Pro-apoptotic effect of *lippia nodiflora* leaf extract against the skin cancer cell line

Running Title: Evaluation of pro-apoptotic effect of *Lippia nodiflora* leaf extract against the skin cancer cell line.

The type of article: Original Research Article

ABSTRACT:

Background: Skin cancers are cancers that develop on the surface of the skin. They occur as a result of the formation of irregular cells with the potential to infiltrate or migrate to other areas of the body. Natural medicinal resources, such as the traditional herbal remedy *Lippia nodiflora*, have long been used to treat dermatological disorders including skin inflammation and melanogenesis. Apoptosis serves as a promising pathway in controlling cancer. However there are minimal amounts of studies exploring its proapoptotic activity of *Lippia nodiflora* in skin cancer cells.

Aim: The aim of this present study was to evaluate the pro-apoptotic effect of *Lippia nodiflora* leaf extract against the skin cancer cell line.

Materials and method: MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays were used to determine the cytotoxic effect of *Lippia nodiflora* leaf extract against skin cancer cells (B16-F10). The skin cancer cells were treated with different concentration *Lippia nodiflora* leaf extract for 24h. We calculated the optimum (IC-50; 20µg/ml) using the MTT assay, which was then used for further analysis. A phase-contrast microscope is used to analyse changes in cell morphology. AO/EtBr dual staining under a fluorescence microscope was used to determine the effect of *Lippia nodiflora* on B16-F10 cell death.

Result: In our research, the cell viability of the B16-F10 skin cancer cell line was dramatically decreased after treatment with different concentrations (5-60 µg/ml) of *Lippia nodiflora* for 24 hours. 50% inhibition was detected at a concentration of 20 µg/ml, which was determined to be an inhibitory concentration (IC-50) value and used in subsequent studies. The viable cell will possess a uniform bright green nucleus. Early apoptotic cells will have bright orange areas of condensed or fragmented chromatin in the nucleus. Late apoptotic cells will have uniform bright red nucleus.

Conclusion: The findings of this analysis revealed that *Lippia nodiflora* leaf extract inhibits the cell viability and indces apoptosis of skin cancer cells. Thus, *Lippia nodiflora* exhibits anti-cancer effects against skin cancer cells, therefore it raises new hope for anti-cancer therapy.

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Keywords: *Lippia nodiflora*, Skin cancer, Cytotoxicity, Pro-apoptotic activity

1. INTRODUCTION

Cancer remains one of the most feared diseases of the 20th century, continuing to spread and rising in prevalence in the twenty-first century. It now accounts for 25% of all human deaths, and chemoprevention of cancer has led to a new trend of research. Human skin cancer accounts for about 30% of all new cancers and is by far the most common malignancy. Skin cancer is caused by exposure to radiation, including ultraviolet-B (UV-B) radiation, as well as other chemical or biological factors (1). The most prevalent malignancy is skin cancer, which includes both malignant melanoma (MM) and non-melanoma skin cancer (NMSC). Both MM and NMSC are on the rise, with a 0.6 % in MM for adults over 50 years old (2). Skin cancer is becoming more common across the world, necessitating the development of alternative therapeutic methods. It is also important to comprehend the aetiology and pathogenesis of skin cancer in order to find a successful treatment for this prevalent condition. Plants and phyto products are still important in the treatment of a variety of diseases, like cancer.

Plants and plant derivatives have long been recognized as effective and versatile chemopreventive agents in the treatment of a variety of cancers (3). The widespread use of plant products of cancer in Indian medicine's traditional context. Increasing evidence suggests that using medicinal plants to target cancer cells is more effective than using purified drugs (4). This is because medicinal plants contain a diverse range of bioactive compounds capable of initiating different actions rather than focusing on a single target (5). Phytochemicals have been linked to a variety of biological processes that support human health, according to several reports. Plants contain phytochemicals in the form of primary and secondary metabolites (6). Plant drug discovery is a multidisciplinary process that incorporates botanical, ethnobotanical, phytochemical, biochemical, and chemical isolation techniques (7). Despite these findings, it is important that more than 60% of commonly used anticancer agents come from natural sources, such as plants, aquatic animals, and microorganisms (8). The apoptosis mechanism is well-known as a successful and promising method for killing cancer cells. The therapeutic use of apoptosis is now being seen as a paradigm for antitumor drug production (9). Apoptosis in cancer cell lines has been confirmed to be induced by a variety of natural plant extracts and phytochemicals (10). When cells are no longer required or are severely compromised, apoptosis is the ability of a cell to cause self-destruction by activating an inherent cellular suicide programme (11). Many cancer treatments have demonstrated that inducing apoptosis of tumour cells is the most common anticancer mechanism (12).

Many phytochemicals of dietary origin have been identified as important possible chemopreventive agents as a result of a remarkable increase interest in chemoprevention science (13). Pro-apoptotic strategies can contribute to significant therapeutic progress against skin cancer, according to researchers. Proapoptotic activity is defined as involvement in a cascade that is activated in response to pro apoptotic signals that culminates in the cleavage of a group of

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proteins, leading to cell disassembly (14). Importantly, the function of various pro-apoptotic pathways in different tumour types must be elucidated and understood in order to assess drug efficacy and alter and optimise therapeutic approaches (15). *Lippia nodiflora* (Verbenaceae) is a creeping annual herb that grows locally in moist areas and is thought to have many medicinal properties in herbal medicine. The Verbenaceae family includes *Phyla nodiflora or Lippia nodiflora*, also known as frog fruit. Turkey Tangle is another name for it, as is Poduthalai in Tamil, Bukkan in Hindi, and Guo Jiang Teng in Chinese (16). Nodifloretin, nodifloridin, sitosterol, stigmasterol, hispidulin, halleridone, hallerone, and eupafolin are some of the phytochemicals found in this herb (17). *Lippia nodiflora* is said to have acrid, cooling, aphrodisiac, astringent, anthelmintic, alexiteric, emmenagogue, bactericide, diuretic, antiseptic, antitussive, antipyretic, and anti-inflammatory properties, and is useful for bronchitis, respiratory disorders, inflammation, fever, dyspepsia, hookworm, gonorrhoea, ulcers,diaper rash, erysipelas, neuralgia, sores, spasms and vertigo (18). Children with indigestion and women after birth are given an extract of the leaves and tender stalks. *Lippia nodiflora* ethnopharmacological significance for skin disorders and folk cosmetics, such as pimples, carbuncles, and skin burns, has also been discovered (19). The aerial portions of *Lippia nodiflora* contained flavonoids, hormones, glycosides, alkaloids, terpenoids, quinols, quinol glucosides, steroids, phenylpropanoids, resin, volatiles, tannins, and phenolics, according to preliminary phytochemical screening (20). As a result, these phytochemicals are thought to be responsible for the plant's pharmacological properties. Flavonoids are found in plants and have a variety of roles (21). Flavonoids are also required in higher-order plants for UV filtration, nitrogen fixation, cell cycle inhibition, and chemical messengers (22). They work on a variety of targets, including scavenging reactive oxygen species (ROS), cell cycle regulation, DNA repair mechanism activation, apoptosis induction, and metastasis inhibition (23). Numerous studies have indicated that increasing flavonoid-rich fruits and vegetables protects normal skin from carcinogens including UV-B radiation by protecting DNA (24). Flavonoids were also shown to have the ability to cause cell death pathways in melanoma, the most dangerous form of skin cancer (25). Our team has extensive knowledge and research experience that has translate into high quality publications (26). The aim of this study is to check whether *Lippia nodiflora* leaf extract has a pro-apoptotic impact on a skin cancer cell line.

2. MATERIALS AND METHODS

2.1. Reagents:

Gibco, Canada, provided the Dulbecco's Modified Eagle Medium (DMEM), Phosphate Buffered Saline (PBS), Trypsin-EDTA, and Fetal bovine serum (FBS). Sigma Chemical Pvt Ltd, USA, supplied the acridine orange (AO), ethidium bromide (EtBr), dimethyl sulfoxide (DMSO), [3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT). All other chemicals were obtained from Sisco Research Laboratory (SRL) in India and were extra pure molecular grade.

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2.2. Cell line maintenance:

The National Centre for Cell Science in Pune provided B16-F10 skin cancer cell lines. T25 culture flasks containing DMEM supplemented with 10% FBS and 1% antibiotics were used to cultivate the cells. The cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

2.3. Preparation of the herbal extract:

The present study used *Lippia nodiflora* leaf extract obtained from IMPCOPS (Chennai, India). In a static condition, 50 g of *Lippia nodiflora* leaf powder was soaked in 500 mL of 95 % ethanol and held at room temperature for three days. The solution was then filtered with crude filter paper before being filtered with whatmann paper. Following evaporation of the fine filtrate, 3 g of the substance was collected. A vacuum evaporate was used to concentrate the complete ethanol extract, which was then stored at 4°C.

2.4. Cell viability (MTT) assay:

The MTT assay was used to determine the cytotoxic effect of *Lippia nodiflora* extract in B16-F10 cells. The assay is focused on metabolically active cells converting soluble yellow tetrazolium salt to insoluble purple formazan crystals. B16-F10 cells were plated in 48 well plates at a concentration of $2x10^4$ cells/well. After 24 hours, the cells were washed twice with 500µl of serum-free medium and starved for 3 hours at 37°C by incubating in serum-free medium. After starvation, cells were given various concentrations (5, 10, 20, 30, 40, 50 and 60 µg/ml) of *Lippia nodiflora* for 24 hours. The medium from control and *Lippia nodiflora* treated cells was discarded at the end of therapy, and 200µl of MTT containing DMEM (0.5 µg/ml) was applied to each well. In the CO_2 incubator, the cells were then incubated for 4 hours at 37°C. The cells were then washed in 1x PBS after discarding the MTT-containing medium. After that, the crystals were dissolved by applying 200µl of solubilization solution, which was correctly blended by pipetting up and down. The formazan crystals were then dissolved in 200µl of dimethyl sulfoxide and incubated in the dark for an hour. The intensity of the colour developed was then measured at 570 nm using a Micro ELISA plate reader. The proportion of viable cells cultured in serum-free medium was used to calculate the number of viable cells. Without any therapy, cell viability in the control medium was represented as 100%. The IC50 was determined as the sample concentration used to reduce 50% of the absorbance. The cell viability is calculated using the formula: % cell viability = [A570 nm of treated cells/A570 nm of control cells]×100.

2.5. Morphology study:

Based on MTT assay we selected the optimal doses (IC-50: 20 µg/ml) for further studies. A phase contrast microscope was used to examine changes in cell morphology. *Lippia nodiflora* (20 µg/ml for B16-F10 cells) was used to treat 3×10^4 cells in 6 well plates for 24 hours. The medium was extracted at the end of the incubation cycle, and the cells were washed once with

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phosphate buffer saline (PBS pH 7.4). A phase contrast microscope was used to observe the morphological changes of *Lippia nodiflora* treated cells.

2.6. Determination of mode of cell death by acridine orange (AO)/ethidium bromide (EtBr) dual staining:

AO/EtBr dual staining was used to assess the effects of *Lippia nodiflora* leaf extract on B16-F10 cell death. The cells were treated for 24 hours with *Lippia nodiflora* leaf extract before being harvested and washed in ice-cold PBS. The pellets were resuspended in 5µl of EtBr (1 µg/ml) and 5µl of acridine orange (1µg/ml). A fluorescence microscope was then used to detect the stained cells' apoptotic changes.

2.7. Statistical analysis:

For comparisons between treatment and control values, one-way ANOVA was used, accompanied by Student Newman–Keuls (SNK) experiments. The data was presented as a mean with SD. The statistical significance level was set at $p<0.05$.

3. RESULTS:

3.1. The MTT test was used to determine the cytotoxic capability of *Lippia nodiflora* leaf extract in skin cancer cells.

For 24 hours, the cells were treated with varied concentrations of *Lippia nodiflora* leaf extract (5 -60 µg/ml). At the 24 hour time period, *Lippi nodiflora* leaf extract dramatically reduced the viability of B16-F10 cancer cells compared to control (Figure-1).With increasing concentration, the percentage of viable cells decreased steadily. At a dose of 20 µg/ml, we found a 50% growth inhibition. As a result, the IC-50 dosage (20µg/ml) was chosen for future testing.

3.2. Inverted phase contrast microscope was used to examine the cell morphology of *Lippia nodiflora* leaf extract treated skin cancer cells.
The B16-F10 cells were treated with *Lippia nodiflora* leaf extract (20 µg/ml) for 24 hours, and the treated cells displayed substantial morphological changes that are indicative of apoptotic cells, such as cell shrinkage and lower cell density, as compared to the untreated cells **(Figure 2).** Apoptosis cells also showed additional morphological alterations, such as rounder cells that shrank and lost contact with nearby cells. Some of the plates' sensitive cells were even removed from the surface.

3.3. The induction of apoptosis in *Lippia nodiflora* leaf extract-treated skin cancer cells was confirmed by Acridine orange/Ethidium bromide (AO/EtBr) dual staining (Figure 3).

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The nuclear morphology of apoptotic cells is assessed with AO/EtBrdual staining. *Lippi nodiflora* leaf extract (20 µg/ml) was treated to the cells for 24 hours. Following treatment, the cells were stained for 20 minutes with both AO/EtBr dye and examined under fluorescence microscopy. The results revealed that AO stained both living and dead cells, but EtBr exclusively stained those that had lost membrane integrity. Green staining indicates healthy cells, yellow staining indicates early apoptotic cells, and orange staining indicates late apoptotic cells. Control cells in this study were uniformly green, but *Lippi nodiflora* leaf extract-treated cells displayed yellow, orange, and red signals. According to these findings, *Lippi nodiflora* leaf extract causes apoptosis in skin cancer cells.

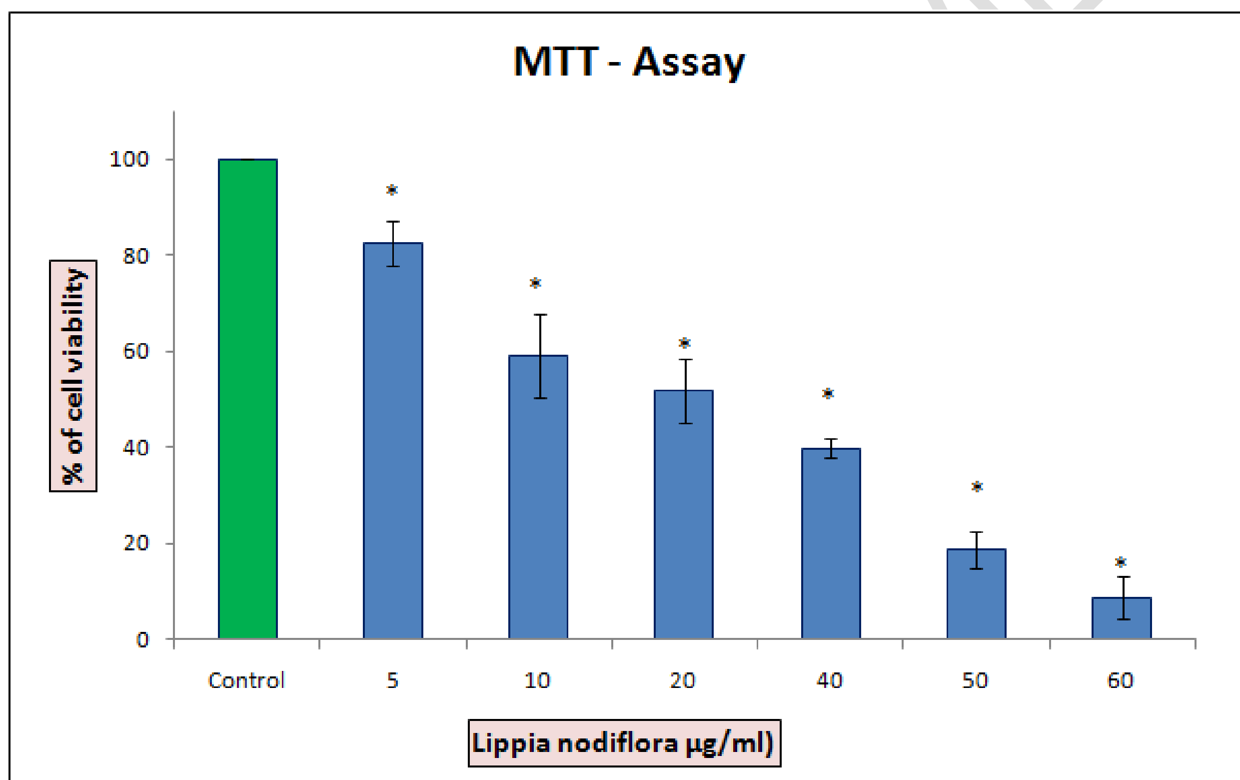

Figure 1: *The MTT assay was used to assess the cytotoxic activity of Lippia nodiflora on the B16-F10 cell line. For 24 hours, the cells were exposed to various concentrations (5, 10,20,30,40,50,60 µg/ml). The inhibitory concentration (IC-50), which was determined to be 50% inhibition at a concentration of 20 µg/ml, (p value: 0.0085) has significance and will be used in future experiments.*' represents statistical significance between control versus treatment groups at $p < 0.05$ level using Student's–Newman–Keul's test.*

20 X Control *Lippia nodiflora* leaf extract (20 µg/ml)

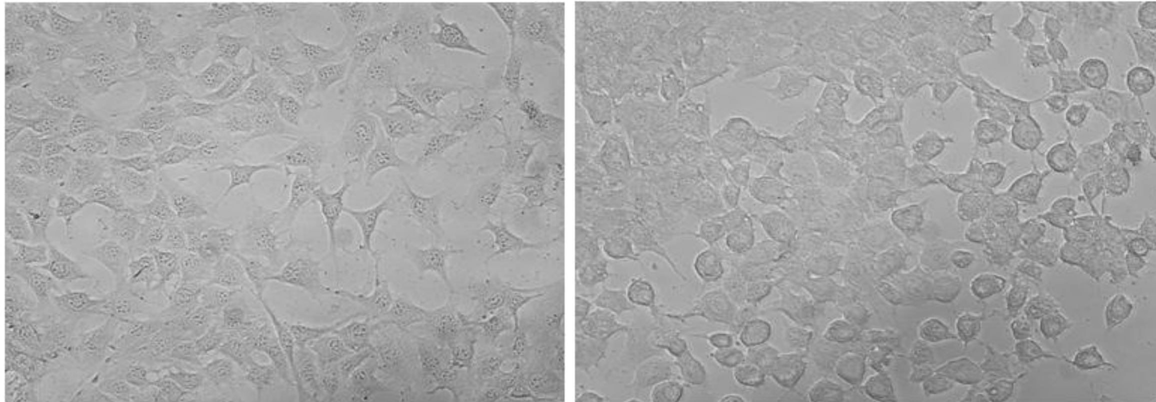

Figure 2: *Indicates the morphological changes in B16-F10 skin cancer cell line upon and without and with the treatment of Lippia nodiflora at 20 µg/ml for 24h by phase-contrast microscope at 20x magnification. The number of cells decreased after treatment and the cells exhibited cell shrinkage and cytoplasmic membrane blebbing.*

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20X Control *Lippia nodiflora* leaf extract (20 µg/ml)

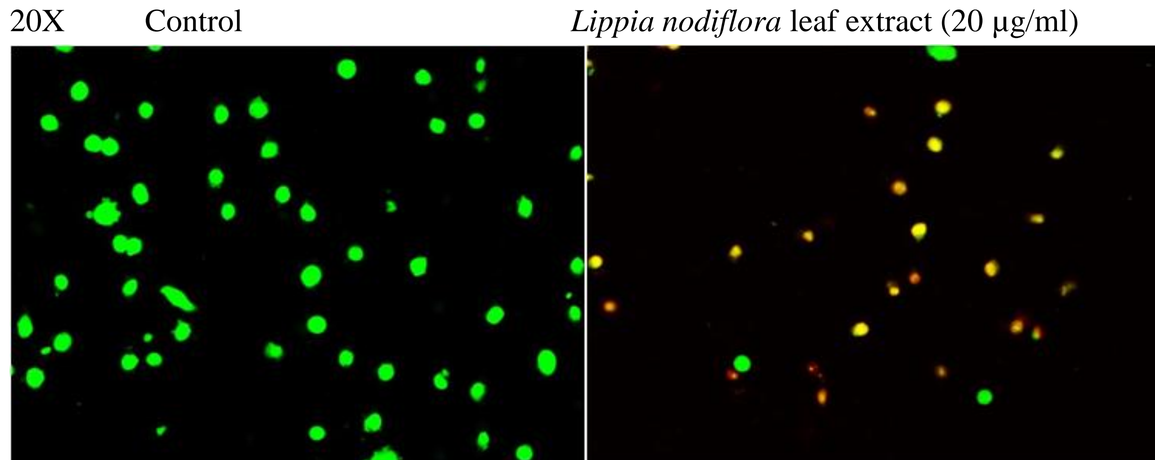

Figure 3: *Indicates the acridine orange and ethidium bromide (AO/EtBr) dual staining of treated B16-F10 skin cancer cell line upon without and with the treatment of Lippia nodiflora at for 24h, viewed under fluorescence microscope at 20x magnification. The viable cell will possess a uniform bright green nucleus. Early apoptotic cells will have bright orange areas of condensed or fragmented chromatin in the nucleus. Late apoptotic cells will have uniform bright red nucleus.*

4. DISCUSSION

Humans first used natural materials present in the ecosystem to treat different illnesses on an objective basis (27). While allopathic medicine has the potential to treat a wide variety of diseases, its high costs and side effects are prompting many people to seek out natural therapies with less side effects (28). *Phyla nodiflora* is an essential member of the verbenaceae family with a wide range of medicinal applications. It may be a source of traditional medicine (29) . The herb was used as an aphrodisiac and diuretic in Ayurveda and Unani medicine to cure heart disease, ulcers, bronchitis, fevers, colds, knee joint pain, and lithiasis. *Lippia nodiflora* (Verbenaceae), also known as Poduthalai in Tamil, is a spreading annual herb that grows locally in moist areas and has many medicinal properties according to traditional medicine. Since human cancer cell lines maintain hallmark characteristics of cancer cells, are easy to propagate, and can be genetically engineered to provide reproducible outcomes, they have become the most widely used laboratory models (30). Results achieved from cell lines are also extrapolated to human tumours *in vivo*. A variety of methods for studying cell viability and proliferation in cell culture have been developed in recent years. From this study we observed the pro-apoptotic activity of *Lippia nodilfora* leaf extract. It was found that IC-50 concentration was found to be 20 µg/ml in skin cancer cells. It was effective in inducing apoptotic activity in the B16-F10 skin cancer cell line which was observed by dual AO/EtBr fluorescent staining. Under a fluorescent microscope, dual AO/EtBr fluorescent staining can be used to detect apoptosis-associated changes in cell membranes during the apoptosis phase.

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This procedure may also differentiate between cells in various stages of apoptosis. As a result, AO/EtBr staining may be used in DSTs (31). The morphological changes in cells treated with *Lippia nodiflora* leaf extract were also detected, and the findings were significant. A previous study found that when cells were treated with *Lippia nodiflora* extract, a proapoptotic factor that induces nuclear disassembly in apoptotic cells, was upregulated. The pro-apoptotic protein is commonly present in the intermembrane space of mitochondria. It will be translocated into the nucleus upon apoptotic activation, causing chromosome condensation and fragmentation (32). Furthermore, its proapoptotic effect has been discovered to be caspase-independent (33). When used at non-toxic concentrations, *L.acutangula* and *L. nodiflora* extracts demonstrated significant antiproliferative effect on lung cancer cells in another study using NCI-H460 cancer cells (19). The American National Cancer Institute (NCI) developed a cytotoxic activity threshold of less than 30μg/ml in the preliminary assay for extracts with possible anti-cancer activity. Both extracts are effective anticancer therapeutic agents because their IC50 concentrations in the NCI-H460 cell line were less than 30 μg/ml (34).

The IC50 concentration in present study was at 20 μg/ml, which was similar to the present study. The interaction of caspase substrate cleavage during apoptosis has been documented in a previous study, and the main morphological alterations including chromatin condensation, nuclear remodelling, and membrane blebbing have been reported by *Lippia nodiflora* treatment. During a 24-hour incubation time, leaf extracts significantly increased intracellular reactive oxygen species (ROS) synthesis on NCI-H460. The phenomenon is reasonable because intracellular ROS aggregation is one of the main mechanisms contributing to early apoptosis (35). Apoptosis pathways, which are regulated by cell-intrinsic responses and immune-mediated extrinsic signals, have effective cancer-prevention mechanisms (Death ligands, such as tumour necrosis factor-alpha (TNF-alpha) and TNF-related apoptosis-inducing ligand (TRAIL), or granzyme B, initiate intrinsic pro-apoptotic pathways. The activation of a caspase cascade results in cell death when these pathways are activated. Inactivation of pro-apoptotic pathways is critical for tumorigenesis and may be the source of therapy resistance (36). As a result, apoptosis-based techniques are critical instruments in the advancement of successful cancer therapies (37). The aim of these treatments is to reactivate p53, decrease anti-apoptotic Bcl-2 proteins, and increase extrinsic, death receptor-mediated pathways. The initial effects of apoptosis-based techniques are encouraging. *P. nodiflora* prevents the growth of MCF-7 cells by causing apoptosis and disrupting cell cycle progression, according to another analysis using separate cell lines (38).

Melanin protects the skin from ultraviolet (UV) light, which can cause skin cancer and burns. Melanogenesis is the mechanism by which melanocytes in the basal epidermal layers develop melanin. Asians, and especially Indians, have a Fitzpatrick scale score of III to IV (39). These skin types have a medium pigmentation and are prone to hyperpigmentation when exposed to UV light, resulting in melasma, chloasma, and solar lentigo (40). Phosphorylation of mitogen-

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activated protein kinases (MAPKs), such as extracellular signal-regulated kinase (ERK), AKT, and p38, effectively modulates the transcription of MITF, resulting in melanin overproduction, according to previous research (41). As a result, *Phyla nodiflora*, a strong skin whitening agent, can reduce melanin production. A previous study found that isolated compound-like flavonoids had outstanding antioxidant efficacy in all antioxidant assays and prevented lipid peroxidation substantially at a concentration of 50 µg/ml (42). The findings suggested that extracts from *L. nodiflora* or phytocompounds extracted from it could be used as a bioactive source of natural antioxidants with beneficial health effects (43). Secondary metabolites found in medicinal plants, such as flavonoids, are now considered to treat a variety of chronic diseases, including inflammation and cancer (44). Flavonoids are divided into flavones, flavonols, isoflavones, flavanones, flavanols, and anthocyanidins, with flavones being the most common (45). Flavonoids found in *Lippia nodiflora* are thought to be responsible for causing these pro-apoptotic effects in skin cancer cells. However, further research is needed to confirm this compound's effect (46). As a result, treating skin cancer with *Lippia nodiflora* leaf extract will be a better option in terms of treating the cancer without causing any adverse side effects (10, 47-60).

6. CONCLUSION:

Overall, the findings of this study showed that the plant extract *Lippia nodiflora* was cytotoxic and caused pro-apoptosis in cancer cells at the 20 µg/ml concentrations and 24h incubation time. However, further research is required to fully comprehend the mechanisms of this plant extract's pro-apoptotic action.

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NOTE:

The study highlights the efficacy of "herbal medicine" which is an ancient tradition, used in some parts of India. This ancient concept should be carefully evaluated in the light of modern medical science and can be utilized partially if found suitable.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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