Chapter 7

To identify insulin sensitizer bioactive compound to ameliorate insulin resistance and steroidogenic dysfunction in human luteinized granulosa cells from PCOS.

7.1 Introduction

Infertility strikes approximately 10-15% of the couples and an approach to decrease this percentage has become a top priority for many health organizations. Polycystic ovarian syndrome (PCOS) is a multifaceted disease characterized by polycystic ovaries, chronic anovulation and hyperandrogenism (Arentz et al. 2014). The life style factors such as physical exercise, psychological stress, high carbohydrate diet and sedentary life that impact fertility are modifiable and are considered to be the first-line of treatment in PCOS (Diamanti-Kandarakis E 2012). Symptom oriented pharmacological intervention such as oral contraceptive to regulate menstrual cycles and decrease hirsutism, spironolactone that block androgen receptors, Finasteride to block production of active form of testosterone and insulin sensitizers such as metformin, thiazilidinediones (TZD's) and D-chiro Inositol are in use for improving insulin sensitivity and lipid levels accompanied by weight loss are in use considered as lifestyle modifiers (Watson 2011; Sharma et al. 2013; Arentz et al. 2014).

Considering the role of insulin resistance (IR) in the interplay of metabolic and reproductive aberrations in infertility, insulin sensitizing drugs (ISD) are expected to have beneficial effects by restoring ovulatory menstrual cycles, reducing insulin resistance and thus being important therapeutic modality for PCOS (Maruthini et al. 2014). Insulin sensitizers such as the TZD's - pioglitazone and rosiglitazone, D-chiro-Inositol and the biguanide metformin are postulated to improve insulin sensitivity and several other aspects of the syndrome, including reproductive abnormalities (Dunaif 2008; Diamanti-Kandarakis et al. 2010). TZD's improve insulin sensitivity in PCOS through decrease in hepatic glucose production (Diamanti-Kandarakis et al. 2010). Studies with TZD's have reported fetal growth restriction as a potential risk in animal experiments and high incidence of weight gain among the users that further hampers their use in obese women with PCOS (Baillargeon et al. 2004; Yki-Järvinen 2004). D-chiro-Inositol has been observed to show effect on serum SHBG levels without affecting testosterone, sex hormone binding globulin (SHBG), fasting glucose, fasting insulin, and ovulation rate (Tang et al. 2011). Biguanide metformin (Fig 7.1) exerts its effects through AMPK activation, inhibition of hepatic glucose production, increase in peripheral

glucose uptake and reduction in fatty acid oxidation. Hence use of metformin in PCOS has been reported as the golden drug as it decreases insulin, testosterone, LH levels, improvement in weight loss and regularizes menstrual cycles, ovulation rates and pregnancy rates (Tang et al. 2011). The role of metformin in increasing the mean number of mature oocytes, cleaved embryos, significantly improved fertilization rates and clinical pregnancy rates is under controversy (Pasquali and Gambineri 2006). Metformin use relate to GI tract problems, malabsorption of vitamin B12. lactic acidosis and cardiovascular effects (Diamanti-Kandarakis et al. 2010). Moreover the metabolic effects of metformin appear to rely primarily upon the improvement of insulin sensitivity in insulin-resistant patients with PCOS and hence is not recommended for normo insulinemic lean or obese PCOS (Palomba et al. 2009). Moreover, in spite of their some role in PCOS, none of the ISD's could increase a chance of having a live birth (Dunaif 2008; Tang et al. 2009; Tang et al. 2012).

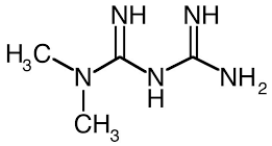


Figure 7. 1: Structure of metformin

Thus, these conventional pharmaceutical drugs show their limitation by the prevalence of contraindications in women with PCOS, non-effectiveness in some circumstances and associated side effects (Arentz et al. 2014). These pharmaceutical treatments appear to be only moderately effective in treating individual symptoms. Moreover with limited understanding of their mechanism, the type of patients, whether only PCOS-IR or even PCOS-NIR would be benefitted by their effects remains to be identified (Rice et al. 2013).

The use of complementary medicine mainly herbals by women has increased in last few years (Smith et al. 2013; Holden et al. 2014). A number of herbal medicines such as *Vitex agnus-castus, Cimicifuga racemosa, Tribulus terrestris, Glycyrrhiza spp., Paeonia lactiflora, Cinnamomum cassia* and *Aloe vera* can improve ovarian function, androgen excess, obesity, insulin resistance, blood lipids and inflammation and exert beneficial effects in PCOS (Watson 2011; Arentz et al. 2014; Radha and Laxmipriya 2015). *Enicostemma Littorale blume* and *Curcuma Longa* are herbal plants that are known for their anti-diabetic effect but their bio-active molecules have been unexplored in the field of PCOS.

Enicostemma Littorale blume (EL) has been widely used by folks since ages for the treatment of diabetes. Since last ten years our lab worked on EL and unravelled its hypo gycemic, hypo lipidemic, anti-inflammatory and insulin sensitizing potentials in different animal models (Maroo et al. 2002; Maroo et al. 2003; Vasu et al. 2005; Vaidya et al. 2009).

Amongst many compounds isolated from this herb, Swertiamarin, is principal compound and proved to be a potent insulin sensitizer in STZ-NA diabetic rat models and different cell lines (Patel et al. 2013) (Patel 2015) (Fig 7.2).

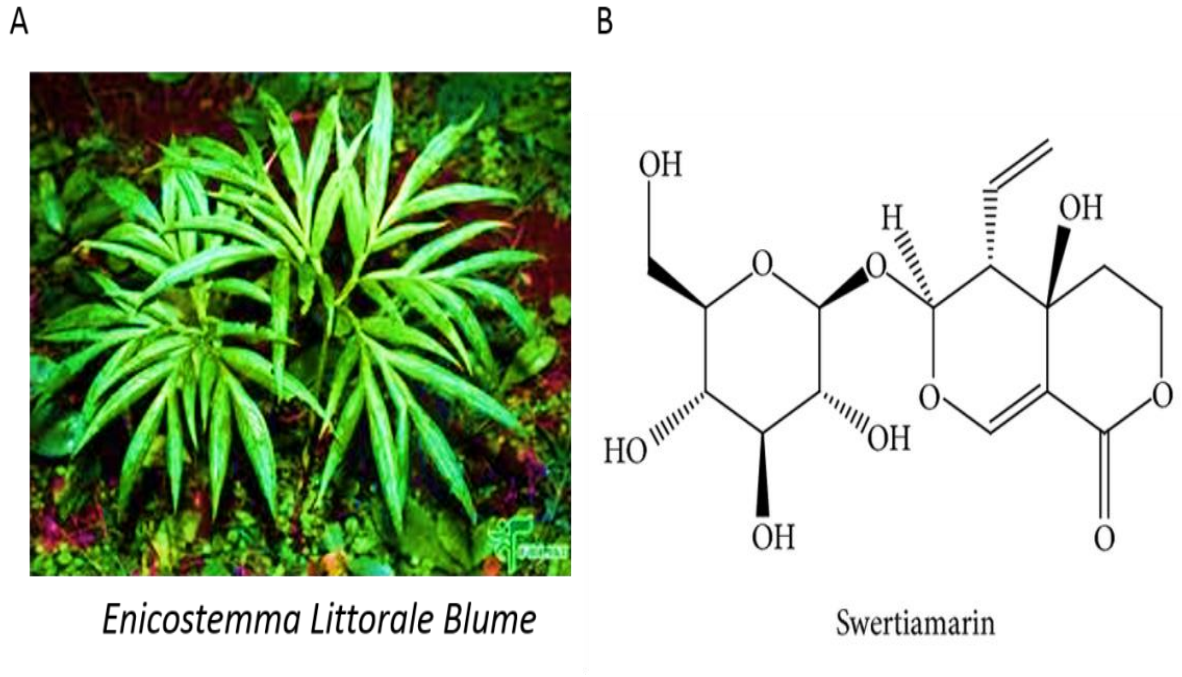


Figure 7. 2: A. Picture of Enicostemma Littorale Blume. B. Molecular structure of swertiamarin

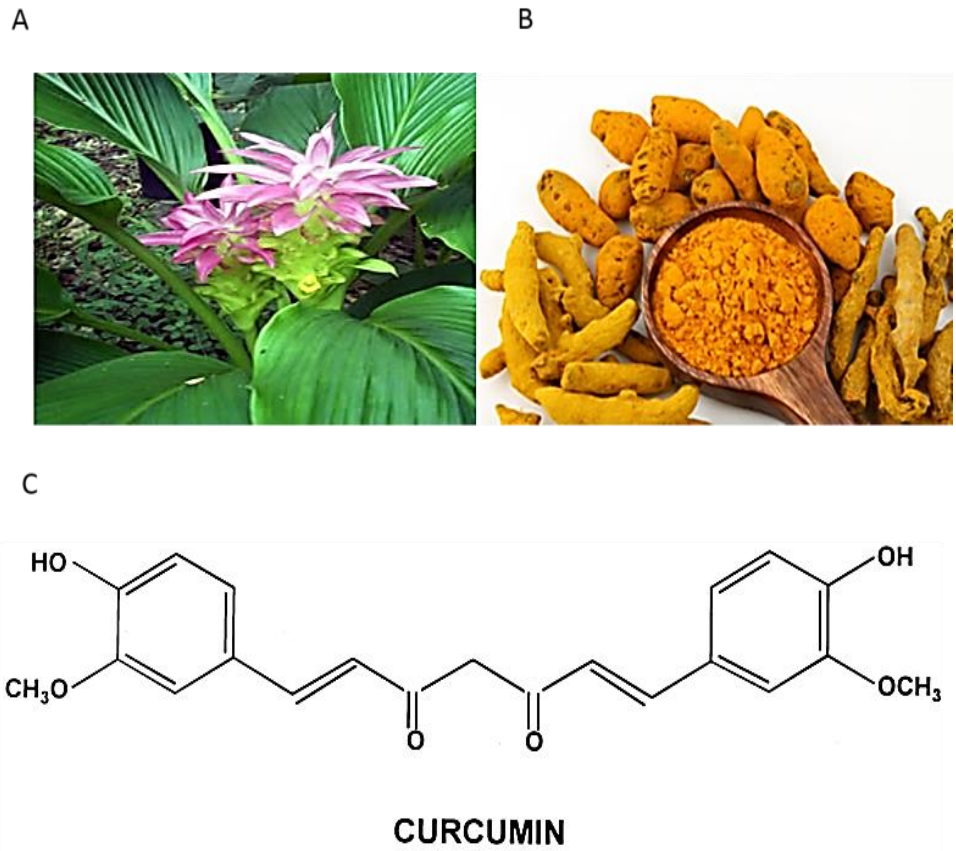
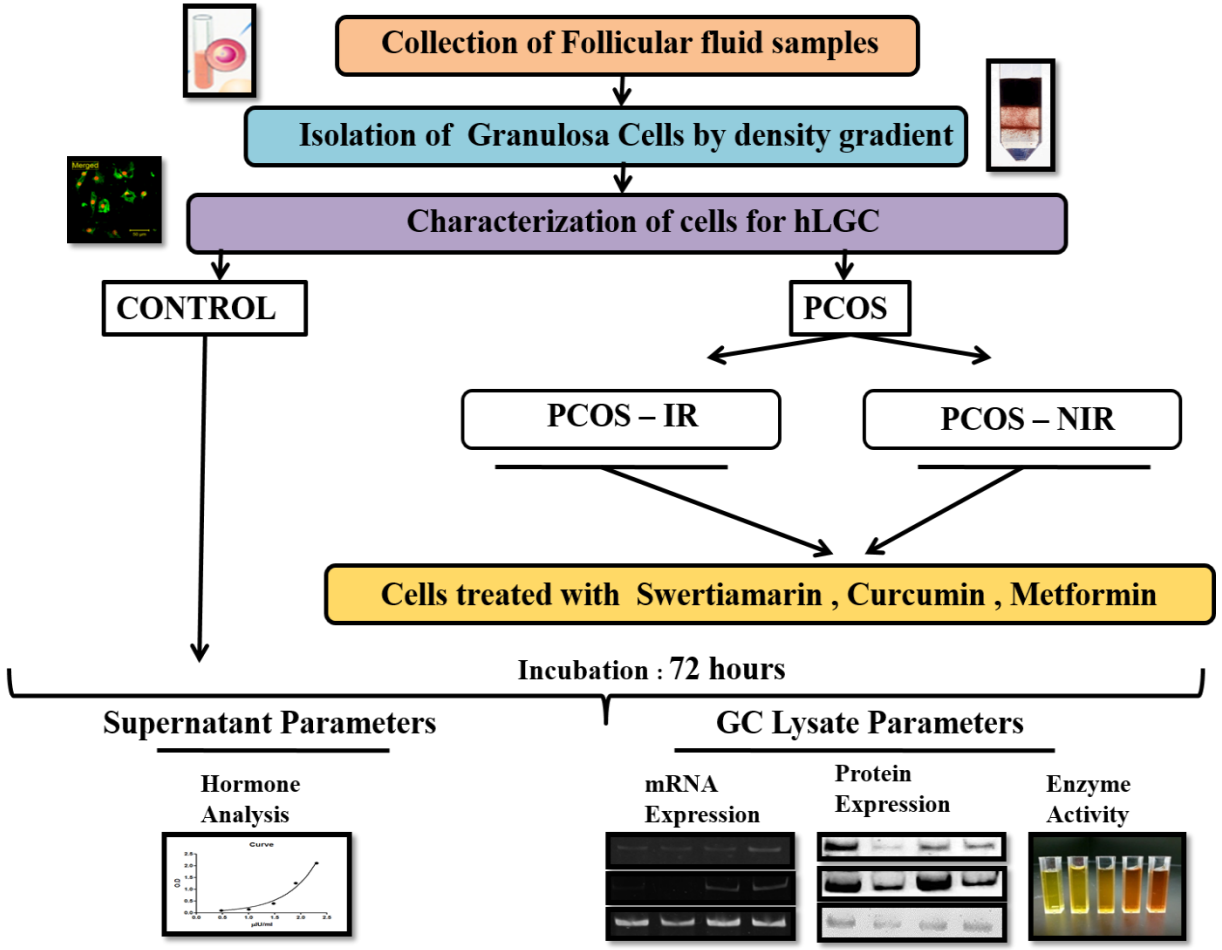


Figure 7. 3: A. Picture of Curcumin Longa B. Powdered form used as spice. C. Molecular formula of curcumin

Curcuma Longa possesses a wide range of physiological and pharmacological properties such as antioxidant, anti-inflammatory, anticancer, neuroprotective and anti-diabetic activities (Fazel Nabavi et al. 2015). Curcumin, a poly phenol is the yellow-colored bioactive constituent and is known to mechanistically exert its beneficial effects via reducing insulin and leptin resistance, attenuating inflammatory cytokine expression, accelerating fatty acid oxidation and increasing antioxidant enzyme (Shao et al. 2012). Recently its use in alleviation of androgen excess in PCOS and damages related to male reproductive system have been demonstrated (Watson 2011; Qin et al. 2015). Hence, the focus of this chapter is to investigate direct effect of bio-active molecules swertiamarin from *Enicostemma Littorale blume* and curcumin from *Curcuma Longa* on reproductive endocrinology for the treatment of women with PCOS (Fig. 7.3).

Though insulin resistance and hyperinsulinemia play a critical role in the pathogenesis of PCOS, certain obese and lean women with PCOS are observed to have normal insulin sensitivity (Marshall and Dunaif 2012). Hence it is interesting to discover potential bio actives for treating PCOS-IR and NIR, considering the side effects observed by conventional pharamaceutical drugs such as metformin. In clinical practice diagnosis for insulin resistance in women with PCOS is not done. Hence in the present chapter PCOS follicular fluid were distinguished for IR on the basis of down regulation of INR-β in luteinized granulosa cell isolated from control and PCOS follicular fluid samples. Thus, in the present chapter role of swertiamarin and curcumin have been explored on granulosa cell death, insulin and steroidogenic signalling, genes involved in fatty acid metabolism and steroidogenic hormones in granulosa cells from PCOS-IR and PCOS-NIR.

7.2 Experimental Design



7.3 Results

The isolated granulosa cells depending upon down regulation of INSR-β were segregated as control, PCOS-IR and PCOS-NIR and were then treated as in chapter 3.3.14. The cells treated with or without swertiamarin (66μM), curcumin (33μM) and positive control-metformin (1mM) for 72 hrs were then analysed for cell viability, expression of insulin signalling proteins, lipogenic genes, steroidogenic proteins, enzyme activity, hormones in supernatant followed by expression of gonadotropin receptor genes and genes involved in IGF system.

7.3.1 Bio active molecules increase hLGC viability from PCOS-IR with no change in PCOS-NIR.

After acclimatization to culture condition for 48 hours, hLGC's from PCOS-IR and PCOS-NIR were treated with swertiamarin, curcumin and metformin for 72 hrs and then compared to control for assessing cell viability by trypan blue staining method. Treatment of swertiamarin and curcumin significantly ($P < 0.001$) ameliorated cell death in PCOS-IR group but no difference was observed because of these treatments in PCOS-NIR group. However, positive control metformin markedly increased the viability in PCOS IR as well as PCOS-NIR group (Fig. 7.4).

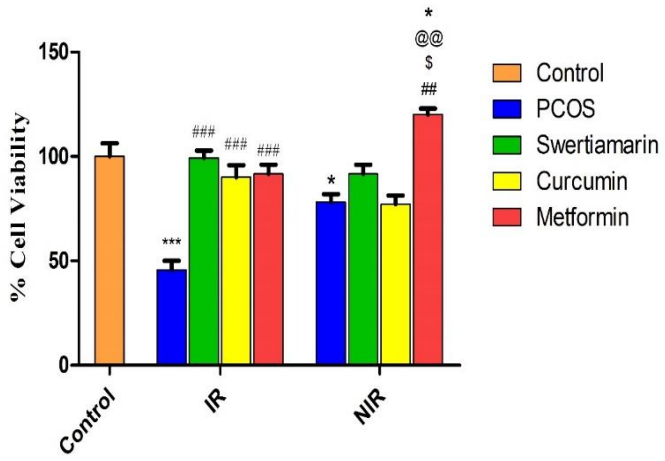


Figure 7. 4: Effect of swertiamarin, curcumin and metformin on cell viability in PCOS-IR and PCOS-NIR. The normalized expression values are represented as mean ± SEM of three independent experiments. * $P < 0.05$, ** $P < 0.01$ vs. C, $^{\#} P < 0.05$, $^{\#\#} P < 0.01$ vs. PCOS-IR and PCOS-NIR, $^{@} P < 0.05$ vs. PCOS-IR+S, $^{\$} P < 0.05$ vs. PCOS-IR+C. n=8 control, n=8 PCOS-IR and n=8 PCOS-NIR

7.3.2 Divergent effects of swertiamarin and curcumin on insulin signalling proteins in hLGC's from PCOS-IR and PCOS-NIR.

The possible effect of swertiamarin and curcumin on protein expression of candidate insulin signalling intermediates viz INSR-β, p-IRS (ser 307), PI(3)K, p-Akt, PKC-ζ, pP38 MAPK, p44/42 MAPK and PPARγ in PCOS-IR and NIR hLGCs was analyzed. Swertiamarin and curcumin treatment significantly ($P < 0.05$) reversed the down regulated expression of IR-β, PI(3)K, p-Akt and PKC-ζ in PCOS-IR hLGCs. Increased expression of p-IRS(Ser 307)- ($P <$

0.01) a hall mark of insulin resistance and PPARγ ($P < 0.05$) – an indicative of PCOS was also reduced remarkably because of the treatment with these bioactive compounds in PCOS IR cells. Swertiamarin and curcumin could significantly decrease protein expression of pP38 MAPK and p44/42 MAPK in hLGC's from both PCOS-IR as well as PCOS-NIR. Suprisingly, swertiamarin and curcumin were unable to show any effect on the candidate insulin signalling intermediates INSR-β, p-IRS (ser 307), PI(3)K, p-Akt, PKC-ζ, and PPARγ in hLGC's from PCOS-NIR group. Moreover, swertiamarin with 66μM was observed to be equally potent to metformin 1mM and curcumin 33 μM (Fig 7.5).

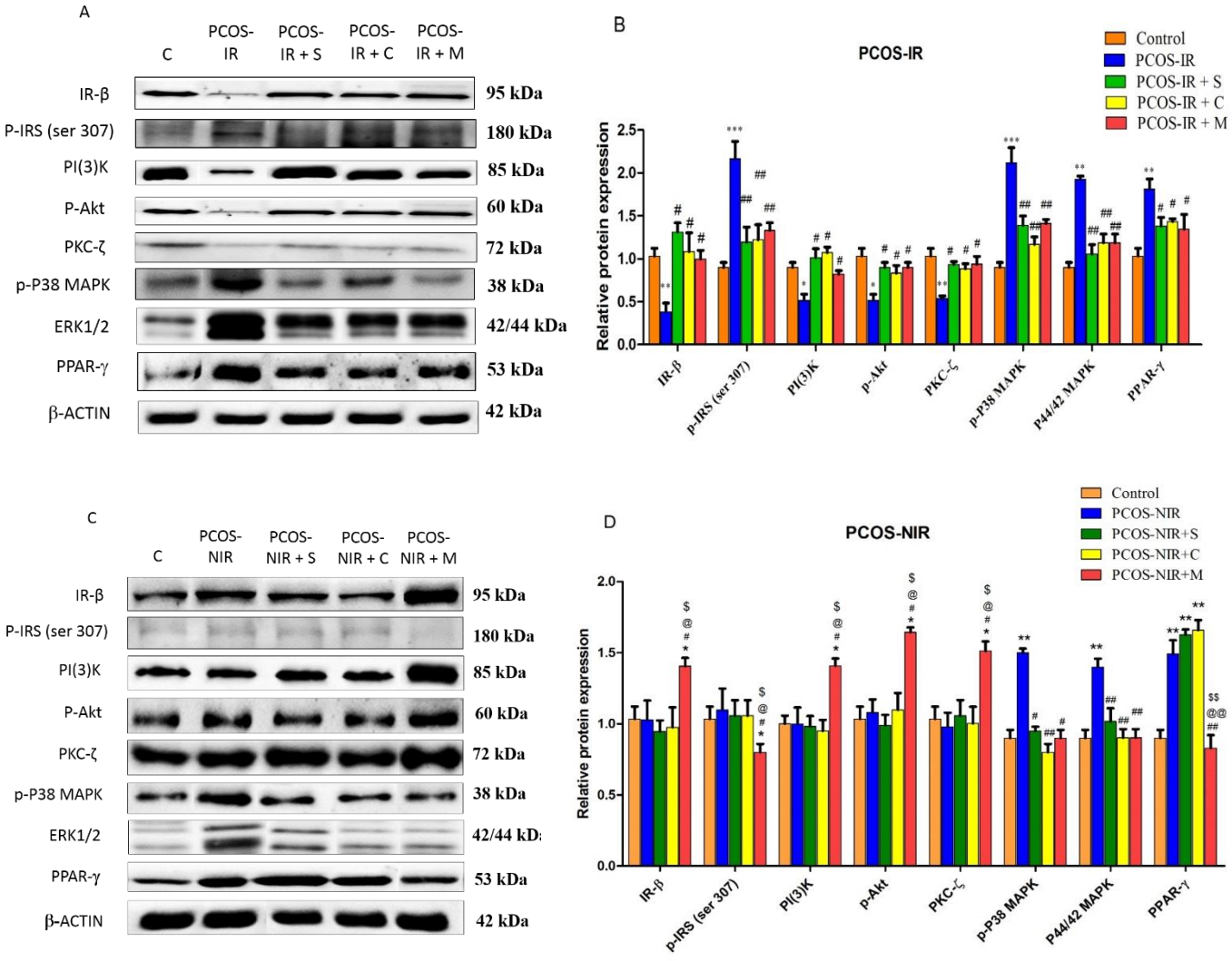


Figure 7.5: Effect of swertiamarin, curcumin and metformin on expression of proteins involved in insulin signalling in hLGC's. A. western blot from PCOS-IR. B. Densitometric analysis for PCOS-IR. C. western blot for PCOS-NIR. D. Densitometric analysis for PCOs-NIR. The relative protein expression values are represented as mean ± SEM of three independent experiments. * $P < 0.05$, ** $P < 0.01$ vs. C, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. PCOS-IR and PCOS-NIR, $^{@}P < 0.05$ vs. PCOS-IR+S, $^{\$}P < 0.05$ vs. PCOS-IR+C. n=8 control, n=8 PCOS-IR and n=8 PCOS-NIR

7.3.3 Swertiamarin and curcumin could reverse variations in the Lipogenic genes

Accumulation of lipids instead of cholesterol in granulosa cells are regulated by lipogenic genes. Hence lipogenic gene profile was assessed after the treatment of swertiamarin, curcumin and metformin in PCOS-IR and NIR hLGC's. Swertiamarin ($P < 0.001$) and curcumin ($P < 0.01$) significantly down regulated the candidate genes viz SREBP1c, FAS, ACC-1 and CPT-1 in PCOS-IR, whereas did not demonstrate any effect in PCOS-NIR hLGC's. Moreover swertiamarin was observed to be more potent than curcumin and equally potent to metformin. The positive control metformin down regulated the basal expression of all the genes in PCOS-NIR hLGC's (Fig 7.6).

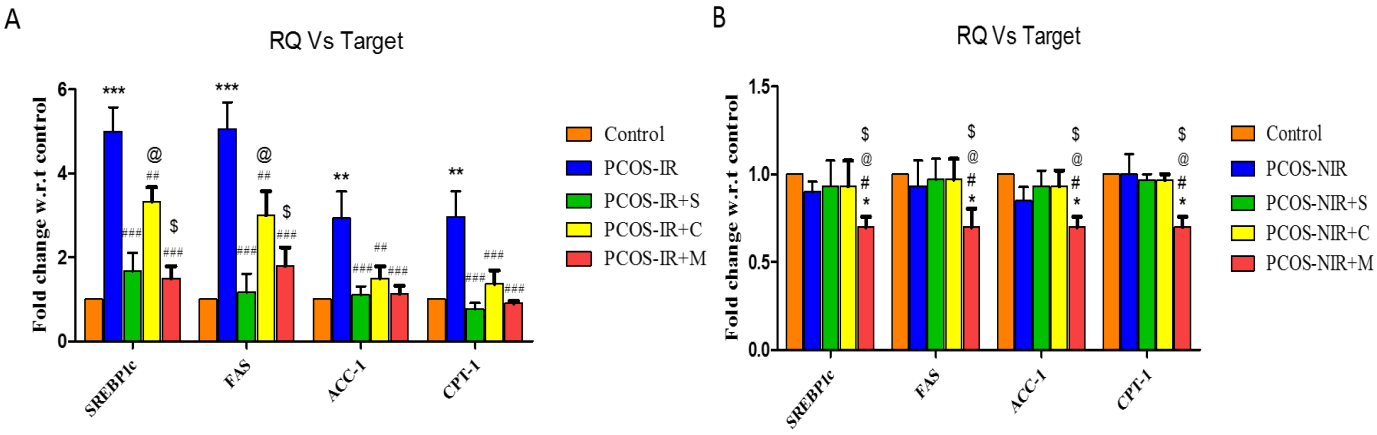


Figure 7. 6: Effect of swertiamarin, curcumin and metformin on mRNA expression of fatty acid metabolism genes in A. PCOS-IR. B. PCOS-NIR. The normalized expression values are represented as mean ± SEM of three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. C, $^{\#} P < 0.05$, $^{\#\#} P < 0.01$ vs. PCOS-IR and PCOS-NIR, $^{@} P < 0.05$ vs. PCOS-IR+S, $^{\$} P < 0.05$ vs. PCOS-IR+C. n=8 control, n=8 PCOS-IR and n=8 PCOS-NIR

7.3.4 Swertiamarin and curcumin reversed the decrease in steroidogenesis in hLGC's from PCOS-IR

The probable effect on steroidogenic pathway in the PCOS-IR hLGCs after 72 hrs of treatment with swertiamarin, curcumin and metformin was assessed. Swertiamarin and curcumin significantly up regulated the mRNA as well as protein expression of StAR, CYP19A1, 17β-HSD and 3β-HSD wherein swertiamarin but not curcumin was observed to be equally potent as compared to metformin. CYP11A1 demonstrated a significant down regulation in mRNA as well as protein expression in all the three treatment groups. Further 17β-HSD and 3β-HSD enzyme activity was also up regulated by swertiamarin ($P < 0.01$) and curcumin ($P < 0.05$). However the results demonstrated that swertiamarin was more potent that curcumin in positively modulation the steroidogenic property that are altered during PCOS IR condition. (Fig 7.7)

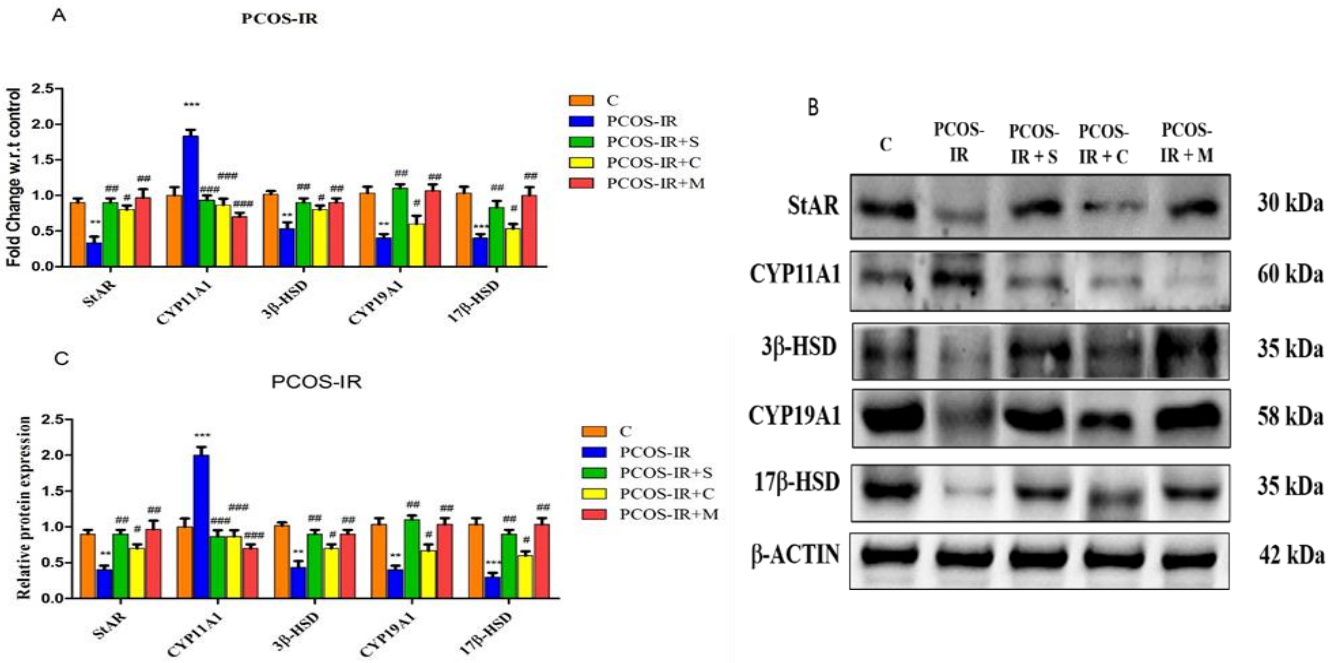


Figure 7. 7: Effect of swertiamarin, curcumin and metformin on steroidogenic machinery in hLGC's from PCOS-IR. A. mRNA expression. B. western blot. C. Densitometric analysis. E. Enzyme activity. The values are represented as mean ± SEM of three independent experiments. * $P < 0.05$, ** $P < 0.01$ vs. C, $^{\#} P < 0.05$, $^{\#\#} P < 0.01$ vs. PCOS-IR, $^{@} P < 0.05$ vs. PCOS-IR+S, $^{\$} P < 0.05$ vs. PCOS-IR+C. n=8 control, n=8 PCOS-IR and n=8 PCOS-IR

7.3.5 Swertiamarin and curcumin had no effect on steroidogenesis in hLGC's from PCOS-NIR

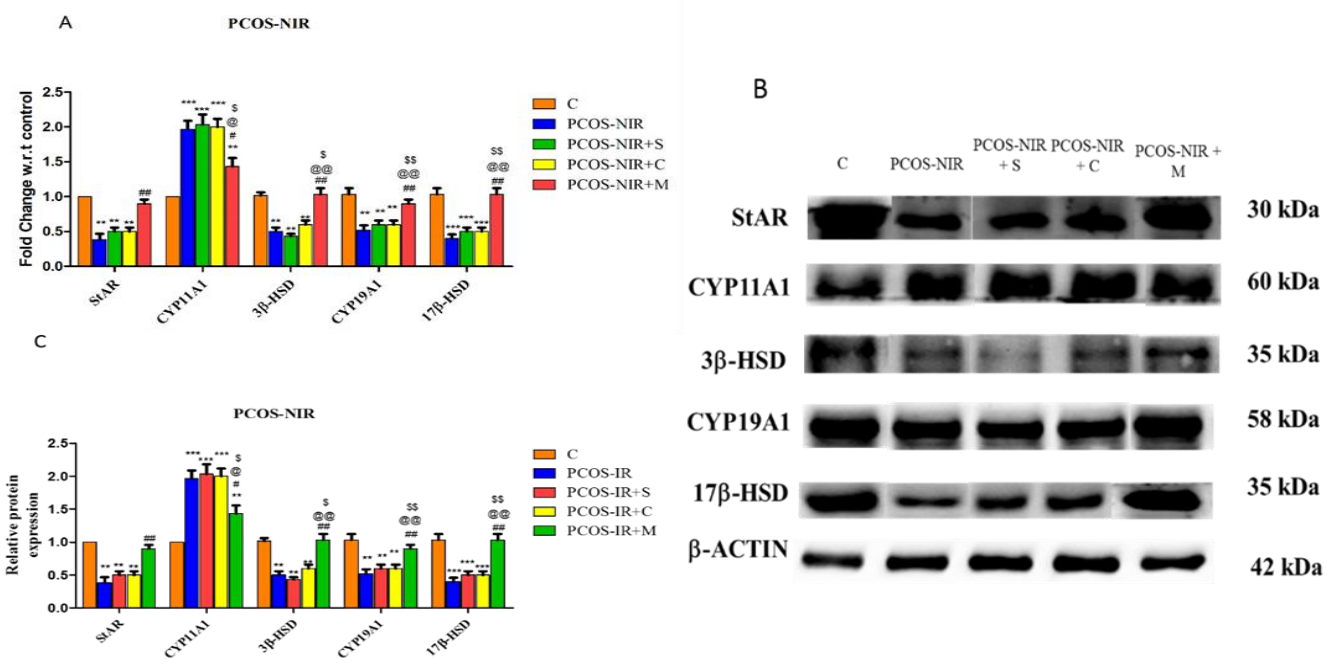


Figure 7. 8: Effect of swertiamarin, curcumin and metformin on steroidogenic machinery in hLGC's from PCOS-NIR. A. mRNA expression. B. western blot. C. Densitometric analysis. E. Enzyme activity. The values are represented as mean ± SEM of three independent experiments. * $P < 0.05$, ** $P < 0.01$ vs. C, $^{\#} P < 0.05$, $^{\#\#} P < 0.01$ vs. PCOS-NIR, $^{@} P < 0.05$ vs. PCOS-NIR+S, $^{\$} P < 0.05$ vs. PCOS-NIR+C. n=8 control, n=8 PCOS-NIR and n=8 PCOS-NIR

Since swertiamarin and curcumin treatment modulated the steroidogenic machinery in the PCOS IR cells, its probable effect on steroidogenic pathway in the PCOS-NIR hLGCs were explored. Swertiamarin and curcumin did not show any effect on mRNA and protein expression of any of the steroidogenic factors which is further supported by no effect on the enzyme activity. However, the gold drug metformin did show significant reversal ($P < 0.01$) of all the genes and proteins along with the enzyme activity with respect to PCOS-NIR group. (Fig 7.8)

7.3.6 Swertiamarin and curcumin reversed the enzyme activity only in hGLC's from PCOS-IR

Following the increase in the gene and protein expression of enzymes we further studied for their activity in the granulosa cell lysate of PCOS-IR and PCOS-NIR treated with swertiamarin, curcumin and metformin. A significant increase in 3β-HSD and 17β-HSD was demonstrated by treatment with swertiamarin ($P < 0.01$, $P < 0.001$)), curcumin ($P < 0.05$, $P < 0.05$)) and metformin ($P < 0.01$, $P < 0.001$)) in PCOS IR hLGCs with respect to untreated PCOS-IR, whereas in PCOS-NIR group only metformin demonstrated a significant increase ($P < 0.01$) in 3β-HSD activity as well as 17β-HSD activity. However, the effect of curcumin was less potent as compared to swertiamarin (Fig. 7.9).

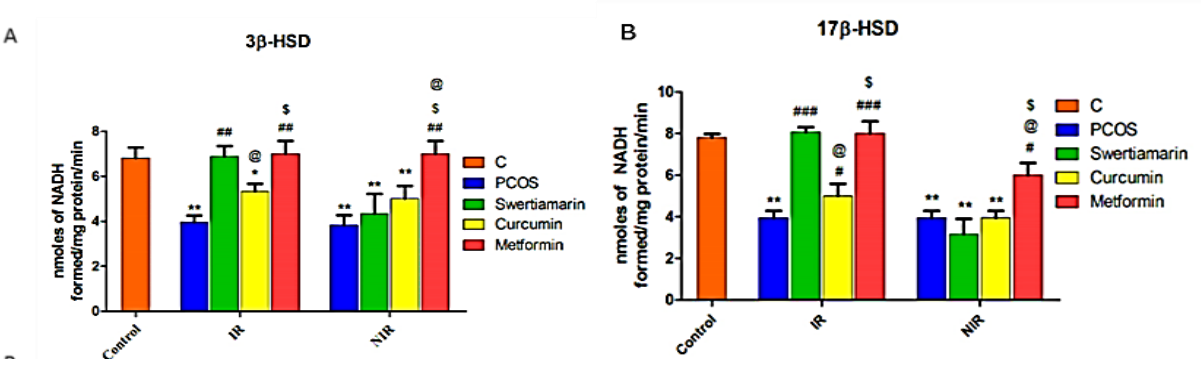


Figure 7. 9: Effect of swertiamarin, curcumin and metformin on enzyme activity in hLGC's from PCOS-IR and PCOS-NIR A. 3β-HSD and B.17β-HSD. The values are represented as mean ± SEM of three independent experiments. * $P < 0.05$, ** $P < 0.01$ vs. C, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. PCOS-NIR, $^{@}P < 0.05$ vs. PCOS-NIR+S, $^{\$}P < 0.05$ vs. PCOS-NIR+C. n=8 control, n=8 PCOS-NIR and n=8 PCOS-NIR

7.3.7 Swertiamarin and curcumin reverted the steroid hormone levels only in hGLC's from PCOS-IR

In vitro secretion of estradiol, progesterone and testosterone were analysed from the conditioned media of PCOS-IR and PCOS-NIR treated with swertiamarin, curcumin and metformin. A significant increase was demonstrated by treatment with swertiamarin ($P < 0.001$), curcumin (P

<0.05) and metformin (P < 0.001) in PCOS IR hLGCs with respect to untreated PCOS-IR, whereas in PCOS-NIR group only metformin demonstrated a significant increase (P < 0.01) in estradiol levels. Further metformin treated PCOS NIR cells could demonstrate significant rise (P < 0.01) in the levels of progesterone secretion relative to PCOS-NIR group. Testosterone levels were not detected in the cell culture supernatant (Fig. 7.10).

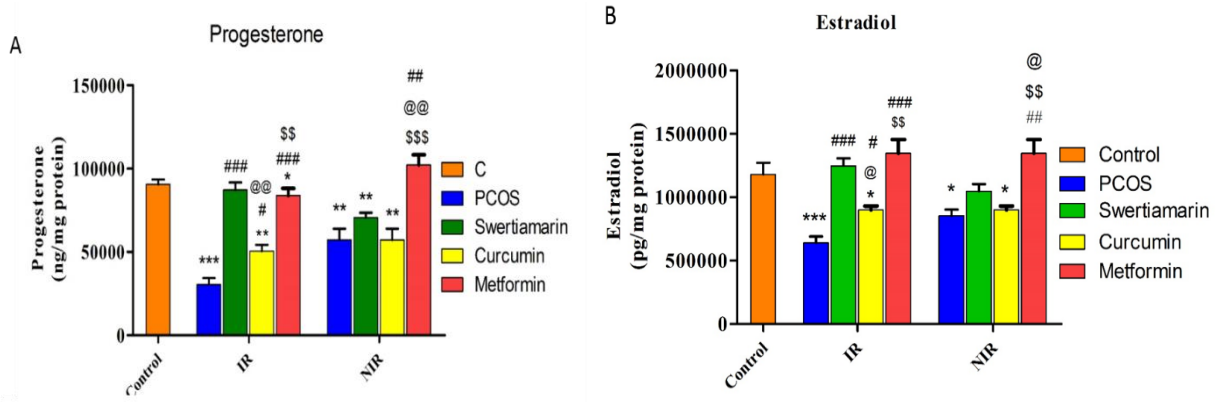


Figure 7. 10: Effect of swertiamarin, curcumin and metformin on steroid hormone levels in hLGC's from. A. PCOS-IR. B. PCOS-NIR. The normalized expression values are represented as mean ± SEM of three independent experiments. * P < 0.05, ** P < 0.01 vs. C, # P < 0.05, ## P < 0.01 vs. PCOS-IR and PCOS-NIR, @ P < 0.05 vs. PCOS-IR+S, $ P < 0.05 vs. PCOS-IR+C. n=8 control, n=8 PCOS-IR and n=8 PCOS-NIR

7.3.8 Swertiamarin and curcumin reversed the gonadotropin hormones only in hGLC's from PCOS-IR

The effect of swertiamarin and curcumin on FSH-R and LH-R gene expression in hLGC's from PCOS-IR and PCOS-NIR was observed. Swertiamarin and curcumin could significantly down regulate expression of both the receptors in PCOS IR hLGCs, again the effect of swertiamarin (P < 0.01) was more profound as compared to curcumin (P <0.05) and equally potent to metformin. As seen in the above results, both the bio active molecules failed to show any effect on the expression of receptors in hLGC's from PCOS-NIR whereas metformin treatment in PCOS-NIR cells did show significant down regulation in FSHR and LHR expression (Fig. 7.11).

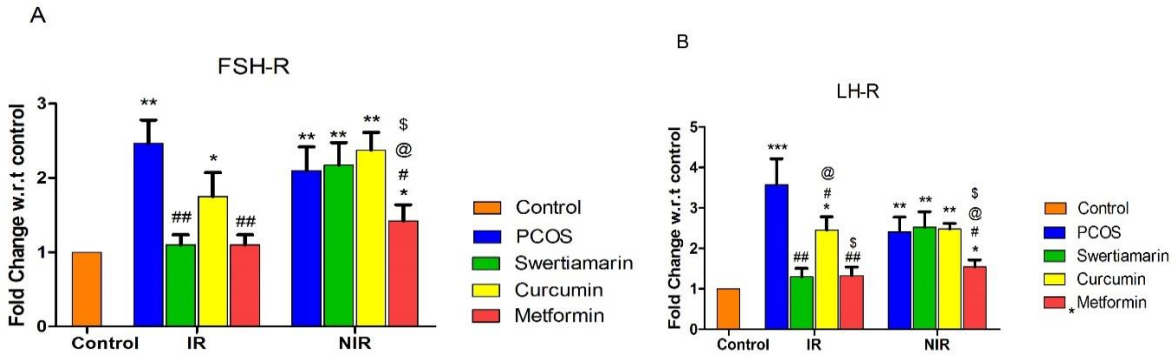


Figure 7. 11: Effect of swertiamarin, curcumin and metformin on mRNA expression of A.FSH-R and B. LH-R in hLGC's from PCOS-IR B. and NIR. The values are represented as mean ± SEM of three independent experiments. *

P < 0.05, ** P < 0.01 vs. C, # P < 0.05, ## P < 0.01 vs. PCOS-NIR, @ P < 0.05 vs. PCOS-NIR+S, $ P < 0.05 vs. PCOS-NIR+C. n=8 control, n=8 PCOS-NIR and n=8 PCOS-NIR

7.3.9 Swertiamarin and curcumin are capable of reversing variations in the IGF system

hLGC's from PCOS-IR and NIR were treated with swertiamarin, curcumin and metformin followed by analysis of gene expression of IGF candidate genes. Swertiamarin and curcumin treatment could significantly (P < 0.05) upregulate the gene expression of IGF-1 in PCOS-IR group. Both the bioactive molecule treatment appreciably down regulated the expression of IGF-1R (P < 0.01), IGF-II (P < 0.05) and IGF-2R (P < 0.01) in hLGC's from PCOS-IR. Although there was no alteration in the IGF system in PCOS NIR group; metformin treatment dramatically reduced the basal levels of all the genes in this group representing the side effect of metformin (Fig. 7.12).

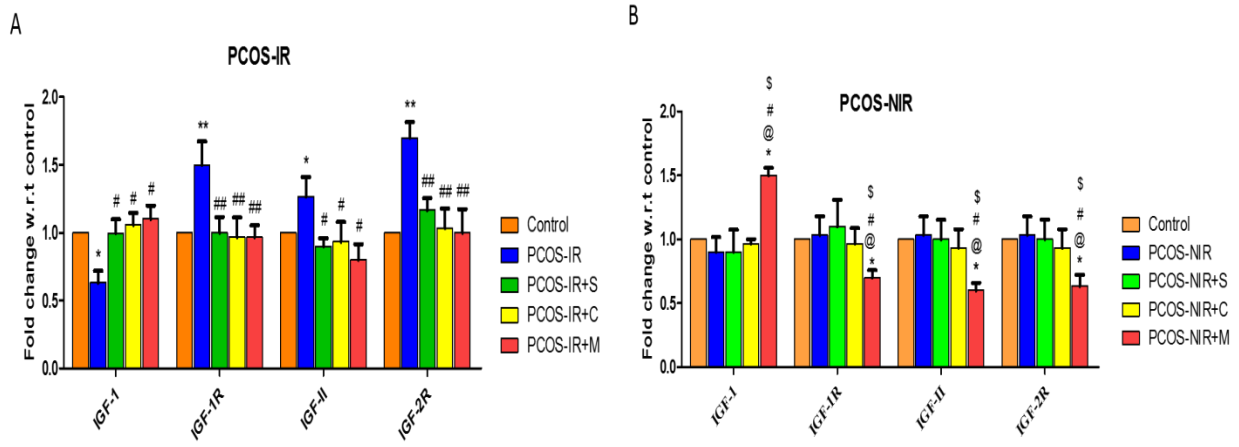


Figure 7. 12: Effect of swertiamarin, curcumin and metformin on mRNA expression of genes involved in IGF system. A. PCOS-IR. B. PCOS-NIR. The normalized expression values are represented as mean ± SEM of three independent experiments. * P < 0.05, ** P < 0.01 vs. C, # P < 0.05, ## P < 0.01 vs. PCOS-IR and PCOS-NIR, @ P < 0.05 vs. PCOS-IR+S, $ P < 0.05 vs. PCOS-IR+C. n=8 control, n=8 PCOS-IR and n=8 PCOS-NIR

7.4 Discussion

Ever since the role of IR in the pathogenesis of PCOS has been established, a positive effect of insulin-sensitizing drugs in the treatment of PCOS has been demonstrated. In accordance with this our study is the first one to report for the potential of swertiamarin and curcumin, the two bio active herbal insulin sensitizers for amelioration of IR and reestablishment of steroidogenesis in hLGC's isolated from follicular fluid of PCOS-IR and PCOS-NIR patients using metformin as a positive control.

Insulin resistance in PCOS has been associated with increase in granulosa cell death (Niu et al. 2014). The recovery of hLGC viability after incubation with swertiamarin and curcumin in our

study may be interpreted as indicative of reduced susceptibility of the PCOS-IR cells to undergo apoptosis as is observed with metformin in earlier studies (Sonntag et al. 2005). However, swertiamarin and curcumin did not show any effect on cell viability in hLGC from PCOS-NIR indicating the process of granulosa cell death in PCOS-NIR to be other than IR.

It is well known that ISD's can modulate insulin signalling that can recruit its downstream docking proteins to activate several other signalling pathways in different cell types (Pasquali and Gambineri 2006). Based on the up regulation of IR-β, PI(3)K, p-Akt, PKC-ζ and PPAR-γ along with down regulation of pIRS(ser 307) by swertiamarin and curcumin in PCOS-IR. Swertiamarin and curcumin are well known for their anti-diabetic and anti-hyperlipidemic effects in various animal models, different cell lines and clinical trials with humans (Pashine et al. 2012; Vaidya et al. 2014; Fazel Nabavi et al. 2015; Patel 2015). Our results for the first time show a direct interaction of swertiamarin and curcumin with key components of the classical insulin-signaling pathway thereby highlighting their ability to sensitize IR condition in hLGC's from PCOS-IR. Moreover curcumin with a dose of 33μM seemed to be the most potent insulin sensitizing drug for reversing insulin sensitivity in PCOS-IR as compared to swertiamarin 66μM and the positive control metformin 1mM. Surprisingly metformin increased the basal expression of key signalling proteins in the insulin signalling pathway which might result in increased insulin sensitivity and decreased insulin levels to less than normal even in PCOS-NIR group. Further, metformin but not swertiamarin and curcumin, increased the basal expression of key proteins involved in insulin signalling in control cells demonstrating its side effects in the long term (data not shown).

The importance of P38 MAPK and ERK1/2 in PCOS granulosa cells has been demonstrated in studies indicating their over expression as a result of oxidative stress and inflammation leading to decrease in the expression of StAR and progesterone synthesis. (Lin et al. 2009; Manna and Stocco 2011; Seto-Young et al. 2011; Tee and Miller 2013). Decrease in the protein expression of pP38 and ERK1/2 MAPK in treated hLGC's from PCOS-IR as well as PCOS-NIR indicating reversal of oxidative stress and thus reversal of cell death. Our findings are in line with the literature where swertiamarin and curcumin could enhance anti-oxidant defense system by suppressing oxidative stress and attenuate inflammation and apoptosis (Galaly et al. 2014; Patel 2015).

As insulin signalling is related to lipid metabolism and lipids are important for oocyte maintenance, we further observed the effect of swertiamarin and curcumin on the lipogenic genes SREBP1c along with enzymes ACC-1, FAS and CPT-1 in PCOS-IR and PCOS-NIR condition. Our data indicated that SREBP-1c protein expression, in addition to the expression of lipogenic target genes ACC1 and FAS and fatty acid oxidation gene CPT-1 were suppressed by swertiamarin and curcumin in hLGC's from PCOS-IR supported by (Um et al. 2013; Patel 2015). The reversal of these processes explain the association of swertiamarin and curcumin supplementations in decreasing lipid accumulation in granulosa cells seemed with an improvement in granulosa cell metabolism via the regulation of SREBP-1c, ACC-1, FAS and CPT-1 expression. However, curcumin was observed to be less potent as compared to swertiamarin and metformin in lowering fatty acid production.

The process of steroidogenesis is very crucial for the development of oocyte, its fertilization and embryo implantation (Doldi et al. 1998). In PCOS condition irrespective of IR, gene and protein expression of steroidogenic factors and their corresponding steroid hormones are lowered (Doldi et al. 1998; Maruthini et al. 2014). In the present study swertiamarin and curcumin could revert back mRNA expression of gonadotropin receptors, mRNA and protein expression of StAR, CYP11A1, CYP19A1, 17β-HSD and 3β-HSD, their enzyme activity along with secretion of the corresponding steroid hormones estradiol and progesterone in hLGC's from PCOS-IR thus improving the process of steroidogenesis. Our results for curcumin are in line with the literature demonstrating alleviation of androgen excess in PCOS (Watson 2011). Metformin, the positive control of the study improves steroidogenesis by down regulating FSH-R and increasing the progesterone secretion by hLGC from PCOS women (Rice et al. 2013; Maruthini et al. 2014). Results further demonstrated curcumin to be weaker bio active in reversing steroidogenesis as compared to swertiamarin and metformin. Moreover swertiamarin at a concentration of 66μM proved to be a better drug in reversing steroidogenesis with the same potency as compared to metformin 1mM in PCOS-IR. In the present study testosterone could not be detected in the cell culture supernatant. The finding was consistent with the literature explaining presence of the enzyme P450c17/CYp17, responsible for converting C21 steroids (progestrogens) to C19 steroids (androgens) solely in theca cells ant not in granulosa cells (Wen et al. 2010).

We further wanted to study the effect of bio actives on IGF (IGF-I, IGF-II, IGF-1R and IGF-2R) system whose involvement is appreciated in the development of preantral to preovulatory

follicles, in the process of follicular atresia and is over expressed in diabetic condition (Silva et al. 2009; Kaur et al. 2012; Mehta et al. 2013; Livingstone and Borai 2014). In the present chapter treatment of swertiamarin and curcumin reversed the aberrant effects of PCOS on IGF-I, IGF-II, IGF-1R and IGF-2R in hLGC's from PCOS-IR. Studies in literature with administration of curcumin and metformin in diabetic patients have reported upregulation of IGF-1 gene and down regulation of its receptor thereby improving insulin sensitivity and glucose uptake (El-Bahr 2013; Markowska et al. 2013). Studies with cancer models have demonstrated role of curcumin in suppression of IGF-1R and IGF-II at transcriptional level, thus preventing development of cancer (Xia et al. 2007; Singh et al. 2010; El-Bahr 2013; Kim et al. 2014).

As observed with the proteins involved in insulin signalling system, metformin reversed the expression of IGF-2R in hLGC's from PCOS-NIR. These findings combined with no change in basal expression of these proteins and genes in control hLGC's as well as PCOS-NIR hLGC's strongly suggest that other than metformin, these bio actives might function only during IR condition.

An additional aim of this study was to determine whether swertiamarin, curcumin and metformin could ameliorate decreased steroidogenesis in hLGC's from PCOS-NIR. Strikingly, swertiamarin and curcumin did not steroidogenesis. On the basis of the findings that swertiamarin and curcumin restored insulin sensitivity in PCOS-IR with no effect on PCOS-NIR, it is quite possible to speculate that swertiamarin and curcumin could be mediating their effects on granulosa cell steroidogenesis only through insulin signaling. Metformin reversed back the decrease in steroidogenesis although with a less pronounced effect as compared to PCOS-NIR. This effect could be attributed to the direct effect of metformin on steroidogenesis probably through a multipathway reaction with ERK1/2, pP38 MAPK or PI(3)K all involved in regulation of steroidogenesis (Moore et al. 2001; Hunzicker-Dunn and Maizels 2006; Stocco 2008). Such direct effects of metformin have been supported by some clinical studies in which the insulin sensitizer increased the ovulation rate, and fertilization rates by having no effect on the basal insulin levels. (Baillargeon et al. 2004; Rice et al. 2009; Marshall and Dunaif 2012). These findings indicate the possibility of some other factors in hLGC's from PCOS-NIR yet unidentified and not associated with insulin signalling to have a role in restoring steroidogenesis by metformin. However few studies with clinical trials have reported increased ovulation rates with decreasing the basal insulin levels in normo insulinemic subjects indicating side effects of

metformin leading to hypoglycaemia if prescribed to PCOS-NIR (Önalan et al. 2005; Marshall and Dunaif 2012). Moreover as the actual treatment time for metformin to induce a clinical ovulation is 6 months, other drugs are preferred over metformin for rapidly inducing ovulation in PCOS (Nestler 2008).

Collectively the results support the notion that, swertiamarin at 66µM and curcumin at 33 µM show equal effect as insulin sensitizers for alleviating IR condition whereas curcumin is less effective at alleviating disorder at fat metabolism and restoring steroidogenesis, thus indicating swertiamarin to be better over curcumin in vitro in hLGC's from PCOS-IR. Swertiamarin and curcumin had no effect on steroidogenesis in PCOS-NIR. Metformin did restore steroidogenesis in PCOS-NIR but it is important however to highlight that it decreases the basal insulin signalling parameters in PCOS-NIR group which might lead to adverse effects in the long-term health (Fig: 7.13). This calls for a proper diagnosis of IR condition in PCOS so that targeted therapy can be prescribed to achieve increased pregnancy rates with decreased time.

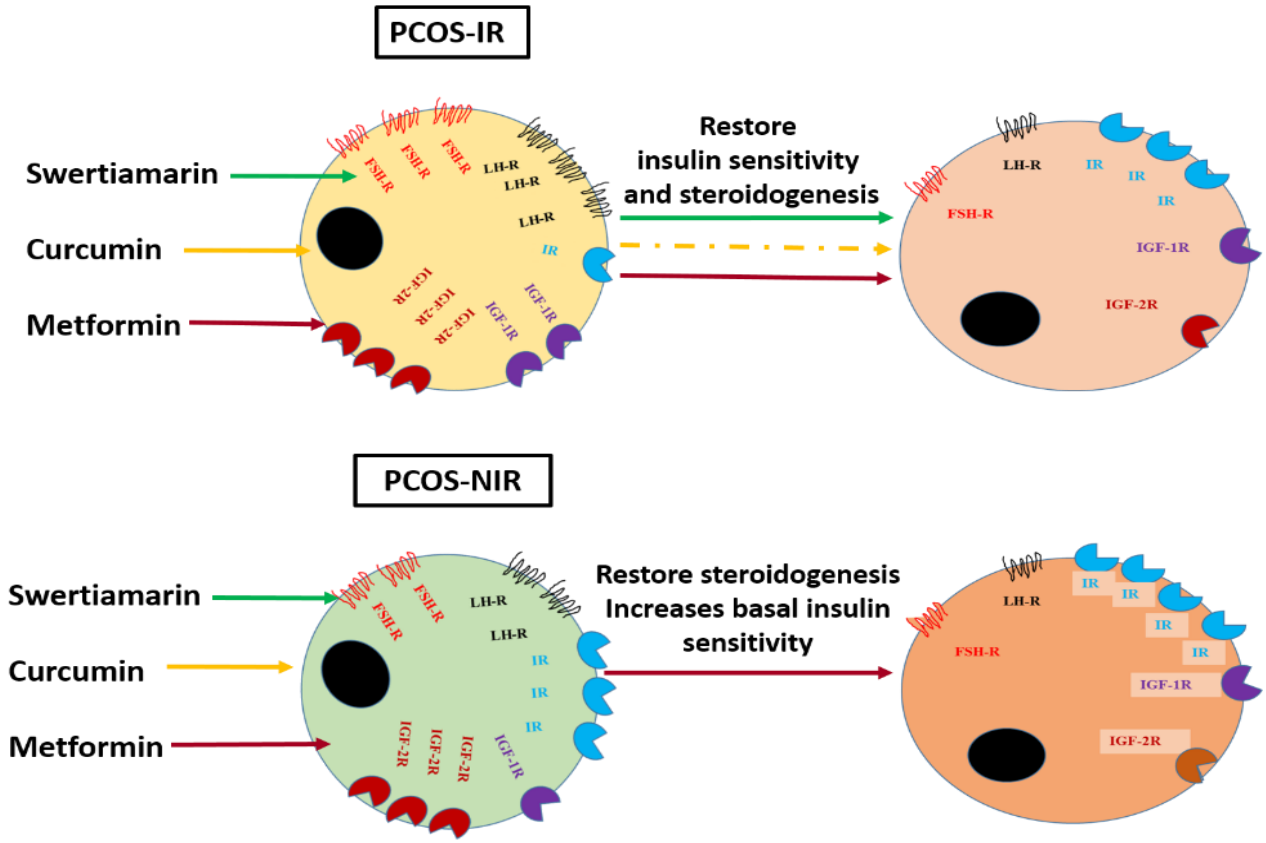


Figure 7. 13: Schematic diagram summarizing potential effect of swertiamarin, curcumin and metformin in PCOS-IR and PCOS-NIR hLGC's.

7.5 References

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