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EFFECT OF NIGELLA SATIVA EXTRACT ON FERTILITY OF NORMAL AND PSEUDOMONAS INFECTED RATS.

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SUMMARY

Nigella sativum has assumed a great importance in folk medicine because it was used as antibacterial agent. Alcoholic extract of *Nigella sativa* was used in this study to detect its antibacterial effect against Gram negative bacteria (*Pseudomonas aeruginosa*) by agar gel diffusion technique as well as its effect on fertility of non infected and infected male rats with virulent strain of *Pseudomonas aeruginosa* (proved to be serum resistance) by using the bactericidal assay. Satisafctory results were obtained by oral administration of the alcoholic extract in doses of 25 and 125 mg/100gm. B. wt. (1/100 & 1/20 of LD_{50}) for 65 successive days to overcome such bacterial infections and improve the sperm characters as well as significantly decreased weight of seminal vesicles, epididymis and prostate gland. Bacteriological examination and biochemical analysis were used for detection of the used strian in either treated or non treated infected male rats to evaluate the using of *Nigella sativa* extract as a bactericidal agent. The LD 50 of alcoholic extract of Nigella sativa was estimated in mice following the subcutanous injection and proved to be of low toxicity (2500 mg/ 100 gm b. wt.).

INTRODUCTION

Nigella sativa has assumed great importince because it was used as anthelmintic, antispasmotic and antibacterial agent (Bedvian, 1936; Perrot and Paris, 1971; Bellakhder, 1978; Hanafy and Hatem, 1991; Salami et al., 1992 and Akhtar et al., 1996).

The role of *Nigella sativa* in fertility, which is considered to be one of the most important economic problem, was conducted against virulent strain of *Pseudomonas aeruginosa* predicted to have great effect on fertility (Naidu et al., 1982 and Jovicin et al., 1992).

The acute toxicity of the *Nigella sativa* constituents was estimated and recorded (Singh Maurya et al., 1983)., but its effect on fertility of normal and infected male rats by *Pseudomonas aeruginosa* which has an effect on fertility, is not studied unitl now.

So the present work was conducted to reveal the toxicity, pharmacological and antimicrobial effect of *Nigella sativa* as well as its effect on the fertility of male rats infected with virulent strain, which is predicated to have a severe effect on fertility, to investigate the role of *Nigella sativa* in such infection.

MATERIAL AND METHODS

Nigella sativa extracts:

500 gm of *Nigella sativa* seeds were extracted till exhaustion by percoltation on 95% ethyl alcohol, evaporated under reduced pressure till dryness by rota vapour, then kept in refrigerator till used.

Laboratory animals:

25 mature mice, mature male rats and ten rabbits were used, they were fed on ordinary ration and water ad-libitum.

Acute toxicity: Acute toxicity was studied in mice as described by Kerber (1941). Five groups of mice (of each of body weight 20-25 gm) were used. The minimum lethal dose and LD50 were estimated by subcutanous (s/c) injection of *Nigella sativa* extract in graded increased doses. Treated and control mice were kept under observation for 24 hour. The symptoms of toxicity were recorded.

In vitro sensitivity test:

The bactericidal activity of *Nigella sativa* was detected by the test diffusion technique according to Finegold and Martin (1982).

The following different dilutions of *Nigella sativa* extract (1/10) 1/20, 1/40. 1/80, 1/100) were used as 5 discs (8 mm in diameter each) impregnated in each dilution. All plates were incubated aerobically overnight at 37°C and the results were recorded by measuring the diameter of inhibition zone.

Detection of pathogenicity of tested organism:

To insure about the virulence of tested strain *(Pseudomonas aeruginosa)* before induce in the infection in male rates, the virulence test, bactericidal assay were applied.

Virulence of Pseudomonas aeruginosa in mice:

Bacteria were grown overnight in tryptic soya broth at 18°C to obtain broth culture of 1.5 x 109 (C. F. U.) using McFarland nephlometer. Ten fold dilution was made and 0.2 ml from the original inoculum and the dilution was used to inject mice i/p in groups, each of 5 mice. One group of mice was used as control. Mortality rate was recorded after 72 hours (Liu, 1966).

Bactericidal assay:

Using guidelines of Taylor (1983), 10 µl sample of a 24 hours brian heart infusion broth culture was used to inoculate 10 ml of sterile brain heart infusion broth warmed to 37°C, then incubated for 2 hour at 37°C to produce bacteria, the bacterial suspension was centrifuged for 20 minutes at 3000 x g and bacterial pillets were suspended in 10 ml gelatin veronal buffer containing 0.15M $CaCl_2$ and 0.5M $MgCl_2$, pH 7.4 .

Reaction mixtures of 250µl containing 25 µl of bacterial suspension (1 x107 C. F. U/ml) by using

McFerland nephlometer standards, and 225 µl of undiluted bovine serum were incubated at 37°C for 3 hours.

50 µl samples collected initially at "0" and at the end of 6 hours incubation were placed in 9 ml gelatin veronal buffer.

Numbers of viable bacteria were determined by plating 10 fold dilutions of this sample on blood agar palte, after incubation the number of colonies were counted, isolate of *Pseudomonas aeruginosa* was classified as serum resistance when the number of colonies was greater than that in "0" hour sample.

Fertility test:

Normal male rats and rats infected with a strain of *Pseudomonas aeruginosa* were used in this assay (Liu, 1966). Broth suspension of 3 x 108 identified strain of *Pseudomonas aeruginosa*, per ml was prepared using McFarland nephlometer (Finegold and Martin, 1982).

To induce bacterial infection, each rat was injected s/c by 1 ml of virulent strain of *Pseudomonas aeruginosa* . Six groups of five mature rats (180-200 gm B. wt.) were used in this experiment . First group was kept as control, second and third groups were administered *Nigella sativa* extract at doses of 25 and 125 mg/100 gm B. wt. Fourth group was challenged by virulent strain of *Pseudomonas aeruginosa* and kept as control positive. Fifth and sixth groups were infected by the same virulent strain of *Pseudomonas aeruginosa* and treated by *Nigella sativa* extract at doses of25 and 125 mg/100 gm B. Wt. orally by stomach tube for 65 successive days to cover complete spermatogenic cycle (Hershberger et al., 1969).

The weight of sexual organs and the epididymal sperm characters were determined according to Bearden and Fluqnar (1980). Blood samples from all used rats were collected for bacteriological examination .

Bacteriological examination:

Re-isolation of the infective organism from all tested male rats (treated or not treated) by examination of blood samples collected from living rats or internal organs of dead rats. All samples were cultured on blood agar medium and Pseudomonas agar medium and incubated aerobically at 37°C for 24 hours. Suspected growing colonies were studied for morphological appearance, haemolytic activity, colonial characters and biochemically according to (Konemann et al., 1992). The blood samples were left to clot and the serum was separated for biochemical analysis and RID test.

RID test:

Redial immunodiffusion (RID) is a technique that is routinely used for measuring the concentration of various soluble antigens (usually protein) in a biological fluid (Mancini and Vearman, 1964).

Biochemical analysis:

The activity of Aspartate aminotrasferase (AST), Alanine aminotransferase (ALT) and Alkaline phosphatase (AP) were determined by the method described by Reitman and Frankel, 1957 and Roy,

1970. Urea and creatinine levels in serum were estimated as explained by (Koplan, 1965) and Husdan and Rapoport, 1968).

Statistical analysis:

Statistical analysis of the obtained data was carried out using student "t" test as explained by (Snedecor, 1969).

RESULTS

Acute toxicity of *Nigella sativa* was carried out in mice revealed that, MLD and LD50 of *Nigella sativa* extract were 1750 and 2500 mg/100 gm B. wt. respectively after subcutanous injection of such extract. The acute toxicity symptoms were characterized by hurried respiration, rapid pulse.

In vitro sensitivity test:

The antimicrobial activity of *Nigella sativa* extract was detected by presence of detectable wide zone of inhibition in the dilution of 1/10 and 1/20 and decreased gradually with the increase of the dilution of the used extract.

Pathogenicity test:

Virulence:

Pseudomonas aeruginosa when injected i/p in rabbit, it produced mortality rate reaching 100%. All rabbits inoculated with that strain died during the experimental period. Weight loss, reduction in daily weight gain, dehydration, dull and severe liquid to watery diarrhoea were observed before their death.

Bactericidal assay:

The serum resistance of *Pseudomonas aeruginosa* strain was determined. Bovine serum was used t test the serum resistance. Isolate of *Pseudomon aeruginosa* was classified as serum resistant, th obtained number of viable *Pseudomonas* in hours sample was greater than that in 0 hou sample.

Fertility test:

The effect of prolonged administration of th studied extract on sexual organs weight an epididymal sperm characters of normal rats an those previously infected with virulent strain o *Pseudomonas aeruginosa* was recorded in Tab (1).

Oral administration of *Nigella sativa* extract doses of 25 and 125 mg/100 gm B. w respectively to mature non infected male rats fo 65 successive days caused a significant decreas in weight of seminal vesicle, prostate glan epididymis gland and insignificant increase i sperm cell count and the percentage o progressive motility if compared with those o control group.

While the oral administration of *Nigella sati* extract in doses of 25 and 125 mg/100 gm B. w respectively for 65 successive days to mature ra previously infected by *Pseudomonas aeruginos* showed siginificant increase in wight of sexu organs, percentage of progressive motility, sper cell count and decrease in the percentages o sperm abnormalities when compared with those o the positive control group (that group infected b *Pseudomonas aeruginosa* without treatmen (Table 1).

Table (1): Showing effect of prolonged administration of Nigella sative extract to infected and non infected male rats for 65 days on the weight of sexual organs and epididymal sperm characters (n = 5).

Group	Dose mg / 100 g B. wt.	Weight of sexual organs (g / 100 gm. B. wt.)				Sperm characters		Abnormality %
		Testis	Edididymis	Seminal vesicle	Prostate	Sperm cell count 10^6 / mm^3	Motility %	
Control - ve	-	1.7 ± 0.06	0.7 ± 0.3	0.8 ± 0.39	0.68 ± 0.049	559 ± 5.89	85 ± 2.3	1.7 ± 0.06
Nigella sativa extract	25	1.69 ± 0.03	0.4 ± 0.02	0.61 ± 0.02 **	0.42 ± 0.01	559 ± 5.89	85 ± 2.5	1.8 ± 0.01
	125	1.65 ± 0.04	0.2 ± 0.02 ***	0.62 ± 0.06	0.2 ± 0.08 ***	576 ± 2.83	88 ± 2.7	2.7 ± 0.07
Infection control +ve	-	0.94 ± 0.03	0.112 ± 0.02	0.57 ± 0.01	0.17 ± 0.02	221 ± 4.24	25 ± 1.04	37.7 ± 1.8
	25	1.4 ± 0.08	0.38 ± 0.02	0.62 ± 0.06	0.4 ± 0.02	316.3 ± 4.06	45.0 ± 2.04	13.5 ± 1.19
	125	1.5 ± 0.05	0.21 ± 0.02	0.6 ± 0.04	0.26 ± 0.02	524.7 ± 9.7	77.5 ± 1.44	9.6 ± 0.9

* $p < 0.01$ ** $P < 0.05$ *** $p < 0.001$

Table (2) : Showing effect of oral administration of Nigella sativa extract on serum enzymatic activity and biochemical constituents in serum of infected and non infected rats (n = 5) .

Group	Dose mg / 100 g B. wt.	AST unit	ALT unit	AP U / L	Urea mg %	Creatinine mg %
Control -ve	-	192 ± 1.2	34.8 ± 0.2	28.7 ± 0.09	38.82 ± 1.8	1.47 ± 0.06
Nagila sativa extract	25	213.2 ± 1.48	43.4 ± 1.03	38.0 ± 1.2	39.02 ± 1.7	1.77 ± 0.11
	125	233 ± 1.24	57.99 ± 0.9	46.6 ± 3.3	43.5 ± 2.7	2.0 ± 0.2
Infection control + ve	-	163 ± 1.25	27.8 ± 0.26	19.2 ± 0.88	55.2 ± 4.5	4.88 ± 0.12
Nigella sativa extract after infection	25	193 ± 1.22	29.2 ± 2.2	29.85 ± 0.91	51.2 ± 2.6	3.3 ± 0.4
	125	222.3 ± 1.1	48.8 ± 1.22	38.05 ± 1.2	47.27 ± 1.1	2.55 ± 0.06

* $P < 0.01$ ** $P < 0.05$ *** $P < 0.001$

The effect of tested extract on serum enzyme activities of normal and infected rats showed significant increase of AST, ALT and AP. While the level of creatinin was significantly increased in serum of normal rats but there is significant decrease in serum of infected group when compared with control negative and positive respective. Insignificant changes in urea level of both normal and infected groups (Table 2).

RID test:

All serum samples were showing no zone of inhibition in agarose gel diffusion if compared with control samples which indicate the absence of *Pseudomonas aeruginosa* in sera of male rats treated with *Nigella sativa* extract.

Bacteriological examination of male rats:

Pseudomonas aeruginosa was isolated fro infected non treated male rats in pure culture o the specific media with same morphological an biochemical characters, but there is no isolatio of *Pseudomonas aeruginosa* from male ra treated with *Nigella sativa* extract by the two us doses.

DISCUSSION

Fertility is one of the problems which considered to play a role in economic proces through its role in animal production.

It is worthy to mention that the strain of *Pseudomonas aeruginosa* used in this study was isolated from bull semen with a record of low fertilizing capacity. This observation is hand-to-hand to hand with that mentioned by Lukacevic et al., 1962; Naidu et al., 1982 and Jovicin et al., 1992 who isolated *Pseudomonas aerugmosa* from low capcity of fertilizing bulls and Pseudomonas aeruginosa was found to be one of the important microorganisms which has a significant role in the capacity of fertilization in males.

Virulence of microorganism is mainly defined as the relative capacity of organism to resist available defence mechanism (Sparling, 1983) and the ability of the organism to resist bactericidal effect of serum is among the most important virulence factors. In this study an in vitro assay (Taylor, 1983) was used to determine the serum resistance of *Pseudomonas aeruginosa* isolate which found was to be serum resistant. Wilson (1968) and Taylor (1983) suggested that bactericidal activity of serum against Gram negative bacteria is mainly mediated by antibody and complement. In addition Pluschke et al. (1983) found also that bacterial surface structure that may be important for serum resistance, including smooth lipopolysaccharides, acidic capsule polysaccharide and outer membrane protein.

The antimicrobial susceptibility of *Pseudomonas aeruginosa* was determined on the extract of *Nigella sativa* using test diffusion technique (Finegold and Martin, 1982) and the results indicate that the seed extract has a good antibacterial activity beside a therapeutic potential for the treatment of some Gram negative bacterial infection (Ferdous et al., 1993).

The effect of *Nigella sativa* extract on fertility of male rats was studied and the result proved that oral aministration of the extract to infected male rats with *Pseudomonas aeruginosa* could overcome the harmful pathological lesion of such organism in addition to significant increase in weight of sexual organs, sperm cell count and progressive motility besides decrease of sperm abnormalities. This result is generally in coincidence with that mentioned by Ferdous et al. (1993).

Oral administration of *Nigella sativa* in doses of 25 and 125 mg/100 gm B. wt. respectively to nrmal mature male rats for 65 successive days caused a significant decrease in weight of seminal vesicle, prostate, epididymes and the testes. When the last dosage of *Nigella sativa* extract was used in infected rats by *Pseudomonas aeruginosa* it showed significant increase in weight of sexual organs, percent of sperm motility and sperm concentration and decrease in the percentage of sperm abnormalities. This effect of *Nigella sativa* extract on sexual organs and sperm characters could be attributed to its direct action on interstitial leydig cells which secrete testosterone hormone which may be due to *Nigella sativa* high content of phosphrus, calcium, protein and vitamin A. These constituents were considered as important factors for fertility and increased spermatogenic cycle. Gleichauf (1967), Masliev and Davtyam (1969), Nockels and Herrick (1969) obtained results proved that LD_{50} of ethanolic

extract of the tested plant was 2500 mg/100 gm B.wt This finding indicates that *Nigella sativa* is of low toxicity as its poisoning manifestations are recorded by very large doses of the tested extract obtained from a larger amount of the studied plant. In this respect Singh Maurya et al. (1983) showed low toxicity at the tested dosage of aqueous ethanolic extract.

The obtained results denoted that alcoholic extract of *Nigella sativa* significantly increased ALT, AST, AP and level of creatinine, this effect is in agreement with Tennekoon et al. (1991) who used *Nigella sativa* extract in treatment of rats infected by *Pseudomonas aeruginosa* and recorded a significant increase in ALT, AST and AP and decrease in the level of creatinine and urea when compared with positive group.

The effect of tested extract on serum enzymatic activity and level of creatinine and urea of rats before and after infection by *Pseudomonas aeruginosa* may be attributed to the influence on the liver and kidney as the plant contains volatile oil which may irritate the renal tissue and increase the level of creatinine.

Thus we can use the extract in treatment of animals infected by bacteria that have an effect fertility and on bulls before mating to increase its activity with successful and satisfactory application.

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