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Summary

Polycystic ovary syndrome (PCOS) is one of the most prevalent endocrine disorders affecting 4-26 % women belonging to reproductive age and is characterized by hyperandrogenism, hyperinsulinemia, oligo-ovulation and multiple peripheral cysts in the ovaries- due to dysregulated steroidogenesis and its signalling (Chatterjee and Bandyopadhyay, 2020; Dunaif and Book, 1997; Abbott et al., 2002; Doi et al., 2005; Wickenheisser and McAllister, 2007). Although the exact etiopathology of PCOS is unclear, it is thought to be a multifaceted condition involving environmental, genetic, metabolic and endocrine abnormalities (Franks et al., 2006). Almost 30-40% of women affected have impaired glucose tolerance, insulin resistance, dyslipidaemia and other complications associated with metabolic syndrome (Chandrasekaran and Sagili, 2018). The treatment of PCOS involves the use of several drugs such as metformin and clomiphene citrate, but they are commonly associated with serious side effects, for instance, increased risk of vascular thromboembolism, digestive complications such as nausea, diarrhoea and dizziness, and Vitamin B12 deficiency upon prolonged usage (Mokaberinejad et al., 2019; Saha et al., 2012). The use of safe and natural therapeutic agent to modulate ovarian activity and enhance reproductive function is an alternative approach. In this direction, many alternative medicine including herbs have been tested and found to improve function of the female reproductive system (Fugh-Berman Kronenberg, 2001; Maharjan et.al., 2010; Yang et al., 2018a; Yang et al., 2018b; Yang et al., 2020).

In this line, data from our lab has demonstrated that *Aloe vera* gel (AVG) could restore ovarian structure-function and decrease associated metabolic complications like hyperglycaemia and dyslipidaemia in PCOS rat model (Desai et al., 2012, Radha et al., 2014). The therapeutic property of AVG towards management of PCOS can be attributed to the non-polar phytochemicals present in it (Dey et al., 2017; Nampoothiri et al., 2015; Radha and Laxmipriya, 2016a). However, the bioactive phytocomponent/s involved in this efficacy has not been still elucidated. **Thereby, the present study was taken up with an aim to isolate and characterize the bioactive non-polar phytocomponent/s of AVG and elucidate their potential molecular targets using *"in-silico"*, *"in-vitro"* and *"in-vivo"* approaches; with**

defining therapeutically possible molecular mechanism that be directed towards the better management of PCOS and its associated metabolic co-morbidities.

In order to isolate the bioactive non-polar phytochemicals from AVG, the petroleum ether extract of AVG was sub-fractionated using column chromatography and five potential partially purified isolates- LP1, LP2, LP3, LP4 and LP5 were obtained. The phytoconstituents of the partially purified isolates was further confirmed by GC/MS analysis and identification was done by comparing with the NIST Library. LP1, LP2, LP3, LP4 and LP5 were found to contain n-Hexadecanoic acid, Cholesta-3,5-diene, Campesterol acetate, b-Sitosterol and Stigmasterol acetate with an abundance of 97.07%, 96.04%, 94.03%, 92.45% and 87.49% respectively, which was also confirmed by performing reverse-phase HPLC by running respective standards of the compounds which was found in abundance.

Apart from the phytochemical analysis, pharmacokinetic and pharmacodynamic assessment was performed in rats to understand absorptive, distributive and clearance of phytocomponents when presented to the body. A potent molecule must reach its target in the body in sufficient concentration and stay there in a bioactive state long enough for the predicted biologic activities to occur for it to be effective as a medication (Daina et al., 2017). Based upon GC/MS profile, it was observed that amongst the different non-polar phyto-compounds, the systemic absorption of n-Hexadecanoic acid, Campesterol acetate, beta- sitosterol, Cholesta-3,5-diene and Stigmasterol acetate was maximum within 5 hours of oral administration of 100 mg/kg body weight of Petroleum ether extract. In addition to this, the above-mentioned phytochemicals exhibited greater affinity towards steroidogenic active tissues such as ovaries, adrenal, uterus and also liver. However, other tissues that play an important role in the etiopathology of PCOS like skeletal muscles, kidney, hypothalamus, pituitary also demonstrated the presence of these phytochemicals, but the concentration was very low. In spite of having low oral bioavailability, the non-polar phytocompounds isolated from *Aloe vera* gel exhibited good pharmacodynamic properties, wherein, they reduced the testosterone levels and increased the estradiol and progesterone levels in the animals within 5 hours of treatment. These results suggest that fatty acid such as n-Hexadecanoic acid and phytosterols such as Campesterol acetate, beta-sitosterol, Stigmasterol acetate or its metabolite like Cholesta-3,5-diene may be responsible for such response.

According to growing scientific data, phytosterols and their derivatives have numerous pharmacological characteristics, including human-wellness-promoting qualities (Salehi et al.,

2021). These health advantages include the capacity to significantly lower total and low-density lipoprotein (LDL) cholesterol levels, lowering the risk of a variety of illnesses (Plat et al., 2019). Phytosterols also have anti-inflammatory, antioxidant, anti-ulcer, immunomodulatory, antibacterial, and antifungal properties, as well as a role in wound healing and platelet aggregation inhibition (Dutta, 2003; Ogbe et al., 2015). Few studies in support of our results, have shown that b-Sitosterol exposure causes reduced circulating hormone, cholesterol concentrations, and intra-mitochondrial cholesterol pools in goldfish (Carassius auratus) owing to changes in gonadal StAR transcript abundance (Sharpe et al., 2006). When young goldfishes were fed a diet enriched with *Aloe*, similar cholesterol-lowering and estrogenic effects were seen (Palermo et al., 2013). Furthermore, fatty acids and phytosterols included in Saw Palmetto extract block the enzyme 5-reductase, which has an anti-androgenic impact on male Syrian Hamsters (Opoku-Acheampong et al., 2015). All these data support our data which suggests the potentiality of whole *Aloe vera gel* as steroid modulator. Further, it was our interest to identify the biological targets of the phytocomponents, which precipitates into above mentioned effects.

In order to elucidate the molecular targets and the magnitude of interaction with the steroidogenic and metabolic targets with the major components of partially purified non-polar phytocomponents (PPNPP) of *Aloe vera* gel. An *"in-silico"* molecular docking analysis was performed using the Glide tool developed by Schrödinger. The docking score, binding free energy, and hydrogen bonds produced with the surrounding amino acids were all utilised to predict the binding affinities and correct alignment inside the active sites of the key steroidogenic and metabolic targets, that included Follicle Stimulating Hormone Receptor (FSHR), Steroidogenic Acute regulatory protein (StAR), 3-beta hydroxysteroid dehydrogenase (3b-HSD), Aromatase, Estrogen Receptor alpha, Estrogen Receptor beta, Androgen receptor, Progesterone receptor, and Phosphorylated Insulin Receptor tyrosine kinase which served as targets. LP1, LP2, LP3, LP4 and LP5 contains n-Hexadecanoic acid, Cholesta-3,5-diene, Campesterol acetate, b-Sitosterol and Stigmasterol acetate with an abundance of 97.07%, 96.04%, 94.03%, 92.45% and 87.49% respectively. Therefore, n-Hexadecanoic acid, Cholesta-3,5-diene, Campesterol acetate, b-Sitosterol and Stigmasterol acetate were considered as the ligands for the "in-silico" studies. Data demonstrate that Campesterol acetate (a significant component of LP3) has the highest affinity for important steroidogenic targets such as the Progesterone receptor, Steroidogenic Acute Regulatory protein, and Aromatase, with docking scores of -9.988, -8.312, and -6.913, respectively. With docking scores of -7.166 and -6.512,

respectively, the Estrogen Receptor beta and 3-beta hydroxysteroid dehydrogenase were shown to have excellent interactions with beta-Sitosterol (LP4) and Stigmasterol acetate (LP5). With docking scores of -7.419 and -3.205, n-Hexadecanoic acid (LP1) was revealed to dock with Androgen Receptor and phosphorylated Insulin Receptor tyrosine kinase, respectively.

Also, it was evident from data that the docking efficiency and H-bond forming capacity of Campesterol acetate (major constituent of LP3) and beta-Sitosterol (major constituent of LP4) are more towards steroidogenic targets like StAR, Aromatase, ER-b and PR. On the other hand, n-Hexadecanoic acid, which was the major component of LP1, was found to strongly interact with Androgen Receptor by forming Hydrogen bonds within the DNA-binding domain of it. LP1 was also found to interact with p- Insulin receptor tyrosine kinase with low docking score, but very low potential energy of binding, suggesting that LP1 confers good affinity towards the target. Furthermore, using the Swiss ADME programme, prediction for physically significant and physiochemical characteristics of prospective therapeutic compounds, assessments of the pharmacokinetic parameters of Absorption, Distribution, Metabolism, and Excretion (ADME) of the non-polar phytochemicals were computed. Our results demonstrate that all the non-polar phytochemicals had all desired properties as per drug criterion and could be developed into potential drug candidates. The phytocomponents poses minimal potential for adverse effects when consumed orally because of their low bioavailability. ***For the first time, the present study has revealed the "drug likeness" properties of non-polar phytochemicals isolated from Aloe vera gel and their potential to modulate important steroidogenic and metabolic target using "in-silico" studies.***

It is important to validate of the results obtained in the *"in-silico"* study. Thereby, following experiments were designed and performed both in *" in vitro" and " in vivo*" approaches. Initial experiments were performed by incubating the phytochemicals with the KGN cell-line-steroidogenic human ovarian granulose-like tumour cell line that has been used to study steroidogenesis, cell proliferation, and FSHR-coupled signalling in human granulosa cells (Havelock, 2004), along with a capacity of secreting estrogen and progesterone (Nishi et al., 2001). Results from the current bioassay study demonstrates the anti-androgenic potential of LP1; Estrogenic and progestogenic nature of LP3. LP1 and LP3 elicit their response by modulating the gene expression of gonadotropin receptors, steroid receptors as well as other key steroidogenic marker proteins. Several studies over the past decade have demonstrated similar bioactive potential of phytosterols, terpenoids and fatty acids isolated from other dietary

sources in birds (Qasimi et al., 2018), fishes (Sharpe et al., 2006; Palermo et al., 2013), rodents (Wang et al., 2018), human adrenocortical H295R cells (Ohno etal., 2002) and pig-derived granulosa cells (Santini et al., 2009). However, reporting such bioactivity for these non-polar phytochemicals isolated from *Aloe vera* gel is novel and holds immense potential for drug development against female endocrinopathies including PCOS.

The steroidogenic and metabolic regulatory potential of LP1 and LP3 were proven in KGN cell-line under normal physiological conditions. However, it would be interesting to study their bioactive potential in a pathological condition like PCOS. In this context, it is to be noted that the granulosa cells, which are the major ovarian cells, are prone to damage by presence of hyperandrogenism and hyperinsulinemia conditions, as observed in case of PCOS (Dey et al., 2021; Belani et al., 2018). The granulosa cells are very important, as it supports the growing oocyte, proliferates, and produces sex hormones and a variety of growth factors, that are necessary factors for follicle development (Tu et al., 2019). Firstly, we developed *"in-vitro"* model of PCO using primary culture of rodent derived luteinized granulosa cells and human derived immortal granulosa cell-line- KGN. Co-administration of 0.1 mIU/mL insulin and 50ng/mL DHT to the primary culture of LGCs and 2.0 mIU/mL insulin and 100 ng/mL DHT to the KGN cell line, respectively, effectively regulated the key genes involved in steroidogenesis, thereby leading to abnormal steroid hormone secretion by the cells. The observed effects were similar to that observed in the granulosa cells of PCOS patients (Wickenheisser et al., 2005; Pigny et al., 2003; Dewailly et al., 2003; Karkanaki et al., 2011; Chaves et al., 2012; Yang et al., 2015; Doldi et al., 2000). The establishment of a novel *"in-vitro"* PCO model is remarkable and has enormous promise for investigating precise downstream cellular signalling as well as therapeutic target screening for ovarian dysfunctions like PCOS. There have been no previous studies that involved screening phytochemicals for their steroidogenic modulatory potential using a PCO-like ovarian microenvironment. The impact of LP1 and LP3 on the regulation of steroidogenic targets and hormone production by PCO-like KGN cells was the emphasis of this study. The gene expression of steroidogenic targets Luteinizing Hormone Receptor (*Lhr*), Steroidogenic acute regulatory protein (*Star*), Aromatase (*Cyp19a1*), Androgen Receptor (*Ar*), and Progesterone Receptor (*Pgr*) was competently modulated by LP1 and LP3, and were comparable with their respective standards. This is the first study to demonstrate the bioactivity of non-polar phytocompound/s of *Aloe vera* gel in an *"in-vitro"* PCO- like model towards restoration of dysregulated steroidogenesis

at the molecular level. Apart from "*in-vitro"* validation, it becomes important to study the efficacy of phytocompounds *"in vivo"*.

"*In-vivo"* research can enhance predictions of safety, toxicity, and efficacy by providing a better knowledge of the drugs' molecular targets and possible interactions with other organs of the body (Schenone et al., 2013). Considering PCOS is a heterogeneous disease that affects various organ systems and has substantial metabolic and reproductive symptoms (Williams et al., 2016), evaluating bio-isolate effectiveness in a *"in vivo"* system is critical. With this background, the current study focused at the effects of LP1 and LP3 in a Letrozole-induced PCOS mouse model, as well as hormonal and metabolic pathways for managing PCOS pathology and its comorbidities. Letrozole (an aromatase inhibitor) has been shown to be effective in instigating PCOS in animals (Kafali et al., 2004). It works by inhibiting aromatase, resulting in a lower conversion of androgens to estrogens and an excess of androgens in the ovary (Garcia-Velasco et al., 2005). The mouse model of PCOS was generated with letrozole (0.5 mg/kg/day orally for 21 days) and matched with the criteria of Rotterdam like hyperandrogenaemia, oligo-ovulation and presence of peripheral cysts and considered as PCOS phenotype (Dey et al., 2017). The PCOS group had a substantial elevated body weight. It has previously been shown that an increase in abdominal fat storage is linked to an increase in body weight and may cause adipocyte malfunction and insulin resistance in PCOS patients (Goodarzi et al., 2011). Furthermore, letrozole-treated mice had glucose intolerance, higher fasting insulin levels, higher HOMA-IR values, and higher circulating triglyceride levels as documented by several researchers (Kauffman et al., 2015; Kelley et al., 2016). According to Desai et al., 2012, the insulin resistance and poor glucose tolerance caused by letrozole are mostly attributable to increased testosterone concentrations.

In addition to this, Letrozole treatment caused reproductive irregularities, as evidenced by a halted estrus cycle during the diestrus stage, increased testosterone levels, and decreased progesterone levels. Furthermore, ovarian sections revealed numerous peripheral cystic follicles, which are typical feature of PCOS. These findings are comparable to those found in previous studies (Rajan and Balaji, 2017; Yang et al., 2018a; Yang et al., 2018b). As a result, this model is appropriate for exploration of both reproductive and metabolic aspects of the condition, and it may be utilised to better understand the etiopathology and assess the bioactivity of the isolated non-polar phytochemicals.

Treatment of PCOS mice with LP3 and CA resulted in substantial weight loss, increased glucose tolerance, lower fasting insulin levels, and lower HOMA-IR readings, which was comparable with that of whole *Aloe vera* gel and Petroleum ether extract of *Aloe vera* gel treated groups. It's possible that non-polar phytocompounds found in *Aloe vera* gel could decrease glucose resistance by regulating glucose homeostasis, increasing insulin production, and potentiating glucose absorption. This supports the hypothesis that isolated bioactives from *Aloe barbadensis* could be identified as a metabolic modulator (Desai et al., 2012). Furthermore, these therapies may be beneficial in lowering the levels of circulating triglycerides in PCOS animals. Studies by Alinejad-Mofrad et al., (2015); Maharjan et al., (2010) and Desai et al., (2012) have reported similar findings. Phytosterols found in *Aloe vera* gel have been shown to be effective in controlling glucose and lipid metabolism in rats in a number of investigations (Tanaka et al., 2006; Misawa et al., 2008; Misawa et al., 2012).

When compared to control animals, PCOS animals displayed an irregular estrus cyclicity, presence of numerous peripheral cysts, higher testosterone levels, and lower progesterone levels. Treatment of the PCOS animals with *Aloe vera* gel, petroleum ether extract of *Aloe vera* gel as well as LP3 restored the ovarian structure-function. The observed bioactivity of LP3 was better than that of Metformin, a conventual drug for PCOS management. Supporting our findings, Demirel and his colleagues (2016) observed that tocopherol, squalene, b-sitosterol, campesterol, and stigmasterol contained in Corylus avellana seed oil produced a substantial decrease in testosterone levels and restored ovarian function in PCOS rats. In PCOS rats, Vitex *agnus-castus* fruit extract had an antiandrogenic impact via enhancing aromatization, resulting in reduced testosterone levels (Jelodar and Askari, 2012). Because abnormally high testosterone levels contribute to the development of PCOS, testosterone downregulation post LP3 therapy might help with managing the reproductive complications associated with PCOS. Recent research has focused on the role of Campesterol and Stigmasterol as progesterone precursors (Janeczko, 2012; Tarkowská, 2019), suggesting that plant sterols may have the ability to influence hormone metabolism.

The results of this investigation showed that LP1 had mainly an anti-androgenic activity. LP3, on the other hand, was shown to be more effective than LP1 at regulating the steroidogenic and metabolic parameters linked to PCOS. The mode of action of LP3, a partly pure non-polar phytochemical produced from *Aloe vera gel*, might be owing to its modulatory impact on steroid receptor transcription, primarily functioning as an anti-androgenic and progestenic

agent. It aided in the restoration of ovarian shape and function in Letrozole-induced PCOS mice by regulating the hormonal environment. Furthermore, therapy with LP3, AVG, and PE reduced metabolic dysfunction associated with PCOS, such as glucose intolerance, insulin resistance, and dyslipidaemia. Surprisingly, LP3 was shown to be as effective as AVG and PE in treating reproductive and metabolic problems in Letrozole-induced PCOS mice, without any toxicity, suggesting that oral administration of LP3 (0.5 g/kg/day) for 60 days is sufficient for PCOS therapy. Paradoxically, the therapeutic potential of LP3 was higher than that of metformin, the most often recommended medication for PCOS.

In conclusion, the overall goals achieved are:

1, Isolation by column chromatography and characterization of five major non-polar phytosterols of *Aloe vera* gel based upon GC/MS analysis - LP1, LP2, LP3, LP4 and LP5, containing n-Hexadecanoic acid, Cholesta-3,5-diene, Campesterol acetate, b-Sitosterol and Stigmasterol acetate with an abundance of 97.07%, 96.04%, 94.03%, 92.45% and 87.49% respectively.

2. Development of a rapid and sensitive HPLC method for simultaneous detection of n-Hexadecanoic acid, Cholesta-3,5-diene, Campesterol acetate, b-Sitosterol and Stigmasterol acetate within the linear range of 5-500 ng/mL.

3. Pharmacokinetic and tissue distribution studies in rats have shown that the non-polar phytochemicals, mainly Campesterol acetate, n-Hexadecenoic acid and b-Sitosterol are absorbed rapidly in the blood and distributed more towards steroidogenic organs like Ovaries and adrenal. However, the percent absorption of these phytochemicals in the blood and various tissues was observed to be low, as compared to the concentration of phytocomponents present in the crude extract which was fed to the animals, suggesting low bioavailability.

4. The pharmacokinetics of these bioactive molecules could be correlated with the pharmacodynamic changes in the hormone levels of the animals. Also, the liver and kidney toxicity markers were found to be within the physiological range with the administered dose, suggesting that the phytochemicals are safe for consumption.

5. Molecular docking studies identified the steroidogenic and metabolic targets of the non-polar phytochemicals of *Aloe vera* gel to be Steroidogenic acute regulatory protein, Aromatase, 3-beta hydroxysteroid dehydrogenase, Androgen Receptor, Progesterone Receptor and

Estrogen Receptor beta and phosphorylated Insulin Receptor tyrosine kinase. Validation in KGN cell-line (granulosa cell-line), further substantiated the *"in-silico"* observations. The observed results demonstrated that LP1 and LP3 have greater bioactivity in terms of modulation of steroidogenic and metabolic targets and suggested that they might be effective drug candidates for management of pathological condition like PCOS.

6. *"In-silico"* ADME prediction revealed that LP1, LP2, LP3, LP4 and LP5 were non-toxic and had desirable drug-like properties.

7. Development of a novel *"in-vitro"* model of PCO that mimics the ovarian microenvironment has been successfully developed by co-induction of hyperinsulinemia and hyperandrogenaemia in the primary culture of luteinized granulosa cells isolated from rat ovaries (Dey et al., 2021) as well as human derived KGN cell-line. Both the models exhibited modulation the key genes involved in steroidogenesis, folliculogenesis and ovulation at molecular level along with abnormal steroid hormone secretion by the cells.

8. Further, the bioactivity of LP1 and LP3 was evaluated in the PCO-like model and they were found to elicit anti-androgenic, estrogenic and progestogenic potential along with modulation of key genes associated with the dysregulated ovarian microenvironment at transcriptional level, as observed in case of PCOS.

9. Studies in Letrozole-induced (0.5mg/kg/day orally for 21 days) mice demonstrated the efficacy of LP1 and LP3 in terms of restoration of ovarian structure-function changes along with improvement of metabolic status, with LP3 (containing 94.03% Campesterol acetate) to be most potential. The observed therapeutic potential could be attributed to the action of LP3 on regulation of gene expression of gonadotropin receptors, steroid receptors and key steroidogenic and metabolic markers.

Overall, this study was helpful in identification of the bioactive components of Aloe vera gel as potential therapeutic alternative for reproductive and metabolic disturbances in PCOS along with its physiological mechanisms for efficacy using "in-silico", "in-vitro" and "in-vivo" models. Also, the current study lays a foundation for converting these phytochemicals from "bench to clinic". Thereby, this scientific study has identified a new herbal-derived drug and its targets for multi organ metabolic endocrinopathy; thus, adding to its overall potential and economic viability at national and international level.