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Research article

Cannabis sativa L. modulates altered metabolic pathways involved in key metabolisms in human breast cancer (MCF-7) cells: A metabolomics study

Ochuko L. Erukainure [a], Omolola R. Oyenihi [a], James F. Amaku [b], Chika I. Chukwuma [c], Adeline Lum Nde [a], Veronica F. Salau [a], Motlalepula G. Matsabisa [a,*]

[a] *Department of Pharmacology, School of Clinical Medicine, Faculty of Health Sciences, University of the Free State, Bloemfontein 9300, South Africa*
[b] *Department of Chemistry, Michael Okpara University of Agriculture, Umudike, Abia State, Nigeria*
[c] *Center for Quality of Health and Living, Faculty of Health Sciences, Central University of Technology, Bloemfontein 9301, South Africa*

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ABSTRACT

The present study investigated the ability of *Cannabis sativa* leaves infusion (CSI) to modulate major metabolisms implicated in cancer cells survival, as well as to induce cell death in human breast cancer (MCF-7) cells. MCF-7 cell lines were treated with CSI for 48 h, doxorubicin served as the standard anticancer drug, while untreated MCF-7 cells served as the control. CSI caused 21.2% inhibition of cell growth at the highest dose. Liquid chromatography–mass spectroscopy (LC-MS) profiling of the control cells revealed the presence of carbohydrate, vitamins, oxidative, lipids, nucleotides, and amino acids metabolites. Treatment with CSI caused a 91% depletion of these metabolites, while concomitantly generating selenomethionine, l-cystine, deoxyadenosine triphosphate, cyclic AMP, selenocystathionine, inosine triphosphate, adenosine phosphosulfate, 5'-methylthioadenosine, uric acid, malonic semialdehyde, 2-methylguanosine, ganglioside GD2 and malonic acid. Metabolomics analysis via pathway enrichment of the metabolites revealed the activation of key metabolic pathways relevant to glucose, lipid, amino acid, vitamin, and nucleotide metabolisms. CSI caused a total inactivation of glucose, vitamin, and nucleotide metabolisms, while inactivating key lipid and amino acid metabolic pathways linked to cancer cell survival. Flow cytometry analysis revealed an induction of apoptosis and necrosis in MCF-7 cells treated with CSI. High-performance liquid chromatography (HPLC) analysis of CSI revealed the presence of cannabidiol, rutin, cinnamic acid, and ferulic. These results portray the anti-proliferative potentials of CSI as an alternative therapy for the treatment and management of breast cancer as depicted by its modulation of glucose, lipid, amino acid, vitamin, and nucleotide metabolisms, while concomitantly inducing cell death in MCF-7 cells.

1. Introduction

Breast cancer accounts for the most common cancer as it is a major contributor to cancer-related mortality and morbidity in women

* Corresponding author.
E-mail address: matsabisamg@ufs.ac.za (M.G. Matsabisa).

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globally, with the low - and middle-income countries having the highest fatality rate [1,2]. This high rate coupled with other diseases such as malaria and tuberculosis pose a huge burden on these countries, particularly in sub-Saharan Africa. This is due to limited economic resources, and poor health infrastructures and services [3,4]. This high rate coupled with other diseases such as malaria and tuberculosis pose a huge burden on these countries, particularly in sub-Saharan Africa [5–7]. Breast cancer is treated in several ways which includes chemotherapy, biological therapy, surgery, radiation therapy and hormonal therapy. The choice of treatment is dependent on the cancer type and its level of metastasis. Complementary and alternative medicine which includes the use of medicinal plants are also employed in the treatment of breast cancer.

Medicinal plants have been employed from time immemorial for the treatment and management of several diseases including cancers. Their use has been attributed mostly to their phytochemical constituents which have been reported for their anticancer efficacies [8,9]. This is further evident from outcomes of clinical trials of medicinal plants and their phytoconstituents as chemosensitizers [10,11].

Cannabis sativa L. is among the medicinal plants reported for its folkloric use in the treatment and management of cancers and its complication. It is a global controversial plant owing to its psychoactive effect but has however started gaining much interests for its therapeutic properties against several diseases such as cancers, diabetes, and epilepsy [12,13]. It is an annual herbaceous plant which belongs to the *Cannabis* genus and the Cannabaceae family. Its common names include marijuana, weed, Indian hemp and ganja. Although *C. sativa* has been reported for the presence of phenolics [14], its major phytochemicals are the phytocannabinoids [15]. Tetrahydrocannabinol (THC), cannabinol, and cannabidiol are the most common and widely studied phytocannabinoids. Phytocannabinoids have been reported for their anticancer properties which include modulation of specific of cannabinoid receptors in tumors, arrest of tumor growth, and mollification of chemotherapy side effects [16]. Phytocannabinoids have also been reported for their antiproliferative activities in breast carcinomas. They bring about this effect by activating the RAF1–MEK–ERK signaling cascade and cycle arrest at the G1/S checkpoint [17–19] as well as induction of apoptosis and autophagy [20].

Anticancer drugs exert their therapeutic effect via several mechanisms. A major anticancer therapeutic mechanism is the alteration of cancer metabolism. Carcinogenesis is an intricate process and involves metabolic changes in cells thereby instigating exacerbated proliferation [21]. Cancer cells have therefore adapted mechanism to meet their increased metabolic requirements to drive tumor proliferation, migration, and metastatic activities [22]. Although *C. sativa* and its phytoconstituents have been reported for their antiproliferative effect on breast cancers, however there is a dearth of their effect on cancer metabolisms. This study was therefore carried out to investigate the apoptotic effect of *C. sativa* leaves infusion and its ability to modulate key metabolisms in human breast cancer (MCF-7) cells using metabolomics tools.

2. Materials and methods

2.1. Chemicals

Formic acid (HPLC grade, ≥95%), Acetonitrile (anhydrous, 99.8%), Fetal bovine serum, Trypsin-EDTA, 3-(4, 5-dimethylthiazolyl-2)-2.5-diphenyltetrazolium bromide, Dimethyl sulfoxide, Doxorubicin, Dulbecco's modified eagle medium, Phosphate buffered saline, Methanol (anhydrous, 99.8%) and Ethanol (HPLC grade, 96%).

2.2. Plant permit approval

Permit approval (Permit No. POS 248/2019/2020) was obtained for the present study from the South African Health Products Regulatory Authority to conduct, collect, posses, transport and store cannabis plant, plant parts and products for research purposes. The study was also conducted to collect cannabis plants in Lesotho under the permit (Permit #: 01/LS/2019/10/02–01).

2.3. Plant material

Cannabis sativa leaves were collected from Mohale's Hoek District, Lesotho (GPS coordinates: 30.333776"S and 27.651201"E) under the permit (Permit #: 01/LS/2019/10/02–01). The leaves were assigned the voucher number BLFU MGM 0018 following identification and authentication at the Geo Potts Herbarium at the University of the Free State, Bloemfontein 9300, South Africa. The leaves were air-dried and blended to powder and stored in air-tight container for subsequent analyses.

2.4. Infusion of C. sativa leaves

The leaves were subjected to aqueous infusion using a previously published method [23]. Twenty grams (20 g) of the blended leaf samples were soaked in boiled water and allowed to extract for 2 h. The infusion was filtered with the aid of a cotton well and funnel. After freezing at −80 °C, the filtrate was freeze-dried. About 6 g of concentrated infusion was obtained and stored in glass vials at 2 °C until further analysis.

2.5. High-performance liquid chromatography (HPLC) of plant extracts

The infusion (500 μg/mL) was subjected to HPLC-diode array detection analysis using an Agilent 1100 series (Agilent, Waldbronn, Germany) instrument equipped with photo diode array, autosampler, column thermostat and degasser. The stationary phase consisted

of A Phenomenex: Luna 5 μm C_{18} (2) (150 × 4.6 mm; 5 μm particle size) column. The mobile phases consisted of water containing 0.1% of formic acid (A) and acetonitrile (B) with a flow rate of 1 ml/min. The gradient elutions were: Initial ratio 95% A: 5% B, keeping for 10 min, changed to 90% A: 10% B for 10 min, changed to 70% A: 30% B for 10 min, to 50% A: 50% B for 10 min, maintained for 0.5 min and back to initial ratio in 0.5 min. Temperature was maintained at 30 °C during the run. The volume of injection was 20.0 μL, while chromatograms were recorded at 254 nm [24].

2.6. Cell culture

MCF-7 human breast cancer cell line was obtained from the American Type Cell Collection (ATCC, Manassas, VA, USA). The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing high glucose (4.5 g/L) and supplemented with glutamine (2 mM) and 10% heat-inactivated fetal bovine serum (FBS) in 5% CO_2 and 37 °C [25]. The growing phase was maintained at 80% confluence with routine passage using 0.025% Trypsin-EDTA. The cells were sub-cultured in 96-well and 6-well plates for 24 h before treatment with infusion.

2.7. Cytotoxicity assay

The cytotoxic effect of the infusion on MCF-7 cells was determined using the MTT (3-(4, 5-dimethylthiazolyl-2)-2.5-diphenyltetrazolium bromide) colorimetric assay [26]. Briefly, the cells (accession number: CVCL_0031) were cultured in 96-well plates (3000 cells per well) and treated with the infusion (30, 60, 120, 240 μg/mL). The control consisted of untreated MCF-7 containing 0.1% (v/v) DMSO, while doxorubicin (10 mM) was used as the standard anticancer drug. Following 48 h incubation, 20 μL of MTT solution (5 mg/mL in PBS) was added to the cells and further incubated for 3.5 h. DMSO was used to solubilize the formazan crystals and absorbance was read at 570 nm using a microplate reader.

2.8. Extraction of cell metabolites

The cell metabolites were extracted using an established protocol with slight modifications [27]. Briefly, MCF-7 cells (5 × 104 cells/mL seeded in 24 well plates) were incubated with the highest concentration (240 μg/mL) of the infusion for 48 h at 37 °C in a humidified atmosphere with 5% CO_2. Doxorubicin served as the standard anticancer drug, while the control consisted of cells incubated without the infusion or doxorubicin. The medium was discarded, and the cells rinsed with PBS. The attached cells were recovered by scraping gently in cold methanol/ethanol (2:8) and collected in 2 mL Eppendorf tubes. The samples were vortexed for 1 min and allowed to incubate in ice for 20 min. They were then after centrifuged at 20,000 g for 10 min at 4 °C. The supernatants were collected into HPLC vials and subjected to Liquid chromatography–mass spectroscopy (LC-MS) analysis for metabolite profiling.

2.9. LC-MS analysis of extracted metabolites

The extracted cell metabolites were subjected to LC-MS (Shimadzu LCMS-2020 Single Quadrupole) analysis. This was carried out by injecting the extracted metabolites (1 mg/mL) directly into the machine via a loop.

The operating parameters of the machine were: **Stop time:** 60 min; **Photodiode Array (PDA) sampling frequency**: 1.5625 Hz; **Operating mode**: low pressure gradient; **Pump A**: LC-2030 Pump; **Mobile Phase A, B, C and D**: 0.1% formic acid, methanol, acetonitrile and water respectively; **Flow rate:** 0.3000 ml/min; **Start and End wavelengths**: 220 and 400 nm respectively; **Oven and Maximum Temperatures**: 40 and 50 °C respectively; **Start and End time**: 0.00 and 60.00 min respectively; **Acquisition mode**: Scan; **Scan Speed**: 5000 u/s; **Polarity**: Positive; **Event Time**: 0.25 s; **Detector Voltage**: +0.00 kV; **Threshold**: 0; **Start and End m/z**: 100.00 and 1000.00 respectively; **Interface**: ESI; **Drying Gas**: 15.00L/min.

The Human Metabolome Database (HMBD) was utilized to identify the metabolites by direct search and comparison of mass spectral (MS) data with that of the database [28].

2.10. Data normalization

The MetaboAnalyst5.0 online server (https://www.metaboanalyst.ca/) was used to normalize the identified metabolites and their values via sample median, cube root transformation and mean centering [29].

2.11. Pathway analysis

The relevant metabolic pathways were identified by subjecting the identified metabolites to pathway enrichment analysis using the MetaboAnalyst5.0 online server [29]. The Small Molecule Pathway Database (SMPDB) was used in mapping metabolites with significant changes using their HMBD numbers [30,31].

2.12. Apoptosis analysis

Phosphatidylserine exposed apoptotic cells was evaluated with Flow cytometry following double staining with V-FITC and PI. Viable cells, early apoptotic (Annexin V^+, PI^-), late apoptotic (Annexin V^+, PI^+), and necrotic (Annexin V^-, PI^+) cells were quantified

and distinguished from one another using Annexin V and PI labelling [32]. Briefly, following sub-culture in 75 cm^2 flasks, cells were treated with *C. sativa* infusion (100 μg/mL) for 24 h and harvested. The cells were stained in accordance with the manufacturer's manual (Clontech, USA) and subjected to flow cytometry analysis using the Becton Dickinson FACS Melody (BD Biosciences, Waltham, MA), with FITC and PI as signal detector. Identification of the cells were done based on forward scatter area (FSC-A) versus side scatter area (SSC-A), and doublet exclusion (SSC–W vs. SSC-H and FSC-W vs. FSC-H). BD FACSChorus software (BD Biosciences, Waltham, MA) was used to analyze the data.

2.13. Molecular docking studies

Molecular docking studies were carried out to determine the molecular interactions of the HPLC-identified compounds of *C. sativa* infusion with key regulating proteins (CDK6 and CDK2) of cell cycle. The 3D structure of human CDK6-Vcyclin in complex with inhibitor LQQ and the 3D structure of CDK-2 in complex with indazole were retrieved from the Protein Data Bank with ID 2EUF and 3EZR respectively. The structure of human CDK6-Vcyclin has two distinct chains A and B however due to computational cost and time, the singular chain B of the protein where the LQQ compound is bound was prepared for the docking studies. The preparation of the proteins were achieved using the UCSF Chimera interface where hydrogens were affixed to the protein and removed from the ligand. On the other hand, the 3D structure of CDK-2 constitutes a single chain and was prepared using similar approach. The grid box that defines the binding site region of CDK6-Vcyclin and CDK-2 were generated using the AutoDock Vina functionality on UCSF Chimera. The grid box size and center coordinates for CDK6-Vcyclin and CDK-2 were (x (13.9618, 31.4847), y (18.0536, 22.956) and z (9.7558, 60.7321)) and (x (10, −0.6777), y (13.159, 28.7851) and z (12.0707, 10.7103)) respectively. Meanwhile, the chemical structures of cannabidiol, rutin, ferulic acid and cinnamic acid were retrieve from PubChem thereafter optimized and employed for the docking studies.

2.14. Statistical analysis

Data were presented as mean ± SD, following analysis with one-way analysis of variance (ANOVA). Significant differences between means were determined at $p < 0.05$ using the Tukey's HSD-multiple range post-hoc test. The statistical analysis was carried out using the IBM Statistical Package for the Social Sciences (SPSS) for Windows, version 23.0 (IBM Corp., Armonk, NY, USA). The normalized data of the LC-MS identified cell metabolites were subjected to clustering analysis which covers for heat maps and principal component analysis (PCA) using the MetaboAnalyst 5.0 online server (https://www.metaboanalyst.ca/). Pathway enrichment values for identified pathways were presented as raw p.

3. Results

As shown in Fig. 1 and S1 and Table 1, HPLC analysis revealed the presence of the phytocannabinoids: cannabidiol and phenolics: rutin, cinnamic acid and ferulic acid in *C. sativa* infusion.

Treatment of MCF-7 cells with *C. sativa* infusion led to 21.2% inhibition of cell growth which depicted 78.8% viable cells compared to the control as shown in Fig. 2. The inhibition was however significantly ($p < 0.05$) lower than that of the standard anticancer drug, doxorubicin.

LC-MS analysis of the untreated, the infusion- and doxorubicin – treated cells led to the identification of 23, 16 and 19 metabolites, respectively as shown in Table S1. LC-MS profiling of untreated MCF-7 cells (control) revealed the presence of carbohydrate, vitamins, oxidative, lipids, nucleotides and amino acids metabolites as shown in Table S1. Except for the lipid metabolites: cardiolipin and phosphorylethanolamine, treatment with *C. sativa* caused 91% depletion of these metabolites, with concomitant generation of selenomethionine, l-cystine, deoxyadenosine triphosphate, cyclic amp, selenocystathionine, inosine triphosphate, adenosine phosphosulfate, 5'-methylthioadenosine, uric acid, malonic semialdehyde, 2-methylguanosine, ganglioside GD2 and malonic acid. MCF-7 cells treated with doxorubicin retained only triglyceride, deoxyribose 5-phosphate, glycerol 3-phosphate, and 2-Methylguanosine compared to the control, with concomitant generation of carbamoyl phosphate, methylmalonic acid, deoxyuridine monophosphate (dUDP), 2-Methyl-1-hydroxypropyl-ThPP, glycolaldehyde, cysteamine, urea, hydrogen peroxide and uridine 5'-monophosphate.

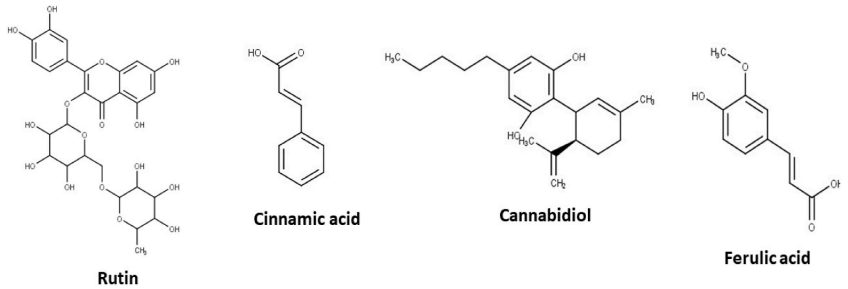

Fig. 1. HPLC identified compounds in *C. sativa* infusion.

Table 1
HPLC identified compounds in *C. sativa* leaf infusion.

Compounds (Retention time)	Parameters	*C. sativa*
Rutin (1.44 min)	RT (min)	1.36
	DFS (min)	0.08
Cinnamic acid (2.05 min)	RT (min)	2.14
	DFS (min)	−0.09
Cannabidiol (7.71 min)	RT (min)	7.04
	DFS (min)	0.67
Ferulic acid (1.61 min)	RT (min)	1.66
	DFS (min)	−0.05

RT = retention time; DFS = difference in retention time.

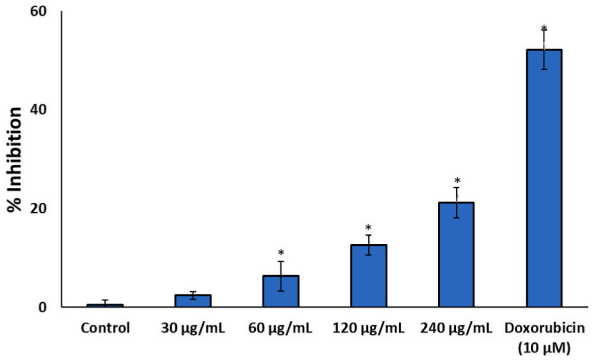

Fig. 2. Cytotoxic effect of *C. sativa* on MCF-7 cancer cells. Values = mean ± SD; n = 3. *Statistically significant to control.

Treatment with doxorubicin also led to the generation of selenomethionine, cyclic AMP, selenocystathionine and 5'-Methylthioadenosine.

As shown in Fig. 3, pathway enrichment analysis revealed carbohydrate metabolism in the untreated MCF-7 cells as depicted by

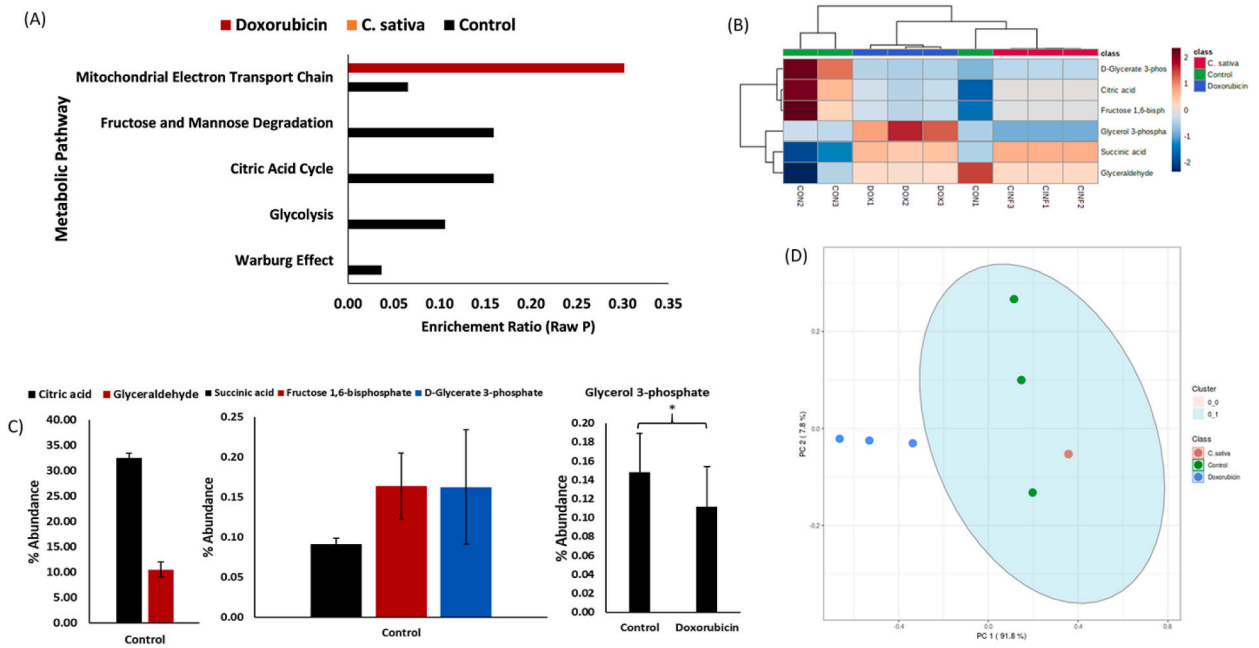

Fig. 3. Effect of *C. sativa* on glucose metabolism in MCF-7 cells. (A) Glucose metabolic pathway enrichment; (B) Heat map of LC-MS identified metabolites involved in glucose metabolism in MCF-7 cells; (C) LC-MS identified metabolites involved in glucose metabolism in MCF-7 cells; and (D) PC scores of LC-MS identified metabolites. Values = mean ± SD; n = 3. *Statistically significant to each other. CON = control; CINF = *C. sativa*; and Dox = doxorubicin.

Warburg effect, glycolysis, citric acid cycle, fructose and mannose degradation and mitochondrial electron transport chain pathways (Fig. 3A). These pathways were inactivated in both *C. sativa* and doxorubicin treated cells which indicates an arrest of carbohydrate metabolism and mitochondrial oxidation. The negative values and color intensity of the heatmap indicates distinct changes in the identified metabolites of these pathways in the cells (Fig. 3B). These pathways are attributed to the presence of citric acid, succinic acid, fructose 1,6-bisphosphate, glyceraldehyde, D-glycerate 3-phosphate and glycerol 3-phosphate (Fig. 3C). Distinct changes in the identified metabolites were further revealed by principal component (PC) analysis as indicated by the pairwise score plots between selected PCs (Fig. 3D).

Pathway enrichment analysis revealed glycerolipid metabolism, phosphatidylcholine biosynthesis, phospholipid biosynthesis, de novo triacylglycerol biosynthesis, glycerol phosphate shuttle, cardiolipin biosynthesis, sphingolipid metabolism, phosphatidylethanolamine biosynthesis, ketone body metabolism, butyrate metabolism, carnitine synthesis, transfer of acetyl groups into mitochondria, oxidation of branched chain fatty acids and phytanic acid peroxisomal oxidation pathways in untreated MCF-7 cells depicting an active lipid metabolism as shown in Fig. 4A. Treatment with *C. sativa* led to the inactivation of these pathways except phosphatidylcholine biosynthesis, sphingolipid metabolism, phosphatidylethanolamine biosynthesis pathways, while concomitantly activating fatty acid biosynthesis and propanoate metabolism pathways.

Doxorubicin inactivated ketone body metabolism, butyrate metabolism, carnitine synthesis, transfer of acetyl groups into mitochondria, oxidation of branched chain fatty acids, and phytanic acid peroxisomal oxidation pathways, while concomitantly activating plasmalogen synthesis and arachidonic acid metabolism pathways. Heatmap analysis of the responsible metabolites revealed distinct changes in their distribution between the different treatment groups as compared to the control (Fig. 4B). Phosphorylethanolamine was identified as the common metabolite in the control and treated cells, with the control having significant ($p < 0.05$) higher concentration compared to the treated cells (Fig. 4C). The pairwise score plots between the selected PCs following PC analysis of the metabolites further depicts distinct changes in the identified metabolites in the control and treated cells (Fig. 4D).

As revealed by pathway enrichment analysis, amino acid metabolism in MCF-7 cells were altered in *C. sativa* treated cells as depicted by the inactivation of glutamate metabolism, arginine and proline metabolism, amino sugar metabolism, beta-alanine metabolism, glycine and serine metabolism, and valine, leucine and isoleucine degradation pathways, while concomitantly activating selenoamino acid metabolism, aspartate metabolism, spermidine and spermine biosynthesis, sulfate/sulfite metabolism and methionine metabolism pathways as shown in Fig. 5A. These pathways were also activated in doxorubicin treated cells, with concomitant inactivation of amino sugar metabolism pathway while also activating D-arginine and D-ornithine metabolism, urea cycle, taurine and hypotaurine metabolism, phenylalanine and tyrosine metabolism, lysine degradation, ammonia recycling, histidine metabolism and tryptophan metabolism pathways. Distinct changes in the distribution of the identified metabolites among the control and treated cells were revealed following heatmap analysis (Fig. 5B). Among the identified metabolites, selenomethionine, selenocystathionine, 5'-methylthioadenosine were common to both *C. sativa* and doxorubicin treated cells (Fig. 5C). The concentrations of

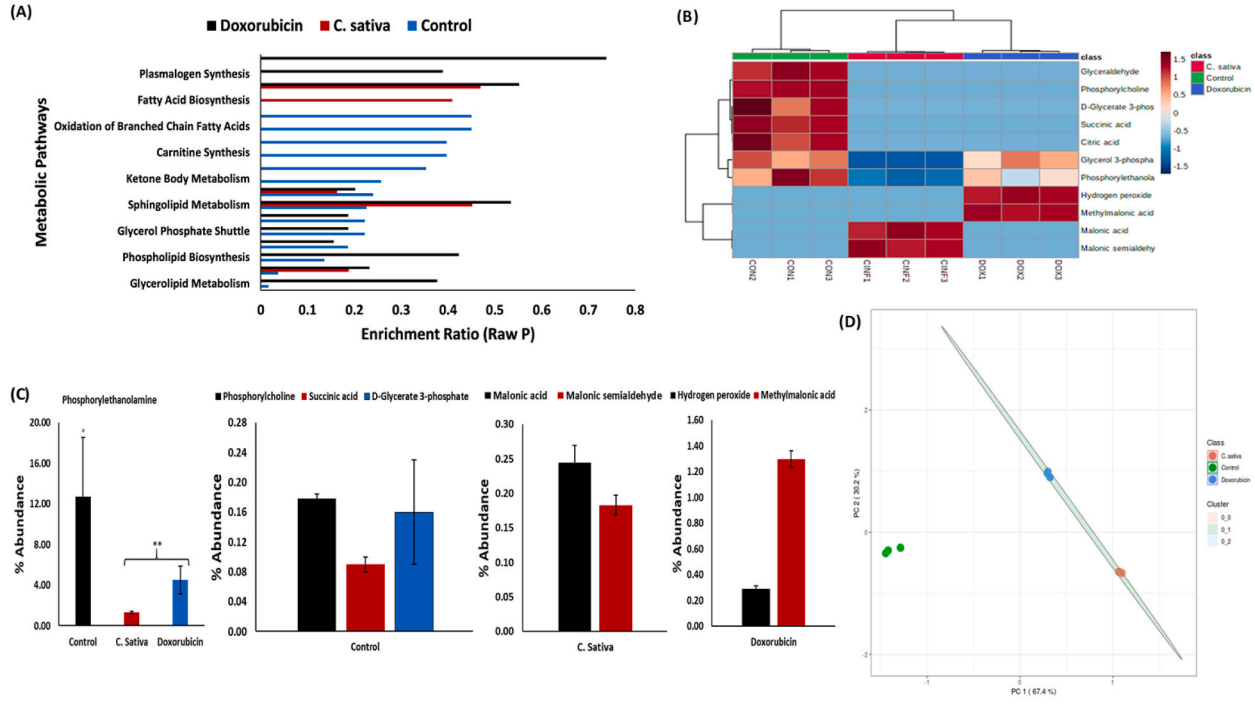

Fig. 4. Effect of *C. sativa* on lipid metabolism in MCF-7 cells. (A) Lipid metabolic pathway enrichment; (B) Heat map of LC-MS identified metabolites involved in lipid metabolism in MCF-7; (C) LC-MS identified metabolites involved in lipid metabolism in MCF-7 cells; and (D) PC scores of LC-MS identified metabolites. Values = mean ± SD; n = 3. *Statistically significant to each other. CON = control; CINF = *C. sativa*; and Dox = doxorubicin.

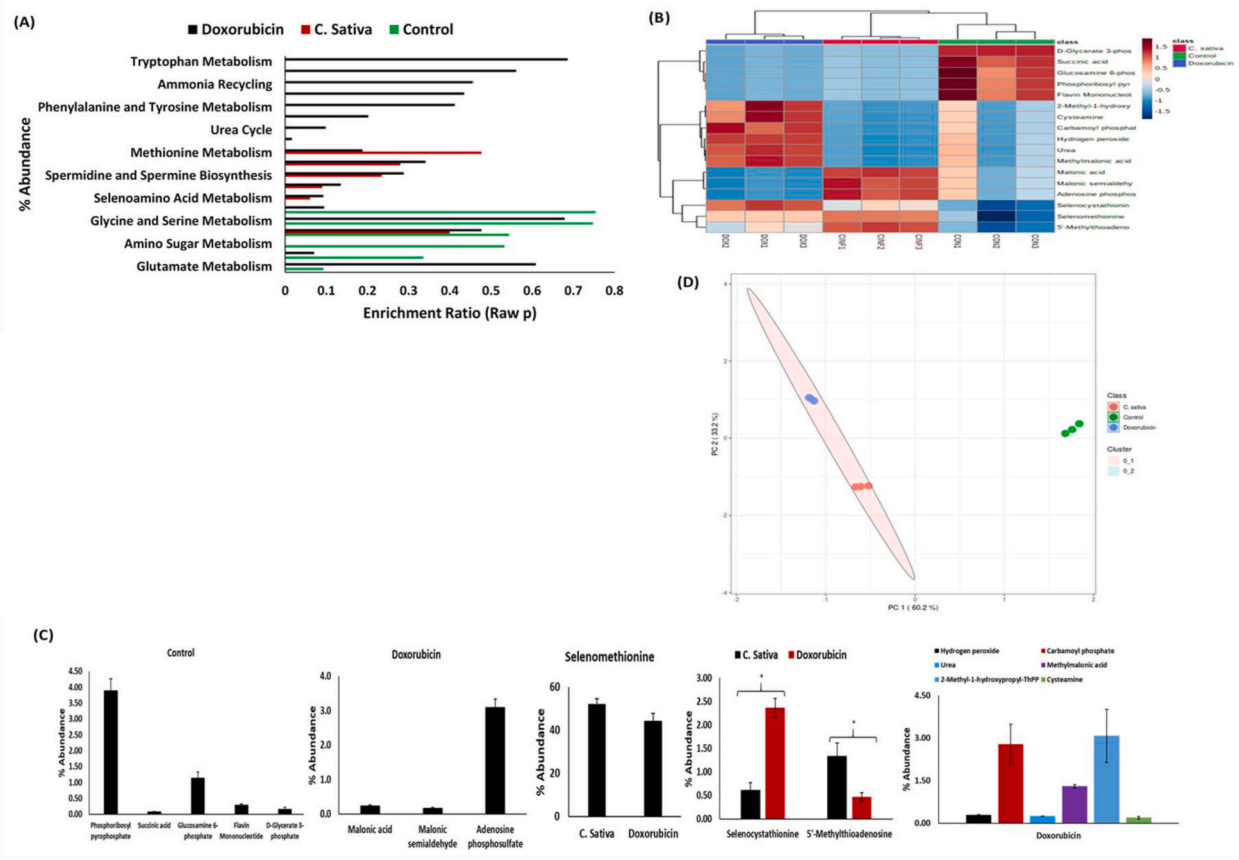

Fig. 5. Effect of *C. sativa* on amino acids metabolism in MCF-7 cells. (A) Amino acids metabolic pathway enrichment; (B) Heat map of LC-MS identified metabolites involved in amino acids metabolism in MCF-7; (C) LC-MS identified metabolites involved in amino acids metabolism in MCF-7 cells; and (D) PC scores of LC-MS identified metabolites. Values = mean ± SD; n = 3. *Statistically significant to each other. CON = control; CINF = *C. sativa*; and Dox = doxorubicin.

selenocystathionine, 5'-methylthioadenosine were significantly ($p < 0.05$) different between both treated cells. The selected PC scores of the metabolites further depicts their distinct distributions among the control and treated cells.

As shown in Fig. 6A, pathway enrichment analysis revealed vitamin metabolism in the untreated MCF-7 cells as depicted by nicotinate and nicotinamide metabolism, vitamin b6 metabolism, riboflavin metabolism and pantothenate and CoA biosynthesis pathways. These pathways were inactivated following treatment with *C. sativa*. However, treatment with doxorubicin did not alter the metabolomics but further activated vitamin K metabolism pathway. The negative values and color intensity of the heatmap indicate distinct distribution of the identified metabolites between the control and doxorubicin treated cells (Fig. 6B). None of the identified metabolites were common to the control and doxorubicin treated cells (Fig. 6C). PC score analysis of the metabolites revealed their distinct distributions between the cells (Fig. 6D).

An intact nucleotide metabolism depicted by pyrimidine metabolism, purine metabolism, inositol metabolism and pentose phosphate pathways were observed in the untreated MCF-7 cells following pathway enrichment analysis as shown in Fig. 7A. Except for pentose phosphate pathway, these metabolites were inactivated following treatment with *C. sativa* while treatment with doxorubicin only led to the inactivation of inositol metabolism pathway.

Heatmap analysis of the identified metabolites for these pathways portrayed a distinct distribution among the control and treated cells as depicted by the negative values and color intensity (Fig. 7B). Among the identified metabolites, cyclic AMP were common to *C. sativa* and doxorubicin treated cells while deoxyribose 5-phosphate was common to the control and doxorubicin treated cells (Fig. 7C). The pairwise score plots between the selected PCs of the metabolites also indicate distinct changes in their distribution in the control and treated cells (Fig. 7D).

As shown in Fig. 8A, treatment with doxorubicin led to the activation of oxidative metabolism as depicted by glutathione metabolism and degradation of superoxides pathways, as well as the activation of porphyrin metabolism pathway in MCF-7 cells. Heatmap analysis revealed the distribution of the identified metabolites to be distinctly distributed in doxorubicin treated cells only (Fig. 8B). The identified metabolites for these pathways were methylmalonic acid and hydrogen peroxide (Fig. 8C). PC score analysis showed these metabolites were distinctly clustered in the doxorubicin treated cells (Fig. 8D).

As shown in Fig. 9A and B, treatment with *C. sativa* led to significant ($p < 0.001$) increase in apoptosis in MCF-7 as depicted by the

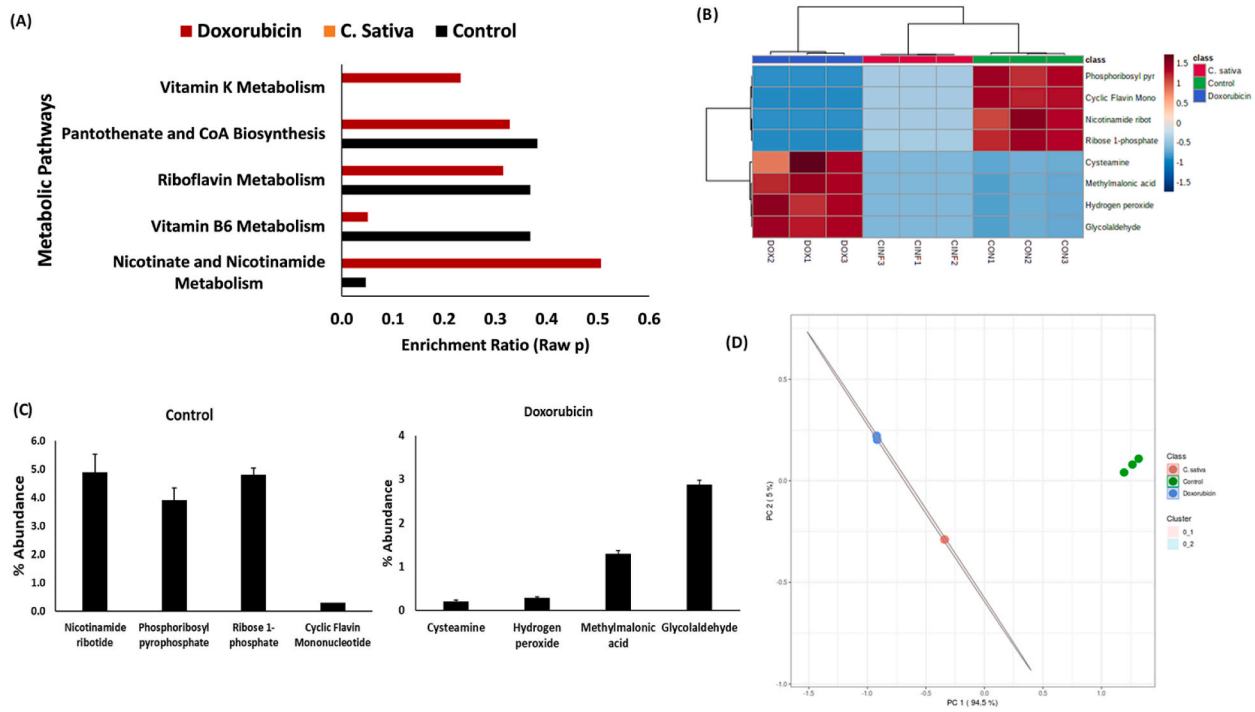

Fig. 6. Effect of *C. sativa* on vitamins metabolism in MCF-7 cells. (A) Vitamins metabolic pathway enrichment; (B) Heat map of LC-MS identified metabolites involved in vitamins metabolism in MCF-7; (C) LC-MS identified metabolites involved in vitamins metabolism in MCF-7 cells; and (D) PC scores of LC-MS identified metabolites. Values = mean ± SD; n = 3. *Statistically significant to each other. CON = control; CINF = *C. sativa*; and Dox = doxorubicin.

movement of 21% of the cells to move to apoptotic phase as compared to the control. It also caused a slight reduction in the number of healthy cells. However, the infusion did not show any significant effect on necrotic activity.

Molecular docking analysis of the HPLC identified compounds of *C. sativa* infusion with CDK6 and CDK2 revealed potent binding affinities between the compounds and the proteins as shown in Table 2. Cannabidiol and rutin had the strongest binding affinities for CDK6 and CDK2, respectively and their molecular interactions are presented in Fig. 10A and B.

4. Discussion

The increasing rate of breast cancer and the cost of its treatment in developing countries have led to a continual search for affordable alternatives. Natural products from plants have over the years been employed as alternative treatments for most diseases including cancers [33,34]. Therapeutic targeting of cancer metabolism has been reported to be a promising mechanism in the treatment and management of cancer, as it prevents the cells from meeting the metabolic requirements needed for tumorigenesis, proliferation and metastasis [22,35]. The present study reports the ability of *C. sativa* infusion to alter key metabolisms vis-à-vis carbohydrate, lipid, amino acid, vitamin, and nucleotide metabolisms as well as induce apoptosis in MCF-7 cells.

Cannabis sativa and its phytoconstituents have been reported for their anticancer activities in various cancer types including breast cancer [36–38]. This is corroborated in the present study by the cytotoxic activity of *C. sativa* infusion in MCF-7 cells (Fig. 2), which depicts an antiproliferative effect against the cells. This activity can be attributed to the synergistic effect of the HPLC-identified compounds in the infusion (Fig. 1 and Table 1). These compounds have been reported for their potent anticancer activities. Cannabidiol has been reported for its ability to inhibit proliferation [19], down-regulate Id-1 gene expression, mTOR and cyclin D1, upregulate PPARg protein expression [39,40], induce apoptosis and autophagy [41], and arrest cell cycle [42] in breast cancer. The antiproliferative effect of cinnamic acid on breast cancer has been attributed to their ability to inhibit 17β-hydroxysteroid dehydrogenase types1 and 5 activities [43,44], inhibit cell growth [45] and induce apoptosis [46]. Rutin has been reported for its anticancer effects in breast cancer which include improving chemosensitivity [47], induction of apoptosis [48], and regulation of tyrosine kinase c-Met receptor [49]. While ferulic acid has also been reported for its anticancer activities in breast cancer which include modulation of HER2 expression [50], regulation of epithelial to mesenchymal transition [51], and inhibition of epidermal growth factor receptor [52].

Increased carbohydrate metabolism characterized by high glycolytic rate and TCA cycle drive is critical to cancer cell survival as the cells utilize glucose to fuel energy production, tumorigenesis, and proliferation [53,54]. This is depicted in the present study by the active glycolysis and citric acid cycle in the untreated MCF-7 cells (Fig. 3). Glycolysis plays a critical role in cancer cells as it provides

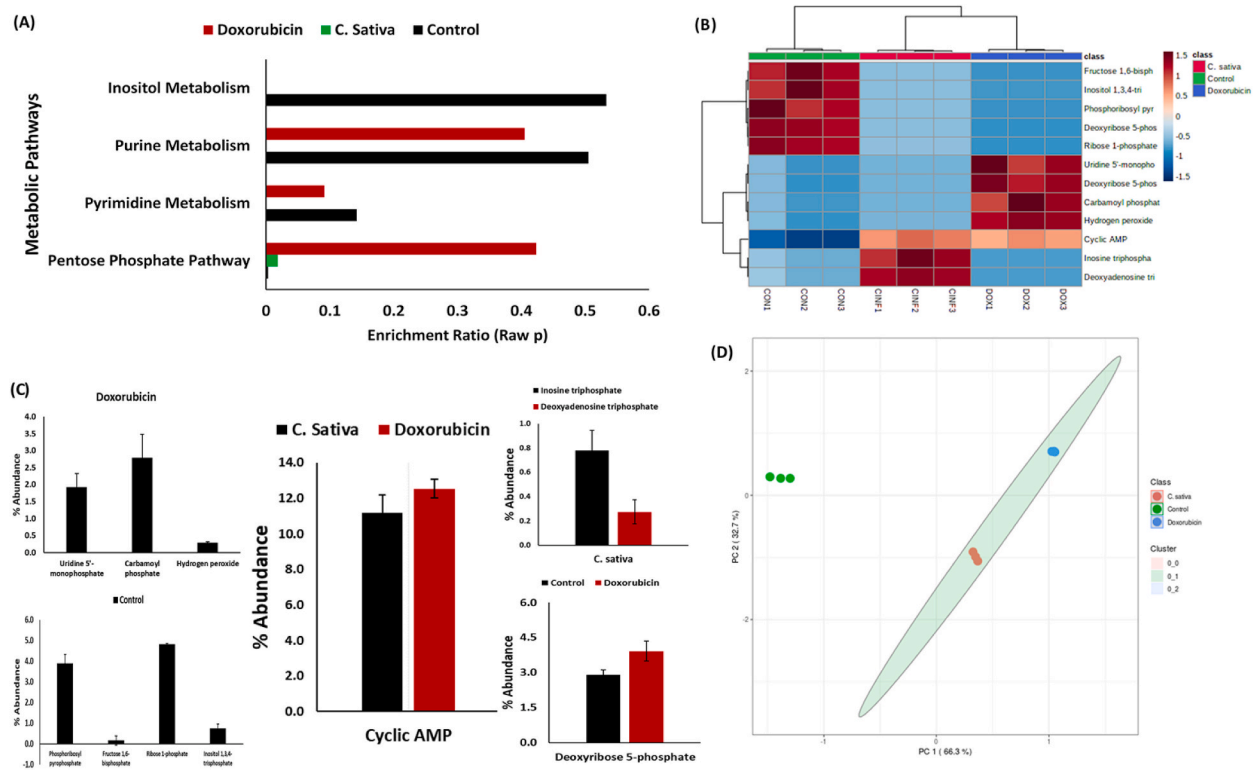

Fig. 7. Effect of *C. sativa* on nucleotide metabolism in MCF-7 cells. (A) nucleotide metabolic pathways enrichment; (B) Heat map of LC-MS identified metabolites involved in nucleotide metabolism in MCF-7; (C) LC-MS identified metabolites involved in nucleotide metabolism in MCF-7 cells; and (D) PC scores of LC-MS identified metabolites. Values = mean ± SD; n = 3. *Statistically significant to each other. CON = control; CINF = *C. sativa*; and Dox = doxorubicin.

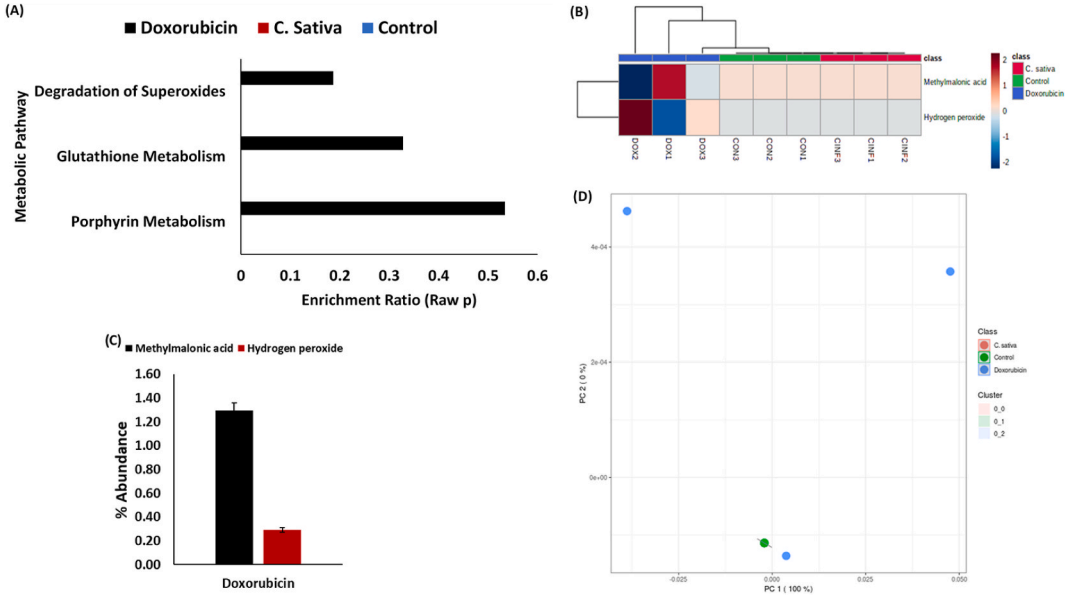

Fig. 8. Effect of *C. sativa* on porphyrin and oxidative metabolisms in MCF-7 cells. (A) porphyrin and oxidative metabolic pathways enrichment; (B) Heat map of LC-MS identified metabolites involved in porphyrin and oxidative metabolisms in MCF-7; (C) LC-MS identified metabolites involved in porphyrin and oxidative metabolisms in MCF-7 cells; and (D) PC scores of LC-MS identified metabolites. Values = mean ± SD; n = 3. *Statistically significant to each other. CON = control; CINF = *C. sativa*; and Dox = doxorubicin.

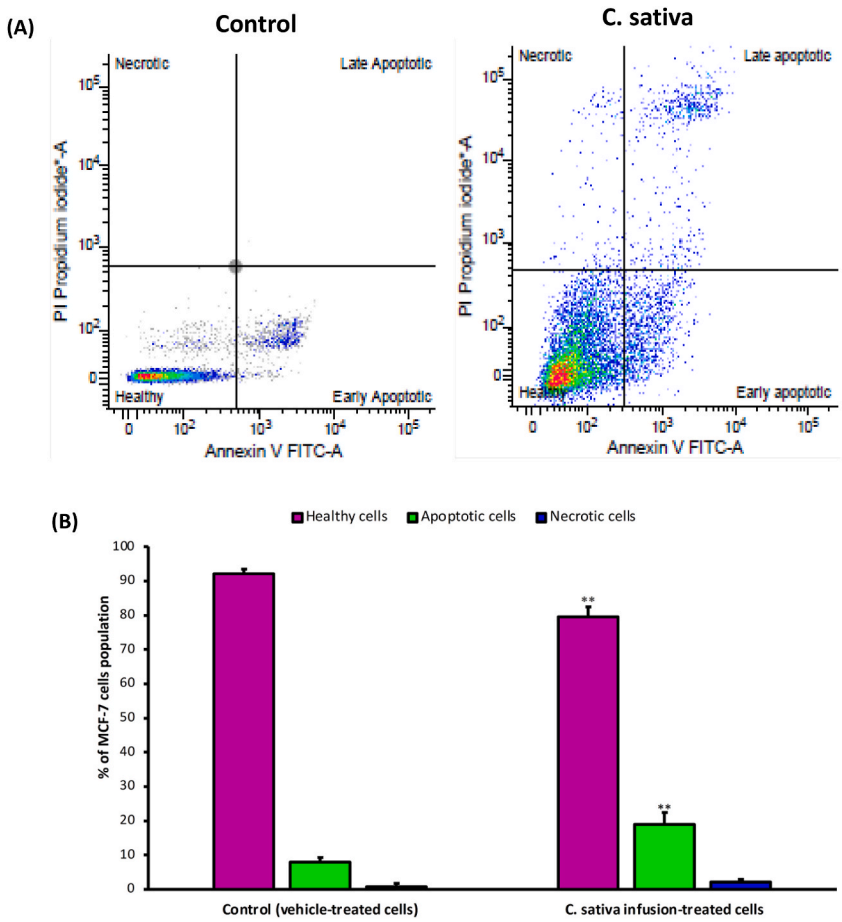

Fig. 9. Effect of C. sativa on apoptosis of MCF-7 cells. Values = mean ± SD; n = 3. **significantly different ($p < 0.001$) to control.

Table 2
Binding affinities (kcal/mol) of compounds with enzymes (bold data denotes highest binding affinity).

Compounds	CDK6 (2EUF)	CDK2 (3EZR)
	(kcal/mol)	
Cannabidiol	**−8.3**	−7.5
Cinnamic acid	−7.5	−6.5
Ferulic acid	−7.3	−6.7
Rutin	−7.5	**−8.2**

major substrates for biosynthetic pathways and ATP production which important for the cell's survival. Regarding ATP production, glycolysis is critical to the Warburg effect which is important in cancer cells' bioenergetics and proliferation [53,55]. These cells tend to adopt a mechanism to generate more ATPs via increased aerobic glycolysis to meet the energy demand required for their survival, a process defined as the Warburg effect. This is also corroborated in the present study as depicted by the active Warburg effect in the untreated MCF-7 cells (Fig. 3). The active fructose and mannose degradation pathway in the untreated MCF-7 cells indicate conversion of fructose 1-phosphate to fructose 6-phosphate catalyzed by phosphofructomutase. This suggests an increased availability of the latter to continuously drive the glycolytic pathway which insinuates an increased rate of the pathway. Oxidative phosphorylation via the citric acid cycle has also been reported to be critical for energy production and macromolecule synthesis in cancer cells [35]. The activation of this pathway in the untreated MCF-7 cells can be attributed to the availability of acetyl-CoA arising from increased glycolytic rate, which feeds the citric acid cycle. Unlike aerobic glycolysis which generates 2 ATPs per glucose molecule, the citric acid cycle generates 38 ATPs. Thus, the activation of this pathway indicates an increased ATP production to meet the energy demand in the untreated cancer cells. This in turn corroborates the activated mitochondrial electron transport chain pathway in the cells, as this pathway uses electrons generated from the citric acid cycle to reduce oxygen and generate ATPs. Inhibition of glucose metabolism is

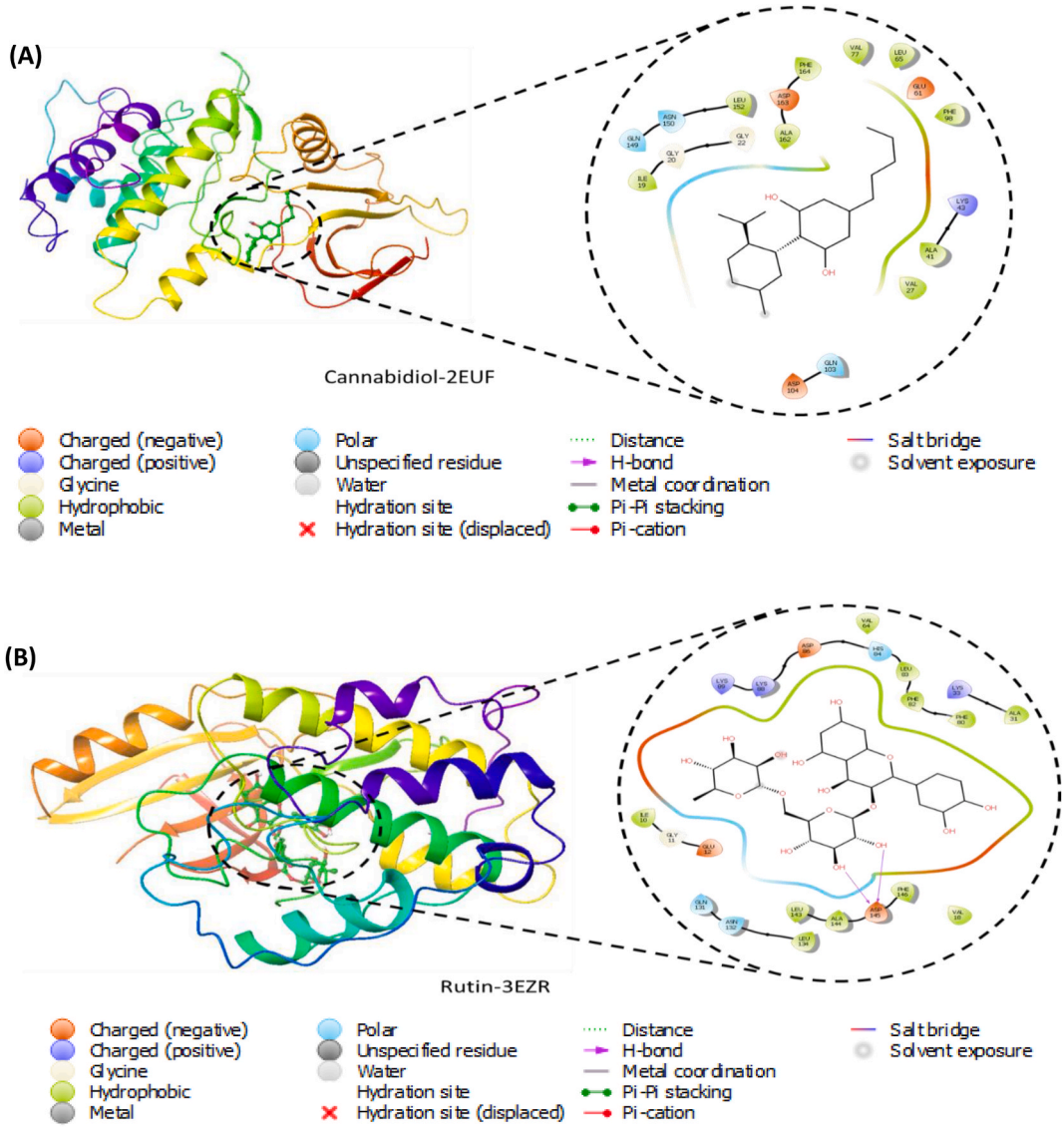

Fig. 10. Molecular interactions of (A) cannabidiol with CDK6; and (B) rutin with CDK2.

lethal to cancer proliferation and survival. The inactivation of these pathways in *C. sativa* treated MCF-7 cells therefore suggests that a therapeutic mechanism of the infusion against breast cancer involves glucose starvation.

Dysregulation in fatty acid metabolism has been reported as an important hallmark of cancers including breast cancer owing to their critical roles in cellular membrane, energy production and secondary messengers [56]. These activities are essential in the proliferation, malignant transformation, migration and invasion of cancer cells [56,57]. This is depicted in the present study by the activated lipid metabolic pathways and the corresponding metabolites in the untreated MCF-7 cells (Fig. 4 and Table S1). The presence of glycerol 3-phosphate, glyceraldehyde, D-glycerate 3-phosphate, succinic acid, citric acid indicates that the glycolytic pathway and citric acid cycle provided the precursors for the lipid metabolic pathways. The presence of phytanic acid peroxisomal oxidation, glycerol phosphate shuttle, oxidation of branched-chain fatty acids, and ketone body metabolism pathways indicates an increased generation of ATPs to meet the high energy demand of the cell needed for proliferation, malignancy, migration, invasion and survival [58–60]. The presence of phosphatidylcholine biosynthesis, phospholipid biosynthesis, cardiolipin biosynthesis, phosphatidylethanolamine biosynthesis, and sphingolipid metabolism suggests an increased synthesis of cellular membrane which may contribute to membrane rigidity [61]. Membrane rigidity has been reported as a possible adaptive mechanism by breast cancer cells to resist chemotherapy [61,62]. Recently, there have been increasing interest in targeting dysregulated lipid metabolism as a plausible adjuvant therapy against breast cancer and other cancers [61,63]. Thus, the ability of *C. sativa* to inactivate these pathways (Fig. 4) suggests its potential as an adjuvant in breast cancers. The inactivation can be attributed to the complete depletion of the corresponding metabolites (Fig. 4 and Table S1).

Cancer cells have been shown to require abundant amino acids supply for survival, proliferation, nucleosides synthesis, energy production, protein synthesis and redox balance [64]. This requirement has been implicated in amino acids dysmetabolism in cancers including breast cancer. This is depicted in the present study by the detected amino acid metabolism pathways and their corresponding metabolites in the untreated MCF-7 cells (Fig. 5 and Table S1). The glutamate metabolism pathway corroborates reports on the role of glutamine in the survival and proliferation of breast cancers [65]. Being the most consumed nutrient after glucose by cancer cells, glutamine is converted to glutamate which can feed the citric acid cycle after conversion to alpha-ketoglutarate [66]. Glutamate has also been implicated in energy production via lactate [67]. Glycine and serine metabolism have also been implicated in cancer cells survival, as serine is converted to glycine which is utilized for de novo biosynthesis of purine [65]. The valine, leucine and isoleucine degradation in the untreated cells also corroborates reports on the roles of essential acid in cancer cells survival and proliferation [64]. It is pertinent to mention that the identified metabolites for these pathways (Fig. 5C and Table S1) are products of glycolysis and citric acid cycle. Over the years, the arrest of amino acid metabolism has been suggested as a plausible therapy against cancers. This is demonstrated in the present study by the ability of *C. sativa* to inactivate glutamate metabolism, arginine and proline metabolism, amino sugar metabolism, glycine and serine metabolism, and valine, leucine and isoleucine degradation, while concomitantly depleting the corresponding metabolites in MCF-7 cells (Fig. 5 and Table S1). The activation of selenoamino acid metabolism following treatment with *C. sativa* may indicate an anticancer mechanism as selenoamino acids have been reported for their ability to induce apoptosis in cancer cells as well as act as chemo-preventive agents [68,69]. The activation and spermidine and spermine biosynthesis in *C. sativa*-treated cells also suggest a therapeutic mechanism as the activation of this pathway and its products have been implicated in proliferation arrest, anti-inflammation, apoptosis and modulation of polyamine metabolism in cancers [70,71].

Alterations in vitamin metabolism in cancer cells have been linked to the roles of the nutrient as major co-factors for many enzyme-catalyzed metabolisms and bioenergetics [72]. This is depicted in the present study by the detected vitamin metabolism pathways and their corresponding metabolites in the untreated MCF-7 cells (Fig. 6 and Table S1). The presence of pantothenate and CoA biosynthesis pathway may insinuate increased synthesis of CoA which is an important substrate in a number of key metabolisms including energy metabolism, fatty acid oxidation, and pyruvate oxidation to acetyl CoA [73]. Of all vitamins, vitamin B6 is often regarded as having the most bodily functions [74] as it functions as a cofactor for over 4% of enzymatic activities [75]. Thus, its pathway in the untreated MCF-7 cells may further insinuate increased energy production, protein and lipid syntheses as the nutrient has been reported as a major cofactor for enzymes catalyzing these biological activities [76]. Riboflavin metabolism pathway in the untreated cells may also insinuate increased energy production from glucose, fatty acids, and amino acids as riboflavin is a major cofactor for the respective metabolizing enzymes [77]. Furthermore, nicotinate and nicotinamide metabolism pathway in the untreated cells suggests increased bioenergetics as the pathway is involved in the generation of NAD^+ and $NADP^+$ which are critical for energy metabolism, signaling and biosynthesis [78]. The absence of these pathways and their corresponding metabolites in MCF-7 cells treated with *C. sativa* (Fig. 6 and Table S1) indicate the potential of the infusion to arrest vitamin metabolism in breast cancers. Thus, insinuating that a possible manipulative mechanism of cancer bioenergetics and metabolism by *C. sativa* may also involve suppression of key enzymes co-factors.

Exacerbated nucleotide synthesis has been reported in cancer cells and has been attributed to the high demand for DNA replication and for RNA production to sustain protein synthesis and cell proliferation [79]. This is depicted in the present study by the detected pathways and metabolites for pentose phosphate pathway, pyrimidine metabolism, purine metabolism and inositol metabolism in the untreated MCF-7 cells (Fig. 7 and Table S1). Increased pentose phosphate pathway has been reported in cancer cells and has been implicated in the generation of NADPH and ribose 5-phosphate (R5P) which are important precursors for the synthesis of nucleic acids, nucleotides, sterols, amino acids, and fatty acids [80]. Pyrimidine and purine metabolisms pathways are respectively responsible for the synthesis of DNA and RNA which is important for cancer cell proliferation [81]. Inositol metabolism also play a key role in the regulation of purine nucleotide synthesis [82]. Its upregulation has been reported in breast cancer where it regulates cell growth, proliferation and survival [83,84]. The inactivation of pyrimidine metabolism, purine metabolism, inositol metabolism and their corresponding metabolites following treatment with *C. sativa* (Fig. 7 and Table S1) insinuates a limited pool of nucleotides for RNA and DNA synthesis, which may contribute to the antiproliferative effect of the infusion. The availability of nucleotide is tightly regulated by the cell cycle [79]. The potent molecular interactions of the HPLC-identified phytoconstituents of *C. sativa* infusion with CDK6 and CDK 2 (Fig. 10A and B; and S2 – S5; and Table 2) may insinuate the ability of the infusion to induce cell cycle arrest and may be attributed to the inactivation of pyrimidine metabolism, purine metabolism and inositol metabolism pathways. However, further wet studies will be required to validate this claim. It is also pertinent to mention that NADPH generated from PPP (Fig. 7) is an important substrate for fatty acid biosynthesis (Fig. 4a) and reductive detoxification. Thus, the flux of metabolites from these alternative metabolic pathways may equip MCF-7 cells with flexibility to adapt to nutrient starvation by *C. sativa* and may partly explain the sustenance of cell growth/viability in 78.8% of *C. sativa*-treated cells while treatment resulted in the death of about 21.2% of cells (Fig. 2).

Induction of oxidative stress has been well reported as a major therapeutic mechanism against breast cancer and other cancer types [85,86]. This therapeutic mechanism has been explored by most chemotherapy as it has been implicated as a major trigger of cell cycle arrest and apoptosis, ultimately leading to antiproliferation and cell death [87–89]. Although in the present study, MCF-7 cells treated with *C. sativa* cells did not display any oxidative metabolic pathway and any corresponding metabolites, treatment with doxorubicin was able to induce oxidative stress as depicted by its activation of glutathione metabolism and degradation of superoxides (Fig. 8). The activation of these pathways can be attributed to the metabolite, hydrogen peroxide (H_2O_2) (Table S1). This result corroborates previous reports on the ability of doxorubicin to generate H_2O_2 and induce oxidative damage in breast cancer cells [90,91].

A schematic pathway of the relevant identified pathways in the present study is presented in Fig. 11.

Induction of cell death characterized by apoptosis and necrosis is one of the main therapeutic mechanisms of most anticancer drugs [92,93]. The induction of apoptosis and necrosis in MCF-7 cells treated with *C. sativa* (Fig. 9) suggests an ability of the infusion to

instigate cell death in breast cancer cells. Both apoptosis and necrosis have been linked to DNA fragmentation and depletion in its synthesis. Thus, their induction may be attributed to the inactivation of pyrimidine metabolism, purine metabolism and inositol metabolism pathways (Fig. 7A) and their corresponding metabolites (Fig. 7C and Table S1). The low percentage increase when compared to the control, may be attributed to the low cytotoxic activity of the infusion (Fig. 2). The inductions can also be attributed to the synergetic effects of the HPLC-identified phytochemical constituents of the infusion, particularly cannabidiol which has been reported as the main cannabinoid responsible for *C. sativa* induced necrosis and apoptosis [94,95]. Rutin [47], ferulic acid [96,97], and cinnamic acid [46,98] have also been reported for their ability to induce cell death in cancer cells.

5. Conclusion

These results portray the antiproliferative potentials of *C. sativa* infusion as an alternative therapy for the treatment and management of breast cancer. This is depicted by the ability of the infusion to modulate (1) glucose metabolism, thereby inhibiting energy production and glycolytic supplies of intermediates for major biosynthetic pathways; (2) lipid metabolism; (3) amino acid metabolism; (4) vitamin metabolism; and (5) nucleotide metabolism, while concomitantly inducing apoptosis and necrosis in breast cancer cells. However, owing to the low inhibition of viable cells when compared to doxorubicin, *C. sativa* infusion may be used an adjunctive therapy to improve breast cancer therapy.

Author contribution statement

Ochuko Lucky Erukainure: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Omolola Oyenihi; James F Amaku; Chika I. Chukwuma: Performed the experiments; Analyzed and interpreted the data.

Adeline Lum Nde; Veronica Folakemi Salau: Performed the experiments.

Motlalepula Gilbert Matsabisa: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Data availability statement

Data included in article/supplementary material/referenced in article.

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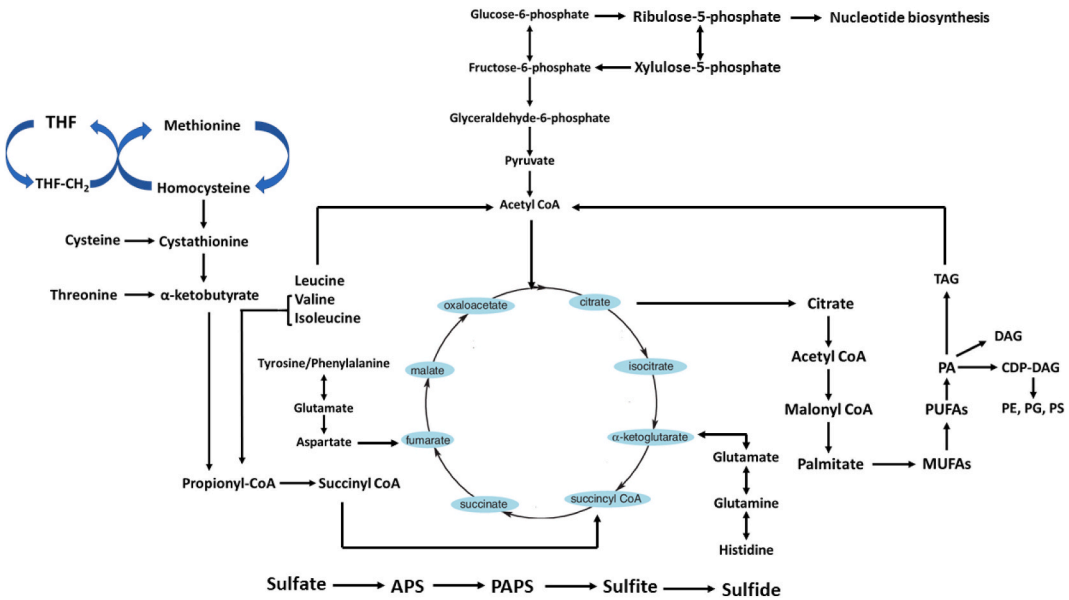

Fig. 11. Schematic pathway of relevant identified pathways and metabolites in the studied MCF-7 cells. THF: tetrahydrofolate; TAG: triacylglycerol; DAG: diacylglycerol; CDP-DAG: CDP- diacylglycerol; PE: phosphatidylethanolamine; PG: prostaglandin; PS: phosphatidylserine; PA: phosphatidic acid; PUFAs: polyunsaturated fatty acids; MUFAs: monounsaturated fatty acids; APS: adenosine phosphosulfate; and PAPS: phosphoadenosine phosphosulfate.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e16156.

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