STRUCTURAL ELUCIDATION OF AN ORGANIC COMPOUND AND ESTIMATION OF ANTI-INFLAMMATORY AND ANTIARTHIRITIC ACTIVITIES OF *BACOPA MONNIERI*

Aye Aye Thant[1], Yi Yi Win[2], Myint Myint Khine[3], Ni Ni Than[4]

Abstract

The present research deals with the analysis to isolate and elucidate an organic compound and to assess the antiarthritic and anti-inflammatory activities of the aerial parts of *Bacopa monnieri* that were collected from Twantay Township, Yangon Region. By column and thin layer chromatography separation, the isolated compound structure was elucidated by physicochemical properties and modern spectroscopic techniques such as FT IR, ^{1}H NMR, ^{13}C NMR spectroscopy, and ESI-MS spectrometry, as well as by comparing it with their reported data. *In vitro*, the anti-inflammatory activity of ethanol and watery extracts was evaluated by a nitric oxide inhibition assay. These results did not significantly affect cellular nitric oxide production or cell viability because of cytotoxicity. The ethanol, watery extract, and an isolated compound (bacosine) were assessed for antiarthritic activity. Diclofenac sodium was used as the reference drug. In the egg albumin method, the bacosine compound (IC_{50} = 105.23 µg/mL) had a more significant antidenaturation effect than the ethanol extract (IC_{50} = 162.44 µg/mL) and watery extract (172.71 µg/mL). In the bovine serum albumin method, the bacosine compound (IC_{50} = 118.73 µg/mL) had a more significant antidenaturation effect than ethanol extract (IC_{50} = 180.58 µg/mL) and watery extract (299.24 µg/mL) compared with diclofenac sodium (IC_{50} = 73.44 µg/mL), respectively.

Keywords: *Bacopa monnieri*, antiarthritic activity, anti-inflammatory activity, bacosine

Introduction

Bacopa monniera (Figure 1) is a plant of the Scrophulariaceae family that is commonly known as Brahmi, thyme-leaved gratiola, water hyssop, herb of grace, and Indian pennywort. Bacopa has been used in traditional Ayurvedic treatments for epilepsy and asthma. It is one of the ingredients in many Ayurvedic formulations used for ulcers, tumours, ascites, splenomegaly, inflammatory disorders, leprosy, anaemia, and gastroenteritis *B. monnieri* also exhibits potent antioxidant, anticancer, antiulcer, calcium antagonist, vasodilatory, mast cell stabilizing, anti-inflammatory, and antistress (Kishore *et al.,* 2017). The ethanol extract of *B. monniera* exhibited marked anti-inflammatory activity against carrageenan-induced paw oedema in mice and rats, an acute inflammatory model. To assess the possible mechanism of anti-inflammatory action against carrageenan, the ethanol extract was used to treat the chemical mediator-induced histamine, selectively inhibited prostaglandin E2-induced inflammation (Channa *et al.*, 2006). The current study estimates the anti-inflammatory potential of crude extracts. For the antiarthritic property, the crude extracts and an isolated compound of *B. monnieri* were examined.

[1] Department of Chemistry, University of Yangon
[2] Department of Chemistry, Mawlamyine University
[3] Myingyan University
[4] Department of Chemistry, University of Yangon

Figure 1. Photograph of *Bacopa monnieri*

Materials and Methods

Plant Material

The aerial parts of the plant were collected from the Twantay Township, Yangon Region, Myanmar, in May 2019 and identified as *B. monnieri*, by the authorized botanist at the Department of Botany, University of Yangon. The sample was dried in the shade for a week, cut into very small pieces, and then ground into a purely fine powder using an electric motor grinder. The powdered sample was stored in airtight containers to prevent moisture changes and contamination.

Extraction and Isolation of Pure Compounds

The dried powdered sample (500 g) was extracted with 95 % ethanol in an air-tight bottle for three days, and then filtered and concentrated by using a rotary evaporator at 70 °C under reduced pressure. This similar procedure was repeated three times until 40 g of the crude ethanol extract was obtained. The ethanol extract was extracted with petroleum ether, followed by ethyl acetate. The ethyl acetate extract (10 g) was subjected to column chromatography on a normal-phase silica gel GF_{254} adsorbent increasing the polarity of the eluent solvent system (PE: EA), and five main fractions (F I to F V) were obtained. Fraction F II (PE: EA 7:1) was subjected to column chromatography with a PE-EtOAc gradient system. It was obtained in six fractions (fractions F-1 to F-6). Fraction F-2 gave an isolated compound (colourless crystals, 65 mg, 0.13 % yield based on the dried sample). The isolated compound was structurally identified by modern spectroscopic methods, including FT IR, ^{1}H NMR, and ^{13}C NMR. The FT IR spectrum was measured at the University of Yangon and ^{1}H NMR, ^{13}C NMR and ESI-MS were measured at Department of Chemistry, Faculty of Science and (NUS) National University of Singapore.

Anti-inflammatory Activity of Ethanol and Watery Extracts by Nitric Oxide Inhibition Assay

The nitric oxide (NO) inhibition assay was used to assess the anti-inflammatory effects of crude ethanol and watery extracts at Toyama University in Toyama, Japan. RAW 264.7 cells were expanded in-MEM supplemented with 10% incubated fetal bovine serum, 1% penicillin

(1000 U/mL), and streptomycin (10 mg/mL). The cells were harvested with a cell scraper and diluted to a suspension in fresh media, after the cell growth reached around 70 % confluent. A 100 µL portion of RAW 264.7 (1×10^4/well) was seeded in 96 well plates and incubated at 37 °C for 24 h. The sample was then treated with 50 mL of each LPS (100 ng/mL) and different concentrations on the cell after a 24 h period of 37 °C incubation. Briefly stated, a fresh plate cell was created and filled with 100 µL of each supernatant from 96 wells. After that, a fresh plate cell was put to an equivalent volume of Griess reagent (0.5% sulphanilamide, 0.05 % naphthylenediamide dihydrochloride, 2.5 % H_3PO_4) (Schmidt, 1996) and allowed it to stand for 10 min at room temperature. A microplate spectrophotometer was used to measure the absorbance at 540 nm. A positive control was employed, which was L-NMMA monoacetate. 100 µL of each 10 % MTT solution (5 mg/mL) in the medium was added to the wells, after the residual medium from the initial plate was removed. The formazan crystal was dissolved with 100 µL each of DMSO after 3 h of incubation, and the absorbance was measured at 570 nm with a microplate reader Jin *et al*, (2012). The following formulae were used to calculate the percentages of NO inhibition and cell viability:

$$\text{NO inhibition (\%)} = [\{A_{\text{control}} - A_{\text{sample}} / A_{\text{control}}\}] \times 100$$

$$\text{Cell viability (\%)} = 100 \times [\{(A_{\text{test sample}} - A_{\text{blank}})/ (A_{\text{control}} - A_{\text{blank}})\}]$$

where, A_{control} = the absorbance of the control group treated by LPC alone,

A_{blank} = the absorbance of the samples.

Assessment of *in vitro* Antiarthritic Activity

The *in vitro* antiarthritic activity was studied using the bovine serum protein denaturation method and the egg albumin denaturation method. The egg albumin denaturation assay is based on the idea that substances with anti-inflammatory qualities may be able to stabilize protein structures and prevent denaturation, which is frequently linked to inflammation and tissue damage. Most biological proteins lose their biological function when denatured.

Protein Egg Albumin Denaturation Inhibition Assay

The assay was performed according to the previous method with slight modifications (Madhuranga and Samarakoon, 2023). The reaction mixture (5 mL) consisted of 0.2 mL of egg albumin (EA) from fresh hen's eggs, 2.8 mL of PBS (pH 6.4), and 2 mL of plant extract at various concentrations. Distilled water, or DMSO, was used as a control. In standard control, diclofenac sodium solutions were used in place of extract solutions. The control and test solutions were incubated at 37 °C for 0.25 h followed by heating at 70 °C for 5 min. After cooling to 37 °C, the absorbance of test and control solutions was determined at 660 nm. The percentage inhibition in protein denaturation was calculated.

$$\text{\% Inhibition} = \frac{A_{\text{control}} - (A_{\text{sample}} - A_{\text{blank}})}{A_{\text{control}}} \times 100$$

where, A_{sample} = absorbance of the sample,

A_{blank} = absorbance of blank (without egg albumin),

A_{control} = absorbance of control (without test sample).

50 % inhibition concentration (IC_{50}) value were calculated by linear regressive excel program.

Protein Bovine Serum Albumin Denaturation Inhibition Assay

The assay was carried out following the previous method with slight modifications Saleem *et al*, (2019). A 0.45 mL of 5 % bovine serum albumin (BSA) was added to 0.5 mL of distilled water to prepare the test control solution. The control solution was prepared by replacing water with an equal volume of extract solution. The test solution (0.5 mL) was composed of 0.45 mL of BSA and 0.05 mL of test extract at 50-1600 mg/mL concentrations. Ethanol and watery extracts, and an isolated compound (bacosine) were dissolved in DMSO in place of water. DMSO served as a control for these extracts and an isolated compound in all *in vitro* antiarthritic assays. The standard drug solution (0.5mL) was comprised of 0.45 mL of BSA and 0.05 mL of diclofenac sodium. The pH of all solutions was set to 6.4. All solutions were incubated at 37 °C for 20 min and then at 60 °C for 3 min in an oven. Solutions were cooled to 37 °C, and 2.5 mL of PB (pH 6.4) was incorporated into each solution. The absorbance of test and control solutions was determined at 660 nm. The assay was performed in triplicate, and the percentage inhibition in protein denaturation was determined.

$$\%\ \%\ \text{Inhibition} = \frac{A_{control}\text{-}(A_{sample} - A_{blank})}{A_{control}} \times 100$$

where, A_{sample} = absorbance of the sample,

A_{blank} = absorbance of blank (without BSA),

$A_{control}$ = absorbance of control (without test sample).

50 % inhibition concentration (IC_{50}) value were calculated by linear regressive excel program.

Results and Discussion

Phytoconstituents Present in Aerial parts of *B. monnieri*

Primary phytochemical screening of *B. monnieri* was performed using the appropriate methods. The phytochemical tests revealed the presence of metabolites such as alkaloids, carbohydrates, glycosides, saponins, organic acids, phenolic compounds, flavonoids, tannins, steroids, and terpenoids. Cyanogenic glycosides and reducing sugar were absent in the sample. These results are summarized in Table 1.

Table 1. Preliminary of Phytochemical analysis on the Aerial Parts of *B. monnieri*

No.	Tests	Extract	Test reagents	Observation	Results
1.	alkaloid	1% HCl	dragendorff's reagent	orange ppt	+
2.	flavonoids	EtOH	EtOH, Mg turning and conc. HCl	pink colour solution	+
3.	glycosides	H_2O	10 % lead acetate	white ppt	+
4.	carbohydrates	H_2O	10 % α-naphthol and conc. H_2SO_4	red ring	+
5.	cyanogenic glycosides	H_2O	sodium picrate	no brick red colour	-

No.	Tests	Extract	Test reagents	Observation	Results
6.	organic acids	H_2O	bromocresol green	green colour	+
7.	phenolic compounds	H_2O	10 % $FeCl_3$	Dark green colour	+
9.	reducing sugars	H_2O	benedict's solution	no brick red ppt	-
10.	saponins	H_2O	distilled water	frothing	+
11.	tannins	H_2O	1% gelatin	white ppt	+
12.	steroids	pet-ether	Ac_2O and conc.H_2SO_4	green colour	+
13.	terpenoids	($CHCl_3$)	Ac_2O and conc. H_2SO_4	pink colour	+

(+) = presence (-) = absence ppt = precipitation

Characterization and Identification of an Isolated Compound

A compound has been isolated from the EtOAc extract of the aerial parts of *B. monnieri* and obtained as colourless crystals with a melting point of 240 °C. It was soluble in chloroform, ethanol, and methanol, but insoluble in pet ether and ethyl acetate. Its R_f value was found to be 0.45 on silica gel TLC with PE: EtOAc (5:1, v/v). It is UV-inactive and according to chemical tests, it did not contain a carbonyl group because the reaction did not give yellow ppt with 2,4-DNP solution. No reaction with a 5 % $FeCl_3$ solution indicated that it was not a phenolic compound. Since an isolated compound gave a pink colour spot when heated along with 5 % sulphuric acid. The pure compound must be a terpenoid compound. The isolated compound also changed the colour of bromocresol to green, indicating that it is an organic acid.

Structure Elucidation of the Isolated Compound

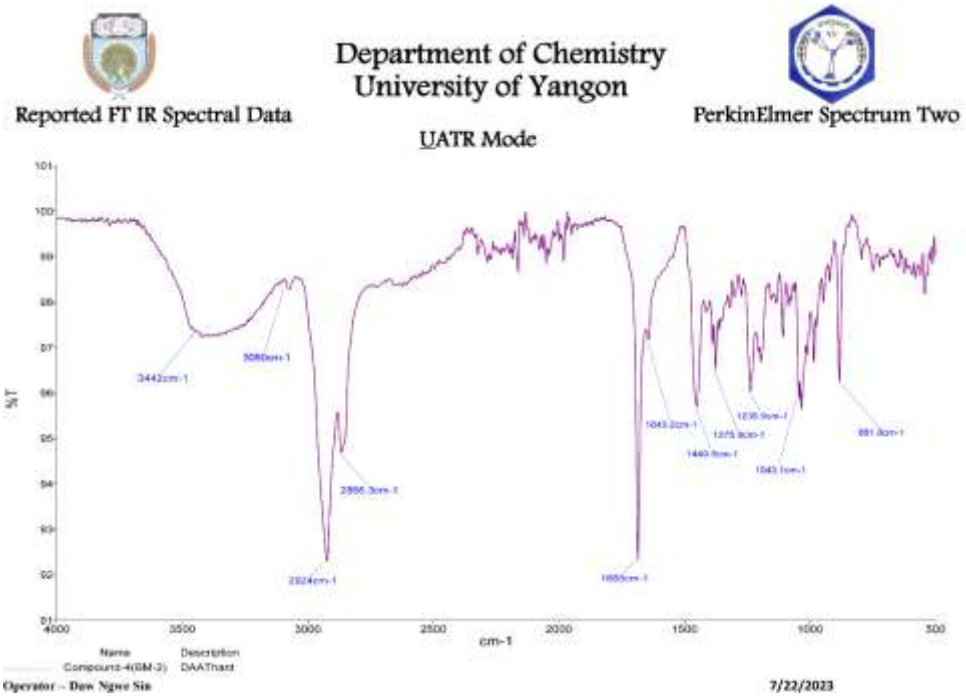


Figure 2. FT IR spectrum of the isolated compound

Table 2. FT IR Spectral Data of the Isolated Compound with Reported Data of Bacosine

Wavenumber (cm^{-1})		Band assignment
Compound	**Bacosine***	
3442	3440	ν_{O-H} of alcoholic O-H group
3080	-	$\nu_{=CH}$ of vinylidene group
2924, 2866	2930, 2850	ν_{C-H} of asym and sym CH_3 and CH_2 group
1685	1680	carbonyl group of esters
1643	1640	$\nu_{C=C}$ of olefinic group
1449	1452	δ_{C-H} of CH_3 and CH_2 group
1375	1368	CH_3 deformation of isopropyl group
1236	1240	ν_{C-O} of carboxylic acid
1043	1040	ν_{C-O} of alcoholic group
882	890	Olefinic C-H out of plane bending

*Ahmed and Rahman, 2000

In FT IR spectrum, the peaks at 3442 cm^{-1}, 3080 cm^{-1}, 1236 cm^{-1}, 1043 cm^{-1} reveals the presence of O-H stretching, C=C bond stretching of alkene, C-O stretching vibrations, commonly found in carboxylic acid, alcohol or esters. (Table 2, Figure 2). The special data are in accordance with reported data of bacosine.

^{1}H NMR and ^{13} C NMR Spectral Data of the Isolated Compound

^{1}H NMR (500 MHz, DMSO-d_6) spectral data is illustrated in Table 3. There are a total of 48 protons, including a double proton at δ 4.27, which indicates the hydroxyl group at C-3. The presence of two signals at δ 4.68 (d, J = 6.5 Hz, α-H-29) and 4.55 (dd, J = 1.4,6.5 Hz, β-H-29) were attributed to two exocyclic methylene protons that established the lupane skeleton for the compound (Figures 3 & 4). Six-methyl signals were resonated at δ 0.76 (Me-23), 0.86 (Me-24), 0.92 (Me-25), 0.87 (Me-26), 0.65 (Me-28) and 1.64 (Me-30). In the ^{13}C NMR spectra, it can be clearly observed in Figure 5 and Table 3 that there are a total of 30 carbons, consisting of one carbonyl carbon, six quaternary carbons, six methine carbons, eleven methylene carbons, and six methyl carbons. one carbinol carbon due to the peak at δ 76.80 (C-3), two olefinic carbons due to the peaks appearing at δ 150.30 and 109.6, and one carboxylic acid group due to the peak at δ 177.2 for the -C=O group (Figure 5). The ^{1}H and ^{13}C NMR values for all the protons and carbons were observed to be identical with the reported data for bacosine (Ahmed and Rahman, 2000), except that some proton chemical shifts (δ_H) were slightly different, which may be due to the solvent effect.

Table 3. ^{1}H and ^{13}C NMR Data of Isolated Compound Compared with Literature Data

Position	Isolated compound (DMSO-d_6, 500 MHz)		*Bacosine ($CDCl_3$, 300 MHz)		Isolated compound δ_C	*Bacosine δ_C
	δ_H (α)	δ_H (β)	δ_H(α)	δ_H(β)		
1	1.34 m	1.79 m	1.35 m	1.70 m	33.90	34.00
2	1.42 m	1.43 m	1.41 m	1.44 m	25.10	25.16
3	-	-	-	-	76.80	76.86
4	-	4.27 m	-	4.27 m	38.70	38.76
5	0.95 m	-	0.97 m	-	48.50	48.61
6	1.48m	1.23 m	1.47 m	1.23m	18.00	18.05
7	1.40 m	1.27 m	1.40 m	1.27 m	36.30	36.42
8	-	-	-	-	40.20	40.43
9	1.53 dd (10, 5Hz)	-	1.53dd (7, 4.5Hz)	-	49.90	50.01
10	-	-	-	-	37.60	37.67
11	2.10 m	1.60	2.08 m	1.59 m	20.50	20.54
12	1.09 m	1.80 m	1.09 m	1.82 m	27.10	27.24
13	2.92 m	-	2.92 m	-	38.50	38.58
14	-	-	-	-	55.40	55.50
15	2.10 m	2.24 m	2.07 m	2.28 m	30,10	30.18
16	1.80 m	2.97 m	1.83 m	2.97 m	31.70	31.79
17	-	-	-	-	42.00	42.08
18	2.12d (10)	-	2.13d (4.5)	-	46.60	46.70
19	2.95 m	-	2.95 m	-	54.90	54.97
20	-	-	-	-	150.30	150.40
21	1.57 m	2.19 m	1.56 m	2.18 m	29.20	29.29
22	2.24 m	1.54 m	2.23 m	1.54 m	36.70	36.81
23	0.76 s	-	0.76 s	-	28.10	28.19
24	0.86 s	-	0.86 s	-	15.70	15.81
25	0.92 s	-	0.93 s	-	15.90	16.04
26	0.86 s	-	0.87 s	-	15.80	15.9
27	-	-	-	-	177.20	177.33
28	0.64 s	-	0.65 s	-	14.40	14.47
29	4.68d (6.5)	4.55d(1.4)	4.68 d (3)	4.5d (1.5)	109.60	109.74
30	1.64 s	-	1.64 s	-	18.90	19.03

*Ahmed and Rahman, 2000

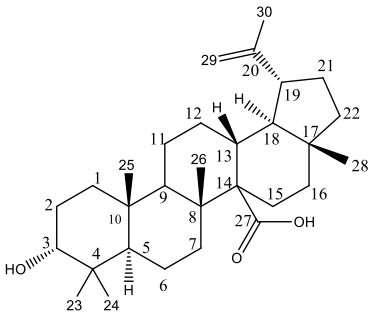


Figure 3. Structure of bacosine

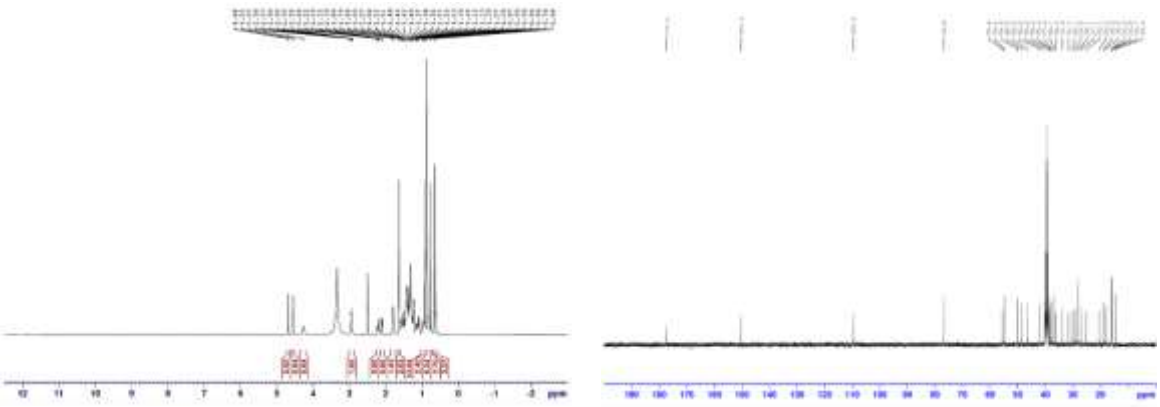
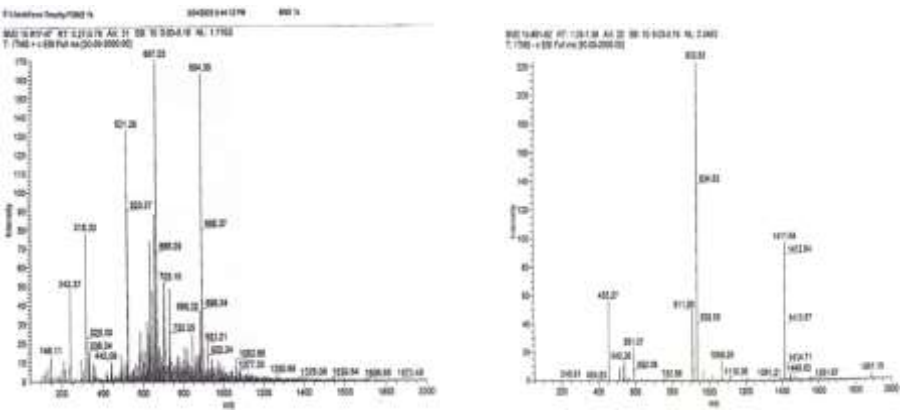


Figure 4. ^{1}H NMR (DMSO-d_6, 500 MHz) and ^{13}C NMR (DMSO-d_6, 125 MHz) spectra of the isolated compound

From the ESI-MS spectrum, $[M+4H+9Na]^+$ was observed, at *m/z* 667 (positive ion mode) (Figure 5a). Consequently, from all of the spectral data analyses, an isolated compound was identified as bacosine with the molecular formula $C_{30}H_{48}O_3$. From the ESI-MS spectrum (Figure 5 b), the molecular mass of the compound depicted the molecular formula $C_{30}H_{48}O_3$, which was deduced from molecular ion peak $[M-H]^-$ at *m/z* 455 (negative ion mode), From all the information, it could be inferred that compound was bacosine with the following structure:



(a) **(b)**

Figure 5. ESI-MS spectra of isolated compound (a) positive ion mode (b) negative ion mode

Anti-inflammatory Activity of *B. monnieri*

Inflammation is a protective or healing response to tissue injury in the body. It is a complex cascade of reactions that can be prompted by several agents, including toxic compounds, microbes, impaired cells, and accumulated exudates (Adegbaju *et al*., 2020). Macrophages are large, specialized cells, present in virtually all tissues. *In vitro* anti-inflammatory activity of the ethanol and watery extracts of BHA was determined by inhibition of NO production against LPS-induced RAW 264.7 cells. NO production is a typical phenomenon that occurs in LPS-stimulated macrophages. It is used as an indicator of a typical inflammatory response. The anti-inflammatory effect of the tested extracts was estimated in comparison with the IC_{50} values of NO inhibition and cell viability. The results of the tested samples are shown in Table 4.

During the 24 h incubation with LPS of tested extracts *of B. monnieri*, the inhibition of NO production in RAW 264.7 cells was dramatically increased at 10 µg/mL and 100 µg/mL concentrations. However, EtOH and watery extracts had higher IC_{50} values of % NO inhibition than IC_{50} values of % cell viability above the 10 µg/mL. Therefore, concentration above 10 µg/mL of two extracts in the sample did not show antiinflammatory activity because of cytotoxicity.

Table 4. % NO Inhibition, % Cell Viability and IC_{50} of Crude Extracts of *B. monnieri* by Inhibition of Cellular NO Production

Sample	Anti-inflammatory activity					
	% NO inhibition			% Cell viability		
	10 µg/mL	100 µg/mL	IC_{50} (µg/mL)	10 µg/mL	100 µg/mL	IC_{50} (µg/mL)
ethanol extract	10.21	60.38	81.38	66.75	60.38	> 100
watery extract	2.82	32.57	> 100	88.20	94.48	< 10
L-NMMA	18.49	50.35	98.25	100.32	92.01	> 100

In vitro Assessment Antiarthritic Activity

Arthritis comprises varieties of joint disorders such as rheumatoid arthritis (RA), osteoarthritis (OA), etc., that affect one joint or multiple joints. Denaturation of tissue proteins is one of the well-documented causes of inflammatory and arthritic diseases. The increments in absorbances of test samples (isolated compounds) with respect to control indicated stabilization of protein, inhibition of heat-induced protein (albumin), denaturation and the reference drug diclofenac sodium. Phytochemical constituents are responsible for maximum protection against protein denaturation and albumin denaturation (Kaushik *et al.,* 2021).

Ethanol and watery extracts and isolated compound bacosine were screened for antiarthritic activity by denaturation of egg albumin and bovine albumin proteins (Tables 5 and 6). Diclofenac sodium was used as the reference drug. In the egg albumin method, the bacosine compound (IC_{50} = 105.23 µg/mL) was more significant anti denaturation effect than ethanol extract (IC_{50} = 162.44 µg/mL) and watery extract (172.71 µg/mL) and in the bovine serum albumin method, the bacosine compound (IC_{50} = 118.73 µg/mL) was more significant anti denaturation effect than ethanol extract (IC_{50} = 180.58 µg/mL) and watery extract (299.24 µg/mL) compared with diclofenac sodium (IC_{50} = 73.44 µg/mL).The present findings exhibited a concentration-dependent inhibition of protein and albumin denaturation.

Table 5. Inhibition at Egg Albumin Denaturation of Crude Extracts and Isolated Compound of *B. monnieri*

Sample	% Inhibition at different concentration (µg/mL)						IC_{50} (µg/mL)
	50	100	200	400	800	1600	
ethanol extract	28.87	36.50	58.12	63.82	74.98	78.32	162.44
watery extract	24.65	31.04	38.89	54.17	61.04	73.37	272.71
bacosine	42.33	49.60	57.24	63.04	78.23	86.38	105.23
diclofenac sodium	45.63	54.95	63.65	72.80	84.53	92.18	73.44

Table 6. Inhibition at Bovine Serum Albumin Denaturation of Crude Extracts and Isolated Compound of *B. monnieri*

Sample	% Inhibition at different concentration (µg/mL)						IC_{50} (µg/mL)
	50	100	200	400	800	1600	
ethanol extract	40.36	42.32	51.85	63.16	66.67	73.69	180.58
watery extract	26.32	38.08	47.37	52.64	56.15	64.92	299.24
bacosine	38.17	47.37	61.41	64.16	68.43	81.93	118.73
diclofenac sodium	45.63	54.95	63.65	72.80	84.53	92.18	73.44

Conclusion

The preliminary phytochemical analysis showed the presence of bioactive secondary metabolites in this plant sample. In this study, the isolation of bacosine ($C_{30}H_{48}O_3$) from the ethyl acetate fraction of *B. monnieri* was accomplished by modern spectroscopic techniques: FT IR, ^{1}H NMR (500 MHz), and ^{13}C NMR (125 MHz). The compound was elucidated as a known compound, in agreement with reference data. The ethanol extract had a higher IC_{50} of NO inhibition than that of cell viability because of cytotoxicity, watery extracts had higher IC_{50} of NO inhibition and cell viability above 100. The ethanol extract exhibited significant inhibitory inflammation however; it can be exhibited less concentration 100 µg/mL. The inhibition of protein denaturation and albumin denaturation was studied to estimate the antiarthritic activity of isolated compounds, ethanol, and watery extracts of the aerial parts *B. monnieri*. The present findings demonstrate the significant antiarthritic activity compared with the standard drug diclofenac sodium.

Acknowledgements

The authors would like to express their profound gratitude to the Department of Higher Education, Ministry of Education, Yangon, Myanmar, for giving them the opportunity to do this research and the Myanmar Academy of Arts and Science for allowing them to present this paper.

References

Adegbaju, O. D., G. A. Otunola, and A. J. Afolayan. (2020). "Anti-inflammatory and Cytotoxic Evaluation of Extracts from the Flowering Stage of *Celosia argentea*". *BMC Complementary Medicine and Therapies,* vol**.** 20 (152), pp 1-7.

Ahmed, B. and A. Rahman. (2000). "Basosterol, a New 13, 14-seco-steriod and Bascosine, a New Triterpene from *Bacopa monniera*". *Indian Journal of Chemistry*, vol.39 B, pp 620-625.

Channa, S., A. Dar, S. Anjum, M. Yaqoob, and A. Rahman. (2006). "Anti-inflammatory Activity of *Bacopa monniera* in Rodents". *Journal of Ethnopharmacology*, vol. 104, pp 286–289.

Jin, S. E., Y. K. Son, B. S. Min, H. A. Jung, and J. S. Choi. (2012). "Anti-inflammatory and Antioxidant Activities of Constituents Isolated from *Pueraria lobata* Roots". *Arch Pharm Res*: vol.5, pp. 823-837.

Kaushik, S., P. Jain, and T. Satapathy. (2021). "Prerna Purabiya1 and Amit Roy Evaluation of Anti-arthritic and Anti-inflammatory Activities of *Martynia annua* L. Ethanolic Extract". *Clinical Phytoscience,* vol.7 (7), pp.1-11.

Kishore, L., N. Kaur, and R. Singh. (2017). "Bacosine Isolated from Aerial Parts of *Bacopa monnieri* Improves the Neuronal Dysfunction in Streptozotocin-induced Diabetic Neuropathy". *Journal of Functional Foods*, vol.34, pp 237–247.

Madhuranga, H. D. T., and D. N. A. W. Samarakoon. (2023). "*In vitro* Anti-Inflammatory Egg Albumin Denaturation Assay: An Enhanced Approach". *Nat Ayurvedic Med*., vol. 7(3), pp. 1-7.

Saleema, A., M. Saleema, and M. F. Akhtar. (2020). "Antioxidant, Anti-inflammatory and Antiarthritic Potential of *Moringa oleifera* Lam: An Ethnomedicinal Plant of Moringaceae Family". *South African Journal of Botany*, vol.128, pp.246-256.

Schmidt, H. and M. Kelm. (1996). *Determination of Nitrite and Nitrate by the Griess Reaction*. In Feelisch, M. and J. Stamler (Eds.) Methods in Nitric Oxide Research. Chichester (UK): John Wiley & Sons, vol. 12, pp.491-497.