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Immunoadjuvant potential of *Azadirachta indica, Butea frondosa* and *Ficus religiosa* against Swine flu vaccine antigen

Shubham S Gaikwad[1], Amit Gupta[1,2*], Bharat Shinde[1,2]

[1]Department of Biotechnology, Vidya Pratishthan's, Arts, Science and Commerce College, Baramati, Maharashtra, India
[2]Department of Zoology, Vidya Pratishthan's, Arts, Science and Commerce College, Baramati, Maharashtra, India

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ABSTRACT

Introduction: In general, primary or secondary metabolites derived from medicinal plant products might be responsible for stimulating or suppressing the immune system against specific protein antigens. The objective of this study was to evaluate the adjuvant potential of aqueous leaves extract of *Azadirachta indica, Butea frondosa* and *Ficus religiosa* against Swine flu vaccine antigen.

Methods: In this study, our group evaluated the antibody (IgG) titre of Swine flu vaccine antigen (2 µg/mL) using variable doses (0.625–5 mg) of aqueous leaves extract of *A. indica, B. frondosa* and *F. religiosa*. In addition, Swiss mice were immunized subcutaneously (100 µL) on day 0 with Swine flu vaccine antigen (1:1000 dilution). Splenocytes were collected on day 7 and cultured with variable doses of aqueous leaves extract of *A. indica, B. frondosa* and *F. religiosa* pertaining to determine the total cellular content and splenocyte proliferation (Swine flu vaccine; Ovalbumin, OVA and Con A) assay. In addition, estimation of Th1 (IFN-gamma and TNF alpha) cytokines in cell culture supernatant containing swine flu vaccine antigen along with aqueous leaves extract were measured.

Results: Aqueous leaves extract of *A. indica, B. frondosa* and *F. religiosa* showed anti-Swine flu titre at higher doses. In *ex vivo* animal model studies these three medicinal plants in the form of aqueous leaves extract enhanced total cellular content at higher doses but increased in splenocyte proliferation (Swine flu vaccine, OVA and Con A) assay at lower doses. Similarly, there was enhancement in Th1 cytokines (IFN-gamma, TNF alpha) with respect to swine flu vaccine antigen containing aqueous extract at lower doses as compared to control group.

Conclusion: Aqueous leaves extract of *A. indica, B. frondosa* and *F. religiosa* showed adjuvant activity against Swine flu vaccine antigen and might be used in manufacturing active adjuvant for vaccine antigen.

Implication for health policy/practice/research/medical education:
Aqueous leaves extracts of *Azadirachta indica, Butea frondosa* and *Ficus religiosa* are capable of enhancing both antibody and cell mediated immune responses against Swine flu vaccine antigen in mice and might be used in manufacturing active adjuvant for vaccine antigen.

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Introduction

Swine influenza (swine flu) is considered to be a respiratory disease of pigs caused by influenza virus (SIV, zoonotic disease). This illness was originally transmitted from pigs to humans but now easily spreads from one person to another. The symptoms of swine flu are totally similar to those of regular influenza and include fever, headaches, chills, diarrhoea, coughing and sneezing (1,2). The pandemic swine flu caused by the SIV subtype H1N1, including other subtypes H1N2, H1N3, H3N1, H3N2 and H2N3 can also cause illness. The virus was termed H1N1 because it mainly exhibited two types of antigens hemagglutinin (H) 1 and neuraminidase (N) 1 (3,4). Several vaccines are now available to prevent and/or against the disease. Generally, a healthy person may become infected by inhaling droplets expelled into the air through coughing or sneezing of an infected person or by transmission of virus-contaminated surfaces. However, the disease is not transmitted directly by eating cooked pork (1-5). The number of cases related to this virus

***Corresponding author:** Amit Gupta,
Email: amitvsbt@gmail.com; amitgupta@vsbt.res.in

shoots up during summer and monsoon season. In this regard, researchers have focused on various medicinal plants to control the burden of this disease.

Immunological exploration of these medicinal plants is totally based on phytochemical screening of primary and secondary metabolites (6). These metabolites of medicinal plants were examined for their immunopharmacological activities and approaches which lead to drug discovery and commonly referred as screening of natural products. Most of the medicinal plant products in the form of secondary metabolites i.e. flavonoids, terpenoids, saponin, etc. have shown anti-inflammatory, anti-microbial and immunosuppressive activities against specific as well as non-specific antigens (7-10). In addition, these metabolites from medicinal plant products are routinely used as raw material for extraction of active ingredients (11) that were used actually in the synthesis and manufacturing of active candidates e.g. adjuvants and vaccine antigens.

Azadirachta indica (Family *Meliaceae*; Common name, Neem) and *Butea frondosa* (Family *Fabaceae*; Common name, Palas) medicinal plants have shown many properties including anti-inflammatory, anti-hyperglycemic, antiulcer, antimalarial, antibacterial and antioxidant properties related to human health (7-13). *Ficus religiosa* (Peepal tree; family *Moraceae*) is found in tropical and subtropical regions of India. As per Ayurveda, isolation and purification of bioactive candidates from these herbal plants have shown various immunopharmacological activities so that they can be used to treat several types of diseases such as asthma, diabetes and inflammatory disorders (14). This study aimed to focus on the immunoadjuvant activities of medicinal plants namely *A. indica, B. frondosa* and *F. religiosa* against Swine flu vaccine antigen.

Materials and Methods

Plant material

Fresh leaves of *A. indica, B. frondosa* and *F. religiosa* were collected from Nakshatra Udyan of Vidya Pratishthan (Baramati, Maharashtra, India). The collected leaves were dried at room temperature and then macerated with a mortar and pestle. Powdered samples (5 g) of each plant were macerated in 50 mL of phosphate buffered saline (PBS, pH 7.2) and centrifuged at 10000 rpm. The supernatant was collected for estimation of various immunological assays. All these samples were stored at 4°C.

Qualitative analysis of secondary metabolites

The presence of secondary metabolites, i.e. terpenoids (using acetic anhydride test); flavonoids (using lead acetate test); saponins (using foam test) and phenolics (using ferric chloride test) was confirmed in aqueous leaves extracts of *A. indica, B. frondosa* and *F. religiosa*.

Swine flu vaccine

Inactivated influenza vaccines (surface antigen IP; 0.25 mL) were used in these studies. The influenza virus surface antigens (haemagglutinin and neuraminidase) were comprised of three different strains i.e. A/California/7/2009 (HIN1) pdm09-like strain (A/California/7/2009, X-181)-7.5 µg HA; A/Hong)-Kong/4801/2014 (H3N2)-like strain (A/New Caledonia/71/2014, x-257A)- 7.5 µg HA; B/Brisbane/60/2008-like strain (B/Brisbane/60/2008, wild type)- 7.5 µg HA with a total volume of 250 µL. All strains were propagated in fertilized hens' eggs from healthy chicken flocks containing haemagglutinin as well as traces of eggs, i.e. OVA, Chicken proteins, etc.

ELISA assay

Indirect ELISA was performed using Swine flu vaccine (1:1000 dilution) as coating antigen. Variable concentrations of aqueous leaves extract of seven different medicinal plants, i.e. *Ficus religiosa, Syzygium cumini, Butea frondosa, Emblica officinalis, Terminalia arjuna, Prosopis spicigera* and *Azadirachta indica* were added and their Swine flu IgG antibody titers were determined. Horse anti-serum was used as secondary antibody and the absorbance in the form of optical density (OD) was measured at 450 nm (15).

Estimation of total cellular content

In *ex vivo* studies, Swiss mice (n = 5) were immunized subcutaneously (100 µL) on day 0 with Swine flu vaccine antigen (1:100 dilution; formulated containing three different strains in a final volume of 250 µL). On day 7, spleen cells were collected from peritoneal cavity of the mouse. Red blood cells from each spleen were then lysed with red cell lysis buffer. Finally, the cells were flushed out with PBS containing 10% FBS in order to prepare a single-cell suspension from the spleen of mouse. Splenocytes (100 µL cells containing 10^5 cells/well) were cultured in 96-well, flat-bottom tissue culture plates for 48. This incubation period was done along with variable doses of aqueous leaves extract of *A. indica, B. frondosa* and *F. religiosa* (0.312-5 mg; 50 µL) in the presence of swine flu vaccine antigen (2 µg/mL; 50 µL), OVA (50 µg/well; 50 µL) and Con A (5 µg/mL; 50 µL). Three different sets of experiments were performed simultaneously. The samples were centrifuged at 15000 rpm. The supernatant was collected in order to determine the total cellular or protein contents using NanoDrop 1000 A280 module. The Beer Lambert equation (A=E*b*c) was applied for all protein calculations to correlate absorbance with concentration.

Splenocyte proliferation assay and analysis of Th1 cytokines

Similarly, in another set of experiment performed MTT assay splenocytes were cultured and incubated at 37°C in 5% carbon dioxide incubator along with variable doses of aqueous leaves extracts in presence of swine flu vaccine antigen, OVA and Con A and determined their proliferation rate. After incubation, the medium was collected, centrifuged for the estimation of Th1 (IFN-gamma and TNF alpha) cytokines and then added fresh PBS containing 10 % FBS. MTT solution (2.5 mg/

ml; 10 µL) was added and then the plate was incubated for another 4 h at carbon dioxide incubator. Formazan crystals were appeared and settled at the bottom and then dissolved in dimethyl sulphoxide (DMSO) solution after centrifuging and discarding the supernatant. The OD was measured at 570 nm (16).

Briefly, 100 µL of diluted capture antibody (IFN-gamma and TNF alpha; 2 µg/mL) was added to each well in 96-well plates and allowed to adhere overnight at 4°C. The plates were washed properly and blocked in 1X PBS supplemented with 10% FBS for 1 hour at room temperature. After washing, serial dilutions of the standard and samples were prepared and incubated for 2 hours. The plates were then washed and the working detector solution (including detector antibody and avidin–horse radish peroxidase reagent) was added into each well. Plates were sealed and incubated at room temperature for 1 hour. After washing, 100 µL of tri-methyl benzidine (TMB) substrate was added into each well. The stop solution (2 N H2SO4) was finally added after incubation in the dark for 30 minutes at room temperature. The absorbance was read at 450 nm. The result was analyzed using softmax program and values were determined against the standard provided by the manufacturer (16).

Haemolytic activity

The haemolytic activity of human whole blood was determined using aqueous leaves extract of *A. indica, B. frondosa* and *F. religiosa*. Distilled water was used as a control. In this study, human whole blood suspension was collected from Mangal Pathology Laboratory, Baramati. Whole blood samples were prepared by diluting in PBS along with variable concentrations of aqueous leaves extracts of *A. indica, B. frondosa* and *F. religiosa*. The samples were incubated in a carbon dioxide incubator for 2 hours. After incubation, the blood samples containing aqueous leaves extract were centrifuged. The supernatants were collected and using spectrophotometer, the free hemoglobin in the supernatants was estimated. The OD was measured at 405 nm (14).

Results

ELISA assay

Determination of antibody production against Swine flu vaccine antigen was done through ELISA. As shown in Figure 1, out of seven, only three aqueous leaves extracts of *A. indica, B. frondosa* and *F. religiosa* showed enhancement in antibody production against Swine flu vaccine antigen as compared to the control group.

Total cellular content

As shown in Table 1, aqueous leaves extracts of *A. indica, B. frondosa* and *F. religiosa* showed enhancement in total cellular content with respect to Swine flu vaccine and OVA, antigen at higher doses as compared to the control group.

Proliferation assay

The effects of aqueous leaves extracts of three different medicinal plants were evaluated to determine splenocyte proliferation assay against Swine flu vaccine antigen, OVA and Con A (Figure 2). The results show that aqueous

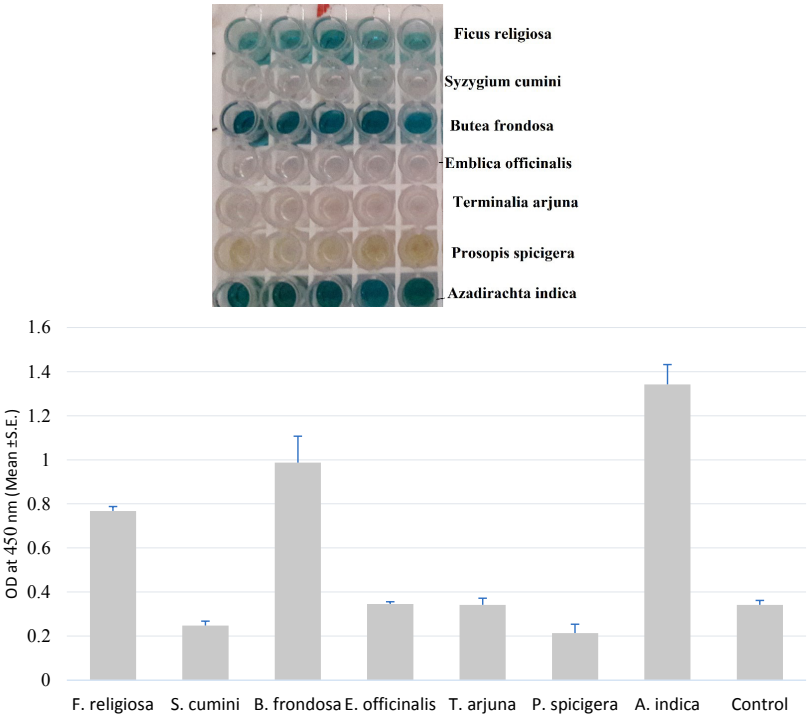

Figure 1. ELISA assay. Indirect ELISA was performed using Swine flu vaccine (1:1000 dilution) as coating antigen. Variable concentration of aqueous leaves extract of seven different medicinal plants were added and determined its Swine flu IgG antibody titre. Horse anti-serum was used as secondary antibody and absorbance in the form of optical density was measured at 450 nm.

Table 1. Effect of variable doses of aqueous leaves extract of medicinal plants on total cellular content in mouse splenocytes

Treatment		Doses (mg/mL)				
		0.156	0.312	0.625	1.25	2.5
Swine flu vaccine						
Control	0.424 ± 0.02	-	-	-	-	-
A. indica	-	0.544 ± 0.04	0.617 ± 0.02	0.982 ± 0.08	1.145 ±0.12	1.342 ± 0.22***
B. frondosa	-	0.750 ± 0.06	0.868 ± 0.14	0.912 ± 0.22	1.022 ± 0.34	1.232 ± 0.42**
F. religiosa	-	0.475 ± 0.02	0.412 ± 0.06	0.448 ± 0.04	0.568 ± 0.06	0.814 ± 0.12*
OVA						
Control	0.377 ±0.02	-	-	-	-	-
A. indica	-	0.562 ± 0.08	0.725 ± 0.06	0.986 ± 0.14	1.023 ± 0.13	1.214 ± 0.18**
B. frondosa	-	0.340 ± 0.02	0.425 ± 0.02	0.827 ± 0.08	0.924 ± 0.16	1.012 ± 0.22*
F. religiosa	-	0.319 ± 0.02	0.464 ± 0.04	0.683 ± 0.04	0.712 ± 0.06	0.784 ± 0.14

Swiss mice were immunized with swine flu vaccine on day 0. Splenocytes were collected on day 7 and cultured with variable doses of aqueous leaves extract and then further exposed with Swine flu vaccine antigen and OVA. Total cellular content was measured after high speed centrifugation and collect supernatant for estimation of total cellular content. Values are expressed as Mean ± S.E. The difference between control and variable doses of aqueous extract is controlled by one-way ANOVA test (Bonferroni multiple comparison test). *$P < 0.05$; **$P < 0.01$ and ***$P < 0.001$.

leaves extracts of *A. indica, B. frondosa* and *F. religiosa* raised splenocyte proliferation at lower doses as compared to the control group. Swine flu vaccine antigen, OVA and Con A were used as standards in this study and showed an enhancement in proliferation as compared to the control group.

Analysis of Th1 cytokines by ELISA

As shown in Figure 3, aqueous leaves extracts of *A. indica, B. frondosa and F. religiosa* raised Th1 (IFN-gamma and TNF alpha) production at lower doses as compared to the control group.

Haemolytic activity

The effects of aqueous leaves extracts of three different medicinal plants evaluated to determine their haemolytic activities in the human whole blood (Figure 4). The results showed that aqueous extracts of three medicinal plants had not any haemolytic effect at higher doses as compared to the control group. On the other hand, distilled water showed higher haemolytic activity.

Discussion

Phytochemicals are bioactive compounds that are present in medicinal plants and applied in traditional herbal medicines. Generally, these herbal medicines are used for curing various human diseases especially diabetes, cancer, HIV etc. The objective of our study was to determine qualitatively screening of phytochemicals within aqueous leaves extracts of three medicinal plants and estimating the antibody titer and proliferation rate (using specific and non-specific antigen) against Swine flu vaccine antigen. These medicinal plants were screened and evaluated in the form of aqueous leaves extracts for adjuvant activity against different vaccine antigens. These adjuvants are needed for vaccine antigen in order to enhance its immunogenicity against various infectious diseases. Searching for those active molecules derived from medicinal plants which are responsible for inducing or stimulating both humoral and cell-mediated immune responses is necessary. For the last several years, only one adjuvant has been approved for human use, i.e. alum which increases humoral response. However it poorly elicits cell-mediated immunity. In addition, emulsion-based adjuvant, i.e. MF59 provokes both humoral and cell mediated immune response. Recently, a trivalent vaccine, namely Fluad, formulated with MF59 (effective for 65 years or more; US approved) has been produced from three strains of influenza (2 subtype A and 1 type B) for prevention of seasonal influenza (17). In view of this, adjuvants are required for vaccine antigen pertaining to increase its immunogenicity, avidity and also help in decreasing the dose of antigen to decrease the toxicity. In this regard, efforts have been made in order to reduce the burden of antigen using various medicinal plants and to use as adjuvant against vaccine antigens. The most important benefit of these adjuvant-based plants is that they would remain stable for longer period of time. Generally, secondary metabolites from medicinal plants, used as adjuvant for vaccine antigen, are still under investigation, e.g. QS21 (saponin from *Quillaja saponaria*); RLJ-NE-299A (glycosides from *Picrorhiza Kurroa*) (11,18). In this study, we got similar results and these medicinal plants in the form of aqueous extract containing various phytochemicals showed enhancement in antibody production against Swine flu vaccine antigen. Further immunological *ex vivo* studies were also conducted in those splenocytes were cultured and exposed to swine flu vaccine, OVA and Con A in aqueous leaves extract and showed dose dependent changes in proliferation and total cellular content as compared to control group. In addition, specific (swine flu vaccine, OVA) and non-specific (Con A) antigens were recognized strongly by aqueous leaves extract containing primary as well as secondary metabolites and antibody-secreting cells from vaccines (11). Studying this interaction will allow us to further elucidate the effectiveness of antigen adjuvant combinations. In contrast, these medicinal plants showed no haemolytic effects as compared to distilled water.

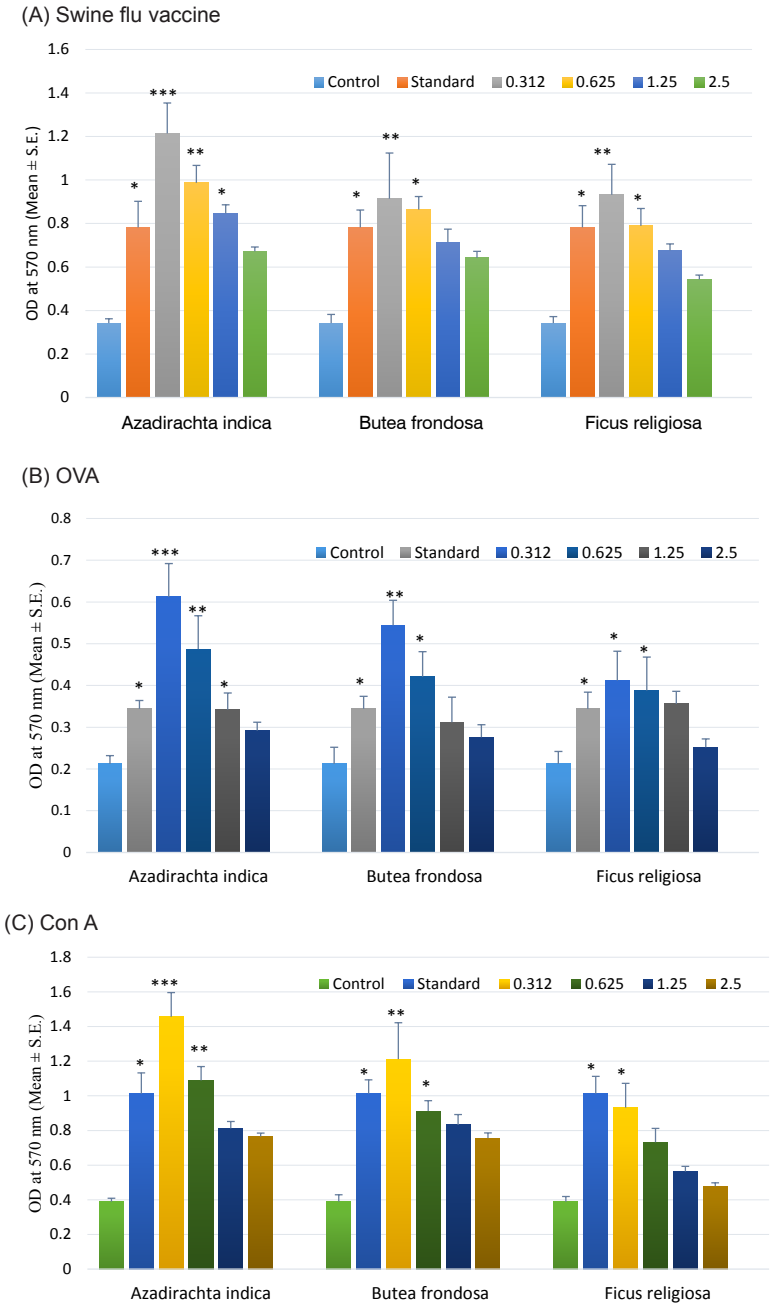

Figure 2. Splenocyte proliferation assay. Swiss mice were immunized subcutaneously on day 0 with swine flu vaccine antigen. On day 7, Splenocytes were collected and cultured with variable doses of aqueous leaves extract (0.312- 2.5 mg/mL; 50 µL) and then exposed to A) swine flu vaccine antigen (2 µg/mL; 50 µL), B) OVA (50 µg/well; 50 µL) and C) Con A (5 µg/mL; 50 µL)and determined its proliferation assay using MTT. Values are expressed as mean ± SE. The difference between control and variable doses of aqueous extract was controlled by one way ANOVA test (Bonferroni multiple comparison test). *$P < 0.05$; **$P < 0.01$ and ***$P < 0.001$.

Furthermore, aqueous leaves extract were able to induce anti-Swine flu, anti-OVA and anti-Con A T-helper cell response as shown by the strong antigen-specific/non-specific cell proliferation. Cytokine determination showed that aqueous extract adjuvanted Swine flu vaccine induced more IFN- gamma and TNF production as compared to control group. In short, we can be concluded on the basis of antibody production, proliferation and cytokines (IFN-gamma and TNF alpha) that aqueous extracts of the plants induce Th1 immune response. These observations suggest that the use of these aqueous extract as an adjuvant for swine flu vaccine antigen may provide better protection against these antigens by eliciting both humoral and cellular immune responses.

Conclusion

The results of this study indicated that aqueous leaves extracts of these plants might be considered as better vaccine adjuvant candidates in order to enhance the immunogenicity or antibody production, proliferation and Th1 cytokines against Swine flu vaccine antigen. The need for exploring alternative plant based formulation

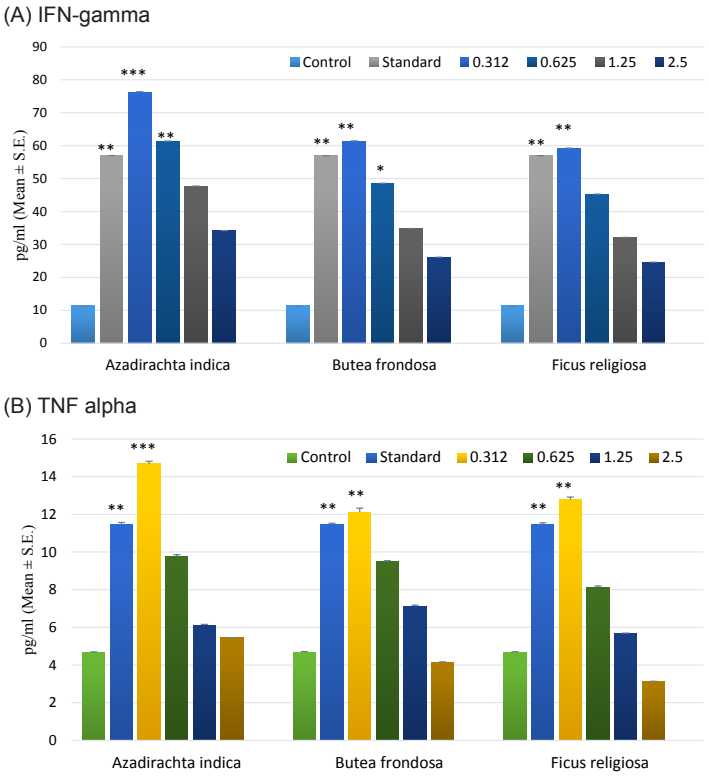

Figure 3. Th1 (IFN-gamma and TNF alpha) cytokines. Swiss mice were immunized subcutaneously on day 0 with swine flu vaccine antigen. On day 7, splenocytes were collected and cultured with variable doses of aqueous leaves extract (0.312- 2.5 mg/mL; 50 µL) and then exposed to swine flu vaccine antigen (2 µg/mL; 50 µL). Incubate the plate for another 24h. The plate was centrifuged and the supernatant was collected for the estimation of Th1 cytokines which determined by Elisa. Values are expressed as mean ± SE. The difference between control and variable doses of aqueous extract was determined by one way ANOVA test (Bonferroni multiple comparison test). **P* < 0.05; ***P* < 0.01 and ****P* < 0.001.

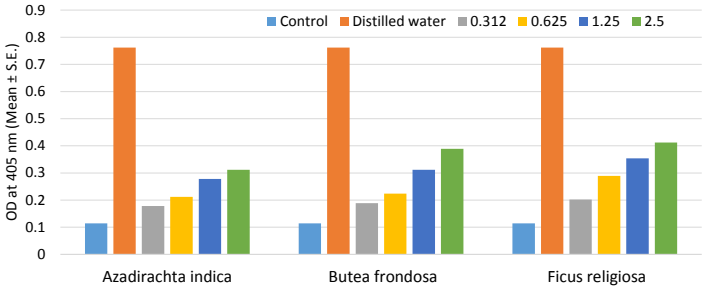

Figure 4. Haemolytic activity. Whole blood samples were diluted in PBS along with variable concentrations of aqueous leaves extracts (0.312- 2.5 mg/mL; 50 µL). The samples were incubated for 2 hours at carbon dioxide incubator. After incubation, the blood samples were centrifuged and the supernatants were collected and the free haemoglobin was estimated in the supernatants using spectrophotometer. The OD was measured at 405 nm.

for Swine flu vaccine antigen may raise the next step in this evaluation to test aqueous extract as an adjuvant with other protein antigens. Further studies with these aqueous extracts adjuvanted with Swine flu vaccine antigen are needed to establish clinically relevant proof of principles.

Authors' contribution

This work was carried out in collaboration of four authors. AG designed the study, wrote the protocol and interpreted the data where SG anchored the field study, gathered the initial data related to his M.Sc. Biotechnology dissertation work under AG guidance and performed preliminary data analysis. AG, SG and BS managed the literature searches whereas AG produced the initial draft. The final manuscript was read and approved by all authors.

Conflict of interests

The authors declared no competing interests.

Ethical considerations

This study was confirmed and conducted under ethical guidelines with registration number 1814/PO/ERE/S/15/CPCSEA.

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