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Investigation of the Effect of *Melissa officinalis* L. on Fetal Development by Ultrasonography in Rats

Elvan Anadol[1], Mürşide Ayşe Demirel[2], İpek Süntar[3], Aydan Avdan Aslan[4], Serap Gültekin[4], Ayris Gökçeoğlu[5], Meltem Bahçelioğlu[6], Elif Gülçiçek Abbasoğlu Topa[7], Ayşen Çalikuşu[8], Gül Fatma Yarim[5]

[1]Gazi University, Laboratory Animal Breeding and Experimental Researches Center, Vocational School of Health Services, Ankara-Türkiye

[2]Gazi University, Faculty of Pharmacy, Department of Basic Pharmaceutical Sciences, Laboratory Animals Breeding and Experimental Research Center, Ankara- Türkiye

[3]Gazi University, Faculty of Pharmacy, Department of Pharmacognosy, Ankara- Türkiye

[4]Gazi University, Faculty of Medicine, Department of Radiology, Ankara- Türkiye

[5]Ondokuz Mayis University, Department of Biochemistry, Samsun- Türkiye

[6]Gazi University, Neuropsyhiatry Education Research and Application Center, and Neuroscience and Neurotechnology Center of Excellence NÖROM, and Faculty of Medicine Department of Anatomy, Ankara- Türkiye

[7]Gazi University, Institute of Health Sciences, Neuropsychiatry Education Research and Application Center, Ankara- Türkiye

[8]Gazi University, Institute of Health Sciences, Neuroscience and Neurotechnology Center of Excellence, Ankara- Türkiye

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Abstract

Medicinal plants have been used to treat many diseases since prehistoric times. With the discovery of new medicinal plants, new therapeutic areas have emerged. On the other hand, the posology of these plants and indications for use during pregnancy or adolescence are still debated. The use of *Melissa officinalis* L. (*M. Officinalis*) in traditional medicine is common, but its effect on fetal development is not clear. Ultrasonographic evaluation of fetal development is critical. Unfortunately, ultrasound probe differences used in pregnancy studies in rats are confusing. Therefore, in the present study, we aimed to search both possible effects of *M. officinalis* on fetal development, and test the effectiveness of human breast ultrasound probes in the ultrasonographic evaluation of rat fetuses. Rats were randomly divided into Control and *M. officinalis* groups. *M. officinalis* was gavaged for 21 consecutive days 24 hours after mating. Rats in the Control group were treated with the extract of *M. officinalis*. Ultrasonography examination was performed with the superficial probe used in breast examination in humans. Gestational sac diameters, biparietal diameters, crown-rump lengths, and heart rates of fetuses were measured in both groups. According to our results, ultrasonographic measurements on the 15th, 17th, and 20th days of pregnancy were higher in the *M. officinalis* group than in the Control group. However, both groups had no statistical significance according to all measurements. As a result, it was concluded that *M. officinalis* extract might not have any effect on the development of rat fetuses in terms of ultrasonographic examination, whereas pregnancy can be diagnosed with human ultrasonography devices and the development of fetuses can be followed in rats.

***Corresponding author:** Elvan Anadol,
e-mail address: elvanadol@yahoo.com

1. Introduction

The World Health Organization estimates that more than 80% of people in developing countries benefit from medicinal plants and traditional treatments, and about 855 traditional medicines contain crude plant extracts. This means that about 3.5 to 4 billion of the world's population rely on herbal sources for medicines. However, the traditional uses of only some of these medicinal plants have been investigated through *in vitro* and preclinical trial studies (Araújo et al., 2016).

Melissa officinalis L. (Lamiaceae), also known as lemon balm, is a medicinal and aromatic plant that is popularly used in the treatment of various diseases. It belongs to the Lamiaceae (mint) family and is common in Europe, Central Asia, and Iran. The benefits of the lemon balm plant, which is often used in meals or consumed as tea, are quite high. Since ancient times, the lemon balm has been known for its sedative/sedative, carminative, antipyretic, antibacterial, spasmolytic, antihypertensive, memory-enhancing, menstrual-inducing, and thyroid-related effects. It also has antiviral and antioxidant activities, antifungal, antiparasitic, and antispasmodic activities. Moreover it is used for treatment of asthma, bronchitis, amenorrhea, heart failure, arrhythmias, ulcers and wounds, headaches, indigestion, colic, nausea, nervousness, anemia, dizziness, syncope, weakness, insomnia, epilepsy. It is also reported to be effective in the treatment of depression, psychosis, and hysteria. However, there are limited studies on its use in pregnancy (Miraj et al., 2017; Moaca et al., 2018; Manolescu et al., 2022).

It is known that there is a relationship between nutrients taken during pregnancy and fetal development (Marshall et al., 2022). The development of fetal tissues and organs requires an adequate supply of nutrients and oxygen, and its reactive forms produced in the mother and fetus have an effect on the replication, differentiation, and maturation of developing cells (Tobola-Wrobel et al., 2020). During pregnancy, many anatomical, physiological, and metabolic changes occur in the mother's body (Ypsilantis et al., 2009; Kirberger et al., 2019). Although various effects of *M. officinalis* on pregnancy have been investigated in many studies (Araujo et al., 2016; Ahandani, 2018; de Abreu Tacon et al., 2020), a study on fetal development parameters in pregnancy by ultrasonography could not be reached. In addition, the ultrasound probe differences used in pregnancy studies in rats are confusing. For this purpose, the hypothesis of our study is to examine whether the ultrasound probe used in humans may be effective in the ultrasonographic evaluation of the effects of *M. officinalis* used during pregnancy on the fetal development process.

2. Material and Methods

2.1. Preparation of the plant extract

The aerial parts of *Melissa officinalis* L. were obtained from Temmuz Organik Çiftliği (Konya, Turkey). In the extraction process of the plant material, 80% aqueous ethanol was used in order to extract both polar and nonpolar secondary metabolite groups. Air-dried powdered plant material (500 g) was extracted with 80% aqueous ethanol (5 L). The obtained extract was concentrated to dryness by using a rotary evaporator at 40ºC under low pressure

***Corresponding author:** Elvan Anadol,
e-mail address: elvanadol@yahoo.com

to obtain dry extract (105 g) with a yield of 21% (Wake et al., 2000).

2.2. Animals

This study was approved by the Experimental Animal Ethics Committee of Gazi University (G.U.ET-22.059). All experimental procedures at the Gazi University of Laboratory Animals Breeding and Experimental Researches Center were conducted in accordance with the ethical guidelines of the Ethics Commission on Animal Use (CEUA) and the Guide for the Care and Use of Laboratory Animals of NIH. Twelve, eight-week-old female, Wistar Albino rats weighing 170–200g were used for experimental procedures. All the rats were housed in polysulfone cages with aspen shavings for bedding, and light-controlled conditions. The animals were fed with a standard pellet diet and tap water *ad libitum* throughout the experimental procedure. The temperature and air humidity were maintained at 21–24°C and 40–45%, respectively.

2.3. Vaginal cytology and mating

The estrous cycle of rats was followed by a daily assessment of vaginal cytology. The rats exhibiting regular (4–5 days) estrous cycles were used in the experimental procedure. The swab was gently inserted into the vagina and the epithelial cells were collected from the vaginal lumen and walls. The cells were then transferred to a glass slide and stained by Giemsa. Smears were examined at a light microscope (Leica CME Microscope, 1349522X, NY, USA, 40x objective lenses). The stages of the estrous cycle were classified as proestrus, estrus, metestrus, and diestrus (Cora et al. 2015). The rats, determined to be in estrus by vaginal cytology, were mated with male rats. Mating was confirmed by the presence of sperm in vaginal smear or seminal plug on the following morning and these females were considered to be at pregnancy day 0.

2.4. Administration of the test materials

Twelve rats were randomly divided into two groups consisting of six rats in each group, as follows: Control and *Melissa* (*M. officinalis)* groups. The administration of test materials was started 24 h after mating and continued for 21 days. The Control group was given 0.5 mL of 0.5% carboxymethylcellulose (CMC). *M. officinalis* crude extract was dissolved in 0.5% CMC aqueous solution and was administered to animals at 0.5 mL volume by oral gavage. The test materials were applied once a day.

2.5. Ultrasonographic evaluations

It is known that the organogenesis period in rats is between the 7th and 13th days of pregnancy (Lohmiller and Swing, 2006). In the present study, following the period of organogenesis, ultrasonographic examination in rats was performed every other day from the 15th to the 20th day after the mating. An ultrasonographic examination was performed by the same investigator. The rats were placed in dorsal recumbency under sedation with medetomidine (0.5 mg/kg, intramuscular). Atipamezole (0.5 mg/kg; subcutaneous) was applied for the recovery from anesthesia. The abdominal area

***Corresponding author:** Elvan Anadol,
e-mail address: elvanadol@yahoo.com

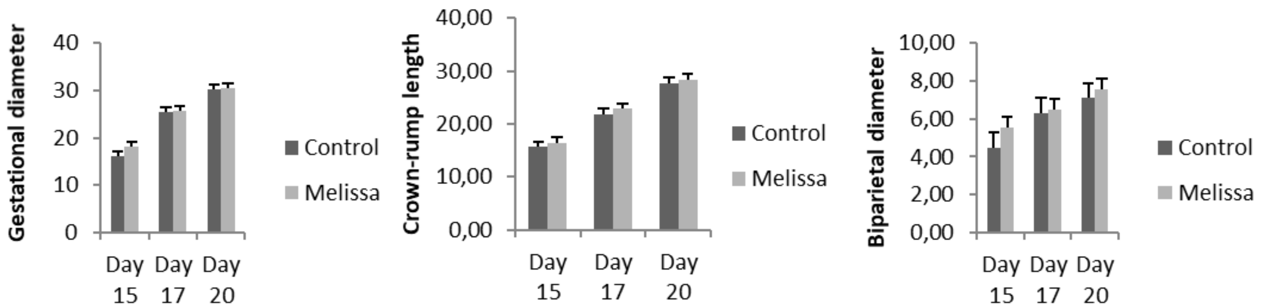
was shaved and cleaned. The uterus was examined transabdominally by using a GE Logiq s8 model ultrasonography device and L8-18i-D linear hockey probe (4-15 MHz). The ultrasound scanning gel was applied to the probe and abdominal surface. Images were examined with a depth of 40 mm and at a high quality and saved on the onboard computer. The procedure for scanning was standardized, with the probe placed in an oblique plane perpendicular to the abdominal wall beginning in the right abdominal area. And then, the left abdomen was examined and finished transversely, beginning caudally with the bladder and descending colon in the midline. During the ultrasonographic examination, the gestational sac (GS), crown-rump length (CRL), biparietal diameter (BPD), heartbeat, vertebral column, ribs, feet, and tail of the two fetuses from each mother were evaluated. The lengths and widths of the anechoic GSs were measured at each examination. The diagnosis of pregnancy was confirmed by ultrasonographic detection of the embryonic heartbeat. The total duration of the examination was approximately 5-10 min.

3. Results

To differentiate the pregnancy diagnosis, embryonic vesicles were distinguished from other round or oval structures such as fecal stools, psoas major muscles, and kidneys of similar echogenicity that was adjacent to the uterus. Early (day 9 or 10) embryonic vesicles were distinguished from fecal stools, which are round or oval structures that do not change shape when the ultrasound probe presses against the abdominal wall.

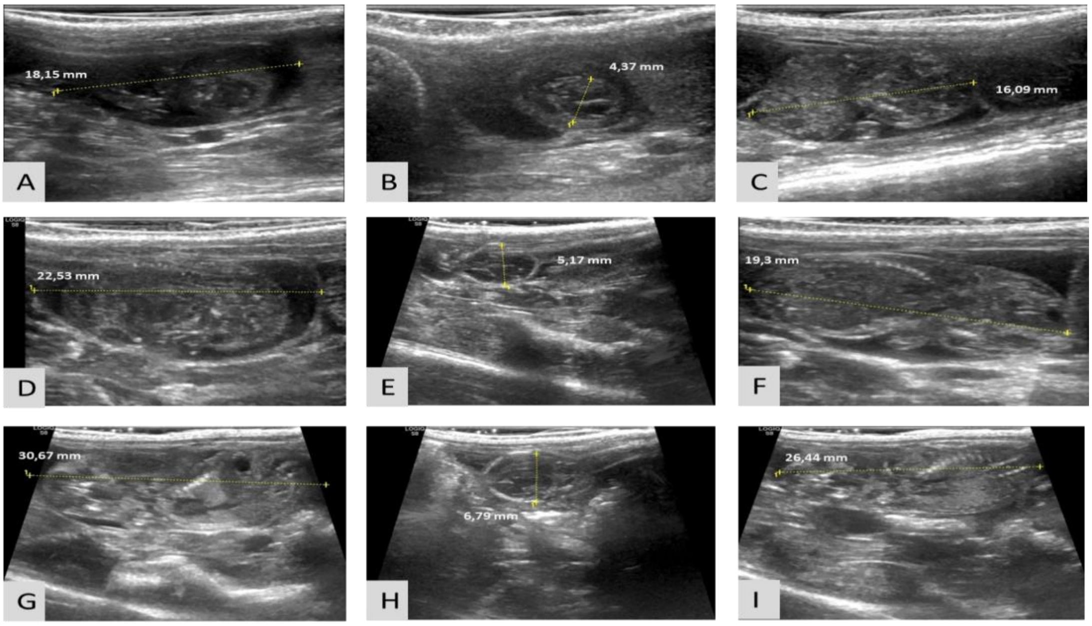
Fetal heart rates on days 15, 17, and 20 were 218±2.58, 208±4.98, and 210±11.13 in the Control group, respectively. Fetal heart rates on days 15, 17, and 20 were 170.66±9.33, 202.66±4.80, and 220±4.61 in the *Melissa* group, respectively. Fetal heart rate was higher in the Control group on the 15th and 17th days of pregnancy compared to the *Melissa* group, and higher in the *Melissa* group on the 20th day compared to the Control group (P>0.05).

Figure 1. Gestational diameters, crown-rump lengths, and biparietal diameters in the Control and *Melissa* groups on the 15th, 17th, and 20th days of pregnancy (P>0.05).



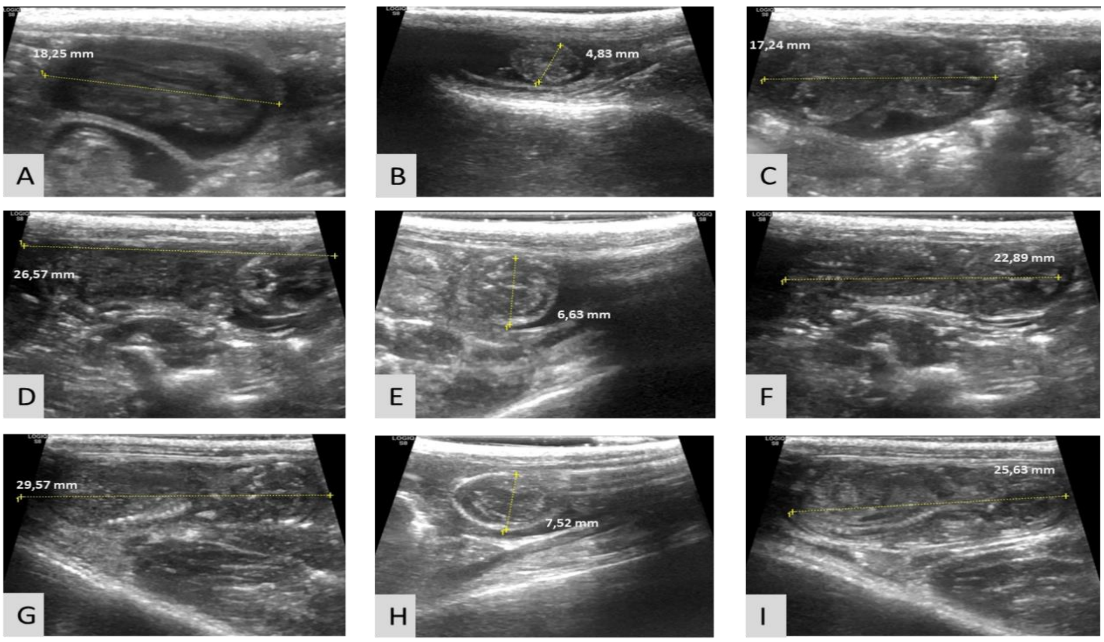
***Corresponding author:** Elvan Anadol,
e-mail address: elvanadol@yahoo.com

Figure 2. Ultrasonographic images on the 15th, 17th, and 20th days of pregnancy in the Control group



A. Longitudinal gestational sac on day 15, **B.** Biparietal diameter of the fetus on day 15, **C.** Crown-rump length of fetus on day 15, **D.** Longitudinal gestational sac on day 17, **E.** Brain ventricles and biparietal diameter of the fetus on day 17, **F.** Crown-rump length of fetus on day 17, **G.** Longitudinal gestational sac on day 20, **H.** Biparietal diameter of the fetus on day 20, **I.** Crown-rump length of fetus on day 20.

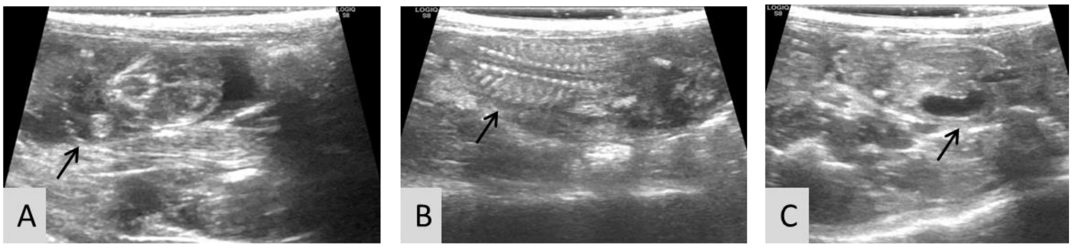
Figure 3. Ultrasonographic images on the 15th, 17th, and 20th days of pregnancy in the *Melissa* group



A. Longitudinal gestational sac on day 15, **B.** Biparietal diameter of the fetus on day 15, **C.** Crown-rump length of fetus on day 15, **D.** Longitudinal gestational sac on day 17, **E.** Biparietal diameter of the fetus on day 17, **F.** Crown-rump length of fetus on day 17, **G.** Longitudinal gestational sac on day 20, **H.** Biparietal diameter of the fetus on day 20, **I.** Crown-rump length of fetus on day 20.

***Corresponding author:** Elvan Anadol,
e-mail address: elvanadol@yahoo.com

Figure 4. A. Ultrasonographic image of a day 17 fetus foot and head (arrow), **B.** Ultrasonographic image of an echogenic medulla spinalis surrounded by anechoic cerebrospinal fluid of a horizontally lying 17-day-old fetus. Thoracic vertebrae and adjacent ribs are visible on the right, and cervical vertebrae are on the left (arrow), **C.** Ultrasonographic image of a day 17 fetus stomach (arrow).



According to the ultrasonographic measurements of fetuses examined, gestational diameter, head-rump length, and biparietal diameter were higher in the *Melissa* group on the 15th, 17th, and 20th days of pregnancy compared to the Control group (Figures 1, 2 and 3). However, it was not statistically significant. In both groups, vertebrae were seen on ultrasound on day 15, and fetal movements, ribs, stomach, and liver on day 17 (Figure 4). The average birth weights of the offsprings were 4.57±0.25 g and 5.05±0.17 g in the Control and Melissa groups, respectively (P>0.05).

4. Discussion

In several pregnancy studies in rats, the diagnosis of pregnancy is mostly made by weight gain and/or abdominal enlargement from day 13, mammary gland development, and nipple enlargement from day 14 (Lohmiller and Swing, 2006; Paronis et al., 2015). Especially in laboratory animals, embryonic structures are very small, which means that high-resolution images cannot be obtained during examinations with ultrasound probes used in humans.For this purpose, ultrasonography devices with expensive probes specific to laboratory animals (20 to 55 MHz) should be used (Jaiswal et al., 2009; Liu et al., 2019). However, in this study, we aimed to determine whether pregnancy can be diagnosed and fetal parameters measured with the ultrasonography device (GE Loqic s8) and 4-15 MHz probe (L8-18i-D linear hockey probe) used in breast examination in humans. Kirberger et al. (2019) measured gestational sac (GS) diameter and crown-to-rump (CR) length in pregnant rats and determined the chronological ultrasonographic appearance of heartbeat and fetal organogenesis (Kirberger et al., 2019). In another study, the ultrasonographic examination technique and findings to be used in the diagnosis of early and mid-term pregnancy in rats were defined (Ypsilantis et al., 2009). The findings of our study were similar to the ultrasonographic measurements in studies performed on pregnant rats.

Therapeutic functions of natural compounds in plants and having relatively low or no side effects when used properly led people to consume these medicinal plants. It is known that health professionals focus on the studies on these plants and have a great interest in diagnosing the therapeutic properties of plants (Araújo et al., 2016). However, there is great confusion about their definition, efficacy, therapeutic dosage, toxicity, standardization, and regulation. It has been reported that herbal medicines with natural

***Corresponding author:** Elvan Anadol,
e-mail address: elvanadol@yahoo.com

basic chemical compounds can cure human ailments and that these natural products, their derivatives, and analogs represent more than 50% of all medicines in clinical use, and natural products from higher plants account for about 25% of the total (Kooti et al., 2014; Araújo et al., 2016; Miraj et al., 2017; Hosseini et al., 2021). In line with these data, we examined the effect of the use of *M. officinalis* extract, which is widely used in daily life for various reasons, during pregnancy on fetal development after organogenesis via ultrasonography.

Some pregnant women prefer to use plants that are thought to be harmless over medicines for fear of harming the baby. However, information on the possible effects of their random use is insufficient (Ahmed et al., 2018; Alinia-Ahandani, 2018; Dillard et al., 2022). It is known that some plant components can reach the fetus by crossing the placenta, and phytochemicals and their metabolites may cause hormone imbalance and uterine contraction, resulting in miscarriage (Bernstein et al., 2020). Exposure to certain substances in the first 3 months of pregnancy, especially before embryogenesis occurs, may pose a risk of congenital malformations (Araújo et al., 2016; Calina et al., 2019). In addition, it has been reported that medicinal plants have some side effects such as excessive uterine contractions, fetal distress, and miscarriage (Godlove, 2011; Ahmed et al., 2018). Therefore, in order to monitor fetal development, we performed ultrasonographic pregnancy diameter, head-rump length, and biparietal diameter measurements from the 15th day of pregnancy in rats given lemon balm extract throughout pregnancy. According to ultrasonographic measurements, gestational diameter, head-rump length, and biparietal diameter were higher in the *Melissa* group on the 15th, 17th, and 20th days of pregnancy compared to the Control group (P>0.05). Although there was no statistical difference between the measurements showing that *Melissa* extract may not affect fetal development physically, the fact that half of the *Melissa* group could not get pregnant suggested that more detailed studies should be conducted. However, similar to other studies, it was determined that fetal parameters were measured accurately in rats with human ultrasound (Ypsilantis et al., 2009; Kirberger et al., 2019).

5. Conclusion

Ultrasonographic imaging of various organs and skeletal structures in this study will help laboratory staff and researchers predict the upcoming birthday quite accurately. Furthermore, in line with the results of this study, it was concluded that the ultrasonography device and breast superficial probe used in humans will enable a wide variety of pregnancy studies to be performed in rats in the future. Although many studies have been carried out on medicinal plants, it has been observed that there is a need for studies on the toxicity of phytotherapeutics during pregnancy, their effects on the fetus, and possible side effects on the mother. Our study revealed that although the physical development of fetuses receiving *M. Officinalis* extract during pregnancy was similar to the rats in the control group, fetal development should be evaluated metabolically in future studies.

Statistical analysis

The data from the study were statistically analyzed using the statistical package software SPSS Statistics V21.0, IBM Corporation, Armonk, NY. Data from

***Corresponding author:** Elvan Anadol,
e-mail address: elvanadol@yahoo.com

the study were shown as mean value±standard deviation. All data were assessed for normality using Shapiro-Wilk before the significance testing. Statistical significance was assumed at the level of P<0.05. For the results that were not normally distributed, which are BPD and heart rate, 2 Independent samples tests were applied. The Independent-samples t-test was used for results with normal distribution, which are GS, CRL, and birth weights of the offsprings.

Conflicts of interest

The authors declare no conflicts of interest.

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Toboła-Wróbel, K., Pietryga, M., Dydowicz, P.,

***Corresponding author:** Elvan Anadol,
e-mail address: elvanadol@yahoo.com

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***Corresponding author:** Elvan Anadol,
e-mail address: elvanadol@yahoo.com