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Full Length Research Paper

Oxidative stabilization of palm olein with isoespintanol (2-isopropyl-3,6-dimethoxy-5-methylphenol) isolated from *Oxandra cf xylopioides*

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Antioxidants are used to retard oxidative processes and improve the sensory and nutraceutical quality of cooking oils. Isoespintanol, a monophenol isolated from *Oxandra cf. xylopioides,* has shown *a* greater free radical scavenger ability than thymol (biosynthetic analog). Isoespintanol antioxidant capacity was evaluated and compared to butylated hydroxytoluene (BHT). Cytotoxicity, thermal stability and effect on olein stability studies were performed. The results revealed that Olein oxidation time was reduced 17.2 and 4.2% in the presence of Isoespintanol and BHT, respectively. Thermogravimetric curves indicated higher thermal resistance for Isoespintanol than for BHT. Finally, cytotoxicity tests for Isoespintanol against murine macrophages revealed no effect on cell viability, indicating their possible use as a safe food additive.

Key words: Oxidative stabilization, isoespintanol, bht, antioxidant capacity, cytotoxicity, safe food additive.

INTRODUCTION

Frying is a wet method of cooking where food is subjected to a quick immersion in a bath of fat or oil at temperatures between 150 and 180°C. The resulting food remains dry, crispy, browned and has the best culinary characteristics. However, frying oils are subjected to elevated temperatures for extended periods of time that leads to oxidative reactions. The reaction of dissolved oxygen and water produces compounds that confer unpleasant flavors to oils and food. Common compounds include volatile carbonyls, hydroxyl acids, keto acids and epoxy acids, providing unpleasant flavors to oils and food (Alvis and Velez, 2008; Wu et al., 2013, Paz et al., 2015).

Oil used for frying should possess chemical characteristics that contribute to its useful life. For instance, saturated and unsaturated fatty acid content ratio affects the stability of the product during the heating

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process. Different studies suggest that oils with high content of monounsaturated fatty acids improve stability of fried food. Oleic acid from palm, olives and sunflower is an example of such oils (Casal et al., 2010; Aladedunye and Przybylski, 2013).

According to the Department of Agriculture of United States of America, palm oil is the second type of oil with higher production volume in the world (USDA, 2004). Palm olein was introduced to the market in the late twentieth century and it has remained in use because of its technical and economic advantages compared to other oils and fats. It is estimated that millions of tons of palm olein and oil in their natural forms or mixtures with other oils, are used annually for domestic and industrial frying. In addition, numerous studies demonstrate the benefits of the use of palm oil in frying because of its greater oxidative stability compared to other oils (Ismail, 2005; Kalogianni et al., 2011; Mozzon et al., 2013). However, palm olein products are susceptible to lipid peroxidation due to the presence of unsaturated fatty acids. For this reason, antioxidants are used to retard oxidative processes and improve the sensory and nutraceutical quality of the products (Sanchez, 2011, Rojano et al., 2008). Moreover, addition of natural metabolites provides extra protection against oxidative stress enhancing the antioxidant system of the consumer. Although antioxidants in food determine the lifetime and reduce the nutritional losses, they never regenerate the quality of a highly-oxidized food.

Butylhydroxyanisole (BHA) and butylatedhydroxytoluene (BHT) are synthetic antioxidants widely used in the food industry; however, they can cause adverse health effects, such as increased cholesterol, liver enlargement and induction of liver cancer. Therefore, the search for natural alternatives with high antioxidant activity and without cytotoxic and genotoxic effects have become a necessity for the food industry (Niaiforovia et al., 2010: Krishnaiah et al., 2011). Recent years have witnessed a growing interest for natural antioxidants. For instance, a study done by Piedrahita et al. (2015) showed that rosemary extracts have shown higher thermal resistance and antioxidant capacity than synthetic antioxidants (BHA, BHT and TBHQ) when working with potato frying processes in olein palm; additionally, some studies have described the stabilizing capacity of rosemary extracts on oily matrices (Upadhyay et al., 2006); Upadhyay and Mishra (2015a) show that the oxidative stability in sunflower oil given by rosemary oleoresin (1200 and 1500 mg/kg) and tertiary butyl hydroquinone (200 mg/kg) are comparable.

Isoespintanol is a isopropylphenol extracted with low polarity solvents from of leaves of Oxandra cf xylopioides *(Annonaceae)* a native plant from Colombia and it has opened new alternatives in the world of fats and oils, due to its chemical structure that makes it soluble in liposoluble media; some studies have shown the antioxidant capacity of Isoespintanol in oily matrices (Rojano et al., 2008a). Likewise, Isoespintanol (2-isopropyl-3,6-dimethoxy-5-methylphenol) have shown a greater free radical scavenging ability than its biosynthetic analog (thymol) (Rojano et al., 2008b).

The industry of frying processes requires antioxidants with high thermal stability that preserve the sensory quality and oxidative stability of oils subjected to high temperatures. In the present study, the authors assess the antioxidant capacity of Isoespintanol to trap reactive oxygen species compared to BHT. We compare the effect of adding Isoespintanol or BHT on the oxidative stability of palm olein under accelerated oxidative conditions. Finally, we calculate the *in vitro* thermal stability of Isoespintanol and BHT and estimate the cytotoxicity of Isoespintanol in biological systems.

MATERIALS AND METHODS

Reagents

Fluorescein, dichlorofluoresceindiacetate, pyrogallol red, AAPH and BHT were bought from Sigma Aldrich through Colombia G & G Successors SAS. Palm olein (antioxidant free) was provided by a local supplier (TECNAS).

Plant material

The *Oxandra cf. xylopioides* species were collected in the municipality of Monteria (Córdoba, Colombia) in coordinates 08°48'17'' north latitude and 75°42'07'' west longitude; in October at warm weather under 24°C. The material was identified by the botanist Francisco Javier Roldan Palacios. A sample under file number 037852 was deposited in the Botanical Garden Joaquín Antonio Uribe, Medellin (Colombia). The plant material was cleaned and stored at 4°C until further analysis.

Extraction and isolation

The powdered leaves (1.0 kg) of *Oxandra cf. xylopioides* were extracted with petroleum ether (15 L) by percolation at room temperature and concentrated in vacuum to obtain a crude extract (150 g). The petroleum ether extract (30 g) was subjected to passage over a silica gel column (5 × 80 cm) eluting with a step gradient of hexane: dichloromethane (95:5, 80:20, 70:30, 0:100 each 1.0 L), to obtain four fractions. Fraction II was dissolved in dichloromethane and compound 4 recrystallized in hexane (3.0 g) (1.5%). The compound was characterized by NMR (1H and 13C COSY bi-dimensional and, NOESY, HMQC, HMBC), MS, UV and IR, and their physical properties; these results were previously reported (Rojano et al., 2007).

Fatty acid composition of palm olein

The fatty acid profile of the oil was performed on an Agilent 6890N GC coupled to an Agilent 5973N MS selective detector equipped with a split/split-less injector. The injector temperature was 300°C and the sample previously derivatized with KOH-methanol. The sample was automatically injected into the split-less mode. A column HP-5ms (5% phenyl methyl siloxane) of 30 m, 0.25 mm with a film thickness of 0.25 µm and at a maximum temperature of

325°C was used. The temperature program started at 50°C and went up to 200°C in 5 min, then it reached a final temperature of 300°C (14 min) at a rate of 10°C/min. Helium was used as carrier at a constant flow of 1 mL/min gas. The detector temperature was 300°C. The software used to calculate all parameters was MSD ChemStation D 02.00.275 Copyright © Agilent Technologies 1989-2005. For determination of saturated and unsaturated fatty acids as methyl esters the database NIST 2005 was used (Zapata et al., 2013).

Oxidative stability of palm olein

The active oxygen method (AOM) was used to monitor the oxidative condition of oil. 30 mL of sample were placed in a heater at a constant temperature of 99 ± 0.1°C and aerated at 1.150 mL. air/min until the time in which a peroxide value (PV) of 100 meq. O_2/kg. oil was reached (Negative Control). Other samples were assessed under the same conditions, one in presence of Isoespintanol and the other in presence of BHT. Both antioxidants were at a concentration of 200 mg/L, and were named as IE200 and BHT200, respectively. The concentrations were defined as the maximum concentration permitted for this application in different countries.

Peroxide value (PV)

The peroxide value (meq.O_2/kg.oil) was determined by spectrophotometry. This method is based on the oxidation of Fe^{+2} to Fe^{+3} by hydroperoxides to form a complex Fe^{+3}-thiocyanate. 0.01 g of sample were added to 3 mL of a solution chloroform: methanol (7:3), the mixture was stirred for 10 s and 1 mL of this solution was mixed with 50 µL of FeSO4-BaCl2 (0.144 M - 0.4 M) and 50 µL of NH_4SCN (0.44 M). The mixture was incubated for 20 min in the darkness; the absorbance was determined at 510 nm in a spectrophotometer Jenway® 6405 UV/Vis (Zapata et al., 2015).

Thermal stability of antioxidants

The thermal stability of the antioxidants was estimated using a thermogravimetric analyzer (Q50, TA Instruments Inc., New Castle, DE). Analyses were carried out by heating the samples in air from 30 to 300°C at a rate of 20°C.min^{-1}. Airflow rate was constant throughout the experiment (100 cm^3/min). Initially, the instrument was calibrated for mass changes and temperature, using nickel as a reference material. For the export of graphical and numerical data, Q Series software was used (Q50-1459 TGA Q-50 @ Mfg-TGA).

Antioxidant capacity - tests ORAC (Oxygen Radical Absorbance Capacity)

The following techniques were used to determine the antioxidant activity of Isoespintanol compared with BHT: ORAC Fluorescein Hydrophilic (H-$ORAC_{FL}$), ORAC Fluorescein Lipophilic (L-$ORAC_{FL}$), ORAC dichlorofluoresceindiacetate ($ORAC_{DCFD}$) and ORAC pyrogallol red ($ORAC_{RPG}$); the ORAC methodologies differ in the target molecule.

H-ORAC$_{FL}$

The method was described in the beginning by Prior et al. (2005) and is currently carried out with modifications described as follows: A volume of 30 µL of the sample was added to 2,899 µL of PBS (75 mM), 21 µL fluorescein 1×10^{-2} M in PBS (75 mM) and 50 µL of AAPH 0.6 M in PBS (75 mM). Temperature and pH were controlled at 37°C and 7.4 respectively. Readings were performed at λ excitation of 493 nm and 10 slit; λ emission of 515 nm and emission slit 15, runs with attenuator 1%. The protective effect of the antioxidant is calculated using the differences in areas under the curve of decay of fluorescein in the absence and presence of sample, and compared against the primary standard Trolox. Results were expressed as µmol Trolox equivalents/100 grams of sample according to the following equation.

$$\mathrm{ORAC} = \frac{(\mathrm{AUC}-\mathrm{AUC}^0)}{(\mathrm{AUC}_{\mathrm{Trolox}-\mathrm{AUC}^0})}\mathrm{f[Trolox]} \quad (1)$$

Where AUC is the area under the curve, in the presence of the sample AUC° is the area under the curve for the control (no sample), AUC Trolox is the area under the curve using Trolox, f is the dilution factor of the sample assessed (Prior et al., 2005).

L-ORAC$_F$

Assay is carried out as explained in the previous section. However, the sample preparation is performed according to the protocol reported by Rautenbach and Venter (2010) described as follows: 0.5 g of sample were dissolved in 4 mL of hexane (lipophilic solution), this solution is mixed with 10 mL of a solution of water: acetone (1:1, v / v) with 7% b-cyclodextrin (micellar agent).

A modified $ORAC_{DCFH}$ method described by Martin-Romero et al. (2008), was used and described as follows. The nonfluorescent probe 2,7 dichlorodihydrofluorescein (DCFH) reacts with reactive oxygen species (ERO's) generated by the azo compound APPH (2,2'-Azobis(2-amidinopropane) dihydrochloride) in an aqueous medium to form 2.7 dichlorofluorescein (DCF), a fluorescent molecule. The compound tested captures free radicals and reduces the fluorescence emitted by decreasing DCF formed. To carry out the reaction; 50 µL of a solution of AAPH 0.3 M were mixed with 50 µL of 2,7-dichlorofluorescein diacetate 2.4 mM, 2850 µL of phosphate buffer - 75 mM pH 7.4 and 50 µL of the sample at 37°C. The intensity of the fluorescence emitted during the first 10 minutes was read (λ emission 490 nm and λ excitation 530 with 10 nm slit emission/excitation). The protective effect of the antioxidant is calculated using the increase in fluorescence in the presence and absence of the compound assessed, and compared with the calibration curve of the primary standard Trolox. Results were expressed as µmol Trolox equivalents/100 g of sample.

The $ORAC_{RPG}$ method described by Lopez-Alarcon and Lissi (2006) was used with the following modifications: Pyrogallol Red (PGR) reacts with reactive oxygen species (ERO's) generated by the azo compound APPH (2,2'-Azobis(2-amidinopropane) dihydrochloride) in an aqueous medium and forms a colorless oxidized product. The compound tested traps free radicals and prevents the reduction of the absorbance due to decreased oxidized RPG formed. To perform the reaction, 10 µL of an aqueous solution of AAPH 0.3 M were mixed with 50 µL of an aqueous solution of Pyrogallol Red (PGR) (5 × 10^{-5} M), 30 µL of sample and 210 µL buffer phosphate (75 mM, pH 7.4), the mixture was incubated for 4000 s and the absorbance was monitored during this time at 540 nm. For the spectrophotometric readings, a Thermo Scientific Multiskan Spectrophotometer UV-VIS Spectrum was used. The protective effect of the antioxidant is calculated using the percentage of inhibition of decay in absorbance when the reaction contains the sample; this value is compared against a primary standard (Trolox). Results were expressed as µmol Trolox equivalents/100 g of sample.

Isoespintanol cytotoxicity

MTT method described by Mosmann (Mosmann, 1983) with murine

Table 1. Fatty acid composition of palm olein

Time (s)	Fatty acid	Carbons: Unsaturation	% Fatty acid
19.4	Palmitic Acid	16: 0	5.91
21.1	Methyl pentadecanoate	15: 0	5.86
22.5	Oleic Acid	18: $1^{\Delta 9}$	31.94
24.9	Stearic Acid	18: 0	10.01
25.3	vaccenic acid	18: $1^{\Delta 11}$	20.00
26.2	Linoleic acid (Omega 6)	18: $2^{\Delta 9,12}$	0.82
27.1	11 - eicosenoic	20: $1^{\Delta 11}$	5.50
29.9	eicosanoic	20: 0	1.04
30.3	docosanoic	22: 0	8.43
31.3	15-tetracosenoic	24: $1^{\Delta 9}$	7.22
35.9	tetradecanoic	24: 0	0.25
36.7	hexacosanoic	26: 0	0.52

macrophages RAW 264.7 was used to measure the cytotoxic effect of Isoespintanol. This assay is based on reduction of 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide (MTT) to Formazan in a reaction catalyzed by mitochondrial succinyl dehydrogenase.

The cells stimulated with LPS (1 µg/mL) were mixed with Isoespintanol (100 µM) and incubated in microplate 96-well at 37°C for 24 h in atmosphere with 5% CO_2 in DMEM medium containing 2 mM L-glutamine (Gibco), 100 U/ml penicillin (Gibco), 100 µg/ml streptomycin without fetal bovine serum. The control contained 0.5% DMSO. After incubation, the culture medium was removed and 100 µL of MTT (0.5 mg/ml) were added. When deposits appeared blue, all liquid was removed and 100 µL of DMSO was added to dissolve Formazan crystals. Absorbance at 490 nm was measured (Spectrophotometer Multiskan Spectrum UV-VIS (Thermo Scientific)). The number of living cells is proportional to the amount of Formazan produced. The increase in absorbance indicates cell viability and this was determined using the following equation:

$$\% \text{ Viability} = \frac{A_p}{A_c} \text{ x } 100 \quad (2)$$

A_p = Arithmetic mean of the absorbance of the test sample; A_c = Arithmetic mean absorbance of control.

Statistical analysis

All the antioxidants experiments were performed four times. Data are presented as the mean ± the standard error (SE) and were processed by one-way analysis of variance (ANOVA). Multiple comparisons of the means were performed using Duncan's New Multiple Range Test ($p < 0.05$). All of the statistical analysis was performed using the Statgraphics Centurion V statistical package for Windows. For testing cytotoxic effect, data was generated with an experimental design of randomized complete block, taking sample from each donor as blocking factor. The analysis of variance and the corresponding mean tests (Duncan) were performed using the statistical software Statgraphics Plus, version 5.0.

RESULTS AND DISCUSSION

Degree of unsaturation is a factor that affects directly the kinetics of degradation of oils (Guillén and Cabo, 2002; Choe and Min, 2006; García-Moreno et al., 2013). Oils with a high content of unsaturated fatty acids, especially polyunsaturated, are more susceptible to oxidation. Palm olein has 12 main fatty acids, with a ratio of unsaturated/saturated (I/S) of 0.71 and with chains of 15-26 carbons. Table 1 summarizes the percentage of fatty acids. The oleic acid (ω9) (31.9%), vaccenic (20%), 15-tetracosenoic (7.22%), stearic (10.01%) and docosanoic (8.43%) percentages are highlighted; also, a low amount of polyunsaturated fatty acids was found (less than 1%).

Palm olein has a high content of monounsaturated (64.66%) and saturated (32.02%) fatty acids and a low content of polyunsaturated fatty acids (0.82%). This gives high stability against oxidative processes as frying, as it is recommended for use in domestic and industrial level (Mozzon et al., 2013).

The ratio of monounsaturated/saturated fatty acids is an indicator of oxidative stability in oils; however, this does not completely define their behavior in the frying process where high temperatures are used for longer periods. In addition, the presence of dissolved oxygen in the oil and water from the food causes a series of reactions where different oxidized compounds are produced. Hence, the addition of antioxidants becomes an important need in the food industry.

Figure 1 shows the formation of hydroperoxides in the palm olein supplemented with Isoespintanol (IE200) and BHT (BHT200) and without antioxidants. The concentration of hydroperoxides increases with time after 60 h of heating and is less in the presence of antioxidants. However, Isoespintanol delays hydroperoxides production in greater proportion than BHT.

The Active Oxygen Method (AOM) (Method Cd 12-57) was used to monitor the oxidative state of palm olein, which determines the time that the oil takes to reach a peroxide value (PV) of 100 meq O_2/kg oil (induction time; IT). Induction times were 89.68, 93.95 and 105.09 h for the control, BHT200 and IE 200, respectively. This shows

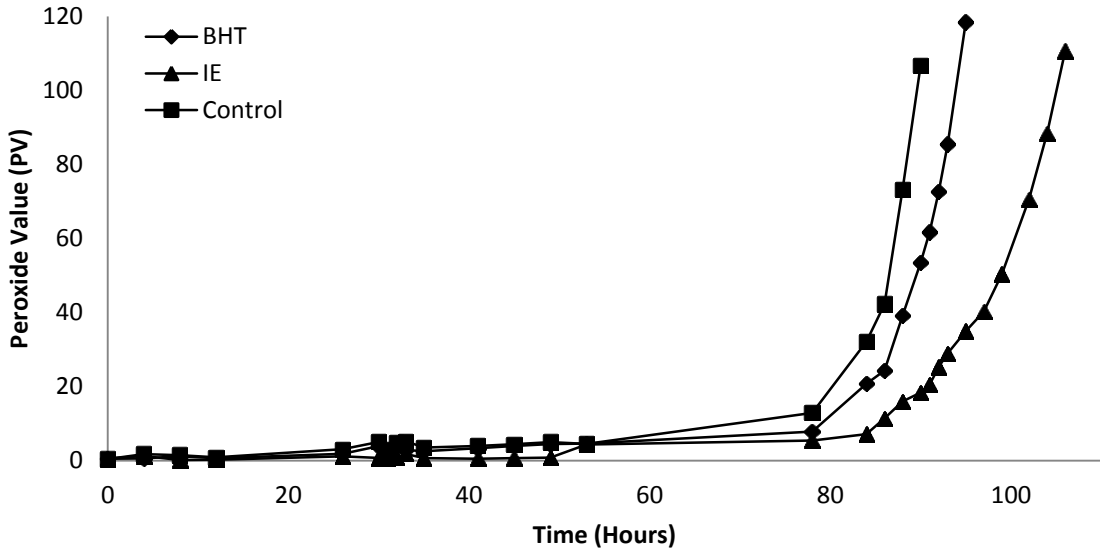

Figure 1. Formation of peroxides in the Palm Olein supplemented with (▲) Isoespintanol 200 mg / L, (○) BHT 200 mg / L and (♦) Control without antioxidant.

that in the presence of Isoespintanol, the induction time is reduced in 17.2% compared to the control, while the use of BHT only implies a reduction of 4.2%. These findings are attributed to the higher capacity of Isoespintanol to trap peroxyl radicals (ROO•) generated in the oxidative process compared to BHT. Previous studies showed that the effectiveness of rosemary extracts in the oxidative stabilization of lipids is greater than commercial mixtures of synthetic antioxidants such as Butyl Hydroxytoluene (BHT), Butyl Hydroxyanisole (BHA) and Propyl Gallate (PG) (Upadhyay et al., 2006; Piedrahita et al., 2015).

Antioxidants are used to improve the oxidative stability of fats and oils that are used at temperatures above 110°C and under aeration for extended periods (Santos et al., 2012; Upadhyay and Mishra 2015b). However, some natural and synthetic antioxidants decompose or volatilize below 110°C, which leads to a decrease in their protective ability. We used thermogravimetry to assess the thermal stability of the studied antioxidants. Dynamic thermogravimetric curves indicated that mass loss is initiated at temperatures below 100°C for both, Isoespintanol and BHT (Figure 2). However, Isoespintanol exhibits a higher overall thermal resistance compared to BHT. The complete evaporation of Isoespintanol occurs at 158.6°C unlike BHT that is volatilized completely at 144.8°C.

The volatility and instability of synthetic antioxidants at high temperatures are known issues in the industry which have prompted the search for natural alternatives. The results suggest that BHT provides lower levels of protection compared to Isoespintanol due to its volatility. Similar results were obtained by Santos et al. (2012), who found that natural antioxidants display higher thermal resistance than synthetic antioxidants, a phenomenon attributed to the difference on chemical bonds type of the natural antioxidants. Although BHT and Isoespintanol have similar molecular masses (220.35 and 210 g/mol, respectively) (Figure 3), the atomic bonding pattern is different. For instance, the type of substitution in the phenolic nucleus makes them energetically different.

H-ORACFL, L-ORACFL, ORACDCFH and ORACRPG values for Isoespintanol and BHT are presented in Figure 4. The results show that the Isoespintanol has substantially greater antioxidant capacity than BHT under all experimental scenarios, which could explain its ability to oxidatively stabilize palm olein under accelerated conditions of oxidation.

ORAC assays using fluorescein (FL) have limitations because they are influenced more by stoichiometry than by reactive factors (Lopez-Alarcon and Lissi, 2006). An additional methodology has been proposed to determine the ORAC value based on pyrogallol red discoloration (RPG), which provides values that are exclusively dependent on the reactivity of the tested compounds rather than the amount; thereof, this concept is important considering the function of an antioxidant. This difference is due to the high reactivity of RPG and the compound concentrations used in the experiments compared to low levels of ORAC probes with fluorescein. One way to check the quantity and the quality of the test compound is to determine $ORAC_{FL}$ and $ORAC_{RPG}$. The results show that Isoespintanol exhibits the perfect setting, between quantity and quality, revealing greater results in both methods compared to BHT. Besides, these results are positive considering that the National Institutes of Health considers that BHT is a promoter of carcinogenesis in humans, so its use as a food additive is not recommended. BHT was toxic for mouse lung cells

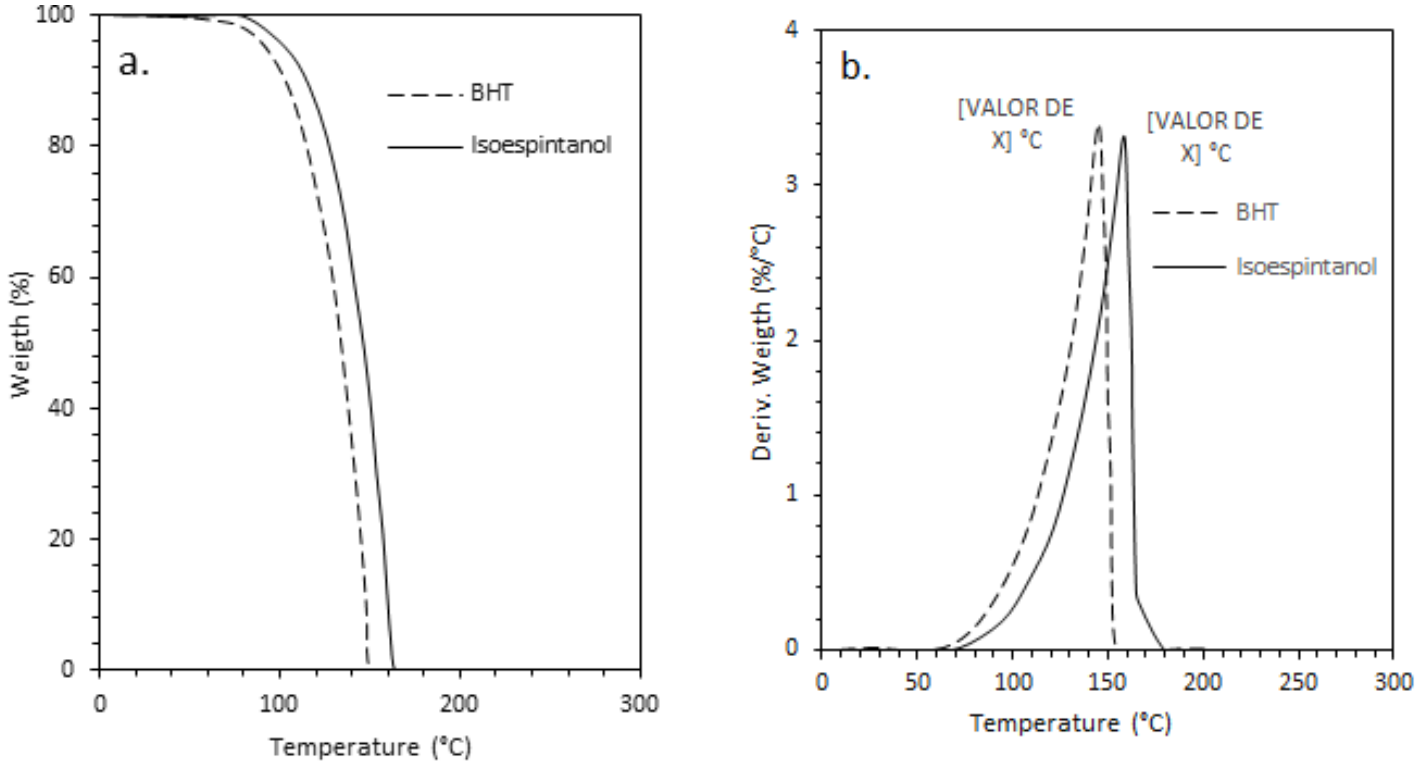

Figure 2. Curves (a) TG and (b) DTG Isoespintanol and BHT.

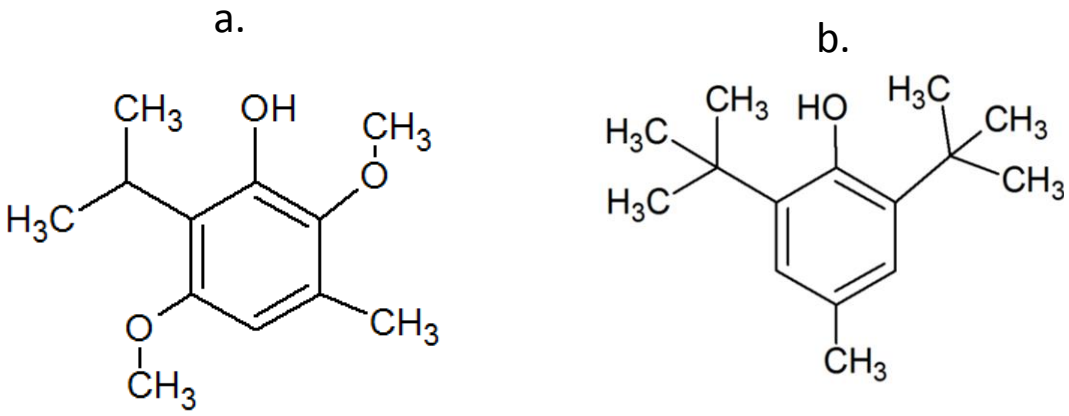

Figure 3. Chemical structure of antioxidants (a) Isoespintanol (IE) and (b) Butylated hydroxytoluene (BHT).

(necrosis type I pneumocytes and endothelial cells), and there is also evidence of hepatotoxicity. BHT also has adverse effects on reproduction and increases the effect of certain carcinogens such as urethane and N-fluorenilacetamida.

This study revealed that Isoespintanol at 100 µM does not have significant cytotoxic effects against murine macrophages (RAW 264.7) (Figure 5). The results are positive considering the possible use of Isoespintanol as a food additive.

Although there is not a clear mechanism that describes the way that the mono- and phenols act on cell protection, we can suggest that due to the high capacity of Isoespintanol to trap oxygen radicals, as evidenced by their ORAC values, the harmful effects of reactive species present in the cell are counteracted (Duthie et al., 2003). Previous studies found that thymol and carvacrol, structural analogues of Isoespintanol, showed protective

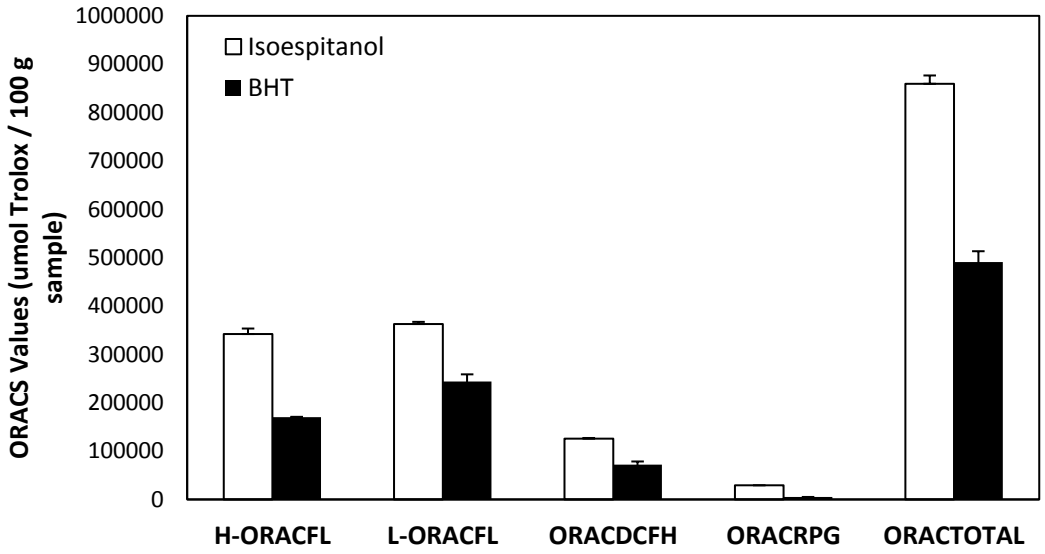

Figure 4. Oxygen Radical Antioxidant Capacity (ORAC) for Isoespintanol and BHA.

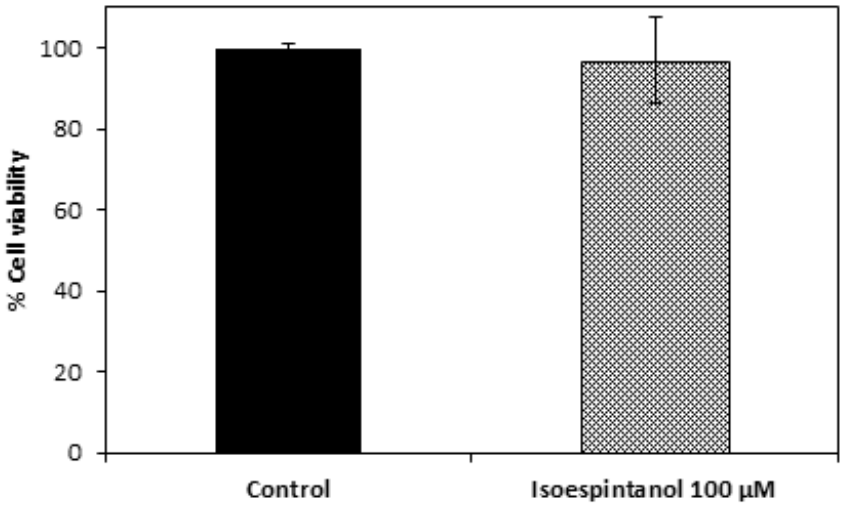

Figure 5. Evaluation by MMT of Isoespintanol Cytotoxicity in murine macrophages (RAW 264.7)

effects on DNA from human lymphocytes at low concentrations (0.5 to 50 mM). However, pro-oxidant effects were observed at concentrations greater than 100 µM (Aydin et al., 2005). Numerous studies have shown that some polyphenolic antioxidants such as quercetin, rutin or epicatechingallate have genotoxic effects at high concentrations (Perez, 2003; Johnson and Loo, 2000) contrary to the findings of this study in respect of Isoespintanol, which represents a great advantage.

Conclusions

Palm olein has a high content of monounsaturated and saturated fatty acids (96.68%) and a negligible content of polyunsaturated fatty acids (0.82%), reflecting stability against oxidative phenomena. However, processes such as frying are adverse and accelerate deterioration reactions. For this reason, the addition of antioxidants becomes an important step in the food industry. The results revealed that hydroperoxides production under oxidation accelerated conditions in palm olein is 39% slower in the presence of Isoespintanol (200 mg/L) and only 10% slower in the presence of BHT. These results are attributed to the superior antioxidant capacity observed for Isoespintanol and demonstrate the ability of this molecule to extend the conservation process in oils subjected to high temperatures. The dynamic thermo-

gravimetric curves for Isoespintanol and BHT indicated that the mass loss for both antioxidants is initiated at temperatures below 100°C, however, Isoespintanol exhibited higher overall thermal resistance than BHT, since complete decomposition took place at 157 and 144, respectively. Finally, cytotoxicity tests with 100 µM Isoespintanol against murine RAW 264.7 macrophages showed no effect on cell viability, indicating their possible use as a safe food additive.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Effects of *Commiphora swynnertonii* on weight and plasma cholesterol levels in *Rattus rattus*

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Experimental studies that aimed to determine the effects of crude aqueous resin extracts of the *Commiphora swynnertonii* on plasma cholesterol levels and weight changes were carried out in rats (*Rattus rattus).* A total of 24 experimental rats divided into four groups with equal sample size (n=6) were used. Group one (G1) served as negative control that received 0.5ml of distilled water (0 mg/kg) orally. Groups 2 (G2), 3 (G3) and 4 (G4) received 50, 100, and 200 mg/kg body weight orally on daily basis for 21 days respectively. Results revealed a significant decrease ($P < 0.05$) in the body weight and on cholestrol levels between the treated and the control groups in a dose dependent manner ($R^2 = 0.89$). *Commiphora swynnertonii* resin lowered cholesterol level by 54, 76 and 79% and weight changes by 18, 31 and 23% for the exposed rats at concentrations of 50, 100 and 200 mg/kg BW respectively. The rats were able to tolerate resin at concentrations lower than 100 mg/kg BW. At higher (>100 mg/kg) doses, few rats showed signs of illness including diarrhoea and finally death. Based on these results, *C. swynnertonii* has a potential to serve as an anti-cholesterol agent with body weight lowering properties.

Key words: Oltemwai, Cardiovascular diseases, rats, Tanzania, blood chemistry.

INTRODUCTION

Cardiovascular diseases associated with increased levels of blood cholesterol particularly the low density lipoprotein cholesterol are increasingly becoming a worldwide health challenges that lead to human deaths. Such disorders are treated, controlled and prevented using different methods including medicinal plants and herbs (Kochhar et al., 2006). Several *Commiphora* species have been studied to assess their activities as anti-lipidemic, anti-cholesterolaemic and anti-atherosclerotic, thus reducing serum cholesterol concentrations without causing any detrimental side effects (Adebayo et al., 2006). In various studies *C. mukul* has been claimed to decrease atherosclerosis and to lower serum cholesterol by 27% and triglycerides by 31% (Singh et al., 1994). Guggulipid, a product from *C. mukul*, increased the high density lipoprotein cholesterol (HDL) (Singh et al., 1994). It exerts its activity by lowering the level of cholesterol by reducing total cholesterol, low density lipoprotein cholesterol (LDL-c), and very low density lipoprotein (VLDL-c) cholesterol at the same time

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elevating the high density lipoprotein cholesterol (HDL-c) (Adebayo et al., 2006). *Commiphora mukul* contains guggulsterone, a compound that act by antagonizing the effect of the nuclear farnesoid × receptor (F×R) (Huang et al., 2003; Adebayo et al*.,* 2006). This receptor is identified as a bile acid receptor and biological sensor for the regulation of bile acid biosynthesis (Huang et al., 2003). Farnesoid × receptor regulates cholesterol metabolism in two ways: (i) chenodeoxycholic acid (CDCA), a primary bile acid, binds directly to and activates F×R, which then mediates the feedback suppression by bile acids of cholesterol 7 alpha-hydroxylase (CYP7A1), the rate-limiting enzyme in bile acid biosynthesis from cholesterol. (ii) Farnesoid × receptor participates in the activation of intestinal bile acid binding protein (IBABP), which is involved in the enterohepatic circulation of bile acids. Thus F×R constitutes a potential therapeutic target that can be modulated to enhance the removal of cholesterol from the body (Tu et al*.,* 2000). The other mechanism reported by Wang et al. (2004), is through the presence of ketosteroid , an active compound of *C. mukul* which acts by stimulating the thyroid gland and has also found to reverse the decrease of catecholamine and dopamine – p- decarboxylase activity that is involved with anticholesterolaemia (Wang et al*.,* 2004). This is done by improving the liver's ability to process, metabolize and excrete cholesterol and improving thyroid function by increasing T_3 and T_4 conversion (Wang et al*.,* 2004). Ethanolic leaf extract of *C. africana* and *C. myrrha* were also shown to exhibit hypolipidaemic activity in experimental rats (Adebayo et al*.,* 2006). Though several studies reported anti-cholesteremic effect of several *Commiphora* spp, relatively little has been investigated in clinical trials on the effect of *C. swynnertonii*. Therefore, this study aimed at evaluating the effect of *C. swynnertonii* on the weight gain and plasma cholesterol levels using rat's model.

MATERIALS AND METHODS

Plant materials

Commiphora swynnertonii plant materials were collected from Simanjiro district in Manyara Region. The plant was identified by a botanist as *Commiphora Swynnertonii* from the family Burseraceae. A voucher specimen (reference number CK 6489) was prepared and preserved at Tanzania National Herbarium, in Arusha (Kayombo, 2009 Personal communication). Tanzania and transferred to Sokoine University of Agriculture (SUA) for processing and use in the experiments. One hundred grams (100 g) of the resin was brewed in 750 ml distilled water and thereafter allowed to stand for 30 minutes. The mixture was filtered using filter paper No 3 and stored in a clean bottle before administered to rats. Twenty mls aliquots of the decoction were evaporated to dryness using an electric heater at 60 to 70°C. The residues were used to determine the concentrations of C. *swynnertonii* extracts which were administered to different groups of experimental rats (Edem et al., 2009).

Sample size determination for experiment animals

The sample size for the experimental animals was determined according to Kirkwood and Sterne (2003).

Experimental design

Twenty four white albino rats (*Rattus rattus*) of seven months old of both sexes weighing 125.7 to 180 g were used in this study. The rats were obtained from the small animal unit at Sokoine University of Agriculture Faculty of Veterinary Medicine. Once in the experimental house, all rats were assessed for signs of diseases. They were caged and maintained on grower mash and drinking water *ad libitum*. The rats were left for three weeks to acclimatize with experimental environment. Following acclimatization, they were weighed, tagged and randomly assigned into four experimental groups of six rats each.

Treatment allocation

The animals were randomly assigned into four groups of six rats (n=6) each. All rats were housed in well-ventilated cages. Groups; G2 - G4 rats were given different doses of aqueous resin extract orally for 21 days consecutive days. G1 remained as negative control that received distilled water only. Blood samples were collected for evaluation of haematological and biochemical parameters.

Preparation of plasma and analysis

At baseline, the body weights of the rats were recorded. About 3 mls of blood samples for plasma preparation were collected from the tail artery using sterile syringes and blood samples were stored in EDTA sterile vacutainer tubes. The blood samples were thereafter centrifuged at 1300 × g for 5 minutes using a bench top centrifuge model to obtain the plasma. The plasma was stored in a refrigerator for analysis of biochemical parameters.

Cholesterol analysis

Total plasma cholesterol level was determined according to Erba Mannheim protocol (Trinders, 1969; Erba, 2010). All analyses on plasma were completed within 24 h after blood collection as recommended (Goji et al., 2009).

Statistical analysis

The data obtained were compiled, coded and analysed using Microsoft excel statistical package (2007) and SAS (Statistical Analysis System) program (Version 8.3) for Window^R. Results from experimental Tests for differences between the means were done and compared by Duncan's Multiple Range Test (DMRTS) at ($p < 0.05$).

RESULTS

Signs of toxicity

Animals used in the study in the negative control group

Table 1. Grouping and treatment allocation

Group (n =6)	Treatment given	Dose
GI	Distilled water	0.5 ml
G2	Resin	50 mg/kg
G3	Resin	100 mg/kg
G4	Resin	200 mg/kg

Table 2. Weight (g) changes following resin administration in rats during the study

Time (weeks)	Dose rate in mg/kg body weight			
	0	50	100	200
0	159.53±6.76[b]	137.13±6.76[b]	169.75±6.76[b]	152.23±6.76[ab]
1	163.63±7.50[b]	114.50±7.50[a]	129.07±7.50[a]	117.70±7.50[a]
2	164.67±6.27[b]	115.65±6.27[a]	118.63±7.14[a]	119.54±8.17[a]
3	165.88±6.36[b]	112.18±6.36[a]	117.08±7.24[a]	117.70±8.29[a]
Percentage (%) change	+4	-18	-31	-23

[a] Means ± SEM based on Weight, [abc] Means in row wise with different superscript are significance different at p< 0.05. *Percentage change refer to positive (+) increase and negative (-) decrease in cholesterol levels.

were apparently in good health condition, as they remained alert, consumed food (growers mash) and water freely and exhibited normal weight increase over time. Depression and diarrhoea were observed in all groups treated with resin extract. The symptoms of toxicity observed with extract administration were dose dependent. Three to seven days after administration of extract all rats in the various groups were very weak. Signs observed before death included loss of appetite, diarrhoea, blindness and coma.

Mortality rate

Mortality was observed in one rat from G3 and two rats from G4 by day eight following an oral administration of resin extract. The rats treated in G2 and G3, were depressed and less active compared to the rats in the other groups during the day.

Changes in body weight

Animals in control group (G1) maintained their weight gain throughout the experimental period (Table 1). The body weights of rats receiving the oral dose of *C. swynnertonii* extract decreased significantly in a time and dose dependent manner. Rats receiving 50 mg/kg (G2) body weight lost about 16.5% of their average body weight during the first week, 15.7% during the 2nd and 18.2% during the 3rd week. Rats receiving 100 mg (G3) of *C. swynnertonii* extract the weight losses were 24.0% (week 1), 30.0% (week 2) and 31.0% (week 3). Likewise, for rats receiving 200 mg of *C. swynnertonii* extract, weight losses were 23.0% (week 1), 21.5% (week 2) and 23.0% (week 3). Overall, animals receiving the *C. swynnertonii* extract lost weight in the range 18 to 31% (Figures 1 and 2).

Changes in the levels of plasma cholesterol

The plasma cholesterol levels of rats before and after administration of *C. swynnertonii* extract at three different doses are shown in Table 2. There was a significant ($P < 0.05$) decrease in plasma cholesterol levels in all rats in the treated groups in a dose dependent manner (R^2-0.89: P=0.03). The body weight for rats in G2, decreased significantly ($P < 0.05$) from week 2 of treatment with resin extract. The plasma cholesterol levels for rats in G2, decreased by 54 % while in G3 and G4, the decrease was by 76 and 79 % respectively. There was a significant positive correlation between weight and cholesterol at different concentrations of *C. swynnertonii* (Figure 3). The correlations were r = 0.432 and P=0.035 for control group, and for exposed groups, the values were r = 0.432 and P=0.009; r = 0.712 and P=0.000 and r = 0.487 and P =0.282; for G1, G2 and G3 treated rats, respectively.

DISCUSSION

The present study has demonstrated the effect of aqueous crude resin extracts from *C. swynnertonii* on plasma total cholesterol in rats. Results revealed that rats in a negative control group were clinically healthy and

Table 3. Changes in the cholesterol level (mmol/L) following administration of resin orally.

Time (weeks)	Dose rate in mg/kg body weight			
	0	50	100	200
0	5.65 ± 0.46[a]	5.29 ± 0.46 [a]	4.30 ± 0.46 [a]	5.49 ± 0.46 [a]
1	5.71 ± 0.37[b]	3.94 ± 0.37[a]	3.19 ± 0.37[a]	3.89 ± 0.37[a]
2	5.50 ± 0.42[d]	3.25 ± 0.42[bc]	1.77 ± 0.48[b]	2.20 ± 0.54[ab]
3	5.78 ± 0.41[c]	2.42 ± 0.41[b]	1.03 ± 0.47[b]	1.13 ± 0.54[ab]
Percentage (%) change	+2.3	-54	-76	-79

*abc Means in row with different superscript are significance different at p< 0.05. *Percentage change refer to positive (+) increase and negative (-) decrease in cholesterol levels.

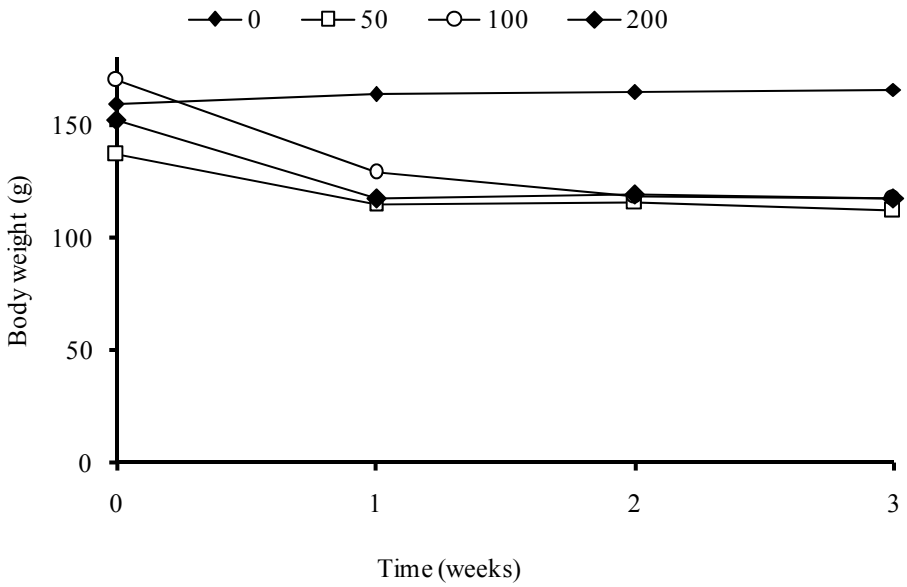

Figure 1. Weight changes over time following oral administration of resin extract in the rats.

their body weights increased significantly all along the study period. Diarrhoea and deaths observed in few treated rats implies that prolong administration of resin extract could be lethal and toxic to the rats. Similar observations were reported in previous studies (Ruitang, 2007) in which extracts from *C. mukul* lead to gastrointestinal discomfort such as loose faeces, mild nausea, and hiccup. The reduced weight gain in a dose dependent manner could be due to reduced feed consumption since the animals were depressed, inactive and with lost appetite. Studies done by Bakari et al. (2012a,b) and Scott, (2005) reported the association between the reduction in weight and the plasma cholesterol and glucose levels through stimulation of thyroid hormone (T3 and T4) function thus interfering with basal metabolic rate leading to loss of body weight (Scott, 2005). Thyroid hormones (T3), stimulates the production of RNA polymerase I and II and, therefore, increases the rate of protein synthesis, potentiates the effects of the β-adrenergic receptors on the metabolism of glucose (Guyton and John, 2006). Therefore, it increases the rate of glycogen breakdown and glucose synthesis in gluconeogenesis. Also stimulates the breakdown of cholesterol and increases the number of LDL receptors, thereby increasing the rate of lipolysis (Guyton and John, 2006).

In the current study, administration of resin from *C. swynnertonii* significantly lowered blood cholesterol. This finding is significant in managing conditions such as coronary heart and atherosclerosis. This was also demonstrated by Helal et al. (2005 and 2006); Ojha et al. (2008); Goji et al. (2009) whereby the application of extracts from *C. myrrha, C. africana* and *C. mukul* using rats lowered the blood cholesterol and glucose while maintaining the myocardial membrane integrity thus preventing myocardial impairment. The effect of *C. swynnertonii* may also base on its ability to bind bile acids in the intestinal lumen and to interrupt the entero-hepatic

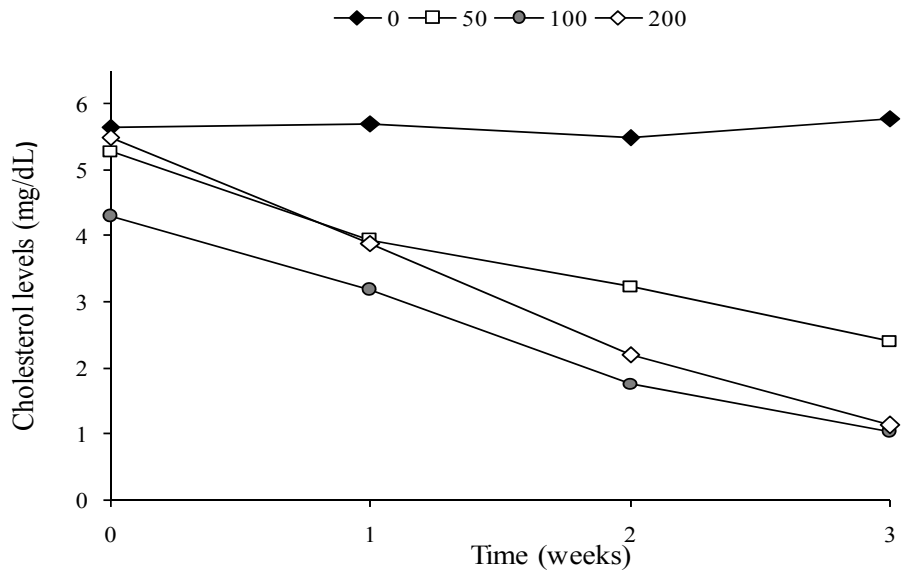

Figure 2. Cholesterol level changes over time following oral administration of resin extract in the rats.

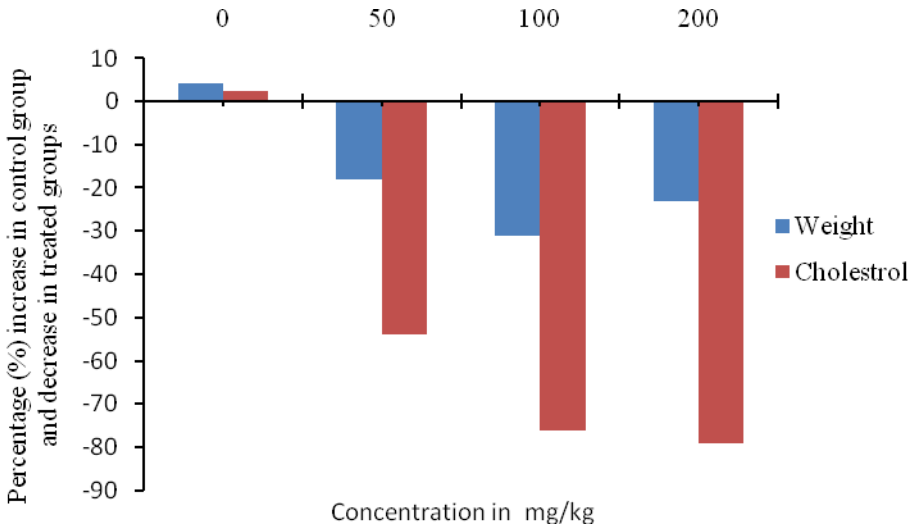

Figure 3. Percentage change in weight and cholesterol in the rats during the experiment

circulation of bile acids, leading to increased excretion of steroids (Singh et al., 1994). Depletion of the hepatic sterol pool may cause compensatory increases in cholesterol biosynthesis, which may cause increased catabolism of LDL particles from plasma (Singh et al., 1994). *Commiphora mukul* contains guggulsterone, a compound which act by antagonizing the effect of the nuclear farnesoid X receptor (FXR) (Huang et al., 2003; Adebayo et al., 2006). This receptor is identified as a bile acid receptor and biological sensor for the regulation of bile acid biosynthesis (Huang et al., 2003). Thus, according to Tu et al. (2000), FXR constitutes a potential therapeutic target that can be modulated to enhance the removal of cholesterol from the body. Another possible mechanism is through the presence of ketosteroid, an active compound of *C. mukul* which acts by stimulating the thyroid gland and has also found to increase the activity of catecholamine and dopamine –p-decarboxylase that are involved in lowering plasma cholesterol (Wang et al., 2004). Some secondary plant metabolites such as coumarin, flavonoid, terpenoid, arginine and glutamic acids have been shown to confer

cholesterol lowering effects in various experimental animal models (Akah and Okafor, 1992; Marles and Farnsworth, 1995). The significant anticholesterol observed in the current study can therefore be explained by the fact that *C. swynnertonii* contain remarkable amounts of saponins terpenoids and flavanoids. Saponins are glycoside components often referred to as "natural detergent" because of their foamy nature (Edeoga et al., 2005) and are reported to posses health benefits such as cholesterol lowering activity (Edeoga et al., 2005). The observed reduction in body weight of resin-treated rats is well connected with the observed levels of plasma cholesterol. High carbohydrate (glucose) and cholesterol intake are known to increase body fats, hence increased body weight and eventually obesity (Scott, 2005).

In conclusion, this study has demonstrated that rats were able to tolerate oral administration of *C. swynnertonii* resin at doses lower than 100 mg/kg bodyweight. Administration of higher doses had negative effects thus causing diarrhoea and genera body weakness in rats. The observed anti-cholesteremic and body weight lowering effect warrants further studies on potentials of this plant.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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