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Exploration of Active Metabolites from Aegle Marmelos and Emblica Officinalis for Determining its Immunosuppressive Properties

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1. Abstract

As per the literature, bioactive compounds isolated or purified from medicinal plant products that are generally used for curing various human diseases. The present study involves two different medicinal plants i.e. Aegle marmelosand Emblica officinalis were locally available in Baramati region of Maharashtra, India. The leaves of these medicinal plants were used for phytochemical analysis in aqueous extract using phosphate buffered saline (PBS; pH 7.2) in order to find out the active metabolites using liquid chromatography mass spectrometry (LC-MS). For these studies, aqueous leaves extract of these two medicinally plants were tested for immunological studies in mice model studies especially Swiss mice pertaining to determine splenocyte proliferation assay using hepatitis B vaccine containing surface antigen (HBsAg) and also determined pro-inflammatory cytokines (IFN-gamma and TNF alpha) by Elisa. The results showed that aqueous leaves extract showed declined in splenocyte proliferation assay and proinflammatory cytokines (FN-gamma and TNF alpha) at higher doses as compared to control samples. HBsAg (20 µg/ml) used as standard for these studies. Overall, the results showed that aqueous leaves extract of Aegle marmelosand Emblica officinalis showed immunosuppressive activity against HBsAg as compared to control.

Keywords: Aegle Marmelos; Emblica Officinalis; Proinflammatory; Hepatitis B Vaccine.

2. Introduction

India has a rich history of using various medicinal plant products for treating various diseases. Most of the medicinal plants are investigated or reported for its beneficial use against different types of diseases and reported as well as published in numerous scientific journals [1, 2]. In other words, use of medicinal plant products as a source of medicine against human diseases and showed its importance in human health care system in India [3]. Currently, most of the medicinal plants are recommended for various diseases having no side effects. As per Indian traditional system, these medicinal plants are continuously used for therapy related to cardiovascular disease and inflammation [4, 5].Several medications that are available to suppress over-reactive T lymphocytes, but many of the currently marketed drugs produce severe and life-threatening side-effects [6]. The most commonly used immunosuppressive drugs especially Cyclophosphamide, betamethasone etc. and historically one of the oldest are the glucocorticoids [6, 7]. These immunosuppressive drugs are generally restricted and inhibited the synthesis and secretion of inflammatory cytokines and support the activation of anti-inflammatory signalling cascades. In addition, these immunosuppressive drugs showed various side effects including toxic effect as well [6, 7]. However, many of immunosuppressive drugs cause unwanted and severe side-effects in patients and therefore there is a high demand for medicinal plant products that showed less-toxic immunosuppressive pharmaceuticals [8, 9].

One of the medicinal plants i.e. Aegle marmelos (Bael; family Rutaceae) and Emblica officinalis (Amla; family Euphorbiaceae) showed various immunopharmacological properties i.e. anti-inflammatory, anti-oxidant, antidiabetic, antimicrobial, proteases etc. [10-12].In view of this, we focused on various medicinal plant products especially aqueous leaves extract of Aegle marmelos and Emblica officinalis in order to determine its immunosuppressive effect against HBsAg.

3. Methodology

3.1. Plants Material

Fresh plant leaves of Aegle marmelos and Emblica officinalis were collected in January 2016 from Baramati region, Maharashtra, India. These medicinal plants were identified by Late Dr Sharadini Dahanukar (founder of Nakshatra udyan, Vidya Pratishthan's) for mentioning the importance and medicinal use of medicinal plant especiallyAegle marmelos and Emblica officinalis in Marathi book language (Nakshatra vriksha).

3.2. Preparation of Aqueous Extracts

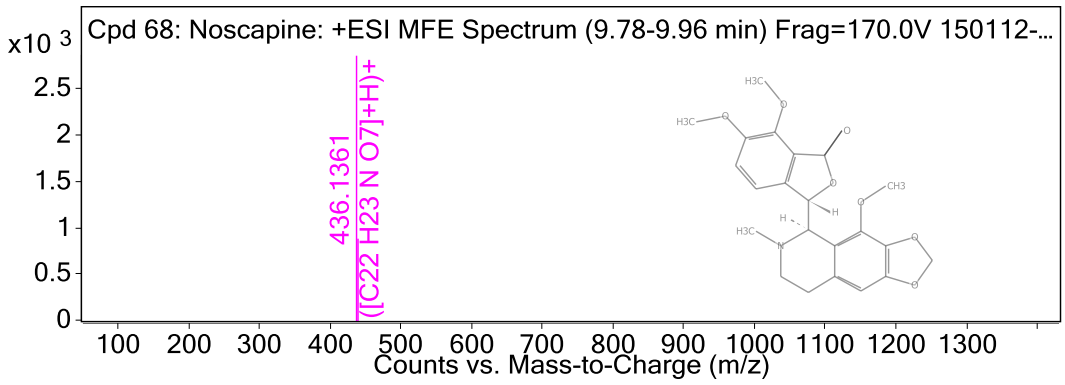
Five grams (5 g) of powdered plant material of Aegle marmelos and Emblica officinalis were extracted or macerated with phosphate buffered saline (PBS, 50 mL) using mortar and pestle.

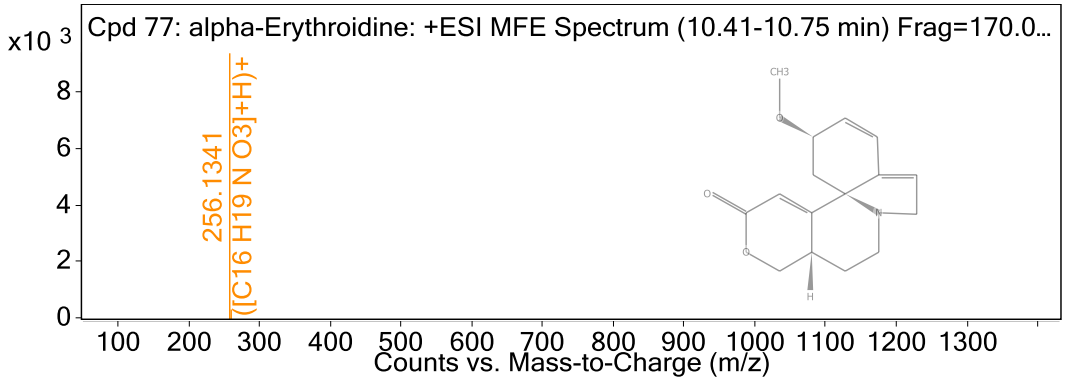
During filtration, extract residues were removed and collect the supernatant for the estimation of secondary metabolites in the aqueous leaves extract.

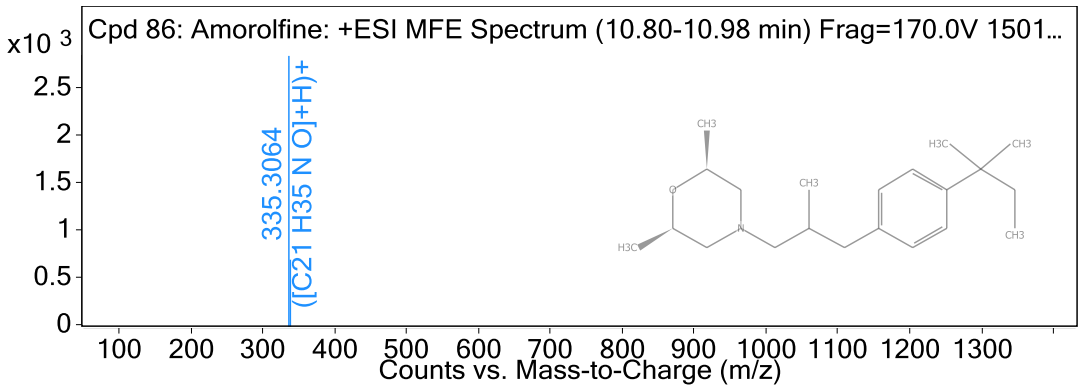
3.3. LC-MS Analysis

Aqueous extract of Aegle marmelos and Emblica officinalis (**Fig.1**) were subjected to LC-MS (LC: Agilent 1260 binary LC System; Column: Agilent Zorbax SB 18 RRHT column, 100×2.1 mm, 1.8µm); Flow rate: 0.3mL/min; Run time: 30 min; Injection Vol: 1µL and MS: 6540 ultra-high definition accurate mass QTOF LC/MS system; Mobile phase A: Water, 0.1% Formic acid; Mobile phase B: Acetonitrile) analysis and compounds were distinguished based on their mass spectra, using precursorand fragment ions as well as comparison of the fragmentation patterns with molecules described in the molecular database. All mass spectrometric (MS) acquisitions were performed in the positive electrospray ionization modewhereas capillary, cone and fragment or voltage were recorded at different set points i.e. 4 kV, 45V and 170V, respectively. The gas temperature was set at 325 ºC. Data was acquired at scan rate of 3Hz in mass range 100-100 m/z. Further data was analysed with Mass hunter qualitative software and METLIN database.

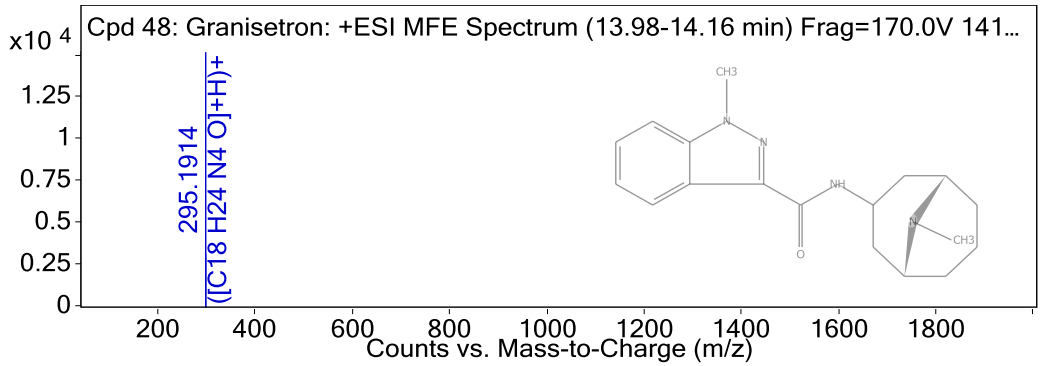
A) Aegle marmelos

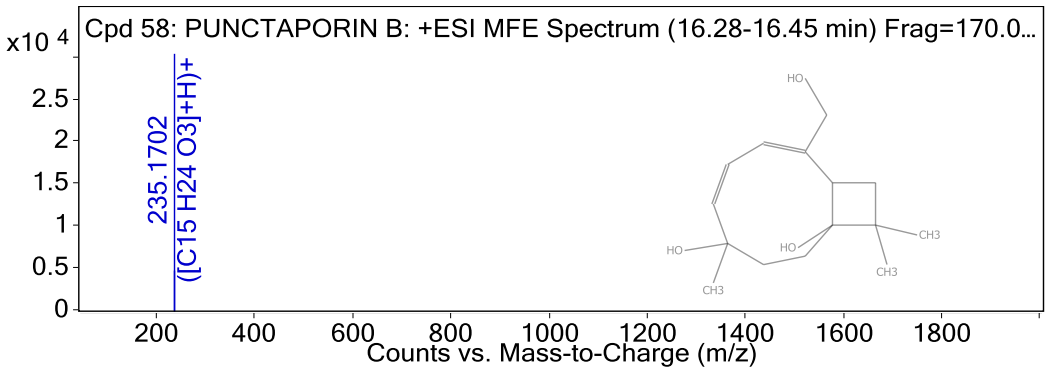


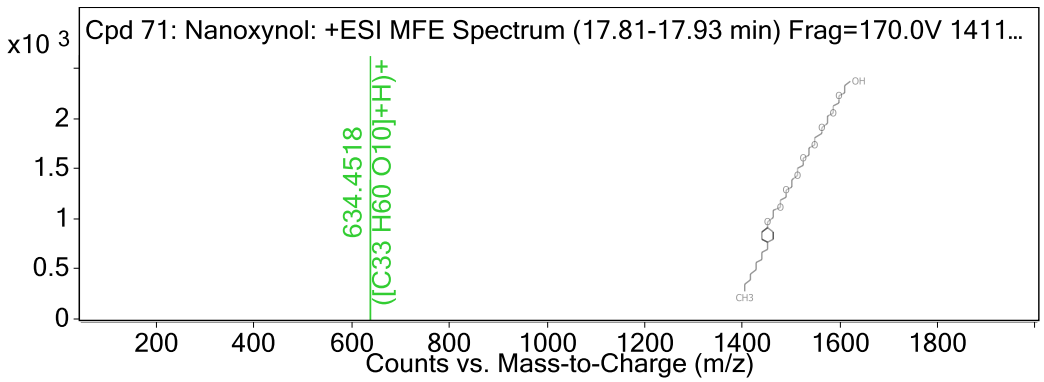




B) Emblica officinalis







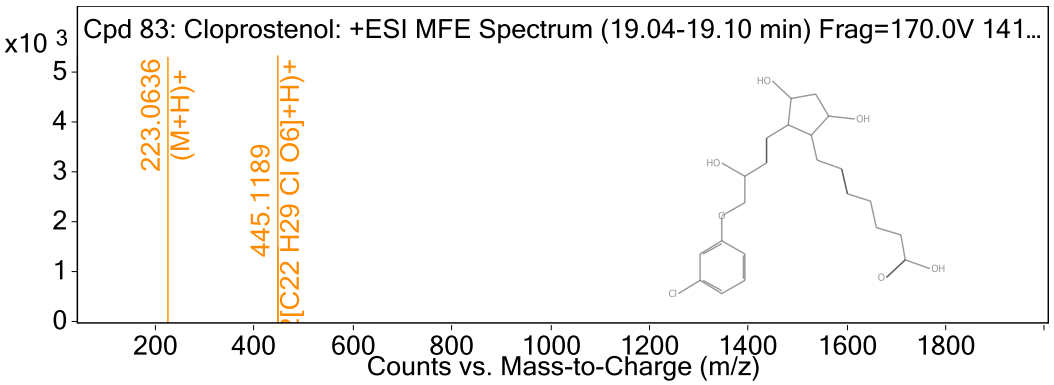


Fig.1. MS spectra for representative bioactive phytochemicals identified in Aegle marmelos and Emblica officinalis.

3.4. Animals Handling

Swiss mice (22-26 g; female) were used for this study. All animals were kept in preclinical research centre, VSBT Baramati under controlled conditions of 12-h light/and 12 h without light and 25°C. Experiments on these animals especially Swiss mice were performed according to the protocols already approved by Animal ethics committee and met the international standards for animal study.

3.5. Splenocyte Proliferation Assay

Splenocytes were collected by careful dissection from the abdominal cavity of mouse and then prepared single cell suspension(using froster slides) and then passed through cell strainer (70 µm) pertaining to remove cell debris.

For these studies, spleen cells were cultured at 2×10^6 cells/ml, 100 µl in RPMI 1640 medium containing 10% heat-inactivated Fetal calf serum (FCS) along with penicillin (100 IU/ml) and streptomycin (100 µg/ml) in 96 well tissue culture plate. Spleen cells were cultured with HBsAg (2 µg/ml) along with serial dilution of aqueous leaves extract of Aegle marmelos and Emblica officinalis (62.5 – 500 µg, 50 µl) for 48 h incubation. Afterwards, splenocyte culture supernatant was collected for the estimation of Th1 cytokines and were stored at −70°C until analysis of cytokine concentrations by ELISA. Afterwards, add fresh medium in 96 well plate and then add MTT (2.5 mg/ml; 10 µl) solution. Incubate the plate for another 4 h at carbon dioxide incubator. After incubation, centrifuge the plate and observed formazon crystals settled at the bottom and then discard the supernatant. Dissolve the formazon crystals in dimethyl sulphoxide (DMSO) solution and then analyzed its optical density (OD) at 570 nm [13, 14].

3.6. Estimation of Th1 Cytokines by ELISA

Th1 (IFN-gamma and TNF alpha) cytokine concentrations in the splenocyte cell culture supernatant were determined by ELISA (BD optia kit). This study was performed according to the manufacturer's instructions [13, 14].

3.7. Statistical Analysis

The difference between controls, standard and aqueous leaves extract of Aegle marmelos and Emblica officinalis is determined by one way ANOVA test (Boniferroni multiple comparison test).

4. Results

4.1. Splenocyte Proliferation Assay

The effect of aqueous leaves extract of Aegle marmelos and Emblica officinalis on splenocyte proliferation assay containing HBsAg as shown in **Fig.2.** The results showed that aqueous leaves extract showed reduction in HBsAg proliferation at higher doses as compared to control.

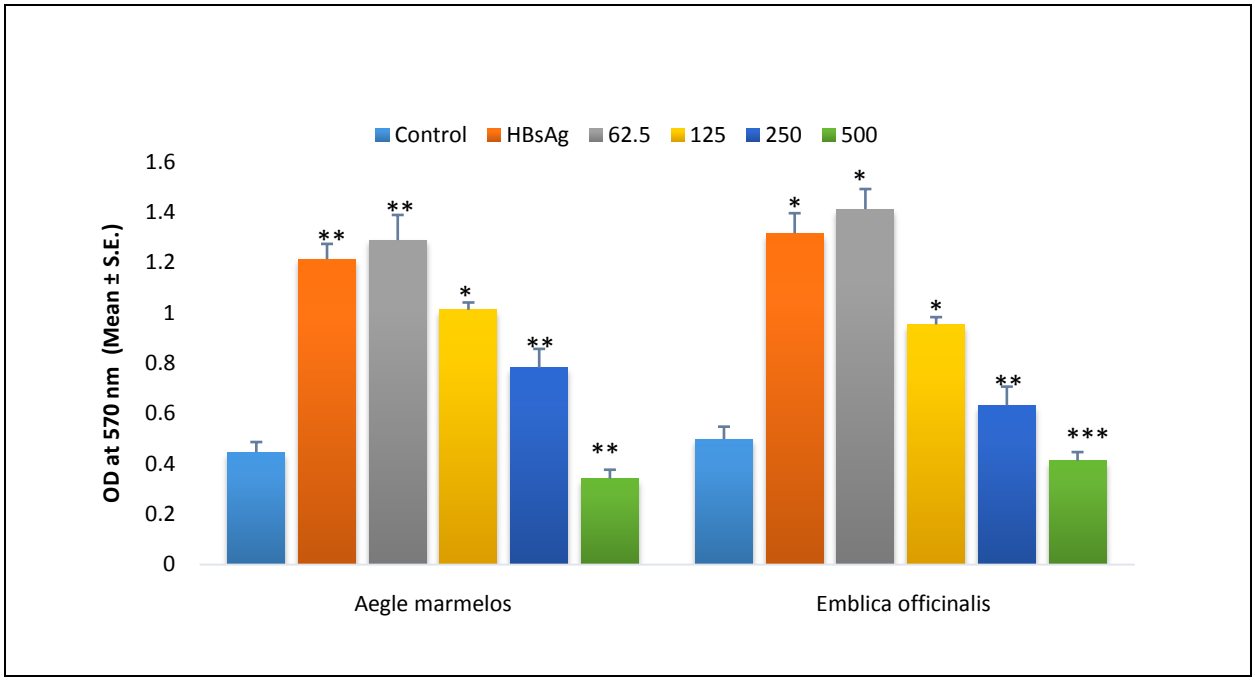
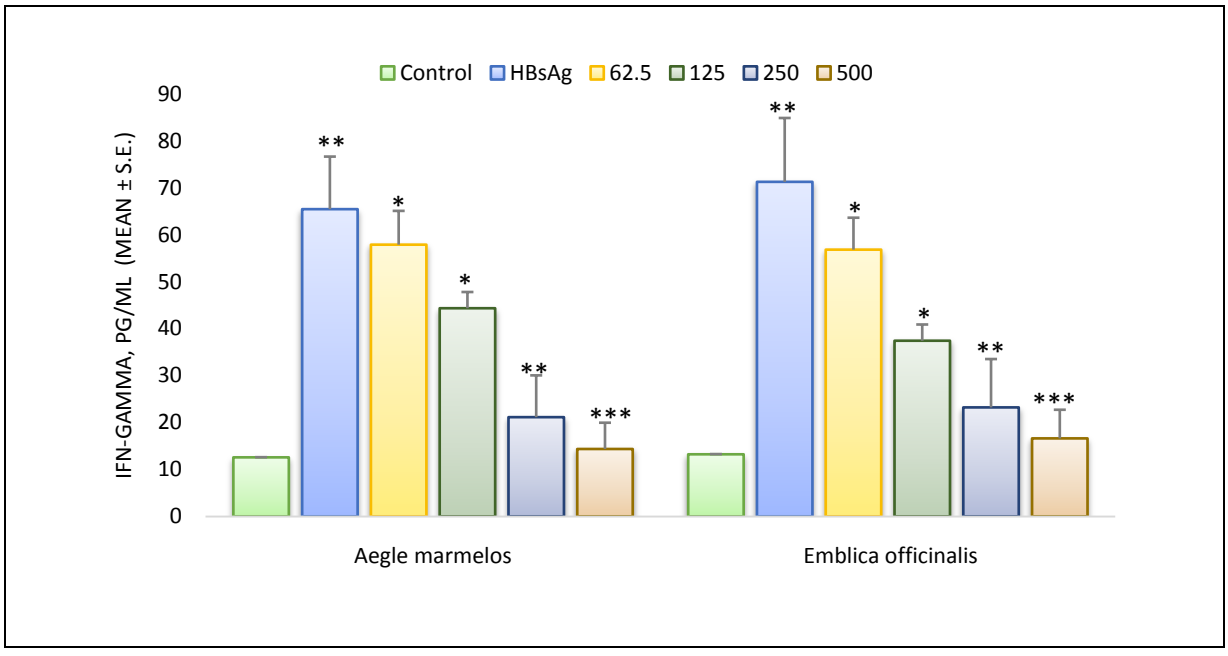


Fig.2. Effect of variable doses of aqueous leaves extract on splenocyte proliferation assay in mice model studies. Spleen cells (10^6 cells/ml; 100 µl) were treated with variable doses of aqueous leaves extract of Aegle marmelos and Emblica officinalis (62.5 – 500 µg, 50 µl) in presence HBsAg (as already described in materials and methods section). Values are expressed in Mean ± S.E. The difference between the control, standard and aqueous leaves extract is determined by one way ANOVA test. *P<0.05, **P <0.01 and ***P< 0.001

4.2. Th1 Cytokines

The effect of aqueous leaves extract of Aegle marmelos and Emblica officinalis on Th1 (IFN-gamma and TNF alpha) cytokines from splenocyte cell culture supernatant containing HBsAg as shown in **Fig.3**. The results showed that aqueous leaves extract showed inhibition in Th1 cytokines at higher doses as compared to control.



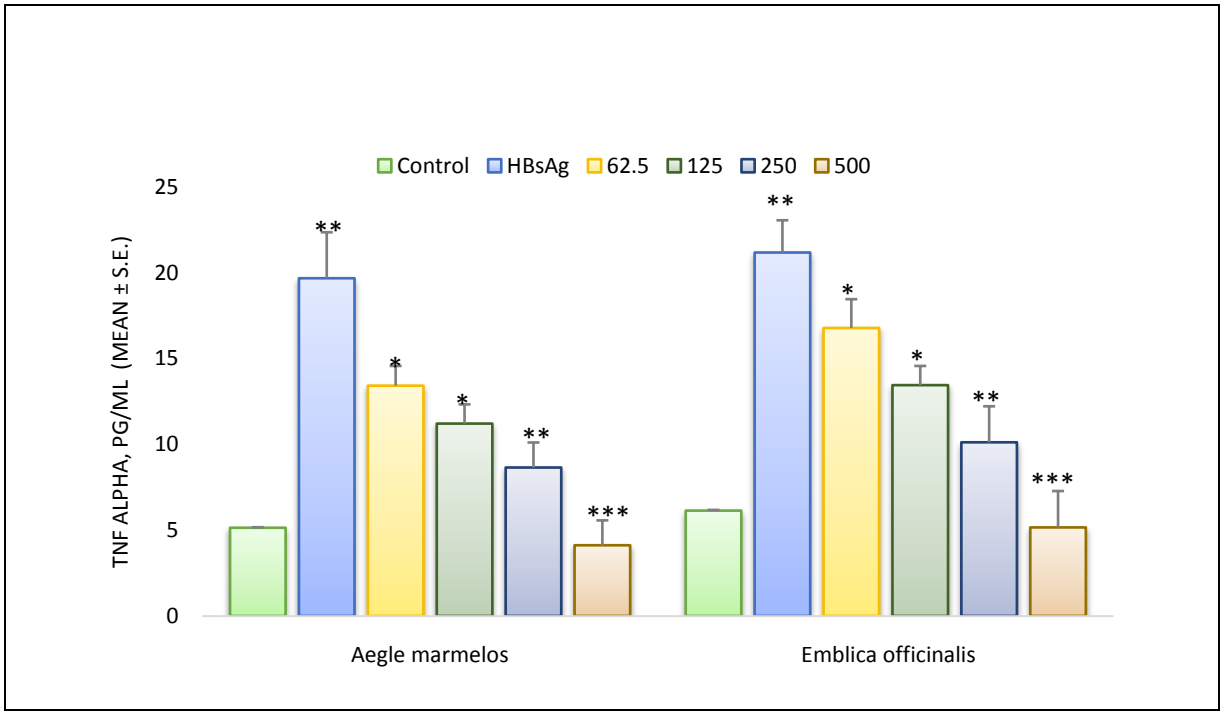


Fig.3. Effect of variable doses of aqueous leaves extract on Th1 cytokines from splenocyte cell culture supernatant. Spleen cells (10^6 cells/ml; 100 µl) were treated with variable doses of aqueous leaves extract in presence HBsAg and cell culture supernatant was collected for the estimation of cytokines (IFN-gamma and TNF alpha). Values are expressed in Mean ± S.E. The difference between the control, standard and aqueous leaves extract is determined by one way ANOVA test. *P<0.05, **P <0.01 and ***P< 0.001

5. Discussion

Medicinal plants especially Aegle marmelos and Emblica officinalis has been shown to possess many important medicinal benefits including anti-inflammatory, anti-oxidant, anti-bacterial, and anti-fungal activities [10-12]. Plant metabolites in the form of aqueous leaves extract that mediate these immunological effects and their mechanism of action are poorly understood. In order to achieve this objective, we focused on active metabolites that are present in aqueous leaves extract that are responsible for immunosuppressive activity. During phytochemical investigation using LC-MS, number of phytoconstitutents are identified (based on the mass to charge ratio ofrespective phytoconstituent) inAegle marmelosand showed various bioactive molecules such as Noscapin at retention time (rt) 9.78-9.96 min and $[M+H]^+$ ion at m/z 436. Noscapin is an Opioid alkaloid, used as an antitussive agent and a tubulin-binding antitumor agent. Embelin is well known anti-tumor agent having wound healing property and anti-proliferative activities [15-18]. Embelin having rt 10.34-10.67 min and shows prominent peak of $[M+H]^+$ ion at m/z 277. LC-MS could identify α-Erythroidine at rt 10.41-10.75 min showing $[M+H]^+$ ion at m/z 256. Amorolfine is showing activity against fungal nail infections, eluted at rt 10.80-10.98 min, $[M+H]^+$ ion at m/z 335.

Similarly, active phyto-molecules were identified from the aqueous extract of Emblica officinalis are Granisetron, a 5-hydroxytryptamine 3 (5-HT(3))-receptor antagonist indicated for the prevention of nausea and/or vomiting[19] shows rt 13.98-14.16 min $[M+H]^+$ ion at m/z 295. Punctaporin B with rt 16.28-16-45 min and $[M+H]^+$ ion at m/z 235, which is reported for antitumor activity [19]. Nanoxynol is widely used in contraceptives for its spermicidal properties. It was eluted at rt 17.81-17.93 min with $[M+H]^+$ ion at m/z 634. Cloprostenol used as a luteolytic agent which causes functional and morphological regression of the corpus luteum followed by return to oestrus and normal ovulation in cattle. It may also be used for the induction of parturition in pregnant cows, sows and mares showing rt 19.04-19.10 min and $[M+H]^+$ ion at m/z 223.

In India, numerous varieties of medicinal plants weregrown because of climatic and geographical variations. More than 1000 medicinal plant species were recorded and showed various immunopharmacological properties. As per the literature, different species of medicinal plants such as Azadirachta indica, Ficus racemosa, Syzygiumcumini, Ficus benghalensisetc. [20-23] have been shown to have appreciable anti-inflammatory and immunosuppressive activity. Modulation of immune cell responseusing medicinal plant products may lead to discovery of new drugs for human against various infectious diseases. In this study, we aimed to evaluate splenocyte proliferation assay using HBsAg and determined Th1 cytokines from cell culture supernatant. The results showed that aqueous leaves extract showed

markedly decline in HBsAg proliferation and Th1 (IFN-gamma and TNF alpha) cytokines at higher doses as compared to control. Overall, the data showed that these medicinal plants especially Aegle marmelos and Emblica officinalis showed immunosuppressive effect.

Cytokines played an important role in the pathogenesis of various immunological disorders especially Th1 type of cytokines i.e. IFN-gamma and TNF alpha, has been the subject of intense investigation in the past few years [13,14]. Recently, animal models provide the most advanced tool available for analysing the relationship between cytokines and immunological disorders. In this study, we determined as well as evaluated the expression of proinflammatory cytokines (IFN-gamma and TNF alpha) against HBsAg and somewhat unexpectedly, these models have also revealed that cytokine factors can act both as potentiating and inhibitory agents, depending upon the site and timing of exposure. Cytokinesi.e. IFN-gamma and TNF alpha are well known to play an important roles for eliminating various viral infections or intracellular pathogens [13, 14]. In this study, we found significant decrease in IFN-gamma and TNF alpha productionin splenocyte cell culture supernatant and also suppressed HBsAg-mediated proliferation at higher doses in splenocytes. Overall, this data indicated that proinflammatory mediators serve as a biomarker for immunosuppressive activity against specific protein antigen. These observations indicated that aqueous leaves extract of these two medicinal plants have potential effects in downregulating the immune system and might be developed as a better and useful immunosuppressive agent in treating undesired immune responses.

6. Conclusion

The results of aqueous leaves extract of these two medicinal plants clearly indicates its immunosuppressive effectagainst HBsAgi.e. decline in splenocyte proliferation and Th1 (IFN-gamma and TNF alpha) cytokines production. Overall studies showed that immunosuppressive activity of Aegle marmelos and Emblica officinalis could be because of bioactive phytoconstituents present in aqueous extract which were identified through LC-MS.

Authors' Contributions

This work was carried out in collaboration between Dr Gupta, Dr Ajam Shaikh and Dr Chaphalkar. All the authors designed the study, wrote the protocol and interpreted the data. Dr Gupta anchored the field study, gathered the initial data and performed preliminary data analysis whereas Dr Ajam Shaikh for LC-MS analysis. All the authors managed the literature searches and produced the initial draft. All authors read and approved the final manuscript.

Conflict of Interests

Authors have declared that no conflict of interest exists.

Ethical Considerations

These studies should be conducted under ethical guidelines with registration no. 1814/PO/ERE/S/15/CPCSEA

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