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Original Research

Bioactive compounds in *Costus afer* Ker Gawl leaves and stem fractions protect against calcium ion-induced mitochondrial membrane permeability transition

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ABSTRACT

Objective: *Costus afer* Ker Gawl is an African ethnomedicinal plant used as a therapeutic for the treatment of oxidative stress-related diseases. This study was designed to identify the most active fractions of *C. afer* leaves and stem against calcium(II) (Ca^{2+})-induced mitochondrial membrane permeability transition (MPT). **Methods:** *C. afer* leaves and stem were extracted using 70% methanol and subsequently subjected to successive solvent partitioning method to separate it into hexane, ethyl acetate, *n*-butanol, and aqueous fraction of *C. afer* leaves and stem. Rat liver mitochondrial fractions were isolated using differential centrifugation technique, and mitochondrial MPT was assessed using a spectrophotometric method. **Results:** Data showed that *n*-butanol fractions of *C. afer* leaves and stem exhibited a significantly high inhibition of Ca^{2+}-induced mitochondrial MPT than other test fractions and standard spermine. **Conclusion:** *n*-butanol fractions of *C. afer* leaves and stem possess bioactive compounds with chemoprotective activity against Ca^{2+}-induced mitochondrial MPT. Furthermore, it is suggested that *n*-butanol fractions of *C. afer* leaves and stem could be harnessed for bioactive compounds that can be used in the treatment of mitochondria-associated neurodegenerative diseases.

KEY WORDS: Bioactive compounds, calcium ion, *Costus afer*, mitochondria

INTRODUCTION

Medicinal plants have been proved to be a major source of drugs due to the presence of diverse bioactive compounds [1]. Most of these medicinal plants are used in developing countries, especially rural settlers, by making concoctions or chewing part of the plant including stem or fruits for therapeutic purposes [2]. Bioactive compounds with antioxidant property are capable of preventing and fighting oxidative stress-related diseases.

Costus afer Ker Gawl of the family of Zingiberaceae, now known as Costaceae, is an herbaceous, rhizomatous monocotyledon which can be found in the Forest belt of Tropical Africa such as Senegal, South Africa, Ghana, Guinea, Cameroon, and Nigeria. The common names for C. *afer* include bush cane, ginger lily, or spiral ginger [3]. C. *afer* leaves and stem as medicinal plant parts have received a lot of attention as a result of its potential in treating a vast number of disease conditions including malaria, rheumatoid arthritis, diarrhea, diabetes mellitus, stomach ache, cough, and cold [4]. C. *afer* is well known for its antioxidant, anti-inflammatory and hepatoprotective activities due to the presence of bioactive compounds [5,6]. Studies have shown that C. *afer* contains steroidal sapogenins, saponins aferosides A-C and dioscin, all of which have important therapeutic applications [7]. Other bioactive compounds found in C. *afer* leaves and stem including polyphenolics, saponin, alkaloids, and glycosides are known to elicit various cellular responses [8].

Mitochondria are highly dynamic membranous organelles that play a major role in energy homeostasis important for various biosynthetic signaling and cell death pathway and they are found in eukaryotic cells. Mitochondria have been implicated in several

human diseases associated with mitochondrial disorders [9]. The enclosed organelle is divided mainly into two compartments which are the outer and inner membrane. In the outer membrane, ions and sugar molecules can easily penetrate [10]. Transporters located in the inner mitochondrial membrane make it possible for certain substrates to enter into the mitochondrial matrix that normally cannot pass through, because it's highly impermeable to molecules. Two main transporters involved are sodium-calcium (Na^+Ca^{2+}) exchanger that aids in the release of Ca^{2+} from the mitochondria into the cytosol and Ca^{2+}-ATPase that allows the uptake of Ca^{2+} into the mitochondria. Thus, maintaining homeostasis between the matrix and the cytosol of the mitochondria [11]. The concentration of Ca^{2+} is tightly regulated in all cells. The normal concentration of intracellular Ca^{2+} is 75-200 nmol/l and an increase to 1-10 μmol/l causes excessive reactive oxygen species (ROS) generation, thus, damaging mitochondrial proteins causing oxidative stress, mitochondrial membrane permeability transition (MPT) and further altering mitochondrial Ca^{2+} homeostasis [12].

Mitochondrial MPT results in the opening of mitochondrial megapore, which is a non-selective mitochondrial pore that opens under certain conditions including elevated matrix Ca^{2+} ion levels, depolarization of mitochondria membrane and excessive production of ROS. The opening of mitochondria megapore can lead to the swelling and eventual rupture of the mitochondria resulting with mitochondria damage [11]. Mitochondria MPT pore is proposed to comprise three proteins, namely, cyclophilin D found in the matrix, adenine nucleotide translocator in the inner membrane, and voltage-dependent anion channel found in the outer membrane of the mitochondria [13]. Opening of the mitochondria MPT pore can trigger the release of cytochrome c which further results in the activation of caspase-9 and downstream cleavage of caspases 3, 6, or 7 leading to apoptosis [14].

This study was, therefore, designed to investigate the potential of *C. afer* leaves and stem fractions to protect against Ca^{2+}-induced mitochondrial MPT. This was performed with the rationale to isolate the most potent fraction with mitochondria membrane protective property.

MATERIALS AND METHODS

Collection of Plant Material

C. afer plant was collected from a farmland located at Irolu, Ikenne local government area, Ogun State, Nigeria. A voucher sample was deposited at the Forestry Herbarium, Ibadan, Oyo State, Nigeria, with the number of FHI-108001.

Plant Processing and Extraction

C. afer leaves were washed and dried in a hot air oven at 40°C. Dried leaves were pulverized using a mechanical blender and kept in an airtight container. Pulverized sample (75 g) was weighed out and soaked in 600 ml of 70% methanol, capped and mixed intermittently for about 48 h. The suspension was filtered using Whatman No. 1 filter paper and the filtrate was subsequently concentrated to dryness using a rotary evaporator at 40°C. The concentrate was then reconstituted in 100 ml distilled water and was fractionated with a separating funnel using successive solvent partitioning method into hexane, ethyl acetate, *n*-butanol fractions, and the remaining portion was considered as aqueous fraction. The four fractions obtained were further concentrated using rotary evaporator at 40°C. The concentrates obtained were then stored in a refrigerator at 4°C until further use.

Animal Care and Handling

Ten male albino rats (Wistar strain) weighing between 150 and 200 g were obtained from an inbred colony in the Animal Facility, Babcock University. Animals were housed under diurnal lightening conditions and fed with standard rat chow and water *ad libitum*. The animals were cared for and maintained following the National Institute of Health Laboratory Guideline on Animal Care and Use [15]. Ethical approval was obtained from Babcock University Health, Research and Ethics Committee (BUHREC) with certification number BUHREC356/17.

Isolation of Rat Liver Mitochondria

The rat liver mitochondria were isolated according to the method of Johnson and Lardy [16]. The animals were anesthetized using petroleum ether and sacrificed by cervical dislocation. They were dissected to harvest the liver. The rat liver was quickly excised, trimmed to remove extraneous tissues, washed with buffer solution (210 mM mannitol, 70 mM sucrose, and 5 mM 2-(4-[2-hydroxyethyl] piperazin-1-yl) ethane sulfonic acid [HEPES]) at pH 7.4 and 1 mM ethylene glycol tetraacetic acid until a clear solution was seen. The liver samples were minced with a pair of clean scissors and subsequently homogenized using homogenizer until a paste was made. The liver paste was then dispensed carefully into Eppendorf tubes and centrifuged at 2500 rpm for 5 min to sediment the nuclear fraction and cellular debris. The supernatant obtained was carefully dispensed into clean Eppendorf tubes and centrifuged again at 2500 rpm for 5 min.

The supernatant obtained was collected in clean Eppendorf tubes, and they were centrifuged at 12500 rpm for 10 min to precipitate mitochondria. The supernatant was discarded while the precipitated mitochondria were washed with HEPES buffer at pH 7.4 and centrifuged at 13000 rpm for 10 min. The supernatant was discarded while the brown mitochondria sediment was immediately suspended in an equal volume of storage buffer (mannitol, sucrose, and HEPES-potassium hydroxide [KOH] pH 7.4). All procedures were carried out under an ice-cold medium to preserve and retain the integrity of the mitochondria.

Determination of Mitochondrial Protein Content

Mitochondrial protein content was estimated following the procedure of Lowry *et al.* [17] using bovine serum albumin as a standard.

Effect of *C. afer* Leaves Fractions on Mitochondrial MPT

Effects of different concentrations of *C. afer* leaves fractions on mitochondrial MPT was determined according to the method of Lapidus and Sokolove [18]. Isolated mitochondrial fraction (0.4 mg/ml) was suspended in a medium containing 210 mM mannitol, 70 mM sucrose, 5 mM HEPES-KOH (pH 7.4), 0.8 μM rotenone, and 5 mM succinate. Preloaded mitochondrial fraction (0.4 mg/ml) was incubated in Eppendorf tubes in the presence of 15, 30 and 50 $\mu g/\mu l$ *C. afer* leaves and stem, respectively. Mitochondrial MPT was triggered using Ca^{2+} while spermine, a polyamine, served as an inhibitor of MPT. The change in absorbance of mitochondria was monitored at 540 nm in a double beam ultraviolet-visible spectrophotometer (T80 Model, PG Instruments, Leicestershire, and UK).

Statistical Analysis

Statistical analysis was performed with the aid of Statistical Package for the Social Sciences version 17.0 (SPSS Inc., Chicago, IL, and USA) to determine the difference between mean using analysis of variance. $P < 0.05$ was considered significant. All studies were conducted in triplicate and reported as a mean ± standard error of the mean.

RESULTS

Data in Table 1 summarizes that an intact mitochondrial fraction was isolated with a change in absorbance of mitochondrial MPT of $\Delta_{540\,nm} = -0.041 \pm 0.003$ at 12 min in the absence of Ca^{2+} in a normal respiration sucrose phosphate buffer. A change in absorbance of $\Delta_{540\,nm} = -0.064 \pm 0.003$ showed a mitochondrial undergoing MPT in the presence of Ca^{2+}, while spermine significantly ($P < 0.05$) inhibited Ca^{2+}-induced mitochondrial MPT with a change in absorbance of $\Delta_{540\,nm} = -0.038 \pm 0.001$ when compared with mitochondria incubated in the absence and presence of Ca^{2+}.

Table 2 summarizes that varying concentrations of *C. afer* leaf fractions significantly ($P < 0.05$) inhibited Ca^{2+}-induced mitochondrial MPT when compared with an untreated mitochondrial fraction in the presence of Ca^{2+}. *C. afer* at 15 $\mu g/\mu l$ inhibited Ca^{2+}-induced mitochondrial MPT in the following order of magnitude: *n*-butanol > hexane > ethyl acetate > aqueous fractions by 84.38, 75, 65.63, and 29.69%, respectively. *C. afer* leaf fractions at 30 $\mu g/\mu l$ inhibited Ca^{2+}-induced mitochondrial MPT in the following order of magnitude: *n*-butanol > hexane > ethyl acetate > aqueous by 85.94, 85.94, 56.25, and 9.38%, while *C. afer* leaf fractions at 50 $\mu g/\mu l$ inhibited Ca^{2+}-induced mitochondrial MPT in the following order of magnitude: *n*-butanol > hexane > ethyl acetate > aqueous by 73.44, 67.18, 59.40, and 18.75%, respectively.

Table 3 summarizes that varying concentrations of *C. afer* stem fractions significantly ($P < 0.05$) inhibited Ca^{2+}-induced mitochondrial MPT when compared with an untreated mitochondrial fraction in the presence of Ca^{2+}. *C. afer* stem fractions at 15 $\mu g/\mu l$ inhibited Ca^{2+}-induced mitochondrial MPT in the following order of magnitude: *n*-butanol > ethyl acetate > hexane > aqueous by 88.7, 77.73, 74.1, and 72.02%, respectively. *C. afer* stem fractions at 30 $\mu g/\mu l$ inhibited Ca^{2+}-induced mitochondrial MPT in the following order of magnitude: Hexane > *n*-butanol > ethyl acetate > aqueous by 88.61, 88.1 75, and 50.26%, respectively. *C. afer* stem fractions at 50 $\mu g/\mu l$ inhibited Ca^{2+}-induced mitochondrial MPT in the following order of magnitude: Order *n*-butanol > ethyl acetate > aqueous > hexane by 79.8, 78.2, 72.02, and 64.77%, respectively.

Table 1: Effect of absence and presence of Ca^{2+} on mitochondrial membrane permeability transition energized by sodium succinate and inhibited by spermine monitored as swelling at $\Delta_{540\,nm}$ for 12 min

Test samples	Mean±SEM
Mitochondria+absence of Ca^{2+}	−0.004±0.000
Mitochondria+presence of Ca^{2+}	−0.064±0.003
Mitochondria+presence of Ca^{2+}+spermine	−0.038±0.001

SEM: Standard error of the mean

Table 2: Inhibitory effects of 15, 30, and 50 $\mu g/\mu l$ *n*-butanol, hexane, ethyl acetate, and aqueous fractions of *Costus afer* leaves on Ca^{2+}-induced mitochondrial membrane permeability transition at $\Delta_{540\,nm}$ for 12 min

Concentration ($\mu g/\mu l$)	*Costus afer* fractions	Mean±SEM	Percentage
15	*n*-butanol	−0.010±0.001	84.38
	Hexane	−0.016±0.001	75
	Ethyl acetate	−0.028±0.002	65.63
	Aqueous	−0.045±0.002	29.69
30	*n*-butanol	−0.009±0.001	85.94
	Hexane	−0.009±0.001	85.94
	Ethyl acetate	−0.028±0.002	56.25
	Aqueous	−0.058±0.003	09.38
50	*n*-butanol	−0.017±0.001	59.4
	Hexane	−0.021±0.001	67.18
	Ethyl acetate	−0.026±0.001	59.4
	Aqueous	−0.052±0.002	18.75

SEM: Standard error of the mean

Table 3: Inhibitory effects of 15, 30, and 50 $\mu g/\mu l$ *n*-butanol, hexane, ethyl acetate, and aqueous fractions of *Costus afer* stem on Ca^{2+}-induced mitochondrial membrane permeability transition at $\Delta_{540\,nm}$ for 12 min

Concentration ($\mu g/\mu l$)	*Costus afer* fractions	Mean±SEM	Percentage
15	*n*-butanol	−0.007±0.009	88.7
	Hexane	−0.017±0.008	74.1
	Ethyl acetate	−0.015±0.008	77.73
	Aqueous	−0.018±0.001	72.02
30	*n*-butanol	−0.008±0.005	88.10
	Hexane	−0.007±0.042	88.61
	Ethyl acetate	−0.016±0.009	75
	Aqueous	−0.018±0.001	72.02
50	*n*-butanol	−0.013±0.001	79.8
	Hexane	−0.023±0.013	64.77
	Ethyl acetate	−0.014±0.008	78.2
	Aqueous	−0.018±0.001	72.02

SEM: Standard error of the mean

DISCUSSION

In this study, mitochondria pre-incubated in the presence of Ca^{2+} were found to undergo mitochondrial MPT which was inhibited by spermine. This observation was in agreement with the statement that accumulation of intracellular Ca^{2+} in mitochondria induces the formation of mitochondrial MPT pore which is indicated as mitochondrial swelling [19]. This mitochondrial swelling can be inhibited by spermine in a normal respiration sucrose phosphate buffer [18].

Furthermore, it was observed that the *n*-butanol fractions of *C. afer* leaves and stem had a high inhibitory effect on Ca^{2+}-induced mitochondrial MPT when compared with the other fractions and standard spermine. This indicates that *n*-butanol fraction of *C. afer* leaves could contain bioactive compound(s) with the potential to protect mitochondria against factors that could elicit mitochondrial MPT. Previous studies had implicated mitochondrial MPT with a number of oxidative stress-related diseases [20,21]. In most neurodegenerative diseases including malaria, ischemia, reperfusion, diabetes and Alzheimer's disease, and death signals are transduced to the mitochondria, which induce mitochondrial MPT leading to the release of cytochrome c which activates proapoptotic signals resulting in cell death [22]. Hence, a bioactive compound that could inhibit the formation of mitochondrial permeability transition pore could serve as an important therapeutic drug in the treatment and management of neurodegenerative diseases.

In addition, the *n*-butanol fraction mitochondrial protective effect was closely followed by hexane fraction of *C. afer* leaves and stem. This is also in agreement with our previous study that the hexane fraction of *C. afer* leaves possesses mitochondrial protective property [23]. The possible mechanism for the observed inhibition of mitochondrial MPT by *C. afer* fraction could be attributed to the bioactive compounds present in *C. afer* binding directly to specific components of the mitochondrial MPT pore complex and interfering with its activity or by reducing the ROS-mediated oxidative stress due to the induction of mitochondrial MPT pore by Ca^{2+}. The bioactive compound(s) in *C. afer* could possess Ca^{2+}-chelating capacity and membrane stabilizing property thus inhibiting mitochondrial MPT.

Phytochemicals present in the fractions of *C. afer* leaves and stems such as polyphenols, tannins, and saponins have been previously shown to bind cations and thereby stabilize biological membranes and macromolecules [24]. Gas chromatography-mass spectrometry (GC-MS) data showed bioactive compounds in *n*-butanol fractions of *C. afer* leaves and stem previously shown to exhibit membrane stabilizing and cation chelating properties. *n*-butanol fraction of *C. afer* leaves contains indolizine, 2-methoxy-4 vinyl phenol, phytol, hexadecanoic acid-methyl ester, and oleic acid, while *n*-butanol fraction of *C. afer* stem contains benzofuran 2,3-dihydro, 2-methoxy-4 vinyl phenol, campesterol, stigmasterol, hexadecanoic acid-methyl ester, and cis-vaccenic acid [25].

Findings from this study showed that n-butanol fractions of *C. afer* leaves and stem generally exhibited a high mitochondrial protection against Ca^{2+}-induced mitochondrial MPT pore when compared with the other fractions. It is therefore recommended the n-butanol fraction could be harnessed for pharmaceutical drug development against neurodegenerative diseases. This study could also provide a subcellular basis for the ethnomedical use of this plant as a potential therapeutic for the treatment of oxidative stress-induced disorders.

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