Original Article

Evaluation of Anticancer Potential of *Eleusine indica* Methanolic Leaf Extract through Ras- and Wnt-related Pathways Using Transgenic *Caenorhabditis elegans* Strains

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Abstract

Background: In the Philippines, many accounts have resurfaced claiming different herbal and therapeutic advantages of *Eleusine indica*. One of these advantages is its anticancer potential. Despite some studies showing that the crude extract has cytotoxic and radical scavenging activity, it is still insufficient and further scientific evidence is needed to support this claim. **Aim:** Hence, we evaluate the anticancer potential of *E. indic*a methanolic leaf extract (EMLE) by focusing on two cancer-related pathways, Ras and Wnt pathways. **Subjects and Methods:** We used wild-type and transgenic *Caenorhabditis elegans* strains which have an irregular Ras or Wnt signaling. We determined the average number of eggs laid of each strain and the multivulva development of the Ras-mutant strain. **Results:** Our experiments show that EMLE does not affect the number of eggs laid of the wild-type, Ras-mutant-and Wnt-mutant worms. Furthermore, EMLE was not able to reduce Ras-mutant population demonstrating multi-vulva. **Conclusion:** Taken together, our data suggest that the anticancer potential of EMLE may be independent of Ras and Wnt signaling pathways.

Keywords: Anticancer, *Eleusine indica,* ras pathway, wnt pathway

Introduction

Nowadays, cancer treatment may come in the form of chemotherapy, immunotherapy, radiation therapy, and stem cell therapy.[1] However, to those marginalized individuals from developing countries, only few can afford to pay for these treatments resulting them to rely on herbal medicine. Thus, this moved us to provide scientific evidence for the medicinal value of these plants. Recently, *Eleusine indica* (*E. indica*) also known as "Paragis or goose grass" has grown recognition due to several studies claiming that this plant has potential to treat various diseases. As a matter of fact, in the Philippines alone, there are several practices which utilized parts of this plant to take advantage of its diuretic and anti-inflammatory properties in treating kidney problems and arthritis.[2,3] Evidently, previous studies have shown that *E. indica* also has antiviral, antiplasmodial, antidiabetic, antioxidant, and antibacterial properties.[4] On top of these various medicinal advantages, *E. indica* became popular due its anticancer potential claims, as supported by several studies demonstrating its antioxidant, proapoptotic, and cytotoxic properties.[5-10]

With these premises, we are interested to identify a potential cancer-related signaling pathway where *E. indica* crude leaf extract may be associated with. We have chosen to use its methanolic leaf extract due to its high total phenolic compound and efficient radical scavenging activity.[11] Reactive oxygen species have long been associated with cancer and found to have a promising impact during the activity of different

Access this article online

Quick Response Code:

Website: www.pnrjournal.com

DOI: 10.4103/jpnr.JPNR_7_20

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Submitted: 23-Apr-2020 **Revised:** 15-Jun-2020
Accepted: 23-Jun-2020 **Published:** ***

How to cite this article: Nas JS, Dangeros SE, Chen PD, Dimapilis RC, Gonzales DJ, Hamja FJ, *et al*. Evaluation of anticancer potential of *Eleusine indica* methanolic leaf extract through Ras- and Wnt-related pathways using transgenic *Caenorhabditis elegans* strains. J Pharm Negative Results 2020;11:XX-XX.

drugs.[12] Two of the most important cancer-related pathways are Wnt and Ras signaling pathways. Dysregulation in Wnt pathway has been linked to colorectal cancer, ovarian cancer, and breast cancer.[13-15] Meanwhile, irregularities in Ras pathway are associated with myelomonocytic leukemia, ovarian cancer, colorectal cancer, and cervical cancer.[16-18] We used two transgenic *Caenorhabditis elegans* strains which exhibit mutations in Ras and Wnt pathways. Humans and *C. elegans* share comparable Ras and Wnt signaling pathways, especially their downstream targets.[19]

Our study evaluates the potential of *E. indica* methanolic leaf extract (EMLE) on affecting mutations in Ras and Wnt signaling pathways of transgenic *C. elegans* strains.

Subjects and Methods

Preparation of *Eleusine indica* methanolic leaf extract

E. indica leaves were collected in Cuyapo, Nueva Ecija, Philippines. The methanolic extraction follows the protocol from a previous study but with some modifications.[11] The plant was dried under the sun for 2–3 days before pulverized and extracted. It was submerged in 95% methanol for 7 days and filtered using Whatman No. 41 filter paper. The solvent was evaporated in a rotary evaporator and the extract was kept at 4°C until used. The extract was dissolved in 0.5% dimethyl sulfoxide (DMSO) to come up with the different concentrations of the extract.

Phytochemical screening

For the qualitative evaluation of EMLE to determine the presence of flavonoids, phenolics, saponins, tannins, alkaloids, triterpenes, and steroids, 1 ml of 10 mg/ml EMLE in 0.5% DMSO was used following the protocols from recent experiments.[20-22]

Procurement and maintenance of *Caenorhabditis elegans* strains

In the study, all Ras-mutant *C. elegans* strain MT-2124, WNT-mutant strain JK3476, Bristol wild-type N2 strain, and OP50 *Escherichia coli* (*E. coli*) were provisioned by *Caenorhabditis* Genetics Center, University of Minnesota, USA. All strains were grown on NGM plate at 25°C following the protocol from a previous study.[23] Worms were transferred every day on a new Nematode Growth Medium plate seeded with OP50 *E. coli*.

Egg laying assay

We placed twenty L1 worms of either wild type, Ras mutant, or WNT mutant in a plate seeded with OP50 *E. coli*. A total of 5 plates per strain were assigned with the positive control, negative control, and varying concentrations of EMLE, namely 10 mg/ml, 1 mg/ml, and 0.1 mg/ml respectively. On our end, we used 10 mg/ml as the highest concentration since it is the highest possible concentration which can be dissolved with 0.5% DMSO. In addition, we used 20 µg/ml sorafenib in 0.5% DMSO (Bayer Healthcare Pharmaceutical Inc, Leverkusen, Germany) as the positive control and 0.5% DMSO as the negative control. The total number of eggs in each plate was counted every day for 4 days post-L4. The entire experiment was repeated at least twice.

Multivulva reduction assay

For this assay, we followed the same protocol as previously mentioned, but instead of using all the three strains, we only used Ras-mutant (MT2124) *C. elegans* strains. This transgenic strain develops pseudo vulvas as depicted by protrusions on the body of the worm. To get the percentage of worms developing multivulva, we counted the number of worms with a protrusion on the abdomen and divided it by the total number of worms observed for the specific treatment. This assay was done with two independent trials.

Statistical analysis

All the data are presented as mean ± standard deviation of all the trials. An average egg laid was assessed using analysis of variance through GraphPad Prism version 8 (GraphPad Software, San Diego, CA, USA). Tukey's test was used for *post-hoc* analysis on treatment groups found to have significant difference. The level of significance was set at $P < 0.05$.

Results

We were able to screen secondary metabolites such as tannin, flavonoid, alkaloids, and phenols found in EMLE through different colorimetric assays. With this knowledge, we were ready to assess possible biological activities of EMLE in wild-type and transgenic *C. elegans* strains.

We first tested the effects of EMLE on the egg-laying ability of wild-type *C. elegans*. As shown in Figure 1a, varying concentrations of EMLE did not pose any positive nor negative effects on the number of eggs laid by the wild-type *C. elegans* on four different day post-L4. These data suggest that EMLE as much as 10 mg/ml has no effect on the normal egg-laying physiology of *C. elegans*.

Wnt-mutant *C. elegans* are sterile when grown at 25°C. We were able to show on Figure 1b that the worms were still capable to grow eggs but in a lower number compared to the wild type. Egg laying was still evident in our experiment which may be attributed to the fluctuating temperature inside the chiller. Minimal changes (±2°C) from the actual temperature may eventually lead to partial sterility. Moreover, the average number of eggs laid during days 1–4 which was treated with different concentrations of EMLE which was comparable with the negative control. The lack of significance in our result indicates that as high as 10 mg/ml, EMLE exhibits no significant effect on the egg laying of transgenic *C. elegans* with altered Wnt signaling.

Similarly, Ras-mutant nematodes treated with 0.5% DMSO performed comparably well with those given with various EMLE concentrations as shown in Figure 1c. Evidently, we were able to demonstrate in Figure 1d that different concentrations of EMLE were not able to significantly reduce number of individuals developing multivulva. In addition, we included in Figure 1e images of the representative individuals

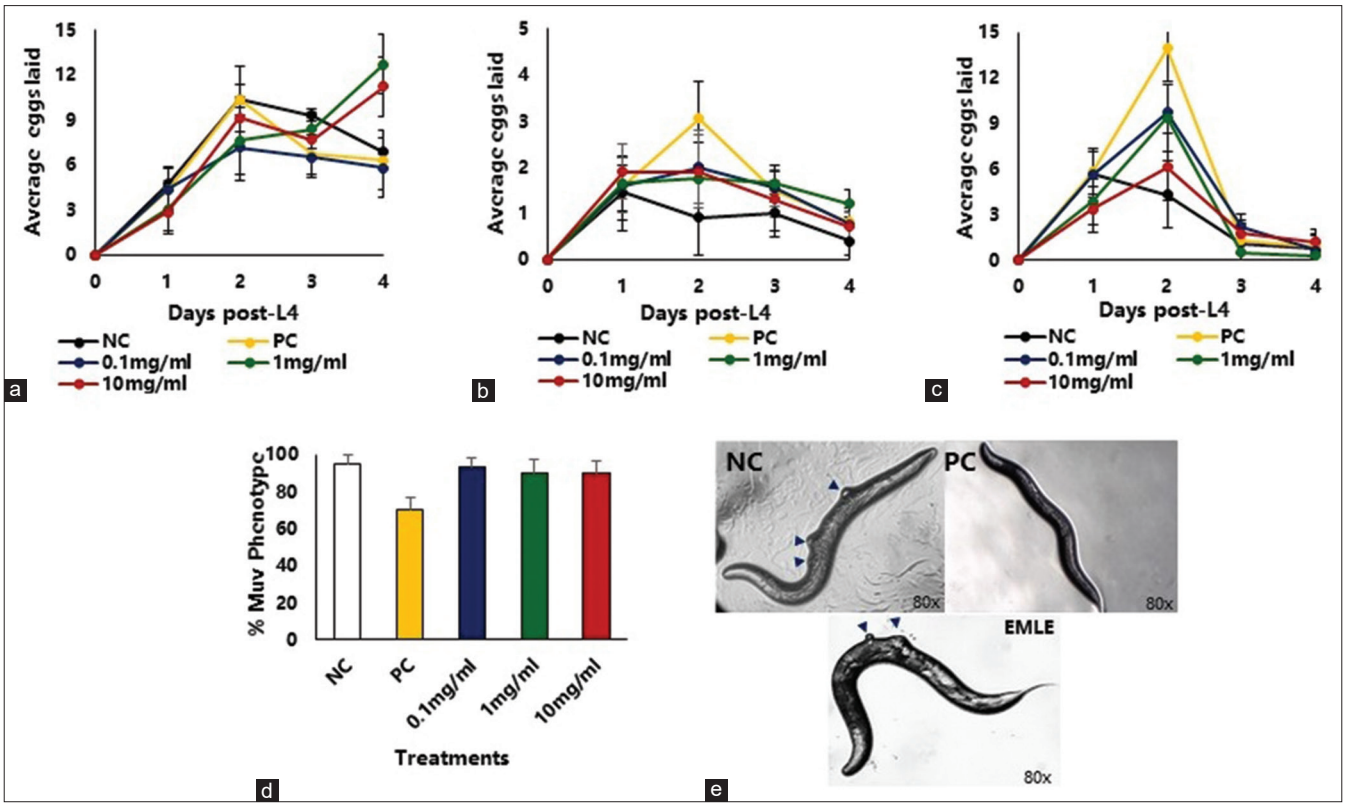

Figure 1: Varying concentrations of *Eleusine indica* methanolic leaf extract did not affect fecundity of wild-type, Ras-mutant, and Wnt-mutant *Caenorhabditis elegans*. Twenty nematodes were assigned various levels of *Eleusine indica* methanolic leaf extract. The negative control is only 0.5% DMSO with 0 mg/ml concentration of *Eleusine indica* methanolic leaf extract and the positive control contains 0.5% dimethyl sulfoxide and 20 μg/ml of sorafenib. The effects of *Eleusine indica* methanolic leaves extract were tested on the (a) wild-type, (b) Wnt-mutant, and (c) Ras-mutant *Caenorhabditis elegans* for 4 days post-L4. (d) The percentage of worms developing multivulva phenotype was determined during the 1st day of adulthood of the worms, where (e) representative images of the nematodes which developed pseudo vulvas were shown. Values are expressed as mean ± standard deviation ($P < 0.05$)

with the presence of pseudo vulvas, as indicated by the arrows. These data confirm our hypothesis that as much as 10 mg/ml of EMLE may have not affected the egg-laying and multivulva development in Ras-mutant *C. elegans*.

Discussion

We investigated the effects of EMLE on the egg laying of wild-type, Wnt-mutant, and Ras-mutant *C. elegans*. We are interested in the feasibility of EMLE to affect two major cancer-related signaling pathways, Wnt and Ras. In comparison with other studies, the reported concentrations of *E. indica* crude extract that show inhibitory action on various cancer cell lines are between 100 μg/ml and 3.125 mg/ml.[5-9] Hence, the concentrations used by the previous experiments fals within the range of the concentrations we used in this study. It seems that even though the crude extract reveals to have cytotoxic activity as previously reported, the mechanism of action involved may be independent of Wnt and Ras pathways. Interestingly, another study shows that EMLE has no cytotoxic activity on selected human cancer cell lines.[11]

Previous studies have associated the abnormal activation of Wnt pathway during the development of cancer.[24] Permanent activation of this pathway leads to a high probability of formation of cancer.[25] In a typical cell, β-catenin is joined by Axin and adenomatous polyposis coli to form a protein complex which later be degraded through ubiquitinylation.[24] Conversely, when Wnt pathway is activated, it sends signals to arrest Axin which allows β-catenin to enter the nucleus and interact with Tcf protein and transcribe c-MYC.[24] c-MYC is a pro-oncogenic gene which is responsible for rapid proliferation of cells.[26] In *C. elegans*, Wnt signaling pathway is associated with the somatic gonads, primarily the distal tip cell (DTC).[27] The absence of Wnt signals produces sterile nematodes.[28] The Wnt-mutant strain used, ceh-22(q632) has an impaired DTC which results to partial sterility, and previous study suggests that it is a downstream target of β-catenin.[29] In human, the counterpart of ceh-22 is Nkx2-5 proteins.[29,30] The regulation of this protein is associated with cardiomyocyte cell differentiation, endocardial fate, and cardiac diseases.[31-33] Our data reveal that varying concentrations of EMLE fail to show significant changes both in the egg-laying of Wnt-mutant *C. elegans* which suggests that its anticancer effect may not intercede with Wnt signaling pathway.

On the other hand, the Ras pathway is associated with the mitogen-activated protein kinase (MAPK) pathway which is

also responsible for various cellular activities like cell growth, proliferation, apoptosis, and migration.[34] The activation of Ras leads to the cascade of activation of the MAPK family starting from Raf, MEK, ERK, and ETS and followed by SIAH which will regulate tumorigenesis and metastasis.[35] Several studies have demonstrated that the Ras pathway and its downstream targets in human are conserved from *Drosophila* and *C. elegans*.[19,35] In *C. elegans*, the Ras activation starts when let-60 receives a signal from let-23 (epidermal growth factor receptor) which allows the signaling be passed down toward lin-45(Raf), mek-2(MEK), mpk-1(ERK), and lin-31 (Erythroblast Transformation Specific) which promotes vulva differentiation.[36] The MT2124 *C. elegans* mutant used has a dysregulation in let-60 causing the development of pseudo vulva.[37] Furthermore, we were able to observe that EMLE does not affect egg-laying and multivulva development in the Ras-mutant nematode. Hence, the possible mechanism of action involved in the anticancer potential of EMLE may have no evident effect on the Ras signaling pathway.

We have mentioned that EMLE may have not significantly affected the egg laying of *C. elegans* with abnormal Ras and Wnt pathways, but this does not neglect its anticancer potential. We recognize the limitation of our experiments in terms of mutant genes, ceh-22 and let-60, affected. This led us to gain interest in evaluating other mutated genes as well. In fact, in cancer development, there are various other genes involved.[38] Moreover, Notch signaling pathway may be further evaluated as it is also an important pathway associated with breast cancer and lung cancer.[39-41] The Notch receptors and Notch ligand-receptor interactions are commonly used as the target for various therapeutic agents in which impede metastasis and tumorigenesis.[42-44] Furthermore, this would still require further investigation which may be achieved using different transgenic *C. elegans* strains or even other model organisms such as *Drosophila* and mice.

Conclusion

Despite the detected secondary metabolites in our extract, EMLE was not able to affect the egg-laying ability of wild-type, Wnt-mutant, and Ras-mutant *C. elegans* strains. Moreover, EMLE was not able to affect multivulva formation in Ras-mutant *C. elegans*. Overall, our study suggests that the potential anticancer property of EMLE may be independent on Wnt and Ras signaling pathways.

Acknowledgment

We would like to thank the staff of University Research Center-FEU Manila for the support given to our research group. We would also like to extend our gratitude to Dr. Medina and the members of Biological Models Laboratory UP Manila for all the help.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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