INVESTIGATION OF ACUTE TOXICITY, ANTIMICROBIAL ACTIVITY, ANTIOXIDANT ACTIVITY OF THE STEM BARK OF *Dalbergia cultrata* Graham Ex Benth. (Yin Daik)

May Theint[1], Kay Khaing Win[2], Tin Myo Thant[3], Myo Thida Chit[4]

Abstract

One of Myanmar's medicinal plant, *Dalbergia cultrata* Graham Ex Benth. was selected for chemical investigation and partial structure elucidation. Firstly, the phytochemical screening of the stem bark of *D. cultrata* was performed by standard method, indicating the presence of α-amino acids, carbohydrates, flavonoids, glycosides, phenolic compounds, reducing sugars, saponins, tannins, steroids and terpenoids. In addition, the OECD Guidelines of Limit Test (425) examined the acute oral toxicity test to determine a test sample's median lethal dose (LD_{50}). Based on the result, a 2000 mg/kg ethanol extract dose showed no signs of acute toxicity in all the experimental mice. However, one animal died at a dose of 5000 mg/kg, indicating that this dosage is approaching or surpassing the LD_{50} threshold. The administration of 2000 mg/kg ethanol extract to the test animals showed the results of normal to significant weight gain in all the animals. However, at 5000 mg/kg, animal 3 showed considerable weight loss, which showed that the dose may have caused adverse effects in some individuals. The Agar well diffusion method evaluated the antimicrobial activity of *n*-hexane, dichloromethane, ethyl acetate, watery, and ethanol extracts. It was found that ethanol and ethyl acetate extracts showed high activity on all organisms except *Bacillus substilis* and *Escherichia coli.* The antioxidant activity of ethyl acetate and ethanol extracts was evaluated using the DPPH assay method. It was found that ethyl acetate extract showed higher antioxidant activity than ethanol extract.

Keywords: *Dalbergia cultrata*, acute toxicity, antimicrobial activity, antioxidant activity, phytochemical

Introduction

Dalbergia is a large genus of trees, shrubs, lianas, and woody climbers in the pea family, the Fabaceae, consisting of approximately 274 accepted species widely distributed in the tropical and subtropical regions of the world, such as South Africa and India, Laos, Myanmar, Thailand and Vietnam in Asia (Aye *et al*., 2019). Members of the genus enjoy several traditional uses all over the world. Various species are widely used in the treatment of different ailments like aphthae, bleeding piles, cough, diarrhoea, dysentery, dyspepsia, epigastric, epistaxis, gonorrhoea, haemorrhages, leprosy, malaria, rheumatism, scabies, scalding urine, stomach ache, syphilis, traumatic injuries, and ulcers, etc. (Saha *et al.*, 2013). Species are also used for their analgesic, anthelmintic, anti-inflammatory, antimicrobial, antipyretic, anti-spermicidal, anti-ulcerogenic, aphrodisiac, astringent, expectorant, and larvicidal activities in traditional medicine (Bekker *et al*., 2002). Some species are validated through pharmacological investigations, while others require scientific investigations to rationalise their traditional uses. The plant family is known to contain isoflavonoids and neoflavonoids (Chan *et al*., 1997). Several phytochemical constituents like isoflavonoids, neoflavonoids, glycosides, cinnamyl phenols, quinones, and furans have been isolated from different species of *Dalbergia* (Innocent, 2012).

D. cultrata was selected for chemical and pharmacological activity analyses in this research work. The present research aims to investigate the acute toxicity, antimicrobial activity, and antioxidant activity of various crude extracts of the stem bark of *D. cultrata*.

[1] Department of Chemistry, Yadanabon University
[2] Department of Chemistry, University of Mandalay
[3] Department of Chemistry, University of Mandalay
[4] Department of Chemistry, University of Mandalay

Family - Fabaceae

Scientific name - *Dalbergia cultrata* Graham Ex Bentham

English name - Rosewood

Common name - Yin daik

Part used - Stem bark

Medicinal uses - Leishmania diseases and cancer diseases

The trunk and leaves of *Dalbergia cultrata* Grahm Ex Benth. are described in Figure 1.

Botanical description of *D. cultrata*

Figure 1. Trunk and Leaves of *Dalbergia cultrata* Grahm Ex Benth.

Materials and Methods

Collection and Preparation of Samples

The stem bark of *D. cultrata* was collected from Pyin Oo Lwin Township, Mandalay Region. Firstly, the collected bark sample was cleaned, then chopped into small pieces and allowed to air dry in the ventilated room for about two weeks. This air-dry sample was kept in a glass bottle with a stopper and it was used throughout the experiment.

Phytochemical Investigation of the Stem Bark of *D. cultrata*

Phytochemical investigation of the stem bark of *D. cultrata* was performed by the standard method (Harborne, 1998).

Extraction of Crude Extracts from the Stem Bark of *D. cultrata*

About 2.2 kg of airdried sample was percolated with 6.5 L of 95 % ethanol thrice for four days. The obtained solution was filtered and the filtrate was removed by a rotatory evaporator then this extract sample was evaporated at room temperature and the ethanol crude extract was obtained. The obtained ethanol crude extract was dissolved by (1:1) v/v of ethanol and *n*-hexane and then stirred using a magnetic stirrer. The obtained solution was partitioned with 200 mL *n*-hexane, with a solution of 200 mL H_2O and 200 mL dichloromethane, and with 200 mL ethyl acetate by using a separating funnel three times for each respectively. Then 10.50 g of *n*-hexane extract, 10.36 g of dichloromethane extract, 3.99 g of ethyl acetate extract, and 10.50 g of watery extract were obtained.

Determination of Acute Toxicity of Ethanol Extract from the Stem Bark of *D. cultrata*

The acute toxicity of an ethanolic extract of *D. cultrata* stem bark was determined using the OECD Guidelines of Limit Test (425) in an in vivo mouse model. This experiment was done at the Department of Biotechnology of Mandalay Technological University in the Mandalay Region.

In this study, the acute oral toxicity test followed the OECD Guidelines of Limit Test (425) which aims to determine the median lethal dose (LD_{50}) of a test substance. The test involved administering two different doses of *D. cultrata* ethanol extract (2000 mg/kg and 5000 mg/kg) to the tested mice and monitoring for signs of mortality and clinical symptoms over a specific observation period. A control group was also included, receiving 2 mL of 20 % ethanol.

Procedure: The study begins with the determination of a starting dose, often chosen based on existing information or estimates. Alternatively, a limit test may be conducted when the LD_{50} is assumed to be higher than the limit dose (e.g., 2000 mg/kg). The first animal is administered the test substance at the chosen dose level (e.g., 2000 mg/kg) and observed for toxicity or death. After administering the dose, the animal is monitored for signs of toxicity over 48 h. Observations include behavioural changes, weight loss, or death. If no lethal effects are observed, another animal is tested at a higher dose to determine the threshold of toxicity. If lethal effects are observed, the dose is adjusted downward for the next animal. If toxicity is observed at either a dose of 2000 mg/kg or 5000 mg/kg, the dose is reduced for the next animal, and the process continues following the "Up-and-Down" method. The process of dosing and observation continues, with doses increased or decreased based on the response of the animals. More animals are tested following the up-and-down procedure to narrow down the LD_{50} estimate.

The study is concluded when sufficient data is gathered to make a reliable estimate of the LD_{50} (the dose at which 50 % of the animals die) or to classify the test substance based on its toxicity. The results are analyzed, and the LD_{50} is determined based on the cumulative data. This information helps to assess the substance's acute toxicity classification. This method minimizes the number of animals used while ensuring a precise estimate of the test substance’s toxicity.

Determination of Antimicrobial Activity of the Stem Bark of *D. cultrata*

The antimicrobial activity of *n*-hexane, dichloromethane, ethyl acetate, watery, and ethanol extracts of the stem bark of *D. cultrata* was determined by applying the agar well diffusion method in the Department of Chemistry, University of Mandalay. The test microorganisms used for the antibacterial activity screening were *Agrobacterium tumerfaciens, Bacillus substilis, Micrococcus luteus, Pseudomonas fluorescens, Escherichia coli, Salmonella typhi.*

Procedure: The crude extract ($20\ \mu L$) was used to check the antimicrobial activity against test organisms by the agar well diffusion method. Well, having an 8 mm diameter was utilised for antimicrobial assay. The assay medium was prepared with 1 % glucose, 0.3 % polypeptone, 0.1 % KNO_3, 1.8 % agar, and 100 mL distilled water at pH 6.5 was used for the antimicrobial activity test. One percent of the test organism was added to the assay medium and then poured into plates. After solidification, wells impregnated with sample (crude extract) were filled on the agar plates and were incubated for 24 – 36 hours at room temperature. Clear zones (inhibitory zones) surrounding the test well indicate the presence of bioactive metabolites which inhibit the growth of test organisms (Collins *et al*., 2004).

Determination of Antioxidant Activity of Crude Extracts of the Stem Bark of *D. cultrata* by DPPH Assay Method

In this present research work, the determination of the antioxidant activity of ethanol extract and ethyl acetate extract of the stem bark of *D. cultrata* was performed by the DPPH Assay method at the Department of Chemistry, University of Mandalay.

Procedure: DPPH radical scavenging activity was determined by the UV-visible spectrophotometric method. The control solution was prepared by mixing 0.002 % DPPH solution (2 mL) and 70 % ethanol (2 mL). The sample solution was prepared by thoroughly mixing 0.002 % (w/v) DPPH solution (2 mL) and the test sample solution (2 mL). The solution was allowed to stand at room temperature for 30 minutes. Absorbance of the solution was measured at 517 mm by using a spectrophotometer and percent inhibition was calculated by using the following equation:

$$\% \text{ Inhibition} = \left[\frac{A_{DPPH} - (A_{Sample} - A_{Blank})}{A_{DPPH}}\right] x\ 100$$

where, % Inhibition = percent inhibition of test sample

A_{DDPH} = absorbance of DPPH in EtOH solution,

A_{Sample} = absorbance of sample and DPPH solution,

A_{Blank} = absorbance of sample and EtOH solution

Results and Discussion

Phytochemicals of the Stem Bark of *D. cultrata*

Preliminary phytochemical analysis of the bark of *D. cultrata* detected the presence of flavonoids, glycosides, phenolic compounds, saponins, α-amino acids, reducing sugars, tannins, steroids, and terpenoids, and the absence of alkaloids. These results are shown in Table 1.

Table 1. Results of Phytochemical Screening of the Stem Bark of *Dalbergia cultrata*

No.	Constituents	Extract	Reagents used	Observation	Results
1	alkaloids	1% HCl	Dragendorff's Wagner's Mayer's	no orange ppt no reddish brown ppt no white ppt	-
2	α-amino acids	DW	Ninhydrin	violet colour spot	+
3	flavonoids	EtOH	Mg turning and conc. H_2SO_4	red colour solution	+
4	glycosides	EtOH	10% lead acetate	yellow ppt	+
5	phenolic	EtOH	10% $FeCl_3$	brown colour ppt	+
6	reducing sugars	DW	Benedict's solution	brick red ppt	+
7	saponins	DW	distilled water	frothing solution	+
8	steroids	Pet-ether	acetic anhydride and conc: H_2SO_4	green colour solution	+
9	tannins	EtOH	10% $FeCl_3$ and conc. H_2SO_4	white ppt	+
10	terpenoids	EtOH	$CHCl_3$, acetic anhydride and conc. H_2SO_4	red ppt	+

(+) = presence, (-) = absence, ppt = precipitate

Acute Toxicity of the Ethanol Extract from the Stem Bark of *D. cultrata*

The acute oral toxicity test followed the OECD Guidelines of Limit Test (425), which aims to determine the median lethal dose (LD_{50}). The test involved administering two doses of *D. cultrata* ethanol extract (2000 mg/kg and 5000 mg/kg) to the tested mice and monitoring for signs of mortality and clinical symptoms over a specific observation period. A control group was also included, receiving 2 mL of 20 % ethanol. These results are shown in Table 2.

Table 2. Observations of Acute Oral Toxicity in Different Dosages of Ethanol Extract of *D. cultrata* Compared with the Control Group

Dose of sample (mg/kg)	Total mice	Observation	Number of mice alive/dead on day by day									
			1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
2000	5	alive	5	5	5	5	5	5	5	5	5	5
		dead	0	0	0	0	0	0	0	0	0	0
5000	5	alive	4	4	4	4	4	4	4	4	4	4
		dead	1	1	1	1	1	1	1	1	1	1
20 % ethanol 2 mL (control)	5	alive	5	5	5	5	5	5	5	5	5	5
	5	dead	0	0	0	0	0	0	0	0	0	0

The data suggests that *D. cultrata* extract at 2000 mg/kg does not exhibit acute toxicity, as no mortality was observed in this group. However, at 5000 mg/kg, one subject died, indicating that this dosage is close to or exceeds the LD_{50} threshold. The single mortality at 5000 mg/kg could indicate inter-individual variability in response to the extract. The control group experienced no mortality or adverse effects, confirming the non-toxicity of the 20 % ethanol solution.

Table 3. Observations of Individual Body Weight Data after Treatment of Ethanol Extract of *D. cultrata* (2000 mg/kg)

Animal	Body weight (g) before and after treating with ethanol extract		
	Day 0	**Day 7**	**Day 14**
1	25.73	26.10	26.62
2	25.76	27.7	28.69
3	25.61	25.83	28.3
4	26. 3	27. 93	28. 61
5	26.1	26.37	29.31

The individual body weight (g) of the tested mice after administering 2000 mg/kg ethanol extract of *D. cultrata*. It was observed that normal to significant weight gain in all animals is shown, indicating that the dose may have no adverse effect. This result is shown in Table 3.

Table 4. Observations of Individual Body Weight Data after Treatment of Ethanol Extract of *D. cultrata* (5000 mg/kg)

Animal	Body weight (g) before and after treating with ethanol extract		
	Day 0	Day 7	Day 14
1	27.01	-	-
2	27.13	27.3	27.8
3	27.04	24.9	24.9
4	27.90	29.83	30.61
5	27.43	31.59	32.1

The individual body weight (g) of the tested mice after administering 5000 mg/kg ethanol extract of *D. cultrata*. It was found that animal 3 showed significant weight loss, indicating that the dose may have adverse effects in some individuals. This result is shown in Table 4.

Antimicrobial Activity of the Stem Bark of *D. cultrata*

The antimicrobial activity of *n*-hexane, dichloromethane, ethyl acetate, watery and ethanol extracts of the stem bark of *D. cultrata* was determined by applying the agar well diffusion method on six selected organisms (*Agrobacterium tumerfaciens, Bacillus substilis, Micrococcus luteus, Pseudomonas fluorescens, Escherichia coli, Salmonella typhi)*). The results are shown in Table 5 and Figure 2.

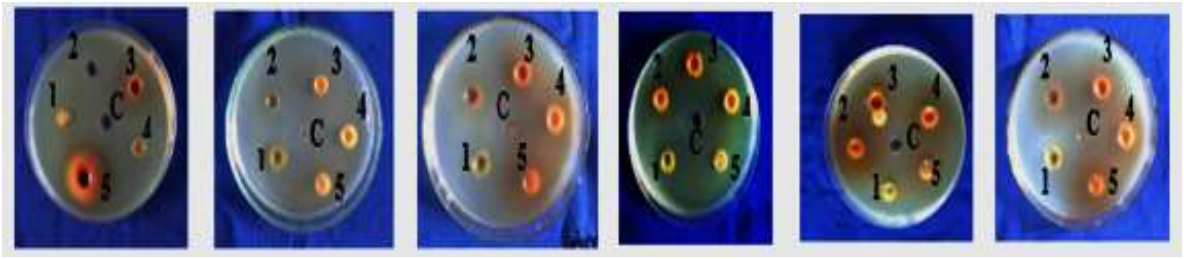
Table 5. Antimicrobial Activity of the Stem Bark of *D. cultrata*

Extracts	Inhibition zone diameters (mm)					
	I	II	III	IV	V	VI
n-Hexane	-	-	-	-	-	-
DCM	-	12 (+)	-	26 (+++)	18 (++)	-
EtOAc	20 (+++)	-	23 (+++)	21 (+++)	-	18 (++)
Watery	-	-	-	23 (+++)	-	23 (+++)
EtOH	24 (+++)	-	23 (+++)	23 (+++)	-	24 (+++)

I – *A. tumerfaciens,* II - *B. substilis*
III - *M. luteus,* IV- *P. fluorescens*
V - *E. coli,* VI- *S. typhi*

Agar well diameter		8 mm
< 12 mm	(-)	no activity
13 mm -15 mm	(+)	low activity
16 mm - 20 mm	(++)	high activity
> 21 mm	(+++)	very high activity

The antimicrobial activity of the crude extracts is shown in Table 5. According to this table, *n*-hexane extract did not show antimicrobial activity on all tested organisms. Furthermore, dichloromethane extract showed very high activity on *Pseudomonas fluorescens*, medium activity on *Escherichia coli*, and low activity on *Bacillus substilis* but had no activity on other test organisms. Moreover, the ethanol and ethyl acetate extract showed very high activity on all tested organisms except *Bacillus substilis* and *Escherichia coli.* Watery extract had very high activity on *Pseudomonas fluorescens* and *Salmonella typhi* but no activity on other test organisms.



Pseudomonas fluorescens | *Salmonella typhi* | *Micrococcus luteus* | *Agrobacterium tumerfaciens* | *Bacillus substili* | *Escherichia coli*

1. *n*-hexane extract 2. DCM extract 3. EtOAc extract

4. Watery extract 5. EtOH extract C. Control

Figure 2. Inhibition zones of the antimicrobial activity of various extracts from the stem bark *D. cultrata*

The inhibition zones of the antimicrobial activity of various extracts are shown in Figure 2. Zone of inhibition (ZOI) is used to determine if the tested organism is susceptible (ZOI produced), intermediate (small ZOI produced), or resistant (no ZOI produced) to the antimicrobial agent. According to these results, *n*-hexane extract showed no ZOI in all the bacterial plates, indicating that it has no activity on all the tested bacteria. DCM extract showed large ZOI in *P. fluorescens*, medium in *E. coli*, small in *B. substilis*, and no ZOI in *A. tumerfaciens, M. luteus, S. typhi*, resulting that DCM extract had very high activity on *P.fluroenscens*, high activity on *E.coli*, low activity on *B. substilis* and no activity on *A. tumerfaciens, M. luteus, S. typhi.* The EtOAc extract had large ZOI in *A. tumerfaciens, M. luteus, P. fluorescens*, medium in *S. typhi*, and no ZOI in *B. substilis* and *E. coli*, indicating that very high activity on *A. tumerfaciens, M. luteus, P. fluorescens*, and high activity on *S. typhi*, and no activity on *B. substilis* and *E. coli*. The watery extract had large ZOI in *P. fluorescens* and *S. typhi*, and no ZOI in *A. tumerfaciens, B. substilis, M. luteus*, and *E. coli*, indicating that watery extract showed very high activity on *P. fluorescens* and *S. typhi*, and no activity on *A. tumerfaciens, B. substilis, M. luteus*, and *E. coli*. The EtOH extract had large ZOI in *A. tumerfaciens, M. luteus, P. fluorescens*, and *S. typhi*, and no ZOI in *B. substilis* and *E. coli*, indicating that very large activity on *A. tumerfaciens, M. luteus, P. fluorescens*, and *S. typhi*, and no activity on *B. substilis* and *E. coli.* According to the results, EtOAc and EtOH extracts have very high and high antimicrobial activity on the tested microorganisms except *B. substilis* and *E. coli*, and *n*-hexane extract has no activity on all tested microorganisms.

Antioxidant Activity of the Stem Bark of *D. cultrata*

IC_{50} values of ethyl acetate extract and ethanol extract from the stem bark of *D. cultrata* were observed to be 10.14 µg / mL and 98.71 µg /mL, respectively. The antioxidant activity of *D. cultrata was* lower than standard ascorbic acid with an IC_{50} value of 5.23 µg /mL. These results are shown in Tables 6, 7, and 8 and in Figures 3, 4, and 5.

Table 6. Antioxidant Activity of Ethyl Acetate Extract of *D. cultrata*

No.	Sample concentration (µg/mL)	Absorbance	(%) Inhibition	IC_{50} (µg/mL)
1	6.25	0.301	43.95	10.14
2	12.5	0.253	52.89	
3	25	0.212	60.52	
4	50	0.158	70.58	
5	100	0.027	94.97	

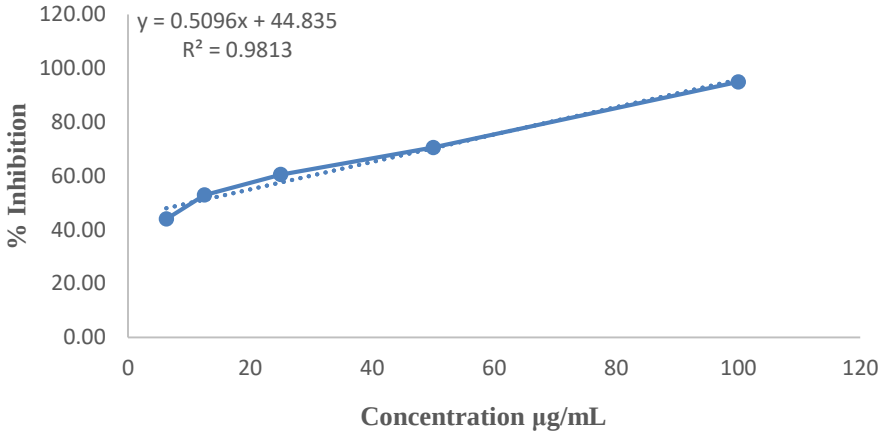


Figure 3. Percent inhibition of ethyl acetate in various concentrations

Table 7. Antioxidant Activity of Ethanol Extract of *D. cultrata*

No.	Sample concentration (µg/mL)	Absorbance	(%) Inhibition	IC_{50} (µg/mL)
1	25	0.341	36.50	98.71
2	50	0.315	41.34	
3	100	0.256	52.33	
4	200	0.171	68.16	
5	400	0.023	95.72	

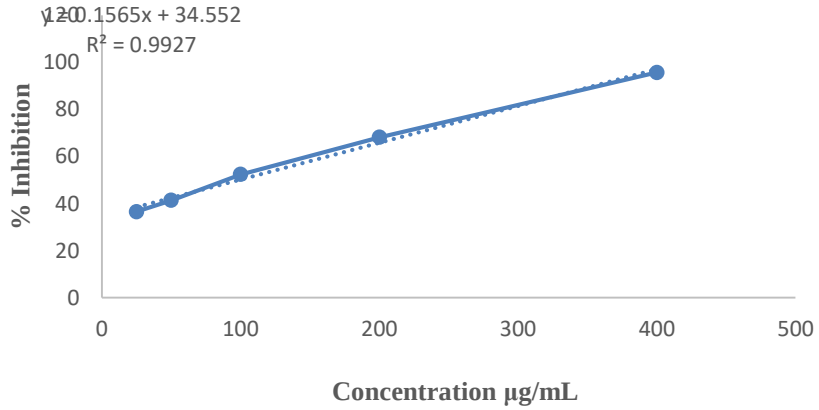


Figure 4. Percent inhibition of ethanol extract in various concentrations

Table 8. Antioxidant Activity of Ascorbic Acid *D. cultrata*

No	Sample concentration (µg/mL)	Absorbance	(%) Inhibition	IC_{50} (µg/mL)
1	1	0.228	32.34	
2	2	0.219	35.01	
3	4	0.178	47.18	5.23
4	8	0.129	61.72	
5	16	0.021	93.77	

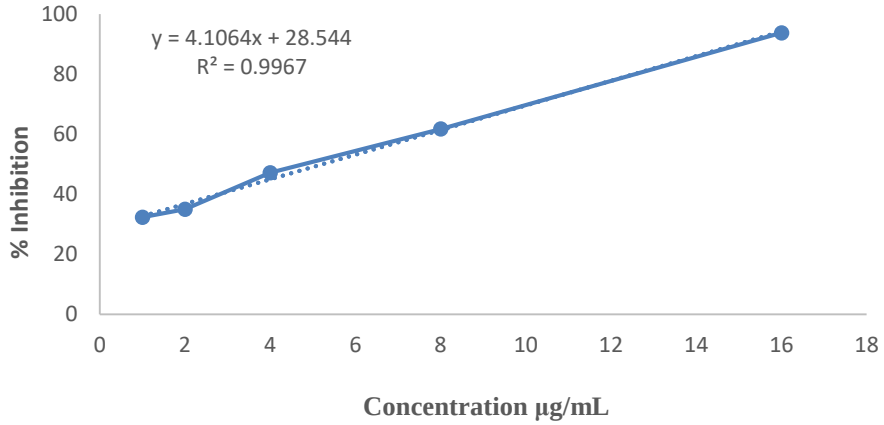


Figure 5. Percent inhibition vs various concentrations of ascorbic acid

From these results, the antioxidant activity of ethyl acetate extract was found to be higher than ethanol extract and it was also found to be lower than that of standard ascorbic acid (IC_{50} = 5.23 µg/mL). Therefore, this result indicates that the ethyl acetate extract of the stem bark of *D. cultrata* has significant antioxidant activity.

Conclusion

The stem bark of *D. cultrata* was chosen for chemical investigation and pharmacological activity screening. The stem bark of *D. cultrata* contains various chemical constituents such as α-amino acids, carbohydrates, flavonoids, glycosides, phenolic compounds, reducing sugars, saponins, tannins, steroids, and terpenes. Additionally, an acute oral toxicity test of 95 % ethanolic extract from the stem bark of *D. cultrata* was carried out following the OECD Guidelines of Limit Test (425), which are designed to determine the median lethal dose (LD_{50}) of a test sample. According to the results, it was found that none of the experimental mice showed signs of acute toxicity at 2000 mg/kg of *D. cultrata* extract. However, at 5000 mg/kg, one animal died, suggesting that this dosage is near or exceeds the LD_{50} threshold. In addition, a 2000 mg/kg dose of the extracted sample resulted in normal to significant weight gain in all animals. In contrast, at 5000 mg/kg, animal 3 experienced considerable weight loss, implying that this higher dose may have caused adverse effects in certain individuals. The antimicrobial activity of *n*-hexane extract, dichloromethane extract, ethyl acetate extract, watery extract, and ethanol extract of the stem bark of *D. cultrata* were investigated by agar well diffusion method. It was found that *n*-hexane extract did not show antimicrobial activity on all tested organisms. Furthermore, dichloromethane extract showed very high activity on *Pseudomonas fluorescens,* high activity on *Escherichia coli,* and low activity on *Bacillus substilis* but had no activity on other test organisms. Moreover, the ethanol and ethyl acetate extract showed very high activity on all tested

organisms except *Bacillus substilis* and *Escherichia coli.* Watery extract had very high activity on *Pseudomonas fluorescens* and *Salmonella typhi* but no activity on the remaining test organisms. The antioxidant activity of ethyl acetate and ethanol extracts of the stem bark of *D. cultrata* was evaluated by the DPPH assay method. It was found that the ethyl acetate extract was more potent than the ethanol extract of the sample in the antioxidant activity.

Acknowledgements

I wish to express my sincere thanks to Professor and Head, Dr. Thinn Myat Nwe, Department of Chemistry, University of Mandalay for their kind permission, invaluable advice and kind guidance on my research work. I wish to express my sincere thanks to Professor and Head, Dr Suu Suu Win, Department of Chemistry, Yadanabon University for their close supervision and kind guidance on my research work.

References

Aye, M. M., H.T. Aung, M.M. Sein, and C. Armijos. (2019). "A Review on the Phytochemistry, Medicinal Properties and Pharmacological Activities of 15 Selected Myanmar Medicinal Plants". *Journal of Molecules,* vol. 24(2), pp.293-297.

Bekker, M., E. Malan, J.A. Steenkamp, and E.V. Brandt. (2002). "An Isoflavanoid–neoflavonoid and O-methylated Isoflavone from the Heartwood of *Dalbergia nitidula"*. *Journal of Phytochemistry*, vol. 59(4), pp.415–418.

Chan, S.C., Y.S. Chang, and S.C. Kuo. (1997). "Neoflavonoids from *Dalbergia odorifera*". *Journal of Phytochemistry*, vol. 46(5), pp. 947–949.

Collins, C. H., P.M. Lyne, J.M. Grange, and J.O. Falkinham. (2004). *Microbiological Methods*. New York: 8th Ed., CRC Publisher, Oxford University Press Inc., pp. 82-90.

Harborne, J. B. (1998). *Phytochemical Methods, A Guide to Modern Techniques of Plant Analysis.* New York: 2nd Ed., Chapman and Hall, pp. 120-160.

Innocent, E. (2012). "A new isoflavone glycoside from *Dalbergia vacciniifolia* (Fabaceae)*". Journal of Scientia Pharmaceutica,* vol. 8, pp. 469-474

OECD, O. "425-Guidelines for the Testing of Chemicals Acute Oral Toxicity-Fixed Dose Procedure." *Animals* (2001).

Saha, S., M. Anisuzzman, M.K. Islam, H. Mondal, and C. Talukder. (2013). "Antibacterial and cytotoxic potential of *Dalbergia spinosa* Roxb. Leaves*". International Journal of Pharmaceutical Sciences and Research*, vol. 4(2), pp. 512-515.