Extraction of phytochemicals betulin and betulinic acid from the chaga mushroom and their effect on MCF-7 Cells

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Abstract

Chaga mushroom (Inonotus obliquus) grows on birch trees found mainly in temperate regions like Canada, Northern United States and Siberia. It contains phytochemicals such as betulin and betulinic acid, which are a pentacyclic triterpenoid along with several bioactive compounds such as β-glucans and phenolic antioxidants. The aim of this study was to enhance betulin (BN) and betulinic acid (BA) extraction from the fungi. It was determined that pre-treatment by water soaking for four days was required before solvent extraction is carried out. Following this extraction using solvents, ultrasonic treatment was done. Ultrasonication dramatically increased betulin and betulinic acid yield. There was considerable variance in the quantity of these products in chaga obtained from different commercial sources. Quantitative analysis for betulinic acid using high performance liquid chromatography (HPLC) method had to be developed. It was demonstrated that pure betulinic acid had significant effect on cell viability and reduction of oxidative stress. However, the combined effect of all the components of the bioactive extract (total phenolics found to be 201mg Gallic acid / 100g of dry sample) was found to be much higher.

Keywords: Chaga, betulin, betulinic acid, cytotoxic, MCF-7 cells

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"If you are not thankful to people, you are not thankful to Allah"

(Prophet Muhammad)

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Abbreviations

ABCA1	ATP-binding cassette transporter
AIF	Apoptosis-inducing factor
Ace	Acetone
AU	Arbitrary units
Bcl-2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma- extra large
BA	Betulinic acid
BN	Betulin
C-20	Carbon 20
C-3	Carbon 3
CA-SP1	Caspase-1
CAT	Catalase
CDK	Cyclin-dependent kinase
ChlC3	Chloroform
DMSO	Dimethyl sulfoxide
ERKS	Extracellular signal–regulated kinases
EtOAc	Ethyl acetate
EtOH	Ethanol
GAE	Gallic acid equivalent
G_0	G_0 phase or resting phase
H_2O_2	Hydrogen peroxide
HIF-1a	Hypoxia inducible factor 1 alpha
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
IC50	The half maximal inhibitory concentration
IKK	I kappa B kinase
IL	Interleukin
JNK	c-Jun N-terminal kinases
MAPKs	Mitogen-activated protein kinase
MeOH	Methanol
MMP	Matrix metalloproteinase
n-BuOH	n-butanol
NA	Not applicable information
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
Nf-*kB*	Nuclear factor- *kappa B*
Nf- *kB*-p65	Nuclear factor NF-kappa-B p65 subunit
NO	Nitric Oxide
p38	P38 Mitogen-activated protein kinases
PARP	Poly ADP ribose polymerase
ROS	Reactive Oxygen Species
RT	Room temperature

Abbreviations (continued)

Smac	Second mitochondria–derived activator of caspases
SIV	Simian immunodeficiency virus
STAT3	Signal transducer and activator of transcription
Sp	Specificity protein1
TLR4	Toll-like receptor 4
TNF-α	Tumour necrosis factor-alpha
VEGF	Vascular endothelial growth factor

Chapter 1

1.1 Introduction

Plants produce two types of substances, primary and secondary metabolites. The primary metabolites are useful for the growth and development of plants and affects their functions. The secondary metabolites are important for the long-term growth of the plant and are often not essential for their growth and normal function. Many of the secondary metabolites protect the plant against foreign substances and organisms. Additionally, they are sometimes utilized for the control and signaling of key metabolic pathways. There is increased focus by researchers on the extraction of such natural products called phytochemicals, which have many beneficial characteristics. These metabolites include polyphenols, alkaloids, and triterpenoids like betulin (BN) and betulinic acid (BA) (Dai et al., 2014; Kessler et al., 2007; Bhatia et al., 2015)

According to Canadian breast cancer society, it is estimated that cancer is the second leading cause of death worldwide. They reported 26,300 women are diagnosed with breast cancer. There has been considerable research and development for many decades to targeted and inhibit the growth of the cancer cells, but there is still a lot of work that needs to be done. Cancer is characterized by unscheduled cell cycle control, cell proliferation, angiogenesis and mutation of tumor suppression gene (Elmore ,2007). Numerous studies have indicated the involvement of oxidative stress and inflammation in tumorigenesis.

Conventional therapies such as chemotherapy, radiation, and hormonal therapy are associated with adverse effects. In addition to targeting cancer cells, conventional treatments may

also damage neighboring normal cells (Bhatia et al., 2015). The ultimate goal in cancer therapy is to develop candidate drugs have the desired effect on cancerous cells with no side effects on normal cells.

Chaga is a type of fungus that mostly grows on birch trees in cold regions like Alaska, Northern Canada, and Siberia. Chaga (Inonotus obliquus) has also been reported to be a potential source of polysaccharides, triterpenoid such as BN and BA for several centuries. They have been found to possess a broad range of medicinal properties (Shashkina et al., 2006). The birch tree (Betula spp., Betulaceae) itself has been reported to be a good source of BA (Moghaddam et al. 2012). The outer layer of birch bark has been reported to have as high as 25 % of BN (Šiman et al, 2016). This BN is considered as a precursor for BA. Moreover, both these compounds exist in chaga along with other bioactive compounds.

The process of isolating BA from such natural sources is very challenging. This is because BA is present along with several triterpenoids, which are structurally related. These include oleanolic acid, erythrodiol, and lupeol (Laszczyk, 2009). Although several studies have reported the presences of BA in chaga; however, this has not been systematically studied and quantified.

1.2 Rationale and objectives of this study

The hypothesis of this study is that the chaga mushroom could potentially have a larger concentration of many useful compound that can be extracted from its host (Hyun et al. 2006; Yin et al., 2008). Based on this presumption it was decided to optimize extraction of betulin and betulinic acid from this mushroom. The process of pretreatment of chaga, use of appropriate solvents and ultrasonication needs to be optimized.

The specific objectives of this study were to:

(1) Investigate the need for pretreatment before solvent extraction of BN and BA from different
   sources of chaga

(2) Enhance the extraction of BA and BN present in the chaga mushroom using solvents and
   ultrasonication

(3) Investigate the effect of the bioactive extract on MCF-7 cancer cell line.

Chapter 2
Literature review

2.1 Importance of Chaga

Chaga is a type of fungus that mostly grows on birch trees in cold regions like Alaska, Northern Canada, and Siberia and has been found to possess a wide range of medicinal value. Though, Chaga has been widely identified as 'mushroom', botanists continue to study its classification to find its suitable category. The presence of BA in chaga and its effects were documented several centuries ago and its healing abilities were recognized by indigenous Siberians who used to have it along with soups and stews (Faass, 2012). For example, Siberians noted that despite of the extreme weather conditions, consumption of chaga prevented the development of degenerative disease. In most cases, chaga was used to attain long life and boost physical stamina. In contemporary Russia, researchers have reported that districts where chaga is regularly used never had any cases of cancer. In Asia, chaga was also considered an important source of medicinal value by ancient people in Korea, China, and Japan. In Korea, chaga was used to regulate energy and relieve stress. In Europe, chaga was used to cure inflammatory skin conditions like eczema and psoriasis. In modern times, the beneficial effects of chaga have been attributed to the presence of BA, which also makes them an important research area in efforts to find alternative sources of cancer therapy (Faass, 2012). However, quantitative extraction and analysis of BA in chaga has not been done systematically.

Considerable research has been carried out to extract anti-cancer drugs from natural products, as exemplified by polyphenols, alkaloids, and triterpenoid like BN and BA (Figure 2.1) (Dai et al., 2014; Kessler et al., 2007; Nikitina et al.,2016). BN and BA are commonly found in the bark of the white birch tree (*Betula alba*). Depending on the extraction method, the birch bark extract can contain up to 22% BN and 1 – 5 % BA (Müllauer, 2011; Baratto et al., 2013).

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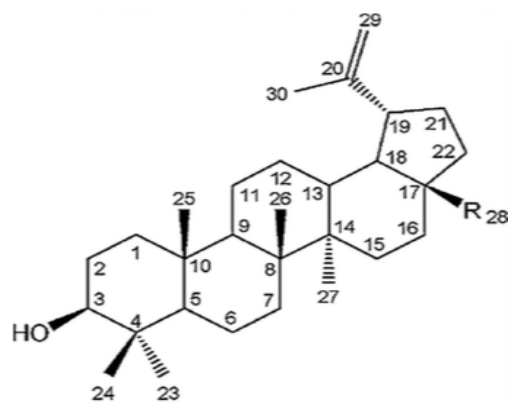
R= CH$_2$OH (BN)

R= COOH (BA)

Figure 2.1: The chemical structure of betulin and betulinic acid(Rizhikovs et al.,2015)

Besides the *Betula alba* (birch tree), BN and BA can also be extracted from several other sources and different parts such as tree stems, leaves, roots, barks, fruits, and in some cases from the whole plant (e.g., *Rhaphidophora hongkongensis*) (Mikhailenko et al., 2011). Table 1 shows some common sources of BA. In addition, BA can be extracted from Syzygium spp. like Myrtaceae, Ziziphus spp., *Ancistrocladus heyneanus*, and *Triphyophyllum peltatum* (Bhatia et al., 2015). Various extraction methods used to obtain BA from different plant species are discussed in subsequent sections. For example, Wu et al. (2011) separated BA from Ligustrum spp. including *Ligustrum lucidum* and *Ligustrum pricei* leaves. Also, Guinda et al. (2011) extracted BA from *Argania spinose* fruit pulp, while Viji et al. (2010) obtained BA from *Bacopa monniera* plant. The botanical sources of BA as shown in Table 2.1 can be grouped into seeds, leaves, stem, stem barks, and bark. For example, BA can be obtained from the leaves of *Dorstenia convexa*, *Malaysian Callistemon*, *Syzrgium claviflorum*, *Vitex negundo*, and *Combretum quadrangulare* plant species.

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Table 2.1: List of the botanical sources of betulinic acid reported in literature

Botanical Name of Plant	Part of Plant	Reference
Melaluca cajuputi	Bark	Mashitoh et al., (2012)
Malaysian Callistemon specious D. E	Leaves	Ahmad et al., (1999)
Combretum quadrangulare	Leaves	Banskota et al., (2000)
Eucalyptus camaldulensis var. obtuse	Leaves	Siddiqui et al. (2000)
Nerium oleanderand	Leaves	Fu et al., (2005)
Dorstenia convexa	Leaves	Poumale et al., (2011)
Vitex negundo L	Leaves	Chandramu et al., (2003)
Syzrgium claviflorum	Leaves	Fujiokaet al., (1994)
Ilex pubescens var. glabra.	Stem	Moghaddam, (2012)
Tetracentron sinense	Stem bark	Moghaddam, (2012)
Physocarpus intermedius	Stem bark	Moghaddam, (2012)
Engelhardtia serrata	Stem bark	Moghaddam, (2012)
Amoora cucullata	Stem bark	Moghaddam, (2012)
Peltophorum africanum	Stem bark	Moghaddam, (2012)
Jacaranda mimosaefolia	Stem bark	Moghaddam, (2012)
Berlina grandiflora	Stem bark	Moghaddam, (2012)
Zizyphus joazeiro	Stem bark	Moghaddam, (2012)
Bischofia javanica	Bark	Moghaddam, (2012)
Avicennia officinalis	Bark	Haque et al., (2006)
Betula platyphylla suk	Bark	Zhao et al., (2007)
Platanus orientalis	Bark	Bastos et al., (2007)
Betula alba	Bark	Müllauer, (2011)

In addition, the common sources of BA from the stem bark of trees can be obtained from

Zizyphus joazeiro, *Tetracentron sinense*, *Berlina grandiflora*, *Physocarpus intermedius*,

Engelhardtia serrata, and *Amoora cucullate*. More detailed information on the different sources

of BA, extraction methods, and separation techniques are discussed in subsequent sections.

2.2 Betulinic acid properties

BA has a hydroxyl group at carbon 3 and an alkene group at carbon-20 (Periasamy et al.,

2014). Wang et al., (2013) reported that BA has three groups at the C-3, C-20, and C-28 where

chemical modifications can be performed to yield derivatives for a structure–activity relationship

studies. When C-3 and C-28 (Figure 2.1) of BA are modified, the anticancer activity of BA is

enhanced. The modified derivative is found to be time and dose dependent (Periasamy et al., 2014).

For instance, chemical modifications at C-20 of BA by converting double bonds from an alkene

to a ketone group give the compound additional cytotoxic effect (Periasamy et al., 2014; Wang et

al., 2013).

BA is also known for its anti-oxidant, anti-inflammatory, anti-cancer and anti-bacterial

properties (Moghaddam et al., 2012). It inhibits tumor growth by stimulating apoptosis and can

trigger activity in the mitochondrial membrane of cancer cells to promote apoptosis. Several

literature (Foo et al., 2015; Viji et al., 2011) reports indicate that BA plays a significant role in

signaling pathways by stimulating or inhibiting the expression of anti-apoptotic, pro-apoptotic

proteins and reactive oxygen species.

2.3 Extraction of betulinic acid from birch bark

Different methods of extracting and isolating BN have been described in several studies.

Most of the studies focus on the isolation of BA from birch bark. One of the easiest techniques

used to extract biologically active BA and other materials is through the deployment of organic

solvents. Some examples of the common solvents used include ethyl acetate, n-hexane, n-heptane,

propanol, ethanol, methanol, and its mixtures with water and ethanol (Boryczka et al., 2013; Jager et al., 2008; Orsini et al., 2015). Ressmann et al. (2012) also used ionic liquids based on imidazole as an extraction solvent.

In order to speed up the extraction process, the bark of the trees is crushed or milled. Additional techniques also include activation and ultrasonic disruption of the bark using steam, microwaves, or superheated steam (Chen et al., 2009; Ferreira et al., 2013). Compared to simple extraction methods, researchers have reported that supercritical extraction of BA using carbon dioxide mixed with ethanol, methanol, or acetone is more difficult. Similarly, extraction of BA after esterification of BN to its dipropionate or diacetate state is even more challenging (Chen et al., 2009; Ferreira et al., 2013). Guidoin et al. (2003) used a sublimation method to extract BA where high vacuum or atmospheric pressure and high temperatures were used. In the purification process, the use of different column chromatography or recrystallization techniques are the primary methods used to purify BN from plant extracts (Joshi et al, 2013; Lugemwa, 2012; Tijjani et al., 2012).

Zhao et al. (2007) have studied the extraction of BA using several solvents. They used 20 g of dried bark in 200 ml dichloromethane, ethyl acetate, acetone, chloroform, methanol, 95% ethanol (aqueous solution, v/v) and refluxed for 2 hours. They have reported that 95% ethanol was found to be the best solvent to extract BA. Mukherjee et al. (2010) extracted BA from rhizomes of *N. mucifera* species using70% ethanol. The thin layer chromatography was done to examine the separation of BA using several solvents, formic acid, methanol, and chloroform in different ratio as mobile phase. Sun et al. (2013) extracted BA from *Sour jujube* by defatting 5kg powder of the plant with petroleum ether. The extraction was undertaken at room temperature using 96% ethyl ether and concentration of supernatants done before being eluted using distilled water. Silica gel chromatography was with 70% ethyl ether was used to concentrate the extract before the dried

fruit was dissolved in methanol and treated using anisaldehyde. Table 2.2 presents a summary of various extraction methods that have been used to extract BA from different sources.

Table 2.2: Extraction conditions for betulin and betulinic acid extraction from different sources

Sr. number	Raw material	Solvents	Condition	Temperature (°C)	Time	Yield BA	Yield BN	References
1		Ethanol	Ultrasound	45	15 min	3.52%	NA	Pinilla et al.,2014
			Soxhlet	45	1.5 h	3.69%	NA	
2		Dichloromethane	Soak & reflux	NA	8 h.	NA	19 g	Bebenek et al., 2016
3	Bark	Several chemical solvents for extraction (200 ml dichloromethane, ethyl acetate, acetone, chloroform, methanol and 95% ethanol, separately)	Reflux	NA	2h	18.6 mg/g	202.2 mg/g	Zhao et al., 2007
4		2-Propanol 400 of 96% ethanol, separately	Soxhlet	NA	6 h	0.4mg	7.36 mg	Dehelean et al., 2012
5		N-hexane	Sonomatic ultrasound bath	NA	NA	NA	NA	Dehelean et al.,2007
		N-hexane & ethyl acetate		NA	NA	1.76%	12%	
		N-hexane (150 ml) & chloroform		NA	NA	2.01%	21.60%	
		N-hexane (150 ml) & dichloromethane		NA	NA	2.15%	23%	
6	Mistletoe	Water	NA	100	12 h	NA	NA	Ko et al., 2016
7	Rhizomes of N. nucifera Gaertn	70% ethanol	Cold maceration	12 h	0.51mg/g.	1.56%	0.11 mg/g	Mukherjee et al.,2010

Table 2.2: Extraction conditions for betulin and betulinic acid extraction from different sources (continued)

Sr. number	Raw material	Solvents	Condition	Temperature (°C)	Time	Yield BA	Yield BN	References
8	Sycamore outer bark	Water	Boiling	100	1 h and replaced the water and kept it for another half hour	NA	NA	Ren& Omori (2012)
		Water as pretreatment &then extracted with methanol	NA	Room temperature	24h	5.70%	NA	
		Water as pretreatment & then extracted with acetone	NA		NA	5.43%	NA	
		Water as pretreatment & then extracted with ethanol	NA		NA	5.46%	NA	
		Water as pretreatment & then extracted with 2-propanal	NA		NA	5.57%	NA	
9	T. potatoria root	Distilled methanol	Soaking	25	48 h.	1.50%	NA	Adesanwo et al.,2013
10	Rosemary leaves	MeOH	Reflux	NA	30 min	32 mg	NA	Abe et al.,2002
11	Syzygium aromaticum	MeOH	Soxhlet	NA	48 h	17%	NA	Aisha et al., 2012

Table 2.2: Extraction conditions for betulin and betulinic acid extraction from different sources (continued)

Sr. number	Raw material	Solvents	Condition	Temperature (°C)	Time	Yield BA	BN	References
12	Sour jujube	Defatted with petroleum ether. Then extracted with 96% EtOH	NA	Room temperature	NA	82 mg	NA	Sun et al., 2013
13	Leaves of Z. spinachristi	They are extracted with methanol, and after that, they extract again with petroleum ether, diethyl ether, chloroform and ethyl acetate separately.	NA	NA	NA	600 mg	NA	Nader & Baraka, 2012

2.4 Factors affecting extraction of betulin and betulinic acid

The quality of raw material, the polarity of the solvents, solid to solvents ratio and the type of treatments used before the extraction are crucial factors that affect the extraction of BN and BA. The quality of raw martial such as chaga, depends on the location of tree, soil, correct collection, storage. It also depends on the section of the tree i.e., middle, or the top of the tree and the presence of fruiting bodies (Shashkina et al., 2006; Laitinen et al., 2005). For instance, low-quality material taken from the bottom of old trees or from dead trees or collected from regions close to industrial area, highways, etc. can contain a considerable amount of heavy metals such as lead and arsenic (Shashkina et al., 2006). For these reasons, chaga must be harvested from the living birch bark trees only (Shashkina et al., 2006).

Besides there are reports (Rizhikovs et al., 2015; Cheng et al., 2011) that the yields of BA and BN is higher in polar solvents compared to nonpolar solvents (Table 2.3). BN and BA have very low water solubility that causes low bioavailability (Król et al., 2015; Soica et al., 2014).

Table 2.3: Yield of betulin from of birch outer bark extracted obtained by various solvents (Rizhikovs et al., 2015)

Solvents	Boiling point	BN yield (%)
Ethanol	78.4 °C	22.3
Methanol	64.7 °C	24.3
Acetone	56.5 °C	25
Chloroform	61 °C	24.3
Dichloromethane	39–40 °C	21.3
Benzene	80.1° C	11.1
Cyclohexane	80.1° C	11.1
n-Heptane	98 ° C	1.9
Petroleum ether	>100–140 °C	4.8
n-Hexane	69 °C	1.5

The solubility of BN in acetone (polar) is 5.2 g /L at 15.2 ° C , whereas in cyclohexane (nonpolar) the solubility is only 0.1 g /L at 35.2 ° C (Rizhikovs et al., 2015). The solubility of BA in polar organic solvents increases with increasing temperature (Cheng et al., 2011). However, higher temperature beyond certain limit shows significant effect on BA. For example, Siddiqui and Aeri (2016) reported that at 54°C, the maximum yield of BA was 2.45% with a raw material to solvent ratio at 1:30 for 6 h. When the temperature was increased above 54.21°C, BA yield was found to decrease proving that higher temperature has significant effect on BA yield as it increases the diffusion coefficient and solubility, although it may also cause compound degradation

(Siddiqui & Aeri, 2016). Most literature reports indicated that no extraction was done beyond the boiling points of the solvents used. In our study, we determined the maximum temperature at which extraction is recommended.

2.5 Need for water pretreatment before solvent extraction

Rizhikovs et al., (2015) have studied the effect of several pretreatment processes on the yield of BA after extraction from birch outer bark. They reported that hot water treatment before the extraction process was found to be effective and improved the quality of extracts. For example, BN yield before water treatment was 61.7 % and it is increased to 72.8%, respectively after 5 h of treatment from (Rizhikovs et al., 2015). Water pre-treatment followed by organic solvent extraction gave 5.70% yield of BA in methanol, 5.43% yield of BA in acetone, 5.46% yield of BA in ethanol and 5.57% yield of BA by 2-propanol (Ren & Omori, 2012). The purpose for using water pre-treatment step before the extraction is associated with the dis-charging of phenolic and monosaccharides substances during the preliminary soaking treatment by hot water treatment. It was found that the presence of admixture of phenolic compounds in birch outer bark extractives makes it difficult to obtain triterpene (Rizhikovs et al., 2015). This step was found to be extremely important for extracting BA from chaga as reported in our studies.

2.6 Separation of betulinic acid by chromatographic methods

Recently, Wu et al. (2011) have reported the extraction of BA from the leaves of Ligustrum spp, *Ligustrum sinensis Lour*, *Ligustrum pricei Hayata,* and *Ligustrum lucidum Ait*. The extraction method first entailed using methanol to extract BA. The resulting extracts were combined and concentrated under low pressure. Determination of the extracted BA 313.43μg/g from the three

Ligustrum spp. was undertaken using high-performance liquid chromatography (HPLC) using the photodiode array detectors (Wu et al., 2011).

In the study conducted by Guinda et al. (2011) BA was extracted from fruit pulp of *Argania spinose* plant using absolute ethanol. Entire fruits of *Argania spinosa* were air-dried for one week followed by maceration in absolute ethanol. The extract was then analyzed for the presence of BA. Viji et al. (2010) also reported air drying of *Bacopa monniera* followed by milling the entire plant. Subsequently, the milled material was defatted using petroleum ether. The defatted substrate was treated with methanol to extract BA before the crude extract was concentrated under vacuum. The obtained residue was suspended in water followed by extraction using chloroform, n-butanol (n-BuOH), and ethyl acetate. Column chromatography was used to separate BA 1mg/5kg from crude mixture.

Kumar et al. (2010) have reported the extraction of BA from *Dillenia indica* L. fruits using methanol. Firstly, the dried fruits were treated with methanol and then the extract was filtered to remove solids. Methanol was evaporated from the solution under vacuum and distilled water was added to the residue. Organic solvents such as n-BuOH and ethyl acetate were added to the aqueous solution and extracted BA from the aqueous suspension. BA was then separated from the crude organic extract using column chromatography and the isolated yield of BA was 95.64%. Joshi et al. (2013) extracted BA from dried leaves of *Pentalinon andrieuxii* using ethanol in the extraction process. Subsequently, after evaporating ethanol, the crude extract was subjected to liquid/liquid partition using butanol, ethyl acetate, and hexane.

Choi et al. (2009) used *Saussurea lappa* roots to isolate BA using methanol. The leaves of *S. lappa* were soaked in methanol for one week. The methanol extract was suspended in water and later partitioned using ethyl acetate, the soluble product was subjected to column chromatography

on silica gel to isolate BA. A similar approach was used by Nader & Baraka, (2012) to extract BA from the *Z. spinachrist* leaves. Table 2.4 summarises the separation methods that have been used in the literature to separate BA from plant products.

Table 2.4: Chromatographic separation techniques used to separate BA from plant solvent extracts

Source	Extraction solvents	Chromatographic method	Reference
Air-dried and whole powdered plant of *Bacopa monniera*	Defatted with petroleum ether	Silica gel chromatography	Viji et al. (2010)
Dried fruits of *Dillenia indica*	Methanol	Silica gel chromatography of ethyl acetate fraction	Kumar et al. (2010)
From the roots of *Saussurea lappa*	Methanol and partitioned with ethyl acetate	Silica gel chromatography	Choi et al. (2009)
From the *Z. spinachrist* leaves	Methanol	Silica gel chromatography	Nader & Baraka, (2012)

2.7 The biological activity of betulinic acid on cancer cell lines

2.7.1 Influence of betulinic acid on oxidative stress

Normal cells are constantly exposed to external and internal stress, including UV light, hypoxia, radiation, and growth factors resulting in reactive oxygen species (ROS) production (Circu & Aw, 2010). Oxidative stress plays an important role in the development of cancer leading to cellular and subcellular damage and ultimately destruction of the cells (Reuter et al., 2010). Tumor cells foster under hypoxic conditions, where healthy cells cannot survive. The factors that promote and inhibit oxidative stress are shown in Figure 2.2. A proper balance between the two factors is required for normal function.

In order to neutralize the ROS effects, antioxidants, particularly vitamins and triterpene compounds, are crucial. Antioxidants are important contributors to the regulation mechanism of proliferative processes (Circu & Aw, 2010). Enzymatic antioxidants protect the cells from oxidative damage by inhibiting ROS production (Reuter, 2011). These enzymes include superoxide dismutase, glutathione peroxidase, glutathione reductase, and catalase (Costa et al., 2014). For instance, hydrogen peroxide (H_2O_2) is largely regarded as a cytotoxic agent whose levels must be minimized by the action of antioxidant defense enzymes (Circu & Aw, 2010). Moreover, UDP-glucose6-dehydrogenase, 6-phosphogluconate dehydrogenase and peroxidase enzyme were involved in the oxidative stress response, which correlates with BA-induced apoptosis of the HeLa cells. The level of ROS production was detected after treatment of the HeLa cells with BA increased significantly. These results suggest that BA induces apoptosis through the activation of ROS in HeLa cells (Xu et al., 2014).
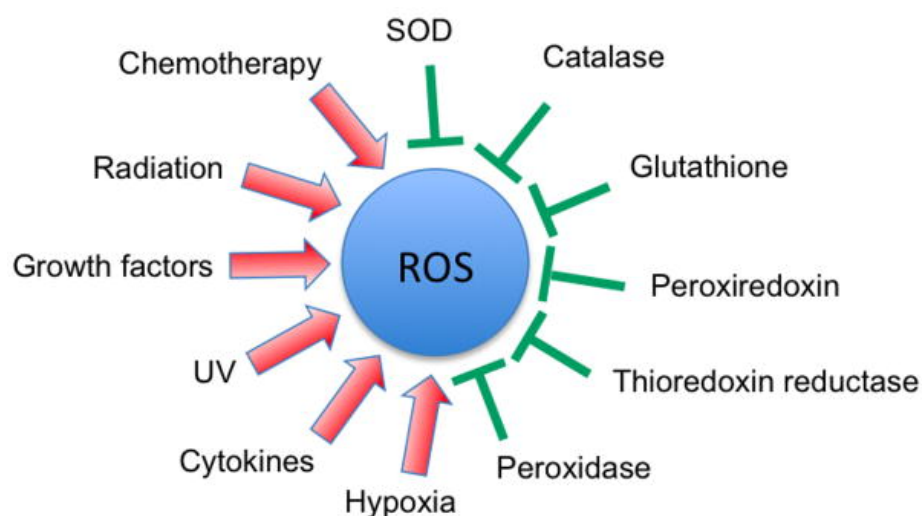
Figure 2.2: Factors promoting and attenuating oxidative stress (Reuter et al., 2010)

BA suppress specificity proteins (Sp) like Sp1, Sp3, and Sp4 that correlated with the induction of ROS such as H_2O_2 (Chintharlapalli et al., 2007). Increased levels of ROS lead to induction of apoptosis in cells incubated with BA. High level of ROS production leads to activate caspases that are involved in apoptosis including the initiator caspases, caspase-9; and the effector caspases, caspases-3 and 7 (Bhatia et al., 2015). Shi (2004) and Tan et al. (2003) reported no detectable change in caspase-8 and caspase-9 after BA was added to UISO-Mel-1 cells. Meanwhile, in colorectal carcinoma cells line, the expression of caspase-3 and 7 is increased, causing chromosome condensation (Aisha et al., 2012).

Cancer cells substantially increase ROS levels to initiate pathways of proliferation, survival, and metabolic activities (Shi, 2004; Viji et al., 2011). However, cancerous cells maintain antioxidant activity to prevent ROS activity that can induce apoptosis. BA, as an antioxidant compound, is able to enhance the type of antioxidant activity (Dash et al., 2015). For example, Qian et al. (2012) found that BA significantly inhibited the increase of ROS level, as well as the decrease of nitric oxide level.

Oxidative stress and inflammation play a key role in the pathogenesis of cancer. In the presence of oxidative stress, cancer cells become inflamed and damaged. In addition, cancer cells trigger the development of metastasis, thereby leading to further progression of the disease (Reuter, 2011). Table 2.5 lists some of the studies on the role of BA on antioxidants, oxidative stress, inflammation, and apoptosis markers. We will study the effect of BA and crude chaga extract on the MCF-7 cancer cell on oxidative stress.

Table 2.5: Role of betulinic acid on markers of oxidative stress, inflammation and apoptosis

A) Oxidative stress

Type of cell lines and tissues	The effect of BA on markers of oxidative stress	Comments	References
HeLa cells cervical cancer	BA decreased the level of UDP-glucose 6-dehydrogenase and 6-phosphogluconate dehydrogenase decarboxylation during oxidative stress BA downregulated antiapoptotic bcl-2 gene and increased proapoptotic bax gene	BA induces apoptosis through the activation of ROS	Xu et al. (2014)
RAW264.7 Immortalized murine macrophages	BA decreased intracellular ROS induced by H_2O_2 and decreased the levels of oxidant stress that induced LPL protein expression and activity from simulated macrophage	BA reduced the cellular lipid production	Peng et al. (2015)

B) Inflammation

Type of cell lines and tissues	The effect of BA on markers of inflammation	Comments	References
VSMCs Vascular smooth muscle	BA increased the expression of cyclins and decreased ROS and NF-κB signaling	The inhibitory dose of BA concentrations has specific effect	Vadivelu et al. (2012)
MDA-MB-468 Breast cancer cells	BA increased the expression of NOS. NF-κB1, TNF-α, TLR4 and STAT3	Each concentration has specific effect on growth factor either by inducing apoptosis or cell arrest	Weber et al. (2014)
THP-1 Macrophage derived foam cells	BA reduced the levels of TNF-a, IL-6 and IL-1b and decreased the phosphoprotein levels of IkBa	——————	Zhao et al. (2013)

C) Apoptosis

Type of cell lines and tissues	The effect of BA on markers of apoptosis	Comments	References
LNCaP, DU145and PC3 Prostate cancer cell	BA increased NF-kB activity because it enhanced degradation of IKB	The inhibitory dose of BA concentrations has specific effect	Reiner et al. (2013)
MCF-7 Breast cancer cell	BA increased the expression of p53 and p21 and G0/G1 phase cell cycle arrest	The increase in Bax/Bcl-2 ratio was mainly due to the down-regulation of Bcl-2	Foo et al. (2015)
PC3 Prostate cancer cell	BA reduced VEGF that induce hypoxia condition and inhibited HIF-1α and STAT	——————	Shin et al .(2011)

2.7.2 Influence of betulinic acid on inflammation

During inflammation, cells produce pro-inflammatory cytokines, such as tumor necrosis factors (TNF)-α and interleukins family (IL), such as IL-1, IL-6, IL-8, and IL-12. The expression

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of all these mediators is regulated by NF-kB which are overexpressed in response to inflammatory cytokines (Hoesel & Schmid 2013). The intensified production of these mediators is the main cause of the deleterious consequences like tissue hypoxia and death (Yadav et al.,2010; Costa et al., 2014). For instance, TNF-a has been found to lead to ROS generation through the activation of NADPH oxidase, and is one of the major mediators of cancer-related inflammation (Dash et al.,2015; Morgan & Liu, 2010). In addition, transcription factors, such as NF-kB and Sp, are involved in the inflammatory process, cell proliferation, and apoptosis. Sp-regulated genes include several signals that are essential for cancer cell proliferation and inflammation.

BA plays a crucial role as a potential anti-inflammatory compound. BA decreased Sp protein expression which reduced the expression of vascular endothelium growth factor (VEGF) (Chintharlapalli et al., 2011). BA also has been found to be potentially effective against a wide variety of cancer cells, including MDA-MB-231 and MDA-MB-468 cells (Weber et al., 2014). Treating the MDA-MB-231 cell with BA decreases the expression of IL-8 and IL-12, but was found to increase the expression of IL-6 and TNF- α (Weber et al.., 2014). On the other hand, Weber et al. (2014) reported that BA also significantly reduced the expression of TNF-α and blocked the inflammatory response in MDA-MB-468 cells. BA also increased the mRNA of G1-phase inhibitors p21 and p27, activators CDK2, CDK6, and cyclin D1 in cancer cell lines (Weber et al., 2014 & Pandey et al., 2010). In addition, BA has the potential for anti-inflammatory activity by decreasing TNF-α expression and increasing production levels of IL-10 that reduce the production of lipopolysaccharide (Costa et al., 2014; Viji et al., 2011; Talmale et al. 2015). Not only did BA significantly reduce the production of TNF-α, but it also decreased the production of IL-6 to a certain extent (Costa et al., 2014), Additionally, macrophages that were treated with BA had less overall pro-inflammatory mediators (Nader & Baraka, 2012).

2.7.3 Influence of betulinic acid on apoptosis

Hypoxia generates ROS that contribute to DNA damage and increase proliferation of tumor cells (Reuter, 2011). ROS are involved in mitochondrial membrane permeabilization and play a critical role in the induction of apoptosis and cell death (Fulda & Kroemer, 2009). ROS can either promote tumor cell survival or act as anti-tumorigenic agents depending on the cell and tissues, the location of ROS production and the concentration of individual ROS (Reuter, 2011).

TNF-a initiates downstream signaling pathways, including activation of NF-kB, Matrix Metalloprotease (MMP) kinase and the induction of apoptosis and necrosis (Morgan & Liu, 2010). TNF-a have an important function in cell death by activating c-Jun N-terminal kinase pathway and produce ROS (Dash et al., 2015). BA blocks TNF-mediated NF-B activation not only by TNF but also by other inflammatory and carcinogenic agents. The inhibitory activity of BA is associated with the suppression of TNF-induced IKK activation, IKB phosphorylation and degradation, p65 phosphorylation and nuclear translocation, and NF-kB-dependent reporter gene transcription (Takada & Aggarwal, 2003).The inhibitory effect of BA was not cell-line specific and there are various ways that BA might inhibit TNF-induced NF-kB activation (Bhatia et al., 2015).

Moreover, BA also inhibited TNF-induced expression of cyclooxygenase-2 and MMP-9 which have NF-KB binding sites in their promoters that regulate their transcription (Takada & Aggarwal, 2003). Furthermore, BA enhanced TNF-induced apoptosis. Kommera et al. (2011) reported that BA was found to cause cell death by induction of apoptosis involving caspases. BA led to loss of the mitochondrial membrane potential, followed by the release of cytochrome c and apoptosis-inducing factors (AIF), leading to activation of caspases and formation of the apoptosome (Rzeski et al., 2006). The release of cytochrome c is determined by the relative amounts of apoptosis-inhibiting Bcl-2 proteins in the outer membrane of the mitochondrion (Bhatia et al., 2015).

P53 is a tumor suppresser gene that controls many genes such as the Bcl-2 family associated proteins that have both pro-apoptotic and anti-apoptotic effects in cancer cells. While bcl-2 suppresses apoptosis; the bax protein accelerates apoptosis. BA activity has been shown be independent of P53 (Fulda et al., 1997). Over-expression of Bcl-2 protected cancer cells from cytotoxic effects induced by BA highlighting the importance of Bcl-2 expression in cell death. BA was initially proposed to have a direct effect on the mitochondria and to induce apoptosis in a BCL-2-dependent manners (Rzeski et al., 2006; Bhatia et al., 2015). However, Bcl-2 overexpression can only provide short-term protection and eventually these cells succumb to apoptosis (Potze et al, 2014; Adams & Cory 2007). BA has also been found to induce apoptosis in multiple myeloma cells when the level of Bcl-xL expression decreases (Pandey et al., 2010). BA affects tumor cell proliferation, migration, apoptosis induction as well as on Bcl-2, bax and cyclin D1 expression.

BA also has been shown to decrease the expression levels of androgen receptor and cyclin D1 proteins in a TRAMP transgenic mouse model and increase the release of cytochrome c, Smac and AIF from the mitochondria in LNCaP and DU145 cells (Reiner et al., 2013). Reiner et al., (2013) reported BA-mediated release of pro-apoptotic proteins from the mitochondria which increase apoptosis in PC cells. BA has been found to induce proteasome-dependent degradation of other transcription factors, such as Sp1, Sp3 and Sp4, which regulate VEGF expression. The latter is also regulated by Signal Transducer and Activator of Transcription 3 (STAT3), suggesting that BA may mediate antiangiogenesis through the downregulation of VEGF (Chintharlapalli et al., 2011).

In addition, BA also induces inhibition of STAT3 activation and suppresses NF-kB activation (Pandey et al., 2010; Takada et al., 2010). It is possible that suppression of JAK activation is the critical target for inhibition of both the NF-kB and STAT3 activation by BA (Pandey et al., 2010).

2.7.4 Antifungal effect of betulinic acid

Dermatomycoses is one of the common cutaneous and superficial fungal infections in humans. Considering the increased challenges related to fungal infections, immediate action is necessary to develop non-toxic and efficient antimycotic agents with specific activities. For the past few decades, BA has been demonstrated to possess a wide range of biological activities against most fungal infections. For instance, Innocente et al. (2014) reported the effect of BA on both cutaneous and mucocutaneous mycotic agents and its minimal inhibitory concentration. These results showed that fungicidal impacts of BA to range from 8 μg/mL to 128 μg/mL.

A betulinic acid derivative called Piperazinyl was observed to have strong antifungal activities similar to Terbinafine, and which was identified to be the most significant derivative to have dermatophyte effects on yeast infections (Innocente et al., 2014). Zhang et al. (2002) identified that BA inhibit the growth of *Candida albicans* with IC_{50} values of 6.5 μg/ml.

BA as an antifungal agent has been found to act by disrupting the fungal cell wall, based on studies using Sorbitol Protection Assays. The results of the assay indicated that minimal inhibitory concentration has slight stress on fungal cells. Also, the minimal inhibitory concentration also contributed to controlled cell growth synthesis in a similar mechanism observed from antifungal drugs such as anidulafungin. In addition, cellular leakage assay indicated that BA inhibited yeast growth by initiating membrane damage, especially compromising the integrity of nucleotides. Also, further analysis using the Ergosterol Effect Assay confirmed that the damage to the fungal membrane after the introduction of BA resulted from binding to ergosterol (Innocente et al., 2014).

Tene et al. (2009) also observed that the mechanism of BA action on fungal cells produces a weak damage to yeast membrane, but did not affect the ergosterol assay. Moreover, the

inhibition mechanism of BA on fungal-like *Fusarium oxysporum* was affected through inhibition of growth, especially in the ethyl ether and n-hexane fractions. Besides, research by Zhang et al. (2002) also noted that BA had inhibitory impacts against *Candida albicans* and secreted aspartic protease with an IC_{50} value of 6.4 μg/ml. These findings indicate that BA has significant antifungal properties that either acts on the cell membranes and limit nucleotide activity or inhibit the growth of yeast cells.

2.7.5 Antibacterial effect of betulinic acid

Recently, the problem of antibiotic resistance bacteria has emerged to be a global concern in both veterinary and human medicine. Often, antibiotic abuse resulting from non-prescription has worsened the situation by accelerating the emergence of super-bacteria that has become a great concern. Hence, the development of new antibiotic medications of therapeutic approaches to combat the problem of multi-drug resistance is an urgent need (Takada & Aggarwal, 2003). In efforts to pursue next generation therapeutics, diverse strategies, including the use of metal nanoparticles, isolating antimicrobial peptide from microorganisms, and extraction of natural plant products to get compound like flavonoids, phenylpropanoids, and triterpenoids are necessary (Tijjani et al., 2012).

Today, several sesquiterpernoids, diterpenoids, and terpenoids have been found to possess synergetic components that act as antibiotics, showing the growing importance of plant-derived chemicals in combination use with the current antibiotic therapeutic agents to combat the existing problem of multi-drug resistant microorganisms (Wu et al., 2011). A recent study by Wang et al. (2016) has shown the increasing antibacterial effects associated with BA against *B. cereus*. Their research further noticed that BA exhibited potential activities against gram-positive bacteria such as *S. aureus*. The mechanism was found to target the inhibition of protein biosynthesis and cell wall synthesis. Other observations noted that BA blocked enzymes that are involved in the cell

wall synthesis and also inhibition of the aminoacyl-tRNA binding to the A site of the 30S ribosomes. Since the structure of the BA is different from that of the antibiotic activities, the pathway of the antimicrobial agents may have a new mechanism and target in suppressing the activities of pathogenic microorganisms, such as *Bacillus subtilis*.

Additional research on the mechanisms of BA demonstrates that the agent can modulate the resistance to oxacillin, ampicillin, and β-lactam antibiotics. BA blocks cell division and thus inhibits the macromolecular and DNA synthesis, especially in *B. subtilis*. Besides, the agent can also inhibit recycling of the murein monomer precursors and also lessen the activity and secretion of β-lactamase. As a result, BA can contribute to increased susceptibility of bacteria to β-lactams and mainly in case of the methicillin resistance strains of *Staphylococcus aureus*. Also, Saelens et al. (2004) have reported active antimicrobial activity by BA towards gram positive bacteria like *L. monocytogenes* and *E. faecalis*. A combination of tetracycline or ampicillin with BA can contribute to synergistic antibacterial effects against resistant strains (Ressmann et al., 2012). The capacity of the BA to enhance the activities of β-lactams might contribute to a valuable group of future therapeutic agents.

Moghaddam et al. (2012) observed that BA had an active effect with a minimum inhibitory concentration of higher than 128 μg/ml on *S. aureus*, *B. subtilis*, and *C. albicans*. Besides, Moghaddam et al. (2012) also reported that the high degree of activity and relatively large abundance of BA are responsible for the bioactivity against pathogenic gram-positive bacteria. The anti-bacterial effect of BA was also tested against *Escherichia coli* and *Bacillus subtilis* using the paper disc method (Tijjani et al., 2012). The study observed that BA has no effect on *Escherichia coli* from lack of zone inhibition, but it has a significant effect on *Bacillus subtilis* at high concentrations of 1000 μg/disc while lacks effective mechanism against *B. subtilis* at concentrations lower than 500 μg/disc.

2.7.6 Antiviral effect of betulinic acid

Besides the reported antibacterial, antifungal, anticancer, and anti-inflammatory effects of BA, other studies have also reported that BA has significant antiviral effects against disease-causing viral particles (Pierson & Doms, 2013). For example, several BA derivatives have been reported to be highly selective and potent inhibitors of HIV-1. Based on precise side-chain modification, BA derivatives have been shown to function by interfering with specific HIV maturation steps and also by inhibiting the fusion of HIV viruses. As such, BA derivatives have substantial therapeutic activities against viruses by inhibiting their integration and entry into host cells (Aiken & Chen, 2005).

Huang et al. (2004) reported various BA inhibitors to be potent inhibitors of viral infections and hypothesized that they act at specific life cycle distinct from host entry. The BA derivatives include PA-457 and 3-O-(30 ,30 -dimethylsuccinyl)-betulinic acid, which has also received more attention because of their potency with an IC_{50} of 5 μM, in addition to their ease of synthesis and low toxicity in cell cultures. Additional research has shown that 3-O-(30 ,30 -dimethyl succinyl)-betulinic acid functions by disrupting viral replication activities in the last step of viral development and also leads to accumulation of incomplete CA-SP1 proteins in the case of HIV-1. Therefore, virus particles that are resistant to RT and PR inhibitors are largely sensitive to these BA derivatives, further stressing the important role that the derivatives play as antiviral agents.

Kanamoto et al. (2001) have elaborated the mechanism of BA in reducing the accumulation of viral antigens in cultured cell lines. The authors also noted that the antiviral action of the agent works by inhibiting maturation or self-assembly of the virus particles. However, other researchers have failed to confirm the effect of some of the BA derivatives on viral particles (Aiken & Chen, 2005). Instead, the authors note that the antiviral activity of BA derivatives is a result of delays in the cleavage of the CA-SP1 junctions, without any additional impact on the breakdown of other

cleavage sites such as the Gag site. However, BA derivatives like PA-457 are effective on specific viruses and does not affect the replication of other viruses even at micromolar concentrations. For example, the 3-O-(30 ,30 -dimethylsuccinyl)-betulinic acid derivative is highly specific to HIV-1 but has no antiviral effect on SIV or HIV-2. Another research by Pierson and Doms (2013) on BA extract from *Syzrgium claviflorum* leaves concluded that the agent has an inhibitory effect on HIV-1 replication in infected lymphocyte cells with an EC_{50} value of 1.4μM. However, on infected H9 cell lines, BA inhibited cell growth with an IC_{50} value of 12.8 μM. These findings further support the antiviral properties that have been reported from various studies on BA.

2.8 Properties of phenolic antioxidants

Phenolic compounds with aldehydes or ketones groups can be found in blueberries, herbs, leaves, rhubarb petioles (Rheum spp), flowering tissues, woody parts such as stems and barks of tree and the fungus chaga (Kähkönen et al., 1999; Dai & Mumper, 2010;Nakajima et al., 2009; Takeoka et al., 2013 & Ju et al., 2010). For instance, phenolic compounds, such as phenolic acids, lignans, stilbenes and lignin are important in the plants for normal growth and development and defense against infection and injury (Kähkönen et al., 1999).

Besides, phenolic compounds have biological effects such as anti-oxidant, anti-inflammatory, anti-cancer properties. For example, phenolic compound are involved insuppressing ROS formation and up- regulating antioxidant defense system (Dai & Mumper, 2010). Nakajima et al. (2009) identified seven phenolic compounds in chaga that showed cytotoxic effects on cancer cells lines.

Beneficial phenolics compound may also be associated with other plants components such as carbohydrate and protein Therefore, there is no common extraction technique suitable for all plant phenolics extraction. Based on the solvent that are used during exaction, an admixture of phenolics soluble and some non-phenolic substances such as organic acids, fats and sugar in the

solvent will be extracted from plant materials. As a result, additional steps may be required to remove those unwanted components (Dai & Mumper, 2010). The total amount of phenolic acids was found to be 327.4 μg/g DW in the petioles extracted by HCl/MeOH. R. undulatum had a level of 198.16 μg/g. Takeoka et al., (2013) have reported phenolic content in the chaga extracts to be 58.7 mg of gallic acid /100 g. But with steam-treated chaga this value increased to 125 mg gallic acid /100 (Ju et al., 2010). We determined the phenolic content of chaga in terms of gallic acid equivalent (GAE) in our studies.

Chapter 3
Materials and Methods

3.1 Materials

Four samples of chaga were investigated in this study. Three of the samples were purchased from 180 Foods Company (LC), Thunder Bay; Tao tea chaga (TT), Toronto; and chaga.ca (CC), Mont-Tremblant, QC, Canada, respectively. The fourth sample was harvested (HC) locally in the Boreal forest at Thunder Bay. Betulin and betulinic acid standards of HPLC grade >97% were purchased from Sigma-Aldrich, Oakville, ON, Canada. A reference betulinic acid standard (90%) assessment of biological activity was also purchased from Sigma-Aldrich, ON, Canada. ACS grade reagents (methanol, hexanol, ethylacetate, acetone, chloroform, and 95% ethanol) and HPLC grade acetonitrile were purchased from Caledon Laboratories Ltd, ON, Canada.

3.2 Preparation of standard solutions calibration curves

3.2.1 Calibration curves for betulin and betulinic acid standard

BN and BA standard curves were developed by weighing 10 mg of the standards into separate volumetric flasks (10 ml each) and filled up to the mark with 95% of Ethanol (EtOH) as a stock solution. Seven different concentrations of BN and BA were formulated and diluted accordingly from the stock solutions as follows: 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, and 0.07 mg/ml. The standard solutions were filtered with 0.22 μm Millipore filter into 2 mL vial before HPLC injection.

3.2.2 Calibration curve for phenolic standard

A stock solution of gallic acid stock solution was prepared by weighing 40 mg of the standard into 100 ml volumetric flask and then filled up to the mark with methanol (MeOH). Six different concentrations of gallic acid standards were prepared from the stock solution to develop the calibration curve as follows: 10, 25, 50, 75, 100, and 200 μg of gallic acid/ml. A stock

solution of Folin–Ciocalteu reagent was prepared by adding 5 mL of the reagent to 50 mL of distilled water at ratio 1:10 (v/v). For Na_2CO_3 stock solution, 6% of Na_2CO_3 was prepared by adding 6 g of the substrate to 100 ml of distilled water and rigorously mixed. For the spectrophotometer analysis, 100 μL of sample was added to 750μL of Folin–Ciocalteu reagent in a 25-mL centrifuge tube and kept in the dark for 5 min. Then, 750 μL of 6% Na_2CO_3 was added and kept in the dark for another 2 h before absorbance analysis on spectrophotometer at 765 nm wavelength. Water was used as blank.

3.2.3 Preparation of betulinic acid solutions for in vitro studies

A stock solution of commercial BA was prepared by adding 50 mg of the standard into 5 ml dimethylsulphoxide (DMSO) (Sigma, USA) to obtain 10 mg/ml concentration of BA solution and stored at 4^0C until use. The stock solution was filtered with 0.22 μm Millipore filter before six different concentrations (0.005, 0.01, 0.05, 0.1, 0.5, and 1 mg /ml) were prepared accordingly by dilution into Dulbecco's Modified Eagle Medium (DMEM) as described by previously Alitheen et al., 2010.

3.2.4 Cell line and culture conditions

Breast cancer MCF-7 cells were purchased from American Type Culture Collection and were cultured in DMEM containing 25 mM glucose, (Sigma-Aldrich, Oakville, ON, Canada) supplemented with 10% fetal bovine serum (FBS) and antibiotic/antimycotic (100 units mL 21 penicillin G sodium, 100 μg/mL streptomycin sulfate, 0.25 μg/ mL amphotericin B). Cell cultures were maintained in T-25 flasks in a humidified atmosphere at 37°C and 5% CO_2. The culture medium was changed every two days and cells were sub-cultured upon reaching ~ 85% confluence.

3.3 Experimental methods

3.3.1   *Effect of water pretreatment*

3.3.1.1 Pretreatment for four days

Water pretreatment of chaga samples was carried out according to previous studies of Rizhikovs et al., 2015 and Oyebanji, et al., 2014. In this procedure, 50 g of powdered chaga was weighed into a round bottom flask, and 500 ml of distilled water was added. The samples were soaked for four days at room temperature with continuous stirring using a magnetic stirrer. At the end of the soaking period, the mixture was divided into centrifuge tubes (50 mL). It was then centrifuged at 4000 rpm for 15 min. The supernatant was slowly decanted while the residue was dried in an oven overnight at 80^0C in preparation for extraction.

3.3.1.2 Water pretreatment for 2h and 5h at 100^0C

A 50 g of powdered chaga was weighed into a round bottom flask followed by addition of 500 ml of distilled water and reflux at 100°C for 2 and 5 h (Rizhikovs et al., 2015). The mixture was continuously stirred by a magnetic stirrer during the reflux for homogeneous mixing and heat distribution. After reflux, the sample was cooled down to room temperature. The mixture was then divided into centrifuge tubes (50 mL) and subjected to centrifugation at 4000 rpm for 15 min. The supernatant was slowly decanted while the residue was dried in an oven overnight at 80^0C in preparation for extraction.

3.3.2   *Effect of temperature on pure betulinic acid degradation*

To study the effect of temperature on BA degradation, 1.2 mg of BA was dissolved in 25 ml of 100% MeOH and 1.6 mg in 25 ml of 95% EtOH. BA solutions were stirred thoroughly to enhance solubility. Then, the samples were filtered using 0.22 mm Millipore filter. From the stock solution, 1.5 mL from each solvent was transferred into HPLC vial to determine the initial peak areas of BA in the solutions. This study was carried out using water-bath at different temperatures

(40, 50, 60, 70, and 80°C). Five samples for each solvent at three sets of times (2, 4, 6 h) were examined. After each time set, the aliquots were injected into the HPLC to determine the current concentration of BA in the solution. Thereafter, % loss of BA at the selected temperature range was estimated using the following equation.

$$\% \, loss \, of \, BA = \frac{Intial \, peak \, area - Final \, peak \, area}{Intial \, peak \, area} \times 100$$

3.3.3 Effect of water pretreatment on betulin and betulinic acid extraction from different chaga sources

Non-pretreated chaga sources (10 g) each was added to 100 ml of 95% EtOH in a ratio (chaga: solvent) 1:10 (m/v), separately into a round bottom flask. The samples were stirred thoroughly and refluxed for 3 h at 50^{0}C (Siddiqui & Aeri, 2016). The supernatant containing the extract was separated from the residue using centrifugation for 15 min at 4000 rpm. The aliquot was filtered with 0.22 µm Millipore filter before HPLC injection. The same protocol was used for the 4 days pretreated chaga from different sources.

3.3.4 Effect of hours of water pretreatment on betulin and betulinic acid yield from different chaga sources

The chaga samples (10 g) pretreated with water for 2 h and 5 h was added to 100 mL of 95% EtOH in a ratio 1:10 (m/v). Each sample was placed in separate round bottom flask. The samples were stirred thoroughly and refluxed for 3 h at 50^{0}C. The supernatant containing the extract was separated from the residue using centrifugation for 15 min at 4000 rpm. The aliquot was filtered with 0.22 µm Millipore filter before HPLC injection.

3.3.5 Effect of different solvents on betulin and betulinic acid yield

In our preliminary study, Tao tea was observed to contain the highest BN and a reasonable amount of BA as compared to other commercial chaga investigated. Effect of different solvents on extraction efficiency was carried out only on Tao tea. Dry water pretreated Tao tea chaga (10 g) was added to 100 ml of selected polar solvents (EtOH, MeOH, and acetone) and non-polar solvent (EtOAc, $CHCl_3$, and hexanol) separately in a 200 ml around bottom flasks. The samples were stirred thoroughly and refluxed for 3 h at 50^0C. The supernatant containing the extract was separated from the residue using centrifugation for 15 min at 4000 rpm. After solvent extraction, other solvents except EtOH were evaporated using rotary evaporator at 50°C to maintain representative chromatogram during HPLC analysis. The analyte was diluted accordingly to standard concentrations and filtered with 0.22 µm Millipore filter before HPLC injection.

3.3.6 Effect of ultrasound-assisted extraction of betulin and betulinic acid from Tao tea chaga

Water pretreated Tao tea chaga (10 g) was added to 100 ml of 95% EtOH in a ratio 1:10 (m/v) in a 50-mL centrifuge tube and sonicated using ultrasound (Branson Digital Sonifier, Branson Ultrasonics) for 20 min. The ultra-sonifier was equipped with a 0.12 in diameter disruptor horn probe capable of up to 90% amplitude. The ultrasound probe was immersed up to 15 mm into the sample solution and centralized for homogeneous cavitation effect in the reactor. The parameters used in the study were 70% amplitude, 5 sec pulse on, and 5 sec pulse off as reported in a previous work (Pinilla et al., 2014). After 20 min of sonication, the sample was stirred thoroughly and refluxed for 3 h at 50^0C. The supernatant containing the extract was separated from the residue by centrifugation for 15 min at 4000 rpm. The aliquot was filtered with 0.22 µm Millipore filter before HPLC injection. This study was carried out in duplicate.

3.4 Analytical Methods

3.4.1 Cell viability (MTT) assay

Using the 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to determine the metabolic activity of living cells, an indication of proliferation and viability. MCF-7 (200 μL; 2.56×10^4 cells per well) were cultured in 96-well microplates and incubated in medium containing 10% FBS. After 24h incubation, MCF-7 cells were treated with a varying of concentrations of BA (0.005, 0.01, 0.05, 0.1 ,0.5 and 1 mg/mL, Sigma-Aldrich, Canada), as used in previous studies (Panndy et al.,2010; Aisha et al.2012; Tiwari et al., 2014; Mullauar,2009). After the cells were treated with BA, they were incubated for a further 24 h at 37^0C and 5% CO_2. After 24 h incubation, the cells were treated with MTT (20 μL; 5 mg/mL in phosphate-buffer-solution, PBS) in the microplate wells and the plate was incubated for 4 h. After that, the incubation medium was aspirated, and 50 μL of DMSO was added per well. Following 10 min of agitation on a Belly Dancer shake, the reduction of MTT was determined spectrophotometrically by measuring the absorbance (A) at wavelengths of 490 and 650 nm, and the difference (ΔA) was used to analyze the results.

The treatment cells were compared with the control cells (cells untreated with BA), and the results were expressed as a percentage of viable cells compared to the control. Based on the results of the MTT assay, a concentration of 0.005 mg/mL and 0.01 mg/mL were chosen to examine the effects of BA on reactive oxygen species (ROS) level, using flow cytometry.

3.4.2 Cell viability (MTT) assay with bioactive compound in crude extract

The crude extract (50 mL) containing 70% EtOH was evaporated to dryness under reduced pressure. The crude extract contained about 0.04 mg of BA. The crude extract containing BA was dissolved in DMSO, and a stock solution of 0.01mg/mL concentration was prepared using DMEM. Another 2 mL was drawn from the stock solution to prepare 0.005 mg/mL of crude extract BA to

treat MCF-7 cells. The final DMSO concentrations in the culture media was estimated as 0.1%. MCF-7 cells were treated with a series of concentrations of BA (0.005 and 0.01mg/mL) in crude extract.

3.4.3 Oxidative stress (CM-H2DCFDA) assay

Using an oxidation-sensitive fluorescent probe CM-H2DCFDA (5- and- 6)-chloromethyl-2′7′-dichlorodihydrofluorescein diacetate, acetyl ester) to measure intracellular reactive oxygen species. A suspension of MCF-7 cells (2.0 × 106 cells/flask) was seeded onto 6-well sterile flat-bottom plates at 37°C and 5% CO_2 to allow them to adhere to the well walls overnight. After incubation for 24 h, the cells were treated with pure BA (0.005 mg/mL and 0.01 mg/mL) for 24 hours at 37^0C and 5% CO_2. Cells incubated with 1 mM H_2O_2 for 4 h to induce the intracellular oxidative stress served as positive control in MCF-7 cells. H_2O_2 was added before fluorescence measurement. Briefly, the CM-H2DCFDA solution was prepared by adding 50 μg CM-H2DCFDA to 856 µL of DMSO and the mixture lightly vortexed. Following this step, 7.70 ml of PBS was added to the tube and the mixture was gently vortexed. The dye solution was next removed by aspiration and the cells were trypsinized and centrifuged at 500 × g for 5 min at 4^0C. Finally, the cells were washed twice with PBS, and kept in the dark on ice before the samples were analyzed by flow cytometry. To analyze the quantity of ROS produced in the MCF-7 cells, the fluorescence of CM-H2DCFDA was measured at 488 nm on the FL1 channel of a BD FACSCalibur Flow Cytometer (BD Biosciences) supported by BD CellQuest Pro Software. 10,000-cells/events were counted, and the results were expressed as the mean intensity of fluorescence.

3.4.4 Determination of total phenolics

The amount of total polyphenol in chaga extracts was determined using modified Folin-Ciocalteu colorimetric method (Takeoka et al., 2013). A crude extract from Tao tea chaga in 95%

EtOH was evaporated by using vacuum rotary evaporator to dryness. The residue was dissolved in 50 ml MeOH as a stock solution. From the stock solution, 100 μL sample extracts were dissolved in MeOH and further diluted appropriately to right concentrations. The sample extracts were oxidized by adding Folin-Ciocalteu reagent (750 μL) After 5 min, neutralization of the mixture was done by 6% Na_2CO_3 (750 μL) and kept in the dark for 2 h. The absorbance was measured at 765 nm using a Genesys 10S UV–Vis spectrophotometer after 2 h in the dark, at room temperature. The total phenolics content was expressed as milligrams of Gallic acid equivalents per 100 g dry weight sample (mg GAE/ 100 g DW), using a standard curve generated to obtain readings with Gallic acid.

3.4.5 Determination of appropriate wavelength for HPLC analysis of betulin and betulinic acid

The absorbances of BN and BA standards were determined using spectrophotometer over its wavelength. The wavelength ranged from 190 to 500 nm and scanned at 1.0 nm interval. The observed maximum absorbances with the corresponding wavelength for BN and BA standards were used to develop analysis protocol on the HPLC. The maximum absorbance was observed at λ 200, 251, and 252 nm. Hence, we ran the standards on HPLC at the observed wavelength λ 200, 251, and 252 nm with maximum absorbance. We observed that both BN and BA were not detectable at λ 251 and 252 nm, but were detectable at λ 200 nm. Thus, λ 200 nm was used to develop the UV-HPLC method.

3.4.6 Quantification of betulin and betulinic acid using HPLC

An HPLC method was developed for identification and quantification of BN and BA in the chaga extracts. HPLC (Agilent Technologies) equipped with an Agilent C18 reversed-phase column (250mm×4.6mmi.d.) and a UV detector was employed for the analysis. An aliquot (20 μL) of each extract was injected into the HPLC and elution was carried out using isocratic mobile

phase (acetonitrile: water 86: 14), at a flow-rate of 1mL/min. The mobile phase was maintained

at 25°C while the UV detector was set at λ=200 nm for a total retention time of 10 mins. Before

injection, each extract was filtered with 0.22 mm Millipore filter. The BN and BA peaks were

identified by comparing the retention times of their corresponding standards. Calibration curves

for BN and BA standards were used to estimate the yield (μg/g) dry basis.

3.5 Statistical analysis

A factorial design was used for each of the experimental sets ran independently to verify

the effect of water pretreatment, temperature effect on pure BA degradation, and impact of

different water pretreatment methods on the BA yield using JMP® 13.0 (Statistical Analysis

Systems, SAS Institute Inc., Cary, NC, USA). Leverage Plot reports the effect of each factor on

the response variable and gives insight on possible multicollinearity observations. The mean

comparison of BA yield at selected water pretreatment method and the significant differences

(confidence level of 95%) between the no water pretreatment and water pretreatment methods

were further analyzed with ANOVA, Student's t-test, and Tukey's studentized range test.

Chapter 4

Results and Discussion

Extraction of valuable phytochemicals is an important component of biorefining. The high value of these chemicals and their potential to act as anticancer agents is a focus of considerable research. The potential of poplar bark phytochemicals as Value-Added co-products from the wood and cellulosic ethanol industry has been reported based on earlier work by Devappa et al. (2015). A number of previous reports have indicated the accumulation of BA in the fungi chaga that develops in the trunk of the Birch tree (Hyun et al., 2006). These studies have mentioned high level of BA did not quantify the amount of BA present in the chaga. However, chaga is sold in the market as a form of health tea, without any details on the amount of such compounds to be expected in the product. This encouraged us to study the extraction of BA from the fungi chaga that grow on the bark of the poplar tree.

Based on this background we decided to carry out detailed studies on the extraction of BA from chaga obtained from poplar bark. Initial experiments indicated that the source of the chaga and the extraction conditions, solvents, etc. would determine the amount of BA that can be extracted. In the following sections, we report the optimization of the conditions of extraction and demonstrate the efficacy of the extract on inhibition of growth of cancer cells lines and reduction in the amount of ROS that may be present in the cells.

4.1 Effect of temperature on betulinic acid extraction with ethanol

Initial studies indicated that there was a loss of BA when the temperature of the reactant mixture was raised. Before going into optimization of a number of other parameters, it was decided to determine the effect of temperature on pure BA which we obtained from a renowned chemical vendor. These experiments were carried out with two solvents, methanol (100%) and ethanol

(95%), which are commonly used in the extraction of such terpenoid compounds. The results of these experiments are given in Table 4.1.

Table 4.1: Effect of temperature on a 97 % solution of pure betulinic acid

Solvents	MeOH			95% EtOH		
	% loss			% loss		
Temperature	2h	4h	6h	2h	4h	6h
40℃	0	0	0	0	0	0
50℃	0	0	0	0	0	1.22
60℃	0	27	39.19	0	0	3.98
70℃	0	44	46.78	0	0	4.63
80℃	0	49	48.74	0	0	10.0

The results of these experiments show that there was no loss of BA up to a temperature of 50^0C. At higher temperatures (60^0C and above) there was higher loss of BA in methanol, when maintained at these temperatures for 4 hours. Loss of betulinic acid in ethanol was lower and high loss (nearly 50%) occurred only at 80^0C for 6 hours in MeOH. Based on these results we decided to carry out extractions at 50^0C or at room temperature with all the solvents which we subsequently accessed.

4.2 Need for pre-treatment before solvent extraction

We obtained chaga from four different sources for our initial experiments; the source of these chaga samples was provided in Materials and Methods section. Initial experiments with these samples, to our surprise, resulted in very low levels of BA. A study of literature about some available chaga products provided some useful insights (Emergent health, 2017). These reports indicated that the cell walls of chaga contain chitin, which is indigestible, so many of its components cannot be reached without heat, or treatment with the enzyme chitinase. Another report indicated that alcohol extraction does not break the chitin either. A pre-treatment step before

the extraction was associated with the discharge of phenolic and monosaccharide substances. It was found that the presence of admixture of phenolic compounds in birch outer bark extractives makes it difficult to get triterpene (Rizhikovs et al., 2015). This was in line with the results we had obtained. We thus concluded that pre-treatment was required, before treating the chaga samples with solvents.

4.2.1 Effect of water pre-treatment at room temperature

We decided to initially treat the four ground samples of chaga at room temperature for 4 days with water. Therefore, stirring with no heat was done to these samples. We wanted to minimize any energy requirement if possible. We hoped that the ground chaga exposed to water treatment for days will increase the accessibility of the solvents to the inherent triterpenes without breaking down the chitin. The centrifuged chaga samples were subsequently treated with 95% ethanol for 3 hours in a reflux system which condensed the evaporating ethanol back into the system. A comparison of the BN and BA obtained by pre-treatment with water at 4 days to samples without the pre-treatment is shown in Figure 4.1.

As observed from these results, there was a considerable increase in the extraction of BN and BA with pre-treatment with all 4 sources of chaga. The locally harvested chaga sample had much higher levels of these compounds compared to the other three commercial samples. The chaga sample bought locally had no BN, but contained a high level of BA. These results indicate that there was certainly a need for pre-treatment so that the solvents could have access to the BN and BA in the samples.
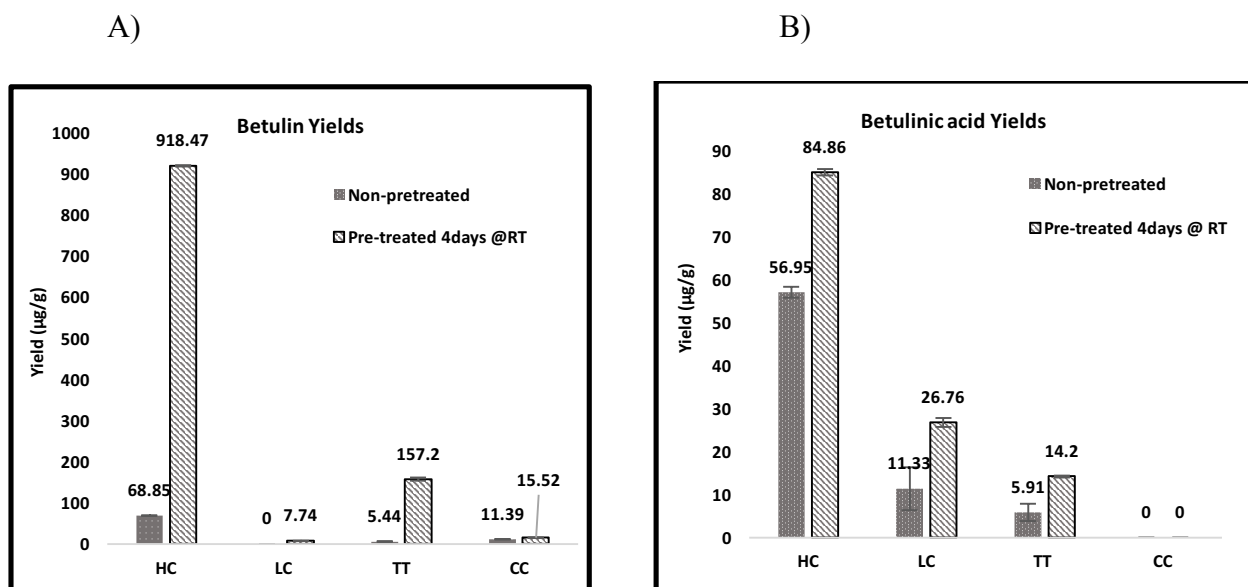
Figure 4.1: Effect of water treatment on betulin (A) and betulinic acid (B) extraction from four different sources of chaga. Pre-treatment for 4 days at room temperature (RT) followed by extraction using 95% EtOH at 50^{0}C. (HC – locally Harvested chaga, LC – Local chaga, TT – Tao tea chaga, CC- chaga.ca)

A closer look at the data showed some interesting findings. The highest levels of both BN and BA was found to be extracted from the samples which we have harvested from the local forests. The levels obtained from the local commercial source (LC) had very low levels of BN, while the samples sources from Tao tea (TT) had reasonable quantities of both BN and BA. At this point we felt that there was a chance to speed up the extraction process from 4 days to a shorter period by heating during the water pre-treatment process.

Figures 4.2 (a-c) show the effects leverage plots of BN yield from chaga based on water pretreatment and no water pretreatment. The plot of actual versus predicted values, which shows the observed data of BN yield against the predicted data of the yield is shown in Figure 4.2a. The figure shows the leverage plot for the whole model with regression coefficient $R^2 = 0.65$, and root mean square error (RMSE) = 210.07 ($p < 0.05$). The effect leverage plot, Figure 4.2b shows the

effect of no pretreatment to be significant ($p < 0.05$) for the extraction of BN from chaga. The effect of water pretreatment was found to be more significant ($p < 0.05$) than no water pretreatment (Figure 4.2c). As observed in Figure 4.1, the statistical analysis confirmed the significance of water pretreatment before solvent extraction on the yield of BN. Further statistical analyzes were carried out using the different comparison of means as shown in Tables 4.3 (a-d). X1 was observed to be significantly ($p < 0.05$) different from X2 (Table 4.2a) from the ANOVA. Student's t-test showed that X1 (L1) was significantly ($p < 0.05$) different from X1(L2) (Table 4.2b). X2(L1) was observed to be significantly ($p < 0.05$) different from X2(L2), X2(L3), and X2(L4) using Student's t-test approach. A slight difference was found using Tukey's HSD test (Figure 4.2d). It was observed that similarity in BN yield X2(L1) and X2(L3) exist. The output confirms our findings in Figure 4.1. The same statistical tests were carried out on BA yield data. Similar trends as BN findings were observed for BA data analyzed as shown in Figure 4.3 (a-c) and Tables (a-d).
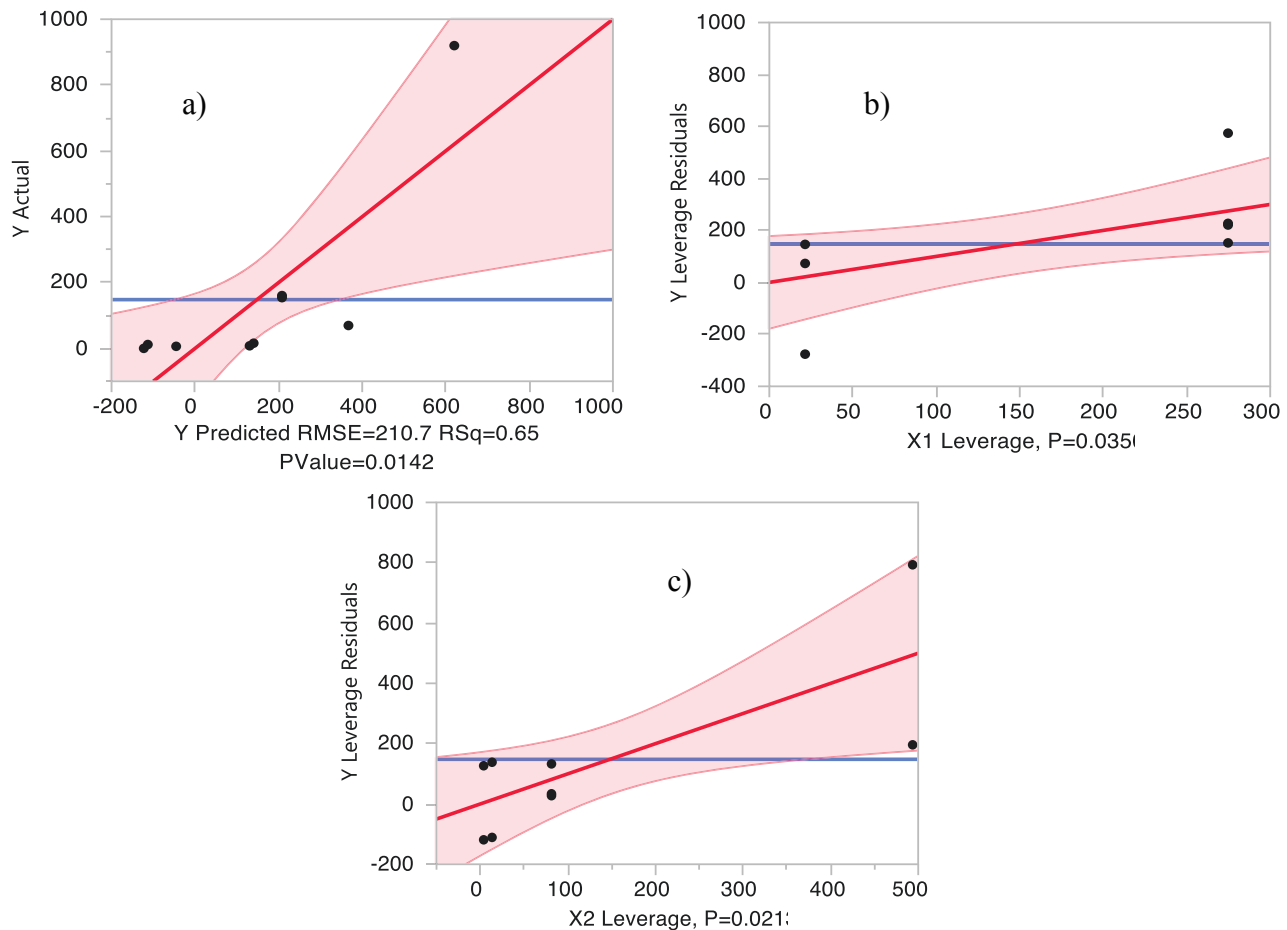
Figure 4.2: Effects leverage plots of betulin yield based on (a) Betulin yield prediction (b) No water pretreatment (c) Water pretreatment.

Table 4.2: Comparison of means of water pretreatment and no water pretreatment and different chaga samples on betulin yield using (a) ANOVA (b) LSMeans Differences Student's t (X1) (c) LSMeans Differences Student's t (X2) (d) LSMeans Differences Tukey HSD (X2). X1(L1) – No pre-treatment; X1(L2) – 4 days pre-treatment; X2(L1) – HC; X2(L2) – LC; X2(L3) – TT; X2(L4) – CC

a)

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
X1	1	1	256670.59	5.7818	0.0350*
X2	3	3	651209.60	4.8898	0.0213*

b)

Level		Least Sq Mean
L2	A	274.73334
L1	B	21.42000

c)

Level			Least Sq Mean
L1	A		493.66000
L3		B	81.32000
L4		B	13.45677
L2		B	3.86990

d)

Level			Least Sq Mean
L1	A		493.66000
L3	A	B	81.32000
L4		B	13.45677
L2		B	3.86990

*Levels not connected by same letter are significantly different

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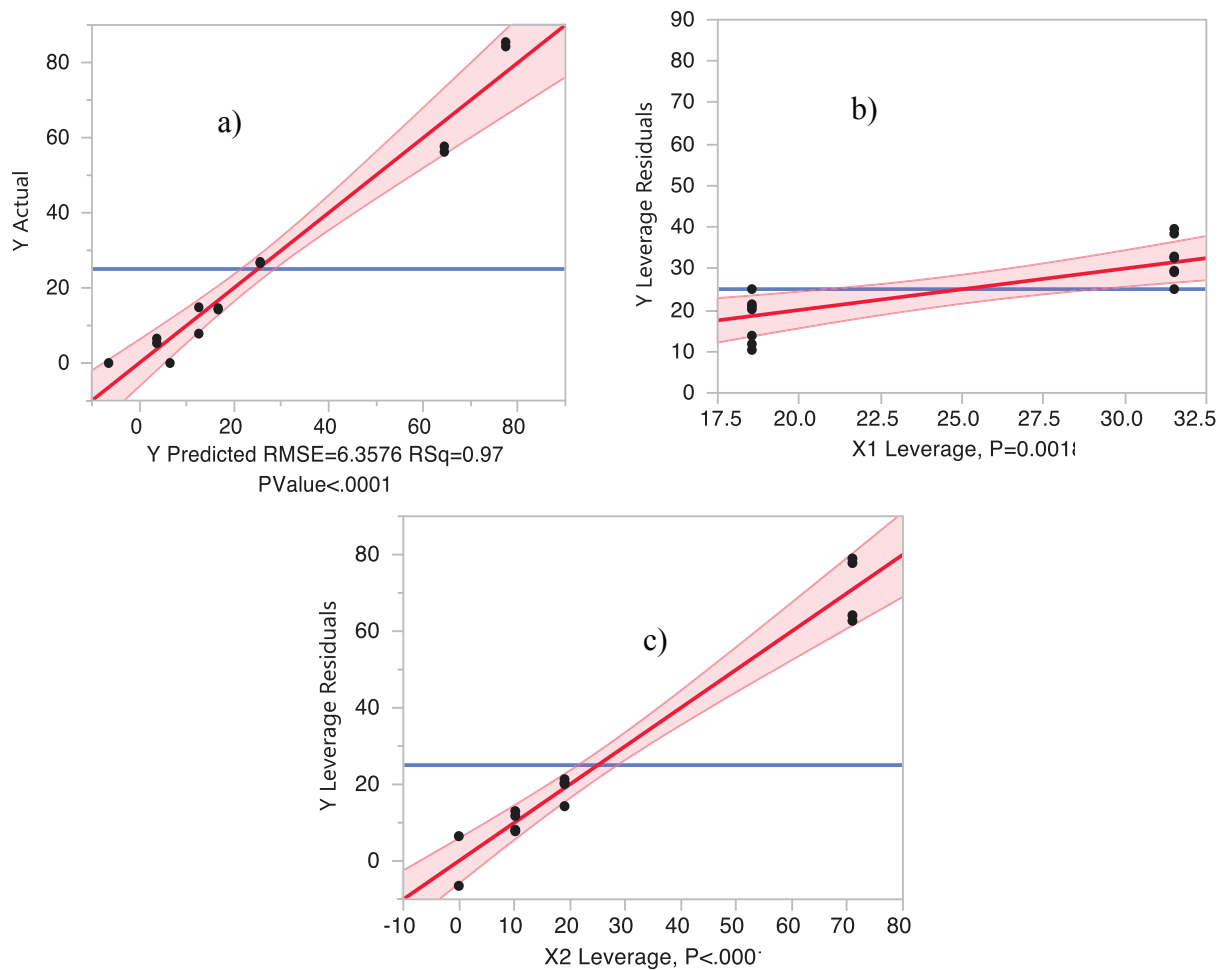
Figure 4.3: Effects leverage plots of betulinic acid yield based on (a) Betulinic acid yield prediction (b) No water pretreatment (c) Water pretreatment.

Table 4.3: Comparison of means of water pretreatment and no water pretreatment and different chaga samples on betulinic acid yield using (a) ANOVA (b) LSMeans Differences Student's t (X1) (c) LSMeans Differences Student's t (X2) (d) LSMeans Differences Tukey HSD (X2). X1(L1) – No pre-treatment; X1(L2) – 4 days pre-treatment; X2(L1) – HC; X2(L2) – LC; X2(L3) – TT; X2(L4) – CC

a)

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
X1	1	1	672.495	16.6382	0.0018*
X2	3	3	11949.885	98.5508	<.0001*

b)

Level		Least Sq Mean
L2	A	31.512500
L1	B	18.546250

*Levels not connected by same letter are significantly different

c)

Level			Least Sq Mean
L1	A		70.902500
L2		B	19.047500
L3		B	10.167500
L4		C	0.000000

d)

Level				Least Sq Mean
L1	A			70.902500
L2		B		19.047500
L3		B	C	10.167500
L4			C	0.000000

4.2.2 Effect of pre-treatment by refluxing at the boiling point of water

In a subsequent set of experiments, all the four samples were pre-treated by mixing the samples in water at 100^0C for 2 and 5 hours. This was compared to the results obtained in the 4-day experiments (Fig. 4.4). The results of these experiments indicated that the pre-treatment for 4 days at room temperature was in most cases better than the shorter treatments times at the boiling

A)   B)
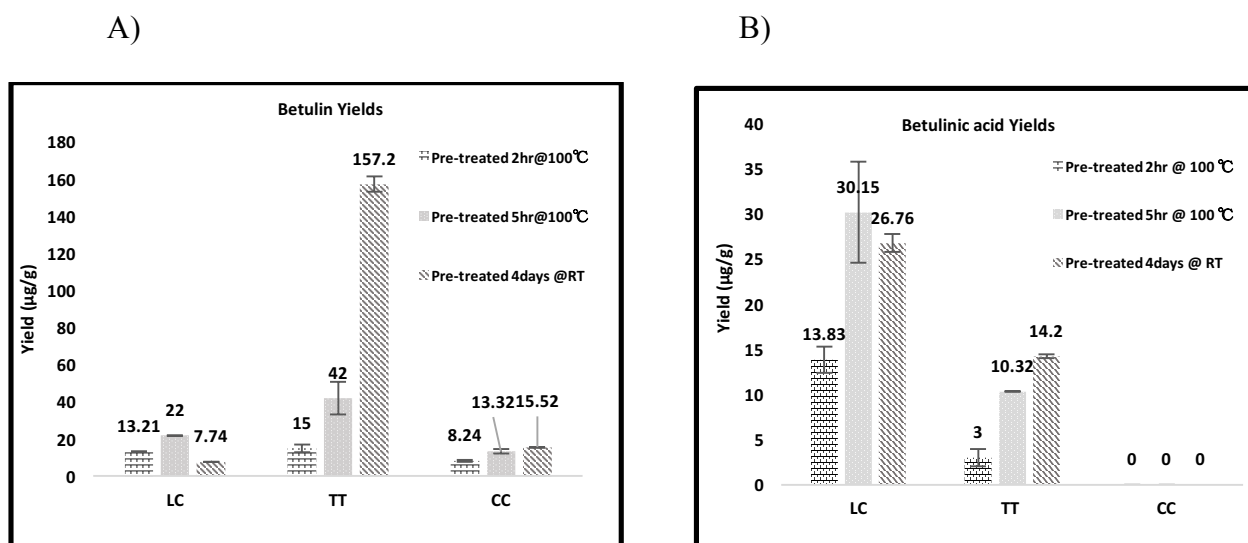
Figure 4.4: Effect of the pre-treatment duration on betulin (A) and betulinic acid (B) extraction from four different sources of chaga using 95% ethanol after pre-treatment in water at 100^0C for 2 and 5 hours and at room temperature for 4 days.

point of water. The only exception was in the case of the BA of the chaga samples obtained from the local commercial source, where the amounts obtained with 5 h were nearly the same.

The results of these experiments also showed large substantial quantities of BN along with BA in most samples. It is known that BN is a precursor to the production of BA and as a triterpene has many characteristics similar BA (Laszczyk, 2009). The total quantities of BN and BA were found to be higher in the samples obtained from Tao tea (TT). Based on these outcomes, it was decided to carry out all subsequent optimization experiments with TT chaga which would be

pretreated at room temperature for 4 days. Moreover, it was also decided to measure the concentration of BN and BA in all samples.

4.3 Effect of solvents on extraction of betulin and betulinic acid

The chaga fungus is made up of an insoluble biopolymer like apple peel which is made of polyphenol esters of long-chain hydroxy-fatty acids, structurally merged with waxy materials, free fatty acids, phenols and small amounts of pectin, chitin cellulose and other compounds. (Huelin and Gallop, 1951). This chemically diverse complex protects the fruit from water loss, insects and other environmental threats. (Kolattukudy, 1984; Ju & Bramlage, 1999). The poor solubility of the triterpenes presents in chaga in environmentally friendly solvents, including alcohol and low-carbon-chain esters is a problem. The inherent ability of each solvent to penetrate the highly lipophilic matrix of such substances is important. The co-extraction of varying amounts of other compounds drastically diminishes the extract's solubility in either organic or aqueous solvents (Jäger et al, 2007) and provides an intractable consistency to the extracts. (Huelin, 1959). Hence this study assesses the extraction of BN and BA from chaga using both polar and non-polar solvents.

Solvents were chosen after considering literature data (Rizhikovs et al., 2015) that suggested the use of polar solvents (95% EtOH, MeOH and Acetone) and non-polar solvent (Choloroform, $CHCl_3$, hexanol and Ethyl Acetate EtOAc). A comparison of the results obtained with different extractants are shown in Fig. 4.5. These experiments were carried out only with Tao tea chaga which had been pretreated for 4 days in water at room temperature. The ground samples were refluxed in different solvents for three hours at 50^0C.

The results showed that BN and BA yields are strongly dependent on the solvents polarities. 95% ethanol extracted the highest concentrations for BN and BA. This was followed by methanol a lower molecular weight alcohol. BN and BA were found to have lower solubility in

non-polar solvents as indicated in numerous literature (Rizhikovs et al., 2015; Zhao et al.,2007; Cao et al., 2007; Cheng et al, 2011). Ethyl acetate can selectively extract BA; as no BN was found in its extract. Chloroform was able to extract both BN and BA but at lower than the polar solvents. As ethanol gave us the highest amount of BN and BA yields it was chosen for further experimentation. As ethanol has been used in earlier experiments during the pre-treatment experiments, no further optimization of those conditions was needed.

A)
B)
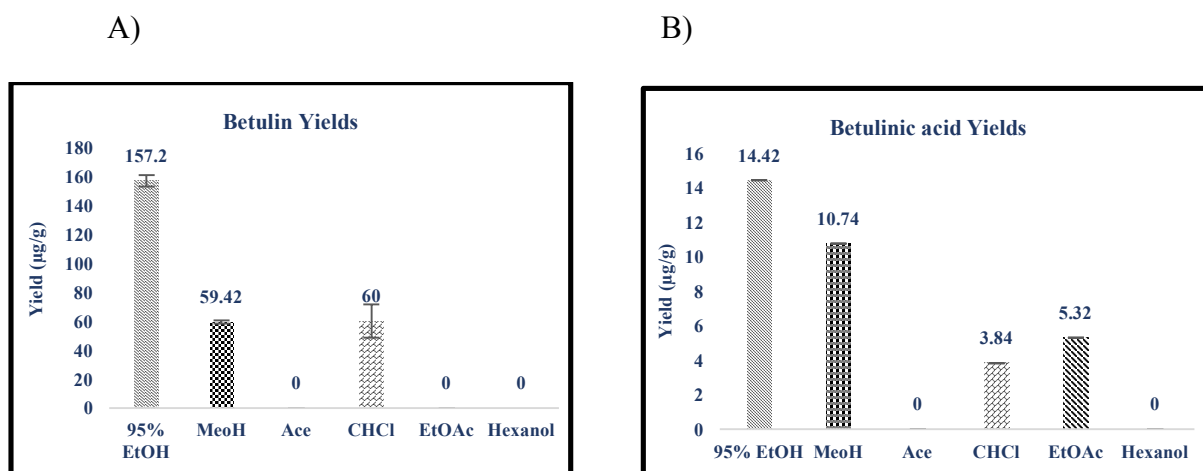
Figure 4.5: Extraction of betulin (A) and betulinic acid (B) from Tao tea chaga on refluxing in polar and polar solvents for 3 hours at 50^0C after pre-treatment in water at room temperature for 4 days.

4.4 Use of ultrasonication for enhanced extraction of betulin and betulinic acid

Cassava roots need to be crushed to release starch it contains. This is known as rasping and is used in many biorefining pre-treatment processes. The starch release, however, is incomplete due to the association of starch within the fiber. Thus, there are ample opportunities to enhance the recovery of starch from cassava chips. The reduction in particle size and opening of cassava's fibrous structures can essentially increase starch yields. One such option which we successfully demonstrated in our labs was the use of ultrasound technology (Nitayavardhana et al, 2008).

Ultrasonic pretreatment of cassava chip slurry resulted in nearly 40-fold reduction in particle size. Chemat et al (2017) presented a good review on current knowledge on ultrasound-assisted extraction in food ingredients and products, nutraceutics, cosmetic, pharmaceutical and bioenergy applications. They provided details about the important parameters influencing its extraction by ultrasound performance. The main benefits are decrease of extraction and processing time, the amount of energy and solvents used and increased yields. We thus decided to investigate the possibility of using this method for extraction of BN and BA from chaga.

4.4.1 Ultrasonication without solvent treatment for betulin and betulinic acid extraction

It was initially reasoned that ultrasonication could reduce the pre-treatment requirement and possibly the need for solvent extraction. Hence initial ultrasonication experiments were done with water pre-treatment for 2 hours and 4 days as optimized earlier. These experiments were done with Tao tea chaga which had given the highest results earlier. Ultrasonication was done at an amplitude of 70% for 20 mins. This was compared to the results obtained by water pre-treatment for 4 days followed by solvent reflux extraction with 95% ethanol. The results of these experiments are given in Table 4.4.

Table 4.4: Comparison between solvent reflux extraction and ultrasonication extraction on betulin and betulinic acid extraction from Tao tea chaga (Ultrasonication for 20 mins at 70 amplitude)

Conditions of samples	BN Yield (µg/g)	BA Yield (µg/g)
Pretreatment & ultrasound extraction	1456.13	72.39
Pretreatment &reflux extraction	157.2	14.2

The results indicated that nearly a 10-fold increase in BN extraction and 5 fold increase in BA extraction occurred on ultrasonication after pre-treatment. This substantial increase in yields was possible even with any solvent treatment. We expected ultrasonic cavitation to assist in the disintegration of chaga cells and to increases its pore sizes that would in would in turn provide organic solvent access and improve adequate contact between the extraction solvent and the desired substance (Pinilla et al., 2014). Hence, we decided to carry out experiments in which the samples would be refluxed in ethanol after pre-treatment and ultrasonication.

4.4.2 Pre-treatment, ultrasonication and solvent extraction of Tao chaga

With the dramatic increase in BN and BA yields with only pre-treatment and ultrasonication we decided to follow this with solvent extraction as well. The following experiment was done using the Tao tea chaga sample and pre-treatment and sonication was followed by refluxing with 95% ethanol. The result reported in Table 4.5 shows that there was a slight increase in yields of BA and BN.

Table 4.5: Comparison of betulin and betulinic acid yields between pre-treatment / ultrasonication and pretreatment / ultrasonication followed by solvent treatment using 95% Ethanol at 50^0C from Tao tea chaga (Ultrasonication for 20 mins at 70 amplitude)

Conditions of samples	BN Yield (µg/g)	BA Yield (µg/g)
Pretreatment / Ultrasound extraction	1456.13	72.39
Pretreatment / Ultrasound followed by 95%EtOH reflux extraction	1490.44	103.42

The results show that ultrasound had played an active role in extracting BN and BA because it can force cell walls to open and promotes better mass and heat transfer. This in turn helps to successfully extract the desired phytochemicals in a short time with high efficiency. The small increase in yields on addition of the solvent extraction step after ultrasonication indicated that most of these compounds must have been extracted out of the biomass. The results obtained indicate the maximum amount of extractable BN in the biomass.

While the high yields of these compounds are very satisfying, it should be noted that the appearance and composition of the chaga rely on several factors such as soil, tree, location and condition. Hassegawa et al. (2016) studied the potential of total phenol, major triterpene and phytosterol found in chaga products from yellow birch (Betula alleghaniensis), evaluating the the wood and bark content and relationship to species' characteristics. The results they obtained showed that most of the studied chemical compounds were related to tree health. The wood of young trees presented the highest phenol level as compared adult trees. The bark of dying trees presented the lowest phenol content, but they also had the highest BN content (6.6 ± 4.2 mg g-1). Fungal fruiting that occur on the tree stem are related to lower values for the BN, lupeol and lupenone. Hassegawa et al. (2016) concluded that the remaining unexplained variation may be attributed to stand-level conditions. In a similar manner, our own results with the different chaga samples indicated that it may be difficult to find correlations between the age of the chaga, the health of the tree on which the chaga existed, the climatic zone of the trees, seasonal variations and many other factors. Under these conditions the consumption of chaga tea extracts and tinctures will have different health benefits depending on where and how they were harvested. This highlights the need to extract compounds and fortify products with known quantities of the compounds, rather than having samples with wide variation of the phytochemicals. Ongoing

research on the synthesis of such compounds further clarifies the need to quantify the amount consumed.

4.5 Studies on biological activity of betulinic acid in cancer cell line

We next decided to demonstrate the bioactivity of the extracted material by demonstrating its activity in MCF-7 cells. We did realize that along with BA and BN there could be a host of other compounds in the extracted solution; hence, we compared the activity of commercial pure BA and our extract on MCF-7 cells.

4.5.1 Effect of bioactive compounds MCF-7 cells viability as determined by the MTT assay

The bioactive compounds were expected to reduce cell viability and growth. Experiments were initially preformed with pure BA in the range of 0.005mg/ml to 1 mg/ml BA. A control was run with no addition of any phytochemical. The results (Figure 4.6) show that there was a large inhibition of cell growth with an increase in BA concentration. At a concentration of 1 mg/ml BA, there was only 16% viable of cells. The IC_{50} values, the concentration at which there was a 50% inhibition of cell growth was found to be about 0.01 mg/ml.

We then carried a similar set of experiments with the extracts obtained from chaga with BA concentration of 0.005 and 0.01mg/ml BA. The results of these experiments are shown in Figure 4.7. The results of these experiments showed that the extracts had a much higher cytotoxicity than the pure BA samples. As the concentrations of the BA were maintained to be equal, the additional cytotoxicity effect could be a result of other compounds present in the mixture. The large difference in the inhibition with the extracts indicated that chaga extract contained large quantities of other compounds.
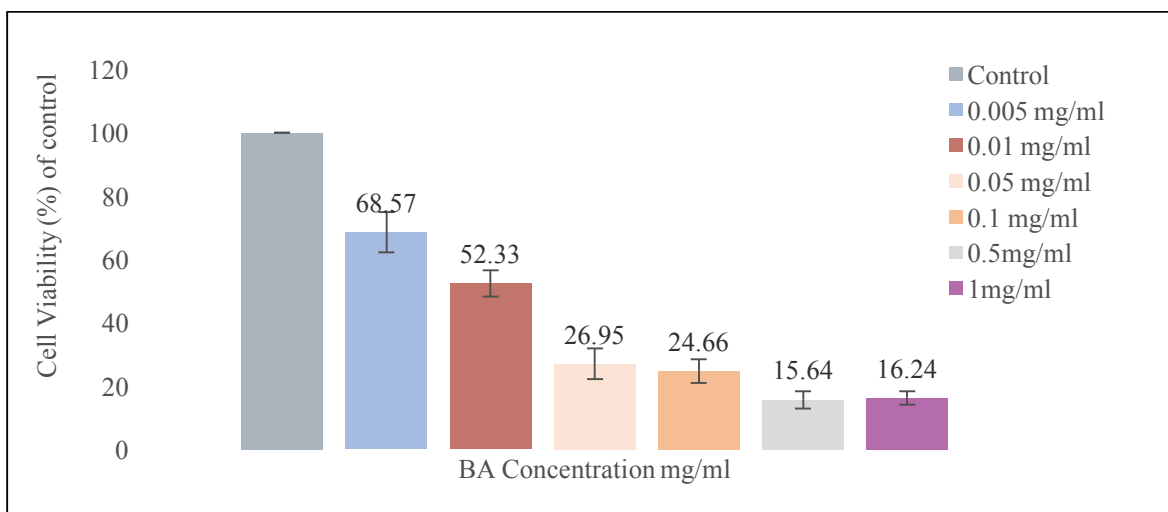
Figure 4.6: Effect of betulinic acid on cells proliferation in MCF-7 cancer cell using the MTT assay.
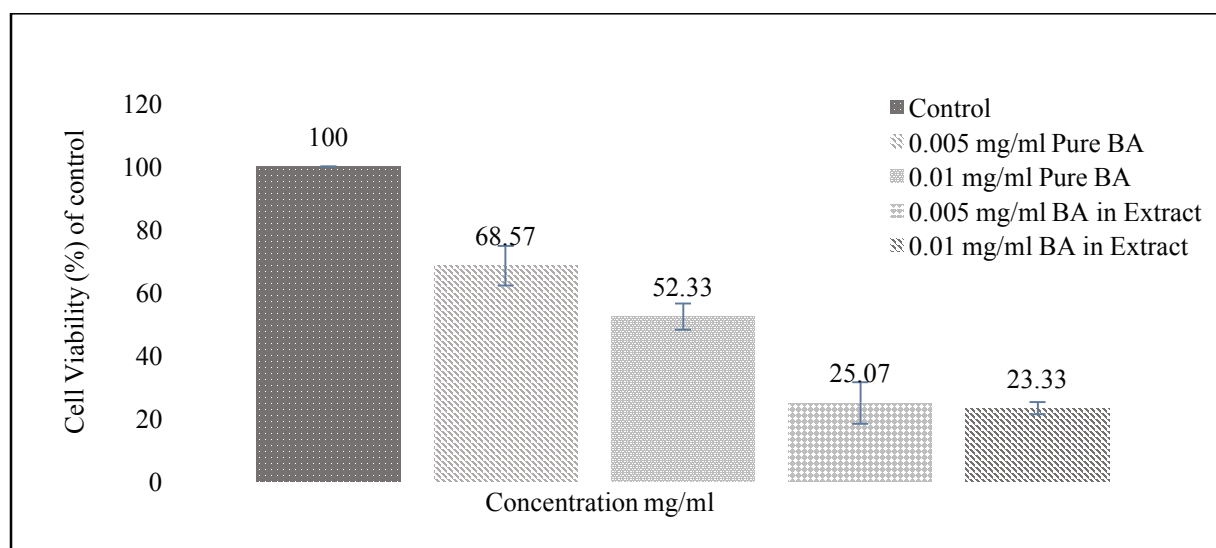
Figure 4.7: Comparison of the effect of pure betulinic acid and extracts from chaga with betulinic acid.

4.5.2 Effect of betulinic acid on reactive oxygen species as determined by CM-H2DCFDA assay.

ROS play a critical role in various cellular function. ROS are implicated in cell proliferation, signaling pathways, oxidative defense mechanisms responsible for killing microorganisms. We chose a fluorescence based method for detection of intracellular ROS in

MCF-7 cells after the cells were exposed to BA and hydrogen peroxide. The cell-permeant 2′, 7′-dichlorodihydrofluorescein diacetate (H$_2$DCF-DA) fluorescent probe is commonly used to detect ROS in cells. It also reacts with several ROS including hydrogen peroxide. The non-fluorescent H2DCFDA passively diffuses within cells, which endogenous cleaved by cellular esterase, and oxides by ROS, which then converted to the highly fluorescent 2′, 7′-dichlorofluorescein (DCF) (Queiroz et al., 2015). We used the CM-H$_2$DCFDA procedure to detect ROS production in MCF-7 cells treated with commercial BA for 24h and measured by flow cytometry. MCF-7 cells treated with BA (0.005 and 0.01 mg/ml) for 24 h decreased ROS levels compared with the control (Fig. 4.6A). We also investigated the effect of BA (0.005 mg/ml and 0.01 mg/ml) in the presence of 1 mM H$_2$O$_2$ for 4 h. The results of these experiments are shown in Figure 4.8.

The results of these experiments were normalized to make the comparison easier. A 30% decrease in fluorescence was obtained with 0.01 mg/ml BA. In the experiment involving the addition of H$_2$O$_2$, the initial control was set to one with H$_2$O$_2$ treated cells with no BA added. In the H$_2$O$_2$ control experiment, a significant reduction in the fluorescence was obtained, with 0.01 mg/ml BA, there was a 90% reduction in fluorescence with 0.01 mg/ml BA.

These experiments demonstrate that BA has a considerable effect on the viability of cancer cells lines and reduces oxidative stress in the cells. It also suggests that the extracts obtained from chaga have many phytochemicals besides BA and BN which act together in producing large effects on the cancer cells.
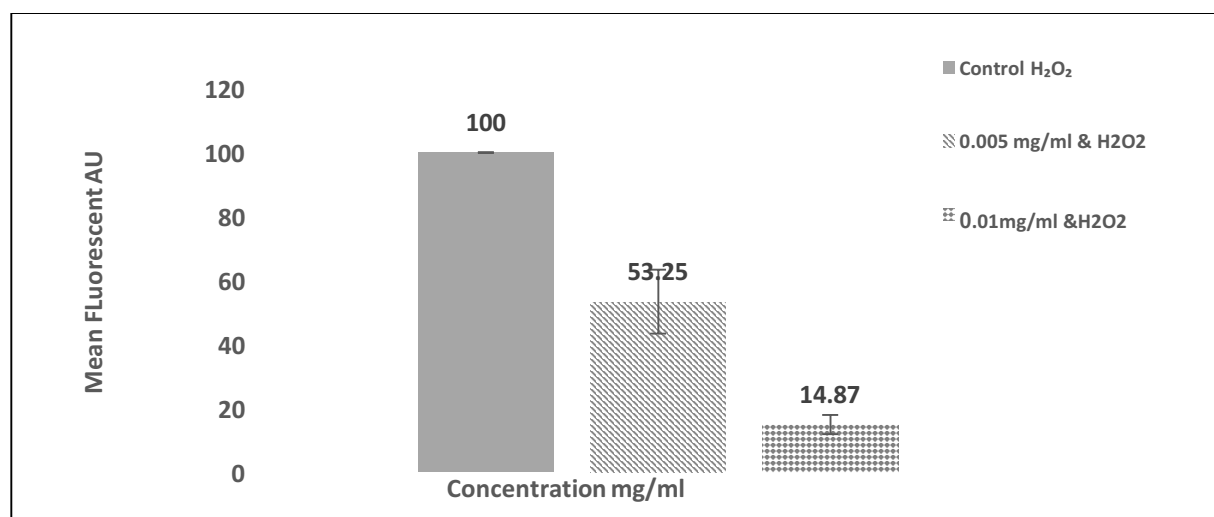
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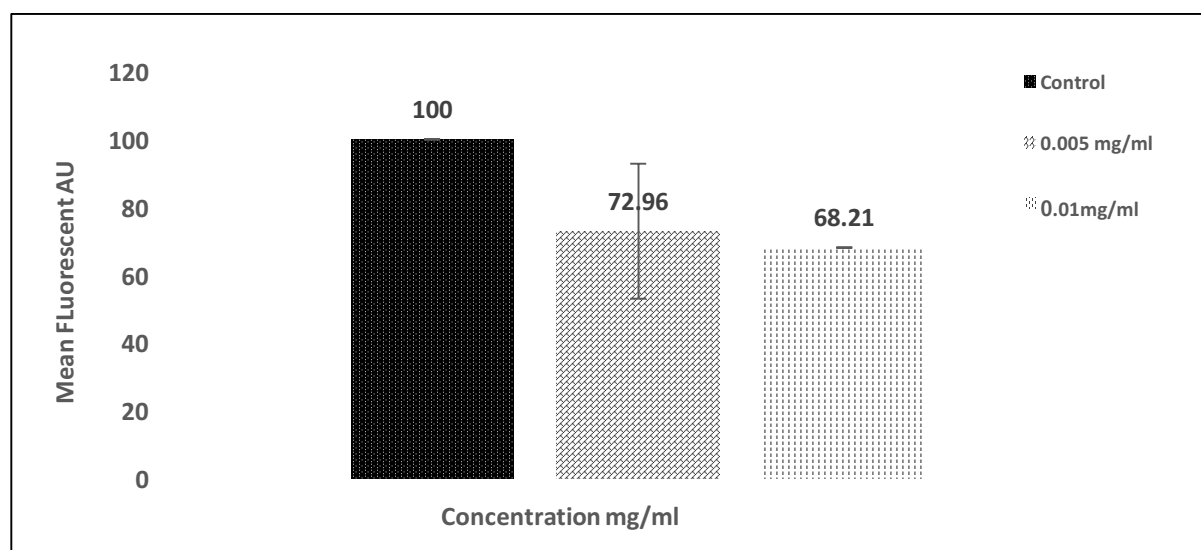
B

Figure 4.8: Effect of betulinic acid on oxidative stress. A) Treated MCF-7 cells with betulinic acid (0.005 and 0.01 mg/ml) for 24 h in the absence of 1 mM H_2O_2. B) MCF-7 cells were treated with BA (0.005 and 0.01 mg/ml) in the presence of 1 mM H_2O_2 for 4 h. ROS was determined by the CM-H_2DCFDA assay and results were expressed as mean fluorescence arbitrary units (AU)

4.6 Determination of anti-oxidant as total phenolic acid content in chaga extracts

From the high cytotoxicity of chaga extracts compared to pure BA sample in MCF-7 cells, it was clear that the extracts were a rich source of other beneficial phytochemicals. We decided to determine the anti-oxidant content of the extracts as these have potential for reducing oxidative stress and subsequently reduce viability of these cell lines.

Total phenolic content was estimated using the Folin-Ciocalteu assay. The amount of total phenolics in plant materials were wide-ranging from 0.2 to 155.3 mg Gallic Acid Equivalent (GAE)/g dry material (Kähkönen et al., 1999). Since ultrasound extraction gave us the highest yield of BN and BA, we estimated the total phenolic acid from samples that underwent pre-treatment and ultrasound. In a second experiment, pre-treatment and ultrasonication were followed by reflux extraction with 95% ethanol, and we found that the total phenolic acids obtained from ultrasounds followed by reflux extraction was 201 mg of GAE /100 dry of dry sample and that without the reflux extraction to have 98.4 mg of GAE / 100 dry of dry sample. These values indicate the high anti-oxidant content of the extract compared to other sources of such compounds. The high cytotoxicity of the bioactive extract from chaga can be attributed to the phytochemical substances.

Chapter 5
Conclusion and Future work

5.1 Conclusion

It was clear that water pre-treatment for four days at room temperature before organic extraction is essential for BN and BA extraction. Solvent extraction with different samples from different sources indicated that there can be large variance in the amount of the phytochemicals, depending on the source. The mushroom chaga provides protection from external factors including parasites. However, the amount of phytochemicals present depends on a number of varying factors such as climate, age of the tree, height from the soil, etc. But once extracted, chaga can have therapeutic use.

Ultrasonication was found to dramatically increase the extraction of BN and BA from the chaga samples. Subsequent solvent extraction brought about further yields. The highest yields of BN and BA obtained were found to be 1490 mg/g and 103 mg/g respectively.

It was demonstrated BN and BA have cytotoxic effects on MCF-7 cell lines both in terms of cell viability and oxidative stress. However, the total extract was found to have a much higher level of inhibition. This indicated that the extract had large quantities of many other bioactive compounds. Analysis of the total phenolic content, a measure of the anti-oxidant content of the extract, indicated that it had 201mg of GAE / 100g of dry sample of total phenolics. This is higher than most other sources of phytochemicals.

The novelty of this investigation showed the quantified amount of BA in chaga. This research has demonstrated that BA has inhibitory effect on cell proliferation and may potentially be used alongside conventional therapies. Additional research is required before chaga can be used for alleviating and curing some of the world's deadliest diseases such as HIV, malaria and a variety of cancers. Currently, BA is under clinical investigation for use as bioavailability treatment for cancer.

5.2 Suggested future studies

There are many different experiments that have not been completed due to lack of time. Listed below are some suggestions for future work with chaga extract:

- Determining the antimicrobial potential of chaga crude extract or isolated BA from chaga on microorganisms.

- Analyzing the biological extract for antioxidant activity and alpha amylase enzyme inhibition capability as a possible way to help diabetic patients.

- Comparing the bioactive activity of our extract with tinctures that are traditionally made with chaga.

- As the long-term aim, increasing the efficacy of betulinic acid by chemical modification and chemical synthesis of betulinic acid.

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