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Research Paper

Scavenger capacity of *Momordica charantia* for reactive oxygen species

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ABSTRACT

In order to quantify total phenolics and flavonoids contents in *Momordica charantia* fruit extract and its total antioxidant activity, the spectrophotometric analyses were employed. Furthermore, to confirm the antioxidant activity of *M. charantia* under biological medium, the cellular test using DCFH, sensor of reactive oxygen species (ROS), was used. This study reports the total antioxidant capacity of *M. charantia* and highlights its scavenger capacity for ROS. The consumption of 100g of *M. charantia* fruit can provide antioxidants equivalent to 145±1.16mg of vitamin C.

Keywords: *Momordica charantia*; Antioxidant activity; ROS; DCFH-DA.

INTRODUCTION

Consequently traditional medicine becomes an interesting alternative to conventional treatment. Many studies have highlighted several pharmacological properties in medicinal plants or their isolated constituents including anti-oxidant, anti-diabetes, antibacterial, antiviral and anti-ulcer activities (Nergard, et al., 2005; Wong, et al., 2006; Meléndez and Capriles, 2006). Plants have formed the basis of traditional medicine systems that have been in existence for thousands of years. Even in modern times, plant-based systems continue to play an essential role in health care. It has been estimated by the WHO that approximately 80% of the world's population from developing countries rely mainly on traditional medicines for their primary health care. Plant products also play an important role in the health care for the remaining 20% in developing countries, and for those in industrialized countries as well (Chivian, 2002).

Medicinal plants are a source for a wide variety of natural products, such as the phenolic acids and flavonoids which are very interesting for their antioxidant properties (Wong, et al., 2006). In addition to their ability to act as an efficient free radical scavengers (Katalinic, et al., 2006), their natural origin represents an advantage to consumer in contrast to synthetic antioxidants which their use is being restricted due to their carcinogenicity (Velioglu, et al., 1998).

Momordica charantia (Family-*Cucurbitaceae*), is commonly known as bitter gourd or bitter melon in English and karela in Hindi. *M. charantia* is cultivated throughout the

world to be used as vegetable as well as medicine. The crude fruit extract has been used in Africa and Latin America as vegetable insulin. In Asia, *M. charantia* has been used as a hypoglycaemic agent (Welinda, et al., 1982). In a recent study, we have demonstrated the antihyperglycaemic effect of *M. charantia* fruit extract in alloxan induced diabetic mice (Rammal, et al., 2009).

In addition to its uses for diabetes (Reyes, et al., 2006; Rammal, et al., 2009), *M. charantia* shows others pharmacological properties like antibacterial, antiviral and anti-ulcer activities (Omoregbe, et al., 1996; Matsuda, et al., 1999; Beloin, et al., 2005).

The purpose of this study was to evaluate the antioxidant power of the aqueous extract of the fruit of *M. charantia*. Consequently, cellular assay of antioxidant activity was used to determine scavenger capacity of the fruit of *M. charantia* for ROS. Furthermore, spectrophotometric analyses were employed for the determination of total phenolics and flavonoids concentrations of the fruit. The total antioxidant activity value was also quantified by the vitamin C equivalent antioxidant capacity (VCEAC) test.

MATERIALS AND METHODS

Chemicals: Halothane was obtained from Laboratoires Belmont, France. Heparine was procured from Sanofi-Synthelabo, France. Aluminium chloride ($AlCl_3$), Catechin, Gallic acid and H_2O_2 were purchased from Across Organics. Lysing and Cellwash solutions were procured from Becton-Dickinson. Ascorbic acid, 2-2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), HBSS buffer, PBS buffer, 2-2'-Azobis (2-methylpropionamidine) dichloride (AAPH), Folin-Ciocalteu's phenol reagent, Sodium carbonate (Na_2CO_3), 2',7'-diacetate dichlorofluorescein (DCFH-DA) and Sodium nitrite ($NaNO_2$) were purchased from Sigma Chemical Company.

Plant material: Fresh fruits of *M. charantia* were obtained from a commercial source and the biological authentication was carried out by Professor Max Henry in the Botanic and Mycology Laboratory, of Nancy University (France). 100g of grinded fruits were macerated in 300ml of pure water for 12h at room temperature, and for 12 h at 37°C temperature. The filtrate was lyophilised, and 5g were obtained (Rammal, et al., 2009).

Animals: Swiss albino male mice that were nine weeks old at the time of delivery from the breeder (Charles River, France) and ranged in weight from 35-40g have been used. They were housed with a 12h light: 12h dark schedule (lights on at 8:00 p.m.) with free access to water and food (SDS Dietex - France) and maintained at a constant temperature (21±2°C) and a relative humidity of 55 ±10%. Experiments began after a 1-week period of acclimatization. All the procedures applied in the experiment, on these mice, were in accordance with the European Communities Council Directive of November 24th, 1986 (86/609/EEC).

Cellular assay of antioxidant activity of *M. charantia* fruit

Apparatus: Flow cytometry technique allows a separation of the different immune cells populations by size (forward light scatter, FSC) and relative granularity (side light scatter, SSC) parameters. FSC and SSC were used after excitation of the immune cells by the 488 nm laser beam of argon. The level of intracellular ROS was measured in the granulocytes by monitoring of the emitted fluorescence into these cells (FACS-Scan, Becton-Dickinson, Immunofluorometry Systems, France).

Procedure: The ROS level was quantified using the DCFH-DA. Non-polar DCFH-DA crosses the cellular membranes, and is hydrolysed by intracellular esterases to form the polar, non-fluorescent dichlorofluorescein (DCFH). Then, this is oxidized to a highly

fluorescent substance, the 2', 7'-dichlorofluorescein (DCF) by intracellular ROS (Shen, et al., 1996; Carini, et al., 2000; Bouayed, et al., 2007; Rammal, et al., 2008a, b; 2010).

The mice were anaesthetized with halothane and sacrificed. The collected blood was heparinized and dispatched in volumes of 100µl in Eppendorff tubes. Erythrolyse was done using 2ml of lysing solution in each tube; the whole was placed in darkness during 10 min. After centrifugation at 4°C (5 min; 2000 rpm), the supernatant was eliminated. Then 2 ml of cellwash solution was added to sediment containing white cells, mixed and followed by a new centrifugation in the same conditions. Supernatant was also eliminated (Bouayed, et al., 2007; Rammal, et al., 2008a, b; 2010).

Three groups of cells belonging to the same blood have been used to assess the intracellular ROS level, the first one was a control without oxidative stress, and the second one was a control with oxidative stress. The third group served to evaluate the antioxidant activity of *M. charantia* extract.

We added 1ml of the HBSS buffer and 5µl of DCFH-DA (50µM) to the white cells. For the control (without oxidative stress): incubation was achieved in darkness during 30 min at 37°C. For the second control (with oxidative stress): incubation was performed in darkness during 15 min at 37°C, after we added 5µl of H_2O_2 (89 mM) to provoke oxidative stress, then the incubation was continued for 15 min. In the third group of cells, several concentrations of MC extract were used for the evaluation of its antioxidant activity. So, to this group in addition to HBSS buffer and DCFH-DA, 5µl of MC extract was added. After 15 min of incubation in darkness, the oxidative stress was induced by the addition of 5µl of H_2O_2 (89mM). The incubation was continued for 15 min at 37°C. As soon as incubation times were completed, the ROS levels were measured by the flow cytometry technique (Bouayed, et al., 2007; Rammal, et al., 2008a, b; 2010).

***Determination of total phenolics*:** was done by using the spectrophotometric analysis (Cary 50 Scan UV-Visible apparatus) with Ciocalteu's phenol reagent (Kim, et al., 2003). Briefly, an aliquot (1ml) of appropriately diluted extract or standard solutions of gallic acid (20, 40, 60, 80 and 100mg/L) was added to a 25ml volumetric flask containing 9ml of ddH_2O. A reagent blank using ddH_2O was prepared. One mililiter of Folin and Ciocalteu's phenol reagent was added to the mixture and shaken. After 5min, 10ml of 7% Na_2CO_3 solution was added with mixing. The solution was then immediately diluted to volume (25ml) with ddH_2O and mixed thoroughly. After incubation for 90min at 23°C, the absorbance versus prepared blank was read at 750nm. Total phenolics content in *M. charantia* fruit was expressed as mg gallic acid equivalent (GAE)/100g fresh sample. Sample was analyzed in three replications.

***Determination of total flavonoids*:** Total flavonoids content was measured according to a colorimetric assay (Zhishen, et al., 1999). A 1 ml aliquot of appropriately diluted sample or standard solutions of catechin (20, 40, 60, 80 and 100mg/L) was added to a 10ml volumetric flask containing 4ml ddH_2O. At zero time, 0.3 ml 5% $NaNO_2$ was added to the flask. After 5 min, 0.3ml 10% $AlCl_3$ was added. At 6 min, 2ml of 1 M NaOH was added to the mixture. Immediately, the solution was diluted to volume (10 ml) with ddH_2O and mixed thoroughly. Absorbance of the mixture, pink in colour, was determined at 510 nm versus the prepared blank. Total flavonoids content in fruit extract was expressed on a fresh weight basis as mg/100g catechin equivalents (CE). Sample was analyzed in three replications.

***Vitamin C equivalent antioxidant capacity assay using ABTS radical*:** ABTS radicals were used to evaluate the antioxidant capacity of *M. charantia* fruit (Kim, et al., 2003). In brief, 1mM AAPH, a radical initiator, was mixed with 2.5mM ABTS in phosphate-

buffered saline (PBS, pH 7.4). The mixed solution was heated in a water bath at 68°C for 13 min. The resulting blue-green ABTS radical solution was adjusted to the absorbance of 0.650±0.020 at 734 nm with additional PBS. 20µl of sample was added to 980µl of the ABTS radical solution. The mixture was incubated in a 37°C water bath under restricted light for 10 min. A control consisted of 20µl 50% methanol and 980µl of ABTS radical solution. The decrease of absorbance at 734nm was measured 10 min later. Total antioxidant capacity of MC fruit, as determined by scavenging blue-green ABTS radicals, was expressed on a fresh weight basis as mg/100g vitamin C equivalent (VCEAC). Sample was analyzed in three replications.

RESULTS

***Cellular assay of antioxidant activity of M. charantia extract*:** The ROS level was detected using a fluorescence probe, DCFH-DA, which could be oxidized to highly fluorescent dichlorofluorescein (DCF) by intracellular ROS (Bouayed, et al., 2007; Rammal, et al., 2008a, b; 2010). Fig. 1 shows a fluorescence intensity (FI) decrease from (C) corresponding to control with oxidative stress to (B) representing the treated cells. This decrease is due to *M. charantia* extract effect on ROS level which is directly correlated with FI.

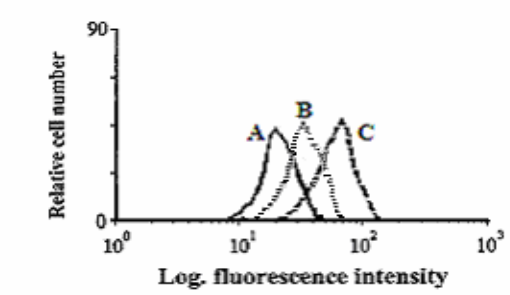

Figure -1: The ROS levels were measured by flow cytometry.

- The fluorescence intensity was expressed as decimal logarithm versus the cell number.
- (A, continuous line) and (C, discontinuous line): Controls corresponding to level of intracellular ROS without and with oxidative stress, respectively. (B, dash line): Treated granulocytes corresponding to ROS level in these cells incubated in presence of *M. charantia* extract at 50mg dry weight (dw)/ml, then subject to the oxidative stress.

Fig. 2 shows linear correlation of scavenger capacity with respect to the concentrations of *M. charantia* extract. At 100mg dw/ml *M. charantia* extract, corresponding to 2g fresh fruit, a reduction of 91.51±4.21% of ROS level generated by exogenous H_2O_2 is observed.

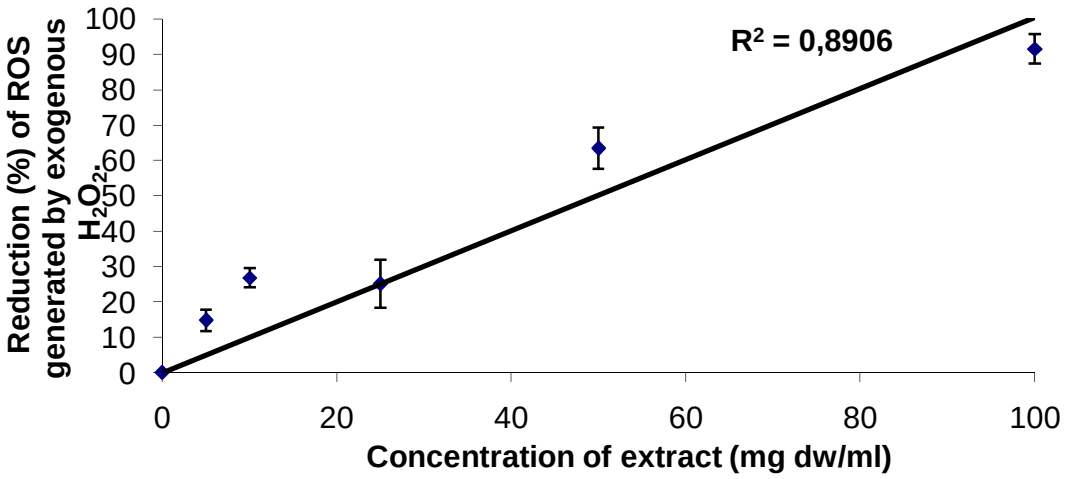

Figure- 2: Concentration–response curve for reduction of ROS generated by exogenous H_2O_2.

- The data are displayed with mean ± standard deviation of three replications.

***Determination of total phenolics, total flavonoids and total antioxidant capacity (VCEAC assay)*:** Table 1 shows that the concentrations of total phenolics and flavonoids in *M. charantia* were 126 ± 0.14 mg GAE and 19.6 ± 0.57 mg CE/100g fresh weight, respectively. It also shows that The VCEAC of fresh *M. charantia* was 145 ± 1.16mg VCE/100g fresh weights.

Table-1: Total phenolics and flavonoids contents in MC fruit and quantification of its total antioxidant activity by VCEAC assay.

	Total Phenolics	Total flavonoids	VCEAC
100g of fresh fruits	126 ± 0.14mg GAE	19.6 ± 0.57mg CE	145 ± 1.16mg VCE

- The data are displayed with mean ± standard deviation of three replications.

DISCUSSION

The short lifetime of the ROS and the variety of antioxidants existing in vivo made extremely difficult the measure of these species (Gomes, et al., 2005). Fluorescent probes are excellent sensors of ROS due to their high sensitivity and simplicity in data collection (Gomes, et al., 2005). DCFH-DA has been used to evaluate the intracellular redox status (Carini, et al., 2000). Oxidative stress is induced by addition of H_2O_2 in the extracellular medium of the granulocytes. Exogenous H_2O_2 diffuses inside the cells, consequently their DCFH oxidation increases compared to oxidation in the control granulocytes without oxidative stress.

Antioxidant activity is a fundamental important property for human life. Many of the biological functions, including antimutagenicity, anticarcinogenicity, and antiaging, among others, originate from this property (Sala, et al., 2002). Recently, Abu Bakar et al. (2009) reported that phenolic compounds have potentially beneficial effects on human health by reducing the occurrence of coronary heart disease, age-related eyes diseases, and artherogenic processes. These compounds also have antioxidant and anti-free-radical properties that allow them to quench free radicals in the body. Moreover, it was reported that antioxidants with ROS scavenging ability have great relevance in the prevention of oxidative stress which is responsible for the majority of diseases (Abu Bakar, et al., 2009). The antioxidant activities recorded using the ABTS test show that *M. charantia* fruit possesses useful antioxidant properties. Under oxidative stress conditions, *M. charantia* led to the reduction of the fluorescence intensity of treated cells. This reduction is directly correlated to the decrease of the ROS level. Our results obtained by cellular assay of antioxidant activity show that *M. charantia* possesses an excellent preventive potential against oxidative stress. These findings implicate that dietary polyphenolics from *M. charantia* may supply substantial antioxidants, which may provide health-promoting advantages to the consumer. Thus, *M. charantia* displays scavenging activity for ROS and is potentially a source of natural antioxidants. Which could be interesting to prevent diseases directly linked to oxidative stress.

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