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EFFECT OF *PIPER BETLE* LEAF EXTRACT AND *SYZYGIUM AROMATICUM* FLOWER EXTRACT AND THEIR ACTIVE COMPONENT EUGENOL ON THE SURVIVAL OF LUNG ADENOCARCINOMA CELLS (A549)

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Keywords:

Piper betle, *Syzygium aromaticum*, Eugenol, Anticancer, Flow cytometry

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ABSTRACT: Medicinal plants are one of the most important resources for developing new lead therapeutic compounds used in various traditional medicines worldwide for thousands of years due to the presence of natural bioactive components, which help in preventing various human diseases. *Piper betle* Linn belongs to the Piperaceae family, commonly known as Betel and Paan. Betel leaves possess several bioactivities, and these are used in traditional medicinal systems. *Syzygium aromaticum* L. (Cloves) is a tree belonging to the Myrtaceae family. Closed flowering buds are used as a spice in food preparations all over the world. Eugenol is an active compound of both plants (*Piper betle* and *Syzygium aromaticum*). Hence, we attempted to study the effect of the methanolic extracts of *Piper betle* and *Syzygium aromaticum*, along with their active component, eugenol, using an *in-vitro* approach. The *in-vitro* cytotoxicity was assessed against lung adenocarcinoma cell lines (A549) using the MTT and SRB assays, and the type of cell death was characterized by differential staining. The results showed that the plant extracts, as well as eugenol, showed good anticancer potential *in-vitro*.

INTRODUCTION: Plants are used in traditional medicine throughout the world for thousands of years due to the presence of bioactive compounds, which help in preventing various diseases. Their low toxicity and highly curative properties have endeared them to recent cancer research [1, 2, 3]. Several synthetic agents are used to cure cancer, but they have many side effects, and hence the research is going on globally to investigate potent natural compounds, the most important being plant-derived compounds.

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Piper betle Linn. belongs to the Piperaceae family, commonly known as Betel and Paan. *P. betle* is mostly found in India, Taiwan, Sri Lanka, Malaysia, Indonesia, Thailand, and Southeast Asian countries [4, 5]. *Piper betle* leaves contain a multitude of phenolics, among which eugenol is a major phenolic [6]. Betel leaves have been shown to exhibit antioxidant, antitumor, immunomodulatory, and anti-inflammatory activities, and anti-bacterial, antiseptic, antimutagenic, antifungal, antiulcer-genic, antiplatelets, antidiabetic, anti-aphrodisiac activity, antiamoebic, antinociceptive, antifertility, and antihyperglycemic effects [7, 8, 9]. *Syzygium aromaticum* L. belongs to the Myrtaceae family. It is widely grown in India, Indonesia, Pakistan, Sri Lanka, Madagascar, and Tanzania. *S. aromaticum* is most commonly called a clove. Cloves are also very rich in plant phenolics, the major constituent being eugenol [10, 11].

S. aromaticum exhibits several pharmacological properties like antibacterial, antioxidant, antifungal, as well as anticancer [12, 13, 14, 15]. The *in-vitro* anticancer activities of three different extracts of clove against several cancer cell lines, like cervical, breast, prostate, and esophageal cancers [16]. Clove has been shown to arrest the division and induce cell death in human MCF-7 breast cancer cell line [17]. Several reports have described the pharmaco-logical effect of clove, such as antiviral, antifungal, anti-inflammatory, antioxidant, antinociceptive, antimicrobial, antimutagenic, and antineoplastic effects [18, 19, 20, 21, 22].

One such phytophenol that has been perceived to have numerous medical advantages is eugenol [23]. Though eugenol is attributed to some anticancer effects, the mechanism of action of the same is largely unknown. Eugenol is a natural phenolic compound, which is the main active component of several plants. Two such eugenol-rich plants are *P. betle* and *S. aromaticum*, which have been chosen for this study. Eugenol is attributed with several pharmacological benefits, like anti-inflammatory, antimicrobial, antioxidant, antiviral, antiulcerogenic as well as anticancer activities [24, 25, 26, 27, 28]. Several reports have described the anticancer properties of eugenol, such as pro-apoptotic, anti-proliferative, tumor-suppressive, and antioxidant effects [29, 30, 31].

The present study was formulated to analyze the anticancer effects of methanolic extracts of these plants, along with their active component, eugenol. The *in-vitro* cytotoxicity was assessed against A549 cell lines using the MTT and SRB assays, and the type of cell death was characterized by differential staining. The results showed that the plant extracts, as well as eugenol, showed good anticancer potential *in-vitro*.

MATERIALS AND METHODS:

Preparation of Betel Leaf Extract: Fresh leaves of Arthur variety of *Piper betle*a nd *Syzygium aromaticum* (clove) dry flowers were purchased from the local market at Coimbatore. The fresh leaves and cloves were washed and air-dried by spreading on tissue paper. The betle leaf sample (10 g) was coarsely cut into small pieces, weighed, and taken in 100 ml of methanol. The cloves (10 g) were ground and suspended in 100 ml of methanol. The extraction was carried out at room temperature, protected from exposure to light to safeguard the themo-labile components, for 24 h with occasional shaking.

The resultant extract was then sieved through Whatman no. 1 filter paper. After filtration, the plant extracts were evaporated at 65 ºC in a water bath for 4 h, and the residue was weighed and dissolved in DMSO. A range of concentrations like 0.1 mg to 1.0 mg in 5 µl DMSO was prepared for the analysis and the plant extracts were stored at 4°C.

PLATE 1: *PIPER BETLE*

PLATE 2: *SYZYGIUM AROMATICUM*

Cell Line and Culture Conditions: The lung adenocarcinoma cell line (A549) used in this study (Plate 3) was received from National Center for Cell Sciences (NCCS), Pune. The cell line was cultured and maintained in Ham's F12 medium (HiMedia) with 10% Fetal bovine serum (FBS) (Gibco) and 1% penicillin-streptomycin (Gibco) using a standard protocol and maintained in a CO_2 incubator. The cells once were grown to confluency and collected by trypsinization into a fresh,

complete medium (with serum and antibiotics) for the analysis of each parameter. An aliquot of the collected cells was counted in a hemocytometer and the proportion of the viable cells was determined with trypan blue. For setting up the different exposure groups, 10^5 line cells were grown overnight in a CO_2 incubator. Prior to seeding, a sterile coverslip was inserted into each well for the convenience of handling the cells. Plate 3 shows the morphology of A549 Cells.

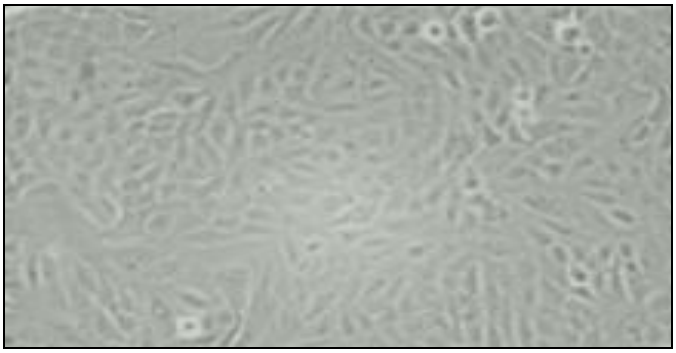

PLATE 3: HUMAN LUNG ADENOCARCINOMA (A549) CELL

Treatment Group's Setup:

- Untreated A549 cells
- Eugenol treated A549 cells
- *P. betle* extract treated A549 cells
- *S. aromaticum* extract treated A549 cells

Parameters Analyzed: The cell viability was assessed by MTT and SRB assays. The characteristic features of apoptotic events were studied by analyzing various parameters like morphological changes (Giemsa staining) and nuclear events (AO/EtBr staining).

MTT Assay: A total of 10^6 cells/ml were seeded into the 96 well plates and exposed for 24 hours plant extracts and bioactive compound eugenol. Cytotoxicity of drugs was assessed by the procedure [32]. The treated cells (100 µl) were incubated with 50 µl of MTT at 37^{o}C for 3 hours. After incubation, 200 µl of phosphate-buffered saline (PBS) was added to all the samples. The liquid was then carefully aspirated. Then added 200µl of Acid-propanol containing 0.04N HCl incubated for 30 min. The absorbance was read at 650 nm in a microtiter plate reader. The optical density of the control cells was fixed to be 100% viability and the percent viability of the cells in the other treatment groups were calculated.

SRB Assay: The treated cells were layered with 350 µl of ice-cold 40% trichloroacetic acid (TCA) incubated at 4 ºC for 1 h. The cells were then washed 5 times with 200 µl of ice-cold PBS. The buffer was removed, and 350 µl of SRB stain was added to each well and left in contact with the cells for 30 min at room temperature. The unbound dye was removed by washing 4 times with 350 µl of 1% acetic acid. Then 10 mM Tris 350µl was added to each well to solubilize the protein-bound dye, and the plate was shaken gently for 20 min. The absorbance was read at 492 nm in a microtitre plate reader. The cell survival was calculated as the percent absorbance compared to the control [33].

Giemsa Staining: The morphological changes in the cells were followed in the presence and the absence of the plant extracts and compound and/ora standard chemotherapeutic drug (etoposide 200µg). The cells were fixed and stained with Giemsa for 10 minutes of incubation. After incubation, the stained cells were visualized under the phase-contrast microscope (Nikon, Tokyo, Japan) as explained [34]. The cells were then observed for morphological changes using a phase-contrast microscope (Nikon, Tokyo, Japan) at 400X magnification. The apoptotic ratio was calculated using the formula,

Apoptotic ratio = Number of apoptotic cells / Number of normal cells

Acridine Orange/Ethidium Bromide Staining: Apoptotic cells were detected with AO/EtBr staining as described [35]. The combination of AO/EtBr staining technique is used to differentiate apoptotic and normal cells. To the treated cells, 10 µl of AO/EtBr was added and spread by placing a coverslip over it. The stained slides were incubated at room temperature for 5 min. The apoptotic cells with condensed chromatin and fragmented nuclei were identified by their red fluorescence, and the normal cells were visualized by their green fluorescence, which was counted by using an upright fluorescent microscope using B2A filter at 400X magnification.

Flow Cytometry: Cells (1×10^6 cells/ml) treated with plant extracts (50 µg of *Piper betle* and 50 µg of *Syzygium aromaticum*) / eugenol (100 µM), as explained above, were collected and washed with PBS. After 24 h incubation, the A549 treated cells

were harvested. Then the cells were incubated with 1ml of the PI reagent and allowed to stain in the dark for 30 minutes. At the end of the incubation period, the cells were subjected and analyzed for the populations of sub-G0, G0/G1, S, and G2/M phases of the cell cycle by flow cytometry (BD FACSVerse, USA).

RESULTS:

MTT Assay: The results of MTT assay revealed that there was a concentration-dependent decrease in the viability of A549 lung adenocarcinoma cells upon exposure to eugenol and eugenol-rich plant extracts (*P. betle* and *S. aromaticum*). Thus, our results showed that 4 different concentrations, namely 10 µM, 25 µM, 50 µM and 100 µM in eugenol or 10 µg, 25 µg, 50 µg and 100 µg of extracts which is depicted in **Fig. 1**. Each dose range was used, based on literature reports, as well as our previous studies, in order to optimize the dose to be used. The MTT assay was used to determine the cell viability for this purpose. The IC_{50} value for both plant extracts was found to be 50 µg, and that of eugenol was found to be 100 µM, which is depicted in **Fig. 2**.

Hence further studies were carried out with these concentrations. After fixing the dose, the cytotoxicity of the test samples was again tested at the selected doses in comparison with the standard chemotherapeutic agent, Etoposide, at the dose of 200µM. All the test samples were comparable to Etoposide in their anticancer activity.

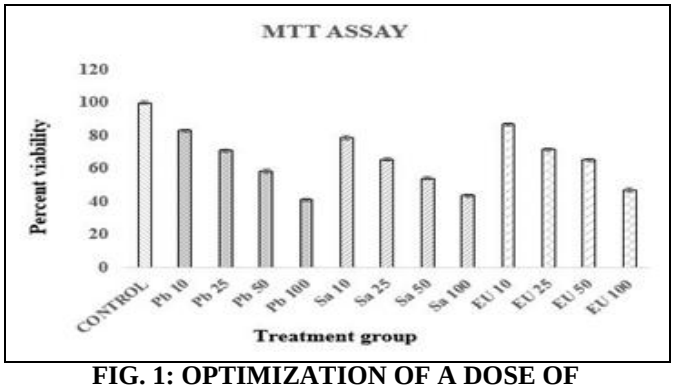

FIG. 1: OPTIMIZATION OF A DOSE OF EUGENOL AND EUGENOL-RICH PLANT EXTRACTS IN A549 LUNG ADENOCARCINOMA CELLS

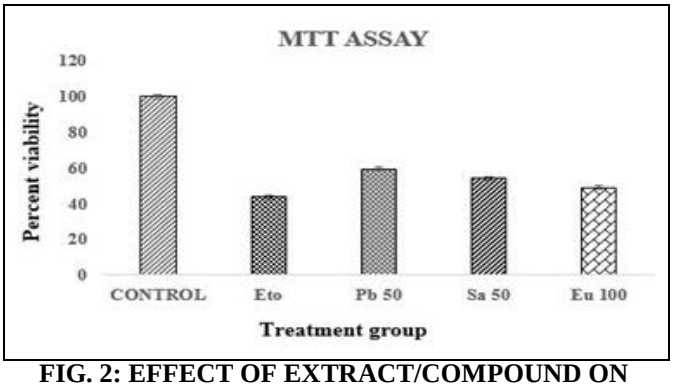

FIG. 2: EFFECT OF EXTRACT/COMPOUND ON THE VIABILITY OF A549 CELLS SUBJECTED TO OXIDATIVESTRESS AS DETERMINED BY MTT ASSAY

SRB Assay: The assay for cytotoxicity using SRB provides a sensitive method for measuring the viability of the cells.

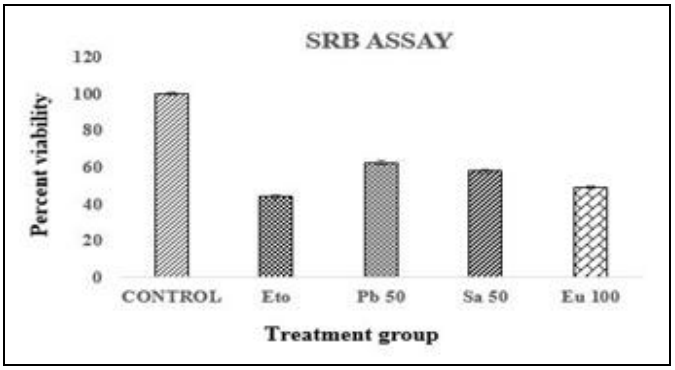

FIG. 3: EFFECT OF EXTRACT/COMPOUND ON THE VIABILITY OF A549 CELLS SUBJECTED TO OXIDATIVE STRESS AS DETERMINED BY SRB ASSAY

In the present investigation, the results of SRB assay showed that the exposure of plant extracts and specific compound eugenol on the viability of lung adenocarcinoma cells was quantified using SRB assay. The percent viability obtained in the various treatment groups in A549 lung adeno-carcinoma cells which are represented in **Fig. 3**.

Morphological Changes as Determined by Giemsa Staining: The morphological changes induced in A549 lung adenocarcinoma cells on the exposure of eugenol and eugenol-rich plant extracts, and/or etoposide, were observed by stained with Giemsa. The type of cell death was further characterized by staining using Giemsa.

All the 4 test samples induced characteristic apoptotic changes like cell shrinkage, membrane blebbing and formation of apoptotic bodies. The number of apoptotic cells was calculated per 100 cells were counted and the apoptotic ratio. These observations showed that the type of cell death

induced is apoptosis. The results are depicted in **Table 1**, **Fig. 4,** and **Plate 4,** respectively.

TABLE 1: EFFECT OF EXTRACT/COMPOUND ON THE MORPHOLOGICAL CHANGES AS DETERMINED BY GIEMSA STAINING

Treatment groups	Number of Apoptotic cells/100 cells Lung cancer cells (A549)
Control	18 ± 4[a]
Etoposide	78 ± 1
Pb 50µg	58 ± 3
Sa 50µg	45 ± 2
Eu 100 µM	60 ± 1

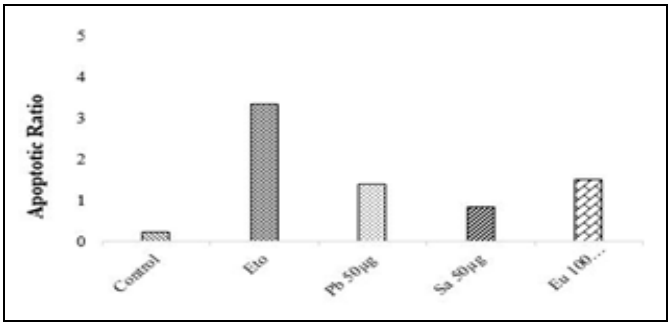

FIG. 4: EFFECT OF EXTRACT/COMPOUND ON THE APOPTOTIC RATIO IN A549 CELLS SUBJECTED TO OXIDATIVE STRESS (GIEMSA STAINING)

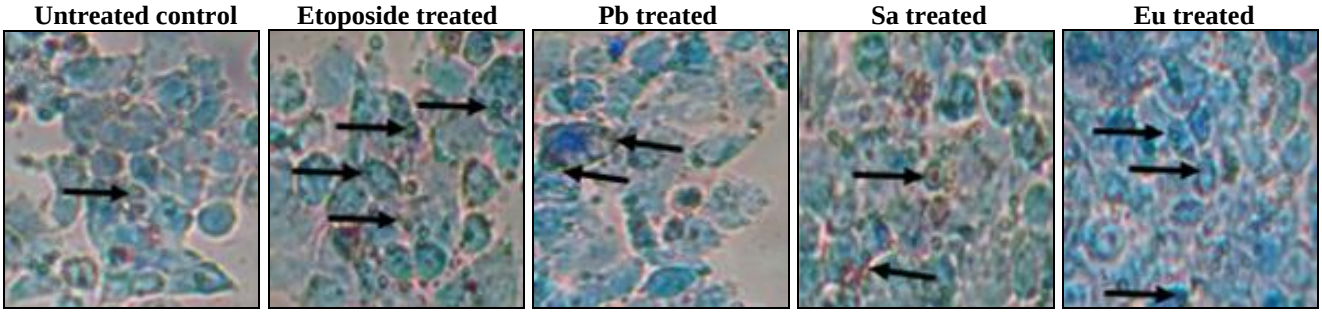

PLATE 4: EFFECT OF EXTRACTS/COMPOUND ON THE MORPHOLOGICAL CHANGES AS DETERMINED BY GIEMSA STAINING

TABLE 2: EFFECT OF EXTRACT/COMPOUND ON THE NUCLEAR CHANGES AS DETERMINED BY AO/EtBr STAINING

Treatment groups	Number of Apoptotic cells/100 cells Lung cancer cells (A549)
Control	20 ± 4
Etoposide	77 ± 3
Pb 50µg	56 ± 2
Sa 50µg	45 ± 1
Eu 100 µM	60 ± 3

Nuclear Changes Induced Oxidative Stress as Determined by AO/EtBr Staining: The nuclear changes that occur during apoptosis in the transformed and untransformed cells exposed to the *P. betle* leaf extract and *S. aromaticum* flower extract and their bioactive component eugenol were analyzed using AO/EtBr. The number of apoptotic cells was counted, and the apoptotic ratio was calculated for each treatment group. It is clear from the results obtained with the viability assays and the staining techniques that the plant extracts and eugenol exhibit an anticancer effect on A549 lung adenocarcinoma cells.

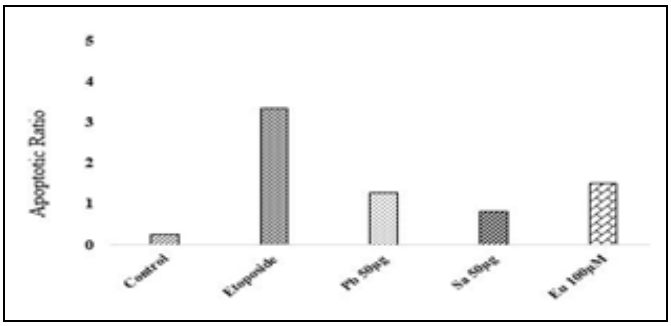

FIG. 5: EFFECT OF EXTRACT/COMPOUND ON THE APOPTOTIC RATIO IN A549 CELLS SUBJECTED TO OXIDATIVE STRESS (AO/EtBr STAINING)

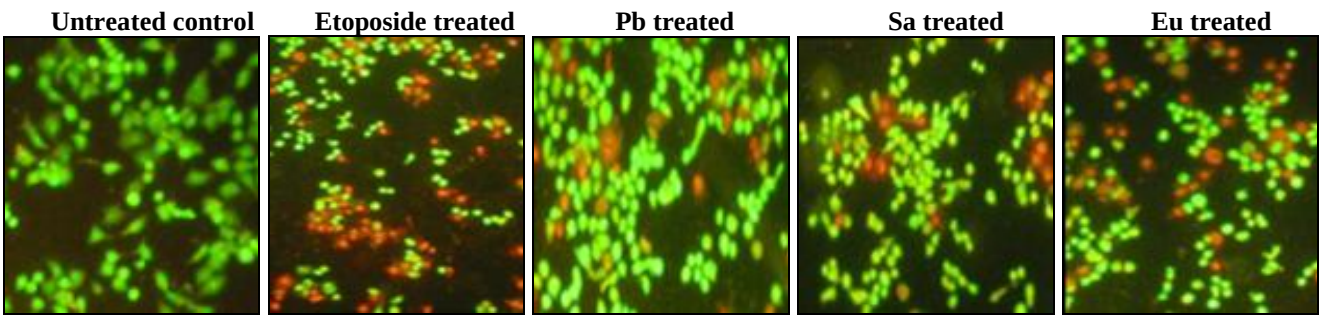

PLATE 5: EFFECT OF EXTRACTS/COMPOUND ON THE NUCLEAR CHANGES INDUCED OXIDATIVE STRESS AS DETERMINED BY AO/EtBr STAINING

Compared to the other plant exposure to the eugenol treated group, there was a drastic increase in the number of apoptotic cells with apoptotic nuclear morphology on A549 cells.

The results are shown in **Table 2,** and the effect of extract/compound on the apoptotic ratio in A549 cells subjected to oxidative stress (AO/EtBr staining) shown in **Fig. 5** and **Plate 5,** respectively.

Cell Cycle Analysis in Lung Adenocarcinoma A549 Cells Exposed to Eugenol and Selected Eugenol-Rich Plant Extracts: The phases of the cell cycle in the treated A549 cells were quantified by flow Cytometry, following propidium iodide staining. The A549 cells were exposed for 24 h to eugenol and selected eugenol-rich plant extracts. The proportions of cells arrested in the G0/G1 phase, S phase, and G2/M phase were quantified.

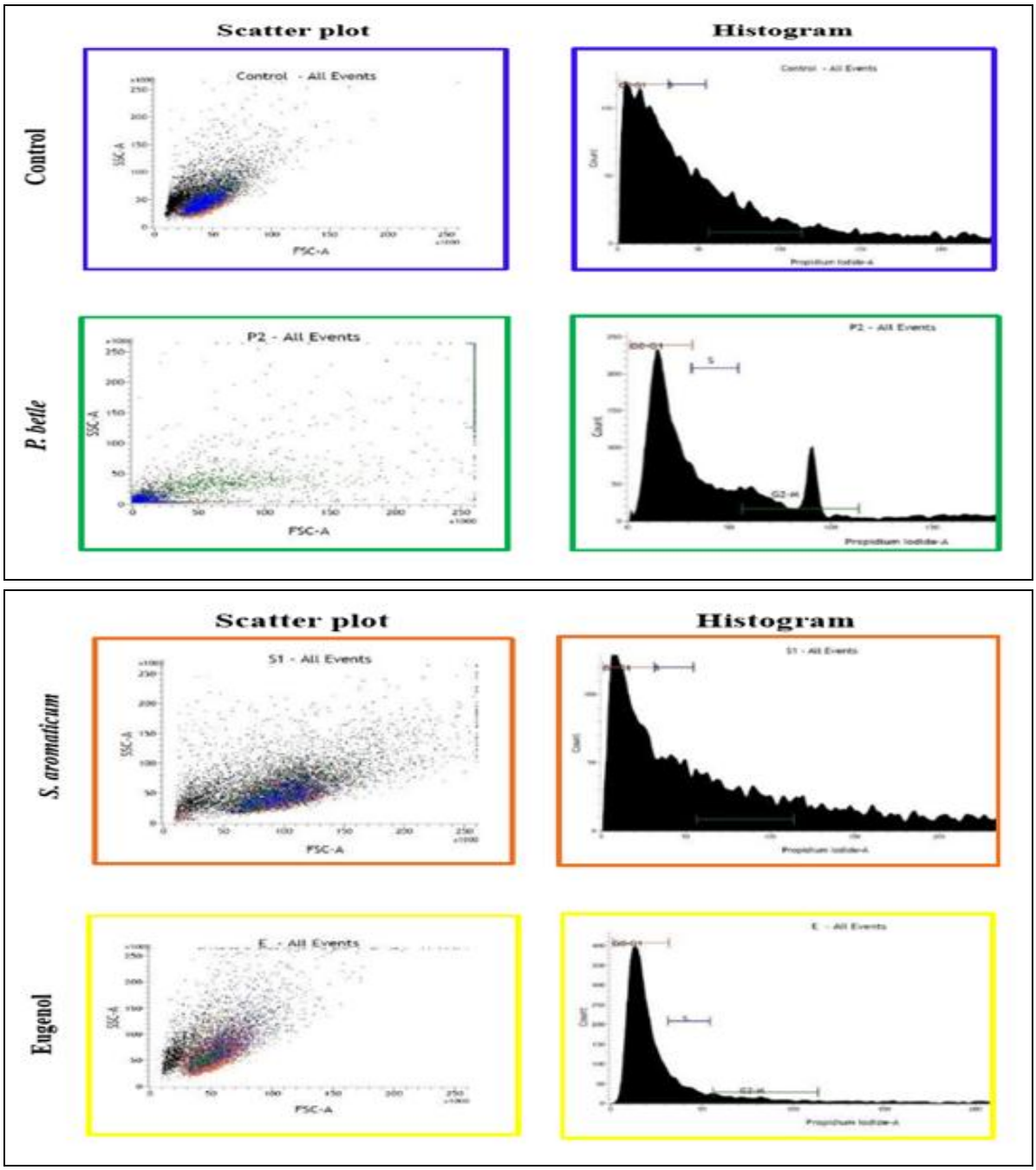

FIG. 6: EFFECT OF EUGENOL AND SELECTED EUGENOL-RICH PLANT EXTRACTS ON CELL CYCLE EVENTS IN LUNG ADENOCARCINOMA A549 CELLS

The scatter plots and histograms recorded in the 3 treatment groups and the control groups are presented in **Fig. 6**.

A majority of A549 cells in the untreated control group were arrested in the G0/G1 phase of the cell cycle. Treatment with eugenol and selected eugenol-rich plant extracts caused a significant portion of the cells to shift to the G2/M phase, which is indicative of early apoptotic death. This observation clearly indicated that a higher proportion of cells commit to death by apoptosis when eugenol and selected eugenol-rich plant extracts were administered. The shift obtained with *P. betle* and eugenol was more prominent than *S. aromaticum*. This suggested that eugenol was very effective in inducing apoptosis, whether administered alone or as plant extracts to cancer cells.

FITC-Annexin Staining in Lung Adenocarcinoma A549 Cells Exposed to Eugenol and Selected Eugenol-Rich Plant Extracts: Following this, using FITC-Annexin staining, the pattern of distribution of the treated cells into early and late apoptotic stages was assessed.

The results showed that, compared to the untreated group of cells, where the number of cells in both stages of apoptosis was negligible, the groups exposed to eugenol (both as a pure compound or as an extract) registered a high proportion of cells in both early and late apoptosis **Fig. 7**.

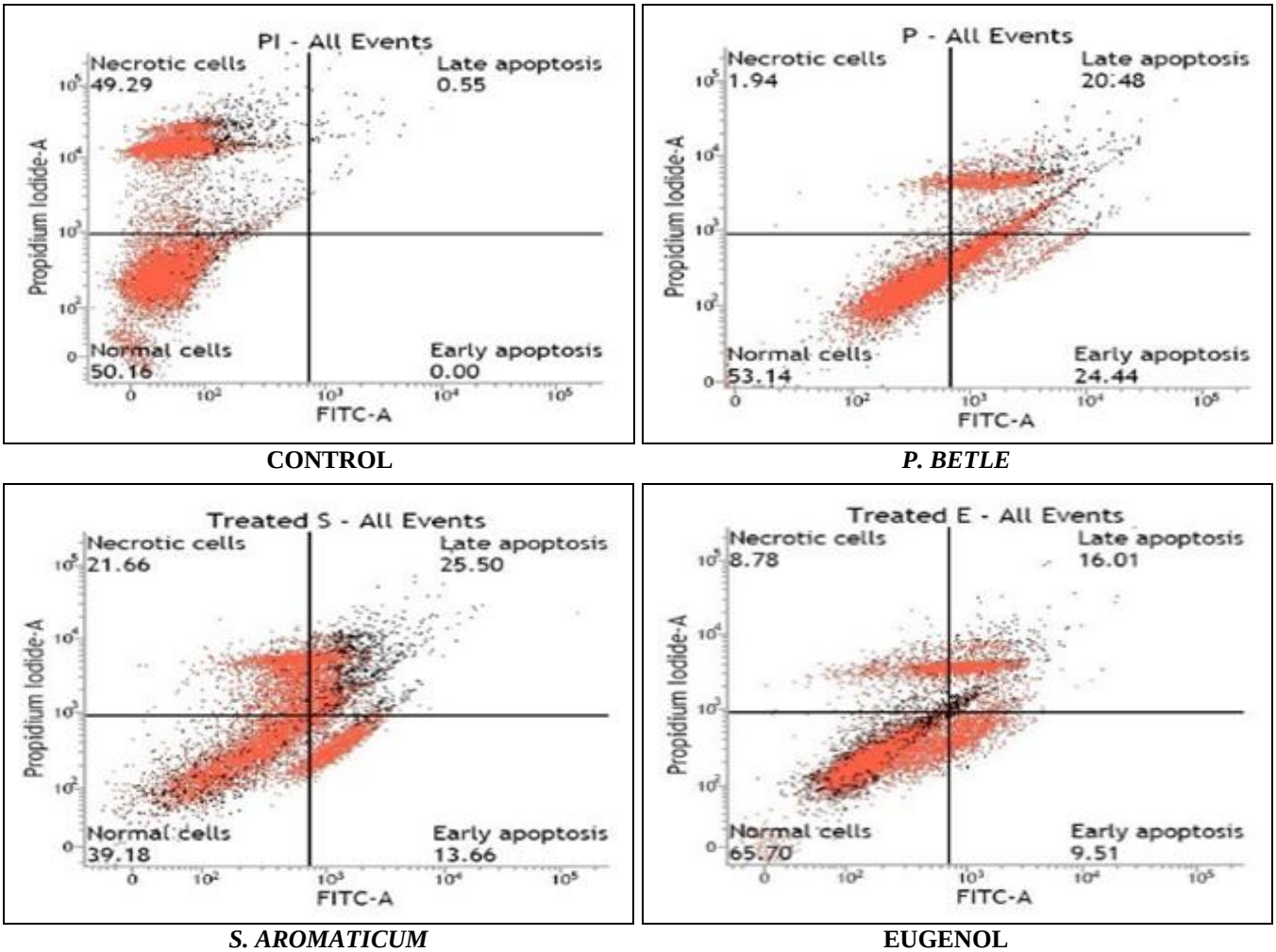

FIG. 7: EFFECT OF EUGENOL AND SELECTED EUGENOL-RICH PLANT EXTRACTS ON APOPTOTIC EVENTS IN LUNG ADENOCARCINOMA A549 CELLS

Thus, the results of the flow cytometric studies reiterated the earlier observations made in this study, that eugenol and eugenol-rich plant extracts induced death by apoptosis in lung adenocarcinoma cells.

DISCUSSION: Cell-based methods like MTT and SRB assays have been developed to rapidly determine the cytotoxicity of a potential chemo-therapeutic drugs in human cancer cell lines [36, 37, 38]. MTT assay estimates the metabolic action,

wherein, live cells decrease the tetrazolium salt to a purple formazan item. The SRB assay gives a sensitive measure of cytotoxicity depending on the cell protein content [39, 40].

In the present study, *P. betle* leaf extract and *S. aromaticum* flower extract were found to significantly decrease the viability of lung cancer cells at all the dose levels tested (10, 25, 50 and 100 µg). The results of SRB assay reiterated the results obtained with MTT assay, confirming the dose-dependent decrease in the proportion of A549 lung adenocarcinoma cells treated with methanolic extract of *P. betle* and *S. aromaticum*. The IC_{50} has been observed to be 50 µg/ml concentration.

A significant dose-dependent decrease in the viability of the MCF-7 human breast cancer cell line, on exposure to *S. aromaticum*, as examined by the MTT test has been reported by [41]. A similar dose-dependent reduction in viability of MCF-7 breast cancer cells on treatment with clove extracts has been documented [42]. Eugenol significantly reduced the viability of different human cancer cell lines (SKBR3, HT29, and HepG2) as measured by MTT assay has been reported [43].

The aqueous extract of *P. betle* leaves exhibited anticancer activity on Hep-2 cells in MTT assay and SRB assay [44]. *Parthenium hysterophorus* Linn. and *Oldenlandia corymbosa* Lam showed anticancer activity (MTT) on K562 human leukemia cancer cell line [45]. SRB assay using neuronal glioblastoma cells (U343) and lung adenocarcinoma cells (A549) were showed significant sensitivity to *Luffa acutangula* and its extracts [46]. *In-vitro* cytotoxicity was reported on C*arissa congesta*, *Polyalthia longifolia* and *Benincasa hispida* crude plant extracts on human colon cancer cell line HCT15, human breast cancer cell line MCF7 and human leukemia cell line MOLT4 by SRB assay [47].

Several authors have reported the usefulness of MTT and SRB assays. Various extracts of *Zea mays* leaves induced apoptosis specifically in cancer (Hep2) cells but evoked protective effect in non-cancerous cells[48]. The cytotoxic effect of the leaves of *P. betle* against the human breast cancer cells lines [49]. In another study, the *in-vitro* anticancer potential of ethanolic extract of clove was shown in human breast cancer (MCF-7) cell lines using MTT assay [50].

The cell viability by MTT assay in which, cancerous (KB (oral cancer) cell line) and non-cancerous (primary cultured human buccal cells) treated with silver nanobioconjugates synthesized from the methanolic extract of *P. betle* leaves and the pure compound eugenol using MTT assay[51]. The MTT assay, a methanolic extract of *Hiptage benghalensis* showed cytotoxicity three different cancer cell lines, namely HeLa, MCF-7 and IMR-32, cells [52].

Morphological analysis by Giemsa on human breast cancer (MCF-7) on the exposure of eugenol showed the induction of apoptosis-like cell shrinkage, vacuole formation membrane blebbing, chromatin condensation, and apoptotic body formation [53]. The characteristic apoptotic morphological highlights including, cytoplasmic shrinkage, atomic buildup, and development of apoptotic bodies in lung adenocarcinoma (A549) cells treated with bufalin, a class of steroids, by Giemsa staining [54].

Studies on the anticancer potential of hydroxychavicol and alcoholic extract of *P. betle* leaves on Chronic Meloid Leukaemia (CML) cell, using Giemsa staining, revealed a significant increase in the number of apoptotic cells, showing membrane blebbing, cell shrinkage, and nuclear fragmentation [55]. The exposure of taxol caused apoptosis-associated morphological changes in different cancer cells as visualized by Giemsa stain [56]. The inhibitory effect of the black turtle bean extract treated with MCF-7 and MDA-MB231 cell, observed characteristic apoptotic morphological changes like cell shrinkage, layer blebbing, cell adjusting and diminished volume, by Giemsa staining [57].

In human cervical cancer cells treated with pinostrobin, observed the characteristic apoptotic morphological features including cytoplasmic shrinkage, nuclear condensation, and formation of apoptotic bodies by Giemsa staining, has been documented [58]. These reports render support to our findings that the plant extracts (*P. betle* and *S. aromaticum*) and their specific compound, eugenol, significantly increase the number of lung

adenocarcinoma (A459) cells displaying apoptotic morphological features.

Several similar studies have been reported in the literature. The human osteosarcoma cell line treated with kappa-selenocarrageenan showed the nuclear changes by AO/EtBr staining [59]. The acetone extract of the *Croton bonplandianus*, has been shown to induce apoptotic features in lung cancer cell line A549 [60]. The greater proportion of MCF-7 and MDA MB231 breast cancer cells showing apoptotic features on treatment with curcumin, a major constituent of turmeric [61].

In two different types of human breast cancer (MCF-7 and MDA MB 231), the exposure of a neem limonoid and neem seed oil showed apoptotic feature s[62].

The cytotoxic effect of lupeol in human adenocarcinomic human cell lines A549 by apoptosis [63]. *Beta vulgaris* silver nanoparticles showed the morphological changes in MCF-7, A549 and Hep-2 cancer cell lines [64].

Morphological analysis of AO/EtBr on human cervical cancer cell line (HeLa) in the presence of essential oil natural derivative of *Atalantia monophylla* showed the induction of apoptosis [65]. The anticancer effect of a decapeptide from *Perinereies aibuhitensis* on lung cancer H1299 cell lineas evaluated by AO/EtBr staining [66]. Another study illustrated that several sulfonamides showed a stronger inhibitory effect on the HeLa (cervical cancer) cells as observed by AO/EtBr staining [67].

Effect of Eugenol and Selected Eugenol-Rich Plant Extracts on Apoptosis and Cell Cycle Analysis in Lung Adenocarcinoma Cell Line: The results showed that untreated lung adenocarcinoma (A549) cells were distributed across all the stages of the cell cycle, whereas, in all the three treatment groups, the cells were arrested in the G0/G1 phase of the cell cycle. Treatment with methanolic extract of *P. betle* and *S. aromaticum* extracts and their bioactive compound eugenol caused a significant portion of the cells to shift to the G2/M phase, which is indicative of early apoptotic death. This showed that the shift obtained with *S. aromaticum* and eugenol and shift was more marked in the case of *P. betle.*

The effect of *S*-allyl mercapto cysteine on human colon cancer cells indicated G2/M phase arrest [68]. *Piper longumine* showed a decrease in cell number in G0/G1 and S phases and an increase in G2/M phase in different types of cholangio carcinoma (KKU-055, KKU-214, and KKU-100) cells lines [69]. In HCT-116 human colorectal cancer cells, isolated active fraction of clove extract (*Syzygium aromaticum*) treatment was shown to result in G2/M phase arrest [70]. Similarly, in A549 and RERF-LC-MS lung cancer cells, a major proportion of the cells were arrested in the G2/M phase of the cell cycle after treatment with ZGDHu-1 (N, N'-di-(m-methylphenyl)- 3,6-dimethyl-1,4-dihydro-1, 2,4,5-tetrazine-1,4-dicarboamide) [71].

The essential oil from the leaves of *Croton matourensis* induced cell cycle arrest of HepG2 human liver hepatocellular carcinoma cell at the G2/M phase after 48 h of treatment [72]. Biochanin A induced the cell cycle arrest significantly at S phase in lung cancer cells (A549 and 95D) [73]. Guercetin induced G2 phase cell cycle arrest and apoptosis in both human cervical cancer HeLa and SiHa cells, accompanied by an increase of p53 and its nuclear signal [74]

CONCLUSION: This study concludes that the methanolic extracts of leaves *P. betle* and *S. aromaticum* and bioactive compound eugenol exert anti-cancer activity in A549 (lung adenocarcinoma) cell line. The MTT and SRB assay was carried out with eugenol and eugenol-rich plant extracts (*P. betle* and *S. aromaticum*) against lung adenocarcinoma A549 cell line, and it illustrates the cell viability with respect to the standard chemotherapeutic agent, Etoposide, and provides us a better IC_{50} value for contrary to A549 lung cancer cell line. Morphological and nuclear changes as determined by Giemsa and AO/EtBr staining. The cytotoxicity assays revealed strong anti-lung cancer activity of all three test samples. Further characterization showed that cell death occurred by apoptosis, and additionally cell cycle in the cancer cells was arrested in the G2/M phase.

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CONFLICTS OF INTEREST: The authors declare that they have no conflict of interest regarding the publication of this paper.

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