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Original Research Article

Anti-endometrial Cancer Activity of *Hedyotis diffusa* Willd and its Phytochemicals by Experimental and *In Silico* Analysis

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ABSTRACT

Hedyotis diffusa Willd (HDW) injection is a clinically approved herbal medicine for various cancer therapies in China. This study aims to investigate HDW injection on endometrial cancer (EC), which is currently not an indication of HDW. Cytotoxicity and flow cytometric analysis were employed to study the inhibition and apoptosis of EC cells under various concentrations of HDW injection, cisplatin, and their combination. *In silico* docking simulations were used to predict the binding affinities between the phytochemicals of HDW and the EC therapeutic target, A_3 adenosine receptors (A_3ARs). FAF-Drugs 4 was used to study the drug-like properties of the phytochemicals. The HDW injection inhibited the growth and caused apoptosis of EC cells. HDW and cisplatin produced more potent inhibition effects than their individual use. The phytochemicals 5-demethylsinensetin, 5-demethylnobiletin and 5-hydroxy-6,7,3,4-quatermethoxyflavonoid obtained the highest docking score with acceptable drug-like properties for oral administration. The HDW injection and the *in silico* identified A_3ARs inhibitors have a high potential for further investigations to develop an effective and safe EC treatment.

Keywords: Cisplatin, Endometrial cancer, *Hedyotis diffusa* Willd, Molecular docking, Phytochemicals.

Introduction

Endometrial carcinoma (EC) is one of the three common malignancies in the female reproductive tract.[1] There are two main types of EC: type I is oestrogen-dependent and accounts for about 80% to 85% of cases;[2] type Ⅱ is non-oestrogen-dependent and mostly occurs in the atrophic endometrium, with poorly differentiated morphology. Nearly 75% of EC patients are menopausal women; however, EC has started to show a trend of increasing incidence at a younger age.[1] In general, the main treatment of EC is hysterectomy with adjuvant therapy, including radiotherapy, chemotherapy and endocrine therapy. Hysterectomies have some potential risks, especially for younger patients, such as sterilization, higher risk of congestive heart failure, coronary artery disease and neuropathy.[3] Thus, instead of hysterectomy, alternative therapies, such as chemotherapy, have become more popular for young patients. The main chemotherapy drugs are cisplatin, carboplatin, doxorubicin and paclitaxel.[4]

Chemotherapy drugs have significant effects in the treatment of EC, but the side effects are generally severe and involve multiple systems, such as the integumentary system, digestive system and circulatory system.[4] Symptoms include chills, high fever, anaphylactic shock, dizziness, nausea and vomiting, abdominal pain, diarrhoea, skin rash, itching, hand-foot syndrome, cardiotoxicity, phlebitis, high blood pressure, dyspnoea, chest tightness, bone marrow suppression, etc.[4]

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These adverse reactions seriously affect the patient's quality of life and long-term treatment compliance.[5] Thus, scientists have been seeking methods to reduce these adverse effects. Studies have confirmed that the combined use of certain herbal medicines and chemotherapeutics can effectively reduce the required dose of the chemotherapeutics and thus reduce their adverse effects.[6,7] Combined use may also reduce the chance of metastasis and recurrence, and improve immunity.[8] Various pharmacological mechanisms of herbal medicines for cancer treatment have been found. They may damage the DNA of cancer cells, block the cell cycle, inhibit tumour angiogenesis, inhibit tumour cell autophagy and cause cancer cell apoptosis. [8]

Hedyotis diffusa Willd (HDW) is one of the herbal medicines that has been clinically used to treat cancers in China.[9] Apart from its anti-tumour effects, HDW also has anti-bacterial, anti-inflammatory and immune-enhancing properties.[10] These properties are produced by different phytochemicals of HDW. The anti-tumour effects of HDW are mainly caused by flavonoids, anthraquinones, terpenes, sterols, polysaccharides, organic acids, alkaloids and volatile oils.[11] HDW can prevent the formation of cancer cells, inhibit cancer cell growth and prevent metastasis through a variety of pharmacological mechanisms, including the regulation of the immune system, inhibition of tumour angiogenesis and lymphangiogenesis, inducing tumour cell apoptosis, regulating cancer-related signal pathways and anti-oxidation.[12–14] Studies have revealed that the use of HDW with chemotherapy agents may help to reduce toxicity and increase the efficacy of chemotherapy, thus improving the quality of life of the patient.[15,16] HDW can inhibit the growth of cervical cancer, ovarian cancer, stomach cancer, breast cancer, colon cancer, liver cancer, leukaemia and lung cancer.[17,18] However, no studies could be found on its effects on EC.

In silico studies have been performed to identify the phytochemicals of HDW and their corresponding anti-tumour targets. For example, Song *et al*. found that 14 compounds of HDW can inhibit or induce various anti-prostate cancer-related targets, including IL1B (Interleukin-1 Beta), IL6 (Interleukin 6), CREB1 (CAMP Responsive Element Binding Protein 1), VEGFA (Vascular Endothelial Growth Factor A), MAPK8 (Mitogen-activated Protein Kinase 8) and more.[19] Liu *et al.* found that the anti-colorectal cancer effects of HDW were caused by affecting the PIK3CA (Phosphatidylinositol-4,5-

Bisphosphate 3-Kinase Catalytic Subunit Alpha), GSK3B (Glycogen synthase kinase 3 beta) and AKT1 (AKT serine/threonine kinase 1) targets, among others.[20] Some of these targets, such as VEGFA, PIK3CA and AKT1, were considered as clinical actionable molecular targets of EC.[21] Thus, we believe the phytochemicals of HDW can also inhibit the growth of EC.

A_3 adenosine receptors (A_3ARs) are a recently identified target for EC.[22] One of the popular phytochemicals that has been proven to induce the activities of A_3ARs and produce anti-cancer effects is cordycepin.[23] This is a phytochemical of *Cordyceps sinensis* and has been used clinically as an anti-inflammatory and anti-cancer agent in traditional Chinese medicine (TCM).[24] Stimulation of A_3ARs can interrupt different tumour cell proliferation pathways, including that of PKA (cAMP-activated protein kinases), MAPK (mitogen-activated protein kinase), VEGF (vascular endothelial growth factor) and more.[25] Activation of A_3ARs can also reduce the activities of telomerase, which is an important factor in cancer metastasis.[26] Thus, we believe the identification of the phytochemicals that can affect A_3ARs can help to develop an EC treatment.

This study investigated the effects of clinically approved HDW injection in EC cells using cytotoxicity MTT assays and flow cytometry. After confirming the *in vitro* anti-EC properties of HDW, *in silico* methods were used to predict the active phytochemicals of the HDW injection that have a high binding affinity with A_3ARs and have favourable pharmacological and pharmacokinetic properties.

Materials and Methods

Cell Culture

An Ishikawa cell line (ATCC, Manassas, VA, USA) was cultured in a humidified 5% CO_2 atmosphere at 37°C in RPMI-1640 (Gibco, 11875, Waltham, MA) supplemented with 10% foetal bovine serum (Gibco, 16000044).

Cytotoxic Assay

An MTT (tetrazolium) assay was used to assess the cytotoxic activity of the HDW injection and cell viability. The MTT assays were performed on 4×10^3 Ishikawa cells seeded in 96-well plates exposed to different concentrations of cisplatin (P4394; Sigma-Aldrich), HDW injection (Z34020595, Keyuan Pharmaceutical Industry, China) and their combination (Table 1) for 24 hours. Cisplatin and a blank group were used as the positive and negative control group, respectively. Phosphate buffer saline (PBS) was used to wash the cells after exposure. MTT (1 mg/mL) solutions were then added to the cells for 4 hours incubation at 37°C in a humidified 5% CO_2 atmosphere. After incubation, the culture media was removed, 150 µL of DMSO (D4540; Sigma-Aldrich) was added and the solution was shaken for 15 minutes to dissolve the formazan crystals. A microplate reader (SPECTROstar Nano; Ortenberg, Allmendgrün, Germany) was used to measure the optical density (OD) of the solution at 550 nm. The percentage of viability and inhibition was calculated using the following equations:[27]

$$Percentage\ of\ viability = \frac{mean\ of\ test\ OD}{mean\ of\ control\ OD} \times 100\%$$

$$Percentage\ of\ inhibition = 100 - percentage\ of\ viability$$

Apoptosis detection

Apoptosis was detected using flow cytometry upon staining the cells with annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI). The experimental protocol started by seeding 1×10^6 Ishikawa cells in 35-mm culture dishes for 1 night. Different concentrations of cisplatin, HDW injection and their combination were added to the cells and left for 24 hours. The cells were harvested, resuspended, washed with PBS and centrifuged for 3 minutes. Then, 500 µL of the binding buffer of the apoptosis kit (556547; BD Biosciences, San Diego, CA, USA) was used to resuspend the cells, followed by the addition of 5 µL of PI and 5 µL of annexin V-FITC to 100 µL. The cell suspension was left at room temperature for 15 minutes for staining. Cellular apoptosis was then analysed using an Applied Biosystems Attune flow cytometer (Waltham, MA).

In silico molecular docking

Molecular docking simulations can predict the binding modes and affinities between protein-ligand interactions. This technique has been used in various drug development studies to identify chemicals that can bind to a target protein for pharmacological activity.[28] Here, molecular docking was used to predict the active phytochemicals of HDW that may have anti-EC effects through binding to the A_3ARs. Overall, 76 phytochemicals of HDW were found from the literature.[11,13,17] Their computational molecular structures were created and optimised using the program GOLD suite v5.5 of the Cambridge structural database system (CSDS).[29] As no X-ray structure of human A_3ARs was available, the homology model template (PDB code: 2YDO), obtained from the Adenosil and platform (http://mms.dsfarm.unipd.it/Adenosiland), was used.[30] The ChemPLP scoring functions of the GOLD suite v5.5 was used to calculate the binding scores between the 76 phytochemicals and A_3ARs. A high score value indicates that the phytochemical has a high binding affinity to A_3ARs and, thus, a high chance of producing a pharmacological effect. In this study, the docking score of the A_3ARs binding ligand (adenosine) in the X-ray structure (PDB code: 2YDO) was used as a positive comparison. The docking methods of this study were the same as that of our previous study and details of the docking procedure and the validation of the docking methods are documented in this previous study,[22] which evaluated the accuracy of the methods using a receiver operating characteristic (ROC) analysis with a dataset of 13,087 compounds. Our docking methods achieved an area under the curve (AUC) value of 0.85, which indicates the high sensitivity and specificity of its predictive power.

Prediction of drug-like properties

Absorption, distribution, metabolism, excretion and toxicity (ADME-tox) are important factors that may affect the clinical efficacy and risk of side effects of a medicine. Computational ADME-tox predictions have become a routine procedure in drug discovery, and many software have been developed for this purpose.[31] Here, the FAF-Drugs 4[32] ADME-tox filtering platform was employed to screen the drug-like properties of the 76 phytochemicals. This study used the FAF-Drugs 4 in house pre-built filters, 'drug-like soft', to define the thresholds of the physicochemical properties that a 'drug-like' molecule should have. For example, the thresholds of log P were -3 to 6, the thresholds of the ratio between the number of carbon atoms and non-carbon atoms were 0.1 to 1.1, and the thresholds of the total charge of a molecule was -4 to 4. Details of the thresholds of the physicochemical properties can be found on the FAF-Drugs4 webpage (https://fafdrugs4.rpbs.univ-paris-diderot.fr/filters.html). The phytochemicals that met these thresholds were considered to have acceptable drug-like properties for oral administration. This study also used the Veber[33] and the Egan[34] rules to access the oral bioavailability of the phytochemicals. The water solubility was estimated by the ESOL method.[35] Both the GSK 4/400[36] and Pfizer 3/75[37] rules were also employed to screen the phytochemicals with favourable ADME-tox properties.

Statistical analysis

Statistical Product and Service Solutions (SPSS) software (version 26.0) and Microsoft Office Excel 2013 were used to analyse the results of this study. The data are stated as the mean ± standard deviation, and a *p*-value of less than 0.05 was considered as statistically significant. One-way ANOVA was used to assess the significance of the differences between 3 or more groups of the apoptosis and the cytotoxic assay. A 2-tailed t-test was used for the analysis of 2 groups.

Results and Discussion

Cytotoxic assays

To explore the effect of HDW on the proliferation of human EC, Ishikawa cells were treated with the HDW injection, cisplatin and their combination at different concentrations (Table 1). After 24 hours of incubation, proliferation of the cells was detected by the MTT method. The results showed that HDW injection at a concentration of 50

μL/mL or higher had an inhibitory effect on the Ishikawa cells, and this effect was dose-dependent (p<0.01). The inhibition rate increased from 7.2% at 50 μL/mL to 96.3% at 200 μL/mL (Table 1). The inhibition rate of cisplatin was also dose-dependent, ranging from 11.5% at 5 μg/mL to 86.4% at 40 μg/mL. The inhibition rate of cisplatin at 40 μg/mL is comparable with the HDW concentration between 150 μL/mL and 20 μL/mL.

The combination of HDW and cisplatin can significantly inhibit the proliferation of Ishikawa cells in a dose-dependent manner (Table 1). The inhibition rate of the combinations was significantly higher than that of the two when administered separately ($p < 0.05$) (Table 1). For instance, the inhibition rate of the low concentration (50 μL/mL) of HDW was 7.2% and that of cisplatin (5 μg/mL) was 11.5%, but the inhibition rate increased to 11.5% when the two were combined. The combination of HDW and cisplatin at 50 μL/mL and 20 μg/mL, respectively, produced an inhibition effect that was comparable with cisplatin alone at 40 μg/mL. The stronger inhibition effect of the combination was also supported by their corresponding IC_{50} values. The combinations obtained lower IC_{50} values than the cisplatin or the HDW alone (Table 2). This suggests that HDW is a potential adjunctive agent for cisplatin, aiming to lower the dose of cisplatin and thus reduce its side effects.

Apoptosis detection

Flow cytometry was used to detect the apoptosis of the Ishikawa cells stained with Annexin V-FITC/PI, caused by HDW, cisplatin and their combination. The results indicated that HDW at ≥50 μL/mL and cisplatin at ≥5 μg/mL caused cellular apoptosis. The total rate of apoptosis caused by 5 μg/mL of cisplatin was 10.0%, while combining it with 50 μL/mL of HDW increased the rate to 17.6% (Figure 1).

Table 1: Inhibition rates on Ishikawa cells by *H. diffusa* Willd, cisplatin and their combination

Compounds (concentration)	Inhibition rate
Cisplatin (5 μg/mL)	11.5% ± 0.8%
Cisplatin (10 μg/mL)	18.1% ± 4.2%
Cisplatin (20 μg/mL)	68.4% ± 9.3%
Cisplatin (40 μg/mL)	86.4% ± 3.6%
H. diffusaWilld (50 μL/mL)	7.2% ± 0.2%
H. diffusaWilld (100 μL/mL)	26.6% ± 4.9%
H. diffusaWilld (150 μL/mL)	77.5% ± 4.8%
H. diffusaWilld (200 μL/mL)	96.3% ± 0.9%
H. diffusaWilld (25μl/ml) plus cisplatin (5 μg/mL)	18.8% ± 3.5%
H. diffusa Willd (25 μL/mL) plus cisplatin (10 μg/mL)	27.9% ± 0.7%
H. diffusa Willd (25 μL/mL) plus cisplatin (20 μg/mL)	75.0% ± 0.6%
H. diffusa Willd (50 μL/mL) plus cisplatin (5 μg/mL)	26.5% ± 1.9%
H. diffusaWilld (50 μL/mL) plus cisplatin (10 μg/mL)	47.9% ± 0.9%
H. diffusaWilld (50 μL/mL) plus cisplatin (20 μg/mL)	81.6% ± 0.4%

The rate was doubled for the combination of cisplatin (20 μg/mL) and HDW (50 μL/mL) compared with 20 μg/mL of cisplatin alone (Table 2).

Both the MTT assay and the flow cytometry experiments proved the ability of HDW to inhibit the survival of EC cells. More importantly, the combination of HDW and cisplatin was shown to produce more potent anti-cancer effects than when used alone. Cisplatin is an effective treatment of EC: its pharmacological mechanism of action is the formation of crosslinks between DNA pairs, thus preventing DNA repair and causing cell apoptosis. However, this action does not only affect cancer cells but also healthy cells that have a high reproductive rate, such as liver, hair and stomach cells. This causes various adverse effects that are unacceptable to some patients, such as alopecia, severe nausea and vomiting, hepatotoxicity, nephrotoxicity and neurotoxicity.[38] The severity of these adverse effects is dose-dependent. This study shows that the use of HDW with cisplatin may reduce its required dose and, thus, may reduce the risk of these side effects.[39]

Chemotherapy resistance is another limitation of using cisplatin. Certain cancer cells have developed resistance mechanisms to various chemotherapy agents, such as efflux pumps, the nucleotide excision repair process, mismatch repair and homologous recombination.[40] For cisplatin, certain cancer cells have developed mechanisms to repair DNA, reduce the intracellular accumulation and inactivate cisplatin.[38] Studies have demonstrated that the use of chemotherapy agents with an adjuvant that has some degree of anti-cancer properties, such as a natural product, can prevent the development of drug resistance.[41] This study revealed the anti-cancer properties of HDW and the possibility of using it as an adjuvant with chemotherapy agents to overcome resistance.

In silico molecular docking and the prediction of drug-like properties

Docking was performed on adenosine and the 76 phytochemicals of HDW to the A_3ARs to predict their binding affinities. The docking score of the natural substrate of A_3ARs, adenosine, was considered to be a positive control, with a score of 63.6. Out of the 76 phytochemicals, 31 achieved a higher binding score than adenosine. This indicates that the anti-endometrial property of HDW may be caused by a 'pool' of phytochemicals that affect A_3ARs. Among these chemicals, daucosterol obtained the highest binding score of 131.24, followed by amentoflavone, 7α-hydroxystigmasterol, stigmasterol, *β*-sitosterol and rutin. Table 3 shows the phytochemicals with the top 20 docking scores and their corresponding drug-like property predictions. Among them, only 4 have adequate physicochemical properties that meet the thresholds of the 'drug-like soft' filters and were considered as acceptable molecules for oral administration. They were 5-demethylsinensetin, 5-demethylnobiletin, diisobutyl phthalate and 5-hydroxy-6,7,3,4-quatermethoxyflavonoid. The high rejection rate was mainly due to an inadequate Log P, the number of rotatable bonds and the number of hydrogen bond acceptors. The rejected molecules are considered here as not suitable for use as oral medication, but other routes of administration, such as injection and vaginal routes, may be appropriate.

Daucosterol was reported to inhibit breast, prostate, colon and liver cancer cell growth by inducing apoptosis via the inactivation of different signalling pathways, including the PI3K/Akt and Wnt/β-catenin pathway.[42–44] It can also suppress cancer survival-related factors, such as VEGF, matrix metalloproteinase-2 (MMP 2), and matrix metalloproteinase-2 (MMP 9).[45] This docking study predicted that daucosterol may also affect A_3ARs. Daucosterol is a bioactive phytochemical of *cordyceps*,[46] which can induce A_3ARs and suppress melanoma, colon carcinoma, fibrosarcoma and lung carcinoma cells.[47] Thus, there is a chance that the A_3AR agonist effects may be partly caused by daucosterol. However, daucosterol violated two of the Lipinski rule-of-five and was thus rejected by the FAF-Drugs 'drug-like soft' filters.

The anticancer effects of amentoflavone are well-documented: it inhibited breast, ovarian, lung and bladder cancer cells.[48] Although no literature can be found on the effect of amentoflavone on EC cells, one of its structurally similar derivatives, ginkgetin (Figure 2) suppressed the growth of Ishikawa EC cells.[38]

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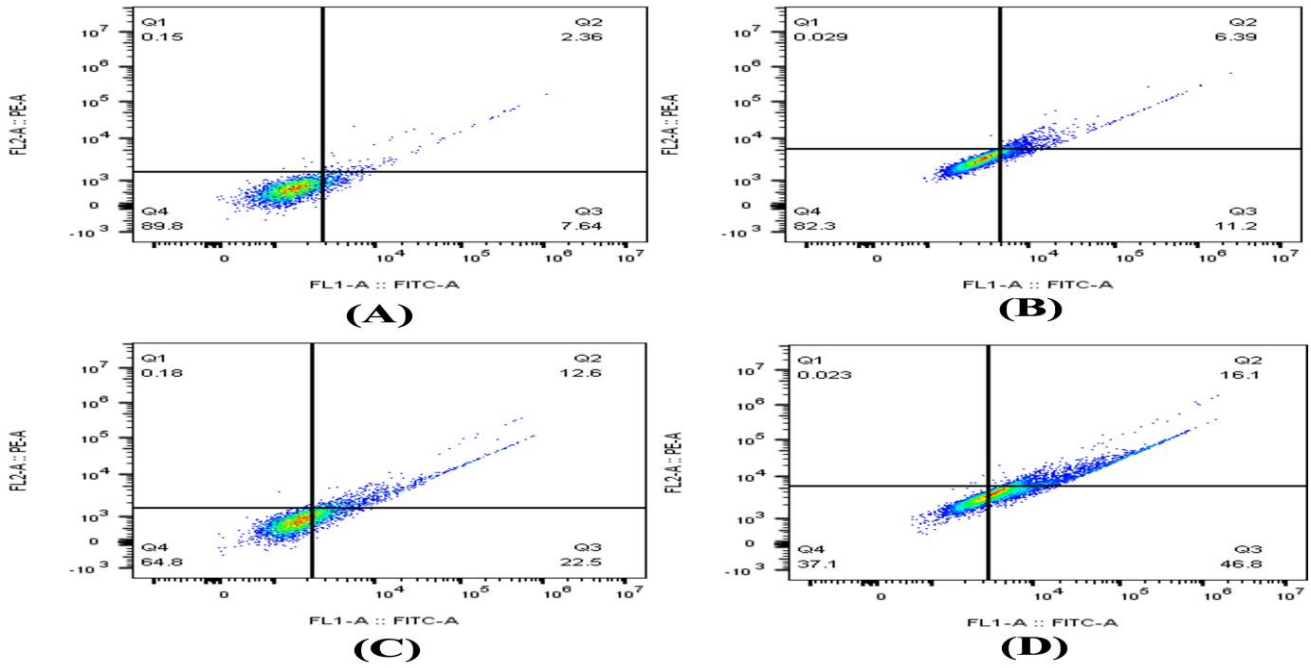

Figure 1: Total rate of cellular apoptosis of Ishikawa cells caused by (A) 5 µg/mL of cisplatin was 10.0%, (B) 5 µg/mL of cisplatin + 50 µL/mL HDW was 17.6%, (C) 20 µg/mL of cisplatin was 34.1% and (D) 20 µg/mL of cisplatin + 50 µL/mL HDW was 62.9%.

The suggested pharmacological mechanism was the suppression of phosphoprotein kinase B, Janus kinase 1, and signal transducers and activators of transcription 3.[49] According to the authors' knowledge, this docking study is the first to suggest the effects of amentoflavone on A_3ARs. However, amentoflavone was also rejected by the 'drug-like soft' filters for use as an oral medication.

The phytochemicals with the third and fourth highest docking scores were 7α-hydroxystigmasterol and stigmasterol. Their structures were very similar (Figure 2), and both of them have documented anti-cancer properties on ovarian cancer and gastric cancer cells.[50,51] A recent study found the inhibition property of stigmasterol on the Nrf2 signal pathway.[52] This pathway was found to be overexpressed in EC cells that were resistant to chemotherapy drugs, such as cisplatin. The study concluded that stigmasterol could overcome chemoresistance in EC therapy. The structure of stigmasterol is very similar to that of 7α-hydroxystigmasterol (Figure 2); thus, it may also inhibit the Nrf2 signal pathway, as both of these are phytochemicals of HDW, which may also be used as an adjuvant therapy to reduce the chemoresistance of EC therapy. Many of the other phytochemicals of HDW listed in Table 3 also have documented anti-cancer properties through various pharmacological mechanisms. The fifth-ranked phytochemical, *β*-sitosterol, suppressed the growth of uterine cervix cancer cells by inhibiting microtubule formation during mitosis.[53]Rutin has shown anti-cancer properties on various cancer cells and may also reduce the adverse effects of chemotherapy.[54] Trimethylpentadecan-2-one is a phytochemical of *Pterocephalus nestorianus* that has shown anti-proliferative effects on six different human cancer cell lines.[55] Lupeol acetate is a dietary triterpene that inhibited lung, liver and skin cancer cells in both *in vitro* and animal studies through modulating the NF-κ B and PI3K/Akt pathways.[56,57]Hexadecanoic acid is a hydroxylated fatty acid that has recently shown its ability in causing apoptosis on breast, cervical and colon cancer cells.[58,59]

5-demethylsinensetin obtained the highest docking score among the phytochemicals with acceptable drug-like properties for oral administration (Table 3). It is predicted to have good oral bioavailability, and its physicochemical properties were within all the thresholds of the FAF-Drugs4 'drug-like soft' filters. 5-demethylsinensetin is a derivative of sinensetin (Figure 2), which has shown anti-cancer properties on gastric, breast and liver cancer cells by suppressing p53-related AMPK/mTOR signalling.[60–62]*In vitro* studies concluded the promising efficacy and minimal toxicity of sinensetin[63] and also found a high binding affinity between sinensetin and A_3Ars.[64] Thus, the authors believe that 5-demethylsinensetin is worthy of further investigation as an oral anti-cancer therapy.

5-demethylnobiletin is another well-studied phytochemical that obtained a high docking score and acceptable drug-like properties in this study (Table 3). It was found to inhibit lung cancer cells by inducing JNK (Jun N-terminal kinases) activation and G2/M cell cycle phase arrest, causing apoptosis.[65] No literature was found to suggest any relationship between 5-demethylnobiletin and A_3ARs; thus, this study is the first to indicate that 5-demethylnobiletin can bind to A_3ARs.

Diisobutyl phthalate is another phytochemical with a high binding score against A_3ARs and with acceptable drug-like properties. However, its effect on EC cells is controversial. Diisobutyl phthalate is generally considered to be an endocrine-disrupting chemical.[66] This means that it can affect human reproductive hormonal activities. Diisobutyl phthalate has been found to induce oestrogen receptors and stimulate the growth of oestrogen-dependent cancers, including ovarian and breast cancer.[67] Overall, 80% of ECs were caused by the overexpression of oestrogen; thus, diisobutyl phthalate may potentially cause EC.[68] On the other hand, diisobutyl phthalate has also been found to have anti-androgenic effects[69] and overexpression of androgen was noticed in 93% of EC patients.[70] This study predicts that diisobutyl phthalate may down regulate the expression of androgens by binding to A_3ARs.

Table 2: IC_{50} values for *H. diffusa* Willd, cisplatin and their combination on Ishikawa cells

Compounds (concentration)	IC_{50}
H. diffusa Willd	126.6µl/ml
Cisplatin	15.8µg/ml
Cisplatin + *H. diffusaWilld* (12.5µg/ml)	12.3µg/ml
Cisplatin + *H. diffusaWilld* (25µg/ml)	9.7µg/ml

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Table 3: Drug-like properties of the *H. Diffusa* Willd components with the top 20 docking scores agonists against A_3Ars

Component[1]	Docking score	LogP	MW	Rotatable bonds	Aqueous solubility	Lipinski	Oral Bioavailability	Prediction[2]
1	131.24	7.74	576.85	9	261.56	2	Good	Rejected
2	116.60	5.04	538.46	3	759.42	3	Good	Rejected
3	115.36	7.45	428.69	5	449.04	1	Good	Rejected
4	113.88	8.56	412.69	5	237.22	1	Good	Rejected
5	113.02	9.34	414.71	6	153.84	1	Good	Rejected
6	96.50	-1.29	610.52	6	46321.52	3	Good	Rejected
7	90.09	6.37	318.49	15	2523.99	1	Good	Rejected
8	88.34	-1.15	550.51	10	79665.88	3	Low	Rejected
9	87.55	3.72	358.34	5	4650.97	0	Good	Accepted
10	86.41	-0.82	564.54	11	65158.18	2	Low	Rejected
11	85.83	7.15	280.45	14	1611.42	1	Good	Rejected
12	85.22	3.23	388.37	6	6224.57	0	Good	Accepted
13	80.30	-1.15	550.51	10	79665.88	3	Low	Rejected
14	79.74	-1.15	550.51	10	79665.88	3	Low	Rejected
15	78.37	6.95	268.48	12	1651.54	1	Good	Rejected
16	78.32	4.11	278.34	8	6659.95	0	Good	Accepted
17	75.44	-0.61	564.54	11	57083.66	2	Low	Rejected
18	75.25	10.45	468.75	3	50.71	1	Good	Rejected
19	72.49	0.51	304.25	1	36804.01	0	Good	Accepted
20	72.38	7.17	256.42	14	1688.61	1	Good	Rejected

[1]Component 1=Daucosterol; 2=Amentoflavone; 3=7α-Hydroxystigmasterol; 4=Stigmasterol; 5=β-sitosterol; 6=Rutin; 7=ZINC3384216; 8=6-O-E-(p-coumaroyl)scandoside methyl ester; 9=5-demethylsinensetin; 10=5-O-p-(methoxycinnamoyl)scandoside methyl ester; 11=(2E,9E)-Octadeca-2,9-dienoic acid; 12=Demethylnobiletin; 13=6-O-Z-(p-coumaroyl)scandoside methyl ester; 14=(E)-6-O-(p-coumaroyl)scandoside methyl ester; 15=6,10,14-Trimethylpentadecan-2-one; 16=Diisobutyl phthalate; 17=(E)-6-O-(p-coumaroyl)scandoside methylester-10-methylether; 18=Lupeol acetate; 19=5-Hydroxy-6,7,3,4-quatermethoxyflavonoid; 20=hexadecanoic acid.

[2]Prediction indicates whether the components met the thresholds of the FAF-Drugs 4 in house pre-built filters, 'drug-like soft'in predicting acceptable drug-like properties.Details of the thresholds can be found on the FAF-Drugs4 webpage (https://fafdrugs4.rpbs.univ-paris-diderot.fr/filters.html).

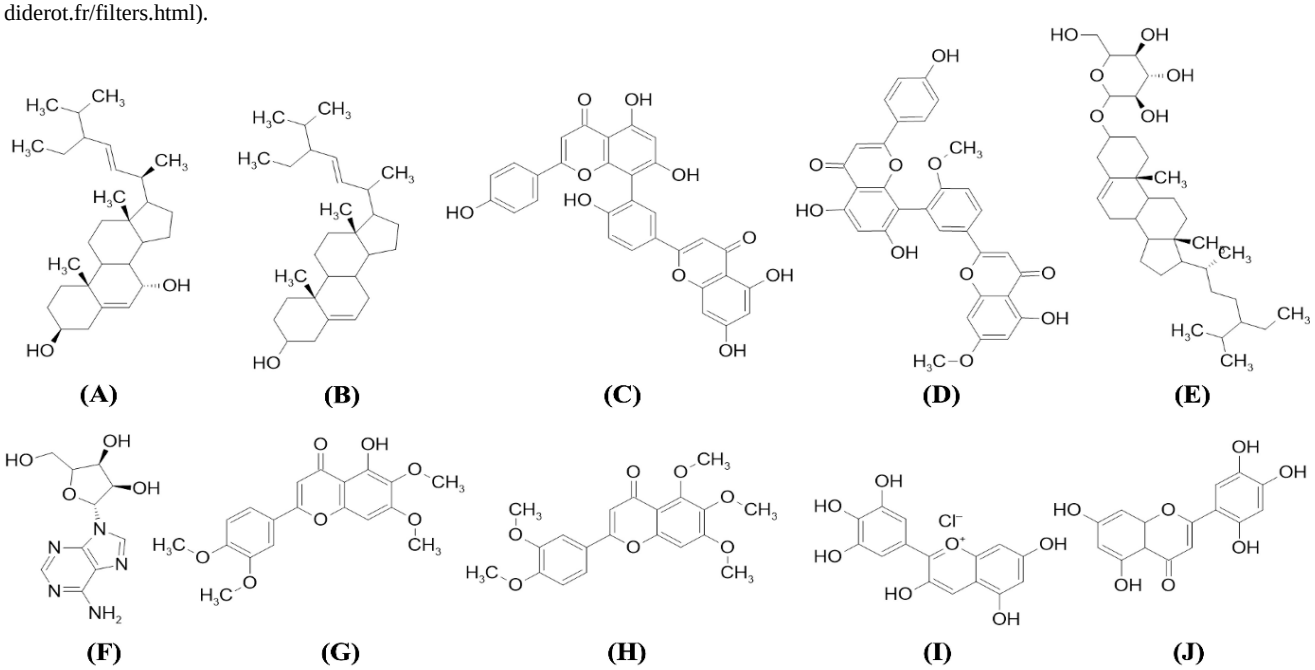

Figure 2: Structures of (A) 7α-hydroxystigmasterol, (B) stigmasterol, (C) amentoflavone, (D) ginkgetin, (E) daucosterol, (F) adenosine, (G) 5-demethylsinensetin, (H) sinensetin, (I) delfinidin and (J) 5-hydroxy-6,7,3,4-quatermethoxyflavonoid.

Another phytochemical of HDW with a high docking score and acceptable drug-like properties is 5-hydroxy-6,7,3,4-quatermethoxyflavonoid (Table 3). Although no literature can be found on its effect on cancer, its structurally similar compound, delfinidin (Figure 2), demonstrated *in vitro* and *in vivo* anti-cancer activities on breast, prostate and lung cancer cells through various mechanisms, including the suppression of VEGF and MMP 9, and the inhibition of angiogenesis.[71,72] Due to the high structural similarity between these two phytochemicals, 5-hydroxy-6,7,3,4-quatermethoxyflavonoid may also have anti-cancer properties similar to delfinidin. This study suggests that it may also affect A_3ARs, leading to apoptosis of EC cells.

Conclusion

The results of this study support future clinical studies on the extended use of HDW injection on EC by demonstrating that HDW can inhibit the growth of EC cells and cause apoptosis. The combined use of HDW and cisplatin produced more potent anti-cancer effects than their individual use. Thus, using HDW may reduce the required dose of cisplatin and minimise the risk of side effects. HDW may also prevent the development of chemotherapy resistance. HDW contains various phytochemicals that have anti-cancer properties proven by other studies in the literature. Findings from this study suggest that the combined effects of these phytochemicals caused the anti-EC property of HDW. . This study predicted that some of these phytochemicals have high binding affinities to A_3ARs, a therapeutic target for various types of cancer. However, some of these compounds, such as diisobutyl phthalate, may be a potential isolation artefact and this is a limitation of this study. HDW is mainly used clinically as an injection, which could be due to the low oral bioavailability of many of its phytochemicals. This study predicted four phytochemicals of HDW that have both a high binding affinity to A_3ARs and good oral bioavailability.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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