RESEARCH ARTICLE

Aqueous Extracts of *Cannabis sativa*: Cytotoxicity and Effects on Angiogenesis, Oxidative Stress and Nitric Oxide Production

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Abstract *Cannabis sativa* has been used in local medicine to manage cancer, ache, inflammation, diabetes, and other conditions. The aqueous extract of *Cannabis sativa* leaves collected from two geographical locations (South Africa, EC and Lesotho, LS) was assessed for their potential against angiogenesis, nitric oxide (NO) production, oxidative stress and cytotoxicity. The EC extract showed better angiogenesis and vascular endothelial growth factor suppression than the LS extract at 20 and 100 µg/mL. EC and LS displayed their highest NO inhibitory effects (91 and 76%) at the most negligible 1.6 µg/mL concentration. The glutathione and Catalase levels increased significantly in the MCF-7 cells following administration with EC and LS. In contrast, a decrease in the malondialdehyde (MDA) level in the cells' supernatant was found compared to the untreated cells. High-performance liquid chromatography analysis reveals the presence of cannabidiol (CBD) and tetrahydrocannabinoilic acid (THCA), with EC and LS having a considerable number of similar peaks. The extracts were relatively non-cytotoxic within the concentration range evaluated (0.78125 -100–µg/mL), though selective effects on cell survival at some concentrations were observed. The study provides information to support the extensive folkloric use of the aqueous extract of *C. sativa* in cancer and other associated ailments, as well as diseases linked to oxidative stress. Studies on the aqueous extract using animal models or other relevant in vivo models are recommended.

Keywords Angiogenesis · Aqueous extracts · *Cannabis sativa* · Cancer cell lines · Cytotoxicity · Nitric oxide · Oxidative stress

Significance Statement: *C. sativa* has been used locally to treat cancer. The study provides data to support the reported folk use of the aqueous extract of *C. sativa* in cancer, oxidative stress, and other diseases. It also supports the development of medical cannabis.

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Introduction

Cannabis sativa L. (hemp, marijuana, cannabis, dagga) belongs to the family-Cannabaceae. *C. sativa* is one of the global plants that has re-emerged despite being labelled an illicit drug. *C. sativa* has gained wide publicity in recent times following its medicinal and potential source to discover modern drugs to treat and manage several human diseases. Interestingly, the revival of *C. sativa* has also been attributed to its rich repertoire of phytochemicals, amongst other attributes [1]

Traditionally *C. sativa* has been used to treat a myriad of disease conditions and utilized for other non-pharmacological purposes [2]. For centuries, Cannabis has been a narcotic drug [3] and a source of oil, food, fibre and medicine [2]. *C. sativa* has other uses, including its application as a painkiller and a soothing drug for nervous disorders. It is topically effective against corns, sores, varicose veins, gout

and rheumatism [4, 5]. The whole plant is utilized as an antiperiodic, anti-inflammatory, antispasmodic, cholagogue and diuretic. Other uses include as a hypnotic, emollient, hypotensive, laxative, ophthalmic and sedative. The plant can boost appetite and create a feeling of positive attitude in patients undergoing cancer chemotherapy. It is also used to treat glaucoma [4]. An earlier report by Odeyemi and Bradley [6] revealed that *C. sativa* is explored by the people of the Eastern Cape in South Africa to manage high blood sugar levels.

Pharmacologically, many studies have been conducted on the potential application of *C, sativa,* in medicine. Several uses and applications of *C. sativa* in folk medicines may have necessitated the throng of pharmacological studies conducted over the years. Typical pharmacological studies done include antimicrobial and analgesic activities [7], anti-diabetic [8], and anticancer activity [9–11]. Other biological studies include anti-inflammatory, muscle relaxant, and neuro-antioxidative activities [12]. *C. sativa* was also found to inhibit the proliferation of cells and the development of differentiated cells [13].

Most of the pharmacological properties adduced to *C. sativa* have been linked to either the phytocannabinoids, mainly cannabidiol (CBD), Δ9-tetrahydrocannabinol (THC), tetrahydrocannabinolic acid (THCA), cannabidiolic acid (CBDA) or further phytoconstituents including terpenes [9]. However, the cocktail of phytochemicals in terms of number and amount in *C. sativa* collected from different locations may differ, as previously reported [11]. Earlier pharmacological studies of *C. sativa* collected from different geographical regions have revealed a disparity in the biological potential of the pharmacological parameters assessed [10]. It is pertinent to note that the array of studies done on *C. sativa* has been limited to CBD and THC as the main cannabinoids determining the medicinal and psychoactive effects of the plant.

A depth of information exists on traditional medicine practitioners widely used extractant (water) in producing commercialized Cannabis products globally. Reports on previous studies on *C. sativa* show that most scientific validation studies rarely use water as an extractant despite its frequent use in folk medicine. It has been observed that many of the studies have been directly or indirectly necessitated by the folk use of *C. sativa*. This study tends to provide the scientific merits of the biological potential of the aqueous extract, which is currently used in folk medicine as a basis for its possible use in the pharmaceutical formulation of commercial cannabis products against cancer cell growth, its progression and other related predisposing factors.

In this study, the aqueous extracts of *C. sativa* leaves, collected from the Eastern Cape (EC), South Africa and Mohale's hoek, Lesotho (LS), were investigated for anti-angiogenic activity and their effects on nitric oxide (NO),

vascular endothelial growth factor (VEGF) and oxidative stress. The cytotoxic effects of the aqueous extracts on cell growth and proliferation in determining the potential anticancer effects of the plant were considered. Additionally, a comparative analysis of the biological potential of the extracts of *C. sativa* leaves obtained from the two geographical locations (South Africa and Lesotho) was also explored in the study. This study is predicated on the ongoing development of *C. sativa* as a herbal formulation purported against its anticancer uses.

Material and Methods

Collection and Extraction of Plant Material

C. sativa leaves were collected from Mohale's Hoek District, Lesotho (GPS Coordinates: − 30.333776″S and 27.651201″E) with permit number- (Permit #: 01/LS/2019/10/02–01) in July 2016 and Port St Johns, Emampondweni in the Eastern Cape Province of South Africa (GPS Coordinates: –31.63846″ S, 29.53627″ E) with permit number-(Permit No. POS 248/2019/2020) in May 2016 for research. The plant materials were authenticated at the Geo Potts Herbarium, University of the Free State, South Africa, with a voucher specimen number BLFU MGM 0018. The air-dried *C. sativa* leaves at ambient temperature were blended to a fine powder (80 g) prior to extraction in a ratio of 1:5 (w/v) using distilled water (400 mL). The extraction was over 72 h at room temperature with mild agitation in a mechanical shaker set at 100 rpm. The extracts were concentrated using a freeze dryer to yield EC and LS extracts stored in glass vials and kept in a cold room.

Determination of Anti-Angiogenic Activities

Chicken Chorioallantoic Membrane (CAM) Assay

The CAM assay was done following earlier reported methods with slight modifications [10, 14]. Initially, fertile eggs (pathogen-free) were purchased from a farmer in Bloemfontein, South Africa. The eggs were left on the laboratory bench for brief acclimatization, after which they were cleaned with 70% ethanol and candled. For eight(8) days, the eggs (with the sharp end down) were incubated at 37 °C with an average humidity of 64.8%. The eggs were rotated automatically every hour in the incubator with constant circulating air (Surehatch, Model No 450 COM, Serial No. SH 364G). On the 8th day, an approximate 1 cm^2 hole was aseptically opened in the eggs (air space side). The previously sterilized 1 cm^2 Whatman filter papers (Sigma-Aldrich, USA), soaked with the extracts (20 μg/CAM), Tinzaparin (20 μg/CAM—positive control), and L-Arg

(20 μg/CAM- negative control), were then placed on the surface of the exposed developing CAM vessels with the eggs properly labelled and re-sealed with adhesive tape under an aseptic condition in a laminar flow hood. Only the eggs ($n = 3$) with visible CAM development were returned aseptically to the incubator until day 11 without rotation or disturbance. On the 11th day, each of the eggs with CAM was carefully opened in sterile Petri dishes, and the formed blood vessels in each CAM were observed and counted. The blood capillaries extending from the main blood vessel were counted to calculate the average number of blood capillaries (angiogenic index). All CAMs were photographed for future reference.

Vascular Endothelial Growth Factor (VEGF) Inhibition Assay

The extracellular VEGF levels were measured using the supernatant of treated breast cancer (MCF-7) cells with the extracts [10]. Total VEGF content in cultured supernatants was estimated as per the manufacturer's instruction of the Human VEGF ELISA kit (ThermoFisher Scientific, Cat. No KHGO111).

Inhibition of Nitric Oxide (NO) Production in LPS-Induced MCF-7 Cells

Cell Culture

The MCF-7 breast cancer (ATCC® HTB-22™) cells purchased from American Type Culture Collection (ATCC, Virginia, USA) were used in this study and maintained in Eagle's Minimum Essential Media (EMEM) supplemented with 10% Foetal bovine serum (Gibco, ThermoFisher) and 1% penicillin/streptomycin/fungizone solution (PSF) in an incubator set at 37°C and a 5% CO_2 atmosphere. The MCF-7 cells were seeded at 4×10^4 cells/well density into columns 2 to 11 of a sterile cell culture 96-well plate (NEST, Whitehead scientific). The treated plates were kept for 24 h at 37°C in a 5% CO_2 incubator to allow cell adherence. After 24 h of incubation, the media from the plate were aspirated from all the wells and replaced with 200 μL of fresh medium. The cells were treated with lipopolysaccharide (LPS) at 1 μg/mL and the extracts' different concentrations (1.6–100 μg/mL) and incubated for 24 h.

Measurement of Nitrite

The nitric oxide assay was achieved via slight modifications to earlier methods [15]. The amount of nitrite was measured in the culture supernatant using Griess reagent (Sigma-Aldrich). The concentration of nitrite in the culture media was used as an indicator of NO production. The percentage of NO inhibition was calculated relative to the untreated LPS-induced cells, and a NO inhibition greater than 70% was considered potent NO inhibition [16].

Cell Viability Assay

Cell viability was ascertained via the mitochondrial reduction of 3-(4, 5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) to formazan [17]. Following incubation for 24 h with 1 μg/mL LPS and the different concentrations of the extracts, the medium was aspirated from all the wells. The MCF-7 cells were carefully washed with pre-warmed PBS (200 μL), and 100 μL of fresh media was added. MTT (to a final concentration of 0.5 mg/mL) was added to all the wells, and the cells were incubated for 4 h at 37°C in 5% CO_2. The medium was removed from all the cells, and dimethylsulphoxide (DMSO) was added to solubilize the formazan salt precipitates. The absorbance was measured at a wavelength of 540 nm. The percentage of cell viability was determined relative to the control (untreated cells with LPS taken as 100% viability).

Antioxidative Stress Studies

MCF-7 cells were maintained, incubated, and treated with the extracts (12.5–100 μg/mL) as described above. They were seeded, and the supernatant was used for the antioxidative studies.

Reduced Glutathione (GSH) Level

The level of GSH in the cells was ascertained via Ellman's method [18]. First, the cells were deproteinized with 10% tricarboxylic acid (TCA) and centrifuged at 3500 rpm for 5 min. After that, the resulting supernatants (200 μL) were collected in a 96-well plate and mixed with Ellman reagent (50 μL). After allowing it to stand for 5 min, absorbance was read at 415 nm. A standard curve of GSH concentrations was used in extrapolating the GSH level.

Catalase Activity   The cells' catalase activity was defined using Aebi's adapted method [19]. Briefly, 10 μL of the cells were incubated with 340 μL of 50 mM sodium phosphate buffer (pH 7.0) and 150 μL of 2 M H_2O_2 for 5 min. Absorbance was measured at 240 nm at 1 min intervals for 3 min.

Lipid Peroxidation Levels   The cells' lipid peroxidation levels were determined by assaying for the thiobarbituric acid reactive substances (TBARS) expressed as malondialdehyde (MDA) equivalent [20].

HPLC (High-Performance Liquid Chromatography) Analysis of *C. sativa* Extracts

The Eastern Cape, South Africa (EC) and Lesotho (LS) aqueous leaf extracts were analysed using HPLC for fingerprinting and detection of cannabidiol (CBD) and tetrahydrocannabinoilic acid (THCA). HPLC coupled with diode array detector (DAD) analysis was carried out using an Agilent 1100 series (Agilent, Waldbronn, Germany) instrument equipped with a photodiode array and autosampler column thermostat, and degasser. The extracts and standards (CBD and THCA) were analysed at a 1 mg/mL concentration. The stationary phase was a Luna omega 3 μm C_{18} (150×4.6 mm; 5 μm particle size) column. The mobile phase was combined 75% acetonitrile (A) and water with 0.1% Formic acid (B). An isocratic run of (A) and (B) at a 1 ml/min flow rate for 20 min was performed at 22 °C. An injection volume of 50 μL was utilized, and chromatograms were recorded at 254 nm.

Cytotoxicity

Cell Lines and Cell Culturing

The normal human embryonic kidney (HEK) 293 (ATCC® CRL-1573™), human DU-145 prostate cancer (ATCC® HTB-81™), and MCF-7 breast cancer (ATCC® HTB-22™) cells were obtained from the American Type Culture Collection (ATCC, Virginia, US). The cell lines were maintained as previously described elsewhere [17].

Cell Proliferation Assay

The 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) method was utilized to determine the effect of the extract on cell viability [21]. Cells were seeded in 100 μL medium in 96-well microtiter plates at 1×10^5 cells/well. Serial dilutions of the plant extract in a range of 100–0.78125 μg/mL were made to achieve target final concentrations. Afterwards, cells were exposed to the extracts and the controls, including vehicle-treated cells exposed to distilled water (dH₂O), propagated cells in the growth medium, and exposed cells to the positive control, doxorubicin, within the same concentration range used for the samples. After the 24 h treatments, the cells were subjected to the MTT reagent (0.5 mg/mL). The colorimetric reaction was measured using a plate reader (Multiskan Go, Thermo Fischer Scientific) set at 570 nm wavelength. The samples were assessed in three independent experimental repeats, and each was evaluated in triplicate. The results represent the average percentage inhibition of all the experimental

repeats. The fifty per cent inhibitory concentrations (IC_{50} values) were determined with the GraphPad Prism 4 program (version 4).

Statistical Analysis

Values are presented as mean ± SEM or mean ± SD where applicable. A standard curve and linear and nonlinear regression curves using excel were plotted where necessary to determine and extrapolate IC_{50} and other assessed parameters. Percentages were also determined. Further statistical analysis with One Way ANOVA was done to ascertain the differences in the means of the different tested samples with Post-Hoc tests (LSD, Turkey HSD, and Duncan's multiple range test) using SPSS 27.0 computer software package. Differences at $P \leq 0.05$ were considered statistically significant.

Results and Discussion

One of the claimed possible health benefits with well-documented pharmacological studies of *C. sativa* is its use in treating and managing cancer and diabetes [6, 10, 11]. Literature reveals that past studies on *C. sativa* rarely use water as an extractant despite its everyday use in traditional medicine practices. It has been observed that many of the studies have been necessitated by the folk use of *C. sativa*. This research tends to determine the biological capacity of the aqueous extracts, commonly explored in folk medicine, to support their possible use in the pharmaceutical formulations of commercial cannabis products.

Percentage Yield of *Cannabis sativa*

The percentage yield (7.10–11.08%) of the extracts utilizing an extraction ratio 1:5 of *C. discuss* was reported by Bala et al. [18], compared to a range of 1.91–7.45% of *C. sativa* samples collected from similar locations albeit at different times. The variations could be from organic solvents like methanol, dichloromethane, and n-Hexane or the extraction ratios (1:1 and 1:3) used.

Effect of *C. sativa* Extracts on CAM

The average fertility rate of the pathogen-free eggs was 83.33%, where $n = 3$. A widely known angiogenesis inhibitor, Tinzaparin, showed the highest inhibitory effect against the formation of blood vessels at a concentration of 20 μg/mL with an angiogenic index of 14 (Fig. 1d and 2). Of the two collected samples of *C. sativa*, EC with an angiogenic index of 21 (Fig. 1c and 2) had a better effect on the inhibition of blood vessel formation than the LS sample (angiogenic index, 32) (Fig. 1b and 2). L-Arginine, used
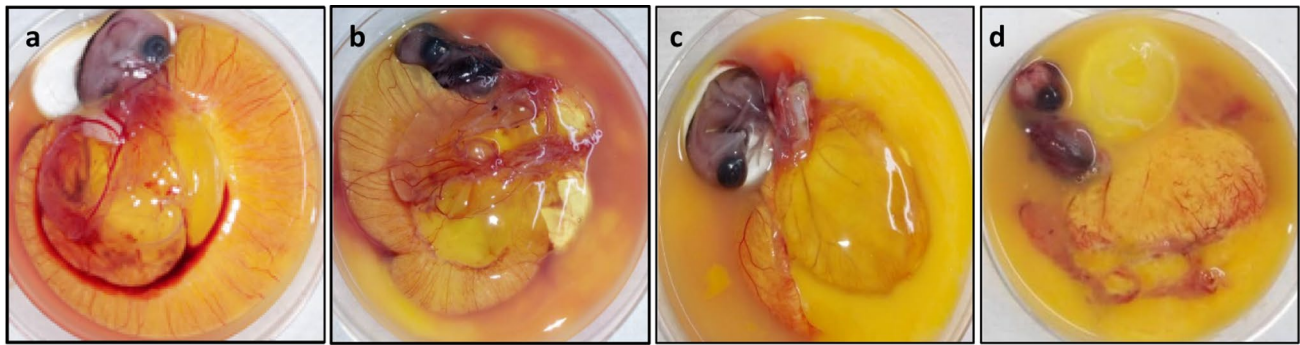
Fig. 1 Typical results of the Chicken chorioallantoic membrane (CAM) assay with images of the treated eggs opened on day 11. **a**: L-Arginine (20 µg/mL), **b**: LS (20 µg/mL), **c**: EC (20 µg/mL), **d**: Tin- zaparin (20 µg/mL). EC, aqueous extracts of leaves collected from Eastern Cape, South Africa, and LS, aqueous extracts of leaves col- lected from Lesotho, $n = 3$
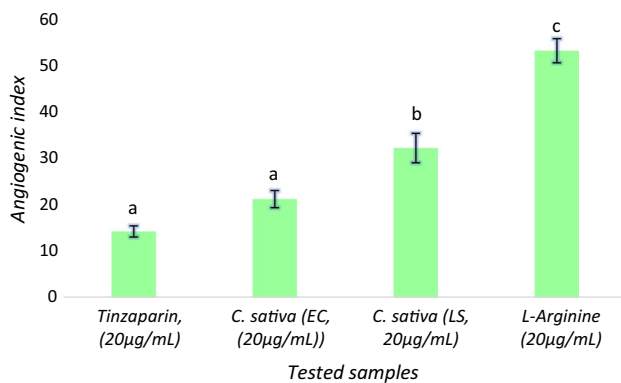

Fig. 2 Angiogenic index of *C. sativa* aqueous extracts collected from Eastern Cape (EC), South Africa, and Lesotho (LS) compared to Tin- zaparin and L-Arginine (positive and negative controls). Angiogenic index (number of blood capillaries extending from the blood ves- sels' main branch). Values with similar alphabets are non-significant ($P \geq 0.05$), while values with different alphabets are significantly dif- ferent ($P \leq 0.05$), $n = 3$

for inducing blood vessel formation, had the highest angio- genic index of 53 (Fig. 1a and 2). The angiogenic inhibitory effect of Tinzaparin was significantly not different ($P \geq 0.05$) from EC. Still, the effect of the duo (tinzaparin and EC) was significantly ($P \leq 0.05$) different from LS and L-Arginine (Fig. 2).

Of the many drug targets for cancer chemotherapy, the tumour vasculature has increasingly become a voluptuous target rather than the tumour cells due to proven advantages [22]. Following this knowledge, a couple of angiogenesis assays were developed [23]. Of these assays, the CAM has become a primary method to assess the development of angi- ogenesis and evaluate the effect of pro-angiogenic and anti- angiogenic factors [14]. In this study, the extracts (EC and LS) of *C. sativa* exhibited a considerable level of inhibitory effect against the formation of blood vessels when compared to the positive control (Tinzaparin) and L-Arginine (blood

vessel formation stimulator), as displayed in Figs. 1 and 2. Comparatively, the EC extract had a better anti-angiogenic effect than the LS extract (Figs. 1 and 2). This result corrob- orates earlier reports elsewhere [10] on the anti-angiogenic potential of *C. sativa* leaf methanolic and DCM extracts col- lected from Eastern Cape, South Africa. However, the anti- angiogenic reports on the methanol extract of *C. sativa* leaf collected from Lesotho by Bala et al. [10] were less effective than the aqueous extracts in the current study for preventing blood vessel formation. This disparity may likely be due to the synergistic activities of the cocktail of phytochemicals extracted by the solvents. Sustained angiogenesis has been reported to be a sinequanon in tumour growth, invasion, and metastasis [24]. The ability of EC and LS to stall the growth of blood vessel formation probably confirms earlier claims of the use of *C. sativa* in cancer treatment [10, 11, 13]. The anti-angiogenic effects of the EC sample may be a probable mechanism for halting tumour growth and progression. LS sample may be developed for managing diabetic-induced gangrene following its poor anti-angiogenic effects (stimula- tion of angiogenesis) (Figs. 1 and 2).

Effect of *C. sativa* Extracts on VEGF

A standard curve of VEGF (0–1.5 ng/mL) with an R^2 of 0.9146 was utilized in estimating the extracellular VEGF. The VEGF levels in the MCF-7 cultured cell superna- tant were determined by extrapolating the standard curve ($y = mx + b$). At 100 µg/mL, EC had the greatest inhibi- tion against the VEGF level (Fig. 3). The LS at a 50 µg/mL concentration was also found to reduce the VEGF levels compared to the control (distilled water). At 50 and 100 µg/ mL, both extracts (EC and LS) had no significant differ- ence ($P \geq 0.05$) in their inhibitory effect against extracellular VEGF levels except at 12.5 and 25 µg/mL. EC and LS were significantly different ($P \leq 0.05$) across the concentrations from the control (Fig. 3).
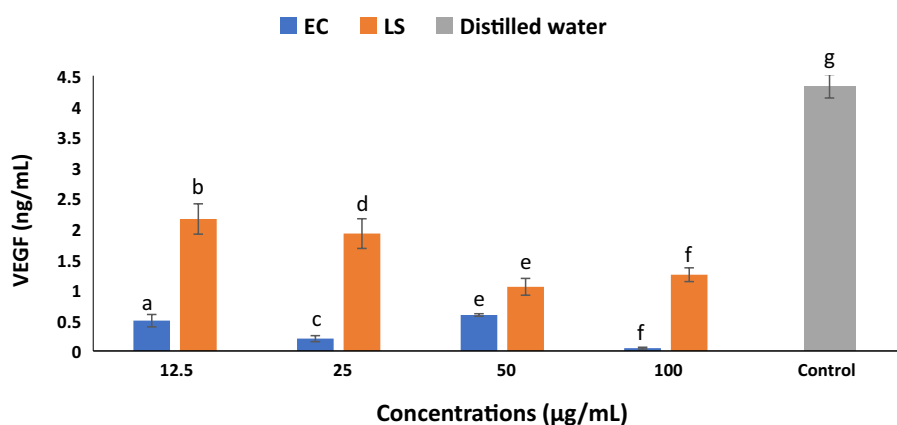
Fig. 3 Inhibition of VEGF in MCF-7 cultured supernatant at different concentrations of *C. sativa* extracts. **EC**, the aqueous extract of *C. sativa* leaf from Eastern Cape, South Africa; **LS**, the aqueous extract of *C. sativa* leaf from Lesotho. **Control** = distilled water. Low VEGF concentration (ng/mL) indicates high inhibition by samples. Different alphabets at a given concentration between the extracts and comparatively with control indicate a significant difference ($P \leq 0.05$). Experiment is repeated three times in duplicates

Vascular endothelial growth factor (VEGF) is a signalling protein involved in one of the multi-steps of vasculogenesis and angiogenesis. Though profoundly associated with the proliferation of cancer cells, VEGF has been linked to other ailments like diabetes, bronchial asthma, and inflammation [25]. Generally, VEGF, expressed in certain cancers, enables the cancerous cells to grow and metastasize. Furthermore, angiogenesis inhibition represents an appealing emergent target to prevent the uncontrollable proliferation of cancer cells. CBD, a major phytochemical of *C. sativa,* has effectively inhibited angiogenesis, as indicated elsewhere [13]. Though EC and LS exhibited an inhibitory effect against VEGF levels compared to the control, the EC extract expressed a better VEGF inhibition at 100 µg/mL. At 50 µg/mL, LS had its most significant VEGF inhibition, indicative of twice the VEGF inhibition compared to EC. However, these effects were non-concentration-dependent inhibition of VEGF (Fig. 3). Earlier reports [10, 11] on the dichloromethane extract of *C. sativa* leaves collected from South Africa, had inhibitory activity against VEGF, which corroborates the results of this current study.

In contrast, LS activities against VEGF in the current study were dissimilar to the inhibitory activity of the extracts of LS reported by Bala et al. [10]. The capacity of the extracts to inhibit extracellular VEGF is indicative of their probable ability to inhibit angiogenesis, which remarkably is one of the many mechanistic steps for tumour growth, invasion, and metastasis in most cell types. Thus, further indicating the anti-angiogenic effect of the *C. sativa* aqueous extract.

Nitric Oxide Inhibition

The inhibitory activity of the aqueous extracts of *C. sativa* on NO production in LPS-induced MCF-7 cells is presented in Table 1. The EC had the best inhibitory activity on NO production (91.89% inhibition/ 93.90% cell viability) at 1.6 µg/mL, compared to LS with 76.19% inhibition and 93.43% viability at the same concentrations, respectively. The percentage viability of the cells ranged from 79–93%. Interestingly, the EC extract stimulated NO production but significantly inhibited NO production, similar to the LS extract at lower concentrations.

In the current study, both EC and LS, at differing concentrations, had moderate to high nitric oxide (NO) inhibitory activities against LPS-stimulated NO production in MCF-7 cells (Table 1). As reflected by the percentage viability, the

Table 1 Inhibitory activities of the aqueous extracts of *C. sativa* (EC and LS) leaf on LPS-induced NO production in MCF-7 cells

Samples	Concentrations (µg/mL)	Nitric oxide Inhibition (%)	Cell viability (%)
C. sativa (EC)	1.6	91.89 ± 8.43	93.90 ± 2.13
	12.5	19.82 ± 3.55	87.74 ± 2.85
	50	< 0	85.97 ± 4.71
	100	< 0	90.77 ± 2.32
C. sativa (LS)	1.6	76.19 ± 7.02	93.43 ± 0.55
	12.5	74.07 ± 0.36	83.30 ± 0.22
	50	54.93 ± 9.53	79.04 ± 0.97
	100	43.25 ± 2.66	93.62 ± 5.40
*Control		0.00	100.00

Mean ± SEM; $n = 3$, EC – *C. sativa* leaves collected from Eastern Cape, South Africa; LS- *C. sativa* leaves collected from Lesotho; *Untreated LPS-induced cells

survival of the cells dispels any doubt that the NO suppression was due to the inhibition of cell growth and proliferation by the aqueous cannabis extracts (Table 1). Previous reports have demonstrated NO's importance in many biological processes and diseases like cancer, hypertension, and oxidative stress [26]. NO plays a critical role in VEGF-induced angiogenesis. The attenuation of angiogenesis has been linked to reduced NO bioactivity [27–29]. This also indicates that VEGF stimulates the release of NO [29, 30]. Also, NO is said to influence angiogenesis by suppressing the synthesis of angiostatin [31]. Contrastingly, NO production by the endothelium suggests arterial health, yet it mediates angiogenesis. Despite the conflicting role of NO in some biological processes, the potential of cannabis extracts to inhibit NO expression is supportive of their probable ability against the inhibition of VEGF and angiogenesis, as indicated in this study. This probably explains the claimed use of *C. sativa* in cancer treatment.

Effect of *C. sativa* Extracts on Oxidative Stress in MCF-7 Cells

As depicted in Fig. 4, treatment with the *C. sativa* aqueous extract led to a significant increase (P ≤ 0.05) in the cells' GSH levels compared to the untreated cells (Fig. 4). Both extracts (EC and LS) at 12.5 and 25 μg/mL were significantly ($P \geq 0.05$) not different from the positive control (doxorubicin). There was a corresponding significant increase in catalase activity (Fig. 5), with concomitant depletion of MDA levels (Fig. 6) in extract-treated cells. At 12.5 and 25 μg/mL, the increase in catalase activity of EC and LS extracts were significantly ($P \geq 0.05$) different

from each other. In contrast, at 50 and 100 μg/mL, no significant ($P \leq 0.05$) difference was noticed. The depletion of MDA levels in extract-treated cells at each of 12.5, 50 and 100 μg/mL of both extracts were significantly ($P \geq 0.05$) not different.

Oxidative stress has been connected to the pathogenesis and progression of breast cancers, playing a significant role in their proliferation [32]. The untreated cells' decreased GSH level and catalase activity depict oxidative stress (Figs. 4 and 5). This is further supported by the high MDA level, which indicates lipid peroxidation (Fig. 6). The reversion of these levels and activity following treatment with *C. sativa* extracts depicted the antioxidative effects of the extracts in MCF-7 cells. This corroborates a previous report on the antioxidative activity of medicinal plants in breast cancer cells [33]. In a different postulation, VEGF is said to induce endogenous synthesis of reactive oxygen species (ROS) via NADPD (nicotinamide adenine dinucleotide phosphate) oxidase (Nox) [34]. The constant production of ROS causes an imbalance in redox homeostasis, leading to oxidative stress, which has been implicated in the promotion of angiogenesis [35]. This is characterized by suppressed levels of GSH and catalase activity, with concomitant production of the peroxidative marker MDA [36]. Thus, leading to the progression and proliferation of cancer cells. This assertion further supports the potential of *C. sativa* extract in combating oxidative stress, cancer and associated diseases, as displayed in this study. Additionally, this antioxidative activity may contribute to the antiproliferative mechanism of *C. sativa* aqueous extract because antioxidants have been reported for their antiproliferative effect against cancers [37].
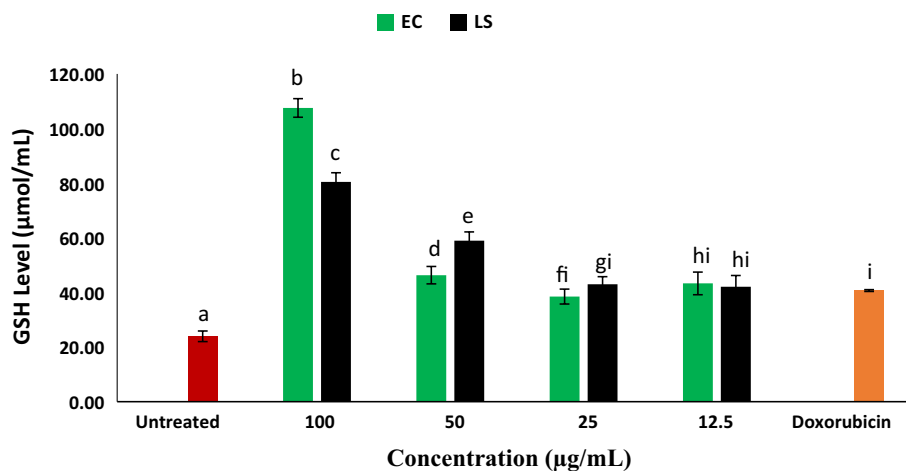

Fig. 4 Effect of aqueous extracts of *C. sativa* leaf on GSH levels in the supernatant of treated MCF-7 cells. $n = 3$. **EC,** *C. sativa* leaves collected from Eastern Cape, South Africa; **LS-** *C. sativa* leaves collected from Lesotho. **Doxorubicin,** 100 μg/mL. Different alphabets at a given concentration between the extracts and comparatively with the control (untreated) indicate a significant difference ($P \leq 0.05$). A similar alphabet between doxorubicin and any samples shows a non-significant difference ($P \geq 0.05$). Experiment is repeated three times in triplicates
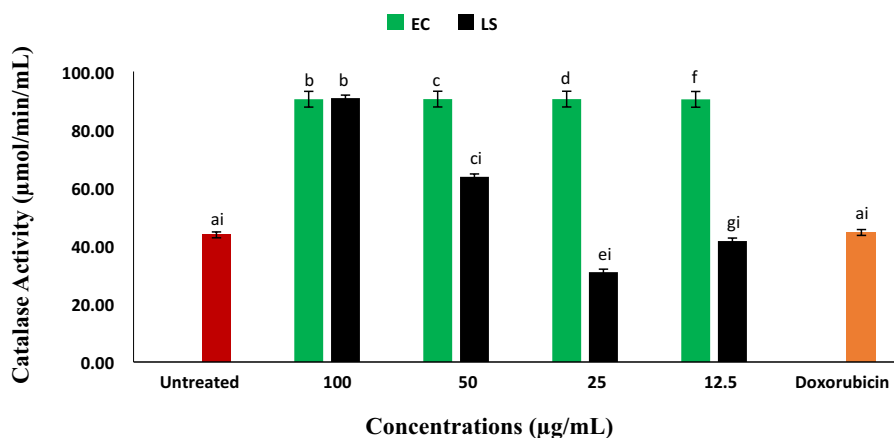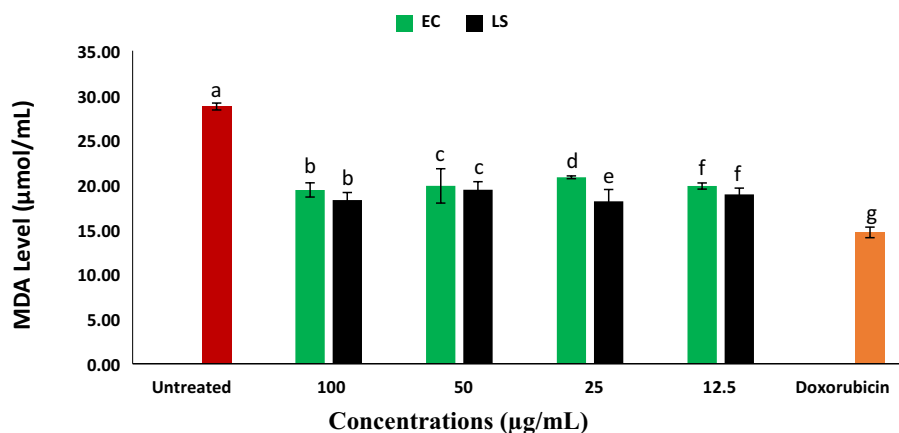
Fig. 5 Effect of aqueous extracts of *C. sativa* leaf on Catalase levels in the supernatant of treated MCF-7 cells. $n = 3$, **EC-** *C. sativa* leaves collected from Eastern Cape, South Africa; **LS**- *C. sativa* leaves collected from Lesotho. **Doxorubicin,** 100 µg/mL. Different alphabet(s) at a given concentration between the extracts and comparatively with the control (untreated) indicate a significant difference ($P \leq 0.05$). Similar alphabet(s) between doxorubicin and any of the samples suggests a non-significant difference ($P \geq 0.05$). Experiment is repeated three times in triplicates

Fig. 6 Effect of aqueous extracts of *C. sativa* leaf on MDA level in the supernatant of MCF-7 cells. $n = 3$. **EC,** *C. sativa* leaves collected from Eastern Cape, South Africa; **LS**- *C. sativa* leaves collected from Lesotho. **Doxorubicin,** 100 µg/mL. Different alphabet(s) at a given concentration between the extracts and comparatively with the control (untreated) indicate a significant difference ($P \leq 0.05$). Similar alphabet(s) between doxorubicin and any of the samples indicates a non-significant difference ($P \geq 0.05$). Experiment is repeated three times in triplicates

HPLC Fingerprinting of *C. sativa* Extracts

The HPLC chromatograms of the aqueous extracts of *C. sativa* collected from Eastern Cape (EC) and Lesotho (LS) are presented in Supplementary Figures S1 and S2. CBD and THCA were respectfully identified in EC and LS at slightly varying retention times (Table 2).

HPLC spectra of the aqueous extract of EC and LS displayed several peaks with similar and slightly differing peak retention times. EC had 42 peaks compared to LS, with 39 peaks (Supplementary Figures S1 and 2). The similarity in peaks of the two extracts indicates the plant's relatively close taxonomic hierarchy. The slight dissimilarity in peaks

Table 2 HPLC retention times of identified CBD and THCA in the aqueous extracts of EC (Eastern Cape, South Africa *C. sativa* leaves) and LS (Lesotho *C. sativa* leaves)

Compounds	Parameters	Extracts	
		EC	LS
CBD (RT: 6.716)	RT (min)	6.755	6.811
	DFS (min)	+0.039	+0.095
THCA (RT:14.956)	RT (min)	14.902	15.125
	DFS (min)	-0.054	+0.169

RT, Peak Retention Time; *CBD*, Cannabidiol (CBD); *THCA*, tetrahydrocannabinoilic acid; *DFS*, Difference between the peak retention time of the compounds and the extracts

may be due to the different collection locations. A myriad of peaks suggests the probable potential of the extracts to have a cocktail of bioactive constituents responsible for the array of biological activities.

Cytotoxicity of *C. sativa* Extracts

The effect of the aqueous extracts of *C. sativa* leaf against a normal Human embryonic kidney (HEK) 293 cell line and two cancer cell lines (DU-145 and MCF-7) are shown in Table 3. The extracts had little or no antiproliferative effect against the three cell lines with IC_{50} values greater than 100 μg/mL compared to the positive control-doxorubicin (an antibiotic that slows or stops the proliferation of cancer cells) with an IC_{50} ranging between 12–50 μg/mL (Table 3). The extracts had a selective growth inhibitory activity against the cancer cell lines (Supplementary Figure S3). The EC extract showed growth inhibitory activity at 50 μg/mL (26.69%) against the DU-145 cell line. The LS had the best growth inhibitory effect on the DU-145 cells with 33.54% cell growth and proliferation inhibition at 50 μg/mL (Supplementary Figure S3a), while at 12.5 and 100 μg/mL, had selective growth inhibitory values of 6.55% and 16.98% against MCF-7 cells (Supplementary Figure S3b).

The safety levels of herbal medicines such as Cannabis products are of huge concern as minimal studies are available on the safe use of these products [38]. In the current study, the aqueous extracts (EC and LS) of *C. sativa* had low to moderate cytotoxic activity against the three cell lines (HEK-293, MCF-7 and DU-145) with IC_{50} values above 100 μg/mL (Table 3). This report is similar to an earlier study by Bala et al. [10, 11] on the dichloromethane extracts of *C. sativa* with no profound cytotoxic effect against MCF-7 and MDA-231 cell lines after exposing the cells to the extracts for 24 h. Additionally, some cytotoxic activity was observed on the same extracts in the same cell lines after 48 h. This could indicate that *C. sativa* extracts might have a time-dependent

antiproliferative effect on breast cancer cell lines. It was also noted that the EC and LS extract similar to those in previous studies [10, 11] of *C. sativa* against MCF-7 and MDA-231 cancer cells stimulated the proliferation of the MCF-7 and DU-145 cell lines at specific concentrations (Supplementary Figure S3). The low cytotoxicity of the extracts and their inhibitory effects on NO, VEGF, and blood vessel formation makes them very useful for progressive drug development.

Conclusion

Summarily, the present study has shown the potential application of the aqueous extracts of *C. sativa* collected from two distinct geographical locations (South Africa and Lesotho) against angiogenesis, VEGF and NO expression and oxidative stress. The leaves of *C. sativa* collected from the Eastern Cape, South Africa (EC), had a better over-arching biological capacity against the assessed pharmacological parameters than those collected from Lesotho (LS). These differences could be attributed to the different climatic and soil conditions under which these plants were grown as one possible explanation. The genetic makeup of these plants needs to be studied to determine any similarities or differences that may also account for the observed slight pharmacological activity difference between EC and LS plants. The study has provided useful information that will significantly assist in the herbal formulation of the plant in managing an array of diseases. Therefore, this study supports the traditional use of *C. sativa* as an anticancer agent and the associated biological processes of other diseases. However, further research is required to confirm these activities in an in vivo experimental model. It is hoped that the study will spur more research on the aqueous extracts of *C. sativa* and their application in medical cannabis development. Further studies using in vivo models and clinical studies are strongly advocated.

Table 3 Cytotoxicity (IC_{50}) of the aqueous extracts of *C. sativa* leaf collected from two geographical areas (Lesotho and South Africa) against HEK-293, DU-145, and MCF-7 cells

Samples	IC_{50} (μg/mL)		
	HEK-293	DU-145	MCF-7
Cannabis sativa (EC)	>100	>100	>100
Cannabis sativa (LS)	>100	>100	>100
*Doxorubicin	12.64 ± 2.61	15.69 ± 3.80	50.08 ± 3.50

*Positive control, Values in bold represent the highest concentration of the extracts evaluated where **IC_{50}** values exceeded the highest concentration. Experiment is repeated three times in triplicates

Declarations

Conflict of interest   The authors affirm that they have no conflict of interest.

Ethical Approval   Not Applicable.

Consent to Participate   Not Applicable.

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