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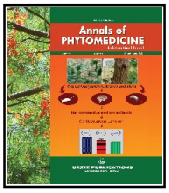

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Comparative investigation of Ashwagandha FMB extract and standardized extract for their antioxidant, anti-inflammatory and immunomodulatory potential

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Abstract

Essential trace elements (ETEs) are present in different forms, and the body needs them to perform cell functions at biological, chemical and molecular levels. These elements act as cofactors for many enzymes and centres for stabilising enzyme and protein structures as well as mediating critical biological events. The objective of the present study is to fuse ETEs into standardized extract of Ashwagandha (*Withania somnifera* (L.) Dunal) to develop novel herbal extract (ASH-FMB) by utilizing an advanced technology, *i.e.*, FMB [(fusion (F), micronization (M), bioligation (B)] and investigate their antioxidant, anti-inflammatory and immunomodulatory activities. ETEs and the bioactive compounds were also identified using atomic absorption spectrometry and HPLC, respectively. The elemental analysis confirmed that FMB technology significantly replenishes iron, zinc and calcium while phytochemical analysis confirmed the presence of withanoside-4 and withanolide-A. Results of the *in vitro* activities revealed that ASH-FMB remarkably scavenged the free radicals and also showed a significantly higher anti-inflammatory activity compared to standardized extract (ASH). In addition, *in vitro* immunomodulatory activity showed that ASH-FMB significantly stimulates the spleen cell proliferation as compared to ASH and similar results were found in pinocytic activation. Conclusively, the current study concludes that FMB techniques can provide higher therapeutic value to conventional standardized extracts, and thus prove to be a better alternative to them.

1. Introduction

Essential trace elements (ETE) are required for the human body to maintain various normal physiological processes. A healthy diet, which includes vital trace elements, antioxidants, vitamins, and minerals, is crucial for overall health, coping with oxidative stress in bodily tissues, and maintaining enough immunity against infections (Bhattacharya *et al.*, 2016). Consequently, its malfunctioning directly influences the metabolic and physiologic processes may result in the development of pathologic states and diseases (Wada, 2004). For millennia herbal medicines are of great importance throughout the globe as a key therapeutic regimen or adjuvant with modern medications and have blossomed into orderly-regulated systems of medicine (Babich *et al.*, 2020). Herbs are one of the bygone friends of human kind hence, in the recent past, a huge number of the population around the globe affectionately turned to herbal medicine (HM) because they trust plant-based remedies, have no side effects or lesser side effects as compared to synthetic medicines (Ibrahim *et al.*, 2021a). Moreover, a report published by WHO, about 75-85 per cent population of the world relies on HM for the management or treatment of disease, especially in primary healthcare setup (Ekor, 2014). It is surprising to notice that many curative effects of medicinal plants employed in traditional medicine are attributable to the presence of very low amounts of ETE. While phytoconstituents identified as markers or bioactive compounds are usually attributed to providing therapeutic effects, essential trace elements are equally important in plant-based formulations.

Ashwagandha (*Withania somnifera* (L.) Dunal) is a native plant to India and the Asian subcontinent. For millennia, it has been utilized as a remedy in traditional systems of medicine to cure numerous diseases and disorders in various parts of the world (Ravishankar and Shukla, 2007). It contains a huge number of biologically active molecules like withanoside-4 and withanolide-A as well as essential trace elements which are mainly responsible for its pharmacological potential such as cardioprotective, antidiabetic, antioxidant, anti-inflammatory, antispasmodic, antiperiodic, antiallergic, anticancer and many more (Girme *et al.*, 2020; Khare and Naharwar, 2020; Shirin *et al.*, 2010). Apart from bioactive compounds, it is observed that essential trace elements have immunomodulatory effects. It is also found that most of the trace elements have antioxidant and anti-inflammatory properties.

The immune system of every human being is accountable for the defence against microbes and pathogens, by making instant reactions through specific receptors (Chaplin, 2010). Generally, pathogenic microbes and infections frequently interact with the host, leads to disrupting homeostasis, increasing oxidative stress and inflammations and ultimately weakening the normal defence system of the body. Moreover, oxo-inflammation is a pathological condition

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characterized by the well-documented systemic and chronic oxidative stress associated with mild-chronic inflammation (Valacchi *et al*., 2018). The occurrence of a long-term sustained oxo-inflammation contributes to generating a permanent loss of the body's immunity and ultimately damages various tissues and organs (Chaplin, 2010). An immunomodulator having properties to maintain normal physiology either by increasing immunity and antioxidant potential or decreasing oxo-inflammation status in the body by destroying oxidizing agents that harm the immunity is the need of the hour.

The current method of manufacturing herbal extracts involving the use of water or other solvents focus on extracting the phytoconstituents while leaving out the ETEs. This leads to the lower pharmacological and therapeutic potential of the herb. In the present study, we have fused ETEs into standardized extract of Ashwagandha (*W. somnifera*) to achieve better therapeutic potency of the herb. The process is called FMB (fusion (F), micronization (M) and bioligation (B). This technique is very easy and it can be utilized to develop ready-to-use extract/formulation in terms of phytochemical constituents and ETEs and achieve higher pharmacological potentials like antioxidant, anti-inflammatory and immunomodulatory activity as compared to the standardized extract alone. This study was carried out to comparatively evaluate FMB extracts of Ashwagandha vs. conventional extracts on these parameters.

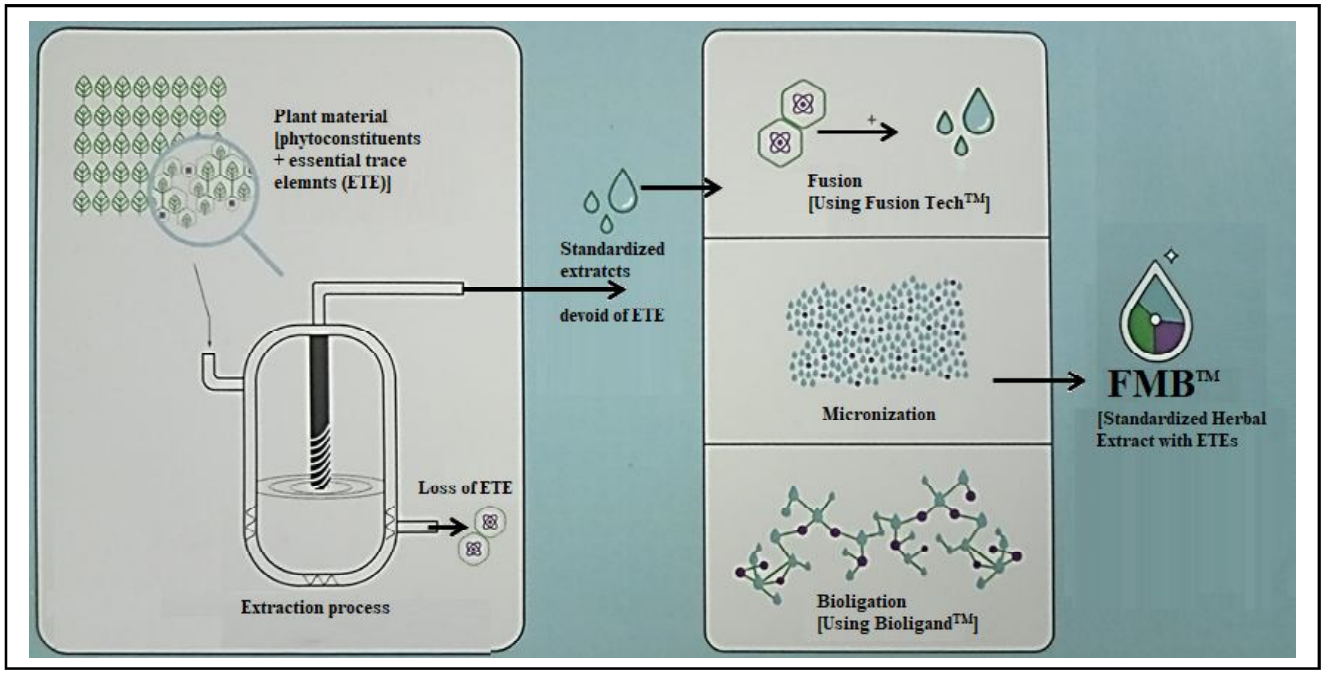


Figure 1: Typical flow process of FMB techniques to develop the novel herbal extract.

2. Materials and Methods

2.1 Chemicals

Analytical grade chemicals and reagents were employed throughout the experiment. Amplicon Biotech, Delhi, India, provided commercial reagent kits for the assessment of biochemical parameters and enzymatic tests.

2.2 Preparation of standardized extract

The standardised root extract of Ashwagandhab (ASH) was made using a hydroalcoholic medium according to the defined methodology (Ahmad *et al*., 2019).

2.3 Development of novel herbal extract (ASH-FMB) by utilizing FMB technology

Step 1: Separation of ETEs (iron, zinc and calcium, *etc*.) from Ashwagandha raw material (roots).

Step 2: Replenish the ETEs into standardized extract to develop novel herbal extractas follows:

1. **Fusion:** Involves replenishment of standardized herbal extract with the ETE lost during the process of extraction.
2. **Micro-fixation:** Micro-fixation of standardized extract and ETEs is done through an incremental and continuous process of micronization.
3. **Bioligation:** Both fusion and micro-fixation help to form natural ligands (bioligands) complex between ETEs, and bioactive compounds present in the standardized extract.

2.4 Determination of ETE

The operating conditions for determining ETE in the samples were carried out in accordance with the protocol (Dileep *et al*., 2013). In a microwave digester, 0.5 g of each sample was digested in 5 ml of ultrapure metal-free nitric acid. After that, the contents were diluted to 12.5 ml with double distilled water, and ETEs were calculated using an inductively coupled plasma and an optical emission spectroscope (ICP-OES). To quantify ETEs such as iron, zinc, and calcium, the digested sample was aspirated into ICP-OES. The experiment was done three times and the results were provided as mean and standard deviation.

2.5 Identification of bioactive markers using HPLC

Bioactive markers (standard) in ashwagandha root extract was analysed by HPLC. Instrumentation detailed is mentioned in the table mentioned below. Briefly, all the standards and test samples were filtered through 0.22 µm (millipore) nylon membrane filters. Prior to the use of solvents, it was ultrasonically degassed and filtered throughthe above filters. The wavelength (λmax) was set to 227 nm for the standards.

Separation of standard and compounds in the test samples was achieved on Phenomenix Luna 5µ, C18 column (4.6 mm × 250 mm particle size). The mobile phase consisted of acetonitrile: buffer (35:65) (v/v) in a gradient elution with a flow rate of 1.5 ml/min 20 µl of the standard and test samples was injected into the HPLC system. The column temperature was kept 25 ± 2°C and the duration time was set to 40 min. Finally, the detection of standards in the test sample was confirmed by comparing it to the standards retention time.

Table 1: Optimized HPLC conditions for identification of bioactive markers

Parameters	Values	
Mobile phase	Solvent A (0.5% formic acid in water) and solvent B (acetonitrile)	
Stationary phase	Phenomenix Luna C18 column	
Wavelength	227 nm	
Column temperature:	25 ± 2°C.	
Solvent flow rate	1.5 ml/min	
Injection volume	20 µl	
Mode of Operation	Gradient elution	
Run time	40 min	
Gradient	Time (min)	Gradient ratio (Solvent A: Solvent B)
	Initially	10: 90
	00-05	20: 80
	05-12	30: 70
	12-20	40: 60
	20-25	60: 40
	25-30	70: 30
	30-35	60: 40
	35-40	90: 10

2.5.1 Preparation of standard

Before analysis, standard solutions of withanoside-4 and withanolide-A were made by dissolving 1.0 mg accurately weighed and dissolved in UPLC grade methanol (0.1 mg/ml) and passing over a 0.22 m (millipore) nylon membrane. The prepared solutions were further used as per the requirement.

2.5.2 Preparation of test sample

Ashwagandha extract (10 mg) was weighed and mixed in one ml UPLC grade ethanol (10 mg/ml) to prepare the working solution. Further, the prepared solution was filtered through 0.22 µm (millipore) nylon membrane filters to remove any particles before analysis.

2.6 DPPH radical scavenging assay

For the presence of DPPH as a free radical, the DPPH radical scavenging activities of different concentrations of ASH-FMB and ASH (25-250 µg/ml) were measured using a spectrophotometric technique. Briefly, different concentrations of tested samples ranging from 25-250 µg/ml of FMB and ASH were mixed with DPPH solution and incubated in a dark place at room temperature for 30 min. Finally, absorbance was recorded at 517 nm. All the experiments were performed in triplicate (Ibrahim *et al.*, 2021b).

2.7 Cell culture and treatments

$HepG_2$ cells were grown in sterile Dulbecco's modified eagle medium (DMEM), supplemented with 10% foetal bovine serum (FBS) and antibiotic solution. Cells were plated for 24 h before being subjected to various treatments. The $HepG_2$ cells were treated with ASH-FMB and ASH in a serum-free medium. Throughout the investigation, all groups were grown in a CO_2 incubator (Thermo Scientific, USA) with 5% CO_2 at 37°C in DMEM.

2.7.1 Cytotoxicity effect of ASH-FMB and ASH on $HepG_2$ cells

$HepG_2$ cells were seeded into 96-well plates in different groups. Cytotoxic assays with ASH-FMB and ASH were explored separately as per described protocol (Ibrahim *et al.*, 2021a). Different concentrations (65-1000 µg/ml) were poured in 96-well plates containing $HepG_2$ cells and 200 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added. Then plates were incubated for 48 h in a 5% CO_2 incubator, respectively. After incubation, dimethyl sulfoxide (DMSO) was added to each well and gently swirled to mix and left for 45 sec. The development of purple colour at 595 nm, measured using a UV spectro-photometer against DMSO as a blank, indicated the existence of live cells. Each sample was tested three times.

2.7.2 Assessment of anti-inflammatory activity

Cells were divided into different groups as per designed protocol and seeded as 1×10^5 cells/well/2 ml in a 12-well microtitration plate (37°C, 5% CO_2, 48 h). Cells were grown in glucose-rich media (50 mM glucose) as a negative control, 50 mM glucose (high glucose) plus test samples (ASH-FMB and ASH, respectively), and normal control for 48 h, respectively. Further, supernatants and cell lysates of the treated cells were collected after 48 h. Supernatants (2 ml conditioned media/1×10^5cells/well) were pooled and collected into individual prelabelled centrifuge tubes concerning each treatment. They were centrifuged at 2000 rpm for 20 min and the supernatant was collected leaving the pellet undisturbed. Following this, the cells in the wells were washed twice with PBS and were trypsinized with Trypsin-EDTA solution (200 µl/well). Cell suspension of each treatment pooled and collected into separate prelabelled centrifuge tubes. It was centrifuged at 1500 rpm for 10 min to obtain a cell pellet. Pellet was suspended in ice-cold cell lysis buffer (25 µl/ $1x10^5$ cells/well) and centrifuged at 13,000 rpm for 5 min. The cell pellet was discarded and the supernatant was collected having a

final concentration of $1x10^5$ cells/25 µl of the buffer. Collected cell supernatants and lysates were stored at –80°C until the further expression of TNF-α level using the respective ELISA kit (Aggarwal *et al*., 2018).

2.8 Immunomodulatory activity

Swiss albino mice (6 weeks old, 25 ± 5 g) were procured from Jamia Hamdard (Animal Approval Number 1551). All of the animals were housed in a polypropylene cage and transported to the experimental room, where they were given a week to acclimate before the test. Throughout the experiment, an air conditioning unit (with 10% air exchange perhrs) was kept in the animal home facility, coupled with a relative humidity of 50-10 RH, a 12/12 h light-dark cycle, and a temperature of 25 ± 2°C.

2.8.1 Splenocyte proliferation assay

Six to eight weeks old healthy albino mice were euthanized, and the spleen was aseptically removed for isolation of splenocyte. The spleen was further homogenised by passing it through a mesh (0.4 microns) and centrifuged at 300 g for 5 min in sterile phosphate-buffered saline (PBS). By adding 500 l of lysis buffer to the cell pellet, the RBCs were lysed (Tris-HCl-NH4Cl, pH 7.2). The reaction was halted by adding an RPMI medium (RPMI-1640, millipore Sigma, India), which was then rinsed twice to remove any debris. The cell pellet was suspended in RPMI medium to get 3.0×10^6 cells/ml, and 100 µl of cell suspension was seeded in a 96 well plate per well, 25 µl of mitogen Con A (2.0 µg/ml) and 25 µl of the ASH-FMB and ASH, respectively (65-1000 µg/ml) was added to the cells per well-containing splenocyte and incubated at 37ºC in a CO_2 incubator for 72 h. After incubation, 20 µl of tetrazolium dye MTT 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide and incubated at 37ºC, 5% CO_2 for another 6 h. After 6 h of incubation, tetrazolium crystals generated in liver cells were dissolved, and cell proliferation was assessed using a microplate reader at 490 nm (Shi and Fu, 2011).

2.8.2 Pinocytic activity assay

Three mice were given one ml of thioglycolate (1.0 mg/ml in PBS) intraperitoneally, and peritoneal macrophages were extracted 48 h later by injecting PBS into the peritoneal cavity. The separated macrophages were washed twice with media and suspended in RPMI medium containing 10% FBS at 1.0×10^6 cells per ml. To allow macrophages to stick to the plate, 200 µl of cells were put onto 96 well plates and cultured overnight at 37°C, 5% CO_2. The non-adherent cells were gently rinsed with RPMI medium before each well was filled with 100 µl of fresh RPMI medium. Additionally, 25 µl of ASH-FMB and ASH (65-1000 g/ml) were applied to the cells in each well and cultured for 48 h at 5% CO_2 and 37ºC. After incubation, 100 µl of neutral red solution (0.1 per cent in 10 mM PBS) was added, and after 2 h of incubation, the cells were gently washed with PBS to eliminate any remaining neutral red solution. The macrophages were further lysed by incubating at room temperature overnight with 100 µl of neutral red detainer (ethanol and 0.1 per cent acetic acid in a 1:1, v/v) and measuring optical density at 540 nm the next day (Shi and Fu, 2011). In the absence of test samples, RPMI medium was employed as a negative control, and the results were expressed as a comparison to the negative control.

2.9 Statistical analysis

All of the data is presented using the mean and standard deviation. ANOVA was used to analyse the data using a statistical programme (Graph Pad Prism version 5). $p<0.05$ was used to establish a statistically significant difference between mean values.

3. Results

3.1 Elemental analysis

Results presented in Table 2 showed the levels of ETEs (iron, zinc and calcium) concentration in ASH-FMB and ASH, respectively. From the result, it is ascertained that ETEs were successfully replenished into standardized extract, *i.e.*, ASH-FMB.

Table 2: The content of ETEs analyzed by ICP-OES (n = 3)

Essential trace elements (ETE)	ASH-FMB (Units in ppm)	ASH (Units in ppm)
Iron	195.93 ± 6.01	12.00 ± 0.61
Zinc	26.89 ± 2.14	18.69 ± 0.97
Calcium	30600.00 ± 60.49	139.66 ± 2.04

3.2 Identification of withanoside-4 and withanolide-A using HPLC

HPLC chromatographic analysis established the presence of with Anoside-4 and with anolide-A in the extract. The RTs of the reference standard (withanoside-4; 15.383 min and withanolide-A; 22.297 min) were matched with the test sample to confirm the presence of withanoside-4 and withanolide-A (Figure 2).

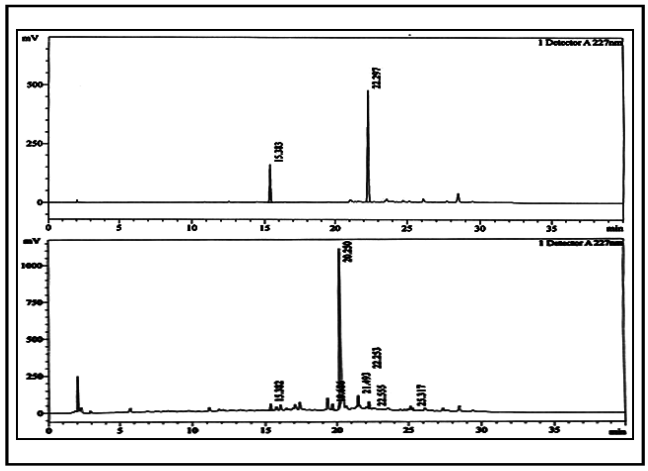


Figure 2: HPLC chromatograms of standards and ashwagandha extract.

3.3 DPPH radical scavenging assay

The DPPH test is commonly used to assess the antioxidant activity of individual chemicals and plant extracts and many more. DPPH, a purple colour solution, reacts with antioxidant compounds present in the test samples (ASH-FMB and ASH) and is reduced to yield a light yellow colour. Results of the present study revealed, ASH-FMB showed significant inhibitory potential in graded dose-response on DPPH free radicle at a tested concentration as compared to ASH (Table 3).

Table 3: DPPH scavenging activity

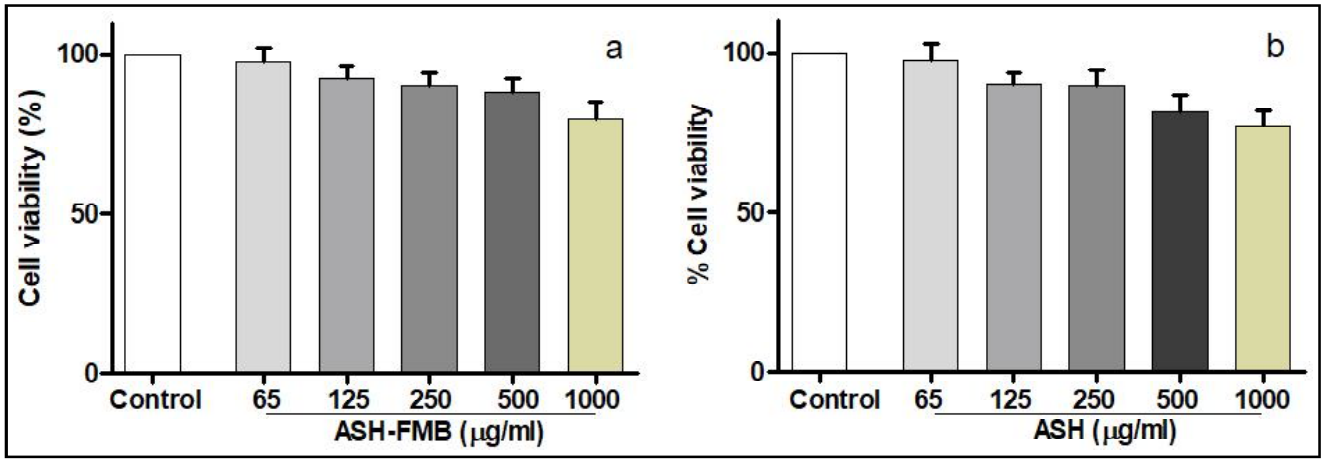
Concentration (µg/ml)	Percentage inhibition with IC_{50} value		
	ASHFMB	ASH	Ascorbic acid
25	22.86	18.72	24.18
50	31.60	26.43	36.49
100	42.70	30.77	59.15
150	63.32	55.89	74.98
250	81.96	68.70	94.59
IC_{50} value	120.68	157.99	105.75

3.4 Cytotoxicity assay in $HepG_2$ cells

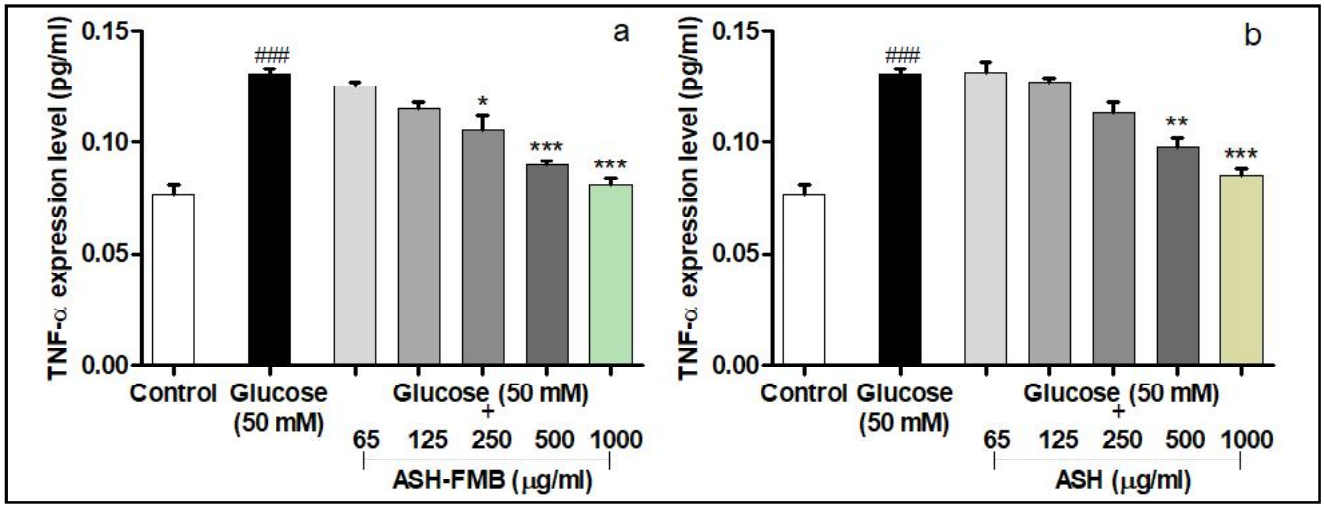
In order to assess, the nontoxic concentrations of ASH-FMB and ASH alone, we carried out MTT based cytotoxicity assay in $HepG_2$ cells for 48 h. Both ASH-FMB and ASH did not cause any significant toxicity to the $HepG_2$ cells upto 1000 µg/ml, and cell viability was maintained about 70 per cent throughout the study (Figures 3a and b).

3.5 Effect of the herbal extracts (ASH-FMB and ASH) on the expression of TNF-α

The level of TNF-α was assessed in experimental groups against high glucose-induced stress. After treatment with the extracts, the level of TNF-α was diminished. Both ASH-FMB and ASH showed the anti-inflammatory response in a dose-dependent manner. Interestingly, ASH-FMB showed better activity as compared to ASH alone (Figures 4a and b).



Figures 3a and b: The effects of ASH-FMB and ASH on the viability of $HepG_2$ cells.



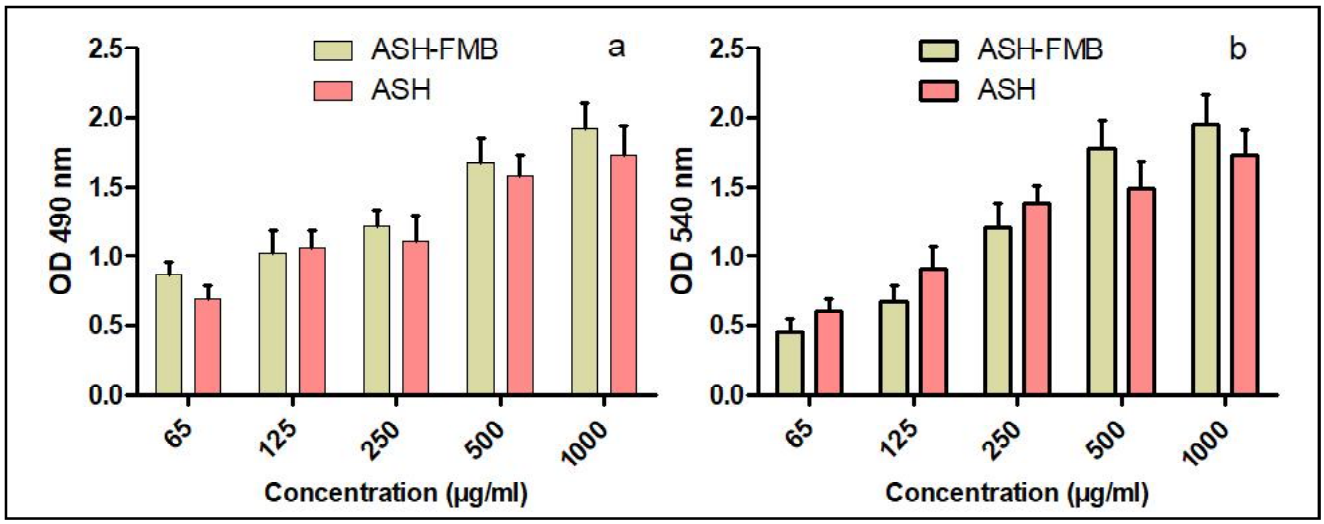
Figures 4 a and b: The effects of ASH-FMB and ASH on tumour necrosis factor-α (TNF- α).

3.6 Immunomodulatory activity of herbal extracts (ASH-FMB and ASH)

3.6.1 Splenocyte and pinocytic assay

It was observed that when splenocytes were treated with the extracts, they proliferated more than cells that were not. Interestingly, at every concentration, the splenocyte proliferation was more pronounced in ASH-FMB as compared to ASH. The test samples were evaluated at five different doses (65-1000 g/ml) in a splenocyte proliferation experiment, and it was discovered that there was a dose-dependent increase in cell proliferation (Figure 5a). Herbal extracts (ASH-FMB and ASH, respectively) treatment

enhanced the pinocytic rate in a dose-dependent manner (Figure 5b). The figure clearly shows that initially pronounced activity was observed in ASH while at higher concentration impressive results were obtained in ASH-FBM. We cultured mouse peritoneal macrophages in RPMI medium with or without external stimuli and measured pinocytic activity using neutral red, which is readily taken up by macrophages, after 48 h of incubation. Macrophages were lysed after being incubated with neutral red to evaluate the concentration of neutral red and the level of pinocytic activity by the macrophages.



Figures 5a and b: Splenocyte proliferation assay and pinocytic activity of the ASH-FMB and ASH, respectively.

4. Discussion

Growing evidence reveals that consumption of ETEs and bioactive compounds exert better immunity and their collective intake is more beneficial as compared to the solitary application of each (Monnerat *et al*., 2021). Moreover, withanoside-4 and withanolide-A are the most common bioactive compounds present in ashwagandha. Analysis of these bioactive compounds assures quality control of extract and its pharmacological potential.

Both experimental and clinical studies suggest that oxidative stress plays a major role in the pathogenesis of various diseases. The diet, especially natural products or food supplements, contains a vast number of bioactive compounds with antioxidant activity, which may have cumulative/synergistic antioxidant effects (Mancini *et al*., 2018). Concurrently, samples with reducing capacity suggest that ASH-FMB is a rich source of bioactive metabolites including trace elements (Wolonciej *et al*., 2016). Apart from scavenging the free radicals, it has been reported that the bioactive compounds and trace elements in the plants powerfully protect the generation of free radicles in the living organism and also have an additive effect on the endogenous scavenging compounds (Gaurav *et al*., 2020; Zhang *et al*., 2021).

Previous studies reported that concurrent administration of ETE including iron and zinc also reduces oxidative damage and increase antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase (Bodiga and Krishnapillai, 2007). Interestingly, it is reported that calcium enhances the activities of antioxidant enzymes and reduces lipid peroxidation of cell membranes (Jiang and Huang, 2001). The results obtained from *in vitro* antioxidant assay clearly indicated that ASH-FMB possesses strong antioxidant potential as compared to ASH alone because of trace elements that synergize the action of bioactive metabolites present in the extract.

Kong *et al*. (2016) described that cytotoxicity or cytoprotective effect of plant extracts is largely reliant on their quantity, bioavailability, and complicated interactions among phytochemicals (Kong *et al*., 2016). However, current results strongly suggested that both ASH-FMB and ASH maintain cell growth and viability.

Among various inflammatory cytokines, TNF-α contributes its decisive role inducing tissue-specific inflammation which may weaken the defence system of the body. Several anti-inflammatory chemicals have been discovered to improve immune reactivity underlying numerous pathogeneses by directly modulating immune system production/function/activation and reducing tissue damage (Bhavya and Haridas, 2017). Previous literature revealed that both withaferin and withanolides have excellent anti-inflammatory and immunomodulatory effects and they play a key role in modulating cancer, diabetes and arthritis, *etc.* (Kanjilal *et al*., 2021). Many experimental studies reported that bioactive constituents of ashwagandha attenuate the inflammatory markers like TNF-α, IL-1β, IL-6 and also suppresses neuroinflammation to keep the body healthy (Gupta and Kaur, 2016).

Moreover, it is reported that ETEs increase the anti-inflammatory cytokine contents in the body's inflammatory response, playing an important role in stabilizing the immune defence system and also having good antioxidant potential (Manangi *et al.*, 2015). Zinc deficiency has been shown to enhance oxidative stress and inflammatory TNF-α, which has been partially reversed by zinc supplementation in rat models (Biaggio *et al.*, 2010). Similarly, iron is another essential element that is required in many physiological processes in the body including erythropoiesis, immune function and host defence, as well as essential cellular activities such as DNA replication and repair, and, enzymatic reactions which require iron as a cofactor. On the other hand, malfunctions in iron, homeostasis result in anaemia or inflammation that eventually leads to chronic disease (Cronin *et al*., 2019). In addition, it is reported that

supplementation of calcium with vitamin D reduces inflammation and bacterial dysbiosis (Zeng *et al*., 2021).

The spleen is the largest immunological organ in the body, and it may produce a high number of lymphocytes. The proliferation of splenocytes is an important step in the activation of both cellular and humoral immune responses (Zhang *et al*., 2020). Earlier studies reported that ashwagandha significantly enhances T cell and B cells (Tharakan *et al*., 2021). Fast T cell proliferation in response to antigen stimulation is governed by T cell differentiation into effector cells in a balanced immune system because T cells manage the immune response and are responsible for cell-mediated immunity (Luckheeram *et al*., 2012). A report published by Loder and his team clearly demonstrated that B cells are found exclusively in the spleen and play a key role in boosting immunity (Loder *et al*., 1999). Macrophages are the first line of defence against the antigen, and pinocytic activity is one of the distinguished methods to determine macrophage activation (Wang *et al*., 2018). The increase in neutral red concentration in cells treated with external stimuli relative to untreated cells was used to measure the enhancement of pinocytic activity by macrophages.

Zinc is an important trace element that aids antiviral immunity, lowers the risk of viral infection, and has anti-inflammatory properties (Read *et al*., 2019). It is also noticeable that calcium plays a pivotal role in the activation of cells of the immune system (Grinstein and Klip, 1989). Importantly, iron and immunity are closely linked. Iron aids pathogen development and virulence, while it is also essential for immune cell activation and multiplication. Overall, ETEs are found in soil, plants, and living organisms in very small quantities but they act as enzyme catalysts in the systems to boost immunity, reduce inflammation and oxidative stress. Moreover, ETEs synergizes the mobility and solubility of the bioactive compounds, improving the pharmacokinetic profile and the cellular uptake (WHO, 1996).

5. Conclusion

It is concluded that ETEs are equally essential as bioactive compounds in herbal ingredients. Moreover, in the present study, we have successfully replenished the ETEs into standardized extract to develop novel herbal extract by utilizing the FMB technology. Our biological screening justify that such techniques are beneficial for the herbal pharmaceutical industry with possible higher therapeutic potential for better efficacy and results.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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