ARTICLE

MicroRNA-21, C-C chemokine receptor type 7, and twinfilin actin binding protein 1 expression in fresh frozen breast tissue samples

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ABSTRACT

Breast cancer ranks second worldwide as the most common cancer in both sexes and is the leading cancer in women. Individuals at high risk of developing breast cancer are recommended mammography to detect early-stage disease. Although this technique was shown to reduce mortality, its usefulness is limited by high false-positive rates due to its inability to differentiate benign from malignant tumors. These problems highlight the need for markers to enhance breast cancer screening strategies. Proposed biomarkers, microRNA-21 (miRNA-21), C-C Chemokine Receptor Type 7 (CCR7), and Twinfilin Actin Binding Protein 1 (TWF1), were evaluated based on their role in cancer progression. In this retrospective analytical cross-sectional study, sixty-four fresh frozen breast tissue samples from the Human Cancer Biobank of St. Luke's Medical Center Quezon City (i.e., 32 breast tumor samples with paired adjacent non-tumor tissue) were retrieved to evaluate the relative expression of miRNA-21, CCR7, and TWF1 using TaqMan® assays. MiRNA-16 was used for miRNA normalization, and 18S rRNA was used for gene expression normalization. Receiver operating characteristic (ROC) curve analysis was performed to examine the diagnostic capabilities of the studied biomarkers. MiRNA-21 (P<.001), CCR7 (P=.003), and TWF1 (P=.003) expression had a significant difference in breast tumors from the paired non-tumor tissue. Additionally, miRNA-21 and TWF1 were able to differentiate early-stage (P<.001; P=.008) and late-stage (P<.001; P=.009) breast tumors from non-tumors. In contrast, CCR7 only differentiated early-stage breast tumors (P=.014). No significant differences were observed between early and late-stage breast tumors. Furthermore, ROC curves of miRNA-21, CCR7, and TWF1 were all statistically significant with area under the curve values of 0.951, 0.697, and 0.733, respectively. MiRNA-21 testing offers to augment the current cancer diagnostic strategy if less invasive samples, such as whole blood, become the sample of choice for testing. However, larger cohorts are needed to

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validate the clinical implications of the studied biomarkers in breast cancer monitoring.

INTRODUCTION

Globally, breast cancer is the leading cancer type in women and the second most common cancer in both sexes (Ferlay J et al., 2024). Etiologically, breast cancer is a complex multifactorial disease triggered by genetic causes such as mutations, copy number variations, and genetic rearrangements, which are just some of the consequences of genomic instability contributing to breast cancer development (Ray and Mukherjee, 2022). Over the years, epigenetic mechanisms such as DNA methylation, histone modifications, and microRNAs (miRNAs) have been identified as influencing factors in breast cancer pathogenesis (Kashyap et al., 2023). MiRNAs are small, approximately 18-25 nucleotides, non-coding RNA molecules that regulate gene expression by binding to the 3' untranslated region of the mRNA, thereby controlling many biological processes, which include cell proliferation, metabolism, differentiation, and apoptosis (Kookli et al., 2024).

MiRNA-21 is one of the most upregulated miRNAs in solid and hematological malignancies (Singh et al., 2021) and is often described as a cellular anti-apoptotic factor (Sothivelr et al., 2022). Moreover, miRNA-21 expression levels in Filipino colorectal cancer malignant tissues and plasma samples were upregulated compared to the control samples (Fellizar et al., 2023). Another overexpressed gene of interest in published literature is the C-C Chemokine Receptor Type 7 (CCR7) gene, which was first reported in 2001 to be highly expressed in 12 primary human invasive lobular or ductal breast carcinomas (Müller et al., 2001). Subsequently, current studies on CCR7 and its knockdown in breast cancer cells inhibited AKT expression and cell epithelial-mesenchymal transition (EMT), migration, and invasion (Giotopoulou et al., 2023). On the other hand, fewer published literature is available regarding the role of Twinfilin Actin Binding Protein 1 (TWF1) in cancer. However, upon TWF1 inhibition in breast cancer cells, the ability for self-renewal and invasion was inhibited (Samaeekia et al., 2017). A pan-cancer study of TWF1 also concluded that its upregulated expression in patient tumors generally predicted poor overall survival in cancer patients (Huo et al., 2021).

The main objective of this study was to determine miRNA-21, CCR7, and TWF1 expression in Filipino breast tissue samples using quantitative real-time reverse-transcription PCR (qRT-PCR) and evaluate the proposed biomarkers as possible early diagnostic tools in breast cancer.

MATERIALS AND METHODS

Samples

The Human Cancer Biobank of St. Luke's Medical Center Quezon City retrieved thirty-two pathologically confirmed invasive ductal carcinoma tissue samples with paired histologically normal adjacent tissue less than 2 cm from the tumor margin. Twenty early-stage, defined as Stages I, II, and IIIA, and twelve late-stage, stages IIIB and IIIC (American Joint Committee on Cancer, 2017), sample pairs were collected between January 2016 and March 2018 and stored at -80°C. Sixty-four breast tissue samples were included in this study to achieve a confidence interval of 95% and a power of 95%. OpenEpi version 3.01 was used to estimate the sampling size.

Ethical Considerations

The Institutional Ethics Review Committee of St. Luke's Medical Center – Quezon City approved all procedures, following the 1964 Helsinki Declaration and its later amendments, under protocol number SL-21200 dated 08 July 2022. All participants provided written informed consent before enrollment.

Breast tissue disruption and homogenization

Breast tissue samples weighing approximately 30 mg were removed from the -80°C freezer and lysed in 300 µL of lysis/binding buffer provided with the mirVana™ miRNA Isolation Kit with phenol (Ambion™). Breast tissue disruption and homogenization were done using the Biospec Tissue-Tearor® Tissue Homogenizer set at rotor speed 10 for 15-20 seconds.

Total RNA extraction

In the same tube used for breast tissue homogenization, 30 µL of miRNA Homogenate Additive was added to 300 µL of breast tissue lysate and incubated on ice for 10 minutes. Next, 300 µL of Acid-Phenol Chloroform was added to the initial lysate volume, vortexed for 30 seconds to mix, and centrifuged for 5 minutes at 14,000 rpm at room temperature to separate the aqueous and organic phases. The aqueous upper phase, volume noted, was transferred to a new tube, and the lower phase was discarded. Before the RNA isolation step, the eluent used for the procedure was pre-heated to 95°C. Then, 375 µL of absolute ethanol was added to the upper aqueous phase, and the lysate/ethanol mixture was allowed to pass through a filter cartridge. It was centrifuged for 15 seconds at 10,000 rpm, and the flow-through was discarded. Next, 700 µL of miRNA wash solution 1 was added to wash the filter; flow-through was discarded after the 15-second centrifugation. A second and third washing was done using 500 µL of wash solution 2/3. After washing, the flow-through was discarded, and the filter assembly was centrifuged for 1 minute to remove residual fluid. Finally, 100 µL of 95°C elution solution (0.1 mM EDTA, pH 8.0, and RNase-free) was added to the filter and centrifuged at 13,500 rpm to recover the RNA. The eluates were stored at –20°C until use.

MicroRNA assay

The procedure for the preparation of miRNA cDNA was based on Applied Biosystems™ TaqMan™ Advanced miRNA cDNA synthesis kit. This method added a poly (A) tail (3') and an adaptor (5') to amplify all miRNAs in a single reverse transcription reaction. Then, a 1:10 dilution of the cDNA template was prepared by adding 2.5 µL of the miR-Amp reaction product to 22.5 µL 0.1X TE buffer. Next, the reaction mix was vortexed and centrifuged, and in each 0.2 mL reaction tube, 7.5 µL of the reaction mix was dispensed, and 2.5 µL of the diluted cDNA template was added to each tube. The total volume of each reaction tube was 10 µL. The reaction tubes were vortexed, centrifuged, and loaded in the QuantStudio™ 5 Real-Time PCR System. The appropriate fast-cycling mode experiment settings and thermal cycling conditions were set at 95°C for 20 seconds, followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds.

Gene expression assay

The SuperScript™ VILO™ cDNA Synthesis Kit generated the first-strand cDNA for qRT-PCR. The procedure for the cDNA preparation for mRNA analysis was based on the manufacturer's protocol. TaqMan™ assay reagents (CCR7 [Hs01013469_m1], TWF1 [Hs00702289_s1], and 18S rRNA [Hs99999901_s1]) were thawed on ice, gently vortexed, and briefly centrifuged to spin down the contents. The appropriate volume of reaction mix was transferred to each well of the 8-tube strip. One µL of cDNA template was added in each reaction well. The 8-tube strip was sealed and briefly centrifuged to eliminate air bubbles. The Fast 8-tube strips were loaded in the QuantStudio™ 5 Real-Time PCR System. The appropriate fast-cycling mode experiment settings

and thermal cycling conditions were set at 50°C for 2 minutes and 95°C for 2 minutes, followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds.

Normalization and statistical analysis

In order to translate qRT-PCR data into expression data, fold change was calculated using the $2^{-\Delta\Delta CT}$ method. Fold change values were reported as means and corresponding standard deviation. MiRNA-16 normalized miRNA-21 expression, and 18S rRNA normalized CCR7 and TWF1 expression. Log2 transformation was done to make highly skewed distributions less skewed.

In comparing paired samples, a paired t-test was used for normally distributed data, and the Wilcoxon signed-rank test was used for non-normally distributed data. When comparing across stages, one-way ANOVA with Tukey HSD was used for normally distributed data, and the Kruskal-Wallis H test with Mann-Whitney U test was used for non-normally distributed data. Receiver operating characteristic (ROC) curve analysis was performed to examine the diagnostic capabilities of the studied groups. SPSS version 21 was used for statistical analysis, and GraphPad Prism version 9 was used for data visualization.

RESULTS

The clinical characteristics of breast cancer patients in this study were summarized in **Table 1**, and normalized log2 expression levels of the studied biomarkers in tumor and paired adjacent non-tumor samples were visualized using a heatmap (see **Figure 1**). MiRNA-21 expression in tumors was consistently upregulated compared to its non-tumor counterpart, and TWF1 expression also exhibited the same upregulated pattern but with a more heterogeneous gene expression profile. CCR7 expression, on the other hand, was downregulated in most samples, with upregulated expression in some tumor tissues.

Table 1: Characteristics of breast cancer patients compared against miRNA-21, CCR7, and TWF1 expression.

Characteristics, n = 32	n (%)	*P* value		
		miRNA-21	CCR7	TWF1
Age, years	52.5 ± 10.26			
≤45	8 (25.0)	0.952	0.496	0.948
>45	24 (75.0)			
Menopausal Status				
Premenopausal	16 (50.0)	0.992	0.091	0.093
Postmenopausal	16 (50.0)			
Axillary Lymph Node Metastasis				
None	13 (40.6)	0.218	0.122	0.939
Yes	19 (59.4)			
Estrogen Receptor				
Negative	4 (12.5)	0.935	0.616	0.393
Positive	28 (87.5)			
Progesterone Receptor				
Negative	5 (15.6)	0.025*	0.174	0.055
Positive	27 (84.4)			
Human Epidermal Growth Factor Receptor 2				
Negative	15 (46.9)	0.395	0.476	0.997
Positive	10 (31.3)			
Equivocal	7 (21.9)			
Tumor Grade				
T1	10 (31.3)			
T2	13 (40.6)	0.841	0.705	0.542
T3	3 (9.4)			
T4	6 (18.8)			
Nodal Involvement Grade				
N0	11 (34.4)			
N1	8 (25.0)	0.207	0.429	0.963
N2	4 (12.5)			
N3	8 (25.0)			
NX	1 (3.1)			
Neo-adjuvant Therapy				
No	29 (90.6)	0.831	0.393	0.821
Yes	3 (9.4)			

*P-value is significant

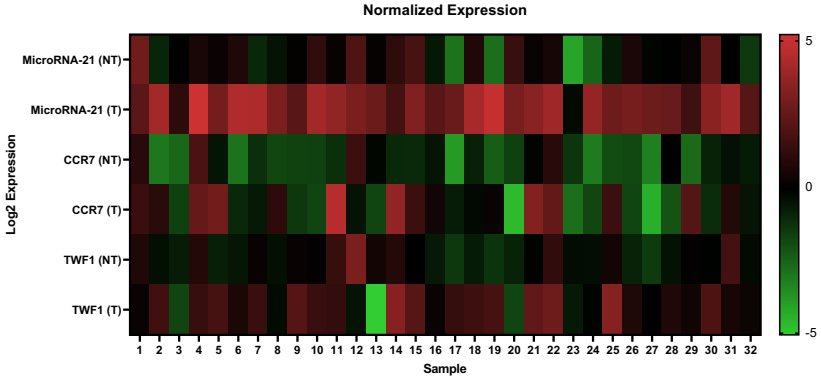

Figure 1: The heatmap displays the normalized log2 expression levels across different samples. Rows represent studied biomarkers in breast tumor (T) and non-tumor (NT) samples, while columns represent different patients (1-32). The red color indicates upregulated expression, while the green color indicates downregulated expression. The data was generated using normalized qRT-PCR results.

MiRNA-21 (*P*<.001), CCR7 (*P*=.003), and TWF1 (*P*=.003) were able to differentiate breast tumors from adjacent non-tumor tissues (see **Figure 2**). Furthermore, MiRNA-21 and TWF1 successfully differentiated early-stage (*P*<.001; *P*=.008, respectively) and late-stage (*P*<.001; *P*=.009, respectively) tumors from non-tumors. On the other hand, CCR7 only differentiated early-stage tumors (*P*=.014) from non-tumors. There were no statistically significant differences observed between early-stage and late-stage breast tumors in miRNA-21 (*P*=.299), CCR7 (*P*=.653), and TWF1 (*P*=.876) (see **Figure 3**). The ROC curve illustrates the predictive model's performance in distinguishing tumor and non-tumor samples. The diagnostic values of miRNA-21, CCR7, and TWF1 were all statistically significant in differentiating the tumor from its adjacent non-tumor tissue (see **Figure 4**). However, among the three biomarkers, miRNA-21 showed the highest sensitivity at 94% and specificity at 88% based on the highest Youden Index value.

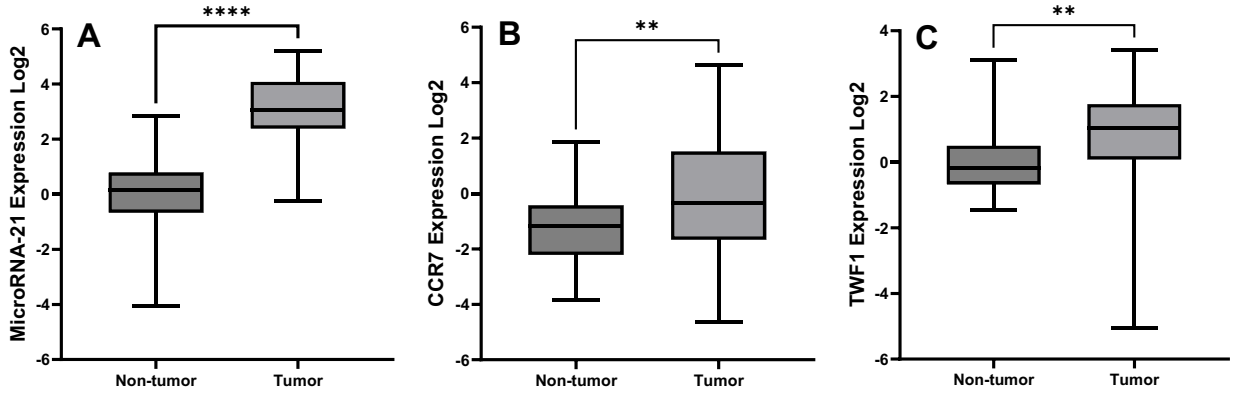

Figure 2: The boxplot displays the relative expression levels in breast tumors and adjacent non-tumor tissues. Each box represents the interquartile range (IQR), with the median indicated by the horizontal line inside the box. (A) MiRNA-21 expression was upregulated in tumors (*M* = 3.10, *SD* = 1.15) than non-tumors (*M* = 0.01, *SD* = 1.52) (*P*<.001). (B) CCR7 expression was downregulated in non-tumors (*M* = -1.26, *SD* = 1.34) than tumors (*M* = 0.01, *SD* = 2.25) (*P*=.003). (C) TWF1 expression was upregulated in tumors (*Mdn* = 1.05) than non-tumors (*Mdn* = -0.17) (*Z* = -2.936, *P*=.003).

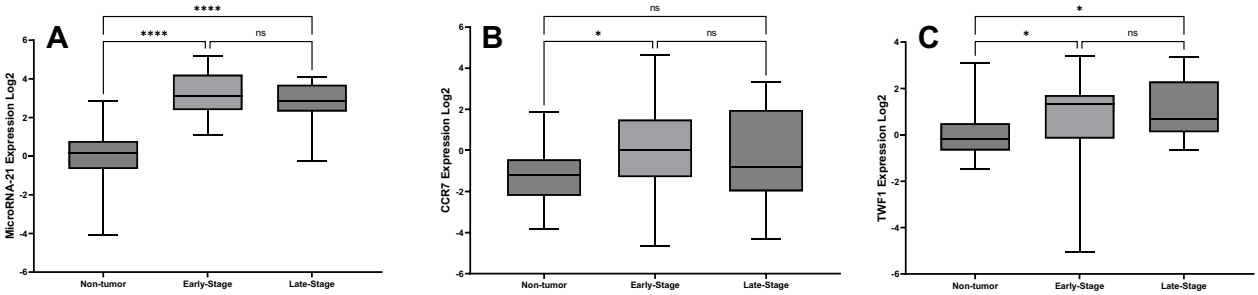

Figure 3: The boxplot displays the relative expression levels in adjacent non-tumor tissues and early-stage and late-stage breast tumors. Each box represents the IQR, with the median indicated by the horizontal line inside the box. (A) MiRNA-21 differentiated early-stage (*M* = 3.28, *SD* = 1.10) (*P*<.001) and late-stage tumors (*M* = 2.80, *SD* = 1.22) (*P*<.001) from non-tumors. (B) CCR7 only differentiated early-stage tumors (*M* = 0.27, *SD* = 2.17) (*P*=.014) from non-tumors. (C) TWF1 also differentiated early-stage (*U* = 180.0, *P*=.008) and late-stage tumors (*U* = 93.0, *P*=.009) from non-tumors.

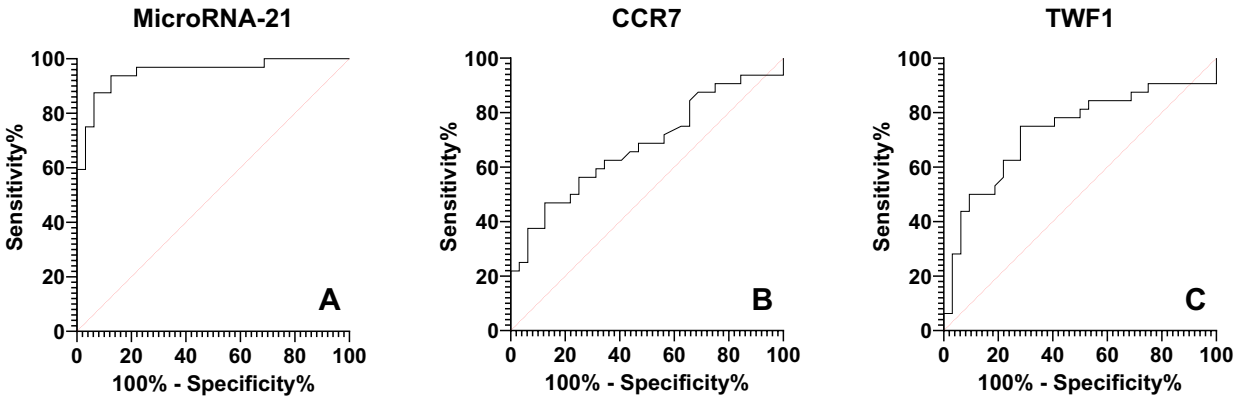

Figure 4: The x-axis represents the false positive rate (1 - specificity), while the y-axis represents the true positive rate (sensitivity). The diagonal line represents the performance of a random classifier, while the curve represents the predictive model's performance. The area under the curve (AUC) quantifies the model's discriminatory power, with higher values indicating better performance. In this analysis, the AUC values were calculated as (A) 0.951 (95%CI=0.899-1; *P*<.001) for miRNA-21, (B) 0.697 (95%CI=0.568-0.827; *P*=.007) for CCR7, and (C) 0.733 (95%CI=0.606-0.861; *P*=.001) for TWF1.

DISCUSSION

Identifying miRNAs in different sample types has been used in clinical studies to profile several pathological conditions. For example, unique miRNA expression patterns in poorly differentiated tumors offer an accurate strategy to identify the tissue of origin and differentiate histological subtypes. The similarity of specific miRNA patterns in tissues and biological fluids presents a lesser invasive testing strategy when using the latter sample type; however, its use in diagnostics has yet to be established.

One of the most studied miRNAs is miRNA-21, whose oncogenic role is attributed to its post-transcriptional regulation of tumor suppressor genes such as TPM1 (Kumar et al., 2023), PTEN (Chawra et al., 2024) and PDCD4 (J. Wang et al., 2023). MiRNA-21 upregulation was first reported in human glioblastoma tumor tissues and early-passage cultures; likewise, miRNA-21 inhibition in glioblastoma cell lines resulted in caspase activation, which eventually led to cell death (Chan et al., 2005). In our study, we observed an upregulation of miRNA-21 expression in Filipino invasive ductal carcinoma tumor samples similar to previous reports in other ethnicities (Bautista-Sánchez et al., 2020; Ding et al., 2021). Additionally, we were able to stratify the tumors according to the stage and found out that miRNA-21 expression was upregulated even at the early stage of breast cancer. Similarly, Fellizar and colleagues reported the same observation in Filipino colorectal cancer patients (Fellizar et al., 2023).

To stop cancer cells from becoming more metastatic and aggressive, anoikis, an apoptosis subset, occurs due to cellular detachment from other surrounding cells and the extracellular matrix (Malagobadan et al., 2020). When CCR7 binds to CCL19, its cognate ligand, processes that ensure tumor survival and proliferation, such as anoikis reduction, will be initiated (R. Ma et al., 2023). In the past, researchers reported CCR7 upregulation in human-invasive lobular and ductal carcinoma (Müller et al., 2001), and we observed the same CCR7 upregulation in early-stage tumors only but were generally downregulated in both sample pairs (see **Figure 1** and **Figure 3B**). Upon further investigation using TargetScan 8.0, we found that CCR7 had one putative seed match site in its 3' untranslated region with miRNA-21-5p and miRNA-590-5p. Furthermore, a recent study concluded that miRNA-21-5p inhibited the progression of the CCR7/STAT3/NF-κB signaling pathway in human chondrosarcoma cells (Li et al., 2021), which may explain the downregulated expression of CCR7 in our samples.

Another critical aspect in tumor progression and metastasis is cell EMT, wherein cells become nonadherent with their neighboring cells and develop mesenchymal properties, increasing their motile abilities (Q. Ma et al., 2024). TWF1, an actin-binding protein, plays a significant role in cytoskeleton-related functions (Y. Wang et al., 2023) and was reported to promote breast cancer cell EMT through the activation of mesenchymal lineage transcription factors, MKL1 and SRF (Samaeekia et al., 2017). In our study, we observed TWF1 upregulation in breast tumors similar to lung adenocarcinoma tissues (Zhai et al., 2023), and due to the mechanistic nature of TWF1, its morphological role in processes such as cell motility, drug sensitivity, and cancer progression has remained controversial.

In future studies, the authors suggest that samples from Stage 0 through IV with varying sample types, e.g., tissue, serum, plasma, and core-needle aspirate, should be included to give a more comprehensive perspective on how these biomarkers relate throughout the various stages of cancer.

CONCLUSION

MiRNA-21, CCR7, and TWF1 expression significantly differed between breast tumors and adjacent non-tumor tissues. Across stages, early-stage and late-stage tumors were differentiated from non-tumors, highlighting the ability to detect malignancy as early as stages 1 and 2. However, our study has some limitations: the sample size was small, which limited further analyses of biomarkers with breast cancer molecular subtypes, and the sample type was homogenous, i.e., tissue. Therefore, the authors recommend large-scale studies to confirm the findings and critically appraise the proposed biomarkers' diagnostic capabilities.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could inappropriately influence the work reported in this paper.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

Study Conceptualization: Romar G. Dabban, Michael O. Baclig, Ma. Luisa D. Enriquez

Data visualization, statistical analysis, laboratory work, and manuscript preparation: Romar G. Dabban

Manuscript Review: Michael O. Baclig, Ma. Luisa D. Enriquez

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