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Genotoxic potential of *Dianthus superbus* var. superbus and *Petasites paradoxus* (Retz.) Baumg. methanolic extracts in Chinese hamster ovary cells

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Dianthus superbus **var. *superbus* (*D. superbus*) and *Petasites paradoxus* (Retz.) Baumg. (*P. paradoxus*) are traditionally used medicinal plants from the Italian flora. This study presents the first investigation into the cytotoxic and genotoxic potential of methanolic leaf extracts from both species. Genotoxicity was assessed using the automated cytokinesis-block micronucleus (CBMN) assay in Chinese Hamster Ovary (CHO-K1) cells. Both extracts exhibited dose-dependent cytotoxic effects, with IC_{50} values of 27 µg/mL for *D. superbus* and 56 µg/mL for *P. paradoxus*. At the lowest tested concentration (6.3 µg/mL), *D. superbus* extract did not significantly affect micronucleus frequency in the presence or absence of mitomycin C (MMC). However, at the highest concentration (25 µg/mL), *D. superbus* extract significantly increased micronucleus frequency under both conditions compared to control cells. For *P. paradoxus*, the lowest concentration tested (12.5 µg/mL) did not alter micronucleus frequency in the absence of MMC, but notably reduced micronucleus formation when co-administered with MMC, suggesting potential antigenotoxic activity. Conversely, the highest concentration of *P. paradoxus extract* (50 µg/mL) induced a significant increase in micronucleus frequency compared to MMC treatment alone. These results indicate that both extracts possess cytotoxic and genotoxic properties. Future research should focus on elucidating the mechanisms underlying these genotoxic effects and identifying the specific phytochemicals responsible.**

Medicinal plants are a rich source of pharmacologically active compounds and have been used therapeutically for centuries[1]. The World Health Organization (WHO) estimates that up to 80% of the global population relies on plant-based remedies for primary healthcare, largely due to their affordability, accessibility, and perceived lower incidence of side effects compared to conventional pharmaceuticals[2]. From a drug discovery perspective, bioactive constituents isolated from these plants serve as invaluable lead compounds for the development of novel therapeutics[3]. Ensuring the safe use of medicinal plants requires a careful balance between their therapeutic efficacy and potential toxicity[4], necessitating rigorous toxicological investigations to establish evidence-based safety guidelines. The scientific literature emphasizes the critical importance of evaluating the genotoxic and carcinogenic risks associated with certain plant extracts and their derived bioactive compounds, as these factors can significantly impact their safe and effective application[5].

The genus *Dianthus* (Caryophyllaceae) encompasses roughly 300 species distributed across Europe, Africa, Asia, and North America[6]. Many *Dianthus* species have a history of use in traditional medicine, with applications across various regions for treating diverse infections and diseases[7]. Phytochemical analyses indicate that *Dianthus* species are rich in compounds such as triterpenes, phenolics, flavonoids, anthocyanins, alkaloids, cyanogenic glycosides, coumarins, ecdysteroids, and essential oils[8,9]. *Dianthus superbus* var. *superbus* (*D. superbus*) has been traditionally used as a diuretic and anti-inflammatory agent in the treatment of urinary

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infections, carbuncles, and carcinoma[10,11]. Previous studies report that *D. superbus* exhibits antioxidant, antimicrobial, anticarcinogenic, and anti-inflammatory properties[12,13]. Additionally, it has been shown to suppress immunoglobulin E production and prevent peanut-induced anaphylaxis[14,15]. Furthermore, stimulate immunosuppressive effects, and demonstrate cytotoxic activity against cancer cells[16,17]. Phytochemical studies of *D. superbus* have revealed diverse bioactive compounds, including dianthosaponins, dianthramides, flavonoids, coumarins, triterpenoids, pyran-type glycosides, and cyclic peptides[4,18]. Cyclic peptides, flavones, and triterpene saponins are reported as predominant constituents[19]. More recent research has focused on isolating and characterizing novel compounds, such as quinolone alkaloids, cyclopeptides, and triterpenoid saponins[20], with comparatively fewer studies providing a comprehensive analysis of the plant's bioactive phytochemical profile.

The genus *Petasites* L. (Asteraceae), commonly known as butterbur or coltsfoot, consists of herbaceous perennial plants characterized by thick, creeping rhizomes and large, hat-shaped leaves[21]. The genus includes 19 widely recognized species[22]. Historically, *Petasites* species have been used in folk medicine to treat conditions such as migraines, hypertension, respiratory disorders, gastrointestinal issues, and genitourinary problems[23,24]. Today, their most extensively studied therapeutic effects focus on anti-migraine and antiallergic properties[25,26]. Phytochemical investigations reveal the presence of sesquiterpenes, pyrrolizidine alkaloids, polyphenols, and essential oils[23]. *Petasites paradoxus* (Retz.) Baumg. (*P. paradoxus*) is endemic to Alpine regions, often cohabiting with *Tussilago farfara*[27]. Its bioactive constituents include kablicin, angelyljaponicin, kablikopetasin, furoeremophilane, and an unidentified triterpenic alcohol[28]. Recent study characterized the leaves methanolic extracts of *D. superbus* and *P. paradoxus* using untargeted metabolomic analysis. The major compounds identified in *D. superbus* extract were flavonoids, oleanane triterpenoids. The major compounds identified in *P. paradoxus* extract were hydroxycinnamate esters and otonecine-type pyrrolizidine alkaloids[29].

Genotoxicity assessment is a crucial component of evaluating the safety of medicinal plant extracts[30]. Despite the traditional use of *D. superbus*, its genotoxic potential has not been investigated. There is limited information available in the literature regarding the genotoxicity of *Dianthus species*. A pronounced protective effect was observed for several *Dianthus* species originated from Turky, namely *D. carmelitarum*, *D. ancyrensis*, *D. kastembeluensis*, and *D. lydus* against the mutagenic effects of sodium azide and 4-nitro-o-phenylenediamine in Salmonella typhimurium TA100 and TA98 strains, respectively[31]. The toxicity of plants within the genus *Petasites* is primarily associated with the presence of pyrrolizidine alkaloids (PAs). PAs isolated from *P. japonicus* demonstrated rapid absorption of neopetasitenine and its subsequent conversion to petasitenine following oral administration in rats. Extrapolation of the metabolic profiles to humans indicated that potentially hazardous levels of petasitenine could accumulate in plasma if *P. japonicus* were consumed daily as a dietary component[32]. Acute oral toxicity studies of methanolic extracts from *P. hybridus* rootstocks revealed no observable toxic effects on the skin, fur, eyes, or behavioral patterns in both rats and mice[33]. Moreover, data on the genotoxic potential of *Petasites* species remain limited. Results from the Ames Salmonella assay, in vivo micronucleus assay, and chromosome aberration test indicate that *P. japonicus* does not exhibit genotoxic effects[34]. Similarly, research on *P. paradoxus* is limited, and its genotoxic potential remains unknown.

This study contributes to the broader initiative at the Italian National Biodiversity Future Center (NBFC)[35], which focuses on bioprospecting Italian flora to identify plant-derived secondary metabolites with bioactivity relevant to food, pharmaceutical, cosmetic, and materials science sectors. Our laboratory is specifically screening the genotoxic and antigenotoxic potential of medicinal plants, with leaves of *D. superbus* and *P. paradoxus* included in this investigation. This study presents the first evaluation of the genotoxic potential of leaf extracts from *D. superbus* and *P. paradoxus* using in vitro models. Cytotoxicity and genotoxicity were assessed in Chinese Hamster Ovary K1 (CHO-K1) cells via the cytokinesis-block micronucleus (CBMN) assay, a well-established method for detecting DNA damage and genotoxic agents[36]. CHO-K1 cells, which are widely recognized as a reference model for such assessments[37]. CHO-K1 cells were chosen due to their rapid growth, genetically stable karyotype (22 ± 2 chromosomes)[38] and well-characterized response profile. Previous studies have reported that CHO-K1 cells exhibit approximately 79% sensitivity to known carcinogenic compounds[39], underscoring their reliability for genotoxicity testing. Therefore, CHO-K1 cells provide a scientifically robust and regulatorily relevant model for assessing genotoxic potential in accordance with OECD TG 487 guidelines. The cytokinesis-blocked micronucleus (CBMN) assay has been frequently used to detect potential genotoxicity and to investigate compounds potentially able to prevent genotoxicity and cytotoxicity[35]. We employed an automated in vitro CBMN protocol integrating widefield fluorescence microscopy and ImageStreamX imaging flow cytometry to enhance the reliability of our genotoxicity assessments. Furthermore, considering the known phytochemical profiles of *D. superbus* and *P. paradoxus*, we hypothesized that their leaf methanolic extracts may demonstrate genotoxic and/or antigenotoxic effects in the in vitro CBMN assay.

Results

Cytotoxicity and concentration selection

The cytotoxic effects of crude methanolic extracts of *D. superbus* and *P. paradoxus* on CHO-K1 cells were evaluated using the MTT cell viability assay, with results shown in Fig. 1A,B. For the *D. superbus* extract, no significant cytotoxicity was observed in CHO-K1 cells after 24 h of exposure to concentrations below 6.25 µg/mL (Fig. 1A). However, concentrations between 6.25 and 100 µg/mL caused a significant, dose-dependent reduction in cell viability. At the highest tested concentration (100 µg/mL), cell viability decreased to less than 10% of the untreated DMSO control. Similarly, the *P. paradoxus* extract exhibited no significant cytotoxic effects at concentrations below 7.80 µg/mL (Fig. 1B). Concentrations ranging from 7.80 to 250 µg/mL resulted in a significant, concentration-dependent reduction in cell viability, with approximately 10% viability observed at 200 µg/mL relative to the DMSO control. The IC_{50} values were determined to be 27 µg/mL for *D. superbus* and 56 µg/mL for *P. paradoxus*.

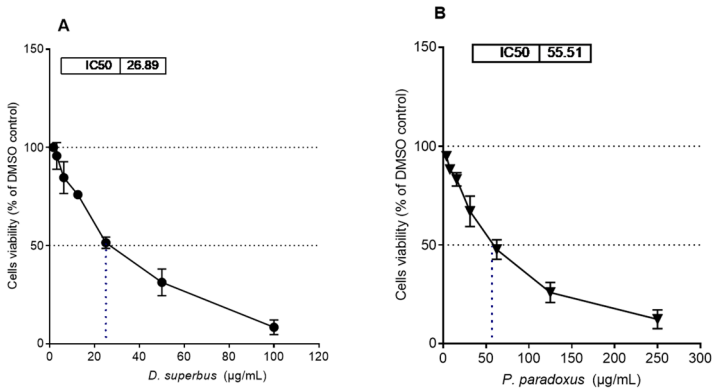

Fig. 1. Viability of CHO-K1 cells after 24 h of treatment with *D. superbus* (**A**) and *P. paradoxus* (**B**) expressed as a percentage of the DMSO control (set to 100%). Data are presented as mean ± STD from three independent experiments.

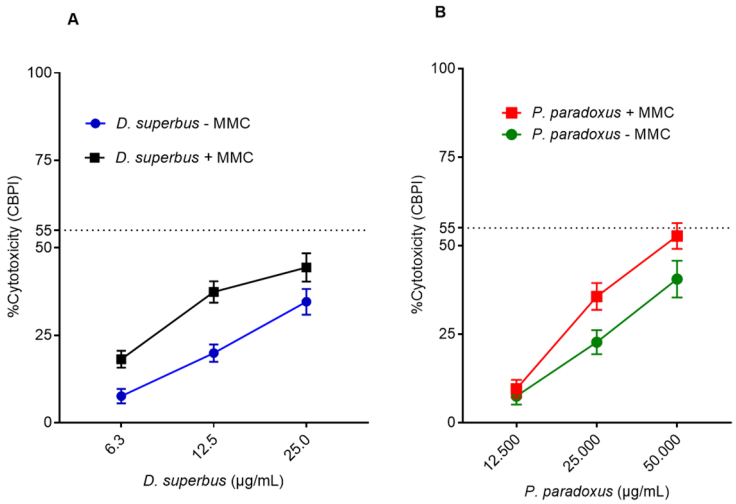

Fig. 2. The cytotoxic effects of *D. superbus* extracts (**A**) and *P. paradoxus* (**B**) extracts on CHO-K1 cells under the CBMN assay in the absence and presence of MMC. Data represents the mean values from three independent experiments.

Genotoxicity assessment

Cytotoxicity evaluation under the CBMN assay

In genotoxicity assays, it is essential to consider cytotoxicity, as excessive cytotoxicity can obscure or confound the detection of genotoxic effects. According to established guidelines, cytotoxicity should not exceed 55 ± 5% at higher tested concentrations relative to the untreated control to minimise the risk of false-positive results[40]. In line with these recommendations and the standard requirement to perform a minimum of three concentration tests per substance in micronucleus assays[41]. Three concentrations were selected for each extract based on their respective IC_{50} values determined by the MTT assay. For the *D. superbus extract* (IC_{50} = 27 µg/mL), concentrations of 6.3, 12.5, and 25 µg/mL were selected. For the *P. paradoxus* extract (IC_{50}= 56 µg/mL), concentrations of 12.5, 25, and 50 µg/mL were employed. To assess the genotoxic potential of the extracts, cytotoxicity was concurrently evaluated using cytokinesis-block proliferation index (CBPI), in the same cell populations analysed for micronuclei, as previously described[42]. The CBPI reflects the proportion of mononucleated, binucleated, and multinucleated cells in cultures treated with cytochalasin B and is sensitive to mitotic inhibition caused by cytotoxic agents, following OECD Test Guideline 487[40].

The OECD guidelines recommend test concentrations ranging from 55 ± 5% cytotoxicity to minimal or no cytotoxicity. As a result, cytotoxicity was assessed as part of the MN experiment in the same cells used to score micronuclei, using CBPI%. The CBPI represents the average number of cell cycles per cell upon exposure to cytochalasin B, which should be between 1.5 and 2 cycles. A decrease in CBPI compared to the negative control indicates an inhibition of cell proliferation[43]. As indicated by OECD guidelines[40], the evaluated chemicals should not have a cytostasis percentage more than 50%. In this study, The *D. superbus* extract induced a dose-dependent increase in cytotoxicity, as measured by the CBPI%, following treatment with *D. superbus* extract, with values of 7.58 ± 2.05%, 19.88 ± 2.48%, and 34.55 ± 3.69% at 6.3, 12.5, and 25 µg/mL, respectively (Fig. 2A

blue color). When cells were co-treated with D. superbus extract in combination with mitomycin C (MMC), a notable increase in cytotoxicity was observed compared to MMC treatment alone with cytotoxicity percentage of 18.15 ± 2.42%, 37.37 ± 3.05%, and 44.69 ± 4.03% were observed in cells treated with *D. superbus* at 6.3, 12.5, and 25 µg/mL, respectively compared with MMC alone (8.66 ± 2.25%) (Fig. 2A black color). As a result, all the 3 selected concentrations, 6.3, 12.5, and 25 µg/mL, of *D. superbus* extracts were chosen for micronuclei evaluation.

The *P. paradoxus* extract also induced a dose-dependent increase in cytotoxicity, with values of of 7.48 ± 2.38%, 22.74 ± 3.40%, and 40.58 ± 5.17% at 12.5, 25, and 50 µg/mL, respectively (Fig. 2B, green color). In the presence of MMC, cytotoxicity showed a dose-dependent increase following treatment with *P. paradoxus* extract, yielding values of 9.52 ± 2.55%, 35.66 ± 3.79%, and 52.76 ± 3.66% in cells treated with *P. paradoxus* at 12.5, 25, and 50 µg/mL, respectively, compared to MMC alone (8.67 ± 2.25%) (Fig. 2B, red color).

Genotoxic effects of D. superbus

In this experiment, binucleated cells and micronuclei were captured using fluorescence microscopy and automatically detected with CellProfiler software (version 4.2.6), as described in the Materials and Methods section. Our study evaluated the genotoxic effects of *D. superbus* extract on CHO-K1 cells. As a positive control, 0.025 µg/mL MMC significantly increased micronuclei frequency to 4.03 ± 0.68% after 24 h, compared to 0.72 ± 0.09% in negative control (NC) cells (Fig. 3A). The lowest concentration of *D. superbus* extract (6.3 µg/mL) showed no significant increase in micronuclei frequency compared to NC cells (Fig. 3A). In contrast, *D. superbus* extract at 12.5 µg/mL and 25 µg/mL significantly induced micronuclei formation, with frequencies of 1.73 ± 0.16% and 2.36 ± 0.29%, respectively, demonstrating its genotoxic potential at these concentrations. However, the genotoxicity observed with *D. superbus* extract was significantly less potent than that induced by MMC (4.03 ± 0.68%). These findings indicate a concentration-dependent genotoxic effect of *D. superbus* extract at higher concentrations, correlating with its cytotoxicity. Representative binucleated cells with micronuclei are shown in Fig. 3B.

In a separate experiment, cells were treated with *D. superbus* extract in combination with MMC. The results revealed a substantial increase in micronuclei frequency in cells co-treated with *D. superbus* extract at 12.5 and 25 µg/mL compared to cells treated with MMC alone (4.03 ± 0.68%) (Fig. 4A). A significant increase in micronuclei frequency was observed in cells treated with *D. superbus* extract at 25 µg/mL (6.95 ± 0.32%) compared to the MMC control (4.03 ± 0.68%) indicating an additive genotoxic effect relative to MMC treatment alone. Representative images of micronuclei formation in binucleated CHO-K1 cells after exposure to MMC alone or in combination with the highest tested concentration of *D. superbus* extract is shown in Fig. 4B.

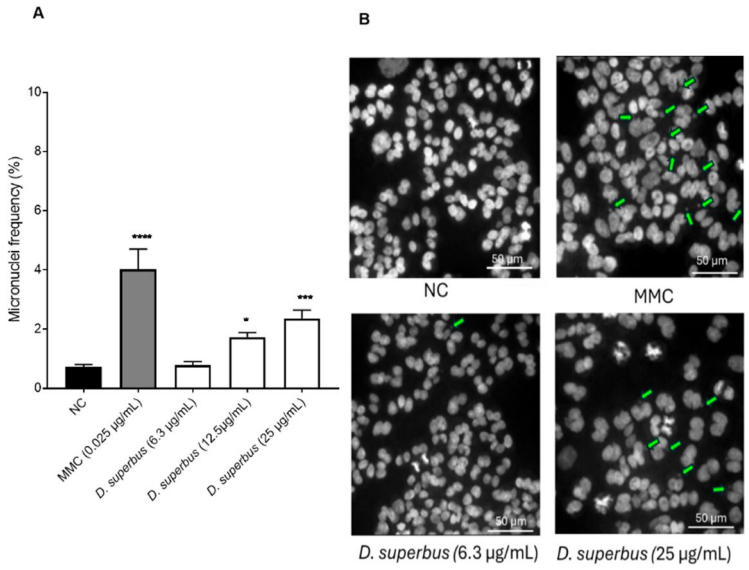

Fig. 3. (**A**) The effect of different *D. superbus* extract treatments on the micronuclei frequency (%) in CHO-K1 cells. Cells were treated for 24 h either with DMSO at 0.3% (NC), MMC at 0.025 µg/mL or 3 different concentrations of *D. superbus* extract, 6.3, 12.5 and 25 µg/mL. Then cells were incubated with 3 µg/mL of cytochalasin B for another 24 h. Graphs represent data collected from 3 independent experiments. One-way ANOVA, Tukey's multiple comparisons test using GraphPad Prism 7 software, was applied to calculate statistical significance in comparison with the NC control. (*$p = 0.0155$, ***$p = 0.0006$, ****$p < 0.0001$). Micronuclei frequency (%) was calculated from the following Equation: Micronuclei frequency (%) = binucleated cells with MN /binucleated cells *100. (**B**) Representative microscopic images showing micronuclei formation in binucleated CHO-K1 cells, observed with a 40× objective. CHO-K1 cell DNA was stained with Hoechst dye. Green arrows indicate the micronuclei, and the white line, labelled 50 µm, represents the scale bar in the image.

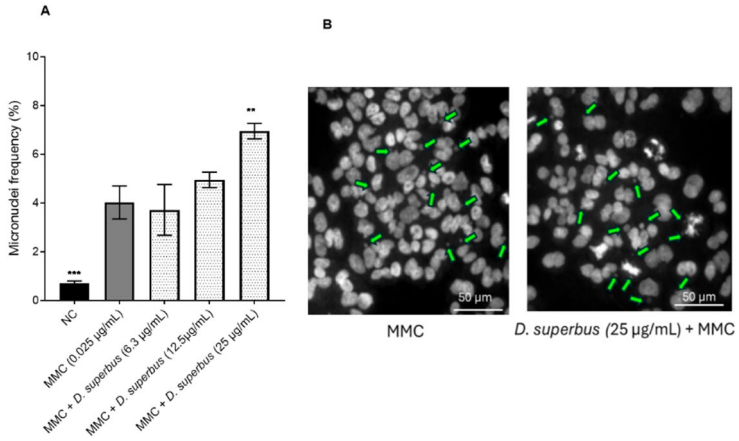

Fig. 4. (**A**) The effect of different *D. superbus* extract treatments on the micronuclei frequency (%) in CHO-K1 cells in the presence of MMC. Cells were treated for 24 h either with DMSO (NC), MMC at 0.025 µg/mL or in combnation of MMC and of *D. superbus* extract at 3 different concentrations 6.3, 12.5 and 25 µg/mL 24 h, then incubation with 3 µg/mL of cytochalasin B for another 24 h. Graphs represent data collected from 3 independent experiments. One-way ANOVA, Tukey's multiple comparisons test using GraphPad Prism 7 software, was applied to calculate statistical significance in comparison with the NC control. (*$p = 0.0155$, ***$p = 0.0006$, ****$p < 0.0001$). Micronuclei frequency (%) was calculated from the following Equation: Micronuclei frequency (%) = binucleated cells with MN /binucleated cells *100. (**B**) Representative images illustrating micronuclei formation in binucleated CHO-K1 cells after exposure to MMC alone or in combination with the highest tested concentration of *D. superbus* extract.

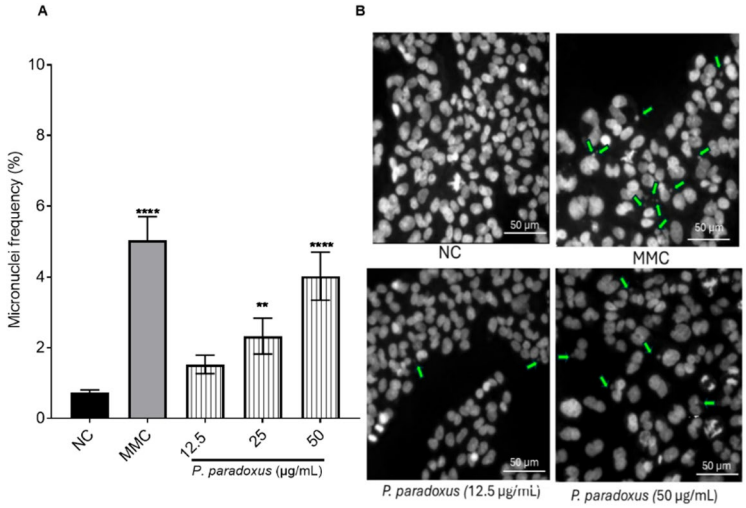

Fig. 5. (**A**) The effect of different *P. paradoxus* extract treatments on the micronuclei frequency (%) in CHO-K1 cells. Cells were treated for 24 h with 3 different concentrations of *P. paradoxus* extract, 12.5, 25 and 50 µg/mL or MMC at 0.025 µg/mL, then followed by 24 h incubation with 3 µg/mL of cytochalasin B. Graphs represent data collected from 3 independent experiments. One-way ANOVA, Tukey's multiple comparisons test using GraphPad Prism 7 software, was applied to calculate statistical significance in comparison with the NC control. (*$p = 0.0435$, **$p = 0.0019$, ***$p = 0.0007$, ****$p < 0.0001$). (**B**) The micronuclei formation in binucleated CHO-K1 cells, observed with a 40× objective. CHO-K1 cells' DNA was stained with Hoechst dye. Green arrows indicate the micronuclei, and the white line, labelled 50 µm, represents the scale bar in the image.

Genotoxic effects of *P. paradoxus* extract

Fluorescence microscopy and cellprofiler analysis of micronuclei

Treatment with *P. paradoxus* at a concentration of 12.5 µg/mL did not result in a significant change in micronuclei frequency when compared to NC cells (Fig. 5A). In contrast, at concentrations of 25 and 50 µg/mL, *P. paradoxus* treatment induced a significant increase in micronuclei frequency, with values of 1.99 ± 0.32% and 2.99 ± 0.32%, respectively, compared to 0.72 ± 0.09% in NC cells. These findings indicate a genotoxic potential of *P. paradoxus* extract at higher concentrations under the tested conditions. Notably, the micronuclei frequency observed at

50 µg/mL (4.00 ± 0.32%) was comparable to that induced by MMC at 0.025 µg/mL (4.03 ± 0.68%),%), reinforcing the extract's genotoxic effect. Overall, our results suggest a link between cytotoxicity and genotoxicity upon exposure to *P. paradoxus* extract, particularly at 50 µg/mL. Representative images of micronuclei formation in binucleated CHO-K1 cells after 24 h of incubation with MMC or *P. paradoxus* extract are shown in Fig. 5B.

When CHO-K1 cells were treated with MMC at 0.025 µg/mL in combination with the lowest concentration of *P. paradoxus* extract (12.5 µg/mL), a significant reduction in micronuclei frequency was observed (2.577 ± 0.40%) compared to cells treated with MMC alone (4.03 ± 0.68%). (Fig. 6A). The reduction in micronuclei frequency in cells treated with MMC in combination with *P. paradoxus* at 12.5 µg/mL (2.58 ± 0.40%) was statistically significant ($p < 0.0001$) compared to cells treated with MMC alone (4.03 ± 0.68%). Furthermore, the micronuclei frequency of the MMC-treated cells in this study aligns with the historical control of our lab range for MMC-induced micronuclei frequency under identical conditions using CHO-K1 cells (4.5 ± 0.68% to 5.60 ± 0.94%), as reported in our previous studies[42,44]. In accordance with OECD TG 487 criteria, this historical comparison was included to assess biological relevance and enhance transparency in interpreting the observed antigenotoxic effect.

In contrast, treating the cells with a higher concentration of *P. paradoxus* extract (50 µg/mL) along with MMC resulted in a significant increase in micronuclei frequency (6.95 ± 0.32%), far exceeding the frequency induced by MMC alone (4.03 ± 0.68%). These findings indicate a dual effect of *P. paradoxus* extract: at low concentration, it may exert a protective or antigenotoxic influence suggesting that the extract might mitigate MMC-induced DNA damage, however the proposed involvement of might mitigate MMC-induced DNA damage remains a hypothesis that requires further investagation. Whereas at higher concentration it appears to act synergistically with MMC, markedly enhancing genotoxicity. Representative images of micronuclei formation in binucleated cells following 24 h of incubation with MMC alone or in combination with the highest concentration of *P. paradoxus* extract are shown in Fig. 6B.

P. paradoxus extract also induced a dose-dependent increase in cytotoxicity. In the presence of MMC, cytotoxicity showed a dose-dependent increase following treatment with *P. paradoxus* extract, yielding values of 9.52 ± 2.55%, 35.66 ± 3.79%, and 52.76 ± 3.66% in cells treated with *P. paradoxus* at 12.5, 25, and 50 µg/mL, respectively, compared to MMC alone (8.67 ± 2.25%). The concentrations used in the antigenotoxicity assessment were within the acceptable cytotoxicity limits (55 ± 5%) recommended by OECD TG 487 and we acknowledge that subtle cytotoxic effects could still influence micronucleus outcomes, particularly at higher concentrations where a synergistic response with MMC was observed.

Micronuclei analysis using imaging flow cytometry

To validate our findings, the frequency of micronuclei was also analyzed using the ImageStreamX imaging flow cytometer coupled with IDEAS software (version number 6.2, Amnis Corporation software, USA). This analysis was performed on CHO-K1 cells treated with the lowest (12.5 µg/mL) and highest (50 µg/mL) concentrations of the *P. paradoxus* extract. As shown in Fig. 7A, treatment with MMC for 24 h, resulted in a significant increase in micronuclei frequency compared to NC cells. Similarly, the *P. paradoxus* extract at 50 µg/mL induced a significant increase in micronuclei frequency relative to NC cells, confirming its genotoxic potential. Notably, the frequency of micronuclei at this concentration was even higher than that induced by MMC alone, highlighting its genotoxic effect. In contrast, treatment with the extract at the lowest concentration of 12.5 µg/mL showed no significant difference in micronuclei frequency compared to the NC cells, mirroring the dose-dependent effect

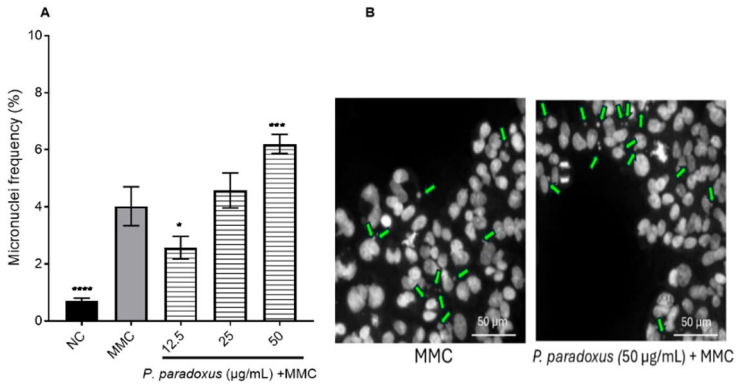

Fig. 6. (**A**) The effect of different *P. paradoxus* extract treatments on the micronuclei frequency (%) in CHO-K1 cells. Cells were treated for 24 h with 3 different concentrations of *P. paradoxus* extract, in the presence of MMC, then followed by 24 h of incubation with 3 µg/mL of cytochalasin B. Graphs represent data collected from 3 independent experiments. One-way ANOVA, Tukey's multiple comparisons test using GraphPad Prism 7 software, was applied to calculate statistical significance in comparison with NC control. (*$p = 0.0435$, **$p = 0.0019$, ***$p = 0.0007$, ****$p < 0.0001$). in micronuclei frequency (%) was calculated from the following Equation: (**B**) Representative microscopic images showing micronuclei formation in binucleated CHO-K1 cells, observed with a 40× objective after 24 treated with 0.025 µg/mL MMC alone or MMC + *P. paradoxus* extract at 25 µg/mL. CHO-K1 cell DNA was stained with Hoechst dye. Green arrows indicate the micronuclei, and the white line, labelled 50 µm, represents the scale bar in the image.

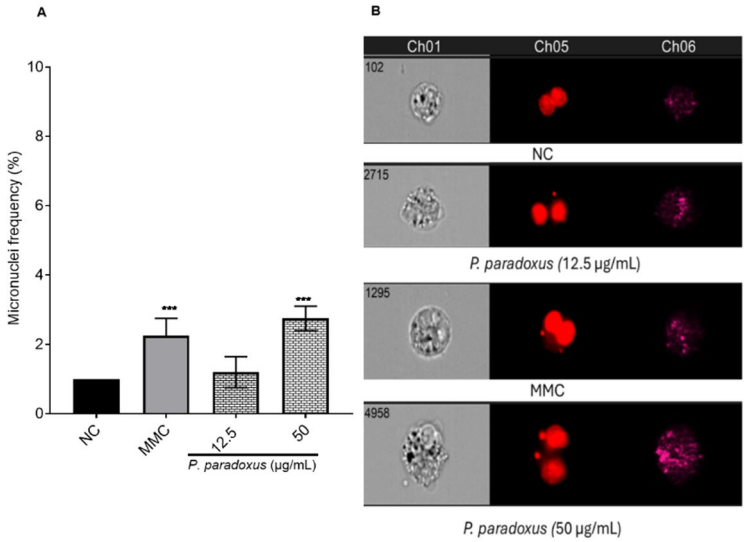

Fig. 7. (**A**) Micronuclei frequency (normalized with NC) per 2000 binucleated CHO-K1cells after 24 h incubation with 2 different concentrations, 12.5 and 50 µg/mL of *P. paradoxus* extract, or MMC at 0.025 µg/mL then followed by 24 h incubation with 3 µg/mL of cytochalasin B. Graphs represent collected data of 3 independent experiments. One-way ANOVA, Tukey's multiple comparisons test using GraphPad Prism 7 software, was applied to calculate statistical significance in comparison with the NC control, $^{***}p = 0.0001$. (**B**) Representative images acquired using the ImageStreamX imaging flow cytometer, display: (a) a brightfield view of single cells; (b) cells from Channel 05 (Ch05), specifically illustrating Draq5-stained binucleated cells, with or without micronuclei, from negative control (NC) samples, *P. paradoxus* at tested concentrations of 12.5 and 50 µg/mL, and MMC at 0.025 µg/mL; and (c) the corresponding side scatter (SSC) image for each cell.

observed in our previous analysis. These results from ImageStreamX imaging flow cytometer are consistent with the findings from fluorescence microscopy, providing a validation for the genotoxic effects of the *P. paradoxus* extract at higher concentrations. Representative images acquired by the ImageStreamX are presented in Fig. 7B.

Discussion

Plant extracts have gained increasing attention as alternative or complementary agents in medical treatments, particularly for their potential chemopreventive properties. However, their genotoxicity remains largely unexplored[45]. Indeed, several studies have reported that certain plant extracts can exert genotoxic, toxic, or even carcinogenic effects[46,47]. In the present study, we evaluated the cytotoxicity and genotoxicity of methanolic extracts derived from *D. superbus* and *P. paradoxus*, both in the presence and absence of MMC. To our knowledge, this is the first study to investigate the genotoxic potential of these specific methanolic extracts. The limited availability of data on the genotoxicity of *D. superbus* and *P. paradoxus* extracts makes direct comparison with previously published findings challenging.

Cytotoxicity assessment is a crucial step in determining the safety profile of plant extracts. This is particularly relevant in genotoxicity studies, as DNA damage can occur secondarily to cytotoxicity, potentially leading to misinterpretation of genotoxic effects[48]. In this study, we performed cell viability screening to evaluate the cytotoxic effects of the extracts and to define suitable concentration ranges for subsequent genotoxicity assessments. Our results demonstrated that both *D. superbus* and *P. paradoxus* methanolic extracts reduced CHO-K1 cell viability, with IC_{50} values of 28 µg/mL and 56 µg/mL, respectively.

Previous studies support the cytotoxic potential of *D. superbus*. For example, an ethyl acetate fraction from dried *D. superbus* exhibited strong cytotoxic activity against various cancer cell lines, with IC_{50} values of 9.5 µg/mL (HeLa), 9.6 µg/mL (SKOV), 13.8 µg/mL (Caski), and 69.9 µg/mL (NCL-H1299)[49]. Similarly, an ethyl acetate fraction extracted with 95% ethanol from the whole plant displayed cytotoxic effects against Bel-7402, HepG2, and HeLa cells, with IC_{50} values of 35.6 ± 0.9, 22.6 ± 1.0, and 20.5 ± 0.8 µg/mL, respectively[50]. Moreover, the petroleum ether extract of *D. superbus* and its isolated compounds showed cytotoxicity at 100 mg/L against HeLa, SMMC-7721, HepG2, SK-hep1, A549, and Bel-7402 cell lines[40]. In contrast, no prior studies have reported the cytotoxicity of *P. paradoxus* extract. Therefore, our findings provide the first evidence of the cytotoxic effect of *P. paradoxus* extract on CHO-K1 cells.

MMC, a widely recognised carcinogenic substance, is a well-established genotoxic agent in both in vitro and in vivo mammalian systems[51]. Consistent with its established effects, MMC produced a clear positive response at the selected concentration, validating the sensitivity and reliability of the CBMN assay. The concentration of MMC was chosen based on our previous research, as reported[42,44]. According to OECD guidelines, a substance is considered genotoxic if at least one tested concentration causes a statistically significant increase in micronuclei frequency compared to the negative control. In this study, the results for the methanolic leaf extracts from both *D. superbus* and *P. paradoxus* met this criterion. Specifically, the genotoxic effects were evident at the two highest

concentrations tested in the CBMN assay for both plant extracts. In contrast, the lowest concentrations of both extracts did not induce micronuclei formation, indicating a dose-dependent genotoxic potential. This confirms that these extracts possess genotoxic activity in CHO-K1 cells at higher concentrations.

According to the OECD, Test Guideline 487[40], for the in vitro micronucleus assay, the observed induction of micronuclei was considered biologically relevant based on multiple criteria. First, a statistically significant increase in micronucleated cells was detected in cells treated with *D. superbus* and *P. paradoxus* extracts at higher concentrations compared with the solvent control (DMSO). Second, a clear concentration-related increase in micronucleus frequency was observed, supporting a true positive response. Third, the findings were reproducible, as the increase in micronucleus frequency was consistently observed across three independent experiments and replicate cultures. Cytotoxicity and exposure adequacy were also assessed in accordance with OECD recommendations. Only cells that had undergone division during or after treatment were analyzed, and cytotoxicity was quantified using the CBPI. The highest tested concentration resulted in approximately 50 ± 5% cytotoxicity, which is within the recommended upper cytotoxicity limit for the assay. Consequently, higher concentrations were not evaluated to avoid excessive cytotoxicity that could confound the assessment of genotoxicity. Taken together, these observations satisfy the OECD criteria for biological relevance in the in vitro micronucleus assay.

Genotoxicity studies on plant extracts frequently reveal a complex, concentration-dependent duality. At different doses, these extracts can act as either genotoxic or antigenotoxic agents. This dual effect is a common finding in natural product research. For instance, the extract of *Staphylea pinnata* L. showed genotoxic properties at higher concentrations while exhibiting antigenotoxic effects at lower doses[44]. Conversely, the methanolic extract of *Cistus monspeliensis* L. displayed antigenotoxic activity at lower concentrations with no effect at higher ones[42]. Moreover, extracts like those from *Artemisia vulgaris* L. and *Artemisia alba* Turra, have demonstrated both genotoxic and antigenotoxic effects at the same tested concentrations[52]. These examples underscore the intricate and often non-linear dose-response relationships inherent in phytochemical-rich extracts, highlighting the need for wide dose-ranging studies to fully understand their biological activity.

The diverse biological activities of plant extracts arise from the complex interactions of their many metabolomic compounds[53]. Numerous studies have shown that various phenolic compounds, for example, can exhibit both antioxidant and prooxidant properties depending on their concentration[54]. Consequently, individual compounds or their combinations can display either genotoxic or antigenotoxic effects[52]. Recent analysis by NBFC collaborators[29] characterized the methanolic extracts of *D. superbus* using untargeted metabolomic analysis. The major compounds identified were flavonoids, oleanane triterpenoids. Among the flavonoids presented in the *D. superbus* extract are luteolin and its derivate[55]. Luteolin, a flavonoid, has been shown to induce cytotoxicity and genotoxicity in various cell types. It showed cytotoxic and genotoxic effects in human lymphoblastoid TK6 cells[56]. In addition, luteolin induced sister chromatid exchanges and micronuclei in human lymphocytes[57]. Furthermore, Luteolin exhibited clastogenic effects in Chinese hamster V79 cells[58]. The genotoxic effects of *D. superbus* extract observed in our study might linked to the presence of some flavonoids like luteolin and its co-eluting metabolites. However further study is needed to identify the responsible phytochemicals for the genotoxic effect in methanolic extract used in this study.

Beyond flavones, the saponins identified in *D. superbus* methanolic extract aas reported prevoiusly[20] may also contribute to its observed genotoxic effect. Studies on other plant extracts have reported genotoxic activity associated with saponins. For instance, a mixture of saponins from Nauclea species demonstrated synergistic in vitro chromosome mutations and DNA damage in mammalian cells[59]. The DNA-damaging effect of hopane-type saponin-containing fractions from *Glinus lotoides* L. has also been documented[60]. Different classes of saponin compounds identified in *Pterolobium stellatum* (Forssk.) Brenan extracts have been proposed as responsible for their genotoxic effects[61].

Research on the *P. paradoxus* plant extract is limited in the literature, making it challenging to compare findings with previous studies. Phytochemical analysis of *P. paradoxus* revealed the presence of various terpenoid compounds, including kablicin, angelyljaponicin, kablikopetasin, furoeremophilane, and an unidentified triterpenic alcohol[28]. In addation, recent study[29] characterized the methanolic extracts of *P. paradoxus* and reported that the major compounds identified in the *P. paradoxus* extract were hydroxycinnamate esters and otonecine-type pyrrolizidine alkaloids. The genotoxic effects observed in our study might be associated with the presence of otonecine-type pyrrolizidine alkaloids. Pyrrolizidine alkaloids (PAs) are common constituents of many plant species worldwide. Numerous PAs have tested positive in micronucleus, chromosomal aberration, and sister chromatid exchange assays, which detect chromosomal damage and indicate the likely induction of chromosomal mutations[62]. Otonecine-type PAs, such as clivorine and senkirkine, have been identified as mutagenic in various biological systems, including Salmonella typhimurium (TA100) and mammalian cell cultures[62].

Several studies have reported genotoxic effects of plant extracts rich in terpenoids, including monoterpenes, sesquiterpenes, diterpenes and sesquiterpene lactones. Notable examples include *Smallanthus sonchifolius*[63], *Hemidesmus indicus* (L.) R.Br.[64], and *Pterolobium stellatum*[61], where terpenoids were suggested as potential contributors to their genotoxic effects. The genotoxic effects observed in our study with *P. paradoxus* plant extract may be attributed to the presence of terpenoid compounds. However, further investigation is needed to characterise the methanolic extract of *P. paradoxus* and to identify the specific compounds responsible for the genotoxic effect.

The *P. paradoxus* leaf extract notably exhibited a protective effect against MMC-induced genotoxicity in CHO-K1 cells. This phenomenon, known as hormesis, characterised by beneficial effects at lower tested concentrations and adverse effects at higher doses, which has been previously described. For instance, the ethanolic root extract of *Hemidesmus indicus* (L.) R.Br., demonstrated genotoxic effects at higher concentrations while concurrently exhibiting antigenotoxic properties at lower concentrations[64]. Correspondingly, the

lowest concentration of wastewater extracts from olive mill demonstrated an antigenotoxic hormetic effect on HepaRG™ cells[65]. Furthermore, *Gentiana lutea* extract, at lower tested doses, reduced the genotoxicity in genetically modified *Salmonella typhimurium* TA1535[66]. These observations highlight the complex and often non-linear dose-response relationships inherent in phytochemical-rich extracts. Conversely, oil and water extracts from *Eugenia uniflora* L. were found to be mutagenic at lower concentrations and antimutagenic at higher concentrations, illustrating a concentration-dependent duality in their genotoxic effects[67].

Based on the in vitro results, neither *D. superbus* nor *P. paradoxus* extracts can be considered entirely safe for human use without further safety evaluation. While the findings presented herein offer new insights into the cytotoxic and genotoxic potential of *D. superbus* and *P. paradoxus* extracts, some limitations warrant consideration. A primary limitation is the absence of a chemical characterization for these specific extracts, which would facilitate the identification of their precise metabolic profiles. Furthermore, all experiments were conducted in vitro, utilizing a single cell line (CHO-K1) and employing only one positive control (MMC). While providing valuable mechanistic insights, the use of a solitary model limits the generalizability of the results across different tissue types and species. Future research employing a battery of diverse cell lines or primary cultures is required to confirm the systemic safety of the extract.

Consequently, in vivo studies are warranted to fully assess the potential toxicological outcomes, including histopathological alterations and changes in biochemical parameters. Future research should also aim to elucidate the underlying mechanisms responsible for the observed genotoxic effects and to identify the specific phytochemicals driving these properties precisely. Furthermore, this study evaluated the genotoxicity of the extract in a direct-acting context only. Since many phytochemicals act as promutagens requiring metabolic transformation, the absence of an exogenous metabolic activation system (S9 liver fraction) represents a limitation. Consequently, the results may not account for genotoxic metabolites formed in vivo." While the present study identifies the cytotoxic and genotoxic potential of these species, further investigation into the chemical characterization of the methanolic extracts is currently underway to identify the specific secondary metabolites responsible for these activities.

Materials and methods

Chemicals

MMC and cytochalasin B were supplied by D.B.A. Italia s.r.l. (Milan, Italy), 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) was obtained from BioSigma (USA). Hoechst 33,342 Staining Dye Solution, provided by Prodotti Gianni Srl (Italy) through Abcam, was also used. Cell culture media, supplements, consumables, dimethyl sulfoxide (DMSO; 99.9%), and formaldehyde solution were sourced from Euroclone S.p.A. (Milan, Italy). Methanol (HPLC grade) was purchased from Merck KGaA (Darmstadt, Germany).

Collection and identification of plant material

D. superbus and *P. paradoxus* plants were purchased from the institutional nursery "Centre for Biodiversity and Outside-Forest Activities" of Veneto Agricoltura (Montecchio Precalcino, Vicenza, Italy). Species selection and nomenclature were verified using the official checklist of the Italian flora via the Portal to the Flora of Italy (available at: https://dryades.units.it/floritaly/index.php, accessed on 1 November 2022). The plants were cultivated at the Department of Biotechnology, University of Verona. For each species, a representative voucher specimen (exsiccatum) was deposited at the Herbarium of the University of Verona (Verona, Italy) under the following accession codes: BDV138-A-AST-as4-Pepa (*P. paradoxus*) and BDV207-A-CA-ca8-Disu-su (*D. superbus* subsp. superbus). For experimental research, three biological replicates were established; each replicate consisted of pooled leaves collected from two individual plants during the vegetative phenological stage. The leaf tissue was immediately frozen in dry ice, ground into a fine powder using an A11 Basic Analytical Mill (IKA-Werke, Staufen, Germany), and stored in light-protected tubes at − 80 °C.

Preparation of plant extract

Methanolic extracts of plant material were prepared based on the method described[44], with minor modifications. Briefly, 20 g of each frozen leaf powder was extracted with methanol at a solid-to-solvent ratio of 1:10 (w/v) in sealed glass containers. The mixture of each plant was continuously agitated using a magnetic stirrer for 48 h at room temperature. Following extraction, the resulting solution was filtered through quantitative filter paper (ArtiGloss; particle retention range: 12–15 µm) and subsequently concentrated using a rotary evaporator (BUCHI R-210) at 40 °C for 30–50 min. To ensure the complete removal of residual methanol, the concentrate was left under a fume hood for 24 h and then freeze-dried using liquid nitrogen. The dried extract was transferred to amber glass bottles and stored at 4 °C until further analysis. The extraction yield for the methanolic extract of *D. superbus* was approximately 2%, and 3% for the *P. paradoxus* extract. The extraction yield was calculated using the standard formula presented in Eq. 1.

$$\text{Yield } \% = \frac{\text{weight of obtained extract (g)}}{\text{weight of dried plant sample used (g)}} \times 100. \quad (1)$$

Cytotoxicity assessment

Cytotoxicity and Genotoxicity of *D. superbus* and *P. paradoxus* methanolic extract were evaluated using the Chinese Hamster Ovary K1 (CHO-K1) cell line. The CHO-K1 cell line (603480) was purchased from CLS Cell Lines Service (GmbH, Germany). Cells were cultured in Ham's F12 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% glutamine. A stock solution of both plants, *D. superbus* and *P. paradoxus* methanolic extracts, were prepared by dissolving the extract in dimethyl sulfoxide

(DMSO) at a concentration of 50 mg/mL. This stock solution was subsequently diluted in cell culture media to generate seven series of two-fold dilutions, ranging from 100 µg/mL to 1.6 µg/mL of *D. superbus* and from 0 to 250 µg/mL of *P. paradoxus* extract. Each of these seven concentrations was tested in triplicate, and the entire experiment was independently replicated three times. All experimental procedures were conducted within a safety cabinet hood to maintain sterility. The cytotoxicity of *D. superbus* and *P. paradoxus* methanolic extracts was assessed using the MTT assay. Briefly, CHO-K1 cells were seeded in 96-well plates (Primo® Multiwall plates, 96-flat bottom, Euroclone S.p.A., Italy) at a density of 3,000 cells per well and incubated for 24 h at 37 °C in a 5% CO_2 atmosphere. Following incubation, cells were treated for 24 h with various concentrations of plant extracts alongside a 0.3% DMSO control and a negative control (cell medium). After the 24-h treatment, 20 µL of MTT solution (5 mg/mL in PBS) was added to each well, and the plates were incubated for an additional 4 h at 37 °C in a 5% CO_2 atmosphere in 5% CO2 atmosphere. Subsequently, the medium was carefully removed, and 100 µL of 100% DMSO was added to each well to dissolve the formed formazan crystals. Absorbance was measured at 570 nm, with background correction at 690 nm, using a microplate reader (Synergy). Cell viability was calculated as a percentage relative to the control wells using the following formula:

$$\%\ \text{Viability of CHO} - \text{K1 cells} = \frac{\text{Mean absorbance of the sample}}{\text{Mean absorbance of the control}} \times 100 \tag{2}$$

Genotoxic and antigenotoxic study

The in vitro micronucleus assessment was conducted using the cytokinesis-block micronucleus (CBMN) assay according to established protocols[44], and in compliance with OECD guidelines[40]. CHO-K1 cells were seeded at a density of 3000 cells/well in 96-well plates and maintained in a humidified atmosphere (37 °C, 5% CO_2) for 24 h prior to treatment. Following this pre-incubation period, cells were exposed to negative control (0.3% DMSO), positive control, MMC at 0.025 µg/mL, *D. superbus* extract at three concentrations (6.3, 12.5, 25 µg/mL) and *P. paradoxus* at 3 different concentrations, 12.5, 25, 50 µg/mL both independently and in combination with MMC (0.025 µg/mL). Treated cells were incubated for an additional 24 h under standard conditions (37 °C, 5% CO_2). Post-treatment, cells were washed with phosphate-buffered saline (PBS) and supplemented with fresh medium containing cytochalasin B (3 µg/mL) to block cytokinesis. Following a 24 h incubation with cytochalasin B, the medium was aspirated, and cells were fixed with 4% formaldehyde for 15 min. After fixation, cells were subjected to two 3-minute PBS washes. Nuclear staining was performed by applying 100 µL/well of diluted Hoechst 33342 and incubating for 30 min at room temperature under light-protected conditions.

The concentration range for *D. superbus* and *P. paradoxus* extract evaluation was established based on MTT cytotoxicity assay results, ensuring that the highest test concentration induced cytotoxicity below the OECD-recommended threshold (55 ± 5%). Concentrations exceeding 25 µg/mL for *D. superbus* and 50 µg/mL for *P. paradoxus* demonstrated cytotoxicity above 50% in CHO-K1 cells; consequently, these concentrations were selected as the maximum test concentrations. The minimum concentration (6.3 µg/mL and 12.5 µg/mL) for *D. superbus* and *P. paradoxus* extracts were determined to be non-cytotoxic to CHO-K1 cells. All experimental conditions were evaluated in triplicate, with a minimum of 2000 binucleated cells scored per concentration in each experimental run. The entire experimental protocol was independently replicated three times.

Cytotoxicity evaluation under CBMN assay

In vitro tests frequently fail to identify genotoxic substances unless the tested amounts cause some level of cytotoxicity. Excessive cytotoxicity, on the other hand, may produce false-positive results and cause incorrect data interpretation. Therefore, at the maximum concentration tested, it is advised that the cytotoxicity level not exceed 55 ± 5%[40]. Considering these factors, it is recommended to assess a minimum of three different concentrations of the test substance in the CBMN assay[41]. To address this issue in our experiments, we evaluated cytotoxicity as an integral part of the CBMN procedure, using the same cells that were utilised for counting micronuclei for the tested 3 concentrations using for CBMN assay and applying the cytokinesis-block proliferation index (CBPI), which reflects the average number of cell cycles that take place during exposure to cytochalasin B. This index is associated with a decreased ratio of binucleated to mononucleated cells. The percentage of cytotoxicity (CBPI) was calculated according to the formulas provided in Eqs. (3, 4).

$$\text{CBPI} = 100 \cdot \frac{[\text{N}_1 + 2 \times \text{N}_2 + 3 \times \text{N}_3]}{\text{total number of cells}} \tag{3}$$

$$\%\ \text{Cytotoxicity (CBPI)} = 100 - \left[100 \times \frac{\text{CBPI of treated cells} - 1}{\text{CBPI of untreated cells} - 1}\right] \tag{4}$$

where N1 is the number of mononucleated cells, N2 is the number of binucleated cells, N3 is the number of multinucleated cells.

Fluorescence microscopy

Fluorescence microscopy was performed using a Leica DMi8S inverted microscope (Leica Microsystems). Fixed and stained cells in 96-well plates were observed using a 40x objective lens. The microscope was equipped with a TIRF module and a FURA filter wheel, enabling rapid switching between excitation wavelengths (20–28 ms). Images were acquired under blue light using a microscopic digital camera (type to be specified) controlled by the LAS X Office software (Leica Microsystems). For each well, images were captured from 64 distinct positions. Each sample concentration was tested in three independent wells, and a total of over 2000 binucleated cells were

scored per concentration in each experimental run. All acquired images were serially numbered and saved using the LAS X Office software.

Detection of micronuclei using CellProfiler software

An accelerated and more precise assessment of the micronucleus assay can be achieved through the automation of micronuclei analysis using image processing software. In this study, binucleated cells and micronuclei were automatically detected using CellProfiler software (version 4.2.6) as described previously[42]. CellProfiler is an open-source, modular image analysis application designed to handle large volumes of images. It offers a versatile platform for image analysis experts to collaborate, experiment, and develop innovative methodologies[68]. The software operates within a pipeline framework consisting of individual modules that process images in various ways, organized sequentially to generate a comprehensive workflow. Initially, images acquired using a wide-field microscope were enhanced with the Leica Lightning deconvolution tool to improve the signal-to-noise ratio, facilitating the identification of nuclei and micronuclei by CellProfiler. Subsequently, images from the same experimental conditions were analysed concurrently using Cell Profiler, as the method described in our published studies[42].

In vitro CBMN of P. paradoxus extract using the ImagestreamX imaging flow cytometer

In this part of the experiment, the detection of micronuclei was determined using the ImagestreamX imaging flow cytometer as described prevoiusly[44].

Cell culture and treatment protocol

CHO-K1 were seeded at a density of 3×10^4 cells per well in 6-well plates, using 1 mL of culture medium per well. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 24 h before treatment. Subsequently, cells were exposed to one of the following conditions for 24 h: 0.3% DMSO (negative control), MMC (positive control) at 0.025 µg/mL, or *P. paradoxus* extracts at concentrations of 12.5 and 50 µg/mL. Treatments with plant extracts were conducted both in the presence and absence of 0.02 µg/mL MMC. After the 24-hour treatment period, culture media were removed, and cells were incubated for an additional 24 h with 3 µg/mL cytochalasin B. Following incubation, cells were washed with phosphate-buffered saline (PBS), detached using trypsin, and centrifuged at 300 × g for 3 min. Supernatants were discarded, and cell pellets were resuspended in 100 µL of calcium- and magnesium-free PBS in Eppendorf tubes. For nuclear staining, samples were stained with Draq5 dye (Life Technologies, Milan, Italy) to a final concentration of 50 µM.

Nucleus and micronucleus scoring via ImageStreamX and IDEAS software

The ImageStreamX flow cytometer allows a quick capture of single cell images in multiple fluorescence channels as previously reported[42]. After cell treatment with the lowest (12.5 µg/mL) and highest (50 µg/mL) concentrations of the *P. paradoxus* extract and MMC, the cells were collected, stained and then examined. Draq5-stained cells were run in the flow cytometry and acquired by the manufacturer's software "Inspire". The acquisition was performed selecting in focus and single cells, discarding events not representing the individual cells. The manufacturer's software, IDEAS, was then used for the imaging analysis. In order to identify and score total cells, binucleated cells, mononucleated cells, multinucleated cells and micronuclei, an optimized analysis template, defining new features and masks, was developed and created in IDEAS. Briefly, the following functions were combined through Boolean logic to create the final mask: Threshold, Spot, Range, LevelSet, Dilate. Binucleated cells (BNC) population gates were derived from the strategy reported in Fig. Xa-f and in particular employing the following parameters: Gradient_RMS (focus), Aspect Ratio, Area, Intensity, Lobe Count, Homogeneity Mean, Compactness. Details analysis steps are shown in Figs. 8 (panels a-g) and 9.

Enhanced gating strategy and exclusion criteria

The sequential gating strategy used to isolate single, high-quality cells while excluding background noise and artifacts. First, to eliminate aggregates (doublets/clumps) and debris, a bivariate plot of Area versus Aspect Ratio was generated. Single cells were identified as the population with an Aspect Ratio greater than 0.5 and a mid-range Area. This population was subsequently used to define bi-nucleated cells in the histogram analysis. To ensure accurate morphometric scoring, an In-Focus gate was applied using the Gradient RMS feature. Only events with a Gradient RMS value greater than 80 were included in the analysis to exclude out-of-focus events. Additionally, small fragments and apoptotic bodies were removed by excluding events with low Draq5 intensity and a nuclear Area below the 5th percentile of the control population. This sequential approach ensured that only intact, well-focused single cells were included in the final dataset. To ensure consistency across the different treatment groups (*P. paradoxus* and MMC), compensation and normalization procedures were applied. Spectral compensation was performed using single-stained control samples to eliminate fluorescence spillover between channels, ensuring that the Draq5 signal did not interfere with other measured parameters. Furthermore, Draq5 fluorescence intensity was normalized to the mean intensity of the untreated control group within each experimental run. This step minimized inter-run variability and allowed for accurate comparison between treatment conditions.

Statistical analysis

All data were statistically analyzed using GraphPad Prism 7.0. Results are presented as the mean ± standard deviation. Comparisons between means were performed using a one-way analysis of variance (ANOVA), followed by Tukey–Kramer post hoc tests with a 95% confidence interval. Statistical significance was defined as $P < 0.05$, with $P < 0.01$ indicating high statistical significance.

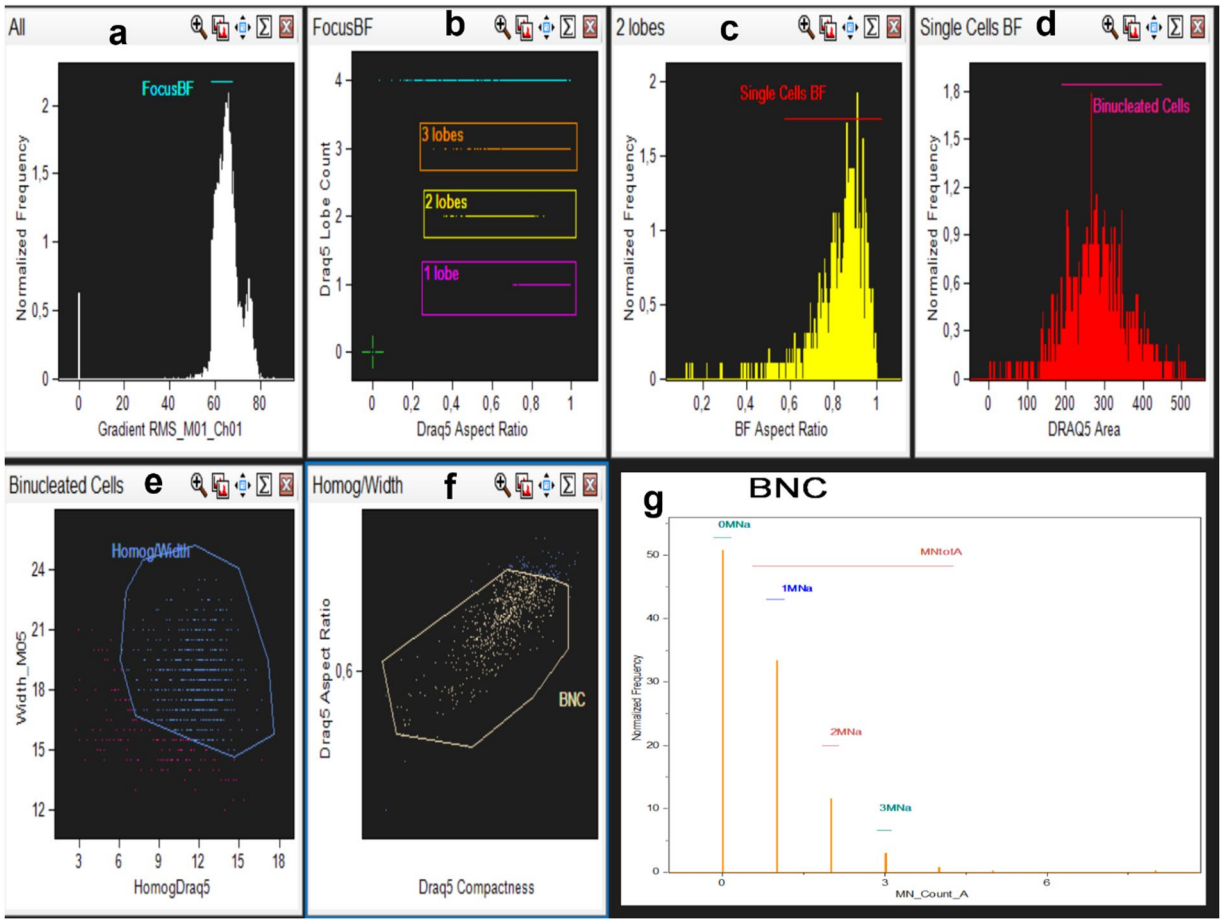

Fig. 8. Representative analysis steps of micronuclei using the IDEAS Software. (**a**) During the analysis, first selection was based on Gradient_RMS parameter to confirm in focus-events in the brightfield channel (BF, Ch01). (**b**) A dot plot of Draq5 lobe count versus aspect ratio was created: the reported gates include all cells with two Draq5-stained nuclei, 2-lobes, single lobe, and cells with more than two nuclei. (**c**) Represents a histogram of BF Aspect Ratio, displaying the gate used to discriminate single cells: all events with Aspect Ratio higher than 0.5 were considered for defining bi-nucleated cells in the histogram reported in (**d**) that used Draq5 Area for this purpose. (**e**) dot plot of Draq5 Width versus Homogeneity parameters where the blue gate includes cells with more uniform distribution of Draq5 stain: the majority of the events of interest showed Homogeneity greater than 10 and Width greater than 15. (**f**) Previous Homog/Width population was considered in a dot plot of Draq5 Aspect Ratio intensity versus Draq5-Compactness to finally define the gate that encompasses the acceptable BNC population (yellow gate). Each sample was checked to better define each single gates, evaluating each single event. (**g**) A histogram of our specific spot count feature (micronuclei _Count A) generated over the masks described in materials and methods and in supplementary data (2). The linear gates over each bar display the number of BNCs without micronuclei (0 micronuclei) and respectively with 1, 2 and 3 micronuclei. The normalized frequency represents the percentage of each type of cell among the total number of cells in the BNC population. BF= brightfield channel, BN= binucleated cells, BNC= binucleated cells with micronuclei. (Reproduced from 42).

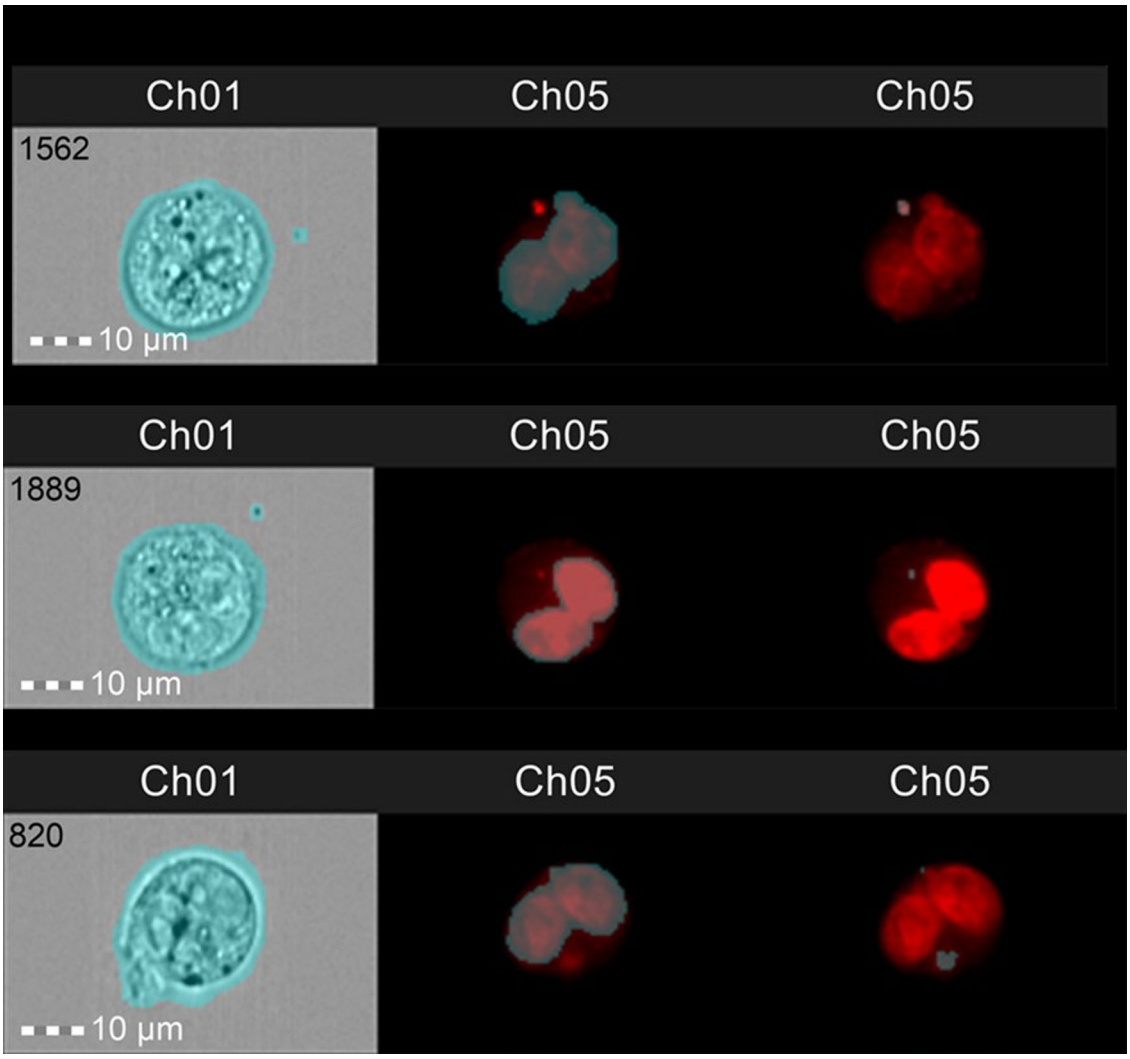

Fig. 9. Masks staining (blue shadows over the images): (**a**) Ch01, brightfield (BF) image with the default mask applied that stains all the picture; (**b**) Ch05, binucleated cells with micronuclei (BNC) with a single micronuclei stained with Draq5 with the nuclear mask applied (micronuclei _MaskB_Step3, see supplementary data); (**c**) Ch05, BNC with a single micronuclei stained with Draq5 with the complete micronuclei mask applied. (Reproduced from 42).

Data availability

Data are contained within the article.

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Ghanya Al-Naqeb contributed to the experimental designing, conceptualization, data curation, investigation, visualization, writing original draft, writing and review & editing.; Rachele De Giuseppe contributed to review and editing, administration, resources and conceptualization; Aliki Kalmpourtzidou and Linda Avesani contributed to contributed to review, and editing and Hellas Cena contributed to review and editing, supervision, project administration, funding acquisition, resources and conceptualization.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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