Investigation the Effect of Traditional Chinese Medicine on Stevia Robustness

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***Abstract*— Stevia is a sweetener derived from the leaves of *Stevia rebaudiana.* As a zero calorie and natural sugar substitute that has negligible effect on blood glucose levels, Stevia offers possibilities for diabetics and patients with hyperglycemia who are conventionally controlled by their highly restricted diets. With projections of the increasing global prevalence of diabetes, there is merit to exploring ways to increase throughput of Stevia. This project thus sought to evaluate ways to improve Stevia robustness to allow for greater crop yields and higher survival rates of Stevia plants. One angle this project decided to take was to investigate the effects of Traditional Chinese Medicine (TCM). Past literature has shown that TCM is effective both as an insecticide and as an antimicrobial. Thus, TCM holds prospects to achieve Stevia robustness. Well diffusion tests, colony forming unit tests and minimum inhibitory concentration tests were conducted to investigate the antimicrobial properties of 16 TCM extracts. Promising TCM extracts were further tested in flour disk bioassays and sugar cube tests to investigate their insecticidal properties. Finally, the extracts were tested on the leaves of purchased Stevia plants to further confirm the findings. It was determined that extracts from *Tasmannia lanceolata* and *Radix achyranthis bidentatae* show greatest potential to improve Stevia robustness when applied topically and systemically.**

Keywords-stevia; traditional chinese medicine; antibacterial; insecticidal

INTRODUCTION

Stevia rebaudiana is a plant commonly grown for its sweet leaves. Its leaves are the source of steviol glycosides, which are about 250 to 300 times sweeter than sugar (Abdullateef & Osman, 2012). Extracted and used as an artificial sweetener, Stevia is currently being employed as a zero calorie alternative to sugar. The general structure of a steviol glycoside is shown with 2 variable groups (denoted R1, R2 in Figure 1).

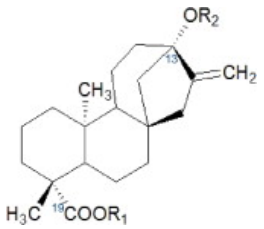


Figure 1. Steviol Glycoside Chemical Structure (Morlock et al., 2012).

Due to its properties, Stevia is of special interest to the rising population of diabetics (Danaei G. *et al*., 2011) and patients with hyperglycemia. In 2011, 347 million people worldwide were reported to have diabetes (Danaei G *et al*., 2011) and in 2012, diabetes contributed to around 1.5 million deaths (World Health Organisation, 2012). In addition, the World Health Organisation (2011) projected that diabetes will be the 7th leading cause of death in 2030.

With the escalating prevalence of diabetes, it comes as no surprise that developments in the field of artificial sweeteners have been gaining rising importance and recognition. B. Ahmed *et al.* (2011) concluded in their research that Stevia's pharmaceutical properties merits developments to increase the throughput of Stevia and its derivatives.

However, common problems faced by farmers and gardeners are infestation by ants and aphids as well as bacterial and viral infections to the plants, reducing their harvest potential (Smart Gardeners, n.d.).

Past literature has shown that Traditional Chinese Medicine (TCM) is effective both as an insecticide and as an antimicrobial. The results of the experiment conducted by Liu and co-workers in 2007 showed that a number of TCM extracts (*Dictamnus dasycarpus*, *Rhododendron molle, Sophora flavescens* and *Tripterygium wilfordii*) were able to deter the insects tested for (*Sitophilus zeamais* and *Tribolium castaneum*) and some even having toxicity effects on them. They also had a Feeding Deterrent Index of more than 50%. Researches by Tan & Vanitha in 2004 and by Yuen *et al.* in 2011 found that TCM extracts had potent antimicrobial activity against bacteria. In the research by Yuen *et al.*, *Rhizoma coptidis* and *Rhus chinensis* exhibited potent antimicrobial activity against the four oral bacteria tested.

Furthermore, TCM is non-synthetic and natural, allowing for safe consumption and chemical-free plants to be grown. Therefore, our project aims to investigate the antibacterial and insecticidal effects of different TCM extracts on Stevia robustness to allow for greater crop yields and higher survival rates of Stevia plants.

METHODOLOGY

Preparation of extracts

All traditional Chinese herbs were purchased and grounded to particles using a dry blender. 1g of each herb

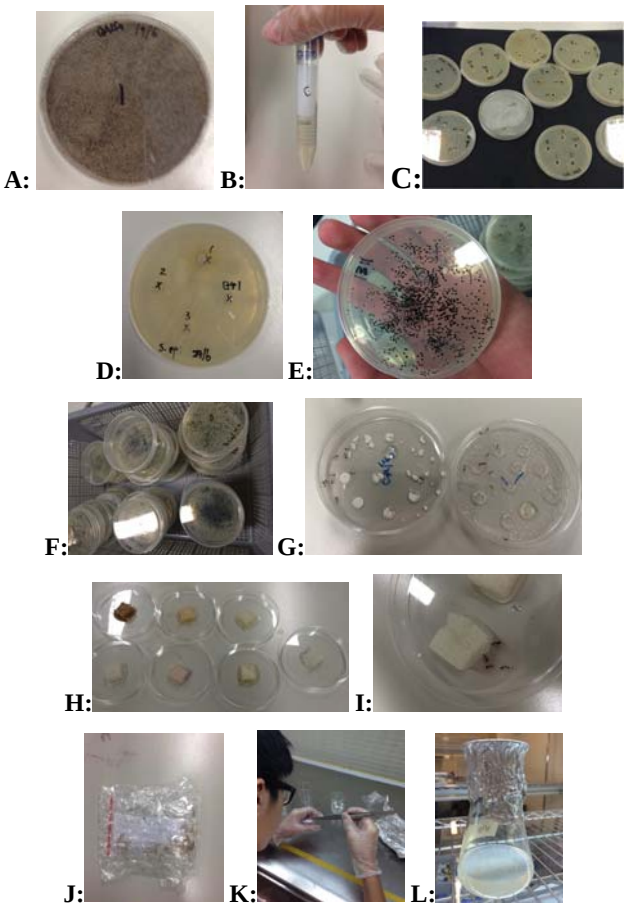


Figure 2. **A:** Dry powdered extract. **B:** Extract dissolved in aqueous medium. **C:** Agar plates for well diffusion test. **D:** Zoomed in view of an individual plate for the well diffusion test. **E:** Colony forming unit test agar plate. **F:** Colony forming unit test plates. **G:** Flour Disk Bioassay. **H:** Sugar cube test. **I:** Ants consuming the control sugar cube. **J:** Stevia seedlings. **K:** Surface sterilization of Stevia seedlings. **L:** Stevia grown in agar media.

powder was then prepared in 10 mL of sterile water using a mortar and pestle. The extracts were centrifuged at 7000 rpm for 10 min and the supernatant collected were then filter-sterilised through a 0.45 µm micro-filter and stored for future use (Figure 2A; 2B).

Well diffusion test

Agar plates were swabbed with bacteria from the liquid broth evenly using sterile cotton swabs. Wells were made in each agar plate and filled with 100 µl each of the 16 TCM extracts. 100 µl of bleach and water was added in separate wells for positive and negative control respectively. The plates were incubated at 30 degrees Celsius for 24 hours. They were then examined for ZOI and the diameter of zone was measured (Figure 2C; 2D). Triplicates were performed.

Colony forming unit test

The colony forming unit (CFU) test was performed to investigate the effect of the extracts on the actively dividing phase of test organisms. 0.5 ml of bacteria culture was mixed with 7.5 ml of LB broth. For the control set-up, 2 ml of sterile water was added, while for the test set-ups, 2 ml of TCM extracts was added to the mixture. The set-ups were then left in a shaking incubator at 30 degrees Celsius for 24 hours.

After 24 hours had passed, 1.0 ml of each mixture was transferred into cuvettes. Blanks containing 0.2 ml of extracts and 0.8 ml of broth were prepared for the test set-ups and 0.2 ml of sterile water and 0.8 ml of broth were prepared for the control set-ups. The absorbance of the mixtures was taken in a spectrophotometer at 595 nm. The absorbance was proportional to cell density; hence the extent of inhibition of growth of bacteria would be indicated by comparing the difference in absorbance between test and control set-ups.

The mixtures were then serially diluted using LB broth. 100 µl of the diluted solutions were spread evenly on agar plates. The plates were incubated at 30 degrees Celsius for 24 hours. The colonies were counted the next day (Figure 2E; 2F).

Minimum Inhibitory Concentration test

A 98-well microtitre plate was used for the above test. 0.2 ml of extract was micropipetted to the first well and was serially diluted by a factor of 2 across the row. This was repeated for the other 3 extracts to be tested as well as sterile water (negative control). 0.2 ml of respective bacteria that was incubated in LB broth overnight was then added to all the wells containing the extracts. In a separate set-up, the aforementioned set-up was repeated replacing bacteria with LB broth zeroing of absorbance reading. The microtitre plate was then read for absorbance readings at 0min and 30min.

Sugar cube test

Despite the established protocol for the flour disk assay, (Jbilou, R., et al. 2008), we were unsuccessful in applying this methodology to our experiments. We therefore decided to improvise our own method to measure the effect of TCM on feeding deterrence. In our tests, one sugar cube was placed at the center of a petri dish. 1 ml of TCM extract solution was micropipetted to the sugar cube and left to dry in an oven overnight. The petri dishes with the sugar cube were then collected and weighed (Figure 2H; 2I). 10 insects was added to each petri dish and left overnight in a culture room. The insects were removed after 24 hours before the petri dishes are reweighed.

In vivo testing of Stevia robustness

Sterile sand was first obtained via autoclaving. 1 spatula of sterile sand was added onto the surface of the Stevia leaves. The leaves were then abraded 20 times on the top surface using the sand previously added. Using a cotton bud, the damaged leaves were then swabbed twice with the extracts. The leaves were then checked after 24hours and the number of leaves surviving was recorded.

Table 1.

No.	Common Name	Scientific Name
1	Bai Xian Pi (白鲜皮)	*Cortex dictamni*
2	Ku Shen (苦参)	*Sophora flavescens*
3	Lei Gong Teng (雷公藤)	*Tripterygium wilfordii*
4	Yang Zhi Zhu (羊踯躅)	*Rhododendron molle*

5	Shan Hu Jiao	(山胡椒)	*Tasmannia lanceolata*
6	Bian Xu	(萹蓄)	*Herba polygoni avicularis*
7	Bai Bu	(百部)	*Radix stemonae*
8	Qin Pi	(秦皮)	*Cortex fraxini*
9	Wu Wei Zi	(五味子)	*Fructus schisandrae chinensis*
10	Niu Xi	(牛膝)	*Radix achyranthis bidentatae*
11	Huang Qin	(黄芩)	*Radix scutellariae*
12	Mu Xiang	(木香)	*Radix aucklandiae*
13	Sheng Di Huang	(生地黄)	*Radix rehmanniae*
14	Chuan Xin Lian	(穿心莲)	*Andrographis paniculata*
15	Lian Qiao	(连翘)	*Fructus forsythiae*
16	Xia Ku Cao	(夏枯草)	*Spica prunellae*

RESULTS AND DISCISSUON

Well Diffusion Test

In the Well Diffusion Test, we investigated the effect of TCM on inhibiting bacteria growth. Table 2 and 3 shows the effect of TCM on the ZOI of the listed bacteria. As not all the TCM showed inhibitory effects, only those that formed a ZOI were shown in the tables. Standard error was calculated by taking the standard deviation of the 3 samples divided by the square-root of 3.

From Graph 1, Sophora flavescens, Cortex fraxini, Fructus schisandrae chinensis and Radix achyranthis bidentatae all showed observable differences with the negative control as the error bars do not overlap with that of the negative control. The error bar of Sophora flavescens does not overlap while error bars of the other extracts overlap with the positive control. Hence, it can be deduced that Sophora flavescens showed a greater inhibitory effect on S. epidermis than 10% bleach while Cortex fraxini, Fructus schisandrae chinensis and Radix achyranthis bidentatae showed an inhibitory effect similar to 10% bleach.

From Graph 2, it can be seen that Sophora flavescens showed an observable difference from the negative control for E. coli. As the error bar of Sophora flavescens does not overlap with that of positive control, it can be observed that it showed an inhibitory effect on E. coli; however, the inhibitory effect is observed to be less than 10% bleach.

As only a small number of extracts showed inhibitory effects to bacterial growth contrary to that of literature review, we postulate that the active compounds in the TCM were too large to diffuse through the agar mesh.

Colony Forming Unit Test

The CFU test was conducted to reassess the inhibitory effects of TCM extracts on bacteria. As we previously postulated that some of the active compounds of the TCM extract would not diffuse readily through agar, we therefore mixed the TCM extract directly with the bacteria.

As seen from Table 4, along with overlaps from the results obtained from the well-diffusion test, Tasmannia lanceolata, Herba polygoni avicularis, Radix stemonae, Sophora flavescens, Fructus schisandrae chinensis and Radix achyranthis bidentatae showed possible inhibitory effects on bacteria cell division. Hence, the respective mixtures were serial diluted and plated.

As seen from Graph 3 and 4, all the extracts chosen showed observable differences with the negative control (sterile water). Hence, all of the extracts were observed to inhibit bacteria cell division.

Statistical analysis was also conducted. T-test was conducted between the extract and the control set-up. As seen from the p-values obtained in Table 5, all the extracts except Radix achyranthis bidentatae showed a significant difference (p value<0.05) in inhibiting bacteria cell division of E. coli. From Table 6, all the extracts except Radix achyranthis bidentatae showed a significant difference in inhibiting bacteria cell division of S. epidermis. Hence, it can be concluded that Radix achyranthis bidentatae showed the greatest effectiveness as an anti-microbial agent, followed by Sophora flavescens and Fructus schisandrae chinensis.

Minimum Inhibitory Concentration

The Minimum Inhibitory Concentration test was conducted to further investigate if the extracts could be bactericidal in addition to being able to inhibit bacteria growth.

We decided to focus on the extracts which showed promising inhibitory effect in the CFU test and whose active compounds would likely be small enough to act systemically in Stevia. Sophora flavescens, Tasmannia lanceolata, Herba polygoni avicularis, Radix stemonae, Fructus schisandrae chinensis and Radix achyranthis bidentatae were tested in a microtitre plate.

However, as seen from results from Table 7 and 8, the absorbance reading did not show a trend when the extracts were diluted. This suggests that the extracts were unable to lyse the bacteria or inhibit the bacteria growth rate significantly. A possibility for this is that the extracts were far too dilute.

Sugar Cube Test

Given the inconclusive results of the Flour Disk Bioassay, the sugar cube test was conducted to continue with our investigation. As seen from Table 10 and Graph 5, all the extracts tested for led to a greater change in mass of sugar cube compared to the control. Hence, the extracts were capable of deterring the ants from consuming the sugar cube. Since the change of mass of sugar cube is proportional to the feeding deterrence of the extract, Radix achyranthis bidentatae showed greatest feeding deterrence followed by Tasmannia lanceolata and Herba polygoni avicularis.

In vivo testing of Stevia robustness

The in vivo testing on Stevia was done to investigate the effect of TCM on bacteria on a Stevia plant. Out of the many leaves tested, most curled up and wilted. Only 5 leaves remained alive with signs of infection. After close monitoring, the 5 leaves recovered over the next day. 2 out of the 3 leaves coated with the Radix achyranthis bidentatae extract survived

while all of the leaves coated with the *Tasmannia lanceolata* extract survived.

Table 2.

E. *coli*

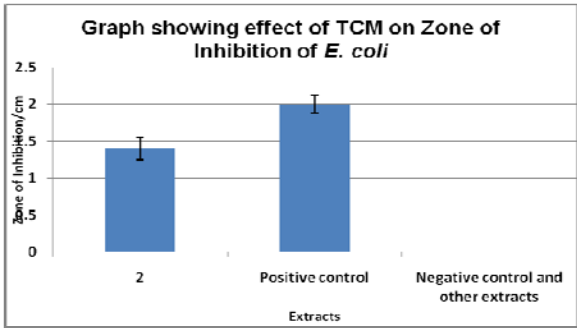
TCM	Zone of Inhibition/cm			Mean/cm	Std Error
2	1.3	1.7	1.2	1.4	0.15275
+	2	1.8	2.2	2	0.11547

Table 3.

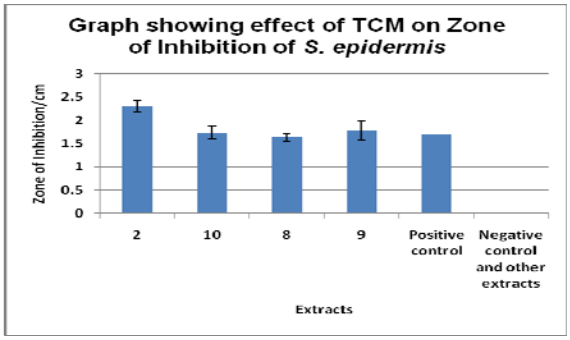
S. *epi*

TCM	Zone of Inhibition/cm			Mean/cm	Std Error
2	2.3	2.5	2.1	2.3	0.11547
10	1.7	2	1.5	1.733333	0.1453
8	1.5	1.8	1.6	1.633333	0.08819
9	1.6	2.2	1.55	1.783333	0.20883
+	1.7	1.7	*failed*	1.7	0

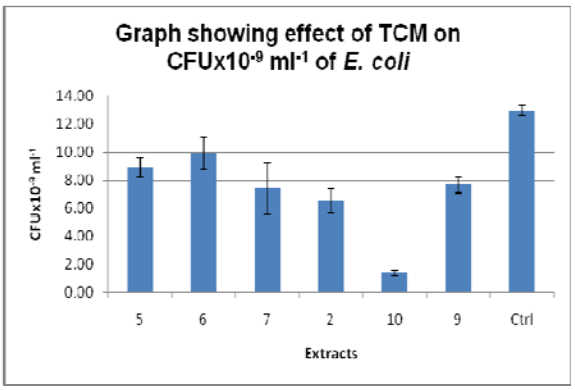
Graph 1.



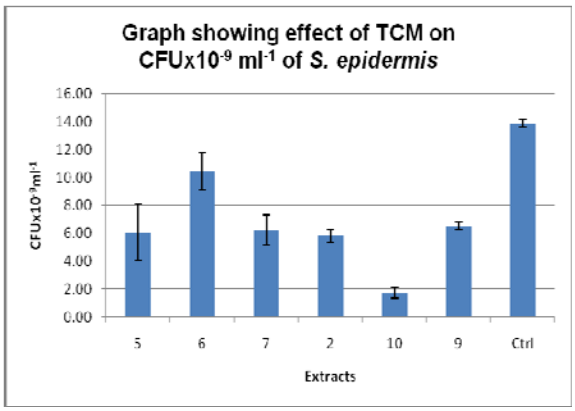
Graph 2.



Graph 3.



Graph 4.



Graph 5.

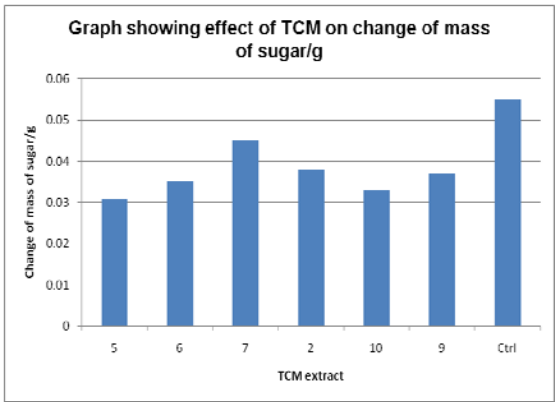


Table 4.

	E.coli	*S.epi*
1	0.5414	0.4407
2	0.6249	0.6666
3	0.5098	0.5396
4	0.7671	0.6368
5	0.475	0.4125
6	0.2418	0.2375
7	0.473	0.468

8	0.516	0.9246
9	0.5225	0.4949
10	0.084	0.1752
11	0.481	0.4183
12	0.6538	0.5989
13	0.6866	0.4847
14	0.643	0.6034
15	0.4766	0.4755
16	0.5273	0.1171
Ctrl	0.7146	0.6752

Table 5.

E.coli

TCM	CFUx10^{-9} ml^{-1}			Mean	Std Error	p-value
5	10.08	8.87	7.81	8.92	0.66	2.60E-02
6	11.25	10.91	7.67	9.94	1.14	2.53E-02
7	4.22	7.54	10.47	7.41	1.81	8.68E-02
2	5.62	5.67	8.33	6.54	0.90	1.50E-02
10	1.50	1.62	1.12	1.41	0.15	1.07E-02
9	6.67	8.55	7.84	7.69	0.55	7.36E-02
Ctrl	13.28	12.64	NIL	12.96	0.32	NIL

Table 6.

Table 7.

	1	1/2	1/4	1/8	1/16
2	0.297	0.358	0.340	0.265	0.303
2*	0.277	0.333	0.384	0.328	0.295
8	0.308	0.296	0.356	0.301	0.352
9	0.286	0.328	0.326	0.281	0.315
10	0.345	0.345	0.312	0.309	0.265

Table 8.

	1	1/2	1/4	1/8	1/16
2	0.306	0.416	0.428	0.355	0.370
2*	0.261	0.377	0.375	0.361	0.331
8	0.353	0.313	0.389	0.332	0.363
9	0.294	0.355	0.344	0.310	0.358
10	0.446	0.373	0.341	0.337	0.309

Table 9.

S.epi

TCM	CFUx10^{-9} ml^{-1}			Mean	Std Error	p-value
5	6.06	9.51	2.58	6.05	2.00	7.44E-02
6	8.86	9.38	13.11	10.45	1.34	6.66E-04
7	4.54	5.87	8.28	6.23	1.09	1.45E-02
2	5.41	6.69	5.31	5.80	0.44	1.50E-02
10	1.32	1.39	2.50	1.74	0.38	5.99E-03
W	6.17	7.07	6.33	6.52	0.28	7.87E-03
Ctrl	13.63	14.17	NIL	13.90	0.27	NIL

Table 9.

TCM	Mass/g			
	Petri dish	Petri dish + flour (before)	Petri dish + flour (after)	Change
5	9.23	9.3	9.28	0.02
6	9.24	9.33	9.33	0
7	8.53	8.64	8.64	0
2	9.23	9.33	9.32	0.01
10	9.27	9.33	9.33	0
9	9.25	9.34	9.34	0
Ctrl	8.15	8.25	8.25	0

Table 9.

TCM	Mass/g		
	Petri dish + sugar (before)	Petri dish + sugar (after)	Change
5	13.5	13.469	0.031
6	13.69	13.655	0.035
7	14.37	14.325	0.045
2	13.56	13.522	0.038
10	13.41	13.377	0.033
9	13.67	13.633	0.037
Ctrl	14	13.945	0.055

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