Available online on www.ijtpr.com
International Journal of Toxicological and Pharmacological Research 2011; 3(4):7-12

ISSN: 0975-5160

Research Article

Immunomodulatory Activity of Aqueous Extract of *Murraya Koenigii, L* in Experimental Animals

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ABSTRACT
Murraya koenigii (Linn) Spreng (Indian curry leaf) is a highly valued culinary plant for its characteristic aroma with the aqueous extract of its leaves reported to exhibit diverse biological activities viz., hyperglycemia, inflammation and hepatitis. The objective of this study was therefore to investigate effects of antioxidant-enriched nutritional dietary herb on immunological parameters that are found to be affected in diseased state including alcoholic liver disease (ALD). Acute toxicity studies were carried out on female Swiss mice and LD_{50} was evaluated with its effects on mortality or any changes in autonomic disturbances and behavioral pattern. The aqueous extract was subjected to *in vitro* DPPH activity at different concentrations against ascorbic acid and high doses of 1 g/kg and 2 g/kg (MK1 and MK2) was objectively evaluated for its immunomodulatory potential in Wistar rats. MK1 and MK2 produced decrease in cell-mediated immune response (30.05% and 55.36% suppression of inflammation, respectively) whereas MK2 extract comparatively reduced antibody titer on all 7th, 14th and 21st days. In conclusion, the extract exhibited good immunomodulation through antioxidant and immunosuppressant mechanisms that could be crucial in treatment of ethanolic liver injury wherein immune stimulation or autoimmunity is involved in its pathogenesis.

Keywords: Curry leaves; dietary antioxidants; antibody titer; delayed-type immunity; alcohol-induced liver disease; immunosuppressant

INTRODUCTION

The immune system is a complex system, to protect the host from invading and to eliminate diseases. [1] Many xenobiotics are metabolically activated to electrophilic intermediates that form covalent adducts with proteins; the mechanism of toxicity is either intrinsic or idiosyncratic in nature. Many intrinsic toxins covalently modify cellular proteins and somehow initiate a sequence of events that leads to toxicity. Major protein adducts of several intrinsic toxins have been identified and demonstrate significant decreases in enzymatic activity. Idiosyncratic toxicities are mediated through either a metabolic or immune-mediated mechanism. Xenobiotics including alcohol that cause hypersensitivity/autoimmunity appear to have a limited number of protein targets, which are localized within the subcellular fraction where the electrophile is produced, are highly substituted, and are accessible to the immune system. [2]

Liver inflammation is the hallmark of ethanolic hepatitis that leads to the release of chemotactants by the liver cells attracting phagocytes from blood to the damaged tissue. These activate Kupffer cells that release tumor necrosis factor, vasoactive eicosanoids and cytotoxins like reactive oxygen free radicals resulting in liver injury. [3, 4]

Adducts formed from acetaldehyde and free radicals with blood and liver proteins [5] are perceived as foreign by the body's immune system contributing to ethanolic liver injury, by stimulating immune mechanism that in turn attacks healthy liver cells [6]. It has been proposed that allo- and autoimmune reactions associated with oxidative stress might contribute to fueling hepatic inflammation.

Murraya koenigii (*Linn*) *Spreng* (Family: Rutaceae), commonly known in India as curry leaf plant, has its characteristic aroma and medicinal properties [7]. It is used as a natural flavoring agent for centuries across India. The leaf contains alkaloid, volatile oil, alpha-selinene, beta-bisabolene, glycolozoline and xanthotoxin. The nutritional values of curry leaves per 100 g includes 38.43% total xanthophylls, 50.25% total carotenoids and total pro vitamin A carotenoids of 11.82 % mg per 100 g of dry weight [8].

The plant is used in Indian system of medicine to treat various ailments including detoxifying action, anti-inflammatory [9] and anti hyperglycemic activities [10]. *M. koenigii* leaves mixed with fat separated butterfat used for the treatment of amoebiasis, diabetes and hepatitis in Ayurveda [11]. The aqueous extract of *M. koenigii* has favorable effect in bringing down the severity of diabetes [12]. Reports mention that fresh crude curry leaf aqueous extract could modulate the levels of drug metabolizing phase-I and phase–II enzymes, antioxidant parameters, lactate dehydrogenase and lipid *per oxidation* changes [13]. Stimulation of immune system by free radicals as in ALD

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Table 1. DPPH radical scavenging activity of SAMK expressed in terms of percentage inhibition against time in minutes. (mean ± SEM, n=3)

Time in min	**Vitamin C**	***M. koenigii***
	25 µg / ml	**25 µg / ml**
	% Inhibition	
0.5	97.08 ± 2.34	42.64 ± 1.45
1	97.21 ± 1.67	47.8 ± 1.1
1.5	97.20 ± 1.78	51.6 ± 2.72
2	100 ± 2.7	57.15 ± 2.34
2.5		59.30 ± 1.79
3		61.26 ± 1.41
3.5		62.81 ± 1.98
4		64.45 ± 2.91
4.5		65.75 ± 2.31
5		66.16 ± 3.12

† % inhibition of absorbance's indicates the DPPH free radical scavenging activity that can directly be correlated to antioxidant activity.

led to the hypothesis that dietary antioxidants could protect the liver cells against ethanol-induced toxicity. It was observed from the *in vivo* and *in vitro* studies that the aqueous extract of *M. koenigii* exhibited potential hepatoprotective property in terms of improvement in liver functional markers because of synergism of various phytochemicals. The resultant immune stimulation in the free radical-mediated liver injury and the reported anti-inflammatory action of the herb encouraged us to investigate the aqueous extract of the leaves of *M. koenigii* for its effects on immune modulation in experimental animals. The present study was design with a

n objective to investigate whether the antioxidant property of the aqueous extract of the nutrient enriched *M. koenigii* could be significant for its effects on the cell and humoral immune responses for claiming its probable hepatoprotective potential.

Methods and materials: *Plant material*: Leaves of *M. koenigii* (Herbarium Sample no. 120401) were obtained from the local market, Mumbai. The sample was suitably authenticated by Dr. Harshad Pandit from Department of Botany, Guru Nanak Khalsa College, University of Mumbai, India. The aqueous extract was standardized for its markers including carbazole alkaloids and tannins by TLC technique. The markers identified using chloroform: methanol (75:25) with Dragendorff's solution as a detection reagent and ethyl acetate: methanol (75:25) solvent systems respectively. The phytochemical markers were isolated using column chromatography using silica gel (60-120 mesh size) with elution rate of mobile phase as 6-12 drops/min. Maceration technique was used to prepare aqueous extract by using 500 g of powdered leaves in 1 liter of distilled water for 6-8 h. The slurry was boiled, concentrated, cooled, and filtered through a muslin cloth. The filtrate was further concentrated by boiling. The concentrated slurry was freeze dried to obtain dried aqueous extract. Its percentage yield was calculated and subjected to a battery of qualitative chemical tests including the Dragendorff test (alkaloids), Borntrager's test (glycosides), Molisch's test (carbohydrates), Liebermann-Bur chard test (phytosterols), ferric chloride test (tannins), foam test (saponins) and alcoholic precipitation test for the presence of gums and mucilage. The presence of alkaloids and tannins were confirmed by using TLC technique.

Evaluation of Antioxidant Activity using DPPH assay : The antioxidant activity in terms of percentage inhibition of 1, 1-diphenyl-2-picryl hydrazyl (200 µM DPPH) by AQUEOUS EXTRACT (20 µg / ml) was calculated at 517 nm at regular intervals of 30 seconds for five minutes using the formula [14]:

% DPPH = A (DPPH) – A (DPPH + sample) X 100/ A (DPPH).

Animals and experiment design :All animal experiments were conducted after the approval of Institutional Animal Ethics Committee at ICT (IAEC Approval No: ICT /PH/IAEC/1204/19).

Acute toxicity studies :The acute oral toxicity was carried out as per the guidelines of OECD 423 received from committee for the purpose of control and supervision of experiments on animals (CPCSEA, Ministry of social Justice and Empowerment, Government of India).

The extract was dissolved in water as it had very good solubility and administered per oral in a single dose following period fasting. Animals were weighed before and after dosing and food was withheld for a further 3-4 h. Animals were observed initially after dosing at least once during the first 30 minutes, periodically during the first 24 h. Additional information like changes in fur, skin, mucous membranes, and also respiratory, autonomic

Table 2. Delayed type hypersensitivity reaction (DTH) is measured by suppression of inflammatory response by MK1 and MK2 on the 10th day after the challenge of second dose of antigen ovalbumin dissolved in 0.1 ml of normal saline emulsified with equal volume of Freund's complete adjuvant.

Thickness of Paw in mm in experimental animals				
Treatment	**Left hind foot paw**	**Right hind foot paw**	**Different in mm between left and right hind paws**	**% Inhibition of thickness of paw**
Vehicle control	3.732 ± 0.23	2.8 ± 0.07	0.932	-----
MK1	3.462 ± 0.08	2.81 ± 0.05	0.652 (69.95%)	30.05
MK2	3.256 ± 0.03	2.84 ± 0.02	0.416 (44.63%)	55.37

DTH is expressed in terms of thickness of paw of mice. 0.932 mm rise in the thickness is 100% inflammation. 0.652 mm rise in the thickness by MK1 indicates 69.95 % inflammation. Thus, M. koenigii at 1 g/ kg exhibited 30.05 % inhibition of thickness of paw. Similarly, 0.416 mm rise in the thickness by MK2 indicates 44.63 % inflammation. Thus, 55.37 % inhibition of thickness of raw paw exhibited by M. koenigii at the dose of 2 g/ kg

and CNS systems and behavioral patterns. Attention was given to any tremors or convulsions, observed if any.

Reagents: Freund's complete adjuvant was purchased from Genei, Bangalore. Ecoline diagnostic kits manufactured by Merck Ltd, Mumbai were used for biochemical estimations. All chemicals and reagents used were of analytical grade (S.D. Fine Chemicals, India).

Evaluation of immunomodulatory activity: With the high LD_{50} of more than 5 g/kg body weight of rats as found in the acute toxicity studies, two selected doses namely MK1 and MK2 of 1 g/kg and 2 g/kg of body weight respectively were evaluated for immunomodulation in rats.

Humoral and cell-mediated immune responses were evaluated *in vivo* with the aqueous extract, as per the standard procedures [15]**.** Wistar mice procured from Haffkine Institute, Parel Mumbai were acclimatized to ICT animal house conditions, fed with standard food and distilled water *ad libitum*

Antibody- mediated (humoral) immune response: Male/ female mice were divided into three groups of six animals each and they were given the dose of drug to be evaluated (aqueous extract at 1 g/kg and 2 g/kg). Dosing to the treatment group continued up to 21 days with a control group kept without any treatment. On the second day of dosing, the animals were immunized subcutaneously with an antigen ovalbumin, 3 mg dissolved in 0.1 ml of normal saline emulsified with equal volume of Freund's complete adjuvant (Bangalore Genei) , considered as Day 0. Blood was collected by retro-orbital puncture on day 7, 14 and 21-post challenge with the antigen. Serum was separated and preserved at – 20° C until analysis. The serum antibody titer to ovalbumin was estimated by enzyme linked immuno sorbent assay [16].

Cell- mediated immune response (delayed type hypersensitivity reaction) : The experimental methodology was carried out as per Ray and Banerjee (1996). Animals were divided into three groups. Group I received no treatment but only distilled water; Group II and group III received *M. koenigii* in the dose of 1 g/kg and 2 g/kg body weights. The two groups were dosed for 5 days as above. On the 6th day, the animals were immunized subcutaneously with 3 mg of ovalbumin antigen (dissolved in 0.1 ml of normal saline emulsified with equal volume of Freund's complete adjuvant). Dosing both the groups was continued up to 10 days in a similar way. On the 10th day, the animals from both the groups were challenged with second dose of the antigen (50 μg ovalbumin in 0.05 ml of phosphate buffer saline), in the left hind foot paw subcutaneously. The right hind foot paw was injected with 0.05 ml of vehicle, served as control. The inflammatory response was measured after 24 h of challenge with the help of vernier caliper. The degree of delayed type hypersensitivity reaction was expressed as the percentage increase in the footpad thickness (L-R) over the control value.

Statistical analyses: All data was expressed as mean ± SEM. Statistical analyses were carried out using one-way analysis of variance and their level of significances determined by Dunnet's method of comparison. The statistical significance of the differences between treated groups and vehicle control for each parameters were calculated with sample size (n=6) using the latest version of statistical software for Windows (Instat, India).The level of significances are denoted by * at $P < 0.05$; ** at $P < 0.01$ and *** at $P < 0.001$.

Table 3. Antibody mediated immune response by two different concentrations of *M.koenigii* of MK1 and MK2 are expressed in terms of antibody titers on 7, 14 and 21 days against vehicle control

Treatment	Antibody titer		
	7^{th} Day	14^{th} Day	21^{st} Day
Vehicle control	10.79 ± 0.1	11.68 ± 0.05***	11.59 ± 0.04
MK1	10.08 ± 0.33	9.87 ± 0.25	10.19 ± 0.14
MK2	9.65 ± 0.08	9.34± 0.10	10.03± 0.06

**** indicates level of significance at P < 0.001 with control as the group of comparison*

RESULTS AND DISCUSSIONS

Phytochemical studies: The aqueous extract from *M. koenigii* exhibited the presence of alkaloids, glycosides, saponins and tannins along with carbohydrates with the percentage yield of the crude extract being 0.6% w/w. The extract was standardized using markers including tannins and carbazole alkaloids that were comparable to their standards using TLC technique.

Antioxidant status: Exogenous chemicals-induced oxidative stress is the result of the combined impairment of antioxidant defenses and the production of reactive oxygen species by the mitochondrial electron transport chain [17]. It was found that 20 µg/ml of the aqueous extract exhibited 57.15 % inhibition of DPPH activity to 100 % inhibitory activity of 25 µg/ml of ascorbic acid in the time period of 2 min (Table 1).The considerable DPPH radical scavenging activity of the aqueous extract in comparison to ascorbic acid must be due to presence of antioxidant phytoconstituents like tannins present in it.

*Immunomodulatory potential***:** To find out the immunomodulatory potential of aqueous extract, delayed type hypersensitivity was evaluated to assess the cell-mediated immune response (delayed-type immunity) on the 10^{th} day and humoral type of immunity by measurement of antibody titer on the 7^{th}, 14^{th} and 21^{st} days (Tables 2 and 3). The results of the study were compared with the control group (no treatment) on specified time points.

Substantial evidence suggests a possible immune mechanism involved in alcoholic hepatitis [18] due to adduct formation that stimulates the immune system [19]. Alcoholic liver injury causes activation of Kupffer cells by endotoxin as host defense mechanisms [20]. Acetaldehyde, the major toxic metabolite of ethanol form adducts with the proteins of hepatocytes [21], and initiates an immunological response leading to inflammation. Any drug to be effective in ethanolic hepatitis should be able to reverse any of the above mechanisms of toxicity of ethanol.

Inflammation is recognized increasingly as having an important role in the pathogenesis of alcoholic liver disease (ALD). Nonetheless, the mechanisms by which alcohol maintains hepatic inflammation are still characterized incompletely. Several studies have demonstrated that ethanol-induced oxidative stress promotes immune responses in ALD by stimulating both humoral and cellular reactions against liver proteins adducted to hydroxyethyl free radicals and several lipid peroxidation products. Moreover, ALD patients have autoantibodies targeting cytochrome P4502E1 and oxidized phospholipids. In both chronic alcohol-fed rats and heavy drinkers, the elevation of IgG against lipid peroxidation-derived antigens is associated with tumor necrosis factor-α production and the severity of liver inflammation. [22].

Innate immune cells, particularly dendritic cells, have a pivotal role in sensing pathogens and initiating adaptive immune responses by activation and regulation of T-lymphocyte responses [23].

The toxic potential of the chronic ethanol consumption and its clinical social relevance serve as an impetus to search for a potential dietary supplement in the ethanol-induced hepatotoxicity.

In this study, the aqueous extract has shown to produce decrease in cell-mediated immune response as indicated by 30.05 % and 55.36 % suppression of inflammation at 1 g/kg and 2 g/kg dose, respectively. As far as antibody (humoral) mediated immune response is considered, control showed significant elevation of antibody titer ($P < 0.001$) on the 14^{th} day, whereas MK1 and MK2 extract showed a non-significant reduction in antibody titer of 9.87 and 9.34 respectively on 14^{th} day. The antibody values on 21^{st} day did not show any appreciable changes in control as well as MK1 treated group. MK2 extract reduced the antibody titer on all, 7th, 14^{th} and 21^{st} days in comparison to MK1 and control.

While most of the investigations into the causative events in the development of ALD are focused on multiple factors, increasing interest has been centered on the possible role of immune mechanisms in the pathogenesis and perpetuation of ALD. Considering the reactions of free radical and lipid *per oxidation* products with hepatic proteins that stimulate both humoral and cellular immune reactions, which contributes to the chronic hepatic inflammation, provided a rationale to study their effects on the immune response. The aqueous extract produced decrease in cell-mediated immune response. In addition, the antibody titer to the specific antigen was reduced with the administration of extracts in a manner with MK2 showing promising results in terms of suppression of inflammation of paw in delayed type hypersensitivity and decreased antibody production as in humoral immune

response. Thus, the extract indicated a trend towards immunosuppression. This could be one of the mechanisms, through which it inhibits the inflammatory changes produced in alcoholic liver injury. This protection related to the presence of antioxidant phytoconstituents such as tannins in the extract and the immunosuppressive trend exhibited by the extract can aid in reducing the inflammation of the liver during ethanolic hepatitis.

The results obtained relates to the previous study wherein the aqueous extract and the crude isolates from *M. koenigii* exhibited excellent hepatoprotective activity on liver carcinoma cell lines (Hep G2) [24]. This immunosuppressant property of *Murraya koenigii* (Indian curry leaves) that we investigated in can be used as a supportive data to these earlier studies as a dietary health supplement in alcoholic hepatitis.

The present investigation has also opened avenues for further research especially with reference to the development of potent phytomedicine for hepatoprotection from the commonly consumed *M. koenigii* (Curry) leaves

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