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Comparative evaluation of Metal Chelating, Antioxidant and Free Radical Scavenging activity of TROIS and six products commonly used to control pain and inflammation associated with Arthritis

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Abstract
Arthritis is an inflammation of joints due to infectious, metabolic or constitutional cause usually accompanied with pain, swelling, and stiffness resulting from infection, trauma, degenerative changes, metabolic disturbances, or other causes. Free radicals damage biomolecules, including lipids, proteins and nucleic acids leading to various diseases including but not limited to arthritis. The objective of the study was to investigate and compare the free radical scavenging, metal chelating, DNA repair potential and nitric oxide inhibitory property of Trois (a nano-technology based research product indicated for the treatment of pain and inflammation associated with arthritis) and with six commercial brands.

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INTRODUCTION

Free radicals play an important role in the biological system. They are highly reactive, unstable molecules formed when oxygen interacts with certain

molecules. Their chief danger comes from the damage they can do when they react with important cellular components such as DNA, or the cell membrane. Cells may function poorly or die if this occurs. They are reactive atom or group of atoms that has one or more unpaired electrons that is produced in the body by natural biological processes or introduced from an outside source (as tobacco smoke, toxins, or pollutants) [1]. They are either in the form of reactive oxygen species (ROS) or reactive nitrogen species (RNS). These molecules play an important role in the biological system and damage biomolecules, including lipids, proteins and nucleic acids leading to various diseases such as atherosclerosis, arthritis, neuro-degenerative disorders, cancer etc. [2,3].

These damages can be neutralized with natural antioxidants such as glutathione peroxidases, superoxide dismutases, catalase etc. and nutritional antioxidants from diet such as vitamins E, C, carotenoids etc. [4].

Arthritis is an inflammation of joints usually accompanied by pain, swelling, and stiffness resulting from infection, trauma, degenerative changes, metabolic disturbances or Reactive Oxygen Species. There are more than 100 types of arthritis including Osteoarthritis, gout, rheumatoid arthritis, septic arthritis, Juvenile idiopathic arthritis, Spondyloarthritis, Gonococcal arthritis, Psoriatic arthritis, Reactive arthritis (Reiter syndrome), Ankylosing spondylitis, Scleroderma, Systemic lupus erythematosus (SLE) and many more [5,6,7,8,9,10,11,12]. During arthritis inflamed human joint is subject to cycles of ischaemia followed by reperfusion. Ischaemic reperfusion cycles lead to iron decompartmentalization which promotes oxygen radical damage [13]. Reactive 0_2 species have the capacity to cause damage to a variety of biomolecules in the joint, eventually leading to a persistent and locally destructive inflammatory process [13]. They are capable of damaging cellular components and may contribute to various disease entities including inflammatory joint disease such as arthritis [14].

Oxidative stress may be defined as an imbalance between cellular production of reactive oxygen species (ROS) and antioxidant defense mechanisms. Hydroxyl radical is a form of ROS and is constantly produced as a result of metabolic reactions in living systems [15]. Hydroxyl radical causes damage to immunoglobulin G in patients with rheumatoid arthritis [16].

Chelation therapy is the administration of chelating agents to remove heavy metals from the body [17]. Disruption of iron ions homeostasis may lead to oxidative stress, a state where increased formation of reactive oxygen species (ROS) overwhelms body antioxidant protection and subsequently induces DNA damage, lipid peroxidation, protein modification and other effects, all symptomatic for numerous diseases, involving cancer, cardiovascular disease, diabetes, atherosclerosis, neurological disorders (Alzheimer's disease, Parkinson's disease), chronic inflammation and others [18].

Trois is a nano-technology based research product for topical application developed by Sunev Pharma Solution (Pvt) Limited, India that contains wintergreen oil (*Gaultheria precumbens*) and Eucalyptus oil (*Eucalyptus globulus*) as major ingredients. It is indicated for the treatment of pain and inflammation associated with different types of arthritis including osteoarthritis, gouty arthritis, rheumatoid arthritis, ankylosing spondylities, Juvenile idiopathic arthritis, psoriatic arthritis, sprains & backache.

The objective of the study was to investigate the free radical scavenging, metal chelating, DNA repair potential as well as nitric oxide inhibitory property of Trois and it's comparison with six commercial brands.

MATERIAL AND METHODS

Chemicals

DPPH (1,1–diphenyl – 1,2 –picryl hydrazyl), TPTZ(2,4,6,-tripyridy-s-triazine), potassium ferricyanide, trichloroacetic acid (TCA), $FeCl_3$, sodium nitroprusside, sulphanilamide, napthylethylenediamine dihydrochloride, TPTZ(2,4,6,-tripyridy-s-triazine), NBT (nitroblue tetrazolium), PMS (phenazine methosulfate), sulphuric acid (H2SO4), ammonium molybdate, ammonium persulphate, ascorbic acid, Butylated Hydroxytoluene (BHT), sodium nitrite, sodium EDTA etc. were purchased from Sigma-Aldrich (India) & Merck India. Trois was procured from Sunev Pharma Solution (Pvt) Limited, India whereas six commercial products A, B, C, D, E & F (Table 1) were purchased from local market. The study was performed at Venus Medicine Research Centre, Baddi , India.

Methods

Superoxide radical scavenging assay

Superoxide Dismutase (SOD) catalyzes the dismutation of the superoxide anion (O^2) into hydrogen peroxide and molecular oxygen and is one of the most important antioxidative enzymes. The effect of sample on superoxide generated in a non-enzymic system was measured spectrophotometrically.

The method as elaborated by Gow-Chin and Hui-Yin (1995) was adapted and modified in the lab [19]. The reaction mixture consisted of 0.75 mg/ml of 1 ml sample, 1ml 60µM phenazine methosulphate (PMS), in phosphate buffer (0.1M, pH 7.4) and 150 µM 1ml nitroblue tetrazolium (NBT) in phosphate buffer. Incubation at ambient temperature followed for 5 minutes, and the resultant colour was read spectrophotometrically at 560 nm against a blank (water). BHT was taken as standard (1mg/ml).The effect of Butylated Hydroxytoluene (BHT) was also determined by replacing plant extract with 1ml BHT (1mg/ml) in methanol in the reaction mixture.

$$\% \text{ inhibition} = \frac{(\text{Absorbance of control} - \text{Absorbance of sample})}{(\text{Absorbance of control})} \times 100$$

Nitric oxide radical scavenging assay

Principle : At physiological pH, nitric oxide generated from aqueous sodium nitroprusside (SNP) solution interacts with oxygen to produce nitrite ions, which may be quantified by the Griess reaction [20].

Preparation of Standard

Take 1.5 ml [Sodium nitroprusside (10 mM) in Phosphate buffered saline 1 M (pH 7.4)] and 1.5 ml ethanolic Sodium Nitrite (0.25-2.5 mg/ml). Incubate at 37°C for 60 minutes. Add 50 µL freshly prepared Greiss reagent. (1% sulphanilamide, 0.1% naphthylethylenediamine dichloride and 3% phosphoric acid).

Preparation of test solutions

1.5 ml [Sodium nitroprusside (10 mM) in Phosphate buffered saline 1 M (pH 7.4)] + 1.5 ml sample (1 mg/ml to 50 mg/ml) = 3 ml reaction mixture. Incubate at 37°C for 60 minutes. Add 50 µL freshly prepared Greiss reagent. (1% sulphanilamide, 0.1% naphthylethylenediamine dichloride and 3% phosphoric acid). Absorbance was taken at 546 nm.

Percentage inhibition was calculated:

$$\text{Inhibition \%} = \frac{\text{Absorbance of Control} - \text{Absorbance of Sample}}{\text{Absorbance of Control}} \times 100$$

Metal chelating activity

The chelation of ferrous ions by sample was estimated by method of Dinis et al [21].

Briefly, 50 µl of 2 mM $FeCl_2$ was added to 1 ml of concentrations (0.05-0.8 mg/ml) of the sample. The reaction was initiated by the addition of 0.2 ml (200 µl) of 5 mM ferrozine solution. The mixture was vigorously shaken and left to stand at room temperature for 10 min. The absorbance of the solution was there after measured at 562 nm.

NaEDTA was used as positive control (dissolved in water). Calibration curve conc. of positive control was 0.05 mg/ml, 0.1 mg/ml (OD 0.496), 0.2 mg/ml, 0.4 mg/ml & 0.8 mg/ml.The percentage inhibition of ferrozine–Fe2+ complex formation was calculated as :

$$\text{Inhibition \%} = \frac{(\text{Absorbance of Control} - \text{Absorbance of Sample})}{\text{Absorbance of Control}} \times 100$$

DPPH free radical scavenging activity

Principle : The antioxidant activity of the samples was determined on the basis of their scavenging activity of the stable 1, 1-diphenyl-2-picryl hydrazyl (DPPH) free radical. DPPH is a stable free radical containing an odd electron in its structure and usually utilized for detection of the radical scavenging activity in chemical analysis.

1 ml of each solution of concentration (10-100 µg/ml) of sample was added to 3 ml of 0.004% ethanolic DPPH free radical solution. After 30 minutes the absorbance of the preparations were taken at 517 nm by a UV spectrophotometer which was compared with the corresponding absorbance of standard ascorbic acid concentrations (10 µg/ml, 25 µg/ml, 50 µg/ml, 75 µg/ml & 100 µg/ml (OD 0.711). Then the % inhibition was calculated by the following equation:

$$\text{\% Inhibition} = \frac{(\text{Absorbance of control - Absorbance of scavenging activity sample})}{(\text{Absorbance of control})} \times 100$$

At first, 5 test tubes were taken to make aliquots of 5 concentrations (10 µg/ml, 25 µg/ml, 50 µg/ml, 75 µg/ml & 100 µg/ml) with the samples. Sample and ascorbic acid were weighed accurately and dissolved in ethanol to make the required concentrations by dilution technique. Here ascorbic acid was taken as positive control.DPPH was weighed and dissolved in ethanol to make 0.004% (w/v) solution. To dissolve homogeneously magnetic stirrer was used. After making the desired concentrations 3 ml of 0.004% DPPH solution was applied on each test tube by pipette.The room temperature was recorded and kept the test tubes for 30 minutes in dark to complete the reactions.DPPH was also applied on the blank test tubes at the same time where only ethanol was taken as blank.After 30 minutes, the absorbances of each test tube were taken by a UV spectrophotometer [22].

Hydroxyl radical scavenging assay

Scavenging of the hydroxyl free radical was measured by the method of Halliwell et al [23] with some modifications. All solutions were prepared freshly. 200µL of 2.8 mM 2-deoxy-D-ribose, 100 µL samples (200 µg/ml), 400µL of 200µM $FeCl_3$, 100µL 1.00 mM EDTA (1:1V/V), 200µL of H_2O_2 (1.0mM) & 200µL ascorbic acid (1mM) was mixed to form a reaction mixture (Fenton reaction**)**. After an incubation period of 1 hour at 37°C the extent of deoxyribose degradation. 1.5ml of 2.8% TCA was added in the reaction mixture and kept for 30 minutes at 100°C to develop the pink color taking Vitamin C as positive control. Calibration curve conc. were 50 µg/ml, 100 µg/ml, 150 µg/ml & 200 µg/ml. After cooling, the absorbance was measured at 532 nm against an appropriate blank solution. Percentage inhibition was calculated :

$$\text{\% inhibition} = \frac{(\text{Absorbance of control - Absorbance of sample})}{(\text{Absorbance of control})} \times 100$$

RESULTS & DISCUSSION

Trois is a nanotechnology based research drug (under patent protection) for topical application developed by Sunev Pharma Solution (Pvt) Limited, India that contains Wintergreen oil (*Gaultheria precumbens*) and Eucalyptus oil (*Eucalyptus globulus*) as major ingredients. It is indicated for the treatment of different types of arthritis including osteoarthritis, gouty arthritis, rheumatoid arthritis, ankylosing spondylities, Juvenile idiopathic arthritis, psoriatic arthritis, sprains & backache after topical application. Anti-inflammatory property, skin penetration rate & dermal toxicity studies of Trois has already been reported [24,25].

This study was done to find out the antioxidant activity, metal chelation, nitric oxide inhibition, hydroxyl radical inhibition and super oxide anion radical scavenging activity of Trois and it's comparison with six commercial leading brands in order to access the efficacy and effectiveness to treat pain and inflammation associated with arthritis & related disorders.

Superoxide radical scavenging activity

SOD is an enzyme that catalyzes the conversion of superoxide into hydrogen peroxide and oxygen [26]. According to merriam-webster [27], it is a metal-containing antioxidant enzyme that reduces harmful free radicals of oxygen formed during normal metabolic cell processes to oxygen and hydrogen peroxide (www.merriam-webster.com). The superoxide anion a form of ROS which leads to tissue damage associated with inflammation in joint diseases such as rheumatoid arthritis, osteoarthritis, and crystal-induced arthropathies [28]. It acts as an endogenous cellular defense system in oxidative stress to degrade superoxide (O2-) into oxygen and hydrogen peroxide which makes SOD as a potentially useful therapeutic agent for treatment of inflammatory disorders [29]. Herbs or natural products do not actually possess SOD enzyme, but they may contain SOD-mimic activity with the help of which superoxide dismutases converts superoxide to hydrogen peroxide, which is then removed by glutathione peroxidase or catalase [28]. Trois showed highest superoxide anion radical inhibition 71.92% which was found to be 12.63% more than A in which lowest inhibition was recorded i.e., 59.29%. B showed second highest inhibition 71.74% followed by E (71.08%), D (70.53%), C (69.95%) & F (67.37%) (Table 1).

Nitric oxide radical scavenging activity

According to Merriam-Webster [30](2011), Nitric oxide is a colorless poisonous gas NO formed by oxidation of nitrogen or ammonia that is present in the atmosphere and also in mammals where it is synthesized from arginine and oxygen and acts as a vasodilator and as a mediator of cell-to-cell communication [30](www.merriam-webster.com). Nitric oxide (NO) is an important mediator of diverse physiologic and pathologic processes, including arthritis [31,32]. According to Jang and Murrell [33], Nitric oxide's (NO) production by chondrocytes, its involvement in various biochemical events of cartilage metabolism, and the in vivo suppression of experimental arthritis by NO synthase inhibitors implicated NO in arthritis. High levels of NO production results in direct tissue toxicity and contributes to various carcinomas and inflammatory conditions [34,35,36]. Highest nitric oxide inhibition was seen in Trois (64.84%) followed by B (54.34%), A(52.28%), D(50.91%), C(49.77%), E (46.35%) & F(45.21%) (Table 1).

Metal Chelating activity

Chronic inflammatory processes cause a significant change in iron metabolism with a drop in serum iron and a redistribution of iron to the activated reticulo-endothelial system. Fe (iron) overload appears to selectively worsen joint inflammation whilst nutritional Fe deficiency has the converse effect. Inflammatory synovial fluid contains Fe in a form capable of generating the hydroxyl radical.

The effect of synovial iron on the progression of rheumatoid disease has been reported by Blake et al [37]. This may be correlated to metal chelation in patients with synovial iron. It has been suggested that iron may play an important part in acute and chronic phases of the arthritic inflammatory process. Iron may catalyse free radical production in the joints, leading to lipid peroxidation and membrane disruption. An abnormal accumulation of iron may promote an infiltration of lymphocytes and macrophages into the synovium of affected joints [38].

There are reports of metal chelation activity of some topical products such as Characteristics of chelation ability of chosen metal ions by protective ointments containing Na_2H_2EDTA [39]; An in vitro study of the use of chelating agents in cleaning nickel-contaminated human skin: an alternative approach to preventing nickel allergic contact dermatitis [40]; A cream containing the chelator DTPA (diethylenetriaminepenta-acetic acid) can prevent contact allergic reactions to metals [41]. Trois is a topical product and metal chelation of Trois was found to be 48.59% followed by B **(**48.19%), A (45.77%), F (33.87%), C (32.86%), E (19.35%) & D (12.70%). Metal chelating activity of Trois was 29.24% more than E and 35.89% better than D (Table 1).

DPPH free radical scavenging activity

Reactive oxygen species (ROS) which are produced by all aerobic organisms can easily react with most biological molecules including proteins, lipids, lipoproteins and DNA. This ROS can generate oxidative stress and produce many patho-physiological disorders such as arthritis, diabetes, inflammation, cancer and genotoxicity [42,43,44].

Free radical scavenging activity of Trois was seen. Highest nitric oxide inhibition was seen in Trois (61.54%) followed by E (60.82%), A(60.12%), F (56.35%), C(55.28%), B (53.49%) & D (39.18%).

Free radical scavenging activity of Trois was found to be 22.36% more than D where lowest inhibition was seen (Table 1).

Hydroxyl radical scavenging activity

Hydroxyl radical is a form of ROS and is associated with arthritis [45,46]. It is cytotoxic, mutagenic and genotoxic and is involved in disease pathogenesis [47]. There are reports of hydroxyl radical scavenging by topical products. According to Koo et al [48], Genipin, active principal of Gardenia possesses anti-inflammatory and is a specific hydroxyl radical scavenger topically. The discovery of 6-[1-[2-(hydroxymethyl)phenyl]-1-propen-3-yl]-2,3-dihydro-5-benzofuranol, a potent topical anti-inflammatory agent [49]; Structure-activity relationships for the formation of secondary radicals and inhibition of keratinocyte proliferation by 9-anthrones [50]; Anti-oxidant actions of oxymethazoline and xylomethazoline [51]; Rosmarinic acid inhibits epidermal inflammatory responses: anticarcinogenic effect of *Perilla frutescens* extract in the murine two-stage skin model [52]. Highest inhibition of hydroxyl radical was done by Trois (75.66%) which was almost comparable to D (75.26%). It was 10.85% more than A, 28.04% more than B, 38.82% more than C, and 31.35% more than that of E (Table 1).

Conclusion

The results of present study demonstrates that Trois has potential of inhibiting nitric oxide, superoxide anion radical, hydroxy radical, DPPH radical as well as metal chelation in vitro. NO inhibition activity of Trois was highest among tested groups, where as Metal Chelation was comparable to group A&B, DPPH activity was comparable to group A&E, Hydroxy radical activity was comparable to group D only. This clearly indicates that none of the compared groups had equivalent prefromance to Trois. Other marketed products could comptete in maximmum two to three parameters but fail to provide activity equivalent to Trois in all the parameters studied. Trois exhibits better efficacy as compared to other six commercial brands due to its potent antioxidant effects also observed in current study.

Table 1 : Trois Vs six other brands

Sample Code	Major Ingredients	NO (% Inhibition)	Superoxide anion radical (%Inhibition)	Metal chelating activity (%)	Free radical scavenging activity (%DPPH activity)	Hydroxyl radical scavenging activity (%)
Trois	Wintergreen oil, Eucalyptus oil & Menthol	64.84%	71.92%	48.59%	61.54%	75.66%
A	Wintergreen oil, Mint, Camphor oil, Eucalyptus oil & Tarpeen oil	52.28%	59.29%	45.77%	60.12%	64.81%
B	Wintergreen oil, *Mentha arvensis*, *Pinus roxburghii*, *Cinnamomum zeylanicum* etc.	54.34%	71.74%	48.19%	53.49%	47.62%
C	Methyl Salicylate, Menthol & Diclofenac	49.77%	69.95%	32.86%	55.28%	36.84%
D	Wintergreen oil, Pudine ka phool, Tarpeen oil & Eucalyptus oil	50.91%	70.53%	12.70%	39.18%	75.26%
E	Methyl Salicylate, Menthol & Aceclofenac	46.35%	71.08%	19.35%	60.82%	48.54%
F	Methyl Salicylate, Menthol & Diclofenac	45.21%	67.37%	33.87%	56.35%	44.31%

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