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Toxicity Study of Malaysian Rubber (*Hevea brasiliensis*) Seed Oil as Rats and Shrimps Tests

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Abstract: The lipid fraction of Malaysian rubber seed was extracted and analyzed for toxicological compounds such as linamarin. Analytical methods (rat's toxicological test and brine shrimps test) were applied to determine the presence of such compounds. Malaysian rubber seed oil was extracted using different solvents (hexane and chloroform+methanol). The oil was found not to contain unusual fatty acids and it is rich in essential unsaturated fatty acids, particularly linoleic (37.28±0.10%) and linolenic (19.22±0.21%). Malaysian rubber seed oil showed no toxic potential toward the rats. Bioassay experiments, in which shrimps were used as test organisms, were performed to evaluate the toxicity of linamarin extracted from the oil. The median lethal concentration (LC_{50}) was found to be 211.70%. The results showed no acute toxicity effects, probably since Malaysian rubber seed oil was not found to contain any hazardous linamarin.

Key words: Rubber seed oil, linamarin., rats, shrimps

INTRODUCTION

Among the ancillary resources obtained from rubber (*Hevea brasiliensis*) plantations (i.e., wood and seeds), seed has the greatest potential use. However, rubber seeds are not currently much in use, so they are abundant and wasted (Achinewhu and Akpapunam, 1985).

The seeds of the rubber tree have been found to be rich in oil. Rubber seed oil is a yellow, semi-drying oil (Aigbodion and Bakare, 2005). At the present time, the production of Rubber Seed Oil (RSO) is increasing greatly in both quantity and quality in Malaysia because of its important role in different industrial processes. The oil does not contain any unusual fatty acids and it is a rich source of essential fatty acids $C_{18:2}$ and $C_{18:3}$ that make up 52% of its total fatty acid composition (Ghandhi *et al*., 1990).

Polyunsaturated fatty acids (PUFAs) consist of omega-6 (linoleic acid, γ-linolenic acid) and omega-3 (α-linolenic acid, eicosapentanoic acid, docosahexaenoic). The importance of PUFAs were highlighted when these fatty acids proved to be essential to human health to reduce cholesterol in the body and risk of heart attack and stroke. However, due to the inability of the human body to synthesize these fatty acids, nutritional PUFAs are obtained from marine life, plant sources and vegetable oils (Yong and Jumat, 2006).

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However, many studies of rubber seeds have indicated that the use of RSO for nutritional purposes faces various vital challenges, one of which is the presence of toxins in RSO. It is well known that some concentration of poisons will always be found in the seeds of all types of plants, including the seeds of the rubber plant. Rubber seeds known to contain linamarin (Duke and Ducellier, 1993).

A linamarin is a cyanogenic glucoside. The hydrolysis or cyanogenesis of linamarin by the endogenous enzyme linamarase (β-glucosidase) results in the formation of glucose and acetonecyanohydrin, which later decomposes into hydrogen cyanide (HCN) and acetone (Idibie *et al*., 2007; Sornyotha *et al*., 2006). Linamarin has been demonstrated to protect the plant from herbivores, both animals and generalized insect feeders (Siritunga and Sayre, 2004).

In this study, analysis of RSO for the detection of linamarin using rats and shrimps tests plays a vital role in the determination of the value of Malaysian RSO (MRSO) for inclusion in a healthy diet for animals and human beings. Hence, the evaluation of the levels of the toxic compound linamarin from MRSO must be done before MRSO can be approved for consumption by both animals and human beings.

MATERIALS AND METHODS

Seed Material and Oil Extraction

Rubber seeds were collected from Malaysia's Rubber Research Institute, Sungai Buloh, Selangor, Malaysia in May 2009. The seeds were shelled and dried in the oven at 105°C for 30 min and then milled using a grinder. The seeds were then kept in the refrigerator. The MRSO was extracted from the 500 g rubber seeds by soxhlet extractor using hexane and a mixture of chloroform+methanol (1:1) as solvents at 60°C for 6 h.

Physicochemical Characteristics

The physicochemical properties of MRSO such as color, free fatty acid content (FFA%), acid value, saponification value, iodine value and unsaponifiable matter content were determined according to the guidelines of the American Oil Chemical Society (AOCS).

Gas Chromatography (GC)

The fatty acid composition of RSO was determined using its fatty acid methyl esters. The GC analysis was performed on a Shimadzu GC equipped with flame ionization detector and capillary column (30 m×0.25 mm×0.25 μm films). The detector temperature was programmed for 280°C with flow rate of 0.3 mL min^{-1}. The injector temperature was set at 250°C. Nitrogen was used as the carrier gas at a flow rate of 20 mL min^{-1}.

Rats Toxicological Test

Nine male white rats (Rumah Haiwan Laboratories, Universiti Kebangsan Malaysia) weighing between 248.1-248.8 g were used for toxicity study as in Nwokolo *et al*. (1988). The male white rats were individually housed in stainless steel cages in a room with controlled temperature (30-35°C) and lighting (alternating 12 h periods of light and darkness). The male white rats were fed a pelleted commercial laboratory feed for 3 months. The mortality, color and the behavior of the male white rats were recorded daily, but the food consumption was recorded every two weeks. The food consumption, food efficiency, body waist measurement and body length measurement were also determined.

Three experiments were conducted to determine the toxicological response of rats fed rubber seed oil. In experiment 1, 3 rats were fed a rubber seed oil that had been extracted with hexane. In experiment 2, 3 rats were fed a rubber seed oil that had been extracted with chloroform+methanol. In experiment 3, 3 rats were fed a normal food were used as blank control. The rubber seed oil to be fed to rats was stored at 4°C. Toxicological evaluation of the rubber seed oil extracted with these two solvents was carried out by an acute oral toxicity limit test to assess the acute toxicity potential of each oil.

Shrimps Test

Sample Extraction

The samples of linamarin were extracted from two different MRSO (100 g) using water as a solvent in a separating funnel. After gentle shaking, the mixture was left a few minutes to allow emergence of two phases: oil phase and water phase. The oil phase was removed and the water phase was kept for further analysis.

HCN Hydrolysis

To conserve cyanide, the samples were supplemented with 4 mL 10 M NaOH. The sample was distilled without further pretreatment. HCN was recovered in the presence of 10 mL zinc acetate buffer (pH 4.5). The remaining cyanide was subsequently recovered by distillation after addition of 5 mL of $MgCl_2$ and 5 mL sulfamic acid plus 5 mL 50% H_2SO_4 later added to obtain pH 1-2 for converting to HCN during distillation (ASTM, 1998). After 3 min, 45 mL 50% H_2SO_4 was added and the solution boiled under reflux for 90 min (Bjarnholt *et al*., 2008; Dzombak *et al*., 2006). After distillation, the samples were put in a drying vacuum to evaporate the solvents. The released HCN was determined using the shrimp acute toxicity method.

Shrimp Acute Toxicity Method

Shrimp used in this study were obtained from a local breeder and transported immediately to the laboratory within 20 min. In the laboratory, a total of 450 shrimps were kept in an 80 L glass aquarium containing filtered and dechlorinated tap water (pH 6.2-6.4, dissolved oxygen concentration 7.3-8.1 mg L^{-1}, conductivity 64-68 μS cm^{-1} and ammonia <0.5 mg L^{-1}). The shrimp aquarium was equipped with a water-cycling device and the water was continuously aerated for one week to remove chlorine before the shrimp were introduced. Shrimps were acclimated for 14 days (26-27°C with 12 h light: 12 h darkness) and fed daily. Care was taken to keep the mortality rate less than 5% for the whole acclimatization period.

The acute toxicity test was performed according to the OECD (1993) and APHA (1998) recommendations. Laboratory static tests were conducted to determine the median lethal concentration (LC_{50}). Ten shrimps of similar size were placed in the test chambers. Shrimp were exposed for 96 h to one of the different concentrations of 5, 10, 50, 75 and 100% of the samples which were extracted from MRSO and rubber seed. The test chambers were aerated throughout the test period. Physiochemical parameters of the water in the chambers such as pH, conductivity, dissolved oxygen and temperature were measured for each solution. The tests were repeated three times for both the control and each test solution. During the experiment, dead shrimps were removed and mortality of the shrimps exposed to various concentrations of samples was recorded after 6, 12, 18, 24, 48, 72 and 96 h. The LC_{50} was calculated based on Finneys Probit Analysis Method (US EPA, 1999). The palm oil was used as blank control.

Statical Analysis

Data collected were subjected to analysis of variance while the Significant of difference between means were determined by Duncan's Multiple Range Test (DMRT), where ($p<0.05$) was considered for significant difference. Each value was determined by at least three replicates. Results were given as Mean±SD (SAS, 1996).

RESULTS AND DISCUSSION

The physicochemical properties of MRSO which was extracted by using different solvents such as hexane ($MRSO_h$) and chloroform+methanol ($MRSO_{ch+mth}$) were determined are given in Table 1. The present FFA% (7.55±0.02 and 8.76±0.03, respectively) and acid value (15.03±0.04 and 17.43±0.06, respectively) show that the MRSO has a high FFA% since it had not been neutralized. $MRSO_h$ presents as a pale yellow oil (33.98±0.08), but lighter (higher L* value) than $MRSO_{ch+mth}$ (30.91±0.6) because of the high FFA% in $RSO_{ch+mthr}$. The MRSO shows high iodine value (135.79±0.33 and 134.44±0.31, respectively) compared with the iodine value of palm oil (52) (Onyeike and Acheru, 2002) due to the high content of unsaturated fatty acids such as oleic acid (22.95±0.15 and 25.31±0.13%, respectively) are shown in Table 2.

The saponification values of $MRSO_h$ and $MRSO_{ch+mth}$ (182.12±0.27 and 183.32±0.29, respectively) are with average saponification numbers in the range of 175-250 (Gunstone *et al*., 1994). The value of unsaponifiable matter of MRSO is 1.83±0.01% and 2.19±0.03, respectively (Table 1). This value is in agreement with the value for RSO reported in by Gandhi *et al*. (1990).

The fatty acid composition of the RSO is shown in Table 2. The Fatty Acids (FA) of $MRSO_h$ and $MRSO_{ch+mth}$ consist of saturated FA 19.12±0.28 and 21.64±0.21%, respectively and unsaturated FA 79.45±0.31 and 77.40±0.26%, respectively. The saturated FA consist mainly of palmitic acid 8.56±0.07 and 9.10±0.06%, respectively and stearic acid 10.56±0.02 and 12.63±0.01%, respectively and the unsaturated FA consist mainly of oleic acid 22.95±0.15% and 25.31±0.135, respectively, linoleic acid 37.28±0.10 and 36.31±0.09%, respectively and

Table 1: Physicochemical properties of MRSO

Analysis	$MRSO_h$	$MRSO_{ch+mth}$
FFA% (as oleic)	7.55±0.02	8.76±0.03
Acid value	15.03±0.04	17.43±0.06
Iodine value	135.79±0.33	134.44±0.31
Saponification value	182.12±0.27	183.32±0.29
Unsaponifiable matter	1.83±0.01	2.19±0.03
Color		
a*	0.86±0.01	0.60±0.01
b*	0.47±0.04	2.91±0.05
L*	33.98±0.08	30.91±0.60

$MRSO_h$: Extracted using hexane as solvent; $MRSO_{ch+mth}$: Extracted using a mixture of chloroform and methanol

Table 2: Fatty acids composition of MRSO

Analysis	$MRSO_h$	$MRSO_{ch+mth}$
Saturated		
Palmitic acid	8.56±0.07	9.10±0.06
Stearic acid	10.56±0.02	12.63±0.01
Unsaturated		
Oleic acid	22.95±0.15	25.31±0.13
Linoleic acid	37.28±0.10	36.31±0.09
Linolenic acid	19.22±0.21	15.78±0.18

$MRSO_h$: Extracted using hexane as solvent; $MRSO_{ch+mth}$: Extracted using a mixture of chloroform and methanol

linolenic acid 19.22±0.21 and 15.78±0.18, respectively (Table 2). The FA composition of RSO can be used as indicator of the type of each fatty acid (Aigbodion and Bakare, 2005). The physicochemical properties and FA composition don't show any significant ($p<0.05$) difference between $MRSO_h$ and $MRSO_{ch+mth}$.

Toxicological evaluation of the MRSO was carried out in white male rats by performing an acute toxicity limit test to assess its acute toxicity potential in a 3-month feeding study. Two different types of rubber seed oil were extracted by using hexane ($MRSO_h$) and chloroform+methanol ($MRSO_{ch+mth}$) as solvents under the same extracting condition. A total of 9 rats, 3 for each experimental condition were used. Table 3 shows the mortality, color and the behavior of the experimental rats. No acute toxic potential was observed with MRSO extracted with both types of solvents. The rats displayed no behavioral changes and there was no mortality in any of the groups during the 3-month feeding study. The color of the male white rats didn't appear to change during the feeding study.

Neither $MRSO_h$ nor $MRSO_{ch+mth}$ had any adverse effect on food consumption. A similar increase in the average daily gain of rats fed $MRSO_h$ and $MRSO_{ch+mth}$ was also observed; differences between the 3 groups of male white rats was not statistically ($p<0.05$) significant. The 3 groups of rats showed no significant ($p<0.05$) differences in body weight gain, food efficiency, body waist measurement and body length measurement. The growth rates of the 3 groups of rats is shown in Table 4 and Fig. 1. These results would indicate that $MRSO_h$ and $MRSO_{ch+mth}$ had no toxic or antipalatability effects agreement with the value for RSO reported in by Gandhi *et al.* (1990) and Nwokolo *et al.* (1988).

Table 5 shows the relation between the samples of the linamarin that was extracted from the MRSO which was extracted using different solvents such as hexane ($MRSO_h$) and

Table 3: Mortality, color, and behavior of the male white rats

Activity	Blank	$MRSO_h$	$MRSO_{ch+mth}$
Color	White	No changes	No changes
Behavior	Normal	No changes	No changes
Mortality	No	No	No

$MRSO_h$: Extracted using hexane as solvent; $MRSO_{ch+mth}$: Extracted using a mixture of chloroform and methanol

Table 4: Food consumption, body weight gain, food efficiency, body waist measurement and body length measurement

Activity	$MRSO_h$	$MRSO_{ch+mth}$	Blank/control
Initial body weight (g)	248.20±0.60	248.50±0.70	248.80±0.20
Final body weight (g)	482.10±0.20	480.80±0.50	480.10±0.70
Body weight gain (g)	234.40±0.60	232.80±0.20	232.10±0.60
Food consumption (g)	1800.20±0.70	1792.60±1.20	1789.80±1.70
Food efficiency ratio (weight gain/food intake)	0.13±0.07	0.13±0.01	0.15±0.02
Initial body waist (cm)	14.50±0.20	14.50±0.10	14.50±0.60
Final body waist (cm)	20.50±0.70	20.50±0.30	20.20±0.20
Body waist gain (cm)	6.00±0.70	6.00±0.10	5.70±0.60
Initial body length (cm)	23.10±0.50	23.70±0.30	23.40±0.30
Final body length (cm)	24.50±0.20	24.20±0.70	24.30±0.90
Body length gain (cm)	1.50±0.70	1.20±0.20	1.30±0.70
Condition*	Normal	Normal	Normal

*Condition was assessed by visual appearance

Table 5: Mortality (%) of shrimps at various concentrations of samples of the linamarin extracted from $MRSO_h$, $MRSO_{chl+mth}$ and palm oil

Conc. (mL)	No. exposed	No. response of $MRSO_h$	No. response of $MRSO_{chl+mth}$	No. response of palm oil
5	10	0	0	0
10	10	0	0	0
50	10	0	0	0
75	10	1	1	1
100	10	1	2	2

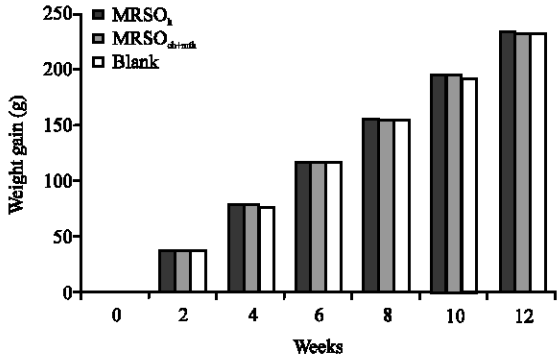

Fig. 1: Body weight gain of rats fed MRSO and blank control

Table 6: Estimated LC values and confidence limits of toxicity on shrimps of samples of linamarin extracted from the MRSO$_h$, MRSO$_{chl+mth}$ and palm oil

Point	Exposure conc. of MRSO$_h$	Exposure conc. of MRSO$_{chl+mth}$	Exposure conc. of palm oil
LC 1.00	45.60	51.59	51.59
LC 5.00	71.49	69.03	69.03
LC 10.00	90.86	80.63	80.63
LC 15.00	106.82	89.52	89.52
LC 50.00	211.70	139.40	139.40
LC 85.00	419.53	217.05	217.05
LC 90.00	493.22	241.03	241.03
LC 95.00	626.85	281.50	281.50
LC 99.00	982.80	376.63	376.63

chloroform+methanol (MRSO$_{ch+mth}$) in the same extracting condition, concentration and the mortality rate of the shrimps. Palm oil was used as blank control for comparison with MRSO. The estimated LC values and their confidence limits that resulted from the acute toxicity testing on freshwater shrimps using samples of the linamarin extracted from the MRSO$_h$, MRSO$_{chl+mth}$ and palm oil are listed in (Table 6). Based on Finneys Probit Analysis Method (using EPA software program), the mean LC_{50} value of samples of the linamarin extracted from the MRSO$_h$, MRSO $_{chl+mth}$ and palm oil using shrimps was found to be 211.70, 139.40 and 139.40%, respectively.

In this study, shrimp has, for the first time, been used as test organism for acute toxicity of linamarin in MRSO. The results showed that samples of the linamarin extract from the MRSO$_h$ and MRSO$_{chl+mth}$ have no toxic effects on shrimps (LC_{50} 211.70 and 139.40%), indicating that no hazardous linamarin was found in MRSO. The results for MRSO were compared with those for palm oil. The palm oil did not show any toxic effect as indicated by its LC_{50} (139.40%). These results would indicate that MRSO $_h$ and MRSO $_{ch+mth}$ had no acute toxicity toward shrimp, a result which supported the results obtained with the other method used (rats toxicological test).

CONCLUSION

The current study has shown that, from the nutritional and toxicological aspects, MRSO could be considered for edible use. These initial results indicate that the use of MRSO as an edible oil will not be restricted by toxic or antipalatability factors.

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REFERENCES

Achinewhu, S.C. and M.A. Akpapunam, 1985. Physical and chemical characteristics of refined vegetable oils from rubber seed (*Hevea brasiliensis*) and bread-fruit (*Artocarpus altilis*). Qual. Plant Foods Hum. Nutr., 35: 103-107.

Aigbodion, A.I. and I.O. Bakare, 2005. Rubber seed oil quality assessment and authentication, Nigeria. J. Am. Oil Chem. Soc., 82: 465-469.

APHA., 1998. Standard Methods for the Examination of Water and Wastewater. 20th Edn., American Public Health Association, Washington, DC., ISBN: 0875532357, pp: 1270.

ASTM, 1998. D 2036-98 Standard Test Methods for Cyanides in water. ASTM International, West Conshohocken, Philadelphia,.

Bjarnholt, N., M. Laegdsmand, H.C.B. Hansen, O.H. Jacobsen and B.L. Mollar, 2008. Leaching of cyanogenic glycosides and cyanide from white clover green manure. Chemosphere, 72: 897-904.

Duke, J.A. and J.L. Ducellier, 1993. CRC Hand Book of Alternative Cash Crops. CRC Press, USA.

Dzombak, D.A., R.S. Ghosh and G.M. Wong-Chong, 2006. Cyanide in Water and Soil: Chemistry, Risk and Management. CRC Press, USA., ISBN-10: 1-56670-666-1.

Ghandhi, V.M., K.M. Cherian and M.J. Mulky, 1990. Nutritional and toxicological evaluation of rubber seed oil. J. Am. Oil Chem. Soc., 67: 883-886.

Gunstone, F.D., J.L. Harwood and F.B. Padley, 1994. The Lipid Hand Book. 2nd Edn., Chapman Hall, London, ISBN: 0412433206.

Idibie, C.A., H. Davids and S.E. Iyuke, 2007. Cytotoxicity of purified cassava linamarin to a selected cancer cell lines. Bioproc. Biosyst. Eng., 30: 261-269.

Nwokolo, E., D.D. Kitts and J. Kanhai, 1988. Serum and liver lipids of rats fed rubber seed oil. Plant Foods Hum. Nutr., 38: 145-153.

OECD, 1993. Organization for Economic Co-operation and Development. OECD, Paris.

Onyeike, E.N. and G.N. Acheru, 2002. Chemical composition of selected Nigerian oil seeds and physicochemical properties of the oil extracts. Food Chem., 77: 431-437.

SAS., 1996. User's Guide Statistics. Version 5, Statistical Analytical System Institute Inc., Cary, NC. USA.

Siritunga, D. and R. Sayre, 2004. Engineering cyanogens synthesis and turnover in cassava (*Manihot esculenta*). Plant Mol. Biol., 56: 661-669.

Sornyotha, S., K. Laykyu and C.K. Ratanakhanok, 2006. Purification and detection of linamarin from cassava root cortex by high performance liquid chromatography. Food Chem., 104: 1750-1754.

US EPA., 1999. LC_{50} Software Program. Version 1.00. Center for Exposure Assessment Modeling (CEAM) Distribution Center, UK.

Yong, O.Y. and S. Jumat, 2006. Characteristics of *Elateriospermum tapos* seed oil as a new source of oilseed. Ind. Crops Prod., 24: 146-151.