**In vitro Neutralisation Potential of Metal-Herbal (Copper-Leucas zeylanica) Nanocomposite (MHNC) against Naja naja and Bungarus caeruleus Venoms**

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**ABSTRACT**

Snake-bite gives rise to significant socio-economic, and limitations of antivenom have raised the necessity for the development of novel neutralising compounds. The present study concentrates on evaluating the neutralisation potential of Copper nanoparticles, Leucas zeylanica leaf extracts and Metal-Herbal Nanocomposite (MHNC) against Naja naja and Bungarus caeruleus venoms. The antigenic potency of snake venoms was determined by gel precipitation test. Leucas zeylanica leaves were used for extraction, and extracts were further purified by column chromatography. Size of Leucas zeylanica extract was found to be 86.7±7.1nm and 249.4±27.3nm for MHNC. Zeta potential values determined the Stability of the MHNC. MHNC showed higher neutralisation to venoms than the Leucas zeylanica extracts. In direct hemolysis, the efficiency of Naja naja and Bungarus caeruleus venoms treated with Leucas zeylanica leaf extract was found to be 30% and 27.2%, whereas MHNC showed 16.1% against Naja naja venom and 17.1% Bungarus caeruleus venom. From Indirect analysis, Naja naja and Bungarus caeruleus venom were able to produce 28mm and 26mm diameter hemolytic halo zones. On treatment with MHNC, the zones were reduced to 15mm and 11mm than Leucas zeylanica extract. This result indicates the MHNC can be used for the treatment of snake bites.

**INTRODUCTION**

Snake-bite gives rise to a significant medical and socio-economic problem in various parts of tropical and subtropical countries of the world. The world health organisation (WHO) recognised the typical acute medical emergency in majority rural areas of the subtropical and tropical countries like India is due to venomous snake bite. As per the world health organisation, nearly 4,21000 snake-bite cases are recorded all over the world in which 20000 are lethal. In a year, nearly 35000-50000 deaths are caused due to the snake bite. The statistical analysis conveys that in India alone, nearly 25000 people die each year, mainly in rural areas (Warrell, 2005).

India has a rich diversity of snake fauna, of which 242 species have been distinguished including 57 poisonous or dangerous species. There are four major ubiquitous venomous snakes in India is well known as “big four” which is responsible for
life-threatening envenomation all over the country. They include the common krait (Bungarus caeruleus), Indian cobra (Naja naja), the saw-scaled viper (Echis carinatus) and Russell’s viper (Daboia russelli). In general, snake venom is classified into three groups (Neurotoxin, Cytotoxin, Hemotoxin) based on its mode and action. The Hemotoxins affect the blood functions and cardiovascular system (Kini, 2003). Cytotoxic venom targets particular cellular sites or muscles. Phospholipase A2, an active venom enzymatic component, was described to be responsible for many toxic effects such as heart failure, deformations, renal failure, and amputations. The venoms of cobra and krait are neurotoxic so that it affects the victim’s central nervous system and cause heart failure (Gopi et al., 2011).

The requirement of antivenom all over the world is increased due to the death rate. The snake venom is required for the development of antivenom for the treatment of potentially lethal snake bite. India has a large unit of the multivalent antivenom production unit at Shimla, central research unit and Kasauli. Even though antivenom plays an essential role in snake bite, the researchers have emphasised its drawbacks with justifications. Generally, the antivenom is costly and available in limited quantity. They are maintained in the freeze-dried ampules and may not maintain stability in other storage conditions. Intramuscular mode of administration may not be substantially effective (Alam and Gomes, 2003). The liquid antivenom gets changed to opaque due to the precipitation of protein at the bottom; this indicates the inefficiency of antivenom activity which increases the defined response against humans. Antivenom doesn’t provide complete protection against bleeding, death, kidney failure induced during snake-bite. Hence, finding novel methods to prevent the multiple poisonousness-caused post envenomation is a vital task to the researchers (Thwin et al., 2010). So there is a necessity for the development of new compounds which can act as antivenom for the neutralisation of venom activity from various new sources.

The production of antivenom from the plant extracts is an ancestral story, which is well known to the people who face the challenge from the snakes except for common people. The plant extract is rich in active pharmacological components which antagonise several snake species venom. Numerous medicinal plants are suggested for treating the snake bite (Gopi et al., 2014). Leucas zeylanica is an annual, erect plant which has branched stems of 20 - 60cm tall. These plants are harvested from the forest, used as food and medicine. Leucas zeylanica is widely used for the snake bite by the people, and it has many uses (Napagoda et al., 2018).

Nanotechnology is a developing source which has the potential application in the field of medicine. The metal nanoparticles are used as the alternative for the drug, which has a broad range of activities. Feynman (1960) introduced the theory of nanoparticles, and they are the size between 1nm and 100nm, which has at least a single dimension. Norio Taniguchi coined the term "Nanotechnology" in 1974 (Salata, 2004). It is used for the treatment as Nanomedicine. There is various metal nanoparticle which has a wide range of uses, and their properties are unique because of their physio-chemical characteristics. Recently the researches had discovered that nanoparticles neutralise the toxicity and venom of different snakes (Saha and Gomes, 2017).

Thus, the present study focuses on the development of Metal-Herbal Nanocomposite (MHNC) with antivenom activity against Naja naja and Bungarus caeruleus venoms. Copper nanoparticles were synthesised, and composites were prepared with extracts of Leucas zeylanica. In vitro Assessment of Venom Toxicity and Neutralization Potential of herbal extracts and Metal-Herbal Nano, composite were determined.

MATERIALS AND METHODS

Procurement of Snake Venom
Irula’s Snake Catchers Industrial Cooperative Society Limited Chennai obtained the lyophilised forms of Naja naja and Bungarus Caeruleus venoms and stored them at 4°C. A stock solution was prepared with 1 mg lyophilised venom dissolved in 1ml physiological saline (1mg / ml).

Determining Snake Venom Antigenic Potency
The antigenic capacity of the venomous snake against commercially available horse antiserum was determined by gel precipitation test. 1% solution of agarose was prepared in saline (0.85%). This solution was steamed for 15 minutes and transferred to a water bath at 50°C. The solution was poured on a Petri plate evenly. Three wells were made at a distance of 7.5mm and the diameter of well-being 3mm and marked A, B and C.

By using micropipettes, Naja naja and Bungarus caeruleus venoms and polyvalent horse antivenom were applied respectively to wells A, B and C. Gel slides were incubated overnight in a moist chamber at 40°C and the results were read on a dark background to look for the precipitation line.

Medicinal Plants and Preparation of Extract
Leucas zeylanica leaves were collected and dried at
room temperature. The leaves were finely grounded and stores in sterile containers. By soaking 180ml of carbonated water in a beaker, about 20g of the powdered sample of the herb was removed, stirred for about 6 minutes and left overnight. The solution was subsequently filtered with filter paper (tWhatman No. 1) and plant extracts evaporated to dryness at a reduced pressure of 40°C rand in dry weight the plant extracts were expressed (Uhegbu et al., 2005).

**Purification of Extracts by Column Chromatography**

The column chromatography technique purified the plant extracts. A long cylindrical glass column (450mm X 20mm) should be stand firm on a column chromatography stand was selected For a column chromatography stand was selected. For the latest report. With the help of hexane, silica gel (60-120 mesh) was packaged without any air bubbles. The *Leucas zeylanica* extracts were distilled dried and finely powdered form for essay distribution of the sample in the already packed silica gel column. Sample powdered mass was placed atop the prepacked silica column, and a sheet of cotton covered the sample. Then solvents (100% hexane) to fractionate the sample extract were passed through the column at a uniform rate under gravity. For further analysis, each fraction was gathered separately in a test tube and counted consecutively, and about seven different fractions were obtained.

**Identification Of Phytochemical Compounds By Thin Layer Chromatography**

For the study of phytochemical compounds (tannins, phenols and flavonoids) through Thin Layer chromatography, one of the best fractions was chosen (Raaman, 2006).

**Test for Tannins-**

With 1 ml of carbonated water, about 1ml of plant extract as stirred, filtered, and a few drops of 1 per cent ferric chloride were applied to the filtrate.

**Test for Phenols-**

Ferric chloride test- Approximately 50 mg was dissolved from the sample in 5 ml of distilled water. 55% of the neutral ferric chloride solution was applied to these few drops. A dark green colour designates the presence of phenolic compounds.

**Test for Flavonoids-**

Tested alkaline reagent: 2ml of the extract’s aqueous solution was handled with a solution of 10 per cent ammonium hydroxide 1ml. Yellow fluorescence has defined the existence of flavonoids.

**Synthesis Of Nanoparticles**

Solution (1): Approximately 6.9 g of copper pentahydrate sulphate was dissolved in 100 ml of distilled water. Solution (2): Dissolved about 34.6 g of sodium-potassium tartrate and 12 g of sodium hydroxide in 100 ml of Carbonated water, 50ml of solution I and 50ml of solution II were combined with a vigorous stirring agent and 5 g of glucose (reducing agent) was added. Then the mixture was stirred vigorously for 10 minutes and then held for 10 minutes in 60°C boiling water bath. Then the mixture collected is centrifuged and washed twice with distilled water and twice with ethanol, and dry air and the powdered material was used for further study (Ghulam et al., 2013).

**Metal-Herbal Nanocomposite Preparation**

In 1:1 combinations, Herbal and metal nano companies have been prepared to achieve those ratios, single intensity concentration of herbal and copper oxide metal nanoparticles was used. 100 mg of *Leucas zeylanica* extracts were dispersed in 1ml of sterile distilled water for 1:1 herbal metal nanocomposites, applied dropwise to 100 mg of nanometal solution. At the rate of 1ml/min, the herbal solution was applied. The composites were stirred continuously, and the formed MHNC was used for future analysis (Meenakshi and Gandhi, 2016).

**Characterisation of *Leucas zeylanica* Extracts and Nanocomposites**

The structural morphology of the nanocomposites was examined by Scanning Electron Microscopic (SEM) using TM-1000, Hitachi, Japan. To classify the functional groups present in the plant extract, FTIR analysis was performed. Particle Size Analyser (PSA) analysed the particle size of the formed nanocomposites. The Zeta potential is a critical parameter for nanoparticle suspension stability characterisation.

**Direct Hemolysis Assay**

RBC was used in vitro to study the haemolytic activity of venomous *Naja naja* and *Bungarus caerus* venoms and plant extracts. In short, for 10 minutes, 5ml of citrate blood had been centrifuged at 900rpm. The supernatant was discarded off and washed with a solution of physiological salt the pellet twice. For 5ml of saline and 0.5 ml of the RBC mixture, control was given. 5ml of distilled water with 0.5 ml of washed RBC was used for 100 per cent hemolysis. As an experimental sample, 5 ml of venom/extract and 0.5 ml of washed RBC were used. The tubes were put in a thermostat for 1 hr at 37°C and centrifuged at 2000 rpm for 20 mts. The supernatant fluid was poured out into separate tubes to estimate the optical density using the spectrophotometer at a wavelength of 540nm. The estimation of haemolysis was calculated using a formula—experimental sam.
Antigenic Potency of Snake Venom

The antigenic potency of *Naja naja* and *Bungarus caeruleus* venoms was determined by mixing constant amounts of venom (µg) with different amounts of plant extracts (µl) and incubation for 30 min at 37°C in agarose egg yolk, sheep erythrocytes gels, then adding 10 µl aliquots to the mixtures to the wells. Without plant extracts, the control is fulfilled by the venom. Plates at 37°C were incubated for 20 hours. Counteraction denoted as the percentage of mg of plant extract/mg of venom that can reduce the diameter of the haemolytic halo by 50% compared to the effect of venom alone (Gutiérrez et al., 1988).

### RESULTS AND DISCUSSION

#### Antigenic Potency of Snake Venom

The antigenic potency of *Naja naja* and *Bungarus caeruleus* venoms were checked by gel precipitation test. Snake venom and antivenom were added in the appropriate wells, and the gel slides were incubated overnight in a moist chamber at 4°C, and the results were read on a dark background. Precipitation arcs were observed against venom and antivenom, which shows antigenic potency of snake venom.

#### Purification and Identification of Compounds in *Leucas zeylanica* Extract

About seven fractions were collected from the column chromatography. The collected fractions were presented in the. The 5th fraction was used for the analysis of phytochemical compounds using Thin Layer Chromatography. From the TLC analysis, the plant extracts showed the presence of Tannins, Alkaloids and Flavonoids.

#### Functional group analysis using FTIR

FTIR spectrum of the *Leucas zeylanica* extract was analysed and presented in Figure 1. FTIR spectra of *Leucas zeylanica* extract showed several bands and broadband at 3745.76 cm⁻¹ due to –OH stretching frequency of the hydroxyl group. A similar peak at 3053.32 cm⁻¹ attributed for simple –OH (hydroxyl) stretching. The appearance of broad peaks at 1647.21 cm⁻¹ was attributed to alkenyl C=C stretching vibrations frequency. Other peaks corresponding to each of its functional groups were presented in Table 1.

#### Scanning Electron Microscopic Analysis

The topographical analysis of the developed MHNC was performed using Scanning Electron Microscopy at 15,000, and 25,000 magnifications (Figure 2), aggregates with an average nanoparticle diameter varying from 1µm have been theoretically observed in different types. Aggregation refers to the array of nanoparticles and compounds of extracts that Vander Waals and electrostatic interaction bring together. CuO nanoparticles may form aggregates or agglomerates in varying forms under ambient conditions. Ghulam et al. (2013) obtained CuO nanoparticles aggregates when observed under Scanning Electron Microscopy. Thus, from this topographical study, we find that, in addition to its diameter, the irregular aggregated form of CuO nanoparticles at 10000 magnifications varies from 1.5 µm and 0.2-1µm, respectively. The visual inspection of the SEM showed that aggregates are formed by nanoparticles and plant extracts, deciding the successful preparation of nanocomposites.

#### Particle size and stability of the Composites

The size and stability of the composites were examined by Particle Size analyser and Zeta potential. The size of the copper nanoparticle was observed to be 86.7±7.1nm, and composites were observed to be 249.4±27.3nm. The Stability of the Metal–Herbal Nanocomposite was observed to be -1.3mV. Thus the developed MHNC were stable and can be used for the medical applications.

#### Direct hemolysis assay of the herbal extract and Herbal-Metal Nano Composite

From the direct hemolysis method, the neutralisation potential of the plant extracts and MHNC against venoms were determined. The hemolysis (%) was found to be reduced on treatment with herbal extracts and MHNC (Figure 3). The hemolysis observed for venoms treated with *Leucas zeylanica* extract was found to be 30% and 27.2% against *Naja naja* and *Bungarus caeruleus* venoms. Similarly, the venoms treated with MHNC showed 16.1% against *Naja naja* venom and 17.1% against *Bungarus caeruleus* venom. Significant reduction in hemolysis was observed on Nanoparticle compos-
Figure 1: FTIR Spectrum of the *Leucas zeylanica* extract

Table 1: FTIR analysis of the *Leucas zeylanica* extract

| S. No | Peaks (cm⁻¹) | Functional groups                      |
|-------|--------------|----------------------------------------|
| 1     | 609.51       | Halogen compounds (C-Cl stretching)     |
| 2     | 860.25       | Carbonate (C=O)                         |
| 3     | 925.83       | Carboxylic acid (O-H stretching)        |
| 4     | 1022.27      | Ether (C-O stretching)                  |
| 5     | 1240.23      | Alkyl compounds (C-H in plane bending)  |
| 6     | 1319.31      | Aromatic amines (CN stretching)         |
| 7     | 1400.32      | Aromatic amines (CN stretching)         |
| 8     | 1448.54      | Methyl CH asym bend                     |
| 9     | 1543.05      | Nitrogen compounds                      |
| 10    | 1647.21      | Alkenyl C=C stretch                     |
| 11    | 2455.38      | Amine groups                            |
| 12    | 2926.01      | Terminal C-H stretch                    |
| 13    | 3053.32      | Alcohol (Simple OH stretch)             |
| 14    | 3745.76      | Hydroxy group, H-bonded OH stre         |

Indirect hemolysis assay of the herbal extract and Herbal-Metal Nano Composite

Indirect hemolysis activity was determined by Phospholipase A2 inhibition activity of the venoms. The zones produced by *Naja naja venom* was observed to be 28mm and zones produced by *Bungarus caeruleus* venom was to be 26mm. Treatment with *Leucas zeylanica* extracts showed 26mm against *Naja naja venom* and 21mm against *Bungarus caeruleus* venom. Similarly, treatment with MHNC showed 15mm reduction in zones against *Naja naja venom* and 11mm against *Bungarus caeruleus* venom respectively.

DISCUSSION

In many countries, the snake bite is endured as public health concern still it is tough to know the accurate number of cases (Chaudhary, 2011). Medicinal plants are treating various disease, including the envenomation by animal bites for many years. Snake-bites are still a major socio-medical crisis for the tropical and subtropical countries. The Anti-snake venom serum (ASVS) is the only available source, but it has a specific adverse effect and limitation. There is no particular dosage of ASVS for the neutralisation of specific snake venom. Toxicologists have developed an interest in finding an alternative for the ASVS. In developing the low-
cost phytotherapeutic agent, the traditional usage of plants for experimental validation is an important step. There are many Indian medicinal plants which are recommended for treating snake bite, and some are inspected for neutralisation of venom. Few Herbal antagonists have recognised the alternative for the ASVS, but it is a failure. The failure of the herbs accounts for several causes, of which “availability, toxicity, geographical variation and effectiveness are essential”. In a particular instance, pure herbal compounds have shown less safety than the entire extract. As per Ayurveda, the bioactivity of the herb is enhanced by the conjugation of metal. Studies have shown that the use of metal in ayurvedic formulation turn them into a nanosized particle. The conjugation of nanoparticle with the herb or herbal compounds can improve the effectiveness of the herbs or herbal compounds. Researches have shown when the copper nanoparticles conjugated with the herbal compounds, they act as efficient drug delivery vector and has improved cellular absorption (Patra et al., 2018).

Leucas zeylanica is an antisnake venom herb. In this research, leaf extracts of Leucas zeylanica are used for the preparation of the Herbal metal nanocomposite (MHNC) against Naja naja and Bungarus caeruleus venoms. The formation of precipitation arcs towards venom and antivenom indicates the antigenic efficacy of snake venom. The samples have been further extracted by column chromatography. The segment was subjected to TLC assay for Flavonoids, phenols and tannins and used for the preparation of the composites. The FTIR spectra study revealed the functional group belongs to Leucas zeylanica extract. The functional groups of compounds found in the plant extract have been iden-
tified. SEM image shows the morphology and size of nanoparticles, which indicates the formation of aggregates. The size of the Leucas zeylanica extract was observed to be 86.7±7.1nm and 249.4±27.3nm for MHNC in the research. The variation in the size is due to the formation of aggregates. The zeta potential is measuring the stability of the colloidal particles. The high positive or negative zeta potential induce a repulsion force between particles, which increase the stability of the particle. The zeta value indicated significant particle stability (Chakrabarty et al., 2019; Wadood et al., 2012).

The direct hemolysis with sheep's RBCs was tested for Naja naja and Bungarus caeruleus venoms, and it was observed that both the snake venom were capable of lysing RBCs. The hemolysis effectiveness of Naja naja and Bungarus caeruleus venoms treated with extracts was found to be 30% and 27.2% respectively. The hemolysis venom is treated with MHNC which tend to reduce to 16.1% against Naja naja venom and 17.1%. This shows that the nanoparticles have high neutralisation capacity than a plant extract. The 20μg of Naja naja venom and Bungarus caeruleus venom produced 28mm and 26mm diameter hemolytic halo zone by phospholipase production (PLA2). MHNC was able to suppress the PLA2 dependent RBC sheep hemolysis caused by snake venom, and the zones were decreased to 15mm and 11mm against Naja naja venom and Bungarus caeruleus venom. Phospholipases A2 is responsible for numerous pathophysiological disorders like cardiotoxicity, oedema, neurotoxicity, haemolysis, necrosis, anti-coagulation and amputation. Besides, the production of free radicals with the reactive oxygen produced toxicity of phospholipases in snake bite victim (Chethankumar et al., 2010). The proteolytic enzyme plays a significant role in the digestive reaction, phospholipase A and B involved in the degradation of lipids into free fatty acids which cause lysis and apoptosis of cell (Wadood et al., 2012). The enzyme Phospholipases A2 is present in the snake venom, which plays an important role in immobilisation and killing of prey or victim (Pithayanukul et al., 2009). Therefore the side effects are reduced by decreasing the activity of Phospholipases A2.

Hence it has been observed the metal-herbal nanocomposite provides more neutralisation against Naja naja and Bungarus caeruleus venoms than the Leucas zeylanica extracts. The neutralisation is due to the conjugation of copper nanoparticle with the Leucas zeylanica extracts. The conjugation improves the availability of compounds, enhance cellular uptake and repairs the damage caused by the venom. MHNC has antagonised (directly/indirectly) the activity of Naja naja and Bungarus caeruleus venoms -induced organ toxicity. Even though the composite can be produced at low cost and it is found to be effective on neutralising venom, MHNC can be used to treat the snake bites.

CONCLUSION

The antigenic potency of snake venoms was determined by gel precipitation test. Leucas zeylanica leaves were used for extraction, and extracts were further purified by column chromatography. Size of Leucas zeylanica extract was found to be 86.7±7.1nm and 249.4±27.3nm for MHNC. Zeta potential values determined the Stability of the MHNC. MHNC showed higher neutralisation to venoms than the Leucas zeylanica extracts. In direct hemolysis, the efficiency of Naja naja and Bungarus caeruleus venoms treated with Leucas zeylanica leaf extract was found to be 30% and 27.2%, whereas MHNC showed 16.1% against Naja naja venom and 17.1% Bungarus caeruleus venom. From Indirect analysis, Naja naja and Bungarus caeruleus venom were able to produce 28mm and 26mm diameter hemolytic halo zones. On treatment with MHNC, the zones were reduced to 15mm and 11mm than Leucas zeylanica extract. This result indicates the MHNC can be used for the development of drugs for the treatment of snake bites.

Conflict of Interest

The authors declare that there is no conflict of interest for this study.

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