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Effect of rooibos (*Aspalathus linearis*) on the female rat reproductive tract and liver and kidney functions in vivo

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ABSTRACT

Looking for an animal model to investigate the potential estrogenic effect of indigenous rooibos (*Aspalathus linearis*) on females with functional ovaries, we used ovary-intact mature female Wistar rats. Rats were exposed to unfermented (Ur) or fermented (Fr) rooibos (2% and 5%, respectively) as sole source of drinking for 21 days. Unfermented rooibos (5%) significantly increased relative uterus weight while fermented tea (5%) caused a significant decrease in relative ovary weight. Although statistically not significant, all rooibos treatments caused a trend to increased serum FSH but decreased LH level. Histological sections revealed no adverse changes in the ovary, uterus, kidney and liver of all treated groups. Endometrium thickness was enhanced, whereas myometrium was unchanged. No signs of inflammation were observed. In serum, ferric reducing antioxidant power (FRAP) did not change whereas 2% Ur caused a significant drop in ALT activity. Fermented rooibos, however, induced an increase in AST activity ($P < 0.01$; 5%) and creatinine level ($P < 0.05$; 5%). No effects on total body weight gain or relative kidney weight were observed while relative liver weight was significantly increased by Ur. Antioxidant activities of CAT, SOD and GSH and MDA levels in the kidney and liver remained unchanged ($P > 0.05$) while liver CAT activity was significantly improved by 5% Ur. In conclusion, *Aspalathus linearis* might exhibit some estrogenic property and may thus be beneficial to boost female fertility. Rooibos is able to maintain antioxidant levels in the serum, kidney and liver. No major adverse in vivo effects could be observed. However, it seems that especially fermented rooibos may affect kidney and liver tissue as there is a significant increase in AST and creatinine values and a trend to dose-dependent rises in ALT activity. Though, the potential clinical relevance needs further investigations.

1. Introduction

Aspalathus linearis, commonly known as rooibos, grows naturally in the Cederberg area in the Western Cape Province of South Africa and is known for its commercial use as herbal tea or tisane (McKay and Blumberg, 2007). It is manufactured in two different forms: unfermented and fermented rooibos, with the previous containing higher level of antioxidants (von Gadow and Joubert, 1997). Antioxidants scavenge and suppress the formation of reactive oxygen species (ROS) and lipid peroxidation (Sikka, 1996). A physiological level of ROS is required to play a regulatory role in folliculogenesis, oocyte maturation, corpus luteum and uterine function, embryogenesis, embryogenic implantation and fetoplacental development through various signaling transduction pathways (Agarwal et al., 2008). However, elevated ROS concentrations, depleted total antioxidant activity or both can result in oxidative stress such as damage to cellular lipids, proteins and DNA (Pasqualotto et al., 2008).

Various types of phytoestrogens are present in plants (Michel et al., 2013). They are structurally and functionally similar to isoflavones (17β-oestradiol) or synthetic estrogens such as diethylstilbestrol (lignins), and exert their biological activity by mimicking the action of endogenous estrogens, acting as estrogen antagonists, altering the pattern of synthesis and metabolism of endogenous hormone or modifying hormone receptor values (Whitten et al., 1995; Sonnenschein and Soto, 1998). Phytoestrogens were shown to exhibit uterotrophic effects which may involve retention of uterine fluid in the lumen and hyperplasia of the endometrium (El Samannoudy et al., 1980; Whitten et al., 1992). Several rooibos compounds exhibit estrogenic activities; of its main constituents, nothofagin showed phytoestrogenic activity comparable to genistein, whereas aspalathin exerted only half the activity of nothofagin (Shimamura et al., 2006).

As rooibos enjoys increasing popularity as refreshment and health tea, the aim of this study was to investigate the phytoestrogenic and antioxidant effects of *Aspalathus linearis* on the sexually mature female reproductive system, together with exploring its safety based on its effect on liver and kidney functions.

Abbreviations: ALT, alanine transaminase; AST, aspartate transaminase; CAT, catalase; Fr, fermented rooibos; FRAP, ferric reducing antioxidant power; GSH, glutathione; MDA, malondialdehyde; SOD, superoxide dismutase; Ur, unfermented rooibos.

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Table 1
Average daily intake (ADI) parameters of female rats exposed to *Aspalathus linearis* for 21 days.

	Tea extract		Soluble solids [SS]		Total polyphenols		Flavonol		Flavanol	
	ADI [mL]	ADI [mL/100 g BW]	[mg/mL]	ADI [mg/100 g BW]	[% SS]	ADI [mg/100 g BW]	[% SS]	ADI [mg/100 g BW]	[% SS]	ADI [mg/100 g BW]
C	32.36 ± 0.02^{a}	14.53 ± 1.23^{a}	–	–	–	–	–	–	–	–
2% Fr	28.83 ± 0.11^{b}	13.24 ± 1.46^{b}	2.05 ± 0.55^{a}	27.1 ± 3.00^{a}	40.10 ± 2.41^{a}	10.88 ± 0.65^{a}	2.02 ± 0.35^{a}	0.55 ± 0.09^{bd}	3.78 ± 1.01^{ceg}	1.03 ± 0.27^{c}
5% Fr	27.65 ± 0.75^{b}	12.65 ± 1.30^{b}	5.69 ± 0.54^{b}	71.97 ± 7.41^{b}	33.47 ± 2.07^{b}	24.09 ± 1.49^{b}	0.49 ± 0.05^{b}	0.35 ± 0.04^{bgi}	3.21 ± 0.44^{dg}	2.31 ± 0.32^{de}
2% Ur	32.41 ± 0.10^{a}	13.05 ± 1.11^{b}	2.55 ± 0.60^{c}	33.28 ± 2.84^{c}	40.06 ± 4.69^{a}	13.33 ± 1.56^{c}	0.80 ± 0.16^{c}	0.27 ± 0.05^{acf}	8.82 ± 2.89^{aef}	2.94 ± 0.96^{ae}
5% Ur	24.14 ± 1.56^{b}	12.25 ± 1.36^{b}	7.10 ± 0.47^{d}	86.97 ± 9.64^{d}	32.16 ± 5.25^{b}	27.97 ± 4.57^{b}	0.30 ± 0.09^{d}	0.26 ± 0.08^{agi}	8.73 ± 0.85^{bef}	7.60 ± 0.74^{b}

Each value represents the mean ± SD of four different tea preparations measured in triplicate. Means in column followed by the same letters do not differ significantly and if letters differ, then $P < 0.05$. ADI = average fluid intake during 21 days [mL] / 100 * % SS; % SS = 100 / soluble solid of corresponding tea [mg/mL] * total polyphenols/flavonol/flavanol of corresponding tea [mg/mL]; BW = body weight.

2. Materials & methods

2.1. Chemicals

Dimethylsulfoxide (DMSO), ethanol, glacial acetic and hydrochloric acid, phosphoric acid, potassium dihydrogen phosphate, potassium dihydrogen orthophosphate, and sodium pyruvate were purchased from Merck Chemicals (Johannesburg, South Africa). Ammonium acetate, α-ketoglutarate, bovine serum albumin, (+)-catechin, creatinine, 4-(dimethylamino)-cinnamaldehyde (DMACA), 2,4-dinitrophenylhydrazine, Folin–Ciocalteu reagents, gallic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, iron(III) chloride, L-alanine, L-aspartic acid, methanol, p-formaldehyde, phosphate buffered saline, quercetin, sodium acetate, sodium carbonate (Na_2CO_3), and 2,4,5-tri(2-pyridyl)-S-triazine (TPTZ) were obtained from Sigma Aldrich (Seelze, Germany).

2.2. Plant material, extract preparation and analysis

Unfermented and fermented rooibos (*Aspalathus linearis*, Ur and Fr) were gifts from Rooibos Ltd (Clanwilliam, South Africa). Unfermented or fermented rooibos were infused in freshly boiled tap water for 5 min, to final concentrations of 2% and 5%, respectively (Opuwari and Monsees, 2014). Followed by filtration through cheese cloth and by Whatman's filter paper (nos. 4 and 1, respectively) using a vacuum system. The aqueous extracts were allowed to cool to room temperature and fed to rats ad libitum. Fresh tea was prepared every second day. Phytochemical analysis was performed as described (Opuwari and Monsees, 2014). In brief, aliquots from the different tea preparations were kept at −20 °C until chemical analysis. At least four samples per tea and concentration, prepared on different days, were investigated in triplicate. Soluble solids of each tea sample (1 mL at 120 °C dried overnight) were collected randomly during the 21 day period of treatment. Total polyphenol content of extracts was analyzed using Folin–Ciocalteu method (Singleton and Rossi, 1965). Flavonol/flavone content using quercetin as standard (Mazza et al., 1999) and flavanol/proanthocyanidin with catechin as standard (McMurrough and McDowell, 1978) were also determined. The results obtained were expressed as a percentage of soluble solids.

2.3. Animals and treatment

Thirty female Wistar rats were bred in the animal facility of the Medical Bioscience department, University of the Western Cape. Rats were individually identified by tail marking and housed three per cage. All animals were housed under standard conditions of 21–24 °C with constant 12 h light/dark cycle. They had access to standard rat chow ad libitum. All protocols were reviewed and approved by the ethics committee of the University of the Western Cape (registration no. 11/7/40).

Sexually mature, but virgin, female Wistar rats (84 day-old; 6 animals per group) weighing 180–230 g were randomly assigned to five different groups: Group 1 received tap water and served as control. Groups 2 and 3 received 2% and 5% unfermented rooibos; and groups 4 and 5 received 2% and 5% fermented rooibos, respectively, as sole source of drinking for 21 days (Opuwari and Monsees, 2014). Over 10–12 days before treatment, the estrous cycle of female rats was determined according to Marcondes et al. (2002) and only the normal cycling ones were used for the purpose of this study. Treatment began on the first day of diestrous and was terminated as they entered pro-estrous. The fluid intake was noted daily and body weights were taken weekly. At the end of treatment, animals were euthanized with CO_2. Final body weight of animals was taken and blood was collected by cardiac puncture and allowed to clot at room temperature for 30 min and centrifuged (3000 ×*g*, 15 min). Serum was stored at −80 °C for further use. Weights of ovaries, uterus, liver and right kidney were taken. Parts of liver and left kidney were frozen at −80 °C for biochemical assays.

2.4. Histology

The ovaries, uterus, liver and kidney were fixed in 10% buffered paraformaldehyde, cut in small sections, dehydrated through a series of graded ethanol, cleared in xylene and infiltrated in wax in an automated Leica TP 1020 tissue processor (Leica Biosystems, Germany) using a 18 h cycle. Following that, tissues were embedded in paraffin wax and cut into sections of 6-μm and stained using hematoxylin & eosin. The heights of uterine epithelium, endometrium and myometrium were measured using ScopeTek ScopePhoto software (Hangzhou Opto-Electric Co, Ltd; Zhejiang Province, China).

Table 2
Total body weight gain, relative organ weight and thickness of endometrium, uterine luminal epithelium and myometrium of female rats exposed to *Aspalathus linearis* for 21 days.

	TBWG (g)	Ovaries (g)	Uterus (g)	Endometrium (μm)	Epithelium (μm)	Myometrium (μm)	Liver (g)	Kidney (g)
C	17.80 ± 7.55	0.08 ± 0.01	0.26 ± 0.07	398.2 ± 153.7	41.6 ± 7.3	167.5 ± 51.1	4.63 ± 0.25	0.95 ± 0.08
2% Fr	18.81 ± 1.59	0.07 ± 0.01	0.34 ± 0.06	ND	ND	ND	4.81 ± 0.27	0.91 ± 0.05
5% Fr	24.19 ± 6.30	0.06 ± 0.01*	0.23 ± 0.05	557.5 ± 114.4*	55.1 ± 11.6	156.6 ± 36.9	4.67 ± 0.22	0.95 ± 0.07
2% Ur	19.52 ± 7.63	0.08 ± 0.01	0.40 ± 0.14	ND	ND	ND	5.07 ± 0.28*	0.94 ± 0.04
5% Ur	17.81 ± 3.47	0.07 ± 0.01	0.40 ± 0.05$^{\#}$	526.16 ± 127.6*	60.8 ± 14.3	168.8 ± 44.1	4.98 ± 0.24*	0.95 ± 0.05

Values are represented as mean ± SD after 21 day treatment. Number of rats per group = 6. $^{*}P < 0.05$; $^{\#}P < 0.01$; compared with the control group. Abbreviations: C, control; Ur, unfermented rooibos; Fr, fermented rooibos; TBWG, total body weight gain; relative organ weight = organ weight / final body weight × 100; ND, not determined.

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