Phytochemical Screening, Total Phenolic Content, Antioxidant Activities and Cytotoxicity of *Dendrobium signatum* Leaves

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Abstract. In the present work, the leaves of *Dendrobium signatum* were extracted with ethanol by maceration; called M; and by sonication-maceration for 30 and 45 mins; called MS30 and MS45. Some biological activities of extracts were investigated. The results showed that the sonication for 30 mins and then maceration 72 hr gave the highest yield at 7.32%. The phytochemical investigation revealed that the bioactive compound from M, MS30 and MS45 were similar which composed of carbohydrates, coumarins, alkaloids, flavonoids, phenolics, sterols and glycosides, respectively. The total phenolic contents were assessed by Folin – Ciocalteau method using gallic acid as the chemical standards. The results showed that MS30 displayed the highest total phenolic contents at the value of 8.83 gGAE/100 g extract. The antioxidant activity was measured using DPPH assay. The results showed that MS30 had the stronger free radical scavenging activity than M and MS45 and had moderate radical scavenging ability compared to ascorbic acid. The cytotoxicity was performed against human cancer cell lines using MTT assay. The results showed that all ethanol extracts displayed weak anticancer activity against MCF-7 and NCI-H187 cell lines, compared to the standard drug doxorubicin. In addition, they showed no activity against KB cell lines.

1 Introduction

The compound which synthesized from the secondary metabolisms was secondary metabolites. Secondary metabolites play a role in reinforcement of many plant tissues. These metabolites are not essential for growth but they produce in order to protect against insects, diseases, and plant regulation (plant hormones). The secondary metabolites compose of a wide range of chemical structures and biological activities. Phytochemicals play an important role for study many bioactive profile of plants and other interesting mircoorganism. Several phytochemical screenings have been carried on for detecting the various groups of naturally occurring phytochemicals. The bioactive compound considers effective in many biological activities A number of phytochemicals including alkaloids, flavonoids, bibenzyl derivatives, phenanthrenes was found in the orchid constituents and suggested various medicinal properties which provided the antimicrobial, antitumor, anti-inflammatory and antiviral activities. Moreover, various other phytochemical constituents have been reported from many orchid species.

In recent decade, the interesting numerous studies have been undertaken to search for the most effective antioxidants because the antioxidants will reduce the oxidative stress in cells and be useful in various treatments of the human diseases [1-3]. Most of plant cells produce the secondary metabolites which have significant potential as antioxidants. Chemical classes of antioxidant secondary metabolites were found such as polyphenol, isoprenoids, tocopherols. Many assay methods were used to estimate the antioxidant content were DPPH method, ABTS method, ORAC assay, PCL assay, β-Carotene linoleic acid bleaching assay, Reducing power assay, Total flavonoid content, Folin-Ciocalteu method [1-3]. Nowaday, the natural antioxidants from plant and other tissues represent the high potentially antioxidant property and less side effect. These can be the alternative for using the natural antioxidants instead of the synthetic antioxidants in the food processing industry and also using in the other pharmacies. Several compound from orchid family were found to have strong antioxidative property. The natural crude obtained from stem, leaves, roots and other parts of orchid which extracted with polar solvents was reported that it presented the significant antioxidant properties. Moreover, several compound were isolated from the Dendrobium orchid showed high and moderate antioxidative properties such as dihydrostilbene isoamoenylin from roots of *Dendrobium amoenum*, Cis- Cismelilotoside, dihydromelilotoside and trans-melilotoside from stems of *Dendrobium aurantiacum*. In addition, The ethanolic extract of *Dendrobium nobile* was found to exhibit antioxidative property equivalent or higher to ascorbic acid. Alkyl ferulates and quercetin were antioxidative compound which were isolated from *Dendrobium monoliforme* and *Dendrobium tosaense* [1-3, 4].

Cancer is the cause of more than 20% of all deaths of one-third of the world's population. Cancer occurs from

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various evidences such as infection, chemicals, environment factors etc. It is important to investigate new herbal medicine which potential as anticancer or antitumor agents for cancer treatment. Nowadays, the bioactive compounds extracted from the parts of plants and various herb were studied and reported that they had the anticancer activities as called the anticancer plants for example *Panex ginseng*, Paclitaxel (Taxol), Turmeric (Curcumin), Carotenoids, Mushrooms, Green tea, Resveratrol [5]. Moreover, the plants with high anticancer activities are undergoing clinical trial. The species of orchids have been studied and a number of bioactive compound with antitumor activity have been isolated. *Dendrobium nobile*, *Dendrobium chrysanthum*, *Dendrobium fimbriatum* have been a good source of compounds with anti-tumor activity [4-6]. The anticancer or antitumor activity of plant extracts was performed the cytotoxicity using MTT method against various cell lines such as human lung carcinoma, human ovary adenocarcinoma, humanpromyelocytic leukemia cell lines, Hela, MCF-7 cell lines.

Dendrobium, a genus of orchid, has been possessed the useful therapeutic activities including antitumor, anticancer, antimicrobial and antioxidant. In this research, *Dendrobium signatum* was used as the plant study. *D. signatum* was one of the species of genus Dendrobium [4, 6]. The flowers and leaves of *D. signatum* were shown in Figure 1 and Figure 2. The leaves were dark green and the flowers had the white sepals and petals. The lip was white with a yellow center. *D. signatum* bloomed from winter to summer. *D. signatum* can grow in Thailand, Los, Vietnam and India. The information of biological activities of *D. signatum* is not reported. The study of this plant has been focused on the analysis of sequence diversity through internal transcribed spacers to identify various Dendrobium species [7]. Therefore, the objective of this study was to investigate the biological activities of *D. signatum* leaves extracts including phytochemical analysis, antioxidant activities and total phenolic contents and to determine cytotoxic activities of extracts of *D. signatum* leaves.

Figure 1. The flowers of *D. signatum* in Sansai, Chiang Mai, Thailand.

Figure 2. The leaves of *D. signatum* in Sansai, Chiang Mai, Thailand.

2 Materials and Methods

D. signatum was grown and collected from Sansai orchid farm, Chiang Mai Province in April 2015, and authenticated by Faculty of Science, Department of Biotechnology, Maejo University, Chiang Mai, Thailand. The dry leaves in the same size and age were used in these experiments.

2.1 Preparation of crude extraction

The first method of crude extraction was performed under the maceration. The dry leaves of *D. signatum* (100 g) were cut and grind into small pieces and then macerated for 72 hours with 95% ethanol (200 mL) at room temperature under the scheme of extraction shown in Figure 3. The agitation was done every 12 hours for 15 mins. The extraction solution was filtered and concentrated under reduced pressure, lyophilized to yield a residue and stored at −20 °C. All crude ethanol extracts from this method were called “M” and used to determine the phytochemical screening, total phenolic contents, antioxidant potential and *in vitro* cytotoxic activities, respectively.

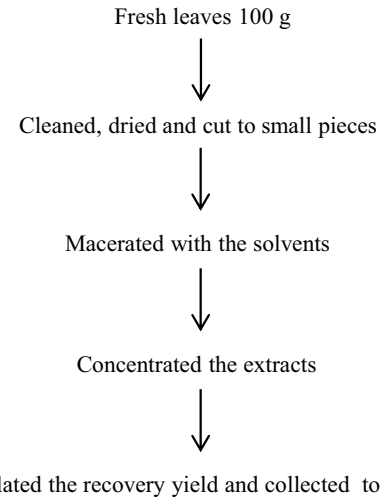

Figure 3. A procedure of the maceration of *D. signatum* leaves extraction.

The second methods of preparation of crude extraction were prepared by using both maceration and sonication. The dry leaves of *D. signatum* (100 g) were cut and grind into small pieces and then sonicated for 30 and 45 mins with 95% ethanol (200 mL) before maceration for 72 hours with the same condition as the first maceration method. The scheme of preparation of the second method was shown in Figure 4. The agitation was done every 12 hours for 15 mins. The extraction solution was filtered and concentrated under reduced pressure, lyophilized and stored at −20 °C. All crude ethanol extracts obtained from the sonication and maceration for 30 or 45 mins were called “MS30 and MS45” and used to determine the phytochemical screening, total phenolic contents, antioxidant potential and *in vitro* cytotoxic activities, respectively.

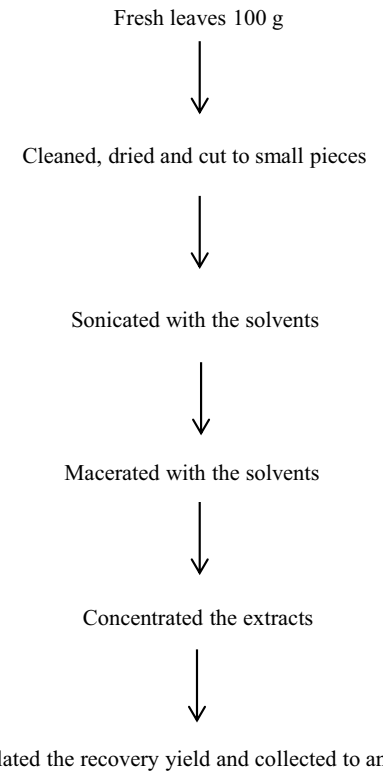

Figure 4. A procedure of the sonication and maceration of *D. signatum* leaves extraction.

2.2 Phytochemical investigation

Phytochemical investigation was achieved in order to preliminary screen the active compound in the ethanoic of leaves extract of both extraction methods. The dried sample was tested following the standard and applied methods for screening the active compound including the carbohydrates, coumarins, alkaloids, flavonoids, phenolics, sterols, glycosides, tannins, saponins, respectively [8-13].

2.3 Determination of total phenolic contents

The total phenolic contents of ethanol extracts were achieved by Folin–Ciocalteu method. About 0.5 ml of ethanol extracts (10 μg/ml) was added to 2.5 ml of Folin–Ciocalteu solution. After 2 min reaction time, 7% of sodium carbonate solution (2.5 ml) was added to the mixture. Then the mixture was incubated for 30 min at 30 °C to complete the reaction. The absorbance of the resulting mixture was measured at 760 nm against blank. The gallic acid was used as standard. The results were calculated the total contents of phenolic compounds in ethanol extracts in gram gallic acid equivalents (gGAE) to 100 gram of extracts [14, 15].

2.4 Determination of DPPH radical scavenging ability

DPPH is 1, 1-diphenyl-2-picrylhydrazyl. DPPH radical-scavenging ability of ethanol extracts was evaluated. In brief, an aliquot of 1.5 ml of 0.05% DPPH solution in ethanol and 0.1 ml of extract or standard (ascorbic acid) at various concentrations in μg/ml were mixed. The mixture of DPPH and standard or DPPH and extracts was shaken vigorously and incubated for 50 min in the dark at room temperature. Decolourization of DPPH was determined by measuring the absorbance at 517 nm. using a spectrophotometer. Ethanol serves as a blank. The percentage inhibition activity was calculated by using the following formula. [% DPPH radical scavenging activity = [{(Absorbance of control – Absorbance of sample or standard}/ Absorbance of control]*100. The results can express as IC_{50} value in μg/mL [14, 15].

2.5 Cytotoxicity studies

The ethanol extractions were tested for cytotoxic activity against the human oral carcinoma (KB), breast cancer (MCF-7) and human small lung (NCI-H187) lines for the evaluation of growth inhibition. Cell line growth was monitored using the MTT assay as reported [16], and compared with doxorubicin as a positive control. The results were expressed as IC_{50} in μg/mL.

3 Results and Discussion

3.1 Preparation of crude extraction

The ethanol extracts from the leaves of *D. signatum* had the dark green residues. The percentage yield of extracts was presented in Table 1. The results showed that the extraction procedure of sonication and maceration (M30, M45) gave higher yield than that of only maceration (M). These suggested that sonication can help to enhance the extract yields which ethanol as solvent can penetrate and eluted some of the active compound from the small pieces *D. signatum* leaves. It should affect to the qualitative and quantitative of the other biological activities of the extracts.

Table 1. The percentage yield and characteristic of the ethanol extractions (M, MS30, MS45)

Compounds	Percentage yield (%)	Colour
M	5.45±0.79	Dark green
MS30	7.32.±0.43	Dark green
MS45	6.87±0.19	Dark green

The results showed that the extraction procedure of sonication and maceration (M30, M45) gave higher yield than that of only maceration (M). These suggested that sonication can help to enhance the extract yields which ethanol as solvent can penetrate and eluted some of the active compound from the small pieces *D. signatum* leaves. It should affect to the qualitative and quantitative of the other biological activities of the extracts.

3.2 Phytochemical screening

The results of the phytochemical screening of ethanol extracts were shown in Table 2.

Table 2. Phytochemical screening of the ethanol extractions. Meanings are + as "Present" and - as "Absence".

Compounds	Ethanol extracts		
	M	MS30	MS45
Carbohydrates	+	+	+
Coumarins	+	+	+
Alkaloids	+	+	+
Flavonoids	+	+	+
Phenolics	+	+	+
Sterols	+	+	+
Glycosides	+	+	+
Tannins	-	-	-
Saponins	-	-	-

In general, several bioactive compounds including alkaloids, flavonoids, bibenzyl derivatives, phenanthrenes were investigated in the orchid constituent [4, 6]. For Dendrobium species, various organic solvents including petroleum ether, hexane, acetone, ethyl acetate, ethanol and water were used to extract the bioactive substances from the different parts of Dendrobium orchid. In this work, ethanol was used to extract the bioactive compound from *D. signatum* leaves using maceration and sonication – maceration procedures. The M extract was obtained from the maceration. For, MS30 and MS45 were obtained from the sonication – maceration procedures in different sonication time of 30 and 45 mins. Those extracts were tested the phytochemical screening. The results revealed that the bioactive compound from M, MS30 and MS45 were similar which composed of the carbohydrates, coumarins, alkaloids, flavonoids, phenolics, sterols and glycosides, respectively. The tannins and saponins were absent from these ethanol extracts. These phytochemical studies of ethanol extraction from *D. signatum* leaves were the first reported. The phytochemical analyses of Dendrobium have been performed in various parts and organic solvents. Physico-chemical and phytochemical evaluation of *Dendrobium macraei* roots revealed the presence of carbohydrates, coumarins, alkaloids, phytosterols, flavonoids and phenolic compound in the methanol extracts [8]. Qualitative preliminary phytochemical analysis of whole plants of *Dendrobium ovatum* (L.) Kraenzl reported the bioactive compound including flavonoids, alkaloids, triterpenoids, glycosides, steroids and carbohydrates [9]. The phytochemical analysis of *Dendrobium macrostachyum* Lindl revealed the presence of alkaloids, flavonoids, glycosides, sterols, tannins and phenols [10]. Moreover, the qualitative preliminary phytochemical analysis of ethanol extracts of *Dendrobium ochreatum* leaves presented the steroids, alkaloids, flavonoids, saponins, and tannins [11]. In addition, the phytochemical studies of ethanol extract from *D. pandurantum* leaves found that the bioactive compound composed of the tannin, phenols and flavonoids, respectively [12]. From the different types of Dendrobium species which tested the phytochemical screening, it can be concluded that most of all extracts presented flavonoids, alkaloids, sterols, glycosides and tannins, respectively [13].

3.3 Determination of total phenolic contents

The first report of total phenolic contents of three ethanol extracts including M, MS30, MS45 were assessed by Folin – Ciocalteau method using gallic acid as the chemical standard. All of the value showed in Table 3. The results showed that MS30 performed the highest total phenolic contents at 8.83 gGAE/100 g extract. Both MS30 and MS45 had higher total phenolic contents than M. The total phenolic contents of each Dendrobium species were different because of the solvent extraction and also parts of orchids. For example, the previous report of total phenolic contents of the methanol extract of *D. ovatum* (L.) Kraenzl. was 4.51±0.4 g GAE/100 g [9].

Table 3. The total phenolic contents of the ethanol extractions.

Compounds	Total phenolic contents (gGAE/100 g extract)
M	5.52±0.28
MS30	8.83±0.07
MS45	7.43±0.37

This revealed that this species had the high total phenolic contents than all of ethanol extracts (M, MS30 and MS45). Several factors such as the species, growing and the extraction method may be affected to the different of the total phenolic contents in various Dendrobium species. Moreover, a study on the total phenolic content of pollinated and unpollinated flowers of *Dendrobium Sonia* and *Dendrobium Savin* revealed that higher total phenolic contents presented in the unpollinated flowers [17]. The high level of polyphenols and flavonoids was reported in the Chinese medicinal orchid, *D. nobile*, in the methanolic extract of stem and leaves, which was significantly higher than that of chloroform and acetone extracts. Additionally, it was reported that phenolic compound are associated with antioxidant activity and play a significant role in stabilizing lipid peroxidation [18, 19]. The high total phenolic contents and antioxidant activities of different *Dendrobium officinale* extracts were reported in polar solvents [20]. The methanol extracts of stems of *Dendrobium herbaceum* Lindl. were performed the high total phenolic contents using tannic acid, gallic acid as standard and also exhibited the high antioxidant activities [21]. Similar observations were indicated in the methanolic extracts from *Dendrobium denudans* D. Don stem [22]. In this work, the extraction procedures had the influence to the results of total phenolic contents. The sonication of ethanol extract (M30, M45) before the maceration can help to enhance the quantitative of the total phenolic contents which exhibit the value of 8.83 and 7.43 gGAE/100 g extract when sonicating for 30 and 45 mins, respectively. For the extract obtained from the maceration, the lower total phenolic contents presented the value of 5.52 g GAE/100 g extract.

3.4 Determination of DPPH radical scavenging activity

The antioxidant potential of three ethanol leaves extracts including M, MS30 and MS45 were measured using

DPPH assay using ascorbic acid as the chemical standard to identify the DPPH radical scavenging activity. All of the value showed in Table 4.

Table 4. DPPH radical scavenging activity of the ethanol extractions (IC_{50}, μg/mL)

Compounds	IC_{50} (μg/mL)
M	112.35±0.36
MS30	97.21±0.07
MS45	109.25±0.28
Ascorbic acid	21.67±0.11

The highest value of radical scavenging ability was observed from the IC_{50} value of 97.21 μg/mL which was of MS30. For M and M45 showed the lower IC_{50} value of 112.35 μg/mL and 109.25 μg/mL than M30. For, the IC_{50} value of ascorbic acid was determined at 21.67 μg/mL. It can be concluded that all extracts displayed the moderate free radical scavenging activity when compared to standard ascorbic acid. The sonication and sonicated time had effect to the DPPH radical scavenging activity of the extracts. The M30 had stronger free radical scavenging activity than M45 whereas both M30 and M45 exhibited higher antioxidant activity than M. This effect appears the results as same as the total phenolic contents studies. In contrast, the long-time of sonication before maceration may not help to improve the total phenolic contents or bioactive antioxidant compounds from the *D. signatum* leaves extract. In this work, the DPPH radical scavenging activity was studied form the ethanol extract of *D. signatum* leaves. Many Dendrobium species was extracted the bioactive compound from various parts of Dendrobium such as stem, leaves, whole plant and roots and then observed the antioxidant activity using the DPPH assay, ABTS and also ORAC [23-25]. In general, orchids were shown to scavenge DPPH free radicals. The extracts from *D. aqueum* [26], have shown to scavenge DPPH free radicals. The DPPH radical scavenging activities of fractions from *D. aurantiacum* var. *denneanum* showed the moderate antioxidant activity in butanol extract (IC_{50} = 49.50 μg/mL) compared to α-tocopherol (IC_{50} = 25.00 μg/mL) [23]. The phenanthrenes and lignans were isolated from *D. nobile* were determined the scavenging DPPH free radicals. The results revealed that these active substances displayed the strong and moderate antioxidant when compare to ascorbic acid and BHT [25]. In addition, various polysaccarides were extracted and isolated using the hot water or other polar organic solvents and determined the antioxidant activity. For example, it has been reported previously that various polysaccharides isolated from *D. ochreatum* possess the antioxidant properties [27]. The antioxidant activity value was different because of the Dendrobium species, isolated substances or fraction, extraction methods and assay [28-33]. The lower IC_{50} value indicated better scavenging activity when compared to the chemical standard such as α-tocopherol, ascorbic acid, Trolox. In this study, a positive correlation was observed between the total phenolic contents and DPPH radical scavenging activity of ethanol extracts from *D. signatum* leaves. The MS30 possessing high total phenolic contents will exhibit the scavenging of DPPH free radicals. This evidence of such correlations was observed in earlier studies [34]-[36].

3.5 Cytotoxicity studies

The cytotoxicity studies were performed against human cancer (KB, MCF-7 and NCI-H187) cell lines using MTT assay to analyse the cell growth inhibition. The results displayed in Table 5. It revealed that the ethanol extract of M, M30 and M45 displayed weak anticancer activity against MCF-7 cell lines with the IC_{50} value of more than 100, 87.45 and 94.22 μg/mL, compared to the standard drug doxorubicin, which had IC_{50} value of 48.33 μg/mL. These results were similar to the cytotoxicity which tested in NCI-H187 which indicated the IC_{50} value of more than 100, 95.22 and more than 100 μg/mL, compared to the standard drug doxorubicin which had IC_{50} value of 0.10 μg/mL.

Table 5. Cytotoxicity (as the inhibition of cell growth) of the ethanol extractions from *D. signatum* leaves.

Extracts	IC_{50} (μg/mL)		
	KB	MCF-7	NCI-H187
M	-	>100	>100
MS30	-	87.45	95.22
MS45	-	94.22	>100
Doxorubicin	2.55	48.33	0.10

In addition, three extracts showed no activity against KB cell lines in all of ethanol extracts. Nevertheless, it can be indicated that the condition of sonication for 30 mins gave the higher cytotoxicity than the other ones. This can be explained that the optimize condition for bioactive substance extraction from *D. signatum* leaves should combine the two procedures of sonication at the appropriate time before the maceration. However, this work was tested only three human cancer cell lines. However, the other cell lines have to test and evaluate such as the colon carcinoma (SW620), gastric carcinoma (KATO), lung carcinoma (CHAGO), breast carcinoma (BT474) and hepato carcinoma (HEP-G2). The bioactive of antitumor and anticancer of Dendrobium extracts were performed in many preliminary researches such as various substances isolated from *D. nobile* showed cytotoxicity against human lung carcinoma, human ovary adenocarcinoma and leukemia cell lines. Erianin obtained from the stem of *Dendrobium chrysanthum* was found to be a potent inhibitor of proliferation of HL-60 cells. Moreover, the water soluble polysaccharides from *D. denneanum* had strong anti-tumor and immunomodulation abilities. Cytotoxic bibenzyl dimers isolated from the stems of *Dendrobium fimbriatum* Hook exhibited the broad-spectrum and cytotoxic activities with IC_{50} values ranging from 2.2 to 21.2 μM against human cell lines such as HL-60 SMMC-7721, A-549, MCF-7 and SW480, respectively [4, 6, 37-39].

4 Conclusion

In this work, this is the first report of the biological activities of ethanol extracts from *D. signatum* leaves. From the results of the phytochemical analysis and other biological activities such as total phenolic contents, antioxidant activity and cytotoxic activities of ethanolic extracts of *D. signatum* leaves revealed that both antioxidant activity and total phenolic contents of ethanolic extract of *D. signatum* leaves had correlated with the phenolics, flavonoids, coumarins and also phytosterols which found in the phytochemical analysis. Moreover, these bioactive compounds have been reported the high efficiency of antioxidant activity and free radical scavenging. The isolation of these active compounds was the future work in order to characterize the chemical components of ethanolic extract of *D. signatum* leaves and evaluate the other bioactivities such as antibacterial and antifungal activities, respectively.

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