Article

Identification of *Hemerocallis* in vitro culture and estimation of its physiological activities

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Abstract

Plants, both ornamental and medicinal, have been used for a variety of purposes for millennia. Ornamental plants may benefit the environment, the economy, and people's lifestyles in a variety of ways. Despite the growing popularity of aesthetic plants, some are grown for medical purposes because they contain a variety of bioactive components such as phenolic compounds, carotenoids, antioxidants, essential oils, and other secondary metabolites. A daylily, often known as a day lily, is a flowering plant in the genus *Hemerocallis*, which belongs to the Asphodelaceae family. Daylilies are perennial plants whose name refers to the short lifespan of their blossoms. The gorgeous flowers and hardiness of many varieties make this species popular all throughout the world.In this study, the plant species was identified by molecular tools (18s rDNA), the total physiological parameters were measured, the fractions were identified using liquid chromatography - mass spectroscopy LC-MS, and the antimicrobial activity were evaluated resulting in 7 fractions. All the defined compounds were proved to have many activities *Hemerocallis*. These compounds were used for treatment of many diseases and in traditions applications.

Keywords *Hemerocallis*; tissue culture; LC-MS; phenolics; flavonoids; chlorophyll pigmentation; 18S rDNA; antimicrobial assay.

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1 Introduction

Ornamental plants may benefit the environment, the economy, and people's lifestyles in a variety of ways. Ornamental plants are cultivated in homes, offices, institutions, and other locations to embrace the landscape because of their magnificent flowers and attractive leaves (Milstein, 2005; Zhang, 2017). Many attractive plants are utilised as indoor plants to bring freshness into homes, hospitals, and other buildings. It has been

observed that patients who have plants in their wards recover more quickly. Plants and flowers have a long-term positive and constructive impact on people's thoughts (Kennedy and Wightman, 2011; Hicks et al., 2016).

Hemerocallis is an East Asian genus that includes hardy plants that have survived in North American zones. There are about 80,000 *Hemerocallis* varieties in the world, according to the American Daylily Society. They're frequently the result of interspecific, intraspecific, or interploidy crossbreeding. These perennial plants are grown as decorative species in Europe and North America, as well as vital food and medical ingredients (Szewczyk et al., 2019).

Daylily crude extracts have been used in Asia as diuretic, anthelmintic, antiemetic, antispasmodic, sedative, and antiphlogistic remedies. Polyphenols, carotenoids, anthraquinones, naphthalene glycosides, steroidal saponins, and lactams have been isolated from the aerial parts and roots of these plants. Pharmacological research has revealed that *Hemerocallis* species have biological activities such as antioxidant, neuroprotection, anti-inflammatory, antidepressant, and anticancer(Guo et al., 2011; Szewczyk et al., 2019).

Extracts from plants are a valuable source of physiologically active chemicals. Plant raw extracts have been shown to have antibacterial, antiviral, antifungal, and anti-inflammatory properties in numerous research investigations. In recent years, cosmetics incorporating them have grown in favour. When used in cosmetic preparations, they produce far greater outcomes than when single physiologically active substances are used. When looking for compounds with anti-inflammatory and antioxidant capabilities, one should focus on plant substances. Materials high in polyphenols, such as phenolic acids and flavonoids, can act as natural antioxidants to limit free radical formation. The principal polyphenols produced by plants are phenolic acids, which have a preventive function in many diseases such as cancer, inflammation, and cardiovascular disease, and are particularly well-known for their powerful antioxidant ability(Oua and Wu, 2020; Li et al., 2021a).

Different plant tissue culture techniques are used to produce medicinal and ornamental plants. Such techniques depending on the final product and evaluate the trends of plant culture techniques (Costa et al., 2019). These techniques allow to maintain the same genotypic characteristics of the original plant, however due to stresses of in vitro culture conditions, culture media and various factors as genotype and explant source, genetic characteristics of regenerants may change (Dias, 2016). Therefore, in vitro techniques might be a useful tool for generating genetic variability, i.e., somaclonal variations because of genetic and epigenetic changes at different levels - morphological, cytological, cytochemical, biochemical, and molecular (Krishna et al., 2016; Costa et al., 2019)

The aim of this study is to identify the in vitro culture of *Hemerocallis* and define its specific species and identify some essential secondary metabolites beside its antimicrobial activities.

2 Materials and Methods

2.1 Plant materials

Hemerocallis species was applied for this study as ornamental and medicinal plant. A complete identification, characterization, and fingerprinting was performed for this species. It was purchased from Tissue culture Center, Genetic Engineering & Biotechnology Research Institute, Sadat City, Egypt.

2.2 Tissue culture conditions

MS basal medium contains required nutrients of macro- and micro-elements for the *in vitro* cultured plants as described by Murashige and Skoog (1962). The medium was allocated into incubation jars where each jar contained 60 ml. Stem nodal cultures were incubated at 25 ± 2°C and satisfactory fluorescent light of 3000 Lux for 16 hour photoperiod provided by cool, white, fluorescent lamps.Resulted shoots from establishment were excised and transferred into multiplication medium of MS supplemented with 0.075 mg/l of BAP in order

to obtain micro-shoots required for multiplication experiment (Fig. 1).

2.3 Molecular identification

2.3.1 DNA extraction

DNA was isolated according to the protocol of Doyle and Doyle (1990) as follows: 500 ml of CTAB buffer (2% CTAB, 2% polyvinylpyrrolidone, 1.4 M NaCl, 20 mM EDTA pH 8.0, 100 mM Tris HCl pH 8.0) was added to 0.5 g of specimen's leaflet. The sample was heated in a water bath at 65^{o}C for approximately 1 h. One volume of chloroform – isoamyl alcohol (24:1) was added to the sample and mixed by inversion for 10 min and then centrifuged for 30 min at 13,200 g. The aqueous phase was collected into a clean microcentrifuge tube and the rest was discarded. Two volumes of absolute ethanol were added with 0.1 volumes (approximately 50 ml) of sodium acetate 3 M pH 5.2 and mixed gently. The sample was left for at least 20 min at -20^{o}C and then centrifuged for 30 min at 13,200 g. The supernatant was discarded, and the pellet was rinsed in 70% ethanol and dried at room temperature. The pellet was dissolved in 50 µl of TE buffer (1 mM Tris HCl pH 8.0, 0.1 mM EDTA pH 8.0).

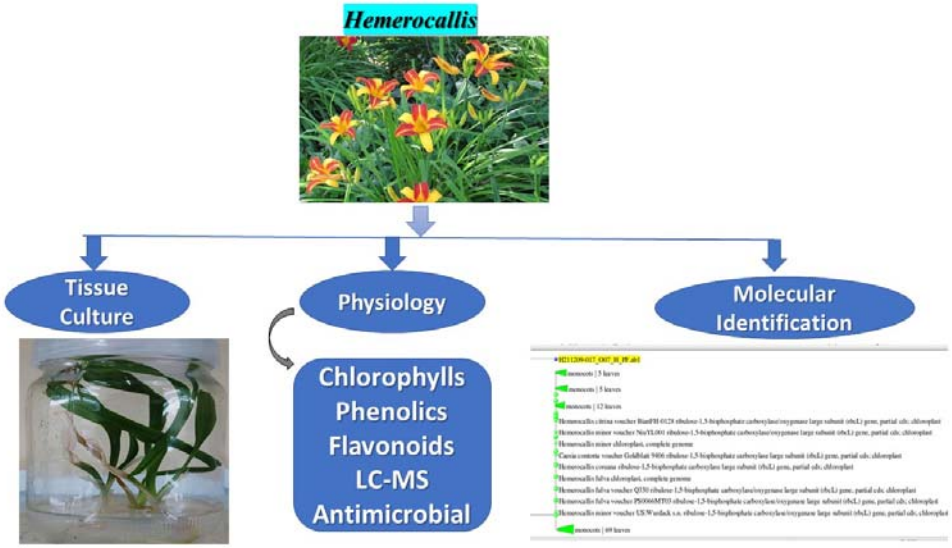

Fig. 1 Methods outline.

2.3.2 18S rDNA PCR Amplification

PCR amplifications of the species 18S rDNA gene, from the purified genomic DNA, were carried out using the primer sets, EUK1.f, 5′-AGCGGAGGAAAAGAAACTA – '3; and EUK2.r, 5′-TACTAGAAGGTTCGATTAGTC – '3. PCR reaction mixture 25 µl, contained 12.5 µl of master mix, 1 µl of each primer and 50 ng DNA template. PCR was performed with an initial denaturation step of 5 min at 95^{o}C. The PCR reaction continued with 35 cycles of 30 sec at 94^{o}C, 30 sec at the annealing temperature of 54^{o}C, and 1 min extension at 72^{o}C. The final extension was at 72^{o}C for 10 min for one cycle. The purified 18S rRNA gene amplicons were analyzed directly by sequencing, using ABI 3730xl DNA sequencer by using forward and reverse sequences, by combining the Traditional Sanger with the new 454 technology (GATC Company, Germany).

2.4 Physiological parameters

2.4.1 Chlorophyll pigmentation

The chlorophyll A, B and carotenoids for the three plant samples were estimated according to Metzener et al.

(1965): A known weight of these leaves (0.5 g) was homogenized in 85% acetone, then centrifuged and transfer into a new tube. After that, the extract was measured using a colorimeter against a blank of 85% pure acetone at three wavelengths (i.e., 452, 645 and 664 nm). The concentrations of chlorophyll a, b and carotenoids were calculated as μg/ml using the following equations:

$$\text{Chl. a} = 10.3\,\text{A664} - 0.918\,\text{A645}$$

$$\text{Chl. b} = 19.7\,\text{A645} - 3.87\,\text{A664}$$

$$\text{Carot.} = 4.3\,\text{A452} - (0.0265\,\text{Chl. a} + 0.426\,\text{Chl. b})$$

where A= absorbance at different wave lengths.

After that, the fractions were calculated as mg/g fresh weight:

$$\frac{\text{Fraction} \times \text{dilution}}{1000}\ \text{mg/g}$$

where Fraction: results from previous three equations. Dilution: how many times solution is diluted from stock.

2.4.2 Total phenolic content (TPC)

Total phenolic content was estimated using the Folin–Ciocalteu colorimetric method as described elsewhere (Wang et al., 2003) with minor modification. 0.2 ml of each extract (1 mg/ml) was transferred into a 5 ml volumetric flask and swirled with 3 ml of deionised water. A 0.25 ml of Folin-Ciocalteu's reagent was added and swirled. After 3 min, 0.75 ml of 20% (w/v) sodium carbonate solution was added and mixed. This was recorded as time zero. Deionized water was added to make up the volume to 5 ml exactly. The solution was mixed thoroughly and allowed to stand at ambient temperature for 2 h until the characteristic blue color developed. Quantification was done on the basis of the standard curve of gallic acid at 760 nm by a spectrophotometer (Perkin-Elmer Lambda EZ-210 UV/VIS). All tests were conducted in triplicate and averaged. Results were expressed as gram of gallic acid equivalent (GAE) per 100 g of dry material.

2.4.3 Total flavonoid content (TFC)

The protocol of Chang et al. (2002) was as follow "stock solution of quercetin (0.5 mg/ml) prepared in methanol was diluted with 80% (v/v) ethanol to construct the calibration curve. A 0.5 ml of standard solutions or the crude extract were separately transferred into a 5 ml volumetric flask containing 2 ml of 80% ethanol, 0.1 ml of 10% (w/v) aluminum chloride and 0.1 ml of 1 M potassium acetate, diluted with distilled water to the mark and mixed. After 30 min, the absorbance was measured at 415 nm with a Perkin-Elmer Lambda EZ-210 spectrophotometer. The TFC in the crude extracts (1 mg/ml) were determined as above method."

2.4.4 Extraction procedure

The aerial parts (5 g) of each sample were extracted separately using a shaker device and 10 ml methanol at room temperature for 24 hours. The recovered fractions were filtered and evaporated entirely under vacuum after being washed with 50 ml of solvent.

The dried and powdered aerial portions of various plants (0.2 g) were placed in a screw-capped extraction tube and extracted for 60 minutes with 10 ml of 70% aqueous methanol at 75^{o}C. 3 ml of supernatant was transferred to a 5 ml vial after centrifuging at 4000 rpm for 5 minutes. The solution was filtered through a 0.2-micron membrane syringe filter for LC-MS measurement of apigenin and apigenin 7-glucoside in plant material (Haghi and Hatami, 2010).

2.5 Antimicrobial bioassay of ethanol extract

Gram-negative bacterial strains *Escherichia coli* (DH5) were evaluated using an ethanol extract of *Hemerocallis* leaves. According to Kavanagh, this assay was carried out utilising the agar plate diffusion method (1972). One millilitre of standardised bacterial stock solution (108-109 CFU/ml) was mixed with 100 millilitresof molten sterile nutritional agar kept at 45°C. The inoculation media was divided into 20 ml aliquots and placed onto sterile Petri-dish plates (9 cm). The bacterial cultures were kept on nutrient agar, and 100 l of plant ethanol extract was injected into wells before being incubated overnight at 37°C. In the control bioassay, just ethanol was inoculated in the wells.

2.6 Statistical analysis

In Minitab 19, the data was subjected to an analysis of variance test. Standard deviations, and mean averages were calculated. Excel 2019 was applied for histogram drawing.

3 Results and Discussion

3.1 Plants culture

The in vitro rooted culture of the three tested samples on MS media supplemented with phytohormones (Fig. 2) for *Hemerocallis* species.

Tissue culture has the advantage of very high propagation rates but is generally a labor-intensive propagation method for which high investigations and special trained personal are necessary. Therefore, its introduction in nurseries is restricted by economic factors (Liu and Liu, 2010).

Fig. 2 The in vitro rooted culture of *Hemerocallis* species on MS media

3.2 Molecular identification

The *Hemerocallis* species was identified using 18S rDNA and the identifications carried the accession number of (MN872235.1). The phylogenetic tree was performed on NCBI to determine the ancestor for this species and confirm that it is *Hemerocallis citrina* (Fig. 3).

The genus *Hemerocallis* was first classified as a member of the lily family (Liliaceae). However, for 20th century treatments of themonocotyledonous plants, Dahlgren et al. (1985) recognized that this classification was actually flawed and *Hemerocallis* was placed in aseparate family, Hemerocallidaceae. Recently, Rodriguez-Enriquez and Grant-Downton (2013) proved that molecular studies have shown evidence supporting this placement of the genus away from the Liliaceae.

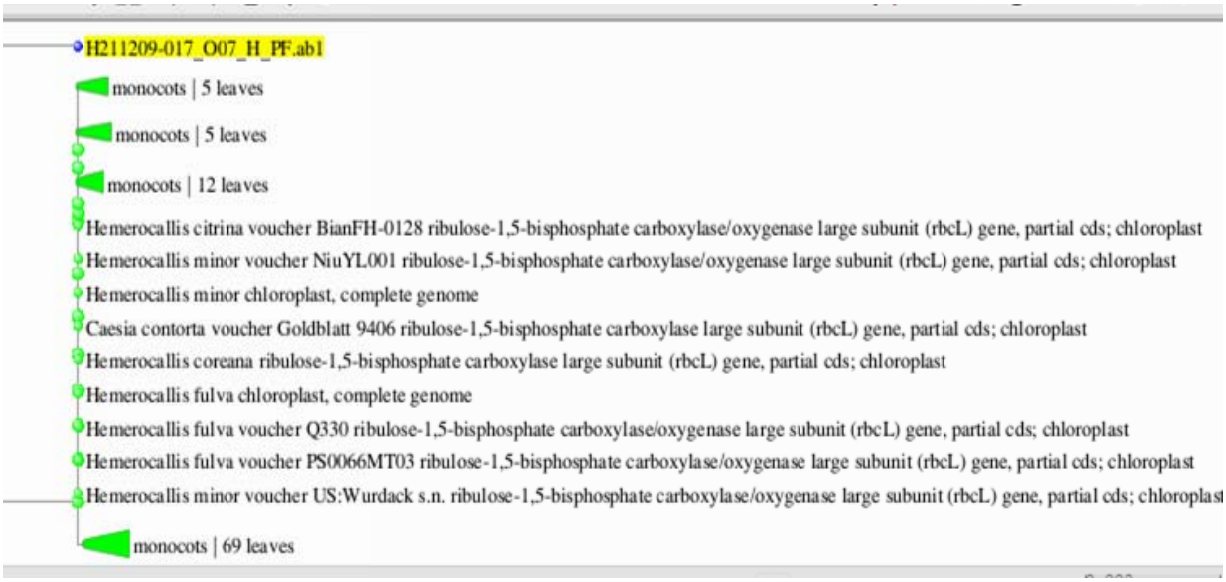

Fig. 3 Blast tree of *Hemerocallis* species

3.3 Physiological parameters

The photosynthetic pigmentation of *Hemerocallis citrina* includes chlorophyll A, B and carotenoids. These parameters were identified for the plant after 5 weeks of in vitro culture (Fig. 4). Also, the essential secondary metabolites of total phenolic compounds and total flavonoids were estimated and illustrated in Fig. 4. The results showed that carotenoids are the highest content in this species, where the other metabolites are more or less the same concentrations.

The estimation of the different physiological parameters defined the different activities of the plant species. These activities included the functions of both primary and secondary metabolites. In this study, we manipulated some essential metabolites like pigmentation, carotenoids, phenolics and flavonoids.Yang et al. (2016) measured the total flavonoids in *Hemerocallis citrina* to estimate its activity. Also, Li et al. (2019) estimated both flavonoids and anthocyanin in different *Hemerocallis* accessions. *Hemerocallis* species havepharmacological activities and Liang et al. (2021) proved that *Hemerocallis* species demonstrate the biological activities, such as antioxidant neurological,cytoprotection, anti-inflammatory, antidepressant, and anticancer.

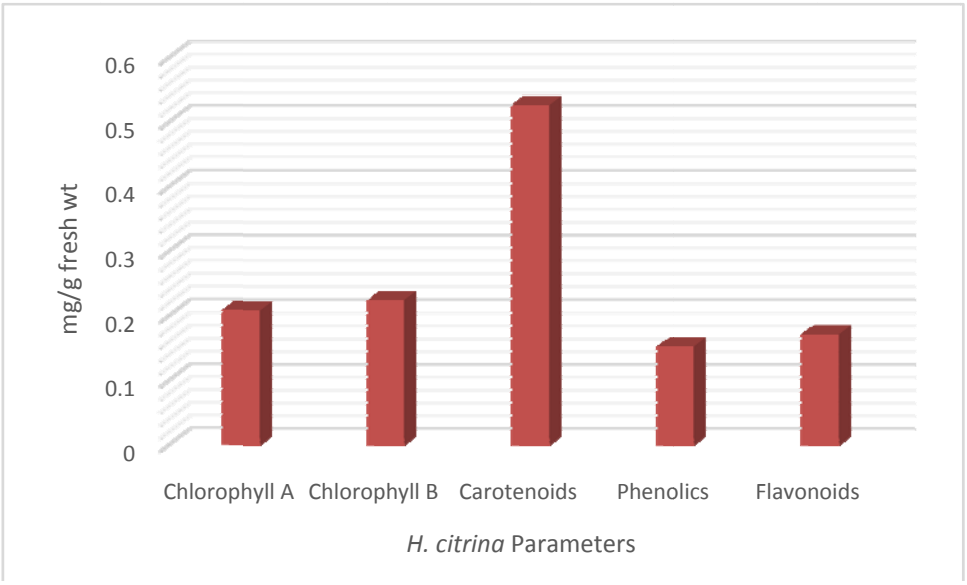

Fig. 4 Histogram illustrating the different physiological parameters of *Hemerocallis citrine*.

3.4 LC-MS

The identification of the total fractions (phenolic and flavonoid) in *Hemerocallis* plant were identified using LC-MS analysis. The identified fractions were tabulated in Table 1 and Fig. 5. The analysis showed 7 fractions from different secondary metabolites, with different activities.

The application of LC-MS chromatography for fraction analysis was performed by many researchers, like Clifford et al. (2006) who used LC-MS to identify different fractions of chlorogenic acids. Lu et al. (2008) approved the application ofmass spectroscopy to identify the different physiological fractions in plants. Besides, Yao et al. (2017) measured different physiological parameters of *Hemerocallis* flower buds. Also, Li et al. (2021, b) who identified flavonoids and anthocyaninsofdifferent *Hemerocallis* accession.

Table 1 The identification of the different compounds in *Hemerocallis* plant.

No.	Fraction/Compound	Fragment size m/z
1	3-O-caffeoylquinic acid	135
2	Guanosine	133
3	Adenosine	134
4	3-O-p-coumaroylquinic acid	41
5	5-O-p-coumaroylquinic acid	51
6	Orcinol-β-D-glucopyranoside	123
7	4-O-feruloylquinic acid	71

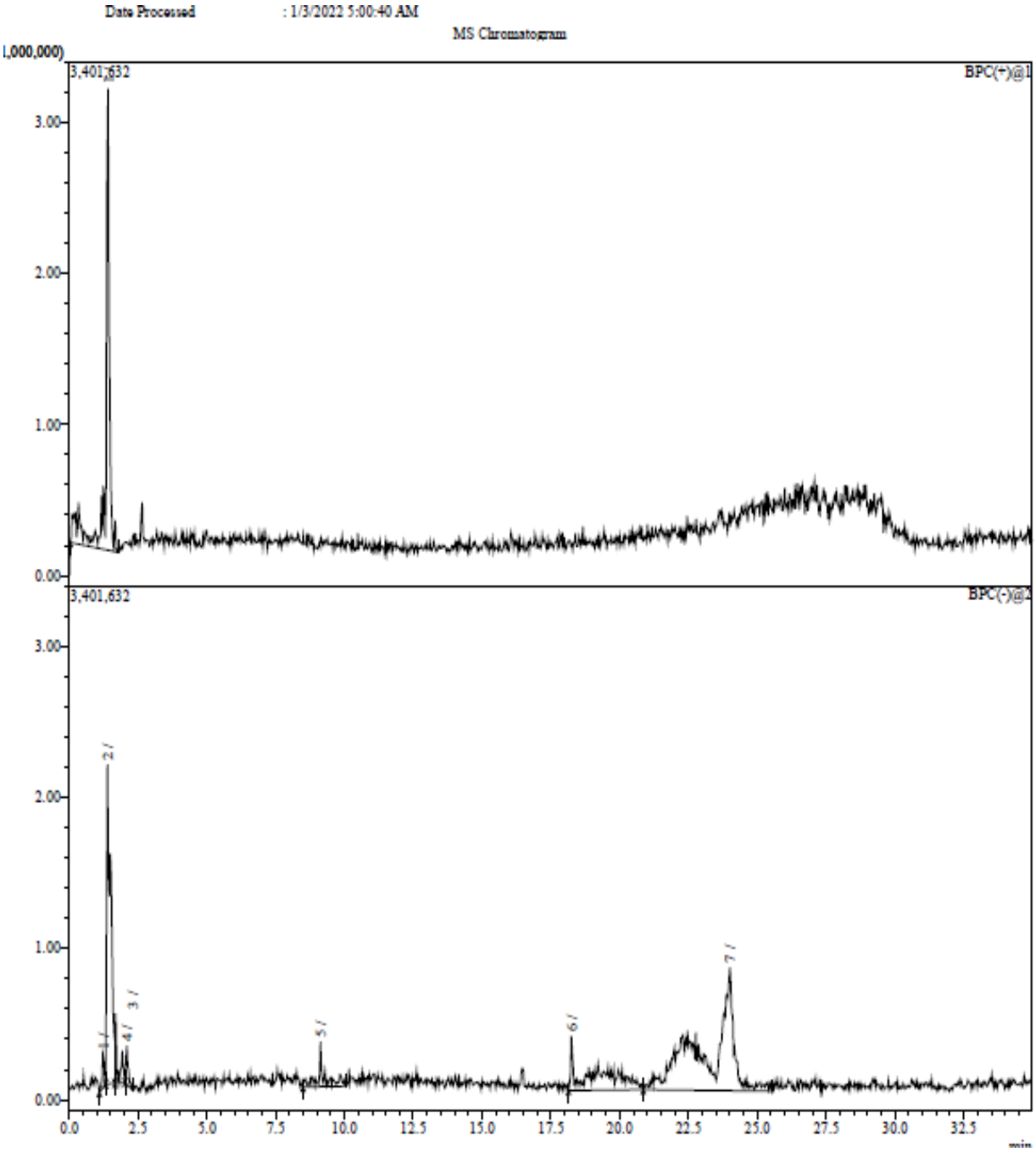

Fig. 5 LC-MS chromatogram for the fractions of *Hemerocallis* plant.

3.5 Antimicrobial bioassay

The antimicrobial activity of *Hemerocallis*species was estimated against Gram negative *E. coli*. These plants proved the antimicrobial activity via the clear zones shown which is represented graphically in Fig. 6. The control ethanol showed a clear zone of 0.75 cm, where the *Hemerocallis* extract showed 1.35 cm. this resulted in that the effect of *Hemerocallis* is only about 0.6 cm. This could be a good result confirming the natural extracts of plants as a safe alternative for antibiotics which is no longer effective against bacteria.

Many studied were performed to prove the concept of using plant extracts as a natural alternative for antibiotics. For example, Sarg et al. (2008) isolated different compounds from *Hemerocallis*: chrysophanol, methyl rhein, l,8-dihydroxy-3-methoxy-anthraquinone and rhein; and approved their antimicrobial activity.

Another survey of antibacterial activity of *Hemerocallis* was performed by Szewczyket al. (2019) who approved that the extract of *Hemerocallis* has antibacterial activity against Gram-positive bacteria (*Staphylococcus aureus* and *Staphylococcus epidermidis*) and Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*). Besides, Balla et al. (2017) applied the extract of *Mentha viridis* against two Gram-negative bacterial strains *Escherichia coli* and *Pseudomonas aeruginosa*, and fungal strains *Candida albicans*. Also, Hussien et al. (2021) used the mint extract as inhibitor for two bacterial strains, Gram-negative *Escherichia coli* (DH5-α) and *Pseudomonas aeruginosa*.

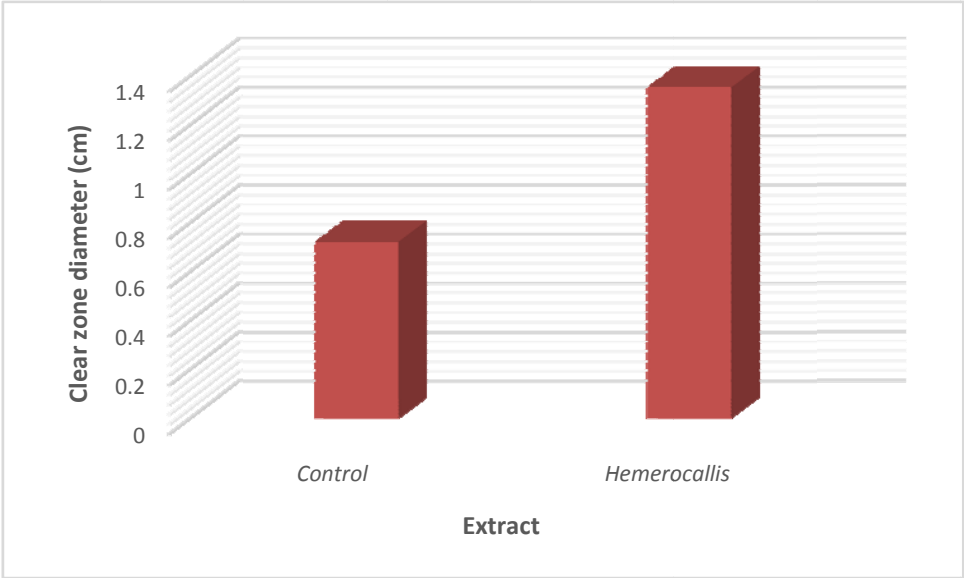

Fig. 6 Histogram showing the antimicrobial activity of *Hemerocallis*species extract against *E. coli* comparing to control ethanol.

4 Conclusion

Secondary metabolites have been demonstrated to have a variety of biological effects, providing a scientific foundation for the use of herbs in traditional medicine in many ancient cultures. They have antibacterial, antifungal, and antiviral properties, and hence can protect plants against infections. Furthermore, they contain crucial UV-absorbing chemicals, preventing major light damage to the leaves. In this study, an ornamental and medicinal plant, *Hemerocallis*, was in vitro cultured on MS media and defined using the molecular technique (18S rDNA). The physiological parameters were measured to define the plant's different activities: antimicrobial, anticancer, anti-inflammatory, antioxidant, etc. The fractions of these metabolites were defined using LC-MS. The antimicrobial activities of this species were estimated against *E. coli* and showed clear zone larger than control one.

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