Original Research Article

CannabisSativa-mediated synthesis of Copper-doped Zinc Oxide nanoparticles (Cu_2O-ZnONPs), characterisation and biomedical activity assessment

ABSTRACT

The escalating emergence of antibacterial resistance to most organic drugs by common pathogenic microbes necessitates the search for alternatives. Inorganic metal oxide nanoparticleshave displayed in many studies their suitability as substitute treatments for organic antibiotic-resistant pathogens. The aim of this study was to biosynthesise Copper-doped ZnOnanocomposites(Cu_2O-ZnONPs)mediated by the polyphenolic extracts of "wild' *Cannabis sativa*.Upon legalization in many countries, cultivars of *C. sativa L*have gained interest and attention due to their uses in traditional medicinal practices.Their purportedrichness in polyphenolic compounds also make them good candidates to mediate in the bio reduction capping and stabilisation of biosynthesised nanocomposites. In this study we utilisedwildly growing *C. sativa*as a mediator because wild plant species are reported to naturally possess more protective secondary metabolites due to increased environmental stresses and threats. The *C.Sativa* was qualitatively and quantitatively investigated for its secondary metabolites and compared to commercial cultivars prior to the biosynthesis. The biosynthesised nanoalloyswere structurallycharacterised and theanti-inflammatory, antioxidant andantibacterialactivity was investigated. Our results confirm the increased abundance of numerous secondary metabolites in hydroethanolic extracts of the aerial parts of the wild *C.Sativa* compared to the artificially propagated cultivar. Thelyophilised extracts of the wild *C. Sativa* and the Cu_2O-ZnONPs demonstrated high efficacy against microbes withzones of inhibition between 19 and 27mmat all tested concentrations. UV-Visspectra identified the fabricated nanostructures as Cu_2O-ZnONPs, Transmission electron microscopyconfirmed the formation of both spherical and cubic nanostructures. Dynamic light scattering estimated the average particle size to be 44nm. Our studies confirm that the wildly growing variety is medicinally superior to the domestic *C.Sativa*specimen. We also concluded that highly potent, anti-bacterial, anti-inflammatory and antioxidantplatforms combining synergistic Cu_2O-ZnONPs and polyphenols can be biosynthesised using*C.Cativa*.

Key words, Cu_2O-ZnONPs, Zinc doping, *Cannabis Sativa*, biosynthesis, antibacterial, antioxidant

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1 Introduction

1.1 Naturalised *Cannabis Sativa L* in Zimbabwe

Native to Central Asia, *Cannabis Sativa* (*Cannabis*) is widely naturalised in many parts of the world, including Southern Africa, where it is frequently encountered as an occasional wild weed, mostly results as from old cultivation[1]. The annual herb can grow to up 3m high in the tropical climate with angular stems that may be branched and palmitate digitate leaves. The herb's flowers are mostly in greenish-white clusters in the upper leaf axils. The plant's reproductive systems are unisexual on different plants and the male plants which are usually larger than the females are more ecologically viable, living longer and possessing more flower clusters[2]. Marijuana or *Dagga* as it is commonly known is perhaps the most widely and often illegally cultivated herb in Southern Africa due to the recreation drug accolades associated with its resin. In Zimbabwe, this angiosperm, belonging to the *Cannabaceae* family has been grown for hundreds of years since the 10th century AD for its fibres, seed oil, medicinal and recreational use[3]. Of major interest to this study, for centuries, Cannabis has always been an integral part of traditional medicine in Zimbabwe. Originally known locally as *'mupanjere'* which literally translates to 'wisdom imparting tree', it is revered by Zimbabweans both ancient and current for its healing properties for many ailments. It was, and still is used in religious rituals[3]. Zimbabwe's cannabismajor cultivation and naturalised area is the Binga district in Northwestern Zimbabwe, where the Tonga people place great cultural value on the plant to the point of reverence. Cannabis is also widely naturalised in the Southeastern parts of Zimbabwe and the Eastern highlands. From these habitats the herbs both natural and cultivated are for domestic local consumption in medicinal practices, though small quantities are sometimes smuggled outside the areas for recreational use[4]. In 2018, Zimbabwe's government legalised the cultivation of research and medicinal cannabis through the Dangerous Drugs (Production of Cannabis for Medicinal and Scientific Use) Regulations (Statutory Instrument 62: 2018), becoming only one of two African nations to do so by then[3,4]. This therefore opened room for systematic scientific studies on wildly growing Cannabis specimen like this one. However, recreational use remains illegal since the herb is still categorised as a "dangerous drug' under the country's current legal structures.

Figure 1: Naturalised, wildly growing C. Sativa L in Zimbabwe's eastern highlands

1.2 Ethno pharmacological significance of *C.Sativa*

C.Sativa is perhaps the most famous or infamous plant ever globally propagated by humans, with a rich history of both use and abuse due to its complex primary and secondary metabolic biology and chemistry. Globally, the extracts have found multiple curative biomedical applications such as, analgesic, anti-bacteria, anti-tumour, anti-inflammatory, anti-emetic, anti-epileptic, anti-parasitic and many others[5]. Almost every part of the plant, seeds, leaves, roots, bark and stems are used in ethnomedicinal practices. The most common extractions are seed oils and juice from the leaves. Kala et al describes 25 different diseases that can be cured by the leaf extracts[6]. Over 500 different chemical entities have been identified from various extracts of *C.Sativa* including at least 114 Phyto cannabinoids and an even larger array of terpenes and terpenoids[6]. More than Ten different Phyto cannabinoids have been isolated, profiled and extensively studied including a recent inclusion called Δ9-tetrahydrocannabiphorol (Δ9-THCP)[7]. From the flowers and leaves, over 50 terpenoids, numerous flavonoids and many cannabinoids have been profiled for their medicinal attributes. Cannabis is also

rich in polyphenols, including flavonoids, phenolic acids, phenol amides, and lignan amides, well known for their therapeutic properties[8]. Studies by Izzo et al (2020) determined high total polyphenol content in Cannabis, with pelargonin being the most abundant polyphenol (1.51 mg/g) in *C. sativa* extracts[9]. Other studies report high antioxidant potential of cannabis plant extracts and concluded that the plant is an excellent source of polyphenols for biomedical and neutraceutical purposes supplementation purposes. The traditional medicinal attributes of *C.Sativa* have largely been translated into tangible products with currently available treatments for hiccups, pain, epilepsy, nausea, anxiety, depression and sleep disorders. In the western world, dozens of treatments are recognised and registered as effective treatments for numerous conditions with the FDA registering Cannabis based drugs in treatments for nerve damage, cancer, multiple sclerosis, and rheumatoid arthritis[10].

1.3 Polyphenols and skin integrity

Polyphenols are natural biochemicals originating from Three biosynthetic pathways which are; the Shikimate pathway (a seven-step metabolic pathway connecting the central carbon metabolism to the biosynthesis of aromatic compounds); the Phenylpropanoid pathway (metabolites biosynthesis catalysed by phenylalanine ammonia-lyase), and the Polyketide pathway (metabolites assembled by polyketide synthase (PKS) enzymes)[11]. Polyphenols as the name implies consist of several phenolic functional units without any affiliated or cojoined nitrogen-based groups. Their range and scope are broad, incorporating biomedically relevant compounds, such as stilbenes, phenolic acids, flavonoids and lignans[12]. The functional moieties in polyphenols and their interactive capacities are the primary contributors to their cardinal importance in human health restoration. Numerous scientific papers have validated that plant phenolics, whether administered topically or orally play crucial roles in the prophylaxis and treatment of many skin disorders[13]. Polyphenols have proven biological effects such as antioxidant, anti-inflammatory, anti-microbial properties. Their free radical scavenging capabilities have been greatly utilised in anti-aging cosmeceuticals and skin photoprotection[14]. Over and above these benefits, they are actively involved in restoration of the skin's barrier function integrity and the natural moisturising factor (NMF) through balancing out trans epidermal water loss (TEWL)[15]. All these functions are essential for skin structure and function as well as the pathogenic surveillance and defence system. Polyphenols have been demonstrated to have positive synergistic influence on both the gut and skin microbiomes.

1.4 Polyphenol mediated Biosynthesis of nanoparticles

Nanoparticles (NPs)for different applications can feasibly be fabricated using different physical and chemical techniques. Most common is the sol gel synthesis and co-precipitation methods due to the inherent ability to manipulate the morphology and characteristics of the resultant Nanoparticles[16]. These methods require precursors, solvents and reducing agents to obtain the precipitated Nanoparticleswhich are further calcined at high temperatures around 500°C. The resultant Nanoparticles are a function of the precursor concentration and its ratio to the reducing agent, the process reaction pH as well as the lyophilisation and calcination temperatures[17]. From a biomedical standpoint, these reactions employ unsafe solvents and also generate huge toxic by-products from side reactions and degradation as well as unreacted or excess reagents. The toxicity concerns and potential threats to human health have slowed the use of novel Nanoparticles in biomedical applications since the purification process is very expensive[18]. Numerous studies have demonstrated the capacity of the polyphenolic functional groups to mediate in the biosynthesis of novel metallic Nanoparticles from their salts, acting as bio reducing, capping and stabilising agents in a one pot ecofriendly technique. This green synthesis route guarantees safer Nanoparticles at lower fabrication costs[19]. The proliferation of numerous –OH groups in polyphenols increases, the reducing efficiency in the synthesis of NPs, increases nucleation points that result in smaller Nanoparticles free from toxic secondary by-products since water can be used as a medium. Polyphenols therefore present ecofriendly, safer and more efficient NPs synthesis due to their selectivity and economic reaction points and functional moiety count[20].The polyphenolic biosynthesis technique has found application in fabricating NPs from many different plant extracts and with amendments can feasibly synthesise NPs from any plant source since almost all plants have secondary metabolites which include polyphenols for their adaptation and survival in the face of environmental threats. Due to the need for resilience to increased and uncontrolled threats, wild and naturally growing plants have demonstrated higher abundances of secondary metabolites compared to artificially propagated varieties.

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1.5 Zinc Oxide Nanoparticles(ZnONPs) and their potential skin applications

Human civilisations have used Zinc in skin care including treating wounds and burns for over 2000 years[21]. Ancient Indian medicinal scripts dating as far back as the year 500BC describe the formulation of a natural skin healing zinc salve referred to as the pushpanjan[22]. 2000 years later Zinc oxide still remains a key biomedical component in many skin treatments including sunscreens, rash creams and calamine lotions. The anti-inflammatory use of ZnO is the subject of numerous scientific papers where it proved efficacious in rashes, allergies and irritations[23]. The photoprotective and broad-spectrum protection from actinic damage are widely reported and ZnO and TiO_2 are the only Two out of Sixteen approved sunscreens that have been accorded GRASE (generally recognised as safe and effective) status by the FDA[24]. ZnO has demonstrated prophylactic protection from neoplasia including Squamous and Basal cell carcinomas through retardation in the development of solar keratosis[25]. The anti-bacterial effects of the metallic oxide and the ability to aid wound healing and damaged tissue recovery from burns have also found use in registered and over the counter pharmaceutical treatments[26]. The cosmeceutical uses of ZnO in acne and warts treatments and anti-dandruff agents are not only reported in scientific studies but are evident in the many retailed products[27]. The only drawback to its wide use has been its insolubility in water and aqueous systems which leaves a white cast on application. Nanotechnologies recently resolved this debacle, and many studies have confirmed the development of clear translucent products from ZnONPs[28]. This development further broadens the benefits of the metallic oxide and centrally positions ZnONPs as one of the most versatile, efficacious and widely applicable skin disorders remedies.

1.6 Copper Oxidenanoparticles (CuONPs) and their potential skin applications

The use of copper and its oxides in skin care is as old as mankind. The Smith Papyrus (circa 2400BC), an ancient Egyptian medical text report the use of copper in treating wounds[29]. Similarly, another Egyptian text Ebers papyrus (circa 1500BC) extols the virtues of copper to treat burns and itching[30]. Hippocrates (circa 400BC), prescribed copper for cutaneous ulcerations related to varicose veins and the ancient Greeks are known to have used copper on open wounds[31]. The references from the ancient world are many and represent structured application of copper in skin care. Much of this has been confirmed by modern research with copper now being referred to as the fountain of youth due to its numerous uses. In skin care the essential antimicrobial mineral plays significant roles in many metabolic processes, including angiogenesis and skin generation[32]. The development of Cu_2ONPs using modern tools has optimised properties and further extended these applications to numerous biomedical settings, such as skin carcinomas. Natural polyphenolic derived Cu_2ONPs are inexpensive and easily up scalable. It has been reported that bioactive Cu_2ONPs participate in modulation of several cytokines that are involved in most stages of the wound healing process[33]. The importance of Cu_2ONPs in nanomedicine is notable due to the antibacterial, antifungal and anti-tumour potency. In skin integrity Cu_2ONPs have fungicidal effects against specific fungus strains. In skin care, Cu_2ONPs play cardinal roles in the formation of collagen proteins, and thereby slowing the wrinkling process. It has been confirmed scientifically that pillowcases, containing copper oxide, reduce wrinkles and that skin elasticity can be promoted by socks containing copper oxide, which also eliminate the development of tinea pedis[34].

1.7 Doped Zinc oxide nanoparticles

As discussed above, ZnONPs have found numerous biomedical application due to their GRASE status. The biomedical properties and efficacy of Nanoparticles for drug development and delivery are a function of various parameters at play. The beauty of nanotechnology is the ability to manipulate and further fine tune these parameters to amplify or compound efficacy, through modulation of the physicochemical characteristics of the Nanoparticles[35]. Several modifications are available for this improvement but perhaps one of the easiest and most cost effective is doping. Synthetic doping is the deliberate practical insertion of a different element into the empty crystal lattices of another element to modify or optimise its properties[36]. This technique has found importance as a standard method amplifying and modifying Nanoparticles' physico-chemical parameters and bioactivity. Rare earth metals and transition elements including gold, copper, silver, manganese, cobalt, nickel have been used as dopant materials that boost the standard bioactivity of ZnONPs by imparting their own attributeslike anti-microbial activity and anti-cancer activities to the new alloy[37]. Doping green synthesised Nanoparticles is a very attractive option because there are no added special requirements, the processing conditions are standard and economical. We present here the Cu_2ONPs doping of ZnONPs so as to amplify and compound the novel properties required for biomedical skin applications of the Two materials.

2 Materials and methods

2.1 Materials, equipment and facilities

All chemicals, associated reagents, equipment and facilities, the biosynthesis and the activity determinations were obtained or done at the University of Zimbabwe, College of health sciences laboratories, Harare, Zimbabwe. For the Nanoparticles' characterisations, all chemicals and equipment were availed by the University of California, Los Angeles, Department of chemistry and biochemistry.

2.1.1 C.Sativa plant preparation

Naturalised, wildly growing *C. Sativa* plants were obtained from the Chirinda forest, Chipinge district in Southeastern Zimbabwe (20°25′27″S 32°41′40″E). The plant was taxonomically authenticated by the National Herbarium and Botanical Garden in Harare, Zimbabwe. The plant materials were washed separately with fresh water to remove dirt and other contaminants, shade-dried for three weeks until constant weight. The dried leaves were separated from the stems and ground to fine powders.
The hydroethanolic extraction was done by adding 400g plant powder into 1000ml of 70% (v/v) ethanol in a 2-litre sterile amber bottle and macerated for 3 days with 3minute physical shaking twice a day. The extracts were filtered (Whatman filter paper number 1) and evaporated under low pressure (Rotavapor® R-300, Buchi, Switzerland), followed by lyophilization (Lyovapor I-200, Buchi, Switzerland) under 140Pa pressure and -50 °C.

2.2 Secondary metabolite Qualitative phytochemical Screening of *C.Sativa*

In a 200ml round bottomed flask, 15g of the lyophilized hydro-ethanolic leaf extracts of *C.Sativa* were dissolved in 100g of distilled water and subjected to various phyto-screening techniques to confirm the presence or absence of relevant phytoconstituents of pharmacological interest to this study. The following qualitative tests were conducted on the extract liquor.

2.2.1 Detection of Phenolic compounds and Tannins

The gelatin test was used to determine the presence of phenolic compounds and tannins. A 1mlaliquot of the lyophilised extract solution was added to 5mL distilled water, 1g of a 1% gelatin solution and 0.5g of a 10% NaCl solution were added. A positive result was identified by a white precipitate forming which indicates the presence of tannins and phenolic components[38].

2.2.2 Tests for alkaloids

The Dragendroff's/Kraut's test was used to determine the presence of alkaloids. In this assay, 1 mL of Dragendroff's reagent was added to 2 mL of lyophilised extract solution. A positive test result was identified by the appearance of an orange-red precipitate indicating the presence of alkaloids[39].

2.2.3 Test for flavonoids

Flavonoids were detected by means of Shibata's reaction/Cyanidin test. In this assay 1 mL of the 15% lyophilised hydroethanolic ethanolic extract solution was added to a test tube containing 2g of magnesium clippings. Then, 0.5 mL of concentrated HCl was added. Positive tests were identified by the appearance of a red or orange colour indicating the presence of either flavonols or flavanones respectively[40].

2.2.4 Test for terpenoids and steroids

The Liebermann Burchard Reaction was used to screen for sterols and terpenoids. Briefly, 1ml of the lyophilised extractssolution was dissolved in 0.5 mL of dichloromethane (DCM) and 0.5 mL of acetic anhydride. After homogenization, 1 mL of concentrated H_2SO_4 was slowly added. The formation at the interface of the two liquids of a reddish-brown or reddish-purple ring with a greenish or purplish supernatant is a positive indication for the presence of steroids and terpenoids[41]

2.2.5 Detection of Lignins

The Labat test was used to detect the presence of lignins. A 2mlaliquotof the lyophilised extract solution was dissolved in 5 mL water and then added to 1 mL of gallic acid. The development of an olive-green colour signals the presence of lignins[42]

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2.2.6 *Total Phenolic content Quantitative analysis*

The total phenolic content of the extracts was determined according to the Folin-Ciocalteu spectrophotometric method with some modifications. To prepare a calibration curve, phenol (Gallic acid) stock solution (5 mg/mL) was added into 100 mL volumetric flasks, and then diluted to volume with water. From each calibration solution, 0.25 mL was mixed with 1.25 mL of 10-fold diluted Folin-Ciocalteu's phenol (1 mL Folin reagent and 9 mL deionized water) reagent and allowed to react for 5 min. Then, 1 mL of 7.5% Na_2CO_3 solution was added, and the final volume was made up to 5 mL with deionized water. After 1 h of reaction at room temperature, the absorbance at 760 nm was determined by spectrophotometry (Lambda 35 UV/Vis-Spectrometer, Perkin Elmer Instruments)[38]. The test was done in triplicate. A calibration curve was plotted to determine the level of phenolics in the samples. The test was done in triplicate. The results were expressed as Gallic acid equivalents (GAE, mg/g) of the plant extract and Cu_2O-ZnONPs.

2.2.7 *Anti-oxidancy evaluation*

The antioxidant activity of all the plant extracts was determined using the amended DPPH free radical scavenging assay. To identical bottles, 50 µL of the lyophilised plant extract solutionin concentrations from 5 to 150 mg/ml was added followed by 5 ml of 0.004% (w/v) solution of DPPH. The resultant mixture was vortexed and incubated for 30min at room temperature in a dark cupboard and then read using a UV spectrophotometer (Lambda 35 UV/Vis-Spectrometer, Perkin Elmer Instruments) at 517 nm. The blank was 70% (v/v) methanol. Ascorbic acid (Vitamin C) was used for comparison. Measurements were taken in triplicate.
DPPH scavenging effect was calculated using the following equation 1:

Equation 1

$$\text{DPPH scavenging effect (\%)} = \left\{\frac{A^{\circ}-A}{A^{\circ}}\right\} \times 100$$

Where A^0 is the absorbance of negative control (0.004% DPPH solution) and A is the absorbance in presence of extract.
The results were reported as IC_{50} values and ascorbic acid equivalents (AAE, mg/g) of *C. sativa* extract and Cu_2O-ZnONPs.

2.3 Biosynthesis of Cu doped ZnO Nanoparticles (Cu-ZnONPs)

The Cu_2O-ZnONPs were prepared based on a modified biosynthesis route using the lyophilized extracts from *C. sativa* as bio reducing, caping and stabilization agents. 2g of the lyophilised extract was dissolved in 50ml distilled water and stirred for 15minutes and heated at 50°C. To this liquor 1g of zinc acetate-2-hydrate ($Zn(CH_3COO)_2{\cdot}2H_2O$) salt was added. The brown solution was mixed with constant stirring at this temperature for approximately 30minutes. Then, 0.6 g copper (II) nitrate trihydrate ($Cu(NO_3)_2.3H_2O$ was added into the complex solution, stirred for a further 30 minutes at 60℃. 0.05 mol NaOH solution was titrated into the mixture. Finally, 0.1 mol/L ascorbic acid solution was added to reduce the chelated cupric ions in situ. The resultant thick precipitate of the formed nanocomposite was collected by simple centrifugal separation, washed with 70%v/v ethanol, and vacuum dried at 80°C for 8 hours. The dried Cu_2O-ZnONPs were calcined at 450°C for three hours in a muffle furnace to get pure Cu_2O-ZnONPs.

2.3.1 *Characterisation of Cu_2O-ZnONPs*

The identities of the nanostructures were confirmed by UV-Vis spectrophotometry (Hitachi, UH5300) and their morphology and configuration was further analysed by Transmission electron microscopy(TEM) (A LEO912 AB OMEGA TEM). For the TEM analysis the nanostructures samples were diluted to various concentrations and dropped onto the carbon-coated copper grids covered with a formvar film, which were then allowed to dry in air. Once dry, the samples were loaded onto the specimen holder. The TEM measurements were conducted at a voltage of 100 kV. To determine the dimensions and average particle sizes, Dynamic light scattering (DLS) (Zetasizer ultra) was used.

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2.4 Anti-inflammatory effect of the lyophilised leaf extract and Copperdoped-ZnONPs

The anti-inflammatory capacity of the lyophilised leaf extract and the Cu_2O-ZnONPswas determined using the protein denaturation assay as described by Chifamba et al (2024)[43]. The test assessed the ability of the extract and the Nanoparticles to hinder egg albumin denaturation in PBS. In our assay 0.5 mL of 3% egg albumin solution from a free-range domesticated hen (*Gallus domesticus*) was added to 10ml of PBS (pH 7.2) and mixed with 5ml solutions of varying concentrations of the colloidal Cu_2O-ZnONPs and the lyophilised leaf extracts. The test concentrations used in the assays in µg/ml were 125, 250, 500, 1000, and2000. The resultant mixtures were then incubated (Shel lab SRI3 Low Temperature BOD Incubator) at $37{\pm}2°C$ for 30 minutes and then heated at 65°C for 10 min. After cooling, their absorbance was determined at 660 nm (UV spectrophotometer, Lambda 35 UV/Vis-Spectrometer, Perkin Elmer Instruments) using the vehicle as blank. Diclofenac sodium was used as a comparative reference standard at related concentrations of (µg/ml) 125, 250, 500, 1000, and 2000 respectively. The vehicle was used as the control. The percentage inhibition of protein denaturation was calculated by using the following formula in equation 2. The Half-maximal inhibitory concentration (IC50) value was determined to be the anti-inflammatory inhibition of 50% concentration

$$\text{Inhibition}(\%) = \frac{A_{control} - A_{sample} \times 100}{A_{sample}}$$

Equation 2

Where:

$A_{s\ ample}$ is the absorbance of samples and $A_{control}$ is the absorbance of the blank.

2.5 Antibacterial inhibition evaluation of lyophilised*C.Sativa* and the Cu-ZnONPs

2.5.1 Test organisms

Pure cultures of *Staphylococcusaureus*(ATCC25923), *Escherichia coli*(ATCC 25922), *Pseudomonas aeruginosa* (NCTC 10662) and *Enterococcus faecalis* (ATCC 29212)were donated bythe University of Zimbabwe Department of Microbiology. To obtain a stock solution of 10,000 µg/ml solutions, 0.005g of the Lyophilised extract and the Cu_2O-ZnONPs were each dissolved in 0.5ml of Dimethyl sulphoxide (DMSO) in a sterilized vile. Stock portions were obtained by further diluting the stock solution to obtain concentrations of50µg/ml, 100µg, 250µg and 500µg/ml.Portions of Ciprofloxacin were prepared in the same manner using pure ciprofloxacin powder.The standardstock solution of Ciprofloxacin was prepared at a concentration of 100µg/ml in sterilewater. The Ciprofloxacin stock solution was used as the positive control and DMSO was used as the negative control.

2.5.2 Broth preparation and culturing

The broth was prepared by dissolving 1.95g of nutrient broth in 300ml of distilled water andheated to 60°C, then 10ml aliquots of theprepared nutrient broth were autoclaved at 120°C for 15 minutesinpolypropylene bottles. After cooling, the bacterialstrains were introduced into the nutrient broth using inoculating loops and the media wasinoculated at 36.7°C for 48 hours.

2.5.3 Agar well diffusion method

To prepare the agar solution, 8g of Tryptone soya Agar was suspended in 200ml of distilledwater and mixed thoroughly at boiling temperature.The prepared agarsolution was divided into 20ml cutting bottles and the medium was sterilized by autoclavingat 120°C for 30minutes. After cooling, 0.1 ml of a bacterial suspension and 20mlof molten agar was then poured into petri dishes, tilted to allow mixing of the bacteria andallowed to settle in an aseptic environment. The stock solution to be tested was prepared bydissolving the extracts at concentration of 50,100, 250 and 500µg/ml in DMSO. A sterile corn borer was used to punch holes on the agar and the holes were filled with0.05ml of stock solution. The plates were kept for an hour at room temperature and thenincubated for 24 hours in the incubator at 37°C. After incubation, the bacterial inhibition zonediameters were measured.

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3 Results and discussion

3.1 Qualitative phytochemicals screening of the wild *C.Sativa*

The secondary metabolites naturally produced by plants are diverse, almost half of them are phenolics (which include polyphenolics, flavonoids, tannins etc), about a third of them are terpenoids and steroids and the remainder are alkaloids and other minor chemical groups. Despite the fact that, these secondary metabolites are non-essential to plant growth, they are the vital materials mediating in a plant's ecological interaction with diseases, competitors, solar radiation as well as other environmental threats and stresses[43]. The realisation that these phytochemicals in various forms can also be administered to protect other organisms including humans from the same threats is the basis of most traditional herbal medicinal practices. In modern medicine, the purported safety and efficacy of plant sourced ingredients has brought renewed attention and focus on secondary metabolites as medical remedies in mainstream medicine. *C.Sativa* L has always been known as a prolific source of phytochemicals with well-known therapeutic use and psychoactive abuse. For its biomedical application, attention has always focused on the major cannabinoids. Most studies therefore related on phytochemical extractions from the seed oil and flowers which are the major sources of these cannabinoids. Our literature search confirmed that over 90% of the published studies on cannabis were on extractions from the seed oil and flowers[45]. However limited phytochemical characterization of other plant parts exhibited the high presence of numerous non-cannabinoid secondary metabolites including, polyphenolics, flavonoids, liganins, spiroindans, steroids, alkaloids and many others which are therapeutically relevant[46]. Our study is a departure from most studies on Cannabis because we used exclusively leaf extracts for two reasons. The first being that in Zimbabwean traditional medicine practices, the antibacterial, anti-inflammatory and anti oxidancy of Cannabis is attributable to leaf extracts use. Secondly in the biosynthesis of nanostructures, polyphenolic compounds are the ones that function as the bio-reducing, capping and stabilising agents. Cannabinoids do no not possess multiple phenolic groups to actively participate in biosynthesis. The biosynthesis mediating capacity of the secondary metabolites of Cannabis should therefore be from the other non-cannabinoid polyphenolic metabolites. Our hypothesis is also that, of the more than 500 phytochemicals present in Cannabis, the secondary defence metabolites for the plant will be more concentrated in non-occasional plant structures like leaves, roots and bark rather than perishable flowers and fruits. Our screening tests identified the huge presence of pharmacologically relevant groups as shown in table 1. This tallies very well with most studies that have identified numerous phytochemicals belonging to the groups identified[44,46]. The presence of flavonoids and alkaloids corresponds well with the antibacterial and other medicinal benefits observed in our studies. The presence of polyphenols is perhaps the reason why it was feasible to fabricate the nanostructures. Our observation is that Cannabis has high prevalence of pharmacologically active secondary metabolites with huge potential for biomedical applications and participation in biosynthesis of metallic Nanoparticles.

Table 1:Phytochemicals present in C.Sativa hydro-ethanolic and distilled water extracts

Test	Presence in hydro-ethanolic extract	Presence in distilled water extract
Alkaloids	+++	+
Phytosterols	+	+
Flavonoids	+++	+
Lignins	+++	+
Phenolic compounds	+++	+
Tannins	++	++
Terpenoids	+++	+
Saponins	++	+
Glycosides	+	+

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3.2 Quantitative phytochemical analysis

3.2.1 Total Phenolic content Quantitative analysis

The total phenolic content of the lyophilised *C. Sativa* extract was 78.5 µg GAE/g, which was lower than the 93.8 and 94.6 µg GAE/g obtained for the hydroethanolic and hydromethanolic extractions respectively (figure 2). The Total phenolic content (TPC) is an approximate number of phenolic compounds constituting a plant's secondary metabolites[47]. Natural Phenolics which are believed to number over 8000 entities, share a prevailing identical sub structure composed of an aromatic hydroxyl moiety. The antioxidant, health benefits of phenolic compounds are well reported in literature.Apart from their roles as reducing agents, phenolic compounds also act as hydrogen donors,

and quenchermolecules for reactive oxygen species (ROS), thereby protecting living structures from protein peroxidation andDNA oxidative damage[48]. In our study the TPC was approximated usingthe Folin-Ciocalteu method. This technique is based on forming coloured complexes that can be measured spectrophotometrically through reacting the present phenolic compounds with a reagent[38]. The TPC was calculated from the regression equation of the calibration curve ($R2 = 0.989$, $y = 0.009x + 0.0464$), expressed as gallic acid equivalents (GAE) milligrams per gram of the extract or fraction (mg GAE/g extract or fraction)[38]. Various studies have confirmed the dependence of the TPC on plant species,plant parts used as well as the solvent of extraction. Our results confirm the same with a lower yield for hydro extraction as compared to hydroethanolic and hydromethanolic extractions. Our results also confirm that the differences between the ethanolic and methanolic extractions are not very significant and hence due to safety reasons we prefer the ethanolic extractions asthe route for extractions for this study. Interestingly the naturally growing Cannabis specimen extractions yielded a higher TPC than the cultivated cannabis. Secondary metabolites are produced in response to environmental threats and stresses. Wild Cannabis varieties face more stresses and threats than the commercially grown varieties which grow in controlled environments and therefore synthesise more protective phenolic compounds. Differences in secondary metabolites among different cultivars of *C.Sativa* have been also reported by Juliano et al[47]. Our results therefore confirm the same phenomenon. The high TPC reported here corelates well with the numerous uses of *C.Sativa* in traditional medicine as a remedy for many ailments.

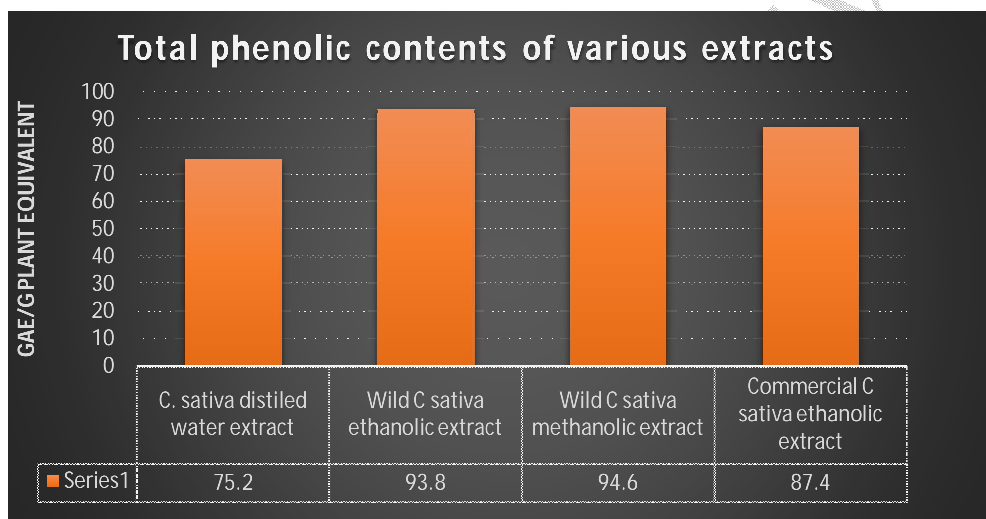


Figure 2: TPC for distilled water, hydro-ethanolic and hydro-methanolic extracts ofwild C. sativa, and the hydroethanolic extracts of commercially grown C.Sativa extracts

3.3 Biosynthesis and characterisation of *C.Sativa mediated*Cu_2O-ZnONPs

In these studies, the successful biosynthesis of Cu_2O-ZnONPs from the leaf extract of *C. sativa* through an eco-friendly easily scalable, sustainable single pot method was achieved. In the reported investigation, the multiple polyphenols in the leaf extract were the reducing and capping agents for the biosynthesis. The bio reduction mechanism involves reducing the metal ions constituting the oxides to zero valence metal NPs by the phenolic secondary metabolite's functional groups,[48]. Participating phytochemicals include polyphenolic compounds, terpenoids, flavonoids, alkaloids, steroids, saponins, tannins etc. All these were reported above to be prevalent in the lyophilised leaf extracts of *C.Sativa.* Through coordinate bonds, the -OH functional groups in these metabolites may be responsible for the bio-reduction of Zn and Cu oxides to metal oxide Nanoparticles. Through hydrolytic interactions the metal ions will be incorporated into a three-dimensional matrix with flavonoids. This matrix can break down at elevated temperatures resulting in the formation of various other polyphenolic compounds and alkaloids[49]. The biosynthesis success was first noted by the colour change which was observed during the process from dark brown to reddish brown which indicated the formation of the nanocomposites. The colour change in the material denoting formation of Cu_2O-ZnONPs, which was due to the excitation of surface plasmon vibrations was further

confirmed by the UV-Vis spectra which noted spectroscopic confirmation of ZnONPs formation with distinctive Cu_2ONPs noises within the obtained spectra. The UV–Vis absorption spectra confirmed the presence of ZnONPs, Cu_2ONPs as well as Cu_2O-ZnONPs nanocomposites. Peaks between 300 and 400 nm which are characteristic of ZnO Nanoparticles were noted. The appearance of uncharacteristic distinct peaks of low absorption intensity alongthe usual ZnONPs spectra between 345 and 370 nm and the reduction in the expected ZnONPs peak intensity confirm the inclusion of Copper in the nanocomposites[50]. The morphology and particle structure determinations were done using TEM. The TEM images of pure and copper-doped ZnONPs as shown in figure 3 confirm formation of both spherical and cubic structures. The image shows marked difference with ZnONPs from other studies and the observed influence of Cu-doping on the ZnONPs corelates very well with related findings by of Labhane*et al*[51]. The particles nano structures had diameters between 40 and 60nm but were heavily agglomerated, it was therefore very difficult to determine the average particle sizes of the Cu-doped ZnO samples. The DLS equipment gave inconsistent measurements which were statistically unrelated and cannot be reported here. The agglomeration was directly proportional to the increase in concentration of the dopant.

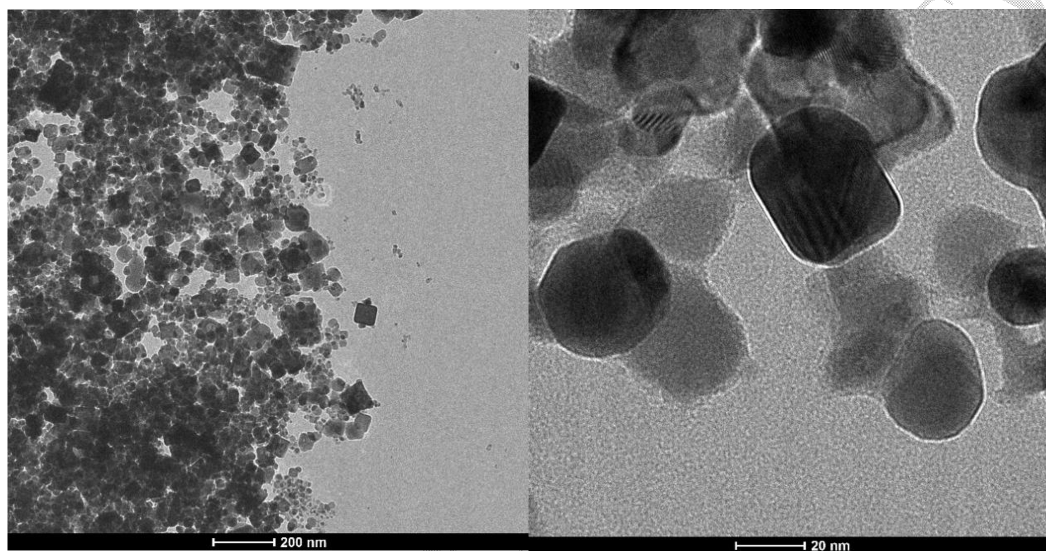


Figure 3: TEM images of C. Sativamediated Cu_2O-ZnONPs

3.4 Anti-inflammatory evaluation

Acute inflammation is usually caused by injuries and infections while chronic inflammation is mostly due to environmental toxins, lifestyle and autoimmune conditions[52]. The Inflammations which may be cutaneous, organ or joint are mostly consequent of activation by cytokines involved in enzymatic processes, fluid clearance, vasodilation as well as apoptosis and tissue damage[53].Many studies have demonstrated that extracts from medicinal plants impart activity towards the treatment of different inflammatory conditions of skin inflammation. Protein denaturation, the phenomenon that occurs when a protein loses morphology and characteristics in the presence of unfavourable conditions, is controlled by the same variables that control inflammation and follows related pathways regarding inflammatory responses. Protein denaturation is a connected response to structural damage or disease infection, or autoimmune situations characterised by a series of cellular and molecular changes aligned to inflammation[54].Plant extracts which demonstrate inhibitory action against protein denaturation are therefore potential anti-inflammatory agents. Various studies have confirmed that isolated flavonoids from plants have demonstrated inhibition of protein denaturation and haemolysis[55]. Our assay confirms that *C. Sativa* lyophilised extracts on their own have considerable anti-inflammatory activity, they also confirm that the biosynthesised Cu_2O-ZnONPs demonstrate anti-inflammatoryactivity almost comparable to the standard diclofenac. These results were expected and co relate well with published results for experiments from other studies

3.4.1 Anti‐inflammatory bioassay in vitro

Both the lyophilised leaf extracts of *C. sativa* andtheir bio mediated Cu_2O-ZnONPs at all doses above 250 g/ml exhibited significant anti‐inflammatory activities (Table 2)

Table 2: Effects of C Sativa, Cu_2O-ZnONPs and Diclofenac against protein denaturation

Concentration	% Inhibition	% inhibition	% Inhibition
µg/ml	*C.Sativa*	Cu_2O-ZnONPs	Diclofenac
250	15± 0.07	26±0.20	31±0.24
500	30±0.09	53±0.78	56±0.72
1000	88±0.14	186±2.25	204±3.82
2000	230±1.16	570±4.37	640.20± 6.24

3.5 Anti-oxidancy evaluation

DPPH scavenging activity was 93.4% for ascorbic acid (the standard used) at 150 µm/ml. While *C. Sativa* extract was 53.3% and 83.1% for the Cu_2O-ZnONPs. Activity was determined from the absorbance obtained at specific concentrations (figure 4). The IC50 values (figure 5) were 28.5µg/ml, 120.5µg/ml and 49.86µg/ml for ascorbic acid, cannabis lyophilised extract and the Cu_2O-ZnONPs,respectively.The DPPH free radical scavenging method used in our studies has been extensively utilized in determination of the antioxidant activity of plant extracts and purified compounds for decades[56,57]. This simple assay whereby plant extracts are mixed with the DPPH solution and their absorbance is measured has not undergone much modification since its development by Blois in 1958[58].Antioxidants activity with regards to DPPH radical scavenging is perhaps as a result of general antioxidants hydrogen donating capacity.The test observations involve the emergence of a stable diamagnetic molecule which vanishes the absorption band at 517 nm due to the acceptance of afree electron or a hydrogen atom. The free reactive oxygen scavenging activity therefore corresponds to the remaining DPPH in the solution in an inverse manner[57,58]. All Three samples of participating extracts and standard exhibited excellent inhibitory performance with respect to the DPPH radical. The highest activity was obtained from the standard ascorbic acid followed by the Cu_2O-ZnONPs and the lyophilised cannabis sample. The free radical scavenging activities of thesesamples corelate well with their TPCs above. It is therefore theorised that the stronger antioxidant activities of Cu_2O-ZnONPs incomparison with the lyophilisedextract is attributed to the conjugation of ZnO and the high content of phenolic compounds in wildly growing Cannabis.

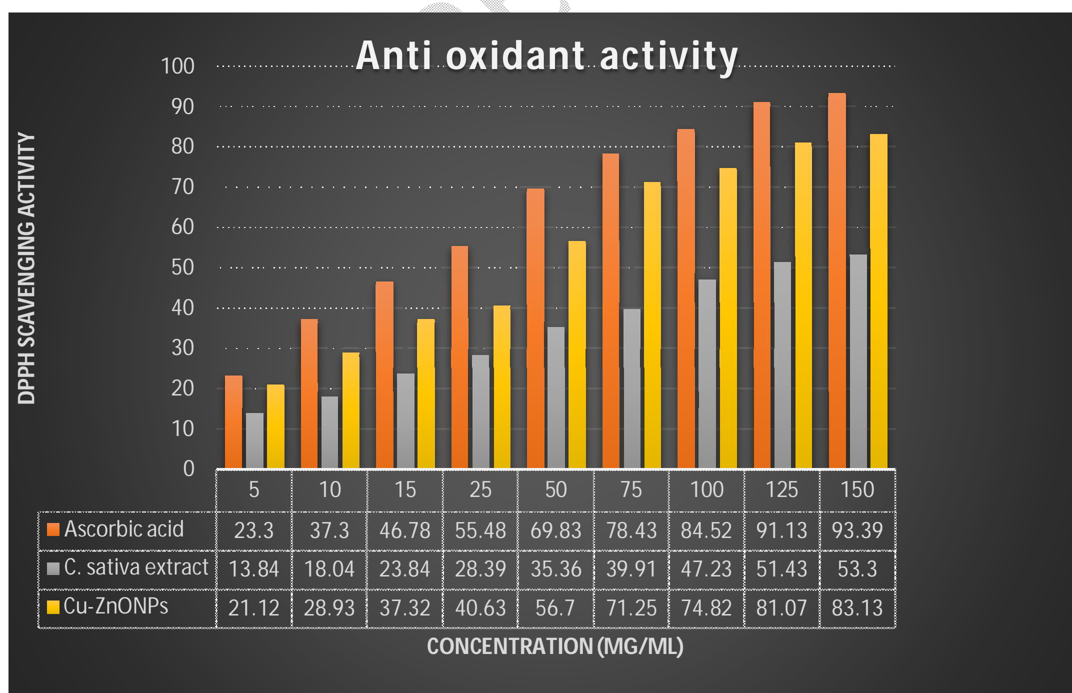


Figure 4: DPPH scavenging assay for C. sativa extract and Cu_2O-ZnONPs

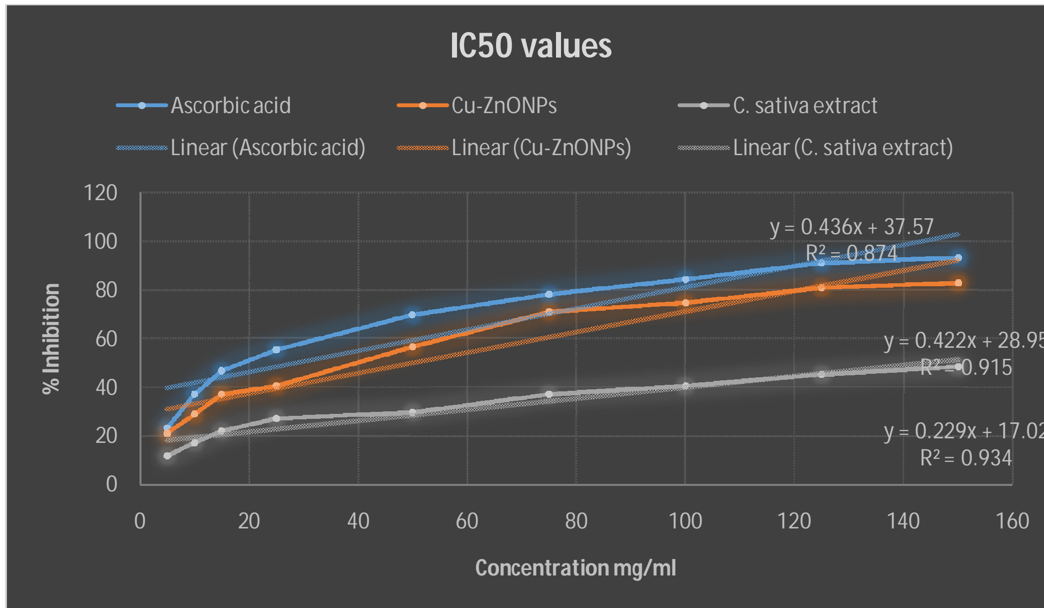


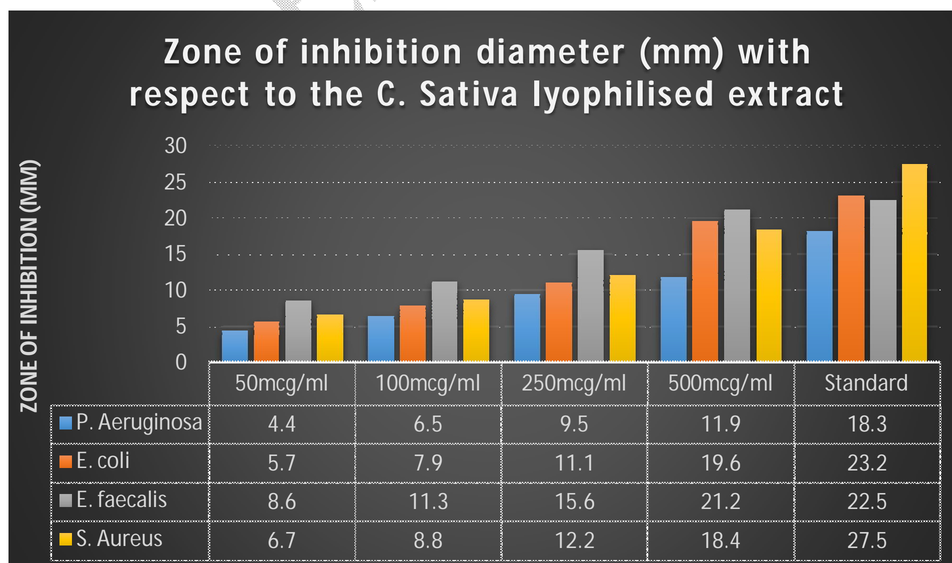
Figure 5: C. sativa and Cu_2O-ZnONPs IC_{50} values

3.6 Antibacterial evaluation

In this study, the lyophilised hydroethanolic extract of *C.Sativa* and the Cu_2O-ZnONPs, were screened for their antimicrobial activity against two Gram positive organisms (*E. faecalis, S.Aureus*), and two Gram negative organisms (*E.Coli*, *P.Aeruginosa*).

The lyophilised extracts *exerted* pronounced antibacterial activity (21.2, 18.4, 19.6mm) against *E. Faecalis,S.Aaureus*and *E.Coli*,respectively,as well as moderate activity (11.9 mm) against *P.Aeruginosa*(figure 6).The Cu_2O-ZnONPs *exerted* high antibacterial activity (19.9-24.9mm) against all tested bacterial strains (figure 7).

Figure 6 below illustrates the antibacterial activity of the lyophilised extract of *C. Sativa*. The antibacterial activity of the ethanolic extract increased with an increase in concentration of the herbal extract. Although the antibacterial activity increased with increase in concentration the standard proved to be more potent as it exhibited higher zones of inhibition against all four strains. The lyophilised extract proved to be more potent against *E. faecalis* than any other bacterial strain that was tested.These results are closely related to those reported by Novak *et al*[59]as well as Ali *et al*[60] on similar studies involving *C.Sativa* aerial plant parts extracts.



	50mcg/ml	100mcg/ml	250mcg/ml	500mcg/ml	Standard
P. Aeruginosa	4.4	6.5	9.5	11.9	18.3
E. coli	5.7	7.9	11.1	19.6	23.2
E. faecalis	8.6	11.3	15.6	21.2	22.5
S. Aureus	6.7	8.8	12.2	18.4	27.5

Figure 6: C. sativa antibacterial activity

In the case of the Cu_2O-ZnONPs, an increase in antibacterial activity (Figure 7) was observed across all four bacterial strains with an increase in concentration. The highest zone of inhibition was recorded against *E. faecalis* at 500mcg/mL. The results of the Cu_2O-ZnONPs were very closely related to the standard. The standard however recorded lower antibacterial activity against all *P. aeruginosa* and *E faecalis*. The confirmation of high antibacterial activity of Cu_2O-ZnONPs corresponds to similar studies done by Hassan *et al*.[61]

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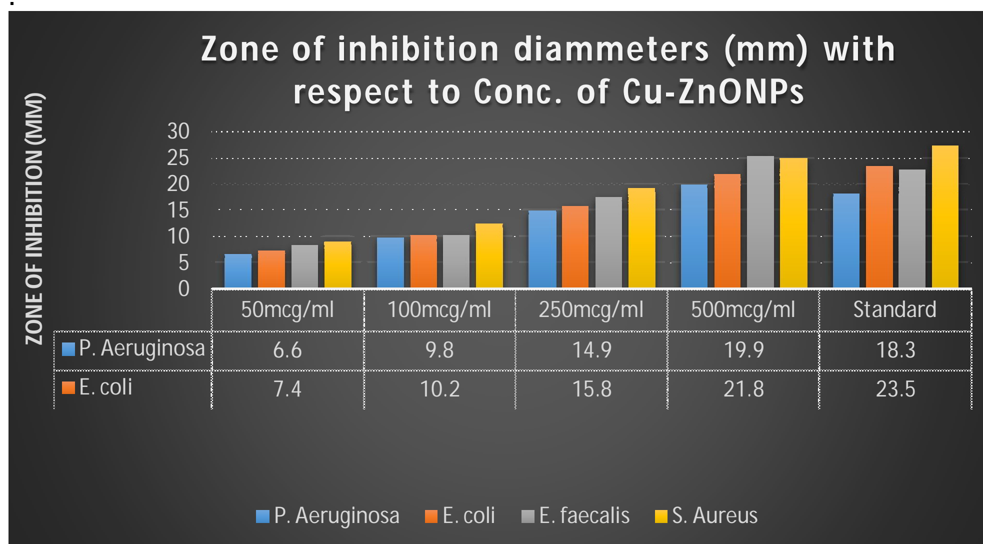

Figure 7:Antibacterial activity of Cu_2O-ZnONPs

4 Conclusions

Most studies have reviewed the biomedical potential of *C.Sativa*, biased towards the cannabinoids from seed oils and flower extracts. Our experiments were based on the biomedically forgotten leaf extracts which are apparently the basis of the use of the plant in folklore medicine. We therefore confirmed the availability of biomedically relevant secondary metabolites in the lyophilised leaf extracts as well as the abundance of functional groups capable of mediating in the biosynthesis of metal nanocomposites. ZnONPs have been largely investigated due to their physical and chemical properties which make them biomedically relevant. However conventionally obtained ZnONPs have also demonstrated limited stability, toxicity and infectivity in varying biological environments. To augment the applications of ZnONPS and to circumventthe limitations and bolster their use in nanomedicine, the environmentally friendly biosynthesis mediated by *C.Sativa* and doping seem to represent a promising solution. We therefore displayed the feasibility of biosynthesising doped ZnONPs with equally biomedically relevant CuONPs to make alloyed nanostructures with enhanced functions. We demonstrated here the amplified biomedical applications such as enhanced antimicrobial activities, anti-inflammatory, anti oxidancy as well as stable biocompatible nanostructures. The results presented above confirm that the doping of ZnONPs withCu_2ONPS is a feasible and promising tool to improve the biomedical properties and applications of both oxides.

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