International Journal of PharmTech Research
CODEN (USA): IJPRIF ISSN : 0974-4304
Vol.5, No.1, pp 271-274, Jan-Mar 2013

Assessment Of Antigen Stabilizing Potential Of Saponin Enriched Extract From Trigonella foenum graecum At Water In Oil Interface For Encapsulation In Polymeric Microspheres

Reeta Bardiya[1], Vivek Shrivastava[1] and U. K. Jain[1]*

[1]Bhopal Institute of Technology and Science - Pharmacy, Bhopal, India.

Corres.author : ukjain65@gmail.com
Mob: +919826075570

Abstract : Background and the purpose of the study: Microspheres have been evaluated as alternative vaccine adjuvants. Despite encouraging results, concerns about stability of antigens in microspheres have held back the rapid progress of polymeric delivery systems. The first emulsification step during microencapsulation is considered a primary cause for protein denaturation and aggregation. The aim of work was to investigate the capabilities of saponin enriched extracts from different parts of plant fenugreek (*Trigonella foenum graecum*) to act as surfactant & stabilize the model antigen at the interface.
Methods: Saponin enriched extracts were prepared from different parts of the plant *Trigonella foenum graceum.* Simulation studies were performed to evaluate the ability of saponin enriched extract to prevent inactivation and loss of test antigen HBsAg upon its exposure to water/dichloromethane interface.
Results: All the extracts (roots, seeds, stems and leaves) showed antigen stabilizing potentials in simulation studies. Root extract showed highest antigen stabilizing capabilities followed by seed extract and leaf extract.
Major Conclusion: Herbal saponins if utilized to stabilize antigen during microencapsulation, they may play dual role i.e. stabilize antigen during microencapsulation and at the same time serve as vaccine adjuvant upon *in vivo* administration.
Keywords: Saponin enriched extract; *Trigonella foenum graecum*; W/O/W interface; Antigen stabilization; Microencapsulation.

Introduction:

The goal of vaccination is to stimulate a strong, protective and long-lasting immune response to the administered antigen. For the achievement of these objectives, potent adjuvants and novel vaccine strategies are required to make the vaccine sufficiently immunogenic (1). In many cases, because of their low immunogenicity, vaccines require adjutants. Biodegradable polymeric microparticles have been widely studied these days as vaccine adjuvant (2). Water in oil in water double emulsification technique is popularly used for microencapsulating antigen in polymeric microparticles. Despite encouraging results, concerns about stability of antigens in microspheres have held back the rapid progress of polymeric delivery systems. The first emulsification step during microencapsulation is considered a principal cause for protein denaturation and aggregation. Therefore, development of strategies to stabilize antigen during primary w/o emulsion step would be of prime significance. Surfactants are among various

additives which are being added to overcome this problem. Surfactants preferentially get adsorbed at interface and prevent loss of antigen by aggregation. Saponins are natural surfactants containing steroid or triterpenoid nucleus and are found in wild or cultivated plants, lower marine animals and some bacteria. Saponins are high-molecular-weight glycosides consisting of polar (sugar moiety) and non polar (aglycone) moiety & this explains their soap-like behavior in aqueous solutions. Apart from surfactant property, recent studies on saponin have demonstrated that they have adjuvant activity and saponin based adjuvants have the ability to modulate the cell mediated immune system as well as to enhance antibody production and have the advantage that only a low dose is needed for adjuvant activity (1 & 3) & Currently safety is the main concern in the development of new adjuvant (4)Thus, the study was designed to assess the antigen stabilizing capabilities of extract containing saponins at aqueous/organic interface. By doing so, the study aims at developing vaccine formulation that can ensure antigen stability during microencapsulation. We hypothesize to prepare saponin rich extracts from different parts of plant *Trigonella foenum graceum*, which upon encapsulation along with antigen in polymeric microparticles; will inhibit interface induced aggregation of the model antigen (HBsAg).

Trigonella foenum graecum is an aromatic annual herbaceous plant, native of India, Africa to central Asia commonly known as Fenugreek, Methini, Methi and belongs to family Fabaceae. Fenugreek is rich source of saponins such as diosgenin, yamogenin, gitogenin,. Other bioactive constituents of fenugreek include mucilage, volatile oils, and alkaloids such as choline and trigonelline (5). There are a variety of plants which serves as rich source of saponin. Fenugreek (*Trigonella foenum graecum*) was selected as it contains fairly good amount of saponin, it is freely available and its safety is established, as it has been consumed as vegetables.

Materials and Methods

Collection of Plant

The plant material was collected as Whole plant of *Trigonella foenum graecum* in the month of October to February from the farms in Bhopal, Madhya Pradesh, India. The herbarium file prepared was submitted in the Department of Botany, Saifia Govt. PG College Bhopal, M.P. The Whole plant was authenticated as the *Trigonella foenum graecum*. The Reference no. is: 196 /Bot /Safia /10. Seeds were air dried in shade with the precaution of contamination from dust. The completely dried material was grinded to moderately coarse powder and stored in air tight container for further use.

Preparation of Saponin Enriched Extract

Extraction of powdered seed for saponin isolation was done according to previously described protocol (6). Briefly dried powdered seeds were defatted with n-hexane & marc was extracted with Methanol by maceration. Solvent was evaporated and dried Methanolic extract was partitioned between Water/ n-Butanol layers. To the n-Butanol fraction, Diethyl ether was added which resulted in precipitation of saponins. The precipitate was dried and plant saponins were collected.

Qualitative test for characterization of saponin in enriched extracts

General test (Froth test, foam test) & specific test like sulpher powder test were performed to characterize the presence of saponins in various extracts (7). Briefly, about 2 ml of sample was mixed with 3 ml of distilled water in a test tube and shaken vigorously for about 30 seconds. It was allowed to stand for half an hour and the height of honeycomb froth produced was observed and recorded. Foam test was performed by mixing 2 ml of sample with 3 ml of distilled water and was shaken vigorously for a stable persistent froth. The froth was mixed with 3 drops of olive oil and again shaken vigorously and the formation of emulsion was observed (8). Sulfur powder test was performed by taking small amount of sulfur powder to the sample and it was observed whether it sinks to bottom or not (7).

Assessment of surfactant property of saponin enriched extract

Surfactant property was assessed by measuring reduction of surface tension & percentage of haemolysis. Surface tension was determined by drop count method (9). Hemolytic activity test (10) was carried by centrifuging human blood sample at 3000 rpm for 15 min, RBC's were separated and washed with normal saline (0.9 %W/V) until a clear, colourless supernant was obtained above cell mass. The cells were re-suspended in normal saline to obtain a haematocrit of 5 %. To 1 ml of RBC's suspension taken in centrifuge tube, 5 ml of distilled water was added, which was considered as producing 100 % hemolysis. Similarly, 5 ml of normal saline was added to 1 ml of RBC's suspension in another tube producing no hemolysis, hence acting as blank. To 1 ml of

RBC's suspension taken in centrifuge tube, 1 ml of saponin enrich extract was added and volume was made up to 5 ml with normal saline. The tubes were allowed to stand for 1 hr at 37 °C with continuous shaking. All the tubes were then centrifuged at 3000 rpm for 15 min. UV-Visible spectroscopic analysis was carried out to estimate % haemolysis at 540 nm, taking normal saline as blank & distilled water as 100 % hemolytic standard.

Simulation studies

Simulation studies (11) were performed according to previously reported method. Simulation studies, which mimic the primary emulsification step involved in preparation of microspheres, were done to assess the antigen stabilizing capabilities of saponin enriched extract isolated from *Trigonella foenum graecum* at primary emulsification stage (water/dichloromethane interface). Briefly antigen solution along with test extract was included in buffer and was emulsified with dichloromethane (organic phase) to yield a Primary w/o emulsion. Saponin enriched extract was co-dissolved with HBsAg at 1**:**1, 2:1, 3:1 & 4:1 mass ratios and emulsified. HBsAg without using saponin enriched extract was also emulsified (Control).

Antigen Recovery

After emulsification, the aqueous and organic phase was separated by centrifugation at 3000 rpm for 15 min. The aqueous phase was carefully collected and filtered. The concentration of protein in filtrate was determined by employing UV spectroscopy at 280 nm.

Statistical Analysis

Data analysis was performed with Graph Pad Instat Software.

Results

The saponin enriched extract was prepared from seed of plant fenugreek and the percentage yield was found to be 0.67± 0.04 %. The presence of saponin in extract was confirmed by performing qualitative tests like froth test, foam test and sulfur powder test which are specific test for steroidal saponin. All these tests were found to be positive and the presence of saponin in extract was demonstrated.

The surfactant behavior of extract was assessed by performing hemolytic activity test and determination of Surface tension. Hemolysis observed in root extract was 38.04 ± 1.1 % and the decrease in surface tension was also recorded (58.96 ± 2.9 dynes/cm) (Table 1). The percentage of antigen recovered after performing simulation studies was found to be highest (72.9 ± 3.3 %) when Saponin:HBsAg mass ratio was 2:1, followed by mass ratio of 3:1 (72.3±3.7%) and 4:1 (65.4 ± 2.9 %).

Table 1: Assessment of surfactant potentials of Saponin extracts

Surface Tension (dyne/cm) Determination	
Water	72.80 ± 3.4
Saponin Enriched Extract	61.29 ± 4.3
Percentage Haemolysis	
Normal Saline	9.38
Saponin Enriched Extract	32.68 ± 1.1
Distilled water	100

Discussion

It was demonstrated that the immunogenicity of HBsAg was damaged by its exposure to organic solvents (2), thus the aim of work was to investigate the surfactant behavior of saponin extract isolated from fenugreek at water in oil interface. Although surfactants have been demonstrated to have protein stabilizing capabilities at interface (12), yet the surfactant from plant sources (saponins) may prove the safer option for stabilizing antigens. By doing so the study aims at exploring the capabilities of saponin enriched extract to stabilize the antigen at water in oil interface during microencapsulation. Besides this, some saponins of herbal origin are also known to have adjuvant properties, So we assume that if antigen could be stabilized successfully at water in oil interface by saponins from *Trigonella foenum graecum* during microencapsulation, they will serve dual purpose i.e. they will stabilize antigen at interface (primary emulsion) during microencapsulation & at the same time they will enhance immune responses against antigen when such microspheres will be administered *in vivo*.

To evaluate this, simulation studies were performed and results revealed that saponin enriched extract was able to prevent antigen aggregation at oil/water interface. Various mass ratios of Saponin / HBsAg (0, 1:1, 2:1, 3:1 & 4:1) were evaluated. The percentage of recovered antigen was increased as the mass ratio (Saponin: HBsAg) was increased from 1:1 to 2:1. Further increase in mass ratio to 3:1 or 4:1 did not show considerable differences in antigen recovered (Figure 1). The results showed that the saponin extract substantially reduced the magnitude of emulsification induced aggregation of HBsAg antigen.

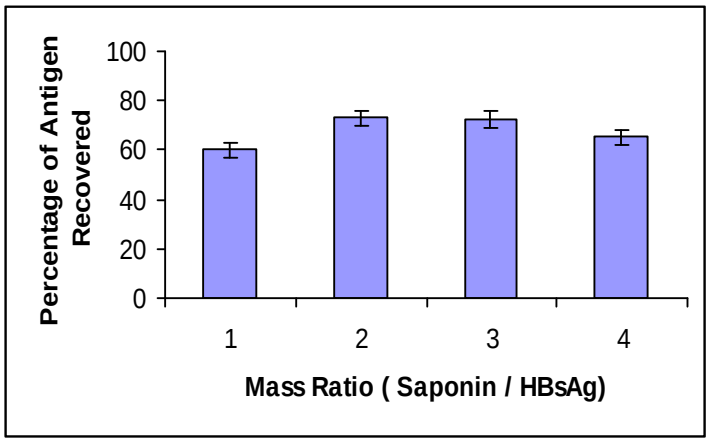

Figure 1: - The percentage of recovered antigen after performing simulation studies.

Summary & Conclusion

Saponins enriched extract from plant *Trigonella foenum graceum* were demonstrated to have antigen stabilizing properties at water in oil interface. Thus if herbal saponins are used to stabilize antigen during microencapsulation of antigen, they may play dual role i.e. they can stabilize antigen as well as serve as vaccine adjuvant, although the adjuvant property of *Trigonella foenum graceum* extract need to be further evaluated.

Acknowledgement

Authors are thankful to Dr K S Jaganathan, Shantha Biotech Ltd, Hyderabad, AP (India) for providing bulk HBsAg antigen for the study.

References

1. Bomford R, Stapleton M, Winsor S, Beesly JE, Jessup EA, Price KR and Fenwick GR. Adjuvancity and ISCOM formation by structurally diverse saponins. Vaccine. (1992); 10: 572-577.
2. Singh M, Li X-M, McGee JP, Zamb T, Koff W, Wang CY, O'Hagan DT. Controlled release microparticles as single dose hepatitis B vaccine: evaluation of immunogenicity in mice. Vaccine 1997:15:475-481.
3. Oda K, Matsuda H, Murakami T, Katayama S, Ohgitani T, Yoshikawa M. Adjuvant and haemolytic activities of 47 saponins derived from medicinal and food plants. *Biol Chem.* (2000); 381(1): 67–74.
4. Gupta RK, Siber GR. Adjuvants for human vaccines-current status, problems and future prospects. Vaccine, (1993); 13: 1263–76.
5. Mukherjee PK. Quality control of herbal drugs: an approach to evaluation of botanicals.(2002). Business Horizons. : 182-422.
6. Kokate CK, Purohit AP, Gokhale SB. Pharmacognosy, Nirali prakashan (2005); 593-597
7. Harborne JB. Phytochemical Methods. Springer, 3rd Edition: Fourth Indian Reprint: 135-136
8. Moore HN and Hajare AA. Practical physical pharmacy, Carrier publication, 1st ed, (2007); 21-25.
9. Yang ZG, Sun HX, Fang WH. Haemolytic activities and adjuvant effect of Astragalus membranaceus saponins (AMS) on the immune responses to ovalbumin in mice. Vaccine. (2005); 23(44): 5196–5203.
10. Hongkee S. Stabilization of proteins against methylene chloride/water interface-induced denaturation and aggregation. Journal of Controlled Release (1999); 58: 143-151.
11. Sun HX, Pan HJ, Pan YJ. Haemolytic activities and immunologic adjuvant effect of Panax notoginseng saponins. Acta Pharmacol Sin. (2004); 24(11): 1150-4.
12. Yoon HS, Kwoon YS, Sung JH, Lee TG, Park EN, Cho HS, Lee BM, Cho JM, Ryu WS. Effect of a novel saponin adjuvant derived from Quillaja saponaria on immune response to recombinant hepatitis B surface antigen. Mol Cells. (1997); 7(2): 178–186.

*****