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HYDRO-ETHANOLIC EXTRACT OF *GANODERMA LUCIDUM* (HEGL) SHOWS ANTI-INFLAMMATORY ACTIVITY ON THP1 CYTOKINES AND NF-κB P65 RESPONSE

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Keywords:

Hydro-ethanolic extract of *Ganoderma lucidum* (HEGL); anti-inflammatory; antioxidant; LPS

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ABSTRACT: The present study is aimed to elucidate the antioxidant property and anti-inflammatory activity of hydro-ethanolic extract of *Ganoderma lucidum* (HEGL) known to possess medicinal activity against numerous diseases. The chemical composition of HEGL was quantified by colorimeteric technique in terms of total phenol and flavonoid content. Antioxidant activity was determined by 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 2,2-diphenyl-1-picryl-hydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) assays. The extract possesses strong anti-oxidant property in terms of high content of phenols, flavonoids and reducing power. Coumarin a known antioxidant and anticoagulant compound was identified and quantified by HPLC. Further, a dose dependent *in vitro* study for HEGL extract was carried out to observe anti-inflammatory activity in THP1 cells. HEGL extract significantly suppressed lipopolysacharide (LPS) – induced release of TNF-α, IFN-γ and IL-1β in THP1 cells and significantly suppressed nitric oxide (NO) release in macrophages cells without causing any toxic effect. Additionally, HEGL extract also decreased NF-kB expression in LPS-treated cells indicating anti-inflammatory activity of hydro-ethanolic extract of *Ganoderma lucidum* (HEGL) at the optimized dose of 100µg/ml. These results predict that the consumption of HEGL may be clinically useful to protect against inflammatory diseases.

INTRODUCTION: Inflammation is a normal protective response to tissue injury. This phenomenon is associated with pain which includes increase in vascular permeability, increase of protein denaturation and membrane alteration [1]. Basically, inflammation is a protective mechanism by organism which removes injurious stimuli as well as initiates the healing process for the tissue [2]. But, if inflammation is not treated it results into various diseases like vasomotor rhinorrhoea, rheumatoid arthritis and atherosclerosis [3].

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Numerous synthetic drugs such as opoids and non-steroidal anti-inflammatory drugs (NSAIDS) are available, but these existing synthetic drugs increase the incidence of adverse cardiovascular thrombotic effects [4]. Therefore, a fruitful and logical research strategy is required for search of new anti-inflammatory drug which may have minimal drawbacks. Medicinal plants have a wide variety of chemicals from which novel anti-inflammatory agents can be discovered. Naturally occurring compounds from plants, fungi and microbes are still used in pharmaceutical preparations in pure or extracted forms.

Traditionally, *Ganoderma lucidum* (Ganodermataceae) is highly valued as folk medicine and functional food for its beneficial

activities. It is medicinal mushroom which has been widely used in China (named Ling Zhi) and Japan (named Reishi Mannentake) for hundreds of years for various diseases [5, 6]. Numerous pharmacological properties such as hypertension, hyperglycemia, hepatitis, chronic bronchial asthma, liver protection are reported for fruiting body and cultured mycelia of *Ganoderma lucidum* [5, 7]. Beside these properties, It is also found medically active in numerous therapeutic effects including antitumor [8], antiviral, antibacterial [9], antiparasitic, antihepatitis B [10], blood pressure regulation, cardiovascular disorders, kidney tonic, nerve tonic, sexual potentiator and chronic bronchitis [11, 12].

Numerous studies have been carried out on fruiting body of *Ganoderma lucidum* however, limited data on the studies using whole body of *Ganoderma lucidum* is available. In addition to this, the antioxidant and anti-inflammatory effects of hydro-ethanolic extract of high altitude Indian variety of *Ganoderma lucidum* is also unknown. Therefore, in the present study, we planned to investigate the effect of hydro-ethanolic extract of *Ganoderma lucidum* on inflammatory cytokines, NF-kB activity and its antioxidant property.

MATERIALS AND METHODS:

Reagents: RPMI and fetal bovine serum (FBS) were purchased from HiMedia, India. TNF-α, IL-1β and IFN-γ ELISA kits were purchased from Cayman Chemicals, New Orleans, Louisiana, USA, Trolox, 1,1-diphenyl-2-picrylhydrazyl, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), Lipopolysaccraide (LPS), were purchased from Sigma, USA.

Preparation of hydro-ethanolic extract of *Ganoderma lucidum* by accelerated solvent Extraction method: Accelerated solvent Extraction system ASE 350 equipped with a solvent controller unit from Dionex Corporation (Sunnyvale, CA, USA) was used for the extraction. Extraction was carried in 33 ml extraction cells, containing 2gm of sample in triplicate using 70% ethanol as a solvent at 25°C±2°C for 15 min. The heat-up time was changed according to extraction temperature and is automatically fixed by the equipment [13].

Determination of total flavonoids and phenolic content:

Total Flavonoid Content: Total flavonoid content was determined as per the reported method [14]. A mixture of 1.0 ml of the extract (1mg/ml), 2.0 ml of nanopure water and 0.15ml of 5% $NaNO_2$ was prepared and allowed to react for 6 min. 0.15 ml of 10% $AlCl_3$ solution was added to the above mixture and mixed thoroughly. After 6 min, 2.0 ml of 4% NaOH solution was added and allowed to stand for another 15 min. Absorbance of the mixture and the blank was measured at 510 nm using Spectrophotometer (SmartSpec 3000, Bio-Rad, CA, USA). Rutin was used as a standard compound for the quantitation of flavonoid content. Results were expressed in mg of rutin equivalents/gm of HEGL extract.

Total Phenolic Content: Total phenolic content was determined with the Folin-Ciocalteu reagent according to the method described by Singleton and Rossi [15]. A mixture of 150 µl of the extract (0.2 mg/ml), 2400 µl of nanopure water and 150 µl of 0.25N Folin-Ciocalteu reagent was prepared and allowed to react for 3 min. Then 300 µl of 1N Na_2CO_3 solution was added into the reaction mixture. After incubation for 2 h at room temperature, the absorbance relative to that of prepared blank was measured at 725 nm using spectrophotometer (SmartSpec 3000, Bio Rad, CA, USA). Gallic acid was used as a reference standard and the results were expressed as mg gallic acid equivalents/gm of HEGL extract.

Antioxidant activity determinations: Antioxidant activity of plant extract cannot be evaluated by only a single method due to the complex nature of phytochemicals. Therefore, in the present study antioxidant activity was evaluated by three different assays viz. 2,2'-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and ferric reducing antioxidant power (FRAP).

DPPH (2,2'-diphenyl-1-picrylhydrazyl) Assay: The DPPH assay determination was carried out according to the method of Brand-Williams et al.,[16] with some modifications. The stock solution was prepared by dissolving 24 mg DPPH with 100mL methanol and stored at −20°C until required.

The working solution was prepared by mixing 10 ml stock solution with 45 ml methanol to obtain an absorbance of 1.1±0.02 units at 515 nm using the spectrophotometer. The extract (150 µl) was allowed to react with 2850 µL of the DPPH solution for 24 h in the dark. Then the absorbance was recorded at 515 nm. The standard curve was linear between 25 and 800 µM Trolox. Results are expressed in µM Trolox equivalents/gm of HEGL extract.

ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) Assay: The method of Arnao *et al.,*[17] with some modifications was used for the determination of ABTS assay. The stock solutions used were 7.4 mM $ABTS^{\bullet +}$ solution and 2.6 mM potassium persulfate solution. The working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 ml $ABTS^{\bullet +}$ solution with 60 ml methanol to obtain an absorbance of 1.1±0.02 units at 734 nm wavelength using the spectrophotometer. Fresh $ABTS^{\bullet +}$ solution were prepared for each assay. The extract (150 µl) was allowed to react with 2850 µL of the $ABTS^{\bullet +}$ solution for 2 h in dark condition. Then the absorbance was taken at 734 nm using the spectrophotometer. The standard curve was linear between 25 and 800 µM Trolox. Results are expressed in µM Trolox equivalents/gm of HEGL extract.

FRAP (Ferric Reducing Antioxidant Power) Assay: The FRAP assay was done according to Benzie and Strain [18] with some modifications. Briefly, the stock solutions included 300mM acetate buffer (3.1 g $C_2H_3NaO_2.3H_2O$ and 16 ml $C_2H_4O_2$), pH 3.6, 10mM TPTZ (2, 4, 6- tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3.6H_2O$ solution. The fresh working solution was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ solution, and 2.5 ml $FeCl_3.6H_2O$ solution and then warmed at 37°C before using. The extract (150 µl) were allowed to react with 2850 µL of the FRAP solution for 30 min in the dark condition. Readings of the colored product (ferrous tripyridyltriazine complex) were then measured at 593 nm.

The standard curve was linear between 25 and 800 µM Trolox. Results are expressed in µM of Trolox equivalents/gm of HEGL extract.

HPLC fingerprinting: HPLC analysis of hydro-ethanolic extract of *Ganoderma lucidum* (HEGL) was performed using model YL9100 HPLC system (Younglin Co., Korea) using photo diode array detector (waters 2998) with autosampler and column oven. A 10µl sample of 1mg/ml solution of the extract was injected followed by implementation of HPLC grade solvent, methanol and 0.01 M KH_2PO_4 (10:90 v/v) at 1 ml/min. Reverse phase C-8 column (25X4.6 mm, particle size 5µm) were used to separate the component at 272 nm absorbance.

***In vitro* Immunomodulatory activity:**

1. **Cell Line and Tissue Culture Media***:* THP1 cell line was obtained from National Centre for Cell Sciences (NCCS), Pune, India. Cell were grown in RPMI 1640 media containing 25mM HEPES, 2mM l-glutamine (Sigma, St. Louis, MO), 10% heat inactivated fetal bovine serum, penicillin (100 U/ml), and streptomycin (100ug/ml) in a humidified atmosphere of 5% CO_2 at 37°C.

2. **MTT Assay for cell proliferation/viability:** The cell viability was tested using yellow tetrazolium salt, MTT [19]. The assay was performed in 96-well tissue culture plates. In brief, THP-1 cells ($0.2X10^6$ cells/ml) were incubated for 24 h in a humidified atmosphere of 5% CO_2 at 37°C with varying concentration (25, 50, 100, 250 and 500 µg) of HEGL in the presence and absence of LPS (0.2 µg/ml) (Sigma). After incubation, 20 µl of 5mg/ml MTT solution (MTT dissolved in 0.1 M Tris-buffered saline and filtered to remove any insoluble matter) was added and incubated for additional 4 h under the same conditions. Supernatant was removed and the blue formazon crystals were solubilized in 200 µl of dimethyl sulfoxide (DMSO) under agitation. After dissolving the crystals, optical density were obtained using microtiter plate reader (BioTek, USA) at 570 nm. Proliferation activity was represented by absorbance at 570 nm.

3. **Determination of TNF-α, IFN-γ and IL-1β:** THP-1 cells (0.2X10^6cells/ml) were incubated with different concentrations of HEGL in the absence and presence of LPS (0.2µg/ml) for 24 h in a humidified atmosphere of 5% CO_2 at 37°C. Supernatant was collected and stored at -80°C until measurements of TNF-α, IFN-γ and IL-1β was performed.

   TNF-α, IFN-γ and IL-1β levels in supernatant were determined by using commercial enzyme-linked immunosorbent assay (ELISA) kits (Cayman, USA) following manufacturer's instructions.

4. **Mouse peritoneal macrophage isolation and culture for nitric oxide (NO) determination:** The mice were injected (*i.p.*) with 1.5 ml of 2% starch suspension prepared in phosphate buffered saline (PBS). After 3 days, peritoneal fluid was collected in the cold phosphate buffer saline (PBS). Collected peritoneal fluid was centrifuged at 400g for 10 min; the resulting pellet was suspended in complete RPMI-1640 medium at a concentration of 0.5×10^6 cells per ml.

   Mouse macrophages were cultured in RPMI1640 media with 25mM HEPES, 2mM glutamine, 10% fetal bovine serum, penicillin (100 IU/ml), and streptomycin (100 IU/ml). Cultured cells were incubated with HEGL with or without LPS in a humidified incubator at 37°C in 5% CO_2. Nitrite production, an indicator of NO synthesis, was determined by the Griess reaction after 48 h treatment. The supernatant of mouse macrophage cell culture was mixed with an equal volume of Griess reagent (1% sulfanilamide and 0.1% naphthylenediamine in 5% phosphoric acid). The optical density at 550 nm (A_{550}) was measured and calculated against a sodium nitrite standard curve.

5. **Nuclear Transcription Factor-kB expression (NF-kB):** 1.0X10^7 cells/ml were suspended in RPMI 1640, centrifuged at 300Xg for 5 min at 4°C and the supernatant was discarded. Then, nuclear extract was prepared as per instructions given in Cayman's Nuclear Extraction Kit, U.S.A.

   Then NF-kB was estimated by ELISA kit (Cayman, USA). All tests were performed in triplicates.

Statistical Analysis: Data are presented as Mean ± SEM. One way Analysis of Variance (ANOVA) followed by Berferroni's multiple comparisons test using Graph Pad Instat3 (Graph Pad Software Inc, La Jolla, CA). A p-value < 0.05 was considered statistically significant.

RESULTS:

Extraction Yield: In this study, 70% ethanol was used as extraction solvent and 18% yield of extracted material was obtained from *Ganoderma lucidum* fungus **(Fig. 1a)**. Further, hydro-ethanolic extract of *Ganoderma lucidum* (HEGL) was found to be rich in total phenolics and flavonoid contents **(Fig. 1b)**.

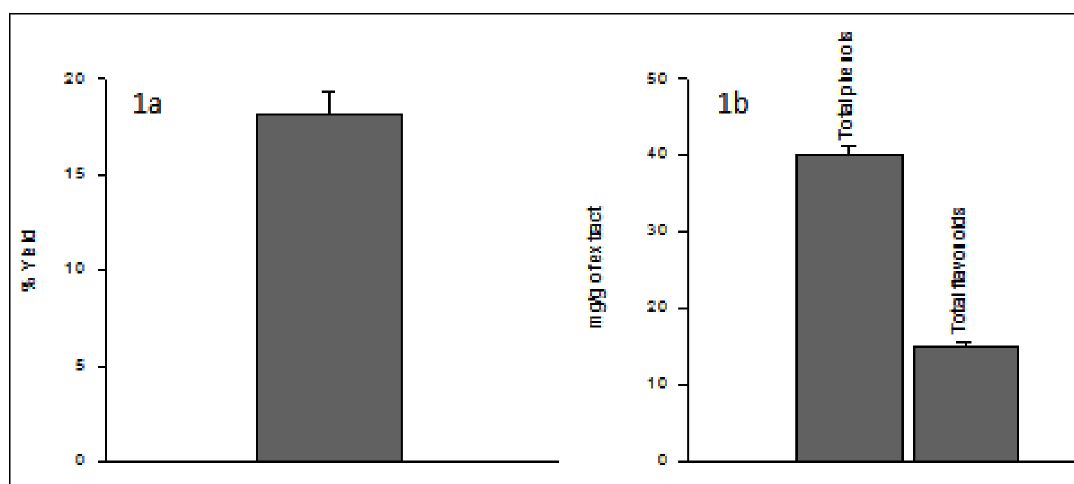

FIG. 1: YIELD OF HYDRO-ALCOHOLIC EXTRACT OF *GANODERMA LUCIDUM (1A) AND* TOTAL PHENOLS AND FLAVONOID CONTENT OF HYDRO-ALCOHOLIC EXTRACT OF *GANODERMA LUCIDUM (1B)*

Antioxidant activity: Hydro-ethanolic extract of *Ganoderma lucidum* (HEGL) exhibited potent antioxidant activity when analyzed by DPPH, ABTS and FRAP assay as indicated in **Table 1**.

TABLE 1: DETERMINATION OF ANTIOXIDANT ACTIVITY OF HYDRO-ETHANOLIC EXTRACT OF *GANODERMA LUCIDUM* (HEGL)

Sample	Hydro-ethanolic extract of *Ganoderma lucidum* (HEGL)
DPPH (µM of Trolox equivalents/gm)	16.02±0.12
ABTS (µM of Trolox equivalents/gm)	69.50±0.78
FRAP (µM of Trolox equivalents/gm)	17.10±1.42

Data represent Means ± SEM of three independent experiments carried out in triplicates

Identification of coumarin by HPLC: A simple and gradient elution-based reverse phase high performance liquid chromatography (RP-HPLC) method was developed for the quantitative analysis of coumarin. For the development of an effective mobile phase, various solvent systems, including different combinations of acetonitrile, methanol and water with orthophosphoric acid were tried. Finally, a solvent system consisting methanol: 0.01M KH_2PO_4 (10:90 v/v) was proved to be successful as it allows the separation of coumarin with good resolution. Identification was carried out by integration of the peak using an external standard method. The results for retention time and major peak area are shown in **Fig. 2 & Table 2.**

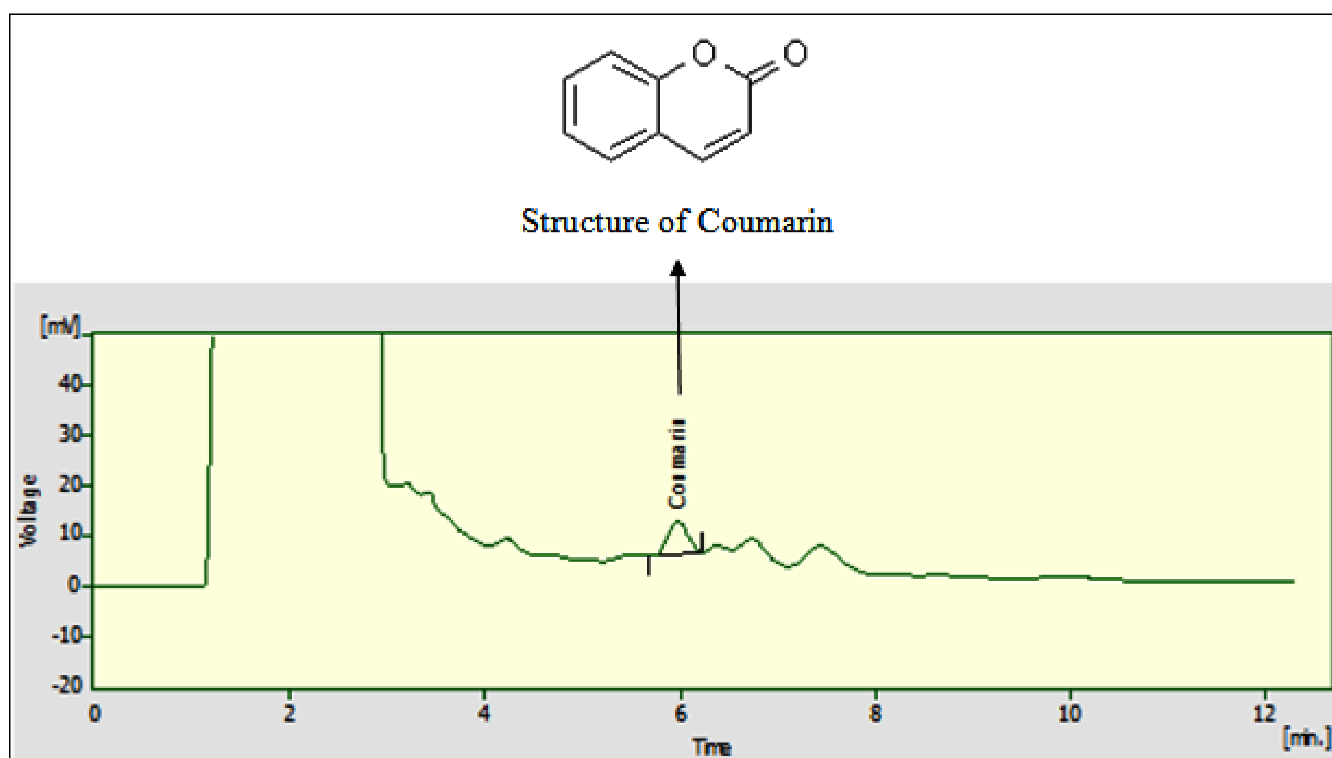

FIG. 2: HPLC FINGERPRINTING PROFILE OF HYDRO-ETHANOLIC EXTRACT OF *GANODERMA LUCIDUM* (HEGL)

TABLE 2: CONCENTRATION OF COUMARIN IN HYDRO-ETHANOLIC EXTRACT OF *GANODERMA LUCIDUM* (HEGL)

Peak Name	RT	Concentration
Coumarin	5.983	0.107 % w/w coumarin

Effect of hydro-ethanolic extract of Indian variety of *Ganoderma lucidum* on cell viability: In the same experiment, we also evaluated if the inhibitory effect on cytokine release was due to direct toxicity of THP1 cells by hydro-ethanolic extract of *Ganoderma lucidum*. The viability of THP1 cells were measured by MTT assay.

No significant change was observed in unstimulated, stimulated and HEGL treated cells which demonstrate that hydro-ethanolic extract of *Ganoderma lucidum* was not cytotoxic either with or without LPS (0.2 µg/ml) **(Fig. 3)**.

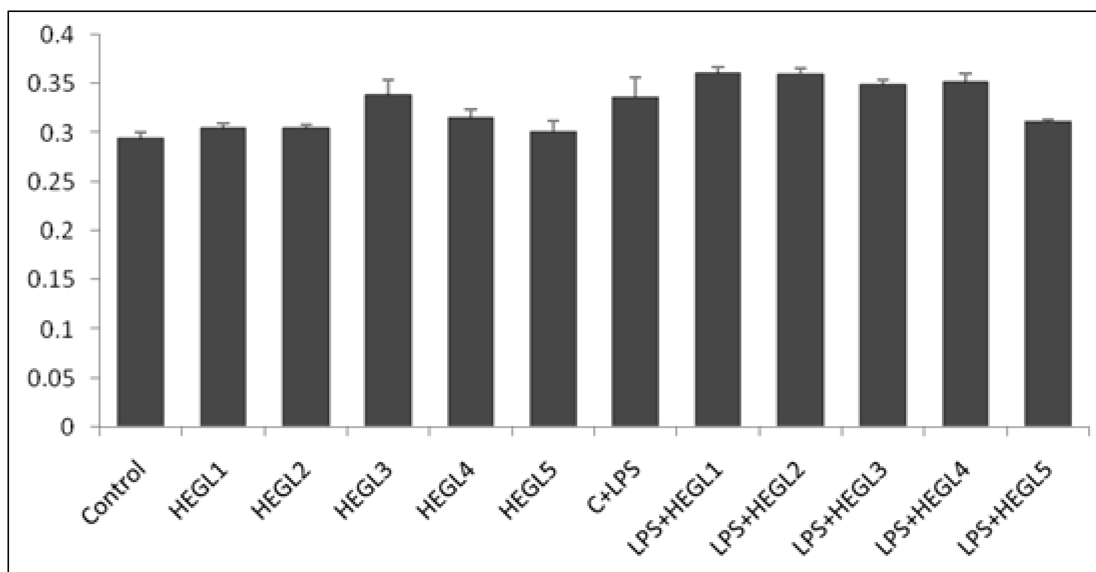

FIG. 3: MTT ASSAY IN THP1 CELLS AFTER 24 H TREATMENT WITH VARIOUS CONCENTRATIONS OF HYDRO-ETHANOLIC EXTRACT OF *GANODERMA LUCIDUM* (HEGL) WITH AND WITHOUT LPS (0.2µg/ml). CELL VIABILITY WAS DEFINED AS ABSORBANCE OF UNSTIMULATED, LPS-STIMULATED AND HYDRO-ETHANOLIC EXTRACT OF *GANODERMA LUCIDUM* (HEGL) TREATED THP1 CELLS. Values represent Means ± SEM of three independent experiments carried out in triplicates.

HEGL1: hydro-ethanolic extract of *Ganoderma lucidum* (25µg/ml); **HEGL2**: hydro-ethanolic extract of *Ganoderma lucidum* (50µg/ml); **HEGL3**: hydro-ethanolic extract of *Ganoderma lucidum* (100µg/ml); **HEGL4**: hydro-ethanolic extract of *Ganoderma lucidum* (250µg/ml); **HEGL5**: hydro-ethanolic extract of *Ganoderma lucidum* (500µg/ml); **C+LPS** - Control + Lipopolysaccharide (0.2µg/ml); **LPS+HEGL1**: Lipopolysaccharide (0.2µg/ml) + hydro-ethanolic extract of *Ganoderma lucidum* (25µg/ml); **LPS+HEGL2**: Lipopolysaccharide (0.2µg/ml) + hydro-ethanolic extract of *Ganoderma lucidum* (50µg/ml); **LPS+HEGL3** - Lipopolysaccharide (0.2µg/ml) + hydro-ethanolic extract of *Ganoderma lucidum* (100µg/ml); **LPS+HEGL4** - Lipopolysaccharide (0.2µg/ml) + hydro-ethanolic extract of *Ganoderma lucidum* (250µg/ml); LPS+HEGL5 - Lipopolysaccharide (0.2µg/ml) + hydro-ethanolic extract of *Ganoderma lucidum* (500µg/ml).

Effect of hydro-ethanolic extract of *Ganoderma lucidum* (HEGL) on TNF-α, IFN-γ and IL-1β release: In this study, human THP1 cell line was used to identify the actions of various immunomodulatory markers [20]. To study the anti-inflammatory effects of HEGL, proinflammatory cytokines, TNF-α, IFN-γ and IL-1β were measured in unstimulated and LPS-stimulated THP-1 cells.

The results related to cytokines release demonstrate that unstimulated THP-1 cells cultured for 24 h produced 6.62±.45 pg/ml of TNF-α, 14.90±0.32 pg/ml of IFN-γ and 25.45±0.16 pg/ml of IL-1β. However, treatment of cells with different doses of HEGL (25, 50, 100, 250 and 500 µg/ml) released TNF-α, IFN-γ and IL-1β in the insignificant amount **(Fig. 4, Fig. 5 and Fig. 6).**

Stimulation with LPS (0.2 µg/ml) for 24 h caused a substantial increase in the release of pro-inflammatory cytokines (102.87±14.42 pg/ml of TNF-α, 28.70±1.24 pg/ml of IFN-γ and 43.21±1.65 pg/ml of IL-1β) that is significant with unstimulated THP1 cells ($p<0.05$). In spite of this, when THP1 cells were treated with LPS (0.2 µg/ml) in the presence of HEGL a significant inhibition of cytokines release (TNF-α, IFN-γ and IL-1β) were observed.

The inhibition of TNF-α due to treatment with HEGL was 5.9%, 5.22%, 42.98%, 46.16% and 60.18% at the dose of 25, 50, 100, 250 and 500µg/ml. At the same time, 28.87%, 22.51%, 31.35%, 35.91% and 31.61% decrease in IL-1β was noted at the doses of 25, 50, 100, 250 and 500µg/ml of HEGL.

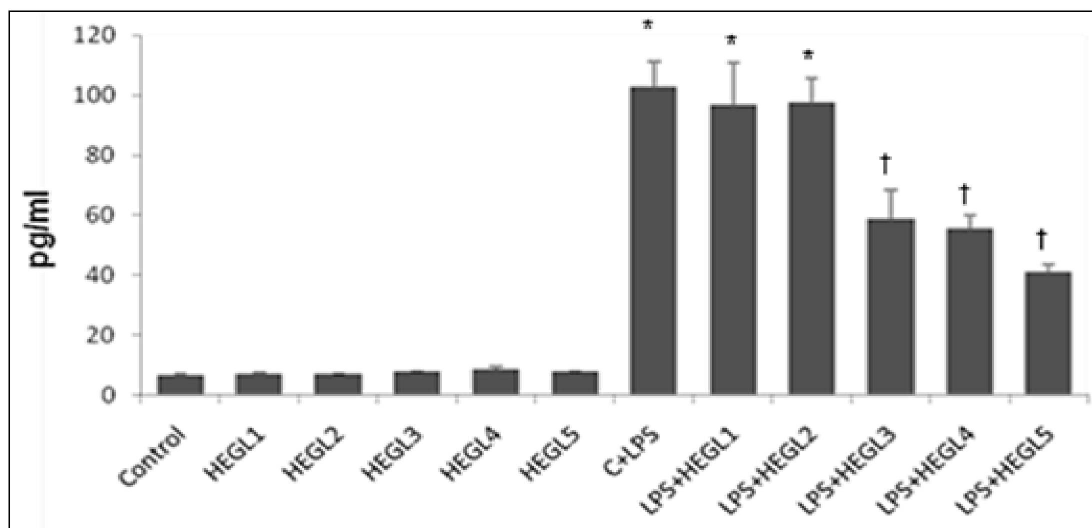

FIG. 4: EFFECTS OF HYDRO-ETHANOLIC EXTRACT OF *GANODERMA LUCIDUM* ON THE PRODUCTION OF TNF-A IN THP1 CELLS. Data represents Means ± SEM of three independent experiments carried out in triplicates. Statistically significant difference in cytokines release ($p<0.05$), as compare with the LPS-treated (*) and LPS+HEGL treated (†) groups, respectively.

HEGL1: hydro-ethanolic extract of *Ganoderma lucidum* (25µg/ml); **HEGL2**: hydro-ethanolic extract of *Ganoderma lucidum* (50µg/ml); **HEGL3**: hydro-ethanolic extract of *Ganoderma lucidum* (100µg/ml); **HEGL4**: hydro-ethanolic extract of *Ganoderma lucidum* (250µg/ml); HEGL5: hydro-ethanolic extract of *Ganoderma lucidum* (500µg/ml); **C+LPS** - Control + Lipopolysaccharide (0.2µg/ml); **LPS+HEGL1**: Lipopolysaccharide (0.2µg/ml) + hydro-ethanolic extract of *Ganoderma lucidum* (25µg/ml); **LPS+HEGL2**: Lipopolysaccharide (0.2µg/ml) + hydro-ethanolic extract of *Ganoderma lucidum* (50µg/ml); **LPS+HEGL3** - Lipopolysaccharide (0.2µg/ml) + hydro-ethanolic extract of *Ganoderma lucidum* (100µg/ml); **LPS+HEGL4** - Lipopolysaccharide (0.2µg/ml) + hydro-ethanolic extract of *Ganoderma lucidum* (250µg/ml); **LPS+HEGL5** - Lipopolysaccharide (0.2µg/ml) + hydro-ethanolic extract of *Ganoderma lucidum* (500µg/ml)

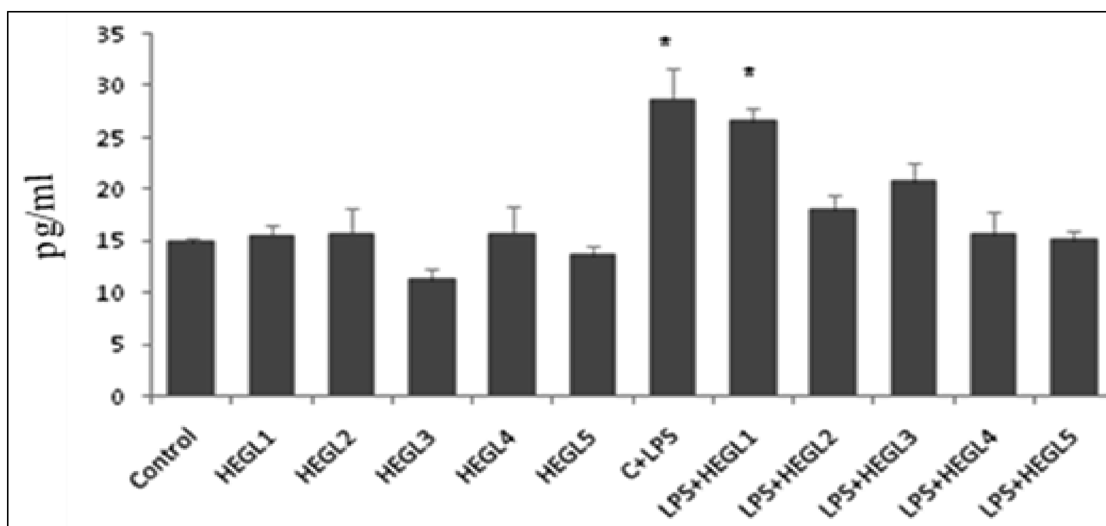

FIG. 5: EFFECTS OF HYDRO-ETHANOLIC EXTRACT OF *GANODERMA LUCIDUM* ON THE PRODUCTION OF IFN-γ in THP1 CELLS. Data represents Means ± SEM of three independent experiments carried out in triplicates. Statistically significant difference in cytokines release ($p<0.05$), as compare with the LPS-treated (*) group.

HEGL1: hydro-ethanolic extract of *Ganoderma lucidum* (25µg/ml); **HEGL2**: hydro-ethanolic extract of *Ganoderma lucidum* (50µg/ml); **HEGL3**: hydro-ethanolic extract of *Ganoderma lucidum* (100µg/ml); **HEGL4**: hydro-ethanolic extract of *Ganoderma lucidum* (250µg/ml); HEGL5: hydro-ethanolic extract of *Ganoderma lucidum* (500µg/ml); **C+LPS** - Control + Lipopolysaccharide (0.2µg/ml); **LPS+HEGL1**: Lipopolysaccharide (0.2µg/ml) + hydro-ethanolic extract of *Ganoderma lucidum* (25µg/ml); **LPS+HEGL2**: Lipopolysaccharide (0.2µg/ml) + hydro-ethanolic extract of *Ganoderma lucidum* (50µg/ml); **LPS+HEGL3** - Lipopolysaccharide (0.2µg/ml) + hydro-ethanolic extract of *Ganoderma lucidum* (100µg/ml); **LPS+HEGL4** - Lipopolysaccharide (0.2µg/ml) + hydro-ethanolic extract of *Ganoderma lucidum* (250µg/ml); **LPS+HEGL5** - Lipopolysaccharide (0.2µg/ml) + hydro-ethanolic extract of *Ganoderma lucidum* (500µg/ml)

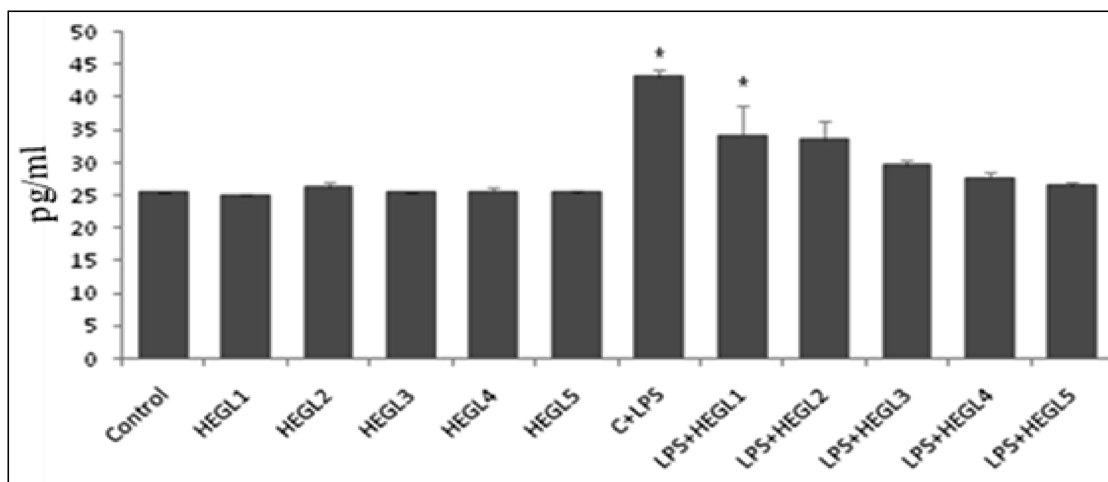

FIG. 6: EFFECTS OF HYDRO-ETHANOLIC EXTRACT OF *GANODERMA LUCIDUM* ON THE PRODUCTION OF IL-1β in THP1 CELLS. Data represents Means ± SEM of three independent experiments carried out in triplicates. Statistically significant difference in cytokines release ($p<0.05$), as compare with the LPS-treated (*) group.

HEGL1: hydro-ethanolic extract of *Ganoderma lucidum* (25µg/ml); **HEGL2**: hydro-ethanolic extract of *Ganoderma lucidum* (50µg/ml); HEGL3: hydro-ethanolic extract of *Ganoderma lucidum* (100µg/ml); **HEGL4**: hydro-ethanolic extract of *Ganoderma lucidum* (250µg/ml); **HEGL5**: hydro-ethanolic extract of *Ganoderma lucidum* (500µg/ml); **C+LPS** - Control + Lipopolysaccharide (0.2µg/ml); **LPS+HEGL1**: Lipopolysaccharide (0.2µg/ml) + hydro-ethanolic extract of *Ganoderma lucidum* (25µg/ml); **LPS+HEGL2**: Lipopolysaccharide (0.2µg/ml) + hydro-ethanolic extract of *Ganoderma lucidum* (50µg/ml); **LPS+HEGL3** - Lipopolysaccharide (0.2µg/ml) + hydro-ethanolic extract of *Ganoderma lucidum* (100µg/ml); **LPS+HEGL4** - Lipopolysaccharide (0.2µg/ml) + hydro-ethanolic extract of *Ganoderma lucidum* (250µg/ml); **LPS+HEGL5** - Lipopolysaccharide (0.2µg/ml) + hydro-ethanolic extract of *Ganoderma lucidum* (500µg/ml)

Inhibition of LPS induced Nitric oxide production from mouse peritoneal macrophages by hydro-ethanolic extract of *Ganoderma lucidum* (HEGL): Fig. 7 shows that unstimulated mouse macrophages cultured for 48 h produced 2.72±0.48 µM of nitrite, and stimulation with LPS (0.2µg/ml) for 48 h caused a significant increase in the level of nitrite to 49.05±3.98 µM. When mouse macrophages were stimulated with LPS (0.2µg/ml) together with different doses of HEGL (25, 50, 100, 250 and 500 µg/ml) for 48 h, the levels of nitrite was decreased to 44.82±1.79 µM, 36.92±1.98 µM, 30.89±3.30 µM, 27.72±1.27 µM and 16.25±1.10 µM respectively and these decrease in NO production was significant when compared with LPS-stimulated mouse macrophages. In addition, treatment of cells with different doses of HEGL alone induced NO production in the insignificant amount at the dose of 25, 50, 100, 250 and 100 µg/ml.

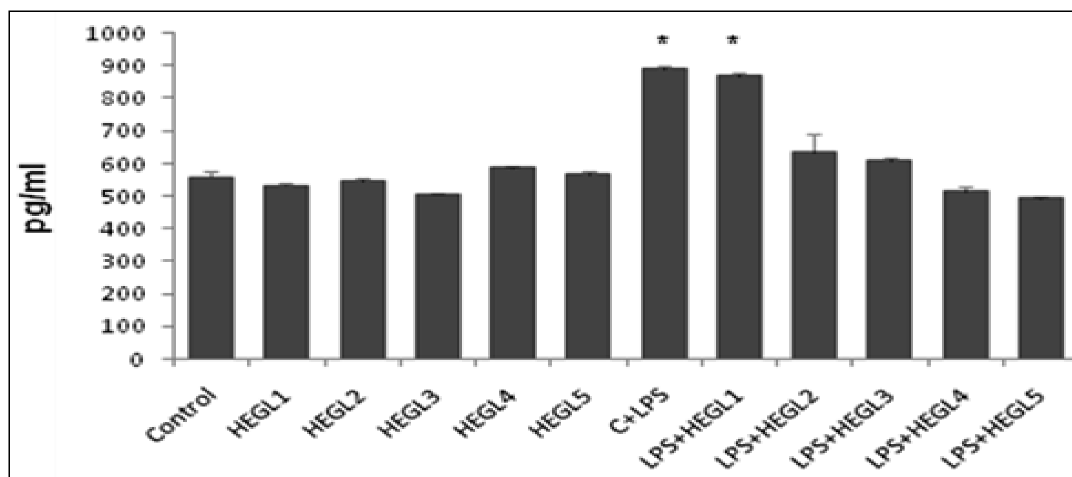

FIG. 7: EFFECTS OF HYDRO-ETHANOLIC EXTRACT OF *GANODERMA LUCIDUM* ON THE PRODUCTION OF NITRIC OXIDE (NO) IN MOUSE PERITONEAL MACROPHAGES. Data represents Means ± SEM of three independent experiments carried out in triplicates. Statistically significant difference in nitric oxide (NO) release ($p<0.05$), as compare with the LPS-treated (*) and LPS+HEGL treated (†) groups, respectively.

HEGL1: hydro-ethanolic extract of *Ganoderma lucidum* (25µg/ml); **HEGL2**: hydro-ethanolic extract of *Ganoderma lucidum* (50µg/ml); **HEGL3**: hydro-ethanolic extract of *Ganoderma lucidum* (100µg/ml); **HEGL4**: hydro-ethanolic extract of *Ganoderma lucidum* (250µg/ml); **HEGL5**: hydro-ethanolic extract of *Ganoderma lucidum* (500µg/ml); **C+LPS** - Control + Lipopolysaccharide (0.2µg/ml); **LPS+HEGL1**: Lipopolysaccharide (0.2µg/ml) + hydro-ethanolic extract of *Ganoderma lucidum* (25µg/ml); **LPS+HEGL2**: Lipopolysaccharide (0.2µg/ml) + hydro-ethanolic extract of *Ganoderma lucidum* (50µg/ml); **LPS+HEGL3** - Lipopolysaccharide (0.2µg/ml) + hydro-ethanolic extract of *Ganoderma lucidum* (100µg/ml); **LPS+HEGL4** - Lipopolysaccharide (0.2µg/ml) + hydro-ethanolic extract of *Ganoderma lucidum* (250µg/ml); **LPS+HEGL5** - Lipopolysaccharide (0.2µg/ml) + hydro-ethanolic extract of *Ganoderma lucidum* (500µg/ml).

Effect of hydro-ethanolic extract of *Ganoderma lucidum* (HEGL) on Nuclear Transcription factor (NF-kB): A significant increase in NF-kB P^{65} activity was observed when cells were treated with lipopolysaccharide alone. This increment was converted into a significant decrease ($p<0.05$) in NF-kB P^{65} activity if combined treatment of LPS and HEGL was given. While no significant change was observed in NF-kB activity when HEGL extract given alone **(Fig. 8).**

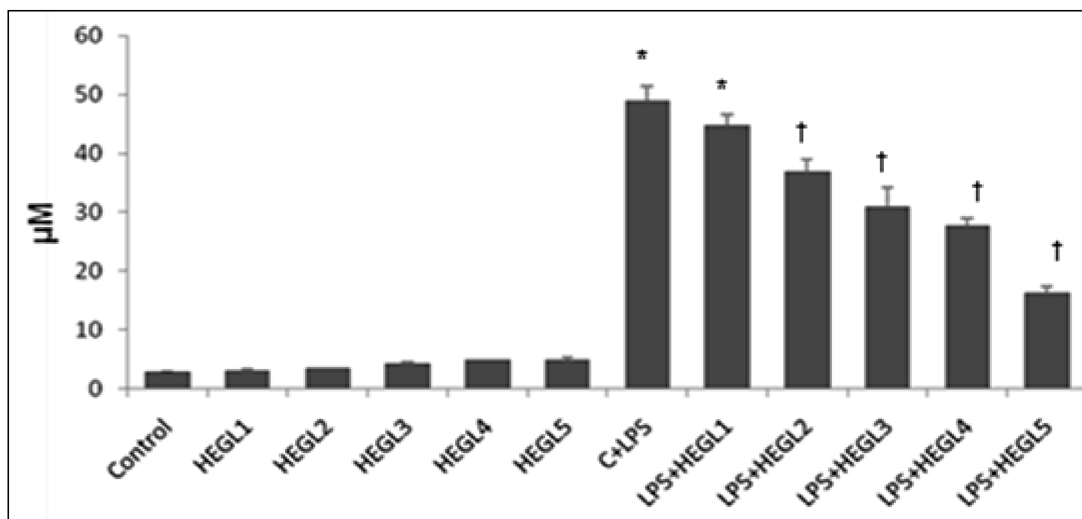

FIG. 8: EFFECTS OF HYDRO-ETHANOLIC EXTRACT OF *GANODERMA LUCIDUM* ON THE EXPRESSION OF NF-κB IN THP1 CELLS. Data represents Means ± SEM of three independent experiments carried out in triplicates. Statistically significant difference in NF-κB expression ($p<0.05$), as compare with the LPS-treated (*) group.

HEGL1: hydro-ethanolic extract of *Ganoderma lucidum* (25µg/ml); **HEGL2**: hydro-ethanolic extract of *Ganoderma lucidum* (50µg/ml); **HEGL3**: hydro-ethanolic extract of *Ganoderma lucidum* (100µg/ml); **HEGL4**: hydro-ethanolic extract of *Ganoderma lucidum* (250µg/ml); **HEGL5**: hydro-ethanolic extract of *Ganoderma lucidum* (500µg/ml); **C+LPS** - Control + Lipopolysaccharide (0.2µg/ml); **LPS+HEGL1**: Lipopolysaccharide (0.2µg/ml) + hydro-ethanolic extract of *Ganoderma lucidum* (25µg/ml); **LPS+HEGL2**: Lipopolysaccharide (0.2µg/ml) + hydro-ethanolic extract of *Ganoderma lucidum* (50µg/ml); **LPS+HEGL3** - Lipopolysaccharide (0.2µg/ml) + hydro-ethanolic extract of *Ganoderma lucidum* (100µg/ml); **LPS+HEGL4** - Lipopolysaccharide (0.2µg/ml) + hydro-ethanolic extract of *Ganoderma lucidum* (250µg/ml); **LPS+HEGL5** - Lipopolysaccharide (0.2µg/ml) + hydro-ethanolic extract of *Ganoderma lucidum* (500µg/ml)

DISCUSSION: Inflammation is the first response of the immune system to infection or irritation. It is caused by pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-1β and nitric oxide (NO). Thus, inhibitors of these cytokines could be considered as candidate of anti-inflammatory drugs. Monocytes/macrophages are key mediators of inflammation and widely distributed in the body [21]. Therefore, in the present study, inflammation and immunomodulaory activity of hydro-ethanolic extract of *Ganoderma lucidum* (HEGL), a fungi was investigated with special reference to inflammatory cytokines (TNF-α, IFN-γ and IL-1β), nitric oxide (NO) and NF-kB using monocytic cell line THP1. Beside this, antioxidant activity of extract also observed due to presence of higher amount of phenols, flavonoids and coumarin.

TNF-α and IL-1β are biologically active peptides produced by monocytes when induced by endotoxins and other stimuli [22]. In addition to cytokine cascade, other inflammatory mediators such as nitric oxide (NO) and NF-kB also play an important role during inflammation [23].

Nitric oxide (NO) synthesis by inducible nitric oxide synthetase (iNOS) is increased in inflammatory diseases and leads to cellular injury. The present study also demonstrated that the hydro-ethanolic extract of *Ganoderma lucidum* markedly decreases nitric oxide (NO) synthesis; this may support its anti-inflammatory activity.

Nuclear Factor-kB (NF-kB), a transcriptional factor controls the expression of genes involved in immune-responses, apoptosis and cell proliferation. NF-kB exists within the cytoplasm in an inactive form associated with regulatory proteins, called inhibitors of kB (I-kB). During stimulation by lipopolysacchrides (LPS), signal cascades result into phosphorylation of I-kB, which is then ubiquitinated, thereby releasing NF-kB dimmers from the cytoplasmic NF-kB-I-kB complex, and allowing them to translocate to the nucleus [24].

In the present study, we demonstrated that HEGL (doses 100, 250 and 500 µg/ml) decreased the production of TNF-α, IFN-γ, IL-1β, NO production and NF-kB expression in LPS-stimulated THP1 cells. The downregulation of these inflammatory mediators are not associated with cell cytotoxicity as no significant change was observed in MTT assay at any dose of HEGL on LPS stimulated and unstimulated THP1 cells. We also observed that HEGL at the dose of 100, 250 and 500 µg/ml may directly activate THP1 to reduce TNF-α, IFN-γ, IL-1β, NO and NF-kB expression. It is clearly seen that a platue was observed at the dose of 100 µg and on other higher doses of extract. This inhibition in LPS-stimulated THP1 cells indicates a possible beneficial effect of HEGL on immunity.

As part of phytochemical analysis, hydro-ethanolic extract of *Ganoderma lucidum* (HEGL) contains high phenolic and flavonoid compounds. It is well known fact that phenols and flavonoids are the major plant components for antioxidant activity. Phenols are involved with redox properties that play a crucial role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides [25]. Flavonoids form a class of benzo-γ-pyrone derivatives include flavones, flavanes, flavanols, anthocyanidines and catechines. These components are also engaged with antioxidant activity in the form of free-radical scavenging activity [26, 27].

The present investigation also showed that hydro-ethanolic extract of *Ganoderma lucidum* (HEGL) has ability to scavenging ABTS and DPPH radicals. This ability describes its proton-donating ability and might serve as free radical inhibitors or scavengers, activity possibly as primary antioxidant [28]. FRAP assay for hydro-ethanolic extract of *Ganoderma lucidum* (HEGL) also supported the DPPH and ABTS assay results which ultimately confirms the antioxidant potentials of *Ganoderma lucidum*.

Ganoderma lucidum contains 90% water and remaining 10% consists 26-28% carbohydrate, 3-5% crude fat, 59% crude fibre and 7-8% crude protein [29]. Beside these, *Ganoderma lucidum* also contains bioactive constituents such as terpenoids, steroids, phenols, glucoproteins and polysaccharides [30, 31].

HPLC fingerprinting profiling from our study also identified and quantified one of the novel compounds, coumarin in HEGL **(Fig. 2b).** Due to its biochemical properties coumarin were proposed for use in clinical medicine. It was evaluated for the treatment of various clinical conditions like high protein edema (HPE) [32], malaria [33], skin cancer treatment [34], Diabetes [35], liver diseases [36], blood coagulation and anticoagulant [37].

In addition to, Coumarin and their derivatives are highly effective against inflammatory response [38]. Therefore, the presence of coumarin could be responsible for antioxidant and anti-inflammatory property of the extract.

It is also believed that plants having more phenolic and flavonoid content show good antioxidant activity and there is direct correlation between total phenol content and antioxidant activity [39, 40, 41].

Our results of this study were also in agreement with the previous reports which confirm that the presence of terpenoids, steroids, phenols, glucoproteins, coumarin compound in extract, along with antioxidant activity of extract could be attributed to the anti-inflammatory activity of hydro-ethanolic extract of *Ganoderma lucidum* (HEGL).

CONCLUSION: Based on this study it can be concluded that hydro-ethanolic extract of *Ganoderma lucidum* (HEGL) suppressed LPS – induced release of TNF-α, IL-1β, IFN-γ and NO in human monocytic THP1 cells and this could be found beneficial against inflammatory diseases .

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