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The use of alfalfa root instead of streptolysin O in ASO diagnostic test

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Abstract
Alfalfa (*Medicago sativa*) has been used as nutritious animal fodder for over years. Streptolysin O (SLO) is one of the several toxic immunogenic exoenzyme produced by group A β-hemolytic Streptococci. ASO is a routine test for post streptococcal infections diagnosis. This study explored the application of Alfalfa root extract instead of SLO. Hemolytic effect of the Methanolic extract of Alfalfa roots was determined by using RBC suspension. Immunological properties of Alfalfa root extract was evaluated by some serological techniques. This study showed that hemolytic effect of Alfalfa roots extract is similar to SLO and the ASO was inhibited hemolysis activity of the extract. Ouchterlony test demonstrate its antigenic similarity and Slide Agglutination Inhibition and neutralizing methods prove its efficiency similarity with SLO. There is significant similarity of antigenic property in both Alfalfa extract and SLO. Thus purified extract of Alfalfa can used as alternative suitable reagent instead of SLO for ASO diagnostic test.

Keywords: Alcoholic extract, Alfalfa, Streptolysin O, Anti Streptolysin O

1. Introduction
S. pyogenes infections are common and include pharyngitis, scarlet fever, skin infections, and other septic infections, in addition rheumatic fever and glomeronephritis may occur as a result of infection with group A streptococci [1, 2]. *S.pyogenes* produces many enzymes and toxins, that one of these toxins is streptolysin O (SLO). Anti-streptolysin O (ASO) is the antibody made against streptolysin O, an immunogenic, oxygen-labile hemolytic toxin produced by most strains of group A and many strains of groups C and G streptococci. A raised or rising levels can indicate past or present infection, also can show the effectiveness of treatment [2, 3, 4, 5]. Different tests can use for ASO assay include Neutralization, passive Agglutination, turbidimetry and ELISA that purified SLO is necessary for these methods [5, 6]. SLO obtains from *S. pyogenes* culture supernatants or recombinant DNA techniques. SLO reagent is very unstable and labile to oxidation, therefore should be used soon after preparation [4, 5, 6, 7, 8]. Alfalfa (*Medicago sativa*), is a perennial flowering plant in the pea family Fabaceae cultivated as an important forage crop in many countries around the world [9]. Alfalfa is high in vitamin and minerals content and can be used as a nutritional supplement. It is also helpful for treatment of many disease such as infection and high cholesterol, asthma, rheumatoid arthritis, diabetes and cancer [10, 11]. In this study we use alfalfa root extract instead of SLO for in vitro diagnosis test.

2. Materials and methods
2.1 Preparation of Extract
The Alfalfa roots were collected from Robat Karim city 25 kilometer from *Tehran*. Then, plants were cleaned and washed with water and dried in the dark place at room temperature and were powdered. The air-dried powdered roots were preserved in clean plastic containers, kept away from light, heat and moisture until use.

2.2 Maceration method
50 g of powdered roots were placed in a container with 200 ml of methanol 80% for 24 h at room temperature with frequent agitation in 4000 rpm. The methanolic extract was filtered through whatman filter paper. These steps were repeated three times. Then the filtrates were concentrated using rotary evaporator until a paste was formed. Finally, the extract was maintained at 4 °C throughout the experiments.

2.3 Evaluation of Alfalfa roots Extract hemolytic effects

0.25 g/cc suspension of the extract was prepared in distilled water and then 0.5cc of 5% RBC suspension was added to this solution and incubated in 37 °C for 1 hours. Then the RBCs were checked for hemolysis.

2.4 Evaluation of Alfalfa roots Extract antigenic similarity with streptolysin O

2.4.1 Ouchterlony assay

In the Ouchterlony assay, solutions of Alfalfa Root extract, SLO and ASO were separately placed in three nearby holes were punched in a thin layer of 1% agarose, and allowed to stand for a day. During that time Antigens and antibody were diffused independently toward each other, and were observed visible line of precipitation. The pattern in which adjacent lines cross one another yields considerable information about the antigenic relationships between different antigens.

2.4.2 Slide Agglutination Inhibition technique

50 µl of Standard serum containing ASO with Agglutination severity of 3+ was mixed with 50 µl Alfalfa extract (0.25 g /cc) on slide; after 1 minute, latex particles that have been coated with Streptolysin O was added and read the result after 2 minutes of circular rotation. positive control include 50 µl ASO standard serum control & 50 µl Normal Saline and one drop of SLO reagent of passive agglutination method that were mixed on another slide.

2.4.3 Neutralization technique

A vial of 300 titer lyophilized ASO standard serum was received from reference lab for neutralization method. Serial dilution of Standard serum was prepared with buffer (3.17 g KH_2PO_4, 1.8 g Na_2HPO_4 and 7.4 g NaCl in 1 liter of distilled water and pH of 6.5) in 10 test tubes; 1:12, 1:24,1:48,1:96,1:192,1:384,1:768, 1:1536, 1:3072. Two tubes were considered as positive control (buffer) and negative control (standard serum).

0.5cc of the extract 0.25 g/ml was added to all test tubes and were incubated in 37 °C for 15 minutes. Then 0.5cc, 5% washed group O RBC suspension was added to all tubes and incubate for another 45 minutes in 37 °C, then centrifuge it for 1 minute with 1500 rpm and evaluate the hemolysis.

3. Results and Discussion

Alfalfa has a long history of medical usage but there is no evidence supporting the use of its root in medicine. This research is the first study for using Alfalfa root extract in diagnosis tests of infectious disease. Our study showed that Alfalfa root Extract hemolytic effect is similar to SLO by using RBC suspension. The antigenic similarity of Alfalfa root Extract and SLO were showed by the ouchterlony test, with observation of two precipitation bands that join together as continuous arc. (fig. 1)

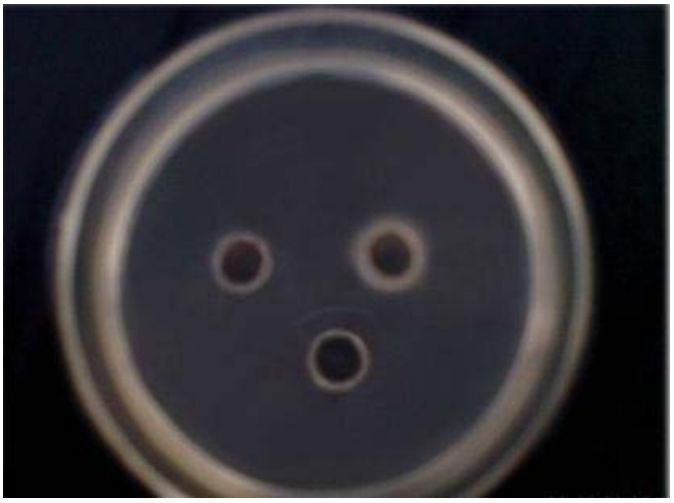

Fig 1: Ouchterlony test: Arc line precipitation between Alfalfa roots extract and SLO showed the similarity between these two antigens

The evaluation of Alfalfa root extract efficiency similarity by agglutination inhibition method showed that the Extract can attach to ASO serum and neutralize it, so that there is not free ASO in test. Therefore after adding SLO latex particle, no agglutination was seen. (fig. 2)

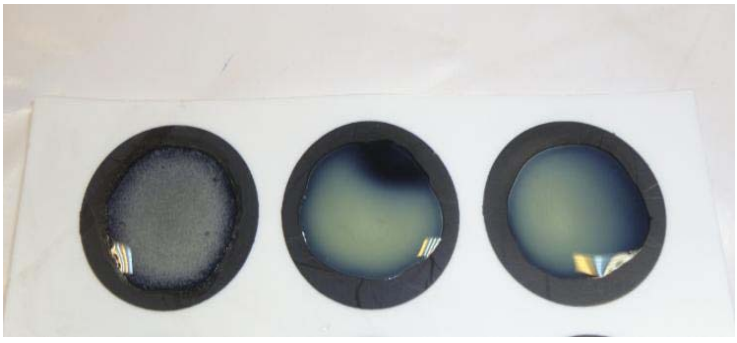

Fig 2: Agglutination inhibition test: reaction between ASO and SLO coated on latex particles (Left). alfalfa roots extract Neutralization reaction with ASO and inhibition the reaction between ASO and SLO-Latex particles (Middle, Right)

In neutralizing technique, there was no hemolysis from 1:12 to 1:384 dilutions. Because Alfalfa roots extract has neutralized with ASO and inhibited the hemolysis. While serum was diluted above 1:384, there was not antibody remained so Alfalfa extract was not neutralize and hemolysis observed in tubes (fig. 3).

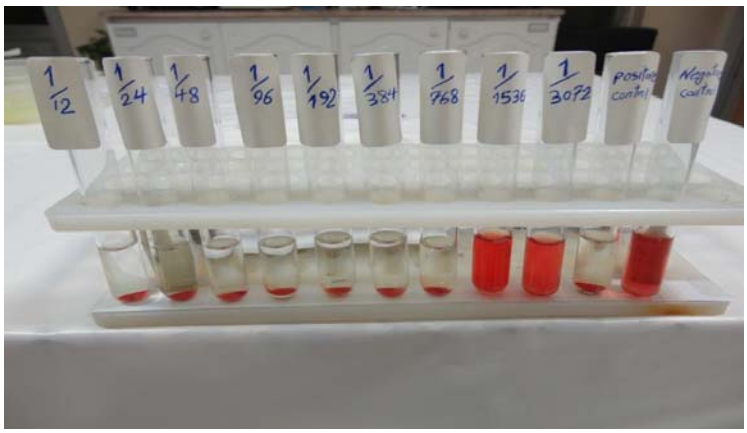

Fig 3: Neutralization Technique: Serial dilution of ASO serum, inhibition of hemolysis was observed to 1/768 test tube.

Since the SLO purification and recombinant DNA techniques are difficult to perform and technically demanding and the yield is low, therefore an alternative material is necessary. Our results suggest that Alfalfa root extract is suitable reagent which can replace the SLO reagent in diagnostic assays, because preparation of this extract is simple & cost effective.
Further investigation requires for developing industrial scale production of Alfalfa roots extract and mass production of diagnostic kits for S.pyogenes infections.

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