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Effect of acetone extract from stem bark of *Acacia* species (*A. dealbata*, *A. ferruginea* and *A. leucophloea*) on antioxidant enzymes status in hydrogen peroxide-induced HepG2 cells



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HepG2 cells;
Superoxide dismutase

Abstract *Acacia* species are multipurpose trees, widely used in the traditional systems of medicine to treat various ailments. The major objective of the present study was to determine the gene expression of enzymatic antioxidants by acetone extract from the stem bark of three *Acacia* species (*Acacia dealbata*, *Acacia ferruginea* and *Acacia leucophloea*) in hydrogen peroxide (H₂O₂)-induced human hepatoma (HepG2) cells. The expression of antioxidant enzymes such as superoxide dismutase containing copper–zinc (CuZnSOD)/manganese (MnSOD), catalase (CAT) and glutathione peroxidase (GPx) in HepG2 cells was evaluated by real-time PCR. The results of antioxidant enzyme expression in real-time PCR study revealed that the H₂O₂ (200 μM) challenged HepG2 cells reduced the expression of enzymes such as SOD, GPx and CAT. However, the cells pre-treated with acetone extracts of all the three *Acacia* species significantly ($P > 0.05$) up-regulated the expression of antioxidant enzymes in a concentration dependent manner (25, 50 and 75 μg/mL). In conclusion, the findings of our study demonstrated that the acetone extract of *Acacia* species effectively inhibited H₂O₂ mediated oxidative stress and may be useful as a therapeutic agent in preventing oxidative stress mediated diseases.

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1. Introduction

Reactive oxygen species (ROS) are the endogenous free radicals of normal cellular metabolism. In living organisms, moderate concentrations of ROS benefit physiological functions such as intra-cellular signaling, cellular defense against

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infective agents, and induction of a mitogenic response. However, the excessive productions of free radicals can cause cellular lipid peroxidation, protein degradation, and DNA mutation, resulting in several degenerative diseases, including inflammation, cardiovascular diseases, cancer, diabetes, and neurological disorders (Valko et al., 2007; Carvalho et al., 2014). Generally, all the organisms are well protected against free radical damage by endogenous oxidative enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) and catalase (CAT). However, these enzymes are commonly insufficient when it comes to completely preventing degenerative diseases and other health problems (Borneo et al., 2009; Soares et al., 2009). In addition, several non-enzymatic antioxidant compounds such as phenolics, ascorbic acid, tocopherol, glutathione and other dietary compounds play an important role in defending the body against free radical damage by scavenging or neutralizing the oxidizing molecules and maintaining redox balance (Tachakittirungrod et al., 2007). Recent studies have reported that the plant kingdom offers a wide range of natural antioxidant molecules including phenolic acids, flavonoids, and other secondary metabolites and they can also be used for the treatment of various human disorders (Slusarczyk et al., 2009).

Acacia trees (Family – Mimosaceae) are known as a versatile source of bioactive components. The genus *Acacia* comprises about 1350 species widely distributed from tropics and to some extent in the temperate regions, especially in Australia, Africa, India and America. A number of biologically active metabolites have been reported from various *Acacia* species including phenolics, flavonoids, terpenes, tannins, amines, and alkaloids, (Seigler, 2003). The different plant parts of *Acacia* species are widely used as indigenous drugs to treat various ailments. Especially, the bark is traditionally used for the treatment of several diseases in ayurvedic system of medicine (Parrotta, 2001; Kirtikar and Basu, 2003). *Acacia ferruginea* DC., *Acacia dealbata* Link. and *Acacia leucophloea* (Roxb.) Willd. are important medicinal plants with various therapeutic properties. Acetone and methanol extracts of *A. leucophloea*, *A. ferruginea*, *A. dealbata*, and *Acacia pennata* barks registered very strong antioxidant properties under different *in vitro* chemical assays (Sowndhararajan et al., 2013). In Indian traditional medicine, the stem bark of *A. leucophloea* is used to treat inflammation, bronchitis, cough, biliousness, skin diseases, leucoderma, pruritus, erysipelas, vomiting, wounds, ulcers, diarrhea, dysentery, stomatitis, intermittent fevers, leprosy, and toothache. *A. ferruginea* bark is used for the treatments of itching, leucoderma, ulcers, stomatitis, and diseases of the blood (Parrotta, 2001). Ethyl acetate fraction from acetone extract of *A. ferruginea* stem bark exhibited antiulcer activity against ethanol-induced gastric ulcer in rats (Sowndhararajan and Kang, 2013). *A. dealbata* yields a gum, resembling gum arabic, used in bronchial troubles (Prajapati, 2005). Recently, the human hepatoma cell line (HepG2) has been extensively used for examining *in vitro* oxidative damage and xenobiotic metabolism (Lee et al., 2015). Based on the highly acclaimed properties of *Acacia* species, the present study was carried out to investigate the effect of acetone extracts of *A. leucophloea*, *A. ferruginea* and *A. dealbata* stem barks on the expression of antioxidant enzymes (SOD, GPx and CAT) in H₂O₂-induced HepG2 cells.

2. Materials and methods

2.1. Chemicals and materials

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), trypan blue, hydrogen peroxide (H₂O₂) and penicillin–streptomycin solution were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Dulbecco's modified eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from HyClone Laboratories, Inc. (Utah, USA). RNeasy Mini kit and SYBR green master mix were purchased from Qiagen-GmbH (Hilden, Germany). SuperScript III First-Strand synthesis system was purchased from Invitrogen (Carlsbad, CA, USA). All other reagents were of the highest available purity analytical grade.

2.2. Preparation of extracts

Fresh barks of *A. dealbata*, *A. ferruginea* and *A. leucophloea* were collected from Coimbatore, Tamil Nadu state, India. The plants were authenticated and herbarium specimens deposited in the Botany Herbarium, Bharathiar University with voucher numbers: BUBH-6140, BUBH-6141, and BUBH-6142, respectively.

The plant materials were washed thoroughly in tap water, shade dried at room temperature (25 °C), powdered, and used for solvent extraction. The plant samples were extracted with petroleum ether (for disposing lipid and pigments), followed by acetone using soxhlet apparatus. The solvents were evaporated using a rotary vacuum-evaporator (RE300, Yamato, Tokyo, Japan) at 50 °C and the remaining water was removed by lyophilization (4KBTXL-75, VirTis Benchtop K, NY, USA). The dried extracts were kept at –20 °C prior to use in the cell culture experiments.

2.3. Quantification of target gene expression by real-time PCR

2.3.1. Cell culture

Human hepatoma cell line (HepG2) was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured and maintained in DMEM supplemented with 10% FBS, 50 units/mL penicillin and 50 µg/mL streptomycin. The cells were incubated at 37 °C in a 5% CO₂ incubator (HERAcell 150, Thermo Electron Corp., Waltham, MA, USA).

2.3.2. MTT cell viability assay

The mitochondrial-dependent reduction of MTT to formazan was used to measure cell respiration as an indicator of cell viability. Briefly, HepG2 cells were seeded in 96-well plates at the density of 5×10^4 cells/well. After 24 h of incubation, the cells were treated with various concentrations of the extracts (6.25, 25, 50, 100 and 200 µg/mL, eight wells per concentration). Twenty-four hours later, after changing the medium, MTT was added to a final concentration of 0.5 mg/mL, and the cells were incubated for 4 h at 37 °C and 5% CO₂. The medium was then removed and the formazan precipitate was solubilized in DMSO. The absorbance was measured at 550 nm on a microplate reader (Biotek, Winooski, VT, USA). The results were

expressed as the percentage of viable cells in comparison to the control (100%).

2.3.3. RNA isolation and first-strand cDNA synthesis

One day before treatment with extracts, HepG2 cells were trypsinized and seeded at a density of 5×10^5 cells/well in a 24-well plate. The media were discarded after 24 h, and fresh FBS-free DMEM were added along with extracts with various concentrations. 200 μ M H_2O_2 was treated after 2 h exposure of extracts. After 24 h of incubation, culture media were aspirated and the cells were collected.

Total cellular RNA was isolated with a commercial kit (RNeasy Mini kit, Qiagen), according to the manufacturer's instructions. One microgram of total RNA was reverse-transcribed using oligo (dT) and SuperScript III Reverse Transcriptase (Invitrogen). cDNA synthesis was carried out according to the manufacturer's instructions, and the resulting cDNA was stored at $-20^\circ C$.

2.3.4. Quantification of mRNA levels by real-time PCR

Using cDNAs as the template, quantitative real-time PCR was carried out using the SYBR Green PCR Master Mix (Qiagen) in a real-time PCR (Rotor-gene Q, Qiagen), according to the manufacturer's instructions, using specific oligonucleotide primers for human CuZnSOD, MnSOD, GPx and CAT genes (Table 1). β -Actin cDNA was used as an internal control. A dissociation cycle was performed after each run to check for non-specific amplification or contamination. After initial denaturation ($95^\circ C$ for 5 min), 40 PCR cycles were performed using the following conditions: $95^\circ C$, 5 s; $60^\circ C$, 10 s at the end of PCR reaction, samples were subjected to a temperature ramp (from 60 to $95^\circ C$, $1^\circ C/s$) with continuous fluorescence monitoring. For each PCR product, a single narrow peak was obtained by melting curve analysis at specific temperature. Relative expression levels were estimated using the method described by Pfaffl (2001). Analysis was performed with relative quantification software (Rotor-Gene Q series 2.0.3 software). Antioxidant enzyme activities were expressed as percentages of the control (100%).

2.4. Statistical analysis

The values expressed are means of three replicate determinations \pm standard deviation. The statistical analysis was carried out by an analysis of variance (ANOVA) followed by Tukey's test. The data were evaluated with SPSS 20.0 (SPSS Inc., Chicago, IL, USA).

3. Results and discussion

The viability of HepG2 cells was evaluated by MTT assay based on the intensity of the purple color. MTT is reduced by mitochondrial dehydrogenase to produce formazan, an insoluble purple color compound. To determine the viability of HepG2 cells in the presence of acetone extracts, cells were incubated for 24 h with the extracts at a wide range of concentrations. The acetone extracts of *Acacia* barks inhibit the growth of HepG2 cells with the IC_{50} value of $> 100 \mu g/mL$. Further, the acetone extracts did not show cytotoxicity in HepG2 cells up to $60 \mu g/mL$. Based on the results, concentrations of sample (cell viability $> 85\%$) were selected for subsequent antioxidant enzyme expression experiment.

The human HepG2 cells retain their endogenous expression of different antioxidant enzymes, and have been used as an ideal tool for studying the mechanisms of oxidative damages and protection against oxidative stress (Lee et al., 2012, 2015). In the present study, to evaluate the protective effect of acetone extract of *Acacia* stem barks on H_2O_2 challenged HepG2 cells, antioxidant enzymes levels of CuZnSOD, MnSOD, CAT and GPx were determined. These enzymes are considered as the first line of the antioxidant defense system against ROS mediated oxidative stress (Bak et al., 2012). The gene response for the CuZnSOD, MnSOD, CAT and GPx was monitored by quantitative real-time PCR in HepG2 cells examined upon treatment with 25, 50 and 75 $\mu g/mL$ of acetone extracts of bark for 24 h. The cells treated with H_2O_2 (200 μM) alone significantly reduced the expressions of CuZnSOD (54.7%), MnSOD (49.3%), CAT (43.3%) and GPx (53.3%) enzymes, when compared with the untreated control (Figs. 1–4). However, HepG2 cells pre-treated with 25, 50 and 75 $\mu g/mL$ of acetone extract exposure to H_2O_2 effectively up-regulated the expression of SOD, CAT, and GPx enzymes in a concentration-dependent manner (Figs. 1–4). The acetone extracts from three *Acacia* stem barks showed appreciable and similar trend of protective potential against oxidative damage induced in HepG2 cells. The highest concentration (75 $\mu g/mL$) of acetone extract from all the three *Acacia* barks showed more potent inductions of CuZnSOD (86.0–91.3%), MnSOD (75.0–88.7%), CAT (68.3–72.0%), and GPx (71.3–74.3%) enzymes.

Antioxidant enzymes are believed to be the most important in cellular defenses because they balance the redox status in cells by removing the excessive free radicals (Valko et al., 2006). H_2O_2 is regarded as an important intermediary of oxidative stress-induced cytotoxicity (Chiu et al., 2013). It is

Table 1 Nucleotidess sequences of PCR primers used for quantitative real-time PCR.

Primer	Oligonucleotide sequence	Temperature ($^\circ C$)	Length (bp)
β -actin	5'-CGGGAAATCGTGCGTGACAT-3' 5'-GGACTCCATGCCAGGAAGG-3'	60	198
CuZnSOD	5'-ACGGTGGGCCAAAGGATGAA-3' 5'-TCATGGACCACCAGTGTGCG-3'	60	151
MnSOD	5'-AGAAGCACAGCCTCCCCGAC-3' 5'-GGCCAACGCCTCCTGGTACT-3'	60	152
GPx	5'-TCGGTGTATGCCTTCTCGGC-3' 5'-CCGCTGCAGCTCGTTCATCT-3'	60	150
CAT	5'-CCAACAGCTTTGGTGCTCCG-3' 5'-GGCCGGCAATGTTCTCACAC-3'	60	180

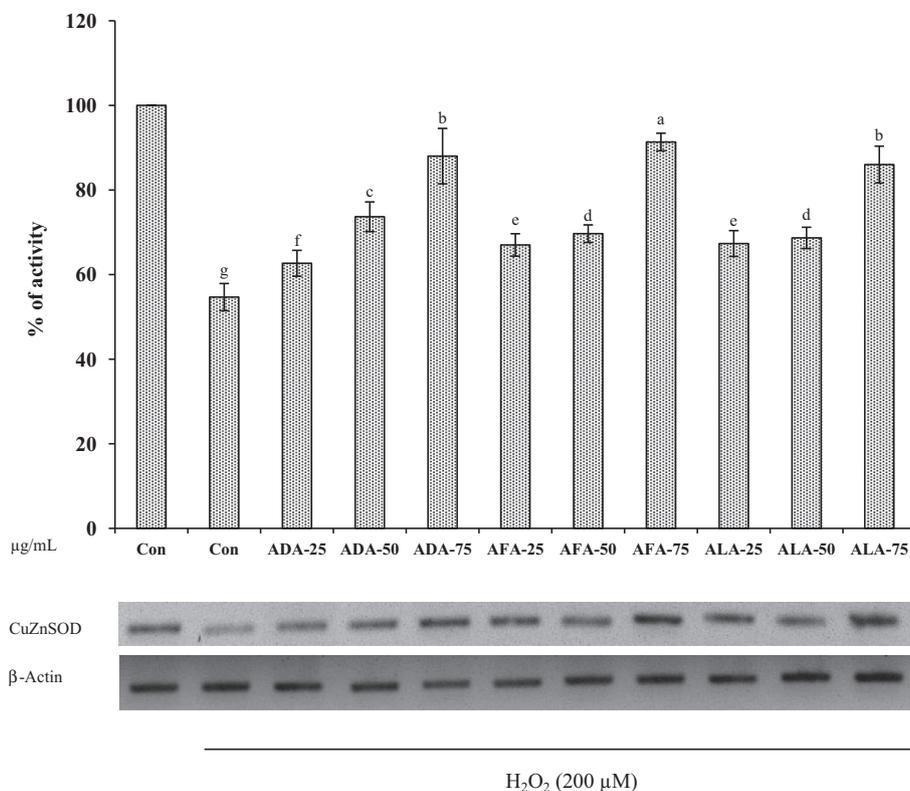


Figure 1 Effect of different concentrations (25, 50 and 75 µg/mL) of acetone extracts of *Acacia* barks on CuZnSOD activity in H₂O₂-stimulated HepG2 cells. ADA, *A. dealbata*; AFA, *A. ferruginea*; ALA, *A. leucophloea*. Values are mean of three replicate determinations ($n = 3$) ± standard deviation. Bars having different letters are significantly different ($P < 0.05$).

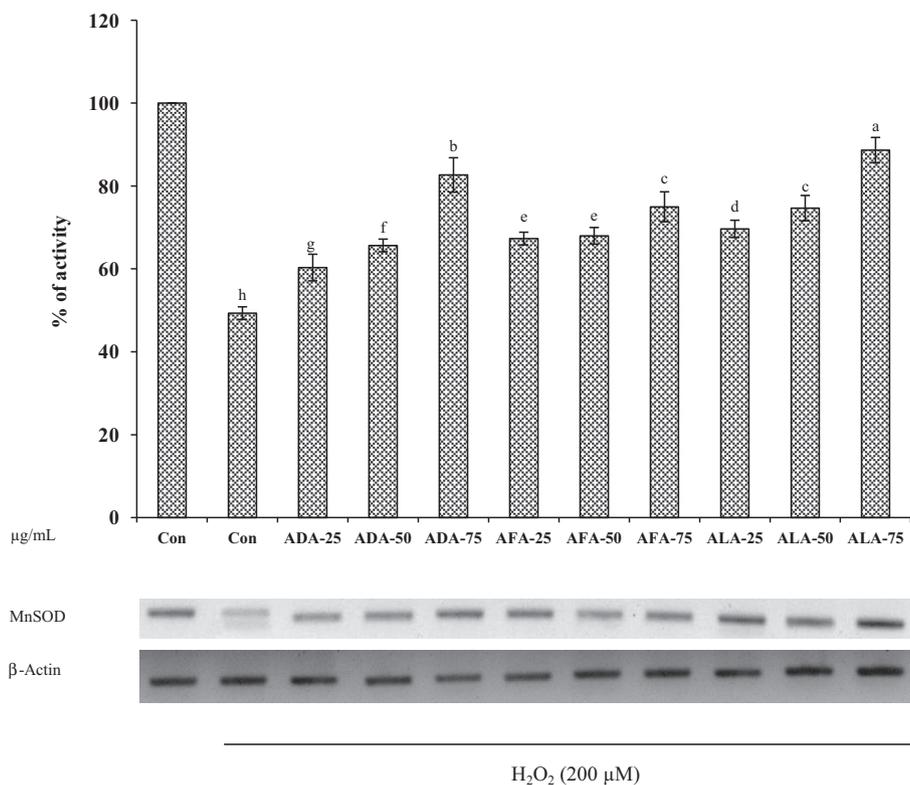


Figure 2 Effect of different concentrations (25, 50 and 75 µg/mL) of acetone extracts of *Acacia* barks on MnSOD activity in H₂O₂-stimulated HepG2 cells. ADA, *A. dealbata*; AFA, *A. ferruginea*; ALA, *A. leucophloea*. Values are mean of three replicate determinations ($n = 3$) ± standard deviation. Bars having different letters are significantly different ($P < 0.05$).

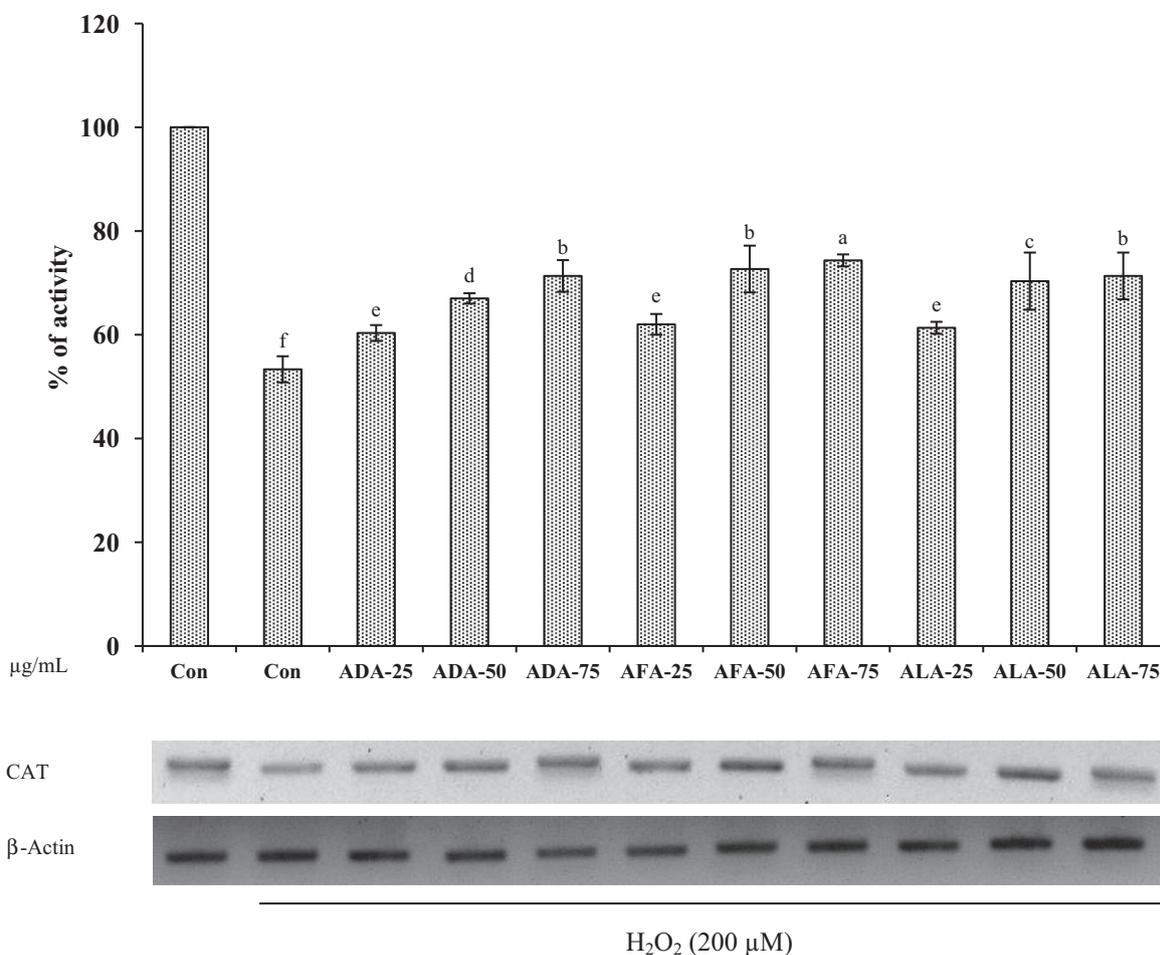


Figure 3 Effect of different concentrations (25, 50 and 75 µg/mL) of acetone extracts of *Acacia* barks on CAT activity in H_2O_2 -stimulated HepG2 cells. ADA, *A. dealbata*; AFA, *A. ferruginea*; ALA, *A. leucophloea*. Values are mean of three replicate determinations ($n = 3$) \pm standard deviation. Bars having different letters are significantly different ($P < 0.05$).

formed from nearly all sources of the oxidative cycle and has the ability to diffuse in and out of cells and tissues (Slamenova et al., 2013). Various enzymes including SOD, CAT and peroxidases are effectively involved in H_2O_2 modulation. Previously, several authors have reported that up-regulating the expression of antioxidant enzymes in HepG2 cells promotes a protective effect against cytotoxicity or apoptosis induced by oxidative stress (Bak et al., 2011; Carvalho et al., 2014). The antioxidant defense enzymes, such as SOD and CAT protect aerobic cells against O_2 toxicity and lipid peroxidation. SOD reacts with superoxide anion radical to produce oxygen and the less-reactive H_2O_2 . H_2O_2 is subsequently converted to water by CAT and GPx (Gao et al., 2012). Therefore, SOD could protect cells from the toxicity of superoxide radicals. Landis and Tower (2005) reported that the level of catalase is lowered in several tumor cells, which results in a decreased detoxifying capacity for H_2O_2 in tumors. Hence, induction of expression of these antioxidant enzymes seems to be essential for prevention of various free radical-mediated diseases such as cancer, atherosclerosis, and chronic inflammation.

The effect of acetone extracts of *Acacia* barks on the expression activity of antioxidant enzymes that modulate H_2O_2 levels, such as superoxide dismutase (CuZnSOD and MnSOD), CAT and GPx in HepG2 cells had not been previously investigated. Sowndhararajan et al. (2013) studied the total phenolic and flavonoid contents and antioxidant properties of acetone and methanol extracts of barks of *A. leucophloea*, *A. ferruginea*, *A. dealbata*, and *A. pennata*. Acetone extracts of barks exhibited higher contents of total phenolics and flavonoids. Further, the acetone extracts showed higher free radical scavenging activities than methanol extracts. In addition, biological activities of the sample are directly related to their chemical composition. Previous studies have reported that the plant bioactive compounds can act as direct antioxidants through scavenging or neutralizing ROS and, also, as indirect antioxidants through up-regulation of endogenous antioxidant defenses (Dinkova-Kostova and Talalay, 2008; Carvalho et al., 2014). It is possible that the antioxidant properties of plant extracts are utilized by the cells, thus releasing the intracellular antioxidant enzymes (Kozics et al., 2013). Hence, the expression of antioxidant enzyme activity by

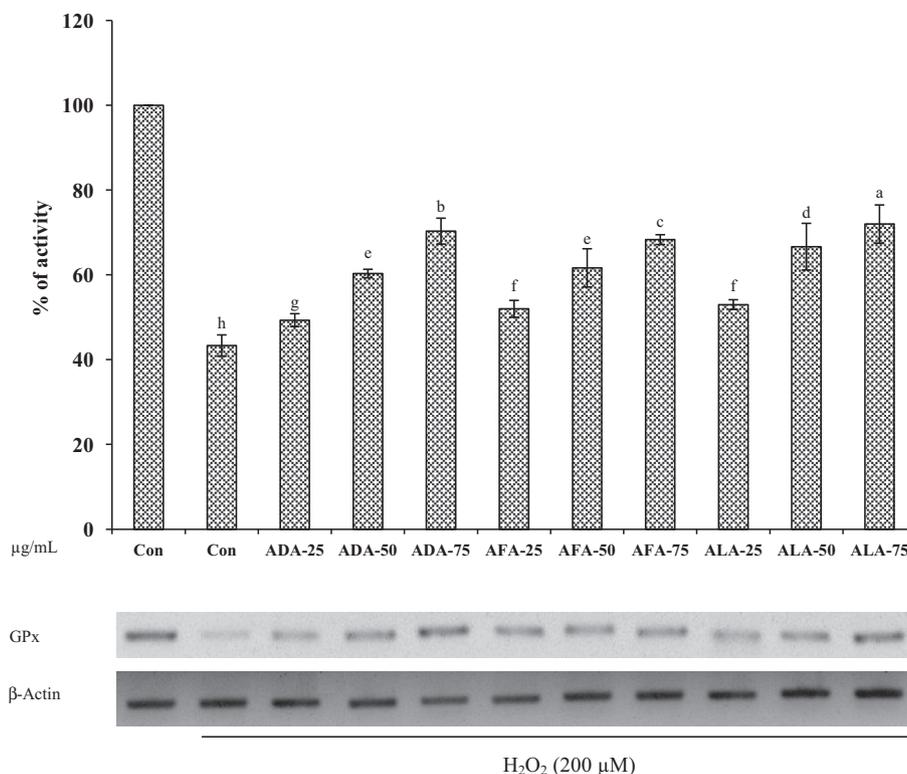


Figure 4 Effect of different concentrations (25, 50 and 75 µg/mL) of acetone extracts of *Acacia* barks on GPx activity in H₂O₂-stimulated HepG2 cells. ADA, *A. dealbata*; AFA, *A. ferruginea*; ALA, *A. leucophloea*. Values are mean of three replicate determinations ($n = 3$) \pm standard deviation. Bars having different letters are significantly different ($P < 0.05$).

acetone extract of *Acacia* barks might be attributed to the presence of bioactive metabolites and their antioxidant potential.

4. Conclusions

Acetone extracts from all the three *Acacia* species effectively up-regulate the expressions of SOD, CAT and GPx enzymes in a concentration dependent manner. These findings strongly suggest that the acetone extracts of *Acacia* barks may participate in cellular protection as an antioxidant molecule and stimulate the expression of antioxidant enzymes. It could be concluded that the barks of *Acacia* appear to be a useful source of a therapeutic agent for the treatment of oxidative stress mediated disorders. Further studies are needed in relation to the mechanism of action and isolation of responsible chemical components from the extracts.

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