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MICROCYSTIN-BASED NANO DRUG FROM *ANABAENA CYLINDRICA* FOR TREATMENT OF HEPATOCELLULAR CARCINOMA

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Keywords:

Microcystin, Hepatocellular carcinoma, *Anabaena cylindrical*, HepG2, Silver nanoparticles

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ABSTRACT: Hepatocellular carcinoma (HCC) is liver cancer characterized by high prevalence and low survival rate. Hence, there is an urgent need to screen resources to develop therapeutic drugs to treat patients suffering from HCC. In this context, the current study involves developing a microcystin conjugated nano-drug from cyan bacteria Anabaena cylindrica to target the tumor liver cells. The microcystin molecules in the aqueous algal extract were quantified by High-performance liquid chromatography (HPLC) and used as reducing and capping agents at a concentration below the toxic limit to synthesize silver nanoparticles. These nanoparticles were characterized by Nanoparticle Tracking analysis (NTA), Fourier Transform Infra-red (FTIR), Transmission emission microscopy (TEM), and their anti-tumor activity was evaluated on *HepG2* cell line by MTT assay. The highest nanoparticle synthesis was obtained at pH 8.5 after 120 min of incubation. NTA and TEM analysis showed mean particle size of 30nm. FTIR spectrum confirmed the presence of functional groups related to proteins conjugated to silver nanoparticles. The nanoparticle formulation exhibited enhanced to toxic activity in *HepG2* cell line compared to the extract and a standard drug. The present investigation is significant as it demonstrates a green method of synthesis of nano-drug from a biological source with potent therapeutic activity against HCC.

INTRODUCTION: *Cyanobacteria*, also known as blue-green algae, are photoautotrophic bacteria commonly found in fresh and brackish waters. They are a prolific source of biologically active metabolites, including compounds that exhibit toxic and enzyme-inhibiting activities. *Cyanobacteria* also known as, blue-green algae are photoautotrophic bacteria commonly found in fresh and brackish waters. They are a prolific source of biologically active metabolites, including compounds that exhibit toxic and enzyme-inhibiting activities. Microcystins are one such frequently occurring and the best known bioactive metabolites produced by *Cyanobacteria*.

The species most commonly associated with microcystin production in *Microcystis aeruginosa* [1], although other species of genera *Anabaena*, *Planktothrix*, *Anabaenopsis*, *Haplosiphon*, and *Nostoc* have also been reported to synthesize low concentrations of microcystins [2]. Chemically, microcystins are monocyclic heptapeptides with a general structure: cyclo-(D-Alanine1-X2 -D-MeAsp3-Z4 -Adda5-D-Glutamate6-Mdha7. The amino acid Adda (3-amino-9-methoxy-10-phenyl-2, 6, 8-trimethyldeca-4,6-dienoic acid) is essential

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for its biological activity [3] **Fig. 1**. Over 80 variants of microcystin have been reported with amino acid variations and modifications. The best known are MC-LR, MC-RR, and MC-YR, with different combinations of leucine (L), arginine (R), or tyrosine (Y). The biological activity of microcystins is directed towards the inhibition of serine/threonine-specific protein phosphatases (PPs) such as PP1 and PP2A through binding to these enzymes [4]. It leads to excessive phosphorylation of proteins followed by alterations in the cytoskeleton, loss of cell shape with subsequent destruction of liver cells causing intra-hepatic haemorrhage or hepatic insufficiency [5].

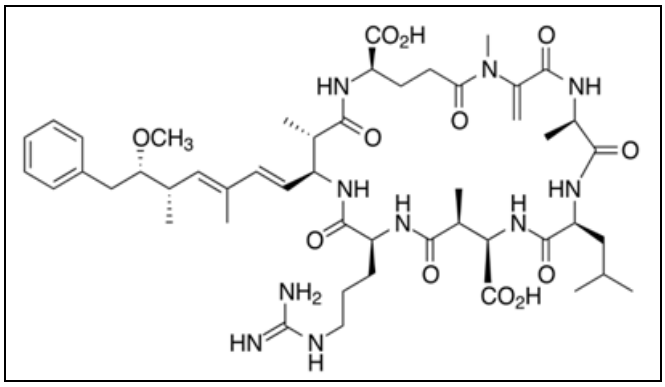

FIG. 1: STRUCTURE OF MICROCYSTIN

Hepatocellular carcinoma, also known as malignant hepatoma, is the fifth most common type of cancer and the third leading cause of cancer-related deaths [6]. Most cases of HCC are secondary to either a viral hepatitis infection or cirrhosis [7]. Therapeutic approaches for HCC treatment are either curative - surgical resection, liver transplantation, and percutaneous ablation [8] or palliative-systemic chemotherapy, immunotherapy, and hormonal compounds [9], both of which aim to improve survival. Drugs used for the palliative treatment of HCC are agents inhibiting vital signalling pathways in the tumor cells and angiogenesis. Combined therapy, which involves using two-or-more drugs targeting tumors through different mechanisms, has been tried [10]. Several clinical trials have been conducted using therapeutic drugs, but there is currently no standard formula found to be effective against advanced HCC [11]. In these circumstances, the application of Transarterial chemoembolization (TACE) therapy uses an infusion of a cytotoxic agent deployed inside the artery followed by the embolization of blood vessels that supply the tumor resulting in a cytotoxic and ischemic effect, can be a good option. TACE can be used to manage unresectable and multifocal HCC and to downstage lesions before liver transplantation [12].

Most chemotherapy drugs like Sorafenib, Doxorubicin, and Bevacizumab currently used for HCC treatment are synthetic. Many plant metabolites like taxol, vincristine, and vinblastine have been reported to exhibit anti-tumor activity. Compounds of biological origin are preferred for treatment due to their biocompatibility with the human system. With this perspective, microcystins that are molecules of biological origin with proven cytotoxic activity specific to tumorous hepatic cells can be an effective chemotherapeutic agent. Although microcystins can be toxic at high concentrations, smaller doses can achieve the desired cytotoxic effect if precisely targeted to tumor cells. Nanotechnology can play a critical role in attaining this objective. Nanoparticles are utilized in cancer therapies for their small sizes, which help in precise tumor targeting and better drug penetration into the tumor. A microcystin-nanoparticle conjugated drug that can be formulated and deployed is Transarterial Chemoembolization (TACE) therapy to inhibit HCC cells. The current research is based on the rationale of green synthesis of microcystin conjugated nano-scale particles in algae *Anabaena cylindrica* and assessing their anti-tumor activity in HCC cell lines. The proposed concept applies nanotechnology principles to biological organisms like algae resulting in the synthesis of therapeutically active biomolecule metabolite which can be a potent chemotherapy drug.

MATERIALS AND METHODS:

Culturing and Biomass Development of *Anabaena cylindrica* for Microcystin Synthesis: The unialgal species of algae *Anabaena cylindrica* was selected for its ability to synthesize microcystin. It was obtained from the National Facility for Marine Cyanobacteria (NFMC) culture collection, Bharathidasan University, Trichi. The organism was cultured in BG-11 media under controlled growth conditions, *i.e.*, temperature 15 ± 2^{o}C, light intensity 1500-2000 lux, and light period 16 hr light / 8 hr dark. The organism was incubated for 7-8 days till sufficient biomass was obtained.

Extraction of Microcystins from Algal Biomass: The algal cells were harvested from growth media by centrifugation at 10,000 rpm for 15 min at 4°C. This biomass was used to extract microcystins following the method reported by Metcalf and Codd [13].

Briefly, the algal cells were suspended in Milli Q-water and subjected to ultrasonication for 1 min, leading to cellular metabolites' release.

This was followed by centrifugation for 10 min at 1400 rpm; the supernatant was collected and evaporated in a water bath. Finally, microcystins were extracted using 100% methanol.

HPLC Analysis for Qualitative and Quantitative Estimation of Microcystins: Microcystin concentration in extracted samples was determined by HPLC with a UV detector following the method of Massey *et al.*[14]. The HPLC instrumentation unit consisted of an Agilent system. The stationary phase was Symmetry C_{18} column (250 x 4.6 mm I.D., 5µm particle size), while the mobile phase consisted of Milli-Q water (solvent A) and acetonitrile (solvent B), both acidified with 0.05% TFA. The mobile phase flow rate was 1ml/min at a column temperature of 38^{o}C. The sample injection volume was 50 µl. Column development was done using a linear gradient, as depicted in **Table 1.**

TABLE 1: LINEAR GRADIENT FOR COLUMN DEVELOPMENT OF HPLC

Time in min	0	10	40	42	44	46	55
Solvent A [%]	70	65	30	0	0	70	70
Solvent B [%]	30	35	70	100	100	30	30

Green Synthesis of Microcystin Conjugated Silver Nanoparticles: To prepare silver nanoparticles, 5 ml of microcystin containing algal extract was mixed with 45 ml of 1 mM $AgNO_3$ solution and incubated at 24°C with constant stirring at 120 rpm [15]. The formation of nanoparticles was detected by monitoring the change in color of the solution using a UV-visible spectrophotometer (Shimadzu UV-1800). The effect of pH and time on nanoparticle synthesis was also evaluated by altering the pH of the algal extract (5.5, 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5) and incubation time (60 min, 90 min and 120 min).

Characterization of Microcystin-nanoparticles by NTA, TEM, FTIR: Nanoparticle tracking analysis (NTA-Nanosight UK-LM20) was performed to estimate the density and size of nanoparticles. The synthesized nanoparticle solution was briefly sonicated, and 10 µl of the sample was injected using a sterile syringe.

Transmission Electron Microscope assessed nanoparticle morphology (TEM - PHILIPS CM 200) operated at an accelerating voltage of 200 kV with the resolution of 0.22 nm. Sample preparation for TEM involved sonication of nanoparticle solution followed by placing a drop of colloidal solution on carbon-coated copper grids. Fourier Transform Infra-Red (FTIR-3000 Hyperion Microscope with Vertex 80) spectroscopy was performed to identify functional groups constituting the biomolecules involved in nanoparticle synthesis and acting as surface capping reducing agents. The methodology involved mixing 2–3 drops of colloidal solution of silver nanoparticles with KBr powder to generate a pellet which IR analyzed.

Evaluation of Anti-cancer Activity of Microcystin Nanoparticles on *HepG2* Cell Line: The microcystin nanoparticles were tested on hepatocellular carcinoma cell line *HepG2* for anti-cancer potential. *HepG2* cells were seeded in a 96-well microtitre plate and were allowed to attain confluence by incubating at 37°C under 5% CO_2 overnight. The cells were then treated with a standard anti-cancer drug, algal extract (1 µg/l), and microcystin conjugated nanoparticle formulation (1 µg/l) and incubated at 37°C under 5% CO_2 overnight. The anti-tumor activity of the samples was determined by MTT assay. In this assay, 10 µl of 5 mg/ml MTT reagent was added to the treated cells in the microtitre plate and incubated for 4 hr. The color change was recorded at 595 nm, and the anti-cancer activity was expressed as IC_{50}.

RESULTS: In the present study, cytotoxic protein molecule microcystins extracted from *Anabaena cylindrical* were used for the green synthesis of silver nanoparticles. This was followed by the characterisation and evaluation of anti-tumor

properties of the synthesized nanoparticles on the *HepG2* cell line.

The results demonstrated that cytotoxic protein molecule microcystin synthesized in the algal cells can be formulated as nanoparticles *via* the green route using silver salts. Their characterization studies showed promising results to illustrate their biological activity under *in-vitro* conditions.

Extraction of Microcystins from Algal Biomass and Quantification by HPLC: *A. cylindrical* cells cultured in BG-11 media showed adequate biomass after incubation for 8 days.

The increase in biomass was monitored colorimetrically at 660nm, and culture was grown till the stationery phase was reached. The dry weight obtained was 3.16g l^{-1} of media. Microcystin molecules extracted from the algal biomass were identified and quantified by the HPLC-UV technique. The chromatogram of algal extract exhibited two prominent peaks at retention times (R_t) of 36.63 min and 37.86 min **Fig. 2A** while the standard MC-LF graph showed a peak at R_t of 36.83 min **Fig. 2B**. This observation implies that microcystin-LF was the prominent microcystin protein detected by HPLC, and its presence was confirmed by comparing the retention time with the standard microcystin-LF molecule. Further quantitative estimation revealed that the concentration of microcystins in the extract was 105.02 µg ml^{-1}.

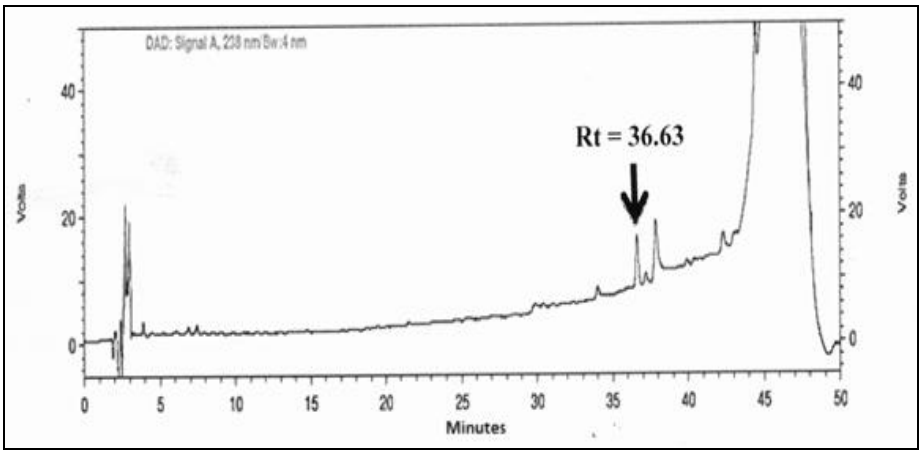

FIG. 2A: GRAPH OF ALGAL EXTRACT

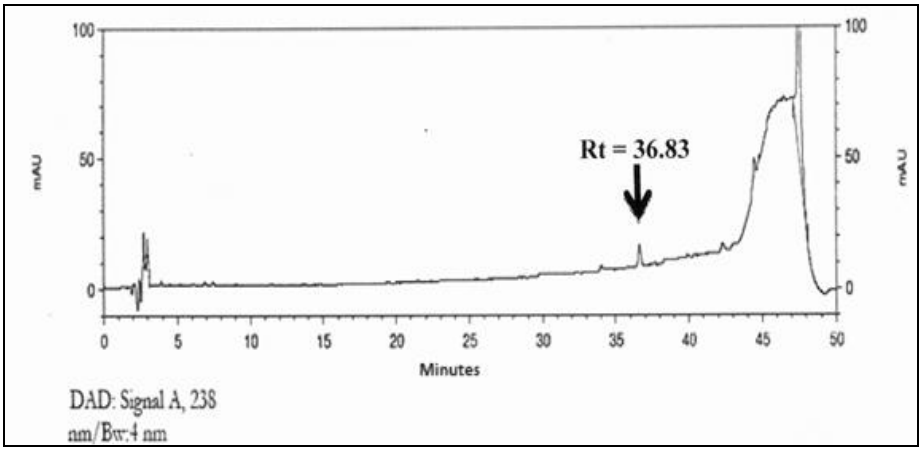

FIG. 2B: GRAPH OF STANDARD MICROCYSTIN

Nanoparticle Synthesis and UV-visible Spectrophotometry Analysis: Reduction of silver ions by the algal extract occurred in the presence of visible light. The microcystin protein acted as the coating and stabilizing agent for the silver nanoparticles formed. A change in colour of the nanoparticle solution was observed from colourless to dark brown which indicated the formation of silver nanoparticles. The effect of pH and time on nanoparticle synthesis was studied to optimize the conditions favourable for nanoparticle synthesis. Nanoparticle synthesis was not observed when the

pH of the solution was acidic (5.5, 6.0, 6.5, 7.0), but synthesis commenced when the pH was shifted to the alkaline range (7.5, 8.0, 8.5). **Fig. 3A** indicates silver nanoparticles synthesized at varying pH values. It is evident that maximum nanoparticle synthesis was observed in a solution with pH 8.5. Similarly, the time for nanoparticle synthesis was also evaluated, and maximum nanoparticle synthesis was observed on 120 min of incubation. Further, incubation did not show any significant increase in nanoparticle synthesis **Fig. 3B.**

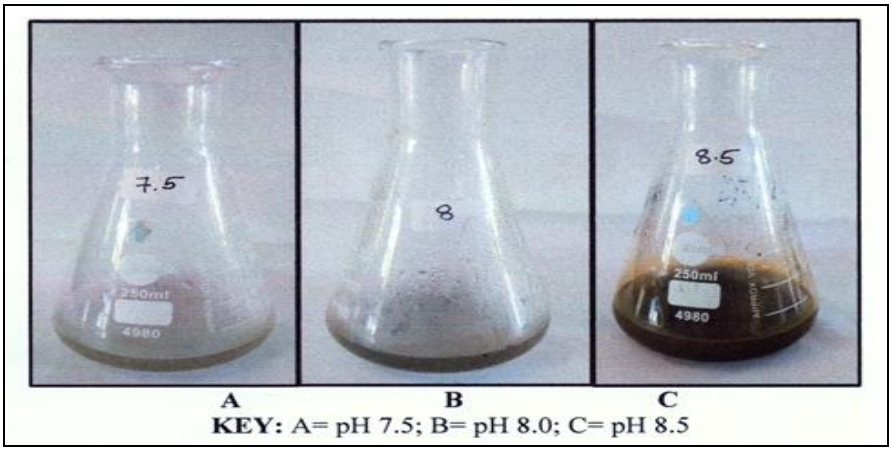

FIG. 3A: NANOPARTICLES SYNTHESIZED AT VARYING pH

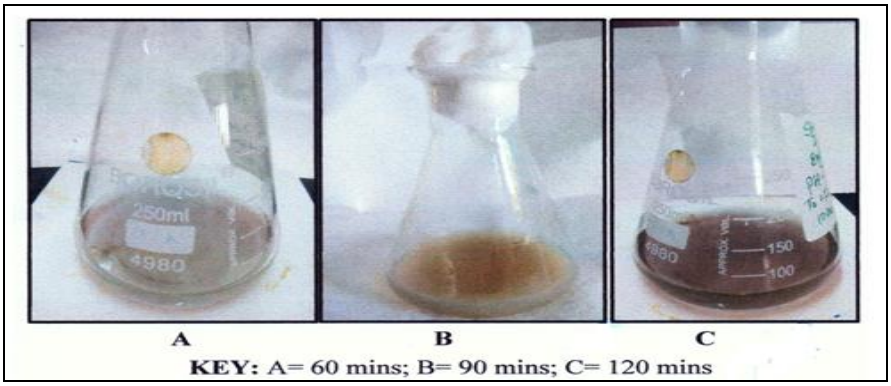

FIG. 3B: NANOPARTICLES SYNTHESIZED AT VARYING TIME

UV-Visible spectrometric analysis was further carried out to confirm the presence of silver nanoparticles. The spectrum was characterized by peaks in the 200nm to 220 nm range.

Fig. 4 shows the UV-Visible spectrum of synthesized silver nanoparticles with varying pH values after 120 min of incubation.

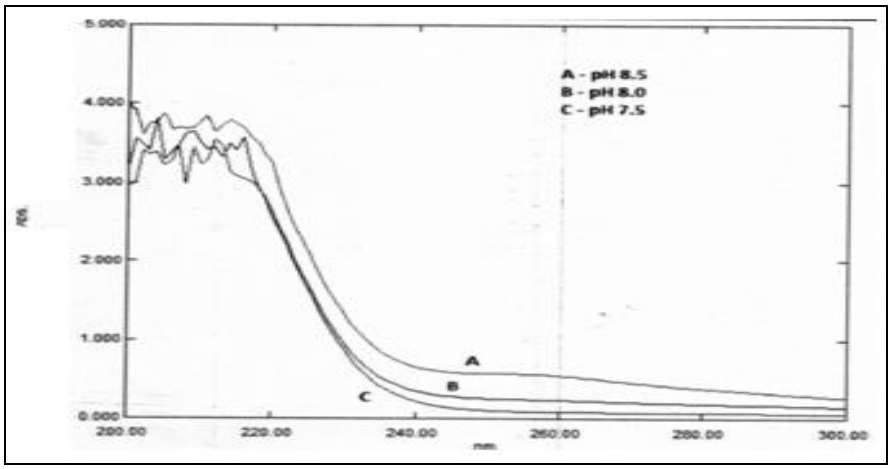

FIG. 4: UV-VISIBLE SPECTRUM OF SYNTHESIZED SILVER NANOPARTICLES

Characterization Studies of Nanoparticles: NTA analysis gave defined estimates of the synthesized nanoparticles' size, size distribution, and concentration.

Fig. 5 indicates the plot generated by NTA for microcystin-silver nanoparticles. It showed a mean particle size of 30nm and particle concentration of 8.48 X 10^8 particles per ml.

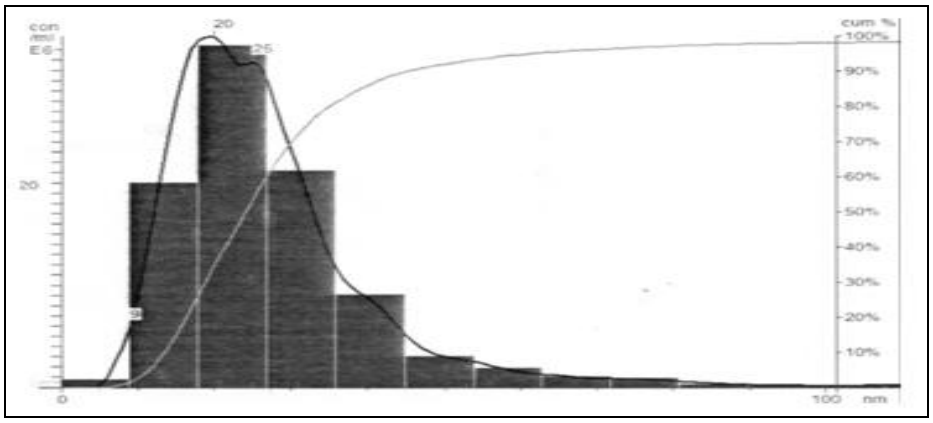

FIG. 5: PLOT OF NTA SHOWING PARTICLE SIZE DISTRIBUTION

TEM images demonstrated the formation of spherical silver nanoparticles homogeneously distributed across the polymer matrix. At low magnification, a very large density of silver nanoparticles was seen **Fig. 6A**. The size range was calculated at 10nm to 40nm. Morphologically, they were irregularly shaped, although majority of the particles were spherical in shape **Fig. 6B**. These nano-scale particles appeared to have assembled into closely packed quasi-linear superstructures **Fig. 6C**. The dark spots visualized indicated the bio-organic compounds capping the silver nanoparticles.

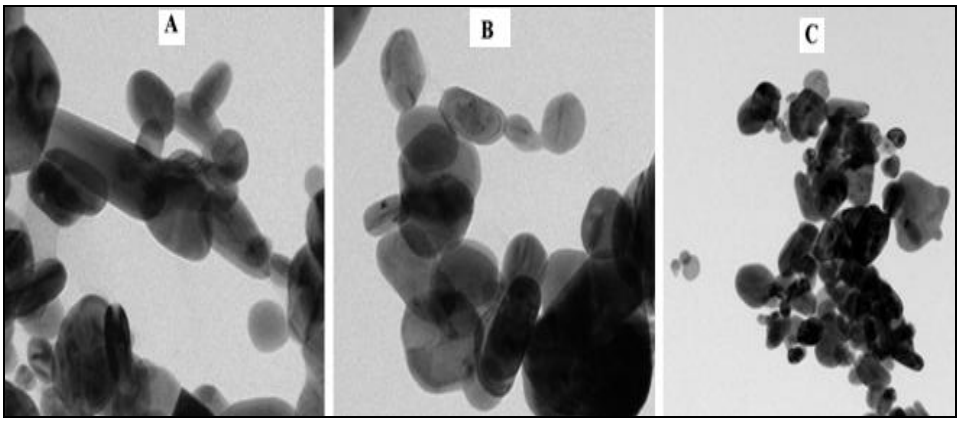

FIG. 6: NANOPARTICLES VISUALISED UNDER TEM

FTIR analysis was carried out to identify the biomolecules responsible for surface coating and stabilization of the silver nanoparticles. The FTIR spectra of algal extract and synthesized nanoformulation were compared. The FTIR spectrum for extract showed bands of several absorption peaks **Fig. 7A**. The prominent peaks were observed at 1096.55 cm^{-1} and 1784.19 cm^{-1}characteristic of C-O and N-H, respectively. The other peaks at 3899.15 cm^{-1}, 3850.48 cm^{-1}, 3703.42 $cm^{-1,}$ and 3564.85 cm^{-1} were due to C-H, C-N, O-H, and COO-groups, respectively, peaks at 704.53 cm^{-1} and 457.35 cm^{-1} showed the presence of aromatic and alkene groups respectively in the extract. **Fig. 7B** shows the FTIR spectrum for the nanoformulation with peaks at 3422.41cm^{-1}, 2923.19cm^{-1}, 1739.29cm^{-1}, 1637.31cm^{-1}, 1382.87$cm^{-1,}$ and 1215.07cm^{-1} corresponding to COO-, C-H, C-O, C-C and C-N groups respectively. Peaks at 708cm^{-1}, 1080$cm^{-1,}$ and 1739cm^{-1} correspond to aromatic, C-O, and N-H groups which were also identified in the algal extract.

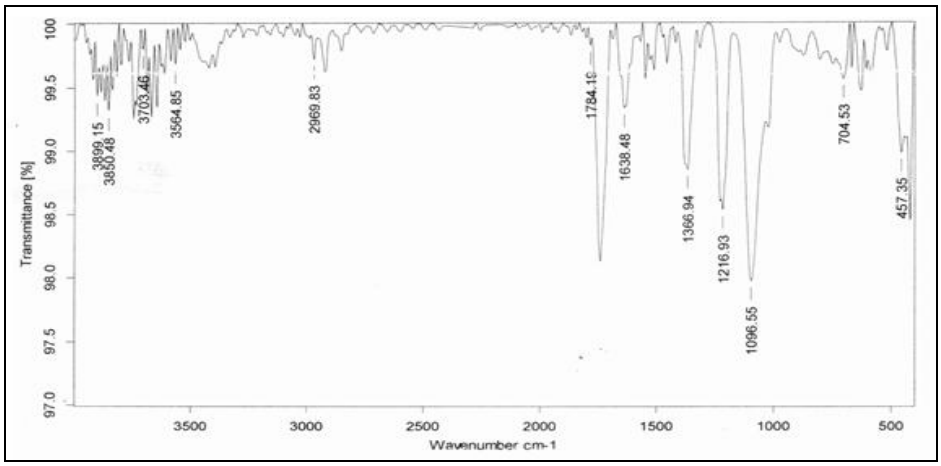

FIG. 7A: FTIR SPECTRUM OF EXTRACT

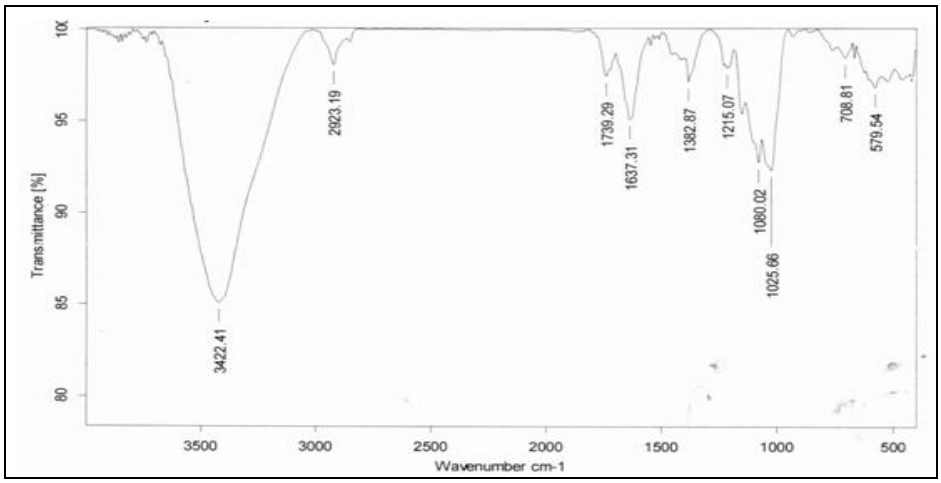

FIG. 7B: FTIR SPECTRUM OF NANOPARTICLES

Assessment of Anti-cancer Activity of Microcystin Nanoparticles against *HepG2* Cell line: The anticancer activity of silver nanoparticles was tested against *HepG2* cell line. Microcystins have been reported to show inhibitory activity against liver enzymes active in cellular metabolism activities. Hence, *HepG2* cell line, i.e. hepatocellular carcinoma cell line, was selected to evaluate the synthesized nanoparticles' anti-cancer activity. MTT assay was performed, and results were expressed as $IC_{50.}$ The anti-cancer activity was determined of the algal extract, synthesized nanoparticles, and a standard anti-cancer drug as reference to perform a comparative study. **Table 2** summarizes the IC_{50} value of the three samples.

TABLE 2 IC_{50} VALUES OF THE THREE SAMPLES ASSESSED AGAINST *HEPG2* CELL LINE

Sample	IC_{50} value (µg ml^{-1})
Standard drug	3.0±0.06
Algal extract	0.93±0.15
Nanoparticle formulation	0.68±0.33

Values are the mean +S.D. and n=3.

DISCUSSION: The results showed that microcystin conjugated silver nanoparticles can be synthesized from *A. cylindrica* aqueous extract via the green route. The quantification of microcystin molecules extracted from algal biomass was performed using a UV detector at 238 nm. The Adda residue of microcystins consists of two conjugated ð-bonds responsible for the characteristic absorbance at 238 nm [16].

During HPLC analysis, another peak was observed at retention time 37.86 min in the algal extract. This retention time corresponds to microcystin-LW. However, the UV absorbance maxima of microcystin-LW are observed at 222 nm [16] and so the possibility of the presence of another variant of microcystin was eliminated. The synthesis of silver nanoparticles was estimated by UV-visible spectrophotometry analysis. The time-dependent shift in the absorbance peak indicated the initiation of reaction and synthesis of silver nanoparticles in bulk amount [17]. The studies performed to determine

the optimum pH of nanoparticle synthesis demonstrated that nanoparticle synthesis accelerated with an increase in pH. This observation complies with the work of Singh *et al.* [18]. Also, the nanoparticles' synthesis was investigated until pH 8.5 only since microcystin molecules degrade at pH above 8.5 [3]. NTA and TEM analysis studied the morphological characteristics of nanoparticles. It was found that the majority of nanoparticles were spherical shaped and had a size in the range of 10 - 40 nm. This observation is critical since nanoparticles' morphology greatly influences their bioactivity in the target cell [19]. Further, it is reported that spherical nanoparticles in the size range of 10 nm - 100 nm showed enhanced permeability and retention (EPR) effect in the tumors [20]. The morphological characteristics of nanoparticles synthesized in the current study comply with the reported scientific work and imply that the nanoparticles are ideal for delivering microcystin molecules into the tumorous liver cells.

The current investigation has revealed the anti-hepatocarcinoma activity of microcystin-coated silver nanoparticles probably because microcystins inhibit serine / threonine-specific protein phosphatases (PPs) PP1 and PP2A [4]. This leads to excessive phosphorylation of proteins and alterations in cytoskeleton, loss of cell shape with subsequent destruction of liver cells [5]. Microcystins are also responsible for increasing oxidative stress in cells which subsequently can trigger apoptotic processes [21]. Results showed that silver nanoparticles-microcystin formulation is more effective than *A. cylindrica* extract and the standard chemotherapy drug in tumor suppression of *HepG2* cells. This outcome could be attributed to the fact that is owing to its size; the nanoformulation had better excess and penetrability into the tumor cells. Also, the conjugation of microcystin proteins with silver nanoparticles would have enhanced their anti-tumor activity [22]. In this context, the anti-tumor activity of microalgal species has been widely investigated. *Chlorella* has been reported to possess anti-oxidative, anti-inflammatory and anti-tumor properties both *in-vitro* and *in-vivo* [23]. An investigation by Czerwonka *et al.* (2018) demonstrated the anticancer effect of *Spirulina* on human lung cancer cell lines [24]. These reports indicate that the metabolites synthesized by algal species are potent anti-cancer agents and the current work is in compliance with the work of these investigators.

CONCLUSION: The current work combined the applicability of bioactive natural products with nanotechnology to develop a chemotherapeutic nanodrug for the treatment of hepatocellular carcinoma. The microcystin-based nanomedicine demonstrated in this study was more potent than the standard drug, implying that it can be an alternative biocompatible source for the treatment of liver cancer. Optimal standardization of dosage and precise targeting of the nano-sized drug through technology like TACE will lead to optimum activity with negligible side effects. This can then prove to be a significant breakthrough in the field of systemic therapy for cancer treatment. The current study thus opens new avenues in the field of cancer therapy for hepatocellular carcinoma patients.

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CONFLICTS OF INTEREST: The authors hereby declare that they have no conflict of interest.

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