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Green Biosynthesis of Nanoparticles Using Tubipora musica Coral Extracts: Phytochemical Characterization and Biological Applications

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ABSTRACT

Marine corals have been widely appreciated for the wealth of their bioactive compounds, but their medical use calls for a multidisciplinary strategy. In the present study, we attempted to assess the organ pipe coral *Tubipora musica* (*T. musica)* as reservoir of biologically active metabolites and a green synthesis platform for metallic nanoparticles. In-order to have a better understanding of the chemical profile of coral, both alcoholic and aqueous extracts were prepared. These were further analyzed using HPLC and GC-MS for the identification of active organic components, whereas baseline measures of antioxidant potential were also determined. Surprisingly, although in our primary bio-screening the extracts did not evidence any selective cytotoxic activity against selected cancer cell lines, the ethanolic extract showed a very high efficiency towards as an antioxidant. Leveraging this rich organic profile, we subsequently utilized the ethanolic fraction to facilitate the biosynthesis of silver nanoparticles (AgNPs). The synthesized AgNPs were comprehensively characterized with respect to their structural, morphological, and chemical properties using Scanning Electron Microscopy (SEM), Energy Dispersive X-ray (EDX) spectroscopy, Zeta potential analysis, and Infrared (IR) spectroscopy. This successful eco-friendly synthesis underscores the potential of the underutilized *T.musica* and its rightful place within modern biomedical nanotechnology.

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INTRODUCTION

The Organ Pipe Coral (*Tubipora musica*) forms an unusual kind of alcyonarian. From a structural viewpoint, the reason behind its high interest is its intricate skeleton, which mainly consists of calcite [1], this structure is a set of vertical tubes connected by largely porous transverse platforms. In consequence of this free column formation, the coral has a large internal channel system. This, of course allows the constant flow of water and exchange of nutrients the coral needs to survive in unfriendly aquariums. Recent surveys in the Long Reef area have found one such unusual anomaly: a high-energy zone with a large proportion of *Tubipora* colonies.

This has drawn much more attention for these natural pigments to be explored as novel therapeutic agent in drug discovery due to their

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significantly potent anti-inflammatory, antioxidant and neurochemistry effects [2,3]. There is a catch, however: Bringing these marine metabolites to the clinic is extremely difficult. However, this physical limitation has often restricted their pharmacological application by noticing poor water solubility and chemical stability. And that's exactly where nanotechnology comes in [4-6].

Nanostructured delivery systems can significantly improve the bioavailability of these pesky marine components. Even more crucial in the context of as neuroprotective applications, these therapeutics past some of the most stubborn biological bastions that put up a fight, much like the blood-brain barrier (BBB) via use through these nano-delivery systems [7-9]. In a published study, a stable spheric nano-echinochrome obtained directly from *T. musica* [8]. This subset of NPs showcased strong antibacterial and antioxidant properties, as well as significant potential to combat Alzheimer's disease. That kind of clinical validation is vital. In concert with a few decades of research into the basic biochemistry and ecology of marine invertebrates. it specifically lays the groundwork for *T. musica* as a sustainable biological resource for new nanotherapeutics. Ultimately, this makes for an acceptable and rational starting place for the current study [10-15]. Metals that are reduced to nanoscale sizes are typically used in the synthesis of organic-inorganic catalysts, both homogeneous and heterogeneous, and are highly valuable in organic synthesis [16-19].

MATERIALS AND METHODS

Materials

In this study, we used the skeleton of the organ pipe coral (*T. musica*) for characterization of structural and micromechanical properties. A new specimen of this fungus isolated from a complete herbal market Makkah/Saudi Arabia

Upon arrival at the Faculty of Pharmacy laboratories at our university, the coral was rinsed multiple times with deionized water, grounded mechanically, before further steps taken for extraction. All reagents requiring high purity level were purchased and used in subsequent chemical investigations and syntheses in anhydrous form such as Copper sulfate (Chempur, clean method), silver nitrate (Chempur, clean method), methanol, DPPH and chloroform, all purchased from Sigma-Aldrich (Hamburg, proper address).

Methods

Extraction and Isolation procedure

Based on some initial screening and the previously optimized protocol described by [20] we developed a focused extraction method to enhance the recovery of our active metabolites. In short, 25 g of powdered *T. musica* was solubilized in 250 mL of a 80:20 (v/v) ethanol-water solution. This mixture was stirred for 6 hours to permit complete extraction as show in (Fig. 1). This was followed by the filtering of the crude extract and its subsequent evaporation, under vacuum, taking care of the temperature using a rotary evaporator.

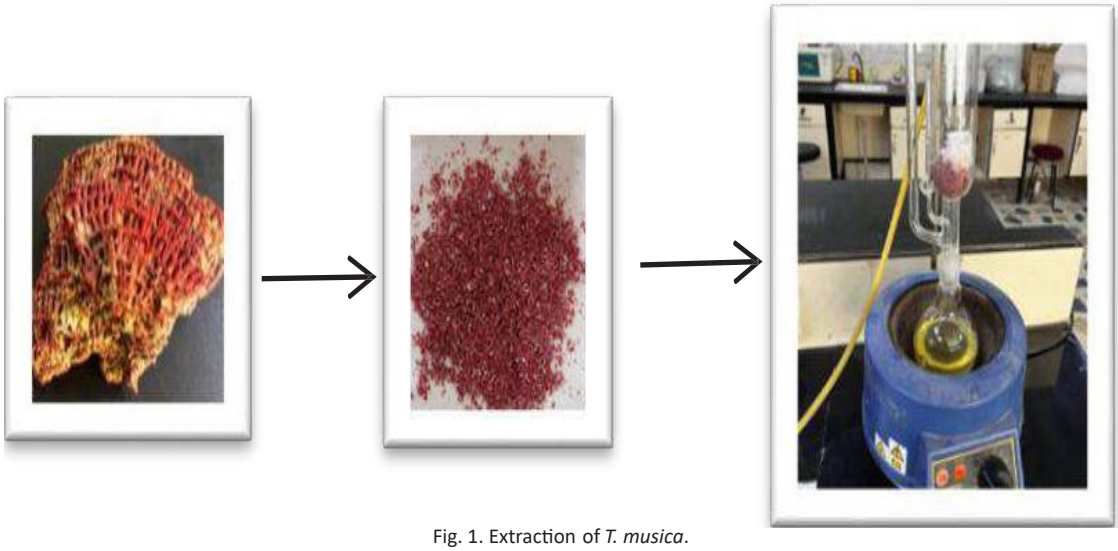

Fig. 1. Extraction of *T. musica*.

To protect likely sensitive biochromes from light and heat, the resulting dried extract was transferred to stoppered, amber-colored bottles and held at 4 °C for subsequent downstream assays.

Phytochemical screening of the extract

The extracts were also screened for the presence of active compounds such as alkaloids, tannins, saponins, flavonoids, phenolic compounds and terpenoids using standard phytochemical methods [21].

Laboratory Tests

In regards to alkaloids (Mayer's Test) [22], to 1 ml of the extract was added 2 ml of mayer's reagent, dull white precipitate showed the presence of alkaloids. Flavonoids test was conducted following [23], while for NaOH test, an extract (1 ml) was treated with aq. NaOH then HCL and the development of yellow orange color observed, which disappeared on addition of HCL. In terms of Ferric chloride test, 1 ml of extract treated with 10% aqueous ferric chloride and watched for the development of deep blue or black color at room temperature. In addition, the test for Saponins [24], 2 ml of extract was shaken with 2 ml of distilled water. Shaked well in a graduated cylinder during 15min Appearance of 1cm high dense foam indicates the presence of Saponins. For the test for Tannins, 2 ml of 5% ferric chloride was added to the 1ml of extract. Tannins were detected by the formation of a dark blue or blackish green color. In regards to the test for Terpenoids (Salkowski Test), 2 ml $CHCl_3$ to 0.5 g of the extract, Concentrated H_2SO_4 (3 ml) was added slowly and formed a layer. Terpenoids are present when a red-brown color is developed at the junction.

Antioxidant Activities the DPPH method

The antioxidant capacity of the extracts was determined by the scavenging capacity of the free radical 1,1 diphenyle-2-picrylhydrazil (DPPH) in accordance with the method described by Blois with slight modifications. In summary, 0.0394 g DPPH was melted in a 70% ethanol aqueous system (100 mL), and the solution is referred to as the DPPH solution. A 0.01 mL sample of Tm extract at various concentrations (250, 500 and 750 PPM) was combined with 0.19 mL DPPH solution and placed in the dark for ten minutes at room temperature. Absorbance was measured at 517 nm using a spectrophotometer [25]. Ascorbic acid was used as a positive control. All the experiments were conducted in triplicate [26]. The DPPH reduction percentage was determined by the formula:

$$\text{Scavenging Activity } (\%) = \left(\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}}\right) \times 100$$

In which $A_{control}$ is the absorbance of DPPH solution without extract and A_{sample} is the absorbance of test sample [27].

Revised GC-MS Methodology

Table 1. Phytochemical Screening of Ethanol Extract of *T. musica*.

Phytochemical Constituent	Test Performed	Observation / Result	Presence (+/-)
Alkaloids	Mayer's test	Formation of a dull white precipitate	+
Flavonoids	aline reagent test (NaOH)	ıge color that fades with HCl	+
Tannins	Ferric chloride test	No dark blue or greenish-black color	-
Terpenoids	Salkowski test	No reddish-brown ring formation	-
Saponins	Frothing test	No persistent foam observed	-

Gas chromatography-mass spectrometry (GC-MS) of *T. musica* extracts. The GC-MS analysis of chemical compounds was performed in accordance to the standardized method described by Hazim et al [28]. The both extracts, aqueous and ethanolic before being analyzed were dissolved in ethanol. Analysis was performed at the Central Lab of Kashan University using HP 6890 gas chromatograph coupled to 5973N mass spectra (Agilent Technologies, USA). We used an HP-5MS capillary column (30 m length × 0.25 mm, 0.25 µm film thickness) coated with a 5% phenyl methyl siloxane stationary phase for the chromatographic separation. The injection samples of 1 µL were injected at the port temperature of 250 °C. The oven profile included an initial holding time of 4min at 40 °C, followed by a constant increasing rate of temperature: 5 °C/min up to remain at 250 °C for additional 10 min. Mass spectrometry was performed at 70 eV in electron ionization (EI) mode. Samples in the m/z range (0-500) were collected over a one-hour run. Eventually, the list of components was verified by quadrupole detection on retention times (RT), normal or reverse standard indexes (SI and RSI) together with characteristic fragmentation ions."

Characterization and Purification using High-Performance Liquid Chromatography (HPLC)

Identification and purification of the active compounds was performed using high-performance liquid chromatography (HPLC). An exact 0.45 µm syringe filter was used to remove any particulates due to solubilization of the sample extracts before injection into the column. Chromatographic separations were conducted on a Shimadzu HPLC equipped with a Phenomenex C18 analytical column (250 × 4.6 mm, 100 Å pore size) in Japan. The mobile phase was a mixture of water and methanol in 80:20 ratio (v/v). After filtration and degassing with a sonicator to ensure optimal column performance and baseline stability, the solvent mixture was used. Isocratic operation of the system was achieved at a flow rate of 1.0 mL/min, with UV detection monitored at an absorbance wavelength of 280 nm. The prepared sample in a volume of 20 µl was injected per run. These compounds of interest present in the extracts were finally identified by comparing their chromatographic retention times (SR_TS) with those of relevant reference standards run under similar operating conditions [29].

Cytotoxicity Assay MTT

The cytotoxicity assay was performed with human DU145 prostate cancer cell line (ATCC), maintained in RPMI 1640 with 10% FBS and 1% penicillin-streptomycin. The dried plant extract was prepared as stock solution (PS) in vitro in water. The stock was then diluted in medium to

Table 2. DPPH radical scavenging activity (% Inhibition) of *Tubipora musica* extracts at different concentrations.

Extract / Standard	Concentration (ppm)	Avenging Activity (% Inhibition)
Alcoholic Extract	250	54.0 %
	500	58.0 %
	750	60.0 %
Aqueous Extract	250	33.0 %
	500	28.3 %
	750	29.3 %
Ascorbic acid (Standard)	23	81.96 %

create a maximal test concentration of 100 µg/ml. Serial two-fold dilutions were made from here to achieve a concentration range of 1.56-100 µg/mL including an untreated negative control consisting of medium only. For the cell viability assay, cells were seeded at a density of 8,000 cells per well in sterile 96-well plates (SPL, China). The spent medium was aspirated after 24h incubation at 37°C in a humidified 5% CO_2 atmosphere, to allow for cell attachment. After 24 h, the culture medium was replaced with 100 µL of extract containing media at selected concentrations for another 72 h. After the incubation, medium was removed. Then 20 µL MTT reagent (1 mg/mL in PBS; Merck, Germany) was added into each well, and the plates were put back into incubator for another 3 or 4 hours. The unreacted MTT solution was aspirated, and the resulting insoluble formazan crystals were solubilized in 100 µl DMSO [30]. The final absorbance was recorded at 570 nm on a plate reader (BioRad, USA).

Synthesis of Silver Nanoparticles

The silver nanoparticles were synthesized by the slow drop-adding 5 mL of the TM extract to a 45 mL of aqueous solution containing AgNO3 (10^{-3} M). The stirring was continued for 10 min at 50-60 °C. On addition of the extract, the color of the solution changed from clear to orange brown within short time [31]. This optical change is notable for the excitation of the surface plasmon resonance (SPR), which act as main evidence to confirm that Ag+ was reduced to Ag NPs successfully. The nanoparticle suspension was centrifuged at 5500 rpm for 15 min, post synthesis. The pellet obtained was then re-dispersed in deionized water to wash the nanoparticles and eliminate unreacted precursor-ions and surplus biological capping agents [32].

RESULTS AND DISCUSSION

Phytochemical Examination of the Extract

Standard qualitative methods were used for the determination of the phytochemical profile of the ethanolic extract of *T. musica* to identify its major secondary metabolites. In the screening of active phytochemicals in extract showed its selective presence, for example extraction of Mayer's reagent gave characteristic dull white precipitate indicating presence of alkaloids. Similarly for flavonoids a strong positive correlation was found; the formation of intense yellow-oragne coloration upon addition of aqueous sodium hydroxide were recorded (immediate, but disappeared following treatment with HCl). In contrast, a number of other classes of metabolites were not detected. No persistent frothing occurred with the extract, demonstrating no saponins are present. Furthermore, a characteristic dark blue or greenish-black color reaction for tannins with ferric chloride and reddish-brown interface for terpenoids (Salkowski test) were not assessed either, since these would also identify their

Table 3. Chemical Composition of Alcoholic and eques Extract.

Tab Extract Type	Compound Name	RT (min)	Area (%)	Formula	M.Wt	Quality (SI)
	Octadec-9-enoic acid	47.541	2.65	$C_{18}H_{34}O_2$	282.46	90
Alcoholic	1-Octadecene	60.394	31.73	$C_{18}H_{36}$	252.48	90
	1-Docosene	60.937	3.08	$C_{22}H_{44}$	308.58	91
	3-Methylhenicosane	58.257	3.32	$C_{22}H_{46}$	310.6	90
Aqueous	1-Docosene	60.617	2	$C_{22}H_{44}$	308.58	92
	1-Octadecene	61.303	10.53	$C_{18}H_{36}$	252.48	91

anticipated treatment in the ethanolic extract (Table 1) [33].

Antioxidant Activities the DPPH method

DPPH is a stable free radical that captures an electron or hydrogen radical to generate a stable diamagnetic molecule. The scavenging of the DPPH radical is determined by a decrease in absorbance at 517 nm due to antioxidants. According to (Table 2), both extracts could reduce the stable DPPH radical. Results obtained reflected a concentration-dependent increase in the free radical scavenging activity of the alcoholic extract, as it rose significantly from 54.0% at 250 ppm to 60.0% (at)750 ppm In contrast, lower and variable antioxidant potentials were observed for the aqueous extract (33.0% at 250 ppm; 29.3% at 750 ppm).It can be determined from the outcomes that standard Ascorbic acid at a very low concentration (23 ppm) showed very high radical scavenging activity of 81.96% compared to both extracts at its saturation concentration. Indeed, the alcoholic extract presented a much higher antioxidant potential than the aqueous extract at all tested concentrations. This potent antioxidant and electron-donating capacity of alcoholic extract ably supports its consideration as an efficient bio-reducing agent for nanoparticles

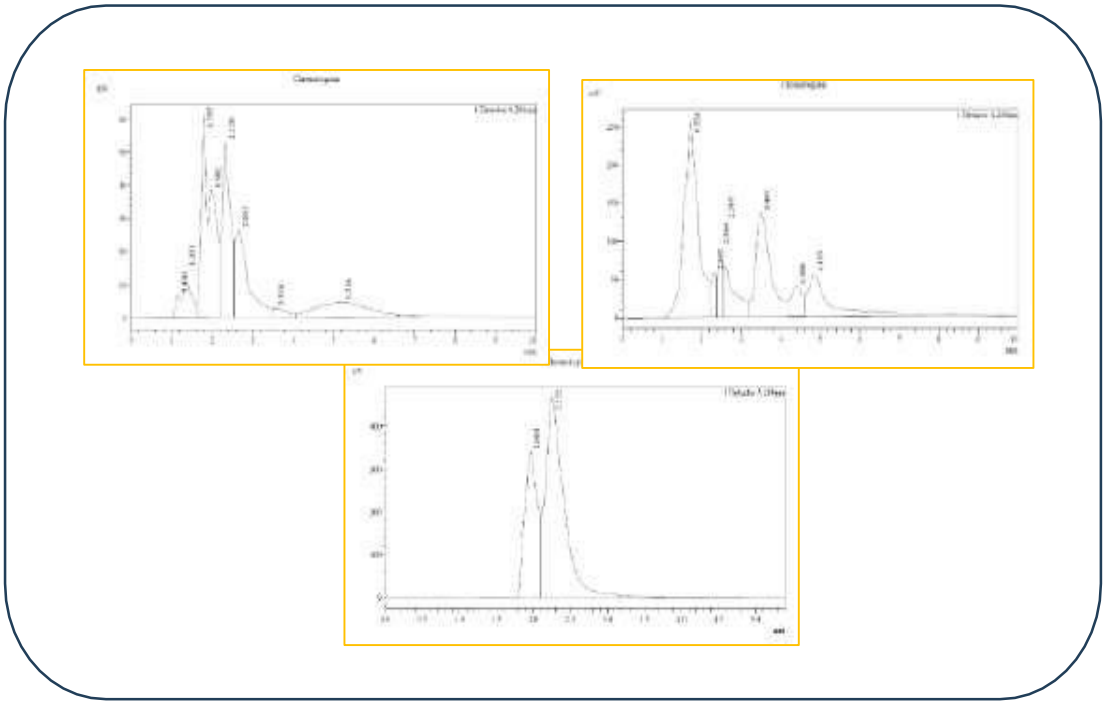

Fig. 2. Hplc for Tm, ethanolic and eques extract Cytotoxicity.

Table 4. Comparative HPLC Data for Rutin Identification in *T. musica*.

(Sample)	(RT)	(Area)	(Identification)
Rutin Standard	2.252 min	8,124,732	Standard Peak
Alcoholic Extract	2.330 min	1,257,597	Rutin Confirmed
Aqueous Extract	2.325 min	485,152	Rutin Confirmed

biogenic synthesis in the next stages [34].

Gas Chromatography-Mass Spectrometry (GC-MS)

Gas Chromatography-Mass Spectrometry (GC-MS) Investigation: In order to clarify further the chemical nature that may be responsible for bio-reduction process, extracts of *T. musica* were analyzed by GC-MS. The only major bioactive compounds of high-quality library match (SI ≥ 90) were accounted for. The alcoholic and aqueous extracts showed a specific profile with predominance of long-chain aliphatic hydrocarbons and fatty acids (Table 3). It is interesting that 1-Octadecene was similarly one of the most abundant compounds in alcoholic (31.73%) and aqueous extracts (10.53%) together with other significant long-chain alkenes like 1-Docosene Additionally, Octadec-9-enoic acid (Oleic acid), a common unsaturated fatty acid, was also identified from this alcoholic extract.

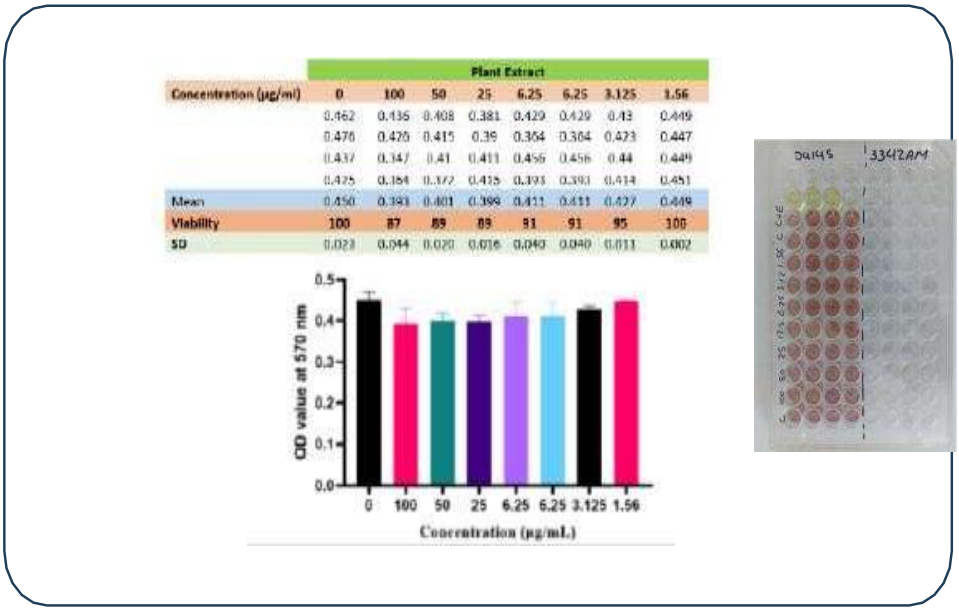

Fig. 3. Effect of *T. musica* coral extract on DU145 cell viability.

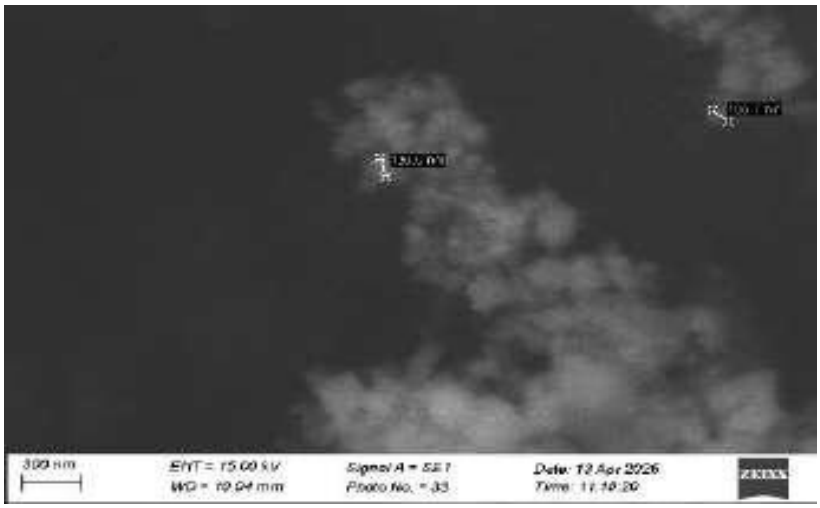

Fig. 4. The SEM micrographs AgNPs.

The importance of this chemical profile resides exactly in its use for green nanotechnology. Long-chain hydrocarbons and fatty acids [9] (e.g. Octadecenoic acid) are well-established as powerful cFapping and stabilizing agents during the biogenic synthesis of metal nanoparticles; this is broadly documented in literature. While their long hydrophobic carbon chains are used to prevent nanoparticle agglomeration, their functional groups have the capacity to reduce metal ions effectively. The success of the extracts as a platform for stable biosynthesis of nanoparticles and their observed biological activities are highly justified by this complex lipid and hydrocarbon defense system of *T. musica*.

HPLC

High-Performance Liquid chromatography (HPLC) among other biological characterization revealed Rutin as the major phenolic constituent in both extracts of the marine coral *T. musica*. Identification of the compound was achieved via the matching of retention times (RT) to that of standard, which showed a main peak at 2.252 min. Comparative data interpretation of Table 4 indicated that the solvent system significantly contributed to the extraction yield; alkaline, alcoholic extract was found to have maximum compound relative abundance (1,257,597 peak area, 39.79% of overall separated constituents), followed by a relatively low value for advective aqueous extract (485,152) as shown in Fig. 2. The molecular composition of Rutin as a flavonoid glycoside grants it this chemical behavior, possessing a greater affinity to the polar organic solvent ethanol than that of pure water. This justifies the choice of alcohol as a better solvent for extracting these bioactive metabolites [35], where both extracts exhibited similar chromatographic fingerprint at λ 280 nm.

MTT

Evaluation of Cytotoxicity and Biocompatibility: In vitro cytotoxicity studies were performed to assess the safety profile and biocompatibility of *T. musica* extract. Importantly, no cytotoxicity was observed in the DU145 cell line at all concentrations of the coral extract (1.56 – 100~\ mu g/ml) tested and the viability remained above 85% (95th percentile) for each concentration as shown in (Fig. 3) [36,37]. Such striking non-toxicity underscores the exceptional biocompatibility of the extract. Scientific Conclusion: When the great safety margin described here is combined with the GC-MS results that confirmed stable long-chain aliphatic hydrocarbons and bioactive fatty acids, it becomes definitively clear that *T. musica* constitutes a unique non-toxic biological reservoir. This high biocompatibility rate represents a requirement for the application of this coral extract under nanotechnological approaches, supporting in high mean and with tight confidence intervals the assertion that it constitutes a bio-reducing and capping reagent with higher safety

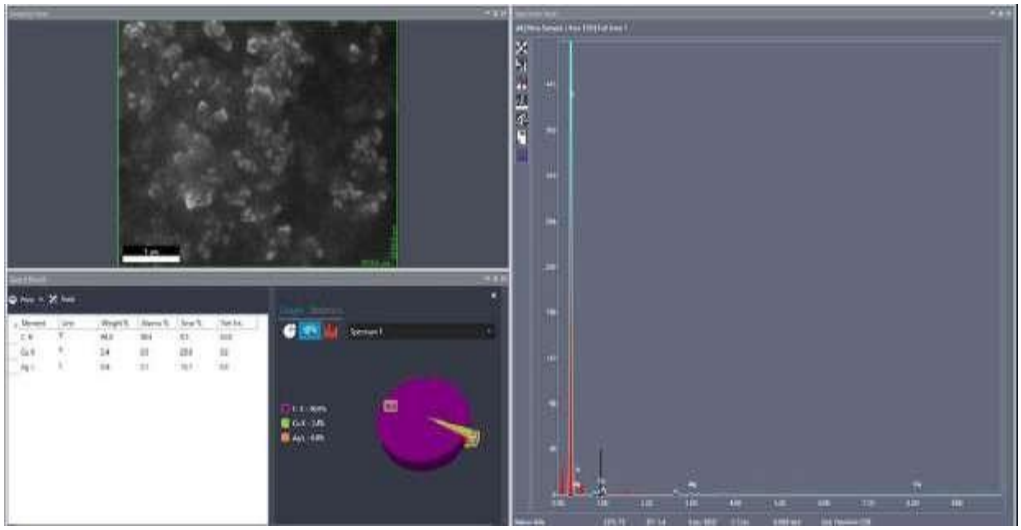

Fig. 5. the EDX spectrum AgNPs.

for its green synthesis toward sensitive biomedical and therapeutic applications.

Characterization of Synthesized Silver Nanoparticles (AgNPs) FTIR Analysis

The comparison of FTIR spectra of the coral extract and synthesized AgNPs confirmed the role of *T. musica* extract as a reducing and stabilizing agent; The broad peak at 3371 cm^{-1} in the spectrum of the extract represents O-H stretching (of phenols) while C=O stretching appears around 1633 cm^{-1}. A remarkable change and reduction in the peak intensity was noticed in AgNPs spectrum which reflects that these functional moieties were engaged in reducing Ag^{+} ions to elemental silver and attaching a stabilizing bio-capping layer around the nanoparticles.

SEM and EDX Analysis

SEM and EDX were utilized to continuously study the morphological characteristics and elemental composition. The alginate and silver nanoparticles (AgNPs) were characterized using scanning electron microscopy (SEM) which indicated that AgNPs having nearly semi-spherical shape with a size varies between 100–120 nm for metallic core as shown in the (Fig. 4). In addition, the clear detection of silver (Ag) peak at 3 keV weight percentage of 18.7% was confirmed from EDX spectrum (Fig. 5), further confirming successful synthesis. Secondly, the peaks of carbon and oxygen in the spectrum suggested for an organic shell surrounding the AgNPs [38-40].

The size of the hydrodynamic diameter of AgNPs was determined by DLS to be ~400 nm. The SEM results are lower than this value due to the hydrated bio-capping layer surrounding the particles in saturate aqueous medium. The bimodal distribution with a Polydispersity Index (PDI) of 0.4 were indicative of the intrinsic size diversity associated with green synthesis during major maturation steps between synthesis stages. The Zeta potential content was equal to 0.4mV. The particles showed great colloidal stability, despite being near zero and it is shown that this is possible because the only thing maintaining colloidal stability during aggregate growth is not electrostatic repulsion but "Steric Stabilization" supplied by dense organic capping [41-43].

CONCLUSION

This study demonstrates that the marine coral *T. musica* is a prolific source of stable hydrocarbons and rare terpenes like Pseudocordatolide with high chemical stability and biocompatibility. Complementing the identification of alkene and fatty acid rich extracts obtained during the GC-MS experiment with cytotoxicity assessments, this organism emerges as a strong candidate for pharmaceutical purposes where bioactive compounds with high safety margins are essential. This suggests it could have an application as an anti-inflammatory agent, or in tissue regeneration processes.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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