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RESEARCH ARTICLE

Investigation of adjuvant effect of viscum album and aesculus hippocastanum in FMD vaccines

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Şap aşılarında viscum album ve aesculus hippocastanum'un adjuvant etkisinin araştırılması

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Öz

Amaç: Kobaylar üzerinde yapılan bu çalışmada, şap hastalığına karşı uygulanan aşılara at kestanesi tohumu tozu ile ökseotunun kurutulmuş yaprak tozunun eklenmesiyle elde edilen nötralize edici antikor titreleri arasındaki ilişkiler araştırılmış, veri analizinde En Küçük Kareler Regresyonu (QLS) kullanılarak bu yöntemin veteriner aşı çalışmalarında uygulaması gösterilmiştir.

Gereç ve Yöntem: Deneysel şap aşıları deri altı yolla üç gruba uygulandı. Üç gruptaki nötralize edici antikor titre seviyelerinin (NATL) homologuos virüs nötralizasyon testine (VNT) göre farklı olup olmadığı istatistiksel olarak analiz edildi. Aşılama sonucunda hayvanlarda oluşan NATL'yi ölçmek için hayvanlardan kan örnekleri alındı. Bu kan örnekleri aşılamadan sonra altı kez (7, 14, 28, 60, 120 ve 270. günlerde) genel anestezi altında alındı ve NATL ölçümleri yapıldı. Elde edilen boylamsal verilere ilişkin analizler QLS ve Genelleştirilmiş Kestirim Eşitlikleri (GEE) yöntemleri ile gerçekleştirildi.

Bulgular: NATL değişkeninde (yanıt değişkeni), ölçüm sürelerinin (gün) hem QLS hem de GEE yöntemlerine göre anlamlı olmadığı görüldü (p> 0.05). Benzer şekilde her iki yöntemde de grup etkisi de anlamlı bulunmadı (p> 0.05).

Öneri: NATL değişkeni üzerindeki grup değişkeni etkisinin istatistiksel olarak anlamlı bulunmaması, şap aşılarına at kestanesi tohum tozu ve ökse otu kurutulmuş yapraklarının eklenmesinin gruplar üzerindeki etkilerinin farklı olmadığı anlamına gelmektedir.

Anahtar kelimeler: Şap hastalığı, aşı, *viscum album*, *aesculus hippocastanum*, yarı en küçük kareler regresyonu

Abstract

Aim: In this study on guinea pigs, the relationships between neutralizing antibody titers produced by the addition of powder of Horse Chestnut seed and powder of Mistletoe dried leaves to the vaccines against Foot- and- Mouth Disease (FMD) were investigated and Quasi-Least Squares Regression (QLS) method in data analysis was introduced for the veterinary vaccine studies.

Materials and Methods: The experimental FMD vaccines were administered to three groups by subcutaneous route. Whether the neutralizing antibody titer levels (NATL) in the three groups were different according to the homologuos virus neutralization test (VNT) was statistically analyzed. Blood samples were taken from the animals to measure NATL generated in animals as a result of vaccination. These blood samples were taken under general anaesthesia, six times after vaccination (7, 14, 28, 60, 120 and 270 days), NATL measurements were made. Analyzes on the longitudinal data obtained were performed with QLS and Generalized Estimation Equations (GEE) methods.

Results: It was observed that the measurement times (days) in NATL variable (response variable) was not significant in both QLS and GEE methods (p >0.05). Similarly, the group effect was not found to be significant in both methods (p> 0.05).

Conclusion: The fact that the group variable effect on NATL variable was not found to be statistically significant means that the effects of the addition of powder of Horse Chestnut seed and Mistletoe dried leaves to the FMD vaccines on the groups were not different.

Keywords: Foot-and-mouth disease, vaccine, *viscum album*, *aesculus hippocastanum*, quasi-least squares regression

Introduction

Foot- and- Mouth Disease (FMD) is a highly contagious viral disease of cloven-hoofed animals. The disease, characterized by high fever and vesicles in the mouth and feet, can cause sudden death in young animals. In addition to productivity losses, restrictions on international trade may cause significant economic losses. Vaccination is a common method to combat the disease in endemic countries. FMD vaccines used today are inactive whole virus vaccines. FMD vaccine prepared either by aluminium hydroxide gel or mineral oils as an adjuvant requires repeated administrations to maintain the protection (Doel 1999).

Various methods have been tried to increase the strength and duration of the immune response to FMD vaccines. Some of these are the use of new adjuvants or immunomodulators. Some of the immunomodulators are of plant origin. The most known and widely used of them are saponins (Ragupathi et al 2008). A well-known saponin for its adjuvant effect is Quil-A produced from a tree called Quilllaja saponaria. The synergistic effect of saponin and oil adjuvant for FMD vaccines have been shown in various studies (Xiao et al 2007, Smitsaart et al 2004). The mechanism of this synergy was explained by depot effect of oil and the immunomodulatory effect of saponin (Xiao et al 2007). On the other hand search for other less toxic plant origin substances is going on. Horse chestnut (*Aesculus hippocastanum*) and Mistletoe (*Viscum album*) are plants commonly used in traditional medicine. Their adjuvant and immunomodulatory effects have been demonstrated in various studies (Lavelle et al 2002, Elluru et al 2008, Salinas et al 2019). Although many herbal extracts are claimed to have an adjuvant effect, most of them have no scientific basis (Ragupathi et al 2008). It was demonstrated that some of the adjuvant activities came from bacterial lipoproteins that was present on the plants (Pugh et al 2008).

The active ingredient of *Aesculus hippocastanum* seed is penta-cyclic triterpene known as Escin. This compound is a saponin mixture. Recent studies have shown that it induces apoptosis in different types of cancer. It is very safe and abundant in nature hence, ecological footprint would be low as a raw material comparing to Quillaja saponins which is widely used as adjuvant.

Another potential herbal adjuvant is *Viscum album*, known as mistletoe. It is an evergreen semi-parasitic plant. Various preparations are used in complementary therapy in diseases such as cancer, cardiovascular diseases and arthritis. *Viscum album* has active ingredients such as lectins, alkaloids, viscotoxins and polysaccharides. However, its use in humans is limited due to its toxicity (Kienle et al 2011).

It has been shown that mistletoe lectins can be used as a mucosal adjuvant in herpes simplex vaccination, thereby increasing the immune response (Lavelle et al 2002). Viscum album extracts have been shown to induce activation and maturation of human dendritic cells, thereby providing an immune response (Elluru et al 2008). Mistletoe preparations have been shown to induce interleukine-5 and interferon-gamma in healthy humans (Huber et al 2005).

Guinea pigs are the animals commonly used for FMD vaccine evaluation. Although natural infection with FMD is not possible in this species, potency testing can be performed in guinea pigs as an alternative to target species. The protective virus neutralization antibody titers was detected as 3 (Log_2) in this species (Nermeen et al 2018). One of the issues that scientists are working on is reducing experimental errors. For this, they make multiple measurement on the same observation unit to see the time-dependent changes. This type of data obtained is called longitudinal data. An example longitudinal data table can be seen in Table1.

Table 1. Data structure for longitudinal data

			Response variable
ID	Grup	Time	$NATL_{vnt}$
5	1	7	7.00
5	1	14	4.00
5	1	28	7.00
5	1	60	7.00
5	1	120	7.58
5	1	270	.
9	2	7	6.49
9	2	14	6.00
9	2	28	7.00
9	2	60	8.00
9	2	120	7.58
9	2	270	4.00
22	3	7	5.49
22	3	14	4.00
22	3	28	3.46
22	3	60	5.00
22	3	120	6.00
22	3	270	.

Longitudinal data are in a clustered data structure. Clusters consist of repeated measurements of each observation unit at different times. There is a correlation between the measurements. Taking this correlation into account in data analysis studies will make the study more reliable. It will not be correct to use traditional data analysis methods when there are missing in the measurements and the measurements are not made at equal time intervals. There are special methods developed for these situations (Shults and 2014, Hedeker and Gibbons 2006, Hardin and Hilbe 2013, Agresti 2007, Kim and Shults 2010). Two of these methods are Quasi-Least Squares Regression (QLS) and Generalized Estimation Equations (GEE) methods.

QLS, which enables longitudinal data analysis, is a correlated data analysis method developed by Shults and Chaganty. The development of the QLS was based on GEE. As for GEE, it is an analysis method developed from Generalized Linear Models. GEE is one of the methods widely used in modelling studies in longitudinal analysis. One of the most important features of QLS and GEE methods is that it takes into account the correlation between repeated measurements for each observation unit in the estimation of regression parameters.

Correlation coefficients, which are frequently used in statistical analysis studies, are measurements that provide information about the degree and direction of the relationship between the examined features (Alpar 2016). In some regression methods, working correlation structures are used when creating a regression model. Two of these methods are QLS and GEE methods. Working correlation structure (Ri(α)) defines the pattern of the relationship between the repeated measurements of i-th observation based on the estimated correlation coefficient "α". So, Ri (α) is an "α" based matrix (Shults and Hilbe 2014, Davis 2002).

Let i:1,2,.....,m and j:1,2,.....,n_i be the indices for observations and repeated measurements, and t is the actual time of measurement. In the literature, working correlation structures (Ri (α)) are defined in various ways and names as follows (Wang 2014, Shults et al 2014, Ziegler 2011).

Exchangeable Working Correlation Structure

$$\begin{pmatrix} 1 & \alpha & \cdots & \alpha \\ \alpha & 1 & \cdots & \alpha \\ \vdots & \vdots & \ddots & \vdots \\ \alpha & \alpha & \cdots & 1 \end{pmatrix}_{n_i \times n_i}$$

This correlation structure is based on the assumption that the correlation between repeated measurements of the same unit is the same at all measurement times.

Tri-diagonal Working Correlation Structure

$$\begin{pmatrix} 1 & \alpha & 0 & \cdots & 0 \\ \alpha & 1 & \alpha & \cdots & 0 \\ 0 & \alpha & 1 & \cdots & 0 \\ \vdots & \vdots & \vdots & \ddots & \vdots \\ 0 & 0 & 0 & \cdots & 1 \end{pmatrix}_{n_i \times n_i}$$

In this structure, the correlation between measurements of the same unit is calculated according to the difference between the measurement order. If the difference between the measurement ranges is equal to 1, "α" is taken into account; if the difference is not equal to 1, zero is taken into account.

The First-Order Autoregressive Working Correlation Structure (AR-1)

$$\begin{pmatrix} 1 & \alpha & \alpha^2 & \cdots & \alpha^{n_i-1} \\ \alpha & 1 & \alpha & \cdots & \alpha^{n_i-2} \\ \alpha^2 & \alpha & 1 & \cdots & \alpha^{n_i-3} \\ \vdots & \vdots & \vdots & \ddots & \vdots \\ \alpha^{n_i-1} & \alpha^{n_i-2} & \alpha^{n_i-3} & \cdots & 1 \end{pmatrix}_{n_i \times n_i}$$

In this working correlation structure, the correlation matrix is established by taking the exponent of the correlation coefficient "α" of the difference between measurement orders.

Markov Working Correlation Structure

$$\begin{pmatrix} 1 & \alpha^{t_{i2}-t_{i1}} & \alpha^{t_{i3}-t_{i1}} & \cdots & \alpha^{t_{in_i}-t_{i1}} \\ \alpha^{t_{i2}-t_{i1}} & 1 & \alpha^{t_{i3}-t_{i2}} & \cdots & \alpha^{t_{in_i}-t_{i2}} \\ \alpha^{t_{i3}-t_{i1}} & \alpha^{t_{i3}-t_{i2}} & 1 & \cdots & \alpha^{t_{in_i}-t_{i3}} \\ \vdots & \vdots & \vdots & \ddots & \vdots \\ \alpha^{t_{in_i}-t_{i1}} & \alpha^{t_{in_i}-t_{i2}} & \alpha^{t_{in_i}-t_{i3}} & \cdots & 1 \end{pmatrix}_{n_i \times n_i}$$

Markov working correlation structure takes into account actual measurement times unlike other working correlation structures. This correlation structure is a structure developed from AR-1 working correlation structure.

Markov working correlation structure can be used in QLS. However, this structure cannot be used in GEE. The use of this structure in QLS gives QLS an application advantage over GEE (Shults and Hilbe 2014, Propst et al 1998, Xie and Shults 2009, Smith and Smith 2018, Aydın 2014).

In our study, it was investigated whether the plants used with all its components without extracting have a synergistic effect on the immune response in the current vaccine formulation. The powders of Horse Chestnut seed and Mistletoe dried leaves were incorporated in Montanide™ ISA 206 adjuvanted FMD vaccines. In order to compare the effect of the addition of the plant powders on the neutralizing antibody

response to FMD virus in guinea pigs, a regression model created with QLS method was used.

Material and Methods

Preparation of the experimental vaccines

Montanide™ ISA 206 (Seppic, France) oil adjuvant was used to form a double oil emulsion (w/o/w). The water phase was a suspension of type A FMD inactivated antigen, received from FMD Institute Ankara. The volume of the one dose of the vaccines was adjusted to be 1,0 mL, and the antigen concentration in the vaccines was adjusted to be 1µg/dose. Desired adjuvant/antigen phase (v/v) ratio (55:45 (oil/water) (v/v)) was obtained by dilution of the antigen phase with phosphate buffer solution (PBS) (pH=7.6). Both phases were kept at 30ºC in an incubator to balance the temperature. The powdered plants in the second and the third group vaccines were added to the oil phase which was under constant stirring during the overall preparation procedures, thus ensuring the suitable dispersion of the powder. The final powder concentrations in the vaccines was adjusted to be % 1.0 (m/v). The kernel of the horse chestnut (Aesculus hippocastaneum) was grinded by an ordinary grinder. The powder of dried mistletoe leaves was obtained by pounding in a mortar with liquid nitrogen.

In order to prepare the first group of the vaccines, the standard vaccine formulation used in the control animal group as the reference, the water phase was added to the oil phase at the rate of 10 mL/min under constant stirring by a magnetic stirrer (500 rpm). Then stirring was increased to 1000 rpm for additional 5 minutes.

In order to prepare the second group of the vaccines, powder of the finely grinded kernel of the horse chestnut was mixed with the Montanide™ ISA 206 and this mixture was used as the oil phase. The water phase was added to the oil phase at the rate of 10 mL/min under constant stirring by a magnetic stirrer (500 rpm). Then stirring was increased to 1000 rpm for additional 5 minutes.

In order to prepare the third group of the vaccines, the powder of dried mistletoe leaves was mixed with the Montanide™ ISA 206 and this mixture was used as the oil phase. The water phase was added to the oil phase at the rate of 10 mL/min under constant stirring by a magnetic stirrer (500 rpm). Then stirring was increased to 1000 rpm for additional 5 minutes.

All formulations were emulsions of w/o/w type. The type and the quality of emulsions was confirmed by the droplet test and measurements of the conductivity and viscosity.

Animal groups and administration of the vaccines

The animals were six months old, conventional, unvaccinated, Duncan-Hartley male guinea pigs weighing 300-500g. The animals were kept under high biosecurity conditions of the Institute's small animal experiment unit during the study. The guinea pigs with these similar characteristics were randomly divided into three groups, eight animals for each group. To determine the number of animals to be included in the three groups for analysis of variance in repeated measurements, a priori power analysis was performed with G * Power software (version 3.1.9.7). This analysis was carried out so that the power of the test was 0.80, the effect size was 0.25, and the significance level was 0.05. Due to the fact that the obtained data is not in a balanced structure (ie with missing measurements), GEE and QLS were used instead of variance analysis in data analysis. The number of animals (eight) determined for the analysis of variance were also used in the data analyzes made with GEE and QLS, taking into account the ethical committee and animal studies in the literature.

The experimental FMD vaccines were administered to these three groups by subcutaneous route. Standard FMD vaccine formulation was administered to the first group of the animals. The second group of vaccine, formulation with *Aesculus hippocastaneum* seed powder, was administered to the second group of the animals. The third group of vaccine, formulation with *Viscum album* dried leaves powder, was administered to the third group of the animals. The animal experiments were done according to EU directive 2010/63EU with an approval of Institute's local ethics committee.

Blood samples and immune response

Blood samples were taken from the animals to measure the neutralizing antibody titer levels (NATL) generated as a result of vaccination. These blood samples were taken from the saphenous vein of animals with 20GX1 ½'' needles to 1.5 ml Eppendorf tubes under general anesthesia, six times after vaccination (7, 14, 28, 60, 120 and 270 days). Following incubation at room temperature for 10 minutes the sera were separated by centrifugation at 3000 rpm for 10 minutes. The sera were stored at -20C until the tests.

VNT was performed according to FMD terrestrial manual (OIE 2011) using A/AS/G-VII, vaccine strain obtained from virus bank of the Institute. Briefly, the sera were inactivated at 56ºC for 45 minutes. Two-fold dilutions of the sera were made in cell culture microplates using Glasgow Minimal Essential Medium. 100 TCID50 homologous virus was added to the wells. The plates were incubated in a 37ºC CO_2 incubator. Following incubation cell suspension containing 600 000 BHK-21 cells (HUKUK WDCM756: Culture Collection of Animal Cells, Foot and Mouth Disease Institute-ANKARA) per ml

was added to the wells. After incubation 48 hours in 37°C CO_2 incubator, cells were stained by crystal violet dye. Cytopathological effects (CPE) were observed under a microscope. The reciprocals of the last dilution of the sera that inhibit CPE formation accepted as the titer of the serum. The reliability of the assay is confirmed routinely by interlaboratory proficiency tests among the FMD laboratories of Europe. The test results were evaluated blindly to avoid bias.

Statistical analysis

Whether NATLs in the three groups were different according to VNT was statistically analyzed. Statistical analysis was performed on the data obtained with results of VNT for type A vaccine strain. When the obtained data was examined, it was seen that a data with six repeated measurements and missing measurements was obtained. In data analysis, analysis of variance in repeated measurements could not be used because the data were not in a balanced structure. QLS and GEE regression methods, which are two methods that can be used in data sets of this structure, have attracted attention and analyzes were carried out with these two methods. In this context, regression models were established by using Stata (version 14.1) software, considering the working correlation structures. Comments and evaluations were made according to the 5% significance level. In these regression models, the NATL obtained according to the vaccine strain used in VNT were taken as response (dependent) variable (NATLvnt). In addition, three-group "group" variable and six-measure "time" variable were integrated into these models as independent variables. In this process, the significance of the interaction term (time * group) on dependent variable was also examined. So, the interaction term was not found to be significant. Therefore, the interaction term was not included in the regression model. Y; response variable, β's are regression coefficients; The regression model is written as: $Y=\beta_0+\beta_1\times$ time$+\beta_2\times$ group .

Results

Within the scope of the study, regression models were established according to QLS and GEE methods by using NATL, time and group variables. In the estimation of the coefficients in the model, the correlation between the measurements was also taken into account. The results obtained according to the regression models explained above and in the previous sections are given in Table 2. Summarizing from this table; for both methods and each working correlation structure, it is concluded that the group and time effects on NATL are not statistically significant ($p> 0.05$).

Looking at the data studied, it was seen that the measurements were not made at equal time intervals. It was Markov working correlation structure that can be used in QLS while modelling and allows us to use real measurement times. In this context, the correlation values obtained for each working correlation structure used as a parameter during modelling are given in Table 3. These correlation values used in the estimation of the correlation coefficients give the degree and direction of the relationship between the NATL measurements. It was seen that the highest value among the correlation values obtained for each working correlation structure was the value obtained using Markov working correlation structure.

Table 2. The results of the regression model based on GEE and QLS methods

Response variable	Working correlation structure	Coefficient, error, p	GEE			QLS		
			constant	time	group	constant	time	group
$NATL_{vnt}$	Exchangeable	Regression Coefficients	6.4431	0.0017	-0.0541	6.8356	0.0009	-0.0247
		Standard Error	0.2657	0.0012	0.1136	0.3520	0.0012	0.1609
		p value	0.0000	0.1630	0.6340	0.0000	0.3990	0.6250
	AR-1	Regression Coefficients	6.6062	-0.0004	-0.0611	6.5960	-0.0001	-0.0680
		Standard Error	0.3920	0.0012	0.1715	0.3652	0.0012	0.1605
		p value	0.0000	0.7310	0.7220	0.0000	0.9670	0.6720
	Tri-diagonal	Regression Coefficients	6.6023	-0.0001	0.0696	6.6566	0.0003	0.0829
		Standard Error	0.3684	0.0012	0.1605	0.3690	0.0012	0.1621
		p value	0.0000	0.9600	0.6650	0.0000	0.8080	0.6090
	Markov	Regression Coefficients	*	*	*	6.5762	0.0007	-0.0357
		Standard Error	*	*	*	0.3253	0.0012	0.1402
		p value	*	*	*	0.0000	0.5470	0.7990

* : Can not be applicable in GEE.
$NATL_{vnt}$: Antibody titer level obtained according to the vnt test

Table 3. Correlation values obtained in QLS according to response variable and working correlation structures

Working Correlation Structure	Response Variable
	$NATL_{vnt}$
Exchangeable	0.1466
AR-1	0.2649
Tri-diagonal	0.3559
Markov	0.3879

Discussion

Studies are carried out on plants to derive immune potentiators. Plant-origin immune stimulators consist of a wide range of small molecules or large polysaccharides. Saponins, tomatines and inulins are among to most important ones. Various substances have been used up to now to improve the immune response to various vaccines. These are mainly saponins from different sources such as Quillaja saponaria, Panax ginseng, Polygala senega, Cochinchina momordica plants. The effect of saponins on the response to FMD vaccines have been demonstrated in various studies (Smitsaart et al 2004, Xiao et al 2007). In this experimental study, the effect of the addition of powders of horse chestnut (*Aesculus hippocastanum*) seed and Mistletoe (*Viscum album*) leaf to Montanide™ ISA 206 oil adjuvanted FMD vaccines on neutralizing antibody response in guinea pigs was investigated. The powder forms of the plants were selected to utilize all potentially active immunomodulators that could be soluble either in the water or oil phase. It was also demonstrated that QLS can be used in analyzing such longitudinal data in the veterinary field.

Due to the characteristics of the data we are working on (missing measurement, unequally spaced measurement times), data analysis was performed using QLS and GEE methods. Analysis of variance is also among the methods that can be used for data based on repeated measures. However, in order to get correct results from this analysis method, the data studied should not contain missing measurements. Another method used in data analysis is GEE. In this method, even if the measurements are not made at equal time intervals, the measurement time intervals are considered equal and this may have a negative effect on the data analysis. In QLS, the other method used in the study, Markov working correlation structure could be used. The fact that this correlation structure, which uses the real time that the measurements are made, can be used in the QLS, makes the data analysis with QLS more powerful.

It was observed that the time variable measured on six different days did not have a statistically significant effect on the NATL variable. This means that the measurement times (days) do not have a significant difference in NATL change. NATLs of all three groups were above the protective levels described by Nermeen et al 2018 from the day 7 post-vaccination in this study. However, the effect of the group variable on the NATL variable was not found to be significant. This showed us that there was no significant difference between the NATLs of the three groups. According to this result, the plant powders used in this study had no significant effect on the neutralizing antibody response which is the most utilized parameters to estimate the protection against the disease. This finding is concordant with of Li et al (2015) that showed anti-inflammatory effect of escin which is the main active substance in *Aesculus hippocastanum* seeds. However, the immunological effects of *Viscum album* compounds are well-known especially in cancer therapy (Oei et al 2019). Perhaps one of the main reasons why these powders did not have an effect on antibody response was the method of vaccine formulation. The powder form of plants might have reduced the bio-availability of the active substances in oil adjuvant. In the other studies generally ethanol extraction is used to obtain the active substances from plants. On the other hand, Ragupathi et al. 2008 suggested that herbal adjuvants have an immunostimulating potential beyond the effect of known active ingredients. Also, according to the immunostimulation against the specific antigen the scientific evidence was missing for many of them (Hong et al 2011).

In the QLS method, in all working correlation structures used in the analysis for all NATL variables, the correlation value obtained with Markov working correlation structure was found to be the highest correlation value obtained. In this context, similar results have been obtained in some studies in the literature. One of these belongs to an obesity study in children after kidney transplantation by Shults et al. in 2006. Among the correlation values obtained in this study, the highest value is the value obtained with Markov working correlation structure (Shults et al 2006).

In cases where the data structure is unequal time-measured and missing-measured, Markov working correlation structure, which is among the working correlation structures, takes into account the actual measurement times, which brings a great privilege to this structure and the QLS.

Conclusion

When the effects of different plant powders added to vaccines were evaluated on the NATL variable, no statistically significant difference in effect was found. So, it was understood that the immunostimulating effect of plant powders in this form was not detected. According to the results of the study, it could be concluded that the presence of these plants in powder form do not have a positive or negative effect on the current vaccine formulation regarding the immune response. Therefore, efforts should be made to develop extraction methods to obtain active substances from the plant and use them in optimal formulation for immune system activation. Instead of the crude plant powders, known active substances can be tested for future studies.

In terms of data analysis methods used in the study, it can be said that QLS and GEE are the leading methods that can be used in cases where repeated measurements are made in unequal time intervals and there are missing measurements. Using real measurement times instead of measurement order in Markov working correlation structure increases the quality of the study. While this working correlation structure can be applied in QLS, it cannot be applied in GEE, making QLS a more suitable analysis method.

In addition to future studies, it can be said that software will be developed that will enable the use of Markov working correlation structure, which can be applied in QLS, in the GEE method.

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Conflict of Interest

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Ethical Approval

This study was carried out with the approval and permission of the ŞAP Institute Ethics Board, dated January 30, 2018 and numbered 2018/01.

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