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Citrullus colocynthis loaded zinc nanoparticle (C.cZnO): A potential gout healer in modulating antioxidant status and lysosomal enzymes in MSU crystal Induced PBMC cells

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Abstract---The aim of the present investigation is to study the effect of *C.colocynthis* loaded zinc nanoparticle (C.cZnO) on the antioxidant status and lysosomal enzymes in PBMC cells induced with monosodium urate crystals. The Zinc nano particles of *C.colocynthis* was prepared. Approximately 1×10^6 cells/well seeded human PBMC were divided in to five groups and treated in the following manner. Group I served as control, Group II cells were treated with 1mg/ml MSU crystals, Group III and Group IV cells were induced with MSU crystals and received 250 μ g/ml and 500 μ g/ml of *C. colosynthis* zinc nanoparticle (C.cZno) respectively and were incubated at 37°C for 24 hrs in the CO₂ incubator. Group-IV cells were treated with colchicine (1 μ g/ml) the positive control used in the present study. The enzymatic and non enzymatic antioxidants as well as lysosomal enzymes were analysed using the established procedures. The extent of lipid peroxidation was also measured. It is observed that there is a significant decrease in the level of the superoxide dismutase, catalase, glutathione S transferase and glutathione peroxidase and non-enzymatic antioxidant activities such as GSH, Vitamin C, Vitamin E and Vitamin A as well as an increase in the level of antioxidant enzyme glutathione reductase and lysosomal enzymes in MSU treated group II lymphocytes when comparable (P<0.01) to control group of

lymphocytes. C.cZnO treatment corrected the imbalance in these enzymes in MSU treated lymphocytes. It can be concluded that by improving the antioxidant status the *Citrullus Colocynthis* stem extract loaded zinc nanoparticle can be used to reduce the oxidative stress induced by MSU crystals.

Keywords---antioxidant enzymes, *Citrullus Colocynthis*, zinc oxide nanoparticles.

Introduction

Nano particles as the name implies are usually smaller than 100 nm in size are employed in a range of applications such as medical products, pharmaceutical, industrial, chemical, and electronics industries (Rafique, et al., 2020). These small particles are generated by green chemistry method and is regarded to be more efficient than the others due to its low cost and lack of hazardous ingredients (Gur, et al., 2020). Zinc oxide nanoparticle are being routinely synthesized using microbes, plant extract, sponge-related extract, and terrestrial microorganisms because they are naturally renewable, cheap, and beneficial in a range of applications including anti-oxidant, anti-malarial, anti-bacterial, anti-cancer, anti-viral drugs (Mary, et al., 2022). Plant-mediated zinc oxide nano particles are considered as an effective nanoparticle due to their high absorption capacity and makes use of important chemical components found in plants (Sharma, et al., 2020).

Gouty arthritis is the most painful type of arthritis, caused by an inflammatory reaction induced by the deposition of uric acid crystals due to hyperuricemia in joints and tissue, resulting in acute inflammation and intense pain (Zhang, et al., 2022). According to epidemiological research, gouty arthritis affects 5% of the population in developed countries, with males and postmenopausal women being the most affected (Ferreira, et al., 2022). To treat acute gouty arthritis, oral colchicine, nonsteroidal anti-inflammatory drugs (NSAIDs), and glucocorticoids are commonly utilized. Despite significant advances in knowledge and potential therapeutic breakthroughs, gout treatment remains inadequate due to unfavorable side effects such as gastrointestinal toxicity, bleeding, diarrhoea, and cardiovascular events (Yaqub, et al., 2022).

The use of plant-derived medications used to treat arthritis has recently resurfaced as a topic of discussion. *Citrullus Colocynthis* L belongs to the family Cucurbitaceae is been grown in Asian regions is considered as a traditional medicine for the treatment of inflammation, diabetes, cancer and bacterial infections (Moncef Chouaibi, et al., 2020). *C.colocynthis* is a strong free radical scavenger and active antioxidant to treat oxidative stress-related diseases. *C.colocynthis* stem-based foods and juices, include flavonoids as secondary metabolites which have the capacity to combat oxidative stress.(Sheng , et al., 2022). With this above context the present investigation is planned to study the ameliorative effect of *C.colocynthis* loaded zinc nanoparticle (C.cZnO) on the antioxidant status and lysosomal enzymes in PMC cells induced with monosodium urate crystals.

Materials and Methods

Collection and Preparation of Stem Extract and Synthesis of Zinc Oxide Nanoparticles (C.cZnO)

The stem of *Citrus colocynthis* were obtained from Madurai. The stem was cleaned 2-3 times in distilled water and dried in the shade for 7–15 days. The shade-dried stem was being ground into a fine powder and was used for the preparation of nano particles. The *C. colocynthis* stem extract loaded Zinc oxide nanoparticles were prepared according to the method of (Saka A, et al., 2022). One gram of *C. colocynthis* stem powder was weighed accurately and boiled in 100 ml distilled water for 15 minutes. After that the extract was filtered through Whatman No. 1 filter paper and 10 mL of filtrate was added to 100mL of 0.594mM Zinc nitrate solution. Then the reaction mixture kept on magnetic stirrer at 30°C for 4h. Zinc Oxide nanoparticles pellets reinforced with *C.colocynthis* stem were obtained after centrifugation.

Monosodium Urate crystals Synthesis

MSU crystals were prepared according to Seegmiller *et al.*, 1962. 24 g of sodium hydroxide was solubilized in 200 ml of pyrogen free double distilled water. 1 g of uric acid was added into the above solution and the pH was adjusted to 7.2 using 1N HCl. This solution was heated to 120°C for 6-hrs with gentle stirring. After incubation, the solution was left to cool at room temperature and kept in 4°C for overnight. The sedimented MSU crystals were collected by filtration and washed with absolute ethanol. Washed MSU crystals were dried under vacuum and sterilized by autoclaving at 121°C for 20 min. The sterilized crystals were prepared as 20mg/ml stock in PBS and used for assay.

Human PBMC Isolation and treatment protocol for Antioxidant and lysosomal enzyme assays

About 10 ml of Blood was collected from the healthy volunteer in EDTA containing tube and mixed well. The PBMC was washed by 1X PBS and centrifuged at 1300 rpm for 10 minutes at room temperature. The washing step was repeated and the PBMC pellet was washed again with plain RPMI 1640 medium and repeated once again. After the final wash, the PBMC pellet was resuspended in RPMI-1640 containing 10% FBS. A quantity of 1×10^6 cells/well of human PBMC were plated in 400µl volume on 24-well tissue culture plate and incubated for 30 min before compound treatment at 37°C under a humidified atmosphere of 95% air and 5% CO₂. The cells were divided into groups and received the following treatment. Group -I control cells which received 100 µl of complete RPMI-1640 medium. Group II cells served as inducer which received MSU crystals at the concentration of 1mg/ml. Group III and Group IV cells received the MSU crystals along with 250 µg/ml and 500 µg/ml of *Citrus colocynthis* zinc nanoparticle (C.cZnO) respectively. Group-IV cells were treated with colchicine (1µg/ml) the positive control used in the present study. All the treated cells were incubated at 37°C for 20 hrs. The treated cells were trypsinised using Trypsin-EDTA Solution and centrifuged for five minutes at 3000rpm to collect the pellet. By using 200µl of lysis buffer (0.1M tris, 0.2M EDTA, 2M NaCl, 0.5% Triton) the

collected pellet was re suspended and further incubated for 20 mins at 4°C and was used for the analysis of enzymatic and non enzymatic antioxidants . In the cell-free supernatant, the activity of lysosomal enzymes was measured

Estimation of Antioxidant status

The enzymatic antioxidants Superoxide dismutase (Misra and Fridovich 1972), Catalase (Sinha, 1972), Glutathione reductase (Dobler and Anderson 1981), Glutathione-S-transferase (Habig, et al.,1974) and Glutathione peroxidase (Necheles et al., 1968)were estimated in the cell lysate. The levels of non-enzymatic antioxidants GSH,(Sedlak, and Lindsay,1968,) ascorbic acid (Omaye et al., 1969) and α Tocopherol (Desai, 1984,) were also estimated. The LPO concentration in cell lysate was quantified using the (Bacon K et al., 2021) technique using thiobarbituric acid. All the enzymatic and non-enzymatic assays were read using Shimadzu spectrophotometer, UV-1601 model.

Determination of Lysosomal Enzymes

In the cell-free Supernatant, the activity of lysosomal enzymes was measured. The activity of acid phosphatase was measured according to the method of Sivasangari et al., 2021 with the application of disodium phenyl phosphate as the substrate. The assay of β -galactosidase was performed using the Tkachenko et al., 2021 technique. The N-acetyl glucosaminidase enzyme activity was performed by the method of Hong X, et al., 2021. The Cathepsin D activity was measured using Arunkumar et al.,2021 method.

Statistical analysis

The statistical significance was carried out using analysis of variance (ANOVA) followed by Dunnet's 't' test. Values reported are Mean \pm SD. The p values of < 0.05 were considered as significant.

Results

The effect of the stem of *C.cZno* nanoparticle on antioxidant enzymes such as catalase, superoxide dismutase ,Glutathione S transferase ,Glutathione Peroxidase and Glutathione reductase in monosodium crystal induced status were depicted in Table-1.It is observed that there is a significant decrease in the level of the superoxide dismutase, catalase ,glutathione S transferase and glutathione peroxidase as well as an increase in the level of glutathione reductase antioxidant enzymes in MSU treated group II lymphocytes when comparable ($P < 0.01$) to control group of lymphocytes which indicates the presence of oxidative stress. The MSU induced and *C.colosynthis* zinc nanoparticle treated group III and group IV cells exhibited a reversion to normalcy in these antioxidant enzymes indicating antioxidant enzymes promoting action of the drug.

Table-1 Effect Of C.cZno stem On Enzymatic Antioxidant Activity of different experimental groups

Parameter	Control	MSU 1mg/ml	MSU+CcZnO 250µg/ml	MSU+CcZnO 500 µg/ml	MSU+ Colchicine 1µg/ml
CAT	5.23±0.25a**	2.9±0.02	4.64±0.07 b*	5.12±0.12 c **	5.19±0.04 d**
SOD	3.72±0.08 a**	1.18±0.05	2.20±0.06b*	2.73±0.01c*	3.11±0.02d**
GST	1.82±0.03 a**	0.43±0.04	1.20±0.09b**	1.22±0.08c**	1.40±0.02d**
GPx	2.57±0.65 a**	1.12±0.0	1.66±0.4b*	1.71±0.05c**	2.667±0.07d**
GR	2.74 ±0.24 a**	4.13±0.48	3.82±0.34b*	3.79±0.04c*	3.77±0.03d*
LPO	2.21±0.03 a**	5.25±0.09	4.90±0.2b*	4.45±04 c*	4.1±0.02d*

Values are mean \pm SEM of of three parallel measurements in each group Statistical significant test for comparison was done by ANOVA followed by Dunnet's "t" test. Comparison between a-Group I vs Group II, b-Group II vs Group III, c-Group II vs Group IV and d-Group II vs Group V. P values---- *p<0.05, * *p<0.01 and NS-Not Significant

Units

SOD -units/mg protein

CAT -µmoles of H₂O₂ decomposed/min /mg protein

GR -µmoles of NADPH oxidized /min/mg protein

GPx -µg of glutathione utilized/min/mg protein

GST -µmoles of CDNB conjugate formed/ min/ mg protein

Table-2 Effect Of C.cZno stem On Non-Enzymatic Antioxidant Activity of different experimental groups

Parameter	Control	MSU 1mg/ml	MSU+CcZnO 250µg/ml	MSU+CcZnO 500 µg/ml	MSU+ Colchicine 1µg/ml
Glutathione (µmoles of GSH/ mg of protein)	0.25±0.30 a**	0.065±0.02	0.16±0.03 b**	0.18±0.04 c**	0.18±0.06 d**
Vitamin C (µg/ml)	6.58±1.23 a**	1.69±0.90	4.16±0.24b**	4.23±0.34 c**	4.45±0.66c**
Vitamin E (µg/ml)	7.10±1.23a* *	1.67±0.24	3.13±1.15b**	3.59±0.34c**	4.74±0.23d**
Vitamin A (µg/ml)	7.33± 1.73a**	1.10±0.55	3.18 ±1.38b*	3.88 ± 0.16c**	5.13 ± 0.52d**

Values are mean \pm SEM of of three parallel measurements in each group Statistical significant test for comparison was done by ANOVA followed by Dunnet's "t" test. Comparison between a-Group I vs Group II, b-Group II vs Group III, c-Group II vs Group IV and d-Group II vs Group V. P values---- *p<0.05, * *p<0.01 and NS-Not Significant.

The effect of stem of C.cZno nanoparticle on non-enzymatic antioxidant activities such as GSH, Vitamin C (ascorbic acid), Vitamin E (Tocopherol), Vitamin A were shown in Table – 2. It is been noted that there is a drastic decrease in the non-enzymatic antioxidant level in MSU induced group II lymphocytes comparable P(< 0.01) to control group which clearly indicates the status of oxidative stress. The MSU induced and C.cZno nanoparticle treated group III and IV cells exhibited an increase in these non-enzymatic antioxidant, indicates the active antioxidant property of the nanoparticle.

Table-3 Effect Of C.cZno stem On lysosomal Activity of different experimental groups

Parameter	Control	MSU 1mg/ml	MSU+CcZnO 250µg/ml	MSU+CcZn O 500 µg/ml	MSU+ Colchicine 1µg/ml
Acid Phosphatase	4.12±0.3 a**	6.43 ±0.04	4.24±0.03b*	5.14±0.03c*	5.4±0.12d*
β-galactosidase	2.26±0.02 a**	4.64 ±0.02	2.54±0.02 b**	2.20±0.21c* *	1.20±0.11d**
N-acetyl-β-D glucosaminidase	4.67 ±0.04 a**	8.40 ±0.02	4.11±0.02b*	3.03±0.18c* *	4.41±0.13d**
Cathepsin D	3.85 ±0.02 a**	8.29 ±0.04	6.26±0.02 b*	6.45±0.09c*	5.30±0.10d*

Values are mean ± SEM of of three parallel measurements in each group Statistical significant test for comparison was done by ANOVA followed by Dunnet's "t" test. Comparison between a-Group I vs Group II, b-Group II vs Group III, c-Group II vs Group IV and d-Group II vs Group V. P values---- *p<0.05, **p<0.01 and NS-Not Significant

Units

Acid Phosphatase - µmoles of phenol released per minute per milligram of protein
 β-galactosidase - moles of p-nitrophenol liberated/h/ mg protein
 N-acetyl-β-D glucosaminidase - moles of p-nitrophenol formed/h/mg of protein
 Cathepsin D - moles of tyrosine liberated/h/mg of protein

The assessment on the release of lysosomal enzymes due to the MSU as well as stem of C.cZno nanoparticle drug treatment was performed in the cell free supernatant and it was shown in the Table -3. There is a significant (p<0.01) rise in the lysosomal enzymes in the MSU treated group II cells when comparable to control cells due to the increased degradative activity. The C.cZno treated group III and group IV lymphocytic cells showed a decrease in the concentration of these enzymes indicating the protective nature of the drug against the deleterious effects of MSU.

Discussion

Gout is a chronic disorder characterized by the deposition of monosodium urate crystals in many joint tissues in particular the metatarsophalangeal joints causing inflammation and severe pain leading to functional disability (Tang, 2018). In the effective management gouty arthritis many drugs are commonly used which includes xanthine oxidase inhibitors, cyclooxygenase inhibitors, uricosuric agents, anti-inflammatory drugs and steroids. Though these drugs are potential in reducing the arthritic attacks they cause gastrointestinal disturbances, renal and hepatic toxicity as well as hypersensitive reactions (Mehmood et al., 2019). So there is always there is a need for a plant based alternative therapy which is easily available with cost effective nature and should produce less side effects.

According to Kim et al., the deposition of MSU crystals seems to be the triggering factor in generating the reactive oxygen species (ROS) and NLRP3. Besides that the activities of xanthine oxidase and NADPH oxidase produce superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) radicals which in turn promotes the degradation of connective tissues leading to joint damage.

Free radicals act as an intracellular second messenger by targeting cysteine and methionine residues on downstream transcription factors such as NF- κ B (Karakurum et al., 2021). Several cell types, particularly macrophages and neutrophils, have been shown to directly generate free radicals during inflammation, which can contribute to lipid peroxidation and degrade antioxidant status via oxidative stress and the generation of reactive oxygen species (Acioğlu et al., 2020). Since the release of free radicals is necessary for the maintenance of tissue injury and inflammation, reducing the release of these free radicals would be beneficial in the treatment of acute gouty arthritis. C.cZnO therapy resulted in a significant decrease in intracellular free radicals, by increasing the concentrations of enzymatic and non enzymatic antioxidants as well as decreasing the lipid peroxidation in MSU crystals, stimulated lymphocyte cells. Marzouk et al., 2009 have studied the analgesic and anti-inflammatory activity of the seed extract and reported the presence of alkaloids, iridoids, flavonoids, steroids in the extract are responsible for the above said activity. According to Kumar et al., 2008 the presence of three flavonoids namely isosaponarin, isovitexin and isoorientin 3-O-methyl ether, contribute significantly towards its free radical scavenging property.

The MSU crystal stimulation causes lysosomal membrane rupture and the release of lysosomal enzymes. These enzymes are capable of degrading proteins, glycosaminoglycans, nucleic acids, and lipids. It is observed that any change in the lysosomal enzyme activity will alter the phagocytic activity especially altering the extracellular matrix turnover and remodeling suggesting their involvement in gouty arthritis. Ghosh, et al., 2011 has reported that oxidative stress is the primary cause of apoptosis or necrosis mediated tissue injury and inflammation due to the early lysosomal rupture and extracellular release of lysosomal enzymes. In this context reduction in the release of lysosomal enzymes is considered as a preventive agent in the management of tissue injury and inflammation. In the present investigation the *C. Colosynthis* stem extract loaded

zinc nanoparticles considerably reduced the lysosomal release indicating the phyto chemicals present in the extract has the potential to keep the lysosomal membrane intact from the MSU crystal damage thereby prevent the tissue injury. *Citrullus colocynthis* has been shown in animal studies to enhance urate excretion in the urine and lower plasma urate levels by Bustanji et al., 2011. Kaushik et al. 2015 have reported the presence of active triterpenoid compounds namely cucurbitacins responsible for the anti-inflammatory activities. Moreover Marzouk et al., 2013 have isolated a new compound from seeds namely 11-deoxocucurbitacin-I-2-O- β -d-glucoside possess anti-inflammatory and antioxidant effect. The presence of the same compound in the stem may be responsible for the observed antioxidant activity in the present investigation.

Conclusion

The complicated immunochemical reactions which is triggered due to the deposition of MSU crystals is the major cause of pain and joint destruction in the gouty arthritis. So the management of gouty arthritis requires a multiple therapeutic approach including the improvement of antioxidant status. The phyto chemicals present in *Citrullus colocynthis* stem loaded Zinc nanoparticles has the potential to reduce the inflammatory reactions by reducing the oxidative stress through the enhancement of antioxidant enzymes and can be employed for the management of gouty arthritis.

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