Original Research Article

Chemopreventive effect of Maha Vallathy leghiyam on 4-Nitroquinoline-1-oxide induced oral carcinogenesis in wistar rats

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International Archives of Integrated Medicine, Vol. 6, Issue 7, July, 2019.

Available online at http://iaimjournal.com/
ISSN: 2394-0026 (P) **ISSN: 2394-0034 (O)**
Received on: 15-06-2019 **Accepted on:** 19-06-2019
Source of support: Nil **Conflict of interest:** None declared.
How to cite this article: Priyanka G, Kayalvizhi E, Madan Kumar A, Chinmayi Sri Amulya Y, Rajajeyakumar Manivel. Chemopreventive effect of Maha Vallathy leghiyam on 4-Nitroquinoline-1-oxide induced oral carcinogenesis in wistar rats. IAIM, 2019; 6(7): 28-36.

Abstract

Background: Siddha medicine is one of the oldest herbal medication after Ayurveda. For many centuries, siddha medicine is being used to cure or prevent many diseases including cancer. MahaVellathyLehyam is one such Siddha medicine possessing characteristic nature against cancer. The main objective of this study is to explicate the potential of MVL as a chemopreventive agent by analysing the alterations in lipidperoxidation, membrane bound enzymes and glycoconjugates against 4-Nitroquinoline-1-oxide (4NQO) induced rat oral carcinogenesis.

Materials and methods: The male wistar rats were subjected to the carcinogen 4NQO and the activity of MVL against the carcinogenic cells was studied through lipid peroxidation, membrane bound ATPases (Na+ /K+ ATPase, Ca2+ ATPase and Mg2+ ATPase) and protein bound carbohydrate components (protein bound hexose, hexosamine, sialic acid and fucose).

Results: The obtained result was visualized as an increase in the expression of the lipid peroxidation of the tongue tissue, membrane bound ATPases and glycoconjucates indicating oxidative stress induced by the carcinogen 4QNO.

Conclusions: The administration of MVL has caused a significant decrease in these aspects suggesting the nature of MVL against the carcinogenic nature of 4NQO by efficiently decreasing the oxidative damage.

Key words

MahaVallathyLeghiyam, Hematology, Acute toxicity, Rats, Biochemical analysis.

Introduction

Oral carcinoma is a major complex multifocal process which occurs in the squamous epithelium that gets affected by several genetic alterations and metastases vigorously [1]. It is reported to be the sixth most common cancer in the world and is prevalent more in men than in women. India, Papua New Guinea, Taiwan and China are the areas with highest incidence and mortality where chewing of betel quids with tobacco or without tobacco or areca nut chewing is common along with chewing tobacco and consumption of alcohol is extremely high [2]. Precancerous and cancerous lesions of oral carcinogenesis have been effectively studied in the male wistar albino rats previously due to the resemblance in human oral cancer morphology and histopathology [3, 4]. Due to its resemblance, this model is widely used to assess the chemopreventive potential of many synthetic and natural compounds [5, 6].

Lipid peroxidation is a process by which oxidants such as free radicals attack lipids containing carbon-carbon double bond(s), especially polyunsaturated fatty acids (PUFAs) [7]. Since lipids play a key role in maintaining the integrity of cellular membranes, a widespread peroxidation of lipids disrupts and rearranges the composition, morphology and dynamics of the lipid membranes [8, 9]. As the lipid peroxides are highly reactive, these also hold the capability of propagating further generation of ROS or get reduced into reactive compounds that have the capacity of cross linking the DNA and proteins [10, 11]. As the lipid peroxidation progresses, it has been reported that proliferation rate of the cell decreases [12]. Glycoproteins being the most important constituents of the cell, play a major role in cell proliferation, cell differentiation and the cell-cell interaction [13, 14]. Hence, the serum glycoproteins of the precancerous and cancerous lesions should be measured to bring about a narrowed diagnosis.

Maha Vallathy Leghiyam is plant derivative which is a siddha medicine with a vast compositional range of ingredients that carry biological importance [15]. It is a derivative with a wide extent of application which aids in treating various ailments including as an anti-AIDS drug besides its anticancer nature. In this study, the effect of MVL on lipid peroxidation, membrane bound enzymes and cell surface abnormalities will be analyzed by measuring the extent of glycoconjugates present in the oral carcinogenesis induced by 4NQO to confirm the chemopreventive nature of MVL.

Materials and methods

Preparation of Aqueous extract of MVL

MVL was procured form Indian Medical Practitioners Co-operative Pharmacy and Stores Ltd., (IMPCOPS, Thiruvanmiyur, Chennai, India), an authoritative source of Indian medicines (http://www.impcops.org/), and its composition as per the ancient scripts [16].

The MVL was checked for its solubility in different solvent system and it was confirmed water was better solvent to completely dissolve MVL in time dependent manor. The aqueous extract of MVL was used for further studies to check the effective chemopreventive assessment.

Experimental Procedure

Male wistar albino rats (Rattus norvegicus) weighing 120–140 g were used for evaluating the chemopreventive nature of MVL. The rats were obtained from Sathyabama Institute of Science and Technology, Centre for Laboratory animal

technology and research, Chennai, India. The animals were acclimatized to laboratory conditions for seven days prior to the experiments. The rats were maintained at a room temperature of 22–24°C, with a 12 h light/dark cycle and humidity around (50 ± 5)%. During acclimatization, the rats were randomized into experimental and control groups and housed individually in sanitized polypropylene cages housed with sterile paddy husk as bedding. Animals were given free access to standard pellet diet and water ad libitum. All experimental procedures were in compliance with the Animal Ethical Committee, Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and were approved by Sathyabama Ethical Committee with an approval number SU/CLATR/IAEC/X/082/2018.

Reagents and chemicals

4NQO was purchased from Sigma Aldrich Chemicals Pvt. Ltd, Bangalore, India. All other chemicals used were of analytical grade, purchased from SRL Chemicals Pvt. Ltd, Mumbai, India.

Experimental design

The experimental animals were divided into five groups, each groups comprising of six animals.

Group 1- Control animals treated with corn oil thrice a week orally for 20 weeks.

Group 2- Oral carcinoma was induced by administration of 50 ppm 4NQO dissolved in drinking water for 20 weeks.

Group 3- Animals were treated with Maha Villathi Lehiyam (MVL) (100 mg/Kg b.wt.) dissolved in water thrice in a week orally. MVL treatment was started one week prior to the first dose of 50 ppm 4NQO administration (as in group 2) and continued till end of the experimental period.

Group 4- Animals were treated with Quercetin (50 mg/Kg b.wt.) dissolved in water thrice in a week orally. Quercetin treatment was started one week prior to the first dose of 50 ppm 4NQO administration (as in group 2) and continued till end of the experimental period.

Group 5- Animals were treated with MVL (100 mg/Kg b.wt.) dissolved in water thrice in a week orally for 20 weeks to assess the cytotoxicity if any, induced by MVL, and rats were referred as drug control.

After the experimental period, the rats were fasted overnight and anesthetized using diethyl ether and sacrificed by cervical decapitation. A portion of tongue was used for was homogenized in 0.1M Tris-HCl buffer pH-7.4 and centrifuged. The supernatant was used for biochemical studies, and total protein in serum and tissue homogenate was done [17]. LPO was assayed [18], with malondialdehyde (MDA) release serving as the index of LPO.

Determination of membrane-bound enzymes

Na^+/K^+ ATPase activity was determined [19]. The activity of Ca^{2+} ATPase [20] and Mg^{2+} ATPase [21] was assayed.

Assay of glycoproteins

Assay of glycoproteins were performed based on the previous report of Madankumar, et al. [22]. Hydrolysis of glycoprotein for the determination of hexose, hexosamine and fucose was carried out. A known amount of defatted tissue was put into a test tube to which 1 ml of 2N HCl was added, and the tubes were sealed. Hydrolysis was completed by keeping the sealed tubes at 100°C for 16-18h. After hydrolysis, the contents were neutralized with NaOH and made up to known volume, and aliquots were used for hexose, hexosamine and fucose determination [23]. Sialic acid was determined by the method of Warren [24].

Statistical analysis

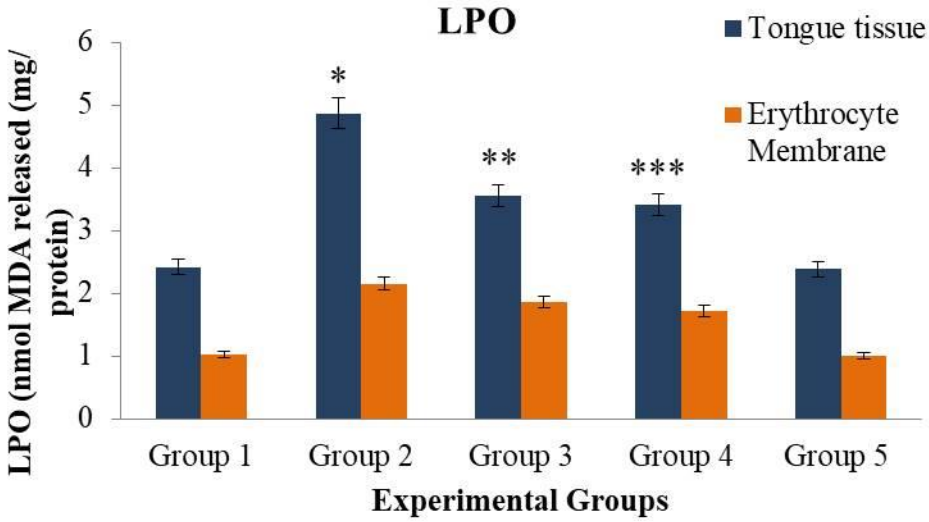
All the grouped data were evaluated with SPSS/10 software. Hypothesis testing methods included one way analysis of variance (ANOVA) followed by Duncan's multiple range test. P values of less than 0.05 were considered to indicate statistical significance. All these results were expressed as mean ± S.D for six animals in each group.

Results

Effect of MVL on Lipid peroxidation (LPO)

The levels of LPO in the tongue and erythrocyte membrane of experimental animals were studied to evaluate the efficacy of the aqueous extract of MVL as expressed in **Figure - 1**. A highly significant (P<0.05) increase in the levels of LPO measured in terms of melondialdehyde were observed in group 2 4NQO induced cancer bearing rats when compared to group 1 control normal healthy rats. On the other side, treatment with MVL significantly normalized the levels of tongue and erythrocyte LPO in group 3 animals. Similar protective effect was compare with standard drug control Quercetin (50 mg/Kg b.wt.) Group 4 rats compared to group 2 experimental rats. However, there was no noticeable change observed in Group 5 MVL alone treated animals when compared to group 1 control animals.

Figure – 1: showed the Level of Malondialdehyde (MDA) in tissue and erythrocyte membrane of control and experimental rats. Results were expressed as mean ± S.D for six rats in each group. Statistical significance at P < 0.05 compared with *group 1, **group 2, and ***group 2 based on Duncan's multiple range tests.



Effect of MVL on Membrane Bound ATPases

The effect of MVL administration on the activity of tongue ATPases in the control and experimental groups The activity of tongue ATPases, such as Na^+ /K^+ ATPase, Mg^{2+} ATPase and Ca^{2+}ATPase of control and experimental animals are presented in **Figure - 2**. A significant decrease in the levels of Na^+ /K^+ ATPase, Mg^{2+} ATPase and Ca^{2+}ATPase were observed in group 2 4NOQ induced Oral cancer bearing animals when compared to group 1 control healthy animals. Pretreatment of MVL to 4NQO treated rats normalized the levels of tongue ATPases in group 3 animals. These results were compared with the positive control Quercetin treatment in group 4 rats, which showed better activity. However, there was no significant change in Group 5 MVL alone treated animals when compared to group 1 control animals.

Effect of MVL on cell surface glycoconjugates

Figure - 3 and **Table - 1** shows the levels of glycoconjugates (protein bound hexose, hexosamine, total sialic acid and fucose) in the tongue and plasma of control and experimental rats in each group, respectively. The levels of glycoconjugates in the tongue tissue and plasma were significantly increased in animals treated with 4NQO alone (group 2) as compared to control (group 1) animals. Oral administration of MVL to 4NQO treated rats (group 3) brought back the levels of above said glycoconjugates to

near normal range. These results were compared with the positive control Quercetin treatment in group 4 rats with group 2 oral cancer bearing rats, which showed significant activity. No significant difference was noticed in the levels of tongue tissue and plasma glycoconjugates in MVL alone (group 5) treated animals as compared to control (group 1).

Figure – 2: The effect of MVL on administration for the activity of ATPases of tongue tissue in the control and experimental groups. Results were expressed as mean ± S.D for six rats in each group. Statistical significance $p<0.05$ compared with *group 1, **group 2, and ***group 3 based on Duncan's multiple range test. Units: Na^+/K^+ATPase, Mg^{2+}ATPase and Ca^{2+}ATPase are expressed µmol of inorganic phosphate formed / min (mg /protein).

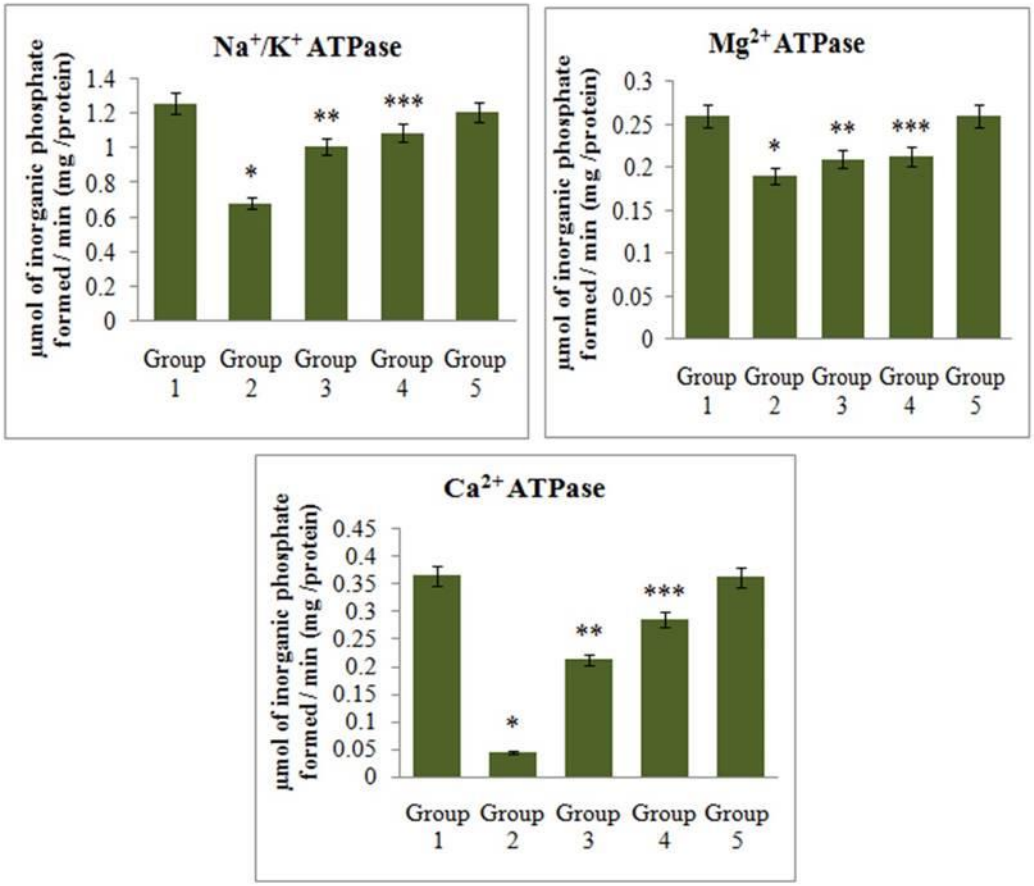
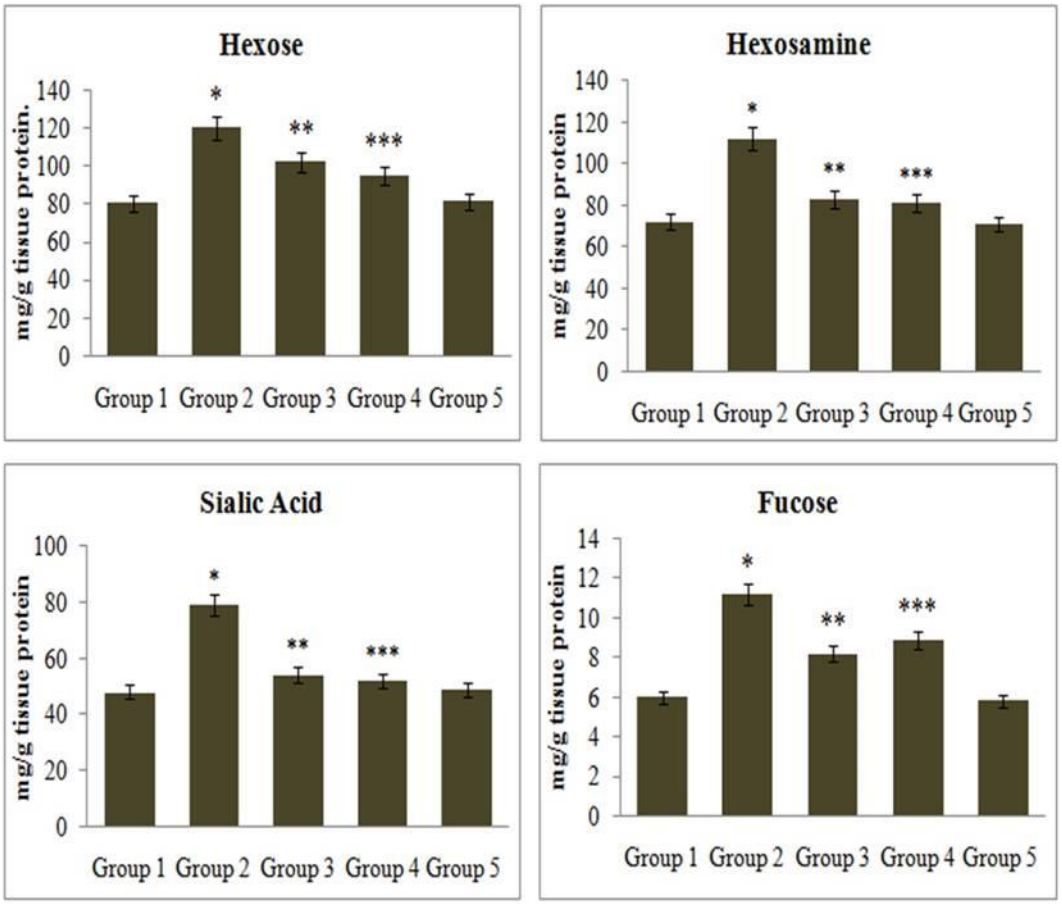


Table – 1: The Level of glycoconjugates such as protein bound hexose, hexosamine, total sialic acid and fucose in the plasma of control and experimental rats.

Glycoconjugates	Group 1	Group 2*	Group 3**	Group 4***	Group 5
	Control	4NOQ	4NQO+MVL	4NQO + Quercetin	MVL alone
Hexose	79.25±2.5	96.08±2.4	83.54±3.5	81.33±3.1	74.09±2.1
Hexosamine	74.12±1.9	91.18±1.4	81.16±1.2	80.09±0.9	73.25±1.4
Total sialic acid	43.21±2.1	61.55±3.1	54.2±1.1	51.31±1.8	41.22±2.6
Fucose	1.02±0.2	2.14±0.7	1.49±0.2	1.32±0.8	1.12±0.9

Results were expressed as mean ± S.D for six rats in each group. Statistical significance $p<0.05$ compared with *group 1, **group 2, and ***group 3 based on Duncan's multiple range test. Units: Hexose, hexosamine, total sialic acid and fucose units are expressed mg/dl protein.

Figure – 3: Showed the effect of MVL on tissue glycoprotein's like hexose, hexosamine, total sialic acid and fucose in the control and experimental groups. Results are expressed as mean ± S.D for six rats in each group. Statistical significance $p<0.05$ compared with *group 1, **group 2, and ***group 3 based on Duncan's multiple range test. Units: Hexose, hexosamine, total sialic acid and fucose units were expressed mg/g tissue protein.



Discussion

The anticancer properties of siddha medicine have been well recognized through many previous reports. The chemopreventive nature of these siddha medicines have not been well explored to its capacity and hence, this study focuses upon evaluating the chemopreventive ability of Maha Vellathy Lehyam (MVL), a siddha medicine [25]. This can be proved by analyzing the major mechanism of lipid peroxidation which exhilarates when a carcinogen is induced causing oxidative damage [26]. In this case, lipid peroxidation has been significantly increased due to the oral carcinogenesis caused by the carcinogen 4NQO which affected the lipid assembly in the cellular membrane, further exploiting the cellular membrane potential. This was further reported to cause a significant production of free radicals which will compromise the defense system of the cellular antioxidation [27]. Once MVL was administered, the LPO levels have been comparably decreased possibly due to free radical scavenging activity. The membrane of a cell plays a crucial role even at the times of malignancy by stimulating the adhesiveness of the cell, regulating its mortality followed by its proliferation [28, 29]. While the drug administration, it is important that the viability of these membranes remains intact without damaging them [30]. If the cell is under requirement of additional nutrition or is under pathological circumstances, the activity of the

membrane bound enzymes like Na+/K+ ATPase are highly responsible for the energy consumption of the cell [31]. The membrane permeability and the cell function are altered when the membrane bound enzyme loses its activity due to the peroxidation of the membrane lipids [32, 33]. This study has shown a considerably reduced activity of these membrane bound enzymes of the cells of 4NQO induced oral carcinogenic animals. Once the siddha derivative MVL was administered to these carcinogenic cells, the activity of this membrane bound enzymes have returned to their normal state indicating the scavenging property of MVL. A cell becomes malignant when there is an abnormal glycosylation occurring in the process [34]. Oral carcinogenesis often reports for such kind of uncharacteristic glycosylation and carbohydrate degradation present on cell surface [35]. At times of carcinogenesis, these glycoproteins are synthesized abnormally and eventually enter the blood stream [36]. There have also been reports revealing increased levels of plasma proteins during the tumorigenic conditions where the glycoconjugates are expressed in excess leading to shedding of them into the plasma. The *in vivo* studies of animal experiments revealed that the synthesis of glycoproteins has been elevated under malignancies that hold the strength to enter into the circulation. In this study, the serum was detected with increased levels of glycoconjugates in the oral carcinogenesis induced animals. These levels were reduced when MVL (50 mg/Kg b.wt.) was administered indicating the ability of this siddha derivative as a potential suppressor of malignancy where it successfully regulates the cellular alteration without abnormality and there-by protecting the cell wall. Further moleculer mechanisms of action of MVL are needed to be explored for the anticancer and chemopreventive effect. This study supports with the preliminary data as reports the chemopreventive effect of Maha Vellathy Lehyam for 4NQO induced oral carcinogenesis in rats.

Conclusions

The administration of MVL has caused a significant decrease in these aspects suggesting the nature of MVL against the carcinogenic nature of 4NQO by efficiently decreasing the oxidative damage.

Acknowledgments

I would also like to express my sincere and profound thanks to Dr. Selvaraj, Scientist and the management of Sathyabama Institute of Science and Technology, Centre for Laboratory animal technology and research, Chennai, India for the kind help and support to do animal research in this study.

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