SCREENING OF PHYTOCHEMICALS AND SOME BIOLOGICAL ACTIVITIES OF THE AERIAL PARTS OF *BACOPA MONNIERI* (L.) WETTST. (BYONE-HMWE)

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Abstract

The aim of the study was to screen some phytochemical constituents of *Bacopa monnieri* (L.) Byone-hmwe such as total phenolic and total flavonoids contents, and some biological activities such as antioxidant, antimicrobial, and antiproliferative activities. The total phenolic content was determined by Folin-Ciocalteu's assay and expressed as GAE equivalent. An aluminium chloride colorimetric assay was used to calculate the total flavonoids content, which was then expressed as QE equivalent. The antioxidant activity of ethanol and water extracts of *B. monnieri* was determined by DPPH assay. These assays found that the ethanol extract (IC_{50} = 470.15 µg/mL) was more potent than the water extract (IC_{50} =770.54 µg/mL) in antioxidant activity. The ethyl acetate extract of the plant was found to have high potent antimicrobial activities against all six tested microorganisms, with inhibition zone diameters (21-26 mm) determined by agar well diffusion method those of pet-ether, ethanol and water extracts. The *in vitro* antiproliferative activity of ethanol extract was more potent against A549 (lung) and Hela (cervical) human cancer cell lines (IC_{50} = 115.03 µg/mL and IC_{50} = 129.95 µg/mL) than the water extract determined by MTT assay.

Keywords*: Bacopa monnieri*, antioxidant, antimicrobial and antiproliferative activities

Introduction

Byone-hmwe, a member of the *Scrophulariaceae* family, is a small, creeping herb with numerous branches, small oblong leaves and light purple or white flower (Bone, 1996). It grows in grassland occurring in aquatic sites, sand and wet soil occupying the edges of freshwater or brackish pools, streams and lake beds. Flowers and fruits appear in summer, and the entire plant is used medicinally. According to World Health Organization (WHO), the majority of the World's population uses traditional medicines for their primary health care needs. Plant secondary metabolites possesses biological properties such as antiapoptosis, antiaging, antiatherosclerosis, cardiovascular protection, inhibition of angiogenesis and cell proliferation activity (Han *et al.*, 2007). It is also used in analgesic and antipyretic activity to treat asthma, insanity epilepsy, hoarseness, enlargement of spleen, snake bite, rheumatism, leprosy, eczema and ringworm. It is used as a diuretic appetitive and cardio tonic (Mishra *et al.*, 2015). Byone-hmwe contains alkaloids, glycoside, flavonoids and saponins (Saraswati *et al.*, 1996). Byone-hmwe has antianxiety, anticancer, antidepressant, antidiabetic, antihypertensive, anti-lipidemia; anti-inflammatory, antimicrobial, antioxidant, hepatoprotective, gastrointestinal protective and neuroprotective activities (Natthawut *et al*., 2016). Byone-hmwe is an important medicinal herb in Ayurveda for the treatment of a number of health problems. The purpose of the present study is to screen the antioxidant, antimicrobial and anti-proliferative activities of some crude extracts of the aerial parts of Byone- hmwe.

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Botanical Aspect of *Bacopa monnieri* (L.) Wettst (Byone-hmwe)

Family : Plantaginaceae

Botanical name : *Bacopa monnieri* (L.) Wettst.

Myanmar name : Byone-hmwe

Part used : the aerial parts

Bacopa monnieri

Materials and Methods

Plant Material

The sample was collected from Yangon Region, Myanmar in the month of May 2019 and identified as *B. monnieri* by the authorized botanist at the Department of Botany, University of Yangon. The aerial parts including flowers and leaves was dried under the shade for a week, cut into very small pieces and then ground into purely fine powder using an electric grinder. The powdered sample was stored in the airtight containers.

Chemicals

95 % Ethanol, ethyl acetate, pet-ether, 2,2-diphenyl-1-picryhydrazyl (DPPH), dimethyl sulfoxide (DMSO), trypticase soy broth from Difco U.S.A, tryticase soy agar from Becton, U.S.A, Muller-Hinton agar (Hi-Media) and triple sugar iron sugar from Becton, U.S.A. phosphate buffer saline (PBS) powder, fetal bovine serum (FBS, Sigma 172012), trypsin, alcohol (70 % ethanol), Minimum Essential Medium (α MEM, Wako 135-15735), 0.1 mM non-essential amino acid (NEAA, Gibo 11140-050),1 mM sodium pyruvate (SM, Gibco-11360-070)

Instruments

Quartz cuvette (4 mL), UV-visible spectrophotometer (UV-7504), a stirrer, an autoclave (Tomy Seiko Co., Ltd, Tokyo, Japan), a constant temperature bath (Yamato Scientific Co., Ltd, Japan), sterile petri-dish, spirit burner, polyethylene plastic bag, a refrigerator and an incubator multipipette, 96 well plate, aluminum foil, centrifuge tube, Haemacytometer, microscope and vibrator.

Preparation of Extracts

Each 50 g of the dried Byone-hmwe powder was extracted with ethanol, petroleum ether, ethyl acetate and water by sonication using each solvent (100 mL,1 h) at 70 ℃. Each filtrate was evaporated under reduced pressure by a rotatory evaporator to yield different solvent extracts.

Preliminary Phytochemical Tests

Preliminary phytochemical tests on the powdered sample were carried out according to the reported methods in order to classify the types of organic constituents present in the samples such as alkaloids, α-amino acids, carbohydrates, cyanogenic glycosides, flavonoids, glycosides, organic acids, phenolic compounds, reducing sugars, saponins, starch, steroids, tannins and terpenoids by appropriate reported methods (M-Tin Wa, 1972).

Determination of Total Phenol Contents by Folin-Ciocalteu Method

Total phenolic contents of ethanol and water extracts were estimated by the Folin-Ciocalteu method as described by Kaveti. *et al* (2011). A dilute extract (1 mL) of gallic acid used as a standard was mixed with Folin-Ciocalteu reagent (5 ml, 1:10 diluted with water) and 4 mL of 1 M of

aqueous sodium carbonate. The mixture was left to stand for 30 min at 25 °C for the colour to develop. Absorbance was measured at wavelength 765 nm UV-spectrophotometer (Shimadzu, USA). Sample of extracts were evaluated at a concentration of 1000 mg/mL. The total phenolic contents were expressed in terms of gallic acid equivalent, GAE (standard curve equation: $y = 0.0177x + 1.0207$, $R^2 = 0.9918$), mg of GAE/g of dry extract. The experiment was repeated three times at each concentration.

Determination of Total Flavonoids Contents by Aluminum Chloride Colorimetric Assay

Total flavonoids contents of ethanol and water extracts were determined by using aluminum chloride colorimetric assay described by Kaveti *et al*. (2011).1 mL of samples/standard (Quercetin) was mixed with 1.5 mL of methanol. A 0.1 mL of 1 % $AlCl_3$ and 0.1 mL of 1 M potassium acetate and 2.8 mL distilled water was added to the mixture and left at RT for 30 min. The absorbance of the mixtures was measured at wavelength 415 nm. The total flavonoids contents were expressed in terms of quercetin equivalent, QE (standard curve equation: $y = 0.0039x + 0.0199$, $R^2 = 0.9986$), mg of QE/g of dry extract. The experiment was repeated three times at each concentration.

Antioxidant Action by DPPH Free Radical Scavenging Assay

Preparation of DPPH solution

DPPH (4.732 mg) was thoroughly dissolved in 95 % ethanol (100 mL). This solution was freshly prepared in the brown-coloured reagent bottle and stored in the fridge for no longer than 24 h. Each tested sample (20 mg) and 20 mL of ethanol were thoroughly mixed by shaker. The mixture solution was filtered and the stock solution (1000 µg/mL) was obtained. By adding ethanol, DPPH solutions with different concentrations of 500–62.5 µg /mL were prepared from the stock solution.

Determination of antioxidant activity

The effect of Byone-hmwe on the DPPH radical was determined by using the method of Marinova and Batchvarov (2011). The control solution was prepared by mixing 1.5 mL of 120 µM DPPH solution and 1.5 mL of 95 % ethanol using shaker. The test sample solution was also prepared by mixing thoroughly 1.5 mL of 120 µM DPPH solution (Absorbance = 0.8941 used for control) and 1.5 mL of each sample solution (1000-62.5 µg/mL). The mixture solutions were allowed to stand at room temperature for 30 min. Then, the absorbance of these solutions was measured at 517 nm by using UV-7504 spectrophotometer. Absorbance measurements were made in triplicate for each concentration and then mean values so obtained were used to calculate percent inhibition of oxidation by the following equation:

$$\% \text{ RSA} = \frac{A_{DPPH-}(A_{smaple-}A_{blank})}{A_{DPPH}} \times 100$$

where, % RSA = % radical scavenging activity

A_{DPPH} = absorbance of DPPH in EtOH solution

A_{Sample} = absorbance of sample and DPPH solution

A_{blank} = absorbance of sample and EtOH solution

The experimental results were performed in triplicate. The data were recorded as mean ± standard deviation.

Screening of Antimicrobial Activity of the Samples by Agar Well Diffusion Method

The antimicrobial activity of four crude extracts such as pet-ether, ethyl acetate, ethanol and water extracts from the aerial parts of Byone-hmwe was determined against six strains of microorganisms such as *Bacillus subtilis* (IFO 90571), *Staphylococcus aureus* (AUH5436), *Pseudomonas aeruginosa, Salmonella typhi* (AUH8465), *Candida albicans* (NITE09542) and *Escherichia coli* (AUH5436) by employing agar well diffusion method (Anibijuwon and Udeze, 2009). These tests were screened at the strains' storage facility, Sagaing University.

Preparation of nutrient agar medium

A mixture of meat extract (0.5 g), peptone (0.5 g), sodium chloride (0.25 g) and 1.5 g of agar powder was placed in a sterilized conical flask, 100 mL of sterile distilled water was added to obtain nutrient agar medium. The resulting mixture was heated to dissolve the contents. Then, the pH of this solution was adjusted to 7.2 with 0.1 M sodium hydroxide solution. It was sterilized in an autoclave at 121 ℃ for 15 min.

Determination of antimicrobial activity by agar well diffusion method

Agar well diffusion method was used to evaluate the antimicrobial activities of the extracts against bacteria and fungi. Different extracts (1 mg each) were dissolved in 1 mL of their respective solvent. After inoculation, plates were dried for 15 min. A hole with a diameter 8 mm was punched aseptically with a sterile cork or a tip and volume 0.1 mL of extract solution at desired concentration was introduced into the well. Then, agar plates were incubated under suitable conditions depending upon the microorganisms. The antimicrobial agent diffuses in the agar medium and inhibits the growth of the microbial strain tested. The plates were incubated for 24 h to allow the extract to diffuse through the agar media to form zones of inhibition. The extent of antimicrobial activity was measured by the diameter of inhibition zone.

Investigation of Antiproliferative Activity of Ethanol and Water Extracts against Human Cancer Cell Lines

Antiproliferative activity of ethanol and water extracts of Byone-hmwe was investigated by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT) to formazan at Division of Natural Product Chemistry, Institute of Natural Medicine, and University of Toyama, Japan. The cell lines used were Hela (human cervix cancer) and A549 (lung cancer). Minimum essential medium with L-glutamine and phenol red (α-MEM, Wako) were used for cell cultures. All media were supplemented with 10 % fetal bovine serum (FBS, sigma) and 1 % antibiotic antimycotic solution (Sigma). From the above medium solution, 100 mL of this supplemented medium was mixed with 1 mL of non-essential amino acid (NAA) for A-549. The *in vitro* antiproliferative activity of the crude extracts was determined by the procedure described by (Win *et al.,* 2015). Briefly, each cell line was seeded in 96-well plates (2×10^3 per well) and incubated in the respective medium at 37 °C under 5 % CO_2 and 95 % air for 24 h. After the incubation, the cells were washed with PBS, serial dilutions of the tested samples were added. The sample solution in wells with cells were incubated in an incubator for 72 h. The sample solution with cell and medium was added with 100 µL MTT reagent. And then the wells were incubated in an incubator for 3 h, After the incubation, cells in the medium were aspirated with aspirator. The cell was washed with PBS (5 mL) 2 times. Then, DMSO was added about 100 µL per well and the 96 well plates were placed in the dark for 15 min. And then, the absorbance of each solution was measured at 570 nm by using UV-visible spectrophotometer. The concentrations of the crude extracts were 200, 20 µg/ mL and 20, 10, 2 mM for positive control were prepared by serial dilution. Cell viability was calculated from the mean values of the data from three wells using the

equation below and antiproliferative activity reagent was expressed as the IC_{50} (50 % inhibitory concentration) value, 5-fluorouracil (5FU) was used as a positive control.

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

where,

$A_{(\text{test sample})}$ = absorbance of test sample solution

$A_{(\text{control})}$ = absorbance of DMSO solution

$A_{(\text{blank})}$ = absorbance of MTT reagent

Results and Discussion

Phytoconstituents in Byone-hmwe

The phytochemical screening of Byone-hmwe was preliminarily carried out by the appropriate methods and the results are shown in Table 1. The phytochemical tests revealed the presence of the secondary metabolites such as alkaloids, α-amino acids, carbohydrates, glycosides, saponins, phenolic compounds, flavonoids, tannins, steroids and terpenoids but cyanogenic glycosides, starch, and reducing sugars were absent in the aerial parts of Byone-hmwe.

Table 1 Results of Preliminary Phytochemical Tests on Byone-hmwe

No.	Test	Extracts	Test Reagent	Observation	Remark
1	Alkaloids	1% HCl	(i)Wagner's	reddish brown ppt	+
			(ii) Dragendorffs'	orange ppt	+
			(iii) Mayer's	white ppt	+
			(iv) Sodium picrate	yellow ppt	+
2	α-amino acids	H_2O	Ninhydrin	violet spot	+
3	Carbohydrates	H_2O	10 % α-naphthol & conc:H_2SO_4	red ring	+
4	Cyanogenic glycosides	H_2O	Sodium picrate solution	no brick red colour	-
5	Flavonoids	EtOH	Mg ribbon & conc: HCl	pink colour	+
6	Glycosides	H_2O	10 % lead acetate	white ppt	+
7	Phenolic compounds	EtOH	1 % $FeCl_3$	green colour	+
8	Reducing sugars	H_2O	Fehling's solution A & B	no brick red ppt	-
9	Saponins	H_2O	distilled water	frothing	+
10	Starch	H_2O	iodine solution	no blue colour	-
11	Steroids	PE	acetic anhydride & conc:H_2SO_4	green colour	+
12	Tannins	EtOH	1 % gelatin	white ppt	+
13	Terpenoids	$CHCl_3$	acetic anhydride & conc:H_2SO_4	pink colour	+

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

Total Phenolic and Flavonoids Contents

In the present study, the resulted total phenolic and flavonoids contents in ethanol and water extracts of Byone-hmwe aerial parts are shown in Table 2 and Figure 2. The aerial parts of Byone-hmwe contained significant total flavonoids contents. The ethanol extract with mean value of 7.435 mg GAE/g extract had higher potency than water extract with mean value 3.596 mg GAE/g extract and total flavonoids content expressed that ethanol extract with mean value of 24.05 mg QE/g was more potent than water extract with mean value of 15.23 mg QE/g extract, respectively.

Table 2 Total Phenolic and Flavonoids Contents of Extracts of Byone-hmwe Aerial Parts

Extracts	TPC (mg GAE/ g ± SD)	TFC (mg QE /g ± SD)
Ethanol	7.435 ± 0.029	24.05 ± 0.004
Water	3.596 ± 0.07	15.23 ± 0.002

* mg gallic acid and quercetin equivalent /g extract. each concentration

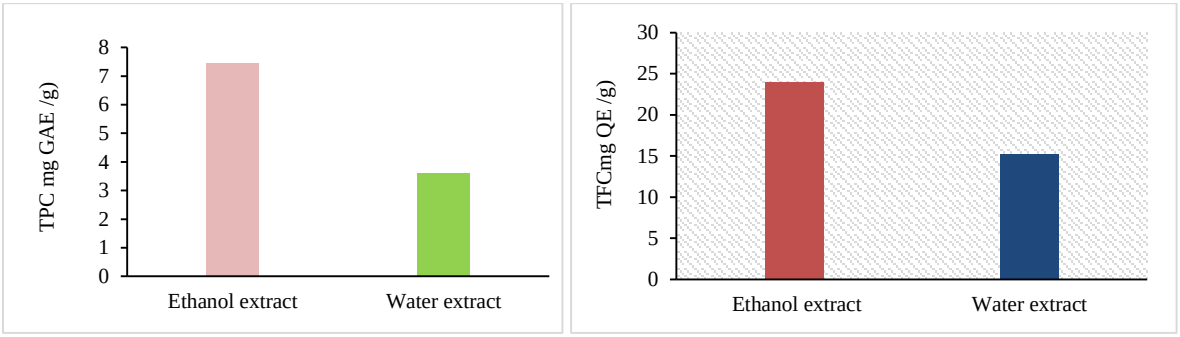

Figure 2 Total phenolic and flavonoids contents of ethanol and water extracts of the aerial parts of Byone-hmwe

In vitro Antioxidant Activity

Free radicals scavenging capacity of the extract was determined using the stable free radical containing DPPH. It can accept an electron or hydrogen radical. The odd electron in it makes the solution to appear deep violet in colour. The absorption vanishes when DPPH accepts an electron resulting in delocalization. DPPH radical scavenging ability of an antioxidant is supposed to be due to hydrogen donating property (Soares *et al*., 1997). In the DPPH free radical scavenging assay, 62.5-1000 µg/mL of ethanol and water extracts of the aerial parts of Byone-hmwe were used. A decrease in absorbance exhibits increase in radical scavenging activity. The radical scavenging activities of two crude extracts were expressed in terms of % RSA and IC_{50} (50 % inhibitory concentration). The IC_{50} values of ethanol and water extracts of the aerial parts of Byone-hmwe were found to be 470.15 µg/mL and 770.54 µg/mL, respectively. Similarly, the reference ascorbic acid (6.25-100 µg/mL) showed significant free radicals scavenging activities ranging from (26.9- 88.34 %) and IC_{50} value was 11.9 µg/mL. Since the lower the values, the greater the antioxidant activity of the samples. The ethanol extracts of the aerial parts of the plant had higher antioxidant activity than water extracts. The results are shown in Table 4 and Figure 3, 5.

Table 3 Antioxidant Activity of Crude Extracts from the Aerial Parts of Byone-hmwe

Crude extracts	% RSA ± SD at different concentrations (µg/mL)					IC_{50} (µg/mL)
	62.5	125	250	500	1000	
Water extract	11.36 ± 0.33	20.13 ± 1.12	30.13 ± 0.56	40.38 ± 0.48	58.02 ± 0.37	770.54
Ethanol extract	25.2 ± 0.57	35.75 ± 0.98	46.3 ± 0.66	57.3 ± 0.3	67.14 ± 0.26	470.15

Table 4 % RSA (Radical Scavenging Activity) of Standard Ascorbic Acid

Sample	% RSA ±SD at different concentrations (µg/mL)					IC 50 (µg/mL)
	6.25	12.5	25	50	100	
Ascorbic acid	26.9 ± 0.04	52.41 ± 0.01	70.55 ± 0.01	88.34 ± 0.01	94.62 ± 0.01	11.9

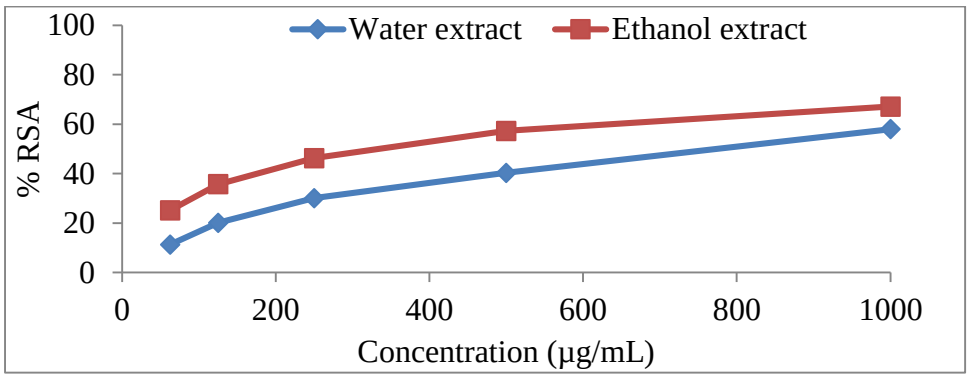

Figure 3 DPPH radical scavenging activities of crude extracts of Byone-hmwe at different concentrations

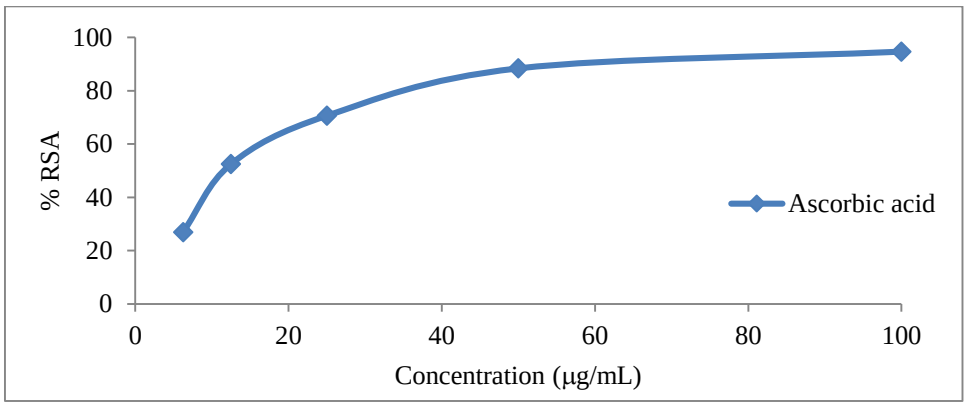

Figure 4 DPPH free radical scavenging activities of standard ascorbic acid

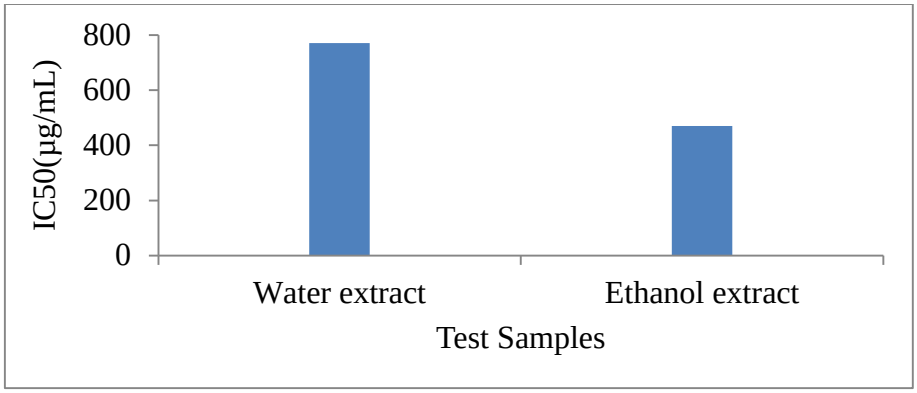

Figure 5 Comparison of percent inhibition and IC_{50} value of crude extracts of Byone-hmwe

Antimicrobial Activity

The antimicrobial activity was determined by measuring the diameter of the zone of inhibition and recording it (Table 5). According to the method described by Selvamohan *et al*. (2012), in this investigation, four extracts (pet-ether, ethanol, ethyl acetate and water of Byone-hmwe were tested against two-gram negative bacteria (*P. aeruginosa and E. coli*), three-gram positive bacteria (*B. subtilis, S. typhi and S. aureus*) and *C. albicans* fungus by agar well diffusion method. The measurable zone diameter, including the well diameter, shows the degree of completeness of the antimicrobial activity. In this experiment, the well diameter was set as 8 mm (Figures 6 and 7). The greater zone diameter, the greater the activity of the tested organisms. According to the results, it was found that water and ethanol extracts of Byone-hmwe exhibited activity against all six microorganisms, with the zone diameter inhibition range between 14-19 mm. Moreover, ethyl acetate extract (the inhibition zone diameter 21-26 nm) and pet–ether extract (the inhibition zone diameter 11-14 mm) were also tested against six microorganisms, water and ethanol extracts showed medium activity on selected microorganisms, and pet ether extract showed low activity against *B. subtilis, C. albicans, E. coli, P. aeruginosa, S. aureus and S. typhi.*

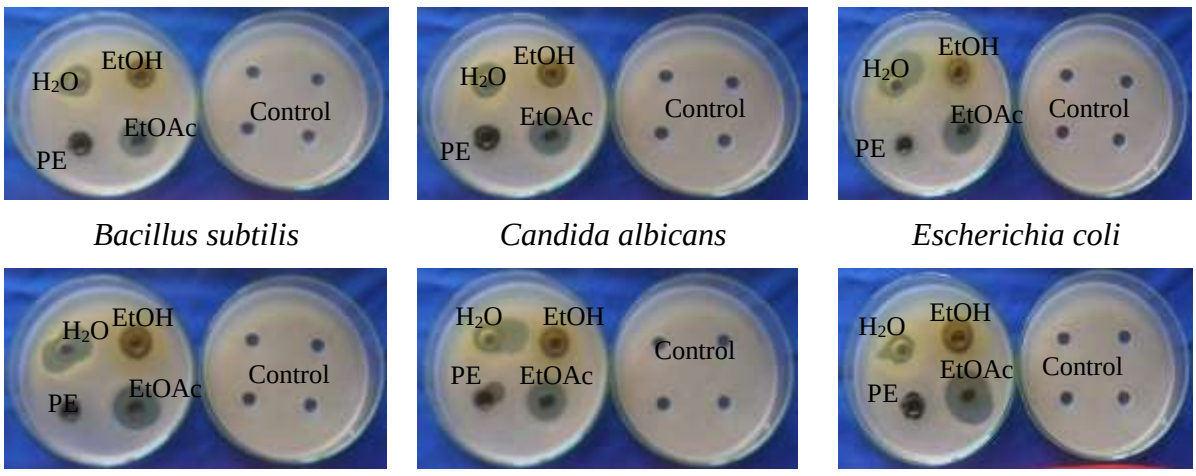

Figure 6 Screening of antimicrobial activities of the aerial parts of *Bacopa monnieri*

Table 5 Inhibition Zone Diameter of the Aerial Parts of *Bacopa monnieri* Against Six Different Microorganisms

Extracts	Inhibition zone diameter (mm) against six microorganisms					
	B. subtilis	***C. albicans***	***E. coli***	***P. aeruginosa***	***S. aureus***	***S. typhi***
Water	15 (++)	16 (++)	14 (+)	15 (++)	16 (++)	16 (++)
EtOH	15 (++)	15 (++)	14 (+)	17 (++)	16 (++)	19 (++)
EtOAc	21 (++)	22 (+++)	22 (+++)	26 (+++)	21 (+++)	26 (+++)
PE	13 (+)	14 (+)	11 (+)	11 (+)	11 (+)	14. (+)

Diameter of agar well = 8 mm
9 mm-14 mm = (+) Low activity
15 mm- 20 mm = (++) Medium activity
21 mm above = (+++) High activity

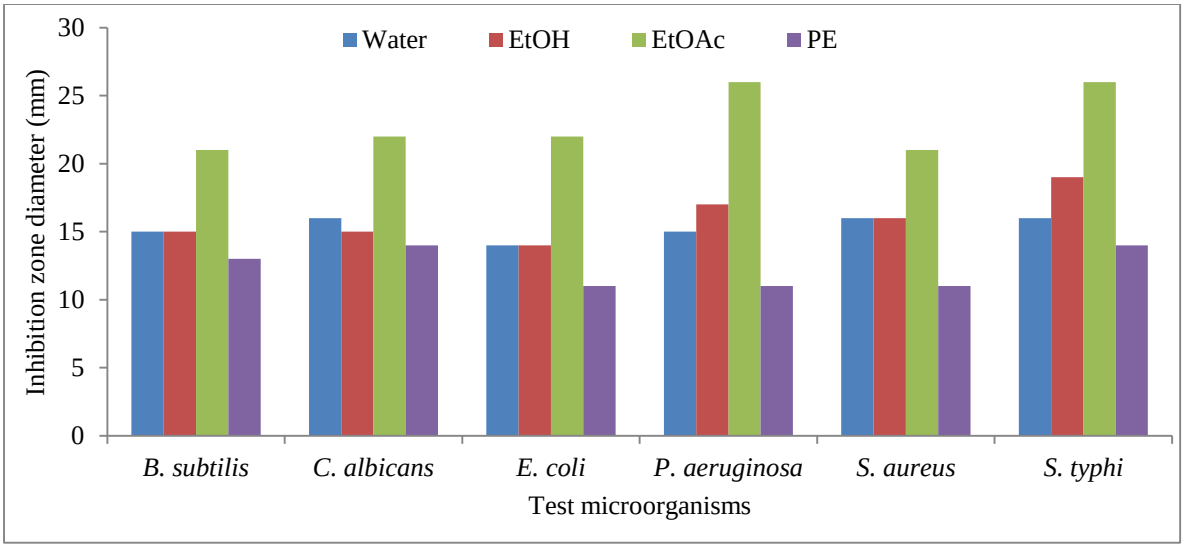

Figure 7 Inhibition zone diameters for crude extracts of the aerial parts of Byone-hmwe

Antiproliferative Activity of the Ethanol and Water Extracts Against One Human Cancer Cell lines

Antiproliferative activity is the activity relating to a substance used to prevent or retard the spread of cells, especially malignant cells, into surrounding tissues. The MTT assay was used to assess the antiproliferative activity of ethanol and water extracts of the Byone-hmwe on two cancer cell lines, A 549 (lung cancer cell line) and Hela (Cervix cancer cell line). The anticancer effect was expressed as IC_{50} values (50 % inhibitory concentration). The lower the IC_{50} values, the higher antiproliferative activity is. According to these results, the ethanol extract of the Byone-hmwe showed antiproliferative activity with the IC_{50} values of 115.03 µg/mL (for the A549 cell line) and 129.95 µg/mL (for Hela cell line). But the ethanol extracts of Byone-hmwe possessed weaker antiproliferative activity for two cancer cell lines (A 549 and Hela) which compared with water extracts because of their IC_{50} values of >200 µg/mL. The test samples had weaker antiproliferative activity when compared with standard 5 FU (Table 6 and Figure 8).

Table 6 Antiproliferative Activity of Crude Extracts of Byone-hmwe

Text Sample	Antiproliferative activity					
	Lung			Cervix		
	20 (µg/mL)	200 (µg/mL)	IC_{50} µg/mL	20 (µg/mL)	200 (µg/mL)	IC_{50} (µg/mL)
Ethanol extract	96.47 ± 17.04	8.45 ± 0.07	115.03	72.25 ± 9.19	6.59 ± 0.00	129.95
Water extract	72.23 ± 0.64	91.71 ± 7.07	>200	96.83 ± 0.64	91.29 ± 0.71	>200

Table 7 Antiproliferative Activity of Standard of 5- Fluorouracil

Positive Control	2 (µg/mL)	10 (µg/mL)	20 (µg/mL)	IC_{50} (µg/mL)
5- Fluorouracil (Lung cancer cell line)	136.24 ± 12.94	70.45 ± 5.59	47.89 ± 8.21	19.06
5- Fluorouracil (Cervix cancer cell line)	91.44 ± 24.93	85.22 ± 4.95	24.93 ± 0.28	15.84

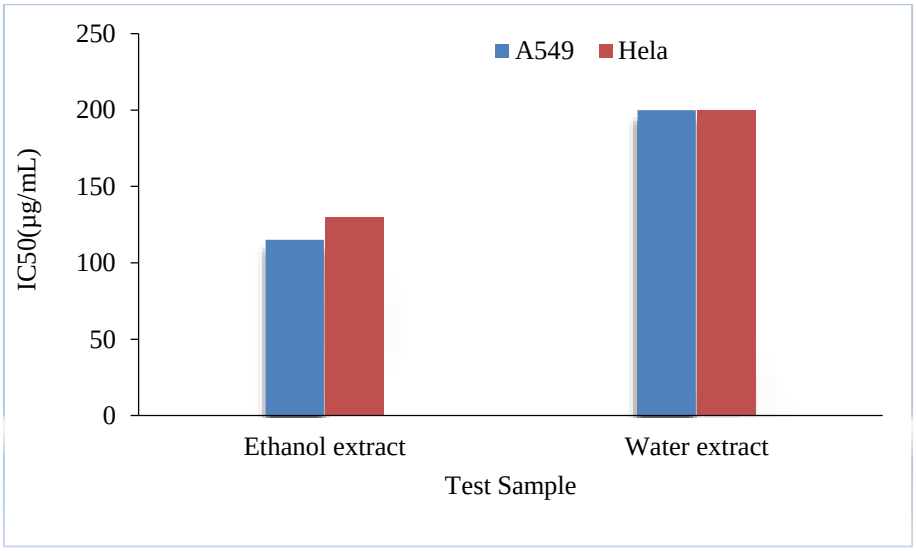

Figure 8 IC_{50} values of crude extracts of the aerial parts of Byone –hmwe

Conclusion

From the preliminary phytochemical results, the aerial parts of Byone-hmwe extracts had bioactive secondary metabolites such as alkaloids, phenolic compounds, steroids, flavonoids and terpenoids. (Jun, *et al*.,2017 and Gharech, *et al.*,2014). Therefore, ethanol, water, ethyl acetate and pet-ether extracts were examined antimicrobial activity, and water and ethanol extracts were screened for antioxidant and antiproliferative activities. The pharmacological activities based on extraction was carried out the ethanol, water, pet-ether and ethyl acetate extracts, the aerial parts of Byone- hmwe. The ethanol extract had higher phenol contents (7.435 ± 0.029 mg GAE/g) and flavonoids contents (24.05 ± 0.004 mg QE/g). The antioxidant activity of ethanol extract (IC_{50} = 470.15 µg/mL) is higher than that of water extract (IC_{50} = 770.54 µg/mL). The ethyl acetate extract of Byone-hmwe had the highest antimicrobial activity (inhibition zone diameter 21-26 mm) compared to the pet-ether, water and ethanol extracts. According to the results, the ethanol extracts was found to possess mild antiproliferative activity against human cancer cell lines such as A549 lung cancer cell line (IC_{50} = 115.03 µg/mL and Hela cervical cancer cell line (IC_{50} = 129.95 µg/mL).

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