Phytochemical, Botanical, Microbiological, Biochemical and Biotechnological Studies of *Naregamia alata*

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ABSTRACT

The phytochemical compounds attributing to antimicrobial, antioxidant and anticancer properties of *Naregamia alata* leaf extracts were selected as the primary aim in this study. Phytochemical analysis were carried out for methanol, acetone, petroleum ether and water extracts. Minimal inhibitory concentration and antibacterial activity was determined against significant test bacteria causing nosocomial infections. Antioxidant activity was investigated using three different scavenging assays. Anticancer activity of the methanol extracts were evaluated in MCF_7 cell lines using apoptosis and DNA fragmentation studies. Phytochemical analysis revealed the presence of alkaloids, phenols, flavonoids, tannins and terpenoids in higher level for methanol extracts of *Naregamia alata*. Free radical scavenging tests conducted in this study revealed the *in vitro* antioxidant properties of the *Naregamia alata* extract based on the obtained IC_{50} values. Minimal inhibitory concentration of 400µg/ml and 500µg/ml was recorded for the test organisms. Antibacterial activity showed significant inhibitory zones ranging from 16mm to 21mm for *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Enterobacter aerogenes and Klebsiella pneumoniae*. Anticancer potential of *Naregamia alatai* leaf extracts was confirmed by EtBr staining. Results showed that methanol extracts of *Naregamia alatai* were highly efficient at inducing apoptosis in cancer cells. DNA fragmentation analysis revealed that DNA migrated as discrete bands and gave a ladder for the cells exposed with plant extracts which significantly considered to be a hall mark of apoptosis. In conclusion, the present findings emphasized the potential of *Naregamia alatai* as a resource for the discovery of novel antimicrobial and anticancer agents.

Keywords: *Phytochemical, Antibacterial, Antioxidant, Anticancer, MCF_7, Apoptosis*

INTRODUCTION

Medicinal plants have been used in traditional health care systems since prehistoric times and are still the most important health care source for the most of the world's population (Mathew George et al., 2017). Indian medicinal plant *Naregamia alata* Wight belonging to family Meliaceae is a shrub which is mainly found in the rocky and grass slopes. In traditional medicine they can be used for the treatment of chronic fever, rheumatism, itch, wounds, emesis, halitosis, anaemia, ulcers, vitiated conditions of varta and pitta, pruritis, dysentery (Raja et al., 2016). The macroscopical studies revealed that the leaves were alternate, three foliate with unrigged petioles, the leaflets were small, cuneate, obovate. The root is a cholagogue, emetic and expectorant and contain an alkaloid called naregamin (Uphof, 1959). The plant has anti-rheumatic activity and the leaf and stem are anti-bilious. The plant is used in prescriptions for malarial and chronic fevers, anemia and enlarged spleen. The root and stem gave heneicosane, beta-sitosterol, stearic and palmitic acids (Chopra et al., 1986).

The previous studies on this herb have shown its pharmaceutical (Shinya et al. 2012), pharmacological (Jacob et al. 2012), antioxidant (Anagha, 2013), and antimicrobial (Mukesh et al. 2011) activities. The phytochemical constituents detected in the root extract of *Naregamia alata* could contribute to the traditional therapeutic use of the whole root. The compounds were found to exhibit various important pharmacological activities anti-oxidant, anticancer, antibacterial, antifungal, antiviral, anti-inflammatory, anti-malarial, activities. Hence, different phytochemical compounds of the plant extracts was thus investigated for its antibacterial activity against *Staphylococcus aureus, Escherichia coli, Enterobacter* sp, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* in the present study.

These phytochemical antioxidant compounds can be isolated and it is used for the prevention and treatment of free radical disorders (Middleton et al., 2000). The antioxidants play a vital role in delaying, intercepting or preventing oxidative reactions catalyzed by free radical. Antioxidants eradicate chemicals in the body and protect against heart disease, arthritis, cancer and many other chronic diseases. Plant-derived antioxidant compounds can effectively eliminate the free radicals mediated toxicity, and many of these activities have been already reported for a wide range of plants (Sulekha et al. 2009) Therefore, search for natural antioxidant has significantly been increased in the recent scenario. As there are no much scientific references regarding the antioxidant and anticancer activity of *Naregamia alata*, the present investigation was undertaken to examine the antioxidant activities by DPPH (2, 2 –diphenyl -1-picryl hydrazyl radical) free radical scavenging assay, Hydroxyl radical scavenging assay, and Nitric oxide scavenging assay, and anticancer activity by Apoptosis Fluorescence Studies and DNA fragmentation studies.

Medicinal plants represent a rich source of antimicrobial agents. A wide range of medicinal plant parts are used for extract as raw drugs and they possess varied medicinal properties (Uniyal et al., 2006). Although hundreds of plant species have been tested for antimicrobial properties, the vast majority of have not been adequately evaluated (Balandrin et

al., 1985). As per literature survey only scanty information was available on the antibacterial effect of *Naregamia alata*. It has antimicrobial property against gram-positive and gram negative bacteria. The phytochemical compounds, like terpenoids, tannins, phenols, flavonoids, etc were found attributing to the antibacterial properties of plant extract.

MATERIALS AND METHODS

Collection and processing of *Naregamia alata* (Nella naragam) leaves

Naregamia alata leaves were collected from the farm house, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India. The leaves were pre-washed in deionised water and kept under shade until completely dried for milling.

Soxhlet extraction of *Naregamia alata* (Nella naragam) leaves

In the Soxhlet extraction method, finely ground sample - *Naregamia alata* leaf powder was placed in a porous bag or “thimble” made from a strong filter paper or cellulose, which is placed, is in thimble chamber of the Soxhlet apparatus. Extraction solvent is heated in the bottom flask, vaporizes into the sample thimble, and condenses in the condenser and drip back. When the liquid content reaches the siphon arm, the liquid contents is emptied into the bottom flask again and the process is continued. For the study, infusion method of Soxhlet Extraction had been adopted. The powdered herbs of *Naregamia alata* were filled in the thimble and placed in the soxhlet extractor. The extractor had been filled with solvent solution of ethanol and the temperature of 60ºC was set and left for 6hours. Slowly and steadily the temperature was increased upto 100ºC. The extract from the thimble was collected in the round bottom flask kept in the heating mantle below by passing through a side arm tube.

Qualitative phytochemical analysis of *Naregamia alata* (Nella naragam) extracts

Phytochemical analysis of the ethanol extracts of *Naregamia alata* was subjected to detect the presence of alkaloids, flavonoids, tannins, saponins, steroids, terpenoids, glycosides, phenols, carbohydrates and proteins using standard method described by Ramaan (2006).

Antioxidant activity of herbal extract

Free radical scavenging activity on DPPH

The antioxidant activity of the sample was determined in terms of hydrogen donating or radical scavenging ability, using the stable radical DPPH, according to the method of Blois (1958). *Naregamia alata* extracts at various concentrations (20 - 100µg) was taken and the volume was adjusted to 100µl with methanol. 5ml of 0.1mM methanolic solution of DPPH was added and allowed to stand for 20min at 27°C. The absorbance of the sample was measured at 517nm. The capability to scavenging the DPPH radical was calculated by using the subsequent formula.

$$\% \text{ inhibition} = \{(A0 - A1)/A0)*100\}$$

Where, A0 is the absorbance of the control reaction, and A1 is the absorbance in presence of all of the extract samples and reference. All the tests were performed thrice and the results were averaged.

The analysis was performed in triplicate. The sample concentration providing 50% inhibition (IC_{50}) under the assay condition was calculated from the graph of inhibition percentage against sample concentration. Data were analyzed using the statistical analysis system SPSS (SPSS software for windows release 17.5; SPSS) Estimates of mean, standard error for aforesaid parameters were calculated.

Hydroxyl radical scavenging activity

Deoxyribose assay was used to determine the hydroxyl radical scavenging activity in an aqueous medium. The reaction mixture containing $FeCl_3$ (100µM), EDTA (104µM), H_2O_2 (1 mM) and 2-deoxy- D-ribose (2.8mM) were mixed with *Naregamia alata* extracts at various concentrations (20 - 100µg) in 1 ml final reaction volume made with potassium phosphate buffer (20mM, pH 7.4) and incubated for 1hr at 37°C. The mixture was heated at 95°C in water bath for 15min followed by the addition of 1ml each of TCA (2.8%) and TBA (0.5%). Finally the reaction mixture was cooled on ice and centrifuged at 5000rpm for 15min. Absorbance of supernatant was measured at 532nm. All readings were corrected for any interference from brown color of the extract or antioxidant by including appropriate controls. The negative control without any antioxidant or CPLL was considered 100% deoxyribose oxidation. The percentage hydroxyl radical scavenging activity of test sample was determined accordingly in comparison with negative control using the above formula. Ascorbic acid was taken as the positive control.

Superoxide scavenging assay

As described by Srinivasan *et al* [11] the superoxide anion scavenging activity was measured. The superoxide anion radicals were produced in 3.0 ml of Tris – HCL buffer (16 mM, pH 8.0), containing 0.5 mL of NBT (0.3 mM), 0.5 mL NADH (0.936 mM) solution, 1.0 mL extract of different concentration (20 - 100µg), and 0.5 mL Tris – HCl buffer (16mM, pH 8.0). The reaction was started by adding 0.5 mL PMS solution (0.12 mM) to the mixture, kept warm at 25oC for 5 min. The absorbance was measured at 560 nm against a blank sample, ascorbic acid. The percentage inhibition was computed by comparing the results of the test with those of the control using the above formula.

Minimum inhibitory concentration (MIC) of *Naregamia alata* extracts against bacteria

Minimum inhibitory concentration (MIC) of extracted *Naregamia alata* was determined by standard Micro broth dilution method. The test organisms (*Staphylococcus aureus*, *Escherichia coli*, *Enterobacter* sp, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*) were grown in 5ml of sterile Nutrient broth (Peptone: 5g/L, Yeast extract: 5g/L, Beef extract: 3g/L, Sodium chloride: 5g/L, and Final pH - 7.0± 0.2) for 24hr at 37°C. The growth of the organism was measured based on the turbidity in the test culture tubes. The turbidity of microbial suspension was compared and adjusted to 0.5 on the McFarland scale.

To determine the MIC of *Naregamia alata*, a set of Mueller-Hinton broth tubes (5 tubes) were prepared under sterile conditions for each test organism. About 100µl of extracted *Naregamia alata* at different concentrations (100µg/ml, 200µg/ml, 300µg/ml, 400µg/ml and 500µg/ml) was added to each Mueller-Hinton broth tubes. Followed by about 10µl of test culture suspension was added to the broth separately and observed for turbid growth. All the tubes were incubated for 24hr at 37°C. The lowest dilution inhibiting the growth of test organism was selected as the minimum inhibitory concentration (MIC) of *Naregamia alata*.

Antibacterial activity of *Naregamia alata* extracts

The antimicrobial activity of *Naregamia alata* was evaluated against the test organisms by well diffusion method. All the test cultures (*Staphylococcus aureus*, *Escherichia coli*, *Enterobacter* sp, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*) were inoculated in a sterile Nutrient broth (Peptone: 5g/L, Yeast extract: 5g/L, Beef extract: 3g/L, Sodium chloride: 5g/L, and Final pH - 7.0± 0.2) and allowed to attain the growth for 24 to 48 hours. Sterile Mueller-Hinton Agar (Acid hydrolysate of Casein: 17.5g/L, Starch: 1.5g/L, Sodium chloride: 5.0g/L, Agar 17.0g/L, and Final pH - 7.0 ± 0.2) plates were prepared and allowed to solidify. About 0.1% inoculum suspensions of each test organisms were swabbed uniformly over the agar surface separately. Under sterile conditions, 6mm wells were cut on the agar surface of each NA plates. About 20µl of each herbal extract fractions were loaded into the well and the plates were incubated at 37ºC for 24h. The antimicrobial activity was evaluated in terms of zone of inhibition around the wells in all the inoculated NA plates. The inhibition clear zones were measured and recorded in millimeter.

Anticancer studies

Apoptosis fluorescence

Cell culture

MCF_7 (Breast cancer cells) cell line were cultured in liquid medium (DMEM) supplemented 10% Fetal Bovine Serum (FBS), 100µg/ml penicillin and 100µg/ml streptomycin, and maintained under an atmosphere of 5% CO_2 at 37°C.

Apoptosis fluorescence assay

MCF_7 (Breast cancer cells) is an established and well-characterized cell line that has demonstrated reproducible results. RPMI-1640 medium supplemented with fetal bovine serum used as culture medium. The cells were grown in RPMI-1640 medium supplemented with 10% FBS. Cells (~10^5) were seeded in the well and incubated at 37°C for 24h. Samples were added at different concentration in triplicate. Cells without sample served as control. The plate was incubated for 24h. After incubation, the medium was completely removed and centrifuged. To the pellet EtBr stain (1µg/ml) was added in and observed under inverted phase contrast microscope.

DNA Fragmentation

Cell culture

MCF_7 (Breast cancer cells) cell line were cultured in liquid medium (DMEM) supplemented 10% Fetal Bovine Serum (FBS), 100µg/ml penicillin and 100µg/ml streptomycin, and maintained under an atmosphere of 5% CO_2 at 37^oC.

DNA fragmentation assay

Briefly, MCF_7 cells were seeded in a six-well plate at a density of 1 x 10^6 cells/well and incubated for 24h at 37°C in a humidified 5% CO_2 incubator. The wells were washed with sterile PBS and treated with 200µg/ml and 400µg/ml of plant extract samples in a serum free DMEM medium and incubated for 24h at 37°C in a humidified 5% CO2 incubator. The HepG2 cells were harvested by trypsinization in a 1.5ml tube and centrifuged at 10,000rpm for 10min. After centrifugation the supernatant was discarded and 500µL of lysis buffer was added to the cell pellet, incubated in room temperature for 1hr. Then 700µL of phenol-chloroform-iso amyl alcohol was added and mixed by inversion, and then centrifuged at 10000 rpm for 5min. The aquatic (upper) phase was transferred into a new eppendorf tubes. An equal volume of cold isopropanol was added into tubes, and mixed gently by inversion. The tubes were then centrifuged at 10000 rpm for 5min and discarded the supernatant followed by the pellet was air-dried for 30min. Then, the dried DNA was dissolved in 50µL distilled water. Furthermore, the extracted DNA was quantified by UV spectrophotometer using optical density (OD) at 260nm. Then the equal amount of each DNA samples was run at 0.8% of agarose gel electrophoresis along with 100bp ladder. The image was captured by gel documentation system (BioRad USA).

RESULTS AND DISCUSSION

Extraction of *Naregamia alata* plant extracts

Naregamia alata extracts were prepared using 4 different solvents in a standard soxhlet apparatus. The collected leaves and powdered parts of plant was presented in Fig. 1. The extracts of each solvents from soxhlet were presented in Fig.2. The extracts were found clear in screw cap bottles; stored in dark cool place to avoid sunlight and humid.

Phytochemical analysis of *Naregamia alata* extracts

Naregamia alata belongs to Meliaceae family; its shrub are used in the ancient days for treating wounds, ulcers, chronic fever, rheumatism, dysentery and anaemia. The extract was used as anti-rheumatic agents; its phytochemical constituents play a vital role in the field of pharmacological science as antioxidant, anticancer and antibacterial compounds. In the present study, phytochemical analysis of the plant extracts revealed the presence of different compounds. In Table-1, the presence and absence of the compounds were expressed in terms of positive and negative signs. From the obtained results it was evident that alkaloids, phenols, flavonoids, tannins and terpenoids were found present in higher level for methanol extracts of *Naregamia*

alata. Acetone extracts and petroleum ether extracts showed the presence of alkaloids, phenols, flavonoids and terpenoids comparatively lesser levels than methanol extracts. Water extracts showed the presence of only phenols and tannins. The presence of many significant phytochemical compounds were found attributing to different biological properties like antibacterial, antifungal, antioxidant, and anticancer. As per literature survery, terpenoids were found to exhibit various important pharmacological activities anti-inflammatory, anticancer, anti-malarial, inhibition of cholesterol synthesis, antiviral and antibacterial activities (Kappers et al. 2005). Tannins have immense attention in many fields especially in the fields of nutrition, health and medicine due to their antioxidant, antimicrobial and anti-inflammatory activity (Buzzini et al. 2008). Flavonoids, a group of polyphenolics, are free radical scavengers, super antioxidants that have anti-inflammatory activity, prevent oxidative cell damage through their water soluble property and also possess strong anti-cancer activity (Gurib-Fakim, 2006). The results obtained confirm earlier reports of some of the phytochemical constituents found in the leaf extracts of *Naregamia alata* (Shinya *et al.* 2012). The researchers concluded that phytochemical analysis conducted on the plant root extract revealed the presence of constituents such as alkaloids, glycosides, terpenoids, phenols, tannin, flavanoids, and coumarin. More research is required to determine the specific roles of these phytochemical constituents present in *Naregamia alata.*

Anti-oxidant activity of *Naregamia alata* extracts

***In vitro* antioxidant assays**

Free radical scavenging tests conducted in this study sought to establish the in vitro antioxidant properties of the *Naregamia alata* extract. The detection of phytochemical compounds and other secondary metabolites in the extracts strongly suggests the possible antioxidant activity of the *Naregamia alata*. Phytochemcial analysis in the previous section thus revealed the presence of phenolic compounds which attributes to phenolic antioxidants. Several concentrations ranging from 12.5–200µg/ml of the methanolic leaf extract of *Naregamia alata* was tested for their antioxidant activity. It was observed that free radicals were scavenged by the test compounds in a concentration dependent manner up to the given concentration. The percentage scavenging and IC_{50} values were calculated for all models.

DPPH radical scavenging activity

The effect of antioxidants on DPPH radical was thought to be due to their hydrogen donating ability to the free radicals and reducing it to nonreactive species (Wang *et al.* 2008). The antioxidant compounds reduce the purple coloured DPPH radical to yellow. Here the reduction capability of DPPH radicals was determined by the decrease in its absorbance at 517nm induced by antioxidant. The DPPH radical scavenging activity of the leaf extract at 100µg/ml was 75 ± 1.05% and that of the standard ascorbic acid was 69 ± 1.05% (Table-2). The results showed that the extract exhibited strong DPPH radical scavenging ability (IC_{50} value of 28.34µg/ml) than the ascorbic acid (IC_{50} value of 48.56µg/ml. These observations support the earlier reports of Mohammed *et al.* (2012) who established the fact that methanol extract of *Naregamia alata* showed strong antioxidant activity with IC_{50} value of 7.5µg/ml.

Hydroxyl radical scavenging activity

The hydroxyl radical can induce oxidative damage to DNA, lipids, and proteins. At a concentration of 100µg/ml, the scavenging activity of methanol extract and the standard ascorbic acid was found to be 62 ± 0.75% and 57 ± 1.05% respectively (Table-3). Of these, the leaf extract (IC50 value of 24.6µg/ml) was found to be more efficient compared with the standard ascorbic acid (IC50 value of 30.4µg/ml) in quenching the hydroxyl radicals produced in the reaction mixture.

Superoxide radical scavenging activity

The maximum inhibition was found to be 69 ± 1.05% for methanolic leaf extract and 60 ± 0.57% for ascorbic acid at 100µg/ml (Table-4). The leaf extract exhibited an IC_{50} value of 21.4µg/ml and standard ascorbic acid was 28.6µg/ml (Figure 5). The obtained extract was found to be an efficient scavenger of superoxide radical. Superoxide anion radical is one of the strongest ROS among the free radicals and gets converted to other harmful reactive oxygen species such as hydrogen peroxide and hydroxyl radical, damaging biomolecules which results in chronic diseases (Al-Mamun *et al*. 2007).

The obtained results from three scavenging assays suggested that the methanol leaf extracts of *Naregamia alata* have many bioactive compounds which show antioxidant activity. Extracts of *Naregamia alata* exhibited significant antioxidant activity compare to standard ascorbic acid. The leaf extract might be helpful in preventing and slowing the progress of various oxidative stress induced diseases. It might be useful for the development of newer and more potent natural antioxidants. The findings of the present study suggest that *Naregamia alata* could be a potential source of natural antioxidant that could have great importance as therapeutic agents in preventing or slowing the progress of oxidative stress related degenerative diseases.

Minimum inhibitory concentration (MIC) of *Naregamia alata* extracts

Minimum inhibitory concentration studies were performed using the plant extracts using five different concentrations against 5 test bacteria. Broth dilution method was used in the present study. The visible growth of the bacteria was confirmed based on the turbidity obtained; and further the turbidity was compared with the McFarland solution. Based on this, the test cultures (*Staphylococcus aureus, Escherichia coli* and *Enterobacter* sp) produced visible growth in the first three tubes with respective concentrations of 100µg/ml, 200µg/ml and 300µg/ml. But no growth was observed from the concentration 400µg/ml for the same respective test bacteria. Interestingly, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* showed evident turbid growth till concentration of 400µg/ml; but no growth was observed from the concentration 500µg/ml. From the obtained results (Table-5), it was proved that the MIC of 400µg/ml and MIC of 500µg/ml was recorded for the respective test organisms.

Antibacterial activity of *Naregamia alata* extracts

The antibacterial activity of *Naregamia alata* methanol extracts against five test bacteria, *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Enterobacter aerogenes and Klebsiella pneumoniae* was evaluated by agar diffusion method. Selected MIC concentration was used to determine the antibacterial activity against each organism. Significant inhibitory zones were found evident for the methanolic plant extracts against all five test organisms. In Table-6, the zone of inhibition measured in millimetre for the extract samples, negative control and positive control samples were presented. In Fig. 3, the inhibitory zones for positive control and sample extracts were found much evident.

The minimal inhibitory concentration and the antimicrobial activity of *Naregamia alata* extracted from the leaves using methanol revealed the mode of action of phytochemical constituents present in it on the microbial cell constituents. Phytochemical analysis of the leaf extracts showed the presence of almost all compounds attributing to antimicrobial and antioxidant properties. These characteristics were found well supportive when compared to the details studied from literatures. According to Rosas-Pinon et al., (2012), antibacterial inhibitory zones obtained during the analysis revealed that the pharmacological active compounds present in the herbal extracts disrupt the cell membrane of the microbes through the physical and ionic phenomenon. The compounds present in the extract thus inhibited growth of test organisms by using an electrochemical mode of action to penetrate and disrupt their cell walls. When the cell walls are penetrated, leakage of metabolites occurs and other cell functions are disabled, thereby preventing the organism from duplication (Rosas-Pinon et al., 2012). The mechanisms of action of the plant extracts include, degradation of the cell wall (Helander et al., 1998), damaging the cytoplasmic membrane, cytoplasm coagulation (Ultee et al., 2002), damaging the membrane proteins, increased permeability leading to leakage of the cell contents (Lambert et al., 2001), and reducing the proton motive force (Ultee et al., 2001). Shan et al., (2007) highlighted that the significant activity exerted in this present research was due to the fact that the natural bioactive compounds responsible for the antibacterial activity are mostly extracted in the solvents and these active compounds may be able to penetrate the thick cell walls through general diffusion channels formed by the bacterial porins present therein, and affect the bacterial enzymes that are responsible for survival and virulence of organisms resulting in cellular lysis (Shan et al., 2007).

The results in our present research were found to be highly significant in terms of antimicrobial activity against bacteria and fungi. The above facts on the mode of action of *Naregamia alatai* extracts on microbial cell contents were found to be the reason for our obtained results. And hence the action of *Naregamia alatai* extracts and their components on bacteria or fungi remains a focal area for future research. The study of the synergistic effects among *Naregamia alatai* extracts and their phytochemical compounds could ensure the antimicrobial properties with reduced concentrations required for hygienic, safety and health-care.

Anticancer studies of *Naregamia alatai* extracts

Apoptosis fluorescence

In addition to the observation of antioxidant assays, the anticancer potential of *Naregamia alatai* leaf extracts was confirmed by EtBr staining in each treatment group and the results were presented. In Fig. 4, apoptotic MCF_7 (Breast cancer cells) showing membrane and nuclear changes when stained with ethidium bromide after exposing to 10µg/ml, 25µg/ml, 50µg/ml and 70µg/ml of extract concentration at 100× magnification were observed. These pictures indicate that the *Naregamia alatai* were highly efficient at inducing apoptosis in cancer cells. The plant extract samples selected at different concentrations found to exhibited characteristic morphological changes typical of apoptosis, including cell shrinkage, plasma membrane blebbing, chromatin condensation, and nuclear fragmentation, when compared to control cells with prominent rounded nuclei and defined plasma membrane contours (Fig. 4A and 4B).

The phytochemicals isolated from *Naregamia alatai* extracts are glycosides, tannins, phenols and steroids and are predicted to be responsible for this anticancer activity. Many plant extracts possessing anti-oxidant principles were reported to have anticancer in nature. Based on this, it was intended to work on this plant; and the present study showed that there is a reduction in the cancer cell number count after the usage of plant leaf extracts.

Similar anticancer activity was observed for methanol extracts of *Naregamia alatai* roots by Velayudhan et al., (2016). The researchers evaluated the anticancer activity in A_{549} (Lung), $HepG_2$ (Liver) and HeLa (Cervical) cell lines. The IC_{50} values were found to be 45µg/ml and 67.5µg/ml for root methanol extract against $HepG_2$ and HeLa cell lines, compared with the standard drug doxorubicin were 16.25µg/ml and 25µg/ml by MTT assay. The researchers concluded that the extract showed dose dependent anticancer activity with root extract being more potent against $HepG_2$ cell lines than A_{549} and HeLa cell lines. This result was found supportive to the present research analysis in terms of anticancer activity.

DNA fragmentation

DNA fragmentation is a process, which damages DNA, leading to cell death that occurs via the activation of certain intrinsic agents. This cleavage produces ladders of DNA fragments that are the size of integer multiples of a nucleosome length (180–200bp). The DNA fragmentation is initiated by caspase-3 activation of inactive caspase-activated deoxyribonuclease (CAD), through removal of its inhibitors, such as inhibitor of CAD (Green and Reed, 1998). As a biochemical hallmark of intrinsic apoptotic cell death, DNA fragmentation was used to determine whether the anticancer effect of *Naregamia alatai* leaf extracts on cells occurs via the activation of caspases, mainly caspase-3.

In the present study, the electrophoretic analysis of MCF_7 DNA treated with 200µg/ml and 400µg/ml of plant extracts, and MCF_7 (untreated) were presented in Fig.5. The DNA from untreated cells was not fragmented, but the *Naregamia alatai* extract treated samples showed

positive DNA fragmentation for both concentrations. The DNA migrated as discrete bands and gave a ladder. Such DNA ladders are considered to be a hall mark of apoptosis. Continuous smears observed in the image for both concentration treated also indicates the fragmentation of DNA due to apoptosis.

Conclusion

The findings of the present study suggest that *Naregamia alata* could be a potential source of natural antioxidant that could have great importance as therapeutic agents in preventing or slowing the progress of oxidative stress related degenerative diseases. The phytochemical compounds obtained attributed the antimicrobial, antioxidant and anticancer properties of *Naregamia alata* leaf extracts. Minimal inhibitory concentration of 400µg/ml and 500µg/ml was recorded for the test organisms. Antibacterial activity showed significant inhibitory zones ranging from 16mm to 21mm against test bacteria. Anticancer potential of *Naregamia alatai* leaf extracts showed that methanol extracts of *Naregamia alatai* were highly efficient at inducing apoptosis in cancer cells. These results might be helpful for providing the platform for researchers for the development of precious medicines which will be helpful for treatment of oxidative stress generated diseases. Present findings emphasized the potential of *Naregamia alatai* as a resource for the discovery of novel antimicrobial and anticancer agents.

Table 1. Phytochemical analysis of *Naregamia alata*

S. No	Phytochemicals	*Naregamia alata* extracts			
		Methanol	Acetone	Petroleum ether	Water
1	Alkaloids	++	+	+	-
2	Phenols	++	+	+	+
3	Flavonoids	+	+	+	-
4	Tannins	++	-	+	+
5	Saponins	-	-	-	-
6	Terpenoids	++	+	+	-
7	Steroids	+	-	-	-
8	Glycosides	-	-	-	-

+present, -absent

Table-2: DPPH radical scavenging activity of *Naregamia alata* extracts

S. No.	Concentration (µg/ml)	Inhibition (%)	
		Ascorbic acid	Herbal extract
1	20	48 ± 0.57	34 ± 1.05
2	40	58 ± 0.75	46 ± 0.57
3	60	63 ± 1.05	58 ± 0.57
4	80	69 ± 0.57	65 ± 0.75
5	100	75 ± 1.05	69 ± 1.05
IC_{50} Value (µg/ml)		28.34µg/ml	48.56µg/ml

Table-3: Hydroxyl radical scavenging activity of *Naregamia alata* extracts

S. No.	Concentration (µg/ml)	Inhibition (%)	
		Ascorbic acid	Herbal extract
1	20	36 ± 0.75	32 ± 1.05
2	40	42 ± 0.57	38 ± 0.57
3	60	50 ± 0.57	44 ± 0.75
4	80	54 ± 1.05	48 ± 0.75
5	100	62 ± 0.75	57 ± 1.05
IC_{50} Value (µg/ml)		24.6µg/ml	30.4µg/ml

Table-4: Superoxide free radical scavenging assay of *Naregamia alata* extracts

S. No.	Concentration (µg/ml)	Inhibition (%)	
		Ascorbic acid	Herbal extract
1	20	46 ± 0.57	39 ± 0.75
2	40	54 ± 0.75	45 ± 0.75
3	60	58 ± 1.05	51 ± 0.57
4	80	64 ± 0.57	58 ± 1.05
5	100	69 ± 1.05	60 ± 0.57
IC_{50} Value (µg/ml)		21.4µg/ml	28.6µg/ml

Table-5: Minimum inhibitory concentration (MIC) of *Naregamia alata* extracts

S. No.	Test Bacteria	Minimal Inhibitory Concentration (µg/ml)					
		100	200	300	400	500	600
1	*Staphylococcus aureus*	+	+	+	(MIC)	-	-
2	*Escherichia coli*	+	+	+	(MIC)	-	-
3	*Klebsiella pneumoniae*	+	+	+	+	(MIC)	-
4	*Pseudomonas aeruginosa*	+	+	+	-	(MIC)	-
5	*Enterobacter sp*	+	+	+	(MIC)	-	-

+ Turbidity (Growth), - No Turbidity (No growth)

Table-6: Antibacterial activity of *Naregamia alata* extracts

S. No.	Test Bacteria	Zone of Inhibition (mm)		
		PE	S	NC
1	*Escherichia coli*	19	20	0
2	*Staphylococcus aureus*	21	21	0
3	*Enterobacter aerogenes*	20	19	0
4	*Pseudomonas aeruginosa*	16	16	0
5	*Klebsiella pneumoniae*	18	19	0

PE: plant extract, S: Positive control, NC: negative control

Fig. 1: *Naregamia alata* leaves and milled leaf powders

Fig. 2: Soxhlet apparatus and obtained solvent extracts stored in screw cap bottles

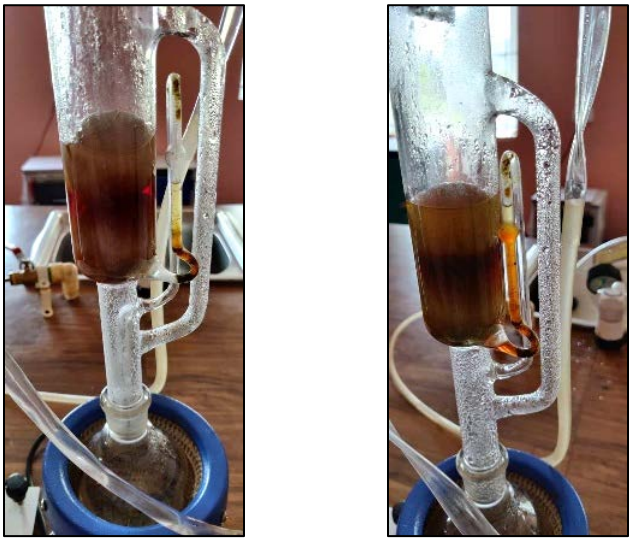


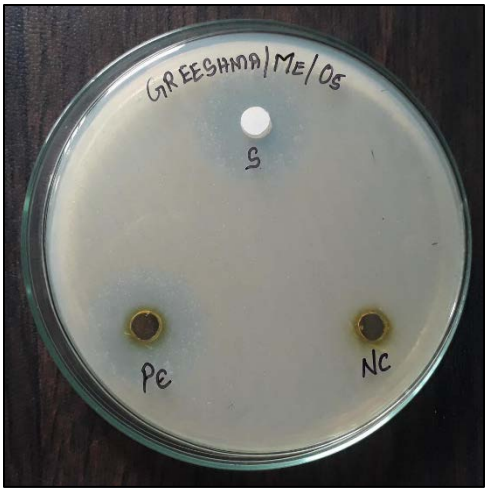
Fig. 3: Antibacterial activity of *Naregamia alata* extracts

Escherichia coli

Staphylococcus aureus

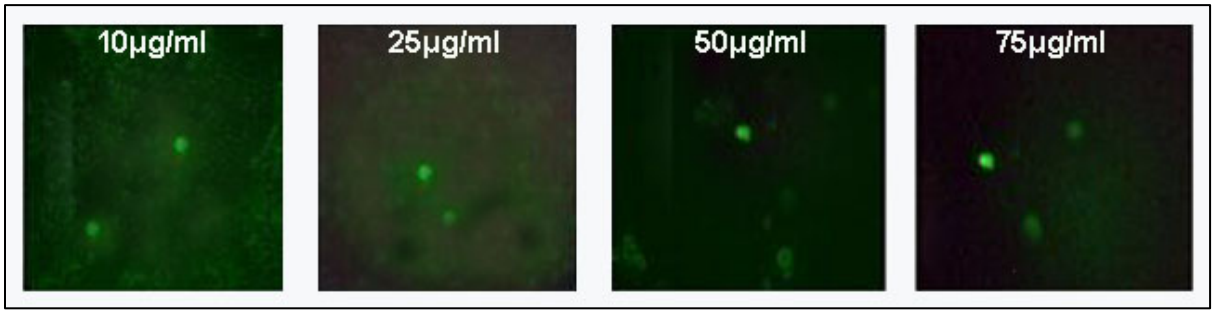
Enterobacter aerogenes

Pseudomonas aeruginosa



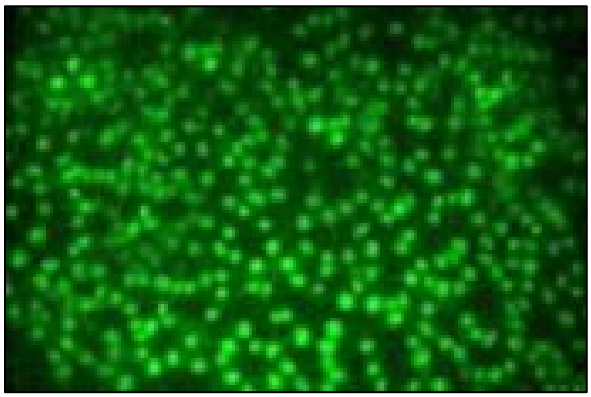
Klebsiella pneumoniae

Fig. 4A: Plant extract treated at different concentrations to study the apoptosis effect on MCF_7 cells



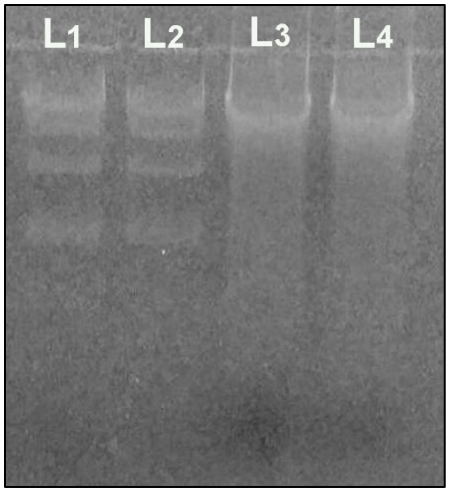
Cells are not evident in plant extract treated samples

Fig. 4B: Control cells



Cells are evident in control

Fig. 5: DNA fragmentation studies of plant extracts on MCF_7 cells



L1: Fragmented DNA of MCF_7 cells after treating with 200µg/ml of Plant extracts)
L2: Fragmented DNA of MCF_7 cells after treating with 400µg/ml of Plant extracts)
L3 and L4: DNA profile of MCF_7 cells (undigested)

Reference

Al-Mamun M, Yamak K, Masumizu T, Nakai Y, Saito K and Sano, H. Superoxide Anion Radical Scavenging Activities of Herbs and Pastures in Northern Japan Determined Using Electron Spin Resonance Spectrometry. *International Journal of Biological Science*. 2007; 3:349-355.

Anagha A. Rajopadhye and Anuradha SU. Determination of phenolic content and in-vitro antioxidant potential of ethanol extract of seven sources of Ayurvedic drug "Pittapada". *Indian Journal of Natural products and Resources*. 2013; 4(1):81-87.

Balandrin MF, Klocke JA, Wurtele ES, Bollinger WH. Natural plant chemicals: Sources of Industrial and Medicinal materials. *Science*. 1985; 228:1154-1160.

Buzzini P, Arapitsas P, Goretti M, Branda E, Turchetti B, Pinelli P, Ieri F and Romani, A. Antimicrobial and antiviral activity of hydrolysable tannins. *Mini Reviews in Medicinal Chemistry* 2008; 8(12):1179-1187.

Chopra RN, Nayar SL and Chopra IC. Publisher Council of Scientific and Industrial Research, New Delhi. 1986. Glossary of Indian Medicinal Plants.

Gurib-Fakim A. Medicinal plants: traditions of yesterday and drugs of tomorrow. *Molecular Aspects of Medicine*. 2006; 27(1):1-93.

Helander IM, Alakomi HL, Latva K, Mattila-Sandholm T, Pol I, Smid EJ, Gorris LG and von Wright A. Characterization of the action of selected essential oil components on Gram-negative bacteria. *Journal of Agriculture and Food Chemistry.* 1998; 46:3590–3595.

Jacob S, John JA, Thomas L and Sabulal, B. In vitro pharmacological activity of the whole plant Naregamia alata. *Asian Journal of Research in Chemistry*. 2012; 5(2): 265-269.

Kappers IF, Aharoni A, Herpen TW, Luckerhoff LL and Dicke M. Genetic engineering of terpenoid metabolism attracts bodyguards to Arabidopsis. *Science*. 2005; 309:2070-2072.

Lambert RJ, Skandamis PN, Coote PJ and Nychas GJ. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. *Journal of Applied Microbiology.* 2001; 91:453–462

Mathew George, Lincy Joseph, Anju V. Phytochemical and Invitro Anti-Inflammatory Activity Studies of *Naregamia Alata* Aerial Parts. *International Journal of Innovative Science and Research Technology*. 2017; 2(10):288-293.

Middleton EJ, Kandaswami G and Theoharidos TC. The effects of plant flavonoids on mammalian cells: Implications for inflammation, heart disease and cancer. *Pharmacological Review*. 2000; 52:673-751.

Mohammed FA, Srinivasa AR, Shaik RA and Mohammed I. Phytochemical studies and antioxidant activity of Melia azedarach Linn leaves by DPPH scavenging Assay. *International Journal of Pharmaceutical Applications*. 2012; 3(1):271- 276.

Mukesh S, Vimal Ohan M, Maneesha A, Joshy PJ and Drishya KR. Antimicrobial screening of different extracts of South Indian medicinal plants of Meliaceae. *Journal of Medicinal Plants Research*. 2011; 5(5):688-695.

Raja R. Jency Esther GD and Nalini Mabel. Evaluation of wound healing activity of the whole plant ethanolic extract of *Naregamia alata* Wight & Arn. *International Research Journal of Pharmacy*. 2016; 7(1): 29-32.

Shinya RV, Jomy J and Padmaja V. Pharmacognostical and Biotechnological studies on *Naregamia alata. Journal of Pharmaceutical and Biomedical Science.* 2012; 25(2):246-251.

Sulekha M, Salish Y, Sunita Y and Rajesh KN. Anti-oxidants: a review. *Journal of Chemical and Pharmaceutical Research*. 2009; 1:102-104.

Ultee A, Bennik MH, Moezelaar R. The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen *Bacillus cereus*. *Appl. Environ. Microbiol.* 2002; 68:1561–1568.

Uniyal SK, Singh KN, Jamwal P, Lal B. Traditional use of medicinal plants among the tribal communities of Chhota Bhangal, Western Himalayan. *Journal of Ethnobiology Ethnomedicines*. 2006; 2:1-14.

Uphof JC. Dictionary of Economic Plants. Th. Publisher Weinheim, 1959.

Velayudhan VK, Supratha PT and Saj OP. *In vitro* anticancer activity of methanolic extract of roots of *Naregamia alata* W&A. *International Journal of Advanced Research*. 2016; 4(12):176-180.