A novel anti-triple negative breast cancer compound isolated from medicinal herb *Myrothamnus flabellifolius*

Jaspal Brar\(^1\), Norman Fultang\(^1\), Kayla Askey\(^2\), Maria Cristina Tettamanzi\(^3\) and Bela Peethambaran\(^1\)*

\(^1\)Department of Biological Sciences, University of the Sciences in Philadelphia, PA 19104, USA.  
\(^2\)Department of Pharmacy, University of the Sciences in Philadelphia, PA 19104, USA.  
\(^3\)Department of Chemistry, Temple University, Philadelphia, PA 19122, USA.

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Triple negative breast cancer (TNBC) is a disease that impacts millions of people around the world. There is an urgent need to find new therapies, specific to triple negative cancer cells with minimal effect on normal cells. This study explores the use of *Myrothamnus flabellifolius* as an anti-TNBC. Using High Performance Liquid Chromatography, the extract was fractionated and each fraction was tested against TNBC cells BT-549 and MDA-MB-231, and a non-malignant breast cell line MCF-10A. The results showed that fraction 7 inhibited the growth of TNBC cells starting at a dry weight concentration of 31.125 μg/mL while there was no significant cell death of the MCF-10A cell line. Using analytical techniques such as Mass Spectrometry (MS-MS) and Nuclear Magnetic Resonance (NMR), the major compound in fraction 7 was determined to be a derivative of galloyl glucose hexahydroxydiphenic acid. The results suggest that this novel compound is the primary anti-TNBC compound in *M. flabellifolius* and might be a potential candidate for targeted TNBC therapy.

**Key words:** Triple negative breast cancer, *Myrothamnus flabellifolius*, natural products, galloyl glucose hexahydroxydiphenic acid derivative.

**INTRODUCTION**

Triple negative breast cancer (TNBC) is the most lethal type of breast cancer and is the most difficult to treat. One in eight women is diagnosed with breast cancer each year, and of these, 15 to 20% of cases are considered to be triple negative (Donepudi et al., 2014). The name triple negative refers to the absence of three receptors which include estrogen, progesterone, and human epidermal growth factor receptor 2 (Anders and Carey, 2008). Therefore, many chemotherapeutic drugs such as tamoxifen, which are used to treat certain types of breast cancer, are not effective against triple negative cells. This is because tamoxifen selectively binds to estrogen receptors, which in turn prevents estrogen from attaching to its receptor (Manna and Holz, 2016). Estrogen is required for some breast cancer cells to grow; hence, blocking the hormone will prevent the tumor...
from growing. This is not the case in triple negative breast cancer cells since it lacks the estrogen receptor altogether, and is one of the main reasons why TNBC is extremely difficult to treat. Current treatment for triple negative breast cancer includes chemotherapy, which has been shown to damage both cancer cells and non-cancerous cells alike (Wahba and El-Hadaad, 2015). In addition, the prognosis for TNBC is poor, especially when compared with other types of cancers. A study found that women who received treatment for non-TNBC have a 1.20 times higher odds of surviving than those who received treatment for TNBC (Pal et al., 2014). There is a clear need to find a treatment for TNBC that provides better survival rate and less cytotoxicity to normal cells.

This study focused on herbs as sources of anti-cancer medicines. In this study, a South African herb *Myrothamnus flabellifolius* was evaluated for possible anti-cancer properties using TNBC cells. Previous studies have shown that *M. flabellifolius* has phytochemicals that are antimicrobial (Moore et al., 2005a, b). It is mainly being used in teas and as decoctions in African culture, as well as an inhaler to treat chest pains and cough; it is used as tea for kidney disorders and masticated leaves to treat scurvy, halitosis and Vincent's gingivitis (Moore et al., 2007). Its main component is a 3,4,5-tri-O-galloylquinic acid, which in addition to protecting the membranes of the plant from desiccation, has been shown to protect against damage from reactive oxygen species (Moore et al., 2005). Major essential oils reported to be present include carvone and perillic acid (Da Cunha et al., 1974), 1,8-cineole and diosphenol (Gibbs, 1974), trans pinocarvone, pinocarvone, α-pinene and β-selinene (Chagonda et al., 1999) and pinocarvone and trans-pinocarvone (Viljoen et al., 2002). *M. flabellifolius* has also been shown to have anti-cancer, anti-viral and anti-fungal properties (Gescher et al., 2011). An extract of *M. flabellifolius* was shown to be significantly cytotoxic in HL-60 human leukemia cells at an IC₅₀ of 62.5 μg/ml, with minimal effect on TK6 normal human lymphocytes (Dhillon et al., 2014). Given the myriad of medicinal properties that *M. flabellifolius* has been shown to have the plant would have the ability to kill TNBC cells without having significant damage on normal breast cells.

**MATERIALS AND METHODS**

**Extraction and fractionation**

The stems and leaves of the *M. flabellifolius* obtained from our collaborator Dr. Ronald Balsamo (Dillon et al., 2014) were ground to a fine powder using a mortar and a pestle. For every 0.1 g of the plant, 1 mL of methanol was added to dissolve the powder. Samples were then centrifuged and the supernatant was collected. A rotary evaporator and SpeedVac technology were used to dry the supernatant to a final volume of 3 mL. Then, in order to fractionate the extract, high performance liquid chromatography (HPLC) was used following a previous method adopted to isolate another compound from the same extract (Badiab et al., 2016). Briefly, the Agilent 1100 series HPLC was used and reverse phase chromatography was performed using a C18 column with a polar mobile phase (which was a mixture of 0.1% folic acid in water and 1% folic acid in acetonitrile). Overall, 100 μL of the extract sample was inserted and 30 fractions of 300 μL were labeled and collected. 300 runs of HPLC were performed and each fraction was collected at 1 min interval. UV fluorescence was used to determine absorbance of the samples at 280 nm.

**Cell culture**

Two TNBC cell lines BT-549 (ATCC® HTB-122™) and MDA-MB-231 (ATCC® HTB-26™) and one normal breast epithelial line MCF-10A (ATCC® CRL-10317™) were used. All cells were kept in an incubator with 5% CO₂ at a temperature of 37°C. BT-549 cells were given the RPMI-1640 Media (ATCC® 30-2001™) along with 10% Fetal Bovine Serum (ATCC® 30-2020™) and insulin, MDA-MB-231 cells were given EMEM media (ATCC® 30-2003™), 10% Fetal Bovine Serum (ATCC® 30-2020™), 1 mM sodium pyruvate, insulin, and glutamine. MCF-10A cells are given MEBM media (ATCC® PCS-600-030™) with horse serum (5% of final volume), epidermal growth factor (EGF), hydrocortisone, insulin, and isopropanol. Both cancerous and non-cancerous cells were treated with serially diluted amounts of the fraction for 72 h.

**Cell viability assay**

XTT Assay (ATCC® 30-1011K™) was performed on both cancerous and non-cancerous cells. Cells were first treated with .25% trypsin in order to cleave and suspend the cells. The cells were then counted using a hemocytometer and it was made sure that between 15,000 and 20,000 cells were present. Viable cells, which remained in their respective media, were added to the first three rows of a 96 well plate at a volume of 100 μL per well. The plate was incubated overnight for 24 h. Then 6 serially diluted amounts of fraction concentration were added to the cells (in columns 1 to 6): 1000, 500, 250, 125, 61.25, and 31.125 μg/mL. Three wells per row (columns 7-9) contained 100 μL of a 0.3% DMSO solution. The last 3 wells per row (columns 10-12) just contained the 100 μL of media and cells. The well plate was put in the incubator for 72 h. Next, 100 μL of a solution containing 0.1 mL reactivating agent (N-methyl dibenzoprazine methyl sulfate): 5 μL XTT reagents was added to each well and left to incubate for 1 h. Each reading was made using the mass spectrophotometer and SoftMax Pro technology. Three readings were performed at 1 h interval at two wavelengths: 475 and 660 nm. Cell viability was measured based on absorbance, since the XTT dye is colorless but turns orange upon reduction. Viable cells undergo the electron transport chain in which NADH and coenzyme-Q transport electrons that become reduced when the reactivating agent is present. The reduced form of the reactivating agent then passes off its electrons to the XTT reagent, which will turn a deeper orange proportional to the amount of electrons being reduced. Results are run in triplicate and averaged.

**Cell viability calculation**

Cell viability was calculated according to the manufacturer's instruction (ATCC®). Briefly, specific absorbance for each experimental and control well was obtained by subtracting non-specific absorbance (660 nm) from the 475 nm absorbance values. Cell viability was computed as percentage of experimental specific absorbance values to specific absorbance of control untreated
Figure 1. The graph shows the cell viability after treatment with Fraction 7 for 72 h. The IC$_{50}$ calculated for TNBC cells MDA-MB-231 was 66.81 µg/ml, BT-549 ast 172.7 µg/ml and the non-malignant cells MCF-10A does not converge at the above concentrations. This shows that the MF extract is non-toxic to MCF-10A.

**Statistical significance**

Results are presented as an average ± standard deviation (SD) of 3 trials. Two-sided t-tests were performed and p-values that are less than 0.05 were deemed statistically significant.

**RESULTS**

**Fraction 7 collected at 7th minute using HPLC was the most potent to TNBC cells**

Out of the 30 fractions that were collected using HPLC, none of the fractions from fraction 1 to 6 showed anti-TNBC effect. Fraction 7 treatment was lethal to showed killing of the TNBC cells (MDA-MB-231, BT-549) with least harm to non-malignant cell line MCF-10A (Figure 1). XTT assays were used to determine cell viability for both cancerous and non-cancerous lines. Cells were treated with fractions of *M. flabellifolius* for 72 h and experiments were run in triplicate. The impact that fraction 7 had on all three cell lines, two cancerous lines and one non-cancerous line is shown subsequently.

Even in small concentrations of Fraction 7 (31.25 µg/mL), the cell viability of BT-549 and MDA-MB-231 was both less than 65 and 50%, respectively. MCF-10A cells were 100% viable at this concentration and showed no signs of damage (Figure 1). Therefore, it can be concluded that Fraction 7 has a potential compound that selectively targets cancer cells and does not harm non-malignant breast cells. To find the major compound in Fraction 7, the fraction was further sub-fractionated using High Performance Liquid Chromatography (Figure 2). The major compound shown at the seventh minute was collected and the identity of the compound was analyzed using NMR and mass spectrometry.

**Identification of the major compound in Fraction 7 using MS-MS and NMR**

Fraction 7 as shown in Figure 2 had only one major compound and this compound was collected using HPLC. The solvent from the collected compound was dried and prepared for identification using NMR and MS-MS (Figure 3).

The ESI QTOF-MS analysis of compound 1 showed a [M-H]$^-$ ion at m/z 1267.11, which corresponded to the structure derived using NMR having a molecular formula...
Figure 2. HPLC of methanolic extract showing the peak that corresponds to Fraction 7. The peak for fraction 7 is boxed which had selective potency against TNBC cells as shown in Figure 1.

Figure 3. The mass spectrometry data showed a mass signal of [M-H] m/z 1267.11, which was an indication of the presence of a dimeric hydrolysable tannin, consisting of 2 hexoses, 2 HHPP (Hexahydroxydiphenic acid) and 2 galloyl residues Fraction 7 show as well the fragment m/z = 633.0546.

C_{54}H_{43}O_{36} and a calculated weight 1267.90 (Figure 4). The signal 1267.11 indicated dimeric hydrolysable tannin having 2 hexose, 2 hexahydroxydiphenic acid and 3 galloyl residues (Figure 4a). The fragments m/z 633 and 169 (Figure 4a) can also be seen in the mass spectrum and correspond to the fragments as described in Figure 4. The m/z in ESI-negative mode of 483 was assumed to be due to a hexose esterified with two gallic acid units (Figure 4).

The total number of isolated galloyl groups in these types of compounds is evidenced by singlet signals integrating for two hydrogen atoms at 6.9 to 7.1 ppm in their 1H-NMR spectra (Table 1). These characteristic galloyl signals were found at 7.01 and 7.06 ppm...
Figure 4. (a) Derived anti-TNBC compound, $[M-1] = 1267.11$, is an isomer of tri gallic acid that is attached to a hexose sugar. HHDP: Hexahydroxydiphenic acid; (b) Fragment $m/z=633$. Chemical name deduced is (3-O-galloyl-4,6-[(S)-hexahydroxydiphenoyl]-β-d-glucopyranose). Molecular formula: $C_{54}H_{43}O_{36}$ (calculated MW: 1267.90).

(Engelhardt et al., 2016; Serna et al., 2015). HSQC spectrums were analyzed to see the protons present in the sugar moiety of the compound (Figure 5). The anomeric centers presented chemical shift at 5.43.
and 5.13 ppm, indicating β-configuration. Each HHDP moiety is depicted as a pair of sp<sup>2</sup> singlet signals between 6 and 6.8 ppm, in the isolated compound corresponding to signals at 6.44 and 6.59 ppm. The HHDP residue was proved to be in the S-configuration. HMBC was used for determination of the respective substituted position. The anomeric proton from the glucose (5.43 ppm) shows an HMBC correlation with one of the HHDP carbonyl signals (162.7 ppm), as well as a correlation with its C-2 of the glucose (71.4 ppm). The H<sub>2</sub> and H-6 of the galloyl moieties showed an expected HMBC correlation with its C-3 and C-4 (Table 1).

**DISCUSSION**

Phytochemical analysis of the compounds present in *M. flabelilfolius* Welw. showed that the novel anti-TNBC compound is similar to myrodigamin-I and -II (Engelhardt et al., 2016). There are differences between anti-TNBC...
compound and myrodigamin-I and -II especially in their molecular weight. Myrodigamin-II has not been implicated in any biological activity and so the compound is novel as it demonstrates anti-cancer activity to TNBC cells. Fraction 7 had both monomeric and dimeric forms of the HHDP derivative. To ensure that the anti-TNBC compound is not an artefact, this compound was isolated using HPLC after treating the leaves with liquid nitrogen and subsequent lyophilization. HPLC showed that the compound is present in both dry tissue and the tissue that was treated with liquid nitrogen indicating that this compound is not artefact created due to the processing of the sample. Hence, it would be valuable to know the ratio of the monomeric to dimeric in these fractions for an effective anti-TNBC therapy.

To understand how the isolated HHDP derivative has anti-breast cancer activity, this work looks into all the possible derivatives that could be metabolized from this compound. Similar metabolites that are known to be present in *M. flabellifolius* extract are also looked into. One of the compounds that is known to be in *M. flabellifolius* extract is galloylquinic acids; it has anti-viral properties, as it could inhibit HIV-1 reverse transcriptase (Bokesch et al., 1996) and DNA polymerase (Koonjul, 1999). Inhibition of DNA polymerase may be one of the potential anti-cancer mechanisms of *M. flabellifolius* extracts. In addition, these acids have been shown to have wound-healing capabilities and they prevent oxidative stress by scavenging free radicals.

Hydrolysis of the monomeric form shown in Figure 4b can produce compounds that are derivative of galloyl glucose which is already implicated in prostate cancer as anti-cancer compound (Hu et al., 2008), lung cancer (Huh et al., 2005) and breast cancer (Hua et al., 2006). It is hypothesized that the isolated HHDP may be undergoing metabolism to produce different forms of galloyl glucose; example, ellagic acid or pentagalloyl glucose to induce anti-cancer effects. Ellagic acid, one possible metabolite that can be hydrolyzed from HHDP, contains two galloyl residues having oxidative linkages but lacking any sugars. Ellagic acid has shown promising effects on both TNBC cell line MDA-MB-231 and receptor positive MCF-7 cell line (Kim et al., 2009). A metabolite produced from ellagic acid by the gut bacteria is Urolithin A (Uro-A) which has shown potential to sensitize colon cancer cells to 5-Fluorouracil and 5’Dihydroflorouracil (González-Sarrias et al., 2015). The specific mechanism of action of the novel anti-TNBC compound to induce selective cell death is unique and needs further study.

Conclusions

Overall, the results of this study demonstrate that *M. flabellifolius* could be potentially used as a source of non-toxic, targeted anti-TNBC agents to complement existing TNBC treatment regimes. The selective cytotoxicity of anti-TNBC compound on breast cancer cells with least damage to non-malignant breast cells also provides a promising solution to the issue of toxicity of chemotherapeutic agents.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests

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