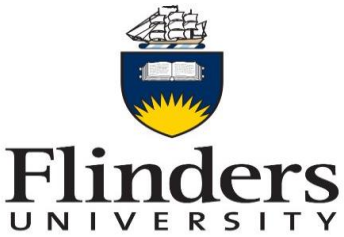
Extraction of polysaccharides from *Lentinula edodes* grown in *Astragalus membranaceus* supplemented bed and determination of cytotoxicity in colorectal carcinoma cell line HCT-116

A thesis submitted for the fulfilment of the degree of

Master of Biotechnology

by

Bunu Tamang



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Declaration

I, Bunu Tamang, declare that the work in this dissertation entitled "Extraction of polysaccharides from *Lentinula edodes* grown in *Astragalus membranaceus* supplemented bed and determination of cytotoxicity in colorectal carcinoma cell line HCT-116" is my own original research that has been carried out by me in the Department of Medical Biotechnology, Flinders University. No section of this thesis was previously published or submitted for the award of another degree or diploma or any other credentials at this or any other University. Where I have quoted other's work, I have always acknowledged by citation of the source made in text.

Bunu Tamang

11 Dec, 2020

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List of Abbreviations

Abbreviation	Explanation
%	Percentage
^0C	Degree Celsius
5-FU	5 Fluorouracil
A. membranaceus	*Astragalus membranaceus*
AMP	*Astragalus membranaceus* Polysaccharides
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
BSA	Bovine serum albumin
CO_2	Carbon dioxide
Da	Dalton
DEAE-Sephadex	Diethylaminoethyl - sephadex
DMSO	Di-methyl sulfoxide
DNA	Deoxy Ribonucleic Acid
FBS	Fetal bovine serum
FSC	Forward scatter
g	Gram
g (centrifugation)	Relative centrifugal force
HPLC	High performance liquid chromatography
hrs	Hours
Hz	Hertz
IC_{50}	50 percent inhibitory concentration
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
L. edodes	*Lentinula edodes*
LPS	*L. edodes* Polysaccharides
M	Molar
MA	Microwave assisted
MEA	Microwave-enzyme assisted

mg	Milligram
MilliQ water	Millipore qualitative water
min	Minutes
ml	Millilitre
ml/min	Millilitre per minute
mM	Millimolar
MPa	Milli Pascal
MTT	(3-(4, 5 dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide)
MUA	Microwave-ultrasound assisted
MUEA	Microwave-ultrasound-enzyme assisted
n	Number
NaCl	Sodium Chloride
nm	Nanometre
NO	Nitric Oxide
PI	Propidium Iodide
PMP	Phenyl methyl pyrazolone
RB	Retinoblastoma
rpm	Rotation per minute
Se	Selenium
SSC	Side scatter
TCM	Traditional Chinese Medicine
TNF	Tumor Necrosis Factor
UEA	Ultrasound-enzyme assisted
W	Watt
w/v	Ratio of weight to volume
w/w	Ratio of weight to weight
WHO	World Health Organization
µg/ml	Microgram per millilitre
µl	Microlitre
µl/min	Microlitre per minute

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Abstract

The chemical constituents and nutritional composition of mushrooms alter significantly when grown on different substrate conditions. With reference to this fact, Shanxi Yulongxiang Agricultural Development Co. Ltd., China, has grown Shiitake mushrooms in the substrate beds supplemented with an edible medicinal plant *Astragalus membranaceus* and expected to have some bio-active components transferred from *A. membranaceus* into the mushroom. The so grown Shiitake mushrooms in the beds supplemented with *Astragalus* under specific conditions (not mentioned here to protect IP of the company) have been named as HAS-A and HAS-B in this project.

Since one of the major bio-active components in both Shiitake and *Astragalus* are polysaccharides, the major aim of this project was to determine any changes in yield, content and anti-proliferative effects of polysaccharides extracted from HAS-A and HAS-B when compared to that of Shiitake grown under controlled conditions. Interestingly, the yield of polysaccharides in HAS-B and HAS-HAS-A rose significantly higher in every extraction method (~45% w/w and ~31%w/w more than that of controlled shiitake, respectively). However, the anti-proliferative activity was totally dependent on the methods and conditions of extraction. For instance, microwave assisted (MA) and ultrasound-enzyme assisted (UEA) extracts of HAS-B have a comparatively higher anti-proliferative activity of HCT-116 than that of controlled Shiitake when determined by MTT assay and Flow-Cytometry. But the polysaccharide extracts from HAS-B and Shiitake with other extraction methods viz. microwave-ultrasound assisted (MUA), microwave-ultrasound-enzyme assisted (MUEA) and microwave-enzyme assisted (MEA) extraction have no significant difference in inhibiting proliferation of HCT-116. Moreover, the modified UEA extraction method in this study showed significantly higher anti-proliferative activity of HCT 116 compared to those

reported in the previous literature. For example, this study showed ~ 89%, ~77% and ~ 94% inhibitory effect of HCT 116 with 1.6 mg/ml of UEA Shiitake, HAS-A and HAS-B polysaccharides, respectively while the literature reported a maximum inhibition of only ~71% with 1.8 mg/ml. Meanwhile, all the enzyme assisted methods of extraction had significantly higher purity when compared to that of non-enzymatic extractions.

The monosaccharides composition in HAS-A and HAS-B were mannose (Man), ribose (Rib), rhamnose (Rha), glucuronic acid (GlcAc), galacturonic acid (GalAc), glucose (Gluc), galactose (Gal), xylose (Xyl), arabinose (Ara) and fucose (Fuc). This composition was found similar to that of Shiitake but the amount of glucuronic acid and glucose was significantly higher in HAS-A and HAS-B when compared to Shiitake. In contrast to previous findings (which suggest maximum anti-proliferative activity with higher molecular weight polysaccharides, > 50 k Da), our findings showed the maximum cytotoxic effect of HAS-B and Shiitake against HCT 116 in the molecular weight range of ~2.5 – 832.7 k Da and ~ 2.5 – 10.5 k Da, respectively.

In conclusion, this project has demonstrated that Shiitake mushrooms grown in the beds supplemented with *A. membranaceus* produce a higher amount of polysaccharides and these polysaccharides have higher anti-proliferative activities when compared to those produced under controlled conditions. Innovative green extraction technique such as UEA extraction method can be used for the efficient, effective and economic extraction of bio-active polysaccharides from mushrooms. The optimization of the UEA extraction method and subsequent efficient purification in the future would further help to potentiate the activity of HAS-B polysaccharides, eventually leading to the development of effective complementary treatment to overcome the drawbacks of current chemotherapy.

1. Introduction

1.1 Cancer

Cancer is the second major cause of death worldwide (WHO, 2018). In 2018, the number of deaths due to cancer reached around 9.6 Million globally which corresponds to 1 in 6 deaths due to cancer only (WHO, 2018). In simple words, cancer is a disease of cells that often relates to abnormal expression of DNA (Mutch et al. 2018) and incorporates a wide range of medical conditions (Rehman 2018). These conditions are characterized by uncontrolled growth of abnormal cells that can invade into the surrounding tissues or metastasize to different organs, which often leads to death if not treated on time (Khan et al. 2019). One of the major causes of cancer is attributed to gene mutations (Griffiths et al. 2000). Whereas, other factors such as viruses (Cook et al. 2014) and socio-environmental factors like smoking, alcoholism, eating habits, stress, long exposure to chemicals are also considered as the risk factors (Blackadar 2016). The genes that are basically related to the development of cancer are oncogenes, tumor suppressor genes and mismatch repair genes (Mutch et al. 2018). The mutation or dysfunction of these genes often leads to a cancer. For example, the mutation or over expression of proto-oncogenes such as Ras, Myc or Wnt often leads to activation of oncogenes that leads to cancer (Todd & Wong 1999). Similarly, the mutation or inactivation of tumor-suppressor genes such as p53, retinoblastoma (RB), BRACA1, BRACA2 or BCL2 is also associated with cancer (Sherr 2004).

1.2 Drawbacks of current cancer treatment

Several treatment strategies have been developed so far for treating cancer, such as chemotherapy, radiotherapy, immunotherapy, hormonal therapy and surgery and these have been proved to be highly effective in certain circumstances (Calaminus et al. 2004; Chester et al. 2004; Restifo et al. 2012; James et al. 2017). However, the underlying truth about these treatments is that patients have to face severe side effects and go through extremely painful conditions (Rehman 2018). Chemotherapy has been used extensively in treating cancer to date but its severe drawbacks in patient's health and immune system remain far beyond consideration (Joseph et al. 2018; Nurgali, Jagoe & Abalo 2018). The very common disadvantage of using chemotherapy is often a non-specific treatment (Agarwal 2016). It tries to kill all the rapidly growing cells including even the healthy/normal cells such as cells in blood and bone marrow, which is the main reason for reduced immunity (Cheok 2012). Apart from this, it causes numerous other side effects like mucositis (inflammation and damage of gastro-intestinal mucosa) (Stringer et al. 2009), hypersensitivity reactions (Yoon et al. 2013) , alopecia (Crown et al. 2017), increased risk of cardiovascular disease (Altena et al. 2009), genetic polymorphism and development of resistance, etc. (Housman et al. 2014; Nurgali et al. 2018) . These evidences, undoubtedly suggest that the development of highly effective anti-cancer agents with minimal or no side effects are of utmost significance.

1.3 Natural polysaccharides interventions for the drawbacks of chemotherapy

In recent years, polysaccharides from biological origin are getting much attention in the medical field due to their strong therapeutic efficacy and effectiveness for a wide range of diseases such as cancer, cardiovascular diseases, diabetes, lungs and kidney associated diseases (Li et al. 2018). Numerous studies provide solid evidences of their efficient bioactivity against various types of

cancers. For example; polysaccharides from the mushroom *Grifola frondosa* and Mongolian milkvetch (*Astragalus membranaceus*) induced significant apoptotic activity against breast cancer cell lines via increased expression of Bax, Caspase 3 and macrophages (Zhang et al. 2017;Li et al. 2019); polysaccharides from boxthorn (*Lycium barbarum*) has shown strong anticancer effect against human colon cancer cell lines via cell cycle arrest in G0/G1 phase; similarly, anticancer effect of polysaccharides from the seaweed (*Laminaria japonica*) was investigated on mice with liver cancer, where they showed profound antitumor effect by increasing the levels of interleukin (IL-2) and tumor necrosis factor (TNF-α) (Zhu et al. 2016). In addition, natural polysaccharides have been used as adjuvant with chemotherapies in-order to potentiate their effect and reduce or counteract the adverse events of chemotherapy. When polysaccharides extracted from magnolia (*Schisandra chinensis*) were combined with 5-fluorouracil (5-FU), it enhanced anticancer effect in Heps bearing mice and reduced the side effects (immunosuppressant effect) induced by 5-fl by enhancing the release of IL-2 and TNF-α (Zhao et al. 2014). Likewise, the combination of the Ginseng polysaccharides with 5-FU not only enhanced the antitumor effect in Sarcoma-180 bearing mice, but also attenuated the side effects caused by 5-FU by enhancing the level of lymphocytes and macrophages (Ni et al. 2010). A summary of some key scientific evidences on the anti-cancer effect of natural bio-active polysaccharides are listed in the Table 1.1.

In summary, these published scientific evidences present the great potential of natural polysaccharides for treating or preventing cancer, either alone or as an adjuvant to chemotherapy with improved efficacy and reduced side effects.

Table. 1.1 List of some representative natural polysaccharides exhibiting anti-cancer properties.

Polysaccharide source	Polysaccharides	Cancer model	Mechanism for anti-tumor effect	References
Ganoderma lucidum	β – (1 -3) Glucan	HCT-116, human colorectal carcinoma cell lines	-activation of caspase-3 and caspase -8 mediated pathways	(Liang et al. 2014)
Glycyrrhiza glabra	α-(1-4) Glucan	H22- hepato-carcinoma bearing mice	-upregulation of TH1/TH2 cytokines ratio -decreased expression for Foxp3 and TGF – β levels.	(He et al. 2011)
Cordyceps sinensis	α-(1-3) Glucan, β – (1 -3) Glucan	HCT-116, human colorectal carcinoma cell lines	-upregulation of AMPKa and down-regulation of PI3K -bafilomycin induced apoptosis	(Qi et al. 2020)
Lycium barbarum	β – (1 -4) Glucan, β – (1 -6) Glucan	SW480, Caco-2 – human colon cancer cells	-cell cycle arrest at G_0/G_1 phase. -decreased level of cyclin D, cyclin E and CDK2.	(Mao et al. 2011)
Lentinus edodes	β – (1 -3) Glucan	H22- bearing mice	-increased expression for IL-2 and TNF-α -decreased ALT and AST activities -decreased expressions for VEGF	(Li et al. 2018)
Astragalus membranaceus	α-(1-4) Glucan	4T1-breast cancer cells	-activation of macrophages -cell cycle arrest at G2 phase	(Li et al. 2020)
Glycyrrhiza uralensis	α-(1-4) Glucan	CT 26 –colon carcinoma bearing mice	-enhanced level of IL-2, IL-6 and IL-7 -activation of CD4 and CD8 lymphocytes	(Ayeka et al. 2017)

1.4 General background of the Project

Many previous studies have proven the anti-tumor properties of natural bio-active polysaccharides as discussed above. Among them the Shiitake mushroom *Lentinula edodes* and the herb *Astragalus membranaceus* are very popular Traditional Chinese Medicines with well-documented immune-enhancing and anti-tumor properties (Li et al. 2019; Zhang et al. 2007). The industry collaborator of this project, Shanxi Yulongxiang Agricultural Development Co. Ltd., China, hypothesized that growing Shiitake in the substrate beds supplemented with *A. membranaceus* under specific conditions (not mentioned here to protect IP of the company) would help to transfer some bioactive components of *A. membranaceus* to Shiitake. The shiitake mushrooms grown by such cultivation approach have been named as HAS-A and HAS-B in this project which are expected to have some bio-transformed components of *Astragalus*. Since the polysaccharide components of both *Astragalus* and Shiitake have been proven to provide high anti-proliferative properties, this study has focused on determining changes in the extraction yield and anti-proliferative properties of HAS-A and HAS-B polysaccharides when compared to the polysaccharides of Shiitake grown under controlled condition.

To ensure the polysaccharide-rich extracts could be used for human consumption, this project has tested a range of green innovative extraction technologies of polysaccharides including microwave assisted (MA), microwave-ultrasound assisted (MUA), microwave-ultrasound-enzyme assisted (MUEA), microwave-enzyme assisted (MEA) and ultrasound-enzyme assisted (UEA) extraction method. The significances of these techniques are more environmentally friendly, highly effective, and economically viable for the extraction of bio-active components (Yin et al. 2018; Zhao et al. 2018).

1.5 Rationale of the study

Despite the fact that cancer is taking millions of lives yearly worldwide, the only very effective treatment extensively used so far is chemotherapy. But the severe drawbacks, side-effects and toxicities of chemotherapy to human health and immune system are very critical as discussed earlier in this chapter. As mentioned in section 1.3, many scientific evidences prove the efficacy and potency of natural polysaccharides in treating and preventing cancer with reduced side effects and toxicities of chemotherapies when natural polysaccharides were used as adjuvant therapies with chemo drugs. Thus, identifying more effective natural polysaccharides with minimal side effects to human health and immune system is of most significance where the outcomes of this project would directly or indirectly contribute to achieve this goal.

1.6 Aims, hypothesis and novelty of the project

1.6.1 Aim

The aim of this project was to test a range of green extraction methods to improve both the yield and bioactivities of polysaccharide-rich extracts from the mushroom *Lentinula edodes* (Shiitake) grown in the substrate beds supplemented with *Astragalus membranaceus* and to determine its cytotoxicity in human colorectal carcinoma (HCT 116).

1.6.2 Hypothesis

We hypothesized that the *L. edodes* (Shiitake) grown in the substrate beds supplemented with *Astragalus membranaceus* would have higher polysaccharide content and superior cancer cytotoxicity compared to those grown under normal (controlled) conditions.

1.6.3 Rationale behind the hypothesis

Many previous studies have shown that nutritional composition and biological activity of the constituents in mushroom alter significantly when grown on different substrates (Özçelik & Pekşen 2007). Economou et al. (2017) conducted a research where the spent mushroom substrate was supplied along with other nutrients in the cultivation of five different mushroom strains including *L. edodes*. As a result of such cultivation technique, it enhanced the concentration of polysaccharides and biomass of the mushrooms. Similarly, a recent research was conducted to enhance the immunomodulatory effect of Lentinan (Shiitake polysaccharides) by growing on Selenium (Se) rich supplemented substrate bed. As selenium has immune-stimulating property, the research group hypothesized that growing mushroom in Se rich environment would give them Se-enriched Lentinan with superior anti-cancer activity compared to the non Se-enriched control. As a result, Se was transformed from the substrate into the mushroom polysaccharides which provided superior anticancer effect (Kaleta et al. 2019). Another research was conducted by Siwulski et al. (2019) where they cultivated six different mushroom species (including *L. edodes*) in different substrates in order to determine their effect on growth and nutritional accumulation in mushroom. Their study revealed that the substrate's chemical composition has a direct effect on both yield and composition of nutritional elements. Thus, these previous findings support the fact that - changes in substrate medium could change the concentration, composition and bio-activity of mushroom.

Therefore, we hypothesized that if *L. edodes* (Shiitake) is grown in *A. membranaceus* supplemented substrate, there is a possibility of transforming bio-active chemical constituents including anti-cancer metabolites from *Astragalus* to Shiitake, which in turn, would further potentiate its cytotoxic effect on cancer cells.

1.6.4 Research novelty

Although the cancer cytotoxicity of *L. edodes* has been established by many researchers, no previous studies have been conducted on potentiating the cancer cytotoxicity of *L. edodes* by growing in *A. membranaceus* supplemented bed yet, which accounts for its complete novelty. In addition, the enzyme mixture used in this project for enzymatic extraction of *L. edodes* is new to the best of my knowledge.

1.7 Significance of the project to human health and biotechnology

This research can further support the discovery of anticancer agents that are more natural, safe, patient compliable and effective in treating or preventing a deadly disease, cancer. Cancer patients would have to suffer less as chemotherapy, radiotherapy and surgery could lead them to severe side effects and painful situations. Secondly, this research will also help to determine efficient methods for the extraction of polysaccharides from shiitake mushroom using green technology. Eventually it helps in developing more environmentally friendly, cost effective and sustainable methods of extraction for Shiitake polysaccharides, for future commercialization. Thirdly, further research could be conducted to isolate and purify novel polysaccharides; determine molecular mechanism of their anti-proliferative activity in cancer cells; modify their chemical structure and determine their structure activity relationship studies and formulate/develop a suitable drug delivery system for improved efficacy and effectiveness.

Finally, there are numerous other functional foods and medicinal plants in the world whose bio-activities are yet to be determined. Thus, this research would also open a new gateway to use the same cultivation approach to transform bioactive components from one source to another that would ultimately help to enhance the health benefits.

Chapter 2: Literature Review

2.1 Natural polysaccharides and their mechanism of anti-proliferative action

Polysaccharides are biopolymers formed by the glycosidic linkage of several monosaccharides. These compounds are one of the important cellular components in plants, algae, fungi and yeasts that help them to provide specific structure and physiological functions (Ji, Peng & Wang 2018). Polysaccharides are hydrophilic in nature and easily hydrolyzed with the aqueous acids into corresponding monosaccharides (van Dam, van den Broek & Boeriu 2017). Their biological functions typically depend upon their chemical structures such that most bioactive polysaccharides have α or β oriented D-glucan units linked by (1,4), (1,6) or (1, 3) glycosidic linkages (Khan et al. 2019).

Several mechanisms have been identified by which they show anti-cancer effects (Fig. 2.1) such as; i) Arresting specific stages of the cell cycle. For example, polysaccharides from *Cordyceps militaris* arrest the G0/G1 or G2/M phase of cancer cell lines (Chen et al. 2018). ii) Mitochondrial membrane depolarization: the depolarization of a mitochondrial membrane leads to release of cytochrome c into the cytoplasm (which is otherwise present in the mitochondrial intermembrane space), this in turn triggers the release of caspase 3 and 9 that induces apoptotic action (Tian et al. 2016). iii) Upregulating inducible Nitric oxide synthase (iNOS): Several reports have shown that functional polysaccharides stimulate overexpression of iNOS. Upregulation of iNOS stimulates macrophages to over-release Nitric Oxides (NO) which simultaneously shows antineoplastic activity through DNA damage, lipid peroxidation, inhibiting essential enzymes or reducing antioxidant stores (Somasundaram et al. 2017; Vannini et al. 2015). In addition, the stimulated macrophages not only trigger NO pathways but also may induce the production of cytokines such as interleukins (IL) and tumor necrosis factors (TNF) (Rong et al. 2019). iv) Immunomodulation: Immune stimulation is one of the key mechanisms by which polysaccharides provide an antitumor

effect. Numerous studies have reported for its immune stimulating activity via the production of macrophages, dendritic cells, cytokines mainly interleukins (IL-1, IL-2, IL-6, IL-10 etc.), TNF-α and Lymphocytes (T and B cells) (Bamodu et al. 2019; Bao et al. 2013; Wang, C et al. 2017).

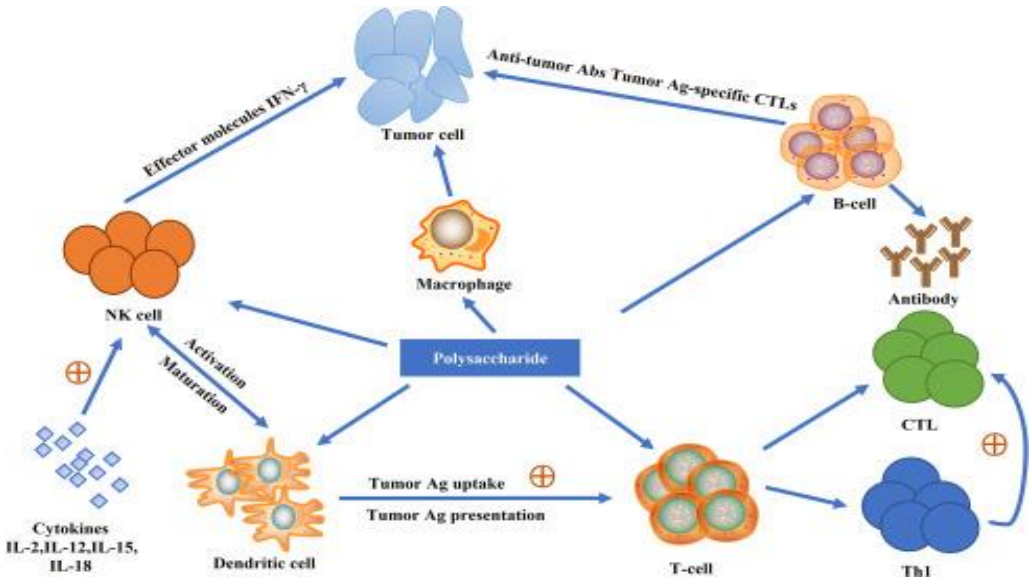


Fig. 2.1 Several mechanisms by which polysaccharide induces anti-cancer effects. Retrieved from: (Zeng et al. 2019)

2.2 *Lentinus edodes* (Shiitake) polysaccharides

Lentinus edodes commonly known as Shiitake is one of the most popular and edible mushrooms native to East Asian countries like China and Japan (Hobbs 2000). *L. edodes* has high nutritional value and also contains therapeutically active polysaccharides that stimulate immune response and therefore, has been very popular in Traditional Chinese Medicine since a long time ago (Alzorqi et al. 2017; Miyaji et al. 2006). The dried mushrooms have a high content of carbohydrates (58-60 %) followed by proteins (20-23 %), fats (3-4 %) and fibers (9-10 %) (Wasser 2005). However, these compositions may vary depending upon mushroom strains, nature of substrates and cultivation conditions (Wasser 2005). The key polysaccharide in *L. edodes* is commonly known

as Lentinan, a water soluble compound. Its chemical structure mainly consists of branched chains of β-D glucans such as β-1, 3 glucopyranoside as a main chain and β-1, 6 glucopyranoside as a branched chain as shown in Fig.2.2 (Unursaikhan et al. 2006). This β-D glucan structure of lentinan polysaccharide gives rise to their anti-cancer effect in-vitro or in-vivo (Ziaja-Sołtys et al. 2020). One study by Zhang et al. (2005) further suggests the relationship between the structural conformation of lentinan and their anti-cancer activity. According to their report, the triple-helix conformation of lentinan has a higher anti-cancer effect whereas the corresponding single flexible chain structure has minimal effect (Zhang et al. 2005).

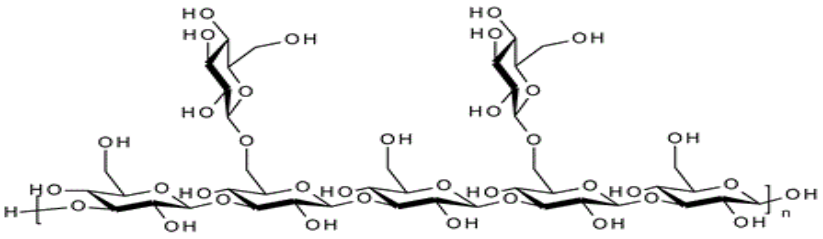


Fig. 2.2 Chemical structure of lentinan with β-1, 3 glucopyranoside as a main chain and β-1, 6 glucopyranoside as a branched chain. Retrieved from: (Zhang et al. 2018)

2.3 Anti-proliferative effects of lentinan

Chihara et al. (1969) discovered the anti-proliferative effects of polysaccharides in *L. edodes* for the first time. Based on this finding, numerous *in-vitro* and *in-vivo* studies have been conducted to determine the anticancer effect of lentinan either alone or in combination with other therapies (Table 2.1). The β glucans isolated from *L. edodes* had a significant cytotoxic effect against breast cancer cells (MDA-MB-231) mediated by activated THP-1 macrophages as shown in Fig. 2.3 (Morales et al. 2020). There is another evidence where lentinan suppressed the proliferation of colorectal carcinoma- HT-29 cells in-vitro as well as in-vivo in mice by upregulation of Bax/Bcl-

2 ratio and Caspase -3, -9 and -8 mediated apoptotic pathway (Wang et al. 2017). Similarly, Finimundy et al. (2018) reported the cytotoxic effect of lentinan in Larynx carcinoma (HEp-2) cell lines mediated by cell cycle arrest at G0/G1 phase. In addition, it's synergistic action in combination with chemotherapies has also been proved in several clinical trials (Ina et al. 2011; Oba et al. 2009). Lentinan injection was approved for its use as an adjuvant with chemotherapy in gastric cancer treatment in Japan in 1985 (Bisen et al. 2010). It has also been used as an adjuvant with paclitaxel and cisplatin (chemo drugs) that are used for treatment of gastric cancer (Akazawa et al. 2010; Hori et al. 2011). Similarly, combination therapy of lentinan with other drugs such as ok-432 (picibanil) and docetaxel has also been proven effective in the treatment of gastric cancer without serious adverse reactions (Yamasaki et al. 2009; Yoshino et al. 2010). Lentinan in combination with cisplatin against H22 and Hep G2 cell lines has significant synergistic anti-tumor action (Zhang et al. 2016). The anticancer effect was mediated as a result of Caspase – 3, -6, -7 and -8 activation, increased Bax/Bcl-2 ratio and decreased expression of Stat -3 and NF-κB. Very importantly, they also reported that the combination therapy had markedly reduced the side effects of cisplatin than when used alone.

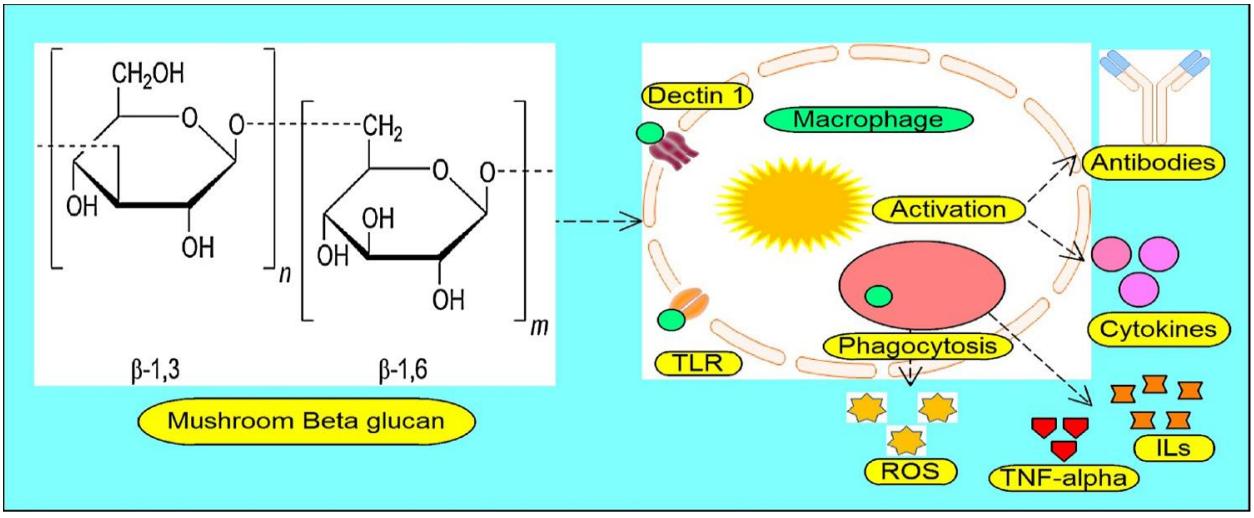


Fig. 2.3 Molecular structure of β-D glucan (Left) and mechanism of action by which the β-glucans trigger anti-proliferative action on cancer cells (right). Retrieved from: (Kothari et al. 2018)

Table 2.1 Examples of anti-cancer effect of *L. edodes* polysaccharides (alone) and in combination with other anti-cancer drugs.

Anticancer agent(s)	**Cancer model**	**Mechanism for anti-tumor effect**	**References**
L. edodes Polysaccharides (LPS)	Human breast cancer cells (MDA-MB-231)	- activated THP-1 macrophages	(Morales et al. 2020)
LPS	Colorectal carcinoma-HT-29 cells	- upregulating Bax/Bcl-2 ratio, Caspase -3, -9 and -8 activity and TNF-α level	(Wang et al. 2017)
LPS	Larynx carcinoma (HEp-2) cell lines	- cell cycle arrest at G0/G1 phase	(Finimundy et al. 2018)
LPS	Human breast cancer cells (MCF-7)	- Increased level of cytosolic Cytochrome c, Caspase 7 activity and Bax/Bcl-2 ratio	(Li et al. 2018)
LPS + Cisplatin	Mice bearing human ovarian cancer cell (HEY cells), Prostate cancer cells (LNCaP) and Lung Cancer cells (A549)	- increased expression of IL-6, TNF- α, TGF-β in all three cases	(Liu et al. 2019)
LPS + oxaliplatin	Hepatocellular carcinoma H22 and Hep G2 cells	- Caspase – 3, -6, -7 and -8 activation, increased Bax/Bcl-2 ratio - decreased expression of Stat -3 and NF-κB	(Zhang et al. 2016)
LPS + Paclitaxel + Cisplatin	Gastric cancer patients (in – vivo)	- activation of Programmed cell death- ligand -1 (PD – L 1) and decreasing release of Prostaglandins E2	(Ina et al. 2016)

1. LPS + Paclitaxel + Gemcitabine 2. LPS + Paclitaxel + alimta 3. LPS + FOLFOX	Pancreatic Cancer Patients Pancreatic cancer patients Colorectal Cancer Patients	- Increased level of T-cells, TNF, Cytokines and macrophages	(Del Buono et al. 2016)

2.4 *Astragalus membranaceus* polysaccharides

Astragalus membranaceus is one of the most common Traditional Chinese Medicines (TCM) that has been used for centuries as an immune modulator (Fan et al. 2012; Wu et al. 2017). One of the major active constituents providing anti-tumor effect in *Astragalus* is attributed to its polysaccharides (Jin et al. 2014; Liu et al. 2018; Yang et al. 2013). In addition to the anticancer effect, other important pharmacological properties like antioxidant (Li et al. 2012), anti-inflammatory (He et al. 2012), anti-diabetic (Liu et al. 2010), anti-viral (Jiang et al. 2010), neuroprotective (Zhang et al. 2012), hepatoprotective (Jia et al. 2012) and cardio-protective actions (Yang et al. 2010) have also been reported. The polysaccharides in *Astragalus* are primarily extracted from roots and stems (Zeng et al. 2019) and mainly consist of hetero-polysaccharides (Wang et al. 2006). These hetero-polysaccharides consist of the nine monosaccharides such as glucose, arabinose, mannose, galactose, xylose, rhamnose, ribose, fucose and fructose (Jin et al. 2014). The major glycosidic linkage responsible for the anticancer effect is α-(1, 4)-D-Glucan backbone with α (1, 6)-linked branch after every ten residues as shown in Fig. 2.4 (Niu et al. 2011).

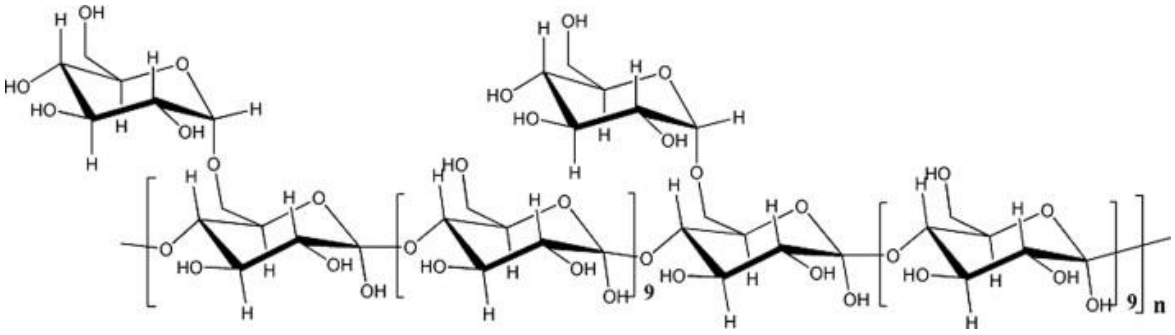


Fig: 2.4 Chemical structure of *Astragalus membranaceus* polysaccharides. Retrieved from: (Niu et al. 2011)

2.5 Anti-proliferative effects of *Astragalus membranaceus* polysaccharides

Many studies have demonstrated the anti-proliferative properties of *Astragalus* polysaccharides (APS) both *in-vitro* and *in-vivo* (Table. 2.2). Most of the studies have reported that their anti-cancer activity is due to enhanced immune response, including alteration in the expression level of Interleukin factors (IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-6, IL-10, IL-11 and IL-12), macrophages and Tumor Necrosis Factor (TNF-α and TNF-β) (Bamodu et al. 2019; Wang et al. 2017; Zheng et al. 2020). Lai et al. (2017) observed the anti-tumor effect of APS on H22 (Hepatoma cells) bearing mice and the anti-tumor effect was attributed to the increased expression for IL-2, IL-6, TNF α and Bax proteins. Similarly, Wu et al. (2017) reported a significant anti-tumor effect of APS on lung cancer cell lines (A549 and NCI-H358) via Nuclear Factor kappa B (NF-κB) inhibition. Chao et al. (2012) determined the anticancer effect of APS in a gastric cancer cell line (MKN45) where anti proliferative action of APS was found to be mediated through cell cycle arrest at the G0/G1 phase. Recent studies have suggested that the anti-cancer effect of APS in breast cancer cell lines (MCF7 and MDA-MB-231), is a result of increased expression for tumor suppressor gene (P53) and decreased expression for Cyclin B1 and CDC6 genes (Liu et al. 2019).

Table 2.2 Examples of anti-cancer effect of *A. membranaceus* polysaccharides alone and in combination with other anti-cancer drugs.

Anticancer agent(s)	**Cancer model**	**Mechanism for anti-tumor effect**	**References**
Astragalus membranaceus Polysaccharides (APS)	Hepatoma carcinoma cells (H22) - bearing mice	- increased expression for IL-2, IL-6, TNF α and Bax proteins	(Lai et al. 2017)
APS	H22 cell line	- suppressed expression for NOTCH 1 by siRNAs, - increased expression for Bcl-2, Bax and Caspase 3 and Caspase 8	(Huang et al. 2016)
APS	Lung cancer cell lines (A549 and NCI-H358)	- nuclear Factor kappa B (NF-κB) inhibition	(Wu et al. 2017)
APS	Intestinal intra-epithelial γδT cells (*in-vitro* model) S-180 tumor bearing mice (*in-vivo*)	- increased proliferation of intestinal intra-epithelial γδT cells and increased expression for IFN-γ, GrB mRNA and FasL - improved activity of γδT cells (increased production of TNF-α and IFN-γ)	(Sun et al. 2014)
APS	Gastric cancer cell line (MKN45)	- cell cycle arrest at G0/G1 phase	(Chao et al. 2012)
APS	Breast cancer cell lines (MCF7 and MDA-MB-231)	- increased expression for tumor suppressor gene (P53) - decreased expression for Cyclin B1 and CDC6 genes	(Liu et al. 2019)

APS + Adriamycin	H22 bearing mice	- increased level of IL-1α, -2, -6, and TNF-α - decreased expression for *MDR1* mRNA and P-glycoproteins	(Tian et al. 2012)
APS + Cisplatin	Lewis lung cancer bearing mice	- increased level of IL -2, -6, -12 and TNF-α in mice blood serum	(Ming et al. 2014)
APS + Cisplatin	Human Nasopharyngeal carcinoma cell lines SUNE-1, CNE-1 and CNE-2	- increased level of Caspase 3, Caspase-9 and Bax protein	(Zhou et al. 2017)

2.6 Extraction of polysaccharides from *L. edodes*

Optimum extraction condition is one of the prime factors that determines the high recovery of bio-active polysaccharides. Many studies have been carried out for extracting polysaccharides from *L. edodes* using different technologies including conventional hot water extraction, alkaline and solvent extraction methods and recent green technologies using ultrasound, microwave, enzyme-assisted and subcritical water extraction techniques. This review section will discuss some of the most common extraction techniques that are recently used for the extraction of polysaccharides from *L. edodes*.

2.6.1 Hot water extraction

Hot water extraction is one of the simplest, safest and cheapest methods of polysaccharide extraction from Shiitake mushrooms. However, this traditional method has certain

disadvantages such as heavy time consuming and deterioration of polysaccharide structure and biological functions due to high temperature (Liu, Yang & Huang 2019). The general method involves the following steps (Fig. 2.5): pretreated powder sample - hot water extraction - filtration - centrifugation of extract solution - collect supernatant - alcohol precipitation (left overnight at 4^0C) – centrifugation & collection of precipitate – drying - crude Shiitake polysaccharide.

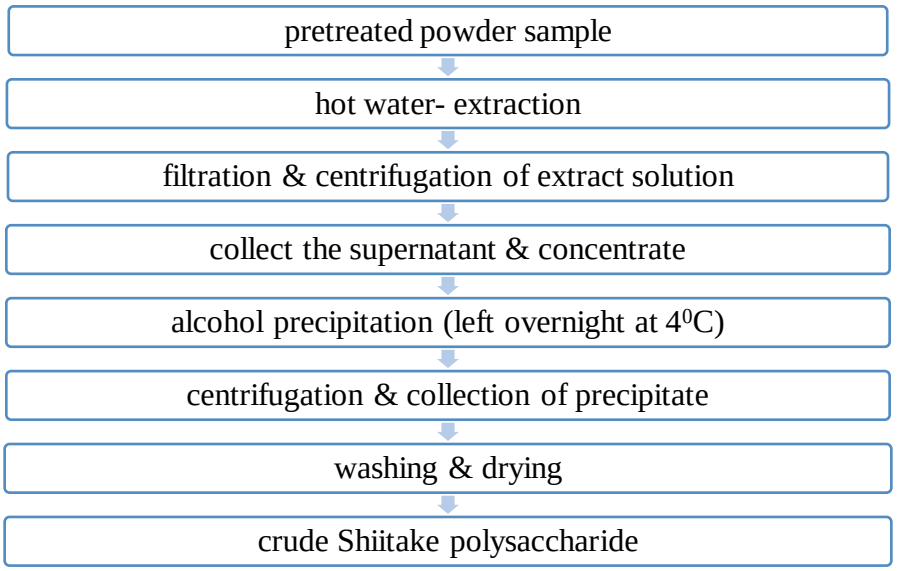


Fig.2.5 Flowchart for the extraction of polysaccharides from Shiitake powder sample using hot water extraction method.

The optimum material to water ratio for the extraction would be 1:30 (w/v) (Gil-Ramírez et al. 2019; Yin et al. 2018). The extraction can be carried out at the temperature ranging from 22^0C to 100^0C (Miyaji et al. 2006) for one to six hours (Finimundy et al. 2013; Yin et al. 2018). The maximum yield reported with hot water extraction is 10.68% w/w when extracted for 6 hrs at 100^0C (Zhao et al. 2016). Usually the centrifugation is done for 10 to 15 min at 5000 g. The volume of ethanol (95% to absolute ethanol) used for precipitation could be as much as three to four times the volume of extract (Gil-Ramírez et al. 2019). The collected precipitate can be dried by freeze drying or hot air drying. Freeze drying is suitable for promoting free radical scavenging property

of Shiitake polysaccharides whereas hot air drying is more favorable for promoting immuno-enhancing property (Liu et al. 2020).

2.6.2 Microwave assisted extraction

Microwave assisted extraction is one of the recently developed extraction technologies which combines microwave energy with traditional solvent extraction technique in order to enhance the extraction efficiency (Gil-Ramírez et al. 2019). One of the advantages of microwave assisted extraction is that the electro-magnetic waves directly heats the extraction solvent rather than simultaneous heat transfer from the vessel which allows minimal temperature gradient during the extraction. Yin et al. (2018) extracted Shiitake polysaccharides at microwave powers ranging from 100 W to 700 W, with the powder sample to liquid ratio of 1:30 (w/v) and for the duration of 8 – 12 minutes. The optimal conditions for microwave extraction were at the microwave power of 450 W with the extraction time of 10 minutes, which led to the maximum yield of 7.91 % (w/w). In another study, Shiitake polysaccharides were extracted using microwave assisted technique at a fixed microwave power of 850 W but varying the temperature and time between (50 – 180^0C) and (5 – 30 min), respectively, with the material to liquid ratio of 1:30 (w/v) (Gil-Ramírez et al. 2019). The optimum microwave conditions in this experiment were 180^0C for 30 min with microwave power of 850 W which were significantly different from the experiment of Yin et al. (2018).The yield of *L. edodes* polysaccharides within this optimized condition was 15.4% (w/w).

2.6.3 Ultrasound assisted extraction

Ultrasonic extraction method is one of the widely used methods in recent years because of its advantages over other methods such as rapid extraction within lower temperatures (Tian et al. 2012). Thus this method is suitable for heat sensitive materials where biological properties may be

compromised due to high extraction temperatures. The highly effective extraction is due to the transient cavitation formed on the surface of materials (Alzorqi et al. 2017). Due to such cavitation, the liquid bubbles near solid surfaces collapse vigorously, creating strong micro-jets that help to disrupt the cells and thus provide efficient extraction. Zhao et al. (2018) optimized the extraction conditions for shiitake polysaccharides using sonication method with the optimal conditions being ultrasonic power of 290 W at 45^0C for 21 min, which led to the maximum polysaccharide yield of 9.75% (w/w). Morales et al. (2019) also performed the extraction of Shiitake polysaccharides using ultrasonic power of 550 W at 50^0C for 60 min. The amount of total polysaccharides obtained using this technique was as much as double (10.14% w/w) when compared to hot water extraction (5.02% w/w).

2.6.4 Enzymatic extraction

Enzymatic extraction method is considered as one of the highly efficient, environmentally friendly and simple technique for the extraction of polysaccharides from mushroom (Cheng et al. 2015). As the fungal cell wall is mainly composed of cellulose, hemicellulose, pectin, chitin and proteins, the use of enzymes such as cellulases, proteases and pectinases will help to degrade the cell wall and thus release the internal cellular components into the surrounding medium (Zhao et al. 2016). This method has further advantage of extracting the polysaccharides with high purity and bioactivity compared to other techniques that utilize high heat and pressure (Chen et al. 2016). Previously the combination of three different enzymes (cellulose, papain and pectinase in the ratio 1:1:1) has been used for the extraction of polysaccharides from shiitake (Yin et al. 2018). The amount of enzymes used was 2% of the powder weight and the extraction was carried out between 36^0C to 60^0C for 2 hours at the pH between 4.5 to 6.5. The optimum enzymatic temperature and

pH were 54^0C and 5.0, respectively, which led to the maximum polysaccharide yield of 15.65 % (w/w).

2.6.5 Vacuum extraction

Vacuum extraction is an effective technique for extracting at a lower temperature. Li et al. (2019a) used vacuum extraction technique to extract the polysaccharides from Shiitake mushroom under vacuum pressure of 0.02 to 0.08 MPa, temperature from 30 to 70^0C, stirrer speed from 800 to 2400 rpm, powder/liquid ratio from 1:5 to 1:30 (w/v) and time duration from 5 to 30 min. The optimal conditions were vacuum pressure of 0.08 MPa at 62^0C, for 25 min, at the stirrer speed of 1200 rpm and powder/liquid ratio of 1:26 (w/v). The extraction yield of polysaccharides under these optimal conditions was 4.28% (w/w).

2.6.6 Microwave assisted aqueous-two-phase extraction

Aqueous-two-phase extraction technique has gained a lot of attention in recent years for extraction and purification of biomolecules (Iqbal et al. 2016). This method has an advantage over other extraction techniques such as more environmentally friendly, easy to scale up, low cost, high yield of the product and able to extract a wide range of polysaccharides because of its multi-phase system (Chen et al. 2016). Lin et al. (2019) applied this technique to extract polysaccharides from shiitake where they used ethanol and ammonium sulfate as the two-phase solvents. The experiment was carried out for different concentration of ammonium sulfate (17 – 21 % w/w) at different extraction temperatures (70 – 90)^{0}C and time (15 – 25 min). The optimum extraction conditions were at the ethanol and ammonium sulfate concentration of 26% and 19.58% w/w, respectively, at 80^0C for 20 min and the powder sample/solvent ratio of 1:50 (w/v). Under these optimal conditions the polysaccharide yield from the top and bottom phase was 2.12% and 11.16 % (w/w), respectively.

When compared to the mono-solvent extraction technique, this method showed improved yield as well as extracted diverse polysaccharides in a single step process.

2.6.7 Enzyme-microwave-ultrasound assisted extraction

Yin et al. (2018) extracted shiitake polysaccharides by combining enzyme, microwave and ultrasound method. The total polysaccharides yield by this technique was 9.38% w/w. In addition, they compared the polysaccharides yield of each extraction technique involved separately. Their study revealed that the combination of these three techniques improved polysaccharides yield compared to microwave, ultrasound, enzymatic and hot water extraction alone by 8.56%, 16.38%, 26.59% and 50.32%, respectively. The optimum temperature, pH, microwave power and exposure time were found to be 48^0C, 5.0, 440W and 10 min, respectively which led to the total polysaccharide yield of 9.38% (w/w) (Yin et al. 2018).

Table 2.3 Different extraction methods exhibiting maximum yield of Shiitake polysaccharides.

Method of extraction	**Maximum (%) yield of Shiitake polysaccharides (w/w)**	**Reference**
Hot water extraction	10.68	(Zhao et al. 2016)
Microwave assisted extraction	15.4	(Gil-Ramírez et al. 2019)
Ultrasound assisted extraction	10.14	(Morales et al. 2019)
Enzymatic extraction	15.65	(Yin et al. 2018)
Vacuum extraction	4.28	(Lin et al. 2019)
Microwave/ aqueous-two-phase extraction	13.28	(Lin et al. 2019)
Enzyme-microwave-ultrasound assisted extraction	9.38	(Yin et al. 2018)

In summary, as shown in Table 2.3, among seven different extraction methods reported in the literature, the highest shiitake polysaccharide yield was achieved by enzymatic extraction method under the optimal extraction conditions as described by Yin et al. (2018) mentioned in section 2.6.4.

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials and Reagents

The dried sample powders of *A. membranaceus*, (*L. edodes*) Shiitake, HAS 5% - Hengshan *Astragalus* bio-transformed Shiitake (HAS-A) and HAS 20% (HAS-B) were provided by Shanxi Yulongxiang Agricultural Development Co. Ltd., China. The enzymes Alcalase and VinoTaste® Pro were obtained as gift samples from NOVOZYMES Pty Ltd, Australia. Other enzymes and chemicals including cellulase, pectinase, DEAE-Sephadex-CL 6B, dialysis membrane (molecular weight cut off – 12,000 Da), glucose, Bio-Rad dye reagent and Bovine serum albumin (BSA) were purchased from Sigma Aldrich, Australia.

3.2 Cell lines and cell culture

For cell culture and cytotoxicity assay, the human colorectal carcinoma cells, HCT-116 were obtained from American Type Culture Collection (ATCC) and all other media and reagents including McCoy's 5A medium, DMSO and MTT were purchased from Sigma Aldrich, Australia. The McCoy's 5A media was supplemented with 10% fetal bovine serum (FBS), 1% penicillin (10,000 U/ml) – streptomycin (10,000 μg/ml) (Sigma Aldrich) and 1% glutamax (Thermo Fisher). The apoptosis detection kit, Alexa Fluor 488 Annexin V/Apoptosis kit was purchased from Invitrogen, Australia. All the reagents used in the experiments were of analytical or reagent grade.

3.3 Methods

The experiments were divided into two main sections; A) investigation of the effect of five different extraction methods on total polysaccharide yield, content (purity), molecular weight distribution, monosaccharide composition and *in-vitro* cytotoxicity analysis in HCT-116 cell lines extracts; B) preliminary purification and characterization of polysaccharides of most active extracts. A brief summary of the experimental design used in this study is outlined below (Fig.3.1).

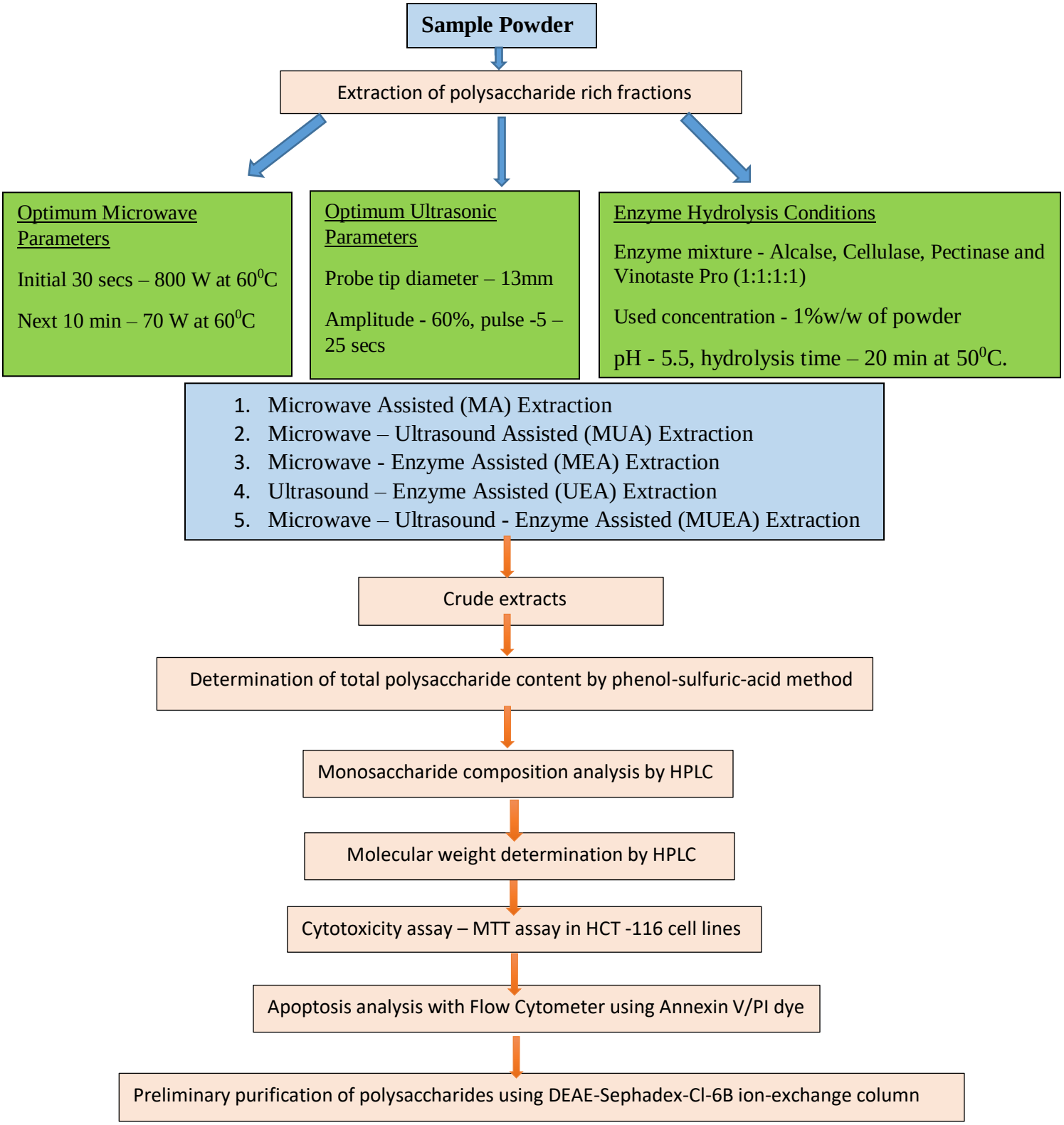


Fig.3.1 Workflow summary of experiments designed for this project.

Section A

3.3.1 Extraction of polysaccharides

Extraction was carried out using five different extraction methods including microwave assisted (MA), microwave-ultrasound assisted (MUA), microwave-ultrasound-enzyme assisted (MUEA), microwave-enzyme assisted (MEA) and ultrasound-enzyme assisted (UEA) extraction method. All the parameters used in the extraction were taken from the previously optimized parameters from literature with some modifications after preliminary studies.

3.3.1.1 Microwave assisted extraction

The extraction was carried out based on the previously described method of Yin et al. (2018) with some modifications. The powder sample (3 g) was mixed with 90 ml of distilled water (1:30 w/v). The suspension was vortexed to form a homogenous mixture and then microwaved (Milestone startSYNTH microwave extractor) under the microwave power of 800 W for initial 30 seconds to reach 60^0C followed by 70 W for another 10 minutes maintaining the same temperature (Gil-Ramírez et al. 2019). After cooking for 10 minutes, the mixture was allowed to cool, centrifuged at 5000 g for 10 minutes and then the supernatant was collected. The supernatant was precipitated with three volumes of absolute ethanol and stored at 4^0C overnight. The precipitates were then collected by centrifugation at 5000 g for 5 minutes and dried in a freeze dryer (Fig. 3.2).

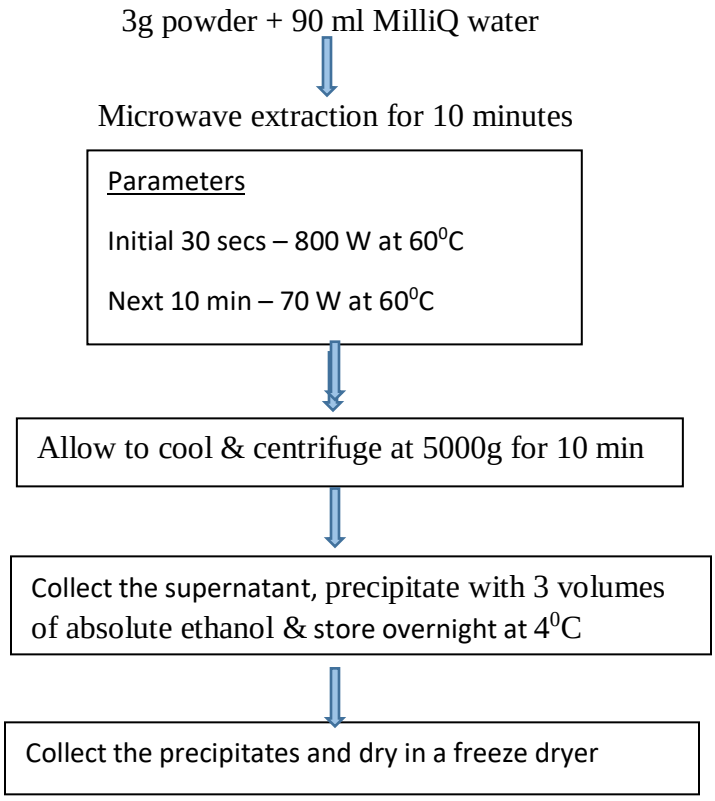


Fig.3.2 Flowchart of polysaccharide extraction using microwave assisted technique.

3.3.1.2 Microwave and ultrasound assisted extraction

The extraction was carried out based on the previously described methods of Zhao et al. (2018) and Morales et al. (2019) with some modifications. As shown in Fig. 3.3, the powder sample (3 g) was mixed with 90 ml of distilled water (1:30 w/v) and vortexed to form homogenous suspension. The sample-liquid mixture was then exposed to pulsed ultrasonic power (750 Watt & 20 kHz Ultrasonic Processor) with 60% amplitude for 10 minutes. This extract mixture was once again extracted with microwave for another 10 minutes using the same extraction conditions described in 3.3.1.1. The extract was then cooled, centrifuged, precipitated and freeze dried as in 3.3.1.1.

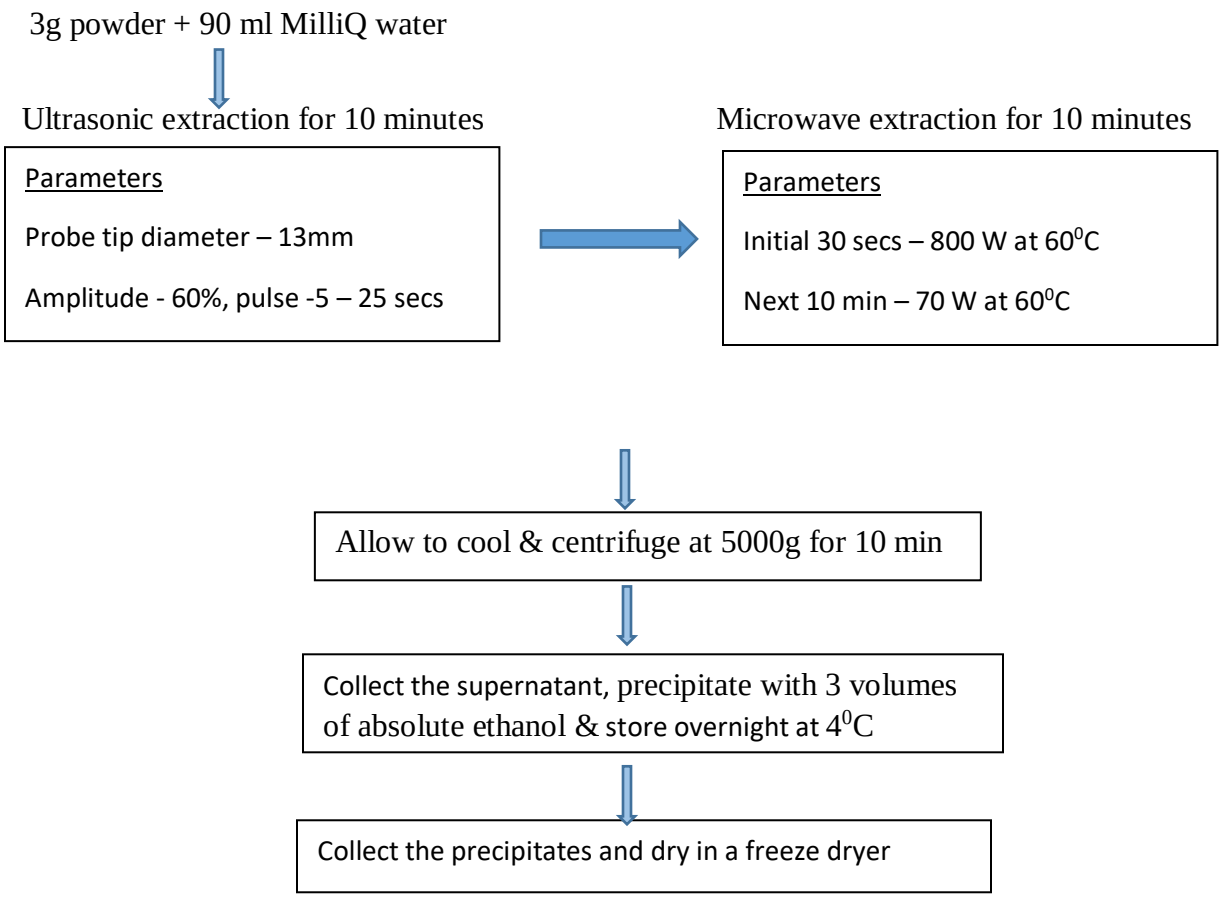


Fig.3.3 Flowchart of polysaccharide extraction using microwave and ultrasound assisted technique.

3.3.1.3 Microwave-ultrasound-enzyme assisted extraction

The extraction was carried out based on the previously described method of Yin et al. (2018) with some modifications. As shown in Fig. 3.3, initially the extract was prepared following the same procedure as microwave-ultrasound assisted extraction method. The pH of the extract was maintained at 5.5 with 0.1 M citric acid and 0.2 M disodium hydrogen phosphate buffer (Amigh & Taghian Dinani 2020). Then the extract was hydrolyzed at 50^{0}C for 20 minutes using the mixture of enzymes (in the ratio 1:1:1:1) Alcalse, Cellulase, Pectinase and VinoTaste® Pro in a concentration of 1% (w/w) of powder. This is a new enzyme mixture prepared in this study while

most of the previous studies had used only the three enzyme mixture of Alcalase, Cellulase and Pectinase in a ratio 1:1:1. After 20 min of hydrolysis, the enzymes were deactivated by heating them in a water-bath to a high temperature of 90^0C for 5 minutes. The extract was then cooled, centrifuged, precipitated and freeze dried as in 3.3.1.1.

3.3.1.4 Microwave-enzyme assisted (MEA) extraction

The extraction was carried out based on the previously described method of Cheng et al. (2015) with some modifications. Initially, the samples were extracted in the same manner as stated in microwave assisted extraction (Fig. 3.2). Then the pH of the extract was maintained 5.5 and hydrolyzed using the same enzyme mixture and under the similar extraction condition as described in 3.3.1.3 for enzymes. Finally, the extract was cooled, centrifuged, precipitated and freeze dried as in 3.3.1.1.

3.3.1.5 Ultrasound and enzyme assisted (UEA) extraction

The extraction was carried out based on the previously described method of Yin et al. (2018) with some modifications. The powder sample solution was prepared in distilled water in the ratio 1:30 (w/v). This mixture was first exposed to pulsed ultrasonic power of 60% amplitude for 10 minutes then further hydrolyzed with enzyme mixture under the similar conditions as described in 3.3.1.3 for enzymes (Fig. 3.4).

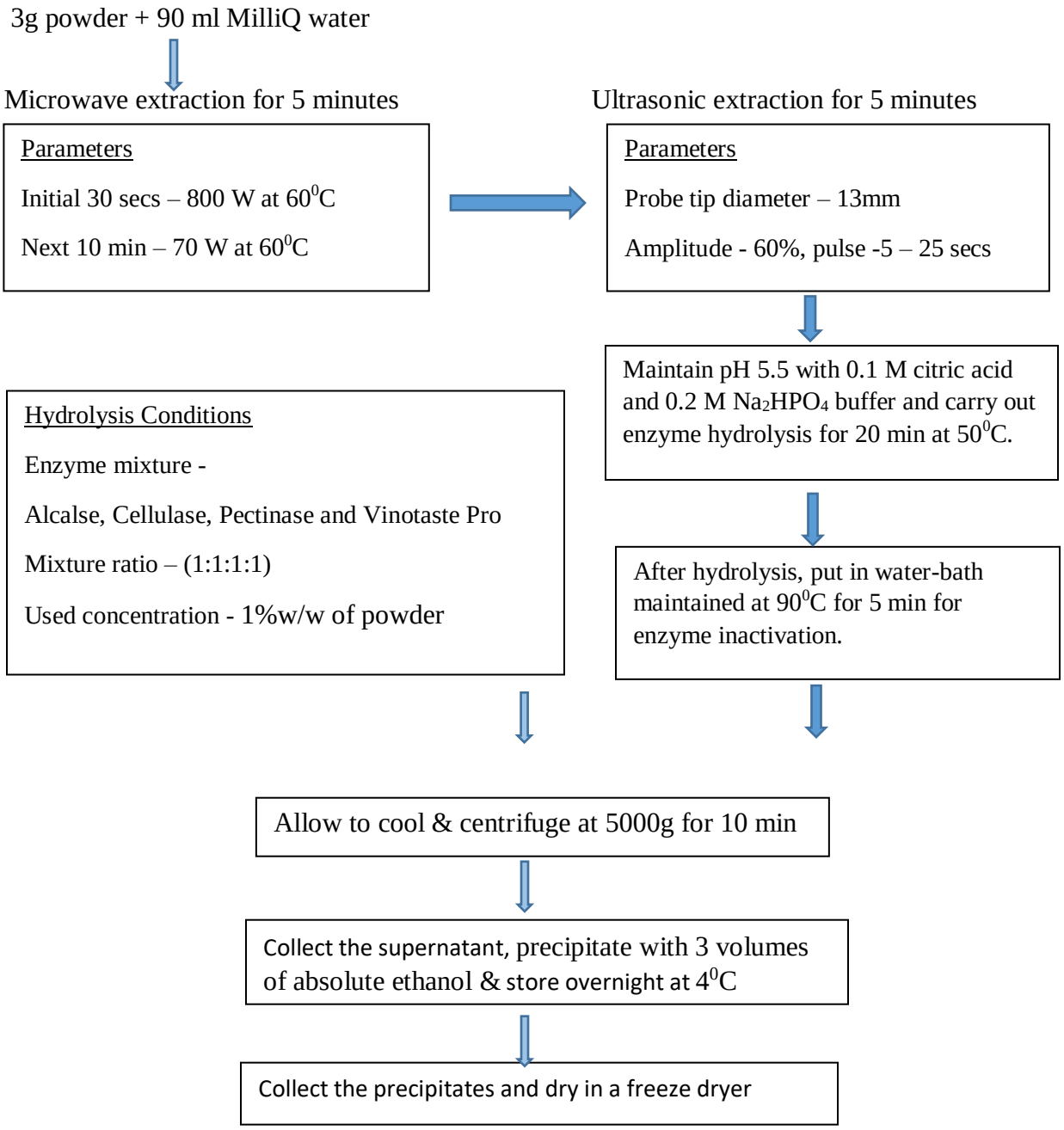


Fig.3.4 Flowchart of polysaccharide extraction using microwave-ultrasound-enzyme assisted technique.

3.3.2 Determination of extraction yield

The extraction yield of total polysaccharides from each extraction technique was determined by dividing the total weight of dried polysaccharide extracts obtained from each extraction by the total weight of sample powder taken for that particular extraction.

$$\%\ Yield = \frac{\text{total weight of polysaccharide extract (g)}}{\text{total weight of sample powder used (g)}} \times 100$$

3.3.3 Total polysaccharide content (purity)

The total polysaccharide content in each crude extract was determined by the previously described phenol-sulfuric acid method using D-Glucose as a reference (Chen et al. 2016). The glucose standard curve was prepared with the concentration ranging from (50 – 150) µg/ml. The assay was conducted in a 96 well microplate where 50 µl of sample/standard solution was taken for each analysis. To this, 30 µl of 5% Phenol solution was added followed by rapid addition of 150 µl of 98% sulfuric acid. The addition of sulfuric acid should be directly to the liquid surface rather than the side wells because the rate of reaction is directly proportional to the heat generated (Nielsen 2010). The reaction mixture was then heated in a water-bath maintained at 90^0C for 5 minutes and absorbance was taken at 490 nm using a plate reader. The percentage of polysaccharide content was calculated as below:

$$\%\ \text{Polysaccharide} = \frac{\text{glucose equivalent concentration } \left(\frac{\mu g}{ml}\right) \text{ in test sample}}{\text{powder (sample) concentration } \left(\frac{\mu g}{ml}\right) \text{ used}} \times 100\%$$

3.3.4 Protein content

Protein content was determined using the Bradford method for protein estimation and bovine serum albumin (BSA) as a standard (Kruger 2009). Five different concentrations of BSA (0.1, 0.2, 0.4, 0.6 and 0.8 mg/ml) were used to prepare a standard curve. The Bio-Rad dye reagent was prepared by diluting one part of dye to four parts of distilled water and protected from direct contact with light. The assay was performed in a microplate and to every 5 µl of sample solution, 250 µl of diluted Bio-Rad reagent was added, left for 15 – 45 minutes in the dark and the absorbance was taken at 595 nm using a plate reader. The percentage of protein content was calculated as below:

$$\%\ \text{Protein} = \frac{\text{protein equivalent concentration } \left(\frac{\mu g}{ml}\right) \text{ in test sample}}{\text{powder (sample) concentration } \left(\frac{\mu g}{ml}\right) \text{ used}} \times 100\%$$

3.3.5 Monosaccharide composition analysis

The composition of monosaccharides in each sample was determined using HPLC with Phenomenex Kinetex C18 column (2.6 um 3 × 100mm 100 A). Initially the samples (10 mg) were hydrolyzed with 100 µl of 72% sulfuric acid at 30^0C for 1 hour. To the above sample solution, distilled water was added to bring it down to the concentration of sulfuric acid to 1 M and further incubated for 3-4 hours at 100-110^0C for complete hydrolysis. After the samples were hydrolyzed, the samples were spun at top speed (16,000 × g) for 5 min to settle down the particles, then 20 µl of each sample and standards were transferred in a 1.5 ml Eppendorf tube. To each of the above tubes, 20 µl of 0.5 mM 2-deoxy Glucose was added as an internal standard. This was followed by the addition of phenyl methyl pyrazolone (PMP)/Ammonia mixture (0.5 M PMP and 1 M

ammonium hydroxide solution) and this mixture was again incubated at 70^0C for 1 hour. To the above reaction mixture, 20 µl of 10 M formic acid was added followed by 1 ml of di-butyl ether. This mixture was shaken vigorously for a minute and later the top di-butyl ether layer was removed carefully. Likewise, the addition, vigorous mixing and removal of di-butyl ether were repeated twice-thrice until the reaction mixture was clear enough. Then the above samples were transferred into a rotary evaporator for the removal of any traces of di-butyl ether. These samples were micro-centrifuged at the top speed for 5 minutes and then injected into HPLC column for monosaccharide analysis (Yang et al. 2005; Lorbeer et al. 2015).

3.3.6 Molecular weight determination

The molecular weight of polysaccharide extracts from each sample was determined by size exclusion chromatography using HPLC (*PL aquagel-OH Mixed-H 8um 300* x *7.5mm* column). Dextran with molecular weight ranging from 1.1, 4.4, 20, 65, 195, 400 and 1050 k Da were taken as reference standards and 0.1 M sodium nitrate as a mobile phase. Initially, the samples were dissolved in MilliQ water at the concentration of 5mg/ml and then micro-centrifuged at the top speed (16,000 × g) for 5 minutes. 100 µl of above clear supernatant was mixed with 100 µl of 0.2 M sodium nitrate solution. This mixture was again spun at top speed for 5 minutes and 150 µl of this solution was injected into HPLC column for molecular weight analysis (Lorbeer et al. 2015).

3.3.7 *In-vitro* cytotoxicity assay in HCT-116 cells

The *in-vitro* antiproliferative activity (cytotoxicity) was determined in colorectal carcinoma cells, HCT-116. The cells were maintained in McCoy's medium at 37^0C with 5% CO_2 supplemented

with 10% Fetal Bovine Serum, 1% penicillin/streptomycin and 1% Glutamax. Cells were seeded into 96 well plate at a density of 5×10^3 cells per well and incubated for 24 hours (You et al. 2013). These cells were then treated with each sample extract between the concentration of 3200 – 200 µg/ml. During treatment, 5-Fluorouracil, 1% DMSO in media and media alone was taken as the positive control, solvent control and negative control, respectively. The treated cells were incubated for 48 hours at 37^0C with 5% CO_2 supplemented. Subsequently 100 µl of MTT (5mg/ml diluted in media in a ratio of 1:10) was added to each well and again incubated for 4 hours. The formazan crystals formed were then dissolved with 100 µl of DMSO and analyzed in the plate reader at 570 nm (Yang et al. 2020). The percentage inhibition was calculated as below:

$$\text{Percentage (\%) inhibition} = \left(1 - \frac{\text{test sample absorbance}}{\text{control sample absorbance}}\right) \times 100\%$$

3.3.8 Apoptosis detection by Flow Cytometry

Apoptosis was determined by Alexa Fluor 488 Annexin V/PI based apoptosis kit by Invitrogen (catalog no. V13241). The experiment was conducted in six well plates. The cells (HCT – 116) were seeded at the density of 3×10^5 cells /3ml in each well and the treatments were done in the same manner as in MTT assay mentioned above. For harvesting, cells were first washed with PBS then trypsinized (1ml trypsin /well) for 3 -5 minutes at 37^0C and 5% CO_2. The cells were then split into two halves and placed in a 5 ml flow tube. These cells were washed with complete medium in order to inactivate trypsin and spun down at 1400 × g for 5 minutes. The supernatant was poured off and 1ml of Annexin binding buffer was added to resuspend the cells. The cell suspension was again centrifuged for 5 minutes at the same speed (1400 × g) and the supernatant was poured off. The tubes were flicked for the resuspension of cells and to each tube 5 µl of Annexin V was added.

These cells were incubated at room temperature (in the dark) for 15 minutes and 5 µl of Propidium Iodide was added. Finally, to each of the above tubes, 400 µl of Annexin binding buffer was added and immediately analyzed by the CytoFLEX S, Beckman Coulter, Flow Cytometry (Vermes et al. 1995).

For the quantification of live and apoptotic cells, firstly, the live cell gate was defined by adjusting forward scatter (FSC) and side scatter (SSC), then the fluorescence was emitted at 530 nm and 575 nm using an excitation wavelength of 488nm. The flow rate of sample was adjusted at 60 µl/min and total of 10,000 cells were analyzed for each sample. The percentage of live and apoptotic cells were analyzed by CytExpert software version 2.3.

Section B

3.3.9 Preliminary purification of selected polysaccharide extracts by ion exchange column

Based on the outcomes from the extraction and characterization of polysaccharide-rich extracts, three crude extracts exhibiting the highest cytotoxicity were selected for further purification, with the hypothesis that high purity polysaccharides will further improve their cytotoxicity. Thus, the three crude extracts of UEA-HAS-B, UEA-Shiitake and MA-*A. membranaceus* were further purified by anion exchange column chromatography using DEAE – Sephadex CL – 6B as an anion exchanger. MilliQ water was used as an equilibrating buffer and different concentrations of Sodium chloride (0.1 to 2 M) were used as an eluting buffer. Firstly, the column was filled with water to check the flow rate (0.4 – 0.5 ml/min). The resin was filled in the column and equilibrated by washing it with at least five column volumes of MilliQ water. Eventually 50 mg of crude extract was dissolved in 10 ml of MilliQ water, spun at the top speed, loaded into the column slowly

without disturbing the upper resin layer and as soon as loading was done, 4 ml of eluate was collected from the bottom in the collecting tubes. Once the loading was finished, the column was washed with at least five column volumes of MilliQ water and collection of eluent was continued. Then the elution was carried out using 0.1, 0.3, 0.5 1.0, 1.5 and 2.0 M NaCl. All the extracts were analyzed for total polysaccharide content by phenol-sulfuric acid method and the main extracts were combined and dialyzed (molecular weight cut-off of 12,000 Da) against distilled water for 24 hours (Chen et al. 2012).

3.3.10 Determination of total polysaccharide content (purity)

The total polysaccharide content (purity) of the purified extracts was determined by phenol-sulfuric acid method as described in section 3.3.3 above.

3.3.11 Analysis of cytotoxic effect in HCT 116

The cytotoxic effect of the purified extracts in HCT 116 was determined following the method described in section 3.3.7.

3.3.12 Determination of molecular weight

The relative molecular weight of the purified extracts was determined by size exclusion HPLC, as described in section 3.3.6.

3.3.13 Statistical analysis

The data for each experiment were collected as three independent replicates and expressed as mean ± standard errors. Data were calculated and analyzed by Microsoft excel 2016 and GraphPad Prism 8.0. The values with $p < 0.05$ were determined as statistically significant values, analyzed by one-way ANOVA and followed by Dunnett's and Tukey's test.

CHAPTER 4: RESULTS

4.1 Effect of different extraction methods on yield of polysaccharide extracts

The total polysaccharide yields from five different extraction methods for four different samples are shown in Fig. 4.1. The yield of total polysaccharide extracts from HAS-A and HAS-B was significantly high than that of Shiitake in every extraction method. The yield increased in the following order, HAS-B > HAS-A > Shiitake > *A. membranaceus*. The maximum yield was obtained from the combination of microwave and ultrasound assisted extraction technique (MUA) where the yield of HAS-B, HAS-A, Shiitake and *Astragalus* were 18.34 ± 0.55%, 16.5 ± 0.42%, 13.64 ± 0.4% and 3.6 ± 0.36 % w/w, respectively (Fig. 4.1). The second best results were obtained from Microwave assisted extraction (MA) method where the yield for the same was 14.6 ± 0.59%, 12.3% ± 0.15, 10.03% ± 0.44 and 2.8% ± 0.13 %w/w, respectively. This trend was similar in case of all the enzyme assisted extractions (MUEA, MEA and UEA), however the yield of total polysaccharides from these techniques dropped to almost half when compared to MA and MUA. For instance, the highest yield using enzyme was shown by UEA where the percentage yield for HAS-B, HAS-A, Shiitake and *Astragalus* was 11.1 ± 0.29%, 8.07 ± 0.35%, 6 ± 0.13% and 2.5 ± 0.08 % w/w, respectively (Fig. 4.1).

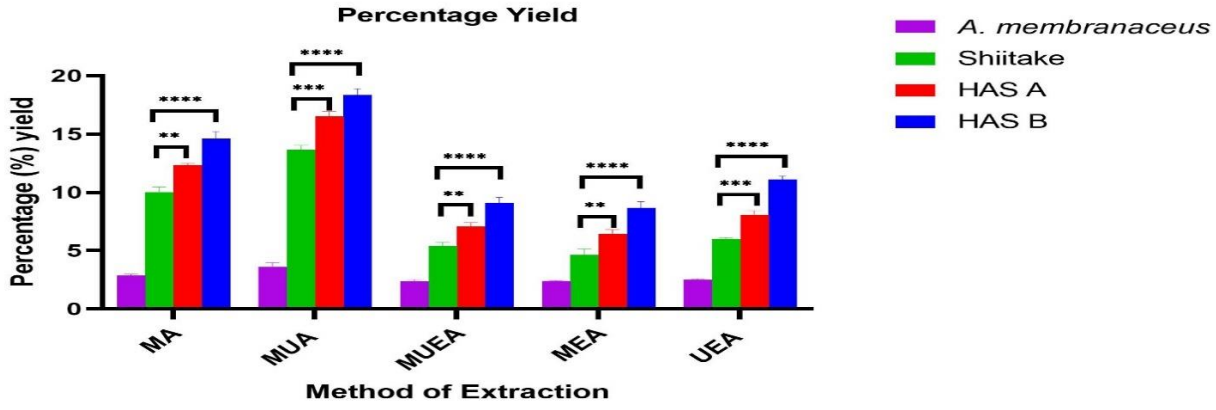


Fig. 4.1: Effect of five different extraction methods on the yield of total polysaccharides from dry powder samples of *A. membranaceus*, Shiitake, HAS-A and HAS-B. The extraction methods are: MA – Microwave assisted, MUA – Microwave/Ultrasound assisted, MUEA – Microwave/Ultrasound/Enzyme assisted, MEA- Microwave/Enzyme assisted and UEA- Ultrasound/Enzyme assisted. HAS-A and HAS-B significantly different from controlled Shiitake with P<0.01 indicated as **, P<0.001 as *** and P<0.0001 as **** while 'ns' represent not significant. Detailed experimental conditions are described in Chapter 3.

4.2 Effect of different extraction methods on total polysaccharide content

The total polysaccharide contents from five different extraction methods for four different samples are shown in Fig. 4.2. The total polysaccharide content in all the enzyme assisted extracts (UEA, MUEA and MEA) was significantly higher when compared to MA and MUA. Although the total polysaccharide yield from MA and MUA was higher, the total polysaccharide content was very low. MUA had comparatively higher polysaccharide content than MA where the polysaccharide content in HAS-B, HAS-A, Shiitake and *Astralagus* was 28.7 ± 3.6%, 23.3 ± 2.2%, 18.8 ± 1.5% and 13.3 ± 0.5 % w/w of dried extract powder, respectively (Fig. 4.2). The polysaccharide content in the same samples obtained by MUEA was 66.2 ± 6.3%, 60.4 ± 1.6%, 48.5 ± 1.3% and 29.3 ± 4.05% w/w, respectively. There was no significant difference in these values between the other enzymatic extraction methods (UEA and MEA).

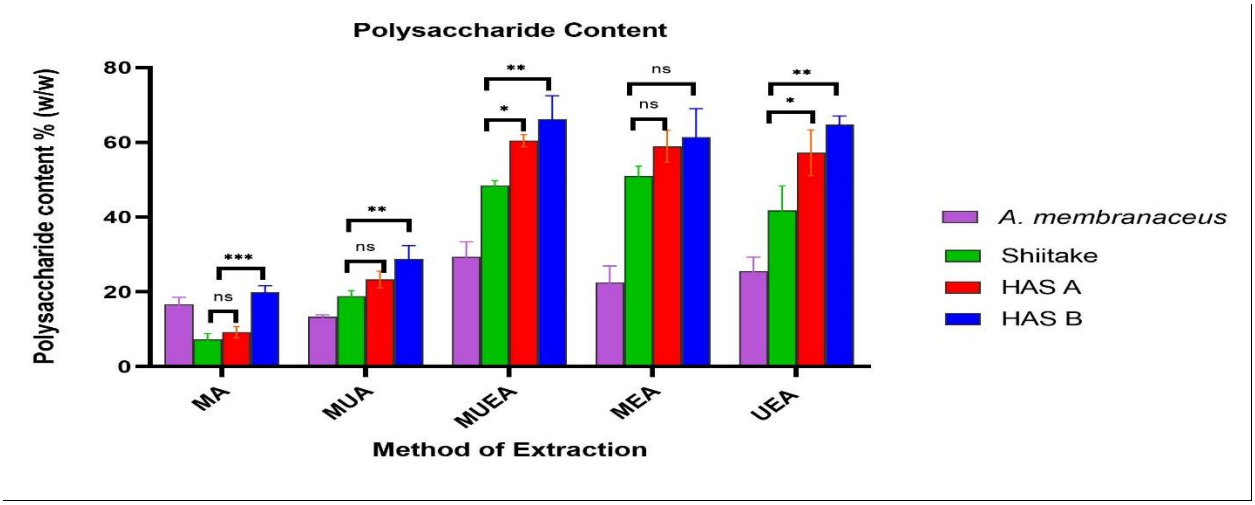


Fig. 4.2: Effect of five different extraction methods on the total polysaccharide content in polysaccharide extracts of *A. membranaceus*, Shiitake, HAS-A and HAS-B. MA – Microwave assisted, MUA – Microwave/Ultrasound assisted, MUEA – Microwave/Ultrasound/Enzyme assisted, MEA- Microwave/Enzyme assisted and UEA- Ultrasound/Enzyme assisted. HAS-A and HAS-B significantly different from controlled Shiitake with P<0.05 indicated as *, P<0.01 as ** and P<0.001 as *** while 'ns' represent not significant. Detailed experimental conditions are described in Chapter 3.

4.3 Effect of different extraction methods on protein content

The protein contents from five different extraction methods for four different samples are shown in Fig. 4.3. There was a significant decline in protein content in all the enzyme assisted extracts (UEA, MEA and MUEA) compared to non-enzymatic ones (MA and MUA). The protein content in MA extracts was the highest (4.5 ± 0.12 %, 3.5 ± 0.18 %, 3.9 ± 0.71 % and 6.1 ± 0.57 % w/w for *Astragalus*, Shiitake, HAS-A and HAS-B respectively) while the UEA extracts had the lowest protein content for the same samples 1.5 ± 0.1 %, 1.7 ± 0.51 %,1.3 ± 0.27 % and 0.6 ± 0.01 %w/w, respectively (Fig. 4.3).

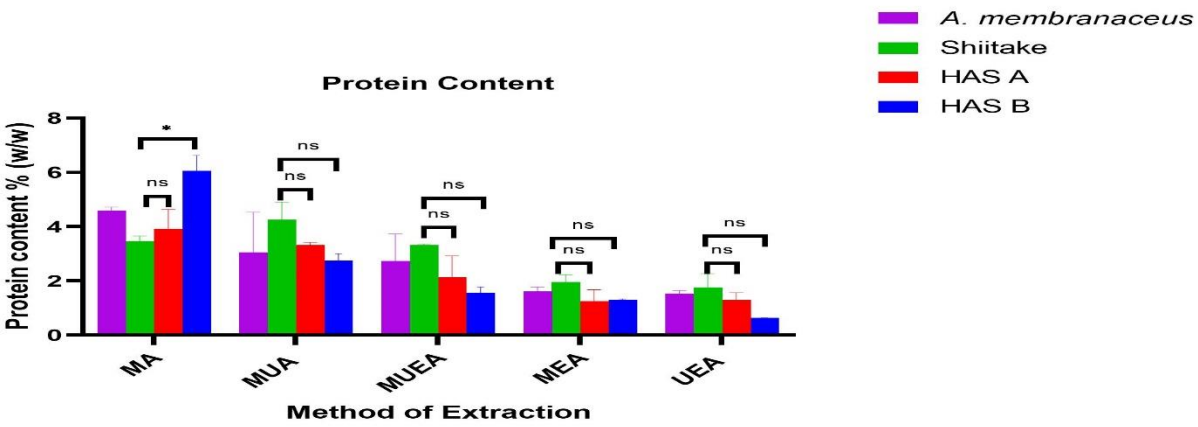


Fig. 4.3: Percentage protein content in crude extracts of *A. membranaceus*, Shiitake, HAS-A and HAS-B using different methods of extraction. MA – Microwave assisted, MUA – Microwave/Ultrasound assisted, MUEA – Microwave/Ultrasound/Enzyme assisted, MEA- Microwave/Enzyme assisted and UEA- Ultrasound/Enzyme assisted. HAS-A and HAS-B significantly different from controlled Shiitake with $P<0.05$ indicated as * while 'ns' represent not significant. Detailed experimental conditions are described in Chapter 3.

4.4 Effect of different extraction methods on monosaccharide composition of polysaccharide extracts analyzed by HPLC

The monosaccharide composition of polysaccharide extracts from five different extraction methods for four different samples are shown in Table 4.1. The monosaccharides such as mannose (Man), ribose (Rib), rhamnose (Rha), glucuronic acid (GlcAc), galacturonic acid (GalAc), glucose

(Gluc), galactose (Gal), xylose (Xyl), arabinose (Ara) and fucose (Fuc) were present in all the extracts. However, the concentration of these monosaccharides varied among all extracts with different methods of extraction.

For instance, in UEA extracts of HAS-B and HAS-A, the amounts of glucuronic acid (GlcAc) and glucose (Gluc) were significantly higher (GlcAc ~ 551.1 ± 1.5 µg/ml and Gluc ~ 2084.1 ± 1.5 µg/ml in HAS-B and GlcAc ~ 487.3 ± 1.8 and Gluc ~ 1876.8 ± 2.6 µg/ml in HAS-A) compared to the control shiitake (GlcAc ~ 346.6 ± 0.8 µg/ml and Gluc ~ 1131.6 ± 2.3 µg/ml) ($P < 0.05$, Appendix A). Meanwhile, the amounts of mannose (Man) and xylose (Xyl) in the same extracts were slightly lower (Man ~ 127.1 ± 1.5 µg/ml and Xyl ~ 12.8 ± 1.2 µg/ml in HAS-B and Man ~ 160.3 ± 1.8 µg/ml and Xyl ~ 19.4± 0.7µg/ml in HAS-A) compared to control shiitake (Man ~ 171.3 ± 1.8 µg/ml and Xyl ~ 24.3 ± 1.9 µg/ml). A similar pattern was observed in all the five extraction methods for the above three extracts, the details are summarized in Table 4.1.

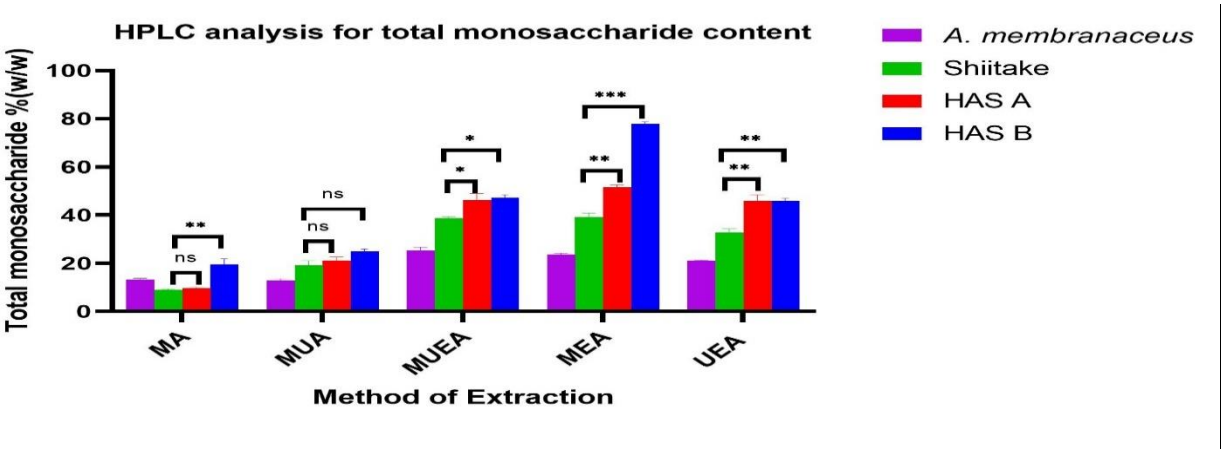


Fig. 4.4: Effect of different extraction methods on total monosaccharide content in the polysaccharide extracts of *A. membranaceus*, Shiitake, HAS-A and HAS-B, determined by HPLC. MA – Microwave assisted, MUA – Microwave/Ultrasound assisted, MUEA – Microwave/Ultrasound/Enzyme assisted, MEA- Microwave/Enzyme assisted and UEA- Ultrasound/Enzyme assisted extraction methods. HAS-A and HAS-B significantly different from controlled Shiitake with $P<0.05$ indicated as *, $P<0.01$ as ** and $P<0.001$ as *** while 'ns' represent not significant. Detailed experimental conditions are described in Chapter 3.

In addition, the total monosaccharides in each of the four samples (HAS-A, HAS-B, Shiitake and *Astragalus*) increased significantly ($P < 0.01$) for all the enzyme assisted extraction methods (MUEA, MEA and UEA) compared to non-enzymatic MA and MUA extracts (Fig. 4.4, Table 4.1 and Appendix B).

In HPLC chromatogram, two unknown peaks were observed in every extract samples which could not be identified due to lack of reference standards for those peaks (Appendix C). In case of *A. memranaceus*, one peak was observed at the retention time (Rt) of 5.35 min and another peak at 6.85 min, while in case of Shiitake, one peak was observed at the Rt of 5.15 min and another at 6.85 min. HAS-A and HAS-B also had the first peak at Rt of 5.35 min (similar to that of *A. memranaceus*) and the second peak at 6.85min. However, the intensity of the peak (Rt - 5.35 min) was higher in HAS-B compared to HAS-A.

Table 4.1 Composition and concentration of monosaccharides in MA, MUA, MUEA, MEA and UEA extracts of *A. membranaceus*, Shiitake, HAS-A and HAS-B, determined by HPLC.

		Concentration µg/ml ± S.D (n=3)									
	Extracts	**Man**	**Rib**	**Rha**	**GlcAc**	**GalAc**	**Gluc**	**Gal**	**Xyl**	**Ara**	**Fuc**
	A. membranaceus	36.7±0.74	10.3±0.59	39.7±4.8	119.9±0.04	38.6±7.4	281.0±9.1	259.6±10.6	32.3±0.27	146.4±5.9	10.0±5.59
MA	**Shiitake**	65.9±0.04	12.7±1.253	8.2±0.75	103.6±7.77	15.5±2.23	317.1±12.4	131.2±6.35	7.0±0.23	1.5±0.66	9.2±4.87
	HAS-A	49.9±0.01	18.8±2.26	8.6±0.32	124.3±16.9	12.3±1.3	313.6±21.0	154.1±3.5	3.6±0.74	2.1±0.35	23.6±0.7
	HAS-B	53.5±6.85	75.5±10.6	8.4±0.83	227.9±16.6	12.6±4.9	783±104.8	243.6±30.0	3.2±0.1	1.5±0.87	38.8±10.3
	A. membranaceus	34.9±1.1	12.8±0.4	40.2±1.4	157.9±3.7	36.4±0.8	286.0±2.1	235.4±2.9	29.2±0.6	134.8±0.5	11.4±2.9
MUA	**Shiitake**	128±12.6	12.7±3.8	8.4±1.6	183.0±18.7	11.7±4.1	543.252.5	455±46.2	13.5±0.7	2.6±0.1	60.7±5.9
	HAS-A	108.1±0.9	22.6±1.5	9.1±0.9	201.7±1.5	9.1±0.5	599.7±5.2	471.5±4.6	9.6±0.2	2.0±0.7	64.4±0.3
	HAS-B	78.1±4.6	32.0±5.5	7.5±0.2	289.4±11.1	13.8±4.0	996.4±44	364±19.2	4.5±1.9	2.3±0.6	61.6±3.3
	A. membranaceus	53.7±2.9	9.1±1.6	44.8±2.5	298.3±25.9	45.1±5	901.7±37.7	313.1±14.6	27.5±0.48	169.0±9.1	16.3±0.5
MUEA	**Shiitake**	193.4±3.8	9.7±2.5	9.2±0.8	385.7±3.8	25.8±0.2	1365.3±23.8	749.2±15.1	21.5±0.46	3.4±1.3	103.5±2
	HAS-A	149.2±7.3	40.7±5.5	7.0±0.9	501.4±43	19.0±8.7	1892.1±97	697.0±35.5	17.5±2.2	3.9±2.2	101.9±5.8
	HAS-B	94.8±9.4	29.2±5.3	10.4±0.2	533.1±40.8	11.0±2.5	2066.0±61	512.9±45.3	10.2±0.21	3.5±0.8	89.8±8
	A. membranaceus	60.0±0.8	14.8±0.2	43.6±1.1	254.5±7.1	61.1±2	730.4±15	303.6±4.9	59.8±1.1	209.8±3	18.5±0.3
MEA	**Shiitake**	194.5±6.3	13.0±1.5	9.6±0.8	392.8±37	22.2±2.4	1352.7±47	785.1±28.3	22.4±0.5	3.2±0.7	109.2±4.1
	HAS-A	158.6±4.2	24.3±1.8	11.8±0.3	566.4±23.9	13.7±9.6	2235.4±70	688.9±22	17.9±3.5	5.7±0.2	97.6±2.7
	HAS-B	174.6±0.8	37.8±3.6	11.1±2.4	858.0±6.6	21.4±12.1	3581.7±27	911.1±2.2	17.1±4.6	3.6±0.3	156.0±6
UEA	***A. membranaceus***	67.9±0.6	8.5±0.4	53.4±1.2	165.1±1	49.5±0.2	634.8±2.5	374.9±6.7	30.9±0.1	192.9±3.2	19.0±0.2
	Shiitake	171.3±1.9	7.7±0.6	8.9±1.1	346.6±0.9	25.1±1.5	1131.7±2.4	663.0±36	24.4±1.9	3.4±0.3	90.8±4.7
	HAS-A	160.3±1.9	9.5±1.3	10.1±1.4	487.3±1.9	21.1±3.9	1876.9±2.6	736.6±41.4	19.4±0.7	4.7±1.3	105.8±6.2
	HAS-B	127.1±1.5	10.0±0.1	13.9±13.1	551.1±1.5	10.7±1.2	2084.1±1.5	557.1±63	12.8±1.2	5.0±0.6	100.7±12

4.5 Effect of different extraction methods on cytotoxicity of polysaccharide extracts in HCT 116 cells

The cytotoxicity of polysaccharide extracts from five different extraction methods for four different samples are shown in Fig. 4.5. The MTT results showed anti-proliferative activity of HAS-A and HAS-B in HCT 116 colorectal carcinoma cells after the treatment of 48 hrs. The growth inhibition response was observed in a dose dependent manner. The highest cytotoxicity for the samples HAS-A, HAS-B and Shiitake (except *A. membranaceus*) was obtained by Ultrasound and Enzyme assisted (UEA) extraction where the extracts at 1.6 mg/ml caused 77 ± 4.24%, 94 ± 2.12% and 89 ± 1.41% of cell death, respectively (Fig. 4.5 F). The IC_{50} value was 0.758 ± 0.037 mg/ml, 0.367 ± 0.044 mg/ml and 0.659 ± 0.043 mg/ml, respectively (Table 4.2). Interestingly, these results clearly showed relatively higher activity of UEA HAS-B compared to control shiitake. However, the difference between these values was not statistically significant (Fig. 4.6 E). On the other hand, the cytotoxicity of above three samples (HAS-A, HAS-B and Shiitake) with other extraction methods (MUEA, MEA, MUA and MA) was very low with IC_{50} values more than three times to that of UEA extraction method (Table 4.2).

In contrast to above extracts, all the enzymatic extracts (MUEA, MEA and UEA) of *A. membranaceus* had lower cytotoxicity compared to non-enzymatic MA and MUA extracts (Fig. 4.5). The microwave assisted (MA) extraction was the best technique in terms of cytotoxicity for *A. membranaceus* where the extract killed 91.5 ± 2.12% of cells at 1.6 mg/ml (Fig. 4.5 B). Interestingly, the MA extracts of HAS-B had also significantly higher anti-proliferative activity ($P<0.01$) with IC_{50} value of 1.276 ± 0.151 mg/ml when compared to shiitake with IC_{50} 2.529 ± 0.252 mg/ml (Fig 4.6 A and Table 4.2).

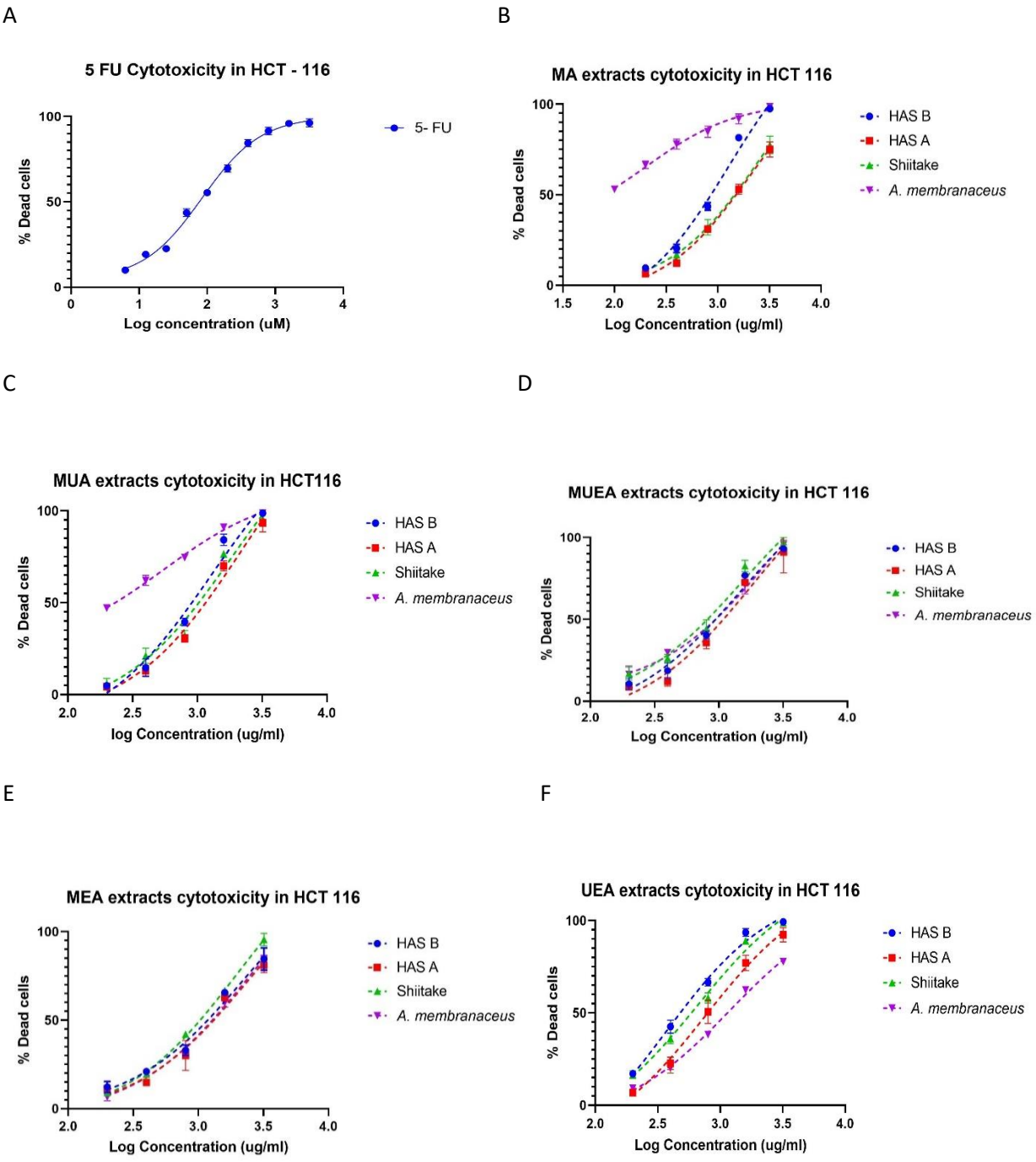
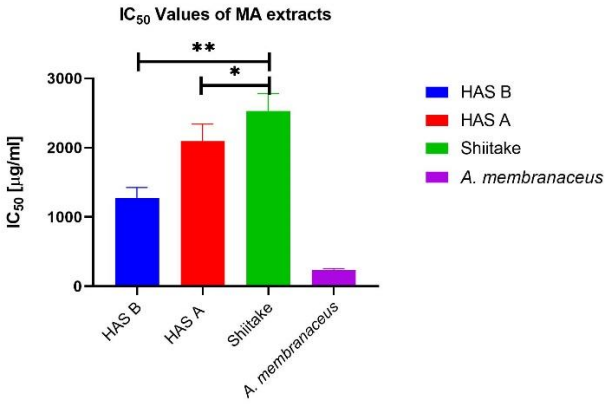


Fig. 4.5: Effect of five different extraction methods on cytotoxicity in HCT 116 after 48 hrs treatment with (200 – 3200) µg/ml of HAS-A, HAS-B, Shiitake and *A. membranaceus* extracts, determined by MTT assay (n=3 with mean ± SD). A) Cytotoxicity of positive control, 5-FU. B) Cytotoxicity of MA extracts. C) Cytotoxicity of MUA extracts. D) Cytotoxicity of MUEA extracts. E) Cytotoxicity of MEA extracts. F) Cytotoxicity of UEA extracts. Detailed experimental conditions are described in Chapter 3.

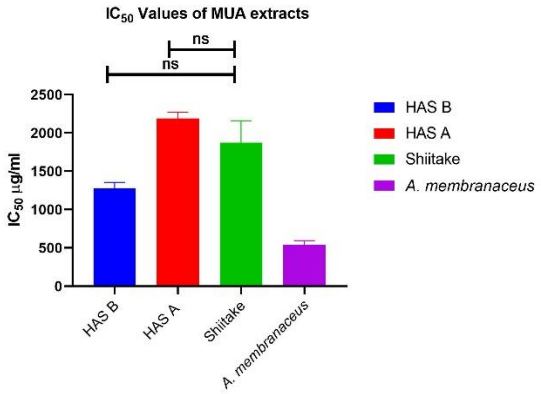
Table 4.2: Effect of five different extraction methods on IC_{50} values for the extracts of HAS-A, HAS-B, Shiitake and *Astragalus* in HCT 116 determined by MTT assay

Extracts	IC_{50} (mg/ml) ± S.D (n=3)			
	HAS-B	**HAS-A**	**Shiitake**	***A. membranaceus***
MA	1.276 ± 0.151	2.095 ± 0.246	2.529 ± 0.252	0.208 ± 0.024
MUA	1.280 ± 0.075	2.188 ± 0.084	1.875 ± 0.284	0.543 ± 0.052
MUEA	1.451 ± 0.303	1.876 ± 0.691	1.508 ± 0.231	2.413 ± 0.542
MEA	1.972 ± 0.240	2.406 ± 0.419	2.001 ± 0.360	2.345 ± 0.147
UEA	0.367 ± 0.044	0.758 ± 0.037	0.659 ± 0.043	1.191 ± 0.183

A.



B.



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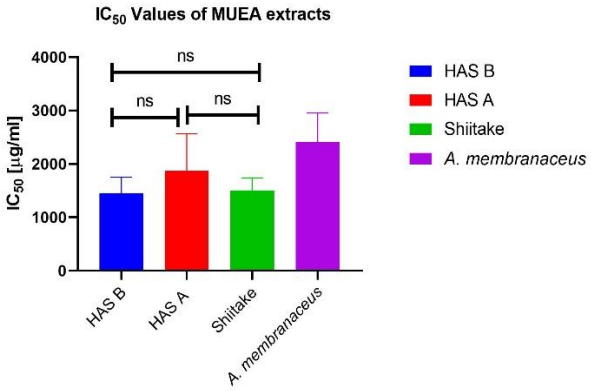
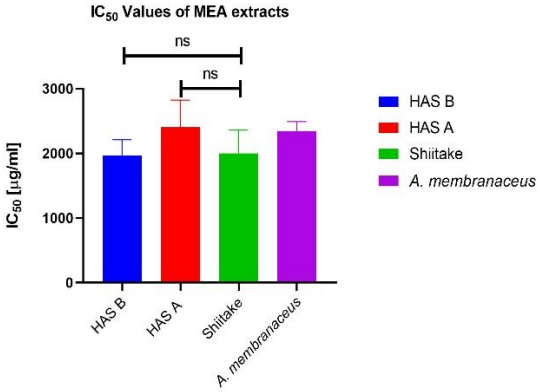


D.



E.

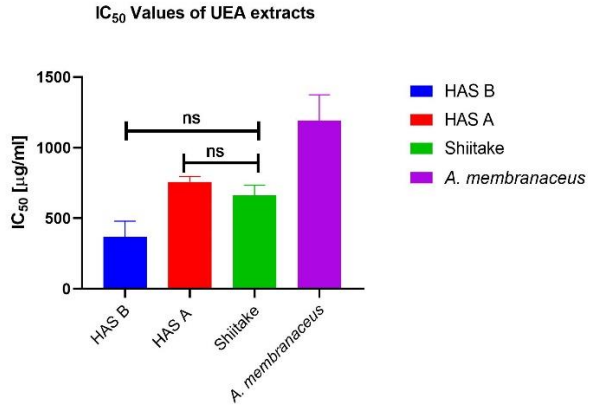


Fig. 4.6: Comparison of the half maximal inhibitory concentration (IC_{50}) values of MA, MUA, MUEA, MEA and UEA extracts of HAS-A, HASB, Shiitake and *A. membranaceus* in HCT 116 cells after 48 hrs treatment, determined by MTT assay. A. (IC_{50}) values for MA extracts where (IC_{50}) value of HAS A and HAS-B is significantly lower than that of shiitake ($P<0.05$ indicated as * and $P<0.01$ indicated as ** respectively). B. (IC_{50}) values for MUA extracts. C. (IC_{50}) values for MUEA extracts. D. (IC_{50}) values for MEA extracts. E. (IC_{50}) values for UEA extracts. P values of HAS-B in all other methods (except MA) are not significantly different to that of shiitake indicated as 'ns'. Detailed experimental conditions are described in Chapter 3.

4.6 Effect of different extraction methods on molecular weight profile of polysaccharide extracts

The molecular weight profiles of polysaccharide extracts from five different extraction methods for four different samples are shown in Table 4.3, 4.4 and Appendix D. The relative molecular weight profile of polysaccharide extracts was different with different extraction methods. For instance, the relative molecular weight of HAS-B ranged from 2.2 – 477.5 k Da for MA extracts, 2.2 – 550 k Da for MUA extracts, 2.7 – 822.8 k Da for MUEA extracts, 2.6 – 814 k Da for MEA extracts and 2.5 – 833 k Da for UEA extracts. Similarly, these values were almost similar for HAS-A (Table 4.3) but the percentage composition of higher molecular weight polysaccharides were less compared to HAS-B. On the other hand, for Shiitake polysaccharide extracts, these values ranged from 2.5 – 12 k Da for MA, 2.2 – 12.34 k Da for MUA, 2.7 – 10.5 k Da for MUEA, 2.8 – 10.4 k Da for MEA and 3.2 – 12.72 k Da for UEA extracts. These results show that HAS-A and

HAS-B contain higher molecular weight polysaccharides, while the same is not present in control Shiitake.

Table 4.3: Relative molecular weight range of MA, MUA, MUEA, MEA and UEA extracts of HAS-B, HAS-A, Shiitake and *A. membranaceus* determined by size exclusion chromatography by HPLC.

Extracts	**Relative Molecular weight range (M_w, k Da)**			
	HAS-B	HAS-A	Shiitake	*A. membranaceus*
MA	2.2 – 477.5	2.3 – 500	2.5 – 12	2.5 – 113
MUA	2.2 – 550	2.3 – 515	2.2 – 12.3	2.2 - 110
MUEA	2.7 – 822.8	2.8 – 915.7	2.7 – 10.5	2.6 – 98.7
MEA	2.6 – 814	2.8 – 1093	2.8 – 10.4	2.2 - 70
UEA	2.5 – 833	2.5 - 976	3.2 – 12.7	2.5 – 100

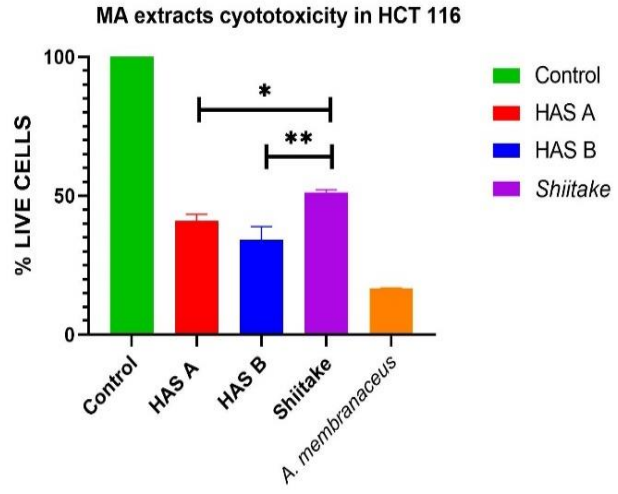
Table 4.4: Relative percentage composition of the major polysaccharide peaks (expressed as M_w, kDa) of HAS-B, HAS-A, Shiitake and *A. membranaceus* eluted by size exclusion chromatography using HPLC with their retention time (Rt)

Extracts	Major Peaks for HAS-B			Major Peaks for HAS-A			Major Peaks for Shiitake			Major Peaks for *Astragalus*		
	Rt, mins	% Concen-tration	M_w, kDa	Rt, mins	% Concen-tration	M_w, kDa	Rt, mins	% Concen-tration	M_w, kDa	Rt, mins	% Concen-tration	M_w, kDa
MA	13.329	2.934	477.450	13.29	2.044	500.355	16.409	30.537	11.797	14.531	9.777	112.648
	15.911	68.246	21.461	16.304	71.551	13.384	17.106	31.443	5.106	16.379	13.396	12.230
	17.812	11.15	2.186	17.772	10.797	2.294	17.673	17.908	2.584	17.093	27.275	5.186
	18.288	9.003	1.234	18.28	10.044	1.246	18.29	18.024	1.231	17.717	29.163	2.451
MUA	13.21	7.555	550.836	13.266	7.246	514.993	16.372	73.144	12.334	14.55	25.281	110.106
	16.042	61.659	18.335	16.349	64.711	12.679	17.808	9.604	2.197	16.232	17.865	14.593
	17.782	12.833	2.266	17.779	11.51	2.275	18.312	10.926	1.199	17.801	24.695	2.215
	18.308	8.442	1.205	18.303	8.386	1.212	19.752	6.327	0.213	18.306	11.753	1.208
MUEA	12.876	20.234	822.817	12.787	4.685	915.679	11.375	75.276	10.500	15.304	1.029	44.501
	15.953	53.484	20.404	16.503	70.536	10.537	16.506	12.763	2.698	16.733	57.15	7.993
	17.638	8.581	2.695	17.604	9.554	2.807	17.637	10.199	1.213	17.671	25.882	2.590
	18.305	6.126	1.209	18.29	4.454	1.231	18.302			18.311	12.152	1.200
MEA	12.885	12.901	813.967	12.64	0.031	1092.567	16.514	80.829	10.399	15.055	1.953	60.021
	16.015	33.56	18.940	16.693	72.565	8.387	17.605	10.873	2.804	16.665	56.546	8.674
	17.037	22.164	5.547	17.604	12.339	2.807	18.307	6.821	1.206	17.82	26.399	2.165
	17.655	10.848	2.640	18.315	7.68	1.195				18.318	10.229	1.190
	18.316	10.973	1.193									
UEA	12.866	12.983	832.763	12.734	0.194	975.885	16.35	20.508	12.72	14.629	4.399	100.135
	16.028	25.044	18.646	17.687	78.288	2.541	17.17	42.988	4.73	17.088	29.413	5.218
	17.719	36.966	2.445	18.326	16.625	1.179	17.5	22.04	3.19	17.706	39.937	2.483
	18.344	15.217	1.154				18.41	12.25	1.05	18.333	11.824	1.169

4.7 Apoptosis determination of HCT 116 treated with selected polysaccharide extracts by Flow-cytometry

The apoptosis of HCT 116 cells induced by selected polysaccharide extracts was further quantified by Flow cytometry using Annexin V/PI and DilC1(5) dye. Only MA and UEA extracts were selected as these extracts showed significant anti-proliferative properties in HCT – 116 through MTT assay. This assay also showed the same order of increasing cytotoxicity to HCT 116 with MA extracts such as, *A. membranaceus* > HAS-B > HAS > Shiitake (Fig.4.7 A). MA HAS-B extract had significantly higher anti-proliferative property ($P < 0.05$) when compared to MA Shiitake extracts, while the same was not significant with HAS-A. The percentage of live cells after 48 hrs treatment with 1.6 mg/ml MA-HAS-B, HAS-A, Shiitake and *A. membranaceus* were found to be 34.11 ± 4.8%, 41.01 ± 2.3%, 51.18 ± 0.99% and 16.69 ± 0.15%, respectively which was comparable and consistent with the MTT results (Fig. 4.7 A and Fig. 4.8 B-E).

A



B

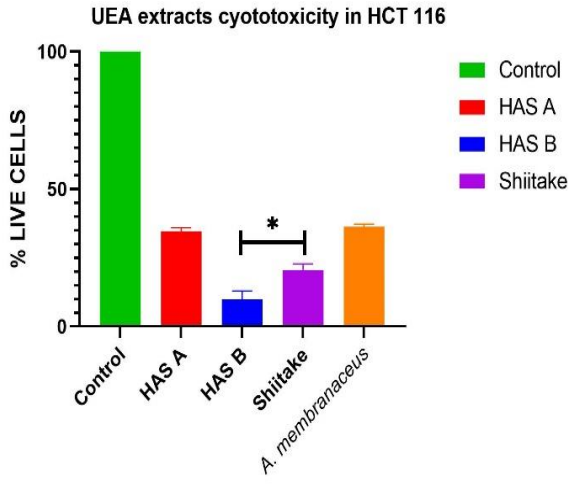


Fig. 4.7 Comparison of apoptosis induced with 1.6 mg/ml of MA and UEA extracts of HAS-A, HAS-B, Shiitake and *A. membranaceus* in HCT 116 after 48 hrs treatment using Flow-Cytometry. A) MA extracts cytotoxicity in HCT 116 where cytotoxicity of HAS-B was significantly high compared to shiitake ($P < 0.01$ indicated as **). B) UEA extracts cytotoxicity in HCT 116 where cytotoxicity of HAS-B was significantly high compared to shiitake ($P < 0.05$ indicated as *).

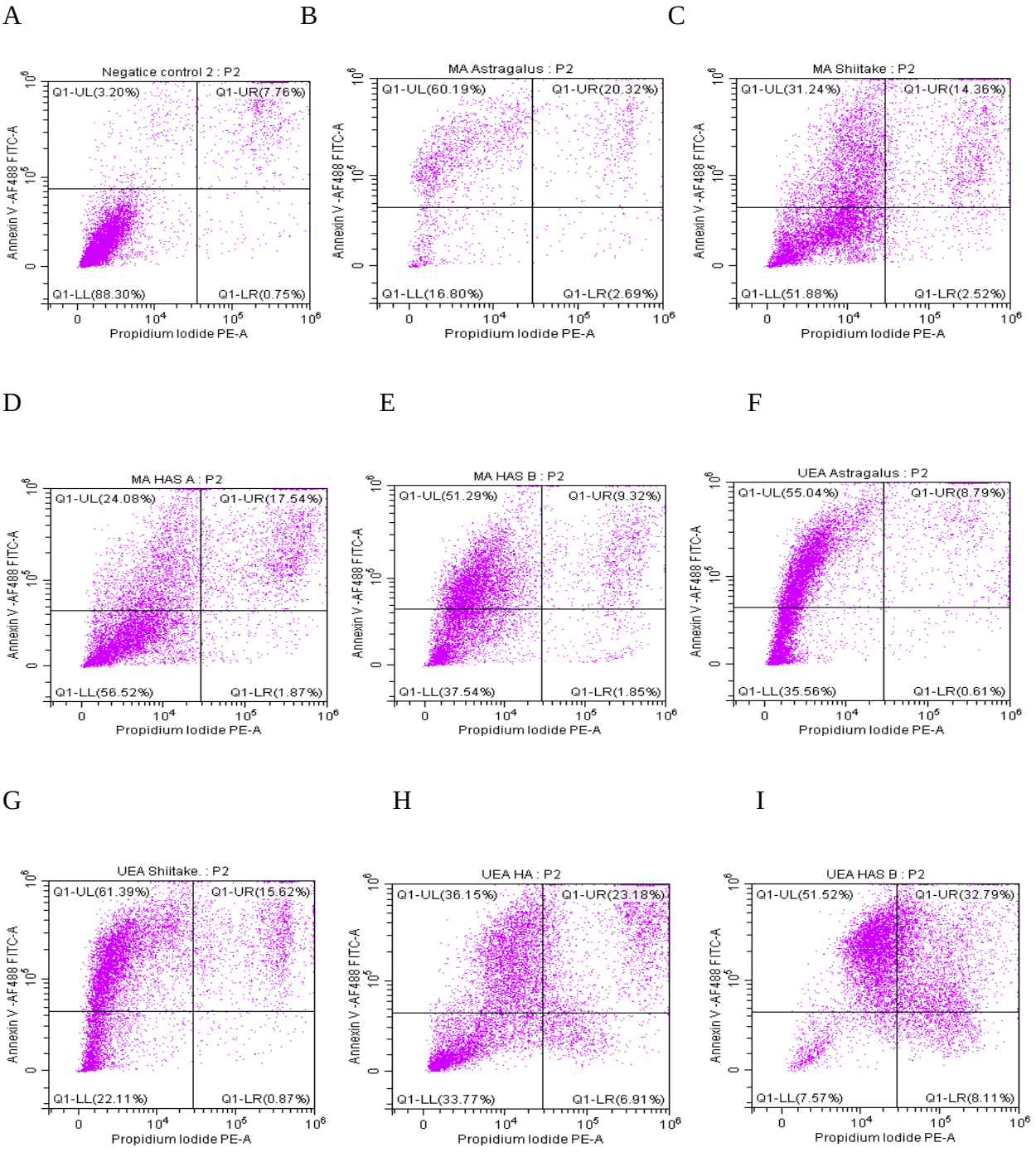


Fig. 4.8 Apoptosis detection in HCT 116 after 48 hrs. treatment with MA and UEA extracts of HAS-A, HAS-B, Shiitake and *A. membranaceus* using Flow-Cytometry/Annexin V/PI dye. (A) Negative control. (B-E) treatment with 1.6 mg/ml MA extracts and (F-I) treatment with 1.6 mg/ml UEA extracts of HAS-A, HAS-B, Shiitake and *A. membranaceus* respectively.

The apoptosis results of UEA extracts were also very similar to that of MTT assay, where the percentage of live cells after 48 hrs treatment with 1.6 mg/ml UEA HAS-B, HAS-A, Shiitake and *A. membranaceus* were found to be 9.78 ± 3.13 %, 34.66 ± 1.26 %, 20.55 ± 2.19 % and 36.28 ± 1.01 %, respectively (Fig. 4.7 B and Fig. 4.8 F-I). The anti-proliferative effect of UEA-HAS-B was significantly higher ($P < 0.05$) compared to shiitake, which was shown not significant during MTT assay (Fig. 4.7 B). Furthermore, the results from flow cytometry as shown in Fig. 4.8 also indicate that most of the cells were in early apoptotic stage after the 48 hrs treatment with each polysaccharide extracts.

4.8 Effect of preliminary purification of selected polysaccharide extracts on purity and cytotoxic activity in HCT 116

Based on the cytotoxicity of all the extracts as summarized in Table 4.2, only the three extracts (extracts of UEA-HAS-B, UEA-Shiitake and MA-*A. membranaceus*) with the lowest IC_{50} values i.e. highest cytotoxicity against HCT 116 were chosen for initial purification. After purification at the specific conditions, the total polysaccharide content (purity) of all these three extracts increased significantly ($P < 0.05$) compared to their respective crude extracts (Fig. 4.9 A). The total polysaccharide content, hence purity for UEA-HAS-B, UEA-Shiitake and MA-*A. membranaceus* increased from 65.4 ± 1.41% to 84.5 ± 3.53%, 46.2 ± 1.4% to 64.5 ± 3.53% and 17.3 ± 2.16% to 24.03 ± 1.12%, respectively (Fig. 4.9 A).

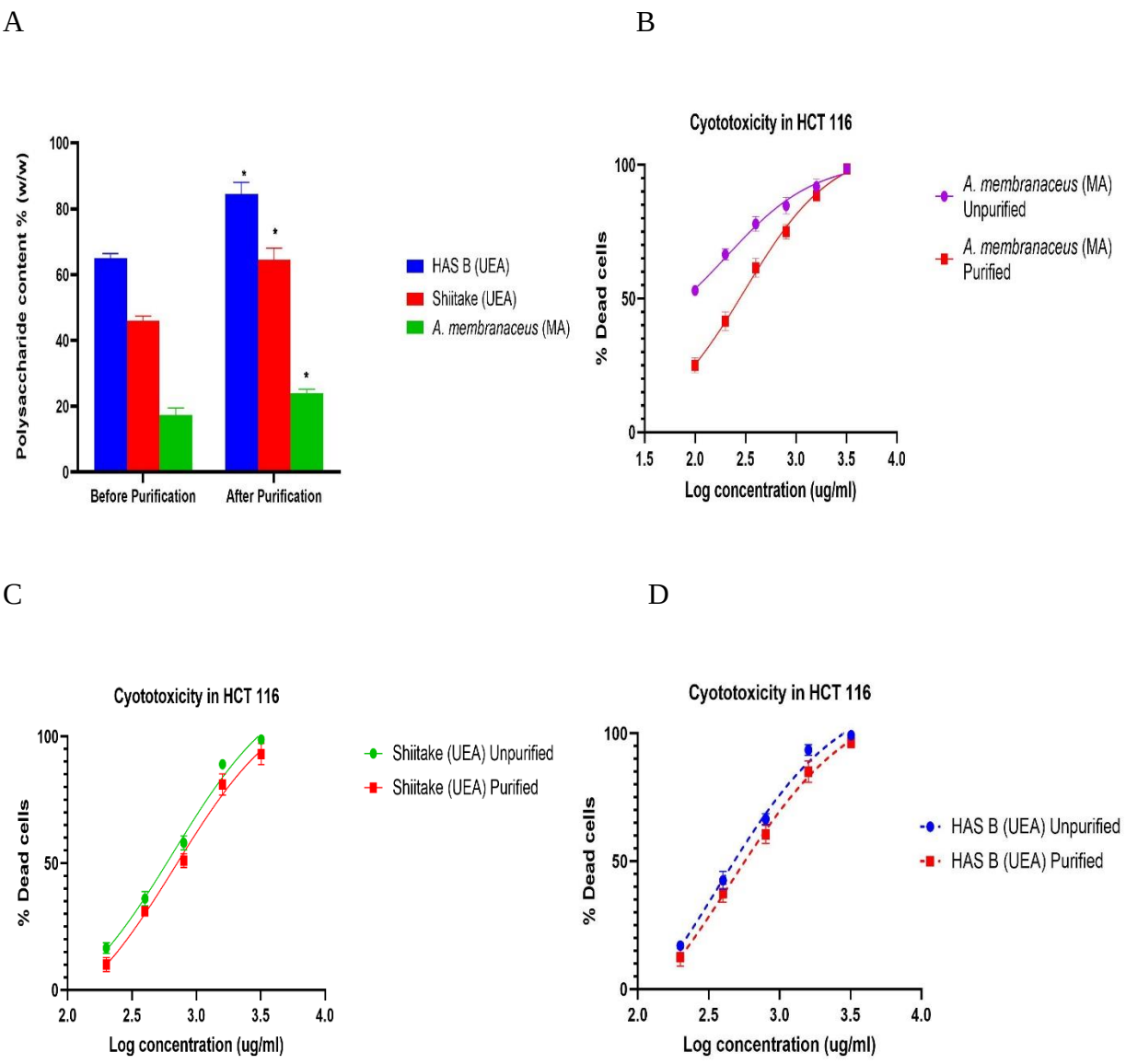


Fig: 4.9 Effect of purification on total polysaccharide content and cytotoxicity in HCT 116. A) Total polysaccharide content of UEA-HAS-B, UEA-Shiitake and MA-*A. membranaceus* extracts before and after purification. All three purified extracts had significantly increase in total polysaccharide content ($P < 0.05$ indicated as *). B), C) and D) show the comparison of cytotoxicity of purified and crude extracts of MA-*A. membranaceus*, UEA-Shiitake and UEA-HASB in HCT 116, respectively. All the purified extracts have shown comparatively low cytotoxicity activity in HCT 116.

Despite significant increase in total polysaccharide content (purity), all these purified extracts had shown comparatively less anti-proliferative activity in HCT 116 than their respective crude extracts (Fig. 4.9 B, C & D). The IC_{50} values of these purified extracts increased from 207.5 µg/ml

to 309.6 µg/ml for MA-*A. membranaceus*, from 659.3 µg/ml to 687.8 µg/ml for UEA-Shiitake and from 367.1 µg/ml to 447.7 µg/ml for UEA-HAS-B against HCT 116 (Appendix E). The same experiment was repeated in three replicates with consistent results. Hence, due to limited time for this Master project, further purification was not carried out.

4.9 Effect of preliminary purification on molecular weight of the extracts

The relative molecular weight of the purified extracts is summarized in the Table 4.5 below. Surprisingly, the molecular weights of the purified extracts were lower compared to the unpurified extracts. The molecular weight range of UEA-HAS-B decreased from 2.5 – 833 k Da to 1.9 – 664.39 k Da. Similarly, in case of UEA-Shiitake it decreased from 3.2 – 12.72 k Da to 1.96 – 11.28 k Da and likewise from 2.5 – 113 k Da to 1.8 – 100.01 k Da in case of MA – *A. membranaceus*.

Table 4.5 Relative molecular weight range of UEA-HAS-B, UEA-Shiitake and MA-*A. membranaceus* extracts before and after purification determined by size exclusion chromatography by HPLC.

Extracts	**Relative Molecular weight range (M_w, kDa)**	
	Before Purification	**After purification**
UEA-HAS-B	2.5 – 833	1.9 – 664.39
UEA-Shiitake	3.2 – 12.72	1.96 – 11.28
MA- *A. membranaceus*	2.5 – 113	1.8 – 100.01

CHAPTER 5: DISCUSSION

The results from our experiments suggest that, HAS-A and HAS-B have a significantly higher amount of polysaccharides than the shiitake grown under controlled conditions. The increase of total polysaccharides in HAS-B and HAS-A was nearly ~45% w/w and ~31%w/w, respectively compared to that in shiitake. The anti-proliferative studies conducted against HCT 116 also suggest that the polysaccharides from HAS-B have significantly higher anti-proliferative activity compared to Shiitake, however, this effect was not significantly different in the case of HAS-A. In addition, the anti-proliferative effects of the polysaccharide extracts changed with different methods and conditions of extraction. Our studies showed that the Ultrasound-Enzyme assisted (UEA) method was the best method for the extraction of HAS-B, HAS-A and Shiitake in terms of exhibiting higher cytotoxic activity against HCT 116. The major findings of this project are discussed in detail below.

5.1 Significant impact of different extraction methods on polysaccharide extraction yield, polysaccharide content and apoptotic activity

Different methods of extraction and different extraction conditions had significant effects on the total polysaccharide yield (Figure 4.1). This study found that MUA extraction was the best method to achieve the highest yield for HAS-B, HAS-A and Shiitake of 18.34 ± 0.55%, 16.5 ± 0.42% and 12.6 ± 0.4 % w/w, respectively. However, all the enzymatic assisted extractions (MUEA, MEA and UEA) were not effective in terms of increasing the polysaccharide yield. In contrast to our study, Yin et al. (2018) had shown the highest yield of shiitake polysaccharides using microwave-ultrasound-enzyme (MUEA) assisted method which was 9.38% (which was comparatively lower than our highest yield, 13.6 ± 0.4 % w/w using MUA method).

The reason for the decreased yield using enzymes in our case would be likely the modifications in previously reported extraction conditions. In previous experiments (Cheng et al. 2015; Yin et al. 2018), the enzymatic hydrolysis was carried out for two hours before exposing to microwave or ultrasonic power. However, during our preliminary study, these conditions worked well only in terms of increasing the polysaccharide yield and did not work in terms of providing higher anti-proliferative activity. For example, the yield using the previous method of Yin et al. (2018) in UEA for HAS-B was very high at 18.12% w/w but its IC_{50} value against HCT 116 was ~ 2.56 mg/ml. While at the same time, our modified method had a comparatively low yield of 11.1% w/w but had significantly higher antiproliferative activity in HCT 116 with IC_{50} value of 438.6 μg/ml. Hence, the modified condition in our experiment (exposing to microwave or ultrasonic power initially followed by enzyme hydrolysis for 20 min.) was used with comparatively lower yield but significantly higher anti-proliferative activity ($P < 0.001$, in case of UEA HAS-B) compared to that of previously reported methods (data shown in Appendix D). Therefore, since the enzymes were added in the sample/solvent mixture after exposing them to microwave/ultrasound in MEA, MUEA and UEA extractions, these enzymes mixture would have caused partial hydrolysis of the extracted polysaccharides that led to a decrease in total polysaccharide yield (Cheng et al. 2015).

In contrast to the yield, the total polysaccharide content of the crude extracts increased significantly with all enzyme assisted extraction. This can be attributed to the fact that the use of enzymes helps in easy degradation of cell wall thus enhancing the release of intracellular components in the extraction medium (Zhang et al. 2017). Among all the enzymatic extracts (MUEA, MEA and UEA), MUEA extracts had the highest polysaccharide content which could be due to the synergistic actions of enzyme, microwave and ultrasound that makes the parenchymal cells ultrathin and then disrupts the cells to release the cellular components in the external environment

(Cheng et al. 2015). Compared MEA and UEA, MEA extracts had higher polysaccharide content. This indicates that MEA extraction inputs more energy to the materials to rupture the cells and hence release more intracellular components than UEA extraction. Similar effects were observed in the experiments of Gil-Ramírez et al. (2019) where MEA extraction had caused a severe rupture of the cell walls in a sample compared to UEA (when observed under SEM) that helped to release more polysaccharides.

More importantly, it was expected that extracts with higher polysaccharide content would also exhibit higher cytotoxicity activity but such a pattern was not observed in our experiments. Although MUEA and MEA extraction methods had higher polysaccharide content compared to UEA extracts, they had lower cytotoxicity activity compared to UEA extracts. This could be due to either the conformational changes in the polysaccharide structure, or the molecular weight changes caused by highly efficient microwave irradiation (Zhao et al. 2016). As indicated in Table 4.1 and 4.3, these changes in monosaccharide composition and molecular weight profile are evident. The changes in structural conformation/configuration of a molecule could be determined in the future work.

5.2 New enzyme mixture does not affect the total polysaccharide yield but increase their cytotoxicity activity

During our preliminary experiment, we had compared the polysaccharide yield and anti-proliferative effect of extracts prepared from previously reported enzyme mixture (Papain: Cellulase: Pectinase in a ratio 1:1:1) by (Yin, et al. 2018; Zhao et al. 2016) with our new enzyme mixture (Alcalase: Cellulase; Pectinase: VinoTaste Pro® at a ratio of 1:1:1:1). This test was only conducted in UEA-HAS-B sample. There was no statistically significant difference in the total

polysaccharide yield between the extracts prepared from the previously reported and new enzyme mixture. But interestingly, the effect of extracts prepared from our new enzyme mixture had a significantly higher anti-proliferative activity ($P < 0.01$, for UEA HAS-B) compared to the previously reported enzyme mixture (data shown in Appendix E). For example, the IC_{50} value of UEA-HAS-B extract using the previously reported enzyme mixture (Papain: Cellulase: Pectinase in a ratio 1:1:1) was 1,809.5 µg/ml while the same sample extracted with our new enzyme mixture (Alcalase: Cellulase; Pectinase: VinoTaste Pro® in a ratio of 1:1:1:1) had IC_{50} value of 438.6 µg/ml (data shown in Appendix E). Therefore, the new enzyme mixture was chosen for all the enzyme assisted extractions in this project. The reason for higher anti-proliferative activity could be the use of extra enzyme VinoTaste Pro®, the exo - (1,3) β glucanase, which would have cut the long (1,3) β glucan chain into a relatively shorter chain. The relatively smaller molecular weight polysaccharides can easily cross the biological membranes and show improved biological activity compared to the larger ones (Li et al. 2016; Shang et al. 2018).

5.3 Preliminary purification increases total polysaccharide content but decreases the cytotoxicity activity

The purification process and conditions increased the total polysaccharide content of all the three selected crude extracts (UEA-HAS-B, UEA-Shiitake and MA-*A. membranaceus*) significantly ($P<0.05$) compared to their unpurified crude extracts, but their antiproliferative activity decreased (Fig. 4.9). Despite the increase in total polysaccharide content, the relative decrease in cytotoxicity activity may be attributed to the possible change in conformation or molecular weight profiles of the polysaccharides due to the high ionic strength of the elution buffer, NaCl. The ionic strength of NaCl buffer used in our experiment was between 0.1 – 2.0 M concentration which was chosen based on the previous studies (Chen et al. 2012; Peng et al. 2016). This concentration was

comparatively higher than that of other experiments (Yin et al. 2017; You et al. 2013) where the highest concentration of NaCl buffer used for elution of polysaccharides was 0.3 and 0.5 M respectively. Furthermore, the study of Carneiro-da-Cunha et al. (2011) demonstrated the significant decrease in size and change in conformation of a polysaccharide molecule with the increasing ionic concentration. Eventually, when we conducted the molecular weight determination of these purified extracts, their molecular weights were significantly lower when compared to their unpurified crude extracts. Thus, it can be inferred that high ionic concentrations can cause a change in the molecular weight profile and/or conformation of polysaccharides, leading to lower cytotoxicity.

5.4 Correlation between methods of extraction, polysaccharide content and cytotoxicity

The total polysaccharide content of the extracts increased significantly with enzyme assisted extractions (Fig. 4.2 and Fig. 4.4). This can be attributed to the fact that the use of enzymes helps in easy degradation of cell wall thus enhancing the release of intracellular components in the extraction medium (Zhang et al. 2017). Among all the enzymatic extracts (MUEA, MEA and UEA), MUEA extracts had the highest polysaccharide content likely due to the synergistic actions of enzyme, microwave and ultrasound that make the parenchymal cells ultrathin and then disrupt the cells to release the cellular components in the external environment (Cheng et al. 2015). Compared to MEA and UEA, MEA extracts had higher polysaccharide content. This result indicates that MEA extraction inputs more energy to the materials to rupture the cells and hence release more intracellular components than UEA extraction. Similar effects were observed in the experiments of Gil-Ramírez et al. (2019) where MEA extraction had caused a severe rupture of

the cell walls in a sample compared to UEA (when observed under SEM) that helped to release more polysaccharides.

It was expected that extracts with higher polysaccharide content would exhibit higher cytotoxicity but such a pattern was not observed. Although MUEA and MEA extraction methods had higher polysaccharide content compared to UEA extracts, they had lower cytotoxicity compared to UEA extracts. The same results have been observed when three selected crude extracts were purified. The purified polysaccharides had lower cytotoxicity when compared to their respective crude extracts. Our analysis of molecular weight profile and also the monosaccharide composition indicates significant changes in size and composition of polysaccharides likely caused by high efficient microwave irradiation (Zhao et al. 2016) or the high salt concentration in purification. Further work to determine these specific changes would be required in the future in order to optimize the extraction and purification conditions for improved cytotoxicity.

5.5 Correlation between M_w and Cytotoxicity of polysaccharide extracts

According to Zhang et al. (2005), the anti-proliferative activity of a polysaccharide molecule is directly related to its molecular weight and triple helix conformation. Wang et al. (2017) further added that glucans with the molecular weights below 50 kDa cannot form a triple helix structure and hence cannot show prominent anti-proliferative activity. Liu et al. (2018) also concluded that the glucans with molecular weight between 290 to 2420 kDa exist as a triple helix conformation and that with the molecular weight of more than 1×10^6 Da had better immune-enhancing property. However, our studies had shown significantly different results compared to the above mentioned literatures. In our study, the highest cytotoxicity of HAS-B, HAS-A and *A. membranaceus* in HCT 116 was obtained with the polysaccharide extracts of M_w ~2.5 – 832.7 kDa, ~ 2.5 – 976 kDa and

~ 2.5 – 113 k Da, respectively. Meanwhile, this molecular weight range was even lower in case of Shiitake where the highest cytotoxicity was shown by the polysaccharide extracts of M_w ~ 3.2 – 12.7 k Da (Table 4.3 and 4.4) which is completely different (extremely low) than that mentioned in the literature. Therefore, in the future, determining the relationship between the molecular weight and bioactivities including cytotoxicity of these polysaccharides would be critical. Nonetheless, many literatures reported that high molecular weight polysaccharides show better bio-activity only *in-vitro* but when it was given *in-vivo*, the immune system was highly triggered, and the molecules were engulfed by the phagocytes and macrophages and completely cleared off (Hao et al. 2016; Lemmon et al. 2012). This suggests that the polysaccharide extracts with lower molecular weights in our study, would have better anti-proliferative effects *in-vivo*, which is to be determined in the future studies.

5.6 Comparing cytotoxic effect of the polysaccharide extracts with literature

When the cytotoxic effect of polysaccharide extracts from Shiitake, HAS-A and HAS-B was compared with the literature, the extracts from this study showed comparatively higher cytotoxic activity than that of previously published reports. Zhao et al. (2016) reported nearly 71% cell death in HCT 116 and nearly 73% cell death in Hela cells at 1.8 mg/ml of Shiitake polysaccharides. Another experiment conducted by Jeff et al. (2013) had shown the highest cytotoxicity of Shiitake polysaccharides at 5 mg/ml where the percentage inhibition of S-180, HCT 116 and HT 29 with their best fraction was ~ 97%, ~ 64% and ~ 53%, respectively. Furthermore, Unursaikhan et al. (2006) generated O-sulfonated derivative of lentinan for better solubility and anti-proliferative activity where the so modified derivative had the highest inhibition of ~ 68% at 5 mg/ml in S-180 cells.

The results from our studies showed better anti-proliferative activity i.e. ~ 89% and ~ 77% inhibitory effect in HCT 116 with 1.6 mg/ml of UEA-Shiitake and HAS-A polysaccharides. In the meantime, this inhibitory effect was more prominent with UEA-HAS-B polysaccharides i.e. ~ 94% of inhibition at 1.6 mg/ml (Fig. 4.5). This indicates that the modifications made in our experiments during extraction are effective for extraction of bioactive polysaccharides from Shiitake, HAS-A and HAS-B.

5.7 Conclusion

In conclusion, shiitake mushrooms grown in the substrate beds supplemented with *Astragalus membranaceus*, (designated as HAS-A and HAS-B in this project) have a significantly higher amount of polysaccharides when compared to Shiitake grown under controlled conditions. The anti-proliferative activity of the polysaccharide extracts from HAS-A is not significantly different from that of the control Shiitake. However, the polysaccharide extracts from HAS-B has significantly higher anti-proliferative activity compared to the control Shiitake when optimum extraction conditions are used. Compared to MA, MUA, MUEA, MEA and UEA extracts, UEA extracts had significantly high anti-proliferative activity in HCT 116. In addition, the extracts from UEA extraction method of the current study had significantly higher anti-proliferative activity in HCT 116 compared to previously reported studies. Likewise, the preliminary purification significantly improved the polysaccharide content, but decreased the cytotoxicity, likely due to the changes in molecular conformation, composition and molecular weight. Hence, optimization of UEA extraction method and subsequent efficient purification in the future would help to further potentiate the activity of HAS-B which may be eventually developed as a complementary cancer treatment that is more natural with less side effects.

5.8 Limitations

While this is a 9-months Master research project, it is expected that several limitations would impact on our research outcomes to be comprehensive and conclusive. These limitations include:

- Although MA and MUA extraction methods provided a very high yield of polysaccharides, these polysaccharide powders when dissolved in water at room temperature were not completely soluble (Appendix I). The solution had very finely suspended particles that would give false reading unless otherwise centrifuged and passed through a membrane filter. This problem was severe with *A. membranaceus* powder. The problem could be either due to not enough centrifugation power/time during the collection of supernatant or insolubility of the extracted polysaccharides at or below room temperature.
- The above problem disappeared for all the samples after the use of enzymatic extraction methods except for *A. membranaceus*. The extracts would form a solution at room temperature but when stored at a cold temperature (4^0C) over night, fine particles appeared and then sedimented at the bottom which caused a loss of extracts.
- The high ionic concentration of NaCl used during preliminary purification led to a decrease in molecular weight of a compound and hence decreased cytotoxic activity compared to an unpurified crude extracts. Hence the purification condition needs to be optimized in future experiments.
- Apoptosis detection using Flow cytometry could be performed for only MA and UEA extracts (in just two replicates) due to time constraints. Therefore, in the future, we expect to optimize purification process and also perform enough replicates to produce more reliable/strong data.

- The previously optimized extraction conditions are good only in terms of enhancing yield but not effective in terms of providing higher cytotoxicity. Therefore, further optimization of extraction conditions is necessary in-order to produce highly bioactive polysaccharides that are effective both *in-vitro* as well as *in-vivo*.
- During HPLC analysis for monosaccharide composition analysis, two unknown peaks were found in the samples which could not be determined due to the lack of reference standards at high molecular weight for those peaks. Hence in the future samples would be subjected to structural identification using IR and NMR spectroscopy techniques.
- While determining molecular weight of the extracts using size exclusion HPLC, the polysaccharide extracts with molecular weight more than 1050 k Da could not be quantified since the highest molecular weight (Dextran) standard used in our experiment was 1050 k Da. Therefore, in the future a more reliable method, MALDI – MS could be utilized in order to determine the precise molecular weight of the extracts.

5.9 Future prospects

- Further optimization of the UEA extraction method needs to be carried out which would help to increase the yield and improve biological activity at the same time.
- Optimization of purification conditions and complete purification of HAS-B extract is highly essential for precise structural and physicochemical elucidation which would further help to correlate between molecular weight, structural conformation and cytotoxic activities in more scientific and justifiable manner.

- RT-PCR, Western blot and various immunofluorescence assays could be conducted to determine gene expression and immune modulation properties.
- *In-vivo* anti-proliferative activities could be determined by implanting tumor xenografts in mice that would help to assess the similarity or differences in treatment between *in-vitro* and *in-vivo* conditions.
- Finally developing these polysaccharides into suitable formulations that would have higher efficacy and effectiveness against cancer cells.

References

Akazawa, N, Taguchi, K, Imai, A, Kikuchi, H, Minato, M & Iwaki, H 2010, 'A case of advanced gastric cancer responding to S-1/paclitaxel/lentinan as neoadjuvant chemoimmunotherapy', Gan to Kagaku Ryoho. Cancer & Chemotherapy, vol. 37, no. 7, pp. 1365-7.

Altena, R, Perik, PJ, Van Veldhuisen, DJ, De Vries, EG & Gietema, JA 2009, 'Cardiovascular toxicity caused by cancer treatment: strategies for early detection', The Lancet Oncology, vol. 10, no. 4, pp. 391-9.

Alzorqi, I, Sudheer, S, Lu, T-J & Manickam, S 2017, 'Ultrasonically extracted β-d-glucan from artificially cultivated mushroom, characteristic properties and antioxidant activity', Ultrasonics Sonochemistry, vol. 35, pp. 531-40.

Amodu, OA, Kuo, K-T, Wang, C-H, Huang, W-C, Wu, AT, Tsai, J-T, Lee, K-Y, Yeh, C-T & Wang, L-S 2019, '*Astragalus* polysaccharides (PG2) enhances the M1 polarization of macrophages, functional maturation of dendritic cells, and T cell-mediated anticancer immune responses in patients with lung cancer', Nutrients, vol. 11, no. 10, pp. 2264.

Amigh, S & Taghian Dinani, S 2020, 'Combination of ultrasound-assisted aqueous enzymatic extraction and cooking pretreatment for date seed oil recovery', Heat and Mass Transfer, pp. 1-10.

Ayeka, PA, Bian, Y, Githaiga, PM & Zhao, Y 2017, 'The immunomodulatory activities of licorice polysaccharides (*Glycyrrhiza uralensis* Fisch.) in CT 26 tumor-bearing mice', BMC Complementary and Alternative Medicine, vol. 17, no. 1, pp. 536.

Bao, X, Yuan, H, Wang, C, Liu, J & Lan, M 2013, 'Antitumor and immunomodulatory activities of a polysaccharide from *Artemisia argyi*', Carbohydr Polym, vol. 98, no. 1, pp. 1236-43.

Bisen, P, Baghel, RK, Sanodiya, BS, Thakur, GS & Prasad, G 2010, *'Lentinus edodes*: a macrofungus with pharmacological activities', Current Medicinal Chemistry, vol. 17, no. 22, pp. 2419-30.

Blackadar, CB 2016, 'Historical review of the causes of cancer', World Journal of Clinical Oncology, vol. 7, no. 1, pp. 54-86.

Calaminus, G, Bamberg, M, Jürgens, H, Kortmann, R, Sörensen, N, Wiestler, O & Göbel, U 2004, 'Impact of surgery, chemotherapy and irradiation on long term outcome of intracranial malignant non-germinomatous germ cell tumors: results of the German Cooperative Trial MAKEI 89', Klinische Pädiatrie, vol. 216, no. 03, pp. 141-9.

Carneiro-da-Cunha, MG, Cerqueira, MA, Souza, BW, Teixeira, JA & Vicente, AA 2011, 'Influence of concentration, ionic strength and pH on zeta potential and mean hydrodynamic diameter of edible polysaccharide solutions envisaged for multinanolayered films production', Carbohydrate Polymers, vol. 85, no. 3, pp. 522-8.

Chao, L, Zhou, J, Zhu, X-x, Ni, W-p, Wang, H-d & Wang, Q 2012, 'Effect of *Astragalus* polysaccharides on proliferation and cell cycle of human gastric carcinoma cell line MKN45 [J]', Chinese Archives of Traditional Chinese Medicine, vol. 11.

Chen, F, Ran, L, Mi, J, Yan, Y, Lu, L, Jin, B, Li, X & Cao, Y 2018, 'Isolation, characterization and antitumor effect on DU145 cells of a main polysaccharide in pollen of chinese wolfberry', Molecules, vol. 23, no. 10, pp. 2430.

Chen, G-t, Ma, X-m, Liu, S-t, Liao, Y-l & Zhao, G-q 2012, 'Isolation, purification and antioxidant activities of polysaccharides from *Grifola frondosa*', Carbohydrate Polymers, vol. 89, no. 1, pp. 61-6.

Chen, Z, Zhang, W, Tang, X, Fan, H, Xie, X, Wan, Q, Wu, X & Tang, JZ 2016, 'Extraction and characterization of polysaccharides from *Semen cassiae* by microwave-assisted aqueous two-phase extraction coupled with spectroscopy and HPLC', Carbohydrate Polymers, vol. 144, pp. 263-70.

Cheng, Z, Song, H, Yang, Y, Liu, Y, Liu, Z, Hu, H & Zhang, Y 2015, 'Optimization of microwave-assisted enzymatic extraction of polysaccharides from the fruit of *Schisandra chinensis* Baill', International journal of biological macromolecules, vol. 76, pp. 161-8.

Cheok, CF 2012, 'Protecting normal cells from the cytotoxicity of chemotherapy', Cell Cycle (Georgetown, Tex.), vol. 11, no. 12, pp. 2227-8.

Chester, JD, Hall, GD, Forster, M & Protheroe, AS 2004, 'Systemic chemotherapy for patients with bladder cancer – current controversies and future directions', Cancer Treatment Reviews, vol. 30, no. 4, pp. 343-58.

Chihara, G, Maeda, Y, Hamuro, J, Sasaki, T & Fukuoka, F 1969, 'Inhibition of mouse sarcoma 180 by polysaccharides from *Lentinus edodes* (Berk.) sing', Nature, vol. 222, no. 5194, pp. 687-8.

Chikari, F, Han, J, Wang, Y & Wenmei, A 2020, 'Synergized subcritical-ultrasound-assisted aqueous two-phase extraction, purification, and characterization of mushroom polysaccharides', Process Biochemistry, vol. 95, pp. 297-306.

Cook, LB, Melamed, A, Niederer, H, Valganon, M, Laydon, D, Foroni, L, Taylor, GP, Matsuoka, M & Bangham, CR 2014, 'The role of HTLV-1 clonality, proviral structure, and genomic integration site in adult T-cell leukemia/lymphoma', Blood, The Journal of the American Society of Hematology, vol. 123, no. 25, pp. 3925-31.

Crown, J, Walshe, J, Fennelly, D, Long, J, Cairney, S, McDonnell, D, Ballot, J, Wildes, D, Sills, E & Gullo, G 2017, 'Incidence of permanent alopecia following adjuvant chemotherapy in women with early stage breast cancer', Annals of Oncology, vol. 28, no. 5, pp. 362-63.

Del Buono, A, Bonucci, M, Pugliese, S, D'ORTA, A & Fioranelli, M 2016, 'Polysaccharide from *Lentinus edodes* for integrative cancer treatment: immunomodulatory effects on lymphocyte population', WCRJ, vol. 3, no. 1, pp. 1-7.

Economou, CN, Diamantopoulou, PA & Philippoussis, AN 2017, 'Valorization of spent oyster mushroom substrate and laccase recovery through successive solid state cultivation of *Pleurotus*, *Ganoderma*, and *Lentinula* strains', Applied Microbiology and Biotechnology, vol. 101, no. 12, pp. 5213-22.

Fan, Y, Hu, Y, Wang, D, Liu, J, Zhang, J, Zhao, X, Liu, X, Liu, C, Yuan, J & Ruan, S 2012, 'Effects of *Astragalus* polysaccharide liposome on lymphocyte proliferation *in vitro* and adjuvanticity *in vivo*', Carbohydrate Polymers, vol. 88, no. 1, pp. 68-74.

Finimundy, T, Gambato, G, Fontana, R, Camassola, M, Salvador, M, Moura, S, Hess, J, Henriques, J, Dillon, A & Roesch-Ely, M 2013, 'Aqueous extracts of *Lentinula edodes* and *Pleurotus sajor-caju* exhibit high antioxidant capability and promising in vitro antitumor activity', Nutrition Research, vol. 33, no. 1, pp. 76-84.

Finimundy, TC, Scola, G, Scariot, FJ, Dillon, AJ, Moura, S, Echeverrigaray, S, Henriques, JP & Roesch-Ely, M 2018, 'Extrinsic and intrinsic apoptotic responses induced by shiitake culinary-medicinal mushroom *Lentinus edodes* (Agaricomycetes) aqueous extract against a larynx carcinoma cell line', International Journal of Medicinal Mushrooms, vol. 20, no. 1, pp. 1-14.

Gil-Ramírez, A, Smiderle, FR, Morales, D, Iacomini, M & Soler-Rivas, C 2019, 'Strengths and weaknesses of the aniline-blue method used to test mushroom (1→ 3)-β-d-glucans obtained by microwave-assisted extractions', Carbohydrate Polymers, vol. 217, pp. 135-43.

Griffiths, AJ, Miller, JH, Suzuki, DT, Lewontin, RC & Gelbart, WM 2000, 'Relation between mutagens and carcinogens', in An Introduction to Genetic Analysis. 7th edition, WH Freeman.

Hao, L, Sheng, Z, Lu, J, Tao, R & Jia, S 2016, 'Characterization and antioxidant activities of extracellular and intracellular polysaccharides from *Fomitopsis pinicola*', Carbohydrate Polymers, vol. 141, pp. 54-9.

He, X, Li, X, Liu, B, Xu, L, Zhao, H & Lu, A 2011, 'Down-regulation of Treg cells and up-regulation of TH1/TH2 cytokine ratio were induced by polysaccharide from Radix Glycyrrhizae in H22 hepatocarcinoma bearing mice', Molecules, vol. 16, no. 10, pp. 8343-52.

He, X, Shu, J, Xu, L, Lu, C & Lu, A 2012, 'Inhibitory effect of *Astragalus* polysaccharides on lipopolysaccharide- induced TNF-α and IL-1β production in THP-1 cells', Molecules, vol. 17, no. 3, pp. 3155-64.

Hobbs, C 2000, 'Medicinal value of *Lentinus edodes* (Berk.) Sing.(Agaricomycetideae). A literature review', International journal of medicinal mushrooms, vol. 2, no. 4, pp. 287-302.

Hori, T, Ikehara, T, Takatsuka, S, Fukuoka, T, Tendo, M, Tezuka, K, Dan, N, Nishino, H & Hirakawa, K 2011, 'Combination chemotherapy of S-1/low-dose CDDP/lentinan for advanced gastric cancer', Gan to Kagaku Ryoho. Cancer & Chemotherapy, vol. 38, no. 2, pp. 293-5.

Housman, G, Byler, S, Heerboth, S, Lapinska, K, Longacre, M, Snyder, N & Sarkar, S 2014, 'Drug resistance in cancer: an overview', Cancers, vol. 6, no. 3, pp. 1769-92.

Huang, W-H, Liao, W-R & Sun, R-X 2016, 'Astragalus polysaccharide induces the apoptosis of human hepatocellular carcinoma cells by decreasing the expression of Notch1', International Journal of Molecular Medicine, vol. 38, no. 2, pp. 551-7.

Ina, K, Furuta, R, Kataoka, T, Kayukawa, S, Ina, H & Yoneda, M 2016, 'Chemo-immunotherapy using lentinan for the treatment of gastric cancer with liver metastases', Medical Sciences, vol. 4, no. 2, pp. 8.

Ina, K, Furuta, R, Kataoka, T, Kayukawa, S, Yoshida, T, Miwa, T, Yamamura, Y & Takeuchi, Y 2011, 'Lentinan prolonged survival in patients with gastric cancer receiving S-1-based chemotherapy', World Journal of Clinical Oncology, vol. 2, no. 10, pp. 339.

Iqbal, M, Tao, Y, Xie, S, Zhu, Y, Chen, D, Wang, X, Huang, L, Peng, D, Sattar, A & Shabbir, MAB 2016, 'Aqueous two-phase system (ATPS): an overview and advances in its applications', Biological Procedures Online, vol. 18, no. 1, pp. 18.

James, ND, de Bono, JS, Spears, MR, Clarke, NW, Mason, MD, Dearnaley, DP, Ritchie, AW, Amos, CL, Gilson, C & Jones, RJ 2017, 'Abiraterone for prostate cancer not previously treated with hormone therapy', New England Journal of Medicine, vol. 377, no. 4, pp. 338-51.

Jeff, IB, Yuan, X, Sun, L, Kassim, RM, Foday, AD & Zhou, Y 2013, 'Purification and in vitro anti-proliferative effect of novel neutral polysaccharides from Lentinus edodes', International Journal of Biological Macromolecules, vol. 52, pp. 99-106.

Ji, X, Peng, Q & Wang, M 2018, 'Anti-colon-cancer effects of polysaccharides: A mini-review of the mechanisms', International Journal of Biological Macromolecules, vol. 114, pp. 1127-33.

Jia, R, Cao, L, Xu, P, Jeney, G & Yin, G 2012, *'In vitro* and *in vivo* hepatoprotective and antioxidant effects of *Astragalus* polysaccharides against carbon tetrachloride-induced hepatocyte damage in common carp (*Cyprinus carpio*)', Fish Physiology and Biochemistry, vol. 38, no. 3, pp. 871-81.

Jiang, J, Wu, C, Gao, H, Song, J & Li, H 2010, 'Effects of *Astragalus* polysaccharides on immunologic function of erythrocyte in chickens infected with infectious bursa disease virus', Vaccine, vol. 28, no. 34, pp. 5614-6.

Jin, M, Zhao, K, Huang, Q & Shang, P 2014, 'Structural features and biological activities of the polysaccharides from *Astragalus* membranaceus', International Journal of Biological Macromolecules, vol. 64, pp. 257-66.

Joseph, TP, Chanda, W, Padhiar, AA, Batool, S, LiQun, S, Zhong, M & Huang, M 2018, 'A preclinical evaluation of the antitumor activities of edible and medicinal mushrooms: A Molecular Insight', Integrative Cancer Therapies, vol. 17, no. 2, pp. 200-9.

Kaleta, B, Górski, A, Zagożdżon, R, Cieślak, M, Kaźmierczak-Barańska, J, Nawrot, B, Klimaszewska, M, Malinowska, E, Górska, S & Turło, J 2019, 'Selenium-containing polysaccharides from *Lentinula edodes*—biological activity', Carbohydrate Polymers, vol. 223, pp. 115-78.

Khan, T, Date, A, Chawda, H & Patel, K 2019, 'Polysaccharides as potential anticancer agents—A review of their progress', Carbohydrate Polymers, vol. 210, no. 15, pp. 412-28.

Kruger, NJ 2009, 'The Bradford method for protein quantitation', The Protein Protocols Handbook, Springer, pp. 17-24.

Lai, X, Xia, W, Wei, J & Ding, X 2017, 'Therapeutic effect of *Astragalus* polysaccharides on hepatocellular carcinoma H22-bearing mice', Dose-Response: An International Journal, vol. 15, no. 1, pp. 1-6.

Lemmon, HR, Sham, J, Chau, LA & Madrenas, J 2012, 'High molecular weight polysaccharides are key immunomodulators in North American ginseng extracts: Characterization of the ginseng genetic signature in primary human immune cells', Journal of Ethnopharmacology, vol. 142, no. 1, pp. 1-13.

Li, Q, Niu, Y, Xing, P & Wang, C 2018, 'Bioactive polysaccharides from natural resources including Chinese medicinal herbs on tissue repair', Chinese Medicine, vol. 13, no. 1, pp. 7.

Li, S, Wang, A, Liu, L, Tian, G & Xu, F 2019a, 'Extraction of polysaccharides under vacuum condition from *Lentinus edodes* stipe and their antioxidant activities *in vitro'*, Food Science and Biotechnology, vol. 28, no. 3, pp. 759-67.

Li, W, Hu, X, Wang, S, Jiao, Z, Sun, T, Liu, T & Song, K 2020, 'Characterization and anti-tumor bioactivity of *Astragalus* polysaccharides by immunomodulation', International journal of biological macromolecules, vol. 145, pp. 985-97.

Li, W, Song, K, Wang, S, Zhang, C, Zhuang, M, Wang, Y & Liu, T 2019, 'Anti-tumor potential of *Astragalus* polysaccharides on breast cancer cell line mediated by macrophage activation', Materials Science and Engineering: C, vol. 98, pp. 685-95.

Li, W, Wang, J, Hu, H, Li, Q, Liu, Y & Wang, K 2018, 'Functional polysaccharide Lentinan suppresses human breast cancer growth via inducing autophagy and caspase-7-mediated apoptosis', Journal of Functional Foods, vol. 45, pp. 75-85.

Li, X, Wang, L, Wang, Y & Xiong, Z 2016, 'Effect of drying method on physicochemical properties and antioxidant activities of *Hohenbuehelia serotina* polysaccharides', Process Biochemistry, vol. 51, no. 8, pp. 1100-8.

Li, X-T, Zhang, Y-K, Kuang, H-X, Jin, F-X, Liu, D-W, Gao, M-B, Liu, Z & Xin, X-J 2012, 'Mitochondrial protection and anti-aging activity of *Astragalus* polysaccharides and their potential mechanism', International Journal of Molecular Sciences, vol. 13, no. 2, pp. 1747-61.

Liang, Z., Guo, Y.T., Yi, Y.J., Wang, R.C., Hu, Q.L. and Xiong, X.Y., 2014. '*Ganoderma lucidum* polysaccharides target a Fas/caspase dependent pathway to induce apoptosis in human colon cancer cells', Asian Pacific Journal of Cancer Prevention, vol. 15, no. 9, pp.3981-86.

Lin, Y, Zeng, H, Wang, K, Lin, H, Li, P, Huang, Y, Zhou, S, Zhang, W, Chen, C & Fan, H 2019, 'Microwave-assisted aqueous two-phase extraction of diverse polysaccharides from *Lentinus edodes*: Process optimization, structure characterization and antioxidant activity', International Journal of Biological Macromolecules, vol. 136, pp. 305-15.

Liu, A-j, Yu, J, Ji, H-y, Zhang, H-c, Zhang, Y & Liu, H-p 2018, 'Extraction of a novel cold-water-soluble polysaccharide from *Astragalus membranaceus* and its antitumor and immunological activities', Molecules, vol. 23, no. 1, pp. 62.

Liu, C, Li, H, Zhuang, J, Gao, C, Liu, L, Feng, F, Zhou, C, Wang, K & Sun, C 2019, 'Identifying the antiproliferative effect of *Astragalus* polysaccharides on breast cancer: coupling network pharmacology with targetable screening from the cancer genome atlas', Frontiers in Oncology, vol. 9, pp. 368.

Liu, M, Wu, K, Mao, X, Wu, Y & Ouyang, J 2010, '*Astragalus* polysaccharide improves insulin sensitivity in KKAy mice: regulation of PKB/GLUT4 signaling in skeletal muscle', Journal of Ethnopharmacology, vol. 127, no. 1, pp. 32-7.

Liu, X-d, Li, M, Li, W-x, Wang, Q-y & Zhang, H-x 2019, 'Combined Effect of Lentinan and Cisplatin on Cytokines IL-6, TNF-α, and TGF-β in Tumor Therapy', International Journal of Polymer Science, vol. 2019, pp. 1-10.

Liu, Y & Huang, G 2019, 'Extraction and derivatisation of active polysaccharides', Journal of Enzyme Inhibition and Medicinal Chemistry, vol. 34, no. 1, pp. 1690-6.

Liu, Y, Luo, M, Liu, F, Feng, X, Ibrahim, SA, Cheng, L & Huang, W 2020, 'Effects of freeze drying and hot-air drying on the physicochemical properties and bioactivities of polysaccharides from *Lentinula edodes*', International Journal of Biological Macromolecules, vol. 145, pp. 476-83.

Liu, Y, Tang, Q, Zhang, J, Xia, Y, Yang, Y, Wu, D, Fan, H & Cui, SW 2018, 'Triple helix conformation of β-d-glucan from *Ganoderma lucidum* and effect of molecular weight on its immunostimulatory activity', International Journal of Biological Macromolecules, vol. 114, pp. 1064-70.

Lorbeer, AJ, Lahnstein, J, Fincher, GB, Su, P & Zhang, W 2015, 'Kinetics of conventional and microwave-assisted fucoidan extractions from the brown alga, *Ecklonia radiata*', Journal of Applied Phycology, vol. 27, no. 5, pp. 2079-87.

Mao, F, Xiao, B, Jiang, Z, Zhao, J, Huang, X & Guo, J 2011, 'Anticancer effect of *Lycium barbarum* polysaccharides on colon cancer cells involves G0/G1 phase arrest', Medical Oncology, vol. 28, no. 1, pp. 121-6.

Ming, H, Chen, Y, Yonghao, H, Dong, X, Jing, G & Yang, L 2014, 'Effects of *Astragalus* polysaccharide (APS) on cytokine and immune function impairment induced by cisplatin in mice bearing Lewis lung cancer', Acta Laboratorium Animalis Scientia Sinica, no. 5, pp. 44-8.

Miyaji, C, Poersch, A, Ribeiro, L, Eira, A & Colus, I 2006, 'Shiitake (*Lentinula edodes* (Berkeley) Pegler) extracts as a modulator of micronuclei induced in HEp-2 cells', Toxicology *in vitro*, vol. 20, no. 8, pp. 1555-9.

Morales, D, Rutckeviski, R, Villalva, M, Abreu, H, Soler-Rivas, C, Santoyo, S, Iacomini, M & Smiderle, FR 2020, 'Isolation and comparison of α-and β-D-glucans from shiitake mushrooms (*Lentinula edodes*) with different biological activities', Carbohydrate Polymers, vol. 229, pp. 1-11.

Morales, D, Smiderle, FR, Villalva, M, Abreu, H, Rico, C, Santoyo, S, Iacomini, M & Soler-Rivas, C 2019, 'Testing the effect of combining innovative extraction technologies on the biological activities of obtained β-glucan-enriched extracts from *Lentinula edodes*', Journal of Functional Foods, vol. 60, pp. 1-10.

Mutch, DG, Babb, SA & Di Saia, PJ 2018, '19 - Genes and Cancer: Genetic Counseling and Clinical Management', in PJ DiSaia, WT Creasman, RS Mannel, DS McMeekin & DG Mutch (eds), Clinical Gynecologic Oncology (Ninth Edition), Elsevier, pp. 493-527.

Ni, W, Zhang, X, Wang, B, Chen, Y, Han, H, Fan, Y, Zhou, Y & Tai, G 2010, 'Antitumor activities and immunomodulatory effects of ginseng neutral polysaccharides in combination with 5-fluorouracil', Journal of Medicinal Food, vol. 13, no. 2, pp. 270-7.

Nielsen, SS 2010, 'Phenol-sulfuric acid method for total carbohydrates', in Food Analysis Laboratory Manual, Springer, pp. 47-53.

Niu, Y, Wang, H, Xie, Z, Whent, M, Gao, X, Zhang, X, Zou, S, Yao, W & Yu, L 2011, 'Structural analysis and bioactivity of a polysaccharide from the roots of *Astragalus membranaceus* (Fisch) Bge. var. mongolicus (Bge.) Hsiao', Food Chemistry, vol. 128, no. 3, pp. 620-6.

Nurgali, K, Jagoe, RT & Abalo, R 2018, 'Editorial: Adverse effects of cancer chemotherapy: Anything new to improve tolerance and reduce sequelae?', Frontiers in Pharmacology, vol. 9, pp. 245.

Oba, K, Kobayashi, M, Matsui, T, Kodera, Y & Sakamoto, J 2009, 'Individual patient based meta-analysis of lentinan for unresectable/recurrent gastric cancer', Anticancer Research, vol. 29, no. 7, pp. 2739-45.

Özçelik, E & Pekşen, A 2007, 'Hazelnut husk as a substrate for the cultivation of shiitake mushroom (*Lentinula edodes*)', Bioresource Technology, vol. 98, no. 14, pp. 2652-8.

Peng, Y, Han, B, Liu, W & Zhou, R 2016, 'Deproteinization and structural characterization of bioactive exopolysaccharides from *Ganoderma sinense* mycelium', Separation Science and Technology, vol. 51, no. 2, pp. 359-69.

Qi, W., Zhou, X., Wang, J., Zhang, K., Zhou, Y., Chen, S., Nie, S. and Xie, M., 2020, '*Cordyceps sinensis* polysaccharide inhibits colon cancer cells growth by inducing apoptosis and autophagy flux blockage via mTOR signaling', Carbohydrate polymers, vol. 237, pp. 1-11.

Rehman, S 2018, 'An Overview of Cancer Treatment Modalities', Neoplasm, Intechopen Limited London.

Restifo, NP, Dudley, ME & Rosenberg, SA 2012, 'Adoptive immunotherapy for cancer: harnessing the T cell response', Nature Reviews Immunology, vol. 12, no. 4, pp. 269-81.

Rong, Y, Yang, R, Yang, Y, Wen, Y, Liu, S, Li, C, Hu, Z, Cheng, X & Li, W 2019, 'Structural characterization of an active polysaccharide of longan and evaluation of immunological activity', Carbohydrate Polymers, vol. 213, pp. 247-56.

Shang, H, Chen, S, Li, R, Zhou, H, Wu, H & Song, H 2018, 'Influences of extraction methods on physicochemical characteristics and activities of *Astragalus cicer* L. polysaccharides', Process Biochemistry, vol. 73, pp. 220-7.

Sherr, CJ 2004, 'Principles of Tumor Suppression', Cell, vol. 116, no. 2, pp. 235-46.

Siwulski, M, Rzymski, P, Budka, A, Kalač, P, Budzyńska, S, Dawidowicz, L, Hajduk, E, Kozak, L, Budzulak, J, Sobieralski, K & Niedzielski, P 2019, 'The effect of different substrates on the growth of six cultivated mushroom species and composition of macro and trace elements in their fruiting bodies', European Food Research and Technology, vol. 245, no. 2, pp. 419-31.

Somasundaram, V, Basudhar, D, Greer, M, Cheng, RY, Ridnour, LA & Wink, DA 2017, 'NO in cancer—carcinogenesis, metastasis, and therapy', in Nitric Oxide, Elsevier, pp. 385-402.

Stringer, AM, Gibson, RJ, Bowen, JM & Keefe, DM 2009, 'Chemotherapy-induced modifications to gastrointestinal microflora: evidence and implications of change', Current Drug Metabolism, vol. 10, no. 1, pp. 79-83.

Sun, S, Zheng, K, Zhao, H, Lu, C, Liu, B, Yu, C, Zhang, G, Bian, Z, Lu, A & He, X 2014, 'Regulatory effect of astragalus polysaccharides on intestinal intraepithelial γδT cells of tumor bearing mice', Molecules, vol. 19, no. 9, pp. 15224-36.

Tian, Q-E, Li, H-D, Yan, M, Cai, H-L, Tan, Q-Y & Zhang, W-Y 2012, '*Astragalus* polysaccharides can regulate cytokine and P-glycoprotein expression in H22 tumor-bearing mice', World Journal of Gastroenterology, vol. 18, no. 47, pp. 7079-86.

Tian, Y, Zeng, H, Xu, Z, Zheng, B, Lin, Y, Gan, C & Lo, YM 2012, 'Ultrasonic-assisted extraction and antioxidant activity of polysaccharides recovered from white button mushroom (*Agaricus bisporus*)', Carbohydrate Polymers, vol. 88, no. 2, pp. 522-9.

Tian, Y, Zhao, Y, Zeng, H, Zhang, Y & Zheng, B 2016, 'Structural characterization of a novel neutral polysaccharide from *Lentinus giganteus* and its antitumor activity through inducing apoptosis', Carbohydrate Polymers, vol. 154, pp. 231-40.

Tin, MM, Cho, C-H, Chan, K, James, AE & Ko, JK 2007, '*Astragalus* saponins induce growth inhibition and apoptosis in human colon cancer cells and tumor xenograft', Carcinogenesis, vol. 28, no. 6, pp. 1347-55.

Todd, R & Wong, DT 1999, 'Oncogenes', Anticancer Res, vol. 19, no. 6a, pp. 4729-46.

Unursaikhan, S, Xu, X, Zeng, F & Zhang, L 2006, 'Antitumor activities of O-sulfonated derivatives of (1→ 3)-α-D-glucan from different *Lentinus edodes*', Bioscience, Biotechnology, and Biochemistry, vol. 70, no. 1, pp. 38-46.

van Dam, JE, van den Broek, LA & Boeriu, CG 2017, 'Polysaccharides in human health care', Natural Product Communications, vol. 12, no. 6, pp. 821-30.

van Engeland, M, Ramaekers, FC, Schutte, B & Reutelingsperger, CP 1996, 'A novel assay to measure loss of plasma membrane asymmetry during apoptosis of adherent cells in culture', Cytometry: The Journal of the International Society for Analytical Cytology, vol. 24, no. 2, pp. 131-9.

Vannini, F, Kashfi, K & Nath, N 2015, 'The dual role of iNOS in cancer', Redox Biology, vol. 6, pp. 334-43.

Vermes, I, Haanen, C, Steffens-Nakken, H & Reutellingsperger, C 1995, 'A novel assay for apoptosis flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled annexin V', Journal of Immunological Methods, vol. 184, no. 1, pp. 39-51.

Wang, C, Feng, L, Su, J, Cui, L, Liu, D, Yan, J, Ding, C, Tan, X & Jia, X 2017, 'Polysaccharides from *Epimedium koreanum* Nakai with immunomodulatory activity and inhibitory effect on tumor growth in LLC-bearing mice', Journal of Ethnopharmacology, vol. 207, pp. 8-18.

Wang, J, Li, W, Huang, X, Liu, Y, Li, Q, Zheng, Z & Wang, K 2017, 'A polysaccharide from *Lentinus edodes* inhibits human colon cancer cell proliferation and suppresses tumor growth in athymic nude mice', Oncotarget, vol. 8, no. 1, pp. 610.

Wang, Q, Sheng, X, Shi, A, Hu, H, Yang, Y, Liu, L, Fei, L & Liu, H 2017, 'β-Glucans: Relationships between modification, conformation and functional activities', Molecules, vol. 22, no. 2, pp. 257.

Wang, SC, Shan, JJ, Wang, ZT & Hu, ZB 2006, 'Isolation and structural analysis of an acidic polysaccharide from *Astragalus membranaceus* (Fisch.) Bunge', Journal of Integrative Plant Biology, vol. 48, no. 11, pp. 1379-84.

Wang, X, Ding, J, Feng, Y, Weng, L, Zhao, G, Xiang, J, Zhang, M & Xing, D 2017, 'Targeting of growth factors in the treatment of hepatocellular carcinoma: The potentials of polysaccharides', Oncology Letters, vol. 13, no. 3, pp. 1509-17.

Wasser, SP 2005, 'Shiitake (*Lentinus edodes*)', Encyclopedia of Dietary Supplements, pp. 653-64.

World Health Organization 2018, Cancer. Retrieved from https://www.who.int/news-room/fact-sheets/detail/cancer

Wu, C-Y, Ke, Y, Zeng, Y-F, Zhang, Y-W & Yu, H-J 2017, 'Anticancer activity of Astragalus polysaccharide in human non-small cell lung cancer cells', Cancer Cell International, vol. 17, no. 1, pp. 115.

Yamasaki, A, Shoda, M, Iijima, H, Nagai, S, Wada, J, Suzuki, H, Chikazawa, N, Tasaka, T, Kameda, C & Tanaka, H 2009, 'A protein-bound polysaccharide, PSK, enhances tumor suppression induced by docetaxel in a gastric cancer xenograft model', Anticancer Research, vol. 29, no. 3, pp. 843-50.

Yang, B, Xiao, B & Sun, T 2013, 'Antitumor and immunomodulatory activity of *Astragalus membranaceus* polysaccharides in H22 tumor-bearing mice', International Journal of Biological Macromolecules, vol. 62, pp. 287-90.

Yang, M, Qian, X-H, Zhao, D-H & Fu, S-Z 2010, 'Effects of *Astragalus* polysaccharide on the erythroid lineage and microarray analysis in K562 cells', Journal of Ethnopharmacology, vol. 127, no. 2, pp. 242-50.

Yang, S, Sun, S, Xu, W, Yu, B, Wang, G & Wang, H 2020, '*Astragalus* polysaccharide inhibits breast cancer cell migration and invasion by regulating epithelial-mesenchymal transition via the Wnt/β-catenin signaling pathway', Mol Med Rep, vol. 21, no. 4, pp. 1819-32.

Yang, X, Zhao, Y, Wang, Q, Wang, H & Mei, Q 2005, 'Analysis of the monosaccharide components in *Angelica* polysaccharides by high performance liquid chromatography', Anal Sci, vol. 21, no. 10, pp. 1177-80.

Yin, C, Fan, X, Fan, Z, Shi, D & Gao, H 2018, 'Optimization of enzymes-microwave-ultrasound assisted extraction of *Lentinus edodes* polysaccharides and determination of its antioxidant activity', International Journal of Biological Macromolecules, vol. 111, pp. 446-54.

Yin, Z, Zhang, W, Zhang, J & Kang, W 2017, 'Isolation, purification, structural analysis and coagulatory activity of water-soluble polysaccharides from *Ligustrum lucidum* Ait flowers', Chemistry Central Journal, vol. 11, no. 1, pp. 1-10.

Yoon, S-Y, Robinson, CR, Zhang, H & Dougherty, PM 2013, 'Spinal astrocyte gap junctions contribute to oxaliplatin-induced mechanical hypersensitivity', The Journal of Pain, vol. 14, no. 2, pp. 205-14.

Yoshino, S, Yoshida, S, Maeda, N, Maeda, Y, Maeda, K, Hazama, S & Oka, M 2010, 'Clinical evaluation of the combination treatment of intrapleural or intraperitoneal administration of lentinan and OK-432 for malignant effusion', Gan to Kagaku Ryoho. Cancer & Chemotherapy, vol. 37, no. 12, pp. 2798-800.

You, L, Gao, Q, Feng, M, Yang, B, Ren, J, Gu, L, Cui, C & Zhao, M 2013, 'Structural characterisation of polysaccharides from *Tricholoma matsutake* and their antioxidant and antitumour activities', Food Chemistry, vol. 138, no. 4, pp. 2242-9.

Zeng, P, Li, J, Chen, Y & Zhang, L 2019, 'The structures and biological functions of polysaccharides from traditional Chinese herbs', Prog. Mol. Biol. Transl. Sci, vol. 163, pp. 423-44.

Zhang, H, Pan, N, Xiong, S, Zou, S, Li, H, Xiao, L, Cao, Z, Tunnacliffe, A & Huang, Z 2012, 'Inhibition of polyglutamine-mediated proteotoxicity by Astragalus membranaceus polysaccharide through the DAF-16/FOXO transcription factor in Caenorhabditis elegans', Biochemical Journal, vol. 441, no. 1, pp. 417-24.

Zhang, L, Li, X, Xu, X & Zeng, F 2005, 'Correlation between antitumor activity, molecular weight, and conformation of lentinan', Carbohydrate Research, vol. 340, no. 8, pp. 1515-21.

Zhang, M, Cui, S, Cheung, P & Wang, Q 2007, 'Antitumor polysaccharides from mushrooms: a review on their isolation process, structural characteristics and antitumor activity', Trends in Food Science & Technology, vol. 18, no. 1, pp. 4-19.

Zhang, X-G, Lu, Y, Wang, W-N, Liu, Z-Y, Liu, J-W & Chen, X-Q 2017, 'A novel enzyme-assisted approach for efficient extraction of Z-ligustilide from *Angelica sinensis* plants', Scientific Reports, vol. 7, no. 1, pp. 9783.

Zhang, Y, Li, Q, Wang, J, Cheng, F, Huang, X, Cheng, Y & Wang, K 2016, 'Polysaccharide from *Lentinus edodes* combined with oxaliplatin possesses the synergy and attenuation effect in hepatocellular carcinoma', Cancer Letters, vol. 377, no. 2, pp. 117-25.

Zhang, Y, Sun, D, Meng, Q, Guo, W, Chen, Q & Zhang, Y 2017, *'Grifola frondosa* polysaccharides induce breast cancer cell apoptosis via the mitochondrial-dependent apoptotic pathway', International Journal of Molecular Medicine, vol. 40, no. 4, pp. 1089-95.

Zhang, Y, Zhang, M, Jiang, Y, Li, X, He, Y, Zeng, P, Guo, Z, Chang, Y, Luo, H, Liu, Y, Hao, C, Wang, H, Zhang, G & Zhang, L 2018, 'Lentinan as an immunotherapeutic for treating lung cancer: a review of 12 years clinical studies in China', Journal of Cancer Research and Clinical Oncology, vol. 144, no. 11, pp. 2177-86.

Zhao, T, Mao, G, Zhang, M, Zou, Y, Feng, W, Gu, X, Zhu, Y, Mao, R, Yang, L & Wu, X 2014, 'Enhanced antitumor and reduced toxicity effect of *Schisanreae* polysaccharide in 5-Fu treated Heps-bearing mice', International Journal of Biological Macromolecules, vol. 63, pp. 114-8.

Zhao, Y-m, Wang, J, Wu, Z-g, Yang, J-m, Li, W & Shen, L-x 2016, 'Extraction, purification and anti-proliferative activities of polysaccharides from *Lentinus edodes*', International Journal of Biological Macromolecules, vol. 93, pp. 136-44.

Zhao, Y-M, Yang, J-m, Liu, Y-h, Zhao, M & Wang, J 2018, 'Ultrasound assisted extraction of polysaccharides from *Lentinus edodes* and its anti-hepatitis B activity *in vitro'*, International Journal of Biological Macromolecules, vol. 107, pp. 2217-23.

Zheng, Y, Ren, W, Zhang, L, Zhang, Y, Liu, D & Liu, Y 2020, 'A review of the pharmacological action of *Astragalus* polysaccharide', Frontiers in Pharmacology, vol. 11, pp. 349.

Zhou, Z, Meng, M & Ni, H 2017, 'Chemosensitizing effect of *Astragalus* polysaccharides on nasopharyngeal carcinoma cells by inducing apoptosis and modulating expression of Bax/Bcl-2 ratio and caspases', Medical Science Monitor : International Medical Journal of Experimental and Clinical Research, vol. 23, pp. 462-9.

Zhu, Q, Chen, J, Li, Q, Wang, T & Li, H 2016, 'Antitumor activity of polysaccharide from *Laminaria japonica* on mice bearing H22 liver cancer', International Journal of Biological Macromolecules, vol. 92, pp. 156-8.

Ziaja-Sołtys, M, Radzki, W, Nowak, J, Topolska, J, Jabłońska-Ryś, E, Sławińska, A, Skrzypczak, K, Kuczumow, A & Bogucka-Kocka, A 2020, 'Processed fruiting bodies of *Lentinus edodes* as a source of biologically active polysaccharides', Applied Sciences, vol. 10, no. 2, pp. 470.

Appendices

Appendix A: Comparing the glucuronic acid and glucose concentration of HAS-A and HAS-B with Shiitake.

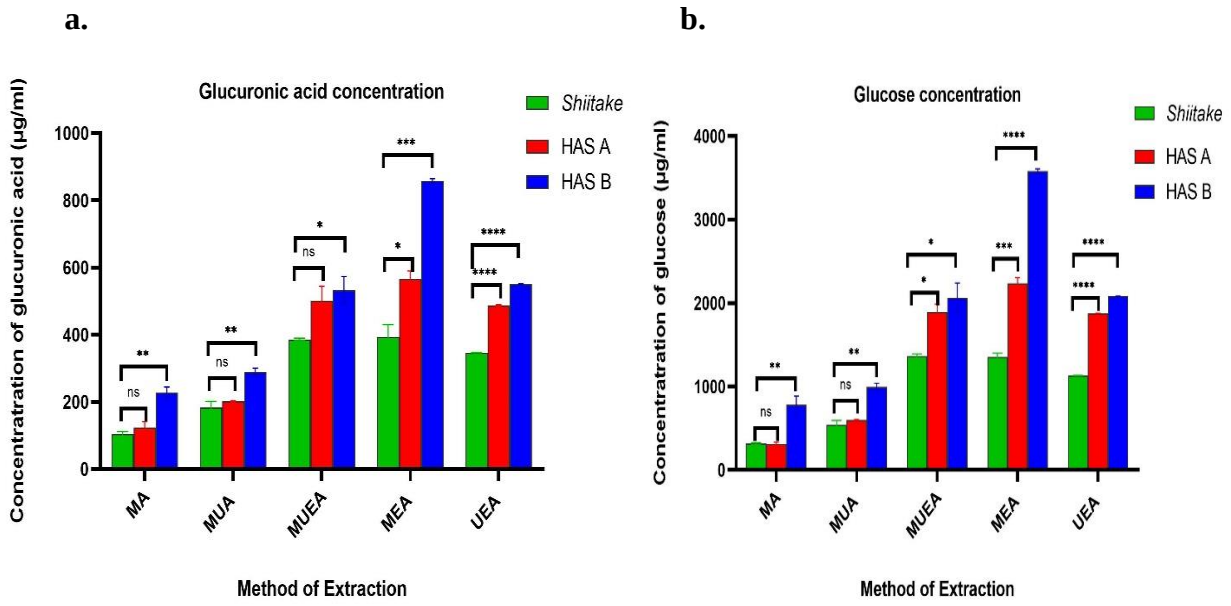


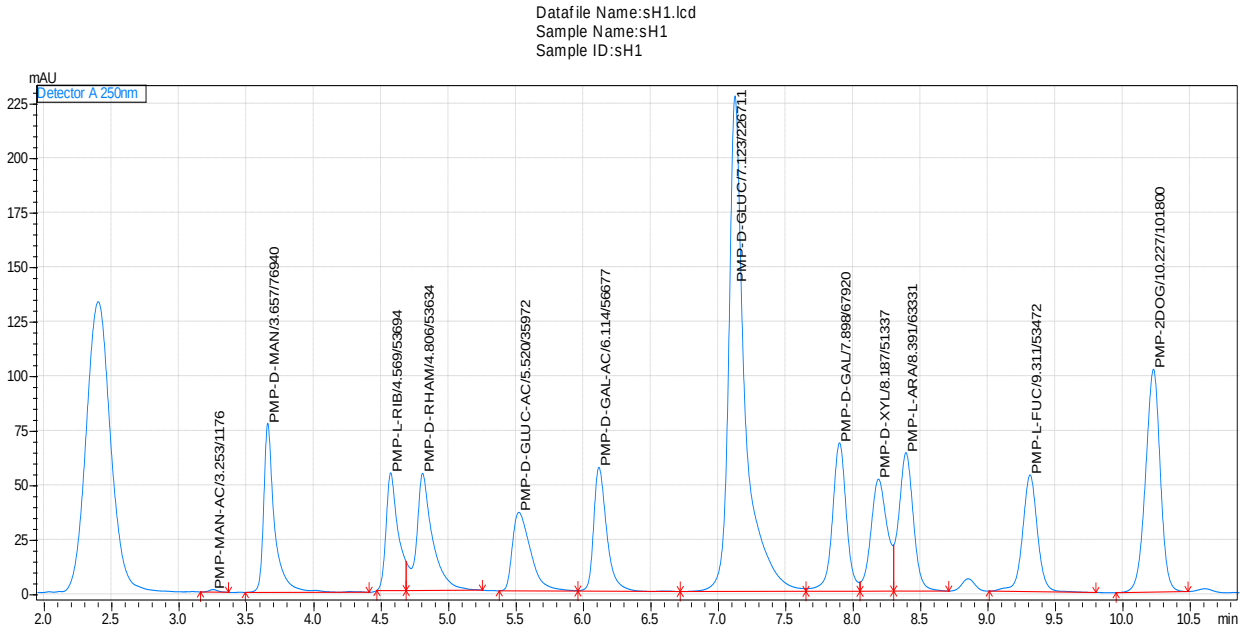
Fig. A1 Comparison of glucuronic acid and glucose concentration of HAS-A and HAS-B with Shiitake, with different method of extraction. a) Glucuronic concentration b) Glucose concentration. $P < 0.05$ is indicated as *, $P < 0.01$ as **, $P < 0.001$ as ***, $P < 0.0001$ as **** and $P > 0.05$ as 'ns'.

Appendix B: Total monosaccharide content determined by HPLC.

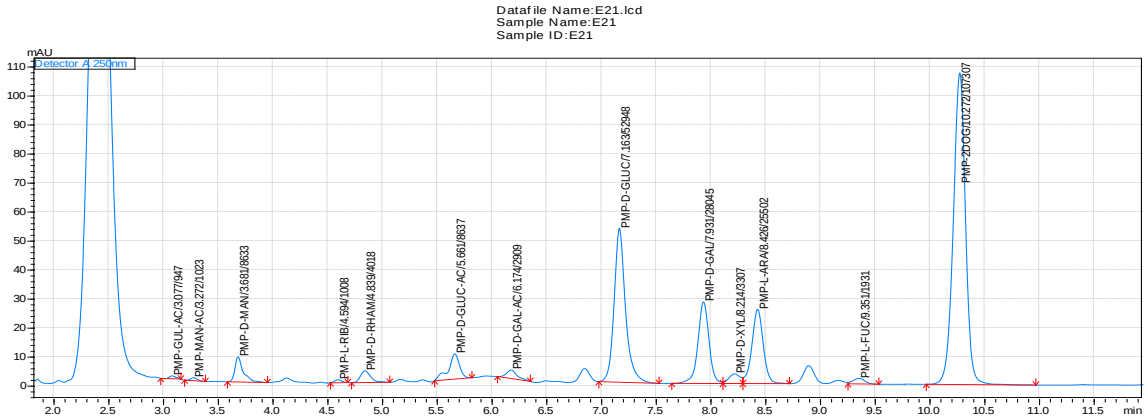
Extracts	Total monosaccharide content % (w/w) ± SD (n=3)			
	A. membranaceus	**Shiitake**	**HAS-A**	**HAS-B**
UEA	21.235±0.18	32.78±1.48	45.905±2.58	45.925±1.11
MEA	23.71±0.48	39.215±1.65	51.575±0.97	77.87±0.81
MUEA	25.36±1.32	38.7±0.68	46.305±2.69	47.27±1.20
MUA	12.805±0.80	19.155±1.97	20.22±0.00	24.97±0.98
MA	13.16±0.59	9.07±0.34	9.595±0.56	19.545±2.35

Appendix C: HPLC chromatograms showing composition of monosaccharides.

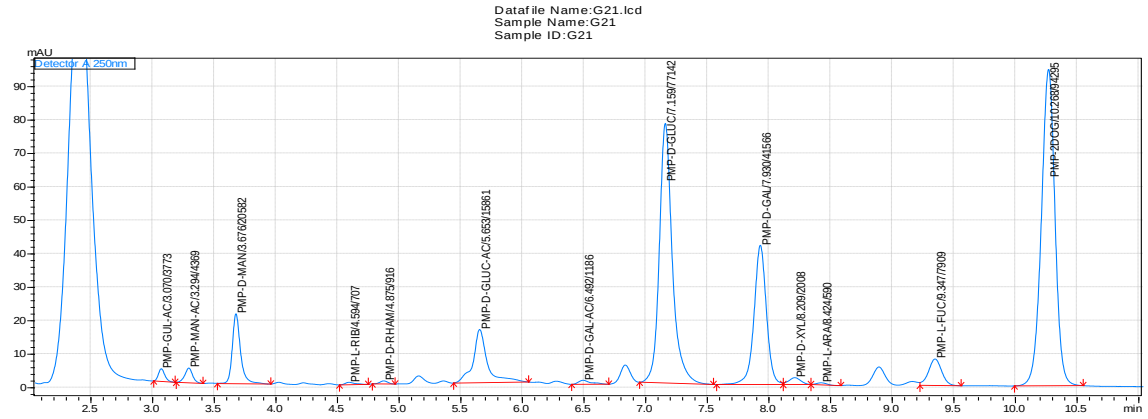
Standard graph



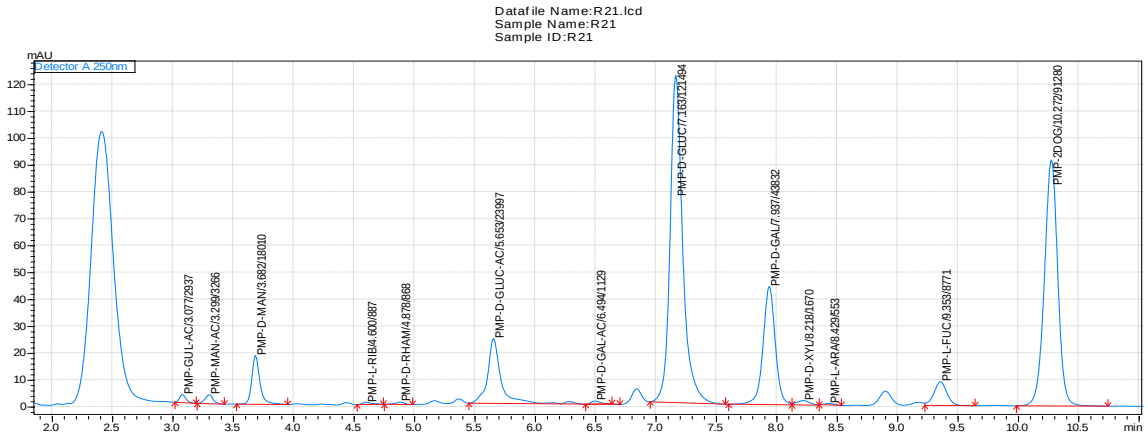
A. membranaceus (UEA)



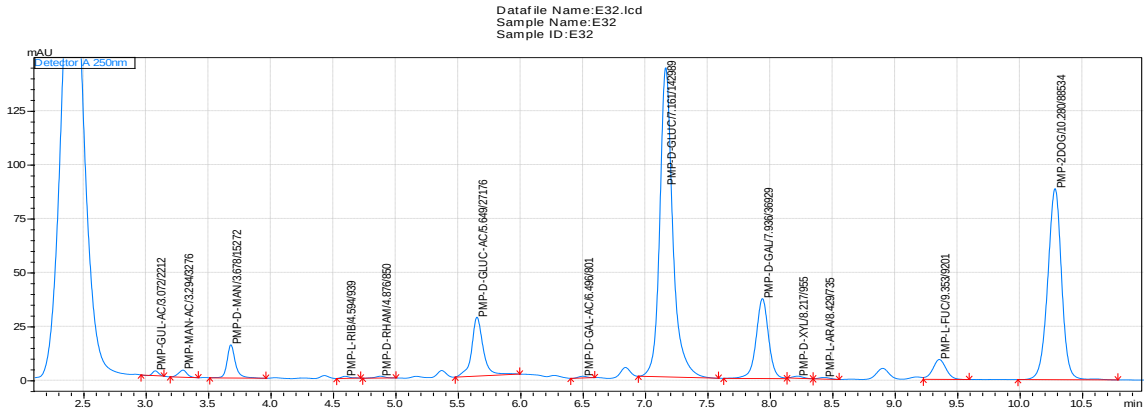
Shiitake (UEA)



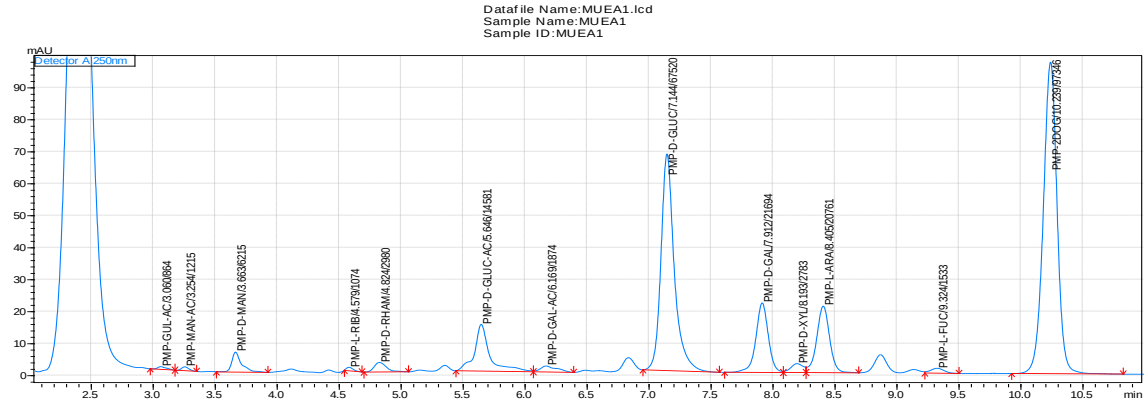
HAS-A (UEA)



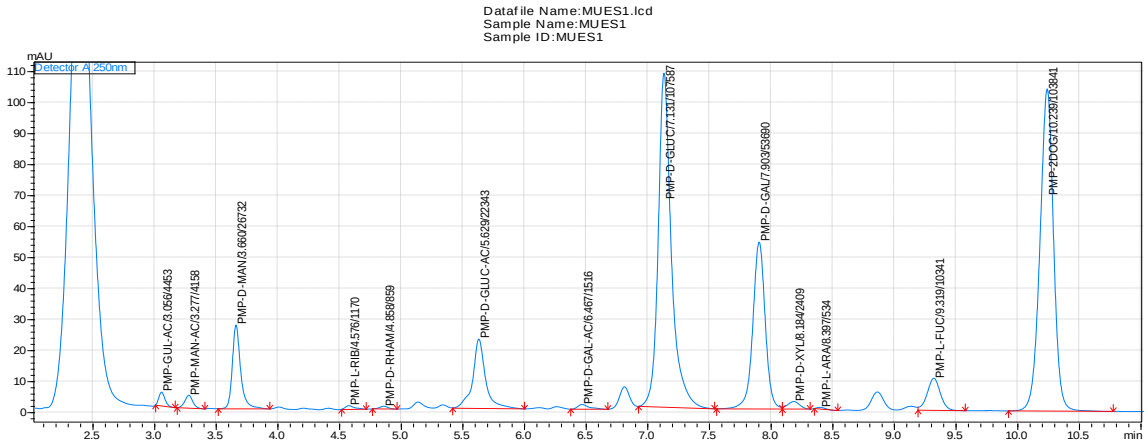
HAS-B (UEA)



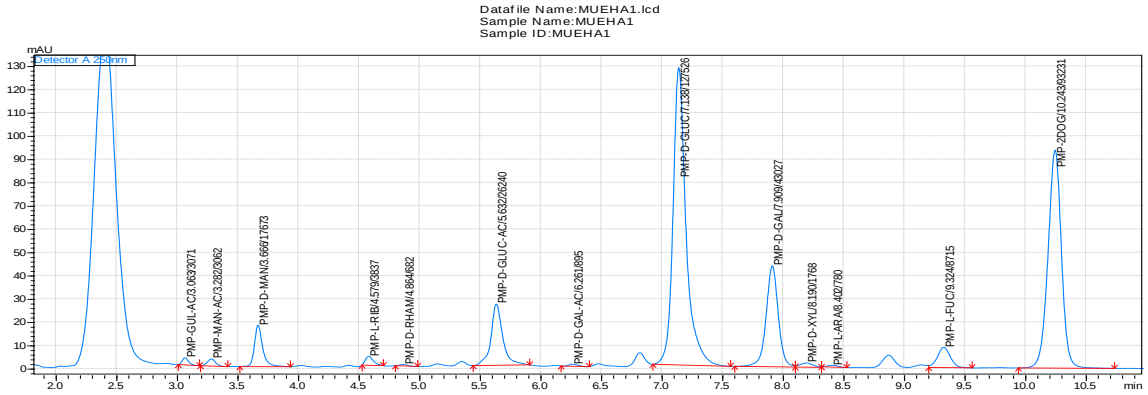
A. membranaceus (MUEA)



Shiitake (MUEA)



HAS-A (MUEA)



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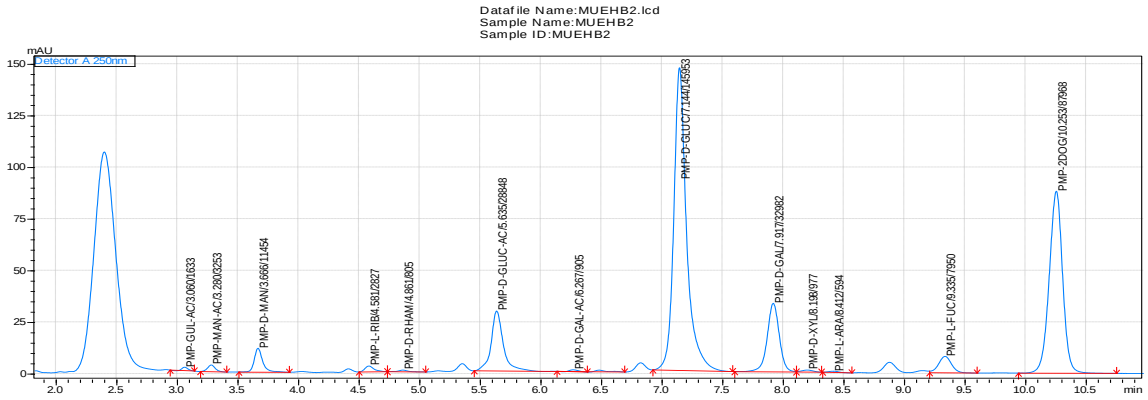
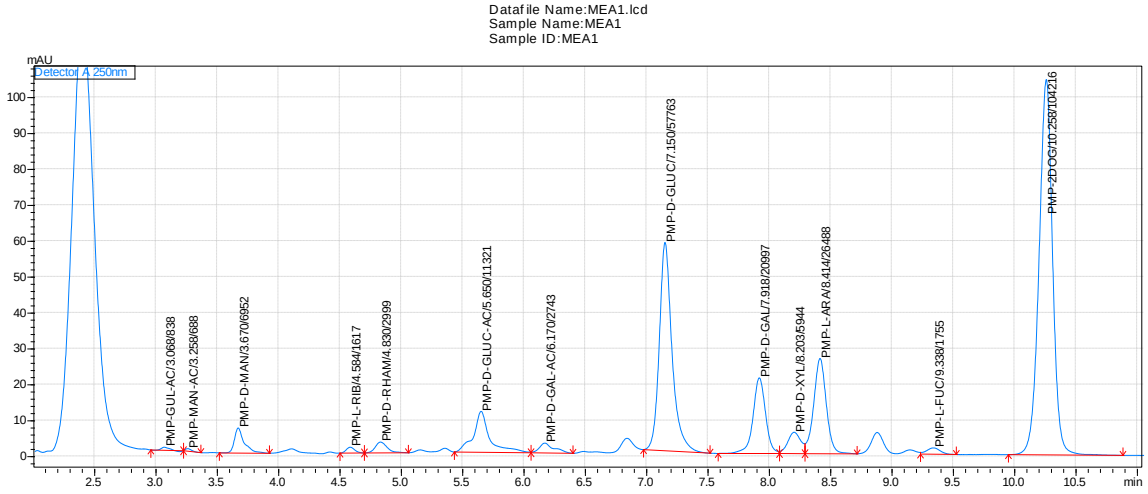
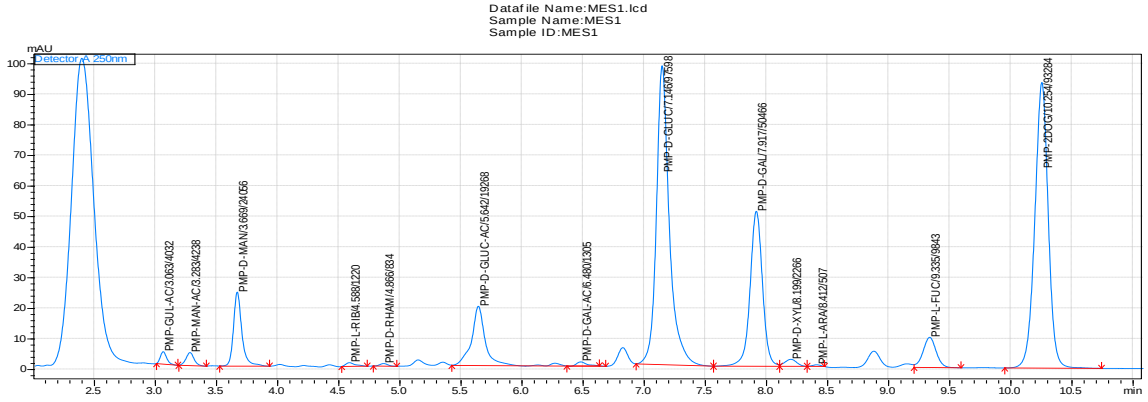


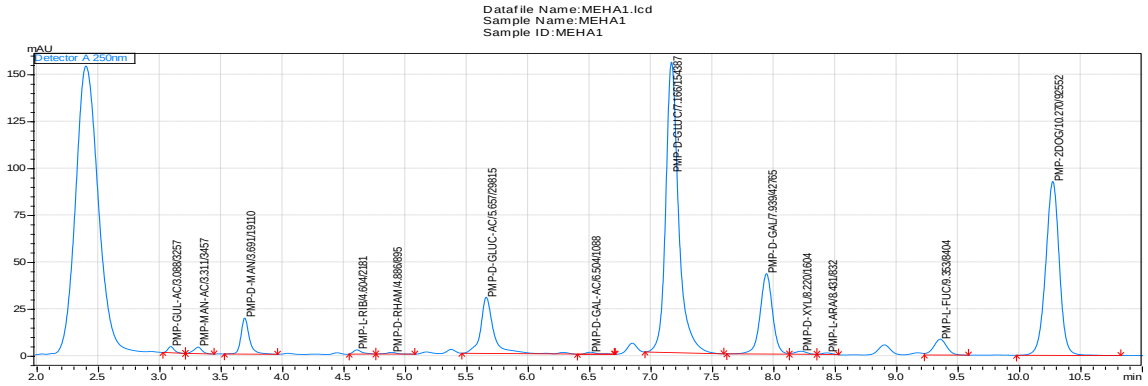
A. membranaceus (MEA)



Shiitake (MEA)



HAS-A (MEA)



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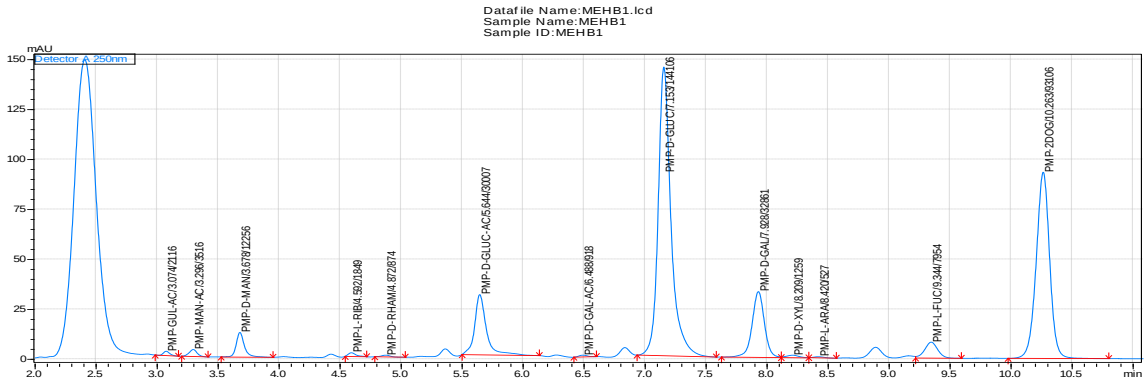
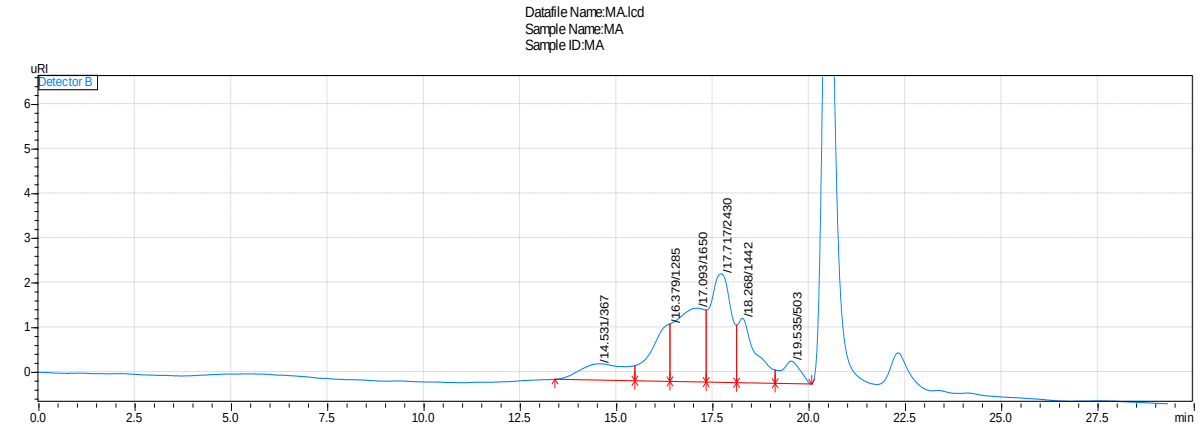


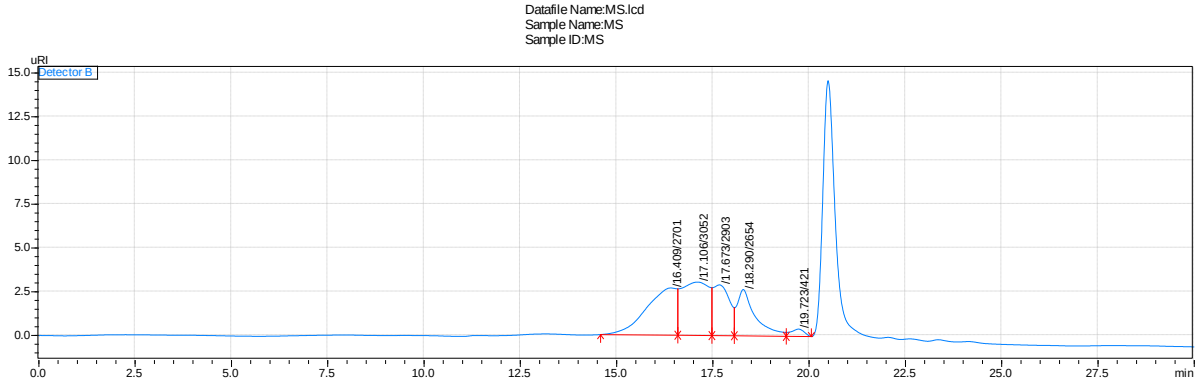
Fig. A2 HPLC chromatograms for monosaccharide composition analysis of *A. membranaceus*, Shiitake HAS-A and HAS-B. MA – Microwave assisted, MUA – Microwave/Ultrasound assisted, MUEA – Microwave/Ultrasound/Enzyme assisted, MEA- Microwave/Enzyme assisted and UEA- Ultrasound/Enzyme assisted extracts.

Appendix D: Relative molecular weights determined by size exclusion HPLC technique.

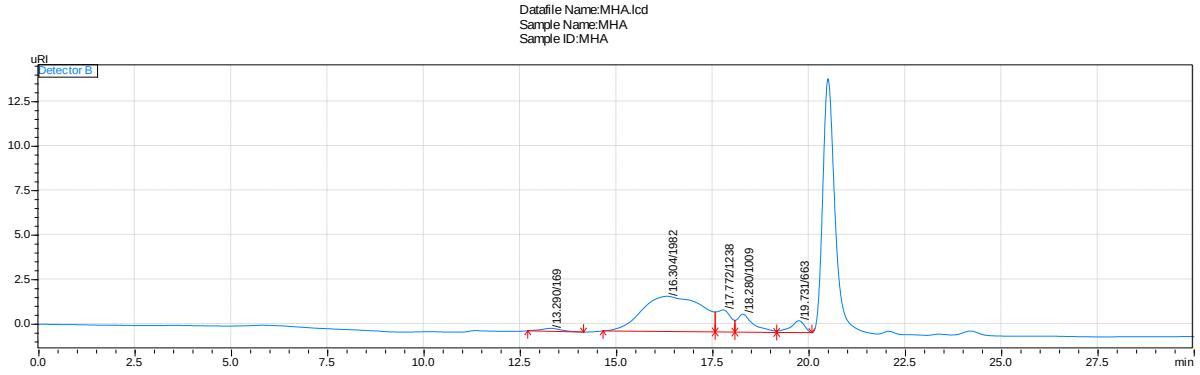
MA *A. membranaceus*



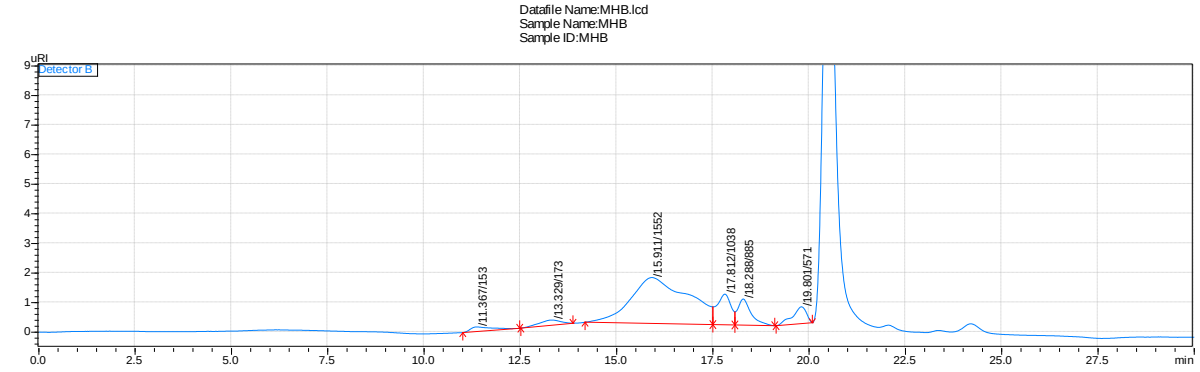
MA Shiitake



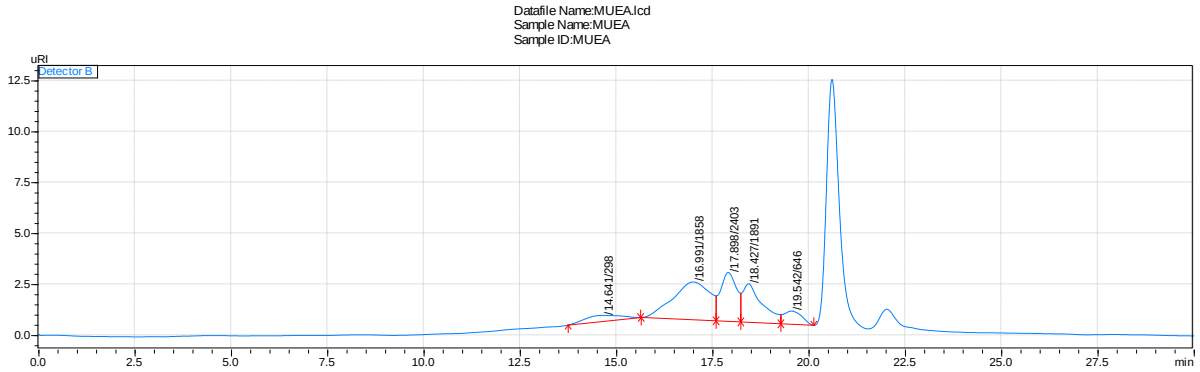
MA HAS-A



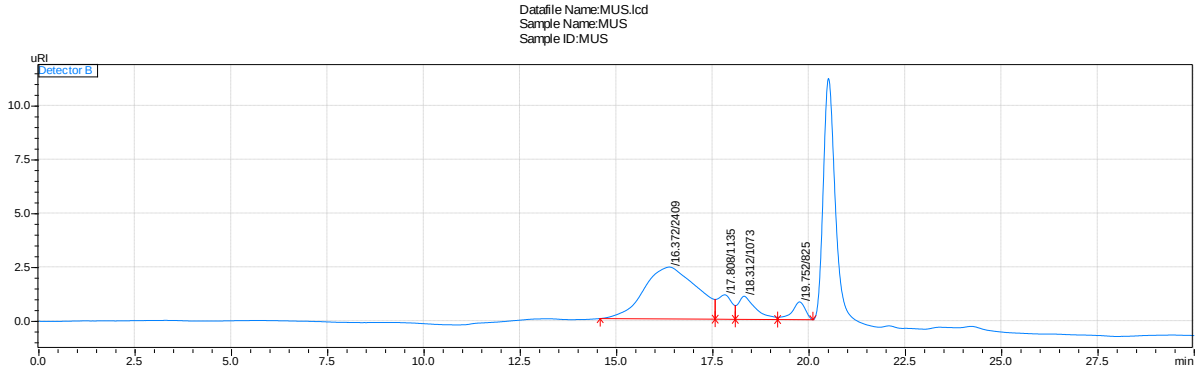
MA HAS-B



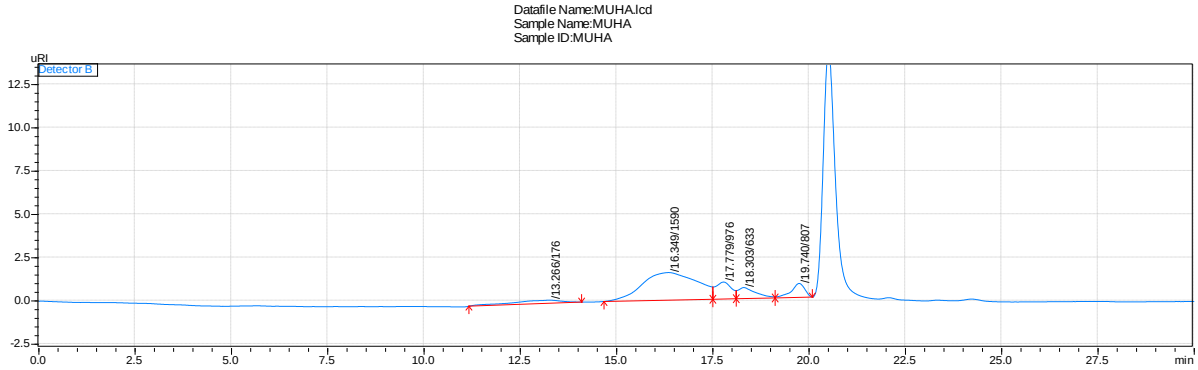
MUA *A. membranaceus*



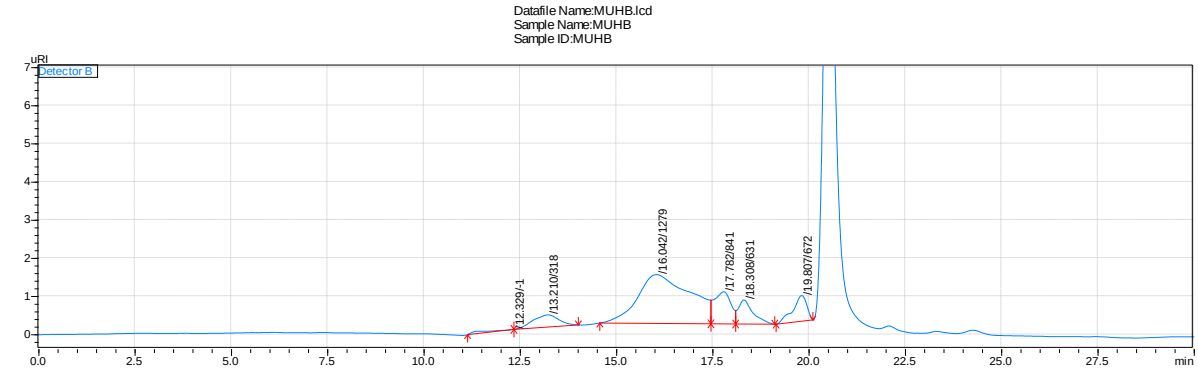
MUA Shiitake



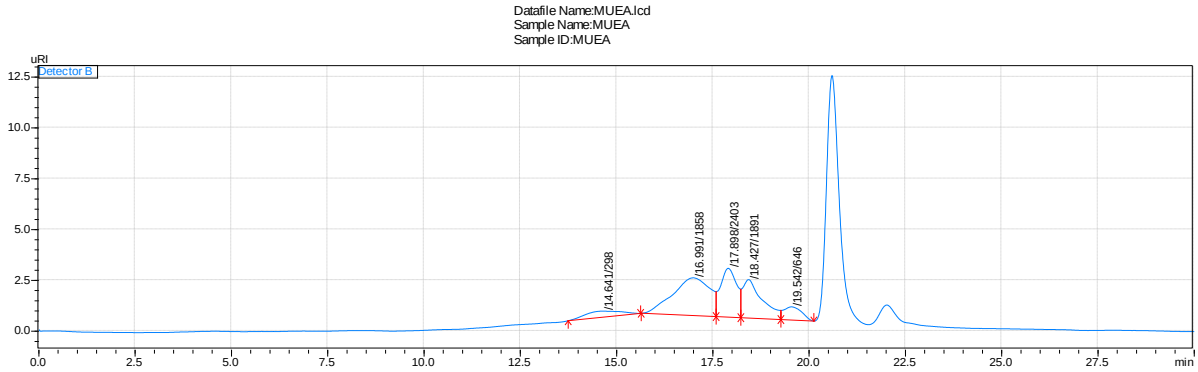
MUA HAS-A



MUA HAS-B



MUE *A. membranaceus*



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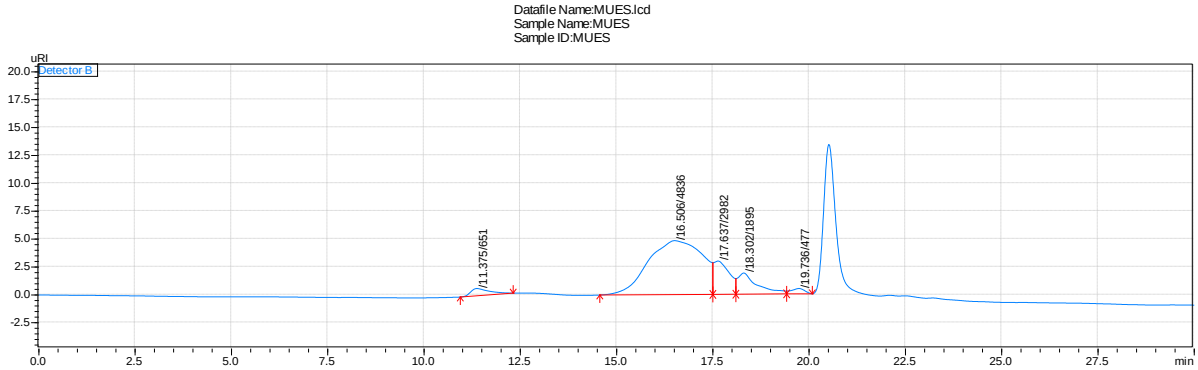

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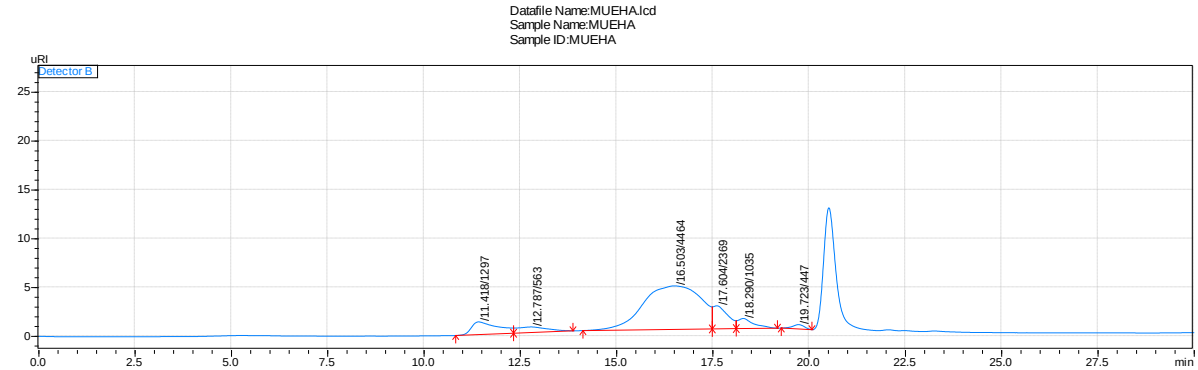

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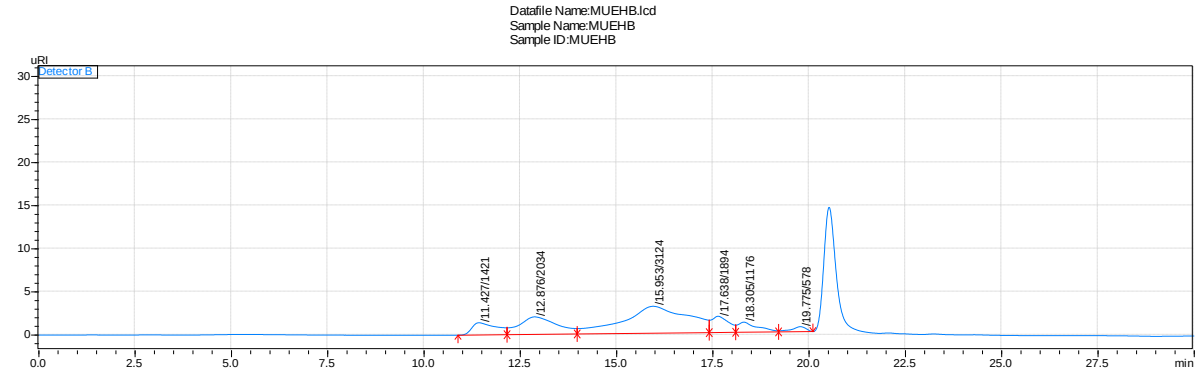
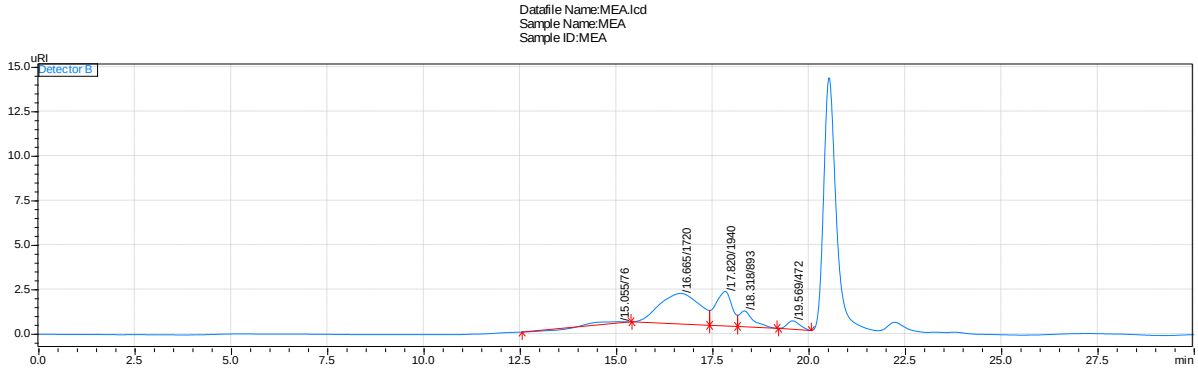
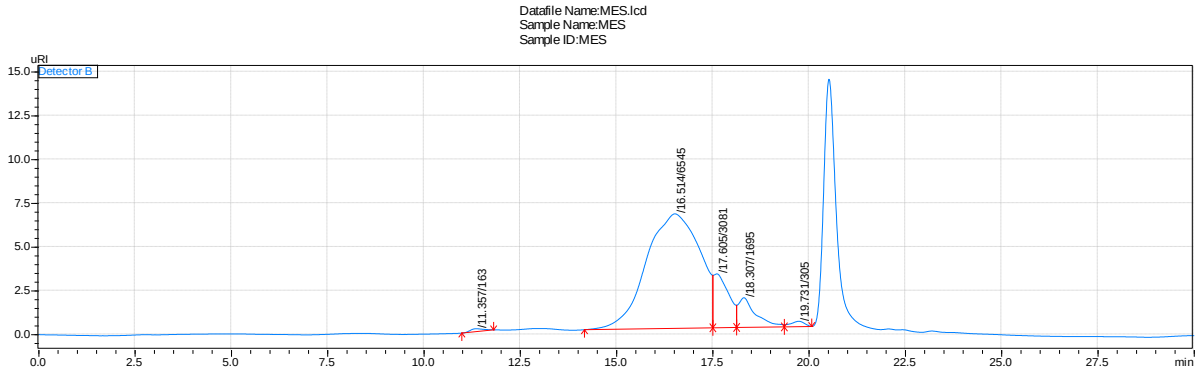


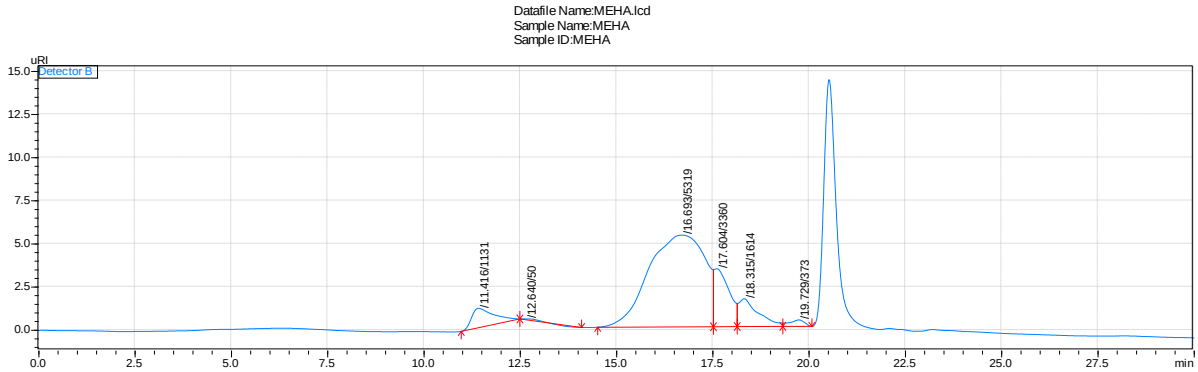
ME *A. membranaceus*



MEA Shiitake



MEA HAS-A



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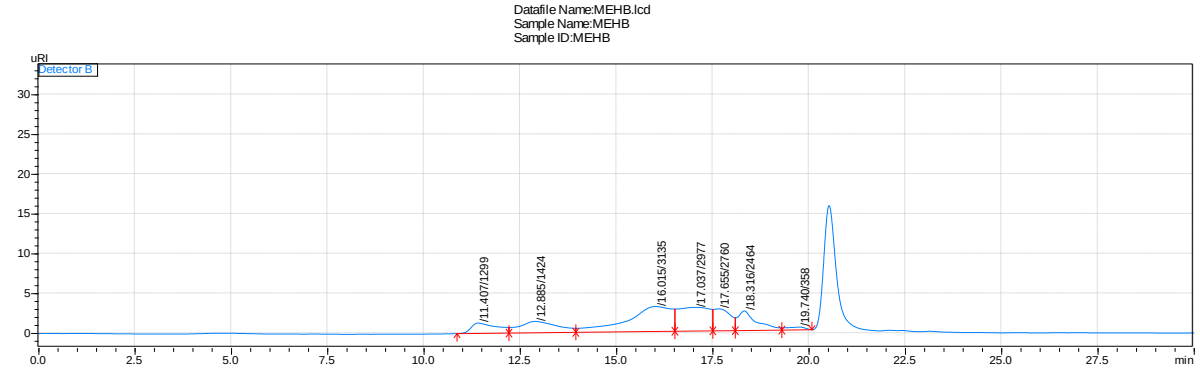
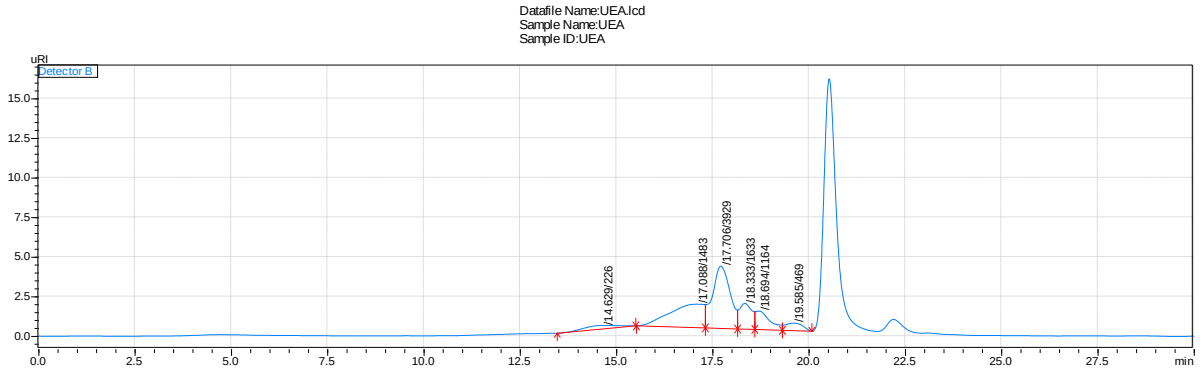
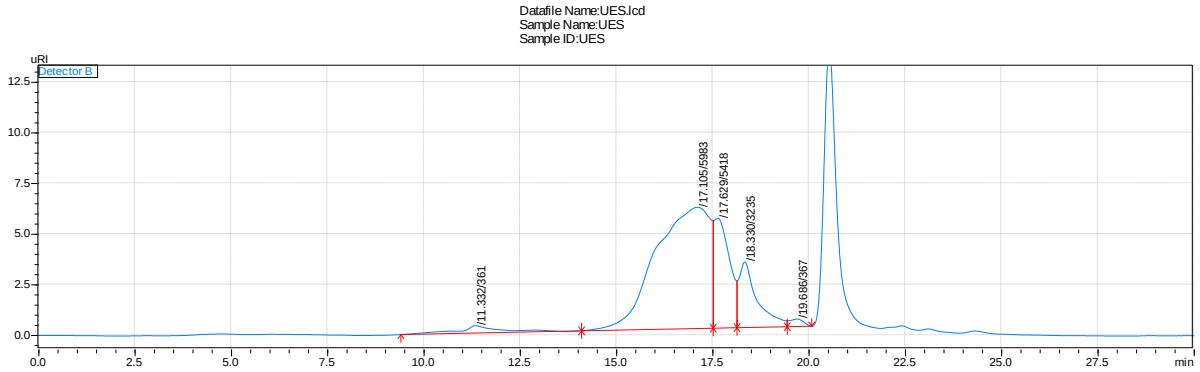


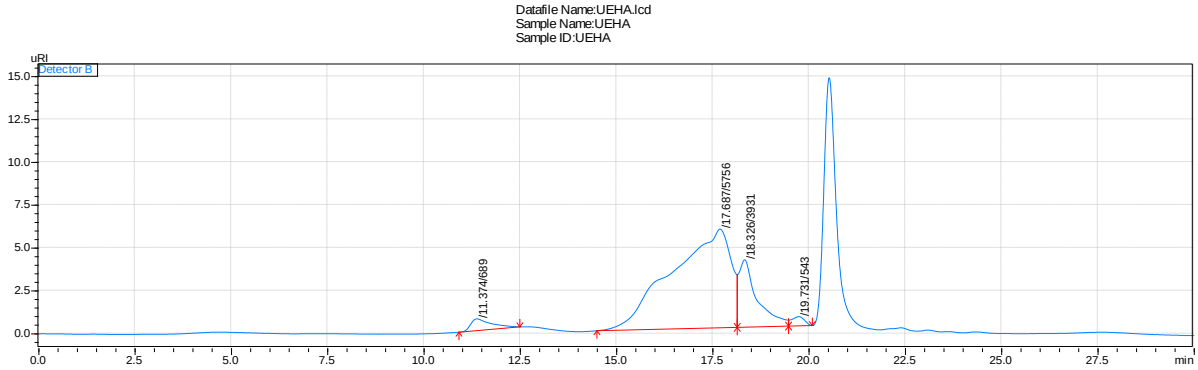
UEA *A.membranaceus*



UEA Shiitake



UEA HAS-A



UEA HAS-B

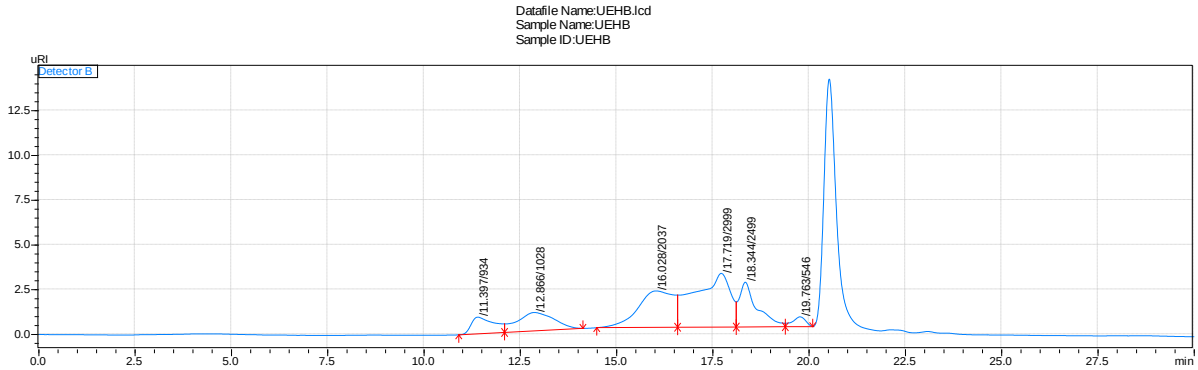


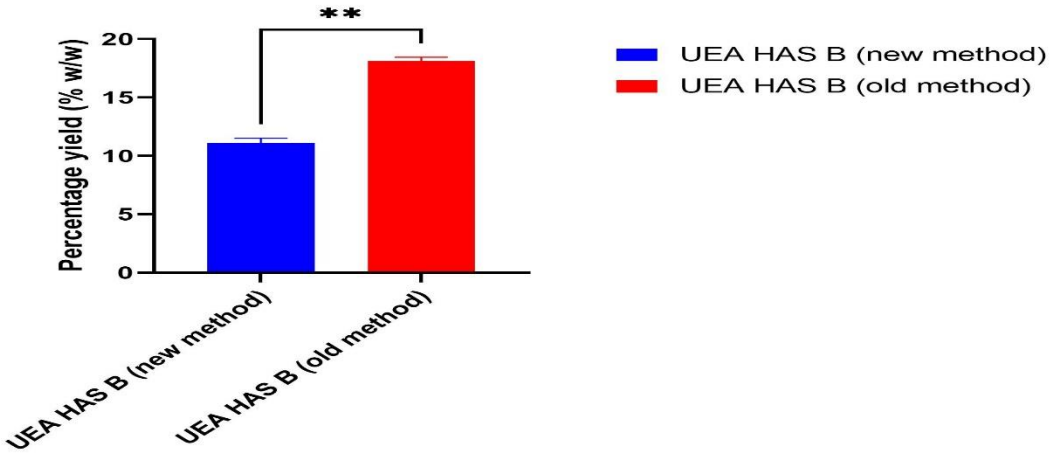
Fig. A3 HPLC chromatograms showing relative molecular weights of different extracts of *A. membranaceus*, Shiitake HAS-A and HAS-B. MA – Microwave assisted, MUA – Microwave/Ultrasound assisted, MUEA – Microwave/Ultrasound/Enzyme assisted, MEA- Microwave/Enzyme assisted and UEA- Ultrasound/Enzyme assisted extracts.

Appendix E: IC_{50} values of extracts before and after purification.

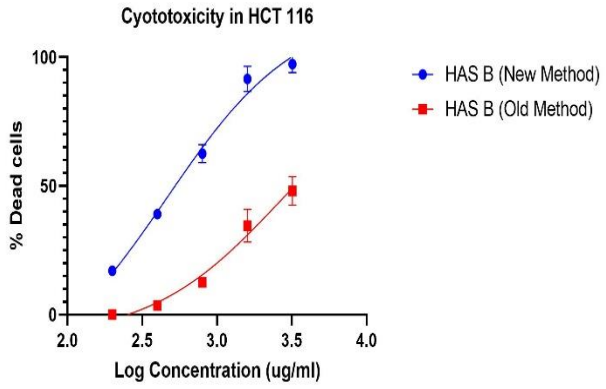
Extracts	**IC_{50} Values (µg/ml)**	
	Before Purification	**After Purification**
MA *A. membranaceus*	207.5	309.6
UEA Shiitake	659.3	687.8
UEA HAS-B	367.1	447.7

Appendix F: Effect of old reported extraction technique and new extraction technique (developed in this study) on the total polysaccharide yield and cytotoxic activity. (Data shown for UEA HAS-B)

A.



B.



C.

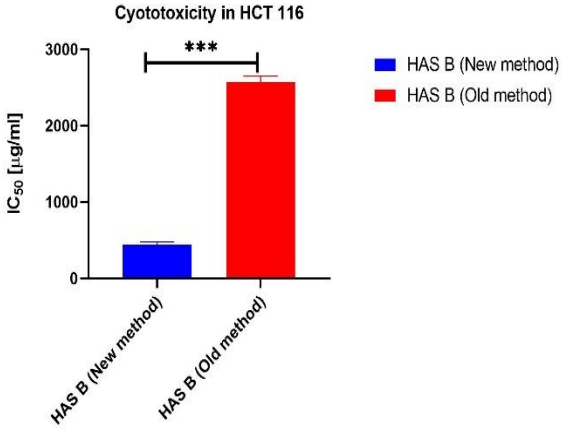


Fig. A4 Effect of old and new enzyme extraction techniques in yield and cytotoxic activity against HCT 116. A) Old extraction technique had significant increase in yield ($P < 0.01$) compared to new extraction technique applied in this study. B) New extraction technique developed in this study showed significantly high cytotoxicity activity ($P< 0.001$) compared to old reported technique.

Appendix G: Comparison of cytotoxicity of UEA HASB extracts extracted from different enzyme mixtures.

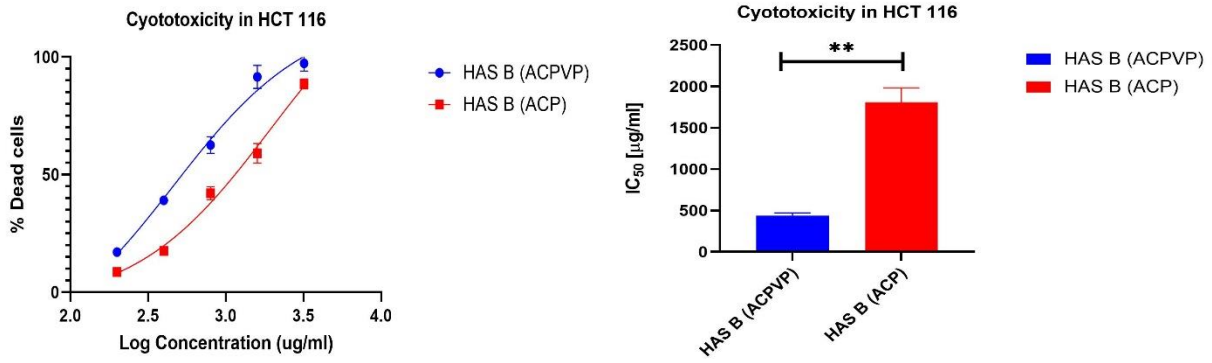


Fig. A5 Comparison of cytotoxicity of UE-HASB extracts extracted from two different enzyme mixtures. ACPVP is the mixture of four different enzymes; Alcalase, Cellulase, Pectinase and Vinotaste-pro in a ratio 1:1:1:1 and ACP is the mixture of three different enzymes; Alcalase, Cellulase and Pectinase in a ratio 1:1:1. The HAS-B extract, extracted from new enzyme mixture ACPVP (developed in this study) had shown significant increase in cytotoxic activity against HCT 116 ($P < 0.01$) compared to previously reported enzyme mixture ACP.

Appendix H: Standard curve for MTT assay.

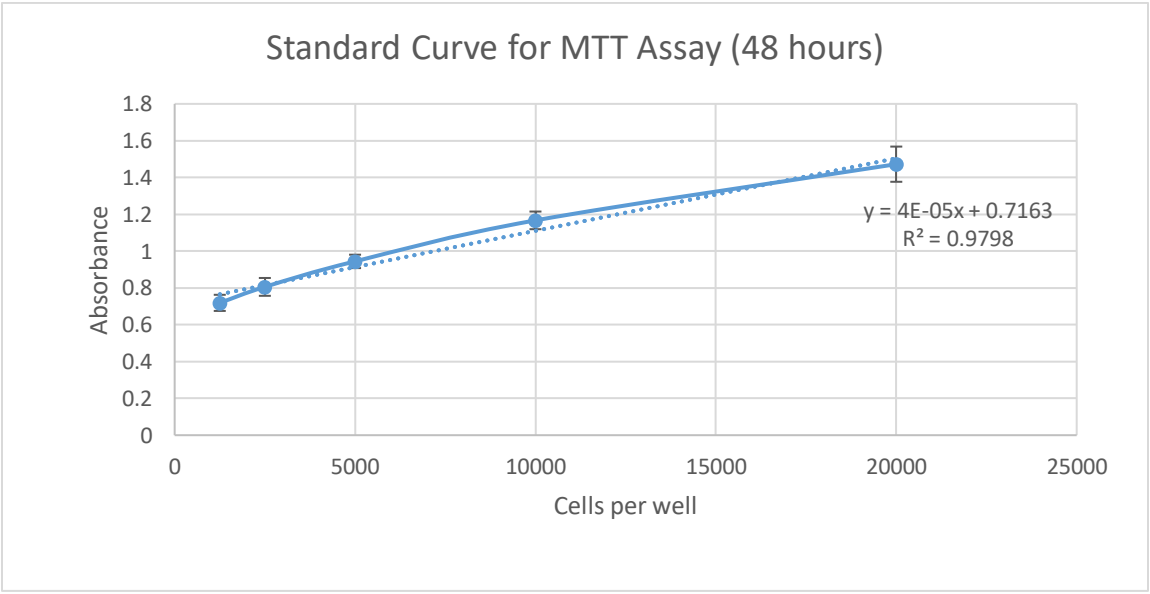
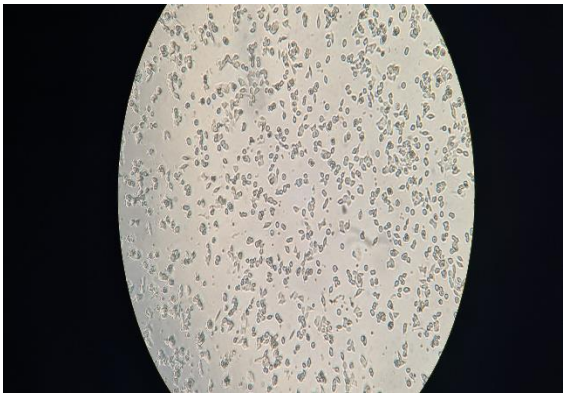
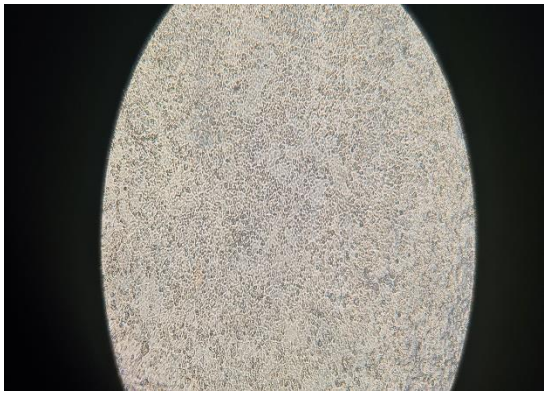


Fig. A6 Standard curve for MTT assay against HCT 116 for the incubation period of forty-eight hours.

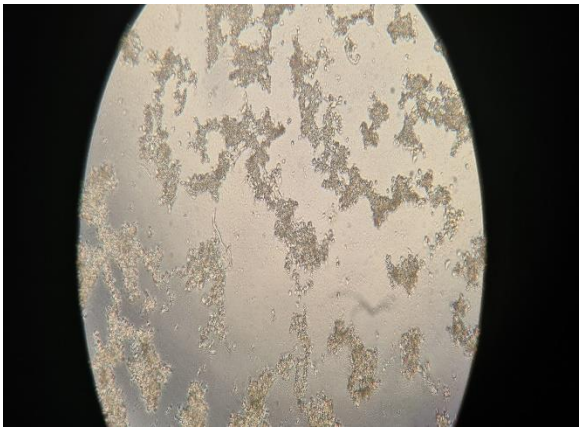
Appendix I: HCT 116 cells before and after treatment with UEA HAS-B extracts.



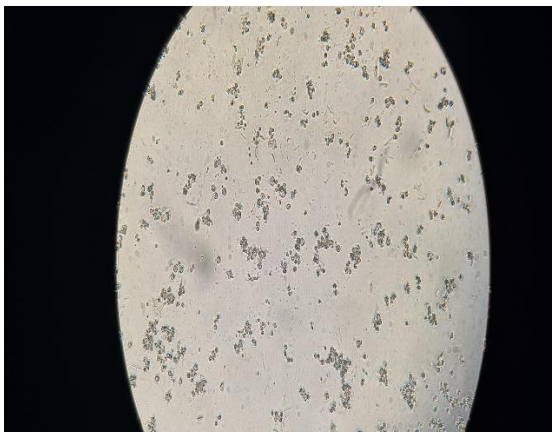
A. Seeding of cells



B. Cells after 48 hours of incubation (Negative control)



C. Cells after 24 hours of treatment with 1600 µg/ml of UEA HAS-B



C. Cells after 48 hours of treatment with 1600 µg/ml of UEA HAS-B

Fig. A7 Microscopic images of cells before and after treatment with UEA HASB (1600 µg/ml) extract.

Appendix J: Challenges faced during extraction.

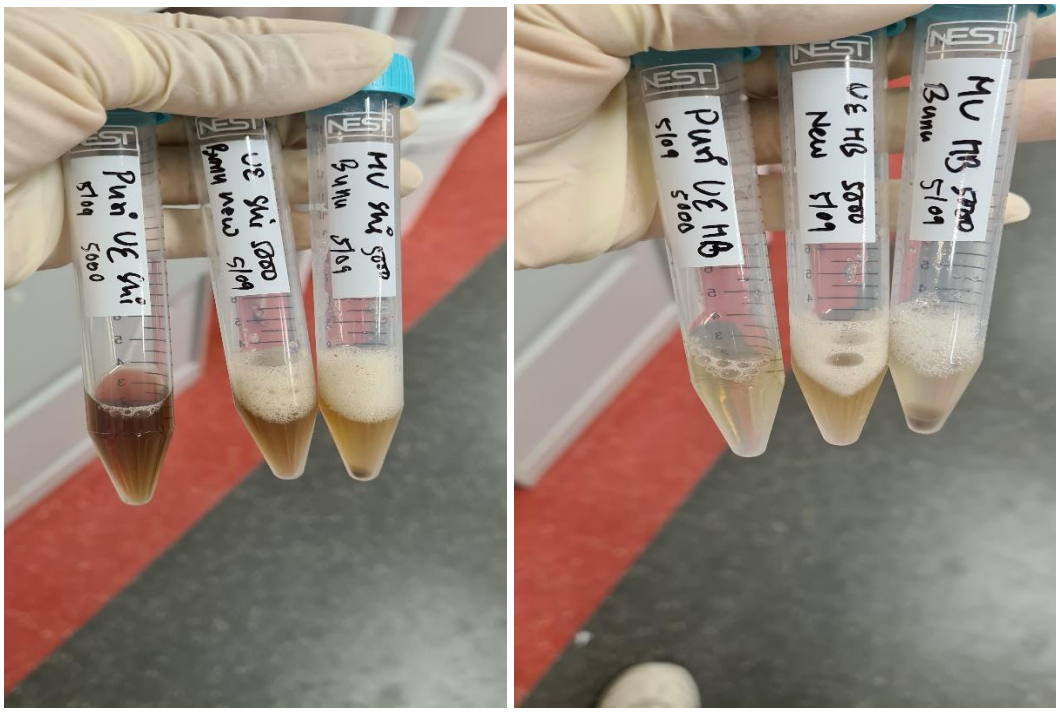
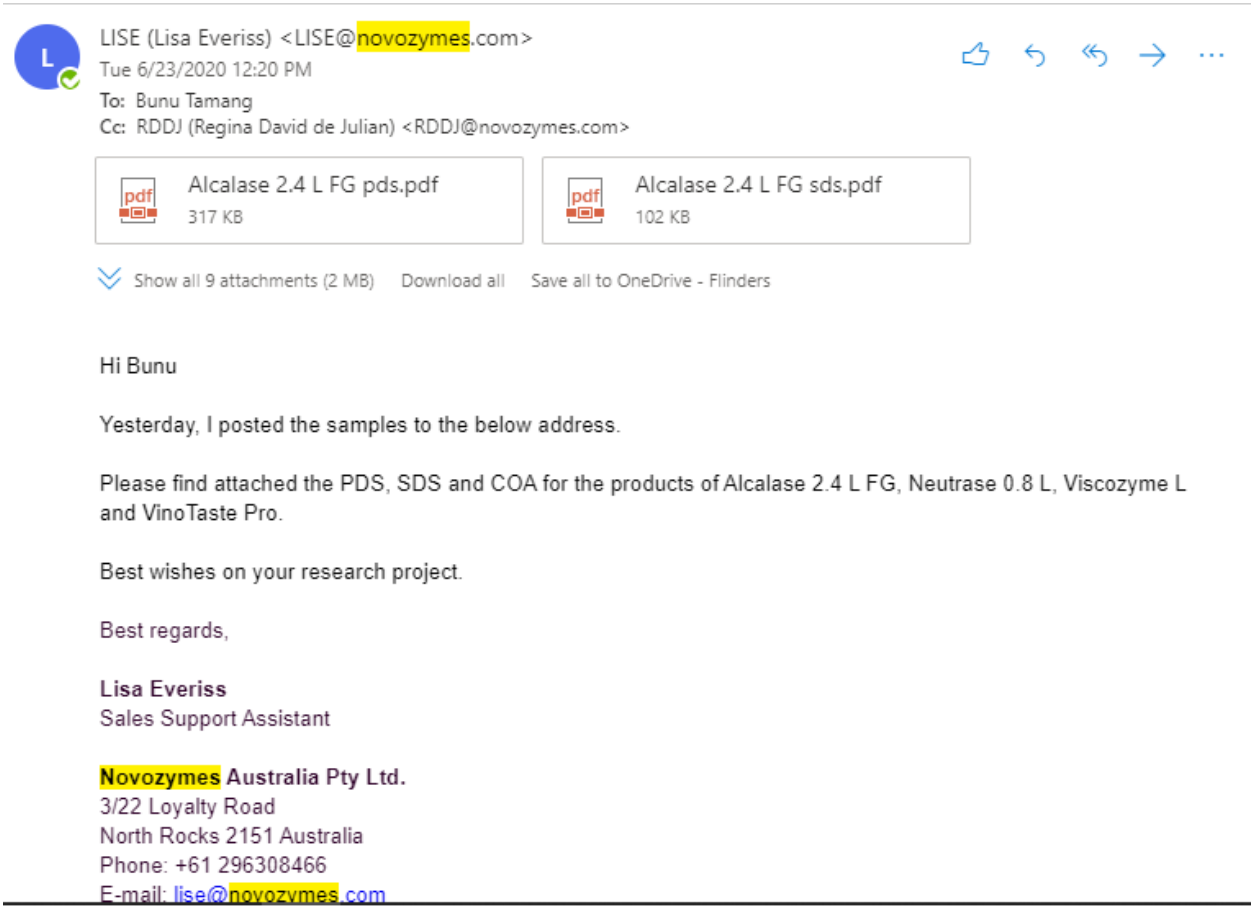


Fig. A8 Prolem in solubility of MA and MUA extracts in water. Fine suspended particles were observed which used to settle down at the bottom when left for a long time. The problem was solved when the same was extracted with mixture of enxymes under optimal condition.

Appendix K: Evidence for the use of free enzyme samples obtained from NOVOZYMES Pty Ltd.



LISE (Lisa Everiss) <LISE@novozymes.com>
Tue 6/23/2020 12:20 PM
To: Bunu Tamang
Cc: RDDJ (Regina David de Julian) <RDDJ@novozymes.com>

Alcalase 2.4 L FG pds.pdf
317 KB

Alcalase 2.4 L FG sds.pdf
102 KB

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Hi Bunu

Yesterday, I posted the samples to the below address.

Please find attached the PDS, SDS and COA for the products of Alcalase 2.4 L FG, Neutrase 0.8 L, Viscozyme L and VinoTaste Pro.

Best wishes on your research project.

Best regards,

Lisa Everiss
Sales Support Assistant

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