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EVALUATION AND NEUROBEHAVIORAL ASSESSMENT OF THYMOQUINONE IN SPRAGUE DAWLEY BY ORALLY ADMINISTRED

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ABSTRACT

Thymoquinone is an active ingredient isolated from *Nigella sativa* and has various pharmacological activities, such as protection against oxidative stress, inflammation, and infections. In addition, it might be a potential neuropharmacological agent because it exhibits versatile potential for attenuating neurological impairments. The use of observational and functional tests for screening in toxicology assessments gained popularity after several expert panels and scientists recommended such in the late 1970s and early 1980s. Their use is based on the premise that behavior represents the integrated sum of activities mediated by the nervous system and is a sensitive marker of nervous system dysfunction. Taken from the Irwin screening battery that is widely used in drug development (Irwin, 1962, 1968), test batteries for behavioral evaluations in neurotoxicology, broadly known as functional observational batteries (FOB; Moser, 1989; McDaniel and Moser, 1993), were developed and validated in subsequent years. Functional neurotoxic effects include adverse changes in the structure or function of the central and/or peripheral nervous system. The effect of thymoquinone on the central and peripheral nervous systems was evaluated using the functional observational battery in male and female rats orally administered at dose level of 5, 10, 20.0 mg/kg body weight for 28 days repeatedly. Additionally, there was a group that received vehicle alone. The FOB assessment included: home-cage, hand-held, and open-field observations as well as sensory, neuromuscular, and physiological measurements. The rats treated with the positive control, Chlorpromazine exhibited the expected response of decrease in mobility / motor activity and body temperature, low arousal and sluggish response to stimuli and an decrease in fore limb foot splay. Post dose administration thymoquinone did not produce any sign of toxicity, mortality, pathological changes and significant blood parameters changes. The investigated result showed that the thymoquinone (20 mg/kg b.wt) significantly ($p<0.05$) decrease mobility and low arousal levels were observed among the male rats at the oral dose of 20 mg/kg post dose. In addition, a decrease in motor activity and body temperature were observed at the dose of 20 mg/kg. The FOB assesses several neurobiological domains including neuromuscular (weakness, coordination, gait and tremor), sensory (auditory, vision and somatosensory), and autonomic (pupil response and salivation) functions. Under the experimental conditions, thymoquinone did not sstatistically significant ($p<0.05$) change any parameter assessed in the study.

KEYWORDS: Thymoquinone, Neurobehavioral, Orally Gavage

Thymoquinone (*Nigella sativa* seed) is a member of the ranunculaceae family growing in countries bordering the Mediterranean Sea, India, Pakistan, and Iran. For many centuries, *Nigella sativa* seeds (also called black seeds or black cumin) have been used as a food additive as well as for medicinal purposes in many countries (Jansen, 1981). This plant is one of the most extensively studied, both phytochemically and pharmacologically (El-Sayed, 1998) (Riaz *et al.*, 1996) (Siddiqui and Sharma, 1996) (Worthen *et al.*, 1998). Most properties of whole seeds or their extracts are mainly attributed to quinone constituents, of which thymoquinone is more abundant compound (Mahfouz *et al.*, 1960) (Filippo D'Antuono *et al.*, 2002). Pharmacological action of *Nigella sativa* has been investigated including immune stimulant, anti-inflammatory, anticancer, antimicrobial antiparasitic and antioxidant (Ibrahim, 2007) (Burits, 2000). Growing evidence concerning the versatile thymoquinone pharmacological activities has been established. In a number of preclinical studies, it elicits antitussive, gastroprotective, anti-inflammatory, antinociceptive, antihistaminic, antibacterial, anthelmintic, antioxidant, immunomodulatory, anticancer, hepatoprotective, cardioprotective, antidiabetic, ototoxicity protective, and nephroprotective effects. Thymoquinone have many medicinal properties with relevance to various neurological illnesses. For instance, they have anticonvulsant, anxiolytic, antidepressant, and antipsychotic potential. Moreover, many recent studies have described the neuropharmacological properties of Thymoquinone such as anti-inflammatory and antioxidative roles in designed neurological models. Besides, it alleviates neuropathy in an experimental peripheral diabetic model. Yet, the pharmacological potential and delivery prospects of thymoquinone this battery is designed to be used in

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conjunction with general toxicity studies and changes should be evaluated in the context of both the concordance between functional neurological and neuropathological effects, and with respect to any other toxicological effects seen. The central and peripheral nervous systems using the functional observational battery motor activity, and neuropathology consists of noninvasive procedures designed to detect gross functional deficits in animals and to better quantify behavioral or neurological effects was not evaluated/detected to date. Therefore, in this article, considering the neurotoxicity screening battery consists of a functional observational battery consists of noninvasive procedures designed to detect gross functional deficits in animals and to better quantify behavioral or neurological effects, motor activity, and neuropathology techniques are designed to provide data to detect and characterize histopathological changes.

MATERIALS AND METHODS

Male and female albino wistar rat (*Rattus norvegicus*) were maintained at 22 ± 3ºc, relative humidity between 60 to 70 % and a light/dark cycle of 12 hr. The rats were provided with rat pellet feed (amrut brand, pranav agro Pune) and drinking water filtered through aquaguard water filtration system *ad libitum* throughout the study period. All groups of rats were acclimatized 6 days prior to the start dosing. The thymoquinone sample was purchased from Sigma Aldrich India and peanut oil from local market. Two rats per cage were housed in standard, polysulfone cages, with stainless steel top grill having facilities for holding pelletted food and drinking water in polycarbonate bottles. Cages were provided with sterilized rice husk as a bedding material. Food and water were provided to animals *adlibitum.*

Dose Selection, Dose Volume, Route of Administration

After randomization male and female rats (110 ± 20 g body weight) were divide in to six groups (I to VI) each group consist 5 male and 5 female rats. At the time of dose commencement animals were least 6 to 7 weeks old. Three dose levels 5, 10, 20 mg/kg body weight were selected based on the preliminary studies. In addition, a concurrent vehicle and positive control were included. The oral route is chosen for the test item since it is the intended route of administration in humans. The dose volume maintained at 10 ml/kg body weight.

Dose Formulation Preparation and Administration

Based on the preliminary toxicity study in rats. Group I served as control group and group II served as vehicle control group given a daily dose of normal saline and 0.5% CMC and 0.25 % tween 80 (based on the higher dose volume). The rats of group III, IV and V were given thymoquinone mixed in 0.5% CMC and 0.25 % tween 80 via gavage at dose level 5, 10, 20 mg/kg body weight respectively for 28 consecutive days. Group VI served as standard drug (Chlorpromazine) and given 5.0 mg/kg body weight. Rats were observed for all groups. The FOB assessment included: home-cage, hand-held, and open-field observations as well as sensory, neuromuscular, and physiological measurements.

Functional Observation Battery

All animals in a given study were observed carefully by trained observers who are unaware of the animals' treatment, using standardized procedures to minimize observer variability. If this is not. The animals were removed from the home cage to a standard arena for observation. Effort was made to ensure that variations in the test conditions are minimal and are not systematically related to treatment.

OBSERVATION

All observations were treatment blinded and was carried out in an area separate from the dosing area. Post observations, the animals were placed back in housing area with the original identification number. The treatment was carried out on 28 consecutive days.The predose observation for all groups was carried out one day before the first day of treatment. Each group was subjected to the FOB at 1, 2, 3, 4 and 6 hours (±20 minutes) post dosing. The FOB assessment included: home-cage, hand-held, and open-field observations as well as sensory, neuromuscular, and physiological measurements.

Home Cage Observation

Body position, abnormal vocalization, convulsion and Ease of removal from the cage.

Hand Held Observation

Ease of Handling animal in hand, Respiration, Lacrimation, Salivation, Piloerection, Palberal closure, Eye/Skin/fur examination, Muscle tone.

Open Field Observation

Gait, Posture, Mobility, Arousal level, Tonic/Clonic movement, Stereotypic behavior, Bizzare behavior, Urination/Defecation, Rearing, vocalization,

Sensory Reactivity Measurement

Approach Response, Touch response, Tail-pinch response, Aerial Righting Reflex, Pupil response, Click response. Land hind/fore limb footsplay, Grip performance, Motor activity,

Organ Body Weight Ratio

The vital organ such as liver, kidney, brain, heart, lung, spleen, adrenal of rats and the male sex organ(testis, epididymis, prostrate and seminal vesicle) and female sex organs (ovary, uterus, cervix and vagina) were quickly removed and weigh individually. The organ to body weight ratio was calculated.

Biochemical Estimation

Different biochemical parameters like Alkaline phosphatase (ALP) marker for bone destruction, Acid Phosphatase (ACP) the lysosomal enzyme activity, Serum glutamate oxalo acetate transaminase (SGOT) and Serum glutamate pyruvate transaminase (SGPT) were estimated by using ALP,ACP, SGOT and SGPT kit in Erba Mannhein EM 200 Clinical Chemistry Analyser. Bood samples were collected by sublingual rout, centrifused and supernatant serum was collected. Different enzyme reagents were added to the serum and estimated in an auto analyser.

Haematological Study

Blood collected in EDTA tube was analyzed for red blood cells (RBC) and white blood cells (WBC) counts were determined according method of Winfrobe and Landsberg whereas, haemoglobbin and differential leucocytes counts (DLC) were measured according to procedure of Kolmer *et al.*, (1995).

Necropsy

All the animals were anesthetized by using isoflurane anesthesia. The rats' brains were carefully dissected out and immediately fixed in 10% formal saline. Thereafter the temporal lobes of the rats' brain were dissected out using a surgical blade, processed for routine histological method and stained with H and E.

Statistical Analysis

Statistical significance were presented between control and experimental values as mean ± SEM (n=5). Statistical comparison of body weight changes was made using one way ANOVA (Seigel, 1996).

RESULTS

Home Cage Observation

No abnormal posture, movements or activity were observed in any of the thymoquinone treated rats at any of the time-points when compared with the vehicle control group.

Hand Held Observation

As in the home cage observation, all rats treated with the thymoquinone did not exhibit any abnormality when compared with the vehicle control group.

Open Field Observation

No significant difference was observed in any thymoquinone treated groups when compared to the vehicle control group**.**

Sensory Observations

No significant difference was observed in the thymoquinone treated groups, in any of the sensory parameters at the specified time points, when compared with the vehicle control group.

Neuromuscular Observations

No significant difference was observed in the thymoquinone treated groups, in any of the neuromuscular observations at the specified time points, when compared with the vehicle control group.

Physiological Observations

No significant difference was observed in any thymoquinone treated groups, except mid dose group showing statistically significant ($p<0.05$) decrease in foot splay measurement at 4 hour post dose time point when compared to the control group animals. Though statistically significant, this difference in the foot splay measurement falls within the control range.

Body weight, Organ Body Weight Ratio

The body weight, absolute body weights of thymoquinone treated male and female rats no significant changes were observed while, comparable to controls rats. The relative organ weights (organ to body weight ratio) of animals exposed to different dose of thymoquinone did not indicate any significant changes and value are shown.

Biochemical Study

The results of serum biochemical parameters of male rats. There was no change in clinic-chemical parameters of male and female rats exposed to different dose of thymoquinone for 28 days and the values were comparable to controls rats.

Haematology

The results of haematological parameters in male and female rats exposed to different doses thymoquinone. There was no significance changes in Hb RBC, WBC and differential leukocyte count (DLT).

Histopathology

Autopsy of treated animals on 29 days of post exposure revealed no significance change in their vital organs. Microscopic examination of liver, kidney, brain, testes, and ovary of rats treated with the different doses of thymoquinone for 28 days did not shown any significant tissue damage and were comparable with those of controls rats. While, the gross pathological examination observed slightly uterus distention in two control and one treated female rat which, spontaneous and is physiological/cycle nature and did not effect on outcome of study.

Table 1: Relative Organ Body Weight of Male Rats Orally Administration Thymoquinone for 28 days

	Dose (mg/kg body weight)					
Organ	**Control**	**Vehicle Control**	**Low Dose**	**Mid Dose**	**High Dose**	**Standard Drug**
Liver	3.12 ± 0.23	2.92 ± 0.21	3.07 ± 0.24	3.07 ± 0.27	3.14 ± 0.24	2.96 ± 0.24
Kidney	0.74 ± 0.22	0.78 ± 0.34	0.76 ± 0.03	0.78 ± 0.07	0.77 ± 0.34	0.76 ± 0.26
Lungs	0.72 ± 0.01	0.72 ± 0.22	0.72 ± 0.03	0.71 ± 0.05	0.72 ± 0.21	0.70 ± 0.28
Brain	0.76 ± 0.02	0.89 ± 0.19	0.77 ± 0.03	0.82 ± 0.05	0.89 ± 0.19	1.48 ± 0.41
Testis	1.11 ± 0.67	1.19 ± 0.64	1.14 ± 0.09	1.11 ± 0.13	1.19 ± 0.63	1.18 ± 0.05
Epididymis	0.32± 0.54	0.37± 0.43	0.42± 0.64	0.38± 0.52	0.38± 0.22	0.36± 0.63
Seminal Vesicle	0.45± 0.56	0.47± 0.41	0.54± 0.61	0.55± 0.12	0.53± 0.21	0.49± 0.54
Spleen	0.23 ± 0.01	0.23 ± 0.01	0.245± 0.03	0.27 ± 0.06	0.25 ± 0.01	0.51 ± 0.24
Heart	0.28 ± 0.01	0.31 ± 0.02	0.31± 0.03	0.32 ± 0.03	0.34 ± 0.02	1.45 ± 1.41
Adrenal	0.01 ± 0.00	0.32 ± 0.07	0.02 ± 0.01	0.04 ± .004	0.03 ± 0.07	0.18 ± 0.31

Table 1 (continue): Relative Organ Body Weight of Female Rats Orally Administration Thymoquinone for 28 days

	Dose (mg/kg body weight)					
Organ	**Control**	**Vehicle Control**	**Low Dose**	**Mid Dose**	**High Dose**	**Standard Drug**
Liver	2.95 ± 0.18	2.87 ± 0.16	3.05 ± 0.27	3.01 ± 0.21	2.98 ± 0.15	2.87 ± 0.16
Kidney	0.74 ± 0.06	0.72 ± 0.08	0.75 ± 0.05	0.74 ± 0.06	0.74 ± 0.07	0.72 ± 0.08
Lungs	0.71 ± 0.81	0.68 ± 0.06	0.71 ± 0.04	0.73 ± 0.08	0.72 ± 0.05	0.68 ± 0.07
Brain	0.81 ± 0.00	0.79 ± 0.03	0.79 ± 0.02	0.77 ± 0.03	0.80 ± 0.04	0.80 ± 0.02
Ovary	0.06 ± 0.01	0.08 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.73 ± 0.01	0.08 ± 0.00
Uterus	0.10 ± 0.01	0.11 ± 0.01	0.11 ± 0.02	0.12 ± 0.02	0.11 ± 0.01	0.11 ± 0.03
Spleen	0.24 ± 0.00	0.22 ± 0.02	0.24 ± 0.03	0.25 ± 0.04	0.25 ± 0.02	0.24± 0.00
Heart	0.23 ± 0.02	0.29 ± 0.03	0.32 ± 0.02	0.31 ± 0.03	0.32 ± 0.02	0.30 ± 0.03
Adrenal	0.29 ± 0.26	0.02 ± 0.00	0.03 ± .004	0.03 ± 0.01	0.09 ± 0.00	0.04 ± 0.01

Table 2: Serum Biochemical parameter in rats treated orally with thymoquinone for 28 days

Parameter	Dose mg/kg body weight					
	Control	Vehicle Control	Low Dose	Mid Dose	High Dose	Standard Drug
AST	19.47 ±14.45	16.62±14.50	15.94±19.28	21.04±19.67	16.94±30.27	17.84±29.27
ALT	67.70 ±10.20	67.64±10.25	57.62±10.50	57.42±10.52	62.67±11.16	64.77±12.16
ALP	60.17 ±14.45	60.62±14.50	60.94±19.28	61.04±19.67	53.94±30.27	55.44±28.27
S-Bilirubin (mg %)	1.21 ±0.21	1.08±0.11	1.05±0.16	1.38±0.12	1.41±0.19	1.31±0.21
S-Cholesterol (mg %)	45.81 ±5.01	46.00±5.43	56.00±11.04	54.80±10.13	51.50±9.48	52.54±9.58
S-Albumin (g%)	4.26 ±0.19	4.18±0.19	4.48±0.31	4.48±0.28	4.29±0.18	4.31± 0.19
S-Protein(g/dl)	7.443 ±0.21	7.42±0.26	7.66±0.19	7.56±0.11	7.50±0.22	7.66±0.19

Table 3: Haematological parameters in rats treated orally with thymoquinone for 28 days

Parameter	Dose mg/kg body weight					
	Control	Vehicle Control	Low Dose	Mid Dose	High Dose	Standard Drug
Hb (mg/dl)	14.69±0.28	14.23±0.38	15.11±0.48	15.29±0.38	14.89±0.26	15.99±0.29
RBC (x10 6/µL)	8.13±0.18	7.92±0.22	7.18±0.08	7.52±0.28	6.93±0.21	6.83±0.24
WBC (mm3)	9.07±1.48	9.15±1.28	9.48 ± 2.12	12.41 ±2.48	14.78±1.71	14.66±1.61
Neutrophil (%)	41.47±3.18	39.77± 2.78	37.72± 2.12	36.52± 2.02	36.02± 1.98	36.82± 2.01
Leucocytes (%)	28.01±1.22	29.12±1.52	24.45±1.62	22.82±1.72	18.82±2.78	19.01±2.68
Monocyte (%)	0.35±0.12	0.55±0.55	0.39±0.18	0.52±0.28	0.72±0.24	0.74±0.23
Eosionophil (%)	1.12±0.13	0.94±0.22	0.71±0.28	0.98±0.07	1.0±0.25	1.0±0.25

Table 4: Histopathological Observation in tissue of rats treated orally with thymoquinone for 28 days

Tissue	Number of Lesion											
	Dose mg/kg body weight											
	Control		Vehicle Control		Low Dose		Mid Dose		High Dose		Standard Drug	
	M	F	M	F	M	F	M	F	M	F	M	F
Liver	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
Kidney	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
Lungs	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
Brain	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
Ovary	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
Testis	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
Spleen	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
Intestine	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
Heart	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD

NAD= No Abnormality detected, M= Male, F=Female

Table 5: Effect of thymoquinone on Hind Limb Foot Splay (cm)

Control	Vehicle Control	Low Dose	Mid Dose	High Dose	Standard Drug
8.11 ± 0.49	8.01 ± 0.49	8.32 ± 0.35	7.52 ± 0.39	7.95 ± 0.41	8.38 ± 0.37
7.51 ± 0.28	7.43 ± 0.28	8.06 ± 0.38	8.00 ± 0.42	7.65 ± 0.31	7.71 ± 0.46
7.52 ± 0.23	7.52 ± 0.23	7.72 ± 0.22	7.71 ± 0.33	7.11 ± 0.22	7.21 ± 0.32
7.12 ± 0.21	7.11 ± 0.21	7.43 ± 0.21	7.71 ± 0.35	7.06 ± 0.20	7.23 ± 0.22
7.04 ± 0.29	7.04 ± 0.29	7.63 ± 0.17	7.21 ± 0.42	6.61 ± 0.23	6.95 ± 0.21
7.01 ± 0.27	7.11 ± 0.27	7.12 ± 0.18	6.92 ± 0.34	6.85 ± 0.29	6.75 ± 0.23

Table 6: Effect on Grip Strength

Control	Vehicle Control	Low Dose	Mid Dose	High Dose	Standard Drug
465.16± 26.98	465.56 ± 26.96	445.18± 35.65	446.75± 29.42	484.47± 35.13	507.96 ± 44.94
472.00 ± 16.39	472.30 ± 16.39	485.42± 34.54	536.71± 29.84	539.67± 50.21	504.75 ± 35.56
540.11 ± 20.57	540.21 ± 20.56	499.22± 27.35	485.67± 33.39	593.41± 45.39	475.33 ± 24.89
509.21 ± 49.58	509.31 ± 49.57	497.29± 31.03	538.52± 46.07	512.67± 43.47	550.96 ± 40.38
449.13 ± 20.35	449.33 ± 20.34	460.16± 20.89	471.00± 22.50	519.46± 55.79	525.71 ± 25.73
475.08 ± 30.90	475.48 ± 30.95	455.24± 18.96	476.82± 32.72	453.42± 29.69	504.54 ± 37.59

Table 7: Effect on Motor activity

Control	Vehicle Control	Low Dose	Mid Dose	High Dose	Standard Drug
1297.15± 126.94	1296.25± 126.94	1786.13 ± 96.35	1516.38± 235.01	1762.00± 194.66	1197.75 ± 125.18
526.25 ± 70.00	526.25 ± 70.00	286.38 ± 68.41	658.25 ± 133.23	339.13 ± 44.89	513.63 ± 68.09
192.11 ± 41.18	192.30 ± 41.18	159.38 ± 37.11	211.13 ± 62.54	209.18 ± 94.42	120.27 ± 36.50
329.11 ± 81.22	329.35 ± 81.22	194.35 ± 40.55	185.88 ± 66.15	236.63 ± 91.43	182.38 ± 39.44
236.15 ± 59.46	235.28 ± 59.46	136.20 ± 42.58	181.63 ± 44.37	264.13 ± 87.65	229.23 ± 60.97
131.84 ± 40.85	132.28 ± 40.85	95.63 ± 21.95	202.88 ± 53.44	404.10 ± 189.74	160.27 ± 84.49

DISCUSSION

The functional observation battery assesses several neurobiological domains including neuromuscular (weakness, coordination, gait and tremor), sensory (auditory, vision and somatosensory), and autonomic (pupil response and salivation) functions. The measures of a neurobehavioral screening battery have been divided into specific functional domains. For example, lacrimation, salivation, pupil response, palpebral closure, defecation and urination are measures of some aspects of the autonomic system. Similarly, the neuromuscular domain is assessed based on the gait/mobility score, landing foot splay, grip strength and righting reflex; the sensorimotor domain is based on response to tail pinch and click/touch/approach response; the central nervous system excitability domain is based on ease of removal and handling of the animal, clonic/tonic movements, arousal and vocalization; the central nervous system activity domain is based on home cage posture, palpebral closure, rearing and motor activity; and the physiological domain is assessed based on the body weight, body temperature and piloerection.

The neurobehavioral effect of thymoquinone on male and female Sprague Dawley rats was assessed by functional observation battery. Under the experimental conditions, thymoquinone did not significantly change any parameter assessed in the study except high dose group at 20 mg/kg showed statistically significant ($p<0.05$) decrease in foot splay measurement post dose administration when

compared to the control group animals. Though statistically significant, this difference in the foot splay measurement falls within the range.

CONCLUSION

In conclusion, based on the neurobehavioral activity results following a single oral dose of thymoquinone in this study the no-observed-effect level (NOEL) is 20 mg/kg The result presently conducted study revealed that daily orally administration thymoquinone (volatile of Kalonji Seed) found to no exhibit significant altering the pathogenesis during assessment without exerting any side effect during the repeated treatment and proved itself to be the traditionally used and recommended by the practitioner best for the treatment for neurobehavior disorder when compare to refernce drug.

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