Research Article

Mutagenicity Effect of *Hydrocotyle Bonariensis* Extracts in *Salmonella*/Microsome Assay

Florinsiah, L.[1,2], Farida Zuraina, M. Y.[1*], Nur Shahirah, N.[1,2], Norfazlina, M. N.[1,2], Suziana Zaila, C.F[1,2], Rajab, N.F.[2],
[1]Faculty of Applied Sciences, Universiti Teknologi MARA, 40450 Shah Alam, Selangor, Malaysia.
[2]Biomedical Science Programme, Faculty of Health Sciences, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, 50300, Kuala Lumpur, Wilayah Persekutuan, Malaysia.
***Corresponding author's E-mail:** fzuraina@salam.uitm.edu.my

Accepted on: 02-04-2013; **Finalized on:** 31-05-2013.

ABSTRACT

Several medicinal plants are traditionally used in the treatment of a variety of ailments including cancer. However, many of these herbs have not been scientifically assessed for its efficacy or safety. The mutagenic effect of aqueous and methanolic extracts obtained from aerial parts (leaves and stem) and roots of *Hydrocotyle bonariensis* were assayed using the *Salmonella typhimurium* assay or Ames test. The pre-incubation method was used on *S. typhimurium* TA 98 and TA 100 bacterial strains in the presence and absence of metabolic activator S9 system. The results showed that all aqueous extracts of the aerial parts and roots of *H.bonariensis* were not mutagenic towards TA98 and TA100 strain with and without S9 activation using all concentration. On the other hand, the methanolic extracts of aerial parts and roots were also not mutagenic in the absence of metabolic activation against both strains. However, with metabolic activation, only methanolic extracts of *H.bonariensis* leaves and stems showed mutagenic effect at the highest concentration (50mg/ml) towards TA98 strain but both were not mutagenic for TA100 in all concentration studied. In conclusion, *H.bonariensis* extracts were not mutagenic on both *S. typhimurium* strains and has the potential to be used as part of traditional medicine.

Keywords: *Hydrocotyle bonariensis, Salmonella typhimurium,* Mutagenicity effect, Ames assay.

INTRODUCTION

Nowadays, it is more than 80% of the world's population use plants as their primary source of medicinal agents[1] because of the high cost of Western pharmaceuticals. From a cultural and perspective view, the traditional medicines are generally more acceptable as compared to synthetic medicines[2]. Furthermore, majority of the world population especially in developing countries are still relies and depends on herbal medicines to meet their health needs because sometimes the synthetic medicine itself cannot reduce the hard to cure illnesses of the patients[3]. Previous studies stated that, even though plant extracts are widely used to treat various kind of diseases but in nature many plants synthesize toxic substances in order to defence itself against infections, insects and herbivores [4]. It is also showed that some substances that present in some medicinal plants are potentially toxic and carcinogenic [5] and some of it may have a genotoxic potential [6-8]. Hence, it is very important to evaluate the safe use of herbs before utilize it as part of herbal medicinal.

Hydrocotyle bonariensis L. (Apiacae) or locally known as *Pegaga Embun* is a perennial prostrate herb and is found mostly in tropical and subtropical regions of the world [9]. It is a rhizomatous perennial herb common to the barrier islands of Southern United states and valued for treating various kinds of diseases such as tuberculosis, relieving the pain of rheumatism and arthritis, to increase brain capacity and for longevity [10]. Previous study has been reported that this herb has its medicinal use as emetics, diuretics and laxatives [11]. Besides that, studied done by [12] showed that, leave of the plant is rapidly gaining popularity by some local people in Western Nigeria because it can treat various symptoms of ophthalmic diseases. However, there is still no scientific proof has been sighted in the literature to support this statement, since there are no scientific data have been reported before.

Therefore, the aim of this study was to evaluate the mutagenic effect of *H.bonariensis* in different extracts by studying their mutagenic effect on two histidine requiring *S. typhimurium* in the absence and presence of a liver metabolizing system since there is still little report on mutagenic effect on this plant.

MATERIALS AND METHODS

Plant material

The whole plant of *Hydrocotyle bonariensis* was originally collected in Kota Marudu, Sabah, Malaysia. The plants were washed three times before cut into different parts which contain the aerial parts (leaves and stems) and roots before dried at room temperature. Voucher specimens (UKMB 30009) were deposited at the Herbarium University National Malaysia, Bangi, Faculty of Science and Technology.

Extraction method

The fresh aerial parts (leaves and stems) and roots of *H.bonariensis* were dried at room temperature and reduced to ground powder by using the microfiner grinder. The aqueous extract was performed as described by [13] with some modification. One hundred fifthy grams (150 g) of the powdered aerial parts and roots were soaked separately in 1.5 L of distilled water and stored

overnight at 4°C in order to prevent microbial activity of the samples. The samples were then filtered out using Whatman filter paper under vacuum before freeze dried to obtain crude extract. Then, the dry extract was kept at 4°C in a schott bottle prior to the bioassays. For the methanolic (MeOH) extract it was performed as described previously[14] with some modification where both samples (150 g each) were soaked separately in 1.5 L of solvent for three days. The mixtures was then filtered out using Whatman filter paper under vacuum and the solvent was evaporated using a rotary evaporator (Buchi Rotavapor R-114, Switzerland) at 50°C. Then, it was freeze-dried (Heto LyoLab 3000, Denmark) to obtain the crude extracts before kept at 4°C in a Schott bottle prior to the bioassays.

Bacterial strains

Tester strains (TA 98 and TA 100) which are histidine requiring mutant as previously described by [15] was obtained from Molecular Toxicology (Moltox) Inc. The genotypes of test strains were checked routinely for their histidine and biotin requirement, deep rough (*rfa*) character, UV sensitivity (*uvr*B mutation) and presence of the R factor (pKM101) plasmid and it were stored at -80°C. *S. typhimurium* TA 98 is frame shift strain which contain the his3052 mutation and *S. typhimurium* TA100 contain the base-pair substitution mutation hisG46.

Mutagens

2-Nitrofluorene (2-NF), sodium azide (NaN_3) and 2-aminoanthracene (2-AA) were purchased from Sigma-Aldrich and dissolved in dimethyl-sulfoxide (DMSO).

Mutagenicity assay

The mutagenicity assay with *S. typhimurium* was performed as described by previous method[16]. The test is based on the plate incorporation method, using *S. typhimurium* tester strains (TA100, TA98) with and without an exogenous metabolic system: S9 fraction in S9 mix. The S9 Microsome fraction were purchased from Molecular Toxicology (Moltox) Inc. and stored at −80 ◦C. The test strains from frozen cultures were grown overnight for 12–16h at 37 ◦C in the Oxoid Nutrient Broth No. 2. All concentrations studied of each extracts were added to 2 ml of top agar, supplemented with 0.5 mM L-histidine and 0.5 mM D-biotin, mixed with 100 µl of bacterial culture (approximate cell density 2×10^8–5×10^8 cells/ml) and then poured onto a plate containing glucose minimum agar. The plates were incubated at 37 ◦C for 48 hours. After 2 days of incubation, his+ revertant colonies were counted. The influence of metabolic activation was tested by adding 500 µl of S9 mixture. Negative and positive control cultures gave number of revertants per plate that were within the normal limits found in the laboratory. Data were collected with a mean ± SEM of three plates (n = 3).

Statistical analysis

Data were collected and expressed as mean ± SEM of three independent experiments (n=3) and analyzed for statistical significance from control. The data were tested for statistical differences by one-way ANOVA and the criterion for significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

H. bonariensis aerial parts and roots in aqueous and methanolic extract was evaluated for its mutagenic inducing ability in Ames test using two *S. typhimurium* strains namely TA98 and TA100 in the absence and presence of S9-mix, respectively as shown in Table 1-4. The Ames test is recommended for initial screening of medicinal plants since studies have shown that the proportion of carcinogens identified as mutagens ranges from about 50 % to 90 % [17]. Compound tested with Ames test have mutagenic effect when there is more than 2-fold increase in the number of revertant colonies over negative control[15]. In this study, all positive controls that were used in this experiment resulted in significant increase (>2-fold) of mean number of revertant when compared with negative control. Table 1 and Table 2 tabulate that both aqueous extracts of the sample were not mutagenic on the tested strains even in the presence of metabolic activation for all concentration used as it showed no significance difference ($p<0.05$) when compared with negative control in both strains. Since there is no mutagenic effect showed in both extracts when tested without the metabolic activation (-S9), therefore, for test with metabolic activation (+S9), only three concentration was tested which is the lowest concentration (3.125 mg/ml), the middle (12.5 mg/ml) and the highest concentration (50 mg/ml) as shown in Table 1-4. The results showed that, when the samples was tested with metabolic activation (+S9), only methanolic extracts of *H.bonariensis* aerial parts (leaves and stems) showed mutagenic effect at the highest concentration (50mg/ml) towards TA98 strain as it showed significance difference ($p<0.05$) over negative control which is 36.7 ± 6.4 as shown in Table 3. The presence of mutagenic and antimutagenic effects of plants have been related to the presence of certain phytochemical subtances in the plants itself [17]. However, both aerial parts and roots were non-mutagenic for TA100 in all concentration studied when tested with metabolic activation (+S9). The absence of a mutagenic response by *H.bonariensis* against *S. typhimurium* bacterial strains in the Ames assay is a positive step towards determining the safe use of this plant utilized in traditional medicine. However, the absence or no mutagenic effect cannot be considered as safe to be in all natural products, since previously studied showed that other medicinal plants assayed with Ames test, with or without the S9, have resulted positive for mutagenicity.[18-20] Therefore, for this herb, further investigation on phytochemical screening should be done to screen the bioactive compounds that might be present in this herb. The evaluation of bacterial mutagenicity is of particular

importance as an initial test for complex mixtures because of the possibility that one or more components can be positive[21, 22]. A positive response in any single bacterial strains either with or without metabolic activation is sufficient to designate a substance as a mutagen[23]. On the other hand, a protective action of plants or their metabolites on genetic material has been reported, leading to its repair or to preserve its integrity [24-27].

Table 1: Mutagenic effects of Aqueous extract of *H. bonariensis* aerial parts (leaves and stems) without metabolic activation S9 (-S9) and with metabolic activation S9 (+S9)

Strains	Concentration	Mean±SEM (-S9); Mean±SEM (+S9)	
TA98	Positive control	318.0 ± 2.3*	401.5 ± 49.5*
	Negative control	15.7 ± 0.3	15.5 ± 1.5
	3.125 mg/ml	12.0 ± 1.0	13.7 ± 1.8
	6.25 mg/ml	16.3 ± 3.9	-
	12.5 mg/ml	11.7 ± 1.7	15.3 ± 1.5
	25 mg/ml	15.3 ± 1.2	-
	50 mg/ml	15.0 ± 1.5	18.0 ± 2.3
TA100	Positive control	814.3 ± 43.1*	1641.5 ± 118.5*
	Negative control	127.0 ± 8.5	212.5 ± 6.5
	3.125 mg/ml	127.3 ± 20.5	234.0 ± 9.9
	6.25 mg/ml	135.7 ± 20.9	-
	12.5 mg/ml	130.3 ± 27.1	231.7 ± 0.3
	25 mg/ml	127.7 ± 10.9	-
	50 mg/ml	109.0 ± 11.2	234.3 ± 5.5

Negative control = Sterile distilled water, Positive control; strain TA 98 (-S9 = 2-NF, +S9 = 2-AA), strain TA 100 (-S9 = NaN_3, +S9 = 2-AA); The results are the means ± SEM of three separate experiments (n=3); *p < 0.05 vs. negative control.

Table 2: Mutagenic effects of Aqueous extract of *H. bonariensis* roots without metabolic activation S9 (-S9) and with metabolic activation S9 (+S9)

Strains	Concentration	Mean±SEM (-S9); Mean±SEM (+S9)	
TA98	Positive control	319.0 ± 2.5*	401.5 ± 49.5*
	Negative control	14.3 ± 1.2	15.5 ± 1.5
	3.125 mg/ml	12.0 ± 2.0	16.0 ± 3.8
	6.25 mg/ml	10.7 ± 1.5	-
	12.5 mg/ml	9.7 ± 1.2	17.0 ± 2.1
	25 mg/ml	10.7 ± 0.7	-
	50 mg/ml	9.7 ± 0.3	15.7 ± 3.3
TA100	Positive control	794.0 ± 53.4*	1641.5 ± 118.5*
	Negative control	127.0 ± 8.5	212.5 ± 6.5
	3.125 mg/ml	111.0 ± 6.8	239.0 ± 4.6
	6.25 mg/ml	106.0 ± 6.8	-
	12.5 mg/ml	105.0 ± 5.2	223.7 ± 2.9
	25 mg/ml	114.3 ± 3.7	-
	50 mg/ml	111.7 ± 7.1	244.0 ± 9.2

Negative control = Sterile distilled water, Positive control; strain TA 98 (-S9 = 2-NF, +S9 = 2-AA), strain TA 100 (-S9 = NaN_3, +S9 = 2-AA); The results are the means ± SEM of three separate experiments (n=3);*p < 0.05 vs. negative control.

Table 3: Mutagenic effects of Methanolic extract of *H. bonariensis* aerial parts (leaves and stems) without metabolic activation S9 (-S9) and with metabolic activation S9 (+S9)

Strains	Concentration	Mean±SEM (-S9); Mean±SEM (+S9)	
TA98	Positive control	378.3 ± 36.8*	420.0 ± 14.0*
	Negative control	17.0 ± 0.6	14.0 ± 1.0
	3.125 mg/ml	16.3 ± 3.3	16.7 ± 1.8
	6.25 mg/ml	16.3 ± 2.4	-
	12.5 mg/ml	18.0 ± 1.5	19.7 ± 4.3
	25 mg/ml	15.0 ± 1.5	-
	50 mg/ml	18.3 ± 2.2	36.7 ± 6.4*
TA100	Positive control	814.7 ± 40.7*	1395.0 ± 15.0*
	Negative control	135.0 ± 3.8	208.0 ± 3.0
	3.125 mg/ml	122.3 ± 12.3	224.7 ± 4.8
	6.25 mg/ml	124.3 ± 7.4	-
	12.5 mg/ml	129.0 ± 14.2	228.0 ± 1.0
	25 mg/ml	121.7 ± 15.9	-
	50 mg/ml	135.0 ± 12.2	242.0 ± 11.1

Negative control = Sterile distilled water, Positive control; strain TA 98 (-S9 = 2-NF, +S9 = 2-AA), strain TA 100 (-S9 = NaN_3, +S9 = 2-AA); The results are the means ± SEM of three separate experiments (n=3); *p < 0.05 vs. negative control.

Table 4: Mutagenic Effects of Methanolic extract of *H. bonariensis* roots without metabolic activation S9 (-S9) and with metabolic activation S9 (+S9)

Strains	Concentration	Mean±SEM (-S9); Mean±SEM (+S9)	
TA98	Positive control	319.0 ± 2.5*	420.0 ± 14.0*
	Negative control	14.3 ± 1.2	14.0 ± 1.0
	3.125 mg/ml	11.0 ± 1.0	15.7 ± 0.9
	6.25 mg/ml	9.7 ± 1.2	-
	12.5 mg/ml	8.7 ± 1.2	16.0 ± 1.5
	25 mg/ml	9.0 ± 1.2	-
	50 mg/ml	12.0 ± 2.1	18.7 ± 1.9
TA100	Positive control	794.0 ± 53.4*	1395.0 ± 15.0*
	Negative control	127.0 ± 8.5	208.0 ± 3.0
	3.125 mg/ml	131.3 ± 5.8	219.0 ± 10.4
	6.25 mg/ml	121.7 ± 9.2	-
	12.5 mg/ml	135.0 ± 7.2	208.3 ± 6.7
	25 mg/ml	110.0 ± 4.6	-
	50 mg/ml	134.0 ± 4.9	224.0 ± 1.2

Negative control = Sterile distilled water, Positive control; strain TA 98 (-S9 = 2-NF, +S9 = 2-AA), strain TA 100 (-S9 = NaN_3, +S9 = 2-AA); The results are the means ± SEM of three separate experiments (n=3); *p < 0.05 vs. negative control.

CONCLUSION

In conclusion, both extracts of *H.bonariensis* aerial parts and roots have no mutagenic effect on both *Salmonella typhimurium* strains, with and without the presence of metabolic activation and therefore, this herb has the potential to be used as part of traditional medicine.

The source(s) of financial support of study: This study was supported by research grant from Universiti Teknologi MARA, Shah Alam, Selangor, Malaysia and Ministry of Science, Technology and Innovation (MOSTI), Malaysia.

REFERENCES

1. Cordell G, Natural products in drug discovery creating a new vision, Phytochem. Rev., 1, 2002, 261-273.
2. Bent S, Ko R, Commonly used herbalmedicines in the United States: A review, American Journal of Medicine, 16, 2004, 478–485.
3. Yu F, McGuire PM, Li R, Wang R, Effects of *Hydrocotyle sibthorpioides* extract on transplanted tumors and immune function in mice, Phytomedicine, 14, 2007, 166–171.
4. Demma J, Engidawork E, Hellman B, Potential genotoxicity of plant extracts used in Ethiopian traditional medicine, Journal of Ethnopharmacology, 122, 2009, 136–142.
5. De Sa Ferreira ICF, Vargas VM, Mutagenicity of medicinal plant extracts in *Salmonella*/microsome assay, Phytotherapy Research, 13, 1999, 397–400.
6. Sohni Y, Davis C, Champs A, Kale P, Frameshift mutations in *salmonella* induced by the extracts of medicinal herbs *Lannea edulis* (sond.) engl. and Monotes glaber Sprague, Environ. Mol. Mutagen, 25 (1), 1995, 77-82.
7. Basaran A, Yu TW, Plewa MJ, Anderson D, An investigation of some Turkish herbal medicines in *Salmonella typhimurium* and the Comet assay in human lymphocytes, Teratogenesis Carcinogenesis and Mutagenesis, 16, 1996, 125–138.
8. Romero-Jimenez M, Campos-Sanchez J, Allana M, Munoz-Serrano A, Alonso-Moraga A, Genotoxicity and antigenotoxicity of some traditional medicinal herbs, Mutation Research, 585, 2005, 147–155.
9. Vimala S, Adenan MI, Ahmad AR, Shahdan R, Nature's choice to wellness: Antioxidant vegetables/ulam, Forest Research Institute Malaysia: Selangor, 2003.
10. Masoumian M, Arbakariya A, Syahida A, Maziah M, Flavonoids production in *Hydrocotyle bonariensis* callus tissues, Journal of Medicinal Plants Research, 5 (9), 2011, 1564-1574.
11. Evans JP, The Effect of Local Resource Availability and Clonal Integration on Ramet Functional Morphology in *Hydrocotyle bonariensis,* Oecologia, 89 (2), 1992, 265-276
12. Edeoga HO, Okwu OE, Mbaebie BO, Phytochemical constituents of some Nigerian medicinal plants, African Journal of Biotechnology, 1, 2005, 685-688.
13. Wong SP, Lai PL, Jen HWK, Antioxidant activities of aqueous extracts of selected plants, Food Chem, 99, 2006, 775-783.
14. Saha P, Ashok Kumar D, Bibhuti BK, Thamil Selvan V, Mazumder VK, Gupta M,Anticancer activity of methanol extract of *ipomoea reptans* in eac bearing mice, Pharmacologyonline, 2, 2008, 808-820.
15. Maron D, Ames B, Revised methods for the Salmonella mutagenicity test, Mutat. Res./Environ. Mutagen. Relat. Subj, 113(3-4), 1983, 173-215.
16. Ames BN, McCann J, Yamasaki E, Methods for detecting carcinogens and mutagens with the Salmonella/ mammalian-microsome mutagenicity test, Mutation Research, 31 (6), 1975, 347.
17. Zeiger E, Mutagens that are not carcinogenic: faulty theory or faulty tests?, Mutation Research, 492, 2001, 29 -38.
18. Kul L, Kook KHY, Effect of bacterial growth-inhibiting ingredients on the Ames mutagenicity of medicinal herbs, Mutat. Res. Lett, 192(2), 1987, 99-104.
19. Sohni YR, Mutangadura-Mhlanga T, Kale PG, Bacterial mutagenicity of eight medicinal herbs from Zimbabwe, Mutation Research, 322, 1999, 133–140.
20. Diana AN, Modulating effects of flavonoids on food mutagens in human blood and sperm samples in the comet assay, Teratogen. Carcinogen. Mutagen, 17 (2), 1997, 45-58.
21. Lee NJ, Choi JH, Koo BS, Ryu SY, Han YH, Lee SI, Lee DU, Antimutagenicity and cytotoxicity of the constituents from the aerial parts of *Rumex acetosa*, Biological and Pharmaceutical Bulletin, 28 , 2005, 2158–2161.
22. Reid KA, Maes J, Maes A, Van Staden J, De Kimpe N, Mulholland DA, Verschaeve L, Evaluation of the mutagenic and antimutagenic effects of South African plants, Journal of Ethnopharmacology, 106, 2006, 44–50.
23. Ferreira ICDF, Vargas VMF, Mutagenicity of medicinal plant extracts in *Salmonella*/microsome assay, 13, 1999, 397 – 400.
24. Abdelwahed A, Bouhle I, Skandrani I, Valenti K, Kadri M, Guiraud P, Study of antimutagenic and antioxidant activities of gallic acid and 1,2,3,4,6-pentagalloylglucose from *Pistacia lentiscus* Confirmation by microarray expression profiling, Chemico Biological Interactions, 165, 2007, 1–13.
25. Ammar RB, Kilani S, Bouhlel I, Ezzi L, Skandrani I, Boubaker J, Antiproliferative, antioxidant and antimutagenic activities of flavonoid-enriched extracts from (Tunisian) *Rhamnus alaternus* L. Combination with the phytochemical composition, Drug and Chemical Toxicology, 31, 2008, 63–82.
26. Hayder N, Ben Ammar R, Abdelwahed A, Kilani S, Mahmoud A, Ghedira K, Chekir-Ghedira L, Antibacterial and antimutagenic activity of extracts and essential oil from (Tunisian) Pistacia lentiscus, Toxicological and Environmental Chemistry, 87, 2005, 567–573.
27. Kilani S, Ben Ammar R, Bouhlel I, Abdelwahed A, Hayder N, Ghedira- Chekir L, Investigation of extracts from (Tunisian) *Cyperus rotundus* as antimutagens and radical scavengers, Environmental Toxicology and Pharmacology, 20, 2005, 478–484.

Conflict of Interest: None.