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COMPARATIVE ANALYSIS OF ANTIMICROBIAL ACTIVITY OF LEAF EXTRACTS FROM THREE SPECIES OF GENUS *HYDROCOTYLE* ON PATHOGENS

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ABSTRACT

Medicinal plants have been a major source of therapeutic agents for alleviation and cure diseases. The antimicrobial activity of the extracts of three species of genus *Hydrocotyle* was analyzed by using well diffusion method paper disk diffusion method and minimum inhibitory concentration. In ethanoic extract of *H. ramiflora*, maximum inhibition zone diameter among all the tested microorganisms was obtained in *Enterobacter cloaceae* with diameter of 6.1±0.5 mm. *H. ramiflora* showed maximum inhibition zone with diameter of 6.3±0.3 mm in *Escherichia coli*, 6.1±0.3 mm in *Enterobacter aerogenes*, and 6.4±0.4 mm in *Klebsiella pneumoniae*. For all the tested microorganisms, ethanol extracts of *H. maritima* showed maximum antibacterial activity in *E. coli*. Maximum inhibition zone diameter of *H. maritima* were obtained in *E. cloaceae* and in *E. coli* with diameter 6.2±0.4 mm and 6.4±0.5 mm, respectively. In ethanolic extract of *H. nepalensis*, maximum inhibition zone diameter among all the tested microorganisms was obtained in *E. coli* DD12 with diameter of 6.4±0.5 mm (Table 3). *H. nepalensis* showed maximum inhibition zone with diameter of 6.2±0.4 mm in *E. cloaceae*, 6.3±0.4 mm in *E. aerogenes*, and 6.3±0.3 mm in *K. pneumoniae*. Although the two stock strains are different, there was not significant differences ($p > 0.05$). In addition, for each strain, there was not significant differences among four various concentrations ($p > 0.05$).

KEYWORDS: Genus *Hydrocotyle*, Paper Disk Diffusion Method, Antibacterial Activity.

INTRODUCTION

Many traditional medicine plants were used to cure various disease in the world. Egypt, China, Korea, India, and Greece followed these medical practice from ancient times and a wide variety of modern drugs have been derived from these systems.[1] The advantages of traditional medicine include its widespread accessibility and relative cheapness, when most people in low-income countries pay for medicine out of their own pockets. Plants are rich source of effective and safe medicines that often used in the treatment of various ailments. Traditional Chinese medicine is a broad range of medicine practices sharing common concepts which have been developed in China and are based on a tradition of more than 2,000 years, including various forms of herbal medicine, acupuncture, massage (Tui na), exercise (qigong), and dietary therapy.[2] There are many published reports from different parts of the world on the antimicrobial properties of medicinal plants, and as a result, plants are still recognized as the bedrock for modern medicine to treat infectious diseases.[3]

Folk medicine is the mixture of traditional healing practices and beliefs that involve herbal medicine, spirituality and manual therapies or exercises in order to diagnose, treat or prevent an ailment or illness. The World Health Organization states that it is mostly practiced by indigenous or native populations and as much as 80% of the population in certain countries within Asia and Africa rely on it for primary care.[4]

However, folk medicine needs rigorous, scientific data to demonstrate its efficacy. It also needs evidence-based standards for quality and safety evaluation to support its appropriate regulation. Vast amount of basic research has been conducted on traditional Chinese medicine herbs, mostly in characterizing the multiple-herb constituents, isolating active components from the herbs, and testing their pharmacological activities in the pursuit of new drug discovery.[5] The US Pharmacopeial Convention published the Herbal Medicines Compendium, which is an online database that provides such standards for herbs. Each herbal monograph contains specifications including tests for critical quality attributes of the herbal ingredient, as well as analytical test procedures and acceptance criteria for specified tests.

The genus *Hydrocotyle* L. is a plant belonging to the subfamily Hydrocotyloideae and the family Araliaceae.

The *Hydrocotyle* genus has between 75 and 100 species that grow in tropical and temperate regions worldwide.[6] The species of genus Hydrocotyle distributed in Korea are composed of five species; *H. sibthorpioides, H. ramiflora, H. nepalensis, H. maritima* and *H. yabei*. Plants of Hydrocotyle have been known to have diuretic, detoxifying, and anti-inflammatory effects and have been used for many years by folk remedies in Korea. The *Hydrocotyle* species were the important original plants of Pai-Tsao-Tsa, which was a kind of popular folk drink inherited from ancient time in Taiwan.[7] In North East India, the plants of genus *Hydrocotyle* were reported to be used traditionally in the treatment of rheumatic troubles, skin diseases including syphilis and liver complaints.[8]

In a recent work, antioxidant activities of ethanol extracts of three *Hydrocotyle* species was evaluated which showed positive results.

MATERIALS AND METHODS

Plant material and extraction: The three species of genus *Hydrocotyle, H. ramiflora, H. maritima* and *H. nepalensis,* were collected from the vicinity of Busan-ci and Juju-do, the South Korea. The samples of *Hydrocotyle* were washed. 100 g powdered samples of each were extracted with 70% ethanol and 50% ethanol in a successive manner to produce crude extracts containing wide range of active compounds. The extracts were prepared by maceration of the plant material with the solvents in a shaker for one day. The respective extracts were filtered using Whatman No.1 filter paper. The sample was evaporated to remove solvent under reduced pressure and controlled temperature by using rotary vacuum evaporator (N-1001S-W, Eyela, Tokyo, Japan). Thereafter, to get dry powder, samples were placed in vacuum chamber a low temperature. A powder sample lyophilized was used in the experiment.

Antimicrobial Activity Essay: The antibacterial potential of ethanoic extracts of three species of genus *Hydrocotyle* was studied using the paper disc diffusion method of Kil et al.[9] Four gram-negative pathogenic bacteria (*Escherichia coli, Enterobacter cloacae, Enterobacter aerogenes, Klebsiella pneumoniae*) were obtained from the Microbiology Division of Institute of College of Medicine, Yensei University in Korea and then grown in nutrient broth medium to yield a final concentration of 10^7 colony forming unit (CFU)/ml. The test bacteria (0.1 ml) were streaked on Mueller Hinton medium plates using sterile cotton swab. Sterilized filter paper discs (6.0 mm) were soaked in ethanoic extracts (100 mg/ml) and were then placed in the center of test bacteria plates. The plates were incubated for 24 h. Bacterial growth inhibition was determined as the diameter of the inhibition zones around the wells. The growth inhibition diameter was an average of four different measurements. Tetracycline disc (30 µg) and DMSO were used as the positive and negative controls, respectively. All measurement were performed in triplicate and mean values ± SD were recorded.

The difference of the mean values by sample concentrations was analyzed by Duncan's multiple range test at $p < 0.05$.[10] Statistical analysis was performed using SPSS (v22.0, SPSS Inc., Chicago, IL, USA) statistical program.

RESULTS

The antimicrobial activity was determined using paper disc diffusion method and summarized in Tables 1, 2, and 3. The antimicrobial potential of the experimental plants were evaluated according to their zone of inhibition against various pathogens and the results (zone of inhibition) were compared with the activity of the standards (Figures 1, 2, and 3). The results revealed that plant extracts are absent from potent antimicrobial against four of the bacteria strains studied.

In ethanoic extract of *H. ramiflora*, maximum inhibition zone diameter among all the tested microorganisms was obtained in *E. cloaceae* with diameter of 6.1±0.5 mm (Table 1). Similarly, ethanol extract showed maximum inhibition zone with diameter of 6.3±0.3 mm in *E. coli*, 6.1±0.3 mm in *E. aerogenes*, and 6.4±0.4 mm in *K. pneumoniae.* Although the two stock strains are different, there was not significant differences ($p > 0.05$). In addition, for each strain, there was not significant differences among four various concentrations ($p > 0.05$).

Table 1. Antimicrobial activity of methanol extract of *Hydrocotyle ramiflora* by well diffusion method.

Stock No.	Strain	Concentration (ug/ml)				t-test
		500	1000	1500	2000	
DD-11	*Enterobacter cloaceae*	6.0±0.2	6.1±0.4	6.1±0.4	6.1±0.5	ns
DD-12	*E. cloaceae*	6.0±0.0	6.0±0.1	6.1±0.3	6.1±0.2	ns
DD-21	*Escherichia coli*	6.1±0.1	6.1±0.2	6.3±0.2	6.3±0.3	ns
DD-22	*E. coli*	6.0±0.2	6.2±0.2	6.2±0.3	6.2±0.4	ns
DD-31	*Enterobacter aerogenes*	6.0±0.3	6.0±0.4	6.1±0.2	6.1±0.2	ns
DD-32	*E. aerogenes*	6.0±0.2	6.1±0.2	6.1±0.3	6.1±0.3	ns
DD-41	*Klebsiella pneumoniae*	6.2±0.2	6.3±0.3	6.3±0.4	6.4±0.4	ns
DD-42	*K. pneumoniae*	6.0±0.2	6.1±0.2	6.1±0.3	6.1±0.4	ns

ns: non-significant ($p < 0.05$).

For all the tested microorganisms, ethanol extracts of *H. maritima* showed maximum antibacterial activity in *E. coli* (Table 2). Maximum inhibition zone diameter of *H. maritima* were obtained in *E. cloaceae* and in *E. coli*

with diameter 6.2±0.4 mm and 6.4±0.5 mm, respectively. Similarly, ethanol extract showed maximum inhibition zone with diameter of 6.3±0.4 mm in *E. aerogenes* and 6.3±0.3 mm in *K. pneumoniae*. Although the two stock strains are different, there was not significant differences ($p > 0.05$). In addition, for each strain, there was not significant differences among four various concentrations ($p > 0.05$).

Table 2. Antimicrobial activity of methanol extract of *Hydrocotyle maritima* by well diffusion method.

Stock No.	Strain	Concentration (ug/ml)				t-test
		500	1000	1500	2000	
DD-11	*E. cloaceae*	6.0±0.0	6.0±0.1	6.0±0.2	6.1±0.2	ns
DD-12	*E. cloaceae*	6.1±0.2	6.1±0.2	6.2±0.4	6.2±0.4	ns
DD-21	*E. coli*	6.1±0.1	6.1±0.2	6.3±0.2	6.3±0.3	ns
DD-22	*E. coli*	6.1±0.2	6.3±0.2	6.3±0.4	6.4±0.5	ns
DD-31	*E. aerogenes*	6.0±0.1	6.1±0.4	6.2±0.3	6.3±0.4	ns
DD-32	*E. aerogenes*	6.0±0.0	6.0±0.2	6.1±0.4	6.1±0.5	ns
DD-41	*K. pneumoniae*	6.1±0.1	6.2±0.2	6.3±0.3	6.3±0.3	ns
DD-42	*K. pneumoniae*	6.0±0.0	6.1±0.3	6.1±0.4	6.2±0.3	ns

In ethanoic extract of *H. nepalensis*, maximum inhibition zone diameter among all the tested microorganisms was obtained in *E. coli* DD12 with diameter of 6.4±0.5 mm (Table 3). Similarly, ethanol extract showed maximum inhibition zone with diameter of 6.2±0.4 mm in *E. cloaceae*, 6.3±0.4 mm in *E. aerogenes*, and 6.3±0.3 mm in *K. pneumoniae*. Although the two stock strains are different, there was not significant differences ($p > 0.05$). In addition, for each strain, there was not significant differences among four various concentrations ($p > 0.05$).

Table 3. Antimicrobial activity of methanol extract of *Hydrocotyle nepalensis* by well diffusion method.

Stock No.	Strain	Concentration (ug/ml)				t-test
		500	1000	1500	2000	
DD-11	*E. cloaceae*	6.0±0.0	6.0±0.1	6.0±0.2	6.1±0.2	ns
DD-12	*E. cloaceae*	6.1±0.2	6.1±0.2	6.2±0.4	6.2±0.4	ns
DD-21	*E. coli*	6.1±0.1	6.1±0.2	6.3±0.2	6.3±0.3	ns
DD-22	*E. coli*	6.1±0.2	6.3±0.2	6.3±0.4	6.4±0.5	ns
DD-31	*E. aerogenes*	6.0±0.1	6.1±0.4	6.2±0.3	6.3±0.4	ns
DD-32	*E. aerogenes*	6.0±0.0	6.0±0.2	6.1±0.4	6.1±0.5	ns
DD-41	*K. pneumoniae*	6.1±0.1	6.2±0.2	6.3±0.3	6.3±0.3	ns
DD-42	*K. pneumoniae*	6.0±0.0	6.1±0.3	6.1±0.4	6.2±0.3	ns

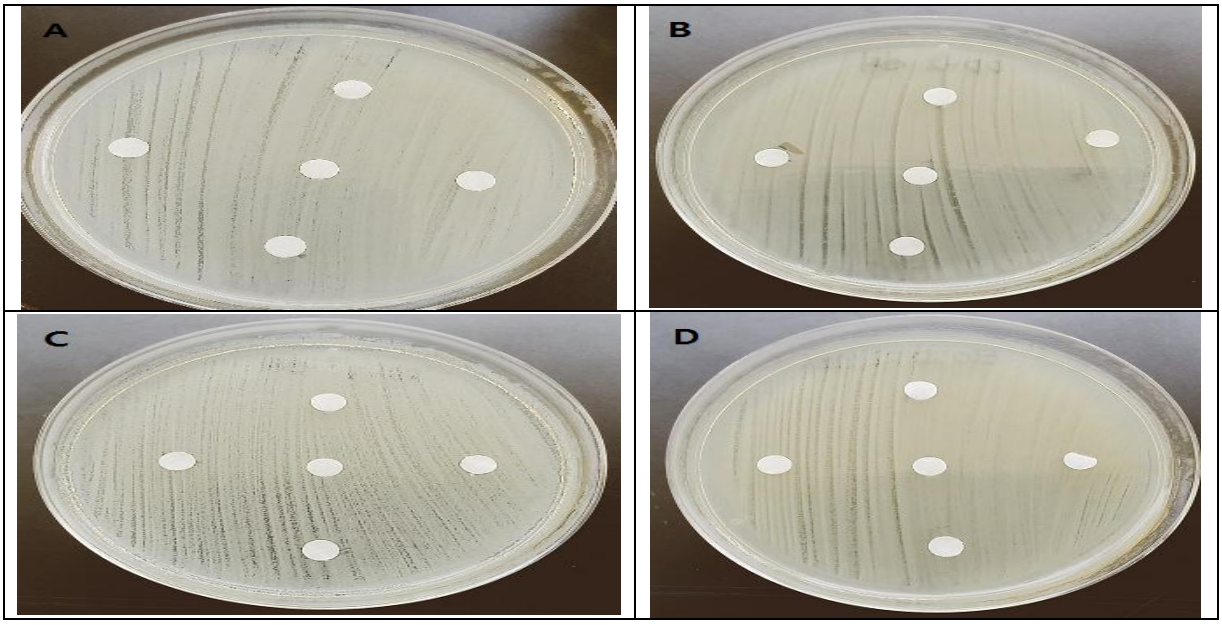

Figure 1. Antimicrobial assay plate for the antibacterial activity of *Hydrocotyle ramiflora* on the growth of (A) *E. cloaceae*, (B) *E. coli*, (C) *E. aerogenes*, and (D) *K. pneumoniae* showing wide zone of inhibition.

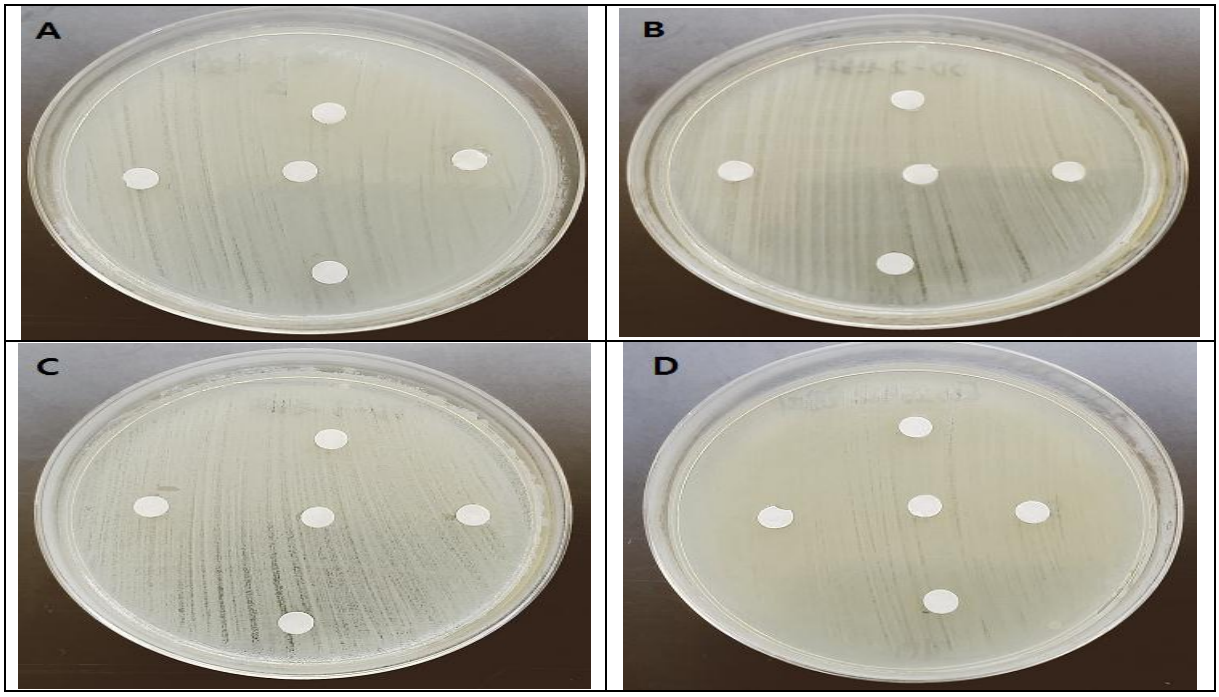

Figure 2. Antimicrobial assay plate for the antibacterial activity of *Hydrocotyle maritima* on the growth of (A) *E. cloaceae,* (B) *E. coli,* (C) *E. aerogenes,* and (D) *K. pneumoniae* showing wide zone of inhibition.

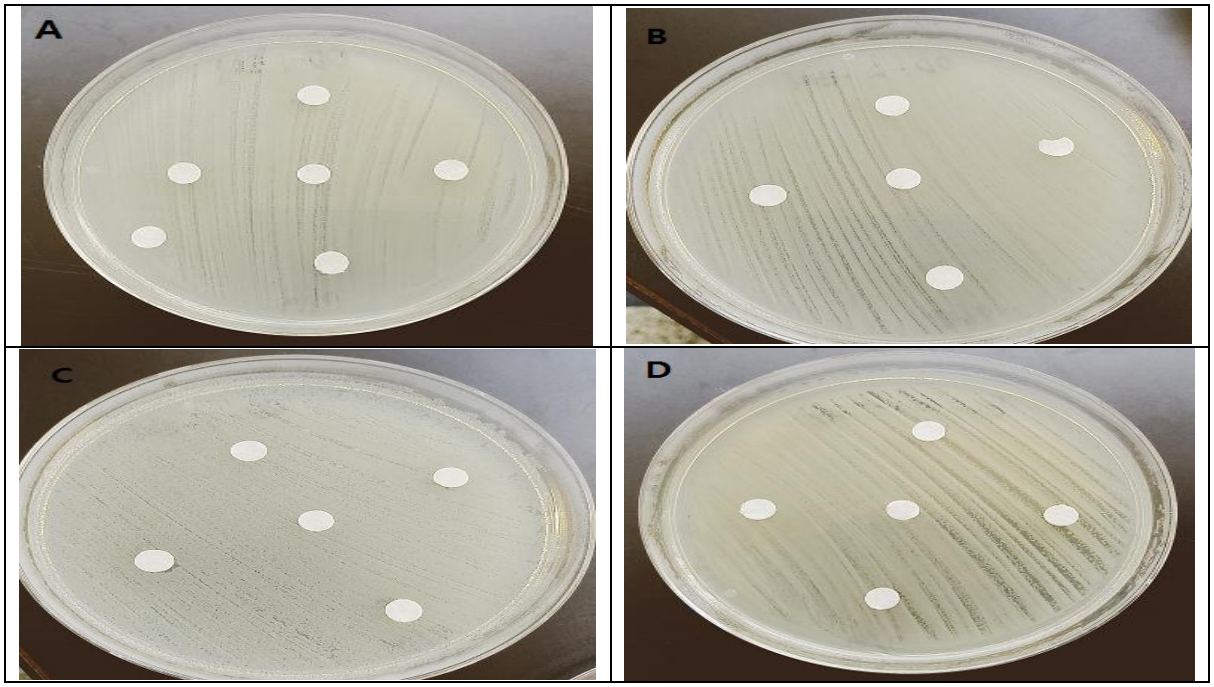

Figure 3. Antimicrobial assay plate for the antibacterial activity of *Hydrocotyle nepalensis* on the growth of (A) *E. cloaceae,* (B) *E. coli,* (C) *E. aerogenes,* and (D) *K. pneumoniae* showing wide zone of inhibition.

DISCUSSION

Folk remedies have been widely used in the past as therapeutic therapies. However, folk remedies are sometimes not scientific. A 2013 editorial by Novella and Colquhoun[11] found that the inconsistency of results of acupuncture studies (i.e. acupuncture relieved pain in some conditions but had no effect in other very similar conditions) suggests false positive results, which may be caused by factors like biased study designs, poor blinding, and the classification of electrified needles as a form of acupuncture. They suggested that given the inability to find consistent results despite more than 3,000 studies of acupuncture, the treatment seems to be a placebo effect and the existing equivocal positive results are noise one expects to see after a large number of studies are performed on an inert therapy. Evidence-based medicine, by integrating individual clinical expertise with the best available clinical evidence from systematic research, has in recent years been established

as the standard of modern medical practice for greater treatment efficacy and safety. [5]

Although the water extracts of the four *Hydrocotyle* species had a higher antiproliferative activity than the ethanol extracts,[7] no antimicrobial activity was observed in three species of genus *Hydrocotyle*, *H. ramiflora*, *H. maritima* and *H. nepalensis* (Tables 1, 2, and 3). The name of the plant in Korea has the meaning of stopping the blood. Thus, their leaves seemed to be used as hemostatic agent. Our present findings are not in accordance with the studies of Sood and Yadav[12] who reported the leaves of *Hydrocotyle javanica* and *Hydrocotyle rotundifolia* to have considerable activity against gram-positive and negative bacteria. The extracts of *Hydrocotyle sibthorpiodes* were found to exert low to moderate antibacterial activity compared to Chloramphenicol which was taken as standard.[13] Earlier, presence of total phenolic compounds, flavonoids and flavonols was reported in *Hydrocotyle* species which was associated with a broad spectrum of chemical and biological activities including radical scavenging properties.[14]. It was also claimed that phenolic compounds are powerful chain breaking antioxidants.[15]

CONCLUSIONS

The present work evaluated and established the antimicrobial activity of the three species of genus *Hydrocotyle* commonly used in traditional medicine. A statistically significant difference ($P < 0.01$) was not observed resistance to all the extracts at the tested concentration among all the extracts.

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