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Synthesis and Characterization of Microbial Metabolites of Abiraterone Acetate and an Evaluation of Their Anticancer Activity against Breast Cancer Cell Line MCF-7

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Abstract: ***Active drug metabolites, rather than the parent drug, are considered commercial drugs because of their potential pharmacological activity. In this study, higher quantities of new microbial metabolites of the anticancer drug, Abiraterone acetate (1), were synthesized using fungi and then purified by column chromatography. The resulting metabolites showed >99% purity on HPLC and were identified as cis-6-hydroxy Δ^4-abiraterone (M2), trans-6-hydroxy Δ^4-abiraterone (M3), cis-6-hydroxy Δ^4-abiraterone N-oxide (M4) by mass, infrared spectroscopy and both ^1H and ^{13}C nuclear magnetic resonance. The microbial metabolites were compared in order to evaluate the highest potential anticancer activities with Abiraterone acetate (1) in the breast cancer cell line, MCF-7. Trans-6-hydroxy Δ^4-abiraterone (M3) exhibited good cytotoxicity compared to the parent drug, Abiraterone acetate (1). However, further studies are required to utilize this anticancer agent for new drug discovery and development against breast cancer.***

Key words: Abiraterone acetate, drug metabolites, anticancer activity, cytotoxicity, breast cancer, MTT assay

I. INTRODUCTION

Cancer is a cluster of diseases that are life threatening throughout the world. The uncontrolled proliferation of abnormal cells in humans is complicated to treat and cure. Breast cancer in women is the second most common cancer in India. The estrogen hormone is the main source for the survival and proliferation of breast cancer cells; almost 75% of breast cancers express estrogen positive receptors (Colditz et al., 2004; Harvey et al., 1999).

Breast cancer can be controlled by the inhibition of estrogen synthesis. Many different types of treatments are provided for breast cancer like chemotherapy, hormone therapy, radiation, surgery and immunotherapy (Florescu., 2011). The drugs used for these treatments were derived from synthetic route compounds and natural products.

To compete with the uncontrolled growth of cancer cells, an effective and specific anticancer agent must be developed that does not affect normal cells, has fewer side effects, and more quickly inhibits the growth of cancer cells. These findings could reduce the cancer mortality rate.

Currently, research focuses on drug metabolites to develop better drugs against diseases (Kevin et al., 2013). Active drug metabolites are marketed as drugs, due to their higher pharmacological activity with less toxicity than parent drugs (Keshetty and Ciddi., 2003; Myung et al., 2010; Kebamo et al., 2015). Active metabolites of anticancer drugs are also under development as drugs and clinical trials are in progress (Mansel et al., 2007).

An anticancer drug, abiraterone acetate (**1**) (17-(3-pyridyl)-5, 16-androstadien-3β acetate), a steroidal anti androgen, is used to treat castration-resistant prostate cancer in men (Barrie et al., 1994). In a research report, the human metabolite of Δ^4-abiraterone (D4A) showed it might have more pharmacological activity against prostate cancer than its parent drug (Li et al., 2015).Now there have been many metabolic research studies and clinical trials of abiraterone acetate are under way for the usage of abiraterone acetate and its metabolites in breast cancer treatment (Ng et al., 2012; Capper et al., 2016; Bonnefoi et al 2016; Shaughnessy et al., 2016).

Given the above, the present study focused on producing a higher quantity of abiraterone acetate **1** microbial metabolites, using fungi, in order to 1) identify the metabolites by spectral analysis and 2) determine which are most likely to have greater activity than abiraterone acetate against breast cancer cell line MCF-7.

II. MATERIALS AND METHODS

Mass spectroscopy was operated in the electron spray ionization (ESI) mode with model: Shimadzu LCMS 2010EV, detector: ion trap detector, operation: positive mode, range: 50-2000 m/z, spray voltage: 3.5kV, capillary temperature: 200° C. The ^{1}H and ^{13}C spectra were measured in JEOLECZ500R/S1 using solvent DMSO-d6 under 500 MHz. Chemical shifts are expressed in δ (ppm) and coupling constants (J) were given in hertz (Hz). Infrared spectra were recorded on a Fourier Transform IR spectrophotometer (Nicolet 5700 FT-IR 8700) using the KBR (vmaxin cm^{-1}) disc method. The HPLC analysis was carried out with our previous method, using a Thermo scientific HPLC system (USA) consisting of Accela 1250 mode, Chromquest Version 5.0, and photodiode array detector. Samples were analyzed by Cyano column (Nucleosil, CN) 4.6 by 250 mm with particle size of 5 µ. Samples were eluted isocratically with a mobile phase consisting of 1% ortho phosphoric acid (OPA) buffer: Acetonitrile (60:40, v/v) at a flow rate of 1 mL/min.

A. Preparative scale synthesis

Cunninghamellaelegans NCIM 690 was purchased from NCL, Pune, India. Saboraud dextrose broth (SDB) (Himedia) was used as media for fungal cultures. The preparative scale synthesis was conducted with a two-stage fermentation method. In the first stage of fermentation, 20*250mL Erlenmeyer flasks each containing 100mL of broth were prepared and autoclaved at 121°C for 20 minutes. In each flask, a loop of fresh mycelium and spore culture was inoculated. The cultures were incubated at 28 °C for 24 h on a rotary shaker operating at 120 rpm. In the second stage of fermentation, a total of 5*5L round bottom flasks containing 2.0L of SDB media were autoclaved at 121°C for 20 minutes. After cooling, 20% of the volume of the first-stage culture was transferred and the cultures were kept in a shaker. The cultures were grown at 28°C for 24 h at 120 rpm. After good growth of cultures was observed, a 100mg/L concentration of abiraterone acetate (1gm) was added directly and the reaction was continued for six days. After microbial transformation, the flasks were removed from the shaker; the collective media were filtered to separate the mycelium. The pH was adjusted to 9.0 with sodium bicarbonate and compounds were extracted with dichloromethane (three times the volume of media). The pooled organic layer was washed with brine, dried over anhydrous Na_2SO_4 and then evaporated under reduced pressure in a rotary evaporator to obtain a brown gummy crude extract, which was separated by column chromatography.

B. Column purification

The gummy brown crude material was prepared with dichloromethane and silica gel 60-120 mesh. A glass column was packed with silica gel with chloroform. Free flow crude slurry was loaded, and cotton was placed on top of slurry. The slurry was eluted with methanol: chloroform solvent system. Elution started with 100 mL of chloroform and then 1%, 1.5%, 2.5%, 5%, 15%, 20%, and 25%. The collected same R_f values of fractions were pooled after monitoring thin layer chromatography. Fractions were concentrated under rotary evaporator at 50^0 Cunder reduced pressure. Four different fractions were achieved and stored at room temperature until further analysis. Fractions were labeled as M1, M2, M3 and M4, indicating their increasing polarity on TLC.

C. Characterization

In our previous research, metabolite M1 was identified as human metabolite Δ^4-abiraterone. In this research, the three new purified metabolites M2, M3 and M4 were analyzed for further characterization. The molecular weight determination, structural conformation, and functional group identification were performed by Mass, IR, ^{1}HNMR and ^{13}CNMR, respectively. The purity of abiraterone acetate1, and unidentified microbial abiraterone acetate metabolites M2, M3 and M4 was analyzed by HPLC.

D. Anticancer activity

1) *Cell culture and cultivation:* The breast cancer cell line MCF-7 used in this study was procured from the National Centre for Cell Sciences, Pune, India. The cells were cultured and passaged in Dulbecco's Modification of Eagle's Medium (DMEM, Himedia, India) supplemented with 10% Fetal Bovine Serum (Himedia, India), and a 1% Antibiotic Antimycotic Solution (Himedia, India). The cells were maintained at 37 °C in a 95% relative humidified atmosphere containing 5% CO_2 in air. Cell growth was measured under a light microscope and 80% confluence of the cells was used in all experiments.
2) *MTT assay:* Cell viability count was performed using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay (Mossman., 1983).Cells were seeded at 2×10^4 cells per well (200 µL/well) in 96-well tissue culture plates and allowed cells to adhere for 24 h at 37 °C in the CO_2 incubator. The culture medium was then replaced with 200 µL/well of the fresh medium for the control group and 200 µL/well of the fresh medium containing the same concentration (1000 µg/mL) of the purified fraction. In brief, the treatment media were removed and the wells were washed once with 1X PBS.Then 100µl of MTT

reagent (5mg/10mL) in 1X PBS was added. Four hours after completion, the reaction mixture was carefully taken out and 100 µL/well of dimethyl sulfoxide (Merck, India) was added. The optical densities (OD) were measured at 570 nm by using DMSO as a blank using micro plate reader (Model-AM 2100, Alere Medical Pvt Ltd., India). Finally, the highest effective anti-breast cancer compound with the highest inhibition concentration was calculated. The nonlinear regression graph was plotted by taking IC_{50} (µg/mL) values of compounds in Y axis and name of compounds in Y axis and IC_{50} was determined with Graph Pad Prism software.

III RESULTS AND DISCUSSION

A. *Synthesis*

In our previous research work, microbial screening and biotransformation of abiraterone acetate 1 was performed using fungi in small-scale and the human metabolite of Δ^4-abiraterone M1 was identified. Along with human metabolite, we found three new metabolites, which we were not able to identify due to their lower yield. In this research work we synthesized these three new metabolites and identified. Next, large-scale synthesis (10L) of biotransformation was performed using fungi in shake flask fermentation. The microbial metabolic pathway of abiraterone acetate 1 by *Cunninghamella elegans* NCIM 690 was shown in Figure

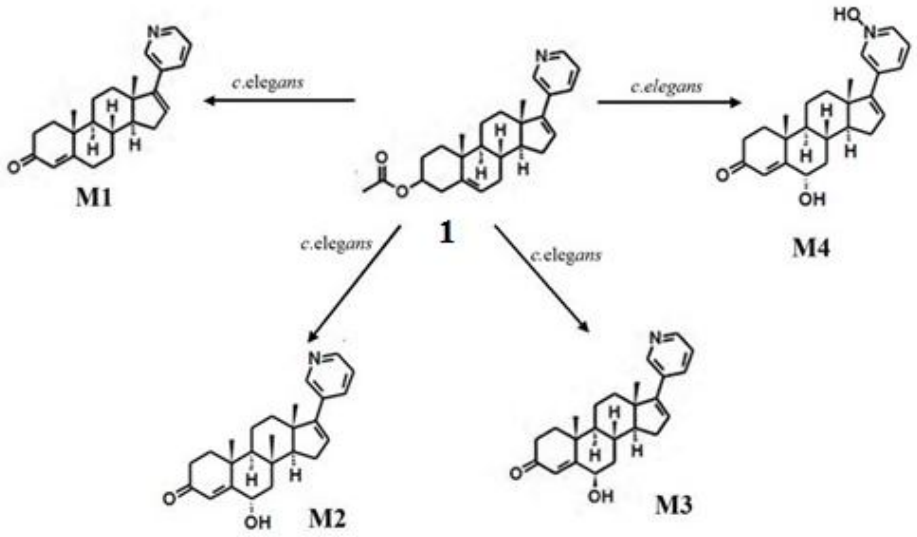

Fig 1 Microbial metabolic pathway of Abiraterone acetate by Cunninghamella elegans NCIM 690

1) Note: - 1-Abiraterone acetate; M1- Δ^4-abiraterone (Human metabolite); M2-cis 6-hydroxy Δ^4-Abiraterone; M3-trans 6-hydroxy Δ^4-Abiraterone; M4-cis 6-hydroxy Δ^4-Abiraterone N-oxide.

After five days of incubation, Abiraterone acetate **1** was completely converted into four products with distinct spots on TLC. The extracted crude metabolites were purified by column chromatography and the purity of metabolites was analyzed by HPLC. In HPLC analysis, all the metabolites were above 99% purity. The TLC retardation factor (R_f), HPLC retention time (R_t) and purity (%) of metabolites are shown in Table 1.

TABLE 1: TLC and HPLC analysis of Abiraterone acetate and its metabolites

Compounds	Retardation Factor R_f	Retention Time R_t(Minute)	Purity (%)
Abiraterone acetate	0.93	7.0	100
Metabolite M2	0.38	4.3	99.70
Metabolite M3	0.29	4.4	99.48
Metabolite M4	0.18	3.8	99.55

B. *Identification*

The structural characterization of three new microbial metabolites was determined by the combined analysis of Mass, IR, ^{1}HNMR, and ^{13}CNMR spectral data. The metabolites M2 and M3 obtained from microbial synthesis have 16 mass units more than the human

metabolite of Δ^4-abiraterone M1, confirming that these two compounds were hydroxylated products of M1. From the spectral data, the compounds were identified as cis-6-hydroxy Δ^4-abiraterone M2 and trans-6-hydroxy Δ^4-abiraterone M3. Metabolite M4 has 16 mass units higher than cis-6-hydroxy Δ^4-abiraterone M2 and confirms the N-oxidation product of M2. The metabolite M4 was identified as 6-hydroxy Δ^4-abiraterone N-oxide.

These three new metabolites were found to be derivatives of Δ^4-abiraterone and reported for the first time. As a steroidal structure of abiraterone acetate 1, it has the potential to form hydroxylated, oxygenated, N-oxidized, sulphated and glucuronidated metabolites (Acharya et al., 2013).

*1) Cis-6-hydroxy Δ^4-Abiraterone (M2).*Off-white Solid, Yield: 10%, Molecular formula: $C_{24}H_{29}NO_2$, ESI-MS (m/z 364.88, [M+H]), IR (KBr, cm^{-1}) -C=O: 1683.89, Aliphatic -CH: 2853.72-3032.52, Aromatic –C=N: 2320-2385, Aromatic –C=C: 1527-1653.7, -OH: 3446.72, *Cis* isomer frequency at 668. ^{1}HNMR (DMSO-d6, 500MHz) δ ppm: 0.9 (6H,s), 1.09 (s)), 1.22 (7H, 1.33 (dt, J = 13.7, 10.2 Hz), 1.61 (ddd, J = 13.3, 3.3, 2.5 Hz), 1.95 (dddd, J = 13.0, 3.5, 3.0, 2.5 Hz), 1.61 (dddd, J = 13.0, 10.2, 10.1, 3.3 Hz), 1.33 (ddd, J = 13.3, 10.2, 3.0 Hz), 1.56 (ddd, J = 10.2, 10.1, 3.5 Hz), 1.56 (qd, J = 10.2, 2.5 Hz)), 1.86-1.95 (2H, 1.92 (ddd, J = 13.7, 3.2, 2.5 Hz), 1.95 (ddd, J = 13.7, 10.2, 3.4 Hz)), 2.05-2.54 (6H, 2.35 (ddd, J = 13.6, 5.2, 4.3 Hz), 2.18 (ddd, J = 13.7, 3.1, 2.4 Hz), 2.12 (ddd, J = 10.2, 9.6, 5.2 Hz), 2.33 (ddd, J = 15.0, 3.4, 2.4 Hz), 2.47 (ddd, J = 15.0, 10.2, 3.1 Hz), 2.31 (ddd, J = 13.6, 9.6, 6.9 Hz)), 4.44 (1H, dd, J = 10.2, 3.2 Hz), 4.66 (1H,s), 5.67 (1H, s), 6.17 (1H, dd, J = 6.9, 4.3 Hz), 7.37 (1H, ddd, J = 8.1, 4.7, 0.5 Hz), 7.80 (1H, ddd, J = 8.1, 1.9, 1.6 Hz), 8.47 (1H, ddd, J = 4.7, 1.9, 1.9 Hz), 8.62 (1H, ddd, J = 1.9, 1.6, 0.5 Hz). ^{13}CNMR (DMSO-d6, 500MHz) δ ppm: (C-1)34.66 , (C-2) 31.99, (C-3) 197.99, (C-4) 123.22, (C-5) 171.03, (C-6) 70.07, (C-7) 34.99 , (C-8) 38.41, (C-9) 53.6, (C-10) 38.41, (C-11) 20.25, (C-12) 33.58, (C-13) 46.9, (C-14) 53.64, (C-15) 34.60, (C-16) 131.7, (C-17) 131.29, Pyridyl ring (C-1) 147.52, (C-2) 133.28, (C-3) 128.89, (C-4) 123.23, (C-5) 148.28.

2) Trans-6-hydroxy Δ^4-Abiraterone (M3). Off-white solid, Yield: 15%, Molecular formula: $C_{24}H_{29}NO_2$, ESI-MS (m/z 364.88, [M+H]), IR (KBr, cm^{-1}) -C=O: 1683.89, Aliphatic -CH: 2853.72-3032.52, Aromatic–C=N: 2320-2385, Aromatic –C=C: 1527-1653.7, -OH: 3396.05, trans isomer frequency at 799.18. ^{1}HNMR (DMSO-d6, 500MHz) δ ppm: 1.04 (6H,s), 1.11 (2H, (s)), 1.29 (7H, 1.39 (dt, J = 13.7, 10.2 Hz), 1.61 (ddd, J = 13.3, 3.3, 2.5 Hz), 1.83 (dddd, J = 13.0, 3.5, 3.0, 2.5 Hz), 1.60 (dddd, J = 13.0, 10.2, 10.1, 3.3 Hz), 1.39 (ddd, J = 13.3, 10.2, 3.0 Hz), 1.60 (ddd, J = 10.2, 10.1, 3.5 Hz), 1.60 (qd, J = 10.2, 2.5 Hz)), 1.83-2.05 (2H, 1.92 (ddd, J = 13.7, 3.2, 2.5 Hz), 1.83 (ddd, J = 13.7, 10.2, 3.4 Hz)), 2.05-2.30 (6H, 2.35 (ddd, J = 13.6, 5.2, 4.3 Hz), 2.20 (ddd, J = 13.7, 3.1, 2.4 Hz), 2.20 (ddd, J = 10.2, 9.6, 5.2 Hz), 2.30 (ddd, J = 15.0, 3.4, 2.4 Hz), 2.30 (ddd, J = 15.0, 10.2, 3.1 Hz), 2.30 (ddd, J = 13.6, 9.6, 6.9 Hz)), 4.38 (1H,s), 5.64 (1H, s), 6.13 (1H, dd, J = 6.9, 4.3 Hz), 7.36 (1H, ddd, J = 8.1, 4.7, 0.5 Hz), 7.77 (1H, ddd, J = 8.1, 1.9, 1.6 Hz), 8.45 (1H, ddd, J = 4.7, 1.9, 1.9 Hz), 8.60 (1H, ddd, J = 1.9, 1.6, 0.5 Hz). ^{13}CNMR (DMSO-d6, 500MHz) δ ppm: (C-1)34.66 , (C-2) 31.99, (C-3) 197.99, (C-4) 123.22, (C-5) 171.03, (C-6) 67.16, (C-7) 34.99 , (C-8) 38.41, (C-9) 53.6, (C-10) 38.41, (C-11) 20.25, (C-12) 33.58, (C-13) 46.9, (C-14) 53.64, (C-15) 34.60, (C-16) 131.7, (C-17) 131.29, Pyridyl ring (C-1) 147.52, (C-2) 133.28, (C-3) 128.89, (C-4) 123.23, (C-5) 148.283.

3) Cis-6-hydroxy Δ^4-Abiraterone N-oxide (M4). Off-white solid, Yield: 20%, Molecular formula: $C_{24}H_{30}NO_3$, ESI-MS (m/z 380.91, [M+H]), IR (KBr, cm^{-1}) C=O: 1683.89, Aliphatic -CH: 2853.72-3032.52, Aromatic –C=N: 2320-2385, Aromatic –C=C: 1527-1653.7, -OH: 3420.80, *Cis* isomer frequency at 710. ^{1}HNMR (DMSO-d6, 500MHz) δ ppm: 1.31 (3H, s), 1.32 (3H, s), 1.39-1.76 (6H, 1.67 (dt, J = 13.7, 10.2 Hz), 1.57 (ddd, J = 13.1, 10.2, 3.0 Hz), 1.62 (dddd, J = 13.2, 3.5, 3.0, 2.5 Hz), 1.48 (dddd, J = 13.2, 10.2, 10.1, 3.3 Hz), 1.57 (ddd, J = 10.2, 10.1, 3.5 Hz), 1.58 (qd, J = 10.2, 2.5 Hz)), 1.78-2.04 (3H, 1.92 (ddd, J = 13.7, 3.2, 2.5 Hz), 1.83 (ddd, J = 13.1, 3.3, 2.5 Hz), 1.97 (ddd, J = 13.7, 10.2, 3.4 Hz)), 2.11-2.54 (4H, 2.47 (ddd, J = 15.0, 10.2, 3.1 Hz), 2.33 (ddd, J = 15.0, 3.4, 2.4 Hz), 2.27 (ddd, J = 10.2, 9.6, 5.2 Hz), 2.17 (ddd, J = 13.7, 3.1, 2.4 Hz)), 2.32 (1H, ddd, J = 13.5, 5.2, 3.7 Hz), 2.47 (1H, ddd, J = 13.5, 9.6, 4.0 Hz), 5.66 (1H, dd, J = 10.2, 3.2 Hz), 6.19(1H, s), 7.3 (1H, dd, J = 4.0, 3.7 Hz), 7.40 (1H, ddd, J = 7.9, 7.8, 0.5 Hz), 7.8 (1H, ddd, J = 7.8, 1.9, 1.7 Hz), 8.49 (1H, ddd, J = 1.8, 1.7, 0.5 Hz), 8.63 (1H, ddd, J = 7.9, 1.9, 1.8 Hz). ^{13}CNMR (DMSO-d6, 500MHz) δ ppm: (C-1)34.66 , (C-2) 31.99, (C-3) 197.99, (C-4) 123.22, (C-5) 171.03, (C-6) 67.16, (C-7) 34.99 , (C-8) 38.41, (C-9) 53.6, (C-10) 38.41, (C-11) 20.25, (C-12) 33.58, (C-13) 46.9, (C-14) 53.64, (C-15) 34.60, (C-16) 131.7, (C-17) 131.29, Pyridyl ring (C-1) 147.52, (C-2) 133.28, (C-3) 128.89, (C-4) 123.23, (C-5) 148.283.

C. Anticancer activity and cytotoxicity effect

In the comparative evaluation, the anticancer activity of abiraterone acetate 1and new microbial metabolites M2-M4 were screened against human breast cancer cell line (MCF-7) using an MTT assay. The MCF-7 cell line was selected because it is an ER-positive human breast cancer cell line.[16]Effective in vitro anticancer activity was analyzed by various concentrations of compounds (25, 50, 100, 250 and 500µg) and tested at an optical density of 570nm to calculate the percentage of inhibition concentration. Each

concentration was tested in triplicate. The data are represented as the mean ± SD. The anticancer activity MTT assay results are shown in Table 2.

TABLE 2: Percentage inhibition concentration of Abiraterone acetate and its metabolites against MCF-7 cell line

Compounds	% Inhibition at different concentrations (µg/mL)				
Concentration (µg/mL)	25	50	100	250	500
Abiraterone acetate 1	3.28±3.00	6.92±5.30	8.29±5.08	10.49±3.32	19.07±5.61
Metabolite M2	27.65±1.77	36.24±2.58	37.77±1.59	38.77±1.35	56.14±2.45
Metabolite M3	31.76±0.94	39.55±5.12	47.76±1.89	52.61±0.52	54.89±0.21
Metabolite M4	18.99±1.51	22.10±0.72	26.37±3.53	30.80±2.81	42.08±3.07

Note: Each Value have been expressed as mean ± standard deviation

In this assay, abiraterone acetate 1 showed only 19% inhibition activity on cell viability against MCF-7. Cis-6-hydroxy Δ^4-Abiraterone M2, trans-6-hydroxy Δ^4-Abiraterone M3 displayed above 50% of inhibition on cell viability at 500µg concentration. Comparatively, the metabolite M3 showed a gradual increase of percentage inhibition concentration from 25µg-500µg, which was more than other metabolites. The half-maximal inhibitory concentration (IC_{50}) was determined by the software Graph pad Prism using non-regression analysis, as shown in Figure 2 and Table 3.

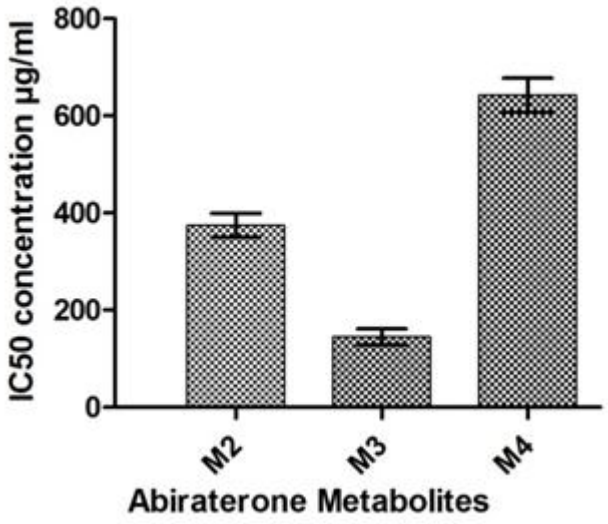

Fig 2 IC_{50} cytotoxicity of Abiraterone acetate metabolites M2-M4 against MCF-7 cell line

(Note: Abiraterone acetate 1 results were not shown, because it didn't show any activity)

TABLE 3: IC_{50} value of Abiraterone acetate and its metabolites on breast cancer cell line MCF-7.

Compounds	MCF-7 (IC_{50}± S.D) µg/ml
Abiraterone acetate 1	NA
Cis-6-hydroxy Δ^4-abiraterone M2	374.67 ± 25.32
Trans-6-hydroxy Δ^4-abirateroneM3	145.21 ± 16.21
Cis-6-hydroxy Δ^4-Abiraterone N-oxide M4	642.45 ± 35.14

IC_{50}= concentration of inhibitor to give 50% inhibition
Each value is the average of triplicate experiments with standard deviation
NA-no activity
With IC_{50} concentration, the abiraterone acetate didn't show any cytotoxicity effect. Among the metabolites, trans-6-hydroxy Δ^4-Abiraterone M3exhibited a more potent cytotoxicity effect against the MCF-7 cell line.Cis-6-hydroxy Δ^4-abiraterone M2 and cis-6-hydroxy Δ^4-Abiraterone N-oxide M4 demonstrated less activity when compared to metabolite M2 and M3 and more than abiraterone

acetate 1did.From the above results, trans-6-hydroxy Δ^4-abiraterone M3, a hydroxylated metabolite having the potential cytotoxicity effect against ER-Receptor positive breast cancer cell line MCF-7 than the parent drug.

IV. CONCLUSION

In conclusion, the preparative scale synthesis of microbial metabolites of abiraterone acetate 1 was performed in this study. A greater quantity of metabolites with high purity was obtained, sufficient for further characterization and for study in biological experiments. The anticancer activity of parent drug and metabolites were performed by MTT assay on breast cancer cell line MCF-7. When compared to its parent drug, the metabolite M3 showed more potent anticancer activity and could be a new anticancer agent. However, prior to use of this metabolite as an anticancer agent, further in vivo and in vitro studies are required.

V. ACKNOWLEDGEMENTS

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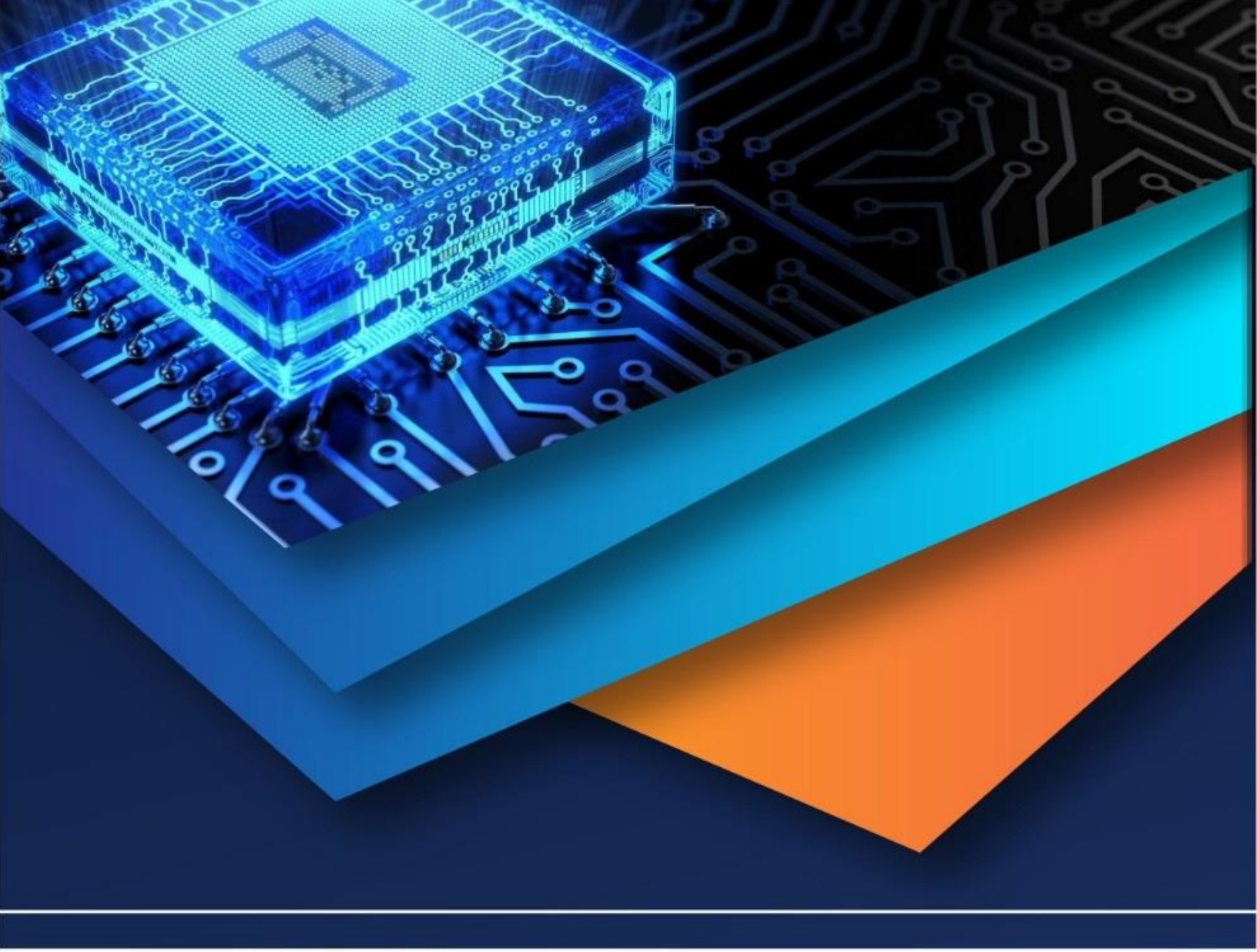

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