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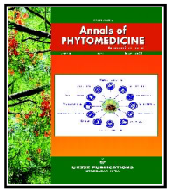

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Fruit juice of *Garcinia indica* Choisy modulates dyslipidemia and lipid metabolism in cafeteria diet based rat model

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Abstract

There is a significant rise in the incidences of dyslipidemia, leading to obesity. The therapeutics available for dyslipidemia are limited and associated with major side-effects. Thereby, researchers are shifting towards nutraceuticals compounds. In the current study, *Garcinia indica* Choisy, which is an endemic species of Western Ghats of India was evaluated for its anti-dyslipidemic properties in cafeteria diet fed obese rat model. Firstly, cafeteria diet fed rat model was developed and validated. After successful development of the model, the rats were orally fed with 1 ml *G. indica* fruit juice for 4 weeks and parameters such as OGTT, lipid profile, hormone levels of insulin and leptin, HMG CoA reductase and LCAT enzyme activities and toxicity parameters were evaluated. Identification and quantification of the hydroxycitric acid in *G. indica* fruit juice was done by HPLC method. Toxicity parameters like SGPT and creatinine were performed to evaluate the toxicity of the dose. Results showed that cafeteria diet fed animals exhibited increased body weight, increased food intake, decreased water intake, increased glucose intolerance and dyslipidemia at 10 weeks. Treatment with *G. indica* fruit juice for 4 weeks, reduced the body weight, improved the metabolic parameters like glucose sensitivity, dyslipidemia, insulin and leptin levels and lipid metabolizing levels without causing toxicity. Oral dosage of *G. indica* fruit juice for 4 weeks exhibits antiobesity potential in cafeteria diet fed dyslipidemic rats. The results obtained were better than orlistat, which is a standard mode of chemotherapy for management of dyslipidemic obesity.

1. Introduction

The prevalence of dyslipidemia leading to obesity is rapidly increasing, however, limited medications are presently available in the market (Birari and Bhutani, 2007). Obesity is a dyslipidemic disorder, wherein, derangement in lipid metabolism has been seen along with abnormal lipid levels (Bays *et al.*, 2013), often associated with higher storage of lipid in adipocytes (Arner *et al.*, 2011). It is interesting to note that dyslipidemia is associated with a cluster of diseases thereby, being a central player in development of metabolic syndrome (Jung and Choi, 2014). The manifestation of syndrome has decreased life expectancy and its quality (Katz *et al.*, 2000; Pimenta *et al.*, 2015; Taylor *et al.*, 2013). Several nutritional theories have implicated diet as an important contributor in development of abnormal lipid levels (Kamran *et al.*, 2016). It is stated that over nutrition with low metabolic output is major cause of developing dyslipidemia (Grundy and Barnett, 2004). In this context, cafeteria based diet which is enriched with high refined sugar and high fat serves as a powerhouse of excessive calories and when used in animal models mimics all features of metabolic syndrome (Gomez-Smith *et al.*, 2016; Sampey *et al.*, 2011). Data from several reports also indicate that people increase the intake of high energy snack foods when stressed, thereby leading to dyslipidemic obesity (Anderson *et al.*, 2011; Shori *et al.*, 2017). This abnormal lipid status has affected immensely other metabolic pathways leading to cardiovascular risk. This has led to urgent need for developing the new antiobesity drugs that could manage dyslipidemia with fewer side effects. Currently, potential use of nutraceuticals for the management of dyslipidemia is not fully explored and could be an outstanding substitute approach for developing safe and effective dyslipidemic drugs. India, especially north-eastern region is a rich source of various medicinal plants. In this context, several herbs have been explored, amongst which *Garcinia* is of utmost importance due to its several important biological properties. Plants from the genus *Garcinia* have been reported from Asia, Africa and Polynesia. Anti-inflammatory, antinociceptive, antioxidant, antitumoral, antifungal, anticancer, antihistaminic, antiulcerogenic, antimicrobial, antiviral, vasodilatory, hypolipidemic, hepatoprotective, nephroprotec-tive and cardioprotective properties of plants from this genus have been reported (Santo *et al.*, 2020). *G. indica* commonly known as “Kokum” is one of the *Garcinia* species used in traditional medicine in Asian countries as folk medicine to treat various ailments. In Ayurveda, it is known as Vrikshamla. Juice obtained from Kokum fruit or extracts prepared from aril or rind is used in the preparation of drugs in

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Indian, Chinese, Thai and Malaysian systems of medicine. Kokum finds its place in Ayurvedic systems of medicine and is considered to be beneficial for health (Swamy *et al.*, 2014). Also, infusion prepared from the Kokum is used to treat piles, dysentery and infections. Furthermore, Kokum is known to strengthen the cardiovascular system and stabilize liver function (Braganza *et al*., 2012). Prophylactic potential of *G. indica* fruits for ailments as varied as rheumatism, rickets, enlargement of spleen, uterine complaints and in animal disorders have been described in *Ayurveda*. Its fruit juice or syrup is used as a coolant and helps to reduce body weight (Braganza *et al*., 2012). It is also used for getting relief in stomach and liver disorders (Bhat *et al*., 2005; Krishnamurthy, 1984; Krishnamurthy *et al.*, 1982; Mishra *et al.*, 2006). The present investigation was undertaken to investigate the efficacy of fruit juice of *G. indica* as a hypolipidemic agent in cafeteria based diet dyslipidemic rodent model.

2. Material and Methods

2.1 Chemicals

Hydroxycitric acid (HCA), calcium salt was purchased from Natural Remedies, Bengaluru, India. HPLC grade solvents (acetonitrile, trifluroacetic acid, TFA, and water) were purchased from Merck, Mumbai, India. Cholesterol (HiMedia Laboratories) and Orlistat (German Remedies) were purchased locally. Metabolic profile involving oral glucose tolerance test, lipid profile were performed using GOD-POD kit and lipid profile test kit was purchased from Enzopak (Rankem). Trizol, cDNA kit was procured from Takara Inc (PrimeScript 1st strand cDNA Synthesis Kit). Primers for metabolic enzymes were designed by primer express and synthesized by integrated DNA technologies. Serum hormones-insulin and leptin were assayed using ELISA kits (DBC Canada).

2.2 *G. indica* fruit juice

Mature fruits of *G. indica* were collected. Fruit pulp was compressed to get the juice. Collected juice was stored in glass bottles under refrigerated conditions till further use.

2.3 Apparatus and chromatographic conditions for profiling of HCA in *G. indica* fruit juice

Quantification of hydroxycitric acid was carried out using HPLC (Waters, USA) system consisting of quaternary pumps, an in-line vacuum degasser, and a photodiode array detector (PDA). The instrumentation was controlled by using Empower 3.0 software (Waters). The chromatographic separation was achieved using Sunfire™ C18 Column (4.6 x 250 mm, 5 µm, Waters, Milford, MA, USA) at ambient temperature. The mobile phases consisted of a mixture of solvents: 0.1 % trifluoroacetic acid in water (A) and 0.1 % trifluoroacetic acid in acetonitrile (B). The optimised HPLC condition for gradient elution mode is as follows: elution was initially started with 90 % of solvent (A) and 10 % solvent (B) with a flow rate of 0.8 ml/min. Further, after 10 min solvent (A) was decreased to 80 % and solvent (B) was increased to 20 %. After 25 min of solvent (A) was decreased to 70 % and solvent (B) was increased to 30 %. Solvent (A) was gradually decreased to 60 % and solvent (B) was increased to 40 % after 30 min. At 31 min composition of solvent (A) and solvent (B) was brought back to 90 % and 10 %, respectively. The flow rate through out the run was 0.8 ml/min and run time was 35 min (Kureshi *et al.*, 2018). Detection wavelength was 211 nm. Injection volume was 20 µl (Kureshi *et al.*, 2018).

2.4 Animals

Forty adult virgin female Charles Foster rats (3-4 months) showing regular estrous cyclicity were chosen for the study. Animals were housed in a standard controlled animal care facility (22-25^{o}C and 45% humidity) in cages (one rats/cage) under equal dark and light cycle (1:1). Standard nutritional and environmental conditions for animals were maintained throughout the experiment (Chidrawar *et al*., 2011). All the experiments were carried out between 9:00 and 16:00 hours, at ambient temperature. The Nations Control and Supervision of Experiments on Animals (CPCSEA) guidelines were strictly followed and all the studies were approved by the Institutional Animal Ethical Committee (IAEC) (Protocol Number: BC/11/2017).

2.5 Induction of dyslipidemia through diet

The cafeteria diet was as described by Chidrawar *et al*. (2012). It consisted of 3 different diets: diet 1-condensed milk (8 g) + bread (8 g); diet 2-chocolate (3 g) + biscuits (6 g) + dried coconut (6 g); diet 3-cheese (8 g) + boiled potatoes (10 g) for each animal. The three cafeteria diets were fed to each rat of the group that had 6 animals on days one, two, and three, respectively with repetition in the same succession for 10 weeks along with standard pellet diet. Cholesterol rich high fat diet (Kumar *et al*., 2008) was given to each rat four weeks prior to the *G. indica* fruit juice treatment.

2.6 Treatment with *G. indica* fruit juice

Animals were divided into six major groups, Group I (C) had animals that received lab chow diet and were considered as control. Further, animals fed with cafeteria diet and high-fat diets were divided into five groups, Group II (CD) consisted of animals which were fed with cafeteria diet and were considered to be dyslipidemic animals; Group III (CD + O) consisted orlistat (standard antiobesity drug) treated animals fed with cafeteria diet; Group IV (CD + G) were *G. indica* fruit juice treated animals fed with cafeteria diet; Group V(HF) animals were high fat diet treated animals, which served as positive control for dyslipidemia and Group VI (HF + G) were high-fat diet fed animals, which received *G. indica* fruit juice treatment. All *G. indica* fruit juice treatment was given daily orally for 30 days at a dose of 1 ml.

2.7 Parameters analysed

During 30 days of *G. indica* fruit juice treatment, all animals were continuously monitored for body weight and food intake. Oral glucose tolerance test (OGTT) was performed after 12 h fasting in all rats (Buchanan *et al.*, 1991). Glucose (2g/kg body weight) was orally fed to the rats and blood samples was collected in sodium fluoride (NaF) and EDTA coated tubes considered as 0 minute. After that blood sample was collected at different time intervals (30, 60, 90, and 120 minutes) and plasma was used for the estimation of glucose. Analysis of the lipid profile was done by Enzopak kits which estimated total cholesterol, triglycerides, HDL-C, and LDL-C from serum. Serum hormones like insulin and leptin were assayed by ELISA method. Homeostatic model assessment, Insulin Resistance (HOMA-IR) was calculated based on the fasting blood glucose and fasting insulin values using the following formula HOMA IR = (Fasting insulin x Fasting glucose)/405. Normal insulin resistance: < 3; moderate insulin resistance: between 3-5; severe insulin resistance: > 5. In addition to

this, 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA reductase) activity was carried out according to the protocol described by (Rao and Ramakrishnan, 1975). Also, plasma lecithin cholesterol acyl transferase (LCAT) activity was assayed by the method described by Hitz (Hitz *et al.*, 1983). In addition to this, gene expression of cholesterol biosynthetic enzymes like Acetyl-CoA carboxylase (ACC) was evaluated by RT-PCR. RNA was isolated using trizol reagent following which 2 microgram of RNA was subjected to cDNA preparation. cDNA was later used for expression studies. Primer details are indicated in Table 1. Serum glutamate pyruvate transaminase (SGPT) and creatinine were analyzed to understand the toxicity of *G. indica* fruit juice.

Table 1: Sequences of target gene specific primers

Gene name	Accession number	Sequence of primers
Acetyl-coenzyme A carboxylase	NM_022193.1	F-5'ATGGTCTACATTCCCCCACA 3'
		R-5'ATCACAACCCAAGAACCACC 3'
GAPDH (internal control)	NM_017008	F-5'CAAGGTCATCCATGACAACTTTG 3'
		R-5'GTCCACCACCCTGTTGCTGTAG 3'

2.8 Statistical analysis

Comparison of different groups was done using analysis of variance (ANOVA) and Student *t*-test. The analysis was carried out using GraphPad version 5.0. $P \geq 0.05$ was considered significant. All results are expressed as the mean ± SEM for 6-7 animals in each group.

3. Results

3.1 HPLC analysis of *G. indica* fruit juice

Identification and quantification of the HCA in *G. indica* fruit juice was carried out with the comparison of retention time and PDA spectra of the peak of standard HCA (Figure 1A). Peak of HCA eluted at 5.3 min. Furthermore, identity of peak of HCA was also confirmed by spiking studies. Quantification of HCA was carried out using a calibration curve prepared from the different concentration of standard HCA and the content of (-)-hydroxycitric acid (HCA) in *G. indica* juice was 16.57 % (Figure 1B).

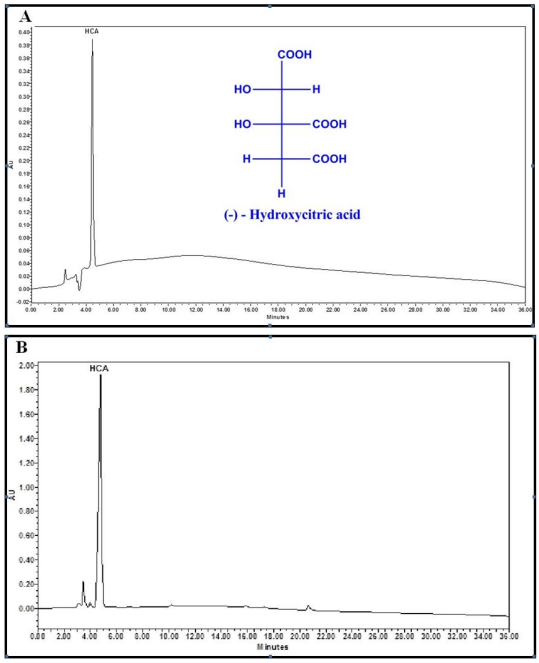
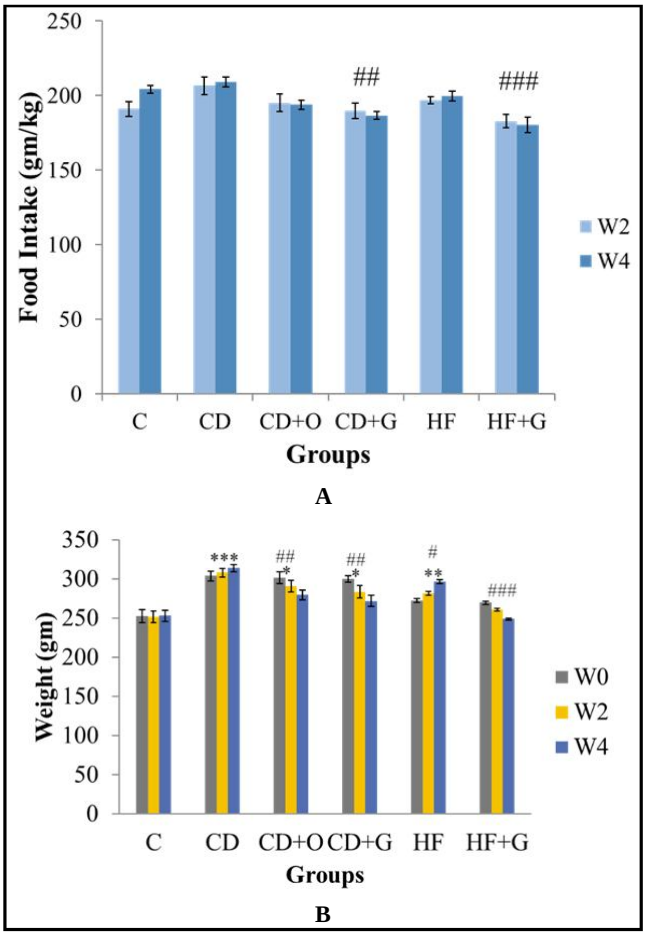


Figure 1: HPLC analysis of *G. indica* fruit juice. A peak of standard HCA B. Quantification of HCA in *G. indica* fruit juice.

3.2 Effect of *G. indica* fruit juice on body weight, food and water intake

Figure 2A. represents the effect of *G. indica* fruit juice on the food consumption of the animals. There was significant increase in body weight observed after 10 weeks of treatment in cafeteria group as compared to control group as shown in Figure 2B. After 4 weeks of treatment with *G. indica* fruit juice, it was observed that there was significant decrease in body weight as compared to non-treated cafeteria group as well as orlistat treated cafeteria group. Thereby, treatment of *G. indica* fruit juice for 4 weeks was effective in reduction of body weight in the animals.



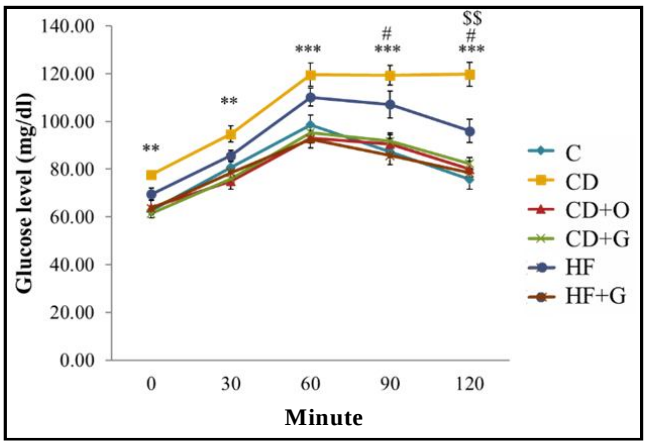


Figure 2: Effect of *G. indica* fruit juice on A. food intake, B. body weight. * comparison with control, # comparison with cafeteria diet, N=5, values are Mean ± SEM, # $p<0.05$, ## $p<0.01$, ### $p<0.001$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, W0, W2, W4 represents week 0 (before treatment), week 2 and week 4 (after treatment) C. glucose tolerance. * comparison with cafeteria diet, # comparison with high-fat diet, $ comparison between cafeteria diet and high-fat diet N=5, values are Mean ± SEM, # $p<0.05$, ** $p<0.01$, *** $p<0.001$, $$ $p<0.01$.

3.3 Effect of *G. indica* fruit juice on metabolic parameters

High fat affects insulin sensitivity. Thereby, oral glucose tolerance test was performed after *G. indica* fruit juice treatment. Figure 2C demonstrated that the treatment with *G. indica* fruit juice for 4 weeks significant decrease in glucose intolerance was recorded. This data was comparable to group treated with orlistat, thereby, suggesting that *G. indica* fruit juice has potential to improve glucose sensitivity. However, cafeteria group and high fat group were glucose intolerant. In addition to this, serum hormone profile for insulin and leptin along with HOMO-IR demonstrated that there was significant increase in serum insulin level in cafeteria treated group (Table 2). Upon treatment with *G. indica* fruit juice, there was significant decrease in serum insulin level. Similar results were observed in orlistat treated group. In case of high-fat group, no significant change was observed as compared to control group. Serum leptin levels were significantly very high as compared to control group in cafeteria treated group. *G. indica* fruit juice treatment showed significant decrease in its level. While calculating HOMO-IR, it was observed, there is severe insulin resistance in cafeteria treated group. After treatment with *G. indica* fruit juice and orlistat, its value decreased but still it was showing moderate insulin resistance similar to control group. Thereby, it could be possible that long term treatment may improve glucose sensitivity.

Table 2: Effect of *G. indica* fruit juice on hormone profile

Groups	Insulin (mIU/ml)	Leptin (ng/ml)	HOMO-IR
C	24.09	0.74	3.73
CD	36.80**	1.17***	7.06
CD+O	32.10#	0.84###	5.07
CD+G	28.47##	0.76###	4.31
HF	29.37*##	0.98**##	5.04
HF+G	27.36	0.81	4.29

* Comparison with Control, # Comparison with cafeteria diet. n=5, Values are represented as Mean ± SEM, # $p<0.05$, ## $p<0.01$, ### $p<0.001$* $p<0.05$,** $p<0.01$,*** $p<0.001$.

Table 3 represents lipid profile after administration of *G. indica* fruit juice for 30 days. There was significant decrease in serum total cholesterol, serum tryglycerides and LDL-cholsterol and increase in HDL-cholesterol. It suggested that dyslipidemic rat model was reverting back to normal phenotype. These data when compared to standard drug shows similar result. Cafeteria treated group and high-fat treated group both shows significant alteration in lipid profile.

Table 3: Effect of *G. indica* fruit juice on lipid profile.

Groups	Total cholesterol (mg/dl)	HDL-cholesterol (mg/dl)	Triglycerides (mg/dl)	LDL-cholesterol (mg/dl)
C	55.55 ± 3.54	32.94 ± 1.09	80.83 ± 4.05	6.445 ± 4.63
CD	95.12 ± 2.90 ***	22.06 ± 0.86 **	141.9 ± 6.36 ***	44.69 ± 4.03 ***
CD + O	78.65 ± 3.75 #	32.16 ± 0.57 ##	97.98 ± 6.64 ##	26.9 ± 4.47 ##
CD + G	80.32 ± 1.58 #	31.3 ± 0.43 ##	95.3 ± 2.33 ##	29.96 ± 1.37 ##
HF	86.16 ± 2.10 ***	28.72 ± 0.78 *	118.1 ± 7.61 **	33.83 ± 1.88 **
HF+ G	82.92 ± 2.37	30.29 ± 0.96	107.5 ± 6.90	31.14 ± 1.23

* Comparison with Control, # Comparison with cafeteria diet n=5, Values are represented as Mean ± SEM, ** $p<0.01$, *** $p<0.001$, # $p<0.05$, ## $p<0.01$.

3.4 Effect of *G. indica* fruit juice on lipid metabolizing enzymes

Figure 3A represents the activity of key lipid metabolizing enzyme HMG-CoA reductase. Upon treatment of *G. indica* fruit juice, HMG-CoA reductase activity was decreased, thus indicated a decreased cholesterol biosynthesis. Ideal decrease in activity was seen on treatment with orlistat. High-fat group does not show significant change suggesting no change, in HMG-Co reductase activity. Figure 3B shows the effect of *G. indica* fruit juice on LCAT activity. On administering *G. indica* fruit juice, a significant increase in LCAT activity was observed as compared to the cafeteria group. This activity was not increased in case of orlistat treated group. While high-fat treated groups showed decrease in LCAT activity. No significant change was observed in *G. indica* fruit juice treated group as compared to high-fat treated group. Figure 3C represents transcript level of enzyme acetyl Co-a carboxylase, a lipid metabolizing enzyme. As seen in the figure, cafeteria diet treated group shows significant increase in ACC level as compared to control.

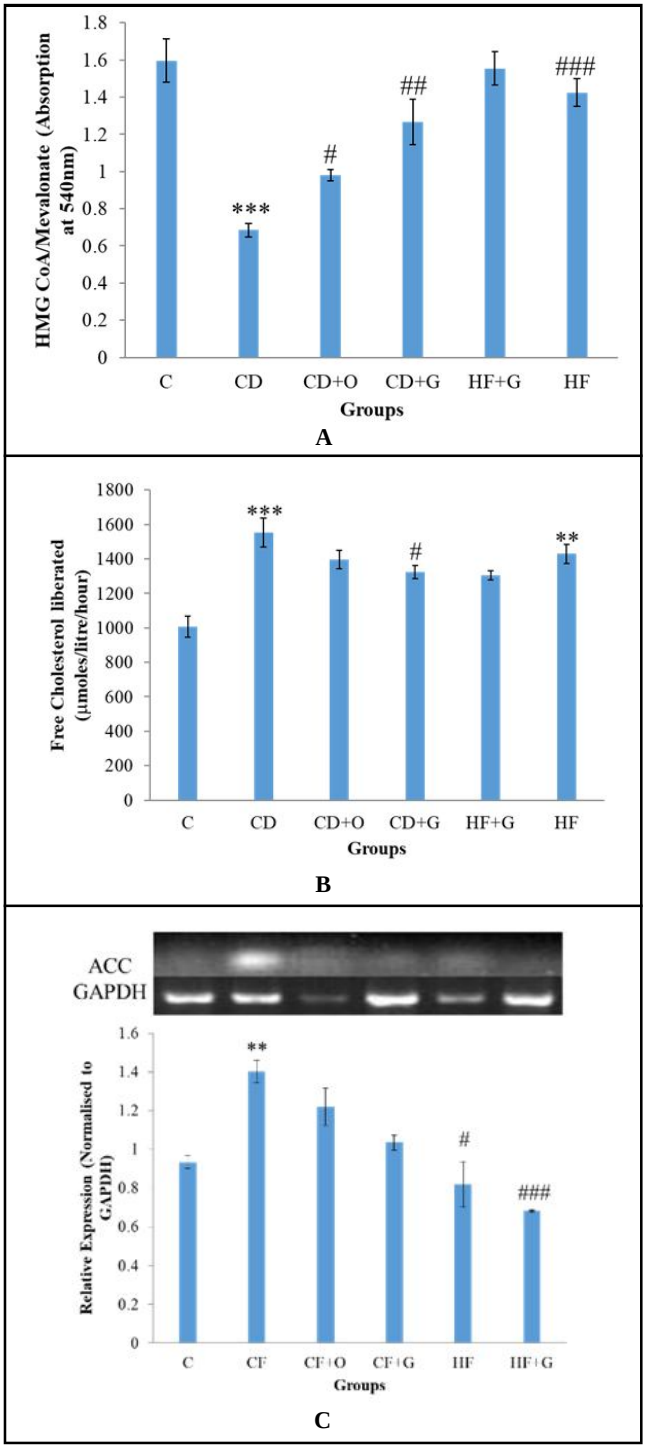


Figure 3: Effect of *G. indica* fruit juice on lipid metabolizing enzymes A. HMG CoA reductase activity B. LCAT activity and C. gene expression level of ACC enzyme. * comparison with control, # comparison with cafeteria diet, N=5, Values are Mean ± SEM, # <0.05, # *p*<0.01, ## *p*<0.001, ** *p*<0.01, * *p*<0.001, C. relative gene expression of ACC.* comparison with control, # comparison with cafeteria diet. n=5, values are Mean ± SEM, # *p*<0.05, ### *p*<0.001, ** <0.01.**

After treatment of *G. indica* fruit juice for 30 days, there was marked decrease in its level. The data is similar to the orlistat treated group. High-fat diet treated group does not show significant change in ACC level as compared to control group.

3.5 Effect of *G. indica* fruit juice on toxicity parameters

Figure 4 describes the toxicity parameters of liver and kidney upon administration with the *G. indica* fruit juice. As seen in Figure 4A, there was significant increase in SGPT activity, suggesting liver toxicity due to cafeteria diet treatment as compared to control. Upon the administration of *G. indica* fruit juice, there was significant decrease in its activity. However, there was no significant change in high-fat treated group. Figure 4B represents serum creatinine level and as seen in the Figure, there was significant increase in serum creatinine level in cafeteria treated group and the level decreased in *G. indica* fruit juice treated group. In case of high-fat treated group, significant increase in serum creatinine level was recorded.

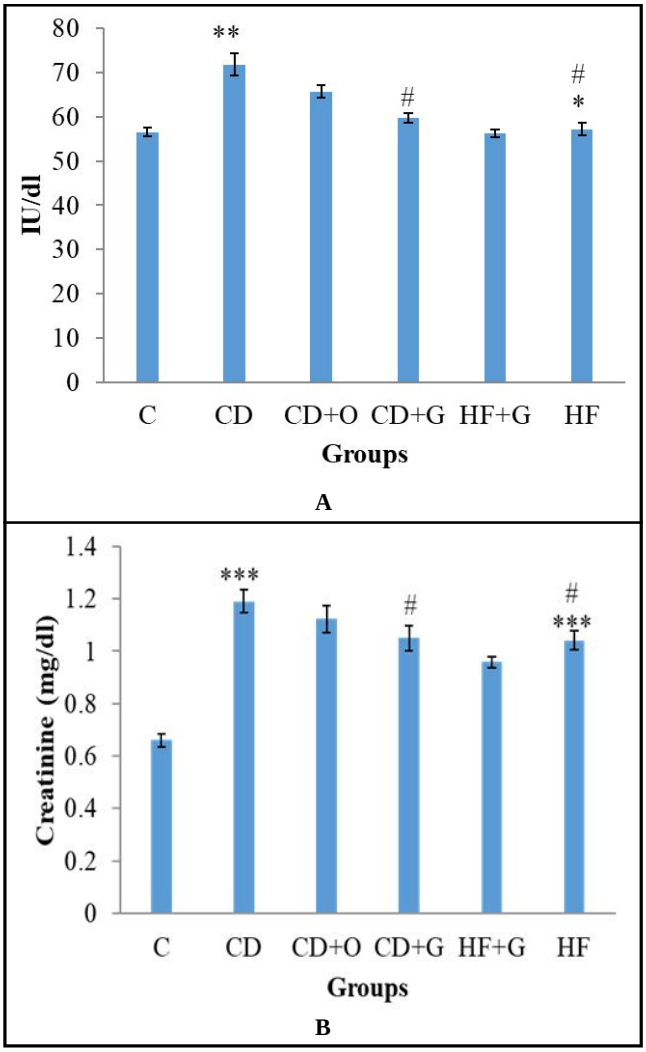


Figure 4: Effect of *G. indica* fruit juice on toxicity parameters of A. liver by SGPT activity and B. kidney by creatinine level. *comparison with control, # comparison with cafeteria diet. N=5, values are Mean ± SEM, ** *p*<0.01, * *p*<0.001, # *p*<0.05.**

4. Discussion

Obesity is a one of major dyslipidemia associated metabolic syndrome. Dyslipidemia is one of the initial steps for the development of obesity. In present study, cafeteria diet was used to generate a metabolic syndrome model for dyslipidemia to study initial stages for obesity. During dyslipidemia, there is increase in glucose intolerance, which is one of the important factor of metabolic syndrome and this may be due to increase in free fatty acid (Nambi *et al.*, 2002). OGTT profile was observed upon cafeteria diet administration, suggesting that enriched fat diet for 10 weeks is sufficient to cause dyslipidemia. In dyslipidemia, lipoprotein metabolism is altered which results in either overproduction or deficiency in certain lipoprotein molecule (Meisinger *et al.*, 2006). There may be alteration in the levels of either one or more lipoprotein molecules followed by elevated levels of total cholesterol, low density lipoprotein and triglycerides along with decreased levels of high density lipoprotein which later on can be developed in obesity (Mishra *et al.*, 2005; Misra *et al.*, 2006; Snehalatha, 2003; Vikram *et al.*, 2003). In our model, it was observed that there was significant increase in total cholesterol, LDL-cholesterol and triglycerides. Also, significant decrease in HDL-cholesterol was also observed. These all parameters are major characteristics of dyslipidemia (Nambi *et al.*, 2002).

HMG-CoA-Reductase catalyses the rate limiting step of liver cholesterol biosynthesis (Bucher *et al.*, 1960; Siperstein,. 1966). In dyslipidemia, it was observed that HMG-CoA-reductase activity was higher as compared to control group (Wu *et al.*, 2013). In our study, it was observed that there was significant decrease in substrate to product ratio suggesting an increase in HMG-CoA-reductase activity. Increase in its activity has resulted in increase in serum cholesterol levels after 10 weeks of treatment. LCAT is an enzyme that catalyses transfer of fatty acids from phosphatidylcholine to the hydroxyl residue (Assmann *et al.*, 1978). Acetylation of cholesterol is helpful for clearance of excess cholesterol. LCAT promotes maturation of HDL particles in plasma and transport cholesterol maintaining a concentration gradient for the diffusion of cellular un-esterified cholesterol to HDL-cholesterol (Shigematsu *et al.*, 2001). In diet induced obese rats, it was observed that there was significant decrease in LCAT activity which correlates with decrease in HDL-cholesterol (Subash and Augustine, 2014). Current study demonstrates that in dyslipidemia, rats there was significant decrease in LCAT activity which can be correlated with low HDL-cholesterol.

Dyslipidemia is a major symptom in development of metabolic syndrome (Mishra *et al.*, 2007). There are several medications available for dyslipidemia. Two major classes are statins and fibrtaes (Wierzbicki *et al.*, 2003). But, due to high cost and hazardous side-effects, there is need for new and better therapy in management of dyslipidemia. Plant derived products or nutraceuticals can be potential targets for the development of new drugs (Chidrawar *et al.*, 2012), as it renders few side-effects. In this context, several plants have been explored for the hypolipidemic effects like *Aloe barbadensis* Mill., *Enicostemma littorale*, *G. cambogia* (Desai *et al.*, 2012; Oluyemi *et al.*, 2002; Vasu *et al.*, 2005). *G. indica* is an indigenous plant however, medicinal properties of this plant are not well studied. Thereby, our present study, involves evaluating efficacy of *G. indica* for its hypolipidemic property.

After the treatment of *G. indica* fruit juice, there was significant decrease in body weight as compared to non-treated groups, suggesting that the *G. indica* fruit juice could be used for the development of anti-dyslipidemic drugs. Similar result was observed in the rats treated with standard drug. Similarly glucose intolerance was reduced in the *G. indica* fruit juice treated group as compared to non-treated group. In case of positive control (high-fat) group, no significant change in the body weight was observed as compared to control group, suggesting that dyslipidemic rat model was not developed. Glucose intolerance is a characteristic feature of insulin resistance, therefore, serum insulin levels were measured (Vikram *et al.*, 2003). There was significant increase in serum insulin level in cafeteria group as compared to control group. After the treatment with *G. indica* fruit juice, there was significant reduction in insulin level. It can be correlated with serum triglycerides level. HOMO-IR is used for evaluation of insulin resistance. While calculating HOMO-IR, it was found that the animals treated with cafeteria diet showed significant increase in index suggesting severe insulin resistance. While treatment with *G. indica* fruit juice resulted in decrease in index but the values were still in the range of moderate insulin resistance. During dyslipidemic obesity, there is increased level of circulating leptin level (Dobrian *et al.*, 2000). Serum leptin levels were elevated in cafeteria treated and high-fat diet treated groups as compared to control. After treatment with *G. indica* fruit juice, it was observed that there was significant reduction in serum leptin levels.

Lipid profile showed significant change in the *G. indica* fruit juice treated groups as compared to non-treated ones. Serum total cholesterol, triglycerides and LDL-cholesterol were back to normal in *G. indica* fruit juice treated group as compared non-treated group. It suggests that *G. indica* fruit juice is altering the lipid metabolism pathway resulting in normal phenotype.

HMG-CoA-reductase and LCAT activity were measured to check effect of *G. indica* fruit juice on lipid metabolising enzymes. After treatment, it was observed that in there was significant increase in the ratio of substrate to product resulting in decreased HMG-CoA-reductase activity. This might be the reason for decreased cholesterol level. Group treated with standard drug also showed the similar result, but to a lower extent. LCAT activity was significantly increased in *G. indica* fruit juice treated group as compared to non-treated group. In case of group treated with standard drug, no significant change was observed. mRNA transcripts levels of acetyl-coenzyme A carboxylase (ACC) were also checked. ACC is an enzyme responsible for the production and regulation of fatty acid. It is predominately expressed in the liver and it is inducible in response to feeding. Transcript level of ACC is increased in obesity resulting in production of free fatty acid (Jeffery *et al.*, 2013). mRNA levels of ACC were increased in cafeteria treated group. As a result of administration of *G. indica* fruit juice, significant reduction in its level was observed, suggesting decrease in free fatty acid levels. However, other transcriptional factors need to be evaluated for further confirmation. Thereby, present study has clearly shown the *G. indica* fruit juice could be a strong candidate for a hypolipidemic drug. Further, investigations needs to be done to mark the product as a dyslipidemic nutraceutical. Liver and kidney function tests showed that after 10 weeks of treatment with cafeteria diet there was significant increase in SGPT activity and serum creatinine, respectively, suggesting that prolong dose of cafeteria diet can alter liver and kidney function (Chidrawar *et al.*, 2012). After treatment with *G. indica* fruit juice, it was observed that there was significant

decrease in SGPT activity and serum creatinine levels. It suggests that *G. indica* fruit juice may have potential to restore liver and kidney functions. These results were not observed in the group treated with standard drug.

5. Conclusion

Increasing evidences are suggesting the significant impact of plant derived phytocomponents in the regulation of different aspects of human physiology and thereby, aid in the treatment and management of cardiovascular diseases and metabolic syndrome like obesity and dyslipidemia. In the current study, cafeteria diet induced obesity model was successfully developed which exhibited the associated complications of metabolic syndrome such as increased body weight, dyslipidemia, and insulin resistance after 10 weeks. Also, the potential of *G. indica* fruit juice to ameliorate dyslipidemia was evaluated in cafeteria diet fed obese rat model. It was observed that treatment with *G. indica* fruit juice at a dose of 1 ml for 4 weeks could restore the body weight, dyslipidemia, glucose sensitivity, insulin resistance and expression and activities of key lipid metabolizing enzymes in cafeteria diet induced dyslipidemic rat model. The observed therapeutic potential was better than orlistat, which is the standard drug used for treatment of dyslipidemia and obesity. Thereby, suggesting that phytocomponents such as HCA present in *G. indica* fruit juice is a potent anti-dyslipidemic agent without inducing toxicity. However, phytocompounds from *G. indica* can be further analysed for understanding its molecular targets towards amelioration of dyslipidemia.

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Conflict of interest

The authors declare that there are no conflicts of interest relevant to this article.

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Synergistic Interplay of Hyperandrogenism and Hyperinsulinism on Primary Culture of Luteinized Granulosa Cells – an "*in-vitro*" Model Mimicking Ovarian Microenvironment of Poly-Cystic Ovary Syndrome (PCOS)

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Abstract

Poly-Cystic Ovary Syndrome (PCOS) is the most prevalent endocrine disorder, characterized by hyperandrogenism and hyperinsulinemia, both at systemic and ovarian levels. This study investigated the synergistic effect of hyperinsulinemia and hyperandrogenism on the regulatory mechanism of ovarian steroidogenesis using Luteinized Granulosa Cells (LGCs). LGCs were isolated from 40 weaning female Charles Foster rats by superovulation by PMSG and characterized for purity and stability in modified DMEM: F12 media. The isolated cells were divided into following groups- control, hyperinsulinic group (0.1-2 mIU/mL of insulin), excess androgen (10-100 ng/mL of DHT) and combination of both. One-way ANOVA was performed with a Bonferroni post-hoc test. Results demonstrate that the LGCs exhibit reduced expression of FSHR and CYP19A and increased expression of LHR, StAR and CYP17A1 at 72 hours. There was reduction in cell viability of LGCs when induced with hyperinsulin and hyperandrogen doses individually or in combination.0.1 mIU/mL of insulin and 50 ng/mL of DHT in combination were the minimum effective dose in inducing PCO like ovarian microenvironment in the primary culture of LGCs. There was exaggerated androgen biosynthesis, reduced progesterone secretion and non-significant change is estradiol levels in the LGCs. The abnormal steroidogenesis can be attributed to upregulation of key genes such as StAR, CYP17A1, AMH and SREBP1-c and down-regulation of genes like CYP19A1, HSD3B2, IGFBP1 and SHBG. This model can be used to study downstream signaling pathways involved with dysregulated ovarian microenvironment as observed in PCOS at cellular level and for screening of drug targets for such pathological conditions.

Keywords: Hyperinsulinemia, Hyperandrogenemia, Luteinized Granulosa Cells, Polycystic Ovary Syndrome, Primary Culture, Steroidogenesis

1. Introduction

Poly-Cystic Ovary Syndrome (PCOS) is defined by the presence of polycystic ovaries, chronic anovulation and hyperandrogenism, leading to metabolic syndrome and infertility. Pathological etiologies of this syndrome are extremely complex as there is link between the intrinsic and extrinsic factors further leading to complication of hyperandrogenemia and hyperinsulinemia[1]. It is reported that women suffering from PCOS exhibit selective insulin resistance, i.e., they have defective insulin signaling due to enhanced insulin receptor (IR) and insulin receptor substrate-1(IRS-1) serine phosphorylation[2]. Also, there is excess insulin production in these women

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due to hyper secretion of Luteinizing Hormone (LH), thereby, stimulating IR and Insulin like growth factor 1 receptor (IGFR1) further leading to perturbed ovarian steroidogenesis[3]. The serum levels of insulin are increased in PCOS women[4] along with an increase in the follicular fluid[5]. Hyperinsulinemia reinforces local insulin-like growth factor (IGF)-I activity in the ovary and, as a result, this induces an androgenic composition in theca cells[6]. Both increased IGF-I activity and a high androgenic environment appear to be involved in the ovulation disorder in PCOS patients[7].

Another important cause of hyperandrogenism in PCOS is modulation of LH and over expression of Luteinizing Hormone Receptors /Human Chorionic Gonadotropin Receptor (LHR/hCGR), further rendering them to be hypersensitive to LH stimuli along with hypersecretion of androgens in theca cells[8]. In addition to this, reduction in inhibition of Gonadotropin Releasing Hormone (GnRH) pulse frequency by progesterone, promotes the development of the PCOS phenotype[9]. Recent studies have demonstrated that 65% of PCOS patients exhibit high titres of androgens, reduced estrogen-to-testosterone (E2/T) ratio as well as insulin resistance in the follicular fluid in spite of having normal serum androgen levels[10]. Normal physiologica androgen levels in follicular fluid is found to be 5-20 ng/mL[11], whereas clinical samples of PCOS patients are known to have 10-100 ng/mL androgen levels in the follicular fluid[12]. The microenvironment of the ovary critically influences the follicular development and hence, it is still unclear as to how hyperandrogenemia and hyperinsulinemia present in the PCOS ovarian microenviroment synergistically causes ovarian dysfunction associated with PCOS. Therefore, it would be interesting to develop an *"in vitro"* model wherein, there is co-exposure of insulin and androgen, which would allow us to study independent as well as cross-talk events at cellular levels. The present study has attempted to develop an *"in vitro"* ovarian microenvironment model of PCO phenotype.

2. Materials and Methods

2.1 Chemicals

Pregnant Mare's Serum Gonadotropin (PMSG), Insulin (40IU), Bovine Serum Albumin (BSA), 5-Androsten-3b-ol-17-one sulfate sodium salt and Transferrin were procured from Sigma Aldrich India. DMEM: F12 (1:1) and Penstrep solution were procured from GIBCO®. Human Menopausal Gonadotrophin (HMG) [HUMOG®-150 IU] was procured from Bharat Serums and Vaccines Limited. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), EGTA [ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid], sucrose, trypan blue and di-methyl sulf-oxide (DMSO) were procured from HiMedia Laboratories Pvt. Ltd. Hank's Balanced Salt Solution (HBSS) and Phosphate Buffered Saline (PBS) were prepared manually in the laboratory using the standard recipe. Sterile cell culture plastic wares were procured from Corning Inc. RNAiso Plus was procured from Takara Inc. High-Capacity cDNA Reverse Transcription Kit was procured from Applied Biosystems. Primers for key steroidogenic and metabolic genes were designed by primer blast tool of NCBI and synthesized by INTEGRATED DNA TECHNOLOGIES (IDT). Hormones- testosterone, estradiol and progesterone were assayed using direct ELISA kits (DBC Canada).

2.2 Animals

Forty weaning Charles Foster female rats were chosen for the study which were housed in standard controlled animal care facility, in cages (four rats/cage), and maintained in a temperature-controlled room (22-25°C, 45% humidity) on a 12: 12-hour dark-light cycle. The animals were maintained under standard nutritional and environmental conditions throughout the experiment. All the experiments were carried out between 9:00 and 16:00 hours, at ambient temperature. The CPCSEA guidelines were strictly followed and all the studies were approved by the Institutional Animal Ethics Committee (IAEC), Department of Biochemistry, The M. S. University of Baroda, Vadodara (Ethical Committee Approval Number (MSU/BIOCHEMISTRY/IAEC/2018/06)).

2.3 Luteal Granulosa Cell (LGC) Isolation from Rat Ovaries

Forty weaning female rats were super-ovulated in batches of four animals, by injecting 10 I.U of pregnant mare's serum gonadotropin (PMSG) subcutaneously. After 48 hours, 50 I.U of Human Chorionic Gonadotropin (hCG) was injected to the animals intra-peritoneally (ip). After 24 hours, the ovaries from four super-ovulated rats were excised and processed for isolation of granulosa- lutein

cells by the Campbell method[13]. In this procedure, ovaries were initially punctured and then incubated in EGTA followed by hypertonic sucrose at 37°C. The ovaries were then gently squeezed to release the cells into Hank's Balanced Salt Solution (HBSS) and further centrifuged over 45% Percoll to remove RBCs. Luteal cells were isolated from the interface, washed 3-4 times with HBSS and resuspended in 1 mL of modified serum- free DMEM/F12 media (Media Composition: DMEM/ F12 (1:1), 0.1% BSA, Insulin (10 ng/mL), hMG (50 ng/mL), 5 µg/mL Transferrin, 1% Penstrep (100 U/mL Penicillin, 100 µg/mL Streptomycin) and the cell yield was estimated using trypan blue exclusion method[14].

2.4 Characterization of Isolated Luteinized Granulosa Cells

Cultures of luteinized granulosa cells were tested for cell type purity by analysis of the transcript abundance of key molecular targets such as Follicle-stimulating Hormone Receptor (FSHR), Luteinizing Hormone Receptor (LHR), Steroidogenic Acute Regulatory protein (StAR), Cytochrome P450 family 19 subfamily a member 1 (CYP19A1) and Cytochrome P450 Family 17 subfamily A member 1 (CYP17A1). The crude cell isolates were counted with a hemocytometer and diluted to 1×10^5 cells/ mL and cultured in 1.0 mL of modified media in 35-mm polystyrene tissue culture treated dishes. Cells were collected at different time intervals of 24 hours upto 72 hours and their RNA was extracted using RNAiso Plus reagent and reverse transcribed to form cDNA. The relative expression of the molecular targets mentioned above was studied by Real-time quantitative Polymerase Chain Reaction (qPCR). The details of RNA isolation, cDNA preparation, primers and Real time qPCR are explained below.

2.5 Culture and Development of PCOS Conditions in Luteinized Granulosa Cells Primary Culture

1×10^6 cells were cultured in modified serum- free DMEM/ F12 medium and the culture conditions consisted of humidified atmosphere of 5% CO2 and 95% air at 37^0C for 24 hours. Thereafter, the cells were incubated with insulin [0.1 – 2 IU/mL (0.7 – 13.9 nM)] and Di-Hydro-Testosterone (DHT) [10 – 100 ng/mL (34.4 – 344 µM)] treatments separately as well as in combinations for 36 hrs. The concentrations of insulin and DHT used in these experiments were significantly greater than the concentrations present in the normal women. However, the concentrations used in these experiments were within an order of magnitude of the concentrations present in the patients with severe insulin resistance[15]. In order to exclude the effect of conversion of testosterone to estradiol, we utilized DHT, instead of testosterone, as the androgen for assessment of the appropriate AR action. At the end of the treatment period, spent medium was collected at different time intervals and stored at -20°C until assayed for hormonal content. For gene expression analysis, treated and control cells were lyzed for RNA extraction.

2. 6 Cell Viability Assay by MTT

Cells were seeded in a 96-well flat-bottom microtiter plate at a density of 1×10^4 cells/well and allowed to adhere for 24 hours at 37°C in a CO2 incubator. After 24 hours of incubation, the culture medium was replaced with a fresh medium. Cells were then treated with various concentrations of insulin and DHT for 36 hours at 37°C in a CO2 incubator. After 36 hours of incubation, the culture medium was replaced with a fresh medium and 10µL of MTT working solution (5 mg/mL in phosphate buffer solution) was added to each well and the plate was incubated for 2-3 hours at 37°C in a CO2 incubator. The medium was then aspirated, and the formed formazan crystals were solubilized by adding 100µL of DMSO per well for 30 min at 37°C in a CO2 incubator. Finally, the intensity of the dissolved formazan crystals (purple color) was quantified using the ELISA plate reader at 570 nm and 620nm.

2.7 RNA Isolation and Real time qPCR

Total RNA was obtained using RNAiso Plus reagent as per the manufacturer's instructions. The quantification was performed using NanoVue Plus spectrophotometer (GE Healthcare Life Sciences) at a wavelength of 260nm. RNA integrity was assessed by electrophoresis in a 1.2% agarose gel stained with ethidium bromide. Purity was assessed through absorption rate OD260/OD280 and samples showing a value less than 1.8 were discarded. The reverse transcription reaction to cDNA was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to manufactures instructions. Real-time qPCR was performed using

QuantStudio 5 Real Time PCR system using SYBR Green (Power SYBR Green PCR Master Mix - Life Technologies, USA). All samples were run in triplicate and accompanied by a non-template control. Thermal cycling conditions included initial denaturation in one cycle of 2 min at 95°C, followed by 40 cycles of 15s at 95°C, 1 min at 60°C and 1 min at 72°C. After amplification, the melting curves were analyzed to verify the amplification of only one product. The relative mRNA expression and fold change was calculated based on the amplification of the reference gene beta act in (ACTB). The primers used for the amplification are given in Table 1. The fold changes in expression levels of less than 0.5 and greater than 2 were considered to be biologically significant.

2.8 Measurement of Hormone Levels

Spent media were collected after giving treatments and lyophilized and then dissolved in Phosphate buffered saline. It was used as samples to estimate the testosterone, estradiol and progesterone levels using kit-based direct ELISA, procured from Diagnostics Biochem Canada (DBC) as per the manufacturer's instructions. Each sample was assayed in duplicate. Sensitivity of the kits was 0.022 ng/mL, 10 pg/mL and 0.1 ng/mL for testosterone, estradiol and progesterone kits respectively. The working range was 0.08 to 116.7 ng/mL, 20 to 3200 pg/mL and 0.3 to 60 ng/mL of testosterone, estradiol and progesterone respectively. The intra-assay Coefficient of Variation (CV) was between 6.6% and 9.6%, 4.6% and 9.3% and 10.2% and 10.6% for testosterone, estradiol and progesterone kits respectively. The inter-assay CV was between 6.1% and 7.3%, 6.2% and 10.1% and 10.2% and 12.6%, for testosterone, estradiol and progesterone kits respectively. The recovery range was between 80.5% and 110.1%, 90.3% and 116.2% and 78% and 124% for testosterone, estradiol and progesterone kits respectively.

2.9 Statistical Analysis

The values are presented as mean ± standard error of mean in all the experiments. Statistical analysis was done using one-way analysis of variance followed by Bonferroni multiple comparison test (GraphPad Prism 5 software, La Jolla, CA). The p values when less than 0.05 were considered to be statistically significant at 95% confidence limit.

Table 1. List of primers used in the study along with the amplicon size.

Gene	Accession Number	Sequence (5' →3')	Product Size	Annealing Tempera-ture
FSHR	NM_199237.1	F:ACGCCATTGAACTGAGGTTT	148	58
		R:TTGGGTAGGTTGGAGAACAC		
CYP19A1	NM_017085.2	F:CCTGGCAAGCACTCCTTATC	199	58
		R:CCACGTCTCTCAGCGAAAAT		
CYP17A1	NM_012753.2	F: ATGATCCAAAACTGACCGCC	132	60
		R:AACCCTTTATCACCTCCAAGCC		
HSD3B2	NM_017265.4	F:GGATCCTTTGCAGAGACCAG	147	58
		R:TGGAGATGCTCAGCCACA		
SHBG	NM_012650.1	F:TATTCTGAGCCACTGGGT	153	60
		R:GAGCACTCTGGATAGGGTCAAT		
SREBF1-c	NM_001276708.1	F: TCAGTTCCAGCATGGCTACC	174	60
		R:TGGGAAGGGGTCTCTCAGTT		
IGFBP1	NM_013144.1	F:CACAGCAAACAGTGCGAGAC	162	60
		R:GGAGGGAGGAAACAACTTTCAG		
StAR	NM_031558.3	F:AGTGACCAGGAGCTGTCCTA	216	58
		R:GCGGTCCACCAGTTCTTCATA		
ACTB	NM_007393	F: ACTGTCGAGTCGCGTCC	88	60
		R:TCATCCATGGCGAACTGGT		

3. Results

3.1 Isolation and Characterization of Luteinized Granulosa Cells

Results demonstrate that $10.94\times10^6 \pm 0.7708$ cells were obtained when ovaries from four animals were pooled. The isolated cells were further cultured for 72 hours and their characteristics such as cell viability and molecular markers were analyzed upto 72 hours. Cell viability assay by MTT demonstrated that around 50% of luteinized granulosa cells (LGCs) were viable up to 72 hours (Figure 1). There was a time-dependent decline in the cell viability which was not perfectly linear. The results were further confirmed using trypan blue exclusion assay. Characterization of the isolated LGCs (Figure 2) demonstrated the presence of FSHR and CYP19A1 in the isolated cells up to 72 hours suggesting that the isolated cells were indeed granulosa cells. However, the expression of FSHR decreased over time, which might be attributed to the fact that the cells might be undergoing luteinization. In addition to this, there was an increase in the expression of LHR, StAR and CYP17A1 from 48 hours onwards, suggesting that the isolated granulose cells have undergone luteinization. The LGCs are essential for normal oocyte development and steroid hormone production. Therefore, they are a good model for the current study.

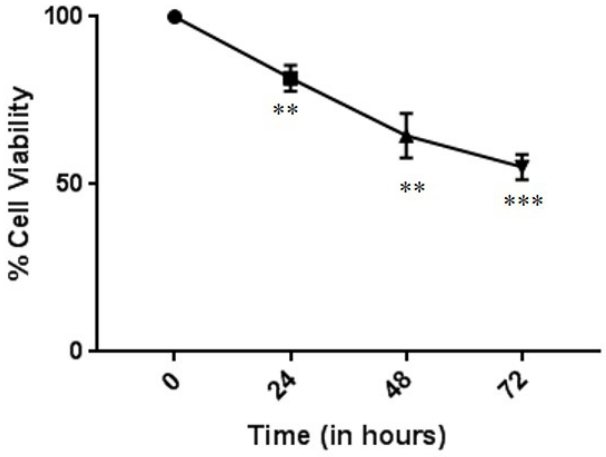

Figure 1. Cell viability of isolated luteinized granulosa cells in a time –dependent manner when grown in modified serum free media by Trypan blue exclusion assay. The values are represented as Mean plus/minus SEM. N=6-8. **p<0.01, ***p<0.0001 as compared to 0 hour.

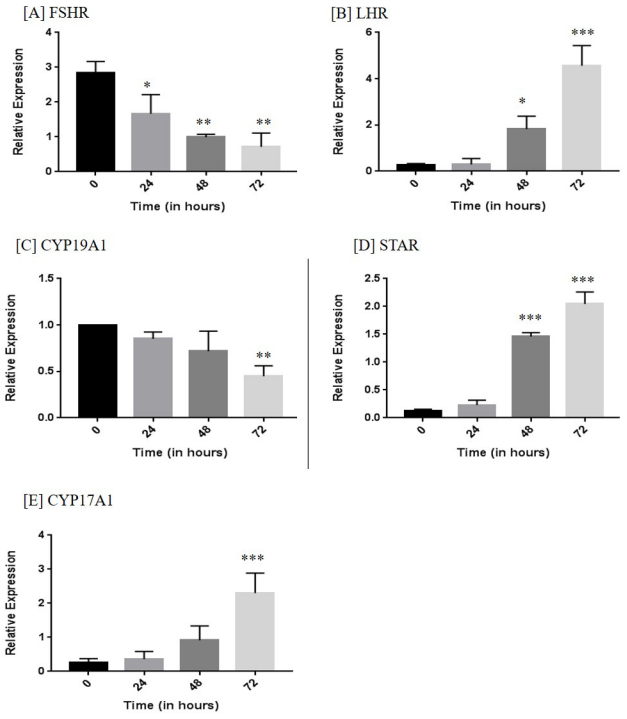

Figure 2. Characterization of isolated luteinized granulosa cells markers [**A**] *FSHR*, [**B**] *LHR*, [**C**] *CYP19A1*, [**D**] *STAR* and [**E**] *CYP17A1* by estimating their transcript abundance. The values are represented as Mean plus/minus SEM. N=6-8. *p<0.05, **p<0.01, ***p<0.0001 as compared to 0 hour.

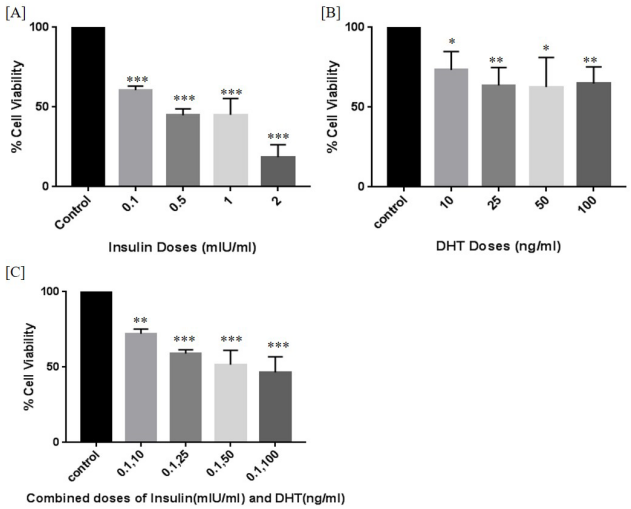

Figure 3. Cell viability assay by treating the isolated luteinized granulosa cells with [**A**] insulin doses (0.1, 0.5, 1.0 and 2.0 mIU/mL); [**B**] DHT doses (10, 25, 50 and 100 ng/mL) and [**C**] Combined doses of insulin and DHT. The values are represented as Mean plus/minus. N=7. *p<0.05, **p<0.01, ***p<0.001 as compared to control.

3.2 Effect of Hyperinsulinemia on Cell Viability of Luteinized Granulosa Cells

Results from MTT assay demonstrated that LGC exhibited significant dose- dependent decline in cell viability when incubated with insulin (Figure 3A). The dose of 0.1mIU/mL of insulin was found to be the minimum effective dose; therefore, it was used for further co-administration studies along with varying concentrations of androgen for development of "*in-vitro*" PCO condition.

3.3 Effect of Hyperandrogenemia on Cell Viability of Luteinized Granulosa Cells

Results revealed that all the treatment groups i.e. 10, 25, 50, and 100 ng/mL of DHT dose significantly decreased the cell viability when compared to the control (Figure 3B). It is important to note that results werenot significant as observed in case of hyperinsulinemia. Moreover, androgens are known to act differentially when they are present in high levels along with other growth factors like insulin or insulin like growth factor due to synergistic effects[16]. Therefore, all the four doses (i.e. 10, 25, 50 and 100 ng/mL) of DHT were selected for the further co-administration studies.

3.4 Synergistic Effect of Hyperinsulinemia and Hyperandrogenemia on Cell Viability and Morphology of Primary Culture of Luteinized Granulosa Cells

Insulin at 0.1 mIU/mL of insulin was considered as the optimum dose for inducing hyperinsulinemia along with varying concentrations of androgen (10, 25, 50 and 100 ng/ml of DHT) for induction of hyperandrogenemia in the ovarian microenvironment. Results from MTT assay demonstrated that all the treatment groups exhibited greater than 50% cell viability (Figure 3C) suggesting that the doses are non-toxic to the cells. The cells were observed under inverted light microscope at 20X magnification and it was observed that the primary culture of granulose cells had normal and healthy morphology in all the treatment groups (Figure 4).

3.5 Synergistic Effect Of Hyperinsulinemia and Hyperandrogenemia on the Transcript Levels of Key Steroidogenic Targets in Primary Culture of Luteinized Granulosa Cells

Results showed that, gene expression of *StAR, CYP17A1, SREBP1-c* and *AMH* increased significantly when the primary LGCs were co-administered with 0.1 mIU/mL of Insulin along with 50 and 100ng/mL of DHT. However, a significant decrease in the gene expression of *CYP19A1, HSD3B2, SHBG* and *IGFBP1* was observed when primary LGCs were co-administered with 0.1 mIU/mL Insulin and 50 ng/ml DHT (Figure 5). Thereby, minimum effective dose of Insulin and DHT to mimic ovarian microenvironment present in PCO using an "*in vitro*" primary culture of LGCs was found to be a combined dose of 0.1 mIU/mL and 50 ng/mL respectively. Further validation of the same can be done on the basis of hormone secretion by the primary culture of LGCs.

3.6 Synergistic Effect of Hyperinsulinemia and Hyperandrogenemia on the Hormones Secretion by Primary Culture of Luteinized Granulosa Cells

Table 2 reveals that there was an increase in the secretion of testosterone by the primary culture of LGCs, when they are subjected to hyperinsulin (0.1mIU/ml) and hyperandrogen (50 and 100 ng/mL) conditions ($p<0.05$ and $p<0.001$ respectively). On the other hand, there was a significant decrease in the secretion of progesterone by the LGCs upon co-administering them with 0.1mIU/mL of insulin and 50 and 100 ng/mL of androgen ($p\leq0.5$ and $p\leq0.05$). However, no significant difference was found in the levels of secreted estradiol. Data suggests that there is altered steroidogenesis by the primary culture of LGCs when they are subjected to "*in-vitro*" PCO like ovarian microenvironment. Thus, we can conclude that co-administration of 0.1 mIU/mL of insulin and 50 ng/mL of DHT is the minimum effective dose for developing "*in-vitro*" PCO like condition in LGCs.

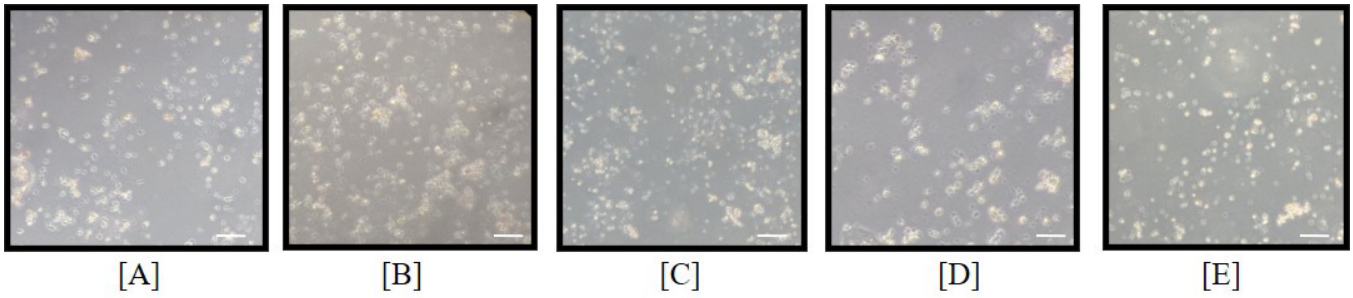

Figure 4. The morphology of the isolated luteinized granulosa cells as observed at 40x magnification of inverted light microscope. [**A**] control; [**B**] 0.1 mIU/mL Insulin,10 ng/mL DHT; [**C**] 0.1 mIU/mL insulin, 25 ng/mL DHT; [D] 0.1 mIU/mL insulin, 50 ng/mL DHT; [E] 0.1 mIU/mL insulin, 100 ng/mL DHT.

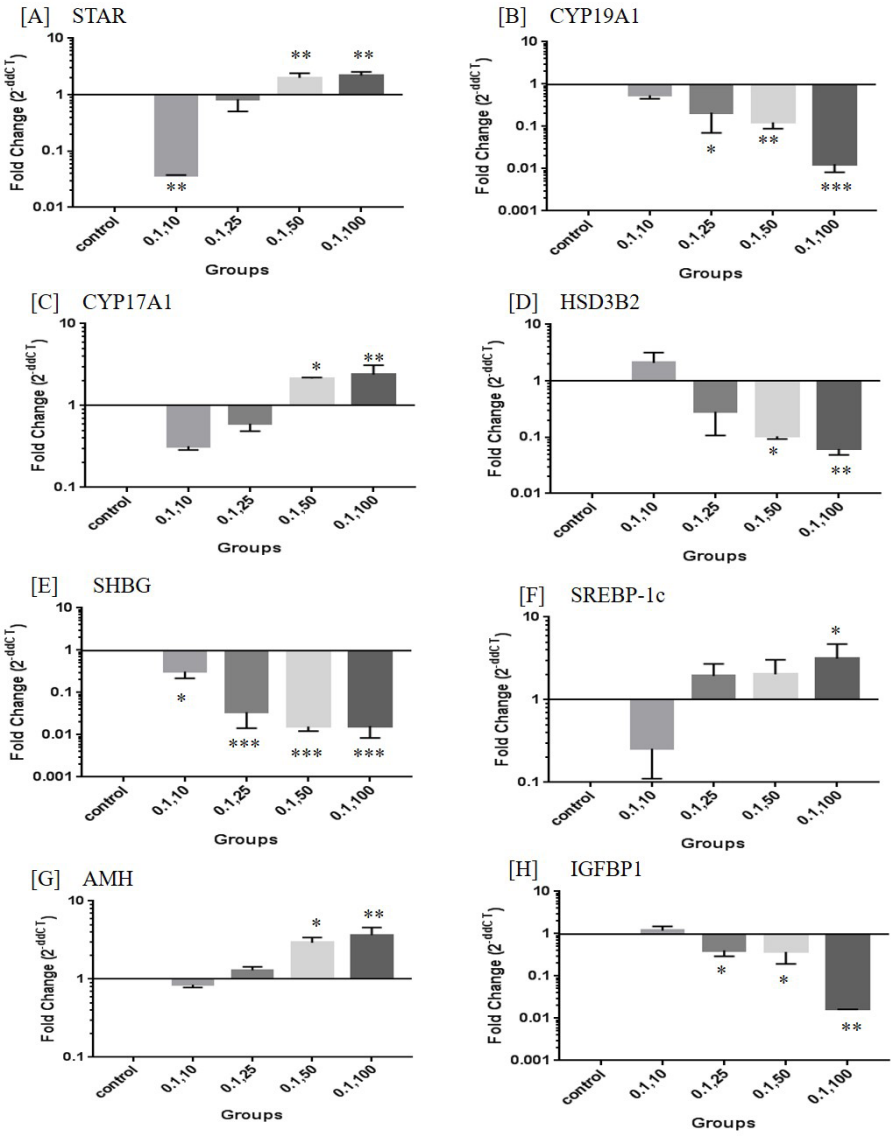

Figure 5. Synergistic effect of hyperinsulinemia and hyperandrogenemia on the transcript levels of key steroidogenic targets [**A**] STAR, [**B**] CYP19A1, [**C**] CYP17A1, [**D**] HSD3B2, [**E**] SHBG, [**F**] SREBP1-c, [**G**] AMH and [**H**] IGFBP1 of isolated luteinized granulosa cells. The values are represented as Mean plus/minus SEM. N=6-8. *p<0.05, **p<0.01, ***p<0.0001 as compared to control.

Table 2. Synergistic effect of hyperinsulinemia and hyperandrogenemia on the hormone secretion by the primary culture of luteinized granulosa cells

	Control	0.1,10 (mIU/mL Insulin, ng/mL DHT)	0.1,25 (mIU/mL Insulin, ng/mL DHT)	0.1,50 (mIU/mL Insulin, ng/mL DHT)	0.1,100 (mIU/mL Insulin, ng/mL DHT)
Testosterone (ng/ml)	0.11+ 0.003	0.9+0.010	7.93+0.005**	28.47+ 0.93***	36.4+ 0.43***
Estradiol (pg/ml)	142+ 3.679	121+0.041	150+ 4.952	140+0.284	135+ 11.53
Progesterone (ng/ml)	58+4.75	42+9.10	20+2.46*	17.15+ 0 .62**	25.80+ 0.035**

The values are represented as Mean+ SEM. N=6-8. **p<0.01, ***p<0.0001 as compared to Control.

4. Discussion

PCOS is the most common endocrinopathy of women in reproductive age. Since quite long, it has been debated that the ovarian dysfunction in PCOS is a consequence of high levels of insulin and androgens in circulation[17]. However, recent reports have shown that ovarian microenvironment plays a crucial role in maintenance of the ovarian dynamics[18]. Therefore, any alteration in the ovarian microenvironment would lead to to perturbed follicular development, oogenesis, ovulation and steroidogenesis. Therefore, the present study was aimed at developing an *"in-vitro"* model of PCO that mimics their ovarian microenvironment and study its implication on the most important cells of the ovary- granulosa luteinized cells. In this context, the luteinized granulosa cells were isolated from weaning female rats by superovulation and characterized for their purity by studying the molecular markers like *FSHR, CYP19A1, LHR, StAR* and *CYP17A1*. Data showed that letting animals superovulate on treatment with PMSG and hCG, lead to high yield of LGCs. Similar procedures have also been performed in the past for obtaining granulosa cells from rats[19], mice[20], rabbits[21], buffaloes[22] and pigs[23], suggesting that this is the most standard protocol of granulosa cell isolation.

The isolated luteinized granulosa cells were grown in serum free conditions using modified DMEM/F12 medium and the cells were found to be stable in the culture for at least 72 hours. In this regard, results from literature have shown that luteinized granulosa cells are stable upto 8 days, when grown in serum free conditions containing FSH[24]. Therefore, the luteinized granulosa cells are a good and stable *"in-vitro"* model to study the signaling cascades involved in steroidogenesis, folliculogenesis and ovulation. The isolated LGCs demonstrated the presence of *FSHR* and *CYP19A1* during the course of experiment. However, there was a decline observed in the relative expression of these genes beyond 24 hours. On the other hand, there was an increase in the gene expression of *LHR, StAR and CYP17A1* after 48 hours of culture condition, suggesting that the cells might be undergoing luteinization. Similar results have been observed when granulosa cells isolated from human derived follicular fluid were cultured in serum free conditions for a prolonged time[25]. After establishment of the conditions for luteinized granulosa cell primary culture, an induction of hyperandrogen and hyperinsulin was given to cells.

The isolated LGCs demonstrated a dose dependent decline in the cell viability when treated with 0.1 mIU/mL-2 mIU/mL of Insulin and 10-100 ng of DHT independently. 0.1 mIU/mL of Insulin was found to be the minimum effective dose for induction of hyperinsulinemia in the primary culture of LGCs. There is a long debate regarding the varying levels of androgens present in the follicular fluid of PCOS patients[26]. Therefore, a dose dependent study was performed by varying DHT concentrations in combination with 0.1 mIU/mL of Insulin. It was observed that a dose dependent decrease in cell viability in the cultured LGCs upon co-administration of hyperinsulin and hyperandrogen conditions. Similar studies have not been performed in the past. However, in some isolated studies it has been shown that incubating granulosa cells with IGF1 and/or DHT lead to induction of hyperinsulinemia and hyperandrogenic condition in the cells[27,28].

Ovarian microenvironment influences the ovarian structure-function. The follicular fluid of PCOS patients contains high androgen and high insulin titers, along with disturbed steroidogenesis[29]. Intra-ovarian hyperandrogenism may be causatively linked with anovulation in PCOS[30]. It has also also studied that hypersecretion of insulin is responsible for excess production of androgens in theca interna cells by impaired tyrosine kinase Ser-phosphorylation of inositolglycan[31] and also reduced hepatic production of sex hormone binding globulin, resulting in higher concentrations of free androgens in circulation[32]. In this regard, it would be interesting to study the impact of hyperandrogenic and hyperinsulinemic condition in combination on the important cells of the ovaries- luteinized granulosa cells. Data demonstrates that there is an alteration in the markers of follicular development, steroidogenesis and luteinization, mainly upregulation of key genes such as *StAR*, *CYP17A1, SREBP1-c* and *AMH* and down-regulation of genes like *CYP19A1, HSD3B2, SHBG* and *IGFBP1* has been observed when primary culture of LGCs were induced with "*in-vitro*" PCO-like microenvironment. It has been demonstrated in the ovary that *StAR* expression highly correlates with steroidogenic activity, because the increased production or concentration of *StAR* may result in abnormal steroidogenesis found in PCOS[33]. Evidence suggests that patients with PCOS may have an abnormality in androgen biosynthesis, with StAR as one possible target of abnormality. Kashar-Miller, *et al.*[34] hypothesized that increased production or concentration of StAR may be responsible for the increased ovarian and adrenal androgen found in PCOS of some patients. In addition to StAR, differential activity of the cytochrome p450 17α-hydroxylase (CYP17A1) gene promoters have been observed in PCOS theca cells[35,36].

There are several reports in this regard, which show higher expression of *CYP17A1* at both transcriptional and post-transcriptional level in the ovaries of PCOS women and it is partly responsible for the perturbed steroidogenesis[37]. Also, 4-folds greater *CYP17A1* promoter activity was observed in the theca interna cells of human polycystic ovaries[38]. Recent studies have linked aberrant regulation of cholesterol mobilization in PCOS, through up-regulation of SREBP1-c, suggesting that this pathway might play a role in the manifestation of PCOS[39]. The gene expression of *SREBP1-c* has been found to significantly increase in the endometrium of women with PCOS and endometrial cancer compared with controls[40].

Another important regulator of ovarian activity is Anti-Mullerian Hormone (AMH). It plays plays a crucial role in the follicular recruitment[41,42]. Studies from the past decade have shown that AMH is modulated by hyperandrogenism and hyperinsulinemia, leading to abnormal follicular dynamics in PCOS pathology[43,44]. AMH levels in the serum of PCOS patients are two or three times higher than average women[45]. It is surprising to note that AMH production by granulosa cells in the polycystic ovary is 75 times higher compared to healthy women[46]. Additionally, histological analysis of ovaries of PCOS patients have shown an increased AMH expression in the granulosa cells, which coincide with an increased number of preantral and small antral follicles, 2–3 times greater than that found in normal ovaries[47]. In women with PCOS, elevated levels of AMH appears to play an important role in long term disruption of ovarian physiology[48], with greater AMH concentrations being linked to poor fertility outcomes[49].

The down-regulation of genes like *CYP19A1, HSD3B2, SHBG* and *IGFBP1* can be considered to be responsible for the elevated androgen biosynthesis and reduced progesterone secretion. Also, high levels of LH and hyperactive theca cells as well as altered granulosa cell activity results into decreased estradiol and progesterone production[50]. Aromatase is encoded by *CYP19A1* gene and is responsible for conversion of androgens (androstenedione and testosterone) to estrogens (estrone and estradiol) during steroidogenesis. Insulin stimulates aromatase activity and induces the development of antral follicles due to the greater sensitivity of granulosa cells to FSH[51]. There is evidence that high concentrations of insulin result in premature differentiation of the granulosa cells and follicular arrest[52]. Also, follicular hyperandrogenism downregulates aromatase in luteinized granulosa cells in PCOS women[53]. Clinical studies demonstrate that luteinizing granulosa cells from follicles of patients with PCOS have a reduced capacity to synthesize progesterone "*in-vitro*" due to reduced 3BHSD gene expression[54]. In addition to this, mutations in 3BHSD and SHBG are responsible for the excess androgen production in the ovaries of PCO patients[55]. Also, PCOS patients demonstrate low serum concentration of IGFBP1, which has been found to be associated with high concentrations of circulating androgens and a greater risk of endometrial hyperplasia and neoplasia[56]. These reports clearly suggest that PCO like phenotype has been developed in the primary culture of

LGCs induced by co-administration of hyperandrogen and hyperinsulin

5. Conclusion

In the present study, an "*in-vitro*" model of PCO that mimics the ovarian microenvironment has been successfully developed by co-induction of hyperinsulinemia and hyperandrogenemia in the primary culture of luteinized granulosa cells isolated from rat ovaries. Co-administration of 0.1 mIU/mL of Insulin and 50 ng/mL of DHT to the LGCs could effectively modulate the key genes involved in steroidogenesis, folliculogenesis and ovulation at molecular level along with abnormal steroid hormone secretion by the cells. The development of an "*in-vitro*" PCO model is novel and holds huge potential towards studying detailed downstream cellular signaling as well as screening drug targets for ovarian dysfunctions like PCOS, ovarian cancer and infertility.

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7. Conflict of Interest

The authors declare that they have no conflict of interest.

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Review Article

Nutraceuticals as Therapeutic Agents for Management of Endocrine Disorders - Sources, Bioavailability and Mechanisms Underlying their Bioactivities

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Abstract

Nutraceuticals refer to health promoting bioactive compounds found in some foods or fortified food products/plant derived products. There is an increase in the consumption of nutraceuticals by mankind over the past decade due to their enriched biological activities and low or no side effects. Nutraceuticals can include inorganic mineral supplements, vitamins, probiotics, prebiotics, dietary fibres, antioxidants, phytochemicals like phytosterols, fatty acids, phenolics, flavonoid, isoprenoids, proteins etc. Dietary consumption of these neutraceuticals has shown to be effective endocrine modulators. However, detailed review with respect to endocrine modulators has not been clearly documented. Thereby, present review focuses on the sources, bioavailability and pharmacological properties of neutraceuticals towards endocrine modulation in detail.

Keywords: Endocrinology; Diabetes; Nutraceuticals; Obesity; Reproductive Dysfunctions

Abbreviations

PUFA: Polyunsaturated Fatty Acids: HbA1c: Glycated Haemoglobin; CRP: C-Reactive Protein; SOD: Superoxide Dismutase; GSH-Px: Glutathione Peroxidise; MDA: Malondialdehyde; IRS-1:Insulin Receptor Substrate 1; IR: Insulin Receptor; IGFBP4: Insulin-like growth factor binding protein 4; IGFBP5: Insulin-Like Growth Factor Binding Protein 5; AMPK: 5' Adenosine Monophosphate-Activated Protein Kinase; MAPK: Mitogen-Activated Protein Kinase; SIRT1: NAD-Dependent Deacetylase Sirtuin-1; LPS: Lipopolysaccharides; COX-2: Cyclooxygenase 2; NF-kB: Nuclear factor kB; IL-1β: Interleukin 1β ; IL-6:Interleukin 6; PPAR: Peroxisome Proliferator-Activated Receptors: FFA: Serum Free Fatty Acid; TG: Triglycerides; GLUT4: Solute Carrier Family 2: Facilitated Glucose Transporter Member 4; LXR-α: Liver X Receptor-alpha; LXR-β: Liver X Receptor-beta; ABCA1: ATP-binding Cassette Transporter A1; ABCG5: ATP-binding cassette transporter G5; ABCG8: ATP-Binding Cassette Transporter G8; SREBP1C: Sterol-Regulatory Element Binding Protein 1C; SCD1: Steroyl CoA Desaturase 1; FASN: fatty Acid Synthase; ACC: Acetyl-CoA Carboxylase; ZDF: Zucker Diabetic Fatty; CYP3A4: Cytochrome p450: Family 3: Subfamily A: Polypeptide 4; CYP1B1: Cytochrome p450: Family 1: Subfamily B: Polypeptide 1; CYP1A1: Cytochrome p450, Family 1, Subfamily A, Polypeptide 1; 16-OHE1: 16-Hydroxyestrone; 2-OHE1: 2-hydroxyestrone; 3β-HSD: 3β-Hydroxysteroid dehydrogenase; 17b-HSD: 17β- Hydroxysteroid Dehydrogenase; LHR: Luteinizing Hormone Receptor; P450scc: Cholesterol Side-Chain Cleavage Enzyme; ER- α: Estrogen Receptor alpha; AR: Androgen Receptor; StAR: Steroidogenic Acute Regulatory Protein; PCOS: Polycystic Ovary Syndrome.

Introduction

The word "neutraceutical" has been derived by combining two essential domains: "nutrition" and "cure", for the large number of plant derived compounds that possess both dietary importance and potential therapeutic activity. There has been an exponential increase in the number of studies focused on the various physiological roles of neutraceuticals, along with their contribution as a therapeutic agent towards management of various diseases [1-3], amongst which endocrine disorders are of major concern. Endocrine disorders are a major burden for the health care system all across the world and it is predicted that the prevalence and oc-

currence of metabolically incorrect pathologies like obesity, diabetes, polycystic ovarian syndrome and other endocrine disorders is going to increase in both developed as well as developing countries in the forthcoming decade [4]. The currently used conventional medications and surgical interventions are falling short of effectively controlling the unbridled spread of endocrine disorders. Additionally, these medications pose severe side-effects upon long term usage. In this regard, dietary interventions using natural bioactive food compounds have emerged as promising therapeutic tools for endocrine and metabolic disorders, with limited deleterious side effects. Phenolic compounds, flavonoids, fatty acids, phytosterols, and carotenoids of plant origin have been well-documented to exhibit protective effects against the prevention of chronic diseases such as coronary heart disease [5] as well as obesity [6], dyslipidemia [7], polycystic ovarian syndrome [8,9] and other endocrine disorders. In this context, the current review aims to understand the role and mechanisms of these neutraceuticals towards modulation of reproductive, metabolic and endocrine system. The neutraceuticals/plant derived biomolecules majorly fall over following categories like fatty acids, polyphenols and catechins, flavonoids, phytosterols, dietary fibers etc. The sources, bioavailability and pharmacological activities of each of these neutraceuticals towards endocrine modulation are reviewed in details.

Fatty acids

Fatty acids are straight chain hydrocarbons possessing a carboxyl (COOH) group at one end. Most of the fatty acids required for the proper functioning of the body are synthesized by the body itself. However, two essential fatty acids, linoleic and alpha-linolenic, which are responsible to build specialized fatty acids like omega-3 and omega-6 fatty acids, cannot be synthesized by the body and have to be obtained externally via food.

Sources

Fatty acids are mostly present in all different foods. The omega 6 fatty acids, Linoleic acid is present in abundance in corn oil, sunflower oil, soybean oil, animal meat etc whereas, Arachdonic acid can be found only from animal sources like meat, eggs etc [10]. Omega 3 fatty acids: alpha-linolenic acid is abundantly present in several vegetable oils, such as chia, linseed oil, walnut oil, faxseed oil, almonds etc. Omega-3 fatty acids like eicosapentaenoic acid and docosahexaenoic acid are found in abundance in cold water fishes such as salmon, mackerel, herring, and tuna. Fatty acids present in fishes, krills and their derived oils are mainly found in the triacylglyceride and free fatty acid forms [11]. Omega-3 fatty acids are present in smaller quantities in nuts, seeds, beans, vegetables, whole grains and soy products.

Bioavailability

The dietary intake of Omega-3 long chain polyunsaturated fatty acids ranges from 250 mg per day up to 2 g per day. After oral intake of fats, they get broken down into fine droplets due to the peristaltic movement of the stomach. The fat droplets get emulsified in the small intestine due to the action of bile. Emulsification is essential for the action of pancreatic lipases to cleave the triglyceride bound fatty acids. Fatty acid transport protein 4, present at the intestinal mucosal membrane are responsible for the transport of fatty acids to the lymph and blood. The bioavailability of long chain omega 3 fatty acids depends on numerous factors like the type of chemical bond, the fat content of the food, presence of calcium ions etc. Calcium ions can form complex with free fatty acids and thereby, reduce its bioavailability. The apparent bioavailability of ethyl ester fatty acids is the lowest whereas it is greatest for free fatty acids [12]. The omega-3 fatty acids are transported by the blood to the target tissues, where they are primarily incorporated in membranes of heart muscle cells, the brain/nervous system and retina cells [13].

Pharmacological activities towards endocrine disorders

There is a strong association of fatty acids with the normal functioning of the endocrine system. Dietary fatty acids have significant impact on hormone, neuropeptide concentrations as well as their receptors [14]. The bioactivities depend upon the type of fatty acids; saturated and trans-fatty acids were found to decrease insulin concentrations hence, leading to insulin resistance. On the other hand, polyunsaturated fatty acids (PUFA) increased plasma insulin concentration and decreased insulin resistance [15]. Insulin resistance lies in the core of all metabolic and endocrine disorders. Hence, supplementation with PUFAs can lead to amelioration of the symptoms associated with pathologies like diabetes and obesity.

Diabetes

Clinical studies have demonstrated that Type 2 Diabetes patients when supplemented food enriched with α-lipoic acid and omega-3 Poly Unsaturated Fatty acids (PUFA), they exhibited improvement in the body mass index, fasting plasma glucose, post-prandial glucose, glycated haemoglobin (HbA1c), fasting plasma insulin and HOMA-IR, lipid profile, high sensitivity C-reactive protein (Hs-CRP) and oxidative stress markers such as superoxide dismutase (SOD), Glutathione peroxidise (GSH-Px) and

malondialdehyde (MDA) [16]. Omega 3 fatty acid supplementation (4g/day) maintained renal function in patients suffering from diabetic nephropathy and hypertriglyceridemia [17]. Additionally, daily consumption of 500 mg/day of long-chain omega-3 polyunsaturated fatty acids reduces the incidence of severe diabetic retinopathy in older adults [18]. In addition to this, the components of the flaxseeds were also found to downregulate targets of insulin signalling pathway- IRS-1, IGFBP4, IGFBP5, AKT and NF-kB signalling [19]. These data suggest that α-linolenic acid can potentiate several metabolic targets. Nutritional supplementation of omega 3 PUFAs in early life can prevent the onset of Type 1 diabetes. Also, prolonged intake of these phytochemicals can suppress inflammation, regenerate pancreatic islets and reduce markers of autoimmunity and thereby, help in amelioration of type 1 diabetes [20]. These reports suggest that fatty acids play an important role in management of diabetes.

Obesity

With the rising epidemic of obesity, its management poses a greater threat to the Medicare system, leading to identification of alternative therapeutic options. Clinical trials demonstrate that daily intake of fish or omega-3 supplementation increased adiponectin levels in the blood by 14-60% [21]. Short-chain fatty acids suppress food intake by activating vagal afferent neurons [22]. Eicosapentaenoic and docosahexaenoic acids have been reported to improve chronic inflammation, insulin resistance and dyslipidaemia associated with obesity [23]. Dietary intakes of fish oil (400 mg/kg/day) for 4 weeks could restore brain alterations in high-fat diet-induced obesity model by partially restoring the inflammatory and oxidative damage parameters [24].

Reproductive endocrinology

Apart from their potential to modulate metabolic disorders, Omega-3 polyunsaturated fatty acids, present in abundance in fish oil was found to ameliorate high-fat diet induced reproductive dysfunction in male C57BL/6 mouse by modifying the rhythmic expression of testosterone synthesis related genes [25]. Studies in mice have shown that dietary intake of omega-3 and omega-6 fatty acids has positive association with the implantation rate by modifying the uterine phospholipid fatty acid composition and arachidonic acid levels [26]. Dikshit., *et al.* [27] demonstrated that α-linolenic acid, a major component of flaxseeds was found to reduce the inflammatory and pro-carcinogenic environment in the ovaries of normal hens by significantly decreasing inflammatory prostaglandin E2, ER-α, CYP3A4, CYP1B1, 16-OHE1. However, it increased CYP1A1 and 2-OHE1. These reports clearly suggest that fatty acids have efficacy to modulate steroidogenic targets.

Polyphenols

Epidemiological studies and meta-analyses robustly recommend that long term consumption of diets rich in plant polyphenols provides protection against development of cancers, cardiovascular diseases, diabetes, osteoporosis and neurodegenerative diseases [28]. Polyphenols and other related compounds are being researched immensely due to their varied bioactivities on several aspects of human physiology.

Sources

Polyphenols are secondary metabolites of the plants, derived from phenylalanine or shikimic acid prescursors. Majorly they are found in conjugated form, either with polysachcharides or monosachcharides or carboxylic and organic acids, amines, lipids or other phenols. They are present in abundance in fruits, vegetables, whole grains, cereal, legumes, tea, coffee, wine and cocoa. Fruits like grapes, apple, pear, cherries and berries contains up to 200–300 mg polyphenols per 100 grams fresh weight [29]. Typically a glass of red wine or a cup of tea or coffee contains about 100 mg polyphenols. Tea leaves contain up to 4.5 g/kg fresh wt of gallic acid [30]. Greater than 8000 polyphenolic compounds, including phenolic acids, flavonoids, stilbenes, lignans and polymeric lignans have been identified in whole plant foods [31]. Hydroxybenzoic acid and hydroxycinnamic acid are the major classes of phenolic compounds, amongst which hydroxybenzoic acid is found in less quantities in few fruits like strawberries, raspberries and black berries, black radish and onions [32]. On the other hand, hydroxycinnamic acids are more common and abundantly present in coffee, kiwis, plums, cherries, apples and blueberries [33]. Wheat grains contain 0.8–2 g/kg dry weight of ferulic acid, another phenolic compound found mostly in cereal grains [34].

Bio availability

It is estimated that dietary intake of polyphenols is approximately 1 g/day [35]. Inspite of being present abundantly in foods, polyphenols are not significantly bioactive, owing to their poor absorption from the intestines, high metabolization and rapid elimination from the body. Most of the polyphenols present in food are found to be conjugated with esters, glucosides or any other form which cannot be absorbed as such [36]. Hence, the metabolites those are present in the blood and the tissues vary from their native component in terms of biological activities. The gastrointestinal absorption of polyphenols is not well studied. The polyphenols are highly hydrophilic in nature and hence, don't passively diffuse through the gut wall. However, they can be taken up by Na- dependent membrane carriers [37].

Pharmacological activities towards endocrine disorders

Several biological activities and beneficial properties have been documented for dietary polyphenols, and some of the more well known ones include antioxidant [38], antiallergic [39], anti-inflammatory [40], anti-viral and anti-microbial [41], anti-proliferative [42], anti-mutagenic and anti-carcinogenic [43,44], free radical scavenging [45], regulation of cell cycle arrest [46] and apoptosis [47]. In addition to these, polyphenols are known to modulate endocrine disorders such as obesity and diabetes.

Diabetes

Green tea treatment (500mg/kg body weight) for 12 weeks in high- fat diet fed mice was found to increase the energy expenditure, reduce the body weight, alleviate insulin resistance and inflammation by down regulating the expression of miR-335, thereby, improving adipose tissue metabolism [48]. Revesterol, the main polyphenolic component of grapes was found to improve glucose homeostasis, decrease insulin resistance, protect pancreatic β- cells and improve insulin secretion by increasing the expression and/or activities of AMPK and SIRT1 in tissues of type 1 diabetes patients [49,50]. Revesterol could restore the skeletal muscle dysfunction by increasing the mitochondrial biogenesis, fatty acid metabolism, GLUT4 expression and decreasing the expression of NF-kB, IL-1β and IL-6 in muscle cells [51]. The therapeutic effect could be attributed to the anti-oxidative as well as anti-inflammatory activities of revesterol [52]. Animal studies have shown that revesterol can effectively ameliorate the glucose homeostasis in the liver of type 1 diabetic animals by decreasing the activity of phosphoenolpyruvate carboxykinase [53], lactate dehydrogenase and increasing the activities of hexokinase and pyruvate kinase [54]. Numerous studies have demonstrated dose and time dependent action of Revesterol in type 2 diabetes patients [55-57] and experimentally induced animal models of type 2 diabetes [58- 60]. Clinical studies demonstrate that 10 to 250 mg/day of revesterol for 1-3 months respectively improve insulin sensitivity and glucose homeostasis and thereby, manage Diabetes pathophysiology [61].

Obesity

Studies demonstrate that polyphenols present in bilberry [62,63], grapes [64], soy beans [65] have shown to possess anti-obesity property and act as an anti-inflammatory agent by modulating the body weight, BMI, adiposity, fat pad, adipocyte differentiation and decreased expression of NF-κB, TNF-α, IL-6, PPAR, SREBP-1C, ACC and CRP. These bioactive phytocomponents modulate cell signaling through the AMPK, MAPK and G-protein coupled receptor 120 signalling pathways and improve the balance of pro- and anti-inflammatory mediators secreted by adipose tissue and hence lowers systemic inflammation and risk for metabolic diseases [66]. Gingerols, the main pungent component of ginger, possesses excellent anti-inflammatory properties by inhibiting prostaglandin synthetase and Arachidonate 5-lipoxygenase activity in RBL-1 cells [67] and LPS induced COX-2 expression in U937 cells [68]. Saravanan., *et al*. [69] reported anti-obesity properties of gingerol through the inhibition of dietary fat absorption in the gastrointestinal tract, and its hypophagic and hypolipidaemic activity in male rats in the High fat diet-induced model of dietary obesity. "*In-vitro*" studies on mouse 3T3-L1 pre-adipocytes demonstrate that gingerols improve adipocyte differentiation and insulin-dependent glucose uptake [70].

Reproductive endocrinology

Currently, potential of plant derived compounds to improve fertility in humans and animals has been a burning topic of research. Polyphenols play an important role in the maintenance of healthy pregnancy by regulating targets associated with inflammation and oxidative stress [71]. Supplementation with Hydroxytyrosol in the maternal diet (1.5mg/kg of feed) during the early pregnancy bore offsprings having higher mean birth weight, suggesting that maternal supplementation with polyphenols improve the pre- and post natal development of the offsprings [72]. This can be attributed to the anti-oxidant, anti-inflammatory, immune-modulatory properties of polyphenols. Recently, dietary polyphenols like Epigallocatechin-3-gallate (EGCG) supplementation has been reported to improve the quality of male and female gametes, principally due to their ability to quench reactive oxygen species [73]. In addition, High doses (50 μM) of Quercetin, catechin and resveratrol can increase antioxidant activity in human and animal semen; thereby polyphenols are useful tools for semen cryopreservation. They improve the sperm motility, survival and integrity of the DNA in cryopreserved samples [74]. On the contrary, polyphenols derived from green tea reduced the testosterone levels in the animals by decreasing the activities of steroidogenic enzymes [75] or by direct or indirect inhibition of P450scc and 17β-HSD in a dose dependent manner. This property of polyphenols can be effectively used to design herbal drugs for reproductive endocrine disorders like polycystic ovarian syndrome (PCOS), wherein, hyperandrogenism lies in the core of the pathology.

Phytosterols

Phytosterols, products of the isoprenoid biosynthesis pathway are non-polar molecules with a molecular weight around 400-415 g/mol. They are highly hydrophobic and have very low solubility in water. The term phytosterols refers to more than 200 different compounds which are found in various plants and marine sources [76]. The most biologically relevant phytosterols are sitosterol, campesterol, stigmasterol and brassicasterol. Dietary consumption of phytosterols has shown to reduce cholesterol absorption. Phytosterols also affect other aspects of cholesterol metabolism and interfere with steroid hormone synthesis [77], suggesting it to be a potent herbal derived therapeutic agent towards management of metabolic and endocrine disorders.

Sources

Phytosterols may exist as free phytosterols or as conjugated phytosterols including steryl esters and steryl glycosides [78]. As a membrane component of plant cells, phytosterols are found in many different lipid-rich and fibre-rich fractions of all plant products. In particular, vegetable oils and products made from oils like spreads and margarine are good sources of plant sterols [79]. Canola oil contains 4.6-8.1 mg/g of total phytosterols, soybean oil contains 2.4-4.0 mg/g and sunflower oil contains 2.1-4.5 mg/g [80]. Corn oil contains even higher amounts of phytosterols with 8 to 15 mg/g [81]. Other foods which contribute to the daily intake of plant sterols are cereal grains, cereal based products, nuts, legumes, vegetables and fruits [82]. In general, the consumption of plant sterols in Asian cultures (including algae in their diets) and vegetarians is much higher.

Bioavailibility

The transport and metabolism of phytosterols within the human body is not yet completely understood. The absorption of phytosterols is promoted by the presence of a double bond. Moreover, the nature of the side chain plays an important role in this process -the more complicated the side chain is, the less phytosterol passes into the enterocytes in the gut [83]. When dietary sterols were supplemented at 2–3 g/day, the serum sitosterol and campesterol levels increased by 34–73% [84]. Phytosterols upon ingestion get emulsified by the action of bile acids in the small intestine to form micelles. The esterified phytosterols are converted to free phytosterols with the help of cholesterol esterase and pancreatic lipase enzymes [85]. Free phytosterols are then absorbed into enterocytes by ATP-binding cassette transporters that are encoded by ABC G5 and G8 genes [86]. Post absorption, the phytosterols form chylomicrons by combining with cholesterol, triacylglycerol and apolipoproteins [87]. It is in this form, that the transportation of phytosterols takes place through the lymph and bloodstream, where they are transformed to chylomicron remnants after the uptake of triacylglycerol by cells and transported to the liver. In the liver, the phytosterols may either be used for synthesis of bile salts [88] or be incorporated into very low density lipoproteins and be secreted into the blood, from where they are converted to low-density lipoproteins and presented to cells for uptake [89,90]. In the tissues, phytosterols are incorporated into the cell membranes [91] and have been found to be highly concentrated in the lungs, adrenal cortex, intestinal epithelia and ovaries [92].

Pharmacological activities towards endocrine disorders

Dietary phytosterols are thought to have a number of health benefits in humans.Small changes in the structure of sterol molecules confer them to highly distinct biological activities [93].

Diabetes

The phytosterols isolated from various plant and fungal sources have been extensively researched for their anti-diabetic potential (Table 1).

Obesity

Phytosterols can decrease the serum cholesterol by inhibiting the cholesterol absorption in the intestine [103]. Also, plant sterols activate nuclear hormone receptors in order to increase cholesterol excretion to the intestinal lumen [104]. The nuclear hormone receptors: LXRα and LXRβ are established regulators of cholesterol, lipid, and glucose metabolism and are attractive drug targets for the treatment of diabetes and cardiovascular diseases [105]. Extensive data from literature clearly suggests the phytosterols such as Stigmasterol and Ergosterol and their modified derivatives have the potential to assume a key role in the modulation of lipid metabolism and glucose homeostasis by altering LXR-alpha and beta expression by strongly inducing the expression of ABCA1 and inadequately/not activating the lipogenic genes SREBP1C and SCD1 or FASN, respectively [106]. Obesity is closely associated with inflammation; hence, a potential therapeutic agent should be in a position to combat the consequences associated with inflammatory responses. In this regard, phytosterols are known to suppress inflammatory responses, either by activating the genes that encode anti-inflammatory proteins or by suppressing the genes that are under the control of pro- inflammatory transcription factors [107].

Sl. No.	Plant Name	Active ingredients	Disease Model	Dose	Effect	References
1.	*Aloe barbadensis* MILLER (gel from the leaves)	lophenol, 24-methyl-lophenol, 24-ethyl-lophenol, cycloartanol, and 24-methylene-cycloartanol	Type 2 diabetic BKS. Cg-m$^{+/+}$Lepr$^{db/J}$ (db/db) male mice	1 µg of isolated phytosterols and 25µg of plant extract for 28 days	Reduction in the fasting and random blood glucose levels and HbA1c levels.	[94]
2.	*Aloe barbadensis* MILLER (gel from the leaves)	lophenol (Lo) and cycloartanol (Cy)	Zucker diabetic fatty (ZDF) male rats	25µg/kg for 44 days	Hypoglycemia, Reduction in the HbA1c levels, Improvised glucose tolerance, Reduction in the serum free fatty acid (FFA), triglyceride (TG) levels and visceral fat accumulation. No change in the total cholesterol levels.	[95]
3.	Unidentified sources	5-campestenone	Zucker diabetic fatty (ZDF) male rats	0.6% Dietary exposure	Reduction in plasma HbA1c, Total Cholesterol, TG and FFA, non esterified fatty acid (NEFA) and improved the glucose tolerance, visceral fat accumulation	[96]
4.	*Urena lobata* (leaves)	mangiferin, stigmasterol and β-sitosterol	In-vitro studies	IC_{50} for water extract- 6489.88 µg/ml IC_{50} for ethanolic extract- 1654.64 µg/ml	Inhibition of dipeptidyl peptidase IV activity	[97]
5.	*Erythrina indica* (Stem bark)	Oleanolic acid	Alloxan and dexamethasone induced diabetes in adult albino Wistar rats	200 and 400 m/kg aqueous and alcoholic extract orally for 3 weeks.	Hypoglycemic effect	[98]
6.	*Cassia alata* (leaves and flowers)	Kaempferol 3-O-gentiobioside			Anti diabetic	[99]
7.	*Pelvetia siliquosa* (sea weed)	Fucosterols	Streptozotocin-induced diabetic rats	30 mg/kg orally Single dose	Antidiabetic, inhibition of Sorbitol accumulation in the lenses.	[100]
8.	*Platycladus orientalis* (Leaves)	carbohydrates, proteins, amino acids, fixed oils, fats, phytosterols, tannins, phenolic compounds and	Streptozotocin-induced diabetic rats	100 and 200 mg/kg daily (aqueous extract) for 28 days orally	Reduced TC, TG, LDL, VLDL; increased HDL levels. Antioxidant property	[101]
9.	Chrozophora Plicata (seed oil)			IC_{50} value of 287.12 µM.	Inhibitors of α-Glucosidase and α-amylase	[102]

Table 1

Reproductive endocrinology

Data from literature shows that β-Sitosterol has estrogen-like effects and modulates the steroidogenic pathway by altering the P450scc activity, thereby, impairing the conversion of cholesterol to pregnenolone [108]. Fish exposed to phytosterols display altered sexual development, changes in hormone production, decreased egg production and decreased spawning rate [109]. Goldfish exposure to 75 µg/L 13 phytosterols resulted in a reduction of reproductive steroid levels and changes in gonadal steroidogenesis [110]. In subchronic rodent studies, it has been observed that even at small doses (0.5–50 mg/kg/day) phytosterols caused changes in the weights of reproductive organs. For example, when b-sitosterol was administered subcutaneously, it lead to reduced testicular

weight and sperm density in rats [111] and increased uterine weights in rats [112,113] and immature sheep (Ovis aries) [114]. Also, administration of 28- homobrassinolide (333 mg/kg body weight) to streptozotocin- induced diabetic male rats by oral gavage for 15 consecutive days to diminished LPO, increased antioxidant enzyme, 3β-HSD and 17β-HSD activities, and elevated StAR and Androgen Binding Protein expression and Testosterone level in rat testis [115]. Chronic effects of a dietary phytosterols mixture (5mg/kg/day), containing mainly beta sitosterol, on the reproduction of the mouse demonstrate increased the plasma levels of testosterone and decreased the relative uterine weights in the pups of F (2) and F (4) generations. Furthermore, phytosterol exposure increased the concentrations of plasma estradiol in the female pups of F (3) generation and testicular levels of testosterone in the male pups of F (2) generations [116]. Limited evidence from animal studies suggests that very high phytosterol intakes can alter testosterone metabolism by inhibiting 5a-reductase, a membrane-bound enzyme that converts testosterone to dihydrotestosterone, a more potent metabolite [117]. Dietary intake of Coumesterol decreases the amplitude of LH pulses in ewes [118]. Thereby, suggesting that phytosterols and its metabolites may act as GnRH modulators. Also, lower doses of phytosterol have found to be effective in treatment of Polycystic Ovarian Syndrome (PCOS). PCOS is a female endocrine disorder which is characterized by hyperandrogenism, insulin resistance and presence of multiple peripheral cysts in the ovaries. Maharjan and Nampoothiri [119] demonstrated that Letrozole induced PCOS rats when treated with phytosterol containing Non-polar extract of *Aloe vera* gel (25 µg/kg body weight) for 60 days, could effectively manage the reproductive as well as metabolic complication associated with PCOS. PCOS rats demonstrated a decrease in serum testosterone and insulin level with improved estradiol and progesterone levels after treatment with Non-polar extract of *Aloe vera* gel. Also, decrease in transcripts level of StAR, LHR, AR, Aromatase and IR as well as relative protein expression of StAR, 3β-HSD and aromatase expression was observed. Additionally, consumption of phytosterols is getting popularity among menopausal women [120] with increasing evidence that they are beneficial in relieving symptoms as well as in protection of certain cancers. Considerable emerging evidence supports the inhibitory actions of phytosterols on lung [121], ovarian [122] and breast [123] cancer. Phytosterols seem to act through multiple mechanisms of action, including inhibition of carcinogen production, cancer-cell growth, angiogenesis, invasion and metastasis, and through the promotion of apoptosis of cancerous cells [124]. Phytosterol consumption may also increase the activity of antioxidant enzymes and thereby reduce oxidative stress [125].

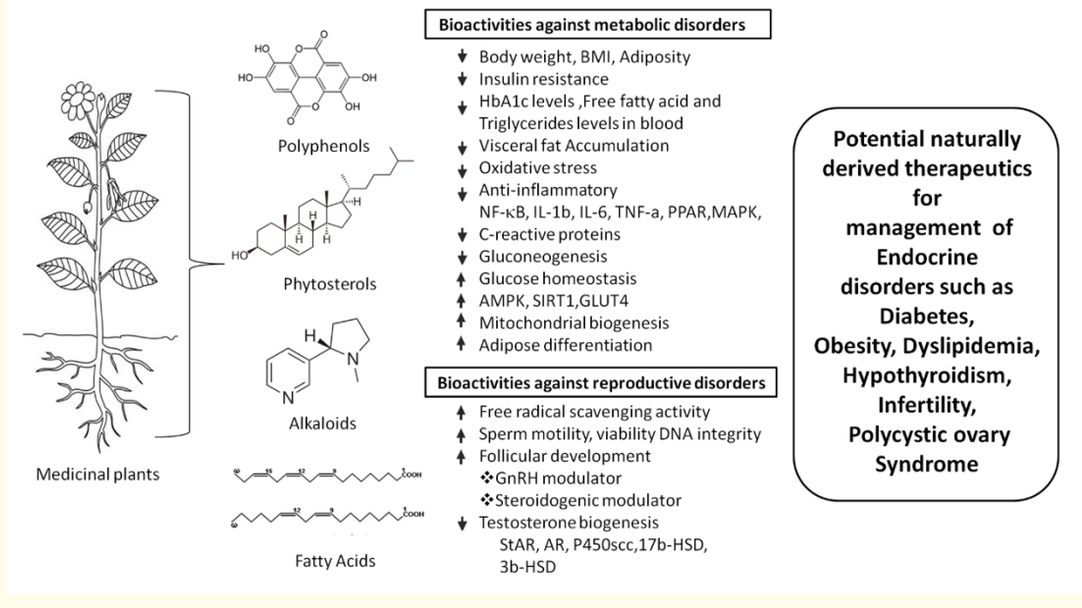

Figure 1: Schematic representation of the possible mechanism underlying endocrine modulation by plant derived nutraceuticals. Nutraceuticals derived from medicinal plants exhibit various health promoting activities, amongst which its role towards endocrine modulation has been described in this figure.They modulate several metabolic parameters linked with lipid and glucose homeostasis. Phytocomponents play an important role in the maintenance of endocrine disorders by regulating targets associated with inflammation and oxidative stress. Also, these phytocomponents can directly act as GnRH and steroidogenic modulators and thereby, manage the pathophysiological symptoms associated with reproductive disorders. Hence, suggesting that neutraceuticals are excellent naturally derived targets for management of endocrine disorders.

Conclusion

Dietary nutraceuticals appear to play an important role in the regulation of different aspects of human physiology. Recent studies have shown that these neutraceuticals are implicated in the treatment and management of cardiovascular diseases, cancers and metabolic syndromes. In this review, detailed description on the various sources of nutraceuticals, their chemical and physical properties, their bioavailability and the role and mechanisms of these phytochemicals towards modulation of reproduction and endocrine system has been summarized. The studies from several decades have clearly shown the potential of modulation of endocrine function and hence, can be effectively designed as drug targets for endocrine disorders. However, evidence for such promising effects is still at an elementary stage and more research is clearly needed to draw firm conclusions.

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Conflict of Interest Statement

The authors have declared no conflict of interest.

Highlights

- Plant derived neutraceuticals are potential therapeutic agents for management of endocrine disorders such as diabetes, obesity, dyslipidemia and reproductive disorders.
- Omega-3 PUFAs can modulate the glucose and lipid homeostasis in diabetes, obesity and dyslipidemia, also it can directly influence the testosterone biosynthetic genes.
- Plant derived polyphenols provides protection against development of cancers, cardiovascular diseases, diabetes, osteoporosis and neurodegenerative diseases due to their anti-inflammatory and anti-oxidant properties.
- Dietary intake of phytosterols restores the metabolic alterations associated with endocrine disorders like diabetes and obesity by directly activating nuclear receptors- LXR α and LXR β.
- Phytosterols and its metabolites can potentially act as GnRH modulators.

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Phytosterols isolated from *Aloe barbadensis Mill*., restore reproductive and metabolic complications in Letrozole-induced PCOS mouse model

Arpi Dey, Radha Maharjan and Laxmipriya Nampoothiri
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Statement of the Problem: Polycystic Ovary Syndrome (PCOS) is a multifactorial reproductive disorder which affects 4-12% of women and is a leading cause of female infertility worldwide. PCOS is related to dyslipidemia, hyperandrogenism and hyperinsulinemia. Treatment of these dysfunctions is by administration of steroid analogues and insulin sensitizers. However, the use of these synthetic drugs gives rise to severe side effects. Hence, there is an immediate pre-requisite of an alternative herbal therapy. The purpose of this study is to understand the role of phytosterols isolated from Aloe barbadensis Mill., towards management of PCOS.

Methodology & Theoretical Orientation: Aloe vera gel was extracted used petroleum ether and the extract thus obtained was fractionated using silica gel column chromatography. Phytochemical screening, thin layer chromatograph, HPTLC and GC/MS were done to characterize the obtained phytosterols. Letrozole (0.5 mg/kg body weight) induced PCOS mouse model was fed orally with the isolated phytosterol for 60 days. After completion of the treatment, several metabolic as well as reproductive parameters were evaluated.

Findings: Detailed phytochemical screening demonstrated the presence of several phytosterols such as beta-sitosterol, stigmasterol and campesterol. Upon treatment with the isolated phytosterols, letrozole induced PCOS mouse model showed an improvement in the glucose sensitivity, restored lipid profile and estrus cyclicity, decreased the serum testosterone levels and increased the serum progesterone and estradiol levels. The animals also exhibited disappearance of the peripheral cysts, which is a hallmark of PCOS. The phytosterols elicit their effectiveness by getting bio-transformed into oxysterols, which can further influence molecular pathways that mainly regulate steroidogenesis and lipid metabolism.

Conclusion & Significance: This study is first of its kind which demonstrates the direct effect of phytosterols isolated from Aloe vera gel at molecular level towards management of PCOS, thereby, adding to its overall potential and economic viability at national and international level.

Biography

Arpi Dey is currently a PhD student at the Department of Biochemistry, The Maharaja Sayajirao University of Baroda, India. She has been extensively trained in the domain of reproductive endocrinology. She has contributed greatly to the development of novel herbal therapeutics by exploring the knowledge of Indian heritage of Ayurveda. She has expertise in animal handling, phytochemistry, molecular biology and cell-culture techniques.

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Female Reproduction

Pharmacokinetics and bioavailability of non-polar phytocomponents of Aloe vera gel and their role as an endocrine modulator in letrozole induced PCOS rat model

Arpi Dey, Shweta Vasoya & Laxmipriya Nampoothiri

Background and aim: Polycystic ovary syndrome (PCOS), the most common endocrine disorder in women of reproductive age with an estimated prevalence of 5–10%, is one of the most common causes of female infertility. The therapeutic options for PCOS are limited. Thereby, traditional knowledge of Ayurveda has been exploited to identify an herbal therapeutic target for PCOS. Pharmacokinetic (PK) studies on bioactive constituents of herbal drugs provide valuable information on bio-transformed metabolites, dosage form, doses and potential herb–drug interactions. Thereby, the aim of the study was to study the pharmacokinetics and bioavailability of the non-polar phytocomponents of *Aloe vera* gel and their role as an endocrine modulator in PCOS pathology.

Methodology: PCOS was induced in rat using letrozole and validated for structural and metabolic characteristics of PCOS. Blood and tissues (ovaries, adrenal, hypothalamus, pituitary, uterus and liver) were collected upto 48 h after petroleum ether extract of dried *Aloe vera* gel (1.0 g) were administered orally to rats. Metabolites of Phytosterols were identified using GC/MS and quantified using HPLC. Progesterone and estradiol were analysed using ELISA. Steroidogenic enzymes as well as important enzymes responsible for biotransformation were evaluated along with the toxicity parameters.

Results: PCOS animals demonstrated altered estrus cyclicity, serum testosterone levels and oral glucose tolerance test profile when compared to controls. An increase in the estradiol ($P<0.01$) and progesterone levels ($P<0.05$) were observed in the plasma after 24 h. Several phytosterols and their modified oxysterols were identified in the plasma and tissues which could be well-correlated with the functional changes observed in hormone profile.

Volume 49

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Vasoya Shweta

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Conclusion: This study elucidates the bioavailability and functionality of phytosterols/oxysterols obtained from Aloe vera gel towards management of PCOS. This study will be helpful in identification of a naturally derived drug target and add to its economic viability at national and international level.

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Reproduction, endocrine disruptors and signalling

Implication of non polar phytocomponents of aloe vera gel in management of polycystic ovarian syndrome

Laxmipriya Nampoothiri, Radha Maharjan & Arpi Dey

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Background: Aloe vera gel has been proven to have hypoglycaemic, hypolipidemic, and steroid enzyme regulating properties. These are of clinical significance in reproductive pathology namely polycystic ovary syndrome (PCOS). Preliminary, '*in-vitro*' studies suggested that non-polar component is responsible for the steroid enzyme modulating effect. This study investigates the role of non polar phytocomponent's of aloe vera gel, which could be responsible in management of this pathology.

Aim: The aim of this study was to evaluate the effects of non-polar phyto-components on the PCOS pathology using rodent model.

Design and methods: Rodent model was developed using letrozole (0.5 g/kg body weight daily orally for 21 days) and validated for PCOS pathology. Later, the non-polar component, extracted using petroleum ether (containing phytosterols validated by HPTLC) was administered to PCOS rats daily (~25 µg/kg body weight) for 2 months, with respective controls (Aloe and vehicle). After the course of treatment, ovarian expression profile (mRNA and protein) of major steroidogenic genes namely StAR, 3 beta-hydroxysteroid dehydrogenase (3β-HSD), aromatase, LHR, and FSH-R were monitored in addition to biochemical parameters. Parallely, phytochemical elucidation of non-polar fraction was evaluated.

Results: Expression of StAR, 3β-HSD, and LHR was found to be decreased (P<0.01), both at transcript as well as protein level in non-polar fraction treated PCOS group as compared with untreated PCOS control while other genes studied did not show any

Volume 37

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significant change. Phytochemically, aloe vera non-polar fraction exhibited the presence of β-sitosterol in major quantity, along with minor amounts of stigmasterol, lupeol, and its derivatives.

Conclusion: Non-polar fraction of aloe vera gel has a potential to manage PCOS phenotype, by modifying steroidogenic targets. This study may serve as platform for designing drugs by exploring these novel targets.

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