

Rapid and Safe Relief Detox Formulation Against Tick Parasites for Cattle Using Bionanocomposite

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Abstract

There exist various parasites around the globe, one such parasite that has been a big deal to humans is the ticks that live on domestic animals. They live on their skin and cause serious issues to animals as well as humans by transmitting the disease from the host to mankind. The present study focuses on assessments to determine the potential of the synthesized bionanocomposite (GO/Ag) using aqueous leaf extract of *Nerium oleander*, against ticks. The synthesized bionanocomposite was confirmed by various characterizations. The ticks were exposed to different concentrations of GO/Ag bionanocomposite and the maximum acaricidal efficiency was observed against dog ticks at 0.01 g/10 ml and against cow ticks at 0.075 g/10 ml respectively. Later the study was extended to infer the toxicity of the synthesized bionanocomposite and that conveyed suitable for use. This is considered as an innovative and alternative approach to serve against ticks.

Keywords

Ticks, Bionanocomposite, Acaricidal efficiency, *Nerium oleander*, Toxicity, Zebrafish

Introduction

The term nanotechnology refers to the ability to measure, manipulate and organize matter at the nanoscale level. The scale classically refers to the matter in the size ranging from 1 - 100 nm is classically referred to as the nanoscale, but it is often extended to include particles in size of below 1 μm [1]. Nanomaterials find their wide application in the field of veterinary medicine depending on various parameters. Those parameters include nanoparticle size, shape and structure, Origin of respective nanoparticles, methodologies adopted for their preparation, etc. [2]. Ticks are ectothermic, hematophagous arthropods. Ambient temperature and biotic and abiotic environmental conditions influence their life cycle, reproduction, phenology, ability to survive, etc. Different species require different suitable conditions to start and end their life cycle [3]. Ticks in dogs and cattle have to be controlled due to various reasons. Dogs and cattle were being reared as domestic animals for various purposes. They should be free from any kind of parasites so that they can be indirectly free of the risk of transmission of pathological agents from them to humans. Within 24 h, each female parasite can lay up to 50 eggs. The eggs fall off the dog into the surrounding area. The next generation of ticks is developed in the places where the dog goes. Some dogs bearing ticks on their skin do not show any skin disease, whereas others can show hair loss, severe irritation, and inflamed or secondarily infected skin [4].

Nanoparticles are nowadays preferred because of their smaller size and larger surface area. Nanoparticles are more targeted in their action than their metal

counterpart. Green synthesis of nanoparticles using leaf extract can overcome many of the destructive effects of physical and chemical techniques [5]. In recent times, silver nanoparticles (Ag NPs) have gained major interest due to their varied potential applications. Ag NPs show less toxicity to human health but, they are highly toxic to microorganisms and parasites like ticks, fleas, etc. [6]. Recent research show that graphene and graphene-related nanomaterials/nanocomposites lead to a pro-inflammatory response in various organs and cause genotoxicity and DNA damage. Even they adversely impact the function of the various organs. Moreover, depending on the size, their oxidative status, and the concentration of nanoparticles, graphene, graphene oxide (GO), and reduced graphene oxide (rGO) have shown different levels of cytotoxicity [7]. Here, in this study, we synthesized GO nanoparticles, Ag NPs and GO/Ag nanocomposite using the leaf extract of *N. oleander*. Oleander leaves and flowers contain various phytochemicals such as phenols, tannins, flavonoids, coumarins, sterols and triterpenes, and alkaloids [8]. Because of its good reducing property, commercial availability, and presence of minor toxic nature, it is preferred to be used as a reducing and capping agent in the study. The required nanoparticles and the nanocomposite were synthesized, and it is tested for its acaricidal activity against tick parasites of both dogs and cows. The activity is performed by testing in different concentrations over the parasite and periodically monitoring the mobility and mortality of the ticks. In this way, the acaricidal effect of the synthesized powder is detected and confirmed. The synthesized bionanocomposite is tested for its toxic effects on humans. After this *in vitro* study, the decided concentrations were further taken for testing its toxicity. This study was carried out in *Danio rerio* (Zebra fish), whose genetic makeup was similar to that of humans by ~71%. Moreover, Zebrafish is an excellent model for toxicological studies among various other experimental models [9]. After successful testing of the bionanocomposite, the bionanocomposite is formulated into a detox powder and this study serves as a good scope for the tick-borne animals to get relief once they were infected.

Materials and Methods

Chemicals

99% pure AR-grade chemicals from Hi media were used for the entire experiment. The chemicals used include silver nitrate (AgNO_3), plant extract (*N. oleander*), graphite powder, sulphuric acid, hydrochloric acid and other required chemicals. All the procedures were conducted using high-grade borosil glass apparatus and they were thoroughly sterilized.

Apparatus used

The primarily required apparatus included a weighing balance, autoclave, centrifuge, hot air oven, magnetic stirrer, ice maker, shaker incubator, UV-Visible spectroscopy and various needed instruments.

Collection of plant material

Fresh leaves of the young and growing *N. oleander* plant were collected in and around the surrounding area, especially in the town of Tiruchengode, Namakkal, Tamil Nadu, India. *N.*

oleander is commercially available in the state of Tamil Nadu.

Preparation of *N. oleander* powder

Initially, the leaves were soaked in 70% ethanol to remove the surface contaminants. Then the leaves were washed with water to remove the dirt particles [10]. They were kept at room temperature for about 15 days, and it is allowed to dry under shade [11]. Dried leaves were ground into powder using an electric mixer and the dried powder is made into a fine powder using a sieve. This fine powder is then stored in an air-tight container for further experimental purposes.

Preparation of plant extract

Standard: 10 g in 100 ml of aqueous solution.

The plant extract was prepared by the decoction method. 10 g of the finely ground powder was weighed accurately using a weighing balance. The weighed material is then added into a clean and dry 200 ml beaker. The beaker is filled with 100 ml of deionized water. The solution is heated and boiled in a heating mantle for about 15 min at a high temperature (80 °C). The solution is allowed to cool for some time. Once after reaching the bearable warmth, the solution is filtered using Whatman No. 1 filter paper into another conical flask. This filtrate is now ready to use. Every time it is good to prepare a fresh extract.

Synthesis of Ag NPs

The entire procedure for the synthesis of Ag NPs must be carried out in a dark environment. Ag NPs were synthesized from AgNO_3 . 1 mM AgNO_3 solution was prepared. This is prepared by taking 0.0169g of AgNO_3 in a clean and dry conical flask and dissolving it in 100 ml of deionized water.

$$\text{Molarity} = \frac{\text{Molecular weight} \times \text{Required molarity} \times \text{Required volume}}{1000}$$

From this prepared AgNO_3 solution, 5 ml was drawn and added into a new clean and dry conical flask covered with an aluminium foil sheet and dark environment. To this solution, 8 - 10 ml of the plant extract was added and mixed. Finally, 2 ml of ammonia solution is added to the conical flask. The mixture is stirred thoroughly by placing it in a shaker incubator at 35 °C and at 200 rpm for 48 h. The formation of Ag NPs is identified by the color change from pale yellow to a dark brown-colored solution [10]. At this point, the solution is centrifuged at 5000 rpm for 10 min. The pellet is washed with distilled water to remove any suspended materials, and it is allowed to dry completely with no moisture in it at room temperature. The dried powder is the synthesized Ag NPs and stored for further characterization and experimental studies.

Synthesis of GO nanoparticles

Synthesis of GO from graphite

The required chemicals and their respective composition for the synthesis of GO are listed in table 1. 1 g graphite powder, 1 g NaNO_3 and 50 ml concentrated H_2SO_4 are added to a 500 ml volumetric flask. The ice bath was prepared manually using the ice cubes from the ice maker. The flask containing the mixture is placed into the prepared ice bath and it is stirred continuously for 2 h. The temperature of the bath is main-

Table 1: Chemical composition for graphite oxide synthesis.

Chemicals	Composition
Graphite powder	1 g
NaNO ₃	1 g
Concentrated H ₂ SO ₄	50 ml
KMnO ₄	3 g
HCl	10%

tained between 0 °C to 5 °C for the entire 2 hours. After 2 h 3 g KMnO₄ was added gradually (0.5 g for every 5 min), maintaining below 15 °C. After 2 days, add 100 ml deionized water, the appearance of brown color is seen. This is to dilute the sample. After stirring for 5 minutes, the mixture is further diluted with 100 ml of deionized water. Finally, the mixture is treated with 8 ml hydrogen peroxide (H₂O₂) that results in the formation of the yellow color solution indicates the formation of GO. The solution was washed several times with 10% HCl and deionized water. The solution is centrifuged at 5000 rpm for 15 - 20 min with dilute HCl. The solution is centrifuged and washed with dilute HCl for 5 times. Then the solution is centrifuged at 6000 rpm for 30 minutes for 4 times with deionized water. Finally, the pellet is collected and dried until there is no moisture content present. The dried content is considered as the GO and is collected in the petri plate [12, 13]

Synthesis of rGO

A colloidal dispersion of 1 g of synthesized GO in 100 ml of deionized water was prepared. It is exfoliated by ultrasonication for 30 min. To the resulting suspension, 10 ml of the leaf extract is added, and it is subjected to ultrasonication in an ultrasonicator bath for about 30 min. Later the suspension is centrifuged, and the pellet is collected as mentioned above. The pellet is allowed to dry completely into a dry powder [14, 15].

Synthesis of GO/Ag bionanocomposite

1 g of the synthesized GO powder was taken in a clean dry beaker and to this 100 ml of deionized water is added. The solution is sonicated in an ultrasonicator bath for 30 min with ON and OFF time of about 8 sec and 2 sec respectively. To this GO suspension, 50 ml of 0.1 Mm AgNO₃ solution is gradually added in a dropwise manner. And the sonication is continued for about 60 min. 100 ml of prepared leaf extract is added in drops to the sonicated mixture and the entire solution is again sonicated for about 30 min. Finally, the solution is centrifuged at 5000 rpm for 15 min. The supernatant is discarded, and the pellet is collected and dried completely at 50 °C. The collected powder is described as GO [16, 17]. The mixture is stored for further experimental application.

Physico-chemical analysis of the synthesized nanoparticles and the nanocomposite

UV-Visible spectroscopy

Using UV-Visible spectroscopy, optical properties, quantitative formation and the size of nanoparticles can be studied [18]. Dispersion of plasmonic nanoparticles gives one or more

peaks due to the surface plasmon resonance (SPR) property of nanoparticles. Those peaks can be used to obtain information regarding the shape, size and size distribution of nanoparticles [19]. UV-Visible analysis determines nanoparticle formation and its stability [20]. The synthesized nanoparticles were re-suspended on deionized water in a quartz cuvette on the double-beam spectrophotometer to confirm the reduction of nanoparticles. The wavelength scan was performed by setting different wavelength intervals for the respective nanoparticles. The peak value for each was noted and the graph was drawn using the obtained values.

Particle size analysis

Particle size analysis can be done through various techniques and diffusion limited aggregation (DLA) is one such approach. Dynamic light scattering (DLS) was carried out to ascertain the size and distribution of particles in the synthesized sample [21]. DLS measurement also gives the average hydrodynamic diameter of the particles through particle Brownian Movement and the monodispersity or polydispersity index (PI) that describes the width of the particles in a colloidal system [22].

X-ray diffraction (XRD)

The atomic structure of materials is determined using an X-ray diffractometer. XRD was mainly used to study the phase structure and the material identification of nanoparticles. XRD is a technique used to characterize and acquire accurate information regarding the composition, crystal structure, and crystalline grain size of nanoparticles [23]. The formed nanoparticles were clearly confirmed using XRD. XRD characterization was performed at sophisticated analytical instrument facility, Cochin.

Fourier transform infrared spectroscopy (FTIR)

FTIR uses infrared radiation to pass through a sample. The principle of detecting functional groups by FTIR was used for analyzing the nanomaterials. The details of functional groups and appropriate surface modification strategy can be understood using FTIR [24]. The dried powders of nanoparticles were used for FTIR analysis, and the result was noted at its various frequencies.

Scanning electron microscopy (SEM)

Using SEM, the shape, size, morphology and distribution of synthesized nanomaterials were characterized. The morphology and topography of synthesized materials were analyzed using SEM [25]. The powdered nanoparticles were mounted on a copper mesh and by using a gold sputtering unit, a 3 nm gold coating was performed [26].

Energy dispersive X-ray analysis (EDAX)

EDAX analysis was carried out along with the analysis of SEM. SEM was additionally fitted with the EDAX instrument for analyzing the samples. The atomic weight percentage, in short, the elemental composition of synthesized GO, Ag and GO/Ag was determined using EDAX results [27]. EDAX characterization was mainly used to perform the qualitative analysis of the samples [28].

Tick collection and identification

The dogs and cows with ticks were identified and the ticks were collected from different dogs and cows. For research purpose, it was collected from the ones with good health and from the ones with very few health issues, the ones with medium symptoms and the ones with weak health. Infected dogs' symptoms vary from asymptomatic to severe [29]. Dog ticks were collected from Tiruchengode, Namakkal, Tamil Nadu and cow ticks were collected from Kumbakonam, Tamil Nadu. Ticks found were manually detached and pooled into a clean and sterile labelling tube (vials). The sterile labelling tubes were clean and dry without any moisture content which may affect the parasite (Tick) present inside. Tick samples were reared and stored following laboratory guidelines. Samples of ticks that were collected from dogs and cows were individually inspected for the various developmental stages (i.e., larval, nymph, and adult) and gender (female and male), etc. [14, 30]. The collected ticks were sent for detailed analysis to the Department of Veterinary Parasitology at Tamil Nadu Veterinary and Animal Sciences University (TANUVAS), Namakkal.

Tick behavioral test against bionanocomposite

Two concentric circles were drawn over the Whatman no.1 filter paper labelling A (2 cm from the center) and B (1 cm from A) and the excess was cut off. B is considered as a finish line. Two petri plates were taken and lined with labelling filter paper. 0.05 g of bionanocomposite was dissolved in 5 ml of d.H₂O and it was applied using a dropper in the space outside the finish line (B) over the petri dish (Figure 1a). One was maintained as a control using only distilled water (Figure 1b). A tick was placed at the center of the filter paper and the time spent by the tick to travel from the center to line A and from A to B was recorded. This was used to understand the basic behavior of ticks against the synthesized bionanocomposite [31].

In vitro bioassay test

The aim of bioassay is to predict the efficacy of the synthe-

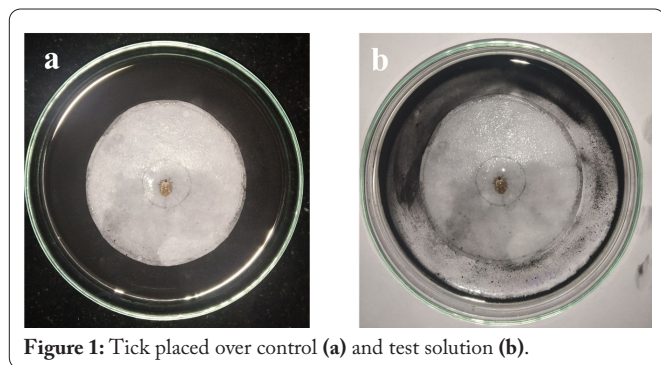


Figure 1: Tick placed over control (a) and test solution (b).

sized acaricide against ticks and to determine the appropriate dosage to be used in further studies. The ticks used for the tests should preferably be of the same species to reduce the scale of variability [32].

Different concentrations of the synthesized bionanocomposite solution were prepared. Whatman no.1 filter paper was impregnated into the respective solutions, and they were

Table 2: Selected bionanocomposite concentrations.

Samples	Concentration (10 g/ml)			
	Control	0.002	0.005	0.01
Dog tick samples	Control	0.002	0.005	0.01
Cow tick samples	Control	0.05	0.1	0.2

placed over the four different Petri plates. The control plate was maintained using distilled water. 5 ticks were placed over the moist filter paper and the motility and mortality of the ticks were observed for 24 h and the obtained results were tabulated [33-35]. For dog and cow ticks the bioassay test was carried out separately following the same procedure with only variation in the concentration of the bionanocomposite (Table 2).

Dose-response bioassay

Based on the preliminary screening results from the bioassay, the synthesized bionanocomposite was subjected to a dose-response bioassay. The desired concentration for acaricidal activity was directly taken from the bioassay results and the dose-response bioassay was carried out by adopting a similar procedure for both dog and cow ticks individually. The number of dead ticks were counted after 24 h of exposure, and the percentage mortality was reported. The death of the parasites was confirmed when there was a cessation of motility or wagging of the appendages upon touching with a needle [36, 37].

Toxicity assay

Collection of experimental animals

In order to test the toxic effect of the developed bionanocomposite, a toxicity study was carried out. The subject animal for this toxicity testing was an adult shortfin wild type of Zebra fish which was bought from Siragua Aqua Agri Farm, Mettur. The fish were allowed to acclimate in the laboratory for about 3 days.

Behavioral analysis of Zebra fish

Here in the study, the toxicity in zebrafish was determined using an adult breed. 3 square glass aquarium tanks, each of dimension 15.1 cm × 15.1 cm × 15.4 cm (L × W × H) were taken for the study, and they were washed thoroughly. The tank was filled with 2 L of fresh water as Zebra fish is a freshwater fish. The tank was then equally divided into two horizontal halves, which are entitled as top and bottom sections. The position of the fish was enlisted by this separating line. The components and the labelling of the 3 tanks were given in table 3.

For dog and cow tanks, the corresponding concentration of the bionanocomposite was dissolved in the water and is left for about 1 h. After one hour of complete dissolution, the fish was allowed to free flow in the tank. The behavior and the moment of each fish in the respective tank were video recorded for about 5 min individually and some of the erratic movements were performed with accuracy through human observations. The behavior recorded was analyzed through TRACKER software. The time spent by each fish in the top and bottom layers was also recorded [38].

Table 3: Labeling and composition of the tank.

Name	Components	No. of fish in each tank
Control	2 L fresh water only	5
Dog	2 L fresh water + desired conc. of GO/Ag bionanocomposite	
Cow	2 L fresh water + desired conc. of GO/Ag bionanocomposite	

Survival test

After behavioural analysis, the subjected fish were taken for testing its survival. 5 fish were left together freely in the respective tanks, and it is monitored for its surviving capacity for about 2 days.

Formulation of tick detox

Formulating a powder involves a good understanding of the properties of the ingredients used and their interaction with each other. The toxicologically tested GO/Ag bionanocomposite was formulated into the form of detox powder for easy application and effective results. The first step is to reduce the particle size which is done through the process called trituration. Trituration is, grinding the chemical in a mortar and pestle by applying a strong and downward press. The formulation process generally involves the combination of various agents such as a carrier, a stabilizer, a preservative, etc., a carrier is used to dilute or disperse the active ingredient in the powder and also helps to prevent clumping as well as improves the flowability of the powder [39]. The stabilizer is used to prevent the powder from clumping together and the preservative is used to prevent any kind of microbial growth.

Result and Discussion

Leaf powder and extract

The collected leaves were dried under shade and ground into a powder, and it was sieved for their fineness (Figure 2). Later the powder was stored in an air-tight container and used whenever necessary. The leaf extract was prepared by dissolving 10g of the powder in 100 ml of deionized water. The obtained crude mixture is then filtered using Whatman no. 1 filter paper. The final greenish-brown liquid remaining after extraction was used as a reducing agent in the experimental processes.

Formation of Ag NPs

Adding *N. oleander* leaf extract to the AgNO_3 solution reduces the metallic silver to Ag NPs due to the phytochemical reduction between the extract and the silver metal. As the reaction proceeds the color of the solution gradually changes from yellow to yellowish brown and to dark brown color from 24 h to 48 h. Ag NPs were confirmed by their SPR and the synthesized Ag NPs were centrifuged and collected in powder form. The primary confirmation of the synthesis was carried out by UV-Visible spectroscopy.

Formation of GO nanoparticles

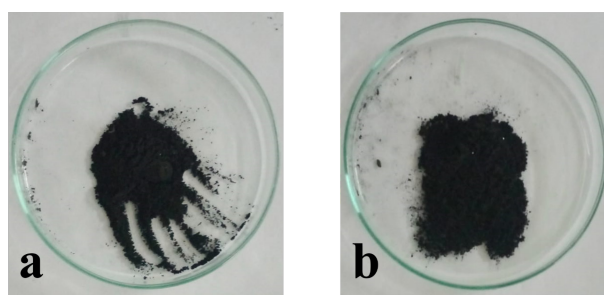
GO nanoparticles were synthesized in two steps. The

**Figure 2:** Nerium oleander leaves fresh (a), dried (b) and powdered (c).

first step involved the synthesis of GO from graphite powder by adopting Hummer's method and the next step involved the synthesis of GO nanoparticles by exfoliation of the GO powder by ultrasonication in an ultrasonicator bath. The addition of a reducing agent during the exfoliation step reduces the bulk graphite to GO nanoparticles by the phytochemical activity. Finally, the GO was synthesized in dark black color and the solution was centrifuged and washed to remove the impurities. The final product is dried in a hot-air oven (Figure 3) and stored.

Synthesis of GO/Ag bionanocomposite

Bionanocomposite was synthesized directly from the GO powder from Hummer's method and the AgNO_3 solution. Each solution after appropriate mixing was subjected to ultrasonication for a respected time duration and this facilitated the reducing agent to act on the metals and the phytochemicals present in it enabling them to reduce into their ionic forms and to the nanomaterials. The final solution retrieved after complete ultrasonication was visually identified as bionanocomposite by the dark black color. It was centrifuged, washed, dried in a hot-air oven (Figure 3) and stored in an air-tight container for experimental application.

**Figure 3:** Synthesized GO NP (a) and GO/Ag NC (b).

Analysis of ticks

Two dog ticks and two cow ticks were submitted to Veterinary College and Research Institute, Namakkal for species identification and the obtained results were represented in table 4.

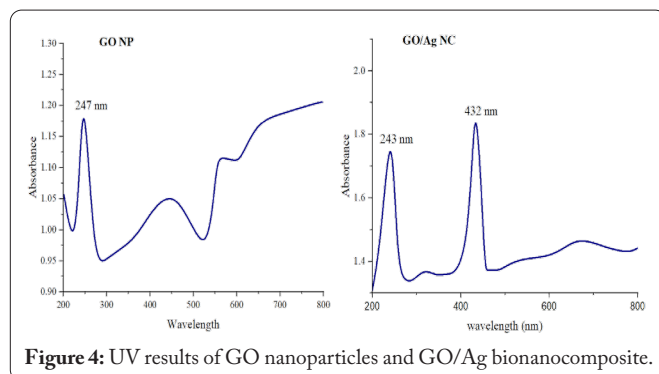
Characterization of nanomaterials and bionanocomposite

UV-Visible Spectroscopy

The UV-Visible spectra were utilized to observe the formation of GO and Ag nanoparticles and GO/Ag bionanocomposite. The UV-Visible spectrum results of the synthesized samples were shown in figure 4. The wavelength was set between 200 nm – 600 nm. The UV spectra showed a peak value at 247 nm for the synthesized GO nanoparticles, cor-

Table 4: Tick characterization results.

Kind of animal	Kind of material	Results
Cow	Ectoparasites	<i>Rhipicephalus (Boophilus) microplus</i>
Dog		<i>R. sanguineus</i>

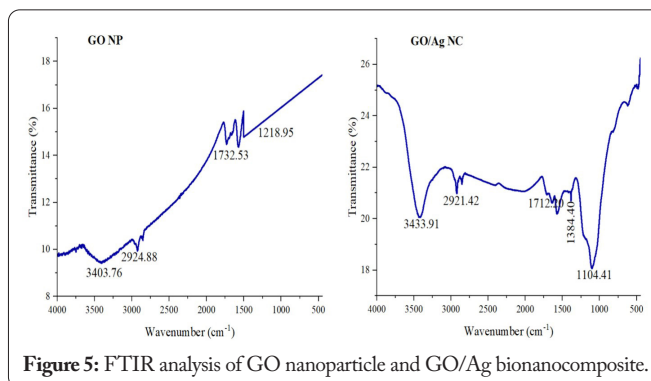
**Figure 4:** UV results of GO nanoparticles and GO/Ag bionanocomposite.

responding to the GO nanoparticle value. Also, this was in accordance with the result obtained [40]. This primarily confirmed the reduction of GO to the formation of GO nanoparticles. This is attributed mainly due to $\pi \rightarrow \pi^*$ transition.

The absorption bands of the synthesized bionanocomposite (GO/Ag) using pure *N. oleander* leaf extract as a reducing and capping agent appeared at 243 nm which correlated to the peak value of the GO and another peak was seen at 432 nm and this correlated to the peak value of Ag. These values were due to SPR, and these peaks confirmed the reduction of GO and Ag ions and the formation of the corresponding nanocomposite. It is concluded that the biologically synthesized sample contained the GO nanoparticles and the GO/Ag nanocomposite.

FTIR

FTIR yields conclusive results on the presence of various vibrational modes as well as the functional groups contained in the synthesized nanoparticles and the nanocomposite. 7 peaks resulted between 528.81 cm^{-1} and 4000 cm^{-1} for the synthesized GO nanoparticle and the obtained result is shown in figure 5 and table 5. The strong and broad peak was seen at 3403.76 cm^{-1} and that indicated the availability of alcoholic OH stretching vibration. The peak at 2924.88 cm^{-1} showed the medium C-H stretching of the alkane group. The strong peak at 1732.53 cm^{-1} indicated the aldehydic C=O stretching vibration. The peak at 1218.95 showed the strong C-O stretching of the vinyl ether group. Figure 5 and table 6 show the FTIR results of the bionanocomposite. 7 peaks were reported between 471.97 cm^{-1} and 4000 cm^{-1} for the prepared GO/Ag bionanocomposite. The peak at 3433.91 cm^{-1} shows strong and broad alcoholic OH stretching. The peak at 2921.42 cm^{-1} indicated the medium C-H stretching of the aldehydic group. The presence of a strong carboxylic acid group with C=O stretching is indicated in the peak at 1712.20 cm^{-1} and a peak at 1384.40 cm^{-1} showed the medium aldehydic C-H bending. The peak at 1104.41 cm^{-1} indicated the presence of strong C=O stretching of the secondary alcohol.

**Figure 5:** FTIR analysis of GO nanoparticle and GO/Ag bionanocomposite.**Table 5:** FTIR spectral results of GO nanoparticles with their vibrational modes.

Absorption frequency	Appearance	Chemical groups	Compound class
3403.76	Strong, broad	O-H stretching	Alcohol
2924.88	Medium	C-H stretching	Alkane
1732.53	Strong	C=O stretching	Aldehyde
1571.34	-	-	-
1218.95	Strong	C-O stretching	Vinyl ether
621.05	-	-	-
528.81	-	-	-

Table 6: FTIR spectral results of GO/Ag bionanocomposite with their vibrational modes.

Absorption frequency	Appearance	Chemical groups	Compound class
3433.91	Strong, broad	O-H stretching	Alcohol
2921.42	Medium	C-H stretching	Alkane
1712.20	Strong	C=O stretching	Carboxylic acid
1574.95	-	-	-
1384.40	Medium	C-H bending	Aldehyde
1104.41	Strong	C-O stretching	Secondary alcohol
471.97	-	-	-

Particle size analysis

The PSA data was displayed in figure 6 and the particle size distribution was carried out using a PS analyzer at $25 \text{ }^\circ\text{C}$. It is particularly used to measure the hydrodynamic diameter. The particle size of the bionanocomposite (GO/Ag) was measured under conditions such as diluent: water, refractive index: 1.3328, viscosity: 0.8878 cP scattering intensity: 31400 cps. The Particle size results showed that the average particle size of the sample was about 95.9 nm with PI of 0.334. The graph showed that the particles are polydisperse in nature and the particles are at constant size with stability.

XRD

XRD characterization was performed to understand the crystal structure (Crystal/amorphous), the crystal size, the lattice strain and the path difference (d) present in the synthesized nanoparticles and the nanocomposite. The results

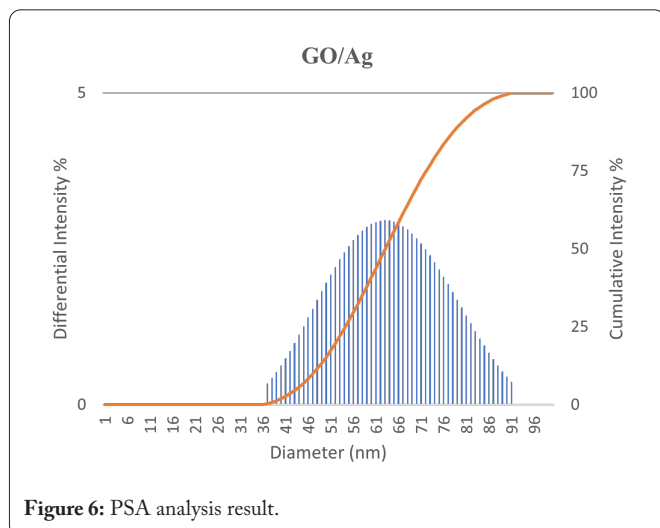


Figure 6: PSA analysis result.

were calculated and tabulated in table 7 and the obtained graphs were displayed in figures 7a and figure 7b. The crystal size and the lattice strain were calculated from the Debye-Scherrer equation and interplanar spacing from Bragg’s equation.

The obtained graphical result confirmed that the synthesized GO and GO/Ag were crystal in their structure. The average crystal size was calculated from the table and was found to be 13.45 nm for GO nanoparticles and 24.046 nm for GO/Ag nanocomposite. Using these results, the synthesis of GO nanoparticles and GO/Ag bionanocomposite was confirmed.

SEM

SEM characterization elucidated the surface morphology, size and shape of the synthesized nanoparticles and the bionanocomposite. The SEM image depicted that the taken reducing agent (*N. oleander*) has good reducing capacity and significantly reduced the metal ions to the nanometric form. The SEM images of GO nanoparticles represent that the particles are irregular and rod-like structures. Meanwhile, the GO particles are very much dense and closely organized.

For GO/Ag bionanocomposite, the structure was a combination of spherical and broken flakes with a rough surface. These structures were so tightly arranged one over the other with minute spaces between them. The SEM images of the developed GO and GO/Ag bionanocomposite were displayed in figure 8a and figure 8b.

EDAX

The EDAX analysis provided the details of elements

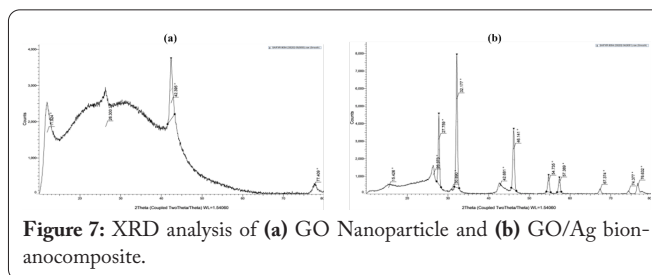


Figure 7: XRD analysis of (a) GO Nanoparticle and (b) GO/Ag bionanocomposite.

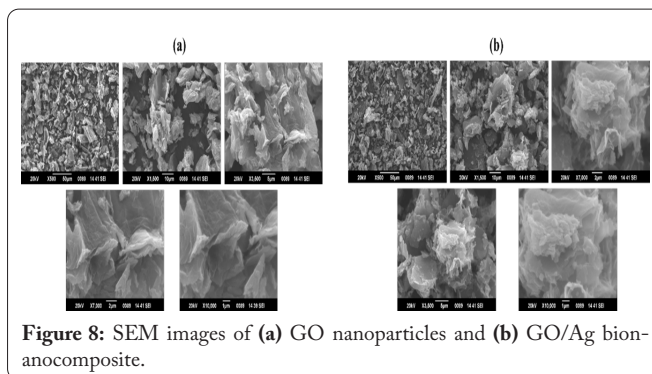


Figure 8: SEM images of (a) GO nanoparticles and (b) GO/Ag bionanocomposite.

present in the synthesized nanoparticles and nanocomposite in terms of weight and atomic percentages. The obtained results were represented in table 8 and table 9. SEM was attached with EDAX equipment to carry out the EDAX characterization (Figure 9a and figure 9b).

Table 8: EDAX results of GO nanoparticle.

Element	Line type	Weight %	Atomic %
C	K Series	70.06	76.44
O	K Series	28.16	23.07
Al	K Series	0.38	0.16
S	K Series	0.16	0.06
Cl	K Series	0.19	0.06
Cu	K Series	0.57	0.12
Zn	K Series	0.47	0.09

Table 9: EDAX results of GO/Ag bionanocomposite.

Element	Line type	Weight %	Atomic %
C	K series	56.99	75.8
O	K series	18.91	18.89
Cl	K series	5.75	2.59
Ag	L series	18.35	2.72

Table 7: XRD results and the crystal sizes of the synthesized particles.

Particles	Peak position (2θ)	FWHM	Crystalline size, Dp (nm)	Lattice strain	Interplanar spacing, d (nm)
GO	42.436	0.704	13.45	0.00796	0.21
GO/Ag	27.756	0.313	29.08	0.00553	0.32
	32.177	0.358	25.67	0.00542	0.28
	46.150	0.438	21.89	0.00449	0.20
	54.729	0.472	21.04	0.00398	0.17
	57.381	0.446	22.55	0.00356	0.16

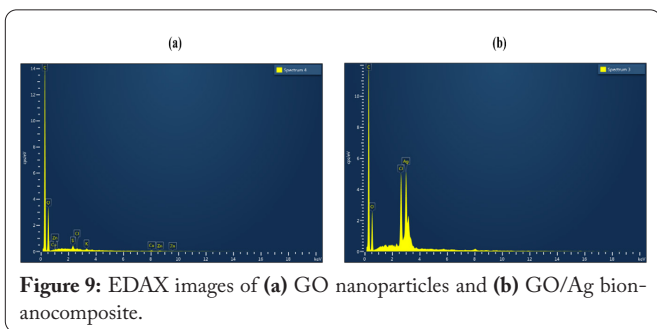


Figure 9: EDAX images of (a) GO nanoparticles and (b) GO/Ag bionanocomposite.

GO is comprised mainly of carbon and oxygen. The presence of other elements in trace quantity comes from the presence of used leaf extract during the synthesis process. The EDAX analysis revealed the bionanocomposite to contain C, O and Ag in different compositions. The presence of C, O and Ag confirmed the formation of the GO/Ag bionanocomposite. Comparing table 8 with table 9, table 8 shows a significant increase in C and O content. The EDX spectrum revealed the formation of GO/Ag nanocomposite with lower oxygen content that equals silver composition (Acar Bozkurt, 2017).

Effect of bionanocomposite on ticks

Table 10.1 and table 10.2 depicts the observation of the mortality of the ticks with varying concentrations of the bionanocomposite at 24h and 48h respectively. The results obtained from the biological assay (Figure 10) were then subjected to a dose-response assay. Here the dose-response assay (Figure 11) was carried out in two stages and the results of the dose-response assay were tabulated in table 11.1, table 11.2, table 11.3 and table 11.4 [41].

From this bioassay test on *R. (B) microplus* and *Rhipicephalus sanguineus*, 0.1 g/10 ml and 0.01 g/10 ml were chosen as desired (Figure 12) and it is subjected to dose-response because the mortality rate of the ticks was high at that concentration compared with the other two taken concentrations. Similar to bioassay tests, the desired concentration that showed high mortality of the ticks in the bioassay was taken for the first dose-response and from the first dose-response results, the second dose-response test was carried out. It is finally concluded from the observation that 0.01 g/10 ml of bionanocomposite was effective against dog ticks and 0.075 g/10 ml of bionanocomposite was effective against cow ticks (Figure 13). And the ticks that were maintained as control showed 0% mortality in both cases.

Toxicity analysis of bionanocomposite

The movement of each zebrafish in the respective tanks was tracked in the form of a graph using the TRACKER Software and the obtained results are given in figure 14.

Movement X and Y axis graph

The movement of the fish in the tank during the recorded time was shown by the movement graph. The graphs in figure 14 represent the movement of the fish after treating the water with the bionanocomposite and the graph in figure 14 shows the control. Comparing the control with other bionanocomposite crude samples, the crude samples depicted similar results as the control, and this confirms that the concentration used has no notable effects and hence less toxic.

Table 10.1: Mortality and the corrected % mortality detected from the bioassay test.

Concentration	Bioassay test			
	Day 1	Corrected % mortality	Day 2	Corrected % mortality
Control	0	0	0	0
0.005 g/10 ml	1	20	2	40
0.1 g/10 ml	2	40	4	80
0.2 g/10 ml	1	20	2	40

Table 10.2: Bioassay test on dog (*R. sanguineus*) ticks.

Concentration	Bioassay test			
	Day 1	Corrected % mortality	Day 2	Corrected % mortality
Control	0	0	0	0
0.002 g/10 ml	0	0	0	0
0.005 g/10 ml	1	20	2	40
0.01 g/10 ml	2	40	4	80

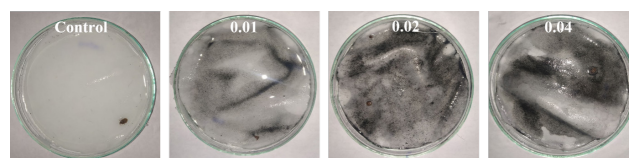


Figure 10: Ticks subjected to varying concentration.

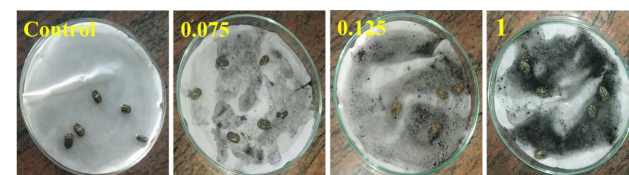


Figure 11: Ticks subjected to determined concentration.

Table 11.1: First dose-response test on cow (*R. (B) microplus*) ticks.

Concentration	Dose-response test			
	Day 1	Corrected % mortality	Day 2	Corrected % mortality
Control	0	0	0	0
0.075 g/10ml	2	40	4	80
0.1 g/10ml	2	40	3	60
0.125 g/10ml	1	20	3	60

Table 11.2: Second dose-response test on cow (*R. (B) microplus*) ticks.

Concentration	Dose-response test			
	Day 1	Corrected % mortality	Day 2	Corrected % mortality
Control	0	0	0	0
0.005 g/10ml	0	0	1	20
0.075 g/10ml	1	20	4	80
0.1 g/10ml	2	40	3	60

Table 11.3: First dose-response test on dog (*R. sanguineus*) ticks.

Concentration	Dose-response test			
	Day 1	Corrected % mortality	Day 2	Corrected % mortality
Control	0	0	0	0
0.005/10 ml	0	0	2	40
0.01/10 ml	2	40	4	80
0.015/10 ml	1	20	3	60

Table 11.4: Second dose-response test on dog (*R. sanguineus*) ticks.

Concentration	Dose-response test			
	Day 1	Corrected % mortality	Day 2	Corrected % mortality
Control	0	0	0	0
0.007/10 ml	0	0	2	40
0.01/10 ml	3	20	4	80
0.012/10 ml	2	40	3	60

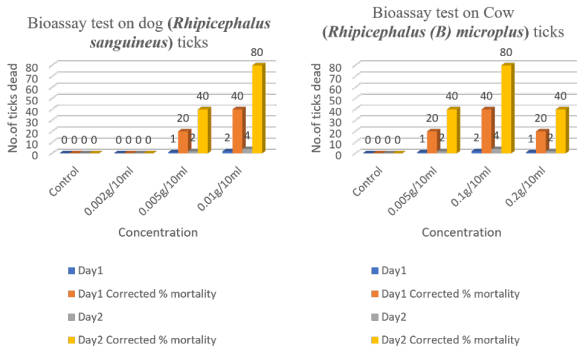


Figure 12: Bioassay test results.

Time and Y- axis graph

The Time and Y-axis graph is the record of the position of the fish during the time intervals. Figure 15 represents the position of each fish inside the tank during the tenure of different concentrations of bionanocomposite treatment. The time spent by each fish in the upper half is maximum and almost equal while comparing with the control group. This maximum time spent in the top layer conveys that the fish experience no stress or normal. Table 12 represents the time taken by the fish to reach the top layer immediately after introduction into the treated water and the time spent by the fish in the top layer for the entire duration of 5 min. The data was in accordance with that of the control group and it explains that the synthesized bionanocomposite was suitable for usage.

Analysis of the survival capacity

The survival analysis was carried out for 2 days and the obtained observation was positive. All the test-subjected fish survived well in the treated solution and were even active compared with the control group. No fish showed mortality, and this confirmed that the developed bionanocomposite is less toxic and good in its action.

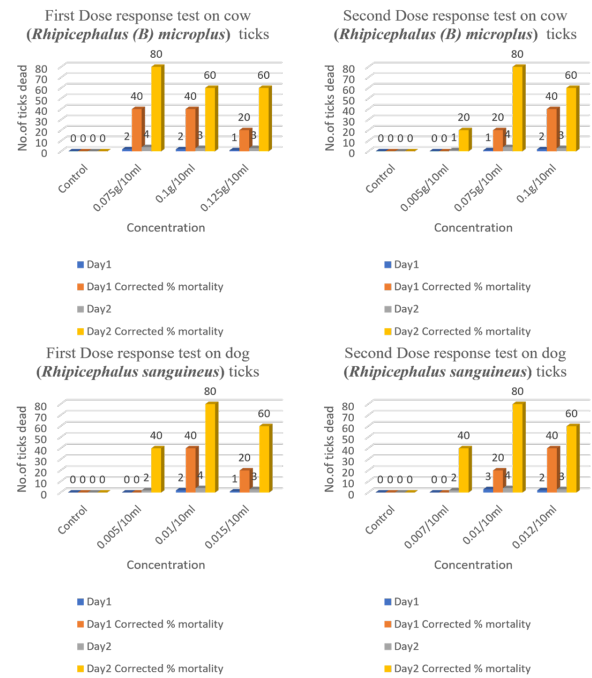


Figure 13: Results of dose-response assay.

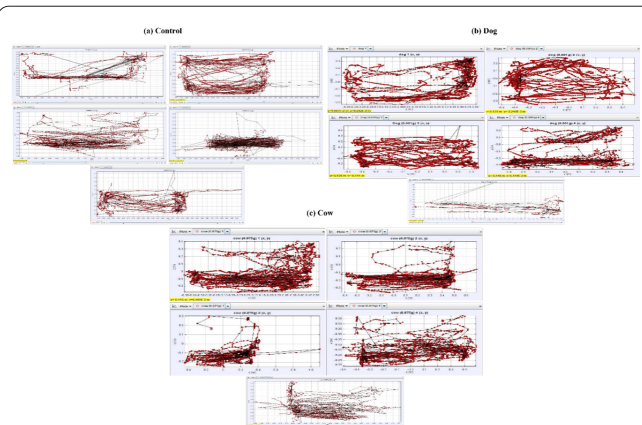


Figure 14: Movement X and Y axis graph of zebrafish treated with bionanocomposite and control.

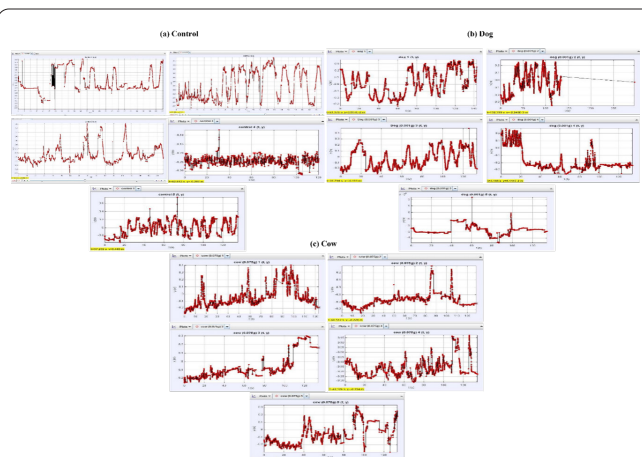


Figure 15: Time and Y-axis graph displaying the position of each treated zebrafish.

Table 12: Average duration taken and spent by each Zebrafish in the top layer.

Name	Average time taken to reach top layer (sec)	Average time spent in top layer (sec)
Control	70	109.6
Dog	52.6	151.6
Cow	59.4	84.2

Formulation of tick detox

The tick detox was developed using a carrier substance, a stabilizer and a preservative. The composition and the quantity (in terms of %) of substances was represented in table 13. Here, Corn starch is used as a carrier because it is insoluble in water at room temperature and also it is good at carrying aqueous substances. It functions as a delivery system, a carrier of other ingredients in a formulation, or a functional ingredient in a personal care formula. A similar compound was used in the study performed [42]. Sucrose is used as a stabilizer in powder formulation since it is used in biotherapeutics as an essential element of formulation and stabilization [43]. Sucrose prevents the active ingredient from clumping together during storage and usage. Sodium benzoate is a commonly used antimicrobial preservative that plays a key preservation role in cosmetics, foods and pharmaceuticals. It prevents the growth of bacteria and other microorganisms in the powder.

Conclusion

The present study focuses on environment-friendly green synthesis of nanoparticles and bionanocomposite and for the synthesis process *N. oleander* is used as an effective reducing agent. The developed bionanocomposite was confirmed through various characterizations such as UV-visible spectroscopy, PSA, FTIR, XRD and SEM-EDAX and the obtained results showed that the particles were rough and tightly arranged with a combination of spherical and broken flakes. The peak spectra displayed the nature of the particle as crystalline, and the size of the particle was found to be in the approximate range of 24.046 nm. The functional groups of organic acids, alcohols and aldehydes in the sample confirmed the metal reduction. The ticks were collected from Dogs at Tiruchengode and Cows at Kumbakonam and their species was identified at TANUVAS, Namakkal. The ticks were subjected to bioassay tests and dose-response assay. On the basis of 48 h motility and mortality observations, 0.01 g/10 ml was found effective against dog ticks (*R. sanguineus*) and 0.075 g/10 ml showed high effectiveness against cow ticks (*R. (B) microplus*). The detected concentration was evaluated for its toxicity in zebrafish. The observations included the movement and behavior of the fish and the time spent by the fish in the top layer. The toxicity examination in comparison with the control groups conveyed that the fish has no stress and the synthesized bionanocomposite was suitable for use. The formulated bionanocomposite is highly stable and has significant tick acaricidal activity. In the future, this study can be used against any higher-end parasite by making slight modifications and usage of the appropriate composition of substances.

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Table 13: Ingredients to formulate tick detox.

Composition	Ingredients	Quantity
Active ingredient	GO/Ag bionanocomposite	35
Carrier	Corn starch	35
Preservative	sodium benzoate	15
Stabilizer	Sucrose	15
Total		100

STAR (BT/HRD/11/09/2018), DBT-PG Scheme (BT/HRD/01/07/2020), DST-FIST (SR/FST/College-235/2014), DST-FIST (SR/FST/College/2023/1423) for financial and instrumentation support. Moreover, the authors are grateful to Dr. R. Gopalakrishnan, Principal, K.S.Rangasamy College of Technology, Dr. B. Kalpana, Associate Professor and Head, Department of Biotechnology, Dr. J. Philip Robinson, Professor and Head, Department of Food Technology for their rendered support and the facilities to carry out this research work.

Conflict of Interest

None.

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