RESEARCH ARTICLE

SCREENING AND EVALUATION OF ANTIMICROBIAL ACTIVITY OF THYMUS VULGARIS USING IN VIVO AND IN VITRO METHODS

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Manuscript Info	*Abstract*

There is a great effort to discover plant-derived antibacterial against multi-drug resistant bacteria. The objective of the study to the possibility of organic production of Thymus vulgaris herb under sandy soil and investigate the in vitro and in vivo antibacterial activities of thyme essential oil that was analyzed and its constituents were identified by GC/MS. The major components were thymol (45.15%) and p-cymene (25.31%), borneol (5.06%), myrecen (3.42%) and α-pinene (3.21%). A Staphylococcus aureus-infected mice model was subjected to meet the study objective. The physiological, biochemical and histopathological markers of possible effects of this essential oil were studied using various standard methods. The extract had a significant effect on the number of viable Staphylococcus aureus recovered from spleen where 4MIC group was the best group in inhibiting the bacteria followed by 2MIC and 1MIC. There were no side effects on vital organs observed even in using 4MIC dose. The obtained results indicate that the essential oil of thyme has the potential to provide an effective treatment for S. aureus infection. It is necessary to further investigate the interaction between the pathogenic bacteria and thyme essential oil and its impact on the development of resistance.

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Introduction:-
Regarding the developing countries, traditional medicine considered the most important source of drugs that used for treating many types of infectious microorganisms. The rise of multidrug-resistant bacteria, expensive price of synthetic drugs and many adverse side effects are factors encouraged the pharmaceutical companies to investigate alternative sources to produce novel therapeutic agents. Medicinal plants have been considerable attention in this issue where these plants have the capability to produce a variety of secondary metabolite compounds (Jaber*et al*., 2012) that be consumed to protect themselves against the attack of different pathogenic microorganisms (Chaudhry and Tariq 2009).

Thyme (*Thymus vulgaris*) is a member of family Lamiaceae and locally known as 'Zaatar'. Since many decades, Thymus essential oil (EO) has been broadly used as a medicinal plant in Egyptian flock medicine, alternative medicine, and natural therapeutics. As a valuable medicinal plant, Thymus vulgaris possesses a wide range of biological activities including carminative, antispasmodic, expectorant, antiseptic, and antioxidative properties

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513

(Omidbaigi and Nejad 2000; Dapkevicius*et al* 2002 and Vardar, *et al*., 2003). Common thyme contains 0.8-2.6% volatile oil. The main constituents of thyme include thymol, carvacrol and flavonoids often thought to have an anti-bacterial effect (Barnes *et al*., 2002). Earlier work has been approved that essential oils of some plants display antimicrobial properties especially against pathogenic bacteria (Prabuseenivasan*et al.,* 2006). This study was planned for estimating in vitro and in vivo antibacterial activities and possible side effects of essential oil of thymus vulgaris against *Staphylococcus aureus* (*S. aureus*) strain.

Materials and Methods:-
Bacterial strains:-
S. aureus strain operated in this work was obtained from microbiology laboratory, department of animal health, Desert Research Center.

Collection and preparation of plant sample:-
Thyme plant was obtained from Sekem farm in the desert of Belbais, Egypt. The collected plant was dried and stored in a dry place until extraction.

Extraction process:-
The dried plant was subjected to the hydro-distillation process for about 3 hours by using Clevenger apparatus as stated by European Pharmacopoeia (1996). Finally, the obtained essential oil (EO) was dehydrated in a desiccator containing anhydrous sodium sulfate and then stored in the refrigerator at 4°C until operated.

Phytochemical analysis:-
Gas chromatography mass-spectrophotometer analysis of thyme essential oil:-
The gas chromatography-mass spectrometry (GS-MS) analysis for oil samples from the second season was carried out at the Central Laboratory ofNational Research Center, Giza. Essential oil GC/ Mass analysis was performed using a Hewlett-Packard 5890 A series 11 instrument equipped with flame ionization detector (FID) and a carbon wax fused silica column (50 m x 0.25mm. i. d., film thickness 0.32 µm). The initial column temperature was 50 Cº and held for 3 minutes, then raised to 60 cº by rate 3.0 Cº per minute and raised to 260 Cº by rate 3.0 Cº per minute and hold at 260 Cº for 5 minutes.The volatile oil components wereidentified by comparing their retentiontimes and mass spectrum with those ofstandards, NIST library of the GC-MSsystem, and literature data. Identifications were made by library searches combining MS and retention data of authentic compounds by comparison of their GC retention indices (RI) with those of the literature or with those of standards available in our laboratories (Adams, 2007).

Antibacterial sensitivity test:-
*S. aureus*was investigated against 12 different antibiotics (Oxoid, UK) via Kirby-Bauer disc diffusion method.

Assessing of antibacterial activity:-
The antibacterial assay was carried out according to Alderman and smith 2001 with some changes. *S. aureus*suspension in PBSwas adjusted to be matched with 0.5 McFarlane standard tubes that equivalent to $1x10^{8}$ CFU/ml. One hundred microliters were spread onto Mueller-Hinton agar (MHA, Oxoid, UK) plates on which wells with 8mm diameter were made by cork borer. After 24 hours incubation, the inhibition zone diameter was measured in millimeters. The test was achieved in triplicates. The results were interpreted as weak action (<10 mm), moderate action (10-15 mm) and strong action (>15 mm) according to Adithepchaikarn et al., 2008.

Estimation of minimum inhibition concentration (MIC) via micro-dilution technique:-
Thyme oil was diluted in methanol to be in a concentration of 640 mg/ml and then to concentration 6400 µg/ml in Muller Hinton broth (MHB, Oxoid, Basingstoke, Hampshire, UK). Afterward, 100 µl of the MHB dilution were diluted serially in 96-well plates (Corning Inc., Corning, NY, USA) to be ranged from 3200 to 100 µg/ml, according to CLSI 2008. Next, 10 µl of *S. aureus* suspension in saline ($1x10^{8}$ CFU/ml) were added to each well of the micro-dilution plates. The inoculated plates were incubated at 37°C for 24 hours. The MIC was estimated as the lowest concentration at which no growth was shown in comparison with positive control wells (broth only).

In vivo assessment of antibacterial activity of thyme oil:-

Forty-eight Swedish mice (*Musmusculus*) were divided into six groups of eight mice each as the following: Control (injected with 100 µl saline), *S. aureus* infected (injected with 100 µl of 1×10^8 colony-forming units (CFU) of *S. aureus* suspension), *S. aureus* infected plus antibiotic (Gentamycin, Sigma, USA) in dose 40 mg/kg/day, *S. aureus* infected plus 1 x MIC, *S. aureus* infected plus 2 x MIC and *S. aureus* infected plus 4 x MIC. To facilitate the infection, all treated groups were injected I/P with cyclophosphamide (Sigma, USA) (30mg/kg body weight) at 72 hours prior to infection. All groups were infected via subcutaneous injection. Five days later, the animals were sacrificed and spleen from individual animals was weighted and homogenized in phosphate-buffered saline (PBS), serially diluted 10-fold and streaked on Baird Parker plates (Oxoid, Basingstoke, Hampshire, UK), and incubated at 37°C for 24 hours to measure the bacterial load. The results were expressed as log (CFU/gm) of the spleen (Barquero-Calvo*et al*., 2013). Blood was collected and sera were prepared for estimation of hematological parameters and organ function tests, respectively.

Haematological and Biochemical assays:-

The collected blood was subjected to analysis by using automated blood analyzer (Abbott Labs Cell-Dyn 1800, USA) to determine the white blood cells count (WBCs), red blood corpuscles (RBCs), and haemoglobin (Hb) concentration. The above-prepared sera were subjected to estimate the following parameters using commercially available kits: alanine aminotransferase (ALT), total protein (TP), Albumin (Alb) (TECO DIAGNOSTICS, ANAHEIM, CA), urea and serum creatinine (BIOASSAY SYSTEM, HAYWARD, CA).

Histopathological assessment:-

Liver and kidneys morphology were characterized by light microscopy using routine hematoxylin and eosin (H&E) staining. After sacrificing the mice, small pieces of liver and kidney were fixed in 10% formol-saline solution. The fixed tissues were embedded in paraffin wax, sectioned, and stained.

Statistical analysis:-

The obtained data were expressed as mean ± SEM. The means of different groups were compared by using one-way analysis of variance (ANOVA) with Turkey's post-hoc test. The statistical analysis was performed by using SPSS version 21.0 (Chicago, IL). A *p*-value of <0.05 was considered statistically significant.

Ethics:-

The work was done with the respect recommended by WHO*for the animal welfare.

Result:-

The chromatographic analysis of the thyme essential oil revealed that Thymol (45.15%) and ρ-cymene (25.31%) are the major constituents as illustrated in Table (1).

Table 1:-The main constituents of the essential oil% of *Thymus vulgaris*

Compound	Percentage
Thymol	45.15
ρ-cymene	25.31
Borneol	5.06
Linalool	4.10
Myrecen	3.42
α-Pinene	3.21
γ-Terpinene	2.80
Terpinol	2.62
trans-Caryophyllene	0.75
Cadinene	0.55
Camphor	0.51
Germacrene-D	0.42
Carvacrol	0.19
Caryophyllen oxide	0.13
Carotol	0.11
β-Bourbonene	0.05
Total	94.38%

The antibiotic susceptibility of S. aureus revealed that the used strain was sensitive to (CN, TOB, TET, VA, CTX and CIP) while was resistant to other used antibiotics. Based on the definition of Multidrug resistance (MDR) by Magiorakos et al 2012 as when the isolate is non-susceptible to at least one agent in ≥ 3 antimicrobial categories, the used *S. aureus* strain is considered a multi drug resistant strain as shown in Table 2.

Table 2:-Antibiotic susceptibility of *S. aureus*

Antibiotic	Disc content	Inhibition zone/mm	Antibiotic	Disc content	Inhibition zone/mm
Amoxicillin- Clavulanic Acid (AMC)	30 μg	7	Gentamycin (CN)	10 μg	38
Ciprofloxacin (CIP)	5 μg	20	Penicillin (P)	10 units	6
Clindamycin (DA)	2 μg	5	Piperacillin (PRL)	100 μg	18
Oxacillin (OX)	5 μg	8	Tetracycline (TE)	30 μg	30
Cefotaxime (CTX)	5 μg	24	Tobramycin (TOB)	10 μg	34
Erythromycin (E)	15 μg	6	Vancomycin (VA)	30 μg	30

The activity of the thyme oil as antimicrobial agent against *S. aureus* strain was determined by using disc diffusion and MIC techniques. It is obvious that the oil had a strong antibacterial activity particularly at the concentration 100% whereas the inhibition zone was 28 mm in diameter. As the MIC was calculated as the lowest concentration causing inhibition of the visible growth of tested organism, the MICin our study was 0.025%, as shown in Table 3.

Table 3:-Antibacterial effect of thyme oil against *S. aureus*

Bacteria	Concentration/ inhibition zone in mm					MIC (V/V)%
S. aureus	100%/ 28	75%/ 22	50%/ 19	25%/ 16	12.5%/ 11	0.025%

Thyme oil and bacterial count:-
As can be illustrated in fig (1), thyme essential oil confirmed to have in vivo antibacterial effect on tested *S. aureus* strain. Whereas there is no significant difference (P>0.05) between the result obtained from 2MIC and 4MIC treated groups with those of antibiotic treated group while 1MIC treated group showed lesser activity.
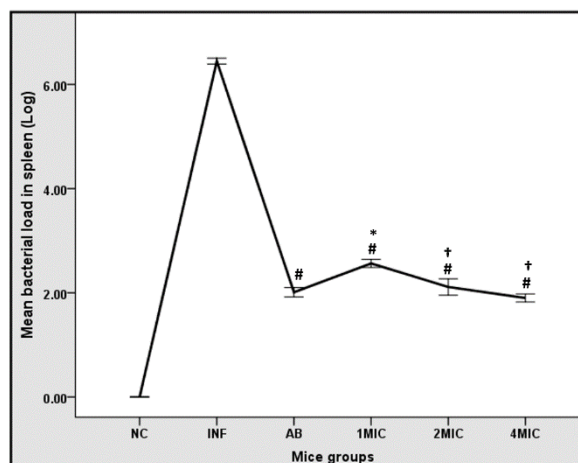

Fig 1:-Effects of double doses of MIC of thyme essential oil on splenic bacterial number.

Effect of Thyme essential oil on hematologicalparameters:-
As it is shown in fig (2), there were significant (*P<0.05*) differences between the negative control group (INF) and all treated groups in terms of decrease Hb and RBCs and increase WBCs. Concerning Hb concentration and RBCs, 4MIC group showed significance (*P<0.05*) decrease when compared with NC while WBCs, it showed significance decrease when compared with 1MIC.
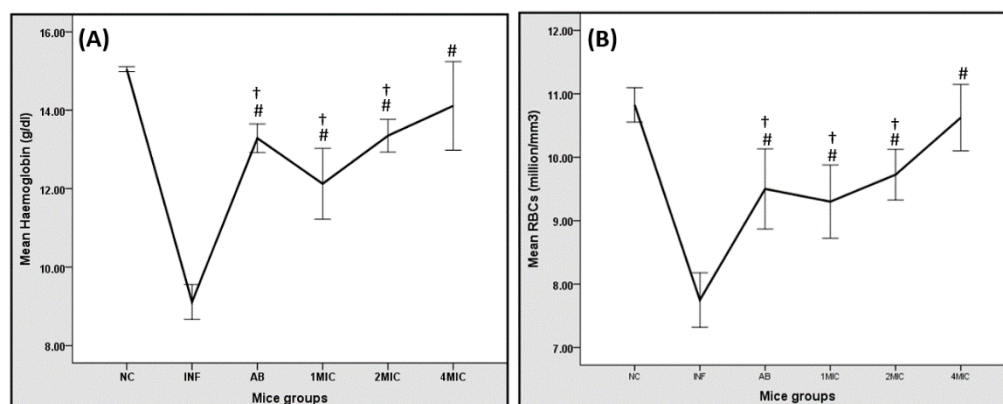
Fig 2:- Effects of double doses of MIC of thyme essential oil on: hemoglobin (g/dl). (B) RBCs (10^6/mm^3), and (C) WBCs (10^3/mm^3).

Effect of thyme extract on liver and kidney function tests:-
As illustrated in fig 3, concerning ALT, there was no significant difference ($P > 0.05$) between treated groups except in case of 4MIC group whereas it recorded the highest level. It was found that the infected group (INF) recorded the highest activity of the enzyme when compared with the other groups and followed by 4MIC group. Regarding serum creatinine, there was significant difference ($P < 0.05$) between the infected (INF) and the all treated groups. However, 2MIC group recorded the nearest enzyme activity when compared with the antibiotic treated (positive control) and normal (neutral) groups and followed by 4MIC group.
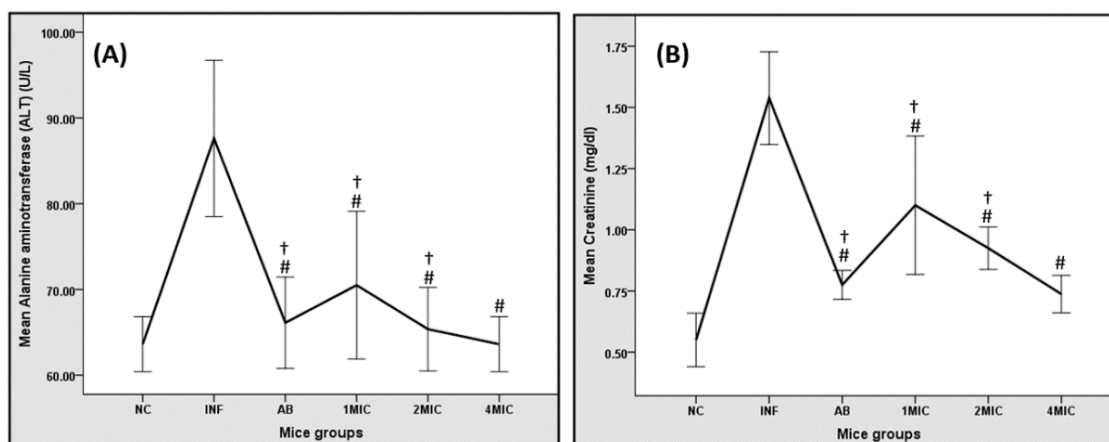

Fig 3:- Effects of double doses of MIC of thyme essential oil on liver (ALT) and kidney (creatinine) functions.Diverse symbols show significant differences (P < 0.05) in different dosage groups in Duncan's multiple range test.

Effect of thyme extract on the histology of liver and kidney sections:-
Histopathological examination of hepatic sections (fig 4) revealed normal hepatocytes architecture of non-infected and all treated groups in different levels ranged from completely normal sections with normal hepatocytes with normal cytoplasm, nucleus, and central vein, mild signs of inflammation as congestion of central vein and hepatoportal blood vessels to vacuolar degeneration of the hepatocytes. The infected group showed the highest degree of inflammation and degeneration while 4MIC showed the best normal sections.
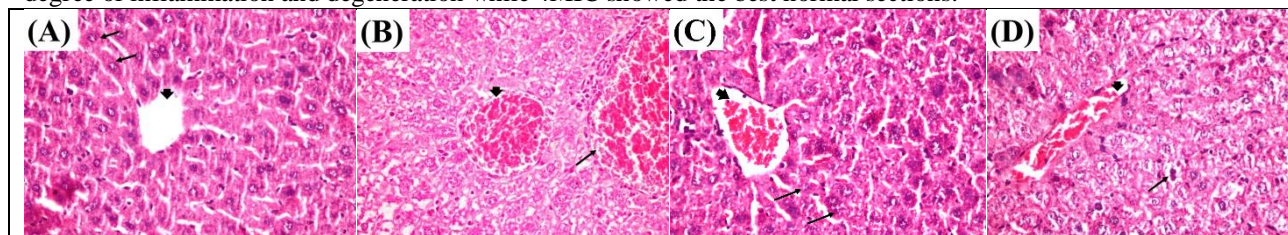

Fig 4:-Histopathological lesions in liver of treated and non-treated groups.

Normal hepatocytes architecture(A), congestion of the central vein (arrow head) and hepatoportal blood vessel (arrows) (B), degenerated hepatocytes (arrow) with congested central vein (arrow head) (C) and vacuolation (ballooning degeneration) of the hepatocytes (arrows) and congested central vein (arrow head) (D) (H&E X 400).
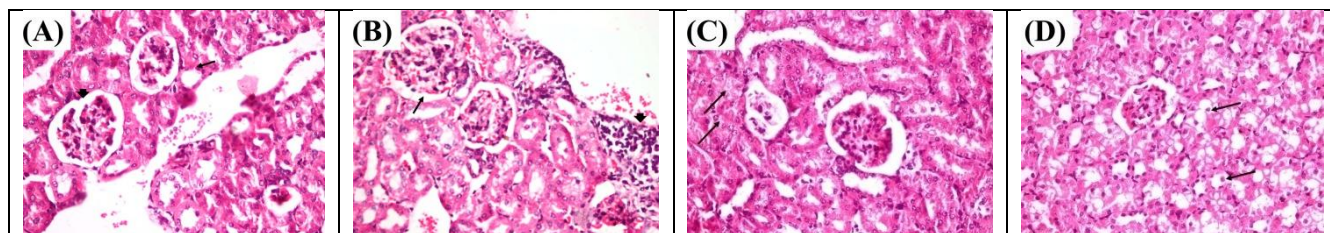

Fig 5:-Histopathological lesions in kidney of treated and non-treated groups.

Normal renal architecture (A); Kidneys showing congested glomerular capillaries (arrows) and leucocytes infiltrations (arrow head) (B); Kidneys showing degeneration of some renal tubules (arrows) (C) and Kidneys showing vacuolation of renal tubular epithelium (arrows) (D).

Almost the same trend of hepatic sections was shown in renal sections (fig 5) where all treated groups resemble to large extent the control group especially 4MIC group while 1MIC and 2MIC groups showed moderate signs of inflammation in the form of congestion of the interstitial blood vessels and degeneration of some convoluted tubules whereas infected group displayed the highest vacuolar degeneration of renal tubules.

Discussion:-
Essential oils have many applications in veterinary medicine such as insecticidal, anti-parasitic and treatment of infectious diseases in animals. Studies deal with antimicrobial properties of herbal plants essential oils against microorganisms with veterinary importance in vitro and in vivo are as yet restricted. Essential oils were applied on a large scale, in spite of their mechanism of action was not well clear. Because of their antimicrobial activity, they were mainly used practically to treat infections (Tiwari*et al.*, 2009; Bakkali*et al.*, 2008 and Burt 2004). From our foregoing findings on antimicrobial sensitivity testing of thyme essential oil extract against S. aureus, it was detected that the thyme essential oil has bactericidal activity with MIC 0.025% but their effectiveness varied according to the concentration (Table 3). Almost the same result was obtained in other studies (Ivanovic*et al*,.2012) against *E. coli* and *Salmonella* strains with MIC of 640 µg mL-1 and in contrast with others (Imelouane*et al.,* 2009) who did not show any inhibition or selectivity towards Gram positive bacteria. In our work, this antimicrobial activity may be due to the presence of the greater concentration of phenolic compounds (Cetin *et al.*, 2011; Rota *et al.,* 2007. Skocibusic*et al*., 2006), thymol, ρ-cymene, borneol and α-pinene (Tabanca*et al.*, 2001; Vardar *et al*., 2003) in a percentage of 45.15, 25.31, 5.06, and 3.21, respectively.

In the present work, the antimicrobial activity of thyme essential oil may be attributed mainly to the high content of thymol (45.15%) and p-cymene (25.31%). Thymol had been established as bactericidal (Sikkema*et al.,* 1995) by a variety of mechanisms of action result in structural and functions modifications in the cytoplasmic membrane by affecting its permeability causing the release of K+ ions and ATP. This in turn leads to cytoplasm coagulation, the denaturation of many enzymes involved in various carbon and nitrogen sources catabolism as well as the loss of ions and metabolites and then cellular death (Nazzaro*et al.,* 2013; Burt and Reinders., 2003; Xu*et al.*, 2008; Walsh *et al*., 2003). Regarding p-cymene, it is a monoterpene, some previous work revealed that it has antimicrobial activity when it is used alone (Bagamboula*et al*., 2004; Aligiannis*et al*., 2001 and Mann *et al*., 2000) and others had a different opinion when they described its antimicrobial effect as cofactor by increasing the antibacterial activity of other compounds such as, its derivative carvacrol (Rattanachaikunsopon*et al*., 2010 and Ultee*et al*., 2002). P-cymene does not affect the membrane permeability but accepts its antimicrobial activity as it has a high affinity for bacterial membranes causing them to expand and decreasing their melting temperature (Cristani*et al*., 2007), and affecting the membrane potential of intact bacteria (Ultee*et al*., 2002).

According to other studies (Sivropoulou*et al*., 1997; Carson and Riley., 1995 and Sur *et al*., 1991) camphor, terpinen and 1, 8-cineole was principally responsible for the antibacterial activities of the plant essential oils. It is worth mentioning that the antimicrobial activities of essential oils are difficult to correlate to a specific compound due to

their complexity and variability where their constituents concentrations may be affected by several factors (Nevas*et al.*,2004; Cakmakcı*et al.*, 2009 and Osman *et al.*, 2009) such as water stress, the seasonal condition, chemical polymorphism, the origin, the stage of the collected plant material, and the method of extraction where there is no an international standardized technique for the evaluation of essential oils allows the use of different protocols, hampering the comparison of the results (Smith-Palmer *et al.*, 1998 and Viuda-Martos*et al.*, 2008). So, we used the M31-A3 protocol (CLSI; 2008) in our work as it is the most recommended among the technique found in the literatures. What is more, the activity of the essential oil was superior to the compound alone (Santurio*et al.,* 2014). Such finding is explained by the fact that the high antimicrobial activity showed by essential oils results from the synergism of the major components (Höferl*et al.,* 2009) and even trace elements as yet unidentified (Tepe*et al.,*2005 ; Goze, *et al.,*2009).

It is obvious from the result of disk diffusion test that the used strain of *S. aureus* considered a multi-drug resistant bacterium. This resistant may be attributed to many factors; one of those is possessing penicillinase enzyme (Kumar *et al.,* 2011) which renders the bacteria resistant to β-lactam antibiotics (MRSA). Other factors of antibiotic resistance in the bacteria may be back to the genetically presence of antibiotic resistance genes on its chromosomes or plasmids, or getting them via a variety of genetic means as transformation, conjugation, and transduction or selecting the bacteria containing resistance genes by unwise use of penicillin or other antibiotics. All these processes promote and enhance the presence of antibiotic resistance and pathogenic *S. aureus* strains (Kumar *et al.*, 2011).

Depending on the established data provided by in vitro antibacterial test results, in vivo study was conducted with a view to confirming the therapeutic effectiveness of the extract. The present study results revealed that the administration of thyme essential oil in different doses inhibited the growth, and reduced the viable *S. aureus* numbers. This ability of inhibition may be attributed to the existence of phytochemical groups as aforementioned in Table 1. This result recommended that the antibacterial activity against *S. aureus* obtained in vitro was continued in vivo.

To check whether there were any statistically significance differences in the spleen bacterial load achieved by different treatments against *S. aureus*, two-way analysis of variance followed by Turkey`s test was applied. This analysis discovered significance variations (5% level) among the different treatments where the effect of this essential oil against *S. aureus* gave a significant ($P<0.05$) result as an antibacterial agent when comparing infected control groups with all treatments where 4MIC group was the best group in inhibiting the bacteria followed by 2MIC and 1MIC. This indicates that the thyme essential oil has inhibitory effect on *S. aureus* and the bacterial inhibition increased directly with the thyme essential oil concentration, as shown in Fig. 1

On the other hand, it must be taken into account that the highest thyme oil concentration (4MIC) had no bad effect on the hemoglobin concentration and RBCs count (Fig. 2) as well as the vital organs as liver and kidneys as indicated by the normal ALT and creatinine levels which commonly used as markers of liver and kidney degeneration (Olaleye*et al.*, 2014; Suganthi*et al.*, 2013 and Bidie*et al.*, 2010), respectively (Fig. 3). High level of this enzyme in the infected untreated group was backed to the infection. The hepatic damage was further supported by histopathological findings in liver and kidney sections (Fig. 4 and 5). The hepatic sections of infected untreated groups revealed signs of inflammation in the form of dilatation and congestion of hepatoportal blood vessels with ballooning degeneration of hepatocytes. At doses of 1MIC, 2MIC and 4MIC liver and kidney sections revealed a significant difference (*P<0.05*) from the infected untreated group and showed the normal appearance similar to large extent the control group particularly in the case of 4MIC group. Therefore, the thyme essential oil at these doses not only overcome the infection but might correct the harmful effect of infection on hepatic and renal cells. Nonetheless, the major signs of hepatic and renal damage as necrosis and apoptosis (Eroschenko, 2000) were not detected in our study, the same findings were observed in another previous study (Tala*et al.*, 2015).

Conclusion:-
The inclusive results of our study provide baseline data for the possible use of essential oils in the remedy of *Staphylococcus aureus* infection and can be considered the best alternative to the antimicrobial drugs in facing the drug resistance development. Beside the antimicrobial activity, the information recorded from using doubled doses showed that the thyme essential oil considered safe in the treatment of bacterial infection. This effect was dose dependent, therefore, there is need to conduct experiments on large scale and on large animals, both in vitro and in vivo, to establish the maximum safe level of this EO against bacteria and also, it is necessary to further investigate

the interaction between the pathogenic bacteria and thyme essential oil and its impact on the development of resistance.

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