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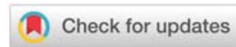

Research Article

Adverse Effects of *Saccharum officinarum* Molasses on Rat Testicular Cells

Abstract

Introduction: *Saccharum officinarum* is the source of the popularly used refined sugar, with reported anti-androgenic effects. *Saccharum officinarum* Molasses (SOM), a sweet byproduct obtained during sugar production, rich in phenolic compounds, minerals and organic acids is being explored as a substitute sweetener for refined sugar due to its nutritional advantage. However, paucity of knowledge of its effects on reproductive functions prompts this study designed to assess activities of testicular cells cultured in SOM fractions.

Methods: Blackstrap® *Saccharum officinarum* molasses was fractionated using methanol and water. Constituents of SOM methanol (SOMMF)and aqueous fractions (SOMAqF) were identified using Gas Chromatography Mass Spectrometry. Testicular cells from twelve rats were isolated, cultured and incubated with SOMMF, SOMAqF, components of SOMMF (Lupeol) and SOMAqF (Diethyl Phthalate). Cell viability, proliferation and testosterone biosynthesis were quantified using MTT assay and ELISA. Data were analyzed using ANOVA at $p<0.05$.

Result: Major constituents from SOMMF and SOMAqF were lupeol (87.2%) and diethyl phthalate (47.4%), respectively. Testicular cell proliferation increased in SOMMF (2.86±0.22) compared to control (1.89±0.18). Lupeol and diethyl phthalate decreased cell proliferation (1.07±0.11 and 1.11±0.17) respectively, compared to the control (1.89±0.18). Testicular cell testosterone biosynthesis was reduced by SOMMF, SOMAqF, Lupeol and Diethyl Phthalate as compared to the control.

Conclusion: *Saccharum officinarum* molasses adversely altered testicular cell activities, this may be linked to its constituents; lupeol and diethyl phthalate.

Introduction

Saccharum officinarum-derived refined sugar is a popularly consumed sweetener worldwide [1]. Refined sugar was previously believed to be non-lethal and wholly safe for consumption [2]. However, other reports have associated refined sugar consumption with both local and systemic adverse effects such as dental caries, appendix and gall bladder disorders, early aging, cardiovascular, autoimmune as well as other metabolic diseases [3]. Refined sugar consumption has also been associated with reproductive dysfunction in males [4].

Consumption of sugar-sweetened snacks and drinks was correlated with low spermatozoa concentration [5]. This, and other similar reports led to the substitution of refined sugar with artificial (non-sugar) sweeteners or food additives with low food energy that replicate the effect of sugar in taste because they contain saccharin, sucralose and aspartame [6]. These substitutes however, presented direr effects such as bladder cancer, weight gain and brain tumors [7]. In order to curb these effects there was yet another shift to the use of natural products. Natural products are believed to have more beneficial effects on health status because of their additional nutritive values [8,9]. *Saccharum officinarum* molasses (SOM) is a natural sweetener and a byproduct obtained during the processing and production of refined sugar from sugarcane juice. *Saccharum officinarum* molasses is now used as an ingredient in the human diet [10].

The production of sugar involves boiling of the *Saccharum officinarum* juice at various temperature and stages. At the 3rd boiling stage the sugar crystallizes and a viscous byproduct is obtained known as *Saccharum officinarum* molasses [11]. Molasses was imported into the United States from the Caribbean Islands. Over the years molasses has been used

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in feeding animals due to its ability to eradicate dust and reduce feed wastage as well as an important source of dietary energy [12,13]. Molasses is used in sweetening of hot drinks and alcoholic beverages [14]. Studies report that *Saccharum officinarum* molasses may be beneficial for treatment of bone defects [15], protection against deoxyribonucleic acid (DNA) oxidative damage [16]. *In vivo* exposure of animals to molasses produced an immunosuppressive effect [14].

The prevalence of male reproductive dysfunction shows an alarming increase [17]. Lifestyle and dietary habits have contributed to antiandrogenicity and hormonal imbalance in men in relation to reproductive disorders. High intake of added sugars has been related with occurrence of reproductive dysfunction in men [4], such as prostate problems, impotence, low sex drive and low sperm count having a direct association with hormonal imbalance [18]. *Saccharum officinarum* molasses is becoming a popular substitute sweetener for sugar [19], but there are evidences that *Saccharum officinarum* molasses may be a causal factor of endocrine disruption [20]. There is paucity of knowledge on the actions of *Saccharum officinarum* molasses on male reproductive physiology. This led to the design of this study to assess the activities of testicular cells cultured with *Saccharum officinarum* molasses fractions.

Materials and Methods

Micro/tissue culture plate, Micropipette (Fisher brand, Ex K08813251) and multipipette (Fisher brand, 9002118). Microplate reader (thermo scientific multiskan SN: 3550904177). Microscopes, camera, computer monitor (Axiocam 1Cc1 [195-040316) and Carl Zeiss Axiovision software. Incubator (Schutzarts DINEN 60529-IP20). SterilGARD class II biological safety cabinet (model SG403 A-HE-INT), centrifuge (Thermo Electron LED GMbH D-37520). Dissecting sets, Neubauer hemacytometer, cover slips, hand gloves, cotton wool, sterile bottles, Bijou bottles and petri dish.

Reagents: Bovine serum albumin, Glutamax, Streptomycin, Penicillin, Amphotericin B, RPMI–1640 (modified) medium (all were tissue culture grade), CO_2 gas, ethanol, Dimethyl Sulfoxide (DMSO), Testosterone ELISA kit (Calbiotech Inc. USA), Luteinizing hormone ELISA kit (Fortress diagnostics, UK), MTT Cell Proliferation Assay kit (Sigma, USA), Deionized water and 70% alcohol.

Fractionation of *Saccharum officinarum* molasses

Blackstrap® *Saccharum officinarum* molasses (Old English Incorporated, USA) was used for the study. The extraction and fractionation were done as described by [21,22] were used in its extraction and fractionation. The *Saccharum officinarum* molasses (SOM) was poured into a separatory funnel, exhaustively extracted using consecutive liquid/liquid partition with methanol and water. Two fractions were obtained, namely; *Saccharum officinarum* methanol fraction (SOMMF) and *Saccharum officinarum* aqueous fraction (SOMAqF), they were concentrated separately using a rotary evaporator. Gas Chromatography Mass Spectometry (GC-MS) of SOMMF and SOMAqF was performed using standard methods [23].

Animals

Ethical consent was obtained from the National Veterinary Research Institute, Animal Use and Care committee (NVRI/EC/20/016) and all procedures involving the use of animals conformed to National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, 1978) guidelines. Twelve adult male Wistar rats (180-200g) were gotten from the Small Animal House, National Veterinary Research Institute, Vom, Plateau State, Nigeria. They were given feed and water *ad libitum* and acclimatized for 2 weeks before commencing the experiment.

Sacrifice and cell preparation

i. The rats were euthanized and the testes were removed under sterile conditions [24]. The cells in suspension were prepared freshly using the method of [14], but with a little modification. Briefly;Pulverized testes were placed in serum free medium (SFM) which comprised of bovine serum albumin (0.2%), glutamax (1%), 1% combination of penicillin, streptomycin and Amphotericin B for prevention of contaminants and RPMI-1640.

ii. The supernatant containing the cells was decanted into a tube.

iii. Centrifugation was done at 1000 rpm for 10 minutes, supernatant was decanted, cell pellet was suspended in SFM made up to 20 mL. Incubation was done at 37°C in 5% CO_2 for an hour.

iv. Cells were centrifuged for 10 minutes at 1000 rpm, supernatant was discarded, cell pellet re-suspended in 20 mL SFM, incubation was done at 37°C in 5% CO_2 for 30 minutes.

v. The cell centrifugation was done for 10 minutes at 1000 rpm, the supernatant was discarded, cell pellet was re-suspended in 20 mL SFM. Incubation was done for 20 minutes at 37°C in 5% CO_2 to ascertain the baseline testosterone biosynthesis of cells.

vi. The prepared cells were either activated or not using luteinizing hormone (LH) enzyme conjugate at 10 mµ/mL and counted using a Neubauer hemacytometer for seeding of cells.

vii. Five dilution ranges (15.625µg/mL, 31.25µg/mL, 62.5ug/mL, 125µg/mL and 250µg/mL) of:

- Distilled water control (5µl/well).
- *Saccharum officinarum* molasses methanol fraction in distilled water (5µl/well).
- *Saccharum officinarum* molasses aqueous fraction in distilled water (5µl/well).
- Diethyl acetate in 0.5% of Dimethyl sulfoxide (DMSO) in distilled water (5µl/well).

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- Lupeol in 1% alcohol and distilled water containing 0.5% DMSO (5µl/well). All the above were prepared and pipette into a 96 well culture plate, the cell suspensions were seeded. 100 µl/well (from vii i.e. the LH stimulated and unstimulated) was aliquot into the plate and incubated for 4 hours with 5% CO_2 at 37°C (U.S. EPA, 2005). Two methods were employed to determine the half maximal effective concentration (EC_{50}).
- Architecture of the layer formed by the cultured cells: whereby the suspension cells were cultured for a period of time and viewed under an inverted microscope at intervals and observations were noted.
- Hormonal: whereby after 4 hours' incubation period, the supernatant was collected and assayed for testosterone biosynthesis.

ix Fresh cell preparations were made, part of the suspension cells were stimulated with LH and the other part unstimulated. The suspension cells (100µl/well) were pipette into culture plate follow by 31.25ug/mL of each of the samples (which was the EC_{50}) at 5µl/well. The plates were incubated for 4 and 24 hours, after which the supernatant was aspirated and assessed for testosterone with Enzyme-linked immunosorbent assay (ELISA) kits.

MTT cell proliferation assay

Cell suspension was prepared comprising 0.1-1.0×10^6 cells/mL in medium. 100µl of cell suspension per well was added to a 96-well cell culture plate with or without samples and culture for 24 hours at 37°C in 5% CO_2 in a humidified incubator. To each well, 100µl of the CytoSelect™ MTT Cell Proliferation Assay Reagent was added. The plate was further incubated at 37°C in 5% CO_2 for 3-4 hours until purple precipitate was visible. 100µl of detergent Solution was added per well. Incubation was done at 4 hours protected from light. Reading of absorbance was done at 570nm as the primary wavelength.

Statistical analysis

Data were expressed as mean ±SEM. The differences in mean were compared by one-way analysis of variance and $p \leq 0.05$ was considered statistically significant. Graphpad prism 5 statistical software was used for statistical analysis of all data.

Results

Gas chromatography mass spectometry of the methanol and aqueous fractions of *Saccharum officinarum* molasses

The figures 1,2 represent the gas chromatogram of *Saccharum officinarum* molasses methanol fraction (SOMMF) and *Saccharum officinarum* molasses aqueous fraction (SOMAqF) respectively. Two constituents were identified in SOMMF, the most abundant constituent was 87.19% of Lup-20(29)-en-3-ol, acetate, (3. Beta) at retention time of 44.623s (Table 1). Nine constituents were identified in the SOMAqF as shown in Table 2, the most abundant constituent was 47.43% Diethyl phthalate at retention time of 13.444s. All the constituents were identified on the basis of their retention indices determined with a reference to a homologous series of n-alkanes.

Effects of SOM methanol and aqueous fractions, diethyl phthalate and lupeol administration on absorbance

There was a directly proportionate significant increase ($P<0.05$) in absorbance value relative to the increase in number

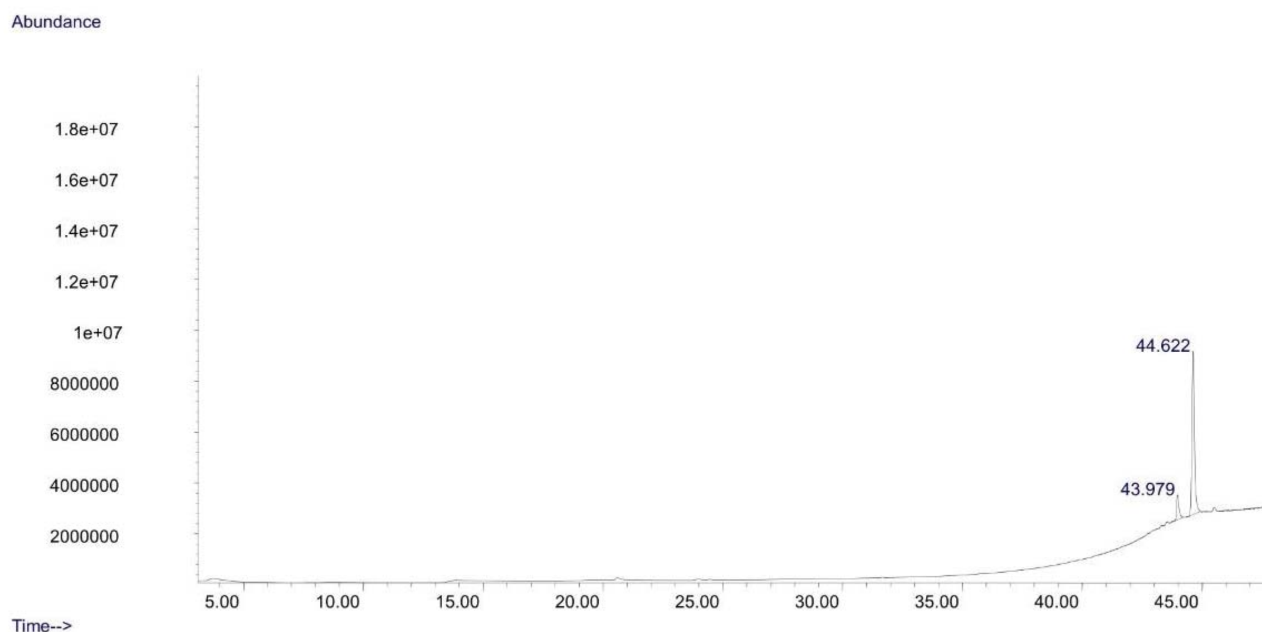

Figure 1: Gas chromatogram of the chemical constituents in *Saccharum officinarum* molasses methanol fraction.

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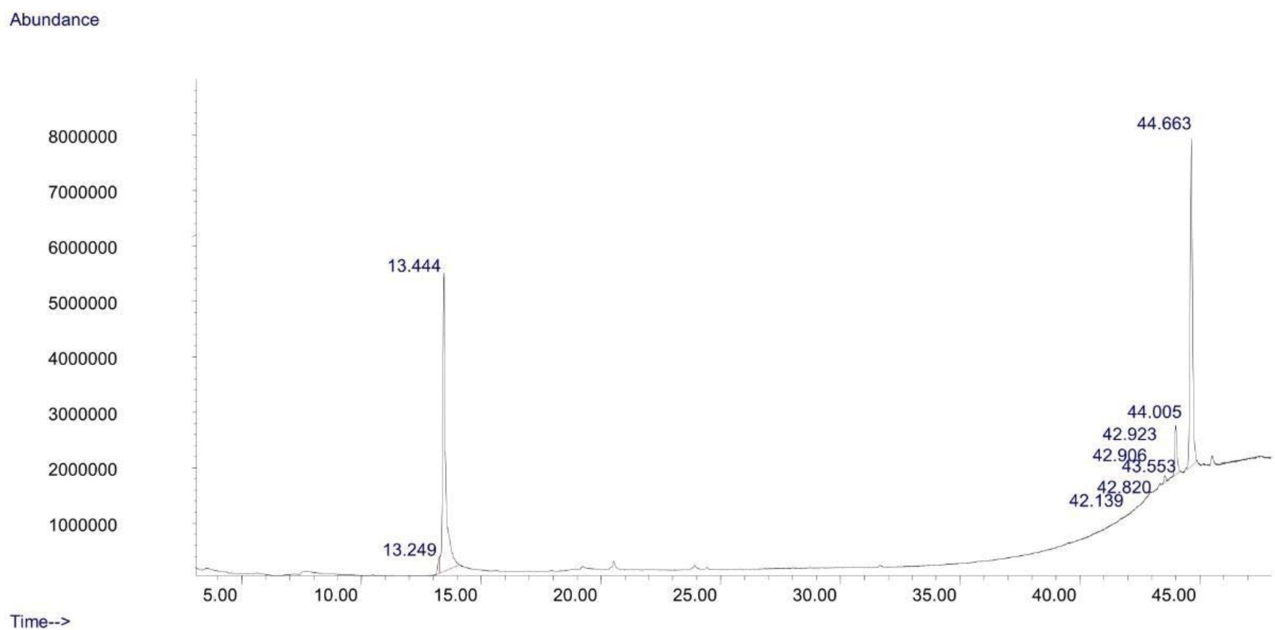

Figure 2: Gas chromatogram of the chemical constituents in *Saccharum officinarum* molasses aqueous fraction.

Table 1: Nomenclatures of constituents of *Saccharum officinarum* molasses.

A. Constituents of Saccharum officinarum molasses methanol fraction						
Pk #	**R.T. min**	**Area%**	**Library/ID**	**Ref#**	**CAS#**	**Qual**
1	43.982	12.81	Olean-12-en-3-ol, acetate, (3.beta)	261153	001616-93-9	90
2	44.623	87.19	Lup-20(29)-en-3-ol, acetate, [3.beta.]	261156	001617-68-1	99
B. Constituents of Saccharum officinarum molasses aqueous fraction						
Pk #	R.T. min	Area%	Library/ID	Ref#	CAS#	Qual
1	13.249	1.67	Diethyl Phthalate	85001	000084-66-2	98
2	13.444	47.43	Diethyl Phthalate	84999	000084-66-2	98
3	42.139	0.15	2-Ethylacridine	71643	055751-83-2	55
4	42.820	0.10	(Z)-Decyl icos-9-enoate	256742	1000414-43-4	58
5	42.906	0.07	Octasiloxane,1,1,3,3,5,5,7,7,9,9,11,11,13,13,1515-hexadecamethyl- 4-Dehydroxy-N-(4,5-methylenedioxy-2-nitro benzylidene) tyramine	272253 157264	019095-24-0 1000111-66-9	74 74
6	42.923	0.03	Octasiloxane, 1,1,3,3,5,5,7,7,9,9, 11,11,13,13,15,15-hexadecamethyl-	272253	019095-24-0	53
7	43.553	0.74	Octasiloxane, 1,1,3,3,5,5,7,7,9,9, 11,11,13,13,15,15-hexadecamethyl-2 -	272253	019095-24-0	58
8	44.005	5.97	Olean-12-en-3-ol, acetate, (3.beta)	261153	001616-93-9	93
9	44.663	43.85	Lup-20(29)-en-3-ol, acetate, (3.beta.)	261156	001617-68-1	99

of cells per well (Table 3). At 250ug/mL administration, SOMMF significantly increased (P<0.05) the absorbance value compared to the control. Diethyl phthalate and lupeol significantly decreased (P<0.05) the absorbance value compared to the control (Table 4).

Effects of varying dilutions of *Saccharum officinarum* molasses methanol and aqueous fractions, diethyl phthalate and lupeol on *in vitro* testosterone biosynthesis after 24 hours of incubation.

SOMMF of 62.5, 125 and 250 µg/mL caused significant decreases in testosterone biosynthesis of LH-stimulated cells and the unstimulated cells compared with their control. Lupeol of 62.5 and 125µg/mL reduced testosterone biosynthesis of LH-stimulated cells compared with the control. SOMAqF of 62.5, 125 and 250µg/mL caused significant reduction in testosterone biosynthesis of the LH-stimulated cells relative to control. Also, diethyl phthalate of 62.5, 125 and 250µg/mL reduced testosterone biosynthesis of the LH-stimulated cells compared with the control. Diethyl phthalate (125µg/mL) significantly reduced testosterone biosynthesis of unstimulated cells relative to control (Table 3). There were significant decreases in the testosterone biosynthesis of unstimulated cells treated with SOMAqF compared with control after 4 hours of incubation,

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this became insignificant after 24 hours of incubation. Lupeol reduced testosterone biosynthesis of LH-stimulated cells compared with control after 24 hours of incubation (Figure 3).

Discussion

Cellular metabolic activity and cell membrane integrity are characteristics of cell proliferation. Assessing cell proliferation and viability is a basis for several *in vitro* assays of a cell population's reaction to external stimuli [25]. One reliable method used for assessment of cell proliferation is incubation of the cells with a tetrazolium salt which is reduced by metabolically active cells in active mitochondria to produce an intracellular purple formazan whose absorbance is measured [26]. There was increased absorbance value in response to increase in number of cells seeded per well after 24 hours of culture, implying that the more the number of cells seeded per well, the higher its absorbance value per well as it was observed 24 hours after culture in the absence of any external factors. Lupeol and Diethyl phthalate are synthetic compounds of the main constituents from SOMMF and SOMAqF, their effects were compared with SOMMF and SOMAqF respectively. The significant increase in absorbance by SOMMF infers that there was an increase in metabolic activity of the cells which reduced the yellow tetrazolium to purple formazan revealing that there was a stimulation of the testicular cells and this may be beneficial, since the cells still retained their viability as they proliferate during culture. However, lupeol caused significant decrease in the absorbance. Studies have shown that the pharmacological activities of lupeol includes antiprotozoal [27], antimicrobial [28] and anti-inflammatory [29], among others. The SOMAqF did not alter absorbance but diethyl phthalate caused a significant reduction in the absorbance value. It is plausible that diethyl phthalate adversely altered the intracellular activities and decreased growth rate resulting in apoptosis or necrosis with a reduced metabolically active cells, hence, the low absorbance value interpreted as decreased cell viability. Study reports that phthalates generally alter germ cell development [30,31].

The SOMMF and SOMAqF did not cause significant alterations in the morphology of the cells as compared to the control which appeared round or flattened and refractile with no visible lesion [32]. Diethyl phthalate and lupeol significantly altered the morphology of cells with notable visible lesions. Diethyl phthalate caused the appearance of smaller, darker and non-refractile dead cells that were detached from the monolayer [32]. Phthalates impaired Leydig cell distribution

Table 2: Effects of MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) on cell proliferation by varying number of cells seeded per well after 24 hours of incubation.

Cells/well	Absorbance
0	0.1475±0.0091
113000	0.2848±0.0140*
125600	0.3545±0.0103*
141250	0.4265±0.0108*
161400	0.543±0.01998*
188300	0.5456±0.0107*
226000	0.5696±0.0089*
285000	0.7721±0.0070*
376700	0.8715±0.0115*
565000	1.377±0.0234*
1130000	2.306±0.0131*

Data represents mean±SEM of absorbance Number of replicates (N) =16, *P<0.05 compared with the medium (0).

Table 3: Effects of SOM methanol and aqueous fractions, diethyl phthalate and lupeol administration at 250ug/mL on cell proliferation seeded at 1.26 x 10^6 and after 24 hours of incubation.

Cells/well	Medium	Medium + Cells (control)	SOMMF	SOMAqF	0.5% DMSO	Dietyl Phthalate	1%alco + 0.5% DMSO	Lupeol
1.26×10^6 cells/mL	0.28±0.03	1.89±0.18	2.86±0.22*	1.83±0.21	2.21±0.07	1.11±0.17*	2.13±0.04	1.07±0.11*
0.32×10^6 cells/mL	0.30±0.02	0.60±0.02	0.80±0.04*	0.56±0.02	0.58±0.01	0.63±0.03	0.58±0.03	0.62±0.03

Data represents mean±SEM of absorbance. Number of replicates (N)= 8, 1% alcoh+0.5% DMSO = Vehicle for lupeol. 0.5% DMSO = Vehicle for Diethyl phthalate. *P<0.05 compared with the cells in the medium (control).

Table 4: Effects of various dilutions of Saccharum officinarum molasses methanol and aqueous fractions, diethyl phthalate and lupeol on in vitro testosterone biosynthesis after 24 hours of incubation.

Group	Medium + Cells	15.625µg/mL	31.25µg/mL	62.5µg/mL	125µg/mL	250µg/mL
Unstimulated + SOMMF	1.207±0.001	1.204±0.003	1.212±0.001	1.195±0.001*	1.166±0.003*	1.163±0.001*
LH- stimulated + SOMMF	1.232±0.004	1.209±0.003	1.197±0.003	1.170±0.002*	1.166±0.002*	1.166±0.002*
unstimulated + SOMAqF	1.207±0.002	1.208±0.002	1.216±0.003	1.208±0.002	1.180±0.005	1.202±0.005
LH- stimulated + SOMAqF	1.230±0.001	1.205±0.009	1.189±0.009	1.16±0.0025*	1.158±0.003*	1.174±0.003*
Unstimulated cells + Vehicle (1% alcoh + 0.5% DMSO) + Lupeol	1.196±0.009	1.207±0.008	1.225±0.003	1.219±0.004	1.180±0.005	1.199±0.006
LH- stimulated + Vehicle (1% alcoh + 0.5% DMSO) + Lupeol	1.205±0.010	1.195±0.009	1.226±0.010	1.181±0.004*	1.177±0.009*	1.195±0.007
Unstimulated + Vehicle (0.5% DMSO) Diethyl Phthalate	1.195±0.003	1.205±0.003	1.217±0.005	1.208±0.0024	1.164±0.002*	1.186±0.004
LH-stimulated + Vehicle (0.5% DMSO) Diethyl Phthalate	1.203±0.002	1.201±0.009	1.193±0.007	1.163±0.008*	1.154±0.004*	1.177±0.004*

Data represents mean±SEM. Number of replicates (N) = 4. 1% alcoh+0.5% DMSO = Vehicle for lupeol. 0.5% DMSO = Vehicle for Diethyl phthalate. Unstimulated = Cells not activated with hormone before incubation. LH-Stimulated = Cells activated with luteinizing hormone enzyme conjugate before incubation. *P<0.05 compared with the cells in the medium (control).

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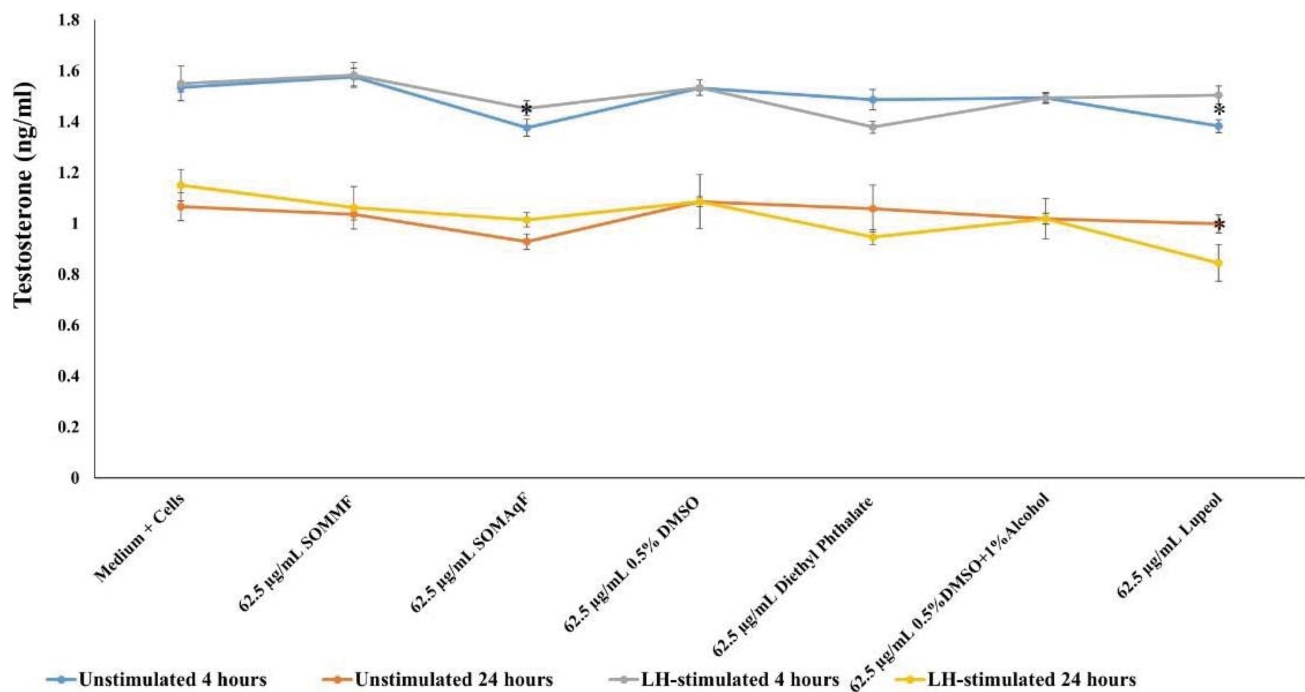

Figure 3: Effect of SOM methanol and aqueous fractions, diethyl phthalate and lupeol administered at 62.5µg/mL on *in vitro* testosterone biosynthesis of unstimulated and LH- stimulated cells after 4 and 24 hours of incubation. Lines represents mean±SEM, Number of replicates (N) = 4, 1% alcoh+0.5% DMSO = Vehicle for lupeol. 0.5% DMSO = Vehicle for Diethyl phthalate. *p<0.05 as compared with the cells in the medium (control).

and steroidogenesis in utero and also possess carcinogenic, teratogenic, and endocrine effects [33]. Lupeol caused a visible accumulation of dead, swollen and non-refractile cells. Lupeol deters proliferation of prostate cancer cells and possesses cancer-preventive and anti-cancer effects *in vitro* and *in vivo* [34]. It is plausible that both diethyl phthalate and lupeol may have exerted necrotic effect on these cells.

The Gas chromatography mass spectrometry [GC-MS] of *Saccharrum officinarum* molasses methanol fraction showed abundance of Lup-20(29)-en-3-ol, acetate [Lupeol acetate] which is a non-polar compound and a triterpene known to have vast occurrence in diverse plant families [35]. It has been reported to possess antiprotozoal [28], antimicrobial [29], anti-inflammatory (30), antitumor and anti-prostate cancer activities [36], it also has potential antifertility effects as well [37]. The GC-MS of *Saccharrum officinarum* molasses aqueous fraction showed abundance of diethyl phthalate. Diethyl phthalate has been reported to possess anti-microbial activity [38]. Phthalates have generally been reported to have carcinogenic, teratogenic, hepatotoxic and endocrine effects [33]. Previous studies have also shown the adverse effects of phthalates on germ cell development [30,31].

In order for the male reproductive tract to grow and properly develop, it requires the presence of testosterone [39]. Biosynthetic pathway is moderated by gonadotropins, steroidal hormones and important enzymes that later becomes the target of various endocrine disruptors [40]. Parameters which modifies these regulatory mechanisms/enzymes, can as well alter the synthesis of hormones causing a negative effect on male reproductive function. The *Saccharum officinarum* molasses caused a general decrease in testosterone biosynthesis which was significant at higher concentrations (i.e. from 62.5ug/mL–250ug/mL) in both the LH-stimulated and unstimulated cells after 4 hours of incubation. Higher doses of SOMMF showed significant response within a shorter period of incubation, it is probable that its action is time and dose dependent. Also, the ability of SOMMF to decrease the biosynthesis of testosterone in both LH-stimulated and unstimulated cells in culture, suggests that its activity is not only in the presence of gonadotropin but also directly on the testicular cells, since the same effects were observed in the presence or absence of Luteinizing hormone contrasting [14], who showed that molasses increased production of testosterone by LH activated testicular cells alone.

After 4 hours of incubation, lupeol caused a significant decrease in testosterone biosynthesis of unstimulated cells. It caused significant decreases at both 4 and 24 hours of incubation in the LH-stimulated cells. This ability of lupeol to alter testosterone level *in vitro* is implicit that its actions are either indirectly (through gonadotropin) or directly on the testicular cells and may not depend on period of incubation with cells but may be dose dependent, since the response observed was significant at higher concentration irrespective of time. This study agrees with [37], who showed that lupeol acetate (an active constituent) caused antifertility effect in male Wistar rats.

The SOMAqF and diethyl phthalate at higher concentrations (i.e. from 62.5ug/mL–250ug/mL) caused significant decrease

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in testosterone biosynthesis in both LH-stimulated and unstimulated cells after 4 hours of incubation, but after 24 hours of incubation no significant difference was observed. They probably exerted time dependent actions through the gonadotropins or directly on the testicular cells [33], since there were no significant alterations at a longer period of incubation of the samples with cells possibly due to a short half-life. The GCMS result show that diethyl phthalate is the abundant constituent present in the SOMAqF, both of them may possess similar chemical characteristics.

Conclusion

Saccharum officinarum molasses increased cell proliferation but decreased testicular cell testosterone biosynthesis during culture and this may be linked to its constituents; lupeol and diethyl phthalate. Consumption of *Saccharum officinarum* molasses may adversely alter reproductive functions in male Wistar rats.

Acknowledgement

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