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Production de composés bioactifs par des levures et évaluation de leurs propriétés biologiques envers le cancer colorectal

Par

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À la douce mémoire de ma mère

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RÉSUMÉ

La chimioprévention est un processus par lequel l'utilisation d'agent chimiopréventif naturel ou synthétique permet de prévenir la progression du cancer. Son importance dans la lutte contre le cancer colorectal est justifiée par le fait que cette maladie représente le troisième cancer le plus répandu au Canada malgré les différents traitements offerts et plus d'une décennie de recherche. Il a été démontré que l'alimentation est liée à l'incidence d'environ 70% des cancer colorectaux. Donc, la consommation d'agents à fort potentiel chimiopréventif pourrait conduire à une réduction de l'incidence de ce cancer.

Les parois cellulaires de levures sont étudiées pour leurs différentes propriétés anticancer et immunomodulatrices en plus de démontrer un potentiel d'application dans les domaines pharmaceutiques, médical et alimentaires. Nonobstant plusieurs décennies de recherche investiguant leurs capacités thérapeutiques, relativement peu d'études ont rapporté le potentiel chimiopréventif des composantes de parois cellulaires de levures envers le cancer colorectal humain.

Cette étude avait pour but de démontrer le potentiel chimiopréventif des composantes de parois cellulaires de levures en modèle *in vitro* et *in vivo* envers le cancer colorectal. En premier lieu, il a été démontré que les β-glucanes insolubles de *Saccharomyces cerevisiae* var. *boulardii* possédaient une excellente capacité à induire la quinone réductase (enzyme impliquée dans la prévention du cancer) et démontraient une meilleure capacité à inhiber la croissance de cellules du cancer colorectal humain en comparaison aux autres extraits de *S. boulardii* et que ceux de *Kluyveromyces marxianus*. De plus, l'extrait de mannoprotéines de *S. boulardii* a présenté une excellente capacité antiradicalaire et antiproliférative envers les cellules cancéreuses. La quantification des polysaccharides au sein des échantillons suggère que les β-glucanes ont constitué la composante contribuant le plus significativement à l'activité biologique observée.

En second lieu, le potentiel chimiopréventif de ces deux meilleurs extraits a été évalué chez des rats traités au 1,2-dimethylhydrazine. Les résultats ont démontré que seuls les β-glucanes insolubles de *S. boulardii* étaient en mesure de réduire le nombre de lésions précancéreuses (cryptes aberrantes) considérées comme étant la première étape de la carcinogenèse du cancer colorectal. L'étude du mécanisme sous-jacent suggère une diminution du temps de transit des carcinogènes au sein du tractus digestif via une modulation de l'activité spécifique de la quinone réductase au niveau hépatique et d'une enzyme fécale (β-glucuronidase).

Finalement, des huiles essentielles de *Boswellia carterii* (Frankincense) et de *B. sacra* (Sacrée) ont été ajoutées aux extraits de β-glucanes insolubles de *S. boulardii* afin d'évaluer la possibilité d'une synergie potentielle pour les propriétés chimiopréventives entre les types de composés. Alors que l'huile Sacrée a démontré un effet cytotoxique spécifique aux cellules cancéreuses, la combinaison entre les β-glucanes insolubles de *S. boulardii* a induit la perte de cette activité. De plus, l'huile Frankincense combinée aux β-glucanes insolubles a été en mesure de démontrer une induction de la quinone réductase supérieure via un effet additif en comparaison avec celle de cette même huile testée séparément. Cette étude a permis de démontrer que les β-glucanes insolubles extraient de la paroi cellulaire de *S. boulardii*, souche de levure considérée comme probiotique et à fort potentiel industriel, pourraient être utilisés dans la fabrication d'un produit nutraceutique ou comme ingrédient fonctionnel dans une optique de prévention du cancer colorectal humain ou même sous la forme de fibre naturelle.

LISTE DES ABRÉVIATIONS

AK-BA	acétyl-keto-β-acide boswellique
AB	acide boswellique
CCR	cancer colorectal
CD	concentration doublant le niveau d'induction de la NAD(P)H : quinone réductase
CI	combinatory index
DMH	N,N-diméthylhydrazine
DMSO	Diméthylsulfoxyde
DPPH	α,α-diphényl-β-picrylhydrazil
EDTA	acide éthylènediaminetétraacétique
EOs	essential oils
FCA	foyers de cryptes aberrantes
HE	huile essentielle
IC_{50}	concentration inhibant 50% de la croissance cellulaire
IP	inhibition percentage
kDa	kilo Dalton
K-AB	keto-β-acide boswellique
NMP	N-méthylpyrrolidone
PMMA	polyméthyl méthacrylate
QR	NAD(P)H : quinone réductase
SC_{50}	concentration captant 50% l'accumulation de radicaux libre formée
Tween-80	polyoxyéthylène sorbitan monooléate
Mw	molecular weight
X/XO	xanthine/xanthine oxydase
5-FU	5-fluorouracil

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CHAPITRE 1 – INTRODUCTION

Revue bibliographique

1.1. Généralités sur le cancer colorectal (CCR)

Le CCR reste toujours un enjeu de taille en matière de santé au Canada malgré plusieurs années de recherches intensives. En 2014, ce cancer fût reconnu comme étant le second cancer le plus meurtrier chez les hommes et le troisième chez la femme en plus d'être le troisième plus répandu chez les deux sexes (Canadian Cancer Society's Advisory *et al.*, 2014, Canadian Digestive Health Foundation, 2016). Le CCR est le résultat d'une série de changements histopathologiques débutant par l'apparition de foyers de cryptes aberrantes et aboutissant généralement à un adénocarcinome (Figure 1.1) (Karoui *et al.*, 2007). La carcinogenèse du CCR s'explique par plusieurs facteurs de risque dont la plupart sont environnementaux et/ou liés au mode de vie. Parmi ces derniers notons un régime alimentaire constitué principalement de viande rouge et pauvre en fibres, fruits et légumes ainsi qu'un trop faible niveau d'activité physique (Haggar *et al.*, 2009, Stevens *et al.*, 2007). Nonobstant ces facteurs de risque bien identifiés, le CCR représente encore aujourd'hui une dépense économique importante pour le Canada sans parler des dommages émotionnels infligés aux familles canadiennes.

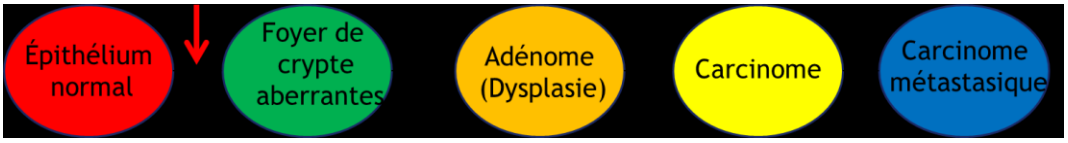

Figure 1.1 : Carcinogenèse du cancer colorectal. L'inhibition ou l'atténuation de la formation des foyers de cryptes aberrantes (flèche rouge) constitue une approche clef dans la prévention du CCR. Image modifiée de Karoui *et al.* (2007).

1.2. Aperçu des traitements contre le CCR disponibles au Canada

Les traitements contre le CCR sont soit de nature local ou systémique. Les traitements locaux impliquent essentiellement en la chirurgie et la radiothérapie et visent l'ablation ou la destruction des cellules cancéreuses dans la région touchée. En contrepartie, les traitements systémiques englobent les chimiothérapies et les thérapies ciblées. Le choix du traitement à accepter peut-être un fardeau pour le patient (et sa famille), et dépend fortement de la taille, du site et de l'étendue de la tumeur ou du polype. De plus, différentes variantes de chaque traitement existent et visent soit la guérison, la prolongation de la vie ou la limitation des effets secondaires (traitements du contrôle ou maintient) ou plutôt à l'amélioration de la qualité de la vie du patient (traitement palliatif) (Colorectal Cancer Association of Canada, 2017).

Parmi tous les traitements disponibles, la chirurgie constitue encore le principal traitement utilisé contre le CCR (toutes méthodes confondues) et est envisageable à différents stages de la carcinogenèse, soit l'ablation du polype bénin ou une colectomie qui vise à enlever une section complète du colon. Alors que la chirurgie permet d'éliminer la tumeur ou le polype, la principale limite de ce traitement est l'impossibilité de s'assurer de l'élimination de toutes les cellules cancéreuses en périphérie de la zone atteinte. La radiothérapie, tant qu'à elle, agit en endommageant l'ADN des cellules cancéreuses visées ce qui limite leur division cellulaire mais est aussi connue pour atteindre les cellules normales ce qui constitue la principale limite de ce traitement. Les traitements de radiothérapies sont soit externe, interne ou peropératoire. La radiothérapie externe, souvent utilisé en combinaison avec la chimiothérapie, est le type de radiothérapie la plus commune et consiste à faire pénétrer des rayons de fortes énergies au travers de la peau en direction des cellules cancéreuses à partir d'une source externe. La radiothérapie interne, utilisé chez les patients qui ne pourront pas supporter la chirurgie, consiste à implanter ou à injecter un composé radioactif à proximité de la tumeur et contraste avec la radiothérapie peropératoire qui est administré durant une chirurgie et consiste, tant qu'à elle, en une seule dose massive de radiothérapie externe (Canadian Cancer Society, 2017a, Colorectal Cancer Association of Canada, 2017).

Les produits utilisés dans le cadre des traitements systémiques sont essentiellement administrés via le sang ou de manière orale et consiste en des molécules anticancéreuses qui inhibent la croissance cellulaire (arrêt du cycle cellulaire, apoptose) ou induit un effet cytotoxique chez les cellules cancéreuses ciblées. Même si l'effet cytotoxique est ciblé envers les cellules cancéreuses, il n'en reste pas moins que les cellules saines en sont affectées puisque plusieurs composés

chimiothérapeutiques ciblent en fait les cellules à forte division cellulaire (ex. cellules des follicules pileux) ce qui se traduit en de très lourds effets secondaires. En revanche, les traitements de thérapie ciblés impliquent l'utilisation de composés (ex. anticorps monoclonaux) qui tentent d'intervenir au niveau d'une étape ou d'une composante spécifique à la biologie des cellules cancéreuses diminuant ainsi les effets secondaires. Cependant, de tels traitements ne sont disponibles que pour les cas de CCR métastasiques. En plus d'être dispendieux, ils nécessitent d'être combinés à des traitements de chimiothérapies pour en augmenter leur efficacité (Canadian Cancer Society, 2017b, Colorectal Cancer Association of Canada, 2017).

Tableau 1.1 : Résumé des traitements contre le CCR disponible au Canada accompagnés de leurs effets secondaires courants

Traitement	Exemples	Objectif	Effets secondaires courants
Chirurgie	Excision locale, résection intestinale, colostomie, iléostomie, exentération pelvienne	Ablation de la tumeur	Douleur, saignement, infection, problème de digestion et urinaire, fatigue, etc.
Chimiothérapie	5-fluorouracil, Capécitabine, Oxaliplatine, Irinotécan, Raltitrexed	Éliminer chimiquement les cellules cancéreuses	Perte de cheveux, faiblesse, vomissement, étourdissement, atteinte de la moelle osseusse, constipations, perte d'appétit, diarhée/constipation, douleur musculaire, etc.
Radiothérapie	Radiothérapie externe, interne et peropératoire	Destruction des cellules cancéreuses par rayons X	Brûlure (douleur), fatigue, problème d'intestin et de vessie, atteinte de la moelle osseuse, cicatrice sur la peau, etc.
Traitements ciblés (biothérapies)	Bévacizumab, Cétuximab, Panitumumab, Régorafénib	Attaquer une cible ou une étape particulière du processus tumorigénèse	Réaction cutanée, asthénie et fatigue physique, faibles taux de minéraux dans le sang, perte d'appétit, réaction à la perfusion (fièvre, maux de tête, frisson, démangeaisons, urticaire, nausée, dyspnée, anomalies aux ongles des mains ou des pieds pouvant entraîner leur chute

Le Tableau 1.1 présente une liste non exhaustive des effets secondaires des différents traitements mentionnés ci-haut. C'est donc de dire que les traitements utilisés contre le CCR sont synonymes d'effets secondaires majeurs, en plus d'être invasif, coûteux, et éprouvant pour le patient et ces proches. En ce sens, une approche préventive et naturelle semble être une meilleure option qu'une approche thérapeutique dans la lutte contre le CCR.

1.3. L'option de la *chimioprévention*

Bien que la détection précoce d'un CCR ne prévienne pas nécessairement son développement, plusieurs études ont été menées sur la prévention primaire de cette maladie (tabagisme, mode de vie, alimentation, activité physique, etc.) dans l'optique de réduire l'incidence de ce cancer. Cependant, plusieurs études ont souvent débouché sur des conclusions contradictoires ouvrant ainsi la voie à l'option de la chimioprévention dans la lutte contre le CCR (Manzano *et al.*, 2012). La chimioprévention est un processus par lequel l'utilisation d'agents chimiopréventifs naturels ou synthétique permet de prévenir la progression du cancer (Hakama, 1998). Il est estimé qu'environ 200 composés ont été identifiés comme étant potentiellement chimiopréventifs envers le CCR dont les fibres diététiques, les probiotiques ainsi que les antioxydants (Czadek, 2016). Le caractère chimiopréventif d'un composé implique souvent plus d'un mécanisme sous-jacent et est propre à lui-même justifiant l'importance d'avoir une large gamme de tests pour démontrer son potentiel chimiopréventif. La première étape de la carcinogenèse du CCR implique, entre autres, l'exposition des cellules saines à des carcinogènes et à des espèces chimiques réactives de l'oxygène (ERO) ainsi qu'à la perte des équilibres entre apoptose/mitose, enzymes de phase I/phase II et bactéries bénéfiques/nocives de l'environnement intestinal (Caillet *et al.*, 2012, Hanahan *et al.*, 2011, Prochaska *et al.*, 1988b, Simon *et al.*, 1986).

1.3.1. Les enzymes de phase II

Les enzymes de détoxification cellulaire de phase II, telles que la NAD(P)H quinone réductase (QR), sont connues pour protéger le milieu intracellulaire des métabolites électrophiliques toxiques directement impliqués dans les premières étapes de la carcinogenèse. En ce sens, la QR réduit ou inhibe la toxicité des carcinogènes chimiques via leur transformation en une forme moins toxique ou en un métabolite inerte (Kang *et al.*, 2004). L'activité de la QR est régulée par les protéines Keap1/Nrf2/ARE où l'inducteur potentiel, possédant une fonction (α-β)-cétone insaturée, permet l'alkylation des résidus cystéines hautement réactifs au niveau de la protéine Keap1. L'activation de Kelch-like ECH-Associated Protein 1 (Keap1) permet à son tour l'entrée de Antioxydant Response Element (ARE) dans le noyau cellulaire et induit alors la transcription des gènes codant pour la QR (Dinkova-Kostova *et al.*, 2002). Par la suite, l'enzyme résultante pourra servir à détoxifier le milieu intracellulaire. Depuis plusieurs années, les analyses d'induction de la QR en

système *in vitro* représentent une manière efficace, facile et rapide pour la découverte de nouvelles molécules possédant des propriétés chimiopréventives potentielles (Cuendet *et al.*, 2006, Kang *et al.*, 2004, Misico *et al.*, 2002).

1.3.2. Les espèces réactives de l'oxygène (ERO)

Les ERO sont des espèces chimiques possédant au moins un atome d'oxygène ou appartenant à une forme chimique de l'oxygène et qui ont une activité chimique supérieure à celle de l'oxygène. L'anion superoxide (anion O_2^-) et l'anion oxyde nitrique figurent parmi les ERO les plus importants, de par le fait qu'ils représentent des précurseurs aux espèces chimiques responsables des transformations cellulaires, tel le peroxyde d'hydrogène, le radical hydroxyde et l'anion peroxynitrite. L'effet négatif des ERO, connu sous le terme du stress oxydatif, survient lorsque la formation intracellulaire de ces espèces surpasse les capacités de défense du système antioxydant cellulaire. L'effet du stress oxydatif dans le milieu intracellulaire mène à la peroxydation des lipides, des dommages aux barrières de la muqueuse intestinale et à l'ADN ainsi qu'à des réponses inflammatoires pro-tumorale pouvant résulter en l'augmentation des risques de développer un CCR (Hanahan *et al.*, 2011, Wang *et al.*, 2016, Young *et al.*, 2001). En ce sens, un composé pouvant réduire ou empêcher la formation ou l'accumulation des ERO démontre un potentiel chimiopréventif pertinent.

1.3.3. La cytotoxicité cellulaire et les tests antiprolifératifs

Depuis quelques dizaines d'années, l'établissement d'un système de criblage de composés anticancers devient une nécessité face à l'augmentation des cas de cancer répertorié. En ce sens, l'*US National Cancer Institute* (NCI) a développé une méthode pour cribler un grand nombre de composés potentiellement cytotoxiques envers les cellules cancéreuses. Un composé cytotoxique peut essentiellement induire la mort cellulaire par nécrose ou apoptose conduisant en une diminution de la prolifération cellulaire de la population. Alors que la nécrose implique souvent une perte d'intégrité membranaire, l'apoptose consiste en la mort cellulaire programmée (voir section 1.2.4). Les tests antiprolifératifs, maintes fois optimisés dans la littérature, sont souvent basés sur la capacité des cellules métaboliquement active après traitement à cliver le sel de tétrazolium (MTT) en cristaux de formazan violet permettant de quantifier l'effet antiprolifératif d'un composé en comparaison avec des cellules non traitées. Cette méthode *in vitro* facile et rapide permet de cribler un grand nombre de composés à de multiples concentrations envers différents

types de lignées cellulaires cancéreuses humaines. En ce sens, les tests antiprolifératifs tels que celui-ci basés sur le MTT renseignent davantage sur les propriétés anticancer et thérapeutiques que sur leurs propriétés chimiopréventives. Les tests antiprolifératifs peuvent aussi bien être réalisés sur des cellules saines que sur des cellules cancéreuses. Dans cette optique, il est possible d'évaluer la toxicité d'un composé en déterminant ces valeurs IC_{50} (concentration d'échantillon pouvant inhiber la croissance cellulaire de 50%) envers des cellules saines et des cellules cancéreuses. Un composé possédant une faible toxicité ($IC_{50cancéreux} > IC_{50non\text{-}cancéreux}$) est considéré comme intéressant dans une approche thérapeutique (Alley *et al.*, 1988, Boyd, 1997, Denizot *et al.*, 1986, van Meerloo *et al.*, 2011, Vistica *et al.*, 1991).

1.3.4. La mort cellulaire programmée (apoptose)

L'apoptose est définie comme un processus de mort cellulaire hautement régulé et organisé chez les organismes multicellulaires œuvrant à plusieurs stades du développement. Parmi les phénomènes apoptotiques les plus connus notons l'élimination des lymphocytes autoréactifs ainsi que l'élimination de cellules infectées par des virus et des cellules malignes. Ce phénomène naturel forme une balance homéostatique avec le processus de mitose. Une perte de l'équilibre fragile entre ces deux processus est en partie à l'origine de la transformation cellulaire, de la carcinogenèse et de la formation de tumeurs. Le phénomène d'apoptose est donc une barrière au développement du cancer (Hanahan *et al.*, 2011, Matsuzawa *et al.*, 2001). En ce sens, un composé en mesure de réduire la viabilité des cellules cancéreuses et d'induire le processus d'apoptose dans les cellules cancéreuses démontre des propriétés à la fois chimiopréventives et anticancer.

1.3.5. Le microbiote intestinal et les enzymes fécales

Le côlon contient le plus grand nombre de bactéries métaboliquement actives chez l'humain adulte en plus de représenter un écosystème des plus complexes et en constante évolution. Nonobstant l'existence de variabilité intrinsèque dans la composition des microbiotes humains, l'alimentation semble être une des causes majeures de changement dans la proportion des genres bactériens chez l'adulte. De plus en plus d'études rapportent de fortes corrélations entre des profils spécifiques de microbiotes intestinaux et l'augmentation de l'incidence de plusieurs maladies et syndromes dont le CCR (Louis *et al.*, 2014). Les composantes non digérées du régime alimentaire, comme par exemple les polysaccharides non amylacés, peuvent se retrouver intactes au niveau du côlon et ainsi permettre de faire varier la capacité de fermentation du microbiote intestinal, la durée du transit et

le pH intestinal aboutissant à un changement dans la composition des espèces bactériennes présentes dans le côlon. Cette composition du microbiote intestinal a une importance cruciale dans la nature des métabolites retrouvés dans le côlon. En effet, plusieurs espèces bactériennes endogènes du microbiote sont reconnues pour produire des acides gras à courte chaînes (AGCC) considérés comme des métabolites protecteurs envers le CCR principalement de par leurs actions anti-inflammatoires (Chang *et al.*, 2014, Fung *et al.*, 2012, Louis *et al.*, 2014, Smith *et al.*, 2013).

En contrepartie, certaines enzymes bactériennes telles que la β-glucuronidase et la β-glucosidase sont connues pour déconjuguer les toxines et les carcinogènes qui ont été préalablement détoxifiés dans le foie pour être sécrétés dans l'intestin via la bile. En ce sens, l'effet de ces enzymes bactériennes constitue la contrepartie des enzymes de détoxification cellulaire telle que la QR. La β-glucuronidase et la β-glucosidase sont produites dans le côlon par une large gamme de bactéries nocives : *Clostridium sp.* (*C. paraputrificum, C. clostridioforme, C. perfringens*), *Bacteroides sp.* (*B. vulgatis, B. uniforme, B. fragilis*), *Enterococcus* sp*., Peptostreptococcus* sp*., Staphylococcus* sp*., Ruminococcus* (gnavus), *Eubacterium* sp. et *Escherichia coli*. La présence de ces enzymes peut mener à une forte concentration localisée de carcinogènes au niveau du côlon ayant pour effet d'augmenter le risque de développer un CCR. De manière générale, la détoxification d'un composé toxique ou carcinogène débute dans le foie où le composé est conjugué à un acide glucuronique ou un autre composé glyconé par les enzymes de phase II pour être ensuite excrété dans le côlon via la bile. À ce niveau, la β-glucuronidase et la β-glucosidase agissent à contresens en déconjuguant ces composés nocifs préalablement détoxifiés (Dabek *et al.*, 2008, Louis *et al.*, 2014, McBain *et al.*, 1998, Simon *et al.*, 1986). Plus spécifiquement, la β-glucuronidase hydrolyse l'acide glucuronique du conjugué relâchant ainsi le métabolite nocif. Pour sa part, la β-glucosidase hydrolyse les liens glycosidiques unissant le métabolite hétéroside détoxifié produisant un résidu glucosyl terminal non réducteur et le métabolite nocif. Dans tous les cas, la détoxification d'un carcinogène augmente le temps de transit de ce dernier dans le côlon augmentant ainsi les risques de carcinogenèse (Gill *et al.*, 2002, Ketudat Cairns *et al.*, 2010, Mroczynska *et al.*, 2013). D'un point de vue clinique, une plus grande activité enzymatique de la β-glucuronidase a été reportée chez des patients atteints du CCR en comparaison avec des patients sains démontrant ainsi l'importance de ces enzymes bactériennes (Kim *et al.*, 2001). La présence d'une faible activité de ces enzymes bactériennes découlant en grande partie de la composition du microbiote intestinal et donc de la balance entre les bactéries bénéfiques et néfastes est d'une grande importance dans la prévention du CCR.

1.4. Les parois cellulaires de levures

Les levures ont depuis toujours été utilisées par l'humain pour leur capacité à produire de l'alcool ainsi qu'à apporter des arômes aux matrices alimentaires. L'industrie alimentaire moderne utilise largement les levures dans plusieurs domaines. Cependant, la biomasse de levures ainsi que leur paroi cellulaire sont souvent considérées comme un déchet industriel. Cet état des faits a amené, il y maintenant quelques décennies, la communauté scientifique à se pencher sur le potentiel bioactif de la biomasse de levures et de leur paroi cellulaire dans les domaines médicaux et des suppléments alimentaires naturels (dos Santos Mathias *et al.*, 2014, Giavasis, 2014, Laroche *et al.*, 2007). Ces années de recherches ont permis, entre autres, de soulever une attention accrue à différents types de polysaccharides présents dans la paroi cellulaire de levures notamment de par leurs propriétés antitumorales et immunomodulatrices chez l'humain.

1.4.1. L'architecture et la composition des parois cellulaires de levures

Les levures sont considérées comme des champignons unicellulaires et eukaryotiques (diamètre d'environ 8µm) se reproduisant par reproduction sexuée et par bourgeonnement. Les levures industrielles appartiennent majoritairement au genre *Saccharomyces* (par exemple, *S. cerevisiae* et *S. cerevisiae* var. *boulardii*) alors que des membres du genre *Kluyveromyces* (par exemple, *K. marxianus*) sont surtout utilisés dans l'industrie laitière. Les parois cellulaires de levures assurent quatre fonctions majeures au sein de la cellule : i) pression osmotique interne, ii) protection contre les stress mécaniques en agissant comme une couche protectrice, iii) maintien de la forme cellulaire elle-même changeante selon les conditions de croissance, le cycle cellulaire ainsi que les réponses aux phéromones, IV) échafaudage formé de polysaccharides permettant la présence d'une couche de protéines à la face extérieure de la cellule (Klis *et al.*, 2006). Les parois cellulaires de levures sont des structures dynamiques et sensibles aux conditions de croissance ainsi qu'aux stress (Aguilar-Uscanga *et al.*, 2003). Cela dit, ces structures cellulaires sont typiquement composées des mêmes polysaccharides échafaudés dans un ordre relativement redondant. En effet, les parois cellulaires de levures sont typiquement composées de chitine, de (1→3)-β-D-glucanes possédant des ramifications (1→6)-β-D-glucanes et de mannoprotéines reliés par des liaisons covalentes (figure 1.2).

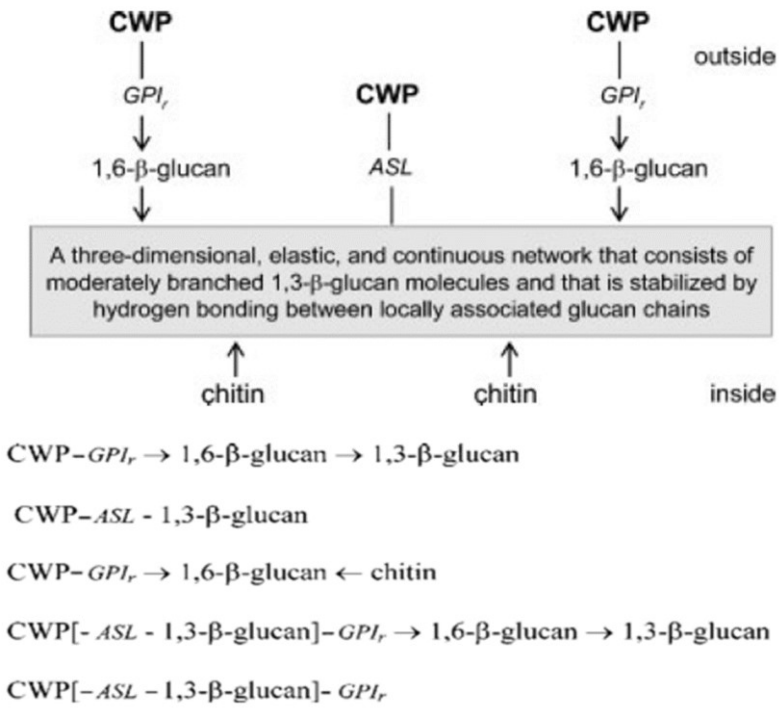

Figure 1.2 : Organisation typique des parois cellulaires de levures (S. cerevisiae). Les termes outside et inside réfèrent au milieu externe et interne de la cellule respectivement. CWP; Cell Wall Proteins. GPI; Ancre de glycosylphosphatidylinositol. ASL; Alkali-Sensitive Linkage. Image modifiée de Klis et al. (2006).

Typiquement, les parois cellulaires de levures sont formées de trois épaisseurs de β-glucanes ; une couche interne insensible aux traitements alcalins connectée à la chitine, une couche intermédiaire sensible aux traitements alcalins et une couche externe de glycoprotéines possédant des résidus mannoses phosphorylés. La teneur en ces macromolécules (tableau 1.2) varie énormément selon différents paramètres tels que la souche de levure, les conditions et la phase de croissance ainsi que la méthode d'extraction (Aguilar-Uscanga *et al.*, 2003, Ahmad *et al.*, 2012, Klis *et al.*, 2002, Mantovani *et al.*, 2008, Pinto *et al.*, 2014). Alors que les β-glucanes et la chitine sont des polymères de glucose et de N-acétyle-glucosamine respectivement, les mannoprotéines sont typiquement constituées de mannanes (polymère de mannose) et de protéines reliées par des liaisons glycosidiques (tableau 1.2).

Tableau 1.2 : Teneurs relatives des différentes composantes typiquement retrouvées dans les parois cellulaires de levures (*S. cerevisiae*)

Composante	Masse sèche relative au poids des parois cellulaires (%)
(1→3)-β-D-glucanes	30-55
(1→6)-β-D-glucanes	5-10
Mannoprotéines	30-50
Chitine	1.5-6

Données compilées d'après Klis *et al.* (2006) et Kwiatkowski *et al.* (2012).

Les β-glucanes adoptent une structure en hélice triple maintenue par des liaisons hydrogènes et possédant des ramifications habituellement formées de (1→6)-β-D-glucanes. La structure en hélice triple et le taux de ramification des β-glucanes ainsi que leur poids moléculaire, leurs charges et leurs degrés de polymérisations (longueur du polymère) dictent la solubilité de ce polysaccharide une fois en solution. Tous ces paramètres ont une importance dans l'activité biologique et dépendent de la méthode d'extraction utilisée. De manière générale, une méthode d'extraction rude affectera l'intégrité de la structure des β-glucanes diminuant aussi leurs activités biologiques (Mantovani *et al.*, 2008, Zekovic *et al.*, 2005). Malgré sa faible teneur, la chitine est souvent reconnue comme étant responsable de la nature insoluble des parois cellulaires. Le rôle de ce polysaccharide est d'ancrer la paroi cellulaire à la membrane plasmique. Cependant, la faible quantité de chitine présente dans la paroi cellulaire est principalement retrouvée au niveau des cicatrices de bourgeonnements. De plus, sa teneur est connue pour augmenter grandement en condition de stress. Finalement, la nature des protéines retrouvées au niveau des mannoprotéines est très large et dynamique. La majorité des mannoprotéines sont modifiées par une ancre GPI et sont donc liées de manière covalente aux (1→6)-β-D-glucanes. Une faible proportion des protéines de la paroi cellulaire est directement liée aux (1→3)-β-D-glucanes via une liaison sensible aux traitements alcalins.

1.4.2. Propriétés anticancer et chimiopréventives des parois cellulaires de levures et de ces composantes

Puisque le traitement du CCR peut s'avérer coûteux, invasif et douloureux pour les patients et les gouvernements, il semblerait que la prévention soit l'approche la plus efficace. En ce sens, l'impact du CCR sur la santé publique au Canada et aux États-Unis a soulevé une demande pour la consommation de produits naturels dans la lutte pour la prévention de cette maladie. Dans cette optique de prévention du CCR, la biomasse de levures ainsi que toutes ses composantes cellulaires furent intensivement étudiées. Cet engouement pour la biomasse de levures et ses parois cellulaires est justifié par sa facilité d'obtention, sa disponibilité ainsi que son caractère naturel en plus d'être d'actualité scientifique.

Étant initialement des déchets de l'industrie agroalimentaire, les parois cellulaires de levures furent utilisées pour nourrir le bétail où ils démontrèrent des capacités à renforcer le système immunitaire des animaux. Il est maintenant accepté que les polysaccharides contenus dans les parois cellulaires soient majoritairement responsables de l'activité biologique de ces dernières. Plus particulièrement, les β-glucanes et les mannoprotéines sont reconnus pour leurs propriétés anticancer, immunomodulatrices et antiradicalaires (Aguilar *et al.*, 2012, Bohn *et al.*, 1995, Chan *et al.*, 2009, Falch *et al.*, 2000, Jaehrig *et al.*, 2008, Kobayashi *et al.*, 2005, Luhm *et al.*, 2006, Oliveira *et al.*, 2013, Rand *et al.*, 2010, Stier *et al.*, 2014, Yoon *et al.*, 2008). Les propriétés immunomodulatrices des β-glucanes sont explicables par le fait que cette macromolécule soit absente chez les cellules des mammifères. Or, les macrophages reconnaissent les β-glucanes comme des motifs moléculaires caractéristiques des pathogènes (MMCP) via le récepteur dectin-1 ce qui induit leur activation et conduit à une réponse immunitaire innée (Rand *et al.*, 2010, Tsoni *et al.*, 2008). En ce sens, l'activité anticancer *in vitro* des β-glucanes est due à l'implication des cellules immunitaires, notamment à l'augmentation de l'infiltration des cellules T activées dans l'environnement tumoral ainsi qu'à l'augmentation de l'activité antitumorale des « cellules tueuses naturelles » dites cellules NK (Adams *et al.*, 2008). De plus, il a été rapporté que les β-glucanes sont en mesure d'induire des phénomènes d'apoptose au niveau des cellules humaines du cancer colorectal pouvant aussi expliquer leurs propriétés anticancer et chimiopréventives (Kim *et al.*, 2009).

Les mannoprotéines de levures ont, quant à eux, démontré des capacités à ralentir la croissance de différentes lignées cellulaires cancéreuses humaines en plus de démontrer des propriétés antiradicalaires. Ces dernières sont probablement dues à la présence de résidus aromatiques et de groupes thiols au sein des glycoprotéines leurs conférant des propriétés antiradicalaires (Jaehrig *et*

al., 2007, Liu *et al.*, 2011). Finalement, des complexes de protéines-polysaccharides (CPP) furent souvent rapportés comme ayant des propriétés antitumorales où un phénomène de cytotoxicité direct serait en cause davantage qu'un effet de réponse immunitaire (Moharib *et al.*, 2014, Ooi *et al.*, 2000, Wang *et al.*, 1995).

1.5. Les huiles essentielles (HE)

Une huile essentielle (HE) est un mélange complexe de composés chimiques au sein duquel les différentes composantes agissent ensemble procurant un effet biologique. Ce mélange, obtenu des plantes, forme un liquide hydrophobe contenant des composés aromatiques volatiles propres à la plante en question et typiquement obtenu par hydro-distillation ou par distillation à la vapeur. Au sein de la plante, les HE ont un rôle antibactérien, antiviral, antifongique, insecticide en plus d'attirer certains insectes pollinisateurs. Dès le Moyen Âge, les HE ont déjà commencé à être utilisées en raison de leurs propriétés médicinales (i.e. antiseptique, anti-inflammatoire, analgésique local), ainsi que dans les pratiques d'embaumement et dans la préservation des aliments. De nos jours, les HE et leurs constituants sont utilisés dans plusieurs produits ainsi que dans une large gamme d'applications ; parfums, maquillages, produits sanitaires, dentisterie, agriculture, préservatif alimentaire ainsi que comme remède naturel. L'utilisation de certaines HE dans le domaine de la médecine semble encore être sujet de discorde dans la communauté scientifique. Plusieurs HE ou bien leurs composantes majoritaires ont démontré des effets anticancer et chimiopréventives très intéressantes qui seront développés dans les sections 1.4.2. et 1.4.3. du présent document (Bakkali *et al.*, 2008, Burt, 2004, Gautam *et al.*, 2014).

1.5.1. Les constituants chimiques des HE

Les différents effets biologiques connus des HE sont attribuables aux espèces chimiques qui les composent. Les HE étant des mélanges complexes, il n'est pas rare d'observer qu'une HE possède une activité biologique supérieure à celle de son composé majoritaire ce qui témoigne d'un effet synergique entre ses composantes. Plusieurs paramètres peuvent faire varier la composition d'une même HE; l'espèce végétale, la partie de l'arbre utilisée, la méthode d'extraction, la composition du sol, l'étape du cycle végétatif de la plante, la saison, le climat et le lieu géographique de la récolte. Par contre, certaines familles de composés chimiques sont en grande partie responsables des propriétés biologiques des HE. En effet, les terpènes (mono, di et sesquiterpènes), les cétones, les esters, les phénols et les lactones ont largement été cités en ce sens (Bakkali *et al.*, 2008).

1.5.2. L'activité anticancer et chimiopréventive des HE

Plusieurs HE ainsi que leurs constituants majoritaires ont démontré des propriétés anticancer et chimiopréventives. La nature lipophile de plusieurs composantes des HE leur permettent de facilement traverser la membrane plasmique des cellules leur donnant ainsi accès au cytosol. L'effet des HE sur les cellules cancéreuses peut impliquer différents mécanismes de nature nécrotique, apoptotique ou induire l'arrêt du cycle cellulaire. Les effets nécrotiques des HE dans les cellules eukaryotiques sont dus à une dépolarisation de la membrane mitochondriale affectant la chaîne de protons des électrons ainsi que les réserves d'ATP. De plus, les HE peuvent augmenter la perméabilité des membranes cellulaires et mitochondriales résultant en une perte de fluide. Les mitochondries contiennent différentes ERO dans le but d'accomplir leurs fonctions cellulaires. En ce sens, la perturbation de la membrane mitochondriale mène à une perturbation des flux d'électrons et donc à une accumulation de ERO qui résulte en une oxydation et endommage les lipides, les protéines et l'ADN causant la mort cellulaire. En revanche, certaines HE ont démontré la capacité à induire des phénomènes d'apoptose dans différentes lignées cellulaires cancéreuses. En effet, la modulation des gènes reliés à l'apoptose, l'arrêt du cycle cellulaire ainsi que la modulations de plusieurs protéines ou complexes protéiques intimement impliquées dans les différentes voies signalétiques du mécanisme apoptotique (l'activation des protéines caspases, BCL-2, MAPK et l'inactivation de la protéine AKT) font partie des manifestations les plus fréquentes des HE aboutissant à l'apoptose (Gautam *et al.*, 2014).

Certaines HE démontrent aussi des propriétés chimiopréventives *in vitro* et *in vivo* notamment envers la capacité à capter les radicaux libres (DPPH, O_2^-, OH^-) et à agir comme inducteur des enzymes de phase II. Nonobstant du fait qu'il est complexe d'attribuer la responsabilité d'une composante d'une huile à une activité biologique spécifique, il n'en reste pas moins que de telles propriétés augmentent le potentiel chimiopréventif d'une huile essentielle.

1.5.3. Les HE de *Boswellia spp*.

Les HE du genre *Boswellia spp (*famille des *Burséracées)*, aussi connues comme « arbre à encens », sont traditionnellement utilisées en médecine, en aromathérapie et en soins cosmétiques ainsi que dans plusieurs pratiques religieuses. Les HE produites par la résine de ces arbres (Figure 1.3) sont connues sous le nom de Frankincense et possèdent une odeur de bois épicée et sont généralement transparentes ou légèrement jaunâtres. L'utilisation de ces HE dans les populations humaines remonte à l'Égypte antique où elles étaient utilisées en embaumement et sont encore

largement utilisées dans les cérémonies religieuses modernes juives, catholiques romaines et grecs orthodoxes jusqu'à devenir encore aujourd'hui unes des huiles essentielles les plus commercialisées au niveau du marché international (Van Vuuren *et al.*, 2010).

Figure 1.3 : Apparence d'une gomme de résine utilisée pour la production des huiles Frankincenses. Figure modifiée de Woolley *et al.* (2012).

Les HE de *Boswellia spp.* sont riches en monoterpènes (α/β-pinène, limonène, α-thujène, sabinène, myrcène, p-cymène, α-copaène, δ-cadiène) ainsi qu'en triterpène tel que l'acide boswellique (BA), l'acide keto boswellique (K-BA) et l'acide acétyl-keto boswellique (AK-BA). Cependant, des variations importantes dans la composition de ces huiles provenant de différentes espèces de *Boswellia spp* furent rapportées (Woolley *et al.*, 2012). Dans ce contexte, plusieurs botanistes, scientifiques et gouvernements considèrent toujours *B. carterii* et *B. sacra* comme étant une seule et même espèce alors que certaines études tentent de prouver le contraire. En effet, Woolley *et al.* (2012) ont rapporté que l'huile essentielle de *B. sacra* diffère de celle de *B. carterii* de par les valeurs supérieures de rotation optique (+30.1 et -13.3° respectivement), les ratios énantiomériques et les teneurs en α-pinène (79.0 et 48.2% respectivement). Ces variations dans la composition de ces deux huiles ne sont pas seulement attribuables au fait qu'elles proviennent de deux espèces différentes, mais aussi au fait que ces arbres ne poussent pas dans la même région géographique. En effet, il est généralement accepté que *B. carterii* est retrouvé en Somalie alors que c'est au Yémen et en Oman que l'on retrouve *B. sacra*.

1.5.4. Activités anticancer des huiles de *Boswellia carterii* et de *B. sacra.*

Une riche littérature démontre les propriétés pharmaceutiques des huiles essentielles de *B. sacra* et *B. carterii*. En effet, ces huiles sont utilisées pour leurs propriétés anti-inflammatoires, immunomodulatrices ainsi qu'en santé cutanée et plus récemment pour leurs propriétés anticancer. La capacité des huiles essentielles de *B. carterii* et de *B. sacra* à inhiber ou ralentir la croissance de différentes lignées cellulaires cancéreuses a été rapportée maintes fois dans la littérature (Chen *et al.*, 2013, Dozmorov *et al.*, 2014, Frank *et al.*, 2009, Hakkim *et al.*, 2015, Ni *et al.*, 2012, Suhail *et al.*, 2011, Xia *et al.*, 2016). Par exemple, Hakkim *et al.* (2015) ont démontré qu'une huile essentielle de *Boswellia* obtenu en Oman fût en mesure d'inhiber totalement la croissance de cellule humaine du cancer du sein jusqu'à une concentration de 1 :3250 (265 ppm) via un test antiprolifératif au MTT. Les auteurs ont en plus démontré un changement dans la morphologie cellulaire des cellules après traitement résultant en une perte de leur adhérence ainsi qu'à l'adoption d'une morphologie sphérique ce qui témoigne d'un arrêt de leurs cycles cellulaires. Les composantes majoritaires de leurs huiles furent l'α-pinène (61.56%) et l'α-amyrine (20.60%). Toujours à titre d'exemple, Ni *et al.* (2012) ont démontré qu'une huile essentielle de *B. sacra* fût en mesure d'inhiber efficacement la viabilité cellulaire de différentes lignées humaines du cancer du pancréas. Les auteurs ont rapporté des valeurs d'IC_{50} variant de 1:240 (3583 ppm) à 1:1560 (551 ppm) en plus d'identifier les composantes majoritaires comme étant l'α-pinène, la limonène, la myrcène et l'acide boswellique. Alors que ces études renseignent sur la capacité des huiles à inhiber la croissance de cellules cancéreuses, elles n'indiquent pas leurs effets sur les cellules saines. En ce sens, un composé affectant de manière similaire des cellules cancéreuses et des cellules saines dans un contexte *in vitro* pourrait s'avérer nocif lorsque administré en modèle *in vivo*. En revanche, un composé présentant des valeurs d'IC_{50} supérieures envers des cellules saines en comparaison à des cellules cancéreuses démontre un excellent potentiel anticancer *in vitro* et serait donc un candidat de choix pour des analyses *in vivo*. Les HE de *Boswellia* spp. sont reconnues pour démontrer une cytotoxicité spécifique aux cellules cancéreuses. En effet, Suhail *et al.* (2011) ont démontré qu'une HE extraite de *B. sacra* est en mesure d'inhiber plus efficacement la croissance de cellules humaines du cancer du sein en comparaison à des cellules du sein non-cancéreuses. Dans le même ordre d'idées, Frank *et al.* (2009) ont démontré qu'une HE de *B. carterii* fût aussi en mesure d'inhiber plus efficacement la croissance de cellules humaines du cancer de la vessie en comparaison avec des cellules saines témoignant de la capacité de cette huile à avoir une spécificité cytotoxique pour les cellules cancéreuses.

Une telle activité antiproliférative spécifique aux cellules cancéreuses a fait l'objet de plusieurs études dans le but de déterminer le mécanisme sous-jacent. Alors qu'une sensibilité des cellules pourrait être responsable d'une telle spécificité envers les cellules cancéreuses, plusieurs études ont

rapporté l'importance de l'apoptose dans ce phénomène. En effet, Dozmorov *et al.* (2014) ont testé la capacité d'une HE extraite de *B. carterii* à induire un effet pro-apoptotique dans des cellules humaines du cancer de la vessie via l'étude de l'expression des gènes modulés par cette huile. Après avoir confirmé la spécificité de l'huile pour les cellules cancéreuses, les auteurs ont observé une modulation de plusieurs gènes reliés aux voies moléculaires de l'apoptose. Frank *et al.* (2009) ont aussi démontré que l'activité anticancer spécifique aux cellules cancéreuses de la vessie était due à un phénomène d'apoptose par une analyse de micromatrice d'ADN (microarray) confirmant qu'un grand nombre de gènes furent modulés par l'huile de *B. carterii*. Finalement, Ni *et al.* (2012) ont démontré que l'HE de *B. sacra* était en mesure d'induire l'apoptose dans des cellules humaines du cancer du pancréas via la voie dépendante de caspase, via une induction rapide et transitoire des protéines AKT (Protéine Kinase B) et Erk1/2 (Extracellular signal-regulated kinases 1 and 2) ainsi que via la diminution de l'expression de complexe protéique lié au cycle cellulaire (D1/cdk4).

Tenter de déterminer la/les composante(s) responsable(s) de l'activité cytotoxique et apoptotique des EHs de *Boswellia spp.* est un sujet d'actualité scientifique. Tel que discuté, les terpènes seraient principalement responsables de l'activité biologique des EHs. Or, ceux de *Boswellia spp.* ne font pas exception. En effet, quelques études semblent démontrer que l'acide boswellique (BA) et ses dérivés seraient responsables des propriétés apoptotiques des EHs de *Boswellia spp*. Liu *et al.* (2002) ont démontré que BA, K-BA et AK-BA sont en mesure d'induire l'apoptose en une réponse de dose-dépendance via l'activation des caspases dans des cellules humaines du cancer colorectal. Les auteurs ont rapporté que cette activité apoptotique était observable à des concentrations en BA, K-BA et en AK-BA supérieures à 25µM. De plus, Ni *et al.* (2012) ont clairement démontré que la méthode d'extraction à une profonde influence sur la composition de l'huile essentielle obtenue et donc sur l'activité biologique. En effet, les auteurs ont obtenu 4 HE à partir de la résine de *B. sacra* en utilisant 4 températures d'hydrodistillation différentes pour ensuite tester la capacité des extraits à inhiber la croissance de cellules cancéreuses humaines du pancréas et à induire l'apoptose. Il en a résulté que les deux extraits contenant la plus forte teneur en BA et en composés à hauts poids moléculaires présentaient de meilleures valeurs d'IC_{50} en plus d'induire l'apoptose. Les auteurs n'ont cependant pas trouvé de corrélation entre l'activité biologique et la teneur en monoterpènes, ce qui contraste avec les études de Bhattacharjee *et al.* (2013), Chen *et al.* (2015) et de Kusuhara *et al.* (2012) qui ont tous démontré des effets anticancer ou chimiopréventif de l'α-pinène. Cette différence dans les corrélations entre les activités biologiques et la composition des HE témoigne de l'effet synergique des composantes de l'huile. En ce sens, il est juste d'avancer que les terpènes sont majoritairement responsables de l'activité anticancer des HE de *Boswellia* puisqu'ils constituent les composantes majoritaires. Par contre, déterminer le composé exact donnant aux

huiles de *Boswellia* leurs propriétés anticancer peut s'avérer ardu. De plus, la présence d'une synergie entre les composantes des HE peut être la clef de l'explication de leurs propriétés biologiques. En effet, Turgis *et al.* (2009) ont montré qu'une HE de thym contenant des teneurs en carvacrol, en γ-terpinène et en p-cymène de 23.25%, 18.09% et de 20.38% respectivement était plus efficace pour ces propriétés antimicrobiennes que le carvacrol pur.

Les HE de *Boswellia spp*. ont aussi une activité antiradicalaire. Bien que celle-ci soit modeste, il n'en reste pas moins que les composantes de ces huiles sont en mesure de capter l'anion O_2^- ainsi que le radical DPPH (Al-Harrasi *et al.*, 2013, Mohamed *et al.*, 2015).

Les HE de *Boswellia* ont aussi démontré des effets thérapeutiques chez l'humain. Deux rapports de cas cliniques ont utilisé l'huile essentielle de *B. sacra* dans le but de réduire ou d'éliminer totalement des tumeurs chez des patients. La première étude rapporte la capacité de l'huile à éliminer totalement une lésion carcinomateuse sur le bras gauche ainsi qu'une régression significative d'une seconde lésion au niveau de la poitrine d'un patient mâle âgé de 56 ans traité pendant 20 semaines à raison de plusieurs applications cutanés par jour (Fung *et al.*, 2013). La seconde étude suggère que la même huile a été en mesure d'éliminer complètement un carcinome à grade supérieur invasif de la vessie chez un patient mâle âgé de 52 ans traité pendant 2 ans à raison d'une prise orale (3 ml) par jour. Ces deux cas cliniques témoignent donc du fort potentiel anticancer des huiles de *Boswellia spp* (Xia *et al.*, 2016).

1.6. Les traitements combinés dans la lutte contre le cancer

L'utilisation d'une combinaison de composés dans une optique médicale est utilisée dans la lutte contre plusieurs maladies complexes telles que le SIDA et le cancer. Dans ce dernier cas, les tumeurs sont souvent constituées d'une hétérogénéité de types cellulaires cancéreux. Alors que l'utilisation d'un seul composé sera en mesure de bloquer une voie moléculaire bien précise résultant en une résistance de la tumeur envers le traitement, une combinaison de composés pourra agir de concert pour ainsi bloquer plusieurs voies moléculaires et donc atteindre la viabilité d'un plus grand nombre de cellules au sein de la tumeur. Alors que cette rationnelle explique le grand succès de la chimiothérapie combinatoire, cette approche semble maintenant standard dans la lutte contre le cancer (National Library of Medicine, 2003).

L'utilisation de composés anticancers en combinaison avec des produits naturels a déjà été rapportée dans la littérature. En effet, Baldwin *et al.* (2010) ont démontré qu'un mélange de bactéries probiotiques (*Lactobacillus acidophilus* et *L. casei*) en combinaison avec le 5-Fluouracil

(5-FU), un agent chimiothérapeutique, fût en mesure d'induire l'apoptose dans des cellules humaines du cancer colorectal plus efficacement que le mélange probiotique utilisé seul. Cette étude démontre donc le potentiel et de la pertinence d'utiliser des produits naturels en traitement combinatoire dans la lutte contre le cancer.

1.6.1. Les β-glucanes de levures utilisées en combinaison dans la lutte contre le cancer

Les β-glucanes de levures sont utilisés comme adjuvants en combinaison avec des anticorps monoclonaux (ACm) dans la lutte contre le cancer mettant ainsi à profit leurs propriétés immunomodulatrices. En effet, les ACm sont utilisés dans une optique d'induire un mécanisme de cytotoxicité au niveau de la cellule cancéreuse. Les mécanismes *in vitro* sous-jacents impliquent une cytotoxicité cellulaire dépendante aux anticorps et une cytotoxicité dépendante du complément. Cependant, les ACm utilisés seuls sont peu efficaces pour induire une cytotoxicité dépendante au récepteur du complément 3 (CD11b/CD18) puisque ce mécanisme est normalement induit par des levures et certains microorganismes exposant des molécules de β-glucanes à leur surface. En ce sens, les β-glucanes permettent d'induire un troisième mécanisme de cytotoxicité cellulaire dans les cellules cancéreuses et donc d'agir comme adjuvant lorsque utilisés en combinaison avec des ACm (Akramiene *et al.*, 2007, Chan *et al.*, 2009). Dans la lutte contre le cancer, les β-glucanes sont donc typiquement utilisés en traitement combinatoire.

1.6.2. Les HE utilisées en combinaison dans la lutte contre le cancer

Les HE sont de plus en plus utilisées en combinaison avec des agents thérapeutiques dans le but de combattre la multi résistance de certains cancers aux médicaments et de réduire les effets secondaires du traitement en soi. En effet, les HE sont de plus en plus utilisées comme suppléments diététiques dans une optique de chimiothérapie combinatoire en raison de l'importante quantité de composés chimiques. Leurs constituants peuvent ainsi agir en synergie (Gautam *et al.*, 2014). Dans cette optique, Chen *et al.* (2013) ont démontré qu'un mélange composé d'huiles de l'arbre à myrrhe et de *B. carterii* (en ratio 1 :1) présente un effet synergique envers la capacité d'induire l'apoptose dans des cellules humaines du cancer du sein. Aucune étude à ce jour n'a été réalisée pour évaluer la synergie potentielle entre des composantes membranaires de levures et des HE.

2. But, hypothèses et objectifs

Le développement et la mise en marché de composés bioactifs et naturels ayant des effets bénéfiques pour la santé sont des avenues très importantes pour l'industrie alimentaire considérant l'impact du cancer colorectal en Amérique du nord. En ce sens, ce travail a pour but d'étudier le potentiel chimiopréventif et anticancer des extraits de parois cellulaires de levures et des huiles essentielles de *Boswellia carterii* et de *B. sacra* envers le cancer colorectal.

Les hypothèses de ce travail sont les suivantes :

i) Les extraits de parois cellulaires de levures et les huiles essentielles de *Boswellia* ont des propriétés chimiopréventives et anticancer envers le cancer colorectal,

ii) Les β-glucanes et les mannoprotéines contribuent significativement à l'activité biologique des extraits de levures,

iii) Les extraits de levures et les huiles essentielles de *Boswellia* utilisées en combinaison démontrent un effet synergique

Les objectifs de ce travail sont :

i) Déterminer le potentiel chimiopréventif et anticancer des extraits de parois cellulaires de levures (Chapitre 2) ainsi que des huiles essentielles de *Boswellia* (Chapitre 4) en en modèle *in vitro*,

ii) Déterminer la/les composante(s) contribuant significativement à l'activité biologique des extraits de parois cellulaires de levures (Chapitre 2),

iii) Déterminer le potentiel chimiopréventif des meilleurs extraits de parois cellulaires de levures en modèle *in vivo* (Chapitre 3),

iv) Évaluer la capacité du meilleur extrait de paroi cellulaire à améliorer le potentiel chimiopréventif et anticancer des huiles essentielles *de Boswellia* en modèle *in vitro* (Chapitre 4)

Les moyens utilisés pour atteindre les objectifs sont :

i) Les extraits de parois cellulaires de levures ont été obtenus par autolyse de la biomasse levurienne suivie d'une étape d'extraction à l'eau chaude alcaline et d'une précipitation à l'éthanol selon une méthode modifiée de Nguyen *et al.* (1998) et de Suphantharika *et al.* (2003)(Chapitre 2),

ii) La démonstration du potentiel chimiopréventif et anticancer *in vitro* des extraits de parois cellulaires de levures a été effectuée avec les tests d'activité de la quinone réductase, de captation de l'anion superoxide et des tests antiprolifératifs sur cellules cancéreuses en utilisant des méthodes spectrophotométriques tel que décrit par Prochaska *et al.* (1988a), Gerhäuser *et al.* (2003) ainsi que Vistica *et al.* (1991) (Chapitre 2),

iii) Le poids moléculaire des extraits de parois cellulaires de levures ainsi que leurs teneurs en sucres totaux, en β-glucanes, en mannanes et en chitine ont été déterminés par HPLC et par des méthodes colorimétriques tel que décrit par Dubois *et al.* (1956), Pérez *et al.* (1983) et basé sur les travaux réalisés à Industrial Microbiology laboratory de CUCEI-UdG (Mexique). La mise en relation de la caractérisation des extraits avec leurs activités biologiques a été réalisée dans le but de déterminer le(s) composante(s) contribuant significativement à leurs activités (Chapitre 2),

iv) La démonstration du potentiel chimiopréventif et anticancer *in vivo* des extraits de parois cellulaires de levures a été effectuée par l'administration orale des meilleurs extraits à des rats traités au 1,2-dimethylhydrazine suivi du compte des cryptes aberrantes au niveau de leurs côlons selon les travaux de Bird (1995). Le mécanisme sous-jacent à la réduction du nombre de cryptes aberrantes a été déterminé par le dosage de l'activité de la quinone réductase au niveau du foie ainsi que le dosage de deux enzymes bactériennes (β-glucuronidase et β-glucosidase) au niveau du caecum basé sur des versions modifiées des travaux décrits par Prochaska *et al.* (1988a) et Park *et al.* (1998)(Chapitre 3),

v) La démonstration du potentiel chimiopréventif et anticancer *in vitro* des huiles essentielles de *Boswellia* a été effectuée avec les tests d'activité de la quinone réductase, de captation des radicaux O_2^- et DPPH, des tests antiprolifératifs sur cellules cancéreuses ainsi que leurs capacités à induire l'apoptose en utilisant des méthodes spectrophotométriques et la cytométrie en flux tel que décrit par Prochaska *et al.* (1988a), Gerhäuser *et al.* (2003), Blois (1958) et Vistica *et al.* (1991) (Chapitre 4),

vi) La capacité du meilleur extrait de paroi cellulaire à améliorer le potentiel chimiopréventif et anticancer *in vitro* des huiles essentielles de *Boswellia* a été mesurée par les tests d'activité de la quinone réductase et les tests antiprolifératifs sur cellules cancéreuses en utilisant des méthodes spectrophotométriques tel que décrit par Prochaska *et al.* (1988a) et Vistica *et al.* (1991) (Chapitre 4)

CHAPITRE 2 - PUBLICATION 1

Cancer chemopreventive, antiproliferative and superoxide anion scavenging properties of yeast cell wall components

Running headline: Chemoprevention of yeast cell wall

Cancer chemopreventive, antiproliferative and superoxide anion scavenging properties of yeast cell wall components

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La publication 1 a été soumise le 17 Janvier 2017 dans le journal Nutrition and Cancer. En ce sens, la version longue et originale est présentée au chapitre 2 alors que la version courte et nouvellement soumise dans le Journal Nutrition and Cancer est présentée en annexe 1. Cette version courte a été préparée afin de répondre aux exigences du journal.

2.1. Contribution des auteurs

J'ai réalisé toutes les expériences, mis sur pied le protocole de captation de l'anion superoxide, rédigé le manuscrit scientifique et supervisé 6 stagiaires qui m'ont assisté dans les analyses préliminaires et finales qui ont mené à la réalisation de ces résultats. Dr. Aguilar-Uscanga m'a supervisé dans la caractérisation des extraits en plus d'avoir participé aux discussions scientifiques ainsi que d'avoir révisé le manuscrit. Dr. Vu m'a montré les différentes méthodes pour quantifier l'activité biologique des extraits, en plus de m'avoir supervisé durant l'élaboration du protocole de captation de l'anion superoxide, d'avoir participé aux discussions scientifiques et d'avoir révisé le manuscrit. Mr. Salmieri m'a aidé dans la rédaction du manuscrit en plus d'avoir participé à quelques discussions scientifiques et d'avoir révisé le manuscrit. Dr. Lacroix, coordonnatrice et responsable du projet, a supervisé l'élaboration du protocole de captation de l'anion superoxide et les discussions scientifiques entourant ce projet en plus d'avoir révisé le manuscrit.

2.2. Résumé en français

Propriétés chimiopréventives, antiprolifératives et de captation de l'anion superoxide des composantes de la paroi cellulaire de levure envers le cancer colorectal

Objectif : Cette étude a investigué les propriétés chimiopréventives, antiprolifératives et antiradicalaires d'extraits de polysaccharides provenant de *Saccharomyces boulardii* et de *Kluyromyces marxianus*. Les teneurs en β-glucanes, mannanes et chitines ont été quantifiées pour identifier le(s) polysaccharide(s) responsable(s) de l'activité biologique observée.

Méthodes et résultats : Des extraits de β-glucanes solubles, β-glucanes insolubles et de mannoprotéines ont été obtenus de la paroi cellulaire via une extraction unique à l'eau chaude alcaline. La quantification des sucres totaux, β-glucanes, mannanes et de la chitine présents dans les extraits a été réalisée par des méthodes spectrophotométriques et par chromatographie liquide à haute performance. La captation de l'anion superoxide (capacité antiradicalaire) ainsi que des tests d'induction de la quinone réductase (QR) et antiprolifératif sur cellules cancéreuses ont été réalisés pour déterminer les propriétés biologiques des extraits. Les résultats ont montré que les β-glucanes insolubles de *S. boulardii* ont été en mesure d'augmenter l'activité de la QR de manière dose-dépendante en plus de présenter la meilleure inhibition de croissance contre des cellules humaines du cancer colorectal.

Conclusion : Les β-glucanes insolubles et les mannoprotéines de *S. boulardii* ont démontré les propriétés biologiques les plus pertinentes. De plus, de fortes teneurs en β-glucanes, des

ratios β-glucanes/sucres totaux élevés et des ratios chitine/β-glucanes faibles se sont avérés avoir un impact sur l'augmentation des propriétés chimiopréventives et antiprolifératives des extraits.

Importance et impact de l'étude : À notre connaissance, cette étude est la première à démontrer une induction de la QR par des composantes de la paroi cellulaire de levures. Cette découverte enrichit les applications potentielles des parois cellulaires de levures dans le domaine médical.

Mot clé : β-Glucanes, chimioprévention, antiradicalaire, antiprolifératif, levure, paroi cellulaire, caractérisation

2.3. Abstract

Aim: This study investigated the cancer chemopreventive, the antiradical and the antiproliferative properties of polysaccharide extracts from the cell wall of *Saccharomyces boulardii* and *Kluyromyces marxianus*. β-glucan, mannan and chitin were also quantified to identify the most important extract responsible for these biological properties.

Method and results: Soluble and insoluble β-glucans as well as mannoprotein were extracted from cell walls the using single hot-alkaline method. Quantification of total sugars, glucan, mannan and chitin of those extracts were performed using spectrophotometric methods and high-performance liquid chromatography (HPLC). Superoxide anion (O_2^-) scavenging (antiradical capacity), quinone reductase (QR) induction and antiproliferative assays were done for evaluation of biological properties of those extracts. Results showed that the insoluble glucan from *S. boulardii* increased QR activity as a dose-dependent function and exhibited the highest growth inhibition against colorectal cancer cells.

Conclusion: Insoluble glucan and mannoprotein of *S. boulardii* exhibited the most relevant biological properties. Moreover, high amount of glucan, high glucan/total sugars ratios and low chitin/glucan ratios were shown to have an impact on enhancing cancer chemopreventive and antiproliferative properties.

Significance and Impact Study: To our knowledge, this is the first study that demonstrates an induction of QR activity by yeast cell wall components. This evidence enriched the potential applications of the yeast cell wall in the medical field.

Keywords: β-Glucan, chemoprevention, antiradical, antiproliferative, yeast, cell wall, characterisation

2.4. Introduction

Colorectal cancer (CRC) is the second leading cause of deaths due to cancer in males and the third in females (Canadian Cancer Society's Advisory *et al.*, 2014). It is also the third most prevalent cancer in Canada (Canadian Digestive Health Foundation, 2016). Since treatment for CRC can be expensive and invasive for patients, prevention methods still seem to be the most efficient approach. It has been shown that life style plays an important role in the incidence of many cancers and diet has been related to almost 70 % of CRC incidence (Aggarwal *et al.*, 2013). Thus, the consumption of a diet containing agents with CRC preventive properties could reduce the risks of CRC incidence. The impact of CRC on the health population in Canada and USA triggered a demand of natural products with CRC preventive properties to prevent or reduce the development of this disease. Among natural agents, yeast cell wall components have shown increased interest due to their anticancer and immunomodulatory properties which can be utilized in nutrition, in pharmaceutical and in medical applications (Laroche *et al.*, 2007).

Yeasts are largely used in industrial domains especially in the food industry. However, cell walls of spent yeasts are often discarded after the collection of fermented broth or used in many applications such as the production of yeast extract or nutraceutical food supplements (dos Santos Mathias *et al.*, 2014, Giavasis, 2014). Many studies have investigated the natural properties of yeast cell walls and demonstrated that there are strong immunomodulatory properties of yeast glucan in *in vitro* and *in vivo* models in addition to their ability to prevent DNA damage which is the first step in carcinogenesis (Karoui *et al.*, 2007, Oliveira *et al.*, 2013). These properties depend on the physicochemical nature and

integrity of the glucan structure (Akramiene *et al.*, 2007, Chan *et al.*, 2009, Volman *et al.*, 2008) which varies according to growth conditions, extraction methods and yeast species (Aguilar-Uscanga *et al.*, 2003, Ahmad *et al.*, 2012, Akramiene *et al.*, 2007, Chan *et al.*, 2009, Klis *et al.*, 2006, Mantovani *et al.*, 2008, Pinto *et al.*, 2014, Stier *et al.*, 2014).

Yeast cell walls are organized with approximatively the same polysaccharides consisting of mannoprotein, chitin and (1→3)-β-D-glucan with (1→6)-β-D-glucan ramifications (Klis *et al.*, 2006). The sugar composition of the cell walls is mainly responsible for their biological and chemical properties (Aguilar *et al.*, 2012).

Saccharomyces boulardii (S. cerevisiae var. *boulardii)* and *Kluyveromyces marxianus* are well-known yeasts with diverse industrial applications and relevant biological properties. Indeed, *S. boulardii* is considered as a probiotic strain known to generate preventive effects on the occurrence of antibiotic-associated diarrhoea, beneficial effects against different enteric pathogens and the production of different anti-inflammatory molecules including *Saccharomyces* anti-inflammatory factor (Czerucka *et al.*, 2007, Kelesidis *et al.*, 2012). Otherwise, *K. marxianus* is known to possess a relevant potential in biotechnology due to its capacity to synthesis β-galactosidase and pectinase in addition to the reduction of lactose content in food products. Furthermore, *K. marxianus* exhibits a higher ethanol production compared to *S. cerevisiae* due to its highly thermotolerant properties (Anderson *et al.*, 1986, Falcão Moreira *et al.*, 1998, Fonseca *et al.*, 2008).

Since the immunomodulatory properties of cell wall extracts (glucan, chitin and mannoprotein) of *S. cerevisiae* are well characterized, this study focused on the chemopreventive, antiradical and antiproliferative properties of cell wall extracts of *S. boulardii* and *K. marxianus*. First, the content, the relative concentrations and the molecular weight of the polysaccharides typically found in yeast cell wall were determined. Then, the cancer chemopreventive potential of the extracts was investigated and compared with commercial insoluble glucans of *S. cerevisiae*. The nicotinamide adenine dinucleotide phosphate hydrogen: quinone reductase (NAD(P)H:QR) assay was been selected as a cancer chemopreventive test for the role of its phase II detoxification enzyme known to protect against toxic electrophilic metabolites directly involved in the very first stage of carcinogenesis whereas O_2^- anion was selected since it is one of the most important reactive oxygen species (ROS) due to its possibility to generate hydrogen peroxide and $OH^\cdot$ radicals (Young *et al.*, 2001). The determination of NAD(P)H:QR activity using murine hepatoma 1c1c7 cell line, the antiradical activity by measurement of O_2^- anion scavenging capacity and the antiproliferative potential against human CRC cell line HT-29 was evaluated in order to reveal structure-function relationships between the cell wall composition and their biological activities.

2.5. Material and methods

2.5.1. Material

Chemicals and media were obtained as follows: dextrose, essential amino acids, sodium pyruvate, fetal bovine serum (FBS), minimum essential medium-Earle's balanced salt solution (MEM-EBSS), Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12

(MEMF/12), Hank's balanced salt solutions (HBSS), trypsin, Pierce®BCA Protein assay, glycine, N-methylpyrrolidone (NMP) and 96-wells microplates were purchased from Fisher Scientific (Ottawa, ON, Canada). Commercial insoluble β-glucan, activated carbon, β-naphthoflavone, digitonin, bovine serum albumin (BSA), glucose-6-phosphate, thiazolyl blue tetrazolium bromide (MTT), menadione, glucose-6-phosphate deshydrogenase,2,3-*Bis*(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt (XTT), xanthine, sodium carbonate buffer (pH 10.2), xanthine oxydase, superoxide dismutase, phenol, sulphuric acid, lyticase from *Arthrobacter luteus*, chitinase from *Streptomyces griseus*, tetraporate potassium, 4-(dimethylamino)benzaldehyde, N-acetylglucosamine, mannan, lithium chloride (LiCl), nicotinamide adenine dinucleotide phosphate (NADP), dimethyl sulfoxide DMSO) and flavin adenine dinucleotide (FAD) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Yeast extract, bacterial peptone and agar were purchased from Alpha Bioscience (Baltimore, MD, USA). Polyethylene glycol and polymethyl methacrylate (PMMA) were purchased from Agilent technologies (Mississauga, ON, Canada).

2.5.2. Yeast strains and growth conditions

Kluyveromyces marxianus ATCC 10022 and *Saccharomyces cerevisiae* var. *boulardii* ATCC MYA-796 were purchased from the American type culture collection (ATCC) (Manassas, VA, USA). The yeast strains were stored at -80ºC in sterile yeast peptone dextrose (YPD) (10 g/l dextrose, 5 g/l yeast extract, 3 g/l, bacterial peptone, 0.8 g/l, $MgSO_4$, 1 g/l KH_2PO_4) containing 10% (w/v) sterile glycerol. One ml of culture cells in cryovials (10^8 cells/ml) from each strain were thawed and inoculated in 25 ml of YPD medium then

incubated for 18 h at 30ºC under agitation at 200 RPM (Forma Scientific, Orbital shaker, Model; EQ-069, USA). 2.5 ml of the resulting cell suspension was inoculated in a final volume of 50 ml of YPD medium for 24 h at 30ºC under agitation. Finally, 12.5 ml of this second cell suspension was inoculated in a final volume of 250 ml of YPD using the latter conditions. At the end of second and third fermentation, 1 ml of fermented broth was serially diluted in sterile peptone water and plated on YPD agar in order to confirm lack of contamination in cell suspension.

2.5.3. Growth kinetics

To determine the growth phase of the yeast species, optical density and dry biomass weight were monitored for 24 h. The fermentation was conducted using the same growth conditions as mentioned above except that 1 ml of cell suspension was collected every 2 h and diluted in sterile medium, to measure growth by optical density at 600 nm (Varian Canada inc., Mississauga, ON, Canada). In parallel, 1 ml of cell suspension was also collected every 2 h and placed in sterile preweighted tubes then centrifuged at 2000 *g* for 10 min at 4ºC. The supernatant was discarded and the pellet was washed with sterile water. Finally, the washed biomass was dried at 60ºC for 48 h and the tubes were weighted. A linear relation (equation) of dry biomass weight and optical density was established and the resulting equation was used to estimate the dry biomass in time function.

2.5.4. Biomass collect and cell wall preparation

Fermented broths were centrifuged at 9000 *g* for 10 min at 4ºC. The supernatant was discarded and the pellet was washed twice with sterile phosphate buffer 50 mmol/l, pH 7.2.

To extract sugars from cell walls with respect to its integrity, yeasts cells were autolyzed by suspending the wet biomass in 15% (w/v) sterile water and were autolyzed for 24 h at 50ºC with agitation at 200 rpm. Autolyzed cells were then centrifuged at 9000 *g* for 10 min at 4ºC. The supernatant was discarded and the autolyzed cells were entirely used for glucan and mannoprotein extraction.

2.5.5. Extraction of yeast glucan and mannoprotein extracts

The method of extraction of β-glucan and mannoprotein was based on work of Nguyen *et al.* (1998) and Suphantharika *et al.* (2003) with some modifications. A quantity of 500 ml of 1 mol/l NaOH was mixed with 100 g of autolysed cells for 1 h at 90ºC without stirring in order to avoid glucan degradation. Then, the suspension was centrifuged at 9000 *g* for 10 min at 4°C. The precipitate was washed twice with distilled water, freeze-dried and the resulting extract was considered as insoluble glucan. The supernatant was mixed with 95% ethanol in 1:4 proportions, left overnight at 4ºC and centrifuged. The resulting pellet was washed twice with distilled water, then freeze-dried and the obtained extract was referred to as soluble glucan. Finally, ethanol in the supernatant was evaporated using a vacuum concentrator (Savant, Automatic environmental speedVac® system, Model; AES1010, Farmingdale, NY, USA) under full vacuum, low speed and at ambient temperature for 8 h and then freeze-dried to obtain the mannoprotein extract.

2.5.6. Total sugars quantification

The total sugars content in each extract was determined following a method of Dubois *et al.* (1956). Briefly, 10 mg of freeze-dried samples were mixed with 1 ml of milli-Q water. Then, 200 µl of this solution were mixed with 200 µl of 5% phenol and 2 ml of sulphuric

acid. The mixture was roughly vortexed and left 5 min at room temperature before reading absorbance at 490 nm with a Cary 1 spectrophotometer (Varian). A standard curve was made using a sugar solution (ranging from 0.02 to 0.1 g/l) which contained 40% mannose and 60% dextrose in order to roughly imitate sugar proportion in the *S. cerevisiae* cell wall. The resulting equation was used to extrapolate the total sugars content in the extracts. All quantifications were made in triplicate (n=3) and results were expressed as average ± standard deviation (SD).

2.5.7. Glucan and mannan quantification by HPLC

Glucan and mannan were quantified by HPLC (Varian Pro Star 210) equipped with a refractive index detector (RID). Polysaccharides were separated in a Metacarb CA-PLUS (30 cm x 7.8 mm) isothermal column at 90°C, using water as mobile phase and a flow rate of 0.6 ml/min. A calibration curve was performed at concentrations between 1 to 0.1 g/l of glucan and mannan. All quantifications were made in triplicate (n=3) and results were expressed as average ± SD. This method was established for analysis of polysaccharides in Industrial Microbiology laboratory of CUCEI-UdG (México).

2.5.8. Chitin quantification

The quantification of chitin in cell wall extracts was done using a modified method of Pérez *et al.* (1983) and Roncero *et al.* (1988). Briefly, 100 mg of freeze-dried extracts were suspended in test tubes containing 5 ml of 6% KOH, heated at 80ºC for 90 min and cooled on ice for 1 min. Afterwards, 500 µl of acetic acid was added and the tubes were centrifuged at 1500 *g* for 2 min. The resulting pellet was washed twice with distilled water and once with 50 mmol/l phosphate buffer (pH 6.3). Then, 500 µl of phosphate buffer, 50 µl of 5

U/ml lyticase and 50 µl of 5 U/ml chitinase were added to the pellet and incubated at 37ºC for 20 h at 200 rpm. Afterward, 100 µl of this solution was mixed with 150 µl of Milli-Q water and with 250 µl of 0.27 mol/l potassium tetraborate (pH 9). Tubes were boiled for 8 min and cooled down on ice for 2 min. Finally, 3 ml of Reissig reagent 1X (1% (w/v) 4-(Dimethylamino)-benzaldehyde, 0.12 mol/l HCl and 1.52 mol/l glacial acetic acid) was added to each tube and incubated for 40 min at 37ºC. Absorbencies were read at 585 nm (Varian). A standard curve of N-acetylglucosamine ranging from 20 to 100 mmol/l was made and resulting equation was used to calculate the amount of chitin in tested samples. All quantifications were made in triplicate (n=3) and results were expressed as average ± SD.

2.5.9. Molecular weight determination by gel permeation chromatography (GPC)

The molecular weights (Mw) of polysaccharides was analysed by gel permeation chromatography (GPC) using an Agilent HPLC (Agilent Technologies 1260 infinity series, Waldbronn, BW, Germany), equipped with a quaternary pump (Model G1311B), a manual injector with a sample loop of 20 µl and a refractive index detector (Model G1362A). Insoluble glucan was treated separately from soluble glucan and mannoprotein extracts. For soluble glucan and mannoprotein extracts, two identical PL Aquagel-OH mixed-M 8 µm 300 X 7.5 mm columns were used in series to increase resolution. Water containing 0.02% (w/v) sodium azide was used as mobile phase with a flow rate of 1 ml/min and both columns and detector were set at 30ºC. Freeze-dried mannoprotein and soluble glucan were rehydrated by solubilizing 2.5 mg/ml in mobile phase and filtered through a 0.2-µm filter.

Polyethylene glycol was used as a standard and prepared as recommended by the manufacturer. Concerning insoluble glucan, 2 identical PLgel 5 µm Mixed-D 300 X 7.5 mm columns were used in series as explained above. The mobile phase consisted of 100% NMP containing 5% (w/v) LiCl was used with a flow rate of 0.5 ml/min and both columns and detector were set at 60ºC. The method for preparation of insoluble glucan for injection was mainly based on Austin (1977), Yilmaz *et al.* (2003) and Chakrabandhu *et al.* (2008) with some modifications due to the insoluble properties of chitin. Freeze-dried insoluble glucan was mixed in NMP at a concentration of 2.5 mg/ml into a glass bottle. The suspension was stirred for 48 h at 60ºC and filtered through a 0.2-µm nylon filter. PMMA was used as a standard for insoluble glucan and was prepared as indicated by the manufacturer. The equation obtained by plotting Mw with retention times of standards was used to calculate the Mw of each peak obtained by GPC of the extracts. All extracts were injected in triplicate (n=3) and expressed as Mw range.

2.5.10. Cancerous cell lines and cell maintenance

Hepa 1c1c7 (ATCC CRL-2026) and HT-29 (ATCC HTB-38) cell lines were purchased from the American type culture collection (ATCC) (Manassas, VA, USA). The cell lines were cultivated in 25 cm^2 cellular flasks (Corning, Manassas, VA, USA) in a humidified incubator at 37ºC in an atmosphere of 5% CO_2 and 95% air. Hepa 1c1c7 cells were grown in complete MEM-EBSS medium which contained 0.1% essential amino acids, 0.1% sodium pyruvate, 10% (v/v) FBS and completed with MEM-EBSS solution whereas HT-29 cells were grown in complete MEMF/12 medium which contained 0.1% essential amino acid, 0.1% sodium pyruvate, 10% (v/v) FBS and completed with MEMF/12 solution. The

maintenance of cells was performed at a confluence of 80-90% by discarding cultured medium and washing adherent cells with HBSS solution. Then, cells were treated with 1.5 ml of 1X trypsin for exactly 30 s. Trypsin was poured off and cells were incubated for exactly 12 min in a humidified incubator at 37ºC in an atmosphere of 5% CO_2 and 95% air. Finally, 2 ml of respective medium was added to trypsinized cells and 1 ml of cell suspension was used to inoculate into 5 ml of fresh completed medium.

2.5.11. NAD(P)H:QR assay

The NAD(P)H:QR (QR) assay was based on studies from Prochaska *et al.* (1988a) and Talalay (1989) with some modifications. The aim of this assay was to determine the concentration of extracts required to double the QR induction (CD) since a basal expression level of QR occurs in Hepa 1c1c7 cells. Freeze-dried extracts were rehydrated in 10% (v/v) dimethyl sulfoxide (DMSO) containing 20 ppm of sodium azide (as antimicrobial agent) in order to avoid sterilization of extract solution by filtration or by heating which could lead to a loss of biological activities. A 96-well plate was seeded with Hepa 1c1c7 cells at a density of 10^4 cells/ml (200 µl per well) in completed MEM-EBSS medium. This microplate, named QR plate, was then incubated in a humidified incubator at 37ºC for 24 h in an atmosphere of 5% CO_2 and 95% air. Afterward, different concentrations of extracts previously serial diluted were added and each well was completed to 200 µl with carbon activated MEM-EBSS cells for 48 h. Cells were then washed with HBSS solution and 50 µl of 1.6% (w/v) digitonin were added in each well. The microplate was incubated for 10 min at 37ºC, then placed on an orbital shaker for 10 min at room temperature. A 20 µl sample of this suspension was added to a new 96-well microplate for further total protein

determination. Then, 200 µl of complete reaction mixture (0.25 mol/l Tris-HCl pH 7, 4.67% (w/v) BSA, 0.01% tween-20, 5 mol/l FAD, 1 mmol/l glucose-6-phosphate, 30 mol/l NADP, 34.8 µmol/l MTT, 50 µmol/l menadione and 2 mU/µl glucose-6-phosphate deshydrogenase in distillated water) was added to each well of the QR plate and optical densities were read after 5 min at 595 nm using a microplate reader (Biotek, Model EL800, Winooski, VT, USA). Protein assays were made using Pierce®BCA reagents and was performed as suggested by the manufacturer. Negative and positive controls consisted of 1% (v/v) DMSO containing 20 ppm sodium azide and 2 µmol/l β-naphthoflavone respectively whereas medium was used as blank. Specific activity of QR was defined as nmol of blue formazan formed per mg protein per minute. Fold induction of QR was calculated as follow:

QR Fold Induction (treated on control) = Specific activity of QR in treated group / Specific activity of QR in negative control group

Fold induction ≥ 2 was considered as relevant in order to determine whether extracts can induce QR. All measurements were done in triplicate (n=3) and results were presented as average ± SD.

2.5.12. Antiradical assay (O_2^- anion scavenging activity)

The capacity of samples to scavenge O_2^- was measured using xanthine/xanthine oxydase system (XTT color assay) based on the method of Gerhausser et al. (2003). This assay aims to determine whether soluble sugars extracts possess radical scavenging properties by

quantifying their ability to scavenge O_2^- anion. Freeze-dried samples of yeast cell wall extracts were rehydrated in 10% (v/v) DMSO at desired concentrations. Then, serial dilutions of extracts were performed in 10% (v/v) DMSO and 20 µl of those extracts were loaded in 96-well microplates. Then, each well was completed to 200 µl with reactional mix (1 mmol/l XTT, 1 mmol/l EDTA, 1 mmol/l xanthine, 50 mmol/l sodium carbonate buffer (pH 10.2) and 3 mU/ml xanthine oxydase) and the optical density was read at 490 nm (Biotek) after 20 min. The negative control consisted of DMSO 1% (v/v) whereas the positive control corresponded to 30 U/ml of superoxide dismutase. Scavenging activity (%) was calculated as follows:

Scavenging Activity (%) = [(sample OD - Negative control OD) / (Positive control OD - Negative control OD)] X 100

Concentrations exhibiting a scavenging activity of 50% were referred to as SC_{50} values. Insoluble glucan was not used for this assay since perfectly soluble samples were required. All measurements were done in triplicate (n=3) and results were presented as average ± SD.

2.5.13. Antiproliferative assay

The antiproliferative effect of different yeast cell wall extracts against the HT-29 cell line was measured using MTT color assay based on the method of Vistica *et al.* (1991). The cell proliferation was determined by the ability of the metabolically active cells to cleave the tetrazolium salt to purple formazan crystals. Concentrations that inhibiting 50% of the

cellular growth (IC_{50} values) were calculated for all extracts. In a 96-well plate, HT-29 cells were seeded at 2 x 10^4 cells per 200 µl of complete MEMF/12 medium. After 24 h of incubation at 37°C under an atmosphere of 5% CO_2 and 95% air, the medium was replaced with 100 µl of fresh medium containing 10 µl of each extract previously serial diluted. The negative control consisted of 1% (v/v) DMSO containing 20 ppm sodium azide and the blank consisted of 100µl of fresh medium. After 48 h of incubation, the culture medium was decanted and replaced with 200 µl of fresh MEMF/12 containing 25 µl of 0.5% (w/v) MTT. The microplate was incubated for 4 h at 37°C in an atmosphere of 5% CO_2 and 95% air. Then, the medium was carefully removed and 200 µl of DMSO plus 25 µl of Sorensen buffer pH 10.5 (0.1 mol/l glycine, 0.1 mol/l NaCl) were added to each well. Absorbencies were measured at 562 nm (Biotek) and the cellular growth inhibition was calculated as follows:

Cellular Growth Inhibition (%)= 100 - [(Sample OD / Negative control OD) X 100]

Equations obtained by plotting the linear portion of growth inhibition versus concentrations of extracts were used to determinate IC_{50} values. All measurements were done in triplicate (n=3) and results were presented as average ± SD.

2.5.14. Statistical analysis

Amounts of total sugar content, chitin, glucan and mannan in extracts as well as IC_{50} values were analyzed by one-way analysis of variance (ANOVA) using PASW statistics 18 software (IBM Corporation, Somers, NY, USA) and differences between samples were

analyzed with post hoc Duncan's multiple-range test. Significance was considered at $P \leq 0.05$.

2.6. Results

2.6.1. Growth kinetics

Growth kinetics of yeasts was performed to collect yeast biomass in stationary phase in order to reflect spent yeast at the end of industrial fermentation processes. As presented in Figure 2.1, the initial amounts of biomass from *K. marxianus* (0.8 g/l) and *S. boulardii* (1.0 g/l) were similar. Afterwards, dry biomass of both *K. marxianus* and *S. boulardii* was increased to 1.5 g/l after 8 h and 10 h respectively, indicating that *K. marxianus* presented a shorter latency phase than *S. boulardii*. Despite the fact that *K. marxianus* produced more biomass than *S. boulardii* (6.3 and 3.2 g/l respectively), both species biomass were collected in stationary phase after 24 h growth. Since growth phase is a major factor in terms of biological activity and composition of yeast cell wall, these results indicate that further comparisons between *S. boulardii* and *K. marxianus* should be carried out.

2.6.2. Solubility and visual appearances of yeast cell wall extracts

The solubility of these extracts is briefly described in Table 2.1 Results showed that insoluble glucan was partially soluble in high concentrations of DMSO whereas it were totally soluble in pure NMP after stirring and heating for 48 h in presence of 5% (w/v) lithium chloride. Soluble glucan and mannoprotein were readily soluble in water and at all concentrations of DMSO. In 10% DMSO, insoluble glucan yielded a white and turbid suspension; soluble glucan yielded a transparent solution whereas mannoprotein yielded a transparent and yellow solution.

2.6.3. Quantification of total sugars, chitin, glucan, mannan in the extracts and Mw determination

The quantification of total sugars is presented in Table 2.2 Results showed that the content of insoluble glucan, soluble glucan and mannoprotein of *S. boulardii* (42.73%, 39.13% and 0.4% respectively) are similar to their respective counterparts in *K. marxianus* (51.38%, 32.55% and 0.52% respectively) suggesting that both species possessed a similar amount of total sugars in cell walls. Results also showed that insoluble glucan from both species contained significantly more total sugars than all other extracts evaluated in this study ($P \leq 0.05$). Also, insoluble extracts of both species contained the highest amounts of glucan (49.17 and 40.54% for *K. marxianus* and *S. boulardii* respectively) whereas the soluble glucan of *S. boulardii* contained the lowest amount of glucan (23.99%). Furthermore, combined amounts of glucan in soluble and insoluble extracts represented 65% and 80% for *S. boulardii* and *K. marxianus* respectively, which demonstrates a noticeable difference in their cell wall composition. The content of mannoprotein is presented in Table 2.2 The results showed that the content of mannoprotein of *S. boulardii* contained significantly less mannan (3.17%) than their *K. marxianus* counterparts (9.14%) ($P \leq 0.05$). Moreover, each extract contained 4-10 times less mannan than glucan. These observations may be due to the presence of mannan covalently bound to glucan despite the extraction. The total content of chitin found in the three extracts of *S. boulardii* (1.51%) was found to be higher than content found in all extracts of *K. marxianus* (0.89%), which also demonstrated typical differences in cell wall composition between each strain. Finally, higher chitin contents were found in both soluble and insoluble extracts of *S. boulardii* (0.66 and 0.58%

respectively) as compared to 0.30 and 0.35% in *K. marxianus*. The GPC analysis showed that the Mw range in insoluble glucan of *S. boulardii* is 1921 kDa and 2085 kDa in *K. marxianus* showing higher Mw than soluble glucan and mannoprotein extracts, which corroborates the fact that solubility partially depends on Mw (Table 2.2). Mannoprotein extracts of *K. marxianus* showed a Mw from 0.48-77 kDa and from (0.72-87 kDa) in *S. boulardii*. Also, Table 2.2 indicates that insoluble glucan in both strains showed a high amounts of total sugars and glucan in addition to high Mw.

2.6.4. Determination of relative concentrations in total sugars, chitin, glucan and mannan content in the extracts

Determination of relative concentration using ratios may lead to a better analysis of extracts composition and to a better understanding of their structure-function relationship. Table 2.3 shows that insoluble glucan of *S. boulardii* possessed a glucan/total sugars ratio (0.94) that was significantly higher ($P \leq 0.05$) then their soluble counterparts (0.61) whereas no differences ($P > 0.05$) in the glucan/total sugar ratios were observed between insoluble and soluble glucan of *K. marxinaus*. Mannan/total sugars ratios were higher in mannoprotein of *S. boulardii* (7.03) and *K. marxianus* (24.94) whereas mannan/glucan the ratios showed no significant differences ($P > 0.05$) between soluble and insoluble glucan of both strains. In addition, chitin/total sugars ratios in insoluble glucans of both yeast strains (6.83×10^{-3} and 13.66×10^{-3} for *S. boulardii* and *K. marxianus* respectively) were the lowest as compared to other extracts whereas the ratios in mannoprotein of both strains were the highest (471.85×10^{-3} and 685.14×10^{-3} for *S. boulardii* and *K. marxianus* respectively), hence suggesting the presence of a higher amount of chitin in mannoprotein extracts which

1335 does not corroborate with the information in Table 2.2 More importantly, the insoluble
1336 glucan of both strains exhibited low chitin/glucan ratios compared to other extracts whereas
1337 this ratio was significantly higher ($P \leq 0.05$) regarding soluble glucan of *S. boulardii* (27.40
1338 x 10^{-3}), hence suggesting that soluble extract of *S. boulardii* possessed more chitin and less
1339 glucan than both insoluble extracts. Also, the chitin/mannan ratios suggest that the
1340 insoluble glucan of both strains contains high amounts of chitin whereas the soluble glucan
1341 of *S. boulardii* exhibited the highest chitin/mannan ratio, which is in agreement with Table
1342 2.2 Analysis of all ratios for each extract and each strain suggests that insoluble glucan
1343 possesses high glucan/total sugars ratios, low chitin/total sugars ratio and low chitin/glucan
1344 ratios for both strains. By contrast, soluble glucan of *S. boulardii* exhibited the lowest
1345 glucan/total sugars ratio and the highest chitin/glucan ratio therefore suggesting a low
1346 proportion of glucan in this extract. Finally, the results presented in Table 2.3 suggest a
1347 lack of direct correlation regarding mannan/glucan ratio and mannan/total sugars ratio in
1348 all extracts, which is congruent with results in Table 2.2.
1349

1350 ### 2.6.5. Biological activities of commercial β-glucan

1351 The QR activity, antiradical and antiproliferative tests were conducted to evaluate some
1352 biological activities of all extracts to estimate the chemopreventive potentiel of the yeast
1353 cell wall. In this aspect, commercial insoluble glucan from *S. cerevisiae* was used for QR
1354 and antiproliferative assays for comparison purposes and results which are presented in
1355 Figures 2.2 and 2.3 respectively. Figure 2.2 indicates that negative control (1% DMSO +
1356 20 ppm sodium azide) and untreated cells did not induce any QR activity (1.0 fold
1357 induction) whereas β-naphthoflavone (positive control) quadrupled the QR activity (3.9

fold induction). Figure 2.2 also shows that commercial insoluble glucan from *S. cerevisiae* induced QR activity in a dose-dependent manner and possessed a CD value of around 125 µg/ml. Figure 2.3 showed that commercial insoluble glucan from *S. cerevisiae* inhibited the growth of HT-29 cells following a dose-dependent function and reached an IC_{50} value of 344.2 µg/ml. Therefore, results from Figure 2.2 and 2.3 demonstrate relevant biological activities of commercial yeast β-glucan and will further be used for comparison with the yeast cell wall extract obtained in this study.

2.6.6. Cancer chemopreventive activity of yeast cell wall extracts

Results of QR assay and CD values of the yeast cell wall extracts are presented in Figure 2.4. Results show that only insoluble glucan from both yeast species could induce QR activity and reach CD values of 500 µg/ml. Also, Figure 2.4 shows that both insoluble glucans exhibited a dose-dependent response. Moreover, Figure 2.4 suggests that the CD value of the commercial insoluble glucan (125 µg/ml) is 4 times lower than those obtained for the insoluble extracts of *S. boulardii* and *K. marxianus* (500 and 500 µg/ml respectively), which might be explained by a higher purity of the commercial glucan. Those results show that the insoluble glucan of *S. boulardii* and *K. marxianus* possess a relevant chemoprevention potential since these extracts were abled to increase the specific activity of the NAD(P)H quinone reducatse.

2.6.7. Antiradical activity of yeast cell wall extracts

The capacity of water-soluble extracts (soluble glucan and mannoprotein extracts) of yeast cell walls to scavenge O_2^- anion was determined and results are presented in Figure 2.5.

Results show that soluble glucan of *K. marxianus* reached a SC_{50} value of 3000 µg/ml as opposed to soluble glucan of *S. boulardii* that did not reached an SC_{50} value. Mannoprotein of *K. marxianus* reached a SC_{50} value (1500 µg/ml) twice as low as found for mannoproteins of *S. boulardii* (3000 µg/ml). Finally, Figure 2.5 also demonstrates that all extracts exhibited a dose-dependent response to scavenge O_2^- anion except for the soluble glucan of *S. boulardii*. Those results demonstrate that mannoprotein and soluble glucan of *K. marxianus* scavenged O_2^- species more efficiently as compared to their *S. boulardii* counterparts.

2.6.8. Antiproliferative activity of yeast cell wall extracts

The antiproliferative activities of cell wall extracts of *S. boulardii* and *K. marxianus* against HT 29 cells are presented in Figure 2.6. Results show that all extracts were able to reach 50% of growth inhibition at the tested concentrations (ranging from 3000 to 125 µg/ml). Results also show that all extracts exhibited a dose-dependent response (Figures 2.6a and 2.6b). Figure 2.6c indicates that extracts of *S. boulardii* showed lower IC_{50} values than their *K. marxianus* counterparts. Indeed, the insoluble extract of *S. boulardii* possessed the lowest IC_{50} value (108.3 µg/ml) among glucan extracts whereas mannoprotein of *S. boulardii* possessed the lowest IC_{50} value (251.0 µg/ml) among mannoprotein extracts. In fact, only the insoluble glucan of *S. boulardii* exhibited an IC_{50} value significantly ($P \leq 0.05$) lower than the commercial insoluble glucan (344.2 µg/ml) whereas soluble glucan and mannoprotein of *K. marxianus* were found to possess the highest IC_{50} values (856.0 and 1403.0 µg/ml respectively) as compared to all extracts.

2.6.9. Relationship between the contents of yeast cell wall polysaccharide and their biological activities

Figure 2.4, 2.5 and 2.6 indicate that insoluble glucan from both yeast strains constitute the most relevant extract regarding biological activities. The insoluble glucan of *S. boulardii* is the most relevant extract based on its QR inductive activity with the CD value of 500 µg/ml and its antiproliferative activity against HT 29 cells with the lowest IC_{50} values of 108.3 µg/ml. However, soluble glucan of *S. boulardii* exhibited the lowest biological activities based on an IC_{50} value of 356.1 µg/ml against HT 29 cells as the unique detected biological activity.

Determining the combination of polysaccharides found in the yeast cell wall that are potentially responsible for cancer chemopreventive, antiradical and antiproliferative properties is essential to understand the major component involved in the biological activities of extracts. Overall, results from characterization and biological activities suggested that insoluble glucan possessed the most relevant biological activities which correspond to high contents in total sugars, glucan and chitin as well as a high Mw, a high glucan/total sugars ratio, low chitin/total sugars and chitin/glucan ratios. In contrast, the soluble extract of *S. boulardii* presented the weakest biological activity and corresponded to a low content in glucan, low glucan/total sugars ratio and high chitin/glucan ratio. These assessments suggest a typical relationship between biological activities and sugars quantification that aims to determine the relative importance of each polysaccharide in biological activities: % chitin < % glucan ≈ % total sugars. This relation shows that extracts majorly need to contain glucan to possess biological properties whereas chitin seems to be

statistically less influential on chemopreventive and anticancer properties against CRC *in vitro*. Finally, this relation suggests that mannan was not responsible for the biological activities of the extracts.

2.7. Discussion

Since growth phase can modulate the yeast cell wall composition drastically, biomass was collected in stationary phase in this study in order to reflect the cellular state of spent yeasts. Hudson *et al.* (2014) observed that wild type *S. boulardii* MYA-797 grown in YPD medium containing 2% dextrose reached the very late exponential phase after 24 h of growth. In this study, *S. boulardii* reached stationary phase (Figure 2.1) after approximately 24 h of fermentation. This could be due to the fact that only 1% dextrose was used instead of 2 % dextrose as used by Hudson *et al.* (2014). Moreover, Mitterdorfer *et al.* (2001) demonstrated that a strain of *S. boulardii* reached the early stationary phase after approximately 25 h of growth using Sabouraud media containing 1% dextrose which is also comparable with our results.

Similarly, the growth kinetics of *K. marxianus* obtained in this study is comparable with the results of Falcão Moreira *et al.* (1998) considering growth conditions. The authors reported that *K. marxianus* ATCC 10022 cells entered stationary phase after approximately 28 h of growth using YPD medium containing 2% dextrose and a growth temperature of 26°C. It has been demonstrated that growth temperature and dextrose concentration lower than 30°C and 2% can lead to a decrease of biomass and thus to a reduction of the time needed to enter stationary phase (Margaritis *et al.*, 1983, Rodrussamee *et al.*, 2011).

Consequently, time needed for *K. marxianus* to enter stationary phase as found in the present study is in good agreement congruent with the scientific literature.

In general, cell walls obtained from yeast biomass collected in stationary phase consists mainly of 3 biopolymers: chitin, mannan and (1→3)-β-D-glucan and (1→6)-β-D-glucan. Their proportions change according to many parameters such as strains, growth conditions, growth phase, method of cell wall preparation, extraction and chemical derivatization of natural biopolymers (Aguilar-Uscanga *et al.*, 2003, Klis *et al.*, 2006, Zekovic *et al.*, 2005). Glucan amounts found in soluble and insoluble extracts obtained in this study are similar to those obtained by Suphantharika *et al.* (2003). These authors used a single hot alkaline extraction approach to recover glucan from baker's yeast cell wall and obtained insoluble extracts constituted of approximately 50% of glucan. Moreover, the theoretical amount of total β-glucans represents between 58 to 72% of yeast cell wall dry weight (Kwiatkowski *et al.*, 2012), which is in accordance with the total amount of β-glucan obtained in this study. In contrast with glucan quantification, the total amount of mannans in extracts was inferior for both yeast strains as compared to mannoprotein complex found in the literature (35 to 40%) (Klis *et al.*, 2002, Kwiatkowski *et al.*, 2012). This divergence might be due to the relatively important content of proteins in the complex which was not quantified since this study focused on polysaccharide characterization. Klis *et al.* (2006) suggested that *S. cerevisiae's* cell wall contains between 1 and 6% of chitin which is congruent with our results. The presence of chitin in soluble and insoluble glucan extracts might be due to residual N-acetyl-glucosamine branched to (1→3)-β-D-glucan and (1→6)-β-D-glucan.

Indeed, chitin in stressed cell walls tends to branch directly with (1→6)-β-D-glucan instead of the typical (1→3)-β-D-glucan (Fontaine *et al.*, 2000, Klis *et al.*, 2006).

The content of chitin, glucan and mannan revealed the divergence in cell wall composition of *S. boulardii* and *K. marxianus* which has already been reported elsewhere (Backhaus *et al.*, 2010). The authors concluded that *S. cerevisiae* tends to decrease the amount of glucan in the cell wall under stress conditions whereas a member of *Kluyveromyces spp.* Shows unchanged glucan content under the same conditions. Those observations seem to be in agreement with our results, since the amounts of glucan and glucan/total sugars ratios were significantly higher for *K. marxianus*. Moreover, chitin/total sugars ratios were lower in soluble/insoluble glucan as compared to mannoprotein extracts, hence suggesting the presence of a higher amount of chitin in mannoprotein extracts. This inconsistency might arise from the phenol-sulfuric acid method used to quantify neutral sugars. Indeed, it may be that sugars in mannoprotein extracts are mainly charged, which could explains the low total sugars contents and high chitin/total sugars ratios.

Glucans with a Mw between 100 and 200 kDa are known to exhibit important biological activities such as tumor growth inhibition *in vivo* and antiproliferative effect on cancerous cells *in vitro* (Mantovani *et al.*, 2008, Saitô *et al.*, 1991, Wang *et al.*, 2004). In the present study, the Mw of insoluble glucan from *S. boulardii* (1921 kDa) and *K. marxianus* (2085 kDa) was found to be higher when compared to other extracts and also exhibited the most relevant biological activities (i.e., exhibited CD values and lowest IC_{50} values). High Mw β-glucan has been reported to possess biological activities. Using hot-alkaline extraction,

Mork *et al.* (1998) produced a soluble β-glucan that exhibited a Mw of approximatively 1000 kDa and showed capacity in activating macrophages. In addition, Kelly (2001) described an insoluble glucan that presented a Mw range of 1000-3000 kDa and exhibited a capacity to heal skin wounds in rats. These studies confirmed that insoluble glucan with molecular weights higher than 1000 kDa possesses biological activities and thus corroborates findings from the present study.

The determination of polysaccharides mainly responsible for biological activities observed in this study revealed that β-glucan was the principal component of yeast cell walls that exhibited important biological activities whereas chitin was important to a lesser extent. Those results corroborate results obtained by others investors. Indeed, *S. cerevisiae's* β-glucan is known to exhibit antimutagenic and antigenotoxic effects *in vivo* (Oliveira *et al.*, 2013), to inhibit the cellular growth of colorectal cancer cells *in vivo* (Yoon *et al.*, 2008) and to exhibit apoptosis properties in colorectal cancer cells *in vitro* (Kim *et al.*, 2009). Furthermore, β-glucan immunomodulation properties have been intensively reported for many decades. For example, it was shown to increase NK cell activity and T cells-mediated cytotoxicity, trigger inflammatory response, strong interferon (IFN) and interleukin (IL) release, enhance phagocytic activity of neutrophils and stimulate macrophages (Aguilar *et al.*, 2012, Bohn *et al.*, 1995, Chan *et al.*, 2009, Falch *et al.*, 2000, Luhm *et al.*, 2006, Rand *et al.*, 2010, Stier *et al.*, 2014).

Chitin covalently bound to glucan in soluble/insoluble extracts might have led to a decrease of glucan biological function. Indeed, glucan branched to chitin or mannan above certain

proportions might cause a decrease in glucan's biological properties, which could explain the importance of high chitin/glucan ratio in the soluble glucan of *S. boulardii*. However, the chitin content was found to be high in insoluble glucan which demonstrated the importance of the relative concentration of chitin and glucan. In this regard, Zhang *et al.* (2004) extracted different water-soluble polysaccharides from fungal sources and determined the amount of N-acetyl glucosamine, glucose and total sugars in extracts and then investigated their effect on growth inhibition of HL-60 leukemic cells and antitumor activities *in vivo*. The authors observed that extracts with higher glucose/total sugars ratios and lower N-acetyl glucosamine/glucose ratios exhibited the most relevant anticancer properties both *in vivo* and *in vitro*, which is in good agreement with our findings.

Since our results suggested the importance of glucan in reaching relevant CD, SC_{50} and IC_{50} values, the mechanism of glucan in such activities is worth further discussion. QR is a phase II detoxification enzyme recognized to protect against toxic electrophilic metabolites directly involved in the very first stage of carcinogenesis. QR decreases chemical carcinogenicity by transforming those compounds into a less toxic form or into inactive metabolites (Kang *et al.*, 2004). The capacity of cell wall extracts obtained from *S. boulardii* and *K. marxianus* to induce QR activity seems to be a novel biological activity highlighted in the present study. Despite the fact that compounds exhibiting a CD value below 10 µg/ml are considered as highly relevant inducers (Kang *et al.*, 2004), high CD values obtained by insoluble glucan (500 µg/ml) can be considered as medium and even low inducers for *in vivo* studies using yeast cell wall compound (Li *et al.*, 2010, Samuelsen *et al.*, 2014). The mechanism of induction depends on whether the inducer is mono- or

1541 bifunctional inducer. Monofunctional inducers increase the specific activity of phase II
1542 enzymes whereas bifunctional inducers increase both phase I and phase II specific activities
1543 (Prochaska *et al.*, 1988b). Further studies will be needed to determine whether insoluble
1544 glucan is a mono or bifunctional inducer.
1545
1546 As opposed to the QR assay, several studies demonstrated the antiradical scavenging
1547 activity of polysaccharides from fungal and yeast sources (Jaehrig *et al.*, 2007, Krizkova
1548 *et al.*, 2006, Saiki *et al.*, 2011, Tsiapali *et al.*, 2001). The dose-dependent response of
1549 soluble glucan and mannoprotein extracts might be explained by a hydrogen atom transfer
1550 (HAT) mechanism of anomeric hydrogen (Tsiapali *et al.*, 2001). Indeed, Tsiapali *et al.*
1551 (2001) demonstrated that antiradical activities of glucan are higher than dextrose and
1552 mannose most probably due to the greater abstraction lability of the anomeric hydrogen
1553 from internal glucose units rather than from the reducing end. In addition, the presence of
1554 a substituted carboxymethyl group in gluco-pyranose structure could also explain the
1555 antiradical scavenging activity observed in the present study (Saiki *et al.*, 2011). Our results
1556 also demonstrated that mannoprotein of *K. marxianus* exhibited the most relevant SC_{50}
1557 value. This observation might be due to a higher presence of aromatic amino acids and
1558 thiol groups in mannoprotein which are known to exhibit high antioxidant activities
1559 (Jaehrig *et al.*, 2007). Moreover, those antioxidant residues in the mannoprotein extract of
1560 *K. marxianus* may be more accessible than in other extracts due to an increased sensitivity
1561 of proteins denaturation during extraction. Indeed, Jaehrig *et al.* (2007) revealed that the
1562 treatment of mannoprotein with dithiothreitol released free thiol and aromatic side chains
1563 which increased the antioxidant activities compared to untreated mannoprotein whereas

native cell walls of *S. cerevisiae* did not generate antioxidant activities. In contrast, all soluble extracts obtained in the present study revealed relatively high SC_{50} values and thus can be considered to possess a low antiradical potential, which is in accordance with the literature since sugar compounds were reported to exhibit low antiradical properties (Machová *et al.*, 2013, Tsiapali *et al.*, 2001). Finally, it has been reported that yeast glucan possesses a weaker antiradical scavenging capacity compared to mannoprotein extracts (Jaehrig *et al.*, 2007, Jaehrig *et al.*, 2008), which is congruent with our results.

Proliferation of cancerous cells can be inhibited through different mechanisms including cell cycle arrest, apoptosis and necrosis (Hanahan *et al.*, 2011, Ren *et al.*, 2012). Although further investigation will be necessary to investigate whether necrosis or apoptosis was involved in growth inhibition of HT-29 cells observed in this study, the fact that all extracts exhibited antiproliferative activities is in agreement with the literature (Zekovic *et al.*, 2005). Indeed, lentinan, a well-known β-glucan from fungi, was reported to inhibit 50% of the growth of sarcoma 180 solids tumors *in vitro* at a concentration of approximately 500 µg/ml (Zhang *et al.*, 2005). Moreover, β-glucan derived from a mutant strain of *Aureobasidum* inhibited 52.6% of the cellular growth of human colon cancer cells SNU-C4 at a concentration of 150 µg/ml and triggered apoptosis at 100 µg/ml (Kim *et al.*, 2009). These biological activities can be linked to β-glucan insolubility, molecular weight, nature of branching, presence of glycoprotein bound to glucan and triple-helix structure of glucan (Wang *et al.*, 2004, Zhang *et al.*, 2005). Indeed, schizophyllan, a homopolysaccharide of glucose from fungal sources, possesses a triple-helix structure and demonstrates the capacity to inhibit tumor growth. However, alkaline-treated schizophyllan adopted a

single-helix structure and showed a reduced ability to inhibit tumor growth as compared to its unmodified counterpart suggesting that the triple-helix structure plays an important role for antitumor activities (Ooi *et al.*, 2000, Ren *et al.*, 2012). Zhang *et al.* (2005) also proposed that triple-helix of β-glucan presents a stiff chain conformation which provides a greater growth-inhibition effect *in vivo*.

The relative proportion of polysaccharides and proteins is also an important factor and has been intensively reported to explain the antiproliferative properties of yeast cell wall extracts. In this context, Wang *et al.* (1995) prepared a polysaccharide-peptide (PSP) complex of *Tricholoma sp.* that exhibited immunomodulatory and anticancer activities at a higher level than crude powder from fruiting bodies. A more recent study isolated polysaccharides from *Pleurotus sajor-caju*, an edible mushroom, which induced excellent cytotoxic activities against human colon cancer HCT 116 cells *in vitro*. After partial characterization of the named polysaccharides PS1, the authors detected significant amounts of proteins and lipids which might have been responsible for these observed anticancer properties (Moharib *et al.*, 2014). Finally, Ooi *et al.* (2000) reviewed the antitumor mechanisms of polysaccharide-protein complexes (PSK) both *in vitro* and *in vivo* with a focus on fungi. The authors found that homopolysaccharides and PSK used different antitumor mechanisms. Typically, lentinan and schizophyllan are triggered by macrophages and T-cells whereas the mechanism of PSK is more direct with no substantial effect on immune responses, hence suggesting that the unique feature of both PSP and PSK is possibly due to the involvement of proteins that increases the structural diversity between active components (Ooi *et al.*, 2000). Conjointly, these investigations brought evidence

indicating that the integrity of the glucan network and the glucan-protein complex might be highly relevant in anticancer properties of yeast's cell walls. Thus, further studies on the structure of glucan to find relation with biological properties are necessary.

This study demonstrated that extracts obtained via a simple and fast extraction method from cell walls of *S. boulardii* and *K. marxianus* suitable for industrial applications exhibited cancer chemoprevention, antiradical and antiproliferative properties in a dose-dependent manner. In addition, this study also revealed for the first time the capacity of insoluble glucan from *S. boulardii* and *K. marxianus* to induce QR activity, a phase II detoxification enzyme. Our results suggested that insoluble glucan and mannoprotein extracts from *S. boulardii* exhibited relevant biological activities toward CRC. Finally, the characterization of extracts demonstrated that high amounts of glucan, high glucan/total sugars ratios and low chitin/glucan ratios in extracts have a major impact in biological properties of yeast cell wall extracts. Thus, insoluble glucan and mannoprotein extracts of *S. boulardii* could be used in animal models in order to investigate their CRC chemopreventive properties in *in vivo* experiments.

2.8. Acknowledgements

This work was supported by the Ministère de l'Économie, de l'Innovation et de l'Exportation du Québec (MEIE). Olivier Fortin is a scholarship recipient of Fondation Armand-Frappier.

Table 2.1: Solubility and visual appearance of yeast cell wall extracts obtained after single hot-alkaline extraction for *S. cerevisiae* and *K. marxianus*.

Extracts	Solubility in solvents						Appearance in 10% DMSO
	Water	DMSO 10%	DMSO 50%	DMSO 75%	DMSO 100%	NMP*100 %	
Insoluble	-	-	-	+/-	+/-	+	White, high turbidity
Soluble	+	+	+	+	+	ND	Transparent
Manno	+	+	+	+	+	ND	Transparent, yellow

* 48 h with agitation at 60ºC and additioned with 5% lithium chloride.

(-) Insoluble.

(+/-) Partially soluble.

(+) completely soluble.

ND Not determined, NMP 1-methyl-2-pyrrolidone.

Soluble, insoluble and manno extracts refer to soluble glucan, insoluble glucan and mannoprotein respectively.

Table 2.2: Sugars quantification and Mw range determination of extracts.

Strain	Extracts	Total sugars (%)	Glucan (%)	Mannan (%)	Chitin (%)	Mw range (kDa)
	Insoluble	51.38±6.02 ^{b}	49.17±0.13^{d}	3.14±2.78^{a,b}	0.35±0.08^{a,b}	2085
K. marxianus	**Soluble**	32.55±2.42 ^{b}	30.38±0.30^{b}	8.49±3.75^{a,b}	0.30±0.03^{b}	0.74-165
	Manno	0.52±0.38 ^{a}	ND	9.14±0.94^{b}	0.24±0.02^{a}	0.48-77
	Insoluble	42.73±7.64 ^{b}	40.54±0.07^{c}	9.72±1.96^{a,b}	0.58±0.03^{c}	1921
S. boulardii	**Soluble**	39.13±5.63 ^{b}	23.99±0.29^{a}	3.99±3.46^{a,b}	0.66±0.02^{c}	0.73-160
	Manno	0.45±0.03 ^{a}	ND	3.17±0.11^{a}	0.27±0.01^{b}	0.72-87

ND Not determined.

Soluble, insoluble and manno extracts refer to soluble glucan, insoluble glucan and mannoprotein, respectively.

Within each column, means bearing a different lowercase letter are significantly different ($P \leq 0.05$).

Table 2.3: Ratios in glucan, mannan and chitin from all extracts.

Strain	Extracts	glucan / total sugars	mannan / total sugars	mannan / glucan	chitin / total sugars (10^{-3})	chitin / glucan (10^{-3})	chitin / mannan
	Insoluble	0.96±0.11^{b}	0.06±0.01^{a}	0.06±0.05^{a}	6.83±1.54^{a}	7.12±1.62^{a}	0.112±0.025$^{c, d}$
K. marxianus	**Soluble**	0.93±0.07^{b}	0.26±0.02^{b}	0.28±0.01^{a}	9.34±0.82^{a}	10.03±0.88$^{a, b}$	0.036±0.003^{b}
	Manno	ND	10.55±1.07^{c}	ND	471.85±38.45^{c}	ND	0.026±0.002^{a}
	Insoluble	0.94±0.16^{b}	0.23±0.04^{a,b}	0.24±0.05^{a}	13.66±2.94^{b}	14.38±3.09^{b}	0.059±0.0129$^{b, c}$
S. boulardii	**Soluble**	0.61±0.09^{a}	0.10±0.01^{a}	0.16±0.14^{a}	16.81±0.48^{b}	27.40±0.79^{c}	0.165±0.005^{d}
	Manno	ND	7.03±0.53 ^{c}	ND	685.14±14.25^{d}	ND	0.086±0.002^{c}

ND Not determined.

Soluble, insoluble and manno extracts refer to soluble glucan, insoluble glucan and mannoprotein respectively.

Within each column, means bearing a different lowercase letter are significantly different ($P \leq 0.05$).

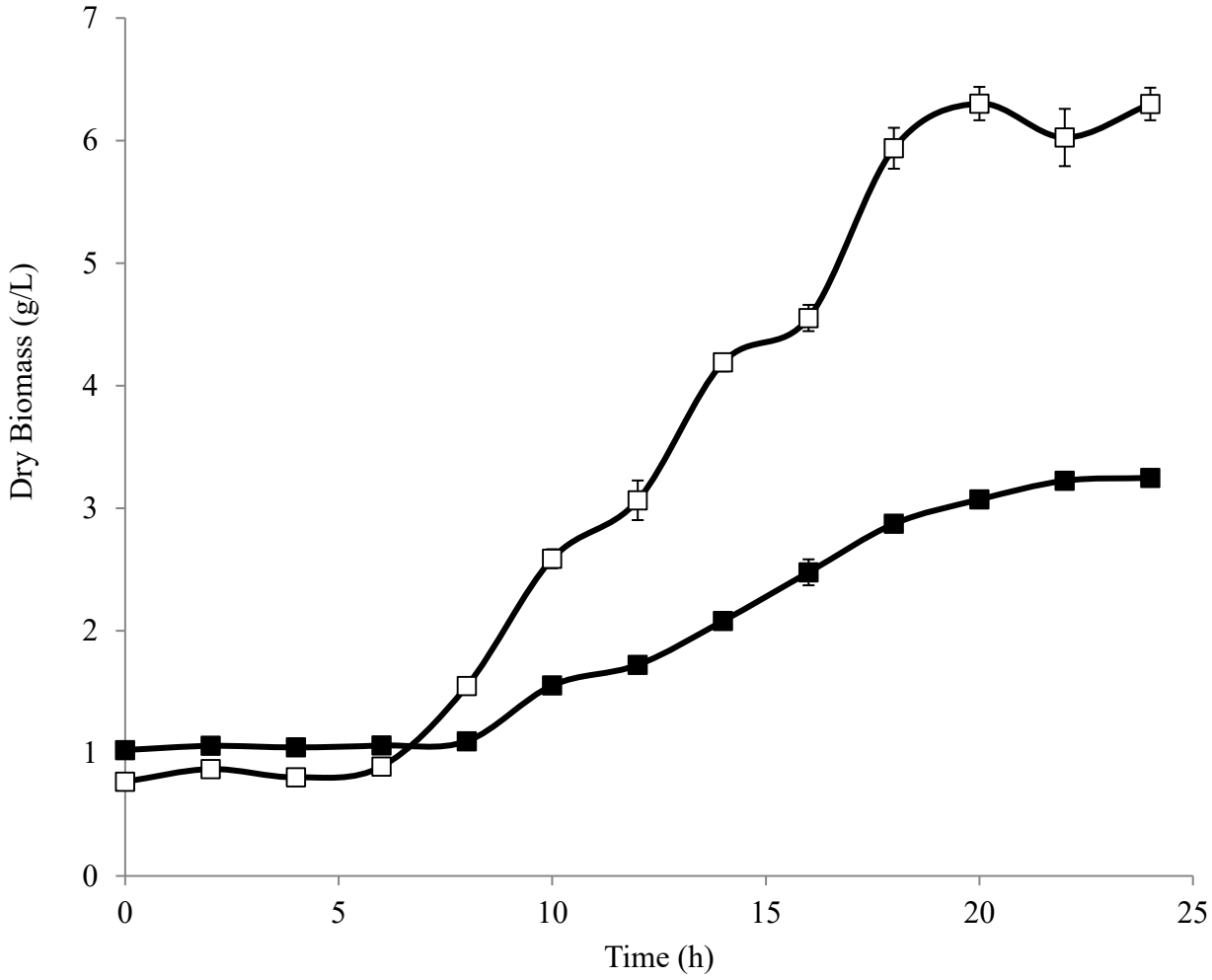


Fig 2.1. Growth kinetics of *S. boulardii* and *K. marxianus* based on dry biomass in time function. (■) *S. boulardii*, (□) *K. marxianus*.

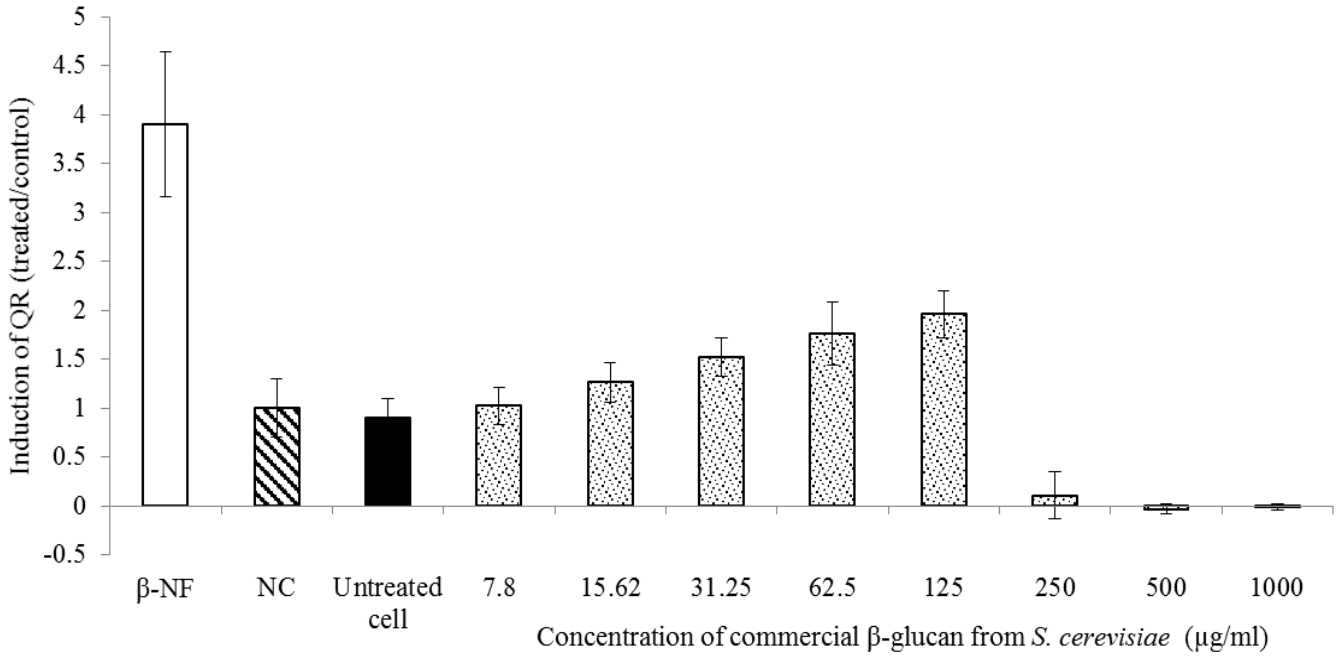


Fig 2.2. Induction of NAD(P)H: quinone reductase of untreated cells, negative control, β-Naphthoflavone and commercial insoluble glucan extracted from *S. cerevisiae* (bakery strain) using alkaline extraction method. (□) β-Naphthoflavone (β-NF), (■) Untreated cells, (▨) 1% DMSO + 20 ppm sodium azide (NC), (▩) Commercial glucan.

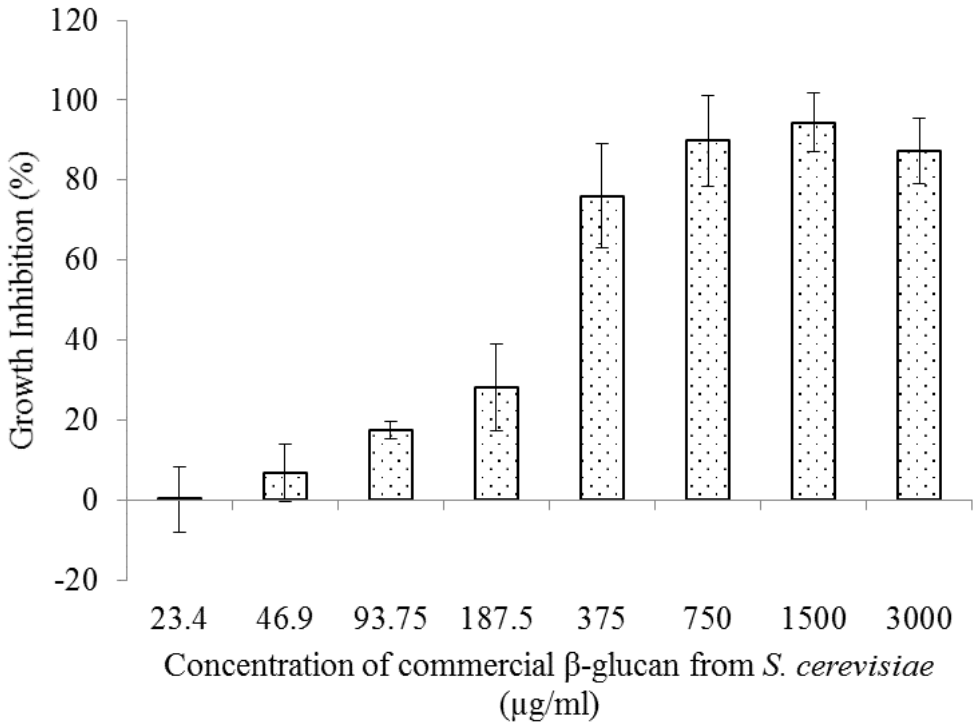


Fig 2.3. Antiproliferative assay against HT-29 cells using increasing concentration of commercial insoluble glucan from *S. cerevisiae*. (▨) Commercial glucan.

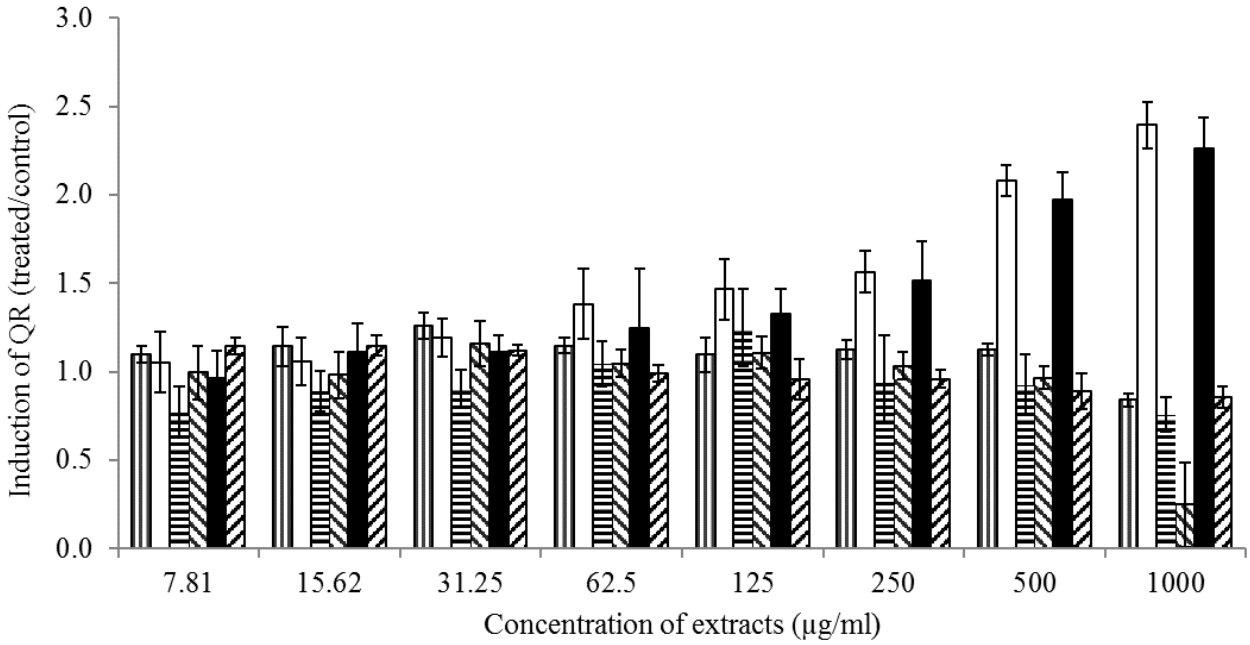


Fig 2.4. Induction of NAD(P)H quinone reductase with increasing concentration of all extracts obtained from both yeast strains. (▨) *S. boulardii* soluble extract, (■) *S. boulardii* insoluble extract, (▧) *S. boulardii* mannoprotein extract, (▤) *K. marxianus* soluble extract, (□) *K. marxianus* insoluble extract, (▥) *K. marxianus* mannoprotein extract.

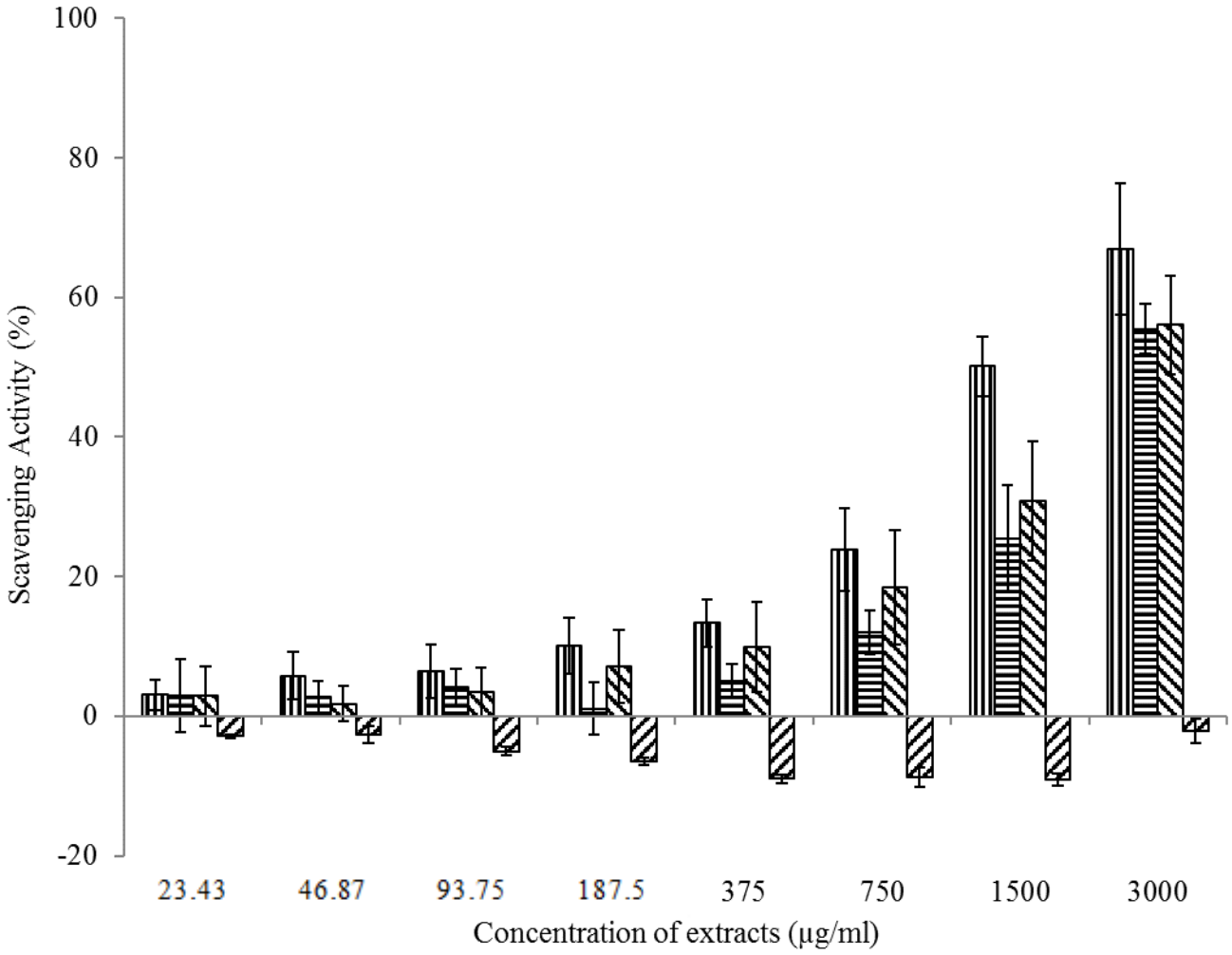
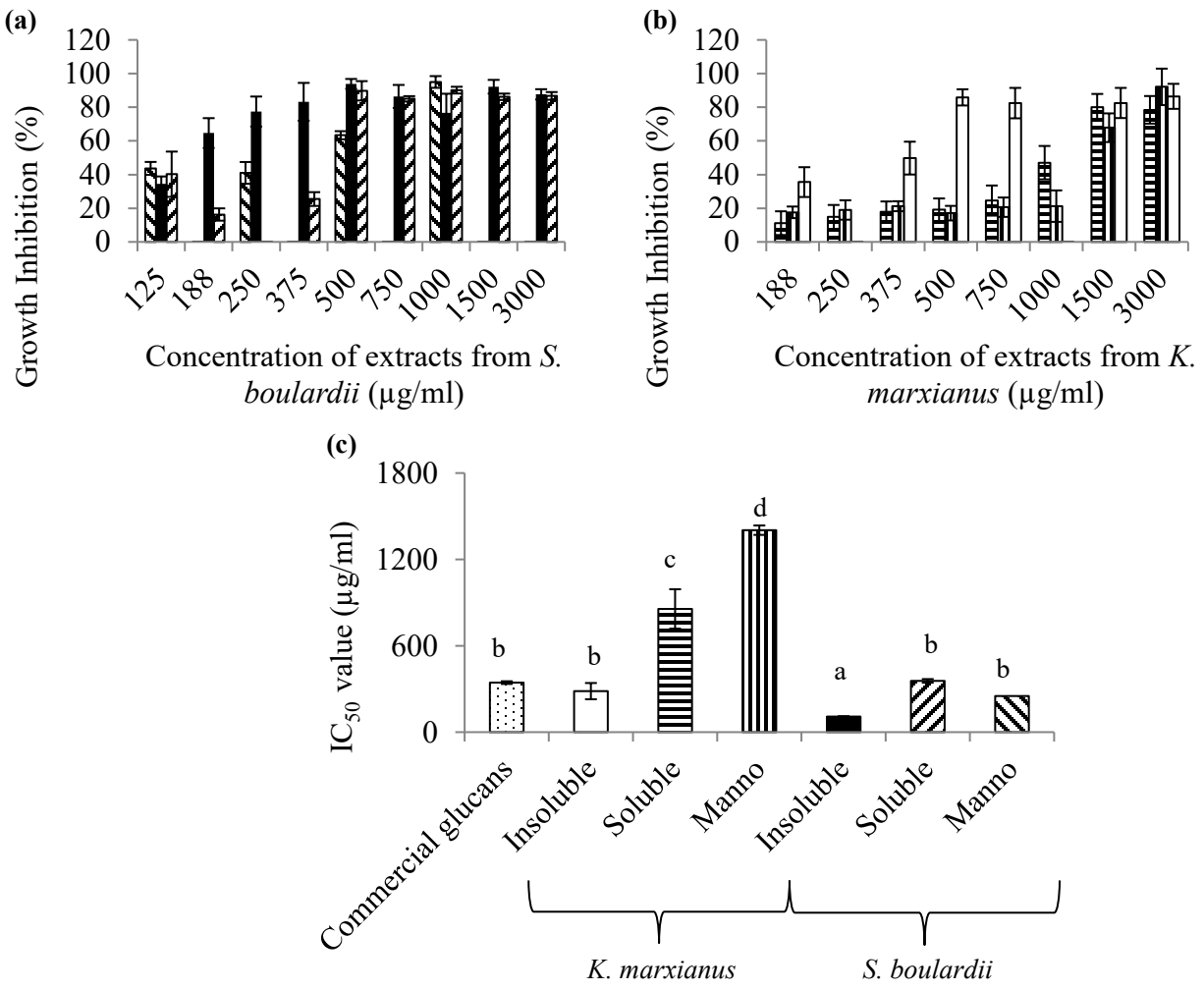


Fig 2.5. Percentage of scavenging activity of increasing concentration of all extracts obtained from both yeast strains against superoxide anion. (▨) *S. boulardii* soluble extract, (▧) *S. boulardii* mannoprotein extract, (▤) *K. marxianus* soluble extract, (▥) *K. marxianus* mannoprotein extract.



1685

1686 **Fig 2.6.** Growth inhibition of HT-29 cell line using a) increasing concentration of extracts
1687 obtained from *S. boulardii* and b) from *K. marxianus* along with c) IC_{50} values for each
1688 extract. (▨) *S. boulardii* soluble extract, (■) *S. boulardii* insoluble extract, (▧) *S. boulardii*
1689 mannoprotein extract, (▤) *K. marxianus* soluble extract, (□) *K. marxianus* insoluble
1690 extract, (▥) *K. marxianus* mannoprotein extract, (▩) Commercial glucan. Soluble,
1691 insoluble and manno extracts refer to soluble glucan, insoluble glucan and mannoprotein
1692 respectively. Different lowercase letter are significantly different ($P \leq 0.05$). IC_{50}
1693 Concentration that inhibits 50% of cellular growth.

1694

CHAPITRE 3 – PUBLICATION 2

Effect of β-glucan and mannoprotein extracted from cell wall of *Saccharomyces boulardii* on colon cancer prevention in male F344 rats treated with 1,2-dimethylhydrazine.

Effect of β-Glucan and Mannoprotein Extracted from Cell Wall of *Saccharomyces boulardii* on Colon Cancer Prevention in Male F344 Rats Treated with 1,2-Dimethylhydrazine

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3.1. Contribution des auteurs

J'ai réalisé les expériences et rédigé le manuscrit scientifique. Dr. Aguilar-Uscanga a participé aux discussions scientifiques et a aussi révisé le manuscrit. Dr. Vu a participé aux discussions scientifiques et a révisé le manuscrit. Mr. Salmieri m'a aidé dans la rédaction du manuscrit en plus d'avoir participé à quelques discussions scientifiques et a révisé le manuscrit. Dr. Lacroix, coordonnatrice et responsable du projet, a supervisé les discussions scientifiques entourant ce projet en plus d'avoir révisé le manuscrit.

3.2. Résumé en français

L'Effet des β-Glucanes et des Mannoprotéines Obtenus de la Paroi Cellulaire de *Saccharomyces boulardii* Envers la Prévention du Cancer Colorectal Chez des Rats F344 Mâles Traités au 1,2-Diméthylhydrazine.

L'effet des extraits de la paroi cellulaire de *Saccharomyces boulardii* envers la prévention du cancer colorectal chez des rats traités au diméthylhydrazine ainsi que les possibles mécanismes sous-jacents ont été investigués. Des extraits bruts de β-glucanes insolubles (0.5 et 1.0 mg/kg/jour) ainsi que de mannoprotéines (0.3 et 3.0 mg/kg/jour) ont été administrés à des rats par gavage pendant 12 semaines, le tout accompagné d'une diète forte en gras et faible en fibres après quoi les rats furent sacrifiés et les foyers de cryptes aberrantes (FCA) ont été comptés au niveau du côlon. De plus, les activités enzymatiques de la NAD(P)H: quinone réductase (QR) et de deux enzymes fécales (β-glucosidase et β-glucuronidase) furent quantifiées au niveau du foie et du caecum respectivement. Les résultats ont montré que seuls les rats traités aux β-glucanes insolubles démontraient une réduction du compte de FCA, une réduction de l'activité de la β-glucuronidase et une augmentation de celle de la QR. À notre connaissance, cette étude est la première à révéler qu'un extrait brut de β-glucanes insolubles obtenu à partir de la paroi cellulaire de *S. boulardii* est en mesure de démontrer d'importantes propriétés de prévention du cancer colorectal en modèle *in vivo* via des modulations enzymatiques.

Mots clés : β-glucanes insolubles de levures, prévention du cancer, foyers de cryptes aberrantes, NAD(P)H:quinone réductase, β-glucuronidase, cancer colorectal

3.3. Abstract

The effect of *Saccharomyces boulardii* cell wall extracts on colon cancer prevention in rats treated with 1,2-dimethylhydrazine and their possible mechanisms was investigated. A crude insoluble glucan (0.5 and 1.0 mg/kg/day) and a crude mannoprotein extract (0.3 and 3.0 mg/kg/day) were administered in rats by gavage for 12 weeks along with a high fat low fiber diet whereupon rats were sacrificed and aberrant crypt foci (ACF) were counted in the colon. Moreover, NAD(P)H: quinone reductase (QR) and harmful fecal enzymes (β-glucosidase and β-glucuronidase) were quantified in the liver and in the caecum, respectively. Results showed that only rats treated with insoluble glucan had a decreased β-glucuronidase activity and an increased QR activity which led to a reduction in ACF counts. To our knowledge, this is the first study that demonstrates that crude insoluble glucan obtained from the *S. boulardii* cell wall exhibited colon cancer prevention properties *in vivo* via an enzymatic modulation.

Keywords: yeast, β-glucan, cancer prevention, aberrant crypt foci, experimental

3.4. Introduction

Colorectal cancer (CRC) is the second most deadly cancer for males and the third for females (Canadian Cancer Society's Advisory *et al.*, 2014) in addition to being the third most prevalent cancer in Canada (Canadian Digestive Health Foundation, 2016). Multiple risk factors are known for CRC. However, it is now well established that environmental factors and life-styles which include smoking, obesity, physical inactivity, diet rich in saturated fat and red meat along with a diet low in fiber, fruits or vegetables are involved in CRC (Haggar *et al.*, 2009, Stevens *et al.*, 2007). Many studies suggest that diet habits have an important impact on CRC carcinogenesis (Gill *et al.*, 2002, Haggar *et al.*, 2009), therefore, dietary modification by consumption of natural products with cancer chemopreventive properties could reduce the incidence of CRC.

Polysaccharides represent the major part of the dry weight of yeast cell walls which mainly consist of mannoprotein, chitin and (1→3)-β-D-glucan with (1→6)-β-D-glucan ramifications (Klis *et al.*, 2002). Yeast cell walls are usually considered as industrial by-products and are often discarded after fermentation processes or used in many applications such as health and food supplements (dos Santos Mathias *et al.*, 2014). For instance, Dadrass *et al.* (2014) demonstrated that the *S. cerevisiae* cell wall modulates enzymatic activities in the liver upon chemically-induced CRC. Moreover, yeast cell wall polysaccharides demonstrated interesting chemopreventive and anticancer properties *in vivo*. Indeed, insoluble (particulate) yeast glucan orally administrated in mice has been reported to induce IL-12 cytokine production which induced a switch from a T-helper (Th)2 to Th1 response. Th1 cells can enhance cytotoxic T lymphocyte response resulting in an improved immunotherapy of cancer (Baran *et al.*, 2007). Moreover, orally

1794 administered insoluble glucan at doses ranging from 100 to 400 µg per day in tumor-
1795 bearing mice could reduce tumor diameter and increase survival rate in a dose-dependent
1796 manner (Li *et al.*, 2010). In contrast, very few studies have focused on *in vivo*
1797 chemoprevention properties of yeast mannoprotein. Liu *et al.* (2011) reported the capacity
1798 of yeast mannoprotein obtained from different extraction methods in exhibiting
1799 antineoplastic effect against several cancerous cell lines (HepG2, HL-60, and Eca109).
1800 In a previous study, Fortin *et al.* (2017a) extracted soluble and insoluble glucan and
1801 mannoprotein from the cell wall of *Saccharomyces boulardii* and *Kluyveromyces*
1802 *marxianus* and their chemopreventive and anticancer potential properties *in vitro* were
1803 determinated. It has been reported that *S. boulardii's* insoluble glucan induced NAD(P)H:
1804 quinone reductase (QR) activity and presented high antiproliferative activity expressing
1805 through a lower concentration required to inhibit 50% cell growth (lower IC_{50} value)
1806 toward CRC cells. Moreover, among mannoprotein extracts, *S. boulardii's* mannoprotein
1807 exhibited the lower IC_{50} value toward CRC cells in addition to revealing the hability to
1808 scavenge superoxide anion. Thus, those two extracts present excellent chemopreventive
1809 potential.
1810 CRC prevention is typically assessed *in vivo* by the evaluation of the capacity of a bioactive
1811 agent to reduce counts of precancerous lesions known as aberrant crypt foci (ACF) in rat
1812 colon, in which CRC is chemically induced by using 1,2-dimethylhydrazine (DMH). The
1813 formation of precancerous lesions is considered to be the very first stage of CRC
1814 carcinogenesis, thus making ACF an excellent target for CRC prevention (Karoui *et al.*,
1815 2007). The exact mechanisms of chemopreventive effects of dietary agents against
1816 colorectal carcinogenesis are not known; however, some possible mechanisms have been

proposed. For example, it has been suggested that an increase in QR activity could be responsible for the detoxification and excretion of carcinogens (Kang *et al.*, 2004) or the decrease of bacterial enzyme activities such as β-glucosidase and β-glucuronidase which are able to catalyse the conversion of procarcinogenic substances into carcinogenic substances (Ketudat Cairns *et al.*, 2010, Simon *et al.*, 1986). DMH is metabolized into azoxymethane (AOM) and methylazoxymethanol (MAM) in the liver. MAM is then transported to the colon via the bloodstream and the bile where it is transformed into methyl cation. In the colon, methyl cation targets the colonic mucosa resulting in the formation of oxidative stress and DNA damage. The severity of that damage partially depends on the transit time of those carcinogens in the colon which can be lowered by a reduction of β-glucosidase and β-glucuronidase activities (Fiala, 1977, Rosenberg *et al.*, 2009).

In this context, the aim of this study was to investigate the *in vivo* chemopreventive potential of insoluble glucan and mannoprotein extracts obtained from the *S. boulardii* cell wall. It was assessed by the reduction of ACF, the induction of QR activity in liver and to the decrease β-glucosidase and β-glucuronidase activities in caecum content using male F344 rats treated with DMH.

3.5. Methods

3.5.1. Materials

N,N-dimethylhydrazine (DMH), methylene blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), menadione, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, ethylenediaminetetraacetic acid (EDTA), flavin adenine di-nucleotide (FAD), bovine serum albumin (BSA), nicotinamide adenine dinucleotide

phosphate (NADP), Tween 20, Tween-80, NaOH, 4-nitrophenol, p-nitrophenyl β-D-glucopyranoside, p-nitrophenyl β-D-glucuronide and 10% formalin were purchased from Sigma-Aldrich Canada Ltd (Oakville, ON, Canada). Dextrose and 96-well microplates were purchased from Fisher Scientific (Ottawa, ON, Canada). Yeast extract and bacterial peptone were purchased from Alpha Biosciences (Baltimore, MD, USA).

3.5.2. Extraction of insoluble glucan and mannoprotein extracts from yeast cell walls and their preparation for administration by gavage

Extraction of insoluble glucan and mannoprotein extracts was carried out as described in our previous study (Fortin *et al.*, 2017a). *Kluyveromyces marxianus* ATCC 10022 and *Saccharomyces cerevisiae* var. *boulardii* ATCC MYA-796 were purchased from American Type Culture Collection (ATCC) (Corning, Mananssas, VA, USA) and were grown in yeast peptone dextrose (YPD) (10 g/l dextrose, 5 g/l yeast extract, 3 g/l bacterial peptone, 0.8 g/l, $MgSO_4$, 1 g/l KH_2PO_4) for 24 h at 30ºC under 200 rpm agitation. Biomass was autolysed at 50ºC for 24 h at 200 rpm. Then, autolyzed cells were centrifuged at 9000 *g* for 10 min at 4ºC (Thermo Scientific, Sorvall ST16 centrifuge, Langenselbold, Hesse, Germany) and used for glucan and mannoprotein extraction. The extraction was performed by single hot alkaline extraction using 1 mol/l NaOH for 1 h at 90ºC without stirring yielding insoluble glucan (precipitate) and soluble glucan-mannoprotein complex (supernatant). The mannoprotein extract was separated from soluble glucan by alcohol precipitation using 95% ethanol followed by the evaporation of ethanol residue using a vacuum concentrator (Savant, Automatic environmental speedVac® system, Model; AES1010, Farmingdale, NY, USA) under full vacuum, low speed and at ambient temperature for 8 h. Both insoluble glucan and mannoprotein extracts were freeze-dried

(Labconco, FreeZone® 2.5 Liter Freeze Dry Systems, model 7670521, Kansas City, MO, USA). To administer both extracts by gavage, freeze-dried glucan and mannoprotein extracts were stirred for 30 min at desired concentration in 0.9% (w/v) saline containing Tween-80 in 1:4 proportions.

3.5.3. Animals, housing and diet

Forty-eight F344 male rats of five weeks old and with an average weight of 122.85 g were obtained from Charles River Laboratories (Laval, QC, Canada) and housed 3 rats per cage. The temperature and the relative humidity were maintained at 22°C and 55% respectively. The light–dark cycles were 12 h each and all animals had free access to water and rat diet commercial pellets provided by Envigo (Madison, WI, USA). The composition of the high fat low fibre diet was 39.8% corn starch, 20% casein, 10% maltodextrin, 10% corn oil, 12% lard, 2% cellulose, 4.375% mineral mixture, 1.25% vitamin mixture, 0.3% choline bitartrate, 0.3% L-cystine and 0.0044% TBHQ. All experimental animal care and treatments followed the guidelines set by the Canadian Council on Animal Care (CCAC) (Desrouillères *et al.*, 2015).

3.5.4. Experimental design

The rats received a one-week period of acclimation and were randomly divided into 6 groups of 8 animals. All rats were fed with the high fat low fibre diet (Harlan). Group 1 and 2 served as negative (NC) and positive controls (PC) respectively and received 0.9% saline water containing Tween-80 (in 1:4 proportion) by gavage. Rats in groups 2 to 6 were injected with DMH dissolved in saline solution (30 mg/kg subcutaneously) once a week

for six weeks (Desrouillères *et al.*, 2015). In addition to the diet, rats in groups 3 to 6 (G3 to G6) were treated by gavage with different doses of insoluble glucan (G3 and G4) or mannoprotein extracts (G5 and G6). G3 and G4 were treated with 0.5 and 1.0 mg/kg/jour of insoluble glucan respectively whereas G5 and G6 received 0.3 and 3.0 mg/kg/jour of mannoprotein extract respectively. Animals were observed daily for general health and body weights were documented once a week. After an experimental period of 12 weeks, rats were sacrificed in a CO_2 chamber (CNBE, Laval, QC, Canada). The entire colon segment was collected for AC analysis whereas the liver and caecum were collected and stored at -80°C for subsequent enzymatic assays. Figure 3.1 summarizes the experimental design used in this study.

3.5.5. Aberrant crypt analysis

The count of aberrant crypt (AC) and aberrant crypt foci (ACF) per colon was based on a procedure described by Bird (1995). The entire colon of each animal was collected and washed with saline (0.9% NaCl) until no fecal residue were observed. Colons were cut longitudinally and laid flat on Whatman paper in a Petri dish and fixed in 10% buffered formalin solution for at least 24 h. Then, colons were stained with 0.2% methylene blue for exactly 3 min and placed in saline overnight to remove excess staining. Stained colons were placed on glass slides and examined under a microscope (Carl Zeiss inc., Gottingen, Basse-Saxe, Germany) using 20 X objective for counting of ACF as well as the number of AC per focus. Moreover, the number of ACF containing a multiplicity of AC was also recorded and presented as total ACF containing 2-3 AC or containing 4-5 AC per colon.

3.5.6. Liver and caecum processing

Rat livers were weighed and samples ranging from 1.5 to 2 g were cut from each liver. A volume of 5 ml of sucrose solution (0.25 mol/l) was added into each sample and homogenization was processed using a tissue homogenizer purchased from Fisher Scientific (Ottawa, ON, Canada). The homogenized samples were centrifuged at 5000 *g* for 30 min at 4 °C. The obtained supernatants were mixed with 1 ml of calcium chloride solution (0.1 mol/l) and were centrifuged at 27,000 *g* for 20 min at 4°C. The obtained supernatants were filtered through a 0.2 µm filter membrane and were used for QR activity in rat liver (Desrouillères *et al.*, 2015).

Rat caecal contents from different treatment groups were weighed and mixed with 10 ml of 100 mmol/l phosphate buffered saline. The suspensions were homogenized using a tissue homogenizer (Fisher Scientific, Ottawa, ON, Canada) and then centrifuged at 1500 *g* for 15 min at 4°C. The obtained supernatants were centrifuged at 10000 *g* for 15 min at 4°C. The clear obtained supernatants were filtered throughout a 0.2 µm filter membrane and used for β-glucosidase and β-glucuronidase activities (Desrouillères *et al.*, 2015).

3.5.7. Protein estimation

Supernatants of rat liver and caecum were used for the determination of total proteins content which is essential for enzymatic assays (Desrouillères *et al.*, 2015). Total proteins determination was assessed using Pierce BCA protein assay kit (Fisher Scientific, Ottawa, ON, Canada) according to the manufacturer's protocol. Briefly, 25 µl of each sample were added into each well of a 96-well microplate and then 200 µl of BCA solution were added

to the samples wells. The blank consisted of 225 µl of heat-inactivated liver or caecum supernatants. All microplates were incubated for 30 min at 37°C and the absorbance was measured at 562 nm using a microplate reader (Biotek, Model EL800, Winooski, VT, USA). BSA was used as standard to determine protein concentration in samples.

3.5.8. QR assay

The QR assay was based on Prochaska *et al.* (1988a) and Talalay (1989) with some modifications to the *in vivo* assays. Briefly, 30 µl samples of liver supernatants were placed in a 96-well microplate. Then, 200 µl of the reaction mixture (0.25 mol/l Tris-HCl pH 7, 4.67% (w/v) BSA, 0.01% Tween-20, 5 mol/l FAD, 1 mmol/l glucose-6-phosphate, 30 mol/l NADP, 34.8 µmol/l MTT, 50 µmol/l menadione and 2 mU/µl glucose-6-phosphate dehydrogenase) was added into each well. The microplate was incubated for 5 min at room temperature and absorbance values were measured at 595 nm using a microplate reader (Biotek). Blank consisted of 30 µl of heat-inactivated liver supernatants. Specific activity of QR was defined as nmol of blue formazan formed per mg protein per minute. The induction activity of QR enzyme was calculated by dividing the enzyme specific activity of a treated group by the enzyme specific activity of the control group.

3.5.9. β-glucosidase and β-glucuronidase assay

β-glucosidase and β-glucuronidase assays were based on Park *et al.* (1998) with some modifications. Enzymatic activities of β-glucosidase and β-glucuronidase were determined using *p*-nitrophenyl β-D-glucopyranoside and *p*-nitrophenyl β-D-glucuronide as substrates, respectively. Briefly, 30 µl samples from faecal supernatants were added in a 96-well

microplate. A volume of 20 µl of 2 mmol/l respective substrate was added into each well and the microplate was incubated at 37°C for 15 min. Then, the reaction was stopped by adding 250 µl of 10 mmol/l NaOH. Absorbance values were measured at 405 nm using a microplate reader (Biotek). Blank consisted of 30 µl of heat-inactivated caecum supernatants. Based on the fact that one activity unit is defined as the quantity of enzyme required to hydrolyse substrate into one µmol/l of *p*-nitrophenol per minute, a standard curve of *p*-nitrophenol ranging from 0 to 300 µmol/l was used to calculate the specific activities of both enzymes which were expressed as units of *p*-nitrophenol formed per min per mg protein of caecum supernatant.

3.5.10. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA), using PASW statistics 18 software (IBM Corporation, Somers, NY, USA) and expressed as means ± standard deviation. Differences among the groups were analyzed with a post-hoc Duncan's multiple-range test.

3.6. Results

3.6.1. Determination of total number of aberrant crypt (AC) and aberrant crypt foci (ACF) per colon

The cancer preventive effect of yeast cell walls obtained from *S. boulardii* was assessed *in vivo* via the capacity of extracts to prevent the formation of precancerous lesions. As presented in Figure 3.2, rats in the PC group showed significantly more AC (403·9 AC per colon) and ACF (263·6 ACF per colon) as compared to rats in NC group (66·1 AC per

colon and 49·6 ACF per colon) ($p \leq 0.05$). Animals treated with insoluble glucan (G3 and G4) showed a 42% and 45% respective reduction of the total count of AC (Figure 3.2a) per colon as compared to the PC group. In addition, rats in groups G3 and G4 showed a 40% and 50% respective total count reduction of ACF (Figure 3.2b) per colon as compared to the PC group, hence suggesting that insoluble glucan demonstrated cancer prevention effects toward CRC. In contrast, no reduction in the count of total AC per colon nor in the count of total ACF per colon was observed in rats treated with mannoprotein extract (G5 and G6) as compared to the PC group. Moreover, Figure 3.2 suggest that animals treated with high dose of mannoprotein extract (3·0 mg/kg/jour) presented a significantly higher count of total AC per colon and total ACF per colon as compared to the PC group ($p \leq 0.05$). Also, no significant differences ($p > 0.05$) were observed between animals in groups G3 and G4 nor between animals in groups G5 and G6 regarding total counts of AC and ACF.

3.6.2. Determination of the number of aberrant crypt foci (ACF) containing a multiplicity of aberrant crypt (AC)

Since there is a direct correlation between the ACF size and the probability of colon carcinoma development, the number of AC per ACF was also evaluated. As presented in Figure 3.3, rats in the PC group had significantly more total number of ACF containing 2-3 AC per focus and containing 4-5 AC per focus ($p \leq 0.05$) as compared to rats in the NC group which is congruent with results from Figure 3.2, hence confirming the relevance of the experimental design of this study. Moreover, rats treated with insoluble glucan (G3 and G4) showed a 38% and 61% respective reduction of the total number of ACF containing

2002 2-3 AC per focus as compared to the PC group. In addition, data from Figure 3.3 also
2003 demonstrated that rats in both groups G3 and G4 showed a 73% significant reduction of
2004 the total number of ACF containing 4-5 AC per focus as compared to the PC group ($p \leq$
2005 0.05). It is of interest to find that there was no significant difference ($p > 0.05$) in the total
2006 number of ACF containing 4-5 AC per focus among G3, G4 and NC groups (Figure 3.3).
2007 These results suggest that insoluble glucan reduced the total number of ACF containing a
2008 multiplicity of AC, hence decreasing the probability of colon carcinoma development in
2009 addition to confirm the cancer prevention effect of insoluble glucan. In contrast, no
2010 reduction in total counts of ACF containing 2-3 AC per focus nor containing 4-5 AC per
2011 focus was observed in animals treated with mannoprotein extract (G5 and G6). Moreover,
2012 animals treated with high dose of mannoprotein extract (3·0 mg/kg/jour) presented a
2013 significantly higher count of ACF containing 2-3 AC per focus as compared to the PC
2014 group ($p \leq 0.05$), hence suggesting a negative effect of this extract on the digestive tracts
2015 at high concentration.
2016

2017 **3.6.3. Induction of QR activity determined in liver supernatant**

2018 Analysis of the reduction of precancerous lesions revealed that insoluble glucan possesses
2019 *in vivo* cancer prevention properties. The mechanism involved in such chemopreventive
2020 properties has been investigated via the effect of insoluble glucan and mannoprotein
2021 extracts on the activity of a phase II detoxification enzyme. Results presented in Figure 3.4
2022 show that only animals in G3 (0.5 mg/kg/jour of insoluble glucan) were able to induce by
2023 68% the QR specific activity as compared to the PC group ($p \leq 0.05$). Animals in group
2024 G4 (1.0 mg/kg/jour of insoluble glucan) showed an increased QR specific activity by 29%

in comparison with the PC group. However, QR induction of animals treated with a high dose of insoluble glucan (1·04 fold induction) was not statistically different than the QR induction of animals in the PC group (0·75 fold induction) ($p > 0.05$), hence suggesting that QR induction might not be the only responsible factor for cancer prevention effect observed since animal in G3 and G4 similarly reduced ACF counts. Moreover, all rats treated with mannoprotein extract (G5 and G6) were not able to significantly ($p > 0.05$) induce QR activity as compared to control groups (G1 and G2) which is congruent with results from Figures 3.2 and 3.3 where no reduction of AC and ACF were observed for mannoprotein extract.

3.6.4. β-glucuronidase and β-glucosidase activities determined in caecum content

The activities of β-glucuronidase and β-glucosidase were also quantified and results are presented in Figure 3.5 and 3.6 respectively. Results demonstrated that rats in the NC group (186.1 units per mg protein of caecum content) presented a β-glucuronidase activity significantly lower than rats in the PC group (294.2 units per mg protein of caecum content) ($p \leq 0.05$). Also, data from Figure 3.5 show that rats in groups G3 (168.9 units per mg protein of caecum content) and G4 (147.9 units per mg protein of caecum content) were able to significantly reduce ($p \leq 0.05$) the activity of β-glucuronidase in a dose-dependent manner. A reduction of 43% and 50% was observed in groups G3 and G4, respectively, as compared to the PC group, hence suggesting that β-glucuronidase was implied in the cancer prevention effect of insoluble glucan. Moreover, results in Figure 3.5 also demonstrated that all rats treated with mannoprotein extract (G5 and G6) significantly increased ($p \leq 0.05$) the β-glucuronidase activity (396.5 and 454.5 units per mg protein of Caecum

respectively) as compared to the PC groups, which is also in accordance with results presented in Figures 3.2 and 3.3 where no reduction of AC and ACF were observed for the mannoprotein extract. In contrast, results in Figure 3.6 demonstrated that all rats treated with insoluble glucan (155.1 and 211.0 units per mg protein of caecum content) or mannoprotein extracts (145.1 and 154.6 units per mg protein of caecum content) did not significantly reduce ($p > 0.05$) the activity of β-glucosidase activity as compared to the PC group (190.0 units per mg protein of caecum content), hence suggesting that β-glucosidase might not be implicated in the cancer prevention effect observed by the reduction of the count of AC and ACF presented in Figures 3.2 and 3.3.

3.7. Discussion

The objectives of this study were to evaluate the effect of the consumption of insoluble glucan and mannoprotein extracted from the cell walls of *S. boulardii* on the reduction of ACF formation in male F344 rats treated with DMH. Then, the effects of those extracts on the QR activity in the rat liver and on two faecal enzymes were evaluated. The results suggest that only the insoluble glucan was able to reduce the initial step of CRC development. Several studies presented the effect of β-glucan from different sources to prevent the formation of precancerous lesions (Lahouar *et al.*, 2012, Watanabe *et al.*, 2013). For instance, Bobek *et al.* (2001) investigated the effect of pleuran (β-glucan from *Pleurotus ostreatus*) on DMH-induced precancerous lesions in rat colon and observed a reduction of ACF count in the group treated with pleuran as compared to a control group. The authors attributed this effect to the fibrous (insoluble) nature of pleuran which participates in the sequestration of bile acid and other carcinogens resulting in the

acceleration of their excretion. Moreover, the well-known immunomodulatory properties of β-glucan may also be responsible for the reduction of ACF. Indeed, the releasing of IL-12 and IL-10 cytokines induced by β-glucan treatment has been reported to play an important role in cancer therapy (Chan *et al.*, 2009, Stier *et al.*, 2014). The fact that mannoprotein extract did not reduce the count of ACF reflects its lack of chemoprevention effect *in vivo*. Very few studies investigated the *in vivo* chemoprevention of yeast mannoprotein whereas many studies focused on its anticancer properties *in vitro*. Indeed, Liu *et al.* (2011) revealed that the molecular weight (Mw) of *S. cerevisiae's* mannoprotein, which depends on the extraction method, is important to obtain relevant antineoplastic activities (inhibition ratios) against cancerous cells. The authors observed the highest inhibition ratio using hot water extracted *S. cerevisiae* mannoprotein (181·127 kDa). The mannoprotein extract used in the present study exhibited a Mw range ranging from 720-87000 Da which is lower than Mw of mannoprotein obtained by Liu *et al.* (2011). In this respect, the low Mw range of mannoprotein extract used in the present study may explain the lack of chemoprevention effect observed. Moreover, mannoprotein extracts were found to have different *in vivo* and *in vitro* antitumor activities, depending on their monosaccharide composition, protein content, molecular mass and chain conformation which also vary according to extraction method (Bland *et al.*, 2004).

CRC carcinogenesis is a multistage process that begins with the transformation of crypt stem cells that remain located at the bottom of crypt while fuelling a growing microadenoma (Barker *et al.*, 2009). The importance of phase II detoxification enzymes such as QR in CRC chemoprevention concerns its capacity to decrease chemical

carcinogenicity by transforming those compounds in a less toxic form or into inactive metabolites (Kang *et al.*, 2004). In this context, an enhancement of QR activity is considered to enhance excretion of carcinogens. Thus, a bioactive agent with the capacity to induce QR activity demonstrates a chemopreventive potential. In the present study, rats treated with insoluble glucan induced QR activity whereas rats treated with mannoprotein did not show such an effect. These results are in accordance with our previous study in which those extracts exhibited the same pattern in QR induction *in vitro (Fortin et al., 2017a)*. Results from the present study also showed that rats treated with high dose of insoluble glucan (1.0 mg/kg/jour) showed no significant QR induction whereas rats treated with low dose (0.5 mg/kg/jour) showed a significant ($p \leq 0.05$) induction of QR induction as compared to rats in the PC group. This result suggests that QR induction might not be the only mechanism responsible for ACF reduction in rats treated with insoluble glucan since both doses similarly reduced the formation of ACF as presented in Figures 3.2 and 3.3. Indeed, it has been reported that QR is interconnected with other phase II enzymes in the liver (Cuendet *et al.*, 2006, Desrouillères *et al.*, 2015, Kang *et al.*, 2004). Thus, a high dose of insoluble glucan may trigger the induction of a second phase II enzyme and/or another chemopreventive mechanism that detoxifies an organism in the sense that if one can detoxify the organism, no QR induction will be observed. Since this study is the first time reporting an induction of QR by yeast cell walls *in vivo*, there is a great interest to investigate the effect of insoluble glucan on other phase II detoxification enzymes using a wider range of doses ranging from 50 to 400 mg/kg/jour for instance (Samuelsen *et al.*, 2014).

β-glucosidase and β-glucuronidase are two bacterial enzymes who are able to deconjugate toxins and/or carcinogens that have been previously detoxified in the liver and secreted into the gut via the bile. Those enzymes may lead to high local concentrations of carcinogens in the gut, hence increasing risk of CRC carcinogenesis (Dabek *et al.*, 2008, Simon *et al.*, 1986). More specifically, β-glucuronidase removes glucuronic acid from a compound detoxified by phase II enzymes hence releasing the carcinogenic compound in the colon (Gill *et al.*, 2002). Similarly, β-glucosidase hydrolyses glycosidic bonds releasing non-reducing terminal glucosyl residues from an aglycon compound which may be carcinogenic or toxic for the colonic environment. Since glycosides are hydrolyzed in the colon by bacterial β-glucosidases, potentially toxic substances may be formed in the large intestine (Ketudat Cairns *et al.*, 2010, Mroczynska *et al.*, 2013). Those bacterial enzymes are mostly produced by many *Clostridium* sp. (*C. paraputrificum, C. clostridioforme, C. perfringens*), *Bacteroides* sp. (*B. vulgatis, B. uniforme, B. fragilis*), *Enterococcus* sp*., Peptostreptococcus* sp*., Staphylococcus* sp*., Ruminococcus* (gnavus), *Eubacterium* sp*. and Escherichia coli* (Dabek *et al.*, 2008, de Moreno de LeBlanc *et al.*, 2005, Nakamura *et al.*, 2002). For those reasons, a reduction in the activity of β-glucosidase and β-glucuronidase can lead to a reduced exposure to carcinogenic substances and thus lead to a reduction in the incidence of CRC.

Our results demonstrated that rats treated with insoluble glucan significantly had a reduced activity of β-glucuronidase which is congruent with previous studies by Shen *et al.* (2012). Indeed, the authors reported that β-glucan present in cereals can decrease β-glucuronidase activity in a dose-independent manner, increase *Bifidobacteria* and *Lactobacilli* counts in

addition to decrease those of colonic *Enterobacteriaceae*. The authors proposed that insoluble compounds contained in oat and barley β-glucan may pass in undigested forms into the large intestine and act as prebiotics, hence stimulating the growth of beneficial bacteria such as *Bifidobacteria* and *Lactobacilli*. In this context, insoluble glucan may have acted as a prebiotic in the colon leading to the increase of *Bifidobacteria* and L*actobacilli* which may have decreased the bacterial population responsible for β-glucuronidase production by competitive exclusion. However, our results demonstrated that insoluble glucan did not reduce bacterial β-glucosidase activity. These results may be due to the different mechanism that dictated change in β-glucosidase and β-glucuronidase activities. Relatively to this assessment, McBain *et al.* (1998) reported that β-glucosidase activity is mostly related to a substrate-induced modulation of bacterial metabolism rather than the number of bacteria as opposed to β-glucuronidase activity. In this context, insoluble glucan used in the present study might have enhanced a competitive exclusion phenomenon to the detriment of β-glucuronidase producing bacteria and might not have acted as a substrate for β-glucosidase producing bacteria. In contrast, mannoprotein extract did not exhibit the capacity to reduce bacterial enzymes as compared to the positive control group which is also congruent with the absence of the capacity to reduce ACF counts. In this perspective, yeast mannoprotein extract obtained from alkaline extraction did not show any chemoprevention properties *in vivo* which highly contrasts with the insoluble glucan. This lack of CRC prevention properties might be due to an intrinsic incapacity of yeast mannoprotein to exhibit such properties since no study has reported, to our knowledge, such capacities.

This study demonstrated that a crude extract containing β-glucan as a major polysaccharide obtained from the cell walls of *S. boulardii* using a single hot alkaline extraction could have a preventive effect against colon carcinogenesis by decreasing the total number of ACF in DMH-treated rats. The mechanisms involved in this cancer prevention effect were found to be the induction of QR activity and the reduction of β-glucuronidase activity which could result in a reduced retention time of carcinogenic compounds in the organism. These findings together with our previous work demonstrates that this crude insoluble glucan from the cell walls of *S. boulardii* possesses both *in vivo* and *in vitro* cancer preventive effect against CRC, which provides further evidence on the chemopreventive potential of yeast cell walls. Further studies will be needed to investigate the immunomodulatory properties of this specific extract which will involve recognizing and eliminating the vast majority of incipient cancer cells and thus nascent tumors (Hanahan *et al.*, 2011). Moreover, the apoptosis properties and the underneath mechanisms need to be assessed in order to determine the specific cytotoxicity of the crude insoluble glucan against cancerous cells.

3.8. Acknowledgements

This work was supported by the Ministère de l'Économie, de l'Innovation et de l'Exportation du Québec (MEIE). Olivier Fortin is a scholarship recipient of Fondation Armand-Frappier.

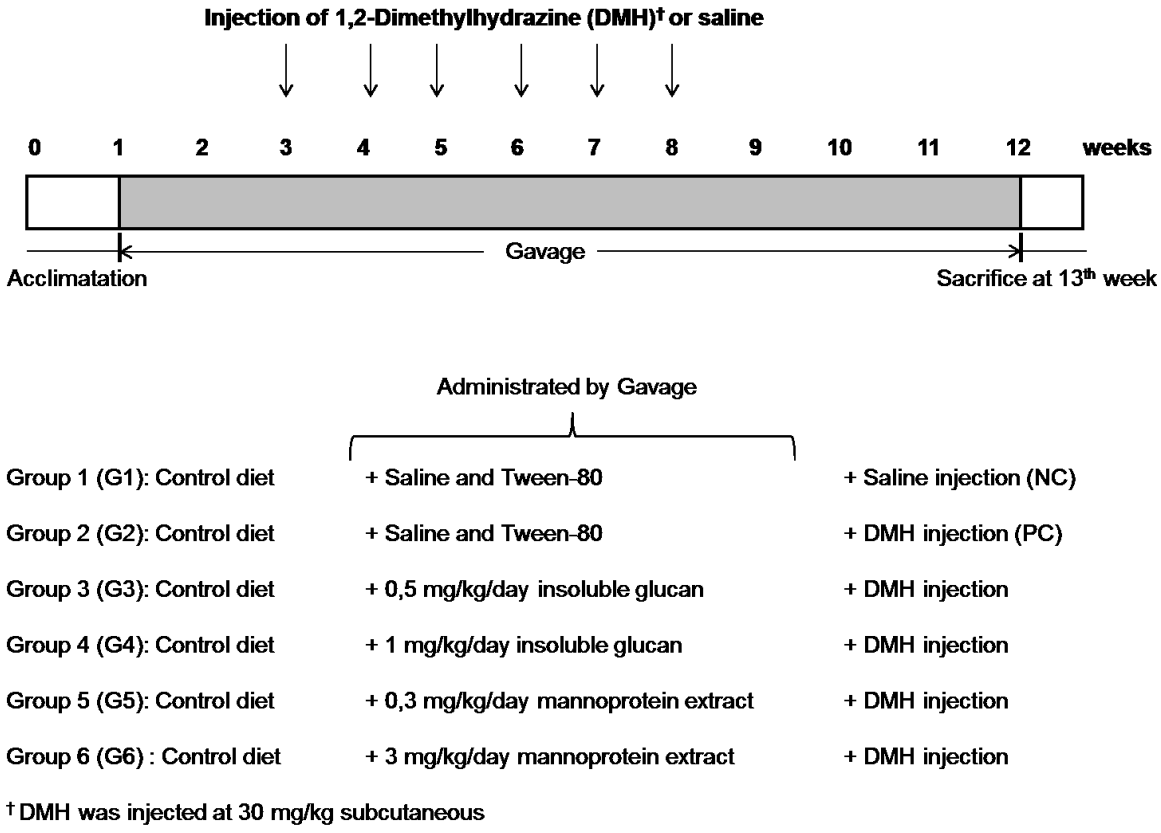

Fig 3.1. Diagram representing the experimental design. Forty-eight rats were divided in 6 groups and were fed with high fat low fibre diet for 13 weeks. After one acclimation week, all rats were injected with saline water (G1) or DMH (G2 to G6) and received by gavage Tween-80 solution (G1 and G2) or different concentration of insoluble glucan (G3 and G4) and mannoprotein extract (G5 and G6). At the 13th week, all rats were sacrificed then colon, caecum and liver were collected for subsequent analysis.

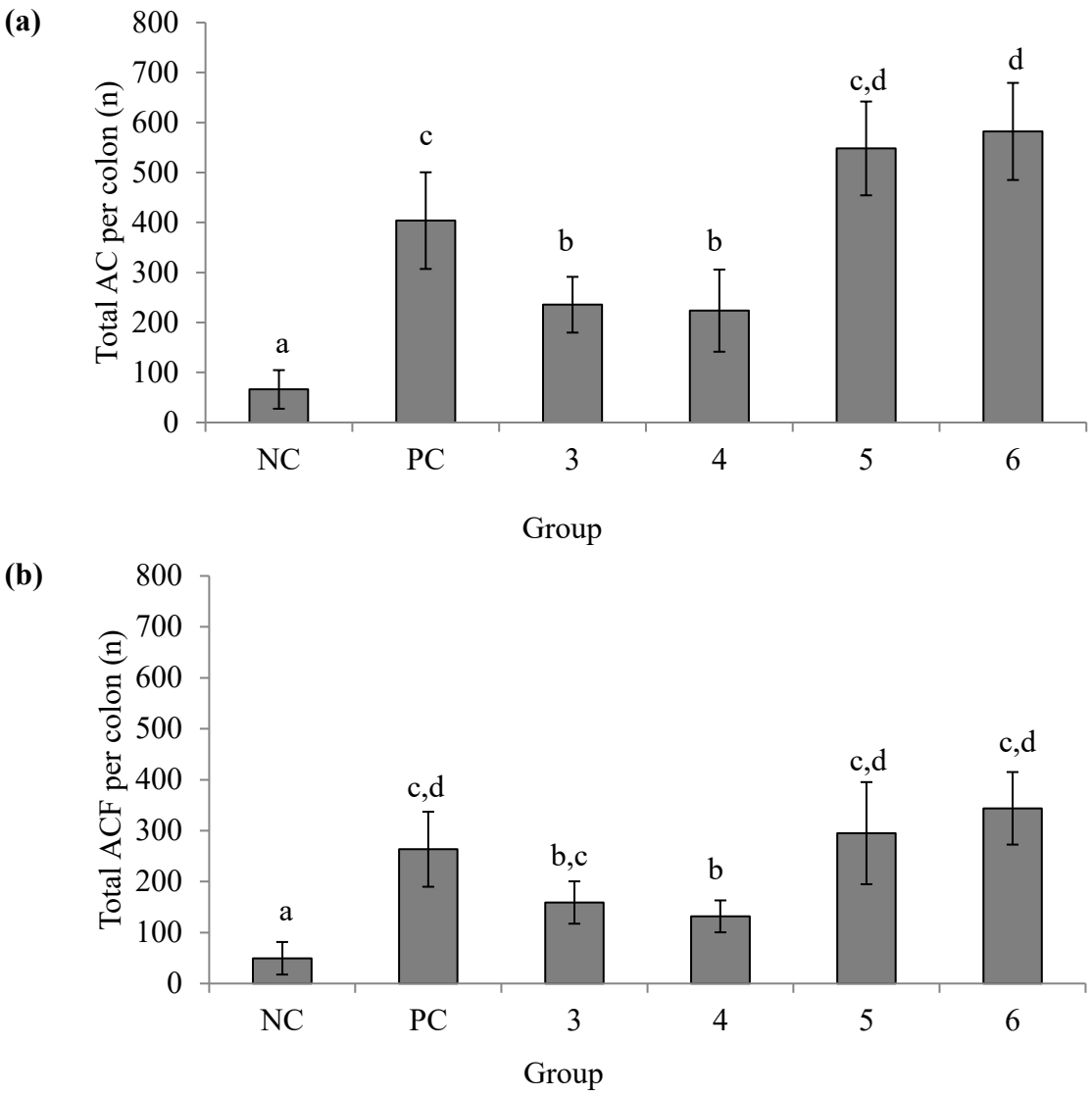

Fig 3.2. Numbers (n) of precancerous lesions induced by DMH in rats treated with insoluble glucan and mannoprotein extracts regarding a) total number of aberrant crypt (AC) and b) total aberrant crypt foci (ACF) per colon. NC: Saline + Tween only, PC: Saline + Tween + DMH, G3: DMH + 0.5 mg/kg/jour insoluble glucan, G4: DMH + 1.0 mg/kg/jour insoluble glucan, G5: DMH + 0.3 mg/kg/jour mannoprotein extract, G6: DMH + 3.0 mg/kg/jour mannoprotein extract. Error bars represent the standard deviation of the mean

2199 total AC and AFC per colon obtained from 8 rats in each group. Different letters are
2200 significantly different ($p \leq 0.05$).

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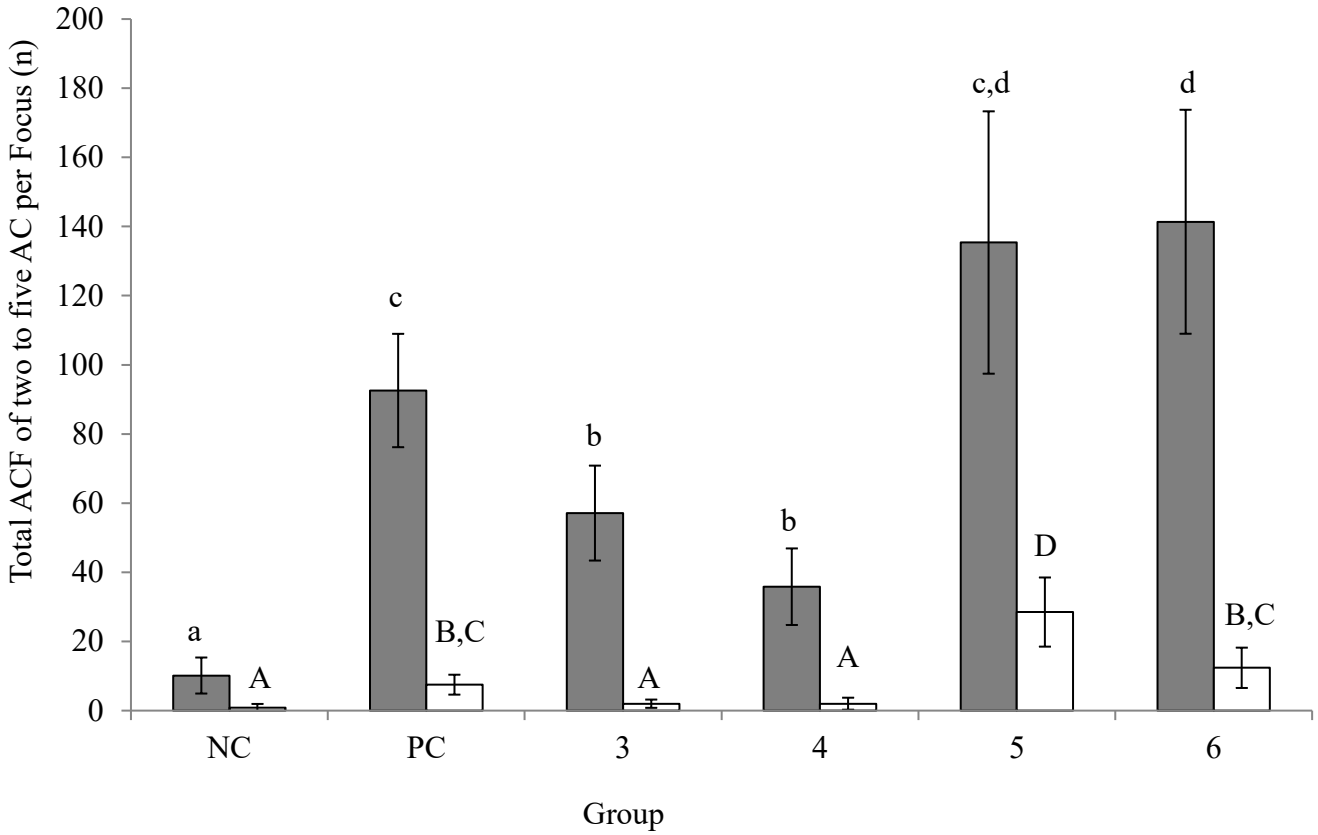

Fig 3.3. Numbers (n) of precancerous lesions induced by DMH in rats treated with insoluble glucan and mannoprotein extracts regarding number of aberrant crypt foci (ACF) containing a multiplicity of AC per focus. (■) ACF containing two to three AC, (□) ACF containing four to five AC. NC: Saline + Tween only, PC: Saline + Tween + DMH, G3: DMH + 0.5 mg/kg/jour insoluble glucan, G4: DMH + 1.0 mg/kg/jour insoluble glucan, G5: DMH + 0.3 mg/kg/jour mannoprotein extract, G6: DMH + 3.0 mg/kg/jour mannoprotein extract. Error bars represent the standard deviation of the mean total AFC containing 2-5 AC per focus obtained from 8 rats in each group. Lowercase and uppercase letters concerns ACF containing 2 to 3 AC per focus and 4 to 5 AC per focus respectively. Different letters are significantly different ($p \leq 0.05$).

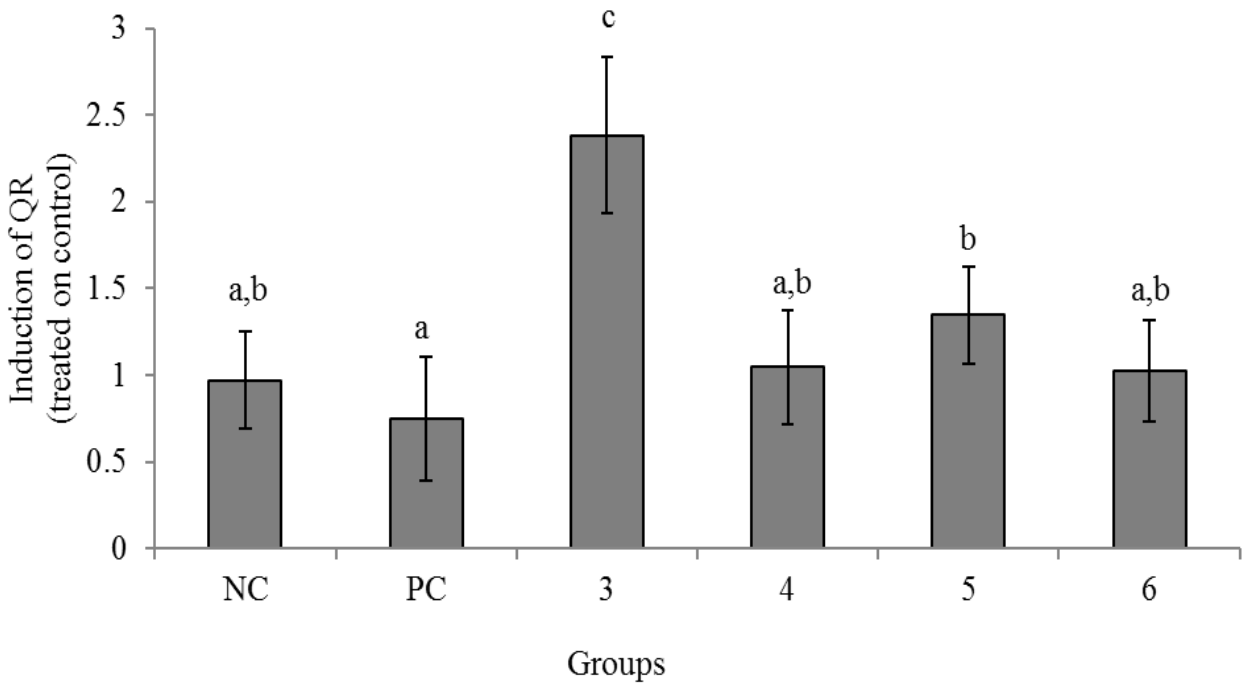

Fig 3.4. Induction of QR activity in liver supernatant of DMH treated rats treated with insoluble glucan and mannoprotein extracts. NC: Saline + Tween only, PC: Saline + Tween + DMH, G3: DMH + 0.5 mg/kg/jour insoluble glucan, G4: DMH + 1.0 mg/kg/jour insoluble glucan, G5: DMH + 0.3 mg/kg/jour mannoprotein extract, G6: DMH + 3.0 mg/kg/jour mannoprotein extract. Error bars represent the standard deviation of mean QR induction obtained from 8 rats in each group. Different letters are significantly different ($p \leq 0.05$).

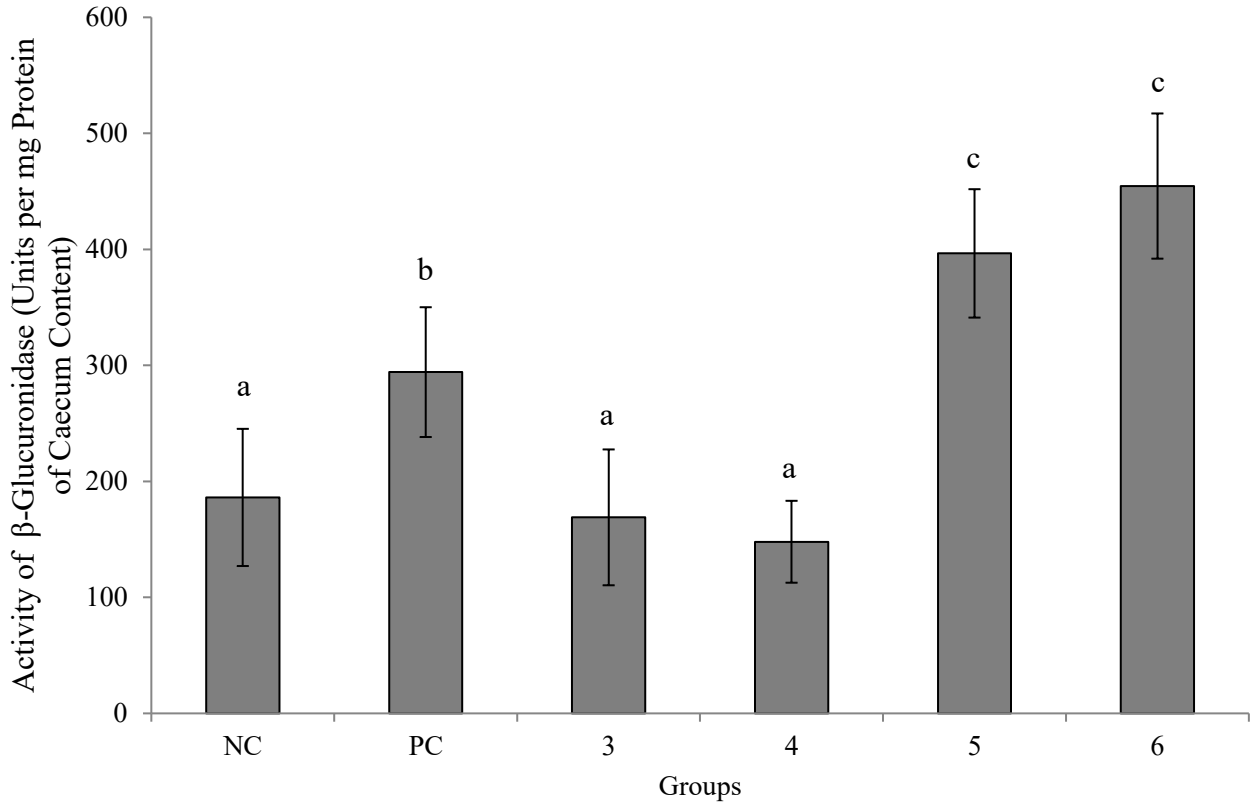

Fig 3.5. β-glucuronidase assay in caecum content of DMH treated rats treated with insoluble glucan and mannoprotein extracts. NC: Saline + Tween only, PC: Saline + Tween + DMH, G3: DMH + 0.5 mg/kg/jour insoluble glucan, G4: DMH + 1.0 mg/kg/jour insoluble glucan, G5: DMH + 0.3 mg/kg/jour mannoprotein extract, G6: DMH + 3.0 mg/kg/jour mannoprotein extract. Error bars represent the standard deviation of the mean β-glucuronidase activity obtained from 8 rats in each group. Different letters are significantly different ($p \leq 0.05$).

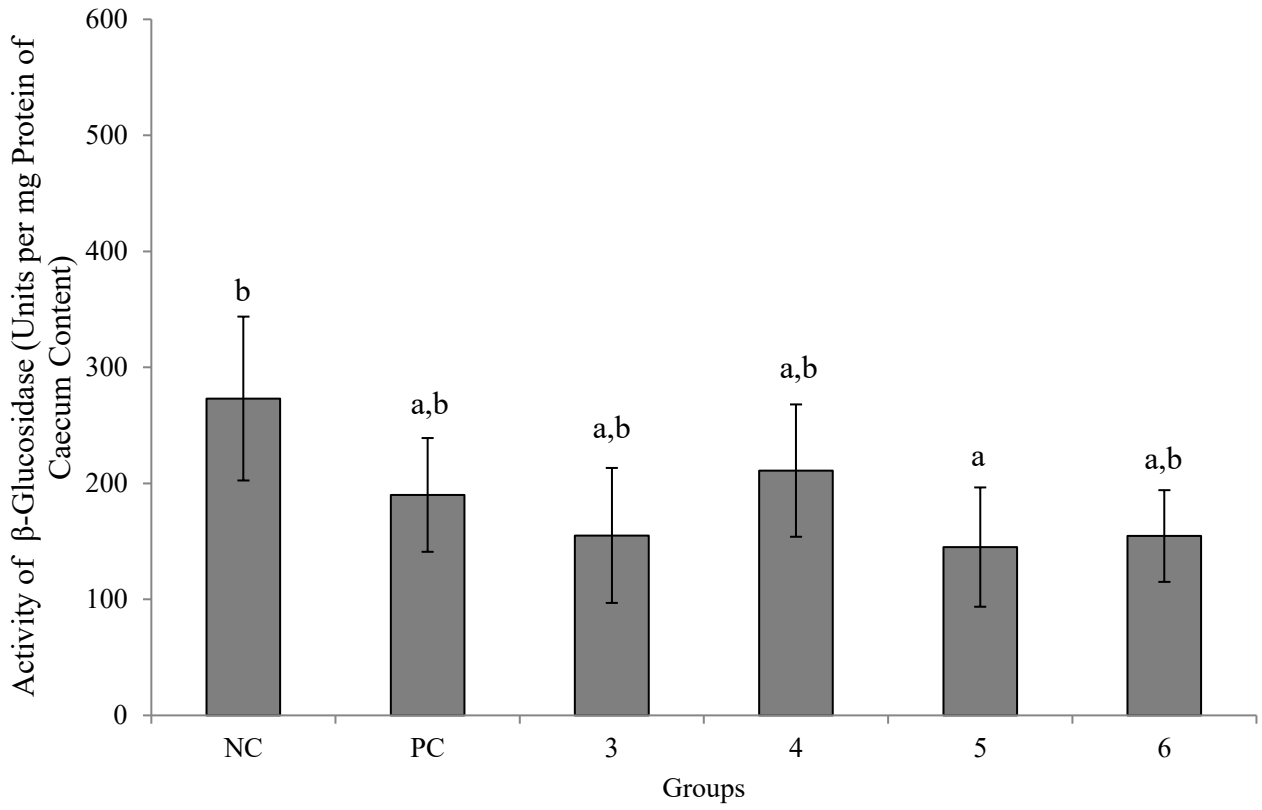

Fig 3.6. β-glucosidase assay in caecum content of DMH treated rats treated with insoluble glucan and mannoprotein extracts. NC: Saline + Tween only, PC: Saline + Tween + DMH, G3: DMH + 0.5 mg/kg/jour insoluble glucan, G4: DMH + 1.0 mg/kg/jour insoluble glucan, G5: DMH + 0.3 mg/kg/jour mannoprotein extract, G6: DMH + 3.0 mg/kg/jour mannoprotein extract. Error bars represent the standard deviation of the mean β-glucosidase assay obtained from 8 rats in each group. Different letters are significantly different ($p \leq 0.05$).

CHAPITRE 4 - PUBLICATION 3

Chemopreventive, antiradical and antiproliferative properties of essential oils obtained from *Boswellia carterii* and *B. sacra* in combination with yeast cell wall extract.

Chemoprevention, antiradical and antiproliferative properties of essential oils obtained from *Boswellia carterii* and *B. sacra* in combination with yeast cell wall extract.

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Abbreviations: EOs, essential oils; 5-FU, 5-fluorouracil; BCA, bicinchoninic acid protein; BSA, bovine serum albumin; CRC, colorectal cancer; FAD, flavin adenine dinucleotide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NADP, nicotinamide adenine dinucleotide phosphate; QR, NAD(P)H: quinone reductase; Tween-80, polyoxyethylene sorbitan monooleate; FBS, fetal bovine serum; MEM-EBSS, minimum essential medium-Earle's balanced salt solution; HBSS, Hank's balanced salt solutions; X/XO, Xanthine/Xanthine Oxidase; DPPH, α,α-diphenyl-β-picrylhydrazyl; PI, propidium iodide; CI, combination index; IC_{50}, concentration that inhibits 50% of the cellular growth; SC_{50}, concentration that scavenges 50% of formed radicals; MIC, minimal inhibitory concentration; BA, boswellic acid; K-BA, keto-β-boswellic acid, AK-BA, acetyl-keto-β-boswellic acid

4.1. Contribution des auteurs

J'ai réalisé les expériences et rédigé le manuscrit scientifique. Dr. Aguilar-Uscanga a participé aux discussions scientifiques et a aussi révisé le manuscrit. Dr. Vu a participé aux discussions scientifiques et a révisé le manuscrit. Mr. Salmieri m'a aidé dans la rédaction du manuscrit en plus d'avoir participé à quelques discussions scientifiques et a révisé le manuscrit. Jingcheng Zhao a participé à la réalisation des expériences. Dr. Lacroix, coordonnatrice et responsable du projet, a supervisé les discussions scientifiques entourant ce projet en plus d'avoir révisé le manuscrit.

4.2. Résumé en français

Les propriétés chimiopréventives, antiradicalaires et antiprolifératives des huiles essentielles (HE) de *Boswellia carterri* (huile Frankincense) et de *B. sacra* (huile Sacrée) utilisées seules ou en combinaison avec des extraits de parois cellulaires de levures ont été investiguées en modèle *in vitro* contre le cancer colorectal (CCR). Les huiles essentielles ont été testées pour leur capacité à induire la NAD(P)H : quinone réductase (QR), à capter des radicaux libres et à inhiber la croissance de cellules cancéreuses humaine du CCR. Les résultats ont démontré que les HE de *B. sacra* et de *Boswellia carterri* sont en mesure de capter l'anion superoxide et inhibent de manière similaire la croissance de deux lignées cellulaires cancéreuses humaines du CCR. Cette étude a aussi mis en évidence pour la première fois que l'augmentation de l'activité de la QR est un mécanisme d'action de ces HE dans la prévention du cancer et a démontré que des extraits de parois cellulaires de levures améliorent la capacité de l'huile Frankincense à augmenter l'activité spécifique de la QR. Finalement, l'huile Sacrée a capté efficacement l'anion superoxide en plus d'exprimer une cytotoxicité spécifique aux cellules cancéreuses contrairement à l'huile Frankincense.

Mots clefs : Huiles essentielles de *Boswellia*, chimioprévention, antiradicalaire, antiprolifératif, NAD(P)H : quinone réductase, β-glucanes, apoptose

4.3. Abstract

The *in vitro* chemopreventive, antiradical and antiproliferative effects of essential oils (EOs) from *Boswellia carterii* (Frankincense oil) and *B. sacra* (Sacred oil) used alone and in combination with yeast cell wall extracts on colorectal cancer (CRC) were investigated. Essential oils were assayed for their capacity to increase the specific activity of NAD(P)H: quinone reductase (QR), scavenge radicals and inhibit growth of human CRC cells. Results demonstrated that EOs from *B. sacra* and *B. carterii* scavenged superoxide anions and similarly inhibited growth of two human CRC cell lines. This study also reported the increase of QR activity as a novel mechanism of action of these EOs in cancer prevention and demonstrated that yeast cell wall extract enhanced the capacity of Frankincense oil to increase QR specific activity. Finally, Sacred oil efficiently scavenged superoxide anions and expressed cancerous cell-specific cytotoxicity when opposed to Frankincense oil.

Keywords: *Boswellia* essential oils, chemoprevention, antiradical, antiproliferative, NAD(P)H: quinone reductase, β-glucan, apoptosis

4.4. Introduction

The colorectal cancer (CRC) is the second most deadly cancer in males and the third for females (Canadian Cancer Society's Advisory *et al.*, 2014) and is the third most prevalent cancer in Canada (Canadian Digestive Health Foundation, 2016). The increase of CRC in Canada and USA has generated an increased interest in the consumption of natural products to prevent the development of this disease. Thus, prevention seems to be the most efficient approach since treatments for CRC can be expensive and invasive for patients. Chemoprevention consists of using natural or synthetic materials to prevent the progression of cancer (Czadek, 2016). Many essential oils (EOs) and their constituents have been reported to be chemopreventive agents due to their abilities to affect phase I and II enzymes, prevent lipid peroxidation, suppress cyclooxygenase-2 activity and exhibit anticancer properties such as *in vivo* antitumoral activities, apoptosis and cancerous cell specific cytotoxicity (Gautam *et al.*, 2014, Jayakumar *et al.*, 2012). More specifically, EOs from *Boswellia spp.* are well known for their tumor cell specific cytotoxicity and their capacity to induce apoptosis in cancerous cells (Dozmorov *et al.*, 2014, Frank *et al.*, 2009). In this aspect, the use of *Boswellia spp.* EOs as chemopreventive agents toward CRC appears to be relevant. Moreover, EOs exhibiting enhanced chemopreventive properties obtained by combination with known chemopreventive agents is also a relevant approach to reduce CRC development.

EOs obtained from *Boswellia* trees have been used for many centuries in religious rituals and medicinal applications such as inflammation, immune support, skin health and more recently cancer treatment. As found with others EOs, the biological properties of Frankincense (obtained from *Boswellia carterii*) and Sacred (obtained from *B. sacra*) EOs

vary according to many factors such as plant species, plant organs, extraction methods, soil composition, vegetative cycle stage, season and climate of harvesting (Bakkali *et al.*, 2008). In this context, many scientists, botanists and governments tend to consider *B. carterii* and *B. sacra* as the same species whereas several studies tend to prove the opposite using chemical characterization (Woolley *et al.*, 2012). Despite these evidences, very few studies have investigated differences between EOs from *B. carterii* and *B. sacra* regarding their biological activities toward colorectal cancer (CRC).

Yeast β-glucans found in the cell wall are known for their strong immunomodulatory properties (Samuelsen *et al.*, 2014) and can be easily extracted from spent yeast (Suphantharika *et al.*, 2003). In previous studies, the chemopreventive potential *in vitro* and *in vivo* of insoluble glucan from *S. boulardii* cell wall was demonstrated (Fortin *et al.*, 2017a, Fortin *et al.*, 2017b). Notably, this specific extract appeared to be an inducer of NAD(P)H: quinone reductase (QR), a phase II detoxification enzyme (EC 1.6.99.2) *in vitro* and *in vivo* and could significantly reduce aberrant crypt count in 1,2-dimethylhydrazine-treated rats.

To substantiate differences between EOs from *B. sacra* and *B. carterii*, this study investigated the differences and the mechanism of action of Frankincense and Sacred EOs regarding chemopreventive, antiradical and antiproliferative properties toward CRC. In this context, EOs were evaluated for their capacity to induce QR activity which has never been evaluated before and to scavenge superoxide anions (O_2^-) and DPPH radicals. Moreover, EOs were tested for their antiproliferative activities against cancerous and non-cancerous cells to reveal a cancerous cell specific cytotoxicity then an apoptosis assay was conducted to determine if this mechanism was involved. Finally, efforts were invested to

2368 enhance biological activities of EOs through addition of insoluble glucan to Frankincense
2369 and Sacred EOs.

4.5. Method

4.5.1. Chemicals

Chemicals and media were obtained as follows: essential amino acids, sodium pyruvate, fetal bovine serum (FBS), minimum essential medium-Earle's balanced salt solution (MEM-EBSS), Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (MEMF/12), Ham's F-12 medium, Dulbecco's Modified Eagle Medium low glucose, Hank's balanced salt solutions (HBSS), trypsin, Pierce®BCA Protein assay, glycine, 25 cm^2 flask, 96-well and 6-well microplates were purchased from Fisher Scientific (Ottawa, ON, Canada). Activated carbon, digitonin, bovine serum albumin (BSA), glucose-6-phosphate, thiazolyl blue tetrazolium bromide (MTT), menadione, glucose-6-phosphate dehydrogenase, nicotinamide adenine dinucleotide phosphate (NADP) and flavin adenine dinucleotide (FAD), Tween-80, sodium azide, 2,3-*Bis*(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt (XTT), xanthine, sodium carbonate buffer (pH 10.2), xanthine oxidase, superoxide dismutase, N-methylpyrrolidone (NMP), α,α-diphenyl-β-picrylhydrazyl (DPPH) and 5-fluorouracil (5-FU) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Lecithin was purchased from ADM (Calgary, AB, Canada). Frankincense and Sacred EOs were graciously provided by Young Living Essential Oils (Lehi, UT, USA). Annexin V-FITC/PI Dead Cell Apoptosis kit was purchased from Invitrogen (Burlington, ON, Canada). Polymethyl methacrylate (PMMA) was obtain from Agilent technologies (Mississauga, ON, Canada).

4.5.2. EOs preparation

Boswellia carterii and *B. sacra* were harvested in Kenya and Oman respectively to obtain Frankincense and Sacred EOs using the steam distillation method and were kindly provided by Young Living Essential Oils (Lehi, UT, USA). Frankincense and Sacred EOs were prepared under oil-in-water emulsion (termed emulsifying solution) containing 1% (v/v) Tween-80 and 1% (w/v) lecithin as emulsifying agents. The EO emulsion was stirred until complete homogenization and then filtered through a 0.2 µm filter. For combined treatments containing EOs and insoluble β-glucan, an EO emulsion was prepared as mentioned above but without filtration through a 0.2 µm filter, and sodium azide (20 ppm) was added to prevent microbial contamination since an insoluble extract was used. For assays, EOs were serial diluted in anhydrous ethanol to a final concentration of 34400 ppm.

4.5.3. Extraction of insoluble glucan from *S. boulardii* cell wall

Extraction of insoluble glucan was performed as described by Fortin *et al.* (2017a). Briefly, *S. boulardii* cells were grown in yeast peptone media containing 1% (w/v) dextrose and collected in early stationary phase. The cell suspension was centrifuged at 9000 x *g* for 10 min at 4ºC and the resulting biomass was washed twice with sterile phosphate buffer 50 mM, pH 7.2. Then, the wet biomass was suspended in sterile water (15% w/v) and autolyzed for 24 h at 50ºC under agitation at 200 rpm. The autolyzed biomass was then centrifuged at 9000 x *g* for 10 min at 4ºC and 500 ml of 1 mol/l NaOH was mixed with 100 g of wet autolyzed cells for 1 h at 90ºC without agitation. Finally, the resulting suspension was centrifuged as described above and the precipitate was washed twice with distilled water and then freeze-dried.

4.5.4. Cancerous cell lines and cells maintenance

Hepa 1c1c7 ATCC CRL-2026, HT-29 ATCC HTB-38, CHO-K1 and Caco-2 cell lines were purchased from American type culture collection (ATCC) (Manassas, VA, USA). All cell lines were cultivated in 25 cm^2 cellular flasks (Corning, NY, USA) at 37ºC in a humidified incubator with an atmosphere of 5% CO_2 and 95% air. Hepa 1c1c7 and HT-29 cells were grown in complete MEM-EBSS and complete MEMF/12 media, respectively (0.1% essential amino acids, 0.1% sodium pyruvate, 10% FBS). Caco-2 cells were grown in Dulbecco's Modified Eagle Medium low glucose (0.1% essential amino acids, 0.1% sodium pyruvate, 20% FBS) and CHO-K1 cells were grown in Ham's F-12 media (20% FBS). At a confluence of 80-90%, cells were treated with 1X trypsin-EDTA for 12 min at 37ºC in presence of 5% CO_2. Finally, trypsin was inactivated with 2 ml of respective media and 1 ml of the resulting suspension was used to inoculate 5 ml of fresh media.

4.5.5. Antiradical assays (O_2^- and DPPH radicals scavenging activity)

The capacity of Frankincense and Sacred EOs to scavenge O_2^- anions was measured using the xanthine/xanthine oxidase (X/XO) system (XTT color assay) based on Gerhäuser *et al.* (2003) with modification. A 20 µl sample previously diluted in ethanol was loaded in a 96-well microplate and completed to 200 µl with reactional mix (1 mmol/l XTT, 1 mmol/l EDTA, 1 mmol/l xanthine, 50 mmol/l sodium carbonate buffer (pH 10.2) and 3 mU/ml xanthine oxidase). The optical density (OD) was read at 490 nm after 20 min. Negative and positive controls consisted of ethanol and 30 U/ml of superoxide dismutase respectively. Scavenging activity was calculated as follows:

Scavenging Activity (%)= [(*sample OD* - *Negative control OD*) / (Positive control OD - *Negative control OD*)] x 100 (Equation 1)

The capacity of Frankincense and Sacred EOs to scavenge DPPH was based on the method of Blois (1958) and Kedare *et al.* (2011) with some modifications. Briefly, 1 ml of 40 µM DPPH previously dissolved in anhydrous ethanol was added to 250 µl of serial diluted EOs (also diluted in anhydrous ethanol). The solution was mixed and kept at room temperature for 1 hour producted from lights then, optical density was read at 517 nm. The blank consisted of 1.25 ml anhydrous ethanol whereas control consisted of 250 µl of anhydrous ethanol and 1 ml of DPPH solution. The inhibition percentage (IP) of free radicals was measured by the equation proposed by Megdiche-Ksouri *et al.* (2015):

IP (%) = ([*Control OD – Sample OD*] / *Control OD*) * 100 (Equation 2)

For both assays, concentrations that exhibited a scavenging activity of 50% (SC_{50} values) were determined.

4.5.6. NAD(P)H: QR assay

QR assay was based on methods from Prochaska *et al.* (1988a) and Talalay (1989) with some modifications. Hepa 1c1c7 cells were seeded at a density of 2 x 10^3 cells/well in a 96-well plate using complete MEM-EBSS media and were incubated at 37°C in a humidified incubator with 5% CO_2. Afterward, the media was removed using a multichannel micropipette and serial diluted samples were added, then the microplate was incubated for 48 h as mentioned above. Cells were washed with 200 µl HBSS solution and

50 µl of 1.6% (w/v) digitonin were added to each well followed by a 20 min incubation. Then, 20 µl of samples were removed using a multichannel micropipette and used for total protein quantification whereas 200 µl of complete reaction mixture (0.25 mol/l Tris-HCl pH 7, 4.67% (w/v) BSA, 0.01% Tween-20, 5 mol/l FAD, 1 mmol/l glucose-6-phosphate, 30 mol/l NADP, 34.8 µmol/l MTT, 50 µmol/l menadione and 2 mU/µl glucose-6-phosphate dehydrogenase) were added to each well then incubated at room temperature for 5 min. The microplate was read at 595 nm. A protein assay was conducted using Pierce®BCA reagents using the manufacturer's instruction. Controls consisted of emulsifying solution whereas media was used as blank. Specific activity of QR was defined as nmol of blue formazan formed per mg protein per minute. Fold induction of QR was calculated as follows:

QR Fold Induction (treated on control) = *Specific Activity of QR in Treated Group* / *Specific Activity of QR in Negative Control Group* (Equation 3)

4.5.7. Molecular weight determination by gel permeation chromatography (GPC)

Molecular weights (Mw) of insoluble glucan treated with Sacred EO was analyzed by gel permeation chromatography (GPC) (Agilent Technologies 1260 infinity series, Waldbronn, BW, Germany), equipped with a quaternary pump (Model G1311B), a manual injector with a sample loop of 20 µl and a refractive index detector (Model G1362A). Two identical PLgel 5 µm Mixed-D 300 X 7.5 mm columns were used in series and mobile phase consisted of 100% N-methylpyrrolidone (NMP) containing 5% (w/v) LiCl at a flow rate of 0.5 ml/min. Both columns and detector were set at 60ºC. Insoluble glucan (5 mg)

and EOs were suspended in 5 ml of emulsion as described in section 2.2. in a proportion of 5:1 for 48 h. Then, 2 ml of 100% NMP was added to obtain a relative concentration in insoluble glucan of 2.5 mg/ml and the suspension was stirred for 48 h at 60ºC, filtered through a nylon 0.2-µm filter and injected in the column. Polymethyl methacrylate (PMMA) was used as a standard and was prepared as indicated by the manufacturer. The equation obtained by plotting Mw with retention times of standards was used to calculate Mw of insoluble glucan. All extracts were injected in triplicate (n=3).

4.5.8. Antiproliferative assay

Antiproliferative properties were determined by the ability of the metabolic active cells to cleave the tetrazolium salt to purple formazan crystals based on Vistica *et al.* (1991). Different cell lines were seeded at 2 x 10^4 cells/well of media in a 96-well plate (200 µl/well) and were incubated for 24 h at 37°C in 5% CO_2. Spent media were removed using a multichannel micropipette and 100 µl of fresh media containing 10 µl of sample previously serial diluted was added and microplate was then incubated for 48 h as mentioned above. Afterward, samples were removed using a multichannel micropipette and replaced with 225 µl of fresh media containing 25 µl 0.5% (w/v) MTT followed by incubation for 4 h at 37°C in 5% CO_2. Finally, the media was carefully removed using a multichannel micropipette and replaced with 225 µl of DMSO containing 25 µl of Sorensen buffer containing 0.1 mol/l glycine and 0.1 mol/l NaCl at a pH of 10.5. The microplate was then read at 562 nm. The negative control and blank consisted of emulsifying solution and media respectively. Growth inhibition was calculated as follows:

Cellular Growth Inhibition (%) = 100 – (([*Sample OD*] / *Negative control OD*) x 100)

(Equation 4)

Equations obtained by plotting the linear portion of growth inhibition versus increasing concentrations of samples were used to calculate concentrations that inhibit 50% of cellular growth (IC_{50} values). For combined treatments, concentrations corresponding to IC_{50} values when tested separately were serial diluted and assayed.

4.5.9. Assessing interaction between EOs and insoluble glucan regarding antiproliferative and NAD(P)H: QR assays

The assessment of interactions in combined treatments regarding QR and antiproliferative assays differed due to the nature of measured effects. The determination of combined effects concerning antiproliferative assay was based on combination index (CI) as used by Hossain *et al.* (2016) with different upper and lower bounds suggested by Berenbaum (1977) following the equation:

$$CI = [Dx / IC_{50}x] + [Dy / IC_{50}y] \quad \text{(Equation 5)}$$

where *Dx* and *Dy* represent concentrations of components used in combination that reached IC_{50} values whereas $IC_{50}x$ and $IC_{50}y$ represents concentrations of components x and y that reached IC_{50} values when tested separately. Based on CI values, different combined effects can be classified: CI value < 1 was interpreted as a synergistic effect, a CI value equal to 1

was interpreted as an additive effect and a CI > 1 was interpreted as an antagonistic effect. Concerning the QR assay, concentrations that exhibited an induction of 1.5 when tested separately were used for combined treatment assays. Determination of the combined effect was based on fold induction and assessed as follows: Fold induction of 1.5 to 3.0 was interpreted as an additive effect, fold induction ≈ 1.5 as no interactive effect and fold induction <1.5 as an antagonistic effect. Fold induction calculated in combined treatments was obtained as described in equation 3.

4.5.10. Apoptosis assay

HT-29 cells were seeded in a 6-well plate at 3 x 10^5 cells/well (3 ml/well) and incubated as described in section 2.8. for 24 h. Then, cells were incubated for 48 h at 37°C in 5% CO_2 in the presence of 450, 900 and 1800 ppm of Frankincense or Sacred EOs in a final volume of 3 ml in order to surround IC_{50} values obtained for this cell line. Cells present in the supernatant were harvested by centrifugation at 500 x *g* for 10 min at 4°C. Adhered cells were treated with 1 ml of 1X trypsin-EDTA for 12 min at 37°C. Then, 2 ml of complete MEM/F12 medium was added and cells were harvested by centrifugation as described previously. The cell-containing pellets (from the supernatant and the adhered cells) were washed twice with PBS containing 0.25% EDTA to avoid clumping and the apoptosis evaluation was performed by using Annexin V-FITC and PI double staining assays. Harvested cells were diluted in 1X binding buffer at 10^6 cells/ml and Annexin V-FITC/PI staining was performed according to the manufacturer's instructions with a total of 10,000 events by flow cytometry (Coulter Epics XL-MCL, Beckman Coulter Canada, Inc., Mississauga, ON, Canada). 5-fluouracil (5-FU) was used as positive control and emulsifying solution was used as negative control.

4.5.11. Statistical analysis

All measurements were done in triplicate (n=3) and results are presented as average ± standard deviation. QR fold induction, IC_{50} values and percentage of apoptotic and necrotic cells were analyzed by one-way analysis of variance (ANOVA) using PASW statistics 18 software (IBM Corporation, Somers, NY, USA) and differences among treatments were analyzed with a post hoc Duncan's multiple-range test. Significance was considered at $P \leq 0.05$.

4.6. Results

4.6.1. Evaluation of the antiradical properties of EOs

Antiradical properties of EOs from *Boswellia spp.* were investigated via their capacity to scavenge O_2^- and DPPH radicals and the results are presented in Fig. 4.1 Sacred and Frankincense EOs demonstrated a dose-dependent response in antiradical activities and were found to scavenge 50% of O_2^- anion at 4300 and 34400 ppm respectively in addition to demonstrate a dose-dependent response (Fig. 4.1a). These results suggest a strong capacity of Sacred EO to scavenge O_2^- anion known to be involved in CRC carcinogenesis (Wang *et al.*, 2016). In contrast, both EOs were not able to scavenge 50% of DPPH radical despite their high concentration ranging from 33.59 to 34400 ppm. However, a dose-dependent response was also observed suggesting a weak capacity of Sacred EO (18% at 34400 ppm) to scavenge DPPH radical (Fig. 4.1b).

4.6.2. Effect of EOs in combination with insoluble glucan on the induction of NAD(P)H: QR and molecular weight

To determine the chemopreventive potential of Frankincense and Sacred EOs, their effects on QR activity was evaluated and results are shown in Table 4.1 Cells treated with emulsifying solution (control) showed a 0.77-fold induction which corresponded to a basal expression of QR in Hepa 1c1c7 cells. Moreover, Frankincense and Sacred EOs reached maximum fold inductions of 1.50 and 1.39 at a concentration of 27 and 54 ppm respectively. In addition, both EOs tested separately demonstrated a maximum fold induction significantly higher than control (0.77-fold induction) ($P \leq 0.05$). Those results demonstrated that Frankincense EO (27 ppm) is twice as efficient as Sacred EO (54 ppm) to induce QR. In order to increase the chemopreventive potential of Frankincense and Sacred EOs, they were combined with insoluble glucan of *S. boulardii* which has demonstrated an excellent chemopreventive potential against colorectal cancer evaluated *in vitro* and *in vivo (Fortin et al., 2017a, Fortin et al., 2017b)*. Concentrations in EOs and insoluble glucan that exhibited similar fold inductions were mixed to determine the combined effect of EO + insoluble glucan on QR activity and these results are also presented in Table 4.1 The combination of Sacred EO+ Frankincense EO showed a similar QR activity (1.39-fold induction) as compared to each EO tested separately, hence suggesting there was no interactive effect between two EOs. In contrast, the combination of Sacred EO + insoluble glucan revealed a QR activity (0.36-fold induction) which was significantly ($P \leq 0.05$) lower than of Sacred EO (1.39-fold induction) and insoluble glucan (1.52-fold induction at 250 ppm) alone, hence suggesting an antagonistic effect. The combination of Frankincense EO + insoluble glucan revealed a QR activity (1.99-fold induction) which was significantly ($P \leq 0.05$) higher than that of Frankincense EO (1.50-

fold induction) or insoluble glucan (1.52-fold induction) alone, hence suggesting an additive effect. These results demonstrated that insoluble glucan improves the capacity of Frankincense EO in inducing QR activity. The antagonistic effect of Sacred EO when combined with insoluble glucan on QR activity led to a hypothesis that Sacred EO might degrade insoluble glucan. The hypothesis was verified by comparing the molecular weight (Mw) of insoluble glucan before and after addition of Sacred EO. Results demonstrated that insoluble glucan possessed a Mw (1921±13 kDa) similar as if it was combined with Sacred EO (1904±297 kDa), hence suggesting that EOs did not affect the yeast cell wall extract.

4.6.3. Effect of EOs in combination with insoluble glucan on the cellular proliferation of different cell lines

The effect of Frankincense and Sacred EOs on the growth inhibition of human CRC HT-29 and Caco-2 cells as well as on non-cancerous Cho-K1cells were evaluated and the results are presented in Table 4.2 Regarding HT-29 cells, IC_{50} values of 1447 ppm and 1348 ppm were found for Frankincense and Sacred EOs respectively. Against Caco-2 cells, IC_{50} values of 1424 ppm and 1138 ppm were found for Frankincense and sacred EOs respectively. Concerning the non-cancerous CHO-K1 cells, Frankincense EO exhibited an IC_{50} value of 1689 ppm whereas Sacred EO showed no effect on this non-cancerous cell line at tested concentrations ranging from 21.5 to 2752 ppm. The results hence suggest that Sacred EO exhibited cancerous cell-specific cytotoxicity. In addition, no significant difference between IC_{50} values of Frankincense and Sacred EOs tested separately were observed regarding HT-29 and Caco-2 cell lines ($P > 0.05$).

In order to increase the chemopreventive potential of Frankincense and Sacred EOs, these EOs and insoluble glucans were used in combination to evaluate their effect on the growth of different cell lines. Table 4.2 indicates that the combination of Frankincense and Sacred EOs against HT-29 cells exhibited a combination index (CI) of 1.05 and an IC_{50} value in total EOs of 1465 ppm, thus suggesting an additive effect. In contrast, Sacred and Frankincense EOs in combination with insoluble glucan (which showed an IC_{50} value of 108 ppm when tested separately) presented a CI values of 2.61 and 1.75 respectively. However, combined treatments revealed that IC_{50} values of insoluble glucan (93 ppm for both EOs) and EOs (1279 and 1282 ppm respectively) were not significantly different from IC_{50} values obtained when tested separately ($P > 0.05$). Those results suggest that EOs combined with insoluble glucan generated antagonistic effects against HT-29 cells.

In case of the Caco-2 cells, combination of Frankincense and Sacred EOs exhibited a CI value of 0.82 and an IC_{50} value in total EOs of 1032 ppm, hence suggesting an additive effect which is congruent with results observed with HT-29 cells. However, Sacred and Frankincense EOs in combination with insoluble glucan (which showed an IC_{50} value of 634 ppm when tested separately) presented CI values of 0.97 and 1.06 respectively. However, Sacred and Frankincense EOs in combination with insoluble glucan showed that IC_{50} values of insoluble glucan (307 and 337 ppm respectively) and EOs (551 and 756 ppm respectively) are lower than IC_{50} values obtained when tested separately. Those results suggest that EOs combined with insoluble glucan demonstrated additive effects against Caco-2 cells.

Table 4.2 also indicates that the combination of Frankincense and Sacred EOs against CHO-K1 cells exhibited an IC_{50} value in total EOs of 704 ppm, hence suggesting a

synergistic effect. Moreover, Sacred and Frankincense EOs in combination with insoluble glucan revealed IC_{50} values of insoluble glucan (611 and 796 ppm respectively) and EOs (561 and 448 ppm respectively), hence suggesting a synergistic effect since insoluble glucan and Sacred EOs assayed separately showed no IC_{50} values against CHO-K1 at tested concentrations ranging from 21.5 to 2752 ppm. These results suggest that CHO-K1 cells revealed to be highly sensitive to a combination of insoluble glucan and EOs, possibly due to an important cytotoxic effect on non-cancerous cells and the loss of cancerous cell-specific cytotoxicity.

4.6.4. Effect of essential oils on the level of apoptosis in human colorectal cancer cells

Since a cancerous cell-specific cytotoxicity was observed with Sacred EO tested separately, Annexin V-FITC/PI double staining was performed to determine was apoptosis is involved in the growth inhibition of cancerous cells by EOs. HT-29 cells were chosen for this test since IC_{50} values of Sacred and Frankincense EOs were not significantly ($P > 0.05$) different against HT-29 and Caco-2 cell lines (Table 4.2). Further, the effect of Frankincense and Sacred EOs on apoptosis induction in HT-29 cells has never been investigated previously. Data shown in Fig. 4.2a demonstrated that at the tested concentrations (450, 900 and 1800 ppm), neither Sacred EO (percentage of apoptotic cells of 2.38%, 2.96% and 3.90%, respectively) nor Frankincense EO (percentage of apoptotic cells of 4.70%, 4.06% and 4.34%, respectively) were able to significantly ($P > 0.05$) induce apoptosis (Annexin $V^+ PI^-$ cells) in HT-29 cells at concentrations surrounding IC_{50} values compared to the negative control (percentage of apoptotic cells of 2.61%). In contrast, data

shown in Fig. 4.2b demonstrated that at the tested concentrations (450, 900 and 1800 ppm), Sacred EO (percentage of apoptotic cells of 18.43%, 25.02% and 24.76%, respectively) and Frankincense EO (percentage of apoptotic cells of 24.11%, 22.62% and 26.17%, respectively) were able to significantly ($P \leq 0.05$) increase the percentage of necrotic cells as compared to negative control (percentage of necrosis cells of 7.57%) which corresponds to annexin $V^+ PI^+$ cells. Those results suggests that Frankincense and Sacred EOs induced cytotoxicity in HT-29 cells via necrosis rather than apoptosis, based on the analysis of externalization of phosphatidylserine on the surface of the cell membrane using Annexin V-FITC and PI double staining.

4.7. Discussion

4.7.1. Evaluation of the antiradical properties of EOs

Antiradical assays revealed that Sacred EO was more efficient than Frankincense EO to scavenge O_2^- anion. This fact underlies the differences between biological activities between them even if both EOs were ineffective to scavenge DPPH radical. Many studies reported the weak capacity of EOs obtained from *B. sacra* and *B. carterii* to scavenge DPPH radical, which is in congruence with our results (Ali *et al.*, 2013, Mohamed *et al.*, 2015, Wang *et al.*, 2008). Indeed, Wang *et al.* (2008) found that *B. carterii* EO reached approximately 12% scavenging activity at 10000 ppm whereas Ali *et al.* (2013) found that *B. sacra* EO reached 8% scavenging activity at 1000 ppm. Moreover, Mohamed *et al.* (2015) reported that an EO from *B. carterii* scavenged 50% of DPPH radical at 15210 ppm (15.21 mg/ml), which is at very high concentration. In addition, Al-Harrasi *et al.* (2013) reported that an EO from *B. sacra* exhibited a greater capacity to scavenge O_2^- anion (56.40

%) as compared to DPPH radical (16.30 %) at tested concentrations, similarly to results obtained in the present study. This difference in capacity to scavenge O_2^- anion more efficiently than DPPH radical can be explained by the lower reactivity of DPPH radical compared to reactive oxygen species (ROS) such as O_2^- anion (Dizhbite *et al.*, 2004). Likewise, it is generally accepted that ROS, especially the O_2^- anion, are the most important free radicals in many diseases including cancer (Nishikawa, 2008, Young *et al.*, 2001). Such O_2^- anion scavenging activity of Sacred EO may be due to its high content of terpenes as compared to Frankincense EO, such as α-pinene (Woolley *et al.*, 2012), as reported by Singh *et al.* (2009).

Although, Sacred and Frankincense EOs were harvested in two geographical regions, may vary according to soil composition, climate and season (Bakkali *et al.*, 2008). Al-Saidi *et al.* (2012) obtained four EOs from *B. sacra* harvested in different geographical regions in Oman. Despite the fact that α-pinene was the main compound in all EOs, the authors observed important variations in their amounts ranging from 46.8 to 76 %. Moreover, their results suggested that EOs exhibited different MIC values against gram-negative bacteria which meant that the geographical location of harvesting may influence the biological activities of EOs from *Boswellia spp*.

4.7.2. Effect of EOs in combination with insoluble glucan on the induction of NAD(P)H: QR and Mw determination

The QR assays results showed that Sacred and Frankincense EOs were able to induce QR activity at low concentrations (54 and 26 ppm respectively). An increase of the QR specific activity by *Boswellia* spp. EOs has never been reported, hence revealing a novel

chemopreventive property of these EOs. Thus, mechanisms by which EOs from *Boswellia spp.* induce QR are worthy further discussion. The increase of gene transcription coding for phase II enzymes depends on the destabilization of Keap1/Nrf cytoplasmic complex which triggers antioxidant response element (ARE) release. This destabilization is related to an α,β-unsaturated ketone moiety of an inducer reacting with the cysteine thiol of Keap1 (Dinkova-Kostova *et al.*, 2002). Unsaturated ketones (enones) are known to be present in EOs from *Boswellia spp*. Indeed, Niebler *et al.* (2016) detected traces of rotundone and mustakone, two sesquiterpene ketones (aromatic enones), in *B. sacra* EO. Moreover, EOs from *Boswellia spp.* contain keto-β-boswellic acid (K-BA) and acetyl-keto-β-boswellic acid (AK-BA) which also possess aromatic enone functional groups (Suhail *et al.*, 2011). Thus, such molecules in Frankincense and Sacred EOs might explain the increase of QR activity observed in the present study especially since other organic acids were proven to be QR inducers such as fumaric acid derivatives and coussaric acid A (Kang *et al.*, 2004, Spencer *et al.*, 1990).

In order to increase the chemopreventive potential of Frankincense and Sacred EOs, they were combined with insoluble glucan of *S. boulardii's* cell wall. In previous studies, this insoluble glucan showed the most relevant chemopreventive properties *in vitro* and *in vivo* (Fortin *et al.*, 2017a, Fortin *et al.*, 2017b). The combination of yeast cell wall extract and *Boswellia* EOs as potential chemopreventive agents has never been tested before. Our results showed that insoluble glucan enhanced QR activity induced by Frankincense EO whereas a similar was not observed for Sacred EO. The degradation of β-glucan by Sacred EO was found not to be responsible for the observed differences between EOs since the Mw of insoluble glucan was similar despite mixing with Sacred EO. Moreover, many

studies demonstrated that EOs may be encapsulated in polysaccharide-based gels without negatively affecting their properties (Ahmed *et al.*, 2016, Anchisi *et al.*, 2006, Beyki *et al.*, 2014), which rejects the hypothesis of glucan degradation. However, divergence of combinatory effect between EOs regarding the QR assay may be due to an increased sensitivity of Hepa 1c1c7 cells toward Sacred EO leading to a weak induction. Thus, future studies on this aspect are necessary.

4.7.3. Effect of EOs in combination with insoluble glucan on the cellular proliferation of different cell lines

Many studies demonstrated that EOs from *Boswellia spp.* exhibit cytotoxic effects (antiproliferative activity) toward different cancerous cell lines. Suhail *et al.* (2011) demonstrated that an EO from *B. sacra* exhibited IC_{50} values varying from 1:1680 (1264 ppm) to 1:1800 (477 ppm) toward human breast cancer cells. Moreover, Ni *et al.* (2012) obtained 4 EO fractions from *B. sacra* exhibiting IC_{50} values varying from 1:270 (3185 ppm) to 1:1560 (551 ppm) toward human pancreatic cancer cells. Finally, Dozmorov *et al.* (2014) demonstrated that an EO from *B. carterii* exhibited an IC_{50} value of 1:1250 (688 ppm) toward human bladder cancer cells whereas Frank *et al.* (2009) obtained an IC_{50} value of 1:600 (1433 ppm) against the same cell line. These investigations confirm the congruence of IC_{50} values obtained in the present study with the scientific literature.

It is largely accepted that *B. carterii* is merely a synonym for *B. sacra* (Woolley *et al.*, 2012). However, Woolley *et al.* (2012) observed significant differences in the composition of EOs obtained from these plants. The authors reported that EOs from *B. sacra* differed from EOs from *B. carterii* on higher optical rotation values (+30.1 and -13.3° respectively),

enantiomeric ratios values and α-pinene content (79.0 and 48.2% respectively), which confirmed that both species are distinct. Terpenes contained in EOs from *Boswellia spp.* are known to influence cancerous cell-specific cytotoxicity. Indeed, Suhail *et al.* (2011) extracted 2 EOs from *B. sacra* and observed that EOs with higher boswellic acid (BA) content exhibited higher cancerous cell-specific cytotoxicity in breast cancer cells. Hakkim *et al.* (2015) extracted an EO from *B. sacra* containing a high concentration of α-pinene (62%) and α-amyrin (21%) which exhibited a more efficient antiproliferative effect on a human breast cancer cell line as compared to a similar EO containing less α-amyrin, hence suggesting a combined effect of terpenes. Moreover, enantiomeric ratios can influence biological activities of EOs. Indeed, Rivas da Silva *et al.* (2012) demonstrated that α and β-(+)-pinenes, which are the most abundant terpenes in EOs from *Boswellia spp.*, exhibited minimal inhibitory concentration (MIC) values against *Candida albicans*, *Cryptococcus neoformans*, *Rhizopus oryzae* and Methicillin-resistant *Staphylococcus aureus* whereas α and β-(-)-pinenes showed no effect at tested concentrations. Those investigations might explain the divergence in biological activities between EOs observed in the present study notably regarding cancerous cells specific cytotoxicity of Sacred EO.

Combined treatments demonstrated that the effect of EOs and insoluble glucan had different effects on the viability of Caco-2 and HT-29 cell lines whereas no difference ($P > 0.05$) was observed when tested separately. Since no study has investigated the combined effect of EOs and yeast cell wall extracts on cell viability, it may be hypothesized that combining these compounds might create different chemical species that acted differently on cell lines. Since it was confirmed that EOs did not affect the Mw of insoluble glucan, further investigation will be necessary to understand such an effect. Finally, results showed

2785 that all combinations exhibited IC_{50} values toward CHO-K1 cells, hence suggesting that
2786 combinations affected the growth of non-cancerous cells since insoluble glucan and Sacred
2787 EO exhibited cancerous cell-specific cytotoxicity when tested separately. Such synergistic
2788 effects in CHO-K1 cells might be due to multiple mechanisms triggered by combined
2789 treatments in non-cancerous cells, which suggests important side effects of these
2790 combinations.

2791

2792 ### 4.7.4. Effect of EOs on the level of apoptosis in human colorectal cancer
2793 cells

2794 Cytotoxic activities of EOs from *Boswellia spp.* are known to be mainly due to pro-
2795 apoptotic properties (Dozmorov *et al.*, 2014, Frank *et al.*, 2009, Ni *et al.*, 2012, Suhail *et*
2796 *al.*, 2011). However, it is interesting that no apoptotic activity was detected in the present
2797 study. Such divergence with the scientific literature may be explained by the fact that no
2798 study investigated the apoptosis activities of whole *Boswellia* EOs (which contains many
2799 bioactive compounds) on HT-29 cells. However, Liu *et al.* (2002) demonstrated that
2800 boswellic acid (BA), keto-β-boswellic acid (K-BA) and acetyl-keto-β-boswellic acid (AK-
2801 BA) induced apoptosis in a dose-dependent manner in HT-29 cells, hence suggesting that
2802 Frankincense and Sacred EOs used in the present study did not contain sufficient amounts
2803 of those specific triterpenes to induce apoptosis in HT-29 cells. Indeed, BA and its
2804 derivatives have been frequently reported to correlate with apoptotic activity depending on
2805 cell lines and concentrations used. Suhail *et al.* (2011) measured BA content of two EOs
2806 from *B. sacra* obtained at different temperatures and investigated their apoptotic activity
2807 in human breast cancer cells. Extracts obtained at 100°C exhibited the highest content in

BA (30.1 mg/ml) and showed the most relevant results regarding DNA fragmentation, caspase activation and cell cycle arrest as compared to EOs obtained at 78°C (19.6 mg/ml boswellic acid). Moreover, Ni *et al.* (2012) obtained 4 EO fractions from *B. sacra* gum resins and reported that fractions (III and IV) containing high content in BA exhibited apoptosis activity in four different human pancreatic cancer cells. The authors also noticed that expression patterns in time function of pAkt, cdk4 and cyclin D1 proteins differ among those four human pancreatic cancer cell lines using cell cycle arrest assay upon treatment with fractions III and IV. Those studies revealed that induction of apoptosis by EOs from *Boswellia spp.* may vary upon terpene composition and cancerous cell lines.

4.8. Conclusion

This study confirmed that EOs from *B. sacra* and *B. carterii* exhibited different chemopreventive, antiradical and antiproliferative properties toward CRC. This study also reported for the first time that Frankincense and Sacred EOs could induce QR activity which is one of the important mechanisms in cancer chemoprevention. Frankincense EO was more effective than Sacred EO in increasing QR specific activity and combining Frankincense EO with insoluble glucan obtained from cell walls of *S. boulardii* enhanced the capacity of this EO to increase QR specific activity. Moreover, Sacred EO efficiently scavenged O_2^- anion and demonstrated cancerous cell-specific cytotoxicity as opposed to Frankincense EO. Also, additive antiproliferative effects were observed by combining Frankincense and Sacred EOs toward human colorectal cancer cells and by combining EOs with insoluble glucans toward Caco-2 cells. It is worth noting that even Sacred EO or insoluble glucan alone expressed the cancerous cell-specific cytotoxicity effects, their

combinations were found to be more efficient in affecting the viability of CHO-K1. Finally, based on the Annexin V-FITC/PI double staining assay, the induction of apoptosis in colorectal cancer cells might not be responsible for the cell growth inhibitory effect observed by both EOs.

4.9. Acknowledgements

We acknowledge Young Living Essential Oils for kindly providing Frankincense and Sacred EOs. We also acknowledge Young Living Essential Oils, the Fondation Armand-Frappier and the Ministère de l'Économie, de l'Innovation et de l'Exportation du Québec (MEIE) for their financial contribution to this project. Olivier Fortin is a scholarship recipient of the Fondation Armand-Frappier.

Conflict of Interest

The authors have no conflict of interest to declare.

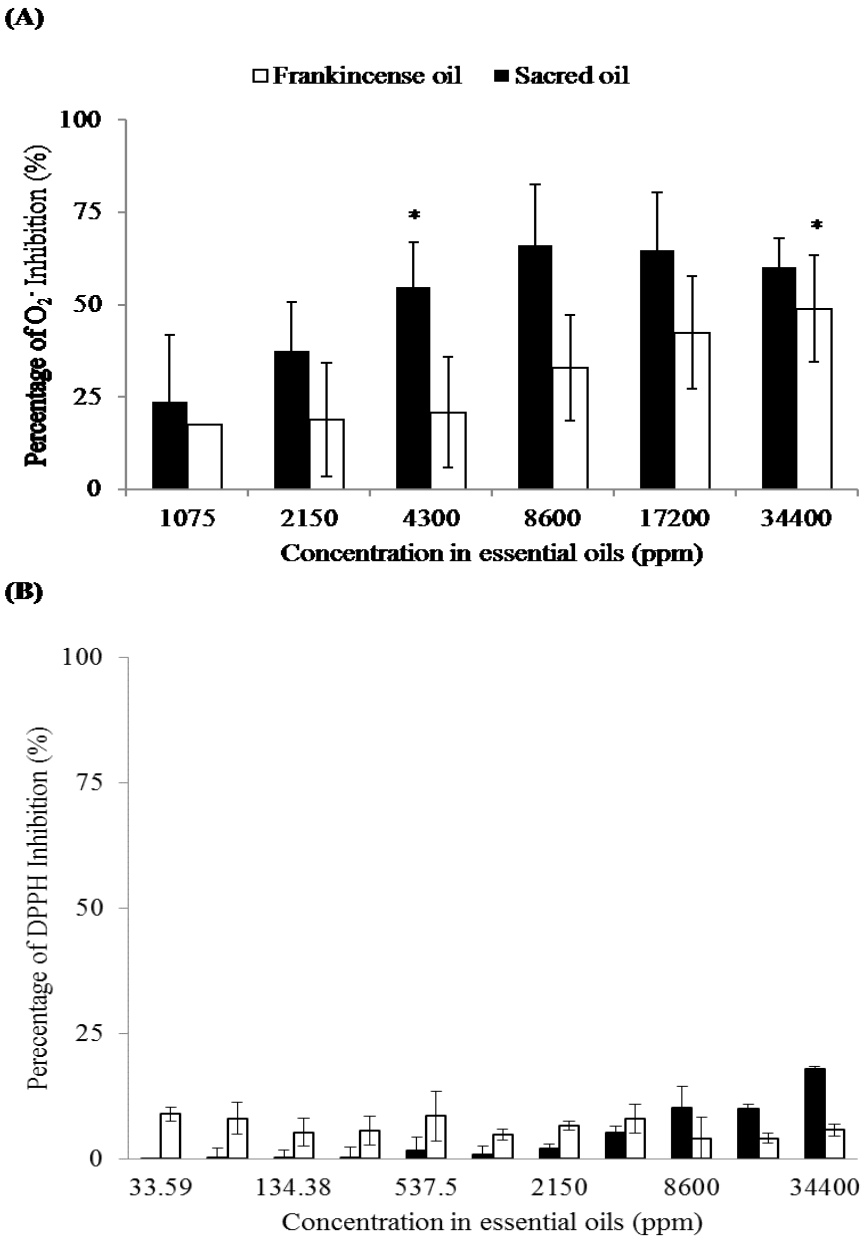


Figure 4.1: Effect of increasing concentration of Frankincense and Sacred EOs on the percentage of **(A)** O_2^- and **(B)** DPPH radical inhibition using xanthine/xanthine oxidase (X/XO) and DPPH methods respectively. Asterisk (*) indicates the concentration that scavenged 50% of radicals formed (SC_{50} value) above respective EO. Error bars represent the standard deviation of the mean of at least 3 independent experiments.

Table 4.1: Effect of EOs used separately and in combination with insoluble glucan on the induction of QR

Samples	Concentration (ppm)		Fold Induction	Effect
	Insoluble glucan	Total EOs		
Control	NA	NA	0.77±0.32[b]	NA
Sacred EO	NA	54	1.39±0.11[c]	NA
Frankincense EO	NA	27	1.50±0.15[c]	NA
Insoluble glucan	250	NA	1.52±0.22[c]	NA
Frankincense EO + Sacred EO †	NA	81	1.39±0.23[c]	I
Insoluble glucan + Sacred EO	250	54	0.36±0.24[a]	A
Insoluble glucan + Frankincense EO	250	27	1.99±0.52[d]	AD

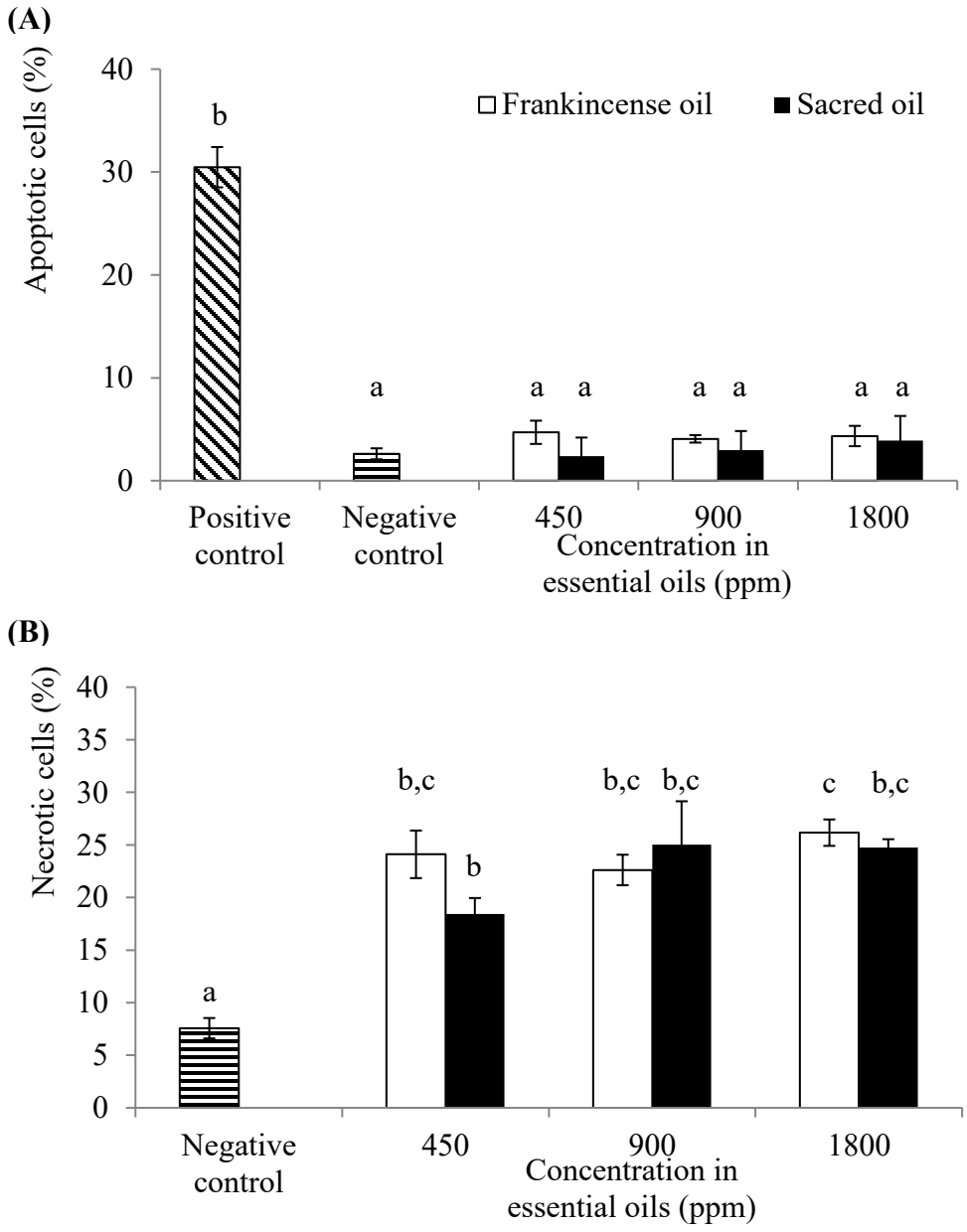
For essential oils (EOs) tested separately, tested concentrations ranged from 3 to 1720 ppm. † 27 ppm of Frankincense and 54 ppm of Sacred EOs were combined. Concentrations for combined treatments were chosen based on the highest induction of each compounds tested separately. Additive effect (AD): 1.5 <Fold induction >3.0. No interactive effect (I): Fold induction ≈ 1.5. Antagonistic effect (A): 1.5 >Fold induction. NA: Not applicable. Means followed by different letters are significantly different ($p \leq 0.05$). Results are presented as average ± standard deviation of at least 3 independent experiments.

Table 4.2: Effect of EOs used separately and in combination with insoluble glucan on the cellular proliferation of different cell lines

Samples	IC_{50} (ppm)											
	HT-29				Caco-2				CHO-K1			
	Insoluble glucan	Total EOs	CI	CE	Insoluble glucan	Total EOs	CI	CE	Insoluble glucan ‡	Total EOs ‡	CI	CE
Sacred EO	NA	1348 ± 107^{B}	NA	NA	NA	1138 ± 57^{B}	NA	NA	NA	NR	NA	NA
Frankincense EO	NA	1447 ± 86^{B}	NA	NA	NA	$1424\pm206^{A,B}$	NA	NA	NA	1689 ± 22^{B}	NA	NA
Insoluble glucan	108 ± 33^{a}	NA	NA	NA	$634\pm242^{a,b,c}$	NA	NA	NA	NR	NA	NA	NA
Frankincense EO + Sacred EO	NA	$1465\pm276^{A,B}$	1.05	AD	NA	$1032\pm8^{A,B}$	0.82	AD	NA	704 ± 108^{A}	CI<1.0	S
Insoluble glucan + Sacred EO	93 ± 6^{a}	1279 ± 87^{B}	2.61	A	307 ± 36^{b}	551 ± 65^{A}	0.97	AD	611 ± 49^{c}	561 ± 45^{A}	CI<1.0	S
Insoluble glucan + Frankincense EO	93 ± 6^{a}	1282 ± 76^{B}	1.75	A	$337\pm54^{a,b}$	756 ± 122^{A}	1.06	AD	$796\pm279^{a,b,c}$	448 ± 157^{A}	CI<1.0	S

EOs: Essential oils. IC_{50}: Concentration that inhibits 50% of the cellular growth. CI: Combination Index. CE: Combinatory effect. NR: Not reached. NA: Not applicable. Concentrations were chosen according with IC_{50} values of each component tested separately. ‡: The highest concentration of insoluble glucan and Sacred EO were selected for combination treatments against CHO-K1 cells since no IC_{50} values were observed when tested separately. Additive effect (AD): CI ≈ 1.0. Antagonistic effect (A): CI > 1.0. Synergistic effect (S): CI < 1.0. Results are presented as average ± standard deviation of at least 3 independent experiments. IC_{50} values of insoluble glucan bearing different

2874 lowercase letters are significantly different ($p \leq 0.05$). IC_{50} values of total EOs bearing
2875 different uppercase letters are significantly different ($p \leq 0.05$).



2876

2877 **Figure 4.2:** Effect of increasing concentration of Frankincense and Sacred EOs
2878 surrounding IC_{50} values on the percentage of **(A)** apoptotic cells (Annexin V^+ PI^- cells) and
2879 **(B)** necrotic cells (Annexin V^+ PI^+ cells) in human colorectal cancer HT-29 cells
2880 determined by flow cytometry using Annexin V-FITC and PI double staining method after
2881 48 h of treatment. Positive control (PC) and Negative control (NC) consisted in cells treated
2882 with 500 µg/ml of 5-fluorouracil and emulsifying solution respectively. Error bars

represent the standard deviation of the mean of at least 3 independent experiments. Means followed by different letters are significantly different ($p \leq 0.05$).

CHAPITRE 5 – DISCUSSION ET CONCLUSION GÉNÉRALES

5.1. Discussion générale

L'objectif global de cette étude était de démontrer le potentiel chimiopréventif et anticancer d'extraits de parois cellulaires de levures envers le CCR. Selon l'hypothèse de départ, les β-glucanes et les mannoprotéines contribuent significativement à l'activité biologique des parois de levures. Le premier sous-objectif fût de sélectionner une méthode d'extraction permettant d'obtenir des extraits ayant une activité biologique intéressante. La séparation des différents polymères retrouvés dans les parois cellulaires de levures est simple et peu couteuse. Par contre, obtenir un extrait d'une grande pureté (>60%) peut s'avérer couteux et difficile pour le secteur industriel (Kwiatkowski *et al.*, 2012). En ce sens, l'extraction des β-glucanes et des mannoprotéines à partir des parois cellulaires de levures est normalement réalisée via des méthodes alcalines, acides, alcooliques, enzymatiques ou à partir de différentes combinaisons de ces dernières (Ahmad *et al.*, 2012). Des tests préliminaires ont montré qu'une méthode d'extraction dite crue était en mesure d'induire la QR de manière plus efficace qu'une méthode d'extraction dite fine démontrant que le respect de l'intégrité des parois cellulaires est un facteur important à prendre en compte dans l'activité biologique. Alors que la méthode d'extraction fine était composée d'un grand nombre d'étapes de purification (deux traitements alcalins, deux traitements acides, une précipitation à l'alcool, deux traitements enzymatiques et l'élimination des β-glucanes de haut poids moléculaire), la méthode d'extraction crue était composée d'un traitement alcalin et d'une précipitation à l'alcool ce qui affectait probablement moins l'architecture des parois et de ses composantes (par exemple, l'hélice triple des β-glucanes). De plus, l'obtention des extraits des parois cellulaires de levures nécessite une étape de destruction cellulaire qui peut être réalisée par autolyse, cisaillement à billes, sonication ou microfluidisation (Geciova *et al.*, 2002). Encore une fois, des tests préliminaires ont montré que l'autolyse permettait d'obtenir des extraits pouvant induire la QR plus efficacement que les méthodes de cisaillement à billes ou de microfluidisation alors que la sonication n'a pas été en mesure de briser les parois cellulaires. Mentionnons que la suspension cellulaire non diluée et obtenue après autolyse fût inoculée sur pétri pour confirmer la perte de viabilité des levures par autolyse. En ce sens, la méthode d'extraction crue précédée d'une étape d'autolyse s'est avérée plus adaptée pour une application potentielle à l'industrie en plus de démontrer une meilleure induction de la QR lors de tests préliminaires. Suite à la sélection d'une méthode d'extraction, il a été confirmé que la biomasse de levures fût collectée en phase stationnaire dans l'optique d'imiter l'état cellulaire des levures après leurs utilisations en milieu industriel (par exemple, industrie brassicole). Cette étape s'est avérée cruciale dans le but de comparer l'activité biologique des

espèces de levures étudiées puisque la phase de croissance influence grandement la composition des parois cellulaires et donc leurs activités biologiques.

L'extraction alcaline a résulté en l'obtention de trois extraits de parois cellulaires tel que présenté dans le chapitre 2. Il est connu que l'extrait insoluble contient majoritairement du (1→3)-β-D-glucanes à fort poids moléculaire et de la chitine alors que l'extrait soluble est connu pour être majoritairement constitué de (1→3)-β-D-glucanes avec ramifications (1→6)-β-D-glucanes. L'extrait de mannoprotéines serait quant à lui constitué de protéines couplées aux mannanes avec présence de β-glucanes en raison de l'absence d'étape de purification subséquente (Kwiatkowski *et al.*, 2012, Mantovani *et al.*, 2008). Puisque la solubilité des extraits est non seulement fonction du type de β-D-glucanes mais aussi de différents paramètres chimiques (degré de ramification et de polymérisation), il serait pertinent d'investiguer la nature chimique des β-D-glucanes obtenues.

L'étude du potentiel chimiopréventif et anticancer des extraits de parois cellulaires de levures a été réalisée en modèle *in vitro* et *in vivo* dans le but de déterminer les meilleurs extraits de β-glucanes et de mannoprotéines tel que présenté aux chapitres 2 et 3. L'extrait insoluble obtenu de la paroi cellulaire de *S. boulardii* s'est avéré être le meilleur extrait de par sa capacité à inhiber efficacement les cellules humaines du cancer colorectal, à augmenter l'activité spécifique de la quinone réductase *in vitro* ainsi qu'à diminuer le nombre de foyers de cryptes aberrantes chez le rat via la diminution de l'activité de la β-glucuronidase au niveau du caecum et l'augmentation de l'activité spécifique de la QR au niveau du foie. La caractérisation des extraits a montré qu'une forte teneur en β-glucanes, des ratios élevés en β-glucanes/sucre totaux ainsi que des ratios faibles en chitine/β-glucanes ont un impact important sur la mise en valeur de leur potentiel chimiopréventif.

Ces résultats confirment le caractère probiotique de *S. boulardii* (Czerucka *et al.*, 2007, Kelesidis *et al.*, 2012). De plus, il semble que les β-glucanes constituent les principales composantes polysaccharidiques responsables de l'activité biologique observée tant *in vitro* que *in vivo* ce qui est conforme à plusieurs autres études (Aguilar *et al.*, 2012, Bohn *et al.*, 1995, Chan *et al.*, 2009, Falch *et al.*, 2000, Luhm *et al.*, 2006, Rand *et al.*, 2010, Stier *et al.*, 2014). La structure des β-glucanes est connue pour avoir un impact majeur sur son activité immunomodulatoire et anticancer. En effet, l'augmentation du poids moléculaire des β-glucanes est proportionnelle avec un fort degré de polymérisation (DP) et un fort degré de ramification (Mantovani *et al.*, 2008). Sachant que les β-glucanes insolubles de *S. boulardii* présentent un fort poids moléculaire, il est possible d'avancer que les β-glucanes contenus dans cet extrait possèdent un DP supérieur à 100 en plus d'être constitués d'une structure (1→3)-β-D-glucanes avec un degré de ramification modéré en (1→6)-β-D-glucanes. Ces dernières caractéristiques chimiques ont été rapportées comme étant

déterminantes dans l'activité biologique des β-glucanes (Zekovic *et al.*, 2005). De plus, des β-glucanes à fort poids moléculaire sont connus pour avoir une meilleure affinité pour le récepteur dectin-1 à la surface des macrophages permettant ainsi l'augmentation de l'infiltration des cellules T activées dans l'environnement tumoral en plus de favoriser l'activité tumoricide des cellules tueuses naturelles (Adams *et al.*, 2008). Il est possible qu'un tel mécanisme ait contribué en partie à l'excellent potentiel chimiopréventif des β-glucanes insolubles en modèle *in vivo*. De plus, il est possible que les β-glucanes aient adopté une structure en hélice triple. En ce sens, le schizophyllan, un homopolysaccharide de glucose de source fongique, possède une telle structure et démontre des capacités anticancer et antitumorales. Cependant, un fort traitement alcalin induit une structure en hélice simple et réduit l'activité biologique du schizophyllan démontrant l'importance de la structure triple des β-glucanes (Ooi *et al.*, 2000, Ren *et al.*, 2012). Zhang *et al.* (2005) ont proposé que l'effet d'une telle structure cause une conformation rigide de la chaîne des β-glucanes leur conférant de meilleures propriétés à inhiber la croissance de cellules cancéreuses (Zhang *et al.*, 2005).

La quantification des composantes polysaccharidiques a permis de démontrer que les extraits ne contenaient pas seulement des polysaccharides. Puisque cette étude se concentrait sur les sucres des parois cellulaires, les autres composantes n'ont pas été quantifiées. Or, les proportions relatives entrent les polysaccharides et les protéines ont été plusieurs fois rapportées comme étant un facteur important dans l'explication de propriétés anticancer des parois cellulaires (Moharib *et al.*, 2014, Wang *et al.*, 1995). Les mécanismes *in vitro* et *in vivo* aboutissant à une diminution des masses tumorales et à une inhibition de croissance de cellules cancéreuses induite par des composés polysaccharidiques purifiés diffèrent de ceux des complexes polysaccharide-protéines (CPP). Typiquement, les polysaccharides purifiés tendent à agir sur l'activation des macrophages et les cellules T alors que les CPP ne tendent pas à induire des effets immunomodulatoires (Ooi *et al.*, 2000). Une faible activité immunomodulatrice des CPP pourrait être en partie responsable de l'absence de potentiel chimiopréventif *in vivo* de l'extrait de mannoprotéines de *S. boulardii* en comparaison à sa forte activité biologique observée en modèle *in vitro*.

L'induction de la quinone réductase par des extraits de parois cellulaires de levures est une découverte réalisée au cours de cette étude (chapitre 2 et 3). Le mécanisme d'induction de cette enzyme dépend de la présence d'un inducteur mono ou bifonctionnel. Alors que les inducteurs monofonctionnels augmentent l'activité enzymatique spécifiquement des enzymes de phase II telle que la quinone réductase, les inducteurs bifonctionnels augmentent l'activité des enzymes de phases II et de phases I (exemples, cytochromes P-450). Les inducteurs monofonctionnels pénètrent

dans le milieu intracellulaire où ils génèrent un signal électrophilique stimulant la transcription de gènes codant pour les enzymes de phase II seulement. En contrepartie, les inducteurs bifonctionnels se lient aux récepteurs d'hydrocarbone aryle ce qui active ensuite les gènes codants pour les enzymes de phase I. La forte activité des récepteurs d'hydrocarbone aryle catalyse la métabolisation de l'inducteur bifonctionnel en composé analogue aux propriétés électrophiliques expliquant alors l'activation des gènes codants aussi pour les enzymes de phase II (Prochaska *et al.*, 1988b). Des études antérieures ont montré que les β-glucanes de source fongique présentaient la capacité d'inhiber les isoenzymes de phase I de la famille du cytochrome P-450 infirmant leur nature bifonctionnelle (Hashimoto *et al.*, 2002, Okamoto *et al.*, 2004). En ce sens, il est possible que les β-glucanes insolubles obtenus dans le cadre de cette présente étude consistent en un inducteur monofonctionnel. Par contre, des analyses subséquentes sont de mises pour confirmer cette hypothèse.

Un second point saillant de cette étude concerne la capacité des β-glucanes insolubles de *S. boulardii* à diminuer le nombre de cryptes aberrantes chez des rats traités au 1,2-dimethylhydrazine (DMH). Il a été déterminé que l'induction de la quinone réductase et la diminution de l'activité de la β-glucuronidase sont les mécanismes sous-jacents à la diminution du nombre de cryptes aberrantes. Ce faisant, un tel patron d'activité enzymatique aurait pu réduire le temps de transit intestinal des carcinogènes ce qui limite la carcinogenèse du CCR (Chapitre 4). Alors que le mécanisme d'induction de la quinone réductase au niveau du foie des rats traités par aux β-glucanes insolubles de *S. boulardii* mérite d'être investigué dans des études supplémentaires, la diminution de l'activité enzymatique de la β-glucuronidase dans le contenu caecal est probablement due aux propriétés prébiotiques de cet extrait. Tel que suggéré dans le chapitre 3, cet extrait aurait pu agir négativement sur la croissance des bactéries excrétant cette enzyme fécale ou agir positivement sur d'autres bactéries bénéfiques (*Bifidobacteria* et *Lactobacilli*) créant un phénomène d'exclusion compétitive. D'ailleurs, il a déjà été rapporté que les (1→3)-β-D-glucanes administrés oralement ne sont pas digérables chez les rats (Chan *et al.*, 2009). Or, il est connu que les β-glucanes insolubles sont majoritairement constitués de (1→3)-β-D-glucanes (Mantovani *et al.*, 2008), suggérant une modification de la composition du microbiote intestinal des rats. De plus, la composition du microbiote peut aussi être modifiée par l'accumulation d'acide gras à courte chaîne (AGCC) par certaines bactéries pouvant être provoqué par la capacité des β-glucanes insolubles de *S. boulardii* à agir comme prébiotique. En effet, Louis *et al.* (2014) ont discuté du fait que les carbohydrates non-digestibles, tels que les β-glucanes, peuvent agir sur la production microbienne d'AGCC

favorisant une modulation du microbiote. En ce sens, ce mécanisme est peut-être responsable de la diminution des populations microbiennes responsables de la production de la β-glucuronidase. Par contre, la nature prébiotique des β-glucanes insolubles de *S. boulardii* devra être confirmée dans des études subséquentes.

Considérant l'importante littérature concernant les propriétés immunomodulatrices des β-glucanes de levures, il est possible qu'un mécanisme immunitaire soit aussi impliqué dans le potentiel chimiopréventif *in vivo* observé au chapitre 3. Par exemple, les (1→3)-β-D-glucanes sont reconnus pour moduler IL-6, ce qui démontre une propriété anti-inflammatoire (Luhm *et al.*, 2006). L'implication d'un mécanisme immunitaire devra faire l'objet d'une étude à part entière d'autant plus que les β-glucanes insolubles de *S. boulardii* n'ont pas été purifiés et que plusieurs mécanismes immunitaires devront être investigués. De plus, Qi *et al.* (2011) ont extrait des β-glucanes solubles de levures et leur étude montre que ces constituants ne présentent aucun effet thérapeutique lorsque utilisé seul alors que sa contrepartie insoluble présentait une forte capacité à ralentir la croissance de tumeur cancéreuse. Les auteurs ont démontré que ces différences étaient dues à la capacité des β-glucanes insolubles à activer les cellules dendritiques et les macrophages via le récepteur dectin-1 ce qui démontre l'importance de la préparation des composantes de la paroi cellulaire de levures et de sa composition dans son activité immunomodulatoire.

En plus de l'excellent potentiel chimiopréventif *in vitro* de l'extrait insoluble de *S. boulardii*, les tests antiprolifératifs effectués sur des cellules non cancéreuses (CHO-K1) ont démontré que cet extrait semble spécifique aux cellules cancéreuses (chapitre 4). En effet, l'extrait insoluble de *S. boulardii* a présenté des valeurs d'IC_{50} lorsque testé envers les cellules cancéreuses HT-29 et Caco-2 alors qu'aucune valeur n'a été obtenue envers les cellules CHO-K1. Ces données suggèrent aussi une faible cytotoxicité de l'extrait insoluble de *S. boulardii* envers les cellules non-cancéreuses ce qui est fort intéressant dans un contexte de thérapeutique. Cette conclusion n'est pas inattendue considérant l'appellation *Generally Recognized as Safe* de S. *cerevisiae* et de l'idée généralement accepté que les bêta-glucanes sont connus comme étant sécuritaire (Czerucka *et al.*, 2007, Kelesidis *et al.*, 2012). En effet, le *European Food Safety Authority* a conduit une étude testant la toxicité chronique, l'allerginicité, la toxicité animale ainsi que la capacité d'absorption/métabolisation et d'extraction de différents bêta-glucanes industriels extrait de *S. cerevisiae* en modèle humain (hommes et femmes âgés entre 1 et 64 ans) et animal en utilisant de fortes doses (jusqu'à 15g/jour chez l'humain et jusqu'à 2g/kg chez le rat). Les conclusions du *European Food Safety Authority* dévoilent que le bêta-glucane de levure, considéré comme un nouvel ingrédient alimentaire, est

sécuritaire à la consommation dans les conditions observées, soit à de très fortes doses ((EFSA), 2011).

En parallèle aux études concernant les parois cellulaires de levures (chapitres 2 et 3), le potentiel chimiopréventif de deux HE considérées par plusieurs auteurs comme étant identiques a aussi été déterminé. Il a été observé au chapitre 4 que l'HE de *B. carterii* était deux fois plus efficace à induire la QR alors que l'HE de *B. sacra* possède une cytotoxicité spécifique aux cellules cancéreuses en plus de présenter de meilleure capacité antiradicalaire. Puisque que les HE sont des mélanges complexes de composés chimiques, les différences dans l'activité biologique de ces deux HE émanent de leurs compositions. En effet, les propriétés antiradicalaires et antiprolifératives des huiles de *Boswellia* ont souvent été associées aux teneurs en terpènes tels que l'acide boswellique, l'α-amyrine et l'α-pinène (Hakkim *et al.*, 2015, Singh *et al.*, 2009, Suhail *et al.*, 2011). Sachant que l'huile de *B. sacra* a été rapporté pour contenir de plus fortes teneurs en terpènes en comparaison avec l'huile de *B. carterii (Woolley et al., 2012)*, il est possible que cette différence soit responsable de la divergence entre les deux HE concernant les tests antiradicalaires et antiprolifératifs. Par contre, la divergence dans l'induction de la QR pourrait être due à une plus grande teneur en composés possédant une fonction cétone α/β-insaturée. Des tests supplémentaires devront être menés pour valider ces hypothèses.

Les tests d'apoptose effectués sur les cellules HT-29 ont suggéré qu'aucune des deux huiles n'a été en mesure d'induire ce phénomène (chapitre 4). Considérant que les terpènes sont les composantes majoritaires des huiles de *Boswellia* qui induisent l'apoptose (Liu *et al.*, 2002, Ni *et al.*, 2012), il est possible que les cellules HT-29 soient plus résistantes aux terpènes (par exemple, acide boswellique) que d'autres lignées cellulaires puisque la présente étude est la première à investiguer l'effet apoptotique des huiles de *Boswellia* sur des cellules HT-29. Il a déjà été montré qu'à l'AK-BA induit l'apoptose dans les cellules HT-29 via une voie moléculaire passant par les protéines PI3K/AKT (Li *et al.*, 2013). Or, il se peut aussi que les teneurs en acide boswellique et ces dérivés retrouvés dans les huiles de *B. sacra* et de *B. carterii* soient inférieurs à ceux utilisées par Li *et al.* (2013). Par contre, il est aussi possible que l'effet apoptotique des huiles de *Boswellia* provienne d'un effet synergique entre ses composantes terpéniques. Des tests supplémentaires s'imposent pour répondre à cette question, notamment une quantification des teneurs en acide boswellique et ces dérivées sur les HE de *B. sacra* et *B. carterii*. Les résultats présentés au chapitre 4 suggèrent que les l'huile de *B. sacra* et de *B. carterii* démontrent un potentiel chimiopréventif différent entre eux ce qui apporte une évidence que ces derniers végétaux n'appartiennent pas à la même espèce.

Considérant l'excellent potentiel chimiopréventif des parois cellulaires de *S. boulardii* dévoilé dans la présente étude (chapitre 2 et 3), ces résultats suggèrent que l'extrait insoluble de cette espèce soit en mesure d'augmenter l'activité biologique des HE. Cette combinaison peut être considérée comme innovatrice et audacieuse puisqu'à notre connaissance, aucune étude n'a investigué l'effet chimiopréventif et anticancéreux d'un extrait de paroi cellulaire de levures combinée à des HE.

Il s'est avéré que la combinaison entre l'huile essentielle de *B. carterii* et les β-glucanes insolubles de *S. boulardii* a été en mesure d'augmenter davantage le niveau d'induction de la quinone réductase en comparaison au niveau d'induction de chaque composé testé seul (Chapitre 4). Par contre, l'huile de *B. sacra* n'a pas montré un tel effet. Ces résultats sont congruents avec la capacité des huiles testées séparément. En effet, l'huile Frankincense a été deux fois plus efficace que l'huile Sacrée pour l'induction de cette enzyme. De plus, l'huile Sacrée possède une plus grande capacité que l'huile Frankincense à affecter la viabilité des cellules Hepa 1c1c7 qui est basée sur la réduction du niveau d'induction de la quinone réductase à forte concentration (108 ppm) ce qui pourrait expliquer la divergence de l'effet observé dans les traitements combinés.

Les tests antiprolifératifs ont démontré que l'effet des différentes combinaisons semble fonction de la lignée cellulaire utilisée puisque chacune d'elles révèle un effet combinatoire différent. Les résultats suggèrent que les différentes combinaisons démontrent une capacité accrue à affecter la viabilité des cellules non-cancéreuses (CHO-K1), ce qui laisse croire que combiner les HE de *Boswellia* avec un extrait de paroi cellulaire de levures est peu favorable quant aux tests antiprolifératifs. Il est peu probable que les huiles aient affecté la structure des β-glucanes contenus dans l'extrait insoluble de *S. boulardii* (considéré comme le composé contribuant significativement à l'activité biologique, voir Chapitre 2). En effet, Carneiro *et al.* (2013) ont démontré que l'encapsulation d'huile de lin avec de la maltodextrine en combinaison avec de la gomme d'arabique ou du concentré de protéine de lactosérum a été en mesure de réduire grandement l'oxidation de l'huile et de démontrer une grande stabilité (taux de cisaillement, densité et humidité interne) des polymères utilisés pour l'encapsulation dans le temps. De plus, plusieurs études ont démontré que des HE peuvent être encapsulées dans un gel à base de polysaccharide sans affecter la fonction biologique des huiles (Ahmed *et al.*, 2016, Anchisi *et al.*, 2006, Beyki *et al.*, 2014). Or, après avoir confirmé que les HE utilisées dans cette étude n'affectaient pas le poids moléculaire de l'extrait insoluble (Chapitre 4), ces résultats pourraient être expliqués par un changement de l'effet synergique intrinsèque des huiles. En effet, les HE sont souvent considérées comme un mélange chimique complexe au sein duquel les différentes composantes peuvent œuvrer de concert pour fournir des propriétés biologiques supérieures à celles de chaque composante testée séparément.

En ce sens, il est possible que l'activité de(s) la composante(s) majoritaire(s) des huiles de *Boswellia* soit modulée par la présence de molécules minoritaires au sein de l'huile ce qui forme un équilibre dans les teneurs des différents composés chimiques (Bakkali *et al.*, 2008). Au cours de cette présente étude, il peut être juste d'avancer que la perte de la cytotoxicité spécifique aux cellules cancéreuses soit due à une déstabilisation de l'équilibre chimique de l'huile Sacrée par l'ajout de l'huile Frankincense et des β-glucanes insolubles causé par une réduction de la disponibilité des groupements fonctionnelles dû aux mélanges. Cette déstabilisation a peut-être engendré des effets antiprolifératifs différents selon la sensibilité de la lignée cellulaire utilisée. Toutefois, les traitements combinés ont permis de mettre en lumière que les β-glucanes insolubles de *S. boulardii* possèdent une cytotoxicité spécifique aux cellules cancéreuses (Chapitre 4) renforçant davantage le potentiel chimiopréventif de cet extrait.

5.2. Conclusion générale

Malgré l'importante quantité d'études utilisant une approche thérapeutique envers le CCR, cette maladie reste encore un enjeu important au Canada puisqu'il représente le troisième cancer le plus répandu au pays. De plus, les traitements typiquement utilisés contre ce cancer sont souvent très invasifs en plus d'être accompagnés d'une large gamme d'effets secondaires. En ce sens, une approche préventive via des moyens naturels semble être nécessaire pour enrayer ce cancer.

Dans ce contexte, ce projet de maîtrise aura permis de dévoiler un excellent potentiel chimiopréventif et anticancéreux *in vitro* et *in vivo* des parois cellulaires de levures et, dans une moindre mesure, celui des HE de *B. sacra* et de *B. carterii*. De plus, ce projet aura permis d'élucider un nouveau mécanisme chimiopréventif (induction de la quinone réductase) des parois cellulaires de levures en plus d'amener des preuves de l'importance cruciale des β-glucanes dans leurs activités biologiques. De surcroît, ce projet aura permis de mettre en évidence la non-toxicité (toxicité spécifique aux cellules cancéreuses) de l'extrait insoluble de *S. boulardii* qui s'est d'ailleurs démarqué des autres extraits par son excellent potentiel chimiopréventif. Cette étude a aussi permis de démontrer un effet additif entre les β-glucanes insolubles de *S. boulardii* et l'huile de *B. carterii* à ce qui attrait à l'induction de la quinone réductase en modèle *in vitro*. Les β-glucanes insolubles extraient de la paroi cellulaire de *S. boulardii*, souche de levure considérée comme probiotique et à fort potentiel industriel, pourraient être utilisés dans la fabrication d'un produit nutraceutique ou comme ingrédient fonctionnel dans une optique de prévention du CCR humain ou même sous la

forme de fibre naturelle. Pour faire suite à cette étude, plusieurs perspectives de recherche sont envisageables. En premier lieu, la détermination du potentiel chimiopréventif d'un composé est basée sur une accumulation de preuves obtenues par plusieurs tests d'activités biologiques de différentes natures. En ce sens, il serait intéressant de déterminer si les β-glucanes insolubles de *S. boulardii* sont en mesure : d'induire l'apoptose, de moduler négativement le gène pro-inflammatoire *cox-2* ainsi que d'investiguer son potentiel immunomodulatoire. En second lieu, il serait possible d'approfondir la connaissance de la structure chimique exacte des β-glucanes contenus dans l'extrait insoluble de *S. boulardii*, notamment en ce qui a trait à la nature des liaisons glycosidiques ((1→3) et (1→6)-β-D-glucanes), le degré de polymérisation et de ramification ainsi que la nature des impuretés (lipides, protéines, etc)). Ces informations seront utiles pour affiner la caractérisation de cet extrait et ainsi mieux comprendre les relations structures-fonctions. D'ailleurs, dans l'optique d'établir une corrélation solide entre les teneurs absolues et relatives en β-glucanes et l'activité biologique, il serait intéressant de comparer le potentiel chimiopréventif *in vitro* des parois cellulaires de *S. boulardii* en phase exponentielle avec ceux de la présente étude collectée en phase stationnaire. Finalement, il serait intéressant d'améliorer le potentiel chimiopréventif via une modification chimique des β-glucanes purifiés de l'extrait insoluble de *S. boulardii*. Pour ce faire, différentes approches sont connues dans la littérature telles que la sulphatation, la méthylation, la carboxyméthylation, l'hydroxylation, l'aminoéthylation et la formylméthylation (Mantovani *et al.*, 2008, Ooi *et al.*, 2000).

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ANNEXE I : VERSION SOUMISE DE LA PUBLICATION 1

Cancer Chemopreventive, Antiproliferative and Superoxide Anion Scavenging Properties of *Kluyveromyces marxianus* and *Saccharomyces cerevisiae var. boulardii* Cell Wall Components

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Abstract

This study investigated the cancer chemopreventive, the antiradical and the antiproliferative properties of polysaccharides extracts from cell wall of *Saccharomyces boulardii* and *Kluyveromyces marxianus*. β-glucan, mannan and chitin were also quantified to identify the most important extract responsible for these biological properties. Soluble and insoluble glucans as well as mannoprotein were extracted from cell wall using single hot-alkaline method. Superoxide anion scavenging (antiradical capacity), NAD(P)H: quinone reductase (QR) (EC 1.6.99.2) induction and antiproliferative assays were done for the evaluation of biological properties of those extracts. The insoluble glucan from *S. boulardii* revealed the most relevant biological properties by increasing QR activity and exhibiting the highest growth inhibition against colorectal cancer cells. Moreover, high amount of glucan, high glucan/total sugars ratios and low chitin/glucan ratios were shown to have an impact on enhancing cancer chemopreventive and antiproliferative properties. To our knowledge, this is the first study that demonstrates QR activity by yeast cell wall components.

Keywords: yeast, cell wall, β-glucan, chemoprevention, experimental

Introduction

Colorectal cancer (CRC) is the second leading cause of deaths due to cancer in males and the third in females (Canadian Cancer Society's Advisory *et al.*, 2014). It is also the third most prevalent cancer in Canada (Canadian Digestive Health Foundation, 2016). Since treatment for CRC can be expensive and invasive for patients, prevention methods still seem to be the most efficient approach. It has been shown that life style plays an important role in the incidence of many cancers and diet has been related to almost 70 % of CRC incidence (Aggarwal *et al.*, 2013). Thus, consumption of diet containing agents with CRC preventive properties could reduce the risks of CRC incidence. The impact of CRC on the health of the population in Canada and USA triggered a demand of natural products with CRC preventive properties to prevent or reduce the development of this disease. Among natural agents, yeast cell wall components have been interesting due to their anticancer and immunomodulatory properties which can be utilized in nutrition, in pharmaceutical and in medical applications (Laroche *et al.*, 2007).

Yeasts are largely used in industrial domains especially in food industry. However, cell wall of spent yeasts are often discarded after fermented broth collected or used in many applications such as yeast extract or nutraceutical food supplements (dos Santos Mathias *et al.*, 2014, Giavasis, 2014). Many studies have investigated the natural properties of yeast cell wall and demonstrated that there are strong immunomodulatory properties of yeast glucan in *in vitro* and *in*

vivo models (Karoui *et al.*, 2007, Oliveira *et al.*, 2013). Those properties depend on physicochemical nature and integrity of the glucan structure which vary according to growth conditions, extraction methods and yeast species (Aguilar-Uscanga *et al.*, 2003, Mantovani *et al.*, 2008, Pinto *et al.*, 2014).

Yeast cell walls are organized with approximatively the same polysaccharides which mainly consist of mannoprotein, chitin and (1→3)-β-D-glucan with (1→6)-β-D-glucan ramifications (Klis *et al.*, 2006). The sugar composition of the cell walls is mainly responsible for their biological and chemical properties (Aguilar *et al.*, 2012).

Saccharomyces boulardii (S. cerevisiae var. *boulardii)* and *Kluyveromyces marxianus* are well-known yeasts with diverse industrial applications and relevant biological properties. Indeed, *S. boulardii* is considered as a probiotic strain known to generate preventive effects on the occurrence of antibiotic-associated diarrhoea, beneficial effects against different enteric pathogens and to produce different anti-inflammatory molecules including *Saccharomyces* anti-inflammatory factor (Czerucka *et al.*, 2007, Kelesidis *et al.*, 2012). *K. marxianus* is known to possess a relevant potential in biotechnology due to its capacity to synthesis β-galactosidase and pectinase in addition to reducing lactose content in food products. Furthermore, *K. marxianus* exhibits an higher ethanol production as compare to *S. cerevisiae* due to its highly thermotolerant properties (Anderson *et al.*, 1986, Fonseca *et al.*, 2008).

Since the immunomodulatory properties of cell wall extracts (glucan, chitin and mannoprotein) of *S. cerevisiae* are well characterized, this study focused on the chemopreventive, antiradical and antiproliferative properties of cell wall extracts of *S. boulardii* and *K. marxianus*. The content, the relative concentrations and the molecular weight of the polysaccharides typically found in yeast cell wall were determined. Then, the cancer chemopreventive potential of the extracts was investigated and compared with commercial insoluble glucans of *S. cerevisiae*. Nicotinamide adenine dinucleotide phosphate hydrogen: quinone reductase (QR) assay has been selected as cancer chemopreventive test for its direct role in protection against toxic electrophilic metabolites directly involved in the very first stage of carcinogenesis process whereas superoxide (O_2^-) anion was selected since it is one of the most important reactive oxygen species (ROS) due to its ability to generate hydrogen peroxide and $OH\cdot$ radicals (Young *et al.*, 2001). Finally, structure-function relationships between the cell wall composition and their biological activities were revealed.

Materials and Methods

Chemicals

Chemicals and media were obtained as follows: dextrose, essential amino acids, sodium pyruvate, fetal bovine serum (FBS), minimum essential medium-Earle's balanced salt solution (MEM-EBSS), Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (MEMF/12), Hank's balanced salt solutions (HBSS),

trypsin (EC 3.4.21.4), Pierce®BCA Protein assay, glycine, N-methylpyrrolidone (NMP) and 96-wells microplates were purchased from Fisher Scientific (Ottawa, ON, Canada). Commercial insoluble β-glucan, activated carbon, digitonin, bovine serum albumin (BSA), glucose-6-phosphate, thiazolyl blue tetrazolium bromide (MTT), menadione, glucose-6-phosphate deshydrogenase (EC 1.1.1.49), 2,3-*Bis*(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt (XTT), xanthine, sodium carbonate buffer (pH 10.2), xanthine oxydase (EC 1.1.3.22), superoxide dismutase (EC 1.15.1.1) , phenol, sulphuric acid, N-acetylglucosamine, mannan, lithium chloride (LiCl), nicotinamide adenine dinucleotide phosphate (NADP), dimethyl sulfoxide DMSO) and flavin adenine dinucleotide (FAD) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Yeast extract, bacterial peptone and agar were purchased from Alpha Bioscience (Baltimore, MD, USA). Polyethylene glycol and polymethyl methacrylate (PMMA) were purchased from Agilent technologies (Mississauga, ON, Canada).

Yeast Strains and Growth Conditions

Kluyveromyces marxianus ATCC 10022 and *Saccharomyces cerevisiae* var. *boulardii* ATCC MYA-796 were purchased from American type culture collection (ATCC) (Manassas, VA, USA). The yeast strains were stored at -80ºC in sterile yeast peptone dextrose (YPD) (10 M dextrose, 5 M yeast extract, 3 M bacterial peptone, 0.8 M $MgSO_4$, 1 M KH_2PO_4) containing 10% (w/v) sterile glycerol. One ml of culture cells in cryovial (10^8 cells/ml) from each strain were

thawed and inoculated in a 125 ml Erlenmeyer containing 25 ml of YPD medium then incubated for 18 h at 30ºC at 200 rpm (Forma Scientific, Orbital shaker, Model; EQ-069, USA). A quantity of 2.5 ml of the resulting cell suspension was inoculated in a 250 Erlenmeyer containing a final volume of 50 ml of YPD medium for 24 h at 30ºC under agitation. Finally, 12.5 ml of this second cell suspension was inoculated in a 1L Erlenmeyer containing a final volume of 250 ml of YPD medium for 24 h at 30ºC under agitation. At the end of second and third growth, 1 ml of fermented broth was serially diluted in sterile peptone water and plated on YPD agar in order to confirm lack of contamination in cell suspension. To obtain sufficient cell wall extract, this procedure was repeated in triplicate (n=3) for each strain.

Growth Kinetics

To determine the growth phase of yeast species, optical density and dry biomass weight were monitored for 24 h. The growth was conducted using the same conditions as mentioned above except that 1 ml of cell suspension was collected every 2 h and diluted in sterile medium, to measure growth by optical density at 600 nm (Varian Canada Inc., Mississauga, ON, Canada). In parallel, 1 ml of cell suspension was also collected every 2 h and placed in sterile pre-weight tubes then centrifuged at 2000 *g* for 10 min at 4ºC. Supernatant was discarded and pellet was washed with sterile water. Finally, washed biomass was dried at 60ºC for 48 h and tubes were weighted. Linear relation (equation) of dry biomass weight and optical density was established and the resulting equation was used

to estimate the dry biomass in time function. For both strains, kinetic were made in triplicates (n=3).

Biomass Collect and Cell Wall Preparation

Fermented broths were centrifuged at 9000 *g* for 10 min at 4ºC. The resulting pellet (biomass) was washed twice with sterile phosphate buffer 50 mmol/l, pH 7.2. The wet biomass was suspended in 15% (w/v) sterile water and was autolyzed for 24 h at 50ºC with agitation at 200 rpm. Autolyzed cells were then centrifuged at 9000 *g* for 10 min at 4ºC. The supernatant was discarded and the autolyzed cells were entirely used for glucan and mannoprotein extraction.

Extraction of Yeast Glucan and Mannoprotein Extracts

Method of extraction of β-glucan and mannoprotein was based on work of Nguyen *et al.* (1998) and Suphantharika *et al.* (2003) with some modifications. The autolyzed cells were mixed with 1 mol/l NaOH (20% w/v) for 1 h at 90ºC without stirring in order to avoid glucan degradation. Then, the suspension was centrifuged at 9000 *g* for 10 min at 4°C. The precipitate was washed twice with distilled water, freeze-dried and the resulting extract was considered as insoluble glucan. The supernatant was mixed with 95% ethanol in 1:4 proportions, left overnight at 4ºC and centrifuged. The resulting pellet was washed twice with distilled water, then freeze-dried and the obtained extract was referred as soluble glucan. Finally, ethanol in the supernatant was evaporated using a vacuum concentrator (Savant, Automatic environmental speedVac® system, Model;

AES1010, Farmingdale, NY, USA) under Full vacuum, low speed and at ambient temperature for 8 h and then freeze-dried to obtain mannoprotein extract. For both strains, extraction was made in triplicate (n=3).

Total Sugars, Glucan, Mannan and Chitin Quantifications

The total sugars content in each extract was determined following a method of Dubois *et al.* (1956) using a mixture of 40% mannose and 60% dextrose (ranging from 0.02 to 0.1 M) as a standard curve. Glucan and mannan were quantified by HPLC (Varian Pro Star 210) equipped with a refractive index detector (RID) using a method established for analysis of polysaccharides in Industrial Microbiology laboratory of CUCEI-UdG (México). Polysaccharides were separated in a Metacarb CA-PLUS (30 cm X 7.8 mm) isothermal column at 90°C, using water as mobile phase and a flow rate of 0.6 ml/min. A calibration curve was performed at concentrations between 1 to 0.1 M of glucan and mannan. The quantification of chitin in cell wall extracts was done using a modified method of Pérez *et al.* (1983) and Roncero *et al.* (1988) using a standard curve of N-acetylglucosamine ranging from 20 to 100 mmol/l. Results were expressed as average ± standard deviation (SD).

Molecular Weight Determination by Gel Permeation Chromatography (GPC)

The molecular weights (Mw) of polysaccharides was analysed by gel permeation chromatography (GPC) using an Agilent HPLC (Agilent Technologies 1260 infinity series, Waldbronn, BW, Germany), equipped with a quaternary pump

(Model G1311B), a manual injector with a sample loop of 20 µl and a refractive index detector (Model G1362A). Insoluble glucan was treated separately from soluble glucan and mannoprotein extracts. For soluble glucan and mannoprotein extracts, two identical PL Aquagel-OH mixed-M 8 µm 300 X 7.5 mm columns were used in series to increase resolution. Water containing 20 ppm sodium azide was used as mobile phase with a flow rate of 1 ml/min and both columns and detector were set at 30ºC. Freeze-dried mannoprotein and soluble glucan were rehydrated by solubilizing 2.5 mg/ml in mobile phase and filtered through a 0.2-µm filter. Polyethylene glycol was used as a standard and prepared as recommended by the manufacturer. Concerning insoluble glucan, 2 identical PLgel 5 µm Mixed-D 300 X 7.5 mm columns were used in series as explained above. Mobile phase consisted of 100% NMP containing 5% (w/v) LiCl. A flow rate of 0.5 ml/min was used and both columns and detector were set at 60ºC. Method for preparation of insoluble glucan for injection was mainly based on Austin (1977), Yilmaz *et al.* (2003) and on Chakrabandhu *et al.* (2008) with some modifications due to the insoluble properties of chitin. Freeze-dried insoluble glucan was mixed in NMP at a concentration of 2.5 mg/ml into a glass bottle. The suspension was stirred for 48 h at 60ºC and filtered through a nylon 0.2-µm filter. PMMA was used as a standard for insoluble glucan and was prepared as indicated by the manufacturer. The equation obtained by plotting Mw with retention times of standards was used to calculate the Mw of each peak obtained by GPC of the extracts. All extracts were injected in triplicate (n=3) and expressed as Mw range.

Cancerous Cell Lines and Cells Maintenance

Hepa 1c1c7 (ATCC CRL-2026) and HT-29 (ATCC HTB-38) cell lines were purchased from American type culture collection (ATCC) (Manassas, VA, USA). The cell lines were cultivated in 25 cm^2 cellular flasks (Corning, Manassas, VA, USA) in a humidified incubator at 37ºC in an atmosphere of 5% CO_2 and 95% air. Hepa 1c1c7 cells and HT-29 cells were grown in complete MEM-EBSS and complete MEMF/12 media respectively containing 0.1% essential amino acids, 0.1% sodium pyruvate, 10% (v/v) FBS. The maintenance of cells was performed at a confluence of 80-90% by washing adherent cells with HBSS solution. Then, cells were treated with 1X trypsin for exactly 12 min in a humidified incubator as mentioned above and inoculated into 5 ml of fresh completed medium.

NAD(P)H: quinone reductase (QR) Assay

QR assay was based on study of Prochaska *et al.* (1988a) with some modifications. The aim of this assay was to determine the concentration of extracts required to double the QR induction (CD) in Hepa 1c1c7 cells. The induction of QR can be easily detected by using this cell line since it possesses the capacity for carcinogen activation and xenobiotic metabolism in addition to possessing a high inducible aryl hydrocarbon hydrolase consequently facilitating metabolic activation of xenobiotics (Fahey *et al.*, 2004). Freeze-dried extracts were rehydrated in 10% (v/v) dimethyl sulfoxide (DMSO) containing 20 ppm of

sodium azide (as antimicrobial agent) in order to avoid sterilization of extract solution by filtration or by heating which could lead to a loss of biological activities. A 96-well plate was seeded with Hepa 1c1c7 cells at a density of 10^4 cells/ml (200 µl per well) in completed MEM-EBSS medium and incubated in a humidified incubator as mentioned above. Afterward, different concentrations of extracts (20 µl) previously serial diluted were added and each well was completed to 200 µl with carbon activated MEM-EBSS media for 48 h. Cells were then washed with HBSS solution and 50 µl of 1.6% (w/v) digitonin were added in each well. The microplate was incubated for 20 min at 37ºC on an orbital shaker. A sample of 20 µl of this suspension was collected for further total protein determination. Then, 200 µl of complete reaction mixture (0.25 mol/l Tris-HCl pH 7, 4.67% (w/v) BSA, 0.01% tween-20, 5 mol/l FAD, 1 mmol/l glucose-6-phosphate, 30 mol/l NADP, 34.8 µmol/l MTT, 50 µmol/l menadione and 2 mU/µl glucose-6-phosphate deshydrogenase) were added in each well and optical densities were read after 5 min at 595 nm using a microplate reader (Biotek, Model EL800, Winooski, VT, USA). Protein assay was made using Pierce®BCA reagents and was performed as suggested by the manufacturer. Control consisted of 1% (v/v) DMSO containing 20 ppm sodium azide whereas medium was used as blank. Specific activity of QR was defined as nmol of blue formazan formed per mg protein per minute. Fold inductions of QR were presented as average ± SD and were calculated as follow:

QR Fold Induction (treated on control) = Specific activity of QR in treated group / Specific activity of QR in negative control group (Equation 1)

Antiradical Assay (O_2^- Anion Scavenging Activity)

The capacity of samples to scavenge O_2^- was measured using xanthine/xanthine oxydase (X/XO) system (XTT color assay) based on method of Gerhäuser *et al.* (2003). This assay aims to determine whether soluble extracts possess radical scavenging properties by quantified their activity to scavenge O_2^- anion. Freeze-dried samples of yeast cell wall extracts were rehydrated in 10% (v/v) DMSO at desired concentrations. Then, serial dilutions of extracts were performed in 10% (v/v) DMSO and 20 µl of those extracts were loaded in 96-well microplate. Each well was completed to 200 µl with reactional mix (1 mmol/l XTT, 1 mmol/l EDTA, 1 mmol/l xanthine, 50 mmol/l sodium carbonate buffer (pH 10.2) and 3 mU/ml xanthine oxydase) and optical density was read at 490 nm (Biotek) after 20 min. Negative control consisted of DMSO 1% (v/v) whereas positive control corresponded to 30 U/ml of superoxide dismutase. Scavenging activity (%) was calculated as follow:

% Scavenging Activity = [(*sample OD* - *Negative control OD*) / (*Positive control OD* - *Negative control OD*)] x 100 (Equation 2)

Concentrations exhibiting a scavenging activity of 50% referred as SC_{50} values. Insoluble glucan was not used for this assay since perfectly soluble samples were required. Scavenging activities were presented as average ± SD.

Antiproliferative Assay

The antiproliferative effect of different yeast cell wall extracts was measured using MTT color assay based on method of Vistica *et al.* (1991). The cell proliferation was determined by the ability of the metabolic active cells to cleave the tetrazolium salt to purple formazan crystals. Human CRC HT-29 cells were chosen for antiproliferative assay since this study focus on CRC. In a 96-well plate, HT-29 cells were seeded at 2 x 10^4 cells per 200 µl of complete MEMF/12 medium. After 24 h as mentioned above, the medium was replaced with 100 µl of fresh medium containing 10 µl of each extract previously serial diluted. Negative control consisted of 1% (v/v) DMSO containing 20 ppm sodium azide and blank consisted of 100µl of fresh medium. After 48 h of incubation, culture medium was decanted and replaced with 200 µl of fresh MEMF/12 media containing 25 µl of 0.5% (w/v) MTT. The microplate was incubated for 4 h as described above. Then, medium was carefully eliminated and 200 µl of DMSO plus 25 µl of Sorensen buffer pH 10.5 (0.1 mol/l glycine, 0.1 mol/l NaCl) were added in each well. Absorbencies were measured at 562 nm (Biotek) and the cellular growth inhibition was calculated as follow:

% Growth Inhibition = 100 – (([*Sample OD*] / *Negative control OD*) x 100)

(Equation 3)

Equations obtained by plotting the linear portion of growth inhibition versus concentrations of extracts were used to determinate concentrations that inhibit 50% of the cellular growth (IC_{50} values). Results were presented as average ± SD.

Statistical Analysis

All quantifications and assays were replicated at least 3 times (n=3). Amounts of total sugar content, chitin, glucan and mannan in extracts as well as QR fold inductions, O_2^- anion scavenging activities and IC_{50} values were analyzed by one-way analysis of variance (ANOVA) using PASW statistics 18 software (IBM Corporation, Somers, NY, USA) and differences between samples were analyzed with post hoc Duncan's multiple-range test. Significance was considered at $P \leq 0.05$.

Results

Growth Kinetics

Growth kinetics of yeasts was performed to collect yeast biomass in stationary phase in order to reflect spent yeast at the end of industrial fermentation processes. As presented in Fig. A.1, the initial amounts of biomass from *K. marxianus* (0.8 g/l) and *S. boulardii* (1.0 g/l) were similar. Afterwards, dry

biomass of both *K. marxianus* and *S. boulardii* was increased to 1.5 g/l after 8 h and 10 h respectively, indicating that *K. marxianus* presented a shorter latency phase than *S. boulardii*. Despite the fact that *K. marxianus* produced more biomass than *S. boulardii* (6.3 and 3.2 g/l respectively), both species biomass was collected in stationary phase after 24 h growth.

Solubility and Visual Appearances of Yeast Cell Wall Extracts

The solubility of those extracts is briefly described in Table A.1 Results showed that insoluble glucan and commercial glucan were partially soluble in high concentrations of DMSO whereas they were totally soluble in pure NMP after stirring and heating for 48 h in presence of 5% (w/v) lithium chloride. Soluble glucan and mannoprotein were readily soluble in water and at all concentrations of DMSO. In 10% DMSO, insoluble glucan yielded a white and turbid suspension; soluble glucan yielded a transparent solution whereas mannoprotein yielded a transparent and yellow solution.

Quantification of Total Sugars, Chitin, Glucan, Mannan in the Extracts and Mw Determination

The quantification of total sugars is presented in Table A.2 Results showed that the content of insoluble glucan, soluble glucan and mannoprotein of *S. boulardii* (42.73%, 39.13% and 0.45% respectively) are similary to their respective counterparts in *K. marxianus* (51.38%, 32.55% and 0.52% respectively) suggesting that both species possessed a similar amount of total sugars in cell

walls. Results also showed that insoluble glucan from both species contain significantly more total sugars than all other extracts evaluated in this study ($P \leq 0.05$). Also, insoluble extracts of both species contained the highest amounts of glucan (49.17 and 40.54% for *K. marxianus* and *S. boulardii* respectively) whereas the soluble glucan of *S. boulardii* contained the lowest amount of glucan (23.99%). Furthermore, combined amounts of glucan in soluble and insoluble extracts represented 65% and 80% for *S. boulardii* and *K. marxianus* respectively, which demonstrate a noticeable difference in their cell wall composition. The content of mannoprotein are presented in Table A.2 The results showed that the content of mannoprotein of *S. boulardii* contained significantly less mannan (3.17%) than their *K. marxianus* counterparts (9.14%) ($P \leq 0.05$). Moreover, each extract contained 4-10 times less mannan than glucan. These observations may be due to the presence of mannan covalently bound to glucan despite the extraction. The total content of chitin found in the three extracts of *S. boulardii* (1.51%) is higher than content found in all extracts of *K. marxianus* (0.89%), which also demonstrate typical differences in cell wall composition between each strain. Finally, higher chitin contents were found in both soluble and insoluble extracts of *S. boulardii* (0.66 and 0.58% respectively) as compared to 0.30 and 0.35% in *K. marxianus*. The GPC analysis showed that the Mw range in insoluble glucan of *S. boulardii* is 1921 kDa and 2085kDa in *K. marxianus* showing higher Mw than soluble glucan and mannoprotein extracts, which corroborates the fact that solubility partially depends on Mw (Table A.2). Mannoprotein extracts of *K. marxianus* showed a Mw from 0.48-77 kDa and

4006 from (0.72-87 kDa) in *S. boulardii*. Also, data shown in Table A.2 indicated that
4007 insoluble glucan in both strains showed a high amounts of total sugars and
4008 glucan in addition to showed high Mw.
4009
4010 ***Determination of Relative Concentrations in Total Sugars, Chitin, Glucan and***
4011 ***Mannan Contents in the Extracts***
4012 Determination of relative concentration using ratios may lead to a better analysis
4013 of extracts composition and to a better understanding of structure-function
4014 relationship. Table A.3 showed that insoluble glucan of *S. boulardii* possess a
4015 glucan/total sugars ratio (0.94) that was significantly higher ($P \leq 0.05$) then their
4016 soluble counterparts (0.61) whereas no differences ($P > 0.05$) in the glucan/total
4017 sugar ratios were observed between insoluble and soluble glucan of *K.*
4018 *marxinaus*. Mannan/total sugars ratios were higher in mannoprotein of *S.*
4019 *boulardii* (7.03) and *K. marxianus* (10.55) whereas mannan/glucan ratios
4020 showed no significant differences ($P > 0.05$) between soluble and insoluble
4021 glucan of both strains. In addition, chitin/total sugars ratios in insoluble glucans
4022 of both yeast strains (6.83×10^{-3} and 13.66×10^{-3} for *S. boulardii* and *K.*
4023 *marxianus* respectively) were the lowest as compared to other extracts whereas
4024 these ratios in mannoprotein of both strains were the highest (470.85×10^{-3} and
4025 685.14×10^{-3} for *S. boulardii* and *K. marxianus* respectively). More importantly,
4026 insoluble glucan of both strains exhibited low chitin/glucan ratios compared to
4027 other extracts whereas this ratio was significantly higher ($P \leq 0.05$) regarding
4028 soluble glucan of *S. boulardii* (27.40×10^{-3}), hence suggesting that soluble

4029 extract of *S. boulardii* possessed more chitin and less glucan than both insoluble
4030 extracts. Also, chitin/mannan ratios suggest that insoluble glucan of both strains
4031 contain high amounts of chitin whereas soluble glucan of *S. boulardii* exhibited
4032 the highest chitin/mannan ratio, which is congruent with Table A.2. Analysis of
4033 all ratios for each extract and each strain suggests that insoluble glucan possess
4034 high glucan/total sugars ratios, low chitin/total sugars ratio and low chitin/glucan
4035 ratios for both strains. Contrastingly, soluble glucan of *S. boulardii* exhibited the
4036 lowest glucan/total sugars ratio and the highest chitin/glucan ratio therefore
4037 suggesting a low proportion of glucan in this extract.
4038
4039 ***Cancer Chemopreventive, Antiradical and Antiproliferative Activities of Yeast***
4040 ***Cell Wall Extracts***
4041 Biological activities of yeast cell wall extracts are presented in Table A.4.
4042 Results showed that only insoluble glucan from both yeast species could induced
4043 QR activity and reached CD values of 500 µg/ml. Also, CD value (125 µg/ml
4044 corresponding to 1.96-Fold Induction) of the commercial insoluble glucan is 4
4045 times lower than those obtained for the insoluble extracts of *S. boulardii* (500
4046 µg/ml corresponding to 1.97-Fold induction) and *K. marxianus* (500 µg/ml
4047 corresponding to 2.08-Fold Induction), which might be explained by a higher
4048 purity of the commercial glucan. Also, fold inductions of insoluble glucan from
4049 both yeast strains were significantly higher than fold inductions of water-soluble
4050 extracts (soluble glucan and mannoprotein extracts) ($P \leq 0.05$). Those results
4051 showed that insoluble glucan of *S. boulardii* and *K. marxianus* are relevant

4052 toward QR induction assay since significant CD values were obtained for those
4053 extracts. The capacity of water-soluble extracts of yeast cell walls to scavenge
4054 O_2^- anion was determined and presented in Table A.4. Results showed that
4055 soluble glucan of *K. marxianus* reached a SC_{50} value of 3000 µg/ml (Scavenging
4056 activity of 55.47 %) as opposed to soluble glucan of *S. boulardii* that did not
4057 reached a SC_{50} value. Mannoprotein of *K. marxianus* reached a SC_{50} value (1500
4058 µg/ml corresponding to a scavenging activity of 51.53 %) twice as low as found
4059 for mannoproteins of *S. boulardii* (3000 µg/ml corresponding to a scavenging
4060 activity of 56.03 %). Also, soluble glucan of *S. boulardii* showed a O_2^-
4061 scavenging activity (Scavenging activity of 6.52 %) significantly lower than all
4062 other extracts ($P \leq 0.05$). Those results demonstrate that mannoprotein and
4063 soluble glucan of *K. marxianus* scavenged more efficiently the O_2^- species as
4064 compared to their *S. boulardii* counterparts. The antiproliferative activities of
4065 cell wall extracts of *S. boulardii* and *K. marxianus* against HT-29 cells are also
4066 presented in Table A.4. Results showed that all extracts were able to reach 50%
4067 of growth inhibition whereas extracts of *S. boulardii* showed lower IC_{50} values
4068 than their *K. marxianus* counterparts. Indeed, insoluble extract of *S. boulardii*
4069 possessed the lowest IC_{50} value (108.28 µg/ml) among glucan extracts whereas
4070 mannoprotein of *S. boulardii* possessed the lowest IC_{50} value (250.98 µg/ml)
4071 among mannoprotein extracts. In fact, only insoluble glucan of *S. boulardii*
4072 exhibited an IC_{50} value significantly ($P \leq 0.05$) lower than the commercial
4073 insoluble glucan (344.18 µg/ml) whereas soluble glucan and mannoprotein of *K.*

4074 *marxianus* were found to possess the highest IC_{50} values (856.05 and 1402.96
4075 µg/ml respectively) as compared to all extracts.
4076
4077 ***Relationship Between the Contents of Yeast Cell Wall Polysaccharide and***
4078 ***their Biological Activities***
4079 Table A.4 also indicates that insoluble glucan from both yeast strains constitute
4080 the most relevant extracts regarding biological activities. The insoluble glucan
4081 of *S. boulardii* is the most relevant extract based on its QR inductive activity
4082 with the CD value of 500 µg/ml and its antiproliferative activity against HT-29
4083 cells with the lowest IC_{50} values of 108.28 µg/ml. In contrast, soluble glucan of
4084 *S. boulardii* exhibited the lowest biological activities based on an IC_{50} value of
4085 356.11 µg/ml against HT-29 cells as the unique detected biological activity.
4086
4087 Determining the combination of polysaccharides found in yeast cell wall that are
4088 potentially responsible for cancer chemopreventive, antiradical and
4089 antiproliferative properties is essential to understand the major components
4090 involved in the biological activities of extracts. Results from the characterization
4091 of biological activities suggested that insoluble glucan possessed the most
4092 relevant biological activities which correspond to high contents in total sugars,
4093 glucan and chitin as well as a high Mw, a high glucan/total sugars ratio, low
4094 chitin/total sugars and chitin/glucan ratios. In contrast, soluble extract of *S.*
4095 *boulardii* presented the weakest biological activity and corresponded to a low
4096 content in glucan, low glucan/total sugars ratio and high chitin/glucan ratio.

These assessments suggest a typical relationship between biological activities and sugars quantification that aims to determine the relative importance of each polysaccharide in biological activities: % chitin < % glucan ≈ % total sugars. This relation shows that extracts mainly need to contain glucan to possess biological properties whereas chitin seems to be statistically less influent on chemopreventive and anticancer properties against CRC *in vitro*.

Discussion

Since growth phase can modulate the yeast cell wall composition drastically, biomass was collected in stationary phase in this study in order to reflect cellular state of spent yeasts. Mitterdorfer *et al.* (2001) demonstrated that a strain of *S. boulardii* reached the early stationary phase after approximately 25 h of growth using Sabouraud media containing 1% dextrose which is also comparable with our results. Similarly, the growth kinetics of *K. marxianus* obtained in this study is comparable with Falcão Moreira *et al.* (1998) considering growth conditions. The authors reported that *K. marxianus* ATCC 10022 cells entered in stationary phase after approximately 28 h of growth using YPD medium containing 2% dextrose and a growth temperature of 26°C. It has been demonstrated that growth temperature and dextrose concentration lower than 30°C and 2% can lead to a decrease of biomass and thus to a reduction of the time needed to enter stationary phase (Margaritis *et al.*, 1983, Rodrussamee *et al.*, 2011). Consequently, time needed for *K. marxianus* to enter stationary phase as found in the present study is considered congruent with scientific literature.

In general, yeast cell wall collected in stationary phase consists mainly of 3 biopolymers: chitin, mannan and (1→3)-β-D-glucan and (1→6)-β-D-glucan. Their proportion change according to many parameters such as strains, growth conditions, growth phase, method of cell wall preparation, extraction and chemical derivatization of naturals biopolymers (Aguilar-Uscanga *et al.*, 2003, Zekovic *et al.*, 2005). Glucan amounts found in soluble and insoluble extracts obtained in this study are similar to those obtained by Suphantharika *et al.* (2003). The authors used a single hot alkaline extraction approach to recover glucan from bakery yeast's cell wall and obtained insoluble extracts constituted of approximately 50% of glucan. Moreover, the theoretical amount of total β-glucans represents between 58 to 72% of yeast cell wall dry weight, which is in accordance with total amount of β-glucan obtained in this study. In contrast, total amount of mannans in extracts was inferior for both yeast strains as compared to mannoprotein complex found in the literature (35 to 40%) (Klis *et al.*, 2002, Kwiatkowski *et al.*, 2012). This divergence might be due to the relatively important content of proteins in the complex which was not quantified since this study focused on polysaccharides characterization. Klis *et al.* (2006) reviewed that *S. cerevisiae's* cell wall contains between 1 and 6% of chitin which is congruent with our results. The presence of chitin in soluble and insoluble glucans extracts might be due to residual N-acetyl-glucosamine branched to (1→3)-β-D-glucan and (1→6)-β-D-glucan.

The content of chitin, glucan and mannan revealed the divergence in cell wall composition of *S. boulardii* and *K. marxianus* which has already been reported elsewhere (Backhaus *et al.*, 2010). The authors concluded that *S. cerevisiae* tends to decrease the amount of glucan in cell wall under stress condition whereas a *Kluyveromyces spp.* member tends to unchanged glucan content under the same condition. Those observations seem to be in agreement with our results, since amounts of glucan and glucan/total sugars ratios were significantly higher for *K. marxianus*.

The Mw of glucan between 100 and 200 kDa are known to exhibit important biological activities such as tumor growth inhibition *in vivo* and antiproliferative effect on cancerous cells *in vitro* (Mantovani *et al.*, 2008). In the present study, the Mw of insoluble glucan from *S. boulardii* (1921 kDa) and *K. marxianus* (2085 kDa) possessed higher Mw as compared to other extracts in addition to exhibit the most relevant biological activities. β-glucan with high Mw glucan have been reported as possessing biological activities. Using hot-alkaline extraction, Mork *et al.* (1998) produced a soluble β-glucan that exhibited Mw of approximatively 1000 kDa and showed capacity in activating macrophages. In addition, Kelly (2001) described an insoluble glucan that presented a Mw range of 1000-3000 kDa and exhibited a capacity to heal skin wounds in rats. Those studies confirmed that insoluble glucan with Mw higher than 1000 kDa possess biological activities and thus corroborates findings made in the present study.

The determination of polysaccharides mainly responsible for biological activities observed in this study revealed that β-glucan was the principal component of yeast cell walls that exhibited important biological activities whereas chitin and mannan were important to a lesser extent. Those results corroborate results obtained by others investigations. Indeed, yeast β-glucan are known to exhibit antimutagenic and antigenotoxic effects *in vivo* (Oliveira *et al.*, 2013) and to inhibit the cellular growth of colorectal cancer cells both *in vitro* and *in vivo* (Kim *et al.*, 2009, Yoon *et al.*, 2008).

Chitin covalently bound to glucan in soluble/insoluble extracts might have led to a decrease of glucan biological function. Indeed, glucan branched to chitin or mannan above certain proportions might cause a decrease in glucan's biological properties, which could explain the importance of high chitin/glucan ratio in soluble glucan of *S. boulardii*. However, content of chitin was proven to be high in insoluble glucan which demonstrated the importance of relative concentration of chitin and glucan. In this regard, those results are congruent with findings of Zhang *et al.* (2004). Indeed, the authors observed that extracts with higher glucose/total sugars ratios and lower N-acetyl glucosamine/glucose ratios exhibited the most relevant anti-cancer properties both *in vivo* and *in vitro*.

The capacity of cell wall extracts obtained from *S. boulardii* and *K. marxianus* to induce QR activity seems to be a novel biological activity highlighted in the present study. Despite the fact that compounds exhibiting a CD value below 10

µg/ml are considered as highly relevant inducers (Kang *et al.*, 2004), high CD values obtained by insoluble glucan (500 µg/ml) can be considered as medium and even low inducers for *in vivo* studies using yeast cell wall compound (Li *et al.*, 2010, Samuelsen *et al.*, 2014). Further studies will be needed to determine the mechanism of QR induction by insoluble glucan.

As opposed to QR assay, several studies demonstrated the antiradical scavenging activity of polysaccharides from fungal and yeast sources (Jaehrig *et al.*, 2007, Krizkova *et al.*, 2006). The antiradical activity of yeast cell wall extracts might be explained by a hydrogen atom transfer (HAT) mechanism of anomeric hydrogen. Indeed, antiradical activities of glucan are higher than dextrose and mannose most probably due to the greater abstraction lability of the anomeric hydrogen from internal glucose units rather than from the reducing end (Tsiapali *et al.*, 2001). Our results also demonstrated that mannoprotein of *K. marxianus* exhibited the most relevant SC_{50} value. This observation might be due to a higher presence of aromatic amino acids and thiol groups in mannoprotein which are known to exhibit high antioxidant activities (Jaehrig *et al.*, 2007). In contrast, all soluble extracts obtained in the present study revealed relatively high SC_{50} values and thus can be considered to possess a low antiradical potential, which is in accordance with literature since sugar compounds were reported to exhibit low antiradicals properties (Machová *et al.*, 2013, Tsiapali *et al.*, 2001). Finally, it has been reported that yeast glucan possess a weaker antiradical scavenging

capacity compare to mannoprotein extracts (Jaehrig *et al.*, 2007, Jaehrig *et al.*, 2008), which is congruent with ours results.

Proliferation of cancerous cells can be inhibited through different mechanisms including cell cycle arrest, apoptosis and necrosis (Ren *et al.*, 2012). Although further study will be necessary to investigate whether necrosis or apoptosis was involved in growth inhibition of HT-29 cells observed in this study, the fact that all extracts exhibited antiproliferative activities is in agreement with literature (Zekovic *et al.*, 2005). Indeed, lentinan, a well-known β-glucan from fungi, was reported to inhibit 50% of the growth of sarcoma 180 solids tumors *in vitro* at a concentration of approximately 500 µg/ml (Zhang *et al.*, 2005). Moreover, β-glucan derived from a mutant strain of *Aureobasidum* inhibited 52.6% of the cellular growth of human colon cancer cells SNU-C4 at a concentration of 150 µg/ml and triggered apoptosis at 100 µg/ml (Kim *et al.*, 2009). These biological activities can be linked to β-glucan insolubility, molecular weight, nature of branching, presence of glycoprotein bound to glucan and triple-helix structure of glucan. Thus, further studies on the structure of glucan to find relation with biological properties are necessary.

Conclusion

This study demonstrated that extracts obtained via a simple and fast extraction method from cell wall of *S. boulardii* and *K. marxianus* suitable for industrial application exhibited cancer chemoprevention, antiradical and antiproliferative

properties. In addition, this study also revealed for the first time the capacity of insoluble glucan from *S. boulardii* and *K. marxianus* to induce QR activity. Results suggested that insoluble glucan and mannoprotein extracts from *S. boulardii* exhibited relevant biological activities toward CRC. Finally, characterization of extracts allowed revealing that high amount of glucan, high glucan/total sugars ratios and low chitin/glucan ratios in extracts have a major impact in biological properties of yeast cell wall extracts. In perspective, insoluble glucan and mannoprotein extracts of *S. boulardii* could be used in animal models in order to investigate their CRC chemopreventive properties and ultimately employ those extracts as food supplements in cancer prevention.

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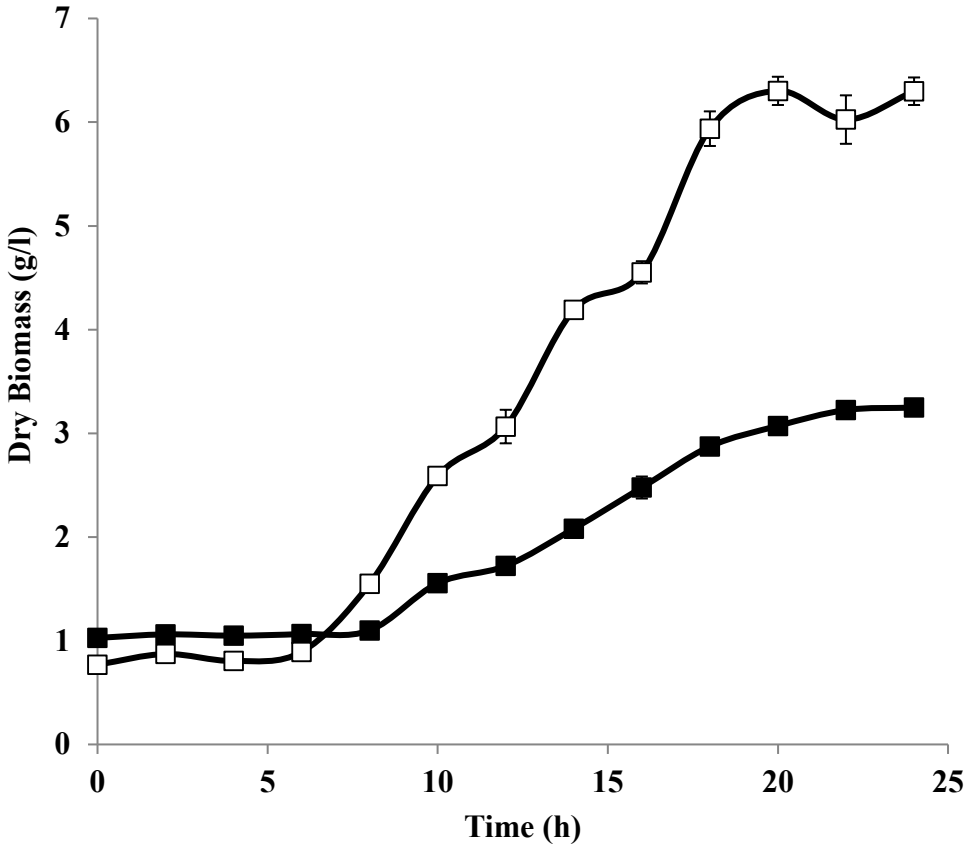
Conflict of Interest

The authors have no conflict of interest to declare.



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4254 **Fig A.1.** Growth kinetics of *S. boulardii* and *K. marxianus* based on dry biomass in time

4255 function. (■) *S. boulardii*, (□) *K. marxianus*.

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Table A.1 Solubility and visual appearance of yeast cell wall extracts obtained after single hot-alkaline extraction for both strains.

Extracts	Solubility in solvents						Appearance in 10% DMSO
	Water	DMSO 10%	DMSO 50%	DMSO 75%	DMSO 100%	NMP* 100%	
Insoluble	-	-	-	+/-	+/-	+	White, high turbidity
Soluble	+	+	+	+	+	ND	Transparent
Manno	+	+	+	+	+	ND	Transparent, yellow
Commercial glucan	-	-	-	-	+/-	+	White, high turbidity

*; 48 h with agitation at 60ºC and additioned with 5% lithium chloride. -; Insoluble. +/-; Partially soluble. +; completely soluble. ND; Not determined. NMP; 1-methyl-2-pyrrolidone. Soluble, insoluble and manno extracts refer to soluble glucan, insoluble glucan and mannoprotein respectively.

Table A.2 Sugars quantification and Mw range of yeast cell wall extracts determined by spectrophotometric method and gel permeation chromatography.

Strain	Extracts	Total sugars (%)	Glucan (%)	Mannan (%)	Chitin (%)	Mw range (kDa)
K. marxianus	Insoluble	51.38±6.02 [b]	49.17±0.13[d]	3.14±2.78[a,b]	0.35±0.08[a,b]	2085
	Soluble	32.55±2.42 [b]	30.38±0.30[b]	8.49±3.75[a,b]	0.30±0.03[b]	0.74-165
	Manno	0.52±0.38 [a]	ND	9.14±0.94[b]	0.24±0.02[a]	0.48-77
S. boulardii	Insoluble	42.73±7.64 [b]	40.54±0.07[c]	9.72±1.96[a,b]	0.58±0.03[c]	1921
	Soluble	39.13±5.63 [b]	23.99±0.29[a]	3.99±3.46[a,b]	0.66±0.02[c]	0.73-160
	Manno	0.45±0.03 [a]	ND	3.17±0.11[a]	0.27±0.01[b]	0.72-87

ND; Not determined. Soluble, insoluble and manno extracts refer to soluble glucan, insoluble glucan and mannoprotein respectively. Within each column, means bearing a different lowercase letter are significantly different ($P \leq 0.05$).

Table A.3 Ratios in glucan, mannan and chitin regarding all yeast cell wall extracts.

Strains	Extracts	Glucan / total sugars	Mannan / total sugars	Mannan / glucan	Chitin / total sugars (10^{-3})	Chitin / glucan (10^{-3})	Chitin / mannan
K. marxianus	Insoluble	0.96±0.11^{b}	0.06±0.01^{a}	0.06±0.05^{a}	6.83±1.54^{a}	7.12±1.62^{a}	0.112±0.025$^{c, d}$
	Soluble	0.93±0.07^{b}	0.26±0.02^{b}	0.28±0.01^{a}	9.34±0.82^{a}	10.03±0.88$^{a, b}$	0.036±0.003^{b}
	Manno	ND	10.55±1.07^{c}	ND	471.85±38.45^{c}	ND	0.026±0.002^{a}
S. boulardii	Insoluble	0.94±0.16^{b}	0.23±0.04^{a,b}	0.24±0.05^{a}	13.66±2.94^{b}	14.38±3.09^{b}	0.059±0.0129$^{b, c}$
	Soluble	0.61±0.09^{a}	0.10±0.01^{a}	0.16±0.14^{a}	16.81±0.48^{b}	27.40±0.79^{c}	0.165±0.005^{d}
	Manno	ND	7.03±0.53 ^{c}	ND	685.14±14.25^{d}	ND	0.086±0.002^{c}

ND; Not determined. Soluble, insoluble and manno extracts refer to soluble glucan, insoluble glucan and mannoprotein respectively. Within each column, means bearing a different lowercase letter are significantly different ($P \leq 0.05$).

Table A.4 Effect of yeast cell wall extracts on quinone reductase induction, superoxide anion scavenging capacity and antiproliferative property.

Strain	Extracts	QR Fold Induction (corresponding CD)†	O_2^- Scavenging Activity (%) (corresponding SC_{50})†	Antiproliferative assay (IC_{50})†
K. marxianus	Insoluble	2.08±0.09^b (500)	ND	284.98±9.31^b
	Soluble	1.25±0.22^a (NR)	55.47±5.57^b (3000)	856.05±56.41^c
	Manno	1.26±0.07^a (NR)	51.53±2.48^b (1500)	1402.96±136.94^d
S. boulardii	Insoluble	1.97±0.16^b (500)	ND	108.28±32.87^a
	Soluble	1.12±0.03^a (NR)	6.52±2.83^a (NR)	356.11±2.82^b
	Manno	1.16±0.13^a (NR)	56.03±4.80^b (3000)	250.98±12.48^b
S. cerevisiae (Bakery strain)	Commercial glucan	1.96±0.24^b (125)	ND	344.18±24.90^b

ND; Not determined. NR; CD or SC_{50} values were not reached. Soluble, insoluble and manno extracts refer to soluble glucan, insoluble glucan and mannoprotein respectively. QR; Quinone reductase. O_2^-; Superoxide anion. CD; Concentration that double the specific activity of QR. SC_{50}; Concentration that scavenges 50% of all O_2^-. IC_{50}; Concentration that inhibits 50% of the cellular growth. †; CD, IC_{50} and SC_{50} values are expressed in µg/ml. Means bearing a different lowercase letter are significantly different ($P \leq 0.05$).

ANNEXE II : COLLABORATION

Desrouillères K, Millette M, Jamshidian M, Maherani B, **Fortin O** & Lacroix M (2016) Cancer preventive effect of a specific probiotic fermented milk components and cell walls extracted from a biomass containing L. acidophilus CL1285, L. casei LBC80R, and L. rhamnosus CLR2 on male F344 rats treated with 1,2-dimethylhydrazine. Journal of Functional Foods 26:373-38