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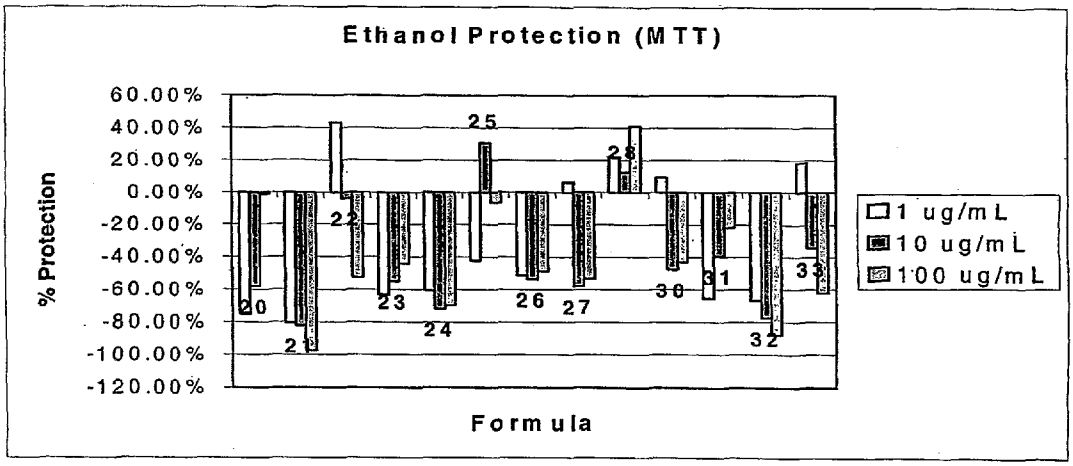

(57) Abstract: The present invention is directed to plant based formulations for improving liver health by protecting the liver from alcohol and chemical insults and/or by inducing phase II enzymes. Formulations according to the present invention include wasabi root fiber powder, artichoke leaf extract, asparagus dehydrate, kudzu root extract, oregano extract, schisandra berry extract, notoginseng (ethanol extract of Panax notoginseng root), sanchi (water extracts from Panax notoginseng root), Gegen root extract (Pueraria omeiensis), spinach dehydrate, or combinations thereof.

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PLANT-BASED FORMULATIONS FOR IMPROVING LIVER HEALTH

BACKGROUND OF THE INVENTION

The present invention is directed to unique formulations of plant-based extracts that work synergistically to aid in good liver health. More specifically, the present invention relates to unique formulations of plant-based extracts that assist in protecting the liver from alcohol and chemically induced damage.

The liver is one of the hardest working organs in the body. Good liver function is important for balanced hormone levels, weight control and maintenance, cholesterol levels, skin health and general health. The liver serves as the body's clearing house and is responsible for the metabolism of a number of substances, including alcohol, and plays an important role in the detoxification of toxins in the body. Phase II enzymes are part of this detoxification process because they aid in the removal of potential carcinogens from the body. As a result of its function in the body, the liver is under constant attack and prone to damage from environmental toxins, impurities, alcohol, prescription and over-the-counter drugs. Many hepatotoxicants such as carbon tetrachloride, nitrosamines, and polycyclic aromatic hydrocarbons are metabolically activated by liver enzymes to form reactive, toxic metabolites that cause injury to the liver in humans. Thus, formulations of plant-based extracts that aid in protecting the liver against alcohol and carbon tetrachloride insults would be useful. Additionally, plant-based formulations that induce phase II enzymes responsible for detoxifying the liver would be useful.

SUMMARY OF THE INVENTION

The present invention is directed to unique formulations that improve liver health by working to protect the liver from carbon tetrachloride and alcohol insults. Formulations of the present invention have shown strong protective abilities on human liver cells as measured by indices such as 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide ("MTT"), which is a tetrazolium salt, and lactase dehydrogenase ("LDH"). In addition, these extracts and their combinations show strong phase II enzyme induction activity. The phase II enzyme induction assay measures a sample's ability to induce quinone reductase (a phase II enzyme) which is indicative of detoxification events.

Accordingly, in one embodiment, the present invention provides a formulation for improving liver health that includes wasabi root fiber powder, artichoke leaf extract, asparagus extract, kudzu root extract, oregano extract, schisandra berry extract, notoginseng

(ethanol extract of *Panax notoginseng* root), sanchi (water extracts from *Panax notoginseng* root), Gegen (*Pueraria omeiensis*), spinach dehydrate, or combinations thereof.

In another embodiment, the present invention provides a method of protecting the liver from carbon tetrachloride ("CCl_4") insults by providing wasabi root fiber powder, artichoke leaf extract, asparagus extract, kudzu root extract, oregano extract, schisandra berry extract, notoginseng (ethanol extract of *Panax notoginseng* root), sanchi (water extracts from *Panax notoginseng* root), Gegen (*Pueraria omeiensis*), spinach dehydrate, or combinations thereof.

In yet another embodiment, the present invention provides methods of protecting the liver from alcohol insults by providing wasabi root fiber powder, artichoke leaf extract, asparagus extract, kudzu root extract, oregano extract, schisandra berry extract, notoginseng (ethanol extract of *Panax notoginseng* root), sanchi (water extracts from *Panax notoginseng* root), Gegen (*Pueraria omeiensis*), spinach dehydrate, or combinations thereof.

In yet another embodiment, the present invention provides methods of inducing phase II enzymes by providing wasabi root fiber powder, artichoke leaf extract, asparagus extract, kudzu root extract, oregano extract, schisandra berry extract, notoginseng (ethanol extract of *Panax notoginseng* root), sanchi (water extracts from *Panax notoginseng root*), Gegen (*Pueraria omeiensis*), spinach dehydrate, or combinations thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a bar graph showing the effect of various formulations in protecting the liver from ethanol insults as measured by MTT.

Figure 2 is a bar graph showing the effect of various formulations in protecting the liver from ethanol insults as measured by LDH.

Figure 3 is a bar graph showing the effect of various formulations in protecting the liver from CCl_4 insults as measured by MTT.

Figure 4 is a bar graph showing the effect of various formulations in protecting the liver from CCl_4 insults as measured by LDH.

DETAILED DESCRIPTION OF THE INVENTION

It is to be understood that this invention is not limited to the particular methodology or protocols described herein. Further, unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs. It is also to be understood that the terminology

used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention, which will be limited only by the claims.

Ingredients for Use in Formulations of the Present Invention

The present invention is based on the surprising discovery that unique combinations of the following ingredients, described more fully in Table 1, improve liver health: wasabi root fiber powder, artichoke leaf extract, asparagus extract, kudzu root extract, oregano extract, schisandra berry extract, notoginseng (ethanol extract of *Panax notoginseng* root), sanchi (water extracts from *Panax notoginseng* root), Gegen (*Pueraria omeiensis*), and spinach dehydrate.

More specifically, the formulations of the present invention improve liver health by protecting the liver against alcohol and carbon tetrachloride insults. Additionally, the formulations improve liver health by inducing phase II enzymes. Phase II enzymes are responsible for removing potential carcinogens by aiding in their removal from the body.

"Protecting the liver against alcohol insults" and "protecting the liver against carbon tetrachloride insults" refers to the ability of a formulation described herein to preserve or improve existing liver function.

Table 1

Ingredient	**Description**
Artichoke leaf extract (*Cynara scolymus* L.)	Extracts from artichoke have been used in folk medicine against liver complaints and such extracts have been claimed to exert ahepatoprotective effect. *Journal of Ethno-pharmacology*, 86: 203-211 (2003). Constituents include flavanoids and sesquiterpenoid bitter compounds of the guaianolide-type. Czygan, Franz-Christian et al., *Herbal Drugs and Phytopharmaceuticals*, 3rd ed., Stuttgart, Germany, Medpharm Scientific Publishers, 2004. p. 174. Artichoke leaf extract can be obtained from Grupo Centroflora, São Paulo, Brazil.

Asparagus dehydrate	*Asparagus racemosus* (commonly known as Shatavari) is recommended in Ayurvedic texts for prevention and treatment of gastric ulcers, dyspepsia and as a galactogogue. *A.racemosus* has also been used successfully by some Ayurvedic practitioners for nervous disorders, inflammation, liver diseases and certain infectious diseases. Animal studies have also demonstrated beneficial antioxidant effects to the liver. *Journal of Ethnopharmacology*, 71: 425–435 (2003).However, no scientific proof justifying the aforementioned uses of the root extract of *A. racemosus* is available so far. *Indian J Med Sci.*, 57(9):408-14 (2003). The chemical constituents so far reported include flavonoids, oligosaccarides, amino acids, sulphur-containing acids, and steroidal saponins. *Id.*
Kudzu root extract (*Pueraria lobata*)	Kudzu or P*ueraria Radix* is the root of *Pueraria lobata* which is a perennial leguminous vine native to eastern Asia. Kudzu has been used for many disorders such as fevers, gastrointerstinal disorders, muscle aches, allergies, respiratory problems, skin problems, high blood pressure, migraine headaches, lowering cholesterol, and treating chronic alcoholism. *Clinical Chimica Acta*, 347: 121-128 (2004). The active principles of *Pueraria lobata* are coumarins, isoflavonoids (puerarin, daidzin and daidzein), and sapoinins (soyasaponins, kudzusaponins).
Notoginseng (*Panax notoginseng)*	Notoginseng as referred to herein is the ethanol extract from *Panax notoginseng* root. *Panax notoginseng* is used to treat coronary heart disease, cardiac angina, and apoplexy. The active constituents of *Panax notoginseng* include saponins (primarily ginsenosides), dencichines, flavonoids, and polysaccharide; however, the levels of these components vary in different geographical regions of growth and also show a seasonal variation. Dong, TT et al., Chemical assessment of roots of *Panax notoginseng* in China: regional and seasonal variations in its active constituents. *J. Agric Food Chem*, 51(16):4617-23 (2003). Notoginseng can be obtained from EUL International Herb Manufacturing, La Verne, California.
Gegen root extract (*Pueraria omeiensis)*	Commonly known as Omei Mountain Kudzu Vine.
Oregano extract	The constituents of oregano include two phenoles carvacrol and thymol (see also thyme and savory.) A variety of monoterpene hydrocarbons (limonene, terpinene, ocimene, caryophyllene, beta-bisabolene and p-cymene) and monoterpene alcohols (linalool, 4-terpineol) have also been found.
Sanchi (*Panax notoginseng)*	Sanchi is referred to herein as the water extract from *Panax notoginseng* root. *Panax notoginseng* is known to contain saponins. Sanchi can be obtained from Draco Natural Products, San Jose, California.

Schisandra berry extract (*Fructus schisandrae)*	Schisandra berry or schizandra berry is commonly known as Chinese Magnoliavine fruit. It is generally used to arrest discharges, promote fluid secretion, tonify the kidney, and induce sedation. See US 6,455,078. Schisandra lignans have been cited to protect the liver against CCl_4 damage. *Planta Medica*, 61 (2); 134-7 (1995).
Spinach dehydrate	Spinach contains betaine which has been used clinically for liver disease. See US2003/0091615. Spinach dehydrate can be obtained from Access Business Group LLC, Ada, Michigan.
Wasabi root	Studies suggest that wasabi (*Wasabia japonica*, syn. Eutrema Wasabi) may generate an increase in the abundance of the protective phase II detoxification enzymes, such as glutathione S-transferase (GST), resulting in hepatoprotection. Recent identification of 6-methylsulfinylhexyl isothiocyanate (6-HITC), an analogue of sulforaphane (4-methylsulfinylbutyl isothiocyanate) isolated from broccoli, has been suggested to be the major GST inducer in wasabi. *J. Biol Chem.*, 1;277(5):3456-63 (2002). It is known that wasabi contains isothiocyanate components. *Id.* Wasabi root extract can be obtained from EUL International Herb Manufacturing, La Verne, California.

Formulations of the Present Invention

Table 2 illustrates representative daily amounts of the ingredients that can be included in the supplement.

Table 2

Ingredient	**Daily Dosage**
Artichoke leaf extract	200 mg – 1000 mg
Asparagus extract	100 mg – 500 mg
Gegen root extract	150 mg – 1000 mg
Kudzu root extract	150 mg – 1000 mg
Notoginseng	100 mg – 500 mg
Oregano extract	20 mg – 500 mg
Sanchi	100 mg– 500 mg
Schisandra berry extract	150 mg – 1000 mg
Spinach dehydrate	20 mg – 500 mg
Wasabi root powder	150 mg – 500 mg

EXAMPLES

The following are illustrative examples of formulations made into tablets according to this invention and it should be understood that they do not limit the scope of the invention.

Example 1

Per: 3 Tablets				
Qty.	**U/M**	**Item #**	**Ingredients**	**%W/W**
350.0	mg	R7462	Sanchi root concentrate	18.88%
300.00	mg	R6911	Asparagus dehydrate	16.18%
150.00	mg	R7464	Gegen root extract, 10%	8.09%
500.00	mg	R7461	Artichoke leaf extract, 5%	26.97%
150.00	mg	NF9824Z	Spinach dehydrate	8.09%
310.0	mg	R4174Q	Microcrystalline Cellulose, Silicified	16.72%
44.0	mg	R3338	Cellulose Gum	2.37%
5.6	mg	R0225	Silicon Dioxide, Fine Powder	0.30%
44.0	Mg	R3512	Stearic Acid, Powder, Vegetable	2.37%
11.0	mg	NF6004	Hydroxypropyl Methylcellulose	

Example 2

Per: 3 Tablets				
Qty.	**U/M**	**Item #**	**Ingredients**	**%W/W**
500.0	mg	R7461	Artichoke powder extract	30.10%
150.00	mg	R7460	Wasabi root concentrate	9.03%
500.00	mg	R7464	Kudzu root, 40%	30.10%
150.00	mg	R6910	Oregano Extract	9.03%
278.0	mg	R4174Q	Microcrystalline Cellulose, Silicified	16.74%
39.0	mg	R3338	Cellulose Gum	2.35%
5.0	mg	R0225	Silicon Dioxide, Fine Powder	0.30%
39.0	mg	R3512	Stearic Acid, Powder, Vegetable	2.35%
11.0	mg	HPMC	Hydroxypropyl Methylcellulose	

Example 3

Per: 3 Tablets				
Qty.	**U/M**	**Item #**	**Ingredients**	**%W/W**
150.00	mg	R7462	Sanchi	8.15%

150.00	mg	R7521	Notoginseng	8.15%
500.00	mg	R7461	Artichoke leaf extract, 5%	27.18%
500.00	mg	R7463	Kudzu root extract	27.18%
150.00	mg	R6910	Oregano Extract	8.15%
300.0	mg	R4174Q	Microcrystalline Cellulose, Silicified	16.31%
42.0	mg	R3338	Cellulose Gum, Modified NF	2.28%
5.5	mg	R0225	Silicon Dioxide, NF Fine Powder	0.30%
42.0	mg	R3512	Stearic Acid, Powder, Vegetable	2.28%
13.0	mg	NF6004	Hydroxypropyl Methylcellulose	

Example 4

Per: 3 Tablets				
Qty.	**U/M**	**Item #**	**Ingredients**	**%W/W**
350.0	mg	R7462	Sanchi	20.42%
300.00	mg	R6911	Asparagus dehydrate	17.50%
500.00	mg	R7461	Artichoke leaf extract, 5%	29.17%
200.00	mg	NF9824Z	Spinach dehydrate	11.67%
280.0	mg	R4174Q	Microcrystalline Cellulose, Silicified	16.34%
39.0	mg	R3338	Cellulose Gum	2.28%
5.0	mg	R0225	Silicon Dioxide, Fine Powder	0.29%
40.0	mg	R3512	Stearic Acid, Powder, Vegetable	2.33%
12.0	mg	NF6004	Hydroxypropyl Methylcellulose	

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Example 5

Per: 3 Tablets				
Qty.	**U/M**	**Item #**	**Ingredients**	**%W/W**
500.00	mg	R7461	Artichoke leaf extract	27.18%
500.00	mg	R7463	Kudzu root extract, 40%	27.18%
150.00	mg	R7521	Notoginseng	8.15%
150.0	mg	R7460	Wasabi root fiber powder	8.15%
150.00	mg	NF9824Z	Spinach dehydrate	8.15%

300.0	mg	R4174Q	Microcrystalline Cellulose, Silicified	16.31%
42.0	mg	R3338	Cellulose Gum	2.28%
5.6	mg	R0225	Silicon Dioxide, Fine Powder	0.30%
42.0	mg	R3512	Stearic Acid, Powder, Vegetable	2.28%
11.0	mg	NF6004	Hydroxypropyl Methylcellulose	

Example 6

Per 3 Tablets				
Qty.	**U/M**	**Item #**	**Ingredients**	**%W/W**
500.0	mg	R7461	Artichoke leaf extract, 5%	23.88%
500.00	mg	R7460	Wasabi root fiber powder	23.88%
500.00	mg	R7464	Gegen Root extract, 10%	23.88%
150.00	mg	R6910	Oregano Extract	7.16%
342.0	mg	R4174Q	Microcrystalline Cellulose, Silicified	16.33%
48.0	mg	R3338	Cellulose Gum	2.29%
6.2	mg	R0225	Silicon Dioxide, Fine Powder	0.30%
48.0	mg	R3512	Stearic Acid, Powder, Vegetable	2.29%
11.0	mg	NF6004	Hydroxypropyl Methylcellulose	

Example 7

Per 2 Tablets				
Qty.	**U/M**	**Item #**	**Ingredients**	**%W/W**
150.0	mg	R7460	Wasabi root fiber powder	8.83%
500.00	mg	R7461	Artichoke leaf extract, 5%	29.43%
300.00	mg	R3490	Schizandra berry ext	17.66%
150.00	mg	R7521	Notoginseng	8.83%
150.00	mg	NF9824Z	Spinach dehydrate	8.83%
346.0	mg	R4174Q	Microcrystalline Cellulose, Silicified	20.36%
49.0	mg	R3338	Cellulose Gum	2.88%
5.1	mg	R0225	Silicon Dioxide, Fine Powder	0.30%
49.0	mg	R3512	Stearic Acid, Powder, Vegetable	2.88%

11.0	mg	NF6004	Hydroxypropyl Methylcellulose	

Example 8

Per 3 Tablets				
Qty.	**U/M**	**Item #**	**Ingredients**	**%W/W**
150.0	mg	R7460	Wasabi root fiber powder	7.62%
500.00	mg	R7461	Artichoke leaf extract, 5%	25.41%
500.00	mg	R7463	Kudzu root, 40%	25.41%
150.00	mg	R7521	Notoginseng	7.62%
150.00	mg	R6910	Oregano extract	7.62%
400.0	mg	R4174Q	Microcrystalline Cellulose, Silicified	20.33%
55.0	mg	R3338	Cellulose Gum	2.79%
5.9	mg	R0225	Silicon Dioxide, Fine Powder	0.30%
38.0	mg	R3512	Stearic Acid, Powder, Vegetable	2.90%
11.0	mg	NF6004	HPMC	

Example 9

Label Per: 2 Tablets				
Qty.	**U/M**	**Item #**	**Ingredients**	**%W/W**
500.00	Mg	R7461	Artichoke leaf extract, 5%	32.16%
500.00	Mg	R7463	Kudzu root extract, 40%	32.16%
150.00	Mg	R6910	Oregano Extract	9.65%
315.0	mg	R4174Q	Microcrystalline Cellulose, Silicified	20.26%
40.0	mg	R3338	Cellulose Gum, Modified NF	2.57%
4.7	mg	R0225	Silicon Dioxide, NF Fine Powder	0.30%
45.0	mg	R3512	Stearic Acid, Powder, Vegetable	2.89%
11.0	mg	NF6004	hydroxypropylmethylcellulose	

The above exemplary tabletted formulations can be manufactured according to typical methods known in the industry. For example, wasabi root fiber powder, artichoke leaf extract, schizandra berry extract, notoginseng and spinach dehydrate are passed through

a SWECO separator equipped with a 20 mesh screen into a 100 cubic foot PK blender. Microcrystalline cellulose is added to the blend in the PK blender. The ingredients are blended for ten minutes. Cellulose gum and silicon dioxide are passed through a SWECO separator equipped with a 20 mesh screen directly into the 100 cubic foot PK blender. The ingredients are blended for ten minutes. Next, stearic acidis passed through a SWECO separator equipped with a 20 mesh screen directly into the 100 cubic foot PK blender. The mixture is blended for an additional five minutes. The resulting mixture is discharged into totes or supersacks, and compressed into tablets.

Methods of Administration

Formulations of the present invention may be formulated in an acceptable carrier and may be prepared, packaged, and labeled for promoting health, liver function, protecting against alcohol and/or chemical insults to the liver, and/or inducing phase II enzymes to promote healthy liver function. The formulations of the present invention and their acceptable carriers may be formulated for oral administration in the form of a pill, tablet, dried or powdered product for reconstitution with water or other suitable vehicle before use, bar, food, solution, syrup, suspension, beverage, lozenge, *etc.* The formulations of the present invention may also be parenterally administered or administered by inhalation or insufflation (either through the mouth or nose).

Liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (e.g., sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (e.g., lecithin or acacia); non-aqueous vehicles (e.g., almond oil, oily esters, or fractionated vegetable oils); and preservatives (e.g., methyl or propyl-p-hydroxybenzoates or sorbic acid). When administered in the form of a beverage, formulations of the present invention may be water-based, milk-based, tea-based, fruit juice-based, or some combination thereof.

Formulations of the present invention may also be orally administered in the form of a solid prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (e.g., pregelatinized maize starch, polyvinyl pyrrolidone or hydroxypropyl methylcellulose; fillers (e.g., lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (e.g., magnesium stearate, talc or silica); disintegrants (e.g., cellulose gum, potato starch or sodium starch glycolate); or wetting agents (e.g., sodium lauryl sulphate).

Formulations of the present invention that are orally administered can further comprise thickeners, including xanthum gum, carboymethyl-cellulose, carboxyethylcellulose, hydroxypropylcellulose, methylcellulose, microcrystalline cellulose, starches, dextrins, fermented whey, tofu, maltodextrins, polyols, including sugar alcohols (e.g., sorbitol and mannitol), carbohydrates (e.g. lactose), propylene glycol alginate, gellan gum, guar, pectin, tragacanth gum, gum acacia, locust bean gum, gum arabic, gelatin, as well as mixtures of these thickeners.

Orally administered formulations of the present invention can contain an effective amount of one or more sweeteners, including carbohydrate sweeteners and natural and/or artificial no/low calorie sweeteners. The amount of the sweetener used in the formulations of the present invention will vary, but typically depends on the type of sweetener used and the sweetness intensity desired.

In addition to the formulations described previously, the compounds may also be a formulated as a sustained and/or timed release formulation. The formulations must be maintained above some minimum therapeutic dose to be effective. Common timed and/or controlled release delivery systems include, but are not be restricted to, starches, osmotic pumps, or gelatin micro capsules.

The formulations may, if desired, be presented in a pack or dispenser device which may comprise one or more unit dosage forms comprising a formulation of the present invention. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

Other useful dosage forms can be prepared by methods and techniques that will be well understood by those of skill in the art and may include the use of additional ingredients in producing tablets, capsules, or liquid dosage forms. The dose, and dose frequency, will vary according to the age, body weight, condition and response of the individual consumer or patient, and the particular formulation of the present invention that is used.

It is intended that the foregoing detailed description be regarded as illustrative rather than limiting. The present invention is further illustrated by the following experimental investigations and examples, which should not be construed as limiting.

Bioassay Study on Individual Ingredients

Several materials were submitted for bioassay testing to try to predict their protective ability against alcohol and chemically induced liver damage. To assess this, human liver cells were treated with the material and an insult, and cell viability was

measured using two different assays. Alcohol damage was mimicked using 2.5% ethanol as the insult. CCl_4 at 0.2% was used for the chemical insult. These concentrations were chosen because they were the concentrations that produced 20% cell death in preliminary experiments, and would therefore not cause the cells to undergo irreversible cell death/damage.

Materials were tested at three concentrations (1, 10 and 100 µg/mL) and an estimation of the EC-50 was determined (see Experimental section below for more details). In addition to the cell viability assays, phase II enzyme induction testing was also conducted to determine if a material had detoxification properties. The phase II enzyme induction assay measures a sample's ability to induce quinone reductase (a phase II enzyme) which is indicative of detoxification events. Phase II enzymes are responsible for removing potential carcinogens by aiding in their removal from the body. Broccoli is known as a good phase II enzyme inducer because of its sulforaphane content. Pure sulforaphane has activity at 10^6 U/g, while broccoli has reported activity from 4000 – 74,000 U/g (dependent on variety, form, and/or extraction conditions). Activity above 30,000 U/g is considered excellent. Activity less than 5000 U/g is considered minimal. Most materials that are considered good phase II enzyme inducers will have activity between 15,000-30,000 U/g.

Tables 3 and 4 summarize the EC-50 values of all materials tested. Table 3 is specifically for results of the ethanol protection, and Table 4 for carbon tetrachloride. Table 5 gives the results of the phase II enzyme induction assay.

Table 3: EC-50 results of the MTT and LDH assays for Ethanol protection assessment.

Sample	**Description**	**EC-50 MTT (µg/mL)**	**EC-50 LDH (µg/mL)**
CD-2808	Hibiscus SD Powder	10-100	None
CD-4230	Picao Pret (Bidens Pilosa) Herb	None	>100
CD-4228	Pueraria (Kudzu) Root 40%	<1	None
CD-3902	Cardoon SD Powder	>100	None
CD-4229	Gegen root extract (*pueraria omeiensis)*, 10%	None	None
CD-4796	Beet Powder	100	>100

CD-4775	Aframomum S/D Powder	None	>100
CD-4788	Schisandra berry Extract	None	>100
CD-4780	Asparagus Dehydrate	None	None
CD-4793	Bidens Pilosa Extract	None	None
CD-4787	Picrorhiza Kurrooa Extract	None	>100
CD-4786	Eclipta Alba Extract	None	None
CD-4795	Curcumin 95%	None	None
CD-4792	Boldo Extract	None	>100
CD-5347	Szechuan Lovage Rhizome	<1	>100
CD-5345	Sanchi	<1	>100
CD-5346	Glycyrrhiza Uralensis Fisch Root	<1	>100
CD-5348	Artichoke Extract Powder, 5% Cynarin	<1	>100
CD-5349	Artichoke Extract Powder, 2.5% Cynarin	<1	>100
CD-5350	Chanca Piedra Extract 3:1	<1	>100
CD-5383	Kudzu Extract Powder 40%	None	None
CD-5384	Tree Peony Bark 1:8	None	None
CD-5385	Gegen Extract	None	None
CD-5386	Pueraria 40%	<1	>100
CD-5442	Curcuma Longa Extract 10:1	None	None
CD-5441	Moutan Extract 12:1	None	None
CD-5440	Notoginseng Extract 10%	None	10-100
CD-5548	Silybin Complex	None	None
CD-5550	Dandelion 4:1	None	None
CD-5549	Milk Thistle 70% Granular	None	None
CD-5624	Oregano S/D Powder	None	None
CD-5728	Holy Basil S/D Powder	None	None
CD5730	Holy Basil S/D Powder	None	None
CD-5741	Sage S/D Powder	None	None
CD-5241	Gegen Extract 10%	None	10-100
CD-5796	Radix Notoginseng	None	10-100

CD-5797	Artichoke Extract Powder 5%	1	>100
CD-5798	Rhizoma Curcumae Longae	None	None
CD-5799	Rhizoma Chuanxiong	None	>100
CD-5800	Semen Armeniacae Amarum	None	>100
CD-5801	Tree Bark Extract 8:1	None	>100
CD-5802	Cortex Moutan	None	None
CD-5803	Radix Glycyrrhizae	>100	>100
CD-5707	*Wasabia japonica* Powder 100%	None	>100
CD-5863	Wasabi root fiber powder P.E. 5:1	>100	100
CD-5864	Wasabi Earhnut P.E. 5:1	10-100	10-100
CD-5865	Wasabi root fiber powder P.E. 5:1	10-100	>100
CD-5866	Wasabi root fiber powder	>100	>100
CD-5867	Wasabi Earthnut Powder	>100	>100
CD-5868	Wasabi root fiber powder	>100	>100

Table 4: EC-50 results of the MTT and LDH assays for CCl4 protection assessment.

Sample	**Description**	**EC-50 MTT (µg/mL)**	**EC-50 LDH (µg/mL)**
CD-2808	Hibiscus SD Powder	None	1
CD-4230	Picao Pret (Bidens Pilosa) Herb	1-10	None
CD-4228	Pueraria (Kudzu) Root 40%	1-10	None
CD-3902	Cardoon SD Powder	None	<1
CD-4229	Gegen (Kudzu Root) 10%	None	<1
CD-4796	Beet Powder	None	1
CD-4775	Aframomum S/D Powder	None	<1
CD-4788	Schisandra Berry Extract	None	1-10
CD-4780	Asparagus Dehydrate	None	<1
CD-4793	Bidens Pilosa Extract	None	<1
CD-4787	Picrorhiza Kurrooa Extract	100	1
CD-4786	Eclipta Alba Extract	>100	<1
CD-4795	Curcumin 95%	None	<1
CD-4792	Boldo Extract	None	<1
CD-5347	Szechuan Lovage Rhizome	>100	None
CD-5345	Sanchi	10-100	1-10
CD-5346	Glycyrrhiza Uralensis Fisch Root	>100	1-10
CD-5348	Artichoke Extract Powder, 5% Cynarin.	100	<1
CD-5349	Artichoke Extract Powder, 2.5%	>100	None

	Cynarin		
CD-5350	Chanca Piedra Extract 3:1	>100	100
CD-5383	Kudzu Extract Powder 40%	10-100	100
CD-5384	Tree Peony Bark 1:8	10-100	1-10
CD-5385	Gegen Extract	10-100	<1
CD-5442	Curcuma Longa Extract 10:1	<1	1-10
CD-5441	Moutan Extract 12:1	1-10	1-10
CD-5440	Notoginseng Extract 10%	<1	<1
CD-5548	Silybin Complex	None	None
CD-5550	Dandelion 4:1	10-100	>100
CD-5549	Milk Thistle 70% Granular	None	>100
CD-5624	Oregano S/D Powder	None	>100
CD-5728	Holy Basil S/D Powder	None	>100
CD5730	Holy Basil S/D Powder	None	None
CD-5741	Sage S/D Powder	None	None
CD-5241	Gegen Extract 10%	10-100	1-10
CD-5796	Radix Notoginseng	1-10	>100
CD-5797	Artichoke Extract Powder 5%	1-10	1-10
CD-5798	Rhizoma Curcumae Longae	None	1-10
CD-5799	Rhizoma Chuanxiong	None	>100
CD-5800	Semen Armeniacae Amarum	None	None
CD-5801	Tree Bark Extract 8:1	>100	>100
CD-5802	Cortex Moutan	10-100	None
CD-5803	Radix Glycyrrhizae	10-100	None
CD-5707	*Wasabia japonica* Powder 100%	>100	>100
CD-5863	Wasabi root fiber powder P.E. 5:1	>100	1-10
CD-5864	Wasabi Earhnut P.E. 5:1	10-100	10-100
CD-5865	Wasabi root fiber powder P.E. 5:1	10	1-10
CD-5866	Wasabi root fiber powder	None	1-10
CD-5867	Wasabi Earthnut Powder	1-10	10-100
CD-5868	Wasabi root fiber powder	10-100	10-100

Table 5: Phase II Enzyme (Quinone Reductase) Induction Assay Results

Sample	**Description**	**U/g**	**Rank**
CD-2808	Hibiscus SD Powder	14,493	++
CD-4230	Picao Pret (Bidens Pilosa) Herb	24,691	++
CD-4228	Kudzu Root 40%	25,641	++
CD-3902	Cardoon SD Powder	Not Tested	Not Tested
CD-4229	Gegen (Kudzu Root) 10%	95,238	++++
CD-4796	Beet Powder	9132	++
CD-4775	Aframomum S/D Powder	Not Tested	Not Tested
CD-4788	Schisandra Berry Extract	30,303	++++
CD-4780	Asparagus Dehydrate	Not Tested	Not Tested
CD-4793	Bidens Pilosa Extract	9132	++
CD-4787	Picrorhiza Kurrooa Extract	5420	++

CD-4786	Eclipta Alba Extract	10,101	++
CD-4795	Curcumin 95%	NR**	NR**
CD-4792	Boldo Extract	13,605	++
CD-5347	Szechuan Lovage Rhizome	11,299	++
CD-5345	Sanchi	13,333	++
CD-5346	Glycyrrhiza Uralensis Fisch Root	14,815	++
CD-5348	Artichoke Extract Powder, 5% Cynarin	27,778	+++
CD-5349	Artichoke Extract Powder, 2.5% Cynarin	44,444	++++
CD-5350	Chanca Piedra Extract 3:1	95,238	++++
CD-5383	Kudzu Extract Powder 40%	NR**	NR**
CD-5384	Tree Peony Bark 1:8	11,111	++
CD-5385	Gegen Extract	6006	++
CD-5442	Curcuma Longa Extract 10:1	15,504	+++
CD-5441	Moutan Extract 12:1	95,238	++++
CD-5440	Notoginseng Extract 10%	16,260	+++
CD-5548	Silybin Complex	NR**	NR**
CD-5550	Dandelion 4:1	NR**	NR**
CD-5549	Milk Thistle 70% Granular	NR**	NR**
CD-5624	Oregano S/D Powder	95,238	++++
CD-5728	Holy Basil S/D Powder	31,746	++++
CD5730	Holy Basil S/D Powder	31,746	++++
CD-5741	Sage S/D Powder	35,088	++++
CD-5241	Gegen Extract 10%	37,037	++++
CD-5796	Radix Notoginseng	15,504	+++
CD-5797	Artichoke Extract Powder 5%	9524	++
CD-5798	Rhizoma Curcumae Longae	83,333	++++
CD-5799	Rhizoma Chuanxiong	27,778	+++
CD-5800	Semen Armeniacae Amarum	22,222	+++
CD-5801	Tree Bark Extract 8:1	17,544	+++
CD-5802	Cortex Moutan	21,505	+++
CD-5803	Radix Glycyrrhizae	11,494	++
CD-5707	*Wasabia japonica* Powder 100%	74,074	++++
CD-5863	Wasabi root fiber powder P.E. 5:1	10,753	++
CD-5864	Wasabi Earhnut P.E. 5:1	83,333	++++
CD-5865	Wasabi root fiber powder P.E. 5:1	12,346	++
CD-5866	Wasabi root fiber powder	30,303	++++
CD-5867	Wasabi Earthnut Powder	5012	++
CD-5868	Wasabi root fiber powder	>100,000	++++

* Inducer units per gram of fresh weight of material. - = Negligible activity, + = Little activity,

++ = Good activity, +++ = Very good activity, ++++ = Excellent activity. **Not reportable due to toxicity to cells.

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Experimental

For the ethanol protection and CCl_4 assessments, stock sample solutions are made in DMSO, then diluted in cell culture media for testing. Treatment of HepG2 cells (human liver cell line) is done by adding 100 µL of sample to each of three wells of a 96-well microtiter plate. After a 4 hour incubation, the insult is added (2.5% ethanol or 0.2% carbon tetrachloride) and an additional overnight incubation period is conducted. The next day, cell viability is measured using two different assays. First, using the CytoTox-ONE Homogenous Membrane Integrity Assay by Promega, the number of non-viable cells is estimated by measuring the release of LDH into the media. LDH leaks out of the cell when the cell membrane is compromised. The second assay is the MTT assay, which measures the reduction of a yellow tetrazolim salt into an insoluble purple formazen product by the mitochondria of viable cells. Following an incubation with the MTT solution, isopropanol is added to solubilize the colored crystals. The amount of color produced is directly proportional to the number of viable cells.

For the phase II enzyme induction assay, stock sample solutions are made in acetonitrile, then diluted in cell culture media for testing. Treatment of Hepa1c1c7 cells (murine hepatoma cell line) is done by adding 150µL of sample to each of three wells, in a 96-well microtiter plate. After 48 hours incubation, induction activity of quinone reductase is established by measuring the NADPH-dependent, menadiol-mediated reduction of MTT. Activity is reported as inducer units per gram of fresh weight of material, where one unit of inducer activity is defined as the amount of inducer required to double the quinone reductase specific activity of Hepa1c1c7 cells.

Bioassay Study on Ingredient Combinations

Several blends of liver detoxification ingredients were submitted for bioassay testing to try to predict their protective ability against alcohol and chemically induced liver damage. These blends were tested alongside two products already on the market for liver health - NUTRILITE® Milk Thistle and Dandelion and China's King Drink. To assess this, human liver cells were treated with the sample and an insult, and cell viability was measured using two different assays. Alcohol damage was mimicked using 2.5% ethanol as the insult. CCl_4 at 0.2% was used for the chemical insult. These concentrations were chosen because they were the concentrations that produced 20% cell death in preliminary experiments, and would therefore not cause the cells to undergo irreversible cell death/damage. Samples were tested at three concentrations (1, 10 and 100 µg/mL) and an

estimation of the EC-50 was determined (see Experimental section below for more details). Materials with EC-50 values at or below 10μg/mL are indicative of being the most efficacious, assuming 10% absorption of the material in 5L of blood (average human volume).

The blends that showed the greatest efficacy against CCl_4 liver cell damage were 8523-25-CI (Example 1), 8523-27-CI (Example 8), 8523-28-CI (Example 2), and 8523-30-CI (Example 3). Other blends that performed well (exhibited 40% protection at ≤10 μg/mL) were 8523-20-CI, 8523-22-CI, 8523-24-CI, 8523-26-CI and 8523-31-CI. None of the blends showed efficacy at ≤ 10 μg/mL against ethanol liver cell damage. The control products (NUTRILITE milk thistle and King Drink) also did not achieve EC-50 values of ≤10 μg/mL. The blends that had EC-50 values >100 μg/mL were 8523-28-CI (Example 2), 8523-30-CI (Example 3), 8523-31-CI (Example 4) and 8523-32-CI (Example 5). Tables 6-9 summarize the results of all the samples tested. These results are also seen in Figures 1-4.

Table 6: Results of the MTT assays for ethanol protection assessment

(* = achieved EC-40 at one or more concentration)

Sample Number	**Formula Number or Sample Name**	**1 ug/mL**	+/-	**10 ug/mL**	+/-	**100 ug/mL**	+/-	**EC-50**
NF6523	8523-20-CI	-73%	6%	-58%	4%	-1%	7%	-
NF6523	8523-21-CI	-80%	5%	-82%	3%	-97%	11%	-
NF6523	8523-22-CI	43%	4%	-4%	4%	-52%	12%	*
NF6523	8523-23-CI	-63%	2%	-55%	4%	-44%	3%	-
NF6523	8523-24-CI	-61%	1%	-72%	2%	-70%	4%	-
NF6523	8523-25-CI	-42%	4%	31%	3%	-7%	1%	-
NF6523	8523-26-CI	-51%	6%	-54%	5%	-49%	11%	-
NF6523	8523-27-CI	6%	6%	-58%	4%	-53%	6%	-
NF6523	8523-28-CI	22%	22%	12%	18%	41%	15%	**>100**
NF6523	8523-30-CI	9%	25%	-48%	17%	-43%	16%	-
NF6523	8523-31-CI	-65%	3%	-39%	4%	-22%	5%	-
NF6523	8523-32-CI	-67%	8%	-77%	8%	-88%	5%	-
NF6523	8523-33-CI	19%	8%	-35%	20%	-62%	8%	-
	Milk Thistle	-53%	3%	-54%	8%	-42%	4	-
CD5439	King Drink	-57%	3%	-13%	17%	-8%	6%	-

Table 7: EC-50 results of the LDH assays for ethanol protection assessment.

(* = achieved EC-40 at one or more concentration)

Sample Number	Formula Number or Sample Name	1 ug/mL	+/-	10 ug/mL	+/-	100 ug/mL	+/-	EC-50
NF6523	8523-20-CI	-60%	20%	-39%	12%	-33%	5%	-
NF6523	8523-21-CI	-53%	17%	-51%	15%	-77%	8%	-
NF6523	8523-22-CI	-27%	4%	-6%	16%	-55%	11%	-
NF6523	8523-23-CI	-82%	7%	-77%	4%	-80%	4%	-
NF6523	8523-24-CI	-69%	2%	-68%	9%	-61%	5%	-
NF6523	8523-25-CI	-62%	8%	-19%	2%	-25%	4%	-
NF6523	8523-26-CI	-65%	7%	-94%	7%	-76%	1%	-
NF6523	8523-27-CI	-17%	6%	-75%	6%	-54%	6%	-
NF6523	8523-28-CI	-35%	15%	-2%	5%	-8%	13%	-
NF6523	8523-30-CI	-62%	10%	-47%	14%	-43%	20%	-
NF6523	8523-31-CI	8%	18%	12%	5%	6%	23%	**>100**
NF6523	8523-32-CI	14%	23%	10%	21%	7%	25%	**>100**
NF6523	8523-33-CI	5%	2%	25%	1%	13%	24%	**>100**
	Milk Thistle	-23%	2%	-24%	6%	-7%	7%	-
CD5439	King Drink	-26%	10%	1%	6%	1%	12%	**>100**

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Table 8: EC-50 results of the MTT assays for CCl4 protection assessment.

(* = achieved EC-40 at one or more concentration)

Sample Number	Formula Number or Sample Name	1 ug/mL	+/-	10 ug/mL	+/-	100 ug/mL	+/-	EC-50
NF6523	8523-20-CI	-42%	10%	-60%	11%	-6%	17%	-
NF6523	8523-21-CI	-95%	15%	-78%	6%	-66%	17%	-
NF6523	8523-22-CI	-47%	22%	-120%	21%	-97%	3%	-
NF6523	8523-23-CI	-56%	12%	-57%	15%	-58%	17%	-
NF6523	8523-24-CI	-12%	12%	-80%	18%	-50%	14%	-
NF6523	8523-25-CI	42%	1%	58%	2%	9%	29%	**1-10**
NF6523	8523-26-CI	-24%	4%	-13%	11%	-38%	22%	-
NF6523	8523-27-CI	8%	13%	-9%	15%	-61%	5%	-
NF6523	8523-28-CI	-66%	8%	58%	7%	-17%	17%	**1-10**
NF6523	8523-30-CI	-84%	19%	54%	11%	-59%	23%	**1-10**
NF6523	8523-31-CI	-33%	7%	-45%	16%	23%	7%	**>100**
NF6523	8523-32-CI	-91%	10%	-39%	5%	4%	16%	**>100**
NF6523	8523-33-CI	-19%	8%	-17%	12%	-25%	18%	-
	Milk Thistle	-18%	12%	-54%	1%	-42%	8%	-
CD5439	King Drink	-67%	13%	33%	6%	-22%	13%	-

Table 9: EC-50 results of the LDH assays for CCl4 protection assessment.

(* = achieved EC-40 at one or more concentration)

Sample Number	Formula Number or Sample Name	1 ug/mL	+/-	10 ug/mL	+/-	100 ug/mL	+/-	EC-50
NF6523	8523-20-CI	44%	11%	35%	5%	25%	14%	*
NF6523	8523-21-CI	20%	20%	35%	11%	22%	15%	-
NF6523	8523-22-CI	22%	8%	42%	27%	34%	2%	*
NF6523	8523-23-CI	36%	13%	38%	5%	26%	6%	-
NF6523	8523-24-CI	42%	2%	39%	26%	39%	3%	*
NF6523	8523-25-CI	38%	9%	23%	1%	47%	11%	**100**
NF6523	8523-26-CI	34%	6%	40%	9%	36%	14%	*
NF6523	8523-27-CI	47%	0%	44%	0%	42%	9%	**1**
NF6523	8523-28-CI	37%	10%	48%	20%	30%	5%	**10**
NF6523	8523-30-CI	32%	6%	39%	2%	40%	4%	*
NF6523	8523-31-CI	43%	11%	31%	2%	22%	6%	*
NF6523	8523-32-CI	-1%	27%	3%	28%	25%	26%	**>100**
NF6523	8523-33-CI	15%	18%	-27%	28%	-20%	28%	-
	Milk Thistle	31%	17%	29%	1%	26%	18%	**>100**
CD5439	King Drink	30%	13%	32%	6%	50%	19%	**100**

In addition to the cell viability assays, phase II enzyme induction testing was also conducted to determine if a material had detoxification properties. The phase II enzyme induction assay measures a sample's ability to induce quinone reductase (a phase II enzyme) which is indicative of detoxification events. Broccoli is known as a good phase II enzyme inducer because of its sulforaphane content. Pure sulforaphane has activity at 10^6 U/g, while broccoli has reported activity from 4000 – 74,000 U/g (dependent on variety, form, and/or extraction conditions). Activity above 30,000 U/g is considered excellent. Activity less than 5,000 U/g is considered minimal. Most materials that are considered good phase II enzyme inducers will have activity between 15,000-30,000 U/g.

All the blends tested had good to excellent phase II enzyme induction activity. The highest activity came from 8523-27 (Example 8) and the lowest from 8523-31 (Example 4). Excellent activity also came from 8523-22, 8523-23, 8523-30 (Example 3), 8523-32 (Example 5) and 8523-33. Table 10 gives the results of the phase II enzyme induction assay for all samples tested.

Table 10: Phase II Enzyme (Quinone Reductase) Induction Assay Results

Sample Number	Formula Number or Sample Name	U/g	Rank
NF6523	8523-20-CI	23,810	+++
NF6523	8523-21-CI	24,690	+++
NF6523	8523-22-CI	51,280	++++
NF6523	8523-23-CI	31,750	++++
NF6523	8523-24-CI	15,500	+++
NF6523	8523-25-CI	26,670	+++
NF6523	8523-26-CI	16,260	+++
NF6523	8523-27-CI	83,330	++++
NF6523	8523-28-CI	19,610	+++
NF6523	8523-30-CI	44,440	++++
NF6523	8523-31-CI	9950	++
NF6523	8523-32-CI	47,620	++++
NF6523	8523-33-CI	60,600	++++
	Milk Thistle	>100,000	++++
CD5439	King Drink	17,540	+++

* Inducer units per gram of fresh weight of material. - = Negligible activity, + = Little activity,

++ = Good activity, +++ = Very good activity, ++++ = Excellent activity. **Not reportable due to toxicity to cells.

Experimental

Stock sample solutions are made in DMSO, then diluted in cell culture media for testing. Treatment of HepG2 cells (human liver cell line) is done by adding 100 µL of sample to each of three wells of a 96-well microtiter plate. After a 4 hour incubation, the insult is added (2.5% ethanol or 0.2% carbon tetrachloride) and an additional overnight incubation period is conducted. The next day, cell viability is measured using two different assays. First, using the CYTOTOX-ONE™ Homogenous Membrane Integrity Assay by Promega, the number of non-viable cells is estimated by measuring the release of LDH into the media. LDH leaks out of the cell when the cell membrane is compromised. The second assay is the MTT assay, which measures the reduction of a yellow tetrazolim salt into an insoluble purple formazen product by the mitochondria of viable cells. Following an incubation with the MTT solution, isopropanol is added to solubilize the colored crystals. The amount of color produced is directly proportional to the number of viable cells.

Protection is determined by first calculating a percent toxicity of each well (1-experimental/negative control), after averaging the three replicates. Percent protection is then calculated by the following: (%Toxicity Positive Control - % Toxicity Sample)/% Toxicity Positive Control, the positive control being either 2.5% ethanol or 0.2% carbon tetrachloride. The concentration that exhibits 50% protection (EC-50) can then be assessed. For the purpose of this experiment, this was categorized as either <1, 1, 1-10, 10, 10-100, 100 or >100 μg/mL.

For the phase II enzyme induction assay, stock sample solutions are made in acetonitrile, then diluted in cell culture media for testing. Treatment of Hepa1c1c7 cells (murine hepatoma cell line) is done by adding 150μL of sample to each of three wells, in a 96-well microtiter plate. After 48 hours incubation, induction activity of quinone reductase is established by measuring the NADPH-dependent, menadiol-mediated reduction of MTT. Activity is reported as inducer units per gram of fresh weight of material, where one unit of inducer activity is defined as the amount of inducer required to double the quinone reductase specific activity of Hepa1c1c7 cells.

Mammalian Studies

Clinical testing can be conducted to confirm the efficacy of the formulations on liver health. It is expected that the formulations will improve liver health by protecting the liver from chemical and alcohol insults. A protocol for such testing follows.

PROTOCOL 1: CCl_4 LIVER INJURY MODEL

1.1 Principles.

When CCl_4 is activated by microsomal enzyme of liver, trichloromethane free radicals ($CCl_3\cdot$) are formed. Covalent combination of this free radical with protein results in impairment of protein synthesis and disorder of lipid catabolism, causing accumulation of triglyceride (TG) in liver cells. $CCl_3\cdot$ also can combine with O_2 rapidly to form trichloromethane peroxide free radicals ($CCl_3O_2\cdot$), leading to lipid peroxidation which causes degenerative injury of cell membrane, leakage of enzymes and various types of pathological changes of cells and even necrosis.

1.2 Experimental animals.

Adult rats or mice of single sex. Each group consists of 8-12 rats (180-220g) or 10-15 mice (18-22g).

1.3 Experimental methods and procedures.

1.3.1 Dosage groups and duration of administration of the test sample.

Three dosage groups, one blank control group and one model control group are set. The dosage of one of the dosage groups is 10 times (mice) or 5 times (rats) the recommended human dosage. CCl_4 (analytically pure) is used to form liver injury model. The method of forming model can use intragastric administration or intraperitoneal injection. The concentration of CCl_4 for intragastric administration in mice is 1%. CCl_4 is diluted with edible vegetable oil and the dosage for intragastric administration is 5mL/kg BW (the dosage in terms of CCl_4 is 80mg/kg BW). The concentration of CCl_4 for intragastric administration in rats is 2%-3% and the dosage is 5mL/kg BW (the dosage in terms of CCl_4 is 160-240mg/kg BW). Positive control group and solvent control group may be set if necessary. The duration of administration of the test sample is 30 days and can be prolonged to 45 days if necessary.

1.3.2 Route of administration of the test sample.

The test sample is given intragastrically. If this is impossible, the test sample can be mixed into the feed or drinking water and the feed intake or water drunk is recorded.

1.3.3 Experimental procedures.

The animals of the experimental group are given intragastrically the test sample daily, while those of the blank control group and the model control group are given distilled water. The animals are weighed twice a week for adjusting the dosage of the test sample. On the eve of day 30 of the experiment, the animals of various groups fast for 16h. The animals of the model group and various test sample groups are given intragastrically single dose of CCl_4, while those of the blank control group are given vegetable oil. The animals of the experimental group continue to receive the test sample until the end of the experiment (the interval between administration of the test sample and CCl_4 is over 4h). After giving CCl_4, the animals are sacrificed 24h or 48h later according to the actual conditions. Blood is taken and serum is separated for measuring ALT and AST. Liver is taken for histopathological examination.

1.3.4 Indices for measurement.

Serum glutamate-pyruvate transaminase (ALT), glutamic-oxaloacetic transaminase (AST), histopathological examination of liver.

1.4 Measurement of ALT and AST.

1.4.1 Measuring method.

Full-automatic biochemical analyzer or Reitman-Frankel method (reagent kit) can be selected.

1.4.1 Data treatment and result assessment.

Variance analysis is used, but variance homogeneity test should be performed first according to the procedures of variance analysis. If the variance is homogeneous, *F* value is calculated. If *F* value is $< F_{0.05}$, the conclusion is that the difference between means of different groups is not significant. If *F* value is $\geq F_{0.05}$ and *P* is ≤ 0.05, the method of paired comparison of means between several experimental groups and one control group is used for statistical analysis. For data with abnormal distribution or variance inhomogeneity, appropriate conversion of variables is performed and the converted data are used for statistical analysis after the requirement of normal or variance homogeneity is fulfilled. If the purpose of normal or variance homogeneity is still not achieved after conversion of variables, rank test is used for statistical analysis.

If ALT and AST of the test sample group are different significantly from those of the model control group, the results of ALT and AST can be assessed as positive respectively.

1.5 Histopathological changes of liver, diagnostic criteria and result assessment.

1.5.1 Experimental materials.

The left lobe of rat liver is fixed with 10% formalin. The liver tissue is taken from the cross section of middle part of the left lobe of liver for routine preparation of pathological section (paraffin embedding, H.E. strain).

1.5.2 Microscopic examination.

Using the 40-fold objective to observe continuously the whole tissue section, the pathological changes of cells are recorded beginning from the visual field of one end of the liver. The degenerative changes of central liver cells of the lobe and necrosis of a few cells can be seen. The main types of pathological changes are ballooning degeneration, fatty degeneration, condensation of cytoplasm, hydropic degeneration and necrosis of liver cells, etc.

1.5.3 Criteria for rating.

Each pathological change accounting for the portion of area of visual field in each visual field is recorded respectively and the total score of pathological changes in the visual fields observed is added up.

Ballooning degeneration of liver cells: (swelling of cells, a little cytoplasm remains)

Roughly normal	0 points
Liver cells with ballooning degeneration account for 1/4 of whole visual field	1 point

Liver cells with ballooning degeneration account for 1/2 of whole visual field	2 points
Liver cells with ballooning degeneration account for 3/4 of whole visual field	3 points
Liver cells with ballooning degeneration account for whole visual field	4 points

Fatty degeneration of liver cells: (distinctly demarcated fat drop vacuoles appear in cytoplasm of liver cells)

Roughly normal	0 points
Liver cells with fatty degeneration account for 1/4 of whole visual field	1 point
Liver cells with fatty degeneration account for 1/2 of whole visual field	2 points
Liver cells with fatty degeneration account for 3/4 of whole visual field	3 points
Liver cells with fatty degeneration account for whole visual field	4 points

Condensation of cytoplasm: (eosinophilic stain is enhanced)

Roughly normal	0 points
Liver cells with condensation of cytoplasm account for 1/4 of whole visual field	1 point
Liver cells with condensation of cytoplasm account for 2/4 of whole visual field	2 points
Liver cells with condensation of cytoplasm account for 3/4 of whole visual field	3 points
Liver cells with condensation of cytoplasm account for whole visual field	4 points

Hydropic degeneration:

No liver cell with hydropic change is seen	0 points
Liver cells with hydropic degeneration account for 1/4 of whole visual field	1 point
Liver cells with hydropic degeneration account for 2/4 of whole visual field	2 points
Liver cells with hydropic degeneration account for 3/4 of whole visual field	3 points
Diffuse liver cells with hydropic degeneration account for whole visual field	4 points

Necrosis of liver cells: (eosinophilic change of cytoplasm, coagulation necrosis)

No necrotic cell is seen	0 points
Sporadic necrotic cells account for 1/4 of whole visual field	1 point
Necrotic cells account for 2/4 of whole visual field	2 points

Necrotic cells account for 3/4 of whole visual field	3 points
Diffuse necrotic cells account for whole visual field	4 points

1.5.4 Data treatment and result assessment.

Variance analysis is used, but variance homogeneity test should be performed first according to the procedures of variance analysis. If the variance is homogeneous, F value is calculated. If F value is $< F_{0.05}$, the conclusion is that the difference between means of different groups is not significant. If F value is $\geq F_{0.05}$ and P is ≤ 0.05, the method of paired comparison of means between several experimental groups and one control group is used for statistical analysis. For data with abnormal distribution or variance inhomogeneity, appropriate conversion of variables is performed and the converted data are used for statistical analysis after the requirement of normal or variance homogeneity is fulfilled. If the purpose of normal or variance homogeneity is still not achieved after conversion of variables, rank test is used for statistical analysis.

Among the pathological changes of liver cells including ballooning degeneration, fatty degeneration, cytoplasm condensation , hydropic degeneration and necrosis of liver cells, if the necrosis of liver cells in any dosage group of test sample is alleviated as compared with that in the model control group with significant difference and other types of pathological changes are alleviated significantly or have no significant difference as compared with those in the model control group, the results of animal pathological experiment can be assessed as positive.

If aggravation and alleviation of the 4 types of pathological changes of liver cells, namely, ballooning degeneration, fatty degeneration, cytoplasm condensation and hydropic degeneration, are present simultaneously with significant difference and necrosis of liver cells is alleviated in any one dosage group of the test sample with significant difference as compared with the model control group, the scores of various pathological changes and double of necrosis score are added together. The total score is used for statistical analysis. If the total score has significant difference, the results of animal pathological experiment can be assessed as positive.

1.6 Assessment of results.

It is expected that any one of the two blood biochemical indices, ALT and AST, and the result of pathological examination will be positive and the test sample will be assessed as assisting in the protection against chemical injury to the liver.

PROTOCOL 2: MODEL OF ALCOHOLIC INJURY OF LIVER

2.1 Principles.

After taking large amounts of ethyl alcohol, massive dehydroxylation catalyzed by ethanol dehydrogenase causes disorder of tricarboxylic acid cycle and weakening of oxidation of fatty acid, thereby influencing fat metabolism and precipitation of fat in liver cells. At the same time, ethyl alcohol can activate oxygen molecules and cause production of oxygen free radicals, leading to lipid peroxidation of liver cell membrane and depletion of reduced glutathione in body.

2.2 Experimental animals.

Adult mice or rats of single sex. Each group consists of 8-12 rats (180-220g) or 10-15 mice (18-22g).

2.3 Experimental methods and procedures.

2.3.1 Dosage groups and duration of administration of the test sample.

Three dosage groups, one blank control group and one model control group are set. The dosage of one of the dosage groups is 10 times (mice) or 5 times (rats) the recommended human dosage. A positive control group may be set if necessary. Absolute ethyl alcohol (analytically pure) is used to form model of liver injury. The concentration of absolute ethyl alcohol is 50% (diluted with distilled water) and the dosage for intragastric administration to mice is 12-14mL/kg BW (equivalent to ethyl alcohol 6000-7000mg/kg BW). The duration of administration of the test sample is 30 days and can be prolonged to 45days if necessary.

2.3.2 Route of administration of the test sample.

The test sample is given intragastrically. If intragastric administration is impossible, the test sample can be mixed in feed or drinking water, and the feed intake and drinking water drunk of each animal is recorded.

2.3.3 Experimental procedures.

The animals of the test sample groups are given intragastrically the test sample every day and those of the blank control group and model control group are given distilled water. The animals are weighed twice weekly and the dose of the test sample is adjusted according to body weight. At the time of completion of administration of the test sample, a single dose of 50% ethyl alcohol 12mL/kg BW is given to the animals of the model control group and three dosage groups, while the animals of the blank control group are given distilled water. After fasting for 16h, the animals are sacrificed for examination of various indices and histopathological examination.

2.3.4 Indices for examination.

Malondialdehyde (MDA), reduced glutathione (GSH), triglyceride (TG) content of liver.

2.4 Method for measuring the degradation product of lipid peroxide malondialdehyde (MDA) in liver homogenate.

2.4.1 Principle.

MDA is one of the final products of peroxidation of lipids of cell membrane. Measuring MDA content can estimate indirectly the degree of lipid peroxidation. When MDA and thiobarbital are heated together in acidic condition, pink-colored complex is formed and its absorption peak is at 535nm, from which the MDA content can be measured.

2.4.2 Instruments and reagents.

Instruments: 721 spectrophotometer, sample micro-applicator, thermostat water bath, ordinary centrifuge, mixing rotator, centrifuge tube with stopper, tissue homogenizer.

Reagents: 0.2M acetate buffer solution, pH 3.5:

0.2M acetic acid solution 185mL

0.2 M sodium acetate solution 15mL

1mmol/L tetraethoxyl propane (stock solution, kept at 4°C for 3 months), diluted with water to 40nmol/mL just before use:

8.1% sodium dodecyl sulfate SDS

0.8% thiobarbital TBA

0.2 M phosphate buffer solution, pH 7.4

0.2 M disodium hydrogen phosphate 1920mL

0.2 M potassium dihydrogen phosphate 480mL

2.4.3 Experimental procedures.

2.4.3.1 Preparation of sample.

Tissue homogenate sample: certain quantity of the organ needed is rinsed with normal saline, wiped to dry, weighed, minced and put into homogenizer. 0.2 M phosphate buffer solution is added and the mixture is homogenized at 2000r/min for 10s. The centrifugation is repeated 3 times with 30s intervals to form 5% tissue homogenate (W/V). The homogenate is centrifugalized at 3000r/min for 5-10min and the supernatant is taken for measurement.

2.4.3.2 Measurement of the sample.

Reagent	Blank tube	Sample tube	Standard tube

5% tissue homogenate		0.1mL	
40nmol/mL tetraethoxyl propane		0.1mL	
8.1% SDS	0.2mL	0.2mL	0.2mL
0.2 M acetate buffer solution	1.5mL	1.5mL	1.5mL
0.8% TBA	1.5mL	1.5mL	1.5mL
H_2O	0.8mL	0.7mL	0.7mL

Mix to homogenize, boiling water bath for 60min protect from light, cooled with flowing water, colorimetry at 532nm

2.4.3.3 Calculation.

$$\text{Lipid peroxide content (nmol/mg tissue)} = \frac{B-A}{F-A} \times C \times K = \frac{B-A}{F-A} \times 40 \times \frac{1}{0.05 \times 1000}$$

Lipid peroxide content (nmol/100mg protein) =

$$\frac{B-A}{F-A} \times C \times K = \frac{B-A}{F-A} \times 40 \times \frac{1}{0.05} \times \frac{1}{\text{Protein (mg)/g tissue}} \times 100$$

A: absorbance of blank tube

B: absorbance of sample tube

F: absorbance of tetraethoxyl propane

C: concentration of tetraethoxyl propane (40nmol/mL)

K: multiple of dilution

2.4.3.4 Data treatment and result assessment.

The data are analyzed with variance analysis, but variance homogeneity test should be performed first according to the procedures of variance analysis. If the variance is homogeneous, F value is calculated. If F value is $< F_{0.05}$, the conclusion is that the difference between means of different groups is not significant. If F value is $\geq F_{0.05}$ and P is ≤ 0.05, the method of paired comparison of means between several experimental groups and one control group is used for statistical analysis. For data with abnormal distribution or

variance inhomogeneity, appropriate conversion of variables is performed and the converted data are used for statistical analysis after the requirement of normal or variance homogeneity is fulfilled. If the purpose of normal or variance homogeneity is still not achieved after conversion of variables, rank test is used for statistical analysis.

Assessment of results

It is expected that the MDA content of test sample groups will be significantly different from that of the model control group and, as such, the result of this index will be assessed as positive.

2.5 Method for measuring reduced glutathione (GSH) in liver homogenate.

2.5.1 Principle.

Reaction between GSH and 5,5'-dithionitroformic acid (DTNB) catalyzed by GSH-Px produces yellow-colored 5-thio-2-nitro-formic acid anion which has maximum absorption peak at wavelength of 423nm. Measuring the concentration of this ion can calculate GSH content.

2.5.2 Reagents.

0.9% normal saline

4% sulfosalicylic acid solution

0.1mol/L PBS solution (pH = 8.0)

Na_2HPO_4 13.452g

KH_2PO_4 0.722g

Distilled water ad 1000mL.

0.004% DTNB solution: DTNB 40mg is dissolved in 1000mL of 0.1mol/L PBS solution (pH = 8.0).

Sodium azide buffer solution.

NaN_3 16.25mg

EDTA-Na_2 7.44mg

Na_2HPO_4 1.732g

NaH_2PO_4 1.076g

Distilled water ad 1000mL. Small amount of HCl and NaOH are used to regulate pH 7.0.

The solution is kept at 4°C.

Standard solution: Reduced GSH 15.4mg is weighed and sodium azide buffer solution is added to 50mL to make the final concentration 1mmol/L. The solution is prepared just before use.

2.5.3 Methods.

2.5.3.1 Measurement of sample.

Normal saline 5mL is added to liver 0.5g. The mixture is well ground to form fine thick liquid (10% liver homogenate). After homogenizing, 4% sulfosalicylic acid 0.5mL is added to the homogenate 0.5mL. After mixing, the mixture is centrifugalized at 3000rpm for 10min at room temperature and the supernatant is the sample.

Reagent	Tube for measurement	Blank tube
Sample	0.5mL	—
4% sulfosalicylic acid	—	0.5mL
DINB	4.5mL	4.5mL

The mixture is mixed, laid aside for 10min at room temperature and its absorbance is measured at 412nm.

2.5.3.2 Standard curve.

Reagent	1	2	3	4	5	6
1mmol/L GSH (mL)	0	0.05	0.10	0.15	0.20	0.25
Normal saline (mL)	0.50	0.45	0.40	0.35	0.20	0.25
DTNB (mL)	4.50	4.50	4.50	4.50	4.50	4.50
GSH content (μmol/L)	0	100	200	300	400	500

2.5.3.3 Calculation.

Sample GSH content (μmol/L liver tissue) = corresponding curve concentration value (μmol/L) ÷50g/L

2.5.4 Data treatment and result assessment.

The data are analyzed with variance analysis, but variance homogeneity test is performed first according to the procedures of variance analysis. If the variance is

homogeneous, F value is calculated. If F value is $< F_{0.05}$, the conclusion is that the difference between means of different groups is not significant. If F value is $\geq F_{0.05}$ and P is ≤ 0.05, the method of paired comparison of means between several experimental groups and one control group is used for statistical analysis. For data with abnormal distribution or variance inhomogeneity, appropriate conversion of variables is performed and the converted data are used for statistical analysis after the requirement of normal or variance homogeneity is fulfilled. If the purpose of normal or variance homogeneity is still not achieved after conversion of variables, rank test is used.

Assessment of results

It is expected that the reduced GSH content of the test sample group will be significantly different from that of the model control group and, as such, the result of this index is assessed as positive.

2.6 Method for measuring triglyceride (TG) in liver homogenate.

2.6.1 Measuring method.

Triglyceride measurement reagent kit (glycerophosphoric acid oxidase peroxidase method) is used to measure the triglyceride content in 10% liver homogenate. Same as the method of measuring serum triglyceride, equal amount of 10% liver homogenate is used instead of serum and the measurement is performed according to the description of operation. The result of measurement is expressed as mmol/g liver weight.

2.6.2 Data treatment and result assessment.

The data are treated with variance analysis, but variance homogeneity test is performed first according to the procedures of variance analysis. If the variance is homogeneous, F value is calculated. If F value is $< F_{0.05}$, the conclusion is that the difference between means of different groups is not significant. If F value is $\geq F_{0.05}$ and P is ≤ 0.05, the method of paired comparison of means between several experimental groups and one control group is used for statistical analysis. For data with abnormal distribution or variance inhomogeneity, appropriate conversion of variables is performed and the converted data are used for statistical analysis after the requirement of normal or variance homogeneity is fulfilled. If the purpose of normal or variance homogeneity is still not achieved after conversion of variables, rank test is used for statistical analysis.

Assessment of results

It is expected that the TG of the test sample group will be significantly different from that of the model control group and, as such, the result of this index will be assessed as positive.

2.7 Histopathological changes of liver, diagnostic criteria and result assessment.

2.7.1 Experimental materials.

Cross section at middle part of left lobe of liver is performed for taking examination material. Frozen section is made and stained with Sudan III staining.

2.7.2 Microscopic examination.

The pathological changes of the cells are recorded beginning from the visual field at one end of the liver. 40-fold objective is used for continuous observation of whole tissue section. The main objects for observation are distribution, range and area of fat drops in liver.

2.7.3 Criteria for rating.

The fat drops in liver cells are sporadic and scarce	0 points
The liver cells containing fat drops do not exceed 1/4	1 point
The liver cells containing fat drops do not exceed 1/2	2 points
The liver cells containing fat drops do not exceed 3/4	3 points
The liver tissue is almost replaced by fat drops	4 points

2.7.4 Data treatment and result assessment.

Variance analysis is used, but variance homogeneity test should be performed first according to the procedures of variance analysis. If the variance is homogeneous, F value is calculated. If F value is $< F_{0.05}$, the conclusion is that the difference between means of different groups is not significant. If F value is $\geq F_{0.05}$ and P is ≤ 0.05, the method of paired comparison of means between several experimental groups and one control group is used for statistical analysis. For data with abnormal distribution or variance in homogeneity, appropriate conversion of variables is performed and the converted data are used for statistical analysis after the requirement of normal or variance homogeneity is fulfilled. If the purpose of normal or variance homogeneity is still not achieved after conversion of variables, rank test is used for statistical analysis.

It is expected that the fatty degeneration in any dosage group of test sample will be alleviated as compared with the model control group with statistical difference and, as such, the result will be assessed as positive.

2.8 Assessment of the results.

It is expected that the following conditions will be fulfilled and, as such, the test sample would be assessed as assisting in protection against alcoholic liver injury:

(a) The results of examination of 3 indices, namely, liver MDA, reduced GSH and TG, are positive.

(b) Any two of the 3 indices, namely, liver MDA, reduced GSH and TG, are positive and the results of histopathological examination are positive.

WHAT IS CLAIMED:

1. A formulation comprising at least two ingredients selected from the group consisting of schisandra berry extract, notoginseng, kudzu root extract, spinach dehydrate, oregano extract, artichoke leaf extract, wasabi root powder, gegen root extract, sanchi, and asparagus dehydrate, wherein the formulation is effective for improving liver health.

2. The formulation of claim 1 wherein the schisandra berry extract is present in an amount from 150 mg – 1000 mg, the notoginseng is present in an amount from 100mg - 500mg, the kudzu root extract is present in an amount from 150 mg – 1000 mg, the spinach dehydrate is present in an amount from 20 mg -500 mg, the oregano extract is present in an amount from 20 mg - 500 mg, the artichoke leaf extract is present in an amount from 200mg - 1000mg, the wasabi root extract is present in an amount from 200 mg -1000 mg, the gegen root extract is present in an amount from 150 mg – 1000 mg, the sanchi is present in an amount from 100 mg 500 mg, and the asparagus dehydrate is present in an amount from 100mg – 500mg.

3. The formulation of claim 1 wherein the formulation comprises 150 mg-500 mg wasabi root powder, 200 mg – 1000 mg artichoke leaf extract, 150 mg – 1000 mg schisandra berry extract, 100 mg – 500 mg notoginseng, and 20 mg – 500 mg spinach dehydrate.

4. The formulation of claim 1 wherein the formulation comprises 150mg - 500mg wasabi, 200mg - 1000mg artichoke leaf extract, 150 mg – 1000mg kudzu root extract, 100mg – 500mg notoginseng, and 20mg – 500mg oregano.

5. The formulation of claim 1 wherein the formulation comprises 200mg – 1000mg artichoke leaf extract, 150 mg -1000 mg kudzu root extract, and 20 mg – 500 mg oregano extract.

6. A method of protecting the liver from carbon tetrachloride insults comprising providing the formulation of claim 1.

7. The method of claim 6 wherein the formulation comprises 150 mg – 500 mg wasabi root powder, 200 mg – 1000mg artichoke leaf extract, 150 mg – 1000 mg schisandra berry extract, 100 mg – 500 mg notoginseng, and 20 mg – 500 mg spinach dehydrate.

8. The method of claim 6 wherein the at least two ingredients are selected from the group consisting of notoginseng, kudzu root extract, spinach dehydrate, oregano extract, wasabi root powder, gegen root extract, sanchi, and asparagus dehydrate.

9. The method of claim 6 wherein the formulation comprises wasabi and one of the ingredients selected from the group consisting of notoginseng, sanchi, kudzu root extract, gegen root extract, spinach, and oregano.

10. The method of claim 6 wherein the formulation is orally administered.

11. The method of claim 6 wherein the formulation is in the form of a tablet.

12. A method of protecting the liver from alcohol insults by providing the formulation of claim 1.

13. The method of claim 12 wherein the formulation comprises 150 mg – 500 mg wasabi root powder, 200 mg – 1000mg artichoke leaf extract, 150 mg – 1000 mg schisandra berry extract, 100 mg – 500 mg notoginseng, and 20 mg – 500 mg spinach dehydrate.

14. The method of claim 12 wherein the formulation comprises artichoke and oregano.

15. The methods of claim 12 wherein the formulation is orally administered in the form of a tablet.

16. A method of inducing phase II enzymes comprising providing the formulation of claim 1.

17. The method of claim 16 wherein the formulation comprises 150 mg – 500 mg wasabi root powder, 200 mg – 1000 mg artichoke leaf extract, 150 mg – 1000 mg schisandra berry extract, 100 mg – 500 mg notoginseng, and 20 mg – 500 mg spinach dehydrate.

18. The method of claim 15 wherein the formulation comprises gegen root extract and one of the ingredients selected from the group consisting of artichoke leaf extract, wasabi root powder, oregano extract, notoginseng, sanchi, and kudzu root extract.

19. The method of claim 15 wherein the formulation is orally administered in the form of a tablet.

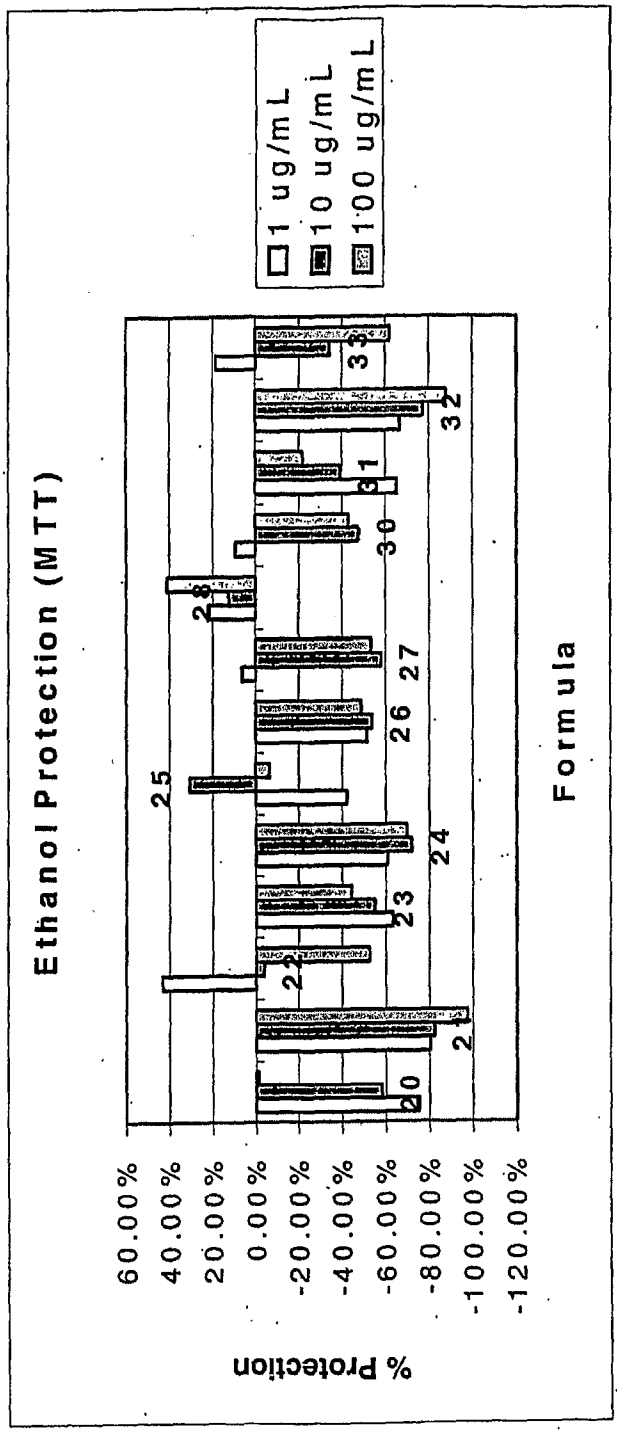
Ethanol Protection (MTT)
1 ug/mL
10 ug/mL
100 ug/mL
% Protection
60.00%
40.00%
20.00%
0.00%
-20.00%
-40.00%
-60.00%
-80.00%
-100.00%
-120.00%
Formula
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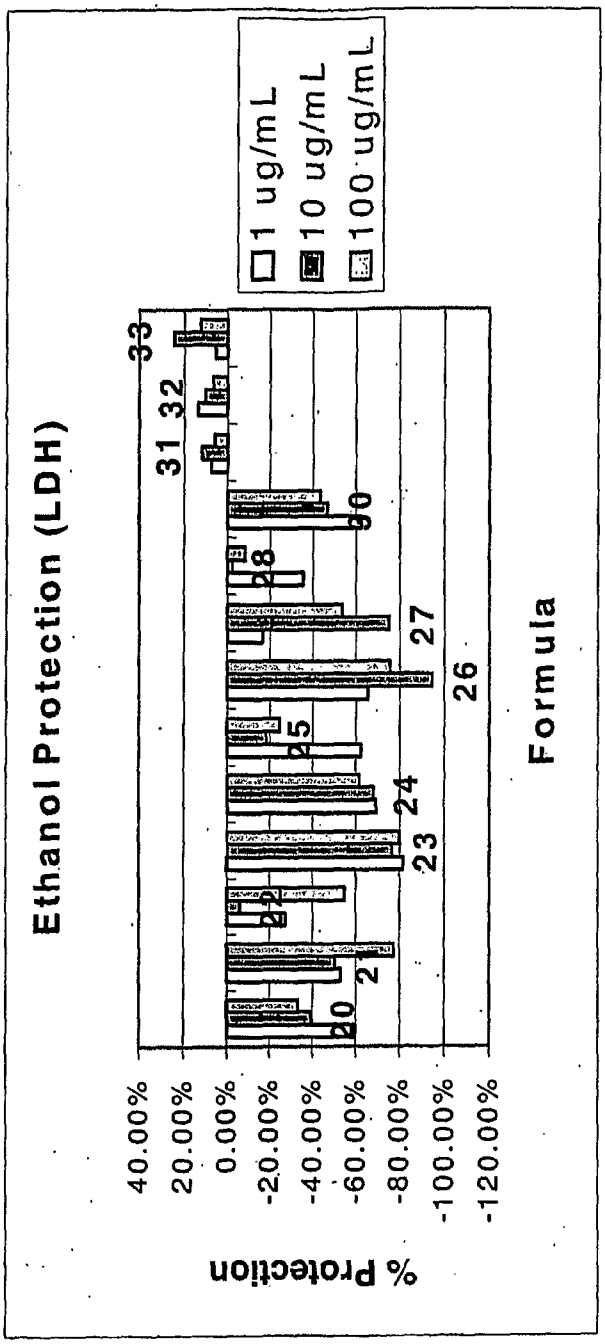
FIGURE 1


Ethanol Protection (LDH)
1 ug/mL
10 ug/mL
100 ug/mL
33
31 32
30
28
27
26
25
24
23
22
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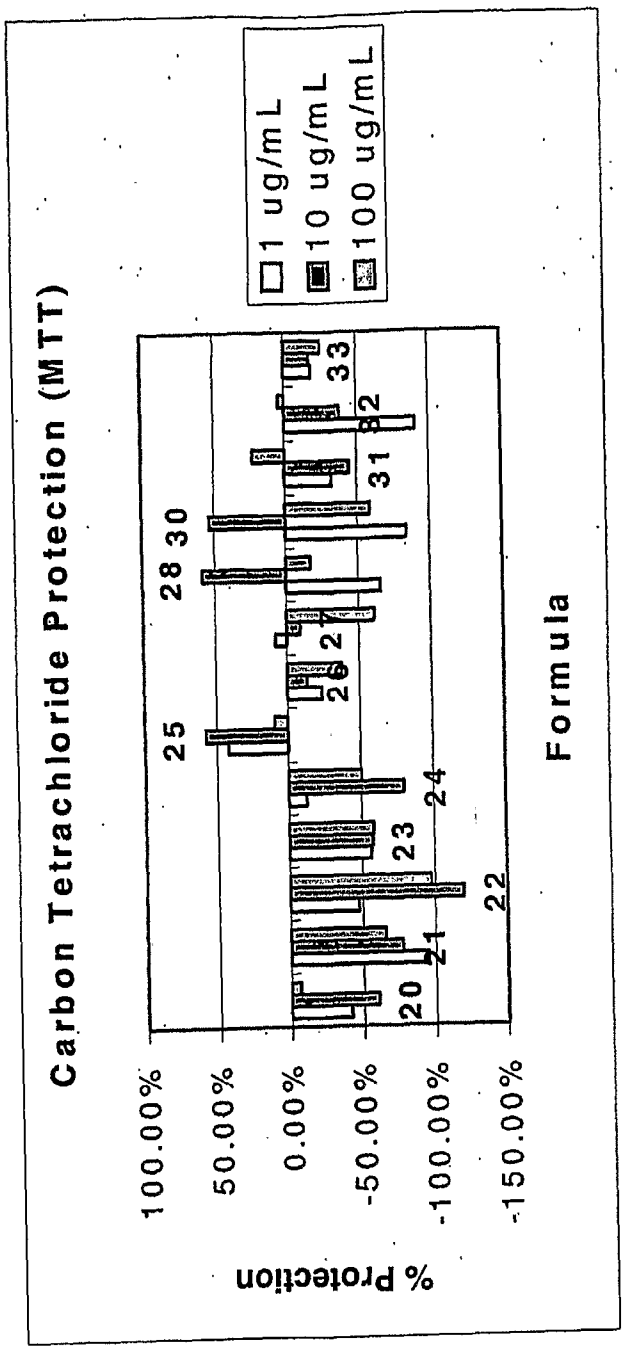
20
40.00%
20.00%
0.00%
-20.00%
-40.00%
-60.00%
-80.00%
-100.00%
-120.00%
% Protection
Formula

FIGURE 2


Carbon Tetrachloride Protection (MTT)
1 ug/mL
10 ug/mL
100 ug/mL
% Protection
100.00%
50.00%
0.00%
-50.00%
-100.00%
-150.00%
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Formula

FIGURE 3

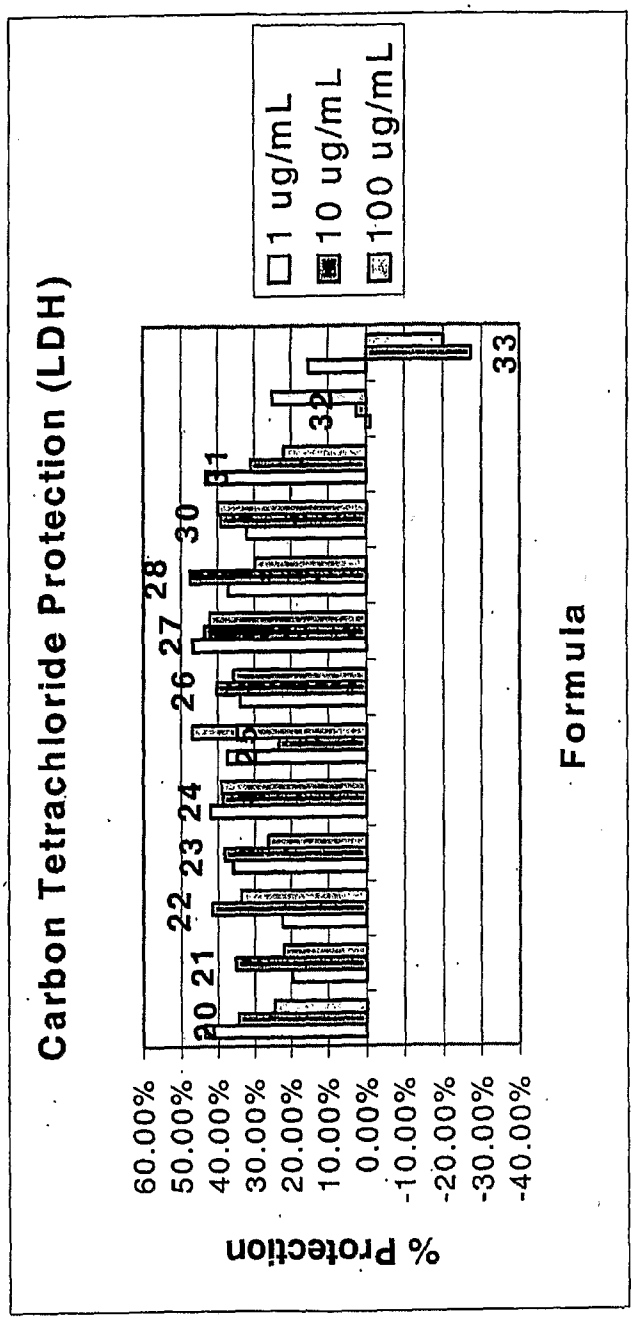
Carbon Tetrachloride Protection (LDH)
1 ug/mL
10 ug/mL
100 ug/mL
% Protection
60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
-10.00%
-20.00%
-30.00%
-40.00%
20 21 22 23 24 25 26 27 28 30 31 32 33
Formula

FIGURE 4