



Preliminary studies on the formulation of immune stimulating complexes using saponin from *Carica papaya* leaves



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ABSTRACT

There have been several modifications in the use of immune stimulating complexes as adjuvants, such as the replacement of phospholipids with saponin content. Not much research has been done on the use of local alternatives. This actually instigated the use of a local alternative saponin source from *Carica papaya* leaves to formulate Iscomatrix adjuvant. The Iscomatrix samples used in this study were formulated using different methods (the rapid injection, the reversed rapid injection, the slow/dropwise injection and the reversed slow/dropwise injection methods). Furthermore, the quantity of the components was also varied. These formulated samples were compared with other adjuvants and analysed for their ability to induce antibody and cell mediated immune responses using animal model i.e. mice. The results showed that the Iscomatrix samples formulated, were able to induce significant humoral and antibody mediated immune response (ranging from 16.7 % - 38.88 %) and they also elicited cell mediated immune response (ranging from 8.33 % - 16.7 %) when compared to the models that were administered with antigen only. Further characterizations were made, such as pH, UV scanning, Scanning Electron Microscopy. The analysis revealed that the samples were slightly soluble in distilled water with a neutral pH ranging from 7.26 – 7.43. The UV analysis also indicated that they all had a close range of absorption peaks (between 266.8-269.37 nm). Saponin from *Carica papaya* leaves can be used to formulate Iscomatrix adjuvant capable of stimulating cell mediated and antibody mediated immune responses.

1. Introduction

Infectious diseases have always been a major problem for humans, animals and they are the most common source of morbidity and mortality. Vaccination has proven to be a valuable and efficacious tool in the prevention of infectious diseases. Vaccines majorly work by capitalizing on the immune system's ability to rapidly respond to microorganisms after a second encounter. The target for vaccination is to stimulate a strong, long-lasting and protective response of the immune system to the antigen [1]. Most subunit vaccine proteins are comparatively weak immunogens on their own, and they usually require repeated boosting and the use of additional substances in order to be able to induce enough/suitable protective immune response [2]. There have been various strategies and techniques employed in the improvement of the immunogenicity and efficacy of vaccines, and an important/prominent one are the use of adjuvants. Adjuvants are capable of boosting immune

responses against a specific antigen and have led to many successful vaccination programmes [3].

An adjuvant is any material that increases the immune response to antigen. They are usually injected along with an antigen in order to enhance the humoral and/or cell-mediated immune response to the antigen. Adjuvants are majorly used in enhancing the immunogenicity of vaccines. They comprise various groups of compounds. There are limited registered adjuvants for human vaccines, such as the aluminum based adjuvants e.g., aluminum hydroxide and the oil/water based adjuvants e.g. MF59. Although these adjuvants are proven to have robust antibody induction and are also considered safe, when it comes to building-cell mediated immunity, which is crucial for anti-cancer vaccines, they appear to be poor adjuvants. In animal vaccines, there are also several adjuvants in use. E.g. Saponin, ISCOMS, oil in water, water in oil emulsions, and Carbopol formulations [4].

Immunostimulating complexes (ISCOMS or ISCOMATRIX) are a class

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of adjuvants which are primarily composed of saponin, cholesterol and phospholipid [4]. They are one of the most successful delivery systems for different varieties of antigen. These antigens could originate from microbes, parasites or viruses [5]. ISCOMs and ISCOMATRIXes are promising adjuvants [3].

Adjuvant technology, especially the use of ISCOMS and ISCOMATRIX, is still being universally harnessed to yield more beneficial discoveries. The saponin majorly used is from a non-indigenous plant source *Quillaja saponaria*. Not much research has been done on the use of local alternatives. This motivated the present study which is targeted at finding a local alternative and indigenous source of saponin for Iscomatrix technology, and also seeks to determine if the formulated Iscomatrix has the ability to produce immunomodulatory effect.

2. Methods

2.1. Materials

Distilled water, analytical grade solvents (ethanol, petroleum ether, butanol), and aluminum hydroxide were all obtained from JHD (China). Cholesterol was obtained from Nice Chemicals (India) and Phospholipid (Hydrogenated phosphatidyl choline from soybean) was obtained from Lipoid (Germany).

2.1.1. Plant material

The leaves of *Carica papaya* were collected from Pawpaw plant at Ado-Ekiti, Ekiti State, Nigeria, in January 2016, and were identified and authenticated by a taxonomist at the Department of Pharmacognosy, faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University (NAU), Agulu, Anambra state, Nigeria. The whole plant was air-dried and then pulverized. A 2kg of the powdered leaves was soaked in 10L of ethanol for 48 h and then filtered through a muslin cloth. The filtrate was concentrated in a rotary evaporator to obtain the crude extract of *C. papaya* leaves. A modification of Hostettmann *et al.* [6] was used to extract saponin from the crude extract. Briefly, the crude extract was defatted using petroleum ether and was further treated by mixing properly with ethyl acetate and separated using a separating funnel. The aqueous fraction was further fractionated with butanol and the butanol fraction was concentrated to yield the crude saponin.

2.1.2. Experimental animals

Both male and female mice about 5–8 weeks old with average weight of 23.2 g were obtained and maintained in the Animal house of the Department of Pharmacology, Faculty of Pharmaceutical Sciences of Nnamdi Azikiwe University Agulu, under standard conditions of room temperature, humidity and light. The animals were allowed to acclimatize for 3 days prior to immunization. The animals were fed with tap water and laboratory chow ad libitum, and exposed to 12 h light or dark cycles. All animal experiments were conducted in compliance with National Research Council (US) Committee guide [7] for care and use of laboratory animals and approved by the Ethical Board for animal studies of the Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University.

2.2. Preparation and characterization of Iscomatrix

2.2.1. Preparation

ISCOMATRIX adjuvant was formulated using the ethanol injection method (Direct and Reverse) according to Lendemans *et al.* [8].

Direct Ethanol Injection: An ethanolic stock solution containing 6.76 mg/ml phospholipid and 3.44 mg/ml cholesterol was prepared. A 0.225 ml volume of ethanolic stock solution was aspirated into a small syringe and injected through a fine needle, into a vial containing 4 mg saponin dissolved in 2.775 ml in phosphate buffer. This was done both rapidly (Rapid Injection) and Slowly (Slow Drop-wise addition at about 5 drops per second, over a period of 20 min).

Reversed Ethanol injection: Saponin (4mg) dissolved in 2.775 ml of

Phosphate Buffer was injected into 0.225 ml of an ethanolic stock solution which contained 6.76 mg/ml phospholipid and 3.44 mg/ml cholesterol. This was also done both rapidly (Rapid Injection) and slowly (Slow Drop-wise addition at about 5 drops per second, over a period of 20 min).

2.2.2. Characterisation of the Iscomatrix

The formulated Iscomatrix was subjected to rotary evaporator to remove ethanol and later freeze-dried (lyophilized) into dry powdered form using a freeze-drying machine (York Scientific, India). The lyophilized Iscomatrix samples were stored in air tight, amber coloured glass containers and kept in a refrigerator at a temperature of about -4 °C, until further use. The morphology of the formulated ISCOMATRIX was examined in a scanning electron microscope. Further characterizations were carried out by dispersing the Iscomatrix samples in distilled water in a concentration of 1 mg/ml. The following characteristics were determined:

- **Solubility:** From the Iscomatrix samples 1 mg was dissolved in 1 ml of distilled and the degree of solubility was determined.
- **pH:** The pH of the Iscomatrix was read using a pH meter (Jenway, UK) at room temperature.
- **UV scanning:** The absorbances of the samples were checked at 1 mg/ml by scanning at different wavelengths, ranging from 200–700 nm using a UV Scanner (Perkin Elmer, Singapore). The highest peak of absorbance was observed for each sample.

2.2.3. Evaluation of the immunogenic potency of Iscomatrix

Total of 24 rabbits (n = 24) were divided into 8 groups. Each group was administered with different formulations according to Table 1.

2.2.3.1. Preparation of antigen. The antigen used for the work is sheep red blood cell (SRBC) obtained from domestic sheep. Sheep red blood cell was used due to ease of availability and preliminary work proved it to be suitable. The SRBCs were collected in Alsever's solution, centrifuged and the supernatant, decanted. The cells were washed three times in normal saline and centrifuged each time to remove the supernatant, until the supernatant was clear [9].

2.2.3.2. Assesment of humoral immune functions. The animals within each experimental group were challenged intraperitoneally, with 0.2 ml of 10 % sheep red blood cells, on the 7th day, after the initiation of the experiment. Administration of the immunogens was continued after challenge for 5 days. Each administration contains 30 µg of the immunogen in 0.1 ml of sterile water. The animals were placed in 8 groups. Blood samples of the animals were collected through the retro orbital plexus on the fifth day after immunization. A hematocrit tube is inserted at the medial canthus of the eye and directed caudally behind the globe to the medial-posterior aspect of the orbit. The tube is lightly twisted to disrupt the vascular plexus at this site and blood was collected as it flows out with the hematocrit tube, and allowed to clot. The blood samples collected were centrifuged at 4000 rpm for 10 minutes to obtain the sera. A 1 % solution of the sheep blood (antigen) was prepared by mixing 0.1

Table 1
The Animal Groups and the formulations administered.

GROUP	Formulation Administered
1	BATCH 1
2	BATCH 2
3	BATCH 3
4	BATCH 4
5	ALUMINIUM HYDROXIDE ADJUVANT
6	SAPONIN
7	SHEEP RED BLOOD CELLS (ANTIGEN) ALONE
8	CONTROL (NO ADMINISTRATION)

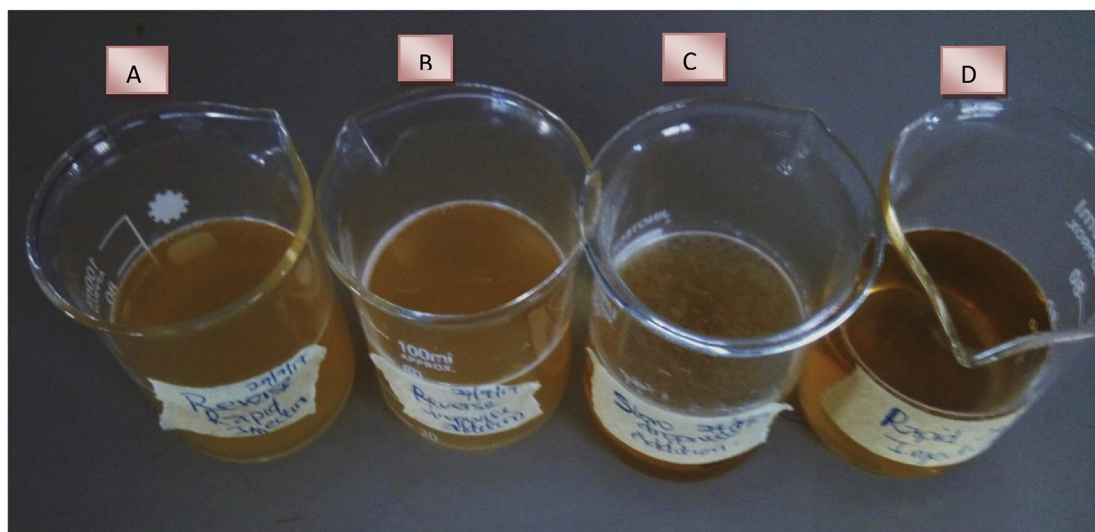


Fig. 1. The Iscomatrix formulations from the four different procedures used. A- Iscomatrix formulated using the Reversed Rapid Injection (batch 2). B- Iscomatrix formulated using the Reversed Drop-wise Injection (batch 4). C- Iscomatrix formulated using the Slow Drop-wise Addition (batch 3). D- Iscomatrix formulated using the Rapid Injection (batch 1).

ml of the blood with 9.9 ml of the PBS. Furthermore, a serial two-fold dilution of the serum was made with 50 μ l of PBS, and mixed with 50 μ l of the 1 % sheep red blood cell suspension (in PBS). After mixing, the tubes were kept at room temperature for 2 h, after which they were observed for agglutination. The antibody titre values were assigned to the highest serum dilution, showing visible hemagglutination [10].

2.2.3.3. Delayed type hypersensitivity response (DTH). The animals were treated for 7 days with 30 μ g of the immunogen in 0.1 ml of sterile water, after which they were immunized with 10 % SRBC intraperitoneally. On the fifth day after immunization, the animals were then again challenged with 1 % SRBC on the left hind footpad. The right footpad was injected with the same volume of normal saline, which serves as the trauma control for non-specific swelling. The increase in the footpad thickness was measured 2 h and 24 h after the challenge and considered as an index of cell mediated immunity/ delayed hypersensitivity. The percentage Inhibition was calculated as % Inhibition = $100(\text{difference in the footpad of control groups minus difference in the footpad of treated group})/\text{difference in the footpad of control groups}$ [10].

2.2.3.4. Data and statistical analysis. Statistical analysis was performed using Graph pad Prism Software Version 5.10. All the results were expressed as Mean $\pm$ Standard Error Mean (SEM). Data were analyzed using One-Way and Two-Way Analysis of Variance (ANOVA) followed by Dunnett multiple comparison test. P-values < 0.05 were considered statistically significant.

3. Results and discussion

3.1. Extraction of saponin

In this present study, the saponin used was obtained from the leaves of *Carica papaya* tree, which were collected from Ado-Ekiti, Ekiti State. The percentage yield of the saponin from the *Carica papaya* leaves was 0.36 %. The extraction process involved various techniques. The outcome of the de-fatting process using petroleum ether indicated that *Carica papaya* leaves contained fat components with the oily nature of the petroleum ether fraction after fractionation. Fractionation was carried out with butanol after treatment with ethyl ether and the butanol fraction yielded more saponin than the aqueous fraction. The extracted saponin was confirmed using the foam test. The result of the whole process is a

confirmation of the presence of saponin in *Carica papaya* leaves. This is in support of earlier works [11, 12] on the phytochemical evaluation of the *Carica papaya* leaves, revealing that they contain saponin which is responsible for the bitter taste in the leaves.

3.2. Preparation and characterization of Iscomatrix

There are several methods of formulating Iscomatrix, such as the Dialysis, Centrifugation, Lipid-Film Hydration, Ethanol Injection and Ether Injection methods. Lendemans *et al.*, [8] compared some of these methods and revealed that the dialysis method has the highest yield of homogenous samples of Iscoms. However, the ethanol injection method is a very good alternative preparation method with advantages such as simplicity, low cost, time -saving and potential for scale up. These benefits of the ethanol injection method instigated its use in this study for the preparation of the Iscomatrix samples.

This study also employed the four sub-procedures of the ethanol injection method as used by Lendemans *et al.* [8]. These include: the rapid injection, the reversed rapid, the slow drop-wise and the reversed slow drop-wise methods. The products from the four formulation sub-procedures are shown in Fig. 1.

3.3. Characterization of the Iscomatrix

3.3.1. Scanning Electron Microscopy

The morphology of the formulated samples (Figs. 2 and 3) was observed using the scanning electron microscope (SEM) and was magnified at 1000 $\times$ (at 536 μ m).

The SEM micrographs of the samples which were formulated using these sub-procedures of the ethanol injection method according to Lendemans *et al.* [8], showed a high number of small spherical ring-like particles and lesser elongated tubular structures. These are somewhat similar to the Transmission Electron Microscope (TEM) micrographs of the ISCOMs formulated with the Reversed Dropwise method by Lendemans *et al.*, [8]. Although the sample 2 (which was produced by the reversed rapid injection method), looked a bit different as it showed lesser spherical particles compared to the other three Iscomatrix samples.

The whole SEM analysis results revealed that the change in the procedure of formulation, and/or concentration of any of the major components of Iscomatrix formulation (i.e. phospholipid, cholesterol or saponin), can significantly affect the rate and homogeneity of the

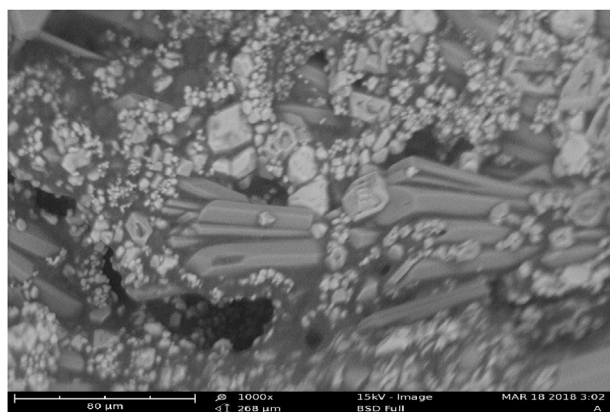


Fig. 2. The SEM micrographs of Sample batch 1 (Rapid injection) at 80 μm showing the scattered, tiny spherical particles with a hollow cage like structure which are more than the bigger elongated tubular structures.

resulting colloidal particles, leading to an effect in the quantitative and qualitative properties of the Iscomatrix.

3.3.2. pH analysis

Further characterization of the samples showed that they are slightly soluble in water, with neutral pH range with an average of 7.32 when measured at an average temperature of 30.13 $^{\circ}\text{C}$. Previous studies have shown that factors such as temperature and pH have a significant effect on the physicochemical properties of adjuvants [13, 14]. Thus, adsorption of antigens to adjuvants was often studied at pH 7.4 in order to be

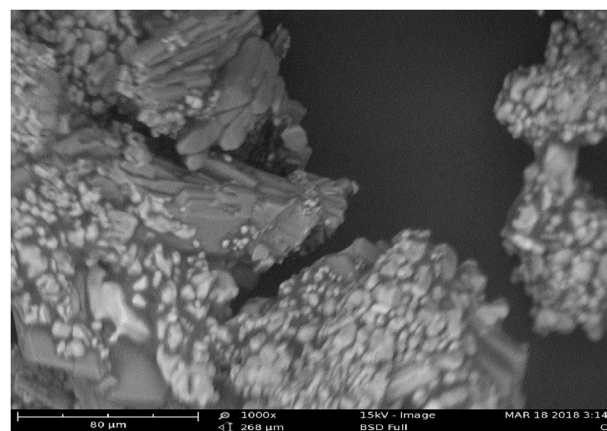


Fig. 3. The SEM micrographs of Sample batch 3 (Slow dropwise injection) at 80 μm showing the higher number of scattered spherical particles and a lesser number of bigger irregular shaped aggregated lump.

close to the physiological conditions of vaccines for parenteral administrations. This implies that the neutral pH range (average 7.32) of the formulated Iscomatrix samples in this study makes it easier for use as an adjuvant in the formulation of vaccines for parenteral administration.

3.3.3. UV scanning

Ultra-violet (UV) radiation has been shown to suppress antigen-specific immune processes in humans and animals through direct and indirect mechanisms [15]. Exposure to sunlight or UV radiation can cause some vaccines to lose their efficacy and potency. Hence there is

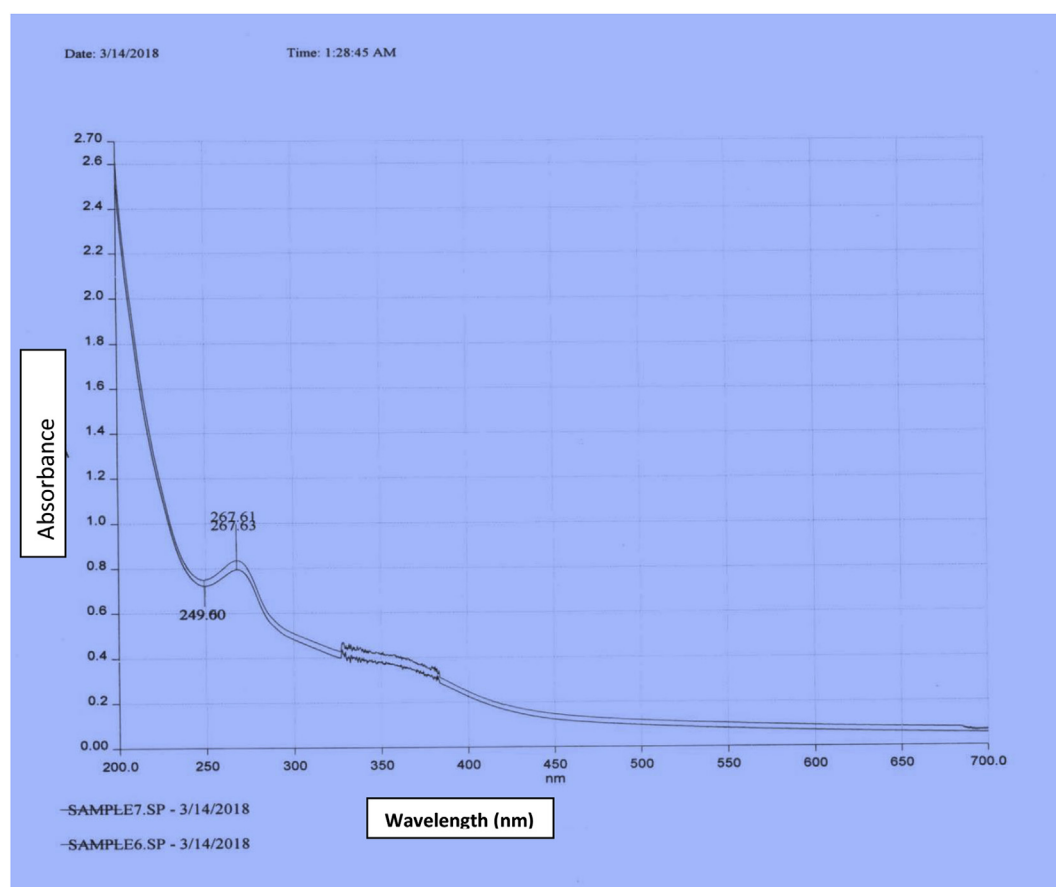


Fig. 4. Comparative absorbances of samples batch No 3 and 4 (Reversed and Slow drop-wise injection) at varying wavelengths, indicating the similarities in the level of absorbance of the two samples having their peaks at a wavelength of 267.6nm.

need to know the maximum wavelength of radiation that a sample can absorb. In this study, the highest wavelength of radiation that each of the samples can absorb was analyzed by scanning through a wavelength range between 200–700 nm. The scanning results (Fig. 4) showed that all the samples have their highest absorption peaks at a very close wavelength range i.e. between 266.8–269.73 nm. This implies that when they are exposed to UV radiation with a higher wavelength, it would significantly affect their stability, durability and efficacy when incorporated into vaccines. Therefore, to avoid degradation due to radiation, it is best to store them in dark/amber coloured unreactive/inert containers.

3.3.4. Evaluation of immunological potential of the Iscomatrix

Immune stimulating complexes have been shown to be able to reduce antibody responses or protective immunity in various animal models [16, 17]. In this study, the formulated Iscomatrix samples were evaluated for their immunological effect. The various groups that were administered with the Iscomatrix samples produced a significant increase in antibody titer values. The reversed dropwise method gave the highest immunomodulatory activity (38.8 %), followed by the reversed rapid (27.79 %), the slow dropwise (22.21 %) and lastly, the rapid injection (16.67 %) methods. In contrast, the groups of animals that were administered with the antigen alone showed no activity i.e. (0 %) (Fig. 6). The implication of this is that the ability of the Iscomatrix samples (formulated in this study) to effect an increase in the antibody titre of the animal groups, makes them suitable adjuvants for prophylactic vaccines. This goes further to support the work of Pearse and Drane, (2004) on the dose-sparing capacity of the Iscomatrix adjuvant (i.e. the ability to generate robust antibody responses at low antigen doses). A Similar study was also carried out by Clements *et al.* [18] on the Comparative analysis of the Iscomatrix adjuvant and other adjuvants. It was observed that only the Iscomatrix adjuvant was able to induce high neutralizing antibody titres at low antigen doses in non-human primates and mice.

Another important property of a good adjuvant is that it should be able to stimulate not only the humoral immune response, but also cell

mediated immunity. In this study, a delayed type hypersensitivity test was carried out in order to ascertain the ability of the formulated Iscomatrix samples to induce T-cell responses. The results showed that the samples formulated with the rapid injection and the reversed drop-wise addition methods both showed an effect of 8.33 % within 2 hours (Fig. 5). This happened to be the same effect observed when compared with that of the aluminum hydroxide adjuvant. The samples from the Reversed Rapid injection and the slow drop-wise addition were also observed to have a slightly higher effect of 16.7 %. On the contrary, the groups of animals that were administered with the antigen alone without any adjuvant, showed no significant effect. This implies that the ability of the formulated Iscomatrix samples to stimulate cell mediated immunity makes them suitable adjuvants for use in vaccines because vaccination against certain infectious diseases is dependent on the efficient in inducing T-cell responses. This is in support of previous studies [19, 20] showing that the ability of the Iscomatrix to induce CT responses in primates and humans makes them suitable as vaccine adjuvants against infectious diseases and also cancer.

3.3.5. Evaluation of the immunogenic potential of crude saponin from *Carica papaya* leaves

Previous studies [21, 22, 23] have shown saponins to be able to modulate cell mediated immunity and antibody production. In this study, the crude saponin that was obtained from the leaves of *Carica papaya* plant was evaluated for its ability to induce cell mediated immunity using the delayed type hypersensitivity test. The groups of animals that were administered with the crude saponin gave the highest value of cell-mediated immune response (37.5 %) after 2 hours (Fig. 5). The hemagglutination test was also used to analyze the ability of the crude saponins to stimulate antibody production. The titer value of the animal group administered with the saponin was observed to be 27.8 % (Fig. 6). This implies that saponin from *Carica papaya* leaves had strong immunological effect, and can stimulate not just cell-mediated immunity, but also humoral immune response by increasing production of antibodies [24, 25, 26].

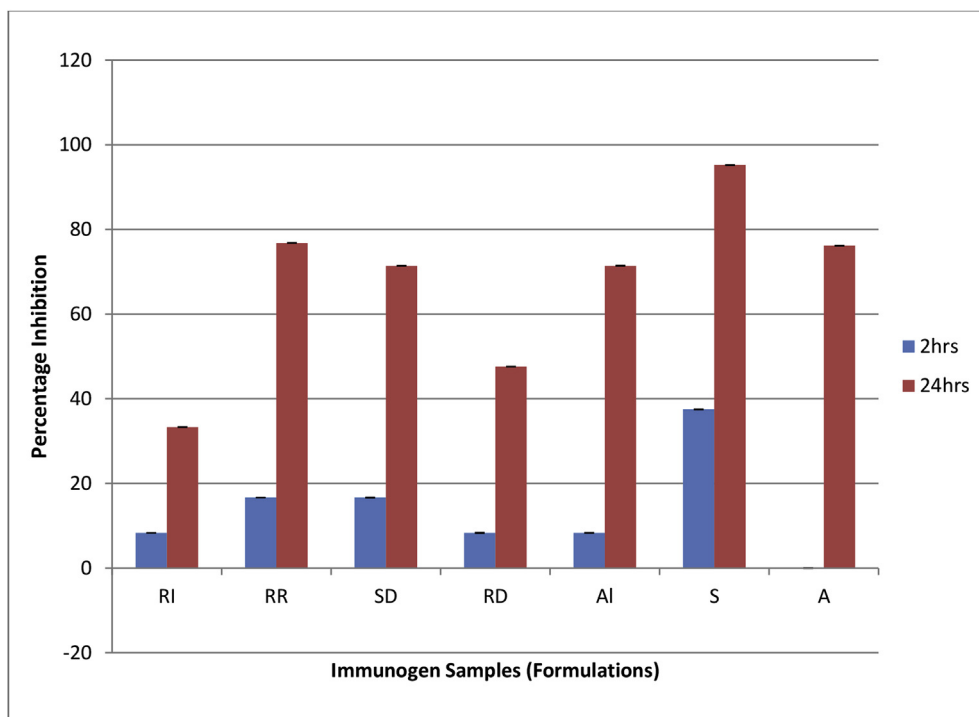


Fig. 5. Bar chart showing % inhibition after 2 and 24 hrs (Delayed Type Hypersensitivity) for the various immunogens/product formulations. KEY: DTH- Delayed Type Hypersensitivity for the various immunogens, RI- Rapid injection (Batch 1), RR-reversed rapid (Batch 2), SD-slow drop-wise (Batch 3), RD-reversed drop-wise (Batch 4), AI –Aluminum, S- saponin, A-antigen only, SRBC- (Sheep red blood cell).

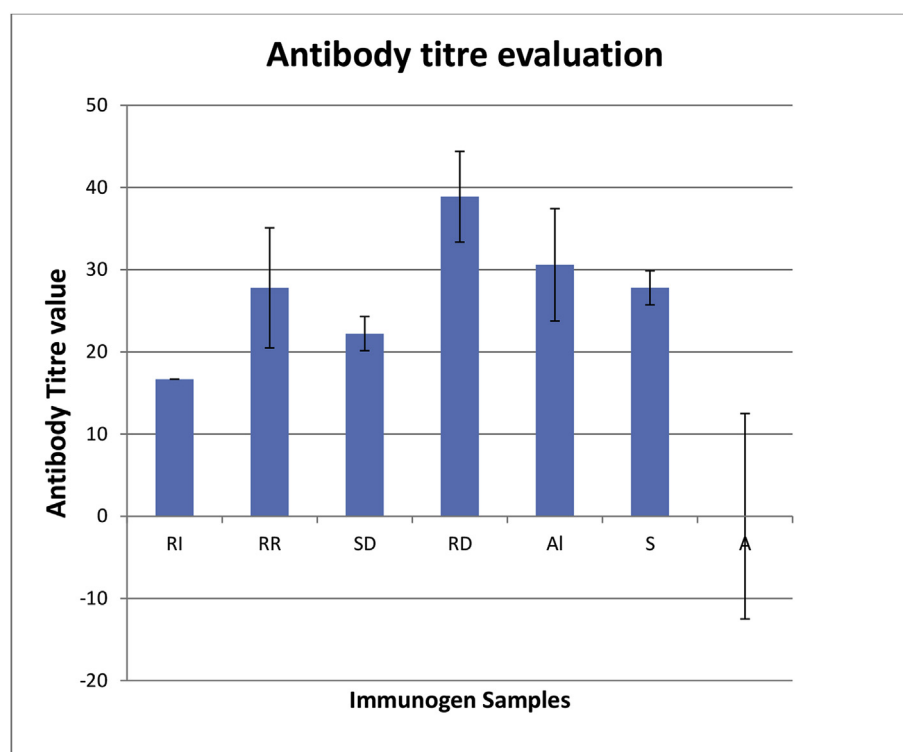


Fig. 6. A multiple bar chart for antibody titre evaluation for the various immunogens.

Data (Fig. 5) of the Delayed Type Hypersensitivity on analysis by 2-way ANOVA showed that the interactions between the duration of observed hypersensitivity and the kind of the immunogens (as appeared in the various the batches of formulations) accounted for 6.25% of the total variance, with a P value <0.0001 . The interaction is considered extremely significant. Also, the duration of the observed hypersensitivity accounted for 74.90% of the total variance with a P value <0.0001 . The effect is considered extremely significant. Again, the kind of the immunogens (as appeared in the various the batches of formulations) accounted for 18.85% of the total variance with a P value <0.0001 . The effect is considered extremely significant.

KEY: RI-Rapid injection (Batch 1), RR – Reversed Rapid (Batch 2), SD-slow drop-wise (Batch 3), RD-reversed drop-wise (Batch 4), RI 2 – Rapid injection 2 (Batch 5), RI 3- Rapid Injection 3, RI 4 – Rapid Injection 4, RI 5- Rapid injection 5, RI 6- Rapid injection 6, AI –Aluminum, S- saponin, A-antigen only, SRBC- (Sheep red blood cell).

Data of the antibody titre (Fig. 6) on analysis using 1-Way ANOVA showed that the antibody titres of the formulation (RD-reversed drop-wise (Batch 4)) with saponins from *Carica papaya* leaves was significantly higher compared with the AI adjuvant and an antigen (Sheep red blood cell) with a P value = 0.0171. Other batches were significantly lower with P value = 0.0193.

4. Conclusion

The results of this work suggest that the use of saponins from local plants such as *Carica papaya*, can serve as a very good local alternative saponin source for formulating immune stimulating complexes (ISCO-MATRIX adjuvant). The crude saponin from the *Carica papaya* leaves was shown to have a strong humoral and cell mediated immune response in the animal models. Furthermore, the Iscomatrix adjuvant samples formulated with this saponin was also able to stimulate strong antibody and cell mediated immunity. The formulated samples also showed

favorable characteristics such as being slightly soluble in water, with neutral pH average range of 7.32, and also having their UV absorption peaks at a very close wavelength range i.e, between 266.8 - 269.73 nm. All the results in this study points to the fact that saponins from *Carica papaya* leaves, can be suitable for the formulation immune stimulating complexes (ISCO-MATRIX) adjuvant.

Declarations

Author contribution statement

Chioma Miracle Ojiako: Performed the experiments; Wrote the paper.

Chibueze Jeremiah Ike: Performed the experiments.

Ebere Okoye, Charles Okechukwu Esimone: Conceived and designed the experiments.

Anthony Attama: Contributed reagents, materials, analysis tools or data.

Angus Oli: Analyzed and interpreted the data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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