Original Article

Role Of Tinospora Cordifolia And Alkaloid Rich Fraction Against Hyperandrogenisam: A In-Vitro Study On Cell Proliferation, Colony Formation And CYP17A1 Gene Expression By Qrt-PCR

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DOI: 10.47750/pnr.2022.13.S05.384

Abstract

Infertility is a global challenge in reproductive health, about 4% to 20% of reproductive-age women are affected with polycystic ovarian syndrome (PCOS).Itis one of the most prevalent endocrine disorders, the underlying principles behind the PCOS development are unknown, and there are no significant therapeutic alternatives or targeted therapeutic interventions towards the disorder. The purpose of this study was to investigate the role of *Tinospora cordifolia* stem extract and alkaloid rich fraction against PCOS.

Method: quantitative real time-polymerase chain reaction (qRT-PCR) was used to assess fold expression. Cell viability was determined by [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] MTT assay. Clonogenic (colony formation), Scratch assay were performed and finally, the obtained values were evaluated using ANOVA**.**

Results: Compared with the other ovarian cancer cells, the MTT assay demonstrated that the alkaloid rich fraction had substantial selectivity against SKOV-3 cells. SKOV-3 cells showed low or no toxicity with the extract as well as alkaloid rich fraction. The efficacy of the extract against MCF-7 cells was further validated by migration and clonogenic experiments.

Conclusion: CYP17A1 mRNA expressionwas more effectively suppressed in SKOV-3 cells, with a highly significant p-value <0.001. The fold expression of CYP17A1 in SKOV-3 cells after treatment with alkaloid rich fraction was 0.182±0.009. Alkaloid rich fraction inhibits the proliferation of the ovarian cancer cell line SKOV3 than *Tinospora cordifolia* stem extract, according to the research. Alkaloid rich fraction could be a possible therapeutic androgen antagonist, according to these data, further scientific *in-vivo* evaluation against metabolic PCOS can be put forth.

Keywords: Estrogen, PCOS, MCF-7 cell lines&SKOV-3 cell lines, Tinospora cordifolia

1. INTRODUCTION

The androgen receptor belongs to the steroid hormone receptor family which is located in the X chromosome and is specifically expressed on reproductive tissues. The primary role of the androgen receptor in regulating androgen is to bind to DNA sequences responsible for androgen-responsive genes. Androgen receptor activation has a significant impact on health and disease. Specific hyperandrogenism is a key factor in the progress of PCOS, breast, and ovarian cancer progression. In each of these conditions hyperandrogenism is a hallmark of disease development. Changes in androgen receptor transcriptional activity are influenced by a variety of factors. In PCOS hyperandrogenism is characterized by acne, hirsutism, and alopecia. Androgens are excessively produced by the ovaries and are involved in the possibility of abnormalities in the steroid–associated pathway. Studies have implicated that alteration of gene expression in androgen synthesis may result in disease progression such as PCOS, and breast and ovarian carcinoma. Epidemiological studies were reported and evidences from other experimental studies stated that a significant rise of androgen and estrogen levels in the PCOS hormone profile indicates an increased risk of breast cancer in estrogen receptor-positive. CYP17A1 consists of 8 exons and 7 introns and is involved in androgen synthesis pathway. As an important gene that contributes to the synthesis pathways of adrenal gland and ovary, it encodes a key qualitative regulatory enzyme called 17-α-hydroxylase/17–20 lyase (P450 17α). Elevated levels of CYP17A1 enhance the synthesis and production of excess androgens. This partially indicating the fact that CYP17A1 is responsible for hyperandrogenism.

To comprehend biological processes, cell lines are often used instead of primary cells. Moreover, since cell lines may not always accurately reproduce primary cells, attention should be paid when interpreting data [1]. MCF-7 ('Michigan Cancer Foundation-7') is an estrogen receptor positive breast cancer cell line [2] and SK-OV 3 is a human ovarian cancer cell line with epithelial-like morphology [3]. *Ovarian cancer* was reported to be *associated with PCOS in* a population-based

cohort study [4]. *Tinospora cordifolia* (T.C) Miers. (Menispermaceae) are one of the few rasayan medicinal plants that have been reported to have immunomodulatory, neuropsychological, and (anti-stress) adaptogenic properties. T.C. is a good example of an herb that has been used for its rasayan medicinal & therapeutic properties since ancient times. It is also used in the clinical efficacy of an Ayurvedic treatment regimen for PCOS infertility [5]. T. C is indeed an excellent adaptogen, but it also has therapeutic potential in the treatment of endocrine diseases such as cognitive impairment, diabetes (IR) [6], anxiety, depression, PCOS, and anti-inflammatory, anti-oxidant, and antibacterial conditions. The enzyme CYP17A1 is involved in steroid hormone synthesis.

In PCOS women, abnormal steroid biosynthesis comprising cytochrome P450 and 17-hydroxylase is considered the main cause (CYP17A1) [7], and elevated CYP17A1 activity enhances androgen [8] production and secretion [9]. The main goal of this study is to see if T.C stem extract and alkaloid-rich fraction can help treat PCOS in SKOV-3 and MCF-7 cancer cells by using a variety of tests, including morphological studies, cell viability, wound healing, and colony formation assays.

2. MATERIALS AND METHODS:

2.1 Plant Materials

Dried stems of *Tinospora cordifolia* were procured from *Ramasamy* Chetty *Naatu Marunthu Kadai*, Rasappa Chetty Street, Park Town, Chennai-600003 India. The plant material was taxonomically identified by Dr. K. N. Sunil Kumar, Research Officer & HOD, Department of Pharmacognosy, *Central Council for Research in Siddha, Ministry of Ayush, Government of India.* T19122001S is the voucher specimen number.

2.2 Extraction of *Tinospora cordifolia* (Stem Extract)

Dried stems of *Tinospora cordifolia* were procured, authentified, and pulverized/milled at an Arul mill near Arumbakam. The coarsely dried stem powder was first defatted with petroleum ether (60-80 °C) for two days before being extracted for 24 hours in a Soxhlet apparatus with a solvent mixture of methanol and water in a 70:30 ratio (2000 ml X 6 cycles) at 40 °C. A rotary evaporator was used to dry the residue under reduced pressure.

2.3 Alkaloid Rich Fraction

Dried coarsely powdered (940g) stem of *Tinospora cordifolia* was defatted with petroleum ether (60–80 ◦C) for 2 days and the dried material was extracted with 70:30 hydro-alcoholic (CH_3OH: H_2O) using a Soxhlet apparatus at 65-70^{0}C for week days. The solvent was retrieved, and the semisolid hydro-alcoholic extract(29.7 g) was acidified using 100ml solution of dilute hydrochloric acid (HCl).Using Whatman filter paper the filtrate was filtered, and rinsed with dilute HCl, until the alkaloid test was passed. With 25 % ammonia, the filtrate and washings were made alkaline (p^H 8.0) and partitioned with (CHCL3) chloroform (3 X 150 ml, 2 X 100 ml). The alkaloid-rich fraction was labelled after the chloroform layer was concentrated.

2.4 Preparation of Sample

The *Tinospora cordifolia* stem extract (T.C.S.E) and alkaloid rich fraction (A.R.F) weighed 100mg and it was dissolved in 1ml of DMSO and stored at -20ºC until use. In a 24-well plate, cells were grown to 70% confluence. DMSOwas kept at a final concentration of less than 0.25 % in the treatment. The data was collected by repeating each experiment at least three times.

2.5 Cell lines and cell culture preparation

Cells of MCF-7 cells and SKOV-3 (metastatic ovarian adenocarcinoma) [10] cells were obtained from NCCS Pune. The cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium) added with 5% FBS (Fetal Bovine Serum) and 100U/ml of penicillin/streptomycin antibiotic solution. Cells were maintained in an incubator at 37C with 5% CO2.

2.6 Cell Viability Assay

Cells were grown in their appropriate media in 96 well plates to attain 70% confluence, and the cells were treated with various concentrations of extract for about 24 hours. After that, the media was removed, and PBS was used to wash the cells. A prepared MTT solution at 0.5mg/ml was added to every well and incubated at 37 C for about 4 hours in the dark. Later, the MTT solution was replaced with a DMSO solution of about 200 µl and kept in the dark for 20 minutes. The plate was allowed to shake at 150rpm for 5 minutes. The optical density was measured at 570 nm using a plate reader.

2.7 Morphology Study

Cells were plated in 24-well plates and treated with an extract T.C.S.E and A.R.F of determined IC 50 concentration for 24 hours. After the treatment period, the cells were observed to visualise the changes using an inverted microscope.

2.8 Colony formation assay

The cell was grown well and reached up to 80% confluence when it was treated with IC50 concentrations of extract for 24 hours. Trypsinization was done and the seed was planted in six well plates to grow for about 14 days. The cells were washed with PBS solutions. 0.5% of crystal violet solution was added and incubated for 30 minutes. Later, the solution was washed and the plates were allowed to dry at room temperature to count the presence of colonies.

2.9 Scratch assay

Cells were grown to 80% confluence in 6 well plates. A uniform scratch was made in each well with a 10 µl pipette tip. The specific concentration of the extract was treated in cells for 24 to 48 hours. The cells were then observed under an inverted microscope.

2.10 Gene Expression

The total RNA was isolated from the control and treated cells using Trizol RNA isolation reagent. The concentration and purity of RNA was determined by measuring the absorbance at 260 and 280 nm. 200ng of RNA was used to synthesize cDNA using High-capacity cDNAreverse transcription kit by following the manufacturer's protocol. The quantitative RT-PCR (qRT-PCR) was performed for the below mentioned genes using SYBR green master mix and gene specific primers mentioned in table below. The Ct values were obtained, normalized with GAPDH and the relative expression (fold change) was calculated. The graphs were plotted and statistical analysis was performed.

Table 1 Sequence of primers used for the gene expression

Gene list		Primer sequence 5'--- 3'
GAPDH	Forward	AATGGGCAGCCGTTAGGAAA
	Reverse	GCGCCCAATACGACCAAATC
CYP17A1	Forward	TGGCTCTCTTGCTGCTTACC
	Reverse	ACGAACCGAATAGATGGGGC

3. RESULTS

Alkaloids are one of the most extensively investigated and diverse classes of secondary metabolites among phytochemicals. Many alkaloids derived from natural sources have been shown to be effective in treating a variety of diseases. Some of these examples are the isoquinoline alkaloid berberine, jatrorrhizine, magnoflorine and palmatine (*Tinospora cordifolia*), carbazole alkaloid from *Murraya koenigii*, and indole alkaloids from *Catharanthus roseus*. In Indian traditional folk medicine, the stem of the *Tinospora cordifolia* (TC) plant is often used to treat diabetes. The antidiabetic properties of the isoquinoline alkaloid rich fraction was related to a number of pathways, including insulin release, insulin sensitization, and gluconeogenesis inhibitory actions. In the current study alkaloid rich fraction exhibits a targeted effect against MCF-7 and SKOV3 [11] cell lines. The effect of T.C.S.E & A.R.F was evaluated by MTT and morphological study. MTT assay revealed the effect of T.C.S.E & A.R.F towards androgen receptor upregulated cell line MCF7 and SKOV-3. The T.C.S.E showed significant cytotoxicity [12] towards MCF7. The IC-50 for MCF-7 cells was calculated to be 25µM, 50µM, and 100Mm. The morphological study, colony formation study, and scratch assays were carried out at the 25µM concentration of the T.C.S.E & A.R.F. Whereas IC-50 for SKOV3 cells was calculated to be 75 µM concentration. The morphological study, colony formation study, and scratch assays were carried out at the 75 µM concentration.

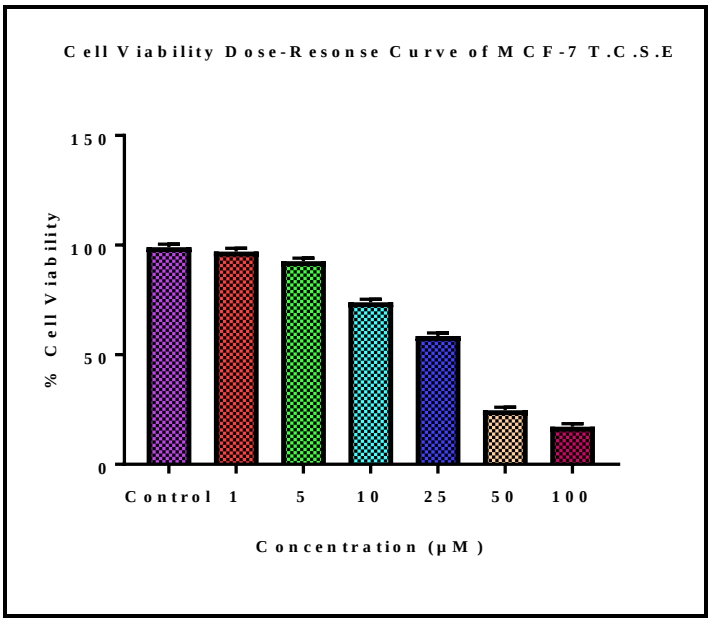

Fig. 1: Cell viability drug response curve for MCF-7 cells of T.C.S.E

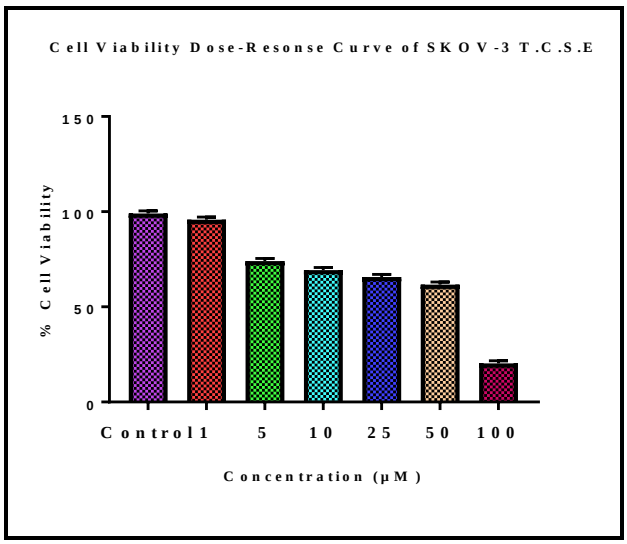

Fig. 2: Cell viability drug response curve for SKOV3 cells of T.C.S.E

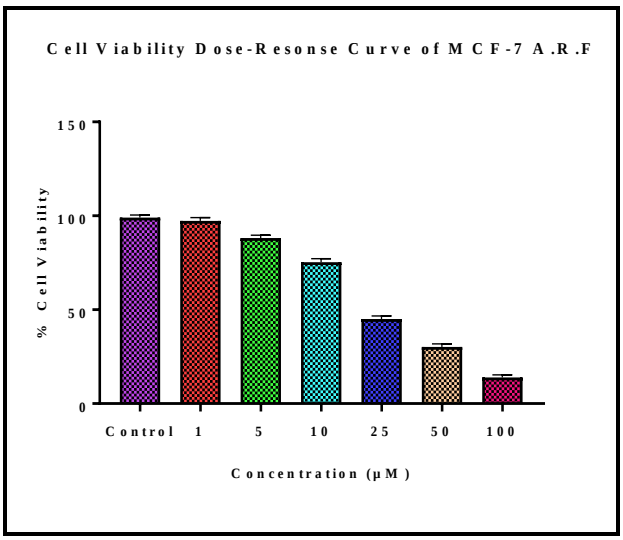

Fig. 3: Cell viability drug response curve for MCF-7 cells for Alkaloid rich fraction

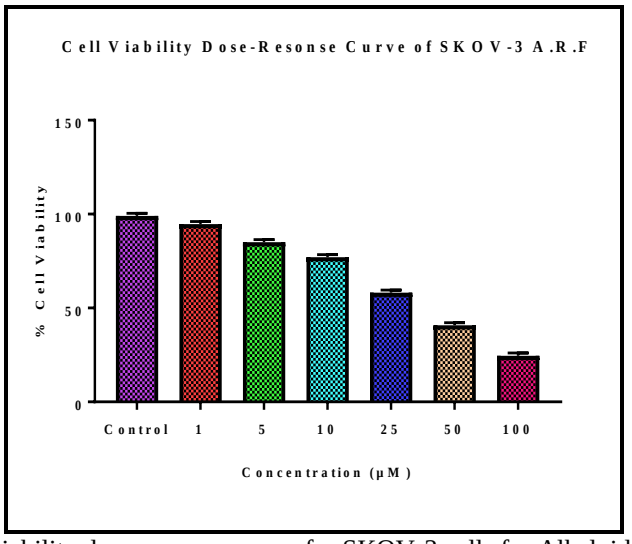

Fig. 4: Cell viability drug response curve for SKOV-3 cells for Alkaloid rich fraction

Morphological studies exhibited significant changes after 24 hours of specific concentration of T.C.S.E and A.R.F treatment cell's morphology was changed compared with DMSO represented in Fig. 5. The number of cells were decreased, detached and shrinked. The morphological differences were analyzed after the treatments with control in SKOV3 cells. The untreated cells were epithelial in shape and the treated cells were irregular and thin, and cell shrinkage was observed under microscope. Besides the size, numbers were also decreased at various concentrations. In addition, to this detached cells were seen in suspension.

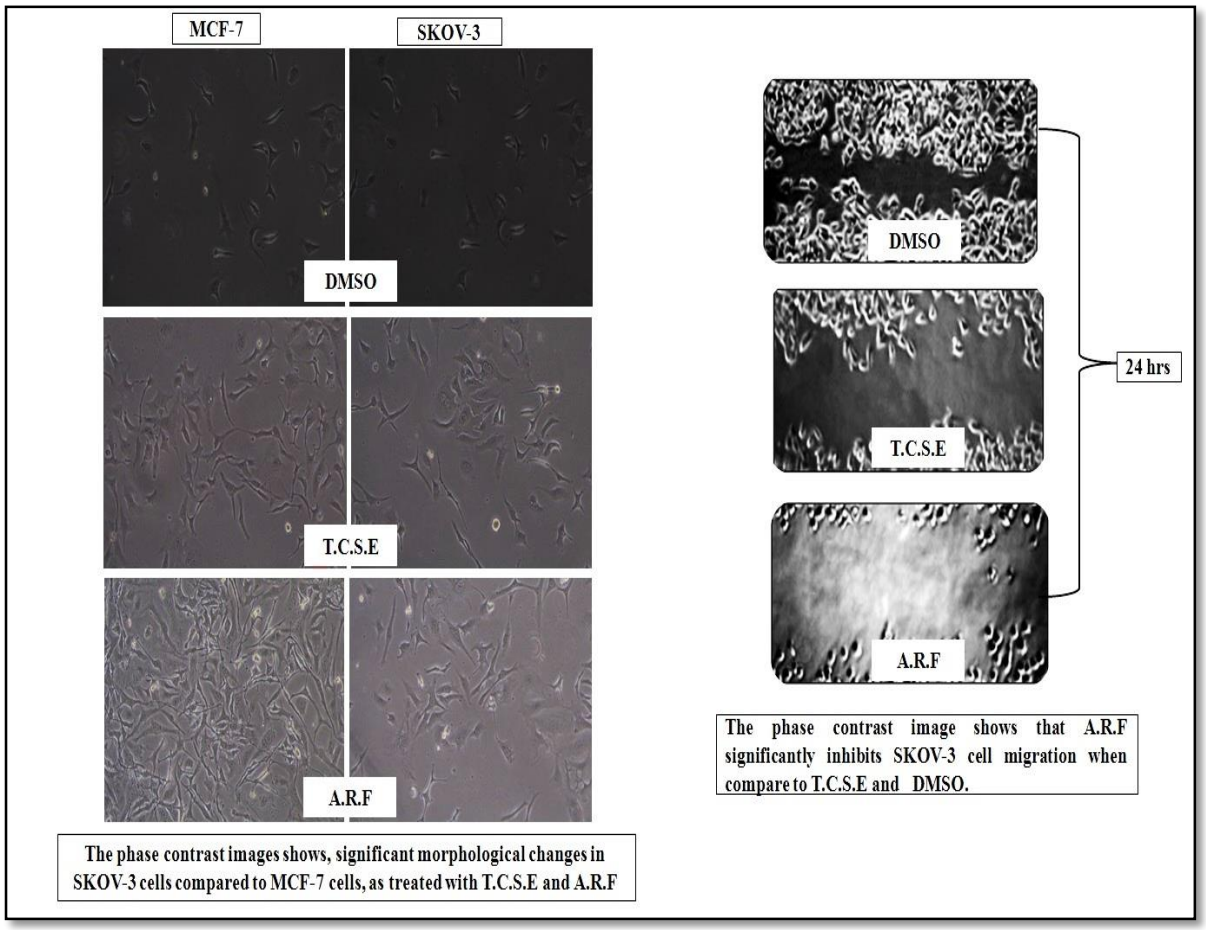

Fig. 5: Phase contrast images shows morphological changes of cells and inhibition of migration of cells by A.R.F in SKOV-3 cells

Colony formation assay results revealed that A.R.F has significantly inhibited the population density of cells compared to T.C.S.E after the treatment in both SKOV-3 & MCF-7 cells. In Fig.5 the number of colonies presented after the treatment compared with the control group shows the effect of A.R.F at 25μM IC 50 decreased the growth of colonies by more than 50%, whereas treatment with A.R.F at 100μM reduced the number of colony formation by 90% in SKOV-3 cells. Similarly, in Fig.6 MCF-7 cells at 75 μM IC 50 concentrations, more than 50% of the colony numbers were reduced in T.C.S.E. Fewer colonies were observed at 100 μM and 150 μM concentrations. Thus, it was suggested that the A.R.F has better anti-proliferative effects in SKOV3 cells.

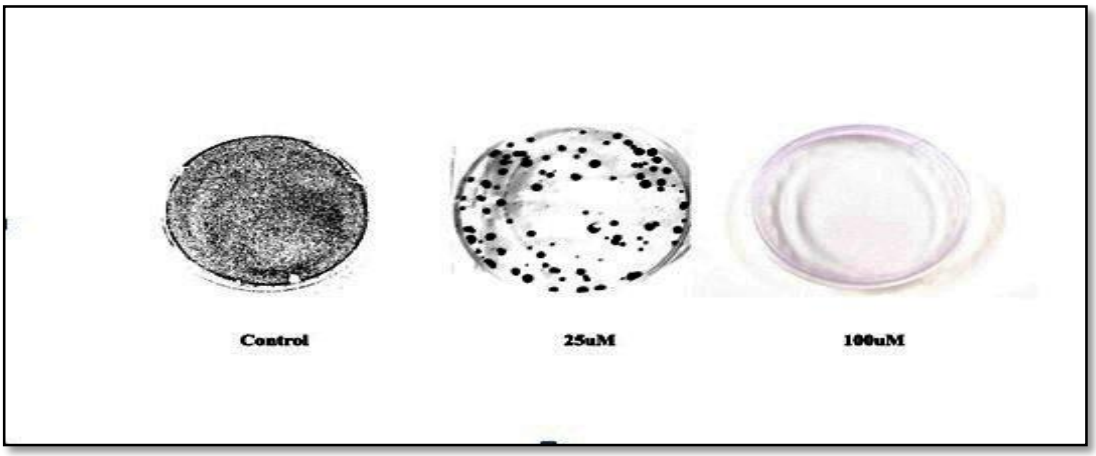

Fig. 6: A.R.F Inhibits colony formation at 25μM showed IC 50 decreased the growth of colonies by more than 50%in SKOV-3 cells at the indicted concentrations compared to the control group.

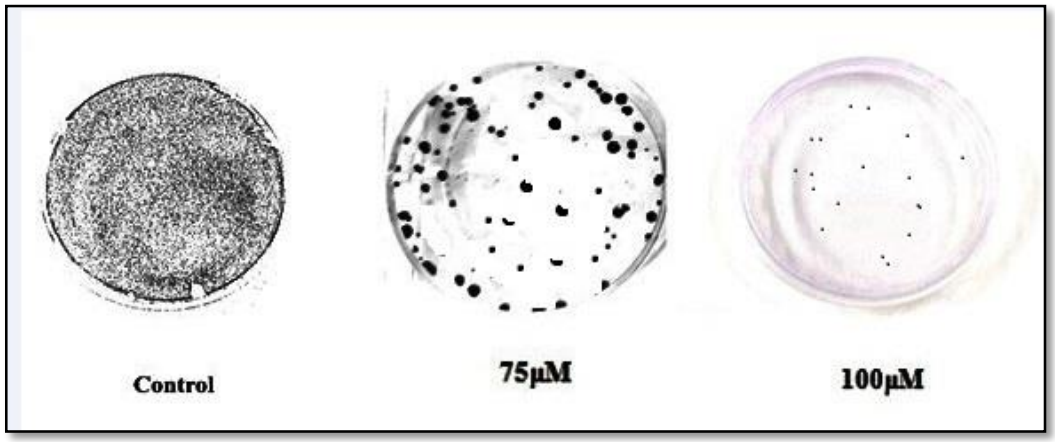

Fig. 7: T.C.S.E Inhibits colony formation at 75µM, showed IC 50 decreased the growth of colonies by more than 50%in MCF-7 cells at the indicted concentrations compared to the control group.

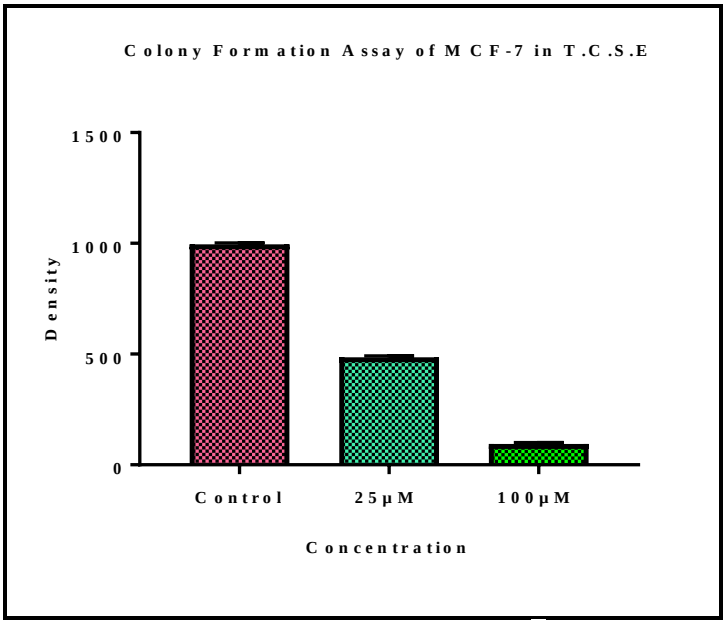

Fig. 8: Colony formation Assay of MCF-7 cells at 25µM IC 50 for T.C.S.E

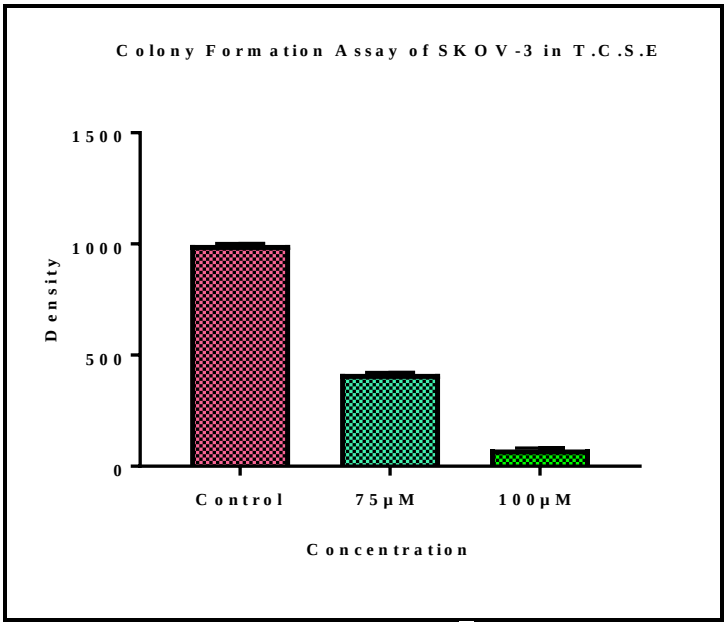

Fig. 9: Colony formation Assay of SKOV-3cells at 75 µM IC 50 concentrations for T.C.S.E

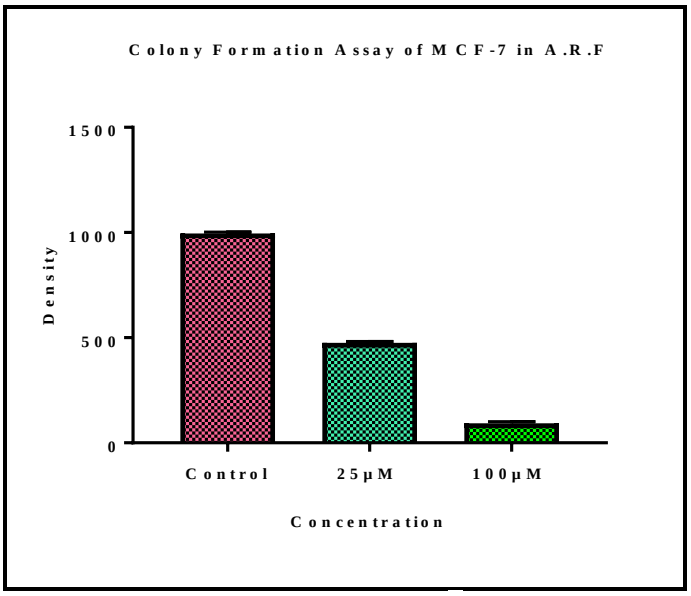

Fig. 10: Colony formation Assay of MCF-7 cells at 25µM IC 50 concentrations for A.R.F

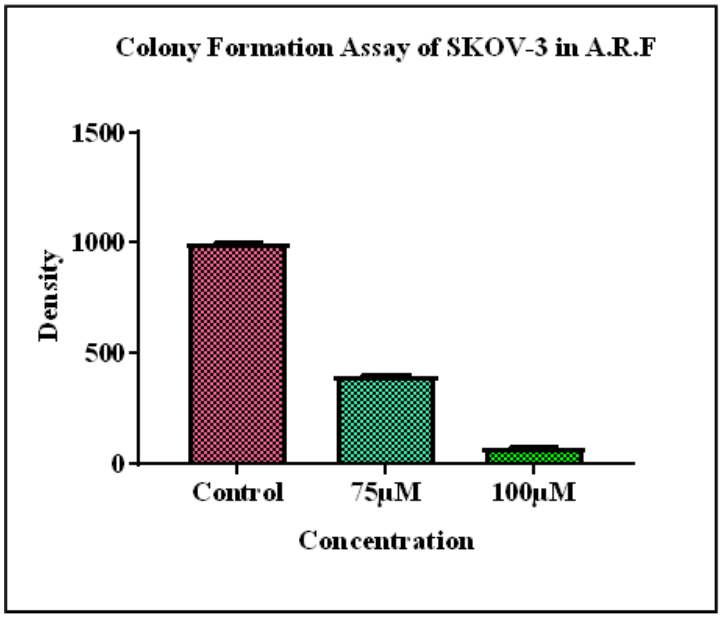

Fig. 11: Colony formation Assay of SKOV-3 cellsat 75 µM IC 50 concentrations for A.R.F

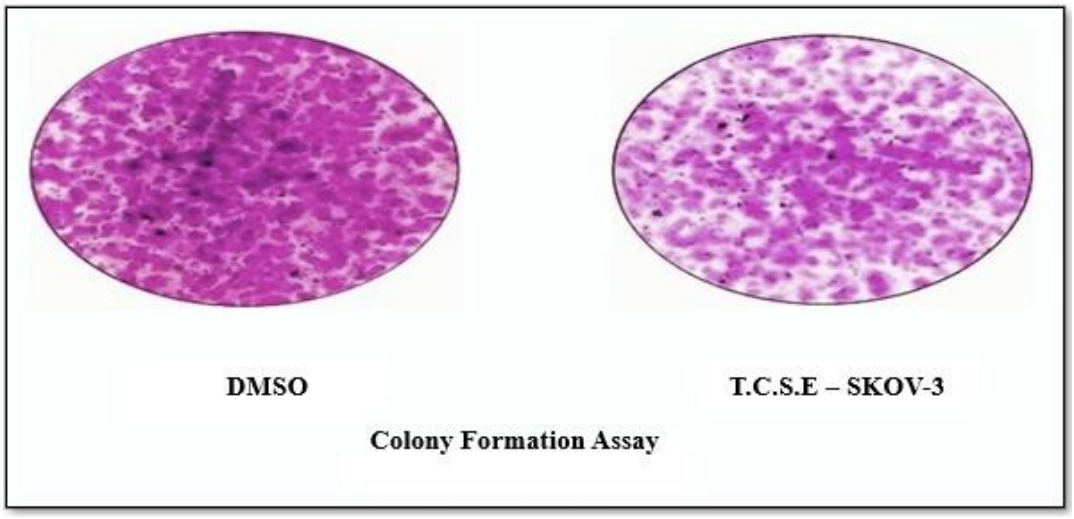

Fig. 12: Colony formation Assay of SKOV-3 cells at 75 µM IC 50 concentrations of T.C.S.E

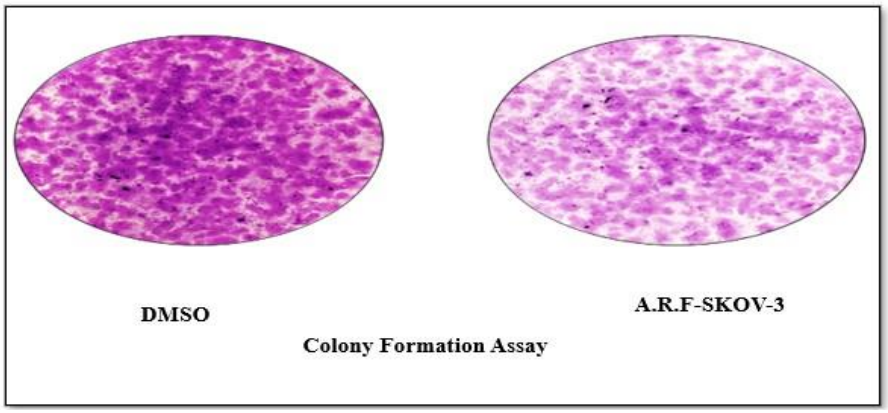

Fig. 13: Colony formation Assay of SKOV-3 cells at 75 µM IC 50 concentrations of A.R.F

3.1 Scratch Assay

A Scratch assay was performed to identify the effect of T.C.S.E & A.R.F on the migration capacity of MCF7 cells and SKOV3 cells. The cells were seeded in a 35 mm Petri dish and treated with a determined concentration of extract for 24 hours. The migration capability of cells was observed under a microscope. The outcome of the experiment explained that the T.C.S.E & A.R.F was significantly decreased in the inhibition of wound healing in the treatment group of 25µM and 100µM, the created space was completely covered in the control group within 48 hours. For SKOV-3cells, the scratch was carried out following an experiment with 75 µM on T.C.S.E & A.R.F for 24 hours. It was achieved by following the perimeter of the wound created before and after the treatments. The results show that the T.C.S.E as well as A.R.F inhibited the cell migration compared to untreated cells.

3.2 Gene Expression analysis of CYP17A1

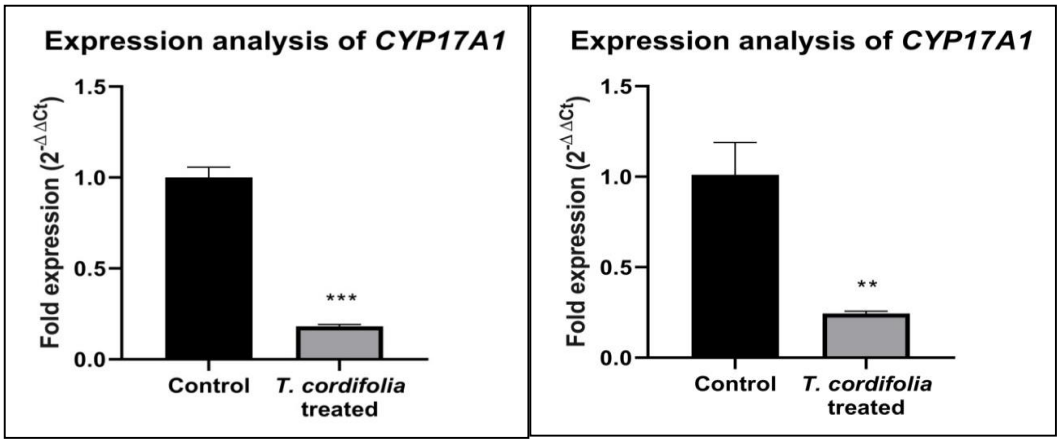

Fig. 14: CYP17A1 gene expression analysis of *Tinospora cordifolia* stems extract

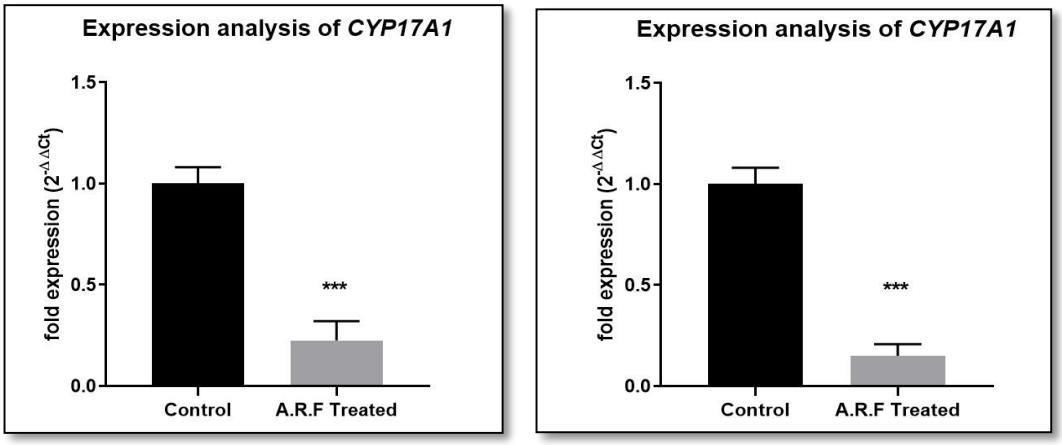

Fig. 15: CYP17A1 gene expression analysis of Alkaloid rich fraction

When analysing gene expression data from RNA-Seq experiments, fold change is frequently used to measure changes in a gene's expression level. A positive fold change value implies an increase in expression for a particular comparison, while a negative fold change value suggests a reduction in expression.

RQ = **Relative quantification** = $2^{-\Delta\Delta Ct}$

The effect of T.C.S.E and A.R.F regulating the mRNA expression of CYP17A1 on MCF7 and SKOV-3 cells using RT-PCR analysis. The A.R.F treatment significantly downregulated the expression of CYP17A1 gene in SKOV-3 cells, and in MCF-7 cells with the significant p-value of 0.001. The fold expression of CYP17A1 on T.C.S.E in MCF7 cells were found to be 0.245±0.006. Similarly, the A.R.F in SKOV-3 cells is more effectively inhibited the expression of CYP17A1 mRNA with a highly significant p-value <0.001. The fold expressions of CYP17A1 on A.R.F treatment in SKOV-3 cells were found to be 0.182±0.009. Thus, the gene expression analysis data suggests that the A.R.F suppressed the expression of CYP17A1 gene in both MCF-7 and SKOV-3 cells which might play a significant role in PCOS condition.

4. DISCUSSION

CYP17A1 gene encodes a member of the cytochrome P450 superfamily of enzyme. The cytochrome P450 enzymes are monooxygenases mostly found in endoplasmic reticulum that catalyse numerous chemical reactions essential for drug metabolism and the production of steroid hormones, cholesterol, and other lipids. It possesses both 17alpha-hydroxylase and 17, 20-lyase activity and essential enzyme in the steroidogenic pathway that synthesize progestins, mineralocorticoids, glucocorticoids, androgens, and estrogens [13]. Estrogen receptor-α modulates the production of CYP17A1 (cytochrome P450, steroid 17-hydroxylase/17, 20 lyase), which in turn mediates an intraovarian negative feedback loop on theca cells steroidogenesis [14]. Cytochrome P450C17 (CYP17) expression, Steroidogenic acute regulatory protein (StAR), P450 side-chain cleavage (P450scc), and 3-hydroxysteroid dehydrogenase (3-HSD) expression are all upregulated in the theca cells under the high pulsatile release of LH, which also results in an increase in steroidogenic activity, resulting in an increased and consistent manner by the high levels of insulin often seen in PCOS women [15]. The data suggests that the CYP17A1 gene expression is significantly downregulated in both MCF-7 and SKOV-3 cells after alkaloid rich fraction treatment. The MTT assay [16], a colorimetric assay for evaluating cell metabolic activity, is frequently used to measure cell viability, proliferation and cytotoxicity. The tetrazolium dye MTT, which is chemically 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, may be reduced by the enzymes to its insoluble formazan, which has a purple hue. The intermediate electron acceptor, 1-methoxy phenazine methosulfate (PMS), is utilised in combination with other closely related tetrazolium dyes such as XTT, MTS, and the WSTs. In this study human ovarian cancer cell line SKOV-3 [17], MCF-7 human breast cancer cells [18], were used, in one study Western blot analysis revealed a strong expression of Androgen Receptor (AR) in MCF-7 [19]. In A.R.F the cell viability was significantly in SKOV-3 cells and MCF-7 cells when compared to SKOV-3 cells and MCF-7 cells of T.C.S.E.

An *in vitro* cell survival assay based on the capacity of a single cell to develop into a colony is known as a clonogenic assay or colony formation assay [20]. A colony is defined as a group of cells with at least 50 cells. The assay effectively determines if each cell in the population has the potential to divide indefinitely. The clonogenic test is the preferred method for determining cell reproductive mortality following ionizing radiation therapy, but it may also be used to assess the efficacy of other cytotoxic agents. A.R.F When compared to the control group, IC 50 inhibited colony formation at 25 μM and reduced colony expansion by more than 50% in SKOV-3 cells at the indicated concentrations. T.C.S.E reduces colony formation at 75 μM, and revealed that at the indicated concentrations, IC 50 reduced colony development by more than 50% in MCF-7 cells compared to the control group. Significant morphological changes in SKOV-3 cells treated with T.C.S.E and A.R.F are seen in phase contrast images when compared to MCF-7 cells. When compared to T.C.S.E and DMSO, the phase contrast picture demonstrates that A.R.F significantly inhibits SKOV-3 cell migration. The 18-mer oligo synthesised shows considerable inhibitory effect against tumour cell migration at the cellular level, according to the Wound Scratch experiment [21].

The hydroxylase enzyme cytochrome P450 17A1 (steroid 17-monooxygenase, 17-hydroxylase, 17-alpha-hydroxylase, 17, 20-lyase, 17, 20-desmolase) is encoded by the CYP17A1 gene [22] on chromosome 10 in humans. Women with polycystic ovarian syndrome (PCOS) have higher androgen levels due to excessive Cyp17A1 expression [23] and changes in gene expression in granulose cells. A.R.F significantly reduces the expression of CYP17A1 gene in SKOV-3 cells highly significant p-value <0.001 and fold expression of CYP17A1 SKOV-3 cells were found to be 0.182±0.009, when compared to MCf-7 cells with the significant p-value of 0.001 and fold expressions were found to be 0.245±0.006. Hence CYP17A1 gene expression plays a major role in manifestation of high level of androgens, which may have an important role in PCOS.

5. CONCLUSION

In this study, preliminary *in vitro* screening assays were done to explore the PCOS activity of *Tinospora cordifolia* stem extract and alkaloid rich fraction. Alkaloid rich fraction has significantly inhibited the population density of cells compared to *Tinospora cordifolia* stem extract, at 25μM showed IC-50 decreased the growth of colonies by more than 50% in SKOV-3, fewer colonies were observed at 100 μM and 150 μM concentrations. Thus, it was suggested that the alkaloid rich fraction has better anti-proliferative effects in SKOV-3 cells. CYP17A1 mRNA expression was more effectively suppressed in SKOV-3 cells, with a highly significant p-value <0.001. The fold expression of CYP17A1 in SKOV-3 cells after treatment with alkaloid rich fraction was 0.182±0.009. The potential PCOS activity of *Tinospora cordifolia* has been illustrated based on inhibition in MCF-7 and SKOV3 cancer cell lines. When compared to *Tinospora cordifolia* stem extract the alkaloid rich fraction has better anti-proliferative, cologenic, and significantly down regulated the expression of CYP17A1 gene expression therefore it will diminished the synthesis and production of excess

androgens. Hence alkaloid rich fraction has better role in suppressing the steroidogenesis CYP17A1 gene against (PCOS) hyperandrogenisam. Together these findings revealed that alkaloid rich fraction of *Tinospora cordifolia* might be a potential therapeutic androgen antagonist.

ACKNOWLEDGEMENTS: The authors wish to express sincere thanks to Dean Dr V. Chitra for our support and motivation towards research.

Author Contribution All authors contributed to the study conception and design. Murali Krishna Moka: conceptualization, methodology, investigation, formal analysis, writing—original draft preparation. Sumithra M: methodology, investigation, formal analysis, writing—review and editing.

Data Availability Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

DECLARATIONS

Ethics Approval Not applicable.
Consent to Participate Yes. All authors consent to participate.
Consent for Publication Yes. All authors have approved the last version of the manuscript for its submission.
Competing Interests The Authors declare no Competing Interests

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