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Isolation and Characterization of a Triterpenoid Compound from Sodhita *Semecarpus anacardium* L. Drupe Ethanolic Extract and its Anticancer Potential *In Vitro*

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Abstract

Semecarpus anacardium is listed in Schedule E1 of the Drugs and Cosmetics Act, of 1940. It is purified (sodhana) and used in traditional medicine to treat various diseases, including gulma (hard mass/cystic growth or lump). Studies on *Semecarpus anacardium* in Non-Small Cell Lung Cancer (NSCLC) cell lines are hitherto unavailable, hence this study was conducted. The drupes of sodhita *Semecarpus anacardium* were extracted with solvents of different polarity, and the half-maximal inhibitory concentration (IC_{50}) of each extract was determined in the NSCLC cell line (A549). The ethanolic extract with an IC_{50} of 47.09±0.032 µg/ml was found to be better than the other extracts and, therefore subjected to compound isolation by wet-pack column chromatography. The fractions (6-9 and 15-19) with a single spot identified by thin-layer chromatography were crystallized (56 mg) and used for spectral techniques. The probable compound structure of $C_{30}H_{50}O_2$, elucidated by FTIR, ^1H and ^{13}C NMR revealed it as a triterpenoid derivative. The IC_{50} of the isolated compound was 15.73±0.019 µg/ml which was further used to investigate anti-cancer properties The treated cells showed apoptotic features like cell blebbing, loss of cell adhesion and membrane integrity. A semi-fragmented piece of unresolved DNA and G2/M phase arrest was observed in the treated cells. RT-qPCR analysis revealed that the expression of the NSCLC biomarkers c-ROS oncogene-1 (ROS1) and proinflammatory factor nuclear factor kappa B (NF-κB) was significantly ($p<0.05$) downregulated in the treated cells. Based on these observations, it was concluded that the isolated compound could be a potential candidate for lung cancer therapy.

Keywords: A549, Cytotoxicity, NSCLC, *Semecarpus anacardium,* Triterpenoid

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1. Introduction

Cancer is an uncontrolled division of mutated cells that causes organ dysfunction. Lung cancer is the second most common type of cancer in both men and women while prostate and breast cancer, respectively are the first common cancer types. The risk of developing lung cancer increases with age. Women are less likely to develop lung cancer than men. About 15% of all lung cancer cases are Small Cell Lung Carcinoma (SCLC) and about 85% are Non-Small Cell Lung Carcinoma (NSCLC)[1]. The survival rate for people with localized NSCLC is higher than for people with metastatic NSCLC. Many drugs used to treat NSCLC develop resistance within a few years of use[2] which increases interest in developing new drugs. Medicinal plants are natural and widely available resources from which a variety of phytochemicals can be extracted.

Following this concept, *Semecarpus anacardium* was identified by applying Ayurvedic wisdom and a literature search to investigate its efficacy on NSCLC. Extensive anticancer studies have been reported for this plant, such as leukemia[3], hepatocellular carcinoma[4], breast cancer[5] and epidermoid laryngeal carcinoma[6], however, to our knowledge no study was reported for nonsmall cell lung carcinoma.

Semecarpus anacardium L. is commonly known as Bhallataka. It is widely distributed in the outer Himalayas and the southeastern coastal regions of the Indian subcontinent. It has been reported that this plant has been used in formulations (vishishta yoga) like Prathama Bhallataka ghrita, and Dwithiya Bhallataka ghrita for the treatment of gulma (hard mass/cystic growth or lumps) based on classical references[7]. Apart from its use as a single drug, Bhallataka is used in various polyherbal formulations compiled in classical texts like Astanga sangraha, Astanga hridaya, Gadani graha etc. for various treatments including arbudha (malignant) and granthi (benign tumor)[8,9]. The literature search revealed that the nut part (drupe) of *Semecarpus anacardium* is known for its antioxidant[10], anti-inflammatory[11], antimicrobial[12], anticancer[13], hypoglycemic and hair growth-promoting properties[14]. The major compounds identified from this nut are phenolic derivatives, bioflavonoids and bhilwanols which were reported to have a wide range of therapeutic benefits[15]. *Semecarpus anacardium* plant is listed in Schedule E1 of the Drugs and Cosmetics Act, of 1940. The traditional practice of medicine therefore recommends purification or detoxification to reduce adverse effects and improve therapeutic benefits[16]. Hence, the present study was carried out to isolate the compound and to study its effects on non-small cell lung cancer.

2. Materials and Methods

2.1 Collection and Identification of Plant Material

The drupes of *Semecarpus anacardium* were procured from the local market in Madurai. It was authenticated in the Department of Botany CSMCARI (CCRAS) M/O Health and Family Welfare, Department of Ayush, Government of India, Arumbakkam, Chennai. The specimen was deposited in the Department of Pharmacology with voucher specimen number *Semecarpus anacardium* L 00001.

2.2 Sodhana Process

The collected drupes were subjected to purification, as described in the classical text[17]. The whole drupes were boiled in cow dung for three hours then washed with water and dried under sunlight. This process was repeated seven times. These drupes were further boiled with tamarind leaves juice, milk, aloe vera juice and tender coconut water respectively and washed with water and dried under sunlight and stored.

2.3 Preparation of Extracts

Sodhita *Semecarpus anacardium* drupes were extracted using the soxhlet apparatus (make- Ausco) with solvents (Rankem) such as hexane followed by chloroform, ethyl acetate, ethanol and water until the colour of the solvent became pale yellow. Each extract was air-dried and analyzed for its half-maximal inhibitory concentration in the non-small cell lung carcinoma cell line (A549).

2.4 Cell Culture

Non-small cell lung adenocarcinoma cells (A549) were purchased from NCCS, Pune. Cells were cultured in RPMI-1640 medium containing 10% FBS, penicillin (100 U/mL) and streptomycin (100 µg/mL). Cells were maintained in a 5% CO_2 incubator (make: RS Biotech (Galaxy B)) at 37 °C. The cells were sub-cultured and used for further studies.

2.5 Cell Proliferation Assay

The MTT assay was performed to measure cellular metabolic activity as an indicator of cell viability, proliferation, and cytotoxicity. Each extract made from low polar to high polar viz., nhexane, ethyl acetate, chloroform, ethanol, and water was dissolved in Dimethyl Sulfoxide (DMSO) and further diluted with media to meet the final concentration of DMSO not exceeding 1%. Each extract was tested in triplicate at linear concentrations of 3.125, 6.25, 12.5, 25, 50 and 100 µg/ml in respective wells seeded with about 10000 cells. Three wells were kept for solvent control (DMSO) and three for negative control (untreated cells). The plate was incubated and the cell viability was measured in the ELISA reader at 570 nm[18].

2.6 Compound Isolation from Ethanolic Extract

From about 1 kg of plant material used for wet column chromatography, a maximum yield of 8 g of potent cytotoxic ethanolic extract was obtained. The ethanolic extract was dissolved in methanol and adsorbed in silica gel (60-120 mesh). The adsorbed extract was loaded into a silica gel column (100-200 mesh) previously packed with hexane solvent. The column was eluted with hexane, followed by gradually increasing polarity with ethyl acetate (90:10; 80:20; 70:30; 60:40; 50:50; 40:60; 30:70; 20:80; 10:90) and finally with 100% ethyl acetate. The column was further eluted with ethyl acetate: Chloroform (90:10; 80:20; 70:30; 60:40; 50:50; 30:70; 20:80), and finally with 100% chloroform. The column was further eluted with chloroform: Methanol (98:2; 96:4; 94:6; 92:8; 90:10; 88:12; 85:15; 80:20). The fractions that showed a single spot on a commercially available pre-coated TLC plate (Merck, silica gel, PF254) were pooled. The resulting crude solid was further treated with activated charcoal in hot ethanol to remove the green hue caused by chlorophyll and filtered with Whatman filter paper under vacuum, and the filtrate was kept for crystallization. The resulting solid was used for spectral analysis.

2.7 Analysis of Compound

The melting point was determined in open glass capillaries using a melting point device (VMP, Veego, India). Fourier-Transform Infrared Spectroscopy (FTIR) spectra were determined using the KBr disc method on a Perkin Elmer FTIR spectrophotometer 1600. Mass spectra were obtained by High-Resolution Mass Spectrometry analysis (HRMS). ^{1}H-NMR and ^{13}C-NMR spectra were recorded using a JEOL spectrometer at 500 MHz and DMSO-d6 as solvent. Spectral techniques were performed by IIT-Bombay.

2.8 IC50 of Isolated Fraction

Linear concentrations of an isolated compound (5, 10, 20, 40 and 80 µg/mL) were tested in triplicate. Each well was seeded with about 10,000 cells and three wells were maintained as a solvent control (DMSO). The plate was incubated and the percentage of cell viability was determined using an ELISA reader at the wavelength of 570 nm[18].

2.9 Reverse Transcriptase PCR (RT-qPCR)

TRIsoln (GeNei labs, India) was used to extract RNA from cells treated with an IC_{50} concentration of an isolated compound. According to the instructions for use, the collected RNA was transcribed into complementary DNA (cDNA) treated with 5X buffer (diluted to 1X), dNTPs, and hexamer primer and stored in a thermocycler at 70°C for 5 minutes. A mixture of reverse transcriptase, DTT, and RNase-free water was then added and spun down before loading into the PCR. The instrument (CFX RTPCR, Bio-Rad) was programmed to 25°C for 5 minutes, 42°C for 45 minutes, and 85°C for 5 minutes for hexamer binding, cDNA synthesis, and denaturation of unconverted RNA. The resulting cDNA was used for real-time PCR analysis.

2.10 Real-Time PCR (qPCR)

The cDNA was used to determine the expression level of the targeted genes together with the housekeeping gene primer actin. The cDNA was treated with the SYBR green RT mix, which consists of a forward primer, a reverse primer (Origene, India, Cat No#: HP206553 (ROS1), HP207409 (NF-kB)) (Table 1) and PCR water. The plate was placed in real-time PCR and programmed for pre-denaturation at 95°C for 1 minute, denaturation at 95°C for

Table 1. Primer sequences

S. No	Primer name	Primer sequence
1.	NF-κB	FS-GCAGCACTACTTCTTGACCACC RS- TCTGCTCCTGAGCATTGACGTC
2.	ROS1	FS- CCAGGACCATATGCTGACGTTG RS- ACGGCACTGATAACTCTTTCTGC

15 seconds, 40 cycles of 95°C for 15 seconds, annealing at 60°C for 15 seconds and extension at 72°C for 45 seconds. The data was recorded during the extension phase using CFX Maestro V4.1 as per vendor protocol.

3. Statistical Analysis

The data were expressed as mean ± SD. Results were statistically analyzed by one-way ANOVA using GraphPad prism V6.01. The difference was considered significant if $p<0.05$.

4. Results

The percentage yield on the extraction of sodhita *Semecarpus anacardium* drupe was found to be 56% for n-hexane, 0.5% for Ethyl acetate, 0.6% for chloroform, 0.8% for ethanolic extract, and 2.3% for aqueous extract. All the extracts were evaluated for their half-maximal inhibitory concentration on NSCLC (A549) cell proliferation. The IC_{50} of ethanol extract was found to be 47.09±0.032 µg/ml and other extracts showed inhibition at the concentration of more than 100 µg/mL. A further evaluation of the ethanol extract was therefore considered.

Fractions of about 30ml each were collected from ethanolic crude extract by wet column chromatography at different gradient compositions of solvents. A total of 98 fractions were collected and each was monitored by TLC (Figure 1). Fractions with a single spot (6-9 and 1519) were combined, and the solvent was evaporated under reduced pressure. The obtained yield was weighed about 56 mg (0.7%) and colourless crystals. The R_f value of the isolated compound was found to be 0.38 in the mobile phase ratio of hexane and ethyl acetate (90:10) on TLC and the melting range was 258-260˚C.

The molecular weight of the isolated compound was determined by high-resolution mass spectrometry and the molecular ion peak appearing at m/z 443.4 (M+1) corresponded to the molecular formula $C_{30}H_{50}O_2$ (Figure 2). Figure 3 shows the spectral data of isolated compound FTIR (KBr cm^{-1}), λmax: 3401 cm^{-1} (-OH group); 2941 cm^{-1} and 2868 (-CH stretch), 1642 cm^{-1} C=C; 1454 cm^{-1} (CH2); 1029 (C-O)[19].

From ^{1}H-NMR (500 MHz, DMSO-d6): δ ppm: 0.6 (s,3H, H28) 0.7 (s, 3H, H29) 0.9-1.0 (m, 19H, H32, H24,25,26,27, H20, H9), 1.3-1.2 (m, 2H, H15) 1.3-1.4 (m,

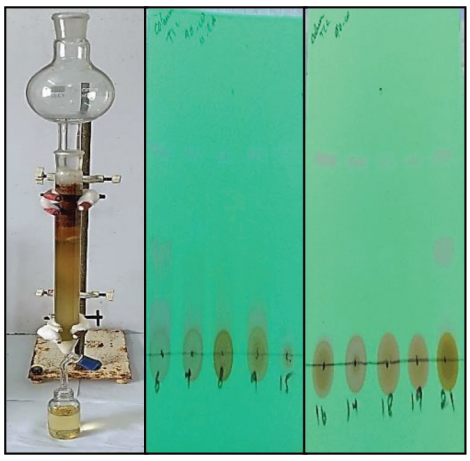


Figure 1. Column Chromatography and TLC plate with a single spot.

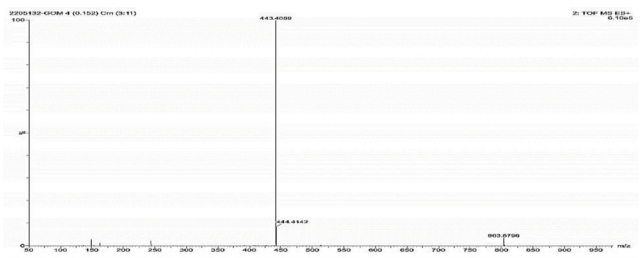


Figure 2. LCMS data of isolated compound.

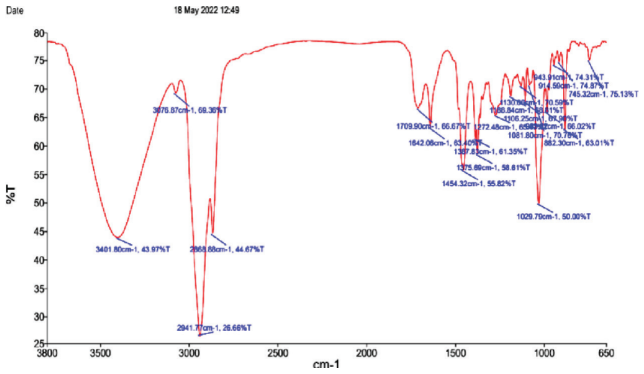


Figure 3. FTIR spectrum of Isolated compound.

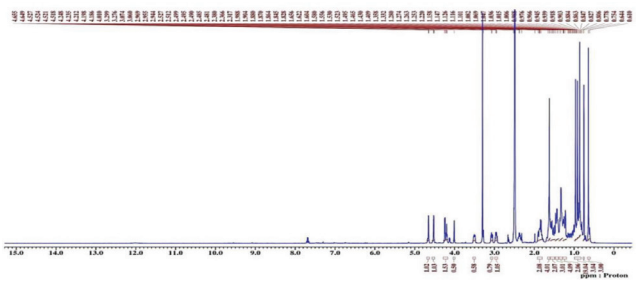


Figure 4. ^{1}HNMR spectrum of Isolated compound.

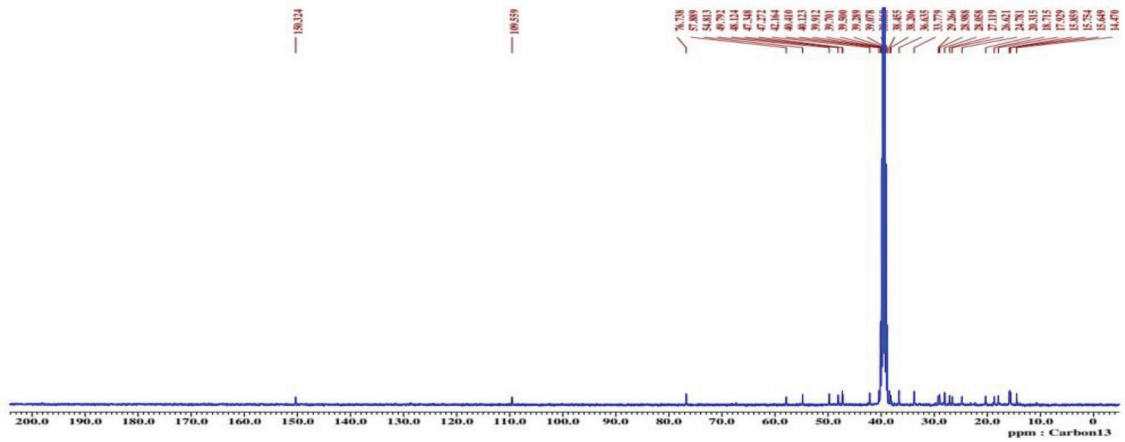


Figure 5. ^{1}CNMR spectrum of Isolated compound.

4H, H21, H7, H3) 1.4-1.5 (m, 2H, H4, H19), 1.6-1.7 (m, 4H, H14, H22), 1.8-1.9 (m, 2H, H12) 2.9-3.5 (m,2H, H18, H16, H13), 4.0 (s, 1H H23, H30) 4.3-4.2 (m, 2H, H11, H1) 4.6-4.5 (d, 2H, H31) (Figure 4). From ^{13}C NMR(500 MHz, DMSO-d6): δ ppm: 150.3 (C17), 109.5 (C31), 76.7 (C1), 57.8 (C11), 54.8 (C10), 49.7 (C2), 48.1 (C5), 47.3 (C6), 47.2 (C8), 42.2 (C18), 40.4 (C3), 39.0 (C7), 38.8 (C4), 38.4 (C9), 38.2 (C16), 36.6 (C14), 33.7 (C12), 29.2 (C15), 28.9 (C21), 28.0 (C13), 27.1 (C20), 26.6 (C22), 24.7 (C19), 20.3 (C32), 18.7 (C28), 17.9 (C24), 15.8 (C29), 15.7 (C27), 15.6 (C25) and 14.4 (C26) (Figure 5)[20].

Based on the spectral data, the isolated compound was confirmed to be a triterpenoid[21-23] and the probable structure is shown in Figure 6. The IC_{50} of the isolated compound was measured to be 15.73±0.019 µg/ml, which is higher than the IC50 of the ethanolic extract of 47.09±0.032 µg/ml.

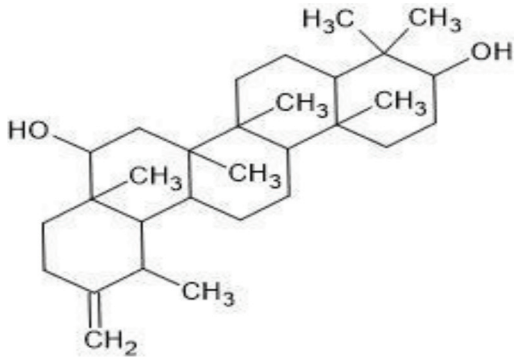


Figure 6. Probable structure of isolated compound.

The same concentration of the isolated compound was further used to investigate the mechanistic screening of anti-cancer properties. The results were compared with the control (untreated) and the cells treated with the reference

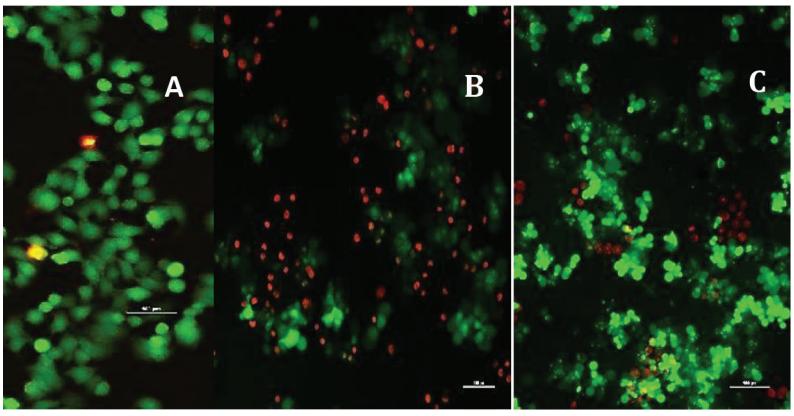


Figure 7. **(A)** control cell. **(B)** Cisplatin treated cells. **(C)** Test compound treated cells (Green colour indicates live cells; orange colour indicates dead cells).

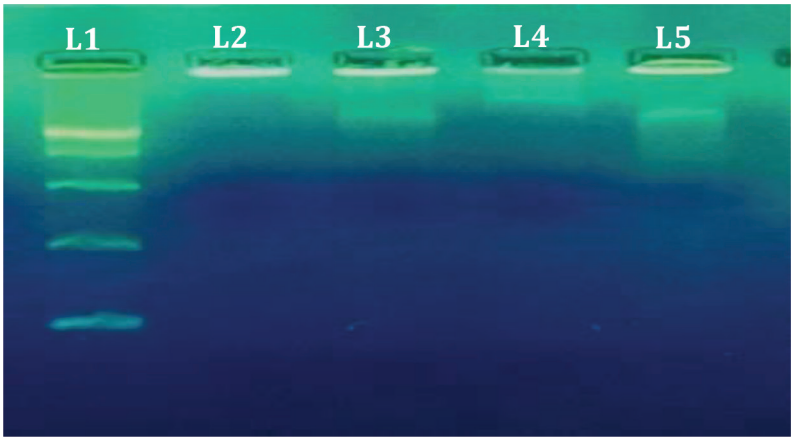


Figure 8. DNA Fragmentation **Lane 1.** DNA ladder, **Lane 2.** Control, **Lane 3.** Cisplatin, **Lane 4.** 7 µg/ml and **Lane 5.** 15 µg/ml of the isolated fraction compound.

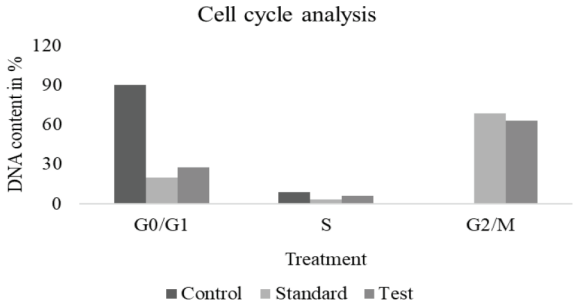


Figure 9. Control, cisplatin (Standard), test compound treated cell.

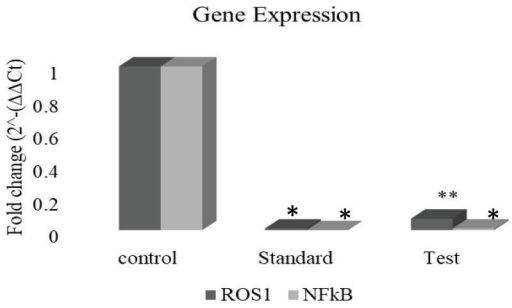


Figure 10. Relative expression of targeted gene ROS1 and NFkB with significant *p-value<0.05 and **p-value<0.01.

compound cisplatin (IC_{50}: 16.48 µg/ml)[24]. The treated cells showed apoptotic features, such as cell blebbing, loss of cell adhesion and membrane integrity in acridine orange/ethidium bromide staining under a fluorescent microscope (Figure 7). In the DNA fragmentation assay, a semi-fragmented piece of unresolved DNA was observed in the treated cells (Figure 8).

The treated cells were subjected to cell cycle analysis in FACS with PI staining. DNA was quantified in the G1, S, and G2/M phases of the cell cycle. It was observed that the quantity of DNA was found higher in the G2/M phase which indicates that the treatment arrests the G2/M phase and prevents further progress of the cell cycle (Figure 9). In gene expression analysis the NSCLC biomarker c-ROS oncogene-1 (ROS1) and a major transcription factor, nuclear factor kappa B (NF-κB) were studied. The relative quantification in terms of fold change (*2^(-ΔΔCT)*) for each gene of interest was measured against the levels of expression of the reference compound. The results revealed that both the genes ROS1 and NFkB were found to be significantly downregulated ($p< 0.05$) in the treated cell (Figure 10).

5. Discussion

The incidence and mortality rate of lung cancer and the development of resistance to chemotherapy have increased in recent years necessitating more research to find alternative drugs for the treatment of NSCLC. *Semecarpus anacardium* was selected for our study as it is one of the familiar plant sources in traditional formulations. Several phytoconstituents have been isolated from this plant, which is reported to have antimicrobial, antifungal, antiarthritic, and antioxidant activities. The stem bark contains some anti-cancer compounds, such as bhilwanols, 3-O-methyl-quercetin, and kaempferol[25]. Studies have shown that nut milk extract and fatty acids have a cytotoxic effect on lung and liver cells as well as an anti-leukemic effect[26]. The methanolic extract of the nut was tested against epidermoid laryngeal carcinomas, while the ethanolic

extract was effective against cervical carcinomas. A Siddha formulation of Kalpamruta containing the ingredient *Semecarpus anacardium* Linn. is used for the treatment of breast cancer[27]. Since there are no detailed mechanistic studies on the efficacy of *Semecarpus anacardium* in NSCLC, the present study was conducted.

Based on traditional practice, the drupes of *Semecarpus anacardium* were purified according to the instructions of the classical text Anuboga Vaidhya Navaneedham and extracted in solvents of different polarities. Based on comparative cytotoxic potential, the ethanolic extract was selected for further analysis. The compounds with different proportions of solvent gradients were isolated from the ethanolic extract and each fraction was tested in TLC. The fractions that eluted a single spot with the same Rf value with the selected mobile phase were pooled and crystallized. The purity of the compound was confirmed by determining its melting range and the structure of the compound was characterized as a triterpenoid by using the spectral data from FTIR for the functional groups, NMR for the number of hydrogen (^{1}H NMR) and carbon atoms (^{13}CNMR). From the HRMS data, the molecular weight of the compound and the empirical formula were determined as $C_{30}H_{50}O_2$. This is the first report of a triterpenoid compound isolated from sodhita drupe of *Semecarpus anacardium*.

A diverse group of terpenoids occurs in nature and is classified based on carbon numbers. Most terpenoids have been reported to have anti-cancer activities via various mechanisms. Some triterpenoids such as ursolic acid, betulinic acid and lupeol[28] have been found to control cancer cell proliferation primarily through the induction of apoptosis.

The best-known genomic alterations in NSCLC are epidermal growth factor receptor (EGFR), Kirsten rat sarcoma viral oncogene homolog (KRAS), echinoderm microtubule-associated protein-like 4-anaplastic lymphoma kinase (EML4-ALK) rearrangements, phosphatidylinositol4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA), proto-oncogene B-Raf (BRAF), proto-oncogene receptor tyrosine kinase MET (MET), liver kinase B1 (LKB1) and protooncogene tyrosine-protein kinase ROS (ROS1) genes[29-32].

Triterpenoids have been found to have a positive effect on EGFR[33,34], KRAS[35,36], but there are no scientific reports on the c-ROS or the proto-oncogene tyrosine-protein kinase ROS (ROS1) gene that we studied. In addition, the expression of the gene NFkB, which regulates proinflammatory signals in many types of cancer, was also investigated.

The IC_{50} of the isolated compound was lower than that of the ethanolic parent extract, confirming that we have isolated a compound that is highly effective for NSCLC cells. The significant reduction in NFkB gene expression correlated with the apoptotic morphologic features of the cells observed in the fluorescence staining methods. The accumulation of DNA observed in the G2/M phase indicates that the isolated compound prevents further progression of the cell cycle from this phase onwards. In the fragmentation assay, a semi-fragmented piece of unresolved DNA was observed in the treated cells, suggesting that the compound causes partial apoptosis and necrosis of DNA.

In gene expression studies, a significant down-regulation of ROS1 and NFkB was observed in the treated cells, suggesting that the drug acts on the targets ROS1 and NFkB. The compound could be an alternative or supportive for the drugs that develop resistance to targets EGFR and KRAS in NSCLC. It has been reported that oil ether extracts of *Premna puberula* roots showed antitumor activity by up-regulating the expression of p21 and cyclin E2, inhibiting A549 cell proliferation through mitochondrial-mediated apoptosis, which arrests the cell cycle in the S phase[37].

6. Conclusion

The anticancer compound isolated from *Semecarpus anacardium* drupe validates the use of this plant in the treatment of non-small cell lung cancer. Furthermore, the effectiveness of the isolated compound could be useful for the development of new anticancer drugs. However, this hypothesis would be confirmed with *in-vivo* studies.

7. Acknowledgement

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