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RESEARCH ARTICLE

EFFECTS OF SILYMARIN PLUS® VERSUS ANTIBIOTICS ON IMPROVING IDIOPATHIC POOR CONCEPTION AND PREGNANCY RATES OF MARES.

***Mohamed El-Raey[1] and Rasha E. Azab[2].**

1. Department of Theriogenology, Faculty of Veterinary Medicine, Benha University, P.O:13736, Tokh, Kaliobia, Egypt.
2. Department of physiology Faculty of Veterinary Medicine, Benha University, P.O: 13736, Tokh, Kaliobia, Egypt.

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Abstract

The current study was carried out to investigate the improving roles of silymarin-plus® versus antibiotics on mare's uterine environment quality (UEQ). Current study was carried on 23 mares aged between 7-13y. Animals were subjected to clinical and gynecological examinations with detailed breeding history that revealed repeat breeding although there were apparently normal mares with normal breeding management. Animals were divided into three groups according to medications used in lavage; G1 (Silymarin-plus®), G2 (Ciprofloxacin + Amikacin), and G3 (mixture between Silymarin plus® and Ciprofloxacin + Amikacin). Jugular blood samples were collected to assess the hormonal patterns (E_2 & P_4) in correlation with monitoring UEQ and follicular growth by ultrasonography. There was non-significant difference ($P<0.05$) between groups concerning the conception rate although G1 and G3 having the highest conception rate. There was highly significant difference ($P<0.05$) between groups regarding the pregnancy rate. Moreover, there were non-significant difference between the treatment groups concerning both follicular growth and E_2 and P_4 levels. Ultrasonography images for silymarin plus® treated groups only revealed presence of unpleasant hyperechoic fine particles that faded on subsequent uterine washing times and before the suspected day of ovulation, where on day 4 of estrus period the washing fluid is totally anechoic. Fine particles in the first uterine lavages were suspected to negatively affect UEQ. On conclusion: Silymarin plus® incorporation in the uterine lavage solution for mares is helpful to improve their pregnancy rate; especially those with idiopathic causes of repeat breeding. Silymarin plus® might have a great role in improving UEQ.

Abbreviations:-
RBS: Repeat breeding syndrome; UEQ: Uterine environment quality; SIL: Silymarin; TNF: Tumor necrosis factor; IFN: Interferon; GM-CSF: Granulocyte-macrophage colony stimulating factor; NAC: Acetylcysteine; ROS:

Corresponding Author:- Mohamed El-Raey.
Address:- Department of Theriogenology, Faculty of Veterinary Medicine, Benha University, P.O:13736, Tokh, Kaliobia, Egypt.

Reactive oxygen species; VTH: Veterinary teaching hospital; LH: Luteinizing hormone; FSH: Follicle stimulating hormone.

Introduction:-

Silymarin is the active ingredient of milk thistle seeds, besides other flavonoids such silybin A, silybin B, isosilybin A, isosilybin B, silychristin, neosilyhermin, silyhermin and silydianin (Khalil, 2002; Seidlová-Wuttke et al., 2003; Karimi et al., 2011and Neha et al., 2016). Silymarin has cyto-protective (Valenzuela and Garrido, 1994); anti-ulcer properties (Borrelli and Izzo, 2000). Furthermore, Silymarin is a powerful antioxidant through its free radical scavenger ability by decreasing the levels of reduced glutathione so preventing lipid peroxidation (Zhao et al., 2015), and inhibiting enzymes that catalyzing the production of leukotrienes and prostaglandins such as 5-lipoxygenase and cyclooxygenase (Valenzuela and Garrido, 1994; Robak and Gryglewski, 1996 and de Groot and Rauen,1998). Also, it is a powerful iron chelator, thereby inhibiting the oxidation of linoleic acid catalyzed by Fe^{2+} salts (Ferenci et al., 1989).

Silymarin (SIL) protects the normal membrane fluidity by direct interaction with cell membrane components (Muriel and Mourelle, 1990); so it stabilizes cell membranes and makes cells more resisted to osmotic lysis (Ramellini et al., 1974). Moreover, Silymarin had structural similarity with the estrogen so it binds with estrogen receptors and modulates its activity (Khalil, 2002). It also stimulates follicle stimulating hormone (FSH) secretion.

Silymarin acts on the uterine wall causing hypertrophy of its endometrial epithelium and increasing the uterine glands number (Khalil, 2002). Additionally, it modulates inflammation by the inhibition of transcription factor NF-jB that was involved in the production of interleukins (IL-1, IL-6), tumor necrosis factor (TNF-a), lymphotoxin, interferon (IFN-c), and granulocyte-macrophage colony stimulating factor (GM-CSF) (Saliou et al., 2001). Moreover, neutrophils migration to the site of inflammation was inhibited by silymarin (Mayer et al., 2005). As well, it inhibited the Kupffer cells, prostaglandins, leukotrienes (Dixit et al., 2007; Loguercio, Festi, 2011 and Vargas-Mendoza et al., 2014). In animal studies, silymarin had been reported to be nontoxic and symptoms free (Karimi et al., 2011).

Acetylcysteine (NAC) is the *N*-acetyl stable derivative of L-cysteine; a precursor of glutathione (Gayatri et al., 2010 and Hu et al., 2016), which functions as an ROS scavenger through increasing intracellular glutathione formation and its thiol-disulfide exchange activity (Zafarullah *et al.* 2003). Furthermore, NAC has anti-inflammatory and mucolytic effects (Conesa et al., 2001 and Sehirli et al., 2003; Ocal et al., 2004 and Gayatri et al., 2010). NAC is safe and well-tolerated mucolytic agent that softens the tenacious mucous secretions that seen in chronic infection (Amin et al., 2008 and Kissinger, 2012). So, NAC decreased the biofilm biomass and aid in killing the bacteria (*E. coli*) within the biofilms (Borlee et al., 2010). Moreover, NAC can bind metals into complexes (Atkuri et al., 2007 and Reinero et al., 2011). Moreover, NAC was found to have no negative epithelial tissue effects in the uterus, besides it aids in reducing post-breeding endometritis especially in mares suffering from low fertility as a result of post-breeding endometritis due to delayed uterine clearance (Melkus et al., 2013).

Vitamin E has an anti-inflammatory, antioxidant, anticoagulant, and antifibroblastic properties and also it decreased collagen production (De La Portilla et al., 2004 and Yildiz et al., 2011). Depending upon all of these properties vitamin E was found to improve the overall uterine health by enhancing early uterine involution (Sattar et al., 2007), combating metritis (Harrison et al., 1984), decreasing days open and services per conception (Sattar et al., 2007; Moeini et al., 2009). Moreover, it prevents tissue adhesions and protects the cellular membranes against lipid peroxidation (Durmus et al., 2011). Additionally, treatments with vitamin E significantly improved endometrial thickness by increasing uterine blood flow and improving the glandular epithelium growth (Takasaki et al., 2010 and Kurniawan et al., 2014).

Selenium is a unique pro-oxidant and antioxidant trace element that minimizing cellular damage by improving the efficiency of the cell antioxidant system (Durmus et al., 2011 and Sengupta and Nandi, 2013). Selenium was found to stimulate uterine defense mechanism (Thatcher et al., 2011), by improving neutrophil functions (Cebra et al., 2003), and general uterine health (Cerri et al., 2009), by supporting uterine tissue remodeling and involution (Rao et al., 2016) through its effect on the uterine motility (Segerson et al., 1980); so, decreasing metritis rate (Harrison et al., 1986), and increasing the rate of endometritis recovery if used synergistically with vitamin E (Sengupta and Nandi, 2013).

The role of vitamin A in conserving the healthy state of uterine epithelium isn't known, but its balance with estrogen is necessary in maintaining uterine epithelium integrity which if disrupted resulting in keratinizing metaplasia (Walter, 1961). Moreover, retinol in general may be involved in the process of uterine gland development (Vallet et al., 1995).

Zinc has a pivotal role in cell biochemistry especially protein and carbohydrate metabolism (Alavi-Shoushtari et al., 2012); and acts as activator and regulator for several enzyme systems mainly those involved in cell replication particularly in nucleic acid metabolism (McDonald et al., 2010). Zinc concentration in the uterine secretion exerts an intra and extracellular cation regulatory mechanism (Burtis et al., 2006). Additionally, Zinc plays a vital role in controlling uterine fluid pH (Leese, 1995).

Vitamin C has an antioxidant, anti-inflammatory and angiogenic effects (Cameron et al., 1979; Berger et al., 1997; Lane et al., 2009 and Du et al., 2012). Besides it has a role in controlling the endometrial thickness depending upon its role as cofactor in collagen synthesis in the luteal extra cellular matrix (Padayatty et al., 2003; Yamauchi et al., 2003 and Eteng et al., 2006). Furthermore, vitamin c not only limits cytokine secretion directly by its radical scavenger function but also prevents cytokine secretion indirectly via intracellular suppression of NFkB (Van Langendonckt et al., 2002; Gonzalez-Ramos et al., 2012a; Gonzalez-Ramos et al., 2012b and Santanam et al., 2013).

Material and method:-

This study was carried out twenty three (23) mares aged 7-13 years, with history of poor conception rate, normal estrous cycle (20-23day), with normal vaginal mucus discharge and normal utrasonographic cervical and uterine image (Image 1, 2, 7, 9, 10 &14) during the estrus and before mating. These animals were classified into three groups each one compromised of 8 animals except the third group involved 7 animals. The breeding history of these animals revealed that these mares were subjected for mating twice during its estrus phase on day 5^{th} morning and day 7^{th} evening (normal adopted schedule ensuring high conception rate in most mares in the current study territory), for two successive estrous cycles; although all mares were mated with fertile stallions with good breeding history.
All of these mares were admitted to Veterinary Teaching Hospital (VTH), faculty of veterinary medicine, Benha University during the period from February 2014 till July 2016. Mares under this study were subjected for detailed Gynaecology examination according to the following:

Hormonal assay:-

Jugular blood samples from 15 mares only (5 animal from each group) were collected in heparinized vacutainer blood collected tubes (Voma Med, vacutainer tubes), following the second trans-rectal ultrasonographic examinations; {from the 4^{th} day of estrus to suspected day of ovulation and for 4 days after (-4, 0, +4)}. Blood samples were centrifuged at 894 $_{\times g}$ for 20 min at 5°C and aliquots were stored in a freezer at -20 °C. Plasma oestradiol and progesterone concentrations were determined using commercial kits (ESTR-CTRIA and PROG-CTRIA, respectively, Cis-bio International, France) according to the attached protocols; using radio-immunoassay in a gamma counter (Léonhardt *et al.,* 2003).

Utrasonographic genital tract examination:-

The healthy condition of mare's genital tract was checked for two times: The first when it was admitted to the Veterinary Teaching Hospital (VTH), Benha University to determine its condition of infertility; and the second check was applied when each mare repeated its estrous cycle after two successive failed conception rate although they were mated with fertile stallion and at optimum mating schedule according to (Conlon and McArdle, 2016) with a modification (i.e. on the 5^{th} day early morning and on the 7^{th} day evening); using B-mode ultrasound machine (*Eickemeyer, Magic 2200, MN-08101509*). The scanner was provided with multi frequency trans-rectal linear transducer 4 and 6 MHZ. Ultrasonic examination was done in the standing position with adequate restraining. Rectum evacuation from fecal balls was done via manual palpation with the aid of lubricated medical paraffin oil, but with ensuring that there was a sufficient rectal relaxation. Gel lubricated transducer was passed into mare's rectum then moved medially and laterally on the uterine horns, body, cervical and vaginal tubes to assess any changes in their thickness or detecting any abnormal contents or secretions (pus or mucus and fluid…..etc) and structures (Images 1, 2, 7, 9 ,10 and 14). Additionally, the ovarian follicular dynamics was assessed to detect its normality (Images 16-17) according to (Ginther and Pierson, 1983).

Ultrasound examination of pregnancy:-
Treated 23 mares were examined for pregnancy at 28-30 days after service and positive diagnosis was based upon the identification of the embryonic vesicle (conception rate). All mares were re-examined again at 40-60 days of pregnancy to detect embryo proper (pregnancy rate) according to (Conlon and McArdle, 2016).

Vaginal and cervical examination:-
Routine vaginoscopy was used to observe signs of inflammation (redness), infection (discharge), urine pooling, scarring and checking cervical competency and integrity (Conlon, McArdle, 2016)

Treatment protocol:-
The treatment protocol was represented in using silymarin plus® + normal saline solution as uterine lavage solution (group, 1), freshly prepared Ciprofloxacin + Amikacin admixture in normal saline as uterine lavage solution (group, 2) and mixture from silymarin plus® + Ciprofloxacin +Amikacin admixture in normal saline as uterine lavage solution (group, 3). Each group according its treatment received uterine wash early morning on the 2nd and 4th days of estrus and again after 6h after a fertile mating. Silymarin plus® (SEDICO-South Egypt Drug Industries Co - Egypt) - (table 1) - was dissolved by rate of one sachet in 1 liter of sterile warmed saline solution (ADWIC, Egypt, Batch No. 17220218), then must be filtered and its pH was adjusted. Ciprofloxacin (Cipromega 1gm XL®, Mash Premiere, Badr City, Egypt) and Amikacin (Amikabiotic® 500mg, Pharco B International-Alexandria-Egypt) mixture was freshly prepared and filtered then used directly at a rate of 2g in each uterine lavage used (Abdel-Raziek, 2006). Prepared antibiotic solution pH was adjusted. In each uterine wash, thoroughly uterine massage was applied rectally for about 5 minutes to ensure efficient washing of the uterine cavity. Oxytocin (Syntocinon®- Novartis pharma, Egypt) 10 I.U./amp/animals was used I/M complementary with uterine lavage (Rasch et al., 1996**;** Knutti et al., 2000 and Pycock, 2009), starting from the second uterine lavage (6h after lavage). On the 5th day of estrus the uterine cavity was examined thoroughly by ultrasonography to ensure that it is free from any fluids, the uterine wall thickness was assessed to ensure its normality. Moreover, ovaries were examined to assure normal follicular growth (Image, 16 & 17) (Kidd et al., 2014). First mating was applied on the 5th day morning and 7th day evening in the same estrus. After 6h from each mating (Knutti et al., 2000), the uterine cavity was washed again using the previous treatments according to each group protocol. Washing fluid drained away again by inclining the washing bag down.

Table (1):- Silymarin plus® compositions according to manufacturing SEDICO Co pharmaceutical industry

Silymarin plus® sachet composition		
1	Silymarin 70%	200 mg
2	Acetylcysteine	200 mg
3	Alfa-tocopherol acetate 50% (Eq. to 5 IU Vit E)	10 mg
4	Beta Carotene (Eq. to 300 IU Vit A)	0.5454 mg
5	Ascorbic acid	30 mg
6	Sodium selenite anhydrous (Eq. to 18.3 microgram Selenium)	0.04 mg
7	Zinc sulfate monohydrate (Eq. to 3.65 mg Zinc)	10.0375 mg

Statistical Analysis:-
Statistical analysis or hormonal data was carried out by Graph Pad Prism software 2007 version 5.03 (Graph Pad Prism, San Diego, CA) to determine the significant difference between treatment groups by one-way analysis of variance (ANOVA); Duncan's test was used to detect the significance of difference between pairs of groups at ($P<0.05$) and determined by superscripted letter. Data of conception and pregnancy rate were represented as percentage. The significant difference between values was determined at ($P<0.05$) using Chi-Square statistics test.

Results:-

The effect of silymarin plus® as additive versus Ciprofloxacin+ Amikacin admixture in the uterine lavage of mare on its conception and pregnancy rate:
Table (2) revealed that there was non-significant difference ($P<0.05$) between the treatment groups concerning the conception rate. However, there was significant difference (0.035) between the same groups regarding the pregnancy rate where silymarin plus® (G, $_1$) and Silymarin plus® + Ciprofloxacin and Amikacin admixture (G, $_3$)

attained the highest percentages (83.33 and 87.5 %, respectively) if compared with Ciprofloxacin and Amikacin admixture in the uterine lavage solution (G, $_2$- 33.33 %).

Table (2):- *Assessment the effect of silymarin plus® uterine lavage versus ciprofloxacin+ Amikacin admixture uterine lavage on mare pregnancy rate*

Kind of treatment used		Animals No./group	Conception rate		Pregnancy rate	
1	Uterine washing using silymarin plus® (G1).	8	5	83.33 %	5	83.33 %
2	Uterine washing using ciprofloxacin and Amikacin admixture (G2).	8	5	55.55%	3	33.33 %
3	Silymarin plus® + ciprofloxacin and Amikacin admixture (G3).	7	7	87.5 %	7	87.5 %

- Percentage was based on animal numbers used in each group.
- Pregnancy rate only was significant between treatment groups at (*P< 0.05*) using chi-square.

Estradiol 17-β (E2pg) and Progesterone ($P_{4\ ng}$) plasma levels before and after ovulation by 4 days in relation to prescribed uterine lavage treatment:

Table (3 and 4) illustrated that there were non-significant differences (*P<0.05*) between the treatment groups (G, $_{1, 2}$ and $_3$) concerning plasma progesterone level (table, 4). Also, the same pattern was adopted by plasma estradiol17-β, except on day (2) preceding and following the ovulation day (table, 3) where there were significant differences between treatments columns as described by superscripted letters.

Table (3):- Estradiol 17 β (E_{2pg}) plasma level before and after ovulation by 4 days in relation to prescribed uterine lavage treatment

Days regarding to mare ovulation		Estradiol 17-β (E_{2pg})		
		G (1)	G (2)	G (3)
Days before ovulation	-4	35.98±0.48^a	35.78±0.98^a	37.29±1.05^a
	-3	37.34±0.16^a	37.32±0.75^a	38.00±0.93^a
	-2	43.29±0.80^b	45.29±0.58^{ab}	45.62±0.69^a
	-1	59.01±0.53^a	58.61±0.76^a	59.93±0.78^a
Ovulation day	0	77.62±1.11^a	77.71±0.91^a	79.57±1.76^a
Days after ovulation	1	39.60±0.27^a	40.48±1.88^a	44.20±0.51^a
	2	38.01±0.72^{ab}	38.78±1.04^a	36.18±1.37^b
	3	34.19±1.09^a	35.20±0.86^a	35.67±1.35^a
	4	31.83±1.13^a	32.95±0.88^a	35.47±1.58^a

- Estradiol measurements were replicated 5 times (five animals in each group).
- Results are presented as mean±SEM; significant difference between treatment groups was not detected at (*P<0.05*) by one-way ANOVA and Duncan's test except on day 2 before and after ovulation.
- Values with ''a and b'' are significantly different between column at (P<0.05).
- G1= Silymarin plus®; G2= Ciprofloxacin + Amikacin; G3= Silymarin plus® + Ciprofloxacin + Amikacin

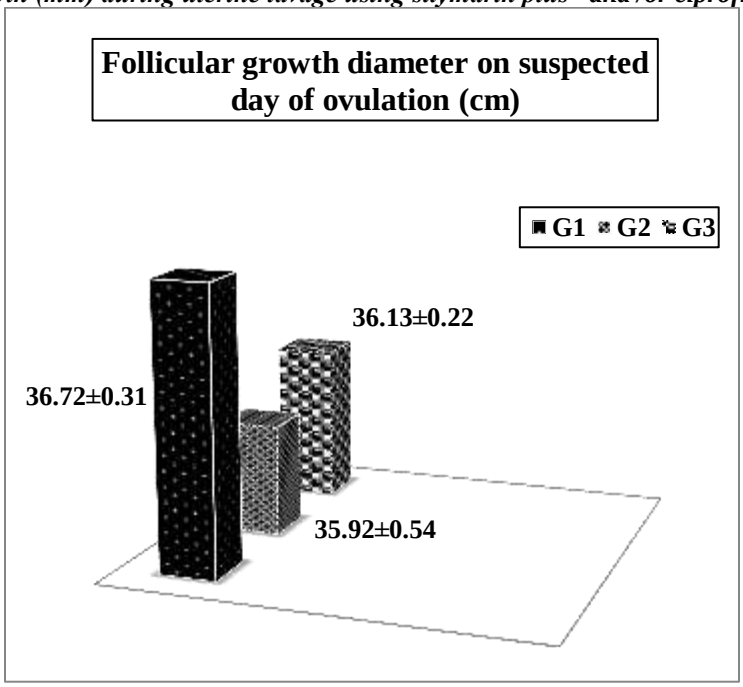
Table (4):- Progesterone ($P_{4\ ng}$) plasma level before and after ovulation by 4 days in relation to prescribed uterine lavage treatment

Days regarding to mare ovulation		Progesterone ($P_{4\ ng}$)		
		G (1)	G (2)	G (3)
Days before ovulation	-4	0.66±0.05^a	0.74±0.04^a	0.68±0.02^a
	-3	0.55±0.05^a	0.54±0.04^a	0.58±0.04^a
	-2	0.45±0.06^a	0.47±0.05^a	0.52±0.05^a
	-1	0.38±0.02^a	0.38±0.03^a	0.44±0.02^a
Ovulation day	0	0.34±0.05^a	0.27±0.04^a	0.22±0.03^a

Days after ovulation	1	0.62±0.02[a]	0.61±0.03[a]	0.66±0.06[a]
	2	1.64±0.14[a]	1.64±0.12[a]	1.68±0.11[a]
	3	1.97±0.10[a]	2.14±0.07[a]	2.06±0.11[a]
	4	2.56±0.12[a]	2.60±0.12[a]	2.46±0.11[a]

- Progesterone (P_{4ng}) measurements were replicated 5 times (five animals in each group).
- Results are presented as mean±SEM; significant difference between treatment groups was not detected at (*P<0.05*) by one-way ANOVA and Duncan's test.
- G1= Silymarin plus®; G2= Ciprofloxacin + Amikacin; G3= Silymarin plus® + Ciprofloxacin + Amikacin.

Mares' Follicular growth (mm) during uterine lavage using silymarin plus® and /or ciprofloxacin + Amikacin



- Figure (3): showing the patterns of follicular growth during the course of uterine lavage. It shows none-significant (P<0.05) differences between groups although G1 and G3 having the highest numerical follicular growth diameter (cm). G1= Silymarin plus®; G2= Ciprofloxacin + Amikacin; G3= Silymarin plus® + Ciprofloxacin + Amikacin.

Discussion:-

Repeat breeding syndrome (RBS) is a major infertility problem that causing substantial economic loss to the equine breeding industry (seasonal breeder). For that, much effort had been attended to clarify the equine pathophysiological mechanisms that involved in poor reproductive performances of mares to save this huge industry.

Uterine lavage with variety of antibiotics and/or antiseptics had much more benefits in augmentation the conception rate among repeat breeding animals (Ahmadi and Dehghan, 2007); through enhancing and clearing the uterine environment for embryo survival (Reddy et al., 2012).

For the first time, the current study explored the effect of silymarin plus® in the uterine wash in comparing with Amikacin+ Ciprofloxacin. The current results revealed that both silymarin plus® alone or in combination with Amikacin+ Ciprofloxacin improved mare conception and pregnancy rates .These results might be due to the powerful antioxidant capability of silymarin plus® due to its components that represented in Silymarin, vitamin E and C, Selenium and Zinc (de Groot and Rauen,1998; De La Portilla et al., 2004; Gayatri et al., 2010; Yildiz et al., 2011; Durmus et al., 2011; Du et al., 2012; Sengupta and Nandi, 2013; Hu et al., 2016). The association between

ROS and various gynecologic and obstetric conditions related to infertility suggests a potential role for oral antioxidant supplementation. The elevated lipid peroxide and decreased SOD in the endometrial cells during the luteal phase (Estrogen and progesterone changing period) might modulate endometrial breakdown by the elevated ROS level (Sugino et al., 2004; Sekhon et al., 2010). Normally the physiological level of ROS may mediate the physiological processes of shedding and implantation (Sugino et al., 2004); but the excess ROS hinder the endometrium (Iborra et al., 2005); by inducing luteal regression resulting in insufficient luteal hormonal support for the continuation of a pregnancy (Agarwal and Allamaneni, 2004). Besides that, silymarin plus® had powerful metal chelation effects due to presence of silymarin and NAC in between its components (Ferenci et al., 1989; Atkuri et al., 2007; Reinero et al., 2011 and Kissinger, 2012). Also, silymarin ensures the membrane stability by protecting the membrane fluidity (Ramellini and Meldolesi, 1974 and Muriel and Mourelle, 1990). Moreover, Silymarin stimulated follicle stimulating hormone (FSH) secretion (Khalil, 2002), and increased the uterine glands number acts by acting on the uterine wall causing its hypertrophy (Khalil, 2002). Also Silymarin plus® due to its components modulated inflammation and faded its signs (Berger et al., 1997; Saliou et al., 2001; Zafarullah *et al.* 2003; De La Portilla et al., 2004; Dixit et al., 2007; Yildiz et al., 2011 and Hu et al., 2016).Silymarin plus® contains NAC that had mucolytic effects (Amin et al., 2008; Gayatri et al., 2010; Kissinger, 2012; Gores-Lindholm et al., 2013; Cheek et al., 2014 and Ferris et al., 2014). So, it decreases the biofilm biomass and aid in killing the bacteria (*E. coli*) within the biofilms (Borlee et al., 2010 and Ferris et al., 2016). Furthermore, NAC was found to reduce post-breeding endometritis especially in mares suffering from low fertility as a result of post-breeding endometritis due to delayed uterine clearance (Melkus et al., 2013). Additionally, NAC inhibited or attenuated the deterioration of the endometrial epithelium (Gao et al., 2017]; by replenishing the stores of the glutathione (Fulghesu et al., 2002). As well as it restored the endometrial receptivity (Gao et al., 2017), depending upon its motivated modulation to the reproductive hormones receptivity (Gao et al., 2017), Moreover, NAC was found to improve pregnancy rate due to its mucolytic action, thus counteracted the negative influence of cervical mucus thus improving cervical mucus quality (Roumen, 1997). At the same time, it had insulin sensitizing effect (Fulghesu et al., 2002 and Kilic-Okman and Kucuk 2004). All of the preceding enhancing effects were reflected in increasing the pregnancy rate not the conception rate (Youssef et al.2006). Furthermore, vitamin E in silymarin plus® was found to improve the overall uterine health (Harrison et al., 1984 and [31]Sattar et al., 2007); so, decreasing days open and services per conception (Sattar et al., 2007 and [33]Moeini et al., 2009). Moreover, it prevents tissue adhesions (Durmus et al., 2011). Additionally, it significantly improved the uterine blood flow and improved the glandular epithelium growth (Takasaki et al., 2010 and Kurniawan et al., 2014). Selenium in silymarin plus® was found to stimulate uterine defense mechanism by improving neutrophil function (Thatcher et al., 2011). Moreover, Vitamin A in Silymarin plus® was necessary in maintaining uterine epithelium integrity (Macpherson and Schlafer, 2015). Moreover, retinol in general may be involved in the process of uterine gland development (Kissinger, 2012). Zinc in silymarin plus® have a pivotal role in controlling uterine fluid pH (Leese, 1995). Vitamin C as component in silymarin plus® has a role in controlling the endometrial thickness depending upon its role as cofactor in collagen synthesis in the luteal extra cellular matrix (Padayatty et al., 2003; Yamauchi et al., 2003 and Eteng et al., 2006).

The current data of assessed hormones during the course of uterine lavage showing non-significant differences between the treatment groups, reflecting that none of the used treatment had remarkable effect on the hormones level or in other-words the pattern of ovarian follicular growth. These results might be due to the different components of silymarin plus® such as silymarin that had no effect on the pattern of steroid production especially estrogen, although it increased the level of follicle stimulating hormone (FSH) not luteinizing hormone (LH), which reflected in increases in the size of growing follicles (Khalil, 2002). Moreover, Křen and Walterová, (2005) and Shah et al. (2005) reported that silymarin and selenium could modulate the activity of estrogenic receptors. Furthermore, zinc and vitamin C in silymarin plus® had positive effects on both estrogen and progesterone secretion (Om and Chun, 1996 and Al-Katib et al., 2013). Especially zinc even it used intrauterine where in An impressive example of the relation between zinc and female reproductive system is that estradiol and progesterone receptors obtained from calf uterus were bound to iminodiacetate-sepharose chelate colons that contained zinc (Vallee and Falchuk, 1993). Moreover, the current data illustrating that theses mares on which the current study applied were cyclic, having normal follicular growth (image 16&17), even though it had unexplained infertility.

Conclusion:-

Silymarin plus® inclusion in uterine wash of cyclic non pregnant mares with unexplained infertility improved its pregnancy rate might be due to its antioxidant activity or by compacting the negative effect of biofilm biomass in mare genital system. Even though, further researches are required to concentrate more attention on this hot point.

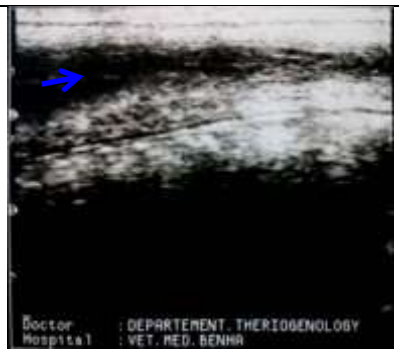
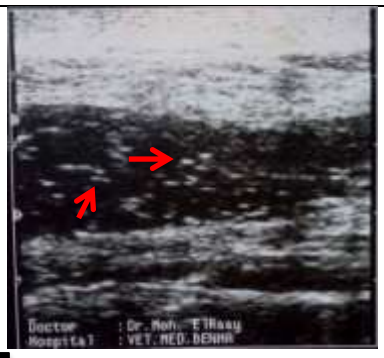
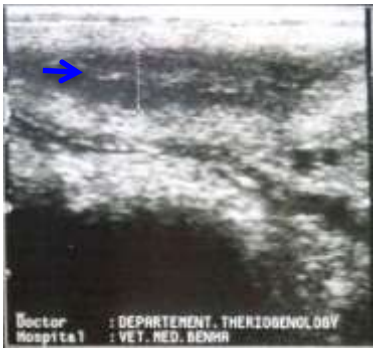
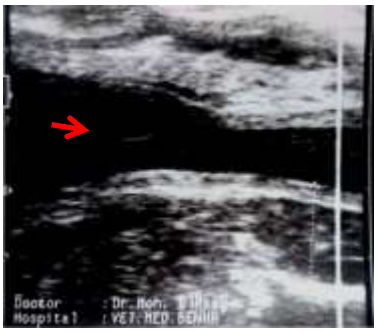
Part of genitalia	*Before uterine washing with Silymarin plus®*	*After uterine washing with Silymarin plus®*
Cervix	 1	 3
	 2	 4
		 5

Image (1 &2): Denotes cervical canal of estrus mare before lavage. Blue arrow means opened cervix contained no pus no abnormal content except slight echogenic strikes of estrus mucus, plus normal homogenous hypoechoic texture of the cervical wall.

Image (3): Show mare's cervix after the first lavage. There is normal hypoechoic pattern of the cervical wall. Red arrows denote the anechoic uterine lavage fluid (Silymarin plus®) containing hyper echoic dots that denote the liquefied contents of the excess mucus and others that might present in the uterus of mare during estrus and responsible for un-suitable uterine environment for pregnancy establishment. Hyper echoic content in the cervical canal decreased in amount on the second uterine lavage (Image, 4), and totally disappear on the third lavage where the cervical canal is entirely an echoic as it contain only the uterine lavage fluid and no liquefied contents (Image 5).

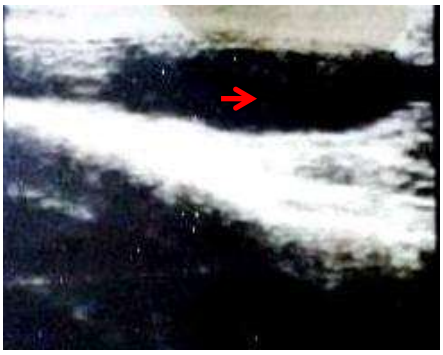
Part of genitalia	*Before uterine washing with Antibiotic mixture only*	*After uterine washing with Antibiotic mixture only*
Cervix	 **7**	 **8**

Image (7): Denotes cervical canal of estrus mare before lavage with antibiotic mixture. Blue arrow means opened cervix contained no pus no abnormal content except slight echogenic strikes of estrus mucus, plus normal homogenous hypoechoic texture of the cervical wall.

Image (8): Show that mare's cervix after the first and second lavages. There is normal echogenic pattern of the cervical wall. A red arrow denotes the anechoic uterine lavage fluid (antibiotic mixture). Notes there are no hyperechoic dots that denote the liquefied mucus and others that might present in the uterus of mare during estrus.

Part of genitalia	*Before uterine washing with Silymarin plus®*	*After uterine washing with Silymarin plus®*
Uterus	 **9**	 **11**

10

12

13

Image (9 &10): A sagittal B-Mode image denotes mare's uterus during estrus before lavage. Blue arrows denote normal homogenous hypo echoic uterine cavity with no pus or abnormal contents with normal hyper echoic uterine wall thickness with no signs of inflammation or fibrosis.

Image (11): Show that mare's uterus after the first lavage. Red arrows denote anechoic uterine lavage fluid (Silymarin plus®) containing hyper echoic fine dots that denote the liquefied contents of the excess mucus and others that present during estrus. Hyper echoic fine contents in the uterine cavity decreased in amount on the second uterine lavage (Image, 12), and totally disappear on the third lavage where the uterine containing only the uterine lavage fluid (an echoic) and no liquefied contents (Image, 13).

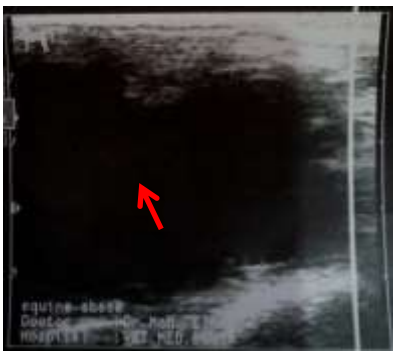
Part of genitalia	*Before uterine washing with Antibiotic mixture only*	*After uterine washing with Antibiotic mixture only*
	 14	 15

Image (14): A sagittal B-Mode image denotes mare's uterus during estrus before lavage. A blue arrow denotes normal homogenous hypo echoic uterine cavity with no pus or abnormal contents with normal hyper echoic uterine wall thickness with no signs of inflammation or fibrosis.

Image (15): Show that mare's uterus after the first and second lavages. A red arrow denotes anechoic uterine lavage fluid (antibiotic mixture). Notes there are no hyperechoic fine dots that denote mucus and others that present during estrus.

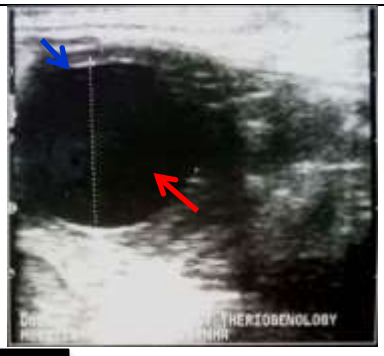
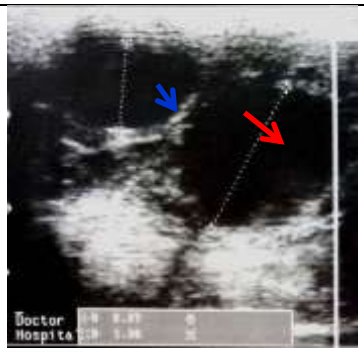
Part of genitalia	*During uterine washing with Silymarin plus®*	*During uterine washing Ciprofloxacin plus Amikacin*
Ovary	 16	 17

Image (16 & 17): Illustrating the patterns of follicular growth in between the treatment groups on the day before the suspected day of ovulation. Red arrows denote an echoic follicular fluid and blue arrows denote the hypo echoic follicular wall.

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