

## Immunostimulant Effect of Egyptian Propolis Extract against Parapox virus in experimental Rats

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**Abstract:** Vaccination has been important in controlling a wide variety of viral infections of man and animals. The most vaccines depend on the association with adjuvant substances. The novel approaches which are presently being implemented to develop more effective vaccines. Evaluation of adjuvant capacity of ethanol and water extract propolis associated to inactivated Parapoxvirus vaccine (IPPV) were tested by ELISA. Inoculation was done intradermal and subcutaneous with inactivated PPV vaccine adjuvant with ethanol and water extract propolis with two doses, two week interval. Serum samples were collected for 4 weeks after the last vaccination till the 7<sup>th</sup> week and the antibody were detected using ELISA assays. Parapox virus vaccine adjuvant with propolis extract higher PPV antibodies levels comparing with antibody titer in rats sera that received commercial PPV vaccine and control negative groups. The ODs ELISA titers increased from 0.255 to 0.755 and this difference remained at the end of experiment at 42 days. The use of propolis extract alone without PPV did not induce significant levels of antibodies. However, it established that it was able to increase immune response, evidenced by the increase in the percentage of antibody titer against PPV. The effect of propolis extract on the humoral immune responses may be exploited for the development of effective vaccines. Both these novel vaccine approaches appear to be more efficacious than live PPV vaccines. The present findings encourage us for using propolis as an immunostimulant with human and animal vaccines.

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### 1. Introduction

Parapoxvirus is a highly contagious, zoonotic, viral skin disease that affects sheep, goats and some other domesticated and wild ruminants (Bhanuprakash et al. 2006). The most PPV infections in humans are localized and heal spontaneously. However, large, poorly healing lesions can occur in people who are immunosuppressed (Mondal et al. 2006). Several studies have pointed out that vaccination is an effective method for controlling the disease (Inoshima et al. 2001 and 2002).

The successful vaccination depends on their association with potent adjuvant which can increase the immunogenicity of vaccine. A better adjuvant can activate specific effectors of the immune system, such as cytotoxic or auxiliary T cells (Th1/Th2) (Leclerc, 2003), and strengthen the humoral and/or cellular immune responses against that antigen (Barr et al. 2006). On the other hand, suitable adjuvant should have lower toxicity and side effects (Aguilar et al. 2007). Therefore, it is urgent to develop a new adjuvant with high efficacy, safety and low cost. Although alum (aluminum hydroxide or aluminum phosphate) is widely used as a vaccine adjuvant, its

role in nephrotoxicity, Alzheimer's disease, subcutaneous local reaction, epitope modifications, its inability to induce T-lymphocyte 1 (Th1) antibody isotypes or cellular immune responses, and its limited adjuvant effect on polysaccharide antigens underscore the need to find new potent safe adjuvants (Aguilar and Rodriguez 2007) and (Heinrich et al. 2008). Propolis is a compound formed by honeybees with their mandible gland secretion and various plant resins. It contains many constituents, such as flavonoid, organic acid, aromatic alcohol, esters, amino acids and enzymes so that it has various biological activities including immune enhancement, antibiosis, antiviral, antioxidation, anticancer, anti-fatigue, hepatoprotection (Sforcin, 2007). Propolis also shows antiviral (Vynograd et al. 2000), antifungal (Sforcin et al. 2001). Propolis extraction methods may influence its activity, since different solvents solubilize and extract different compounds. The most common extracts used in biological assays are ethanol, in different concentrations, ethanol and water (Salom et al. 2004). Its chemical composition is very complex: more than 300 components have already been identified, and its composition is dependent upon the source plant and local flora.

Moreover, propolis composition is completely variable creating a problem for the medical use and standardization (De Castro, 2001). After its administration to mice or to humans propolis does not seem to have side effects (Sforcin, et al. 2002, Burdock, 1998).

Propolis is non-toxic, and its LD50 ranges from 2 to 7.3 g / kg in mice. No significant alterations in total lipids, triglycerides, cholesterol, HDL-cholesterol concentrations, nor in AST and LDH specific activities were observed (Mani et al. 2006). A number of studies have also addressed the use of propolis as an adjuvant for viral vaccines. These studies have demonstrated propolis superiority as a potent adjuvant over oil-emulsion, and lower toxicity than Freund's adjuvant in inactivated Newcastle disease virus in chickens (Mu et al. 2007), its role in increasing the protection index in pigeons immunized by the avian paramyxovirus inactivated vaccine (Zou et al. 2002), its effect in inducing a safe, more efficient vaccine when formulated with the canine distemper virus of mink (Wang, 2008), its role in increasing the protection index of the inactivated hepatitis virus of ducks (Chen et al. 2000), its ability to act as potent adjuvant for the DNA vaccine against the infectious bursal disease virus (Yu et al. 2004), and its use as adjuvant for the inactivated hemagglutinating encephalomyelitis virus vaccine of pigs (Chen et al. 2009) and (Qiao et al. 2011).

In 2012, an Egyptian vaccine against *K. pneumonia* was formulated with the alcoholic extract of the Egyptian brown propolis (Ahmad et al. 2012). White New Zealand rabbits were injected with the propolis formulated vaccine which required a lower dose of the antigen to produce similar levels of antibodies. Moreover, the antibody titer responded earlier and persisted for a longer period (Ahmad, 2012). The immunostimulant effect of the ethanolic extract of the Egyptian propolis was assessed with the inactivated *Pasteurella multocida* vaccine for rabbits. The oral or subcutaneous administration of the formulated vaccine proved to be safe, had no adverse reactions on the rabbit's health, enhanced specific and non-specific immune response of the vaccine, and reduced the severity of adverse clinical symptoms and mortality rate among the tested rabbits (Nassar et al. 2012). The present study aimed to evaluate immune enhancement of propolis adjuvant with inactive PPV compared with commercial PPV vaccine as controls, offer theoretical evidence for exploit propolis into the adjuvant vaccine

## Material and methods

### 1. Preparation of Propolis extract.

Egyptian propolis was commercial purchased Beeswax honeycomb processing. The propolis

adjuvant was prepared as previously described (Paulino et al. 2002). Briefly, the propolis was ground and macerated with absolute ethanol for 10 days, agitation 10 min daily. Then, the solvent was evaporated and the resulting dried matter was dissolved in phosphate buffer solution (PBS, pH 6.2), in a final concentration of 40 mg /ml. Water extract propolis was extracted with water at 50 °C, and its main constituents were previously reported by (Schnitzler et al. 2009).

**Control virus:** Reference strain (orf vaccine, Scabivax UK) strain were used as controls.

### Preparation of Inactive Parapox virus

PPV was supplied from Veterinary Division of National Research Center Cairo Egypt. After it was propagated and titrated in SPF. Inactivated PPV according to the method described by Madbouly et al. (2006), with bromoethylamine BEI (C2H7Br2N, Merck), in a final concentration of 0.02 M and pH 7.5, the viral suspension with titer of 10<sup>6</sup> EID50/ml (Embryo infections dose 50% / ml). The inactivated virus contained 10<sup>6</sup>EID50/ml as the vaccine antigen. After inactivation PPV was adjuvanted with E199&FCS or propolis extract respectively. Their 10<sup>6</sup> EID50/ml virus contents were the same in two vaccine.

### Experimental animals

This study was carried out according to guidelines for animal experimentation and approved by the institutional animal care and use committee, National Research Center animal care unit, Dokki, Giza, Egypt. 25 Albino rats, about 250-300 g body weight purchased from Animal house of National Research Center Cairo Egypt, were randomly divided into 5 groups then housed in wire cages, lighted for 24 h receiving feeding and water, used in experimental vaccine efficacy.

### Experimental Design

A total of 25 Albino rat were assigned into 5 groups (each containing 5 animals). Each group was injected by interdermal(I/D)/subcutaneous(S/C) route(dose of 50 mg/kg B. W) by 0.5 ml / rat, all rats received two doses interval between first and second dose 2 weeks apart as follows:Group1 (G1): injected ID/SC with saline as control negative, Group2(G 2): injected ID/SC with 0.5 ml from PPV antigen, Group3 (G 3): injected ID/SC with 0.5 ml from PPV adjuvant with ethanol propolis extract, Group4 (G 4): injected ID/SC with 0.5 ml from PPV adjuvant with a water propolis extract ID/SC and Group5 (G 5): injected ID/SC with 0.5 ml from PPV vaccine. Blood samples were collected every 0, 3, 7, 9, 14, 21, 28, 35 and 42. The experiment continue for 2 months, collected (0.5 ml / rat) from eye drawn into

Eppendorf tubes and allowed to clot at 37 °C for 1 h. Serum was separated by centrifugation then stored at -20°C until used.

#### ELISA Technique

The indirect enzyme-linked immunosorbent assay (ELISA) technique was used for quantitative of antibodies according to the method described by **Hubschle et al. 1981**. The 96-well plates coated with inactivated purified PPV antigen were incubated with diluted antisera (1:100) followed by incubation with an enzyme labeled preparation of anti-immunoglobulin. The color change in each well was estimated spectrophotometrically at 450 nm.

#### Statistical analysis

Antibody titers were compared using Statistical analysis, We used SPSS software version 16. Data were analyzed significant between groups' small at  $t < 0.05$  and highly significant at  $t < 0.01$  by using T test.

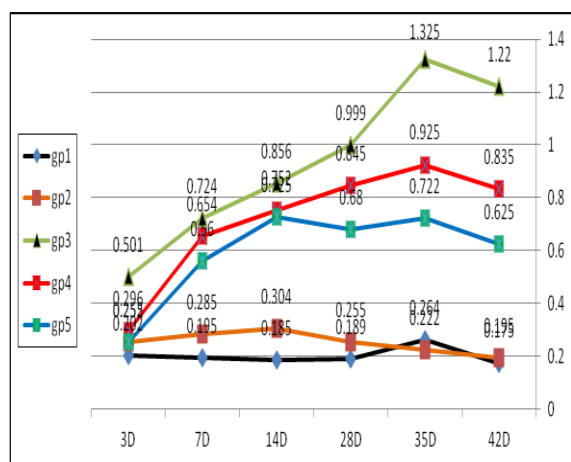
## Results

### Immune response test

Our results revealed that novel adjuvant to enhance antibody titer is an attractive option to improve the PPV vaccine. The results of the indirect ELISA for the measurement of antibodies titers of every group are shown in Table (1). As compared with the control negative G1 and control positive G 5, the antibody titers in all vaccination groups increased significantly from day 7–42. On days 7 and 14 after vaccination, the antibody titers in vaccinated group were higher than those of G 5 and groups ( $t > 0.05$ ). On day 21, 35 and 42, the antibody titers in gp3, gp4 were highest than those of gp5 but there were not significant differences ( $t > 0.05$ ). On day 28, the antibody titer in gp5.

**Table (1): Examined of different collected inoculated rats sera groups by ELISA at wave length of 450 nm.**

Groups	D3 Means ODs ± SD	D7 Means ODs ± SD	D14 Means ODs ± SD	D28 Means ODs ± SD	D35 Means ODs ± SD	D42 Means ODs ± SD
G1	0.202±.0087*	0.195±.003*	0.185±.0050*	0.189±.0050*	0.245±.0195*	0.175±.0047*
G2	0.252±.0180*	0.285±.008	0.304±.0102*	0.255±.0266*	0.222±.0254**	0.195±.0105
G3	0.501±.0270*	0.724±.010	0.856±.0152*	0.999±.0227*	1.325±.0694**	1.22±.0658
G4	0.296±.0076*	0.654±.025	0.752±.0208*	0.845±.0088*	0.925±.0088	0.832±.0435*
G5	0.255±.0110*	0.560±.075	0.700±.0498*	0.680±.0235	0.722±.0386	0.625±.0435



• D: means day

**Figure (1) Means of immune response for different groups against PPV vaccine detected by ELISA at wave length 450 nm.**

Group 1: blank control with saline, group 2: PPV antigen with MEM and FCS, group 3: PPV adjuvant a mixture propolis ethanol extract ID/SC, group 4: PPV

adjuvant with a mixture propolis water extract ID/SC, group 5: control positive PPV vaccine ID/SC

T: Significant between groups at  $t < 0.05$  using CI = 95 % and highly significant at  $t < 0.01$ , by using CI = 99 %, by using analysis of one simple t test, \* High significant at  $t < 0.05$ . Similar letters in the same row means non-significant changes. Different letters in the same row means significant changes at  $t < 0.05$ . \*\* Highly significant at  $t < 0.01$  by using CI = 99 %

Group 1(gp1): blank control injected with saline, group 2 (gp2): PPV antigen injected with MEM and FCS, group 3 (gp3): injected with PPV adjuvant with ethanol propolis extract, group 4(gp4): injected with PPV adjuvant with water propolis extract, group 5 (gp5): control positive injected with PPV vaccine

## Discussion

Vaccines require optimal adjuvants including immuno-stimulants and delivery systems to offer long term protection from infectious diseases in animals and man. The current study was aimed to investigate the immunostimulant effect of propolis extract. Due to the high chemical complexity of propolis, it is

extremely difficult to identify which substances are responsible for its biological activities (**Ozkul et al. 2005**). (**Sforcin, et al. 2005**). The majority of vaccines requires association with adjuvant capable of increasing the potency or stimulating the appropriate immune response. Propolis is a resinous hive product collected by honey bees from various plant sources. It contains more than 160 constituents that have several biological and pharmacological properties such as antimicrobial, antiinflammatory, immunomodularity, and antioxidant effects (**Bankova, 2005, Storni et al. 2005 and Orsi et al. 2006**) have many of the mechanisms of action are still unknown.

Propolis is a natural product and safe for human and animal. In many countries it has been approved as health food or medicine. The development of new vaccines will be highly benefited with the identification of new substances capable of promoting and directing to a proper immune response (**Singh and Hagan, 2002**). Oil adjuvants, which are no longer in use for humans due to their adverse effects (**Lindblad, 2000**) are still largely used in vaccines for veterinary use. These oil emulsions, used specially in association with inactivated antigens, potentiate the immune system through the formation of a deposit at the inoculation site, with slow and long release of the antigen (**Cox and Coulter, 1997**). However, these inactivated vaccines need periodic revaccinations to produce an efficient immunological response (**Fenner et al. 1993**). In the present study, conducted in rats, no adverse effect was observed due to the association of propolis with vaccine against PPV.

Our results revealed that the ethanol and water extract propolis associated with an inactivated PPV were evaluated comparing with control positive reference PPV vaccine by neutering antibody titer. The ELISA positive titer of PPV antibodies in group 3, 4 and 5(vaccinated with PPV vaccine). Antibody titers in groups 3 and 4 treated with the IPPV adjuvanted with ethanol and water propolis extract by SC/ID routes were higher than that of the control positive reference vaccine group5 and may be attributed to the ability of propolis for modulating the synthesis of antibodies as in Table (1) and Fig (1). This finding agreement with (**Orsolich et al. 2005**) found that the vaccine with propolis increased the potency of the humoral immune response when compared to the vaccine without propolis as suggested by (**Cox and Coulter, 1997**). The evaluation of humoral and/or cellular responses induced by a vaccine. Comparing the vaccines with or without propolis, increase percentage of immunized group with the antigen and the propolis extract was also surprising, these results are similar to the ones obtained by (**Banskota et al. 2001**). increase in the humoral as well as the cellular immune response for

PPV adjuvant with water and ethanol extract propolis. Finally the association of propolis increased the potency of the humoral and cellular responses.. Our results revealed that both water and ethanol propolis extract can improve the immune response against PPV vaccine

## Conclusion

The use of an adjuvant to enhance antibody production is an attractive option to increase the vaccine efficacy. A potent immunostimulant effect of mainly water and ethanol propolis extract allow them to be used as new adjuvants for PPV vaccine. The use of propolis extract as an adjuvant might contribute for the efficacy of vaccines. It can also increase the potency of vaccines

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