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Garcinia morella fruit, a promising source of antioxidant and anti-inflammatory agents induces breast cancer cell death *via* triggering apoptotic pathway

Bhaswati Choudhury[a,*], Raghuram Kandimalla[a], R. Elancheran[a], Rupjyoti Bharali[b], Jibon Kotoky[a,*]

[a] Drug Discovery lab, Institute of Advanced Study in Science and Technology, Guwahati, Assam, India
[b] Department of Biotechnology, Gauhati University, Guwahati, Assam, India

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ABSTRACT

A rapid rise in cancer cases worldwide, especially breast cancer in females, instigates the need for more effective and less side effect causing drugs from natural origin. Thereby, in the present study, *Garcinia morella* fruit was investigated for antioxidant, anti-inflammatory and anti-breast cancer activity. Preliminary antioxidant and anticancer evaluation of different fractions and crude methanol extract of *G. morella* fruit suggested chloroform fraction as the bioactive fraction. Time course analysis (by 24 h, 48 h and 72 h) of the bioactive fraction (1.56–25) μg/ml treatment on breast cancer cell lines (MCF7, MDAMB231 and SKBR3) showed dose and time dependent antiproliferative responses. Further, mechanistic studies involving morphological observation and western blotting analysis, revealed its apoptosis inducing effect on breast cancer. P53 dependent up-regulation of Bax and down-regulation of Bcl X_L is suggested as the possible pathway of apoptosis followed by MCF7 cells on exposure to the bioactive fraction. The anti-inflammatory assays revealed that it significantly lowered the release of nitrite and TNF-α level of LPS induced RAW 264.7 cells ($p < 0.05$). Moreover, pre treatment of Carrageenan induced paw edema animals with 20 mg/kg of the bioactive fraction significantly ($p < 0.05$) inhibited paw inflammation and controlled the cytokine and nitrite levels of the edema induced rat. Its main bioactive component was identified to be Garcinol by UHPLC and ESI-MS/MS. Thereby, this study clearly reflects that *G. morella* fruit is a valuable antioxidant and anti-inflammatory gift of nature with the potential to be used against breast cancer. This is also the first report of isolation of bioactive compound Garcinol from *G. morella* fruit.

1. Introduction

Cancer is a complicated disease involving a multi-step process in which due to mutation, one cell of the body starts behaving abnormally and continuously multiplies to produce an unwanted clone of cells. It is a disease with highest mortality rates. According to a report in 2012, 14.1 million cancer cases and 8.2 million cancer associated deaths have been recorded. Breast cancer is one of the three commonly reported cancers in female and alone it was expected to be accountable for 29% of new cancer cases in women in the United States [1]. Although breast cancer was considered to be a disease of the developed world, according to Globacon 2008, 50% of breast cancer occurrence and 58% of death due to breast cancer occur in developing nations.

In 1863, it was first hypothesized by Rudolf Virchow that cancer arises from inflammatory sites and now there is evidence to support that chronic inflammatory diseases raise the risk of some types of cancer [2]. When there is a tissue injury or microbial infection, inflammation occurs at that site and this leads to the production of cytokines and eventually excess free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) [3]. Free radicals initiate oxidative stress in the body which ultimately leads to the occurrence of various diseases such as cardiovascular diseases, inflammatory diseases, atherosclerosis and cancer [4]. Interestingly, in the search for a modern and effective therapy for treatment of cancer, tumor microenvironment is being extensively studied by researchers worldwide and the role of inflammation and immune system has been cited. Natural killer cells and macrophage infiltrate was found in spontaneously developed mammary tumor [5], hence inflammatory

Abbreviations: GF, *Garcinia morella* fruit methanol extract; GFCH, *G. morella* fruit chloroform fraction; GFHEA, *G. morella* fruit hexane: ethyl acetate (9:1) fraction; GFEA, *G. morella* fruit ethylacetate fraction; GFMC, *G. morella* fruit methanol: chloroform (1:1) fraction

* Corresponding authors.
E-mail addresses: bhaswatichoudhury@ymail.com (B. Choudhury), jkotoky@gmail.com (J. Kotoky).

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infiltrate has been propounded as a prognostic marker for breast cancer in the studies conducted in 1990's. Moreover, previous reports hint the contribution of inflammation not only in cancer development but also in affecting the efficacy of chemotherapy [6]. Hence, anticancer agents displaying multi-target potential in inhibiting inflammation as well as blocking the proliferation of cancer cells is most sought at present [7].

There is a considerable development in cancer treatment worldwide, but it involves expensive treatment procedures and drugs. Also, side effects associated with chemotherapeutic drugs, bring misery in the life of the patients. Hence, new approaches finding efficient anticancer drugs with lesser side effects are in high demand. Plants have been considered as a rich source of antioxidant, anti-inflammatory and anticancer compounds. According to Newman and Cragg's drug classification, 78.6% of all approved anticancer drugs are either directly derived from natural products or based thereon, or synthetic molecules that resemble them in some form [8]. Over the last few decades, the scientific community is focussing on the validation of herbal remedies and development of new drugs from this natural therapeutic arsenals. This endeavor has led to the recognition of plants as a prolific source of structurally diverse potential ligands having high demand in the pharmaceutical industry [9].

Garcinia morella (Gaertn.) Desr. belonging to Clusiaceae family is mainly distributed in India, Srilanka and Southern Phillippines. Members of this family are a rich source of neoflavonoids, prenylated xanthones quinines and di pyranocoumarins. Garcinia comprises of 200 species in which almost 20 are available in India and they have been extensively used in Ayurvedic medicinal practices for treating various diseases [10]. Garcinia species are well endowed with secondary metabolites and hence exhibit various activities such as antibacterial [11], antifungal [12], anti-inflammatory [13], antioxidative [14] and anticancer [15]. *Garcinia morella* is a lesser explored species of this family. In the northeastern region of India, *Garcinia morella* desr. fruit locally known as kuji thekera is dried and consumed by preparing authentic curries or simply as coolers during summer. The dried pulps are also stored for years and used as home remedy for stomach ailments, inflammatory disorders and gastritis. *G. morella* fruit is widely used in Northeastern India for its nutritional and medicinal properties. Despite its rich traditional uses, not much scientific study has been undertaken on this lesser known plant of Garcinia family. Thereby, we embarked on a study of antioxidant, anti-inflammatory, antiproliferative and apoptotic activity of *G. morella* fruit against breast cancer. In our previous work, we have reported the anticancer activity of *Garcinia morella* desr. fruit by using Dalton's lymphoma induced mice model. To the best of our knowledge, cytotoxic activities of *G. morella* fruit against breast cancer have not been investigated previously, nor the comparative antioxidant properties of different fractions of *G. morella* fruit. Scientific validation of medicinal properties of natural products is critical to ensure its route to the clinic. Therefore, the present study was focussed on understanding the antiproliferative efficacy of *G. morella* on breast cancer (MCF7, MDAMB-231 and SKBR3) cells. *G. morella* fruit crude extract was fractionated and comparative anticancer activity of the fractions was determined. Furthermore, the bioactive fraction was identified and studied in details to elucidate its molecular mechanism of action on breast cancer. Additionally, the *in vitro* and *in vivo* anti-inflammatory property of this bioactive fraction was investigated to establish its multi-target capacity. This fraction was further purified to isolate and identify the bioactive molecule.

2. Experimental

2.1. Source of fruits

Garcinia morella fruits were collected from Sorbhog, Patchala district of Assam, India. The taxonomical classification of the collected sample was done by taxonomist at Northeast Indian Ayurvedic Research Institute (Government of India), Guwahati, Assam, India. Herbarium (IASST/BCCS/ HNO112/ 2012) was preserved in Drug Discovery laboratory IASST, Guwahati, Assam, India.

2.2. Extraction of G. morella fruit, isolation and identification of active compound

Shade dried samples of *G. morella* fruits were cut into small pieces and grounded to powder. 1 Kg of dried powder was extracted with 1.5 L of methanol by maceration for 3 days by continuous shaking at 37 °C. This step was repeated thrice and all the extracts were pooled together and concentrated under reduced pressure and vacuum dried at 45 °C in a rotor evaporator (Buchi R3). The final yield of dried methanol extract (GF) was 380 g. An aliquot (300 g) of GF was subjected to ordinary phase silica gel column chromatography and separated into Hexane: Ethyl acetate (1:1) (GFHEA), Ethyl acetate (GFEA), Chloroform (GFCH) and Methanol: Chloroform (9:1) fractions (GFMC). All fractions were dried and stored in airtight containers for all biological assays. The chloroform fraction (GFCH) was again chromatographed on silica gel using a gradient of solvent hexane and ethyl acetate. The subfractions obtained were collected separately and air dried. Subfraction-23 was crystallized and was characterized by spectroscopic analysis (UHPLC and ESI-MS/MS). Previous literature was used as a reference to study all data.

2.3. UHPLC-ESI orbitrap MS/MS analysis

UHPLC-ESI Orbitrap MS/MS analysis was performed using UHPLC system coupled with an ESI orbitrap MS/MS following the previously used methodology [16] with slight modification. Lyophilized sample was dissolved in HPLC grade methanol at a concentration of 1 mg/ml. The optimization of the instrument was set as follows: LC condition: UV at 254 nm, the constant flow rate at 0.5 ml/min, gradient chromatographic separation was performed on the sample using a mobile phase of solvent A (water with 0.01% formic acid) and solvent B (100% acetonitrile). Sample injection volume of 2 µl was injected into a Hypersil Gold C18 column (150 × 3.00 mm, Thermo, USA) with 95% solvent A added for initial 2 min, followed by addition of linear gradients of solvent B (5% to 95%) added from the 2nd minute of injection to 8 min, with a hold at 95% solvent B for 1 min and then redirecting back to initial conditions (5% solvent B) for 1 min. The runtime was calibrated for 10 min and re-equilibration of the column was achieved later in solvent B. A PDA detector was used to record the chromatogram while identification of the compounds was achieved through full mass spectral mass Bank Database.

2.4. Comparative antioxidant study of different fractions and crude extract of G. morella

2.4.1. DPPH free radical scavenging assay

Comparative antioxidant capacity of the fractions and crude extract of *G. morella* fruit were determined by DPPH free radical scavenging assay. GF, GFHEA, GFEA, GFCH and GFMC were taken at concentrations of 10, 25, 50, 75 and 100 µg/ml and their ability to inhibit DPPH free radical was determined by following previously used method [17]. Briefly, 0.3 ml of the samples was mixed with 2.7 ml of 0.2 mM DPPH in methanol solution. The reaction mixture was then mixed thoroughly and kept at room temperature for 1 h. Following which the OD of the samples were taken at 570 nm. The free radical scavenging activity was measured by following formula –

$$\text{Scavenging rate} = \{(As - Ai)/As\} \times (100)$$

Where A_S is the absorbance of pure DPPH and A_i absorbance of DPPH in presence of the various samples.