Antimutagenic activity of methanolic extract of *Ganoderma lucidum* and its effect on hepatic damage caused by benzo[a]pyrene

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Abstract

The antimutagenic activity of the methanolic extract of the fruiting bodies of *Ganoderma lucidum* (Fr.) P. Krast. occurring in South India was investigated. The activity was assayed by Ames *Salmonella* mutagenicity test using histidine mutants of *Salmonella typhimurium* tester strains, TA98, TA100 and TA102. The methanolic extract of the mushroom significantly inhibited \((P < 0.001)\) the in vitro sodium azide (NaN₃), \(N\)-methyl-\(N\)′-nitro-\(N\) nitrosoguanidine (MNNG) and 4-nitro-\(o\)-phenylenediamine (NPD), and benzo[a]pyrene (B[a]P) induced his\(^+\) revertants in a dose dependent manner. In vivo antimutagenic activity of extract was also assayed by determining the mutagenicity of the urine of rats administrated with B[a]P as a mutagen. The prior administration of extract markedly inhibited mutagenicity induced by B[a]P. The results indicated that the methanolic extract of *Ganoderma lucidum* occurring in South India possessed significant antimutagenic activity. The effect of B[a]P on hepatic enzymes, such as serum glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT) and alkaline phosphatase (ALP), were also evaluated. The extract prevented the increase of SGOT, SGPT, and ALP activities consequent to B[a]P challenge, and enhanced the levels of reduced glutathione (GSH) and activities of glutathione peroxidase (GPx), glutathione-S-transferase (GST), superoxide dismutase (SOD), and catalase (CAT). The extract also profoundly inhibited lipid peroxidation induced by B[a]P. The results revealed that *Ganoderma lucidum* extract restored antioxidant defense and prevented hepatic damage consequent to the challenge by B[a]P.

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Keywords: Medicinal mushroom; *Ganoderma lucidum*; Antimutagenicity; Antioxidant

1. Introduction

The human body is continuously and unavoidably exposed to a plethora of structurally diverse chemicals with established carcinogenic activity in animal models and/or mutagenic activity in short-term tests (Maron and Ames, 1983). A characteristic of the major classes of chemical carcinogens, namely polycyclicaromatic hydrocarbons, heterocyclic amines, and aromatic amines, are that in order to express their genotoxicity and carcinogenicity, they must be metabolized to reactive intermediates that are capable of interacting covalently with DNA (Weisberger, 1999). Damage to DNA is likely to be a major cause of cancer and other diseases. Hopefully the genotoxic effects of toxicants can be minimized by modulation of the physiological detoxification. Many naturally occurring compounds with antioxidant activity are known to protect cellular components from oxidative damage and prevent diseases (Ferguson, 1994). A number of such compounds can activate the phase II detoxification enzymes, which can remove the toxic elements from the system. Exposure to such phytochemicals is therefore beneficial to human health. A considerable emphasis is being placed on the use of dietary constituents to prevent mutagenesis and carcinogenesis due to their relative non-toxic effects (Stranic, 1994).

Chemoprevention aimed at inhibiting or delaying the onset of carcinogenesis is a rapidly growing area of cancer research. There has been growing interest in the identification of naturally occurring dietary factors as potential anticarcinogens (Namiki et al., 1986). The identification of such dietary components and definition of their antitumor effects could lead to strategies for reducing the risk of human cancer (Gao et al., 2004). Mushrooms are known to possess significant medicinal properties. They have been used in folk medicine throughout the world since ancient times. *Ganoderma lucidum* (Fr.) P. Krast. has been used for thousands of years in traditional oriental medicine and this medic-
inal mushroom has been considered as a panacea in Chinese medicine (Jong and Birmingham, 1992). Our earlier investigation have demonstrated that the fruiting bodies of *Ganoderma lucidum* occurring in South India possessed significant antioxidant, antiinflammatory, antinociceptive and antitumor activities. (Jones and Janardhanan, 2000; Sheena et al., 2003). In this study, the antimutagenic properties of the methanolic extract of this mushroom and its effect on the prevention of hepatic damage caused by benz[a]pyrene, was examined.

2. Materials and methods

2.1. Experimental material

Fruiting bodies of *Ganoderma lucidum* growing on *Delonix regia* Raf. were collected on 15 September, 2000 from the adjoining areas of Thrissur district, Kerala, South India. The specimen was identified by Prof. K.M. Leelavathi, Department of Botony, Calicut University, Calicut, Kerala, India. The type specimen was deposited in the Herbarium of Centre for Advanced Studies in Botony, University of Madras, Chennai, India (HERB. MUBL 3172).

2.2. Chemicals

Glucose-6-phosphate, L-histidine, d-biotin were purchased from Sisco Research Laboratories, Mumbai. Benzo[a]pyrene (B[a]P), 4-nitro-o-phenylene diamine (NPD) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) were purchased from Sigma Chemicals, St. Louis, USA. Amberlite XAD-4 was purchased from Lancaster Synthesis, England. Sodium azide (NaN₃) was purchased from Hi-Media, Mumbai, India. All other chemicals employed in the studies were of analytical reagent grade.

2.3. Bacterial strain

Histidine requiring strains of *Salmonella typhimurium* TA98, TA100 and TA102 were kindly supplied by Prof B.N. Ames, University of California, Berkeley, USA. They were incubated in nutrient broth for 12 h and frozen permanents were prepared by freezing at −70 °C in the presence of 9% dimethyl sulfoxide (DMSO). Fresh cultures were prepared by inoculating 40 ml of frozen permanents in 5 ml of nutrient broth and incubated for 12 h at 37 °C. The cultures thus obtained were used for the experiments.

2.4. Preparation of mutagens

All of the chemical mutagens were dissolved in DMSO except sodium azide, which was dissolved in water.

2.5. Preparation of extract

Fruiting bodies of mushrooms were dried at 45–50 °C for 48 h and powdered. The powdered material (2 Kg) was extracted with petroleum ether in a Soxhlet apparatus for 8–10 h (Suffness and Douros, 1979). The extraction was done in four batches of 500 gm each. The defatted material was then extracted with hot methanol:water (70:30) at 70–80 °C twice. Methanolic extracts were pooled, concentrated and evaporated under vacuum. The extract thus obtained (40.8 gm) was used for the experiments.

2.6. Chemical analysis of the extract

Preliminary chemical analysis of the extract was carried out to determine its major chemical constituents. The extract was tested for reaction with anthrone reagent (Yemm and Wills, 1954) and also the phenol–sulphuric acid reaction (Dubois et al., 1956), for detecting polysaccharide components. Thin layer chromatographic (TLC) analysis of extract was carried out on silica gel G using chloroform:methanol (90:10) as solvent system. The TLC plates were sprayed with vanillin–H₂SO₄ reagent or alcoholic FeCl₃ for detecting terpenes and phenolic compounds.

2.7. Animals

Male Wistar rats (190 ± 10 g) were purchased from the Small Animal Breeding Centre, Kerala Agricultural University, Mannuthy, Thrissur, Kerala, India. They were housed in well-ventilated cages and fed with standard pelleted diet (Sai Durga Agencies, Bangalore, India) and kept at air-controlled rooms. All animal experiments were conducted according to guidelines and following the approval of the Institutional Animal Ethical Committee. Three groups, consisting of six animals each were used for the study.

2.8. Determination of in vitro antimutagenicity

2.8.1. Direct acting mutagens

Antimutagenicity of *Ganoderma lucidum* extract against direct acting mutagens was determined according to the methods of Maron and Ames (1983). For this 2 ml of top agar containing 0.2 ml of 0.5 mM histidine–biotin was mixed with mutagens (NaN₃, MNNG, or NPD), at a concentration given in the Tables 1 and 2. Different concentrations of *Ganoderma lucidum* extract (3, 2, or 1 mg) dissolved in DMSO and 0.1 ml freshly grown *typhimurium* culture (1 × 10⁸ cells/ml approximately) were poured onto minimal agar plates and incubated at 37 °C for 48 h. After the incubation, the revertant colonies were counted using a colony counter.

<table>
<thead>
<tr>
<th>Table 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effect of the methanolic extract of <em>Ganoderma lucidum</em> (GL) to <em>Salmonella typhimurium</em> spontaneous revertants in the presence (+S9) or absence of (−S9) microsomal fraction</td>
</tr>
<tr>
<td>Extract</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>GL (3 mg)</td>
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<tr>
<td>GL (3 mg)</td>
</tr>
<tr>
<td>SR</td>
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<tr>
<td>SR</td>
</tr>
</tbody>
</table>

SR, spontaneous revertants.
Table 2
Antimutagenic activity of the methanolic extract Ganoderma lucidum (GL) against sodium azide (NaN3) and N-methyl-N’-nitro-N-nitosoguanidine (MNNG)

<table>
<thead>
<tr>
<th>Concentration (mg/plate)</th>
<th>Average number of revertants/plate</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TA100</td>
<td>TA102</td>
</tr>
<tr>
<td>NaN3 (0.0025)</td>
<td>1354.7 ± 75.1</td>
<td>431.0 ± 22.6</td>
</tr>
<tr>
<td>NaN3 + GL (3)</td>
<td>658.7 ± 36.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>242.3 ± 42.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>NaN3 + GL (2)</td>
<td>823.7 ± 21.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>321.7 ± 12.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>NaN3 + GL (1)</td>
<td>913.3 ± 17.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>352.6 ± 14.1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>MNNG (0.001)</td>
<td>1105.6 ± 47.6</td>
<td>631.0 ± 19.6</td>
</tr>
<tr>
<td>MNNG + GL (3)</td>
<td>381.3 ± 16.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>421.0 ± 15.0&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>MNNG + GL (2)</td>
<td>671.7 ± 20.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>526.0 ± 22.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>MNNG + GL (1)</td>
<td>761.0 ± 32.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>576.0 ± 17.4</td>
</tr>
<tr>
<td>SR</td>
<td>96.3 ± 7.5</td>
<td>232.6 ± 32.2</td>
</tr>
</tbody>
</table>

Values are mean ± S.D. (n = 3). Average no. of revertants after deducting the SR.
<sup>a</sup> P < 0.001.
<sup>b</sup> P < 0.005.
<sup>c</sup> P < 0.01 with respect to the NaN3 and MNNG treated group.

2.8.2. Mutagens requiring activation
Antimutagenic assay against mutagen that require metabolic activation (B[a]P) was carried out as follows. Liver microsomal fraction (S9) was prepared from Sprague Dawley rat (200 g). The rat was treated with 0.1% phenobarbital in drinking water for 4 days (Ames et al., 1973). After overnight fasting the animal was killed by decapitation, the liver was removed and the homogenate was prepared aseptically (Lakshmi et al., 2003).

The activation mixture was prepared by mixing 50 μl of the S9 fraction, containing 0.25 ml phosphate buffer (0.2 M, pH 7.4), 20 μlNADP (0.1 M), 2.5 μl glucose-6-phosphate (1 M) and 10 μl of 1.65 M MgCl<sub>2</sub>− 0.4 M KCl and various concentration of Ganoderma lucidum extract (3, 2, or 1 mg) mixed with the mutagens at a given concentration poured onto minimal agar plates and incubated for 48 h at 37°C. After incubation, number of revertants was counted using a colony counter. Toxicity of Ganoderma lucidum extract, if any, against bacterial strains was determined by incubating various concentrations of Ganoderma lucidum extract with cultures of different tester strains of Salmonella for 48 h and checking the number of revertants and background lawn. Percent inhibition of mutagenicity was determined by the following formula:

Inhibition (%) of mutagenicity = \(\frac{(R_1 - SR) - (R_2 - SR)}{(R_1 - SR)} \times 100\)

where \(R_1\) is the number of revertants without Ganoderma lucidum extract, \(R_2\) the number of revertants with Ganoderma lucidum extract and SR is the spontaneous revertants. The experiments were carried out in triplicate.

2.9. Determination of in vivo antimutagenic activity
Male Wistar rats were divided into three groups. Group I animals without any treatment. Group II animals were given distilled water for 30 days. Group III animals were fed with Ganoderma lucidum extract orally (500 mg/kg body weight) for 30 days. On the 31st day B[a]P (10 mg/rat i.p.) was administered to Group II and Group III animals. The urine was collected from the animals for 24 h in metabolic cages. The urine thus collected was filtered using Whatman no. 1 filter paper, and 20 ml of urine was passed through Amberlite XAD-4 column (40 mm × 10 mm) to concentrate the mutagen (Yamasaki and Ames, 1997). The weakly anionic components adsorbed were eluted with 10 ml acetone. The eluents were evaporated to dryness at 60°C stored at −20°C and reconstituted in 1.5 ml DMSO just before the antimutagenicity assay (Polosa et al., 1991). S. typhimurium strains TA98 and TA100 were used for the assay. Fresh Salmonella culture (1 × 10⁹ cells/ml) and 0.1 ml of urine concentrate were mixed with 2 ml top agar containing 0.2 ml of 0.5 mM histidine–biotin and poured on minimal glucose agar plate. The revertants were counted after incubation for 48 h at 37°C. The urine collected from animals treated with B[a]P alone, processed similarly and kept as control plate. The assay were done in triplicates. The percent inhibition of mutagenicity was calculated by the above formula.

2.10. Determination of the effect of Ganoderma lucidum extract on serum alkaline phosphatase and transaminases consequent to B[a]P challenge

After collection of urine, the animals were sacrificed by cervical dislocation, blood was collected directly from the heart, and the serum was separated. Serum was used for the determination of glutamate pyruvate transaminase (GPT) (Reitman and Frankel, 1957), glutamate oxaloacetate transaminase (GOT) (Reitman and Frankel, 1957) and alkaline phosphatase activities (ALP) (Kind and King, 1954).

2.11. Determination of the effect of Ganoderma lucidum extract on the antioxidant status of liver consequent to B[a]P challenge

Liver was excised and washed with ice-cold saline (0.89%) and 10% homogenate was prepared in phosphate buffer (50 mM, pH 7). A part of the homogenate was used for the estimation of reduced glutathione (GSH) (Moron et al., 1979) and the
remaining homogenate was centrifuged at 4 °C at 10,000 rpm for 10 min. The supernatant was used for the assay of glutathione-\(S\)-transferase (GST) (Habig et al., 1974), glutathione peroxidase (GPx) (Hafemann et al., 1974), superoxide dismutase (SOD) (Mc Cord and Fridovich, 1969) and catalase (CAT) (Beer and Sieser, 1952) activities. The level of lipid peroxidation was determined by the method of Ohkawa et al. (1979). The protein was estimated by the method of Lowry et al. (1951).

2.12. Statistical analysis

Experimental data were expressed as mean ± S.D. Student’s \(t\)-test was applied for expressing the significance and \(P<0.05\) was considered as significant.

3. Results

3.1. In vitro antimutagenicity of the Ganoderma lucidum extract

The methanolic extract of Ganoderma lucidum at a concentration of 3 mg/plate did not show any effect on spontaneous revertants of any of the Salmonella tester strain in the presence or absence of microsomal fraction (Table 1). The extract at a concentration of 3 mg/plate, inhibited NaN\(_3\)-induced mutagenicity by 51.4% (TA100) and 43.78% (TA102) (Table 2). There was significant difference between strain (\(P<0.001\)). Between concentrations, there was significant difference in activity (\(P<0.01\)). Control showed significantly higher number of revertants compared to 1, 2 and 3 mg extracts. MNNG-induced mutagenicity was inhibited by 65.5% (TA100) and 33.2% (TA102) (Table 2) and NPD-induced mutagenicity by 40.3% (TA98) and 64.2% (TA100) (Table 3), by the extract at a concentration of 3 mg/plate. The methanolic extract of the mushroom was also found to inhibit mutagenicity elicited by B[a]P, a mutagen requiring activation. At a concentration of 3 mg/plate the extract inhibited B[a]P-induced mutation by 65% (TA98) and 70.3% (TA100) (Table 4).

3.2. In vivo antimutagenic activity

Antimutagenicity tests on the urine of animals treated with B[a]P showed that the administration of methanolic extract of Ganoderma lucidum inhibited mutagenicity induced by B[a]P. The extract at a dose of 500 mg/kg of body weight inhibited 59.3% mutagenicity induced by B[a]P (Table 4).

3.3. Effect of Ganoderma lucidum extract on hepatic enzymes after B[a]P challenge

The activity of SGOT, SGPT and ALP was significantly elevated in B[a]P treated group of animals. There was significant difference between concentrations, \(P<0.01\). Control showed significantly higher number of revertants compared to 1, 2 and 3 mg extracts. MNNG-induced mutagenicity was inhibited by 65.5% (TA100) and 33.2% (TA102) (Table 2) and NPD-induced mutagenicity by 40.3% (TA98) and 64.2% (TA100) (Table 3), by the extract at a concentration of 3 mg/plate. The methanolic extract of the mushroom was also found to inhibit mutagenicity elicited by B[a]P, a mutagen requiring activation. At a concentration of 3 mg/plate the extract inhibited B[a]P-induced mutation by 65% (TA98) and 70.3% (TA100) (Table 4).

Table 3

<table>
<thead>
<tr>
<th>Concentration (mg/plate)</th>
<th>Average number of revertants/plate</th>
<th>TA98</th>
<th>TA100</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPD (0.020)</td>
<td>1125 ± 90.1</td>
<td>755.0 ± 24.8</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>NPD + GL (3)</td>
<td>670.6 ± 14.7a</td>
<td>270.0 ± 14.7a</td>
<td>40.4</td>
<td>64.2</td>
</tr>
<tr>
<td>NPD + GL (2)</td>
<td>781.0 ± 27.4b</td>
<td>422.0 ± 23.1a</td>
<td>30.6</td>
<td>44.1</td>
</tr>
<tr>
<td>NPD + GL (1)</td>
<td>823.0 ± 33.0c</td>
<td>615.3 ± 27.3b</td>
<td>26.8</td>
<td>18.5</td>
</tr>
<tr>
<td>SR</td>
<td>38.6 ± 3.5</td>
<td>88.0 ± 5.3</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Values are mean ± S.D. (\(n = 3\)). Average no. of revertants after deducting the SR.

Table 4

<table>
<thead>
<tr>
<th>In vivo</th>
<th>In vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutagen/extract per animal</td>
<td>Average number of revertants per plate</td>
</tr>
<tr>
<td></td>
<td>TA98</td>
</tr>
<tr>
<td>B[a]P (10 mg/rat)</td>
<td>128.4 ± 4.5</td>
</tr>
<tr>
<td>B[a]P + GE (500)</td>
<td>52.8 ± 3.5a</td>
</tr>
<tr>
<td>Untreated</td>
<td>27 ± 2.6</td>
</tr>
<tr>
<td>Spontaneous Revertants</td>
<td>40.5 ± 5.6</td>
</tr>
<tr>
<td>SR</td>
<td>36.6 ± 8.9</td>
</tr>
</tbody>
</table>

Values are mean ± S.D. (\(n = 6\) in vivo, \(n = 3\) in vitro). Average no. of revertants after deducting the SR values are significant. \(^a\) \(P<0.01\) with respect to the B[a]P alone treated group.

\(^a\) \(P<0.001\).

\(^b\) \(P<0.005\).
enzymes were restored to the normal level in animals treated with *G. lucidum* extract (Fig. 1).

### 3.4. Effect of *Ganoderma lucidum* extract on the antioxidant status after B[a]P challenge

The activities of GST, GPx, catalase and the level of GSH were decreased consequent to B[a]P treatment. *Ganoderma lucidum* extract elevated the activity of GST (Fig. 2), catalase (Fig. 3), and GPx (Fig. 4) and the level of GSH (Fig. 5). The activity of SOD (Fig. 6) was significantly reduced in B[a]P-treated group of animals and the level was markedly enhanced in animals that were treated with the extract. The lipid peroxidation (MDA) was elevated in the serum and tissue of B[a]P treated animals compared to animals treated with extract prior to B[a]P challenge (Fig. 7).
3.5. Phytochemical analysis

Phytochemical analysis showed that the extract reacted with the antron reagent and also with phenol—sulphuric acid reagent indicating the presence of polysaccharide as one of the major components. The extract also responded to the Lowry-Folin test indicating the presence of protein. The TLC analysis detected the presence of marked amount of terpenes and traces of flavonoids in the extract. Phytochemical analysis thus, indicate that the major components of the extract are polysaccharide and terpenes.

4. Discussion

Experimental results indicate that the methanolic extract of the fruiting bodies of *Ganoderma lucidum* occurring in South India possessed significant in vitro and in vivo antimutagenic activity. Considerable attention has been focussed on the role of dietary supplements and their constituents as chemopreventive agents in recent years (Shio et al., 1994). Dietary interactions that decrease the mutagenic load and abnormal biological responses appear to be one of the plausible approaches for cancer prevention. Significant correlations have been observed between the carcinogenicity of a series of polycyclic aromatic hydrocarbons (PAH) and their covalent binding to mouse epidermal DNA (Brookes and Lawley, 1964; Muller, 1978; Hoel et al., 1983). Based on extensive evidence accumulated in the last two decades, it is believed that PAH must be metabolically activated to electrophillic intermediates, which can bind to DNA and exert its carcinogenic effects (Pelkonen and Nebert, 1982). B[a]P is metabolized by mixed function oxidase (MFO) of rat liver to active intermediate benzo[a]pyrene-7,8-diol, 9,10-epoxide [BPDB] (Smith and Gupta, 1996). These can attack cellular macromolecules like DNA, RNA, proteins, membranes, etc., and cause dysfunction and damage.

The reactive oxygen species are important as direct and indirect initiators as well as promoters of mutagenesis and carcinogenesis. They also increase the lipid peroxidation, which in turn alter the integrity of membrane bound enzymes (Halliwell and Gutteridge, 1985). The free radical scavenging efficiency of the extract thus might be playing an important role in the antimutagenic activity. The present investigations reveal that the methanolic extract of *Ganoderma lucidum* is able to decrease the levels of B[a]P-mutagenesis. The treatment of animals with B[a]P shows a significant increase in lipid peroxidation. Antioxidants lower the carcinogenicity of B[a]P by acting on antioxidant response elements and thereby increasing the synthesis of enzymes involved in detoxification. The prevention of carcinogenicity of B[a]P might be mediated by the changes in the GSH content, which can detoxify intermediate epoxide of B[a]P (Pietropaole and Weinstein, 1975; Pezzuto et al., 1976; Burke et al., 1981). The significant increase of superoxide dismutase in the extract treated group of animals appears to facilitate removal of superoxide anions, and H₂O₂ formed in the process by GPx and GST which are also increased by the treatment of extract.

The results of the present investigations demonstrate the significant antimutagenic activity of *Ganoderma lucidum* extract and its ability to ameliorate the oxidative damage caused by the B[a]P. The findings suggest the potential of the extracts of *Ganoderma lucidum* occurring in South India as a chemopreventive agent.

Acknowledgement

The valuable help of Prof. K.M. Leelavathi, Department of Botony, Calicut University, Calicut, Kerala, India for the identification of *Ganoderma lucidum* is gratefully acknowledged.

References


