UNIVERZA V LJUBLJANI
BIOTEHNIŠKA FAKULTETA

Tanja PETELINC

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DOKTORSKA DISERTACIJA

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BIOACTIVE COMPOUNDS OF PROPOLIS AND THEIR EFFECT ON YEAST *Saccharomyces cerevisiae* AT CELLULAR AND PROTEOME LEVEL

DOCTORAL DISSERTATION

Ljubljana, 2015

Doktorska disertacija je rezultat dela na Katedri za biotehnologijo, mikrobiologijo in varnost živil ter na Katedri za tehnologijo mesa in vrednotenje živil, Oddelka za živilstvo, Biotehniške fakultete, Univerze v Ljubljani.

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AI Propolis ima številne biološke učinke, vendar ni popolnoma znano katere spojine v propolisu jih povzročajo in kakšni so mehanizmi delovanja bioaktivnih spojin na molekularni ravni. Zato smo v doktorski nalogi kemijsko okarakterizirali izvleček slovenskega propolisa in ga frakcionirali z ekstrakcijo s trdno fazo. Vpliv posameznih frakcij, za katere smo s tekočinsko kromatografijo visoke ločljivosti z detektorjem z nizom diod pokazali, da imajo kvalitativno in kvantitativno različne profile fenolnih spojin, smo analizirali na celični ravni modelnega organizma kvasovke *Saccharomyces cerevisiae*. S tekočinsko kromatografijo visoke ločljivosti s tandemsko masno spektrometrijo smo uspešno identificirali bioaktivne fenolne spojine v izbrani frakciji EL70, ki je izmed vseh frakcij najbolj zmanjšala znotrajcelično oksidacijo in najbolj povečala celično metabolno energijo v celicah kvasovk. Spojine so bile sledeče: izoprenilni ester kavne kisline, benzilni ester kavne kisline, fenetilni ester kavne kisline, cimetni ester kavne kisline, benzilni ester *p*-kumarne kisline, krizin, apigenin, luteolin-metil-eter, pinocembrin, kamferid, ramnetin, pinobanksin-5-metil-eter, pinobanksin-3-*O*-acetat, pinobanksin-3-*O*-propionat, pinobanksin-3-*O*-butirat, pinobanksin-3-*O*-pentanoat in 3-prenil-4-(2-metilpropionil-oksi)-cimetna kislina. Za benzilni ester kavne kisline, fenetilni ester kavne kisline in cimetni ester kavne kisline smo pokazali tudi privzem v kvasno celico. Dodatno smo za fenetilni ester kavne kisline pokazali, da zniža znotrajcelično oksidacijo in poviša celično metabolno energijo v celicah kvasovk. Proteomske analize so pokazale, da bioaktivne fenolne spojine frakcije EL70 med drugim vplivajo tudi na metabolizem ogljikovih hidratov in energetski metabolizem, dinamiko aktinskih filamentov, odziv na oksidativni stres in zvijanje proteinov. Pri tem smo za uporabo na kvasovki uspešno prilagodili protokol za diferencialno detergentno frakcionacijo, s katerim se iz celic ekstrahirajo citosolni, membransko/organelni, jedrni in citoskeletni proteini. Ugotovili smo tudi, da izvleček propolisa in izbrana frakcija EL70 povečata sirtuinsko aktivnost v kvasni celici, in sicer v citosolni in jedrni proteinski frakciji. Ugotovitve v doktorski disertaciji dajejo nov vpogled v razumevanje molekularnih mehanizmov v ozadju različnih aktivnosti, ki jih ima izvleček propolisa bogat s fenolnimi spojinami in predstavljajo dodatno referenco za njegovo nadaljnjo uporabo.

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AB Propolis has several biological effects, but it is still not well known, which compounds of propolis are responsible for these effects and what are the molecular mechanisms behind the activity of the bioactive compounds. Thus, we chemically characterized the extract of Slovenian propolis and fractionated it by solid-phase extraction. The influence of individual fractions, which had qualitatively and quantitatively different profiles of phenolic compounds shown by high performance liquid chromatography with diode array detection, was tested at cellular level of a model organism yeast *Saccharomyces cerevisiae*. Next, by high performance liquid chromatography with tandem mass spectroscopy, we identified bioactive phenolic compounds in the selected fraction EL70 that decreased intracellular oxidation and increased cellular metabolic energy in yeast cells the most among fractions. These were caffeic acid isoprenyl ester, caffeic acid benzyl ester, caffeic acid phenethyl ester, caffeic acid cinnamyl ester, *p*-coumaric acid benzyl ester, chrysin, apigenin, luteolin-methyl-ether, pinocembrin, kaempferide, rhamnetin, pinobanksin-5-methyl-ether, pinobanksin-3-*O*-acetate, pinobanksin-3-*O*-propionate, pinobanksin-3-*O*-butyrate, pinobanksin-3-*O*-pentanoate and 3-prenyl-4-(2-methylpropionyl-oxy)-cinnamic acid. We also showed that caffeic acid benzyl ester, caffeic acid phenethyl ester and caffeic acid cinnamyl ester entered the yeast cell. Additionally, caffeic acid phenethyl ester decreased intracellular oxidation and increased cellular metabolic energy in yeast cells. Proteomic analysis showed that bioactive phenolic compounds in fraction EL70 change the abundance of proteins involved in carbohydrate and energy metabolism, actin filament dynamics, oxidative stress response and protein folding. For these analyses we successfully adjusted the protocol for differential detergent fractionation used for extraction of cytosolic, membrane/organelle, nuclear and cytoskeletal proteins from yeast cell. We also showed that propolis extract and selected fraction EL70 increases sirtuin activity in cytosolic and nuclear protein fractions of the yeast cell. Findings of this doctoral dissertation give a new insight into understanding molecular mechanisms behind the activities of propolis extract rich with phenolic compounds and present an additional reference for its further use.

KAZALO VSEBINE

KAZALO ZNANSTVENIH DEL

str.

KAZALO PREGLEDNIC

str.

KAZALO SLIK

str.

KAZALO PRILOG

OKRAJŠAVE

2-DE	dvodimenzionalna elektroforeza
ATP	adenozin trifosfat (ang. adenosine triphosphate)
CABE	benzilni ester kavne kisline (ang. caffeic acid benzyl ester)
CACE	cimetni ester kavne kisline (ang. caffeic acid cinnamyl ester)
CAE	ekvivalent klorogenska kisline (ang. chlorogenic acid equivalent)
CAIE	izoprenilni ester kavne kisline (ang. caffeic acid isoprenyl ester)
CAPE	fenetilni ester kavne kisline (ang. caffeic acid phenethyl ester)
CFU	kolonijske enote (ang. colony forming units)
DAD	detektor z nizom diod (ang. diode array detection)
DDF	diferencialna detergentna frakcionacija
DMSO	dimetil sulfoksid
DPPH	1,1-difenetil-2-pikrilhidrazil (ang. 1,1-diphenyl-2-picrylhydrazyl)
EDTA	etilendiamintetraocetna kislina (ang. ethylenediaminetetraacetic acid)
EL30-EL70	frakcije izvlečka propolisa, ki so bile pripravljene z ekstrakcijo s trdno fazo, kjer smo pri spiranju s kolone zaporedno uporabili naraščajoče koncentracije etanola (od 30 % do 70 %, s koraki po 10 %)
ETV	elektronska transportna veriga
GAE	ekvivalent galne kisline (ang. gallic acid equivalent)
HPLC	tekočinska kromatografija visoke ločljivosti (ang. high performance liquid chromatography)
Hsp	proteini toplotnega šoka (ang. heat shock protein)
MALDI	ionizacija v matriksu z desorpcijo z laserjem (ang. matrix-assisted laser desorption/ionization)
MS	masna spektrometrija (ang. mass spectrometry)
NADPH	reducirana oblika nikotinamid adenin dinukleotid fosfata (ang. nicotinamide adenine dinucleotide phosphate)
RNS	reaktivne dušikove spojine (ang. reactive nitrogen species)
ROS	reaktivne kisikove zvrsti (ang. reactive oxygen species)
SDS	natrijev dodecil sulfat (ang. sodium dodecyl sulfate)
SELDI	površinsko okrepljena ionizacija z desorpcijo z laserjem (ang. surface-enhanced laser desorption/ionization)
sHsp	proteini toplotnega šoka z molekulsko maso 15-30 kDa (ang. small heat shock proteins)
SOD	superoksid dismutaza
SPE	ekstrakcija s trdno fazo (ang. solid phase extraction)
TOF	masni analizator na čas preleta ionov (ang. time-of-flight)
YEPD	gojišče (kvasni ekstrakt, pepton, glukoza) (ang. yeast extract peptone dextrose)

1 PREDSTAVITEV PROBLEMATIKE IN HIPOTEZE

1.1 PREGLED OBJAV

1.1.1 Propolis

Propolis je aromatična smolnata snov zeleno-rumene do temnorjave barve, ki jo čebele nabirajo na rastlinah in uporabljajo v panju v različne namene. Botanično poreklo propolisa so rastline iz rodu *Populus, Baccharis, Betula, Dalbergia, Cupressaceae, Clusia* in *Macaranga* (Sforcin in Bankova, 2011). Čebele naberejo smolo na različnih delih rastlin, predvsem brstih, cvetovih, sadežih in listih (Bokal in Gregori, 2008; Jürgen, 2010). Smolo prinesejo v panj v koških na nožicah, kjer jo druge čebele odstranijo, ob čemer same popolnoma mirujejo (Meglič, 2004). Čebele nato smolo prežvečijo, ji dodajo encime iz sline, zmešajo z voski in uporabijo v panju (Burdock, 1998).

V panju čebele uporabljajo propolis kot gradbeni material, da zapolnijo razpoke, ki povzročajo prepih in kot dezinfekcijsko sredstvo, da preprečijo okužbe. Z njim prekrijejo notranje stene panja in mumificirajo trupla velikih živali, ki jih ne morejo odstraniti iz panja. Poleg tega s propolisom čebele premažejo vhod v panj, kar so opazili tudi prvi čebelarji. Od tod torej izhaja beseda propolis, ki je sestavljena iz latinskih besed *pro-* , kar pomeni pred in *polis-*, kar pomeni mesto (Bokal in Gregori, 2008; Jürgen, 2010).

Človek uporablja propolis že od egipčanskih časov. Danes z njim lajšamo številne zdravstvene težave, kot so okužbe zgornjih dihal, rane in opekline ter herpes (Wagh, 2013). Poleg tega, da ima propolis številne biološke aktivnosti, se je izkazalo tudi, da s sinergističnim delovanjem poveča učinek cepiv (Ashry in Ahmad, 2012), protimikrobnih učinkovin (Stepanović in sod., 2003) in kemoterapije (Oršolić in sod., 2013).

1.1.2 Kemijska sestava propolisa

Na splošno so približno polovica propolisa fenolne snovi, okoli 30 % je voskov in maščobnih kislin, 10 % eteričnih olj, preostanek pa cvetni prah in druge organske snovi (Kosalec in sod., 2004).

Natančnejša kemijska sestava propolisa je odvisna od njegovega geografskega porekla. Tako poznamo več tipov propolisa, in sicer: (i) topolov tip (Evropa, Severna Amerika, netropska Azija, Nova Zelandija); (ii) zelen tip (Brazilija); (iii) brezov tip (Rusija); (iv) rdeč tip (Kuba, Brazilija, Mehika); (v) mediteranski tip (Grčija, Sicilija, Kreta, Malta); (vi) tip "Clusia" (Kuba, Venezuela) in (vii) pacifiški tip (Okinava, Tajvan, Indonezija). Za topolov tip propolisa so značilni flavoni, flavanoni, hidroksicimetne kisline in njihovi estri.

Zelen tip propolisa vsebuje prenilirane *p*-kumarne kisline in diterpenske kisline. Brezov tip propolisa prepoznamo po flavonolih in flavonih, ki so drugačni kot pri topolovem tipu propolisa. Rdeč tip propolisa vsebuje izoflavonoide (izoflavane in pterokarpane). Mediteranski tip propolisa vsebuje diterpene. Za propolis tipa "Clusia" so tipični poliprenilirani benzofenoni, za pacifiški tip propolisa pa C-prenil-flavanoni (Sforcin in Bankova, 2011).

Izvlečki propolisa lahko vsebujejo hidroksicimetne kisline in njihove estre, stilbene, flavonoide (flavonole, flavone, flavanone, dihidroflavonole, halkone), terpene, aromatične aldehide in alkohole, maščobne kisline in β-steroide (Gardana in sod., 2007). Vendar se predpostavlja, da so glavne bioaktivne spojine v propolisu fenolne spojine, in sicer hidroksicimetne kisline in njihovi estri ter flavonoidi (Castaldo in Capasso, 2002).

Fenolne spojine so definirane kot spojine, ki imajo najmanj en aromatski obroč, na katerega je vezana ena ali več hidroksilnih skupin (-OH). Poznanih je že več kot 8000 fenolnih spojin. So sekundarni metaboliti rastlin in do njihovega nastanka vodijo tri različne biogenetske poti: šikimat/arogenatna pot, acetat/malonatna (poliketidna) pot in acetat/mevalonatna (Strack, 1997). Navadno so te spojine bele barve, pojavljajo pa se tudi v rumeni (flavonoidi) in rdeči barvi (antocianini). Nizko-molekularne fenolne spojine so hlapne in imajo pogosto karakteristične arome po metilsalicilatu, vanilinu in eugenolu. Interakcija hidroksilnih skupin s π-elektroni v aromatskemu obroču daje fenolnim spojinam sposobnost, da se pretvorijo v prosti radikal, ki je stabiliziran z delokalizacijo elektronov (Parr in Bolwell, 2000).

Fenolne spojine ločimo glede na ogljikov skelet v 14 razredov, in sicer preproste fenole (C_6), hidroksibenzoate (C_6-C_1), acetofenone in fenilacetate (C_6-C_2), hidroksicinamate, fenolpropene, kumarine in kromone (C_6-C_3), naftokinone (C_6-C_4), ksantone (C_6-C_1-C_6), stilbene in antrakinone (C_6-C_2-C_6), flavonoide (C_6-C_3-C_6), lignane $(C_6\text{-}C_3)_2$, biflavonoide $(C_6\text{-}C_3\text{-}C_6)_2$, kateholne melanine $(C_6)_n$, hidrolizabilne tanine $(C_6\text{-}C_1)_n$:glukoza, lignine $(C_6\text{-}C_3)_n$ in kondenzirane tanine $(C_6\text{-}C_3\text{-}C_6)_n$. Fenolne spojine lahko razdelimo tudi na flavonoidne in neflavonoidne fenolne spojine (Strack, 1997). Za flavonoide sta značilna dva aromatska obroča, ki sta povezana s tremi ogljikovimi atomi (Crozier in sod., 2009).

1.1.3 Biološke aktivnosti propolisa

Propolis ima številne biološke aktivnosti, in sicer protivirusno (Huleihel in Isanu, 2002; Gekker in sod., 2005; Shimizu in sod., 2008; Búfalo in sod., 2009; Schnitzler in sod., 2010; Nolkemper in sod., 2010), protimikrobno (Stepanović in sod., 2003; Mavri in sod., 2012), protiparazitsko (da Silva Cunha in sod., 2004; Salomão in sod., 2004; Dantas in sod., 2006; Ayres in sod., 2007; Machado in sod., 2007; Monzote Fidalgo in sod., 2011; Santana in sod., 2014), antioksidativno (Nakajima in sod., 2009; Mavri in sod., 2012; Žižić in sod.,

2013; de Sá in sod., 2013; Sun in sod., 2000; Jasprica in sod., 2007), imunomodulatorno (Sforcin, 2007; Chan in sod. 2013), protirakavo (Barbarić in sod., 2011; Sawicka in sod., 2012; da Silva Frozza in sod., 2013; Žižić in sod., 2013) in protivnetno (Sforcin, 2007; Chan in sod., 2013).

1.1.3.1 Protivirusna aktivnost propolisa

Ugotovljeno je bilo, da propolis prepreči okužbo gostiteljskih celic z različnimi virusi, kot so herpes simpleks virus tipa 1 (HSV-1) (Huleihel in Isanu, 2002; Schnitzler in sod., 2010), herpes simpleks virus tipa 2 (HSV-2) (Nolkemper in sod., 2010), virus humane imunske pomanjkljivosti tipa 1 (HIV-1) (Gekker in sod., 2005), virus influence (Shimizu in sod., 2008) in poliovirus tipa 1 (PV-1) oz. virus otroške ohromelosti (Búfalo in sod., 2009).

Propolis zaščiti gostiteljsko celico pred okužbo z virusom tako, da: (i) prepreči vstop virusa v celice (Gekker in sod., 2005; Huleihel in Isanu, 2002; Búfalo in sod., 2009); (ii) prepreči razmnoževanje virusa v celicah (Huleihel in Isanu, 2002; Búfalo in sod., 2009) ali (iii) povzroči razgradnjo nukleinske kisline v virusu preden vstopi v celico ali potem ko se sprosti iz celice (Búfalo in sod., 2009).

Okužbo celične kulture z virusom HSV-1 v 50 % prepreči 0,5 % izvleček propolisa. V 80-85 % so neokužene ostale tudi celice, ki so jim 2 uri po izpostavitvi virusu dodali 10 % izvleček. V organizmu je okužbo preprečil 5 % izvleček (Huleihel in Isanu, 2002). Schnitzler in sod. (2010) so pokazali, da je srednja inhibitorna koncentracija (IC_{50}) izvlečka propolisa za virus HSV-1 med 0,000035 % - 0,0004 %, medtem ko so Nolkemper in sod. (2010) pokazali, da je IC_{50} izvlečka propolisa za virus HSV-2 med 0,0004 % - 0,0005 %. Največja zaščita celične kulture pred virusom HIV-1 s strani izvlečka propolisa je bila zaznana pri koncentraciji 66,6 μg/mL (Gekker in sod., 2005).

Vsi izvlečki propolisa niso enako učinkoviti. Izmed trinajstih različnih izvlečkov, ki so jih dodali organizmu po okužbi z virusom influence, so le štirje preprečili razvoj bolezni (Shimizu in sod., 2008). Na splošno se učinkovitost izvlečka propolisa proti virusom poveča sorazmerno s povečanjem njegove koncentracije (Gekker in sod., 2005; Shimizu in sod., 2008).

Proti virusu PV-1 so učinkovali tudi izvleček rastline *Baccharis dracunculifolia*, ki je vir zelenega propolisa, kavna kislina in cimetna kislina (Búfalo in sod., 2009). Protivirusno aktivnost proti HSV-1 sta imeli fenolni spojini galangin in krizin iz propolisa, medtem ko druge fenolne spojine iz propolisa, kot so kavna kislina, *p*-kumarna kislina, benzojska kislina in pinocembrin niso imele učinka (Schnitzler in sod., 2010).

1.1.3.2 Protibakterijska in protiglivna aktivnost propolisa

Propolis ima tudi protibakterijsko aktivnost in protiglivno aktivnost (Stepanović in sod., 2003; Mavri in sod., 2012). Izkazalo se je, da je propolis bolj učinkovit proti grampozitivnim bakterijam (minimalna inhibitorna koncentracija (MIC) = 0,078 % - 1,25 %) in kvasovkam (MIC = 0,16 % - 1,25 %), kot proti gramnegativnim bakterijam (MIC = 1,25 % - >5 %). Najbolj odporna med grampozitivnimi bakterijami je *Enterococcus faecalis*, med kvasovkami *Candida albincans* in med gramnegativnimi bakterijami *Salmonella* spp. (Stepanović in sod., 2003).

Dodatno so Mavri in sod. (2012) pokazali, da je propolis različno učinkovit tudi znotraj skupine gramnegativnih bakterij. Tako je bolj učinkovit proti bakterijam *Campylobacter* (MIC = 0,27 - 0,38 mg ekvivalenta klorogenske kisline (CAE)/mL), kot proti bakterijam *Salmonella* in *E. coli* (MIC = 0,66 - 0,78 mg CAE/mL). MIC za grampozitivne bakterije so določili med 0,49 - 0,59 mg CAE/mL, za glive pa med 0,61 - 0,72 mg CAE/mL. Posamezne fenolne spojine propolisa, kot so kvercetin, pinocembrin, klorogenska kislina, ferulna kislina in kavna kislina niso imele protimikrobne aktivnosti za *Streptococcus pyogenes*, *Salmonella enteritidis* in *Penicillium* sp. (Mavri in sod., 2012).

1.1.3.3 Protiparazitska aktivnost propolisa

Več avtorjev poroča tudi o protiparazitski aktivnosti propolisa, in sicer, da učinkuje proti praživalim, ki povzročajo tripanosimiazo oz. spalno bolezen (*Trypanosoma cruzi*) (da Silva Cunha in sod., 2004; Salomão in sod., 2004; Dantas in sod., 2006), lišmaniozo (*Leishmania amazonensis*, *L. braziliensis*, *L. chagasi* in *L. major*) (Ayres in sod., 2007; Machado in sod., 2007; Santana in sod., 2014) in trihomoniazo (*Trichomonas vaginalis)* (Monzote Fidalgo in sod., 2011).

Dantas in sod. (2006) so pokazali, da so glavne tarče delovanja propolisa v parazitih mitohondriji in rezervosomi. Medtem ko drugi avtorji poročajo, da je protiparazitska aktivnost propolisa bolj posledica tega, da aktivira imunski sistem gostitelja, kot tega, da ima toksični učinek na zajedavce (Ayres in sod., 2007).

Podobno kot pri protivirusni aktivnosti propolisa, vsi izvlečki propolisa niso enako učinkoviti proti parazitom. Le pet izmed osemnajstih različnih izvlečkov propolisa je zmanjšalo živost praživali *T. vaginalis* pri koncentraciji nižji od 10 µg/mL (Monzote Fidalgo in sod., 2011). Tudi petnajst različnih izvlečkov propolisa je imelo različno učinkovitost proti *T. cruzi* (da Silva Cunha in sod., 2004). IC_{50} različnih izvlečkov propolisa za *Leishmania* spp je bila določena med 2,8 - 229,3 µg/mL (Machado in sod., 2007). Na splošno je učinkovitost proti praživalim odvisna od koncentracije izvlečka propolisa in časa izpostavitve (Ayres in sod., 2007).

Kljub pokazani protiparazitski aktivnosti propolisa je potrebno natančneje ovrednotiti njegov učinek na gostitelja, saj nekateri avtorji poročajo o poškodbah gostiteljskih celic po izpostavitvi propolisu (Dantas in sod., 2006; Monzote Fidalgo in sod., 2011).

1.1.3.4 Antioksidativna aktivnost propolisa

Literatura kaže, da ima propolis antioksidativno aktivnost. Ob izpostavitvi izvlečku propolisa se je znotrajcelična oksidacija zmanjšala v celicah kvasovke *S. cerevisiae* (Mavri in sod., 2012) in človeški celični liniji HCT-116 (rak debelega črevesa) (Žižić in sod., 2013). Pri podganah se je izpostavitev propolisu pokazala v zmanjšani lipidni peroksidaciji celic debelega črevesa (Sun in sod., 2000). Pri zdravih ljudeh se je izpostavitev propolisu v eritrocitih pokazala v zmanjšani koncentraciji malonaldehida (23,2 %) in povečani aktivnosti superoksid dismutaze (SOD) (20,9 %) (Jasprica in sod., 2007).

Izvleček propolisa zaščiti celice tudi pred oksidativnim stresom. Predtretiranje kvasovke *S. cerevisiae* z izvlečkom propolisa je povzročilo, da je po izpostavitvi menadionu in vodikovemu peroksidu nastalo manj ROS in se je zmanjšala lipidna peroksidacija. Obenem se je povečala tudi aktivnost Cu-Zn SOD (de Sá in sod., 2013).

Nakajima in sod. (2009) so pokazali, da je propolis učinkovitejši *in vivo* antioksidant v primerjavi z ostalimi čebeljimi produkti, kot sta matični mleček in cvetni prah.

1.1.3.5 Imunomodulatorna aktivnost propolisa

Pokazano je bilo, da propolis vpliva tudi na imunski sistem, saj poveča mikrobicidno aktivnost makrofagov (Sforcin, 2007; Chan in sod. 2013), litično aktivnost celic ubijalk in produkcijo protiteles (Sforcin, 2007). Chan in sod. (2013) navajajo, da sta za imunomodulatorno aktivnost propolisa najbolj odgovorni njegovi fenolni spojini fenetilni ester kavne kisline (CAPE) in artepilin C.

1.1.3.6 Protirakava aktivnost propolisa

Predmet raziskav je tudi protirakava aktivnost propolisa, ki je bila pokazana na različnih človeških celičnih linijah, kot so Hep-2 (rak grla) (da Silva Frozza in sod., 2013), HeLa (rak materničnega vratu) (da Silva Frozza in sod., 2013; Barbarić in sod., 2011) in HCT-116 (rak debelega črevesa) (Žižić in sod., 2013).

IC_{50} izvlečka propolisa za človeško celično linijo HCT-116 je bila določena med 26,33 - 143,09 μg/mL (Žižić in sod., 2013), medtem ko je bila srednja inhibitorna

koncentracija celične proliferacije (GI_{50}) izvlečka propolisa za človeško celično linijo HeLa 76 μg/mL (Barbarić in sod., 2011).

Protirakava aktivnost propolisa je povezana z zaustavitvijo proliferacije in apoptozo tumorskih celic. Zaustavitev proliferacije poteka preko: (i) inhibicije kompleksov med ciklini in od-ciklina-odvisnimi-kinazami; (ii) povečane ravni proteinskih inhibitorjev p21, p16 in p27 in (iii) zmanjšane ravni β-katenina. Indukcijo apoptoze pa povzroči: (i) aktivacija proteinov Bax, p53, p21, p38 MAPK, JKN kinaz in ERK kinaz; (ii) sprostitev citokroma c v citosol; (iii) aktivacija kaskade kaspaz; (iv) inhibicija transkripcijskega faktorja NF-κB; (v) inhibicija protiapoptotskih proteinov IAP, c-FLIP in Akt kinaze in (vi) aktivacija TRAIL in Fas receptorjev (Sawicka in sod., 2012). Protirakavo aktivnost imata tudi fenolni spojini krizin in CAPE, ki ju najdemo v propolisu. Vendar pri tem Sawicka in sod. (2012) navajajo, da molekularni mehanizmi delovanja teh spojin niso enaki kot pri propolisu.

Posamezne biološke aktivnosti propolisa so med seboj povezane. Tako je posledica znižanja znotrajcelične oksidacije v celicah lahko tudi protitumorsko ali protivnetno delovanje. Tudi stimulacija imunskega sistema lahko vodi v protirakavo delovanje. Posledica zaustavitve proliferacije v normalnih celicah, kot so limfociti, pa je protivnetno delovanje (Chan in sod., 2013; Sforcin, 2007).

1.1.3.7 Sinergistični učinek propolisa

Analiza literature pokaže, da imajo fenolne spojine v propolisu sinergističen učinek in zato večjo učinkovitost kot posamezne fenolne spojine propolisa. Tako je imel izvleček propolisa večjo protivirusno aktivnost in selektivnost kot njegove posamezne komponente (Schnitzler in sod., 2010). Enako za protibakterijsko aktivnost navajajo, da je najbrž posledica sinergističnega učinka spojin v propolisu (Mavri in sod., 2012). Tudi Barbarić in sod. (2011) navajajo, da protirakava aktivnost ni v korelaciji s posameznimi spojinami propolisa, ampak je posledica sinergističnega učinka fenolnih kislin in flavonoidov.

1.1.4 Nekateri metodološki pristopi proučevanja bioloških aktivnosti

1.1.4.1 Določanje protivirusne aktivnosti

Pri določanju protivirusne aktivnosti virus najprej inokuliramo v enega izmed sistemov za razmnoževanje virusov, kar so lahko celične kulture ali poskusne živali. Najpogosteje se za določanje protivirusne aktivnosti uporabljajo celične linije. Tako so npr. za testiranje izvlečka propolisa pri okužbah z virusom HSV-1 raziskovalci izbrali celice Vero (trajna linija ledvičnih celic afriške zelene opice) (Huleihel in Isanu, 2002), z virusom HSV-2

celice RC-37 (trajna linija ledvičnih celic afriške zelene opice) (Nolkemper in sod., 2010), z virusom HIV-1 $CD4^+$ limfocite in mikroglialne celice (Gekker in sod., 2005), z virusom influence celice MDCK (trajna celična linija pasjih ledvičnih epitelnih celic) (Shimizu in sod., 2008) in z virusom PV-1 celice HEp-2 (trajna linija celic humanega karcinoma grla) (Búfalo in sod., 2009).

Z namenom, da se ugotovi kako testirana snov prepreči okužbo celic z virusom, se le-ta lahko doda celični liniji oz. poskusni živali pred, med ali po inokulaciji z virusom oz. se testni snovi pred inokulacijo izpostavi virus (Schnitzler in sod., 2010; Búfalo in sod. 2009).

Pri celičnih kulturah protivirusno aktivnost najpogosteje določamo s štetjem plakov, ki nastanejo v trdnem gojišču po inokulaciji celične kulture z virusom. Plaki so prazna mesta na trdnem gojišču, kjer je virus okužil celice in jih liziral. V primeru, da ima testirana spojina protivirusno aktivnost, se število plakov zmanjša. Rezultati so izraženi kot efektivna koncentracija, ki povzroči 50 % zmanjšanje formacije plakov (EC_{50}) (Shimizu in sod., 2008; Nolkemper in sod., 2010). Ker je protivirusna aktivnost testirane snovi sorazmerna odstotku živih celic po inokulaciji, lahko za njeno določanje uporabimo tudi metode za določanje celične živosti, npr. metodo z barvilom kristal vijolično, ki obarva vse nelizirane celice (Búfalo in sod., 2009). Viruse lahko dokažemo tudi z imunskimi metodami (npr. ELISA), kjer dokazujemo virusne antigene ali protitelesa v krvi okužene živali (Gekker in sod., 2005) in verižno reakcijo s polimerazo (PCR) v realnem času, kjer dokazujemo virusno DNK (Búfalo in sod., 2009).

Včasih se kot sistem za razmnoževanje virusov uporabljajo tudi poskusne živali, kot so miši (Shimizu in sod., 2008), podgane in zajci (Huleihel in Isanu, 2002). Tu se lahko kot indikator okužbe z virusom uporablja število preživelih osebkov ali spremembe v telesni teži (Shimizu in sod., 2008).

1.1.4.2 Določanje protiparazitske aktivnosti

Pri določanju protiparazitske aktivnosti se preverja število okuženih celic in število znotrajceličnih parazitov (Ayres in sod., 2007; Santana in sod., 2014).

Pri bioloških aktivnostih, kot sta protivirusna in protiparazitska, je zelo pomembna selektivnost testiranega vzorca. Zaželeno je namreč, da testiran vzorec učinkuje na virus oz. parazit in ne na gostiteljsko celico.

1.1.4.3 Določanje celične živosti

Z določanjem celične živosti po izpostavitvi testiranemu vzorcu lahko določimo citotoksičnost testiranega vzorca. Slednja je zaželena pri bioloških aktivnostih, kot sta

protimikrobna in protiparazitska. Rezultati se izrazijo kot citotoksična oz. toksična koncentracija testiranega vzorca, ki povzroči 50 % zmanjšanje celične živosti (CC_{50} oz. TC_{50}) (Shimizu in sod., 2008; Nolkemper in sod., 2010).

Poleg tega lahko z določanjem celične živosti po izpostavitvi testiranemu vzorcu določimo tudi vpliv na celično proliferacijo. Celična proliferacija pomeni povečanje števila celic zaradi rasti in delitve in je povezana z rakavimi obolenji. Spojine s protirakavim delovanjem tako zavirajo celično proliferacijo. Rezultati so izraženi kot koncentracija, ki povzroči 50 % zmanjšanje celične proliferacije (GI_{50}) (Žižić in sod., 2013; Barbarić in sod., 2011). Za določanje celične proliferacije se pogosto uporablja MTT (3-4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolijev bromid) test, ki temelji na dejstvu, da žive celice z aktivnim metabolizmom spremenijo MTT v vijoličen produkt formazan, ki ga zaznamo spektrofotometrično (Mosmann, 1983).

Celično živost določimo tudi kadar iščemo ugodne biološke aktivnosti, kot je antioksidativna. V tem primeru je namreč pomembno, da ne izberemo previsokih koncentracij, ki bi povzročile oksidativen učinek in morebitno celično smrt.

Eden izmed komercialno dostopnih kompletov barvil za ugotavljanje celične živosti mikroorganizmov je "Live/Dead FungaLight", ki ga sestavljata dve barvili za nukleinske kisline. Zeleno fluorescentno barvilo SYTO 9 označi vse celice, medtem ko rdeče fluorescentno barvilo propidijev jodid prodre le v celice s poškodovano celično membrano. V primeru, da sta v celici prisotni obe barvili, se zaradi fluorescenčnega resonančnega prenosa energije zmanjša fluorescenca SYTO 9. Merjenje celične živosti tako temelji na poškodbi celične membrane, zato določimo za mrtve tudi celice s poškodovano celično membrano, ki pa so sposobne obnovitve in razmnoževanja. Poleg tega za žive določimo tiste celice, ki imajo nepoškodovano membrano, niso pa sposobne razmnoževanja.

Živost bakterijskih in kvasnih celic lahko določimo tudi s štetjem kolonijskih enot (ang. colony forming units, CFU) ali preko določanja ATP, ki je indikator metabolno aktivnih celic, saj ga živi organizmi porabljajo za rast in vzdrževanje celičnih funkcij (Erecińska in Wilson, 1982). Eden izmed komercialno dostopnih kompletov za določanje ATP je "BacTiter-Glo", ki vsebuje luciferin in encim luciferazo. Encim luciferaza ob prisotnosti ATP in kisika pretvori luciferin v oksiluciferin, ob čemer nastane svetloba. Metoda nam razkrije raven ATP v celici, ne pa če je spremenjena raven ATP posledica spremenjene sinteze ali porabe ATP v celici.

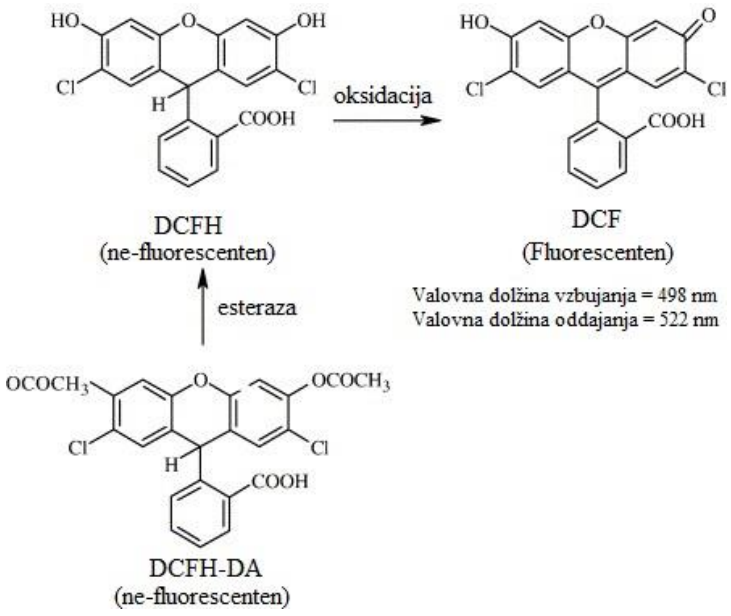
1.1.4.4 Določanje antioksidativne aktivnosti

Antioksidativna aktivnost testiranega vzorca se lahko določa na različne načine. Prvič, lahko se določa preko merjenja znotrajcelične oksidacije oz. ravni reaktivnih kisikovih

zvrsti (ROS) ali reaktivnih dušikovih zvrsti (RNS) v celici. Če se v celici ob izpostavitvi testirani spojini raven ROS/RNS zmanjša, ima le-ta antioksidativno aktivnost.

ROS/RNS v celicah lahko določimo z različnimi fluorescenčnimi barvili. Za določanje superoksidnega aniona ($O_2^{\bullet -}$) se uporabljajo barvila hidroetidin, 1,3-difenilizobenzofuran in 2-(2-piridil)-benzotiazolin. Prisotnost vodikovega peroksida (H_2O_2) lahko potrdimo z barvili 2,7-diklorodihidrofluorescein (DCFH), skopoletin, "Amplex" rdeče, homovanilna kislina in dihidrorodamin 123. Za določanje singlet kisika ($^1O_2$) se uporabljajo barvila 9,10-dimetilantracen, 9-[2-(3-karboksi-9,10-difenil)antril]-6-hidroksi-3H-ksanten-3-on in 9-[2-(3-karboksi-9,10-dimetil)antril]-6-hidroksi-3H-ksanten-3-on. Hidroksilni radikal ($HO^{\bullet}$) se določa z barvili 4-(9-antroiloksi)-2,2,6,6-tetrametilpiperidin-1-oksil, 1,3-cikloheksandion, natrijev tereftalat, kumarin, kumarin-3-karboksilna kislina, N-sukcinimidilni ester kumarin-3-karboksilne kisline, 2-[6-4'-hidroksi)fenoksi-3H-ksanten-3-on-9-il]benzojska kislina, 2-[6-4'-amino)fenoksi-3H-ksanten-3-on-9-il]benzojska kislina in fluorescein. Peroksilni radikal ($ROO^{\bullet}$) zaznamo z barvili cis-parinarna kislina, 4,4-difluoro-5-(4-fenil-1,3-butadienil)-4-bora-3a,4a-diaza-s-indacen-3-undekanojska kislina, lipofilni derivati fluoresceina, dipiridamol, difenil-1-pirenilfosfin, DCFH, β-fikoeritrin, fluorescein in 6-karboksilfluorescein (Gomes in sod., 2005).

Barvilo DCFH se oksidira z različnimi ROS (H_2O_2, $HO^{\bullet}$, $ROO^{\bullet}$) in RNS ($^{\bullet}NO$, $ONOO^-$), zato se pogosto uporablja za določanje antioksidativne aktivnosti testiranih spojin. Celicam dodamo nepolarno obliko spojine 2,7-diklorodihidrofluorescein diacetat (H_2DCFDA), ki difundira skozi celično membrano v celico, kjer jo nespecifične esteraze hidrolizirajo. Nastane 2,7-diklorodihidrofluorescein (DCFH), ki je bolj polarna oblika spojine in se zato se zadrži v celici ter se v prisotnosti ROS in RNS oksidira do fluorescentne oblike 2,7-diklorofluoresceina (DCF). Nastanek fluorescentne oblike barvila je indikator znotrajcelične oksidacije (Slika 1) (Jakubowski in Bartosz, 1997; Gomes in sod., 2005).



Slika 1: De-esterifikacija 2,7-diklorodihidrofluorescein diacetata (DCFH-DA) do 2,7-diklorodihidrofluoresceina (DCFH) in oksidacija le tega z ROS in RNS do fluorescentnega diklorofluoresceina (DCF) (Gomes in sod., 2005)

Figure 1: De-esterification of 2,7-diclorodyhidrofluorescein diacetate (DCFH-DA) to 2,7-diclorodihydrofluorescein (DCFH) and its oxidation with ROS and RNS to fluorescent dicloroflurescein (DCF) (Gomes et al., 2005)

Pri izbiranju najbolj ustreznega fluorescentnega barvila se opremo na naslednje parametre: (i) topnost barvila v vodnem in lipidnem okolju; (ii) prehodnost barvila skozi celične membrane in znotrajcelična porazdelitev; (iii) specifičnost in občutljivost barvila; (iv) inkubacijski čas; (v) zahteve po posebnem ravnanju z vzorcem; (vi) interferenca s pH in topili; (viii) stabilnost barvila in (ix) valovna dolžina vzbujanja in oddajanja (Gomes in sod., 2005).

Drugič, antioksidativna aktivnost testiranih spojin se lahko določa tudi preko merjenja oksidativnih poškodb v celici. ROS/RNS, predvsem superoksidni anion, hidroksilni radikal, vodikov peroksid in singlet kisik, z oksidacijo lipidov, proteinov in nukleinskih kislin poškodujejo sestavne dele celic. V primeru, da se raven oksidativnih poškodb v celici po izpostavitvi testirani spojini zmanjša, ima testirana spojina antioksidativno aktivnost.

Proteine oksidirajo vse ROS/RNS, lahko pa reagirajo tudi s produkti lipidne peroksidacije. Superoksidni anion oksidira 4Fe-4S skupine v encimih, pri čemer se sprosti železo in s tem inaktivira encim. Vodikov peroksid je šibek oksidant in lahko inaktivira encime z oksidacijo tiolne skupine na cisteinu, ki se nahaja v aktivnemu mestu proteina. Oksidacija tiolne skupine vodi v nastanek disulfidov med cisteini v proteinu in tiolnimi skupinami v glutationu, cisteinu ali γ-glutamil-cisteinu. Oksidirani cisteini v proteinih se reaktivirajo z detiolacijo preko redukcije z glutationom, glutaredoksinom, tioredoksinom ali

disulfid-izomerazo. Vodikov peroksid oksidira tudi metionin v metionin sulfoksid ali metionin sulfon. Manj oksidirane proteine lahko popravi metionin sulfoksid-reduktaza z redukcijo metionin sulfoksida v metionin. Oksidacija aminokislin arginina, prolina, lizina in histidina z vodikovim peroksidom vodi v nastanek karbonilov. Nastanek metionin sulfona in karbonilov je ireverzibilen, zato se proteini s takšnimi spremembami razgradijo (Costa in Moradas-Ferreira, 2001).

Oksidacije nukleinskih kislin vodijo v poškodbe baz in sladkorjev, cepljenje verige, abaznih mest in povezav med DNA in proteini. Kljub temu, da superoksidni anion in vodikov peroksid ne reagirata direktno z DNA, iz njiju nastaja hidroksilni radikal, ki poškoduje DNA. Eden od glavnih produktov poškodovanih baz je 8-hidroksigvanin (Moradas-Ferreira in sod., 1996).

Oksidacija lipidov vodi v nastanek krajših maščobnih kislin in s tem večjo fluidnost membrane. Poleg tega ob oksidaciji lipidov nastanejo zelo reaktivni produkti, kot so epoksidi, aldehidi in alkani, ki nadaljnje poškodujejo DNA in proteine (Moradas-Ferreira in sod., 1996).

Tretjič, antioksidativno aktivnost testiranih spojin lahko preverimo tudi preko stanja primarnih in sekundarnih antioksidativnih obrambnih sistemov. Primarni antioksidativni obrambni sistemi v kvasovki *S. cerevisiae*, ki vzdržujejo ROS/RNS na fiziološki varni ravni, tako da nevtralizirajo ROS/RNS, se delijo na encimske in neencimske. Encimski endogeni antioksidativni obrambni sistemi so Cu,Zn SOD (odstranjevanje $O_2^{\bullet-}$ v citoplazmi), Mn-superoksid dismutaza (odstranjevanje $O_2^{\bullet-}$ v mitohondriju), katalaza A (razgradnja H_2O_2 v peroksisomih), katalaza T (razgradnja H_2O_2 v citoplazmi), citokrom-c-peroksidaza (razgradnja H_2O_2 v mitohondriju), glutation-reduktaza (redukcija oksidiranega glutationa), glukoza-6-fosfat-dehidrogenaza (redukcija $NADP^+$ v NADPH) in tioredoksin-peroksidaza (razgradnja H_2O_2 in alkiliranih hidroperoksidov) (Moradas-Ferreira in sod., 1996).

Neencimski antioksidativni obrambni sistemi so glutation (redukcija proteinskih disulfidov, odstranjevanje prostih radikalov, združevanje z elektrofili, vezava Cd), metalotioneini (vezava Cu, preprečevanje Fentonove reakcije, odstranjevanje $O_2^{\bullet-}$ in $HO^{\bullet}$), tioredoksin (redukcija proteinskih disulfidov) in poliamini (zaščita lipidov pred oksidacijo). Med slednjimi je posebno pomemben glutation, ki ima redoks občutljivo sulfhidrilno skupino in zato reagira z oksidanti (Moradas-Ferreira in sod., 1996).

Sekundarni antioksidativni obrambni sistemi, ki popravljajo in odstranjujejo produkte oksidativnih poškodb na DNA, proteinih in lipidih, so 8-okso-gvanin-glikozilaza/liaza (izrezovanje oksidiranih DNA baz), AP-endonukleaza (rezanje apurinskih/apirimidinskih (AP) mest, tvorba 3'-hidroksilnih skupin na AP mestih), metionin-sulfoksid-izomeraza (redukcija metionin-sulfoksidov), protein-disulfid-izomeraza (redukcija disulfidnih mostičkov v proteinih), glutation (redukcija disulfidnih mostičkov v proteinih), tioredoksin

(redukcija disulfidnih mostičkov v proteinih) in proteini toplotnega šoka (Hsp) (sodelujejo pri razgradnji oksidiranih proteinov) (Moradas-Ferreira in sod., 1996).

Nekateri antioksidativni obrambni sistemi so stalno prisotni, medtem ko so drugi inducirani kot odziv na povišane ravni ROS/RNS v celici (Jamieson, 1998). Kvasovke lahko zaznajo povišane ravni ROS/RNS v celici in so se sposobne na to odzvati na molekularni ravni z indukcijo primarnega in sekundarnega antioksidativnega obrambnega sistema. Prilagoditev na stresne razmere vključuje zgodnje odzive, ki zagotovijo takojšnjo zaščito proti subletalnim stresnim pogojem in pozne odzive, ki zagotovijo bolj učinkovito zaščito proti resnemu stresu. Zgodnji odzivi so posttranslacijska aktivacija že prej obstoječih antioksidativnih obrambnih sistemov in aktivacija signalnih poti, ki aktivirajo pozne odzive. Pozni odzivi so induciranje novih antioksidativnih obrambnih sistemov (Moradas-Ferreira in sod., 1996).

1.1.5 Molekularne tarče propolisa

Fenolne spojine so znani lovilci prostih radikalov *in vitro*, zato se je predvidevalo, da so njihove številne biološke aktivnosti posledica antioksidativnega delovanja v celicah, tj. znižanja znotrajcelične oksidacije. Kasneje se je izkazalo, da je delovanje fenolnih spojin *in vivo* veliko bolj kompleksno, zato se je pozornost raziskovalcev usmerila h konceptu fenolnih spojin kot modulatorjev receptorjev, encimov in transkripcijskih faktorjev (Fraga in sod., 2010). Kljub temu, da so v nekaterih študijah že iskali molekularne tarče propolisa na mRNA (Gruber in Holtz, 2010; de Castro in sod., 2012) in proteomski ravni (Barlak in sod., 2011; da Silva Frozza in sod., 2014), njegovi natančni mehanizmi delovanja v celicah ostajajo neznani.

Vpliv propolisa na mRNA so analizirali Gruber in Holtz (2010) in de Castro in sod. (2012). V študiji Gruber in Holtz (2010) so izvlečku propolisa izpostavili človeške kožne celice, in sicer dermalne fibroplaste in epidermalne keratinocite.

V dermalnih fibroblastih so zaznali:

- povišano raven mRNA za adenozin trifosfat (ATP)/citrat-liazo (Acly), akvaporin 1 (Aqp1), kolagen tipa 1 (Col1a1), citokrom c-oksidazo 1 (Cox1), granulin (Grn), dušikov oksid (NO)-sintazo 3 (Nos3), lizin-hidroksilazo 3 (Plod3), receptor za retinojsko kislino α (Rara), tioredoksin (Txn) in
- znižano raven mRNA za dezmoglein 3 (Dsg3), hialuronan-sintazo 1 (Has1), interlevkin 1 (Ilia), protein Klotho (Kl), NO-sintazo 2 (Nos2), receptor za progesteron (Pgr).

V epidermalnih keratinocitih se je pokazala:

- povišana raven mRNA za ATP/citrat-liazo (Acly), akvaporin 1 in 3 (Aqp1, Aqp3), transmembranski protein CD44 (Cd44), kadherin 1 (Cch1), citokrom c-oksidazo 1 (Cox1), fibroblastni rastni faktor 1 (Fgf1), granulin (Grn), protein toplotnega šoka

Hsp27 (Hspb1), keratin 5 (Krt5), NO-sintazo 3 (Nos3), lizin-hidroksilazo 3 (Plod3), histamin sproščajoči faktor (Tpt1), tioredoksin (Txn) in

- znižana raven mRNA za androgenski receptor (Ar), citoglobin (Cygb), epidermalni rastni faktor (Egf), estrogenski receptor 2 (Esr2), fibrilin 1 in 2 (Fbn1, Fbn2), insulinu podoben rastni faktor 1 (Igf1), protein Klotho (Kl), receptor za melanokortin 1 (Mc1r), receptor za progesteron (Pgr), proopiomelanokortin (Pomc), receptor aktiviran s proliferatorjem peroksisomov γ (Pparg), receptor 1 za prostaglandin E (Ptger1), RAD23 homolog A (Rad23), receptor za retinoid X (Rxra), Sod3, 5α-steroid-reduktaza A2 (Srd5a2), telomerazo (Tert), tirozinazi sorodni peptid 1 (Tyrp1).

Tako sta se oba tipa celic na izpostavitev propolisu v raziskavi Gruber in Holtz (2010) odzvala s povišano ravnjo mRNA za ATP/citrat-liazo, akvaporin 1, citokrom c-oksidazo 1, NO-sintazo 3, lizin-hidroksilazo 3, granulin in tioredoksin ter znižano ravnjo mRNA za protein Klotho in receptor za progesteron.

Kasneje so de Castro in sod. (2012) spremembe na ravni mRNA raziskovali v kvasovki *S. cerevisiae*, ki so jo izpostavili letalni koncentraciji propolisa (0,125 %). Pokazali so povišano raven mRNA proteinov vpletenih v:

- transmembranski transport in lokalizacijo: protein 1 za odpornost proti flukonazolu (Flr1), protein 1 za odpornost proti azolu (Azr1), od ATP odvisne permeaze PDR10, PDR12 in PDR15 (Pdr10, Pdr12, Pdr15), od ATP odvisne permeaze YOR1 za odpornost proti oligomicinu (Yor1), Na^+-ATPazi 1 in 2 (Ena1, Ena2), intermembranski protein VMA21, ki sestavlja vakuolarno ATPazo (Vma21), transporterja poliaminov 1 in 4 (Tpo1, Tpo4), protein 22, ki je povezan z avtofagijo (Atg22), podenota F ATPaze tipa V (Vma7), sestavni faktor PKR1 ATPaze tipa V (Pkr1) in
- odziv na stres: monotiolni glutaredoksin 4 (Grx4), glutation S-transferaza (Gtt2), glutation-transferaza 3 (Gtt3), peroksiredoksin TSA2 (Tsa2), tioredoksin 1 (Trx1), 6-P-glukonat-dehidrogenaza 2 (Gnd2).

Znižano raven mRNA so določili za proteine vpletene v:

- celični cikel: protein 4, ki vzdržuje strukturo kromosomov (Smc4), protein 10, ki vzdržuje minikoromosome (Mcm10), protein CIN8, ki je podoben kinezinu (Cin8), protein IBD2, podenota 2 kondenzinskega kompleksa (Ibd2), podenota B kompleksa CBF3, ki se veže na centromere (Cbf3), proteina kinetohora SLK19 in SPC24 (Slk19, Spc24), protein NSL1, ki je povezan z kinetohorom (Nsl1), verjetna histon-deacetilaza HOS2 (Hos2), histon-acetiltransferaza ESA1 (Esa1), podenota MAM1 kompleksa monopolin (Mam1),
- organizacijo kromosomov: podenota p90 v faktorju 1, ki sodeluje pri sestavljanju kromatina (Rfl2), faktor 29 povezan s histon-acetiltransferaznim kompleksom SAGA (Sgf29) in
- metabolizem in sintezo RNA: protein LEO1 povezan z RNA-polimerazo (Leo1), protein SPT21, represor proteina 1 v MSE (Rfm1), podenota 2 proteina INO80

(Ies2), IFH protein, podenota NGG1 v kompleksu, ki sodeluje pri remodeliranju kromatina (Ngg1).

Raziskavi Gruber in Holtz (2010) in de Castro in sod. (2012) sta v celicah tretiranih z izvlečkom propolisa pokazali le eno skupno molekularno tarčo, in sicer tioredoksin, katerega mRNA je imela povišano raven. Tioredoksin sodeluje v različnih redoks reakcijah preko reverzibilne oksidacije dveh tiolnih skupin v njegovem aktivnem mestu v disulfid (Jacquot in sod., 1990).

Vpliv propolisa na proteom so preučevali Barlak in sod. (2011) in da Silva Frozza in sod. (2014). Obe študiji sta bili izvedeni na tumorskih celicah. Barlak in sod. (2011) so propolisu izpostavili človeško celično linijo PC-3 (rak prostate). Uporabili so površinsko okrepljeno ionizacijo z desorpcijo z laserjem z masnim analizatorjem na čas preleta ionov (SELDI-TOF) ter pri tretiranju z izvlečkom propolisa v dimetil sulfoksidu (DMSO) pokazali spremembe, vendar jih niso identificirali.

Da Silva Frozza in sod. (2014) so za analizo vpliva propolisa na proteom uporabili dvodimenzionalno elektroforezo (2-DE). Človeško celično linijo Hep-2 (rak grla) so izpostavili izvlečku propolisa pri koncentraciji 120 μg/mL (IC_{50}) in našli znižano raven proteinov:

- citoskeleta: 1-A veriga tubulina α (Tuba1a), vimentin (Vim),
- metabolizma ogljikovih hidratov: B veriga laktat-dehidrogenaze (Ldhb),
- antioksidativnega obrambnega sistema: peroksiredoksin 2 (Prdx2) in
- proteinov, ki sodelujejo pri zvijanju proteinov: protein iz družine Hsp70 (Grp78).

Ko so da Silva Frozza in sod. (2014) celice izpostavili propolisu pri koncentraciji, ki nima vpliva na celično živost (6 μg/mL), so našli povišano raven proteinov RAD23B, ki popravlja UV poškodbe z izrezovanjem in P0 proteina ribosomske podenote 60S (Rplp0).

1.1.6 Proteomski pristop za proučevanje mehanizma delovanja bioaktivnih spojin

Če želimo razložiti mehanizem delovanja bioaktivnih učinkovin, moramo poznati njihove molekularne tarče. Za to zahtevno nalogo so se razvile t.i. "omike" (transkriptomika, proteomika, metabolomika). To so orodja, ki omogočijo celostni pristop k analizi različnih celičnih gradnikov. Transkriptomika je skupek metod, ki omogoča analizo celokupne mRNA v bioloških sistemih v določenem trenutku, proteomika omogoča analizo celokupnih proteinov v bioloških sistemih v določenem trenutku, metabolomika pa omogoča analizo celokupnih metabolitov v bioloških sistemih v določenem trenutku (Frank in sod., 2006).

Dejstvo, da so proteini neposredno povezani z encimsko aktivnostjo in so nosilci funkcij v celici, govori v prid uporabi proteomike za identifikacijo molekularnih tarč bioaktivnih

spojin. Poleg tega spremembe v ravni mRNA še ne pomenijo sprememb tudi na proteinski ravni (Cyrne in sod., 2003).

V proteomiki se največ uporablja 2-DE, s katero proteine ločimo glede na izoelektrično točko v 1. dimenziji (izoelektrično fokusiranje) in glede na molekulsko maso v 2. dimenziji (poliakrilamidna gelska elektroforeza z natrijevim dodecil sulfatom (SDS)). Običajen postopek iskanja molekularnih tarč bioaktivnih spojin z 2-DE vsebuje naslednje korake: (i) ekstrakcija proteinov iz celic in določanje njihove koncentracije; (ii) 2-DE in detekcija proteinskih lis; (iii) primerjava profilov proteinskih lis izpostavljenih in kontrolnih celic ter iskanje statistično značilnih diferencialno izraženih proteinov; (iv) izrez izbranih proteinskih lis iz gela; (v) razgradnja proteinov v lisah na peptide (ponavadi s tripsinom) in njihova identifikacija z masno spektrometrijo in (vi) iskanje funkcije identificiranih proteinov po bazah podatkov, npr. UniProt.

Proteomika celičnih organelov in struktur omogoča analizo celokupnih proteinov v posameznih organelih in drugih celičnih strukturah, kot so membrane in citoskelet. Tu so proteinski ekstrakti manj kompleksni, v njih pa so obogateni proteini, ki imajo v celicah majhno vsebnost, npr. transkripcijski faktorji in regulatorni proteini (Gauthier in Lazure, 2008).

Organeli so dinamične strukture, saj imajo poleg proteinov, ki se v njih nahajajo večino svoje življenjske dobe, tudi proteine, ki prehajajo preko organelov, da dosežejo nek drug organel, ali pa so v organelu le toliko časa, da opravijo določeno funkcijo (Gauthier in Lazure, 2008). Translokacija proteinov iz enega dela celice v drugega je pomembna za veliko regulatornih poti. Med proteini, ki translocirajo, so npr. proteinska kinaza odvisna od kalmodulina (CaM-PK II), proteinske kinaze C, A in N, aneksin IV, substrat kinaze C, ki je miristoiliran na alaninu, fosfolipaza A2, proteinska fosfataza 1 in 2A, kinaza Src, faktor Rho, dinein, NO-sintaza, kortaktin, paksilin, filamin, spektrin, kalretikulin, proteini toplotnega šoka in miozin (Patton, 1999). Poleg tega se lahko enaki proteini nahajajo v večih organelih in imajo različno funkcijo, ki je odvisna od njihove celične lokacije. Npr. gliceraldehid-3-fosfat dehidrogenaza, ki je sicer glikolitičen encim in se nahaja v citoplazmi, a ima lahko tudi vlogo pri zlitju membran, dinamiki mikrotubulov, fosfotransferazni aktivnosti, transportu RNA v jedro, podvojevanju in popravljanju DNA, kar je odvisno od tega, kje v celici se nahaja (Sirover, 1999).

Pri klasičnih metodah v proteomiki celičnih organelov in struktur se uporablja gradientno ali diferencialno centrifugiranje (Gauthier in Lazure, 2008). Gradientno centrifugiranje temelji na dejstvu, da imajo organeli med seboj različno sestavo in zato različno gostoto (Wiederhold in sod., 2010) (Preglednica 1). Zato se organeli iz celičnega lizata v kontinuirnem ali diskontinuirnem gradientu gostote sedimentirajo tako dolgo, dokler ni njihova gostota enaka gostoti okoliškega medija (Gauthier in Lazure, 2008). Medtem je princip diferencialnega centrifugiranja zasnovan na tem, da gostejši delci sedimentirajo pri manjših pospeških, manjši pa pri večjih. Tako posamezne organele iz celičnega lizata

ločimo tako, da začnemo centrifugirati pri manjših pospeških, pri katerih sedimentirajo le gostejši delci. Supernatant nato ponovno centrifugiramo pri večjih pospeških. S ponavljanjem postopka in večanjem pospeška izoliramo delce z manjšo gostoto (Wiederhold in sod., 2010). Za izolacijo organelov se največ uporablja kar kombinacija gradientnega in diferencialnega centrifugiranja (Patton, 1999).

Preglednica 1: Lastnosti organelov kvasovke *S. cerevisiae* (Wiederhold in sod., 2010)
Table 1: Properties of yeast *S. cerevisiae* organelles (Wiederhold et al., 2010)

Organel	**Razmerje lipidi:proteini (mg/mg)[A]**	**Gostota (g/cm^3)[B]**	**Rang vsebnosti proteinov (kopije/celico)**
Mitohondrij	0,92 (zunanja membrana), 0,17 (notranja membrana)	1,14-1,21	10^1-10^6
Peroksisom	0,46	1,21-1,23 (glicerol in oleinska kilsina), 1,19-1,21 (glicerol), 1,16-1,17 (Nycodenz, oleinska kislina), 1,19-1,2 (oleinska kilsina)	10^2-10^4
Lipidni delci	16,80	<1,000	10^2-10^5
Jedro	0,11		10^1-10^5
Celična stena		>1,3	10^1-10^6
Celična membrana	0,65	1,24-1,26 (z ogljikovodiki), 1,12-1,18, 1,20-1,22 (brez ogljikovodikov)	10^1-10^6
Endoplazemski retikulum	0,13	1,145 (gladki endoplazemski retikulum), 1,17-1,19 (zrnati endoplazemski retikulum)	10^2-10^5
Golgijev aparat	0,08	1,248 (sorbitol)	10^1-10^5
Endosom	1,17 (sekretorni vezikli)	1,12-1,16 (Nycodenz) 1,030-1,075	10^1-10^4
Vakuola	0,58	1,011 (Ficoll)	10^1-10^5

[A] Uporabljena je vsota fosofolipidov in sterolov.

[B] Če ni navedeno drugače, je uporabljen gradient saharoze.

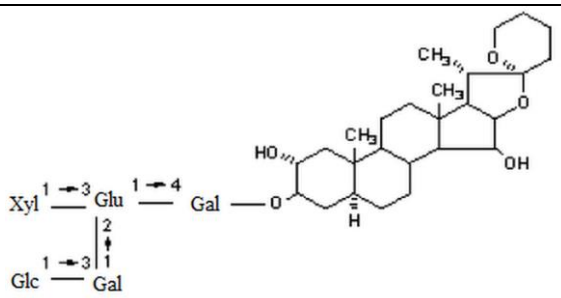
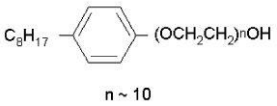
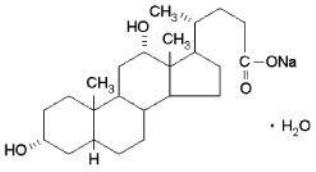
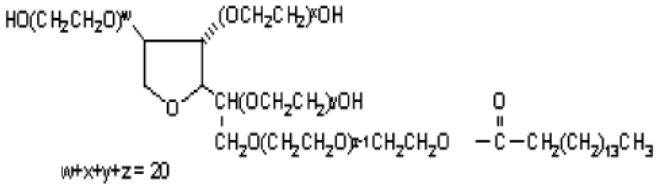
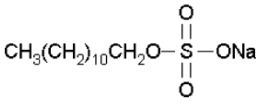
Pri novejših metodah v proteomiki celičnih organelov se poleg gostote uporabljajo tudi druge fizikalno-kemične lastnosti (Gauthier in Lazure, 2008). Postopek pri katerem zaporedno uporabimo več detergentov, ki imajo različno moč, z namenom, da ekstrahiramo proteine iz različnih celičnih organelov in struktur, se imenuje diferencialna detergentna frakcionacija (DDF). Pri tem različne kombinacije detergentov omogočajo detekcijo unikatnih setov proteinov (Rockstroh in sod., 2010), med drugim tudi detekcijo proteinov celične membrane in citoskeleta, ki so še posebno zanimivi za raziskovanje. Celična membrana je meja med notranjostjo in zunanjostjo celice, preko katere poteka izmenjava materiala in informacij, in sicer to omogočajo proteini, ki so vgrajeni vanjo. Zaradi te pomembne biološke vloge so membranski proteini posebno zanimivi kot tarče zdravil (Strachan in sod., 2006). Vendar pa so slabo topni in jih je zato težko ekstrahirati

(Santoni in sod., 2000). Citoskelet ima pomembno vlogo pri regulaciji endocitoze, eksocitoze, mitoze, citokineze, kemotakse, prenosa signalov in biosinteze proteinov (Patton, 1999). Vendar se citoskeletni deli, kot so mikrofilamenti, mikrotubuli in intermediatni filamenti, izgubijo med pripravo celic za gradientno ali diferencialno centrifugiranje, ki vključuje celično lizo. Drugače je pri DDF, kjer se integriteta citoskeleta ohrani.

Ramsby in Makowski (1999) sta z uporabo DDF pridobila citosolno, membransko/organelno, jedrno in citoskeletno proteinsko frakcijo. Postopek vključuje zaporedno ekstrakcijo celic z detergenti digitonin/etilendiamintetraocetna kislina (EDTA), Triton-X-100/EDTA, Tween-40/deoksiholat in SDS (Preglednica 2). Dodatek EDTA detergentu pospeši hitrost permeabilizacije celične membrane (10 min +EDTA, 40 min –EDTA). Dodatno EDTA inhibira citosolne proteaze, ki so odvisne od kalcija, s čimer prepeči proteolizo. Digitonin je steroidna spojina, ki tvori komplekse s holesterolom, kar povzroči permeabilizacijo celične membrane in sprostitev citosolnih komponent. Ostane le prazna celica s težjimi organeli. Pri koncentraciji digitionina 0,015 % se strukture mitohondrijev in endoplazemski retikulum ohranijo, medtem ko koncentracija nad 0,1 %, vodi v poškodbe teh organelov. Triton-X-100 je neionski detergent, ki topi membrane in sprosti vsebino organelov, s čimer pridobimo membranske in orgenelne proteine. Ob uporabi nižjih koncentracij Tritona-X-100 jedro in mikrofilamenti ostanejo nepoškodovani. Kombinacija neionskega detergenta Tween-40 in šibko ionskega detergenta deoksiholata razbije jedro in raztopi proteine jedra in proteine, ki so šibko povezani s citoskeletom. Kar ostane po zadnji ekstrakciji je frakcija bogata z intermediatnimi filamenti, aktinom, proteini, ki interagirajo z citoskeletom, pa tudi jedrnimi proteini iz matriksa in DNA, ki se raztopi v fosfatnem pufru, ki vsebuje SDS (Patton, 1999).

Preglednica 2: Strukture detergentov uporabljenih pri diferencialni detergentni frakcionaciji za ekstrakcijo citosolnih, membransko/organelnih, jedrnih in citoskeletnih proteinov (McCarthy in sod., 2005)

Table 2: Structures of detergents used in differential detergent fractionation for the extraction of cytosolic, membrane/organelle, nuclear and cytoskeletal proteins (McCarthy et al., 2005)

Detergent	Struktura
Digitonin	
Triton-X-100	
Deoksiholat	
Tween-40	
Natrijev dodecil sulfat (SDS)	

1.1.7 Kvasovka *Saccharomyces cerevisiae* v stacionarni fazi rasti kot modelni organizem

V primeru stradanja kvasne celice zaustavijo rast in vstopijo v nebrsteče se stanje, imenovano stacionarna faza rasti. Kvasovka *S. cerevisiae* v stacionarni fazi rasti pridobi lastnosti, ki ji omogočijo, da dlje časa preživi brez hranil. Te lastnosti so upočasnitev rasti, kopičenje glikogena, povečana rezistenca na okoljski stres in odebelitev celične stene (Herman, 2002). Stacionarno fazo rasti imenujemo tudi G_0 faza rasti ali kviescenca (Gray in sod., 2004).

Kvasovka *S. cerevisiae* ima celične organele, ki so tipični za evkariontske celice in je primeren modelni organizem za študij temeljnih metabolnih poti in celičnih procesov v

evkariontih (Ma, 2001). Poleg tega je kvasovka v stacionarni fazi podobna celicam večceličnih organizmov, in sicer z več vidikov. Kot prvo, energijo pridobiva z mitohondrijskim dihanjem. Drugo, celice so v G_0 fazi rasti. In tretjič, skozi čas se v celicah nakopičijo poškodbe (Longo in sod., 1996). Poleg tega je primerjalna genomika pokazala, da ima več kot 40 % kvasnih proteinov podobno aminokislinsko zaporedje kot vsaj en človeški protein (Hughes, 2002), med njimi so tudi taki, ki imajo podobno aminokislinsko zaporedje kot proteini, ki so vpleteni v bolezni pri človeku (Foury, 1997).

V primerjavi s sesalskimi celicami ima kvasovka več prednosti, saj ima hiter celični cikel, lahko raste tako v suspenziji kot na trdnem gojišču in ne potrebuje posebnih sterilnih tehnik ter dragega medija (Sherman, 1991). Zato so eksperimenti s kvasovkami enostavnejši, hitrejši in cenejši od tistih, ki vključujejo sesalske celice (Sturgeon in sod., 2006).

1.2 CILJI RAZISKOVALNEGA DELA

V doktorski nalogi smo si zastavili naslednje cilje:

- kemijsko okarakterizirati izvleček propolisa,
- preučiti vpliv izvlečka propolisa na celični ravni kvasovke *S. cerevisiae*, in sicer na znotrajcelično oksidacijo, celično metabolno energijo in celično živost,
- preveriti celični privzem fenolnih spojin izvlečka propolisa v kvasovko *S. cerevisiae*,
- preveriti vpliv izvlečka propolisa na izražanje citosolnih, membransko/organelnih, jedrnih in citoskeletnih proteinov kvasovke *S. cerevisiae*.

1.3 RAZISKOVALNE HIPOTEZE

Predvidevamo, da:

- antioksidativna učinkovitost v celicah ni vedno povezana z antioksidativno učinkovitostjo *in vitro*,
- imajo nekatere fenolne spojine propolisa večjo antioksidativno učinkovitost v celicah kot druge,
- bomo uspešno identificirali fenolne spojine propolisa, ki vstopijo v celico,
- se bo vpliv posameznih fenolnih spojin odražal na subceličnem proteomu (citosolna, membranska/organelna, jedrna in citoskeletna frakcija) modelnega organizma v smislu spremembe ravni proteinov in/ali interakcij fenolnih spojin s proteini.

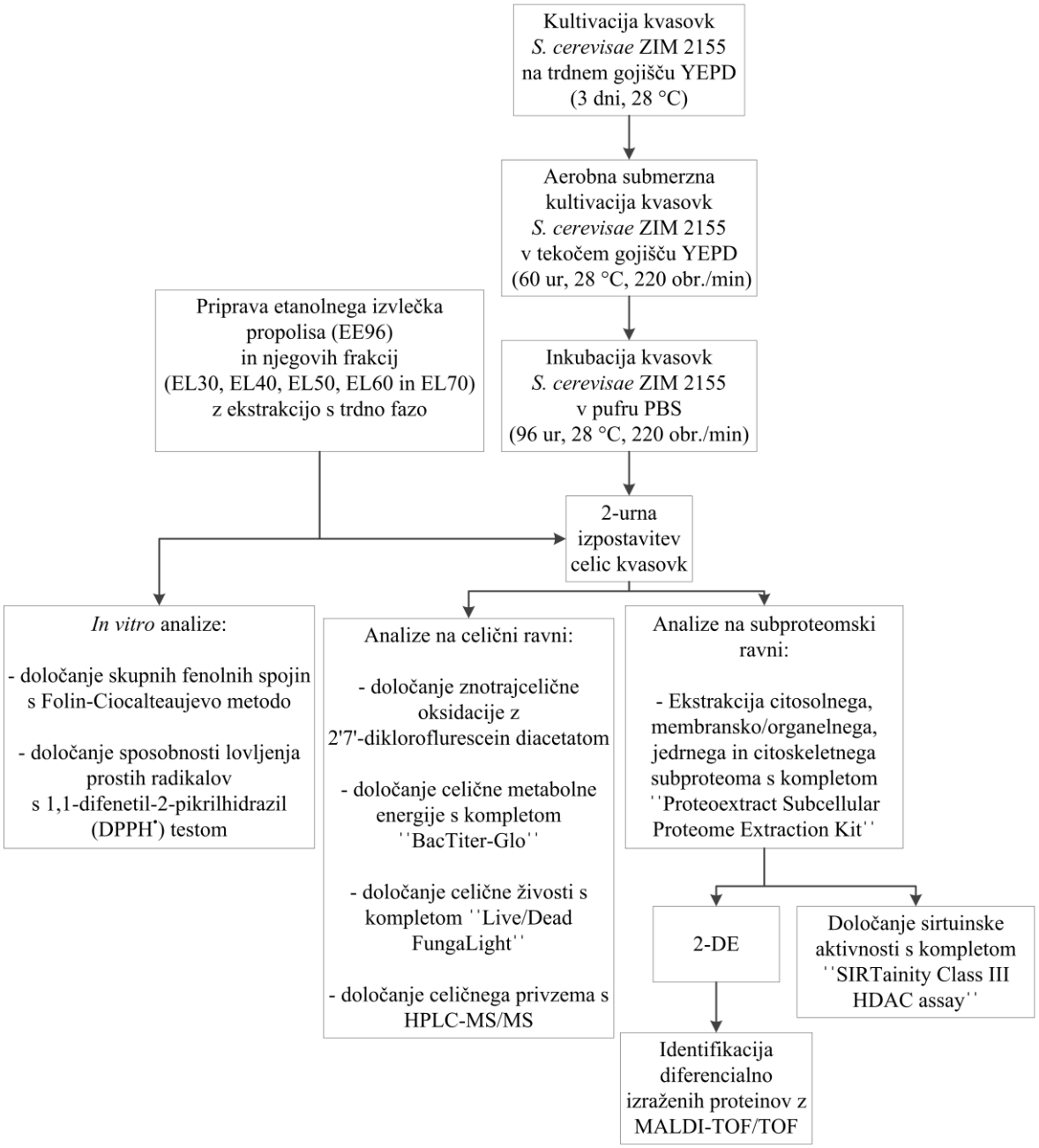
1.4 POTEK RAZISKOVALNEGA DELA

Naše začetne raziskave so pokazale, da ima izvleček propolisa pomemben vpliv na znotrajcelično oksidacijo in celično metabolno energijo ter da se kaže povezava med celičnim privzemom posameznih fenolnih spojin in aktivnostjo izvlečka propolisa v kvasovki *S. cerevisiae*. Na celotnem in mitohondrijskem proteomu tretiranih kvasovk se je pokazalo malo sprememb v ravni proteinov, zaradi česar še ni bilo mogoče podati zaključkov o mehanizmu delovanja izvlečka propolisa v celici (Cigut in sod., 2011).

Zato smo v nadaljevanju fenolne spojine, ki sestavljajo izvleček propolisa, frakcionirali v več frakcij, pri čemer smo uporabili ekstrakcijo s trdno fazo (SPE). Metoda se je izkazala kot uspešna za pridobitev frakcij s kvantitativno in kvalitativno različnimi fenolnimi profili, kar smo potrdili s tekočinsko kromatografijo visoke ločljivosti z detektorjem z nizom diod (HPLC-DAD). Pridobljene frakcije smo kemično okarakterizirali (Folin-Ciocalteau metoda, DPPH test) in analizirali njihov vpliv na celični ravni kvasovke *S. cerevisiae* tako, da smo izmerili znotrajcelično oksidacijo (2,7-diklorofluorescein), celično metabolno energijo (luciferin-luciferaza) in celično živost (SYTO 9/propidijev jodid) v izpostavljenih celicah. V izbrani frakciji smo s tekočinsko kromatografijo visoke ločljivosti s tandemsko masno spektrometrijo (HPLC-MS/MS) identificirali fenolne spojine in določili, katere izmed njih vstopijo v kvasovko (Petelinc in sod., 2013a).

Vpliv izbrane frakcije smo proučili tudi na proteomu kvasovke *S. cerevisiae*, kjer smo uporabili dvodimenzionalno elektroforezo (2-DE). Za ekstrakcijo proteinov smo uporabili diferencialno detergentno frakcionacijo (DDF), s katero se ekstrahira proteine iz različnih celičnih struktur, in sicer citosola, membran/organelov, jedra in citoskeleta ("Proteoextract subcellular proteome extraction kit"). Pri tem smo protokol uspešno modificirali za uporabo na kvasovki, saj je bil sprva namenjen za sesalske celice. Identificirali smo 18 diferencialno izraženih proteinov, ki so bili vpleteni v različne celične procese, kar nam je omogočilo, da smo razvili hipoteze o možnem mehanizmu delovanja izvlečka propolisa v celici, ki se odražajo v spremembah na celični ravni (Petelinc in sod., 2013b). Nazadnje smo določili tudi sirtuinsko aktivnost, saj so spremembe v subproteomu nakazale na možnost, da propolis učinkuje na ta encim.

Potek raziskovalnega dela je prikazan na sliki 2.



Slika 2: Shematski prikaz poteka raziskovalnega dela

Figure 2: Schematic representation of the research work

2 ZNANSTVENA DELA

2.1 OBJAVLJENA ZNANSTVENA DELA

2.1.1 Antioksidativna aktivnost izvlečka propolisa v kvasni celici

Cigut T., Polak T., Gašperlin L., Raspor P., Jamnik P. 2011. Antioxidative activity of propolis extract in yeast cells. Journal of Agricultural and Food Chemistry, 59, 21: 11449-11455

Propolis ima mnoge biološke aktivnosti, med drugim antioksidativno. Večina raziskav antioksidativne aktivnosti propolisa je izvedena *in vitro*, zato smo v raziskavi antioksidativno aktivnost propolisa preverili *in vivo* na modelnem organizmu kvasovki *S. cerevisiae*.

Kvasne celice smo izpostavili izvlečku propolisa v DMSO pri koncentraciji 0,05 g ekvivalentov klorogenske kisline (CAE)/L in po 1 uri izmerili znotrajcelično oksidacijo z 2'7'-diklorofluoresceinom. V izpostavljenih celicah smo pokazali znižano znotrajcelično oksidacijo za 42 %, povišano celično metabolno energijo (za 2,3x) in nespremenjeno kultivabilnost v primerjavi s kontrolo (z DMSO tretirane kvasne celice).

Ker se predvideva, da so za antioksidativni učinek propolisa zaslužne njegove fenolne spojine, smo preverili tudi *in vivo* antioksidativno aktivnost izbranih fenolnih spojin v propolisu, in sicer kavne kisline, *p*-kumarne kisline, ferulne kisline in fenetilnega estra kavne kisline (CAPE). Kvasne celice smo izpostavili izbranim fenolnim spojinam oz. kombinaciji fenolnih kislin v DMSO pri koncentraciji 0,05 g/L in po 1 uri izmerili znotrajcelično oksidacijo z 2'7'-diklorofluoresceinom. Pokazali smo, da izbrane fenolne spojine oz. kombinacija fenolnih kislin niso imele večjega vpliva na znotrajcelično oksidacijo kvasnih celic pri izbrani koncentraciji.

Dodatno smo preverili celični privzem izvlečka propolisa in ugotovili, da v celice vstopijo oz. z njimi interagirajo le manj polarne fenolne spojine izvlečka propolisa. Slednje smo ločili od bolj polarnih fenolnih spojin izvlečka propolisa z ekstrakcijo s trdno fazo (SPE), s čimer smo pridobili dve frakciji (E1-bolj polarne spojine in E2-manj polarne fenolne spojine). Frakcija E1 je tako med drugim vsebovala tudi kavno kislino, *p*-kumarno kislino in ferulno kislino, medtem ko je frakcija E2 med drugim vsebovala tudi CAPE. Slednje se ujema z rezultati celičnega privzema čistih fenolnih spojin (kavna kislina, *p*-kumarna kislina, ferulna kislina, CAPE), kjer smo vstop v/na celice pokazali le za CAPE.

Po 1-urni izpostavitvi kvasnih celic frakcijama E1 in E2 se je izkazalo, da le frakcija E2 zniža znotrajcelično oksidacijo, in sicer za 20 %. Torej je antioksidativna aktivnost izvlečka propolisa povezana le z nekaterimi fenolnimi spojinami v njem.

Vpliv izvlečka propolisa in frakcije E2 smo proučili tudi na ravni celotnega in mitohondrijskega proteoma z dvodimenzionalno elektroforezo (2-DE). Analiza mitohondrijskega proteinskega profila po 1-urni izpostavitvi kvasnih celic izvlečku propolisa pri koncentraciji 0,05 g CAE/L je pokazala spremembe v ravni proteinov vpletenih v sintezo ATP (podenota α mitohondrijske ATP-sintaze), medtem ko je analiza mitohondrijskega proteinskega profila po 1-urni izpostavitvi kvasnih celic frakciji E2 pokazala spremembe v ravni antioksidativnih proteinov (Mn-superoksid dismutaza in peroksiredoksin 1).

Petelinc T. Bioaktivne spojine propolisa in njihov vpliv na kvasovko *Saccharomyces cerevisiae* na celični in proteomski ravni. Dokt. disertacija. Ljubljana, Univ. v Ljubljani, Biotehniška fakulteta, 2015

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Antioxidative Activity of Propolis Extract in Yeast Cells

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ABSTRACT: The antioxidative activities of propolis and its main phenolic compounds, caffeic acid, *p*-coumaric acid, ferulic acid, and caffeic acid phenethyl ester, were investigated in the yeast *Saccharomyces cerevisiae*. After 1 h of exposure of the yeast cells, their intracellular oxidation was measured using 2′,7′-dichlorofluorescein. Yeast cells exposed to 96% ethanolic extracts of propolis in DMSO (EEP) showed decreased intracellular oxidation, with no significant differences seen for the individual phenolic compounds. However, cellular uptake was seen only for a moderately polar fraction of EEP (E2) and caffeic acid phenethyl ester. The EEP antioxidative activity thus resulted from this E2 fraction of EEP. The influence of EEP was also investigated at the mitochondrial proteome level, by analyzing its profile after 1 h of exposure of the yeast cells to EEP and E2. Changes in the levels of antioxidative proteins and proteins involved in ATP synthesis were seen.

KEYWORDS: propolis, phenolic compounds, caffeic acid, *p*-coumaric acid, ferulic acid, caffeic acid phenethyl ester, antioxidative activity, yeast, *Saccharomyces cerevisiae*, proteomics

INTRODUCTION

To prevent oxidative-stress-related diseases, different bee products, such as propolis, have generated considerable interest. Propolis is the strongly adhesive and resinous substance that is collected by the bees, transformed, and used to seal holes in their honeycombs, to smooth the internal walls, to protect the entrance of the beehive against intruders, and to prevent the decomposition of creatures that have been killed by the bees after an invasion of the beehive. Bees collect the resin from cracks in the bark of trees and leaf buds. This resin is masticated by the bees, thus adding salivary enzymes, and the partially digested material is mixed with beeswax and used in the beehive.[1,2]

Propolis has been reported to have anticancer, anesthetic, antimycotic, anti-inflammatory, antioxidant, antiseptic, astringent, antiviral, bacteriostatic, choleric, and spasmolytic properties.[3,4] It is believed that the various pharmacological activities of propolis can be attributed to its phenolic compounds and, in particular, the caffeic acid, caffeic acid derivates, and flavonoids in propolis are of interest for their known antioxidative activities.[4,5]

With the exception of a few studies of the antioxidative activity of propolis in vivo,[6−9] almost all such studies have been carried out in vitro,[4,5,10,11] with the main aim of defining the relationship between the antioxidative activity of propolis and its phenolic compounds.

On the basis of in vitro studies, we cannot assume that the antioxidant compounds in various bioactive substances will show the same activities in the cell. Hence, for any antioxidative evaluation, it is also necessary to understand the pharmacodynamics, which is in the case of phenolic compounds less well-known. Therefore, many studies are being carried out on the molecular and genetic interactions of phytochemicals and other bioactive substances in food and dietary supplements.

In the present study, the antioxidative activity of propolis was investigated in vivo using the yeast *Saccharomyces cerevisiae* in stationary phase. This lower eukaryote *S. cerevisiae* is an appropriate model organism for the study of fundamental eukaryotic cellular processes, such as their stress responses and metabolic pathways.[12−16] Furthermore, in their stationary phase, yeast cells resemble cells of multicellular organisms according to several aspects: (1) most of their energy comes from mitochondrial respiration; (2) the cells are in the G_0 phase; and (3) damage accumulates over time.[17]

To our knowledge, none of the in vivo studies on propolis have investigated the relationships between its antioxidative activity and its phenolic composition. The aim of the present study was to define the antioxidative activities and cellular uptake of propolis and its main phenolic compounds in the yeast cell. To determine whether these yeast cells respond to propolis exposure by changes in protein level, proteome analysis was also carried out, using two-dimensional (2-D) electrophoresis.

MATERIALS AND METHODS

Yeast Strain and Cultivation. The yeast *S. cerevisiae* ZIM 2155 was obtained from the Culture Collection of Industrial Microorganisms (ZIM) of the Biotechnical Faculty, University of Ljubljana, Ljubljana, Slovenia.

The yeast was cultivated in yeast extract (10 g/L; Biolife), peptone (20 g/L; Biolife), glucose (20 g/L; Merck) (YEPD) medium at 28 °C and 220 rpm, until stationary phase. The cells were then centrifuged for 3 min at 4000*g*, washed once with phosphate-buffered saline (PBS) (Merck), and suspended in PBS at a concentration of 1×10^8 cells/mL. The cells were further incubated at 28 °C and 220 rpm for 96 h.

Solid-Phase Extraction (SPE). The present study used a 96% ethanolic extract of propolis of Slovenian origin with solid-phase extraction used to clean this original extract and also to separate it into two elution fractions. Here, 200 μL of the original 96% ethanolic extract of propolis (or for the separation, the "cleaned" 96% ethanolic extract of propolis) was mixed with 200 μL of 20 mM ammonium formate and then added to a Strata-X SPE cartridge (Phenomenex) that had previously been conditioned with 2 mL of methanol (Merck) followed

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by 2 mL of 20 mM ammonium formate. After the sample was loaded, the cartridge was washed with 2 mL of 20 mM ammonium formate in 15% methanol/water (v/v) and vacuum-dried for 3 min.

For the cleaning of the propolis, the cartridge was eluted with 2 mL of 96% ethanol (Merck). Then the ethanol was removed by evaporation and replaced with dimethyl sulfoxide (DMSO) (Fluka). For the separation of the cleaned propolis into eluates E1 (polar fraction, retention time < 30 min) and E2 (moderately polar fraction, retention time > 30 min), the cartridge was eluted with 2 mL of 30% ethanol followed by elution with 2 mL of 96% ethanol. The ethanol was removed from both eluates by evaporation and replaced with 200 μL of DMSO.

Treatment of Yeast Cells. The cleaned 96% ethanolic extract of propolis in DMSO (EEP) was added to the yeast cell suspensions following their 96 h of incubation in PBS, at a concentration of the phenolic compounds of 0.05 g/L (expressed as grams of chlorogenic acid per liter of EEP).

As well as this treatment with EEP, the yeast cells were treated with ferulic acid (Sigma), caffeic acid (Sigma), *p*-coumaric acid (Sigma), and caffeic acid phenethyl ester (CAPE) (Sigma), a combination of phenolic acids (ferulic acid, caffeic acid, *p*-coumaric acid), eluate E1, and eluate E2. These phenolic compounds were also dissolved in DMSO and added to the yeast cell suspensions following their 96 h of incubation in PBS, at a concentration of 0.05 g/L.

After a further 1 h of incubation at 28 °C and 220 rpm, the samples were taken for further analyses: measurements of intracellular oxidation, cellular uptake, and analysis of the mitochondrial proteome (this last only for EEP and eluate E2).

Determination of Intracellular Oxidation. Intracellular oxidation was estimated using 2′,7′-dichlorofluorescein (H_2DCF), which reacts with oxidants, thus revealing the presence of reactive oxygen species (ROS). This was given to the cells as 2′,7′-dichlorofluorescein diacetate (H_2DCFDA), which easily penetrates the plasma membrane and is hydrolyzed inside the cells by nonspecific esterases. The nonfluorescent H_2DCF can then be oxidized to fluorescent 2′,7′-dichlorofluorescin (DCF), which is measured fluorometrically.[18]

The cells from the 2 mL incubations were sedimented by centrifugation (14000*g*, 5 min) and washed three times with 50 mM potassium phosphate buffer (pH 7.8). The cell pellets were finally resuspended in 9 volumes of 50 mM potassium phosphate buffer (to 10%, v/v) and incubated at 28 °C for 5 min. The ROS-sensing dye H_2DCFDA (Sigma) was added from a 1 mM stock solution in 96% ethanol (Merck), to a final concentration of 10 μM. After a 20 min incubation at 28 °C and 220 rpm, the fluorescence of yeast cell suspension was measured, using a Safire II microplate reader (Tecan). The excitation and emission wavelengths of DCF were 488 and 520 nm, respectively.

The experiments were performed in biological duplicates, and within each three technical replicates were done. Data are expressed as the mean of the relative fluorescence intensity ± SE. Individual comparisons were made using Duncan's multiple-range test[19] on a total of six samples. A value of $p < 0.05$ was considered to indicate a significant difference between groups.

Determination of Cell Energy Metabolic Activity. Cell energy metabolic activity was determined by BacTiter-Glo Microbial Cell Viability Assay (Promega) according to the manufacturer's instructions. Briefly, after 1 h of incubation, 100 μL of cell suspension at a concentration of 1×10^7/mL and 100 μL of BacTiter-Glo reagent were placed in a 96-well microplate and mixed. After 5 min, luminescence was measured using the Safire II microplate reader (Tecan). The experiments were performed in biological duplicates, and within each three technical replicates were done. Data are expressed as the mean of the relative luminescence intensity ± SE.

Cell Viability Determination. Cell viability was measured as colony-forming units (CFU). After 1 h of incubation, CFU were determined by plating cell suspension on yeast extract (10 g/L; Biolife), peptone (20 g/L; Biolife), glucose (20 g/L; Merck), agar (20 g/L; Biolife) (YEPD) medium, and then after a 2 day incubation at 28 °C, the number of colonies was counted. The experiments were performed in biological duplicates, and within each three technical replicates were done. Data are expressed as the mean of CFU/mL ± SE.

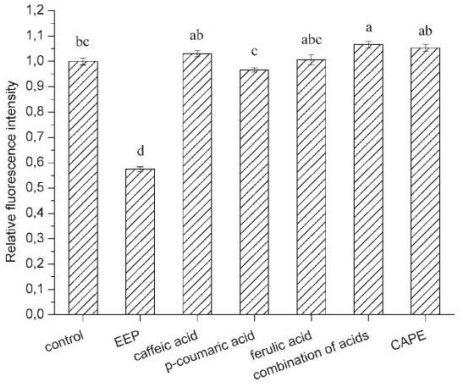

Figure 1. Intracellular oxidation in the yeast *S. cerevisiae* after 1 h of exposure to 96% ethanolic extract of propolis in DMSO (EEP) or to its main phenolic compounds, including caffeic acid phenethyl ester (CAPE), or to a combination of phenolic acids (caffeic, *p*-coumaric, and ferulic acid) at a concentration of 0.05 g/L, in comparison to the control. Data are the mean ± SE, with values not sharing a common letter (a−d) being significantly different (Duncan's multiple-range test; $p < 0.05$).

Cellular Uptake. To study the cellular uptake of phenolic compounds, the phenolic profile was determined both in the PBS and in the yeast cell suspensions 1 h after the addition of EEP or of the particular phenolic compounds. Thus, 1 h after the addition of the test compounds, the PBS and yeast cell suspensions were centrifuged (4000*g*, 3 min). The supernatants obtained were filtered (pore size: = 0.2 μm) and analyzed using liquid chromatography with diode array detection (LC-DAD), as below, to obtain the phenolic profile. All of the experiments were performed in duplicate.

LC-DAD Analysis. The samples were diluted 20-fold with 1% HCOOH in 50% methanol, filtered through a 0.22 μm PTFE filter, and analyzed by LC-DAD. The LC system consisted of an Agilent 1100 model G1312A binary pump and a model G1330B autosampler (Agilent Technologies). This reversed-phase HPLC separation was carried out using a Gemini C18 column (150 mm × 2.0 mm internal diameter; 3 μm particle size), which was protected by a Gemini C18 security guard cartridge (4.0 mm × 2.0 mm internal diameter) (Phenomenex). The mobile phase comprised aqueous 1% HCOOH (A) and acetonitrile (B), and the following gradient was used: 0−5 min, 10% B; 5−50 min, 10% −60% B; 50−52 min, 60%−80% B; 52−60 min, 80% B; 60−70 min, 80%−10% B; 70−80 min, 10% B. The column was maintained at 25 °C, with an injection volume of 20 μL and a flow rate of 0.2 mL/min.

Extraction of Mitochondrial Proteins. To analyze the mitochondrial proteome, 20 mL of yeast cell suspension was centrifuged at 4000*g* for 3 min. The pellet obtained was washed once with PBS and used for the extraction of the mitochondrial proteins using Cytosol/Mitochondria Fractionation kits (Calbiochem), according to the manufacturer's instructions, with a little modification. The yeast cells were disrupted by vortexing with zirconia/silica beads (BioSpec Products), five times for 1 min each, with 1 min intervals for cooling on ice.

Petelinc T. Bioaktivne spojine propolisa in njihov vpliv na kvasovko *Saccharomyces cerevisiae* na celični in proteomski ravni. Dokt. disertacija. Ljubljana, Univ. v Ljubljani, Biotehniška fakulteta, 2015

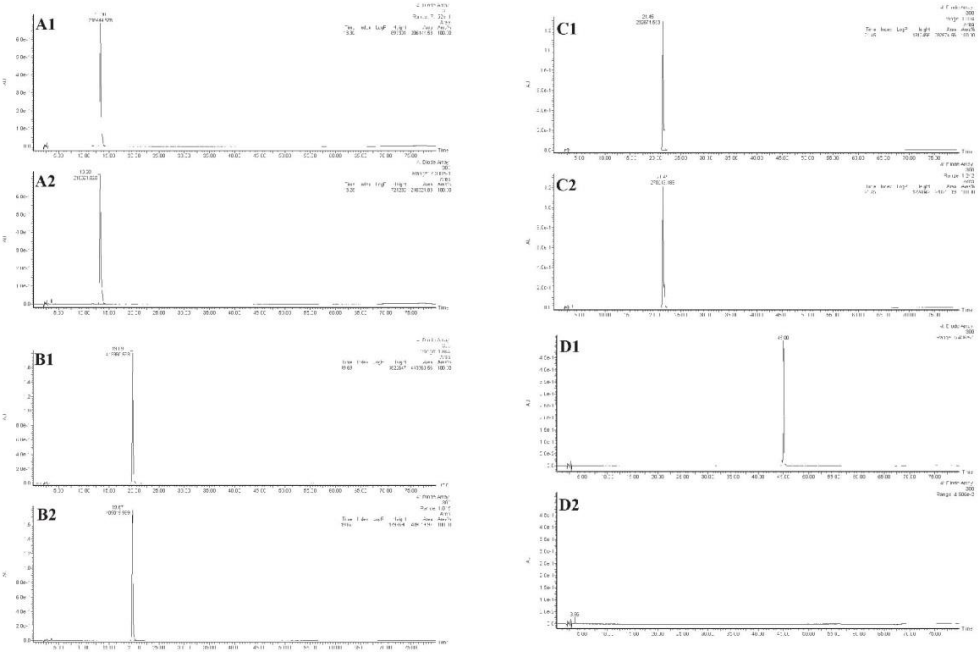

Figure 2. LC-DAD (300 nm) chromatograms of the phenolic compounds in the supernatants after centrifugation of suspensions of the yeast *S. cerevisiae* before (1) and after (2) their 1 h of exposure to caffeic acid (A), *p*-coumaric acid (B), ferulic acid (C), and caffeic acid phenethyl ester (CAPE) (D) at a concentration of 0.05 g/L.

The mitochondrial protein concentrations were determined according to the method of Bradford,[20] using bovine serum albumin (Sigma) as standard.

Two-Dimensional Electrophoresis. The 2-D electrophoresis was performed according to the method of Görg,[21] with minor modifications. The samples (100 μg of protein) were mixed with rehydration solution [7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 2% (v/v) immobilized pH gradient (IPG) buffer (pH 3−10), 18 mM dithiothreitol, and a trace of bromophenol blue] and applied to 13 cm pH 3−10 IPG strips (GE Healthcare). After rehydration, the first dimension of isoelectric focusing was carried out at 20 °C on a Multiphore II system (GE Healthcare). The following voltage program was applied: 0−300 V (gradient over 1 min), 300 V (fixed for 1 h), 300−3500 V (gradient over 1.5 h), and 3500 V (fixed for 5 h). Prior to the second dimension of the 2-D electrophoresis, the IPG strips were equilibrated in sodium dodecyl sulfate (SDS) equilibration buffer [75 mM Tris HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, and a trace of bromophenol blue], containing 1% dithiothreitol, for 15 min, and containing 4.8% iodoacetamide for an additional 15 min. The second dimension (SDS−polyacrylamide gel electrophoresis) was carried out with the 12% running gels on a vertical SE 600 discontinuous electrophoretic system (Hoefer Scientific Instruments), at a constant 20 mA/gel for 15 min and then at a constant 40 mA/gel until the bromophenol blue reached the bottom of the gels. The 2-D gels were stained with SYPRO Ruby (Invitrogen). For each sample, two 2-D gels were run under the same conditions.

Protein Visualization and Image Analysis. After staining, the gels were analyzed using a CAM-GX-CHEMI HR system (Syngene). The gel image analysis was carried out using 2-D Dymension software, version 2.02 (Syngene). Duplicate gels for each sample were matched to provide an average gel sample. The spots were revealed and quantified on the basis of their normalized volumes, as the spot volume divided by the total volume over the whole set of gel spots. Expression changes (fold changes) were considered to be significant if the intensity of the corresponding spot reproducibly differed by >1.5-fold in a normalized volume as a comparison between the control and treated samples and if this was statistically significant (Student's *t* test).

Yeast Proteome Map and Protein Identification. To identify differentially expressed proteins, a previously obtained 2-D mitochondrial proteome map was used, where the protein identities were confirmed by LC−tandem mass spectrometry (MS/MS) using electrospray ionization−ion trap mass spectrometry, at the Cogeme Proteome Service Facility 1 (University of Aberdeen, U.K.; Istenič et al., unpublished results).

RESULTS AND DISCUSSION

Intracellular Oxidation and Cellular Uptake. The yeast cells were treated for 1 h with EEP at a concentration of 0.05 g/L. This had been determined in our previous study (Mavri et al., unpublished results) as a concentration that promoted a significant decrease (42%) in the intracellular oxidation of the cells treated with EEP (Figure 1), as compared to the control

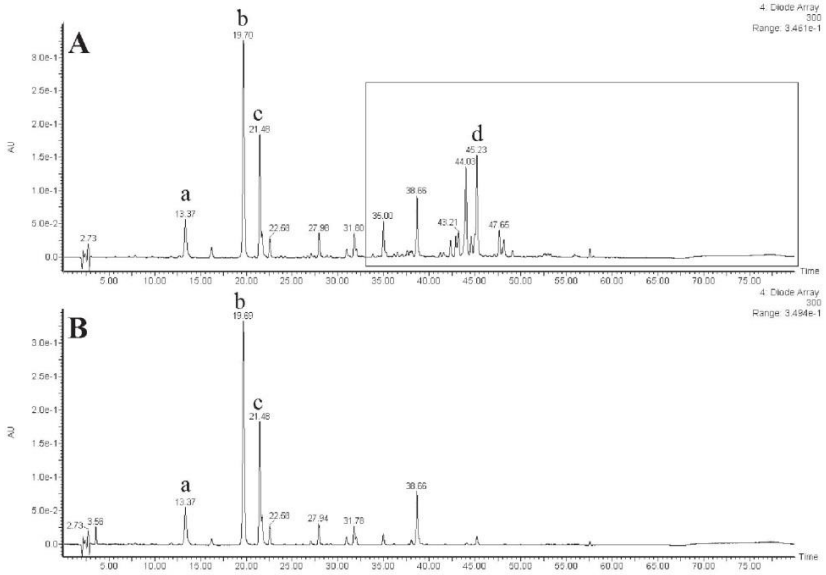

Figure 3. LC-DAD (300 nm) chromatogram of phenolic compounds in the supernatants after centrifugation of suspensions of the yeast *S. cerevisiae* before (A) and after (B) their 1 h of exposure to 96% ethanolic extract of propolis in DMSO (EEP) at a concentration of 0.05 g/L. The marked phenolic compounds are (a) caffeic acid, (b) *p*-coumaric acid, (c) ferulic acid, and (d) caffeic acid phenethyl ester (CAPE), and the frame shows phenolic compounds that entered the yeast cells.

(nontreated cells). Also, there were no effects on intracellular oxidation by DMSO at this concentration, whereas concentrations above 0.05 g/L DMSO caused increased intracellular oxidation (data not shown).

To test if the propolis has any effect on cell viability and activity, we measured cell viability as CFU and cell energy metabolic activity. Results showed that there is no change in CFU between control and treated cells [$(8.5 \pm 0.2) \times 10^7$ and $(7.6 \pm 0.6) \times 10^7$, respectively]. Additionally, in the cells treated with EEP, cell energy metabolic activity increased 2.3-fold compared to control (1.00 ± 0.04, control; 2.30 ± 0.05, treated sample). On the basis of these results it can be concluded that decreased intracellular oxidation reflects the antioxidative activity of propolis.

As the antioxidative activity of propolis is believed to be related to its phenolic compounds,[4,5] we also tested the antioxidative activities of a series of its main phenolic compounds: caffeic acid in our previous study (Mavri et al., unpublished results) and, in the present study, *p*-coumaric acid, ferulic acid, and CAPE, the levels of which in EEP were determined by LC-MS as being higher compared to other compounds (CAPE, 501.32 μg/mL; *p*-coumaric acid, 465.26 μg/mL; caffeic acid, 359.67 μg/mL; ferulic acid, 225.56 μg/mL) (Mavri et al., unpublished results). The yeast cells were thus treated with these particular compounds and with a combination of phenolic acids, each at 0.05 g/L. The individually tested compounds did not show any antioxidative activities compared to the control [control, 1.00 ± 0.01; caffeic acid, 1.03 ± 0.01; *p*-coumaric acid, 0.97 ± 0.01; ferulic acid, 1.01 ± 0.02; combination of acids, 1.07 ± 0.01; CAPE, 1.05 ± 0.01 (Figure 1)].

Therefore, the following question arose: did these compounds even enter the cells? To test the uptake of these particular compounds into these yeast cells, their levels were determined using LC-DAD, before and after exposure of the cells. These data showed that only CAPE entered these yeast cells, whereas for the other compounds there were no changes in their levels before and after exposure (Figure 2). Interestingly, CAPE did not decrease the intracellular oxidation (Figure 1); indeed, considering its polarity, it would have remained in the cell membrane and, therefore, did not show any effects on intracellular oxidation. It is known that phenolic compounds can interact with lipids. These interactions seem to be rather unspecific, based essentially on physical adsorption. Adsorption is the process of accumulation at an interface and should be distinguished from absorption, which implies the penetration of one component, for example, the polyphenol molecule, throughout the membrane.[22]

Furthermore, cellular uptake was also examined for EEP, which demonstrated that only a fraction of the phenolic compounds entered these yeast cells (Figure 3). On the basis of this observation, using solid-phase extraction, EEP was separated into two eluates according to polarity: a polar eluate, E1, and moderately polar eluate, E2.

EEP used in this study was chemically characterized by Mavri et al. (unpublished results). It consists of the following components, from more to less polar: gallic acid, chlorogenic acid, caffeic acid, *p*-coumaric acid, ferulic acid, rutin, ellagic acid, myricetin, luteolin, quercetin, formononetin, caffeic acid benzyl ester, pinobanksin, apigenin, kaempferol, caffeic acid isoprenyl ester, CAPE, pinobanksin-3-*O*-acetate, kaempferide, chrysin,

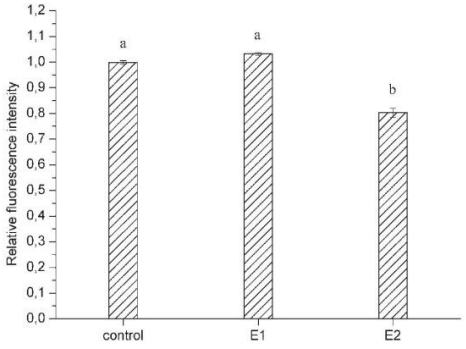

Figure 4. Intracellular oxidation in the yeast *S. cerevisiae* after 1 h of exposure to eluates E1 and E2 from EEP, in comparison to the control. Data are the mean ± SE, with values not sharing a common letter (a, b) being significantly different (Duncan's multiple-range test; $p < 0.05$).

pinocembrin, galangin, and caffeic acid cinnamyl ester. Eluate E1 is mostly composed of hydroxycinnamates, whereas in eluate E2 flavonoids are mostly present.

Then the yeast cells were treated separately with both eluate E1 and eluate E2, and their intracellular oxidation was measured. Eluate E2 corresponded to the fraction that entered the cells, which decreased (20%) the intracellular oxidation, in contrast to eluate E1, with which no cellular uptake or changes in oxidant levels were seen (control, 1.00 ± 0.01; eluate E1, 1.03 ± 0.01) (Figure 4). Difference in the decrease of intracellular oxidation for EEP and eluate E2 can be explained by varieties in preparation procedure. Namely, to prepare eluate E2 solid-phase extraction was used once more compared to EEP, resulting in a greater loss of phenolic compounds.

Mitochondrial Proteome Analysis. The influence of EEP and eluate E2 at the proteome level was investigated using 2-D electrophoresis. Although the effects of both of these treatments on the total proteome were not significant (data not shown), intensive changes were found for the mitochondrial proteome. The main source of ROS in yeast cells is the mitochondrial respiratory chain.[23] Therefore, it is not surprising that the first changes at the proteome level in the presence of exogenous antioxidants are observed in the mitochondria. To analyze the mitochondrial proteome, a subcellular proteomic approach was used.

Subcellular proteomics has the advantage not only of relating proteins to functional compartments within eukaryotic cells but also of reducing the complexity of a whole cell or a tissue protein extract, which can often prevent satisfactory proteomic analysis. Namely, it allows the identification of novel and low-abundance proteins that can otherwise remain masked when total cellular extracts are investigated.[24]

In the case of this EEP exposure, this promoted a reduced level of α subunit of mitochondrial F_0F_1-ATP synthase (Atp1) ($R = -1.547$; $p = 0.022$) (Figure 5). This F_0F_1-ATP synthase is a multisubunit membrane-associated protein complex that catalyzes the phosphorylation of ADP to ATP at the expense of a proton motive force that is generated by the electron transport chain. In some organisms, this enzyme can also work in the reverse direction, by hydrolyzing ATP and generating an electrochemical proton

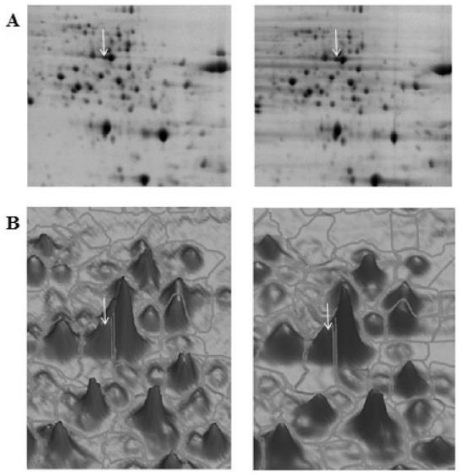

Figure 5. Comparison of mitochondrial protein profiles of control (left) and EEP-treated (right) yeast cells: (A) arrow, spot representing the α subunit of mitochondrial F_0F_1-ATP synthase (Atp1); (B) statistically significant differences in this spot ($R = -1.547$, $p = 0.022$) as shown in the 2-D Dymension gel image analysis software. Arrows indicate spots with levels that are statistically significantly different (Student's t test).

gradient across a membrane, to support locomotion or nutrient uptake. F_0F_1-ATP synthase can be divided into two parts: a soluble globular F_1 catalytic section, where, among others, the α subunit is located, and a membrane-bound F_0 proton-translocating section. F_0F_1-ATP synthase has been suggested to be a good molecular target for drugs in the treatment of various diseases and in the regulation of energy metabolism.[25]

Bioactive compounds can affect proteins through interactions with these proteins. In the case of phenolic compounds, it has been shown that these compounds often target F_0F_1-ATP synthase.[25−27] Gledhill et al.[27] showed that some phenolic compounds can inhibit the rotary mechanism of F_1-ATPase by binding to a site where the upper extremity of the central stalk fits into the hydrophobic annular sleeve of the "bearing" formed by the loop regions below the "crown". In the case of resveratrol, the residues are either within 4 Å of the inhibitor and form hydrophobic interactions, or they are linked to it via hydrogen bond networks.

Whereas in most studies interactions of bioactive compounds with proteins are indicated, changes in gene expression are also observed.[28] Bioactive compounds can alter mRNA and protein levels by altering the activities of transcription factors or by binding to cell receptors, which can result in changed activities of enzymes, including phosphatases and kinases.[29] The expression levels of subunit e of F_0F_1-ATP synthase have been shown to be highly sensitive to diverse physiological changes and stresses.[25] Indeed, it is already known that the intracellular balance of oxidants and antioxidants is a key factor in the regulation of the expression of certain genes.[29]

In the present study, decreased levels of Atp1 were seen in these yeast cells exposed to EEP, which can be explained directly

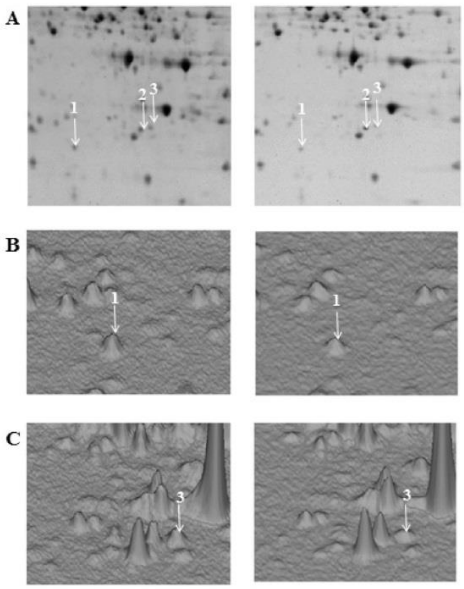

Figure 6. Comparison of mitochondrial protein profile of control (left) and EEP eluate-E2-treated (right) yeast cells: (A) spot 1, Mn SOD; spot 2, peroxiredoxin (Prx1); spot 3, probably an isoform of Prx1; (B) statistically significant differences in Mn SOD ($R = -1.784$, $p = 0.096$) and (C) in spot 3 ($R = -1.740$, $p = 0.026$), as shown in the 2-D Dymension gel image analysis software. Arrows indicate spots with levels that are statistically significantly different (Student's *t* test).

via the binding of the phenolic compounds to Atp1, to cause its modification. It has to be noted that by using a proteomic approach, such chemical interactions can be disrupted due to the use of denaturants in the sample extraction buffer, which can result in the restoring of a protein to its former state or, also indirectly, in the following: (1) binding of the phenolic compounds to transcription factors can modify the regulation of transcription of Atp1 and (2) post-translational modification of Atp1 can occur through, for example, kinases and phosphatases, to cause a shift in p*I*.

In the cells exposed to eluate E2, a reduced level of Mn SOD was seen ($R = -1.784$; $p = 0.096$), along with protein spot 3 ($R = -1.740$; $p = 0.026$) (Figure 6). Spot 3 might be an isoform of peroxiredoxin (Prx1) that has changed its p*I* due to post-translational modifications, such as, for example, phosphorylation. Prx1 has a thioredoxin peroxidase activity with a role in the reduction of hydroperoxides.[30] Both of these proteins (Mn SOD and Prx1) belong to endogenous antioxidant defense systems, and their decreased levels might be due to reduced intracellular oxidation after exposure to exogenous antioxidants (eluate E2) (Figure 5).

These reduced levels of endogenous antioxidant proteins and reduced intracellular oxidation indicate that eluate E2 is likely to be acting directly as a radical scavenger, and not indirectly as a pro-antioxidant. This latter would lead to decreased intracellular oxidation due to increased levels of endogenous antioxidant proteins.[31] Similarly, Jamnik et al.[32] showed a reduced level of Cu/Zn SOD in the yeast *S. cerevisiae* when exposed to bee royal jelly, which decreased intracellular oxidation. On the other hand, changes in mitochondrial proteome of yeast cells exposed to this fraction (Figures 5 and 6) indicate that these compounds might be involved in cellular metabolism or interactions with transcription factors or appropriate proteins.

In the present study, we have focused on propolis. As most of the information relating to the antioxidative activity of propolis arises from in vitro studies, we here investigated the antioxidative activity of propolis extract in cells at both the cellular and proteome levels using the yeast *S. cerevisiae* as our model organism. Only a moderately polar fraction of the EEP was shown to enter these yeast cells and to decrease their intracellular oxidation. Changes were also found at the mitochondrial proteome level, including for antioxidative proteins and proteins involved in ATP synthesis. Further investigations of the phenolic compounds in this moderately polar fraction of EEP will be carried out to provide a better understanding of these antioxidative activities of propolis in vivo.

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ABBREVATIONS USED

CAPE, caffeic acid phenethyl ester; DMSO, dimethyl sulfoxide; EEP, 96% ethanolic extracts of propolis in DMSO; E1, polar fraction of EEP; E2, moderate polar fraction of EEP; PBS, phosphate-buffered saline; DCF, 2′,7′-dichlorofluorescin; LC-DAD, liquid chromatography with diode array detection; Atp1, α subunit of mitochondrial F_0F_1-ATP synthase; Prx1, peroxiredoxin; SOD, superoxide dismutase.

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2.1.2 Frakcionacija fenolnih spojin izvlečka propolisa in njihova aktivnost v kvasovki *Saccharomyces cerevisiae*

Petelinc T., Polak T., Demšar L., Jamnik P. 2013a. Fractionation of phenolic compounds extracted from propolis and their activity in the yeast *Saccharomyces cerevisiae*. PloS ONE, 8, 2: e56104, doi: 10.1371/journal.pone.0056104: 8 str.

Propolis je bogat s fenolnimi spojinami, ki naj bi bile zaslužne za njegove mnoge biološke učinke. Da bi bolje razumeli katere fenolne spojine v izvlečku propolisa so odgovorne za njegovo delovanje v celicah, smo izvleček propolisa frakcionirali in analizirali vpliv posameznih frakcij na celično raven modelnega organizma kvasovke *S. cerevisiae*.

Uporabili smo očiščen etanolni izvleček propolisa, ki je vseboval 20 g propolisa/L. V primeru frakcionacije smo uporabili ekstrakcijo s trdno fazo (SPE) in pridobili pet frakcij. Te frakcije so se ločile po polarnosti fenolnih spojin, ki so jih vsebovale. Glede na vol. % etanola, ki smo ga uporabili pri eluciji posamezne frakcije s kolone, smo jih poimenovali EL30-EL70. Analiza s tekočinsko kromatografijo visoke ločljivosti z detektorjem z nizom diod (HPLC-DAD) je pokazala, da se frakcije razlikujejo po profilu fenolnih spojin tako kvalitativno kot kvantitativno.

V izvlečku propolisa in frakcijah smo določili skupne fenolne spojine (Folin-Ciocalteaujeva metoda) in sposobnost lovljenja prostih radikalov (DDPH test). Pokazalo se je, da je koncentracija skupnih fenolnih spojin v izvlečku propolisa 1,99 g ekvivalentov galne kisline (GAE)/L in da ima sposobnost lovljenja prostih radikalov, ki ustreza 1,88 g GAE/L. Med frakcijami je imela frakcija EL70 največjo vsebnost fenolnih spojin (0,83 g ekvivalentov galne kisline GAE/L) in največjo sposobnost lovljenja prostih radikalov (1,22 g GAE/L).

Nato smo določili tudi *in vivo* antioksidativno učinkovitost izvlečka propolisa in frakcij na kvasne celice. 2-urna izpostavitev izvlečku propolisa pri koncentraciji 1 % (v/v) (≈0,02 g GAE/L) v celični suspenziji je znižala znotrajcelično oksidacijo za 61 % v primerjavi s kontrolo (s 96 % etanolom tretirane kvasne celice). Med frakcijami je najbolj znižala znotrajcelično oksidacijo frakcija EL70 pri koncentraciji 1 % (v/v) (≈0,01 g GAE/L) v celični suspenziji, in sicer za 66 % v primerjavi s kontrolo. Nadaljnje se je izkazalo, da 2-urna izpostavitev izvlečku propolisa ali frakciji EL70 pri koncentraciji 1 % (v/v) v celični suspenziji tudi povišata celično metabolno energijo (za 19 % in 21 %) v primerjavi s kontrolo. Izvleček propolisa in frakcija EL70 pri koncentraciji 1 % (v/v) v celični suspenziji nista imela učinka na celično živost po 2-urni izpostavitvi.

S tekočinsko kromatografijo visoke ločljivosti s tandemsko masno spektrometrijo (HPLC-MS/MS) smo identificirali fenolne spojine v frakciji EL70, in sicer smo v njem našli izoprenilni ester kavne kisline, benzilni ester kavne kisline (CABE), fenetilni ester

kavne kisline (CAPE), cimetni ester kavne kisline (CACE), benzilni ester *p*-kumarne kisline, krizin, apigenin, luteolin-metil-eter, pinocembrin, kamferid, ramnetin, pinobanksin-5-metil-eter, pinobanksin-3-*O*-acetat, pinobanksin-3-*O*-propionat, pinobanksin-3-*O*-butirat, pinobanksin-3-*O*-pentanoat in 3-prenil-4-(2-metilpropionil-oksi)-cimetno kislino.

Za CABE, CAPE in CACE smo pokazali tudi privzem v kvasne celice. S tem smo identificirali fenolne spojine, ki najverjetneje prispevajo k spremembam v znotrajcelični oksidaciji in celični metabolni energiji po 2-urni izpostavitvi celic frakciji EL70.

Petelinc T. Bioaktivne spojine propolisa in njihov vpliv na kvasovko *Saccharomyces cerevisiae* na celični in proteomski ravni. Dokt. disertacija. Ljubljana, Univ. v Ljubljani, Biotehniška fakulteta, 2015

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Fractionation of Phenolic Compounds Extracted from Propolis and Their Activity in the Yeast *Saccharomyces cerevisiae*

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Abstract

We have here investigated the activities of Slovenian propolis extracts in the yeast *Saccharomyces cerevisiae*, and identified the phenolic compounds that appear to contribute to these activities. We correlated changes in intracellular oxidation and cellular metabolic energy in these yeasts with the individual fractions of the propolis extracts obtained following solid-phase extraction. The most effective fraction was further investigated according to its phenolic compounds.

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Introduction

Propolis is a resinous substance that is collected from certain plants by bees. The bees use it as a sealer in their hives and to prevent the decomposition of creatures that invade the hive and the bees can kill, but cannot remove. Although the composition of propolis varies, depending on the place and time of its collection [1], in general it contains resins and balsams (50%), waxes (30%), aromatic and essential oils (10%), pollen (5%) and other organic matter (5%) [2,3]. Propolis has a broad spectrum of biological activities, including antioxidative, anti-inflammatory, immunomodulatory, anticancer, antibacterial, antiviral, antifungal and antiparasitic effects [4,5]. Although propolis is a mixture of compounds, its pharmacological activities are reported to arise from its flavonoids and phenolic acids, and their esters [6].

In the present study, the activity of propolis was investigated using stationary phase *Saccharomyces cerevisiae* yeast as the model organism. In this system in our previous study, we showed that propolis decreases intracellular oxidation, with its antioxidative activity in yeast arising from only a part of it [7]. To better understand this antioxidative activity of propolis in yeast, we have here further investigated the activities of the phenolic compounds of propolis. Therefore, the objectives of this study were to: (1) fractionate a crude propolis extract using polarity-based solid-phase extraction; (2) determine any correlations between the total phenolic content of individual propolis fractions and its antioxidant activity *in vitro*/*in vivo*; (3) determine any correlations between the total phenolic content of individual fractions of propolis and the cellular metabolic energy; and (4) identify the phenolic compounds of any fractions that promote changes in intracellular oxidation and cellular metabolic energy in yeast.

Materials and Methods

Ethics Statement

No specific permits were required for the described field studies. The location is not privately-owned or protected in any way. The field studies did not involve endangered or protected species.

Chemicals and Standards

Folin-Ciocalteu's reagent, 96% ethanol, glucose, methanol, formic acid, sodium carbonate and acetonitrile were from Merck. The 2,2-diprenyl-1-picryhydrazyl radical (DPPH), gallic acid and 2′,7′-dichlorodihydrofluorescein diacetate were from Sigma. Yeast extract and peptone were from Biolife. Ammonium formate was from Fluka.

Sample Preparation

Propolis was collected from bee hive in the Savinjska Valley in Slovenia during the autumn of 2010. The propolis (10 g) was extracted with 70% ethanol (100 mL) by mixing for 1 h at room temperature. The crude extract was recovered by centrifugation (3000 *g*, 5 min) and concentrated under vacuum using a rotary evaporator.

Solid-phase Extraction

Solid-phase extraction (SPE) was used to clean the crude propolis extract, whereby it was separated into five elution fractions according to polarity. Crude propolis extract (200 µL) was mixed with 20 mM ammonium formate (200 µL), and then added to a Strata-X (33 u Polymeric Reversed Phase 60 mg/3 mL 8B-S100-UBJ) SPE cartridge (Phenomenex) that had previously been conditioned with methanol (2 mL) and equilibrated with 20 mM ammonium formate, pH 3.2 (2 mL). After the loading of the sample, the cartridge was washed with 20 mM ammonium

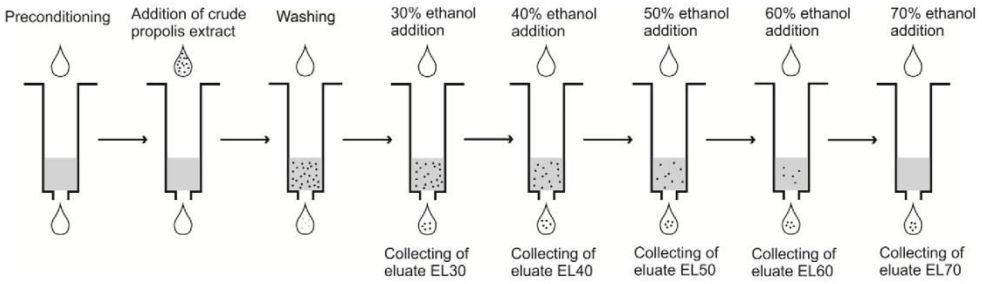

Figure 1. Preparation of the EL30 to EL70 eluates from the crude propolis extract by solid-phase extraction.
doi:10.1371/journal.pone.0056104.g001

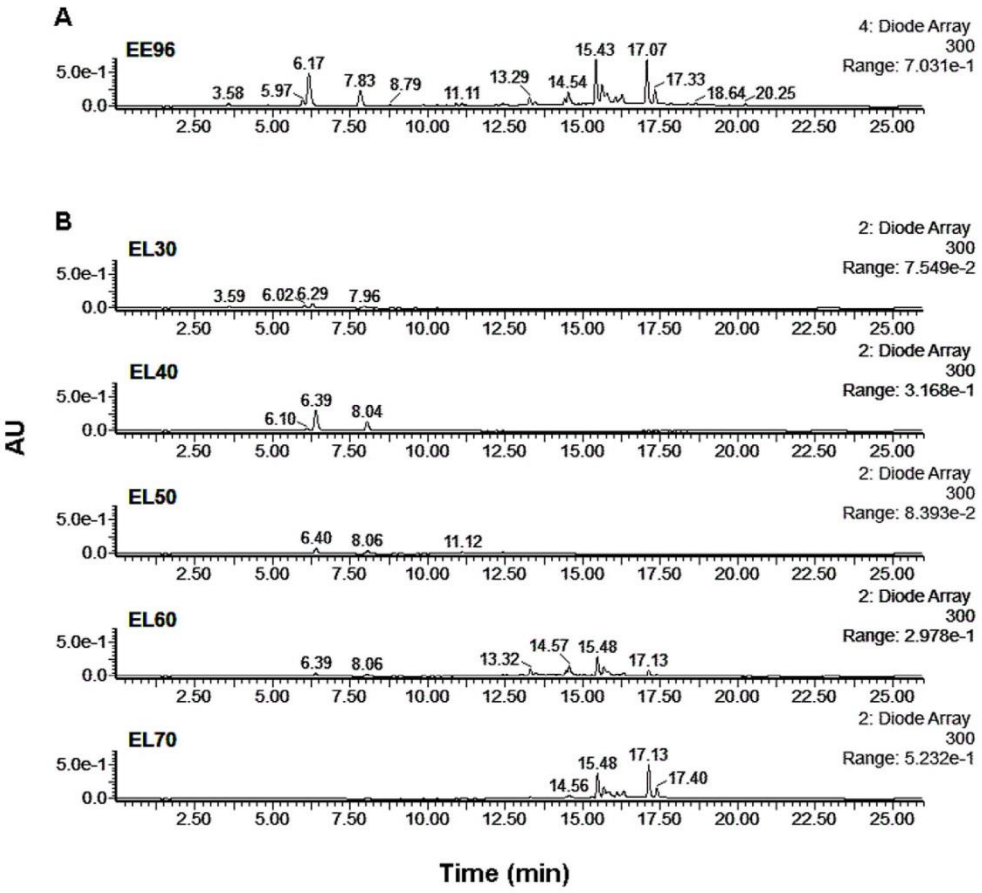

Figure 2. LC-DAD (300 nm) chromatogram of the phenolic compounds following solid-phase extraction without fractionation (A: EE96) and with fractionation (B: EL30–EL70). AU, arbitrary units.
doi:10.1371/journal.pone.0056104.g002

formate in 15% methanol (2 mL), and vacuum-dried for 3 min. For cleaning of the crude propolis extract, a cartridge was eluted with 96% ethanol (2 mL), to obtain the cleaned propolis ethanolic extract (EE96). For separation of the crude propolis extract, a cartridge was eluted with 30% ethanol (2 mL), followed by 40% ethanol (2 mL), 50% ethanol (2 mL), 60% ethanol (2 mL), 70% ethanol (2 mL) (Fig. 1). This thus provided the propolis ethanol eluates, as the 30% (EL30) to 70% (EL70) ethanol eluates for further analysis.

Determination of Total Phenolic Content

The total phenolic contents of the cleaned propolis EE96 and the eluates (EL30–EL70) were determined using the Folin-Ciocalteu method [8]. Each sample (50 µL) was mixed with distilled water (700 µL) and Folin-Ciocalteu reagent (125 µL). After 5 min, 20% (w/v) Na_2CO_3 (125 µL) was added and the samples were mixed. Absorbance was measured after 90 min incubation in the dark at room temperature, using a Safire II microplate reader (Tecan) ($\lambda = 765$ nm). The data expressed as g gallic acid equivalents (GAE)/L were estimated from the calibration curve using gallic acid as standard (absorbance at 765 nm).

Determination of DPPH Free Radical Scavenging Activity

The *in vitro* antioxidative activities of the cleaned propolis EE96 and the eluates (EL30–EL70) were evaluated using the 2,2-diphenyl-1-picryl-hydrazyl (DPPH) free radical scavenging method [9]. Each sample (25 µL) was mixed with distilled water (225 µL) and DPPH solution (0.05 mg/L in 96% ethanol; 1 mL). The absorbance was measured after 30 min incubation in the dark at room temperature, using a Safire II microplate reader (Tecan) ($\lambda = 517$ nm). The data expressed as g GAE/L were estimated from the calibration curve using gallic acid as standard (absorbance at 517 nm).

Yeast Strain and Cultivation

The yeast *Saccharomyces cerevisiae* ZIM 2155 was obtained from the Culture Collection of Industrial Microorganisms (ZIM) of the Biotechnical Faculty, University of Ljubljana, Ljubljana, Slovenia.

The yeast were cultivated in yeast extract (10 g/L), peptone (20 g/L), glucose (20 g/L) (YEPD) medium at 28°C and with agitation at 220 rpm, until their stationary phase. The yeast were then centrifuged at 4000 *g* for 3 min, washed once with phosphate-buffered saline (PBS) and resuspended in PBS at 1×10^8 cells/mL. The yeast was further incubated at 28°C and 220 rpm, for 96 h.

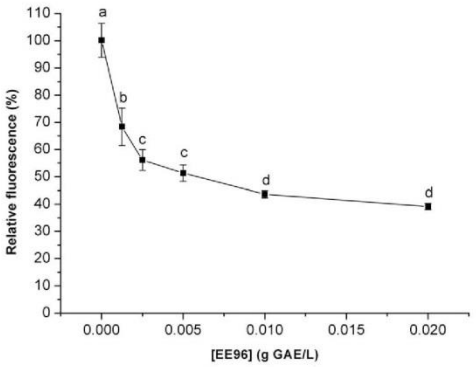

Figure 3. Intracellular oxidation in the yeast *S. cerevisiae* treated with EE96. Data are means (n = 3) and are expressed as fluorescence relative to control. Values followed by the same letter (a–d) are not statistically different ($P<0.05$), as measured by Duncan's test. doi:10.1371/journal.pone.0056104.g003

Yeast Treatment

Following the 96 h of incubation in PBS, the yeasts were treated with either the cleaned propolis EE96 or with the particular eluates (EL30–EL70). After a further 2 h of incubation at 28°C and 220 rpm, samples were taken for the determination of: (1) intracellular oxidation; (2) cellular metabolic energy; (3) cell viability; and (4) cellular uptake of individual phenolic compounds.

Determination of Intracellular Oxidation

Intracellular oxidation was estimated using 2′,7′-dichlorodihydrofluorescein (H_2DCF), which reacts with oxidants, thus revealing the presence of reactive oxygen species (ROS). This was given to the yeast as H_2DCF diacetate, which penetrates the plasma membrane and is hydrolyzed inside cells by nonspecific esterases. The nonfluorescent H_2DCF can then be oxidized to fluorescent 2′,7′-DCF, the levels of which are measured fluorimetrically [10].

After 2 h of treatment, the yeast from 2 mL cell suspensions were sedimented by centrifugation (14,000 *g*, 5 min), and washed three times with 50 mM potassium phosphate buffer (pH 7.8). The cell pellets were resuspended in 9 volumes of 50 mM potassium phosphate buffer (to 10%, v/v) and incubated at 28°C for 5 min. The ROS-sensing dye H_2DCF diacetate was added

Table 1. Total phenolic content and evaluation of the free radical scavenging activity of EE96 and eluates EL30–EL70.

Eluate	Total phenolic content (g GAE/L)	Free radical scavenging activity (g GAE/L)
EE96	1.99 ± 0.01^a	1.88 ± 0.01^a
EL30	0.26 ± 0.01^d	0.18 ± 0.08^d
EL40	0.39 ± 0.01^c	0.33 ± 0.08^c
EL50	0.26 ± 0.01^d	0.30 ± 0.05^c
EL60	0.82 ± 0.01^b	1.20 ± 0.03^b
EL70	0.83 ± 0.02^b	1.22 ± 0.02^b

Data are means ±S.D. (n = 3); values in the same column followed by the same letter (a-d) are not statistically different ($P<0.05$), as measured by Duncan's test.
EE96, ethanolic extract of crude propolis extract prepared using SPE with 96% ethanol elution.
EL30-EL70, eluates of the crude propolis extract prepared using SPE with 30% to 70% ethanol elution.
GAE, gallic acid equivalent.
doi:10.1371/journal.pone.0056104.t001

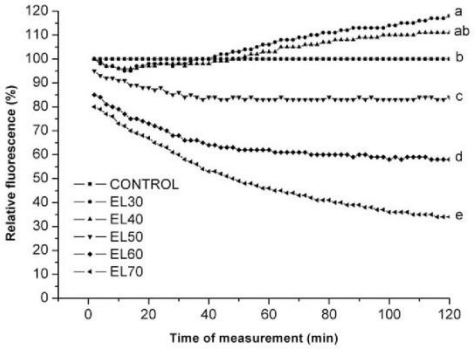

Figure 4. Intracellular oxidation in the yeast *S. cerevisiae* treated with EL30 to EL70 eluates. Data are means (n = 3) and are expressed as fluorescence relative to control. Values followed by the same letter (a–e) are not statistically different ($P<0.05$), as measured by Duncan's test.
doi:10.1371/journal.pone.0056104.g004

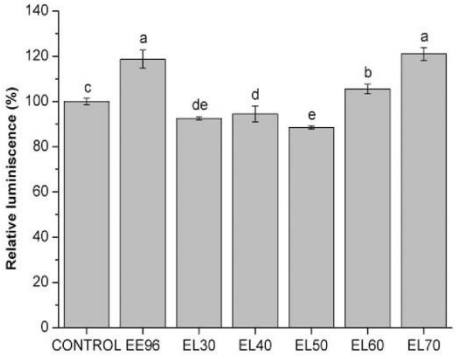

Figure 5. Cellular metabolic energy in the yeast *S. cerevisiae* treated with EE96 and EL30 to EL70 eluates (1%). Data are means ±S.D. (n = 3) and are expressed as luminescence relative to control. Values followed by the same letter (a–e) are not statistically different ($P<0.05$), as measured by Duncan's test.
doi:10.1371/journal.pone.0056104.g005

from a 1 mM stock solution in 96% ethanol, to a final concentration of 10 µM. After a 20 min incubation at 28°C and 220 rpm, the fluorescence of the yeast suspension was measured, using the kinetic mode of a Safire II microplate reader (Tecan). The excitation and emission wavelengths of DCF were 488 nm and 520 nm, respectively. The data are expressed as fluorescence relative to control.

Determination of Cellular Metabolic Energy

The cellular metabolic energy was determined using the BacTiter-Glo™ Microbial Cell Viability Assay (Promega), according to the manufacturer's instructions. After 2 h of treatment, 100 µL cell suspension (washed three times with filtered PBS) at 1×10^7 cells/mL, and 100 µL BacTiter-Glo™ reagent were placed in 96-well microplates and mixed. After 5 min the luminescence was measured using a Safire II microplate reader (Tecan). The data are expressed as luminescence relative to control.

Cell Viability Determination

Cell viability was measured as the cell membrane integrity, using LIVE/DEAD® Funga Light TM Yeast Viability kits (Molecular Probes), according to the manufacturer's instructions. After 2 h of treatment, the yeast from 1 mL cell suspension were sedimented by centrifugation (14,000 *g*, 5 min), and washed three times with filtered PBS. A cell suspension in filtered PBS at 1×10^6 cells/mL was prepared. Then 1 µL SYTO® 9 and 1 µL propidium iodide were added in the dark to 1 mL of cell suspension, and the samples were vortexed and incubated at 37°C for 30 min, with vortexing every 10 min. After the incubations, the fluorescence was measured using a Safire II microplate reader (Tecan). The excitation/emission wavelengths for these two dyes are 480/500 nm for SYTO® 9, and 490/635 nm for propidium iodide. The data are expressed as fluorescence normalized to OD_{650} relative to the control.

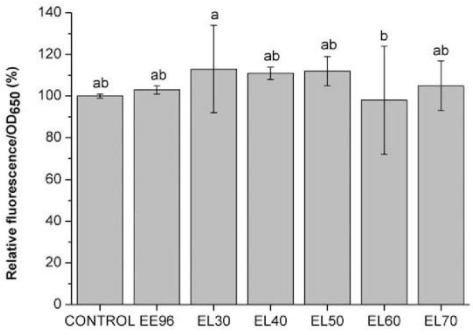

Figure 6. Cell viability of the yeast *S. cerevisiae* treated with EE96 and EL30 to EL70 eluates (1%). Data are means ±S.D. (n = 3), and are expressed as fluorescence normalized to OD_{650} relative to control. Values followed by the same letter (a-b) are not statistically different ($P<0.05$), as measured by Duncan's test.
doi:10.1371/journal.pone.0056104.g006

Determination of Cellular Uptake of Phenolic Compounds

To study the cellular uptake of the phenolic compounds, the phenolic profile was determined in the PBS buffer immediately after the addition of 1% eluates (i.e. before yeast exposure) and after 2 h of exposure of yeast cells to eluates. The samples were centrifuged (4000 *g*, 3 min) and the supernatants obtained were first cleaned using SPE (according to procedure described for EE96) and then analyzed using LC-DAD, to obtain the phenolic profile.

Liquid Chromatography-diode Array Detection Analysis

The samples were diluted 20-fold in 1% formic acid in 50% methanol and analyzed by liquid chromatography-diode array detection (LC-DAD). The LC system consisted of an Agilent 1100

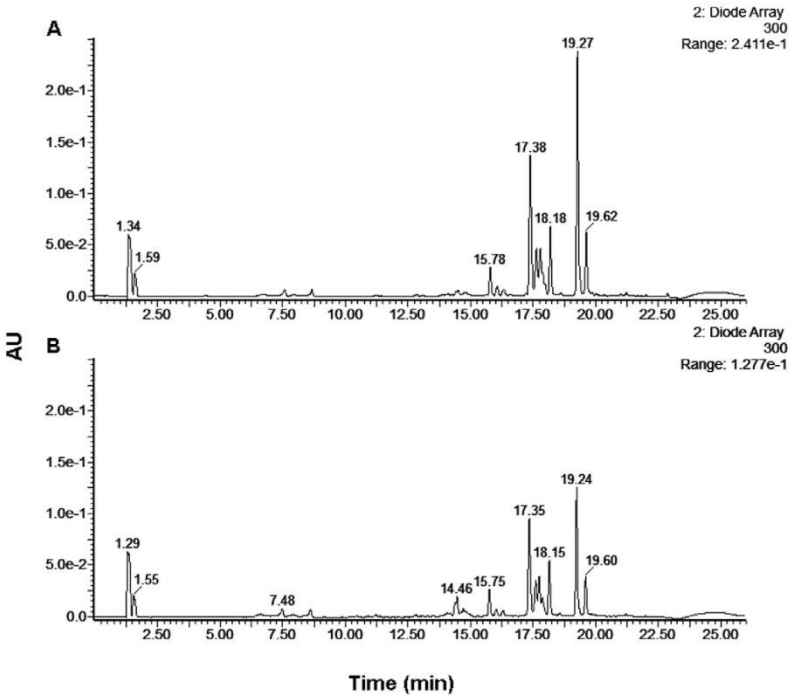

Figure 7. LC-DAD (300 nm) chromatogram of the phenolic compounds in the incubation medium before (A) and after (B) 2 h of exposure of the yeast *S. cerevisiae* to eluate EL70 (1%). AU, arbitrary units.
doi:10.1371/journal.pone.0056104.g007

model G1312A binary pump and a model G1330B autosampler (Agilent Technologies). This reversed-phase HPLC separation was carried out using a Gemini C18 column (150 mm×2.0 mm internal diameter; 3 µm particle size), which was protected by a Gemini C18 security guard cartridge (4.0 mm×2.0 mm internal diameter) (Phenomenex). The mobile phase comprised aqueous 1% formic acid (A) and acetonitrile (B), and the following gradient was used: 0–5 min: 10% B; 5–50 min: 10%–60% B; 50–52 min: 60%–80% B; 52–60 min: 80% B; 60–70 min: 80%–10% B; 70–80 min: 10% B. The column was maintained at 25°C, with an injection volume of 20 µL and a flow rate of 0.2 mL/min.

Liquid Chromatography-mass Spectrometry Analysis

To identify the phenolic compounds, liquid chromatography-mass spectrometry (LC-MS) was used. The samples were diluted 20-fold in 1% formic acid in 50% methanol and analyzed by LC-MS. A Micromass Quattro Micro mass spectrometer equipped with an electrospray ionization source was operated in negative ion mode (Waters, Milford, MA, USA). The mass spectra were recorded with the following operating parameters: capillary voltage, 3.0 kV; cone voltage, 25 V; extractor, 5 V. The source temperature was 100°C, and the desolvation temperature was 350°C. The cone gas flow was set at 50 L/h, while the desolvation gas flow was set to 400 L/h, and the collision energy at 20 V. The phenolic compounds were identified on the basis of m/z of the $[M-H]^-$ and MS^2 ions.

Statistical Analysis

The data are expressed as means ±S.D., as determined from triplicate analysis. Duncan's multiple range tests, at $P<0.05$, determined the significant differences among the means.

Results and Discussion

The present study is a continuation of our previous study [7], where we showed that with propolis, the antioxidative activity is not related to the whole propolis extract, but to only a part of it. To better understand which phenolic compounds are responsible for the antioxidative activity of propolis in cells, here fractionation of phenolic compounds in a crude ethanolic propolis extract was performed using SPE (Fig. 1). Five sequential eluates that varied in polarity were obtained, according to the ethanol used for the elution (30% to 70%): EL30, EL40, EL50, EL60 and EL70, from more to less polar, respectively. For the cleaned ethanolic extract of propolis (EE96) as well for the EL30 to EL70 eluates, the phenolic profiles were determined using LC-DAD (Fig. 2). As expected, these profiles differed between the eluates.

Here, as well as the phenolic profile, the total phenolic content and free radical scavenging activity of EE96 and EL30 to EL70

Table 2. Phenolic compounds identified in eluate EL70 using mass spectrometry detection.

$[M-H]^-$ (m/z)	MS^2 ions (m/z)	Compound	Reference
247	134 179	Caffeic acid isoprenyl ester	[20]
253	143	Chrysin	[19]
253	118	*p*-Coumaric benzyl ester	[21]
255	107 151	Pinocembrin	[20]
269	117 107	Apigenin	[22]
269	134	Caffeic acid benezyl ester	[20]
283	179 135 161	Caffeic acid phenethyl ester	[23]
285	252 138 224 165	Pinobanksin-5-methyl-ether	[19]
285	165 185	Unknown	
285	135 163	CA- and CO-derivate	[19]
295	134	Caffeic acid cinnamyl ester	[20]
299	179 135	CA-derivate	[19]
299	227 255	Luteolin-methyl-ether	[19]
299	284 151 164	Kaempferide	[21]
313	253 107	Pinobanksin-3-O-acetate	[20]
315	271 255	3-Prenyl-4-(2-methylpropionyl-oxy)-cinnamic acid	[21]
315	165 121	Rhamnetin	[24]
327	253	Pinobanksin-3-O-propionate	[20]
341	163 119	CO-derivate	[19]
341	253	Pinobanksin-3-O-butyrate	[20]
355	253	Pinobanksin-3-O-pentanoate	[20]
381	119 135 179 163	CA- and CO-derivate	[19]
404	294	Unknown	
425	163	CO-derivate	[19]
455	163 193	FE- and CO-derivate	[19]
457	179 161 235 295 135	CA-derivate	[19]
471	193 179 235 135 175 161	CA- and FE-derivate	[19]

CA, caffeic acid; CO, coumaric acid; FE, ferulic acid.
doi:10.1371/journal.pone.0056104.t002

were determined (Table 1). The highest total phenolic content was seen for eluates EL60 and EL70 (0.82 g GAE/L and 0.83 g GAE/L, respectively), which also showed the highest *in vitro* antioxidative activities, measured as the free radical scavenging activity (1.20 g GAE/L and 1.22 g GAE/L, respectively). Thus, eluates EL30 to EL50 showed lower total phenolic contents (0.26 g GAE/L to 0.39 g GAE/L), which was reflected in their lower *in vitro* antioxidative activities (0.18 g GAE/L to 0.33 g GAE/L). The sum of the total phenolic content in these eluates was higher compared to the total phenolic content of EE96. This was expected, as the total volume of ethanol used for elution of the eluates (EL30 to EL70) was five-fold higher than the volume of ethanol used for elution of the cleaned propolis EE96 (Fig. 1). Therefore, some of the phenolic compounds from the crude propolis extract were lost in the process of obtaining EE96.

Fractionation of these compounds extracted from propolis has been reported previously [11,12], where nanofiltration [11] and supercritical fluid extraction [12] were used. In the case of nanofiltration, the compounds from an ethanolic extract of propolis were fractionated according to their molecular weights. The DPPH test was performed, and it was shown that the antioxidative activity was proportional to the flavonoid content of the fractions. The same was concluded for the fractions obtained using supercritical fluid extraction.

On the bases of *in vitro* studies, predictions about the activities of the phenolic compounds in the cell are usually done. However, this extrapolation can be misleading and additional *in vivo* studies need to be conducted.

Therefore, the antioxidative activities of the cleaned propolis EE96 and the eluates (EL30–EL70) that showed free radical scavenging activities were here investigated using an appropriate model organism. These antioxidative activities were determined by measuring intracellular oxidation using the yeast *S. cerevisiae*. The yeast was treated for 2 h with EE96 or the EL30 to EL70 eluates. With EE96 used in the yeast suspensions at concentrations from 0.00125 g GAE/L to 0.02 g GAE/L, there was a dose-dependent decrease in the intracellular oxidation (Fig. 3). This decrease continued to 0.01 g GAE/L EE96, after which it reached a plateau.

Next, the yeast suspensions were treated for 2 h with the eluates EL30 to EL70 at 1% (v/v). Preliminary experiments showed that the solvent of 1% (v/v) ethanol has no effects on intracellular oxidation in these yeast suspensions (data not shown). The greatest decreases in the intracellular oxidation were seen when the EL70 eluate was added, followed by eluates EL60 and EL50. In contrast, eluate EL30 showed a trend to an increase in intracellular oxidation, whereas no statistical difference compared to the control was seen for EL40 (Fig. 4). Eluates EL60 and EL70 have

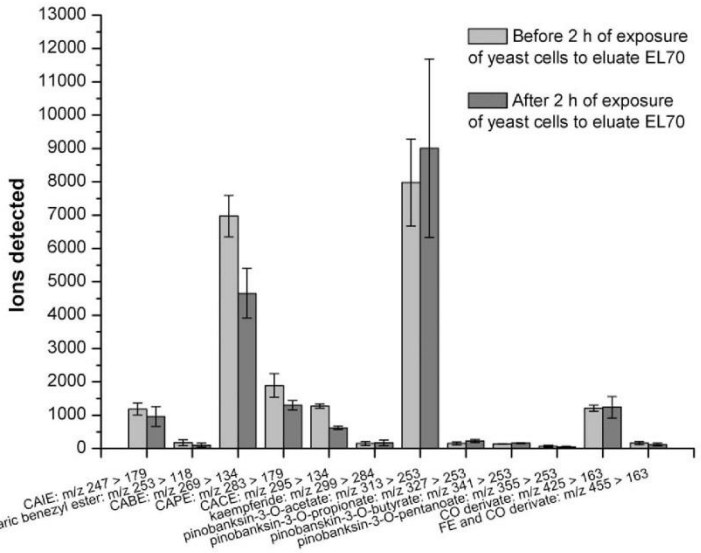

Figure 8. LC-MS/MS of the phenolic compounds in the incubation medium before and after 2 h of exposure of the yeast *S. cerevisiae* to eluate EL70 (1%). Data are means ±S.D. (n = 3), and are expressed as the detected ions. CAIE, caffeic acid isoprenyl ester; CABE, caffeic acid benezyl ester; CAPE, caffeic acid phenethyl ester; CACE, caffeic acid cinnamyl ester; CO, coumaric acid; FE, ferulic acid.
doi:10.1371/journal.pone.0056104.g008

the same total phenolic content and free radical scavenging activities (Table 1), although they have different *in vivo* antioxidative activities determined by 2′,7′-DCF. Eluate EL70 decreased the intracellular oxidation by 66%, whereas eluate EL60 decreased it by 42%. The diversity of the phenolic compounds in these eluates would be a reason for this difference. Eluates EL30 and EL50 both have free radical scavenging activity, although they show different effects on yeast intracellular oxidation. In the case of eluate EL50, the intracellular oxidation decreased by 16%, whereas eluate EL30 increased it by 18%. Therefore, these data indicate that in these yeasts, the free radical scavenging activities of these eluates do not always correlate with their antioxidative activities.

As well as the antioxidative activities, insights into other biological activities are also of importance. Therefore, we investigated the cellular metabolic energy and the viability of these treated yeast.

For the cellular metabolic energy, eluates EL60 and EL70 again showed differences compared to the others, as seen in Figure 5. An increase in cellular metabolic energy was seen when eluates EL60 and EL70 were added to the yeast suspensions at the final concentration of 1% (v/v). Despite the same concentration of total phenolics in these two eluates (Table 1), eluate EL70 showed a greater increase in cellular metabolic energy (21%) than seen for EL60 (6%). This difference in effects between these eluates was already seen in the case of intracellular oxidation. With eluates EL30 to EL50, there were small, but significant, decreases in the cellular metabolic energy compared to the control (Fig. 5). As also seen in Figure 5, the cellular metabolic energy increased (19%) in the yeast treated with the cleaned propolis EE96 at the same final concentration of 1% (v/v).

Cell viability was measured as cell-membrane integrity using the nucleic-acid-specific dyes SYTO®9 and propidium iodide in the LIVE/DEAD® Funga Light TM Yeast Viability kits (Molecular Probes). These data showed no significant changes in the viability of the yeast treated with the cleaned propolis EE96 and the EL30 to EL70 eluates at this final concentration of 1% (v/v) (Fig. 6). It was previously demonstrated that Brazilian propolis induces cell death in *S. cerevisiae* [13]. As stated, no such effect was observed in our study, where stationary yeast cells as a model organism were used. We also tested activity of eluate EL70 in the exponential growth phase, where the same effect (decreased intracellular oxidation and no changes in cell viability) as in stationary growth phase was observed (data not shown). Thus, activity of eluate EL70 is growth-phase independent. Therefore, different results might be due to the differences in the composition of propolis and concentration of its phenolic compounds, since it is known that European propolis differ from Brazilian propolis [1].

As the antioxidative activities of phenolic compounds in the cell have been shown to be connected with their cellular uptake [7], the uptake from the particular eluates by these yeasts was also investigated here. The LC-DAD profile of the incubation medium before and after exposure of these yeast to the various eluates was determined. Here, some of the LC-DAD peaks of the eluate EL70 samples were decreased after treatment (Fig. 7: retention times: 17.32±0.05 min; 18.17±0.02 min; 19.26±0.02 min; 19.61±0.01 min), whereas no changes in these LC-DAD profiles were seen before and after the treatments with eluates EL30 to EL50 (data not shown). These decreased LC-DAD peaks in eluate EL70 were also seen in EL60, but to a lesser extent. This might be a reason for the higher antioxidative activity of eluate EL70 compared to eluate EL60 (Fig. 4). Despite no apparent cellular uptake from eluate EL30, it increased intracellular oxidation

(Fig. 4). This might be explained in terms of many phenolic compounds being unstable in cell-culture media, whereby they can undergo rapid oxidation, to generate hydrogen peroxide and other ROS [14–17]. Among these products, hydrogen peroxide can pass through cell membranes by passive diffusion, which would contribute to an increase in intracellular oxidation [18].

Thus, we have shown initially that there is cellular uptake, a decrease in intracellular oxidation, and an increase in cellular metabolic energy in yeast treated with eluate EL70. This led to further investigation of the individual phenolic compounds in this EL70 fraction of the crude propolis extract. Here we used LC-MS/MS and focussed on the LC-DAD retention times from 17 min to 20 min.

Based on comparison of our m/z of $[M-H]^-$ and MS^2 ions with those described in the literature [19–24], we were able to identified the following phenolic compounds: caffeic acid esters (caffeic acid isoprenyl ester, caffeic acid benezyl ester, caffeic acid phenyethyl, caffeic acid cinnamyl ester), p-coumaric benzyl ester, flavonoids (chrysin, pinocembrin, apigenin, kaempferide, rhamnetin), phenolic acid derivates (caffeic acid derivate, coumaric acid derivate, ferulic acid derivate), pinobanksin derivates (pinobanksin-5-methyl-ether, pinobanksin-3-O-acetate, pinobanksin-3-O-propionate, pinobanksin-3-O-butyrate, pinobanksin-3-O-pentanoate) and luteolin-methyl-ether and 3-prenyl-4-(2-methylpropionyl-oxy)-cinnamic acid) (Table 2). This identification of the individual phenolic compounds enabled investigation of their cellular uptake in more detail. The parent/daughter ions of individual phenolic compounds that were detected before and after 2 h of exposure of the yeast to 1% (v/v) eluate EL70 were compared (Fig. 8). Ions from caffeic acid benezyl ester and pinobanskin-3-O-acetate dominated. Significant decreases in the ions detected were observed for caffeic acid benezyl ester, caffeic acid phenethyl ester, caffeic acid cinnamyl ester. Other phenolic compounds that are not shown were identified in eluate EL70, but were only present at trace levels.

To our knowledge, this is the first study where solid-phase extraction has been used for fractionation of phenolic compounds from crude propolis, with the investigation of the various activities of the individual eluates investigated in yeast as a model organism: intracellular oxidation, cellular metabolic energy, and cell viability. Additionally, we have identified the cellular uptake of individual phenolic compounds that may contribute to the antioxidative and cellular metabolic energy effects of propolis in yeast.

Author Contributions

Conceived and designed the experiments: T. Petelinc PJ T. Polak. Performed the experiments: T. Petelinc. Analyzed the data: T. Petelinc LD. Wrote the paper: T. Petelinc PJ T. Polak.

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2.1.3 Vpogled v molekularne mehanizme aktivnosti propolisa z uporabo proteomike celičnih organelov in struktur

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Propolis je poznan po mnogih bioloških učinkih, vendar molekularni mehanizmi v ozadju le teh v večji meri ostajajo nepoznani.

V naši predhodni raziskavi smo iz etanolnega izvlečka propolisa z ekstrakcijo s trdno fazo (SPE) pridobili več frakcij in za frakcijo EL70 pokazali, da ima največji vpliv na celično raven kvasovke *S. cerevisiae* (Petelinc in sod., 2013a). To pomeni, da je frakcija EL70 pri koncentraciji 1 % (v/v) (≈0,01 g GAE/L) v celični suspenziji po 2- urni izpostavitvi najbolj znižala znotrajcelično oksidacijo (za 66 %) in najbolj povišala celično metabolno energijo (za 21 %) med pridobljenimi frakcijami v primerjavi s kontrolo (Petelinc in sod., 2013a).

V tej raziskavi smo vpliv frakcije EL70 analizirali na subproteomski ravni, pri čemer smo uporabili dvodimenzionalno elektroforezo (2-DE). Iz kvasovke *S. cerevisiae* smo ekstrahirali proteine in jih frakcionirali glede na subcelično lokacijo, pri čemer smo uporabili komplet za diferencialno detergentno frakcionacijo (DDF) "ProteoExtract subcellular extraction kit" (Calbiochem). Komplet je v osnovi namenjen ekstrakciji proteinov iz sesalskih celic, zato smo ga prilagodili za uporabo na kvasovki tako, da smo uvedli dodatno razbijanje celične stene in spiranja. Tako smo pridobili 4 subcelične proteome: citosolni, membransko/organelni, jedrni in citoskeletni.

2-urna izpostavitev kvasovke frakciji EL70 pri koncentraciji 1 % (v/v) (≈0,01 g GAE/L) v celični suspenziji se je odražala v spremembah v ravni 18 proteinov v primerjavi s kontrolo (s 96 % etanolom tretirane kvasne celice). Med temi so bili najbolj zastopani proteini, ki so vpleteni v metabolizem ogljikovih hidratov in energetski metabolizem (45 %). Proteini gliceraldehid-3-fosfat-dehidrogenaza (Tdh1), fruktoza-bisfosfat-aldolaza (Fba1), glukoza-6-fosfat-isomeraza (Pgi1) in piruvat-dekarboksilaza izoencim 1 (Pdc1) so imeli znižano raven, medtem ko so imeli podenota β komponente E1 mitohondrijske piruvat-dehidrogenaze (Pdb1), podenota β mitohondrijske sukcinil-CoA-ligaze (Lsc2), podenote 2 kompleksa III (Qcr2) in podenota β mitohondrijske ATP-sintaze (Atp2) povišano raven. Druga najbolj zastopana skupina diferencialno izraženih proteinov (22 %) so bili proteini, ki so povezani z dinamiko aktinskih filamentov. Aktin (Act1) in fruktoza-bisfosfat-aldolaza (Fba1) sta imela znižano raven, medtem ko sta imela aktinu sorodni protein 2 (Arp2) in protein toplotnega šoka HSP26 (Hsp26) povišano raven. Diferencialno izraženi proteini so bili povezani tudi z odzivom na oksidativni stres (11 %), kjer je imel Hsp26 povišano raven, protein NADPH-dehidrogenaze 2 (Oye2) pa znižano raven. Proteina Hsp26 in fosfomanomutaze (Sec53) sta imela povišano raven in sta

povezana z procesom zvijanja proteinov (11 %). Poleg tega je bil diferencialno izražen tudi protein Ran GTPaza GSP/CNR1 (Gsp1), ki je imel znižano raven.

Na podlagi informacije o identiteti diferencialno izraženih proteinov, njihovi funkciji in subcelični lokaciji smo pridobili boljši vpogled v procese, ki potekajo v celicah, ki so izpostavljene frakciji EL70, pridobljeni iz izvlečka propolisa in bogati z estri kavne kisline.

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Insight into the Molecular Mechanisms of Propolis Activity using a Subcellular Proteomic Approach

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ABSTRACT: The effects of a fractionated 70% ethanolic extract of propolis were analyzed at the subproteome level by two-dimensional electrophoresis. Differential detergent fractionation was used to fractionate proteins from the yeast *Saccharomyces cerevisiae* according to their subcellular localization. Thus, four subcellular proteomes were obtained: cytosolic, membrane/organelle, nuclear, and cytoskeletal. Yeast treatment resulted in changes in the levels of proteins involved in carbohydrate and energy metabolism, antioxidant defense, actin filament dynamics, folding of proteins, and others. On the basis of this information, we can obtain better insights into the processes that are carried out in cells exposed to propolis extract.

KEYWORDS: *Two-dimensional electrophoresis, subcellular fractionation, propolis, yeast*

INTRODUCTION

Propolis is a resinous substance that bees collect and use in their hive as a building material and as a disinfectant. In general, it contains resins and balsams (50%), waxes (30%), aromatic and essential oils (10%), pollen (5%), and other organic matter (5%). Because of various potentially beneficial properties of propolis, including antioxidative, anticancer, antibacterial, antiviral, and antifungal effects, it is an intriguing subject of research.[1−7] However, the molecular mechanisms of propolis activities remain largely unknown.

In our previous study, a 70% ethanolic extract of propolis was fractionated using polarity-based solid-phase extraction. Following exposure of the yeast *Saccharomyces cerevisiae* to the propolis fractions obtained, the effects were analyzed at the cellular level. The fraction termed 'eluate EL70' had the greatest effects on the yeast in terms of decreasing intracellular oxidation and increasing cellular metabolic energy.[8]

Yeast *S. cerevisiae* was used as a model organism, which is an excellent model for investigating fundamental cellular processes, stress responses, and metabolic pathways of the human.[9,10] Comparative genomics studies have shown that 40% of yeast proteins share amino acid sequence similarity with at least one human protein[11] and that 30% of genes with a recognized involvement in human disease have an orthologue in yeast.[12] Yeast presents many technical advantages over human cells. It is well-suited to high-throughput methods because its life cycle is rapid, it can grow as dispersed cells in liquid or as colonies on solid media, and its culture requires neither elaborate sterile technique nor expensive media. It is highly amenable to genetic modifications such as gene disruption, deletion, and replacement.[13] Experiments with yeast are easier technically, more rapid, and much less costly than experiments with human cells.[14] The ease of genetics, combined with the ease of growth, makes *S. cerevisiae* a model organism that beautifully combines the power of genetics with that of biochemical studies.[15]

In the present study, the influence of the eluate EL70 fraction (henceforth referred to as EL70) was further analyzed at the proteome level of yeast *S. cerevisiae* using two-dimensional (2D) electrophoresis. Subcellular fractionation was used to reduce the complexity of the protein extracts as well as for the enrichment of low-abundance proteins. These are poorly represented when a classic proteomic approach with a single extraction buffer is applied, thus losing information about important classes of proteins, such as transcriptional factors and regulatory proteins. Furthermore, subcellular fractionation enables an extracted protein to be associated with its localization in the cell, which also correlates strongly with the protein function.

Conventional subcellular fractionation procedures involve differential and density-gradient centrifugation.[16] However, newer techniques use the stepwise fractionation of cellular proteins that is based on the different solubilities of the different cellular compartments in detergents of increasing solubilization efficiency.[17] There are several kits now available for differential detergent fractionation, one of which is the ProteoExtract subcellular proteome extraction kit. This kit is designed for use with mammalian cells, and it yields four subcellular fractions that are enriched in cytosolic, membrane/organelle, nuclear, and cytoskeletal proteins. Previous studies have shown the use of this kit on a variety of cell types, including the SAOS2 osteosarcoma cell line, the A-431 epidermoid carcinoma cell line,[18] human colon mucosa cells,[19] frozen rat liver and heart tissue,[20] and human pancreatic cancer tissue.[21] Here, to the best of our knowledge, we provide the first report of the application of this kit to yeast cells to provide useful insight into our investigations into the molecular mechanisms of propolis activity.

MATERIALS AND METHODS

Chemicals and Standards. The Bradford reagent was from Bio-Rad. Peptone and yeast extract were from Biolife. Ammonium formate was from Fluka. The immobilized pH gradient (IPG) buffer and 3-[(3-

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cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) were from GE Healthcare. SYPRO Ruby was from Invitrogen. Ethanol, glucose, hydrochloric acid (HCl), and methanol, were from Merck. Phosphate-buffered saline (PBS) was from Oxoid. Modified porcine trypsin was from Promega. Bovine serum albumin, bromophenol blue, dithiothreitol, glycerol, iodoacetamide, sodium dodecyl sulfate (SDS), thiourea, urea, tris(hydroxymethyl)-aminomethane (Tris), and 4-hydroxy-α-cyano-cinnamic acid were from Sigma.

Sample Preparation. Propolis was collected from a bee hive in the Savinjska Valley (Slovenia) during the autumn of 2010. A propolis sample (10 g) was extracted using 70% ethanol (100 mL) by mixing for 1 h at room temperature. The crude propolis extract was obtained after centrifugation (3000*g*, 5 min) of the extraction mixture and concentration of the supernatant under vacuum using a rotary evaporator.

Solid-Phase Extraction. Solid-phase extraction was used to separate the crude propolis extract into five elution fractions according to polarity. The crude propolis extract (200 μL) was mixed with 20 mM ammonium formate, pH 3.2 (200 μL), and then added onto a STRATA-X solid-phase extraction cartridge containing 33 μm polymeric reverse-phase sorbent (60 mg/3 mL tube; catalog no. 8B-S100-UBJ; Phenomenex) that had previously been conditioned with methanol (2 mL) and equilibrated with 20 mM ammonium formate, pH 3.2 (2 mL). After the loading of the sample, the cartridge was washed with 20 mM ammonium formate in 15% methanol (2 mL) and vacuum-dried for 3 min. Next, the cartridge was eluted with 30% ethanol (2 mL) followed by 40% ethanol (2 mL), 50% ethanol (2 mL), 60% ethanol (2 mL), and 70% ethanol (2 mL). This provided the propolis fractions as the 30 (EL30) to 70% (EL70) ethanol eluates.

In the present study, EL70 was used. The composition of EL70 was determined in our previous study using liquid chromatography−tandem mass spectrometry, and the total phenolic content was determined using the Folin−Ciocalteu method (0.83 g of galic acid equivalent/L).[8]

Yeast Strain and Cultivation. The ZIM 2155 *S. cerevisiae* strain was obtained from the Culture Collection of Industrial Microorganisms (ZIM) of the Biotechnical Faculty, University of Ljubljana, Ljubljana, Slovenia.

The yeast cells were cultivated in yeast extract (10 g/L), peptone (20 g/L), and glucose (20 g/L) (YEPD) medium at 28 °C and with agitation at 220 rpm on a rotary shaker until they reached the stationary phase. The cells were then centrifuged (4000*g*, 3 min), washed once with PBS, and resuspended in PBS at 1×10^8 cells/mL. The yeast cells were further incubated at 28 °C and 220 rpm for 96 h.

Yeast Treatment. Following the 96 h incubation in PBS, the yeast cells were treated with EL70 (1% v/v). After a further 2 h incubation at 28 °C and 220 rpm, the yeast samples from three biological replicates were used for protein analysis.

Extraction of Subcellular Proteomes. Yeast suspensions (20 mL) were centrifuged (4000*g*, 3 min). The cell pellet obtained was washed twice with PBS and frozen at −80 °C until the extraction of the subcellular proteomes using the ProteoExtract subcellular proteome extraction kit (Calbiochem).

This kit was used according to manufacturer's instructions with some modifications. Briefly, the thawed yeast cell pellet was washed twice with wash buffer. After centrifugation (300*g*, 10 min, 4 °C), 1 mL of the first extraction buffer (containing 5 μL of protease inhibitor cocktail) was added to the cell pellet. The cells were disrupted with zirconia/silica beads (BioSpec Products) by vortexing five times for 1 min each spaced with 1 min intervals for cooling on ice. The supernatant obtained following centrifugation (1000*g*, 10 min, 4 °C) was stored as fraction 1 (F1), and the pellet was washed three times with 1 mL of the first extraction buffer. Then, following a 30 min incubation of the pellet with 1 mL of the second extraction buffer (containing 5 μL of protease inhibitor cocktail) with gentle agitation, fraction 2 (F2) was recovered by centrifugation (6000*g*, 10 min, 4 °C). The pellet obtained was washed two times with 1 mL of the second extraction buffer. Next, 0.5 mL of the third extraction buffer (containing 5 μL of protease inhibitor cocktail and 1.5 μL of the Benzonase nuclease) was added to the pellet. After a 10 min incubation with gentle agitation, the supernatant following centrifugation (7000*g*, 10 min, 4 °C) was stored as fraction 3 (F3), and the pellet was washed once with 0.5 mL of the third extraction buffer. Finally, fraction 4 (F4) was obtained following addition of 0.5 mL of the final extraction buffer (containing 5 μL of protease inhibitor cocktail) to the pellet and resuspension of the sample by pipetting.

The protein concentration in each of the fractions was determined according to the method of Bradford,[22] with bovine serum albumin as the standard.

Clean-up of the Fractions. Fractions F2−F4 included a cleanup step prior to 2D electrophoresis because of the low protein concentrations in these samples. Thus, 2D clean-up kits (GE Healthcare) were used. In short and according to the manufacturer's instructions, the volume of the samples that corresponded to 100 μg of protein were mixed with three volumes of the precipitant and incubated on ice for 15 min. Afterward, three volumes of the coprecipitant was added followed by centrifugation (8000*g*, 10 min, 4 °C). More of the coprecipitant (80 μL) was added to the pellet, and once it was resuspended, it was incubated on ice for 5 min. After centrifugation (8000*g*, 5 min, 4 °C), double-distilled water (50 μL), chilled wash buffer (1 mL), and the wash additive (5 μL) were added to the pellet, which was then mixed and incubated (30 min, −20 °C), with vortexing every 10 min. Following centrifugation (10 000*g*, 10 min, 4 °C), the pellet was left to air-dry for 5 min and then redissolved in 250 μL of rehydration solution (7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 2% (v/v) IPG buffer (pH 4−7), 18 mM dithiothreitol, and a trace of bromophenol blue) and incubated for 60 min at room temperature, with protein concentrations determined according to the method of Bradford.[22]

Two-Dimensional Electrophoresis. Two-dimensional electrophoresis was performed according to the method of Görg,[23] with minor modifications. The samples (50 μg of protein) were mixed with the rehydration solution (see above) and applied to 13 cm pH 4−7 IPG strips (GE Healthcare). After rehydration, the first dimension of the isoelectric focusing was carried out at 20 °C on a Multiphore II system (GE Healthcare). The following voltage program was applied: 0−300 V as a gradient over 1 min, 300 V fixed for 1 h, 300−3500 V as a gradient over 1.5 h, and 3500 V fixed for 5 h. Prior to the second dimension of the 2D electrophoresis, the IPG strips were equilibrated for 15 min in SDS equilibration buffer (75 mM Tris-HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, and a trace of bromophenol blue) containing 1% (w/v) dithiothreitol and then for an additional 15 min with the addition of 4.8% (w/v) iodoacetamide. The second dimension (SDS polyacrilamide gel electrophoresis) was carried out with 12% running gels on a vertical SE 600 discontinuous electrophoretic system (Hoefer Scientific Instruments) at a constant 20 mA/gel for 15 min and then at a constant 40 mA/gel until the bromophenol blue reached the bottom of the gel. The 2D gels were stained with SYPRO Ruby.

For each fraction, three 2D gels were run under the same conditions.

Protein Visualization and Image Analysis. After staining, the gels were documented using the CAM-GX-CHEMI HR system (Syngene). This gel image analysis was carried out using the 2D Dymension software, version 2.02 (Syngene). The 2D gels of the particular fractions extracted from the treated yeast were compared to the gels of the corresponding fraction from the control cells.

The spots on the gels were quantified on the basis of their normalized volumes as the spot volume divided by the total volume of all of the spots resolved in the gels. The expression changes (as fold changes) were considered significant if the intensity of the particular spot reproducibly differed by >1.5-fold between the control and the treated samples and if this was statistically significant (ANOVA).

Protein Identification and Bioinformatics Analysis. The proteins in the individual spots from the gels were identified using matrix-assisted laser desorption/ionization−time-of-flight/time-of-flight mass spectrometry, at the Proteomics Technology Facility, Department of Biology, University of York (York, United Kingdom), as previously described.[24]

Briefly, a volume of 10 μL of solution containing 0.02 μg/μL of modified trypsin was added to washed and dried gel pieces. Digestion was performed overnight at 37 °C. One microliter of each sample was loaded on a MALDI target plate followed by an equal volume of matrix solution (5 mg/mL of 4-hydroxy-α-cyano-cinnamic acid in 50% (v/v) aqueous acetonitrile containing 0.1% (v/v) trifluoroacetic acid). Positive-ion MALDI mass spectra were obtained using a Ultraflex III MALDI-TOF/TOF (Bruker). The mass spectrometer was externally calibrated with a mixture of des-Arg-Bradykinin, Angiotensin I, Glu-Fibrinopeptide B, ACTH (1-17 clip), ACTH (18-39 clip), ACTH (7-38 clip). The ten strongest peaks of interest were chosen for further MS/MS fragmentation in LIFT mode. FlexAnalysis software (Bruker, version 3.3) was used to perform the spectral processing and peak list generation for both the MS and MS/MS spectra.

Tandem mass spectral data were submitted to database searching using the Mascot program (Matrix Science Ltd., version 2.3) through the ProteinScape interface (Bruker, version 2.1). Spectra were searched against the NCBInr 20101130 database. The following search criteria were applied: *S. cerevisiae* as the species and tryptic digestion with a maximum number of one missed cleavage. The peptide mass tolerance was set to ±100 ppm, and the fragment mass tolerance was set to ±0.5 Da. Additionally, carbamidomethylation and oxidation were considered as possible fixed and variable modifications, respectively. The results were filtered to accept only peptide matches with an expect score of 0.05 or better.

To obtain information about the cellular localization of the proteins identified, the UniProt database was used (http://www.uniprot.org/).

■ RESULTS AND DISCUSSION

In the present study, the effects of a fractionated 70% ethanolic extract of propolis were studied at the proteome level using 2D electrophoresis as a continuation of our previous study[8] where these activities were investigated primarily at the cellular level.

In classic proteomic procedures, a single buffer is used to extract the cellular proteins. Here, in contrast, the fractionation method was based on the different solubilities of the cellular compartments using sequentially added detergent-containing buffers of increasing extraction efficiency. Thus, we reduced the complexity of the extract as well as enriched the low-abundance proteins, which can otherwise be masked by the high-abundance proteins. The commercially available kit used for the differential detergent fractionation allowed the fractionation of the proteins according to their cellular localization, thus providing four subproteomes. According to the manufacturer, the fractions obtained can be defined as cytosolic (F1), membrane/organelle (F2), nuclear (F3), and cytoskeletal (F4). Because this differential detergent fractionation kit was designed for mammalian cells, we modified the procedure for yeast cells. Thus, two additional steps were added: (1) mechanical cell disruption using zirconia/silica beads and (2) pellet washing between the sequential additions of the different detergent-containing buffers to avoid contamination with proteins from the other subcellular compartments; the numbers of washing steps were determined according to the protein concentrations in the eluates (Figure 1). In addition, prior to 2D gel electrophoresis, F2−F4 were purified and concentrated using a 2D clean-up kit.

Analysis of Subproteomic Profiles. Because the application of this differential detergent fractionation kit to protein extraction from yeast is novel, we first examined whether the protein extraction occurred according to distinct subcellular compartments. Thus, characterization of the four subcellular fractions obtained was carried out.

Analysis of these individual subproteomic profiles separated on the 2D gels showed distinct protein patterns for each

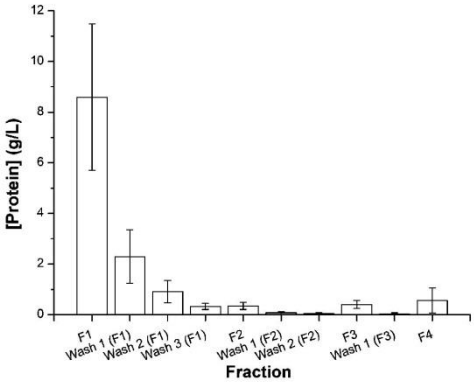

Figure 1. Protein concentrations in the F1−F4 fractions extracted from the yeast *S. cerevisiae* using the subcellular proteome extraction kit.

fraction. Furthermore, some of the protein spots were found only in a single subproteome (Table 1), and as such, they can be used to match each fraction with its specific subcellular compartment. Protein identification and further bioinformatics analysis of the cellular localizations were carried out. As shown in Table 1, the different tentative cellular localizations for the protein spots from each of the single subproteomes were annotated, which also demonstrated that some of the proteins resided in more than one location in the cell.[25] However, this might indicate that a protein shuttles between different compartments to carry out a specific function.[26] On this basis, we evaluated the distributions of the annotations for the cellular localizations of protein spots found in a single, distinct subproteome (Figure 2). Proteins from F2 were annotated to the mitochondrial outer and inner membranes as well as the mitochondrial intermembrane space, whereas the proteins in F3 can be tentatively located to the mitochondrial matrix, mitochondrial nucleoid, and mitochondrial inner membrane. Furthermore, proteins from F3 were also annotated as being of nuclear and chromosomal origins.

By further analyzing the 2D gels, most of the protein spots were found as consensus spots in two or more subproteomic profiles. This might mean that a protein is found in multiple cell locations simultaneously, or it might result from contamination with the proteins from the other subcellular compartments. Proteins can perform different functions depending on their local environment, pH, cofactor availability, protein interactions, and post-translational modifications, such as phosphorylation and proteolysis.[27] Thus, in the case of proteins with multiple locations in the cell, each might imply a different function of the protein. Furthermore, simultaneous fractionation of the cellular proteins according to the subcellular compartments also allowed us to observe dynamic changes in the distribution of these proteins. This is important because proteins can be redistributed in response to a variety of physiological stimuli. Furthermore, activation of numerous cellular regulatory pathways is accompanied by the translocation of key proteins from one subcellular compartment to another.[28] A contamination could be the result of incomplete extraction in the previous step of the extraction procedure, which is important to consider in the case of high-abundance

Table 1. Proteins Expressed in the Single F2 and F3 Subproteomes That Were Extracted from the Yeast *S. cerevisiae* using the Subcellular Proteome Extraction Kit

spot no.	UniProt accession no.	gene name	protein name	theoretical M_r (kDa)/pI	score	matched peptide	sequence coverage (%)	tentative subcellular localization annoted in the UniProt database
F2								
1	P00830	Atp2	ATP synthase subunit beta, mitochondrial	54.8/5.52	666	7(7)	18	Mitochondrion. Mitochondrial inner membrane. Mitochondrial intermembrane space. Cytosol.
2								
3					800	8(8)	21	
					843	8(8)	21	
4	P23644	Tom40	Mitochondrial import receptor subunit TOM40	42.2/5.34	470	5(5)	23	Mitochondrion. Mitochondrial outer membrane. Mitochondrial intermembrane space. Cytosol.
5	P07257	Qcr2	Cytochrome b-c1 complex subunit 2, mitochondrial	40.5/7.67	527	7(7)	23	Mitochondrion. Mitochondrial inner membrane.
6	P08067	Rip1	Cytochrome b-c1 complex subunit Rieske, mitochondrial	23.6/8.24	181	2(2)	13	Mitochondrion. Mitochondrial inner membrane.
7	P00128	Qcr7	Cytochrome b-c1 complex subunit 7	14.6/5.62	379	5(5)	38	Mitochondrion. Mitochondrial inner membrane.
F3								
8	P15705	Sti1	Heat shock protein STI1	66.4/5.45	935	10(10)	21	Cytoplasm.
9					893	10(10)	19	
10					896	10(10)	21	
11	P04147	Pab1	Polyadenylate-binding protein, cytoplasmic and nuclear	64.5/5.71	470	6(6)	12	Nucleus. Cytoplasm. Ribosome. Cytoplasmic stress granule.
12	P16451	Pdx1	Pyruvate dehydrogenase complex protein X component, mitochondrial	45.5/5.55	540	6(6)	22	Mitochondrion. Mitochondrial matrix.
13	P21827	Srm1	Guanine nucleotide exchange factor SRM1	53.5/5.71	112	2(2)	2	Chromosome. Nucleus.
14	P16387	Pda1	Pyruvate dehydrogenase E1 component subunit alpha, mitochondrial	46.7/8.29	186	3(3)	7	Mitochondrion. Mitochondrial matrix. Mitochondrial nucleoid.
15	P07256	Cor1	Cytochrome b-c1 complex subunit 1, mitochondrial	50.3/6.77	317	4(4)	11	Mitochondrion. Mitochondrial inner membrane.
16	P32473	Pdb1	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	40.1/5.23	649	6(6)	23	Mitochondrion. Mitochondrial matrix. Mitochondrial nucleoid.
17					614	6(6)	23	
18	P53228	Nqm1	Transaldolase NQM1	37.3/5.99	330	5(5)	16	Cytoplasm. Nucleus.
19					355	5(5)	17	

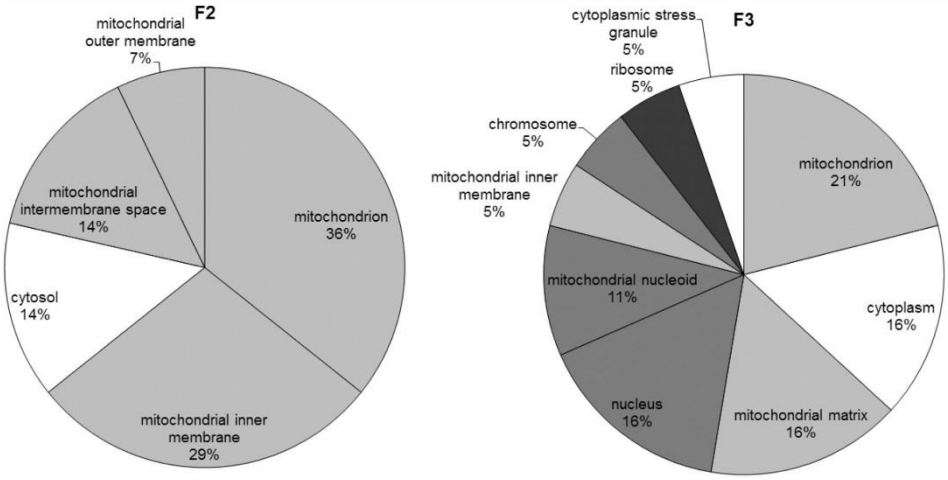

Figure 2. Tentative subcellular localization of the proteins identified that were extracted in a single subproteome, F2 or F3, on the basis of gene ontology annotations.

proteins because these can be present in abundance over the low-abundance proteins by up to 6 orders of magnitude . Even after several washing steps, the quantity of the residue protein might still be high in relation to the low-abundance proteins

Table 2. Differentially Expressed Proteins in Subproteomes F1, F2, and F4 Extracted from the Yeast *S. cerevisiae* using the Subcellular Proteome Extraction Kit[a]

spot no.	UniProt accession no.	gene name	protein name	fold change (treated vs control)	P value (ANOVA)	theoretical M_r (kDa)/p*I*	score	matched peptide	sequence coverage (%)	tentative subcellular localizations annoted in the UniProt database
F1										
20	P00360	Tdh1	Glyceraldehyde-3-phosphate dehydrogenase 1	−2.9	0.004	43.4/5.30	311	5(5)	13	Cytoplasm. Mitochondrion. Lipid particle. Plasma membrane. Cell wall.
21	P60010	Act1	Actin	−1.8	0.06	41.9/5.44	538	6(6)	23	Nucleus. Cytoskeleton. Chromosome. Cytoplasm.
22	P43616	Dug1	Cys-Gly metallodipeptidase DUG1	−1.5	0.03	53.1/5.43	492	6(6)	17	Cytoplasm. Mitochondrion. Ribosome.
23	P14540	Fba1	Fructose-bisphosphate aldolase	−1.5	0.03	39.9/5.51	510	5(5)	23	Cytoplasm. Mitochondrion. Cytosol.
24	P12709	Pgi1	Glucose-6-phosphate isomerase	−1.5	0.03	61.3/6.00	189	3 (3)	5	Cytoplasm. Mitochondrion. Plasma membrane.
F2										
25	P32381	Arp2	Actin-related protein 2	1.7	0.04	44.2/5.54	171	2(2)	5	Cytoplasm. Mitochondrion. Cytoskeleton.
26	P53312	Lsc2	Succinyl-CoA ligase [ADP-forming] subunit beta, mitochondrial	1.7	0.05	47.2/7.09	270	3 (3)	8	Mitochondrion.
27	Q03558	Oye2	NADPH dehydrogenase 2	1.6	0.04	44.9/6.13	151	3(3)	8	Nucleus. Cytoplasm. Mitochondrion.
28	P07257	Qcr2	Cytochrome b-c1 complex subunit 2, mitochondrial	1.5	0.06	40.5/7.67	784	9(9)	31	Mitochondrion. Mitochondrial inner membrane.
F4										
29	P32835	Gsp1	GTP-binding nuclear protein GSP1/CNR1	−2.2	0.01	25.0/6.11	140	2(2)	8	Cytoplasm. Nucleus.
30	P60010	Act1	Actin	−1.9	0.01	41.9/5.44	538	6(6)	23	Nucleus. Cytoskeleton. Chromosome. Cytoplasm.
31	P06169	Pdc1	Pyruvate decarboxylase isozyme 1	−1.7	0.02	61.7/5.80	211	3(3)	7	Nucleus. Cytoplasm. Cytosol.
32	P14540	Fba1	Fructose-bisphosphate aldolase	−1.7	0.03	39.9/5.51	510	5(5)	23	Cytoplasm. Mitochondrion. Cytosol.
33	P14540			−1.6	0.02		616	6(6)	26	
34	P32473	Pdb1	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	2.1	0.03	40.1/5.23	328	4(4)	14	Mitochondrion. Mitochondrial matrix. Mitochondrial nucleoid.
35	P07283	Sec53	Phosphomannomutase	2.0	0.002	29.2/5.14	283	3(3)	16	Cytoplasm. Cytosol.
36	P00830	Atp2	ATP synthase subunit beta, mitochondrial	1.6	0.01	54.8/5.52	530	7(7)	18	Mitochondrion. Mitochondrial inner membrane. Mitochondrial intermembrane space. Cytosol.
37	P15992	Hsp26	Heat shock protein 26	1.5	0.01	23.9/5.31	375	4(4)	28	Nucleus. Cytoplasm.

[a]After a 2 h exposure to the bioactive fraction of the 70% ethanolic extract of propolis, EL70 (1% v/v).

dx.doi.org/10.1021/jf4042003 | *J. Agric. Food Chem.* 2013, 61, 11502−11510

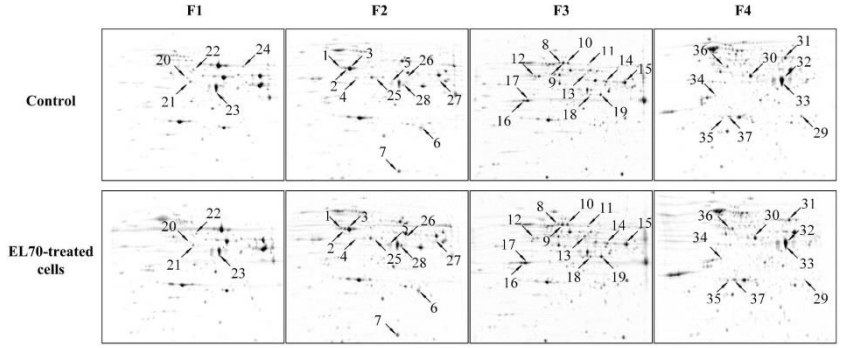

Figure 3. Representative 2D gel images of the four subcellular proteomes extracted from the yeast *S. cerevisiae*, with marked spots for the proteins extracted in a single fraction (1−19) and for the differentially expressed proteins (20−37). Details of the indicated spots are listed in Tables 1 and 2.

extracted in the following step of the procedure. The discrimination between a genuine resident of an enriched cellular compartment or a contaminant is possible by quantification of the consensus protein spots.[25]

Overall, this differential detergent fractionation kit proved to be more suitable for the enrichment of low-abundance proteins from particular cellular compartments rather than to extract the pure subproteomes, which is consistent with previous reports.[18−20]

Propolis Effects on the Yeast Subproteomes. After characterizing these subproteomic profiles, the effects of the EL70 fraction, obtained from the 70% ethanolic propolis extract (Materials and Methods), on these yeast cells was investigated at the proteome level. EL70 is the most active fraction of this 70% ethanolic propolis extract with respect to our previously defined EL70-induced decreases in intracellular oxidation and increases in cellular metabolic energy.[8] Here, a further proteomic study was carried out to understand the activity of EL70 in the cell better and thus to define further the molecular mechanisms of propolis activity.

Recently, propolis effects on yeast cells were examined at the mRNA level.[29,30] However, it should be noted that the abundance of specific mRNAs does not necessarily correlate with the abundance of the corresponding proteins. Therefore, functional changes in cells are best determined by monitoring the proteins that carry out these activities.[28] There was also a study in which the influence of propolis was investigated at the proteome level in cancer cell lines.[31] Differentially expressed proteins were found between the control and treated samples; however, protein identification was not carried out. In the present study, a subproteomic approach was used to study simultaneously the effects of this propolis extract fraction, EL70, on the four fractions extracted from the different cellular compartments by the differential detergent fractionation kit. These protein profiles were compared between the control and the treated samples within the same fraction (e.g., F1 control vs F1 treated). The differentially expressed proteins are summarized in Table 2 and illustrated in Figure 3.

Eighteen proteins showed different levels in the comparisons between the control and treated cells, and these are involved in different cellular processes. Among these, proteins involved in carbohydrate and energy metabolism were the most abundant group (44.4%). The proteins GAPDH (spot 20), FBPA (spot 23), Pgi1p (spot 24), and Pdc1p (spot 31) showed reduced levels upon EL70 treatment, whereas the proteins Lsc2p (spot 26), Qcr2p (spot 28), Pdb1p (spot 34), and Atp2p (spot 36) had increased levels. The second most represented group included proteins associated with actin filament dynamics (22.2%), where Act1p (spot 30) and FBPA (spots 32 and 33) had reduced levels upon EL70 treatment, whereas Arp2p (spot 25) and Hsp26p (spot 37) had increased levels. The proteins identified were also related to oxidative stress responses (11.1%), where Dug1p (spot 22) had decreased levels upon EL70 treatment and Oye2p (spot 27) had increased levels. The proteins Hsp26p (spot 37) and Sec53p (spot 35) had increased levels upon EL70 treatment and are associated with protein folding (11.1%) (Figure 4).

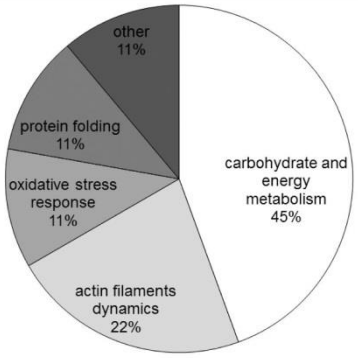

Figure 4. Cellular processes associated with the differentially expressed proteins in the yeast cells treated with EL70 (1% v/v).

In the framework of carbohydrate and energy metabolism, EL70 caused changes in the levels of proteins involved in glycolysis, the tricarboxylic acid cycle, and the mitochondrial respiratory chain. Glyceraldehyde-3-phosphate dehydrogenase 1 (GAPDH) is a key glycolytic enzyme, and it was reduced in the EL70-treated cells. As reported previously, inactivation of GAPDH causes the rerouting of the carbohydrate flux from glycolysis to the pentose phosphate pathway, thus supplying the

cell with NADPH (i.e., providing a reducing equivalent for antioxidant systems).[32] Additionally, we also observed reduced levels of two more glycolytic enzymes: fructose-bisphosphate aldolase (FBPA) and glucose-6-phosphate isomerase. This might indicate the induction of the pentose phosphate pathway in the EL70-treated cells. At the same time, exposure of these cells to EL70 promoted increased levels of pyruvate dehydrogenase E1 component subunit beta (mitochondrial). Pyruvate dehydrogenase converts pyruvate to acetyl-CoA, a substrate for the tricarboxylic acid cycle. In agreement with this, an enzyme that converts pyruvate to acetaldehyde, pyruvate decarboxylase isoenzyme 1, was reduced in these EL70-exposed cells. Furthermore, increased levels were also found for (1) succinyl-CoA [ADP forming] subunit beta (mitochondrial), which is the only enzyme in the tricarboxylic acid cycle that directly produces ATP, (2) cytochrome b-c_1 complex subunit 2 (mitochondrial), which is part of the electron transport chain where the proton motive force is generated, and (3) ATP synthase subunit beta (mitochondrial), which generates ATP in the presence of the proton motive force. These data could explain the increased cellular metabolic energy in yeast treated with EL70 that was shown in our previous study.[8]

The oxidative stress response was also a target of this treatment with EL70, as there were changes in the levels of two proteins associated with antioxidant systems. Exposure of these cells to EL70 promoted reduced levels of the Cys−Gly metallodipeptidase DUG1, which is involved in *S. cerevisiae* degradation of glutathione and which has homologues in bacteria, fungi, plants, and mammals.[33,34] Glutathione degradation has a major impact on the intracellular glutathione concentration, as blocked degradation of glutathione results in an increase in the cellular glutathione content.[35] Therefore, reduced Dug1p can help to maintain the levels of glutathione, an important cellular redox buffer that is involved in responses to sulfur and nitrogen starvation, detoxification of endogenous toxic metabolites and xenobiotics, protection against oxidative stress, and resistance to heavy-metal stress.[36] Moreover, there were increased levels of NADPH dehydrogenase 2 (Oye2p) in cells treated with EL70. An antioxidative role of Oye2p was shown in a study by Odat et al.[37] where the level of reactive-oxygen species (ROS) in cells with overexpressed Oye2p was lower (13%) compared to wild-type cells. Furthermore, NADPH generated in the pentose phosphate pathway can be used by Oye2p[37] or NADPH-dependent glutathione reductase, which maintains the balance between the oxidized and reduced forms of glutathione.[36] Oxidative stress response was expected because enhanced cellular metabolic energy is frequently accompanied by induction of the cellular antioxidant machinery to cope with the increased levels of ROS that are produced during mitochondrial respiration.[37] Thus, decreased levels of Dug1p and increased levels of Oye2p might explain the decreased intracellular oxidation in cells treated with EL70, as reported in our previous study.[8]

Another cellular process that is associated with several differentially expressed proteins is actin filament dynamics. In addition to actin (Act1p) itself, three actin-binding proteins were affected by treatment with EL70: actin-related protein 2 (Arp2p), FBPA, and heat shock protein 26 (Hsp26p). In the case of actin (Act1p), reduced levels were observed in two of the fractions: the cytosolic (F1) and cytoskeletal (F4) fractions. Arp2p is a part of the Arp2/3 complex that promotes actin filament assembly through its capping, nucleating, and branching activity;[38] here, Arp2p had increased levels with the EL70 treatment. Similarly, FBPA is mainly known as a glycolytic enzyme, although it also has a role in the inhibition of actin polymerization;[39] here, FBPA was also decreased in F1 and F4. Finally, for Hsp26p, there were increased levels upon EL70 treatment. Small heat shock proteins (sHSPs) with molecular masses of 15−30 kDa show affinity for actin as well, where it has been shown that the interaction between actin and nonphosphorylated sHSPs inhibits actin polymerization.[40] However, it appears that the binding of phosphorylated sHSPs with actin prevents actin depolymerization.[41] Additionally, Hsp26p might have a role not only in actin dynamics but also as a molecular chaperone that is responsible for correct protein folding under stress conditions.[42] Because the actin cytoskeleton is an early target of ROS,[43] these changes in the levels of actin and actin-binding proteins can be expected.

Other cellular processes were also impacted by EL70 treatment of these yeast. For example, phosphomannose, which catalyzes the conversion of mannose 6-phosphate to mannose 1-phosphate, had increased levels upon EL70 treatment. Together with GTP, mannose 1-phosphate forms GDP-mannose, which is required for the folding and glycosylation of secretory proteins in the lumen of the endoplasmic reticulum.[44] Additionally, reduced levels of the Ran GTPase GSP1/CNR1 were seen. It has been reported that there are increased levels of Ran GTPase in cancer cells.[45,46] Furthermore, it appears that only low levels of Ran GTPase are required for normal cell viability, whereas excessive Ran GTPase activity can deregulate normal cell function, predisposing the cells to tumorigenesis.[47] Thus, the reduced levels of GSP1/CNR1 found in the EL70-treated cells might indicate an antitumor activity of EL70.

On the basis of the information relating to the protein levels and their identities, subcellular localization, and functions, we can obtain better insight into the processes that are carried out in cells exposed to EL70, which results in decreased intracellular oxidation and increased cellular metabolic energy, as was also described in our previous study.[8]

We have used a proteomic approach at the subcellular level to analyze the cytosolic, membrane/organelle, nuclear, and cytoskeletal subproteomes simultaneously in cells exposed to a fractionated 70% ethanolic extract of propolis, EL70. This has provided further insight into the molecular mechanisms behind propolis activity. Most of the changes at the proteome level investigated here were involved in carbohydrate and energy metabolism, actin filament dynamics, oxidative stress response, and protein folding.

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Notes

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2.2 OSTALO POVEZOVALNO ZNANSTVENO DELO

2.2.1 Vpliv fenetilnega estra kavne kisline (CAPE) na kvasno celico

2.2.1.1 Uvod

Rezultati so pokazali, da izmed vseh fenolnih spojin v propolisu v kvasno celico vstopijo oz. z njo interagirajo le estri kavne kisline (benzilni ester kavne kisline, fenetilni ester kavne kisline (CAPE) in cimetni ester kavne kisline) in so zato potencialni kandidati za bioaktivne spojine propolisa, ki vplivajo na spremembe na celični ravni kvasovke, tj. znižano znotrajcelično oksidacijo in povišano celično metabolno energijo (Petelinc in sod., 2013a). V naši preliminarni raziskavi smo pokazali, da CAPE nima vpliva na znotrajcelično oksidacijo pri koncentraciji 0,05 g/L (≈176 μM) v suspenziji kvasovk (Cigut in sod., 2011) po 1-urni izpostavitvi, zato smo se odločili preveriti njegovo aktivnost še pri drugih koncentracijah, in sicer pri 1, 10, 100 in 1000 μM, po daljši 2-urni izpostavitvi.

2.2.1.2 Materiali

Gojišča

- Trdno gojišče YEPD

Preglednica 3: Sestava trdnega gojišča YEPD (Atlas, 1993)
Table 3: Composition of liquid medium YEPD (Atlas, 1993)

Sestavina	**Količina**	**Končna koncentracija**
Kvasni ekstrakt (Biolife)	10 g	1 % (w/v)
Pepton (Biolife)	20 g	2 % (w/v)
Glukoza (Merck)	20 g	2 % (w/v)
Agar (Biolife)	20 g	2 % (w/v)
Dodamo destilirano vodo do 1 L		

Gojišče smo sterilizirali 20 min pri temperaturi 121 °C in tlaku 1,1 bar. Po končani sterilizaciji smo gojišče ohladili na 45 °C in ga razlili v petrijevke.

- Tekoče gojišče YEPD

Preglednica 4: Sestava tekočega gojišča YEPD (Atlas, 1993)
Table 4: Composition of agar medium YEPD (Atlas, 1993)

Sestavina	Količina	Končna koncentracija
Kvasni ekstrakt (Biolife)	10 g	1 % (w/v)
Pepton (Biolife)	20 g	2 % (w/v)
Glukoza (Merck)	20 g	2 % (w/v)
Dodamo destilirano vodo do 1 L		

Gojišče smo sterilizirali 20 min pri temperaturi 121 °C in tlaku 1,1 bar.

Raztopine in reagenti

- Pufer PBS

Preglednica 5: Sestava pufra PBS
Table 5: Composition of PBS buffer

Sestavina		Količina	Končna koncentracija
1 tabletka (Oxoid)	NaCl	0,8 g	8 % (w/v)
	KCl	0,02 g	0,2 % (w/v)
	Na_2HPO_4	0,115 g	1,15 % (w/v)
	KH_2PO_4	0,02 g	0,2 % (w/v)
Dodamo bidestilirano vodo do 100 mL			

Pufer smo sterilizirali 20 min pri temperaturi 121 °C in tlaku 1,1 bar.

- Kalijev fosfatni pufer (pH=7,8)

50 mM kalijev fosfatni pufer smo pripravili tako, da smo zmešali 50 mM kalijev dihidrogenfosfat (KH_2PO_4) (Preglednica 6) in 50 mM kalijev hidrogenfosfat (K_2HPO_4) (Preglednica 7) v določenem razmerju, da smo dosegli pH 7,8. Sterilizirali smo ga s filtracijo (velikost por: 0,2 µm).

Preglednica 6: Sestava 50 mM K_2HPO_4
Table 6: Composition of a 50 mM K_2HPO_4

Sestavina	Količina	Končna koncentracija
KH_2PO_4 (Merck)	3,40 g	50 mM
Dodamo bidestilirano do 500 mL		

Preglednica 7: Sestava 50 mM KH_2PO_4
Table 7: Composition of a 50 mM KH_2PO_4

Sestavina	Količina	Končna koncentracija
K_2HPO_4 (Merck)	4,36 g	50 mM
Dodamo bidestilirano do 500 mL		

- 2',7'-diklorodihidrofluorescein diacetat (H_2DCFDA)

Preglednica 8: Sestava 1 mM založne raztopine 2',7'-diklorodihidrofluorescein diacetata (H_2DCFDA)
Table 8: Composition of a 1 mM stock solution of 2',7'-diclorodyhidrofluorescein diacetate (H_2DCFDA)

Sestavina	Količina	Končna koncentracija
H_2DCFDA (Sigma)	0,0049 g	1 mM
Etanol (96 % (v/v), Merck)	10 mL	

Uporabili smo sveže pripravljeno 1 mM raztopino H_2DCFDA.

Aparature in naprave

Priprava delovnih raztopin CAPE:
- kolona Strata-X 8B-S100-UBJ (Phenomenex)
- binarna gradientna črpalka Agilent 1100, G1312A (Agilent Technologies)
- avtomatski podajalnik Agilent 1100, G1330B (Agilent Technologies)
- predkolona Gemini C18, 3 μL, 150 mm x 2,0 mm (Phenomenex)
- kolona Gemini C18, 4,0 x 2,0 mm (Phenomenex)
- masni spektrometer Micromass Quattro Micro (Waters) z elekrorazpršilno ionizacijo v negativnem načinu (ESI^-)
- program MassLynx 4,0 (Micromass)

Priprava gojišč, raztopin in reagentov:
- pH-meter SevenEasy (Mettler Toledo)
- parni sterilizator - avtoklav (Sutjeska)
- magnetno mešalo MM-540 (Tehtnica)
- tehtnica TE214S (Sartorius)
- tehtnica PS 1200/C/2 (Radwag)

Kultivacija in inkubacija kvasovk *S. cerevisiae*:
- brezprašna komora LFV P122 (Pio)
- centrifuga Centric 200 (Tehtnica)
- centrifuga 322A (Tehtnica)
- inkubator IG 150 (Jouan)

- mikroskop B1 (Motic)
- stresalnik Multitron (Infors HT)
- spektrofotometer MA 9510 (Iskra)

Določanje znotrajcelične oksidacije:

- čitalec mikrotitrskih plošč Safire 2 (Tecan)

2.2.1.3 Metode

Priprava delovnih raztopin CAPE

CAPE je bil sintetiziran na Katedri za tehnologijo mesa in vrednotenje živil, Oddelka za živilstvo, Biotehniške fakultete, Univerze v Ljubljani z esterifikacijo kavne kisline in fenetilnega alkohola. Po koncu reakcije so bili vzorci očiščeni s trdno fazo (SPE). Čistost in donos CAPE v vzorcih je bil določen s HPLC-MS/MS analizo. Iz vzorcev smo nato izpareli topilo in pripravili delovne raztopine CAPE v 96 % (v/v) etanolu s koncentracijami 0,2; 0,02; 0,002 in 0,0002 M.

Kultivacija kvasovk *S. cerevisiae* in izpostavitev CAPE

Za precepljanje kulture smo uporabili trdno gojišče YEPD (Preglednica 3), za aerobno submerzno namnoževanje kvasne biomase smo uporabili tekoče gojišče YEPD (Preglednica 4), za vzdrževanje kvasne biomase v stacionarni fazi rasti pa smo uporabili PBS pufer (Preglednica 5).

3 dni staro kulturo *S. cerevisiae* smo s trdnega gojišča YEPD precepili v 50 mL tekočega gojišča YEPD v 100-mL erlenmajericah s stransko kiveto (Borosilicate z 1 utorom) do optične gostote pri 650 nm (OD_{650}) 0,95. Nato smo prenesli 40 mL brozge v 360 mL tekočega gojišča YEPD v 1-L erlenmajericah s stransko kiveto (Shot duran z 1 utorom). Sledila je kultivacija kvasovk pri 28 °C in 220 obr./min do začetka stacionarne faze rasti.

Po 60 urah smo preverili OD_{650} (≈2,0) in 50 mL brozge ($5x10^8$ celic/mL) centrifugirali 3 min pri 4000 obr./min (centrifuga 322A, Tehtnica), odstranili supernatant ter sediment 1x sprali s PBS. Celice smo resuspendirali v 50 mL PBS in nato 40 mL suspenzije prenesli v 160 mL PBS, da smo dosegli koncentracijo $1x10^8$ celic/mL v 500-mL erlenmajericah s stransko kiveto (Simex z 1 utorom). Sledila je inkubacija kvasovk nadaljnjih 96 ur pri 28 °C in 220 obr./min.

Celični suspenziji (10 mL) smo po 96-urni inkubaciji dodali 50 µL CAPE z ustrezno koncentracijo, tako da smo v suspenziji dosegli koncentracije 0, 1, 10, 100 in 1000 µM.

Po 2-urni izpostavitvi pri 28 °C in 220 obr./min v temi smo določili njihovo antioksidativno učinkovitost v celici z merjenjem znotrajcelične oksidacije, vpliv na celično metabolno energijo in celično živost s štetjem kolonijskih enot (ang. colony forming units, CFU) v primerjavi s kontrolo (s 96 % etanolom tretirane kvasne celice).

Določanje znotrajcelične oksidacije

Znotrajcelično oksidacijo v celicah kvasovk *S. cerevisiae* smo določili s spojino diklorofluorescein (DCF), ki po stiku z ROS/RNS fluorescira. Po 2-urni izpostavitvi smo v 2-mL Eppendorf centrifugirko prenesli 2 mL brozge in centrifugirali 5 min pri 14000 obr./min (centrifuga Centric 200, Tehtnica). Po odstranitvi supernatanta smo sediment 2x sprali s 50 mM kalijevim fosfatnim pufrom (pH 7,8) (Preglednica 6 in 7). Sedimentu smo nato dodali 990 µL 50 mM kalij fosfatnega pufra (pH 7,8) in inkubirali 5 min pri 28 °C. Po končani inkubaciji smo dodali 10 µL sveže pripravljene 1 mM raztopine 2',7'-diklorodihidrofluorescein diacetata (H_2DCFDA) (Preglednica 8). Eppendorf centrifugirke smo prenesli na stresalnik in inkubirali v temi 15 min pri 28 °C. Sledil je prenos vzorcev (200 µL) v črno mikrotitrsko ploščico in merjenje fluorescence na čitalcu mikrotitrskih plošč. Valovna dolžina vzbujanja je bila 488 nm, emisije pa 520 nm. Rezultati so bili izraženi kot relativne vrednosti fluorescenčne intenzitete glede na kontrolo - relativna fluorescenčna intenziteta (RFI).

Določanje celične živosti

Živost kvasovk *S. cerevisiae* smo določili s štetjem CFU. Po 2-urni izpostavitvi smo v 1,5-mL Eppendorf centrifugirko prenesli 1 mL brozge in centrifugirali 5 min pri 14000 obr./min (centrifuga Centric 200, Tehtnica). Po odstranitvi supernatanta smo sediment 2x sprali s pufrom PBS (Preglednica 5). Sedimentu smo nato dodali 1 mL pufra PBS in pripravili rečitve po Kochu. Ustrezno rečitev smo v treh paralelkah nacepili na trdno gojišče YEPD (Preglednica 3), razmazali s paličico po Drigalskem in inkubirali 48 ur pri 28 °C. Po zaključku inkubacije smo izmed nacepljenih petrijevih plošč izbrali številne in prešteli kolonije. Rezultati so bili izraženi kot relativne vrednosti glede na kontrolo.

Določanje celične metabolne energije

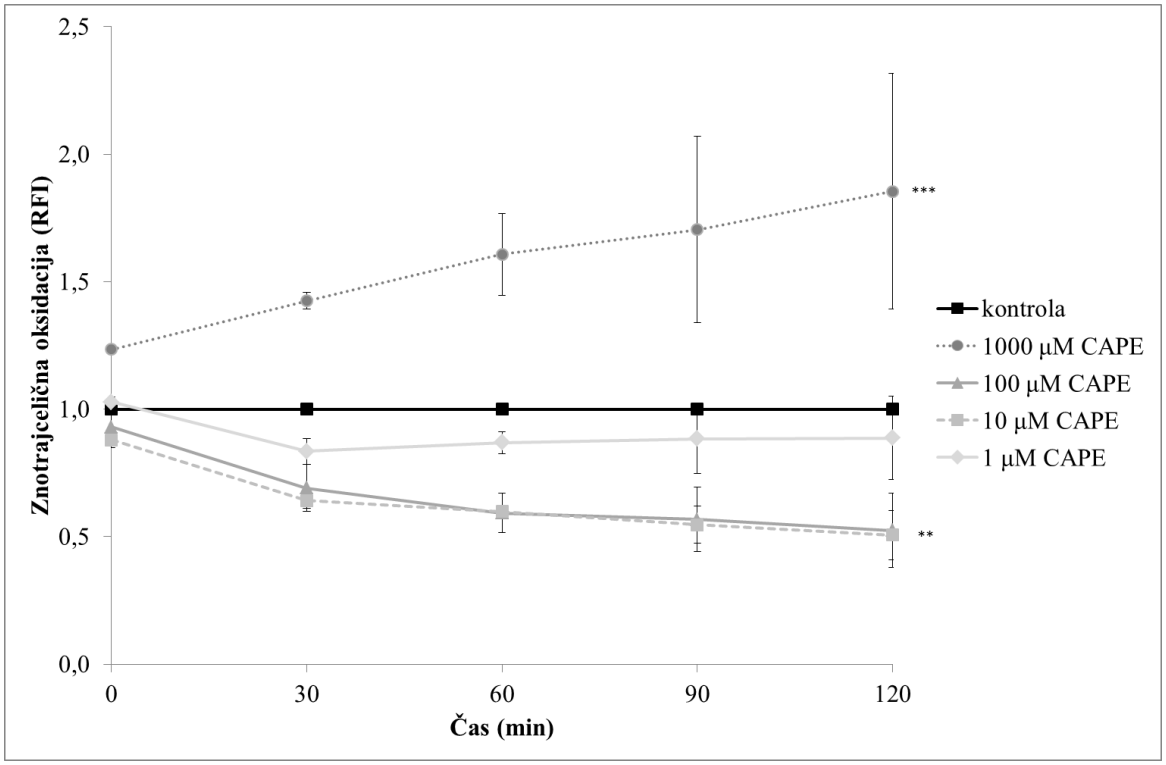
Za določanje ATP v celicah smo uporabili komplet "BacTiter-Glo", ki vsebuje substrat luciferin in encim luciferazo. Luciferaza ob prisotnosti ATP in kisika pretvori luciferin v oksiluciferin, ob čemer nastane svetloba. Po 2-urni izpostavitvi smo v jamico mikrotitrske ploščice nanesli po 100 µL 10-krat redčene tretirane celične suspenzije. V jamice smo nato dodali po 100 µL reagenta, ploščico vstavili v merilec mikrotitrskih plošč, premešali ter po

5 min pomerili luminiscenco vzorcev. Rezultate smo normalizirali na optično gostoto vzorcev pri 650 nm in jih izrazili kot relativne vrednosti glede na kontrolo.

Statistična analiza

Vrednosti so podane kot povprečje s standardnim odklonom vsaj treh neodvisnih eksperimentov. Statistično značilna razlika med kontrolo in tretiranim vzorcem je bila določena s Studentovim dvostranskim t-testom in je na sliki (Slika 3) ter v preglednici (Preglednica 9) označena kot **($p<0,05$) in ***($p<0,01$).

2.2.1.4 Rezultati



Slika 3: Znotrajcelična oksidacija kvasovke *S. cerevisiae* po 2-urni izpostavitvi fenetilnemu estru kavne kisline (CAPE) pri koncentracijah 0, 1, 10, 100 in 1000 µM, izražena kot relativna fluorescenčna intenziteta glede na kontrolo (RFI). Različne oznake pomenijo statistično značilne diferencialne vrednosti v primerjavi s kontrolo (p≤0,05, ***p≤0,01).**

Figure 3: Intracellular oxidation of yeast *S. cerevisiae* after 2-hour exposure to caffeic acid phenethyl ester (CAPE) at concentrations of 0, 1, 10, 100 and 1000 µM. Data are expressed as fluorescence intensity relative to control (RFI). Different marks mean statistically differential values compared to control (p≤0.05, ***p≤0.01).**

Rezultati so pokazali, da po 2-urni izpostavitvi kvasnih celic, CAPE statistično značilno (p≤0,05) zniža znotrajcelično oksidacijo v kvasovki *S. cerevisiae* pri koncentracijah 10 µM (za 49 ± 10 %) in 100 µM (za 48 ± 14 %) v celični suspenziji, medtem ko pri koncentraciji 1 µM na znotrajcelično oksidacijo nima statistično značilnega vpliva. Pri koncentraciji 1000 µM v celični suspenziji CAPE statistično značilno (p≤0,01) poviša znotrajcelično oksidacijo v kvasovki za 85 ± 46 % (Slika 3).

Preglednica 9: Celična živost in celična metabolna energija kvasovke *S. cerevisiae* po 2-urni izpostavitvi fenetilnemu estru kavne kisline (CAPE) pri koncentracijah 0, 1, 10 in 100 µM, izraženi kot relativne vrednosti glede na kontrolo. Različne oznake pomenijo statistično značilne diferencialne vrednosti v primerjavi s kontrolo (p≤0,05, ***p≤0,01).**

Table 9: Cell viability and cellular metabolic energy of yeast *S. cerevisiae* after 2-hour exposure to caffeic acid phenethyl ester (CAPE) at concentration 0, 1, 10 and 100 µM. Data are expressed as relative to control (RFI). Different marks mean statistically differential values compared to control (p≤0.05, ***p≤0.01).**

[CAPE] (µM)	Celična živost (CFU) (%)	Celična metabolna energija (%)
0	100	100
1	100±3	nd
10	83±17	nd
100	89±8	164 ± 7**

nd=ni določeno

Rezultati so pokazali, da po 2-urni izpostavitvi kvasnih celic, CAPE statistično značilno (p≤0,05) poviša celično metabolno energijo pri koncentraciji 100 µM v celični suspenziji, in sicer za 64 ± 7 %. Pri tem CAPE nima večjega vpliva na celično živost pri koncentracijah 1, 10 in 100 µM v celični suspenziji (Preglednica 9).

2.2.2 Vpliv izvlečka propolisa in frakcije EL70 na sirtuinsko aktivnost

2.2.2.1 Uvod

Rezultati so pokazali, da fenolne spojine v izvlečku propolisa in frakciji EL70 zmanjšajo znotrajcelično oksidacijo (61 %, 66 %) in povečajo celično metabolno energijo (19 %, 21 %) v kvasovkah po 2-urni izpostavitvi pri koncentraciji 1 % (v/v) (≈0,02 in ≈0,01 g GAE/L) v celični suspenziji (Petelinc in sod., 2013a). Slednje spominja na učinek kalorične restrikcije (Choi in Lee, 2013; Choi in sod., 2011; Lin in sod., 2002), kjer se spremeni aktivnost proteina sirtuina (Guarente in Picard, 2005; Wood in sod., 2004; Chen in Guarente, 2007). Zato smo dodatno preverili sirtuinsko aktivnost v kvasovki, ki smo jo izpostavili izvlečku propolisa in frakciji EL70.

2.2.2.2 Materiali

Gojišča

- Trdno gojišče YEPD (Preglednica 3)
- Tekoče gojišče YEPD (Preglednica 4)
- Pufer PBS (Preglednica 5)

Reagenti

- Bradfordov reagent

Bradfordov reagent (Bio-Rad) smo pred uporabo 5x razredčili z bidestilirano vodo.

- Goveji serumski albumin (BSA)

- Komplet "ProteoExtract subcellular proteome extraction kit" (Calbiochem)
- Pufer za spiranje
- Ekstrakcijski pufer 1
- Ekstrakcijski pufer 2
- Ekstrakcijski pufer 3
- Ekstrakcijski pufer 4
- Koktajl inhibitorjev proteaz
- Nukleaza

- Komplet SIRTainity Class III HDAC assay (Millipore)
- Pufer
- Acetiliran peptid
- NAD^+
- Nikotinamidaza

Aparature in naprave

Priprava gojišč, raztopin in reagentov:
- pH-meter SevenEasy (Mettler Toledo)
- parni sterilizator - avtoklav (Sutjeska)
- magnetno mešalo MM-540 (Tehtnica)
- tehtnica TE214S (Sartorius)
- tehtnica PS 1200/C/2 (Radwag)

Kultivacija in inkubacija kvasovk *S. cerevisiae*:
- brezprašna komora LFV P122 (Pio)
- centrifuga Centric 200 (Tehtnica)
- centrifuga 322A (Tehtnica)
- inkubator IG 150 (Jouan)
- mikroskop B1 (Motic)
- stresalnik Multitron (Infors HT)
- spektrofotometer MA 9510 (Iskra)

Ekstrakcija proteinov:
- vrtinčnik TTS2 (Ika)
- centrifuga z možnostjo hlajenja 3K3O (Sigma)
- stresalna plošča (Biometra)

Določanje koncentracije proteinov:
- čitalec mikrotitrskih plošč Safire 2 (Tecan)

Določanje deacetilazne aktivnosti
- vrtinčnik TTS2 (Ika)
- centrifuga miniSpin (Eppendorf)
- inkubacijski stresalnik Thermomixer comfort (Eppendorf)
- čitalec mikrotitrskih plošč Safire 2 (Tecan)

2.2.2.3 Metode

Priprava izvlečka propolisa in frakcije EL70

10 g propolisa smo 1 uro ekstrahirali s 70 % etanolom (100 mL) pri sobni temperaturi. Surov izvleček smo centrifugirali (3000 g, 5 min) in supernatant 2x koncentrirali z rotavaporjem. Nato smo ga očistili ali frakcionirali v pet frakcij z ekstrakcijo s trdno fazo (SPE).

SPE smo izvedli z uporabo kolone Strata-X (33 µm polimerna reverzna faza 60 mg/3 mL 8B-S100-UBJ, Phenomenex). Kolono smo aktivirali z metanolom (2 mL), aktivacijsko topilo pa odstranili z 20 mM amonijevim formiatom (2 mL). Sledil je nanos vzorca (200 µL surovega izvlečka + 200 µL 20 mM amonijevega formiata). Nezaželene komponente smo iz kolone odstranili s spiranjem z raztopino 15 % metanola v 20 mmol/L amonijevem formiatu (2 mL), čemur je sledilo sušenje polnila z vakuumom (3 min).

Za čiščenje surovega izvlečka smo kolono eluirali s 96 % etanolom (2 mL). Za ločitev surovega izvlečka v pet frakcij smo kolono eluirali s 30 % etanolom (2 mL), čemur je sledila zaporedna elucija s 40 %, 50 %, 60 % in 70 % etanolom (2 mL). Pridobljene frakcije (EL30-EL70), imenovani po vol. % etanola, ki je bil uporabljen pri eluciji, so se med seboj ločile po polarnosti. Tako je bila frakcija EL30 najbolj polarna in frakcija EL70 najmanj polarna. Za nadaljnje analize smo uporabili frakcijo EL70.

Kultivacija kvasovk *S. cerevisiae* in izpostavitev izvlečku propolisa in frakciji EL70

Za precepljanje kulture smo uporabili trdno gojišče YEPD (Preglednica 3), za aerobno submerzno namnoževanje kvasne biomase smo uporabili tekoče gojišče YEPD (Preglednica 4), za vzdrževanje kvasne biomase v stacionarni fazi rasti pa smo uporabili PBS pufer (Preglednica 5).

3 dni staro kulturo *S. cerevisiae* smo s trdnega gojišča YEPD precepili v 50 mL tekočega gojišča YEPD v 100-mL erlenmajericah s stransko kiveto (Borosilicate z 1 utorom) do optične gostote pri 650 nm (OD_{650}) 0,95. Nato smo prenesli 40 mL brozge v 360 mL tekočega gojišča YEPD v 1-L erlenmajericah s stransko kiveto (Shot duran z 1 utorom). Sledila je kultivacija kvasovk pri 28 °C in 220 obr./min do začetka stacionarne faze rasti.

Po 60 urah smo preverili OD_{650} (≈2,0) in 50 mL brozge ($5x10^8$ celic/mL) centrifugirali 3 min pri 4000 obr./min (centrifuga 322A, Tehtnica), odstranili supernatant ter sediment 1x sprali s PBS. Celice smo resuspendirali v 50 mL PBS in nato 40 mL suspenzije prenesli v 160 mL PBS, da smo dosegli koncentracijo $1x10^8$ celic/mL v 500-mL erlenmajericah s

stransko kiveto (Simex z 1 utorom). Sledila je inkubacija kvasovk nadaljnjih 96 ur pri 28 °C in 220 obr./min.

Celični suspenziji (10 mL) smo po 96-urni inkubaciji dodali 1 % (v/v) izvlečka propolisa ali frakcije EL70 (≈0,02 in ≈0,01 g GAE/L). Po 2-urni izpostavitvi pri 28 °C in 220 obr./min v temi, smo z diferencialno detergentno frakcionacijo (DDF) ekstrahirali 4 proteinske frakcije in v njih določili sirtuinsko aktivnost v primerjavi s kontrolo (s 96 % etanolom tretirane kvasne celice).

Ekstrakcija citosolne, mitohondrijsko/organelne, jedrne in citoskeletne proteinske frakcije

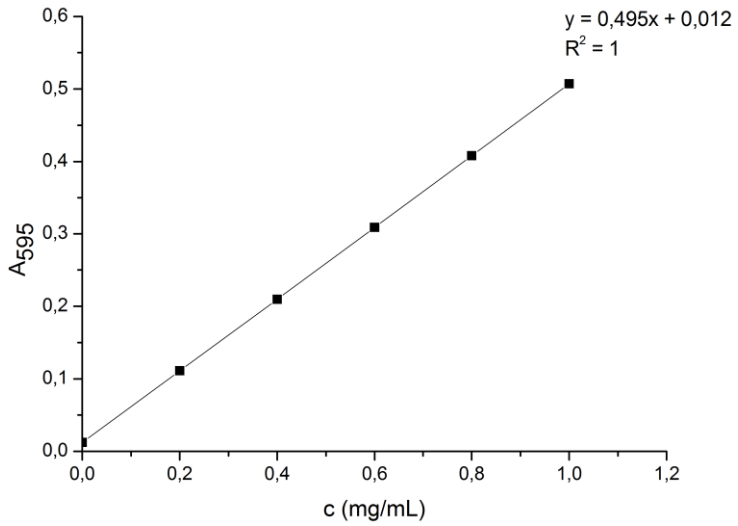
Po 2-urni izpostavitvi kvasovk izvlečku propolisa ali frakciji EL70 pri koncentraciji 1 % (v/v) (≈0,02 in ≈0,01 g GAE/L) v celični suspenziji smo 2x po 10 mL brozge centrifugirali 3 min pri 4000 obr./min (centrifuga Centric 200, Tehtnica). Po odstranitvi supernatanta smo sediment 2x sprali s pufrom PBS (Preglednica 5) in združili 2 vzorca. 20 mL brozge smo nato ponovno centrifugirali 3 min pri 4000 obr./min (centrifuga Centric 200, Tehtnica) in sediment zamrznili v tekočem dušiku ter shranili na -80 °C.

Vzorce zamrznjene na -80 °C smo odtajali in ekstrahirali 4 proteinske frakcije (citosolno, mitohondrijsko/organelno, jedrno in citoskeletno) s kompletom "ProteoExtract subcellular proteome extraction kit" (Calbiochem). Komplet smo uporabili po navodilih proizvajalca z nekaj spremembami. Sedimentu smo dodali 2 mL pufra za spiranje, vse skupaj prenesli v 12,5 mL falkonke z ovalnim dnom in centrifugirali 10 min pri 4 °C in 300 g. Po ponovnem spiranju smo sedimentu dodali 1 mL ekstrakcijskega pufra 1, ki je vseboval 5 µL koktajla inhibitorjev proteaz in 4 žličke cirkonij-kremenčevih kroglic, vrtinčili 5 x 1 min, z vmesnimi 1-min intervali na ledu ter centrifugirali 10 min pri 4 °C in 1000 g. Supernatant smo shranili na -80 °C kot citosolno proteinsko frakcijo. Sedimentu smo nato dodali 1 mL ekstrakcijskega pufra 2, ki je vseboval 5 µL koktajla inhibitorjev proteaz in tekočino nad kroglicami prenesli v 2-mL Eppendorf mikrocentrifugirko. Po 30-min inkubaciji na stresalnem pladnju na ledu smo vzorec centrifugirali 10 min pri 4 °C in 6000 g. Supernatant smo shranili na -80 °C kot mitohondrijsko/organelno proteinsko frakcijo. Sedimentu smo nato dodali 0,5 mL ekstrakcijskega pufra 3, ki je vseboval 5 µL koktajla inhibitorjev proteaz in 1,5 µL nukleaze. Po 10-min inkubaciji na stresalnem pladnju na ledu smo vzorec centrifugirali 10 min pri 4 °C in 7000 g. Supernatant smo shranili na -80 °C kot jedrno proteinsko frakcijo. Sedimentu smo nato dodali 0,5 mL ekstrakcijskega pufra 4, ki je vseboval 5 µL koktajla inhibitorjev proteaz. Po 30-min inkubaciji na sobni temperaturi smo vzorec shranili na -80 °C kot citoskeletno proteinsko frakcijo.

Merjenje koncentracije proteinov v ekstraktih

Koncentracijo proteinov v ekstraktih smo določili po metodi Bradford (Bradford, 1976) na podlagi umeritvene krivulje (Slika 4). Za pripravo umeritvene krivulje smo uporabili BSA

v različnih koncentracijah: 0 0,2; 0,4; 0,6; 0,8 in 1 g/L. 4 µL vsake raztopine z različno koncentracijo BSA smo odpipetirali v jamico prozorne mikrotitrske ploščice in dodali 196 µL 1x Bradfordovega reagenta. Po mešanju in 5 min smo izmerili absorbanco na čitalcu mikrotitrskih plošč Safire 2 pri valovni dolžini 595 nm.



Slika 4: Umeritvena krivulja za merjenje koncentracije proteinov po Bradfordu
Figure 4: Calibration curve for measuring the concentration of proteins by the Bradford method

V primeru vzorcev smo v jamice prozorne mikrotitrske ploščice odpipetirali 4 µL ustrezno redčenega ekstrakta in dodali 196 µL 1x Bradfordovega reagenta. Slepi vzorec je predstavljal ekstrakcijski pufer. Po mešanju in 5 min smo izmerili absorbanco na čitalcu mikrotitrskih plošč Safire 2 pri valovni dolžini 595 nm. Vzorcem smo odšteli slepi vzorec in izračunali koncentracijo proteinov z enačbo umeritvene krivulje. Ekstrakte smo redčili tolikokrat, da so absorbance padle v linearno območje umeritvene krivulje.

Določanje sirtuinske aktivnosti

Sirtuinsko aktivnost smo določili z merjenjem nikotinamida, ki nastane, ko sirtuin v prisotnosti NAD^+ deacetilira peptid (Hubbard in sod., 2013). Za določanje nikotinamida se uporablja dodaten encim nikotinamidaza, ki pretvori nikotinamid v amonijak. Slednji pa po reakciji z *o*-ftalaldehidom fluorescira.

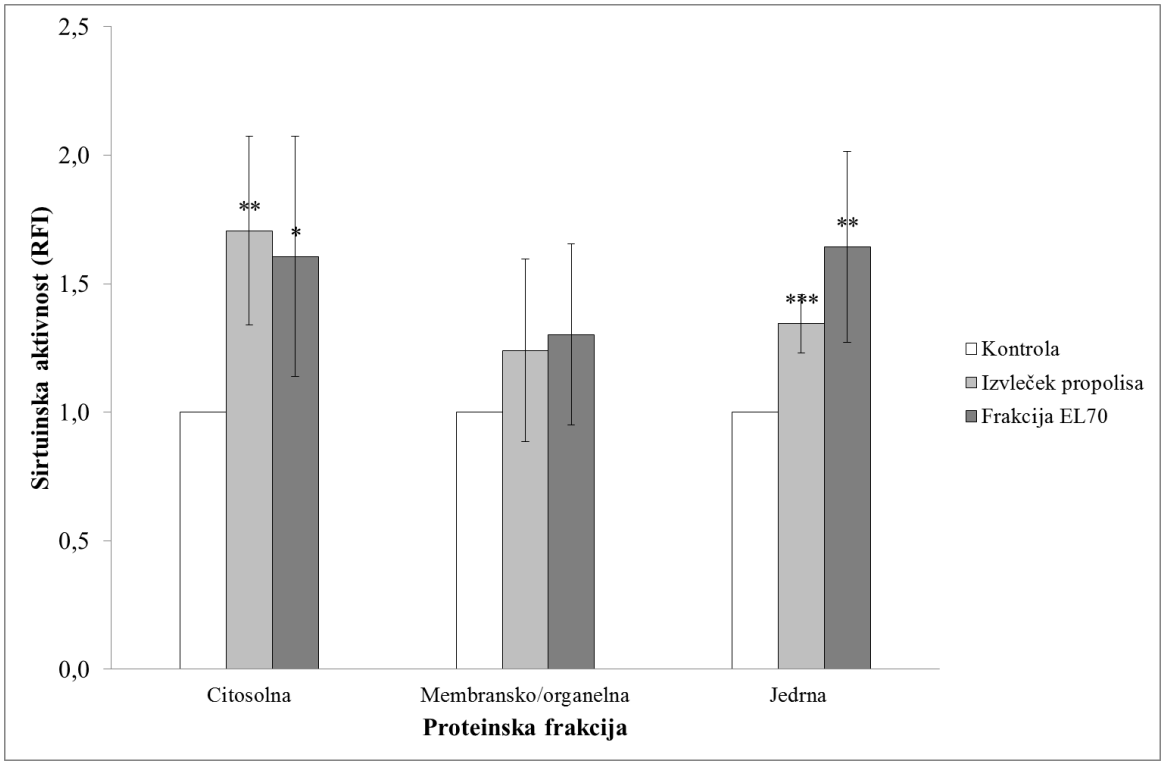
Proteinske ekstrakte zamrznjene na -80 °C smo odtajali in v njih s kompletom "SIRTainity Class III HDAC assay" (Millipore) določili deacetilazno aktivnost. Komplet smo uporabili po navodilih proizvajalca z nekaj spremembami. Reakcije so potekale v 1,5-mL Eppendorf mikrocentrifugirkah. Reakcijska mešanica (25 µL) je bila sestavljena iz proteinskega

ekstrakta (5 µL), 25µM acetiliranega peptida (5µL), 200 µM NAD^+ (5µL), 0,5 µg nikotinamidaze (5 µL) in pufra (5 µL). Reakcija deacetilacije je potekala 1 uro pri 28 °C in 450 obr./min (inkubacijski stresalnik). Nato smo dodali 25 µL razvijalca in nadaljnje inkubirali 1 uro pri 25 °C in 450 obr./min (inkubacijski stresalnik). Sledil je prenos vzorcev (50 µL) v črno mikrotitrsko ploščico in merjenje fluorescence na čitalcu mikrotitrskih plošč Safire 2. Valovna dolžina vzbujanja je bila 420 nm, valovna dolžina emisije pa 460 nm. Rezultati so bili izraženi kot relativne vrednosti fluorescenčne intenzitete glede na kontrolo - relativna fluorescenčna intenziteta (RFI).

Statistična analiza

Vrednosti so podane kot povprečje s standardnim odklonom vsaj treh neodvisnih eksperimentov. Statistično značilna razlika med kontrolo in tretiranim vzorcem je bila določena s Studentovim dvostranskim t-testom in je na sliki (Slika 5) označena kot *($p \leq 0,1$), **($p \leq 0,05$) in ***($p \leq 0,01$).

2.2.2.4 Rezultati



Slika 5: Sirtuinska aktivnost v posameznih proteinskih frakcijah pridobljenih z diferencialno detergentno frakcionacijo iz kvasovke *S. cerevisiae* po 2-urni izpostavitvi izvlečku propolisa ali frakciji EL70 pri koncentraciji 1 % (v/v) skupnih fenolnih spojin v celični suspenziji, izražena kot relativna fluorescenčna intenziteta glede na kontrolo (RFI). Različne oznake pomenijo statistično značilne diferencialne vrednosti v primerjavi s kontrolo (*p≤0,1, **p≤0,05, ***p≤0,01).

Figure 5: Sirtuin activity in particular protein fractions obtained from yeast *S. cerevisiae* with differential detergent fractionation after 2-hour exposure to propolis extract or fraction EL70 at a concentration of 1 % (v/v) of total phenolic compounds in cell suspension. Data are expressed as fluorescence intensity relative to control (RFI). Different marks indicate statistically significant differential values compared to control (*p≤0.1, **p≤0.05, ***p≤0.01).

Rezultati so pokazali, da se v celicah, ki so bile izpostavljene etanolnemu izvlečku propolisa in frakciji EL70 pri koncentraciji 1 % (v/v) (≈0,02 in ≈0,01 g GAE/L) statistično značilno poveča sirtuinska aktivnost v kvasovkah, in sicer v citosolni subproteomski frakciji (etanolni izvleček propolisa, 71 ± 37 %, p≤0,05; frakcija EL70, 61 ± 47 %, p≤0,1) in jedrni subproteomski frakciji (etanolni izvleček propolisa, 34 ± 11 %, p≤0,01; frakcija EL70, 64 ± 37 %, p≤0,05) (Slika 5).

3 RAZPRAVA

Propolis ugodno vpliva na zdravje človeka, saj ima številne biološke aktivnosti, kot so protivirusna, protimikrobna, protiparazitska, antioksidativna, imunomodulatorna, protirakava in protivnetna (Burdock, 1998). Zato se tudi preventivno in terapevtsko uporablja v zdravstvene namene (Castaldo in Capasso, 2002) kljub temu, da njegove bioaktivne spojine in mehanizmi delovanja v celicah niso natančno poznani.

Z namenom, da osvetlimo to področje, smo v doktorski nalogi frakcionirali izvleček propolisa in analizirali vpliv pridobljenih frakcij na celični in proteomski ravni evkariontskega modelnega organizma kvasovke *S. cerevisiae*.

3.1 FRAKCIONIRANJE IZVLEČKA PROPOLISA

Pri našem delu smo uporabili slovenski propolis topolovega oz. evropskega tipa, za katerega so značilne hidroksicimetne kisline in njihovi estri ter dve skupini flavonoidov - flavoni in flavanoni (Sforcin in Bankova, 2011). Izvleček propolisa smo pridobili z ekstrakcijo s 70 % etanolom, za njegovo čiščenje in frakcioniranje pa smo uporabili ekstrakcijo s trdno fazo (SPE).

Posamezne frakcije izvlečka propolisa smo pridobili tako, da smo pri SPE zaporedno povečevali odstotek etanola, s katerim smo spirali spojine s kolone. Ker smo spiranje začeli s 30 % etanolom in odstotek etanola pri vsakem nadaljnjem spiranju povečali za 10 %, smo pridobili 5 eluatov oz. frakcij, ki smo jih poimenovali EL30-EL70. Tako smo najprej pridobili frakcije, ki vsebujejo bolj polarne spojine, kot so hidroksicimetne kisline, zatem pa frakcije z manj polarnimi spojinami, kot so flavonoidi in estri hidroksicimetnih kislin.

Uspešnost frakcionacije izvlečka propolisa smo analizirali tako, da smo med seboj primerjali kromatograme fenolnih spojin, ki smo jih pridobili s HPLC-DAD pri 300 nm. Skoraj vse fenolne spojine imajo namreč maksimum absorpcije v UV območju od 270 nm do 280 nm. Nekatere pa tudi pri 220-230 nm (katehini, hidroksitirosoli) ali 330 nm (hidroksicimetne kisline, flavonoidi). Antocianini imajo celo maksimum absorpcije v območju vidne svetlobe pri 520-550 nm (Miniati, 2007). Primerjava kromatogramov je pokazala, da je SPE ustrezen način za frakcioniranje izvlečka propolisa, saj se fenolni profili pridobljenih frakcij med seboj kvalitativno in kvantitativno razlikujejo (Petelinc in sod., 2013a).

Fenolne spojine v propolisu so frakcionirali tudi Wang in sod. (2004), kjer so uporabili superkritično tekočinsko ekstrakcijo, kasneje pa še Tsibranka in Tylkowski (2011), kjer so fenolne spojine ločili glede na molekulsko maso, in sicer z nanofiltracijo.

3.2 SKUPNE FENOLNE SPOJINE IN SPOSOBNOST LOVLJENJA PROSTIH RADIKALOV IZVLEČKA PROPOLISA IN NJEGOVIH FRAKCIJ

Preden smo vpliv izvlečka propolisa in njegovih frakcij analizirali na celični in proteomski ravni evkariontskega modelnega organizma kvasovke *S. cerevisiae*, smo preverili njihovo učinkovitost *in vitro*. S spektofotometričnimi metodami smo določili skupne fenolne spojine in sposobnost lovljenja prostih radikalov. Kot referenčni standard smo uporabili galno kislino.

Skupne fenolne spojine smo določili s Folin-Ciocalteaujevo metodo, ki temelji na redukciji heteropolnih kislin v Folin-Ciocalteaujevemu reagentu ob prisotnosti fenolnih spojin. Pri tem nastane moder kompleks (Prior in sod., 2005). Rezultati so pokazali, da izvleček propolisa in njegove frakcije vsebujejo fenolne spojine. V izvlečku propolisa smo določili 1,99 ± 0,01 g ekvivalentov galne kisline (GAE)/L. Med frakcijami sta imele največ fenolnih spojin frakciji EL60 in EL70, in sicer 0,82 ± 0,01 g GAE/L in 0,83 ± 0,02 g GAE/L. Frakcije EL30-EL50 so vsebovale manj fenolnih spojin, in sicer od 0,26 ± 0,01 - 0,39 ± 0,01 g GAE/L (Petelinc in sod., 2013a).

Sposobnost lovljenja prostih radikalov smo določili z 1,1-difenetil-2-pikrilhidrazil ($DPPH^{\bullet}$) testom. Molekula $DPPH^{\bullet}$ je stabilen dušikov radikal in ima intenzivno vijolično barvo, ki pa ob redukciji z antioksidantom izgine. Preko zmanjšane obarvanosti raztopine določimo kakšna je sposobnost antioksidanta, da lovi proste radikale (Molyneux, 2004). Rezultati so pokazali, da izvleček propolisa lovi proste radikale (1,88 ± 0,01 g GAE/L). Med frakcijami sta imeli najboljšo sposobnost lovljenja prostih radikalov frakciji EL60 in EL70 (1,20± 0,03 g GAE/L in 1,22 ± 0,02 g GAE/L). Frakcije EL30-EL50 so imele nekoliko slabšo sposobnost lovljenja prostih radikalov (0,18 ± 0,08 - 0,33 ± 0,08 g GAE/L) (Petelinc in sod., 2013a).

Pokazali smo, da izvleček propolisa in njegove frakcije vsebujejo fenolne spojine, ki so znani lovilci prostih radikalov (Rice-Evans, 1996). Poleg tega je med skupnimi fenolnimi spojinami in sposobnostjo lovljenja prostih radikalov v vzorcih linearna povezava. Slednje navajajo tudi drugi avtorji (Russo in sod., 2002; da Silva in sod., 2006; Gregoris in Stevanato, 2010).

Ker le na osnovi *in vitro* študij ne moremo sklepati o aktivnosti izvlečka propolisa v celici, smo v doktorski nalogi opravili dodatne *in vivo* raziskave.

3.3 VPLIV IZVLEČKA PROPOLISA IN NJEGOVIH FRAKCIJ NA KVASOVKO *Saccharomyces cerevisiae*

V okviru doktorske naloge smo določili vpliv izvlečka propolisa in njegovih frakcij na evkariontski modelni organizem kvasovko *S. cerevisiae* na celični in proteomski ravni.

Kulturo kvasovk smo namnožili do stacionarne faze rasti v tekočem gojišču YEPD in jo v pufru PBS vzdrževali v stacionarni fazi rasti 96 ur. V tem času se je kvasovka izkazala za ustrezen model za proučevanje vpliva različnih okoljskih stresnih dejavnikov, saj je nivo znotrajcelične oksidacije in celične metabolne energije v celicah stabilen (Zakrajšek in sod., 2011).

Vpliv bioaktivnih spojin propolisa smo na celični ravni preverili z merjenjem znotrajcelične oksidacije, celične metabolne energije in celične živosti. Preverili smo tudi celični privzem posameznih spojin propolisa v kvasovko. Na proteomski ravni smo z dvodimenzionalno elektroforezo (2-DE) analizirali spremembe v proteinskem profilu citosolne, membransko/organelne, jedrne in citoskeletne frakcije po izpostavitvi izbrani frakciji propolisa. Poleg tega smo preverili tudi sirtuinsko aktivnost po izpostavitvi celic kvasovke izvlečku propolisa in izbrani frakciji propolisa.

3.3.1 Celična raven

3.3.1.1 Znotrajcelična oksidacija

Znotrajcelično oksidacijo v celicah kvasovk *S. cerevisiae* smo določili z 2',7'-diklorodihidrofluorescein diacetatom (H_2DCFDA). Manjša fluorescenca pomeni manjšo vsebnost oksidantov v celici, medtem ko večja fluorescenca pomeni večjo vsebnost oksidantov v celici.

Celice smo za 2-uri izpostavili etanolnemu izvlečku propolisa s koncentracijami v celični suspenziji 0,02; 0,01; 0,005; 0,0025 in 0,00125 g GAE/L. Etanolni izvleček propolisa je znižal znotrajcelično oksidacijo pri vseh testiranih koncentracijah in je zato učinkovit antioksidant. Najboljša antioksidativna učinkovitost se je pokazala pri koncentraciji 0,02 g GAE/L, pri kateri se je nivo oksidantov v celici znižal za 61 ± 1 %. Iz rezultatov vidimo tudi, da je etanolni izvleček propolisa dosegel največji statistično značilen antioksidativni učinek že pri koncentraciji med 0,005 - 0,01 g GAE/L (29 - 59 µM) (Petelinc in sod., 2013a). Slednje je v skladu s predhodnimi ugotovitvami, kjer smo pokazali, da izvleček propolisa v DMSO doseže največji antioksidativni učinek že pri koncentraciji med 0,0125 - 0,025 g ekvivalentov klorogenske kisline (CAE)/L (35 - 71 µM) (Cigut, 2010). Iz tega sledi, da izvleček propolisa v celicah deluje antioksidativno že pri mikromolarnih koncentracijah in to neodvisno od tega, ali za topilo izberemo etanol ali DMSO.

Da je propolis učinkovit antioksidant, ki zaščiti kvasovko *S. cerevisiae* v eksponentni fazi rasti pred oksidativnim stresom, so pokazali tudi de Sá in sod. (2013). Enourno predtretiranje kulture z etanolnim izvlečkom propolisa in naknadna izpostavitev

menadionu ali vodikovemu peroksidu kot induktorju stresa, se je v primerjavi s kulturo, ki ni bila predtretirana, pokazalo v nižji ravni ROS in lipidni peroksidaciji.

Nadaljnje nas je zanimala tudi antioksidativna učinkovitost posameznih frakcij etanolnega izvlečka propolisa, ki smo jih pripravili s SPE. Frakcije smo dodali suspenziji kvasnih celic pri koncentraciji 1 % (v/v) skupnih fenolnih spojin za 2-uri. Izmed vseh testiranih frakcij se je antioksidativna učinkovitost pokazala le pri frakcijah EL50-EL70. Največji učinek je imela frakcija EL70, ki je znižala nivo oksidantov v celici za 66 ± 1 %. Sledili sta frakciji EL60 z 42 ± 5 % in EL50 s 16 ± 2 %. Dodaten eksperiment je pokazal, da frakcija EL70 zniža znotrajcelično oksidacijo tudi v kvasovkah v logaritemski fazi rasti, in sicer za 66 ± 2 % v primerjavi s kontrolo (Petelinc in sod., 2013a).

Frakcija EL30 je imela oksidativen učinek, saj je nivo oksidantov v celici povišala za 18 ± 12 % (Petelinc in sod., 2013a). Kot že omenjeno, začetne frakcije vsebujejo več hidroksicimetnih kislin, ki lahko v kombinaciji povečajo znotrajcelično oksidacijo. Slednje smo pokazali na primeru kombinacije kavne, ferulne in *p*-kumarne kisline, ki poviša nivo oksidantov v celici za 7 ± 1 % (Cigut in sod., 2011). Znano je, da eksogeni antioksidanti v nizkih koncentracijah, kot so prisotni v naravnih snoveh, dopolnjujejo delovanje endogenega antioksidativnega obrambnega sistema. Obratno pa imajo v visokih koncentracijah oksidativni učinek (Bauayed in Bohn, 2012). Vendar, po vsebnosti skupnih fenolnih spojin, frakcija EL30 ni izstopala od ostalih frakcij.

Tako rezultati kažejo, da imajo frakcije z enako vsebnostjo skupnih fenolnih spojin določeno s Folin-Ciocalteaujevo metodo, različen učinek na znotrajcelično oksidacijo določeno z 2,7-diklorofluoresceinom. Frakciji EL60 in EL70, ki imata podobno vsebnost skupnih fenolnih spojin, znižata znotrajcelično oksidacijo za različen odstotek, in sicer za 42 % in 66 %. Frakciji EL30 in EL50 tudi s podobno vsebnostjo skupnih fenolnih spojin, pa na znotrajcelično oksidacijo delujeta nasprotno, saj jo prva frakcija poveča, druga pa zmanjša (Petelinc in sod., 2013a). Iz tega sklepamo, da je antioksidativna učinkovitost posameznih frakcij v celici bolj odvisna od vrst fenolnih spojin v frakciji, kot od vsebnosti skupnih fenolnih spojin. V literaturi navajajo, da poleg vrst fenolnih spojin v vzorcu, na antioksidativno učinkovitost v celicah, vplivajo tudi razmerja med njimi (Slatnar in sod., 2012; Figueiredo-Rinhel in sod., 2013).

Tudi frakcije z enako sposobnostjo lovljenja prostih radikalov določeno z DPPH testom, imajo različen učinek na znotrajcelično oksidacijo določeno s 2,7-diklorofluoresceinom. Frakciji EL60 in EL70, ki imata podobno sposobnost lovljenja prostih radikalov, različno zmanjšata znotrajcelično oksidacijo, in sicer za 42 % in 66 %. Izmed frakcij EL40 in EL50, ki imata tudi podobno sposobnost lovljenja prostih radikalov, le frakcija EL50 zmanjša znotrajcelično oksidacijo za 16 %. Frakcija EL30, ki ima sicer najslabšo sposobnost lovljenja prostih radikalov, znotrajcelično oksidacijo celo poviša za 18 % (Petelinc in sod., 2013a). Rezultati so tako pokazali, da ni povezave med sposobnostjo lovljenja prostih radikalov posameznih frakcij in njihovo antioksidativno učinkovitostjo v celici.

3.3.1.2 Celična metabolna energija

Indikator metabolno aktivnih celic je ATP, saj ga živi organizmi porabljajo za rast in vzdrževanje celičnih funkcij (Erecińska in Wilson, 1982). Za določanje ATP smo uporabili komplet "BacTiter-Glo", ki vsebuje luciferin in luciferazo. Encim luciferaza ob prisotnosti ATP in kisika pretvori luciferin v oksiluciferin, ob čemer nastane svetloba. Metoda nam razkrije raven ATP v celici, ne pa če je spremenjena raven ATP posledica spremenjene sinteze ali porabe ATP v celici.

Suspenzijo celic smo za 2 uri izpostavili etanolnemu izvlečku propolisa in posameznim frakcijam pri koncentraciji 1 % (v/v) skupnih fenolnih spojin v celični suspenziji. Izpostavitev izvlečku propolisa in frakcijama EL60 in EL70 je v primerjavi s kontrolo povišala raven ATP v celici. Frakcija EL70 je povišala raven ATP (21 ± 3 %) podobno kot etanolni izvleček propolisa (19 ± 4 %), medtem ko je frakcija EL60 povišala raven ATP za 6 ± 2 % (Petelinc in sod., 2013a). Slednje je v skladu s predhodnimi ugotovitvami, kjer smo pokazali, da tudi izvleček propolisa v DMSO poviša raven ATP v celici (Cigut in sod., 2011). Iz tega sledi, da izvleček propolisa v celicah poviša raven ATP neodvisno od tega, ali za topilo izberemo etanol ali DMSO.

Ko smo celice izpostavili frakcijam EL30, EL40 in EL50 se je v primerjavi s kontrolo raven ATP v celici zmanjšala za 7 ± 1 %, 6 ± 4 % in 12 ± 1 % (Petelinc in sod., 2013a). V literaturi najdemo, da je nižja raven ATP značilna ob izpostavitvi nekaterim fenolnim spojinam, kot so kvercetin (Takahashi in sod., 1998; Dorta in sod., 2005), galangin (Dorta in sod., 2005) in rutin (Takahashi in sod., 1998). Fenolne spojine znižajo raven ATP v celici, ker zaustavijo ali razklopijo mitohondrijsko respiratorno verigo (Dorta in sod., 2005).

Kljub podobni vsebnosti skupnih fenolnih spojin, določeni s Folin-Ciocalteaujevo metodo, je frakcija EL70 bolj povečala raven ATP (21 %) kot frakcija EL60 (6 %). Tudi frakciji EL30 in EL50, ki imata enako vsebnost fenolnih spojin, različno znižata raven ATP, in sicer za 7 % in 12 % (Petelinc in sod., 2013a). Tako rezultati kažejo, da je vpliv posameznih frakcij na celično metabolno energijo bolj v korelaciji z vrsto fenolnih spojin v frakciji, kot s skupnimi fenolnimi spojinami.

3.3.1.3 Celična živost

V literaturi se pojavljajo navedbe, da je propolis toksičen za celice (Burdock, 1998). Med drugim lahko zasledimo tudi, da inducira celično smrt pri kvasovki *S. cerevisiae* (de Castro in sod., 2011). Po drugi strani nekateri avtorji navajajo, da propolis nima vpliva na celično živost (de Sá in sod., 2013). Zato smo v doktorski nalogi z namenom, da določimo morebitno toksičnost etanolnega izvlečka propolisa na kvasovko, preverili celično živost izpostavljenih celic.

Za ugotavljanje celične živosti smo uporabili komplet "Live/Dead FungaLight", ki ga sestavljata dve barvili za nukleinske kisline. Zeleno fluorescentno barvilo (SYTO 9) označi vse celice, medtem ko rdeče fluorescentno barvilo (propidijev jodid) prodre le v celice s poškodovano celično membrano. V primeru, da sta v celici prisotni obe barvili se zaradi fluorescenčnega resonančnega prenosa energije zmanjša fluorescenca SYTO 9. Merjenje celične živosti tako temelji na poškodovanosti celične membrane, zato določimo za mrtve tudi celice s poškodovano celično membrano, ki pa so sposobne obnovitve in razmnoževanja. Poleg tega za žive določimo tiste celice, ki imajo nepoškodovano membrano, niso pa sposobne razmnoževanja.

Suspenzijo celic smo za 2 uri izpostavili etanolnemu izvlečku propolisa in posameznim frakcijam pri koncentraciji 1 % (v/v) skupnih fenolnih spojin v celični suspenziji. V primerjavi s kontrolo, nobeden od testiranih vzorcev ni imel vpliva na celično živost (Petelinc in sod., 2013a). Da propolis nima vpliva na celično živost, smo predhodno pokazali tudi za izvleček v DMSO (Cigut in sod., 2011).

Naši rezultati so drugačni od rezultatov, ki so jih pridobili de Castro in sod. (2011), kjer je pri kvasovkah v logaritemski fazi rasti propolis povzročil povišanje znotrajcelične oksidacije in indukcijo apoptoznih ter nekroznih procesov, najverjetneje zaradi razlik v koncentraciji fenolnih spojin v obeh raziskavah. V študiji ne zasledimo, kakšno koncentracijo propolisa izraženega na skupne fenolne spojine, so uporabili. Dodatni eksperimenti v našem primeru so pokazali tudi, da frakcija EL70 pri koncentraciji 1 % (v/v) skupnih fenolnih spojin v celični suspenziji v primerjavi s kontrolo nima vpliva na celično živost kvasovke v logaritemski fazi rasti. Namreč, celična živost izpostavljenih celic je bila v primerjavi s kontrolo 100 ± 9 %.

Kadar analiziramo biološke aktivnosti propolisa je pomembno, da opravimo tudi analizo spojin v njem, saj imajo različni tipi propolisa različno sestavo. Zato smo s HPLC-MS/MS identificirali fenolne spojine v frakciji EL70, ki ima med frakcijami najbolj pozitiven učinek na celično raven kvasovke *S. cerevisiae*. Ta frakcija namreč najbolj zniža znotrajcelično oksidacijo (za 66 %) in najbolj poviša raven celične metabolne energije (za 21 %) pri koncentraciji 1 % (v/v) (≈0,01 g GAE/L) v suspenziji kvasovk.

3.3.1.4 Identifikacija fenolnih spojin v frakciji EL70

V frakciji EL70 smo identificirali fenolne spojine iz dveh različnih skupin, in sicer derivatov hidroksicimetnih kislin (estri hidroksicimetnih kislin in prenilirana hidroksicimetna kislina) in flavonoidov. Estri hidroksicimetnih kislin, ki smo jih identificirali, so bili izoprenilni ester kavne kisline (CAIE), benzilni ester kavne kisline (CABE), fenetilni ester kavne kisline (CAPE), cimetni ester kavne kisline (CACE) in benzilni ester *p*-kumarne kisline, prenilirana hidroksicimetna kislina pa 3-prenil-4-(2-metilpropionil-oksi)-cimetna kislina. Med flavonoidi smo identificirali

različne podkupine kot so flavoni (krizin, apigenin, luteolin-metil-eter), flavonoli (kamferid, ramnetin), flavanoni (pinocembrin) in dihidroflavonoli (pinobanksin-5-metil-eter, pinobanksin-3-*O*-acetat, pinobanksin-3-*O*-propionat, pinobanksin-3-*O*-butirat in pinobanksin-3-*O*-pentanoat. Etanolni izvleček propolisa je bil obogaten z CAIE, CAPE, CACE, kamferidom in pinobanskin-3-*O*-acetatom (Petelinc in sod., 2013a).

V izvlečku slovenskega propolisa so bili že predhodno identificirani CAIE, CABE, CAPE, CACE, krizin, apigenin, pinocembrin, kamferid in pinobanksin-3-*O*-acetat, poleg tega pa še druge fenolne spojine, kot so hidroksicimetne kisline (galna, klorogenska, kavna, *p*-kumarna, ferulna in elaginska kislina), flavoni (luteolin), izoflavoni (formononetin), flavonoli (miricetin, kvercetin, pinobanksin, kamferol in galangin) in flavonoli (rutin) (Mavri in sod., 2012).

Med fenolnimi spojinami, ki smo jih identificirali v frakciji EL70, je kar nekaj kandidatov za naslov najbolj bioaktivne spojine v izvlečku propolisa. Po mnenju nekaterih raziskovalcev je CAPE glavna učinkovina evropskega propolisa. Ima namreč mnoge farmakološke aktivnosti, med drugim antioksidativno, protibakterijsko, protivirusno, protiglivno in protivnetno (Omene in sod., 2013). Ker ima CAPE tudi antiproliferacijske in citotoksične učinke na različne tipe rakavih celic, ob čemer pa nima toksičnih učinkov na zdrave celice, se smatra tudi kot učinkovit protirakav agens (Sanderson in Clarke, 2006). Način delovanja CAPE na molekularni ravni ni znan. Predvideva se, da deluje preko modulacije transkripcijskih faktorjev NRF2 (Kim in sod., 2013) in NF-κB (Park in sod., 2013). Po drugi strani pa učinkuje tudi na druge proteine v celici, kot so antioksidativni encimi, npr. SOD. Yasui in sod. (2013) navajajo, da CAPE poveča raven SOD v celici in s tem zniža vsebnost ROS v celici. Nasprotno so Song in sod. (2012) v celicah, ki so jih izpostavili CAPE, pokazali znižano raven SOD.

V frakciji EL70 smo identificirali tudi številne flavonoide, ki imajo različne ugodne učinke na zdravje človeka, med drugim protivnetne, antioksidativne, protitumorske in protivirusne (Xiao in sod., 2011; Lu in sod., 2013; Kumar in Padney, 2013). Bankova (2005) poroča, da z biološko aktivnostjo propolisa korelirata vsebnost skupnih flavonov in flavonolov ter vsebnost skupnih flavanonov in dihidroflavonolov. V našem izvlečku propolisa smo odkrili več predstavnikov teh podskupin flavonoidov, natančneje 3 flavone, 2 flavonola, 1 flavanon in 5 dihidroflavonolov. Literatura navaja, da se metilirani derivati flavonoidov, ki smo jih tudi identificirali v frakciji EL70, bolje absorbirajo iz prebavnega trakta, so stabilnejši in imajo zato večjo aktivnost kot njihove nemetilirane oblike (Wen in Walle, 2006; Walle, 2007).

Dodatno smo v frakciji EL70 našli tudi 3-prenil-4-(2-metilpropionil-oksi)-cimetno kislino. Ta spada med prenilirane hidroksicimetne kisline, ki so značilne za zeleni propolis iz Brazilije (Salatino in sod., 2005). Najbolj poznana fenolna spojina v tej skupini je artepilin C (4-hidroksi-3,5-diprenil-cimetna kislina), ki velja za glavno učinkovino

zelenega propolisa (Messerli in sod., 2009). Artepilin C prepreči lipidno peroksidacijo in nastanek 8-hidroksigvanina v DNA v Caco-2 (rak debelega črevesa) in HepG2 (rak jeter) tumorskih celičnih linijah (Shimizu in sod., 2004).

3.3.1.5 Celični privzem fenolnih spojin iz frakcije EL70 v kvasno celico

Termin celični privzem se uporablja za akumulacijo spojine v citosolu celic ali njene interakcije s celično membrano. Celični privzem fenolnih spojin je pogoj za njihovo biološko aktivnost (Spencer in sod., 2004). Le v primeru celičnega privzema fenolne spojine dosežejo svoje tarče, kot so receptorji, encimi in transkripcijski faktorji (Fraga in sod., 2010).

Že primerjava fenolnih profilov posameznih frakcij izvlečka propolisa pred in po izpostavitvi celicam, ki smo jih pridobili s HPLC-DAD, je pokazala, da imajo fenolne spojine iz frakcije EL70, za katero je bil opažen najbolj pozitiven vpliv na celico, največji celični privzem v celice kvasovk (Petelinc in sod., 2013a).

Izmed vseh fenolnih spojin identificiranih v frakciji EL70 smo že predhodno za CAPE v DMSO pokazali, da po 1-urni izpostavitvi kvasovke pri koncentraciji 176 µM v celični suspenziji (ustreza koncentraciji 0,05 g/L, kjer je izvleček propolisa v DMSO znižal raven ROS/RNS v celici za 42 %) ne spremeni znotrajcelične oksidacije v primerjavi s kontrolo, kljub temu da v celoti vstopi oz. interagira s celico (Cigut in sod., 2011). Možno je, da CAPE ostane na/v celični membrani in tako ne doseže svojih tarč v celici, saj je znano, da nekatere fenolne spojine interagirajo z membrano (Oteiza in sod., 2005; Hendrich, 2006). Izbrana metoda z analizo supernatanta nam poda informacijo katera spojina izgine iz medija, ne pa če le-ta vstopi v celico, ostane v/na celični membrani ali kakorkoli drugače interagira s celico. Po drugi strani lahko, da CAPE deluje le v sinergizmu z ostalimi fenolnimi spojinami. Ali pa, da je bila koncentracija previsoka, da bi imel CAPE v celici antioksidativen učinek.

Tako nas je v nadaljevanju zanimalo, če s celico interagira katera izmed ostalih fenolnih spojin iz frakcije EL70 in povzroči antioksidativni učinek v celici. Analiza s HPLC-MS/MS je pokazala celični privzem le za nekatere fenolne spojine iz frakcije EL70, in sicer za tri estre hidroksicimetnih kislin CABE, CAPE in CACE (Petelinc in sod., 2013a). Tako kvasovka ne interagira z vsemi fenolnimi spojinami, ki so ji na voljo. Slednje so pokazali tudi Slatnar in sod. (2012). V skladu z našimi ugotovitvami, tudi drugi avtorji navajajo privzem CAPE v celice, le da gre za tumorske celice HL-60 (levkemija) (Chen in sod., 2001).

CABE, CAPE in CACE so se izkazali kot močni protirakavi agensi (EC_{50} < 4µg/mL) in so celo aktivne komponente protitumorske aktivnosti nizozemskega propolisa skupaj s flavonoma krizinom in galangin-7-metil etrom. CABE in CACE sta imela močnejše

protitumorsko delovanje kot CAPE. Izkazali so se tudi kot lovilci prostih radikalov, ki imajo podobno moč kot α-tokoferol (vitamin E) in so močnejši od askorbinske kisline (vitamin C). Avtorji povezujejo protitumorsko oz. antiproliferativno delovanje estrov z njihovim antioksidativnim delovanjem (Banskota in sod., 2002). Omene in sod. (2013) so pokazali, da ima CAPE skupaj z ostalimi fenolnimi spojinami v izvlečku propolisa večji protitumorski učinek kot enaka koncentracija čistega CAPE. CAPE igra vlogo pri *in vitro* antioksidativni učinkovitosti propolisa (Russo in sod. , 2002) in ima *in vivo* antioksidativno delovanje (Sud'ina in sod., 1993; Chen in sod., 2009).

Naši dodatni poskusi so pokazali, da po daljši 2-urni izpostavitvi kvasnih celic, CAPE zniža znotrajcelično oksidacijo v kvasovki *S. cerevisiae* pri koncentracijah 10 μM (za 49 %) in 100 μM (za 48 %) v celični suspenziji, medtem ko pri koncentraciji 1 μM na znotrajcelično oksidacijo nima vpliva. Pri koncentraciji 1000 μM ima CAPE oksidativno aktivnost in poviša raven ROS/RNS v kvasni celici za 85 % (Slika 3). To je v skladu z literaturo, ki kaže, da imajo lahko antioksidanti pri visokih koncentracijah škodljive učinke za celice, saj lahko povišajo znotrajcelično raven oksidantov (Bouayed in Bohn, 2012). Poleg tega smo pokazali tudi, da CAPE poviša celično metabolno energijo pri koncentraciji 100 μM za 64 %. CAPE nima večjega vpliva na celično živost pri koncentracijah 1, 10 in 100 μM (Preglednica 9), kjer zniža znotrajcelično oksidacijo (Slika 3).

Omeniti je potrebno, da se celični privzem fenolnih spojin razlikuje med različnimi tipi celic. Prav tako rezultati celičnega privzema fenolnih spojin v celičnih kulturah še ne pomenijo enakega celičnega privzema v višjih organizmih (Spencer in sod., 2004).

Iz naših rezultatov lahko zaključimo, da imajo največji pozitiven učinek na celični ravni kvasovke, fenolne spojine iz frakcije EL70, ki so vstopile v/na celico, in sicer trije estri kavne kisline CAPE, CABE in CACE. Poleg tega smo za CAPE pokazali, da zniža znotrajcelično oksidacijo in poviša celično metabolno energijo pri določenih koncentracijah.

3.3.2 Proteomska raven

Identifikacija proteinskih tarč propolisa je bil eden od ciljev te doktorske naloge z namenom, da osvetlimo molekularne mehanizme v ozadju delovanja propolisa. Kvasovka *S. cerevisiae* je zaradi biokemičnih in molekularnih podobnosti s človeško celico uporaben evkariontski model za odkrivanje novih bioaktivnih spojin in razumevanje načina, kako se celica nanje odzove (Mager in Winderickx, 2005).

Frakcija EL70 se je izkazala za najbolj pozitivno v smislu, da je znižala znotrajcelično oksidacijo (66 %) in povečala celično metabolno energijo (21 %). Poleg tega so določene fenolne spojine iz frakcije EL70 vstopile v kvasno celico (CAPE, CABE in CACE). Zato

smo se odločili, da analiziramo njen vpliv na kvasni proteom (Petelinc in sod., 2013b). Uporabili smo subcelularni proteomski pristop, ki omogoča obogatitev proteinov z nizko vsebnostjo v celici in istočasno pridobitev informacije o lokaciji proteinov v celici. Pri tem smo se odločili za uporabo diferencialne detergentne frakcionacije (DDF).

3.3.2.1 Ekstrakcija citosolnih, membransko/organelnih, jedrnih in citoskeletnih proteinskih frakcij

Proteine smo iz kvasnih celic ekstrahirali z DDF. Uporabili smo komplet "Proteoextract Subcellular proteome extraction kit", ki vsebuje 4 ekstrakcijske pufre, in sicer digitonin/EDTA, Triton-X-100/EDTA, Tween-40/deoksiholat in SDS/ditiotreitol. Zaporedna uporaba detergentov omogoči pridobitev citosolne (F1), membransko/organelne (F2), jedrne (F3) in citoskeletne proteinske frakcije (F4) (Ramsby in Makowski, 1999). Protokol je bil v osnovi zasnovan za sesalske celice, zato smo ga najprej modificirali za uporabo na kvasovki. Pri tem smo upoštevali, da imajo kvasne celice, z razliko od sesalskih celic, celično steno, zato smo jo razbili s cirkonij-kremenčevimi kroglicami (Petelinc in sod., 2013b).

Velika prednost uporabe DDF je, da so detergenti kompatibilni z elektroforetskimi tehnikami (Patton, 1999). Slabost te metode pa je, da se v proteinskih ekstraktih pojavljajo kontaminacije, kar pomeni, da so v ekstraktu prisotni tudi proteini iz drugih organelov ali struktur. Presojo ali je protein kontaminant ali ne, oteži dejstvo, da se isti proteini lahko pojavljajo v večih organelih (Wiederhold in sod., 2010). Zato smo v našem protokolu uvedli dodatne korake spiranja, s čimer smo zmanjšali kontaminacijo v vzorcih (Petelinc in sod., 2013b).

Proteine posameznih frakcij, ki smo jih pridobili z DDF, smo ločili z 2-DE v območju MW 10-220 kDa in pI 4-7 in gele barvali z barvilom Sypro Ruby. Analiza subproteomskih profilov na 2-D gelu je pokazala značilen vzorec proteinskih lis za posamezne frakcije. Nekatere proteinske lise so bile značilne samo za eno proteinsko frakcijo. Te proteinske lise smo identificirali z MALDI-TOF/TOF in računalniškim algoritmom Mascot. Z analizo lokacij identificiranih proteinov, ki so jim anotirane v bazi podatkov UniProt, smo preverili, če lahko posamezne frakcije povežemo z določenim celičnim organelom ali strukturo. Izkazalo se je, da imajo nekateri proteini več lokacij v celici. Anotacije za lokacijo proteinov iz F2 so bile zunanja in notranja mitohondrijska membrana ter intermembranski prostor mitohondrija. Anotacije za lokacijo proteinov iz F3 pa so bile notranja mitohondrijska membrana, mitohondrijski matriks, mitohondrijski nukleoid, jedro in kromosom (Petelinc in sod., 2013b). V kompletu "Proteoextract subcellular proteome extraction kit" je F2 definirana kot mitohondrijska/organelna proteinska frakcija in F3 kot jedrna proteinska frakcija (Ramsby in Makowski, 1999), kar se ujema z našimi rezultati.

Večina proteinskih lis se je pojavljala skozi dve ali več frakcij. To lahko pomeni, da isti protein istočasno najdemo v večih celičnih organelih ali strukturah. Po drugi strani je to lahko rezultat kontaminacije posamezne frakcije s proteini iz ostalih celičnih organelov ali struktur. To je lahko posledica nepopolne ekstrakcije s predhodnim ekstrakcijskim pufrom. Kar je zelo verjetno v primeru proteinov z visoko vsebnostjo v celici (ang. high abundance proteins), saj jih je za 6 redov velikosti več od proteinov z nizko vsebnostjo v celici (ang. low abundance proteins). Kljub spiranju je tako ostanek proteinov z visoko vsebnostjo v celici, večji od proteinov z nizko vsebnostjo v celici, ki so ekstrahirani z naslednjim ekstrakcijskim pufrom.

3.3.2.2 Vpliv frakcije EL70 na kvasni subproteom

Po karakterizaciji posameznih proteinskih frakcij, ki smo jih pridobili z DDF, smo analizirali vpliv frakcije EL70 na kvasni subproteom. Kvasovke *S. cerevisiae* smo za 2-uri izpostavili frakciji EL70 pri koncentraciji 1 % (v/v) in nato proteine ločili z 2-DE v območju MW 10-220 kDa in pI 4-7 ter gele barvali z barvilom Sypro Ruby. Proteinske profile po izpostavitvi frakciji EL70 smo primerjali glede na kontrolo (za vsako frakcijo svoja kontrola) z uporabo računalniškega programa 2-D Dymension. Naredili smo tri biološke ponovitve in za analizo statističnih razlik v ravni proteinov uporabili statistično analizo Anova. Diferencialno izražene proteine smo identificirali z MALDI-TOF/TOF in računalniškim algoritmom Mascot, nato pa njihovo funkcijo poiskali v bazi podatkov UniProt.

Skupno smo iz vseh proteinskih frakcij identificirali 18 proteinov, ki so imeli glede na kontrolo spremenjeno raven v EL70-tretiranih celicah kvasovke. Izmed teh je bilo največ proteinov vpletenih v metabolizem ogljikovih hidratov in energijski metabolizem (44,4 %) (Tdh1, Fba1, Pgi1, Pdc1, Lsc2, Qcr2, Pdb1, Atp2). Druga največja skupina so bili proteini vpleteni v dinamiko aktinskih filamentov (22,2 %) (Act1, Arp2, Fba1, Hsp26). Identificirani proteini so bili vpleteni tudi v antioksidativno obrambo (11,1 %) (Dug1, Oye2), proces zvijanja proteinov (11,1 %) (Hsp26, Sec53) in ostale celične procese (11,1 %) (Sec53, Gsp1) (Petelinc in sod., 2013b).

Spremembe v antioksidativnemu obrambnemu sistemu

Antioksidativni obrambni sistem vzpostavlja redoks homeostazo v celici tako, da odstranjuje presežne ROS. Glavni vir ROS v večini celic je mitohondrijska elektronska transportna veriga (ETV). V primeru puščanja ETV, predvsem s strani kompleksa I in kompleksa III, izgubljeni elektroni nepopolno reducirajo kisik v prosti radikal, in sicer v superoksidni anion ($O_2^{\bullet -}$). Superoksidni anion odstranjuje encim SOD, pri čemer nastane vodikov peroksid, ki pa ga nato v vodo reducirajo drugi encimi, npr. katalaza, glutation peroksidaza ali peroksiredoksin (Gutterman, 2005).

Sklepa se, da večja produkcija ATP pomeni hitrejši prenos elektronov v ETV, ob tem pa se izgubi še več elektronov in nastane več ROS (Karu in sod., 1995). Višja raven ROS spodbudi sintezo antioksidativnih encimov, ki jih odstranijo preden povzročijo škodo v celicah (Kim in sod., 2011). Predvidevamo, da velja tudi obratno, in sicer da ob manjši produkciji ATP nastaja manj ROS, zato ni potreb po antioksidativnih encimih in se njihova raven zniža. V kvasovki izpostavljeni frakciji EL70 smo pokazali višjo raven ATP, zato smo pričakovali povečano raven antioksidativnih encimov. Vendar takih sprememb nismo zaznali. Predhodno smo v kvasovkah izpostavljenim celotnemu izvlečku propolisa našli znižano raven dveh antioksidativnih encimov, in sicer mitohondrijske manganove superoksid-dismutaze (Sod2) ($R = -1,8$; $p = 0,1$) in mitohondrijskega peroksiredoksina 1 (Prx1) ($R = -1,7$; $p = 0,03$), kljub povišani ravni ATP in znižani ravni ROS (Cigut in sod., 2011). Prx2 je poznana molekularna tarča nekaterih fenolnih spojin (Firouzi in sod., 2014). Tudi po izpostavitvi kvasnih celic preparatu gobe *Cordyceps militaris*, ki je povišal celično metabolno energijo, se je aktivnost encima Sod2 zmanjšala glede na kontrolo (Horvat, 2014).

Frakcija EL70 lahko deluje kot lovilec prostih radikalov in ima tako celica, kljub povečani metabolni energiji, znižano znotrajcelično raven oksidantov. Res je, da previsoke koncentracije ROS vodijo do oksidacije bioloških molekul (DNA, lipidov, beljakovin in ogljikovih hidratov) in celičnih poškodb oz. stanja, ki ga v celici imenujemo oksidativni stres. Vendar so ROS tudi bistvenega pomena za življenje, saj so vpletene v vrsto bioloških funkcij (Gomes in sod., 2005), zato bi nižja raven ROS lahko pomenila tudi stres za celico. Npr. superoksidni anion in vodikov peroksid, ki ju odstranjujeta Sod2 in Prx1, sodelujeta pri znotrajcelični signalizaciji in redoks homeostazi (Finkel, 1998).

Znižana raven antioksidativnih encimov je lahko posledica modifikacij, npr. fosforilacije. Fosforiliranemu proteinu se spremeni izoelektrična točka, zato zaznamo horizontalni premik proteinske lise na gelu. Ob odzivu na zunanji stimulans se fosforilira le del molekul. Če se fosforilira le del molekul, ki sestavljajo liso, zaznamo zmanjšano intenziteto lise nefosforiliranih proteinov, medtem ko fosforilirane proteine zaznamo na drugem mestu na gelu oz. jih sploh ne zaznamo, če je njihova vsebnost pod pragom zaznave (Woo in sod., 2010). Prx1 in Sod2 sta tarči fosforiliracije z od-ciklina-odvisnimi kinazami (Chang in sod., 2002; Candas in sod., 2013). Ko od-ciklina-odvisna kinaza fosforilira Sod2, poveča njegovo stabilnost in aktivnost encima ter tako zniža raven ROS v celici (Candas in sod., 2013), medtem ko fosforilacija peroksiredoksina zniža njegovo aktivnost (Chang in sod., 2002). Prav tako je znano, da sta Prx in Sod zelo občutljiva na oksidativne poškodbe (Avery, 2011). Najbolj proučena oksidativna modifikacija proteinov je karbonilacija proteinov t.j. oksidacija do karbonilov, ki vodi v razgradnjo s proteasomom. Ker je v našem primeru v celicah izpostavljenim propolisu raven ROS nižja glede na kontrolo (Cigut in sod., 2011), znižano raven Prx1 in Sod2 ne moremo pripisati njuni oksidaciji.

Po drugi strani smo opazili, da se je po 2-urni izpostavitvi kvasovk frakciji EL70, spremenila raven nekaterih drugih proteinov povezanih z antioksidativnim obrambnim sistemom. Npr. raven cistein-glicinske metalopeptidaze DUG1 (Dug1) se je znižala (R = 1,5; p = 0,03) (Petelinc in sod., 2013b). Protein Dug1 je ohranjen pri bakterijah, glivah, rastlinah in sesalcih (Ganguli in sod., 2007; Kaur in sod., 2009). Kvasni Dug1 je del kompleksa, ki razgrajuje glutation in vpliva na njegovo vsebnost v celici. Npr. če se razgradnja glutationa ustavi, bo v celici prisotnega več glutationa (Baudouin-Cornu in-sod., 2012). Glutation je pomemben antioksidant v celici in je vpleten v: (i) odziv na stradanje ob pomanjkanju žvepla ali dušika; (ii) detoksifikacijo ksenobiotikov in endogenih toksičnih metabolitov ter (iii) zaščito pred oksidativnim stresom ter stresom ob prisotnosti težkih kovin (Penninckx, 2002). Zmanjšana raven Dug1 bi tako lahko pomenila manjšo razgradnjo glutationa in njegovo večjo raven v celici. Povečana vsebnost glutationa je lahko razlog za znižano raven ROS, ki smo jo opazili v kvasovki izpostavljeni frakciji EL70 (Petelinc in sod., 2013a).

V celicah kvasovke izpostavljene frakciji EL70 je bila povečana tudi raven NADPH-dehidrogenaze 2 (Oye2) (R = 1,6; p = 0,04) (Petelinc in sod., 2013b). Odat in sod. (2007) so pokazali, da je v celicah, ki imajo več proteina Oye2, 13 % manj ROS.

Spremembe v metabolizmu ogljikovih hidratov

V celicah kvasovke izpostavljene frakciji EL70 smo našli spremenjene ravni proteinov, ki so vpleteni v glikolizo, metabolizem piruvata in citratni ciklus.

Diferencialno izraženi proteini vpleteni v glikolizo

V EL70-tretiranih celicah se je raven glikolitičega encima gliceraldehid-3-fosfat-dehidrogenaze 1 (Tdh1) znižala (R = -2,9; p = 0,004) (Petelinc in sod., 2013b). Tdh1 katalizira reakcijo: *gliceraldehid-3-fosfat* + P_i + NAD^+ ⇌ *1,3-bisfosfoglicerat* + *NADH* + H^+ (Boyer, 2005), njegova inaktivacija pa povzroči preusmeritev glikolize v pentozafosfatno pot (imenovana tudi fosfoglukonatna pot) (Ralser in sod., 2007). V pentozafosfatni poti se glukoza oksidira do pentoze riboza-5-fosfata, pri čemer nastane reduciran koencim NADPH. Poraba NADPH v celici pospeši oksidacijo glukoze po pentozafosfatni poti in nastanek novega NADPH. NADPH je v celicah potreben za redukcijske reakcije, med drugim ohranja glutation v reduciranem stanju (Boyer, 2005). Ravnotežje med oksidirano in reducirano obliko glutationa namreč vzdržuje od NADPH odvisen encim glutation-reduktaza (Mullins in sod., 1998). To se ujema z našo hipotezo, da v EL70-tretiranih celicah znižana raven Dug1 (Petelinc in sod., 2013b), ki razgrajuje glutation, lahko pomeni višjo raven glutationa in zato nižjo raven ROS v celici, saj bi bile ob tem potrebe po NADPH v celici večje. Z NADPH se reducira tudi protein Oye2 (Odat

in sod., 2007), katerega raven v EL70-tretiranih celicah je bila povečana (Petelinc in sod., 2013b).

V EL70-tretiranih celicah se je znižala raven še dveh encimov glikolize, glukoza-6-fosfat-izomeraze (Pgi1) (R = -1,7; p = 0,03) in fruktoza-bisfosfat-aldolaze (Fba1) (R = -1,7; p = 0,03 in R = -1,6; p = 0,02). Znižano raven Fba1 smo našli v citosolni in citoskeletni proteinski frakciji (Petelinc in sod., 2013b). Pgi1 katalizira reakcijo: *glukoza-6-fosfat* ⇌ *fruktoza-6-fosfat*, Fba1 pa katalizira reakcijo: *fruktoza-1,6-bisfosfat* ⇌ *dihidroksiacetonfosfat* + *gliceraldehid-3-fosfat* (Boyer, 2005). Glikolitični encim Fba1 je molekularna tarča fenolne spojine kurkumina (Firouzi in sod., 2014), njegova raven pa se je tudi znižala v celicah izpostavljenim s polifenoli bogatem izvlečkom morske alge (Ott, 2013). Inaktivacija teh treh glikolitičnih encimov lahko pomeni aktivacijo pentozafosfatne poti, ki celici priskrbi redukcijsko moč za antioksidativne obrambne sisteme.

Diferencialno izraženi proteini vpleteni v metabolizem piruvata

Izpostavitev kvasovk frakciji EL70 je povzročila dvig v ravni podenote β komponente E_1 mitohondrijske piruvat-dehidrogenaze (Pdb1) (R = 2,1; p = 0,03) (Petelinc in sod., 2013b). Piruvat dehidrogenaza je multiencimski kompleks, ki ga sestavljajo encimi piruvat-dehidrogenaza (E_1), dihidrolipoil-transacetilaza (E_2) in dihidrolipoil-dehidrogenaza (E_3). Kompleks katalizira reakcijo: *piruvat* + NAD^+ + *CoASH* ⟶ *acetil-CoA* + *NADH* + H^+ + CO_2, v kateri nastane reduciran koencim NADH in energijsko bogata spojina acetil-CoA, ki vstopi v citratni cikel. Encim piruvat-dehidrogenaza (E_1) katalizira prvo stopnjo reakcije, in sicer dekarboksilacijo. Za svoje delovanje potrebuje koencim tiaminpirofosfat (vitamin B_1) (Boyer, 2005). Raven Pdb1 se je povišala tudi v celicah, tretiranih s preparatom gobe *Cordyceps militaris*, ki je prav tako povzročil povišano raven ATP in respiracije (Horvat, 2014).

V EL70-tretiranih celicah se je znižala raven piruvat-dekarboksilaze izoencima 1 (Pdc1) (R = -1,7; p = 0,02) (Petelinc in sod., 2013b). Pdc1 katalizira prvi del reakcije pri alkoholni fermentaciji: *piruvat* ⇌ *acetaldehid* + CO_2, za kar potrebuje koencim tiaminpirofosfat (vitamin B_1) (Boyer, 2005).

Diferencialno izraženi proteini vpleteni v citratni ciklus

V kvasovki izpostavljeni frakciji EL70 se je povečala raven podenote β mitohondrijske sukcinil-CoA-ligaze (Lsc2) (R = 1,7; p = 0,05) (Petelinc in sod., 2013b). Lsc2 katalizira reakcijo: *sukcinil-CoA* + *ADP* + P_i ⇌ *sukcinat* + *CoASH* + *ATP*, v kateri ATP nastaja v procesu fosforilacije na ravni substrata. Lsc2 je edini encim v citratnemu ciklusu, ki proizvaja ATP (Boyer, 2005). Dvig v ravni Lsc2 je v skladu z našimi rezultati na

celičnemu nivoju, kjer smo pokazali povišano raven ATP v kvasnih celicah izpostavljenih frakciji EL70 (Petelinc in sod., 2013a).

Spremembe v elektronski transportni verigi

V kvasovki izpostavljeni frakciji EL70 smo pokazali višjo raven ATP (Petelinc in sod., 2013a) in ker 95 % ATP v evkariontskih celicah nastane na ETV (Erecińska in Wilson, 1982) smo tu pričakovali spremembe. Glavni proteini na ETV so: NADH:ubikinon-oksidoreduktaza ali krajše NADH-dehidrogenaza (kompleks I), sukcinat:ubikinon-oksidoreduktaza ali krajše sukcinat-dehidrogenaza (kompleks II), ubikinol:(feri)citokrom *c*-oksidoreduktaza ali krajše citokrom *c*-reduktaza (kompleks III) in (fero)citokrom *c*:kisik-oksidoreduktaza ali krajše citokrom *c*-oksidaza (kompleks IV) (Boyer, 2005).

V EL70-tretiranih celicah je bila povečana raven podenote 2 kompleksa III (Qcr2) (R = 1,5; p = 0,06) (Petelinc in sod., 2013b). To je v skladu s predhodnimi ugotovitvami, kjer smo tudi našli povečano raven Qcr2 v mitohondrijski proteinski frakciji po tretiranju s propolisom (R = 1,6; p = 0,03) (Cigut, 2010). Dvig ravni Qcr2 je v skladu z našimi rezultati na celičnemu nivoju, kjer smo pokazali povišano raven ATP v kvasnih celicah izpostavljenih propolisu (Cigut in sod., 2011) in frakciji EL70 (Petelinc in sod., 2013a). Znano je, da s kompleksa III elektroni najpogosteje puščajo z ETV in povzročijo nastanek ROS v celici. Aktivacija ETV poviša raven ROS v celici, ker z ETV pušča več elektronov (Karu in sod., 1995). V našem primeru kljub večji ravni ATP v celici, raven ROS ni povišana, zato predpostavljamo, da bi lahko povišana raven proteinov na kompleksu III preprečila puščanje elektronov. Literatura navaja, da je ETV molekularna tarča različnih fenolnih spojin. Tako je kompleks III tarča genisteina (Salvi in sod., 2002), kompleks I pa kvercetina, kamferola in apigenina (Lagoa in sod., 2011). Izpostavitev propolisu tudi poveča mRNA polipeptida VIII kompleksa IV (Cox8) (de Castro in sod., 2012).

V EL70-tretiranih celicah se je povišala tudi raven podenote β mitohondrijske ATP-sintaze (Atp2) (R = 1,6; p = 0,01) (Petelinc in sod., 2013b). Mitohondrijska ATP-sintaza (kompleks V) je encimski kompleks na ETV, kjer se s pomočjo protonskega gradienta sintetizira ATP. Po drugi strani smo predhodno našli zmanjšano raven podenote α mitohondrijske ATP-sintaze (Atp1) (R = -1,5; p = 0,02) (Cigut in sod., 2011). Atp1 je fosfoprotein in njegova fosforilacija bi lahko povzročila premik dela proteinov na gelu, kar mi zaznamo kot zmanjšano raven (Reinders in sod., 2007). Mitohondrijska F_0F_1-ATPaza je sestavljena iz dveh glavnih funkcionalnih domen ali področij: F_0 in F_1. Domena F_1 štrli iz mitohondrijske membrane v matriks mitohondrija in predstavlja katalitično enoto na kateri poteka sinteza ATP. Domena F_1 je preko središčnih in perifernih pecljev pritrjena na domeno F_0. Slednja je usidrana v membrano, skozi katero potujejo protoni iz medmembranskega prostora v matriks mitohondrija (Gledhill in sod., 2007). Domeno F_1 sestavlja pet različnih podenot, nekatere tudi v večih kopijah ($\alpha_3\beta_3\gamma\delta\varepsilon$) s katalitičnim

mestom na β-podenoti, medtem, ko domeno F_0 sestavlja več membranskih proteinov (a, b, c, d, e, F_6, A6L) in protein Oscp (ang. oligomycin sensitivity-conferring protein) (Zheng in Ramirez, 2000). Literatura navaja, da so nekatere fenolne spojine (stilbeni, flavoni in izoflavoni, katehini, proantocianidini, kurkumin, floretin, teaflavin, taninska kislina) inhibitorji mitohondrijske ATP-sintaze (Hong in Pedersen, 2008). Po drugi strani de Castro in sod. (2012) navajajo, da propolis poveča mRNA nekaterih proteinov vpletenih v sintezo ATP, in sicer Atp17, Atp18, Atp19, Atp20 in Atp21. Pri izpostavitvi kvasovke s polifenoli bogatim izvlečkom morske alge so našli povišano raven Atp2 (Ott, 2013).

Spremembe v dinamiki aktinskih filamentov

Izpostavitev kvasovk frakciji EL70 je povzročila upad v ravni aktina ($R = -1,8$; $p = 0,06$ in $R = -1,9$; $p = 0,01$) (Petelinc in sod., 2013b). Aktinski citoskelet ima pomembno vlogo v vrsti celičnih procesov, kot so vzdrževanje celične morfologije in polarnosti, endocitoza, znotrajcelični transport, gibanje in deljenje celice (Winder in Ayscough, 2005). Aktin je molekularna tarča nekaterih fenolnih spojin, npr. (–)-epigalokatehin-3-galata (Weinreb in sod., 2007) in kurkumina (Firouzi in sod., 2014) ter s polifenoli bogatega izvlečka morske alge (Ott, 2013). Da bi bila znižana raven aktina posledica karbonilacije aktina, ki se sicer pogosto zgodi (Dalle-Donne in sod., 2001), ni verjetno, saj je bila raven ROS v EL70-tretiranih celicah znižana (Petelinc in sod., 2013a).

Aktinski citoskelet je dinamičen sistem, saj se aktinski filamenti nenehno krajšajo in daljšajo, snopi in mreže filamentov pa se oblikujejo ali razpadajo. Struktura in dinamika aktinskih filamentov je odvisna od številnih različnih proteinov, ki se vežejo na aktin (Jezernik in sod., 2012; Winder in Ayscough, 2005). V EL70-tretiranih kvasnih celicah se je spremenila raven treh na aktin vezavnih proteinov, in sicer se je povišala raven aktinu sorodnega proteina 2 (Arp2) ($R = 1,7$; $p = 0,04$), znižala raven Fba1 ($R = -1,7$; $p = 0,03$ in $R = -1,6$; $p = 0,02$) in povišala raven Hsp26 ($R = 1,5$; $p = 0,01$) (Petelinc in sod., 2013b). Arp2 je del kompleksa Arp2/Arp3 in je aktivator polimerizacije aktina (Mullins in sod., 1998), medtem ko je Fba1, sicer bolj poznan kot glikolitičen encim, inhibitor polimerizacije (Schindler in sod., 2001). Hsp26 spada med proteine toplotnega šoka z molekulsko maso med 15-30 kDa (ang. small heat shock proteins, sHsp), ki so šaperoni, a imajo tudi afiniteto do aktina. Fosforilirani sHsp-ji inhibirajo polimerizacijo aktina (Mounier in Arrigo, 2002), nefosforilirani sHsp-ji pa inhibirajo depolimerizacijo aktina (Guay in sod., 1997). Tako povišana raven Arp2 in znižana raven Fba1 nakazujeta na povečano polimerizacijo aktina. Pomembno vlogo pri polimerizaciji ima tudi ATP, saj le ob njegovi prisotnosti aktinske molekule polimerizirajo in oblikujejo prožen filament, sestavljen iz dveh vrvic zaporedno vezanih aktinskih molekul, ki se ena okoli druge ovijata v dvojno vijačnico (Jezernik in sod., 2012).

Spremembe v procesu zvijanja proteinov

Izpostavitev kvasovk frakciji EL70 je povzročila povišanje ravni proteina toplotnega šoka HSP26 (Hsp26) (R = 1,5; p = 0,01) (Petelinc in sod., 2013b). Protein Hsp26 je šaperon in skrbi za pravilno zvijanje proteinov v stresnih pogojih (Jakob in sod., 1993). Literatura kaže, da so proteini toplotnega šoka molekularne tarče nekaterih fenolnih spojin, npr. (–)-epigalokatehin-3-galata (Weinreb in sod., 2007) in kurkumina (Firouzi in sod.; 2014) ter s polifenoli bogatega izvlečka morske alge (Ott, 2013) in preparata gobe *Cordyceps militaris*, ki je povišal metabolno energijo celic (Horvat, 2014).

V kvasovki izpostavljeni frakciji EL70 smo pokazali povišano raven encima fosfomanomutaze (Sec53) (R = 2,0; p = 0,002) (Petelinc in sod., 2013b). Sec53 katalizira pretvorbo manoze-6-fosfata v manozo-1-fosfat. Ta skupaj z GTP tvori GDP-manozo, ki sodeluje pri zvijanju in glikolizilaciji sekretornih proteinov v lumnu endoplazemskega retikuluma (Feldman in sod., 1987).

Spremembe v ostalih celičnih procesih

Dodatno smo v EL70-tretiranih kvasnih celicah opazili znižano raven Ran GTPaze GSP/CNR1 (Gsp1) (R = -2,2; p = 0,01) (Petelinc in sod., 2013b). Povečana raven Ran GTPaz je povezana z nastankom raka (Sanderson in Clarke, 2006; Xia in sod., 2008, Lui in Huang, 2009), zato zmanjšana raven Gsp1 nakazuje na protirakavo učinkovitost frakcije EL70.

Pri tem je pomembno poudariti, da literatura navaja uporabo oksidantov za uničenje rakavih celic, katerih se normalne celice lažje ubranijo, saj imajo že v izhodišču nižjo raven ROS/RNS kot tumorske celice. Zaradi večje proliferacije imajo namreč večje zahteve po energiji in posledično nastaja več ROS/RNS, ki jih morajo odstraniti, da lahko preživijo. Zato je lahko tretiranje tumorskih celic z antioksidanti oz. znižanje ravni ROS/RNS, kot smo jo pokazali v primeru našega izvlečka propolisa in frakcije EL70, še bolj ugodno za razvoj tumorskih celic. Po drugi strani tretiranje normalnih, zdravih celic z antioksidanti lahko prepreči previsoke ravni ROS/RNS in oksidativni stres, zaradi česar bi nastale poškodbe DNA in tumorigeneza (Gorrini in sod., 2013).

3.3.2.3 Vpliv izvlečka propolisa in frakcije EL70 na sirtuinsko aktivnost

V naši raziskavi smo pokazali, da etanolni izvleček propolisa in njegova frakcija EL70 v kvasnih celicah zmanjšata znotrajcelično oksidacijo (61 %, 66 %) in povečata celično metabolno energijo (20 %, 21%) (Petelinc in sod., 2013a), kar spominja na učinek kalorične restrikcije (Choi in Lee, 2013; Choi in sod., 2011; Lin in sod., 2002). Znano je, da so fenolne spojine mimetiki kalorične restrikcije in da imajo sirtuini pomembno vlogo

pri kalorični restrikciji (Guarente in Picard, 2005; Wood in sod., 2004; Chen in Guarente, 2007), zato smo preverili učinek propolisa in frakcije EL70 na sirtuinsko aktivnost.

Sirtuini so histonske deacetilaze razreda III in katalizirajo deacetilacijo histonskih in nehistonskih proteinov v prisotnosti nikotinamid adenin dinukleotida (NAD^+). V reakciji se poleg deacetiliranega substrata tvorita še nikotinamid in 2'-*O*-acetil-ADP-riboza. Ker so sirtuini odvisni od kofaktorja NAD^+, je razpoložljivost le-tega eden od načinov regulacije sirtuinov (Cantó in Auwerx, 2012). Raven NAD^+ upade, ko je okolje v prid njegovi reducirani obliki - NADH. Tako raven NAD^+ naraste v primeru kalorične restrikcije ali vadbe in upade v primeru prehrane z visoko vsebnostjo maščobe (Houtkooper in sod., 2012). Od NAD^+ odvisna narava sirtuinov nakazuje na povezavo med sirtuinsko aktivnostjo in energijskim metabolizmom celic.

Sirtuini so prisotni v prokariontih in evkariontih in se delijo v 5 razredov (I-IV, U). *S. cerevisiae* ima 5 sirtuinov in vsi so razreda I (Sir2, Hst1-4) (Frye, 2000). Najbolj raziskan kvasni sirtuin Sir2 (ang. silent information regulator 2) najdemo v jedru (Gotta in sod., 1997), kjer s spreminjanjem kromatinske strukture sodeluje pri utišanju transkripcije ribosomalne DNA (Gottlieb in Esposito, 1989), telomerne DNA in lokusa paritvenega tipa (Armstrong in sod., 2002). Človek ima 7 sirtuinov, ki pripadajo različnim razredom: SIRT1-3 so razreda I, SIRT4 je razreda II, SIRT5 je razreda III, SIRT6-7 sta razreda IV (Frye, 2000). SIRT1 je homolog kvasnega Sir2. Prisoten je v jedru in citosolu človeških celic (Tanno in sod., 2007). V jedru SIRT1 deacetilira histone, kar povzroči bolj gosto pakiranje DNA in manjšo dostopnost za transkripcijske faktorje (Schemies in sod., 2010). SIRT1 prav tako deacetilira nehistonske proteine v jedru in citosolu. V jedru SIRT1 deacetilira proteine, kot so p53 (Vaziri in sod., 2001), transkripcijski faktorji FOXO (Motta in sod., 2004), transkripcijski faktor NF-κβ (Yeung in sod., 2004), transkripcijski koaktivator PGC-1α (Rodgers in sod., 2005), androgenski receptor (Fu in sod., 2006), estrogenski receptor (Yu in sod., 2011) in protein toplotnega šoka 1 (Westerheide in sod., 2009). Citosolni proteinski tarči SIRT1 sta acetil-CoA sintetaza (Hallows in sod., 2006) in NO-sintaza (Mattagajasingh in sod., 2007). Na celični ravni SIRT1 uravnava procese popravljanja DNA, apoptoze, cirkadialnega ritma, protivnetnih poti, izločanja inzulina in mitohondrijske biogeneze (Hubbard in sod., 2013).

Zaradi omenjenega širokega delovanja so sirtuini zanimive molekularne tarče za zdravljenje različnih bolezni, kot so rak, HIV, sladkorna tipa II, srčno-žilne in nevrodegenerativne bolezni (Schemies in sod., 2010; Kokkola in sod., 2014). Poleg tega so sirtuini predmet raziskav pri regulaciji staranja, saj se je izkazalo, da igrajo pomembno vlogo pri podaljševanju življenjske dobe (Guarente and Picard, 2005).

V naši raziskavi smo za določanje sirtuinske aktivnosti uporabili komplet "SIRTainty Class III HDAC assay" s katerim se meri nikotinamid, ki nastane v reakciji deacetilacije. Določili smo sirtuinsko aktivnost v subproteomskih frakcijah kvasovke *S. cerevisiae* po tretiranju s etanolnim izvlečkom propolisa ali frakcijo EL70. Rezultati so pokazali, da etanolni

izvleček propolisa in frakcija EL70 povečata sirtuinsko aktivnost v kvasnih celicah, in sicer v citosolni subproteomski frakciji (etanolni izvleček propolisa, 71 %, $p \leq 0,05$; frakcija EL70, 61 %, $p \leq 0,1$) in jedrni subproteomski frakciji (etanolni izvleček propolisa, 34 %, $p \leq 0,01$; frakcija EL70, 64 %, $p \leq 0,05$) (Slika 5).

Povečana sirtuinska aktivnost lahko razloži nekatere spremembe v proteinskem profilu kvasnih celic, ki smo jih pokazali po izpostavitvi frakciji EL70 (Petelinc in sod., 2013b). V literaturi navajajo, da je spremenjena aktivnost sirtuinov povezana s spremembami na citoskeletnih komponentah (North in sod., 2003). V našem primeru smo po izpostavitvi frakciji EL70 zaznali spremenjeno raven aktina (Act1) in na aktin vezavnih proteinov (Arp2, Hsp26, Fba1). Poleg tega spremenjena aktivnost sirtuinov vpliva tudi na metabolne encime, in sicer glikolitične encime in encime oksidativne fosforilacije (Houtkooper in sod., 2012; Ralser in sod., 2012). V našem primeru smo po izpostavitvi frakciji EL70 zaznali spremenjeno raven glikolitičnih encimov (Tdh1, Fba1, Pgi1) in proteinov mitohondrijske respiratorne verige (Atp2, Qcr2). Pri tem je potrebno omeniti, da katalitična aktivnost sirtuinov vpliva na celični metabolizem in obratno, da spremembe v metabolizmu celic vplivajo na aktivnost sirtuinov.

Omene in sod. (2013) so predhodno pokazali, da se v rakavih celicah izpostavljenim propolisu akumulirajo acetilirani histoni, kar kaže na zmanjšano aktivnost histonskih deacetilaz. Enako so pokazali za CAPE, fenolno spojino v propolisu. Nasprotno je bilo s fluorescentno označenim substratom pokazano, da nekatere fenolne spojine, kot so resveratrol, kvercetin in piceatanol, povečajo aktivnost sirtuinov (Howitz in sod., 2003), kar je v skladu z našimi rezultati.

Rastline proizvajajo fenolne spojine, ko so v stresu, npr. ob dehidraciji, pomanjkanju hranil, ultravijoličnem sevanju in okužbami z rastlinskimi patogeni. Zato je možno, da rastline s pomočjo fenolnih spojin preko sirtuinov regulirajo stresni odgovor (Howitz in sod., 2003). Evolucijsko so sirtuini zelo ohranjeni, česar posledica je lahko, da eksogene fenolne spojine tudi v glivah in živalih oponašajo endogene aktivatorje sirtuinov.

Pokazali smo, da izvleček propolisa in frakcija EL70 povečata aktivnost sirtuinov v kvasnih celicah. To nakazuje na možnost, da so spremembe na celični ravni (zmanjšana znotrajcelična oksidacija in povečana celična metabolna energija) ter spremembe na proteomski ravni (raven citoskeletnih komponent in metabolnih encimov), lahko posledica delovanja fenolnih spojin na sirtuinsko aktivnost. Vsekakor pa so potrebne nadaljnje raziskave, da se ugotovi natančen mehanizem delovanja fenolnih spojin na sirtuine.

3.4 ZAKLJUČNE UGOTOVITVE

V doktorski nalogi smo postavili več raziskovalnih hipotez o vplivu bioaktivnih spojin propolisa na kvasovko *S. cerevisiae*.

Hipotezo 1, da antioksidativna učinkovitost v celicah ni vedno povezana z antioksidativno učinkovitostjo *in vitro*, smo potrdili. Iz sposobnosti lovljenja prostih radikalov, ki smo jo določili z DPPH testom, se ni dalo predvideti antioksidativno učinkovitost v celici določeno z 2,7-diklorofluoresceinom. Izkazalo se je, da frakcije etanolnega izvlečka propolisa, ki imajo enako sposobnost lovljenja prostih radikalov, različno vplivajo na znotrajcelično oksidacijo. Poleg tega nekatere frakcije, ki imajo sposobnost lovljenja prostih radikalov, celo povečajo znotrajcelično oksidacijo.

Hipotezo 2, da imajo nekatere fenolne spojine propolisa večjo antioksidativno učinkovitost v celicah kot druge, smo potrdili. Rezultati so pokazali, da ni povezave med vsebnostjo fenolnih spojin, ki smo jo določili z Folin-Ciocalteaujevo metodo v posameznih frakcijah izvlečka propolisa in njihovo antioksidativno učinkovitostjo v celici določeno z 2,7-diklorofluoresceinom. Izkazalo se je namreč, da imajo frakcije z enako vsebnostjo skupnih fenolnih spojin, različen učinek na znotrajcelično oksidacijo. Poleg tega imajo nekatere frakcije, ki vsebujejo fenolne spojine, oksidativen učinek na celico. Tako vse kaže na to, da je antioksidativna učinkovitost v celici bolj odvisna od vrst fenolnih spojin, kot od skupnih fenolnih spojin.

Hipotezo 3, da bomo uspešno identificirali fenolne spojine propolisa, ki vstopijo v celico, smo potrdili. S HPLC-MS/MS smo izmed 27 fenolnih spojin, ki smo jih zaznali v etanolnemu izvlečku propolisa, za tri estre hidroksicimetnih kislin pokazali, da vstopijo v celico oz. z njo reagirajo. Te fenolne spojine so CAPE, CABE in CACE.

Hipotezo 4, da se bo vpliv posameznih fenolnih spojin odražal na subceličnem proteomu (citosolna, membranska/organelna, jedrna in citoskeletna frakcija) modelnega organizma v smislu spremembe ravni proteinov in/ali interakcij fenolnih spojin s proteini, smo potrdili. Diferencialno izraženi proteini so bili vpleteni v metabolizem ogljikovih hidratov oz. energijski metabolizem (Tdh1, Fba1, Pgi1, Pdc1, Lsc2, Qcr2, Pdb1, Atp2), dinamiko aktinskih filamentov (Act1, Arp2, Fba1, Hsp26), antioksidativno obrambo (Dug1, Oye2), proces zvijanja proteinov (Hsp26, Sec53) in ostale celične procese (Sec53, Gsp1). Uporaba subproteomike nam je pomagala pri interpretaciji rezultatov, saj je funkcija proteina odvisna od njegove lokacije. Spremembe v proteinih vpletenih v metabolizem ogljikovih hidratov oz. energijski metabolizem in antioksidativno obrambo so osvetlile molekularne mehanizme v ozadju sprememb v ravni ROS in ATP, ki smo jih zaznali na celični ravni. Poleg tega so nekatere molekularne tarče propolisa, kot so aktinski citoskelet (Act1, Arp2, Fba1, Hsp26) in Gsp1 zanimive za razvoj protirakavih učinkovin.

Menimo, da so dobljeni rezultati osvetlili področje bioaktivnih spojin propolisa in njegovih molekularnih mehanizmov delovanja v celici. Poleg tega predstavljajo dodatno referenco za nadaljnjo uporabo propolisa v zdravstvene, kozmetične in prehranske namene.

4 SKLEPI

Na osnovi rezultatov doktorske naloge lahko podamo naslednje sklepe:

- Izvleček propolisa ima antioksidativno učinkovitost v celicah, ki smo jo določili z 2,7-diklorofluoresceinom. Po 2-urni izpostavitvi kvasovke izvlečku propolisa pri koncentraciji 1 % (v/v) (≈0,02 g GAE/L) v celični suspenziji se je raven znotrajcelične oksidacije znižala za 61 %. Obenem se je raven celične metabolne energije povišala za 19 %, medtem ko izvleček propolisa ni imel učinka na celično živost.

- Ekstrakcija na trdni fazi (SPE) je primerna metoda za frakcionacijo fenolnih spojin v izvlečku propolisa, saj se pridobljene frakcije (EL30-EL70) med seboj kvantitativno in kvalitativno razlikujejo v profilu fenolnih spojin, kar smo pokazali s HPLC-DAD analizo.

- Med skupnimi fenolnimi spojinami v posameznih frakcijah izvlečka propolisa, ki smo jih določili s Folin-Ciocalteau metodo, in njihovo sposobnostjo lovljenja prostih radikalov, ki smo jo določili z DPPH testom, obstaja korelacija (R^2= 0,88). Frakcije z večjo vsebnostjo skupnih fenolnih spojin imajo večjo sposobnost lovljenja prostih radikalov.

- Med skupnimi fenolnimi spojinami posameznih frakcij izvlečka propolisa, ki smo jih določili s Folin-Ciocalteau metodo, in njihovo antioksidativno učinkovitostjo v celicah, ki smo jo določili z 2,7-diklorofluoresceinom, ni korelacije (R^2= 0,55).

- Posamezne frakcije izvlečka propolisa imajo lahko v celicah antioksidativen ali oksidativen učinek pri koncentraciji 1 % (v/v) skupnih fenolnih spojin v suspenziji kvasovk, odvisno od vrst fenolnih spojin v njem. Bolj polarne frakcije (EL30) so kazale oksidativen učinek, medtem ko so imele manj polarne frakcije (EL50, EL60, EL70) antioksidativen učinek.

- Posamezne frakcije izvlečka propolisa lahko v celicah povišajo ali znižajo raven celične metabolne energije pri koncentraciji 1 % (v/v) skupnih fenolnih spojin v suspenziji kvasovk, odvisno od vrst fenolnih spojin v njem. Bolj polarne frakcije (EL30, EL40, EL50) so zmanjšale raven ATP, medtem ko so manj polarne frakcije (EL60, EL70) povišale raven ATP.

- Po 2-urni izpostavitvi kvasovke izbrani frakciji EL70 pri koncentraciji 1 % (v/v) (≈0,01 g GAE/L) v celični suspenziji se je raven znotrajcelične oksidacije znižala za 66 %. Obenem se je raven celične metabolne energije povišala za 21 %, medtem ko frakcija EL70 ni imela učinka na celično živost.

- S HPLC-MS/MS analizo smo pokazali celični privzem za tri hidroksicimetne estre, CAPE, CABE in CACE iz frakcije EL70.

- CAPE zniža znotrajcelično oksidacijo v kvasovki *S. cerevisiae* po 2-urni izpostavitvi pri koncentracijah 10 in 100 μM v celični suspenziji, medtem ko pri nižji koncentraciji 1 μM nima vpliva in pri višji koncentraciji 1000 μM poviša znotrajcelično oksidacijo. CAPE poviša celično metabolno energijo pri izbrani koncentraciji 100 μM. CAPE nima vpliva na celično živost pri koncentracijah 1 μM, 10 μM in 100 μM.

- Uspešno smo prilagodili komplet "Proteoextract subcellular proteome extraction kit" namenjen diferencialni detergentni frakcionaciji proteinov iz sesalskih celic za uporabo na kvasovki in pridobili citosolno, membransko/organelno, jedrno in citoskeletno proteinsko frakcijo. To smo potrdili z analizo anotacij celičnih lokacij nekaterih ekstrahiranih proteinov, ki so jim dodeljene v bazi podatkov UniProt.

- Na subproteomu kvasovke, ki smo jo izpostavili frakciji EL70 pri koncentraciji 1 % (v/v) (≈0,01 g GAE/L) v celični suspenziji, smo s proteomsko analizo identificirali 18 diferencialno izraženih proteinov glede na kontrolo. Pokazali smo znižano raven gliceraldehid-3-fosfat-dehidrogenaze (Tdh1), aktina (Act1), cistein-glicinske metalopeptidaze DUG1 (Dug1), fruktoza-bisfosfat-aldolaze (Fba1), glukoza-6-fosfat-isomeraze (Pgi1), Ran GTPaze GSP/CNR1 (Gsp1) in piruvat-dekarboksilaze izoencima 1 (Pdc1) ter povišano raven aktinu sorodnega proteina 2 (Arp2), podenote β mitohondrijske sukcinil-CoA-ligaze (Lsc2), NADPH-dehidrogenaze 2 (Oye2), podenote 2 kompleksa III (Qcr2), podenote β komponente E_1 mitohondrijske piruvat-dehidrogenaze (Pdb1), fosfomanomutaze (Sec53), podenote β mitohondrijske ATP-sintaze (Atp2) in proteina toplotnega šoka HSP26 (Hsp26).

- Na podlagi pregleda literature smo diferencialno izražene proteine povezali s procesi, kot so antioksidativna obramba, metabolizem ogljikovih hidratov in energijski metabolizem (glikoliza, metabolizem piruvata, citratni ciklus, elektronska transportna veriga), dinamika aktinskih filamentov in zvijanje proteinov.

- Rezultati so pokazali, da etanolni izvleček propolisa in frakcija EL70 pri koncentraciji 1 % (v/v) (≈0,02 in ≈0,01 g GAE/L) v celični suspenziji povečata sirtuinsko aktivnost v kvasnih celicah, in sicer v citosolni subproteomski frakciji (etanolni izvleček propolisa za 71 %; frakcija EL70 za 61 %) in jedrni subproteomski frakciji (etanolni izvleček propolisa za 34 %; frakcija EL70 za 64 %).

5 POVZETEK (SUMMARY)

5.1 POVZETEK

Propolis je čebelji produkt, ki ima številne biološke aktivnosti, kot so protivirusna, protimikrobna, protiparazitska, antioksidativna, imunomodulatorna, protirakava in protivnetna. Uporablja se v zdravstvene, kozmetične in prehranske namene, kljub temu, da njegove bioaktivne spojine in molekularni mehanizmi delovanja v celicah niso natančno poznani. V doktorski nalogi smo na modelnemu organizmu kvasovki *S. cerevisiae* analizirali vpliv izvlečka propolisa na celični in proteomski ravni. Identificirali smo bioaktivne fenolne spojine in s pomočjo najdenih sprememb na subproteomu razložili njihov molekularni mehanizem delovanja.

Z ekstrakcijo s trdno fazo (SPE) smo pridobili 5 frakcij izvlečka propolisa, ki smo jih poimenovali glede na vol. % etanola, s katerim smo jih zaporedno eluirali s kolone: EL30-EL70. Frakcijam smo s HPLC-DAD določili fenolne profile, s Folin-Ciocalteaujevo metodo skupno vsebnost fenolnih spojin in z DPPH testom *in vitro* antioksidativno učinkovitost. Pokazali smo, da imajo frakcije različne kvantitativne in kvalitativne profile fenolnih spojin in da obstaja korelacija med vsebnostjo skupnih fenolnih spojin in sposobnostjo lovljenja prostih radikalov $DPPH^{\bullet}$.

V kvasovki je izvleček propolisa pri koncentraciji 1 % (v/v) (≈0,02 g GAE/L) v celični suspenziji po 2-urah izpostavitve zmanjšal znotrajcelično oksidacijo (61 %) in povečal celično metabolno energijo (19 %), medtem ko so frakcije različno vplivale na ta dva parametra. Frakciji EL30 in EL40 sta povečali znotrajcelično oksidacijo za 18 % in 11 % in zmanjšali celično metabolno energijo za 7 % in 5 %. Frakcija EL50 je zmanjšala znotrajcelično oksidacijo za 16 % in celično metabolno energijo za 12%. Frakciji EL60 in EL70 sta zmanjšali znotrajcelično oksidacijo za 42 % in 66 % in povečali celično metabolno energijo za 5 % in 21 %. Iz tega sledi, da frakcije, ki imajo sposobnost lovljenja prostih radikalov, določeno z DPPH testom, lahko tudi povečajo raven ROS v kvasovki. Na celično živost tako izvleček propolisa kot njegove frakcije pri uporabljenih koncentracijah niso imeli večjega učinka.

V izbrani frakciji EL70, ki izmed vseh frakcij najbolj zniža raven ROS in najbolj poviša raven ATP v kvasovki, smo z LC-MS/MS identificirali estre hidroksicimetnih kislin (CAIE, CABE, CAPE, CACE, benzilni ester *p*-kumarne kisline), prenilirano hidroksicimetno kislino (3-prenil-4-(2-metilpropionil-oksi)-cimetna kislina) in flavonoide (krizin, pinocembrin, apigenin, kamferid, ramnetin, pinobanksin-5-metil-eter, pinobanksin-3-*O*-acetat, pinobanksin-3-*O*-propionat, pinobanksin-3-*O*-butirat, pinobanksin-3-*O*-pentanoat, luteolin-metil-eter). Dodatno smo s HPLC-MS/MS za CABE, CAPE in CACE pokazali celični privzem v kvasovko. Poleg tega CAPE zniža znotrajcelično oksidacijo (10 μM in 100 μM) in poviša celično metabolno energijo pri izbrani koncentraciji 100 μM.

Nadaljnje smo vpliv frakcije EL70 analizirali na subproteomski ravni kvasovke *S. cerevisiae*, tako da smo za delo s kvasovko prilagodili protokol za diferencialno detergentno frakcionacijo (DDF), ki je bil prvotno namenjen za ekstrakcijo proteinov iz sesalskih celic. Z dvodimenzionalno elektroforezo (2-DE) smo poiskali diferencialno izražene proteine, ki smo jih identificirali z MALDI-TOF/TOF analizo. Po 2-urni izpostavitvi celic kvasovk frakciji EL70 pri koncentraciji 1 % (v/v) (≈0,01 g GAE/L) v celični suspenziji so se spremembe v ravni pokazale v proteinih vpletenih v metabolizem ogljikovih hidratov in energetski metabolizem (Tdh1, Fba1, Pgi1, Pdb1, Pdc1, Lsc2, Qcr2, Atp2), dinamiko aktinskih filamentov (Act1, Arp2, Fba1, Hsp26), odziv na oksidativni stres (Dug1, Oye2), zvijanje proteinov (Hsp26, Sec53) in ostale procese (Sec53, Gsp1). Tako smo preko proteinskih tarč lahko sklepali na molekularne mehanizme delovanja bioaktivnih spojin propolisa.

Nekatere spremembe na celični ravni in v proteinskem profilu kvasnih celic, ki smo jih pokazali po 2-urni izpostavitvi celic kvasovk frakciji EL70 pri koncentraciji 1 % (v/v), lahko razložimo s povečano sirtuinsko aktivnostjo, ki smo jo pokazali v citosolni in jedrni proteinski frakciji v EL70-tretiranih celicah. Prav tako je bila deacetilazna aktivnost sirtuinov povečana v citosolni in jedrni proteinski frakciji, ko smo celice kvasovk izpostavili celotnemu izvlečku propolisa.

Naše analize vpliva izvlečka propolisa na celični in proteomski ravni modelnega organizma kvasovke *S. cerevisiae* so pripomogle k boljšemu razumevanju nekaterih bioloških aktivnosti propolisa. Identificirali smo bioaktivne spojine propolisa (estri kavne kisline - CAPE, CABE, CACE), ki so najverjetneje odgovorne za aktivnosti, ki smo jih pokazali tako na celični kot tudi na proteomski ravni. Ker je kvasovka *S. cerevisae* primeren modelni organizem za študij osnovnih metabolnih poti in celičnih procesov v evkariontskih celicah, lahko izsledke naših raziskav preslikamo tudi na višje organizme.

5.2 SUMMARY

Propolis is a bee product with several biological activities, such as antiviral, antimicrobial, antiparasitic, antioxidative, immunomodulatory, anticancer and antiinflammatory. Propolis is used in medicinal, cosmetic and nutritional purposes, despite the fact that its bioactive compounds and the molecular mechanisms of action in the cells are not well known. In the doctoral dissertation we analyzed the influence of propolis extract on a model organism yeast *S. cerevisiae* at the cellular and at the proteome level. We identified bioactive phenolic compounds and molecular mechanisms of actions based on the changes found at the subproteome.

From propolis extract we obtained 5 fractions using solid phase extraction (SPE), which were named after the vol. % of ethanol that was used for the sequential elution of the fractions from the cartridge: EL30-EL70. Next, we determined the phenolic profiles of the

fractions using HPLC-DAD, total phenolic content using Folin-Ciocalteau method and *in vitro* antioxidative activity using DPPH test. We showed that fractions have different quantitative and qualitative profiles of phenolic compounds and that there is no correlation between total phenolic content and free radical $DPPH^{\bullet}$ scavenger capacity.

Propolis extract has reduced intracellular oxidation (61 %) and increased cellular metabolic energy (19 %) in yeast at a concentration of 1 % (v/v) (≈0.02 g GAE/L) in cell suspension after 2-hour of exposure. On the other hand, fraction had different effect on these two parameters. Fractions EL30 and EL40 increased intracellular oxidation for 18 % and 11 %, respectively, and also reduced cellular metabolic energy for 7 % and 5 %. Fraction EL50 reduced intracellular oxidation for 16 % and cellular metabolic energy for 12 %. Fractions EL60 and EL70 decreased intracellular oxidation for 42 % and 66 %, respectively, and also increased cellular metabolic energy for 5 % and 21 %. Therefore, fractions that possess free radical scavenger capacity, determined by DPPH test, can also increase ROS level in yeast. On cell viability propolis extract and its fractions had no significant effect at selected concentrations.

In selected fraction EL70 that in yeast decreased ROS level the most and increased ATP level the most among fractions, we identified the following phenolic compounds using HPLC-MS/MS: hydroxycinnamic acid esters (CAIE, CABE, CAPE, CACE, *p*-coumaric acid benzyl ester), prenylated hydroxycinnamic acid (3-prenyl-4-(2-methylpropionyl-oxy)-cinnamic acid) and flavonoids (chrysin, pinocembrin, apigenin, kaempferide, rhamnetin, pinobanksin-5-methyl-ether, pinobanksin-3-*O*-acetate, pinobanksin-3-*O*-propionate, pinobanksin-3-*O*-butyrate, pinobanksin-3-*O*-pentanoate, luteolin-methyl-ether). Using HPLC-MS/MS we also showed that CABE, CAPE and CACE entered the yeast cell. Additionally, CAPE decreases intracellular oxidation (10 μM in 100 μM) and increases cellular metabolic energy at selected concentration of 100 μM.

Furthermore, the effect of the fraction EL70 was analyzed at the subproteome level of yeast *S. cerevisiae* by modification of a protocol for differential detergent fractionation (DDF) that was primarily intended for the extraction of proteins from mammalian cells. Differentially expressed proteins were detected using 2-D electrophoresis and identified using MALDI-TOF/TOF. After 2-hour exposure of yeast to fraction EL70 at a concentration of 1 % (v/v) (≈0.01 g GAE/L) in cell suspension, changes were found in the level of proteins involved in carbohydrate and energy metabolism (Tdh1, Fba1, Pgi1, Pdb1, Pdc1, Lsc2, Qcr2, Atp2), actin filament dynamics (Act1, Arp2, Fba1, Hsp26), oxidative stress response (Dug1, Oye2), protein folding (Hsp26, Sec53) and other cellular processes (Sec53, Gsp1). Based on the identification of protein targets we concluded, which are the molecular mechanisms behind the activity of bioactive propolis compounds.

Certain changes at a cellular level and in the protein profile, that were shown after 2-hour of exposure of yeast cells to fraction EL70 at a concentration of 1 % (v/v), can be

explained with increased sirtuin activity, which was shown in cytosolic and nuclear protein fraction of EL70-treated cells. Additionally, deacetilase activity of sirtuins was increased in cytosolic and nuclear protein fraction of cells treated with propolis extract.

Our analysis of propolis extract influence on cellular and proteomic level of model organism yeast *S. cerevisiae*, contributed to better understanding of some biological activities of propolis. We have identified the bioactive compounds of propolis (caffeic acid esters - CAPE, CABE, and CACE), which are most likely responsible for the activities that were showed on cellular and proteomic level. Also, yeast *S. cerevisiae* is an appropriate model organism for basic metabolic pathways and cellular processes in eukaryotic cells, therefore the findings of our study can also be used for higher organisms.

6 VIRI

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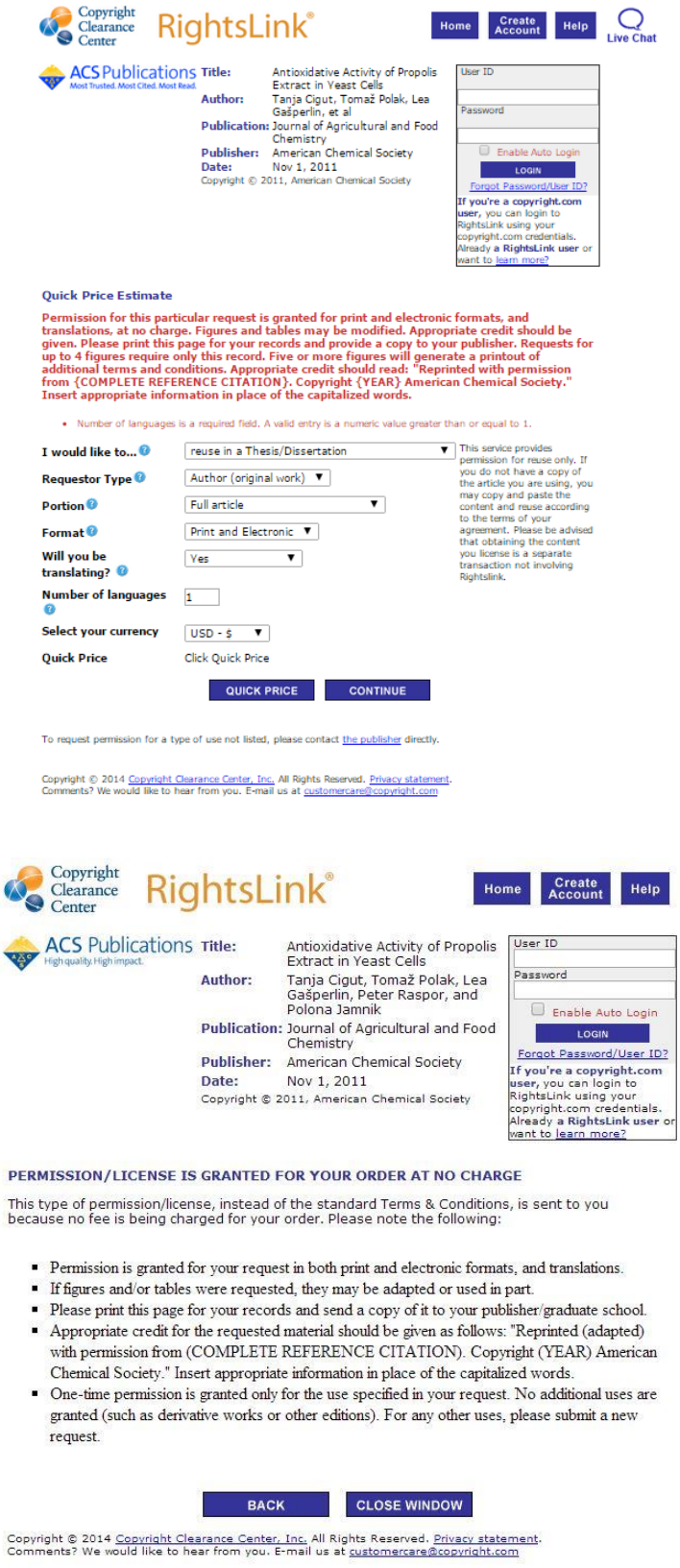

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PLOS ONE

Fractionation of Phenolic Compounds Extracted from Propolis and Their Activity in the Yeast *Saccharomyces cerevisiae*

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Abstract

We have here investigated the activities of Slovenian propolis extracts in the yeast *Saccharomyces cerevisiae*, and identified the phenolic compounds that appear to contribute to these activities. We correlated changes in intracellular oxidation and cellular metabolic energy in these yeasts with the individual fractions of the propolis extracts obtained following solid-phase extraction. The most effective fraction was further investigated according to its phenolic compounds.

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Introduction

Propolis is a resinous substance that is collected from certain plants by bees. The bees use it as a sealer in their hives and to prevent the decomposition of creatures that invade the hive and the bees can kill, but cannot remove. Although the composition of propolis varies, depending on the place and time of its collection [1], in general it contains resins and balsams (50%), waxes (30%), aromatic and essential oils (10%), pollen (5%) and other organic matter (5%) [2,3]. Propolis has a broad spectrum of biological activities, including antioxidative, anti-inflammatory, immunomodulatory, anticancer, antibacterial, antiviral, antifungal and antiparasitic effects [4,5]. Although propolis is a mixture of compounds, its pharmacological activities are reported to arise from its flavonoids and phenolic acids, and their esters [6].

In the present study, the activity of propolis was investigated using stationary phase *Saccharomyces cerevisiae* yeast as the model organism. In this system in our previous study, we showed that propolis decreases intracellular oxidation, with its antioxidative activity in yeast arising from only a part of it [7]. To better understand this antioxidative activity of propolis in yeast, we have here further investigated the activities of the phenolic compounds of propolis. Therefore, the objectives of this study were to: (1) fractionate a crude propolis extract using polarity-based solid-phase extraction; (2) determine any correlations between the total phenolic content of individual propolis fractions and its antioxidant activity *in vitro*/*in vivo*; (3) determine any correlations between the total phenolic content of individual fractions of propolis and the cellular metabolic energy; and (4) identify the phenolic compounds of any fractions that promote changes in intracellular oxidation and cellular metabolic energy in yeast.

Materials and Methods

Ethics Statement

No specific permits were required for the described field studies. The location is not privately-owned or protected in any way. The field studies did not involve endangered or protected species.

Chemicals and Standards

Folin-Ciocalteu's reagent, 96% ethanol, glucose, methanol, formic acid, sodium carbonate and acetonitrile were from Merck. The 2,2-diprenyl-1-picryhydrazyl radical (DPPH), gallic acid and 2′,7′-dichlorodihydrofluorescein diacetate were from Sigma. Yeast extract and peptone were from Biolife. Ammonium formate was from Fluka.

Sample Preparation

Propolis was collected from bee hive in the Savinjska Valley in Slovenia during the autumn of 2010. The propolis (10 g) was extracted with 70% ethanol (100 mL) by mixing for 1 h at room temperature. The crude extract was recovered by centrifugation (3000 *g*, 5 min) and concentrated under vacuum using a rotary evaporator.

Solid-phase Extraction

Solid-phase extraction (SPE) was used to clean the crude propolis extract, whereby it was separated into five elution fractions according to polarity. Crude propolis extract (200 µL) was mixed with 20 mM ammonium formate (200 µL), and then added to a Strata-X (33 u Polymeric Reversed Phase 60 mg/3 mL 8B-S100-UBJ) SPE cartridge (Phenomenex) that had previously been conditioned with methanol (2 mL) and equilibrated with 20 mM ammonium formate, pH 3.2 (2 mL). After the loading of the sample, the cartridge was washed with 20 mM ammonium

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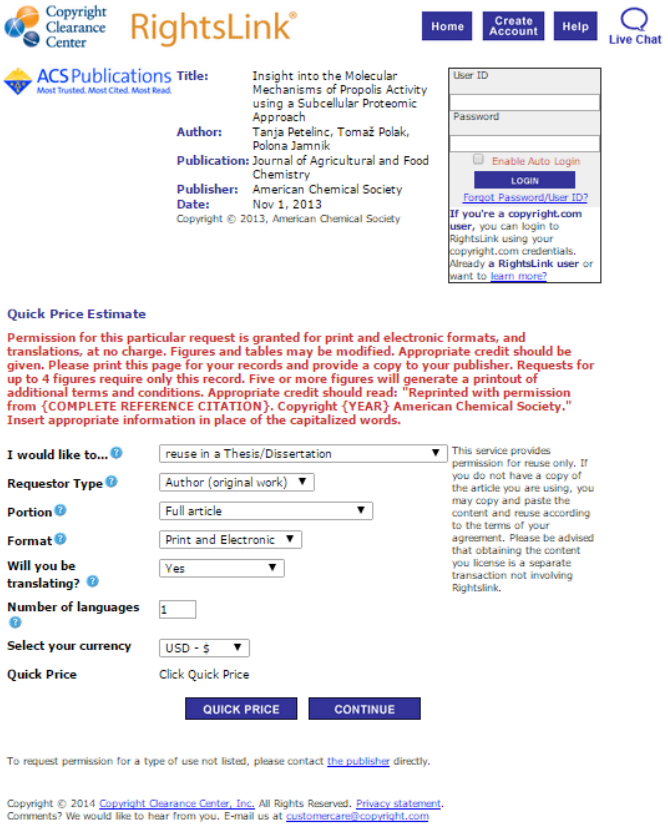

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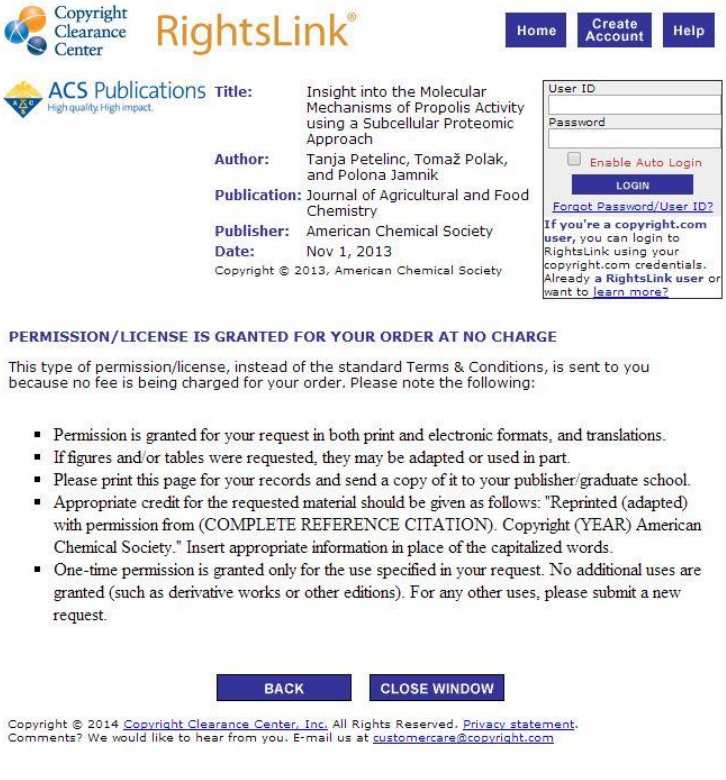

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