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ANTI-INFLAMMATORY EFFECT AND TOXICOLOGICAL EVALUATION OF THYMOQUINONE (VOLATILE OIL OF BLACK SEED) ON ADJUVANT-INDUCED ARTHRITIS IN WISTAR RAT

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ABSTRACT

Inflammatory disorder to be considered autoimmune disease which, affects the joints and is associated with swelling, stiffness and pain. The study was subjected to evaluate therapeutic potential of thymoquinone (volatile oil of black seed) on freund's complete adjuvant induced arthritis in rats. Arthritis was induced in rats by injecting 0.1ml of freund's complete adjuvant into the left hind paw of the rat intradermally for 21 days. Thymoquinone orally administered to male and female wistar rat at dose level of 2.5, 5.0, 10.0 mg/kg body weight for 35 days repeatedly, post induced did not produce any sign of toxicity, mortality, pathological changes and significant blood parameters changes. The investigated result showed that the thymoquinone (10 mg/kgb.wt) significantly ($p<0.001$) inhibited the FCA induced arthritis and showed significant anti-inflammatory activity. Thymoquinone 10 mg/kgb.wt also tends to suppress the inflammation at $p<0.05$ level significance. Therefore, thymoquinone treatment found to possess potent anti-inflammatory activity with no toxicity and the treatment significantly inhibited the development phase of arthritis which, is further supported by its anti-inflammatory effect was comparable to that of standard drug prednisolone (5 mg/kgb.wt).

KEYWORDS : Thymoquinone, Freund's adjuvant induced arthritis, Arthritis activity

Inflammation is a common clinical conditions and rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disorder that affects about 2.1 million (Majithia & Geraci,2007., El-Dakhakhny, 1963) in Indians and Americans etc which, affects the joints and is associated with swelling, stiffness and pain. The drugs commonly in used for the treatment of inflammation and RA include glucocorticoids eg., cortisone, prednisolone and NSAIDS drugs (eg. Ibuprofen), disease-modifying anti-inflammatory and anti-rheumatic drugs (DMAIDs and DMARDs; eg. Methotrexate (MTX) and leflunomide) and biological response modifiers (eg. Tumor necrosis factor-alpha blocking agents). Such therapies are helpful controlling the symptoms of acute RA, but their effect on chronic, prolong RA are unsatisfactory. Moreover, besides their high cost, the prolonged use of many of these drugs is associated with severe adverse reactions and toxicity, including gastrointestinal disturbances and cardiovascular risk.

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 (Riaz et al., 1996; Siddiqui & Sharma, 1996). Most properties of whole seeds or their extracts are mainly attributed to quinone constituents, of which, thymoquinone is more abundant compound (Filippo D'Antuono et al., 2002). Pharmacological action of *Nigella sativa* has been investigated including immune stimulant, antiinflammatory, anticancer, antimicrobial antiparasitic and antioxidant (Ibrahim Tekeoglu et al. 2007; Burits and Bucar 2000).

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 rat 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 rat were acclimatized 6 days prior to the start dosing. The thymoquinone sample was purchased from sigma aldrich India and peanut oil from local market the dose volume maintained at 10 ml/kg body weight.

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Complete Freund's Adjuvant Arthritis

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. On day one, all rats were injected into the sub plantar region of the left hind paw with 0.1ml of freund's complete adjuvant. This consist of mycobacterium butyricum suspended in heavy paraffin oil by thorough grinding with motor and pestle to give a concentration of 6mg/ml. Dosing with the test and standard substance was started on the first day and continued for 21 days.

Anti-inflammatory Studies

Based on the subacute 28 days oral toxicity in rats. Group I served as arthritis control group and group II served as vehicle control group given a daily dose of normal saline and peanut oil. The rats of group III, IV and V were given thymoquinone mixed in peanut oil via gavage at dose level 2.5, 5.0, 10 mg/kg body weight respectively for 35 days post induced arthritis and group VI served as standard drug (prednisolone) and given 5.0mg/kg body weight. Rats were observed for the paw swelling in the injected and contra lateral hind paws of the rats were monitored daily using liquid displacement plethysmometer (Ugo Basile, Italy). Increase in the extent of erythema and edema of the tissues shows the severity of the inflammation. The difference in severity of arthritis between the experimental groups and arthritis control group were statistically analyzed and toxicological effect and mortality throughout the study period. Body weight, food and water of individual rat were recorded weekly for each group. After 56 day treatment as well as controls animals were sacrificed and blood collected directly from jugular vein in ethylene diamine tetra acetic acid (EDTA) solution and non-oxalate tubes for the estimation of haematological and biochemical parameter respectively. All the animals were closely observed for organs like ears, nose, tail, fore paws and hind paw.

Paw Edema

Paw volumes of left hind limb were recorded and measured on day 1^{st}, 2^{nd}, 3^{rd}, 5^{th}, 10^{th}, 14^{th}, 18^{th} and 21^{st} using mercury column plethysmometer. On the day 21^{st}, the rheumatoid arthritis becomes more evident and inflammatory changes.

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).

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

Organ Body Weight Ratio

The 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 in (Table, 1).

Biochemical Study

The results of serum biochemical parameters of male rats are shown. There was no change in clinic-chemical parameters of male and female rats exposed to

Table 1: Relative Organ Body Weight of Male Rats Orally Administration Thymoquinone for 35 days post induced arthritis

Dose (mg/kg body weight)						
Organ	**Arthritis Control**	**Vehicle Control**	**Low Dose**	**Mid Dose**	**Higher Dose**	**Standard Drug**
Liver	3.13 ± 0.24	2.90 ± 0.21	3.07 ± 0.24	3.09 ± 0.27	3.13 ± 0.24	2.96 ± 0.24
Kidney	0.75 ± 0.20	0.77 ± 0.34	0.76 ± 0.03	0.77 ± 0.07	0.77 ± 0.34	0.76 ± 0.27
Lungs	0.73 ± 0.01	0.71 ± 0.21	0.73 ± 0.03	0.72 ± 0.05	0.71 ± 0.21	0.70 ± 0.28
Brain	0.78 ± 0.03	0.89 ± 0.19	0.78 ± 0.03	0.81 ± 0.05	0.89 ± 0.19	1.48 ± 0.40
Testis	1.12 ± 0.68	1.18 ± 0.63	1.13 ± 0.09	1.11 ± 0.13	1.18 ± 0.63	1.17 ± 0.05
Epididymis	0.31± 0.54	0.38± 0.44	0.41± 0.64	0.39± 0.52	0.38± 0.22	0.37± 0.64
Seminal Vesicle	0.48± 0.56	0.48± 0.41	0.52± 0.61	0.54± 0.12	0.52± 0.21	0.49± 0.54
Spleen	0.24 ± 0.01	0.25 ± 0.01	0.24 ± 0.03	0.26 ± 0.06	0.25 ± 0.01	0.52 ± 0.26
Heart	0.29 ± 0.00	0.33 ± 0.02	0.32 ± 0.03	0.31 ± 0.03	0.33 ± 0.02	1.45 ± 1.40
Adrenal	0.02 ± 0.00	0.03 ± 0.07	0.03 ± 0.01	0.03 ± .004	0.03 ± 0.07	0.17 ± 0.31

Table 1 (continue): Relative Organ Body Weight of Female Rats Orally Administration Thymoquinone for 35 days post induced arthritis

Dose (mg/kg body weight)						
Organ	**(Arthritis Control)**	**Vehicle Control**	**Low Dose**	**Mid Dose**	**Higher Dose**	**Standard Drug**
Liver	2.99 ± 0.18	2.88 ± 0.16	3.04 ± 0.27	3.00 ± 0.22	2.99 ± 0.15	2.88 ± 0.16
Kidney	0.74 ± 0.06	0.72 ± 0.08	0.75 ± 0.05	0.74 ± 0.06	0.74 ± 0.06	0.72 ± 0.08
Lungs	0.70 ± 0.80	0.69 ± 0.07	0.71 ± 0.04	0.72 ± 0.07	0.71 ± 0.05	0.69 ± 0.07
Brain	0.81 ± 0.00	0.79 ± 0.03	0.78 ± 0.02	0.77 ± 0.03	0.80 ± 0.04	0.80 ± 0.01
Ovary	0.07 ± 0.00	0.07 ± 0.00	0.06 ± 0.01	0.07 ± 0.01	0.72 ± 0.00	0.07 ± 0.00
Uterus	0.10 ± 0.01	0.11 ± 0.01	0.10 ± 0.02	0.11 ± 0.01	0.11 ± 0.01	0.11 ± 0.02
Spleen	0.24 ± 0.01	0.22 ± 0.02	0.24 ± 0.03	0.25 ± 0.05	0.24 ± 0.01	0.23± 0.00
Heart	0.28 ± 0.02	0.28 ± 0.02	0.31 ± 0.02	0.30 ± 0.03	0.32 ± 0.02	0.30 ± 0.02
Adrenal	0.29 ± 0.26	0.02 ± 0.00	0.03 ± .004	0.03 ± 0.01	0.08 ± 0.00	0.03 ± 0.01

different dose of thymoquinone for 28 days and the values were comparable to controls rats. A marked increase in the activity of membrane marker enzymes ALP, AST and SGP were observed in the serum of arthritis rats group V (Table, 2).

Haematology

The results of haematological parameters in male and female rats exposed to different doses thymoquinone are shown. There was no significance changes in Hb RBC, WBC and differential leukocyte count (DLT) (Table, 3).

Histopathology

Autopsy of treated animals after 56 days of 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, 4).

Ant-inflammatory Effect

The left hind paw injected with complete freund's adjuvant become gradually swollen and reach edits peak at 21st day. The results obtained for the different dose of thymoquinone and the standard drug prednisolone 5.0 mg/kg body weight in the complete freund's adjuvant-induced (FCA) paw edema test at specific time intervals. It was obvious that during 21st day treatment paw edema in disease control inflamed paw is increase in time dependent manner and all administration groups significantly inhibited the development of joint swelling induced by complete Freund's adjuvant. Arthritic index and rheumatoid factor were significantly ($p<0.001$) decreased start on 30 days in

Table 2: Serum Biochemical parameter in rats treated orally with thymoquinone for 35 days post induced arthritis

Parameter	Dose mg/kg body weight					
	Arthritis Control	Vehicle Control	2.5 (Low)	5.0 (Mid)	10.0(High)	Standard Drug
AST	19.67±14.45	16.62±14.50	15.94±19.28	21.04±19.67	16.94±30.27	17.84±29.27
ALT	57.70±10.20	57.14±10.25	57.62±10.50	57.42±10.52	62.67±11.16	64.77±12.16
ALP	60.67±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 %)	1.20±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 %)	46.80±5.01	47.00±5.43	56.00±11.04	54.80±10.13	51.50±9.48	52.54±9.58
S-Albumin(g%)	4.18±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)	7.48±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 35 days post induced arthritis

Parameter	Dose mg/kg body weight					
	Arthritis Control	Vehicle Control	2.5 (Low)	5.0 (Mid)	10.0 (High)	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 35 days post induced arthritis

Tissue	Number of Lesion											
	Dose mg/kg body weight											
	0 (Arthritis Control)		0 (Vehicle Control)		2.5(Low)		5.0(Mid)		10.0(High)		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

treatment with thymoquinone 10.0 mg/kg, and prednisolone 5.0 mg/kg treated animal as compare to disease control treatment (Table, 5 & Figure 1 & 2).

DISCUSSION

Most of the investigators have reported that inhibition of adjuvant-induced arthritis in rats is one of the most suitable test procedures to screen anti-arthritic agents since, it closely resembles human arthritis. Arthritis (RA), one of the chronic inflammatory diseases, systemic inflammatory disorder affecting the synovial joints and typically producing symmetrical arthritis that leads to joint

Table 5: Effect Of Thymoquinone Mean Percentile In Paw Edema Volumes In Male Rats Induced By Adjuvant Freund's Complete Adjuvent (FCA)

Dose (mg/kg body weight)	Days							
	22	23	25	30	35	42	48	56
Arthritis Control	86.56±1.9	86.42±2.1	86.26±1.8	83.85±1.5	81.48±0.83	65.68±3.3	58.8±1.56	58.44±1.2
Vehicle Control	85.67±2.1	85.65±1.8	81.96±1.3	70.56±1.5	64.94±1.9	61.34±1.2	58.90±3.8	55.30±4.9
Low Dose	84.33±2.9	83.95±2.9	83.44±3.2	65.96±15.6	65.04±3.3	40.82±5.4	40.45±5.1	38.85±4.8
Mid Dose	87.14±2.8	86.93±2.6	86.34±2.1	70.56±2.7	66.74±3.3	43.11±4.1	33.47±33.7	31.32±1.4
Higher Dose	86.90±2.1	86.46±2.4	86.16±2.3	56.80±2.7	44.30±3.2	66.67±2.8	35.68±2.5	30.08±2.1
Standard Drug	86.40±1.4	85.32±1.7	84.36±1.7	72.08±0.2	63.05±1.3	39.36±2.6	37.07±2.1	26.52±2.0

Value are expressed as a mean ± S.E.M ($p < 0.001$) as compared to control

Table 5 (continue): Effect Of Thymoquinone Mean Percentile In Paw Edema Volumes In Female Rats Induced By Adjuvant Freund's Complete Adjuvant (FCA)

Dose (mg/kg body weight)	Days							
	22	23	25	30	35	42	48	56
Arthritis Control	86.51±2.0	85.33±1.8	83.21±2.6	82.09±0.2	75.48±5.3	63.88±0.6	58.8±1.5	58.44±1.2
Vehicle Control	85.57±2.0	85.44±1.4	82.44±2.2	81.09±1.5	78.50±2.6	64.14±1.2	44.28±0.6	55.30±4.9
Low Dose	84.13±2.8	83.75±2.6	84.12±3.2	70.68±2.6	64.94±3.2	42.22±5.9	40.45±5.1	38.85±4.8
Mid Dose	86.93±2.7	86.34±2.6	84.17±2.2	72.65±1.9	65.05±1.5	43.31±3.8	39.27±4.8	36.72±5.2
Higher Dose	86.56±2.1	85.26±1.9	83.35±2.0	71.73±1.7	44.54±2.6	40.88±5.3	37.48±3.8	29.68±1.2
Standard Drug	86.22±1.3	84.92±1.4	84.16±1.3	72.16±0.1	43.05±1.5	40.75±2.8	37.07±2.1	25.12±2.8

Value are expressed as a mean ± S.E.M ($p < 0.001$) as compared to control

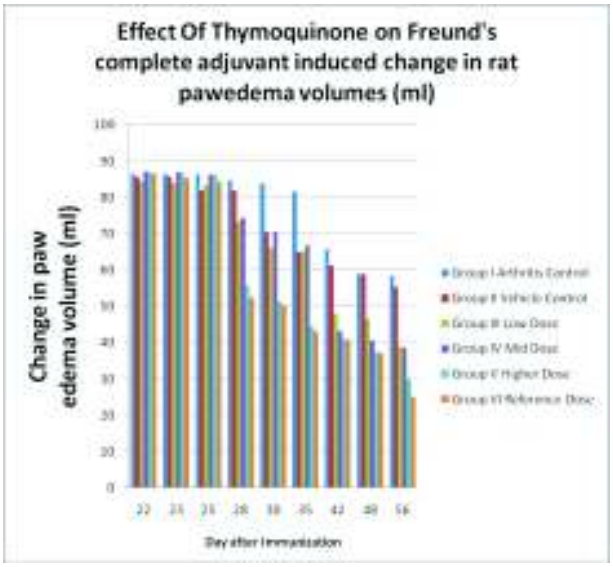

Figure 1 : Mean Percentile change In Paw Edema Volumes (Ml) In Male Rats Induced By FCA

destruction. In this present study, the result demonstrated the effect of thymoquinone and prednisolone on FCA induced arthritis model in rats, selected to evaluate their efficacy against the proliferateive phase of inflammation, the activity of membrane marker enzymes was significantly markedly increased in the adjuvant induced arthritic rats after treated with thymoquinone 10 mg/kg, and prednisolone 5.0 mg/kg when comparable to the arthritis control and vehicle control group. Increased white blood cell count is a common feature of inflammatory reactions, especially those induced by microbial infection. So in arthritic group an increase in total leukocyte number was found. A significant reduction ($p<0.001$) in total leukocyte number was found in case of treated thymoquinone 10 and standard drug 5 mg/kg body weight. In present study it was found that the administration of thymoquinone and standard drug lead to inhibition leukocyte migration which, may have beneficial effect for joint preservation. Traditional medicine has maintained greater popularity all over developing world prompted by the increase awareness and interest in medicinal plant and the use is rapidly increase generation by generation (Daswaniet al., 2006; Ogbonnia et al., 2008). More often, this has led to indiscriminate use with out appropriate dose resulting in abuse. The incidence of adverse effects of these herbal remedies and sometimes life-threatening conditions has been reported among various ethnic groups (Elvin-Lewis, 2001; Chan, 2003). The result presently conducted study revealed that daily orally administration thymoquinone (volatile of Kalonji Seed) found to exhibit significant antiinflammatory and the

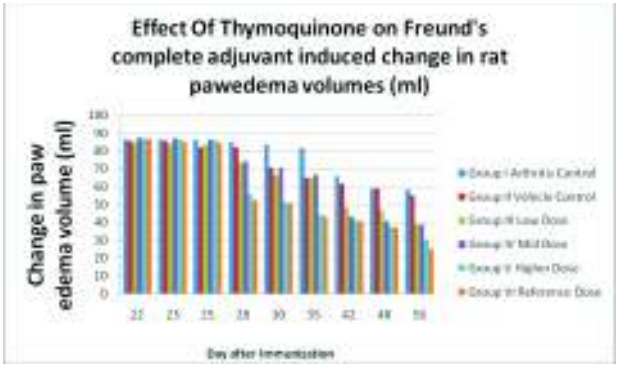

Figure 2 : Mean Percentile change In Paw Edema Volumes (Ml) In Female Rats Induced By FCA

potent anti arthritic activity by significantly ($p < 0.001$) altering the pathogenesis during arthritis without exerting any side effect and did not induce any treatment related observable toxic effects, with regards to the haematological parameters, biochemical parameter and histopathological when compared to its control group of animal treated with corn oil (vehicle) only throughout the study.

CONCLUSION

The result presently conducted study revealed that daily orally administration thymoquinone (volatile of Kalonji Seed) found to exhibit significant antiinflammatory and the potent anti arthritic activity by significantly ($p < 0.001$) altering the pathogenesis during arthritis 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 arthritis when compare to allopathic steroids drug. The study are also give advice to should not be used daily higher amount of kalonji seed in form of kalnoji oil and kalonji majoon which, available in market. But give advise to use daily little amount which, is remedy for all disease except death which, has been proved traditionally practionaer generation by generation.

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