

Characterization of essential fatty acid semicarbazide (EFASC) from *Linum usitatissimum* as a safe and effective adjuvant.

Ahmad Ayad Hady¹, Mayyada F. Darweesh², Hayat khadum salman³

University of Kufa, Collage of science ,IRAQ ^{1,2}

Technical medical institute ,Baghdad³

Correspond author email: Mavada.alsagheeri@uokufa.edu.iq²

E.mail Ahmed.ayad97@yahoo.com¹, E.mail hyatkathem65@gmail.com³

Abstract

Bacterial infections are world- wide problem and resistant bacteria is increasing globally that lead to treatments failure. Thus, it is important to find new techniques that are effective for the treatment of antibiotic-resistant microorganisms. Green medicine used for the medication of different bacterial infection. Therefore, **the study aimed to evaluate the immunomodulatory activity of essential fatty acid semi carbazide (EFASC) compounds extracted from flaxseed to eradicate resistance pathogens**. Crude extracts of *Linum usitatissimum* were extracted by hexane for extraction of EFASC. The results of LD₅₀ appear that a live suspension of *E.coli* was 0.49×10^7 , while HK *E.coli* was 10^8 , 125 mg /ml were the optimum dose in stimulate immune response of EFASC which gave maximum dose of total WBC, Neutrophil and Monocyte count. The immunomodulatory activity appear that *E.coli* + EFASC released moderate levels of IL-4. HK *E.coli* release higher IL-4 lead to increase the hypersensitivity and hyper responses of immune system. HK + EFASC immunogen revealed moderate increase in IL-12 mean level 0.99 pg /ml, HK bacteria recorded 1.49 pg/ml, while the mean of EFASC revealed no significant difference compared with control. An effective protection was observed in immunized groups with HK *E.coli* and EFASC challenged with 100 of LD₅₀ of a live *E.coli*.

Key word : essential fatty acid semi carbazide, LD₅₀, IL-4, IL-12

INTRODUCTION

Bacterial infections are world- wide problem and resistant bacteria is increasing globally in both outpatients and hospitalized patients that lead to treatments failure and this lead to important cause of morbidity and mortality around the world (Li and Webster, 2018). Thus, it is extremely important to find novel antimicrobials or new techniques that are effective for the treatment of infectious diseases caused by antibiotic-resistant microorganisms. Efforts have been demonstrated that plants either contain antimicrobials activity that can operate in synergy with antibiotics or possess compounds that have no intrinsic antibacterial activity but are able to sensitize the pathogen to a previously ineffective antibiotic (Cheesman *et al.*, 2017).

Immunomodulatory agents originate from plant increases the immune responsiveness of the body against a future exposure to pathogens by activating of immune component cells, cytokine and also release of inflammatory mediator to eradicate infection (Chen *et al.*, 2018).

Seeds from *Linum usitatissimum* (Flaxseed), which contain about 36 - 40% of oil, are a rich source of unsaturated essential fatty acids (USFA): linolenic acid (Omega 3), linoleic acid (Omega 6) and oleic acid content, which cannot be synthesized by the human body and must be derived from the diet (Goyal *et al.* 2014). Fatty acid composition can alter the function of innate immunity by affecting on various components. The omega-3 and omega-6 fatty acids effect of on the STAT6 phosphorylation pathway that leads to the production of IgE antibodies when stimulated by cytokines such as IL-4 and IL-13. Such findings are significant in the future design of dietary therapeutics for the treatment of allergies (Ahmed *et al.*, 2016). The extraction of Essential fatty acid Semicarbazide (omega-3-6-fatty acid) from *Linum usitatissimum* revealed broad spectrum antibacterial property of oil compounds against multi-drug resistance *S.aureus*, and *E.coli* isolated from nosocomial infection (Hady *et al.*, 2017).

MATERIALS AND METHODS

1. Laboratory of Animals:

Hundred and thirty-two albino swiss rats (*Rattus rattus*), their age was 8-10 weeks, and their weight 23-27 g, were used as the experimental animals. They were housed in plastic cages with hard wood chips for bedding in an air conditioned room at 30 ± 5 °C. They were maintained on special pellets diet with free access to drink water, with natural light dark. The animal were given a week's time to get acclimatized with the laboratory condition, before experimentation. They were divided into groups, and each group was kept in separate plastic cage.

2 . Plant collection. Flaxseed were obtained from the local market in Al-Najaf city. The seeds were cleaned and the foreign materials were removed. The dried seeds were then powdered, and stored in a refrigerator at 4°C to await further processing (Harborne., 1984).

Preparation of oil. The flaxseed oil was extracted using hexan solvent (1: 4 w: v) in a Soxhlet apparatus (Preciso/,England) for 24 h. Then, EFA was isolated from the oil using a Cleavenger (Shepreth, England), according to (Borhad et al.,2014). Purity and identification of EFA-omega3 compounds by TLC was carried out according to (Harborne., 1984).

Preparation of EFA – Semicarbazide (EFASC).

One gram of EFA-omega-3 were dissolved in 4 ml of methanol and 1 : 1 H₂SO₄, then 4mg of thiosemicarbazide in methanol were added to this solution with constant stirring at room temperature for 4 h. Following this, NH₄OH was added till alkaline, then stirred for about 15 min and kept overnight. The resulting crystals was filtered, dried and recrystallized (Darweesh .,2017 a). A stock solution was prepared by dissolving 500 mg of dried extracts with 1 ml DMSO to give a final concentration of 500 mg/ml, from which a four concentration 62.5, 125, 250 and 500 mg/ml was prepared.

Determination the efficient dose of EFASC in stimulate immune system:

In order to determine the most effective dose in stimulate immune response by using EFASC, different concentrations 62.5, 125, 250 and 500 mg/ml from EFASC of linseed (oil) were testing their ability to stimulate immune system of rats by evaluate total WBC count as well as Neutrophil and Monocyte count counts. Used three rats (triplicate)for each concentration

3 .Determination LD₅₀ of *E .coli* : multidrug resistance *E.coli* bacterium obtained from laboratory in faculty of science and this bacteria diagnostic according to (MacFaddin,2000) . Vitek-2 Compact(Bio Mérieux,France,) used for confirmed identification. *E.coli* used in preparation bacterial suspension and heat killed vaccine. .

*Preparation of bacterial suspension

Brain hart infusion(BHI) broth were inoculated with a loop full of 18 hr of colonies and incubated over night at 37 C . The bacteria were washed twice in PBS with centrifugation for 20 min at 6000 rpm and re-suspended in PBS again , then optical density of absorbency has been read at 6000 nm, at 1 degree which represent 1×10^8 . The suspension was diluted by used PBS solution to prepared series 10-fold dilution (10^5 , 10^6 , 10^7 , 10^8 , 10^9 and 10^{10}) of CFU/ml respectively, six groups of albino rat with five replicates for each group were injected intraperitoneally with 1ml of *E.coli* suspension in one of the serial dilution. The control groups were injected with phosphate buffer. The live and killed rate were estimated proportional to total number of rate for each dose after five days and the **lethal dose (LD₅₀)** was estimated according to (Reed and Muench,1938).

Sterility of suspension, HK-*E.coli*, EFASC. Were determinate by culturing of both vaccines on blood and nutrient agar, while safety were detected by injected 3 rats with 1 ml of each vaccine for 1 week and monitoring clinical feature in rats.

4. Determination of LD₅₀ of heat killed *E.coli*

*Preparation of heat killed *E. coli*

The same steps in preparation *E. coli* suspension but the series 10 fold dilutions (10^5 , 10^6 , 10^7 , 10^8 , 10^9 and 10^{10}) were killed by heating in water bath at 80 C° for 1 h then the concentration of heat killed vaccine compare

with standard series dilution that prepared firstly(yosif *et al.*,2013). The sterility of both suspension was determined by cultured on nutrient agar media for 24 hour at 37°C to make sure all bacteria was killed.

The lethal dose 50% of heat killed *E.coli* was determined by injection of 30 rats that divided randomly into six groups with 5 rats for each group , injected 1ml intraperitoneally(IP) of 10 fold dilutions of the HK *E. coli* respectively , the control group injected with 1ml of sterile PBS , all rats were observed for 5 days .

5. Immunization of Rats

The 36 rats were divided equally into three groups and immunized I.P. with either 1ml of 125 mg/ml of EFASC or 0.1 LD₅₀ HK *E. coli* and third group treated with 0.5 ml of EFASC and 0.5 ml of HK *E.coli* vaccine , 3 rats treated with 1 ml of 0.1 LD₅₀ of *E.coli* suspension as positive control and 3 rats injected with normal saline as negative control group . Injection was given two doses (1st and post dose) at 14 days intervals. At the end of each period, 3 rats were killed from 1,2 and 3 group while the 4 and 5 group gave first dose only then blood samples taken. Each sample was divided into two parts: one kept in ethylene diamine tetra acetic acid (EDTA) tubes in order to evaluate WBC count by (Genx ,USA) and the second part for Sera separation which was stored at -20°C until used for measuring cytokines levels (, IL-4 and IL-12) by ELISA as per manufacturer's instruction (Elabscience, USA) .

6.Challenge test :

After one month from post dose of immunization for each type of (EFASC and HK+EFASC) , ten rats were Challenge Intraperitoneally with 1ml 100 LD₅₀ of *E.cole* suspension while control group give only Challenge dose . The relative degree of protective afforded by vaccine was assessed by the number of rats surviving 5 days after challenge then the rats were sacrificed and drawn the blood (Darweesh.,2017 b) .

Ethical Approved :This study was approved by the ethical and research committee of College of Medicine (No 23024), University of Kufa, Ministry of High Education and Scientific Research

Statistical analysis :The results are presented as means \pm standard error (S.E.) and were analyzed using one-way analysis of variance (ANOVA) test via Graph pad prism 5.04. $p < 0.05$ was considered significant.

Results and Discussion

Determination the efficient dose of EFASC in stimulate immune system:

The result explain that total WBC count , Neutrophil and Monocyte count were significantly increased ($P < 0.05$) in rats treated with 125 mg/ml of EFASC of Flaxseed oil 11.600 ,72.00 \pm 0.57 and 6.14 \pm 0.05 cell/ mm respectively as demonstrated in Table (1) . From these results, the concentration 125 mg/ml flaxseed oils was the efficacy in stimulating the immune response therefore chosen to complete the study and testing the immune response.

Table (1): WBC Count, Neutrophil and Monocyte count to determination the optimum dose of EFASC to stimulate immune response.

| Dose mg/ml | WBC count | Neutrophil count | Monocyte count |
|------------|-------------------|------------------|-----------------|
| 62.5 | 9.600 \pm 0.10 | 70.3 \pm 0.05 | 6.6 \pm 0.057 |
| 125 | 11.600 \pm 0.57 | 72.0 \pm 0.57 | 7.14 \pm 0.05 |
| 250 | 8.300 \pm 0.5 | 70.9 \pm 0.05 | 5.9 \pm 0.05 |
| 500 | 6.900 \pm 0.57 | 68.6 \pm 0.057 | 6.7 \pm 0.057 |
| control | 7.900 \pm 0.5 | 66.5 \pm 0.05 | 6.3 \pm 0.05 |

LSD (0.05) = 0.430

1.659

1.325

Estimation of median lethal dose LD₅₀ of a live bacterial suspension:

The results of LD₅₀ estimation for *E.coli* suspension revealed that the LD₅₀ was 0.49×10^7 CFU/ml

Table (2) LD₅₀ of bacterial suspension of *E. coli*

| No. of bacteria | Number of injected Rat | Number of survived Rat | Number of dead Rat | Accumulated number of survived and dead Rat | | | |
|------------------|------------------------|------------------------|--------------------|---|----------|-----------------------|-------------------------|
| | | | | survived Rat | Dead Rat | survived and dead Rat | Percentage of mortality |
| 10 ¹⁰ | 5 | 0 | 5 | 0 | 15 | 15 | 100 |
| 10 ⁹ | 5 | 1 | 4 | 1 | 10 | 11 | 90.91 |
| 10 ⁸ | 5 | 2 | 3 | 3 | 6 | 9 | 66.66 |
| 10 ⁷ | 5 | 3 | 2 | 6 | 3 | 9 | 33.33 |
| 10 ⁶ | 5 | 4 | 1 | 10 | 1 | 11 | 9.09 |
| 10 ⁵ | 5 | 5 | 0 | 15 | 0 | 15 | 0 |

The results of LD₅₀ for Heat killed *E.coli* revealed that the LD₅₀ was 10⁸ table(3).

Table (3) : LD₅₀ of heat killed *E.coli*

| Concentration of Bound EPS | Number of injected Rat | Number of survived Rat | Number of dead Rat | Accumulated number of survived and dead Rat | | | |
|----------------------------|------------------------|------------------------|--------------------|---|----------|-----------------------|-------------------------|
| | | | | Survived Rat | Dead Rat | survived and dead Rat | Percentage of mortality |
| 10 ¹⁰ | 5 | 0 | 5 | 0 | 12 | 12 | 100 |
| 10 ⁹ | 5 | 2 | 3 | 2 | 7 | 9 | 77.77 |
| 10 ⁸ | 5 | 3 | 2 | 5 | 4 | 9 | 44.44 |
| 10 ⁷ | 5 | 4 | 1 | 9 | 2 | 11 | 18.18 |
| 10 ⁶ | 5 | 4 | 1 | 13 | 1 | 14 | 7.14 |
| 10 ⁵ | 5 | 5 | 0 | 18 | 0 | 18 | 0 |

1: Concentration of Interlukin-4 in Serum of Rat

The Concentration of IL-4 was significantly ($p < 0.05$) increased in the rats treated with HK bacteria (2.09 ± 0.07 pg/ml), EFASC obtained (0.99 ± 0.03 pg/ml) but HK+ EFASC and Bacteria suspension gives moderate raised in IL-4 serum level (0.81 ± 0.07 pg/ml), (0.68 ± 0.02 pg/ml) respectively as compared with control group (0.39 ± 0.03 pg/ml) as shown in figure (1).

The results showed high significance elevated at ($p < 0.05$) in concentration of IL-4 in post dose of HK *E.coli* (2.81 ± 0.04 pg/ml), then EFASC (1.92 ± 0.05 pg/ml), while HK+ EFASC record moderate elevation in IL-4 serum level (1.35 ± 0.12 pg/ml) Table (4).

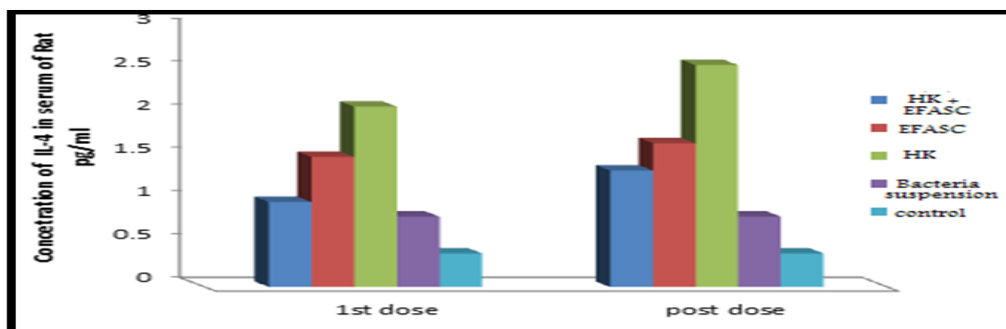
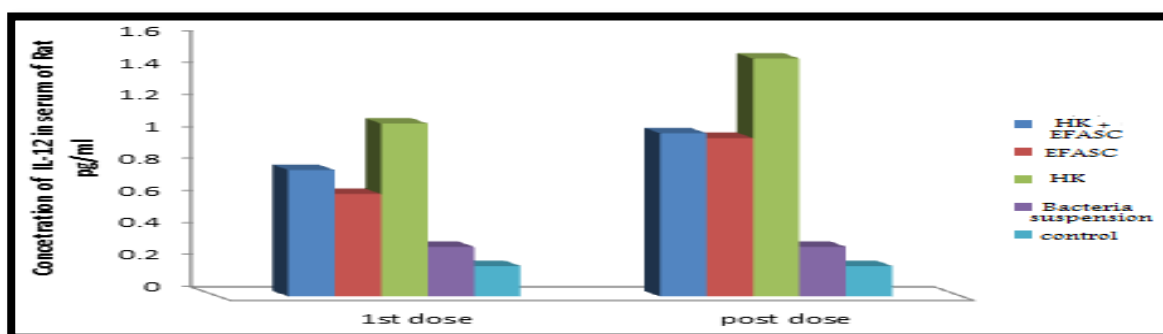


Figure (1) Mean Serum Concentration of IL-4 in rat treated with HK+ EFASC, EFASC, HK, Bacteria suspension and normal saline as control.

2: Concentration of Interlukin-12 in Serum of Rat:

Figure (2) shows the Concentrations of IL-12 in the serum of studied group revealed increased serum level of IL-12 significantly ($p < 0.05$) compared to control group, the rats treated with 0.1 LD₅₀ of HK bacteria gave maximum serum level of IL-12 in first and post doses (1.17 ± 0.14 and 1.49 ± 0.29 pg/ml), while the mean of EFASC revealed no significant different compare with control (0.22 and 0.29 pg/ml) in first and post dose respectively. HK + EFASC immunogen revealed moderate increased in IL-12 mean level (0.81 , 0.99 pg/ml) in compare with control group that recorded (0.16 pg/ml).



Figure(2) Mean Serum Concentration of IL-12 in rat treated with HK+ EFASC, EFASC, HK, Bacteria suspension and normal saline as control.

Table (4) : Concentration of IL-4 ,IL-12 in Serum of Rat treated with :

| | Dose | IL-4pg /ml | IL-12 pg/ml |
|----|-----------------|-------------------|-------------------|
| HK | 1 ST | $2.09 \pm 0.07^*$ | $1.17 \pm 0.14^*$ |

| | | | |
|---------------------|-----------------|-------------------|-------------------|
| | Post | $2.81 \pm 0.04^*$ | $1.86 \pm 0.1^*$ |
| EFASC | 1 st | $0.99 \pm 0.03^*$ | $0.22 \pm 0.05^*$ |
| | Post | $*1.92 \pm 0.05$ | $0.29 \pm 0.06^*$ |
| HK + EFASC | 1 st | 0.81 ± 0.07 | $0.64 \pm 0.03^*$ |
| | Post | $1.35 \pm 0.12^*$ | $0.99 \pm 0.01^*$ |
| Bacteria suspension | D | $0.81 \pm 0.02^*$ | $0.51 \pm 0.03^*$ |
| control | D | 0.39 ± 0.03 | 0.16 ± 0.09 |

*P ≤ 0.05, SE: Standard error.

L.S.D =

0.153

0.068

HK=(Heat killed *E.coli*)

Challeng test

The results explored after challenge dose that vaccinated rats were protected against challenge dose. HK+EFASC give 100% protection against challenge dose, while HK 90% protection, 0% protection for control group this showed in table (5).

Table (5): Percentage of survived Rats after challenge dose

| Groups | Number rats | Number of survived Rats after challenge dose | Percentage of survived Rats |
|------------------------------|-------------|--|-----------------------------|
| HK + EFASC | 10 | 10 | 100% |
| HK | 10 | 9 | 90% |
| Control bacterial suspension | 10 | 0 | 0% |

Discussion

The result of Median lithal dose (LD₅₀) of *E.coli* was similar to results done by Stork *et al.*, (2018) they found that LD₅₀ of *E.coli* was 3×10^7 . The LD₅₀ for *E.coli* suspension were 6×10^6 CFU/rat by (Kun Cai *et al.*, 2015). while Baker, (2015) who found that LD₅₀ of a live *E.coli* suspension was 0.3×10^8 CFU/rat. The reasons for the difference in the LD₅₀ in various studies back to the pathogenesis strain used in the experiment, isolated place, type of experimental animals in addition to the strength of the immune system to hit the animal used in the experiment.

The results of LD₅₀ for heat killed *E.coli* were similar to (Yousif *et al.*, 2013) they found that LD₅₀ of H.K. *E.coli* was 1×10^8 . In same line Darweesh, (2017 b) Who isolated *E.coli* from recurrent UTI observed that Median lethal dose of heat killed *E.coli* suspension that killed half of animal group was 2.3×10^8 .

The results that evaluate the humeral immune response according to type of treatment (HK *E.coli*, Bacterial suspension, EFASC and HK+ EFASC explain that concentration of IL-4 varied with the type of treatment.

Interlukin-4 is a cytokine that participates in the regulation of the immune system at multiple levels, it promotes Th2 lymphocyte (B-cells and T-cells) development, growth, differentiation and survival, also enhance cytotoxic T-lymphocyte activity (Velde *et al.*, 2016). Interlukin-4 is a Th2 cytokine, small upregulation of IL-4 early in the infection since the activation of Th-1 and inhibition of Th-2 provide an ineffective defense against the development of a bacterial infection (Spellberg and Edwards, 2001). These results agree with a study done by AbdAlhussen and Darweesh, (2016) They found that *Pantoea spp* suspension a member of Enterobacteriaceae family, giving low increased in level of IL-4 in comparison with control group, it is also, matching with a study by Vanaja *et al.*, (2016). they found that gram negative bacteria such as *E.coli* and *Serratia merrisense* cause low or not detected increased levels in IL-4 during early infection.

Kaithwas *et al.*, (2011) illustrated anti-inflammatory activities of flaxseed oil against inflammatory mediator like histamine, leukotriene and prostaglandine as well as cytokine mediator like IL-1, IL-6 and TNF- α . The inhibition activity of flaxseed oil could be for α -linolenic acid an omega-3 fatty acid content. Van Amersfoort *et al.*, (2003) pointed out that lipopolysaccharide a component of G-ve bacteria can enhance immune response in animal like increased production of pro-inflammatory cytokine but when these animals fed with conjugated linoleic acid significantly reduced these adverse effects of endotoxin.

Hosseini-Mansoub and Bahrami, (2011) explain that the addition of unsaturated fatty acid to broiler chick's diet may stimulate the development of the immune response by activating humeral immune response ex, IL-4. also result by Wu *et al.*, (2019) who study the effect of omega-3 PUFAs on T cell function in mice, they observed the increase of the percentage of Th2 polarized cells and suppressed Th1 cell proliferation, shifting the Th1/Th2 balance toward a more humoral immune response. In the same line Lee *et al.*, (2019) stated that flaxseed oil rich in omega-3 PUFA and omega-6 PUFA, respectively, significantly improved antibody response to ND vaccination.

Wu and Schauss (2012) revealed that diet can play an important role in attenuating and mitigating chronic pro-inflammatory processes associated with chronic diseases and infection, also they found elevated in IL-4 level and decline in IL-6, IL-1, IL-12, TNF- α during anti-inflammatory dietary factors including fruit, nuts, whole grains (flaxseeds) and fish oil. The activation of the innate immune system by the Western diet is likely caused by an excessive production of pro-inflammatory cytokines associated with a reduced production of anti-inflammatory cytokines, on the other hand certain dietary factors, including adequate omega-3 fatty acids are associated with a lower incidence of chronic diseases and could modulate immunomodulatory and inflammatory processes (Gammone, *et al.*, 2019).

IL-12 is a heterodimeric cytokine produced by activated antigen presenting cells during inflammation by inducing proliferation of natural killer cells, proliferation T-helper and cytotoxic lymphocytes and production of cytokines particularly of IFN- γ (Zheng *et al.*, 2016).

The present study is in accordance with several studies which revealed that gram negative bacteria like *E.coli* , *Veillonella parvula* , *Serratia* induced significantly higher levels of pro-inflammatory cytokines production which are associated with cell-mediated immune response that characterized by production of cytokines such as IL-12 and INF- γ and enhance resistance against reinfection (Ulrik Engelsöy *et al.*,2019). In same line , Ramachandran,(2014) observed that infection with G-ve bacteria like *Salmonella* stimulate Th-1 adaptive immune response by increasing IL-12 , IL-2 production which enhance the resistance against reinfection , this is possibly related to the strong pro-inflammatory effect of LPS component of G-ve bacteria .

In mice, individual dietary omega-3 –PUFA were found to be sufficient to reduction of IL-12 and INF- γ which may have important implications for host infectious disease resistance (Kevin *et al.*,2016).

The results of this study agree Bassaganya-Riera *et al.*,(2012) they found that dietary with USFA , enhanced cellular immunity by modulating effector functions of CD8 T-cells.

Piya *et al.*,(2013) explained that LPS released from the cell wall of gram-negative bacteria induces inflammation through production and release of cytokines(such as IL-6,IL-1 family, IL-12 and TNF- α), leukotrienes, and prostaglandins . PUFA supplementation has been shown to decrease LPS-induced chronic inflammation and explain the ability of omega-3 in altering cytokine production and macrophage recruitment . In same line (Tabbaa *et al.*,2013) confirmed that omega-3 PUFA supplementation helped to resolve LPS-driven inflammation by reducing the macrophage pro-inflammatory response to LPS associated with *E. coli* and was to assist in clearing the pathogenic bacteria .

The results showed that rats group immunized with EFASC+ HK was best in resist the challenge dose where record the 100% protection while 1 rats were lost form HK in contrast with control group that lose all rats. Ruqin *et al.*(2017) demonstrated that mice treated with serum from mice immunized with *E.coli* revealed higher degree of protection against lethal challenges with live *E.coli* in comparison with untreated mice . These results are in the same line with Olsen *et al.*(2017)they found that sera drawn from treated mice with live bacteria showed protective capacity against protein epitopes of antibiotic – exposed *E.coli* and this as a result that treated mice contain antibodies towards protein epitopes. .Minor ,(2015) explain that challenge results depended on the amount of the dose ,time required , type of pathogen , virulent pathogen.

Mayyada F.Darweesh : <https://orcid.org/0000-0001-5901-9396>

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