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RESEARCH ARTICLE

5α-Reductase Inhibitory Potential of *Hibiscus rosa sinensis*: Effective for the Management of Alopecia

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Abstract

The condition known as male pattern hair loss and benign prostatic hyperplasia are both treated with a family of drugs known as 5 alpha reductase (5α-reductase) inhibitors. This study shows that the flower of *Hibiscus rosa sinensis* has 5α-reductase inhibitory action that is helpful in the treatment of androgenic diseases. Phytochemical screening was done using petroleum ether, methanol, and water extracts to identify the phytochemical group present. Then, using quercetin as a reference flavonoid component, the Total Flavonoid Content (TFC) was assessed using a colorimetric technique. The 5α-reductase inhibitory activity was evaluated using several extracts and compared to the well-known 5α-reductase inhibitor finasteride. Finally, the inhibition of different extracts to the enzyme was assessed using a biochemical approach to measure the activity of 5α-reductase. It was determined that the IC_{50} value of *Hibiscus rosa sinensis* methanolic extract, 146.048 ± 0.453 (µg/mL) and quercetin (a chemical biomarker of the plant material) 141.426 ± 1.578 (µg/mL), were promising candidates for future investigation into their antiandrogenic activities.

Keywords: Alopecia, Hair Loss, *Hibiscus rosa sinensis*, NADPH, Quercetin, 5α-Reductase

1. Introduction

Androgens and the androgen receptor interact to have cellular effects that control essential mechanisms involved in the growth, structure, function of prostate, and male pattern baldness. Additionally, androgen may contribute to the emergence of prostate cancer. Suppression of Dihydrotestosterone (DHT) may prevent the formation of cancer, according to various pre-clinical and clinical investigations[1]. Testosterone (T) is converted into Dihydrotestosterone (DHT) by the nuclear membrane bound enzyme steroid 5α-reductase (5αR), which has two subtypes called type 1 (5αR1) and type 2 (5αR2)[2]. 5αR catalyses the NADPH dependent conversion of T to DHT[3]. The 5αR and its metabolite DHT have an effect on a number of human disorders, including male pattern baldness, alopecia, Benign Prostatic Hyperplasia (BPH), prostate cancer, acne, and hirsutism[4,5]. Two popular 5αR inhibitors on the market are finasteride and epristeride, although due to various adverse effects, their usage is restricted. This problem might be solved by using herbal alternatives for 5αR inhibition. To find potential 5αR inhibitors, we evaluated plants for antiandrogenic activity and their potential to block the 5αR.

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Hibiscus rosa sinensis (Malvaceae) is a glabrous shrub that is extensively grown as an ornamental plant in the tropics and comes in a variety of variants with different colored flowers. However, the red-flowered variant is favoured in medicine. In gardens all across India, it is raised as an ornamental plant[6]. Dried flower decoction was used for abortion. Hot water extract of flower was employed as a contraceptive in ayurvedic medicine. Oral intake of flower hot water extract is recommended for menorrhagia, bronchitis, and as an emmenagogue. Hot water extract of aerial part was utilized as an aphrodisiac orally. Together with *Veronia cineria* juice, it was used externally as an emollient. A hot water extract of the root was used internally as a demulcent and cough treatment[7].

The leaves and flowers of *H. rosa sinesis* are well-acknowledged to have hair growth promoting and anti-ageing effects. Furthermore, in India, herbal remedies for hair growth contain extracts from various portions of *H. rosa sinesis*. According to Adhirajan *et al*., the leaf extract of *H. rosa-sinesis* has a potential influence on hair growth development in both *in-vivo* and *in-vitro* approaches[8]. To the best of our knowledge, no scientific research has been conducted on the potentiality of various extracts on hair growth development of *H. rosa sinensis* flower for anti-androgenic activity as 5αR inhibition model and putative mechanism of action. Also, by comparing the results, try to correlate the activity of a probable plant biomarker responsible for hair growth-promoting activity. Time dependent enzyme inhibition will be performed to determine the effect of the extract's inhibitory action on the enzyme after a time interval, which will aid in better understanding the process. In this study, we set out to investigate and establish the rationale for using flowers to treat alopecia.

2. Materials and Methods

2.1 Collection and Identification Plant Material

The flower of the *H. rosa sinensis* plant was collected from Kolkata (W.B.) during February. The flower part was identified and authenticated. The finely pulverized, air-dried plant material was sieved through 10 mesh.

2.2 Preparation of Extracts

Each of the three 1000 mL conical flasks holding 100 g of crushed *H. rosa sinensis* (flower) contained 500 mL of water, 500 mL methanol and 500 mL of petroleum ether separately. In an airtight container, it was kept at 25 to 30 °C for three days. Then it was filtered using ordinary filter paper. Next, the filtrate was stored in a 1000 mL beaker. After filtering, the filtrates were concentrated using a rotary evaporator at a temperature ranging from 40° to 45° C.

2.3 Drugs and Chemicals

NADPH tetrasodium salt was purchased from Sisco Research Laboratory (SRL). Finasteride, tris-HCl buffer, quercetin and Testosterone (T) were purchased from Sigma-Aldrich. Merck, Mumbai provided sodium nitrite, aluminum chloride, sucrose, sodium phosphate, methanol, petroleum ether, and Ethylenediamine Tetraacetic Acid (EDTA). All other chemicals ware of analytical-grade.

2.4 Qualitative Evaluation of *Hibiscus rosa sinensis* Extracts

Extracts were submitted to several qualitative analyses to detect plant components such as saponins, phytosterols, alkaloids, glycosides, carbohydrates, flavonoids, proteins, tannins and phenolic compounds[9].

2.5 Total Flavonoid Content (TFC)[10]

A colorimetric approach utilizing aluminum chloride was used to assess the total flavonoid content. First, 2.5 mL of distilled water, 3 mL of 5% sodium nitrite solution, and 0.5 mL of the extract were added, combined, and left to stand for 3 min; then it was added 0.3 mL of 10% aluminum chloride solution. After adding 2 mL of 1M NaOH solution, the final volume in each tube was adjusted to 10 mL after 6 min. After 60 min, the absorbance was measured at 415 nm against a blank. The calibration curve was generated using a range of concentrations (100-500 µg), with quercetin serving as the standard. Total flavonoid concentration was calculated as mg of quercetin equivalents per gm of the air-dried sample. Triplicate measurements (n = 3) were made.

2.6 Enzyme Inhibition Assay Preparation for 5α-reductase

The method described by Nahata and Dixit, was followed with some modifications[11]. The procedure's details are shown below;

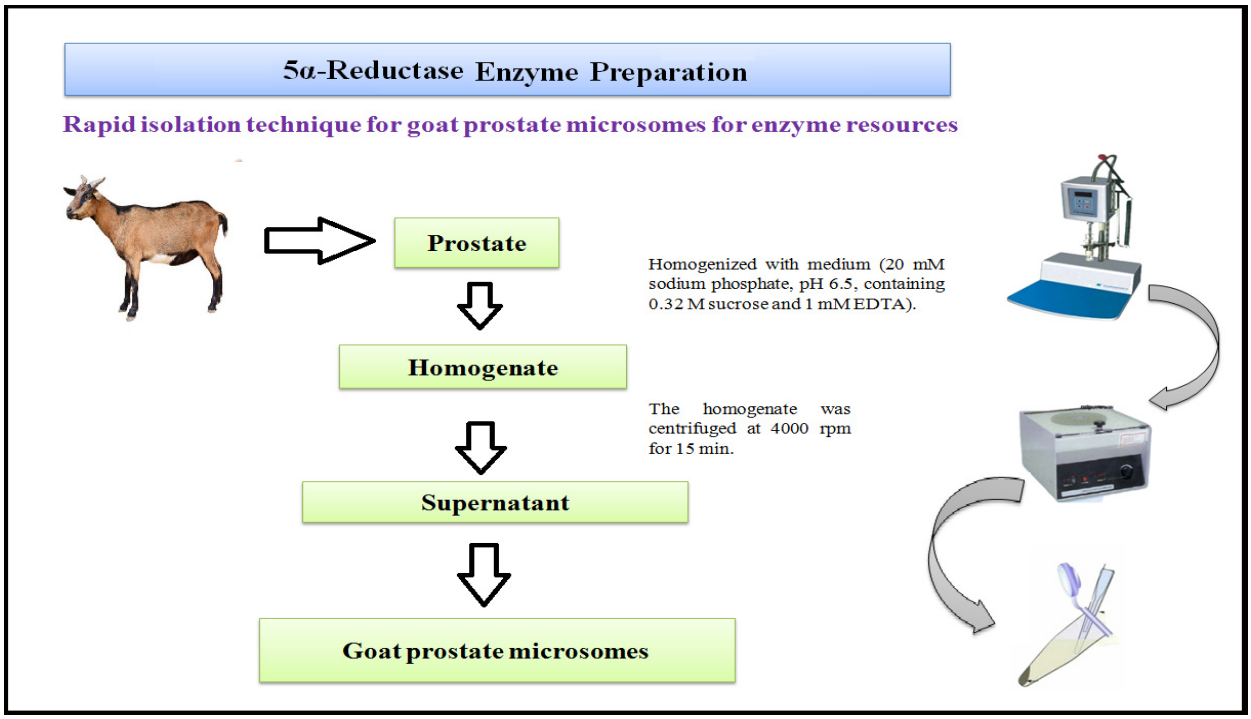

Figure 1. Methods of preparation 5αR solution.

2.6.1 Methods of Preparation 5α-Reductase Solution

Adult male goat prostate was collected from the local slaughter house. The equivalent of 400 mg was taken from it, which was minced into small pieces and then mixed with a 30 mL medium (20 mM sodium phosphate, pH 6.5, containing 0.32 M sucrose and 1 mM EDTA). Then it was centrifuged, the homogenate was for 15 min at 4000 rpm (716 g), and the supernatant was utilized as an enzyme source. The protein content in the supernatant was calculated using the Bradford technique of protein quantification. Bradford technique of protein quantification was used to estimate the amount of enzyme in the supernatant. This method produced a stock Bovine Serum Albumin (BSA) solution of 1 mg/mL in deionized water. From stock, two-fold serial dilutions with a concentration of 0.5, 0.25, and 0.125 mg/mL were made. 5 µL of the produced BSA solution was put to a 96-well microplate at various concentrations. Finally, 200 µL of Bradford reagents were added to the BSA solution. At 592 nm, absorbance was observed. A standard curve was created by plotting the standard concentrations against the absorbance at 592 nm. 5 µL of enzyme homogenate solution was incubated for 5 min with 200 µL of Bradford reagent. The protein concentration was then calculated by using the BSA standard curve. The isolated prostate protein content was 0.68 mg/mL. The solution was further diluted to 100 µg/mL for the enzyme assay using tissue homogenization media (Figure 1).

2.6.2 Preparation of Standard Curve of NADPH

Standard curve of NADPH was prepared in methanol at 340 nm and was found to be linear over the range 1 to 20 µg/mL (r^2 = 0.991, with Y = 0.098x-0.015).

2.6.3 Preparation of Test Solutions

Testosterone (75 µM) and NADPH (22 µM) methanol solutions and Tris-HCl buffer (0.5 M) distilled water solution were prepared.

2.6.4 Preparation of Finasteride Solution

Finasteride stock solution was prepared by dissolving 0.0037 gm of powder in 10 mL methanol (1000 µM). Sonication was carried out for 1 hour and vortexed for 15 min. Then filtration was carried out by 0.45 µ syringe filter, and the filtrate was collected in two separate eppendorf tube. 2 µL of stock finasteride solution was diluted with 1998 µL methanol to obtain 1 µM working solutions. Further dilution was done using 0.1, 0.2, 0.4, 0.6, and 0.8 µM to determine the IC50 value.

2.6.5 Preparation of Plant Extracts and Quercetin

Extracts solutions were prepared by using water, methanolic and petroleum ether as their parent solvent (1 mg/mL). It was preserved for later research after filtering via a 0.45 µ syringe filter. Additionally, dilutions of 25, 50, 75, 100, 150, 200, and 300 µg/mL were carried out. Quercetin (plant biomarker) was prepared in methanol (1 mg/mL). Further dilution was done at 25, 50, 75, 100, 150, and 200 µg/mL.

2.7 Assay Procedure of 5α-Reductase Inhibition of Test samples

Based on the procedure outlined by Nahata and Dixit, 5αR inhibition experiments were carried out[11]. In summary, test samples, enzyme homogenate solution, T, and NADPH were mixed together. Table 1 describes the specific reaction mixtures. All reaction mixtures underwent a 30-mint incubation period at 37 °C. At 340 nm, absorbance was determined spectrophotometrically. The test samples corresponding NADPH concentrations were determined from the NADPH standard curve. NADPH concentrations that remain in the reaction medium were calculated. The NADPH concentration was calculated as a percentage of NADPH scavenging. To calculate the net absorbance of NADPH, blank absorbance was subtracted from the test samples. The 5αR inhibition was calculated for each test substance, showing the test substance's original efficacy against the enzyme. The percentage of NADPH scavenging potential was used to calculate the percent inhibition of 5αR.

All of the extracts were subjected to a blank test to determine their intrinsic *in vitro* antioxidant activity or their capacity to convert NADPH to NADP, which would prevent them from initially being able to inhibit the 5αR present in the reaction medium. Therefore, it was considered that 2 mL of 75 µM of T was transformed into DHT by 3 mL of 22 µM NADPH. The 5αR inhibition was then calculated for each extract to assess the actual activity against the enzyme.

Absorbance at 340 nm, Net absorbance of test = (Test Absorbance – Blank Absorbance)

Calculate the NADPH concentration in each sample from NADPH standard curve prepared previously.

Percentage inhibition = 100 – [(54.78-concentration of NADPH obtained from net absorbance of the test solution)/54.78) × 100]

To measure the percentage inhibition of various test sample concentrations required to establish the IC_{50} value of the test extracts.

2.7.1 Time-Dependent Inactivation of Enzyme

Further, this model uses a graphical representation of enzyme inhibition over the time of each sample to show the properties of time-dependent inactivation. The IC_{50} concentration for each sample was prepared from data on enzyme inhibition, and enzyme inhibition analysis was carried out. The total observation period was 90 min, with 15 mint intervals between samples. The first sample was collected at 30 min.

3. Results

The current findings showed that the water extract contains flavonoids, alkaloids, protein, carbohydrates, tannins, glycosides, and saponins. Flavonoids, alkaloids, carbohydrates, tannins, glycosides, and saponins are all found in methanolic extract. Besides, phytosterols and tannins tests in petroleum ether extract are positive. The findings of phytochemical screening are shown in the

Table 1. Enzyme, substrate and coenzyme mixture

Sample ID	Methanol (mL)	Tris HCL (mL)	NADPH (mL)	Enzyme (mL)	Finasteride (mL)	Test sample (mL)		Testosterone (mL)		Total volume (mL)
Blank Control	4	4	3	1			**Vortex and incubate at 37°C for 10 min**		**Vortex and incubate at 37°C for 30 min**	12
Negative control	2	4	3	1				2		12
Finasteride		4	3	1	2			2		12
Test samples		4	3	1		2		2		12

Table 2. Phytochemical screening of the extracts of *Hibiscus rosa sinensis*

Screening test		Water extract	Methanolic extract	Petroleum ether extract
Phytosterols	Libermann-Burchard Test	-	-	+
	Liebermann's reaction	-	-	+
Glycosides	Keller-Killiani Test	+	+	-
	Bontragger's Test	+	+	-
Flavonoids	Shinoda test	+	+	-
	Ferric chloride test	+	+	-
Alkaloids	Mayer's test	+	+	+
	Dragendroff's test	+	+	+
Protein	Millon's reaction	+	-	-
	Xanthoproteic reaction	+	-	-
Carbohydrates	Molisch's test	+	+	-
	Fehling's test	+	+	-
	Barfoed reagent test	+	+	-
Tannins and Phenolic Compounds	Ferric chloride test	+	+	+
	Lead acetate solution	+	+	+
Saponins	Foam test with water	+	+	-
	Foam test with sodium carbonate	+	+	-

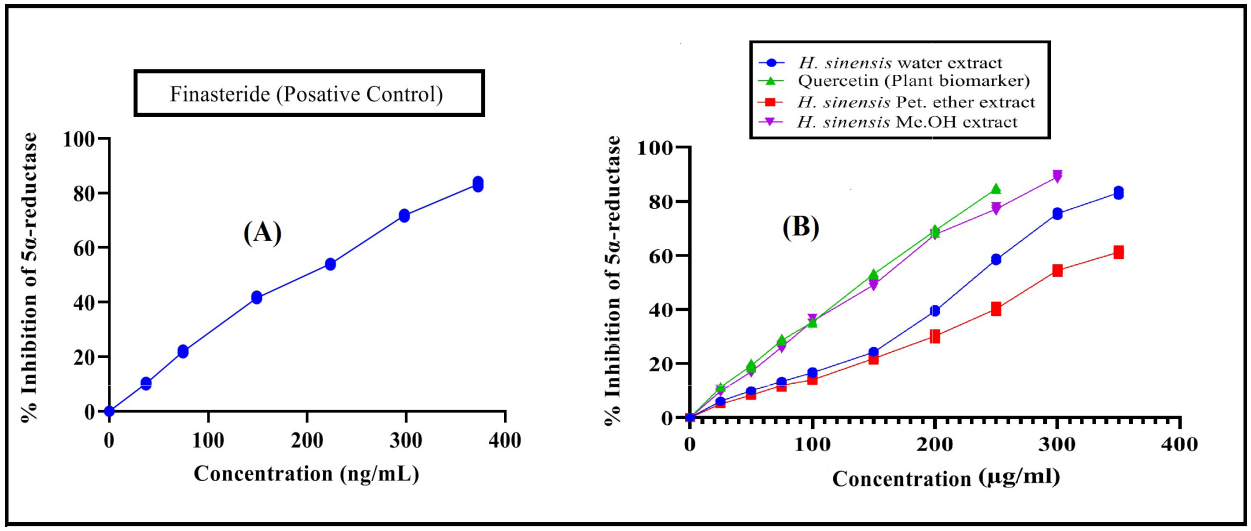

Figure 2. (**A**). 5αR inhibition of finasteride and (**B**). 5αR inhibition of quercetin, *H. rosa sinensis* water, methanolic and petroleum ether extract.

Table 2. TFC results showed that the methanolic of *H. rosa sinensis* contains a high amount of quercetin than water extract. The study found that *H. rosa sinensis* methanolic extract was superior to water extract as a 5αR (type 2) inhibitor. The most effective 5αR inhibitor activity was discovered in quercetin (a chemical marker of *H. rosa sinensis* analysis). The methanolic extract of *H. rosa sinensis* contains a significant amount of quercetin, which has been proven to be a potent 5αR inhibitor (Figure 3).

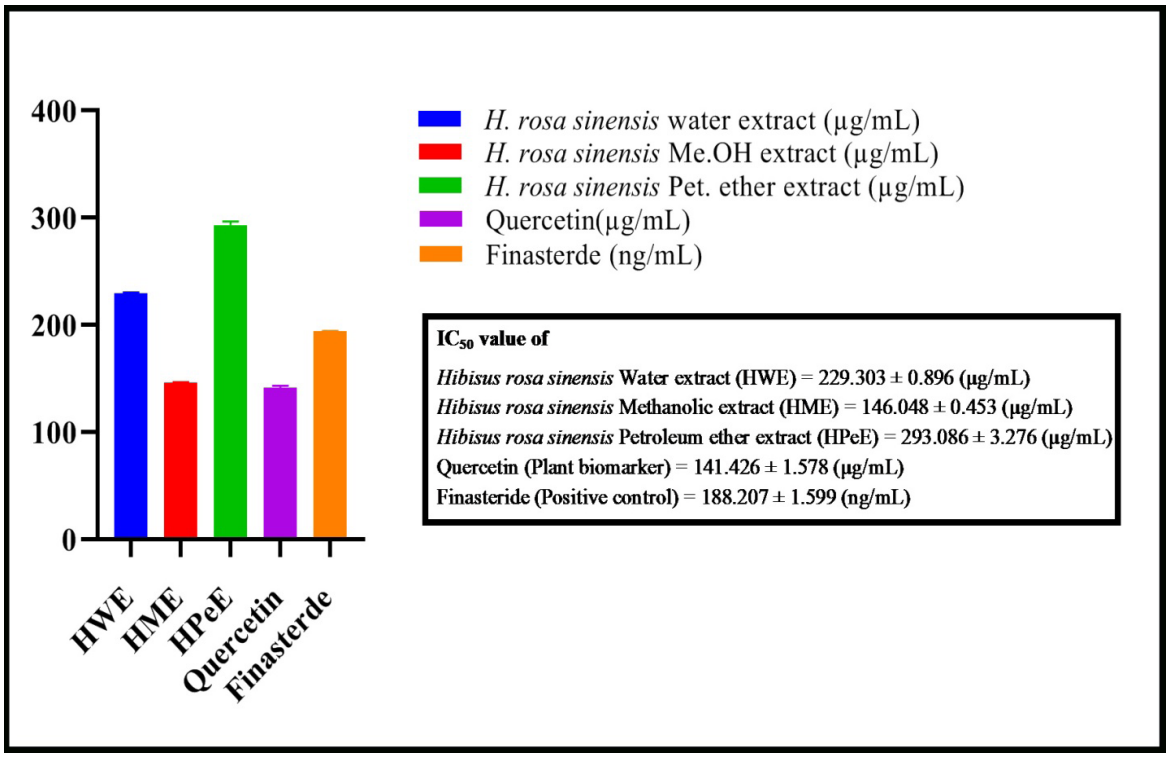

Figure 3. Comparison study of 5α-reductase inhibitory potential.

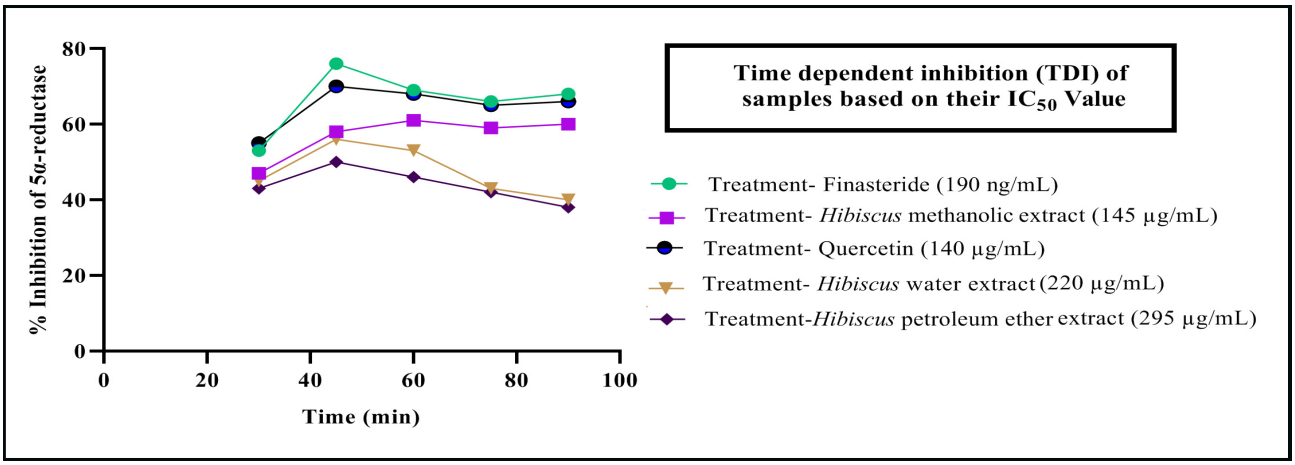

Figure 4. Time dependent inactivation of test samples.

4. Discussion

In water, methanol, and petroleum ether, the extraction yield was 4.3 %, 2.65 %, and 0.81 % w/w, respectively. According to the phytochemical screening results, the water extract contains flavonoids, alkaloids, protein, carbohydrates, tannins, glycosides, and saponins. Flavonoids, alkaloids, carbohydrates, tannins, glycosides, and saponins are all found in methanolic extract. Besides, phytosterols and tannins tests in petroleum ether extract are positive. The findings of phytochemical screening are shown in the Table 2.

According to a literature survey, *H. rosa sinensis* flowers are abundant in flavonoid compounds. Flavonoids have well established health advantages. The sample's total flavonoid concentration was thus calculated and represented as Quercetin (QE). The entire flavonoid content of water, and methanolic extracts of *H. rosa sinensis* were found

to contain 10.62 ± 0.31 and 22.82 ± 0.51 mg equivalent QE/gm of the air-dried sample. The petroleum ether extract of *H. rosa sinensis* contained no flavonoid, as determined by the results of the phytochemical screening. A standard quercetin calibration curve with the equation Y = 0.001x + 0.007 (correlation coefficient = 0.998) was used to establish this.

Statistic analysis is utilized to calculate IC_{50} values. Plotting the curve with the percentage of inhibition vs the concentrations of the several investigations was used to get the IC50 values, which were then represented as mean ± standard deviation (n = 3). The statistical analysis was performed utilizing GraphPad software version 6.0, and one-way ANOVA and the Bonferroni post - hoc test was performed. Compared to the reference standard, the P was deemed to have a significant difference of less than 0.05. IC_{50} value of *H. rosa sinensis* water, methanolic and petroleum ether extract (flower) was found to be 229.303 ± 0.896, 146.048 ± 0.453 and 293.086 ± 3.276 (µg/mL). In contrast, 5αR inhibition of quercetin and finasteride exhibited values of 141.426 ± 1.578 (µg/mL) and 188.207 ± 1.599 (ng/mL), respectively. 5αR inhibition of *H. rosa sinensis* extract, quercetin and finasteride is shown in Figure 2. Figure 3 compares the sample's comparative 5αR inhibitory potential.

Finasteride (190 ng/mL), quercetin (140 µg/mL), *H. rosa sinensis* extracts methanolic (145 µg/mL), water (220 µg/mL), and petroleum ether (295 µg/mL) were made by their standard stock solutions using the IC_{50} concentration obtained by the 5αR inhibitory test. To facilitate dilution, the sample's closest IC_{50} concentration value was taken. To further illustrate the characteristics of time-dependent inactivation, this model employs a graphical depiction of enzyme inhibition across time, shown in Figure 4. The total observation period was 90 min, with 15 mint intervals between samples. The first sample was collected at 30 min. The graph in Figure 4, shows the 5αR inhibitory activity of samples with time. Sample activity was found to be decreasing in the following order:

> Finasteride > quercetin > *Hibiscus* methanolic extract > *Hibiscus* water extract > *Hibiscus* petroleum ether extract.

We demonstrate here that the finasteride (positive control) has the highest level of time-dependent enzyme inactivation out of all the results. Following that, decreasing activity was observed in this manner of quercetin (a chemical biomarker of the plant), *H. rosa sinensis* methanolic, water, and petroleum ether extract, respectively. Also, their potential IC_{50} values resemble the same sequence as decreasing the time-dependent 5αR activity of the samples. In quercetin, we found the second most effective IC_{50}. In addition, the total flavonoid test revealed that methanolic extract contained more quercetin than water extract and found the strongest 5αR inhibitory action, based on the IC_{50} value we got and time-dependent inhibition graph data. It can cause greater quercetin concentration, increasing the 5αR inhibitory action of *Hibiscus* flower methanolic extract among all extracts. As the concentration of quercetin in plant extracts increases, more potential 5αR inhibitory activity was found.

In a prior study, it was shown that administering quercetin to the C3H/HeJ mice model might improve alopecia areata[12]. According to Zhoa *et al.*, topical quercetin administration promotes mice's resting hair follicles to develop with faster follicular keratinocyte proliferation and replenishes perifollicular microvasculature[13]. Hence, it may be assumed that quercetin, a key component of *H. rosa sinensis*, aids in stimulating hair development. Furthermore, the results of the 5αR inhibitory effect *in vitro* also pointed to the claim indicated above.

Therefore, standardizing the quercetin content in *H. rosa sinensis* plant samples significantly may improve the opportunity for plant extraction processes, raw and finished product specifications, and promising therapeutic activity against 5αR inhibition to treat many androgenic diseases.

4.1 Limitations of the Protocol

In our experiment, all reaction mixtures included the same reagents used in the blank and the examined extracts, allowing us to conclude. NADPH (22 µM) was added to the blank solution (3 mL) to observe the absorbance that was seen at time zero in the blank control group. Therefore, at this point, a new NADPH-only group without any test samples was preferred for an adequate comparison. It would have demonstrated NADPH's innate absorbance. So, for a relevant comparison, it is suggested that the experiment in the future add a new group the NADPH control group.

Prospects for this study include a more time-dependent investigation using various fractions that would have provided more information about the plants.

In finding out how precisely the substances affect the enzyme, how well they interact with the androgen

receptor that NADPH binds to, and how they block NADPH, whether by competitive inhibition or site-specific inhibition, more research on the potent substances of the study is necessary. It is also unknown if allosteric modulation has a role in the overall interaction of the enzyme activity.

Whatever the reason, the screening was very effective in locating the powerful 5αR inhibitory agents among the drugs included in the study.

5. Conclusion

Male pattern hair loss and benign prostatic hyperplasia are two conditions that can be treated with 5αR inhibitors. This study shows that the flower of *H. rosa sinensis* has 5αR action that is helpful in treating androgenic diseases. Results of the phytochemical screening showed that the presence of flavonoids in the methanol and water extracts was positive. According to TFC findings, *H. rosa sinensis* methanol extract has a higher concentration of quercetin than water extract. Quercetin was shown to have the strongest 5αR inhibitor action. Since it contains the greatest quercetin, the flower's methanol extract had the most 5αR inhibitory activity. *H. rosa sinensis* can be used for the purpose mentioned above since 5αR inhibition aids in the treatment of androgenic alopecia. The current investigation supports the ethnomedical usage of plants for treating hair loss. Commercial application of *H. rosa sinensis* flower for the treatment of alopecia may benefit from further use of the methanolic extract and its use in a formulation.

6. References

1. Lieberman R. Evolving strategies for prostate cancer chemoprevention trials. World J Urol. 2003; 21(1):3-8. https://doi.org/10.1007/s00345-003-0317-4
2. Wright AS, Douglas RC, Thomas L.N, Lazier CB, Rittmaster RS. Androgen-induced regrowth in the castrated rat ventral prostate: role of 5α-reductase. Endocrinol. 1999; 140(10):4509-4515. https://doi.org/10.1210/endo.140.10.7039
3. Levy MA, Brandt M, Sheedy KM, Holt DA, Heaslip JI, Trill, JJ, Ryan PJ, Morris R, Garrison LM, Bergsma DJ. Cloning, expression and functional characterization of type 1 and type 2 steroid 5α-reductase from Cynomolgus monkey: Comparisons with human and rat isoenzymes. Steroid Biochem. Mol. Biol. 1995; 52:307-319. https://doi.org/10.1016/0960-0760(94)00183-M
4. Guarna A, Poletti A, Catrambone F, Danza G, Marrucci A, Serio M, Celotti F, Martini L. Synthesis of a chemiluminescent probe useful for the purification of steroid 5α-reductase. Bio org Med Chem Lett. 1996; 6(16):1997-2002. https://doi.org/10.1016/0960-894X(96)00360-5
5. Russell DW, Wilson JD. Steroid 5α-reductase: two genes/two enzymes. Annu. Rev Biochem. 1994; 63(1): 25-61. https://doi.org/10.1146/annurev.bi.63.070194.000325
6. Jadhav VM, Thorat RM, Kadam VJ, Sathe NS. *Hibiscus rosa sinensis* Linn-"Rudrapuspa": A review. J Pharm Res. 2009; 2(7):1168-73.
7. Ross IA. *Hibiscus rosa-sinensis*. In medicinal plants of the world. Humana Press, Totowa, NJ. 2003; 253-266. https://doi.org/10.1007/978-1-59259-365-1_12
8. Rathi V, Rathi J, Tamizharasi S, Pathak A. Plants used for hair growth promotion: A review. Phcog Rev. 2008; 2(3):185.
9. Singh R, Kori ML. Phytochemical studies of *Tephrosia purpurea Linn*. and *Martynia annua Linn*. extracts for identification of chemical constituents. Int J Pharm Sci Drug Res. 2022; 14(2):226-232. https://doi.org/10.25004/IJPSDR.2022.140211
10. Sheth F, De S. Evaluation of comparative antioxidant potential of four cultivars of *Hibiscus rosa-sinensis* L. by HPLC-DPPH method. Free radic antioxid. 2012; 2(4):73-78. https://doi.org/10.5530/ax.2012.4.13
11. Nahata A, Dixit VK. Evaluation of 5α-reductase inhibitory activity of certain herbs useful as antiandrogens. Andrologia. 2014; 46(6):592-601. https://doi.org/10.1111/and.12115
12. Wikramanayake TC, Villasante AC, Mauro LM, Perez CI, Schachner LA, Jimenez JJ. Prevention and treatment of alopecia areata with quercetin in the C3H/HeJ mouse model. Cell Stress Chaperones. 2012; 17:267-274. https://doi.org/10.1007/s12192-011-0305-3
13. Zhao Q, Zheng Y, Zhao D, Zhao L, Geng L, Ma S, Cai Y, Liu C, Yan Y, Belmonte JC, Wang S. Single-cell profiling reveals a potent role of quercetin in promoting hair regeneration. Protein and Cell. 2022; 1-18. https://doi.org/10.1093/procel/pwac062