

Testing *Simarouba amara*'s therapeutic effects against weedicide-induced tumor-like morphology in planarians

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SUMMARY

According to the World Health Organization, cancer is a leading cause of death globally. The disease's prevalence is rapidly increasing in association with factors including the increased use of pesticides and herbicides, such as glyphosate, which is one of the most widely used herbicide ingredients. Although chemotherapy is currently the most common option for treatment, its high cost and rates of side effects make it less accessible to economically disadvantaged patients. Natural antioxidants and phytochemicals are being tested as anti-cancer agents due to their antiproliferative, antioxidative, and pro-apoptotic properties. *Simarouba amara* is one such plant and contains several alkaloid derivatives known to inhibit cancerous growth. Thus, we aimed to investigate the potential role of *S. amara* extract as a therapeutic agent against glyphosate-induced toxicity and tumor-like morphologies in regenerating and homeostatic planaria (*Dugesia dorotocephala*). We hypothesized that *S. amara* would reduce and prevent tumor-like lesions and toxicity in planarians exposed to glyphosate. We conducted several assays to determine the efficacy of *Simarouba* extract in reducing tumor-like lesions and morphological changes, increasing survival rate, mitigating blastema reduction, and maintaining locomotive velocity in glyphosate-exposed planarians. In three sets of trials, planarians treated with *S. amara* extract after glyphosate-induced toxicity showed higher negative phototoxicity compared to those treated with spring water after glyphosate exposure. *S. amara* also significantly increased the survival rate, subdued tumor-like lesions, and mitigated blastema reduction in glyphosate-exposed planarians compared to those treated with spring water. The results show that *S. amara* extract possesses therapeutic effects against weedicide-induced toxicity.

INTRODUCTION

Cancer is a leading cause of death globally, causing ten million deaths in 2020 and accounts for about one in six deaths (1). Approximately 70% of these deaths occur in low to middle-income countries, which tend to have agriculture-based economies (2). Cancer is also estimated to have had a global economic cost of over \$1.16 trillion in 2010, and this amount has been increasing over the past 10 years (3).

Cancer emerges from the transformation of normal,

healthy cells into tumor cells in a multistage process due to a person's exposure to carcinogens such as ultraviolet and ionizing radiation, arsenic in water, environmental toxins, or certain viruses (3). The increasing use of pesticides and herbicides in agriculture around the world is linked to an increase in the prevalence of certain cancers (4, 5).

The International Agency for Research on Cancer (IARC) added glyphosate, the most widely used herbicide ingredient in the world, and Glyphosate-Based Herbicides (GBHs) as Group 2A carcinogens ("probably carcinogenic to humans") (7). The IARC reported that there is limited evidence of carcinogenicity in humans, but sufficient evidence of carcinogenicity in experimental animals (7). IARC also reports that there is strong evidence that glyphosate can operate through two key characteristics of known human carcinogens, genotoxicity (DNA damage) and oxidative stress, and that these can be operative in humans (7). A recent study in 2019 reported that using glyphosate increases the risk of non-Hodgkin lymphoma by 41%, which suggests a link between exposures to glyphosate-based herbicides and increased risk for non-Hodgkin lymphoma (8). An Italian multicenter case-control study showed that the risk of follicular lymphoma (FL) was elevated 7-fold in subjects who self-reported with medium-high confidence as ever exposed to glyphosate, 4.5-fold in association with medium-high cumulative exposure level, 12-fold with medium-high exposure intensity, and 6-fold with exposure for 10 days or more per year (9).

While there is limited evidence for glyphosate's carcinogenicity in humans, several studies suggest that glyphosate increases cancer risk in animal models. A recent study showed that glyphosate causes hemangiosarcomas, kidney tumors, and malignant lymphomas in CD-1 mice (10). The same study also showed evidence of hemangiomas in female Swiss albino mice, as well as kidney and liver adenomas, skin keratoacanthomas, basal cell tumors, and adrenal cortical carcinomas in Sprague-Dawley rats after glyphosate exposure (10). Hemangiosarcoma (HSA) affects blood vessels in the spleen, liver, heart, and skin that rapidly advances into aggressive metastasis, and is typically not noticed in animals until later-stage hemorrhagic events (11). Human angiosarcomas (HA) have a similar pathology as HSA, and animal studies can help in finding a treatment that can be translated into human treatment.

Current cancer and tumor treatments include surgery, radiotherapy, and chemotherapy. The average cost of cancer treatment in the United States is \$150k whereas the median household income was \$69k in 2019 (12,13). The two most common treatments – chemotherapy and anticancer drugs – also cause adverse side effects on normal cells/tissue, such as inhibition of bone marrow function, nausea, vomiting, and alopecia. These high costs and severe side effects make

them unfavorable and inaccessible, especially to low-income regions that are typically the most affected by environmental toxins.

Alternate anti-cancer adjuvant therapies using natural antioxidants and several phytochemicals have been recently suggested because of their anti-proliferative, antioxidant, and pro-apoptotic properties (14,15). The search for novel anticancer agents and compounds from plants is underway to find a safe and supplementary cure that is more affordable than current therapeutics.

Alkaloids sourced from the Simaroubaceae family, such as cathinone, have shown antiviral, antiparasitic, antibacterial, cytotoxic, and anti-inflammatory properties. These phytochemicals are used traditionally for the treatment of malaria, and as antitumor, anti-inflammatory, and antiviral supplements (15–19). A recently studied plant from the Simaroubaceae family is *Simarouba amara*, which contains several cytotoxic phytochemicals, such as quassinoids, alkaloids, triterpenes, anthraquinones, and other metabolites (15).

Recent studies indicated that *Simarouba* plants contain several compounds with chemoprotective effects and cytotoxic activity against human cancer cells in a lab setting (20–22). Similarly, antioxidant properties of plant extracts from Simaroubaceae family were also extensively studied using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (23, 24).

Planaria are flatworms with remarkable regenerative ability. The abundance of neoblasts (stem cells) in the body of planaria allows for them to be used for experimental studies of regeneration and stem cell growth in ecotoxicology. Research has shown that various carcinogens can induce tumor growth and lesions in the planarian, *Dugesia dorotocephala*, used in this experiment (25). These lesions are histologically similar to those found in humans and vertebrates (25, 26). A recent study on the toxicity of the herbicide glyphosate to another species of planaria, *Dugesia japonica* showed significant morphological malformations and histopathological changes in them after exposure to glyphosate (27). Planaria have stem cells throughout their body that help them to regenerate at any point from their body, making planaria an ideal candidate for this study.

We hypothesized that *S. amara* would mitigate and prevent tumor-like lesions and toxicity-associated morphological changes in planarians exposed to glyphosate. We also hypothesized that *S. amara* extract can improve the regenerative capacity of planarians with tumor-like lesions and toxicity after exposure to glyphosate.

RESULTS

Before proceeding with the main experiment of testing the efficacy of *S. amara* extract in preventing and treating tumor-like lesions in planarians, the lethal concentration 50 (LC50) of glyphosate in planarians was determined through a pilot experiment. LC50 is the quantity of a substance needed to kill 50% of a test organism during a certain observation period and are used to find the acute toxicity of a chemical. Our results indicate that the LC50 values of glyphosate for planaria at 18 h, 24 h, 48 h, and 72 h were 50 ppm, 40 ppm, 30 ppm, and 20 ppm respectively (Figure 1A). The 18 h LC50 of glyphosate (50 ppm) was used in all experiments conducted for testing the efficacy of *S. amara* extract in preventing and treating tumor-like lesions because it was the shortest time

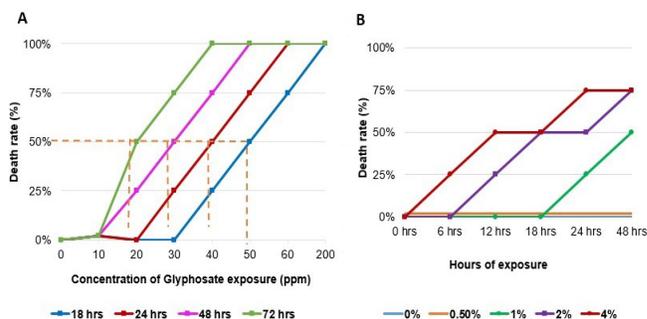


Figure 1: Pilot experiments. A) LC50 of glyphosate for planaria. Line chart showing the relationship between glyphosate concentration and death rate of planarians exposed for 18 hours, 24 hours, 48 hours and 72 hours. LC50 is calculated as the concentration of glyphosate that is lethal to 50 percent of the organisms in a certain time period. LC50 at 18 hours was 50 ppm and this concentration was chosen for experiments. Organisms were exposed to 10 ppm, 20 ppm, 30 ppm, 40 ppm, 50 ppm, 60 ppm and 200 ppm concentrations of glyphosate and observed for up to 3 days (n = 4). B) Maximum Tolerated Dose (MTD) of *S. amara* for planarians. Graph showing the relationship between length of exposure to *S. amara* extract and death rate of planaria (n = 4). MTD at six hours was calculated by determining maximum concentration with zero deaths at six hours.

and it helped to repeat the experiment multiple times. In all experiments, tumor-like lesions were induced in planarians by exposing them to 50 ppm of glyphosate and tumor-like lesions were developed in all parts of the body of planaria.

The safest concentration of *S. amara* for planarians needed to be determined from its maximum tolerated dose (MTD) concentration. We tested the MTD of *S. amara* extract for planarians to determine the highest concentration of *Simarouba* extract in which no worms died after continuous exposure for six hours and no other adverse effects were observed. We tested concentrations of *S. amara* extracts ranging from 0.5% to 4% in comparison to a spring water control (Figure 1B). We determined that the MTD of *S. amara* for six hours was a 2% aqueous solution.

Next, we tested the therapeutic efficacy of *S. amara* using assays to test the negative phototoxicity, regeneration (blastema growth), locomotion velocity (pLmV measured in mm/sec), and survival rate of glyphosate-exposed worms with lesions. We compared the treatments with *S. amara* extract versus those treated in spring water (negative control) in these assays (Figure 2). Planarians developed tumor-like

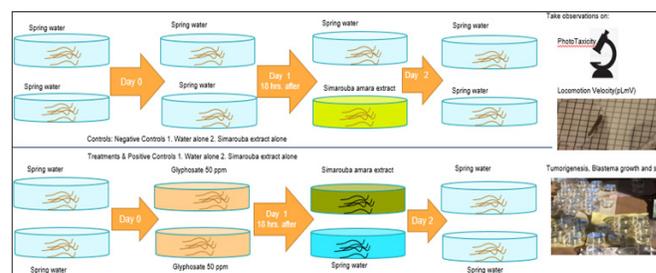


Figure 2. Diagram of experimental design and methodology. Flowchart displaying processes of control and treatment trials (n = 3). Planaria were first observed in spring water control environments before half were exposed to glyphosate for 18 hours. Half of the organisms were then treated with *S. amara* extract for six hours and observed.

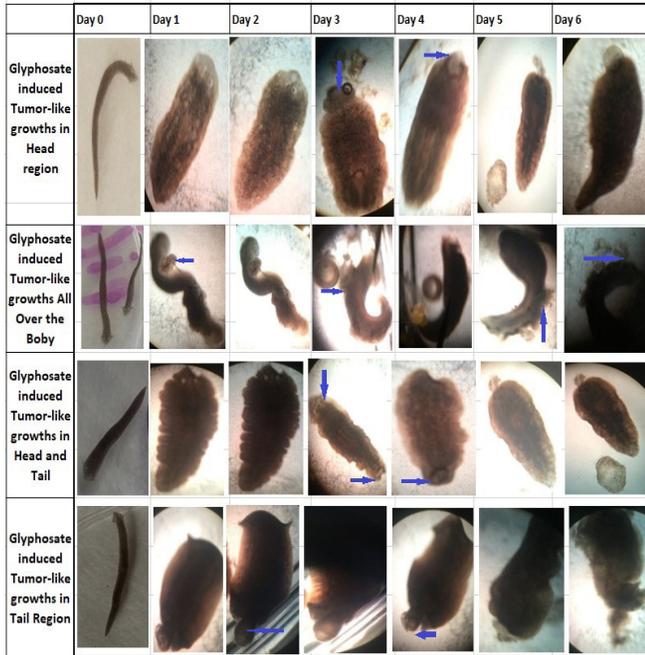


Figure 3. Glyphosate-induced morphological changes including tumor-like lesions in planaria. Chart showing the progress of morphological changes on various parts of planaria bodies over one week (n = 4). Planarians were exposed to glyphosate for 18 hours before being placed in spring water and observed for 6 days.

growths, epidermal blisters, changed morphology (shriveled phenotype), and reduced locomotion (Figure 3). Planarian worms under normal conditions show negative phototoxicity (movement away from direct light). Planarians that developed tumor-like growths did not respond to light exposure or showed much lower negative phototoxicity. In all three sets of trials, worms treated with *S. amara* extract after glyphosate-induced toxicity, regained negative phototoxicity sooner compared to those treated with spring water after exposure to glyphosate.

S. amara significantly subdued glyphosate-induced toxicity in exposed planarians compared to those treated with spring water, which developed more tumor-like growths (two-way ANOVA, $p < 0.001$; Tukey's HSD test, $p = 0.01$). *S. amara* treated planarians experienced fewer tumor-like lesions on Day 1 and were lesion-free by Day 5 post-treatment, while planarians treated with water maintained lesions for nine days (Figure 4A).

The average locomotion velocity of planarians in water was 2 mm/sec. During exposure to glyphosate, their locomotion velocity was reduced to 0.36 mm/sec. After exposure to glyphosate, when these worms were transferred back to normal water, the locomotion velocity was regained back to 1.6 mm/sec in three days (Figure 4B). These changes were not statistically significant (two-way ANOVA, $p = 0.918$).

We observed the survival rate of glyphosate-exposed worms in *S. amara* extract as well as their spring water counterparts for eight days after exposure to glyphosate. The survival rate of *S. amara* treated glyphosate-exposed worms was 80% after 8 days and was significantly higher than the spring water control glyphosate-exposed worms, which was 0% (two-way ANOVA, $p = 0.001$; Tukey's HSD test, $p = 0.01$)

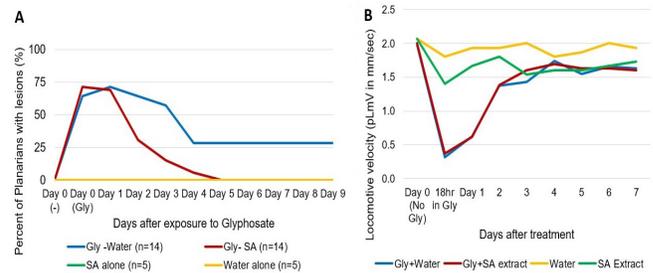


Figure 4. Experimental assays with *S. amara* extract. A) Percentage of planarians with tumor-like lesions. Line chart showing the percentage of glyphosate-exposed planarians who developed tumor-like lesions over time under two treatment conditions – one treated with *S. amara* extract and one with spring water (two-way ANOVA, $p < 0.001$; Tukey's HSD test, $p = 0.01$). Toxicity in negative controls (planarians grown in spring water and *S. amara* extract) were also measured. Planarians were observed for nine days after glyphosate exposure. B) The locomotive velocity of planarians under four treatment conditions – glyphosphate exposed and treated with *S. amara* extract (Gly+SA extract), glyphosphate exposed and treated with water (Gly+Water), planarians in water (negative control), and planarians in SA extract (negative control). Glyphosphate-exposed worms' locomotion velocity reduced in 18 hours of exposure and slowly regained in 3 days (two-way ANOVA, $p = 0.918$). Locomotion velocity didn't change for negative controls.

at eight days (Figure 5). All the worms placed in water after glyphosate-exposure died by Day 6.

Glyphosate-induced toxicity affected blastema growth in regenerating planarians. Blastema growth helps with regeneration of planarians. The tumor-like lesion development in cut planarians exposed to glyphosate was also observed. These lesions were developed at the extremities of the cut ends of planarians. The regeneration was measured as "blastema growth observed". The blastema growth in planarians treated with *S. amara* extract was higher than those treated in spring water. The efficacy of *S. amara* on preventing blastema reduction in regenerating planarians exposed to glyphosate was significantly higher (two-way ANOVA, $p = 0.03$; Tukey's HSD test, $p = 0.03$) than those regenerating in spring water after glyphosate exposure

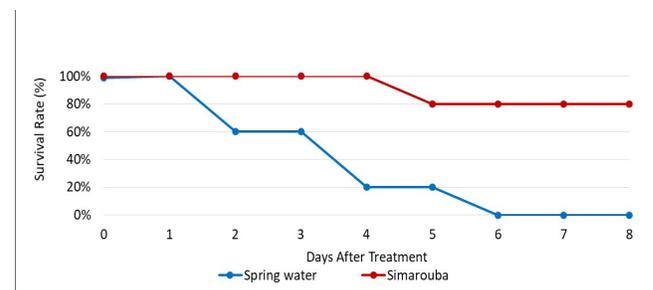


Figure 5. Change in survival rate of planarians after exposure and treatment. Graph displaying survival rates of planaria treated with *S. amara* extract compared to spring water after exposure to glyphosate (n = 10). After 18 hours of exposure to glyphosate, planaria were then treated with spring water or *S. amara* extract before being placed into spring water to be observed. Survival rate is calculated as number of planarians alive divided by the total number of planarians used in each treatment. A two-way ANOVA with time and treatment as two factors showed a significant difference ($p = 0.001$, Tukey's HSD test, $p = 0.01$).

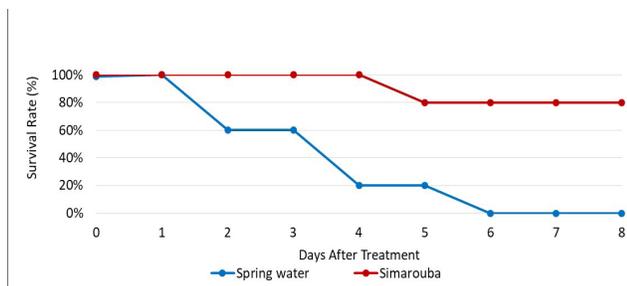


Figure 5. Change in survival rate of planarians after exposure and treatment. Graph displaying survival rates of planaria treated with *S. amara* extract compared to spring water after exposure to glyphosate (n = 10). After 18 hours of exposure to glyphosate, planaria were then treated with spring water or *S. amara* extract before being placed into spring water to be observed. Survival rate is calculated as number of planarians alive divided by the total number of planarians used in each treatment. A two-way ANOVA with time and treatment as two factors showed a significant difference (p = 0.001, Tukey's HSD test, p = 0.01).

(Table 1). *S. amara* extract treatment showed a significant (two-way ANOVA, p = 0.05; Tukey's HSD test, p = 0.05) reduction in tumor-like lesions in regenerating planarians after glyphosate exposure when compared to the spring water control planarians after glyphosate exposure (Figure 6). Data from both these experiments were analyzed using the two-way ANOVA method with time after treatment and treatment group as two factors.

The antioxidant activity of *S. amara* extract was examined using the oscillating Briggs-Rauscher (BR) reaction. In the presence of starch, the BR reaction mixture or solution goes through a series of periodic color changes from colorless to yellow to blue and then back to colorless which are called oscillations. The addition of an antioxidant to the active mixture stopped these oscillations (color changes) and, after a certain time, the oscillations started again. This inhibition time linearly depends on the antioxidative power of the extract and was measured. The results showed that the 2% *S. amara*

Percent of Planarians with Blastema Development under Different Treatments (Average of 3 trials)				
Days before/after exposure	Gly -Water (n = 42)	Gly- <i>S. amara</i> (n = 42)	<i>S. amara</i> alone (+ control) (n = 15)	Water (+ control) (n = 15)
Day 0 (-)	0	0	0	0
Day 0 (Gly)	0	0	50	50
Day 1	0	0	50	100
Day 2	20	42.9	83.3	100
Day 3	20	42.9	100	100
Day 4	60	71.4	100	100
Day 5	63.6	75	100	100
Day 6	100	100	100	100
Day 7	100	100	100	100

Table 1: Percentage of blastema growth in regenerating planarians. Effects in various treatments and control trials on preventing blastema reduction in regenerating planarians. Regenerating organisms treated with *S. amara* extract after glyphosate-exposure displayed less reduction in blastema growth than those regenerating in spring water after glyphosate exposure (two-way ANOVA, p = 0.03; Tukey's HSD test, p = 0.03).

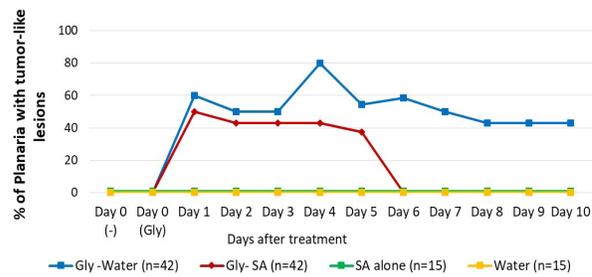


Figure 6. Tumor-like lesions in regenerating planarians. Line graph showing 10-day percentage of planaria in each trial with tumor-like lesions (n = 42). Organisms were exposed to glyphosate for 18 hours before half were treated with *S. amara* extract for 6 hours. Observations were recorded against each other and against both control trials. (two-way ANOVA, p = 0.05, Tukey's HSD test, p = 0.05)

extract had a significantly higher inhibition time than the negative control, distilled water, and a lower inhibition time than the 2% green extract, used as positive control (Table 2) (two-way ANOVA, p < 0.001; Tukey's HSD test, p = 0.01). This shows the antioxidant power of *S. amara* extract.

DISCUSSION

We hypothesized that *S. amara* would have a significant effect in mitigating glyphosate-induced toxicity and tumor-like lesions in planarians. The results from the assays indicated that *S. amara* was able to greatly increase the survival rate of planarians, reduce tumor-like lesions, and prevent blastema growth reduction from glyphosate. The maximum tolerated dose for immersing planarians was 2% aqueous solution of *S. amara*. For higher organisms and humans, the dosage could be much smaller as it will be ingested, and it needs to be estimated using dose-response studies.

In the first pilot experiment, planaria exposed to glyphosate showed severe toxicity symptoms such as tumor-like lesions, blisters, and protruded growths all over their bodies. Most of the tumor-like lesions were at the anterior or posterior extremities (Figure 3). The morphology of these blisters and lesions resembled exactly the tumor blisters and lesions reported from another study, where planarians were exposed to known carcinogen (class 1A), Cd (28). Similarly, results from another study on in vivo tumorigenesis in planarians using known mammalian chemical carcinogens produced tumors of similar morphology in planarians (25, 26). The

Test or Control Solution	Average Inhibition Time In Seconds
1ml water (Negative control)	10
2% <i>S. amara</i> extract	98
2% Green tea extract (Positive control)	108

Table 2. Antioxidant assay using oscillating Briggs-Rauscher (BR) reactions. Effect of *S. amara* extract on BR reaction inhibition time, based on antioxidative capacity. *S. amara* extract was measured against known antioxidant and positive control, green tea extract, to determine length of time that oscillations stop, caused by antioxidant free radicals (Ar-OH) reacting with hydroperoxyl radicals to disturb BR reaction, which is directly proportional to antioxidative power. Two-way ANOVA with repetition and antioxidant as two factors showed significant difference between inhibition times (two-way ANOVA, p < 0.001; Tukey's HSD test, p = 0.01).

development of tumor-like lesions that increased in size, became necrotic, and eventually disintegrated the body of planarians resulting in their death was also noticed in past studies with known carcinogens (25). However, classification of the observed blisters and lesions as malignant tumors would require validation via qPCR to show the presence of cancer-associated markers or histopathological testing of the lesions with H&E staining, which were beyond the scope of this study.

A recent study on the molecular toxicity of glyphosate on planarians reported various morphological abnormalities such as body twisting, contraction, local fester, and head and tail disintegration, as well as differential expression of several genes associated with detoxification, apoptosis, stress response, and DNA repair (20). The toxicity symptoms and tumor-like lesions we observed, might be due to the differential expression of these genes, which need to be confirmed by further studies.

In planarians treated with *S. amara* extract, the lesions disappeared after Day 5, while 28% of those in spring water remained with these lesions. This supports our hypothesis that *S. amara* extract has toxicity-mitigating effects on planarians. The therapeutic and chemoprotective effects of *S. amara* could be due to the quassinoids and several alkaloids present in them that protected planarians against the toxicity effects (20–22). A recent study indicated that quassin and neoquassin from Simarouba plants inhibited the CYP1A1 isoenzyme, an isoform of the P450 cytochrome enzyme known for its carcinogenic activity, thus showing its role as a chemoprotector (20). Another alkaloid from plants, Simalikalactone D showed cytotoxic activity against mammary human adenocarcinoma cells (21). Four canthin-6-one derived alkaloids, isolated from *S. glauca*, showed cytotoxic activity against human colon cancer, human oral epidermoid cancer, human hormone-dependent prostate cancer, and human lung cancer cells (22).

Tricaproin, a quassinoid from *S. glauca*, has been shown to inhibit histone deacetylases and prevent colorectal cancer growth in a lab setting (22). Histone deacetylases are enzymes involved in chromatin remodeling and the oncogenic behavior of cells. When deregulated, they promote cancer cell proliferation, prevent apoptosis, and increase cell migration through the modulation of histone acetylation.

Moreover, *S. amara* had no noticeable negative effects on healthy planarians, supporting the belief that it is a possible alternative to chemotherapy. Similarly, antioxidant properties of plant extracts from Simaroubaceae family were also extensively studied (24). The antioxidant assay showed the high antioxidant power of *S. amara* extract and could also contribute to its tumor mitigating effect. Antioxidants are efficient at removing free radicals from the body. Free radicals are highly reactive chemicals that harm cells and damage all major components of cells, including DNA, proteins, and cell membranes, which may cause the development of cancer (31).

This study will set the stage for understanding glyphosate-associated toxicity and the possibility of tumor growth in stem cells and homeostatic planarians as well as how extracts from *S. amara* affects toxicity and protective effect in homeostatic and regenerating planarians. A past study on human non-small-cell lung cancer, A549 Cells, showed evidence for apoptosis induced by Simarouba (32).

Although the experiment did not test for any comparison to current therapeutic and treatment options, prior studies show that the use of quassinoids from *S. amara* has lower rates of side effects than chemotherapy drugs such as Paclitaxel (32). Findings from another study show that glaucarubinone (GLU), one of the major quassinoids from Simarouba glauca, has the potential to enhance the activity of chemotherapy drugs and suggests it as an alternate treatment strategy for multi-drug resistance for drugs like Paclitaxel (33).

Further studies involving cytotoxicity studies using a concentration and time-dependent cytotoxicity assay should be done to check the cytotoxic effects of *S. amara* extracts in vitro and in vivo to see whether *S. amara* extract is rich in anti-cancer molecules such as phenolic compounds and flavonoids and whether it can exhibit potent anti-proliferative activity compared to any conventional drug. An MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) cytotoxicity assay can determine the cytotoxic effect of *S. amara* extract. Access to a more comprehensive laboratory setting would facilitate using assays to visualize the movement of neoblasts to cancer or injury sites with and without *S. amara* extract. This study helps to understand the toxic effects of glyphosate on stem cells as well as normal cells and could set the stage for future studies on testing phytochemicals for treating pediatric and stem cell cancers.

MATERIALS AND METHODS

The LC50 of glyphosate and maximum tolerated dose of *S. amara* were estimated before proceeding with the main experiment.

Determining the LC50 of glyphosate:

Glyphosate solutions of 10, 20, 30, 40, 50, and 60 ppm were prepared from the 200 ppm glyphosate stock solution (Home Depot) and spring water (HEB). Four planarians (Carolina Biological Supply BioKits Planaria Regeneration Kit) were added to each petri dish (Carolina Biological Supply BioKits Planaria Regeneration Kit). The survival of the planarians in each petri dish was observed at 12, 18, 24, 36, 48 and 72 hours. The LC50 of glyphosate in planarians at 18 hours was determined and used for further experimentation.

Determining the Maximum Tolerated Dose (MTD) of *S. amara* after six hours:

Solutions of *S. amara* bark extract (0.5%, 1%, 2%, and 4%) (Natural-e-Health) were prepared from the 50% stock solution and spring water. Four planarians were added to each petri dish. The survival of the planarians in each petri dish was observed at 6, 12, 18, and 24 hours. The maximum tolerated dose (MTD) of *S. amara* extract for planarians at six hours was determined and used for further experimentation.

Testing the effect of *S. amara* extract on cancerous growth, negative phototoxicity, locomotion, and survival of planarians (homeostatic) treated with glyphosate:

The planarians used for this trial were observed for normal morphology without any lesions and normal gliding movements along the side walls of the petri-dish to make sure they were healthy before beginning the experimentation. Three petri dishes were filled with spring water and two planarians each as negative control. Three petri dishes were filled with 10 milliliters (mL) of the 2% *S. amara* solution

and two planarians each as a control. Fourteen petri dishes were then filled with 10 ml of the 50 ppm glyphosate solution and two planarians each for carcinogen exposure. After 18 hours, the glyphosate solution was removed from the petri dishes and seven dishes were refilled with spring water as a positive control. The other seven petri dishes were filled with 10 ml of 2% *S. amara* solution as treatment. Six hours later, the *S. amara* solution was removed from the petri dishes and the dishes were refilled with spring water. For four more days, daily observations on planarians' locomotion, negative phototoxicity, and survival were taken using a microscope (Meiji Techno Co., Ltd. Stereo Microscope) and noted in a lab notebook. The experiment was repeated once to validate the results (Figure 2).

Locomotion velocity was measured in mm/sec by placing the transparent petri dish (with worms moving around) on a math graph paper with markings and counting the number of gridlines crossed in response to light for two minutes, in eight periods of 15 seconds each, and converting the segments to mm/sec. Negative phototoxicity is measured as the percentage of worms moving away from the light source. The transparent petri dish with 14 worms was placed on white paper, with a circle box marked around the petri dish with a line at the middle to mark two halves. A light source was directed at one half, and the other half was covered at the top with black paper, and number of worms moved from the lighted half to the darker half was measured as negative phototoxicity.

The survival rate of each treatment was measured by counting the number of live planarians remaining alive on the bottles for that treatment on each day and dividing it by the total worms used for that treatment. The survival rate across the eight days was analyzed using two-way ANOVA (Microsoft Office Excel Professional 2013) with time and treatment group as two factors. For those with ANOVA tests significant, Tukey's HSD tests were done with a level of significance set up at $p \leq 0.05$, to find which group, intervention or control, performed better.

Testing the effect of *S. amara* extract on cancerous growth and blastema growth (regeneration) of cut planarians (regenerating) treated with glyphosate:

The planarians being used for this trial were observed to be healthy before beginning the experimentation. Three petri dishes were filled with spring water and one planarian each as negative control. Three petri dishes were filled with 10 ml *S. amara* solution and one planarian each as a control. Ten petri dishes were filled with 10 ml glyphosate Solution and one planarian each. Using several disposable scalpels (Carolina Biological Supply BioKits Planaria Regeneration Kit), each planarian was laterally cut near the pharynx into two pieces. After 18 hours, the glyphosate solution was removed from the petri dishes and five dishes were refilled with spring water as a positive control, and the other five with 10 ml of 2% *S. amara* solution as treatment. Six hours later, the *S. amara* solution was removed from the petri dishes and the dishes were refilled with spring water. Daily observations on planarians for cancerous growth and blastema growth were taken for 10 more days and noted in a lab notebook.

Testing the effect of *S. amara* extract on survival rate of planarians (homeostatic) exposed to glyphosate:

First, 50 ml of 50 ppm glyphosate solution and 10 full planarians were added to each of two culture bottles. After 18 hours, the glyphosate solution was removed from the bottles. Then one bottle was refilled with spring water and the other with 50 ml *S. amara* 2% solution. After six more hours, the *S. amara* solution was removed from the bottle and the bottle was refilled with spring water. Daily observations were taken by counting the number of planarians alive on each bottle on each day. Observations on planarian's survival were taken for 15 days on the first trial and noted in a lab notebook. To validate the results the experiment was repeated three times with the observations on planarian survival taken for up to eight days after treatment with *S. amara* (Figure 1).

Antioxidant assay using oscillating Briggs-Rauscher reactions:

Antioxidant activity of *S. amara* extract was examined using the oscillating Briggs-Rauscher reaction (Fascinating Oscillating Reaction Kit, Educational Innovations, Inc.) (29, 30). Using a beaker, a mixture was prepared of 1 ml starch solution, 2 ml distilled water, 10 ml 3% hydrogen peroxide (Walgreens), 10 ml malonic acid 0.3 M, 10 ml acidic sodium iodate 0.2 M. The beaker was put on white paper and the contents were stirred with a stirring rod before adding 2 ml of manganese sulfate solution. After a few seconds, the mixture started to oscillate changing color from colorless to yellow to blue to colorless again. At the moment in which the mixture became blue for the second time, 12 drops (0.4 ml) of *S. amara* extract was added and a stopwatch started. The oscillation process immediately stopped, and the mixture remained colorless. The time when the blue color ("third blue") reappeared was measured, this was a measure of the inhibition time. This process was repeated three times, and a control experiment adding 1 ml of distilled water instead of the *S. amara* extract was completed as well. Two percent green tea extract (12 drops) was used as a positive control, as green tea extract is known to be an antioxidant. These experiments were repeated three times to check the validity of the results from each trial.

Received: July 26, 2023

Accepted: October 12, 2023

Published: April 26, 2024

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