Deer Velvet Antler Water Extract Demonstrates Non-Cytotoxic Effects and Maintains Viability of Dental Stem Cells

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Abstract

Deer velvet antler (DVA), widely used in traditional Asian medicine, is gaining research interest for its effects on bone health, tissue regeneration, and cancer. However, the fundamental analysis looking into the effects of DVA on stem cells; particularly derived from dental tissues, are limited.

Hence, this research aims to determine the biological effects of DVA water extract on the viability of stem cells harvested from human exfoliated deciduous teeth (SHED). DVA water extract was subjected for small molecule compounds analysis using LC-MS/MS-QTOF. The effects of DVA water extract on SHED were assessed at concentration up to 200 µg/ml for cell viability using MTT assay, cell morphology using inverted microscope and proliferative activity described as population doubling time (PDT) using alamarBlue assay. Although insignificant, DVA water extract at 3.125 µg/ml produced the lowest PDT while maintaining the cell proliferation and viability.

Overall, DVA water extract at concentration up to 200 µg/ml was not cytotoxic, maintained its proliferative effects and the morphology of SHED. The presence of detectable small molecules in water extract which corresponds to the multiple benefits of DVA might be responsible for the demonstrated observations.

Experimental article (J Int Dent Med Res 2025; 18(1): 26-33)

Keywords: Velvet antler; dental stem cells; water extract, biological activity.

Received date: 25 February 2025 **Accept date:** 20 March 2025

Introduction

The antlers of the *Cervidae* family undergo a unique yearly shedding and growth cycle, distinct from all other mammals. Deer velvet antlers (DVA) refer to the pre-calcified antlers covered in velvet during their growth phase, and for centuries, have been used in traditional Chinese medicine due to their many purported health benefits. Recently, there has been renewed interest in DVA research, with several studies reporting on its anti-fatigue effects[1], anti-tumor properties[2], and potentially health-beneficial peptides[3], as well as its role in improving physical performance and promoting bone health[4].

DVA's abundant bioactive compounds and chemical constituents with therapeutic potential may explain its numerous health benefits and pharmacological potential[3]. DVA is rich in amino acids, polypeptides, and proteins, featuring up to 19 amino acids, including glutamic acid, glycine, arginine, and aspartic acid[5,6].

Velvet antler polypeptides (VAPs) are active compounds extracted from DVA that can improve facet-joint osteoarthritis (FJ-OA) induced in beta-catenin conditional activation mice[7]. Moreover, bioactive peptides extracted from deer antlers exhibit beneficial properties for bone metabolism, further supporting DVA's usefulness in bone health and development[3]. Furthermore,

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DVA has anti-osteoporotic activities, protecting bones from estrogen deficiency[8]. IGF-1 is a protein that regulates cell growth, survival, and metabolism by maintaining bone mass and stimulating the osteoblastic differentiation of mesenchymal stem cells (MSCs)[9] and it is present in DVA[10–12].

The utilization of MSC in tissue regeneration has been widely acknowledged. Stem cells harvested from human exfoliated deciduous teeth (SHED), a type of mesenchymal stem cell derived from dental tissues, have emerged as an alternative source in regenerative medicine and dental tissue engineering due to their high proliferation rates, easy accessibility through less invasive techniques, the ability to differentiate into multiple lineages, and their suitability for long-term storage without affecting its stem cell characteristics[13,14].

These observations were obtained when the stem cells were treated with common growth factors or chemical reagents. Therefore, it would be highly advantageous to identify certain non-toxic animal or plant-based natural products that can maintain or promote the growth of SHED. Thus, this study aimed to investigate the biological effects of DVA water extract on the viability of SHED. To address this, cell viability, proliferation activity, and morphological observations were assessed on SHED. Detection of small metabolites from DVA water extract was also performed.

Materials and methods

DVA collection

The powdered sample of the DVA was provided to the researchers by the local company; Regal Malay Capital Berhad, Pahang, Malaysia. Briefly, the antler of the male Malayan deer *(Cervus timorensis)* was collected and cut into small pieces and dried. The dried antler samples were powdered using variable speed laboratory blender (Waring ®, USA).

DVA water extraction

The DVA water extraction (WE) was done following the protocol as previously published with modification[15]. The filtrate was collected and subjected to freeze-drying. The freeze-dried sample was then stored at -20°C for further analysis.

Small molecule compounds analysis using LC-MS/MS-QTOF

The chemical profiles of DVA water extract were analyzed using an Agilent 1290 Infinity Liquid Chromatography system coupled to Agilent 6520 Accurate-Mass Quadrupole Time-of-Flight mass spectrometer with dual electrospray ionization (ESI) source (Agilent Technologies Inc, USA). The analysis was conducted for negative and positive polarity using the Agilent MassHunter Qualitative Analysis B.07.00 software (Agilent Technologies, United States). This analysis was conducted at LCMS Platform, Jeffrey Cheah School of Medicine and Health Sciences, Monash University, Malaysia Campus.

Cell viability by MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl- tetrazolium bromide)

SHED was seeded in 96-well plates at a density of $1x10^3$ cells/well in alpha-MEM (Gibco, USA) media supplemented with 10 to 15 % of FBS (Gibco, USA) and 0.5% of Penicillin-Streptomycin (Gibco, USA) and incubated at 5% CO_2 at 37°C for 24 hours to allow for cell adhesion. Subsequently, SHED was treated with 100 µl of DVA extracts at concentrations ranging from 200 µg/ml to 0 µg/ml, with a two-fold serial dilution. Background controls are wells with growth media only, with no SHED seeded. After 72 hours of incubation, the media from the well are discarded, and cells are washed twice with DPBS. 100 µl of freshly prepared 0.5 µg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl-tetrazolium bromide; Invitrogen, USA) solution was added into all test wells and incubated for 3 hours to allow formazan crystals to form. Afterwards, the MTT solution was discarded, and 100 µl of DMSO was added to the wells to dissolve the formed crystals. The plate was shaken for 30 minutes at room temperature to solubilize the formazan crystals. The absorbance of each test well was measured at 570 nm using a microplate ELISA reader. All experiments were performed in triplicate.

Proliferation activity by alamarBlue assay

The protocol for the alamarBlue assay was provided by the manufacturer (Invitrogen, USA). Like the MTT assay, 100 µl of SHED was seeded in 96-well plates at a seeding density of $1x10^3$ cells/well. After 24 hours of incubation, the

old growth media was discarded and replaced with DVA extracts of concentrations 0 – 200 µg/ml and incubated for a further 24 hours. The proliferation activities of SHED treated with DVA extract were measured every 24 hours for eight days. The media was replenished every 24 hours also. Briefly, 10 µl of alamarBlue reagent was added to each test well and incubated for 4 hours. Then, the media now containing the alamarBlue reagent is transferred into new 96-well plates, and the absorbance is measured on a microplate reader at 570 nm with a reference wavelength of 600 nm. The absorbance value was then converted to percentages of reduction following the equation provided by the manufacturer.

Additionally, a standard curve of cell density against the percentage of reduction was also plotted. Cell densities of 40,000, 20,000, 10,000, 5000, 2500, 1250, 625, 312.5, and 156.25 were seeded in a 96-well plate. Additionally, background control of wells containing only media and no cells was also included on the same plate. After 24 hours of incubation, the proliferation activities of SHED were determined in the same way above.

Determining the population doubling time (PDT) of SHED

Population doubling time (PDT) is the duration, measured in hours, for cells to double in population, determined by the multiplication rate. Multiplication rate (r) is the number of generations that occur per unit time. Typically, r is expressed as population doubling per 24 hours. Cell multiplication rate and population doubling time were calculated according to the cell number obtained from the standard curve above by using the formula from Davis (2002)[16].

Cell Morphology

The morphology of SHED treated with DVA extract was observed under an inverted microscope for a period of 21 days. Observations was done on Day 3, 7, 14 and 21.

Statistical analysis

The data was analyzed using SPSS statistical software (version 30.0.0.0, SPSS Inc., Chicago, IL, USA). The statistical analyses were conducted using ANOVA, followed by Tukey's post hoc test when applicable. A significance level of 0.05 was chosen to determine statistical significance in this study. Results with a p-value of less than 0.05 were considered statistically significant.

Results

Small molecule compounds analysis using LC-MS/MS-QTOF on DVA water extract

The identified non targeted compounds in DAV water extract resulted from positive and negative polarity ionization were tabulated in Table 1 with the following parameters: retention times (RT), molecular mass, molecular formula generation (MFG), MFG Diff (ppm), Database (DB) formula, DB Diff (ppm), hits (DB) and percentage of volume (Vol %). The data shows the presence of 11 small molecule compounds from DVA water extract: 7 identified compounds from the positive polarity and 4 identified compounds from the negative polarity. These compounds contain various functional groups and structural features.

Effect of DVA water extract on cell viability of SHED

MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl- tetrazolium bromide)

The results of the MTT assay indicate no significant difference in SHED viability across the tested concentrations of DVA extract ($p>0.05$) (Figure 1). The viability of control group; SHED cultured in growth medium without DVA extract, was set at 100% viability. Although this difference is not significant, the MTT results suggest that lower dosages of DVA are associated with higher percentages of SHED viability. For instance, concentrations from 0.78125 µg/ml to 3.125 µg/ml produced viability percentages exceeding 100%. However, cell viability appeared to be declined at 200 µg/ml. Nonetheless, statistical analysis reveals that the percentage of viability does not significantly differ from that of the control, thus rendering this concentration non-cytotoxic to SHED.

AlamarBlue Assay

The proliferation activity of SHED treated with DVA extract was observed over the course of eight days via alamarBlue assay. The standard curve of SHED is shown in Figure 2, from which the cell numbers were obtained. A higher percentage of alamarBlue reduction corresponds to a higher cell number.

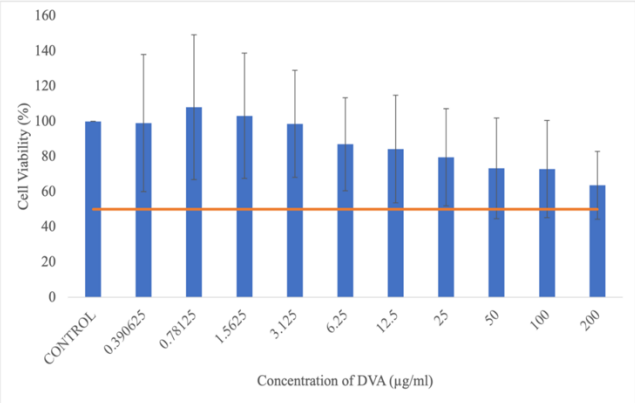

Figure 1. Cell viability of SHED treated with different concentrations of DVA extract. MTT results indicate that all tested DVA extract concentrations are non-cytotoxic to SHED. Data is expressed as Mean ± SD, where n=3. Line indicate the IC_{50}.

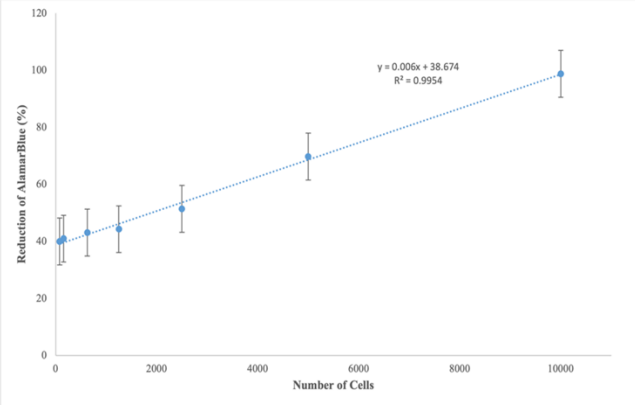

Figure 2. The standard curve of SHED proliferation obtained by alamarBlue analysis. Data is expressed as Mean ± SD, where n=3, and each n constitutes three technical replicates.

The proliferation activities of SHED treated with DVA extract is shown in Figure 3. Overall, all groups with different DVA concentration show increased cell numbers over eight days.

On Day 2, all DVA extract treatment groups yielded no significant difference in cell number compared to the control group ($p>0.05$). 200 µg/ml of DVA extract yielded a cell number of 2438 ± 16, which is higher than the control's 1769 ± 131 by a margin, although still found to be statistically insignificant. Further, cell yield at 200 µg/ml of DVA treatment was found significantly higher than treatment groups of 3.125 µg/ml and 50 µg/ml, which had cell numbers of 1729 ± 89 and 1847 ± 82, respectively.

There is a significant increase in cell number from Day 4 to Day 6, before decreasing approaching Day 8, as shown in Figure 3 ($p<0.05$). The cell numbers for DVA extract treatment at concentrations of 3.125 µg/ml (3783 ± 73) and 200 µg/ml (3856 ± 286) were found to be significantly higher than those of the control group (3163 ± 114). Additionally, these cell numbers do not differ significantly from one another.

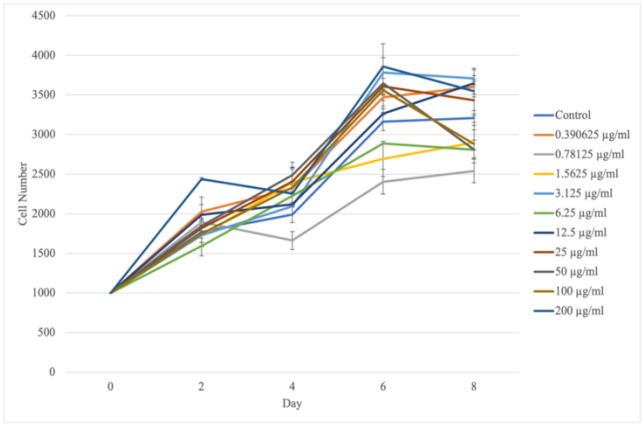

Figure 3. The proliferation activity of SHED treated with DVA extract concentrations of 0- 200 µg/ml, analyzed by alamarBlue assay. The data is expressed as Mean ± SD, where n=3, and each n represents three technical replicates.

DVA Extract (µg/ml)	PDT (hour/doubling)	*p-value*
0.00 (Control)	86.74 ± 2.67	
0.39	80.22 ± 0.77	p>0.05
0.78	114.32 ± 8.04*	P<0.05
1.56	101. 44 ± 8.40*	p<0.05
3.13	75.02 ± 1.09	p>0.05
6.25	94.17 ± 3.20	p>0.05
12.50	84.44 ± 2.16	p>0.05
25.00	78.28 ± 6.14	p>0.05
50.00	77.52 ± 5.58	p>0.05
100.00	78.60 ± 1.12	p>0.05
200.00	74.14 ± 4.07	p>0.05

Table 2. Population Doubling Time (PDT) of SHED treated with DVA extract of different concentration. Data is expressed as Mean ± SD, where *n*=3. Data was analyzed using One-way ANOVA followed by Tukey's post hoc test. A *p-value* of less than 0.05 was taken as significant. Asterisks (*) indicate significantly different PDTs from the control group. Numbers were rounded off to two decimal points.

Population Doubling Time (PDT) of SHED Treated with DVA extract

The PDT of SHED with or without DVA extract treatment was calculated based on the readings obtained on Day 6. The control group was found to have a PDT of 86.73 ± 2.67 hours. The two lowest PDTs on Day 6 were revealed to be treatment group 3.125 µg/ml at 75.02 ± 1.09 hours and treatment group 200 µg/ml at 74.15 ± 4.07 hours. However, these PDTs and all other treatment groups except 0.78125 µg/ml and 1.5625 µg/ml had no significant difference from the control group ($p>0.05$). 0.78125 µg/ml and 1.5625 µg/ml had higher PDTs than the control, indicating slower growth rates.

Cell Morphology of SHED Treated with DVA Extract

There were no detectable changes in the morphology of SHED with DVA extract treatment during the 21-day observation period with DVA concentration at 3.125 µg/ml and 200 µg/ml. SHED maintained their elongated, spindle-shaped, fibroblast-like morphology until Day 21, with or without DVA extract treatment.

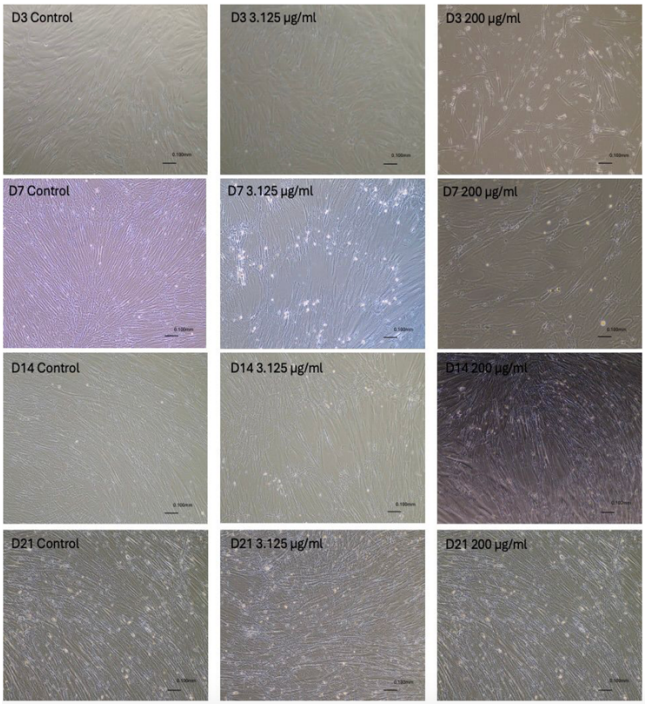

Figure 4. The morphology of SHED cultured in growth medium with and without DVA extract at 3.125 µg/ml and 200 µg/ml over 21 days demonstrates no changes in the morphology of SHED. The images were taken using an inverted microscope with 10X magnification.

Discussion

Presence of small compounds from DVA water extract identified through LC-MS/MS-QTOF

Currently, there is limited research on the effects of the identified compounds in DVA analyzed via LC-MS/MS-QTOF assays. Although limited study was done on dental stem cells, some of the compounds have shown various effects towards other types of cell viability. 4-vinyl cyclohexene diepoxide (VCD) is recognized to be ovotoxic, as there are studies that show VCD can cause damage to ovarian tissues and trigger apoptosis through elevated oxidative stress[17, 18]. Although this pertains to ovarian cells, VCD's pro-apoptotic and oxidative stress-inducing properties could also be detrimental to other cell types, including SHED.

Conversely, gingerol has been reported to exhibit protective effects on certain stem cell types[19]. The authors found that gingerol can protect nucleus pulposus-derived mesenchymal stem cells from oxidative injury by activating autophagy, thus reducing apoptosis and maintaining cell viability. Interestingly, gingerol is also found to inhibit the proliferation of certain cancer stem cells[20]. However, the specific effects of gingerol on SHED have not been explored, and so the effect of this compound on SHED is still uncertain.

Deoxymiroestrol is a phytoestrogen typically derived from the tuberous roots of the *Pueraria candollei var. mirifica* plant, which grows in northern and north-eastern Thailand and Myanmar and can mimic estrogen in the body. While estrogen plays a role in maintaining bone density, specific studies on deoxymiroestrol's impact on bone development are still lacking. Even so, the presence of this component in DVA could be the driving factor that attributes to DVA's apparent ability to protect bones from estrogen deficiency[8].

Another interesting molecule that is present in the DVA water extract is (17Z) - 1α, 25 - dihydroxy - 26, 27 - dimethyl - 17, 20, 22, 22, 23, 23 - hexadehydrovitamin D3, which is a synthetic analog of vitamin D3[21]. This molecule or its analogs has been widely researched for their roles in bone health, immune regulation, and anticancer properties[22, 23]. Overall, each of these molecules has distinct chemical properties based on their functional groups, influencing their

reactivity, solubility, and biological activity. To summarize, although the compounds isolated from DVA are promising, targeted research on their effects on stem cells, including SHED, is still limited.

DVA water extract is non-cytotoxic and maintains comparable viability of SHED

Previous studies indicate that DVA is selectively cytotoxic to cancer cells and wounded cells while non-cytotoxic to healthy cells[2, 24].

From that particular study, the IC_{50} for cancer cells (glioblastoma cell-lines) is 1 mg/ml, while healthy cells (HACAT cells) demonstrated non-toxic effect up to the similar concentration[2].

In the current study, DVA of up to 200 µg/ml is not toxic to SHED as indicated by MTT and alamarBlue analyses. MTT results show that SHED maintains high viability with small DVA concentrations, where the smaller concentrations of DVA yielded higher percentages of viability, even exceeding 100%, although statistically insignificant $p>0.05$. Similarly, even though the viability of SHED decreased considerably from concentrations 6.25 µg/ml to 200 µg/ml, statistical analysis revealed these changes in viability to be insignificant as well. Additionally, MTT results show that SHED viability does not decrease below 50% or IC_{50}. Hence, all concentrations are still considered for downstream analysis to find an optimum concentration of DVA to use for further study.

Based on the MTT results indicating that a DVA of up to 200 µg/ml is not toxic to SHED and the continuous growth of SHED observed up to Day 6, along with having the smallest PDT, it was concluded that 3.125 µg/ml is the optimum concentration for SHED treatment according to this study's analyses. Also, important to note, the quality of SHED used in the current study might have influenced the overall results as an average PDT of SHED is reported to be around 40 – 60 hours [13,25], but this study found the control group SHED to have a significantly higher PDT of 86.74 ± 2.67 hours.

Multiple studies have utilized DVA water extract for their analysis[26–28] while others performed extractions using ethanol or methanol, among other methods[29].

Hung et al. (2021) analyzed the effects of velvet antler (VA) water extracts from different species of deer on anti-inflammatory activity and wound healing in vitro[26]. They also observed that VA water extract, at concentrations ranging from 125 to 500 µg/ml, was not cytotoxic towards CaCo-2 cells. However, in the case of wounds, VA extract could enhance the cell viability of CaCo-2 cells[26].

Therefore, it can be suggested that DVA extract does not possess cytotoxic effects on normal cell lines, including mesenchymal stem cells. Overall, it can be concluded that DVA water extract, at concentrations up to 200 µg/ml, is not cytotoxic to SHED, as demonstrated in the current study.

Conclusions

To the best of our knowledge, this is the first report to document the effects of DVA water extract on biological activity of SHED.

Our findings indicate that DVA water extract is non-toxic to SHED, supports cell proliferation, and preserves cellular morphology at concentrations up to 200 µg/ml. Although the extract did not significantly enhance proliferation; its slight stimulatory effect at the smaller concentration is promising, particularly for tissue regeneration research.

Notably, the presence of multiple bioactive compounds in DVA water extract might be responsible for the demonstrated observations. Interestingly, the presence of these compounds might suggest potential involvement of DVA in the overall process of bone developmental process; among others, further highlighting its therapeutic potential.

Acknowledgements

This study was supported by research grant from Ministry of Higher Education, Malaysia (FRGS/1/2021/SKK0/UIAM/02/12; FRGS21-233-0842).

The authors would like to acknowledge Regal Malay Capital Berhad for providing the DVA sample.

Declaration of Interest

All authors declared no conflict of interests.

No	Name	MFG Formula	MFG Diff (ppm)	DB formula	DB Diff (ppm)	Hits (DB)	Retention time (RT)	Mass	Mass-to-charge ratio (m/z)	Vol (%)
	Identified compounds with positive polarity ionization									
1.	16-hydroxy hexadecanoic acid	C16 H32 O3	5.03	C16 H32 O3	5.03	10	12.323	272.2338	290.2677	0.71
2.	4-Vinylcyclohexene diepoxide	C8 H12 O2	2.04	C8 H12 O2	2.04	10	16.52	140.0834	163.0726	3.2
3.	Deoxymiroestrol	C20 H22 O5	0.31	C20 H22 O5	0.31	7	16.523	342.1466	343.1521	5.34
4.	4-Hydroxy-6-methylpyran-2-one	C6 H6 O3	-5.26	C6 H6 O3	-5.26	9	17.001	126.0324	149.0215	2.02
5.	Bis(2,3-epoxycyclopentyl) ether	C10 H14 O3	-0.87	C10 H14 O3	-0.87	10	17.016	182.0945	205.0837	1.25
6.	3α,12α-Dihydroxy-5β-chol-8(14)-en-24-oic Acid	C24 H38 O4	-2.55	C24 H38 O4	-2.55	10	21.335	390.278	391.2852	1.81
7.	N-Hexadecanoyl pyrrolidine	C20 H39 N O	-0.96	C20 H39 N O	-0.96	8	21.45	309.3035	310.3108	3.62
	Identified compounds with negative polarity ionization									
8.	4-Hydroxyphenylglyoxylate	C8 H6 O4	0.07	C8 H6 O4	0.07	7	7.689	166.0266	165.0194	1.51
9.	Gingerol	C17 H26 O4	-0.79	C17 H26 O4	-2.68	7	13.305	294.1839	293.1766	3.43
10.	N-Undecyl benzenesulfonic acid	C17 H28 O3 S	-1.44	C17 H28 O3 S	-4.61	1	16.617	312.1774	311.1701	8.74
11.	(17Z)-1α,25-dihydroxy-26,27-dimethyl-17,20,22,22,23,23-hexadehydrovitamin D3	C29 H42 O3	-0.62	C29 H42 O3	-5.35	2	19.923	438.3157	473.2848	20.68

Table 1. The identified compounds from DVA water extract with positive polarity ionization (No.1-7) and negative polarity ionization (No. 8-11) identified with LC-MS/MS-QTOF.

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