

Hispolon induces apoptosis against prostate DU145 cancer cells via modulation of mitochondrial and STAT3 pathways

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Abstract: Hispolon, a bioactive polyphenolic entity extracted from *Phellinus linteus*, possesses anticancer, anti-inflammatory and anti-oxidant properties. Despite the reported therapeutic effects of this natural chemical entity, inhibitory potential of hispolon towards prostate carcinoma DU145 cells and mechanism of its action are yet to be explicated. Deregulated STAT3 pathway performs multifaceted functions in facilitating the development of cancer. Here, we have investigated the mechanism of hispolon by which it exerts its anticancer effects in DU145 cells and whether its anticancer activity is mediated by modulation of STAT3. Our outcomes show that hispolon significantly halted the multiplication of DU145 cells as well as arrested cell cycle at S phase. S phase arrest induced by hispolon was associated with downregulation of cyclin B1, cyclin D1 and CDK4 while up-regulation of p21. Moreover, hispolon treatment leads towards induction of apoptosis in a dose-dependent mode in DU145 cells. Hispolon induced modulation of Bcl-2 family proteins lead towards loss of MMP allowing the discharge of cytochrome c from mitochondrial porin channels which triggered the cascade of caspases ultimately causing cellular death. We further investigated the role of hispolon in mediating deregulated STAT3 pathways in DU145 cells. Hispolon has potential to downregulate the p-STAT3 expression with no effect on total STAT3. Contemporaneously, these results represent that hispolon's anticancer mechanism of action proceeds via downregulating the phosphorylation of STAT3 and induction of apoptosis via mitochondrial pathway.

Keywords: Anti-cancer, natural products, polyphenols, hispolon

INTRODUCTION

Prostate cancer has been reported as most frequently diagnosed malignancy of urogenital system in men globally and is turning up as major health issue in developed countries. Prostate cancer has been reported as a principal cause of deaths in USA (Bashir, 2015). People from third world countries with poor standards of living and malfunctioning are also at high risk of this cancer (Hebert *et al.*, 1998; Giovannucci *et al.*, 2007). Occurrence and progression of prostate cancer involves genetic and metabolic alterations, unusual expression of certain oncogenes, inhibition of the tumor suppresser genes, and malfunctioning of signal transduction molecules (Benedettini *et al.*, 2008).

STAT is a family of transcriptional factors which have been known as major players of cellular differentiation, proliferation, angiogenesis, invasion, survival, metastasis, and immune responses (Carpenter and Lo, 2014). Several investigations have illustrated that STAT-3 is constitutively active in various cancers such as solid tumors (breast, prostate, gastric, lung, neck, head, colon,

and hepatocellular) as well as hematological cancers (lymphomas and leukemia) (Kim *et al.*, 2017).

A variety of growth factors and cytokines such as EGF and IL-6 arbitrate the activation of STAT-3 by phosphorylating Tyr705 residue of STAT-3. Induction of STAT3 activation via ligand-receptor association leads to the dimerization of phosphorylated monomers of STAT3 protein to form dimers which are translocated towards the nucleus (Grivennikov and Karin, 2010). Once in the nucleus, STAT3 gets bind to DNA and further controls the pro-proliferative, immune, anti-apoptotic and cell cycle regulatory genes expression. Aberrant as well as persistent expression of STAT3 with modulated levels of various regulatory proteins as well as apoptotic proteins has been implicated in disease progression and malignant transformation of prostate cancer. Moreover, in prostate cancer, STAT signaling is linked with cancer stem cell like-phenotype (Don-Doncow *et al.*, 2014). Therefore, inhibiting the activation of STAT3 could serve as a novel approach for the development of therapeutics to win the battle against prostate cancer.

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Phellinus linteus is a renowned therapeutic fungus from the *Phellinus* genus. *Phellinus linteus* has been utilized since centuries as a conventional medicinal mushroom (Zhu *et al.*, 2008). It is well renowned in countries of Asian region such as Korea, China and Japan where it has been used since many years for the cure of several pathological conditions i.e. gastrointestinal disorders, inflammation, lymphatic diseases, and tumors (Zhu *et al.*, 2008). A range of bioactive constituents such as proteoglycans, polysaccharides, hispolon, and hesperidin have been isolated from *Phellinus linteus* (Zhu *et al.*, 2008). Previous investigations have documented that *Phellinus linteus* represses growth of lung cancer cells by triggering apoptosis (Guo *et al.*, 2007). Hispolon is a yellow coloured polyphenolic entity extracted from *Phellinus linteus* which has been documented as antimicrobial (Chen *et al.*, 2008), anticancer, and anti-metastatic agent (Ho *et al.*, 2017).

Hispolon has ability to induce perceptible cytotoxicity in a range of cancer cells (Arcella *et al.*, 2017; Hsin *et al.*, 2017). Hispolon exerts its anticancer activity via induction of cell cycle arrest and apoptosis by caspase family activation, modulation of p53 and p27 expression, recovering the Bax/Bcl-2 levels, discharge of cytochrome c, and PARP cleavage (Chen *et al.*, 2013; Hsiao *et al.*, 2013; Wu *et al.*, 2014). However, to date, no one has attempted to explore the anti-cancer capability of hispolon against human prostate DU145 cancer cells. Hence, the objective of this investigation was to figure out the anticancerous efficacy of hispolon against DU145 cells and its underlying mechanisms of action *in vitro*.

MATERIALS AND METHODS

Cell culture

DU145 cells were cultured in DMEM medium supplied with 0.5% antibiotics, and 10% FBS (Gibco) in 10 cm² cell culture plates. Cells were placed in a CO₂ incubator (37°C, 5% CO₂). Before further experimentation, cells were allowed to attain 70% confluency. Hispolon was purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Inc., Dallas, TX, USA)

CCK-8 assay

The effect of Hispolon on DU145 cells was examined by CCK-8 assay (Zhang *et al.*, 2018). Seeding of cells was done in wells of 96-well plate with growth medium followed by incubation at a temperature of 37°C and final concentration of cells was 5x10³. After 24 h, treatment of hispolon was given to cells for 24 h and again incubated. Then, each well of 96-well plate was filled with CCK-8 (10µl) solution. After that, incubation was further done for 4 hours. Finally absorbance was checked at 570 nm by utilizing Varioskan Flash Multimode Reader. Results were represented as percentages and IC₅₀ was evaluated by non-linear regression analysis.

Cell cycle analysis

For the determination of hispolon-induced effects on different phases of cell cycle in DU145 cells, FACs was performed (Rasul *et al.*, 2012b). Treatment of hispolon (0, 20, 40, and 80 µM) was given to DU145 cells for 24 h followed by harvesting and then washing of cells with PBS. Then, fixation of cells was done with 70% ethanol for 2 hours and then, staining with the solution of PI containing PI (1mg/ml) and RNase A was done. The fluorescence-activated cancer cells were analyzed by the flow cytometric (FACs) analysis and for the analysis of data, Cell Quest software was used.

Measurement of cell apoptosis

Rate of hispolon-induced apoptosis was assessed by Annexin V/PI assays (Rasul *et al.*, 2013). Staining of DU145 cells were done with Annexin PI and V-FITC and, then, FACs was performed according to the protocol provided by manufacturer (Beyotime, China). Hispolon treated cells (0, 20, 40 and 60µM) were subjected to incubation for 24 h. DU145 cells were collected and PBS was used for washing the cells, then, staining was done twice with 10µl and 5µl of PI and Annexin V-FITC respectively in binding buffer (500µl) for 15mins. Percentage of apoptotic cells was done by FACs and for the analysis of data, Cell Quest software was used.

Mitochondrial aym assay

Seeding of DU145 cells were done in a 6-well plate (8 × 10⁴ cells/ml) and placed in an incubator at 37°C with 0, 20, 40 and 60µM concentrations of hispolon for 24 h. JC-1 staining solution (10µL) was mixed into all wells and again incubation was done for 30mins. After centrifugation at 400×g for about 5 minutes, the supernatant was discarded and cells were subjected to washing for two times with assay buffer (200µl/well). After addition of 200µL assay buffer in all wells, fluorescence was determined at 590 nm with a micro plate reader (Rasul *et al.*, 2012a).

Western blot analysis

Furthermore, to uncover the mode by which hispolon has stimulated apoptosis in DU145 cells, western blot analysis of apoptosis-associated proteins was performed (Rasul *et al.*, 2012a). DU145 cells grown in DMEM containing FBS (10 %) were treated with hispolon (0, 20, 40 and 60 µM) and then placed in incubator for 24 h. PBS was utilized for washing, and cells were collected in a centrifuge tube. The resulting pellet of cells was resuspended and lysed on ice using RIPA buffer with freshly added protease inhibitor for half an hour. SDS-PAGE (10-12% gel) was run for the separation of protein lysate and separated protein lysate on gel was then transferred to PVDF membrane. The membranes were dipped in blocking buffer and then washed with TBST, followed by incubation at 4°C with primary antibodies overnight and then washed thrice using TBST. The resulting blots were again incubated with anti-rabbit

conjugated secondary antibodies at room temperature for 1 h. Finally ECL plus chemiluminescence kit on X-ray film was used for signal detection.

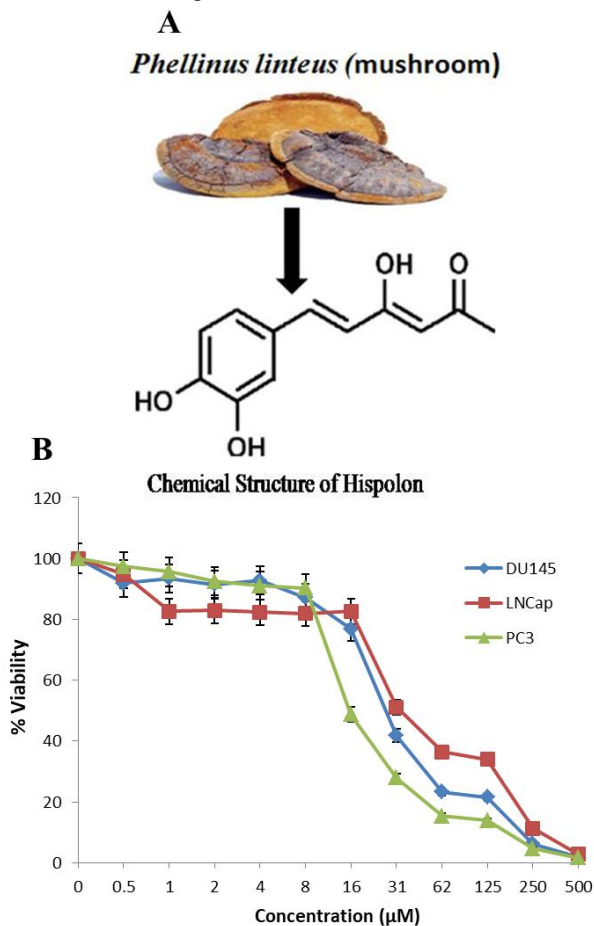


Fig. 1: Structural representation of hispolon and impacts of hispolon on growth of cancer cells: (A) Representation of chemical structure, (B) Hispolon caused inhibition of growth and induced apoptosis in PC3, LNCaP and DU145 cancer cells. Cancer cells were given treatment of 0, 0.5, 1, 2, 4, 8, 16, 31, 62, 125, 250 and 500 μM of hispolon for 24 h. The % viability of cells was measured by CCK-8 assay.

STATISTICAL ANALYSIS

Origin lab 8 was used to perform statistical analysis. Every experiment was performed for at least thrice. Data is represented as the mean ± standard deviation. Paired *t*-test was utilized for the evaluation of statistical significance. Differences were contemplated to be statistically significant at $P < 0.05$.

RESULTS

Hispolon-induced cytotoxic effects on prostate cancer cells

In this investigation, we have explored the role played by hispolon in inducing apoptosis in DU145 cells. Hispolon

is a yellow coloured naturally occurring compound belonging to the family of polyphenols. Chemical structure of hispolon is illustrated in fig. 1 (A). CCK-8 assay was done to assess the effects of hispolon on growth rate of prostate cancer cell lines. Cells were given treatment of different concentrations (0, 0.5, 1, 2, 4, 8, 16, 31, 62, 125, 250 and 500 μM) of hispolon for the duration of 24 h. The growth inhibiting efficacy of hispolon enhanced with increasing concentration of hispolon. Our data confirmed that the IC_{50} values were 31 μM, 32 μM and 28 μM against DU145, LNCap and PC3 respectively (fig. 1B). As shown in fig. 1B, it has been found that hispolon caused growth inhibition in a dose dependent mode in PC-3, LNCap and DU145 cells.

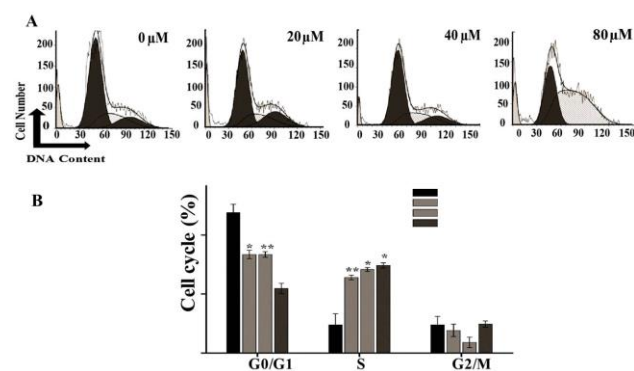


Fig. 2: Cell cycle profiles of DU145 cells after treatment with hispolon assessed by FACS analysis, (A) Results showing S phase arrest in DU145 cells induced by treatment of hispolon (20 μM, 40 μM, and 80 μM) for 24 h (B) Graph showing the percentage of hispolon treated cells at different phases of cell cycle (G0/G1, S, G2/M).

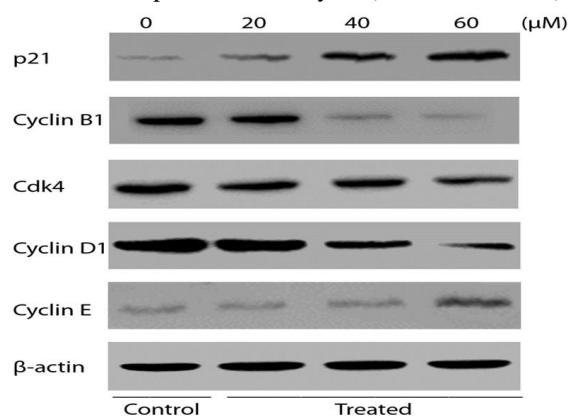


Fig. 3: WB analysis of p21, cyclin B1, CDK4, cyclin D1 and cyclin E. DU145 cells treated with hispolon for 24 h were harvested for WB analysis. β-actin was utilized as loading control.

Hispolon induced cell cycle arrest at S phase in DU145 cells

Next, we have evaluated the impacts of hispolon on cell cycle of DU145 cells. It was examined that hispolon treatment increased the cell percentage in the S phase from 14.34% in the control group to 38.45%, 42.49% and

44.53% (fig. 2) in the cells incubated with 0, 20, 40 and 80 μM of hispolon for 24 h correspondingly. This enhanced percentage was accompanied with reduced numbers of cells in G2/M and G0/G1 phases. The outcomes obviously revealed that hispolon induced arrest at S phase; consequently, our investigations demonstrated that the hispolon treatment leads to cell cycle arrest in DU145 cells which is one of the likely means of hispolon induced anti-cancer activities.

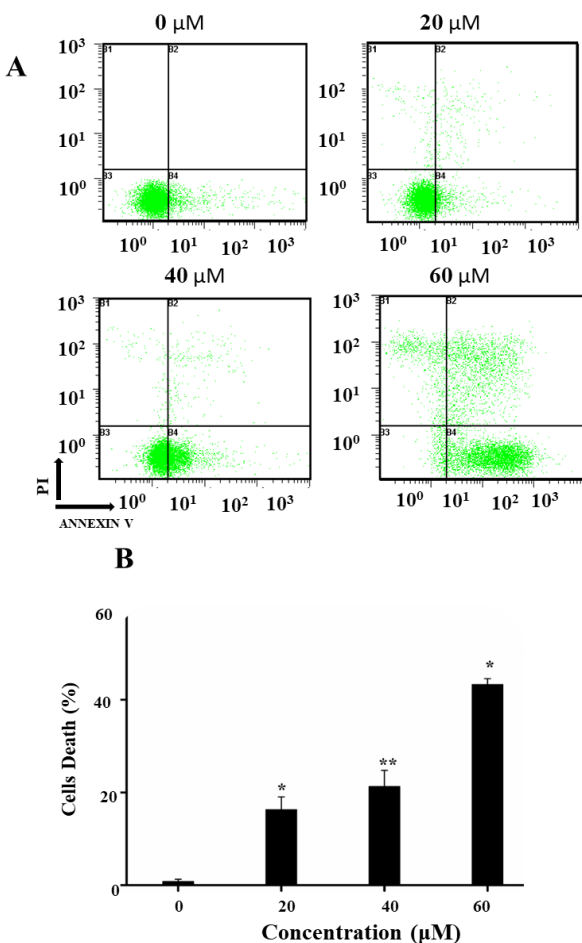


Fig. 4: Analysis of apoptosis in DU145 cells with treatment of various concentrations of hispolon by flow cytometry (A) FACs analysis of DU145 cells with treatment of 0, 20, 40 and 60 μM of hispolon respectively showing percentage of cancer cells in various phases of apoptosis (B) Graph displaying the percentage of cell death in a dose-dependent mode.

Molecular mechanism of hispolon-induced S phase arrest in DU145 cells

In order to interpret the mode by which hispolon induced S phase arrest, we assessed the effects of hispolon on proteins reported to be engaged in cell cycle regulation such as CDK4, p21, Cyclin B1, Cyclin D1, and Cyclin E. WB (Western blot) analysis shows that hispolon treatment leads towards the up-regulation of protein expression of

p21 in a dose-dependent mode (fig. 3). Consequently, we further examined the effects of hispolon on certain cell cycle regulatory proteins in DU145 cells. Our findings confirmed that hispolon treatment slightly up-regulated cyclin E expression. While, the expression levels of G0/G1 phase associated proteins such as cyclin D1 and CDK4 were also slightly down-regulated after 24 h of hispolon treatment, while expression of Cyclin B1 which is G2/M phase-regulating protein was drastically decreased in DU145 cells in a dose-mediated manner (fig. 3).

Determination of hispolon-induced apoptosis in prostate cancer DU145 cells by flow cytometric analysis

Besides arresting of cell cycle, natural compounds also have capability to exert anti-cancer effects via inducing apoptosis. To explore whether hispolon has potential to inhibit cellular proliferation via induction of apoptosis, DU145 cells were given the treatment of 0, 20, 40 and 60 μM hispolon for 24 h and flow cytometric analysis was done utilizing annexin V-FITC/PI staining. Our findings propose that hispolon presents efficacy towards apoptosis induction in DU145 cells. Data obtained from flow cytometric analysis displays the rates of apoptosis as 3.6%, 13.9%, 15.8% and 22.4% in cells with treatment of 0, 20, 40, and 60 μM hispolon, respectively after the treatment of 24 hours (fig. 4A). Treated cells showed an increased trend towards apoptosis. At the dose concentration of 40 and 60 μM , DU145 cells displayed enhanced early and late apoptosis in comparison to 20 μM concentration. Our findings recommend that hispolon inhibited cellular growth in DU145 cells by induction of apoptosis in a dose dependent mode.

Hispolon induced modulation of Bcl-2 family proteins in DU145 Cells

Furthermore, to delineate the mechanism underlying the hispolon-induced apoptosis, we explored the impacts of hispolon on proteins such as Bax and Bcl-2. Western blot analysis has revealed that Hispolon treatment enhanced the Bax level and reduced Bcl-2 level (fig. 5C). So, further we have performed western blot analysis to determine whether hispolon have potency to lower the MMP to trigger the discharge of cytochrome c or not. Our results clarified that the loss of MMP leads towards the discharge of cytochrome c in hispolon treated DU145 cells (figs. 5A-C).

Hispolon induced apoptosis in DU145 cells via mitochondrial apoptotic pathway

As previous studies have reported that hispolon has capability to induce caspase-mediated intrinsic apoptosis (Hsiao *et al.*, 2013), so, further we have examined hispolon effects on death-driving cysteine proteases generally familiar as caspases. As displayed in fig. 5C, hispolon markedly up-regulated the expression levels of cleaved caspase-9 and -3 in DU145 cells. These results demonstrate that the mitochondrial-initiated intrinsic

apoptosis pathway can be stimulated by hispolon treatment in DU145 cells.

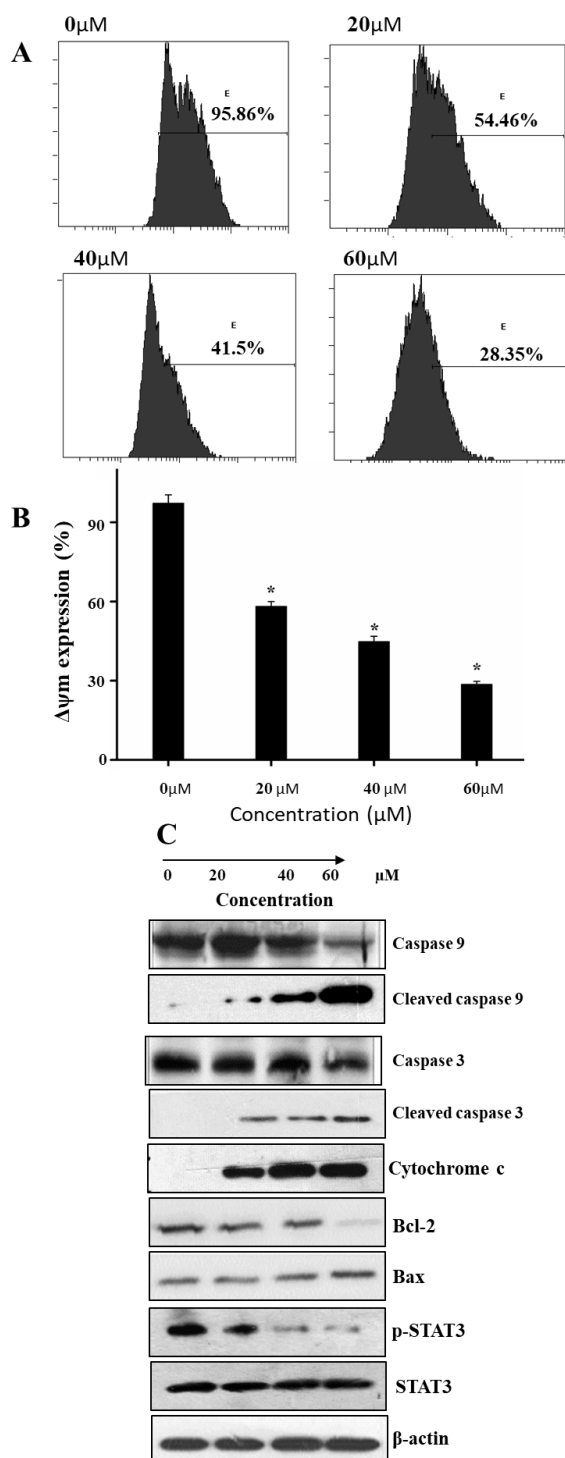


Fig. 5: Results of flow cytometric and western blot analysis of cells with treatment of 0, 20, 40 and 60 μM of hispolon respectively (A) Representation of FACs analysis of mitochondrial membrane potential of DU145 cells treated with 0, 20, 40 and 60 μM of hispolon

respectively (B) Graph displaying the percentage of reduction in MMP in a dose-dependent mode (C) WB analysis representing the levels of cytochrome c, caspase 9, cleaved caspase 9, caspase 3, cleaved caspase 3, cytochrome c, Bcl-2, Bax, p-STAT3 and STAT3 in hispolon treated (0, 20, 40 and 60 μM) cells. β-actin was utilized as loading control

Hispolon down-regulated the phosphorylation of STAT3 in DU145 cells

To investigate the impact of hispolon on STAT3, we have performed western blot analysis. Western blot analysis shows that treatment of hispolon down regulated the expression of p-STAT3 in a dose-dependent way whereas total STAT3 found to be unaffected (fig. 5C). Thus, it can be stated that hispolon triggered apoptosis is linked with the down-regulation of p-STAT3 in DU145 cells.

DISCUSSION

Prostate cancer is the principal cause of mortality in men globally. Treatment of prostate cancer mainly relies on chemotherapeutic drugs such as docetaxel. Side effects associated with the utilization of chemotherapeutic drugs arouse the dire need to discover more selective, safer and economical drugs (Puente *et al.*, 2017). Natural products as novel and rich reservoir of anti-cancer, anti-inflammatory and anti-oxidant compounds have provided the foundation of drug discovery since times. Thus, the search for safer, selective and effective therapeutic drug candidates from natural products is a spotlight issue nowadays (Nageen *et al.*, 2018). Hispolon is a bioactive polyphenolic entity extracted from *Phellinus linteus*. Polyphenols have gained attention of researchers recently as novel and potent anti-cancer agents (Ho *et al.*, 2017). But the molecular mechanisms by which polyphenols exert anti-cancer activities still need to be investigated. Thus, in this study we have explored the anti-cancer mechanisms of hispolon against DU145 cells. It has been found during this study that hispolon caused inhibition of growth of DU145 cancer cells. Similar results of hispolon have also been reported by other researchers in other cancer cell lines (Huang *et al.*, 2010; Huang *et al.*, 2011). Natural compounds exert their anti-cancer effects via arresting cell cycle at different phases. Thus, we have explored whether anti-cancer mechanism of hispolon is linked with arresting of cell cycle or not. Regulation of cell cycle occurs by number of checkpoints in healthy cells. Modification, alteration or deregulation in these adaptable checkpoints is acquired by cancerous cells, thus, leading towards unrestrained cellular proliferation. (Visconti *et al.*, 2016). Hispolon presents its chemotherapeutic potential by induction of cell cycle arrest in various phases of cell cycle in cell line-dependent mode such as in leukemia HL-60 cells at sub G1 phase (Hsiao *et al.*, 2013), in Hep3B cells at S phase (Huang *et al.*, 2011), in nasopharyngeal NPC-039 cells

(Hsieh *et al.*, 2014), and in NB4 human leukemia cells, hispolon induces G0/G1 phase arrest (Chen *et al.*, 2013). Our results are in line with the previous study which reports the induction of S phase arrest in hispolon treated Hep3B cells via up-regulating the expression of p21 while down-regulating cyclin A and CDK2 (Huang *et al.*, 2011). Moreover, hispolon treatment to NB4 cells induced G0/G1 phase arrest via reducing cyclin D1 and CDK4 expression which further support our results (Chen *et al.*, 2013).

Bcl-2 family is categorized into pro-apoptotic (Bax, Bid and Bad) members and anti-apoptotic (Bcl-2 and Bcl-xl) members. Activation of pro-apoptotic members provokes the release of death-driving cysteine proteases known as caspases via decreasing potential across mitochondrial membrane and releasing cytochrome c, thus, ultimately leading towards apoptosis (Rasul *et al.*, 2012a). Modulation of Bcl-2 family proteins via naturally occurring cytotoxic agents lead towards apoptosis and ultimately reduced proliferation in cancer cells (Pellecchia and Reed, 2004). Hispolon has also been reported previously to induce apoptosis in SGC-7901 through intrinsic pathway via enhancing the expression of caspase-9, decreasing MMP and releasing cytochrome c in a dose-dependent mode (Chen *et al.*, 2008).

More than 70% of human cancers overexpress p-STAT3. Phosphorylation of STAT3 triggers transcriptional activation of various cancer-linked genes such as MMP-9, IL-6, Bcl-xl, and cyclin D1, thus, STAT3 acts as molecular hub for multiple anti-apoptotic signaling pathways (He *et al.*, 2015). Hispolon has been known to reduce the phosphorylation of NF- κ B at p65 subunit, thus, blocking its translocation to nucleus (Sun *et al.*, 2015). NF- κ B is a down-stream target of STAT3, however, no one has still reported the role of hispolon in modulating the expression of STAT3. This is the first study reporting that hispolon treatment down-regulated p-STAT3 in a dose-dependent way which ultimately inhibited growth of DU145 cells.

CONCLUSION

Our findings highlights that hispolon has capability to induce cytotoxicity in DU145 cancer cells. To the best of author's knowledge, this is the first reported data that anti-cancer activity of hispolon in DU145 cells is mediated by inhibiting p-STAT3. Thus, hispolon might turn up as a novel and safe anti-cancer natural entity and lead structure for a number of cancers where constitutive expression of STAT3 is favored. Moreover, development and evaluation of synthetic analogues of hispolon will hopefully uncover more potent compounds for practical applications in the cure of cancer. Previous investigations and our results collectively demonstrate that hispolon has capability to modulate multiple deregulated signaling

pathways in cancer. As hispolon inhibited the invasive capabilities of breast cancer via down-regulation of NF- κ B pathway (Sun *et al.*, 2015). Previous data also proclaims that hispolon exerts its anti-proliferative effects via inhibiting the expression of PI3K/Akt pathway (Huang *et al.*, 2010) and through enhancing p53 expression (Arcella *et al.*, 2017). Thus, hispolon might serve as modulator of multiple signaling pathways for cancer treatment. Furthermore, preclinical studies and *in vivo* investigations are clearly needed to decipher its anti-cancer potential in adjuvant and combinatorial therapies for the cure of cancer.

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