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Research article

Exploring targeted apoptosis in huh-7 human liver cancer cell line through *Mentha Officinalis*-mediated gold nanoparticles: an in vitro study

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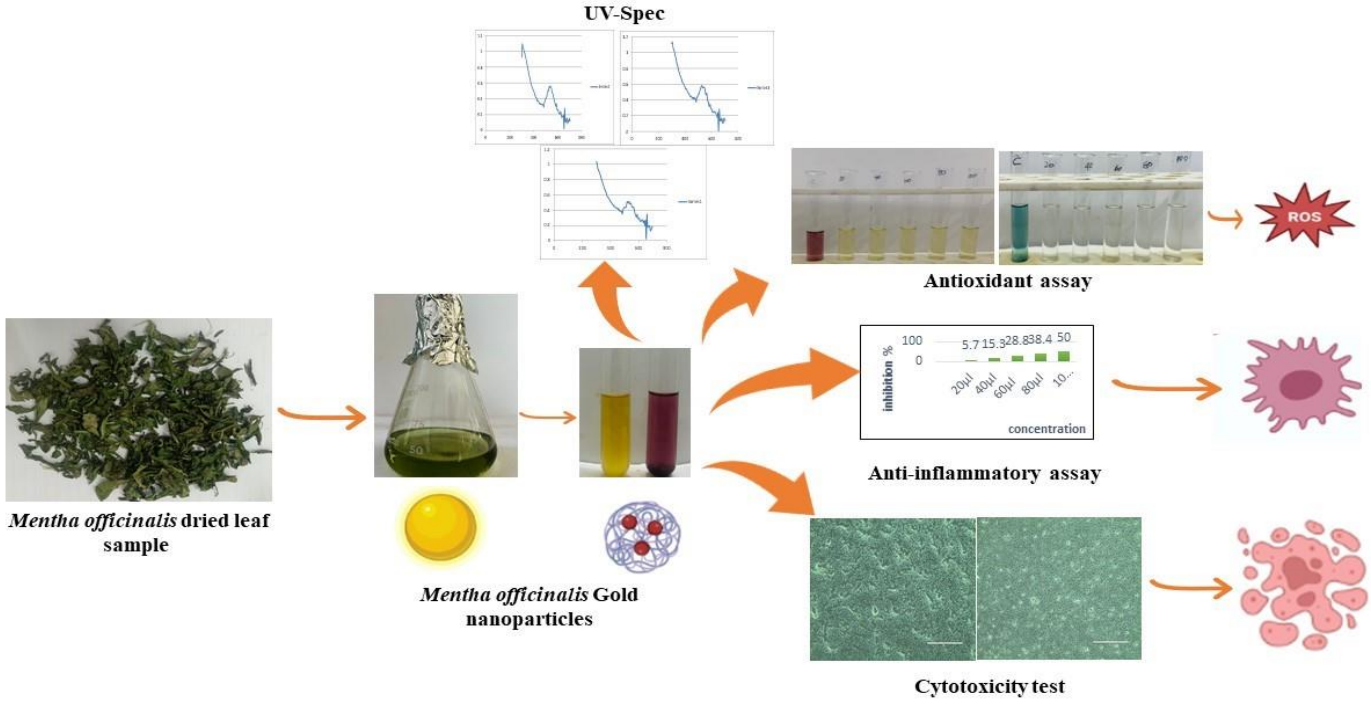
ABSTRACT

Functionalized gold nanoparticles (AuNPs) have emerged as promising tools for targeted cancer therapy due to their unique physicochemical properties and tunable surface chemistry. Research has highlighted hepatocellular carcinoma (HCC) as a significant global health concern, ranking as the fifth most prevalent cancer and the third leading cause of mortality worldwide. Despite advancements in diagnosis and treatment, effectively combating this disease remains challenging.



Anticancer efficiency of *Mentha officinalis* gold nanoparticles

Therefore, there is a pressing need to explore novel diagnostic and therapeutic approaches. Cancer nanotechnology has emerged as a promising field in medicine, aiming to revolutionize cancer diagnosis and treatment. Gold nanoparticles (GNPs/AuNPs) have garnered considerable attention due to their biocompatibility, ability to undergo surface modifications with various ligands, and exceptional optical properties, offering potential avenues for innovation in cancer management. In the present study, the potential of biosynthesized AuNPs functionalized with *Mentha officinalis* aqueous extract (MO-AuNPs) was explored for selectively inducing apoptosis in human liver cancer cell line (Huh-7). This research delves into various aspects of gold nanoparticles (AuNPs) synthesized using *Mentha officinalis* extract, including synthesis, stability, antioxidant properties, anti-inflammatory effects, cytotoxicity, and apoptosis induction potential. The synthesis of MO-AuNPs is described, mechanistic insights into apoptosis induction by MO-AuNPs are provided.

Keywords: GNPs, AuNPs, Cytotoxicity, *Mentha officinalis*, Apoptosis, Anti-inflammatory.

INTRODUCTION

Cancer is one of the leading causes of morbidity and mortality around the globe and is likely to become the major cause of global death in the coming years. As per World Health Organization (WHO) report, every year there are over 10 and 9 million new cases and deaths from this disease [1]. Cancer treatment aiming at eradicating all cancer cells with minimum off-target effects on other cell types. Most drugs have serious adverse effects due to the lack of target selectivity. To overcome this problem, in recent years, nanotechnology-based drug therapies have been explored and have shown great promise in overcoming resistance, with most nano-based drugs being explored at the clinical level [2]. The unique characteristic of nanoparticles i.e. their high surface to volume ratio enables them to tie, absorb, and convey small biomolecule like DNA, RNA, drugs, proteins, and other molecules to targeted site and thus enhances the efficacy of therapeutic agents [3].

Exosomal nanoparticles are cell-derived nano-sized vesicles in the size range of 30-150 nm formed by the inward in-folding of the cell membrane [4]. Their specific structural and inherent properties are helpful in therapeutics and as biomarkers in diagnostics [5].They can also carry chemotherapeutic agents to the target site minimizing their target ability concerns. Chemo immunotherapy (CIT) is a synergistic approach in which chemotherapy and immunotherapy are utilized to benefit each other [6]. Exosomal nanoparticles (NPs) are essential in delivering CIT agents into tumor tissues. During the last decades, a plethora of nanoparticles have been developed and evaluated and a real hype has been created around their potential application as diagnostic and therapeutic agents. Despite their suggestion as potential diagnostic agents, only a single diagnostic nanoparticle formulation, namely iron oxide nanoparticles, has found its way into clinical routine so far [7]. This fact is primarily due to difficulties in achieving appropriate pharmacokinetic properties and reproducible synthesis of monodispersed nanoparticles. Furthermore, concerns exist about their biodegradation, elimination, and toxicity [8]. The present study aims to evaluate the anticancer effectively of *Mentha officinalis-mediated* gold nanoparticles in human liver cancer cell lines.

MATERIALS AND METHODS

Collection and Processing of *Mentha officinalis* (MO)

The mature leaves of MO were collected from the local agricultural farm located near Thiruporur (Chengalpet, Tamil Nadu). The plant species was identified and authenticated by a botanist Dr. P. T. Devarajan, Presidency College, Chennai, India with Accession number 187872. The leaf samples were then washed with sterile distilled water and air-dried at room temperature (25 ± 2 °C) for three weeks. The dried plant materials were ground into fine powders using a high-speed electrical blender and stored in a desiccator at room temperature until further analysis.

Extraction and Phytochemical Analysis

Aqueous extracts of the dried plant material were prepared by mixing 50 g of powdered plant material into 250 mL of distilled water and incubated at room temperature (25°C) for 48 hrs. The samples were filtered using Whatman No. 1 filter paper, the residues were re-extracted under the same conditions and added to the first filtrates. The aqueous extracts were freeze dried using a Usifroid SMH-45 Lyophilizer (Indiana, USA) Freeze Dryer. The dried residues were kept at 4 °C until further experiments [9].

Synthesis of Gold Nanoparticles

This study used *Mentha officinalis* aqueous leaf extract under ultrasonic radiation at room temperature to create a simple, environmentally friendly process for the green synthesis of bioactive gold nanoparticles (AuNPs) [10]. 5 G of *Mentha officinalis* leaf powder was added into 100 ml of deionized water with ultrasonic radiation for 5 hrs. The *Mentha officinalis* leaf extract was filtered and was stored at 4 °C for further studies. The aqueous extract (5 ml) was combined with 5 ml of 10 mM $HAuCl_4$ to biosynthesize AuNPs under ultrasonic radiation and the pure AuNPs was obtained from the mixture after 30 min of centrifugation at 10, 000 rpm. Following many rounds of washing with deionized water, the precipitates were lyophilized for a duration of 12 hrs. A specific concentration of NaOH was used to bring the extract's pH down to the appropriate levels. A pH meter was used to monitor and determine the pH value of mixture, which did not change noticeably after $HAuCl_4$ was added for the reduction reaction. A visual inspection was conducted to observe the formation of AuNPs and a color transformation from pale yellow to ruby-red in the test samples indicated successful AuNP synthesis.

Characterization of Gold Nanoparticles

Using a UV-VIS spectrophotometer (Perkin Elmer LAMBDA™ 365 UV/Vis Instrument, USA) the spectra of the mixture from 200 to 800 nm were periodically taken to track the synthesis of AuNPs.

Antioxidant Properties of Biosynthesized Gold Nanoparticles

DPPH Assay

The antioxidant activity was determined by adding various concentrations of AuNPs (1 ml) into 1 ml of a 1, 1-diphenyl-2-picrylhydrazine solution (DPPH, 2.5 µg·mL−1), it was shaken vigorously and incubated for 30 min in the dark at room temperature[11]. The addition of antioxidative AuNPs would somewhat quench the absorbance of DPPH at 517 nm in ethanol. From each group of the reaction mixture, the absorption of the negative control group (without AuNPs) was deducted. The DPPH scavenging rate could be obtained by the following equation:

Radical scavenging activity (%) = (Abs Control-Abs Sample / Abs Control) ×100

Where:

The Abs Control refers to the absorbance of the control, Conversely, Abs Sample indicates the absorbance of sample

ABTS Assay Method

The antioxidant activity of the *Mentha officinalis* (MO)-AuNPs through ABTS method was determined by the method described by (Chaves et al., 2020) [12]. ABTS•+ was prepared by oxidizing ABTS with potassium persulfate. A 1:1 (v/v) mixture of ABTS (6 mM) and potassium persulfate (3 mM) was prepared and kept in the dark for 16 hrs. At room temperature.

After that, methanol was added to the mixture to dilute it until the absorbance of the mixture at 734 nm was between 1 and 1.5. MO-AuNPs at four different concentrations viz., 0.1, 0.5, 1, and 2 mg/mL (two replicates per sample and concentration) was added with 3.9 mL of the ABTS•+ dilution added. The absorbance drop was measured with a UV-30 spectrophotometer at 734 nm. The results were represented in milligram equivalents of quercetin per milligram of dry weight, with the blank being made with ABTS•+. The following quercetin concentrations were used to create the calibration line: 0.00062, 0.00125, 0.0025, 0.005, 0.01, and 0.032 mg/mL.

Inhibition of Bovine Albumin Denaturation (BSA) Assay

BSA with a concentration of 1 % was prepared, subsequently, different volumes (20, 40, 60, 80, and 100 µl) of MO-AuNPs samples were added to separate tubes containing BSA. Similar volumes of PBS without the test sample served as controls. The mixture was incubated at 37°C for 15 min. Following incubation, the mixture was heated at 70°C for 10 min. to denature proteins. After cooling, the absorbance of the mixture was measured at 660 nm using a UV-30 spectrophotometer [13].

Assessment of Cytotoxicity

The cell viability assay such as MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay quantifies the ability of a test component to kill a target cell [14]. The cytotoxicity assessment of biosynthesized gold nanoparticles (MO-AuNPs) was done using normal Vero cell lines and HUH-7 human liver cancer cell line. Cultured cells were produced at a concentration of 2000 cells/well and seeded onto 96-well plates with 100 µl/well. Each well was added with diluted MO-AuNP extracts at concentrations of 20, 40, 60, 80 and 100 µg/ml. The cells were incubated for 72 hrs. At 37 °C in a 5% CO_2 incubator. The MTT solution was then added to each well, and the incubator was left to incubate for a further 3 hrs. At 37°C, following the solubilization of the purple formazan crystals in dimethyl sulfoxide, an ELISA plate reader was used to assess the optical density of the well at 570 nm. The cytotoxicity was recorded as the drug concentration causing 50% growth inhibition of the tumor cells (IC_{50} value) using the formula given below in Eq.

For Calculating Cell Viability

Cell viability (%) = (Absorbance of sample/Absorbance of control) X 100. and an inhibition graph was plotted. The images of HT-29 and NIH-3T3 before and after treatment were also assessed by inverted microscope attached to a camera system (Nikon, Eclipse, TS100, ElWD 0.3/OD75).

RESULTS

Samples of MO were taken from their natural environment, and careful drying techniques were used to preserve the integrity of their chemical makeup. Upon phytochemical analysis, the dried *Mentha officinalis* samples unveiled a diverse spectrum of compounds, notably the range of antioxidant-rich phytochemicals. This thorough examination highlights its potential in cancer therapy, emphasizing its array of beneficial constituents.

This aqueous extraction process yielded (23 %) a solution rich in bioactive compounds derived from *Mentha officinalis***.** Subsequent phytochemical analysis of this aqueous extract revealed a diverse array of compounds, including menthol and various antioxidant-rich phytochemicals. These findings further underscore the potential therapeutic value of *Mentha officinalis* in cancer therapy.

Synthesis of Gold Nanoparticles

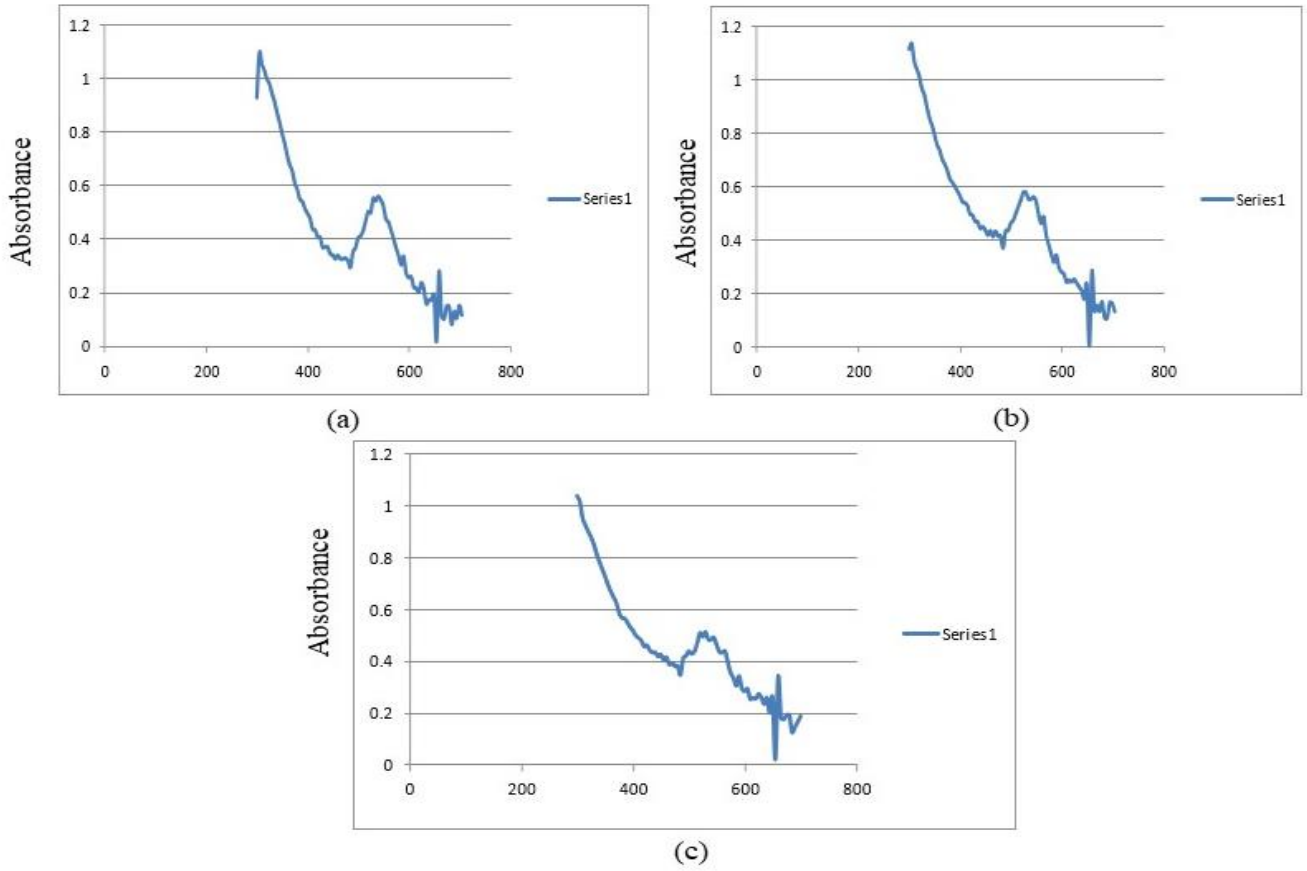
For synthesizing gold nanoparticles, 1 % aqueous plant extract of *M. officinalis* was mixed with $HAuCl_4 \times 4H_2O$ solutions (1000 mg / L of Au) to a final concentration of 200 mg / L. The mixtures were then incubated at 20°C for 24 hrs. After that, a noticeable color change was observed with the post-reaction mixture from the initial pale yellow to a vibrant ruby red. This transformation signifies the successful synthesis of gold nanoparticles (AuNPs) utilizing the *Mentha officinalis* aqueous extract as both a reducing and stabilizing agent [15] [16]. The distinct ruby-red coloration observed is a

hallmark characteristic of colloidal gold nanoparticles, attributed to the excitation of surface plasmon resonance (SPR) within the AuNPs. This phenomenon arises from the reduction of Au (III) ions to Au^0 by phytochemicals inherent in the *M. officinalis* extract, facilitating the formation of AuNPs with unique optical properties and resulting in the observable color shift [17].

UV-Spec Analysis

To assess the longevity of AuNPs, UV-visible spectra were recorded at various intervals post-synthesis. Figure 1 consistently displayed plasmon peaks with maximum absorption wavelengths ranging from approximately 530 to 540 nm across different time points. This unwavering peak position signifies the sustained structural integrity and optical properties of the AuNPs throughout the study duration. Each spectrum had an LSPR (longitudinal plasmon resonance band) that ranged from 520 to 580 nm, supporting the formation of AuNPs and it represents the nanostructures of various shapes, such as triangles, rods, or stars.[18] [19].

Figure 1: UV-Vis absorption spectrum of AuNPs using *M. officinalis*: (a) Stability on 5th day (530 nm - 0.5145); (b) Stability on 6th day (540 nm - 0.5763); (c) Stability on 10th day (530 nm - 0.5145)



Antioxidant Analysis

DPPH Assay

Antioxidants derived from plants may lower a variety of illnesses. The majority of the antioxidant molecules found in a regular diet come from plants, which have a variety of different chemical and physical characteristics [20]. With a distinctive absorbance at 517 nm, DPPH is a persistent free radical molecule that can be used to evaluate the scavenging ability of plants. The antioxidant profile of AuNPs is shown in Figure 2. revealing a considerable dose-dependent scavenging potential of AuNPs at varying concentrations (Table 1). From the results obtained in the present study, the highest scavenging activity was noticed at a concentration of 100 µg/ml. The obtained results are in line with the results obtained by Rauf et al., (2021). [21].

ABTS Assay

The ABTS scavenging activity of the sample was measured by (2, 2-casino-bis-(3-ethylbenzothiazoline-6-sulfonic) acid). It is a widely used compound for determining the total antioxidant capacity (TAC). The AuNP showed the highest ABTS radical scavenging potential at a concentration of 100 µg/ml (Table 1). The antioxidant activity of extracts can be determined using various methods and it is reported to use at least two different methods [22]. In the present study, two methods (Figure 2) were used to determine the antioxidant property of the nanoparticle and both methods presented a significant correlation between them and had high Pearson's correlation coefficient values. This correlation has also been documented in several other research studies [23, 24, and 25].

Figure 2: Determination of antioxidant potential of *M. officinalis* extract mediated AuNPs. (a) DPPH assay; (b) ABTS assay.

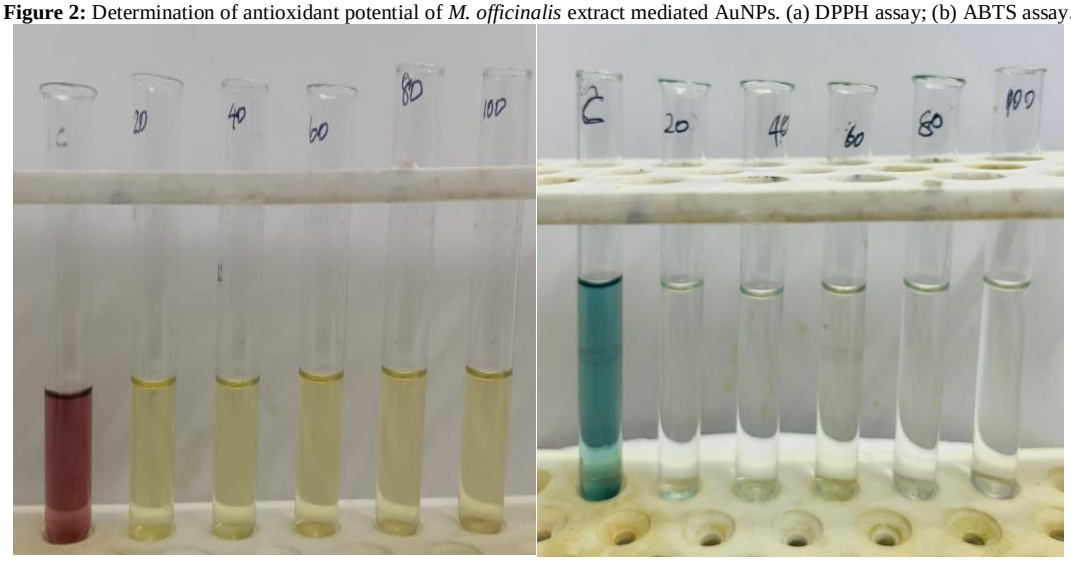


Table 1: Antioxidant potential of *M. officinalis* extract mediated AuNPs

Concentration (µg/ml)	Radical scavenging activity (%) DPPH	Radical scavenging activity (%) ABTS
20	60.8	70.5
40	68.9	78.3
60	75.2	83.5
80	82.4	89.4
100	89.6	93.6
Control	92.3	97.8

BSA (bovine serum albumin) Denaturation Assay

Protein denaturation is used as a marker for inflammation. BSA denaturation assay is a well-recognized and utilized in-vitro protein denaturation assay and is used to assess the anti-inflammatory properties of natural plant extracts. BSA is used to denature proteins at a pH of 6.8. In the present study, the anti-inflammatory properties of AuNPs were determined by subjecting it to inflammation (which is induced by BSA and results in the formation of denatured proteins). A dose-dependent increase in the activity was noticed (Figure 3). The obtained results are similar to that of the results produced by Anyasor et al., (2019) [26].

Table 2: Cytotoxic activity of *M. officinalis* derived AuNPs on Vero cell line

Concentration (µg/ml)	Absorbance		Average	Cell Viability (%)
	I	II		
Control	0.974	0.979	0.9765	100
20	0.963	0.966	0.9645	98.77
40	0.948	0.953	0.9505	97.33
60	0.936	0.941	0.9385	96.10
80	0.921	0.927	0.924	94.62
100	0.905	0.912	0.9085	93.03

Table 3: Cytotoxic activity on Huh-7 cell line

Concentrations (µg/mL)	Absorbance		Average	Cell Viability (%)
	I	II		
Control	0.922	0.915	0.9185	100
20	0.839	0.845	0.842	91.67
40	0.684	0.667	0.6755	73.54
60	0.537	0.529	0.533	58.02
80	0.377	0.383	0.38	41.37
100	0.188	0.195	0.1915	20.84

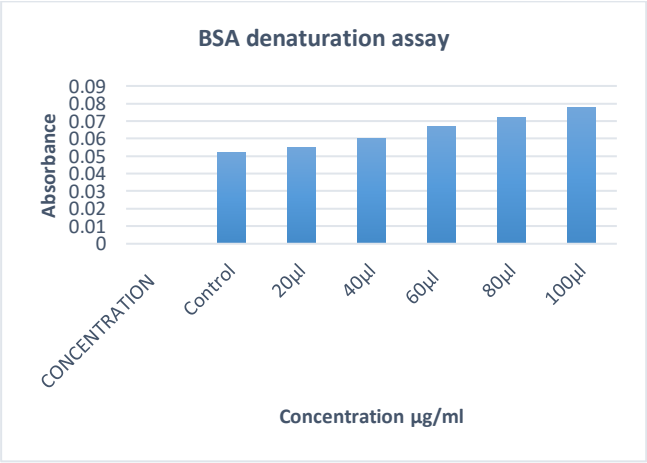


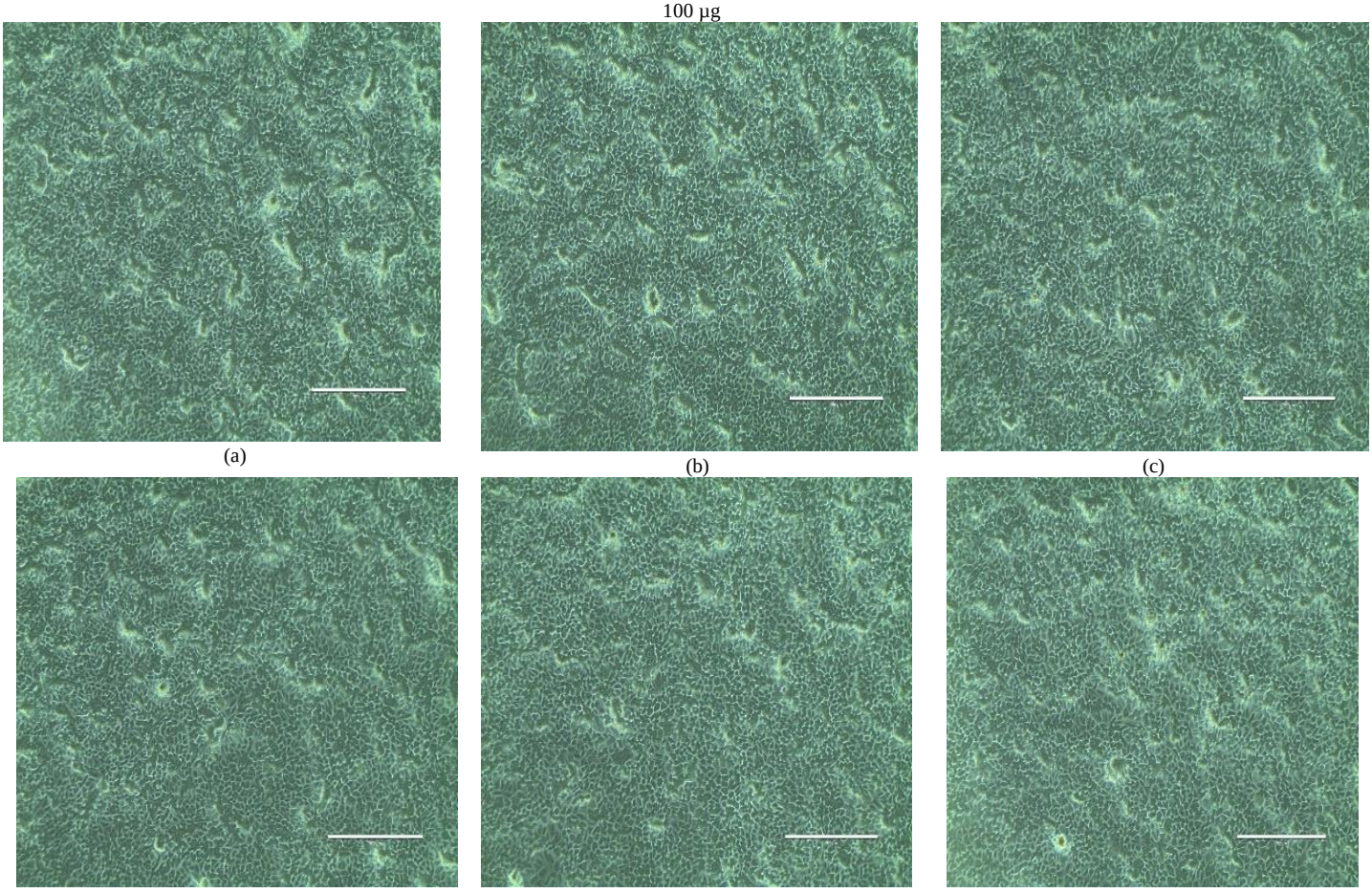
Figure 3. BSA denaturation assay of *M. officinalis* derived AuNPs

MTT Assay

Normal cell line - Vero (African green monkey kidney normal epithelial cell line)

The cytotoxic effect of the sample was tested against Vero cell lines by MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay [27]. The anticancer property of the biosynthesised AuNPs were initially evaluated by subjecting to normal (Vero) cell lines with the doses of AuNPs ranging from 0 to 100 µg/ml for a duration of 24 hrs. The cytotoxicity of AuNPs for Vero cell line was assessed using MTT assay and as depicted in the Figure 4 and Table 2, a significant viability on the normal cell line was exhibited by the synthesized *M. officinalis* derived AuNPs indicating its viability potential. Maximum viability potential was obtained at a concentration of 20 µg/ml of drug exhibiting its comparatively lower percentage of cytotoxicity on the normal cell line (Table 2).

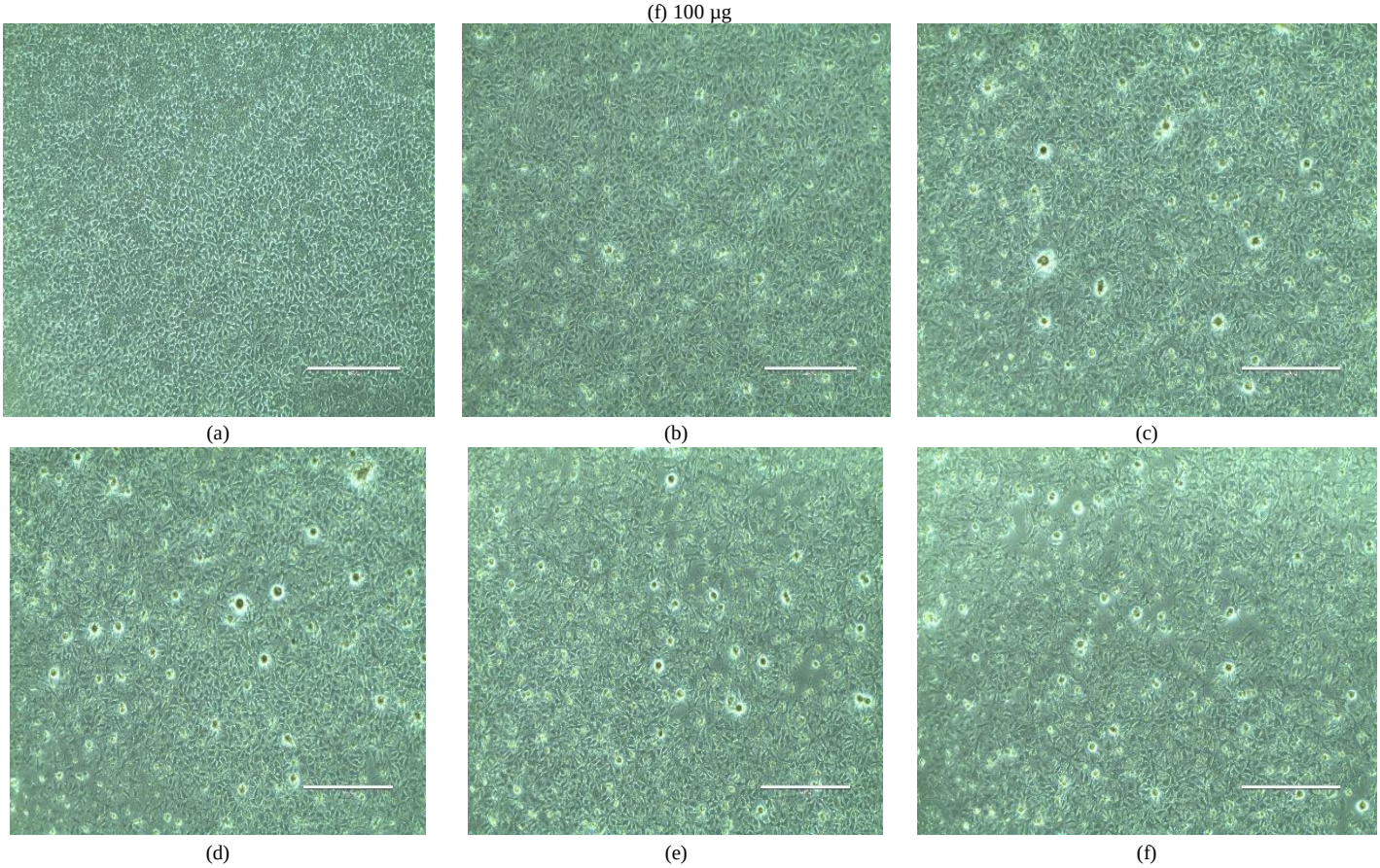
Figure 4: Cell viability study of *M. officinalis* derived AuNPs on Vero cell line (20X magnification - 100µm): (a) Control; (b) 20 µg; (c) 40 µg; (d) 60 µg; (e) 80 µg; (f) 100 µg



(a) (b) (c)

(d) (e) (f)

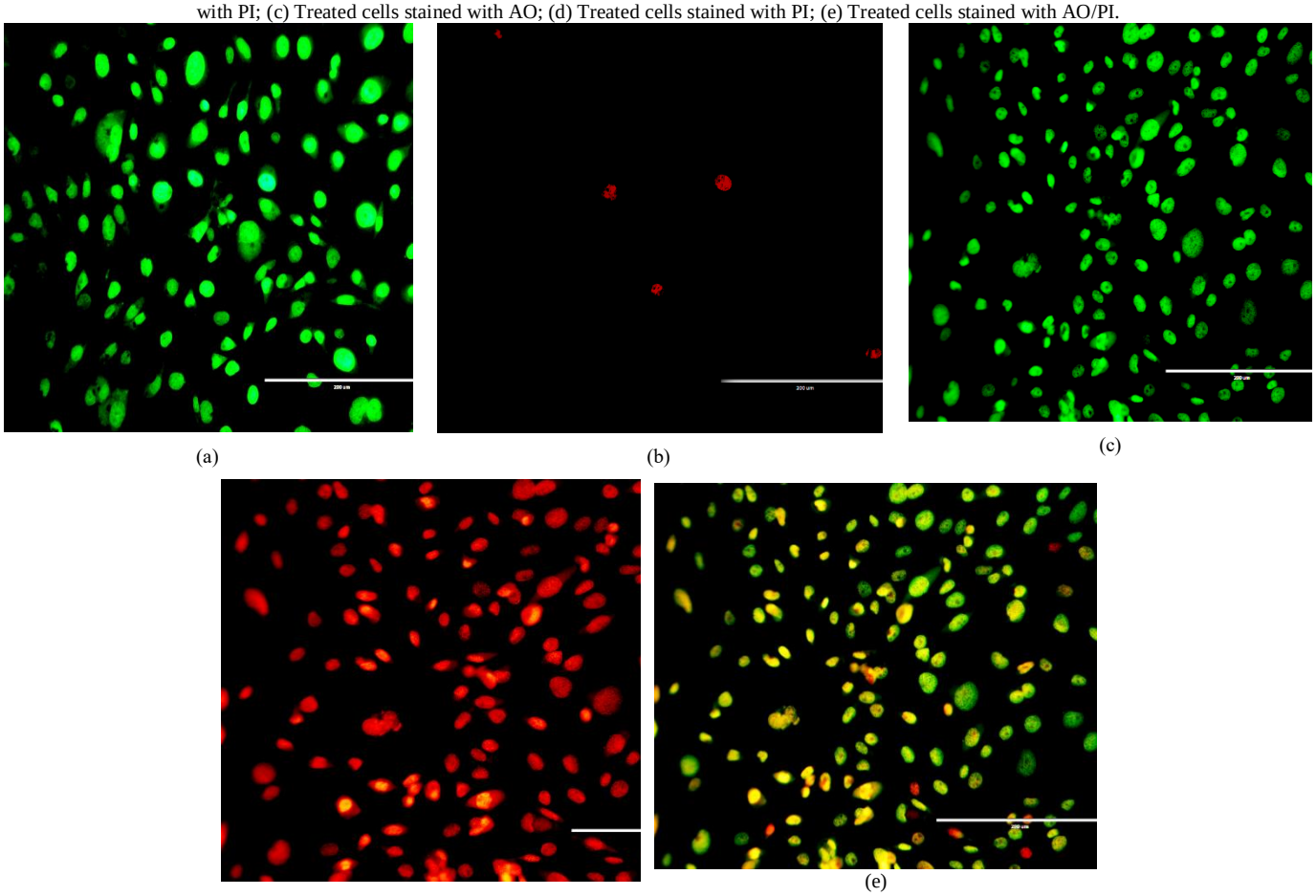
Figure 5: Cell viability study of *M. officinalis* derived AuNPs on Huh-7 cell line (20X magnification - 100µm): (a) Control; (b) 20 µg; (c) 40 µg; (d) 60 µg; (e) 80 µg; (f) 100 µg



(a) (b) (c)

(d) (e) (f)

Figure 6: AO/PI Staining *M. officinalis* derived AuNPs on Huh-7 cell line (20X magnification - 100µm): (a) Control cells stained with AO; (b) Control cells stained with PI; (c) Treated cells stained with AO; (d) Treated cells stained with PI; (e) Treated cells stained with AO/PI.



(a) (b) (c) (d) (e)

Cancer Cell Line - Huh-7 (Human hepatocellular carcinoma epithelial cell line)

The anticancer activities of the biosynthesized AuNPs against the Huh-7 cell line were examined through MTT assay by treating the cancer cell lines to AuNPs at various doses with a range of 0 −100 µg/ml for 24 hrs. As depicted in Figure 5 & Table 3, the cell viability decreased in a dose-dependent manner after exposing the cancer cell lines to AuNPs. The AuNP-treated cell lines exhibited significantly decreased viability with increased doses of AuNPs. Cell viability was significantly decreased to 20.8 % at 100 µg/ml concentration, on the other hand comparatively higher viability 91.6 % was noticed at a concentration of 20 µg/ml indicating the anticancer potential of the *M. officinalis* AuNPs. In the present study, 2.5-fold higher anticancer activity was noticed with the AuNPs synthesized by *M. officinalis* compared to that of the AuNPs synthesized by *M. longifolia* (50.9 % at 100 µg/ml) [28].

CONCLUSION

The *M. officinalis* derived AuNPs displayed a great potential of anticancer activity as confirmed using normal and cancer cell lines. UV-Vis Spec. analysis revealed that the bioactive compounds of *M. officinalis* are attached on to the surface of AuNPs. These results were confirmed by antioxidant, anti-inflammatory and cytotoxic activity of AuNPs produced. AuNPs exhibited antioxidant, anti-inflammatory and considerable cytotoxic activity at low concentrations in Human hepatocellular carcinoma epithelial cell line. Hence, from the obtained results on in vitro studies validate the promising clinical applications of the green synthesized gold nanoparticles in the treatment of cancer.

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