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Quantitative Determination of Free Radical Scavenging, Antitumor and Antimicrobial Activities of Some Myanmar Herbal Plants

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Abstract. Various organic and aqueous extracts of leaves of *Ardisia japonica* Blume, *Ageartum conyzoides Linn., and Cocculus hirsutus Linn Diels.,* obtained by maceration were screened for their antioxidant and anti-tumor and antimicrobial activities in this research. All of these extracts have remarkable antioxidant activities and EC_{50} values were 12.72µg/ml for *A. japonica,* 15.19µg/ml for *A. conyzoide* and 10.68µg/ml for *C. hirsutus* respectively. According to results from bioassay with carrot discs infected with *Agrobacterium tumefaciens*, no gall was detected in carrot disks treated with 100 ppm dose of *C. hirsutus* and *A. japonica* crude extracts and 1000ppm dose of *A.* conyzoides extract after 3 weeks incubation. According to the *in vitro* brine shrimp larvae lethal toxicity test, the LC_{50} value of the crude extract ranged from 890ppm for *A. japonica*, 768ppm for *A. conyzoides* and 587ppm for *C. hirsutus* respectively and that were very much higher than that of potassium dichromate. *A. japonica* was selected to do further experiments as it has highest LC_{50} value among these three plants. In *in vivo* toxicity test with mice model, there is no toxicity of *A. japonica* extract dosage up to 2500mg/kg/day indicating its safety for further experiments. According to antagonistic activities against nine different species of food borne and human pathogenic microorganisms by the agar-well diffusion method, the methanol fraction of crude extract of the leaves of *A. japonica* showed strong inhibitory activity against the food borne pathogenic bacteria *Shigella boydii* with 34mm in diameter. Ethyl acetate fractions also showed best results against other pathogens. The MIC value was in the ranged from 0.625 to 5.0mg/ml and the MBC value was in the ranged from 0.625 to 10.0mg/ml for these tested microorganisms. Therefore, the research clearly indicates that these herbal plants of Myanmar's dry farm land are expectionally advantageous for human health especially *A. japonica* can be used as anti-microbial drug or antioxidant diet or as food preservative.

Keywords: antimicrobial, antioxidant, antitumor, pathogen, preservative

1. Introduction

Medicinal plants are an important source of practical and inexpensive new drugs. Myanmar is rich in varieties of medicinal as well as aromatic plants due to the presence of different climate zone in the country. There are 7000 different plants growing in Myanmar and most of them have been recognized as medicinal plants. Ever green shrubs, *Ardisia japonica* Blume; and *Ageratum conyzoides* L., and ever green climber shrub *Cocculus hirsutus* (L) Diels are widely growing throughout Myanmar. Although species of *Ardisia, Ageratum and Cocculus* are rich sources of novel and biologically potent phytochemical compounds, the utilization of *Ardisia* species or their phytochemical constituents have not been fully explored, resulting in underexploitation of their uses [1]. The aim of my research here is to study on the Myanmar traditional herbs that play a very important role in the development of new drugs. The objective of this research is to find out the potential antioxidant, antitumor and antimicrobial herbal drugs as well as herbal food preservatives.

2. Materials and Methods

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2.1. Preparation of Plant Extract and Phytochemical and Mineral Analysis

A known mass of each air dried leaves powder of *A. japonica*, *A. conyzoides and C. hirsutus* was soaked in ethanol for 1 month. The extracts obtained were then concentrated and stored in sealed vials in the refrigerator prior to further processes. Preliminary phytochemical examination of these plant extracts were analyzed by qualitative method. Ash and mineral contents were also determined quantitatively [2].

2.2. Quantitative Determination of *in vitro* Free Radical Scavenging Activity

In this bioassay, 1 ml of varying concentrations (5, 10, 15, 20 and 25 ug/ml) of each sample extract was mixed with 2 ml of 0.1mM DPPH(1,1-diphenyl-2-picryl hydrazyl radical) solution in methanol for 30min in the dark at room temperature[3]. Each test sample solutions were prepared as blank solutions when negative control was DPPH solution. L-ascorbic acid (Vitamin C) has been used as reference antioxidant and/or as positive control. Green tea extract was also used to study comparatively the antioxidant activity with the selected plant extracts. Absorbance was measured at 518nm using spectrophotometer. Values obtained were converted to percentage antioxidant activity (AOXA%). The antioxidant activity is expressed as effective concentration (EC_{50}) values, the concentration of the sample leading to 50% reduction of the initial DPPH concentration. The results are also expressed as the mg Vit-C equivalents per mg dry weight extract.

2.3. Determination of Anti-tumor Activity by using Carrot Disc Assay

Selected plant extracts were prepared with 100 ppm and 1000ppm concentration. Carrot (*Daucas carota* L.) samples were sterilized with commercial bleach (cocorax) followed by washing with sterilized deionized water for three times. Each disc was overlaid with 100ul of *Agrobacterium tumefaciens* inoculums (10^8cfumL^{-1})[4]. A 50ul aliquot of each extract with different concentration was then added using syringe into disc. Petri dishes were sealed by para-film and incubated at 30˚C. After 3 weeks, the discs were checked for young galls (tumors) developing from the meristematic tissue around the central vascular system.

2.4. Estimation of the Natural Toxin of Crude Extracts by using Brine Shrimp Toxicity Test

One gram of dried cysts of brine shrimp (*Artemia salina*) was hatched into free swimming forms. Each extract sample was prepared as 4,000ppm, 2,000ppm, 1,000ppm, 800ppm, 600ppm, 400ppm, 200ppm and 100ppm respectively. 2 ml of each of the diluted extract solution was added to vials and 20 nauplii were collected with Pasteur pipette from the hatching container and were transferred to each vial carrying over the minimum amount of sea water. The vials with solvent and potassium dichromate solutions were also filled with 20 nauplii as controls. The vials were restored in the dark room while the temperature was controlled at 25 ± 1°C. After 6 hours and 24 hours incubation in the dark room, the vials were taken out for counting of nauplii. Counting of dead nauplii in each vial was made to get LD_{50} of acute toxicity (6hrs) and LD_{50} of chronic toxicity (24hrs) for plant ethanol extract. Nauplii were considered dead if they lay immobilized at the bottom of the vials [5].

2.5. *In vitro* Mouse Model Toxicity Test

Various concentrations of *A. japonica* crude extracts (500, 100, 1500, 2000 and 2500 mg/kg/day) were dissolved in 20% ethanol in volume of 8ml/kg/day. Either sex healthy mice weighed about 20g with an age ranging between 4-6 weeks were kept in optimal experimental condition with free access to food and water and were observed for a period of 7days before use. Animal were housed in colony cages with covers. The animals were grouped into six having 5 mice in same sex in each group. One group was kept for solvent control, five groups for test plant. They received the test drug in the dose level ranging from 500 to 2500 mg/kg/day orally for six days. During administration of the drug, normal feed was given to animals; water was supplied freely. Observation was done for ten days and both dead and alive outcome of animals was daily recorded. The experiment was carried out following the rules and regulations for animal studies [5].

2.6. *In vitro* Antimicrobial Assays by Agar-Well Diffusion Method

All nine strains of food borne and human pathogenic microorganisms used in this study were as shown in Table2. Agar-Well diffusion test was used for testing the antimicrobial activity of crude extracts [2].

Crude *A. japonica* extract was further separated using three solvents, n-hexane, ethyl acetate and methanol. For the antimicrobial test, sterile Muller-Hinton Agar for bacteria and Potato Dextrose Agar for

fungi were used. With sterile technique, four of five similar colonies from the subculture of microorganisms were inoculated by swabbing thoroughly over the entire sterile agar surface of a plate to obtain a confluent lawn of microbial grow and equally spaced wells were made on the agar. Each test sample solution (40 mg/well) was introduced with 50µl pipette into each well as labelled. The solvent only (70% ethanol) was used as control. And then, the plates were placed in an incubator at 37°C for 22-24 hour. After respective incubation time for microorganism, the plates were examined and the diameters of the zones of complete inhibition were measured to the nearest whole millimetre with a ruler.

The minimal inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) were applied to the aqueous extract that had proved to be highly effective against microorganisms by the microdilution and agar- diffusion method. The aqueous extract of *A. japonica* was prepared with decreasing concentrations (from 20 mg/ml to 0.15625mg/ml). The strains were designated arbitrarily as sensitive or resistant and the zones were measured at the end of the incubation time.

3. Results and Discussions

3.1. Phytochemical and Mineral Analysis

The phytochemical analysis of selected plant extracts had showed the presence of glycosides, flavonoids and phenolic compounds but had show the absence of cyanogenic glycosides. It has been mentioned that antioxidant activity of plants might be due to their phenolic compounds [2]. Flavonoids are a group of polyphenolic compounds with known properties which include free radical scavenging, inhibition of hydrolytic and oxidative enzymes and anti-inflammatory action. The presence of polyphenolic compound in the selected herbal plants prompted us to study the free radical scavenging activity. According to the results of mineral analysis, there is absence of lead and arsenic in the selected plants revealed that these plants are potentially safe for further activity test.

3.2. In vitro DPPH Free Radical Scavenging Assay

DPPH radical is scavenged by antioxidants through the donation of proton forming the reduced DPPH. The color changes from deep purple to pink to yellow after reduction, which can be quantified by its decrease of absorbance at wavelength 518 nm. Radical scavenging activity increased with increasing percentage of the free radical inhibition. The degree of discoloration indicates the free radical scavenging potentials of the sample/antioxidant by their hydrogen donating ability. The electrons become paired off and solution loses colour stochiometrically depending on the number of electrons taken up.

Free radical scavenging activity of the selected plant extracts and extract of green tea and the standard antioxidant Vitamin-C are shown in Table 1. From this table, EC_{50} value of *C. hirsutus* extract shows less than that of *A. japonica, A. conyzoides* and green tea (*C. sinensis*) extracts. The results of free radical scavenging activity also showed that *C. hirsutus* have the strongest activity among the three plant extracts with 55.06% at 10.68ug/ml (its EC_{50} value) concentration and first followed by Vit-C. Scavenging capacities of the *A. japonica* and green tea extracts have been found almost equal. 50% and above inhibition DPPH radical is considered as significant for scavenging activity.

TABLE 1. RADICAL SCAVENGING ACTIVITIES OF SELECTED EXTRACTS AND STANDARD ANTIOXIDANTS ON DPPH FREE RADICAL

Sample	EC_{50} (ug/ml) Mean ± SD	I% or Free Radical Scavenging Activity (%)	EC_{50}Value (mg equivalent Vit-C/ mg dry wt. extract)
Vitamin-C	8.31 ± 0.33	61.49	1
C. hirsutus	10.68 ± 0.81	55.06	0.77
Tea (*C. sinensis*)	11.70 ± 0.37	53.61	0.71
A .japonica	12.72 ± 0.02	53.84	0.65
A. conyzoides	15.19 ± 0.11	50.56	0.55

Expressing plant extract's antioxidant activity in mg Vitamin C equivalent has the benefits that the antioxidant activity was quantified and different plant extracts were comparable. Compared to green tea where 1mg of dry weight, had Vitamin C equivalent of 0.71mg was a little lower than that of *C. hirsutus,* 0.77mg. *A. conyzoides* and *A. japonica* showed almost half and over half of the value of antioxidant activity

of Vitamin-C respectively. All selected plant extract here gave positive scavenging capacity (antioxidant activity) with DPPH.

3.3. Anti-tumor Activity on Carrot-Disc Assay

A. tumefaciens is an indigenous soil bacterium known for its phytopathogenic effects. It causes crown gall tumor disease in a wide range of plants including most dicots, some monocots and some gymnosperms. Upon infection, the bacterium transfers part of its plasmid DNA to the plant. The Ti-plasmid causes the plant's cells to multiply rapidly without going through apoptosis, resulting in tumor formation similar in nucleic acid and histology to human and animal cancers [3]. The T-DNA has also been transferred to human cells, demonstrating the diversity of insertion application. The mechanisms by which *Agrobacterium* inserts materials into human cells also by type IV system, is very similar to mechanisms used by animal pathogens to insert materials (usually proteins) into human cells also type IV secretion. This makes *Agrobacterium* an important topic of medical research as well. Besides, it plays a vital role in aspect of antitumor studies. After 3 weeks incubation of *A. tumefaciens* on each carrot disc in this research, negative control which use only for pathogenicity test showed young galls (tumors) developing from the meristematic tissue around the central vascular system. All extracts of selected plants showed anti-tumor activity. No gall was detected in carrot discs treated with 100ppm of *C. hirsutus* and *A. japonica* extracts and 1000 ppm of *A. conyzoides* extract. 70% EtOH treated on the test disc was used in this case as positive control.

3.4. Toxicity Testing of Crude Ethanolic Extract using Brine Shrimp Larvae

Toxicity of *A. japonica* was tested by using brine shrimp (*Artemia salina*) and the results are shown in Table2. From these results, it was found that LC_{50} values of all plants extracts were very much higher than that of potassium dichromate and it reveals the safety of these plants to use as herbal drugs.

TABLE 2. MORTALITY OF BRINE SHRIMP LARVAE TO VARIOUS CONCENTRATION OF SELECTED HERBAL EXTRACT

Extract	LC_{50} (Acute Toxicity-6hrs exposure) (ppm)	LC_{50}(Chronic Toxicity- 24 hrs exposure) (ppm)
A.japonica	1572.33 ± 3.7	890.89 ± 13.86
A.conyzoides	2005.07 ± 4.3	768.72 ± 16.02
C.hirsutus	2345.47 ± 3.2	587.04 ± 15.08
Control ($K_2Cr_2O_7$)	400 ± 2.9	11.69 ± 0.16

3.5. Toxicity Test by using *in vivo* Method

LC_{50} of oral administration of crude extract was essential to be investigated in rodent model before clinical trial was started [5]. According to the highest LC_{50} value of *A. japonica* among the three kinds of Myanmar herbal plants in in vitro assay, which plant was selected to do further researches.

In in vivo test, for acute and subacute toxicity, the crude extract of *A. japonica* was tested with serial doses of 2500, 2000, 15000, 1000 and 500 mg/kg/day given for six days. In this study, LC_{50} values of crude plant extract was found to be more than 2500 mg/kg/day. They were not toxic to the mice up to the highest concentration tested in this experiment, i.e., up to 2500mg/kg/day.

3.6. Antimicrobial Activity by Agar Well Diffusion Test

Crude extract of *A. japonica* was further separated using three solvents (n-hexane, ethyl acetate and methanol). In this investigation, each fraction of ehtanolic extracts *A. japonica* was screened against nine strains of pathogenic bacteria by using Agar Well Diffusion Method. Inhibition zone of diameter in millimeter was represented as the degree of activity. Antimicrobial activities of crude extracts of *A. japonica* are shown in Table 3.

According to the testing results, from those extracts, ethyl acetate fraction of crude extract shows best activity against *S. aureus* (18mm), *B. cereus* (29mm), *P. aerugonisa* (18mm), *P. marneffei* (19mm), and *S. typhi* (17mm) in inhibition zone diameter respectively. Methanol fraction of crude extracts showed good activity against *E.* coli (16mm), *S. boydii* (34mm) and n-hexane fraction also showed good activity against *S. sonnei* (12mm). Although positive control Ampicillin didn't show activity against *V. cholare*, all fractions of crude extract showed activity. Therefore, the crude extract was used to determine the MIC (Minimal Inhibitory Concentration) against nine strains of pathogenic microorganisms by microdilution test. MIC and

MBC (Minimum Bactericidal Concentration) values of crude extracts against tested microorganisms are also showed in this Table3. The MIC values of *A. japonica* against tested microorganisms are in the ranged from 0.625 to 5.0mg/ml and MBC values are in the ranged from 1.25 to 10.0mg/ml respectively. This indicated the distinct growth inhibition and wider spectrum of its potential antimicrobial activity.

TABLE3. ANTIMICROBIAL ACTIVITY OF CRUDE *A. JAPONICA* PLANTS EXTRACTS ON THE TESTED MICROORGANISMS

Gram reaction	Tested Microorganisms	Zone of Inhibition (mm) in diameter						MIC (mg/ml)	MBC (mg/ml)
		1	**2**	**3**	**4**	**5**	**6**		
Gram(+)ve	*Staphylococcus aureus*	16	18	15	11	19	0	0.625	0.625-1.25
	Bacillus cereus	19	29	29	24	13	0	2.5	2.5-5.0
	Pseudomonas aeruginosa	17	18	15	18	14	0	5.0	5.0-10.0
	Penicillium marneffei	15	19	14	17	15	0	5.0	5.0-10.0
Gram(-)ve	*Escherichia coli*	9	10	16	8	12	0	1.25	1.25-2.5
	Shigella boydii	15	10	34	9	10	0	2.5	2.5-5.0
	Salmonella typhi	16	17	14	15	15	0	2.5	2.5-5.0
	Shigella Sonnei	12	10	9	10	9	0	2.5	2.5-5.0
	Vibrio cholare	6	7	5	3	0	0	5.0	5.0-10.0

1.n-hexane fraction; **2**.Ethyl acetate fraction; **3**.methanol fraction; **4**.crude extract; **5**.(+)ve control (Ampicillin 50ug/well)
6. (-)ve control (70 % EtOH) ; # Concentration of all extracted samples – 40mg/ 50 µl per Well

4. Conclusions

Recently, much attention has been directed toward extracts and biologically active compounds isolated from popular plant species. The use of medicinal plants plays a vital role in covering the basic health needs in developing countries.

From this study we can conclude that all of these herbal plants *A. japonica*, *A. conyzoides* and *C. hirsutus* can be used as the source of typical diet or drugs of antioxidant and antitumor activity as having the potential to reduce disease risk. Antioxidants (AOX) are considered a promising therapeutic approach as they may be playing neuroprotective and neurodegenerative roles. Currently available synthetic antioxidants as well as food preservatives like butylated hydroxyl anisole (BHA), butylated hydroxy toluene (BHT), tertiary butylated hydroquinon and gallic acid esters, have been suspected to cause or prompt negative health effects. Hence, strong restrictions have been placed on their application and there is a trend to substitute them with naturally occurring antioxidants. Moreover, the traditional use of *A. japonica* plant for the treatment of infectious diseases, mainly against bacteria is promising.

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