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Anticancer Activity of Bee Venom against Lung Cancer Cell Line (A549 Cells) Enhanced by Iron Oxide Nanoparticles Synthesized from *Syzygium Aromaticum*

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ABSTRACT

Iron oxide nanoparticles were synthesized using aqueous extracts of *Syzygium aromaticum*. Total phenolic content and iron oxide nanoparticles were found to increasing with concentration of extract and had higher antioxidant activity. The UV-Visible spectral analysis showed absorption peaks at 240 nm. SEM image of nanoparticles revealed that they were aggregated and had irregular shape. The signals obtained in EDAX spectrum imply that particles synthesized were iron oxide nanoparticles. The FT-IR spectrum revealed that nanoparticles which is capped by C-H group of alkane. The average size of nanoparticles was 52 nm. The nanoparticles were mixed with bee venom in various ratios to enhance anticancer activity and were identified as 1:1. MTT assay of 1:1 volume ratio of iron oxide nanoparticles and bee venom was found to have cytotoxicity higher than absence of nanoparticles. It confirmed the enhancement of anticancer activity by iron oxide nanoparticles of clove extract.

Keywords: *Syzygium aromaticum;* Green synthesis; Iron oxide nanoparticles; Bee venom; Anticancer activity.

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INTRODUCTION

In the recent years, the study of different types of cancer treatments is one of the important areas in modern science. Cancer may be caused by various factors like genetic problems, incorrect diet, exposing to harmful radiation etc. The common treatment for cancer is unfavourable because it causes many detrimental side effects and lately, there has been a growing resistance towards anticancer drugs, which worsens the future of cancer treatment[1]. Nanotechnology is an interesting field for the researchers. It is one of the recent advancement in the area of cancer research. The use of Nanotechnology in cancer treatment offers some exciting possibilities, including the possibility of destroying cancer tumor with minimal damage to healthy tissue and organs, as well as the detection and elimination of cancer cells before they form tumors. Many natural compounds have anti-cancer activity are used as a drug for the cancer treatment. Targeting the anti-cancer compound using nanoparticles was a promising way to increase the treatment efficiency[2].

An extensive research has been focused on the magnetic nanoparticles for drug targeting. Magnetic nanoparticles can deliver the anticancer agent or drugs in the particular cancer site. It has potential applications such as *in vivo* drug delivery, biosensor, bio separation, magnetic storage, magnetic ink printing, microwave absorption, immune magnetic array, magnetic resonance imaging contrast agents and hyperthermia treatment of cancer[3]. Currently, various clinical trials are in progress to investigate the potential of different magnetic nano systems for pharmaceutical and biomedical applications. Appropriate precursors such as ferric chloride can be used for the reduction of plant extracts[2]. Iron oxide nanoparticle has the ability to kill the cells by two mechanisms. One is by target delivery of cancer drug in the cancer site and other by incorporating the heat to kill the cancer cells.

Syzygium aromaticum (Cloves) was the aromatic dried flower buds of a tree. Eugenol was the major compound present in the cloves. The anti oxidative, cytotoxic and genotoxic effects of eugenol and borneol has been tested and the study reveals that it has the ability to modulate resistance against the damaging effects of hydrogen peroxide on DNA of different strains of human cells[4]. *Syzygium aromaticum* was chosen to synthesize the iron oxide nanoparticles mainly for its anticancer activity[5]. It is used as a carminative to increase hydrochloric acid in the stomach and to improve peristalsis[6]. Besides acting as an antioxidant, cloves also possess many other functions, such as anti-inflammatory, antibacterial and antiseptic which make them an ideal natural source to be developed as an anticancer agent. Cloves have anti-carcinogenic properties, which have been proven to be helpful in controlling lung cancer when it is in the early stages. The scientists have found that cloves inhibit abnormal cell growth in lungs of mice[7].

Bee venom contains a complex mixture of proteins such as melittin, apamin, adolpin and phospholipase A_2. Melittin has the potential to induce cytotoxic, antitumor, immune modulatory and apoptotic effects in different tumor cells *in vivo* or *in vitro*[8]. By specifically targeting the bee venom in the cancer site it can kill most of the cancer cells. Bee venom has been used for centuries in traditional medicine to relieve pain and treat chronic inflammatory diseases[9]. It has also been used in conditions such as arthritis, rheumatism and skin diseases. Melittin is the main component of bee venom and it is a biologically active peptide which constitutes about 50% of bee venom's dry weight[10]. The cytotoxicity of Melittin has been previously attributed to both necrotic and apoptotic cell death[11]. The MEL has cytotoxicity against cell lines like HeLa, WiDr and Vero. The MEL present in the bee venom was a promising anticancer agent[12]. The present study deals with the enhancement of anti-cancer activity of honey bee (*Cerana indica*) venom by mixing iron oxide nanoparticles synthesised from the extracts of *Syzygium aromaticum* against lung cancer cell line.

MATERIALS AND METHODS

Sample collection:

The buds of *Syzygium aromaticum* (25g) was purchased from retail shop in Tiruchengode, Namakkal. The honey bee (*Cerana indica*) venom was collected from EFGC Biological farm office, Sembakkam, Chennai. The A549 cell (Adeno carcinomic human alveolar basal epithelial cell) was purchased from King Institute of Preventive Medicine and Research, ICMR, Chennai, India.

Preparation of *Syzygium aromaticum* extracts (Monalisa and Nayak, 2013):

Syzygium aromaticum buds were washed to remove dust particles and then shade dried and grinded into fine powder. The plant extract was prepared by mixing 5g of *Syzygium aromaticum* powder in 100 mL of distilled water. Then the solution is heated at 70°C for 5 minutes. The supernatant was filtered by using Whatmann filter paper. The filtrate was stored at 4°C for further experiments.

Synthesis of iron oxide nanoparticles from Syzygium aromaticum extracts:

The iron oxide nanoparticles were synthesized by mixing the *Syzygium aromaticum* extract with 10 mM ferric chloride substrate[12]. About 1:10 ratio of *Syzygium aromaticum* extract and 10mM ferric chloride was prepared and heated at 70°C for 5 minutes. It was then incubated at room temperature for synthesis of iron oxide nanoparticles.

Total phenolic content and Antioxidant assay of aqueous extract of *Syzygium aromaticum* and iron oxide nanoparticles:

The total phenolic content of the *Syzygium aromaticum* extract and the sample of synthesized iron oxide nanoparticles were determined. The antioxidant activity of the synthesized iron oxide nanoparticles were determined by DPPH assay[13].

Characterization of iron oxide nanoparticles:

The sample prepared for the synthesis of iron-oxide nanoparticles were characterized by UV-Visible Spectral analysis[14], SEM (Scanning electron microscope) analysis[12] and EDS (Energy Disperse Spectroscopic analysis) analysis[15], FT-IR (Fourier transform infra-red) analysis[12] and XRD (X-ray Diffraction) analysis[16]. Particle size of the iron oxide nanoparticle was determined by using Particle size analyzer. The sample was analyzed for its size in its liquid form. The lyophilized sample was dissolved in distilled water and it was analyzed. The sample was analyzed under the intensity of laser light on the sample particle.

Protein estimation of bee venom:

The protein present in the honey bee venom was estimated[17]. The amount of protein present in the bee venom was identified using standard graph. Bee venom contains a mixture of proteins in Melittin accounts for about 52% of venom peptides.

Mixing of synthesized iron oxide nanoparticles with bee venom in various ratios:

The synthesized iron oxide nanoparticles were mixed with bee venom to enhance the anticancer activity. The iron oxide nanoparticles and bee venom was mixed in the volume ratio of 1:1, 1:2, 1:3, 1:4 and 1:5. The samples containing different composition of synthesized iron-oxide nanoparticles and the bee venom were labelled properly.

Identification of potential mixture of iron oxide nanoparticles and bee venom:

The potential sample in the different mixture was identified by different assays. The potential sample was identified based on its anti-oxidant property. The DPPH (Diphenyl picryl hydrazyl) assay was done to identify the potential mixture[18]. Based on the inhibitory concentration of the different mixture in the above assay were used for the identification of potential composition.

DPPH assay of various ratios of iron oxide nanoparticles and bee venom:

The DPPH (diphenyl picryl hydrazyl) assay was performed for various ratio of iron oxide nanoparticles and bee venom. The increasing concentration of ascorbic acid like 10, 20, 30, 40 and 50µL was used as a standard. 10, 20, 30, 40 and 50µL of test samples were used to determine its antioxidant activity. The absorbance was obtained at 517 nm for each sample. The control was maintained to determine the inhibitory concentration of bee venom and the different ratio of iron oxide nanoparticles and bee venom. The inhibitory concentration was determined by the following formula.

$$\%\text{inhibitory concentration} = \frac{\text{control OD} - \text{sample OD}}{\text{control OD}} \times 100$$

Characterization of the potential mixture of iron oxide nanoparticles and bee venom:

The interaction between the bee venom component and the synthesized iron oxide nanoparticles were studied by the

characterization studies. FT-IR (Fourier transform infrared spectroscopy) analysis was used to determine the functional groups present in the 1:1 ratio (potential mixture) of iron oxide nanoparticles and bee venom which has higher anti-oxidant activity compared to other combinations. The chemical composition of the synthesized iron oxide nanoparticles was studied. The lyophilized sample powder was characterized in the range of 4000-400cm^{-1}KBr pellet method.

Anticancer activity of the potential mixture of iron oxide nanoparticles and bee venom:

The anticancer activity of the potential composition and the bee venom was identified. The cytotoxicity tests were performed for potential mixture and bee venom. The cytotoxicity parameter like IC_{50} was calculated. It requires lung cancer cell line (A549) for the study.

Cell line and culture:

Lung cancer cell line (A549) was cultured in Dulbecco's modified Eagle's medium (DMEM-Hi media lab, Mumbai, India) supplemented with 10% fetal bovine serum and maintained at 5% CO_2 incubator. The anticancer activity of bee venom (*Cerana indica*) and potential mixture 1:1 ratio of iron oxide nanoparticles was studied by MTT assay[11].

Comparing the anticancer activity of bee venom and the 1:1 ratio of iron oxide nanoparticles and bee venom:

The anticancer activity of the potential combination and the bee venom by MTT assay were compared. By comparing these parameters the enhancement of the anticancer activity by the synthesized iron-oxide nanoparticles was identified.

Statistical analysis:

Values are represented as mean ± SD of three replicates. The data were statistically evaluated using GRAPHPAD PRISM 6 software.

RESULTS AND DISCUSSION

Synthesis of iron oxide nanoparticles from extracts of *Syzygium aromaticum*, Total Phenolic content and antioxidant activity:

The iron oxide nanoparticles were synthesized using *Syzygium aromaticum* extract and 10mM ferric chloride as a substrate. The total phenolic content of iron oxide nanoparticles and aqueous extract of *Syzygium aromaticum* were determined. It was increased with increasing concentration of extract. It was determined using the standard curve equation y=0.0095x-0.0057 with the R^2value of 0.9803 (Fig. 1(a)). Iron oxide nanoparticles with the size of 33nm using ferric chloride as a substrate have been reported[12] synthesized Iron oxide nanoparticles were synthesised and reported from *Psoralea corylifolia* seeds[19].

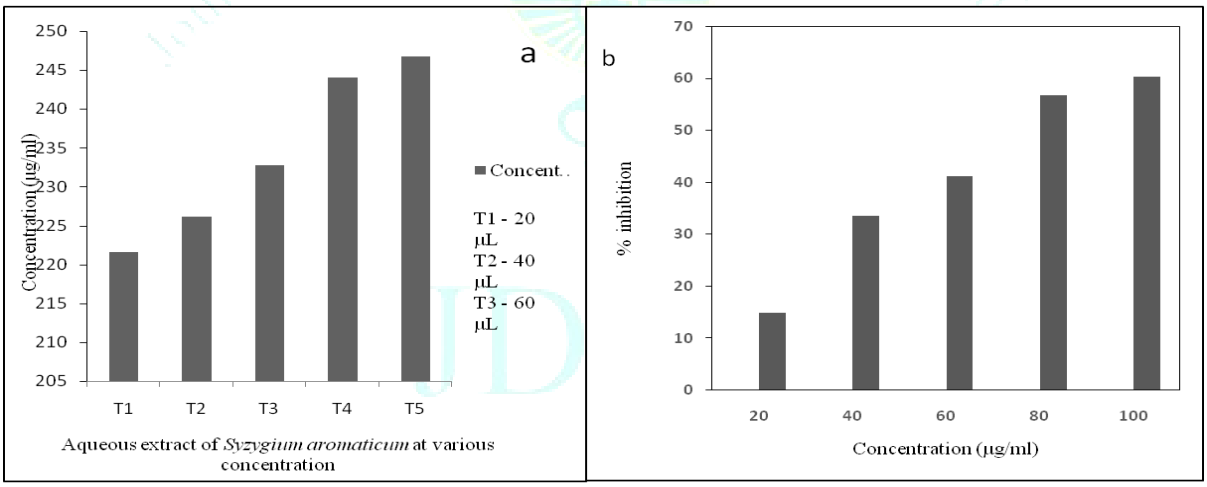

Figure 1: Analysis of *Syzygium aromaticum* extract

(a) Total Phenolic content (b) DPPH assay of iron oxide nanoparticles

The total phenolic content of the synthesized iron oxide nanoparticles were increased with increasing concentration of the aqueous extract (Fig. 1a). Studies of total phenolic content and total flavonoid content present in the selected medicinal plants contributed to the antioxidant activity which in turn aid in anticancer activity was reported[18]. Studies revealed that the biological activities of cloves (*Syzygium aromaticum*) represented the major sources of phenolic compounds [5].

The DPPH assay was performed for iron oxide nanoparticles. The percentage inhibition was found to be increasing with increase in the concentration of the samples. The sample synthesized using 10mM as a substrate had higher antioxidant activity (Fig. 1 (b)). The IC_{50} value of the sample was found to be 75.22 µg/mL. Iron oxide nanoparticles were synthesized via chemical and green route and have effectively compared with the synthesized iron oxide nanoparticles[13]. According to their research the green route showed more free radical scavenging activity than the chemically synthesized iron oxide nanoparticles. The UV visible spectral analysis of iron oxide nanoparticles revealed that the maximum absorption was at 240nm, which indicated the reduction of metal ions to iron oxide nanoparticles (Fig. 2). This might be due to the excitation of surface plasmon vibrations of iron oxide nanoparticles. Reports are available on iron oxide nanoparticles which had the absorption peaks at 216-268 nm regions[6]. It confirmed the synthesis of iron oxide nanoparticles. The characteristic surface plasmon absorption band at 233nm for synthesized magnetite nanoparticles was observed [14].

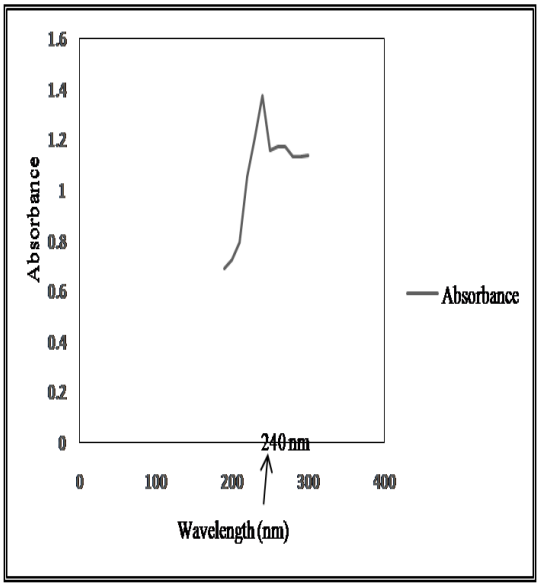

Figure 2: Characterization of nanoparticles synthesized from *Syzygium aromaticum* - UV-Visible analysis

Characterization of iron oxide nanoparticles:

The SEM analysis was done for the iron oxide nanoparticles which had antioxidant activity. The particles were agglomerated at low magnification (2k) which indicated that the size and morphology was irregular (Fig.3a). The magnification of 25k x 17,000 the iron oxide nanoparticles from *Annona squamosa* leaf were spherical structure was reported[20]. The SEM images of iron oxide nanoparticles were found to be aggregated, irregular sphere shapes with rough surfaces[21].

The elemental composition of the synthesized iron oxide nanoparticles which have anti-oxidant activity was done by Energy Disperse Spectroscopic (EDS) or Energy Disperse Analysis of X-rays (EDAX). The EDAX spectrum (Fig. 3b) shows iron, oxygen, chloride and sulphate signals. The chloride and the sulphate signals were mainly due to the substrate (ferric chloride and ferrous sulphate) used for the synthesis of iron oxide nanoparticles. EDS analysis of iron oxide nanoparticles with iron and oxygen peaks and reported the successful synthesis of magnetite (iron oxide) nanoparticles by green method using *Caricaya papaya* leaves [12]. Studies were carried out to optimized microwave assisted green synthesis protocol for iron oxide nanoparticles and studied the elemental composition of synthesized iron oxide nanoparticles using EDS [3].

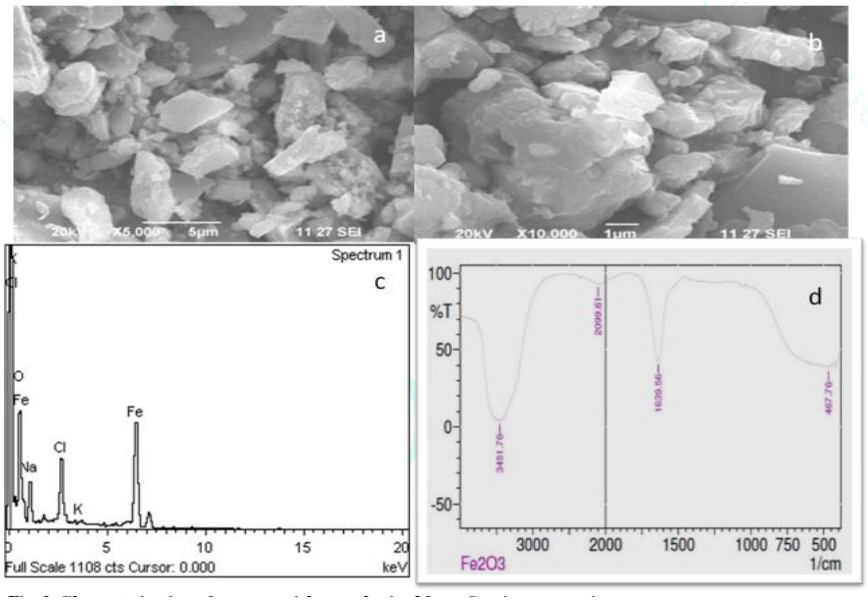

Figure 3: Characterization of nanoparticles synthesized from *Syzygium aromaticum* (a & b)-SEM analysis (c) EDS analysis (d) FT-IR spectrum

FT-IR analysis was done to determine the functional groups present in the sample containing iron oxide nanoparticles. The chemical constituents in the solution containing 10mM ferric chloride and *Syzygium aromaticum* extract was revealed by FT-IR analysis. The peak at 3451.76 cm^{-1} corresponds to the OH stretching of the alcohol and phenol. The signals at 2099.61 cm^{-1} indicates the presence of aliphatic C-H stretching in methylene groups. The signal at 1639.56 cm^{-1} was due the stretching vibration of CO groups in ketones, aldehydes and carboxylic acids (Fig. 3c). The synthesis of iron oxide nanoparticles was characterized by the absorption band at 467.76 cm^{-1}, which corresponds to the Fe-O band. FT-IR analysis confirmed the reduction of ferric chloride into iron oxide nanoparticles. The FT-IR spectrum of *Syzygium aromaticum* extract which showed peaks at 3375cm^{-1}, 2920-2852cm^{-1}, 1730cm^{-1}, 1643cm^{-1}, 1452$cm^{-1}$and 1070cm^{-1} [21]. The absorption band at 467.76cm^{-1} was absent in the spectrum of *Syzygium aromaticum* extract which indicated the presence of iron oxide nanoparticles. The ferric ions were reduced to iron oxide nanoparticles by the component present in the *Syzygium aromaticum* extract which act as a capping material.

XRD analysis was performed to confirm the crystal structure of the synthesized iron oxide nanoparticles. The XRD spectrum of synthesized iron oxide nanoparticles was shown in the picture (Fig. 4a). The peaks at 2θ = 30.20°, 38.35°, 44.48°, 51.55°, 64.15° and 82.55° were attributed to the crystal planes of iron oxide nanoparticles at 122, 004, 140, 115, 312 and 343 respectively. The particle size was

analyzed using particle size analyzer. The average particle size distribution of the synthesized iron oxide nanoparticles was found to be 10-170 nm (Fig. 4b).The average particle size of the synthesized iron oxide nanoparticles was determined by taking the average of the sizes at the peaks and was found to be 52nm. The average particle size distribution of the iron oxide magnetic nanoparticles was found to be 30-80 nm [23]. The particle size distribution of zero valent iron nanoparticles to be 52-120 nm. Average size of the iron oxide nanoparticle synthesized *Psoralea corylifolia* seed extract as 39nm [24 &19].

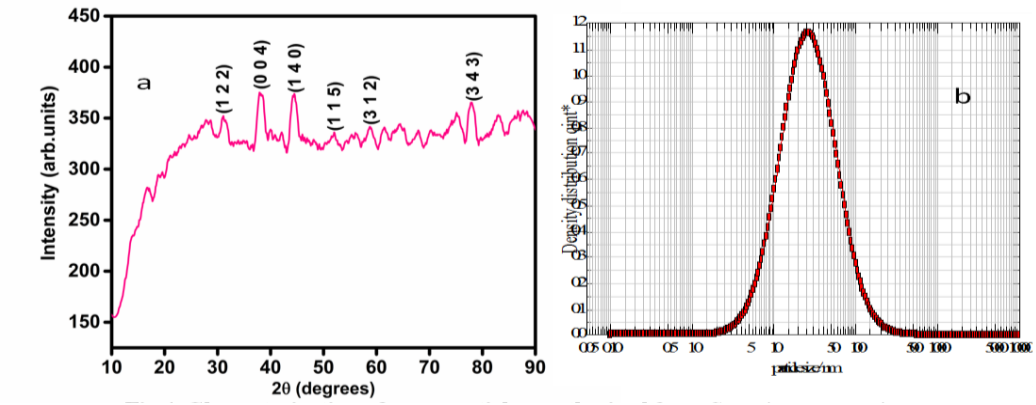

Figure 4: Characterization of nanoparticles synthesized from *Syzygium aromaticum*

(a) XRD analysis (b) Particle size analysis

Total protein estimation of bee venom:

The total protein content present in the bee venom was estimated. The bee venom was taken in the concentration of 20, 40, 60, 80 and 100µg/mL. The total protein in the bee venom was found to be 19.34, 29.057, 51.057, 73.91 and 75.91µg/mL respectively. The total protein present in the venom sac of the three different *Apis* species was reported[25]. According to their report *Apisdorsata* has higher amount of protein than *Apismellifera* and *Apisflorea*.

Mixing of synthesized iron oxide nanoparticles with bee venom:

The iron oxide nanoparticles having higher antioxidant activity and the bee venom was mixed in various volume ratio of 1:1, 1:2, 1:3, 1:4 and 1:5.

Antioxidant activity of bee venom:

DPPH assay was done to determine the antioxidant property of the bee venom. The antioxidant assay of bee venom increases with increase in concentration of 20, 30, 40 and 50µg/mL. The highest % inhibition was found to be 34.84% which was obtained at 50µg/mL. The IC_{50} value of the bee venom was found to be 65.5325µg/mL. In the antioxidant activity of the 1:1 ratio of iron oxide nanoparticles and bee venom the % inhibition at the concentration of 50µg/mL was found to be 46.46. The IC_{50} value of the sample containing 1:1 ratio of iron oxide nanoparticles and bee venom was found to be 55.9493µg/mL. The 1:2 ratio of iron oxide nanoparticle and bee venom showed less antioxidant activity than 1:1 ratio of iron oxide nanoparticles and bee venom. The % inhibitory concentration at 50µg/mL was found to be 30.3. The IC_{50} value was found to be 1.4223µg/mL. The antioxidant activity of the 1:3 ratio of iron oxide nanoparticles and bee venom was less than the 1:1 and 1:2 ratios. The % inhibitory concentration at 50µg/mL was found to be 24.74. The IC_{50} value of the 1:3 ratio of iron oxide nanoparticles and bee venom was found to be 94.6256µg/mL. Similar kind of mixture of nanoparticle with venom was carried out[26]. They have created biodegradable chitosan nanoparticles for loading *Naja najaoxiana* cobra venom and evaluated their (chitosan nanoparticles containing snake venom) potential as antigen delivery systems. The highest % inhibition was found to be 34.84% which was obtained at 50µg/mL. The IC_{50} value of the bee venom was found to be 65.5325µg/mL..

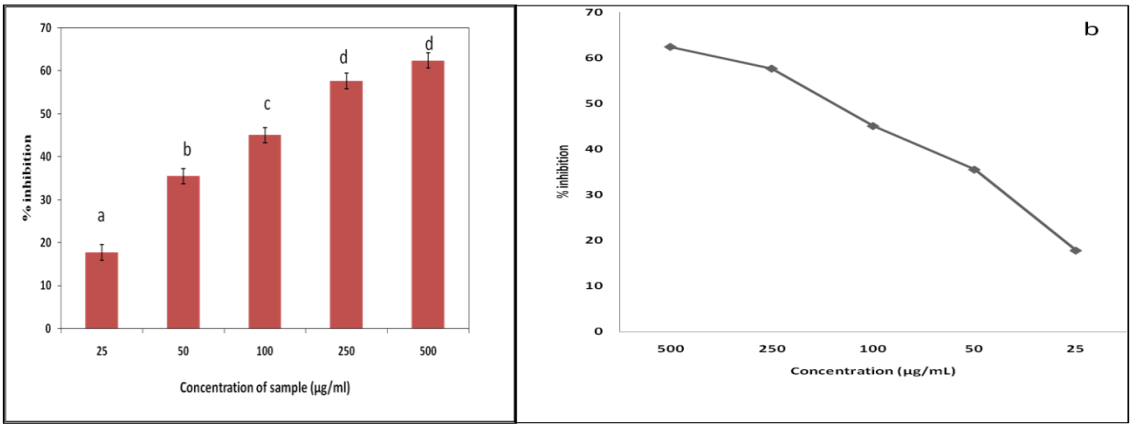

Figure 5: Analysis of 1:1 ratio of Iron oxide nanoparticles and bee venom
(a) Per centage inhibition of A549 cells (b) Cytotoxicity activity
All the data represented in the figure are the average of three replicates

FT-IR analysis of the potential mixture of iron oxide nanoparticles and bee venom:

The functional group present in the 1:1 ratio of iron oxide nanoparticles and bee venom was identified by FT-IR analysis. The peak obtained at 3451.76 cm^{-1} indicated the presence of O-H alcohol stretching and H-bonded. The peak at 2075.5 cm^{-1} represents the presence of aliphatic C-H stretching in methylene groups. The sharp peaks at indicated the aromatic attached to the functional groups of C=N, C=C and NH stretching by multiple combination. The absorption peak at 467.76 cm^{-1} corresponds to the Fe-O band.

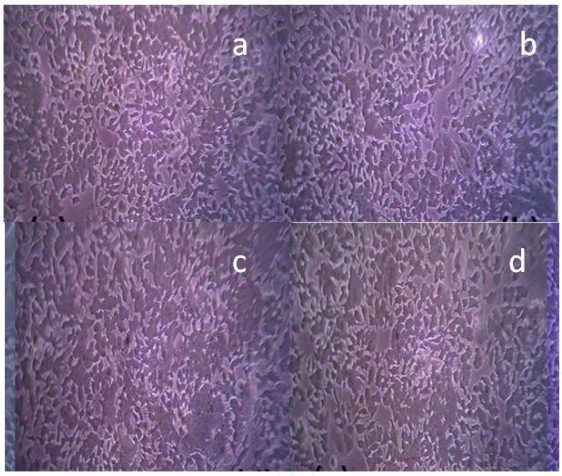

Figure 6: Concentration dependent proliferation of the A549 cell lines

(a) Control (b) 25µg/mL (c) 100µg/mL (d) 250µg/mL

Anticancer activity of 1:1 ratio of iron oxide nanoparticles and bee venom:

The MTT assay was performed to determine the cytotoxicity of the 1:1 ratio of iron oxide nanoparticles and bee venom in which the sample was taken at different concentrations and treated against A549 cells (Adeno carcinomic human alveolar basal epithelial cells). The morphological changes were observed in the cells after the treatment with 1:1 ratio of iron oxide nanoparticles and bee venom. The percentage inhibition of A549 cells was determined at different concentrations. The percentage inhibition was found to be 57.63% at 250µg/mL (Fig.5a & b). The MTT assay of 1:1 ratio of iron oxide nanoparticles and bee venom was studied. The percentage inhibition of A549 cells (Adeno carcinomic human alveolar basal epithelial cells) at various concentrations of the sample at 4 hours incubation was studied. The percentage inhibition at 25µg/mL was found to be 17.74% with the 4 hours of treatment and it was observed with the morphological changes which was clearly visible (Fig.6b). At 50µg/mL, inhibition was 35.49% At 100µg/mL, it was 45.04% and the morphological changes were observed (Fig. 6c). At 250µg/mL, the percentage inhibition was found to be 57.63% and it was near to the IC_{50} value (Fig. 6 d). At 500µg/mL, inhibition was found to be 62.40% and the morphological changes were observed. The Fig. 6 (a) represents the control. Our results were correlated with the study of honey bee venom and *Zingiber officinale extract* had more cytotoxicity against breast adeno carcinoma cells (MCF-7) was reported[27] .

Cytotoxicity of bee venom against lung cancer cell line proved that bee venom induced cytotoxicity at the concentration of 10µg/mL and it exhibited cell shrinkage, cytoplasmic condensation and irregularity in shape after 24 hours of treatment[28]. The presence of iron oxide nanoparticles with the bee venom in the volume ratio 1:1 induces cell death in lung cancer cell line within 4 hours of treatment and this indicated that the iron oxide nanoparticles synthesized using *Syzygium aromaticum* have enhanced the anticancer activity of bee venom.

CONCLUSION

The iron oxide nanoparticles were synthesized from the extracts of *Syzygium aromaticum.* The iron oxide nanoparticles were mixed with various ratios of bee venom and the activity was identified by DPPH assay. In MTT assay of the 1:1 volume ratio of iron oxide nanoaprticles and bee venom was found to have higher cytotoxicity against lung cancer cell line. It confirms the progress of anticancer activity of bee venom by iron oxide nanoparticles synthesized using *Syzygium aromaticum* extract. This study contributes to novel materials as alternative therapeutic treatment for human lung cancer cell line. Hence, further studies are needed to identify the mechanisms of anticancer activity and toxicity of this mixture.

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CONFLICTS OF INTEREST

The authors report no conflicts of interest.

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