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Exploring the Biomarker of Collagen Degradation in Cartilage for Osteoarthritis Management: The Power of Topical Phytotherapy

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ABSTRACT

Background: This 12-week study aimed to investigate the efficacy of the topical application of phytoconstituents derived from nine Indian medicinal plants by regulating the biomarker, 4-hydroxyproline protein, that stimulates the production of collagen in cartilage, in the treatment of knee osteoarthritis (OA), characterized by the gradual breakdown of the cartilage that cushions the joints, leading to pain, stiffness, reduced range of motion, limited mobility, and an increased proportion of obesity.
Methods: A total of 144 patients with OA, verified by the Kellgren-Lawrence scale, were included, with 72 receiving topical phytotherapy (TPT) and 72 receiving nutraceutical supplements. At weeks 0 and 12, IL-10, TNF-alpha, malondialdehyde, creatine kinase-muscle, aldolase-A, and 4-hydroxyproline levels, pain-related outcomes, and anthropometric measurements have been assessed as per the approved protocol and kits for both groups.
Results: The results showed that the TPT group had significantly reduced inflammation, oxidative stress, pain, and joint stiffness and improved cartilage formation compared to the control group. The areas under the ROC curves, risk ratios, and Kellgren-Lawrence scores of the studied risk factors in the TPT group were highly significant ($p<0.0001$) at week 12.
Conclusion: These findings suggest topical phytotherapy for regulating the biomarker, 4-hydroxyproline, of collagen may have great potential in treating OA and provide a novel therapeutic approach for patients suffering from OA compared to the control group, as correlated with the KL scale.

Keywords: Osteoarthrosis; 4-hydroxyproline; Topical phytotherapy; ROC curves.

INTRODUCTION

Osteoarthritis (OA) is a degenerative joint disease characterized by the loss of articular cartilage, subchondral bone changes, and synovial inflammation. It is one of the leading causes of disability and affects around 58 million adults, and it is estimated that by 2040, this number will increase to 78.4 million [1].

The most significant change in this disorder is the breakdown of articular cartilage, which is mostly carried on by the extracellular matrix being broken down by degradation enzymes and the chondrocytes dying through apoptosis or autophagy [2].

According to research, articular cartilage may be lost during the onset and progression of OA because 4-hydroxyproline concentrations in cartilage decline with aging and in OA patients [3].

Hermann Emil Fischer, a German Nobel Laureate in chemistry, isolated 4-hydroxyproline (4-hydroxypyrrolidine-2-carboxylic acid; an imino acid; also known as an amino acid) from acid hydrolyzed gelatine (a translucent, colourless,

unfavorable food ingredient of animal collagen) in 1902 [4].

4-hydroxyproline is a modified (non-standard) amino acid, derived from proline, found in high concentration in collagen. It is the primary structural protein in the extracellular matrix found in the various connective tissues, such as cartilage that covers the ends of bones in joints. Collagen is essential to maintaining the normal structure and strength of connective tissue, such as bones, skin, cartilage, and blood vessels. Collagen consists of 13.3 g of proline, 10.73 g of 4-hydroxyproline, 0.11 g of 3-hydroxyproline, and 25.8 g of glycine residues per 100 g of collagen in humans **[2]**. On the other hand, glycine, proline, and hydroxyproline (Hyp) contribute to 57% of total amino acids in collagen, which accounts for one-third of proteins in animals [5].

4-Hyp has been shown to have several beneficial effects on OA. First, it promotes the synthesis of collagen, which is essential for the maintenance of articular cartilage. Second, it has anti-inflammatory properties that can reduce the synovial inflammation associated with OA. Third, it can inhibit the production of matrix metalloproteinases (MMPs), which are enzymes that break down collagen and contribute to the destruction of articular cartilage in OA [6].

The levels of 4-HPRO in OA are regulated by multiple mechanisms in collagen synthesis and degradation, such as collagen degradation by matrix metalloproteinase-13 (MMP-13) and mechanical loading-induced activation of collagen synthesis pathways of the mitogen-activated protein kinase (MAPK)/ extracellular signal-regulated kinase (ERK) [6].

The exact cause of OA is not fully understood, but it is believed to be the result of a combination of factors, including genetic predisposition, joint injury, and aging. OA is characterized by the breakdown of cartilage, leading to the formation of osteophytes (bone spurs) and the thickening of the subchondral bone as evaluated by the Kellgren-Lawrence scale (KLS) [7-8].

While there is no cure for osteoarthritis, there are several treatment options available that can help manage the symptoms and improve the quality of life for patients. Three different techniques are recommended by current guidelines for treating OA. The first is pharmacological treatment, which is recommended by both the Osteoarthritis Research Society International (OARSI) and the American College of Rheumatology (ACR) [9-10], and involves using non-steroidal anti-inflammatory drugs (NSAIDs), opioids, cyclooxygenase-2 (COX-2)-specific medications, corticosteroid injections, and hyaluronic acid injections to relieve pain, inflammation, and stiffness. Nevertheless, just treating the symptoms without addressing the root cause of the cartilage tissue, only serves a "palliative" purpose. Moreover, conventional medicines may have side effects that could diminish compliance through the emergence of gastrointestinal difficulties, cardiovascular effects, and other problems (particularly when used for a lengthy period) [11].

The second strategy involves lifestyle modification, a non-pharmacological strategy that emphasizes physical exercise, the optimization of an optimal diet, and nutraceutical treatment, which includes rehabilitation to promote a healthy body composition. The most commonly utilized nutraceuticals in OA include chondroitin sulfate, glucosamine sulfate, hyaluronic acid, methylsulfonylmethane (MSM), laboratory-made calcium, vitamin D, even intramuscular injection, and 4-hydroxyproline in the form of collagen. The health of joints and cartilage is thought to be promoted by nutraceutical supplements of collagen like bone broth (a simmered mixture of animal bones and connective tissue typically served as soup), grass-fed red meat, wild-caught salmon fish, and bovine collagen and marine collagen in the form of capsules, tablets, or powders, either alone or in combination in the reduction of

clinical symptoms and in reducing inflammatory indices in people with OA, leading to decreased pain and stiffness syndromes. If medicine and lifestyle changes are ineffective, surgery is a possibility as a third option [12].

It should be highlighted that despite surgery having numerous irreversible side effects, more than 42 percent of patients suffering from acute knee OA choose to get knee replacements as a result of the intense pro-surgical marketing. According to a report by the Organization for Economic Cooperation and Development (OECD) in 2019, knee replacement surgeries were most commonly performed in countries such as the United States, Germany, and Australia [13].

Despite these encouraging outcomes, the use of nutraceutical therapy containing 4-hydroxyproline may be helpful in these circumstances, but there have been conflicting reports of their adverse effects and outcomes that have limited statistical significance, which has prevented them from being used widely in medicine or as oral supplements to treat OA. Besides these, some individuals might not be able to ingest enough 4-hydroxyproline through supplements, especially when 72 percent of research participants live a vegan or vegetarian lifestyle and 25 percent of participants avoid red meat.

Nonetheless, following the discoveries of phytoconstituents "capsaicin" from chili paper for fundamental insights into the mechanisms of pain in the 2021 Nobel Prize and "artemisinin" from the plant Artemisia annua for malaria parasite disease in the 2015 Nobel Prize, people have become increasingly interested in alternative phytotherapy treatment options as opposed to pharmaceutical drugs and nutritional supplements, which have numerous negative effects on long-term OA issues [14].

In this respect, alternative topical use of phytoconstituents derived from nine Indian medicinal plants with an affordable minimum cost and significant duration is suggested, even at the early stage of OA, considering all the pathophysiological risk factors involved in progressive OA.

The main common phenomena in OA are the pain syndrome, as well as the psychometric quality of life, which leads to an oxidative stress factor that indicates the disease progression **[1].** As a result, appropriate pain parameters should be well-thought-out under internationally accepted clinical outcome measures, such as the visual analogue scale (VAS) [15], the Western Ontario and McMaster Universities osteoarthritis index (WOMAC index) [16], the Knee-Injury Osteoarthritis Outcomes Scale (KOOS) [17], and the Lower Extremity Functional Scale (LEFS) [18], as well as anthropometric factors, such as body weight, body mass index (BMI), waist circumference (WC), hip circumference (HC), waist-to-height ratio (WHtR), and waist-to-hip ratio (WHpR) which contribute another major causing factor of pain and disability **[19]**

This degenerative disease is characterized by biochemical composition and structural changes that alter the tissue's biological and biomechanical functions. The biological changes include the loss of matrix proteins, such as proteoglycan and collagen, an increase in macroscopic degenerative fibrillation, and altered water content**.**

Therefore, in pursuance of taking care of the prime tissue's biological factors in OA, namely, inflammation, oxidative stress, connective tissue damage, and muscle degeneration, including muscular dystrophy and skeletal muscle damage, cartilage and bone health, and nerve functions have been appraised by the analyses of biochemical parameters, such as inflammatory cytokines (IL-10 and TNF-alpha), malondialdehyde (MDA) (a biomarker marker of oxidative stress), creatine kinase-muscle (CK-MM), aldolase A, and 4-hydroxyproline.

The present study attempted to normalize the aforementioned risk factors, such as abnormal international-approved clinical outcome measures, anthropometric parameters, and biomarkers, in patients with OA by topical application of a specific

phytotherapeutic treatment protocol for 12 weeks, which has great potential to reduce inflammation, oxidative stress, pain, and joint stiffness, as well as improve cartilage formation by appropriately regulating 4-hydroxyproline, as evaluated by the KL grading scale [8].

2. MATERIALS AND METHODS

2.1 Recruitment of patients: The present study has been undertaken with 252 approached cohorts with the age group of 40–80 years old from August 2022 to January 2023. An Institutional Review Board-approved consent form for the physical examinations, blood sample collections, and radiological images required for the study has been signed by all participants in the first phase of the screening procedure.

2.2 Exclusion criteria: Out of 252 participants, 108 were eliminated because they had additional pathological conditions that might have contributed to the symptoms already present. These conditions included rheumatic diseases, osteochondritis diseases, inter-articular fractures, congenital dysplasia, radicular syndrome, joint symptoms brought on by malignant tumours, Baker's cyst, Perthes disease, Plica syndrome, dermatomyositis, polymyositis diseases, iliopectineal or trochanteric bursitis bone, and joint infectious diseases and ischemic bone necrosis. as well as patients with multiple drug dependencies, a history of cancer, including carcinomatosis and granulocytic leukemia, patients with cuts, wounds, or any type of chronic skin disease, patients with severe neurological diseases, patients with histories of the chronic liver, kidney, and heart diseases, and patients who refused to submit to physical examinations and/or attend weekly follow-up appointments were also excluded from the study.

2.3 Study design: Seventy-two cohorts (62.5% of females) have been assigned to the experimental group (TPT) out of the remaining 144 patients with KOA who have significant pain syndromes, discomfort, obesity (both general and central), cartilage damage, subchondral bone changes, osteophyte formation, muscle weakness, oxidative stress, and inflammation of the synovium tissue as evidenced by elevated levels of biomarkers: interleukin-10 (IL-10), Tumours necrosis factor-alpha (TNF-alpha), malondialdehyde (MDA), creatine kinase-muscle (CK-MM), aldolase-A, and 4-hydroxyproline levels, and radiological images, and the other 72 subjects (56.9% of females) have been assigned to the control group treated with nutraceutical supplements (NS group). Following the prior methodology, each individual completed a baseline questionnaire for demographic information [19–20].

2.4 Evaluation of Clinical Outcome Measures (COM) and Anthropometric parameters (AP): Observation of the patient's perceived symptoms of pain intensity in the last 24 hrs. for pain, stiffness, and functional disability of the individual patient, the patient's opinion about their knee and associated problems under VAS, WOMAC, KOOS, and LEFS, and anthropometric parameters (BMI, WC, HC, WHpR, and WHtR) as per previous study [15-19] for experimental and control groups at 0-week and 12-week. The significance of all the parameters of the COM and AP at 12 weeks was evaluated with the help of ROC curve analysis, meta-analysis, and radar chart and graphically depicted.

2.5 Evaluation of Specific Biochemical Parameters in Blood: The blood samples were centrifuged to obtain serum and analyze biomarkers for subchondral bone health in both the experimental and control groups at baseline and 12 weeks. The biomarkers were measured following standard protocols and kits as per previous studies [20] and rechecked with a biochemical analyzer before reporting the final results. The significance of the studied

biomarkers at 12 weeks was evaluated for the pooled cohorts of the experimental group compared to the control group and graphically depicted.

2.6. Evaluation of knee joints radiographic assessment under the Kellgren–Lawrence grading scale: Bilateral anterior–posterior (AP) weight-bearing knee radiographs were used to identify tibiofemoral OA and patellofemoral OA. The severity of tibiofemoral and patellofemoral conditions was determined using the Kellgren-Lawrence grading scale [8] that includes the formation of osteophytes, narrowing of joint cartilage, sclerosis of subchondral bone, and altered shape of bone ends at baseline and 12 weeks for both groups.

The AP views of knee joint x-ray images of two such experimental patients before and after the treatment at 12 weeks are evaluated separately.

2.7. Evaluation of Receiver operating characteristic (ROC) curve: The analyses of the ROC curves [21], the pooled experimental group-vs-control group at 12 weeks, were evaluated with the help of the following formulae in support of Figure 1 as per the previous study [22].

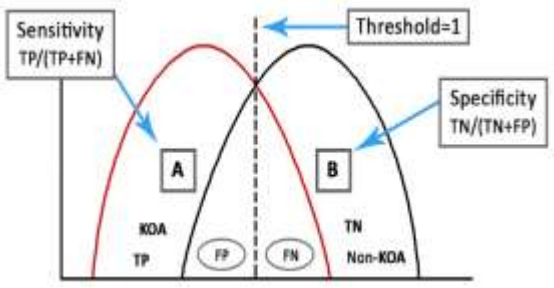


Figure 1. Probability density functions of a hypothetical diagnostic test that gives values on the real line. The density of the diagnostic test is plotted for each of the 2 populations, with KOA (A) and non-KOA (B).

Sensitivity = True positive/ (True positive + False negative).

Specificity= True negative/ (False Positive + True negative).

Positive predictive value (PPV) = True positive/ (True positive +False positive).

Negative predictive value (NPV) = True negative/ (True negative + False negative). Accuracy = (True positive + True negative)/ (True positive + False negative +False positive +True negative).

2.8. Evaluation of Forest plotting for assessing heterogeneity in meta-analysis: Meta-analyses combine results from multiple studies to increase the reliability and accuracy of findings in medical and health care. This research methodology is widely accepted in social and health sciences [21,23].

The significance of all the parameters by analyzing the risk ratios was evaluated between the pooled cohorts of TPT and SM groups at 12 weeks in respect of all the risk factors of KOA and graphically depicted under the respective head of analyses.

The significance of all the parameters along with the heterogeneity of the meta-analysis (Cochran's Q-test statistics, I^2-index, degree of freedom, *p*-values, and $\tau 2$-statistic) was separately evaluated for the pooled cohorts in both the groups (experimental group-vs-control group) in respect of all the risk factors of KOA and graphically depicted under the respective heads of analyses.

2.9. Evaluation of Radar chart: The radar chart tool in Microsoft Excel (version 2011) was used to graphically display on separate axes, with all axes being scaled equally [21].

The mean percentage of improvement at each level of risk factors for the experimental group and control group at the end of 12 weeks allows for more comprehensive discussions on OA's therapeutic effect criteria in healthcare services by using radar charts to present results.

2.10 Management of topical phytotherapy: The treatment protocol, phytochemicals used and their mechanisms of action, and disease prevention have already been evaluated in detail as per previous studies [20].

2.11. Data Collection and Statistical Analysis: The study analyzed data using descriptive statistics including means,

standard deviations, ranges, and frequencies. The results showed a significant relationship between risk factors related to COM, AP, and biochemical analyses, with knee osteoarthritis (KOA) development receiver operating characteristic (ROC) curve analysis conducted to determine the accuracy of various measurements in predicting the performance of KOA between experimental and control groups. The meta-analysis showed that the effect size was calculated as the risk ratios between cohorts with and without KOA. The statistical analysis was performed using Graph Pad Prism software (version 5.0, San Diego, California, USA) with a p-value of less than 0.05 considered statistically significant.

3. RESULTS

3.1 Enrolment and Baseline Characteristics of Patients*:* A total of 144 cohorts out of 252 met the inclusion criteria and were enrolled. Finally, 72 cohorts (62.5% females) were selected for the experimental group (TPT group), and 72 (56.9% females) were in the control group (NS group). The demographic data and baseline characteristics of all cohorts were evaluated as per the previous protocol [9-10]. The gender-wise classifications with their age groups of (40-50), (50-60), (60-70) and (70-80) years for both groups were shown in Figure 2.

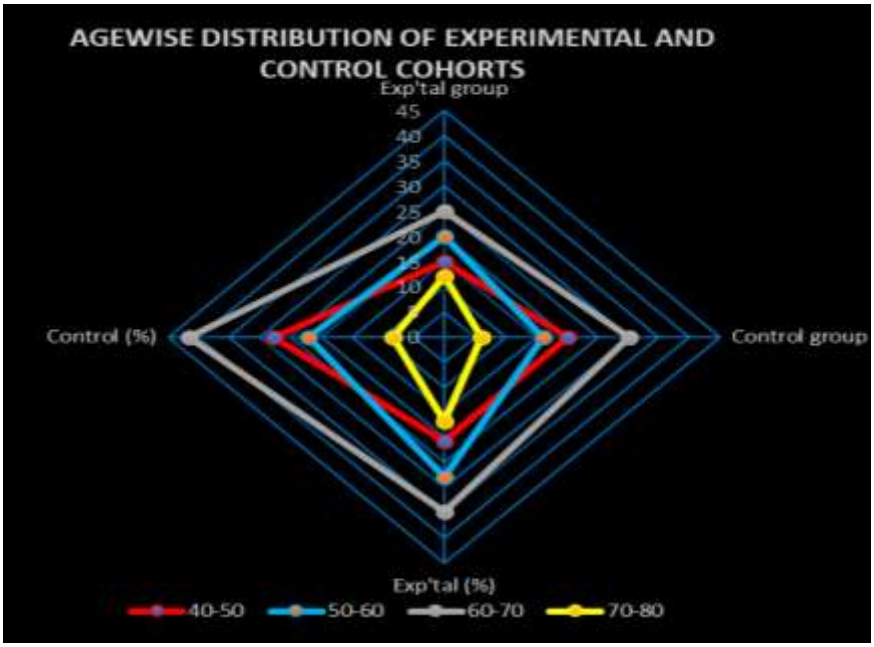


Figure 2: Gender-wise classifications with their age groups for both groups

Risk factors such as pain-related outcomes (COM), anthropometric measurements (AP), and biomarkers (TNF-alpha, IL-10, MDA, CK-MM, aldolase A, and 4-hydroxyproline):

(a)The analyses of ROC curves of all the studied parameters of COM under WOMAC, VAS, LEFS, AP such as BMI, WC, HC, WHpR, and WHtR, and biomarkers namely TNF-alpha, IL-10, MDA, CK-MM, aldolase A, and 4-hydroxyproline for 72 pooled TPT cohorts *versus* 72 pooled NS cohorts at 12 weeks with all significant values of percentage of R-squares, sensitivity, and specificity, areas under the ROC curves (AUCs) were within the range of 0.997-0.915 and their optimum cut-off points showed within the normal ranges as evaluated by Youden's Index (J), indicating a satisfactory treatment policy (Figure 3A-3D).

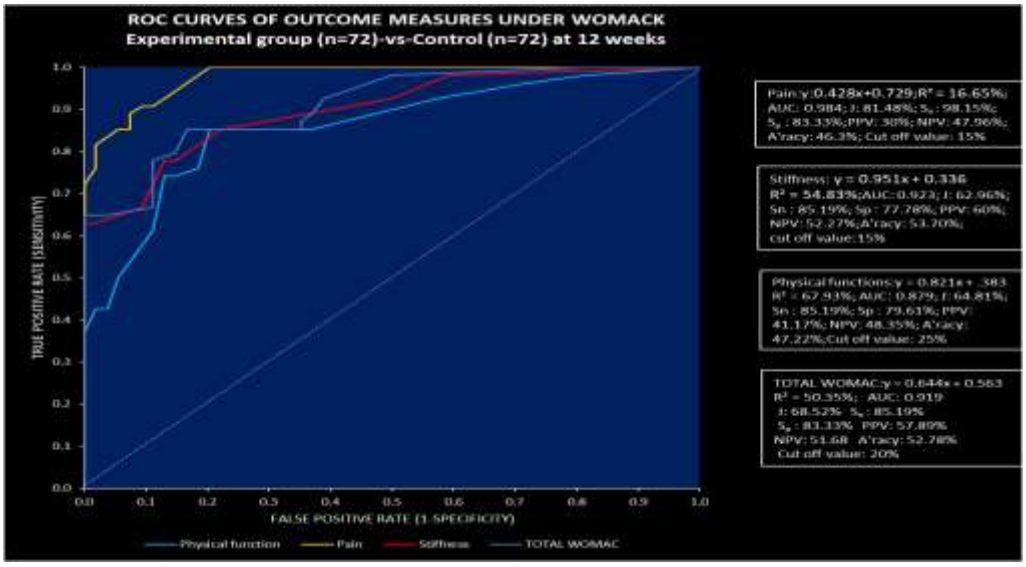


Figure 3A: ROC curves of outcome measures under WOMAC of experimental group (n=72)-vs-control group (n=72) at 12 weeks

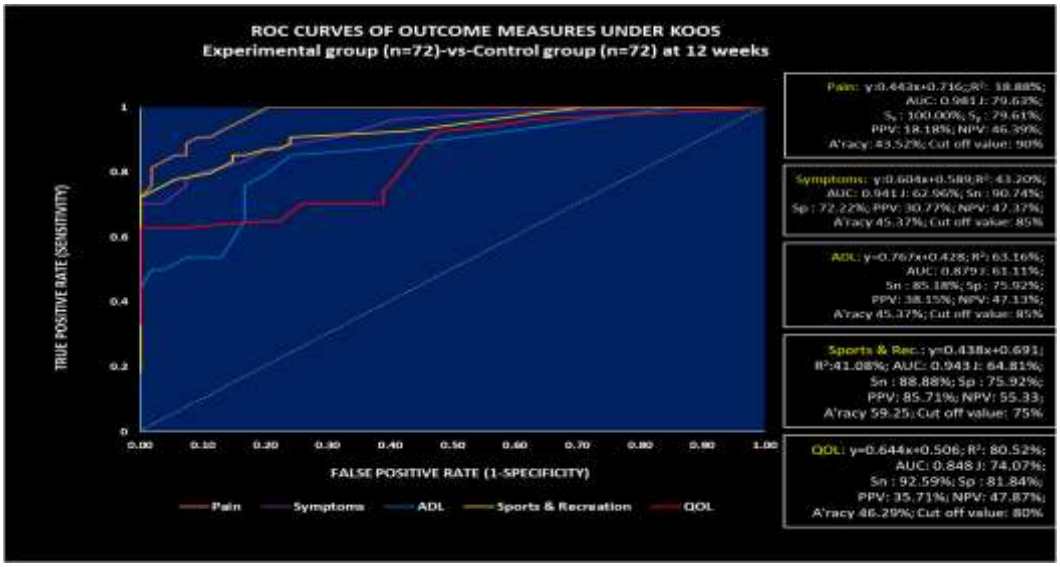


Figure 3B: ROC curves of outcome measures under KOOS of experimental group (n=72)-vs-control group (n=72) at 12 weeks

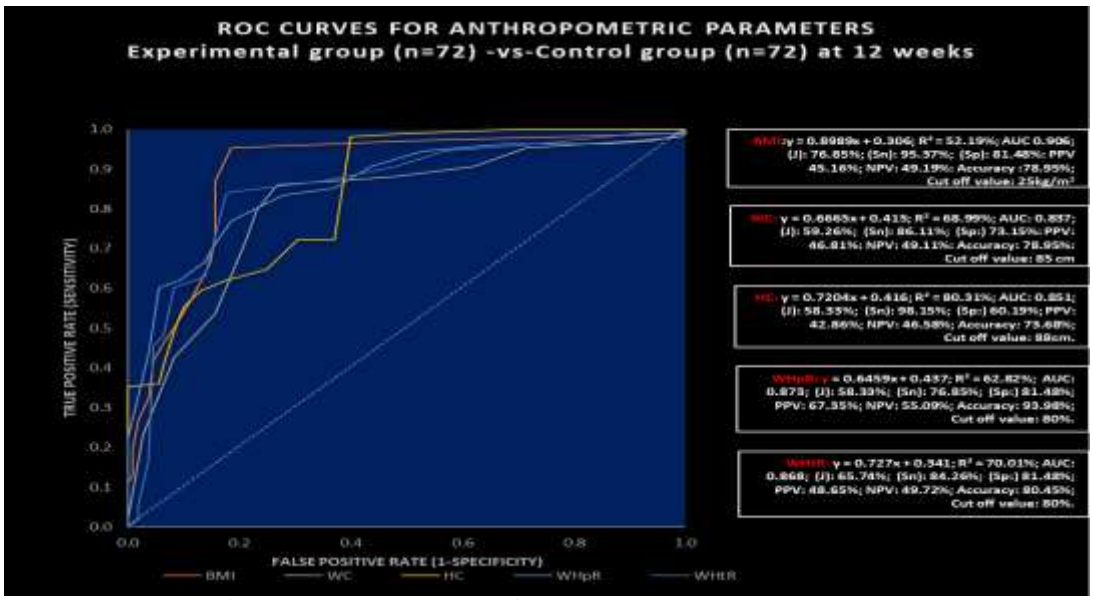
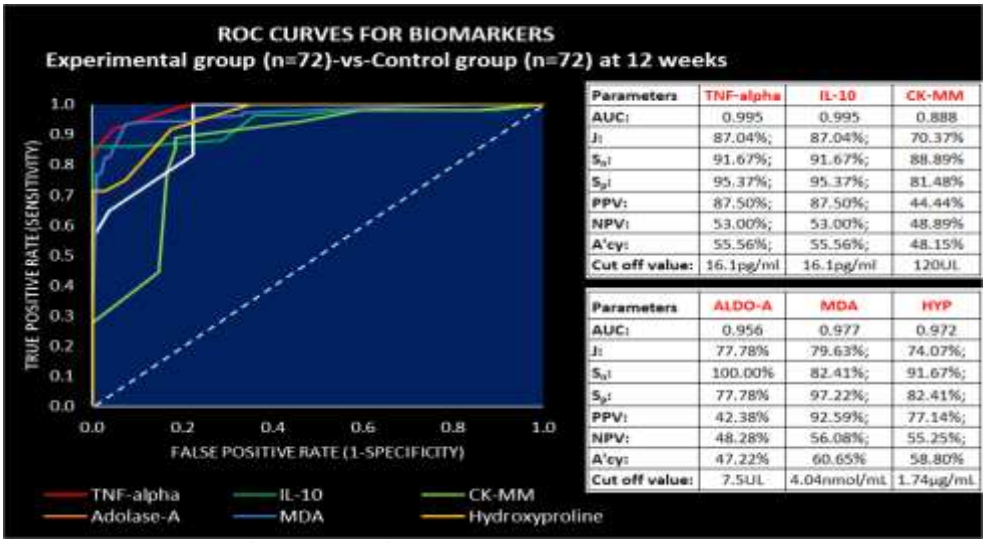


Figure 3C: ROC curves of Anthropometric Parameters of experimental group (n=72)-vs-control group (n=72) at 12 weeks



Parameters	TNF-alpha	IL-10	CK-MM
AUC:	0.995	0.995	0.888
J:	87.04%;	87.04%;	70.37%
S_n:	91.67%;	91.67%;	88.89%
S_p:	95.37%;	95.37%;	81.48%
PPV:	87.50%;	87.50%;	44.44%
NPV:	53.00%;	53.00%;	48.89%
A'cy:	55.56%;	55.56%;	48.15%
Cut off value:	16.1pg/ml	16.1pg/ml	120UL

Parameters	ALDO-A	MDA	HYP
AUC:	0.956	0.977	0.972
J:	77.78%	79.63%;	74.07%;
S_n:	100.00%	82.41%;	91.67%;
S_p:	77.78%	97.22%;	82.41%;
PPV:	42.38%	92.59%;	77.14%;
NPV:	48.28%	56.08%;	55.25%;
A'cy:	47.22%	60.65%	58.80%
Cut off value:	7.5UL	4.04nmol/ml	1.74µg/mL

Figure 3D: ROC curves of Biomarkers of experimental group (n=72)-vs-control group (n=72) at 12 weeks

(b) Figures 4A-4B show that the risk ratios for all the parameters and the heterogeneity (I-squared values exhibited between 81.25-90.45%) at the end of 12 weeks of understudied risk factors between TPT and NS groups were all highly significant ($p<0.0001$).

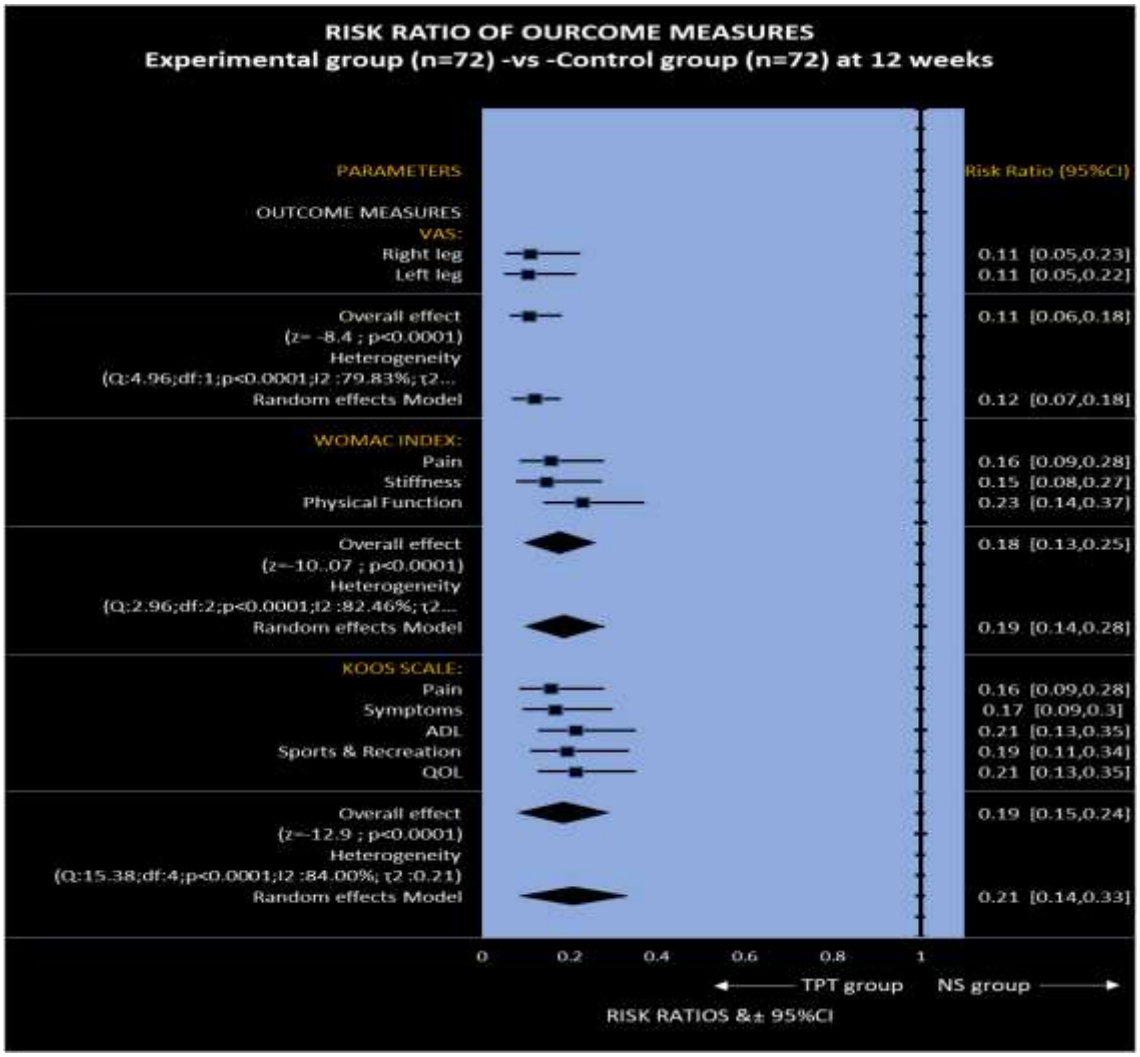


Figure 4A: Risk ratios of outcome measures of experimental group (n=72)-vs-control group (n=72) at 12 weeks

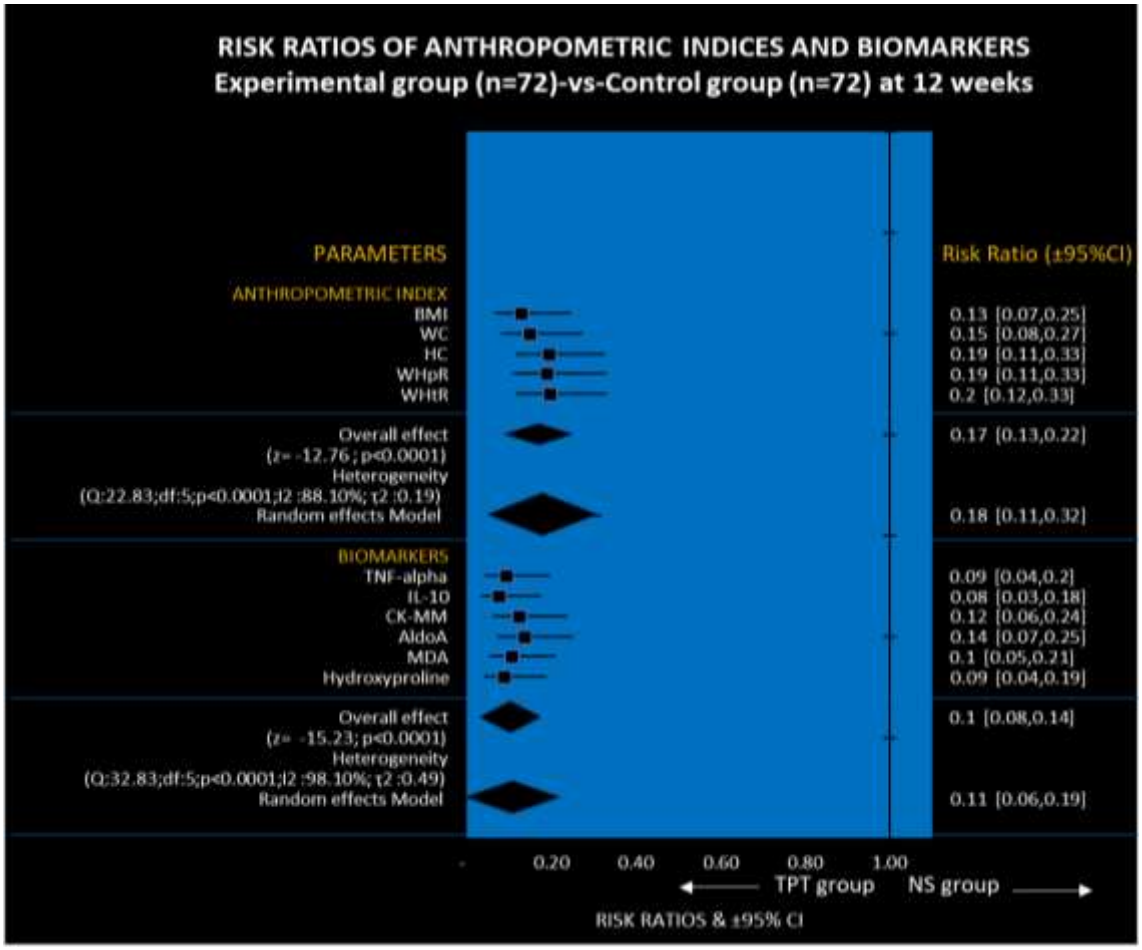


Figure 4B: Risk ratios of Anthropometric Indices and Biomarkers of experimental group (n=72)-vs-control group (n=72) at 12 weeks

(c) Figures 5A-5F show the significantly improved parameters of the studied risk factors such as COM, AP, and biomarkers (TNF-alpha, IL-10, MDA, CK-MM, aldolase A, and 4-hydroxyproline) for the experimental group compared to the control group

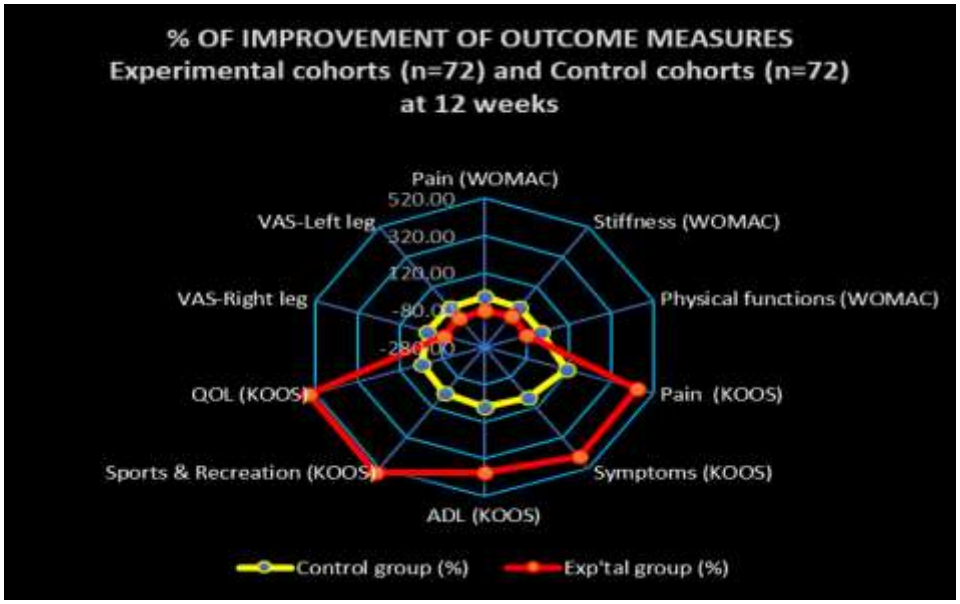


Figure 5A: Percentage of improvement of outcome measures for experimental cohorts (n=72) and control cohorts (n=72) at 12 weeks

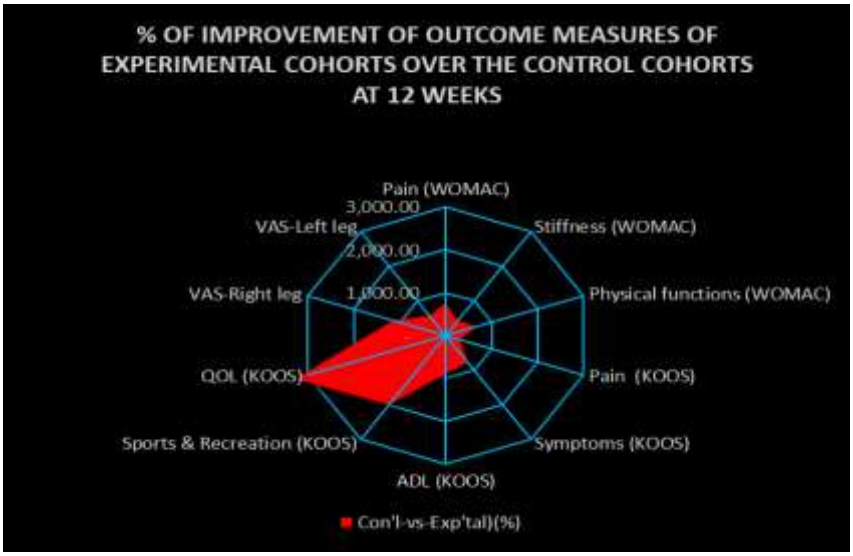


Figure 5B: Percentage of improvement of outcome measures for experimental cohorts over the control cohorts at 12 weeks

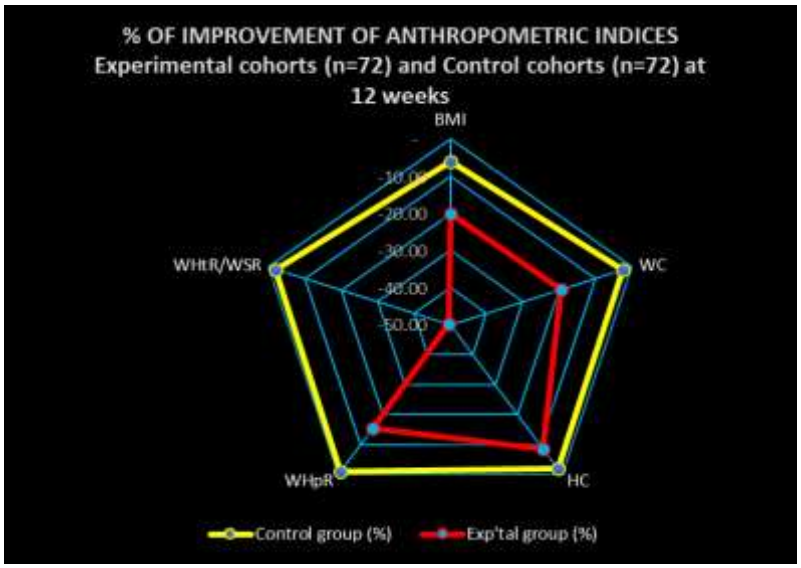


Figure 5C: Percentage of improvement of Anthropometric Indices for experimental cohorts (n=72) and control cohorts (n=72) at 12 weeks

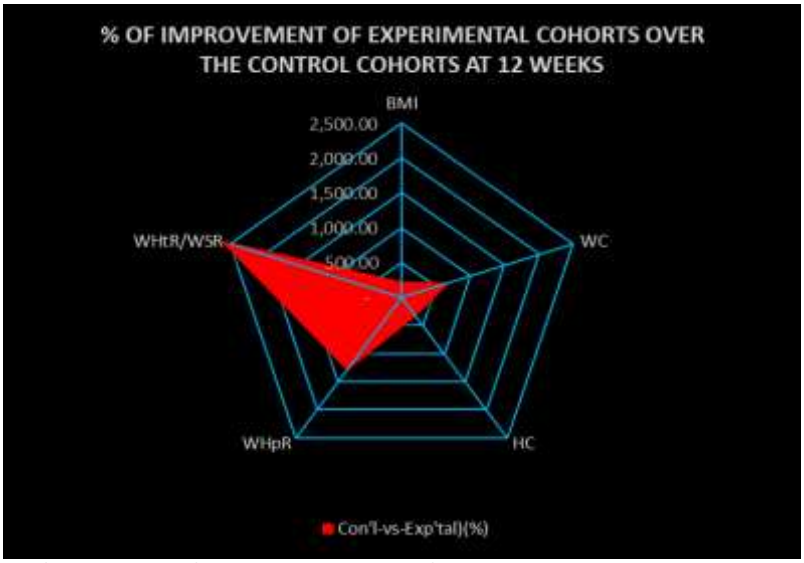


Figure 5D: Percentage of improvement of Anthropometric Indices for experimental cohorts over the control cohorts at 12 weeks

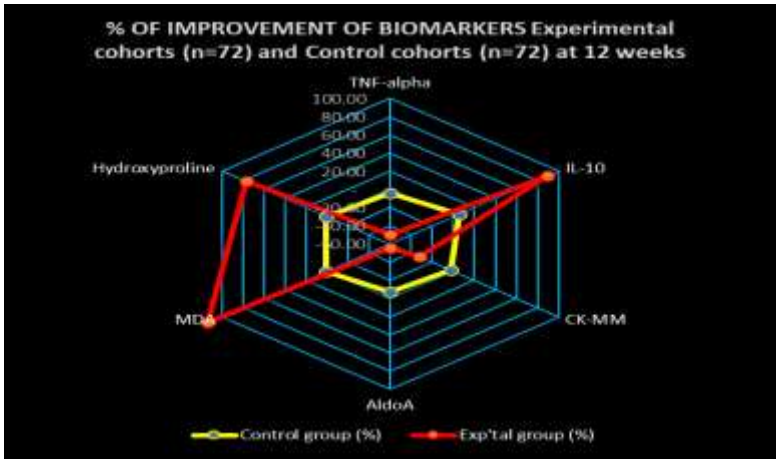


Figure 5E: Percentage of improvement of Biomarkers for experimental cohorts (n=72) and control cohorts (n=72) at 12 weeks

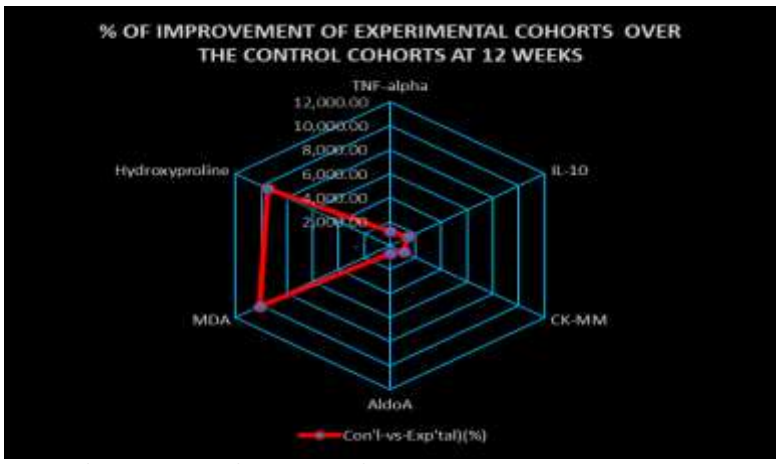


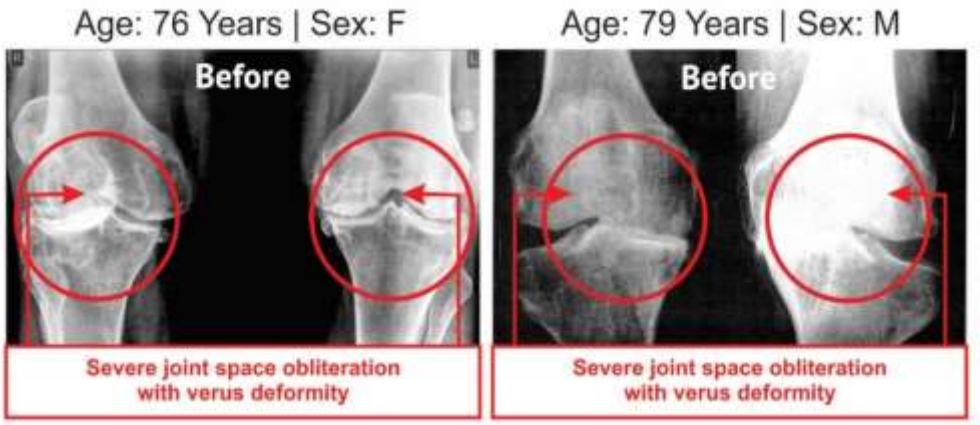
Figure 5F: Percentage of improvement of Biomarkers for experimental cohorts over the control cohorts at 12 weeks

3.3 Upgrading the bone health as per radiological images as assessed by the Kellgren-Lawrence scale:

Bilateral anterior-posterior (AP) views of the x-ray reports of 133 cohorts were used to identify tibiofemoral OA and patellofemoral OA. Table 1 shows the severity of tibiofemoral and patellofemoral conditions including the formation of osteophytes, narrowing of joint cartilage, sclerosis of subchondral bone, and bilateral varus/valgus deformities along with the percentages of deteriorations of grades using the Kellgren-Lawrence grading scale [8] of the experimental group and control group at baseline and 12-week. The AP views of knee joint x-ray images of two such cohorts of the experimental group are separately shown in Figures 6A-6B.

Table 1: Kellgren-Lawrence (KL) grading scale for knee osteoarthritis of experimental cohorts (n=72) and control cohorts (n=72) at weeks 0 and 12

KL Scale	Experimental group				Control group			
	Baseline		After 12-week		Baseline		After 12-week	
	Number	%	Number	%	Number	%	Number	%
	RIGHT KNEE JOINT							
Grade-0	None	0.00	2	2.78	None	0.00	None	0.00
Grade-1	None	0.00	28	38.89	None	0.00	None	0.00
Grade-2	None	0.00	36	50.00	None	0.00	None	0.00
Grade-3	31	43.06	5	6.94	27	37.50	28	38.89
Grade-4	41	56.94	1	1.39	45	62.50	44	61.11
	LEFT KNEE JOINT							
Grade-0	None	0.00	1	1.39	None	0.00	None	0.00
Grade-1	None	0.00	27	37.50	None	0.00	None	0.00
Grade-2	None	0.00	34	47.22	None	0.00	None	0.00
Grade-3	28	38.89	8	11.11	28	15.43	27	21.43
Grade-4	44	61.11	2	2.78	44	3.86	45	5.36



Figures: 6A: Showing the bilateral anterior-posterior (AP) views of the x-ray of two experimental cohorts.

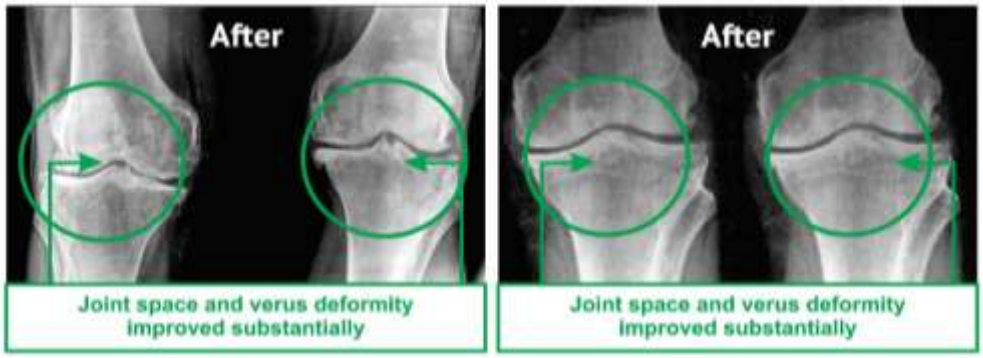


Figure 6B: Showing the bilateral anterior-posterior (AP) views of the x-ray of two experimental cohorts after the topical phytotherapy

4. DISCUSSION

The findings suggest that topical phytotherapy for a twelve-week duration may elevate 4-hydroxyproline concentrations in cartilage in individuals with OA, indicating a potential mechanism of action. This improvement is significantly associated with various risk factors, such as abnormal international pain-related clinical outcome measures, anomalous anthropometric measurements, aberrant biochemical markers of skeletal muscle metabolism dysfunction, joint mobility, cartilage, and bone health, as well as abnormal KL grading scales. To the best of our knowledge, OA has not yet been treated using this protocol.

The role of 4-Hyp in knee osteoarthritis (OA) is not fully understood, but it may involve activating the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway, which regulates the antioxidant response in cells. [24]. Additionally, the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway plays a significant role in regulating collagen synthesis and degradation in knee OA. Activation of the MAPK/ERK pathway can increase collagen production by chondrocytes while also increasing the activity of matrix metalloproteinases (MMPs), which break down collagen and other extracellular matrix components. Inhibiting the MAPK/ERK pathway may be a potential therapeutic target for preventing or treating knee OA [25].

As discussed earlier, collagen is a protein that contains 4- hydroxyproline, sometimes known as hydroxyproline. Proline undergoes hydroxylation to form hydroxyproline, which is a post-translationally changed amino acid. Proline and hydroxyproline are important building blocks of collagen stability. The schematic mechanism is shown to indicate proline hydroxylation at proline to form 4-

hydroxyproline in collagen stability in our body in Figure 7 [26].

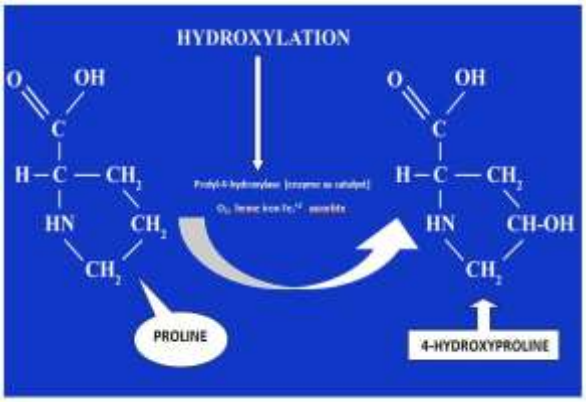


Figure 7: The schematic mechanism is shown to indicate proline hydroxylation at proline to form 4-hydroxyproline in collagen stability

***Evaluation of phytotherapeutic Mechanisms*:** The study suggests using topical phytotherapy for twelve weeks, derived from nine Indian medicinal plants, including *Cissus quadrangularis* (whole plant), *Calotropis gigantea* (root and leaves), *Zingiber officinale* (rhizome), *Rosemarinus officinalis* (leaves and flowers), *Boswellia serrata* (resin), *Curcuma longa* (rhizome), *Capsicum annuum* (fruit), *Mentha arvensis* (whole plant), and *Withania somnifera* (root), to revive collagen synthesis and degradation in knee osteoarthritis (OA). Sesame oil and castor oils, and beeswax are combined with the phytoconstituents to produce a viscous oil free from preservatives or additives [20].

The plants used in the therapy have specific mechanisms of action that eliminate risk factors in OA. For example, curcumin extract from turmeric has anti-inflammatory properties, similar to non-steroidal anti-inflammatories, that affect the signaling of pro-inflammatory cytokines, reduce the breakdown of collagen and proteoglycans, and have properties of mitigating systemic oxidative stress [27-28]. Boswellia serrata contains 5-Loxin and Aflapin® (a novel synergistic composition), which reduce the enzymatic degradation of cartilage with the production of 4- hydroxyproline and pro-inflammatory modulators for controlling inflammatory responses [11]. The extracts from Calotropis gigantea stimulate the production of 4-hydroxyproline in human cartilage cells with active compounds like glycosides and alkaloids [29]. Cissus quadrangularis is cast-off to treat obesity, metabolic syndrome, and obesity-induced oxidative stress. It increases the generation of 4-hydroxyproline, which aids in collagen synthesis [30]. While Withania somnifera, ginger, chili pepper, and wild mint promote collagen synthesis and have analgesic and anti-inflammatory properties, these plants may help to support joint health and increase collagen synthesis in KOA patients [31-34].

Furthermore, it should be noted that the current treatment plan is based on well-established principles and theories as well as the application of well-known chemical, mechanical, and thermal stimuli that enhance the basic qualities of all muscles, including excitability, conductivity, contractibility, elasticity, and viscosity. When the prepared oil is applied topically, strong mechanotransduction is created, which has phytochemicals' synergistic effects [35].

Mechanotransduction, the process by which mechanical stimuli are converted into electrochemical signals, is essential for various biological processes, including neuronal cell development, pain sensation, and red blood cell volume regulation. The said prepared oil penetrates through the deepest layers of the skin through a diffusion process and ends up in the bloodstream which helps: to promote the dissolution of thrombi (fibrinolysin); to prevent or to retard blood from clotting (anticoagulant); to cause body tissues to contract/tighten (astringent); to cause contraction of blood vessel walls (vasoconstrictor); to reduce swellings (resolvent); to reduce inflammation (antiphlogistic); to protect calcium-phosphorous nano-crystallization, to encourage the growth of new cells (cytophylactic); to promote agents that soften, loosen, and facilitate exfoliation of squamous cells of the epidermis

(keratolytic) and also to activate the antipruritic properties; to prevent tissue degeneration (vulnerary); and to safeguard the body from destructive free radicals. Moreover, the author has elaborated on the biochemical mechanism of action and disease prevention of each medicinal plant species used in the therapy for KOA patients in the previous study [19,20,22].

***International accepted pain-related Parameters*:** Patients with musculoskeletal pain, particularly osteoarthritis, experience pain frequently, which enormously distresses their quality of life. Insufficient blood flow, inflammation, stiffness, deterioration of the connective tissues, compression between bones, and physical or mental impairment all contribute to the pain of OA. Previous studies have suggested that the Knee-Injury and Osteoarthritis Outcome Score (KOOS) is a more accurate analysis tool than the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) for patients with OA [19-20].

In the present study, both scales, as well as the Visual Analogue Scale (VAS) and Lower Extremity Functional Scale (LEFS), were used to evaluate patients, which showed highly significant (p<0.0001) for the experimental cohorts compared to the control subjects (Figures 3A–3B, 4A, 5A–5B). As a result, our findings suggest that the TPT treatment is more likely to be effective than the NS treatment.

Moreover, there is an assessment of the indicators of inflammation, obesity, and oxidative stress. According to the study, there is a strong link between obesity (both general and central) and psychosocial factors such as anxiety, stress, depression, body dissatisfaction, binge eating disorder, and a diminished quality of life, including disability. According to the study by Zhang Y et al., the findings also showed that older women of the Indian Malware ethnic group in Kolkata had a higher frequency of knee OA than Punjabi women in Delhi **[36].** The regulation of active biomarkers eventually addressed in the treatment under TPT is shown in the diagram as a summary of the hypothesized obesity-related mechanisms underlying KOA [Figure 8].

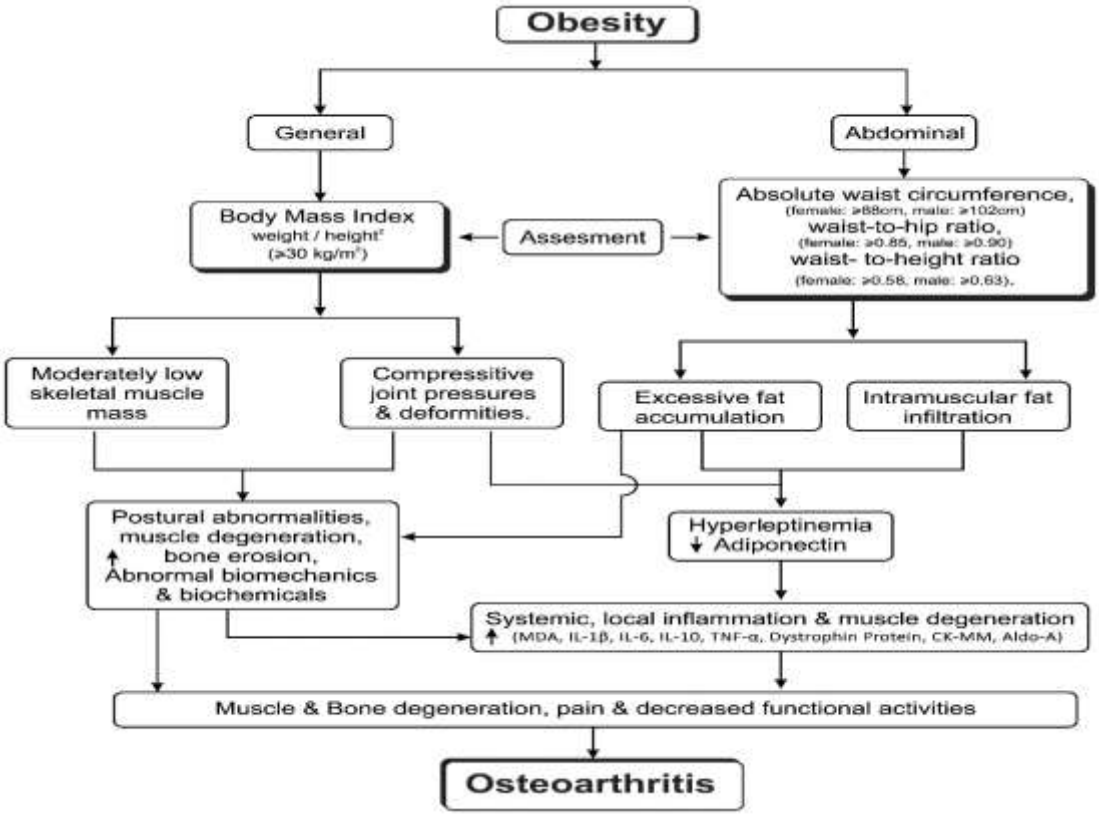


Figure 8: Showing the summaries of the proposed obesity-related mechanisms are underlying KOA

In this contract, it is possible to vouch favourably for the TPT treatment's improvement over the NS treatment in terms of anthropometric indices.

***Evaluation of biochemical parameters*:**
Researchers have established a close association between skeletal metabolic dysfunction and joint mobilization, subchondral bone remodeling factors, and various risk factors such as oxidative stress, inflammation, muscle degeneration, and cartilage-specific protein damage in KOA. The study evaluated biomarkers of inflammatory factors, oxidative stress, muscle degenerative factors, and collagen protein breakdown using ROC curves, meta-analysis, and radar graphs. Finally, the study established a synergistic relationship between oxidative stress and inflammation (MDA, TNF-α, and IL-10), skeletal muscle degenerative and bone erosion factors (CK-MM and Aldolase-A), which mediate several chronic diseases of the muscular and neurological etiologies, especially OA, and collagen protein breakdown factor (4-hydroxyproline) for evaluation of breakdown of the extracellular matrix and the loss of articular cartilage in OA of experimental cohorts compared to the controls at 12 weeks were highly significant ($p<0.0001$) [Figures 3D, 4B, 5E-5F].

Analysis of ROC curves: While the ROC curves have been drawn from the parameters of all the risk factors, such as outcome measures, anthropometric indices, and biomarkers of the cohorts of the TPT group (n=72)-vs- NS group (n=72) with the help of the true positive rate (sensitivity) and false positive rate (1-specificity) at 12 weeks, the respective areas under the ROC curves (AUCs) lie between 0.9 and 0.8, which is considered to be the excellent performance of the protocol used in the treatment for OA. The results further indicate that all the parameters of the risk factors have been significantly improved ($p<0.0001$) by the studied protocol. After the application of the present treatment protocol, the cut-off values of all the measurements of the risk factors are individually shown to be within the normal limits, which indicates a satisfactory treatment policy [Figure 3A-3D]

***Analysis of heterogeneity in the forest plot*:**
With the binary data of the TPT group (n=72) and NS group (n=72), the ratio between risks (risk ratios) and their 95% confidence interval were calculated from the studied parameters and presented graphically and numerically [Figure 4A-4B]. Each line in the graphical display represents a study of each parameter of the study groups, such as outcome measures, anthropometric indices, and biochemical results. The line on the left side of the box represents the 95% lower confidence interval limit, and that on the right side represents the 95% upper confidence limit. Each forest plot contains a vertical line known as “no effect,” which corresponds to the value 1 for the risk ratio on the x-axis. In the present study, as all the boxes (risk ratios) lie on the left side of the line of "no effect", they indicate all the results of outcome measures, anthropometric indices, and biomarkers that indicate the substantial improvement in bone and muscle health have been substantially improved after the studied treatment protocol (i.e., favorable to the post-treatment under TPT). As none of the horizontal lines (±95%CI) is touching the line of "no effect”, therefore, it is firmly confirmed that all the results of the studied parameters for each group are highly significant ($p<0.0001$). In the present study, the technique of meta-analyses has been used to establish an unbiased study design and consistency of effects across studies. Additionally, it is said that values of I-squared in a meta-analysis range from 84.46% to 98.10%, indicating a high degree of heterogeneity among the studies included in the analysis. Hence, it might be emphasized that the reported differences in effect sizes (risk ratios) between studies reflect actual variances in the study populations, interventions, or outcomes

rather than merely variations due to chance. In light of this, compared to the treatment of NS, the study of forest plots would have been seen as an effective treatment strategy under the TPT treatment protocol.

***Evaluation of comparative improvements with the help of radar charts*:** One of the strengths of radar charts is their ability to show patterns and relationships in multivariate data, especially when comparing multiple variables across different categories. They can be particularly useful in decision-making processes, such as assessing performance or identifying areas for improvement, where it is important to understand the relative strengths and weaknesses of different factors. The present study of the analyses of radar charts may be suggested that the improvement of risk factors for KOA is more reliable and visible so far as the treatment under TPT. According to the current study's analysis of radar charts, it may be inferred that the therapy under TPT is more effective at reducing KOA risk factors while also being more observable [Figures 5A-5F].

Effectiveness of radiographic images*: The overall improvement of bone health as per radiological images, especially in the* tibiofemoral and patellofemoral joints, formation of osteophytes, narrowing of joint space, cartilage degradation, sclerosis of subchondral bone, and varus/valgus deformities *evaluated by the Kellgren-Lawrence scale at 12 weeks in two such experimental cohorts treated with TPT compared to the control cohorts treated with NS, are shown in Figures 6A and 6B. This phenomenon may indicate that the treatment with TPT has a higher potential and effectiveness of 4-hydroxyproline for patients with OA than the NS treatment.*

Limitation of the study: Unfortunately, there are several of important limitations to this study. First of all, a small sample size raises the possibility of biased results. We don't know if the therapy would have had the same efficacy and safety over a longer period because it was only evaluated for a short period (around 3 months). Second, x-ray scans were used to determine KOA's evaluation. Magnetic resonance imaging is still necessary despite the improved resolution for more accurate measures of cartilage disease. Meniscal subluxation is a risk factor for cartilage loss and joint space constriction in persons with symptomatic KOA, and there is conflicting evidence that links it to pain. Thirdly, patients are prohibited from receiving treatment if they have any of the following conditions: concurrent illnesses requiring simultaneous multidrug therapy; a history of cancer, including carcinomatosis and granulocytic leukaemia; a history of chronic liver, heart, or kidney diseases; patients who refuse to undergo x-rays, blood tests, a physical examination, and/or attend weekly follow-up appointments; patients with dementia; morbid obesity; pregnancy; previous knee surgery.

5. CONCLUSION

Based on the findings, it appears that TPT treatment, as opposed to TNS, which is linked to the KLS, may have a greater ability to promote cartilage production by controlling 4-hydroxyproline and reduce inflammation, oxidative stress, pain, and joint stiffness in OA patients.

The examination of cartilage oligomeric matrix protein (COMP) and crosslinked C-telopeptides of type II collagen (CTX-II), two biomarkers that may be able to predict the degeneration of articular cartilage in early OA, calls for more research on the biomarkers.

Declaration by Authors

Ethical Approval: Approved

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Conflict of Interest: The author declares that there are no conflicts of interest regarding the present study.

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