Drug delivery Resistance of Blood-Brain-Barrier: *Cajanus cajan* and *Lycopersicon esculentum* down-regulated Multi-drug Resistance1 Expression in Cerebrum of Rats

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P-glycoprotein (multi-drug resistance 1 protein) is a brain endothelial cell protein which functions as a drug efflux transporter and strengthens the shielding effect of the blood brain barrier making drug delivery to the brain a challenging task. This study examined neuroprotective potential of two ethnomedicinal plants, *Cajanus cajan* and *Lycopersicon esculentum*, on P-glycoprotein expression in ethidium bromide-induced neurotoxicity. Ethidium bromide-induced toxicity was achieved via application of 0.5 ml of a solution of 0.5 g/100 ml ethidium bromide in ethanol to the scraped ventral skin of 23 groups (n = 5) of adult rats (day 1). Groups 1 and 2 were post-treated with normal saline and tamsulosin hydrochloride, respectively (days 1-28). Nine groups (CC3-11) were post-treated with *Cajanus cajan* (aqueous, butanolic and ethanolic extracts of seeds, stems and leaves) (days 1-28). Twelve groups (LE3-14) were post-treated with *Lycopersicon esculentum* (aqueous, butanolic, ethanolic and n-hexane extracts of roots, stems and leaves) (days 1-28). Concentrations of P-glycoprotein in homogenates of cerebral cortices were determined using ELISA following anaesthetization using diethyl ether. Data were analysed using ANOVA with Tukey post-hoc test at $p \leq 0.05$. Post-treatments with *Cajanus cajan* and *Lycopersicon esculentum* extracts resulted in significant downregulations of MDR1 in CC3, CC4, CC6-11 and LE3-14 compared with group 1. *Cajanus cajan* and *Lycopersicon esculentum* possess neuroprotective and anti-drug resistance potential.

Key words: Ethidium bromide, neurotoxicity, Multi-drug resistance protein1, *Cajanus cajan*, *Lycopersicon esculentum*

INTRODUCTION

The brain controls the activities of body systems. Different protective mechanisms such as the blood-brain barrier (BBB) with its brain-microvascular endothelial cells protect the brain against exogenous substances (1,2). Brain endothelial capillaries when compared to other body capillaries possess increased mitochondria, fewer pinocytic vesicles and no pores, while their adjoining cells are closely apposed by tight junctions in order to protect the delicate brain organ (1,2). This shielding effect of the BBB also restricts the entry of drugs used in the treatment of central nervous system (CNS) diseases and neurodegenerative disorders (2,3). This makes drug delivery to the brain a challenging task.

The P-glycoprotein or Multi-drug Resistance1 (MDR1) is a brain microvascular endothelial cell protein present in high concentration on apical surfaces of endothelial cells. It works as a

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drug efflux transporter protein thereby strengthening and increasing the shielding effect of the BBB (1,3,4). MDR1 has attraction for hydrophobic compounds and research studies have tried to resolve the efflux effect of P-glycoprotein by employing the usage of several resistance reversal compounds including Cremophor EL, R-verapamil and Tween-80. However, these resistance reversal compounds induced significant adverse effects at doses sufficient for P-glycoprotein suppression (1,3,4). Furthermore, different methods developed to enhance brain penetration of therapeutic agents including drug manipulation, BBB disruption and the use of alternative routes for drug delivery have limitations and are not yet efficient (1,3). In addition, cancer treatment procedures eliminate cancer cells, rather than cancer stem cells (CSCs) which possess shielding and impenetrable mechanisms (5,6) through the overexpression of P-glycoprotein (4).

Cajanus cajan (CC) or pigeon pea is widely grown in Nigeria. CC is used traditionally in the treatments of diseases such as bladder stone, jaundice and sickle cell anemia (7,8). Traditionally, vapour from smoldered leaves of CC is breathed in to relieve asthma and cough (7,8). Furthermore, CC is low in sodium content, hence its use traditionally in the treatment of hypertension (7,8). CC contains folate, thiamin, pantothenic acid, niacin, potassium, riboflavin and calcium (7,8).

Lycopersicon esculentum (LE) or *Solanum lycopersicum* (tomato) is a globally consumed vegetable. Traditionally, tomato juice and cooked tomatoes are used as homemade remedy for oral and other cancers (9,10). Tomato leaves are used in the treatments of painful joints, wound infections, eye weakness and optic nerve diseases including optic neuritis (a component of multiple sclerosis) (9,10). LE contains carotenoids, vitamins, ascorbic acid, phenolic compounds, α-tocopherol and lycopene (9). Lycopene effects the release of phase II enzymes which can eradicate toxins and carcinogens, thereby protecting lipids, proteins and DNA against cellular toxicity (10). Lycopene equally inhibits cancer cell proliferation (11), and blocks changes in cell architecture via decreased cancer cell adhesion (12,13).

Ethidium Bromide (EB) is an effective intercalator, a strong mutagen (14) and possible carcinogen (15,16). Human exposure to EB is via skin contact, inhalation, irritation of the eyes and respiratory system or leakage of EB-contaminated compounds or waste materials into water supplies, rivers, drinking water, garden and agricultural products (17). EB intercalates adjacent base pairs of neuronal cells of CNS, deforms double-stranded DNA (18) and induces multiple sclerosis in animal models (19). In addition, 0.1% EB injection resulted in the destruction of microvessels, carbon leakage and breakdown of the BBB in rats. Hence, EB-induced neurotoxicity will adversely affect concentrations of P-glycoprotein secreted by the microvessels of the BBB (21). Furthermore, EB accumulation by multi-drug resistant mouse T-lymphoma cells upregulated ATP-binding cassette transporter B1 (P-glycoprotein) confirming EB as a substrate for efflux pump (22).

Current conventional drug delivery systems used to enhance brain penetration are not efficient. Similarly, there is no drug known to efficiently eliminate cancer stem cells (CSCs) and drug resistant capacity of cancers. P-glycoprotein expression is more marked in the microvessels of the cerebral cortex (20). Therefore, this study evaluated the effects of aqueous, butanolic, ethanolic and n-hexane fractions of leaves, roots, seeds and stems of CC and LE on MDR1 expression in cerebral cortices of rats in EB-induced neurotoxicity to determine which plant parts possess neuroprotective, anti-drug resistance and anticancer potential.

EXPERIMENTAL

Collection and preparation of plant materials

Freshly cut seeds, stems and leaves of CC

and freshly cut roots, stems and leaves of LE were obtained from the school forest of the University of Ilorin, Nigeria. Attestation and verification of plant materials were conducted at the Herbarium unit of the Department of Plant Biology, University of Ilorin, Nigeria. The plant materials were washed to remove sand and debris and dried under shade at 25-30 °C for three weeks. Thereafter, the plant materials were pulverized and homogenized with an electric blender to increase the surface area for solvent extraction.

About 200 g of CC and LE powdered samples were weighed on an analytical balance (Sartorious BS 124S, Gottingen, Germany) with 0.1 mg accuracy. Aqueous extracts were prepared at 50 mg/l in distilled water by infusing for 5-10 minutes. The extracts were cooled at 25-30°C and centrifuged at 2000 rpm for 10 minutes in a centrifuge (Centribio, MLW T24 D, Janetzki, Leipzig, Germany) to obtain the supernatant. The supernatant was filtered through Whatman No. 4 filter paper to obtain the aqueous extract.

The butanolic extracts were prepared using modifications of previously described procedures (24). Solutions from the pulverized dried seeds were prepared at a concentration of 0.1 g/ml in 70% ethanol by maceration in the dark for 7 days at 25 ± 2˚C. The resulting mixtures were fully dried using a rotary evaporator (Heidolph 4011, Schwabach, Germany). Infusions of the resulting solid were prepared using distilled water to obtain a concentration of 50 µg/ml. The resulting extracts were centrifuged at 2000 rpm for 10 minutes, and the supernatants filtered with Whatman No. 4 filter paper. n-Hexane was added to the supernatant and left to extract for another 72 hours and a separatory funnel was used to separate the n- hexane fraction.

Animal care and feeding

One hundred and fifteen (115) healthy albino Wistar rats (7 weeks old and an average weight of 150 g) were purchased from the Animal House of the Department of Biochemistry of the University of Ilorin, Nigeria. The animals were kept in standard ventilated cages, fed with standard rat feed and granted access to tap water ad libitum in a hygienic environment.

Induction of Ethidium Bromide (EB) neurotoxicity

EB (0.5 g) was dissolved in 100 ml of ethanol. With the aid of a dissecting blade, 7 cm width of the skin of each rat was scraped ventrally in the midline from the neck to the pelvic region, and 0.5 ml of EB solution was applied to the scraped skin area topically (23).

Grouping of rats and treatment

All 115 rats used in the present study were exposed to 0.5 ml of the EB solution. The rats were divided into 23 experimental groups of 5 rats per group as shown in Tables 1 and 2. Following EB-exposure on day 1 of experimental procedure, rats in control group 1 and experimental group 2 were post-treated orally with normal saline and tamsulosin hydrochloride, respectively, for 4 weeks (days 1-28). In addition, following EB-exposure on day 1, 9 groups (CC3-11) were post-treated orally with 40 mg/kg body weight of aqueous, butanolic or ethanolic extracts of the seeds, stems and leaves of CC for 4 weeks (days 1-28) as presented in table 1. Twelve (12) groups (LE3-14) were post-treated orally with 40 mg/kg body weight of aqueous, butanolic, ethanolic or n-hexane extracts of the roots, stems and leaves of LE for 4 weeks (days 1-28) as presented in Table 2.

Evaluation of levels of P-glycoprotein in homogenates of cerebral cortices of rats

Following the completion of experimental procedure, each rat was sacrificed by anaesthetization using diethyl-ether inhalation, and the cranium was exposed and the cerebral cortex removed. Each cerebral cortex was divided into two hemispheres. Homogenates of each excised cerebral hemisphere were evaluated for levels of P-glycoprotein using an ELISA

technique as described by Akinlolu *et al*., 2020 (23).

Data analysis

The data acquired from the microplate ELISA results were analyzed and comparisons between each experimental and control group made by employing one-way ANOVA, while Tukey post-hoc test was employed for groups' comparisons. The statistical level of significance was set at $p \leq 0.05$.

Ethical Approval

The University of Ilorin Ethical Review Committee (UERC) approved the protocols for this study (ethical approval number UERC/ASN/2019/1820). The experimental procedures of this study were carried out according to the guidelines governing laboratory animal use and care as provided for in the policy statements of the University of Ilorin Ethical Review Committee, European Community guidelines (EEC Directive of 1986; 86/609/EEC) and the US guidelines (NIH publication #85-23, revised in 1985).

RESULTS AND DISCUSSION

Effects of *Cajanus cajan* (CC) on MDR1/P-glycoprotein levels

Statistical analyses revealed significantly ($p \leq 0.05$) decreased levels of MDR1/P-glycoprotein (ng/ml) in rats of groups 2 and CC3, CC4 and CC6 - CC11, in comparison with control group 1. However, there was a statistically non-significant ($p > 0.05$) decreased level of MDR1/P-glycoprotein (ng/ml) in rats of group CC5 compared with group 1 (Table 1).

The results of this study implied that EB-induced neurotoxicity resulted in mutagenesis with accompanied increased drug resistance as made evident by the significant increased expression of MDR1 levels in rats of control group 1, when compared with groups CC3, CC4, CC6 - CC11 and LE3 - LE14 (Tables 1 and 2).

Qualitative and quantitative phytochemical screening of fruits, seeds and leaves of CC species grown in Nigeria showed the presence of antioxidant, antimicrobial and anticancer compounds such as anthocyanin, alkaloids, cyanogenic glycosides, flavonoids, glycosides, saponins, tanins and terpenes (8, 25, 26). Hence CC possesses antioxidant and anticancer potential (23).

Post-treatments with aqueous, butanolic and ethanolic extracts of the seeds, stems and leaves of CC ameliorated EB-induced neurotoxicity, drug resistance and mutagenesis via positive immunomodulation and downregulation of MDR1 concentrations in rats of groups CC3 - CC11 (Table 1). However, post-treatment with ethanol seed extract of CC (group CC5) resulted in non-significant downregulation of MDR1/P-glycoprotein levels (Table 1). In addition, MDR1 is a signature molecule of CSCs. Hence, the seeds, stems and leaves of CC possibly possess anti-drug resistance and anti-CSCs potential.

Effects of *Lycopersicon esculentum* (LE) on MDR1/P-glycoprotein levels

Statistical analyses revealed significantly ($p \leq 0.05$) decreased levels of MDR1/P-glycoprotein (ng/ml) in rats in groups 2 and LE3 - LE14, in comparison with control group 1 as presented (Table 2). Qualitative and quantitative phytochemical screening of fruits and leaves of LE species grown in Nigeria showed the presence of antioxidant and anticancer compounds such as anthraquinone, cardiac glycosides, carotenoids, β-carotene, coumaric acid, cyanogenic glycosides, flavonoids, lutein, lycopene, oxalate, phlabotannins, quercetin, steroids, saponins, tannins, terpenoids and zeaxanthin (27-29). Hence, LE possesses antioxidant and anticancer potential (9-13, 23).

Post-treatment with aqueous, butanolic, ethanolic and n-hexane extracts of the roots, stems and leaves of LE ameliorated EB-induced neurotoxicity, drug resistance and mutagenesis via positive

immunomodulation and downregulation of MDR1 levels in rats in groups LE3-14 (Table 2). Likewise, MDR1 is a biomarker of CSCs. Hence the roots, stems and leaves of LE possibly possess anti-CSCs potential. The ethanol stem extract of LE (group LE5) had the best anti-drug resistance and anticancer potential (Table 2), and is highly recommended for further studies.

The ethanol stem extract of LE (group LE5) had the best anti-drug resistance and anticancer potential when compared all LE and CC plant extracts. Post-treatments of EB-induced neurotoxicity with all LE extracts resulted in significant downregulation of MDR1 unlike post-treatment with ethanol seed extract of CC (group CC5) which resulted in non-significant downregulation of MDR1. These observations indicated that LE achieved better anti-drug resistance and anticancer potentials compared with CC.

Table 1: Levels of P-glycoprotein in homogenates of cerebral cortices of rats treated with ethidium bromide and *Cajanus cajan* extracts

Group	EB + NS/Drugs/Extracts	P-gp (ng/ml), Mean ± SD	p-value
1	0.5 ml NS	42.30±0.03	
2	Tamsulosin HCl	32.2±0.03	0.05
CC3	Ethanol leaf	17.62±0.05	0.02
CC4	Ethanol stem	32.95±0.02	0.05
CC5	Ethanol seed	41.21±0.06	0.50*
CC6	Aqueous leaf	32.11±0.01	0.05
CC7	Aqueous stem	14.18±0.05	0.02
CC8	Aqueous seed	17.79±0.06	0.02
CC9	Butanol seed	9.87±0.04	0.01
CC10	Butanol leaf	11.90±0.05	0.02
CC11	Butanol stem	17.27±0.03	0.02

Key: CC = *Cajanus cajan*, NS = normal saline.
$p \leq 0.05$ (*except for CC5): Group 1 vs Groups 2 and CC3 - CC11; All groups were pretreated with 0.5 ml of 0.5% w/v ethanolic ethidium bromide; All extracts and tamsulosin HCl were dosed at 40 mg/kg body weight.

Table 2: Levels of P-glycoprotein in homogenates of cerebral cortices of rats treated with ethidium bromide and *Lycopersicon esculentum* extracts

Group	EB + NS/Drugs/Extracts	P-gp (ng/ml), Mean ± SD	p-value
1	0.5 ml NS	42.30±0.03	
2	TH	32.2±0.03	0.05
LE3	Ethanol root	24.48±0.06	0.03
LE4	Ethanol leaf	26.73±0.04	0.03
LE5	Ethanol stem	3.14±0.03	<0.01
LE6	Aqueous Root	28.31±0.03	0.03
LE7	Aqueous leaf	26.59±0.05	0.03
LE8	Aqueous Stem	10.55±0.04	0.01
LE9	Butanol root	11.76±0.02	0.01
LE10	Butanol leaf	15.02±0.02	0.02
LE11	Butanol stem	16.94±0.03	0.02
LE12	n-Hexane root	19.86±0.04	0.02
LE13	n-Hexane leaf	29.85±0.01	0.03
LE14	n-Hexane stem	20.9±0.06	0.03

Key: LE = *Lycopersicon* esculentum, NS = normal saline,
$p \leq 0.05$: Group 1 versus Groups 2 and LE3 - LE14 ; All groups were pretreated with 0.5 ml of 0.5% w/v ethanolic ethidium bromide; All extracts and tamsulosin HCl were dosed at 40 mg/kg body weight.

CONCLUSION

Treatment with *Cajanus cajan* extracts (seeds, stems and leaves) and *Lycopersicon esculentum* extracts (roots, stems and leaves) conferred neuroprotection against ethidium bromide-induced neurotoxicity, drug resistance and mutagenesis in cerebrum of rats. Treatment with *Lycopersicon esculentum* extracts achieved better results compared with *Cajanus cajan* extracts.

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