RESEARCH ARTICLE

THE EFFECT OF ALKALOIDS, SAPONINSAND THYMOQUINONE OF *NIGELLA SATIVA* SEEDS ON BIOFILM PRODUCTION, MOTILITY, OUTER MEMBRANE PROTEINS AND LIPOPOLYSACCHARIDE OF SOME BACTERIA.

Mohamed Shohayeb[1] and Eman Halawani[2].
1. Taif University, Faculty of pharmacy, Department of microbiology, Taif, Saudi Arabia.
2. Taif University, Faculty of Science, Department of Biology , Taif, Saudi Arabia.

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Manuscript Info	*Abstract*
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Alkaloids, saponinsand thymoquinone of*Nigella sativa*are active against both Gram-positive and Gram-negative bacteria. Subinhibitory concentrations of theseconstituentswere tested for their effect on biofilm production, motility and the expression of the proteins and the lipopolysaccharides of the outer membrane of Gram-negative bacteria. While all the three tested constituents reduced the biofilm formation by*E. coli*, only saponins and alkaloids reducedthe biofilm formation in*K. pneumoniae* and *Sal.* Typhimurium. Nonetheless, alkaloids, saponinsand thymoquinone, enhanced the biofilm formation in *P. aeruginosa* and *Sh. flexneri.* On the other hand,thymoquinone inhibited the motility of *Escherichia coli, P. aeruginosa* and *Sal.* Typhimurium. There were changes in the level of expression of seven and three outer membrane proteins of*E. coli* and *P. aeruginosa,*respectively. Amongst these changes in *E. coli,*the amounts of OmpF apparently decreased and those ofOmpA increased. Also, three outer membrane proteins of *P. aeruginosa* were affected by the phytochemicals includingOprF. The expression of the latter was increased by alkaloids. Electron microscopy revealed some morphological changes in*S. aureus* and *P. aeruginosa.* It may be concluded that thymoquinone, alkaloids and saponins affect several pathogenesis mechanisms in both Gram-positive and Gram-negative bacteria.

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Introduction:-

For centuries, *Nigella sativa* L. (Family *Ranunculaceae)* seeds have been traditionally used in bakery as pungent appetizer and aromatic, and folk medicine in the Middle East, Eastern Europe, Asia, and Africa, as thermogenic, diuretic, expectorant, purgative, stimulant, sudoriferous, sedative, carminative and for many other diseases in different (1, 2). Muslim communities,in particular, considered *N. sativa*seeds as one of the most important prophetic medicinal plants because its healing effects were referred to by the Prophet Muhammad (Peace be upon him) (3).

Nigella sativa seeds contain different pharmacologically active constituents like alkaloids, saponins and essential oil (4). These constituents have been investigated for their pharmacological effects both *in vitro*and *in vivo*(5,6). Theyhave been demonstrated to enhance the immune system (7), and to have galactagogue, carminative, laxative

Corresponding Author:- Mohamed Shohayeb.
Address:- Taif University, Faculty of pharmacy, Department of microbiology, Taif, Saudi
Arabia.

(2), anti-inflammatory (8), anti-cancer (9,10) antimicrobial (11-15), anti-parasitic (16), antioxidant (17), hypoglycemic (18) activities.

The different extracts of *N. sativa* seeds were found to possess broad-spectrum antimicrobial activity against bacteria, viruses and fungi (19). This broad-spectrum of antimicrobial activity may be attributed to their effect on the key biochemical elements of microorgansims (20).

Extracts of *N. sativa* were found to inhibit both Gram-positive and Gram-negative bacteria. Water extracts of the seeds were active against *Staphylococcus aureus*(21, 22). Diethyl-ether,Methanol and chloroform extracts of the seed of *N. sativa* inhibited the growth of *Escherichia coli, Helicobacter pylori,Bacillus subtilis* and *Streptococcus fecalis* (23,24).

The antibacterial activity of crude extracts and phytochemicals of*N. sativa* have also been found to be active against multi-drug-resistant bacteria, including both Gram-positive bacteria like *Staphylococcus aureus* and Gram-negative bacteria like *Pseudomonas aeruginosa* and *Escherichia coli* (15, 25, 26).

Some purified constituents of *N. sativa* have also been tested for their antibacterial activity. The first reported constituent which possessed an anti-bacterial activity was the phenolic fraction of *N. sativa* oil (13). The essential oil of *N. sativa* and some of its components like thymoquinone and hydrothymoquinonefvwere found to be lethal to fungi, Gram-positive and Gram-negative bacteria (27-31). The antibacterial activity of alkaloids and saponins, of *N. sativa,* were evaluated both qualitatively and quantitatively (15, 32, 33).

In a previous study, we demonstrated the antibacterial activity of alkaloids, saponins and thymoquinone of *N. sativa* seeds on a wide range of antibiotic sensitive and resistant bacteria (15).In this report, we investigated the effect of these active constituents on slime production, motility, outer membrane proteins and lipopolysaccharide.

Materials and Methods:-
Bacteria and chemicals:-
The six pathogenic bacteria used were *Escherichia coli ATCC 35218, Salmonella enterica serovar*Typhimurium *ATCC 14028 (Sal.*Typhimurium*, Pseudomonas aeruginosa ATCC 27853 (P. aeruginosa),Klebsiella pneumonia (K. pneumoniae), Shigellaflexneri*(*Sh. Flexneri)*and *Staphylococcus aureus* (*S. aureus*) were clinical isolates of the culture collection of the Department of Microbiology, College of Pharmacy, Taif University. Thymoquinone and organic solvents were purchased from Sigma-Aldrich.

Extraction of alkaloids:-
Powdered seeds of the plant were extracted with 70% methanol. The extract was evaporated under reduced pressure, dissolved into distilled water and acidified with 3% hydrochloric acid. The solution was extracted with petroleum ether. The acidic solution was made alkaline with 25% ammonium hydroxide (pH 9-10) and then extracted with chloroform. The combined chloroform extracts were dried to get the crude *N. sativa* alkaloids (34).

Extraction of saponins:-
Powdered seeds of the plant were defatted with n-hexane. Further extraction was performed with 70% methanol. After evaporation of the methanol under vacuum, the crude residue was acidified with 5% hydrochloric acid and left overnight in the refrigerator. The precipitate was extracted with chloroform: methanol (75:25), to get crude saponins (35).

Biofilm production (36):-
Thymoquinone, saponinsand alkaloids of *N. sativa* were two-fold serially diluted with a minimum medium (M9) to give series of concentrations in sterile 96-well polystyrene microtitration plates. Each series of dilutions was inoculated with 10^4 CFU/ml of the tested bacteria and incubated at 37^oC for 18 hours. Bacteria suspension was aspirated and wells were washed-buffer saline (pH 7.5), ethanol, and stained with 0.1% crystal violet for 30 min. Wells were rinsed with water and filled with ethanol for 30 min and their contents were transferred to another microtiter plate. Plates were read at 595nm using an ELISA reader.

Effect of some constituents on swarming and motility of bacteria (37):-
Bacteria were grown onto swarming or swarming plates containing subinhibitory concentrations of *N. sativa*: Plates were incubated overnight at 30°C and zones of swimming or swarming were measured and compared to those of control plates.

Effect on outer-membrane proteins(38):-
Log phase bacteria treated with sub-inhibitory concentrations of some constituents of *N. sativa* were harvested and sonicated. Debris was harvested by centrifugation and membranes were collected by high-speed centrifugation. Inner and outer membranes were separated by differential solubilisation and with sarkosyl and subjected to SDS-PAGE

Purification of lipopolysaccharide:-
Log phase bacterialcells were lysed at 100°C with SDS in presence of glycerol and β-mercaptoethanol. Lysed cells were treated with proteinase K at 60°C for 1 hr(**39**), subjected to SDS-PAGE (40).

SDS-PAGE:-
Components of different fractions were separated by the SDS-PAGE as described before. Briefly, proteins were stacked in 4.5% acrylamide and separated in 12.5% acrylamide. Gels were run at a constant current of 20 mA per gel, and proteins were visualised with Coomassie blue (40) or silver stain (39, 41).

Electron microscopy:-
Bacterial cells grown at a sub-lethal concentration of the purified constituents were fixed with 2.5% glutaraldehyde and were negatively stained with 2% (w/v) phosphotungstic acid in 0.1 M sodium phosphate buffer (pH 6.5)(42). Bacteria were examined by transmission electron microscope (JEM, Japan).

Statistical Analysis:-
All determinations were carried out in triplicates and the statistical analyses were carried out using SPSS 13.0 and Microsoft Excel programs

Results:-
Biofilm formation by *S. aureus, P. aeruginosa, E. coli, K. pneumoniae, Sh. flexneri and Sal.* Typhimurium, was investigated in presence and absence of the thymoquinone, saponins and alkaloids (Table 1). Biofilm production by *S. aureus* was not very much affected by the three active constituents of *N. sativa*. On the other hand, while biofilm production by *E. coli*, was reduced, its production by *P. aeruginosa* and *Sh. flexneri* was enhanced (Table 1). The percentage of production of biofilm by *E. coli* was 19-85% compared to the control. On the other hand, biofilm production was 210-235% and 117-240% increased as compared with the control in the case of *P. aeruginosa* and *Sh. flexneri* respectively (Table 1). In the cases of *K. pneumoniae* and *Sal.* Typhimurium there was an enhancement of biofilm production by thymoquinone and a reduction of production by both alkaloids and saponins at ½ MIC (Table 5).

Table 1:- Effect of some active contents of *N. sativa* on biofilm production by different types of bacteria (in M9 medium)

Constituent	Conc.	Bacteria					
		Staph. aureus	*Pseudomonas aeruginosa*	*Escherichia coli*	*Klebsiella pneumoniae*	*Shigellafl exneri*	*Salmonella* Typhimurium
		% biofilm production compared to control					
Thymoquinone	1/2 MIC	101.6	125.3	29.9	125.3	235.0	153.4
	1/4 MIC	103.7	112.7	85.1	154.1	240.0	88.6
Saponins	1/2 MIC	106.5	140.6	19.4	30.3	125.4	50.6
	1/4 MIC	95.0	110.2	26.9	25.0	143.4	53.2
Alkaloids	1/2 MIC	107.1	210.7	30.9	54.5	126.0	65.3
	1/4 MIC	104.6	125.3	39.4	102.6	117.7	74.7

The effect of thymoquinone and saponins on swimming capacity of three motile bacteria (*P. aeruginosa, E. coli, and Sal.* Typhimurium) was investigated (Table 1). While thymoquinone inhibited the motility of the tested bacteria, saponins enhanced it (Table 2). The diameters of zone of swarming of untreated bacteria, were 11, 8, and 20mm for *Ps. aeruginosa*, *E. coli* and *Sal.* Typhimuriumrespectively, while their respective swarming diameters in presence of ½ the MICs of saponins were 22, 20 and 50mm (Table 2).

Table 2:- Effect of phytochemicals on swimming of bacteria

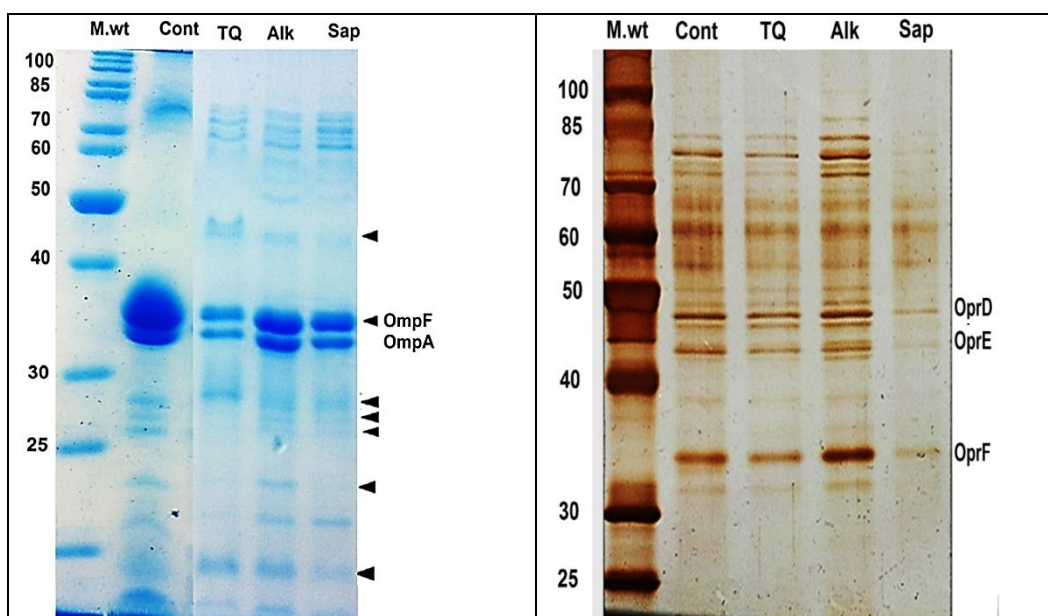| Constituent	Conc.	*Bacteria*		
		Ps. aeruginosa	*E. coli*	*Sal.* Typhimurium
	Zone Diameter (mm)			
Control	0	11	8	**20**
Thymoquinone	($^{1/2}$MIC)	**No S**	**No S**	**No S**
	($^{1/4}$MIC)	13	**No S**	**No S**
Saponins	($^{1/2}$MIC)	**22**	**20**	**50**
	($^{1/4}$MIC)	**18**	**13**	**44**

Fig 3:- Effect of *Nigella sativa* active constituents at ½ MIC concentrations on outer membrane of *Escherichia coli*(left, Coomassie blue stained) and Pseudomonas aeruginosa (right, silver stained). Mw, molecular weight markers; TQ, thymoquinone; Alk, alkaloids; Sap, saponins.

Table 3:- Summary of the effect of *Nigella sativa* active constituents at ½ MIC concentrations on the expression of outer membrane proteins of *Escherichia coli* and *Pseudomonas aeruginosa*

Bacteria	Protein apparent molecular wright (kDa)	Effect of *Nigella sativa* active constituent on protein level *		
		Thymoquinone	Alkaloids	Saponins
Escherichia coli	36 (OmpF)	-	-	-
	46	+	+	+
	28 (OmpA)	+	+	+
	27	-	+	+
	26	-	+	+
	22	NC	NC	-
	17.5	+	NC	NC
Pseudomonas aeruginosa	86	NC	+	NC
	80	-	NC	-
	33 (OprF)	NC	+	NC
*NC, no change; -, decrease in amounts; +, increase in amounts				

The changes of the apparent level of expression of some outer membrane proteins of *E. coli* and *P. aeruginosa* exposed to 1/2 MICs are shown in figure3 and table 3. Changes in the levels of expression of seven proteins in *E. coli* and three proteins in *P. aeruginosa* were observed and

Thymoquinone caused an increase in the expression of 3 proteins in *E. coli* and a decrease in 3 others. On the other hand, it caused an apparent decrease in the amount of a protein which has a molecular weight of 80 kDa in *P. aeruginosa* (Table 3). Alkaloids cause an increase in the level of 4 proteins and a reduction in one protein in *E. coli* and an increase in the level of expression of two proteinsone of which was OprFin *P. aeruginosa* (Table 3). Saponins increased the level of expression of 4 proteins and reduced two in *E. coli* and reduced the level of only one protein in *P. aeruginosa*(Table 3).

Thymoquinone, alkaloids and saponins affected the expression of lipopolysaccharides in *E. coli*and not *P. aeruginosa.* As shown in figure4, there was a decrease in the amount of high molecular weight LPS in *E. coli* treated with thymoquinone, alkaloids and saponins.
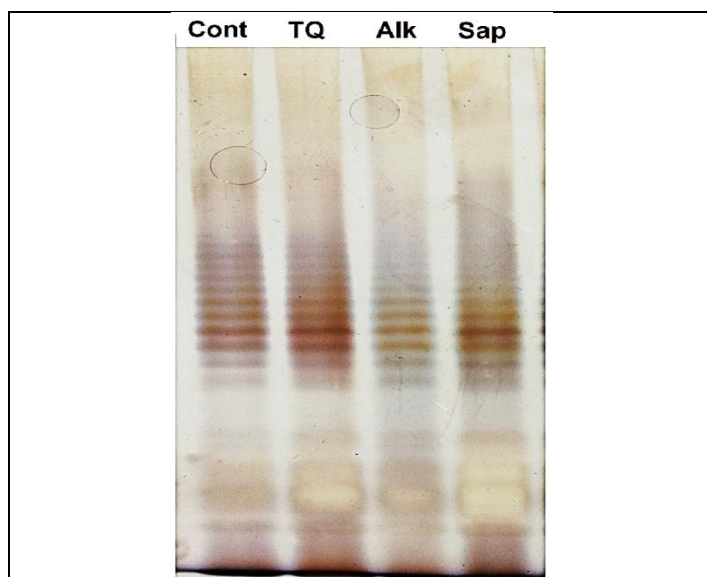

Fig 4: -Effect of *Nigella sativa* active constituents at ½ MIC concentrations on lipopolysaccharide of *Escherichia coli*. Mw, molecular weight markers; TQ, thymoquinone; Alk, alkaloids; Sap, saponins.

S. aureus, E. coli and *P. aeruginosa* as treated with thymoquinone, saponins and alkaloids, were examined for changes in their morphology. While no apparent changes in the morphology of *E. coli*, (data not shown), some changes were observed in both *P. aeruginosa* and *S. aureus.* Thymoquinone and alkaloids caused *P. aeruginosa* cells to become more elongated and thinner compared to the control (Fig. 1). This was more apparent in the case of thymoquinone. On the other hand, *S. aureus* exposed to saponins at sub-inhibitory concentration suffered from protoplasting and some cells suffered from the retraction of their cytoplasmic contents away from the cell wall as shown in Fig 5B.
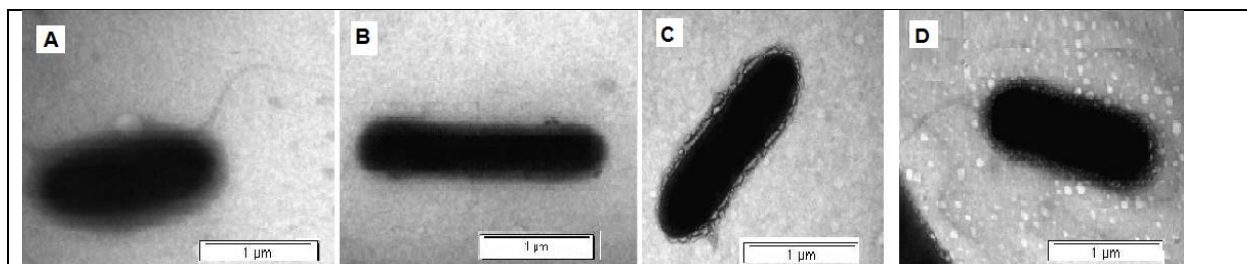

Fig. 1:- Pseudomonas aeruginosa after growth for 24 h in M9 (A) and in M9 containing subinhibitory concentration of thymoquinone (B), alkaloids (C), and saponins (D).
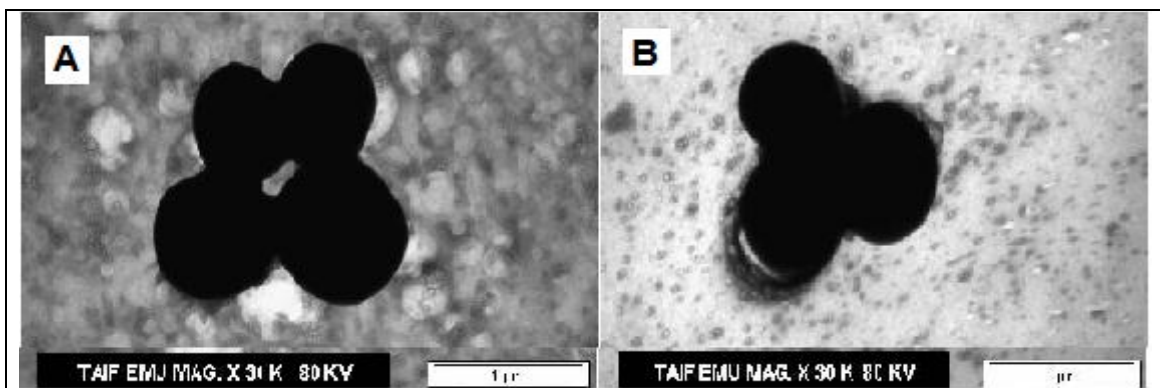
Fig. 2:- *Staphylococcus aureus* after growth for 24 h in M9 (A) and in M9 containing saponins at subinhibitory concentration (B).

Discussion:-

The saponins, alkaloids, and thymoquinone were previously reported to be inhibitory to different types of bacteria even though they might be multi-drug resistant clinical isolates (15, 32).In this study, we investigated the effect of subinhibitory concentrations of saponins, alkaloids, and thymoquinone of *N. sativa* on some pathogenesis mechanisms of some Gram-positive and Gram-negative.

Is this study, *S. aureus, P. aeruginosa*, *E. coli*, *K. pneumonia*, *Sh. flexneri* and *Sal.* Typhimurium*,* were grown at ½ and ¼ the MICs of thymoquinone, saponins and alkaloids and were tested for their capability of forming biofilms.

Biofilms are formed on surfaces of living tissues, medical devices and contact lenses, etc. (43). Biofilms help bacteria to survive and withstand hostile conditions on surfaces and contribute to the persistence of chronic infections (44). Several studies have been performed to find natural antimicrobial agents that influence microbial biofilm formation (45-48).

While there was a reduction in the ability of *E. coli* to produce biofilms by the three tested constituents, only saponins and alkaloids were capable of reducing biofilm formation by *K. pneumoniae* and *Sal.* Typhimurium*.* On the other hand, biofilm formation was enhanced in *P. aeruginosa* and *Sh. flexneri* and was indifferent in the case of *S. aureus.*

The induction of biofilm formation, in *P. aeruginosa*, and the failure to reduce biofilm formation in *S. aureus,* contradicts with Chaieb, *et al.,* (49), who reported a reduction in biofilm formation by both *S. aureus* and *P. aeruginosa* treated with thymoquinone. However, it should be mentioned that the inhibition detected was at concentrations higher than their reported MICs. Therefore, while thymoquinone caused inhibition of biofilm formation at concentrations 22 and >512, for *S. aureus* and *P. aeruginosa* respectively, the MICs reported were 8 and >512 respectively (49).

Motility plays a key role in the colonisation of surfaces by bacteria (50,51). In this study, thymoquinone inhibited the motility and swarming of *Escherichia coli, P. aeruginosa* and *Sal.* Typhimurium. In a previous study,tannins of cranberry fruit and the hydrolysable tannin in pomegranate were reported to inhibit swarming motility but did not block swimming or twitching motilities (51). Sub-inhibitory concentrations of alkaloids like piperine of black pepper and reserpine of snakeroot, decreased bacterial swarming and swimming motilities (52). Tannic acid and epigallocathecingallate were found to block swarming motility in Pseudomonas aeruginosa (53).

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Escherichia coli, P. aeruginosa and *S. aureus* treated with a sub-inhibitory concentration of thymoquinone, alkaloids and saponins were examined under transmission electron microscope for morphological changes. Thymoquinone and alkaloids caused cells of *P. aeruginosa* to become thinner and elongated, compared to the

control. Though, the morphological changes might be attributed, to their effect on penicillin-binding proteins, however, this is not necessary because quinolone antibiotics, which do not bind to penicillin-binding proteins, affect the morphology of *E. coli* (55).

S. aureus exposed to saponins at a sub-inhibitory concentration suffered from protoplasting and retraction of the cytoplasmic contents away from the cell wall. Morphological changes of *S. aureus* exposed to aqueous extracts of green tea (56) or extracted catechingallates (57) have been reported in *S. aureus*. Methanolic extract of a soft sponge, *Haliclona* sp., caused internal shrinkage of methicillin-resistant *S. aureus* and *B. subtilis*cells which finally collapsed after prolonged exposure to the extract (58).

There is evidence that several outer membrane proteins are involved in adherence of bacteria to mammalian cells (59-61). In this study, the effect of thymoquinone, alkaloids and saponins at 1/2 MICs on the outer membrane proteins was examined. There were indeed apparent changes in the levels of expression of seven and three proteins in *E. coli* and *P. aeruginosa* respectively. While in *E. coli*OmpF outer membrane protein apparently decreased in amounts by treatment with the three constituents, OmpA increased.

On the other hand, three outer membrane proteins of *P. aeruginosa* were affected by the tested phytochemicals one of which was OprF. The expression of the latter was increased by the treatment with alkaloids. Recent studies have shown that the expression of 5% of bacterial promoters may be affected by sub-inhibitory concentrations of antibiotics (62). Likewise, it seems that phytochemicals like thymoquinone, alkaloids and saponins affect the expression of bacterial promoters.

The effect of phytochemicals on the expression of some bacterial proteins has been previously documented.Bioactive fraction 9EA-FC-B of*Acalyphawilkesiana*inhibited the production of MRSA by reducing the amount PBP2a in the matrix(63). Proteomic analysis of bacterial expression profiles following exposure to flower extracts of *Melastomacandidum* affected the expression of four proteins in *E. coli* and one protein in *S. aureus* (64).

In this study, alkaloids and saponins were found to reduce the amounts of high molecular weight Lipopolysaccharide (LPS) in *E. coli*.LPS is a main outer membrane component of Gram-negative bacteria (65).It causes pathophysiological effects such as fever, leucopenia, leucocytosis and Shwartzman reactivity (65). It is also involved in the attachment to host cells and it is important for the virulence and pathogenesis of many bacterial species, including *Pseudomonas aeruginosa, Salmonella* species, and *Escherichia coli*(66-68).

LPS is associated closely with the OmpF protein of the outermembrane (69). The low expression of OmpF caused by alkaloids and saponins might have a role in the decreased in the detected amounts of high molecular weight LPS. LPS are essential for the pathogenesis of bacteria. It is an important mechanism to evade complement activation (70). Thisimplies that alkaloids and saponins could affect the pathogenesis of *E. coli*.

Conclusion:-
Thymoquinone, saponins and alkaloids of *N. sativa*at subinhibitory concentrationsaffect motility, biofilm formation and the expression of some proteins and LPS of theouter membrane of bacteria. This presumably would affectthe pathogenesis of bacteria.

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