

Part 8: References

- [1] Zang, M. and C.H. Su. 1990. *Ganoderma comphoratum*, a new taxon in genus *Ganoderma* from Taiwan, China. *Acta Bot. Yunnanica* 12: 395-396.
- [2] Chang, T.T. and W.N. Chou. 1995. *Antodia cinnamomea* sp. nov. on *Cinnamomum kanehirai* in Taiwan. *Mycol. Res.* 99:756-758.
- [3] Wu SH, Ryvarden I, Chang TT. *Antrodia camphorata* (niu-chang-chih), new combination of a medicinal fungus in Taiwan. *Bot Bull Acad Sinica* 1997;38:273-5
- [4] Tzean, Shean-Shong. Authority of *Niu-Chang-Ku*. National Taiwan University 2005
- [5] QC Report of GDAC. 4/09/2007
- [6] Hseu, YC., Yang, HL. 2005. Anti-inflammatory potential of *Antrodia Camphorata* through inhibition of iNOS, COX-2 and cytokines via the NF- κ B pathway. *International Immunopharmacology.* 5 1914-1925
- [7] Physiological Activities of *Antrodia camphorata*, a Unique Fungal Species in Taiwan
- [8] Statement of Response on Selling Distribution. October 2007
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- [10] Ninety-Day Safety Assessment of GD-*Antrodia camphorata*. March 2007
- [11] Genetic Toxicity Assessment of GD-*Antrodia camphorata*. March 2007
- [12] SGS and Analysis Report for *Antrodia camphorata*. April 2007
- [13] Lin, C.H., Tsai, P.H. 2006. The effects of *Antrodia camphorata* supplementation on antifatigue capacity.
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- [16] Mau, J.L., Huang, P.N., 2004. Antioxidant properties of methanolic extracts from two kinds of *Antrodia camphorata* mycelia. *Food Chemistry* 86:25-31

FDA-1995-S-0039

Batch 1

FEB.01.2008 16:48

GOLDEN BIOTECH

#3574 P.003

Attachment

The HPLC analytical of Antroquinonol Fingerprint for GDAC

Sample :

Report No:961121-1
Report Date:2007.04.13

GDAC0703 - Antroquinonol

Sample prepare :

Using 1 g sample add 10ml. EO11 to extract 4hr (15 sec vortex/hr) and put it in 4°C overnight.

Standard: Antroquinonol

HPLC Method :

Column: No.1 (Thermo ODS Hypersil C18 5 μm 4.6*250mm RP)

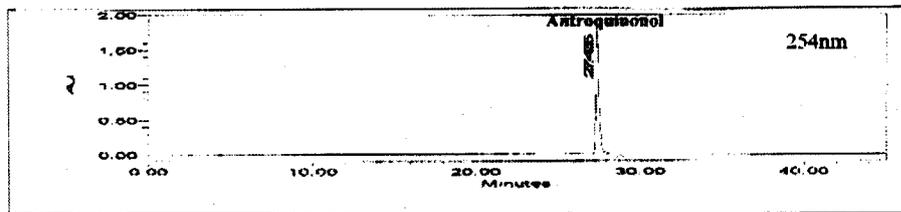
Detector: PDA (190nm-400nm)

Injection: 20 μL

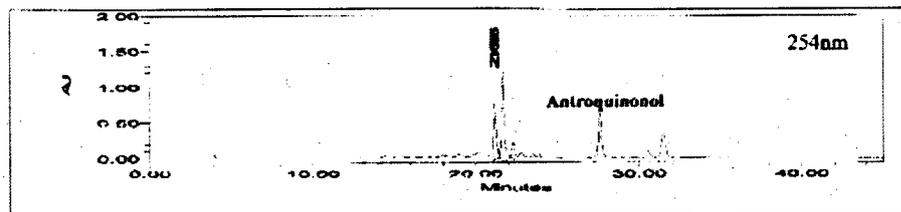
Method name: 0815

Result:

Standard: Antroquinonol



GDAC 0703



RT (min)	Area (V*sec)	Antroquinonol (mg/g)
27.246	5.5164	2.45

Authority Signature

Wu-Che Wen

Wu-Che Wen

Vice President

Batch 2

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#3574 P.002

Attachment

The HPLC analytical of Antroquinonol Fingerprint for GDAC

Sample :

Report No:961121-2
Report Date:2007.04.13

GDAC0704 - Antroquinonol

Sample prepare :

Using 1 g sample add 10mL EOH to extract 4hr (15 sec vortex/hr) and put it in 4°C overnight.

Standard: Antroquinonol

HPLC Method :

Column: No.1 (Thermo ODS Hypersil C18 5 μ m 4.6*250nm RP)

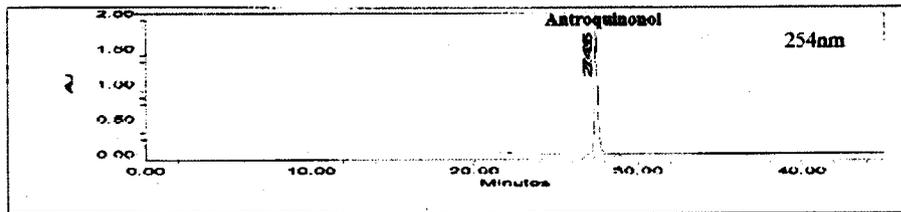
Detector: PDA (190nm-400nm)

Injection: 20 μ L

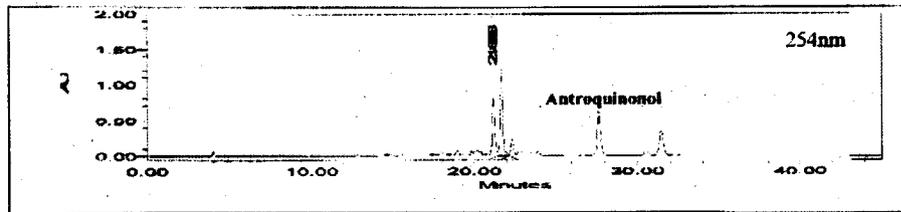
Method name: 0815

Result:

Standard: Antroquinonol



GDAC 0704



RT (min)	Area (V*sec)	Antroquinonol (mg/g)
27.318	4.9073	2.18

Authority Signature

Wu-Che Wen
Wu-Che Wen

Vice President

Batch 3

FEB.01.2008 16:48

GOLDEN BIOTECH

#3574 P.001

Attachment

The HPLC analytical of Antroquinonol Fingerprint for GDAC

Sample :

Report No:961121-3

Report Date:2007.04.13

GDAC0705 - Antroquinonol

Sample prepare :

Using 1 g sample add 10ml. EOII to extract 4hr (15 sec vortex/hr) and put it in 4°C overnight.

Standard: Antroquinonol

HPLC Method :

Column: No.1 (Thermo ODS Hypersil C18 5 μ m 4.6*250mm RP)

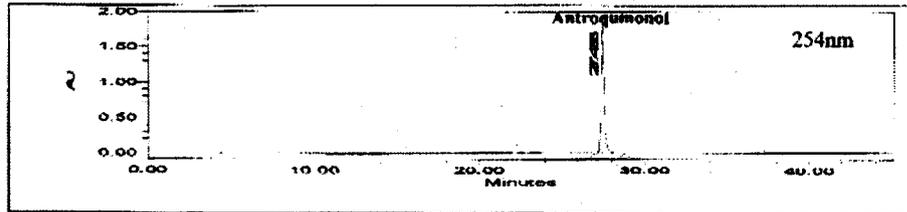
Detector: PDA (190nm-400nm)

Injection: 20 μ L.

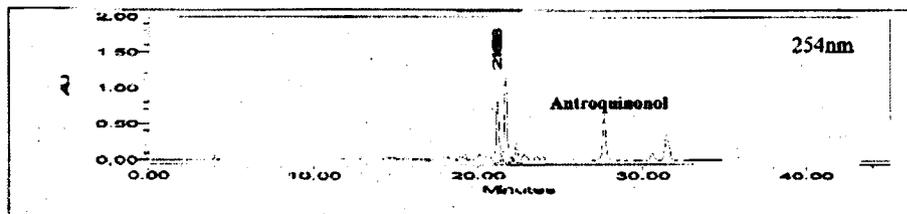
Method name: 0815

Result:

Standard: Antroquinonol



GDAC 0705



RT (min)	Area (V*sec)	Antroquinonol (mg/g)
27.341	4.7380	2.11

Authority Signature

Wu-Che Wen

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Tamsui Town,Taipei 251,Taiwan

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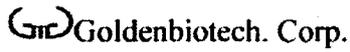
國鼎牛樟芝產品檢驗報告書**QC Report of GDAC****Product name : GDAC****Product No. : GDAC0703****Report No. : RP960413-1****Sampling date : 2007.04.09****Sample : 30g****Report date : 2007.04.13**

Test Item (檢驗項目)	Specification (規格)	Result (結果)	Verifier (檢驗者)
1. General properties (性狀)	The orange/light orange capsule(500mg) (500 mg 橘/淺橘膠囊)		
2. Loss on drying (乾燥減重)	Not more than 13.0 % (13.0 %以下)		
3. Water-soluble extractives (水抽提物)	Not less than 32.0 % (32.0 %以上)		
4. Diluted alcohol-soluble extractives (稀醇抽提物)	Not less than 30.0 % (30.0 %以上)		
5. Total ash (總灰分)	Not more than 10.0 % (10.0 %以下)		
6. Acid-insoluble ash (酸不溶性灰分)	Not more than 5.0 % (5.0 %以下)		
7. Average capsule weight (20 capsule) (平均膠囊重量-20粒膠囊平均)	610±30mg/capsule (610±30mg/膠囊)		
8. The assay of Antroquinonol(mg/g) (安卓奎諾爾 Antroquinonol 含量 mg/g)	2.2±0.5mg/g (2.2±0.5mg/g)		

*Calibration curve of Antroquinonol:

(Linear range : 31.25 ~ 1000µg/mL) Y=0.027X-0.079 R=1

Conc. (µg/mL)	Peak area (V*sec)	Average	SD	CV (%)
31.25	0.8146	0.8199	0.0048	0.6%
	0.8212			
	0.8240			
62.5	1.6467	1.6498	0.0035	0.2%
	1.6492			
	1.6537			
125	3.3454	3.3496	0.0037	0.1%
	3.3519			
	3.3514			

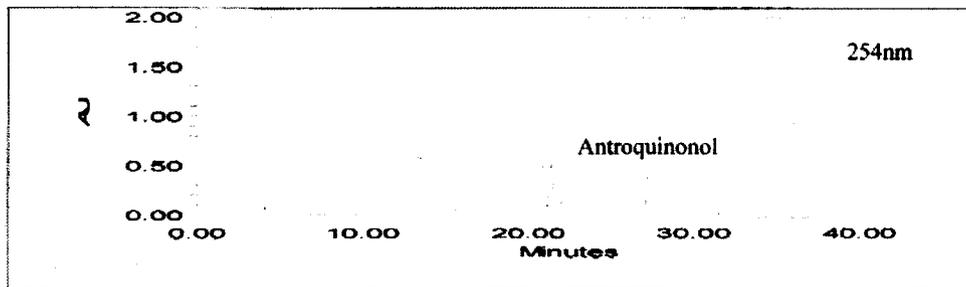
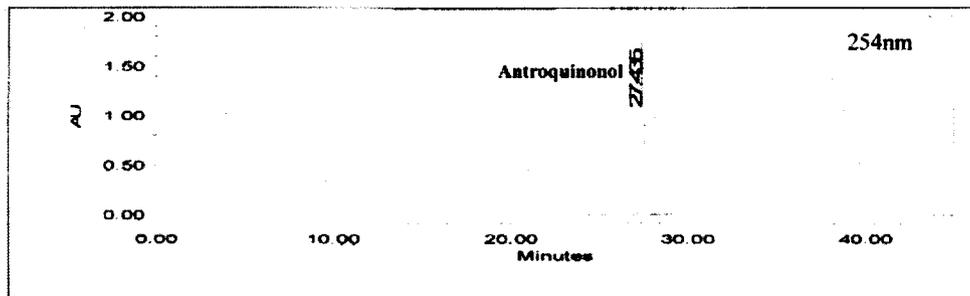


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國鼎牛樟芝產品檢驗報告書

QC Report of GDAC

Conc. (µg/mL)	Peak area (V*sec)	Average	SD	CV (%)
250	6.7635	6.7786	0.0137	0.2%
	6.7817			
	6.7904			
500	13.7412	13.6923	0.0430	0.3%
	13.6751			
	13.6605			
1000	27.3715	27.4980	0.1138	0.4%
	27.5302			
	27.5922			



RT (min)	Area (V*sec)	Antroquinonol (mg/g)
27.246	5.5164	2.45

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國鼎牛樟芝產品檢驗報告書**QC Report of GDAC**

Product name : GDAC		Product No. : GDAC0703	
Report No. : RP960413-1		Sampling date : 2007.04.09	
Sample : 30g		Report date : 2007.04.13	
Test Item (檢驗項目)	Specification (規格)	Result (結果)	Verifier (檢驗者)
9. Divide heavy metal test (個別重金屬檢驗) Pb (鉛試驗) Cd (鎘試驗) Hg (汞試驗) As (砷試驗) Cu (銅試驗)	Pb not more than 2 ppm by ICP-MS test (以 ICP-MS 檢測不得高於 2 ppm 以上) Cd not more than 0.2 ppm by ICP-MS test (以 ICP-MS 檢測不得高於 0.2 ppm 以上) Hg not more than 0.1 ppm by ICP-MS test (以 ICP-MS 檢測不得高於 0.1 ppm 以上) As not more than 1 ppm by ICP-MS test (以 ICP-MS 檢測不得高於 1 ppm 以上) Cu not more than 10 ppm by ICP-MS test (以 ICP-MS 檢測不得高於 10 ppm 以上)		
10. Pesticides (CNS 13570-2, 102 residues) (農藥殘留(CNS 13570-2, 102 種))	Not detect or not more than 0.3ppm (不得檢出或<0.3 ppm)		

Batch 1 page 4

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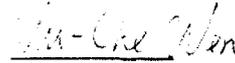
15th Fl., No.27-6,Sec.2,Chung Cheng East Rd.,
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國鼎牛樟芝產品檢驗報告書
QC Report of GDAC

Product name : Antrodia Camphorata		Product No. : GDAC0703	
Report No. : RP960413-1		Sampling date : 2007.04.09	
Sample : 30g		Report date : 2007.04.13	
Test Item (檢驗項目)	Specification (規格)	Result (結果)	Verifier (檢驗者)
11. Total Bacterial Count (總生菌數)	Not detect or not more than 10^4 CFU/g (不得檢出或 $<1 \times 10^4$ CFU/g)		
12. Mold & Yeast (黴菌與酵母菌)	Not detect or not more than 10^4 CFU/g (不得檢出或 $<1 \times 10^4$ CFU/g)		
13. <i>E. coli</i> (大腸桿菌)	Negative or $<1 \times 10$ MPN/g (陰性或 $<1 \times 10$ MPN/g)		

Result :

Authority Signature



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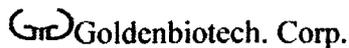
國鼎牛樟芝產品檢驗報告書**QC Report of GDAC****Product name : GDAC****Product No. : GDAC0704****Report No. : RP960413-2****Sampling date : 2007.04.09****Sample : 30g****Report date : 2007.04.13**

Test Item (檢驗項目)	Specification (規格)	Result (結果)	Verifier (檢驗者)
1. General properties (性狀)	The orange/light orange capsule(500mg) (500 mg 橘/淺橘膠囊)		
2. Loss on drying (乾燥減重)	Not more than 13.0 % (13.0 % 以下)		
3. Water-soluble extractives (水抽提物)	Not less than 32.0 % (32.0 % 以上)		
4. Diluted alcohol-soluble extractives (稀醇抽提物)	Not less than 30.0 % (30.0 % 以上)		
5. Total ash (總灰分)	Not more than 10.0 % (10.0 % 以下)		
6. Acid-insoluble ash (酸不溶性灰分)	Not more than 5.0 % (5.0 % 以下)		
7. Average capsule weight (20 capsule) (平均膠囊重量-20 粒膠囊平均)	610±30mg/capsule (610±30mg/膠囊)		
8. The assay of Antroquinonol(mg/g) (安卓奎諾爾 Antroquinonol 含量 mg/g)	2.2±0.5mg/g (2.2±0.5mg/g)		

*Calibration curve of Antroquinonol:

(Linear range : 31.25~1000µg/mL) Y=0.027X-0.079 R=1

Conc. (µg/ml.)	Peak area (V*sec)	Average	SD	CV (%)
31.25	0.8146	0.8199	0.0048	0.6%
	0.8212			
	0.8240			
62.5	1.6467	1.6498	0.0035	0.2%
	1.6492			
	1.6537			
125	3.3454	3.3496	0.0037	0.1%
	3.3519			
	3.3514			

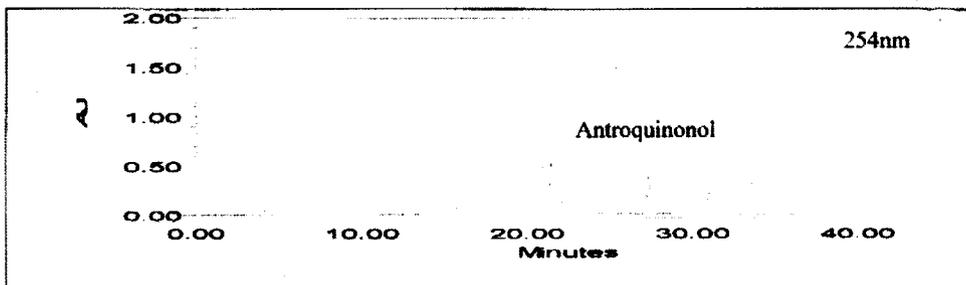
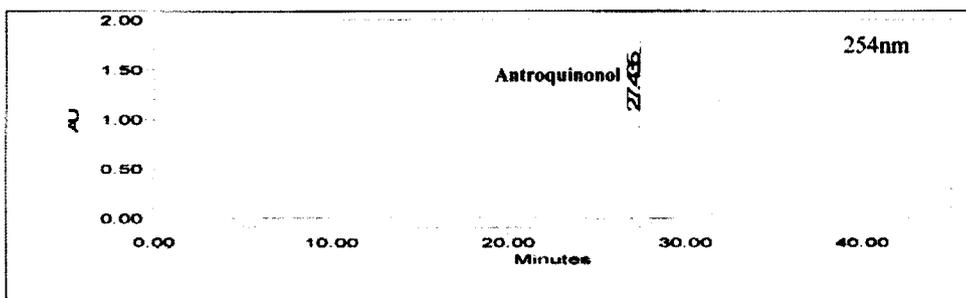


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國鼎牛樟芝產品檢驗報告書

QC Report of GDAC

Conc. (µg/ml.)	Peak area (V*sec)	Average	SD	CV (%)
250	6.7635	6.7786	0.0137	0.2%
	6.7817			
	6.7904			
500	13.7412	13.6923	0.0430	0.3%
	13.6751			
	13.6605			
1000	27.3715	27.4980	0.1138	0.4%
	27.5302			
	27.5922			



RT (min)	Area (V*sec)	Antroquinonol (mg/g)
27.318	4.9073	2.18

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國鼎牛樟芝產品檢驗報告書
QC Report of GDAC

Product name : GDAC		Product No. : GDAC0704	
Report No. : RP960413-2		Sampling date : 2007.04.09	
Sample : 30g		Report date : 2007.04.13	
Test Item (檢驗項目)	Specification (規格)	Result (結果)	Verifier (檢驗者)
9. Divide heavy metal test (個別重金屬檢驗) Pb (鉛試驗) Cd (鎘試驗) Hg (汞試驗) As (砷試驗) Cu (銅試驗)	Pb not more than 2 ppm by ICP-MS test (以 ICP-MS 檢測不得高於 2 ppm 以上) Cd not more than 0.2 ppm by ICP-MS test (以 ICP-MS 檢測不得高於 0.2 ppm 以上) Hg not more than 0.1 ppm by ICP-MS test (以 ICP-MS 檢測不得高於 0.1 ppm 以上) As not more than 1 ppm by ICP-MS test (以 ICP-MS 檢測不得高於 1 ppm 以上) Cu not more than 10 ppm by ICP-MS test (以 ICP-MS 檢測不得高於 10 ppm 以上)		
10. Pesticides (CNS 13570-2, 102 residues) (農藥殘留(CNS 13570-2, 102 種))	Not detect or not more than 0.3ppm (不得檢出或<0.3 ppm)		

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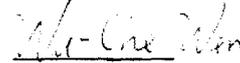
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國鼎牛樟芝產品檢驗報告書
QC Report of GDAC

Product name : Antrodia Camphorata		Product No. : GDAC0704	
Report No. : RP960413-2		Sampling date : 2007.04.09	
Sample : 30g		Report date : 2007.04.13	
Test Item (檢驗項目)	Specification (規格)	Result (結果)	Verifier (檢驗者)
11. Total Bacterial Count (總生菌數)	Not detect or not more than 10^4 CFU/g (不得檢出或 $<1 \times 10^4$ CFU/g)		
12. Mold & Yeast (黴菌與酵母菌)	Not detect or not more than 10^4 CFU/g (不得檢出或 $<1 \times 10^4$ CFU/g)		
13. <i>E. coli</i> (大腸桿菌)	Negative or $<1 \times 10$ MPN/g (陰性或 $<1 \times 10$ MPN/g)		

Result :

Authority Signature



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國鼎牛樟芝產品檢驗報告書**QC Report of GDAC**

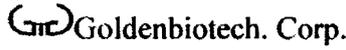
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Report No. : RP960413-3	Sampling date : 2007.04.09
Sample : 30g	Report date : 2007.04.13

Test Item (檢驗項目)	Specification (規格)	Result (結果)	Verifier (檢驗者)
1. General properties (性狀)	The orange/light orange capsule(500mg) (500 mg 橘/淺橘膠囊)		
2. Loss on drying (乾燥減重)	Not more than 13.0 % (13.0 % 以下)		
3. Water-soluble extractives (水抽提物)	Not less than 32.0 % (32.0 % 以上)		
4. Diluted alcohol-soluble extractives (稀醇抽提物)	Not less than 30.0 % (30.0 % 以上)		
5. Total ash (總灰分)	Not more than 10.0 % (10.0 % 以下)		
6. Acid-insoluble ash (酸不溶性灰分)	Not more than 5.0 % (5.0 % 以下)		
7. Average capsule weight (20 capsule) (平均膠囊重量-20 粒膠囊平均)	610±30mg/capsule (610±30mg/膠囊)		
8. The assay of Antroquinonol(mg/g) (安卓奎諾爾 Antroquinonol 含量 mg/g)	2.2±0.5mg/g (2.2±0.5mg/g)		

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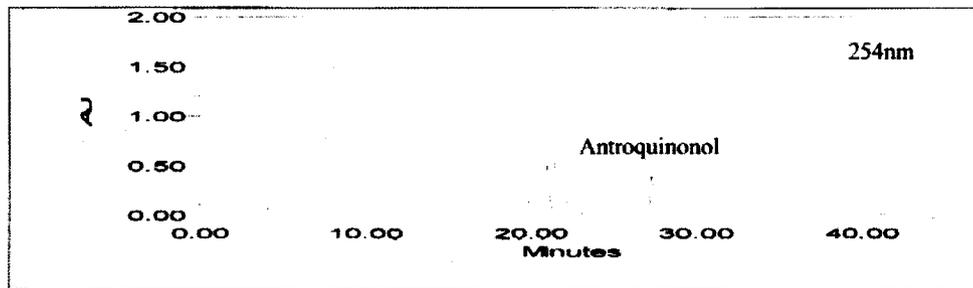
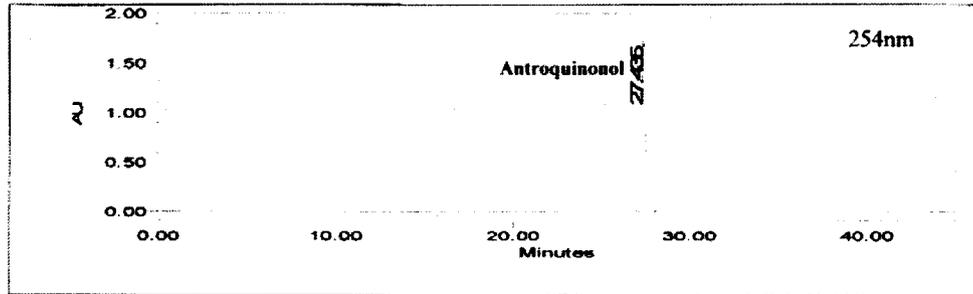


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國鼎牛樟芝產品檢驗報告書

QC Report of GDAC

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RT (min)	Area (V*sec)	Antroquinonol (mg/g)
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國鼎牛樟芝產品檢驗報告書

QC Report of GDAC

Product name : GDAC		Product No. : GDAC0705	
Report No. : RP960413-3		Sampling date : 2007.04.09	
Sample : 30g		Report date : 2007.04.13	
Test Item (檢驗項目)	Specification (規格)	Result (結果)	Verifier (檢驗者)
9. Divide heavy metal test (個別重金屬檢驗) Pb (鉛試驗) Cd (鎘試驗) Hg (汞試驗) As (砷試驗) Cu (銅試驗)	Pb not more than 2 ppm by ICP-MS test (以 ICP-MS 檢測不得高於 2 ppm 以上) Cd not more than 0.2 ppm by ICP-MS test (以 ICP-MS 檢測不得高於 0.2 ppm 以上) Hg not more than 0.1 ppm by ICP-MS test (以 ICP-MS 檢測不得高於 0.1 ppm 以上) As not more than 1 ppm by ICP-MS test (以 ICP-MS 檢測不得高於 1 ppm 以上) Cu not more than 10 ppm by ICP-MS test (以 ICP-MS 檢測不得高於 10 ppm 以上)		
10. Pesticides (CNS 13570-2, 102 residues) (農藥殘留(CNS 13570-2, 102 種))	Not detect or not more than 0.3ppm (不得檢出或<0.3 ppm)		

 Goldenbiotech. Corp.

15th Fl., No.27-6,Sec.2,Chung Cheng East Rd.,
Tamsui Town, Taipei 251, Taiwan
TEL : (886)-2-2808-6006 · FAX : (886)-2-2808-6007

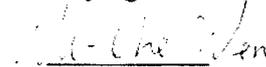
國鼎牛樟芝產品檢驗報告書
QC Report of GDAC

Product name : Antrodia Camphorata	Product No. : GDAC0705
Report No. : RP960413-3	Sampling date : 2007.04.09
Sample : 30g	Report date : 2007.04.13

Test Item (檢驗項目)	Specification (規格)	Result (結果)	Verifier (檢驗者)
11. Total Bacterial Count (總生菌數)	Not detect or not more than 10^4 CFU/g (不得檢出或 $<1 \times 10^4$ CFU/g)	Not detect	Wu-Che Wen
12. Mold & Yeast (黴菌與酵母菌)	Not detect or not more than 10^4 CFU/g (不得檢出或 $<1 \times 10^4$ CFU/g)	Not detect	Wu-Che Wen
13. <i>E. coli</i> . (大腸桿菌)	Negative or $<1 \times 10$ MPN/g (陰性或 $<1 \times 10$ MPN/g)	Negative	Wu-Che Wen

Result :

Authority Signature



Wu-Che Wen
Vice Presiden

Physiological Activities of *Antrodia camphorata*, a Unique Fungal Species in Taiwan

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Antrodia camphorata (Wu, Ryvardeen & Chang) is a unique fungal species found in the forest of Taiwan using *Cinnamomum kanehirai* Hay as its sole host. The fruiting body of *A. camphorata* has long been used as a remedy for various diseases as in folk medicine. The natural produced fruiting body of *A. camphorata* contains large amount of special type triterpenoids and the related components. However, the natural source of the fruiting body is exhausting due to an over-poaching and currently the collection of the fungus is prohibited. Until recently, the artificial production of the fruiting body has been successfully developed after intensive trials. Biological evaluations on in vitro cytotoxicity (5000 µg/ml), genocytotoxicity (5000 µg/ml), and acute toxicity (2000 mg/kg) assured a high safety for the product. Preliminary studies on the cultivated fruiting body showed that the triterpenoid pattern had a high resemblance to that of natural fruiting body and the crude ethanolic extract show strong potency in anti-inflammation that inhibited the expression of COX-2 and INOs at a concentration of 20 µg/ml. HL-60 cell line model for the changes in alkaline phosphatase activity, G1/S/G2M ratio in cell cycle and MTT assay was evaluated to find a 85% reversion rate of the cell line to a non-invasive level at a concentration of 50 µg/ml. Trigger of monocyte into dendritic cell with extracts from *Antrodia camphorata* also demonstrated the presence of CD86 under flowcytometric investigation. In this connection, an intensive study for the cultivated fruiting body from *Antrodia camphorata* in the preventing of aging-associated disorders in the levels of crude extract as well as the pure individual compound is launched.

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Statement of Response on Selling Distribution

Antrodia camphorata (GD-AC) has been released in Taiwan's market from year 2004 until October 2007. The total number distribution of GD-AC was approximately 80,000 bottles from the period of time with around 50,000 consumers nationwide. There were no negative reports or side-effects came into sight on the Taiwan's market.


Eric Chan

General Manager of Goldenbiotech



DEPARTMENT OF HEALTH EXECUTIVE YUAN REPUBLIC OF CHINA

Taipei, Taiwan, R. O. C. P. O. Box: 91-103 Taipei Fax: +886-2-2392-9723

CERTIFICATE OF FREE SALES AND MANUFACTURE

Article:

GOLDEN ANTRODIA CAMPHORATA CAPSULE

(國鼎牛樟芝膠囊)

Name of Applicant:

GOLDEN BIOTECHNOLOGY CORP.

(國鼎生物科技股份有限公司)

**(15F, No. 27-6, Sec. 2, Jhongjheng E. Rd., Danshuei Township, Taipei County
251, Taiwan, ROC.)**

TO WHOM IT MAY CONCERN

This is to certify that the food product mentioned above is produced and sold freely in Taiwan, the Republic of China. This Department does not object to the sale of the above-mentioned product abroad and this document is issued for certifying the export of this product to oversea markets.

Issued on July 13, 2007



Signature of Authorized Officer

David Cheng

David H. Cheng, Ph.D.

Director

Bureau of Food Safety

中華民國行政院衛生署

台北市愛國東路100號

台北市郵局第91-103號信箱

電話 23210151

NINETY-DAY SAFETY ASSESSMENT
and
GENETIC TOXICITY ASSESSMENT of
GD-Antrodia camphorata
MANUFACTURED BY
GOLDEN BIOTECHNOLOGY CORP

Applicant Entity

Golden Biotechnology Corp.

Taipei, Taiwan (R.O.C.)

Investigation Institute

R&D Division, Laboratory Animal Center

College of Medicine, National Taiwan University

Taipei, Taiwan (R.O.C.)

Principal Investigator:

Dr. Ming-Feng Wu

March, 2007

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A. Objective

The aim of this trial is to test for potential toxic effects of GD-*Antrodia camphorata* (herbal supplement, manufactured by Golden Biotechnology Corp, Taipei, Taiwan) on the mammals (mice) tested after repetitive oral administration for 90 days. This trial is to understand the occurrence of toxicity change and the body part that would be affected, and to determine the no-observed-adverse effect level (NOAEL) of this substance at the same time.

B. Trial Substances

Commercial Name: GD-*Antrodia camphorata*

Chemistry generic name: *Antrodia camphorata*

Purity: 100%

Appearance: Light Brown Powder

Stability: Stable in room temperature

Storage condition: dry and cool place

Lot No.: GDACS0703

C. Trial Methods

1. Experimental Animals

a. Reasons for Using the Selected Animals

SPF (Specific Pathogen Free) male and female Balb/c mice (produced by Laboratory Animal Center, College of Medicine, National Taiwan University) were selected in conform to the standard set for 90 days repetitive oral administration of toxic substances by the Department of Health, Taiwan, R.O.C. Monitoring of the health conditions of these animals was performed before subjecting to the trial.

b. Age and Number of Animals When Purchased

Forty male and forty female Balb/c mice, all four-week old of age, were purchased. They were raised without any experiment procedure until they reached six-week old and the trial starts.

c. Taming Time

The animals were kept in the facility without any test for two weeks after they were purchased in order to get accustomed new environment. General states of each animal, including feeding, drinking, motion and external appearance, were monitored everyday. The trial starts when all terms meet the condition for carrying out the trial.

d. Trial-starting Age

Drug administration trial starts when both the male and the female animals reached six-week old.

2. Animal Husbandry

a. Method

(1) Environment

Clean animal room with temperature set at 20 ± 2 °C, humidity set at 75% \pm 15%, and lights-on time set for 12 hours was utilized. The temperature, humidity and light-dark cycle were monitored everyday to ensure the environmental conditions were always within the set range.

(2) Cages

PC cages with dimensions 260 mm in length, 160 mm in width and 120 mm in height (with stainless steel lids) were used for housing the animals. Each cage basically contained 10 mice during the taming period. For experimental purposes, after the animals were numbered, five mice were grouped for each cage. Every cage was labeled with a tag with information of the name of the trial substance, dosage group, animal strain, sex and animal number.

Beddings were changed twice per week (every Monday and Thursday) basically. If the animal generated waste faster than usual, the frequency of the change increased accordingly. Rack space of the cages was exchanged with bedding change to provide equal illumination on all animals. When exchanging, individual cage was moved one level below from the original level on the rack, and the lowest level cage was moved to the top level of the rack.

(3) Grouping of Animals

Trial animals were the healthy population selected from the purchased animals after the taming period. Body weight of each animal was recorded on the start date of drug administration. Ten animals were grouped separately into each of the control group, the GD-*Antrodia camphorata* experimental group of different dosage (low, medium, high) according to the randomized table.

(4) Labeling of the Animals

Individual animal was labeled by applying picric acid solution to a part of the fur of each animal.

b. Feed

The feed used in this trial was the commercially available Laboratory Rodent Diet 5001 manufactured by PMI[®] Nutrition International. Periodic microbial monitoring was performed on the feed.

c. Drinking water and water bottle

Drinking water from Taipei Water Department was supplied ad libitum to animals and was periodically detected for microbes in the water. The water bottles were washed every week by first immersing the bottles in hydrochloric acid and then rinsed with water. After that, it was washed once again with 0.5% Chlorox. The water bottle head was sonicated for 15 minutes in hot water containing one cap of Chlorox.

3. Administration Method And Time of Trial Substances

Administered doses of GD-*Antrodia camphorata*:

the low dose was set at 0.335 mg/0.3 c.c./25g mouse;

the medium dose was set at 16.75 mg/0.3 c.c./25g mouse;

the high dose was set at 26.8 mg/0.3 c.c./25g mouse.

The trial substance was administered through feeding tubes, once per day orally, 0.3 c.c. for 90 consecutive days.

4. Determination Of Dosage And Number Of Animals

Forty male and forty female mice were grouped into 10 animals each of a blank control group that does not have any treatment and 10 animals each of the three experimental groups that each receives different doses of GD-*Antrodia camphorata*.

5. Examination Items

a. Clinical Symptoms

General external appearances such as vivacity and hair color were observed daily. Palpation (including detailed clinical sign examination) of animal was performed at least once per week. Time of discovery, type, level of seriousness and persistence time were all recorded in detail once any clinical signs were seen.

b. Mortality

Mortality of each group was calculated as the ratio using the total number of the animals that died and those which were dying and were euthanized as the numerator, and the number of effective animal as the denominator.

c. Body Weight

Body weights of trial mice were taken at the beginning of the trial before the trial substance was administered and again measured at least once weekly after that.

d. Hematological Examination

Before the experiment starts, blood samples of all the animals were collected by orbital bleeding and subjected to hematological examination. The experiment starts after the animals were determined to have normal hematological profile. After 90 days of consecutive drug administration, all animals again had their blood samples collected by orbital bleeding for hematological examination. A portion (about 20 ml) of the blood collected was taken by EDTA-containing

capillary tubes for the detection of the items listed in Table 1 with Medonic CA530 automated cell analyzer.

RBC(red blood cell concentration) $10^6/\text{mm}^3$	HCT(hematocrit) %	HGB(total hemoglobin concentration) g/dl
RDW% (red blood cell volume distribution width) %	RDW _a (absolute red blood cell distribution width) μm^3	MCV(mean corpuscular volume) μm^3
MCH(mean corpuscular hemoglobin) pg	MCHC(mean corpuscular hemoglobin concentration) g/dl	PLT (platelet) $10^3/\text{mm}^3$
PCT (platelet crit) %	PDW (platelet distribution width) μm^3	MPV (mean platelet volume) μm^3
LPCR (large platelet) %	WBC(white blood cell) $10^3/\text{mm}^3$	LYMF (lymphocyte) $10^3/\text{mm}^3$
GRAN (granulocyte) $10^3/\text{mm}^3$	MID (mid-range cell) $10^3/\text{mm}^3$	

Table 1 Examination Items (Abbreviation)

e. Blood Biochemistry Examination

1. After 90 days of consecutive drug administration, all animals were subjected to blood biochemistry exam. Serum samples were collected from blood samples that were let sit briefly and then centrifuged at 3,000rpm for 5 minutes. The serum samples were subjected detection in reference to items in Table 2 with Arkray Spotchem SP-4410 biochemistry analyzer.

TP (total protein)	BUN (blood urea nitrogen)	Creat (creatinine)	SGPT (ALT)	SGOT (AST)
Glu (glucose)	T-Chol (total cholesterol)	ALB (albumin)	T-Bil (total bilirubin)	

Table 2 Examination Items (Abbreviation)

2. Blood electrolytes (sodium, potassium and chloride) were determined with the above prepared serum by ARKRAY SPOTCHEM SE-1520 electrolyte analysis system.

f. Autopsy and Harvesting of Tissues and Organs

Animals died during the trial period were dissected as soon as possible to grossly observe for changes in tissues and organs. If possible, major organs were weighted separately and histopathological examination conducted to find out the reason of death and the characteristics of toxic changes (such as the level of seriousness). Dying animals are euthanized to obtain toxicity data at a greater extend. Clinical observations of each animal were recorded before they are euthanized. If possible, blood samples were collected for blood and serum biochemical analysis. Necropsy was performed for gross observation of changes in the organ and tissues and for histopathological examination to understand the characteristics of the toxicity change (such as the level of seriousness). The weights of the major organs were recorded if needed for the trial. All animals were euthanized at the end of the trial (the period when trial substances were given). Blood samples were collected before autopsy for blood and serum biochemical analysis. During necropsy, changes in the organs and tissues were observed and recorded. Organs including brain (cerebra, cerebellum, pons, and medulla oblongata), heart, lung, liver, pancreas, kidney (bilateral), intestines, uterus (bilateral), ovary (bilateral), and testis (bilateral) were fixed by 10% neutral formalin.

g. Pathohistological Examination

Pathohistological examination was performed on the organs (lung, liver, spleen, kidney (bilateral), intestines). The result is basically determined by optical microscopic observation of H&E stained sections.

D. Results

1. Clinical Symptoms

As of external features, there was no special difference between the experimental and the control groups. The luster of the animals had no significant change too.

2. Mortality

None of the animals died during the experimental period.

3. Body Weight

No significant weight differences were observed between the control group and the experimental group (figure 1 and figure 2 in Attachment).

4. Feed Efficiency

Comparing the feed uptake between the experimental groups versus the normal control group it was noticed that in the male mice the lower dosage group has a higher food intake than the control group. Decreased food intake was also observed with increased drug dosage. Female mice basically showed the same trend as the male mice. When referred to the glucose value derived from the blood biochemistry tests, it is shown that the decrease in the food intake was relative to the stability of blood glucose, and has lesser relationship to the generation of toxicity by the drug (figure 3 and figure 4 in Attachment).

5. Hematological Test

No significant change was observed in the hematological test when comparing the GD-*Antrodia camphorata* -administered experimental groups to the control group (table 3 and table 4 in Attachment).

6. Blood Biochemistry Examination

No significant change was observed in the GD-*Antrodia camphorata*-administered experimental groups (table 5 and table 6 in Attachment); also, no significant change was observed in the electrolytes examination (sodium, potassium and chloride) (table 7 and table 8 in Attachment).

7. Autopsy

Autopsy of the organs did not show any significant gross pathological change; when comparing the weights of the liver, spleen and kidneys, no significant change was observed too (figure 5 and figure 6 in Attachment).

8. Pathohistological Examination

Major organs were selected for representative microscopic examination. No significant pathohistological change was observed. That is, we did not observed any accumulation of inflammatory cells, hyperplasia of cells or metaplasia of tissues, etc. Other organs not selected were preserved in formalin.

E. Attachments:

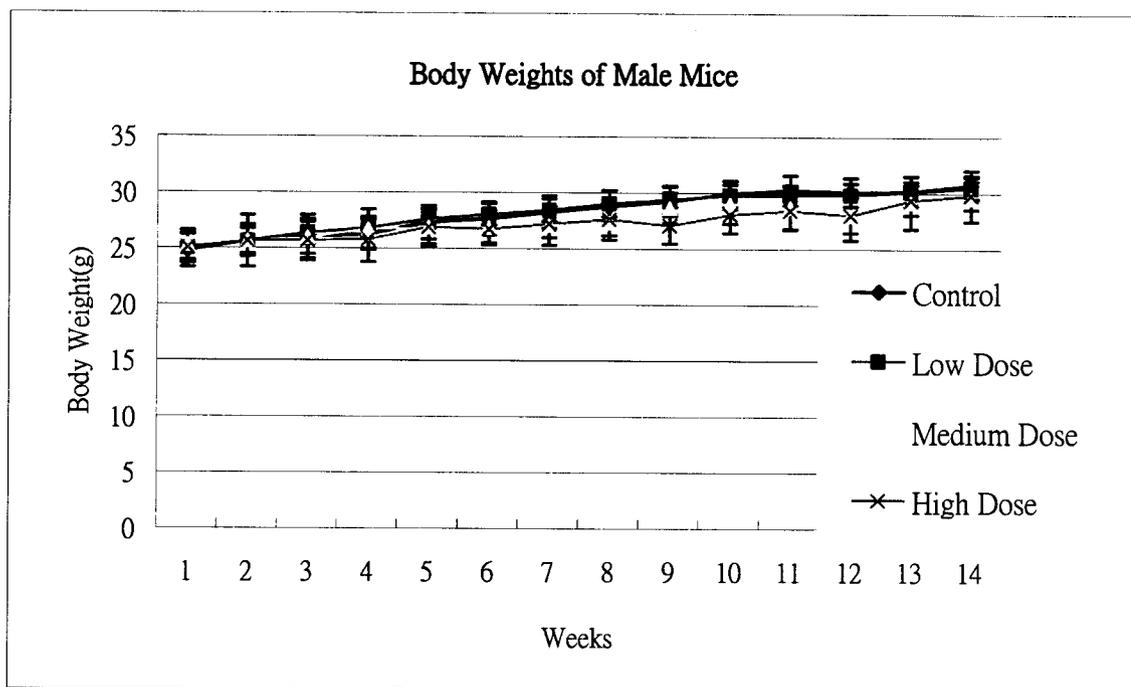


Figure 1 Male mice body weight with respect to AC intakes against time (n=10)

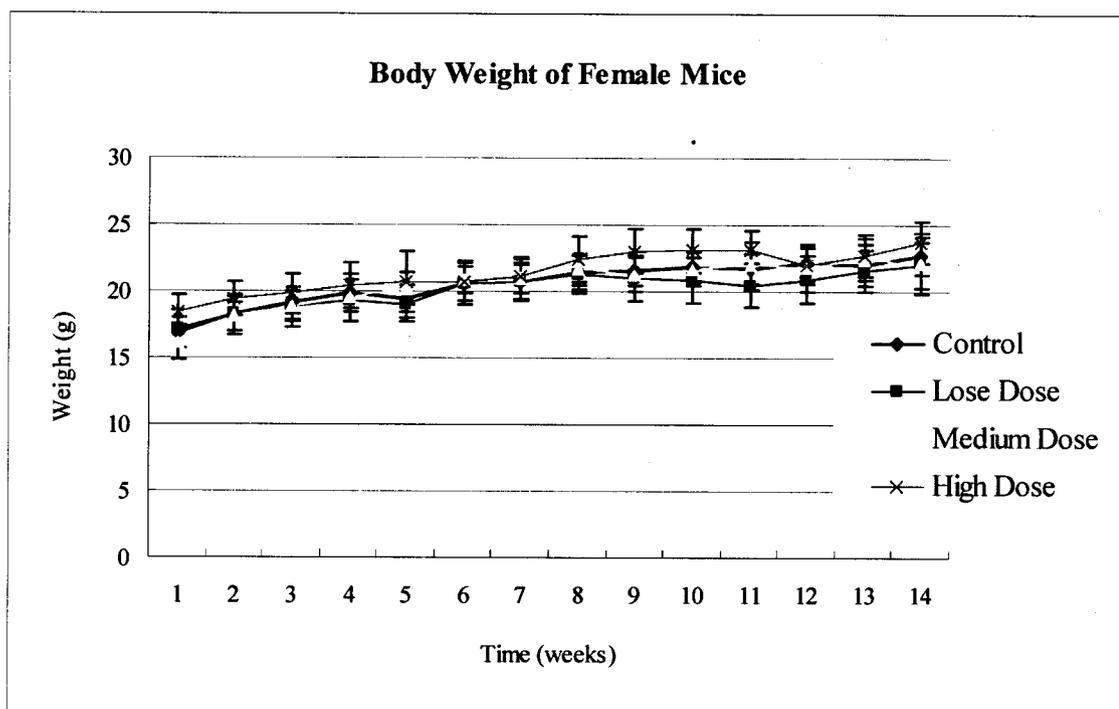


Figure 2 Female mice body weight with respect to AC intakes against time (n=10)

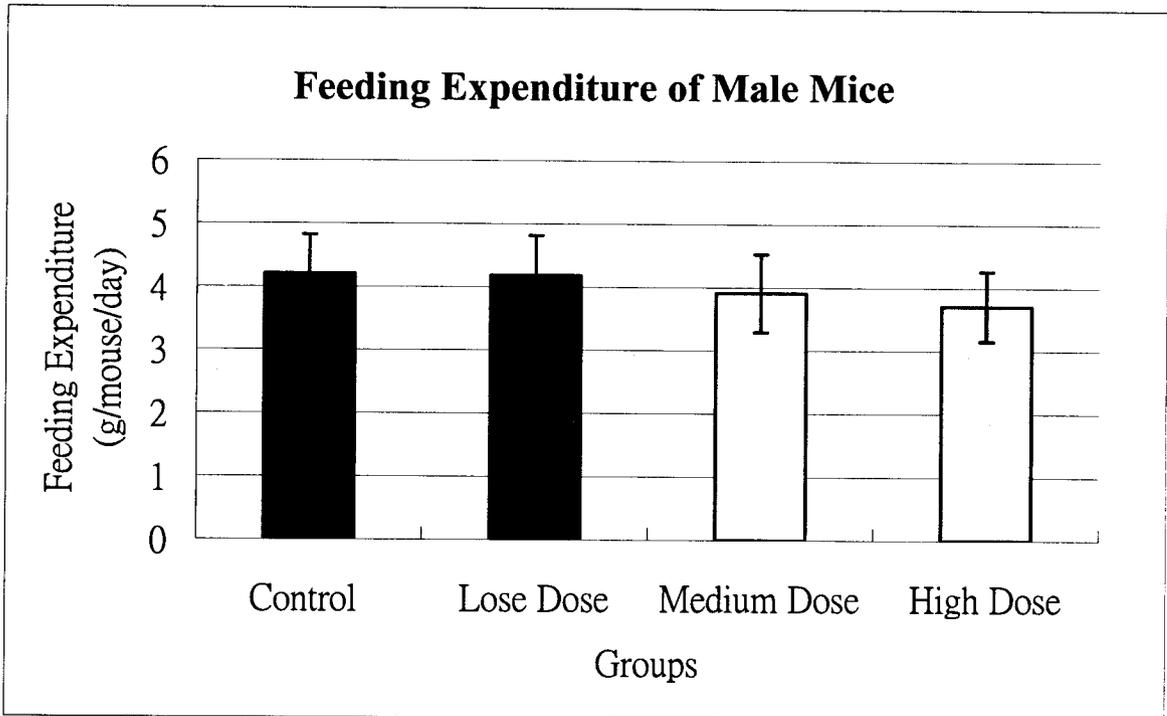


Figure 3 Feeding expenditure of male mice with respect to AC intakes (n=10)

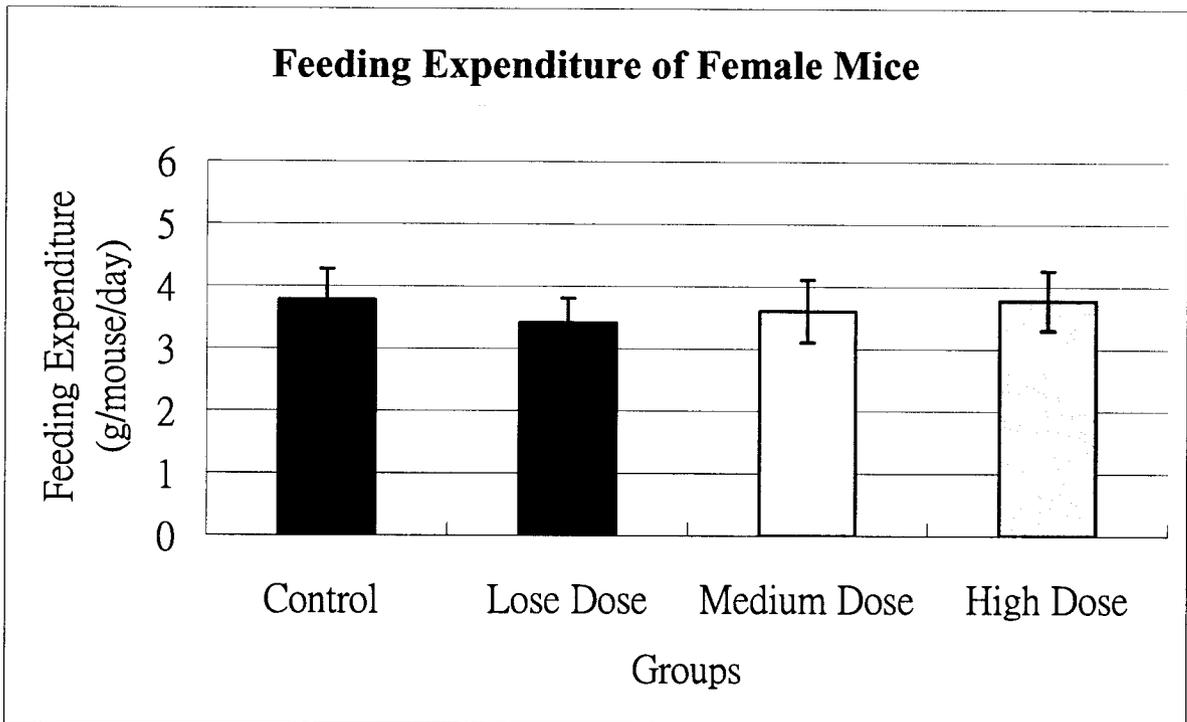


Figure 4 Feeding expenditure of male mice with respect to AC intakes (n=10)

	RBC (mm)	MCV (um)	RDW% (%)	RDWa (um)	HCT (%)	PLT (mm)	MPV (um)	PDW (um)
Control	10.93±0.89	50.64±0.74	15.43±0.73	36.87±1.1	55.37±4.32	817±167.48	6.76±0.21	8.9±0.32
Low Dose	11.21±0.65	51.03±1.44	15.6±1.09	37.37±1.8	57.28±3.61	942.4±183.02	6.73±0.14	8.84±0.19
Medium Dose	11.32±0.86	50.45±0.55	15.51±0.7	36.7±0.78	57.11±4.28	928.9±217.64	6.83±0.25	9.01±0.39
High Dose	11.42±0.97	52.16±1.13	15.52±0.8	38.42±1.24	59.58±5.03	965.08±219.8	6.81±0.21	8.94±0.31

	PCT (%)	LPCR (%)	WBC (mm)	HGB (g/dl)	MCH (pg)	MCHC (g/dl)	LYMF (mm)	GRAN (mm)
Control	0.52±0.14	10.41±1.53	5.53±2.01	15.74±1.25	14.38±0.27	28.45±0.28	2.93±1.2	1.74±0.81
Low Dose	0.67±0.14	10.31±0.9	5±1.36	16.2±0.97	14.43±0.32	28.32±0.29	2.53±1.39	1.66±0.58
Medium Dose	0.68±0.17	10.82±1.99	3.85±0.98	16.28±1.2	14.38±0.39	28.53±0.62	1.73±1.17	1.47±0.31
High Dose	0.66±0.16	11.14±1.53	4.51±1.44	16.37±1.42	14.32±0.32	27.44±0.21	2.22±1.02	1.54±0.57

	MID (mm)	LYMF (%)	GRAN (%)	MID (%)
Control	0.87±0.43	53.34±18.35	31.83±11.32	14.83±7.11
Low Dose	0.82±0.22	48.86±18.21	35.81±14.18	15.33±4.25
Medium Dose	0.58±0.09	44.6±17.45	38.2±12.25	14.74±3.2
High Dose	0.75±0.22	49.16±15.74	35.29±10.33	15.55±5.7

Table 3 Hematological Analysis of Male GD-*Antrodia camphorata*-administered Balb/c Mice (n=10)

	RBC (mm)	MCV (um)	RDW% (%)	RDWa (um)	HCT (%)	PLT (mm)	MPV (um)	PDW (um)
Control	10.46±0.48	51.81±1.04	15.13±0.66	37.55±1.19	54.18±2.04	514.77±29.27	6.36±0.27	8.45±0.44
Low Dose	10.64±0.79	50.22±0.85	14.56±0.59	35.52±1.15	53.42±3.8	537.92±59.25	6.32±0.19	8.42±0.34
Medium Dose	10.76±0.71	50.71±0.88	15.18±0.75	36.58±1	54.59±3.66	552.62±91.14	6.4±0.33	8.54±0.55
High Dose	10.73±0.96	50.31±0.76	14.91±0.85	35.91±1.35	54.04±5.24	598.14±53.32	6.39±0.21	8.43±0.3

	PCT (%)	LPCR (%)	WBC (mm)	HGB (g/dl)	MCH (pg)	MCHC (g/dl)	LYMF (mm)	GRAN (mm)
Control	0.32±0.02	8.67±2.16	5.95±2.05	15.39±0.63	14.71±0.24	28.42±0.27	3.24±1.45	2.19±0.7
Low Dose	0.34±0.04	8.43±1.68	5.66±1.29	15.17±1.04	14.25±0.27	28.42±0.32	2.94±0.93	2.16±0.72
Medium Dose	0.35±0.05	8.62±2.35	4.48±1.06	15.34±0.98	14.25±0.29	28.11±0.38	1.75±0.85	2.24±0.96
High Dose	0.38±0.04	8.68±1.58	5.05±1.97	15.19±1.42	14.14±0.33	28.14±0.36	2.22±0.8	2.41±1.62

	MID (mm)	LYMF (%)	GRAN (%)	MID (%)
Control	0.52±0.16	52.95±9.22	38.26±7.96	8.78±3.88
Low Dose	0.56±0.19	51.88±8.7	38.69±10.51	9.42±3.43
Medium Dose	0.49±0.32	45.14±11.49	45.22±12.36	9.78±5.52
High Dose	0.41±0.12	47.79±12.98	46.51±14.72	8.57±4.39

Table 4 Hematological Analysis of Female GD-*Antrodia camphorata*-administered Balb/c Mice (n=10)

	Glu (mg/dl)	T-Cho(mg/dl)	BUN(mg/dl)	T-Bil(mg/dl)	GOT(IU/L)	GPT(IU/L)
Control	157.25±38.39	70.83±8.34	26.58±2.64	0.31±0.2	264.5±231.29	89.3±97.43
Low Dose	148±22.7	72.17±7.57	26.25±2.42	0.13±0.18	191.5±103.23	63.67±34.18
Medium Dose	144.17±25.88	72.25±6.57	28.5±2.78	0.44±0.4	331.8±182.88	67.9±20.86
High Dose	161.77±20.47	68.77±5.7	30.23±3.94	0.43±0.38	253.09±196.97	59.55±18.44

	T-Pro(g/dl)	Cre (mg/dl)	Alb (g/dl)
Control	5±1.16	0.7±0.23	2.19±0.24
Low Dose	4.58±0.24	0.65±0.07	1.99±0.16
Medium Dose	5.08±0.43	0.68±0.11	2.15±0.21
High Dose	4.77±0.3	0.63±0.08	2.06±0.19

Table 5 Serum Biochemistry Analysis of Male *GD-Antrodia camphorata*-administered Balb/c Mice versus Control Mice (n=10)

	Glu (mg/dl)	T-Cho(mg/dl)	BUN(mg/dl)	T-Bil(mg/dl)	GOT(IU/L)	GPT(IU/L)
Control	109.92±29.52	82.46±13.92	24.08±6.7	0.49±0.34	222.09±215.86	80.18±39.1
Low Dose	108.38±27.53	83.92±17.72	32.38±9.28	0.4±0.37	265.2±131.71	74±16.47
Medium Dose	139.54±38.9	92.77±17.08	25.92±2.99	0.53±0.44	266.9±238.18	94.9±44.42
High Dose	126.21±20.75	95.5±12.49	27.93±4.16	0.44±0.39	262.75±252.82	87.5±39.48

	T-Pro(g/dl)	Cre (mg/dl)	Alb (g/dl)
Control	5.71±0.41	0.5±0.12	2.38±0.13
Low Dose	5.63±0.39	0.58±0.13	2.29±0.19
Medium Dose	5.9±0.64	0.51±0.17	2.38±0.21
High Dose	5.59±0.28	0.44±0.09	2.51±0.13

Table 6 Serum Biochemistry Analysis of Female GD-*Antrodia camphorata*-administered Balb/c Mice versus Control Mice

(n=10)

	Na	K	Cl
Control	132.83±8.21	11.39±1.57	114.45±2.46
Low Dose	136.58±5.26	8.79±1.18	115.25±2.42
Medium Dose	138.83±5.22	8.94±1.4	117.83±2.41
High Dose	134.31±3.77	8.22±0.71	115±2.08

Table 7 Sodium, Potassium and Chloride Analysis of Male GD-*Antrodia camphorata*-administered Balb/c Mice versus Control Mice (n=10)

	Na	K	Cl
Control	130.46±4.98	11.24±1.46	119.38±6.46
Low Dose	135.69±5.33	10.01±0.93	118±3.34
Medium Dose	132.77±3.61	11.12±1.15	119.15±1.99
High Dose	138.71±3.24	10.71±0.85	121.5±2.1

Table 8 Sodium, Potassium and Chloride Analysis of Female GD-*Antrodia camphorata*-administered Balb/c Mice versus Control Mice (n=10)

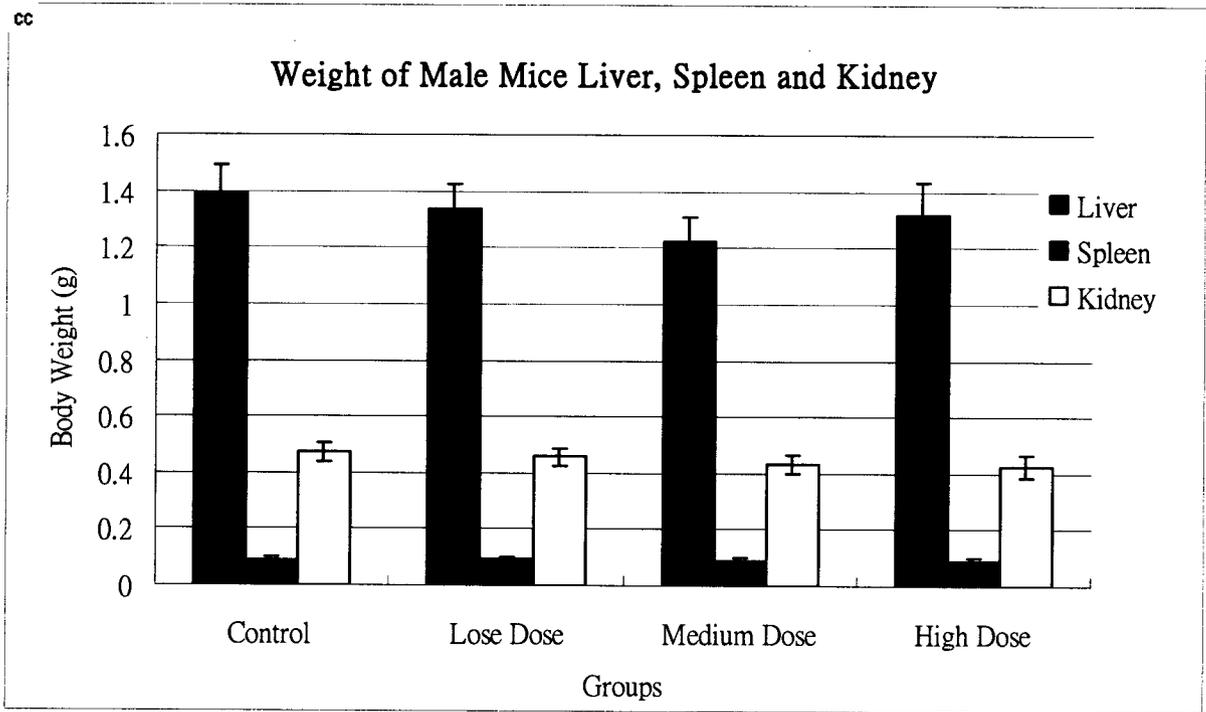


Figure 5 Comparison Chart of Male Mice Liver, Spleen, and Kidney Weights (n=10)

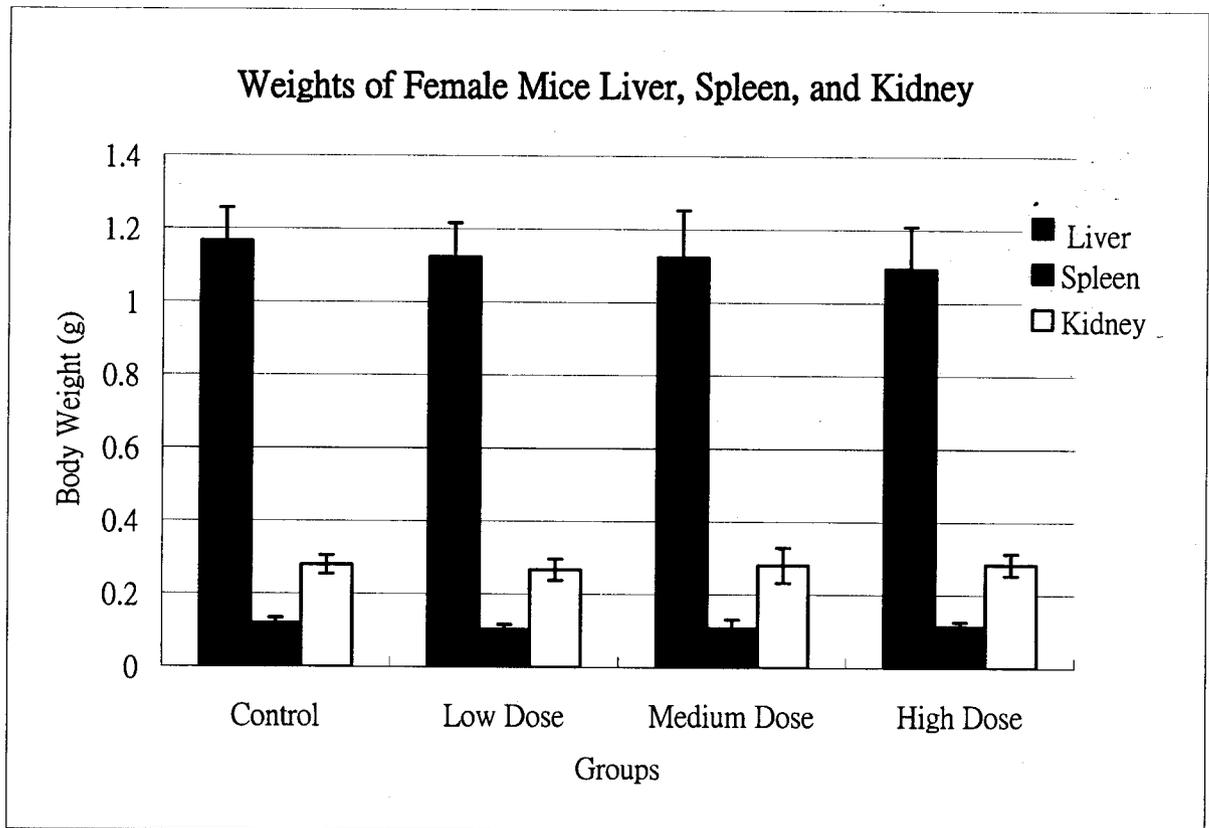


Figure 6 Comparison Chart of Female Mice Liver, Spleen, and Kidney Weights (n=10)

F. Abbreviations:

WBC	Total White Blood Cell
RBC	Total Red Blood Cell
HGB	Hemoglobin
HCT	Hematocrit
MCV	Mean Corpuscular Volume
MCH	Mean Corpuscular Hemoglobin
RDW%	RBC Distribution Width
RDWa	Absolute RBC Distribution Width
MCHC	Mean Corpuscular Hemoglobin Concentration
PLT	Platelet
PCT	Platelet Crit
PDW	Platelet Distribution Width
MPV	Mean Platelet Volume
LPCR	Large Platelet Count
LYMF	Lymphocyte
GRAN	Granulocyte
MID	Mid-Range Cells
Glu	Glucose
T-Cho	Total Cholesterol
BUN	Blood Urea Nitrogen
T-Bil	Total Bilirubin
Cre	Creatinine
T-Pro	Total Protein
Alb	Albumin

GENETIC TOXICITY ASSESSMENT OF
GD-Antrodia camphorata
MANUFACTURED BY
GOLDEN BIOTECHNOLOGY CORP

Applicant Entity

Golden Biotechnology Corp.
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Investigation Institute

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PART I:

BACTERIAL REVERSE MUTATION ASSAY

SUMMARY

This trial studied potential effects of GD-*Antrodia camphorata* samples on bacterial reverse mutation. In the trial, a series of mutated *Salmonella typhimurium* histidine auxotroph was used and reverse mutation were measured during the test. These mutated bacteria require addition of histidine in the culture media for growth. If in the presence of mutagens, the mutated *S. typhimurium* histidine auxotroph would display reverse mutation and convert into wild-type-like phenotype. By counting bacterial colonies on selective histidine-deficient medium, the testing substance would reveal its potency of mutagenesis.

The GD-*Antrodia camphorate* was dissolved in DMSO and prepared to a stock concentration of 50 mg/ml. Concentrations of the GD-*Antrodia camphorata* used in the bacterial gene mutation test were 3 mg/ml, 6 mg/ml, 12 mg/ml, 25 mg/ml and 50mg/ml. Diluted substances were made in DMSO solvent. Solvent control group (used as the negative control) and positive control group were also included. Two experimental treatments were applied to five bacterial strains (TA97, TA98, TA100, TA102, and TA1535). Treatment 1 tested bacterial strain with the trial substances for 30 minutes in the presence of the rat liver activating enzyme system (S9). Treatment 2 tested bacterial strain with the trial substances for 30 minutes in the absence of S9. Bacteria colony counts were conducted after the bacteria clones were added with 2 ml of top agar and incubated in an incubator for 48 hours.

We found that GD-*Antrodia camphorata* at all tested dosages did not cause significant increase in the number of mutagenic colony whether or not S9 is added in the treatment. The positive control group had significantly more revertants than the solvent control group.

Our result of GD-*Antrodia camphorata* tests for gene mutation showed that there was no evidence of causing gene mutation in bacteria.

INTRODUCTION

Taiwan Health Authority passed the Health Food Control Act in August, 1999. The Act requires pharmacological analysis and safety assessments of all marketed health food products. In the Act, there are four classes in safety assessment for health food precuts. The GD-*Antrodia camphorata* belongs to the Class 2. Genetic toxicity assay is a requirement for market clearance of Class 2 health food products. We used 9,000 X g-separated rat liver supernatant to simulate the metabolic activating reaction in the living body. With the action of the enzymes and some co-factors of the liver particulate body present in the supernatant, we analyzed whether GD-*Antrodia camphorata* would increase microbial gene mutation *in vitro*. We also analyzed whether GD-*Antrodia camphorata* would display mutagenicity before and after it is metabolized. This experimental model can provide risk assessment of whether GD-*Antrodia camphorata* is genetically toxic to human.

MATERIALS AND METHODS

Bacterial Strains

This trial used TA97, TA98, TA100, TA102 and TA1535 *Salmonella typhimurium* strains which have different characteristics, in order to verify the sensitivity of this test. These TA97, TA98, TA100, TA102 and TA1535 strains were bought from Hsin-Chu Food Science Research Institute, Hsin-Chu County, Taiwan, R.O.C.

Each of above bacterial strains owns a different histidine operon. Strain TA97, TA98, TA100 and TA1535 are Δ urB bacteria, which are defective in the DNA excision-repair system and display the presence of DNA damaging more easily. Strain TA97, TA98, TA100, TA102 and TA1535 are all rfa bacteria in which the lipopolysaccharide barrier of the cell walls are partially defective. This defectiveness allows for greater permeability of large molecular chemical substances. Meanwhile TA97, TA98, TA100 and TA102 have been transformed with the pKM101 plasmid, these cells, therefore, are apt to show mistake in DNA repairing. The pKM101 plasmid lowers the repair capability of the bacteria and renders the cells more susceptible to the trial substances.

Mice Liver Activating Enzyme Preparation

Beta-naphthoflavone was introduced to the mice to enhance production of

particulate body activating enzymes in the liver. Mouse livers were harvested, weighted, washed with buffered saline, and then cut/abraded into smaller pieces. After the tissue solution is centrifuged at 9,000Xg, the supernatant (S9) were collected and aliquot into cryogenic vials (2 ml/tube) and preserved at -70°C freezer. The supernatant was thawed on ice right before use.

The preparation of the S9 mixture was in accordance to the Dr. Ames' protocol reported in Mutation Research in 1983. Fresh S9 solutions were prepared for each test and leftovers were discarded after each test.

Dosages of Trial Substance

There were five dose groups in this trial: 3mg/ml, 6mg/ml, 12mg/ml, 25mg/ml and 50mg/ml.

Control Groups

The control groups included negative control and positive control groups. In the S9 solution mixture treatment, benzopyrene at 1µg/plate was used as positive mutagen control for TA98, and TA102 strains. 2-aminothracene at 4 µg/plate was used as positive mutagen control for TA97, TA100, TA1535 strain. In the treatment without S9 mixture, 4-nitroquinoline-N-oxide at 0.5 mg/plate was used as positive mutagen control for TA97 and TA98 strains. Mitomycin C at 0.5 mg/plate was positive mutagen control for TA102; sodium azide at 1mg/plate for TA100 and TA1535 strains.

Metabolism Activation

Metabolism activation was mimicked by the presence of S9 solution mixtures in experiments.

Test Method

A plate incorporation method was used here. Mix 0.1 ml trial substance solution with 0.1 ml of a 16-hour incubated bacteria culture broth (for those that underwent activation treatment, 0.5 ml of S9 is also added to the mixture). Add in 2 ml of 45°C soft agar solution that contains 0.2 ml of 0.5M histidine/biotin to the mixture and mix thoroughly. Pour the mixture onto minimal glucose plate. When the agar has harden, place the culture dish upside down in a 37°C incubator and culture

for 48 hours. Count the number of revertants after a 48-hour incubation period.

Preparation of GD-*Antrodia camphorata* Solution

The GD-*Antrodia camphorata* solution was prepared without adding the rat liver activating enzyme system (S9) as the following. For the highest dosage (50mg/ml of GD-*Antrodia camphorata*), 500mg trial substance was dissolved in 10 ml DMSO to make a 50 mg/ml GD-*Antrodia camphorata* solution. Other trial dosages (3 mg/ml, 6 mg/ml, 12 mg/ml, 25 mg/ml GD-*Antrodia camphorata*) were prepared by diluting the highest dosage GD-*Antrodia camphorata* with DMSO.

Verification of Valid Trials

The data was accepted by the trial only when the control results met the following criteria. In counting colonies on the negative control groups and the positive control groups, the colony count of TA97 negative control group bacteria was within 90-180; the colony count of TA98 negative control group bacteria was within 30-60; the colony count of TA100 negative control group bacteria was within 150-240; the colony count of TA102 negative control group bacteria was within 240-320; the colony count of TA1535 negative control group bacteria must be within 15-35; the count for the positive control groups was significantly higher than the negative control groups.

RESULTS AND DISCUSSION

Experimental data are averages of triplicates. Negative controls are solvent control without trial substances and positive controls are mentioned above. Experimental data would be discarded only if the positive control counts were not twice higher than the negative control counts.

Table 1 and Table 2 show effects of GD-*Antrodia camphorata* with or without the S9 treatment. Results of the GD-*Antrodia camphorata*-treated groups are not significantly different from those of the negative control groups no matter whether S9 activating enzyme system is present or absent in the experiment. These results suggest that before and after metabolic activation, the GD-*Antrodia camphorata* does not induce genotoxicity on TA97, TA98, TA100, TA102 and TA1535.

Due to that none of the drug dosage group showed significant increase in toxicity, further dose-response analysis is not necessary in this trial.

CONCLUSION

Based on the results of analysis of microbial gene mutation, we conclude that GD-*Antrodia camphorata*, acting like the negative controls, does not cause significant effect on the microbial gene mutation under the trial conditions.

TRIAL REVIEW

This trial was carried out based on the original trial proposal. No event which could impact quality and integrity of the trial happened during the period. We did not encounter any situation which could influence the trial results or interpretations.

Table 1. Colony counts of *Salmonella typhimurium* strains treated with GD-*Antrodia camphorata* in the presence of S9-induced metabolic activating enzymes.

	TA97	TA98	TA100	TA102	TA1535
Positive Control	610±42	376±17	409±24	556±25	85±2
50 mg sample/plate	130±17	171±5	259±4	336±14	39±1
25 mg sample/plate	129±4	130±11	273±12	302±13	38±8
12.5 mg sample/plate	116±15	110±4	257±21	295±12	31±11
6.25 mg sample/plate	102±3	120±6	239±13	275±12	21±4
3.125 mg sample/plate	110±12	117±3	225±14	253±11	15±3
Negative Control	120±5	106±4	200±21	286±11	44±1

Mutagens used in this test: benzopyrene (1 µg/plate, for TA98 and TA102); 2-aminoanthracene (4 µg/plate, for TA97, TA100 and TA1535).

Table 2. Colony counts of *Salmonella typhimurium* strains treated with GD-*Antrodia camphorata* in the absence of S9-induced metabolic activating enzymes.

	TA97	TA98	TA100	TA102	TA1535
Positive Control	336±17	63±8	107±10	342±8	65±13
50 mg sample/plate	121±4	12±3	58±6	146±1	12±2
25 mg sample/plate	97±7	17±1	59±7	164±11	11±3
12.5 mg sample/plate	127±4	14±2	56±4	145±13	11±1
6.25 mg sample/plate	97±1	13±1	45±5	135±18	10±2
3.125 mg sample/plate	112±8	12±1	49±5	133±7	14±1
Negative Control	100±3	12±3	62±16	185±18	13±4

Mutagens used in this test: 4-nitroquinoline-*N*-oxide (0.5 µg/plate, for TA97 and TA98); mitomycin C (0.5 µg/plate, for TA102); sodium azide (4 µg/plate, for TA100 and TA1535).

PART II:

***IN VITRO* STRUCTURAL CHROMOSOME ABERRATION ASSAY**

SUMMARY

This trial studied potential effects of the GD-*Antrodia camphorata* samples on *in vitro* structural chromosome aberration. The trial assay analyzed frequency of structural chromosome aberration of Chinese hamster ovary cells in metaphase. The obtained frequency was used to assess damaging power on chromosome structures caused by GD-*Antrodia camphorata*.

The GD-*Antrodia camphorata* was dissolved in culture medium and prepared to a stock concentration of 50 mg/ml. After complete dissolved, the sample solution was sterilized by passing a 0.22 µm filter. Five concentrations of GD-*Antrodia camphorata* (3 mg/ml, 6 mg/ml, 12 mg/ml, 25 mg/ml and 50 mg/ml) were prepared in culture medium and applied in the trial. Solvent control group (used as the negative control) and positive control group were also included.

Three different experimental treatments with duplicates were used. The first treatment was that the cells were incubated in the absence of the rat liver activating system (S9) for three hours. The second treatment method was that the cells were incubated in the presence of the rat liver activating system (S9) for three hours. The third treatment was that the cells were incubated in the absence of the S9 for 20 hours. The results of the chromosome smear analysis show that GD-*Antrodia camphorata* at all trial doses did not significantly increase the induction of structural chromosome aberration rate no matter in the absence or the presence of S9. Whereas, the positive control group significantly increased the structural chromosome aberration rate compared to the negative control group.

These results suggested that GD-*Antrodia camphorata*, acting like the negative control, did not induce chromosomal structural mutation in this trial.

INTRODUCTION

Taiwan Health Authority passed the Health Food Control Act in August, 1999. The Act requires pharmacological analysis and safety assessments of all marketed health food products. In the Act, there are four classes in safety assessment for health food products. *GD-Antrodia camphorata* belongs to the Class 2. Genetic toxicity assay is a requirement for market clearance of Class 2 health food products. Structural chromosome aberration analysis is an important assessment to analyze genotoxicity of environmental conditions. In the presence of mutagens, chromosomes in the testing cells display some abnormalities which can be observed by microscopy. This trial apply structural chromosome aberration analysis to study the genotoxicity of *GD-Antrodia camphorate*. Results obtained here can be used as risk assessment of *GD-Antrodia camphorate* in human genetic toxicity.

MATERIALS AND METHODS

Cells

Information of Chinese hamster ovary cells (CHO-K1) used in this trail are listed in the below table. The information includes source, morphology, culture medium, culture condition, modal number, cell growth cycle, mycoplasma test and activating enzyme system. In brief, mycoplasma test result for the cells was negative. The cells were used at the log phase of its growth. Only cells with normal morphology were analyzed in the trial. The CHO-K1 cells were stored on liquid nitrogen for long term storage. The cells were passed for not more than five times after thawed.

Cell Line and Materials	
Cell Line	Chinese hamster ovary cells
Cell Line Source	Hsin-Chu Food Science Research Institute, Taiwan, ROC
Morphology	Spindle shape monolayer
Culture Medium	McCoy's 5A (Sigma, USA) plus fetal bovine serum (10%), sodium bicarbonate (0.22%), L-glutamine (2 mM), streptomycin (100 µg/ml) and penicillin-G (100 units/ml) , pH 6.8-6.9.
Culture Condition	Saturated water vapor, 37±1 °C, 5% CO ₂ incubator.
Chromosome Modal Number	20
Cell Cycle	12 hours
Mycoplasma Test	Negative (by ELISA)
Activating Enzyme System	Postmitochondrial fraction (S9) derived by induction of β-naphthlavone on Wistar rat liver.

Experimental Design

Experimental factors and protocols are described in details in the table below. 1.75×10^6 CHO cells at log phase were inoculated in a T-75 tissue culture flask and incubated in an incubator for 24 hours. Two flasks of cells were used for each trial dosage. The preparation of *GD-Antrodia camphorata* samples and culture media were described below. Three experimental treatments were used in this trial. The first treatment was that the cells were incubated in the absence of the rat liver activating system (S9) for three hours. The second treatment method was that the cells were incubated in the presence of the rat liver activating system (S9) for three hours. The third treatment incubated the cells in the absence of S9 continuously for 20 hours (about 1.5 cell growth cycles). Cells were harvested after 20 hours of treatment. Colcemid, a mitotic-arresting reagent, was added to the cultured cells to a final concentration of 0.1 µg/ml two hours before harvesting. Theoretically, cells were at mitotic phase at the moment of the harvest. The cells were harvested by shake-off method and transferred to hypotonic solution. Cells were fixed with a solution

containing methanol: cold acetic acid (3:1) and then stained on glass slides. Cells were examined under the microscope with 1,000X magnification for chromosomal morphology. Abnormal chromosomal morphology can be concluded into chromosome type and chromatid type abnormalities. Chromosome type includes gap (G), break (B), dicentric (D) and ring(R), whereas the chromatid type includes gap (g), break (b), and exchange (e). Other abnormal morphologies such as polyploidy and endoreduplication were recorded separately. Two hundred metaphase cells for each dosage group were examined, and the percentages of chromosome aberrations in the trial were summarized.

Experimental Conditions	
Cell Number of Inoculation	About 1.75×10^6 cells /75 cm ² tissue culture flask
No. of Replica Per Experimental Dosage	Duplicate culture
Solvent	Cell culture medium or S9 culture fluid
Treatment of GD- <i>Antrodia camphorata</i>	A. Treatment with Agaricus Blazei murrill for 3 hours without rat liver activating system (S9). B. S9 added (+S9), cells treated for 3 hours. C. Without S9 (-S9), cells treated for 20 hours.
Concentrations of GD- <i>Antrodia camphorata</i>	3 mg/ml , 6 mg/ml , 12 mg/ml , 25 mg/ml , 50 mg/ml
Positive Control Group	Groups without S9 treatment: 1μM mitomycin C for 3 hours. Groups with S9 treatment: 40μM cyclophosphamide monohydrate for 3 hours. (DMSO was used as the solvent for negative control group.)
Content of Liver Activating Enzyme (S9)	10 ml serum-free medium, 3.15 mg NADP, 1.52 mg G-6-P and 40 μl S9 per tissue culture flask.
Synchronous Cell Toxicity Analysis Method	Cell at metaphase were harvested. Cells were trypsinized and counted by a cell counter.
Drug to Increase Metaphase	Treatment with 0.1μg/ml colcemid for 2 hours

Cell Number	
Time to Harvest Metaphase Cells	20 hours after treatment
Method of Harvesting Metaphase Stage Cells	Mitotic shake-off technique
Preparation of Chromosomal Smear	Cells were treated with hypotonic solution , fixed with methanol: cold acetic acid (3:1), and processed for Giemsa staining on glass slide.
Standards for Chromosomal Structural Mutation Analysis	Smears were blind coded before observation. Each cell was used as a unit for chromosomal observation. Cell selected for observation were those with both modal no. of 18-22 (normal modal no.±2) and also evenly spread-out chromosomes. Calculate the percentage of cells with abnormal chromosomes. This percentage did not include gap.
Types of structural chromosome aberrations	Includes chromosome gap (G), chromosome break (B), dicentric (D), ring (R), chromatid gap (g), chromatid break (b), and exchange (e), all separately recorded in the data sheet for each tissue culture flask. Polyploidy or endoreduplication were also recorded , but only discussed when there was significant increase and was not included into the percentage rate of cells with morphologically abnormal chromosomes
Analysis of metaphase cell number	200 cells at metaphase are observed.

Preparation of Solution Containing Trial Substances

GD-*Antrodia camphorata* solutions were freshly prepared on the day of experiments. For treatment without the rat activating enzyme system, GD-*Antrodia camphorata* solutions were prepared as follows:

- a. solvent control group (0 µg/ml): tissue culture medium
- b. the highest dose (50 mg/ml): dissolve 500 mg of GD-*Antrodia camphorata* with 10 ml of culture medium.

- c. other testing doses (3 mg/ml, 6 mg/ml, 12 mg/ml, 25 mg/ml): dilute the highest concentration with tissue culture medium accordingly.

All experimental media was sterilized by filtration with 0.22 µm filters.

Verification of Valid Trial

By observing negative control and positive control smears, all data in the trial were accepted as an valid trial when 1) the negative control group showed 0-3% aberrant cells and 2) the positive control group had significantly higher ($p < 0.05$) value than the negative control group,.

RESULTS AND DISCUSSION

Analysis of Chromosome Modal Number of Control CHO-K1 cells

The chromosome modal numbers of CHO-K1 cells treated with the solvent control are listed in Table 3. Fifty cells were randomly selected. In all the observed cells, the chromosome modal numbers were all within the range of 20 ± 2 . The range of modal number is in accord to the cell requirement for chromosomal structural mutation analysis.

Table 3

Modal number	< 18	18	19	20	21	22	> 22
Cell number	0	0	10	36	4	0	0

Structural Chromosome Aberration Analysis

In the first treatment (cells were treated with *GD-Antrodia camphorata* without adding metabolic activating enzyme S9 for 3 hours), there was no increase in the rate of chromosome abnormality induced by each dosage groups (Table 4), compared to the solvent control group. In the second treatment (cells were treated with *GD-Antrodia camphorata* in the presence of the metabolic activating enzyme S9 for 3 hours), there was no increase in the rate of chromosome abnormality induced by each dosage groups (Table 5). No significant difference was observed when the experimental group was compared with the solvent control group. In the third treatment (*GD-Antrodia camphorata* incubation for 20 hours), all stages of a cell cycle may be included. The result of experimental groups did not appear any increase in the rate of chromosome abnormality induced by each dosage groups (Table 6). Data

in Table 4-6 was recorded independently by two groups of observers. Similar results were obtained. From the results of the three treatments, our data concluded that GD-*Antrodia camphorata* did not induce the rate of chromosome abnormality, neither before nor after metabolic activation.

Due to that none of the drug dosage group showed significant increase in toxicity, further dose-response analysis is not necessary in this trial.

CONCLUSION

Data in this trial meets all criteria in data validation of experimental design. Based the result of the *in vitro* chromosomal structural mutation analysis, it suggested that GD-*Antrodia camphorata*, acting like the negative controls, had no significant effect on the structural chromosome aberration of mice in this trial.

TRIAL REVIEW

This trial was carried out based on the original trial proposal. No event which could impact quality and integrity of the trial happened during the period. We did not encounter any situation which could influence the trial results or interpretations.

Table 4. Percentage of chromosomal aberration of CHO cells treated with GD-*Antrodia camphorata* in the absence of S9 for 3 hours.

Treatment (mg/ml)	Aberrant cells (%) ^a	No. of chromosome aberrations/100 cells							
		G ^b	B	D	R	g	b	e	MA
Negative control	1	0	0	0	0	1	0	0	0
50	6	0	0	0	2	1	3	0	0
25	5	0	0	0	1	3	1	0	0
12	5	0	0	0	0	2	3	0	0
6	3	0	0	0	0	1	2	0	0
3	2	0	0	0	0	2	0	0	0
Positive control ^c	13	0	2	0	6	4	1	0	0

^a Aberrant cells were calculated excluding cells with gaps.

^b G: chromosome gap B: chromosome break D: dicentric R: ring
g: chromatid gap b: chromatid break e: exchange MA: multiple aberrations

^c Positive control was 1µM Mitomycin C for 3 h.

Data was recorded independently by two groups of observers.

Table 5. Percentage of chromosomal aberration of CHO cells treated with GD-*Antrodia camphorata* in the presence of S9 for 20 hours.

Treatment (g/ml)	Aberrant cells (%) ^a	No. of chromosome aberrations/100 cells							
		G ^b	B	D	R	g	b	e	MA
Negative control	1	0	0	0	0	1	0	0	0
50	7	0	0	0	1	4	2	0	0
25	5	0	0	0	1	2	2	0	0
12	4	0	0	0	0	3	1	0	0
6	4	0	0	0	1	2	1	0	0
3	1	0	0	0	0	1	0	0	0
Positive control ^c	15	0	3	0	3	5	4	0	0

^a Aberrant cells were calculated excluding cells with gaps.

^b G: chromosome gap B: chromosome break D: dicentric R: ring
g: chromatid gap b: chromatid break e: exchange MA: multiple aberrations

^c Positive control was 40µM cyclophosphamide monohydrate for 20 h.

Data was recorded independently by two groups of observers.

Table 6. Percentage of chromosomal aberration of CHO-K1 cells treated with GD-*Antrodia camphorata* in the absence of S9 for 20 hours.

Treatment (g/ml)	Aberrant cells (%) ^a	No. of chromosome aberrations/100 cells							
		G ^b	B	D	R	g	b	e	MA
Negative control	3	0	0	0	0	2	1	0	0
50	6	0	0	0	0	4	2	0	0
25	6	0	0	0	1	3	2	0	0
12	6	0	0	0	1	3	2	0	0
6	3	0	0	0	0	2	1	0	0
3	1	0	0	0	0	1	0	0	0
Positive control ^c	16	0	4	0	4	6	2	0	0

^a Aberrant cells were calculated excluding cells with gaps.

^b G: chromosome gap B: chromosome break D: dicentric R: ring
g: chromatid gap b: chromatid break e: exchange MA: multiple aberrations

^c Positive control was 1µM mitomycin C for 20 h.

Data was recorded independently by two groups of observers.

PART III:

***IN VIVO* MICRONUCLEUS ASSAY**

SUMMARY

This trial studied potential mutagenic effects of GD-*Antrodia camphorata* samples on animal chromosomes by using peripheral blood micronucleus assay. This analysis served a gene mutation analysis can be used to evaluate the effect of GD-*Antrodia camphorata* on chromosome damaging.

The GD-*Antrodia camphorata* was dissolved in water, prepared to a stock concentration of 100 mg/ml, and completely mixed/grinded by a grinder. The GD-*Antrodia camphorata* solution was administered via feeding tube to eight-week old ICR male mice in the conditions of 26.8 mg/day/each mouse, 16.75 mg/day/each mouse, 0.335 mg/day/each mouse, for 90 days. Water was used as negative control. After administration protocol, mice were bled for blood smear microscopy. At least 1000 red blood cells in each slide were checked. Our results of the peripheral blood smear counts showed that all GD-*Antrodia camphorata* dosage groups did not show significant increase in reticulocyte micronucleus occurrence. Positive control group showed significantly increase in the occurrence of micronucleus over the negative control group.

Our results in this trial suggest that GD-*Antrodia camphorata*, acting like the negative control, has not mutagenic effects on animal chromosomes in rodent peripheral blood micronucleus assay.

INTRODUCTION

Taiwan Health Authority passed the Health Food Control Act in August, 1999. The Act requires pharmacological analysis and safety assessments of all marketed health food products. In the Act, there are four classes in safety assessment for health food products. *GD-Antrodia camphorata* belongs to the Class 2. Genetic toxicity assay is a requirement for market clearance of Class 2 health food products. The micronucleus assay is one of the most widely used tests for mutagenesis and potential carcinogenesis required by regulatory agencies for testing of genetic toxicity in regulated substances, during pre-clinical assessment of pharmaceuticals. We use this approach to examine and evaluate whether *GD-Antrodia camphorata* could possess mutagenicity. *GD-Antrodia camphorata* samples are administered to mice by oral feeding tube for 90 days. Whole blood of the *GD-Antrodia camphorata*-treated mice, negative control mice, and positive control mice are withdrawn and whole blood smear are analyzed by a standard micronucleus method.

MATERIALS AND METHODS

Animal Source

Seven-week old male and female ICR mice were purchased from the Laboratory Animal Center at National Taiwan University Hospital, Taipei, Taiwan. Eighty mice (40 males and 40 females) were randomly assigned into four male groups and four female groups.

Substance Dosage

A water group was used as the negative control. Three trial concentrations were 26.8 mg/mouse, 16.75 mg/mouse, and 0.335 mg/mouse.

Microscopy Slide Preparation

One milligram of Acridine Orange (Sigma, St. Louis, MO, USA) was dissolved in water. 10 μ l solution of acridine orange solution was pipetted onto a glass slide that was pre-cleaned with methanol and pre-warmed to 70⁰C. The solution was spread evenly on glass slides. The glass slides were air dried and set in a desiccator until use.

Trial Methods

Drugs were administered to eight-week old ICR mice via feeding tube. Trial concentrations were 26.8 mg/mouse/day, 16.75 mg/mouse/day, and 0.335 mg/mouse/day. A water group was used as the negative control. Ten mice were randomly assigned for each dosage group. Blood were collected after ninety days of drug administration, by puncturing the mouse tail with a shape razor blade. Five microliter of the blood was pipetted on to the acridine orange-treated slides and spread evenly with a cover slip (24 X 40mm) to make blood smear. The blood smear was put into a sealed box, stored at 4⁰C for at least four hours for staining and fixation steps.

Verification of Valid Trial

The blood smear was observed under a fluorescent microscope. The fluorescent microscopy used blue light (wavelength 488nm) for excitation and the images were taken under a wavelength 515nm filter for yellow-orange light emission. Under the microscope, the micronucleus was stained in red. However, if the whole cell was stained red, the result would not be accepted as positive for micronucleus. In each slide examination, at least one thousand red blood cells were examined. The trial data would be valid only when comparing the negative control blood smear to the positive control blood smear, and that the positive control group had significantly higher count ($p < 0.05$) than the negative control group.

RESULT AND DISCUSSION

As results of peripheral blood micronucleus analysis shown in Table 7 and 8, GD-*Antrodia camphorata*-treated groups at all tested dose levels did not show significant difference, compared to the negative control group. Both genders of tested mice showed similar results. These results suggested that GD-*Antrodia camphorata* did not significantly increase the chromosomal mutation rate of rodents.

Due to that none of the drug dosage group showed significant increase in toxicity, further dose-response analysis is not necessary in this trial.

CONCLUSION

Data in this trial meets all criteria in data validation of experimental design. Based on rodent peripheral blood micronucleus assay, the result suggested that under

the conditions of this experiment, GD-*Antrodia camphorata*, acting like the negative controls, had no significant effect on the chromosomal mutation analysis of rodents in this trial.

TRIAL REVIEW

This trial was carried out based on the original trial proposal. No event which could impact quality and integrity of the trial happened during the period. We did not encounter any situation which could influence the trial results or interpretations.

Table 7. Safety Dosage Trial. Peripheral blood micronucleus analysis for male mice with 90-day GD-*Antrodia camphorata*^b treatment^b.

Groups	Peripheral Blood Micronucleated RBC Count ^a Blood Sample After Treatment
Control (water)	X=1.50/1000
0.335 mg/each mouse	X=1.54/1000
16.75 mg/each mouse	X=1.42/1000
26.8 mg/each mouse	X=1.46/1000

^a The experimental data derives from the number of micronucleated RBC observed per 1000 RBC ; X equals to the average count of each group.

^b The experimental result showed that the treated group when compared with the normal control group, did not show significant increase in the RBC micronucleus number.

Table 8. Safety Dosage Trial. Peripheral blood micronucleus analysis for female mice with 90-day GD-*Antrodia camphorata*^b treatment^b.

Groups	Peripheral Blood Micronucleated RBC Count ^a Blood Sample After Treatment
Control (water)	X=1.35/1000
0.335 mg/each mouse	X=1.41/1000
16.75 mg/each mouse	X=1.42/1000
26.8 mg/each mouse	X=1.42/1000

^a The experimental data derives from the number of micronucleated RBC observed per 1000 RBC ; X equals to the average count of each group.

^b The experimental result showed that the treated group when compared with the normal control group, did not show significant increase in the RBC micronucleus number.

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