Evaluating The Impact of Strigolactone GR24 On *Capparis Spinosa* L. Callus Production And Phenolic Compound Content

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Research Article

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Abstract

The effect of strigolactones on plants, which has been recently described as a new group of plant hormones, has not been fully characterized. *Capparis spinosa* L. callus formation using synthetic strigolactone GR24 (0.1 and 0.2 µM) alone or in combination with 1-naphthalene acetic acid (NAA) (2 mg / L) and 6-benzylaminopurine (BAP) (1 mg / L) and its effect on phenolic substance production were evaluated. 2 mg/L NAA+1 mg/L BAP+0.1 µM GR24 was the medium with the highest callus formation (60.3%) and callus fresh weight (120.8 mg). In the phytochemical analysis, the highest total flavonoid and phenolic substance and the highest rutin, quercetin, chlorogenic acid content were found in this application and their amounts increased at various rates compared to the control. Aromatic substances in caper calluses were grouped as sulfur compounds (66.97% -87.53%), aldehydes (4.88% -7.90%), ketones (0.34% -19.3%), hydrocarbons and derivatives (0.56%-5.8%), alcohols (% 1.62-6.08%), others (0.61% -2.37%) and their amounts varied at various hormone applications. When 0.1 µM GR24 was applied alone, the total sulfur compound in callus samples was 87.53% and the dominant substance was found to be methyl isothiocyanate.

Introduction

Capers are plants from the Capparaceae family, tropical/subtropical, states with more than 350 varieties and can grow naturally in all continents, including Mediterranean countries. Capers as perinnniels and shrubs have 250 species in the world (Musallam et al. 2012), while in Turkey's flora there are two species (*Capparis spinosa L. and Capparis ovata Desf.*) and a total of six different varieties *C. spinosa* var. *spinosa*, *C. spinosa* var. i*nermis* Turra., *C. spinosa* var. *aegyptia* (Lam) Boiss, *C. ovata* var. *palaestina* Zoh., *C ovata* var. *herbacea* (wild) Zoh., and *C. ovata* var. *canescens* (Coss.) Heywood has been detected (Davis 1982).

Numerous chemical compounds such as alkaloids (Capparispine), flavonoider, lipids, polyphenols, terpenes, indoles, and aliphatic glucosinolates are found in various parts of *C. spinosa* (Arena et al. 2008; Rajesh et al. 2009; Wang et al. 2009), in many studies various parts of caper are used in the treatment of different diseases such as rheumatism, hypertension and diabetes. Sher and Alyemeni (2010) reported that *C. spinosa* is a safe plant and there are no studies in the scientific literature showing its toxic effect.

It is difficult to produce *C. spinosa* by common propagation methods. For example, seed propagation of capers is not preferred due to seed dormancy and high heterozygosity. However, vegetative cutting of caper production is less successful due to rooting problem (Musallam et al. 2011). Many studies have been conducted on the seed dormancy caused by the negative ecological conditions during the germination phase and the negativities arising from the structure of the seed and the ways to eliminate it (Sozzi and Chiesa 1995; Bahrani et al. 2008; Germanà and Chiancone 2009).

Nonetheless, the use of *in vitro* culture techniques is seen as a very good option to overcome the propagation problems of capers and also for mass production without threatening natural resources.

There have been many studies showing the effect of these growth regulators BAP, 2,4-D and NAA on callus formation (Tyagi et al. 2010; Kumari et al. 2015). Although it is known that strigolactones regulate plant growth and development especially with other plant hormones such as auxin and cytokinin (Wang et al. 2007; Koltai 2015) there are very few studies evaluating the effect of this hormone concerning *in vitro* success (Wu et al. 2017).

Our study is the first protocol to evaluate the effect of various strigolactone concentrations on callus formation and phenolic content in caper plant. In the context of this study, it was aimed to evaluate the effect of synthetic strigolactone GR24, which will be used in various concentrations with BAP and NAA, on callus formation and fresh weight. It is also aimed to investigate the amount of phenolic and aromatic substances such as rutin and quercetin in the calli formed.

Materials And Methods

Plant material and establishment of callus culture

Caper seedlings (*Capparis spinosa* L.) were used as material in thıs study which was conducted in Süleyman Demirel University Biology Department Plant Biotechnology Laboratory between 2020–2021. The seedlings were planted at a depth of approximately 10 cm, 2 in each pot containing 1/3 sand-peat mixture. From the planting of the seedlings, the plants were under 16 hours / day 8 hours night photoperiod, 135 µmol m^{-2} s^{-1} PAR light intensity, 23–25 ° C temperature and 51–54% humidity (measured with Peak Tech 3695) conditions and were grown in the plant growth cabinet for an average of 3 months.

Young leaves between 1.5 and 2.5 cm, taken from caper seedlings were used as explants. These were cut from the stem and washed with tap water until they were cleared of the soil, then rinsed in 70% ethanol for 10 minutes. Afterwards, they were rinsed 3–4 times with sterile distilled water, 1% sodium hypochlorite (NaOCl) containing 1–2 drops of Tween-20 for 10 minutes, and surface sterilization was completed by rinsing 3–4 times with sterile distilled water.

With a sterile scalpel, explants pieces of the size 0.5–1 cm were inoculated onto the culture medium with pinset. 3% (w/v) sucrose, 0.7% (w/v) agar and MS (Murasige and Skoog 1962) medium (Sigma-M9274) containing NAA, BAP and various concentrations of GR24 were used as the culture medium. Previous studies on capers stated that the NAA and BAP ratio has significant effects on callus formation (Kumari et al. 2015). For this reason, 2 mg/L NAA and 1 mg/L BAP were accepted as the control group and 4 separate combination groups were established as indicated below.

1. NAA (2 mg/L) + BAP (1 mg/L) (Control)

2. NAA (2 mg/L) + BAP (1 mg/L) + GR24 (0.1 µM)
3. NAA (2 mg/L) + BAP (1 mg/L) + GR24 (0.2 µM)
4. GR24 (0.2 µM)

The pH of the culture medium was adjusted to 5.8 using 1 M NaOH and 1 M HCl, and then autoclaved at 103 kPa pressure 121 ° C for 20 minutes. The media were transfered into sterile petri dishes of 9 cm diameter with an average of 20 ml each. An average of 10–15 explants were planted in a petri dish, covered tightly with parafilm tape to prevent contamination and incubated for 4–5 weeks at 22ºC ± 1 temperature under dark conditions. The explants were then subcultured for 10 days in 2 mg/ l NAA, 1 mg / l BAP and 30 g 99% L-rhamnose medium.

For each hormone combination, the number of explants placed in the culture medium and the number of callus formed were recorded. The percentage value was found with the ratio of the number of callus obtained to the number of explants as shown in the formula below.

Callus induction frequency (%) = [(Number of callus) ÷ (Total number of explants)] x 100

The fresh weight of each callus was recorded and taken into eppendorf tubes then stored at -18 ° C until the examination of the chemical accumulation of the phenolic compounds.

Determination of phenolics and aromatics

Sample analysis were performed at the Innovative Technologies Application and Research Center in Suleyman Demirel University. Callus samples (1000 mg) were weighed and homogenized with 10 mL of methanol in a homogenizer (IKA T 25 Ultra-Turrax®, Staufen, Germany). The mixture obtained was filtered with a 0.45-µm filter (Minisart®, Sartorius Stedim Biotech, France) and then evaporated to dryness in an evaporator (Heidolph Hei-VAP G1, Schwabach, Germany) at 40°C. The dry extract was then dissolved in 1 mL of methanol and 20 µL was transfered into the HPLC apparatus. The HPLC system was equipped with a LC-10ADvp pump, SIL–10AD vp auto-sampler and CTO-10Avp column oven (Shimadzu, Kyoto, Japan). Agilent eclipse XDB-C18 (250 × 4.60 mm, 5 µm) column and a mobile phase consisting of methanol and acetic acid (3% v/v) in water. The flow rate was 0.8 mL min^{-1} and the injection volume was 20 µL. Column temperature was set to 30°C. Diode array detector (DAD) worked at λmax = 278 nm, and chromatograms were obtained at various wavelengths according to absorption maxima of the analyzed compounds.

For aromatics, the system was known as fused silica SPME fiber assembly Carboxen/Polydimethylsiloxane (CAR/PDMS) (Sigma-Aldrich®) with a column of Restek Rx-5Sil MS (30 m× 0.25 mm i.d., 0.25-µm film thickness) (Restek Corporation, Bellefonte, PA). The flow rate of helium as a carrier gas was 1.61 mL min − 1. The injector temperature was set to 250°C for splitless injection. After 2 min at 40°C, the system reached 250°C with 4°C increments per minute and waited for 5 min at 250°C. Mass spectra were taken at 70 eV. The sample stood for 30 min with fiber, for 15 min without fiber at 60°C, and desorbed at 250°C. Relative percentage amounts of the separated aromatic compounds were calculated from the total ion chromatograms displayed by the computerized integrator (Shimadzu, Kyoto, Japan).

Statistical analysis

Pattern of Randomized block design was used for the trials and were conducted at least 3 times. The Oneway-ANOVA of the SPSS 23.0 package program was used for the variance analysis of the data, and the Duncan Multiple Comparison Test was used for the comparison of the means. Graphs for all experimental data were constructed to determine whether the mean values between the different treatment concentrations held a significant difference.

Results And Discussion

Callus induction and fresh weight

The effects of strigolactones on plants are not yet fully defined and it is unknown whether the observed effects are universal across plant species. 0.2 µM GR24 used alone in this study significantly reduced both callus formation and fresh weight ($P < 0.05$). While 0.2 µM GR24 used with NAA + BAP significantly reduced the incidence of callus formation, but had no significant effect on fresh weight. Fresh weight measurements showed similar trends in the control group and GR24 combined with NAA + BAP (Fig. 1). Mdodana (2012) found that strigolactone-deficient and insensitive mutants of wild-type *A. thaliana* Col-O were widely used in callus culture, also the media containing various amounts of 2,4-D and kinetin (2: 2 mg / L or 0.5: 0.05 mg / L) were successful and when these calluses were transferred onto media containing 0.1 µM GR24 and auxin or cytokinin, the amount of callus biomass increased in some of the mutants. Grobbelaar et al. (2014) demonstrated that strigolactones (GR24 and Nijmegen-1 (0.1 µM) generally had a minimal effect on the growth of *Salvia frutescens* nodal explants. However, when combined with 1 mg / L NAA, these hormones promoted biomass production. Similar results were also shown by Zulfiqar et al. (2020) in which 0.01 mg/L GR24 was the optimum amount for the callus growth of *Helianthus annuus* L. In this study, the frequency of *in vitro* callus formation and fresh weight were similar in amount to the control group but the highest was found in the 2NAA + 1BAP + 0.1 µM GR24 medium, indicating the synergistic relationship between auxins and strigolactones (Brewer et al. 2009; Hayward et al. 2009; Agusti et al. 2011).

Production of phenolic and aromatic compounds

As the main active compound, flavonoids play a notable role in a variety of pharmacological activities, including antiallergic, anti-inflammatory and antioxidant effects (Trombetta et al. 2005, Panico et al. 2005). Germano et al. (2002), Matthaus and Özcan (2005), Tlili et al. (2009) reported that the caper plant is a rich plant source of flavonoid compounds rutin (rutocide) and quercetin, also different parts of the plants contained phytosterols, tocopherols, carotenoids and glucosinolates. In literature reviews, no study has been found concerning the effect of GR24 on the amount of phenolic compounds in *C. spinosa* callus. In this study, the total flavonoid in the 2 mg L NAA + 1 mg/L BAP + 0.1 µM ($P < 0.05$) treatment increased by 19% when compared to the control. Goda et al. (2017) found that the total flavonoid content in *C. spinosa* callus from the 2,4-D medium was 0.85 mg/100 mg. It has also been stated in many studies that the rutin contained in the aerial parts were dominant flavonoid (Zhou et al. 2011; Argentieri et al. 2012). In our study except 0.2 µM GR24, rutin was the dominant flavonoid in leaf calluses obtained from

all applications, and its content in 2 mg/L NAA + 1 mg/L BAP + 0.1 µM GR24 application increased approximately by 1.5 times when compared to the control group and the amount found was 16.9 µg/g DW ($P < 0.05$). While the GR24 applied alone significantly reduced the rutin content, the low concentration of GR24 used with NAA and BAP positively affected the amount of rutin (Fig. 2).. Tlili et al. (2010) found the rutin content of caper plant leaves collected from Tunisia flora as 13.52 mg/100 g, Behnaz et al. (2013) aslo examined the amount of rutin and quercetin in various parts of the capers and reported that the highest was found in the leaves at 25.2 and 10.4 mg/g respectively.

Palacio et al. (2012) found the amount of quercetin from the callus of *Larrea divaricata* leaves treated with 2 mg/L 2.4 D and 1 mg/L BAP to to be approximately 6 µg/g. In our study, quercetin was almost the same amount in all other applications (5.1–5.4 µg/g DW) except for the 0.2 µM GR24 application (quercetin was zero) (Fig. 2). This suggests that NAA and BAP are effective hormones in the accumulation of quercetin. According to many *in vitro* studies it is recommended to add BAP to the culture medium to increase the production of various secondary metabolites in plants. For example, Al-Ashoush (2017) and Udomsuk et al. (2009) reported that BAP had a positive effect on the amounts of some secondary metabolites in addition to the total isoflavonoids extracted. In addition, it has been documented that kinetin affects polyphenol groups such as quercetin and isomer by interfering with the synthesis of nucleic acids that can affect polyphenol production (Shah et al. 1976).

Kaempferol amount varied between 4.8 and 3.3 µg/g DW in applications (Fig. 2). Tlili et al. (2017) determined the amount of kaempferol in caper leaf extracts as 3.63%. Haifa et al. (2016) could not detect kaempferol in *C.spinosa* leaves in the Tunisian flora.

The total amount of phenolic substance increased by 48.8% compared to the control and the highest was found to be 72.79 µg/g DW in 2 mg/L NAA + 1 mg/L BAP + 0.1 µM GR24 application ($P < 0.05$). In other applications with GR24, this amount was found to be much lower, therefore the increase in the GR24 concentration decreased the amount of phenolic substance compared to the control, regardless of the presence or absence of NAA and BAP (Fig. 3).

Although this is not the case for the amount of chlorogenic acid, the highest amount of chlorogenic acid was found in this application as 46.4 µg/g DW, and also caught our attention as the highest among the analyzed phenolics. GR24 used alone also significantly increased the amount of chlorogenic acid compared to the control (Fig. 3). Rad et al. (2021) obtained 0.680 mg/g DW chlorogenic acid in caper leaves. There are studies analyzing the effects of auxin and cytokinins on the amount of chlorogenic acid in *in vitro* cultures of some plants. Erkoyuncu and Yorgancılar (2021) reported that 1 mg/L 2.4-D + 2 mg/L BAP treated leaf calluses of *Echinacea purpurea* L. contained 0.23 mg g chlorogenic acid; Szopo et .al. (2020) also determined the amount of chlorogenic acid as 20 mg/100 mg DW in *Schisandra rubriflora* microshoot extracts. Siahposuh et al. (2011) reported that kinetin stimulates chlorogenic acid production positively in *Varthemia persica* callus, and replacement of 2,4-D with NAA does not change chlorogenic acid production.

In our study, caffeic acid was not detected in calluses in any of the application, including the control group (data not shown). According to the findings of Oudah et al. (2019) the amount of caffeic acid in caper leaves was 73.542 µg/ml, Rezzan et al. (2013) reported no caffeic acid in the caper leaves collected from Gaziantep/Turkey flora.

Antognoni et al. (2008) reported that the production of α-tocopherol in *Amaranthus caudatus* and *Chenopodium* species calli were approximately 40 times lower than the tocopherol content in the plant leaves and other organs. In some cases, this is due to the lack of specialized cell structures (St. Pierre et al. 1999; Pasqua et al. 2003). In our study, the highest amount of α-tocopherol was found in the control group, and it decreased significantly in all applications with GR24. In callus cultures of *C. spinosa*, α-tocopherol accumulation was approximately 1000 to 5000 times lower than in leaves (20.19 ± 31.71 mg/100g),regardless of the culture medium (Tlili et al. 2009).

In our study, aromatic substance contents of calluses were generally examined under six groups as sulfur compounds, aldehydes, ketones, hydrocarbons and derivatives, alcohols and others (Table 1). In the GR24-only applied group, the total sulfur compound was found to be 87.53% and the dominant substance was methyl isothiocyanate (556-61-6). The pungent aroma of capers is usually caused by the very sharp methyl isothiocyanate released after an enzymatic reaction with a mustard oil glycoside known as glucocaparin (methyl glucosinolate) (Sozzi et al. 2012). El-Ghorab et al. (2007) and Bakr and El Bishbishy (2016) stated that the predominant essential oil in the caper plant collected from the flora was methyl isothiocyanate at 20.0% and 24.66%, respectively. In our study, this rate was found to be higher in all applications in caper calluses. Here, we can clearly state that callus culture is a good method for methyl isocyanate production. Zhang (2004) documented the cancer-preventive activity of a significant number of isothiocyanates, mostly of which occur in plants, especially in cruciferous vegetables. Moreover, glucosinolates via their hydrolysis products are among the most powerful antibiotic substances known from higher plants (Louda and Mole, 1991), with an established correlation between the content of glucosinolates (isothiocyanates) and disease resistance (Esteve 2020). Again, 0.2 µM GR24 application increased the total aldehyde content from 6.09–7.90%.

2NAA + 1BAP + 0.1 µM GR24 increased ketone and hydrocarbon compounds by 10 and 14 folds, respectively, compared to control group. It was realized that the combination of GR24 with the other two plant growth regulators had a positive effect and this effect was directly proportionional to the decrease in GR24 concentration. The dominant compound in ketones was acetoin (513-86-0), and n-hexan (110-54-3) in hydrocarbons and derivatives. The amount of alcohol and other aromatics was reduced in all applications with GR24 compared to the control. This article is the first to present the effect of GR24 on the amount of essential oil in *C. spinosa* callus. Romeo et al. (2007) recorded 8.42% sulfur compounds, 12.8 % hydrocarbons and derivatives and 7.48% alcohol in Eolian capers. Bidabadi and Sharifi (2021) stated that strigolacones (10 µM GR24) increased the essential oil content and yield in *Dracocephalum kotschyi* under drought stress. They also reported that increasing levels of SL application positively influenced essential oil content and yield in *S. nemorosa* where the lowest salt concentration (100mM

NaCl) accompanied with 0.3 µM SL, resulted in the highest essential oil content and yield (Sharifi and Bidabadi 2020).

Table 1
Effect of various GR24 concentrations on the aromatic substance of caper calluses

Aromatic compounds	2NAA + 1BAP (Control) (%)	2NAA + 1BAP + 0,1 µM GR24 (%)	2NAA + 1BAP + 0,2 µM GR24 (%)	0,2 µM GR24 (%)
Sulphur compounds	83.58	66.97	83.27	87.53
Aldehydes	6.09	4.88	6.45	7.90
Ketones	1.32	19.3	6.55	0.34
Hydrocarbons and derivatives	0.56	5.8	0.83	0.62
Alcohols	6.08	1.62	1.86	3.00
Others	2.37	1.43	1.04	0.61

In conclusion, in this study, it was observed that GR24 may be effective in the production of phenolic compounds in caper callus cultures. It was realised that both the formation and wet weights of calluses cultured in the medium containing 2 mg / L NAA + 1 mg / L BAP + 0.1 µM GR24 were higher compared to other media, and this was the best application especially in the accumulation of chlorogenic acid, rutin and quercetin phenolics. It was also determined that GR24 used at lower concentrations was more effective, but much studies on this is required in the future. The lack of comprehensive genomic data for most plants with medicinal or nutraceutical properties has made it difficult to use common genomic (eg, microarray-based) approaches to study metabolic pathways. *In vitro* culture offers an attractive alternative to understanding the regulation of enzymes involved in plant secondary metabolism.

Declarations

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Compliance with ethical standards

Conflict of interest: The authors declare that they have no competing interests.

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Figures

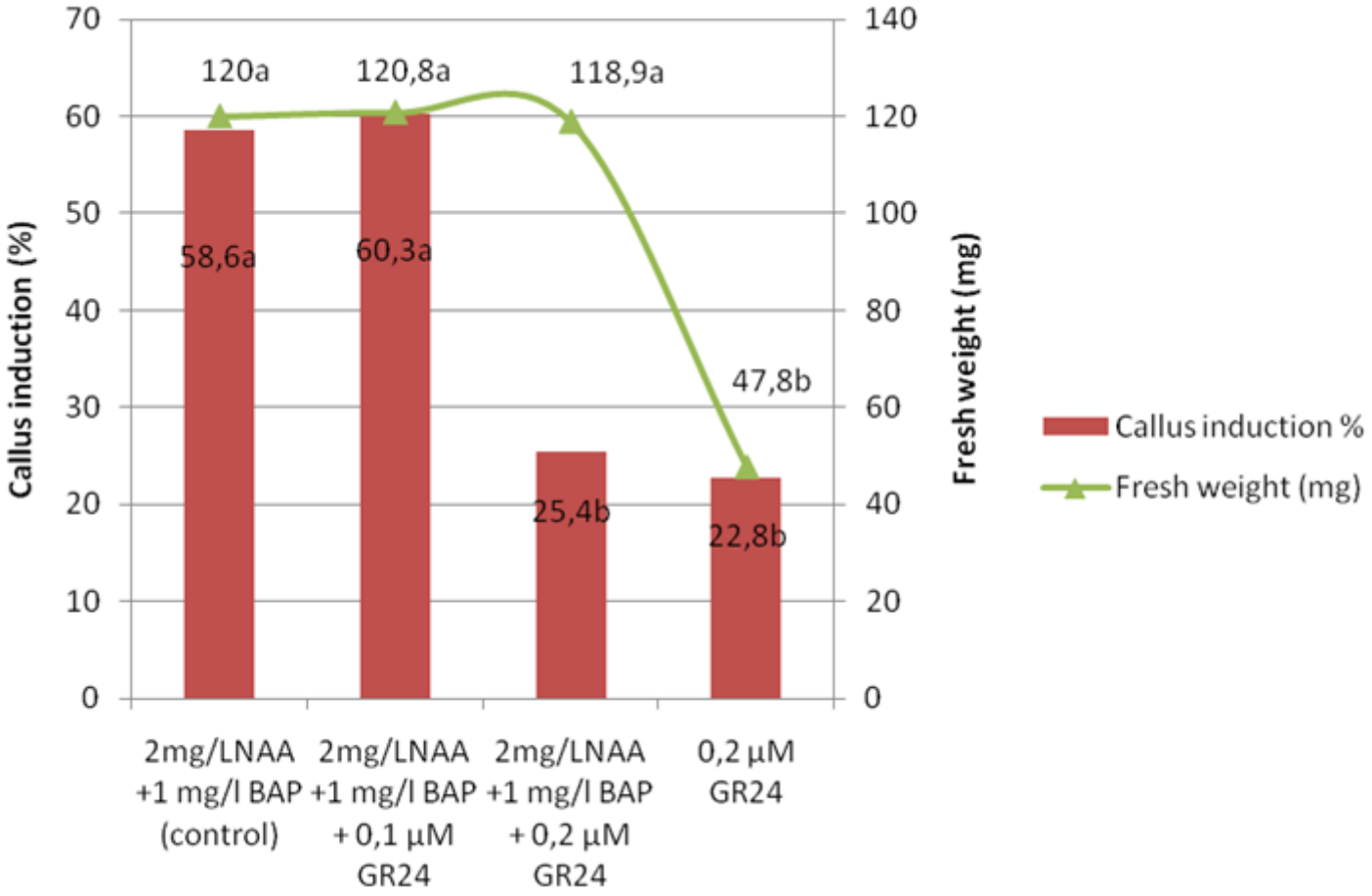

Figure 1

Effect of various GR24 concentrations on caper callus formation and fresh weight * Differences between means shown by individual letters are significant (P <0.05)

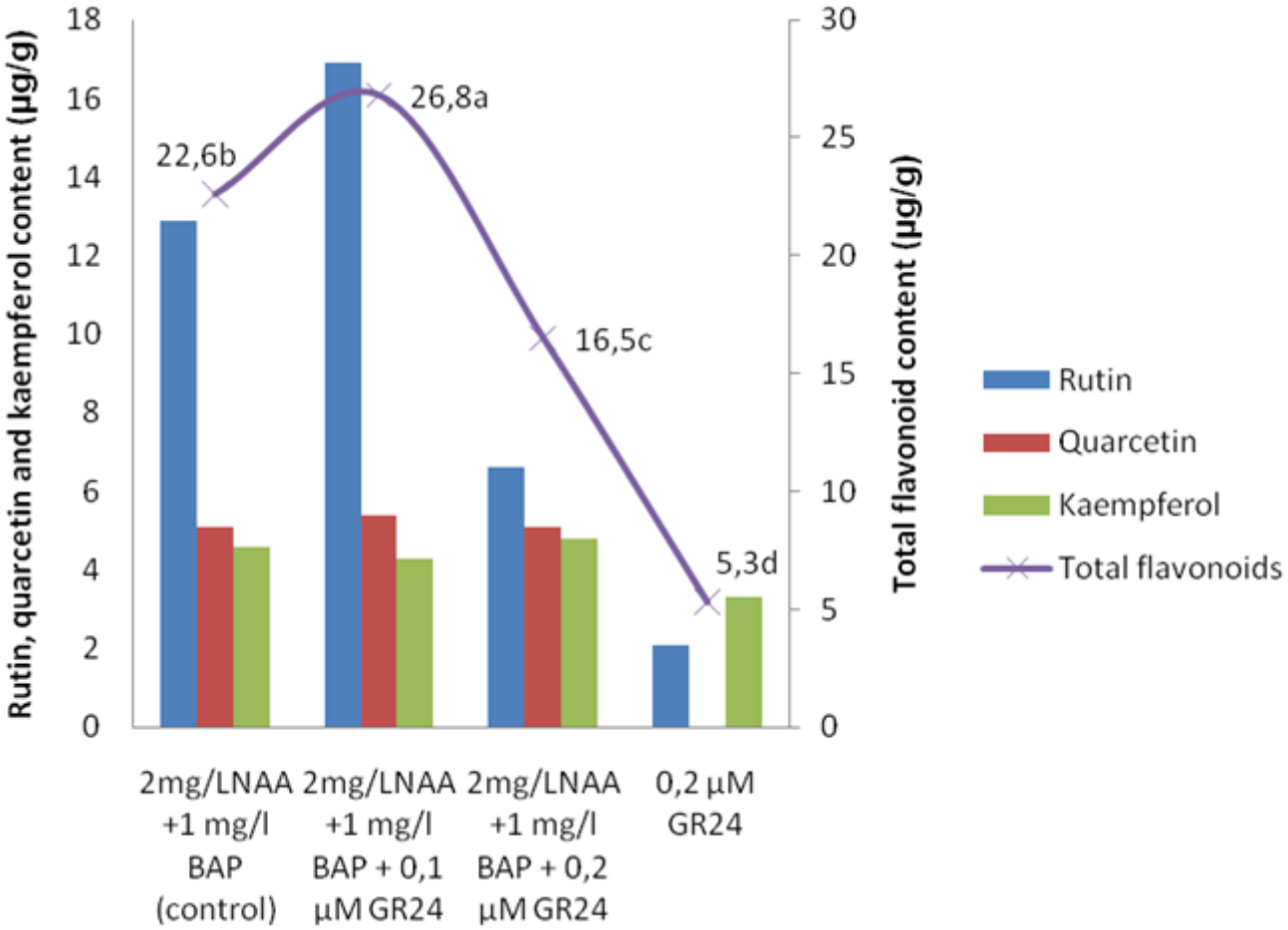

Figure 2

The effect of various GR24 concentrations on the total flavonoid, rutin, quercetin and kaempferol in caper callus. * The differences between the means shown by individual letters are significant (P <0.05)

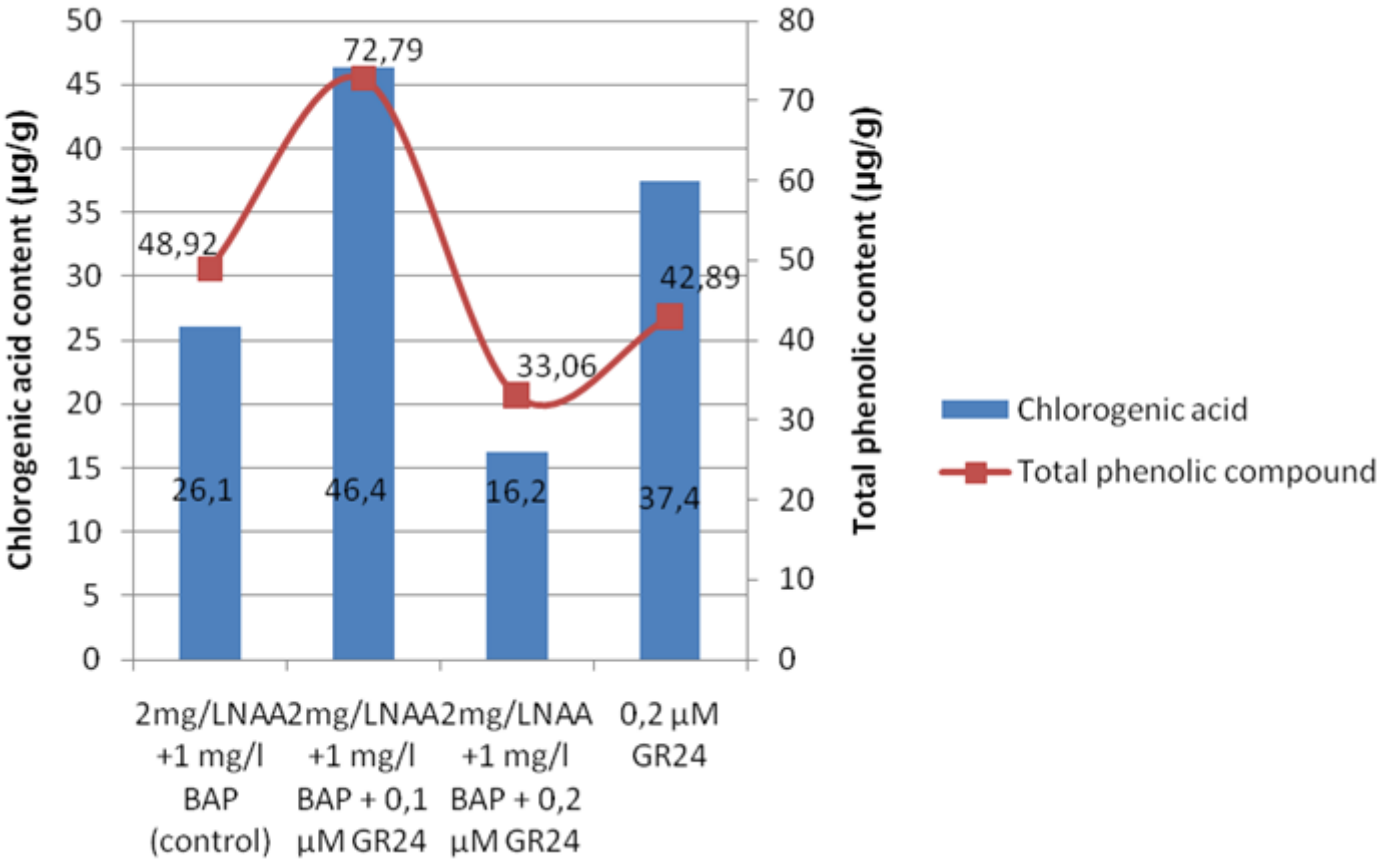

Figure 3

Effect of various GR24 concentrations on the total phenolic substance and chlorogenic acid in caper calluses. * The differences between the means shown by individual letters are significant (P <0.05)