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The Efficacy and Safety of Green-Lipped Mussel Extract Plus Ginkgo Biloba on Anti-Inflammatory Status in Patients with Knee Osteoarthritis

Shukur Wasman Smail; PhD[1,2], Mahdi Khaled Qadir; MSc[3], Omer Ahmed Hamad Amin; MSc[4], Dedawan Rasul; PhD[5], Sonia Elia Ishaq; MSc[1], Zhikal Omar khudhur; MSc[6], Mohammed I. M. Gubari; PhD[7] & Kurosh Djafarian; PhD[*8]

[1] College of Pharmacy, Cihan University-Erbil, Kurdistan Region, Iraq; [2]. Department of Biology, College of Science, Salahaddin University-Erbil, Erbil, Iraq; [3] Department of Physiotherapy, Erbil Technical Health and Medical College, Erbil Polytechnic University, Erbil, Iraq; [4] Department of Rheumatology, Ranya Teaching Hospital, Ministry of Health, Ranya, Kurdistan Region, Iraq; [5] Kurdistan higher council of medical specialties, Erbil, Kurdistan Region, Iraq; [6] Biology Education Department, Tishk International University, Erbil, Iraq; [7] Community Medicine, College of Medicine, University of Sulaimani, Sulaimani, Iraq; [8] Department of Clinical Nutrition, School of Nutrition and Dietetics, Tehran University of Medical Science, Tehran, Iran.

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Corresponding author:
kdjafarian@tums.ac.ir
Department of Clinical Nutrition, School of Nutritional Sciences and Dietetics, Tehran University of Medical Sciences, Tehran, Iran

Postal code: 1417613151
Tel: + 98 912 665 4577

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ABSTRACT

Background: Clinical research on the efficacy of nutraceutical compounds recommended for the relief of osteoarthritis (OA) symptoms has been largely disputed. In addition, no link has been established between its safety and efficacy in Kurdish population. The aim of this study is to determine the efficacy of New Zealand green-lipped mussel extract + Ginkgo biloba (GLME+) in patients with OA, and analyze the effect of GLME+ on inflammation. **Methods:** In an open-label, single-group allocation study, 40 patients diagnosed with knee OA were administrated 1000 mg/day of New Zealand green-lipped mussel extract and 100 mg/day of Ginkgo biloba for eight weeks. The outcome measure was scored using Western Ontario and McMaster Universities arthritis index (WOMAC). The serum concentration of inflammatory chemokine (CCL3) and myeloperoxidase (MPO) were measured. An intention-to-treat analysis was employed and subject data at T_0 and T_8 weeks. **Results:** Results showed a significant improvement in WOMAC score in post-treated OA patients with GLME+ (P<0.001). In addition, CCL3 serum levels were significantly decreased after an 8-week intervention (P<0.001). Moreover, no statistical significance was observed within groups in MPO serum levels (P> 0.05). **Conclusions:** GLME+ improved knee joint pain, stiffness, and mobility in OA patients. Two of GLME+'s chondroprotective properties were the reduction of oxidative damage and the inhibition of inflammation, both of which have been linked to the etiology of OA cartilage destruction. The findings indicated that GLME+ may be useful in the treatment of OA patients.

Introduction

Osteoarthritis (OA) is the most common form of arthritis and can affect any joint in the body, including shoulders, hands, feet, spine, knees, and hips (Katz *et al.*, 2021). The basic goal

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of osteoarthritis treatment is to reduce pain and enhance joint function. Although the actual cause of osteoarthritis is unknown, many studies have revealed that inflammation in early stages of the disease appears to have a role in the disease's development and progression (Felson, 2006).

Analgesics and non-steroidal anti-inflammatory drugs (NSAIDs) are the most common treatment options for rheumatoid arthritis and osteoarthritis, although they usually have unsatisfactory results and are associated with serious gastrointestinal side effects (Hawkins and Hanks, 2000). As a result, medical experts are looking for safer and more effective alternatives to both analgesics and NSAIDs (Walker-Bone, 2003).

The lipid extract of New Zealand's green-lipped mussel *Perna canaliculus* (GLME) is a natural alternative for treating inflammation and arthritis. GLME has been found to have anti-inflammatory, anti-arthritic, and gastro-protective properties in various animal and clinical studies (Brien *et al.*, 2008, Cho *et al.*, 2003, Pollard *et al.*, 2006, Whitehouse *et al.*, 1997). The active ingredients in GLME are thought to be concentrated long-chain n-3 polyunsaturated fatty acids (PUFAs) which can block membrane arachidonic acid (AA) metabolism by inhibiting the cyclo-oxygenase (COX) and lipo-oxygenase (LOX) pathways. Therefore, it reduces the formation of prostaglandins and leukotrienes and the inflammatory sequence (McPhee *et al.*, 2007, Singh *et al.*, 2008).

Ginkgo biloba, also known as the maidenhair tree, is a well-known Chinese plant used for thousands of years in traditional Chinese medicine. Ginkgo biloba extract has been linked to various biological activities and pharmacological effects, such as free radical scavenging, anti-inflammation, anti-tumor, anti-aging, and cardiovascular properties (Chan *et al.*, 2007). Ginkgo biloba extract prevents the production of pro-inflammatory cytokines IL-1β and TNF-α while increasing the production of anti-inflammatory cytokines IL-10 and IL-10R, implying that it has anti-inflammatory characteristics (JIAO *et al.*, 2005). In human articular chondrocyte and OA rats, EGb761, a standard extract of Ginkgo biloba leaves has been found to have anti-inflammatory effects by reducing PGE2 and NO levels in blood, as well as histological changes, COX-2, and nitrotyrosine expressions in cartilages (Chen *et al.*, 2013). According to the new research, bilobalide, a sesquiterpene compound produced from Ginkgo biloba leaves, prevents IL-17-induced inflammatory damage in ATDC5 cells by suppressing microRNA-125a through JNK and NF-KB signaling pathways (Mao *et al.*, 2019); this demonstrates that bilobalide has anti-inflammatory effects on chondrocytes.

The administration of NSAIDs results in gastrointestinal (GI) dysfunction. GLME, besides its anti-inflammatory effects, has gastroprotective effects. Coulson *et al.* documented that the therapeutic efficacy of the GLME extract used was possibly correlated with its effects on GI function by improving gastrointestinal symptom rating scale (GSRS) scores (Coulson *et al.*, 2012). Jhun *et al.* revealed that GLME prevents the progression of cartilage damage in OA-induced rat models through the inhibition of metalloproteinase-3 (MMP-3) (Jhun *et al.*, 2021).

Macrophage inflammatory protein-1 alpha/CC chemokine ligand 3 (MIP-1 α /CCL3) is a chemotactic chemokine released by macrophages. It has various biological activities, including inflammatory cell recruitment, wound healing, stem cell suppression, and immune response maintenance. Activating bone resorption cells causes direct bone destruction. The number of cells that release CCL3 rises in areas of inflammation and bone resorption (Bhavsar *et al.*, 2015). CCL3 is involved in the development of several inflammatory diseases and disorders that cause bone loss, such as periodontitis, multiple myeloma, Sjögren syndrome, and rheumatoid arthritis (Al-Sabbagh *et al.*, 2012, Cuello *et al.*, 1998, Koch *et al.*, 1994, Terpos *et al.*, 2005). Referring to previous studies, CCL3 has abnormal expression in osteoporosis and is closely linked to bone abnormalities (Collins *et al.*, 2017, Fatehi *et al.*, 2017), although its relationship with OA is still unknown.

Myeloperoxidase (MPO) is an oxidative enzyme found in phagocytic cells. MPO is a heme enzyme that converts chloride ions into hypochlorous acid

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(HOCl) and other reactive oxygen species (ROS) by utilizing the oxidizing potential of superoxide and hydrogen peroxide (H2O2) (Winterbourn *et al.*, 2000). Neutrophil-derived HOCl has been implicated in lung injury (Hammerschmidt *et al.*, 2007), renal disease (Maruyama *et al.*, 2004), and rheumatoid arthritis (Wu and Pizzo, 2001). In a recent study, patients with early OA had significantly higher levels of MPO in their synovial fluid (Steinbeck *et al.*, 2007). These findings suggest that MPO could be used as a diagnostic and inflammatory marker for early diagnosis of OA.

The purpose of the present study was to determine the therapeutic effect of New Zealand green-lipped mussel extract + Ginkgo biloba (GLME+) in patients diagnosed with OA in terms of knee pain, stiffness, and function. The second aim of this study is to investigate the anti-inflammatory activity of GLME+ by evaluating serum levels of CCL3 and MPO in OA patients.

Materials and Methods

Participants

The study included 40 participants with knee OA (15 men and 25 women, age 50.7±1.8 year and body mass index 29.3±0.7 kg/m^2) who met the inclusion and exclusion criteria. The sample size was calculated according to the method developed by Chow *et al.(Chow et al., 2017)*. Participants were eligible if their knee OA had been assessed and confirmed by a rheumatologist, and they had not used any antibiotics or herbal/multivitamin/ nutritional supplements in the previous four weeks. Participants were not allowed to enroll if they had uncontrolled systemic disease, were pregnant or breastfeeding, or had shellfish allergies or intolerances. They were also told not to take antibiotics while participating in the research. Before signing informed consent, each patient got written and verbal information about their participation in the study. The researchers chose people from the orthopedics and rheumatology departments at private CMC Hospital. GLME+ treatment (1000 mg of GLME and 100 mg of Ginkgo biloba) was given to participants who met the criteria for inclusion. Redrose Manufacturing Limited in the UK manufactures GLME+ ; it has 500 mg of GLME and 50 mg of Ginkgo biloba, and patients take it twice a day after meals. The patients were not given any NSAIDs or pharmacological drugs along GLME+. They were assessed at baseline (T0) before getting their first treatment and then again at (T8) weeks after treatment when their participation was completed. Blood samples were obtained from the participants before and after therapy, and serum was separated and stored at -20 °C for future investigation.

Outcome measure

The primary outcome measure was Western Ontario McMaster Universities Arthritis Index (WOMAC) (Bellamy, 2002), which was administered by a clinical researcher in an interview format at each participant's visit (T_0 and T_8). The WOMAC is a validated questionnaire that assesses the intensity of knee pain (5 questions), stiffness (2 questions), and physical function limitation (17 questions) in people with osteoarthritis of the knee or hip (Bellamy, 2002). Each subscale's maximum severity values are 20, 8, and 68, respectively. The maximum total score of severity for the WOMAC is 96.

Intervention

Patients received 2 capsules of GLME+ (Redrose Manufacturing Limited, UK) (1000 mg/day of GLME, a proprietary blend of freeze-dried green shell mussel meat (Perna canaliculus) and 100 mg/day of Ginkgo biloba) in opaque white bottles for the duration of the study. The daily consumption of GLME+ by participants was documented in diaries, which were used to track compliance. Patients who did not follow regular treatment or took other medications besides GLME+ were excluded from the study.

Measurements

Chemokine (CCL3) assay: The concentration of CCL3 in serum was measured using a commercial ELISA kit according to the manufacturer's instructions (Elabscience Company, USA). The absorbance was measured at 450 nm using an

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ELISA microplate reader after color development (Molecular Devices, Sunnyvale, CA, USA).

Measurement of serum MPO activity: The method of Klebanoff and Clark (Klebanoff *et al.*, 1978) was used to determine serum MPO activity, which was based on a kinetic measurement of the formation rate of yellowish-orange product of oxidation of o-dianisidne with MPO in the presence of H2O2 at 460 nm. MPO was defined as a substance capable of decomposing 1 µmol of H2O2 per minute at 25 °C. The calculation was done using a molar extinction coefficient 1.3 × 104 M−1 cm−1 of oxidized o-dianisidine. MPO activity was measured in Mmol/dl of serum.

Ethical considerations

The Human Research Ethics Committees of the University of Salahaddin (Ethic Number: R37-111; 78 approved on June 5, 2021) and CMC Hospital (code number: CM25-12; 34 approved on June 15, 2021) gave their permission for the study to go forward.

Data analysis

GraphPad Prism was used for all the statistical analyses (ver. 6.0; GraphPad Software Inc., San Diego, CA, USA). To check the assumption of normality, normality tests were performed on all the data (parameters and scoring variables), which justified the use of paired t-tests. Paired t-tests were used to compare parameters before and after supplementation. P-values of less than 0.05 were considered significant when data was provided as mean + standard error of the mean (SEM).

Results

Paired t-tests were used on WOMAC score between T_0 and T_8 , and WOMAC total score revealed significant changes between T_0 and T_8 (T_0 =61.97±1.809; T_8=52.82±1.924) (P<0.001) as seen in **Figure 1**.

According to **Figure 2**, the serum level of CCL3, a marker of inflammation, was significantly decreased in post-treated OA patients with GLME+ (T_8=83.83±6.211 pg/ml) when compared to pre-treated OA patients (T_0=103.7±4.348 pg/ml) (P=0.01).

MPO serum level was also decreased in post-treated OA patients when compared to pre-treated OA patients, but this was statistically non-significant (P>0.05) (T_0=82.93±13.21 Mmol/dl; T_8=58.56±4.646 Mmol/dl) (**Table 1**, **Figure 3**).

Table 1. The mean±SEM of studied variables before and after intervention.

Variables	Before (T_0)	After (Week 8)	P-value[a]
WOMAC score	61.97±1.80	52.82 ±1.92	0.0009
CCL3 pg/ml	103.70 ± 4.34	83.83 ± 6.21	0.01
MPO Mmol/dl	82.93 ± 13.21	58.56 ±4.64	0.086

***CCL3**: C-C motif chemokine ligand 3; **GLME+**: Green-Lipped Mussel Extract + Ginkgo biloba; **MPO**: Myeloperoxidase; **WOMAC**: Western Ontario and McMaster Universities arthritis index; [a]: Paired t-test.*

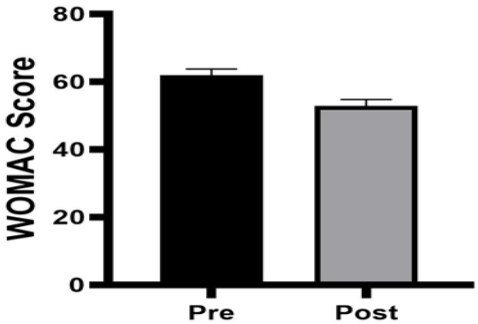

Figure 1: Mean±SEM of WOMAC scores in OA patients in before and after GLME+ supplementation.

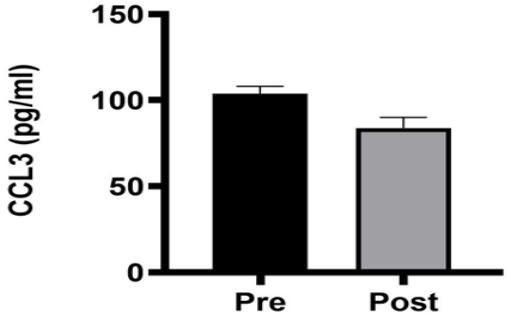

Figure 2. Mean±SEM of serum CCL3 in OA patients in before and after GLME+ supplementation.

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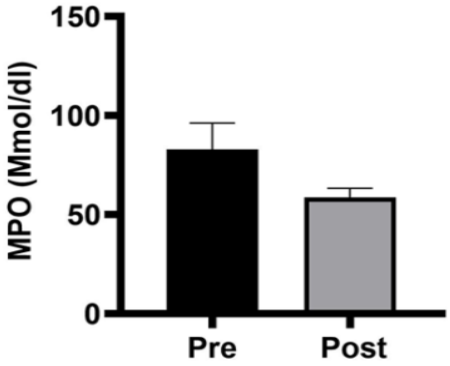

Figure 3. Mean±SEM of serum myeloperoxidase in OA patients in before and after GLME+ supplementation.

Discussion

The aim of this study was to determine whether GLME+ nutritional combination relieves OA symptoms and has an anti-inflammatory effect. GLME+ significantly reduced pain and inflammation by reducing CCL3 and MPO. Therefore, GLME+ simultaneously controlled OA pain and protect cartilage.

For people with OA, painkillers are the only effective treatment option. Nevertheless, long-term use of many analgesics can cause gastrointestinal and cardiovascular side effects. Therefore, food or drink components as well as dietary supplements with moderate immunomodulatory effects, such as resveratrol (Liu *et al.*, 2010), may be viable options for people with OA to gain symptomatic relief. There have been various studies using natural compounds such as cashew nut, hyaluronic acid, and ALIAmide palmitoyl-glucosamine to reduce pain and inflammation in osteoarthritis patients (Cordaro *et al.*, 2019, Di Paola *et al.*, 2016, Fusco *et al.*, 2020, Pérez-Lozano *et al.*, 2021). In this study, the authors used GLME+, a combination of New Zealand green-lipped mussel extract and ginkgo biloba leaf extract. GLME has been found to contain bioactive lipids known as pro-resolving lipid mediators, which help to resolve inflammation by inhibiting pro-inflammatory cytokines, eliminating apoptotic neutrophils, and enhancing wound healing and tissue regeneration (Wakimoto *et al.*, 2011). Ginkgo biloba leaf extract also has anti-inflammatory effects (Ilieva *et al.*, 2004, Ye *et al.*, 2019), and bilobalide, a sesquiterpene lactone molecule, may have pharmacological benefits (Goldie and Dolan, 2013). Gingko biloba extract is commonly used in clinical practice to treat pain, asthma, and diarrhea. It can treat OA patients by removing oxygen's free radicals, promoting cell metabolism, and reducing symptoms (Dubey *et al.*, 2004).

In the present study, GLME+ was shown to reduce pain and cartilage damage in OA patients. Coulson *et al.* observed that giving 3,000 mg of GLM extract to OA patients improved pain, stiffness, and function considerably (Coulson *et al.*, 2012). The chondroprotective effects of GLME+ were associated with the inhibition of oxidative damage and suppression of inflammation, which are essential in the etiology of OA cartilage destruction (Jhun *et al.*, 2021).

Chronic low-grade inflammation has been linked to the formation and progression of OA even in the early stages of the disease. It is recognized by the presence of infiltrating immune cells and the release of a cascade of inflammatory mediators (Berenbaum, 2013, Kapoor *et al.*, 2011, Robinson *et al.*, 2016). Macrophages, the most prevalent immune cell type found in OA synovium, are assumed to be important in maintaining synovial inflammation. According to Dapunt *et al.*, CCL3 increased the development of monocytes into osteoclasts, which was thought to speed up both bone and cartilage destruction (Dapunt *et al.*, 2014). This study revealed that CCL3 levels in OA patients' serum decreased significantly throughout treatment, implying that CCL3 may play a role in the onset and progression of OA.

Zhao *et al.* found that patients with OA have higher levels of CCL2, CCL3, and CCL4 in their synovial fluid compared with healthy controls (Zhao *et al.*, 2020). According to previous studies, neutralizing CCL3 in the affected joint reduced circulating monocyte recruitment and collagenase-induced synovitis. This finding was in line with reports from gene-knockout mice. Mice with CCL3 deficiency were greatly protected from cartilage degradation, synovitis, and mononuclear cell infiltration after intra-articular injection of an anti-

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type collagen II antibody and lipopolysaccharide (Chintalacharuvu *et al.*, 2005).

These findings revealed that in osteoarthritic knees, inflammatory tissue releasing CCL3 may contribute to an increase in macrophage infiltration and synovitis and that treatment with GLME+ significantly reduced CCL3 and relieved osteoarthritic patients' symptoms.

Pro-inflammatory cytokines are thought to indicate cartilage breakdown by stimulating both resident and infiltrating neutrophils and macrophages. When these cells are activated, they produce reactive oxygen species (ROS) like superoxide anion, hydrogen peroxide, hypochlorous acid, and chlorine gas, which all have an oxidative modifying effect on articular cartilage. Daumer et al. have shown that the later products can alter pyridinoline crosslinking of articular cartilage (Daumer *et al.*, 2000), triggering an early event in degradative process. Several investigations have found that patients with early not late OA exhibit overexpression of inflammatory mediators within synovial membrane (Benito *et al.*, 2005, Bonnet and Walsh, 2005, Loeuille *et al.*, 2005).

According to Girish *et al.*, MPO levels in controls were normal because articular cartilage was completely healthy and free of inflammation. Even though there was ongoing inflammation in late OA, there was no cartilage left to destroy, hence MPO levels were within the normal range. However, because active inflammation and articular cartilage were present in early OA, MPO levels were much higher than late OA and controls (Girish *et al.*, 2012). This finding supported the results of this study regarding the fact that serum MPO levels in OA patients treated with GLME+ did not change significantly, which could be because the patients were at different stages of the disease.

The present study had some limitations that must be acknowledged. First, lacking a placebo to compare to the GLME+ intervention was one limitation of the study. Second, most of the patients were middle-aged or elderly females lacking information about their menopausal state, which may affect the results. Third, the lack of information regarding the nutritional status of OA patients may interfere with the anti-inflammatory effects of GLME+. Finally, the sample size was relatively small and it was from one medical center; broader studies should be done with a larger number of patients and a multicenter from different regions of the country. Therefore, these results must be interpreted cautiously.

Conclusion

The authors concluded that GLME+ was effective in reducing OA symptoms and improving function via the reduction of WOMAC scores. GLME+ has an anti-inflammatory effect by lowering inflammatory chemokine levels (CCL3). Patients seeking natural remedies for OA will benefit from the findings of this study. GLME+ has the potential to be an effective OA treatment. Further randomized clinical trial studies are needed to confirm the findings of this study.

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Authors' contribution

Wasman Smail S, Khaled Qadir M, Gubari MIM, khudhur ZO and Elia Ishaq S designed the research. Ahmed Hamad Amin O, Rasul D, and SEI conducted it. The manuscript was written by Wasman Smail S, Khaled Qadir M, and Gubari MIM. The analysis was done by Gubari MIM and rechecked by Djafarian K. The manuscript was revised by Gubari MIM and Djafarian K. All the authors confirmed the final version of the manuscript.

Conflict of interest

The authors declared no conflict of interests.

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