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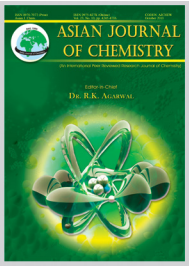

Oxygen-Dependent-Regulation of Ehrlich's Ascites Carcinoma Cell Respiration by Gallic Acid and Rutin Isolated from *Melothria heterophylla* (Lour.) Cogn.

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The effect of gallic acid and rutin isolated from *Melothria heterophylla* (Lour.) cogn on the oxygen consumption of Ehrlich-ascites-carcinoma (EAC)-cell was tested by using different respiratory substrates, electron donors at different segments of the mitochondrial respiratory chain and site-specific inhibitors to identify the specific respiratory complex, which might be involved in the inhibitory effect of gallic acid and rutin on the oxygen consumption by these cells. The results indicate that isolated compounds fail to inhibit ADP-stimulated ascorbate plus *N',N'*-tetramethyl-*p*-phenylenediamine, L-glutamate, like malate and α-keto-glutarate-dependent respiration, but strongly inhibit succinate-dependent respiration. So, gallic acid and rutin possibly inhibit the electron flow through complex II of the Ehrlich-ascites-carcinoma-cell mitochondrial respiratory chain.

Key Words: *Melothria heterophylla*, Oxygen uptake, Oxygraph.

INTRODUCTION

Melothria heterophylla (Lour.) Cogn., family- cucurbitaceae, popularly known as kudari, a scandent herb with tuberous roots found throughout India ascending up to 2,100 m in the hills. They are considered to be used by the tribal of Orissa for their stimulant, invigorating, purgative property[1] and antioxidant activity[2]. The juice of the leaves is applied to the parts inflamed by the application of the marking nut juice (from *Semecarpus anacardium* Linn). In this study, we report that gallic acid (3,4,5-trihydroxybenzoic acid, GA) and Rutin (RU), the major bioactive compounds isolated from ethanol fraction of *Melothria heterophylla* extracts (EEMH).

The flavonoids and phenolic compounds are bioactive constituents, which are reported to acts as an antioxidant and help to protect human cells against oxidative damage. Gallic acid was found to show cytotoxicity against cancer cells, without harming healthy cells[3].

Cancer cells adapt to hypoxic and acidic conditions generated during progressive tumor cell growth by shifting the burden of energy metabolism from mitochondrial oxidative phosphorylation to glycolysis[4].

Although the molecular pathogenic mechanism is currently unknown, it is expected that mitochondria play a crucial role in this transition. Cancer cells have a defective mitochondrial function mainly resulting in high aerobic glycolysis. Here we will study the effects of gallic acid and rutin on respiration, especially of the malignant cells, which will provide us an opportunity to understand precisely the possible alteration of mitochondrial functions in malignant cells. The present study was undertaken to identify the involvement of specific respiratory complex, which inhibits electron flow of EAC cell mitochondria.

EXPERIMENTAL

All the biochemicals were purchased from Sigma Chemicals Co., USA. Rotenone, malonate, antimycin A and *N,N, N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) were the products of sigma. All other chemicals were of analytical grade.

The aerial parts of *Melothria heterophylla* were collected from young matured plants during August-September, from the rural belt of Mayurbhanj district, Orissa, India and identified by taxonomist, Botanical Survey of India, Howrah, India. A voucher specimen (CNH/I-I (65)2006/Tech.II/1661) was deposited in the Department of Pharmaceutical Technology, Jadavpur University. The collected plant material was washed, shade-dried and then milled to course powder by a mechanical grinder for further studies.

Preparation of extracts: The powdered plant material (40 mesh size) was extracted in succession with petroleum ether (40-60 ºC), chloroform, ethyl acetate, ethanol and distilled water using the soxhlet apparatus. The solvent was then removed under reduced pressure, to obtain petroleum ether (PEMH, yield 4.38 %), chloroform (CEMH, yield 2.28 %), ethyl acetate

TABLE-1
RELATIVE RATE OF SUBSTRATE OXIDATION BY MITOCHONDRIA IN DIGITONIN-PERMEABILIZED EAC CELLS

Cell pretreatment*	Relative rate of substrate oxidation**								
	Endogenous substrate	L- glutamate (10 mM)	L- glutamate (10 mM) + ADP (0.1 mM)	L-glutamate (10 mM) + ADP (0.1 mM) + GA	L- glutamate (10 mM) + ADP (0.1 mM) + Rutin	Succinate (10 mM)	Succinate (10 mM) + ADP (0.1 mM)	Succinate (10 mM) + ADP (0.1 mM) + GA	Succinate (10 mM) + ADP (0.1 mM) + Rutin
EAC	100								
EAC + digitonin	50	55	62	30	48	75	82	3	7
EAC + digitonin + Rotenone (40 mM)			10				25		
EAC + digitonin + antimycin (2µg/mg protein)			5				2		
EAC + digitonin + KCN (2 mM)			2				1		

*Relative rate 100 was considered for EAC endogenous oxygen uptake rate to be 15 nmol O_2/min/mg cell proteins. Relative rate values represent the average of three experiments; **EAC cells were treated with 100 µg digitonin/mg cell protein, for 3 min before the addition of oxadizable substrates and/or electron transport inhibitor.

(EAMH, 0.46 %), ethanol (EEMH, yield 5.30 %) and aqueous extract (AEMH, yield 14.39 %), respectively.

Isolation of active constituents: The ethanol extract (3 g) was fractionated over the silica gel column eluted with CH_2Cl_2-MeOH with gradual increasing of the MeOH content and 40 fractions were collected. Fraction 19-21 are mixed together, which shows a single spot having similar R_f and was rechromatographed over the silica gel column eluted with 100 % MeOH to afford 40 mg of compound **1**.

The ethanol fraction (2 g) was fractionated by column chromatography over the silica gel with CH_2Cl_2/MeOH/H_2O = 7:1:0.5 to 12.5:6:2 to yield subfraction (Fr.1-Fr.30). Subfraction Fr. 22 (37.2 g) was further purified on a silica gel column with EtOAc/MeOH/H_2O = (93:4:3) to give compound **2**, which was recrystallized from MeOH to yield pure compound **2** (50 mg) as yellowish amorphous powder. Compound **1** and **2**, which were characterized by spectroscopic analysis and comparison of spectra with published literature as gallic acid[5] and rutin[6].

Toxicity of gallic acid and rutin: Acute administration of gallic acid even at a dose as high as 5 g/kg body weight did not produce any signs of toxicity or mortality[7]. As well as rutin is also considered to a safe[8].

Animals and transplantation of tumors: The EAC cells were grown in the abdominal cavity of Swiss albino mice. The cells were maintained by weekly intraperitoneal inoculation of the cells into recipient mice. Each mouse received 0.2 mL of ascites fluid containing approx. 10^7 cells diluted in sterile normal saline. The cells were harvested after 8-10 days and were initially diluted and washed with 20 mM HEPES, pH 7.3. Erythrocytes occasionally present were removed by washing in 35 mM NaCl.

Protein estimation: EAC Protein estimation was performed by the biuret method in the presence of 0.2 % deoxycholate. One mg of EAC protein corresponds to 1.25×10^8 cells.

Digitonin permeabilization of cell: EAC cells were collected and suspended in HEPES buffer (pH 7.0). Cells were permeabilized with various amounts of digitonin (100 µg/mg protein) and incubated on ice for 10 min. After incubation, the cells were centrifuged at 6000 g for 7 min. The enzymatic activities that were released from the cells were assayed in the supernatant. Pellets were resuspended in the assay buffer.

Respiratory measurements: Rates of oxygen consumption were measured in HEPES buffer at 25 ºC in a water-jacketed DW1 Hansatech oxygraph with the 1-mL glass chamber (Hansatech Instruments Ltd., UK) containing a Clark type polarographic oxygen electrode. The solubility of oxygen in an air-saturated temperature-equilibrated medium was taken to be 480 ng-atoms/mL at 25 ºC and 760 mm Hg[9].

RESULTS AND DISCUSSION

Ehrlich's ascites tumor cells could be selectively permeabilized by using digitonin at 100 µg/mg cell protein. Very little stimulation of oxygen consumption was achieved by the addition of NADH-dependent substrate glutamate to digitonin-permeabilized tumor cells in the presence of ADP (Table-1). Even at a very high concentration of rotenone, glutamate stimulated oxygen uptake was not completely inhibited (Table-1). Both Gallic acid and rutin only partially reduce oxygen uptake. Thus, results of Table-1 suggest the partial activities of complex **I**.

Addition of either antimycin A or cyanide completely blocked glutamate-stimulated oxygen uptake in digitonin permeabilized tumor cells (Table-1). These inhibitors suggest the presence of complexes III and IV in the pathway of electron transport from glutamate to oxygen. In addition, potential flavoprotein-linked substrates like dihydroorotate failed to stimulate phosphorylation in digitonin permeabilized tumor cells (Table-2). Other substrates like malate and α-ketoglutarate also fails to stimulate respiration in EAC cells. Gallic acid, rutin and rotenone fail to inhibit oxygen uptake completely (Table-3).

The inclusion of the respiratory substrate system *N',N'*-tetramethyl-*p*-phenylenediamine (TMPD)/ascorbate, which reduce respiratory complex IV, reinitiated respiration in digitonin permeabilized tumor cells. Addition of ADP to this preparation resulted in no stimulation of respiration. Inclusion of cyanide into this system resulted in complete inhibition. Thus we can conclude that site IV of the mitochondrial electron transport chain of tumor cells is non-phosphorylating. Partial inhibition of TMPD/ascorbate-stimulated respiration in digitonin permeabilized tumor cells by rotenone suggest an alternative site of the cytochrome c-dependent electron transport pathway in tumor cells. Antimycin A had no effect on TMPD/ascorbate stimulated respiration in digitonin-permeabilized tumor cells (Table-1).

TABLE-2
RELATIVE RATE OF SUBSTRATE OXIDATION BY MITOCHONDRIA IN DIGITONIN-PERMEABILIZED EAC CELLS

Cell pretreatment*	Relative rate of substrate oxidation**								
	Endog-enous substrate	Ascorbate (10 nM) +TMPD (0.4 nM)	Ascorbate (10 nM) +TMPD (0.4 nM) + ADP (0.1 mM)	Ascorbate (10 nM) +TMPD (0.4 nM) + ADP (0.1 mM) + GA	Ascorbate (10 nM) +TMPD (0.4 nM) + ADP (0.1 mM) + Rutin	Dihydro-orotate (10 mM)	Dihydro-orotate (10 mM)+ ADP (0.1 mM)	Dihydro-orotate (10 mM)+ ADP (0.1 mM) + GA	Dihydro-orotate (10 mM)+ ADP (0.1 mM) + GA + Rutin
EAC	100								
EAC + Digitonin	50	60	72	38	51	80	88	45	62
EAC + Digitonin + Rotenone (40mM)			20				nd		
EAC + Digitonin + Antimycin (2µg/mg protein)			40				nd		
EAC + Digitonin + KCN (2mM)			4				nd		

Note: n.d., not determined. * Relative rate 100 was considered for EAC endogenous oxygen uptake rate to be 15 nmol O_2/min/mg cell protein. Relative rate values represent the average of three experiments.; ** EAC cells were treated, where indicated with 100 µg digitonin/mg cell protein, for 3 min before the addition of oxadizable substrates and/or electron transport inhibitor.

TABLE-3
RELATIVE RATE OF SUBSTRATE OXIDATION BY MITOCHONDRIA IN DIGITONIN-PERMEABILIZED EAC CELLS

Cell pretreatment*	Relative rate of substrate oxidation**								
	Endogenous substrate	Malate (10 mM)	Malate (10 mM)+ ADP (0.1 mM)	Malate (10 mM)+ ADP (0.1 mM) + GA	Malate (10 mM)+ ADP (0.1 mM)+ Rutin	α- keto glutarate (10 mM)	α- keto glutarate (10 mM)+ ADP (0.1 mM)	α- keto glutarate (10 mM)+ ADP (0.1 mM) + GA	α- keto glutarate (10 mM) + ADP (0.1 mM) + Rutin
EAC	100								
EAC + digitonin	50	72	81	25	42	60	72	40	68
EAC + digitonin + rotenone (40 mM)			12				22		
EAC + digitonin + antimycin (2 µg/mg protein)			8				10		
EAC + digitonin + KCN (2 mM)			2				3		

*Relative rate 100 was considered for EAC endogenous oxygen uptake rate to be 15 nmol O_2/min/mg cell proteins. Relative rate values represent the average of three experiments; **EAC cells were treated with 100 µg digitonin/mg cell protein, for 3 min before the addition of oxadizable substrates and/or electron transport inhibitor

The failure of NADH-dependent substrates and TMPD/ascorbate to support the increase of sufficient oxygen consumption in the presence of ADP in digitonin permeabilized tumor cells suggest that the NADH dehydrogenaseubiquinone (Complex I) and cytochrome c oxidase segment (complex IV) is not functioning properly in EAC cells. Addition of succinate to digitonin-permeabilized cells resulted in profound stimulation of oxygen uptake. The subsequent addition of ADP induced respiration of the tumor cells. Addition of rotenone at 40 µM concentration partially inhibited respiration of succinate in digitonin-permeabilized tumor cells, which was then completely inhibited by antimycin A, cyanide, gallic acid (5mM) and rutin (5mM) (Table-1).

Tables 1-3, data revealed that gallic acid and rutin were devoid of complex IV activity as they fail to consume oxygen. Assay of complex I, II and III suggested the presence of complex II in digitonin permeabilized tumor cells.

Conclusion

From the results, it can be concluded that isolated compounds fails to inhibit ADP-stimulated ascorbate plus TMPD, L-glutamate, like malate and α-keto-glutarate-dependent respiration, but strongly inhibit succinate-dependent respiration. So, gallic acid and rutin possibly inhibit the electron flow through complex II of the EAC-cell mitochondrial respiratory chain.

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