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IN VITRO STUDY OF CYTOTOXIC AND DNA-PROTECTIVE EFFECTS OF *THYMUS GUYONII* EXTRACT

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Abstract

The present work aims to identify some biological activities of *Thymus guyonii* an endemic species belonging to the Lamiaceae family distributed in Algerian Sahara. The targeted activities in this work were the cytotoxic activity evaluated by Thiazolyl Blue Tetrazolium Bromide assay (MTT) using the human leukemia monocytic cell line (THP-1 cells), the DNA damage induced by homocysteine (Hcy) and the protective effect of the extract were revealed using the single cell gel electrophoresis (comet assay). The results obtained allowed to highlight in particular the biological activities of the extract. The MTT test showed that the concentrations of 100µg/mL and 500µg/mL of the extract were not cytotoxic and cell viability decreases only when the concentration is very high (1000µg/mL). The extract of *Thymus guyonii* showed a very potent protection activity against DNA damage induced by Hcy (100µM) at both concentrations 1µg/mL and 10µg/mL. In fact, *Thymus guyonii* extract seems to have a high ability to protect cells against DNA damage induced by Hcy.

Keywords: *Thymus guyonii, Homocysteine, Cytotoxic, DNA protective effect.*

Introduction

During the past decade, a great deal of attention has been focused on natural antioxidants such as vitamins, flavonoids and polyphenols in order to prevent and treat different kinds of diseases. Moreover, many studies have shown that ROS can induce DNA strand breaks as well as structural changes in lipids and proteins causing human disease [1].

Lamiaceae family species are considered important because of their use in folk medicine, culinary and flavoring throughout the world. Their interesting bioactivities are attributed mainly to essential oils, polyphenols and terpenes. It's known that natural antioxidants from plants such as polyphenols play an important role in the protection of cells from oxidative damage and consequently induce anticancer activities including pro-apoptotic, anti-angiogenic, and immunostimulatory effects [2]. Thyme "Zaatar" is one of the most used herbs as tea and spice, added to some food to give an acceptable flavor. It is widely used in traditional medicine in several forms: leaves are used in infusion against cough, in decoction to cure headaches, hypertension and gastritis, externally used for healing [3]. Extracts have been used for the treatment of several respiratory diseases, also as antiseptic, antispasmodic, antitussive, antimicrobial, antifungal, antiviral and antioxidative [4].

Hyperhomocysteinemia or elevated plasma homocysteine (HHcy) is an independent risk factor for atherosclerotic vascular disease and atherothrombosis[5,6]. It has been shown to induce several functional changes in vascular cells, leading to endothelial cell dysfunction [7,8], smooth muscle cell proliferation, monocyte attraction [9], monocyte adhesion to endothelial cells [10] and monocytes differentiation into macrophages[11]. Furthermore, it has been suggested that HHcy may promote cardiovascular disease through the activation of pro-inflammatory factors, endoplasmic reticulum stress and oxidative stress, which are considered underlying mechanisms of DNA damage and carcinogenesis [12-14]. HHcy is also able to induce DNA fragmentation and caspase activation triggering cell necrosis and apoptosis [15,16]. Other researchers have proposed a potential role of HHcy in some other diseases including age-related muscular degeneration, cognitive abnormalities, osteoporosis, neural tube defects, depression, schizophrenia, and inflammatory bowel disease and colon tumors [17].

Pharmacological studies have confirmed that extracts of plants belonging to *Thymus* genus have strong antibacterial, antimicrobial, antifungal, anti-inflammatory, spasmolytic activities and other functions [18,19].

In this study, we tried to investigate new biological activities as well as to evaluate the natural antioxidant potential of *Thymus guyonii* de Noé, an endemic Algerian species belonging to the Lamiaceae family and locally known for its antispasmodic, expectorant and antibacterial activities [20]. The species is distributed mainly in the Sahara, and it has not been sufficiently investigated.

For the first time, we evaluated *in vitro* the cytotoxic, the anti-genotoxic activities of the butanolic extract, these activities were analyzed using THP-1 cells in the presence and absence of the homocysteine which considered as genotoxicagent.

Material and Methods

Plant material

Thymus guyonii de Noé which is an endemic plant of Algerian Sahara was collected from Zelfana- Ghardaia, during the flowering stage, the species was authenticated by Prof. G. De Belair (University of Annaba, Algeria). The leaves were washed and dried for 7 days at room temperature, and then they were powdered by a blender.

Extraction

Dried and powdered aerial parts (500 g) of *Thymus guyonii* were macerated in a hydroalcoholic mixture MeOH-H_2O (7:3 v/v) for 72 h, repeated each 24 h at room temperature. The three hydroalcoholic extracts recovered were combined and

concentrated to dryness in a rotary vacuum evaporator. The crude extract was subjected to successive liquid-liquid extractions with organic solvents of increasing polarity: petroleum ether, chloroform, ethyl acetate and n-butanol. The partitioning was performed in a separator funnel of 1 L by confronting each solvent with the aqueous phase and shaking vigorously with the rotary shaker for 30 min. the aqueous phase and the phase loaded with specific molecules are recovered separately. In the end the 4 organic phases recovered were evaporated under reduced pressure and weighed, the extracts obtained were stored at 4 °C [21].

Cell culture

THP-1 cells (Rockville, MD, USA) cells were grown in RPMI 1640 medium supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 µg/mL streptomycin and 50 U/mL penicillin (Sigma, Milan, Italy) at 37 °C in a 5% CO_2 95% air humidified atmosphere. The cells were maintained at the concentration of $5x10^5$ for two weeks, with the medium being renewed every two days. Then, THP-1 cells were seeded in 24 or 96-well plates and treated for 24 h with Hcy (100 µM) with and without *Thymus guyonii* extract, which was added to culture medium 30 min prior of Hcy addition. *Thymus guyonii* extract was dissolved with dimethyl sulfoxide (DMSO) at the concentration of 5 mg/ml.

Cell viability assay

To evaluate the extract and Hcy effects on cell viability, the MTT (3-(4, 5- methylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide) reduction assay was performed. After treatments, monocyte cells, grown in 96 well culture plates, were incubated with fresh medium containing MTT (0.5 mg/mL) at 37 °C for 4 h. Then, insoluble formazan crystals were dissolved in 100 µl of acidic isopropanol (0,04 M HCl in absolute isopropanol) for 1 h. The optical density in each well was evaluated by spectrophotometrical measurement at 540 nm with a sunrise microplate reader (Tecan Italia, CologNoMonzese, Italy). The percentage of cell viability was measured by the absorbance ratio of treated cell vs untreated cells.

Comet assay

The DNA damage was assessed by the alkaline single-cell gel electrophoresis "comet assay" method as described by Di Pietro [22]. Undamaged DNA migrates in the gel at a slower rate and remains within the confines of the nucleoid and thus appears as an intact comet head. DNA fragments migrate relatively faster and form a comet-like tail whose fluorescent intensity and shape can be related to the level of damage [23]. The extent of the damage can be quantified by measuring the length, shape, intensity of staining of the tail in comparison to the "head" of the comet. After 24 h of treatment, THP-1 monocytes were washed twice with PBS and then immediately mixed with 0.5 % low melting point agarose at 37 °C at the concentration of $10x10^4$ cells, 50 µl of this suspension were pipetted onto the pre-coated glass slides (slides were already dipped briefly into 1% hot (60 °C) normal melting agarose (NMA) prepared in phosphate-buffered saline (PBS). The slides were dried overnight at room temperature and then stored at 4° until used). And allowed to set at 4 °C for 10-15 min. After that, another 50 µl of the low melting agarose (0,5%) was layered onto the previous cell-containing layer and allowed to solidify. The samples were then placed in a freshly prepared lysis solution (100 mM EDTA, 2.5 M NaCl, 10 mM Tris, 1% Triton X-100, pH 10, 4 °C) for at least 1 h. Following lysis, the slides were drained and incubated in electrophoresis buffer (10 N NaOH, 200 mM EDTA, pH >13) for 20 min for DNA unwinding, followed by electrophoresis in the same buffer for 30 min (25 V, 300 mA). After electrophoresis, the slides were washed three times with neutralization buffer (0.4 M Tris, pH 7.5) for 5 min each to remove the alkaline buffer. Microscope slides were dipped briefly into 1.5% hot (60 °C) normal melting agarose (NMA) prepared in phosphate-buffered saline (PBS). The slides were dried overnight at room temperature

and then stored at 40 °C until used. The slides were then stained with Ethidium bromide and analyzed using an epifluorescence microscope DM IRB at 400× magnification (Leica Microsystem, Heidelberg, Mannheim, Germany), equipped with a digital camera (Canon Power Shot S50, Milan, Italy).

Statistical analyses

Statistical analysis were performed by one-way analysis of variance (ANOVA) plus Student multiple comparison test using SPSS software. Each experiment was performed in triplicate, the data were expressed as the mean values±S.E, for comet assay more than 50 cells were analyzed for each group using Graph Pad Prism 5 Demo Software. The value of $*p<0.05$ was considered significant, $*p<0.001$ was considered highly significant.

Results

Cytotoxicity studies

Cell viability study was performed prior in order to select non cytotoxic concentrations of the extract. The tested concentrations range for extract was 100 to 1000 µg/mL. After 24 h of incubation, the results indicated that Hcy at 100 µM did not caused any significant effects on cellular viability. In addition, the tested extract in the concentration range of 100 to 500 µg/mL had no cytotoxic effects on THP-1 cells, cell viability was above 100%. However, briefly increased cell death was observed with the highest concentration (1000 µg/mL) (Figure 1).

DNA fragmentation

To evaluate the effect of Hcy on THP-1 DNA damage, we used the single cell gel electrophoresis (comet) assay which is a rapid and sensitive technique to analyse DNA damage at the individual cell level.

The ability of Hcy to induce DNA strand breaks and the protective effect of our tested plant extract was assessed using comet assay. Tail length, head length, comet length, % tail DNA and % head DNA were the different parameters used to measure DNA damage level.

The results showed that Hcy induced a significant increase in DNA strand breaks in comparison to control cells (*p <0.001). Indeed, values of tail length, comet length and % of DNA in the tail were significantly higher than those of control cells (table 1). Interestingly, the addition of the extract to the medium was able to suppress DNA damage in THP-1 cells, as indicated by the reduction of tail length, comet length and % tail DNA values in the presence of *Thymus guyonii* extract at both concentrations, but the most effective concentration was10µg/mL

The data indicated that the extract could significantly reduce DNA damage induced by Hcy in monocyte THP-1. The maximum protective effect was observed with *Thymus guyonii* extract at 10 µg/mL (87,9%) where the tail length and the amount of tail DNA were very low compared to Hcy and control groups (Figure 2)

Discussion

The aim of this study was to evaluate the capacity of *Thymus guyonii* extract on reducing ROS production and DNA damage generated by a high level of Hcy.

The overproduction of free radicals induced by elevated Hcy is considered as one of the mechanisms causing many pathologic effects [24,25]. Cell damage in the vascular wall, endothelial cell injury, monocytes differentiation into macrophages [6] and neuronal death via apoptosis or necrosis [25]. This is also one of the proposed mechanisms for DNA damage, carcinogenesis and apoptosis [26].

DNA damage induced by ROS is mainly caused by hydroxyl radical and singlet oxygen (however, other ROS like superoxide or H_2O_2 are involved in its production). DNA damage may be in the form of basic sites, base modifications, sugar lesions, base-

protein cross-links or single and double-strand breaks. The hydroxyl radical is a very reactive ROS and reacts with DNA [27].
Our data revealed that Hcy at the concentration (100µM) was able to induce strongly DNA fragmentation, the results were highly significant (*p<0,001) in the presence of Hcy alone, DNA damage was expressed by an increased in comet tail length and DNA tails percentage, the nucleus of the incubated cells with only Hcy appear with a comet tail shape. DNA damage, PARP activation, and p53 induction induced by Hcy have been reported to be involved in neuronal Apoptosis [28].

Previous findings have shown the mechanism by which a high concentration of Hcy (100 µM) induced oxidative DNA damage in leukemia cell line HL-60, resulted in damage principally to guanine residues. The reaction of homocysteinyl radical with dissolved molecular oxygen produces unstable Hcy thiol peroxyl radical, which can undergo further decomposition to Hcy sulfinyl radical, and $^1O_2$. [29].

In the other hand, the comet tails length and the amount of DNA in the comet tails were significantly decreased in all the treated groups with *Thymus guyonii* extract at both concentrations compared to the amount of DNA in the comet tails of cells exposed to Hcy only (*p<0.001) , noting that none of the concentrations was cytotoxic. However, the most effective concentration on reducing DNA damage was 10µg/mL which considered as small concentration. This high potential protective effect against Hcy induced DNA damage may be related to the chemical composition of its polyphenols. The extract at different concentrations showed a potent ability to reduce DNA damage, the potential mechanisms behind these DNA protective properties could be related to the antioxidant activity of this species as it is belonging to the Lamiaceae family which contains high levels of dietary antioxidants especially polyphénols [30,31]. Polyphenols are considered as natural antioxidants playing an important role in the protection of cells from oxidative damage and consequently induce anticancer activities including pro-apoptotic, DNA damaging anti-angiogenic [32].
Our previous data indicated that *Thymus guyonii* extract has a very high antioxidant activity at very low concentrations [33], the total content of phenolic compounds of the extract was reported to be 19.11 g/100 g [34]. The presence of a potent antioxidant activity in medicinal plants is attributed to the total phenolic compounds [35]. A similar work of Kapiszewska *et al*. (2005) [36] showed that the treatment of lymphocytes with *Thymus piperella* extract exhibited a highly significant protective effect against DNA damage induced by H_2O_2, the extract showed a similar linear dependence between the DNA protective ability, polyphenols concentrations and the intracellular ROS reduction.
In the same context, Mapelli *et al*. (2016) [37] had evaluated the protective capacities of *Thymus vulgaris* L leaf extract and thymol against the damages induced by ultraviolet UVA and UVB in a keratinocytes cell line (HaCaT). The alkaline comet assay showed that the two substances were capable to decrease DNA damage but *Thymus vulgaris* L extract was more effective than thymol in decreasing genotoxicity markers. The methanolic extract exhibits an antioxidant action and anti-apoptotic effect by reducing the percentage of the cells (sub-G_1 phase).
The same species (*Thymus vulgaris* L) showed anti-clastogenic and anti-cytotoxic effects against cyclophosphamide in mice bone marrow cells (Salmani *et al*., 2015)[38]. Thymol, carvacrol, linalool, and luteolin are the major components of this species [39].
According to Zeghib *et al*. (2017) [34], the dominant compounds in *Thymus guyonii* are carvacrol (55.55%) and thymol (19.51%). Thymol is a monocyclic-phenolic compound with antioxidant and anti-cancer properties, which protects the DNA from damage [40]. Carvacrol is an aromatic compound with anti-cancer, antioxidant, and neuroprotective activities [41]. It has been reported that the incubation of human cells (HepG2 and Caco-2 cells), with different concentrations of the monoterpènes thymol and carvacrol, did not

induce DNA strand breaks, and showed a significant protection of cellular DNA damage induced by a strong oxidative agent, hydrogen peroxide (H_2O_2) [42] . Another study of Samarghandian *et al*. (2016) [43] indicated that carvacrol ameliorates chronic stress and prevents lipid peroxidation by inducing SOD, GPx, GR, and CAT. Carvacrol efficiently scavenges free radicals such as peroxyl radicals, superoxide radicals, hydrogen peroxide, and nitric oxide [44]. Carvacrol exerts antioxidant effect both *in vitro* and *in vivo* and its antioxidant activity is attributed to the presence of hydroxyl group (OH) linked to the aromatic ring [45]. Indeed, our results agree with the previous references showing that *Thymus* species have a high capacity to reduce DNA damage exerted by cytotoxic and genotoxic agents. The ability of *Thymus guyoniii* to repair DNA damage and reduce oxidative stress induced by Hcy seems to be strongly related to its richness with carvacrol and thymol, however, the mechanisms by which carvacrol and thymol exert their protective effects against DNA damage still not clear and need more investigation.

Conclusion

The present work, for the first time, reveals *in vitro* new biological activities of *Thymus guyonii*, the extract exerted a potent protective effect against DNA damage especially at the concentration of 10µg/mL. This extract seems to have the ability to suppress oxidative stress induced by Hcy in THP-1 and consequently repair the DNA damage.

Conflicts of interests

The authors declare that they have no conflicts of interest.

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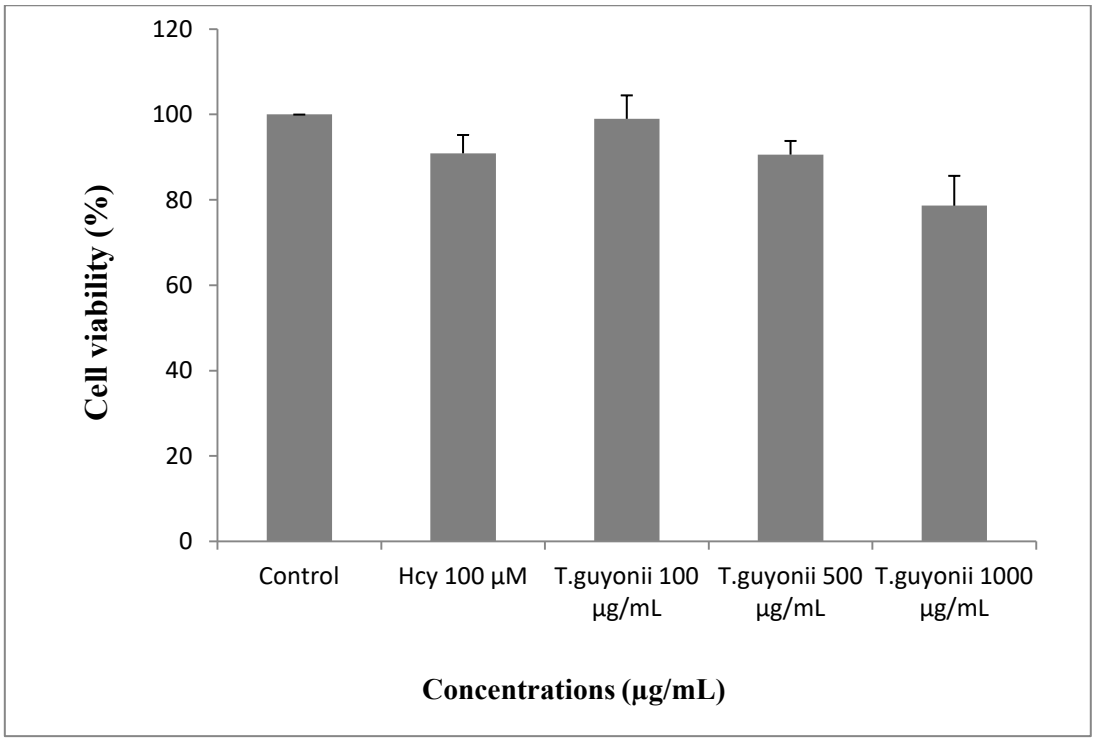

Figure 1. Percentage of cell viability in the presence of Hcy and different concentrations of *Thymus guyonii* extract. Values are mean ±S.E (P >0.05).

Table 1. DNA damage in THP-1 exposed to Hcy and *Thymus guyonii* extract for 24h. Data are reported as mean±SE.

Groups	L Head	L TailL	L Comet	% DNA Head	% DNA Tail	% DNA protection
Control	313,4±6,546	38,6±5,697	352,5±8,93	96,57±0,5188	3,41±0,518	
Hcy 100µM	314,86±4,782	163,034±8,75*	487,48±9,6375*	83,38±0,935*	16,61±0,935*	
T.guyonii 1µg/mL	295±3,115	14,9±1,879#	310,06±4,4974#	99,68±0,068#	0,31±0,066#	
T.guyonii 10µg/mL	301,35±5,81#	30,03±2,69#	332,17±7,0795#	98,36±0,197#	1,63±0,197#	
T.guyonii **72,486** 1µg/mL +Hcy	261,48±4,391#	52,12±3,398#	313,6±5,8782#	95,42±0,323#	4,57±0,323#	
T.guyonii **87,947** 10µg/mL +Hcy	272,58±3,503#	35,98±2,975#	308,54±4,9998#	97,99±0,223#	2,002±0,223#	

The percentage by which *Thymus guyonii* extract exerted the protective effect was calculated according to the equation: 100 - [(TDC for extract+ Hcy)/ TDC Hcy)*100], where TDC is the percentage of DNA in the comet tail. The difference between the mean of TDC values for Hcy and the combined treatment. Values are mean± S.E, *P <0.001 compared to control and #P <0.001 compared to Hcy.

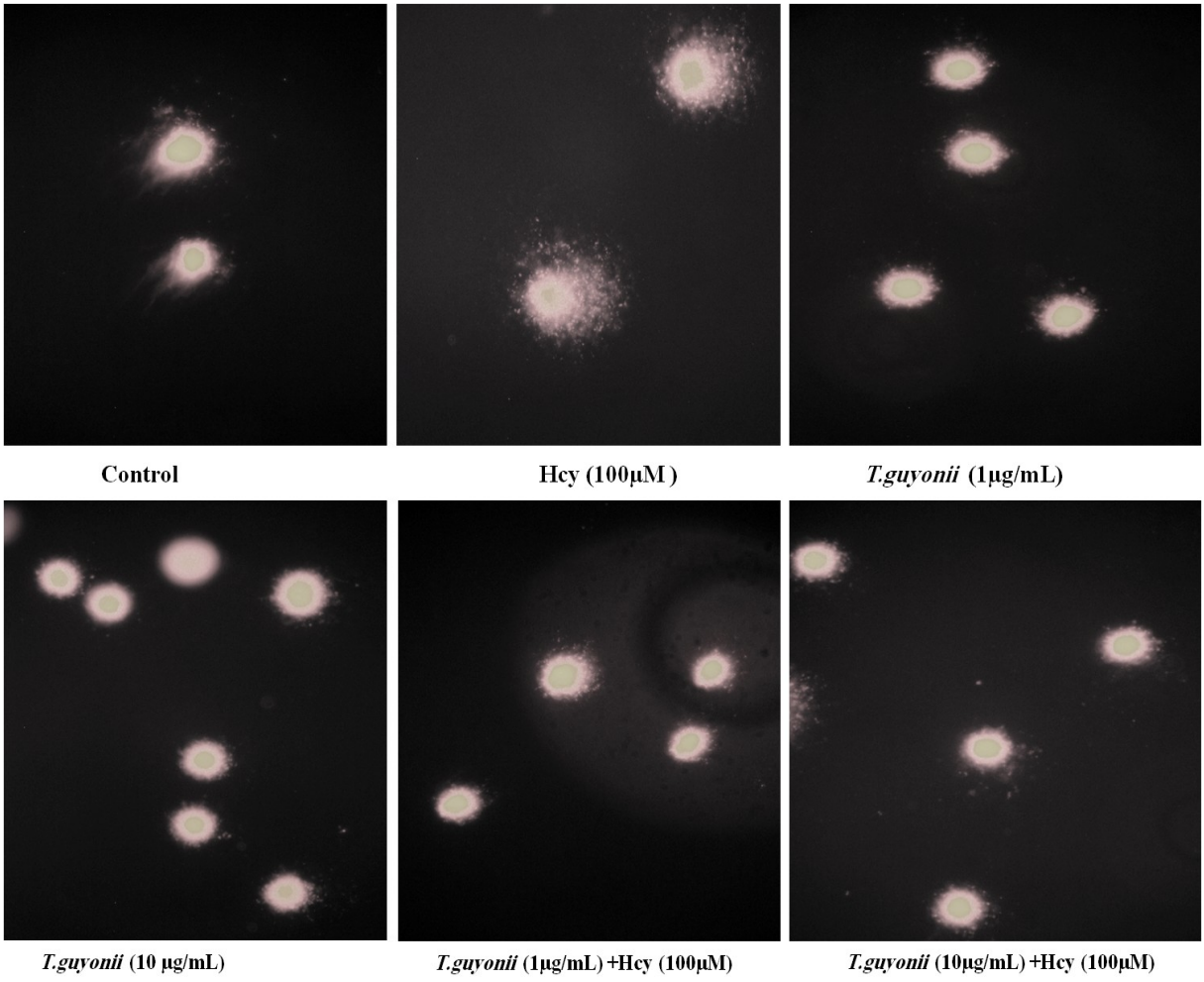

Figure 2. The protective effect of *T.guyonii* extract against DNA damage induced by Hcy in THP-1 cells after 24 of exposition. Cells treated with *T.guyonii* showed a spherical aspect.