Differential response of antioxidant defense in HepG2 cells on exposure of Livotrit®, in a concentration dependent manner

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ABSTRACT

Livotrit®, a polyherbal formulation (Zandu, India) is commonly prescribed for liver health. The present study was undertaken to elucidate possible mechanism of antioxidant potential of Livotrit®. Livotrit® exhibited concentration dependent radical scavenging activity, inhibition of lipid peroxidation as well as activation and gene expression of antioxidant enzymes. Interestingly, lower concentration of Livotrit® (0.05%) significantly increased activities and gene expression of catalase, Glutathione reductase (GR) and Gluthathione peroxidase (GPx), while higher concentration of Livotrit® (0.5%) significantly increased antioxidant enzyme Heme-oxygenase 1(HO-1) and not catalase (CAT), GR and GPx. Transcription factor, Nuclear factor erythroid 2-related factor 2 (Nrf2) required for expression of catalase, GR, GPx and HO-1 was efficiently translocated into the nucleus at both concentrations. Inspite of this, concentration dependent activation of these enzymes was found to be mediated through miRNAs involved in regulation of their gene expression.

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1. Introduction

Free radicals are continuously produced as natural by-products of normal metabolism and play an important role in redox signaling.[1] To counteract an overproduction of reactive oxygen species (ROS), cells have evolved a highly regulated endogenous antioxidant defense system including antioxidant enzymes and molecules. The first line of defence is executed by antioxidant enzymes namely Superoxide dismutase (SOD), CAT and GPx. In addition, inducible HO-1 is also considered as a major contributor of antioxidant defense during stress conditions.[2] The by-products of heme metabolism, catabolised by HO-1, act as potent antioxidants, thus making HO-1 a key player of antioxidant defense. Expression of HO-1 is regulated by Nrf2 which on activation translocates in the nucleus and binds to antioxidant response element (ARE) in the *ho-1* gene promoter and upregulates its expression. Since HO-1 is induced in response to various stimuli, targeted induction of this stress response enzyme is considered as an important therapeutic strategy for protection against various inflammatory and oxidative damage conditions.

A number of natural antioxidant compounds in food and plants have been demonstrated to be effective inducers of HO-1.[3] Due to their lesser side-effects, herbal formulations containing natural antioxidant compounds are becoming increasingly important in modern medicine and lifestyle disorders and thus are used as therapeutic agents in various diseases.[4] Livotrit®, a poly-herbal formulation is recommended as a daily health supplement for protection against hepatic damage. It is manufactured by Emami Limited, Kolkata and marketed by Zandu (India). It consists of the extracts of (i) *Boerhavia diffusa* (ii) *Tinospora cordifolia* (iii) *Eclipta alba* (iv) *Andropraphis paniculata* (v) *Picrorrhiza kurrao* (vi) *Embelia ribes* (vii) *Cichorium intybus* and (viii) *Amoora rohitaka*. Though, it is in use for a long period of time data about its biological activities is not available. Present study was undertaken to check the antioxidant activity of Livotrit® and elucidate its possible mechanism. The antioxidant activity of aqueous extract of Livotrit® was carried out using standard *in vitro* antioxidant assays and ability of livotrit® to modulate intracellular antioxidant defense and HO-1 was checked using HepG2 cells as a model system. We indeed observed strong

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in vitro antioxidant potential to livotrit®. In addition, it also activated antioxidant enzymes and HO-1 in a concentration dependent manner. This differential modulation was mediated through miRNA regulation.

2. Material and methods

2.1. Chemicals

2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tripyridyl-s-triazine (TPTZ), Phenazine methosulphate (PMS), L-ascorbic acid, 2,2-azinobis (3-ethylbenzothiazoline-6- sulphonate) ABTS, 2-thiobarbituric acid (TBA), 1,1,3,3-tetramethoxypropane (TMP) were purchased from Sigma Chemicals Co. St. Louis, MO, USA. Other chemicals were procured from one of the following companies: SRL (New Delhi, India), BDH (Mumbai, India), Hi-media (Mumbai, sIndia) or Merck (Darmstadt, Germany).

2.2. Biologicals

Wistar rats of either sex weighing about 250 ± 20 g were used for isolation of rat liver mitochondria. These were purchased from Institute of Biological Products (IVBP), Pune. These animals were housed in polypropylene cages maintained at 25 ± 2 °C with a 12:12 h light and dark cycle at Department of Zoology, Savitribai Phule Pune University. They were given feed and water *ad libitum*. Prior approval was obtained from the institutional Animal Ethical Committee for protocols involving animals (Registration No. 538/02/c/CPCSEA).

2.3. Preparation of livotrit® extract

Livotrit® tablets were purchased from the market in one batch and were crushed to form powder. 10 g of powder was dissolved in 100 ml of distilled water and stirred on a magnetic stirrer over night. Extraction was carried out three times. The extract was lyophilized and the powder so obtained was stored at -80 °C till use.

2.4. In vitro antioxidant assays

For all radical scavenging assays, Livotrit® at a concentration of 0.05, 0.5, 2.5 and 5% was used. Radical scavenging activity of aqueous extract of Livotrit® against DPPH• (Sigma, USA) was determined spectrophotometrically at 515 nm.[5] The ability of aqueous extract of Livotrit® to scavenge superoxide radicals generated by PMS/NADH system was observed spectrophotometrically using a previously known method.[6] Hydroxyl radical scavenging activity of aqueous extract of Livotrit® was determined by deoxyribose degradation assay.[7] The ability of Livotrit® to reduce the Fe^{3+}- TPTZ complex was assayed using the FRAP assay.[8] Inhibition of ABTS•+ radical formation by aqueous extract of Livotrit® was determined by Ferrylmyoglobin/ABTS·+ assay using previously described method.[9]

2.5. Effect of Livotrit® on lipid peroxidation in rat liver mitochondria

Rat liver mitochondria were isolated following the protocol of Devasagyam et al., 1993.[10] Three months old female wistar rats were sacrificed by cervical dislocation and liver was minced, homogenized in 50 mM sucrose-EDTA buffer on ice and mitochondria were isolated using differential centrifugation. Mitochondria at a final concentration of 0.2 mg/ml were damaged using hydroxyl radical generated by ascorbate-Fe^{2+} for 30 min at 37 °C. The amount of lipid peroxidation end products (mlonialdehyde (MDA)

and other aldehydes) were quantitated by their reaction with TBA and measured at 532 nm. The concentration of TBARs (Thiobarbituric acid reactive substances) was calculated using TMP as a standard.

2.6. Culturing of HepG2 and cell viability

HepG2 cells were purchased from National Centre for Cell Sciences (NCCS), Pune. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were grown in a humidified atmosphere containing 5% CO_2 at 37 °C. 1×10^4 HepG2 cells were treated for 16 h with different concentrations (0.05, 0.5, 2.5, 5%) of aqueous extract of livotrit® and cell viability was checked using MTT assay.[11]

2.7. Measurement of antioxidant enzymes

HepG2 cells were pre-treated with aqueous extract of Livotrit® for 16 h, harvested and pellet was resuspended in thrice the volume of protein extraction buffer (PEB). Cells were then vortexed vigorously for 2 min, repeating the cycle three times. Homogenate was then centrifuged at 10,000 rpm for 10 min. The supernatant was collected and was used to quantitate antioxidant enzymes namely CAT,[12] GR,[13] and GPx[14] using standard protocols. All assays were done atleast in five replicates and values given are average of these replicates.

2.8. Glutathione estimation

Glutathione was estimated using glutathione assay kit (Sigma chemicals, USA). GSH causes a continuous reduction of 5,5'-dithiobis - 2-nitrobenzoic acid (DTNB) to (5- thio-2-nitrobenzoic acid) TNB and the GSSG (oxidized glutathione) formed is recycled by glutathione reductase and NADPH. The reaction rate is proportional to the concentration of glutathione. The yellow product (TNB) was measured spectrophotometrically at 412 nm.[15] The standard curve of reduced glutathione was used to determine the amount of glutathione in cell homogenate.

2.9. Detection of Nrf2 translocation

1×10^4 cells were seeded on glass coverslips and were treated with different concentrations of aqueous extract of Livotrit®. Cells were fixed using methanol-acetone (3:1), blocked in blocking buffer (3%BSA, 0.3% triton x- 100 in 1X PBS) and incubated with anti-Nrf2 primary antibody (Cell Signaling Technologies). After three washes with 1X PBS, cells were incubated with goat anti-rabbit AlexaFlour- 488 (ThermoFisher Technologies, USA), followed by staining with DAPI for 3 min. Coverslips were mounted in anti-fade medium (Vectashield) and examined using confocal microscope (Nikon, Japan).

2.10. RNA isolation and quantitative real- time PCR amplification (qRT-PCR)

1×10^6 cells were treated with different concentrations of aqueous extract of Livotrit® for 16 h. RNA was isolated using Trizol reagent and cDNA was prepared using verso cDNA synthesis Kit Thermo scientific (USA). qRT- PCR was performed using specific primers as follows:

Catalase_ F: 5'-TGCTGAATGAGGAACAGAGG-3'
Catalase_ R: 5'-GTGTGAATCGCATTCTTAGG-3'
GR_ F:5'-CAGTGGGACTCACGGAAGAT-3'
GR_ R: 5'-TTCACTGCAACAGCAAAACC-3'
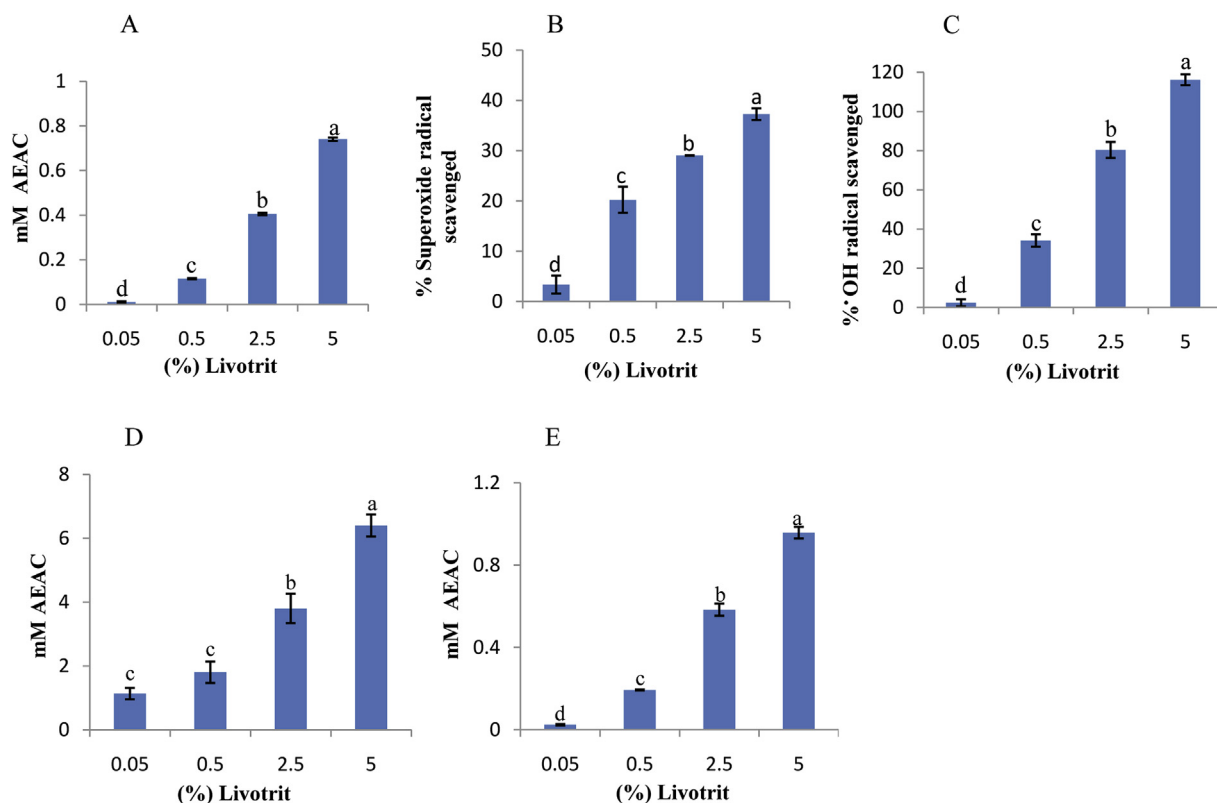
Fig. 1. *In vitro* antioxidant activity of aqueous extract of Livotrit® as assessed by (A) DPPH radical scavenging assay, (B) Superoxide radical scavenging assay, (C) Deoxyribose degradation assay, (D) Inhibition of ABTS⁺ formation, (E) Ferric reducing ability. Values are expressed as mean ± SE of the three independent experiments. Dissimilar alphabets in superscript indicate significant difference at 0.05 level.

GPx_ F: 5′-GCGGCGGCCCAGTCGGTGTA-3′
GPx_ R: 5′-GAGCTTGGGGTCGGTCAT-3′
HO-1 _ F: 5′- ACTGCGTTCCTGCTCAACAT- 3′
HO-1 _ R: 5′-GGGGCAGAATCTTGCACTTT-3′
BACH1_ F: 5′-TGCGATGTCACCATCTT-3′
BACH1_R: 5′-CCTGGCCTACGATTCTTG-3′
GAPDH_ F: 5′-TGGGGAAGGTGAAGGTCGGA-3′
GAPDH_ R: 5′-GGGATCTCGCTGCTGGAAGA-3'.

Fold change values were calculated by comparative C_t analysis after normalizing with GAPDH mRNA. miR specific cDNA was prepared using stem-loop primers and qRT- PCR was performed using miR specific primers.

2.11. Western blot analysis

Proteins extracted using protein extraction buffer and quantitated using Bradford's microestimation assay[16] were analyzed by SDS-PAGE. Proteins were electro-transferred to PVDF membrane, blocked with BSA/non-fat dry milk and reacted with different primary antibodies, namely HO-1 (Assay designs, USA), BACH-1 (Cell signaling technologies, USA) and β-actin (Sigma, USA) and appropriate secondary antibodies tagged with horseradish peroxidase. Signals were detected by chemiluminescence using luminol as a substrate and imaging was carried out using ChemiDoc Imaging System (BioRad, USA).

2.12. Statistical analysis

All experiments were repeated three to four times with each set carried out in duplicate. The data presented are an average of these replicates. One—way analysis of variance (ANOVA) test associated with the Tukey's test was used to determine the statistical significance among experimental groups. A value of $P \le 0.05$ was considered to be statistically different and is represented by different alphabets. All the statistical analyses were done using SPSS 19.0 software.

3. Results

3.1. Antioxidant activity of aqueous extract of Livotrit® and inhibition of lipid peroxidation

Aqueous extract of Livotrit® exhibited significant concentration dependent DPPH (Fig. 1A), superoxide (Fig. 1B) and hydroxyl radical scavenging (Fig. 1C) activity. The highest concentration used (5%) exhibited maximum DPPH, superoxide and hydroxyl radical scavenging activity with a value of 0.740 ± 0.007 mM ascorbic acid equivalent antioxidant capacity (AEAC), 37.29 ± 1.16 % and 116.12 ± 2.718% respectively ($P \le 0.05$). Similarly 5% aqueous extract of Livotrit® significantly ($P \le 0.05$) inhibited ABTS radical formation with a value of 6.4 ± 0.30 mM AEAC (Fig. 1D) as well as showed high ferric reducing activity with a value of 0.957 ± 0.028 mM AEAC (Fig. 1E). Exposure of rat liver mitochondria to ascorbate—Fe^{2+} induced significant lipid peroxidation with a value of 423.23 ± 37.07 nmoles TBARs/mg protein compared to control 3.0 ± 0.6 nmoles TBARs/mg protein (Table 1). This was significantly reduced to 114.74 ± 20.58 nmoles TBARs/mg protein in presence of 5%, followed by 2.5% (234.25 ± 0.09 nmoles TBARs/mg protein) and 0.5% (319.47 ± 3.53 nmoles TBARs/mg protein) of Livotrit®.

Table 1
Inhibition of lipid peroxidation in rat liver mitochondria by livotrit® measured in terms of nmoles of TBARS formed/mg protein.

|	nmoles TBARs/mg protein
Control	3.04 ± 0.68^e
Damage	423.23 ± 37.07^a
0.05% Livotrit®	406.89 ± 34.74^a
0.5% Livotrit®	319.47 ± 3.53^b
2.5% Livotrit®	234.13 ± 0.09^c
5% Livotrit®	114.74 ± 20.58^d

Dissimilar alphabets in superscript indicate significant difference at 0.05 level.

3.2. Cell viability of HepG2 cells on exposure to Livotrit®

Cells treated with aqueous extract of Livotrit® at a concentration of 0.05% and 0.5% for 16 h showed cell viability similar to untreated control cells whereas those treated with 2.5 and 5% showed significant decrease in cell viability ($P \leq 0.05$), indicating cytotoxicity of Livotrit® at higher concentrations (Fig. 2A). Therefore, for all further experiments only 0.05% and 0.5% of aqueous extract of Livotrit® was used.

3.3. Modulation of intracellular antioxidant enzymes and glutathione by Livotrit®

In cells treated with 0.05% concentration of aqueous extract of Livotrit® glutathione increased to 33393.8 ± 3344.28 nmol/mg protein compared to control (25715.56 ± 2770.75 nmol/mg protein), however significant increase was not observed in cells treated with 0.5% concentration (Fig. 2B). In control untreated cells, CAT, GR and GPx activities were 1311.27 ± 101 units/mg protein, 15781.96 ± 795 units/mg protein and 6205.51 ± 117 units/mg protein respectively. Treatment of cells with 0.05% of aqueous extract of Livotrit® significantly increased these activities to 1581.56 ± 107 units/mg protein (CAT), 20280.38 ± 1882 units/mg protein (GR) and 7241.48 ± 234 units/mg protein (GPx), which was not observed at 0.5% concentration of aqueous extract of Livotrit® (Table 2). To further assess whether the increased activities of CAT, GR and GPx is caused by altered transcription, expression of these antioxidant enzymes were quantitated by qRT-PCR. For all three enzymes namely CAT (Fig. 2C), GR (Fig. 2D) and GPx (Fig. 2E) expression increased significantly at 0.05% of aqueous extract of Livotrit® ($P \leq 0.05$) whereas at 0.5% it remained same as that of control. These data confirmed that CAT, GPx and GR levels are activated only at lower concentration of Livotrit®. miRNAs are also known to regulate gene expression. Levels of three miRNAs namely miR 30b, miR 214 and miR 181a known to regulate CAT, GR and GPx
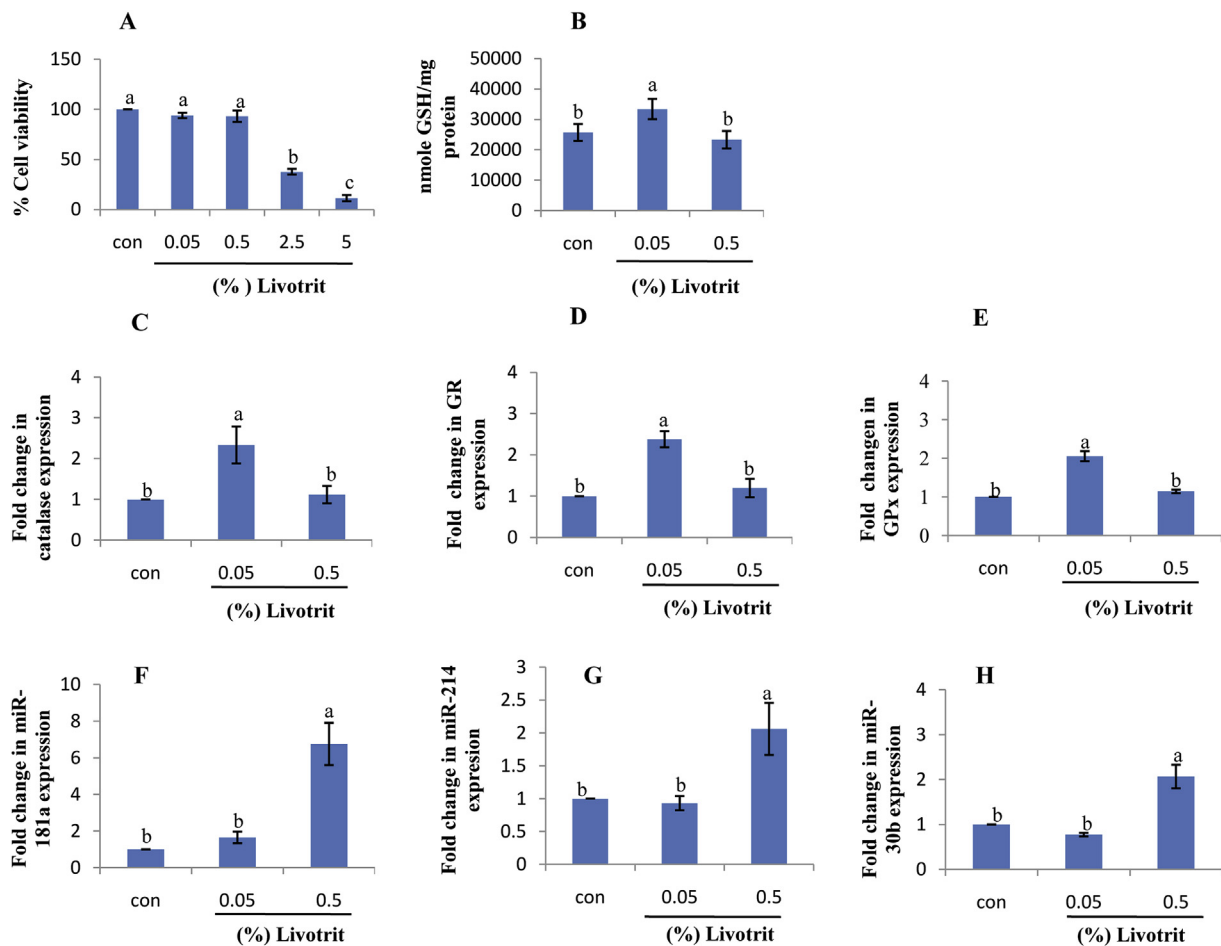
Fig. 2. Effect of aqueous extract of Livotrit® on antioxidant enzymes in HepG2 cells. (A) HepG2 cell viability was examined by MTT assay. Con = control untreated cells, (B) nmole GSH/mg protein in HepG2 cells after treatment with Livotrit®. mRNA expression levels of (C) CAT, (D) GR and (E) GPx in HepG2 cells after treatment with Livotrit® for 16 h. Expression of (F) miR 181a (G) miR 214 and (H) miR 30b in cells treated with Livotrit®. Data are presented as mean ± SE of 3 independent experiments. Dissimilar alphabets in superscript indicate significant difference at 0.05 level.

Table 2
Effect of different concentrations of aqueous extract of livotrit® on enzyme activities of CAT, GR and GPx in HepG2 cells.

	CAT (U/mg protein)	GR (U/mg protein)	GPx (U/mg protein)
Control	1311.27 ± 101^{b}	15781.96 ± 795^{b}	6205.51 ± 117^{b}
0.05% Livotrit®	1581.56 ± 107^{a}	20280.38 ± 1882^{a}	7241.48 ± 234^{a}
0.5% Livotrit®	1202.20 ± 106^{b}	16413.25 ± 281^{b}	5951.67 ± 414^{b}

Dissimilar alphabets in superscript indicate significant difference at 0.05 level.

respectively, were examined on treatment with Livotrit®. Expression of all three miRNAs (Fig. 2F, G and H), increased at 0.5% concentration of aqueous extract of Livotrit® and not at 0.05% concentration.

3.4. Nrf2 is translocated in the nucleus in presence of Livotrit®

Antioxidants enzymes such as CAT, GR and GPx contain ARE elements in their promoter region to which transcription factor Nrf2 binds and activates transcription. This requires translocation of Nrf2 from cytoplasm into nucleus. To check whether the observed increase in the antioxidant gene expression in presence of aqueous extract of Livotrit® is via Nrf2, its localization in the cytoplasm and nucleus was checked using immunostaining. As shown in Fig. 3B and C, treatment with both the concentrations of aqueous extract of Livotrit® led to increased translocation of Nrf2 into the nucleus compared to untreated control cells (Fig. 3A).

3.5. HO-1 is also induced in presence of Livotrit®

HO-1 is an important phase II enzyme, known to be activated by Nrf2. Since significant nuclear translocation of Nrf2 was observed on treatment with Livotrit®, expression of HO-1 was checked using qRT-PCR and Western blot. Interestingly significant increase in RNA expression (Fig. 4A) as well as protein (Fig. 4B and C) was observed only at 0.5% aqueous extract of Livotrit® but not at 0.05%. HO-1 is regulated positively by Nrf2 and negatively by BACH-1. Therefore expression of BACH-1 was checked and was found to be significantly low both at RNA (Fig. 4 D) and protein (Fig. 4E and F) at 0.5% aqueous extract of Livotrit®, while at 0.05% there was significant increase in RNA and protein levels of BACH-1. Thus, at lower concentration of Livotrit®, even in presence of translocated Nrf2, HO-1 is not expressed due to increased expression of its negative regulator BACH-1. This increased expression of BACH-1 was through significant decrease in expression of BACH-1 specific miR 98 (Fig. 4G).
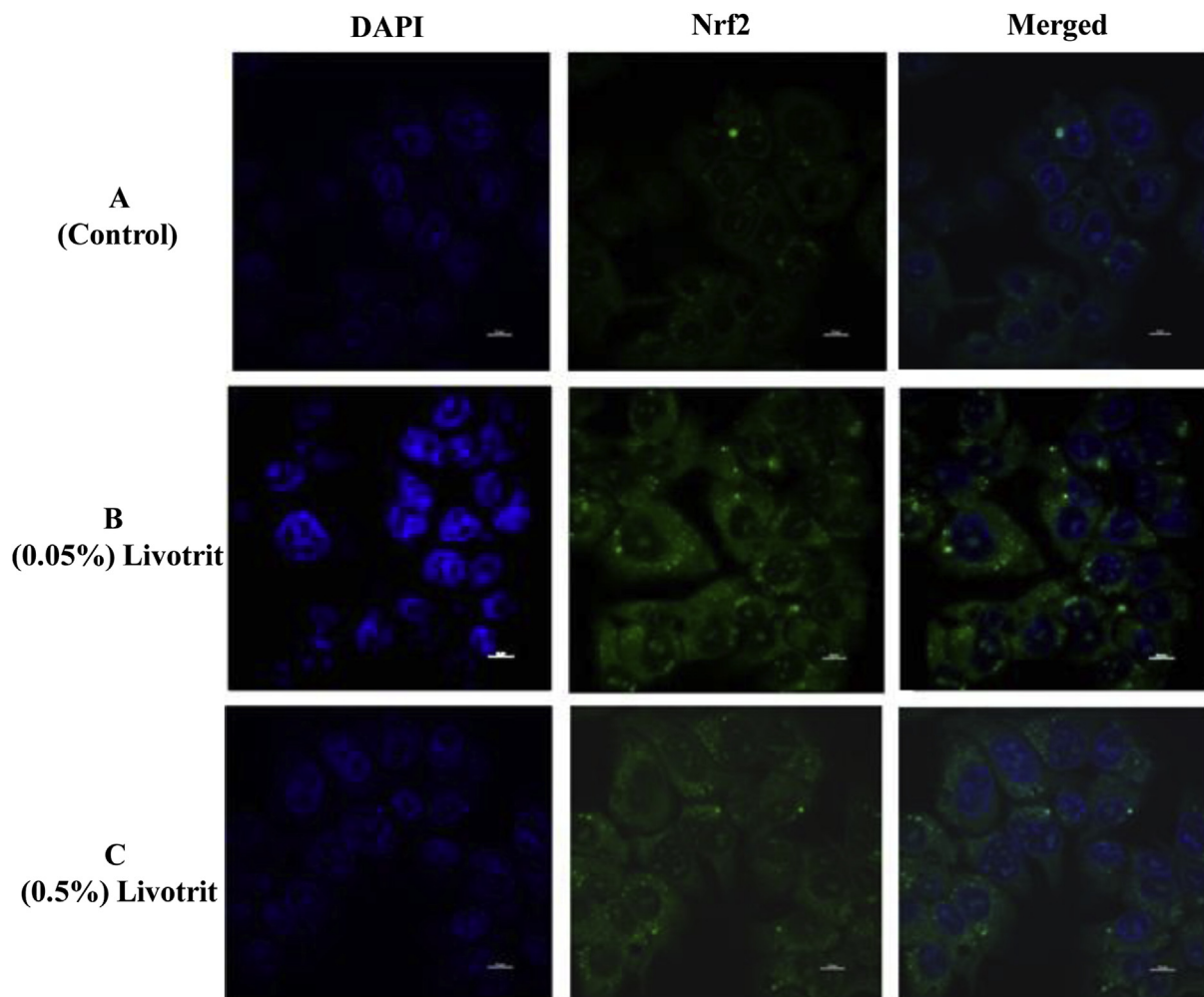
Fig. 3. Nrf2 is translocated on treatment with Livotrit®. HepG2 cells were seeded in 6 well plates on cover slips and were treated with aqueous extract of Livotrit® for 16 h. Confocal images of (A) untreated, (B) 0.05% and (C) 0.5% treated cells stained with anti-Nrf2 antibody, followed by Alexafluor 488-conjugated goat anti-rabbit secondary antibody. Nucleus was counterstained with DAPI. Left panel represents DAPI stained cell nuclei, middle panel represents Nrf2 stained cells and and right panel represents merged images of DAPI and Nrf2 stained cells.
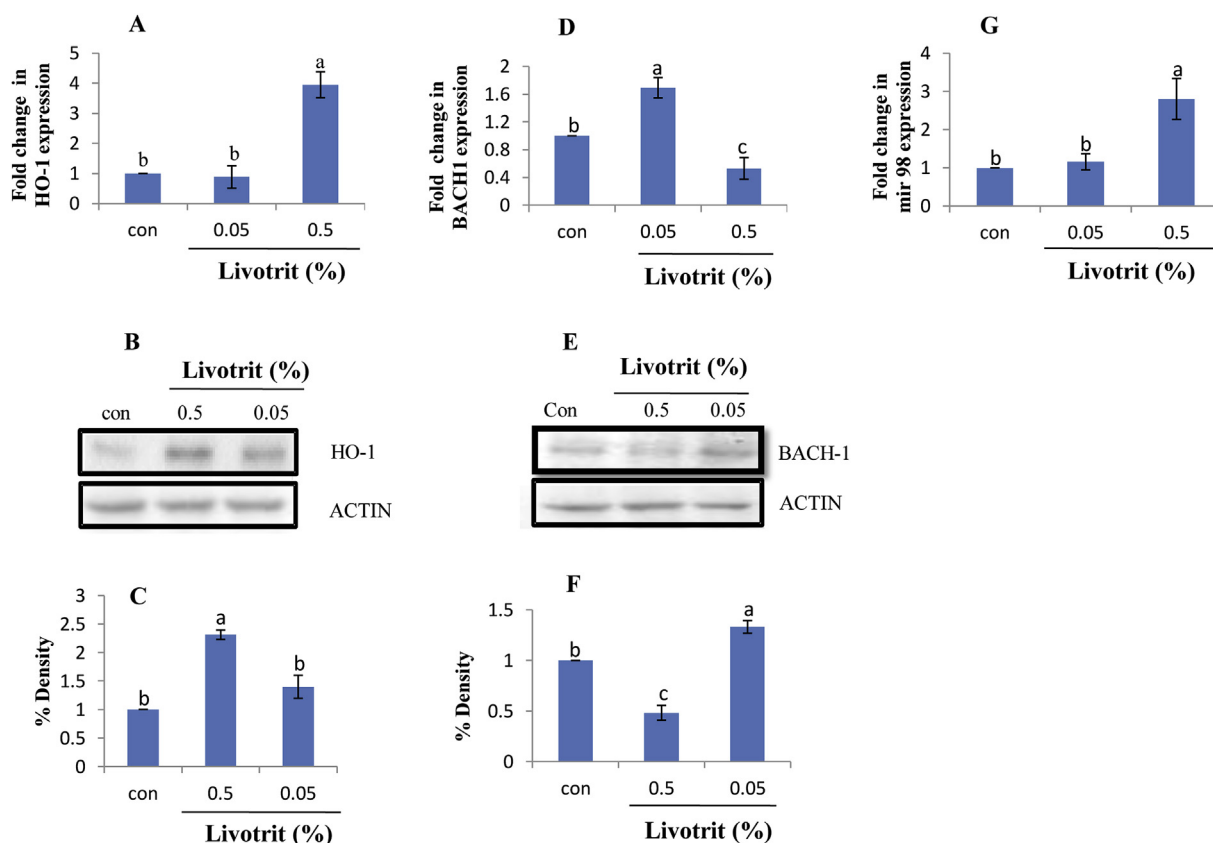
Fig. 4. mRNA expression levels of (A) HO-1, (D) BACH-1, (G) miR 98. Representative western blot of B) HO-1 and E) BACH-1 in HepG2 cells after treatment with 0.05% and 0.5% of aqueous extract of Livotrit® for 16 h. Densitometric analysis of C) HO-1 and F) BACH-1 as normalized to actin. Con = control untreated cells. Data are presented as mean ± SE of 3 independent experiments. Dissimilar alphabets in superscript indicate significant difference at 0.05 level.

4. Discussion

Polyherbal formulations are commonly used and their effects are potentiated when compatible herbs are formulated together. However, its use is often limited due to lack of chemical characterization and experimentally validated scientific data. Livotrit® is recommended as a daily health supplement for good liver health, however data regarding its antioxidant activities is not known.

Major constituents of aqueous extract of Livotrit® were checked by LC-MS/MS (Supplementary Table S1, Fig. S1). Aqueous extract of Livotrit® exhibited concentration dependent radical scavenging and inhibition activity in all the assays performed. Individual constituent herbs of Livotrit® are reported to possess antioxidant potential.[17–19] In addition, aqueous extract of Livotrit® also protected rat liver mitochondria against ascorbate-Fe^{2+} induced oxidative damage in a concentration dependant manner.

To further understand the antioxidant mechanism of livotrit®, HepG2 cells were used. Since Livotrit® at higher concentration was found to be cytotoxic all further experiments were done using lower concentration (0.05 and 0.5%) of Livotrit®. Antioxidants can act either by directly scavenging the free radicals and/or modulating levels of intracellular antioxidant enzymes such as CAT, GPX, GR and molecules such as glutathione. Aqueous extract of Livotrit® at a concentration of 0.05% significantly increased the expression as well as activities of CAT, GPx and GR compared to untreated cells. Similarly the level of glutathione was also increased. Surprisingly at higher concentration of livotrit® this increase was not observed. Instead, another important antioxidant enzyme HO-1 was significantly induced. All these antioxidant enzymes are regulated through transcription factor Nrf2 by binding to ARE element in their promoter region. Nrf2 is normally kept sequestered in cytoplasm by binding to cytoskeleton-associated protein keap1. On activation it dissociates from keap1 and translocates to the nucleus and binds to ARE element.[20] Immunolocalization of Nrf2 indeed showed its nuclear accumulation at both concentrations of livotrit® used. Inspite of having enough Nrf2 translocated in the nucleus, CAT, GR and GPx were induced significantly only at lower concentration and that of HO-1 at higher concentration.

Expression of antioxidant enzymes is also regulated post-transcriptionally by miRNAs.[21] These miRNAs recognize sequences in the 3'untraslated regions (3'-UTR) of target messenger RNAs and induce either mRNA degradation or inhibit translation of proteins. Levels of antioxidant gene specific miRNAs namely miR 30b[22] miR2 14[23] and miR 181a[24] specific for CAT, GR and GPx respectively were found to be significantly increased only at 0.5% co-relating with the observed decrease in antioxidant enzyme gene expression.

Besides antioxidant enzymes, Nrf2 also regulates expression of HO-1. Up-regulation of HO-1 is known to be important for tissues under stress and thus agents which promote this are considered to have good therapeutic value. Livotrit® significantly increased HO-1 expression at higher concentration (0.5%) but not at lower concentration (0.05%). Although Nrf2, a positive regulator of HO-1 was significantly activated at both concentrations, HO-1 was induced only at higher concentration of livotrit®. BACH-1 is a negative regulator of HO-1 and is known to repress the transcription of HO-1 gene by binding to the stress responsive elements (StREs) in the 5'-UTR of the HO-1 promoter.[25] Treatments of cells with 0.5% of

Livotrit® led to significant decrease in BACH-1 expression compared to 0.05%. Additionally, level of BACH-1 specific miR 98 was found to be inversely proportional to the levels of BACH-1 mRNA. Thus, Livotrit® at higher concentration (0.5%) increased HO-1 gene expression by downregulating BACH-1 expression through its specific regulator miR 98.

5. Conclusion

In conclusion, the present data clearly demonstrates the ability of Livotrit® to activate antioxidant enzymes in a concentration dependent manner. At lower concentration it activated CAT, GR and GPx as well as maintained levels of intracellular GSH. While at higher concentration it activated HO-1. The regulation of these enzymes was both through their positive regulators and miRNAs.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgments

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jtcme.2017.08.007.

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