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Full Length Research Paper

The acute and long-term safety evaluation of aqueous, methanolic and ethanolic extracts of *Achillea fragrantissima*

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This study was designed to explore the safety and side effects of the different extracts (water, ethanolic and methanolic) of *Achillea fragrantissima* given acutely or on repeated doses (125 and 250 mg/kg) in rats. Acute and subchronic toxicity, as well as reproductive (fertility, embryotoxicity and teratogenicity, peri- and postnatal study) effects were recorded on treated and control rats. Daily administration of the plant extract revealed no significant changes on the body weights, heart rates, and other physiological parameters. The plant extract induced a significant increase in total proteins and globulins in rats. It did not induce any abnormal liver and kidney functional changes as demonstrated by serum biochemical analysis in rats. Interestingly, the plant extract induced a significant decrease in alkaline phosphatase (ALP), urea and creatinine. Significant decrease in blood glucose level was detected in animals receiving 250 mg/kg of the extract. The plant extract did not affect fertility. Dosed males showed comparable data with the controls when dosed at 250 mg/kg b.wt. It did not cause any embryotoxic, teratogenic or any deleterious effects on the dosed females and their offspring. Litter size, survival rate and weight gain were comparable between groups. In conclusion, *A. fragrantissima* extract is a well tolerated substance and had a wide safety margin. The tested plant extracts did not induce any toxic effects even on repeated administration in rats for 2 months. Additionally, no evidences of impaired fertility, or teratogenic potentials at higher doses up to several times the recommended maximum human doses were detected.

Key words: *Achillea fragrantissima*, physiological parameters, fertility, embryotoxicity, teratogenicity.

INTRODUCTION

The use of herbal medicine has become more prevalent, and the past few decades have witnessed a rapidly increasing demand worldwide. The range of medicinal

plants is very diverse and it has been estimated that around 70,000 different plant species have been used at least once during the history of traditional medicine

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(Ghasemi, 2002). According to a World Health Organization (WHO) report, around four billion people (80% of the world's population) use herbal medicine (Ghasemi, 2002), with eleven different bioclimatic regions and around 7,500 different plant species. Therefore, many folk remedies from plant origin are tested for its potential antioxidant and hepatoprotective liver damage in experimental animal model. Medicinal plant which at least one of its parts contains substances, can be used for therapeutic purposes. *Achillea* is one of the most important genera of the Compositae family and comprises more than 115 species (Saeidnia et al., 2009). Several effects such as anti-inflammatory (Benedek et al., 2007), antihypertensive and anti-hyperlipidemia (Asgary et al., 2000), and antitumoral (Csupor-Löffler et al., 2009) have been reported for *Achillea*. It is widely used in traditional medicine for gastrointestinal disorders, and there are some reports of its effects on gastrointestinal tract such as antispasmodic (Lemmens-Gruber et al., 2006). *Achillea* Sch. Bip. is considered an important medicinal species with high content of essential oils, and is thoroughly studied.

The plant is overexploited by collection for folk medicinal uses, it has the Arabic common name Qaysoom and it is considered endangered in Al-Gouf and Al-Qaseem, Kingdom of Saudi Arabia (KSA). It is used for the treatment of gastro-intestinal disturbances, cough and was reported as carminative, anthelmintic and antiseptic to various infections for the urinary tract (Aboutable et al., 1986; Sincich, 2002). Neither acute nor subchronic toxicity were noticed in mice with ethanolic extracts of *Achillea fragrantissima* (Fleisher and Fleisher, 1993). Moreover, insecticidal and rodenticidal activities of *A. fragrantissima* oils were demonstrated by Hifnawy et al. (2001).

The herb contains essential oil (0.81%) and consists of 59 components of which α and β -thujone, α -pinene, β -pinene, limonene, 1,8-cineole, linalool, carvacrol, eugenol, artemisia ketone, palustrol, sabinene hydrate, α -terpineol and santolina alcohol are the major constituents. Its tannin content reaches 8% such as resorcin, phloroglucin, methyl phloroglucin, and pyrocatechol. Flavonoids were also reported, such as afroside, cirsimartin, chrysoplenol and cirsililol.

Also, fatty acids, lauric, myristic, palmitic, stearic, oleic, linoleic and linolenic as well as sesquiterpene lactones as achilloide A, in addition to taraxasterol and pseudotaraxasterol acetates have been identified (Elgamel et al., 1991; Moustafa et al., 1995; Shalaby and Richter, 1964).

A few studies have been published in the literature regarding the properties of *A. fragrantissima* extract in the kingdom of Saudi Arabia. Therefore, the present study was designed to explore the safety and side effects of the different extracts of *A. fragrantissima* given acutely or on repeated doses in rats.

MATERIALS AND METHODS

Animals

Fifteen male Sprague Dawley rats 2 to 3 month age (175 to 200 g) were obtained from the closed random bred colony maintained in the animal house at College of Veterinary Medicine and Animal Resources, King Faisal University, Kingdom of Saudi Arabia. The rats were acclimatized for a period of 15 days under standard environmental conditions of temperature, relative humidity and dark/light cycle. They were maintained on food and water *ad libitum* and housed in groups of four in isolated cages. Rats in all groups received humane care in compliance with the animal care guidelines of the National Institute of Health, and the local ethical committee approved this study.

Plant description and identification

Achillea is one of the most important genera of the Compositae family. Synonyms: *Santolina fragrantissima* Forssk. Common names: Lavender cotton (English); Garda-robe, aurone gemelle, santoline (French); Cypressengarbe (German); Guardaroba, abrotano femmina, santolina (Italian); Qaysūm (Arabic), other vernacular names: quaysūn, baytaram, bu'aythirān. Description: Fragrant chamaephyte, 50 to 100 cm, many-stemmed from a woody base. Leaves small, oblong or ovate, generally more ovate than in the other Syrian species of *Achillea*. Heads discoid, involucre 5 mm, oblong-ovoid. Flowering: May to August. Habitat: Dry areas, steppe, and desert (El-Shatoury et al., 2009). Bedouin name: gaySuum, qaySuum. Status: Not at risk. This is a low shrub with woody older stems; the stems are white, woolly with hairs, the leaves oblong with an undulate margin; there are clusters of small yellow flower heads, and the flowers lack ray florets. The name gives away its intensely fragrant nature. It is a southern Middle East speciality, but is extremely common in Sinai and hence not at risk (Photo by Mike James (2001) Wadi Gebal). Distribution: Arabian peninsula, most rainy districts of Saudi Arabian Kingdom, especially Al-Jouf, South West of Skaka, Ryadah as well as al Qaseem. *A. fragrantissima* were collected at the flowering stage, dried in the shade, and the leaves were separated from the stem and ground in a grinder into fine powder using electric blender.

Preparation of the crude extracts

Water crude extract was prepared by boiling 100 g of dry powder with 300 ml distilled water for 10 to 15 min, sieved and then the crude extract was evaporated until a paste was obtained and then dried. Solid crude extract was weighted and 10 g dissolved into 100 ml distilled water according to Chaplins'ka and Golovkin (1962).

Ethanolic and methanolic crude extract was prepared by soaking 50 g of dry powder each in 300 ml of each ethanol 95% and methanol 95%, with intermittent shaking till ethanolic and methanolic extracts were obtained, then the crude extract was evaporated until paste was obtained under vacuum using the rotary evaporator. The paste was weighed and diluted in 10 g with 100 ml Tween 80 solution 1% as a solvent.

Acute toxicity studies

Six Sprague Dawley rat groups (6 rats/group) were administered different single doses (1.00, 2, 4 mg/kg bwt) of *A. fragrantissima* crude extract 125, 250, and 500, respectively. Doses of the extract

and the vehicle at the same volumes were given orally by stomach tube to adult albino rats. Clinical signs, symptoms and mortality were recorded during a 14-days observation period. The LD₅₀ values were calculated.

Subchronic toxicity studies

These studies were carried out in Sprague Dawley rats (2 to 3 month age). Four groups each of 10 mature male albino rats (160 to 185 g) were used. The drug was given by stomach tube once daily at a volume of 10 ml/kg bwt for a period of 2 months at two dose levels (from each extract), 125 and 250 mg/kg bwt. Meanwhile, rats of the control group were given only the vehicle. The rats were fed with standard feed and provided with water *ad libitum*. During the experiment, all animals were observed daily for general conditions and behavior. Body weight and food consumption in rats were recorded at week intervals.

After 2 weeks, 1 and 2 months from drug administration, rats from each group were anesthetized with light ether, and blood samples were drawn from their retro-orbital plexus before they were killed by decapitation. Two blood samples were collected from each animal. The first sample was collected on disodium salt of ethylene diamine tetra-acetic acid (EDTA) for hematological studies. The second blood sample was collected without anticoagulant for obtaining serum and kept frozen at -20°C until used for biochemical analysis. Hematological parameters [red blood corpuscles (RBCS), white blood corpuscles (WBCS), packed cell volume (PCV), and hemoglobin (HB)] were investigated according to Dacie and Lewis (1984) using Vet scan 5 HM-machine ABaxis USA analyzer (2010), and serum analysis for liver and renal functions [serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST)] activities were measured colourimetrically according to the method described by Reitman and Frankel (1957).

Alkaline phosphatase (ALP) was measured colourimetrically according to the method described by Kind and King (1954). Total protein and albumin levels were measured by the colourimetric method (Doumas et al., 1971), serum globulin level was determined by subtracting the albumin value from total protein value of the same sample as described by Coles (1974). Serum urea activity was measured by the enzymatic colourimetric method as described by Coulomb and Farreau (1963). Serum creatinine activity was measured by the colourimetric kinetic method as described by Husdan and Rapoport (1968), gamma glutamyl transferase (GGT), glucose and cholesterol by Tietz (1986) were recorded using commercial kits. Kits for all biochemical studies were obtained from BioSystems S.A. Costa Brava 30, Barcelona (Spain). At the end of the experiment (after 2 months), animals were weighed and anesthetized with ether for blood collection. Gross, pathologic changes, and weights of several organs were also recorded.

Reproductive studies

Study of fertility

A fertility study was carried out in 80 male and female Sprague Dawley rats. The extract was given at the dose of 250 mg/kg bwt. once daily to males and females, for respectively 35 and 14 days prior to mating. Dosed males and females were each mated with non dosed counterparts.

Dosed female rats were further treated throughout the gestation period. Control rats received the vehicle only. On day 20 of pregnancy the female rats were sacrificed and fetuses were delivered by caesarean section for further examination.

Study of embryotoxicity and teratogenicity

Three groups of 10 female Sprague Dawley rats each received the tested extracts at doses of 0 and 250 mg/kg bwt twice daily from day 6 to day 15 of pregnancy. Rats were sacrificed on day 20 of pregnancy. Fetuses were delivered by caesarean section. Fetal skeleton and visceral organs were examined. Each fetus was individually identified, weighed, sexed, and given a gross examination for external malformations/variations including observation for palatal defects. Approximately one-half of the fetuses in each litter were evaluated for visceral malformations/variations (Staples, 1974). The fetuses selected for visceral examination were injected abdominally with 0.2 ml Bouin's solution and then placed in Bouin's fixative for overnight, then turned to formalin solution 10% and subsequently sectioned and examined (Wilson, 1965). Whereas, the other fetuses were eviscerated and processed; the ossified skeletal structures were stained with alizarin red S and the cartilage parts were stained with alcian blue stain (Dawson, 1976; El-Ashmawy et al., 2011).

Peri-and postnatal study

In the peri-and postnatal study, 10 pregnant Sprague Dawley rats received the plant extracts at the dose 250 mg/kg bwt once daily. Dosing was started from day 16 of gestation and continued throughout the 3-week-lactation period. Ten other pregnant rats were used as controls. Observations on the offspring were made at birth and at day 4, 14 and 21 after birth.

Statistical analyses

Data were analyzed by the general linear model (GLM) procedure (SAS, 2004). The least square mean (LSM) + standard errors were calculated and tested for significance using the "t" test (Steel and Torrie, 1960).

RESULTS

Acute toxicity studies

The plant extract overdosing was tolerated in rats, up to 3 g/kg bwt, resulting in no fatality, or any signs of toxicity or change in behavior over 14 days following its administration by gavage.

Subchronic toxicity studies

Daily administration of the plant extract by gavage daily at doses of 125 and 250 mg/kg in rats, for 2 months revealed no significant changes on the body weights, heart rates, and other physiological parameters and revealed no histological alterations in different organs, and the following results were obtained:

1. The plant extract did not induce any significant adverse changes in blood hematological parameters in rats (Tables 1 and 2).

Table 1. Hematological parameters of rats given aqueous, methanolic and ethanolic extracts of *Achillea fragrantissima* by gavage once daily for 2 months.

Treatment	Parameter		
	Hb (g/dl)	PCV%	RBCS × 10 ⁶ /Cmm
Control (vehicle)	11.9±0.32	40.0±0.61	6.02±0.25
Aqueous extract (250 mg/kg)			
2nd week	11.6±0.40	40.2±0.60	6.15±0.21
1st month	12.0±0.68	40.4±1.41	6.2±0.36
2nd month	11.8±0.56	40.8±1.31	6.28±0.44
Methanolic extract (250 mg/kg)			
2nd week	12.0±0.56	40.0±1.36	5.88±0.34
1st month	12.2±0.56	41.0±2.0	5.9±0.33
2nd month	11.4±0.57	41.6±2.39	6.4±0.25
Ethanolic extract (250 mg/kg)			
2nd week	12.2±0.64	40.1±1.15	6.3±0.54
1st month	11.5±0.52	40.0±1.05	5.86±0.36
2nd month	11.8±0.88	39.2±0.9	6.3±0.27

Values are mean ± SEN = 6 animals.

Table 2. Hematological and biochemical parameters of rats given aqueous, methanolic and ethanolic extracts of *Achillea fragrantissima* by gavage once daily for 2 months.

Treatment	Parameter		
	WBCS × 10 ³ /Cmm	Glucose (mg/dl)	Cholesterol (mg/dl)
Control (vehicle)	7.1±0.24	66.6±2.09	59.6±1.29
Aqueous extract (250 mg/kg)			
2nd week	7.2±0.31	65.2±1.16	59.8±1.59
1st month	6.92±0.49	66.2±1.93	60.6±1.29
2nd month	6.8±0.37	63.0±3.04	56.8±2.78
Methanolic extract (250 mg/kg)			
2nd week	6.80±0.50	59.0±2.12	57.4±1.89
1st month	7.00±0.44	58.8±2.25	51.8±1.07*
2nd month	6.6±0.51	57.6±2.62	49.2±1.07*
Ethanolic extract (250 mg/kg)			
2nd week	7.1±0.45	56.4±2.06	53.8±2.54
1st month	6.8±0.37	57.2±2.96	51.0±1.52*
2nd month	6.4±0.51	53.6±2.38*	49.6±1.03*

Values are mean ± SEN = 6 animals. *Significantly different compared to control (P < 0.05).

2. The plant extract induced a significant increase in total proteins and globulins in rats (Table 3), yet it did not ex-

ceed the normal reference range in all animals.

3. The plant extract did not induce any abnormal liver and

Table 3. Serum protein profile of rats given aqueous, methanolic and ethanolic extracts of *Achillea fragrantissima* by gavage once daily for 2 months.

Treatment	Parameter		
	Protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)
Control (vehicle)	5.08±0.24	3.26±0.27	1.82±0.15
Aqueous extract (250 mg/kg)			
2nd week	5.12±0.12	3.2±0.14	1.92±0.08
1st month	5.2±0.22	3.28±0.18	1.92±0.08
2nd month	5.28±0.21	3.5±0.22	1.78±0.19
Methanolic extract (250 mg/kg)			
2nd week	5.64±0.27	3.66±0.29	1.98±0.02
1st month	5.48±0.33	3.24±0.44	2.24±0.24*
2nd month	5.48±0.21	2.88±0.41	2.6±0.24*
Ethanolic extract (250 mg/kg)			
2nd week	5.52±0.26	3.34±0.23	2.18±0.22
1st month	6.0±0.27*	3.0±0.27	3.0±0.31*
2nd month	5.7±0.37*	3.0±0.31	2.7±0.20*

Values are mean ± S.E. N = 6 animals. *Significantly different compared to control (P < 0.05).

kidney functional changes as demonstrated by serum biochemical analysis in rats. Interestingly, the plant extract induced a significant decrease in ALP, urea & creatinine in rats (Tables 3 to 5).

4. Although no significant change in blood glucose level was observed in animals receiving the plant extract at the level of 125 g/kg, significant decrease in blood glucose level occurred in animals receiving 250 mg/kg (Table 2).

5. Light microscopic examination of the different organs in rats revealed no significant alterations as compared to the control animals.

Reproductive studies

Fertility study in rats

The plant extract did not affect fertility. Dosed males showed comparable data with the controls when dosed at 250 mg/kg bwt (Table 6).

Embryo toxicity and teratogenicity study in rats

The plant extract did not cause any embryotoxic or teratogenic effect (Table 6 and Figure 1).

Peri- and postnatal study

The plant extract did not cause any deleterious effects on the dosed females and their offspring. Litter size, survival rate and weight gain were comparable between groups (Table 7).

DISCUSSION

The data concerning the effect of the *A. fragrantissima* extract in rats revealed no significant changes on the body weights, heart rates, and other physiological parameters and revealed no histological alterations in different organs. In the present investigation, it was observed that the medicinal plant treatment did not cause significant reduction in the rat body weight when compared to control. This shows the absence of toxic side effect of the plant in the tested animals. The same result has been found in the administration of *Alstonia scholaris* bark extract (Gupta et al., 2002), *Strychnos potatorum* seed extract (Gupta et al., 2006), *Tuniperus phoenica* (Shkukani et al., 2007) to male rats and *Achillea millefolium* flower extract (Montanari et al., 1998) to male mice.

Blood hematology picture, PCV, and Hb did not show

Table 4. Effect of administration of aqueous, methanolic and ethanolic extracts of *Achillea fragrantissima* by gavage once daily for 2 months on serum AST, ALT, and ALP in rats.

Treatment	Parameter		
	AST (U/L)	ALT (U/L)	ALP (U/L)
Control (vehicle)	50.2±1.56	19.2±0.58	81.4±1.12
Aqueous extract (250 mg/kg)			
2nd week	49.4±2.36	16.6±0.98	83.0±1.52
1st month	47.8±2.71	16.6±0.92	82.4±1.60
2nd month	46.8±2.01	16.6±0.46	81.2±1.85
Methanolic extract (250 mg/kg)			
2nd week	48.0±0.44	18.0±0.71	80.6±2.16
1st month	46.4±2.71	17.4±0.68	76.2±2.44
2nd month	45.0±2.10	16.8±1.16	73.6±2.73*
Ethanolic extract (250 mg/kg)			
2nd week	45.6±3.06	17.4±0.60	79.2±1.46
1st month	44.6±2.93	14.0±0.71*	74.4±2.32
2nd month	44.6±2.93	14.0±0.71*	71.6±2.91*

Values are mean ± S.E. N = 6 animals. *Significantly different compared to control (P < 0.05).

Table 5. Effect of administration of aqueous, methanolic and ethanolic extracts of *Achillea fragrantissima* by gavage once daily for 2 months on serum GGT, urea and creatinine in rats.

Treatment	Parameter		
	GGT (U/L)	Urea (mg/dl)	Creatinine (mg/dl)
Control (vehicle)	12.0±0.71	19.2±0.67	0.56±0.05
Aqueous extract (250 mg/kg)			
2nd week	11.8±0.86	19.8±1.07	0.58±0.07
1st month	11.2±1.16	19.4±1.75	0.56±0.05
2nd month	12.5±1.07	18.6±2.07	0.58±0.06
Methanolic extract (250 mg/kg)			
2nd week	12.2±0.73	19.0±0.89	0.58±0.03
1st month	12.2±0.80	19.0±1.23	0.54±0.03
2nd month	11.4±0.98	15.2±1.07*	0.58±0.03
Ethanolic extract (250 mg/kg)			
2nd week	12.6±0.81	17.6±2.32	0.58±0.08
1st month	12.2±1.24	16.2±1.80	0.58±0.08
2nd month	11.0±0.98	15.6±0.93*	0.40±0.04*

Values are mean ± S.E. N = 6 animals. *Significantly different compared to control p < 0.05.

Table 6. Effect of the administration of aqueous extract of *Achillea fragrantissima* by gavage on fertility in rats.

Parameter	Dosage group (mg/kg)			
	0		250	
	C-M	T-M	C-M	T-M
Adult rat data	+	+	+	+
	T-F	C-F	T-F	C-F
No. of treated males (n)	0	10	0	10
No. of treated females (n)	10	0	10	0
No. of pregnant rats (n)	8	9	9	9
No. of surviving females (n)	10	10	10	10
Fertility index (%)	80	90	90	90
Litter data				
Average no. of implantations/No. of pregnancies	8.9	9.1	8.8	9.2
No. of alive fetuses/No. of implantations (%)	99.2	98.4	98.0	99.0
No. of resorbed fetuses/No. of implantations (%)	1.5	1.2	1.2	1.3
Average weight at birth (g)	5.5	5.2	5.6	5.4
Abnormalities (%)	-	-	-	-

C = Control, M = male, T = treated, F = female.

Table 7. Effect of the administration of ethanolic extract of *Achillea fragrantissima* by gavage on the development and viability of first generation (F₁) pups during the pre-weaning period in rats.

Parameter	Dosage group (mg/kg)	
	0	250
Mean number of pups/litter		
Born	8.5±0.3	8.6±0.4
Born alive	8.0±0.4	8.4±0.3
Sex ratio (males)	51.0±2.4	49.5±3.0
Mean pup weight (g)/litter		
Postnatal day 0 (PND) males	5.4±0.2	5.3±0.1
Females	5.1±0.1	5.6±0.1
PND 4 males	9.0±0.4	8.5±0.2
Females	8.3±0.1	8.2±0.1
PND 14 males	29.5±0.9	30.0±0.8
Females	28.2±0.7	28.5±0.6
PND 21 males	47.2±1.1	48.2±1.1
Females	43.7±1.4	44.0±2.3
Survival (%) = $\frac{\text{No. of pups alive on PND 21}}{\text{No. of pups alive on PND 0}} \times 100$	95.0±4.2	97.3±3.2

Values are mean ± SEN = 10 animals.

significance difference between all treated groups when compared with the control. Therefore, the extract of *A.*

fragrantissima may not have had any adverse effect on the bone marrow, liver, kidney and haemoglobin

metabolism, since the value of red blood cells are not affected (Young and Maciejewski, 1997). Similar results were demonstrated by Emadi et al. (2007) and Basavaraj et al. (2011) in the hematological picture of animals and birds.

Serum AST and ALT being the most sensitive hepatic markers employed in the diagnosis of hepatic damage (Sallie et al., 1991) were not significantly different between the treated and control group. Previous study has shown extraction of medicinal plants to be an effective antioxidant under *in vitro* conditions. Rasekh et al. (2001) have demonstrated no changes in these enzymes in male rats, and El-Bagir et al., (2010) indicated that medicinal plants were safe to be included in rat diet as reflected on the above unchanged liver and kidney function biomarkers. Moreover, the non significant change in the levels of AST and ALT is suggestive of the fact that decoction is successful in quenching the free radicals inhibiting lipid peroxidation and protecting the membrane lipids from oxidative damage in the liver of rats (Suboh et al., 2004).

Although no significant changes in blood glucose and cholesterol levels were observed in animals receiving the plant extract at the level of 125 g/kg, significant decrease in blood glucose level occurred in animals receiving 250 mg/kg. A finding that would be helpful while studying cardiovascular diseases such as arteriosclerosis that are caused as a result of hyperlipidemia elevate mortality% (Frangle, 1995); so reducing serum hyperlipidemia is very important. A 1% reduction in serum cholesterol concentration results in a 2% reduction in the prevalence of coronary artery diseases (Frangle, 1995).

In addition, present investigation demonstrated that oral administration of *Achillea millefolium* extract at different doses caused no significant change in fertility parameters in male rats. The animal model used in this work has previously been with minor changes used by several researchers to assess the effect of various extracts obtained from medical plants on reproductive functions in males (Yinusa et al., 2005; Parandin et al., 2008). The present results disagree with the previous studies that *A. millefolium* (200 mg/kg/day intraperitoneally for 20 days) and different variety of *Achillea santolina* (300 mg/kg intraperitoneally for 20 days) have an antispermatic and degenerative changes on mice testes (Montanari et al., 1998; Golalipour et al., 2004).

Conclusion

The study showed that the tested material named *A. fragrantissima* extract is a well tolerated substance and had a wide safety margin as demonstrated from studies performed in this report. The study also confirmed that the tested plant extracts did not induce any toxic effects

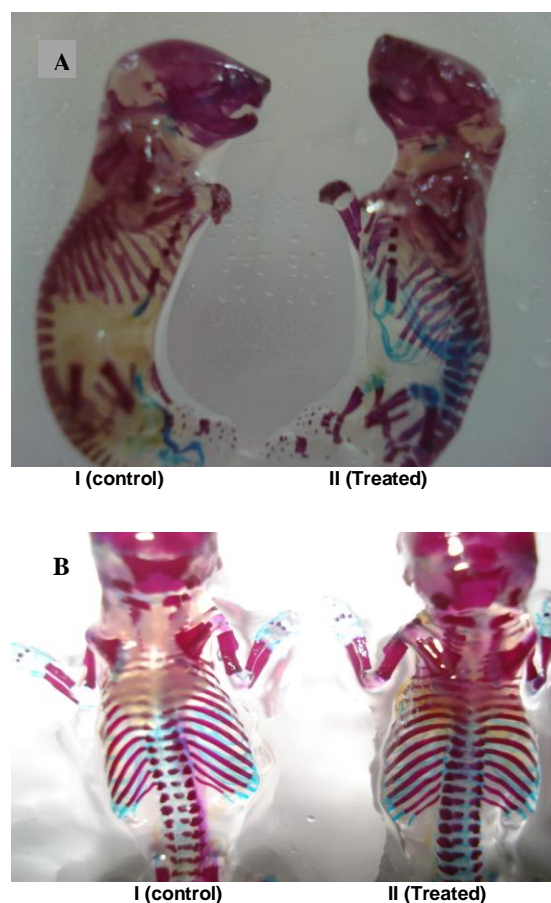


Figure 1. Effect of the administration of ethanolic extract of *Achillea fragrantissima* by gavage once daily from day 6 to day 15 of pregnancy on the fetal skeleton and cartilage development. (A) Normal ossifications of sternbrae and skull; (B) normal cartilage development ossifications of skull, vertebrae, ribs and absence of fused ribs.

even on repeated administration in rats for 2 months.

Additionally, the study confirmed that *A. fragrantissima* extract had no evidences of impaired fertility or teratogenic potentials at higher doses up to several times the recommended maximum human doses.

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Full Length Research Paper

Preliminary evaluation of the immunoenhancement potential of fowl typhoid vaccine formulated as an oral cationic liposome

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Salmonella gallinarum is a non-motile host-specific bacterium in domestic poultry. It causes fowl typhoid, especially in domestic poultry. The study was performed with a view to compare the antibody titre of commercial subcutaneous Fowl typhoid® vaccine with its oral liposomal formulation. The chicken, divided into three groups (n = 20 per group), were inoculated via oral or subcutaneous route. Group A was given commercial subcutaneous Fowl typhoid® vaccine, Group B served as the unvaccinated control and Group C was given the Fowl typhoid® vaccine encapsulated in a cationic liposome. Vaccination of the groups was carried out once at 42 days of age. Blood samples were collected from the jugular vein at 56 days of age to obtain the sera for slide and microplate agglutination test. At 63 days of age, the birds were challenged with a field strain of the *S. gallinarum*. From the microplate agglutination test (mean ± standard deviation (SD), at p < 0.05), it was found that there was no significant difference in the mean antibody titre of the birds by either the subcutaneous or oral vaccination of Fowl typhoid® vaccine.

Key words: Fowl typhoid vaccine, *Salmonella gallinarum*, liposome.

INTRODUCTION

Salmonella gallinarum (SG) is the etiologic agent of fowl typhoid (FT), a severe systemic disease of chickens and other galliform birds (Shivaprasad, 2000). The disease is dose-dependent and differences in pathogenicity may be found depending upon the susceptibility of the infected genetic line of chickens (Oliveira et al., 2005). Mortality and morbidity rates due to fowl typhoid may reach up to 80% (Wigley et al., 2002, 2004). Fowl typhoid is still of considerable economic importance in many countries of Africa, Asia, and Central and South America (Pomeroy and Nagajara, 1991; Lee et al., 2003). Vaccination against SG is commonly used as preventive measure which is available as injections (Lee et al., 2007). However, oral delivery of Fowl typhoid® vaccine has not yet been fully exploited. Delivering Fowl typhoid® vaccine

orally would not only be beneficial to farmers but would reduce the injuries and hazards caused by needle-stick vaccination. Overall, it would encourage rural farmers to adopt vaccination and this would ultimately improve livestock production and ensure food security. Liposomes have therefore, been employed in this research as carriers and adjuvants of Fowl typhoid® vaccine.

Twenty-five years after the discovery of the immunological adjuvant properties of liposomes, they appear now as a major candidate adjuvant, with a liposome based vaccine (against hepatitis A) being licensed for use in humans (Gregoriadis, 1995). Liposomes are small, spherical, self-closed vesicles of colloidal dimension which consist of amphiphilic lipids, enclosing an aqueous core. (Valiante et al., 2003). Cationic liposomes remain

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at the forefront of vaccine design owing to their well documented ability to boost both humoral and cell-mediated immune response. In this study, DOTAP-based liposome was used to entrap a standard fowl typhoid vaccine for possible oral vaccination. The results suggest that fowl typhoid vaccine entrapped in a DOTAP-based liposome might be a suitable alternative to parenteral delivery.

MATERIALS AND METHODS

Cholesterol (Sigma Grade, minimum purity 99%, Sigma Aldrich Chemie, St. Louis, USA), 1,2-dioleoyl-3-trimethylammonium propane (DOTAP (Sigma-Aldrich, USA) phospholipon 90H (GmbH Nattermannallee, Köln, Germany), methanol (extra pure, Scharlau Chemie S.A.), chloroform (Sigma-Aldrich, GmbH Germany). All are of analytical grade.

Chickens

Sixty day old chicks (*Gallus gallus domesticus*) from CHI farms, Ogun State were used. The chicks were raised from day old until termination of the experiment. The birds in the different groups were housed in separate rooms and all cleaning and feeding was planned to minimise the risk of cross-contamination. All animal handling and experiments were conducted following the guidelines stipulated by University of Nigeria Research Ethics Committee on animal handling and use. Results of the study was presented as mean with standard deviations (SD) of each of the parameters.

Vaccine and challenge strain

The 9R Fowl Typhoid® vaccine strain [100 units] (National Veterinary Research Institute (NVRI), Vom, Nigeria) was used in this trial. The challenge strain used was the virulent *S. gallinarum* (1.0 Mc Farland suspension was prepared) strain Gr. D1, 1.9.12 (Istituto zooprofilattico sperimentale delle venezie Legnaro, Italy. It was stored at -20°C.

Experimentals

Preparation of dry lipid films

196 mg of phosphatidylcholine, 96.7 mg of cholesterol and 50 µg of DOTAP were weighed and dissolved in 3 ml of chloroform/methanol system (2:1) in a round bottom flask. The solvent mixture was evaporated at room temperature and the flask rotated until a smooth, thin, dry film on the wall of the flask was obtained (Azmin et al., 1985).

Hydration of dry lipid films

Fowl typhoid® (100 units) vaccine was reconstituted with 20 ml of normal saline. 5 ml volume of the reconstituted vaccine was used to hydrate the dry films of the liposome and agitated gently until multilamellar vesicles were formed.

Vaccination of the birds

Sixty birds were divided into three groups of twenty birds each. At

42 days of age, Group A was given 0.2 ml/bird of the commercial subcutaneous fowl typhoid vaccine, Group B served as the unvaccinated control and Group C was given 0.2 ml/bird of the fowl typhoid vaccine encapsulated in a cationic liposome. The vaccination was carried out once.

Blood collection

At 56 days of age, about 5 ml of blood was collected from the jugular vein of each bird using sterile syringe and emptied into marked individual Ethylenediaminetetraacetic acid (EDTA) tubes. Sera were prepared from blood samples to determine the circulating antibody titres by slide and microplate agglutination test.

Preparation of challenge strain

1.0 McFarland standard was prepared by adding 990.0 ml of 1% sulfuric acid to 10.0 ml of 1% barium chloride. The density of the *S. gallinarum* inoculum was adjusted to match the density of the standard solution. A 0.5 McFarland standard is comparable to a bacterial suspension of 10⁹ cfu/ml.

Slide agglutination test

Clean white square tiles were used. Serum samples from all the birds were tested. One drop (0.05 ml) of crystal violet stained *S. gallinarum* antigen was placed at nine different points equidistant from each other. A drop of the serum samples of the immunized birds from the different groups, one group at a time, was placed next to the drop of antigen. The drops of antigen and serum were mixed using a glass rod which was wiped clean between samples. Then, using a gentle rocking motion the tiles were rotated for 2 min and signs of agglutination observed. The samples that showed agglutination were used for the plate agglutination test.

Plate agglutination test

The O somatic antigen was incubated overnight at 40°C. A 25 µl of 0.5% phenol saline was added to all the wells in a column in a microtitre plate. A 25 µl volume of the chicken serum was added to the first well and two-fold dilutions made. The last well in the row contained only saline as negative control. A 25 µl volume of O somatic diluted antigen was then added to all the wells and left for 4 h to incubate. The wells were examined for signs of agglutination and compared with the negative and positive controls. Statistical analysis was performed at $p < 0.05$, applying a one-way analysis of variance (ANOVA) test with least squared difference (LSD) multiple comparisons.

Challenge and post-challenge observation of chicken

At 63 days of age, ten chickens from each group were peritoneally inoculated with 0.5 ml of 1.0 Mc Farland suspension of the virulent *S. gallinarum*. Necropsy samples of the spleen, liver, bursa and thymus of infected were randomly collected from dead as well as live infected birds from each group for gross examination.

Safety test

The safety test had already been carried out in an earlier experiment using La Sota® vaccine (Onuigbo et al., 2012).

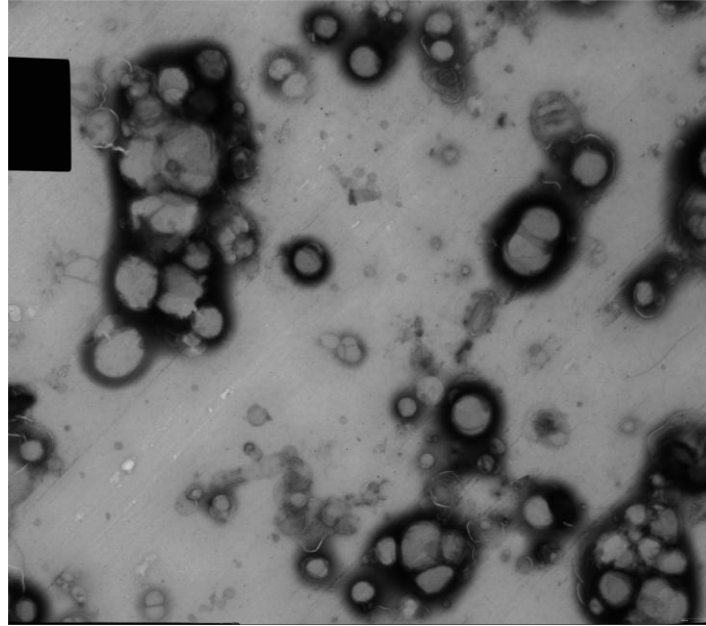


Figure 1. Transmission electron microscopy images of the cationic liposome incorporating the fowl typhoid vaccine ($\times 10500$).

RESULTS AND DISCUSSION

The technique used for the preparation of the liposome was lipid film hydration technique (hand shaking method) which formed a film on the wall of the flask and on hydration with phosphate buffer solution (pH 7.4), produced thick, gel-like, milky colloidal dispersion as shown in Figure 1. These vesicles incorporated the fowl typhoid vaccine within the core of the vesicles and protected it from the harsh environment of the gastrointestinal tract and peptidases which destroy antigen. It also prevents vaccine from being released immediately to the lymphoid tissues, thereby prolonging the contact of the vaccine with the vast mucosal associated lymphoid tissues. The net surface charge of the liposomes was modified by the incorporation of positively charged DOTAP lipid. This positive liposome can fuse with negatively charged cell membranes and deliver the vaccine endosomally.

Using a nanosizer, the particle sizes of the cationic liposome were determined. The mean size distribution was found to peak at 101.1 nm as shown in Figure 2. Particle size is a very important factor in vaccine delivery. Particle size is one of the determining factors for macrophage clearance. Large liposomes are rapidly removed from circulation. If the size of the liposome is below 200 nm, as obtained in the experiment, it would escape phagocytosis and the circulation time will be prolonged (Maurer et al., 2001). If the circulation time is prolonged, there will be more contact time of the liposomes with the immune cells, resulting in higher immunity.

In the slide agglutination test, the sera of the unvaccinated did not agglutinate which indicated negative result. The sera of the positive group and the liposome group showed strong agglutination. The post-vaccination plate agglutination titres of sera samples (mean \pm SD) of the unvaccinated was 1.00 ± 0.0 ; the liposomal group was 7.75 ± 0.25 and positive control group given Fowl typhoid vaccine subcutaneously was 8.00 ± 0.0 . From the microplate agglutination test (mean standard deviation (SD_0 , at $p < 0.05$), it was found that there was no significant difference in the mean antibody titre of the birds by either the subcutaneous or oral vaccination route of fowl typhoid vaccine. The birds in the unvaccinated group were seen in a recumbent position after challenge. They had loss of appetite, looked depressed and had white mucoid stool. On the fifth day post-vaccination, a total of five birds from the unvaccinated group had died. The birds in the positive group and liposomal group looked healthy and none died.

Conclusion

FT vaccine encapsulated in a cationic liposome administered orally to chicken protected them from the field strain of *S. gallinarum* after vaccination. The antibody titre of the commercial FT vaccine administered subcutaneously was not significantly higher than the orally administered FT vaccine encapsulated liposome. More experiments would be carried to ascertain stability of the liposomal FT vaccine at room temperature. Positive results from this would be beneficial to farmers in rural areas and improve

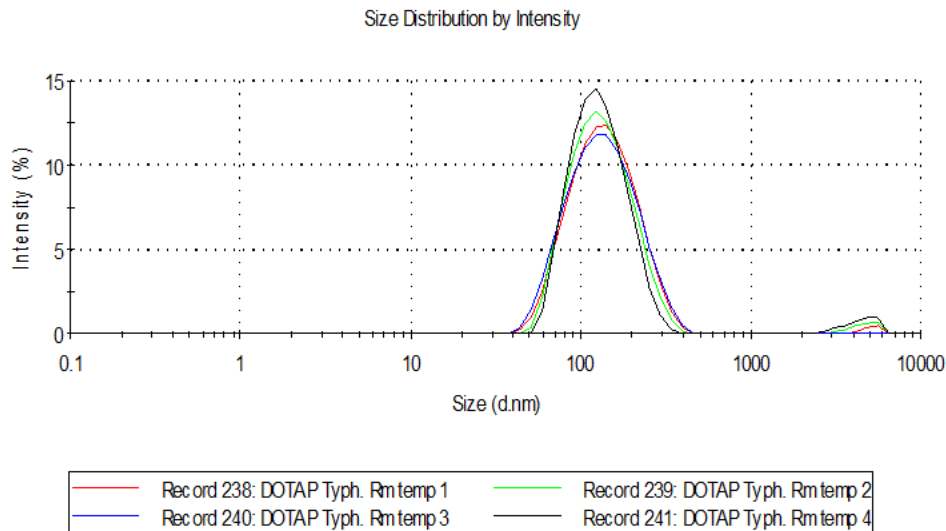


Figure 2. Particle size of the liposome using photon correlation spectroscopy.

vaccination compliance.

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Full Length Research Paper

Effect of *Hibiscus sabdariffa* calyx extract on reproductive hormones in normal rats

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Medicinal plants contain physiologically active principles that over the years had been exploited in traditional medicine for the treatment of various ailments. The present study was undertaken to investigate the effects of ethanolic extract of *Hibiscus sabdariffa* calyx on rat reproductive hormones. The effects on the basal levels of estradiol, testosterone, prolactin and follicle stimulating hormone were conducted in experimental animals. *H. sabdariffa* calyx extract at a dose of 250 mg/kg produced minor effects on rat reproductive hormones, namely testosterone and estradiol while no change was observed on both prolactin and follicle stimulating hormone levels. Moreover, no histological changes were detected on both testes and ovaries of the experimental animals after 28 days of administration. It can be concluded that *H. sabdariffa* calyx extract at a dose of 250 mg/kg caused mild effects on rat reproductive hormones.

Key words: Estrogenic effect, reproductive hormones, *Hibiscus sabdariffa* extract.

INTRODUCTION

Endocrine disrupting compounds (EDCs) are natural or synthetic compounds that have the ability within the body to alter endocrine functions often through mimicking or blocking endogenous hormones (James et al., 2013). These actions on the endocrine system have resulted in developmental deficits in various invertebrate and aquatic species (Crain et al., 2007; Elango et al., 2006) and mammals (Christopher et al., 2012). Exposures in adulthood have consequences but fetal and early life exposures appear to have more severe effects that persist through life (Rubin and Soto, 2009). Among these classes of chemicals are phytoestrogens that show effects suggestive of estrogenicity, such as binding to the estrogen receptors, induction of specific estrogen-

responsive gene products, stimulation of estrogen receptor(s) and positive breast cancer cell growth (James et al., 2013). Through these interactions by acting as agonists or antagonists, EDCs are able to alter the activity of response elements of genes, block natural hormones from binding to their receptors, or in some cases increase the perceived amount of endogenous hormone in the body by acting as a hormone mimic to its receptor (Ze-hua et al., 2010).

Hibiscus sabdariffa Linn (Roselle) is an annual shrub commonly used to make jellies, jams and beverages. The brilliant red colour of its calyx makes it a valuable food product, a part from its multitude of traditional medicinal uses. Infusions of the calyces are considered as diuretic,

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cholorectic, febrifugal and hypotensive, decreasing the viscosity of the blood and stimulating intestinal peristalsis (Salleh et al., 2002). Roselle calyx extract is a good source of antioxidants from its anthocyanins and associated with antitumor and inhibitory effects on the growth of several cancer cells (Ajiboye et al., 2011).

Extracts of *H. sabdariffa* calyces have been reported to be rich in phytoestrogens (Adigun et al., 2006; Orisakwe et al., 2004; Brian et al., 2009; Omotuyi et al., 2011) and some reports indicated that *H. sabdariffa* calyces have estrogenic effects, although exact estrogen-like ingredient is not determined (Ali et al., 1989). This study was undertaken to determine to which extent *H. sabdariffa* calyces extract alters the basal levels of selected reproductive hormones: estradiol, testosterone, prolactin and follicle stimulating hormone as well as the histological features of both testes and ovaries of rats.

MATERIALS AND METHODS

Plants

The dried calyces of *H. sabdariffa* were purchased from the local market in Wad-Medani, Sudan. The plant material was identified by the Department of Pharmacognosy, Faculty of Pharmacy, University of Gezira, Sudan.

Extraction of plant material

One hundred grams of coarsely powdered calyces of *H. sabdariffa* were extracted by maceration using ethanol (70%) in a conical flask for 72 h, kept away from light throughout the extraction period, then filtered and evaporated by a rotary evaporator at 60°C. The resulting solution was freeze dried and placed into a refrigerator until use.

Effect of ethanolic extract of *Hibiscus sabdariffa* calyx on rat reproductive hormones

Experimental animals

The effect of ethanolic extract of *H. sabdariffa* calyx on rat reproductive hormones was conducted based on the method described by Omotuyi et al. (2011). A total of 20 rats (10 each for males and females) were housed in a clean animal house subjected to an intensive nutritional program. Rats were acclimatized for a period of 14 days under standard environmental conditions. The ethical committees, University of Gezira and Ministry of Health, Gezira State, ethically approved the experimental protocol.

Experimental design

Albino wistar rats were divided into four groups each of five. Water control groups for both males and females and the other two groups (either males or females) received 250 mg/kg of plant extract via gastric tube daily for 28 days.

Collection of blood samples

Blood samples were collected from conjunctival veins using capillary tubes at 7-day intervals for 28 days.

Hormonal assay

The hormones were estimated using the standard protocols of enzyme-linked immunosorbent assay (ELISA) kits (Roche, Switzerland) for determination of estradiol, testosterone, prolactin and follicle stimulating hormone (FSH) levels.

Histopathological examination

Twenty-eight days after oral administration of the extract, all experimental animals were anaesthetized using chloroform vapour and dissected. The ovaries and testes were collected and immediately fixed in Bouins fluid for 6 h and transferred to 70% alcohol for histological processing according to Drunny and Wallington (1990). Briefly, following fixation of the right side testes and ovaries from both control and test animals, tissue sections were processed by dehydration in 95% and absolute alcohol, cleared in xylene and embedded in pure clean moltenparaffin wax from which blocks of tissues were made for sectioning. Ribbon slices of about 5.0 µm in thickness were made with the aid of a microtome (delete machine) and the sections picked with slides, which were dried in oven. The slices were stained with haemotoxylin and eosin, and then mounted using DPX onto a light microscope (delete magnification 40x for testes and 10x for ovary) for histopathological and morphological changes.

Data analysis

All the obtained data were expressed as means ± standard deviation and analyzed using analysis of variance (ANOVA). Comparisons with the control groups were made using One-way ANOVA. Differences were considered significant if P-value < 0.05.

RESULTS

Effect of ethanolic extract of *H. sabdariffa* calyx on estradiol levels in female rats

The study revealed that the ethanolic extract of *H. sabdariffa* calyx in a dose of 250 mg/kg exhibited a mild increase (p-value < 0.05) in estradiol level in female rats in time-dependent manner (Table 1). On day 28, estradiol reached more than twice the value observed on day 0 compared to those of water control group.

Effect of ethanolic extract of *H. sabdariffa* calyx on testosterone levels in male rats

Following intragastric administration of 250 mg/kg of ethanolic *H. sabdariffa* calyx extract for 28 days, serum levels of testosterone were significantly reduced (P-value

Table 1. Serum estradiol levels (pg/ml) in female rats (n=5) after oral administration of ethanolic extract of *Hibiscus sabdariffa* calyx and water (SD \pm SEM).

Preparation	Day 0	Day 7	Day 14	Day 21	Day 28
<i>Hibiscus sabdariffa</i>	6.40 \pm 29	8.12 \pm 39	12.93 \pm 40	15.92 \pm 47	16.6 \pm 50
Control (water)	5.39 \pm 30	6.1 \pm 35	6.14 \pm 34	6.34 \pm 36	7.12 \pm 37

*P-value < 0.05.

Table 2. Serum testosterone levels (ng/ml) in male rats (n=5) after oral administration of ethanolic extract of *Hibiscus sabdariffa* calyx and water (SD \pm SEM).

Preparation	Day 0	Day 7	Day 14	Day 21	Day 28
<i>Hibiscus sabdariffa</i>	2.77 \pm 3.66	2.18 \pm 1.89	1.44 \pm 1.02	0.8 \pm 0.53	0.54 \pm 0.16
Control (water)	0.47 \pm 2.64	0.68 \pm 2.37	0.94 \pm 0.64	1.34 \pm 1.06	1.07 \pm 2.39

*P-value < 0.05

< 0.05) in male rats throughout the experimental period compared to those of water control group (Table 2).

Effect of ethanolic extract of *H. sabdariffa* calyx on prolactin and FSH levels in female rats

The ethanolic extract of *H. sabdariffa* calyx in a dose of 250 mg/kg/ml did not cause changes in the serum levels of prolactin or FSH throughout the experimental period in female rats administered the plant extract for 28 days.

Histological effects of *H. sabdariffa* calyx extract on rat testes and ovaries

The extract did not cause histological changes on both testes and ovaries of the experimental animals when the plant extract was administered for 28 days.

DISCUSSION

Certain phytoestrogens such as isoflavones and lignans have been thoroughly investigated for their estrogenic properties (Miksicek, 1995; Collins et al., 1997; So et al., 1997). Extracts of *H. sabdariffa* have been reported to be rich in phytoestrogen (Adigun et al., 2006; Orisakwe et al., 2004; Brian et al., 2009; Omotuyi et al., 2011). There are reports indicated that *H. sabdariffa* calyces have estrogenic effects, although exact estrogen-like ingredient is not determined (Ali et al., 1989). Moreover, plant phenols, anthocyanin isolates and anthocyanin-rich mixture of bioflavonoids possess estrogenic activities (Omotuyi et al., 2011).

The reduction of serum level of testosterone in male rats produced by ethanolic extract of *H. sabdariffa* calyx may be explained by the estrogenic activity of the plant, an evidence raised by Orisakwe et al. (2004). Furthermore, other studies had reported a statically significant decrease in testosterone levels in laboratory animals treated with phytoestrogens (Sharpe et al., 2002; Cline et al., 2004). The precise role that oestrogens play in male reproductive development is unclear, but generally, oestrogens tend to have 'demasculinising' or anti-androgenic effects. In foetal and neonatal life, this probably results from suppression of testosterone production (Haavisto et al., 2001), or loss of androgen receptors (McKinnell et al., 2001). Oestrogens are synthesised from androgens via the action of a single enzyme (aromatase), and there is a close relationship between the actions of these two hormones. Moreover, testosterone may be converted to estrogen by aromatase (Benassayag et al., 2002).

Although dietary phytoestrogens have been implicated in adverse effect upon fertility in various animals, there are few published reports of such effects in human populations consuming large amounts of these substances (Benassayag et al., 2002). In male rats, reduction of testosterone level might impair spermatogenesis and cause male infertility (Orisakwe et al., 2004). It should be noted that in the study of herbal extracts, one could not attribute the observed biological effects to a particular constituents because many other compounds are present in the plant extracts (Saied-Karblay et al., 2010). Factors such as species, age, gender, diet, dose, route of administration and metabolism strongly influence the ultimate biological response to phytoestrogen exposure (Shweta, 2009).

Conclusion

H. sabdariffa calyx extract caused mild effects on rat reproductive hormones and due to the pleiotropic effects of phytoestrogens *in vivo*, a broad panel of *in vitro* assays covering not only estrogenic action but also other regulating processes has to be used to assess the potential of plant-derived compounds to beneficially or adversely affect human health.

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Full Length Research Paper

Effect of guava (*Psidium guajava* Linn.) fruit water extract on lipid peroxidation and serum lipid profiles of streptozotocin-nicotinamide induced diabetic rats

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The prevalence of diabetes is rapidly increasing and it can cause a wide range of health complications affecting almost every part of the body. The aim of this study was to examine guava fruit ability in reducing blood glucose level and preventing diabetic complication of streptozotocin-nicotinamide induced diabetes rat. Guava fruit performance was measured by *in vitro* lipid peroxidation inhibition, *in vivo* blood glucose level, *in vivo* serum lipid profile, and *in vivo* lipid peroxidation inhibition. This study confirmed that guava fruit extracts can significantly reduce blood sugar in type 2 diabetic rats, plasma triglycerides and low density lipoprotein (LDL) concentrations. Most importantly, guava fruit water extract showed impressive efficacy against *in vitro* and *in vivo* lipid peroxidation examinations. These activities may be due to the antioxidants properties offered from phenolic and flavonoid compounds in guava fruit water extract. Guava fruit water extract was found to consist 40.13 mg GAE/g of phenolic content and 18.43 ± 1.22 mg quercetin equivalent/g of flavonoid content.

Key words: Guava fruit, lipid peroxidation, malonaldehyde, serum lipid profile, diabetes mellitus.

INTRODUCTION

Due to the economic conditions improvement, various types of health-oriented food products have gradually been paying attention and diabetic health food is not an exception. The prevalence of diabetes is rapidly increasing in industrialized countries, and type 2 diabetes accounts for 90% of the disease. Diabetes can cause a wide range of health complications, affecting almost every part of the body. However, many of these complications can develop and become quite severe before the patient

even realizes there is a problem brewing; hence the reason diabetes can be called a silent killer among diseases.

Most of western medicines, which are often made of a single chemical compound, are very effective for direct relief of symptoms, such as lowering blood sugar. Chinese medicines are effective not only to treat and prevent diabetic complications but also at the meantime to lower blood glucose level (Li et al., 2004). Therefore,

Chinese doctors often make a combination of traditional medicine with Western medicine, Western medicine for reducing blood sugar, traditional medicine for integrated care of body (Cheng et al., 1998).

Previous studies have found that free radical activity, lipid peroxidation products accumulation and also oxidative stress caused by unbalance antioxidant defense system seems to have important role in diabetes and its complications (Oberly, 1988; Baynes, 1991). Lipid accumulation in the liver has been shown to play a key role in insulin resistance, and alleviation of this condition was suggested to be one mechanism to improve insulin sensitivity (Kim et al., 2001). On the other hand, dyslipidemia (abnormal amounts of serum lipids), associated with insulin resistance can increase the risk for cardiovascular disease (CVD) of diabetic complication (Goldberg et al., 2005).

White guava (*Psidium guajava* L.), as one of traditional Chinese medicines, it is widely cultivated and mostly consumed fresh. Guava leaves, fruit and stem bark were also used as a hypoglycemic agent in folk medicine. Hypoglycemic activity of guava leaves has been well-documented (Maruyama et al., 1985; Shen et al., 2008; Cheng et al., 2009), but not for guava fruit. Cheng and Yang (1983) has proved that guava juice exhibited hypoglycemic effects in mice by examining blood glucose level. As diabetic complication also contribute to ill health, disability, poor quality of life and premature death, observing the potential of natural product on diabetic complication become important. Therefore, the aim of this study was not only to examine hypoglycemic effect of guava fruit extract but also its ability in preventing diabetic complication by examining *in vitro* and *in vivo* lipid peroxidation inhibition and blood lipid profile of streptozotocin-nicotinamide induced diabetes rat.

MATERIALS AND METHODS

Plant and chemicals

Half ripen white guava (*P. guajava* Linn.) fruits were collected from Yanchao Township in Kaohsiung County. Streptozotocin, heparin, bovine serum albumin (BSA), Folin-Ciocalteu's phenol reagent, and thiobarbituric acid (TBA) were purchased from Sigma (MO, USA). Glucose enzymatic kits test were purchased from Audit Diagnostics, Cork, Ireland. All other reagents were of analytical grade. The instruments used were spectrophotometer (HITACHI U-1100, Tokyo, Japan) and freeze dryer (Panchum, FD-5030, Taiwan).

Sample preparation

Guava fruit were washed with clean water, drained, weighed and cut into slices. Slices were kept inside 80°C oven for dehydration. After drying process, the sample was ground into powder to increase contact surface area during extraction. For extraction procedure, 10 ml distilled water to 1 g dried sample ratio were heated inside

incubator for 30 min. The extract was then cooled to room temperature, filtered and centrifuged at 4°C, 9,000 × g for 30 min in order to separate extracted with non-extracted compound. Afterwards, supernatant were collected and freeze-dried into powder. The powders were kept in -20°C freezer.

Proximate chemical composition analysis

General composition analysis was done to measure freeze dried guava fruit water extract composition. It was divided into six parts; moisture test, water activity, ash content, crude fat, crude protein, and total carbohydrate content based on Association of Analytical Communities (AOAC) (1997).

Phenolic and flavonoid contents determination

Total phenolic content was measured according to the protocol by Kujala et al. (2000) by using 0, 50, 100, 150, 200, 250 µg/ml gallic acid as standard. Sample or standard (0.2 ml) were diluted into 2 ml, and 0.1 ml Folin-Ciocalteu's phenol reagent were added into sample. Then, 2.5 ml of 20% (w/v) sodium carbonate solution was added and mixture was allowed to react for 20 min. The absorbance was determined at 765 nm by spectrophotometer. Total phenolic contents were represented as mg gallic acid equivalent per gram dry weight sample (GAE). Flavonoid content was measured by colorimetric reaction as described previously by Christel et al. (2000) by using 0 to 100 µg/ml of quercetin as standard. 95% alcohol (1.5 ml) was added into 0.5 ml standard or sample, and several series of reagents were added: 0.5 ml of 1 M aluminum nitrate, 0.5 ml of 1 M potassium acetate and 2.8 ml distilled water. After stirring and incubating for 30 min, the absorbance values were measured at 415 nm. Flavonoid contents were shown as mg quercetin equivalent per gram dry weight sample.

In vitro Fe²⁺-ascorbic acid induced lipid peroxidation assay

Five week old male normal Wistar rats were sacrificed and 1 g of liver cell was taken and disrupted with 10 ml of 150 mM Tris-HCl (pH 7.2) buffer using homogenizer, followed by centrifugation at 500 × g for 10 min. Supernatant was harvested and diluted with water to make 10% (w/v) liver cell for lipid peroxidation analysis. Lipid peroxidation analysis was done according to Yoden et al. (1980) and Kimura et al. (1981) method. Obtained liver cell (0.25 ml) was added into test tube and following reagents were added: 0.05 ml Tris-HCl buffer (pH 7.2), 0.05 ml of ascorbic acid (0.1 mM), 0.05 ml of FeCl₂ (4 mM) and 0.05 ml sample (25, 50, 100, 200, 400, 800 µg/ml). Freeze dried water extract of guava (80 mg) was diluted with 100 ml of water to obtain sample with the concentration of 800 µg/ml and two times dilution was conducted to obtain the others concentrations. Solution mixture was incubated at 37°C for 1 h after mixing. After incubation, the mixture were added with 0.5 ml HCl (0.1 N), 0.2 ml sodium dodecyl sulphate (SDS) (9.8%), 0.9 ml distilled water and 2 ml (0.6% thiobarbituric acid; TBA). The mixture was then incubated at 95°C for 30 min. After cooling at room temperature, 5 ml n-BuOH was added with intense oscillation and centrifuged again with 1,000 × g for 25 min. Supernatant was taken and observed using fluorescence photometer at 515 nm excitation and 553 nm emissions to measured malondialdehyde (MDA) concentration. Negative control was examined using liver solution that was not treated with FeCl₂ and ascorbic acid. Positive control was examined using liver solution that was treated with FeCl₂ and

Table 1. Guava fruit's proximate chemical composition and phenolic content.

Composition	Content (%)
Water content	11.77±1.85
Water activity	0.44±0.02
ash	17.85±0.16
fat	0.83±0.18
Crude protein	16.48±1.32
Total carbohydrate	35.76±1.74
Phenolic compound	Content (mg/g)
Total phenolic compounds	40.13±2.12
Flavonoids compounds	18.426±1.22

Each value represents mean ± standard deviation (n = 3).

ascorbic acid only (without extract).

In vivo assay

Experimental animals

The five-week Wistar strain male rats were purchased from the Bio Lasco, Taiwan and the rat chows were purchased from Yong Li Company (Taipei, Taiwan) with compositions: 23% crude protein, 4.5% crude fat, 6.0% crude fiber, 8.0% ash, 2.5% minerals, 56% carbohydrate. The Wistar strain male rats were fed *ad libitum*. Body records were recorded weekly, and after body weight reached about 200 to 230 g, the rats were ready for experiment. Induction of streptozotocin plus nicotinamide to generate diabetic rat was done by following Masiello et al. (1998) induction methods by intraperitoneal injection using nicotinamide (180 mg/kg BW) and streptozotocin (STZ) (50 mg/kg BW) with 15 min time interval. Second injections were carried out a week later by the same procedure. Two weeks after second injections, oral glucose tolerance tests were conducted and blood glucose was measured at 0, 30, 60, 120 min. After 120 min of sugar feeding (1.5 g/kg BW), rats with blood glucose level > 200 mg/dl were considered to have severe diabetes type 2. After several weeks of sample or deionized water treatments, the rats were sacrificed after 12 h fasting. Blood and liver of rats were taken for analysis. For animal grouping, rats were randomly divided into four groups (n = 12). Normal group (NC): non-diabetic rats with deionized water feeding, control group (DC): diabetic rats with deionized water feeding, experimental group 2 (DF2): diabetic rat with 200 mg/kg BW sample feeding, experimental group 4 (DF4): diabetic rat with 400 mg/kg BW sample feeding. Guava feeding was carried out daily for six weeks.

Determination of plasma glucose concentration and serum lipid profile

Blood plasma was taken and 10 µl of plasma was mixed with 1 ml of glucose enzymatic kits test and incubated at 37°C for 5 min. The absorbances were measured by spectrophotometer at 505 nm wavelength and glucose content was converted from glucose standard curve. Blood cholesterol, triglycerides, high density lipoprotein (HDL) and low density lipoprotein (LDL) concentrations were detected using commercial kit.

Lipid peroxidation assay in vivo

Thiobarbituric acid reactive substances (TBARs) are low molecular weight compounds formed by decomposition of certain primary and secondary lipid peroxidation products that at low pH and high temperature participate in nucleophilic addition reaction with thiobarbituric acid generating red fluorescent complex (Janero, 1990). Determination was done according to Uchiyama and Mihara (1978) method by adding 9 fold (w/v) of cold 1.15% KCl solution into 0.5 g liver and homogenized in ice bath. After homogenization, 3 ml H₃PO₄ (1%) and 1 ml 0.67% thiobarbituric acid (TBA) were added into 0.5 ml of the homogenized liver as sample or 0.9% saline as blank or 1.1.3.3-tetraethoxy (0 to 10 µg/ml) as standard. After incubation in water bath at 95°C for 45 min, 4.0 ml n-butanol were added and centrifuged at 3,000 rpm (1,570 × g) for 10 min. The supernatant were measured at 520 and 535 nm by fluorescence photometer. Finally, TBARS values were converted from standard curve.

Statistical methods

Experiments were done in three replications for *in vitro* analysis and twelve replications for *in vivo* analysis. One way analysis of variance followed by post hoc testing (Duncan's test) was used for statistical analysis where appropriate, at P < 0.05 by using statistical package for social sciences (SPSS).

RESULTS

Proximate chemical general composition and antioxidant content analysis

The amount of total phenolic content of guava water extracts was 40.13 ± 2.12 mg/g of dry weight sample and the flavonoid content was 18.426 ± 1.22 mg/g of dry weight sample. Proximate chemical general composition of guava leaf water extract is shown in Table 1.

In vitro anti-peroxidation evaluation

In this experiment, lipid peroxidation assayed as malondialdehyde (MDA) production, was catalyzed by ascorbic acid and Fe²⁺ and after reacting with TBA, MDA will form a visible red color product. MDA is one of lipid oxidized derivatives and can be used as a biomarker of oxidative stress. As a result, 25 to 800 ppm guava fruit extracts offer dose-dependent protection from lipid peroxidation *in vitro*. Each concentration was able to effectively reduce MDA formation, and highest inhibition rate was 57.27% by 800 ppm guava fruit extracts (Table.2).

In vivo evaluation

Experiments in 0-, 4- and 6-week after guava fruit extract administration were observed and the changes in blood

Table 2. Effect of guava fruit water extract on Fe²⁺-ascorbic acid induced lipid peroxidation as expressed by MDA amount.

Treatment concentrations	MDA (nmol mg ⁻¹ protein)
Negative control (normal liver)	15.74±0.86 ^a
Positive control (untreated with extract)	117.24±2.29 ^e
25 µg/ml of guava fruit extract	95.06±10.34 ^d
50 µg/ml of guava fruit extract	85.00±4.73 ^c
100 µg/ml of guava fruit extract	67.73±3.50 ^b
200 µg/ml of guava fruit extract	59.92±10.69 ^b
400 µg/ml of guava fruit extract	59.22±0.69 ^b
800 µg/ml of guava fruit extract	57.27±2.10 ^b

Each value represents mean ± standard deviation (n = 3). ^{a-d}Data with different superscripts are significantly different at P < 0.05 (n = 3) analyzed by ANOVA with Duncan's post hoc using SPSS.

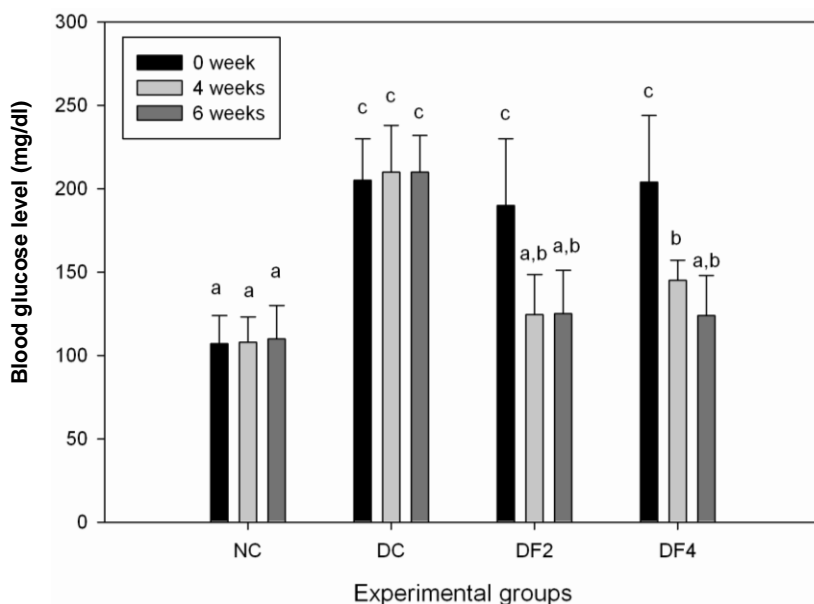


Figure 1 Effect of guava water extract admission on plasma glucose concentration for 4 and 6 weeks treatment. Each value represents mean ± standard deviation (n = 12). NC, normal control; DC, diabetic control; DF2, diabetic rats fed with 200mg guava water extract per kg body weight; DF4, diabetic rats fed with 400 mg guava water extract per kg body weight. ^{a-c}Data with different superscripts are significantly different at P<0.05 (n=3) analyzed by ANOVA with Duncan's post hoc using SPSS.

glucose were recorded (Figure 1). At 0 week, significant damage was evident to DC, DF2 and DF4 rat group which was indicated by higher blood glucose level after streptozotocin injection. The rats in the group which received water extract of guava showed rectification of increased blood glucose level. After 4 weeks of guava extract administration, blood glucose level was found to be decreased significantly, thus indicating that guava

extract can reduce plasma glucose concentrations of diabetic rats. After six weeks, blood glucose levels of the DF2 and DF4 groups were found to be lower than 200 mg/dl, and almost ameliorated the damage caused by streptozotocin to normalcy. Changes of serum lipid profile are shown in Table 3. Plasma triglyceride on DF2 group was reduced and significant reduction was expressed by DF4 group. DF2 and DF4 groups showed decline in

Table 3. Effect of guava fruit water extract admission for two weeks on lipid profile.

Treatment	Lipid profile (mg dL ⁻¹)			
	Triglyceride	Cholesterol	HDL	LDL
NC	53.00 ±6.26 ^{a,b}	67.80±4.46 ^b	17.80±3.56 ^a	11.40±3.58 ^a
DC	65.00±4.08 ^b	61.00±1.89 ^{a,b}	24.00±9.63 ^a	38.50±4.36 ^c
DF2	56.20±6.96 ^{a,b}	61.40±1.14 ^{a,b}	22.40±1.67 ^a	24.20±2.77 ^b
DF4	49.25±6.78 ^a	59.00±6.16 ^a	23.00±2.45 ^a	18.00±6.06 ^{a,b}

Each value represents mean ± standard deviation (n = 12). NC, normal control; DC, diabetic control; DF2, diabetic rats fed with 200 mg guava water extract per kg body weight; DF4, diabetic rats fed with 400 mg guava water extract per kg body weight. ^{a-c}Data with different superscripts are significantly different at P<0.05 (n=3) analyzed by ANOVA with Duncan's post hoc using SPSS performed separately for every analysis.

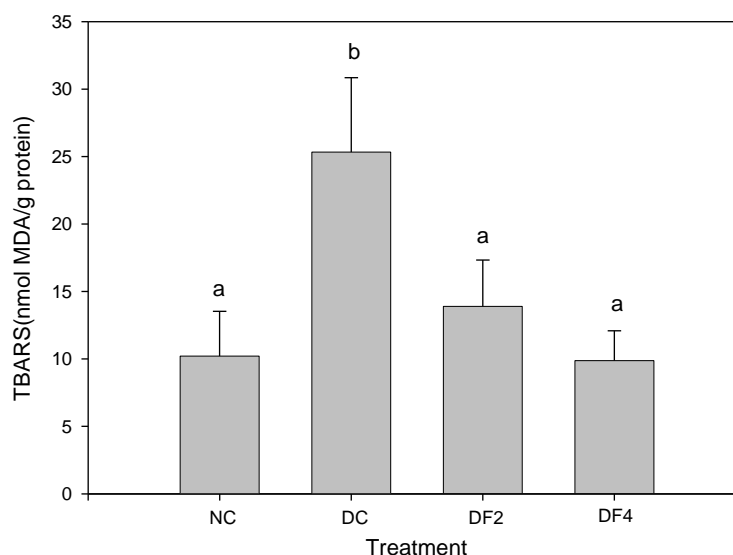


Figure 2. Effect of guava fruit water extract admission on rat liver as expressed by TBARS content. Each value represents mean ± standard deviation (n = 12). NC, normal control; DC, diabetic control; DF2, diabetic rats fed with 200 mg guava water extract per kg body weight; DF4, diabetic rats fed with 400 mg guava water extract per kg body weight. ^{a-b}Data with different superscripts are significantly different at P < 0.05 (n = 3) analyzed by ANOVA with Duncan's post hoc using SPSS.

serum LDL level, especially in DF4 group. Other lipid profiles (cholesterol and HDL) did not show significant difference compared to diabetic control (DC) group. TBARS in the liver are an indicator of damage caused by lipid peroxidation. Figure 2 shows that guava fruit water extract administration on rat can significantly reduce TBARS content that was generated by lipid peroxidation, either in DF2 or DF4 groups compared to DC group.

DISCUSSION

Cheng and Yang (1983) has proved that guava juice

could reduce blood glucose level in mice and human, and this present study also revealed the same blood glucose lowering ability in rat. Anti-hyperglycemic activity of unripe guava peel also showed similar result (Rai et al., 2009). Its anti-hyperglycemic activity may due to its ability to inhibit α -glucosidase enzyme in intestine which in turn reduce glucose formation. *In vitro* α -glucosidase inhibition performed by 0.05 mg/ml guava fruit extract was about 70% (Wongsa and Zamaluddin, 2011).

Lipid peroxidation is the process in which free radical steal electron from double-layer polyunsaturated fatty acids on cell membrane, resulting in toxic aldehyde type

of lipid peroxides, such as malondialdehyde (MDA). In diabetes, MDA can cause protein glycation that leads to further complications occurrence (Slatter et al., 2000; Pariand Latha, 2005). This study found that a higher dose of guava extract has a better inhibition of liver lipid peroxidation effect *in vitro*. *In vitro* evidence of lipid peroxidation inhibition is also supported by its *in vivo* result. This inhibition may occur due to guava's antioxidant activity from ascorbic acid and phenolic compounds. According to Soares et al. (2007), mature white guava fruits contained high amount of ascorbic acid, which was about 168.36 mg/100 g samples and it was mostly found in peel part. Phenolic compounds was a group of compounds well-known for its antioxidant properties. Sa´nchez-Moreno et al. (1999) indicated that the inhibition of lipid oxidation of the phenolic compounds and antioxidant standards followed the order: rutin, ferulic acid > tannic acid, gallic acid, resveratrol > BHA, quercetin > tocopherol > caffeic acid, in a linoleic acid system. These flavonoids act by scavenging superoxide anions, singlet oxygens and lipid peroxy radicals, and through sequestering metal ions that promote oxyradical formation (Rao, 2003). Since guava fruit water extract consisted of 40.13 mg GAE/g phenolic content and 18.43 ± 1.22 mg quercetin equivalent/g flavonoid content, we suspected that phenolic and flavonoid contents in guava fruit extract may have important role in inhibiting lipid peroxidation.

This study found that high doses of guava fruit water extract could also reduce low density lipoprotein (LDL) and triglycerides content in the diabetic rat significantly. Similar research on hypertension patient by Singh et al. (1992) showed that guava fruit intake also caused serum cholesterol (9.9%) and triglyceride (7.7%) reduction and an increase in high-density lipoprotein cholesterol (8.0%). Anti-LDL glycative agents investigated using aqueous decoctions of *P. guajava* ripe fruit revealed that guava fruits exhibit excellent antiglycation effect, being a rather powerful and effective inhibitor of LDL glycation in both glucose and glyoxal induced models (Hsieh et al., 2005). The antiglycation activities of guava fruit were relevantly and directly related to its polyphenolic content. Total grain, whole-grain, total dietary fiber, cereal fiber, and dietary magnesium intakes showed strong inverse associations with incidence of diabetes (Meyer et al., 2000). Pulp and peel of guava fruit showed high content of dietary fiber of 48.55 to 49.42% (Jimenez-Escrig et al., 2001). Dietary fiber was expected to offer some improvement in carbohydrate metabolism, lower total cholesterol and LDL cholesterol, and have other beneficial effects in patients with non-insulin dependent diabetes mellitus (Vinik and Jenkins, 1998).

Beside its dietary fiber content, guava fruits are also rich in magnesium and potassium. From previous research, Singh et al. (1991) stated that it is possible that dietary magnesium may have contributed to the reduction of total

serum cholesterol, LDL cholesterol, and triglyceride, and the marginal rise in HDL cholesterol.

Conclusion

This study confirmed that guava fruit extracts can reduce blood sugar in type 2 diabetic rats, plasma triglycerides and LDL concentrations.

Integrated role from several compounds contained in guava fruit extract, especially phenolic compounds, was expected to give contribution on these activities. From this research, guava fruit water extract was able to show impressive efficacy against *in vitro* and *in vivo* lipid peroxidation, plasma triglycerides and LDL concentration, thus expected not only able in blood glucose reduction but also diabetic complication preventer.

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