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Assessment of Primary Osteoporosis Status in the Postmenopausal Women of Tabriz and the Effect of Curcumin Nanomicelles, *Nigella sativa* Oil, and Curcumin Nanomicelles and *Nigella sativa* Oil Soft Capsules on Cellular-Molecular and Clinical Outcomes: A Study Protocol

Somayeh Abdolalipour[1], Mojgan Mirghafourvand[2], Majid Mobasseri[3], Sakineh Mohammad-Alizadeh[2], Alireza Ostadrahimi[4], Neda Dolatkhah[5], Seyed Kazem Shakouri[5], Safar Farajnia[6], Azizeh Farshbaf-Khalili[5]*

Abstract

Objectives: To determine the status of primary osteoporosis and evaluate the effects of curcumin nanomicelles, *Nigella sativa* oil, and curcumin nanomicelles plus *N. sativa* oil compared to placebo on cellular-molecular and clinical outcomes.

Materials and Methods: The study protocol will be implemented in two phases. The first phase is a cross-sectional study aiming at determining the prevalence of primary osteoporosis and its risk factors, especially cellular-molecular factors in 528 postmenopausal women aged 50-65 years in Tabriz, Iran through simple random sampling. The second phase is a triple-blind factorial randomized controlled clinical trial. In this phase, patients with primary osteoporosis, identified with dual-energy X-ray absorptiometry, will be randomly divided into equal four groups of 30 individuals in a triple-blind factorial randomized controlled trial. The four group included *N. sativa* oil soft capsules (one capsule 1000 mg/d) and placebo- curcumin nanomicelles, curcumin nanomicelles soft capsules (one capsule 80 mg/d) and placebo-*N. sativa*, *N. sativa* oil, and curcumin nanomicelles soft capsules, and both placebos for six months.

Results: The prevalence of primary osteoporosis and its relationship with several parameters will be determined in phase 1, including socio-demographic-obstetric-medical characteristics, anthropometric indices, body composition, lifestyle, osteoporosis-related microRNAs, inflammatory and oxidative biomarkers, bone turnovers, and some gene polymorphisms. Finally, changes in mean bone mineral density (BMD), bone turnovers, inflammatory and oxidative biomarkers, and osteoporosis-related microRNAs will be evaluated in phase 2.

Conclusions: The present study can significantly contribute to the prognosis of the disease and the selection of an appropriate herbal supplement given the cost-effectiveness of herbal compounds as pharmaceutical adjuvants.

Keywords: Postmenopausal osteoporosis, Menopause, Health promotion lifestyle, Quality of life, Dual-energy X-ray absorptiometry, Curcumin

Introduction

Osteoporosis is one of the most common metabolic bone diseases (1). Primary osteoporosis may result from menopause or aging whereas secondary osteoporosis is developed from local infection or inflammation, renal disease, medications (e.g., corticosteroids), systemic inflammation, and the like. Secondary osteoporosis is idiopathic in 30%-40% of cases (2). In postmenopausal women, bone mass decreases by about 3%-9% per year in the first six postmenopausal years (3). According to the International Osteoporosis Foundation, more than 50% of all bone fractures are projected to be of an osteoporotic nature by 2050 in East and Southeast Asia (4).

Oxidative stress has been reported to create major changes in the function of bone cells such as osteocytes. Oxidative stress and inflammatory factors reduce bone mass through excessive osteocyte apoptosis (5).

Bone is also highly responsive to sex hormones, especially estrogen, and its turnover can be regulated by estrogen-like compounds. Estrogen has an important role in skeletal development and bone homeostasis in both men and women (6).

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[1]Nutrition Research Center, Department of Midwifery, Faculty of Nursing and Midwifery, Tabriz University of Medical Sciences, Tabriz, Iran. [2]Social Determinants of Health Research Center, Tabriz University of Medical Sciences, Tabriz, Iran. [3]Endocrinology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran. [4]Nutrition Research Center, Tabriz University of Medical Sciences, Tabriz, Iran. [5]Aging Research Institute, Physical Medicine and Rehabilitation Research Centre, Tabriz University of Medical Sciences, Tabriz, Iran. [6]Biotechnology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.

***Corresponding Author:** Azizeh Farshbaf-Khalili, Tel: +98 9144023216, Fax: +98-41 34796969, E-mail: azizeh_farshbafkhalili@yahoo.com

It has been shown that the acute loss of estrogens leads to an increase in the levels of reactive oxygen species (ROS), activates nuclear factor kB (NF-kB), and stimulates the production of proinflammatory cytokines such as interleukin 1β (IL-1β) and tumor necrosis factor α (TNF-α). The production of proinflammatory agents is the most common characteristic of different types of inflammatory joints and bone disorders (7).

Estrogen receptor 1 (ESR1) gene polymorphism is presumably associated with postmenopausal osteoporosis, thus this gene could be employed as a selection method for identifying peoples with a higher risk of osteoporosis. In addition, ESR gene polymorphisms may be used to select potential therapies and to test the efficacy of the applied drugs in osteoporotic patients (8).

Aging is also associated with changes in body composition (9). Increased fat mass is associated with a decrease in muscles and bone masses (10). Therefore, body composition appears to be another factor affecting the reduction of bone density (11).

Today, osteoporosis risk assessment is conducted by measuring bone mineral density (BMD) and clinical risk factors. Bone turnover markers (BTMs) have been recently used as a new approach for osteoporosis diagnosis, albeit inconsistently (12). The measurement of bone turnover biochemical markers contributes to the detection of high bone turnovers (13).

The existing evidence also points to a link between microRNAs and bone homeostasis, with numerous research groups investigating the relationship between microRNAs and bone disorders such as osteoporosis (14).

There are contradictory results regarding the relationship between polymorphisms in osteoprotegerin (OPG), the receptor activator of nuclear factor KB ligand (RANKL), lipoprotein receptor-related protein 5 (LRP5), ESR1, and the zinc finger and BTB domain containing 40 genes (ZBTB40) according to Genome-Wide Association Studies and BMD, with diverging results among various racial and ethnic groups (15,16).

In postmenopausal women with osteoporosis, hormone therapy is used in addition to conventional treatment for the prevention and treatment of osteoporosis, albeit with serious side effects such as thrombosis, hypertension, and atherosclerosis (17). Therefore, an urgent need is felt for more extensive research on the discovery and development of healthy foods containing natural compounds that can effectively compensate for estrogen deficiency (18).

The essential oil extracted from *Nigella sativa* seeds has a chemical composition of the Ranunculaceae Family. Its mechanism of effect on osteoporosis may be related to its active constituent, namely, thymoquinone (19). It has been shown that the synthesis of prostaglandins and leukotrienes was inhibited by thymoquinone. It seems that this anti-inflammatory action is due to the inhibition of cyclooxygenase and lipoxygenase pathways causing the production of prostaglandins and leukotrienes from arachidonic acid, respectively (20). Further, the production of nitric oxide by macrophages can be suppressed by the anti-inflammatory agent of *N. sativa* (21).

Curcumin nanomicelles is a major active constituent of turmeric, and several mechanisms have been reported for its effect on the prevention of the bone resorption process (22,23). It has also been demonstrated to have efficient anti-inflammatory effects through altering the NF-kB transcription activity and inhibiting prostaglandin E2 production and cyclooxygenase-2 expression (24). Additionally, it is shown that curcumin can suppress the production of pro-inflammatory cytokines (especially IL-1 and TNF-α), and thus prevent or suppress the progression of the bone resorption process (25). The other mechanism of curcumin administration is the amelioration of oxidative stress-induced apoptosis by preserving the mitochondrial functions and activation of Akt-GSK3β signaling pathway in osteoblasts (26). These data provide empirical evidence supporting the clinical use of curcumin for the treatment and prevention of osteoporosis. In addition, a decrease in ESR1 gene expression has been found to be one of the mechanisms of curcumin in the treatment of some malignancies (27,28). No side effects have been reported following the administration of the standard doses of curcumin nanomicelles.

Given the cost-effectiveness of these herbal compounds in comparison with their pharmaceutical counterparts, as well as the lack of sufficient clinical studies regarding the effectiveness of these herbs on postmenopausal osteoporosis, especially in combination together, attempts will be made to evaluate the effects of curcumin nanomicelles and *N. sativa* oil soft capsules, separately and together, on postmenopausal women with secondary osteoporosis.

The main objective of the project was to determine the status (i.e., prevalence, risk factors, and related cellular-molecular biomarkers) of primary osteoporosis in postmenopausal women in Tabriz, Iran, and then to evaluate the effects of three types of intervention (curcumin nanomicelles, *N. sativa* oil, and curcumin nanomicelles plus *N. sativa* oil) on cellular-molecular and clinical outcomes compared to the placebo.

Methods

Study Design

This is a study protocol which will be implemented in two phases.

First Phase

The first phase is a cross-sectional study on 528 postmenopausal women in Tabriz, Iran.

Target Population

All postmenopausal women aged 50-65 years, covered by Tabriz health care centers.

Sample Selection

Sample selection will be carried out in 87 health care centers using the simple random method. The information of all postmenopausal women can be accessed in an integrated manner on the Integrated Health System (SIB) via the Tabriz Health Center. Five hundred and twenty-eight 50-65-year postmenopausal women will be selected from among these individuals using the website www.random.org through the simple random method.

Inclusion Criteria

Postmenopausal women aged 50-65 years, residence in Tabriz, cessation of menstruation for at least 12 consecutive months, the ability to verbally communicate insofar as they can answer the questions, lack of a history of fracture in the last 10 years, no hormone therapy in the last year, and non-occurrence of menopause before the age of 40.

Exclusion Criteria

Renal failure or diseases verified by laboratory examinations and physician confirmation, bone diseases other than osteoporosis, metastatic bone disease, malignancy, hereditary diseases (e.g., hemophilia, thalassemia, and hemochromatosis), and endocrine diseases (e.g., Cushing's syndrome, hyperthyroidism, type 1 diabetes, and primary hyperparathyroidism). In addition, other criteria were a chronic liver disease, digestive diseases (e.g., chronic liver diseases such as primary biliary cholangitis, coeliac disease, Crohn's disease, total gastrectomy, and stomach surgery), body mass index (BMI) of less than 18.5, and consumption of medications that affect bone metabolism, including intravenous bisphosphonate over the past 5 years, and oral bisphosphonate in the last 6 months. Finally, cumulative consumption of oral bisphosphonate for over 3 years or over 1 month (between 6 and 12 months) prior to the study, use of parathormone analogs within 12 months prior to the study, or strontium, fluoride or cathepsin K inhibitors at any time, use of hormonal medications or corticosteroids during the study, and 25(OH)D <20 ng/mL or current hypocalcemia were the other related criteria.

Sample Size

Based on the study of Bayat et al (29), the prevalence of osteoporosis as 25.5%, α=0.05, and d=0.16 (study accuracy), the sample size was calculated 440 women. Considering 20% drop-outs due to secondary osteoporosis)30(which will be diagnosed by the laboratory test, the final sample size was calculated 528 women for the first phase.

Procedures

After obtaining permission from provincial and urban health centers, the selected postmenopausal women will be phone called in order to briefly explain the objectives and methodology of the research. In the case of willingness to participate in the study, participants will be asked to attend the health center at a specified time. Written informed consent forms will be obtained in case of meeting the inclusion criteria. Then, different scales will be completed by the participants. These instruments were the socio-demographic, international physical activity (IPAQ), obstetric and medical, food frequency (FFQ), and 24-hour dietary recall (this questionnaire will be completed by the participant or, in case of illiteracy, a family member on three occasions on two workdays and a holiday during one week). The other scales included the menopausal quality of life (MENQOL), health-promoting lifestyle questionnaires (HPLPQ), and the anthropometric checklist. Anonym and coded questionnaires will be used to protect confidentiality.

Participants' blood pressures will be measured in each visit after 15 minutes of rest using a mercury sphygmomanometer (Riester, Diplomat) with an accuracy of ±3 mm Hg.

At the baseline, patients' height, weight, waist circumference, and hip circumference will be calculated using a manual wall-mounted height gauge (Seca) with a measurement range of 0-220 cm and an accuracy of 1 mm, in a standing position next to a wall with their shoes taken off and the scapulae in a normal position. Other biometric identifiers will be measured with a digital ground scale (Seca) with an accuracy of 100 g, a capacity of 220 kg, and with minimum clothes. The mean value of the two measurements will be used on each occasion. Then, the BMI will be calculated using the formula of weight (kg) divided by height (m) squared. BMIs in the range of 18.5-24.9, 25-29.9, and ≥30 kg/m^2 will be regarded as normal, overweight, and obese, respectively.

Information about the participants' body compositions will be evaluated using a body composition analyzer (South Korea), which can measure protein masses, minerals, mineral tissues, adipose tissues, non-adipose tissues, total body water, body age, basal metabolism, waist-to-hip ratio, and subcutaneous fat.

Next, 10 mL of blood samples will be collected, 2 mL of which will be used for complete blood count with differential (CBC/diff), calcium (Ca), phosphate (P), alkaline phosphatase (ALP), thyroid-stimulating hormone (TSH), creatinine (Cr), fasting blood sugar (FBS), and vitamin D to differentiate the primary and secondary osteoporosis. The rest of each blood sample will be frozen in three forms of whole blood, serum, and plasma specimens at -80°C to examine serum bone turnovers and inflammatory markers, as well as the markers of oxidative stress by the enzyme-linked immunosorbent assay, serum microRNAs by the real-time polymerase chain reaction method, and genetic polymorphisms in relevant laboratories (Biotechnology Research Center and Nutrition Research Center) by the restriction fragment length polymorphism (RFLP) method.

After making necessary arrangements, postmenopausal women will be referred to as the bone density screening center to undergo dual-energy X-ray absorptiometry (DEXA). The final diagnosis and interpretation of osteoporosis will be made by an endocrinologist (Figure 1).

Data Collection Methods

In this study, data will be collected from the inclusion and exclusion criteria checklist, the socio-demographic, obstetric and medical, IPAQ created in 1998 in Geneva as a standard tool for measuring physical activity (31-34), and 24-hour food record to complete a 3-day food diary at home (35). Moreover, other data sources include MENQOL designed by Hilditch et al at the University of Toronto, Canada, which measures the quality of life with 29 questions on the vasomotor, psychosocial, physical, and sexual domains (36), and HPLPQ which has 52 items in 6 domains of health responsibility, spiritual growth, physical activity, interpersonal relationship, nutrition, and stress management (37,38). Additionally, anthropometric checklist, complete analysis of body components, bone densitometry (DEXA), the primary test registration checklist (i.e., Ca, 25-hydroxy vitamin D, Cr, P, ALP, TSH, FBS, and CBC/diff) in addition to serum BTMs (e.g., osteocalcin, procollagen type 1 N-terminal propeptide (P1NP) are used as well. Data were also collected using bone-specific alkaline phosphatase (BSAP), C-terminal telopeptide of type 1 collagen (CTX-1), inflammatory markers (e.g., TNF-α, hs-CRP, and IL-6), oxidative (e.g., superoxide dismutase, malondialdehyde, and total antioxidant capacity), and serological microRNAs (e.g., miR422a, miR-133a, miR-21, and miR-503) associated with inflammation. Finally, genetic tests were also applied, including genetic polymorphisms such as OPG gene rs2062377 polymorphism, RANKL gene rs9533090 polymorphism, LRP5 gene rs3736228 polymorphism, ESR1 gene rs4869742 polymorphism, and ZBTB40 gene rs6426749 polymorphism.

Statistical Analysis

Data will be analyzed using SPSS23 and descriptive statistics including frequency distribution and percentage to determine the prevalence of osteoporosis. In addition, analytical statistics including the chi-square test, independent *t* test, and univariate and multivariate logistic regression analyses were applied to determine associated factors with osteoporosis. These factors were socio-demographic and obstetric and medical characteristics, physical activity, health-promoting lifestyle, food intake, quality of life, anthropometric indicators, inflammatory and oxidative markers, BTMs, serum osteoporosis-related micro-RNAs, genetic polymorphisms, and body component analysis.

Second Phase (Intervention)

The second phase of the study is a triple blind factorial

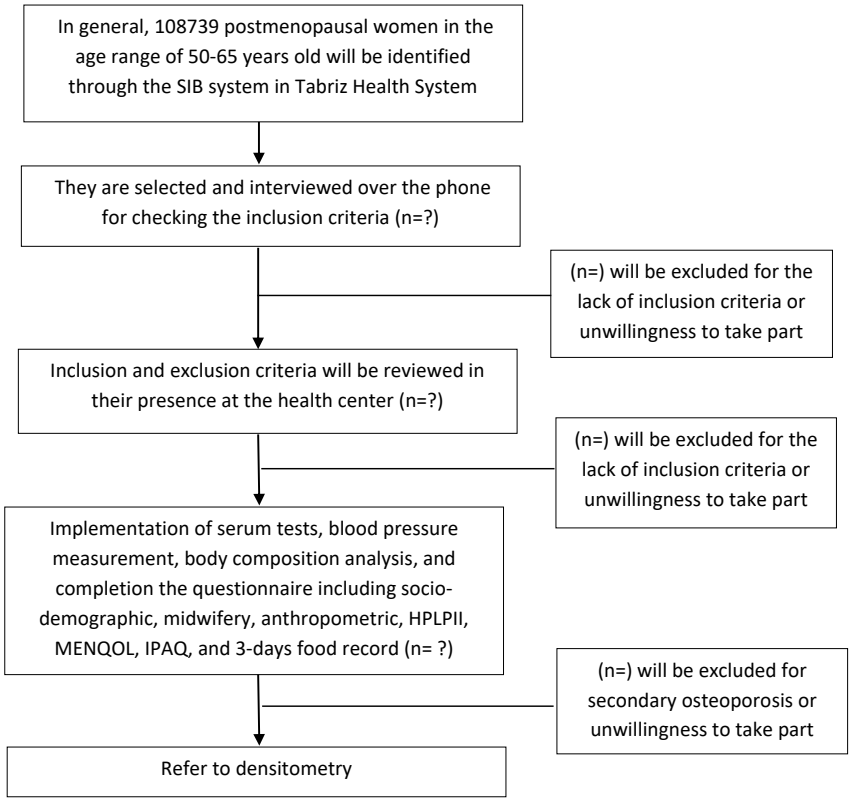

Figure 1. Flow Diagram of Phase One Progress.

randomized controlled clinical trial registered on the Iranian Registry of Clinical Trial website (identifier: IRCT20131009014957N4).

Inclusion Criteria

Low bone density (T-score ≤-2.5) in lumbar vertebrae, pelvis, or femoral neck.

Exclusion Criteria

T-score ≤-4 in lumbar vertebrae, T-score ≤-3.5 in pelvis and femur neck, and a history of hip or vertebral fracture. Taking anticoagulants, beta-blockers, gastric ulcer, and bile duct stones.

Sample Size

In this phase, the sample size will be calculated using G*Power 3.1.2 based on the study of Bayat et al (29) and considering m1 = 0.92 (the mean BMD of the lumbar vertebrae). The sample size was calculated to be 26 women for each group given a 20% increase assumption ($m^2 = 0.104$), sd1 = sd2 = 0.178, two-sided $\alpha = 0.05$, and power = 95%. Taking a 10% attrition rate into account, the final sample size was computed to be 30 women for each of the four groups.

Primary Outcomes

Mean BMD, the serum levels of BTMs (e.g., osteocalcin, P1NP, BSAP, and CTX-1), and the serum levels of some inflammatory factors (e.g., TNF-α, HS-CRP, and IL-6).

Secondary Outcomes

The mean serum levels of osteoporosis-related MicroRNAs (i.e., miR422a ،miR-133a ،miR-21, and miR-503), the quality of life score, body composition analysis score (i.e., percent body fat, mass body fat, soft lean mass, lean body mass, visceral fat mass, total body water, and mineral), the serum levels of some oxidative stress indices (e.g., total antioxidant capacity, superoxide dismutase, and malondialdehyde), treatment response based on the frequency of the rs2062377 polymorphism of the OPG gene, the frequency of the rs9533090 polymorphism of the RANKL gene, frequency of the rs3736228 polymorphism of the LRP5 gene, the frequency of the rs4869742 polymorphism of the ESR1 gene, and the frequency of the rs6426749 polymorphism of the ZBTB40 gene.

Intervention

After signing written informed consent forms, eligible postmenopausal women with osteoporosis will be randomly assigned to four groups through the randomized block design using 4 and 8 blocks and an assignment ratio of 1:1:1:1 in Random Allocation Software. The groups included the recipients of (1) one *N. sativa* oil (1000 mg) and placebo-curcumin nanomicelles soft capsule per day, (2) one curcumin nanomicelles (80 mg) and *N. sativa* placebo soft capsule per day, (3) one *N. sativa* oil (1000 mg) and curcumin nanomicelles (80 mg) soft capsule per day, and (4) one soft capsule from both placebos (containing microcrystalline cellulose) per day (39-42,43). These medicines will be provided by Barij Essence and Exir Nano Sina Pharmaceutical Companies. To blind the participants, researchers, along with care providers' main drugs and their placebos will be the same in shape, size, color, odor, and dose. In addition, drugs will be placed inside similar, opaque, and sealed envelopes. Each participant will be given an envelope containing two types of 60 capsules or placebo every two months. The envelopes will be numbered from 1 to 120 and opened from the first to the last envelope in the order of participant inclusion in this phase. Moreover, they will be provided with drug use and a side effect checklist in order to complete them during consumption. The participants will have access to the researchers' telephone number in order to report any side effects. They will be advised to revisit the health centers two, four, and six months after the beginning of the intervention. Completed checklists and used drug boxes will be taken at each visit and the next envelopes and checklists will be given to them. They will be asked to complete the quality of life and IPAQ questionnaires at baseline (phase 1), two, four, and six months after the intervention. The 3-day food intake record checklists will be provided to be completed at baseline (phase 1), two, and six months after the beginning of the intervention at home. Follow-up phone calls will be made every 15 days to emphasize the regular use of drugs and the completion of checklists. The regular or irregular intake of medicines will be evaluated by the recorded drug use checklist.

A clinician blind to the trial will generate the random allocation sequence and prepare envelopes accordingly. Research team members will enroll participants and assign them to intervention groups. Participants, care providers (researchers), and those assessing outcomes (i.e., researchers) will be blind after assigning participants to interventions in this study.

It is worth noting that all groups will receive weekly alendronic acid (70 mg) tablets and daily calcium (500 mg) and vitamin D calcium (400 IU) supplements, as well as nutritional and physical activity and other lifestyle recommendations. Following the conclusion of the intervention, bone density reassessment will be performed by DEXA, serum biomarkers will be measured again, and body components will be re-analyzed as well. Moreover, the menopausal quality of life questionnaire, three-day food intake record, and satisfaction with medication checklists will be completed following the intervention. The superiority of interventions will be investigated in comparison to placebo (Figure 2).

Data Collection Methods

Data will be collected using the MENQOL questionnaire, 24-hour food record to complete a 3-day food diary (two weekdays and one weekend) at home, side effects and

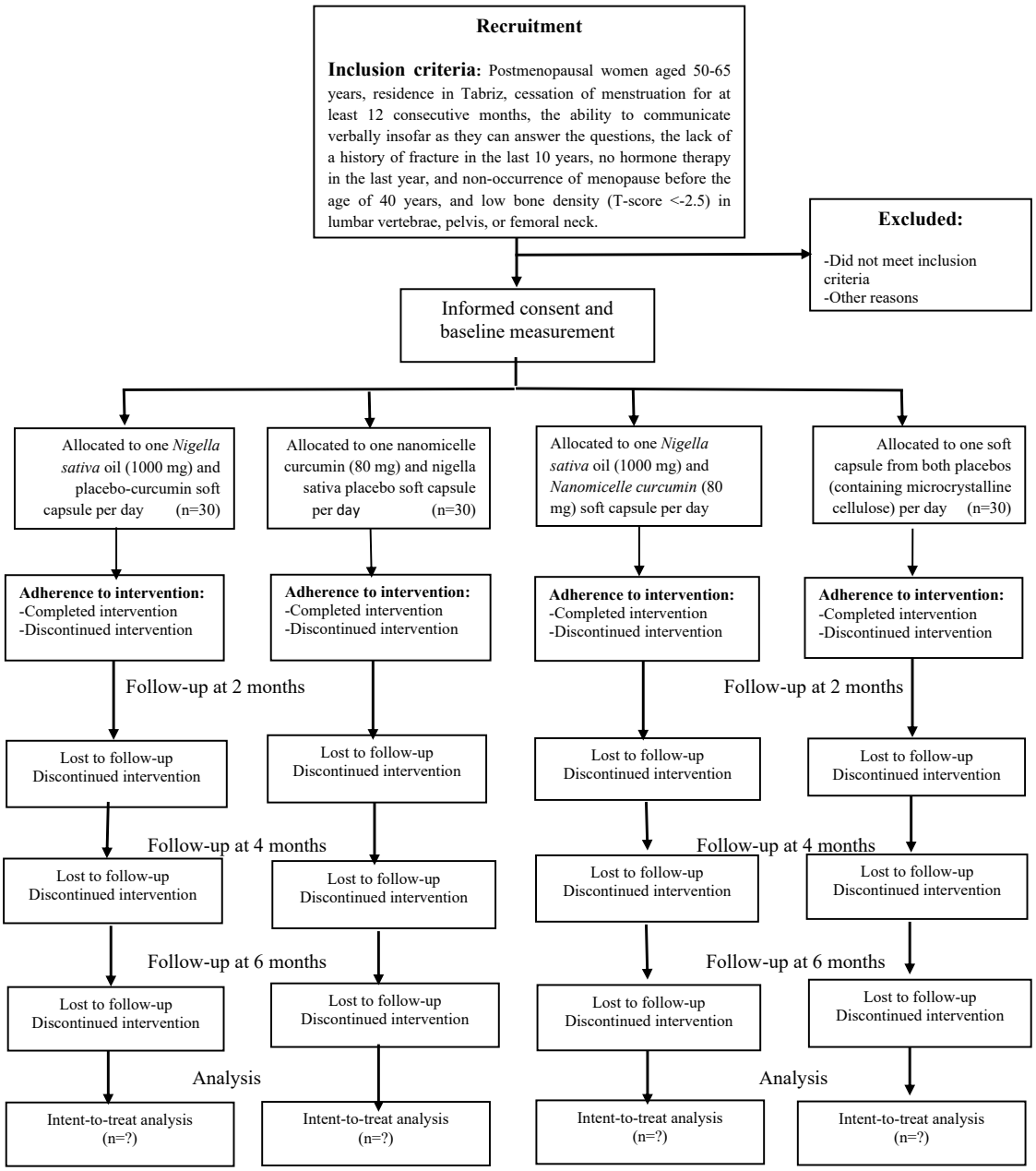

Figure 2. Flow Diagram of Phase Two Progress.

participants' satisfaction with medication checklists, bone densitometry by DEXA, and the collection of blood samples (10 mL) to evaluate inflammatory, oxidative, and BTMs, along with serum bone metabolism-related microRNAs.

Data Analysis

The collected data will be analyzed in SPSS23. The analysis of covariance with baseline control will be performed to compare bone density, serum BTMs, inflammatory and oxidative factors, and serum osteoporosis-related microRNAs among the study groups following the intervention. Repeated measures ANOVA with baseline control and possible confounding variables will be conducted to compare the mean quality of life scores at different assessment times. Additionally, the chi-square test will be used to determine treatment responses based on the genetic polymorphisms of the study groups. All analysis will be done based on intention-to-treat analysis.

Discussion

The prevalence of osteoporosis in Iran was reported about 17% in 2012 (44). This rate is expected to increase owing to the changing demographic patterns in the future, increased consumption of nutrient-poor foods, the prevalence of low-mobility lifestyle (35), and the prevalence of osteoporosis among the elderly, and increment of elderly women in particular. In addition, this imminent increase

will pose a great challenge to the Iranian healthcare system so that the economic burden of osteoporotic fractures on the healthcare system will exceed that of numerous other chronic diseases. On the other hand, the debilitating complications of osteoporosis have turned this disease into one of the major health issues in Iran, which calls for consideration and planning for screening, treatment, and prevention purposes. In postmenopausal women diagnosed with osteoporosis, hormone therapy is used in addition to conventional treatments for the prevention and treatment of osteoporosis although it has serious side effects such as thrombosis, hypertension, and atherosclerosis (45). Therefore, there is an urgent need for more extensive studies on the discovery and development of non-estrogenic natural compounds capable of effectively preventing and treating osteoporosis (46). Alternative treatments such as herbal medicines have attracted the attention of researchers in addition to synthetic drugs (47). However, the significance of herbal compounds in the prevention and treatment of bone disorders remains unanswered.

Herbal compounds are found cost-effective in comparison with their pharmaceutical counterparts. Based on the literature review, there is a lack of studies regarding the prevalence and status of postmenopausal osteoporosis in Tabriz, Iran, as well as a lack of sufficient clinical studies on human subjects suffering from osteoporosis based on the research group's knowledge and inquiries from credible scientific websites. To the best of our knowledge, the present study was the first one to investigate the effect of curcumin nanomicelles *and N. sativa* oil, alone and combined, on cellular-molecular and clinical outcomes in postmenopausal women after surveying the prevalence and risk factors of postmenopausal osteoporosis.

Limitations

- Age restriction of participants (50-65 years old);
- The impossibility of selecting the rural areas of Tabriz due to travel distance;
- The impossibility of free administration of routine osteoporosis medicines due to the lack of funding;
- Use of the RFLP method for detecting the polymorphisms of genes due to the lack of access to the gene sequencing method.

Authors' Contribution

Study concept and design: AFK, MoM, SA, MaM, SMA, SKS, AO, ND, and SF; Acquisition, analysis, and interpretation of data: SA, AFK, MoM, and SF; Manuscript drafting: SA, AFK, MoM, and ND; Critical revision of the manuscript for important intellectual content: MaM, SMA, SKS, AO, and SF; Statistical analysis: SKS, AFK, MoM. All authors have read and approved the manuscript.

Conflict of Interests

The authors declare that they have no competing interests.

Ethical Issues

Written informed consent forms will be obtained from all participants. This protocol was approved by the Ethics Committee of Tabriz University of Medical Sciences (Ethics code: IR.TBZMED.REC.1397.131).

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