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# Bio- and Phyto-chemical Study on *Nannochloropsis oculata* algal Extract Incorporated with Gold Nanoparticles, *in Vitro* Study

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## ABSTRACT

**Background**: *Nannochloropsis oculata* is rich in various active phyto-constituents have high ability to diminish generation of free radicals. These components have high molecular weights. Therefore, they integrated with nanotechnology to facilitate their absorption and hence increase their bioavailability. The present experiment was designed to evaluate role of gold nanoparticles (Au-NPs) incorporation in ameliorating the biological efficiency of *N. oculata* algal extract. **Materials and Methods**: The ultraviolet-visible (UV-VIS) spectroscopy, X-Ray Diffraction (XRD) spectrum, dynamic light scattering (DLS) measurement and transmission electron microscope (TEM) were used for characterizing the biosynthesized Au-NPs. The major phyto-constituents and the *in vitro* biological activities (total antioxidant, free radicals scavenging, anti-diabetic and anti-Alzheimer's activities) were quantified in *N. oculata* algal extract. The cytotoxic activity against growth and progression of human colon carcinoma (CACO-2) and hepatocellular (HEPG-2) cells was assayed. Also, safety of the algal extract after incorporating Au-NPs was evaluated by determining values of the median lethal dose ($LD_{50}$) and compared with that of the extract itself. **Results**: It was found that the *in vitro* biological efficacy (antioxidant, scavenging, anti-diabetic and anti-Alzheimer's activities) increased in *N. oculata* algal extract after incorporating Au-NPs and this was related to elevating concentrations of the active phyto-constituents. Moreover, it ameliorated the cytotoxic activity against CACO-2 and HEPG-2 cells. Moreover, gold algal nano-extract was orally safer as compared to native algal extract itself. **Conclusion**: Incorporation of Au-NPs enhanced the *in vitro* biological activities of *N. oculata* algal extract.

## 1. Introduction

Microalgae belong to the marine organisms that used as important medicinal agents due to the presence of various phenolics, chlorophyll, carotenoids, phycobiliproteins, polysaccharide, vitamins, amino acids and fatty acids that are categorized as the most common biologically active metabolites in these organisms besides the macro and micro elements (Safafar *et al*., 2015; Talero *et al*., 2015). They have attracted more attention and universal interest in the research area to exploit in pharmaceutical usage due to isolation of these active phyto-constituents that have been developed to produce various pharmaceutical products utilized as antiproliferative, anti-inflammatory, antimalarial, antimicrobial and anticancer agents (Enwereuzoh and Onyeagoro, 2014; Khan *et al*., 2018).

Due to ability of microalgae to grow even under deleterious conditions on a large scale in addition their ability to produce significant amounts of fatty acids, they are provided with high nutritional value. Therefore, they are can be used as functional food ingredients (Adarme-Vega *et al*., 2014).

Only few species of microalgae or their derivatives are approved for being included in the human diet, although they were recommended for prevention of aging related to metabolic problems due to the existence of many beneficial and active constituents. The priority should be given to the species under the genus *Nannochloropsis* among the recommended microalgae strains due to the existence of high concentrations of polyunsaturated fatty acids, antioxidants, carotenoids, polyphenols and vitamins in addition to their suitability for intensive culture (Zanella and Vianello, 2020).

It is well known that the green algae *Nannochloropsis oculata* has been explored for biochemical, nutraceutical and pharmaceutical applications (Abdullah *et al*., 2017; Shah and Abdullah, 2018). It is marine eukaryotic unicellular phytoplankton. It is classified as a small green

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microalga genus belonging to the class "*Eustigmatophyceae*". In aquaculture, it is well known by its potency to produce wide range of the biologically active constituents in addition to omega-3 fatty acids (Hamidi *et al*., 2014; Kent *et al*., 2015). *N. oculata* is rich in high concentrations of proteins and polyunsaturated fatty acids in addition to the antioxidant pigments that possess high ability to inhibit overproduction of the free radicals due to increasing capacity of the antioxidant system (Yanuhar *et al*., 2011; Selvendran, 2013). In 2016, Sanjeewa *et al*. documented that *N. oculata* is characterized by high content of the sterols that might aid in enhancing anti-cancer and anti-inflammatory activity.

Most of the active phyto-constituents are characterized by their high molecular weights. Therefore, they have limited ability to cross the cellular membrane. Consequently, this leads to decreasing their bioavailability and efficacy. Absorption of these biologically active components was accelerated by integrating the medicinal plants that used traditionally with nanotechnology to reduce their toxicity and enhance their bioavailability (Mamillapalli *et al*., 2016; Aboulthana *et al*., 2018).

Nanotechnology is considered as a new strategy belonging to the most promising areas of the modern medical science that focused on increasing the bioavailability and efficacy of medicinal plants (Gunasekaran *et al*., 2014). Shape and size belong to the physical properties that provided to the nanomaterials enabling them to be used in both fundamental and applied research fields (Dhas *et al*., 2012). The inherent stability problem of the plant extracts can be solved by incorporating metal nanoparticles (M-NPs) into polymeric matrices during development of nano-extracts. Existence of M-NPs into plant extracts increased the total phenolic compounds. Therefore, it showed a higher antioxidant and antimicrobial activities at lower concentrations compared to native plant extract solely (Johnson *et al*., 2014; Abdel-Aziz *et al*., 2014; Shousha *et al*., 2019). The scientists have come to be interested in preparation of gold nanoparticles (Au-NPs) due to their special physical and chemical properties in extensive practical areas like drug delivery, catalysis, bio-labeling and packaging applications (Pal *et al*., 2013; Youssef and El-Sayed, 2018). They become an emerging nanomedicine due to their significant properties such as high surface reactivity, resistance to oxidation and plasmon resonance in addition to their biocompatibility. They have received great attention due to their therapeutic potential in treatment of various chronic diseases like rheumatoid arthritis, pathological neo-vascularization and neoplastic disorders. Moreover, they are inactivated easily by precipitation and complexation thus limiting their desired functions in human systems (BarathManiKanth, 2010). The present study was designed to appraise the biological activities (*in vitro*) of the most effective *N. oculata* algal extract after incorporating Au-NPs.

## 2. Materials and Methods

### *2.1. Preparation of Microalgal strain*

*Nannochloropsis oculata* alga (NNO-1 UTEX Culture LB 2164) was cultivated and collected from the Algal Biotechnology Unit, Agricultural and Biology Research Institute, National Research Centre, Dokki, Giza, Egypt. The biochemical composition and concentration of this strain were controlled based on the method reported by Nuno *et al.* (2013). The microalgae were precipitated by centrifugation at 897 *g* and 20 °C for 10 min. The recovered biomass was freeze-dried then stored at - 20 °C until use.

### *2.2. Preparation of Nannochloropsis oculata algal extract*

Based on the *in vitro* biological activities carried out by Aboulthana *et al*. (2018), ethyl acetate was the most suitable solvent for preparing *N. oculata* algal extract to be used for Au-NPs synthesis. After drying the vegetative algal material at 50°C for 72 h, the algal material was crushed into powder and defatted in hexane by cold maceration to remove the lipoidal matter followed consequently by the extraction at room temperature in ethyl acetate then filtered. The ethyl acetate extract was dried at 45°C under vacuum rotary evaporator.

### *2.3. Synthesis of gold nanoparticles (Au-NPs)*

#### *2.3.1. Preparation of gold nanoparticles (Au-NPs)*

The Au-NPs were fabricated *via* chemical reduction method as documented by Zhao *et al*. (2013) through two main parts. Part I was concerned with reducing $Au^{+3}$ ($HAuCl_4$) to $Au^0$ in an aqueous solution by the reaction with trisodium citrate ($Na_3C_6H_5O_7.2H_2O$). Part II was the stabilization using cetyltrimethylammonium bromide (CTAB) in order to avoid aggregation of the particles.

#### *2.3.2. Preparation of gold N. oculata* algal *nano-extract*

The dried algal extract was used in combination with Tween 20 (HLB-16.7) (non-ionic surfactant), cellulose nanocrystal (CNC) and water for preparing nano-emulsion *via* spontaneous emulsification method during two steps. The first step was concerned with fabricating organic phase by mixing algal extract with Tween 20 (the chosen surfactant) at the ratio 1:5 followed by adding 3 gm of CNC then sonicating for 30 min. The second step was concerned with adding organic phase (algal extract, Tween 20 and CNC) to water drop by drop (20 mL/min) using separating funnel followed by stirring the system magnetically for 5 hrs at 800 rpm (60 °C). Consequently, the prepared Au-NPs were added to this nanoemulsion (at the ratio 1%) followed by sonicating the mixture for 30 min at 50 °C.

### *2.4. Characterization of biosynthesized nanoparticles*

Spectrum of the Au-NPs was assayed by Shimadzu UV-VIS recording spectrophotometer UV-240 at wavelength (λ) ranged from 200 to 800nm in the samples diluted 10-fold with deionized water. Size and crystalline nature of the biosynthesized Au-NPs were analyzed by a Philips X-ray diffractometer (XRD) (PW 1930 generator, PW 1820 goniometer) equipped with an X-ray source consisting of Cu Kα radiation (45 kV, 40 mA, with λ =

0.15418 nm). The dynamic light scattering (DLS) (Malvern Zetasizer Nano ZS, Malvern Instruments Ltd., Malvern, United Kingdom) was used for determining the average hydrodynamic size of the biosynthesized Au-NPs according to method suggested by Murdock *et al*. (2008). Size and shape of the biosynthesized Au-NPs were determined using Transmission Electron Microscope (TEM) (model JEM-1230, Japan) operated at accelerating voltage of 120 kV, high resolution level (200 KV), with maximum magnification of $600X10^3$ and a resolution until 0.2 nm.

*2.5. Quantitative determination of the major phyto-constituents*

All active components related to the biological activities were assayed in *N. oculata* algal extract incorporated with Au-NPs then compared with the native extract itself (before Au-NPs incorporation). All analyses were carried out in three replicates.

*2.5.1. Total polyphenolic and tannins content*

Concentration of total polyphenols was determined using Folin Ciocalteu reagent according to method suggested by Singleton and Rossi (1965). Total tannins contents were determined using tannic acid as a reference compound based on the method described by Broadhurst and Jones (1978).

*2.5.2. Fourier Transform Infrared Spectroscopy (FT-IR) analysis*

FT-IR analysis was carried out according to the method documented by Weigel *et al*. (2004) using FT-IR technique manufactured by Bruker. The absorbance and transmittance were estimated against blank then the transmittance percent (Trans. %) and relative intensities (Int. %) were calculated.

*2.6. In vitro biological activities*

*2.6.1. Antioxidant activity*

Total antioxidant capacity (TAC) was assessed as mg gallic acid equivalent per gram dry weight using the method suggested by Prieto *et al*. (1999). Total iron reducing power was measured as µg/mL using ascorbic acid as a reference compound according to the method suggested by Oyaizu (1986).

*2.6.2. Free radicals scavenging activities*

The scavenging activities were assessed by measuring ability of the extract to scavenge the free radicals using spectroscopic method. This activity was assessed against 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) radicals by calculating the median inhibitory concentration ($IC_{50}$) according to the method described by Rahman *et al*. (2015). Moreover, it was assayed against 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radicals by determining percent of the ABTS radical inhibition (%) using ascorbic acid as standard according to the method modified by Arnao *et al*. (2001).

*2.6.3. Anti-diabetic activity*

It was assessed by calculating percent of α-amylase enzyme inhibition (%) using acarbose as standard drug based on the 3.5-dinitrosalicylic acid (DNSA) method suggested by Wickramaratne *et al*. (2016).

*2.6.4. Anti-Alzheimer's activity*

It was determined by calculating percent of acetyl cholinesterase (AChE) enzyme inhibition (%) according to Ellman's method (Ellman *et al*., 1961).

*2.6.5. Cytotoxic activity*

The *in vitro* cytotoxic activities against growth of human colon carcinoma (CACO-2) and hepatocellular (HEPG-2) cells were determined using 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as suggested by Vichai and Kirtikara (2006). Percent of cell-growth inhibition (%) and median inhibitory concentration ($IC_{50}$) were calculated using $IC_{50}$ calculation software.

2.7. Determination of the median lethal dose ($LD_{50}$)

Values of the $LD_{50}$ of *N. oculata* algal extract incorporated with were evaluated and compared with the native extract itself according to the methods described by Paget and Barnes (1964). Seventy-two adult albino mice ranged between 20-25 g body weights were divided into 6 groups (6 mice in each group) for calculating the $LD_{50}$ of *N. oculata* algal extract and 6 groups for that of gold *N. oculata* algal nano-extract. All groups were treated orally by stomach tube with increasing the doses (1000, 2000, 4000, 6000, 8000 and 10000 mg/Kg). After 24 hrs of extract administration, number of dead mice was recorded.

## 3. Results and Discussion

The synthetic substances have undesired (deleterious) side effects. Therefore, it was necessary to find accessible natural products obtained easily from the nature with antioxidant and free radicals scavenging activities (Mahfouz, 2011). Different reducing agents (organic and inorganic) are used for reduction of metal ions in solutions (aqueous and non-aqueous) during biosynthesis of M-NPs (Tran *et al*., 2013; Iravani *et al*., 2014). Toxicity and the non-ecofriendly by-products obtained as a result of utilization of these chemicals might be the reason which leads to biosynthesis of M-NPs *via* the green route that does not employ synthetic (toxic) chemicals (Ahmed *et al*., 2016). By mean of green nanotechnology, the plant extracts used as reducing or capping agents for M-NPs biosynthesis (Lakshmanan *et al*., 2018). It is preferred to use different plant extracts for M-NPs biosynthesis in different shapes and sizes because synthesis of the plant-mediated nanoparticles is environmentally friendly, cost-effective and a single-step method for biosynthesis process in addition to its safety for human therapeutic use (Ahmed and Ikram, 2015; Aboulthana *et al*., 2019).

*3.1. Studying the structural properties of the synthesized Au-NPs*

The UV-VIS spectroscopy is simple, fast, discerning for various M-NPs types and requests simply a short period for measurement. As presented in Fig. 1a, there is a sharp peak identified at 550 nm confirming Au-NPs formation.

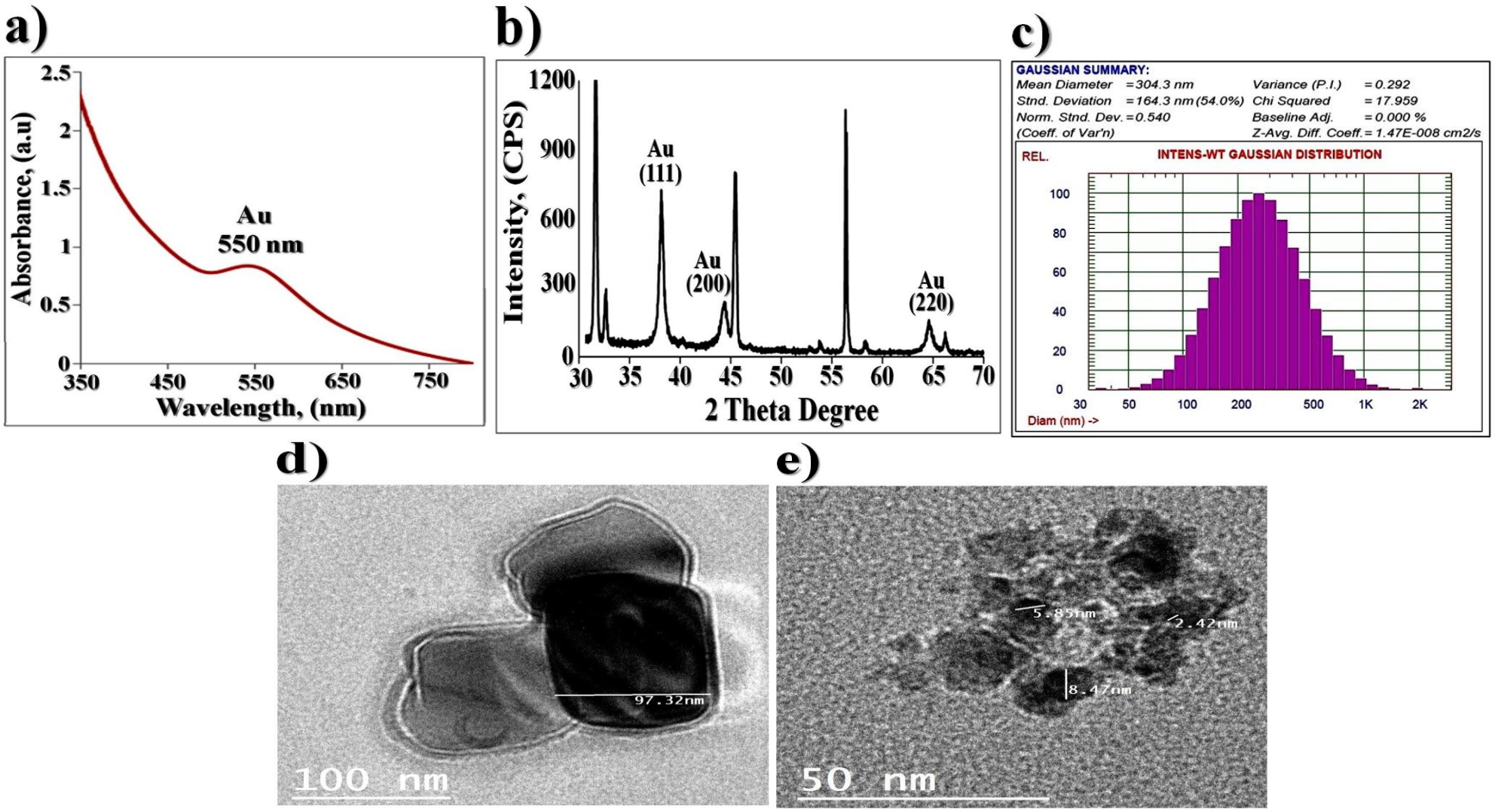


**Fig. 1.** Characterization of the synthesized gold nanoparticles (Au-NPs) showing **a)** Ultraviolet-visible (UV-VIS) spectroscopy, **b)** X-Ray Diffraction (XRD) spectrum, **c)** Dynamic Light Scattering (DLS), **d)** Transmission Electron Microscope (TEM) image of Au-NPs and **e)** TEM image of Au-NPs incorporated into *N. oculata* algal extract.

The XRD and TEM are the most suitable techniques used for studying structural properties of the M-NPs. Biosynthesis of Au-NPs at nano-scale was examined and confirmed by the XRD diffraction pattern that illustrated in Fig. 1b. It was noticed that the fabricated Au-NPs was achieved in the presence of AuCl4- analogous diffraction peaks that are allocated to metallic Au phase with the greatest significant representative peaks at 38°, 43.8° and 65°. This might be attributed to the crystallographic planes (1 1 1), (2 0 0), and (2 2 0), respectively. The peak width of Au-NPs from crystalline plane (1 1 1) and sizes of the Au crystallite were found to be approximately 15 nm for Au-NPs and this agreed with the results obtained by Youssef *et al*. (2014). The average diameter of the M-NPs as well as their size distributions were acquired in aqueous solutions by using DLS that is categorized as a nondestructive technique. The size attained from DLS is frequently larger than that measured by TEM and this might refer to role of Brownian motion. As showed in Fig. 1c, it was noticed that the main diameter of particle size distribution of the fabricated Au-NPs was around 200 nm. The TEM was used to examine morphology of the biosynthesized Au-NPs for determining their shapes and sizes from diameter of whole particles on TEM images. It was found that average Au width was ranged from 2 to 10 nm (not exceeding 100 nm) associated with very little particles of higher and lower size distribution when it is incorporated into *N. oculata* algal extract (Fig. 1d). Furthermore, data of the TEM displayed that Au-NPs exist spherical or round in their shapes as shown in Fig. 1e.

As obtained by El-Feky *et al.* (2017) and subsequently supported by Aboulthana *et al*. (2018), it was found that phenolics, chlorophylls and carotenoids exist with the highest concentrations in *N. oculata* algal (ethyl acetate) extract. Furthermore, flavonoids, pyrogallol and catechine were identified in addition to four phenolic compounds (cinnamic acid, p-coumaric acid, p-hydroxybenzoic acid and gallic acid) by the chromatographic technique. These phenolic compounds showed their antioxidant efficiency through hydrogen atom transfer and / or through single electron transfer (Goiris *et al*., 2012). Also, presence of the different vitamins in addition to α-tocopherol and carotenes (the most abundant fat soluble compounds) play an effective role in decreasing check degenerative diseases and the deleterious effects induced as a result of action of free radicals (Jacab and Sotoudeh, 2002; Della-Penna and Pogson, 2006). The α-tocopherol showed its scavenging activity through the ascorbate-glutathione cycle by combining with singlet oxygen to form the tocopheroxyl radicals that consequently reduced back to α-tocopherol in the presence of ascorbate (Pinheiro-Sant'ana *et al*., 2011). Therefore, it possesses high concentrations of the polyphenolic biomolecules that exert strong antioxidant capacity facilitating reduction of gold ions $Au^{+3}$ to $Au^{0}$ and hence, it is considered a good choice for green synthesis of Au-NPs.

### *3.2. Quantitative determination of the major phyto-constituents*

It was reported that the scavenging activities of the natural extracts against reactive (oxygen and nitrogen) species are related to increasing concentrations of the phenolic compounds (the most common active phyto-constituents) that used as reducing agents due to their ability to act as hydrogen donors (Kalim and Nikalje, 2017). It is well known that the polyphenolic compounds especially tannins are the major phyto-constituents in *N. oculata* algal extract. Therefore, total contents of polyphenolic compounds and condensed tannins were quantified. During the current study, it was noticed that concentrations of total polyphenols and condensed tannins increased after incorporation Au-NPs (Table 1). This was in agreement with Alegria *et al*. (2018) who documented that amount of the polyphenols increased in the extract after preparing nano-extract indicating their effective involvement in M-NPs biosynthesis. Many previous studies supported our findings and revealed that quantity of the active phyto-constituents increased after integrating plant extract with M-NPs. Consequently, this leads to elevating *in vitro* biological activities (antioxidant, scavenging and cytotoxic activites) compared to native extract itself (Abdelhady and Badr, 2016; Aboulthana *et al*., 2019). Mystrioti *et al*. (2016) postulated that the elevation in concentration of these contents might be attributed to interference of Au ions that were utilized for Au-NPs biosynthesis with polyphenolic measurements.

**Table 1:** Contents of the total polyphenols (TPP) and total condensed tannins (TCT) in *N. oculata* algal extract before and after incorporating Au-NPs.

| Extract | Total Polyphenols (mg gallic acid/100 gm) | Total Condensed Tannins (µg/ml) |
|---|---|---|
| Algal Extract | 58.79 ± 0.81 | 25.13 ± 0.09 |
| Gold algal nano-extract | **123.79 ± 0.93*** | **35.09 ± 0.03*** |

*: The most effective extract, Values expressed as mean ± SE of three replicates.

Regarding antioxidant capacity, gold *N. oculata* algal nano extract showed higher antioxidant capacity (93.38 ± 0.52 mg gallic acid/gm) and iron reducing power (38.83 ± 0.42 µg/mL) compared to native algal extract itself before incorporating Au-NPs (72.17 ± 0.33 mg gallic acid/gm and 12.48 ± 0.32 µg/mL, respectively). Consequently, incorporation of the Au-NPs into *N. oculata* algal extract showed higher scavenging activity against DPPH ($IC_{50}$ 4.52 µg/mL) compared to the algal extract itself ($IC_{50}$ 7.33 µg/mL). Moreover, it increased scavenging activity against ABTS free radicals (33.85 ± 0.03 %) at equal concentration (100 µg/mL) as compared to the algal extract itself (24.98 ± 0.10%) (Table 2). Consistent with findings of the present study, inclusion of Au-NPs into extract increased the bioavailability and hence leads consequently to enhancing the antioxidant and scavenging activities compared to native extract itself (Abdel-Aziz *et al*., 2014; Aboulthana *et al*., 2019; Abdel-Halim *et al*., 2020). The antioxidant and scavenging activities are highly correlated to the total polyphenolic content. This is clear in our study where *N. oculata* algal extract is a good source of these biomolecules with high antioxidant capacity making it an excellent candidate for utilizing the green nanotechnology to biosynthesize Au-NPs.

**Table 2:** Antioxidant and radical scavenging activities of *N. oculata* algal extract before and after incorporating Au-NPs.

| Extract | Total Antioxidant Capacity (mg gallic acid/gm) | Iron Reducing Power (µg/mL) | Radical Scavenging Activity | |
|---|---|---|---|---|
| | | | DPPH ($IC_{50}$ µg/ml) | ABTS Inhibition (%) |
| Algal Extract | 72.17 ± 0.33 | 12.48 ± 0.32 | 7.33 | 24.98 ± 0.10 |
| Gold algal nano-extract | **93.38 ± 0.52*** | **38.83 ± 0.42*** | **4.52*** | **33.85 ± 0.03*** |

*: The most effective extract, Values expressed as mean ± SE of three replicates.

*3.3. Fourier Transform Infrared (FT-IR) Spectroscopy analysis*

The chemical composition of the organic compounds can be analyzed by the FT-IR technique. During the current study, it was employed for identifying the possible functional groups in the biomolecules existing in *N. oculata* algal extract and responsible for reducing the Au ions and stabilizing the biosynthesized Au-NPs. The FT-IR results confirmed abundancy of the phenolic and tannins that are considered as the most common bioactive metabolites in the extract (before and after incorporating Au-NPs) (Firoozi *et al*. 2016). As shown in Fig. 2 and compiled in Table 3, the FT-IR spectrum showed that 19 phenolic compounds were identified in native *N. oculata* algal extract (before incorporating Au-NPs). Data presented in Fig. 3 and depicted in Table 4 showed that incorporation of Au-NPs into *N. oculata* algal extract increased number of the identified phenolic compounds into 62. This was in agreement with Alamdari *et al*. (2020) who emphasized that appearance of new absorption bands proved formation of covalent bonds in Au-NPs. Abdelhady and Badr (2016) reported that number of the phenolic compounds that were represented by peaks with specific absorbance and transmittance identified during FT-IR analysis increased as a result of incorporating M-NPs. Shousha *et al*. (2019) emphasized that increasing number of peaks is directly related to increasing number of the polyphenolic compounds. Consequently, the overall *in vitro* biological activities represented during the present study by antioxidant (TAC and iron reducing power), scavenging and cytotoxic activities were strongly related to enhancing concentration of these compounds. Alegria *et al*. (2018) postulated that percentage of the polyphenols consumed during M-NPs biosynthesis is directly correlated with size of the produced nanoparticles. Size of the M-NPs decreased, when amount of the polyphenol increased in the extract during preparation of the nano-extract. This might refer to conceivable regeneration of polyphenols under conditions of the catalytic reaction and possible interference of the small M-NPs with measurement of the polyphenolic compounds (Mystrioti *et al*. 2016).

The potential anti-diabetic activity of the plant extract may be refer to presence of the phenolic compounds that exhibit high antioxidant capacity since oxidative stress affect β- cells of pancreas. The assay that used for measuring anti-diabetic activity of the extract is called α-amylase inhibitory assay (Sekhon-Loodu and Rupasinghe, 2019). During the current study, α-amylase inhibitory assay was carried out for determining the anti-diabetic activity of *N. oculata* algal extract using acarbose as standard drug. As revealed in Fig. 4, it was noticed that gold *N. oculata* algal nano-extract possessed higher inhibitory effect on α-amylase activity (64.33 ± 0.33%) as compared to the extract itself (54.33 ± 0.67%) before incorporating Au-NPs, whereas at the same concentration, the standard drug (acarbose) was about 66.67 ± 0.33%. This was in accordance with Kifle *et al*. (2020) who reported that α amylase inhibitory activity is most likely to be due to the presence of phenolic compounds. Phenolic acids and tannins in addition to flavonoids belong to the polyphenolic compounds that possess inhibitory effect on α-amylase (Kim *et al*., 2000). During this study, the *N. oculata* algal extract is rich in the polyphenolic compounds that belong to the bioactive metabolites and exist in all natural extracts at different concentrations exhibiting an inhibitory effect against α-amylase. The total polyphenolic compounds and condensed tannins that increased as a result of Au-NPs incorporation were responsible for elevating the inhibitory effect against α-amylase.

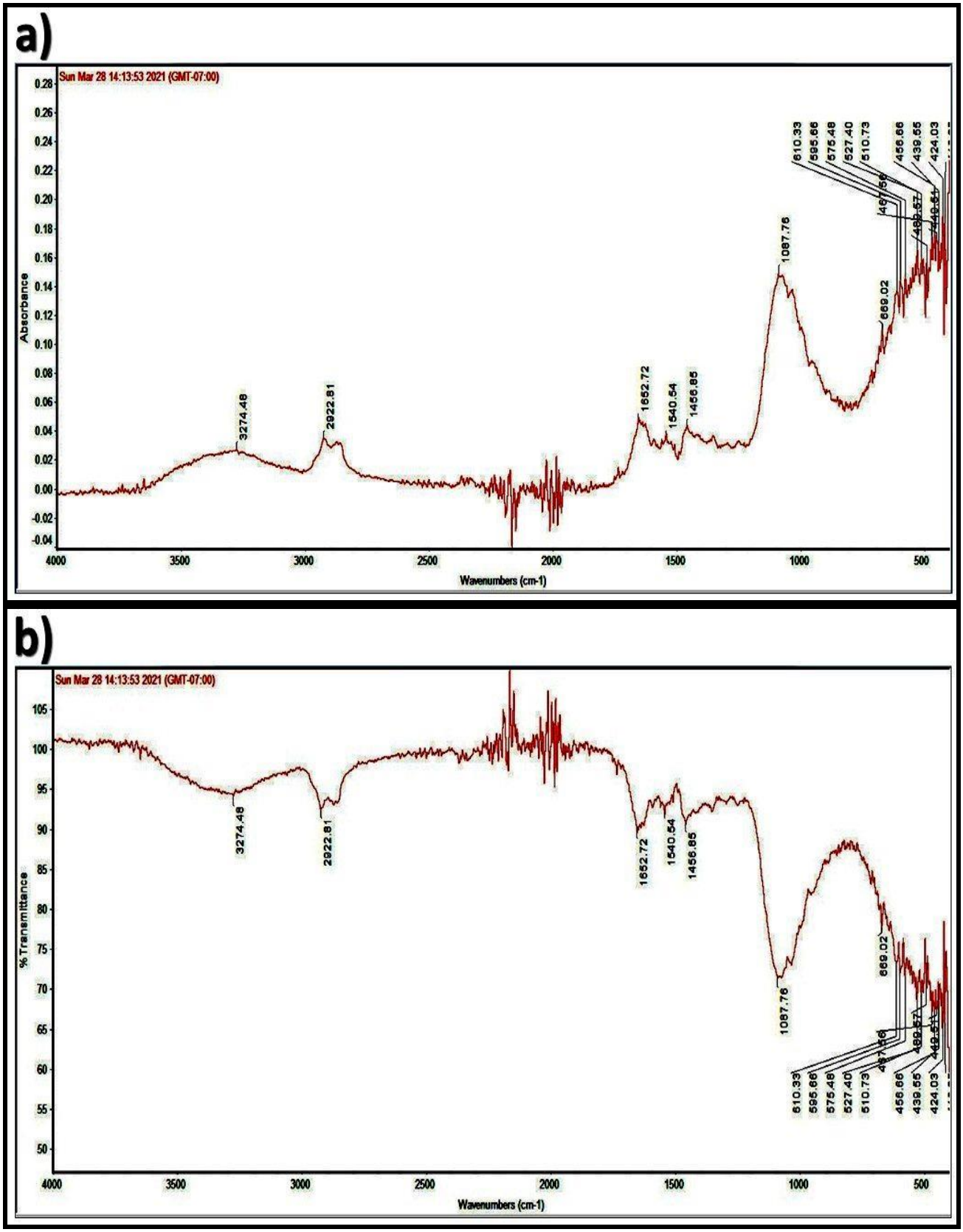


**Fig. 2.** The FT-IR showing **a)** absorbance and **b)** transmittance of the bioactive compounds in *N. oculata* algal extract before incorporating Au-NPs.

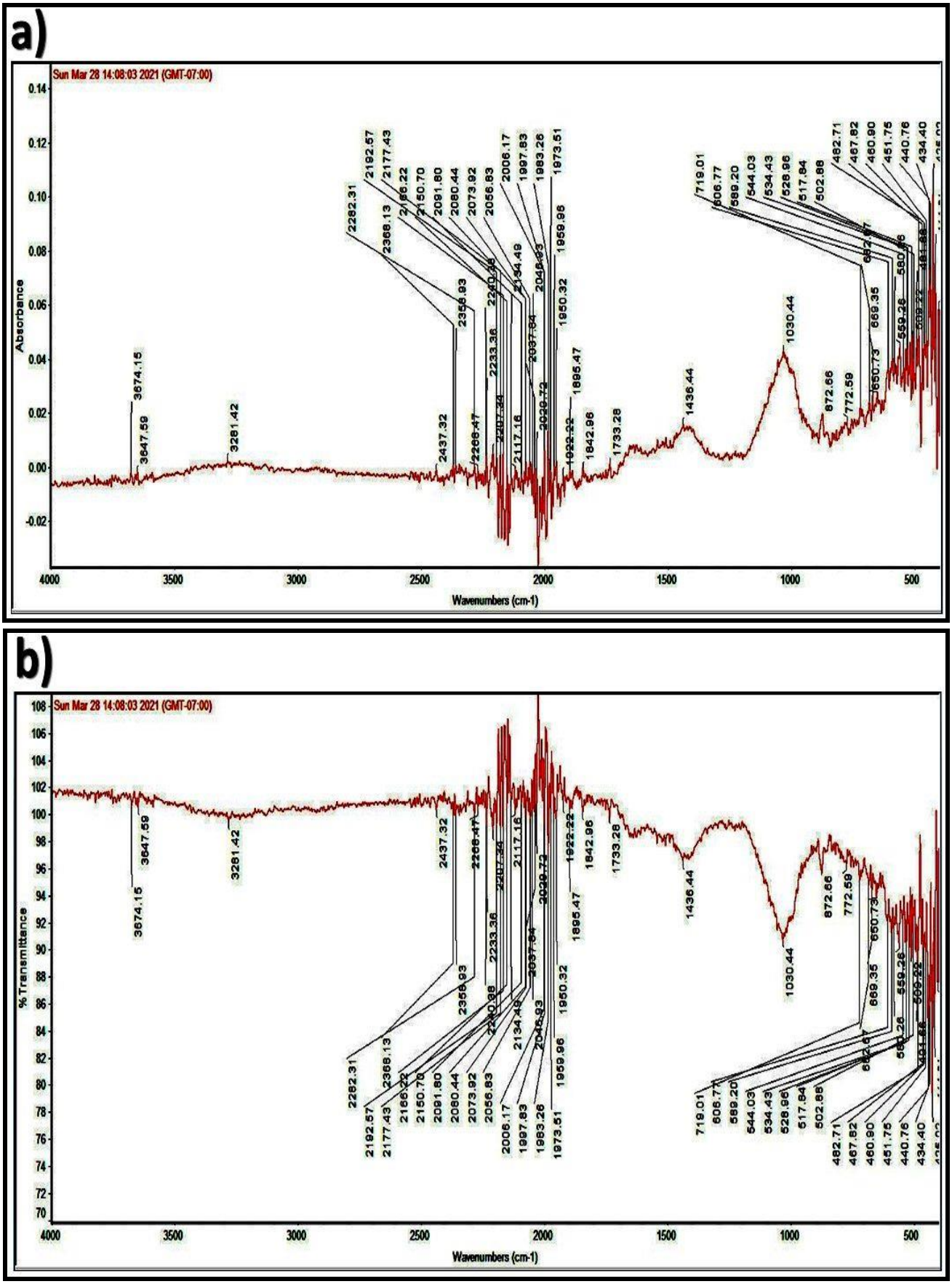


**Fig. 3.** The FT-IR showing **a)** absorbance and **b)** transmittance of the bioactive compounds in *N. oculata* algal extract after incorporating Au-NPs.

**Table 3:** The FT-IR measurements showing transmittance percent (Trans. %) and relative intensities (Int.%) of the bioactive compounds in *N. oculata* algal extract before incorporating Au-NPs.

| Wave number ($Cm^{-1}$) | Trans. % | Int. % |
|---|---|---|
| 416.35 | 65.60 | 4.58 |
| 424.03 | 64.45 | 4.50 |
| 439.55 | 68.56 | 4.79 |
| 449.51 | 67.31 | 4.70 |
| 456.66 | 66.70 | 4.66 |
| 467.56 | 67.00 | 4.68 |
| 489.57 | 69.94 | 4.89 |
| 510.73 | 69.35 | 4.85 |
| 527.4 | 68.34 | 4.78 |
| 575.48 | 71.48 | 5.00 |
| 595.66 | 71.89 | 5.02 |
| 610.33 | 73.19 | 5.11 |
| 669.02 | 77.75 | 5.43 |
| 1087.76 | 71.20 | 4.98 |
| 1456.85 | 90.52 | 6.33 |
| 1540.54 | 91.77 | 6.41 |
| 1652.72 | 89.44 | 6.25 |
| 2922.81 | 92.41 | 6.46 |
| 3274.48 | 94.07 | 6.57 |

**Trans**.: Transmittance, **Int.:** Intensity

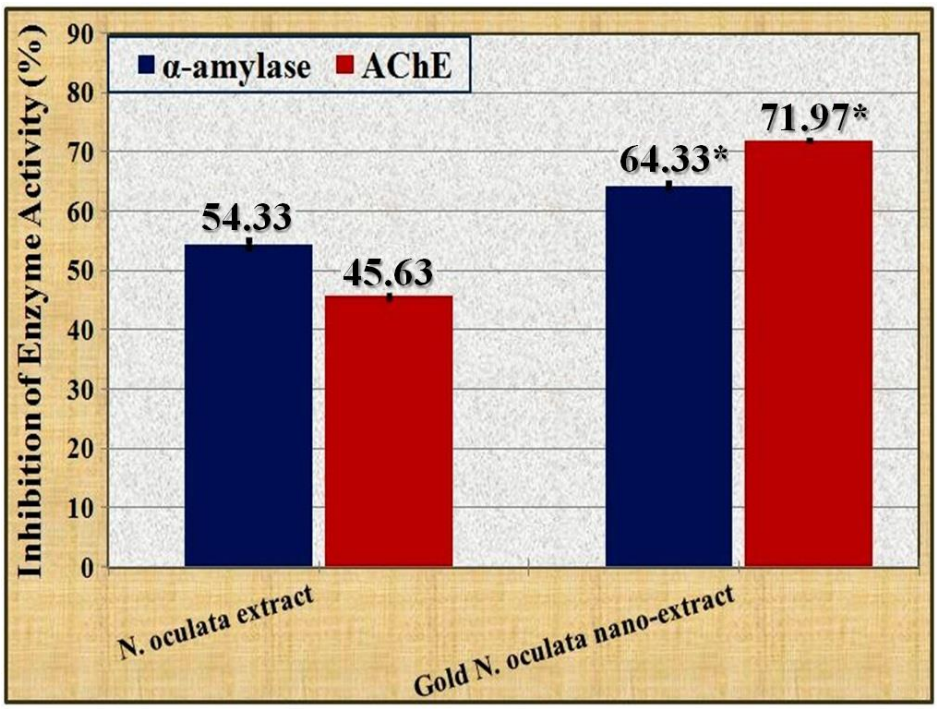


**Fig. 4.** Percent of the inhibitory effect on activities of α-amylase and acetyl cholinesterase (AChE) for **a)** native *N. oculata* algal extract and **b)** gold *N. oculata* algal nano-extract. Values expressed as mean ± SE of three replicates.

* indicates the most effective extract.

**Table 4a:** The FT-IR measurements showing transmittance percent (Trans. %) and relative intensities (Int. %) of the bioactive compounds in *N. oculata* algal extract after incorporating Au-NPs.

| **Wave number (**$Cm^{-1}$**)** | **Trans. %** | **Int. %** |
|---|---|---|
| 402.62 | 87.55 | 1.48 |
| 414.61 | 86.14 | 1.45 |
| 425.02 | 79.27 | 1.34 |
| 434.40 | 85.74 | 1.44 |
| 440.76 | 84.53 | 1.42 |
| 451.75 | 89.27 | 1.50 |
| 460.90 | 90.09 | 1.52 |
| 467.82 | 90.31 | 1.52 |
| 482.71 | 88.98 | 1.50 |
| 491.66 | 89.74 | 1.51 |
| 502.88 | 91.23 | 1.54 |
| 509.22 | 89.35 | 1.51 |
| 517.84 | 90.04 | 1.52 |
| 528.96 | 90.43 | 1.52 |
| 534.43 | 90.48 | 1.52 |
| 544.03 | 91.88 | 1.55 |
| 559.26 | 90.39 | 1.52 |
| 580.26 | 91.52 | 1.54 |
| 589.20 | 91.18 | 1.54 |
| 606.77 | 92.05 | 1.55 |
| 650.73 | 93.80 | 1.58 |
| 669.35 | 94.12 | 1.59 |
| 682.67 | 94.70 | 1.60 |
| 719.01 | 95.03 | 1.60 |
| 772.59 | 96.30 | 1.62 |
| 872.66 | 95.62 | 1.61 |
| 1030.44 | 90.55 | 1.53 |
| 1436.44 | 96.53 | 1.63 |
| 1733.28 | 99.77 | 1.68 |
| 1842.96 | 100.12 | 1.69 |
| 1895.47 | 100.36 | 1.69 |

**Trans**.: Transmittance, **Int.:** Intensity

**Table 4b:** The FT-IR measurements showing transmittance percent (Trans. %) and relative intensities (Int.%) of the bioactive compounds in *N. oculata* algal extract after incorporating Au-NPs.

| **Wave number (**$Cm^{-1}$**)** | **Trans. %** | **Int. %** |
|---|---|---|
| 1922.22 | 100.87 | 1.70 |
| 1950.32 | 99.59 | 1.68 |
| 1959.96 | 100.03 | 1.69 |
| 1973.51 | 100.37 | 1.69 |
| 1983.26 | 96.88 | 1.63 |
| 1997.83 | 98.66 | 1.66 |
| 2006.17 | 100.96 | 1.70 |
| 2029.72 | 100.78 | 1.70 |
| 2037.84 | 100.11 | 1.69 |
| 2046.93 | 100.28 | 1.69 |
| 2056.83 | 99.66 | 1.68 |
| 2073.92 | 99.67 | 1.68 |
| 2080.44 | 100.44 | 1.69 |
| 2091.80 | 100.75 | 1.70 |
| 2117.16 | 100.20 | 1.69 |
| 2134.49 | 101.04 | 1.70 |
| 2150.70 | 100.34 | 1.69 |
| 2166.22 | 98.97 | 1.67 |
| 2177.43 | 99.07 | 1.67 |
| 2192.57 | 99.21 | 1.67 |
| 2207.34 | 98.78 | 1.66 |
| 2233.36 | 99.13 | 1.67 |
| 2240.38 | 100.40 | 1.69 |
| 2268.47 | 100.35 | 1.69 |
| 2282.31 | 99.97 | 1.68 |
| 2358.93 | 99.82 | 1.68 |
| 2368.13 | 99.90 | 1.68 |
| 2437.32 | 100.30 | 1.69 |
| 3281.42 | 99.55 | 1.68 |
| 3647.59 | 100.45 | 1.69 |
| 3674.15 | 100.57 | 1.69 |

**Trans**.: Transmittance, **Int.:** Intensity

The anti-Alzheimer's activity of *N. oculata* algal extract was assayed by measuring its inhibitory effect on AChE activity. Activation of AChE enzyme is one of leading cause of Alzheimer's disease because in neuromuscular junctions or tissue synapses, AChE enzyme performs its action by catalyzing the hydrolysis process through conversion of acetyl choline (a neurotransmitter) into choline and acetic acid. Therefore, inhibition of this enzyme is one of the suitable ways necessary for treatment of Alzheimer's disease (Suganthy *et al*., 2018). During the present experiment, it was noticed that gold *N. oculata* algal nano-extract exhibited higher inhibitory effect on AChE activity (71.97 ± 0.16%) as compared to the extract itself (45.63 ± 0.25%) before incorporating Au-NPs. The M-NPs have high binding affinity to ChEs and they exhibited their inhibitory effect primarily by interacting with AChE protein. This might be attributable to hydrophobicity of the enzyme environment in ChE molecules and / or due to lipophilicity of these M-NPs. The recent studies supported that the mechanism by which the M-NPs interact with ChEs proteins is not completely understood (Wang *et al*. (2009); Aboulthana *et al*. (2022a); Aboulthana *et al*. (2022b)).

*In vitro* cytotoxic activity showed that inclusion of Au-NPs into *N. oculata* algal extract exhibited obvious elevation in the cytotoxic activity against HEPG-2 ($IC_{50}$ 218.50 µg/mL) (Table 5). Fig. 5 showed the $IC_{50}$ and maximum concentration (1000 µg/mL) of *N. oculata* algal extract (before and after incorporating Au-NPs) against CACO-2 cells compared to control CACO-2 cells. Regard to cytotoxic activity against CACO-2, gold *N. oculata* algal nano-extract possessed the highest activity with the lowest $IC_{50}$ value ($IC_{50}$ 185.50 µg/mL) (Table 5). The Fig. 6 revealed the $IC_{50}$ and maximum concentration (1000 µg/mL) of *N. oculata* algal extract (before and after incorporating Au-NPs) against HEPG-2 cells compared to control HEPG-2 cells. The *N. oculata* algal extract incorporated with Au-NPs showed higher cytotoxic effect against growth of CACO-2 cells and this was in agreement with Suganya Devi *et al*. (2012) who supposed that development the invasive cancer effectively was inhibited by the active phyto-constituents that increased after incorporating Au-NPs. Aboulthana *et al*. (2019) added that the cytotoxic activity of the algal extract incorporated with Au-NPs elevated compared to the crude (native) extract itself and this was manifested by their reduced $IC_{50}$ for different used cell lines and this might be related to increasing the antioxidant and scavenging activities against attack of the free radicals (Abdel-Halim *et al*., 2020).

### 3.4. Toxicity of *N. oculata* algal extract incorporated with Au-NPs

The algal extract incorporated with Au-NPs caused no toxicity when administrated orally in experimental animals by stomach tube. It was safer than *N. oculata* algal extract itself. During the present study, the $LD_{50}$ of *N. oculata* algal extract before incorporating Au-NPs was about 5000.00 mg/Kg and the therapeutic dose was about 500.00 mg/Kg. After incorporating Au-NPs, the $LD_{50}$ was about 9333.00 mg/Kg and hence the therapeutic dose was 933.00 mg/Kg (Fig. 7). This was in accordance with Abdel-Halim *et al*. (2020) who supported that green route synthesis of Au-NPs increased safety of plant extract. The urinary excretion is considered as a multifaceted process for removing M-NPs through glomerular filtration, tubular secretion and finally eliminated from the body (Mohanpuria *et al*., 2008). The M-NPs that were eliminated from the body by renal clearance did not follow catabolic pathway completely. Therefore, they were excreted without possible side effects due to minimal degradation rate (Aboulthana *et al*., 2019 ; Aboulthana *et al*., 2022).

**Table 5:** Cytotoxic activity of *N. oculata* algal extract before and after incorporating Au-NPs against human colon (CACO-2) and liver cancer (HEPG-2) cells before and after incorporating gold nanoparticles (Au-NPs).

| Extract | $IC_{50}$% | |
|---|---|---|
| | **CACO-2** | **HEPG-2** |
| Algal Extract | 188.00 | 487.70 |
| Gold algal nano-extract | **185.50*** | **218.50*** |

*: The most effective extract, Values expressed as mean ± SE of three replicates.

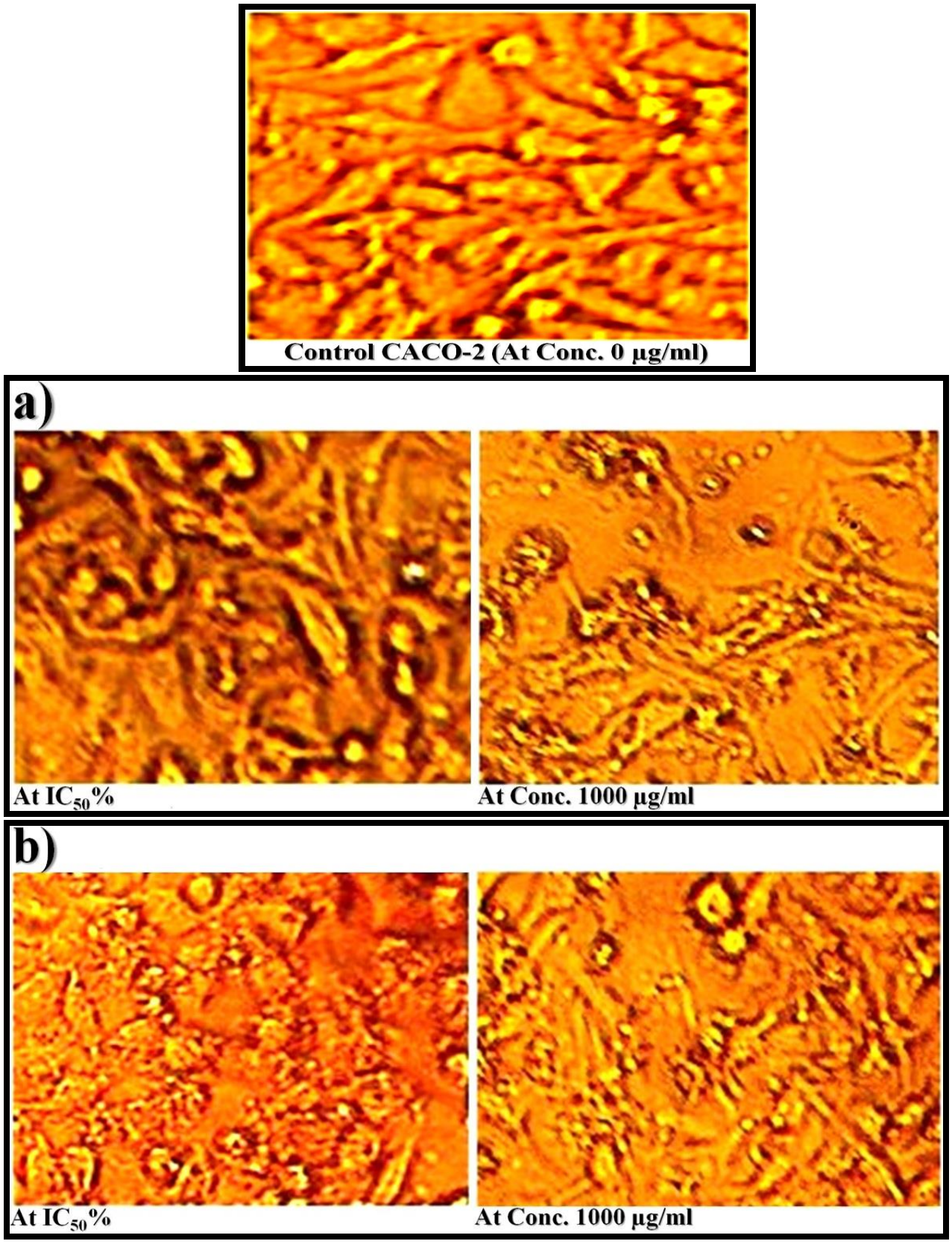


**Fig. 5.** Cytotoxic activity against human colon cancer (CACO-2) cells showing the median ($IC_{50}$) and maximum inhibitory concentrations (Conc. 1000 µg/mL) of *N. oculata* algal extract **a)** before and **b)** after incorporating gold nanoparticles (Au-NPs) compared to control CACO-2 cells.

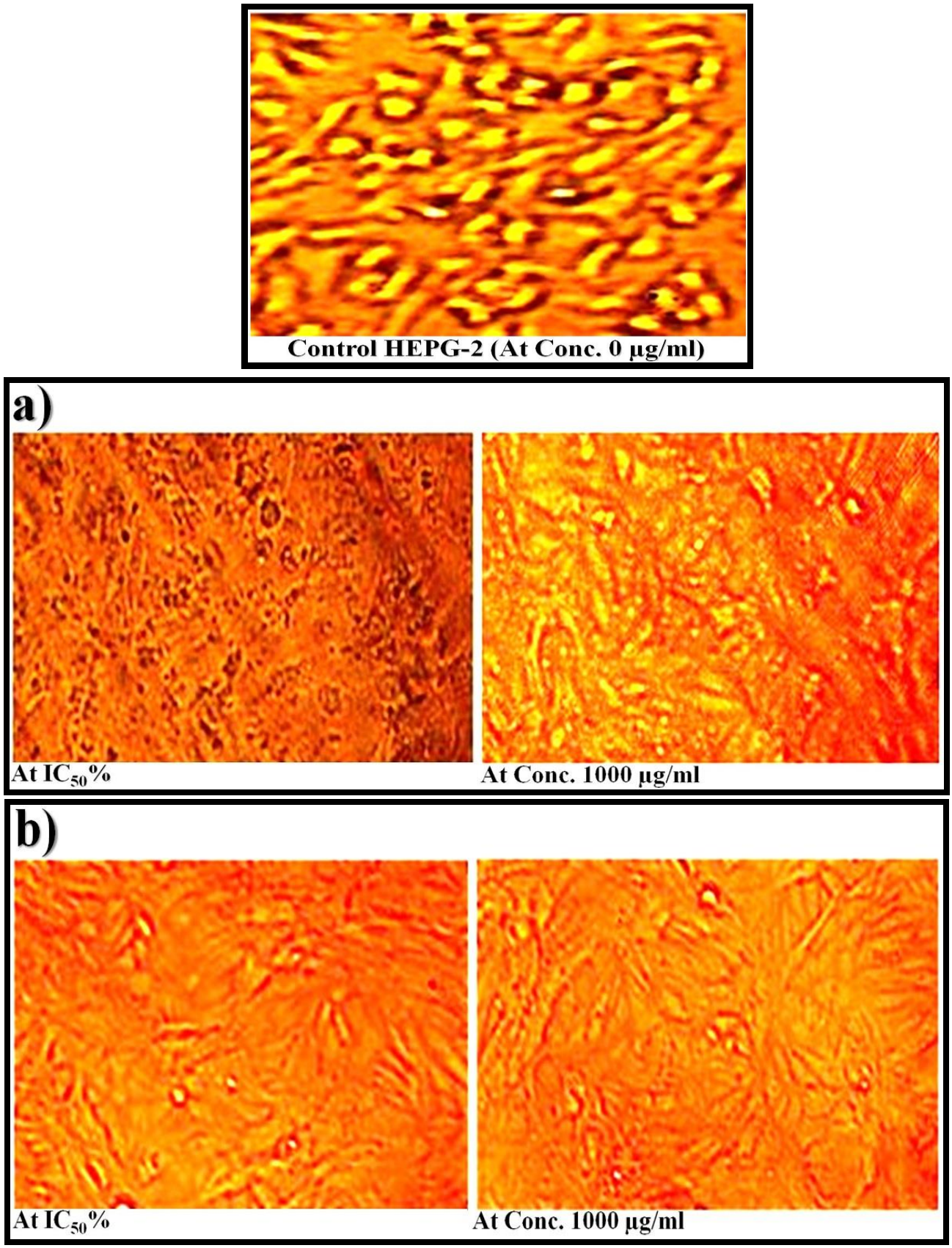


**Fig. 6.** Cytotoxic activity against human liver cancer (HEPG-2) cells showing the median ($IC_{50}$) and maximum inhibitory concentrations (Conc. 1000 µg/mL) of *N. oculata* algal extract **a)** before and **b)** after incorporating gold nanoparticles (Au-NPs) compared to control HEPG-2 cells.

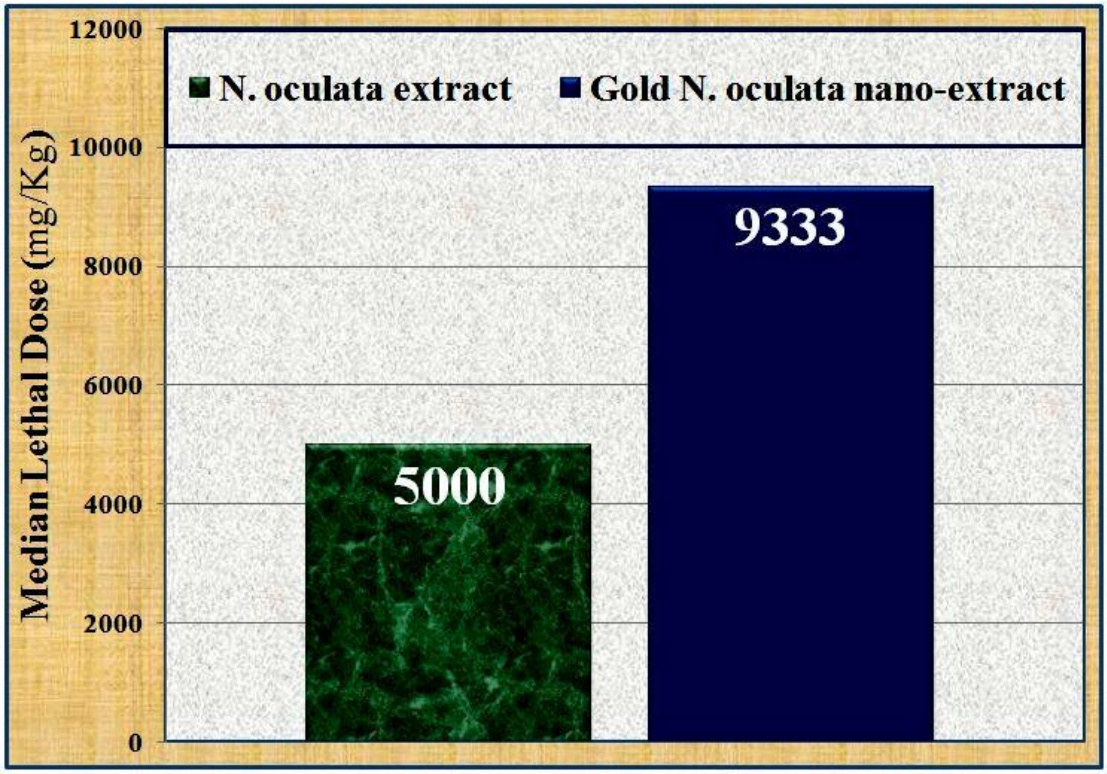


**Fig. 7.** The median lethal doses ($LD_{50}$) of *N. oculata* algal extract before and after incorporating Au-NPs.

## Conclusion

The *N. oculata* algal extract incorporated with Au-NPs possessed higher biological efficacy (antioxidant, scavenging, anti-diabetic and anti-Alzheimer's activities) as a result of increasing concentrations of the major active phyto-constituents (total polyphenolic compounds and total tannins). Furthermore, it showed higher cytotoxic activity against growth of CACO-2 and HEPG-2 cells. The gold *N. oculata* algal nano-extract was orally safer compared to native algal extract itself.

## Declaration of Competing Interest

The authors who responsible for carrying out the experiment declare that there were no conflicts of interest financially or non-financially among them.

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