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Nutritional Value of *Ganoderma* Extract and Assessment of its Genotoxicity and Antigenotoxicity using Comet Assays of Mouse Lymphocytes

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Abstract—The nutritive composition of a hot aqueous extract of wild *Ganoderma* fruit bodies was determined. This extract was assessed for cytotoxicity and *in vivo* genotoxicity by both acute and subchronic exposure of mice (given by mouth at a dose equivalent to extract of 220 g fresh *Ganoderma* fruit body/kg body weight). To test any alleged protection against mutagens by *Ganoderma* treatments, the mice were injected intraperitoneally with the radiomimetic mutagen ethyl methanesulfonate (EMS), and after 24 hr of treatment their lymphocytes were examined using the comet assay. *Ganoderma* extract consisted of Folin-positive material (68.9% of dry weight), but protein comprised only 7.3% of dry weight. Glucose accounted for 11.1% and metals 10.2% of dry weight (K, Mg and Ca being the major components with Ge (often touted as being of value in sales literature for *Ganoderma* preparations) having the fifth highest metal concentration at 489 μg/g). In comparison to rodent chow, *Ganoderma* extract was a modest dietary supplement. No evidence was found for genotoxic chromosomal breakage nor cytotoxic effects by *Ganoderma* extract in the mouse, nor did it protect against the effects of ethyl methanesulfonate. We found no support in this study for the extract having any value in protecting against the test mutagen. © *2000 Elsevier Science Ltd. All rights reserved*

Keywords: genotoxicity; lingzhi; single-cell gel electrophoresis; nutraceutical; *Ganoderma*; comet assay.

Abbreviations: AOAC = Association of Official Analytical Chemists; DMSO = dimethyl sulfoxide; EMS = ethyl methanesulfonate ; GOT = glutamic–oxaloacetic transaminase; GPT = glutamic–pyruvic transaminase; HBSS = Hanks' balanced salt solution; PBS = phosphate buffered saline.

INTRODUCTION

Ganoderma is used extensively in traditional Chinese medicine as ''the mushroom of immortality''. Among cultivated mushrooms, *Ganoderma* is unique in being consumed for its pharmaceutical value rather than as a food. Under the names linzhi or reishi, several *Ganoderma* species of the *G. lucidum* complex provide various commercial brands of nutraceuticals, in the form of health drinks, powders and dietary supplements as well as specific functional agents as mycelial or fruit body extracts, exopolysaccharides, spores, etc. (Jong and Birmingham, 1992; Ying *et al*., 1989). Dietary supplement sale in the United States was over 6.5 billion US dollars in 1996 (http://vm-cfsan.fda.gov). In 1997, the worldwide production of *Ganoderma* was approx. 4300 tonnes, of which China contributed 3000 tonnes (see: http://www.kyotan.com). Traditionally, *Ganoderma* is highly regarded as a herbal medicine which is claimed to alleviate or cure virtually all diseases, and its popularity in China has spread to other Asian countries as well as the wider world (Anonymous, 1998; Stamets, 1993; Zhu *et al*., 1994; see website: http://www.kyotan.com). Current research is focused on purification and characterization of the bioactive components and determination of clinical value, es-

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pecially putative anti-tumour and anti-ageing properties.

Anti-tumour effects may result from immunomodulation by β-glucans or immunosuppressive proteins (Lin *et al.*, 1997; Wang *et al.*, 1997); inhibition of DNA polymerase (Mizushina *et al.*, 1998a,b) or inhibition of post-translational modification of oncoproteins (Lee *et al.*, 1998). The anti-ageing property may depend on inhibition of the cell cycle and apoptosis (Gan *et al.*, 1998) or enhanced mutation repair (Chen *et al.*, 1995; Liu, 1998). Scavenging of free radicals (Barber and Harris, 1994; Stavric, 1994) and/or content of transition metals, especially germanium (Gerber and Leonard, 1997; Lee *et al.*, 1998; Liu *et al.*, 1990; Schimmer *et al.*, 1997), have also been suggested as providing anti-mutagenic and/or anti-carcinogenic properties in *Ganoderma* products.

These recent studies indicate that specific clinically useful activities may be identified eventually, but at the moment almost all *Ganoderma* products in use are complex mixtures or extracts which may contain toxins (e.g. heavy metals such as Hg or Cd; Lin, 1997) as well as useful components. Further, the nature of the traditional remedies concerned ('control' of ageing, disease 'prophylaxis') ensures that users will experience chronic exposure to any such toxins. As the most prized *Ganoderma* are collected from the wild, there is a risk of contamination by heavy metals from the environment which macrofungi readily accumulate (Chiu *et al.*, 1998; Tyler, 1980), by toxins from spoilage microbes and by pathogenic microbes. Yet we have found no recently-published toxicological assays or assessments of the safety of *Ganoderma* products.

In this study, we analysed the nutritive composition of wild *Ganoderma* fruit bodies, and assessed cytotoxicity and *in vivo* genotoxicity of both acute and subchronic exposure in mice (Scassellati-Sforzolini *et al.*, 1997). We also searched for any protective effect against the radiomimetic mutagen ethyl methanesulfonate (EMS) using the comet assay (also called the single-cell gel assay) which directly visualizes DNA damage in individual cells (Böcker *et al.*, 1997; Fairbairn *et al.*, 1995; Hartmann and Speit, 1997; Klaude *et al.*, 1996; McCarthy *et al.*, 1997). Such an assay has gained popularity for environmental biomonitoring, food and nutraceutical hazard analysis, DNA repair and genotoxicity testing and study of apoptosis (Gabelova *et al.*, 1997; Gichner and Plewa, 1998; Green *et al.*, 1994; Mitchelmore and Chipman, 1998; Ralph and Petras, 1998; Sasaki *et al.*, 1997).

MATERIALS AND METHODS

Ganoderma fruit bodies were collected on the campus of the Chinese University of Hong Kong which is located in a rural area. Inner tissues were excised, freeze-dried and ground into 0.5–1 cm pieces. Approximately 1 g freeze-dried powdered extract was obtained from 20 g freeze-dried fruit body tissue. 50 ml of autoclaved (20 min at 121°C), filtered extract was obtained from 100 g *Ganoderma* freeze-dried fruit body fragments.

After ashing according to AOAC (1990), the metal content of the *Ganoderma* extract was determined with inductively coupled plasma (ICP) spectrometry (Atomscan 16 sequential Plasma Spectrometer, Thermo Jarrell Ash) or with atomic absorption spectrophotometry (Hitachi model Z-82000 series polarized Zeeman atomic absorption spectrophotometer). All glass and plastic wares used in metal determinations were acid-treated prior to use.

Commercial kits were used to assay protein (in crude extract to estimate total Folin-positive material, and then after precipitation with trichloroacetic acid to determine high molecular weight protein) and glucose in extract or serum (determined using a hexokinase/glucose-6-phosphate dehydrogenase kit).

Male ICR mice in the weight range 27–30 g were divided into groups of five to 10 individuals and fasted overnight. The treatment group was administered *Ganoderma* extract solution orally at a dose equivalent to 0.9259 g of the freeze-dried extract/kg body weight, which is equivalent to the consumption of half a *Ganoderma* fruit/kg body weight (a dosage recommended in many commercial *Ganoderma* concentrate extract products). Control groups were given an equal volume of ultrapure water. For testing acute toxicity this was a once-only treatment. For subchronic toxicity tests the animals were fed with *Ganoderma* extract (as described above) or with ultrapure water daily for 2 wk. Blood samples were drawn by syringe from the posterior vena cava for further analysis. Serum glutamic–pyruvic transaminase (GPT) and glutamic–oxaloacetatic transaminase (GOT) levels and serum urea nitrogen were assayed using commercial kits (Sigma 505 and Sigma BUN (endpoint) kits, respectively).

For the acute toxicity assay, body weight and food consumption were recorded daily during the 2 wk following treatments with *Ganoderma* extract, to monitor the general health of the animals. After the experimental period all mice were sacrificed by ether inhalation. For the subchronic toxicity assay half the animals in each group were sacrificed after the 2 wk of *Ganoderma* extract treatment. Autopsies were performed; fresh weights of heart, kidney, liver, lung and spleen were measured and sera were collected for further analyses as mentioned above. The other half of each group was observed for 2 more weeks, then the mice were sacrificed for autopsy as before.

Cytotoxicity was examined in organ samples (liver, kidney and spleen) fixed in Bouin's fluid and embedded in paraffin wax. Sections 7 μm thick were

stained with haematoxylin and eosin for microscopy.

Four groups of five mice each were used to examine *in vivo* genotoxicity using the comet assay. Following overnight fasting, two groups were administrated ultrapure water orally and two groups were administrated the *Ganoderma* extract solution for 2 wk (dose equivalent to 0.9259 g freeze-dried extract/kg body weight per mouse). For the positive control group and one of the *Ganoderma* fed groups, on day 15, an ip injection of the mutagen EMS was administered, the dose being 300 mg EMS/kg body weight per mouse). The negative control group received an ultrapure water injection of the same volume. After 12 hr, blood was sampled and examined as before.

The comet cell assay procedure followed Klaude *et al*. (1996) and Scassellati-Sforzolini *et al*. (1997) in combination with the manufacturer's instruction manual and website (http://www.kineticimaging.com). Blood was collected from the cut tails of mice from the control and treatment groups. 10 μl mouse blood was suspended in 540 μl Hanks' balanced salt solution (HBSS) with 20 mM EDTA in HBSS buffer, Ca^{2+} and Mg^{2+}- free; pH 7.2) (composition (g/litre): KCl, 0.4; NaCl, 8; $NaHCO_3$, 0.35; Na_2HPO_4, 0.048; KH_2PO_4, 0.06; D-glucose, 1; EDTA, 7.44). After thorough mixing, the solution was centrifuged at 1800 ***g*** for 3 min. The pellet of lymphocytes was resuspended in fresh HBSS buffer. Washing was repeated once. After adding 100 μl HBSS buffer to the pellet, 60 μl suspension was pipetted out and added to 60 μl melted 1.5% low melting agarose in calcium and magnesium free phosphate buffered saline (PBS) kept at 45°C. 120 μl 1% conventional agarose in PBS was layered onto a fully frosted slide on ice and then covered by a coverslip to solidify. Once the coating was set and the coverslip was removed, the blood cell agarose suspension was pipetted onto the slide with a coverslip to help spreading. When the agarose had set, the whole slide was immersed in freshly prepared cold lysing solution [2.5 M NaCl, 100 mM EDTA, 10 mM Tris, (pH 10), 1% sodium sarcosinate, 1% Triton X-100 and 10% dimethyl sulfoxide (DMSO)] for 2 hr at 4°C in darkness. Then the slide was placed in a horizontal gel electrophoresis chamber containing buffer [300 mM NaOH, 2% DMSO (v/v), 0.1% 8-hydroxyquinoline and 10 mM EDTA (pH 10.0)] and an electric field of 25 V and 300 mA was set and run for 20 min. Drops of neutralizing buffer [0.4 M Tris (pH 7.5)] were added to neutralize the excess alkali. After fixing with absolute ethanol for 5 min, the slides were stained in 2 g ethidium bromide/ml.

Nuclei of 50 lymphocytes per slide per sample were examined at random at 200-fold magnification using an epi-fluorescence microscope with a green filter, an excitation filter of 515–560 nm and a barrier filter of 590 nm. Quantification used a CCD camera and Komet ver. 3.1 image analysis (Kinetic Imaging Ltd, Liverpool, UK).

RESULTS AND DISCUSSION

Crude chemical analysis of the *Ganoderma* extract showed that the bulk of the freeze-dried material consisted of Folin-positive material which amounted to 68.9% of its dry weight. Much of this was low molecular weight since TCA-precipitable protein comprised only 7.3% of the overall dry weight. This distribution of Folin-positive material might be due to the extraction method. Glucose accounted for a further 11.1% of the dry weight. Metals contributed a further 10.2% (101,948 μg/g; Table 1), potassium, magnesium and calcium being the major components with Ge being the fifth highest metal concentration (489 μg/g). The other metals, including Pb, Ni and Zn, were at levels slightly higher than those encountered in common foods (Ybanez and Montoro, 1996). Significantly, bearing in mind that these samples were collected from nature, the metals Cd, Hg, Li, Se and Ti were not detected, suggesting the collection site may be free of contamination. Apart from higher contents of Ge and Na, the amounts of metals determined here generally agree with those reported in the literature (Chen *et al*., 1998; Lin, 1997; Liu *et al*., 1990).

Comparing the nutrient content of rodent chow (supplied by Streges Bates, Ridley Agri product, Australia) and water with *Ganoderma* extract indicates that the latter is a minor contributor to the nutrition of the animals. During the treatment period, the food consumed by mice in the control (with fasting) group was calculated as a fraction of 0.157 of the animals' weight while that for the treatment group was 0.145. For a 30 g 'average' mouse the nutrient intake (rodent chow *vs* *Ganoderma* extract) was: crude protein, 894 mg *vs* 20 mg; glucose, 9 mg *vs* 31 mg; K, 43.4 mg *vs* 23.5 mg; Mg, 10.3 mg *vs* 1.2 mg; Na, 14.8 mg *vs* 0.5 mg; Fe, 751 μg *vs* 32 μg; Zn, 360 μg *vs* 71 μg; Mn, 358 μg *vs* 50 μg; Cu, 74 μg *vs* 13 μg; Co, 2.9 μg *vs* 8.6 μg; Cr, 12 μg *vs* 19 μg. The *Ganoderma* extract supplied marginally more cobalt and chromium than normal food and about three times as much glucose. However, in rodent chow the polysaccharide con-

Table 1. Mineral assays of *Ganoderma* extract

K	84650	Ni	133
Ca	9449	Pb	86
Mg	4480	Cr	69
Na	1612	Bi	71
Ge	489	Cu	47
Zn	257	Co	31
Ga	246	Sr	28
Mn	179	Be	4
Fe	115	Ba	2

Entries shown as μg/g freeze-dried *Ganoderma* extract powder; averages of two determinations. Metal ions determined with inductively coupled plasma spectrometry or atomic absorption spectroscopy. Cd, Hg, Se, Ti and Li not detected.

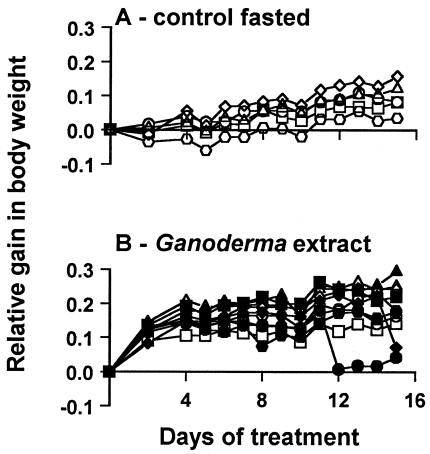

Fig. 1. The effect of *Ganoderma* extract on the relative body weight gain of mouse. Comparison of the body weight change is standardized by a relative scale to the initial body weight of each mouse. (A) control group, (B) treatment group.

tent was 2.5% (predominantly starch) and this calculates to approximately 114 mg/mouse so the normal food supplied much more carbohydrate as well. For the most part, therefore, *Ganoderma* extract was a modest dietary supplement.

The germanium content of *Ganoderma* extract is notable because *Ganoderma* extract is claimed, like ginseng, to contain a comparatively high content of Ge (Anonymous, 1998; Liu *et al*., 1990), and this metal has been associated with antimutagenic, immunomodulatory, antioxidant and antitumour effects (Kolesnikova *et al*., 1997; Lee *et al*., 1998; Schimmer *et al*., 1997). Although prolonged intake can lead to renal failure and injury to other organs (Shamir and Sprung, 1997; Tao and Bolger, 1997), Ge is regarded as an element of rather low risk to man (Gerber and Leonard, 1997) and could be an important contributor to any nutraceutical activity. However, our results shown below do not support any contention that protection is offered against the effects of a test mutagen.

No morbidity or abnormal behaviour was found in any treatment group 1 day after administration of the *Ganoderma* extract. Besides, the serum GOT, GPT and urea nitrogen contents were comparable to those of the control group (data not shown), so there was no indication of acute toxicity. Body weight increased steadily after the day of administration of the concentrate, but no abnormal behaviour or distinctive clinical signs were observed in any group (treatment or control) over the next 14 days and all mice lived to the end of the study (2 months).

For the subchronic toxicity assays, *Ganoderma* extract stimulated the initial body weight gain in comparison to the fasted control (Fig. 1). Yet in the 2 wk treatment time, body weight gained over the initial weight of individual animals was variable. Two animals among 10 in the treatment group

Table 2. The effect of *Ganoderma* extract on organ to body weight ratios and serum biochemistry (glucose, urea nitrogen, GOT and GPT) of mice

	Serum properties				Organ to body weight ratio			
	Urea nitrogen (mg/ml)	Glucose (mg/ml)	GOT (SF unit/ml)	GPT (SF unit/ml)	liver	kidney	spleen	heart	lung
					Treatment period				
Control	0.221 ± 0.025	0.87 ± 0.29	100 ± 38	37 ± 11	0.058 ± 0.004	0.018 ± 0.001	0.003*	0.005*	0.007*
Ganoderma extract	0.190 ± 0.010	1.46 ± 0.46	211 ± 26	69 ± 40	0.057 ± 0.005	0.017*	0.003*	0.005*	0.007*
					Post-treatment period				
Control	0.207 ± 0.020	2.84 ± 0.51	95 ± 23	39 ± 7	0.059 ± 0.004	0.015 ± 0.001	0.003*	0.005*	0.006*
Ganoderma extract	0.181 ± 0.028	3.13 ± 0.39	108 ± 30	30 ± 8	0.054 ± 0.003	0.017 ± 0.001	0.004 ± 0.001	0.005*	0.007 ± 0.001

Data presented as mean ± SE, n = 5 (* = SE is zero). Enhanced GOT during treatment may be a stress response; GOT:GPT ratios not significantly different. In the post-treatment period one mouse had an enlarged spleen, there was no significant difference between the groups.

Table 3. The *in vivo* genotoxicity assays of *Ganoderma* extract and detection of anti-mutagenic effects against ethyl methanesulfonate (EMS) using comet assay of mouse lymphocytes

Treatment	Fluorescence ratio (head: tail)	Tail extent moment	Head mode	Olive tail moment	Comet skewness
Control	7.98 ± 1.01	6.93 ± 0.59	134.57 ± 1.00	3.13 ± 0.39	2.89 ± 0.27
Ganoderma extract	9.65 ± 1.89	6.33 ± 1.23	134.96 ± 3.44	3.09 ± 0.36	3.14 ± 0.18
EMS alone	2.19 ± 0.21	22.97 ± 1.53	160.89 ± 5.35	7.90 ± 1.86	1.65 ± 0.16
EMS plus *Ganoderma* extract	2.48 ± 1.30	23.58 ± 9.67	151.39 ± 1.65	7.54 ± 2.51	1.56 ± 0.57

Data presented as mean ± SE, n = 5. The decrease in comet skewness and fluorescence ratio (head: tail) in EMS and EMS + *Ganoderma* treatments indicates a more even spread of DNA between head and tail (i.e. the treatments fragment the DNA). The increase in head mode in EMS and EMS + *Ganoderma* treatments shows that the diameter of the head was increased (i.e. DNA spread to a larger area as well as leading to the formation and appearance of the comet tail). Increases in tail extent moment and olive tail moment in EMS and EMS + *Ganoderma* treatments are measures of the increase in relative amount of DNA in the tail compared with the head. Overall, *Ganoderma* extract caused no detectable genotoxic effects itself, nor did it protect the animals against the genotoxic effects of EMS.

showed loss in body weight in the later period of the treatment, but overall there was no significant difference between treatment and control groups (Fig. 1). During the treatment period, when animals were fed with *Ganoderma* extract and the post-treatment period, marginally less food was consumed by the group which was given *Ganoderma* extract (on a per unit body weight basis the fraction was 0.157 for the control (fasted) *vs* 0.145 for the treated group during treatment and 0.175 *vs* 0.155 after treatment). Body weight gain was also essentially uniform in the 14-day post-treatment period. The only exception was one mouse which developed an enlarged spleen and lost body weight during the treatment period. However, the weight loss was transient, and even this animal recorded the largest body weight gain in the post-treatment period. The enlarged spleen seemed to be cytologically normal.

Post-mortem analyses revealed no consistent abnormalities in organ weight or organ/body weight ratios. GOT, GPT activities and their ratios were within normal ranges throughout. Although absolute values of these enzyme activities were elevated during administration of *Ganoderma* extract (Table 2), this was probably a transient stress response because the levels were still low compared with those associated with pathologies (e.g. acetaminophen caused GPT and GOT levels to increase to 2000–3000 units/ml; Kroger *et al*., 1997) and returned to normal in the post-treatment period. Low urea nitrogen contents are consistent with freedom from kidney and liver damage. Similarly, histological sections of liver and kidney revealed no abnormalities in tissue cells between the control group (fasted) and the group given *Ganoderma* extract.

The only abnormalities seen throughout the whole study were in mice injected with EMS. Those given EMS and EMS + *Ganoderma* extract were moribund and slow in reaction. In histological sections of both these groups the liver appeared normal but there was evidence of bleeding and necrosis in the kidney (data not shown). Evidently, *Ganoderma* extract offers no protection against the cytopathological effects of EMS.

In the *in vivo* comet assays for genotoxicity in mouse lymphocytes, control (fasted) and *Ganoderma* extract (fasted) were similar to each other, and EMS (injected) and *Ganoderma* extract fed for 2 wk before EMS injection were also similar to each other on all criteria (Table 3). All parameters indicate that more DNA breakage occurred following EMS administration but there was no significant difference between the control group and animals given only *Ganoderma* extract. Thus, *Ganoderma* extract does not show any genotoxic effect detectable by this assay method, nor does *Ganoderma* extract provide any protection against the genotoxicity of EMS.

These experimental results have revealed no cause for concern over the safety of *Ganoderma* extract. No evidence was found for genotoxic chromosomal breakage (Table 3) or cytotoxic effects in the mouse test. The soluble N content including protein and high content of essential minerals such as K, Mg and Ca, and even the content of Ge, may recommend *Ganoderma* extract as a nutraceutical. *Ganoderma* extract did not protect against the radiomimetic mutagen EMS so we find no support for its alleged value in ameliorating cancer or ageing through such a mechanism.

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