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# Research Article
# Antioxidant and *in vitro* Toxicity Assessment of Elegant Explosion Daylily Flower (*Hemerocallis* spp.) Extract in Hepatic Cell Line

[1]J. Johnson, [2]S. Ogutu and [1,3]J. Boateng

[1]Laboratory of Food Safety and Toxicology, Alabama A and M University, Normal Al, 35872, United States
[2]Laboratory of Food Chemistry, Alabama A and M University, Normal Al, 35872, United States
[3]Departments of Food and Animal Sciences, Laboratory of Nutrition Biochemistry, Alabama A and M University, Normal Al, 35872, United States

## Abstract

**Background and Objective:** Edible flowers such as the daylily (*Hemerocallis* spp.) have been shown to have beneficial effects against chronic diseases. This study determined antioxidant activity and polyphenol contents of two cultivars of daylily flowers Barbara Mitchell (BM) and Elegant Explosion (EE). The *in vitro* bioactivities of EE extract were further examined in HepG2 cell lines. **Materials and Methods:** BM and EE daylily flowers extracts were prepared with aqueous ethanol and used to determine Total Phenolic Content (TPC), Total Flavonoid Content (TFC), Total Monomeric Anthocyanins (TMA), Condensed Tannins (CT) and antioxidant assays. Selected phytochemicals were also determined via HPLC/DAD. *In vitro* toxicity, apoptosis and enzymatic activities of EE extracts were assessed in HepG2 cells. **Results:** Results indicated EE showed significantly ($p \leq 0.05$) higher polyphenol contents and antioxidant activity compared to BM. *In vitro* cytotoxicity indicated that EE extracts possess moderately weak toxicity towards HepG2 cells. Significant ($p \leq 0.05$) increases in antioxidant activities were noted. **Conclusion:** With limited studies on polyphenol content and bioactivity of EE daylily flower, these results provide preliminary evidence and contribute to the promotion of edible flowers as a potential dietary supplement and nutraceutical for use as functional foods.

Key words: Edible flowers, daylily, superoxide dismutase, cytotoxicity, nutraceutical, phenolic compounds, endogenous antioxidants

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Corresponding Author: Judith Boateng, Laboratory of Food Safety and Toxicology, Alabama A and M University, Normal Al, 35872, United States
Tel: 256-372-8794 Fax: 256-372-5432



Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

A primary knowledge of edible flower preparations has been documented from accounts described in ancient literature[1-3]. For example, in 16th century Central Europe, fried batter-coated black elder (*Sambucus nigra*) flowers were common, as were dandelion flowers boiled with ersatz honey[2-4]. Ancient Romans enjoyed roses and lavender in sauces[4,5]. In Victorian-era England, roses were used as flavouring agents in teas, sweets, pastries, conserves and sauces[4,5]. Besides the wholesomeness and suitability in most aspects of culinary applications, edible flowers are abundant sources of nutrients such as carbohydrates, protein, fat, minerals and vitamins[6,7]. Additionally, edible flowers contain varying quantities of several bioactive compounds[8-11]. These bioactive compounds with antioxidant properties contribute to the medicinal soundness of edible flowers and thus support increased worldwide demand.

The daylily (*Hemerocallis* spp.) is an edible flower, which dates back thousands of years in sub-tropical Asia. The main diversity of the genus is centred in China, Korea and Japan, where they are used as medicine and food[12]. For culinary and consumption purposes, different parts of the flower are used as vegetables and incorporated into soups, stews, or eaten raw. Daylilies (*Hemerocallis* spp.) have shown strong antioxidant activities *in vitro* and *vivo*[13-15]. These beneficial effects are believed to be due to several novel bioactive compounds including naphthalene glycoside, stelladerol, caffeoylquinic acid derivatives, as well as other antioxidants such as gallic acid, rutin and catechin[16,17]. According to Rodriguez-Enriquez and Grant-Downton[17], *Hemerocallis* spp. could have significant potential as a nutraceutical and the source of new compounds with biomedical activities. Thus, the daylily could be considered as a functional food and used in natural products and supplements to improve health.

Although the consumption of flowers is on the rise, it is concluded that there are still numerous aspects that need to be evaluated. This is especially important if it is to be considered as a functional food or nutraceutical. As highlighted by Granato *et al*.[18], assigning a functional label to a potential food product or nutritional supplement requires completion of a proper assessment to attest its functionality using *in vitro* and *in vivo* methods. Given this, Granato *et al*.[18] indicated that a wide variety of tests, both *in vitro* and *in vivo*, must be performed to determine efficacy and bioactivity as well as to assess the physicochemical and chemical properties of any food before stating it is functional.

This study aims to determine the polyphenolic contents and antioxidative activity of daylily cultivars Barbara Mitchell (BM) and Elegant Explosion (EE) and also, to assess their acute *in vitro* toxicity potential (cytotoxicity, apoptotic and antiproliferative properties) in HepG2 hepatic cell line.

## MATERIALS AND METHODS

**Study area:** The study was carried out in the Department of Food Science at Alabama A and M University, Normal AL, United States from September, 2016-December, 2018.

### Materials and reagents

**Flowers:** Barbara Mitchell (BM) and Elegant Explosion (EE) daylily plantlets were obtained from a local nursery (Bennet Nurseries) that cultivates and sells edible flowers from organic production. (The plantlets were confirmed by Dr. Patti Coggins, who has a long history of working with *Hemerocallis* spp.). The plantlets were cultivated and cared for in the greenhouse at Alabama A and M University, United States until harvesting.

**Chemicals:** All chemicals and reagents that were used in this study were of pure analytical grade and purchased from Fisher Scientific (Suwannee, GA) and Sigma Aldrich (St. Louis, MO). Kits and other supplies were purchased from Promega, Thermo Fisher Scientific (Madison, WI) and Cayman Chemical Company (Ann Arbor, MI). Catechin, Rutin, Gallic acid and Chlorogenic acid standards were acquired from Indofine Chemical Company (Hillsborough, NJ).

**Harvesting and extraction of polyphenols from daylily flowers:** Barbara Mitchell and Elegant Explosion daylily flowers were harvested at full bloom in the morning for a week. The pulling method was used to combine several batches over the days of harvest. Flowers were placed in individual plastic containers with absorbent papers at the bottom to prevent any lymph leakage due to guttation. The harvested flowers were placed on ice and transported to the laboratory in refrigerator bags and stored at -20°C immediately. The flowers were analyzed within 1 week from the collection day.

**Extraction procedure:** Approximately 70 g of flowers were weighed and homogenized in 200 mL of 80% ethanol. The homogenate was stored in the dark overnight. The supernatant was removed by centrifugation (3000 rpm at 4°C for 20 min). The extraction procedure was repeated two more

times and all the supernatants/extracts were pooled. The extracts were concentrated by evaporation and the concentrated extract was stored at -80°C until analysis. The extracts were prepared in triplicates.

### Determination of total polyphenol contents in daylily flowers

**Total Phenolics Contents (TPC) and Total Flavonoids Content (TFC):** The TPC of daylily extracts was determined using the Folin-Ciocalteu method modified for the microplate Gajula *et al.*[19] The TPC was expressed as mg Gallic acid equivalent per gram sample (mg GAE $g^{-1}$ sample). The TFC was determined via colorimetric assay[20] with modifications[19] and was expressed as mg catechin equivalent per gram sample (mg CE $g^{-1}$ sample).

**Condensed tannins and Total Monomeric Anthocyanins (TMA) contents:** Condensed tannins were determined by the Vanillin-HCl/vanillin $H_2SO_4$ assay method[18] and expressed in mg catechin equivalent $g^{-1}$ sample (mg CE $g^{-1}$ sample). The TMA of daylily flowers was determined by the pH differential method with modification[21,22]. Daylily extracts were diluted with two buffers, potassium chloride buffer (0.025 M, pH 1.0) and sodium acetate buffer (0.4 M, pH 4.5). Diluted samples are read against a blank at 520 and 700 nm. The TMA was expressed as mg cyanidin-3-glucoside equivalent $g^{-1}$ sample (mg C3GE $g^{-1}$ dwb).

### Determination of antioxidant activities in daylily flowers

**Trolox Equivalent Antioxidant Capacity (TEAC) assay:** TEAC was determined following previous methods[23]. The TEAC was expressed as a Mmol Trolox $g^{-1}$ sample.

**Ferric Reducing Antioxidant Power (FRAP) assay:** The FRAP of daylily extracts was measured using methods described by Benzie and Strain[24]. The FRAP activity was expressed as an mM $Fe^{2+}$ $g^{-1}$ sample.

**DPPH radical scavenging capacity assay:** The DPPH test was performed following the method of Brand-Williams *et al.*[25], with modifications[26]. The $IC_{50}$ was calculated and the decrease in absorbance at 515 nm was monitored at $T_0$ and $T_{30}$, $T_{60}$ and $T_{90}$. The DPPH scavenging ability was calculated as a percentage using the following Eq.[26]:

$$\text{DPPH scavenging ability (\%)} = \frac{A_{515\,(t=0)} - A_{515\,(t=90\,min)}}{A_{515\,(t=0)}} \times 100 \quad (1)$$

**Metal chelating activity assay:** The Metal chelating activity was measured using a modified method by Wong *et al.*[27] Various concentrations of extracts were prepared and 180 µL of methanol (100%) and 5 µL $FeCl_2$ (2 mM) were added and incubated for 3 min. About 10 µL of ferrozine (5 mM) was added to initiate the reaction and the mixture was incubated for 10 min at room temperature after shaking. Absorbance was read at 562 nm. The percentage of $Fe^{2+}$-ferrozine complex formation was calculated as $Fe^{2+}$ (%) chelating ability[27].

**Determination of selected polyphenols in daylily flowers via HPLC:** High-performance liquid chromatography (HPLC) analysis of polyphenols in daylily flowers was carried out on a Varian Modular Analytical HPLC System. Polyphenol's identification was determined using methods of Schutz *et al.*[28] with modifications. Samples and standards were prepared using 80% ethanol. Mobile phase A was 0.5% (v/v) acetic acid in the water and mobile phase B was 0.5% (v/v) acetic acid in acetonitrile. Separations of polyphenols were conducted at a flow rate of 0.8 mL $min^{-1}$ at 25°C. Catechin and gallic acid were eluted at 284 nm, chlorogenic acid at 320 nm and rutin at 350 nm. Spectral data was collected for each standard and sample. Polyphenols (i.e., rutin, catechin, chlorogenic acid and gallic acid) were selected based on the previous studies[29-32].

### Determination of *in vitro* cytotoxicity of Elegant Explosion (EE) extract

**Cell line and culture conditions of Hep2G cells:** Human hepatocellular carcinoma cell line (HepG2), (ATCC, Manassas, VA) was cultured following previous methods and conditions[33]. HepG2 was seeded at $10^4$ cells $cm^{-2}$ density and allowed to adhere for 48 hrs and cells were used for cytotoxicity determinations on the third day after seeding.

**Determinations of cell viability of EE extract in HepG2 cells:** Cell viability was determined by measuring the metabolic activity of living cells using the PrestoBlue® (Life Technologies Corporation, Grand Island, NY) assay reagent. After the seeding period specified above, HepG2 cells were incubated with different concentrations (0.2, 0.4, 0.8, 1.0, 2.0, 4.0 and 8.0 mg $mL^{-1}$) of EE extract for 24 and 48 hrs. After the incubation period, the culture medium (supernatant) was removed and cell viability was determined.

**Morphological evaluation of EE extract on HepG2 cells:** HepG2 cells ($1\times10^4$) were grown on coverslips and treated with or without daylily extracts for 48 hrs. The medium was discarded and cells were washed once with PBS. Cells were

fixed with MeOH: acetic acid (3:1) and stained with NucBlue® Live reagent (Hoechst 33342) and NucGreen® Dead reagent (Life Technologies). After 15 min of incubation, the dye was removed and the cells were rinsed with PBS and imaged with the EVOS® FL Auto system (Thermo Fisher Scientific, Waltham, MA).

### Determination of antioxidant/detoxification enzymes

**Extraction of protein from cells:** After treatment with daylily extract, cell lysate was collected from HepG2 cells following a protocol from our lab[33,34]. Protein in the cell lysate was determined using a BCA protein assay kit from Pierce (Rockford, IL). The cell lysate was used for enzyme analysis.

**Determination of endogenous antioxidant enzymatic activity:** Glutathione (GSH) was determined according to Griffith[35] and expressed as μmol $mg^{-1}$ protein. Superoxide Dismutase (SOD) was determined by the method outlined by Fernández-Urrusuno *et al.*[36]. Xanthine oxidase was utilized to generate a superoxide flux and samples were measured at 440 nm after the addition of xanthine oxidase. Data were expressed as units of SOD activity per mg protein (U $mg^{-1}$ protein). Glutathione-S-transferase (GST) activity was based on the method of Habig *et al.*[37] and 1-chloro-2, 4-dinitrobenzene was used as the substrate. GST was expressed as μmol $mg^{-1}$ protein. All samples were analyzed in triplicates.

**Statistical analysis:** All experiments were repeated three independent times and results were conducted in triplicates. ANOVA was used to determine any significant differences among daylily cultivars. Data for the antioxidant assays are presented as the mean ± standard deviation of the mean (SD) and those for the cytotoxicity assays are presented as the mean percentages relative to the control ± SD. Significant ($p \leq 0.05$ or 0.1), means were separated using Tukey's Studentized Range Test. Statistical analysis was conducted using SAS, 9.4 tools.

## RESULTS AND DISCUSSION

**Total polyphenol contents in daylily flowers:** The results of TPC, TFC, TMA and CTC are shown in Table 1. The TPC was significantly ($p \leq 0.05$) higher in EE (2.13 mg GAE $g^{-1}$ dwb) compared to BM (1.11 mg GAE $g^{-1}$ dwb). TFC showed a parallel trend between the BM and EE flowers. The TFC was approximately two times higher in EE (1.47 mg CE $g^{-1}$ dwb) than in BM (0.64 mg CE $g^{-1}$ dwb). While the results for TFC in EE and BM are within the range reported elsewhere for edible flowers[38-41], there is high variability in TPC. The TPC reported in this study was lower compared to other studies. This variation may be due to the extraction methodology used or the types of solvent employed. Additionally, several factors such as genotype, environment, geographical location and altitude, UV exposure, season, soil, maturity stage at harvest, post-harvest conditions, etc. could all impact TPC variation. It should be noted that the daylily cultivars utilized in this study were cultivated in the AAMU greenhouse under controlled culture conditions. Flavonoids are well-known antioxidants with importance in human health. It is reported that due to their strong antioxidant capacity, most flavonoids outperform natural and well-known antioxidants including ascorbate (vitamin C) and α-tocopherol (vitamin E) because of their strong ability to donate electrons or hydrogen atoms to neutralize free radicals[42]. This indicates their potential as important therapeutic agents and also for improving the quality of foods[43]. The contents of anthocyanins in edible flowers have been well documented[7,44,45]. Among the most prominent anthocyanins, cyanidin-3-glucoside (C3G) is the major anthocyanin found in most flowering plants. The data of Table 1 shows the TMA content in EE was significantly ($p \leq 0.05$) higher (1.73 mg C3G $g^{-1}$ dwb) compared to BM (1.20 mg C3G $g^{-1}$ dwb). These results are comparable to other studies[46-50]. Anthocyanins, besides their importance as natural colourants or pigments in foods, have various beneficial health benefits[46,49,51-56]. A similar trend was observed whereby CTC was significantly ($p \leq 0.05$) higher in EE (0.024 mg CE $g^{-1}$ dwb) than in BM (0.004 mg CE $g^{-1}$ dwb) (Table 1). Condensed tannins (proanthocyanidins) have found various applications in foods. They are used as additives, they prevent oxidation in foods and enhance the shelf life of beverages including teas, fruit juices and wine[57]. For health purposes, condensed tannins or proanthocyanidins are noted for their antibacterial activity against urinary tract infections, for beneficial regulation of gut microbiota[58,59] as well as anti-inflammatory enhancing properties[57,60,61].

**Antioxidant activities of daylily flowers:** The result of Table 2 show the FRAP, TEAC, DPPH and $Fe^{2+}$ chelating antioxidant activities of EE and BM flowers. When it comes to determining the antioxidant activity of plant extracts there's not a standardized method for evaluation since the extracts may exhibit different antioxidant mechanisms. As such, it is important to perform more than one type of antioxidant activity measurement to take into account the various

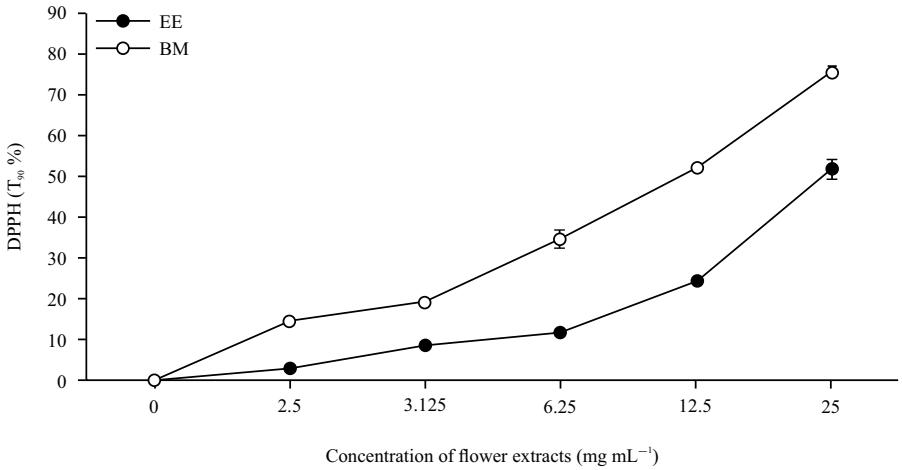


Fig. 1: DPPH (%) of daylily cultivars at $T_{90}$ min

Values are expressed as means±SD and were conducted in triplicates in three independent experiments. Statistical significance was determined by one-way ANOVA followed by Tukey's range test. EE: Elegant explosion, BM: Barbara mitchell

Table 1: Total polyphenol contents in daylily cultivars

| Samples | TPC (mg GAE g$^{-1}$ dwb) | TFC (mg CE g$^{-1}$ dwb) | TMA (mg C3GE g$^{-1}$ dwb) | CTC (mg CE g$^{-1}$ dwb) |
|---|---|---|---|---|
| BM | 1.11±0.06$^{b}$ | 0.64±0.01$^{b}$ | 1.20±0.15$^{b}$ | 0.004$^{b}$ |
| EE | 2.13±0.01$^{a}$ | 1.47±0.02$^{a}$ | 1.73±0.08$^{a}$ | 0.024$^{a}$ |

Values (n = 3) are means±SD, means in the column ($^{ab}$) letter differ ($p \leq 0.05$) using Tukey's Studentized range test. BM: Barbara mitchell, EE: Elegant explosion, GAE: Gallic acid equivalent, DWB: Dry weight basis (dry flowers), TFC: Total flavonoid, TPC: Total phenolic, TMA: Total monomeric anthocyanin content, CTC: Condensed tannins content, CE: Catechin equivalent, GAE: Gallic acid equivalent, C3G: Cyanidin-3-glucoside equivalent

Table 2: Antioxidant activities of daylily cultivars

| Samples | FRAP | TEAC | DPPH ($IC_{50}$ mg mL$^{-1}$) | $Fe^{2+}$ chelating activity ($IC_{50}$ mg mL$^{-1}$) |
|---|---|---|---|---|
| BM | 0.97±0.04$^{b}$ | 4.82±0.02$^{b}$ | 24 | 43.08 |
| EE | 2.48±0.15$^{a}$ | 16.15±0.24$^{a}$ | 14.31 | 37.05 |

Values (n = 3) are expressed as means±SD means in column ($^{ab}$) letter differ ($p \leq 0.05$) using Tukey's Studentized range test. (1) FRAP is expressed in µmol $FeSO_4$ per g of dry flowers, (2) TEAC is expressed in mM Trolox per g of dry flowers. BM: Barbara mitchell, EE: Elegant explosion, DPPH: 2,2-diphenyl-1-picrylhydrazyl, FRAP: Ferric reducing antioxidant power, $Fe^{2+}$: Ferrous ion, TEAC: Trolox equivalent antioxidant capacity

mechanisms of antioxidant action[62,63]. The results in Table 2 show EE had a significantly ($p \leq 0.05$) higher FRAP value (2.48 µmol $FeSO_4$ g$^{-1}$ dwb) compared to BM (0.97 µmol $FeSO_4$ g$^{-1}$ dwb). TEAC activity was higher ($p \leq 0.05$) in EE (16.15 mM Trolox g$^{-1}$ dwb) compared to BM (4.82 mM Trolox g$^{-1}$ dwb). The DPPH assay is a rapid, direct and reproducible procedure widely used to determine the radical scavenging activity, via inhibition (%) or $IC_{50}$ of plant extracts. The $IC_{50}$ was 14.31 mg mL$^{-1}$ ($R^2 = 0.94171$) in EE compared to 24 mg mL$^{-1}$ ($R^2 = 0.99363$) in BM in Table 2. The data of Fig. 1 shows the DPPH expressed as percentage (%) inhibition after 90 min ($T_{90}$) was higher ($p \leq 0.05$) in EE (75.37%) than in BM (51.56%). These results are comparable with other studies investigating the antioxidant activities of edible flowers[3,64,65]. The $IC_{50}$ for $Fe^{2+}$ capacity was indicated at 43.08 ($R^2 = 0.82275$) and 37.05 mg mL$^{-1}$ ($R^2 = 0.70796$) for BM and EE, respectively. In Fig. 2 the Metal chelating ($Fe^{2+}$) capacity in the presence of BM and EE extracts showed a linear activity with increasing concentration. Although this study did not record the statistical correlation between polyphenol contents and antioxidant activity, many authors[6,66,67] have indicated a strong linear relation between the polyphenol contents and the antioxidant activities of edible flower species.

**Selected polyphenols in daylily flowers:** HPLC chromatogram of selected polyphenol standards are shown in Fig. 3. The standards are chlorogenic acid (RT = 6.62 min), (+)-catechin (RT = 4.43 min), gallic acid (RT = 4.45 min) and rutin (RT = 15.04 min). The peaks were confirmed by UV/Vis spectra obtained at λmax 284 nm for (+)-catechin (Fig. 3a) and gallic acid (Fig. 3b), 320 nm for chlorogenic acid (Fig. 3c) and 350 nm for rutin (Fig. 3d). The chromatogram in Fig. 4 shows the peaks for the polyphenolic compounds identified in daylily flower extracts. In Fig. 4a, the chromatogram show (+)-catechin,

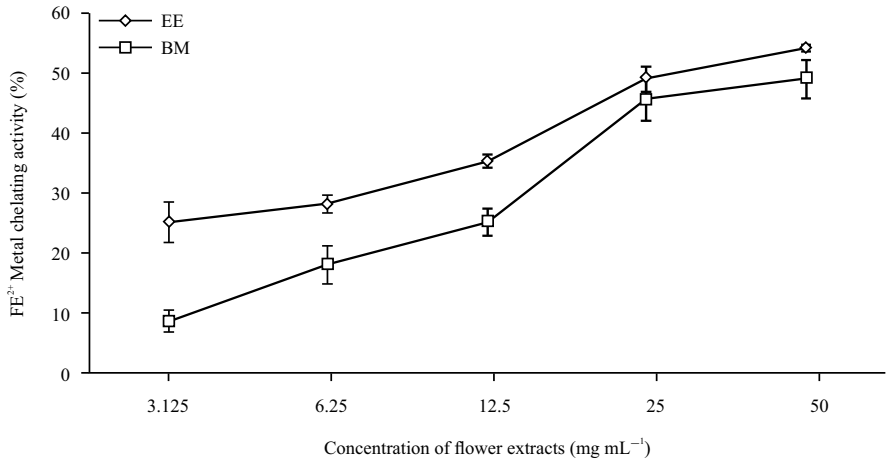


Fig. 2: $Fe^{2+}$ chelating activity of daylily cultivars

Values are expressed as means±SD and were conducted in triplets in three independent experiments. Statistical significance was determined by one-way ANOVA followed by Tukey's range test. EE: Elegant explosion, BM: Barbara mitchell

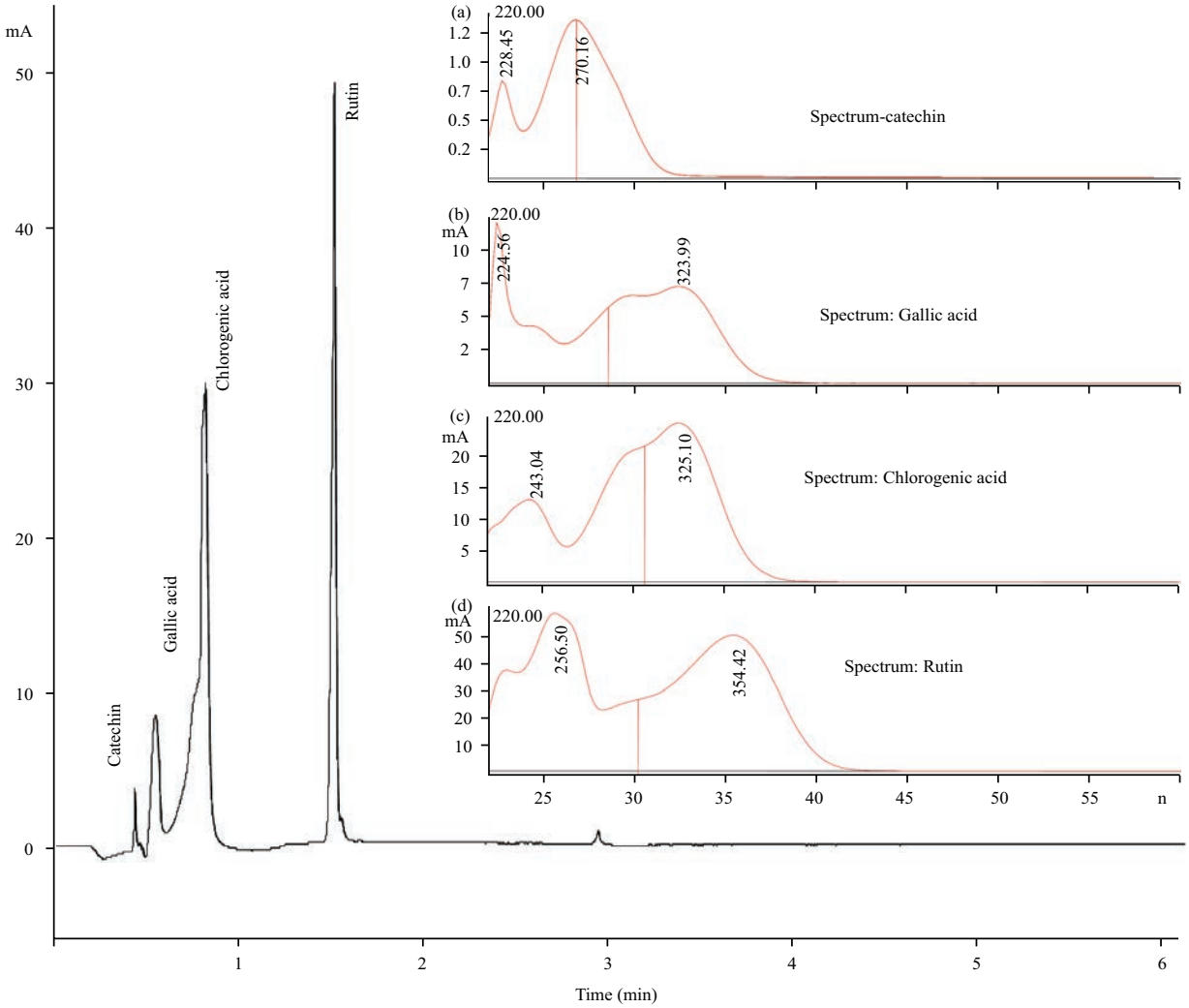


Fig. 3(a-d): HPLC Chromatographs of polyphenolic standards catechin, gallic acid, chlorogenic acid and rutin

UV spectrum was recorded for (a) Catechin (284 nm), (b) Gallic Acid (284 nm), (c) Chlorogenic acid (320 nm) and (d) Rutin (350 nm)

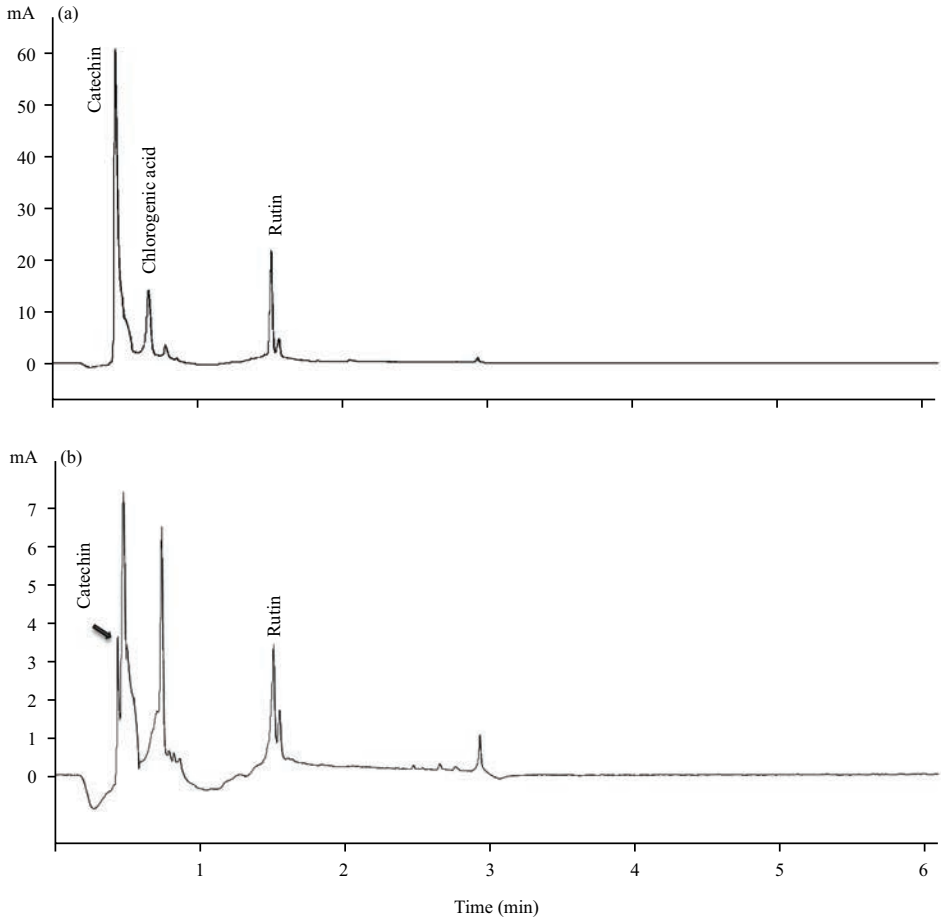


Fig. 4(a-b): HPLC Chromatograms and UV spectrums of selected polyphenols identified in daylily cultivars
(a) Catechin, chlorogenic acid and rutin were identified in Elegant Explosion and (b) Catechin and rutin were identified in Barbara Mitchell

Table 3: Concentrations of selected polyphenol compounds (μg $g^{-1}$ dwb) identified in daylily cultivars

| | Catechin (μg $g^{-1}$) | Rutin (μg $g^{-1}$) | Chlorogenic acid (μg $g^{-1}$) | Gallic acid (μg $g^{-1}$) |
|---|---|---|---|---|
| BM | $13^{b}$ | $11^{b}$ | ND | ND |
| EE | $83^{a}$ | $42^{a}$ | 37 | ND |

Values (n = 3) are expressed as means±SD of three replicates. Means in column ($^{ab}$) letter differ ($p \leq 0.05$) using Tukey's Studentized range test. EE: Elegant explosion, BM: Barbara mitchell, ND: Not detected, dwb: Dry weight basis

chlorogenic acid and rutin were identified in EE. The data of Fig. 4b shows (+)- catechin and chlorogenic were identified in BM. Surprisingly; gallic acid was below the quantification limit and was not detected in any of the flowers in our study. The data in Table 3 shows concentrations of polyphenols in EE and BM. (+)-Catechin and rutin in EE were six times and four times higher compared to BM. Chlorogenic acid was only identified in EE (37 μg $g^{-1}$ dwb). The results show that (+)-catechin was the most represented polyphenol in both the daylily cultivars (83 and 13 μg $g^{-1}$ for EE and BM, respectively). It is reported that in daylily flowers, (+)-catechin represents nearly 75% of all polyphenols[14,68]. The high (+)-catechin, chlorogenic acid and rutin concentration in EE may be responsible for the demonstrated antioxidant activity. Polyphenols such as chlorogenic acid, rutin and (+)-catechin possess several physiological activities. These compounds are shown to inhibit the proliferation of cancer cells[69-72]. They also have cardioprotective[73,74], anti-obesity[75] and anti-hypertensive properties[76-79]. Due to the polyphenol contents, the daylily flowers used in this study could be considered as ingredients for several types of functional food applications with health benefits or as a nutraceutical in dietary supplements. Even though there were some variations in polyphenolic composition compared to other studies[80-82], these variabilities might be due to the daylily flower cultivars, experimental conditions and the type of solvent used for polyphenolic

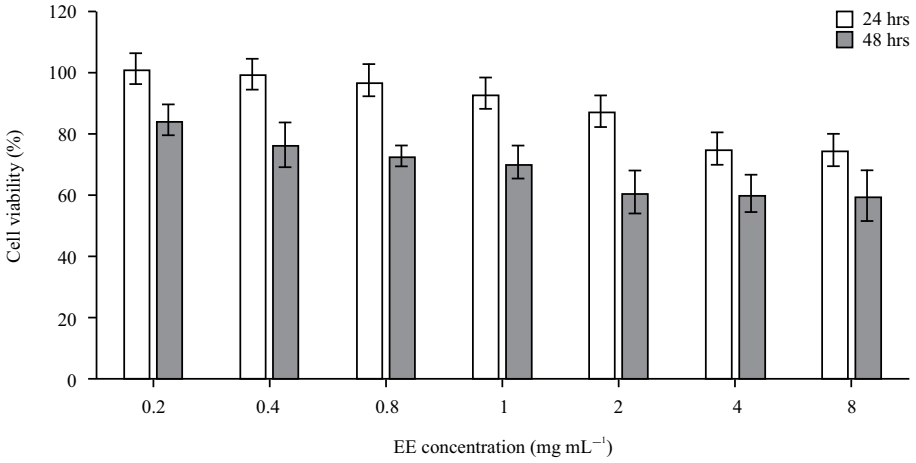


Fig. 5: Cytotoxicity of elegant explosion extracts in HepG2 cells after 24 and 48 hrs exposure
Values (n = 3) are expressed as means±SD of three replicates. EE: Elegant explosion

extractions. All the same, the polyphenol contents estimated in our study are in the range or comparable to those reported by some investigators[14,30,41,66,83,84].

While gallic acid is one of the most common phenolic acids in edible plants, we did not detect this phenolic compound in our samples. Gallic acid and catechin share some structural similarities and as a result, both compounds could have co-eluted together under the HPLC conditions in this study. In any case, our results correspond with Zheng *et al.*[85] and Barros *et al.*[84] who reported similar findings in edible flowers from China and Brazil, respectively. Similar results were also observed with *Calendula officinalis* L., *Chrysanthemum morifolium*, *Jasminum sambac* (L.) Ait and other edible flowers[83]. Wang *et al.*[86] reported that good separation of gallic acid and catechin is best achieved if the concentration of ethanol in the final sample solution for HPLC analysis is less than 15%. In this study, the final sample solution of ethanol was more than 15%.

Overall, the results show EE flowers contained high TPC, TFC, TMA CTC and strong antioxidant properties compared to BM flowers. As such, EE flowers were selected and used to perform *in vitro* toxicity experiments.

**Cytotoxicity of Elegant Explosion (EE) extracts in HepG2 cells:** The cytotoxicity of EE extract in Hep2G cells was achieved by the PrestoBlue® test and results are presented in Fig. 5. The PrestoBlue® assay is a simple and fast live assay to monitor cell viability and cytotoxicity[87]. It is a resazurin-based compound that is quickly reduced by metabolically viable cells[88,89]. Based on the results in Fig. 5, EE extract was non-cytotoxic activity towards HepG2 cells after 24 hrs exposure, as cytotoxicity ranged from 73-100% (8-0.2 mg $mL^{-1}$). The results also show cell viability was slightly decreased with prolonged exposure (48 hrs incubation). After 48 hrs incubation cytotoxicity was 59% at the highest (8 mg $mL^{-1}$) and the $IC_{50}$ was recorded at 10.13 mg $mL^{-1}$. The present results indicate that hydroethanolic EE extract possesses weak to moderate toxicity towards the HepG2 cell line used in this study. This assertion is based on ISO 10993-5[90] which specifies that cell viability above 80% is non-cytotoxic; 80-60% is weakly cytotoxic; 60-40% is moderately cytotoxic and <40% is strongly cytotoxic. The present results are comparable to the previous observations[7,91,92]. The effect could be attributed to the specific phenolic compounds and antioxidant activity of EE. Studies conducted by Cichewicz *et al.*[93] and Lin *et al.*[94] showed that polyphenols compounds anthraquinones and caffeoylquinic acid derivatives from daylily flowers showed weak cytotoxic activities and prevented oxidative stress in the HepG2 cell line. Recent research indicated caffeoylquinic acid derivatives and flavanone glycosides in *Chrysanthemum morifolium* displayed hepatoprotective effects in HepG2 cells[95]. Kwon *et al.*[96] also reported quercetin and kaempferol in *Begonia semperflorens* flowers proved to be protective against oxidative stress in t BHP induced HepG2.

**Morphological changes in Hep2G cells after 48h incubation:** The result of Fig. 6a-d show microscopic observations in Hep2G cells exposed to EE extract after 48 hrs incubation. The results show EE extract induced high apoptosis with increasing concentration (0-4 mg $mL^{-1}$). Chromatin condensation and fragmentations were observed in the HepG2 cells treated with EE extract, while the control untreated cells displayed minimal intact nuclei or apoptosis-inducing effects. Besides, microscopic observation showed

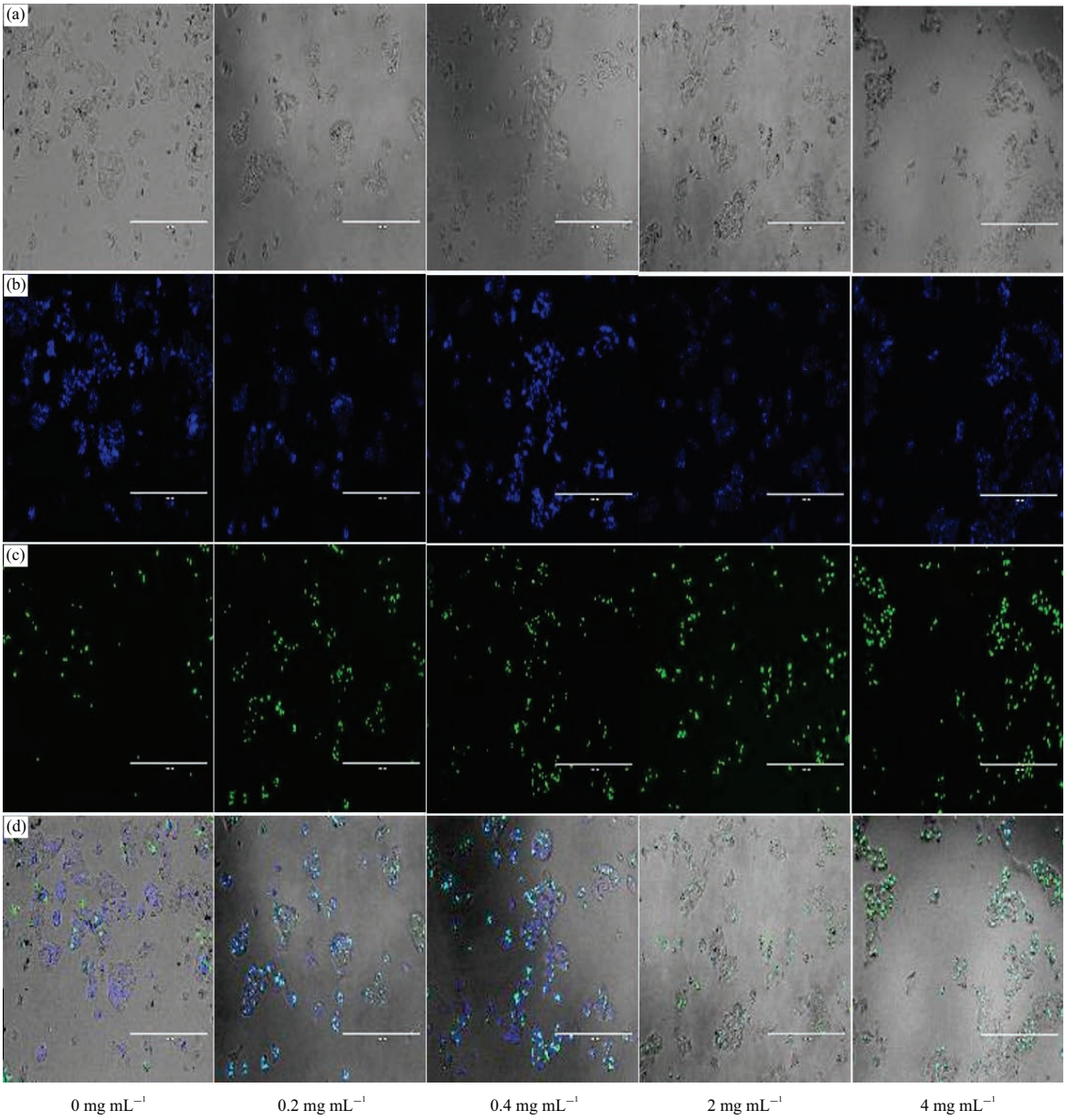


Fig. 6(a-d): Elegant explosion (EE) extract induced morphological changes in Hep2G cells following 48 hrs incubation

(a) Phase-contrast photomicrographs of Hep2G cells treated with EE extract at 0 (control)-4 mg mL$^{-1}$ for 48 hrs, (b-c) Cytotoxicity of EE extract analyzed by live/dead assay. NucBlue® Live (Hoechst 33342) reagent stains the nuclei of all cells while NucGreen® Dead reagent stains only the nuclei of dead cells. A higher percentage of apoptotic cells (live (blue)/dead (green)) are observed in the treated cells than in the control. Green channel signal intensity (dead cell stain) is strong in the cells treated with a high concentration of EE extract. Images are representative of three independent experiments. Cells were imaged on the EVOS® FL Auto system. Images were captured at ×10 magnification. Scale bars: 400

cell confluence was markedly reduced with increasing concentration. Furthermore, EE extract induced obvious morphology changes including cell shrinkage, abnormity and death as compared with control untreated cells (Fig. 6a). It is important to point out that maintenance of membrane integrity is a standard criterion for cell viability. NucBlue® Live (Hoechst 33342) reagent stains the nuclei of all cells (Fig. 6b). Live cells react with the fluorescent reactive dye only on their surface to yield weakly fluorescent cells. The nuclei of dead cells with compromised membranes react with NucGreen® Dead reagent stains only and produce brightly stained green cells (Fig. 6c). The results from cell viability analysis, i.e., presto blue are in line with cell images. The data of Fig. 6d depicts the merge or overlay of Fig. 6a-c.

Table 4: Effects of elegant explosion daylily flowers extracts on endogenous antioxidant enzymes in HepG2 cells

| | SOD (U $mg^{-1}$ protein) | GST (μmol $mg^{-1}$ protein) | GSH (μmol $mg^{-1}$ protein) |
|---|---|---|---|
| **24 hrs endogenous enzyme activity** | | | |
| Control | $1.11 \pm 0.06^{c}$ | $6.36 \pm 0.31^{a}$ | $0.27 \pm 0.01^{b}$ |
| 0.1 | $1.4 \pm 0.04^{a}$ | $5.84 \pm 0.07^{b}$ | $0.40 \pm 0.02^{a}$ |
| 0.2 | $1.48 \pm 0.03^{a}$ | $4.60 \pm 0.13^{bc}$ | $0.30 \pm 0.01^{a}$ |
| 0.4 | $1.46 \pm 0.03^{a}$ | $4.11 \pm 0.12^{bc}$ | $0.32 \pm 0.05^{a}$ |
| 0.8 | $1.47 \pm 0.02^{a}$ | $4.0 \pm 0.16^{bc}$ | $0.40 \pm 0.01^{a}$ |
| 1 | $1.45 \pm 0.05^{a}$ | $3.57 \pm 0.15^{c}$ | $0.34 \pm 0.02^{a}$ |
| 2 | $1.43 \pm 0.02^{a}$ | $3.0 \pm 0.11^{c}$ | $0.35 \pm 0.02^{a}$ |
| 4 | $1.32 \pm 0.07^{b}$ | $3.54 \pm 0.23^{c}$ | $0.37 \pm 0.03^{a}$ |
| **48 hrs endogenous enzyme activity** | | | |
| Control | $0.88 \pm 0.07^{c}$ | $4.08 \pm 0.53^{ab}$ | $0.14 \pm 0.01^{b}$ |
| 0.1 | $1.15 \pm 0.01^{b}$ | $4.68 \pm 1.05^{a}$ | $0.40 \pm 0.01^{a}$ |
| 0.2 | $1.32 \pm 0.03^{a}$ | $3.87 \pm 0.38^{ab}$ | $0.38 \pm 0.01^{a}$ |
| 0.4 | $1.28 \pm 0.04^{a}$ | $3.43 \pm 0.24^{b}$ | $0.35 \pm 0.01^{a}$ |
| 0.8 | $1.22 \pm 0.06^{a}$ | $3.11 \pm 0.18^{b}$ | $0.31 \pm 0.01^{a}$ |
| 1 | $1.17 \pm 0.05^{b}$ | $3.01 \pm 0.05^{b}$ | $0.41 \pm 0.01^{a}$ |
| 2 | $1.13 \pm 0.03^{b}$ | $2.83 \pm 0.15^{b}$ | $0.48 \pm 0.01^{a}$ |
| 4 | $1.02 \pm 0.06^{bc}$ | $2.97 \pm 0.12^{b}$ | $0.46 \pm 0.01^{a}$ |

Values are expressed as means ± SD and were conducted in triplicates. [abc]Letters demonstrate the significant difference ($p \leq 0.05$), comparing the concentrations of the assay at 24 and 48 hrs. Statistical significance was determined by one-way ANOVA followed by Tukey's range test. SOD: Superoxide dismutase, GSH: Glutathione, GST: Glutathione-S-Transferase

**Effect of EE extract on redox biomarkers in HepG2 cells:** The result of Table 4 shows the effect of EE extract on endogenous enzyme activity. SOD activity (U $mg^{-1}$ protein) was increased ($p \leq 0.05$) in HepG2 cells exposed to EE extract (1.32-1.48 U $mg^{-1}$ protein after 24 hrs incubation and 1.02-1.32 U $mg^{-1}$ protein after 48 hrs incubation) compared to control untreated cells (1.11 U $mg^{-1}$ protein and 0.88 U $mg^{-1}$ protein, respectively for 24 and 48 hrs). The results show SOD activity was significantly ($p \leq 0.05$) different after 24 and 48 hrs incubation. While the present data show a high SOD activity after treatment with EE extract, there was no linearity regarding concentrations. Other studies have indicated that SOD activity increase when treated with polyphenols[97,98]. SOD is a family of antioxidant enzymes that convert harmful superoxide radicals into $H_2O_2$, which is subsequently metabolized to water and oxygen by catalase and glutathione peroxidase. The present results could be due to the high antioxidant activity of the EE extract directly retarding the superoxide radical's formation. It is also possible that the EE extract activated the SOD enzyme that catalyzes the conversion of superoxide radicals into hydrogen peroxide and oxygen for subsequent detoxification to water and oxygen.

GST activity (μmol $mg^{-1}$ protein) decreased ($p \leq 0.05$) in HepG2 cells exposed to EE extract for 24 hrs compared with control shown in Table 4. However, the exposure of cells for 48 hrs produced no significant changes compared to the control. The results are in line with others who reported decreased GST activity in HepG2 cells after acute incubation with basil and ginger extracts[33,97] and grape pomace extract[99]. Also, previous studies have detected no change in GST activity after prolonged incubation with polyphenolic compounds in HepG2 cells[100-102].

The results of the GSH level shown in Table 4 followed a similar pattern observed for SOD activity. The results demonstrated no significant difference in GSH levels as EE extract concentration increased. A study by Franco and Cidlowski[103] indicated that GSH depletion is a common feature of apoptotic cell death. GSH participates in the disposal of potentially harmful electrophiles and protects cells from the damaging effects of free radicals[104,105]. Therefore, its depletion renders cells particularly vulnerable to oxidative stress. Since these results indicated no significant associations between cell viability and cellular GSH level, this phenomenon could be due to the tendency for cultured cells to increase protection as the concentration of extracts is increased[106]. Some studies utilizing high concentrations of plant extracts have reported increased GSH levels[97]. Others have suggested that GSH synthesis could be up-regulated during oxidative stress by phenolic compounds if their concentration does not compromise cell viability[107]. This might finally contribute to the paradox that is associated with the protective mechanisms of GSH.

## CONCLUSION

A preliminary study of herbal extracts from daylily flower cultivars Elegant Explosion (EE) and Barbara Mitchell (BM) were evaluated for their polyphenol content and antioxidant activity. Also, the *in vitro* assessment of the safety

(cytotoxicity/cell viability) and bioefficacy (effect on endogenous antioxidant enzymes) was evaluated for EE extract. According to results EE extracts exhibited higher TPC, TFC, TMA, polyphenol contents and strong free radical scavenging inhibitory activities. Furthermore, EE extracts induced weak to moderate cytotoxicity.

## SIGNIFICANCE STATEMENT

The extent of this study is to utilize daylilies for use as functional food ingredients. The results generated from this study will help in gathering baseline information on the potential of daylily flowers to be utilized in the preparation of nutraceutical, functional food products and dietary supplements.

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