Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2018; 6(3): 1050-1057

Received: 22-03-2018
Accepted: 23-04-2018

Sivapriya V
Nano-Bio Translational Research Laboratory, Department of Biotechnology, Bannari Amman Institute of Technology, Sathyamangalam, Erode, Tamil Nadu, India

Abdul Azeez N
Nano-Bio Translational Research Laboratory, Department of Biotechnology, Bannari Amman Institute of Technology, Sathyamangalam, Erode, Tamil Nadu, India

Sudarshana Deepa V
Nano-Bio Translational Research Laboratory, Department of Biotechnology, Bannari Amman Institute of Technology, Sathyamangalam, Erode, Tamil Nadu, India

Correspondence
Sudarshana Deepa V
Nano-Bio Translational Research Laboratory, Department of Biotechnology, Bannari Amman Institute of Technology, Sathyamangalam, Erode, Tamil Nadu, India

Phyto synthesis of iron oxide nano particles using the agrowaste of *Anthocephalous cadamba* for pesticidal activity against *Sitophilus granaries*

Sivapriya V, Abdul Azeez N and Sudarshana Deepa V

Abstract
Evaluation of new pesticide for the management of *Sitophilus granaries* was carried out in Nano – Bio translational research Laboratory, Bannari Amman Institute of Technology, Sathyamangalam during 2017-2018. An attempt was made to control the *Sitophilus granaries* menace by organic modus in combination with nanotechnology. The aqueous extract of *Anthocephalous cadamba* was used as reducing and capping agent for the phyto synthesis of iron oxide nanoparticles. The incorporation of nanotechnology enhanced the target specificity and mortality rate of pests. The antimicrobial activity of phyto synthesized iron oxide nanoparticles (PION) against *Bacillus* sp., (Gram +ve) and *E. coli* (Gram –ve) exhibited the minimal zone of clearance less than chemically synthesized iron oxide nano particles (CION), which implies that the PION is specifically lethal only to the pest. Out of the three, PION exhibited 100% mortality against *Sitophilus granarius*. Hence the iron oxide nano particle in conjugation with the aqueous extract of *Anthocephalous cadamba* has proved to be a target specific effective pesticide.

Keywords: *Anthocephalous cadamba,* iron oxide nanoparticles, agro wastes, *Sitophilus granarius*, pesticidal activity, anti-microbial, target specific

1. Introduction

Horse gram (*Macrotyloma uniflorum*) is a legume of tropics and subtropics, which is an excellent source of iron and molybdenum [1]. Various pests infest the Milled horse gram during storage [2]. Storage and upkeep of agricultural products are very important post-harvest activities. Considerable amount of food grains is being spoiled after harvest due to the lack of sufficient storage and processing facilities leading to an extensive loss in quality and quantity of commercial products as well as the deterioration of seed viability worldwide [3].

Currently, the pests are controlled by various chemical agents such as organophosphates, pyrethroids and fumigants such as methyl bromide and phosphine [4]. These chemicals are effective for pest control but produce several adverse effects on the consumers (humans). Thus, the repellents, fumigants, feeding deterrents and insecticides of natural origin are rational alternatives to synthetic pesticides [5]. Nano particles on the other hand can intensify the lethal property of the compound since the structure of each nanoparticle varies making it difficult for the insects to develop resistance to the compound [6].

Phyto synthesis of the metallic nano particles using agro wastes has advantages like producing wealth out wastes, combinatorial presence of reducing agents, surfactants, compound of interest in single extract. This reduces the cost and time of synthesis by appreciable folds. This novel approach overcomes the limitations of the other conventional methods [7]. The bioreduction of metal by biomolecules extracted from plants act as reducing agent and capping agent that increases the stabilization of synthesized nanoparticles [8-10]. The green synthesis of nanoparticles yields products that are non-toxic [11] environment friendly and much cost effective [12].

Consequently, the nanotechnology created a revolution and will revolutionize agriculture in the 21st century including pest management. Over the next two decades, the green revolution would be accelerated by means of the nanotechnology. Particularly, a few reports reported the synthesis of metallic nanoparticles using leaf extract of *Anthocephalous cadamba* but iron oxide. The aim of our work is to synthesise iron oxide nanoparticles both by chemical and biological means to verify their pesticidal activity against *Sitophilus granarius* and antimicrobial activity on the sequence of Gram-positive and Gram-negative bacteria.

This is the first report that deals with the green synthesized iron oxide nanoparticles with the agrowaste of *Anthocephalous cadamba* which produce 100% mortality over the horse gram pest.

2. Materials and methods

2.1 Insects rearing

Sitophilus granarius pests were collected from the infested horse gram obtained from a local market, Sathyamangalam reared in glass jars at 30 °C ± 1 °C, 75 ± 5% relative humidity (RH) under continuous darkness. The RH was maintained by using a saturated solution of sodium chloride [10]. Immediately after the pupa stage, the adults less than 24 h old were used for the experiments.

2.2 Preparation of plant extracts

The plant materials of *Anthocephalus cadamba* collected from Palani hills, Tamil Nadu, were washed with tap water, shade dried at room temperature and powdered using electrical blender. Aqueous extract from the pulverized leaf materials was obtained with the help of a soxhlet apparatus. The aqueous extract obtained was stored at 20 °C [13].

2.3 Phytochemical analysis

Phytochemical investigation on the presence of active components in aqueous leaf extract of *Anthocephalus cadamba* has been carried out for Tannin, Phlobatanin, Flavonoids, Terpenoids, Proteins and Amino acids, Phenols, Saponins and Steroids [14,15].

2.4 GC-MS analysis

The GC-MS analysis of active fractions of the methonolic extract of *Anthocephalus cadamba* were analyzed using Thermo GC - Trace ultra ver: 5.0, Thermo MS DSQ II gas chromatography equipped with DB 35 - MS capillary standard non - polar column dimension of 30mts and mass detector (EM with replaceable horn) was operated in EMV mode. Helium was used as the carrier gas with the flow rate of 1.0 ml min^{-1}. The injection port temperature was operated at 260 °C. The column oven temperature was held at 70 °C for 2 min then raised to 260 °C at 6 °C/min.

2.5 Phyto Synthesis of Iron Oxide Nanoparticles

The ferric chloride used in this study was obtained from Himedia Laboratories Pvt. Ltd., Mumbai, India. A 0.1 M concentration ferric chloride aqueous solution was added with plant extract till the colour of the solution transformed from brown to black indicating the formation of iron oxide nanoparticles. Then the reaction mixture was centrifuged at 559 × g for 10 min. The supernatant was discarded and the pellets obtained were air dried for the evaporation of aqueous phase [16,17].

2.6 Chemical Synthesis of Iron Oxide Nanoparticles

Ferric chloride aqueous solution of 0.1 M concentration was added with 0.1 M NaOH till the colour of the solution changed from brown to black indicating the formation of iron oxide nanoparticles. Then the reaction mixture was centrifuged at 559 × g for 10 min. The supernatant was discarded and the pellets obtained were air dried for evaporation of aqueous phase

2.7 Characterization of Iron Oxide Nanoparticle

2.7.1 FT-IR Spectroscopy

The vibrational and rotational modes of the motion of a molecule were studied using Fourier transform infrared spectroscopy (FTIR). For FT-IR analysis, the PION was characterized with the Shimadzu FT-IR 8400S and MB3000 in KBr matrix over a spectral width of 4000 to 500cm^{-1} at a resolution of 2cm^{-1} [18].

2.7.2 Scanning Electron Microscopy analysis

The surface morphology of PION was studied by recording their SEM micrographs using scanning electron microscopy (JEOL-MODLE 6390) at 20KV for the magnification range of 7500 [19].

2.7.3 XRD Analysis

In order to obtain the structural information of the product, the crystallographic structure of iron oxide nano particles was analyzed by X-ray power diffraction (XRD). The crystallographic analysis of samples in diffraction patterns was recorded from 10° to 70° with an analytical system diffractometer using Cu Kα (λ = 1.542 Å) with an accelerating voltage of 40 KV. Data were collected with a counting rate of 1°/min [20].

2.8 Pesticidal activity

The adult pests of *Sitophilus granarius* were collected from the local grocery store. These insects were reared with the clean and uninfected rice grains in a jar with sufficient aeration. The pesticidal activities of PION, CION and the aqueous extract of *Anthocephalus cadamba* were carried out against *Sitophilus granarius* at varying concentrations of 0.25mg/ml, 0.50mg/ml and 1mg/ml. Test solutions were swabbed homogenously on a sterile petridish using cotton. The cotton plug was placed in the petridish containing the pest *Sitophilus granarius* along with 1 gram of horse gram and sealed. The death rate of pest was observed after 6 h of exposure. Percentage mortality was calculated using the formula [9, 21].

$$\% \text{ Mortality} = \left(\frac{\text{No of weevil dead}}{\text{no. of weevil introduced}}\right) x\ 100$$

2.9 Antimicrobial activity

The PION was tested for their antimicrobial activity against *Escherichia coli* (gram negative) and *Bacillus subtilis* (gram positive) by the well diffusion method [22-24]. The pure culture of organism was inoculated in the nutrient broth at 35 °C in a rotary shaker at 22.4 × g for 24 h. The sub-cultured organism of 100μl was swabbed on the nutrient agar plates using sterile L-rod. Wells were created on the agar plate using gel puncture. Different concentrations such as 10μl, 20μl and 30μl of the PION, CION and plant extract per ml of the aqueous solution were added to the agar plates. After incubation at 37 °C for 24 h, different extents of zone of inhibition were observed.

3. Results and Discussions

3.1 Phytochemical analysis

Phytochemical investigation on the presence of active components in aqueous leaf extract of *Anthocephalus cadamba* was carried out. Based on the calorimetric readings, the possibility of the isolable compounds in the aqueous extract of *Anthocephalus cadamba* leaves were Tannin, Phlobatanin, Flavonoids, Terpenoids, Phenols, Saponins and

Steroids. Himanshu Gurjar *et al*. and Jeyalalitha *et al*. carried out the phytochemical investigation for the same plant leaf extract and reported the same [25, 26]. This confirms that the leaves offer a wider array of phytochemicals.

Table 1: Phytochemicals in aqueous leaf extract of *Anthocephalus cadamba*

S. No	Phytochemicals	Leaves of *Anthocephalus cadamba*
1	Tannin	+
2	Phlobatanin	+
3	Flavonoids	+
4	Terpenoids	+
5	Phenols	+
6	Proteins and Aminoacid	-
7	Steroid	+
8	Saponins	+

(+ implies positive; - implies negative)

3.2 Formation of Fe_3O_4

On dissolving the ferric chloride in water, the dissociation and association of Fe^{3+} and Cl^- ions reaches equilibrium. By adding the reducing agent and plant extract with ionic functional groups, this equilibrium was disturbed by reducing Fe^{3+} to FeOH which is further oxidized by the reactive plant extracts to yield Fe_3O_4-phyto chemical compounds.

3.3 GC-MS analysis

The GC-MS analysis of *Anthocephalus cadamba* leaves revealed the presence of phytochemical constituents that contribute to the pesticidal activity of the plant. The identification of the phytochemical compounds was confirmed based on the peak area, retention time and molecular formula. The active principles with their Retention time, Molecular formula, Molecular weight and peak area in percentage are presented in Table 2.

Some of the active phytoconstituents of methanolic extract of *Anthocephalus cadamba* are Calconcarboxylic acid, 2-(p-Toluenesulfonyl) methyltetrahydropyran, Dimesitylmethane, 3-ethoxycarbonyl-2-hydroxy-4-oxo-4H-pyrido [1,2-a] pyrimidine-7-carboxylic acid, 2,3,4,6-Tetramethylphenol, Eicosanol-2. Few of these compounds have also been reported in other species. For instance, Humaira Rizwana *et al*. reported the GC-MS analysis of phytoconstituents in methanol and ethanol extracts of *M. aurea* in which 2,3,4,6-Tetramethylphenol was identified [27]. Antara sen and Amla batra reported eicosanol was present methanolic leaf extract of *M. azedarach* [28]. Calconcarboxylic acid was found out as one of the major active compound in the methanolic extract of *Butea monosperma* seed in Thooyavan G and Karthikeyan [29]. The GC-MS analysis of the methanolic extract of *Anthocephalus cadamba* marked the presence of compounds that are responsible for specific activities such as antimicrobial, anti-inflammatory, neuroprotective, potent antioxidant activity and larvicidal activity have also been reported in bioactive compounds of same extract [26].

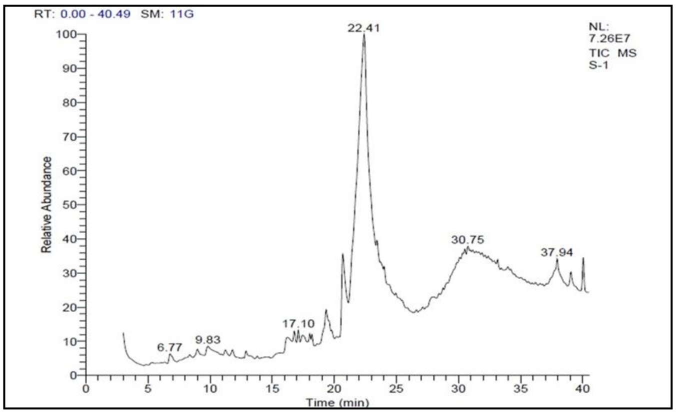
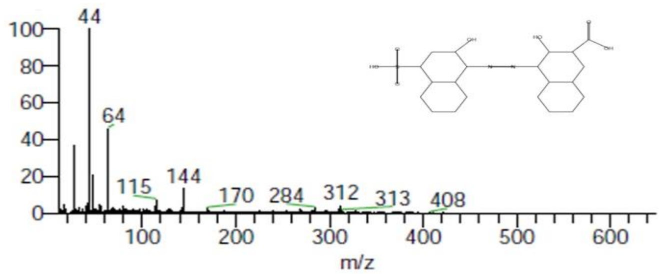
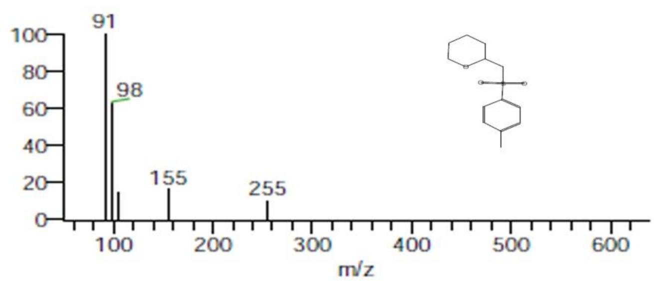
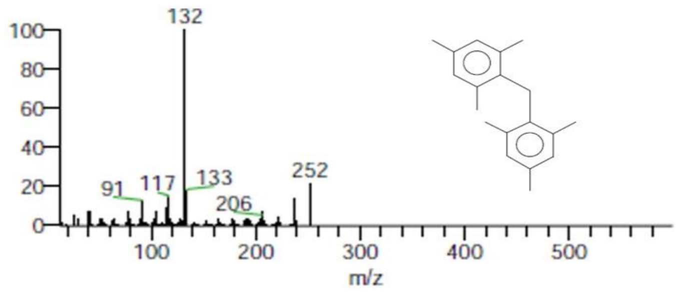


Fig 1: GC-MS analysis of the methanolic extract of *Anthocephalus cadamba*

Table 2: Phytocomponents identified in the methanolic extract of the leaves of *Anthocephalus cadamba* by GC-MS analysis

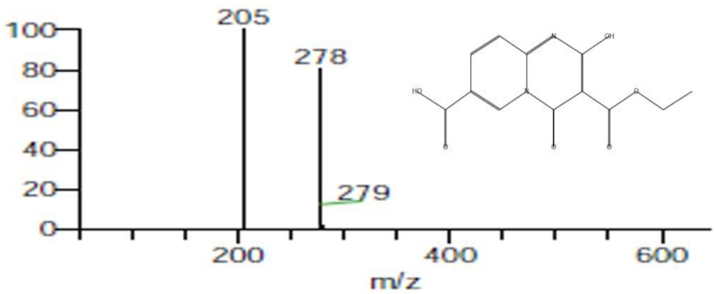
S. No	Retention time	Compound	Molecular formula	Molecular Weight	Area %
1	5.29	Calconcarboxylic acid	$C_{21}H_{14}N_2O_7S$	438	0.33
Structure					
2	6.77	2-(p-Toluenesulfonyl)methyltetrahydropyran	$C_{13}H_{18}O_3S$	254	1.67
Structure					
3	8.36	Dimesitylmethane	$C_{19}H_{24}$	252	0.52

Structure



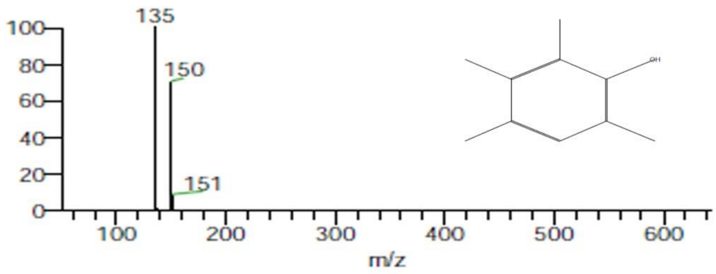
4	9.83	3-ethoxycarbonyl-2-hydroxy-4-oxo-4H-pyrido[1,2-a]pyrimidine-7-carboxylic acid	$C_{12}H_{10}N_2O_6$	278	1.48

Structure



5	11.24	2,3,4,6-Tetramethylphenol	$C_{10}H_{14}O$	150	0.70

Structure



6	5.29	Eicosanol-2	$C_{20}H_{42}O$	298	0.33

Structure

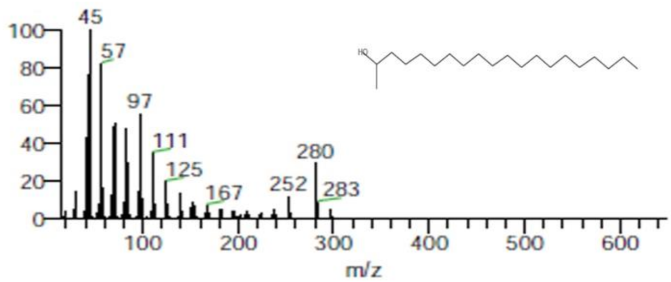


Table 3: Activity of important phytocomponents identified in *Anthocephalus cadamba* extract by GC-MS.

S. No	Retention time	Name of the compound	Physico chemical properties / Bio-Activity
1	5.29	Calconcarboxylic acid	Aromatic, Antioxidant, anti-microbial activity
2	5.29	Eicosanol-2	-OH group at the end of ethyl chain promotes conjugation;
3	6.77	2-(p-Toluenesulfonyl)methyltetrahydropyran	Antioxidant, antimicrobial and bacteriocide, Antipyretic, antiinflammatory activity
4	8.36	Dimesitylmethane	Volatility, Aromatic, Invasive
5	9.83	3-ethoxycarbonyl-2-hydroxy-4-oxo-4H-pyrido[1,2-a]pyrimidine-7-carboxylic acid	Antioxidant, antimicrobialand bacteriocide, anti-inflammatory activity
6	11.24	2,3,4,6-Tetramethylphenol	oral anesthetic/ analgesic, antiseptic

3.4 Characterization of Iron Oxide Nanoparticle

3.4.1 FT-IR Spectroscopy

To determine the functional groups on PION, Fourier Transform –Infra Red analysis was performed. The characterization was carried out to identify the possible biomolecules responsible for the reduction of Fe ions and capping of the reduced iron oxide nanoparticles. The following bands were observed.

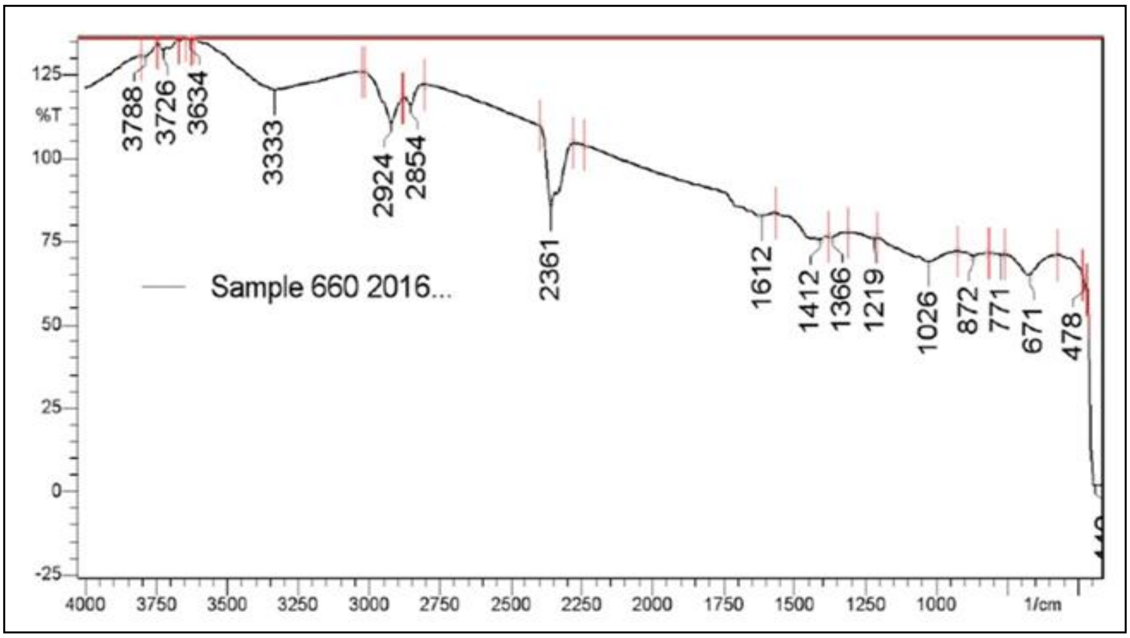


Fig 2: FTIR analysis of iron oxide nanoparticles using leaf extract of *Anthocephalus cadamba*

Table 4: Wave number (cm^{-1}) of dominant peak obtained from absorption spectra from *Anthocephalus cadamba*

S. No	Wave number (cm^{-1})	Functional group
1	671	Fe O stretch
2	771	Aromatic C-H bend
3	872	Aromatic meta C-H bend
4	1026	C-N
5	1219	C-N and C=O
6	1412	COO
7	1612	C=C
8	2854	C-H and carboxylic O-H
9	2924	sp^2 C-H stretch and carboxylic O-H stretch

From the Wave number (cm^{-1}) of dominant peak obtained from absorption spectra from *Anthocephalus cadamba*, it was confirmed that the surface of the particles has aromatic stretches corresponding to tannins and flavonoids, iron oxide stretches, amine group, carboxylic groups, alcohol stretches and sp2 orbital C-H bond vibrations. Two significant peaks found at 478 and 671 correspond to the stretching vibration mode of iron oxide in the absorption spectra. Latha *et al*. reported the formation of iron oxide is characterised by the absorption bands from 418 to 502 cm-1 which corresponds to the Fe–O band while synthesizing nanoparticle using *Carica papaya* leaf extract [30]. Matheswaran Balamurugan *et al.* reported the broad peak observed around $544cm^{-1}$ may due to the organic molecule which was from the *Eucalyptus globules* leaf extract on the surface of iron oxide nanoparticles [31]. Thus the formation of iron oxide nanoparticle is confirmed by the characteristic peaks lying in the region between 400 and 600 cm^{-1} which were corresponds to the iron oxide.

3.4.2 Scanning electron microscopy analysis

A scanning electron microscopy was used to determine the morphology of PION. Fig. 3 represents SEM image of polydispersed PION synthesized using *Anthocephalus cadamba* leaf extract. At 7,500x magnification, the size of PION is found to be less than 2µm. The morphology of the particles was observed to be quasi spherical from the electron micrograph. Balamurughan *et al* performed SEM analysis of iron oxide nanoparticles synthesized from *Ocimum sanctum* at different magnification levels are 10µm and 50µm which reveals that the synthesized iron oxide nanoparticles were aggregated as irregular sphere shapes with rough surfaces [9]. Sudhanshu Shekhar Behera *et al.* reported the size of chemically synthesized iron oxide nanoparticle by SEM analysis is to be 0.5µm at x30,000 magnification which are almost spherical in shape [32].

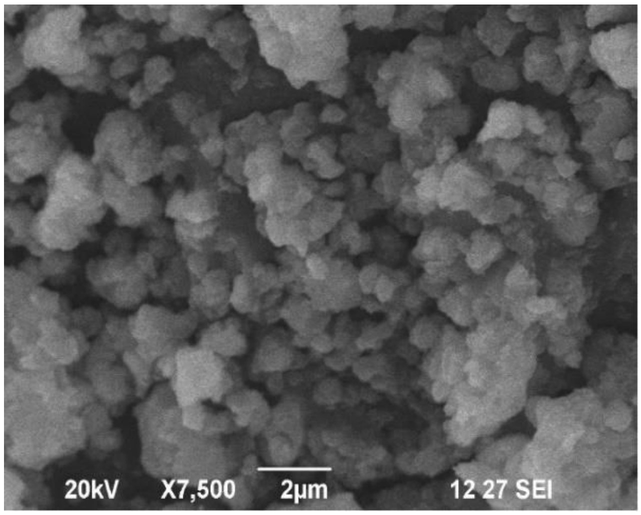


Fig 3: Scanning electron micrographs of photosynthesized iron oxide nanoparticle

3.4.3 XRD analysis

Major X-Ray diffraction peaks were observed at 2θ degrees of 35.23, 39.38 and 46.69 for PION which implies that the diffraction angles of different peaks correspond to the iron oxide nanoparticle. This data is very close to the American Society for Testing and Materials (ASTM) data of iron oxide nanoparticles, which could be a good evidence to prove that the prepared nanoparticles, was made of iron oxide. Zuolian Cheng *et al.* reported the XRD patterns of synthesized iron oxide nanoparticles with different concentrations of $FeCl_3$ and obtained peak at 2θ values of 38.73 and 45.62 indicates that the particle consists of iron oxide [33]. Matheswaran Balamurugan *et al.* reported an additional peak at 2θvalues of 45 is due to the presence of iron oxide nanoparticles and also indicates that the prepared iron oxide nanoparticles are well crystalline in nature [31].

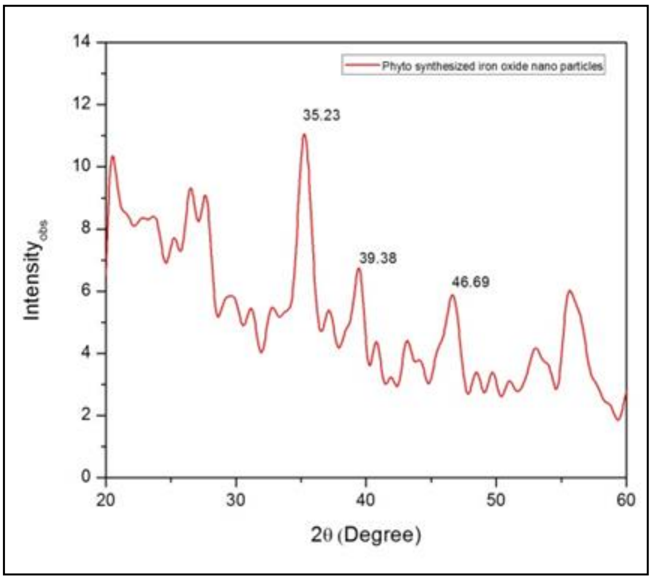


Fig 4: XRD of phytosynthesized iron oxide nanoparticle

3.5 Pesticidal activity

Results from the pesticide activity against *Sitophilus granarius* of PION and CION showed 100% mortality rate after 6 h and were more effective than the *Anthocephalus cadamba* aqueous extract. Various studies have been conducted to control the storage pests using botanicals as the source. Kannan *et al*. compared the methanolic extract of White and Blue coloured flowered leaves of *Clitoria ternatea* and reported the potential pesticidal activity within an hour against *Sitophilus oryzae* [34]. Abduz Zahir *et al*. reported that biosynthesized nanoparticle from mixtures of plant extract exhibited highest mortality rate against the same pest and offered various degrees of protection to plants [35]. Mohammad Rouhani *et al*. reported that the synthesized nanoparticles could be an effective pest control approach for *Aphis nerii* and also claimed that the pest mortality increased significantly with increase in pesticide concentrations [36]. Stadler *et al*. successfully applied nano alumina against two stored grain pests [37]. Thus, the results obtained suggest good potential for the use of PION for pesticidal activity.

Table 5: Pesticidal activity against *Sitophilus granaries*

Plant	Concentration µg/ml	Mortality % at			Confidence interval		Chi square
		2 hours	4 hours	6 hours	Negative	Positive	
Aqueous extract	0.25	0.00	10.00	23.33	22.79	23.88	.000
	0.5	13.33	26.67	56.67	56.12	57.21	
	1	20.00	43.33	66.67	66.12	67.21	
CION	0.25	20.00	50.00	76.67	76.12	77.21	.002
	0.5	33.33	83.33	96.67	96.12	97.21	
	1	56.67	96.67	100.00	100.00	100.00	
PION	0.25	10.00	43.33	70.00	70.00	70.00	.000
	0.5	20.00	60.00	93.33	92.79	93.88	
	1	40.00	83.33	100.00	100.00	100.00	

The α value assigned for null hypothesis was 5% (0.05) for each sample. The three test samples exhibit p value less than 0.05, rejecting the null hypothesis and hence the significant difference in mortality with respect to concentration is proved. The statistical analysis was calculated using one way ANOVA in SPSS v21, IBM.

3.6 Antimicrobial activity

Comparison of antimicrobial activity of *Anthocephalus cadamba* leaf extracts, PION and CION was carried out by agar well diffusion method against gram positive and gram-negative bacteria at different aliquots and found that PION forms minimal zone of clearance. Chandrashekar and Prasanna reported the antimicrobial activity of leaves of *Anthocephalus cadamba* [38]. Hossain *et al*. also reported on preliminary cytotoxicity and antimicrobial activity of methanol extract and its fractions of the stem bark of this plant and similar findings were also reported [39 - 41]. This reveals that the crude extract of *Anthocephalus cadamba* leaf extract has antimicrobial activity whereas PION targets only the pests. This reduction in toxicity can be due to conjugation of iron oxide nano particles with the toxic functional groups of the plant extract.

Table 6: Antibacterial activity against *Bacillus subtilis* (Gram positive) and *Escherichia coli* (Gram negative) at different aliquots and their zone of inhibition

Test sample	Bacterial strain	Zone of inhibition (cm)		
		10µl	20µl	30µl
Aqueous plant extract	*B. subtilis*	0.1	0.2	0.2
	E. coli	0	0	0
CION	*B.subtilis*	0.5	1.2	1.5
	E. coli	0.7	0.8	1.0
PION	*B.subtilis*	0.2	0.6	0.8
	E. coli	0.2	0.3	0.3

4. Conclusion

The iron nanoparticles conjugated with plant extract PION possessing pesticidal property has proved to be better candidate in comparison with chemically synthesized iron nanoparticles CION and crude plant extracts. The results of pesticidal activity support our claim. The agrowastes along that are lethal to the pests is intensified in its action with the addition of metallic nano particles. Also, the conjugation of other free functional groups by the nano particles ensures the specificity of the conjugate towards pest. This work will further be extrapolated to a broader spectrum of pests and aiming to develop a commercial pesticide.

5. Acknowledgement
Authors are thankful to the Bannari Amman Institute of Technology, Sathyamangalam and Department of Biotechnology for the facilities provided to carry out our project.

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