Research Article

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Molecular and docking studies of tetramethoxy hydroxyflavone compound from *Artemisia absinthium* against carcinogens found in cigarette smoke

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Abstract: *Artemisia absinthium* (AA) is an indigenous medicine used for treatment of inflammation of the liver and chronic fever, and is studied as an antimalarial and anticancer agent. The focus of the current investigation was to determine the action and effect of AA on microRNAs (miRNAs) from breast cancer cell lines. Molecular docking is a structure-based drug design process that studies the interaction of small molecule ligands with receptor biomacromolecules to predict binding mechanism and affinity. MiRNA expression profiling was done using microarray technology. Validation of transcripts with regulated expression pattern was done by SYBR-based quantitative real time PCR (qRT-PCR). AutoDock 4.2 programming allots polar hydrogens, bound together total Kollman charges, solvation borders, and fragmental volumes to the protein using auto dock devices in docking research (ADT). As confirmed by SYBR-based RT-PCR, our investigation discovered an upregulation of the miRNA-22 articulation and a downregulation of miRNA-199a*. These findings support and demonstrate the role of AA as a miRNA articulation-influencing factor in human breast cancer progression. AA's tetramethoxy hydroxyflavone (p7F) molecule was found to be effective in the treatment of cancer.

Changes in miRNA expression patterns could be a key pathogenic component in AA's physiological action on cancer cells.

Keywords: *Artemisia absinthium*, docking studies, miRNA, nicotine, smoking

1 Introduction

Artemisia absinthium (AA) is an indigenous medicine locally known as “Tethwen” from the Kashmir Valley, India. Chemical analysis of the extracts of AA has detected it to be rich in antihelmintic oil known as thujone [1]. The active compounds include artemisinin and artemisinic acid and both are mainly used as antimalarial agents. Artemisinic acid has also been shown to exhibit anti-pyretic, anti-tumor, antibacterial as well as anti-adipogenesis effect [2]. The whole extract studies on AA and breast cancer cell lines have detected the active constituents including the oil alpha-thujone and also the artemisinin and artemisinic acid from different parts to harbor the least IC₅₀ values [3]. Increasing the number of research is being focused toward detecting biomedicine and plant-derived active ingredients to be used for cancer treatment to overcome the side effects of chemotherapy. In this regard, AA has been widely studied for its anti-proliferative properties possibly through modification of the BCL-2 family and MEK/ERK signaling pathway [4]. *Artemisia* has been used in Korean herbal medicine for centuries to clear damp heat and treat uteritis and jaundice. Flavonoids extracted from *Artemisia* have anti-inflammatory properties. AA extracts have anticancer efficacy, as they inhibit the proliferation of hepatocellular carcinoma cells [5]. A previous research study extracted tetramethoxy hydroxyflavone (p7F) from AA, and the study revealed that the molecule p7F could be clinically helpful in the treatment

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of inflammatory disorders since it has antioxidant and anti-inflammatory properties [6].

Environmental, genetic, and epigenetic factors make cancer a complex disease and genomic instability has been documented to be a classic hallmark of this condition [7]. The major risk factors studied for cancer including breast cancer is a defective DNA repair mechanism, which includes roughly 5 different pathways and 200 repair genes [8]. Epigenetic analysis has proven efficient in identifying underlying disease mechanisms and microRNAs (miRNAs) have been extensively studied in this regard. MiRNAs are pleiotropic agents and their modified expression pattern in peripheral blood has been connected with various cancer types [7]. MiRNAs are known to be endogenous small RNAs and they regulate gene expression and thus have certain roles in modification of cellular and signaling pathways leading to inducing cancer as well as have role in cancer therapeutics [9]. Though the regulatory mechanism of miRNAs is complicated, computational studies have identified these to control over one-third of total human protein-coding genes [10]. Since their discovery in the year 1993, an approximate of 706 miRNAs has known to be recognized in humans and the miRNA database has a collection of over 5,000 miRNAs from various organisms [11].

Chemically, miRNAs are short non-coding RNAs between 19 and 24 nucleotides in long verily known for their role in cancer and other diseases. Through their interaction with the 3'-untranslated region (3'-UTRs) of protein coding transcripts, they control expression of genes during the post transcriptional level [12]. A single human miRNA is documented to have several hundred targets and over 60% of the protein-coding genetic regions have been predicted to harbor miRNA-interaction sites within their 3'-UTR [13]. Many publications have identified different roles of miRNAs including that in cancer initiation and metastasis, tumor suppression, and oncogenesis, especially the miR-17-92 family that has a key role in progression of carcinogenesis [14]. In breast cancer, the miRNAs have been classified as oncomirs, oncosuppressors, and metastamiRs. The oncomirs have been documented to disturb the expression of oncosuppressors, while the metastamiRs have a role in apoptosis, angiogenesis, and epithelial-mesenchymal transition [4]. Today, miRNA expression profiling can be done by over 30 methods including microarray, bead-based flow cytometry, quantitative real-time PCR (qRT-PCR), and the next-generation sequencing [15]. Of all the technologies, the qRT-PCR has been documented to harbor high sensitivity, specificity as well as throughput, and their utility can also be extended to validate data from assays run on other detection platforms [16]. In case of studies in

breast cancer, the miRNAs including miR145, miR125b, and miR10b have been found to be downregulated, while miR21 and miR155 have been detected to be consistently upregulated [17,18].

The goal of this study was to see if methanolic extracts of AA could modulate the expression of miRNAs in human breast cancer cell lines, as well as to perform computational analysis of the p7F compound from AA against carcinogens found in cigarette smoke.

2 Materials and methods

2.1 Preparation of methanolic extract of AA

Ethnopharmacology was considered as a basis to select the plant material and half a kilogram of the same was sourced regionally. The aerial parts were subjected to air-drying in shade, powdered in a milling machine, and subjected to extraction using methanol by a previously described protocol [19]. In brief, the process involved soaking the raw plant material in methanol, the volume of which was ten times the weight of the plant material. This was followed by extraction, which was done for a total of three times, and each extraction phase lasting for a period of 24 h. In order to obtain the final powder/paste, the resultant methanolic filtrate of AA was evaporated under reduced pressure which was further dried in a rotary evaporator. This extraction process yielded 29.15 g residue from approximately 500 g raw plant material. The final residue was dissolved in dimethyl sulfoxide (DMSO) for further assay.

2.2 Preparation of the extract

The final concentration of 1 mg/mL stock of the methanolic AA extract was prepared in DMSO and further sterilized using autoclaving for a period of 15 min. Further dilution of stock material for test compounds was done using Dulbecco's modified Eagle's medium (DMEM).

2.3 Maintenance of cell lines

MCF-7 and MDA-MB-231 breast cancer cell lines were obtained from the National Centre for Cell Science. After receiving the cell line, it was maintained in DMEM (90%) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Further culturing of the cells from the

cell line was done till an adherent monolayer of confluence around 70–80% was generated. Incubator at 5% CO₂ and a temperature of 37°C were used for maintenance of the cell culture. The harvesting process involved use of trypsin and all the chemicals used for the procedure were of research grade.

2.4 Extraction of miRNA

Total RNA, which contained miRNA, was isolated from both human breast cancer cell lines using a commercial RNA extraction kit (Qiagen, Germany) according to the manufacturer's guidelines in order to collect miRNA for this investigation. Quantification of the extracted total RNA involved spectrophotometer using NanoDrop ND-1000 (Thermo Scientific, USA). The full spectrum of detection of the spectrophotometer involves range between 220 and 750 nm and has a large dynamic range of concentration measurement. Electrophoresis involving 2% agarose gel was utilized to assess quality of the extracted RNA.

2.5 Microarray analysis

Agilent Human miRNA microarray v.2 platform (Agilent Technologies, USA) was used to study the expression profiling of the extracted miRNAs. This particular microarray was found to be best suited for our study as it included features from the Sanger miRBASE database (Release 14.1). Technically, the microarray included *in situ* synthesized 60-mer DNA probes with 15,000 features, which represent 923 human miRNAs. Further labeling and hybridization of RNA were done as per manufacturer instructions. The raw microarray data was generated using the Agilent Feature Extraction software (Agilent Technologies, USA).

2.6 cDNA synthesis and quantification of miRNA

The protocol for quantification of the extraction of miRNA using the YBR-based qRT-PCR system wherein a total of 1 µg of the extracted and quality checked. cDNA was synthesized using miScript Reverse Transcription kit (Qiagen, Germany). ABI PRISM 7500 Fast RT-PCR system (ABI, USA) with the miScript SYBR Green PCR kit (Qiagen, Germany) was used. The final reaction volume of 20 µL included 0.5 mM of each primer, 2 µL of the

cDNA, and 1× QuantiTect SYBR Green PCR Master Mix (Qiagen, Germany). The PCR protocol involved denaturing at 95°C for 15 min, followed by 40 amplification cycles of 94°C for 15 s, 55°C for 30 s, and 70°C for 30 s. The melt curve analysis was done and validation was done using a 2% agarose gel. The miRNA expression levels were normalized to RNU1A (internal control).

2.7 Docking studies using AutoDock 4.2

The structure of p7F compound has been obtained from website (<https://pubchem.ncbi.nlm.nih.gov/compound>) with PubChem CID13942543 and used for docking against three carcinogens found in cigarette smoke of tobacco (Hecht, S.S., 2003) such as tobacco specific nitrosamines as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (PubChem CID 47289), *N'*-nitrososornicotine (NNN) (PubChem CID 12613538), and Nicotine (PubChem CID 8959) as shown in Figures 1–3. For predicting the binding affinities of a variety of ligands, molecular docking techniques are commonly utilized. AutoDock 4.2 software assigns polar hydrogens, unified atom Kollman charges, solvation parameters, and fragmental volumes to the

Figure 1: Shows the 2D and 3D structures of Nicotine.

Figure 2: Shows the 2D and 3D structures of (NNK).

Figure 3: Shows the 2D and 3D structures of NNN.

protein using auto dock tools. AutoDock stored the prepared file in PDBQT format. A grid map was created using AutoGrid and a grid box. The grid center was set to -1.095 , -1.554 , and 3.894 in x , y , z dimensions, with a grid size of $60 \times 60 \times 60$ xyz points and a grid spacing of 0.375 Å. A scoring grid is built utilizing the ligand structure to reduce the calculation time (Figure 4).

2.8 Statistical analysis

The GeneSpring GX 11 software (Agilent Technologies, USA) was used for miRNA microarray data analysis. Data normalization was applied to the entire set followed by quantile normalization and followed by log 2 transformation. To ensure retention of only relevant miRNAs which were expressed in a minimum of one sample, gene expression filters were used and ANOVA statistical test was applied. Cluster analysis of differentially expressed genes was performed by the Manhattan correlation as a measure of similarity. Determination of the cycle threshold (Ct) value for the genes was done by the SDS software v1.2 (Applied Biosystems, USA) and the expression level of miRNA was normalized by calculating the ΔCt value. The relative gene expression was calculated as $2^{-\Delta Ct}$. Student's t -test was used to measure statistical significance, and $P < 0.05$ was considered significant.

Figure 4: Shows the 2D and 3D structures of tetramethoxy hydroxyflavone.

Figure 5: Effects of AA treatment on miRNA expression in MCF-7 cell lines. Microarray profile of miRNA expression in MCF-7 cells treated with AA for 48 h. The relative increase or decrease in the 29 miRNAs that showed a significant change in levels of expression after AA treatment compared with untreated cells.

3 Results

3.1 MiRNA expression profile post AA treatment

The microarray analysis of miRNA expression post treatment with methanolic extract from AA was done with extracted total RNAs containing the enriched-miRNA from both the human cancer cell lines exposed with respective IC_{50} concentrations of AA. Effect of AA on miRNA expression of cell lines MCF-7 and MDA-MB-231 has been highlighted in Figures 5 and 6. Post incubation for 48 h, a total of 11 miRNAs were found to be upregulated and a total of 18 were detected to be downregulated in MCF-7 cell line as highlighted in Figure 5. MiRNA-22 was found to be 65.5% upregulated, while miRNA199a* was found to be 54.2% downregulated. The expression of 5 miRNAs was found to be highly upregulated in the MDA-MB-231 cell

Figure 6: Effects of AA treatments on miRNA expression in MDA-MB-231 cell line. Microarray profile of miRNA expression in MDA-MB-231 cells treated with AA for 48 h. The relative increase or decrease in

Table 1: Docking results of tetramethoxy hydroxyflavone with three carcinogens from cigarette smoke

S. No	Ligand	Receptor	RMSD ^b (Å)	Binding energy (kcal/mol)	Inhibition constant (K_i) (mM)
1.	Tetramethoxy hydroxyflavone	Nicotine	4.391	−1.52	76.50
		NNK	2.148	+5.02	NA
		NNA	4.634	−2.01	33.89

Nicotine – L-nicotine, NNK – 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, NNN – *N'*-nitrosonornicotine.

line, while the expression of ten miRNAs was found to be significantly downregulated, as shown in Figure 6. MiRNA-22 was found to be 68% upregulated, while miRNA-199a* was found to be 71% downregulated. In both the cell lines, the number of miRNA expressions downregulated was higher than the number of upregulated miRNA expressions.

3.2 RT-PCR analysis of differential expression

The upregulated miRNA-22 and the downregulated miRNA-199a* from both the studied cell lines were subjected to the qRT-PCR assay and highlighted in Figure 7a. The miRNA-enriched total RNA from the preparation used for microarray analysis was subjected to reverse transcription and amplification for SYBR detection. As highlighted in Figure 7b, miRNA-22 was upregulated with AA treatment by 60.3 and 68.6% in both cell lines, MCF-7 and MDA-MB-231, respectively. As highlighted in Figure 7c, the miRNA-199a* was downregulated post AA treatment

in both the cell lines, MCF-7 and MDA-MB-231, by 25.1 and 36.4%, respectively. The qRT-PCR results were comparable and confirmed by the microarray data.

3.3 Docking studies

In our present work, we have determined the docking or binding-free energy, RMSD, and inhibition constant as represented in Table 1 and Figure 8, in which the binding energy of p7F with NNN was found to be highest at −2.01 kcal/mol, whereas p7F with nicotine docking results showed binding energy −1.52 kcal/mol with inhibition constant 76.50 mM, and the least binding energy was found with NNK which was +5.02 kcal/mol.

4 Discussion

For thousands of years, natural plants have been utilized to prevent and treat numerous diseases. In recent days,

Figure 7: Comparison of microarray analysis with RT-PCR on the expressions of miRNA-22 and miRNA-199a*. MCF-7 and MDA-MB-231 cells were exposed to AA for 48 h. (a) Microarray analysis; the fold changes of AA-regulated miRNA-22 and miRNA-199a* compared with untreated controls. (b) RT-PCR confirmation of miRNA-22 expression compared with normal control values (mean fold change $\pm$ SE ($n = 3$), $P < 0.05$). (c) RT-PCR confirmation of miRNA-199a* expression compared with normal control values (mean fold change $\pm$ SE ($n = 3$), $P < 0.05$).

Figure 8: Docking results of three compounds (Nicotine, NNK, and NNA) with the ligand showing RMSD and binding affinity.

when numerous diseases are looking for therapy, different herbs are viewed as good sources of bioactive components with health-promoting properties [20]. Tobacco leaves and the smoke produced when they are smoked contain around 4,000 compounds, the most well-known of which is nicotine, which was isolated from tobacco leaves for the first time in 1828 by Posselt and Reimann [21]. Nicotine is the chemical that causes smokers to get addicted to tobacco, and it is lethal even in small doses. Nicotine is rapidly absorbed by each organ of the body when tobacco smoke is inhaled. Nicotine raises blood pressure and heart rate, possibly contributing to the high occurrence of apoplexy and atheroma in smokers. Nicotine substitution therapy is frequently used to help people quit smoking because it protects them from the numerous other harmful substances found in tobacco smoke, such as cancer-causing polycyclic aromatic hydrocarbons and *N*-nitroso compounds; aggravating substances such as acrolein, benzene, formaldehyde, alkali, $(\text{CH}_3)_2\text{CO}$, acidic corrosive, etc. One of the studies has reported that ethanolic extract of leaves from AA on human cerebral cortical cells proved to displace (*N*)-nicotine and (*n*)-scopolamine from nicotinic and muscarinic receptors [22]. Reports from many studies have presented work on the cytotoxicity of various parts of AA on breast cancer cell lines and have detected the whole extract to exhibit good cytotoxic activity against breast cancer [2,23]. In addition, to the role of miRNA in breast-cancer, studies have detected these to be involved in disease propagation. Same as in another comparison analysis involving 76 breast cancer samples and 10 normal samples, the study detected significant deregulated miRNAs to be miR-21, miR-145, miR-125b, and miR-155 [24]. The study, we believe, is a pilot attempt wherein we report the global expression profile of miRNAs in human MDA-MB-231 and MCF-7 breast cancer cells, and the effect

of AA extract on the miRNA expression profile after 48 h of treatment with the same.

Our microarray analysis with the 923 known human miRNAs, a total of 29 and 15 miRNAs were detected to exhibit differential expression post AA treatment in breast cancer cell lines of MCF-7 and MDA-MB-231. The miRNAs detected to be deregulated in our study have not been reported in any previous reports though the fundamental of differentially expressed miRNAs to exhibit altered response to chemical treatment in human and animal cell line models have been shown. The microarray assay in our study revealed that extract of AA upregulated the expression of miRNA-22, while downregulated the expression of miRNA-199a*. These results were confirmed by RT-PCR-based expression patterns of miRNA-22 and miRNA-199a*.

The organic compound p7F is considered as ligand and Nicotine, NNN, and NNK as receptor molecules and docking has been performed using AutoDock 4.2 version [25,26]. p7F, isolated from AA, has been found to be effective in the treatment of inflammatory illnesses and has antioxidant and anti-inflammatory properties. Based on our docking results, it is possible to predict that p7F has anticancer activity against carcinogenic compounds found in cigarette smoke.

Our findings, along with those of other similar studies, imply that the range of miRNAs that respond to pharmacological therapy varies by pharmaceutical class. Investigating the molecular regulatory mechanisms of common and drug-specific miRNAs will help us better understand the therapeutic efficacy of various medications and, as a result, bring fresh insight into drug screening for cancer treatment.

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Ethical approval: The conducted research is not related to either human or animal use.

Data availability statement: Data set are available and will be shared on request to the corresponding email.

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