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Inhibitory Effects of Maharishi-4 and Maharishi-5 on Microsomal Lipid Peroxidation

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DWIVEDI, C., H. M. SHARMA, S. DOBROWSKI AND F. N. ENGINEER. *Inhibitory effects of Maharishi-4 and Maharishi-5 on microsomal lipid peroxidation*. PHARMACOL BIOCHEM BEHAV **39**(3) 649–652, 1991.—The effects of Maharishi-4 (M-4) and Maharishi-5 (M-5) on microsomal lipid peroxidation were examined in vitro. Rat liver microsomes were incubated with an NADPH-generating system or with sodium ascorbate and an ADP-iron complex to stimulate enzymatic or nonenzymatic lipid peroxidation respectively. Alcoholic or aqueous extracts of M-4 or M-5, when added to these incubation systems, inhibited hepatic microsomal lipid peroxidation in a concentration-dependent manner. The aqueous extract of M-4 was the most effective antiperoxidant in these systems. A 10% (w/v) aqueous extract of M-4 inhibited ascorbate or NADPH-induced lipid peroxidation by approximately 50% when added at volumes of 8 μl and 3.5 μl respectively to the incubation mixtures (total incubation volume, 2 ml). These findings suggest that M-4 and M-5, by virtue of their antiperoxidant properties, may be useful in the treatment of free radical-linked drug toxicities and disease states.

Lipid peroxidation Free radicals Maharishi-4 Maharishi-5

THE food supplements, M-4 and M-5, have been shown to inhibit the growth of mammary tumors (16), retard platelet aggregation (14), enhance lymphoproliferative response (1), and modulate opiate receptors in the brain (7). The mechanism(s) underlying these diverse biological effects remain to be elucidated. It has been suggested earlier that M-4 and M-5 have antioxidative properties and may modulate oxygen-linked, free radical-mediated processes (5,16).

Although all aerobic organisms require oxygen for their survival, these organisms are also susceptible to damage by highly reactive, oxygen-derived free radicals (9,18). Over the past few years, free radical mediated reactions such as lipid peroxidation have received considerable attention, following the identification of these processes in various disease states (10–12,19) and in aging (8). Lipid peroxidation has been implicated in post-ischemic diseases (10), head injury and stroke (6), inflammation (10), and carcinogenesis (11,21). Moreover, the toxicity associated with chemicals/drugs such as CCl_4 (20), paraquat (13), ethanol (22), and Adriamycin (4,12) is reported to be linked to free radical generation and lipid peroxidation.

The purpose of this investigation was to evaluate the effects of M-4 and M-5 on rat liver microsomal lipid peroxidation in vitro. The results of this study may help to elucidate the mechanism of action responsible for the diverse effects of these food supplements. This study also suggests novel applications for M-4 and M-5 in the treatment of disease states and drug-induced toxicities associated with oxidative stress.

METHOD

M-4 and M-5 were obtained from Maharishi Ayurveda Products International (Stoneham, MA). The ingredients of M-4 and M-5 have been described earlier (15,16). ADP, NADP, sodium ascorbate, glucose-6-phosphate dehydrogenase and bovine serum albumin were purchased from the Sigma Chemical Co. (St. Louis, MO), and 2-thiobarbituric acid (TBA) was obtained from Eastman Organic Chemicals (Rochester, NY). Male Sprague-Dawley rats (SASCO Inc., Omaha, NE), weighing 190–240 g, were maintained on commercial rat chow (Wayne Pet Food Division, Chicago, IL) and fasted for 24 hours prior to being sacrificed. The livers were excised and placed in ice-cold 1.15% KCl buffer. Microsomes were prepared by differential centrifugation as previously described (3). The microsomal pellet was washed twice and resuspended in 1.15% KCl buffer. Microsomal protein was determined by the Biuret method.

Microsomal lipid peroxidation was induced enzymatically (NADPH-induced) or nonenzymatically (ascorbate-induced) essentially as described earlier (3). Microsomal suspensions (0.5 mg protein/ml) were incubated with a freshly prepared NADPH-generating system (glucose-6-phosphate, 5 mM; NADP, 0.3 mM; 0.5 units of D-glucose-6-phosphate dehydrogenase) in phosphate buffer (15 mM, pH 7.4). Total incubation volume was 2 ml. For induction of nonenzymatic lipid peroxidation, the NADPH-generating system was replaced by ascorbate (0.5 mM) and an ADP-iron complex (0.012 mM $FeCl_3$, 0.4 mM ADP). All incubations were carried out at 37°C for 15 minutes under aerobic conditions. Blanks without NADPH or ascorbate were included.

Aqueous and alcoholic extracts were prepared by adding 1 g M-5 or M-4 to 9 ml of solvent (water or alcohol). The mixture was vortexed for 2 minutes and centrifuged for 10 minutes. The supernatant was filtered, and the filtrate was added to the incu-

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bation systems at the indicated volumes. Zero-time blanks were included to determine interference with the TBA assay at 532 nm. The values of the zero-time blanks at different M-5 or M-4 concentrations were subtracted from the OD_{532} values for the corresponding incubations.

To determine the extracted mass for the M-4 and M-5 preparations, 1 ml of the aqueous extract was lyophilized, and 1 ml of the alcoholic extract was evaporated in a water bath. The residue was weighed. The extracted mass for the alcoholic M-4 and M-5 extracts was 33.4±4.5 mg/ml and 32.8±1.9 mg/ml respectively, and that for the aqueous extract of M-4 and M-5 was 74.3±2.3 mg/ml and 29.5±1.2 mg/ml respectively. Each value is the mean±S.D. of triplicate determinations. The experiments were repeated with two other batches of M-4 and M-5. The antiperoxidant property of M-4 and M-5 from the different batches was very similar.

The TBA assay for malonaldehyde (3) was used to measure lipid peroxidation. After the 15 min incubation period, 35% trichloroacetic acid (1 ml) and 1.5% 2-thiobarbituric acid (1 ml) were added. The mixture was placed in a water bath (90°C) for 20 min, cooled and then centrifuged. The supernatant was filtered, and the pink colored TBA-malonaldehyde product was spectrophotometrically measured at 532 nm. Increase in malonaldehyde levels was linear with respect to time for the 15 min incubation period. Lipid peroxidation was expressed as nanomoles of malonaldehyde per milligram protein. Malonaldehyde values at each point represent the mean±S.D. of 3–5 determinations. A molar extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ at 532 nm was used to calculate malonaldehyde values. The data obtained were evaluated by the Student *t*-test.

RESULTS

The 10% alcoholic or aqueous extract of M-4 inhibited ascorbate-induced lipid peroxidation in a concentration dependent manner (Fig. 1). The aqueous M-4 extract was a more effective antiperoxidant than the alcoholic M-4 extract under our experimental conditions. Incubations containing 10 μl of aqueous or alcoholic M-4 extract had 7.05±0.19 and 14.84±0.14 nmoles malonaldehyde/mg protein respectively. Each of these values is significantly lower ($p<0.05$) than the corresponding control values (Fig. 1). Volumes of 10% alcoholic or aqueous M-4 extracts required to reduce lipid peroxidation by 50% were approximately 25 μl and 8 μl respectively.

The aqueous or alcoholic extract of M-4 significantly inhibited ($p<0.05$) NADPH-induced microsomal lipid peroxidation at all concentrations tested (Fig. 2). 7 μl and 3.5 μl of the 10% M-4 alcoholic or aqueous extract respectively inhibited control malonaldehyde levels by about 50%. The alcoholic or aqueous M-4 extracts, when added at volumes of 20 μl and 25 μl respectively, virtually abolished (>99% inhibition) malonaldehyde formation (Fig. 2).

The 10% alcoholic extract of M-5 inhibited ascorbate-driven lipid peroxidation by approximately 50% and 95% when added at volumes of 20 μl and 50 μl to the incubation mixture (Fig. 3). The aqueous extract of M-5 was relatively less effective as an antiperoxidant and inhibited ascorbate-driven lipid peroxidation by only 31% when added at a volume of 50 μl to the incubation systems (Fig. 3). The alcoholic and aqueous M-5 extracts significantly inhibited ($p<0.05$) NADPH-induced hepatic lipid peroxidation at all concentrations tested (Fig. 4). Volumes of 10% alcoholic or aqueous M-5 extracts required to reduce malonaldehyde formation by 50% were approximately 3 μl and 7.5 μl respectively.

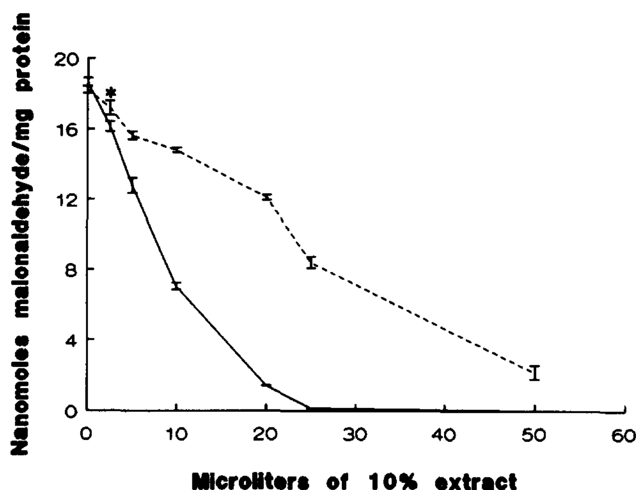

FIG. 1. Effect of M-4 (10% extract) on ascorbate-induced hepatic microsomal lipid peroxidation. The aqueous extract (—) or alcoholic extract (---) was added to the incubation mixture (total incubation volume = 2 ml) described in the Method section. Malonaldehyde values at each point represent the mean ± SD of 3–5 determinations. Values that are not significantly different ($p<0.05$) from the corresponding control value are marked with a * symbol.

DISCUSSION

The mechanism by which M-4 and M-5 inhibits the peroxidation of microsomal lipids is unclear. Analysis of different components of M-4 revealed the presence of various antioxidants (16). It is possible that antioxidants present in these food supplements may additively or synergistically inhibit lipid peroxidation. In the present study, the alcoholic or aqueous extracts of M-4 and M-5 were tested. Since no attempt was made to test

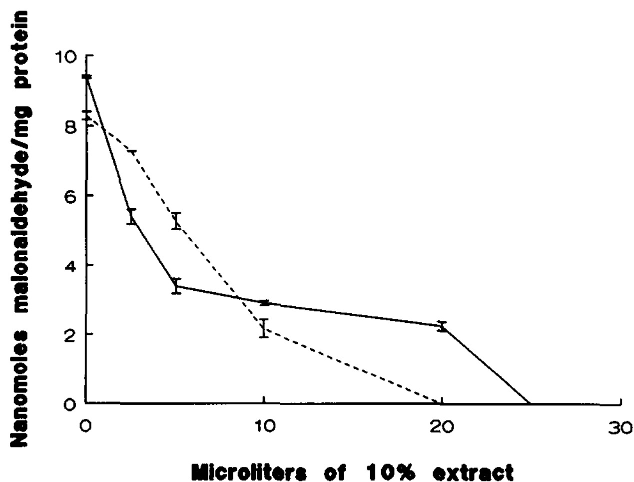

FIG. 2. Effect of M-4 (10% extract) on NADPH-stimulated hepatic microsomal lipid peroxidation. The aqueous extract (—) or alcoholic extract (---) was added to the incubation mixture (total incubation volume = 2 ml) described in the Method section. Malonaldehyde values at each point represent the mean ± SD of 3–5 determinations. Values that are not significantly different ($p<0.05$) from the corresponding control value are marked with a * symbol.

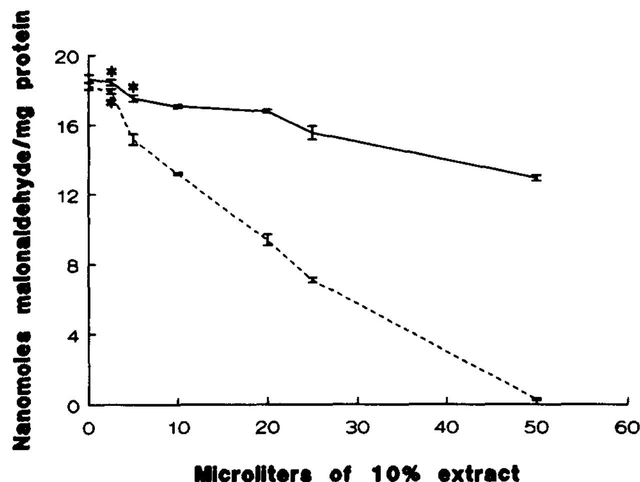

FIG. 3. Effect of M-5 (10% extract) on ascorbate-induced hepatic microsomal lipid peroxidation. The aqueous extract (—) or alcoholic extract (---) was added to the incubation mixture (total incubation volume = 2 ml) described in the Method section. Malonaldehyde values at each point represent the mean ± SD of 3–5 determinations. Values that are not significantly different ($p<0.05$) from the corresponding control value are marked with a * symbol.

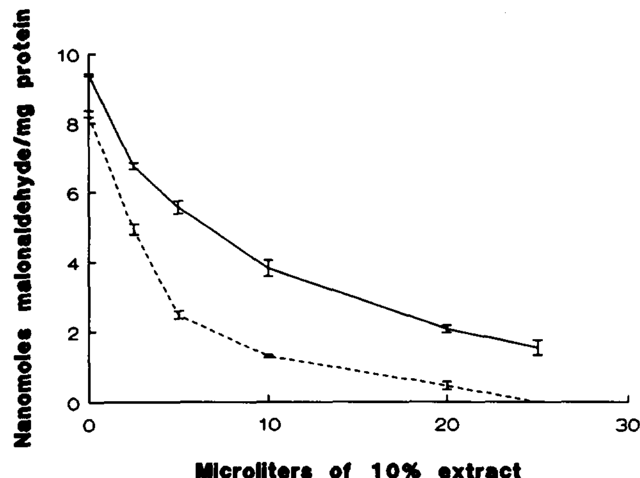

FIG. 4 Effect of M-5 (10% extract) on NADPH-stimulated hepatic microsomal lipid peroxidation. The aqueous extract (—) or alcoholic extract (---) was added to the incubation mixture (total incubation volume = 2 ml) described in the Method section. Malonaldehyde values at each point represent the mean ± SD of 3–5 determinations. Values that are not significantly different ($p<0.05$) from the corresponding control value are marked with a * symbol.

the individual components of M-4 and M-5, it is difficult to elucidate the exact mechanism responsible for the antiperoxidant effects observed. Although studies are underway to identify M-4 constituents responsible for the various biological effects exhibited by this compound, no conclusive information is presently available. It would be of interest to determine whether the M-4 and M-5 components necessary for the antineoplastic effects (2, 16, 17) are identical to those responsible for its antiperoxidative properties.

The present study demonstrates that M-4 and M-5 are effective inhibitors of lipid peroxidation, a free radical-mediated phenomenon (18). It seems plausible that M-4 and M-5 modulate free radical-dependent processes by quenching free radicals or by diverting electrons away from the biological pathway leading to lipid peroxide formation. Recent studies (16) have demonstrated the antineoplastic effects of M-4 against chemically induced mammary tumors in rats. Since free radicals and lipid peroxidation are involved in carcinogenesis (11,21), it is possible that the antiperoxidative properties of M-4 might play a role in the prevention of mammary tumor formation reported earlier (16). The effects of M-4 on free radical formation in in vitro systems are currently being investigated in our laboratory using electron spin resonance (ESR) spectroscopy. The results from the present study also suggest that M-4 and M-5 may be useful in the treatment of disease states and drug-induced toxicities associated with free radical production and lipid peroxidation. Preliminary studies in our laboratory indicate that M-4 inhibits Adriamycin-induced microsomal lipid peroxidation in vitro. Further studies are required to determine the in vivo significance of these observations and also to elucidate the mechanism of action of M-4 and M-5.

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REFERENCES

1. Dileepan, K. N.; Patel, V.; Sharma, H. M.; Stechschulte, D. J. Priming of splenic lymphocytes after ingestion of an Ayurvedic herbal food supplement: Evidence for an immunomodulatory effect. Biochem. Archiv. 6:267–274; 1990.
2. Dwivedi, C.; Sattar, B. C.; Sharma, H. M. Anticarcinogenic activity of an Ayurvedic food supplement, Maharishi Amrit Kalash (MAK). Pharmacologist 30:A121; 1988.
3. Engineer, F. N.; Sridhar, R. Inhibition of rat heart and liver microsomal lipid peroxidation by nifedipine. Biochem. Pharmacol. 38: 1279–1285; 1989.
4. Engineer, F. N.; Sridhar, R. Verapamil protects against Adriamycin-induced peroxidative injury to cardiac membranes in vitro and in vivo. Proc. Am. Assoc. Cancer Res. 31:442; 1990.
5. Fields, J. Z.; Rawal, P. A.; Hagen, J. F.; Todd, I.; Wallace, K.; Tomlinson, P. F.; Scheider, R. H. Oxygen free radical (OFR) scavenging effects of an anticarcinogenic natural product, Maharishi Amrit Kalash (MAK). Pharmacologist 32:A155; 1990.
6. Hall, E. D.; Braughler, J. M. Effect of intravenous methyl-prednisolone on spinal cord lipid peroxidation and Na+, K+-ATPase activity. Dose-response analysis during the first hour after contusion injury in the cat. J. Neurosurg. 57:247–253; 1982.
7. Hanissian, S. H.; Sharma, H. M.; Tejwani,G. A. Effect of Maharishi Amrit Kalask (MAK) on brain opioid receptors. FASEB J. 2:A433; 1988.
8. Harman, D. The free-radical theory of aging. In: Pryor, W. A., ed. Free radicals in biology. vol. 5. New York: Academic Press; 1982: 255–275.
9. Hess, M. L.; Manson, N. H. Molecular oxygen: friend and foe. The role of the oxygen free radical system in the calcium paradox, the oxygen paradox and ischemia/reperfusion injury. J. Mol. Cell. Cardiol. 16:969–985; 1984.
10. McCord, J. M. Superoxide radical: A likely link between reperfusion injury and inflammation. Adv. Free Radical Biol. Med. 2:325–345; 1986.
11. Marks, F.; Furstenberger, G. Tumor promotion in skin: Are active oxygen species involved? In: Sies, H., ed. Oxidative stress. New

York: Academic Press; 1985:437–475.
12. Myers, C. E.; McGuire, W. P.; Liss, R. H.; Ifrim, I.; Grotzinger, K.; Young, R. O. Adriamycin: The role of lipid peroxidation in cardiac toxicity and tumor response. Science 197:165–167; 1977.
13. Saito, M.; Thomas, C. E.; Aust, S. D. Paraquat and ferritin-dependent lipid peroxidation. J. Free Radicals Biol. Med. 1:179–185; 1985.
14. Sharma, H. M.; Panganamala, R. V. Inhibition of human platelet aggregation in vitro by Maharishi Amrit Kalash (MAK). FASEB J. 2:A400; 1988.
15. Sharma, H. M.; Dwivedi, C.; Satter, B. C.; Abou-Issa, H. Antineoplastic properties of Maharishi Amrit Kalash, an Ayurvedic food supplement, against 7,12-dimethylbenz(a)anthracene-induced mammary tumors in rats. J. Res. Ed. Ind. Med., in press; 1990.
16. Sharma, H. M.; Dwivedi, C.; Satter, B. C.; Gudehithlu, K. P.; Abou-Issa, H.; Malarkey, W.; Tejwani, G. A. Antineoplastic properties of Maharishi-4 against DMBA-induced mammary tumors in rats. Pharmacol. Biochem. Behav. 35:767–773; 1990.
17. Sharma, H. M.; Krieger, J.; Dwivedi, C. Antineoplastic properties of dietary Maharishi-4 and Maharishi Amrit Kalash, Ayurvedic food supplements. Eur. J. Pharmacol. 183:193; 1990.
18. Sies, H. Oxidative stress: Introductory remarks. In: Sies, H., ed. Oxidative stress. New York: Academic Press; 1985:1–10.
19. Singal, P. K.; Kirshenbaum, L. A. A relative deficit in antioxidant reserve may contribute in cardiac failure. Can. J. Cardiol. 6:47–49; 1990.
20. Slater, T. F.; Sawyer, B. C. The stimulatory effects of carbon tetrachloride and other halogenoalkanes on peroxidative reactions in rat liver fractions in vitro. Biochem. J. 123:805–814; 1971.
21. Ts'o, P. O.; Caspary, W. J.; Lorentzen, R. J. The involvement of free radicals in chemical carcinogenesis. In: Pryor, W. A., ed. Free radicals in biology. vol. 3. New York: Academic Press; 1977:251–303.
22. Younes, M.; Wagner, H.; Strubelt, O. Enhancement of acute ethanol hepatotoxicity under conditions of low oxygen supply and ischemia/reperfusion. The role of oxygen radicals. Biochem. Pharmacol. 38:3573–3581; 1989.