxy-4-methoxy benzoic acid-induced DNA damage in SK-MEL-28 cells

HMBA-induced DNA damage was analyzed via comet assay. Figure 2 shows the results of comet assay. Statistically significant ($P < 0.05$) level of tail length [Figure 2a], number of (percent) comet-positive cells [Figure 2b], and % DNA in tail [Figure 2c] were observed in HMBA-exposed cells at IC_{25} concentration when compared with control cells. Furthermore, we observed that increased concentration (IC_{50} and IC_{75}) of HMBA increases the level of significance ($P < 0.001$ for IC_{50} and IC_{75}). Figure 2d depicts the representative image obtained from different experimental studies.

Level of 8-hydroxyguanine in control and experimental cells

To analyze the oxidative DNA damage (DNA adducts) caused due to HMBA, we estimated the levels of 8-hydroxyguanine (8-OHG) in control and experimental cells through immunohistochemistry [Figure 3]. As seen in comet assay, 8-OHG ($P < 0.05$) was increased with IC_{25} dose of HMBA when compared with control cells. Upon increasing the concentration of HMBA (IC_{50} and IC_{75}), the level of significance was found to be further increased ($P < 0.001$) than that of control cells.

Early and late apoptotic and necrotic cells in 2-hydroxy-4-methoxy benzoic acid-exposed melanoma cells

The data obtained from comet and DNA adducts show that HMBA might stimulate the DNA break. This result shows that HMBA might cause cell death, which may be apoptosis or necrosis-mediated cell death. To distinguish between apoptotic and necrotic cell death, the experimental cells were stained with annexin V-cy3 and 7-AAD for flow cytometric

analysis [Figure 4a and b]. It is noteworthy that 7-AAD binds with DNA of dead cells. One of the properties observed in the cells undergoing early apoptosis is the translocation of phosphatidylserine from the internal to the external side of the plasma membrane. This translocation could be measured through annexin V-cy3 dye. Therefore, 7-AAD-positive cells depict necrosis, whereas annexin V-cy3-positive cells denote early apoptosis. However, annexin V-cy3 + 7-AAD double-positive depicts late apoptosis. As shown in Figure 4a and b, IC_{25} dose-treated HMBA cells showed statistically significant ($P < 0.001$) amount of annexin V-cy3-positive cells when compared with control cells. Likewise, 7-AAD + annexin V-cy3 double-positive cells were increased from ~ 2% to ~ 8%, whereas there was no change in 7-AAD-positive cells when compared with control cells. Furthermore, exposure of cells to IC_{50} and IC_{75} concentration of HMBA increased the percentage of annexin V-cy3-positive cells (29%—IC_{50} and 37%—IC_{75}) and 7-AAD + annexin V-cy3 double-positive cells (15%—IC_{50} and 31%—IC_{75}). However, we did not observe any statistically significant increase in 7-AAD-positive cells with IC_{50} and IC_{75} concentration of HMBA-exposed cells. These results show that HMBA-induced cell death is mediated through apoptosis and not by necrosis in melanoma cells.

Next, to study the HMBA-induced apoptosis, we performed TUNEL experiment. Figure 5a and b shows the results of TUNEL assay and Figure 5c shows caspase-3 activity. HMBA (IC_{50}) exposed cells showed statistically ($P < 0.001$) significant quantity of TUNEL-positive cells than that of control cells. In this study, we observed [Figure 5c] that HMBA increased the activity of ($P < 0.001$) casepase-3 when compared with untreated cells. These results show that HMBA inhibited the melanoma cell proliferation and further induces apoptosis.

2-hydroxy-4-methoxy benzoic acid-induced acidic vesicle organelles in melanoma cells

Figure 6 shows the acidic vesicular organelles (AVO) in control and experimental cells. As shown in Figure 6, increasing the concentration of HMBA increased the number AVOs in HMBA-treated cells than that of control cells.

Involvement of mitogen-activated protein kinase signaling pathway and autophagy in 2-hydroxy-4-methoxy benzoic acid-treated cells

In this study, we observed a significant dose-dependent increase in the expression of P-ERK, P-p38, and P-JNK ($P < 0.001$) [Figure 7a and b] in the HMBA-treated cells when compared with control cells. Furthermore, we observed that HMBA increased the level of LC3 protein and decreased the level of p62 in SK-MEL-28 cells. Furthermore, we found that there was a time dependent increase in the expression of P-ERK, P-p38, and P-JNK [Figure 8a and b] when compared with non-phosphorylated forms. Similar results were obtained for autophagy proteins [Figure 8c and d].

To understand the contribution of P-ERK, P-p38, and P-JNK in autophagy protein and vice versa, the cells were pretreated with FR180204, SB 203580, and SP600125, which are phosphorylation inhibitors of ERK, p38, and JNK, respectively. Viability assay showed that [Figure 9a] only ERK inhibitor was able to prevent the HMBA-induced cell death and formation of AVOs [Figure 9b]. We also found that HMBA [Figure 9c and d] causes ERK phosphorylation, and it further activates the autophagy proteins such as LC3 and p62.

DISCUSSION

There are numerous advancements in cancer treatment. However, malignant tumors have always posed a challenge. One of the malignant tumors is melanoma. The incidence and mortality due to melanoma are rising worldwide. Furthermore, melanoma shows resistance to conventional chemotherapeutic agents.[23,24] Therefore, many researchers are trying to identify safe and effective anticancer agents from various

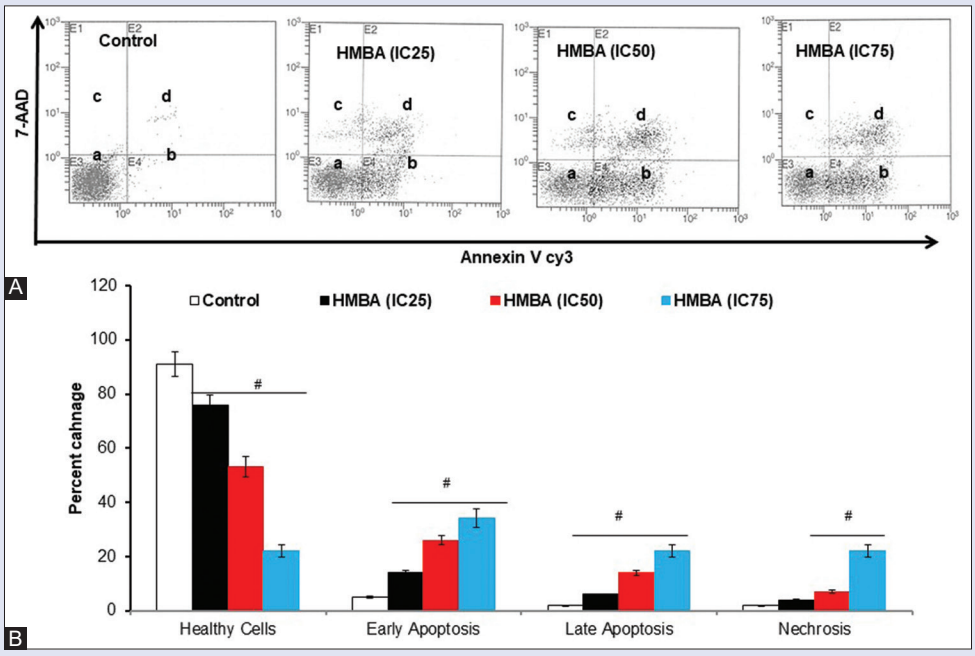

Figure 4: Normal, early apoptotic, late apoptotic, and necrotic cells in control and 2-hydroxy-4-methoxy benzoic acid-treated cells. Representative experimentation showing flow cytometric analysis in terms of percent apoptosis in control and 2-hydroxy-4-methoxy benzoic acid-treated cells. (A) The X-axis shows 7-AAD-positive cells, Y-axis shows annexin V-cy3-positive cells; (a) Annexin V-cy3 and 7-AAD-negative cells (normal cells); (b) Annexin V-cy3-positive cells (early apoptotic cells), (c) 7-AAD-positive cells (necrotic cells), and (d) annexin V-cy3 and 7-AAD-positive cells (late apoptotic cells). Experiments were repeated thrice with a copy each time. (B) Representation of cells in different stages

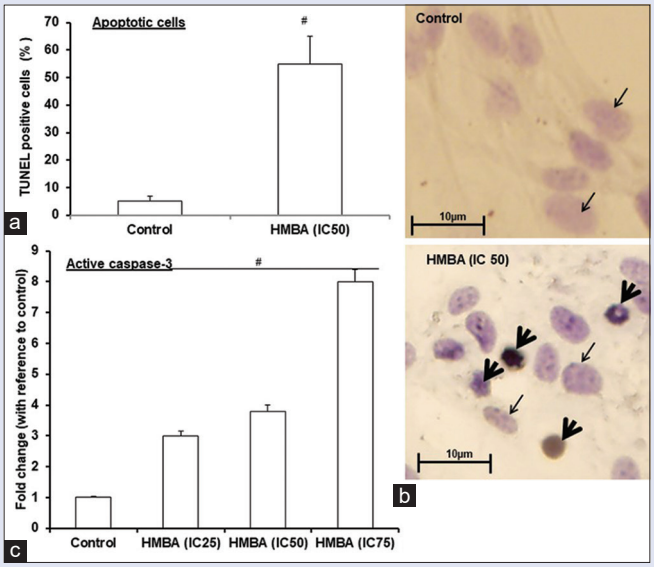

Figure 5: Terminal deoxynucleotide transferase dUTP Nick End Labeling-positive (a and b) and caspase-3 activity (c). Representative picture (a) indicating Terminal deoxynucleotide transferase dUTP Nick End Labeling-positive cells in control and 2-hydroxy-4-methoxy benzoic acid-treated cells. Analyses were repeated thrice with a carbon copy at every occasion. (b): Balance chart of Terminal deoxynucleotide transferase dUTP Nick End Labeling-positive cells. Experiments were repeated twice with triplicate at every occasion. Data are presented as mean ± standard deviation. One-way analysis of variance Tukey's *post hoc* test was employed to test the statistical differences. P <0.05 were considered as statistically significant. * $P < 0.05$, # $P < 0.001$ versus control

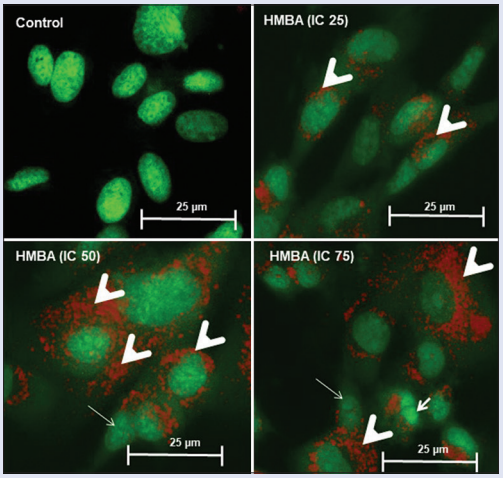

Figure 6: Acridine orange-stained control and 2-hydroxy-4-methoxy benzoic acid-treated cells. The thin arrow shows the nuclei of cells. Thick arrowhead shows acidic vesicular organelles. Experiments were repeated thrice with a copy each time

natural sources.[25,26] To the best of our knowledge, this is the first study to present the anticancer property and the molecular mechanism of HMBA, a major component of *H. indicus*.

HMBA is a phenolic acid, known for its antioxidant, antifungal, and hepatoprotective property.[18–21] However, various phenolic compounds are known for their anticancer property against numerous human cancer cell lines including liver, ovarian, bladder, breast cancers, gastric, and cervical.[27-29] However, the extract of *H. indicus* has shown to have antiproliferative effect.[30,31] The therapeutic effect of HMBA on hepatic toxicity is associated with antioxidant and changes in inflammatory cytokines.[20,21] Moreover, HMBA inhibited the accumulation of lipids in primary rat hepatocytes.[21] Many of the existing drugs developed from natural sources show systemic toxicity. Therefore, researchers use them as lead molecules.[32] However, a group of phenolics compounds is known to have less or no adverse effects in humans.[33] Therefore, researchers are very much interested in establishing phenolic compounds as important targets for the development of anticancer agent. In addition, phenolics are present in around 99% of the edible plants, and they are considered to be widespread components in the human food. Furthermore, recent data shows that phenolics compounds are chemopreventive agents.

Phenolic compounds demonstrate anticancer activity through different mechanisms including arresting cell cycle, induction of apoptosis, neutralizing the activity of cancer-causing agents, suppression of angiogenesis, antioxidative limit, and inversion of multidrug obstruction.[33-37] As HMBA is a phenolic compound, we intended to elucidate its mechanism of action in SK-MEL-28 human malignant melanoma cells. The anticancer property of HMBA is to provoke apoptosis, further, it was correlated with understanding its belongings on MAPK phosphorylation and autophagy. Our results show that HMBA prevents the proliferation of SK-MEL-28 melanoma cells in a portion subordinate way just as a time-subordinate way. According to the results of comet assay, the exposure of SK-MEL-28 melanoma cells to HMBA caused DNA damage. Furthermore, the severity of DNA damage was increased upon increasing the concentration of the drug. These findings indicate that HMBA might cause apoptosis or necrosis in a dose-dependent manner. To identify the reason behind this, control and experimental cells were incubated with annexin V-cy3 and 7-AAD. HMBA-induced apoptosis of SK-MEL-28 melanoma cells was evidence through increased number of cells in annexin V-cy3-positive and 7-AAD + annexin V-cy3 double-positive cells. 7-AAD-positive cells represent the necrotic cells, which was not observed even at IC_{75} concentration of HMBA-treated cells. These findings indicate that even at very high concentration of HMBA, SK-MEL-28 melanoma cells undergo apoptosis-mediated cell death. Apoptosis might be induced by extrinsic (through death receptor commencement) or intrinsic factors, which is mitochondrial cytochrome-c release to cytosol. Activation of procaspase-3 to active caspase-3 is an important mechanism in both pathways of apoptosis. Increased caspase-3 activity and its level in HMBA-treated cells confirms the apoptosis-mediated cell death in this study. Numerous studies have revealed that phenolic compounds might increase apoptosis of cancer cells. For example, Thabrew *et al.* showed that *H. indicus* extract induced apoptosis in HepG2 cancer cells.[30] Our results are in-line with earlier literature that phenolic compounds might induce cancer cell death via induction of apoptosis.[38-42] These findings show that HMBA induces caspase-dependent apoptosis and cell cycle arrest.[32-35]

One of the basal mechanisms that maintains the cellular homeostatic function is autophagy. This property maintains protein and organelle quality control, performing in corresponding with the ubiquitin-proteasome degradation mechanism to put off the gathering of polyubiquitinated and aggregated proteins.[43] Furthermore, this mechanism is also used in the removal of pathogens,[44] as well as the phagocytosis of apoptotic cells.[45] However, the effect of HMBA on these processes in cancer treatment is unknown. Albeit, most proof backs the path of autophagy in continuing cell survival, incomprehensibly, passing out of cells because of dynamic cell utilization has been ascribed

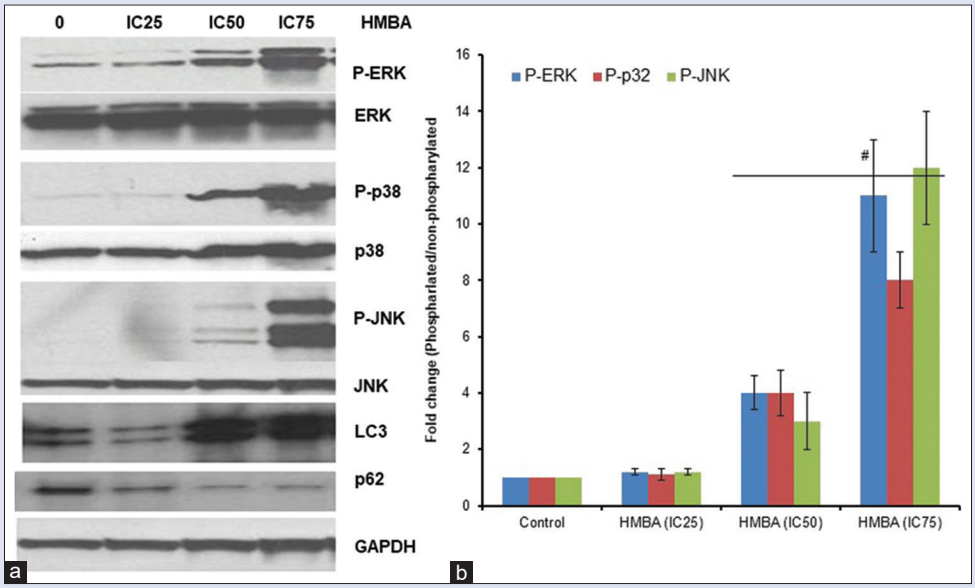

Figure 7: Western blot analysis for P-ERK, P-p38, P-JNK, LC3, and p62 expression. (a) Representative image (3 identical experiment) showing immunoblotting of phosphorylated and nonphosphorylated ERK, p38, JNK, LC3, p62, and NAPDH (as loading control). (b) Graphical representation of ERK, p38, and JNK (phosphorylated/nonphosphorylated ratio). Data are presented as mean ± standard deviation. One-way analysis of variance Tukey's *post hoc* test was employed to test the statistical differences. $P < 0.05$ were considered as significant. $^{\#}P < 0.001$ versus control

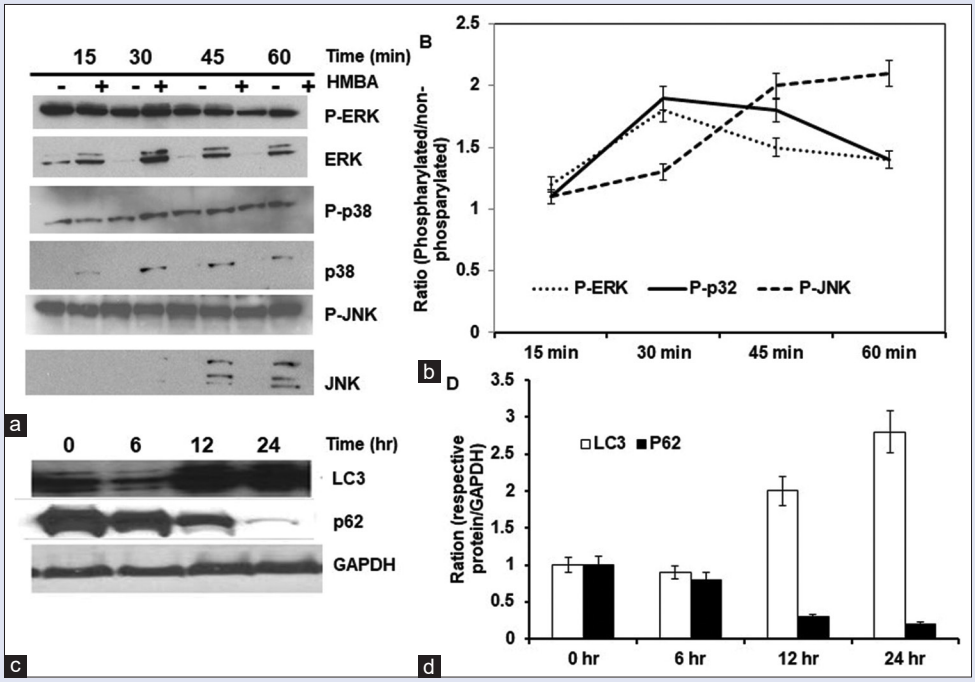

Figure 8: Time depends on Western blot analysis for PERK, Pp38, PJNK, LC3, and p62 expression cells treated with IC_{50} concentration of 2hydroxy4methoxy benzoic acid. (a) Representative image (3 identical experiments) showing immunoblotting of phosphorylated and nonphosphorylated ERK, p38, and JNK. (b) Graphical representation of ERK, p38, and JNK (phosphorylated/nonphosphorylated ratio). (c) Representative image (3 identical experiments) showing immunoblotting of LC3, p62, and NAPDH (as a loading control). (d) The graphical delineation of LC3 and p62. Data are presented as the mean ± standard deviation. Oneway analysis of variance Tukey's *post hoc* test was employed to test the statistical differences. $P < 0.05$ were considered as significant. $^{\#}P < 0.001$ versus control

boundless autophagy.[46,47] A previous study shows that deficiency in autophagy and apoptosis promotes tumorigenesis.[44] These two processes are moderately increased by the increase in necrotic cell death and inflammatory response in tumor cells.[44] Survival during starvation is prevented through autophagy and substandard apoptosis in tumor cells redirect to necrotic cell fate, creates chronically necrotic tumors. In this study, we found that HMBA induces AVOs in addition to DNA damage and DNA adducts.

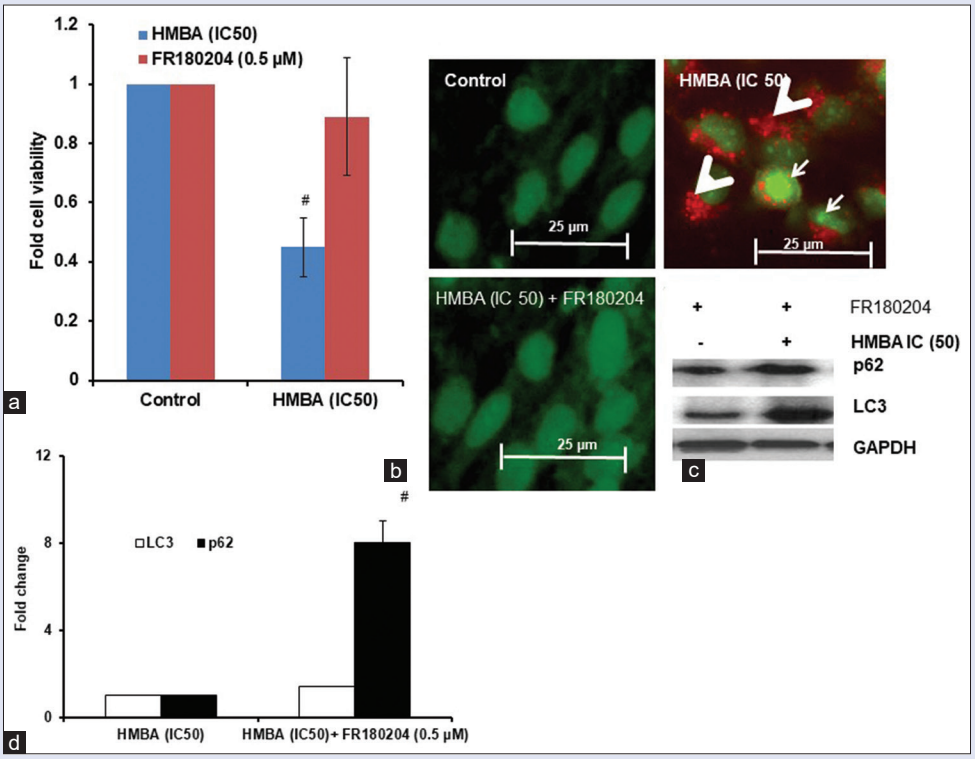

Figure 9: ERK inhibition and autophagy and active caspase3 activity in 2hydroxy4methoxy benzoic acid (IC_{50})exposed cells. Cells were preincubated with ERK phosphorylation inhibitor (FR180204 [0.5 μM]) for 30 min. 2hydroxy4methoxy benzoic acid was then added and the incubated further for 24 h. (a) Cell viability, (b) Acidic vesicular organelles, (c) LC3 and p62 protein by Western blot assay, and (d) densitometric ratio of LC3 and p62. Data are presented as the mean ± standard deviation of three unique trials performed in the copy. Oneway analysis of variance Tukey's $_{post\ hoc}$ test was employed to test the statistical differences. P <0.05 were considered as significant. $^{\#}P < 0.001$ versus control; $^{@}P < 0.05$ versus 2hydroxy4methoxy benzoic acid (IC_{50} dose)

In this study, we observed that HMBA successfully inhibits the proliferation of melanoma cells by actuating caspase-subordinate apoptosis. Apoptosis initiated by the phenolic compounds is regulated by both internal and external cellular pathways.[44-47] Melanoma starts with a dynamic sickness in the local area and it advances into the neighboring tissues. The metastatic capacity of melanoma cells decides the seriousness of this illness hence viewed as a significant objective in harmful melanoma board.[48]

Tumor cells in the case of malignant melanoma start to invade into neighboring tissues. Tumor cells spreading to neighboring tissue indicates the progressive disease. As melanoma is a highly metastatic tumor, it shows the vigorous nature of this tumor and is therefore considered to be an important target in the management of malignant melanoma.[48] Literature shows that various signaling molecules such as ERK/p38 and JNK phosphorylation are highly altered in the case of melanoma cells. In this study, we found that HMBA upregulates the phosphorylation of ERK/p38 and JNK signaling pathways, thereby leading to apoptosis in tumor cells. Different phenolic compounds are known to inhibit the cancer cell proliferation by altering these signaling pathways.[33,34] These pathways have been additionally shown to be significant being developed of harmful melanoma by advancing cell multiplication and metastasis.[49] In order to clarify the molecular mechanism responsible for the harmful effect of HMBA on melanoma cells, we pretreated the cells with ERK/p38 and JNK phosphorylation inhibitors. Inhibition of ERK phosphorylation reverted the anticancer effect of HMBA showing that ERK phosphorylation is essential for the anticancer effect of HMBA. Furthermore, it also reverted autophagy proteins such as LC3 and p62. Ultimately, HMBA could be a progressively effective anticancer operator against dangerous melanoma by at the same time focusing on the ERK and autophagy pathway.

CONCLUSION

In summary, the results of this study show the anticancer, apoptotic, and autophagy activity of HMBA in SK-MEL-28 human malignant melanoma cells. ERK phosphorylation and autophagy activation were initiated by HMBA which leads to apoptosis-mediated cell death. Furthermore, as evidenced by annexin V-cy3 and 7-AAD staining, HMBA did not cause any necrosis-mediated cell death. Thus, HMBA might be recognized as a significant escort molecule.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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