Acta Tropica 191 (2019) 243–247

Contents lists available at ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica

Phytochemical profiling, antioxidant and anticancer activities of *Gastrocotyle hispida* growing in Saudi Arabia

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ARTICLE INFO

Keywords:
Anticancer
Gastrocotyle hispida
MTT
Antioxidant

ABSTRACT

The present study aimed at isolation the phytocompounds from the aerial parts of *Gastrocotyle hispida* and to evaluate its antioxidant and anticancer potential using in vitro assay. *Gastrocotyle hispida* is belonging to the family Boraginaceae used as a refreshing drink like tea. The decoction of the leaves is diuretic and is used in the treatment of rheumatism. Phytochemical study of a methanol extract yielded five known compounds viz: β-sitosterol (**GH-1**), β-sitosterol 3-glucoside (**GH-2**), 1-O-β-glucopyranosyl-1,4-dihydroxy-2-prenylbenzene (**GH-3**), 6-Hydroxy-2,2-dimethyl-3-chrom (**GH-4**) and rosmarinic acid (**GH-5**). Total phenolic and flavonoid contents were calculated for the extract and fractions, the methanolic extract contained the highest content of total flavonoids (178 mg/g, expressed as quercetin equivalents) and total polyphenol (98.4 mg/g, expressed as gallic acid equivalent). Compounds were isolated by using column chromatography. In vitro, antioxidant activity of the extract and isolated compounds was investigated by DPPH and ABTS radical scavenging assays. The four different cell lines HepG2 (Liver), HEK-293 (Kidney) MCF-7 (Breast) and MDA-MB 231 (Breast) were used against the compounds. The isolated compounds showed dose-dependent free radical scavenging property in all tested models with the IC_{50} values of 10.2 µg/mL rosmarinic acid (**GH-5**), 52.1 µg/mL β-sitosterol (**GH-1**) and 85 µg/mL for β-sitosterol 3-glucoside (**GH-2**). The β-sitosterol (**GH-1**) showed significant activity against HepG2 and HEK 293 cell lines. Rosmarinic acid (**GH-5**) possesses potent anticancer activity against breast cancer cells (MCF7) with the IC_{50} value of 4.2 µg/mL. It can be concluded that *Gastrocotyle hispida* has potential antioxidant, anticancer activities and further used as an anticancer agent.

1. Introduction

Cancer is one of the most life-threatening diseases, with more than 100 different types occurring due to some molecular changes within the cell. It is the third leading cause of death worldwide following cardiovascular and infectious diseases. (Shaikh et al., 2014). Cancer is a major public health burden in both developed and developing countries. According to WHO, cancer is one of the leading causes of death worldwide, which accounted for 7.6 million deaths (around 13%) of the world's population annually. Furthermore, WHO estimated that the worldwide deaths are likely to rise to over 11 million in 2030 (Dai and Mumper, 2010). The limited success of the currently used clinical therapies including radiation, chemotherapy, immunomodulation, and surgery in treating cancer, as evident by the high morbidity and mortality rates, indicates that there is an imperative need of new cancer management (Bhandari, 2015). Due to lack of effective drugs, cost of chemotherapeutic agents, and the side effects of anticancer drugs, cancer can be a cause of death. Therefore, efforts are still being made to search for effective naturally occurring anticarcinogens that would prevent, slow, or reverse cancer development. Medicinal plants have a special place in the management of cancer. It is estimated that plant-derived compounds in one or the other way constitute more than 50% of anticancer agents (Karayil, 2016).

The National Cancer Institute collected about 35,000 plant samples from 20 countries and has screened around 114,000 extracts for anticancer activity. It was estimated that 14 cancer drugs of the top 35 drugs in year 2000 based on worldwide sales were natural products and natural product derivatives. Thus, it is urgent to find more and more

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https://doi.org/10.1016/j.actatropica.2019.01.013
Received 6 February 2018; Received in revised form 15 January 2019; Accepted 15 January 2019
Available online 16 January 2019
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safe new compounds that kill cancer cells (Vijayalakshmi et al., 2013; Ghagane et al., 2017). *Gastrocotyle hispida* is belonging to the family Boraginaceae, it is also named as *Anchusa hispida. Gastrocotyle hispida* is an important aromatic and medicinal plant which is traditionally used as medicine for various diseases It is used as a refreshing drink like tea. The decoction of the leaves is diuretic and is used for the treatment of rheumatism. Boraginaceae is one of the most widely distributed families. It has about one hundred sixty genera and more than two thousand species distributed all over the world. It includes herbs, shrubs and trees annuals or perennials lacking essential oils. Members of the family have great variations in their morphological characters. In folk medicine around the world some of these family plants are used for burns and wounds (Gharib and Godarzee, 2017; Taia, 2006).

Gastrocotyle hispida has neuromuscular blocking activity and CNS stimulant activity, the decoction is used for treatment of favor, cough, syphilis and as a diuretic. Some of the boraginaceae family is used in Iran for its antipyretic, antihypertensive, aphrodisiac, treatment of bronchitis, diarrhea, cramps, kidney ailments and asthma (Arm et al., 2013). Biological effects such as anti-inflammatory, antioxidant, antimutagenic, anticancer activities have been attributed to presence of flavonoids and phenolic compounds (Gharib and Godarzee, 2017). The main phytochemical constituents are alkaloids as retronecine, 7-angleloyliotridine, trachelanthamine, supinine, intermedine, lycopsamine and 7-acetyllycopsamine. Another species e.g. Anchusamilleri contains alkaloids, flavonoids, tannins and sterols, flavonoids, polyphenols, terpenoids and phytosterols (El-Shazly et al., 1998).

2. Materials and methods

2.1. Plant material

The aerial part of *Gastrocotyle hispida* (Boraginaceae) was collected from Hail, Saudi Arabia in March 2015. The plant was kindly authenticated by an expert Taxonomist at the Herbarium Unit. A Voucher specimen has been deposited (SY-253/2015) at the Herbarium Unit of the faculty of Pharmacy, King Saud University, Saudi Arabia.

2.2. Extraction of plant material and isolation of the compounds

The air dried plant material (700 g) was powdered and extracted with 2000 ml of 80% methanol and experiment was carried out three times. The extracts were collected and filtered. The combined extract was concentrated using rotary evaporator under reduced pressure (extraction yield was 19.6% of the plant material). The residue was suspended in water and fractionated successively with n-hexane, chloroform and n-butanol to give n-hexane (3.1 g), chloroform (6.2 g), n-butanol (3 g) and water-soluble portions. Fractionation of 5.5 g of chloroform fraction on silica gel column chromatography using different polarity of methanol/chloroform (5%–30% MeOH/ $CHCl_3$) resulted in 8 sub fraction. Repeated column chromatography of sub fraction 2, 5 and 7 on silica gel and chloroform/methanol yielded compounds **GH-2** (24 mg), **GH-3** (16 mg) and **GH-4** (21 mg). Compound **GH-5** (25 mg) was isolated from n-butanol fraction using sephadex-LH-20 and 10% water in methanol as eluent. **GH-1** (30 mg) was isolated from hexane fraction using silica gel column chromatography and eluted with a gradient of ethyl acetate in hexane (5–20%) (Fig. 1).

2.3. NMR data

NMR spectra are recorded on a Bruker-400 MHz spectrometer. The chemical shift values were reported in ppm (δ) and the coupling constants (J) are in Hz.

2.3.1. NMR data compound (1-O-β-glucopyranosyl-1,4-dihydroxy-2-prenylbenzene) (GH-3)

^{1}H NMR (400 MHz, CH3OD) δ 6.45 (H-2), δ 6.55 (H-3), δ 6.64 (H-5), δ 5.33 (H-8), 1.77 and 1.88 (2 H, H-9), 5.34 (H-1′) of the glucose, δ 3.7-3.2 (5 H, sugar). ^{13}C-NMR (CD3OD, 100 MHz): δ 152.1 (C-4), δ 148.7 (C-1), δ 131.8 (C-6), δ 117.03 (C-5), δ 114.4 (C-3), δ 112.4 (C-2), δ 132.6 (C-9), δ 122.6 (C-8), δ 27.8 (C-7), δ 24.54 (C-11), δ 19.48 (C-11), δ 102.6 (C-1′), δ 76.8 (C-5′), δ 76.4 (C-3′), δ 73.7 (C-2′), δ 70.0 (C-4′), δ 61.2 (C-6′).

2.3.2. NMR data of Compound (6-Hydroxy-2,2-dimethyl-3-chrom) (GH-4)

^{1}H NMR (400 MHz, CDCl3) δ 6.62 (H-4), δ 6.60 (H-7), δ 6.53 (H-8) δ 6.18 (H-5), δ 5.22 (H-3) δ 1.5 2H, (H-2). ^{13}C-NMR (100 MHz, CDCl3): δ 149.4 (C-6), δ 146.6 (C-9), δ131.9 (C-3), δ 122.2 (C-4), δ 122.1 (C-10), δ 115.2 (C-7 and C-8), δ 112.8 (C-5), δ 77.3 (C-2), δ 28.2 (CH3),

2.4. Total phenolic content

The phenol concentration was quantified following the method previously described (Taga et al., 1984). In brief, the Folin Ciocalteau method was utilized to determine the content of polyphenols in the extract. A test sample (50 µl) was mixed with 2 ml 2% sodium carbonate and allowed to stand at RT for 2 min. During this time,100 µl 50% Folin Ciocalteau reagent was added; the reaction mixture was kept undisturbed at RT for 30 min and readings were taken at 720 nm. Gallic acid was used as a standard for the calibration curve. The quantity of phenol present in the extract was expressed as Gallic acid equivalents (GAE).

2.5. Determination of total flavonoids

The flavonoid content was determined by the aluminum chloride method using Quercetin as a reference compound (Djeridane et al. 2006) with some modification. In brief, sample (1 mL) was mixed with 1 mL methanolic solution containing 2% aluminum chloride. flavonoid-aluminum complex was formed during 15 min incubation at 30 °C. The complex was measured at 430 nm using a UV–Vis spectrophotometer. Quercetin (0–100 µg mL) was used as a standard for calibration curve preparation. The total flavonoid content was expressed as the Quercetin equivalent (QE) in mg /g of dry weight plant extract.

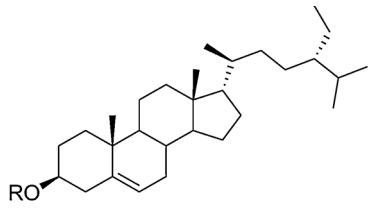
2.6. Antioxidant activity

2.6.1. DPPH radical scavenging activity

The antioxidant activity of the extract was measured based on the scavenging activity of the stable DPPH free radical (Kalaivani et al., 2011a,b). The activity was determined by the method described previously (Shahat et al., 2014). The compounds and fractions were dissolved in 5% DMSO. Plant extracts at concentrations ranging from 20 to 100 µg/mL were added to 3 mL of 0.004% DPPH solution. One milliliter of methanol was used as a control. Absorbance was determined at 520 nm after 30 min, % inhibition was calculated by the following equation: % inhibition = A_0-A_t/A_o ×100 Where At = absorbance of extract, A0 = absorbance of control.

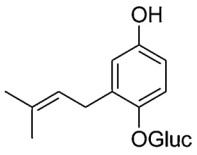
2.6.2. ABTS radical cation scavenging activity

The ABTS radical scavenging activity of the extracts was measured using the method previously described (Shahat et al., 2015). ABTS solution in water (7 mmol/L) and an aqueous solution of potassium persulfate (2.45 mmol/L) were prepared. The two solutions were mixed in a 1:1 ratio and stored in the dark for 6 h at RT to allow the production of ABTS radicals. The ABTS stock solution was diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm and equilibrated at 30 °C. The compounds and fractions were dissolved in 5% DMSO. An aliquot of each plant extract (0.1 mL) was mixed with 2.9 mL of diluted ABTS radical cation solution. After the reaction was incubated at 30 °C for

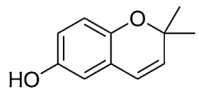


β-Sitosterol (**GH-1); R = H**

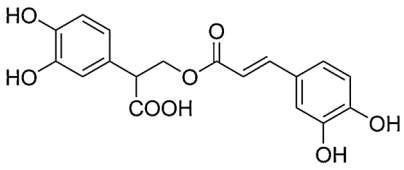
β-sitosterol 3-glucoside (**GH-2); R = Gluc**



1-O-β-glucopyranosyl-1, 4-dihydroxy-2-prenylbenzene (**GH-3**)



6-Hydroxy-2, 2-dimethyl-3-chromen (**GH-4**)



Rosmarinic acid(**GH-5**)

Fig. 1. Chemical structures of the isolated compounds.

20 min, the absorbance at 734 nm was measured. The ability of test material to quench ABTS free radicals was evaluated according to the following formula: Scavenging (%) = [(Ac- Aa/ Ac)] x100, Where Ac = absorbance of control; Aa = absorbance of sample

2.7. In vitro anticancer activity

Human cancer cell lines: HepG2 (Liver), HEK-293 (Kidney) MCF-7 (Breast) and MDA-MB 231 (Breast) were grown in DMEM media supplemented with 10% bovine serum, 1X penicillin-streptomycin (Sigma-Aldrich) at 37 OC in a humidified chamber with 5% CO2. 5-Flurourasil (sigma) was used as the reference drug (standard).

Cells were seeded (1×10^5 cells/well in triplicate) in a 96-well flat-bottom plate (Becton-Dickinson Lab ware) a day before treatment and grown. The compounds and fractions were initially dissolved in 5% DMSO and diluted further by serial dilution and only diluted DMSO were kept as control. Stock solution of extracts, fractions and compounds (1.0 mg/mL) were made with 5% DMSO (Sigma-Aldrich) and further working solutions (100 µg/ml) were prepared in serum-free culture media. Cells were treated with four different doses (6.2, 12.5, 25 and 50 µg/ml; in triplicate) of the compounds, in complete growth media, including reference drug, was further incubated for 48 h (Alajmi et al., 2017; SaiKoushik et al., 2015)

2.7.1. Cell proliferation and viability assay

On day 2 of treatment, cell proliferation and viability test was performed by using MTT Cell Proliferation and Viability Assay Kit (TACS) as per manufacturer's instructions. The relationship between surviving fraction and compound concentration was plotted to obtain the survival curve of cancer cell lines. The response parameter calculated was the IC_{50} value, which corresponds to the concentration required for 50% inhibition of cell viability.

2.8. Statistical analysis

Data are expressed as the mean ± SEM of at least three independent experiments. The paired *t*-test was used to calculate statistical significance between control and treated groups with a $P < 0.05$ considered to be statistically significant.

3. Results and discussion

In recent years, the use of herbal medicines in cancer treatment has received increasing attention due to their varied phyto-metabolic contents with multiple biological activities. Among the different phytochemicals, phenolic compounds have gained attention of different areas of applications such as pharmaceutical, health, food, and cosmetic industries. These compounds are widespread in the plant kingdom as part of our daily diet and are attractive as natural antioxidants. Hence we extracted *Gastrocotyle hispida* with 80% methanol and fractionated extract with series of solvent of different polarity.

After a series of column chromatography five compounds were isolated and purified. The structure of the isolated compounds were elucidated on the basis of spectroscopic methods and finally confirmed directly with the published data. Compound **CH-1** was identified as β-sitosterol (Chaturvedula and Prakash, 2012), compound **CH-2** was identified as β-sitosterol 3-glucoside (Peshin and Kar, 2017), compound **CH-3** was identified as 1-*O*-β-glucopyranosyl-1, 4-dihydroxy-2-prenylbenzene (Góngora et al., 2001), compound **CH-4** was identified as 6-Hydroxy-2, 2-dimethyl-3-chromen (Howard et al., 1979) and **CH-5** was identified as rosmarinic acid (Exarchou et al., 2001). The structures are shown in (Fig. 1).

The amount of total polyphenols and flavonoids in the extract and fractions varied with the solvent used (Figs. 2 and 3). Methanol crude extract had the highest phenolic content. Butanol, chloroform and water fractions showed significantly higher phenolic content than the hexane fractions. The butanol fraction and crude extract showed the significant flavonoid content, followed by the chloroform fraction. The hexane fractions did not show any flavonoid content. Figs. 4 and 5 shows the DPPH and ABTS scavenging activity of crude extract and its fractions. Among the fractions chloroform fraction exhibited significant

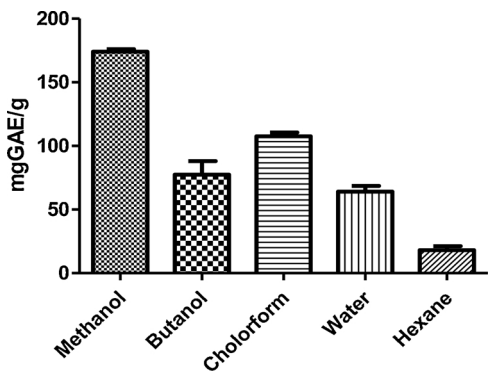


Fig. 2. Total phenolic content of *Gastrocotyle hispida* and its fractions Values are means of three replicates ± SD.

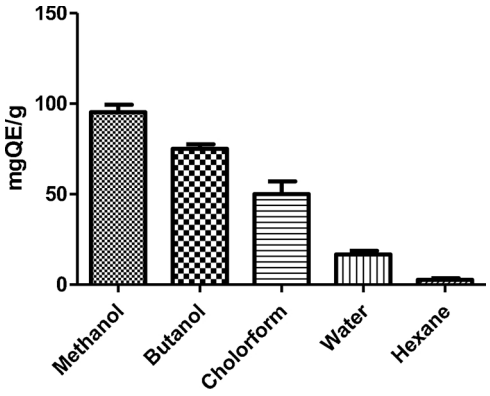


Fig. 3. Total flavonoid content of Gastrocotyle hispida and its different fractions. Values are means of three replicates ± SD.

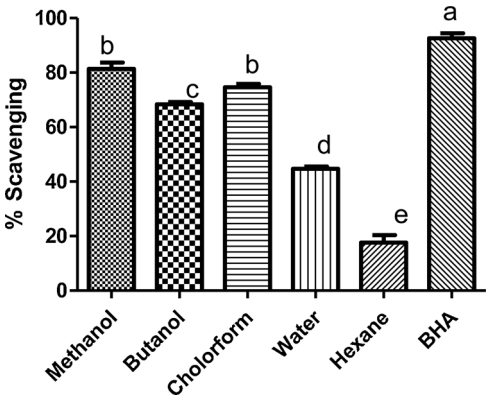


Fig. 4. Comparison of DPPH scavenging activity *Gastrocotyle hispida* extract, fractions and BHA. Values are means of three replicates.

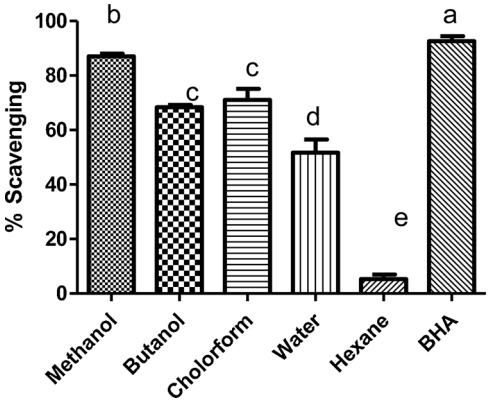


Fig. 5. Comparison of ABTS scavenging activity *Gastrocotyle hispida* extract, fractions and BHA. Values are means of three replicates.

antioxidant activity compared to other fractions. Hence this fraction was used to isolate the compounds responsible for observed activity.

The isolated compounds revealed the excellent antioxidant activities. The order of DPPH free radical scavenging activity is as BHA, **GH-5, GH-1, GH-2, GH-3** and **GH4** (Table 1). GH-5 showed highest scavenging activity compared to the other compounds. **GH-4** showed the lowest activity. The mechanism of DPPH scavenging activity is based on the hydrogen or electron releasing capability of antioxidant molecules to DPPH$^{\cdot}$ molecules to form the nonradical DPPH-H and the measurement of the reducing ability of antioxidants. When DPPH radicals react with a proton donor molecule like an antioxidant, the radicals are scavenged and the absorbance is reduced. During the reduction process, purple DPPH$^{\cdot}$ changes to a colorless diphenyl picryl hydrazine and remaining DPPH$^{\cdot}$ exhibiting the maximum absorption at 517 nm is measured (Erenler et al., 2017). In the present study, the capacity of the isolated compounds to scavenge ABTS is shown in (Table 1).

Table 1
Comparison of IC_{50} values of the isolated compounds for DPPH and ABTS Scavenging activity (μg/ml).

No.	Sample code	DPPH	ABTS
1.	β-Sitosterol (GH-1)	52 ± 2	33.7 ± 2.5
2.	β-sitosterol 3-glucoside (GH-2)	85 ± 32	44 ± 6
3.	1-*O*-β-glucopyranosyl-1, 4-dihydroxy-2-prenylbenzene (GH-3)	124 ± 23	116.2 ± 32
4.	6-Hydroxy-2, 2-dimethyl-3-chromen (GH-4)	234 ± 73	241.8 ± 1
5.	Rosmarinic acid (GH-5)	10.2 ± 2	5.2 ± 0.7
6.	BHA	8.1 ± 0.7	17.4 ± 1.5

The order of activity of isolated compounds is as follows: **GH-5, GH-1, GH-2, GH-3, and GH-4**. Dose dependent scavenging activity was observed in the present study. **GH-5** exhibited significant antioxidant activity compared to other extracts. **GH-4** exhibited lowest activity. ABTS is blue–green color in its native form. Decreasing in color of ABTS measures the antioxidant ability of extracts and compounds. The active compounds have the ability to donate protons/electrons to the ABTS$^{\cdot+}$ radical form. This spectrophotometric technique is easy and simple for screening and routine analyses. This method is based on the inhibition of ABTS$^{\cdot}$ radical form by abstracting proton or electron from antioxidants.

ABTS$^{\cdot+}$ is more reactive than DPPH$^{\cdot}$. All the isolated compounds were evaluated against a panel of four human cancer cell lines *viz* HepG2 (Liver), HEK-293 (Kidney) MCF-7 (Breast) and MDA-MB 231 (Breast) and the results are collected in Table 2. Cells were treated with the test compounds at concentrations ranging from (5–50 μg/ml); in triplicate, for 48 h. The results are expressed in IC_{50} values of the indicated compounds tested against the different cell lines and 5-Flurourasil was used as a reference drug. **GH-1** revealed the highest activity againstHepG2 and HEK 293 cells lines with IC_{50}, 12.2 and 2.3 μg/ ml L compared to other compounds.

Noticeable effect has not been detected for GH-4 on HepG2 and HEK 293 cell lines. The **GH-4** was effective on MCF-7 cell lines as compared to other extracts. **GH-5** exhibited moderate activity onHepG2 and MDA–MB231 cells with IC_{50} values of 20 μg/mL and 25 μg/ml respectively. **GH-1** and **GH-4** exhibited the lowest activity on MDA-MB-231 with 72 and 71 μg/ml respectively. (Park et al., 2000) reported that β-sitosterol increases the activity of caspase-3 through downregulation of Bcl-2 which agrees with the present study. The β-sitosterol also induces endo-reduplication and apoptosis by involving phosphatidylinositol 3-kinase/Akt and ERK-independent pathways. ROS production is the main cause for carcinogeniss many reports have been made on rosmarinic acid as potential antioxidant which might be the mechanism of action in cell lines used in our study.

4. Conclusions

Present study demonstrated the bioactive compounds responsible for the activities. β-sitosterol (**GH-1**) β-sitosterol3-glucoside (**GH-2**), 1-*O*-β-glucopyranosyl-1,4-dihydroxy-2-prenylbenzene (**GH-3**), 6-hydroxy-2,2-dimethyl-3-chromen (**GH-4**) and Rosmarinic acid (**GH-5**) were isolated from this plant for the first time. Rosmarinic acid and β-sitosterol exhibited an excellent antioxidant activities compared to other compounds. β-sitosterol, β-sitosterol 3-glucoside and rosmarinic acid revealed significant activity on different cancer cells lines used in the study. Some of the isolated compounds of *Gastrocotyle hispida* has the potency to be used in food industries as a natural antioxidant as well as pharmaceutical trades.

Table 2
IC_{50} values of different compounds against four human cancer cell lines (μg/ml).

No.	Sample code	HepG2 (Liver)	HEK-293 (Kidney)	MCF-7 (Breast)	MDA-MB 231 (Breast)
1.	β-Sitosterol (GH-1)	12 ± 2	2.3 ± 0.5	6.2 ± 2	72 ± 6
2.	β-sitosterol3-glucoside (GH-2)	25 ± 2	44 ± 6	8.1 ± 1	53 ± 5
3.	1-*O*-β-glucopyranosyl-1, 4-dihydroxy-2-prenylbenzene (GH-3)	24 ± 3	6.2 ± 2	5.7 ± 1	55 ± 5
4.	6-Hydroxy-2,2-dimethyl-3-chromen (GH-4)	13 ± 3	4.8 ± 1	3.7 ± 1	71 ± 6
5.	Rosmarinic acid (GH-5)	20 ± 2	5 ± 1	4.2 ± 1	25 ± 2
6.	Flurourasil	2.1 ± 0.5	2.4 ± 0.5	3.2 ± 0.5	2.5 ± 0.5

Acknowledgement

The authors extend their appreciation to the Deanship of Scientific Research at King Saud University for funding this work through research group no. RGP-262.

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