Dietary soy isoflavones increase metastasis to lungs in an experimental model of breast cancer with bone micro-tumors

Xujuan Yang[1], Aashvini Belosay[1], James A. Hartman[1], Huaxin Song[2], Yukun Zhang[1], Wendan Wang[1], Daniel R. Doerge[3] and William G. Helferich[1,*]

[1] Department of Food Science and Human Nutrition, University of Illinois at Urbana-Champaign

[2] Health Sciences Center, School of Nursing, Texas Tech University

[3] Division of Biochemical Toxicology, National Center for Toxicological Research, U.S. Food and Drug Administration

[*] **Address correspondence to:** Dr. William G. Helferich

580 Bevier Hall, 905 S. Goodwin Ave

Department of Food Science and Human Nutrition

University of Illinois at Urbana-Champaign

Urbana, IL 61801

Tel: 1-217-244-5414, Fax: 1-217-265-0925

email: helferic@illinois.edu

Supplemental Materials and Methods

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Cell viability

Murine 4T1 cells (4×10^4/well) were plated onto 24-well plates. After 24 hours, cells were cultured with genistein, daidzein or (-)-equol at various concentrations in culture medium for 24 hours. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added and optical density was measured at 570nm with an EL800 Universal plate reader (Bio-Tek Instruments Inc., Winooski, VT). The experiment was performed in 6 duplicates and repeated 3 times.

Motility (wound healing) assay

Murine 4T1 cells (1×10^5/well) were plated onto 12-well plates and allowed to form a confluent monolayer. After the cell monolayer was formed, two parallel scratches were made in each well with a P1000 pipette tip. Cells were then cultured with genistein, daidzein or (-)-equol at various concentrations in culture medium. Each scratch was photographed at 3 different positions at the beginning of the experiment and the photographed position was marked for photographing again after 24 hours. The non-cell occupied area between the two edges was measured using AxioVision AC software (Carl Zeiss, Thornwood, NY) and the migration distance was calculated using the formula (measured $\text{area}_{\text{time 0}}$–measured $\text{area}_{\text{time 24}}$)/a fixed width for all graphs (2336 pixels). The experiment was performed in triplicate and repeated 3 times. Data were presented as percentage of migrated distance in 24 h to original distance at time 0h.

Invasion chamber assay

Murine 4T1 cells (1×10^5/insert) were seeded onto Matrigel™ coated cell culture inserts (8 μm) and treated with genistein, daidzein or (-)-equol at 1 μM in culture medium. The inserts were placed on a companion 24-well plate filled with cell culture medium. After 24 hours, cells on the upper surface of the membrane in the insert were removed with a cotton swab and cells on the lower surface were fixed with cold methanol and stained with hematoxylin. The membrane was then cut from the insert housing, mounted onto a micro-slide and observed under an AxioSkop 40 microscope (Carl Zeiss, Thornwood, NY). Each membrane was divided into 9 fields and the invaded cells in each field were counted using ImageJ (NIH, Bethesda, MD). The experiment was performed in duplicate and repeated twice.

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Supplemental Table 1 Composition of the mixed isoflavones

Isoflavones	Percentage by weight
Genistein	0.2 ± 0.004
Total genistein[a]	37.3 ± 1.2[c]
Daidzein	0.2 ± 0.008
Total daidzein[b]	15.8 ± 0.7[d]

Data are presented as mean ± SD (n=8). a: aglycone genistein + conjugated genistein. b: aglycone daidzein + conjugated daidzein. c: percentage of genistein equivalent. d: percentage of daidzein equivalent.

Supplemental Table 2 Primer sets for RT-PCR analysis

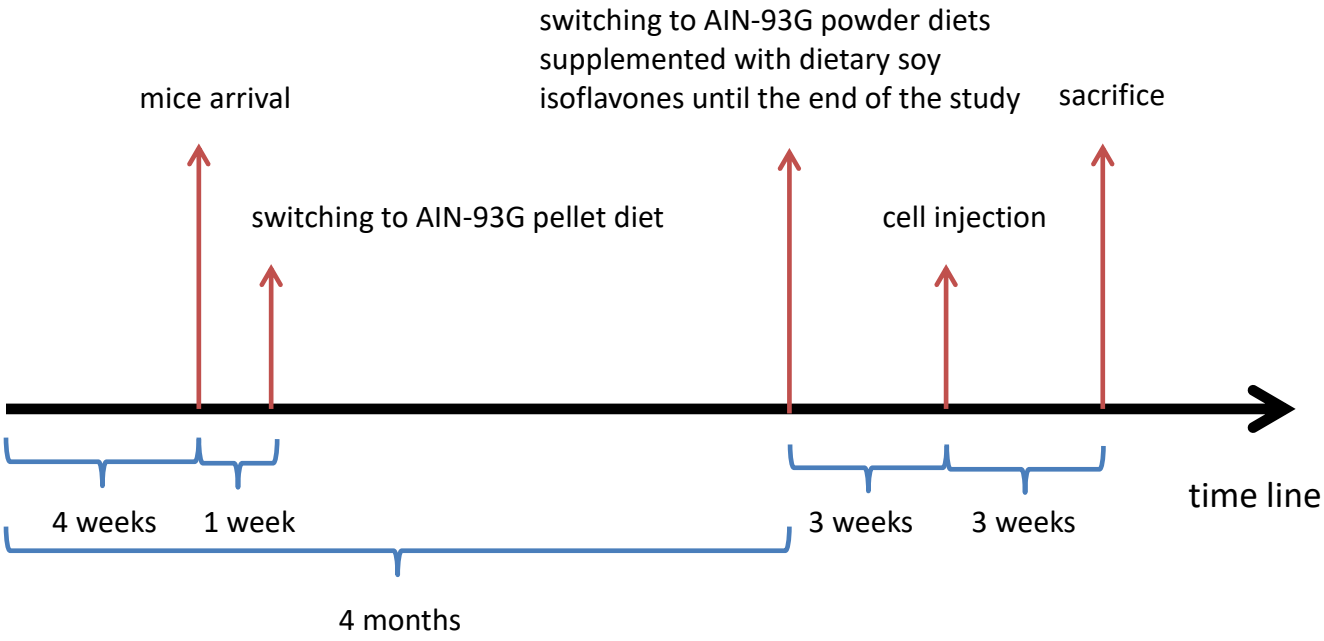
Gene	Forward	Reverse
MMP2 (+1891/+1964)	5′-GAGAAGGCTGTGTTCTTCGCAGG-3′	5′-TTGGGGTATCCTCGCTCCAGAG-3′
MMP9 (+1354/+1430)	5′-TCCAGTATCTGTATGGTCGTGGCTC-3′	5′-TGTCGGCTGTGGTTCAGTTGTG-3′
TIMP1 (+391/+463)	5′-TGGGAAATGCCGCAGATATCC-3′	5′-GACTTGTGGGCATATCCACAGAGG-3′
TIMP2 (+472/+541)	5′-ACCCAGAAGAAGAGCCTGAACCAC-3′	5′-TCATGGGACAGCGAGTGATCTTG-3′
VEGFa (+1095/+1167)	5′-CCCACGACAGAAGGAGAGCAGAA-3′	5′-GGCAGTAGCTTCGCTGGTAGACATC-3′
VEGFb (+584/+653)	5′-CCCTGGAAGAACACAGCCAATGTG-3′	5′-ACCCTGTCTGGCTTCACAGCACTCT-3′
VEGFc (+753/+851)	5′-GCATGAACACCAGCACAGGTTACC-3′	5′-ACTGGTTTGGGGCCTTGTGAGA-3′
BCL2 (+1967/+2045)	5′-CTGGGATGCCTTTGTGGAAC-3′	5′-CAGGGTCTTCAGAGACAGCCA-3′
CCND1 (+388/+463)	5′-AGAAGTGCGAAGAGGAGGTC-3′	5′-TTCTTCAAGGGCTCCAGG-3′
CXCL1 (+196/+265)	5′-TCACCTCAAGAACATCCAGAGC-3′	5′-TATGACTTCGGTTTGGGTGC-3′
EGFR (+3684/+3762)	5′-TATCTCAACACTGCCCAGC-3′	5′-GGTGACTGCCTTTCTGGAT-3′
TFF1 (+211/+291)	5′-TGTTTTGATGACAGTGTCCG-3′	5′-GGGACATTCTTCTTCTTGAGTG-3′
WISP2 (+739/+807)	5′-TGTGTGACCAGGCAGTGATG-3′	5′-GGGCAGAAAGTTGGTGTCCT-3′
CAV1 (+414/+492)	5′-ACCGCTTGTTGTCTACGATCTTCG-3′	5′-TGCAGGAAGGAGAGAATGGCAA-3′
MYO1b (+1300/+1375)	5′-GAACAGTTGAAGCGAAGCAGG-3′	5′-AGGGCATCACGGGCATAAT-3′
L7a (+176/+289)	5′-AACTTCGGCATTGGACAGGACA-3′	5′-TTTGAGCCGCTTGTAGAGGATAGC-3′

Supplemental Table 3 Doses of dietary soy isoflavones supplemented in the AIN-93G diet

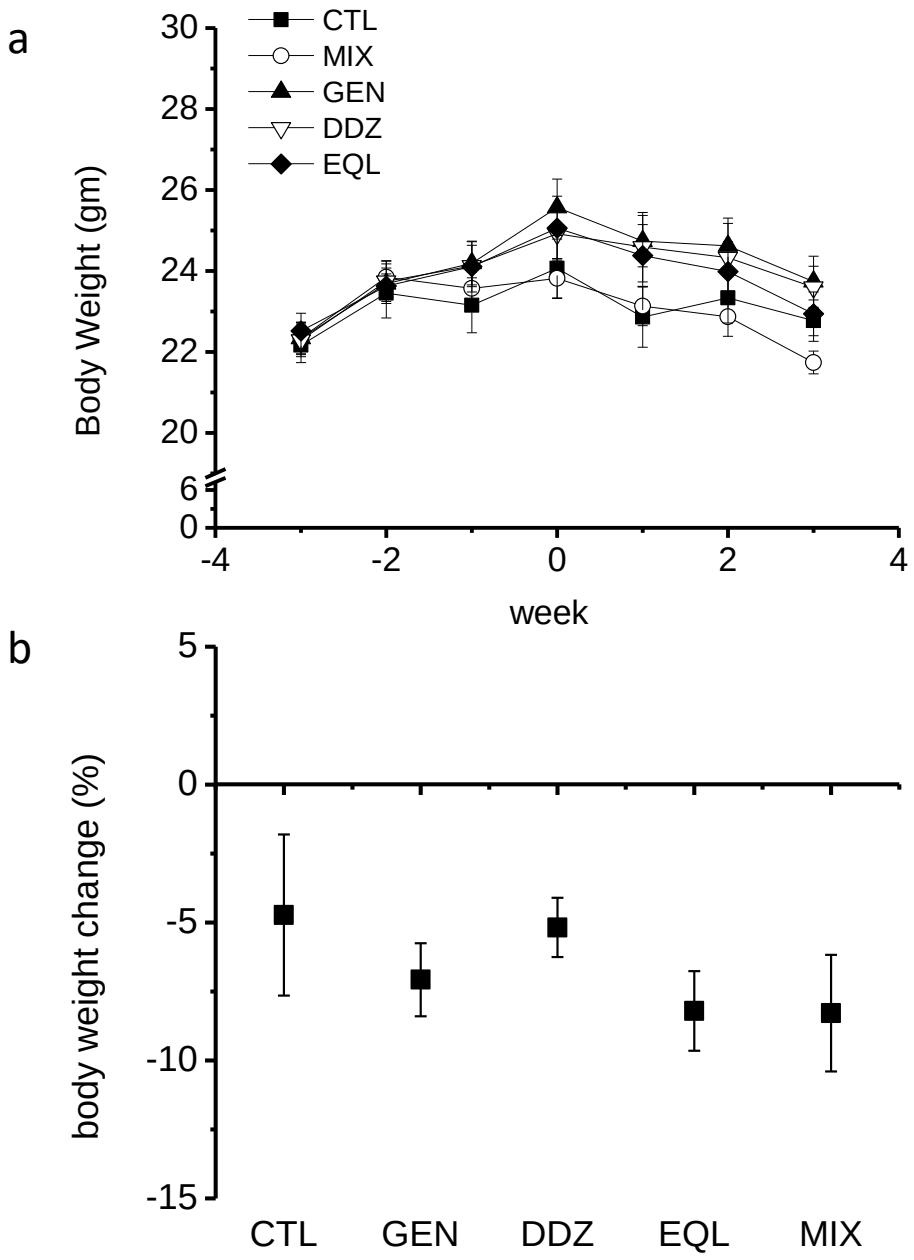
Groups	Genstein (mg/kg)	Daidzein (mg/kg)	(-)-Equol (mg/kg)
CTL	/	/	/
GEN	750	/	/
DDZ	/	750	/
EQL	/	/	750
MIX	750[a]	318[b]	/

CTL: the control group, GEN: the genistein group, DDZ: the daidzein group, EQL: the (-)-equol group, MIX: the mixed isoflavone group. a: the equivalent dose of genistein, b: the equivalent dose of daidzein.

Supplemental Fig. 1 Study design

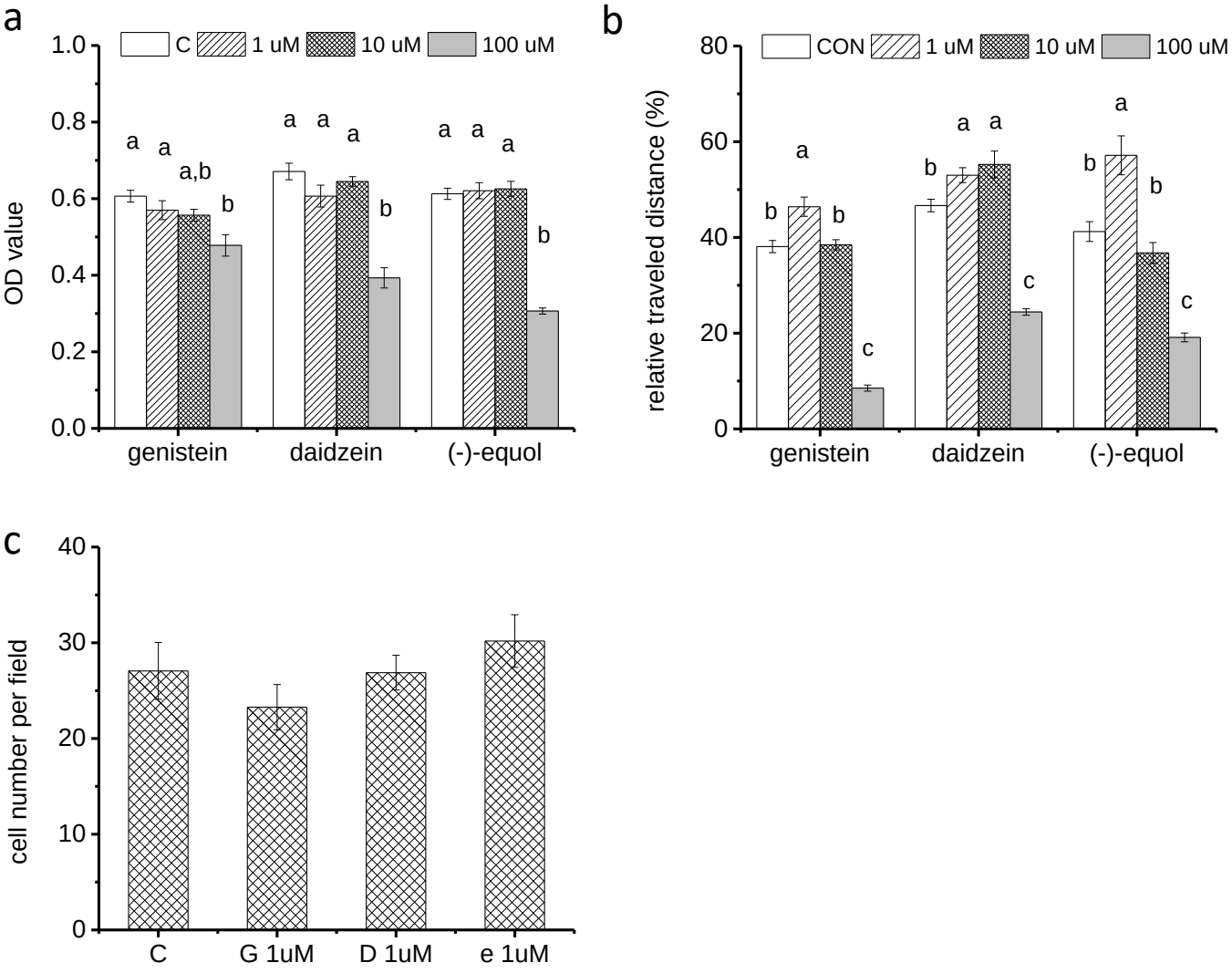


Supplemental Fig. 2 Monitoring of mouse body weights in the duration of the study



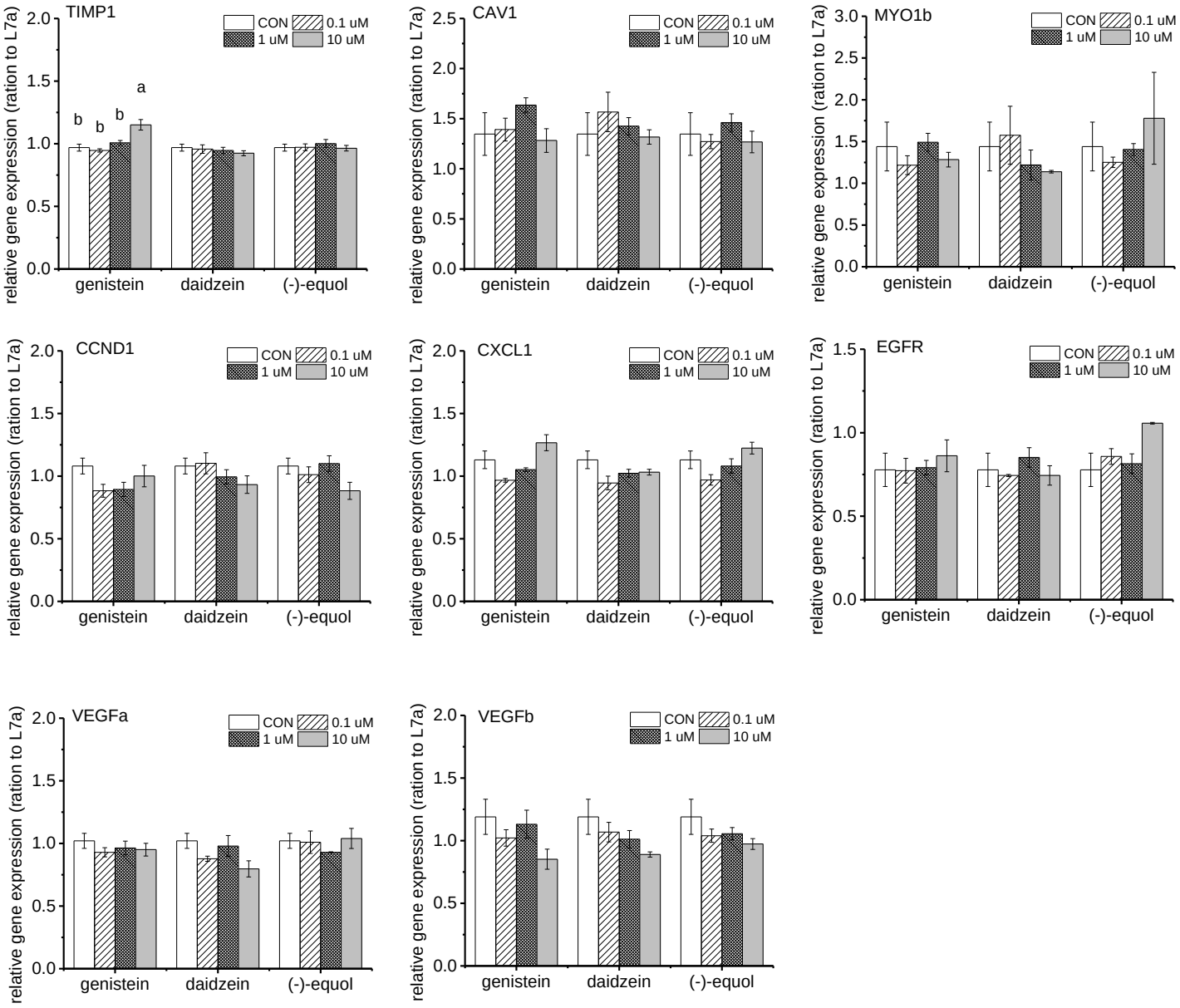
Supplemental Fig. 1a) measurements of mouse body weights over time. Murine 4T1 cells (1,000) were injected into the tibial bone marrow of mice at week 0. 1b) Body weight changes were calculated for the duration of week 0 (cell injection) to week 3 (end of the study) using the formula (body weight at week 3 – body weight at week 0)/body weight at week 0×100%. Data are presented as mean±SEM and analyzed using two-sample *t*-test. No difference was observed between the dietary soy isoflavone-treated groups when compared to the control group.

Supplemental Fig. 3 Effects of soy isoflavones on the growth, motility and invasion of cultured 4T1 cells



Supplemental Fig. 2a) Effects of genistein, daidzein and (-)-equol on 4T1 cell growth and 2b) 4T1 cell motility. Data are presented as mean±SEM and analyzed using one-way ANOVA with post hoc Tukey's test. Different letters indicate significant difference between the means of two groups of the same treatment set ($P<0.05$). 2c) Effects of genistein, daidzein and (-)-equol on the invasion of 4T1 cells. Data are presented as mean±SEM and analyzed using two-sample *t*-test. No difference was observed between the treated groups when compared to the control group.

Supplemental Fig. 4 Effects of soy isoflavones on gene expression of cultured 4T1 cells



Experiments were conducted in triplicates and repeated 3 times. Results were the average of the 3 independent experiments. Data are presented as mean±SEM and analyzed using one-way ANOVA with post hoc Tukey's test. Different letters indicate significant difference between the means of two groups of the same treatment set (P<0.05).