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Pilot study of Gold nanoparticles and CTAB-nanorods to be used in chemopreventive therapy: anti/genotoxicological, antiaging, tumoricidal and epigenetic analysis.

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Cuando la gratitud es tan absoluta, las palabras sobran. Álvaro Mutis.

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"La sonrisa es mía, pero el motivo sois vosotros" Toy Story.

"Vive como si fueras a morir mañana y aprende como si fueras a vivir siempre"
Mahatma Gandhi

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ABSTRACT

Nanoparticles (NPs) are particles with dimensions between molecules and microscopic structures. They have many uses, but one of the most important is in Biomedicine cancer therapies. Due to NPs can be synthesized from different materials (gold, silver, aluminium, etc.), with different shapes (spherical, rods, etc.) and sizes within the nanometric range, it is crucial to know what material, shape and size will serve us in the future to apply them in Biomedicine. *In vivo* tests are needed, and in this study toxicity, antitoxicity and longevity tests have been carried out using *Drosophila melanogaster* as a model organism to evaluate their intervention in some degenerative processes. In addition, *in vitro* cytotoxicity, DNA fragmentation, global methylation and comet assays were performed using the human promyelocytic cell line HL60 to evaluate some biological activities related to chemoprevention.

None of the concentrations tested of AuNPs and CTAB-AuNRs reached the LD_{50} in *D. melanogaster*. On the other hand, only one concentration of AuNPs (0.05 nM) was statistically different respect to the postitive control in the antitoxicity test; However, all tested concentrations except the highest concentration of CTAB-AuNRs showed significant differences regarding positive control. None of the concentrations of both tested substances were genotoxic. As for the longevity tests, a decrease in the *D. melanogaster* survival is induced as concentration increased. According to the *in vitro* tests, none of the substances tested was cytotoxic to promyelocytic HL60 cells as they did not reach IC_{50}. No DNA fragmentation was observed when HL60 cells were treated with AuNPs. In contrast, DNA fragmentation was induced at intermediate concentrations of CTAB-AuNRs. Moreover, genetic damage was observed after the comet assay at certain concentrations of AuNPs (0.100; 0.200 nM) and CTAB-AuNRs (2E-5 and 2E-13 nM). At the epigenomic level, the AuNPs could increase the methylation status of Alu-M1 and LINE-1 repetitive sequences. In contrast, CTAB-AuNRs could decrease the methylation status in Sat-α and increase the methylation status in Alu-M1. Although the results obtained have let some light on the biological activities (safety and chemoprevention), we consider that it is still necessary to deeply carry out tests to determine what type of NPs or NRs are most suitable to be used in Biomedicine.

Keywords: *D. melanogaster*; HL60; AuNPs, CTAB-AuNRs.

RESUMEN

Las nanopartículas (NPs) son partículas con dimensiones comprendidas entre las moléculas y las estructuras microscópicas. Poseen multitud de utilidades, pero una de las más importantes es su uso en Biomedicina en terapias contra el cáncer. Debido a que las NPs pueden ser sintetizadas a partir de diferentes materiales (oro, plata, aluminio, etc.), con diferentes formas (esféricas, bastoncillos, etc.) y diferentes tamaños dentro del rango nanométrico, es de vital importancia conocer que material, forma y tamaño nos servirá en el futuro, para aplicarlas en Biomedicina. Ensayos *in vivo* son necesarios, y en este estudio ensayos de toxicidad, Antitoxicidad y longevidad han sido llevados a cabo usando *Drosophila melanogaster* como organismo modelo para evaluar su intervención en algunos procesos degenerativos. Además, ensayos *in vitro* de citotoxicidad, fragmentación del ADN, de metilación global y del cometa fueron realizados usando la línea celular promielocítica humana HL60 para evaluar algunas actividades biológicas relacionadas con la quimioprevención.

Ninguna de las concentraciones ensayadas de AuNPs y CTAB-AuNRs alcanzó la DL_{50} en *D. melanogaster*. Por otro lado, tan solo una concentración de AuNPs (0.05 nM) fue estadísticamente diferente respecto al control positivo; sin embargo, todas las concentraciones testadas excepto la más alta de CTAB-AUNRs mostraron diferencias significativas respecto al control positivo. Ninguna de las concentraciones de ambas sustancias fue genotóxicas. En cuanto a las pruebas de longevidad, se induce una disminución en la supervivencia de *D. melanogaster* a medida que aumenta la concentración. De acuerdo con los ensayos *in vitro*, ninguna de las sustancias ensayadas fue citotóxica para las células promielocíticas HL60 ya que no se alcanzó la CI_{50}. No se observó fragmentación del ADN en las células HL60 cuando fueron tratadas con AuNPs. Por el contrario, concentraciones intermedias de CTAB-AuNRs indujeron fragmentación del ADN. Además, fue observado daño genético cuando llevamos a cabo el ensayo del cometa con determinadas concentraciones de AuNPs (0.100; 0.200 nM) y CTAB-AuNRs (2E-5 and 2E-13 nM). A nivel epigenómico, las AuNPs podrían incrementar el estado de metilación de las secuencias repetitivas Alu-M1 y LINE-1. Por el contrario, CTAB-AuNRs provocan una disminución del estado de metilación en Sat-α y lo incrementan en Alu-M1. Aunque los resultados obtenidos han permitido poner en claro las actividades biológicas (seguridad y quimioprevención), consideramos que todavía es necesario realizar profundamente pruebas para determinar qué tipo de NPs o NRs son las más adecuadas para ser utilizadas en Biomedicina.

Palabras clave: *D. melanogaster*; HL60; AuNPs, CTAB-AuNRs.

ABREVIATIONS

A.U.	Arbitrary Units
AuNPs	Gold Nanoparticles
AuNRs	Gold Nanorods
CTAB-AuNRs	CetyltrimeThylAmmonium Bromide Gold Nanorods
DNA	Deoxyribonucleic acid
flr^3	*flare* genetic marker
g	Grams
HL60	Human Leukaemia-60 cell line
IC_{50}	Inhibitory Concentration 50
LD_{50}	Lethal Dosis
LINE	Long Interspersed Elements
mwh	*multiple wings hairs* genetic marker
NPs	Nanoparticles
NRs	Nanorods
PEG	Polyethylenglycol
qMSP	Quantitative Methylation-Specific PCR
rpm	revolutions per minute
Sat-α	Satellite-alpha-DNA
SCGE	Single Cell Gel Electrophoresis
SMART	Somatic Mutation and Recombination Test
SPR	Surface Plasmon Resonance
TEM	Transmission Electron Microscopy
TM	Tail Moment
WHO	World Health Organization

1. INTRODUCTION.

Nanotechnology consists of the creation and use of materials or systems whose dimensions are on the nanometre scale (0.1-100 nm^4 range), with some exceptions. The development of this discipline begins in the last decade since the studies carried out proposed an explanation on the interactions between nanoparticles (NP) and biological systems **(Dothager and Piwnica-Worms, 2011)**.

The synthesis process of the NP offers the possibility to manipulate the physical and chemical properties with the purpose of designing molecules that provide a highly specific biological interaction **(Praetorius and Mandal, 2007)**.

NPs are used for many purposes in environment, textile sector, building, transport, fine arts, informatics and electronics, being the therapeutic uses the most important to: **(De Jong and Borm, 2008)**:

- Act as controlled release systems.
- Reach specific tissues / barriers and penetrate in cells unattainable by conventional active principles thanks to its small diameter.
- Decrease the adverse effects and toxicity associated to free drug administration.
- Improve the physical and chemical stability of encapsulated active substances.
- Increasing the absorption of active molecules, which provides a better bioavailability thereof.

Therapeutic nanoparticles were first developed during the 1970s, with the aim of using them as a way of release active molecules and vaccines **(Kreuter, 2007)**. The NP can be classified into **(Morris et al., 2007)**:

- Organic nanoparticles: consist of polymers, repetitive structures, lipid bilayers. Included within this group are polymer nanoparticles, dendrimers, micelles and liposomes.
- Inorganic nanoparticles: consist of metals and materials such as titanium dioxide, hydroxyapatite or silica. Including within this group: gold nanoparticles, silica, copper, iron oxide, cerium oxide, carbon nanotubes, quantum dots, titanium dioxide and zinc oxide being AuNPs the most used.

Because of the numerous applications of NP, we must assume that human exposure to NPs will increase in the next few years, being significant for health and environment **(Nowack and Bucheli, 2007)**. It is obvious the necessity to evaluate and verify the potential risks of the use of NP **(Maynard et al., 2006)**. The new-born Nanotoxicology area intends to know the main factors that define the toxic effects of nanomaterials **(Mateo et al., 2013)**.

1.1. Nanoparticles and cancer.

The World Health Organization (WHO) defines cancer as a process of uncontrolled growth and spread of cells. The WHO points this disease as one of the main causes of mortality, and prevents an increase of 45% of mortality between 2007 and 2030 (from 7.9 to 11.5 million deaths **http://www.who.int/features/qa/15/es/**). Treatments against cancer do not act only on damaged cells but on normal cells as well. In addition, the side effects are very high.

NPs can be produced in different shapes and sizes and therefore can be readily used with a wide range of ligands such as antibodies, polymers, diagnostic probes, drugs, genetic material, etc. **(Mateo et al., 2013).** The NP can be used as dispensers of drugs to increase the response to therapies with anticancer compounds **(Ferrari, 2005; Sinha et al., 2006)**.

The NPs incorporating ligands into their structure are being studied as selective vectors for cancer treatment. The most studied ligand is folic acid, a high overexpression of folate receptors in tumor tissue is observed **(Low et al., 2007)**. A study by **Liu et al. (2012)** shows an improvement in cytotoxicity on tumor cells after the encapsulation of several chemotherapeutic agents if compared to nanoparticles without this ligand. More recent investigations include the combination of chemotherapeutics with magnetic NP to be able to guide them to the tumor tissue using an external magnetic field **(Shen et al., 2012)**. The use of NP in cancer therapy shows a single pharmacokinetics because NPs are internalized and stabilized quickly **(Nguyen, 2011)**. Nanotechnology could help to better develop new methods for diagnosis and treatment of different diseases, including cancer. Nanotechnology allows for more effective anti-cancer drug, but the approval of the nano-drugs in the market is slow.

1.2. Gold Nanoparticles (AuNPs).

This type of NP stands out because of their photothermal properties. When they are activated in the presence of laser light, they give off heat, so they are considered "nano-heaters". They are characterized as phototherapeutic agents in the treatment of cancer. In addition, they are used for manufacturing nanostructures useful to conduct the transport and selective vectorization of therapeutic drugs and macromolecules **(Jain et al., 2008)**. One of the most important goals is to use these AuNPs to elaborate "intelligent conveyor systems", which allow to control, in a space and at a certain time, the release of the drug or compound, since this is usually triggered by a biological stimulus property such as the in/out cellular concentration difference of glutathione **(Hong et al., 2006)** or by the activation of an external stimulus, such as laser light **(Han et al., 2006a)**.

Different types of AuNPs can be found depending on the technique of preparation, shape, size and physical properties. The most used are nanospheres, nano-cylinders, nanocouples, and nanowires **(Grabar et al., 1995)**. A frequent problem is to know what kind of shape of NP is the most appropriate to obtain a therapeutic application as the intracellular uptake of the AuNPs depends on their shape and size.

Despite the numerous applications of nanoparticles, AuNP are characterized by a high number of applications in Biomedicine. Being the most outstanding:

- Releasing of therapeutic drugs and macromolecules **(Mocellin et al., 2007; Polizzi et al., 2007)**.
- Gene therapy **(Han et al., 2006b; Thomas and Klibanov, 2003)**.
- Cancer therapy **(Bannon-Peppas and Blanchette, 2004)**.

It is crucial to know the *in vivo* and *in vitro* toxic possible effects of AuNPs both isolated and as part of conjugates. It should be noted that AuNPs have low toxicity and high biocompatibility. According to a study by **Connor et al. (2005)**, the nuclei of AuNPs are inert and non-toxic. But, now, there is a great controversy since studies realized with *D. melanogaster*; model organism, show that these substances are toxic since they provoke oxidative stress, they intervene in longevity, fertility and even produce malformations **(Vecchio et al., 2012a)**. In addition, these NPs are characterized because they cause DNA damage **(Pompa et al., 2011)**.

Therefore, it is of vital importance to carry out a risk-benefit evaluation since these new products have a high interest both at the industrial level and in the field of Biomedicine.

1.3. CetylTrimethylAmmonium Bromide -Gold Nanoparticles (CTAB-AuNPs).

A “nanorod” is a nanoscale particle with an aspect ratio (length/width ratio) between 1 and ∼20−25. Higher aspect ratio materials are termed as “nanowires” (with diameter <100 nm) **(Murphy et al., 2011)**. The AuNRs show interesting biomedical applications such as biosensors, biomedical imaging tools, drug and diagnostic administration and cancer therapy **(Ma et al., 2013)**. In general, all AuNPs are characterized by a property called surface plasmon resonance (SPR), which results from a coherent resonance between the oscillation of the electrons of their last orbitals and the oscillation of the external electromagnetic field (light) **(Huang et al., 2009)**. The spherical AuNPs have a single SPR bands, in the visible region, around 520 nm. In contrast, AuNRs are characterized by two bands of SPR that correspond to a transverse direction and a longitudinal band. The UV-Visible spectrum of the synthesized AuNRs shows two SPR bands placed at 740 and 520 nm corresponding to the longitudinal and transverse plasmon peaks, respectively **(Figure 1)**.

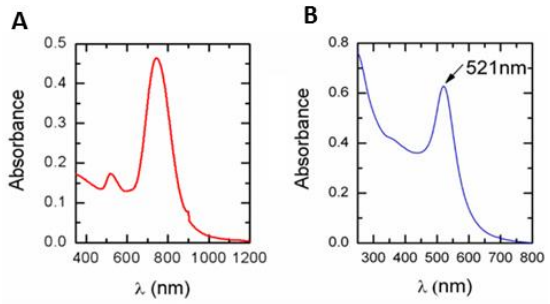

FIGURE 1. (**A**) Absorption spectrum of synthesized CTAB-AuNRs; (**B**) Absorption spectrum of AuNPs, protected by citrate anions. Chemistry-Physics Department.

AuNRs strongly absorb light in the visible and infrared electromagnetic spectrum regions, which confer them some interesting bio-medical applications (near infrared spectrophotometry absorption makes AuNRs ideal candidates for medical applications involving photo-thermal effects) **(Gans, 1915)**.

To properly use AuNRs and provide a good for the society, the most appropriate cover, the size, the concentration, etc. must be know. There a high controversy like in the case of AuNRs. According to **Niidome et al. (2007)**, AuNRs modified with polyethylene glycol (PEG) rather than with CTAB are better for biomedical applications. **Tsoli et al. (2005)** found a high relationship between the size and toxicity of AuNR. In contrast, other studies have shown that cytotoxicity is related to the cell type **(Patra et al., 2007)**. Although the AuNRs present a compromising future especially in the field of biomedicine, there are still issues that need to be assessed.

1.4. *In vivo* assays.

In vivo assays are useful in the study of the mutagenic capacity of substances that can activate various oncogenes or cause mutations in DNA, a critical stage for the onset and development of tumors in the body. Different researchers have developed their studies with the main objective on somatic recombination due to its relation with different types of cancers **(McCann et al., 1975; Romero-Jiménez et al., 2005)**.

1.4.1. Somatic mutation and recombination test (SMART) in D. melanogaster.

The SMART test performed for imaginal wings discs is a sensitive, specific and precise model to perform different genetic *in vivo* studies on *D. melanogaster*. Specifically, the potential of the compounds to exert DNA damage (genotoxicological analysis) is monitored by the loss of heterozygosity of two cellular markers (*mwh* and *flr*), which is translated into a modification in the phenotype of trichomes easily detectable. It also allows the evaluation of the antigenotoxic capacity of a substance performing combined treatments with a genotoxin (antigenotoxicological analysis) **(Katsue et al., 2014).** Considering the methodological advantages of this test we can highlight its simplicity and low cost. *D. melanogaster* has a wide spectrum of metabolic activities to activate a high number of genes as well as to deactivate them. In addition, in this test it is possible to expose thousands of somatic cells to the substance to be analysed **(Graf et al., 1984)**.

1.4.2. Longevity test.

Longevity is a quantitative trait influenced by both endogenous factors, involving numerous genes, such as exogenous factors, the environment **(Leips and Mackay, 2000)**. Mutations affecting genes involved in endocrine, stress response, metabolism and telomere-related pathways may increase life expectancy in model organisms **(Kenyon, 2005)**. Exogenous factors such as environmental conditions or diet are also involved in this type of processes as well. Numerous trials in *D. melanogaster* relate

the caloric increase/restriction with the diminution or the increase of the life expectancy. Despite this, the caloric restriction does not directly is involved in the increase of the life expectancy in model organisms **(Lee et al., 2008)**. In contrast, supplementation with certain foods or beverages such as beer has a positive effect on the longevity of this type of organisms **(Merinas-Amo et al., 2013)**.

Some studies showed that human genes homologous to genes related to longevity in invertebrates can vary their expression in tissues during aging. This fact indicates that underline the genetic control of invertebrates´ lifespan is essential to understand the aging process in humans **(Bell et al., 2009)**.

D. melanogaster is an excellent model organism due to its relatively short lifespan, the high number of individuals that can be controlled and because many aspects of cellular aging are observed that show mammals **(Fleming et al., 1992)**. Therefore, flies have frequently been used to study the physiological and pathological processes that affect life expectancy, and they may help to understand the relationship between the metabolism of nutrients and aging mechanisms **(Li et al., 2010)**.

1.5. *In vitro* assays.

Substances identified with genotoxic or antigenotoxic potential tested in model organisms may be carcinogenic or anticancer agents in humans. Ideally, studies in humans should be carried out to verify the effects, but ethical problems would be highly relevant. It is for this reason that *in vitro* tests are carried out. In our Department, we used the HL60 cell line. This cell line belongs to the undifferentiated immortal lines, as they are cancer or tumour cells. Leukocytes were isolated from the peripheral bloodstream of a human caucasian patient aged 36 who suffered from acute promyelocytic leukaemia **(Collins et al., 1977)**.

1.5.1. Cytotoxicity assay.

The inability of the leukemic cells to undergo a process of cell differentiation, symbolizes a good example of deregulation of maturation as a fundamental characteristic of neoplastic transformation. Because the various therapies used against cancer are currently very toxic and in most cases unspecific, an alternative strategy would imply the use of agents that are capable to induce differentiation in proliferating cancer cells **(Leszczyniecka et al., 2001)**. Among the utilities of the cell line HL60 is the search for substances capable of inducing the terminal differentiation of these cells, a phenomenon that would affect their capacity for proliferation and therefore their immortality, with the consequent onset of apoptosis **(Anazetti et al., 2003)**. Based on the above, cytotoxicity studies have been carried out which have demonstrated the tumoricidal capacity of certain plants as well as a certain number of active components **(Villatoro-Pulido et al., 2013)**.

The cytotoxicity test consists of checking whether a compound causes cell death, regardless of the pathway used (apoptosis, necrosis, etc.). This test can analyze any substance in the diet or the action of a new drug on normal or cancerous cell lines. For example, some coumarin-derived compounds tested on Hela-B75, HL60 and HEP-3B

cancer cell lines have been shown to be cytotoxic against these cell types **(Gacche and Jadhav, 2012)**.
Compounds capable of inducing differentiation and apoptosis in tumor cell lines are candidates to act as chemopreventive or chemotherapeutic agents against cancer **(Fésüs et al., 1995)**.

1.5.2. DNA fragmentation.

DNA internucleosomal fragmentation assay is an *in vitro* test used to detect the ability of a compound to induce cell death by proapoptotic way, a type of active death. This type of cell death is characterised by morphological changes, nuclear condensation and apoptotic body formation bound to the membrane **(Wyllie et al., 1981)**. Furthermore, an outstanding feature of cell death by apoptosis is the degradation of genomic DNA into internucleosomal fragments multiples of 200 pb separated by the same distance **(Gaido and Cidlowski, 1991)**. HL60 human cell line is widely used as a model for inducible cell differentiation **(Breitman et al., 1980)**. This phenomenon might affect the cell ability to proliferate, and therefore their immortality, with the appearance of apoptosis **(Anazetti et al., 2003)**. Compounds capable to induce differentiation and apoptosis are candidates to act as a chemopreventive agents or cancer chemotherapeutic **(Fésüs et al., 1995)**.

1.5.3. Comet Assay.

The Comet assay or Single Cell Gel Electrophoresis (SCGE) has become one of the standard methods for assessing on DNA damage with applications in genotoxicity testing and it is capable of measuring DNA breaks **(Collins, 2004)**. Alkaline comet assay is used to detect both single and double strand breaks for measuring all kind of damage **(Olive and Banáth, 2006)**. Necrosis and apoptosis can be distinguished by comet assay since apoptotic cells provided “hedgehog” tails **(Fairbairn et al., 1995)**. The induction of such comets in cancer cells should be an efficient way to specifically eliminate transformed cells.

1.5.4. Methylation status evaluation.

Epigenetics defines all meiotic and mitotic heritable changes in gene expression that are not coded in the DNA sequences itself. The two main epigenetic marks are 5-methylcytosine (5-meC) and histone acetylation **(Egger et al., 2004).** The DNA methylation in plants and animals is the addition of a methyl group to the fifth carbon position of the cytosine pyrimidine ring via methyltransferase enzymes in Cytosine-phosphate-Guanine (CpG) dinucleotides **(Robertson, 2005)**. It normally is a unidirectional process and will be inherited after cell division, associated with “closed” (inactive) chromatin state and therefore negative regulation of transcription **(Antequera and Bird, 1993)**. It is related to transcriptional gene silencing and plays a vital role during the development and in the genome defence against transposable elements **(Suzuki and Bird, 2008)**.

Since interspersed repetitive elements (LINE-1 retrotransposons and Alu-M1 sequences) as well as tandem repeat centromeric and juxtacentromeric repeats

(satellite sequences) contain numerous CpG, the methylation status of these sequences is relevant for understanding the global DNA methylation. Repetitive elements are heavily methylated in normal somatic tissues, although they are methylated to a lesser extent in malignant tissues, driving the global genomic hypomethylation commonly found in human cancers. The hypomethylation affecting repeat sequences and transposable elements is believed to result in chromosomal instability and increase mutation events **(Wei et al., 2005)**.

Prevention or reversal of hypermethylation-induced inactivation of tumour suppression genes or gene receptors by DNA-Methyltransferase (DNMT) inhibitors could be an effective approach to cancer prevention **(Roman-Gomez et al., 2008)**.

2. OBJECTIVES.

The objective of the present work is to verify the safety of AuNPs and CTAB-AuNRs, with important biomedical applications. For this main purpose:

- We have evaluated the toxicity and antitoxicity, genotoxicity and antigenotoxicity and longevity using *D. melanogaster* as a model organism.
- We have determined their chemopreventive capacity and clastogenic activity of these compounds in the human leukemia cell line HL60 *in vitro* model.

3. MATERIAL AND METHODS.

3.1. Compounds assayed in this study.

To carry out this study we used AuNPs and CTAB-AuNRs. The particles were provided by Dr. Teresa Pineda (Chemistry-Physics Department of the University of Córdoba) and their synthesis was carried out using a classical protocol **(Turkevich et al., 1951)**. Transmission electron microscopy (TEM) provides images of AuNPs that allow analysis of both the mean size and distribution. In **Image 1** TEM micrographs of AuNPs in which an estimate of the average size (12-13 nm) are shown.

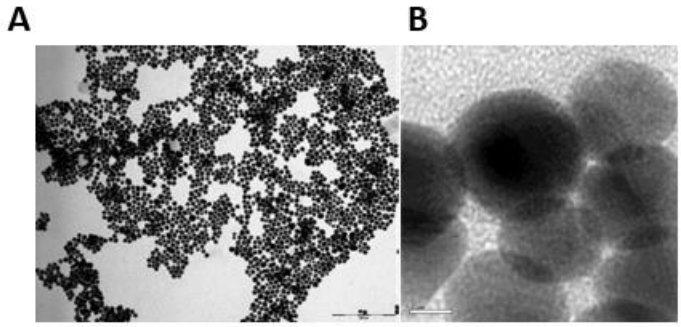

IMAGE 1. TEM and HR-TEM micrographs of AuNPs. The scales on the images correspond to 200 nm (**A**) and 5 nm (**B**). Photograph taken from the Chemistry-Physics Department of the University of Córdoba.

CTAB-AuNRs possessed a CTAB (CetyltrimeThylAmmonium Bromide), cationic surfactant coating that forms a bilayer structure on the surface of the AuNRs and provides positive charges on the surface thereof **(Murphy et al., 2010)**. TEM provides images of the CTAB coated AuNRs that allows analysing their average size and distribution. **Image 2A**, shows the TEM micrographs of the AuNRs. The average size was determined to be 46x11 nm (Aspect ratio 4:1) (personal communication).

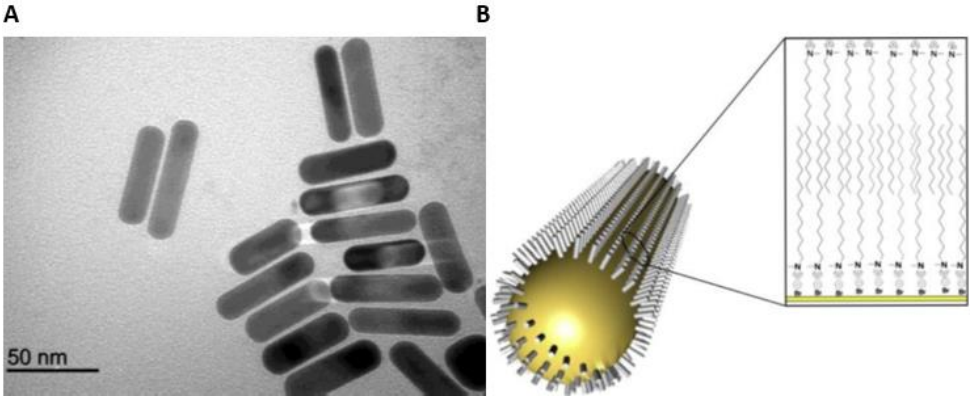

IMAGE 2. A, shows the TEM micrographs of the AuNRs. The average size was determined to be 46x11 nm (Aspect ratio 4:1). **B**, Scheme of the bilayer that constitutes the CTAB coating an AuNR **(Alkilany et al., 2012)**.

3.2.-Preparation of samples.

Samples were dissolved either in distilled water for *in vivo* safety assays or in RPMI medium for *in vitro* assays to obtain the different concentrations. These concentrations were established considering the average daily food intake (1 mg/day) and body weight (1 mg) of *D. melanogaster* to extrapolate the results to humans **(Deshpande et al., 2014)**.

3.3.- *In vivo* safety studies.

In vivo assays were performed from two different strains of *D. melanogaster*, each having a marker for the trichome on chromosome three:

Strain *mwh*. With the *mwh (multiple wing hairs)* recessive mutation, that in homozygosis exhibits multiple trichomes per cell instead of one as in the wild phenotype **(Yan et al., 2008)**.

Strain *flr* (*flr^3/TME Bd^s*). Where *flr^3* is a recessive lethal mutation in homozygosis in the zygote phase; although it may be expressed in adult somatic cells. Its phenotype consists of the appearance of amorphous trichomes. TME is a multiple inversion which prevents natural crossingover. The *Bd^s* gene is a dominant and lethal mutation in homozygosis. The edge of heterozygous wings shows irregular morphology **(Image 3) (Ren et al., 2007)**.

IMAGE 3. Phenotype of the two *D. melanogaster strains*. Upper part phenotype *mwh / mwh*, and lower one phenotype *flr^3 / Bd^3*. Photograph taken from the Genetic laboratory.

3.3.1. Maintenance of D. melanogaster strains.

The strains were maintained at 25°C and 80% humidity in glass tubes with homemade meal (0.5 grams of NaCl, 24 g of agar-agar, 100 g of yeast, 25 g of sucrose, 5 mL of propionic acid and 3.5 mL of 0.2% streptomycin sulfate solution and 1 litre of water) providing fresh medium three times per week.

Virgin females were obtained from these tubes and reciprocally crossed to perform the different assays.

3.3.2. Treatments.

After the crossing, eggs from a cross-breeding of *flr*^{3} and *mwh* parent individuals with optimally fertile flies were collected after 8 hours to obtain synchronised larvae of 72±4 hours. Transheterozygous larvae (*flr/mwh*) were washed with distilled water and separated into groups of 100 individuals. Then, each group was transferred to tubes which were fed on a chronic treatment with the different selected NPs, NRs and concentrations. A range of concentrations was checked for each compound in the assays was: AuNP (0.006; 0.0125; 0.0250;0.050; 0.100 and 0.200 nM) and CTAB-AuNRs (2E-13; 2E-12; 2E-11; 2E-10; 2E-9; 2E-8; 2E-7; 2E-6; 2E-5; 2E-4; 0.125, 0.250, 0.500,1.00 and 2.00 nM). Only the highest and lowest concentration were used in genotoxicity and antigenotoxicity assays. The negative control consisted in 4 mL distilled water and 0.85 g *Drosophila* Instant Medium (DIM, Formula 4-24, Carolina Biological Supply, Burlington, NC). Positive control consisted in 0,15 M de H_2O_2 (Sigma, Nº CAT H1009). The genotoxicity and antigenotoxicity assays were carried out following the method previously described by **(Graf et al., 1984)**. Emerging adult individuals were transferred into tubes which containing 70% ethanol for storage and subsequent analysis.

The toxicity and antitoxicity tests are performed with the same transheterozygous individuals. Emergent individuals were counted in each treatment and finally the survival percentage was determined respect to the negative (toxicity) and positive (antitoxicity) control following the next formula **(Anter et al., 2010)**:

$$\left[\frac{Number\ of\ individuals\ born\ in\ the\ treatment}{Numbers\ of\ individuals\ born\ in\ the\ negative\ control}\right] x\ 100$$

Significant differences with respect to the concurrent control in toxicity assay were assessed using the Chi-square method described previously by **Martínez Becerra and Robles González (1999)** being a concentration toxic when Chi-square values is higher than 5.02.

3.3.3. Mutations scoring.

Wings of transheterozygous individuals (*mwh flr*$^+$ / *mwh*$^+$ *flr*^{3}) and wild phenotype were mounted in slides using Faure´s solution (30 g gum arabic, 20 mL glicerol, 50 g chloral hydrate and 50 mL water) and scored under photonic microscope at 400x magnification. The *D. melanogaster* wing consists of 24.000 cells distributed in two cellular epidermal layers, dorsal and ventral **(Moraga and Graf, 1989)**. When a wing presents the wild phenotype, each cell will show a single trichome. The analysis consists of identifying clones or individual cells showing the *mhw* and / or *flr* mutant phenotype. For each mutation, we note the size and type of clone, the number of wing and the region where it has been located.

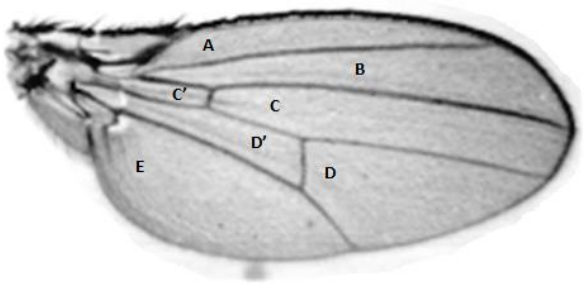

IMAGE 4. Regions of the *D. melanogaster* wings. Photograph taken from the Genetic laboratory.

3.3.4. Data evaluation and statistical analysis.

The wing mutations or spots were grouped in three different categories **(Image 5)**:

Simple small: This mutation corresponding to one or two cells exhibiting the *mwh* phenotype which occurs in the late stages of mitotic division of the larvae and corresponds to gene mutations, somatic recombination and deletions between the two markers.

Simple large: This mutation corresponding to three or more cells exhibiting the *flr*^{3} or *mwh* phenotype whose mutations take place in early stages of the larval growth and corresponds with mutations and recombination's like those previously mentioned.

Twins: This mutation corresponding to two juxtaposed clones, one of them showing the phenotype *mwh* and the other *flr*^{3}. These types of clones only correspond to the recombination between the *flr*^{3} gene and the centromere.

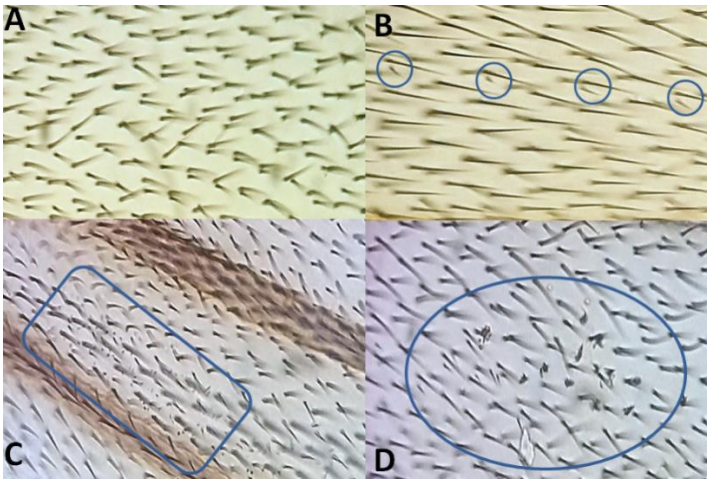

IMAGE 5. Types of clones found in the SMART test. **A**) Wild phenotype; **B**) Simple clone small *mwh*; **C**) Large simple stain *mwh*; **D**) Twin clone. Photograph taken from the Genetic laboratory.

The mutation frequencies were subjected to a multiple decision process which determine positive, inconclusive or negative results respect to the negative control **(Frei and Würgler, 1995)**. The frequencies of each mutant clone per wing were compared with their corresponding negative control using the Kastenbaum-Bowman binomial test with significance levels $\alpha = \beta = 0.05$. The inconclusive or positive results obtained then were analyzed with the non-parametric Mann-Whitney-Wilcoxon U test ($\alpha = \beta = 0.05$). The U-test consider the range of values in controls and treatments

(Jacociunas et al., 2010). From the total spot frequency, the percent inhibition (IP) is calculated for the combined treatments using the following formula **(Abraham, 1994)**:

$$\frac{Genotyxin\ treatment - combined\ treatment}{Genotyxin\ treatment} \times 100$$

3.3.5. Longevity trials.

The flies used in the lifespan assays show the same genetic background as the flies used in the genotoxicity assays to compare both results. The treated adults consisted of the F1 progeny from *mwh* and *flr*^{3} parental strains produced by a 24 h eggs-lying on yeast medium.

All experiments were carried out at 25°C and following the procedure described by **(Fernández-Bedmar et al., 2011)**. Briefly, synchronised 72±12 hours old transheterozygous larvae were washed, collected and transferred in groups of 100 individuals into test vials containing 0.21 g of DIM and 4 mL of the different concentrations of the assayed compounds. Only AuNPs were analysed. The tested concentrations were the same used in toxicity test (0.006, 0.0125, 0.025, 0.05, 0.1 0.2 nM).

Adults emerged from pupae were collected and anesthetised under diethyl ether (Panreac, Nº CAT 212770.0311 stabilized con 6 ppm de BHT QP), and 25 individuals of the same sex were selected and placed into vials containing 0.21 g of DIM (Carolina Biological Supply Company, Burlington, NC) and 1 mL of water solutions of the different tested concentrations. Medium was renovated twice a week until all individuals die and the alive individuals were counted.

3.3.6. Statistical analysis of longevity and quality of life.

Survival curves were plotted as estimated by the Kaplan-Meier method and the statistical significance of curves were assessed using the Log-Rank (Mantel-Cox) method using the SPSS 19.0 statistics software (SPSS Inc., Chicago, IL, USA).

3.4. *In vitro* safety studies.

3.4.1. Cell culture and incubation conditions.

The HL60 promyelocytic human leukaemia cell line was kindly provided by Dr. Villalba (Department of Cell Biology, University of Córdoba, Spain). Cells were grown and incubated in RPMI-1640 (Sigma, R5886) supplemented with heat-inactivated 10% foetal bovine serum (Linus, SO1805), 1% L-glutamine (Sigma, G7513) and antibiotic-antimycotic solution (Sigma, Cat No. A5955) at 37°C in humidified atmosphere of 5% CO_2 (ShelLab, Cornelius, OR, USA) **(Gallagher et al., 1979)**.

HL60 cells were passed twice a week at 2.5 x 10^5 cells/mL density in 40 mL culture bottles with a final volume of 10 mL.

3.4.2. Cytotoxicity assay.

Cytotoxicity assays were established from cell cultures with a density of 10^6 viable cells/mL. HL60 cells (1 x 10^5 cells/mL) were placed in 96 multi-well culture plates and treated with the tested concentrations of AuNPs (0.006, 0.0125, 0.025, 0.05, 0.1 0.2 nM) and CTAB-AuNRs (2E-13; 2E-12; 2E-11; 2E-10; 2E-9; 2E-8; 2E-7; 2E-6; 2E-5; 2E-4 nM) for 72 hours. To detect the IC_{50} a wide range of concentrations was studied. A negative control with RPMI medium was used. Three independent replicates were performed for each treatment. After incubation, cell viability was determined by the trypan blue dye (Sigma, T8154) exclusion test. Trypan blue was added to the cell cultures at 1:1 volume ratio and 10 µL of cell suspension was loaded into Neubauer chamber. Cells were counted with an inverted microscope (AE30/31, Motic) at 100x magnification. In addition, an estimation of IC_{50} was calculated **(Fernández-Bedmar et al., 2011)**.

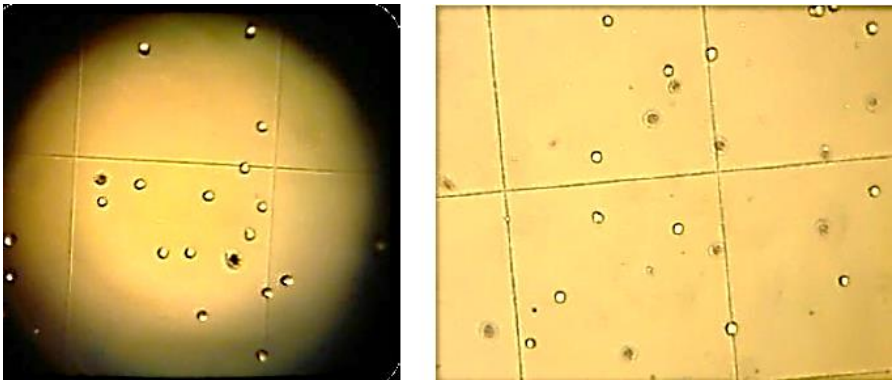

IMAGE 6. HL60 cells. Photograph taken from the Genetic laboratory.

3.4.3. Internucleosomal DNA fragmentation assay.

Apoptosis is the major mechanism of cancer suppression. To determine the mechanism by which the cytotoxic effect occurs in HL60 cells, we examine whether our compounds induce programmed cell death. The fragmentation assay is an *in vitro* test that is used to detect the ability of a compound to induce propapoptotic death, a type of active death characterized by morphological changes in cells, nuclear condensation, and apoptotic bound formation to the membrane **(Wyllie et al., 1981)**.

This assay was performed following the method described by **Merinas-Amo et al. (2013)**. HL60 cells (10^6 cells/mL) were treated with different concentrations of AuNPs (0.006, 0.0125, 0.025, 0.05, 0.1, 0.2 nM) and CTAB-AuNRs (2E-13, 2E-12, 2E-11, 2E-10, 2E-9, 2E-8, 2E-7, 2E-6, 2E-5,2E-4 nM) and incubated for 5 hours. Then, treated cells were centrifuged at 3000 rpm for 5 minutes and DNA was extracted as follows: the cell pellet was resuspended in 900 µL in lysis buffer (10 nM Tris HCl, 5 mM EDTA and 100 mM NaCl) and then added 100 µL of SDS 10% and 25 µL of proteinase K solution (20 mg/mL) and incubated shaking for 5 hours at 55°C. After this, 432 µL of 5 M NaCl was added and the samples were centrifuged at 13.000 rpm for 15 min. Supernatant was recovered into a tube and 750 µL of cold isopropanol was added to precipitate DNA. Then, samples were centrifuged at 13.000 rpm for 10 min, washed with 1 mL ethanol (70%) and DNA was dried and resuspended in 20 µL of deionised water. Finally, 0.6 µL of RNAse was added and incubated at 37°C over night. Extracted DNA was quantified

in a spectrophotometer (Nanodrop ND-1000) and 1200 ng were loaded into agarose gel electrophoresis 2% stained with GelRed and visualised under UV light.

3.4.4. Comet assay.

To perform this test, we used the method described by **Mateo-Fernández et al. (2016)**. Briefly, 10^6 HL60 cells were centrifuged and resuspended in 1.5 mL of AuNPs (0.006, 0.0125, 0.025, 0.05, 0.1, 0.2 nM) and CTAB-AuNRs (2E-10, 2E-9, 2E-8, 2E-7, 2E-6, 2E-5, 2E-4 nM) and were incubated for 5 hours in P-12 plates. After the period, the samples were transferred to eppendorf tubes, centrifuged and washed with PBS. A solid medium was prepared, which is constituted by a low melting point agarose gel and then dilutions (25 µL) were mixed with 75 µL of the agarose gel and loaded onto the slide. Quickly covered with coverslip, let the plates solidify and the slides were then immersed in an alkaline lysis solution for 1 hour and at 4° C. Then, samples were equilibrated with an alkaline electrophoresis buffer for 20 minutes. Subsequently, the electrophoresis was carried out for 8 minutes at 12 V and 400 mA. After this time, samples were washed with Tris media at pH 7.5 and allowed to dry at room temperature.

Gels were stained by adding 7 µL of propidium iodide to each sample and proceeded to the visualization in a Leica DM2500 microscope at 400X magnification.

To perform the statistical analysis of the test results, about 100 single cells of each treatment were analyzed using the Open Comet software ™ **(Gyori et al., 2014)**. The "Tail Moment" (TM), can be defined as the product of the proportion of DNA in the tail and the average distance of its migration in the tail. The data obtained from TM were analyzed using an ANOVA test and the Tukey test. To do this, we used the SPSS statistical package for Windows, version 19.0 to determine the effects of selected components on the DNA integrity of the HL60 tumor line.

3.4.5. Methylation status.

HL60 cells were treated with the highest concentration of AuNPs (0.2 nM) and CTAB-AuNRs (2E-3 nM) for 5 hours. Then, DNA was extracted similarly to previously described DNA following the protocols DNA fragmentation assay. After that, the DNA was converted with bisulphite (EZ DNA Methylation-Gold™Kit). The kit is based on a three-step reaction process between cytosine and sodium bisulphite resulting in cytosine being converted into uracil: 500 ng of DNA, in 20 µL total volume, is mixed with CT Conversion Reagent and thermo cycled at 98°C for 10 minutes and 64°C for 2.5 hours (or 4°C storage up to 20 hours). A binding buffer step following by a wash step is carried out in a IC Column to stick the DNA to the binding solution of the column. Then a desulphonation buffer is used to eliminate otherwise cumbersome precipitations. Finally, a washing buffer step and an elution buffer are used to obtain our bisulphite converted DNA (it should be stored at or below -20°C for late use).

Bisulphite-modified DNA was used for fluorescence-based real-time quantitative Methylation -Specific PCR (qMSP). The final reaction mixture with a total volume of 10

µL consisted of 5 µM of each forward and reverse primer (Isogen Life Science BV), 2 µL of iTaq™ Universal SYBR® Green Supermix (Bio-Rad, it contains antibody-mediated hot-start iTaq DNA polymerase, dNTPs, $MgCl_2$, SYBR® Green I dye, enhancers, stabilizers and a blend of passive reference dyes including ROX and fluorescein) and 25 ng of bisulphite converted genomic DNA.

PCR conditions included initial denaturalisation at 95°C for 3 minutes and amplification which consisted of 45 cycles at 95°C for 10 seconds, 60°C for 15 seconds and 72°C for 15 seconds, taking picture at the end of each elongation cycle. After that, melting curve was determined increasing 0.5°C each 0.05 second from 60 °C to 95 °C and taking pictures.

qMSP was carried out in 48 well plates in MiniOpticon Real-Time PCR System (MJ Mini Personal Thermal Cycler, Bio-Rad) and were analysed by Bio-Rad CFX Manager 3.1 Software. The housekeeping primer Alu-C4 was used as a reference to correct for total DNA input, also negative controls and multiple water blanks were used. Each sample was analysed in triplicate. Alu-C4 and the target repetitive elements Alu-M1, LINE-1 and Sat-α were obtained from Isogen Life Science and their sequences are shown in **Table 1.**

Primer [1]	Forward primer sequence 5´to 3´(N)	Revese primer sequence 5´to 3´(N)
ALU-C4	GGTTAGGTATAGTGGTTTATATTTGTAATTTTAGTA (-36)	ATTAACTAAACTAATCTTAAACTCCTAACCTCA (-33)
ALU-M1	ATTATGTTAGTTAGGATGGTTTCGATTTT (-29)	CAATCGACCGAACGCGA (-17)
LINE-1	GGACGTATTTGGAAAATCGGG (-21)	AATCTCGCGATACGCCGTT (-19)
SAT-α	TGATGGAGTATTTTTAAAATATACGTTTTGTAGT (-34)	AATTCTAAAAATATTCCTCTTCAATTACGTAAA (-33)

TABLE 1. Primers information **(Weisenberger et al., 2005)**.

The results of each CT were obtained from each qMSP. Data were normalised with the housekeeping Alu-C4 using the **Nikolaidis et al. (2012)** and **Liloglou et al. (2014)** comparative CT method (ΔΔCT. One-way ANOVA and post-hoc Tukey´s tests were used to evaluate the differences between the tested compounds, repetitive elements and concentrations with SPSS Statistics for Windows, Version 19.0 (IBM 2010).

3.5. References software and exponential notation.

Ednote X7 software was used to manage all cited references according to the journal "Toxicology letters". Exponential notations were fit to statistics programs.

4. RESULTS.

4.1. *In vivo* safety studies.

4.1.1. Toxicity and antitoxicity assays.

Relative percentage of emerging adults after treatements with different concentrations of AuNPs and CTAB-AuNRs are shown in **Figure 2**. All studied concentrations of AuNPs **(Figure 2.A)** and CTAB-AuNRs **(Figure 2.C)** showed significant differences with respect to the negative control. However, LD_{50} was not reached at any assayed concentration.

The antitoxicity results in *D. melanogaster* are shown in **Figure 2**. After combined treatments with hydrogen peroxide as genotoxine, only AuNP at 0.05 nM was significantly different to the positive control **(Figure 2.B)**. On the other hand, all concentrations of CTAB-AuNRs except the higher one (2 nM), were significantly different with respect to the positive control **(Figure 2.D)**.

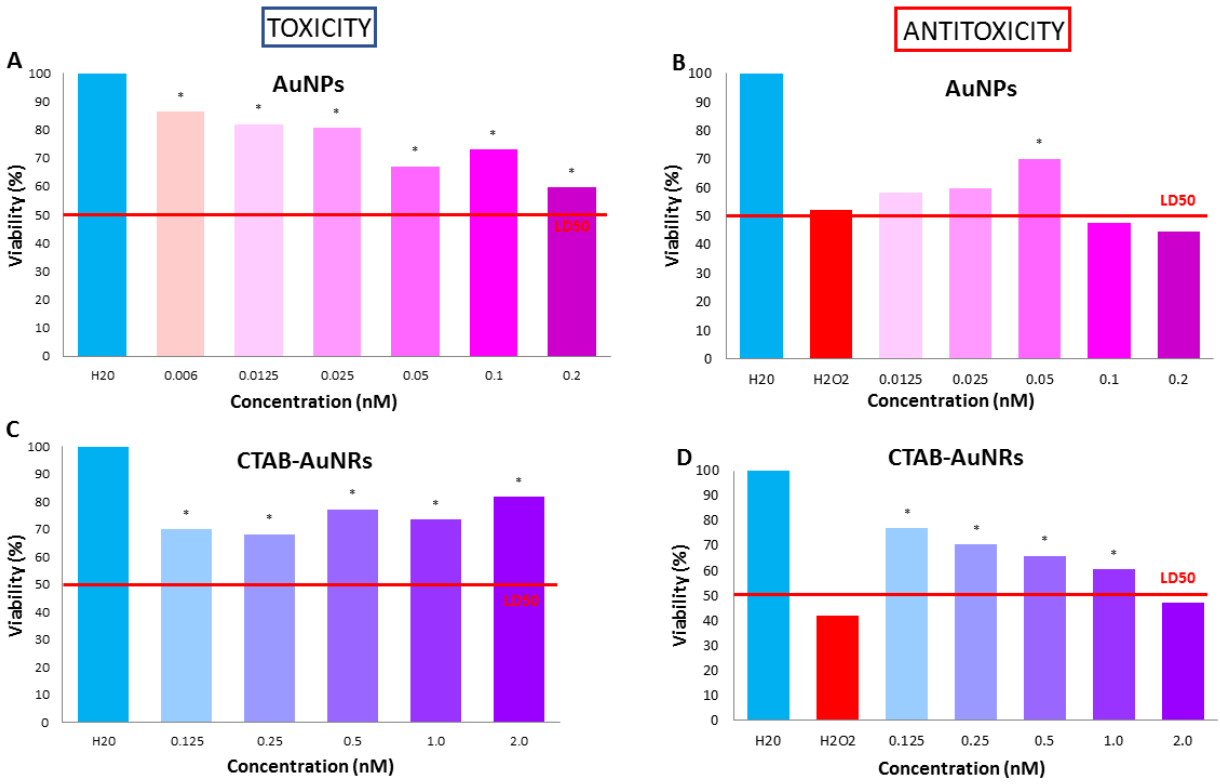

FIGURE 2. Results of toxicity and antitoxicity assays in *D. melanogaster*. Percentage of adults emerging after simple treatment with AuNPs (**A**) and CTAB-AuNRs (**C**). Percentage of adults emerging after combined treatment with H_2O_2 (0.15M) and AuNPs (**B**) and CTAB-AuNRs (**D**). Asterisks (*) indicate differences with respect to the negative control: (*) $\chi^2 > 5{,}02$ **(Martínez Becerra and Robles González, 1999)**.

4.1.2. Genotoxicity and antigenotoxicity.

Table 2 shows the results obtained in the SMART test using *D. melanogaster* as genetic animal model. Distilled water (H_2O) was used as negative control exhibiting a mutation frequency per wing of 0.080. This value is found within the range obtained in previous studies **(Fernández-Bedmar et al., 2011)**, demonstrating the reproducibility of the SMART test in imaginal discs of *D. melanogaster*, and thus allowing the genotoxic study of the tested samples. This genotoxine exerted a high genotoxic level at the tested concentration (0.15 M), with a total mutation frequency of 0.350. Therefore, the accuracy of this assay has been demonstrated. After applying the binomial Kastenbaum-Bowman Test, the genotoxicity results of AuNPs and CTAB-AuNRs were inconclusive. Hence Mann-Whitney U-test was applied to resolve the inconclusive results, simple treatments were non-genotoxic at the assayed concentrations since no significant differences were found with respect to the negative control. According to antigenotoxicity assay, only the higher concentration of AuNPs was non-antigenotoxic at the higher concentration.

Table 2. Genotoxicity and Antigenotoxicity of AuNPs and CTAB-AuNRs in the SMART Test.

	Clones per wings (number of spots) [1]					
Compound	Number of wings	Small single spots (1-2 cells) m = 2	Large single spots (>2 cells) m = 5	Twin spots m = 5	Total spots m = 2	Mann-Whitney Test [3]
H_2O	40	0.005(2)[2]	0.075(3)	0	0.080(5)	
H_2O_2 [0.15 M]	40	0.325(13)	0.025(1)	0	0.350(14) +	*
SIMPLE TREATMENT						
AuNPs (nM)						
[0.006]	38	0.079(3)	0.079(3)	0	0.158(6) i	Δ
[0.2]	38	0.132(5)	0	0	0.132(5) i	Δ
CTAB-AuNRs (nM)						
[0.125]	38	0.079(3)	0.026(3)	0	0.105(4) i	Δ
[2]	38	0.132(5)	0.079(3)	0	0.211(8) i	Δ
COMBINED TREATMENT						
AuNPs (nM)						
[0.006]	32	0.281(9)	0.031(1)	0	0.312(10) β	
[0.2]	24	0.33(8)	0.042(1)	0	0.372(9) λ	Δ
CTAB-AuNRs (nM)						
[0.125]	38	0.158(6)	0(0)	0	0.158(6) β	
[2]	40	0.275(11)	0(0)	0	0.275(11) β	

1)Statistical diagnosis according to **Frei and Würgler (1988)**. + (positive) and i (inconclusive) versus negative control; β (significantly different) and λ (inconclusive) versus positive control. m: multiplication factor. Kastenbaum-Bowman Test without Bonferroni correction, probability levels α = β = 0.05. **2**) Number of spots or clones in parentheses. **3**) Inconclusive results were resolved using Mann-Whitney U-test. Delta marker (Δ) means no differences between the treatments and the concurrent control.

As shown in **Figure 3**, AuNPs highest concentration induced a non-significant synergic effect with the H_2O_2 genotoxine. On the other hand, CTAB-AuNRs exhibited the highest percentage of inhibition (55%) at the lowest assayed concentration in negative dose-dependent effect.

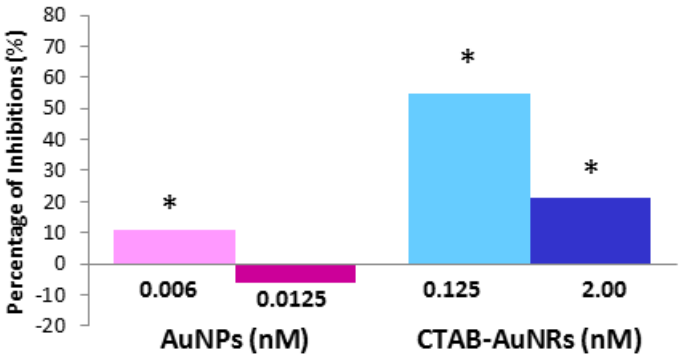

FIGURE 3. Inhibition Percentage of AuNPs and CTAB-AuNRs at different tested concentrations against H_2O_2-induced genetic damage.

4.1.3. Longevity.

Kaplan-Meier method was used to obtain the lifespan curves for each concentration of AuNPs (**Figure 4** and **Table 3)**. The highest tested concentration showed a significant reduction of estimated means of lifespan around 10 days with respect to the concurrent control, decreasing the life expectancy of *D. melanogaster*.

The 25% of individual survival at the top of the lifespan curves was studied for ascertaining on the quality of life of the *D. melanogaster* chronically treated in the longevity assay. The results obtained reported an increase of the estimated mean of quality of life with respect to the concurrent control roughly 9 and 14% at 0.0125 and 0.025 nM (lowest concentrations), respectively. Contrarily, the highest concentrations (0.05 and 0.2 mM) significantly decreased the quality of life around 15 and 7%, respectively.

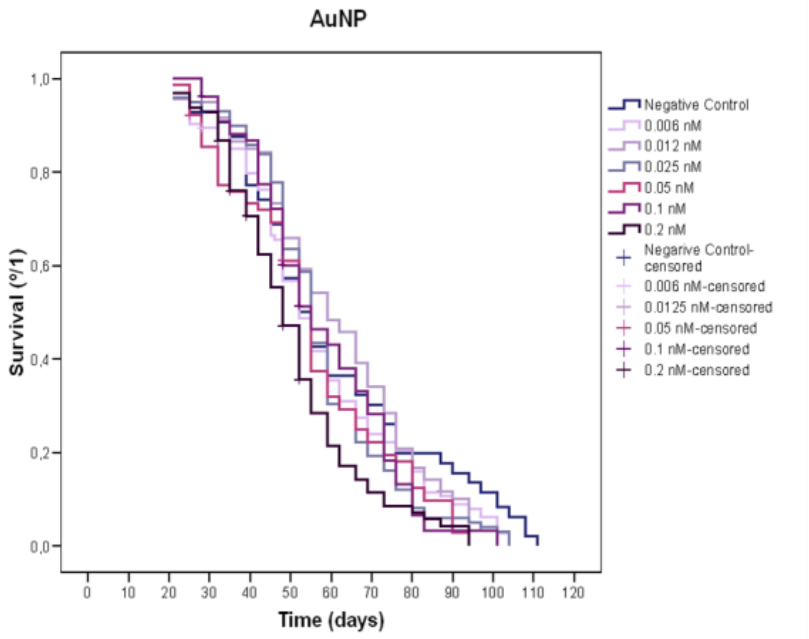

FIGURE 4. Survival curves of *D. melanogaster* fed with different concentrations of AuNPs over time**.**

TABLE 3. Effects of different treatments of AuNPs on the survival time of *D. melanogaster*.

	Lifespan Media (Days)	Difference from lifespan (%) [a]	Healthspan 75th percentile (Days)	Difference from Healthspan (%) [a]
Dose (nM)				
0	59.9±2.5		33.2±1.5	
0.006	56.9±2.0	-5	31.7±1.5	-5
0.0125	61.1±1.9	2	36.1±1.6*	9
0.025	57±1.8	-5	38.0±1.8*	14
0.050	54.6±2.3	-9	28.2±0.8*	-15
0.100	57.8±2.0	-4	36.2±1.3	9
0.200	50.3±1.8***	-16	30.9±0.6*	-7

a) The difference was calculated by comparing individuals treated with concurrent water control. Positive numbers indicate lifespan and healthspan increase and negative numbers indicate lifespan and healthspan decrease. * P≤0.05, ** p≤0.01, *** p≤0.001: Significance obtained using the log rank test (Mantel-Cox).

4.2. *In vitro* safety studies.

4.2.1. Cytotoxicity.

Figure 5 shows the cell viability (%) of HL60 treated with AuNPs and CTAB-AuNRs at different concentrations. None of the tested substances were cytotoxic to the promyelocytic HL60 cell line since the IC_{50} was not reached at the assayed concentrations.

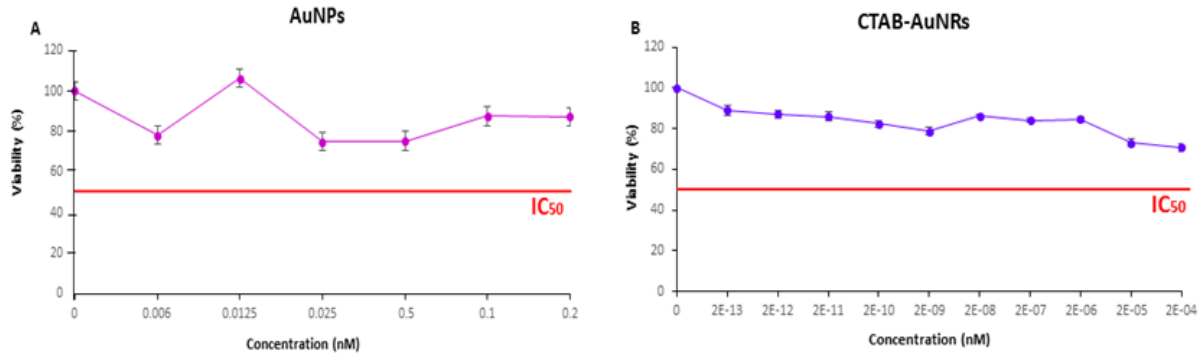

FIGURE 5. Viability of HL60 cells treated with AuNPs (**A**) and CTAB-AuNRs (**B**) for 72 hours.

4.2.2. DNA fragmentation test.

The electrophoresis of genetic DNA integrity in HL60 cells treated with different concentrations of AuNPs and CTAB-AuNRs are shown in **Image 7**. None of the tested concentrations for AuNPs induced the typical ladder pattern of cells with fragmented internucleosomal DNA. **(Image 7A)**. On the other hand, internucleosomic fragment (size: 200bp or multiple of these) were observed after treating with CTAB-AuNRs at median concentrations (2E-10, 2E-9 and 2E-7 nM) **(Image 7B)**.

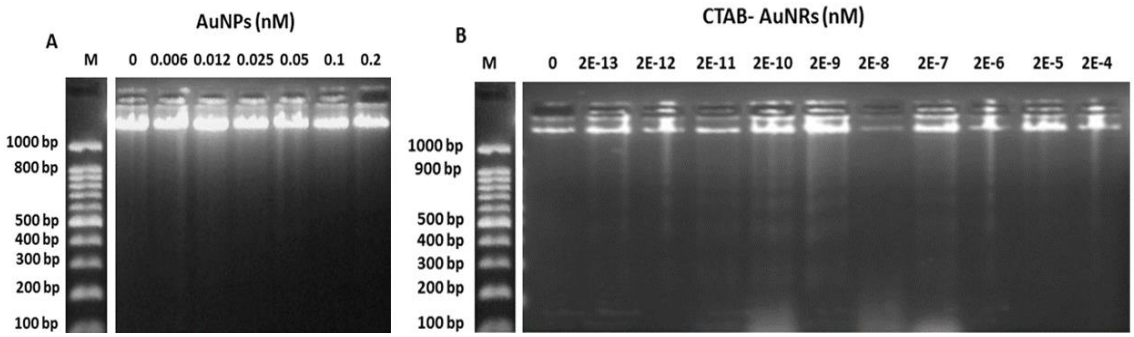

IMAGE 7. Internucleosomal DNA fragmentation after 5 h of treatment with AuNPs (**A**) and CTAB-AuNPs (**B**). Letter M means weight size marker.

4.2.3. Comet assay.

SCGE test was carried out to determine if the selected nanoparticles could induce single-strand breakage in the DNA of the HL60 cells. The tested concentrations were selected based on the results obtained in the previous *in vitro* assays (cytotoxicity and DNA internucleosomal fragmentation). Although all tested concentrations of the studied nanoparticles were different significantly to the negative control after 5 h exposure, only the highest concentrations of AuNPs (0.100 nM and 0.200 nM) and CTAB-AuNRs at 2E-5 and 2E-13 nM could induce an increase in the TM parameter with respect to the negative control **(Figure 6.A; 6B)**. However, all TM value obtained were lower than 1 a.U.

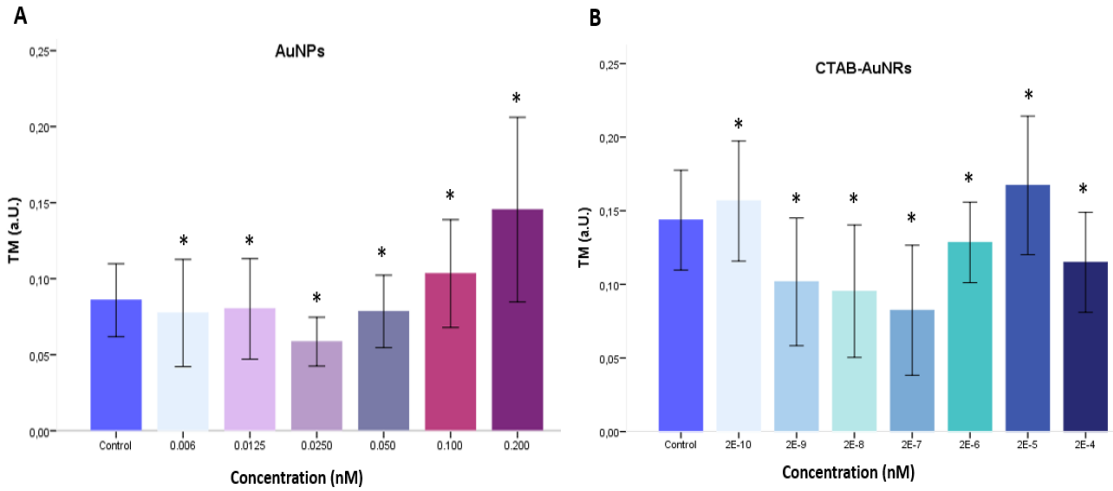

FIGURE 6. Results of comet assay. TM mean value of HL60 cells treated with different concentrations of AuNPs (**A**) and CTAB-AuNRs (**B**). The plot shows mean TM values and standard errors. DNA migration is analyzed with the Tail Moment (TM) parameter. The graph shows the values of TM mean, standard errors and significance compared to the control group, p≤0.05 (*), p≤0.01 (**) and p≤0.001 (***).

4.2.4. Methylation status evaluation.

The relative normalised methylation status (RMS) of the three repetitive sequences (AluM1, LINE-1 and Sat-α) in HL60 cell line treated with the tested compounds is shown in **Figure 7**. AuNPs significantly induced hipermethylation at the tested concentration (0.2 nM) in Alu-M1 and LINE-1 sequences. On the other hand, whereas CTAB-AuNRs decreased the methylation status of Sat-α repetitive element in HL60 cell line, the methylation status of Alu-M1 was increased.

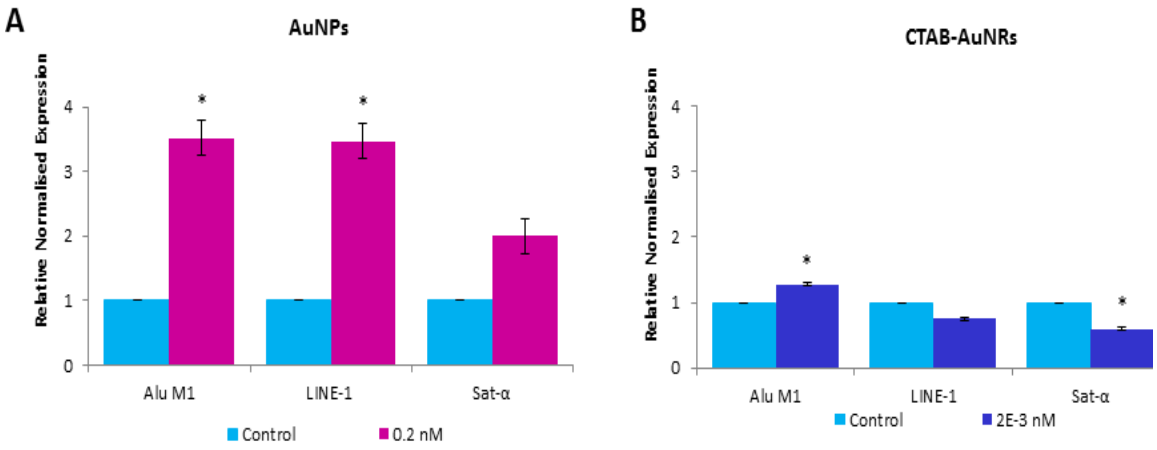

FIGURE 7. Relative normalized expression data of each repetitive element; (**A**) AuNPs and (**B**) CTAB-AuNRs. Asterisks (*) indicate different RMS means compared to the concurrent control for each tested concentration: p≤0.05 (*), p≤0.01 (**).

5. DISCUSSION.

5.1. *In vivo* safety studies.

The expected potential benefits by Nanotechnology in various fields have led to a rapid increase of the presence of engineered nanomaterials contained a high number of commercials goods. The side biological effects of NPs should be known in deep as they are intended to widely use in biomedicine for therapeutic purposes.

To perform the *in vivo* assays, we used the model organism *D. melanogaster*. It has been the most important model organism used in Biology and Biomedicine in the last century. Currently, it is being used in Nanotoxicology studies although it has only been exploited a part of its potential to know the macroscopic/teratogenic damages.

Moreover, this organism can also be used to analyze the molecular interactions pathways involved in the responses between nano-bio agents **(Vecchio, 2015)**. Nowadays it is known that 75% of the genes related to human diseases have a homologous functional fly **(Lloyd and Taylor 2010)**, for instance genes responsible for development and neurological disorders, cancer, cardiovascular and metabolic diseases among others **(Bier, 2005)**. Although phenotypes are not very similar between humans and flies, *D. melanogaster* is considered the choice model organism even in those disciplines where mammals model organisms are considered irreplaceable both in Pharmacology **(Pandey and Nichols, 2011)** and Genotoxicology **(Mukhopadhyay et al., 2004)**.

5.1.1. Toxicity and antitoxicity.

Toxicity tests are the initial step to evaluate the safety of different compounds. There are several parameters to determine the toxicity of a substance, including the most commonly used and known as Lethal Dose 50 (LD_{50}), which determines the concentration at which half of a given population dies **(Shetty Akhila and Alwar, 2007)**. According to this parameter, although all test concentrations of AuNPs and CTAB-AuNRs were significantly different from the negative control, none could be considered as toxic since the results obtained exceed this value. **Vecchio et al. (2012b)** observed that AuNPs induce *in vivo* toxicity in this same model organism but the toxic effects of these NPs are directly related to concentration regardless of size. It is noteworthy that in their case they fed flies with AuNPs 3 µg/g/day.

Size, concentration and chemical surface influences the outcome of toxicity. **Kim et al. (2013a)** found toxicity of AuNPs (0.08 to 50 mg /L) using zebrafish embryos as a model organism. Our results do not agree with these authors due to the different organism model, size, way of administering or even in the shape of synthesis and ligand.

We must consider that the golden nanoparticles with the shape of rod-shaper gold (AuNRs) can also present toxicity. CTAB-AuNRs toxicity results showed significant differences with respect to the negative control at all concentrations in our study. Nevertheless, none could be considered as toxic since they were lower than the LD50. Numerous studies have shown that the toxicity of NRs depends of the concentration, size, shape and coating used. For this reason, there are controversial results about NRs and in *in vivo* assays this fact should be considered with caution. **Wan et al. (2015)** showed that CTAB-AuNRs had low levels of toxicity at 560 µg/kg/day in mice for two weeks. Other study showed that PEG-NRs were less toxic than CTAB-NRs. Thus, it is assumed that PEG-AuNRs are better for biomedical applications **(Niidome et al., 2007)**. Likewise, other authors determined that AuNRs could disrupt genes related to apoptosis, cell cycle, inflammation and circadian rhythm control **(Balasubramanian et al., 2010).**

It should be noted that there are no references on antitoxicity tests carried out with the NPs used.

5.1.2. Genotoxicity and antigenotoxicity.

The genotoxicity assays showed that neither AuNPs nor CTAB-AuNRs were genotoxic. These results disagree with the studies carried out by other authors that showed genotoxic activity for NPs. Dramatic phenotypic changes were observed in *D. melanogaster* offspring after exposing to AuNPs. These effects are also observing even in the *Caenorhabditis elegans* model organism **(Kim et al., 2013b)**. **(Demir et al., 2013)** stated non-genotoxicity of of titanium, aluminum and zirconium nanoparticles in the SAMRT.

Hydrogen peroxide is the best mediator of oxidative stress and a potent mutagenic agent responsible for the prevalence of some kinds of inflammatory cancer- associated processes **(Konat, 2003)**. This genotoxic agent is endogenously produced in our cells during oxidative phosphorylation being a natural source of oxidative damage, causing a spectrum of DNA damage, including both single and double chain breaks **(Benhusein et al., 2010)**.

The genotoxic effects of NPs do not depend only on the size of the NP, the coating and the route of exposure but also the period of exposure time. A genotoxicity assay carried out in rats exposed for 28 days to AgNPs did not prove to be genotoxic **(Klien and Godnić-Cvar, 2012)**. **Vales et al. (2013)** used NPs of cobalt (0.1 to 10 nM) feeding transheterozygous larvae showing a dose-dependent genotoxic effect. It should be noted that there are no references on genotoxicity and antigenotoxicity tests of the CTAB-AuNRs used.

5.1.3. Longevity.

A strong concentration-dependent reduction of lifespan and fertility performance was observed by **Vecchio et al. (2012b)** when administering AuNPs to *D. melanogaster* although different sizes from us were used (5-80 nm). These results agree with those obtained by **Pompa et al. (2011)** using the same model organism and particles despite of the different concentration range used (1.9 to 380E-3 pmol/L). Rodent model let similar adverse effects. Mice injected intraperitoneally AuNPs (8 mg/kg/week) decrease the half-life **(Chen et al., 2009)**. It should be noted that neither the model organisms used nor the sizes of NPs are coincident with that of our assay.

It should be noted that our results show some differences regarding the results obtained by other authors considering that size of the NPs, form of administration, period or even the number of individuals could be the cause of this discrepancy.

5.2. *In vitro* safety studies.

5.2.1. Cytotoxicity.

Numerous cytotoxicity assays have been carried out using different cell types and NPs, both with modifications in size, coverage, shape, etc. And. again, much controversy is found. In our case, neither the AuNPs nor the CTAB-AuNRs showed to be toxic for promyelocytic HL60 cells. These results coincide with those of **Shukla et al. (2005)**,

whose objective was to determine the cytotoxicity and immunogenicity of the AuNPs, against RAW264.7 macrophages cells, obtaining as a result that these nanoparticles are non-toxic since they do not reveal the secretion of pro-inflammatory cytokines. Therefore, the non-cytotoxic and non-immunogenic properties of the AuNPs have been demonstrated. An *in vivo* study by **Shukla et al. (2005)** , whose objective was to study the biodistribution of AuNPs after their intravenous and peritoneal injection to mice, showed that nanoparticle are captured in some way by cell membranes through endocytosis. In addition, nanoparticles do not cross neither the placental barrier nor the blood-brain barrier, and do not accumulate in the kidney, brain, lung, ovaries, placenta or fetal liver **(Sadauskas et al., 2007)**.

The size and surface characteristics of nanoparticles are key points for the possible toxicity. One of the most important studies was carried out by **Pan et al. (2007)**, which was based on determining what relationship existed between the size of the nanoparticles and their toxicity. They used four different cell types (fibroblasts, macrophages, epithelial cells and melanoma cells) and AuNPs of different size (0.8 to 15 nm) and demonstrated that the size of the nanoparticles is involved in the response and mechanism of cell death. In addition, the surface characteristics of NPs have been shown to influence their toxicity: cationic nanostructures usually have a greater toxicity than anionic ones, being anionic ones with a nucleus of almost 2 nm usually non-toxic whereas cationic equivalents are usually toxic **(Goodman et al., 2004)**.

Some authors have demonstrated the cytotoxicity of AuNPs for HeLa cell line **(Pan et al., 2009)** and for red blood cells and even for *E.coli* **(Goodman et al., 2004)**. We must emphasize that neither the size nor the concentration nor the time of exposure are coincident with ours.

Rayavarapu et al. (2010) found toxicity of CTAB-AuNRs in the HL60 cell line. It should be noted that their results do not coincide with ours since the time of exposure of the treatment is not coincident (24 hours in their case and 72 hours in our case). Other studies by **Takahashi et al. (2006)**, **Yu et al. (2007)**, **Wang et al. (2008)** showed that NRs are toxic. It should be noted that the cell lines used respectively were HeLa, MCF10A and HaCat. In addition, the concentrations of CTAB-AuNRs are different in each of the trials along with the exposure time and the procedure. However, a study carried out by **Wan et al. (2015)**, showed that CTAB-AuNRs have low cytotoxicity and do not influence cell death.

5.2.2. DNA fragmentation test.

Apoptosis is the major mechanism of suppression of cancer. With the purpose of determining the mechanism by which the low cytotoxic effect occurs in the HL60 cells, we studied whether our NPs and NRs induce programmed cell death in some extent.

Our results, in which no DNA fragmentation was observed when the cells were treated with AuNPs, did not coincide with the results obtained by **Sabella et al. (2011)** since they observed a significant fragmentation in the DNA. It is important to note that the HeLa cell line was used in this trial. **Wan et al. (2015)** showed that CTAB-AuNRs can

cause cellular apoptosis and autophagy by damaging mitochondria and activating ROS. In our case, not all the concentrations tested induced cellular apoptosis.

Coated AuNPs cause DNA fragmentation in addition to a significant modification in the *Drosophila* expression levels of the genes involved in the responses to stress, thus recognizing DNA damage and apoptosis **(Vecchio et al., 2012b)**.

Mouse fibroblasts (NIH3T3), embryonic mouse and Human acute monocytic leukemia cell line treated with AgNPs undergo apoptosis and generation of reactive oxygen species was observed **(Ahamed et al., 2008).**

5.2.3. Comet assay.

Alkaline SCGE is performed to detect DNA damage **(Forchhammer et al., 2012)**, which are widely used to determine whether cells are undergoing apoptotic and/or necrotic pathways **(Olive and Banáth, 2006)**. It is assumed that apoptosis occurs when treatments induces a TM > 30 (hedgehog pattern) whereas control cells remain lower than 2 (no tails). On the contrary, necrosis shows a short comet-tail pattern since, much of the damaged DNA remains in the comet head **(Fairbairn and O'Neill, 1995)**.

In our case, all concentrations tested for both AuNPs and CTAB-AuNRs showed significant differences from the negative control.

Many nanoparticles induce comets in different cellular types. NPs of silica induce DNA damage in normal cells of *D. melanogaster* larvae **(Demir et al., 2015)**. A comet assay using cells from the lung tissue of Wistar rats prior to administration of AuNPs served to determine that no relevant DNA damage was found **(Schulz et al., 2012)**. Induction of DNA damage, oxidative stress and proapoptotic markers were obtained as a result after applying AgNPs to zebrafish liver cells **(Choi et al., 2010)**. Different tests have been carried out using AuNPs to determine if they can produce damage or not in the DNA. A study carried out with HeLa cells and this type of NPs has served to determine that they are able to produce apoptosis and cell death through necrosis **(Pan et al., 2009)**. It should be noted that there are no references on genotoxicity and antigenotoxicity comet tests with the CTAB-AuNRs used.

5.2.4. Methylation status evaluation.

Cancer cells genomes are globally hypomethylated inducing transposable element activity and thus triggering genome instability **(Lopez-Serra and Esteller, 2008)**. On the other hand, it is known that the silencing of tumour suppressor genes is closely associated with hypermethylation **(Qin et al., 2009)**. Repetitive elements are highly methylated in somatic normal cells contributing to a global genomic hypermethylation **(Weisenberger et al., 2005)** suppressing the transposable activity of repetitive elements. Three different repetitive elements: LINE-1, Alu-M4 and Sat-α were studied. Long interspersed nuclear elements (LINE) are abundant retrotransposons and representing LINE-1 about 17 % of the human genome with a non-random distribution

by accumulating primarily in G-positive bands, which are AT-rich regions of chromosomes **(Lander et al., 2001).** LINE-1 elements are also accumulated in regions of low recombination rate mainly in X-chromosome **(Boissinot et al., 2001)**. Alu elements belong to the SINE family (Short Interspersed Nuclear Elements), being the most abundant (accounting about 10 % of the whole human genome **(Weisenberger et al., 2005)** and predominantly present in non-coding and GC-rich regions **(Lander et al., 2001)**. Sat-α (Satellite alpha DNA repeats are composed of tandem repeats of 170 bp DNA sequences, are AT-rich regions and represent the main DNA component of every human centromere, constituting about 5 % of total human DNA **(Lander et al., 2001)**. Therefore, examination of the methylation status of LINE-1, Alu and Sat-α regions has served as an approach for measuring global methylation levels since 32 % of the human genome has been evaluated **(Martínez et al., 2012)**.

It has been demonstrated that the expression of satellite sequences is associated with a hypomethylation triggering cancer cells. Therefore, methylation process in satellite sequences is a potential mechanism for silencing its satellite expression in transformed cells **(Ting et al., 2011)**. On the other hand, human therapies against cancer are based on hypomethylation agents since this therapy is highly related to gene silencing thus this fact could activate tumour suppressor genes and be a positive highlight **(Wild and Flanagan, 2010)**.

To our knowledge, this is the first attempt evaluating the ability of AuNPs and CTAB-AuNRs for modulating the epigenome thus there is not any information related with assay using AuNPs and CTAB-AuNRs on scientific database.

6. CONCLUSION.

Due to the world-wide use of NPs, it is important to know their biological effects both *in vivo* and *in vitro*.

- AuNPs and CTAB-AuNRs are safe as they are not genotoxic in the SMART Test of *Drosophila*.
- None of the NPs and NRs reached the LD_{50}
- The AuNPs can produce a decrease in the longevity of *D. melanogaster* as we increase the concentration.
- None of NPs exhibit cytotoxic activity against HL60 tumour cells growth.

- AuNPs can induce hypermethylation in repetitive sequences of HL60 cells.
- It would be necessary to deeply carry out tests to determine what type of NPs are most suitable to be used in Biomedicine.

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