BIOMEDICAL AND ENVIRONMENTAL SCIENCES **17**, 469-475 (2004)

Antimutagenicity of Propolis Against Some Mutagens *in vivo* and *in vitro*

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Objective To evaluate the antimutagenicity of propolis *in vivo* and *in vitro*. **Methods** *Salmonella typhimurium* strains TA98 and TA100 were used as a test model *in vitro* against a direct mutagen DMC and an indirect mutagen 2AF with or without S9 mix, and MN formation of mice bone marrow cell and CAs induction of mice testicle cell were applied as a test model *in vivo* against two mutagens CP and MMC. **Results** The present study clearly demonstrated that propolis could inhibit mutagenicity of both DMC and 2AF directly in a dose-dependent manner, and significant antimutagenic effects ($P<0.05$) were obtained in TA98 strain at 2000 and 3000 μg/plate. It also could inhibit mutagenicity of both DMC and 2AF to TA98 strain in a dose-dependent manner, with significant antimutagenic effects ($P<0.05$) appeared at 1000, 2000, and 3000 μg/plate. The results of antimutagenicity test *in vivo* revealed that propolis could inhibit MN formation significantly ($P<0.05$) at the doses of 45.0 and 135.0 mg/kg b. w., and decrease the frequency of chromosome aberrants and chromosome aberrant cells significantly ($P<0.05$) only at the dose of 135.0 mg/kg b. w. **Conclusion** The propolis is a good inhibitor for mutagencity of DMC and 2AF *in vitro*, as well as for CP and MMC *in vivo*.

Key words: Propolis; *Salmonella typhimurium* strain; Mutagenicity

INTRODUCTION

Propolis (CAS No. 9009-62-5) (sometimes also referred to as 'bee glue') is the generic name for the resinous substance collected by honeybees from various plant sources. In general, it is composed of 50% resin and vegetable balsam, 30% wax, 10% essential and aromatic oils, 5% pollen and 5% various other substances, including organic debris[1,2]. Propolis has been mainly used as home remedies and a personal product since 300 BC[3]. In China propolis has also been used as a Chinese traditional medicine for a long time. In modern studies, it is shown to exhibit a variety of biological effects, ranging from antiviral activity[4,5], antimicrobial activity against many Gram-positive and Gram-negative rods and cocci, yeasts and fungi which are associated with various degrees of pathogenicity in human to antiseptic, astringent, choleric, spasmolytic, anti-inflammatory, anesthetic and antioxidant properties[6]. Propolis has been shown to be antimutagenic against some mutagens in *Salmonella typhimurium* strains TA97, TA98 and TA100[7,8]. S.-N. JENG *et al*.[9] reported

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0895-3988/2004
CN 11-2816

that the ethanol extract of propolis was an inhibitor for the mutagenicity of two direct mutagens, 4-nitro-O-phenylenediamine (4-NO) and 1-nitropyrene (1-NP) and two indirect mutagens, 2-amino-3-methylimidazo{4,5-f}quinoline (IQ) and benzo[a]pyrene (B[a]P) in the presence of S9 mix. Propolis and its constituent flavanoids exhibit an antitumor effect both *in vivo* and *in vitro*[10,11].

No reports about antimutagenicity of propolis *in vivo* are available. The present study has provided evidence for the role of propolis in inhibiting antimutagenic activity both *in vivo* and *in vitro*.

MATERIALS AND METHODS

Chemicals and Materials

Mice (Kunming strain),18 g-20 g, clear grade, purchased from the Center of Experimnetal Animals of Zhejiang Province, were used in this study. *Salmonella typhimurium* TA98 and TA100 were provided by the Institute of Food Safety Control and Inspection, Ministry of Public Health of PRC. NaN3, 2-aminofluorene (2-AF), daunomycin (DMC) and mitomycin (MMC) were purchased from Sigma Co. Cyclophosphamide (CP) was purchased from Shanghai Biochemistry Co. and propolis (20%) was obtained from Functional Food Co. (Zhejiang, China).

Antimutagenicity Test in vitro

Improved Ames test Propolis was dissolved in alcohol. The plate preincubation assay with or without S9 mix of Maron and Ames[12] was improved. Briefly, 10.5 mL S9 mix or buffer solution, 0.1 mL propolis and 0.1 mL mutagens were added to test-tubes and then gently mixed. The resultant mixture was transferred into a water-bath at 37℃ for 20 min. After incubation, 0.1 mL TA98 or TA100 (overnight culture) and 2.0 mL molten top agar were added to the mixture and poured onto minimal glucose agar plates. 20.5 mL S9 mix or buffer solution, 0.1 mL propolis, 0.1 mL mutagens and 0.1 mL TA98 or TA100 (overnight culture) were added to test-tubes and then gently mixed. The resultant mixture was transferred into a water-bath at 37℃ for 20 min. After incubation, 2.0 mL molten top agar was added to the mixture and poured onto minimal glucose agar plates. 30.5 mL S9 mix or buffer solution, 0.1 mL mutagens and 0.1 mL TA98 or TA100 (overnight culture) were added to test-tubes and then gently mixed. The resultant mixture was transferred into a water-bath at 37℃ for 20 min, and centrifuged and cleaned by buffer solution. The 0.7 mL mixture remained and 0.1 mL propolis was added to 2.0 mL molten top agar and poured onto minimal glucose agar plates. The plates were incubated at 37℃ for 48 h and the number of revertant colonies was counted. All experiments were performed in triplicate and each assay was performed in duplicate.

Surviving Test of Salmonella typhimurium

Surviving test utilized the similar procedure except that the testing bacteria were diluted 10^6 times with buffer solution and the plate was incubated at 37℃ for 24 h. The number of surviving revertant colonies was counted.

Antimutagenicity Test in vivo

Treatment schedule Propolis was dissolved in refined peanut oil for the improved

micronucleus (MN) test and chromosome aberration (CA) test. Acute oral LD_{50} of propolis (20%) was determined by Horn's method in mice and rats and found to be greater than 10 000 mg/kg b. w. respectively. Three groups of mice (10 mice/group) were exposed through oral gavage to 22.5, 45.0, or 135.0 mg/kg b. w. of propolis for 28 consecutive days. Propolis was dissolved in a manner that each group was administered to a constant volume of 10.0 mL/kg b. w. For reference, one group of mice was orally administered refined peanut oil to serve as vehicle control. All groups were administered mutagens (CP for improved MN test, 60 mg/kg b. w.; dissolved in distilled water, given twice for later 2 days; MMC for improved CA test, 2.0 mg/kg b. w.; dissolved in distilled water, given once after 14 days). Another group was only administered CP as positive control for improved MN test and MMC for improved CA test.

Improved MN Test of Mice Bone Marrow Cells

Six hours after the last CP was given, mice were killed by cervical dislocation and bone marrow smears were stained with Giemsa. To avoid any subjective error, at least 1000 polychromatic erythrocytes (PCE) /animal were scored for MN induction.

Improved Chromosome Aberration (CA) Test in Mice Testicle Cells

Mice were injected with aqueous solution of clochicine (6 mg/kg b. w., i. p.) 6 h prior to scheduled killing by cervical dislocation. The mice testicle cells were obtained by swollen, centrifuged and fixed, and dropped on clean chilled slides. Slides were air-dried and stained with Giemsa. CAs were scored blind-fold and at least 100 well-spread metaphase cells/ mouse were analyzed.

Statistics

The means and standard deviations were calculated. Data obtained in improved Ames test were subjected to one-way analysis of variance and the Student-Newman-Keuls test. Data obtained in improved MN test were calculated and expressed in frequency (‰) and the significance at different dose levels was tested using poisson distribution. Data obtained in improved CA test were calculated and expressed in percentage frequency and the significance at different dose levels was tested by application of one-way analysis of variance and the Student-Newman-Keuls test after sin $(1/P^{1/2})$ transformation. Differences between the groups were considered to be statistically significant at $P<0.05$. All data were analyzed using SPSS 10.0 for windows.

RESULTS

The toxicity and mutagenicity of propolis were investigated. It was found that propolis, at the concentration ranging from 8 to 5000 μg/plate, was non-toxic to *Salmonella typhimurium* TA98 or TA100 strain and exhibited no mutagenicity to two strains.

In the *Salmonella typhimurium* TA98 strain all the test doses regardless of the presence of S9 mix or not were able to inhibit the mutagenicity of both DMC and 2AF directly in a dose-dependent manner. At 200 μg/plate propolis, the relative revertants induced by DMC were decreased by 8.5% as compared with the control group. When the concentration of propolis was further increased to 3000 μg/plate, the relative revertants were inhibited up to 61.6%. In comparison, the relative revertants induced by 2AF were reduced by 46.6% at 3000 μg/plate of propolis. However, significant antimutagenicity ($P<0.05$) was obtained

only at 2000 and 3000 μg/plate (Table 1).

TABLE 1

Antimutagenic Activities of Propolis Against Mutagens Using the *S.typhimurium* Testing System[a]

Propolis Dose μg/plate	No. of Relative Revertants/Plate[b]							
	TA100 -S9		TA100 +S9		TA98 -S9		TA98 +S9	
	NaN3	PI(%)[c]	2AF	PI(%)	DMC	PI(%)	2AF	PI(%)
Vehicle Control	1310±60	3.5	1047±51	2.6	1353±201	5.5	1064±138	4.6
200	1337±133	1.3	1027±68	4.8	1310±124	8.5	993±47	11.1
1000	1279±26	6.2	997±132	8.2	1163±51	19.1	936±41	16.4
2000	1224±18	10.8	981±62	10.0	723±26[d]	50.7	745±64[d]	33.9
3000	1237±48	9.7	968±58	11.5	572±45[d]	61.6	608±66[d]	46.6
NaN3 1.25	1352±74	–	–	–	–	–	–	–
2AF 20	–	–	1070±153	–	–	–	1114±134	–
DMC 10	–	–	1070±153	–	1429±92	–	–	–

Note. [a]Values are $\bar{x}\pm s$, Spontaneous revertants of the TA98 with or without S9 mix are 27±10, 37±8 respectively, and TA100, 180±16, 168±14 respectively; [b]Relative revertants=induced revertants×surviving revertants/surviving vehicle revertants. Results indicated are the average value of triplicate determininations; [c]PI (% inhibition)=[relative revertants (without inhibitor)-relative revertants(with inhibitor)]/[relative revertants (without inhibitor)- spontaneous revertants]×100; [d]Highly significant differences were found between the dose of propolis used and the mutagen positive control (P<0.05, Student-Newman-Keuls test).

In the *Salmonella typhimurium* TA98 strain all the test doses with or without S9 mix were able to inhibit the mutagenicity of both DMC and 2AF to TA98 strain. The PI (%) increased with increasing dose of propolis. At 3000 μg/plate, the relative revertants induced by DMC and 2AF were decreased by 48.3% and 42.7% respectively. Significant antimutagenicity against DMC to TA98 strain (P<0.05) was obtained at the doses of 1000, 2000, and 3000 μg/plate, and was similarly against 2AF to TA98 strain at the doses of 2000 and 3000 μg/plate (Table 2).

TABLE 2

Antimutagenic Activities of Propolis Against Mutagens Using the *S.typhimurium* Testing System[a]

Propolis Dose μg/Plate	No. of Relative Revertants/Plate[b]							
	TA100 -S9		TA100 +S9		TA98 -S9		TA98 +S9	
	NaN3	PI[c](%)	2AF	PI(%)	DMC	PI(%)	2AF	PI(%)
Vehicle Control	1359±64	-0.6	1035±39	3.9	1425±176	0.3	1088±130	2.4
200	1373±146	-1.8	1008±102	7.0	1357±96	5.2	1082±123	2.9
1000	1216±124	11.5	902±174	18.9	1033±119[d]	28.4	956±37	14.5
2000	1249±79	8.7	915±78	17.4	814±74[d]	44.2	717±63[d]	36.5
3000	1241±80	9.4	811±66	29.1	756±67[d]	48.3	650±60[d]	42.7
NaN3 1.25	1352±74	–	–	–	–	–	–	–
2AF 20	–	–	1070±153	–	–	–	1114±134	–
DMC 10	–	–	–	–	1429±92	–	–	–

Note. [a]Values are $\bar{x}\pm s$; Spontaneous revertants of the TA98 with or without S9 mix are 27±10, 37±8 respectively, and TA100, 180±16, 168±14 respectively; [b]Relative revertants=induced revertants×surviving revertants/surviving vehicle revertants. Results indicated are the average value of triplicate determininations; [c]PI (% inhibition)=[relative revertants(without inhibitor)-relative revertants(with inhibitor)]/[relative revertants (without inhibitor)-spontaneous revertants]×100; [d]Highly significant differences were found between the dose of propolis used and the mutagen positive control (P<0.05, Student-Newman-Keuls test).

There was no evidence that the propolis, at concentration ranging from 200 to 3000 μg/plate could effectively inhibit the mutated *Salmonella typhimurium* TA98 and TA100 strain induced by DMC and 2AF. No significant difference was found between propolis and mutagen control (Table 3).

TABLE 3

Antimutagenic Activities of Propolis Against Mutagens Using the *S. typhimurium* Testing System[a]

Propolis Dose μg/Plate	No. of Relative Revertants/Plate[b]							
	TA100 -S9		TA100 +S9		TA98 -S9		TA98 +S9	
	NaN3	PI (%)[c]	2AF	PI(%)	DMC	PI(%)	2AF	PI(%)
V ehicle Control	1350±59	0.2	1090±18	–2.2	1377±75	3.7	1069±149	4.1
200	1307±20	3.8	1035±42	3.9	1442±126	-0.9	1092±68	2.0
1000	1328±46	2.0	1057±99	1.5	1373±189	4.0	1105±93	0.8
2000	1384±74	-2.7	1034±60	4.0	1324±211	7.5	1099±97	1.4
3000	1354±67	-0.2	997±116	8.2	1266±185	11.7	1049±103	6.0
NaN 1.25	1352±74	–	–	–	–	–	–	–
2AF 20	–	–	1070±153	–	–	–	1114±134	–
DMC 10	–	–	–	–	1429±92	–	–	–

Note. [a]Values are $\bar{x}\pm s$; Spontaneous revertants of the TA98 with or without S9 mix are 27±10, 37±8 respectively, and TA100, 180±16, 168±14 respectively; [b]Relative revertants=induced revertants×surviving revertants/surviving vehicle revertants. Results indicated are the average value of triplicate determininations; [c]PI(% inhibition)=[relative revertants (without inhibitor)-relative revertants(with inhibitor)]/[relative revertants (without inhibitor)- spontaneous revertants]×100.

The treatment doses of propolis, 22.5, 45.0, and 135.0 mg/kg b.w. were able to inhibit MN formation. The frequency of MN was also considerably lowered in the mice exposed to 135.0 mg/kg b.w. Significant effects were found (P<0.05) at the doses of 45.0 and 135.0 mg/kg b.w. (Table 4).

The percent frequency of aberrations and aberration cells decreased with increasing dose of propolis. The types of decreased CAs were mainly breaks, reciprocal translocation and precocious disjunction, with only a few gaps and minutes. Only at single dose treatment of propolis 135.0 mg/kg b. w. the frequency of aberrants and aberrant cells were decreased significantly (P<0.05) as compared with the mutagen and vehicle control (Table 5).

TABLE 4

Antimutagenic Activities of Propolis Against Mutagens CP Using MN Induction in Mice Marrow Cells[a]

Treatment Groups mg/kg b.w.	Number of Mouse/Group	Number of PCE Observed	Number of MN	Frequency of MN in PCE (‰)	Value of *P*	Value of *P*
CP	10	1000	259	27.1±4.9	–	–
Refined Oil+CP	10	1000	259	27.1±3.5	1.000	–
22.5+CP	10	1000	222	25.0±2.8	0.565	0.565
45.0+CP	10	1000	193	21.5±2.6	0.011[b]	0.011[c]
135.0+CP	10	1000	195	20.6±1.8	0.003[b]	0.003[c]

Note.[a] Values are expressed as $\bar{x}\pm s$; [b]P<0.05 compared with the positive control; [c]P<0.05 compared with the vehicle control.

TABLE 5

Antimutagenic Activities of Propolis Against Mutagens MMC Using CA Induction in Mice Testicle Cells[a]

Treatment Groups mg/kg b.w.	Metaphase Scored/ Mice	Aberrantion Type								Percent Frequency of Aberrants	Percent Frequency of Aberrant Cells
		Brea[b]	Gap	Minu	Ring	CE	RT	PD			
								X-Y	Automal		
MMC	1000/10	16	6	4	2	6	15	21	10	8.1±2.9	7.5±2.2
Refined Oil+MMC	1000/10	17	7	3	4	5	14	20	9	8.0±2.8	7.5±2.5
22.5+MMC	1000/10	15	6	3	1	4	10	19	8	6.6±1.4	6.4±1.4
45.0+MMC	1000/10	11	5	3	3	6	9	17	9	6.3±3.1	5.8±2.5
135.0+MMC	1000/10	9	4	2	2	6	10	11	6	5.0±2.8[c,d]	4.7±2.3[c,d]

Note. [a] Values are expressed as $\bar{x}\pm s$, [b]Brea: Breaks, Gap: Gaps, Minu:Minutes, Ring: Rings, CE: Chromatid exchange, RT: Reciprocal translocation, PD: Precocious disjunction, [c]$P<0.05$ compared with the positive control, [d]$P<0.05$ compared with the vehicle control.

DISCUSSION

Although no definitive studies *in vivo* of the antimutagenicity of propolis are available, the antimutagenicity of propolis *in vitro* is well known. Rao *et al.*[8] investigated the antimutagenic effect of three caffeic acid esters present in propolis, and synthesized these three caffeic acid esters and tested their ability against the 3, 2-dimehtyl-4-aminobiphenyl -induced mutagenicity in *Salmonella typhimurium* strains TA98 and TA100. Cizmarik *et al*.[7] also showed that propolis was antimutagenic against nitrovin and nitroguanidine in *Salmonella typhimurim* TA97 and TA100. S.-N. Jeng *et al*.[9] also gave an evidence that the ethanol extract of propolis was a good inhibitor for mutagencity of direct mutagens, 1-NP and 1-NO, as well as for the indirect mutagens IQ and B[a]P in the presence of S9 mix. In the present study, using *Salmonella typhimurium* testing system with or without S9 mix, it was further demonstrated that propolis could inhibit no only the mutagenicity of both direct mutagen DMC and indirect mutagen 2AF effectively (Improved Ames test plan①), but also the mutagnecity of both two mutagens to TA 98 strains (Improved Ames test plan②). It was also shown that there was no antimutagenicity of propolis against the mutated *Salmonella typhimurium* TA98 or TA100 strain induced by these two mutagens (Improved Ames test plan③).

Many antimutagencity studies used the *Salmonella* plate test, or its variation, which differs from mutagenicity tests of mammalian cells or other eukaryotes. The most important difference is that in the *Salmonella* test no concurrent measurement of cell survival or cell growth could be directly related to the number of measurable mutant cells[13]. Genetic damage might result from several mechanisms, and no single test system is capable of detecting all possible mechanisms, so the composition of the test battery is critical to the initial data evaluation[14]. It is the author's opinion that a test battery consisting of at least both bacteria and mammalian assays should be used both *in vitro* and *in vivo* assays. Regarding the genetic toxicology, risk can be related to DNA alterations which occur in either somatic or germ cells. Therefore, risk estimations must consider both cell populations. Somatic cell risk is important for the estimation of non-transmissible toxicity such as cancer and teratosis, germ cell damage, which requires the genotoxic agents to reach the gonadal tissue and produce alterations capable of transmitting to the next generation. MN test in

mice marrow cells and CA test in mice testicle cells have been conducted routinely for many years. Findings in the present study also showed that propolis could inhibit MN and CAs formation in mice induced by CP and MMC respectively. It is suggested that propolis has effective antimutagenicity against standard mutagenic chemicals CP and MMC not only in somatic cells but also in germ cells of mammalians.

In conclusion, propolis could inhibit not only the mutagenicity of both direct mutagen DMC and indirect mutagen 2AF effectively, but also the mutagnecity of both two mutagens to TA98 strains. Propolis is a good inhibitor of mutagencity (MN formation) induced by CP in somatic cells and (CAs induction) induced by MMC in testicle cells of mice. The detailed mechanisms of antimutagenicity of propolis and its components are still unknown. To identify which components of propolis exert the antimutagenic effect and their mechanisms awaits further study.

ACKNOWLEDGEMENTS

The authors are grateful to Ms. Yu-Man CHEN for her technical assistance. Thanks are due to Mr. Xi-Peng JIN, Professor, Medical School of Shanghai Fudan University for his expert assistance of this manuscript.

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(*Received January* 9, 2004 *Accepted July* 30, 2004)