



Research article

Cytotoxic effect of selected wild orchids on two different human cancer cell lines



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ABSTRACT

Majority of the orchid species are used in the traditional medicines for the treatment of several diseases. They are the sources of polysaccharides, phenanthrenes, bibenzyl derivatives, reveralter, stilbenoids and polyphenol compounds. This study explored the cytotoxic activity of seven wild orchid species and identification of medicinally active compounds. The extracts of orchid species were screened for cytotoxic effect on the human cervical cancer cells (HeLa) and human glioblastoma cells (U251) using an MTT assay. The medicinally active compounds of high cytotoxic extracts were identified by GC-MS resulting in many stilbenoids and phenolic derivatives. The extract of *Dendrobium transparens* (DTs) and *Vanda cristata* (VCw) showed high cytotoxic effect towards the HeLa and U251 cell lines (IC₅₀ of DTs: 382.14 µg/ml and 75.84 µg/ml respectively and IC₅₀ of VCw: 317.23 µg/ml and 163.66 µg/ml respectively). This study concludes that they could be used as cancer therapeutics.

1. Introduction

More people than ever die from cancer. After cardiovascular diseases, cancer is the second biggest health problem accounting for an estimated 9.56 million deaths worldwide in 2017. The number of cancer deaths increased between 1990 and 2017 by 66%. For example in the same year, 259,671 deaths worldwide accounted for cervical cancer and 247,143 deaths accounted for brain and nervous system cancers, respectively (Stanaway et al., 2018). In 2019, 1,762,450 new cancer cases and 606,880 cancer deaths are projected to occur in the United States (Siegel et al., 2019). Worldwide, one in five men and one in six women develop cancer during their lifetimes. The five-year-prevalence figure is estimated to be 43.8 million cases globally and is expected to rise by 22 million annually within the next two decades (Bray et al., 2018). Treatment of cancer currently includes the surgical removal of cancerous tissue, radiotherapy, chemotherapy, and a combination of chemotherapy and target therapy. The use of anticancer drugs (chemotherapy), while often more beneficial when used in conjugation with radiation therapy or

surgery, and are nonetheless a key line of treatment. Because cytotoxic drugs are the mainstay of chemotherapy, it is important to discover novel cytotoxic agents with diverse activity, a novel mechanism of action and minimal issues of toxicity (Guilbaud et al., 2001; Moraes et al., 2017; Greenwell and Rahman, 2015). Several plant-derived anticancer drugs are currently used in the treatment of cancer. They include vincristine, vinblastine, etoposide, teniposide, paclitaxel (taxol), docetaxel (taxotere), camptothecin, homoharringtonine and elliptinium (Cragg and Newman, 2013; Oberlies and Kroll, 2004; Gali-Muhtasib et al., 2015; Roy et al., 2018). However, cancer cells are capable of developing resistance to the drugs used in chemotherapy. This fact, fact coupled with the increase in the cancer-related death rate has resulted in an ever-increasing demand for new anti-cancer entities (Newman, 2008).

Medicinal orchids have been traditionally used in folk medicine for centuries as natural healing remedies with significant proven therapeutic effects (Singh et al., 2012; Pant and Raskoti, 2013). A large number of phytochemicals, including alkaloids, bibenzyl derivatives, flavonoids, and phenanthrenes, have been found in medicinal orchids (Gutierrez,

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2010; Pant, 2013). Their presence means that they can play a significant role in expressing different biological activities, including anti-microbial, anti-tumour, anti-inflammatory, anti-viral activities. Several orchid species have been studied, and a number of compounds which were isolated have been found to express cytotoxic activity (Singh et al., 2012; Newman, 2008; Attri, 2016; Paudel et al., 2018). It is becoming increasingly clear that the beneficial effects of medicinal orchids are due to a complex interplay of the compounds present in the whole plant rather than to any single constituent operating on its own (Dias et al., 2012; Harvey, 2008; Koehn and Carter, 2005). Chand et al. (2016) previously reported the antioxidant properties of the selected seven wild orchids of this study. Medicinal orchids exhibiting the profile of novel therapeutic and pharmaceutical compounds matching the demand for structurally diverse and unique novel small molecule entities, ultimately guiding lead discovery toward new drugs candidate (de la Torre and Albericio, 2020). The chances of obtaining active traditional medicine from orchids, which have been used to treat various diseases have been known for the past centuries. Thus, it is of great importance to document and screen them (Newman et al., 2000, 2003; Cragg and Newman, 2013). A lot of compounds that have been isolated from orchids demonstrated significant anticancer activities. For example, moscatilin, denbinobin, erianin, dendrochrysanene, fimbriatone, and cirrohopetalanthrin have been isolated from various orchids (Na et al., 1998; Heo et al., 2007; Peng et al., 2007; Xia et al., 2005; Wu et al., 2006; Chen et al., 2007; Attri, 2016). The assessment of the cytotoxic potential of medicinal orchids serve as the baseline for the chemical identification of active molecules may be used as anticancer compounds which in turn could be chemically manipulated into effective anticancer drugs. These drugs could retard the growth of cancer cells and even cure cancer patients. Their creation would contribute positively to the economy of the country that produced them and to the world at large. Obtaining anticancer compounds from orchids should help reduce the unpleasant side effects associated with current cancer treatment methods as natural and semi-synthetic products are considered safer than synthetic drugs (Tripathi and Singh, 2015). They could also be included in primary health care, as encouraged by the WHO (Shoeb, 2006). Several natural products of plant origin are currently being subjected to clinical testing, but the search for new products remains of utmost importance as diseases, including cancer, are constantly developing resistance to existing drugs. This research highlight the screening of extracts of wild orchids for their cytotoxic effect toward the cancer cell lines which could help to promote the implementation of these therapeutically important medicinal orchids.

2. Materials and methods

2.1. Collection of plant materials

The different parts of seven wild orchids: pseudobulbs of *Eria graminifolia* (EGp) and *Otochilus albus* (OAp), stem of *Dendrobium transparens* (DTs), leaves of *Pholidota articulata* (PAL) as well as its pseudobulb (PAP), whole plant of *Gastrochilus distichus* (GDw) and *Papilionanthe uniflora* (PUw) and *Vanda cristata* (VCw) were collected from central Nepal between April and August 2016. Plants were identified by Asst. Prof. Dr. Mukti Ram Paudel and Prof. Dr. Bijaya Pant of Central Department of Botany, Tribhuvan University. The identities of these plants were confirmed concerning the literature, taxonomists and specimens in the Tribhuvan University Central Herbarium (TUCH), and voucher specimens were deposited at TUCH.

2.2. Preparation of extracts

The plant materials were air-dried in shade and then grounded to make powder. The powder was extracted in a sonicator using methanol in the ratio of 1:10 of weight/volume (w/v). The methanol is used as a solvent due to its low, therefore mild, boiling point and other favourable solvent properties applicable to secondary plant compounds. The solvent

was evaporated under reduced pressure using a rotary evaporator and the crude extracts were kept at 4 °C for further biological in vitro test.

2.3. Cytotoxic effect of extracts

The cytotoxic activity of the extracts was evaluated by using a standard MTT (3-[4, 5-dimethylthiazole-2-yl]-2, 5-diphenyl-tetrazolium bromide) colourimetric assay with a slight modification. Human cervical cancer (HeLa) and glioblastoma (U251) cells were cultured in EMEM medium supplemented with 10% FBS, 1% penicillin/streptomycin and 1% L-glutamine and incubated in 5% CO₂ supplemented incubator at 37 °C (Mosmann, 1983). The cells in 100µl medium were seeded in a 96-well plate (1 × 10⁴ to 2 × 10⁴ cells per well) and incubated in the above mentioned condition for 24 h. Thereafter, the cells were treated with different concentrations (50 µg/ml, 100 µg/ml, 200 µg/ml, and 400 µg/ml) of plant extracts for 48 h incubation. After that, the supernatant was replaced by 150 µl of medium with 50 µl of MTT in each well. Following the 4 h of incubation, purple formazan crystals of living cells were produced and they were dissolved by the addition of 100 µl of DMSO (0.1%). The absorbance was measured with a microplate reader at 595 nm. Commercially available cisplatin drug was used as a positive control. The percentage of the cytotoxic activity was calculated using the following formula

$$\% \text{ cytotoxic activity} = \frac{\text{Abs1} - \text{Abs2}}{\text{Abs1}} \times 100$$

Where Abs1 is the absorbance of cells with all components except plant extracts, Abs2 is the absorbance of the cells with all components including plant extracts.

2.4. Identification of compounds using GC-MS

The bioactive compounds of the methanol extracts of *D. transparens* and *V. cristata* were identified by using GCMS-QP2010 Ultra (Shimadzu Europa GmbH, Germany). In GC-MS, an electron ionization system with ionization energy of 70 eV was used. The carrier gas was pure helium (99.99%) with a column-flow rate of 0.95 ml/min. The initial temperature was set at 100 °C and increased at a rate of 3 °C/min after a holding time of about 10 min. Finally, the temperature was raised to 300 °C at a rate of 10 °C/min. One microliter of 1% extract diluted in methanol was injected in a splitless mode. The relative quantity of compound present in the extract was expressed in a peak area produced in the chromatogram. Computer software was used to identify the compounds based on GC retention times and by matching the spectra with standard values.

2.5. Statistical analysis

The cytotoxic activity assay was carried out in triplicate. The values were presented as mean ± standard deviation (SD). The IC₅₀ value of the extract was calculated using a second- or third-order polynomial regression equation.

3. Results and discussion

In the present study, eight methanol extracts of different concentrations (50, 100, 200, and 400 µg/ml) of seven wild orchids, none of whose cytotoxic activity has been previously reported on, were screened for their cytotoxic activities on two cancer cell lines (HeLa and U251) by using the MTT assay. The cytotoxic effect of these orchid extracts and commercial drug cisplatin against the cancer cell lines are presented in Table 1.

The present study found that extracts of *D. transparens* stem (DTs) and *V. cristata* whole plant (VCw) were the most effective cytotoxicity toward both HeLa and U251 cancer cell lines with the lowest IC₅₀ values as much as compared with the IC₅₀ of cisplatin. The formazan crystals of living cells

Table 1. Cytotoxic effect of extracts of selected wild orchids.

Sample	Concentration (µg/ml)	HeLa cells % inhibition Mean ± SD	IC ₅₀ (µg/ml)	U251 cells % inhibition Mean ± SD	IC ₅₀ (µg/ml)
<i>Gastrochilus distichus</i> (GDw)	50	0	No activity	0	No activity
	100	0		0	
	200	0		0	
	400	1.57 ± 0.10		0	
<i>Eria graminifolia</i> (EGp)	50	0	5219.85	0	No activity
	100	0		0	
	200	1.57 ± 0.10		0	
	400	3.16 ± 0.05		0	
<i>Dendrobium transparens</i> (DTs)	50	20.65 ± 1.64	382.14	53.95 ± 0.32	75.84
	100	30.10 ± 0.75		58.93 ± 0.12	
	200	38.41 ± 0.57		64.95 ± 0.63	
	400	49.94 ± 0.71		71.05 ± 0.64	
<i>Papilionanthe uniflora</i> (PUw)	50	0	781.85	0	2585.88
	100	0		0	
	200	6.47 ± 0.03		2.50 ± 0.11	
	400	23.76 ± 0.08		6.58 ± 0.11	
<i>Otochilus albus</i> (OAp)	50	0	2345.19	0	no activity
	100	0		0	
	200	0		0	
	400	7.55 ± 0.16		0	
<i>Pholidota articulata</i> (PAI)	50	0	673.04	0	3170.55
	100	0		0	
	200	12.35 ± 0.07		0	
	400	27.20 ± 0.05		5.52 ± 0.11	
<i>Pholidota articulata</i> (PAp)	50	0	723	0	3862.14
	100	0		0	
	200	10.37 ± 0.17		0	
	400	25.33 ± 0.05		4.49 ± 0.11	
<i>Vanda cristata</i> (VCw)	50	23.30 ± 2.29	317.23	41.24 ± 0.68	163.66
	100	33.41 ± 3.69		47.94 ± 0.63	
	200	45.21 ± 1.79		54.90 ± 0.76	
	400	54.56 ± 1.29		61.86 ± 0.84	
Cisplatin drug	-	-	25.00	-	25.00

were formed after addition of MTT dye and no crystals were formed of cells those were killed by extracts. *P. uniflora* and *P. articulata* were not effective toward the cancer cell lines. Of the selected species, *G. distichus*, *E. graminifolia*, and *O. albus* were the least sensitive against these cancer cell lines.

Against HeLa cells, extracts of *E. graminifolia*, *G. distichus* and *O. albus* showed no evident cytotoxicity. *V. cristata*, *D. transparens*, *P. articulata* and *P. uniflora* exhibited significant cytotoxic activity of 49.56% 54.56%, 23.76% and 27.20%, respectively at the highest concentration (400 µg/ml). The 50% cell growth inhibition concentration (IC₅₀) by the extracts of the selected orchids in the HeLa cell line was determined by using a linear regression equation of the percentage inhibition curve. The extracts of the whole plant of *V. cristata*, the stem of *D. transparens*, the leaf and pseudobulb of *P. articulata* and the whole plant of *P. uniflora* were effective cell growth inhibitors, with IC₅₀ values of 317.23 µg/ml, 382.14 µg/ml, 673.04 µg/ml, 723 µg/ml and 781.85 µg/ml, respectively (Table 1).

In the case of U251 glioblastoma brain tumour cells, *D. transparens* and *V. cristata* were significantly effective in inhibiting growth, with percentage inhibition of 71.05% and 61.86%, respectively and IC₅₀ of 75.84 µg/ml and 163.66 µg/ml, respectively, none of the other extracts demonstrated much cytotoxic activity against U251 cells (Table 1).

The bioactive compounds present in the extract obtained from *D. transparens* stem (Table 2) and *V. cristata* whole plant (Table 3) were detected and identified using GC-MS. The results of the GC-MS analysis of

the other extracts are not shown here because those extracts showed either no or little inhibitory activity. The elution time of compounds with their base mass-to-charge ratio (m/z) and their contents by percentage were also determined.

As major components were identified: palmitic acid (23.51%); 9-methyl-octadecanoate (53.43%); 10-octadecenoic acid, methyl ester (34.32%) and 14-methyl-pentadecanoic acid methyl ester (12.86%). As minor components by abundance were identified: 15-methyl-hexadecanoic acid methyl ester (6.43%), as well as below 5%, 1,2-di-palmitin; hexadecane; 1,1'-hexadecylidenebis-cyclopentane; 6-ethyl-3-trimethylsilyloxydecane; 9-octadecenoic acid; and 1,2,3-propanetriyl ester, which all of them were found to have various biological activities, including cytotoxic activity (Asghar and Choudahry, 2011; Belakhdar et al., 2015; Lee et al., 2007; Hsouna et al., 2011; Panigrahi et al., 2014). Besides these, *alpha*-bisabolol; 2-methyl-(Z, Z)-3,13-octadecadienol; hexadecanoic acid; docosenoic acid; 15-methyl-hexadecanoic acid methyl ester; 10-octadecenoic acid, and its methyl ester were also identified (Table 2). In *D. transparens*, a total of 27 compounds have been detected. Of them, the major components based on abundance were: 10-octadecenoic acid methyl ester (34.32%); 14-methyl-pentadecanoic acid methyl ester (12.86%). Minor components were: 15-methyl-hexadecanoic acid methyl ester (6.43%). Besides these, 1,2-di-palmitin; hexadecane; 1, 1'-hexadecylidene- bis-cyclopentane and 6-ethyl-3-trimethylsilyloxydecane were also present (Table 3).

Table 2. Bioactive compounds detected and identified from *Dendrobium transparens* stem extract.

S.N	Compound Name	RT min	Content %	Base m/z
1	1-hexadecene	6.735	0.12	41.05
2	1-pentadecene	8.217	0.14	41.05
3	Pentadecanal-	8.564	1.17	68.05
4	Phthalic acid butyl undecyl ester	8.867	1.08	149
5	11-octadecenoic acid methyl ester	9.037	1.73	55.05
6	14-methyl-pentadecanoic acid methyl ester	9.156	12.86	74
7	10-octadecenoic acid methyl ester	10.313	34.32	55.05
8	15-methyl-hexadecanoic acid methyl ester	10.46	6.43	74
9	11,14-eicosadienoic acid methyl ester	10.918	2.05	67.05
10	Hexanoic acid 9-decen-1-yl ester	11.381	0.98	41
11	Hexadecane	11.628	1.82	57.05
12	1,2-di-palmitin	11.686	3.17	57.05
13	10-oxo-octadecanoic acid methyl ester	11.749	2.39	55.05
14	2,6,10,15-tetramethyl-heptadecane	12.204	1.11	57.05
15	17-octadecynoic acid	12.744	4.61	55.05
16	Hexadecane	12.79	3.54	57.1
17	(S)-(+)-5-methyl-1-heptanol	12.833	1.25	83.05
18	(Z)-13-docosenoic acid methyl ester	12.874	1.95	55.05
19	1,2-benzenedicarboxylic acid diisooctyl ester	13.225	2.08	149
20	6-ethyl-3-trimethylsilyloxydecane	13.92	2.22	131.1
21	Hexadecane	14.157	1.64	57.05
22	Cholesta-3,5-diene	16.029	2.23	81.05
23	1,1'-hexadecylidenebis-cyclopentane	17.068	3.53	151.1
24	10-undecenoic acid, propyl ester	17.157	1.52	70.05
25	(E,E,E)-9-octadecenoic acid, 1,2,3-propanetriyl ester	18.628	2.02	69.05
26	beta.-sitosterol	20.337	2.49	43.05
27	1-(2-decyldodecyl)-2,4-dimethyl-cyclopentane	21.64	1.56	55.05

Table 3. Bioactive compounds detected and identified from *Vanda cristata* whole plant extract.

S.N	Compound Name	RT min	Content (%)	Base m/z
1	alpha-bisabolol	7.627	0.59	43.05
2	14-methyl-pentadecanoic acid methyl ester	7.757	1.21	74.05
3	10-Nonadecenoic acid methyl ester	9.037	3.55	55.05
4	Palmitic acid	9.172	23.51	74
5	10-octadecenoic acid methyl ester	9.679	0.44	55.05
6	Hexadecanoic acid	9.812	0.37	74
7	9-methyl-octadecenoate	10.344	53.43	55.05
8	15-methyl-hexadecanoic acid methyl ester	10.468	4.86	74
9	Linolelaidoyl chloride	10.621	0.54	67.05
10	9,12-hexadecadienoic acid methyl ester	10.923	0.81	67.05
11	1,2-15,16-diepoxyhexadecane	11.592	0.6	55.05
12	3,7-dimethyl-1,6-octadien-3-ol	11.637	0.52	71.1
13	11-tridecen-1-ol	11.694	2.74	55.05
14	10-oxo-octadecanoic acid methyl ester	11.751	1.05	43.05
15	15-methyl-hexadecanoic acid methyl ester	11.831	0.29	74
16	E-2-octadecadecen-1-ol	12.056	0.47	55.05
17	2-methyl-Z,Z-3,13-octadecadienol	12.748	2.95	55.05
18	Erucic acid methyl ester	12.878	1.3	55.05
19	Docosanoic acid methyl ester	13.012	0.28	74.05
20	Cyclohexane	13.922	0.49	131.05

Dendrobium nobile, for example, showed cytotoxicity against human lung carcinoma, human ovary adenocarcinoma, and human promyelocytic leukemia cell lines (You et al., 1995). *Dendrobium chrysanthum* inhibited the proliferation of HL-60 cells (Li et al., 2001), and *Bulbophyllum kwangtungense* showed antitumour activities against HeLa and K562 human tumour cell lines (Wu et al., 2006). *Bulbophyllum*

odoratissimum was found to be cytotoxic against human cancer cell lines, such as human leukemia cell lines K562 and HL-60, human hepatoma BEL-7402, human lung adenocarcinoma A549 and human stomach cancer cell line SGC-7901 (Chen et al., 2007) and *Dendrobium longicornu*, *D. amoenum*, *D. crepidatum* and *D. moniliforme* were found cytotoxic to HeLa and U251 cell lines (Paudel et al., 2017, 2018; 2019; Paudel and

Pant, 2017). Besides, compounds isolated from *Dendrobium* are found to have various antioxidant, anticancer, and neuroprotective activities (Chand et al., 2016; Ng et al., 2012). The majority of plant-based secondary metabolites consist of phenolic compounds, alkaloids, flavonoids and tannins (Farzaneh and Carvalho, 2015; Wong et al., 2006; Gupta et al., 2004). These natural products possess diverse pharmacological properties, including cytotoxic and cancer chemopreventive effects. Flavonoids, triterpenoids and steroids, in particular, exert multiple biological effects due to their antioxidant properties and free radical-scavenging abilities (Farzaneh et al., 2018; Gupta et al., 2004). Studies have shown antioxidant and cytotoxic activity to be associated with a variety of classes of compounds, such as polyphenols, flavonoids, and catechins (Uddin et al., 2009). Also, almost all orchids inhibit endophytic fungi, surface saprophytes, latent pathogens and mycorrhizal fungi, at some stage of their lives (Petrini and Fisher, 1990; Rasmussen and Rasmussen, 2009; Pant et al., 2016). Endophytes provide a broad variety of bioactive secondary metabolites with unique structures, including alkaloids, benzopyranones, chinones, flavonoids, phenolic acids, quinones, steroids, terpenoids, tetralones, and xanthenes (Tan and Zou, 2001; Shah et al., 2019). The bioactive compounds in plants have a wide range of applications such as agrochemicals, antibiotics, immune-suppressants, and anti-diabetic, anti-inflammatory, anti-parasitic, antioxidants and anti-cancer agents (Farzaneh and Carvalho, 2015; Gunatilaka, 2006; Kharwar et al., 2011; Schulz et al., 2002; Strobel et al., 2004).

The mechanism behind the exhibited biological activity is unknown, however, the following mechanisms may be envisioned. Polyphenol compounds might inhibit cancer cells by xenobiotic-metabolizing enzymes that alter the metabolic activation of potential carcinogens, while some flavonoids may alter hormone production to prevent the development of cancer cells (Chen et al., 2007; Gali-Muhtasib et al., 2015). Phenolics, on the other hand, may disrupt cellular division during the telophase stage of mitosis. Phenolics also reduce the amount of cellular protein, the mitotic index and colony formation during cell proliferation (Li et al., 2001; Wu et al., 2006; Paudel et al., 2019). The more hydroxyl groups there are in a phenolic, the greater is its antioxidant activity. The presence of the 4-carbonyl group in the flavonoid molecule contributes to its anticancer activity (Cragg and Newman, 2013). Also, the presence of 2,3-double bond in a flavonoid molecule correlates with mitochondrial damage and cancer cell death (Prasad et al., 2009). Extracts of orchids have the potential to yield useful antioxidants and anticancer compounds which could lead to the production of valuable drugs and therefore merit more study to isolate such potentially useful drugs (de la Torre and Albericio, 2020).

4. Conclusion

The methanol extracts of particular wild orchids of Nepal, in particular, *D. transparens* and *V. cristata*, showed significant cytotoxic activity against cervical cancer and brain tumour cell lines. Our results provide the basis for further investigation of these orchid species for the potential lead discovery of new chemical entities with therapeutic anti-cancer properties. Elucidating the mechanism of action by which these anti-cancer properties from the identified compounds are derived from, and their optimization towards a drug-like efficacy and safety profile is of crucial future importance.

Declarations

Author contribution statement

Pusp Raj Joshi: Performed the experiments; Wrote the paper.
Mukti Ram Paudel: Conceived and designed the experiments; Wrote the paper.
Mukesh Babu Chand, Basant Pant: Contributed reagents, materials, analysis tools or data; Wrote the paper.

Shreeti Pradhan, Krishna Kumar Pant, Giri Prasad Joshi, Manoj Bohara, Sven H. Wagner: Analyzed and interpreted the data; Wrote the paper.

Bijaya Pant: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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