Original article:

Evaluation of wound healing biomarkers of Interleukin 6 (IL-6), Vascular Endothelial Growth Factor (VEGF) and Matrix Metalloproteinases 9 (MMP-9) in post Lower Segment Caesarean Section (LSCS) patients consuming *Channa Striatus* extract

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Abstract:

***Background*:** Wound healing is a dynamic process which is divided into four phases; haemostasis, inflammatory, proliferation and tissue remodelling phases, that encompasses inflammatory cells, cytokines and growth factors. Interleukin-6 (IL-6), Vascular Endothelial Growth Factor (VEGF) and Matrix Metalloproteinase 9 (MMP-9) involve at the different phases of wound healing. *Channa striatus (C.striatus)* is a fresh water fish that is believed to have natural properties to promote wound healing. Currently, the effects of *C.striatus* on the cytokines and growth factors are not available. ***Objective**:* This study was conducted to evaluate the wound healing biomarkers; IL-6, VEGF and MMP-9 on post Lower Segment Caesarean Section (LSCS) women consuming oral *C.striatus* extract. ***Methods**:* This was a randomised, double-blinded study amongst LSCS women consuming *C.striatus* extract versus a placebo at Universiti Sains Malaysia Hospital and Raja Perempuan Zainab II Hospital from May 2011 to January 2013. After randomization, the treatment group received freeze dried *C.striatus* extract 500 mg daily while the placebo group received maltodextrin 500 mg daily for 6 weeks. Blood samples for IL-6, VEGF and MMP-9 were taken from both groups post-operatively at day 3, week 2, week 4 and week 6. The data were analysed using SPSS version 22. ***Results**:* A total of 39 patients from *C.striatus* and 34 patients from placebo group were included in this study. Within *C.striatus* group, the results of IL-6, MMP-9 and VEGF showed significant differences (P<0.05) for all the study period. Between group comparison showed significant difference (P<0.05) on week 4 and week 6 for IL-6 and MMP-9 whereas VEGF showed significant difference (P<0.05) on day 1, day 3, week 4 and week 6. The trend of IL-6 and MMP-9 exhibit decreasing trend in both groups however, VEGF in *C.striatus* group exhibit increasing trend till week 6 compared to placebo group. ***Conclusion**:* This study showed *C.striatus* extract had effects on IL-6, VEGF and MMP-9 in post LSCS women.

Keywords: *Channa striatus*; lower segment caesarean section; wound healing; Interleukin-6; MMP 9; VEGF

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Introduction

Wound healing is divided into four distinct and overlapping phases of haemostasis, inflammatory, proliferation and tissue remodelling[1]. It encompasses a variety of cells and inflammatory markers. Interleukin-6 (IL-6) involves in the systemic changes during inflammation and infection[2]. It plays a crucial role in inflammation, particularly

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at the early phase of wound healing and during proliferation and remodelling phases and promotes collagen deposition and angiogenesis[3]. Vascular Endothelial Growth Factor (VEGF) is involved in the angiogenesis and recently during epithelization and collagen deposition[4]. It is reported to be the most powerful endothelial cell specific mitogen in the complicated process of angiogenesis[5]. Matrix metalloproteinases regulates collagen remodelling. Matrix Metalloproteinase 9 (MMP-9) is involved directly in the contraction and remodelling of scar tissue in the extracellular matrix forming new and highly organised tissue structure [6].

Channa striatus (C.striatus) or locally known as Haruan is a snakehead fresh water fish is widely consumed for wound healing in Malaysia. It is believed that *C.striatus* has natural properties for reducing pain and inflammation by promoting wound healing especially in post-partum women and post-injury patients [7]. Other than wound healing properties, *C.striatus* has anti-nociceptive, anti-inflammatory, antioxidant and could act as energy booster [8]. *C.striatus* also exhibit on cardiological effects due to high amount of polyunsaturated fatty acids such as eicosapentaenoic acid (EPA) and decosahexaenoic acid (DHA) which regulates the prostaglandin synthesis and hence induces wound healing [9,10]. Other studies also found that *C.striatus* contains vitamin A or retinol with several dietary minerals such as magnesium, copper, calcium, manganese, iron and zinc that aid in the wound healing process [11]. Magnesium, copper, zinc and iron act as cofactors in many enzyme reactions in protein and collagen synthesis and also involved in tissue growth and wound healing [12].

At present, it is acknowledged that cytokines and growth factors, as biological mediators, contribute to the repair of the wound site[13-15]. However the effects of *C.striatus* on the cytokines and growth factors are not available. This study was designed to evaluate the wound healing biomarkers of IL-6, VEGF and MMP-9 on postpartum women who had Lower Segment Caesarean Section (LSCS) consuming oral *C.striatus* extract.

Materials and methods

Study Design and Setting

This study was a randomised, double-blinded, two-arm parallel comparative study amongst women who have undergone LSCS consuming *C.striatus* extract versus a placebo at the Universiti Sains Malaysia Hospital (HUSM), Kubang Kerian, Kelantan, and Raja Perempuan Zainab II Hospital (HRPZ II), Kota Bharu, Kelantan, from May 2011 to January 2013. Ethical permission was obtained from the Human Research Ethics Committee USM (USMKK/PPP/JEPeM [227.3.(05)]. For the HRPZ II, approval from the National Medical Research Register (NMRR) [Ref: NMRR-11-1018-10092] was obtained. All aspects of this study comply with the Declaration of Helsinki.

The inclusion criteria were women aged between 18 and 40 years who had undergone a LSCS with no present active medical, surgical, or gynaecological problems. Women who had taken any form of herbal extract in the previous three months before study entry and used fresh *C.striatus* during the study period were excluded from the study. The patients were then randomized 1:1 into two groups using a computer-generated table of random numbers. One group received freeze dried *C.striatus* extract 500 mg daily and another group received placebo of maltodextrin 500 mg daily for 6 weeks. The orally administered freeze-dried *C.striatus* extract was prepared by a GMP-certified laboratory at the School of Pharmaceutical Sciences, Universiti Sains Malaysia. Both the freeze-dried *C.striatus* extract and maltodextrin were available in capsules of 250 mg. The capsules were taken orally with water once daily and at any time of the day, with or without a meal. Informed consent was obtained from the women 24–48 hours after the elective or emergency LSCS when the women were fully conscious and comfortable. Information on demographic data and past and concurrent medical history was obtained by interviewing the women.

Blood samples were taken on day 3 post-operation from both groups of women for the biochemical markers of IL-6, VEGF and MMP-9. The women were then followed up at post-operative week 2, week 4, and week 6 and at each visit, blood was taken for the above markers. The women's compliance was measured at every visit using the number of capsules taken. Measurement of the safety profiles of liver function, renal function, and full blood count were conducted at baseline and at the last visit (post-operative week 6).

Biomarkers Analysis

IL-6 Assay

Analysis of IL-6 was done on automatic immunoassay analyzer Cobas e-411(Roche). The test reaction

is based on sandwich method and the principle of measurement is using electrochemiluminescence (ECL) and has been standardized against the National Institute for Biological Standards and Control (NIBSC). Reference range for IL-6 in healthy individual used was 0 to 7 pg/ml.

MMP-9 Assay

Analysis of MMP-9 was done by ELISA (Enzyme Link Immunosorbent Assay) method using commercial kit manufactured by Cusabio, China. The measuring range for MMP-9 using this kit was 0.3 to 20.0 ng/ml and the lowest detectable concentration was less than 0.28 ng/ml. Reference range for MMP-9 in human serum is 12.0 ng/ml to 71.0 ng/ml.

VEGF Assay

Analysis of VEGF in serum was done by ELISA method using commercial kit manufactured by Cusabio, China. The measuring range for VEGF using this kit was 31.5 to 2,000 pg/ml with the lowest detectable concentration of less than 7.8 pg/ml.

Statistical Analysis

Sample size of each biochemical markers were calculated based on the objective of the study. The calculations were done using Power and Sample Size Calculation Software [16] for comparing two means between treatment group and placebo group. By using standard deviation of the intended wound healing biomarkers, taking the power of 90%, detectable difference between both groups *(C.striatus* and placebo) based on expert opinion and level of significance of 0.05, the calculated sample size for each group was 30. However, after considering 20% dropout, the sample size for each group was 36. This study used a (modified) intention-to-treat (ITT) analysis approach where all subjects who took at least one dose of study drug and had at least one post-baseline efficacy evaluation were analyzed based on their original randomization. The differences in mean or comparison between the treatment group and the placebo group were analysed using Independent t-test. All values were reported as two-tailed results. The level of statistical significance was set at $P<0.05$. The data were analysed using software IBM SPSS version 22.

Ethical approval

This study was approved by Human Research Ethics Committee USM (USMKK/PPP/JEPeM [227.3.(05)] for Hospital USM and approved by National Medical Research Register (NMRR) [Ref: NMRR-11-1018-10092] for HRPZ II.

Results

A total number of 73 post LSCS Malay women were included in this study. The data for drop-out (13 subjects) was imputed according to the Last Observation Carried Forward (LOCF) principle. Per protocol (PP) analysis was done to 60 subjects who completed all the study visits with adequate compliance for comparison. The demographic data of the study populations are shown in Table 1.

Table 1: Demographic data for overall patients.

	No. of respondents (%)		Mean (SD)	
Variables	***C.striatus***	**Placebo**	***C.striatus***	**Placebo**
Age (years)			28.0 (5.1)	28.5 (5.2)
Mode of caesarean				
a. Elective	3 (7.7)	2 (5.9)		
b. Emergency	36 (92.3)	32 (94.1)		

Results within C.striatus group

The results within *C.striatus* group, IL-6, MMP-9 and VEGF, showed significant differences in all the healing biomarkers between all days/weeks. The highest mean score difference for IL-6 **(Table 2)** was between day 1 and week 6 (41.89 pg/ml, p ≤ 0.001, 95% CI 35.63, 48.15) and the lowest mean score difference was between week 4 and week 6 (4.46 pg/ml, *P*≤0.001, 95% CI 3.31, 5.62).

Table 2: Comparison of Interleukin-6 (IL-6) within *C.striatus* group.

Day/Week	(IL-6) Mean (SD) pg/ml	Mean score difference (95% CI)	t-statistic (df)	p-value *
Day 1 Day 3	52.24(20.76) 34.19(18.49)	18.06(13.25,22.86)	7.61(38)	< 0.001
Day 1 Week 2	52.24(20.76) 23.52(15.62)	28.72(22.38,35.06)	9.17(38)	< 0.001
Day 1 Week 4	52.24(20.76) 14.82(9.00)	37.43(31.58,43.27)	12.96(38)	< 0.001
Day 1 Week 6	52.24(20.76) 10.36(7.89)	41.89(35.63,48.15)	13.54(38)	< 0.001
Day 3 Week 2	34.19(18.49) 23.52(15.62)	10.67(6.89,14.45)	5.71(38)	< 0.001
Day 3 Week 4	34.19(18.49) 14.82(9.00)	19.37(14.59,24.15)	8.20(38)	< 0.001
Day 3 Week 6	34.19(18.49) 10.36(7.89)	23.83(18.88,29.22)	8.95(38)	< 0.001
Week 2 Week 4	23.52(15.62) 14.82(9.00)	8.70(5.06,12.35)	4.84(38)	< 0.001
Week 2 Week 6	23.52(15.62) 10.36(7.89)	13.17(9.01,17.32)	6.41(38)	< 0.001
Week 4 Week 6	14.82(9.00) 10.36(7.89)	4.46(3.31,5.62)	7.82(38)	< 0.001

*paired t test ; significant p<0.05

For MMP-9 **(**Table 3), the highest mean score difference was between day 1 and week 6 (27.99 ng/ml, *P*≤0.001, 95% CI 23.12, 32.87) and the lowest mean score difference was between week 4 and week 6 (3.97 ng/ml, *P*≤0.001, 95% CI 2.87, 5.08).

Table3: Comparison of Matrix Metalloproteinase-9 (MMP-9) for *C.striatus* group.

Day/Week	MMP-9 Mean (SD) ng/ml	Mean score difference (95% CI)	t-statistic (df)	p-value *
Day 1 Day 3	44.19(14.48) 37.05(12.14)	7.13(5.04,9.22)	6.91(38)	< 0.001
Day 1 Week 2	44.19(14.48) 25.84(9.17)	18.35(14.81,21.89)	10.48(38)	< 0.001
Day 1 Week 4	44.19(14.48) 20.17(8.16)	24.02(19.67,28.37)	11.17(38)	< 0.001
Day 1 Week 6	44.19(14.48) 16.19(8.04)	27.99(23.12,32.87)	11.63(38)	< 0.001
Day 3 Week 2	37.05(12.14) 25.84(9.17)	11.22(8.94,13.50)	9.96(38)	< 0.001
Day 3 Week 4	37.05(12.14) 20.16(8.16)	16.89(13.72,20.06)	10.79(38)	< 0.001
Day 3 Week 6	37.05(12.14) 16.19(8.04)	20.86(17.25,24.47)	11.71(38)	< 0.001
Week 2 Week 4	25.84(9.17) 20.17(8.16)	5.67(4.29,7.05)	8.31(38)	< 0.001
Week 2 Week 6	25.84(9.17) 16.19(8.04)	9.65(7.71,11.58)	10.10(38)	< 0.001
Week 4 Week 6	20.17(8.16) 16.19(8.04)	3.97(2.87,5.08)	7.29(38)	< 0.001

*paired t test ; significant p<0.05

VEGF **(**Table 4) had the highest mean score difference between day 1 and week 6 (- 37.22 pg/ml, *P*≤0.001, 95% CI -41.92, -32.52) and the lowest mean score difference was between day 1 and day 3 (-5.61pg/ml, *P*≤0.001, 95% CI -7.00, -4.22).

Table 4: Comparison of Vascular Endothelial Growth Factor (VEGF) for *C.striatus* group.

Day/Week	VEGF Mean (SD) pg/ml	Mean score difference (95% CI)	t-statistic (df)	p-value *
Day 1 Day 3	20.28(11.41) 25.89(12.63)	(-)5.61(-7.00 ,-4.22)	(-)8.16(38)	< 0.001
Day 1 Week 2	20.28(11.41) 35.79(14.82)	(-)15.51(-19.02,-12.00)	(-)8.95(38)	< 0.001
Day 1 Week 4	20.28(11.41) 47.17(17.45)	(-)26.88(-31.31, -22.46)	(-)12.30(38)	< 0.001
Day 1 Week 6	20.28(11.41) 57.50(17.28)	(-)37.22(-41.92, -32.52)	(-)16.02(38)	< 0.001
Day 3 Week 2	25.89(12.62) 35.79(14.82)	(-)9.90(-12.87, -6.94)	(-)6.76(38)	< 0.001
Day 3 Week 4	25.89(12.62) 47.17(17.45)	(-)21.28(-25.16, -17.40)	(-)11.11(38)	< 0.001
Day 3 Week 6	25.89(12.62) 57.50(17.28)	(-)31.61(-36.02, -27.21)	(-)14.53(38)	< 0.001
Week 2 Week 4	35.79(14.82) 47.17(17.45)	(-)11.38(-13.66, -9.09)	(-)10.09(38)	< 0.001
Week 2 Week 6	35.79(14.82) 57.50(17.28)	(-)21.71(-25.27, -18.16)	(-)12.37(38)	< 0.001
Week 4 Week 6	47.17(17.45) 57.50(17.28)	(-)10.33(-13.06, -7.61)	(-)7.68(38)	< 0.001

*paired t test ; significant p<0.05

Results between C.striatus and placebo group

Comparison of IL-6 between *C.striatus* and the placebo group **(Table 5)** showed the mean difference was statistically significant (*P*<0.05) on week 4 (4.17 pg/ml, *P*≤0.001, 95% CI 0.63, 7.71) and week 6 (3.86 pg/ml, *P*≤0.001, 95% CI 0.81, 6.90).

Table 5: Comparison of IL-6 between *C.striatus* and placebo.

Day/Week	IL-6 Mean(SD) pg/ml		Mean diff (95% CI)	t-stat (df)	p-value*
	C.striatus	Placebo			
Day 1	52.24 (20.76)	45.32 (23.34)	6.92 (-3.37,17.22)	1.342(71)	0.475
Day 3	34.19 (18.49)	25.58 (14.84)	8.61 (0.71,16.51)	2.172(71)	0.131
Week 2	23.52 (15.62)	15.09 (9.72)	8.43 (2.25,14.61)	2.720(71)	0.052
Week 4	14.82 (9.00)	10.65 (5.46)	4.17 (0.63,7.71)	2.351(71)	< 0.001
Week 6	10.36 (7.89)	6.50 (4.42)	3.86 (0.81,6.90)	2.524(71)	< 0.001

*Independent t-test; significant level p<0.05

Similarly, MMP-9 **(**Table 6) had statistically significant mean difference on week 4 (3.80 ng/ml, *P*=0.008, 95% CI 0.47, 7.14) and week 6 (4.44 ng/ml, *P*=0.033, 95% CI 1.19, 7.69).

Table 6: Comparison of MMP-9 between *C.striatus* and placebo.

Day/Week	MMP-9 Mean(SD) ng/ml		Mean diff (95% CI)	t-stat (df)	p-value*
	C.striatus	Placebo			
Day 1	44.19	36.09	8.09	2.530(71)	0.104
	(14.48)	(12.60)	(1.71,14.47)		
Day 3	37.05	30.93	6.12	2.18(71)	0.315
	(12.14)	(11.72)	(0.53,11.72)		
Week 2	25.84	22.36	3.47	1.477(71)	0.397
	(9.17)	(10.92)	(-1.22,8.16)		
Week 4	20.12	16.36	3.80	2.276(71)	0.008
	(8.16)	(5.71)	(0.47,7.14)		
Week 6	16.19	11.75	4.44	2.726(71)	0.033
	(8.04)	(5.40)	(1.19,7.69)		

*Independent t-test; significant level p<0.05

VEGF (Table 7) showed statistically significant in the mean difference (P<0.05) on day 1 (-13.72 pg/ml, P=0.010, 95% CI -20.42,-7.02), day 3 (-14.88 pg/ml, P=0.024, 95% CI -22.12,-7.65), week 4 (28.08 pg/ml, P=0.003, 95% CI 21.34,34.74) and week 6 (42.80 pg/ml, P<0.001, 95% CI 36.40,49.20), but not statistically difference (P>0.05) on week 2(8.13 pg/ml, p=0.738, 95% CI 1.43,14.82).

Table 7: Comparison of VEGF between *C.striatus* and placebo

Day/Week	VEGF Mean(SD) pg/ml		Mean diff (95% CI)	t-stat (df)	p-value*
	C.striatus	Placebo			
Day 1	20.48	34.20	(-)13.72	(-)4.08(71)	0.010
	(11.49)	(17.04)	(-20.42,-7.02)		
Day 3	26.09	40.97	(-)14.88	(-)4.103(71)	0.024
	(12.72)	(18.11)	(-22.12,-7.65)		
Week 2	36.06	27.93	8.13	2.420(71)	0.738
	(14.85)	(13.68)	(1.43,14.82)		
Week 4	47.31	19.27	28.08	8.347(71)	0.003
	(17.45)	(9.51)	(21.34,34.74)		
Week 6	57.66	14.86	42.80	13.333(71)	< 0.001
	(17.24)	(7.77)	(36.40,49.20)		

*Independent t-test; significant level p<0.05

The trend of both cytokines IL-6 (Fig. 1) and MMP-9 (Fig.2) exhibit decreasing trend over time in both groups however VEGF (Fig. 3) exhibit differently. In *C.striatus* group, the VEGF exhibit increasing trend till week 6 but in the placebo group, the VEGF exhibit a decreasing trend.

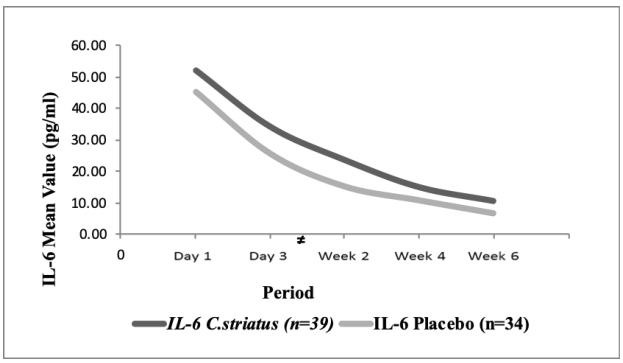

Figure 1. Trend of IL-6 between C.striatus and placebo over period of time.

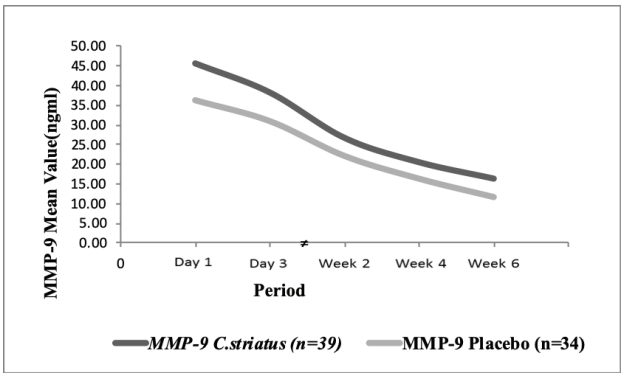

Figure 2. Trend of MMP-9 between C.striatus and placebo over period of time.

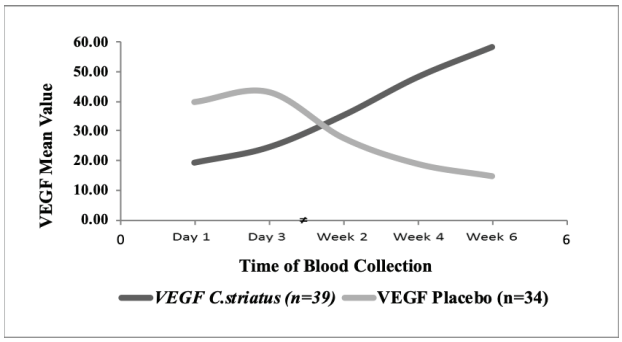

Figure 3. Trend of VEGF between C.striatus and placebo over period of time.

Discussion

Process of wound healing which involves three phases, are regulated by an array of cytokines and growth factors secreted by inflammatory and local cells. IL-6, MMP-9 and VEGF produced by inflammatory and local cells have crucial role in the pathogenesis of inflammations and wound healing [17]. MMP-9 is important in the proteolytic remodeling by degrading most of the structural components of extracellular matrix. Where as VEGF is an important signaling protein which is involve in both vasculogenesis and angiogenesis. Since the practice of traditional or complementary medicine among Malaysian people are still popular and is fully supported by Ministry of Health, the possible role of consuming *C.striatus* extract may have a role in wound healing.

In this study, the results obtained revealed that the levels of three wound healing biomarkers were statistically significant from day 1 to week 6 in patients who consume *C.striatus* extract. These findings suggest that *C.striatus* extract has enhancing and stimulating effects on multiple biological pathways by increasing the cytokines and growth factors in response to tissue injury. Since *C.striatus* is rich in fatty acids and amino acids particularly glycine and arachidonic acid, this could

have attributed to the above findings [7]. *C.striatus* is also known to produce polyunsaturated fatty acids which regulate prostaglandin synthesis inducing wound healing[18]. In this study the level of wound healing markers (IL-6, MMP-9) between group were statistically significant different from week 4 until week 6. IL-6 and MMP-9 levels were higher in *C.striatus* group compared to the placebo group. However, VEGF levels were increasing till week 6. This findings correlated well with the parallel study done by Ab Wahab *et al.* (2014) which showed an improvement in term of wound cosmetic appearance using visual analogue scale among *C.striatus* group at the end of week 6[19,20].This findings indicate that the effectiveness of *C.striatus* extract starts at week 4 onwards enhancing the production of VEGF during proliferation and remodelling phase. At this particular week, keratinocytes migration and collagen production via fibroblast are in progress to establish healing response. Perhaps, the high content of amino acid and fatty acids in *C.striatus* had triggered more productions of VEGF through the influence of IL-6 which play a role in the regulation of VEGF and in turn stimulate angiogenesis and vascularity. Increase vascularity stimulates the endothelial sensitivity to produce growth factors which further induce VEGF expression in a positive feedback loop[4].

Similar pattern was observed in both IL-6 and MMP-9 over time (Figure 1 and Figure 2). Both groups exhibit similar pattern with a peak at the beginning of inflammation phase (1- 3 days) and decreasing gradually till week 6. This confirms that IL-6 and MMP-9 levels are high during haemostasis and inflammation phase that last several days and act as a defence mechanism triggered by pro-inflammatory cells. Both cytokines started decreasing from week 2 onwards until the final phase as the number of pro-inflammatory cells will subsequently be replaced by tissue building cells such as fibroblast, myofibroblast, keratinocytes and epithelial cells.

The trend of VEGF between both groups showed different magnitude where VEGF trend in *C.striatus* showed an increasing trend compared to VEGF in placebo that showed a decreasing trend from day 1 until week 6 (Figure 3). *C.striatus* had triggered more productions of VEGF. A study done by Holmes and Zachary (2005) reported that the biological and signalling roles of the VEGF receptors have not yet been fully defined although there was a significant progress made towards elucidating the mechanisms mediating the angiogenic effects of VEGF[21]. This study was not followed on and the level and trend of VEGF after 6 weeks could not be elicited.

As a conclusion, this study showed that the oral administration of *C.striatus* extract had effects on wound healing process. High concentration of amino acid, EPA and DHA in *C.striatus* extract is believed to exert the effectiveness as wound healing properties. Both cytokines IL-6 and MMP-9 exerted effects at week 4 and above. However, VEGF trend could not be elicited as increasing trend was seen till week 6. Further study is needed to understand the detailed effects of *C.striatus.*

Conflict of interest

We declare that we have no conflict of interest.

Author's contribution

Data gathering and idea owner of this study: Julia Omar, Ahmad Ezam Zainan

Study design: Julia Omar, Ahmad Ezam Zainan, KNS Sirajudeen, Mohamed Rusli Abdullah

Data gathering: Julia Omar, Ahmad Ezam Zainan, KNS Sirajudeen, Noorazliyana Shafii, Mohamed Rusli Abdullah

Writing and submitting manuscript: Julia Omar, Ahmad Ezam Zainan, Noorazliyana Shafii

Editing and approval of final draft: Julia Omar, KNS Sirajudeen, Noorazliyana Shafii

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