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**Research Paper**

# Evaluation of the *in-vitro* anticancer and antimicrobial effectof*Centellaasiatica*(L.)Urb and *Cocciniagrandis*(L.) Voigt leaves

Mithun K Rajan[1], BenittaSoosan Thomas[1], Sujith Shankar[1], Abitharani Jothibasu[1], Venkatesh Rajendran[1]*, Reena.D[2]

[1]*Department of Biochemistry (PG and Research), Kongunadu Arts and Science College (Autonomous), G.N Mills post, Coimbatore-641029, Tamil Nadu, India.*
[2]*Department of Biotechnology, Madurai Kamaraj University, Madurai - 21*
**Corresponding Author- Dr.R.Venkatesh, Assistant professor and Head, Department of Biochemistry (PG and Research), Kongunadu Arts and Science College (Autonomous), Coimbatore-641029, Tamil Nadu, India.*

***Abstract***
*In recent years, there has been a resurgence of interest in drug discovery from medicinal plants for health maintenance in all parts of the world. Although natural products have been shown to be reliable sources of anticancer medicines, there is indeed a consistent demand for new therapeutic natural products for cancer care with reduced toxicity.In this study,Phytochemical analysis of ethanolic extracts of Centellaasiatica (L.) and Cocciniagrandis (L.) leaves revealed the presence of alkaloids, flavonoids, saponins, phenols, and glycosides. This research also highlights the absence of tannins and steroids. The pharmacological interest of these compounds, combined with the plant's use in traditional medicine, prompted researchers to investigate Centellaasiatica (L.) and Cocciniagrandis (L.) for anticancer, antimicrobial, and antioxidant activities. The antioxidant potential of Centellaasiatica (L.) and Cocciniagrandis (L.) was determined using the DPPH (2,2-diphenyl-1-picrylhydrazil) method whichrevealed excellent free radical scavenging activity with $IC_{50}$ values for EECACG is 64.20µg/ml clearly shows high antioxidant activities.It has more effective antimicrobial activity against Staphylococcus aureus, Escherichia coli, Klebsiellapneumoniea and Pseudomonas sp. Based on MTT assay $IC_{50}$ value20 ± 0.3clearly indicates the effective anticancer activity on Centellaasiatica (L.) andCocciniagrandis (L.) against A549 lung cancer cell line.*

***Keywords:*** *Centellaasiatica(L.), Cocciniagrandis(L.),Phytochemicals, Antimicrobial, Antioxidant, Anticancer*

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## I. Introduction

Cancer is the uncontrolled growth of cells and is one of the most deadly diseases in the world, with over 6 million deaths among 22 million cancer victims worldwide. (Sushitra*et al*.,2014). With significant advancements in treatments and preventative measures, cancer has been a continuous global problem.The disease is characterized by cells in the human body that are constantly multiplying and cannot be controlled or stopped, as well as malignant cells with the potential to spread to various organs. (Ochwang'I and Kimwele, 2014).

The worldwide burden of infectious diseases can be reduced with the help of antimicrobial agents (Bhatia andNarain,2010). However, the emergence and spread of multidrug resistant (MDR) strains in pathogenic bacteria has become a significant public health threat because there are fewer, and sometimes no, effective bactericidal agents available for pathogenic bacteria infection. (Boucher*et* al., 2009).Using a standard protocol, a qualitative analysis of the extract was analyzed to detect the existence of phytochemicals such as carbohydrates, proteins, tannins, saponins, flavonoids, phenols, alkaloids, and thiols. (Harbone*et al.*,2015).Many compounds in plant tissues have the potential to act as antioxidants and free radical scavengers. (Mensor, 2001).

Since ancient times, medicinal plants have been widely used to treat a variety of diseases. They have many curative properties, including pain relief, antibacterial, anti-inflammatory, and anti-diabetic properties.(Rosario, 2015).Centellaasiatica (Apiaceae Family) is also known as Centella and Gotu kola. It is

indigenous to India and other parts of Asia, including China, Sri Lanka, Nepal, and Madagascar, and has been used in folk medicine in various countries. (Gohil*et al*., 2010).Centellaasiatica has been reported to have a variety of pharmacological activities, including antimicrobial, anticancer, neuroprotective, immune modulation, and anti-inflammatory properties (Hashim, 2011). Cocciniagrandis is a plant in the Cucurbitaceae family. Telakucha, Tindora, Scarlet-fruited gourd, and Ivy gourd are some of its common names. It is native to India, Asia, and Central Africa (Yadav, 2010). The leaves have anti-diabetic, anti-inflammatory, antipyretic, analgesic, and anti-pasmodic properties (Dewanjee et al., 2007). Therefore the present investigation was conducted in a systematic manner to study the in-vitro anti-cancer activity and antimicrobial effects of ethanolic extract of *Centellaasiatica* and *Cocciniagrandis.*

## II. Materials and Methods

***Plant identification and collection***

Leaves of Cocciniagrandis (L.)Voigt and Centellaasiatica (L.)Urb were collected in various locations throughout Mananthavady, Wayanad District, Kerala, India. The plant samples were authenticated at the Botanical Survey of India's South Circle in Coimbatore, and specimen copies (BSI/SRC/5/23/2022/Tech/454) and (BSI/SRC/5/23/2022/Tech/455) were deposited for future reference

***Plant extract preparation***

The plant material was washed in clean water and dried in the shade at room temperature. After drying, the leaves were ground into a fine powder using a mechanical grinder. The Soxhlet apparatus was used for solvent extraction. About 0.25 gm of dried powder of each plant was separately extracted with a Soxhlet extractor using 250 ml of ethanol. The extract solution was then dried after being evaporated with a rotary evaporator to remove the solvent. The final samples were kept at 40 degrees Celsius for further research (Muhammad ShahzadAslam et al., 2016)

***Polyherbal formulation processing***

In 10 ml of ethanol, 25 mg of each extract was mixed together and labeled as an herbal formulation. The final samples were kept at 40 degrees Celsius for further scrutiny (PradeepKaur et al., 2019).

***Photochemical Screening***

***Qualitative analysis***

To identify the secondary metabolites present in the plant extracts, phytochemical screening of ethanol extracts of herbal plants leaves was performed using standard procedures. The analysis used small amounts of herbal extracts, and the major secondary metabolites analyzed were alkaloids, flavonoids, saponins, phenols, glycosides, tannins, and steroids (Oluwafemi et al., 2019).

***Antioxidant activity***

The Blois method was employed to test the radical DPPH assay's ability to measure the hydrogen donating or radical scavenging capacity of plant extracts. Each test tube received 3 ml of ethanol at various concentrations, and solutions (20, 40, 60, 80, and 100 mg) were prepared using the stock solution (0.01 gm of extract dissolved in 1 ml of ethanol). To make the standard ascorbic acid solution, dissolve 0.001 gm of ascorbic acid in 1 ml ethanol. 3ml of ethanol was kept as negative standard. In another test tube, DPPH (1 ml) was added to all the test tubes. The mixture was vigorously shaken for several minutesAfter incubation in the dark for 30 minutes, observance was measured for the entire mixture, including the negative controlled and standard ascorbic solution (Poornima and Salman, 2021).The DPPH scavenging activity was calculated using the formula,

DPPH scavenging effect (%) = Ac – Ab / Ac * 100

Ac – Absorbance of Negative control

Ab – Absorbance of sample

***Antimicrobial activity***

***Test microorganisms***

Clinical isolates of Ecoli, Salmonella aureus, Klebsiella pneumonia, and Pseudomonas sp. were used in the tests, and they were obtained from the Department of Microbiology at Coimbatore Medical College and Hospital (CMC). The bacterial cultures were subcultured in nutrient broth (NB) and kept for overnight incubation at 37˚C.

***Agar well diffusion method***

The Agar well diffusion method was used to determine the antibacterial activity of ethanolic extracts of the leaves Centellaasiatica (L.) and Cocciniagrandis (L.). Using a sterile cork borer, wells were bored into the agar (6mm diameter). Extracts of varying concentrations were poured into the wells and incubated at 37°C for 24 hours. The standard well was loaded with antibiotic disc. The zone of inhibition was measured after the incubation period (Etminani et al., 2018).

***Anticancer activity***

***Cell culture***

The Human lung cancer cell line (A549) was obtained from the National Center for Cell Sciences (NCCS) in Pune, India. The cancer cells were grown in Dulbecco's modified eagles medium (DMEM) supplemented with 2mM l-glutamine and balanced salt solution (BSS) adjusted to contain 1.5 g/L Na2CO3, 0.1 mm nonessential amino acids, 1 mm sodium pyruvate, 2 mM l-glutamine, 1.5 g/L glucose, 10 mM (4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid) (HEPES) and 10% fetal bovine serum (GIBCO, USA). Penicillin and streptomycin concentrations (100 IU/100g) were changed to 1mL/L. In a humidified CO2 incubator, the cells were kept at 37°C with 5% CO2.

***Evaluation of cytotoxicity***

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used to determine the inhibitory concentration (IC50). Cancer cells (1x104 cells/well) were grown in a 96-well plate for 48 hours to 75 % confluence. The medium was replaced with fresh medium containing serially diluted samples, and the cells were incubated for another 48 hours. 100 mL of MTT [3-(4,5-dimethylthiozol-2-yl)-3,5-diphenyl tetrazolium bromide] (Hi-Media) solution was added to each well after the growth media had been removed, and the wells were then incubated at 37 °C for 4 hours. The formazan crystals were dissolved by adding 50 mL of DMSO to each well after the supernatant had been removed. This process took 10 minutes. An ELISA multiwell plate reader was used to detect the optical density at 620 nm (Thermo Multiskan EX, USA). The following formula was used to get the percentage of viability using the OD measurement.

% of viability = OD value of experimental sample/OD value of experimental control ×100

***Analysis of apoptotic cell death using fluorescence microscopy***

On clean microscope glass cover slip, 1µL of a dye mixture (100 mg/mL acridine orange (AO) and 100 mg/mL ethidium bromide (EtBr) in distilled water) was mixed with 90 µL of cell suspension (1x105 cells/mL). The cancer cells were collected, washed in phosphate buffered saline (pH 7.2), and stained with 10 µL of AO/EtBr. After 2 minutes, the cells were washed twice with PBS (5 minutes each) and examined under a fluorescence microscope (Nikon Eclipse, Inc, Japan) at 20 magnification with a 480 nm excitation filter.

Similarly, the cells were seeded on a glass cover slip in a 24-well plate and treated for 24 hours with the compound. The fixed cells were permeabilized with 0.2 percent triton X-100 (50µl) for 10 minutes at room temperature before being incubated for 3 minutes with 10 µl of DAPI using a cover slip to ensure uniform stain distribution. The cells were examined using a fluorescent microscope (Nikon Eclipse, Inc, Japan).

Likewise, the cells were seeded on a glass cover slip in a 24-well plate and treated for 24 hours with the compound. The cells were then fixed with 70% ethanol for 10 minutes at room temperature before being incubated for 3 minutes with 10l of Propidium Iodide using a cover slip to ensure uniform spreading of the dye. The cells were observed under (Nikon Eclipse, Inc, Japan) fluorescent microscope.

## III. Result and discussion

***Phytochemical analysis***

Phytochemicalanalysis of ethanolic extract of *Centellaasiatica*(L.) and *Cocciniagrandis*(L.) reveals the presence of bioactive compounds such as Alkaloids, Flavonoids, Saponins, Phenols and Glycosides.Tannins and Steroids were absent in ethanolic extract of *Centellaasiatica*(L.)and *Cocciniagrandis*(L.).

**Table-1: Phytochemical analysis of ethanolic extract of *Centellaasiatica*(L.) and *Cocciniagrandis*(L.)**

| S.No | Phytochemical | Extract | Result |
|---|---|---|---|
| 1 | Alkaloids | EECACG | + |
| 2 | Flavonoids | EECACG | + |
| 3 | Saponins | EECACG | + |
| 4 | Tannins | EECACG | - |
| 5 | Phenols | EECACG | + |
| 6 | Steroids | EECACG | - |
| 7 | Glycosides | EECACG | + |

Based on some studies: Freshly prepared crude ethanolic extracts of Centellaasiatica (L.) leaves were tested for the presence of akaloids, flavanoids, saponins, and phenols for preliminary phytochemical analysis. Tannins, steroids, and glycosides are not found in Centellaasiatica (L.) ethanolic extract (Amir et al., 2014).

The fruits of Cocciniagrandis (L.) were extracted with ethanol, and preliminary screening revealed that they contained alkaloids, flavanoids, tanins, and phlobatanins. The ethanolic extract of Cocciniagrandis (L.) lacks carbohydrates (Prashant and Balwantsinh, 2017).

***Antioxidant activity***

The ability of the extract to neutralize donating ability using hydroxyl radical was expressed as a 50% inhibition concentration in g/ml. The extract's IC50 value was determined to be 64.20 g/ml.

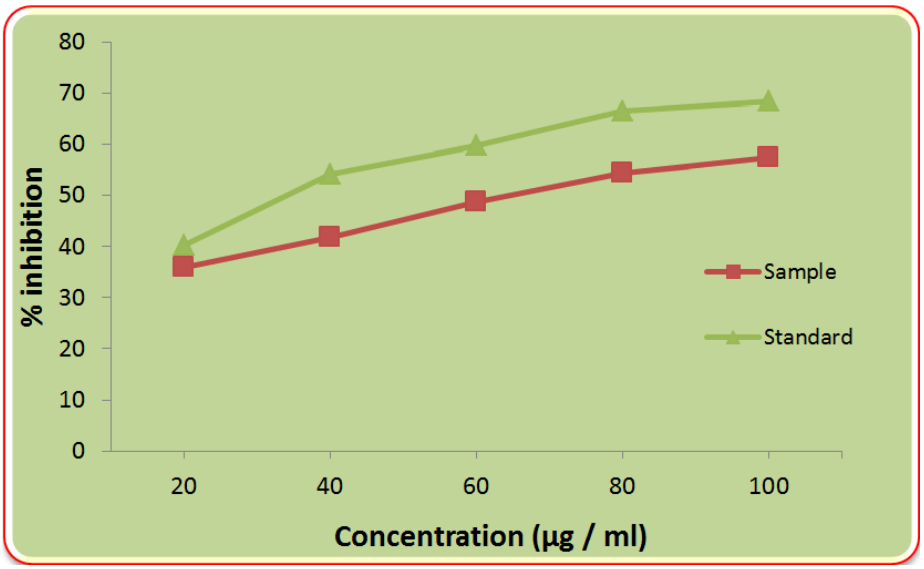



**Figure 1: Antioxidant activity of *Centellaasiatica*(L.) and *Cocciniagrandis*(L.).**

The aqueous extract of fruits outperformed the methanolic and aqueous extracts of fruits and leaves in terms of antioxidant activity. The DPPH radical is a widely used method for assessing the ability of various samples to scavenge free radicals. DPPH is a stable nitrogen-centered free radical whose color changes from violet to yellow when reduced by either hydrogen- or electron-donation. Substances which are able to perform this reaction can be considered as antioxidants and therefore radical scavengers.(Ashish*et al*., 2011).

***Antimicrobial activity***

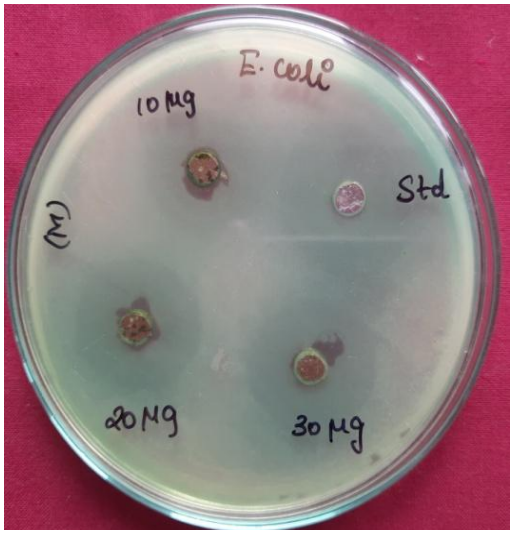
The result of antimicrobial activity of *Centellaasiatica*(L.) and *Cocciniagrandis*(L.) are shows in table 3. Four test organisms were exposed to an ethanolic extract of the plant's leaves. At concentrations ranging from 10 to 30 g, both Gram-positive and Gram-negative bacteria stains were sensitive to the extracts.

**Table 2: Antimicrobial activity of *Centellaasiatica*(L.) and *Cocciniagrandis*(L.)**

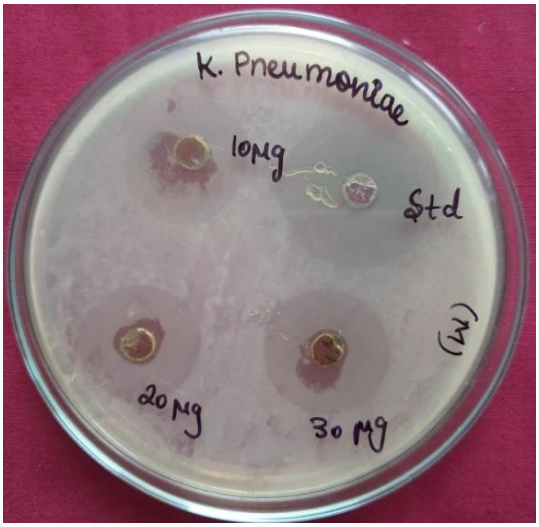
| S.No | Pathogenic bacteria | Zone of inhibition (mm) | | | Standard (Amoxicillin) |
|---|---|---|---|---|---|
| | | 10 µg | 20 µg | 30 µg | |
| 1. | *Staphylococcus aureus* | 10 | 12 | 16 | 24 |
| 2. | *E.Coli* | 08 | 14 | 22 | 24 |
| 3. | *Klebsiella pneumonia* | 08 | 12 | 18 | 24 |
| 4. | *Pseudomonas sp.* | 08 | 10 | 10 | 18 |

The experiment was conducted in triplicates (n=3)

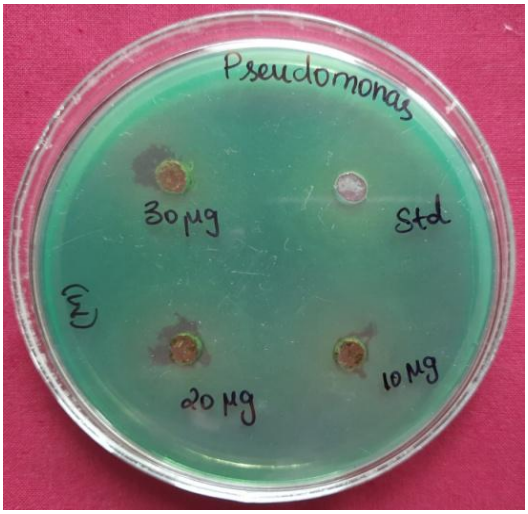

***a) Staphylococcus aureus***




***b) Escherichia coli***




***c) Klebsiella pneumonia***




**d) *Pseudomonas sp.***

**Figure 2: Antimicrobial activity of *Centellaasiatica*(L.)and *Cocciniagrandis*(L.).**
**a) *Staphylococcus aureus*b) *Escherichia coli***
**c) *Klebsiella pneumonia* d)*Psudomonassp***

The results showed that ethanol was the best extractive solvent for anti-microbial properties of Centellaasiatica (L.) leaf and root, followed by chloroform and aqueous. The ethanol extract of Centellaasiatica (L.) root demonstrated the greatest zone of inhibition against bacteria using agar well diffusion and the disc plate method (Muhammad Yusuf Nasution et al., 2018).

***Anticancer activity***

***MTT assay***

In-vitro cytotoxicity testing has recently emerged as a primary screening method for evaluating the anticancer properties of various chemicals and natural substances. The cell viability (MTT assay) of Centellaasiatica (L.) and Cocciniagrandis (L.) leaf extracts was investigated when treated with the A549 lung cancer cell line**.**

***Morphological analysis***

The percentage of cell viability decreases as the concentration of ethanol extract of Centellaasiatica (L.) and Cocciniagrandis increases (L.). The extract's inhibitory concentration (IC50) was determined to be 20 ± 0.3.

**Table 3: IC50- Values of ethanolic extract of *Centellaasiatica*(L.) and*Cocciniagrandis*(L.)**

| Sample | A549 cells (IC 50) |
|---|---|
| Extract | 20 ± 0.3 |
| Doxorubicin (std) | 18 ± 0.5 |

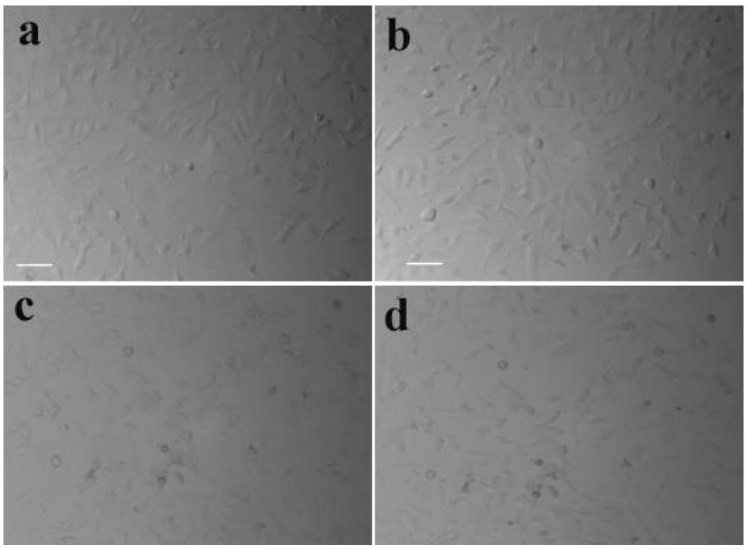



**Figure 3: Morphology of A549 lung cancer cell line treated with ethanolic extract of *Centellaasiatica*( L.)and*Cocciniagrandis*(L.).**

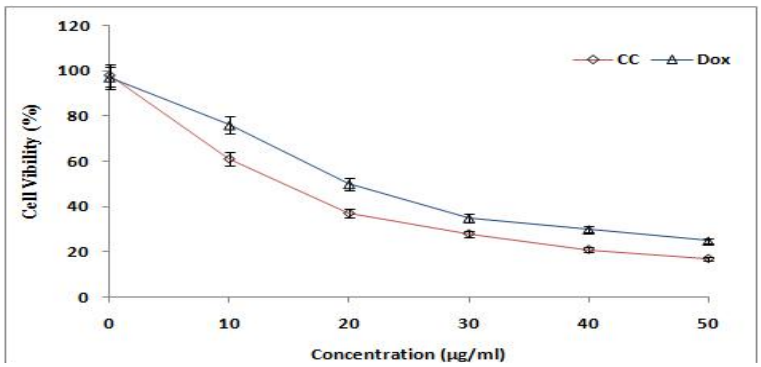



**Figure 4: Cell viability of *Centellaasiatica*(L.)and*Cocciniagrandis*(L.)ethanaolic extract treated with A549 lung cancer cell line.**

The effect of Centellaasiatica (L.) extract on human HepG2 cell line was evaluated using MTT assay, and it demonstrated cytotoxic effects on tumor cells in a dose-dependent manner. On the human HepG2 cell line, more DNA damage and apoptotic cell death were seen. (Hussin et al., 2014).

**Acridine orange/ Ethidium bromide (AO/EtBr) staining method**

Apoptosis is a type of programmed cell death that eliminates precancerous and virus-infected cells. The figure depicts the apoptosis studies of A549 cells treated with ethanol leaves extracts of Centellaasiatica (L.) and Cocciniagrandis (L.). The green cells transform into orange/red cells as a result of induction apoptosis and the nuclear condensation effect on the cells.

The control cell line (figure) showed the green color, indicating that apoptosis was downregulated. Apoptotic activity of different concentrations and plant extracts of Centellaasiatica (L.) and Cocciniagrandis (L.) was shown in the figure, where the color change from green to yellowish orange indicates cell apoptosis. The maximum concentration of plant extract produced a reddish orange color as a result of the cells' vigorous apoptosis. A549 lung cancer cells treated with the highest concentrations of Centellaasiatica (L.) and Cocciniagrandis (L.) showed the strongest reddish orange hue, indicating the highest level of apoptosis. It implies that the plant extract is more efficient on A549 cancer cell line.

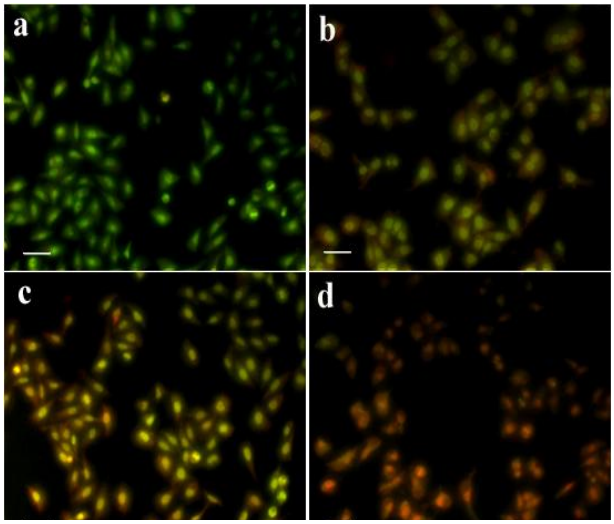



**Figure 5: Apoptosis activity of *Centellaasiatica*(L.)and *Cocciniagrandis*(L.)on A549 lung cancer cell line.**

***PI Staining***

Because PI is less expensive, more stable, and an excellent indicator of cell viability due to its capacity to reject dye in living cells, it is employed more frequently than other nuclear stains (Fried et al., 1976). Due to the presence of an intact plasma membrane, PI cannot stain living or early apoptotic cells, which is determined by the membrane's permeability (Vermes et al., 1995). (Vermes et al., 2000). (Darzynkiewicz et al.,1992). Late apoptotic and necrotic cells have lessened nuclear and plasma membrane integrity (Kroemer et al., 1998) (Bacso et al., 2000), allowing PI to cross the membranes, intercalate into nucleic acids, and exhibit red fluorescence. (Vermes*et al.*, 1995)

The cell cycle distribution in the cisplatin-resistant A549 cancer cell line was determined using PI staining.It stains DNA and RNA inside of dead cells or the ones with universal stain they cross intact membranes and stains nucleic acids (NA) of all cells, thereby enabling to obtain total cell counts (Boulos*et al.*, 1999).The ethanol leaf extracts of Centellaasiatica (L.) and Cocciniagrandis were used to induce apoptosis in A549 cells (L.). The intensity of the red fluorescence color was seen in A549 lung cancer cells treated with the highest doses of Centellaasiatica (L.) and Cocciniagrandis (L.), suggesting the highest level of apoptosis. It implies that the plant extract is more potent against the cancer cell line A549 than other substances.

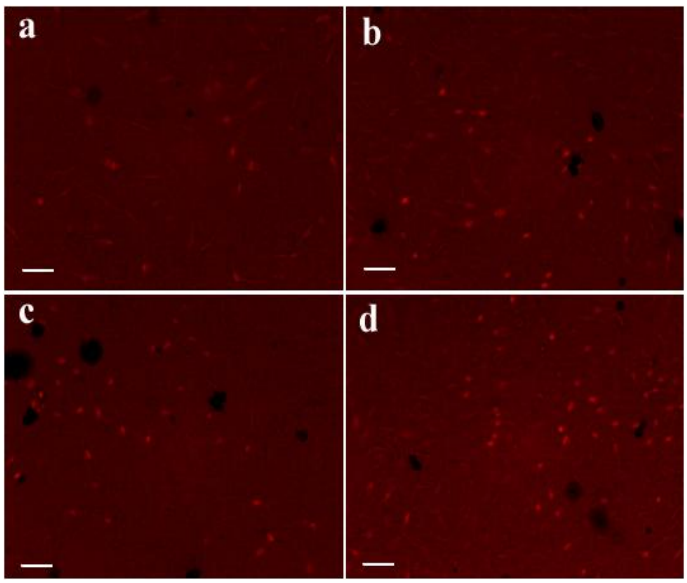



**Figure 6: PI staining assay of plant extract treated A549 lung cancer cell line.**

***DAPI Staining***

Apoptosis assessment and nuclear alteration visualization can both be done using the dye DAPI. Adenine-thymine sections of DNA's minor groove are strongly and specifically bound by DAPI. When DAPI is attached to double-stranded DNA, it absorbs light at 359 nm (ultraviolet light) and emits light at 461 nm

(fluoresces blue). Bound DAPI has a fluorescence intensity that is roughly 20 times greater than free DAPI. The fluorescence is directly proportional to the amount of DNA that is present. More DAPI penetrates the cell, staining it a brighter blue as the apoptotic cell membrane becomes more permeable (Cornelissen et al., 2002).The visual identification of DAPI-stained apoptotic cells is aided by the distinct nuclear morphology of apoptotic cells, such as chromosome condensation and disintegration. Additionally, nuclear blebbing may be observed in apoptotic cells stained with DAPI, which may help in separating them from non-blebbing necrotic cells. The intensity of the blue hue was seen in A549 1 lung cancer cells treated with the highest doses of *Centellaasiatica* (L.) and *Cocciniagrandis* (L.), suggesting the highest level of apoptosis. This suggests that the plant extract is more effective against the A549 cancer cell line.

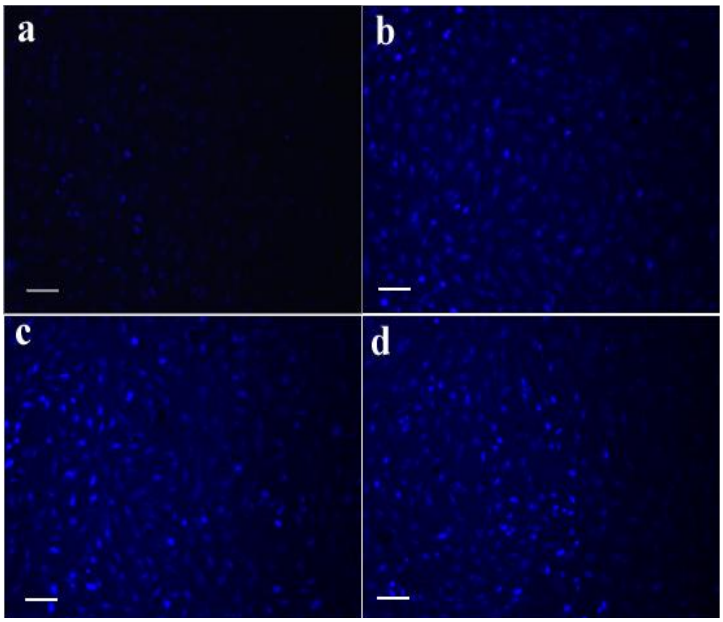



**Figure 7: Nuclear morphology of *Centellaasiatica*(L.)and *Cocciniagrandis*(L.)on A-549 lung cancer cell line.**

## IV. Conclusion

The current study involves the leaf extracts of Centellaasiatica (L.) and Cocciniagrandis (L.), which have anticancer activity based on various assays, making them useful in the treatment of lung cancer. The DPPH assay revealed that ethanolic extracts of Centellaasiatica (L.) and Cocciniagrandis (L.) had high antioxidant activity and antimicrobial activity when tested using the agar well diffusion method. As a result, both of these plants have greater medicinal value. Overall, the study recommended further purification, structural analysis, and in vivo lung cancer activity studies.

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**Conflicts of interest:**
The author declares no conflict of interest.

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