Control of JE vector by organic compounds isolated and green nanoparticles synthesised from leaf extract of *Holoptelea integrifolia* (Roxb.)

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Research

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Abstract

Background: Japanese encephalitis (JE) is a dreadful disease transmitted by Culex vishnui group of mosquitoes. Control of JE vectors at the larval stage is one of the effective approaches in controlling JE.

Methods: Leaves of *Holoptelea integrifolia* were subjected to petroleum ether, ethyl acetate, acetone and absolute alcohol solvent extraction by Soxhlet apparatus. As the ethyl acetate extract showed best mosquito larvicidal activity against *Culex vishnui*, it extract was selected and processed further for isolation of active principle through column chromatography and TLC and then characterization of the active principle by FTIR and GC-MS was done.

Results: Ethyl acetate extract was found to be the most potent larvicide. In the active fraction of isolated compounds from ethyl acetate extract, N-methyl-1-adamantaneacetamide was the major constituent responsible for larvicidal activity of Culex vishnui. Green nanoparticles were synthesised by treating silver nitrate with leaf extract of *H. integrifolia* and were examined for larvicidal activity. Nanoparticles were characterised by UV-VIS spectral analysis, XRD study, TEM, SEM and FTIR spectral analysis. Synthesised nanoparticles were 40-50 nm in size and showed good larvicidal activity on Cx. vishnui mosquitoes at 1.25, 2.25, 5, 7.5 and 10 ppm concentrations. Active principle of plant extract and green nanoparticles showed eco-friendly effect on non-target organisms like *Chironomus circumdatus, Daphnia sp, Diplonychus anulatum* and Tadpole larvae.

Conclusion: Thus it can be concluded that active ingredient as well as green synthesized nano particles from H. integrifolia can be a good alternative of presently used chemical insecticides.

Background

Japanese encephalitis (JE) previously known as Japanese B encephalitis is a disease caused by the mosquito borne Japanese encephalitis virus belonging to the family Flaviviridae [1]. Major outbreaks of JE occur every 2–15 years. Transmissions of JE virus occurs at high frequency in the rainy season when propagation of vector population gets pace. The spread out of JE in new area has been associated with agricultural development and extensive rice cultivation by irrigation schedule [2]. According to World Health Organization (WHO) more than 3 billion people of South-East Asia and Western Pacific regions are at risk of JE transmission. In Asia up to 70,000 instances are reported yearly and mortality proportions extend from 0.3–60% relying on the age and socio economic conditions of populations. The first major epidemic of JE in India was reported from Bankura and Burdwan districts of West Bengal in 1973. Since then, repeated annual outbreaks have occurred especially in the post monsoon season when high mosquito density is observed in West Bengal. Populations of rural territories in endemic locations are at high risk of this disease.

According to NVBDCP (National Vector Borne Disease Control programme) under The Ministry of Health and Family Welfare, Government of India, Culex vishnui group (Culex tritaeniorhynchus, Culex vishnui and

Culex pseudovishnui) is the chief vector of JE in different parts of India. The most important vector of JE in India, Srilanka and Thailand is Culex vishnui mosquito belonging to the family Culicidae [3].

A multitude of prevention and control strategies has been developed against Japanese encephalitis, out of which special emphasis on the control of vector mosquitoes at their larval stages has been found to be rational because they remain confined in some particular habitats. The natural products of plant origin have been experimentally used to control mosquito population because plants are rich source of different secondary biochemicals and are preferred over synthetic insecticides due to their biodegradable and eco-friendly nature.

Holoptelea integrifolia (Roxb.), also known as Ulmus integrifolia (Roxb.), belonging to the family Ulmaceae distributed over tropical and temperate regions of Northern hemisphere including Indian peninsula to Indo China region and Srilanka [4].

In ancient times, H. integrifolia was known for its important medical values. The plant is used traditionally for the treatment of inflammation, gastritis, dyspepsia, colic, intestinal worms, vomiting, wound healing, leprosy, diabetes, dysmenorrhea and rheumatism [5]. Leaves of the tree has been reported to contain various secondary metabolites like steroids, saponins, tannins and phenol [6] which might be responsible for various medicinal activities shown by the tree.

Nanoparticle is a core particle which acts as a whole unit in terms of transport property ranging its size from 1-100 nm. The green synthesis of nano-material from plants can provide safe and beneficial way to the synthesis of metallic nanoparticles as it is easily available, eco-friendly and the rate of production is faster [7]. Out of gold, silver, copper, silicon, zinc, titanium, magnetite and palladium nanoparticle colloids, silver nanoparticles synthesised from plants had exhibited better catalytic and antibacterial property as well as chemical stability [8, 9].

Objective of the present study was to control the spreading of Japanese Encephalitis by control of mosquito larvae at their own habitats through bioactive principle isolated from ethyl acetate extract of H. integrifolia leaf and by green synthesised silver nanoparticles using H. integrifolia leaf extract as reducing agent. Effect of these tools on some non target organisms was also examined.

Material And Methods

Collection of plant leaves

Fresh mature leaves of *Holoptelea integrifolia* were randomly harvested from plants growing at outskirts of Debipur, Purba Bardhaman, West Bengal. Collected leaves were properly cleaned under tap water and then washed with distilled water to remove dust, debris and any kind of impurities on the leaf and soaked in paper towel.

Collection of mosquito larvae

Culex vishnui predominantly breed in rice field water. Egg rafts of *Cx. vishnui* were collected from rice fields surrounding Burdwan University Golapbag campus. The eggs were kept in plastic tray bearing volume of 12.6 x 10 x 6 inches^3 in Mosquito and Microbiology Research Unit, The University of Burdwan. After hatching, larvae were fed with a mixture of dog biscuits and dried yeast powder in the ratio of 3:1.

Preparation of solvent extracts

Finely grounded shade dried leaves of 250 g were put into a Soxhlet apparatus for solvent extraction. Plant extracts were prepared using solvents of increasing polarity (non polar to polar) namely petroleum ether, ethyl acetate, acetone and absolute alcohol, applying successively (extraction period 72 hour in each solvent) with the same leaf sample. The extracts were collected separately and the Soxhlet apparatus was washed with 200 ml water and100 ml of similar solvent as an eluent after each solvent extraction procedure. Eluted material of each extract was concentrated below at 40^0 C temperatures to 100 ml of solution by evaporation in rotary evaporator. Resultant concentrated extract were kept in a deep freeze at -80 ^{0} C (REVCO model No: ULT 790-3-V32) for 24 hour. The resulting freeze dried extract was lyophilized and the solid residue was weighed and then dissolved in suitable amount of sterilized distilled water to make the different graded concentrations. Total yield of ethyl acetate extract was 2.68g.

Bio assay with solvent extracts

The bioassay experiments were conducted on 3rd instars larvae of *Cx. vishuni* according to standard WHO procedure (1981) [10] with slight modification. Tween 20 was used as solubilizer for petroleum ether, ethyl acetate, acetone and absolute alcohol solvent extracts. The quantity of Tween 20 used to prepare the solution had been determined previously by tolerance experiments with *Cx. vishuni* larvae to find the non lethal concentrations. The stock solution of 500 ppm concentration of each extract was prepared. Different concentrations were obtained from this stock solution by addition of distilled water. Later those concentration of each extract was transferred to disposal plastic cups separated to carry out the tests, in which twenty five 3rd instars larvae of *Cx. vishuni* were placed with the help of disposable plastic pipette (WHO/VBC, 2005) [11] and a similar type of bio assay were conducted with only distilled water but without any of the solvent extract of the mature leaves as a control. The dead larvae were counted after every 24 hour up to 72 hour of exposure and percentage mortality was reported from the average of the three replicate taken.

Phytochemical analysis

The leaf extract was subjected to qualitative phytochemical analysis for determination of secondary metabolites using standard methods [12].

Column chromatographic analysis

Column chromatographic analysis was only done with the most effective extract which showed maximum mortality of the *Cx. vishnui* larvae in bioassay experiment. Dried 5 g sample to be analysed

was transferred to the top of the prepared column. Solvent level was kept above the sample by adding the eluting solvent as necessary. Then the column was eluted with single and mixture of different ratios of organic solvents with increasing polarity like petroleum ether, petroleum ether: n- hexane , n- hexane, n-hexane ethyl acetate, ethyl acetate, ethyl acetate: chloroform, chloroform, chloroform: methanol, methanol, methanol: acetone, acetone, acetone: absolute alcohol and absolute alcohol were used as eluting solvent. The flow rate was 2 ml/ min. During the process of separation of the change for the eluting solvent to a more polar system, were previously determined by TLC. Several fractions were collected by combining the same fraction totalling 500 ml. Confirming homogeneity of compounds, the fractions with larvicidal activity against *Cx. vishuni* 3rd instar larvae was detected by bioassay.

Thin layered Chromatography (TLC) analysis

The bio active fractions were monitored by thin layer chromatography silica gel ‘G’ (Merk, India) coated (0.5 mm thickness), using petroleum ether as mobile phase. TLC glass plates were placed in well lidded iodine chamber (21 x 21 x 9 cm) for 1 min to properly detect the bands. The plate was removed and the main band appeared on the subsequent plates with similar R_f (0.357) values were selected afterwards and mixed together and used as apparently purified compound. The R_f value was calculated using formula: R_f = Distance of spot centre from start point/Distance of solvent run from the start point.

IR and GC- MS analyses of bio active principle

The bioactive spots were scrapped from the glass plates and dissolved in absolute alcohol. The alcohol fractions were collected by discarding the silica G and filtered through Whatman No. 1 filter paper. For IR analysis, the sample was kept in vacuum desiccators over KOH pellets for 48 h, and then Infrared (IR) spectral analysis were done with 1 mg sample using potassium bromide (KBr) plates. The pellets were undergoing scanning in Jasco-Fourier Transformer Infrared Spectroscopy FTIR (FT/IR-42 Jasco) with a scanning period of 4 min/ sec and scanning speed of 2 mm sec^{-1}. The purified fraction was analysed directly by GC on a Hewlett Packard (HP; Palo Alto CA, USA) model HP-6890 PLUS GLC and HP-3398a GC Chemstation instrument fitted with a column HP-5 (Capillary column of 0.32 mm in diameter and 30 m in length). The oven temperature programmed was initially 150^0C (4 min) and then 250^0C (4 min). The sample was introduced at 250^0 C.

Bioassay with active principles

Bioassay experiments of active principles were conducted with active principles. Different concentrations of bioactive principles (5, 10 and 15 ppm) were applied on 3rd instar larvae of *Cx. vishnui* mosquitoes for the bioassay experiment according to standard protocol mentioned earlier.

Synthesis of silver nano particles

Ten gram of air dried and crushed leaves of *H. integrifolia* were weighed and put into three separate 500 ml beakers containing 100 ml double distilled water. Each beaker containing mixture of water and plant

leaves was boiled for 10 minutes at 60^0C temperature. Then Whatman filter paper No: 42 were used for filtration of three separate extracts. The filtrates were treated with silver nitrate ($AgNO_3$) solution for the reduction of Ag^+ to Ag^0.The strength of used silver nitrate aqueous solution was 10^{-3}M $AgNO_3$. The mixture was exposed to heat at 60^0C temperature and the colour changes take places within few minutes from colourless to reddish brown colour. The final nano- colloidal solution was centrifuged (twice) at 10,000 rpm for 15 min to isolate the pellet of synthesised nanoparticles from leaves of *H. integrifolia* for further use. After collecting the pellet from centrifuge tube was dried in vacuum desiccators for preparation of different concentrations of aqueous solution of nanoparticle for bio assay experiments.

Characterization of silver nano particles

Characterization of silver nanoparticle was conducted to determine shape and size of nanoparticles. Numbers of techniques were used for this study, including UV-visible spectroscopy, Scanning Electron Microscopy (SEM), Transmission electron microscopy (TEM), Fourior Transmission Infrared Spectroscopy (FTIR) and X-Ray Diffraction (XRD).

The formation of nanoparticles was verified by using UV-VIS due to surface plasmon resonance (SPR) absorption in the UV visible region. The nano particle surface plasmon resonance of the synthesised green nanoparticles in the centrifuged pellets was studied by UV-spectra analysis.

The transmission electron microscopy (TEM) image were obtained using Technai-20 Philips instrument operated at 200 kv and beam current of 104.1µA. Sample for this analysis were prepared by coating the aqueous AgNP on carbon coated copper grids (300 mesh size) by slow evaporation and then allowed to dry in vacuum at 25^0C for overnight.

Scanning electron microscope (SEM) analysis was employed to characterize the size, shape & morphologies of formed nano particles.

The FTIR study by using a FT IR spectrometer (Perkin Elmer Lx10-8873) with scanning range of 450-40000 cm^{-1} at the resolution of 4 cm^{-1} was used to analyse the vibration characteristics of chemical functional groups of the nano particles.

For the study of crystal structure, texture or orientation, X-ray-diffraction (XRD) studies were conducted using Siemens X-ray diffractometer (Japan), operated at 30 kv and 20 mA current with Cu Kα (I=1.54Å). Films of colloidal form AgNP were tested by drop coating on Si (III) substrates and data were recorded. The scanning range was selected between 10^O and 80 ^{O}.

Bioassay of green silver nano particles

In case of bioassay of green nano particles synthesised from leaves of *H. integrifolia*, following concentrations were used i.e. 1.25, 2.5, 5, 7.5 and 10 ppm against all larval instars of *Cx. vishnui*.

Later those concentrations of nano particles were transferred to disposal plastic cups separated to carry out the tests, in which twenty five 1^{st} to 4^{th} instars larvae of *Cx. vishuni* were placed with the help of disposable plastic pipette (WHO/VBC, 2005)[7] and similar type of bio assay were conducted. The dead larvae were counted after 24 hour of exposure and percentage mortality was reported from the average for the three replicates taken.

Toxicity test on non target organism

Active principle of ethyl acetate extract and silver nano particles of *H. integrifolia* leaves were tested against those organisms sharing the habitat of *Cx. vishnui* mosquito i.e. rice field. Some of the non-target organisms are natural predator of *Cx. vishnui* mosquitoes. It is very much essential to determine the effect of synthesised ethyl acetate and silver nano particles extract in laboratory condition on non target organisms to guess the probable effect that should occur after applying it in field conditions. Experiments were conducted on non-targets like *Chironomus circumdatus, Daphnia* sp, *Diplonychus anulatum* and Tadpole larvae.

Results

Bio assay with solvent extracts

Result of bioassay with four different extracts against third instar larvae of Cx. vishnui is depicted in Table 1. Bioassay experiment revealed that all the extracts had larvicidal efficacy against third instar larvae of Cx. vishnui. Ethyl acetate extract showed highest mortality at 35 ppm concentration after 72 h exposure whereas cent percent mortality was recorded at 45 ppm concentration of same extract after 24 h exposure. Result of probit and regression analyses showed that LC_{50} and LC_{90} values gradually decreased with the time of exposure period being lowest at 72 h of exposure and R^2 value approached to 1 in every case (Table 2). Susceptibility of larvae of Cx. vishnui to various concentrations of bioactive principle isolated from mature leaves of H. integrifolia is presented in Table 3. Larvicidal activity of bioactive compound in ethyl acetate extract of H. integrifolia leaf was found statistically significant ($p < 0.05$) comparing the mortality rates of 1st to 4th instars larvae of Cx. vishnui by Multivariate ANOVA analysis (Table 4).

Table 1
Larvicidal activity of different solvent extracts of Holoptelea integrifolia against third instars larvae of Culex vishnui

Solvent extract	Concentration (ppm)	Mortality (Mean ± SD)		
		24 h	48 h	72 h
Petroleum ether	15	8.67 ± 0.33	14.33 ± 0.33	21.67 ± 0.67
	25	16.33 ± 0.67	23.33 ± 0.33	34.67 ± 0.33
	35	32.33 ± 0.67	42.33 ± 0.67	47.67 ± 0.67
	45	42.33 ± 0.33	49.67 ± 0.67	53.67 ± 0.67
Ethyl acetate	15	21.67 ± 0.67	34.67 ± 0.33	71.67 ± 0.33
	25	51.33 ± 0.33	61.33 ± 0.67	83.67 ± 0.33
	35	86.33 ± 0.33	91.67 ± 0.33	100 ± 0.00
	45	100 ± 0.00	100 ± 0.00	100 ± 0.00
Acetone	15	2.67 ± 0.33	5.67 ± 0.67	10 ± 1.54
	25	5.67 ± 0.33	7.67 ± 0.67	13.67 ± 0.67
	35	7.67 ± 0.33	10.33 ± 0.67	18.33 ± 1.33
	45	9.67 ± 0.67	12.33 ± 0.67	15.67 ± 0.67
Absolute alcohol	15	0.00 ± 0.00	2.33 ± 0.67	11.67 ± 0.89
	25	5.33 ± 0.67	7.67 ± 0.67	26.33 ± 0.67
	35	12.33 ± 0.67	16.33 ± 0.33	35.33 ± 0.33
	45	21.33 ± 0.33	25.33 ± 0.67	35.33 ± 0.33

Table 2
Probit and regression analysis of mortality by different solvent extracts on 3rd instars larvae of Culex vishnui

Solvent used	Hour of exposure	LC_{50}	LC_{90}	LCL-UCL(LC_{50})	Regression equation	R^2 value
Petroleum ether	24	54.2346	170.4132	48.424–63.494	Y = 1.17X-10.183	0.9802
	48	45.2039	158.4331	41.077–51.469	Y = 1.25X-5.0833	0.9673
	72	39.337	188.6642	35.525–40.043	Y = 1.09X + 6.7167	0.9712
Ethyl acetate	24	22.19	37.1728	8.457–32.097	Y = 2.7X-16.167	0.9731
	48	19.2151	34.2722	1.907–28.07	Y = 2.2633X + 4.0167	0.9559
	72	11.5769	23.9619	4.132-32.4287	Y = 1.0133X + 58.433	0.8988
Acetone	24	345.7425	2616.732	154.752-3576.76	Y = 0.2433X-1.2167	0.9357
	48	858.2362	21898.6397	225.493-422144.236	Y = 0.2267X + 2.2	0.8758
	72	267.5024	3078.6931	132.94-1684.958	Y = 0.3767X + 0.95	0.9008
Absolute alcohol	24	71.5861	156.2542	61.946–89.765	Y = 0.71X-11.55	0.9792
	48	70.5271	173.3347	61.028–87.738	Y = 0.8167X-11.917	0.9896
	72	62.3821	186.4576	54.646–75.548	Y = 1.0367X-11.35	0.9709

Table 3
Susceptibility of Culex vishnui larvae to the bioactive compound isolated from mature leaves of Holoptelea integrifolia

Instar	Concentration (ppm)	Mortality (Mean ± SD)		
		24 h	48 h	72 h
First	15	59.67 ± 0.33	68.67 ± 0.33	78.33 ± 0.33
	20	66.33 ± 0.67	74.67 ± 0.67	87.33 ± 0.33
	25	76.33 ± 0.67	85.67 ± 0.89	98.67 ± 0.33
Second	15	47.67 ± 0.33	57 ± 0.58	65 ± 0.58
	20	63.33 ± 0.67	69.33 ± 0.67	83 ± 0.58
	25	68.67 ± 0.33	76.67 ± 0.33	91.67 ± 0.33
Third	15	43.67 ± 0.33	50.67 ± 0.33	59.33 ± 0.33
	20	52.67 ± 0.33	62.67 ± 0.33	78.67 ± 0.33
	25	66.67 ± 0.33	77 ± 0.58	92.67 ± 0.33
Fourth	15	34.67 ± 0.33	36.67 ± 0.33	39.33 ± 0.33
	20	40.67 ± 0.33	42.67 ± 0.33	44.67 ± 0.33
	25	46.67 ± 0.33	47.33 ± 0.33	50.67 ± 0.33

Table 4

Multivariate ANOVA for comparing the mortality rates of 1st -4th instars larvae of Culex vishnui where different instars, different concentrations and different hour's acts as variables

Source	Type III Sum of Squares	df	Mean Square	F	Significance
Instar	17854.741	3	5951.580	9888.779	.000
Hour	5178.574	2	2589.287	4302.200	.000
Concentration	7087.185	2	3543.593	5887.815	.000
Instar × Hour	985.204	6	164.201	272.826	.000
Instar × Concentration	753.704	6	125.617	208.718	.000
Hour × Concentration	96.704	4	24.176	40.169	.000
Instar × Hour × Concentration	81.519	12	6.793	11.287	.000
Residual	467310.000	108			
Corrected Total	32080.963	107			

Phytochemical analysis

Qualitative phytochemical analysis revealed a number of phytochemicals including steroids, saponins and tannins (Table 5).

FT-IR and GC- MS analyses of bio active principle

Result of FT-IR analysis showed frequency range and probable functional groups of the compound (Rf 0.357) (Figure 1). The identification of chemical compounds in the active principle of ethyl acetate extract was accomplished by GC-MS by comparing the mass spectra with those of the Wiley and the National Institute of Standard and Technology (NIST) mass spectral database library. Structure of the compound determine by Royal Society of Chemistry structural database. Result of GC-MS analysis depicted in Figure 2 and Figure 3 support the findings of preliminary phytochemical assay and IR analysis and it also revealed presence of 2-(Adamantan-1-yl)-N-methyl acetamide (Figure 4) as principal compound which is supposed to be the active larvicidal compound present in ethyl acetate extract of *H. integrifolia* leaf.

UV–VIS spectroscopy study

The resultant solution showed the typical characteristic of colour changing within minutes from colourless to reddish brown. The bio-reduction (Ag^+ to Ag^o) potentiality of leaves of *H. integrifolia* causes gradual formation of AgNp which results in change in the SPR absorption band. The characteristic surface plasmon absorption spectral band observed at 450 nm. The recode of absorbance were noted after 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 7 h and 8 h (Figure 5). The blank solution contains 20 ml of aqueous 10^{-3} M $AgNO_3$ solution was exposed to sunlight and subjected to analyses by UV–vis study. No colour change was noticed in blank solution, so nanoparticles are not formed in blank.

X-ray diffraction study

X-ray diffraction is a versatile and non-destructive analytical method to uniquely identify the crystalline phases present and to study the structural properties. The condition for diffraction at any observable angle is given by Bragg law.

$$n\lambda = 2d\sin\theta$$

where is the order of diffraction and is the interplaner spacing and the angle is called the Bragg angle. The crystal structure and orientation of the ZnO films were investigated from the X-ray diffraction (XRD) patterns. The x-ray diffraction (XRD) profiles of the samples were recorded using filtered radiation (l=1.5418 Å) from a highly stabilized and automated Philips X-ray generator (PW 1830) operated at 40 kV and 20 mA.

Figure 6 shows the x-ray diffraction pattern of the sample which reveals the formation of phase pure silver. The figure shows the plot of diffracted intensity in arbitrary units against . The major peaks at 37.92 and 44.94^o are in good agreement with the Joint committee on powder diffraction standard (JCPDS) data belonging to silver structure.

Scanning Electron Microscopy:

Scanning Electron Microscopy is done for revealing the surface morphology of particles and the following images were obtained from silver nanoparticles of *H. integrifolia* leaves extract. (Figure 7)

Transmission Electron Microscopy:

TEM analysis was done to visualize the shape as well as measure the average mean size of silver nanoparticles was 20-30 nm and the tiny particles were seemed to be quasi-spherical (or polyhedral) in morphology as shown in the following images. The images also showed the existence of nano-crystalline structure from fresh leaves of *H. integrifolia* (Figure 8).

FT-IR of Nano particles:

FT-IR spectrum of nano particles shows the bonding pattern of compounds of nano particles (Figure 9).

Effect on non – target organisms

The LC_{50} concentration of active principle for 3^{rd} instars larvae had no toxic effect against Chironomid – *Chironomus circumdatus* (Diptere: Chironomidae) larvae, *Daphina* sp, *Diplonychus annulatum* and tadpole larvae after 48 hours of exposure. Although after 72 h of exposure slight toxicity (3.33 %) found against the same *Chironomus circumdatus* larvae and *Daphnia* sp. No toxicity found against tadpole larvae and *Diplonychus annulatum* even after 72 hours of exposure.

No toxicity was recorded to the non-target organisms *Daphnia* sp., *C. circumdatus* larvae, *D. annulatum* and tadpole larvae in the bioassay test with nano particles after 24 exposures. Only 3.33 % mortality of *Daphnia* sp. was recorded after 48 h of exposure. During 72 h time period no death was seen in case of tadpole larvae while 3.33 % mortality of *C. circumdatus* larvae and *D. annulatum* found 72 h post exposure.

Discussion

In recent years natural product of plant origin have been given much priroty as they are cheap, biodegradable and without any ill effect on ecosystem [13]. Larval stage is the softest target for mosquito control program due to their restricted distribution within aquatic habitats. In many parts of world plant derived products have been successfully used for vector control program [14]. Some phyto extracts have been shown as good ecofriendly larvicides, without hamparing lives of non-target organisms [15,16].

Bioassay experiments with solvent extracts (petroleum ether, ethyl acetate, acetone and absolute alcohol) showed that ethyl acetate have maximum potentiality in the mortality of 3rd instars larvae of Cx. vishnui. Out of four tested concentrations (15, 25, 35 and 45 ppm) 45 ppm has highest mortality efficacy after 72 h of exposure. From ethyl acetate extract of mature leaf of H. integrifolia finally single spot with $R_f$value (0.35) was isolated having highest larvicidal activity. At 25 ppm concentration of bioactive compound on different larval instars of Cx. vishnui mosquito show highest mortality of 1st instar larvae with lowest LC_{50}value after 72 hour of exposure.

The LC_{50} concentration of bioactive compound of 3rd instars larvae when applied against non-target organisms those sharing the same habitat of Cx vishnui mosquito have no ill effect on Chironomid-Chironomus (Diptera: Chironomode) larvae, Daphnia sp, tadpol larvae and Diplonychus annulatum after 48 h of exposure. But 3.33% mortality of Chironomus was recorded after 72 h of exposure.Qualitative analyses of mature leaf of H. integrifolia indiact the presensce of saponin, tannins, and steroid as secondary metabolites but absence of alkaloids, flavonoids, free glycoside bound anthroquinone and terpenoids.

The present study revealed that ethyl acetate extract has potential larvicidal property against 3rd instars larvae of Cx. vishnui mosquito and this efficacy is due to the presence of organic compound 2-(Adamantan-1-yl)-N-methyl acetamide in the active principle of ethyl acetate extarct detected through Column and Thin Layer Chromatograghic methods.

Previous reports of different authors regarding mosquito larvicidal activity of green nanoparticles synthesised from different plants are fruit extract of Tanacetum vulgare [17],Rhizophora mucronata leaf [18], Delphinium denudatum root [19], Couroupita guianensis Aubl. leaf & fruit [20] and Achyranthes aspera [21] Drypetes roxburghii fruits [22], leaves and green barriers of Solanum nigrum L (Solanaceae: solanales) [23] and biosynthesized silver nanoparticles using Curcuma zedoaria essential oil [24].

We successfully characterized the biologically synthesized Ag-nano particles from the leaves of H. integrifolia. The UV absorption peak at 421 nm clearly demonstrates the presence of silver nano particles from the extracted collidal solution of plant. The SEM and TEM studies were helpful to study the superficial and morphological shape, size and distribution of synthesised Ag nanoparticles. XRD result confirmed the purity of nano particles. Then truly synthesised silver nanoparticles of H. integrifolia are subjected to study their mosquito larvicidal efficacy against Japanese Encephalitis vector Cx. vishnui mosquitoes and the results indicated significant efficacy of the nano particles.

Conclusion

In the present context, isolated compounds from leaves of H. integrifolia may be very useful in mosquito control programme of Cx. vishnui mosquitoes as it is indigenously available, safe in comparison to chemical insecticides. Further detailed study is needed on the physiological mechanism of toxicity production in mosquito before its commercial use and wide application. Our research study area was periphery of Burdwan town where lots agricultural field are situated. We know that Burdwan is an Encephalitis prone zone, so it is very much essential to control the spreading of this disease through application of a new strategy. It is very effective step to control Cx. vishnui mosquito population by the application of silver nanoparticles synthesised from leaves of H. integrifolia. It is also an eco-friendly product because it could not harm those non target organisms sharing the same habitat of Cx. vishnui mosquitoes. So further research must be need to modulate the product such an away that it could be effectively apply for vector control programme of JE in future.

Abbreviations

LC_{50}: LC_{50} is the concentration of the compound that is lethal for 50% of exposed population.

LC_{90}: LC_{90} is the concentration of the compound that is lethal for 90% of exposed population.

Declarations

Ethics approval and consent to participate:

Ehics approval is not needed for this study as it did not involved human or animal.

Consent for publication:

Authors give the consent for publication in Parasites and Vectors.

Availability of data and materials:

All data generated or analyzed during this study are included in this article.

Competing interests:

Authors have no competing interests.

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Authors' contributions:

SS conducted the experiments and statistical analysis of the study and prepared the first draft of the manuscript. GC designed and supervised the study and also revised the manuscript. Both authors checked the final manuscript and approve the manuscript for communication.

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Figures

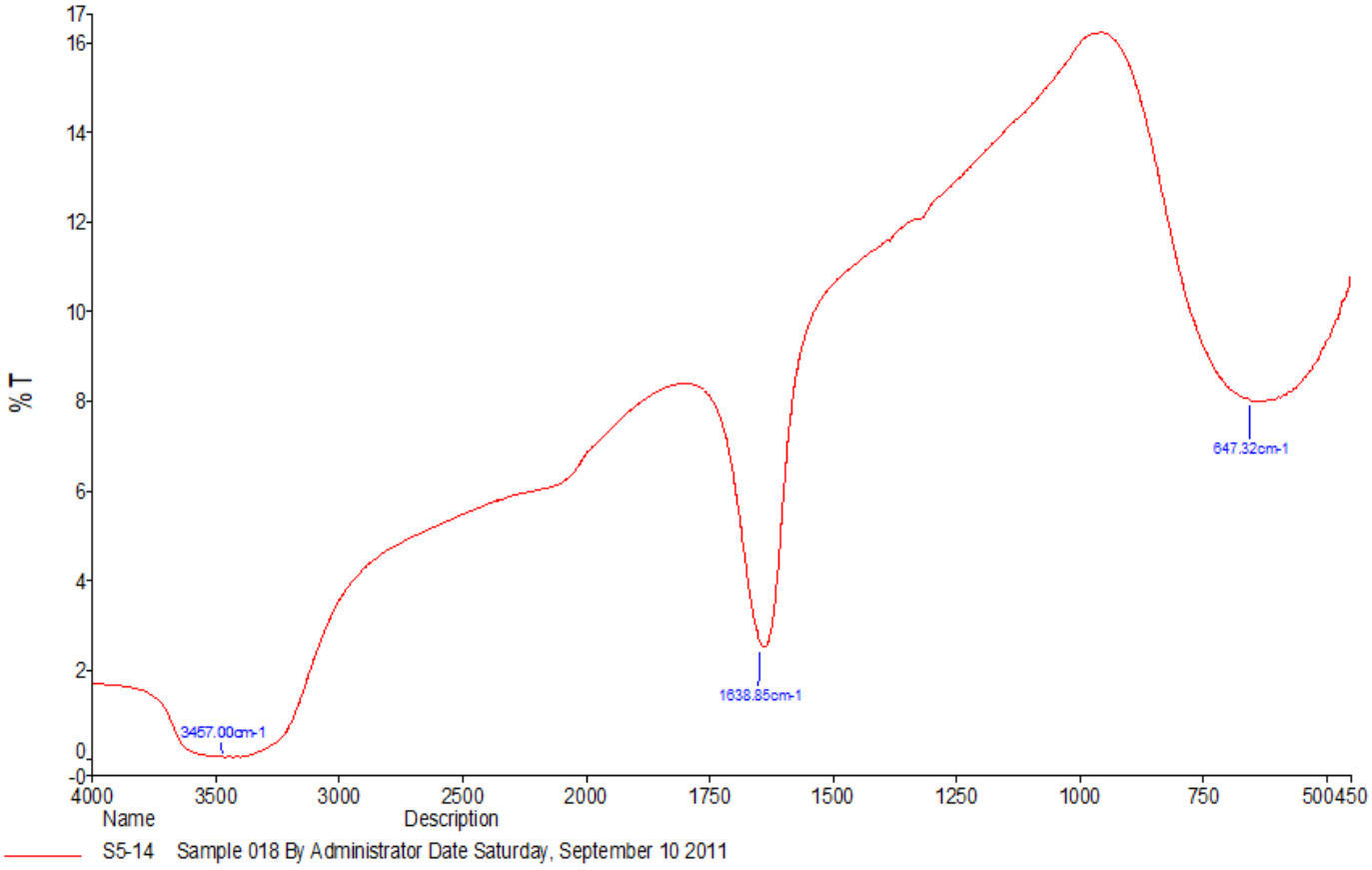

Figure 1

Interpretation of IR spectra of the compound having Rf 0.357. Frequency range and probable functional groups of the compound (Rf 0.357): 3457cm-1 RCH2OH, R2CHOH, R3COH varies OH- stretch; 1638.85 cm-1 C=C stretch (w) NH out of plane (s), C=O stretch (s), C=N(s), NH2 in plane (s) (bend); 647.32 cm-1 S, C-H bend. S =strong and W = weak

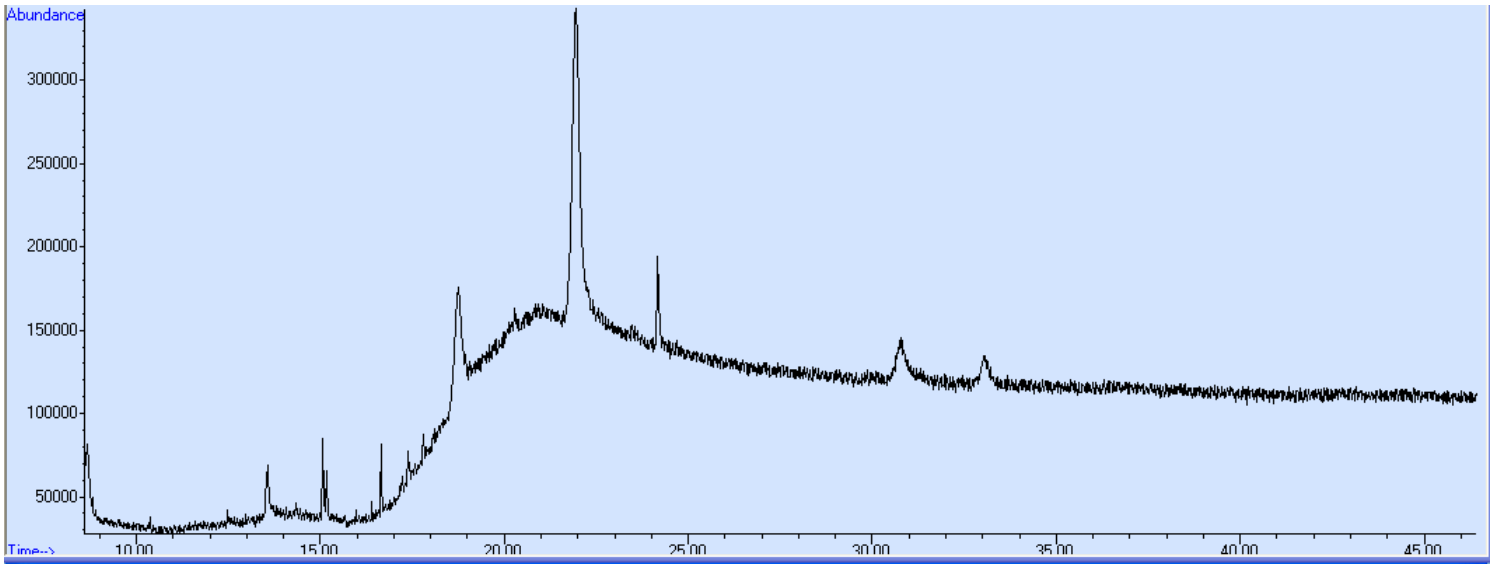

Figure 3

GC analysis of Holoptelea integrifolia leaf ethyl acetate extract

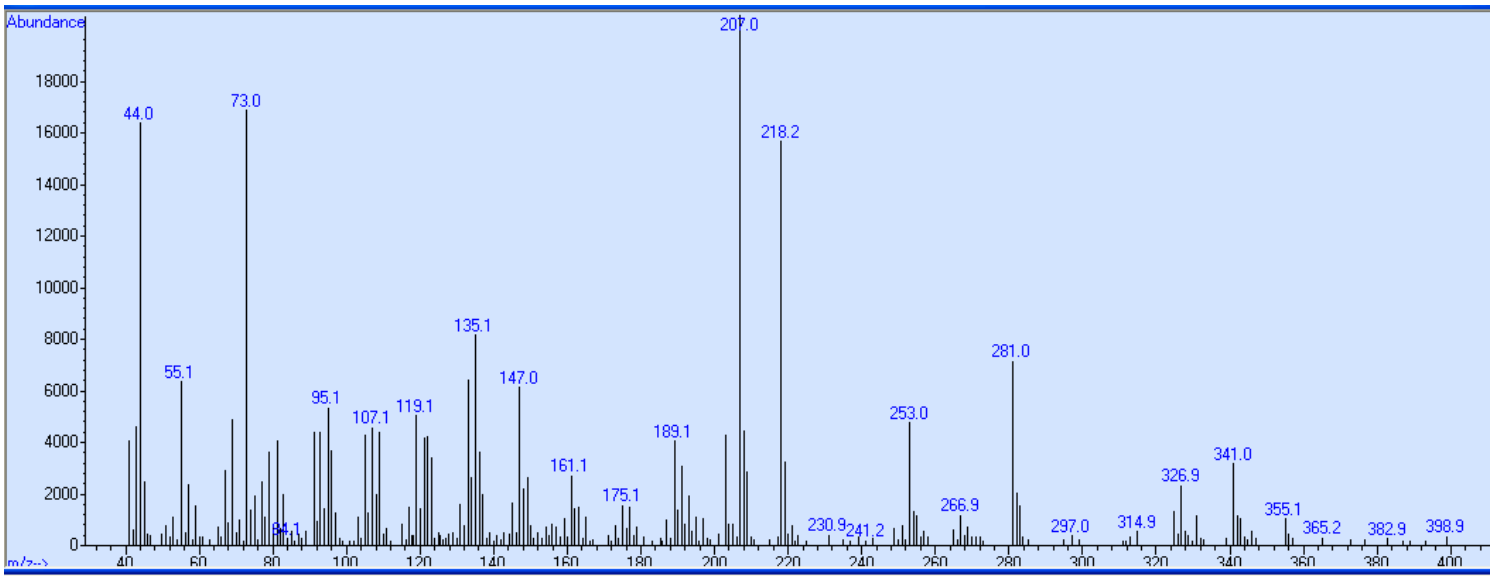

Figure 5

Mass spectra analysis of Holoptelea integrifolia leaf ethyl acetate extract

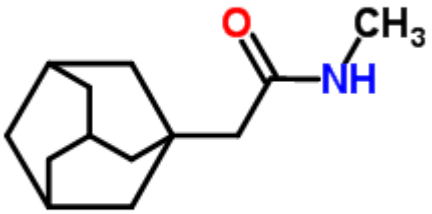

Figure 7

2-(Adamantan-1-yl)-N-methylacetamide Structures of major constituent of active principle of ethyl acetate extract of Holoptelea integrifolia leaf

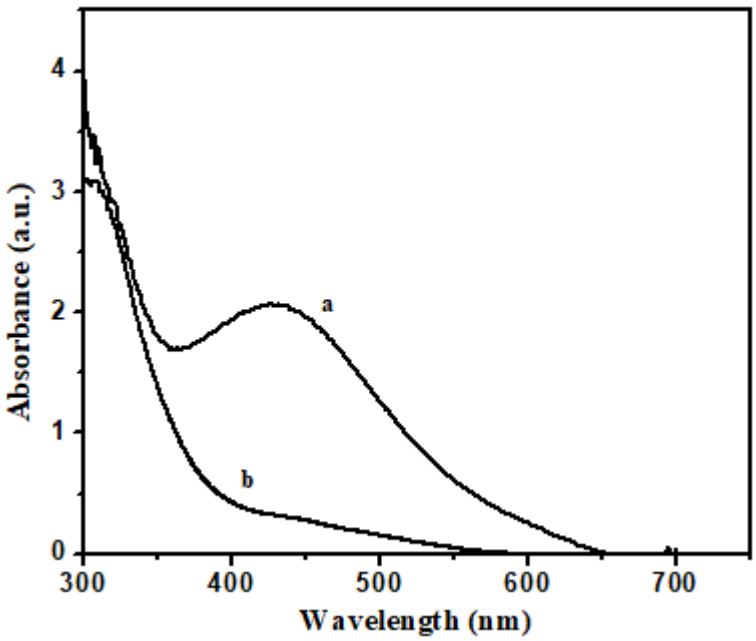

Figure 9

UV –Vis spectroscopic study of synthesised green nanoparticles of Holoptelea integrifolia extract indicating line a -for nanoparticles absorption maximum at 450 nm where as distilled water b- line indicate control absorption spectra

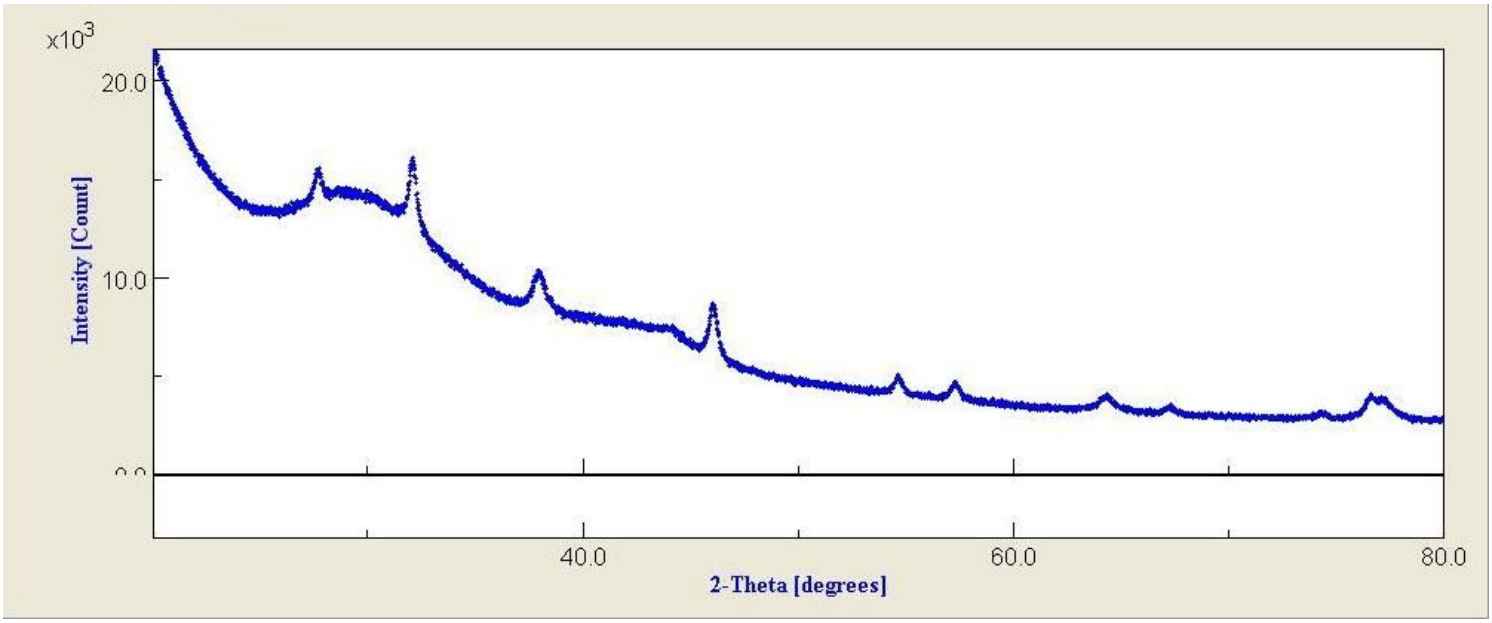

Figure 11

XRD image of silver nano particles of leaves of Holoptelea integrifolia

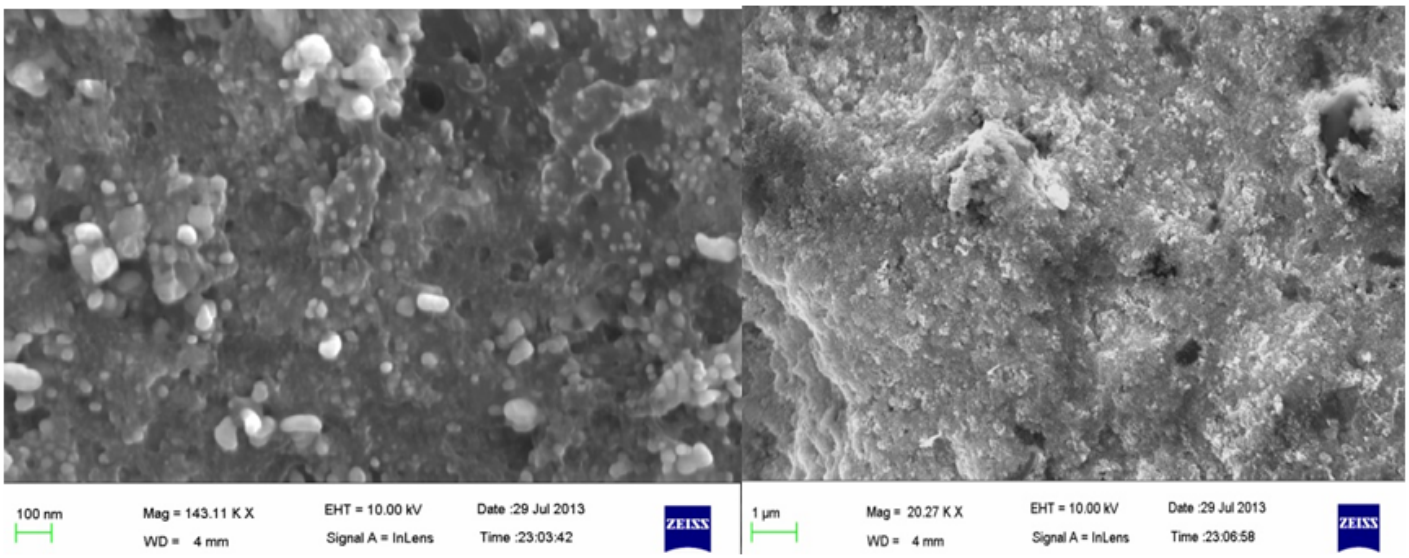

Figure 13

SEM image of Holoptelea integrifolia silver nanoparticle from leave

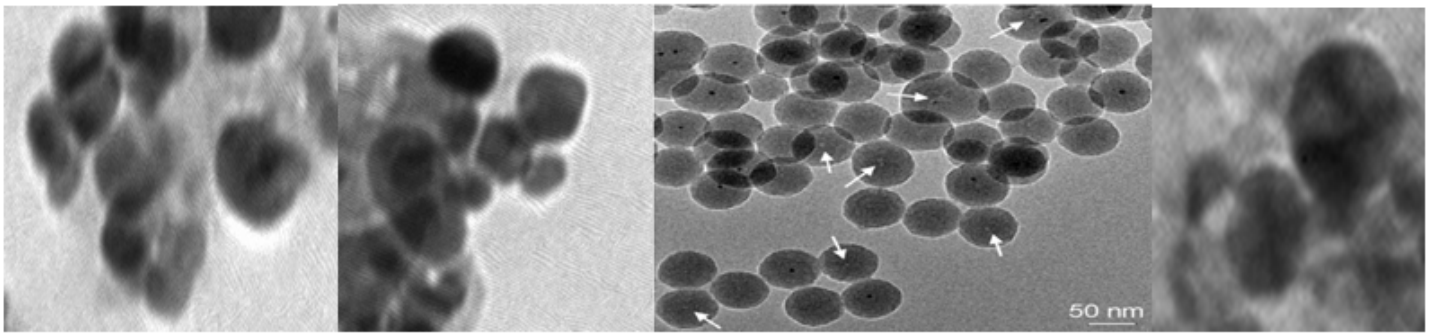

Figure 15

TEM image of silver nanoparticle of Holoptelea integrifolia leaves

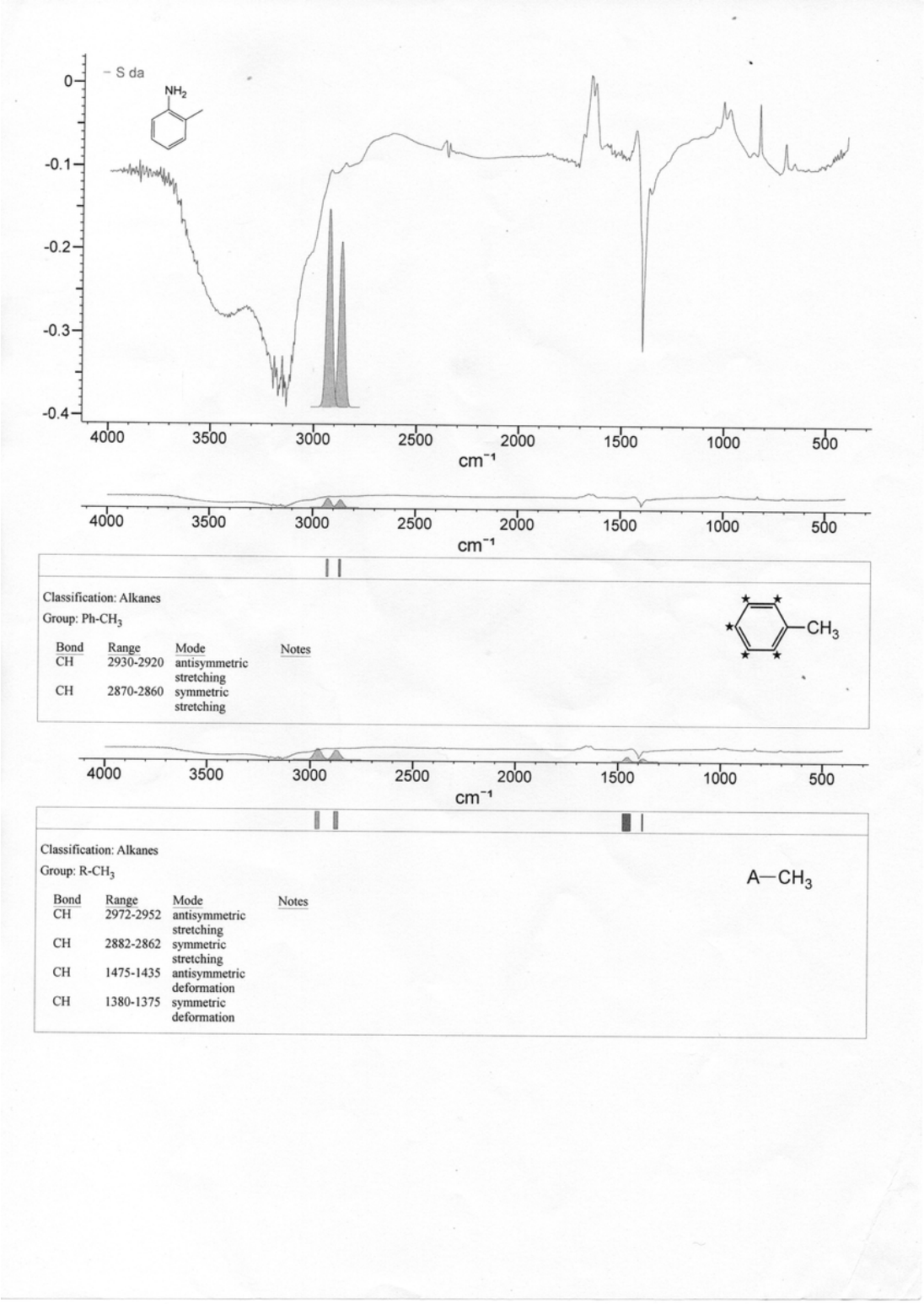

Figure 18

FTIR analysis of silver nanoparticles synthesised from leaves of Holoptelea integrifolia and their chemical stretch