CHAPTER III

YEAST SCREENING FOR TOPOISOMERASE I INHIBITORY ACTIVITY

Topoisomerase I is an important therapeutic target in cancer chemotherapy. We are interested in the continuing discovery of new topoisomerase I-targeted agents from Thai medicinal plants since many people in Thailand have used traditional herbs as an alternative treatment for cancer. The selection of plants used in the screening was based on two approaches. The first one was ethnopharmacology approach which led to plants used for cancer treatment. The second one was the Thai medicinal plants previously reported cytotoxicity against any cancer cell lines.

This chapter, we report topoisomerase I inhibitory activity of selected Thai medicinal plants by screening with yeast cell-based assay. These results support previous used on the efficacy of Thai medicinal plants for anticancer.

3.1. Materials and methods

3.1.1 Plant materials for screening

The plant species were collected from various localities and bought from local crude drug stores in Thailand. They were identified by Associate Professor Thatree Phadungcharoen and Associate Professor Dr. Nijsiri Ruangrungsri at the Department of Pharmacognosy and Pharmaceutical Botony, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok. Voucher specimens were deposited at the Herbarium of Natural Medicine, Chulalongkorn University, Bangkok, Thailand (Table 3.1).

Table 3.1 Plant species used for screening of inhibitory activity on topoisomerase I

No.	Plant Species	Thai name	Family	Voucher number	Plant Part	Biological activity
1.	*Artocarpus gomezianus* Wall.	Hat nun	Moraceae	BCH-08001	Bark	Antiviral (Chattopadhyay and Khan, 2008)
2.	*Betula alnoides* Buch.-Ham.	Kamlang seua khrong	Betulaceae	PRD-08001	Bark	Aphrodisiac (Temkitthawon *et al.*, 2008)
3.	*Bridelia ovata* Decne.	Maka	Euphorbiaceae	PRD-08002	Leaf	Anti-cancer (Saetung *et al.*, 2005)
4.	*Cissampelos pareira* L. var. hirsuta	Krung khamao	Menispermaceae	PRD-08003	Root	Anti-cancer (Amresh *et al.*, 2007)
5.	*Curcuma longa* L.	Khamin chan	Zingiberaceae	PRD-08004	Rhizome	Anti-cancer (Pillai *et al.*, 2004; Johnson and Mukhtar, 2007)
6.	*Curcuma zedoaria* (Berg) Roscoe	Khamin oi	ZIngiberaceae	PRD-08005	Rhizome	Anti-cancer (Saetung *et al.*, 2005)
7.	*Derris scandens* (Roxb.) Benth.	Thao wan priang	Leguminosae	PRD-08006	Stem	Anti-cancer (Saetung *et al.*, 2005)
8.	*Drynaria quercifolia* (L.) Sm.	Kratae tai mai	Polypodiaceae	PRD-08007	Stem	Antimicrobial (Ramesh *et al.*, 2001)
9.	*Garcinia hanburyi* Hook f.	Rong thong	Guttiferae	NSR-08001	Secretion	Antibacterial, Anti-cancer (Sukpondma, Rukachaisirikul, and Phongpaichit, 2005; Lu *et al*, 2007)
10.	*Grangea maderaspatana* (L.) Poir.	Phaya mutti	Compositae	PRD-08008	Whole plant	Anagesic (Ahmed, 2001)

Table 3.1 (continued)

No.	Plant Species	Thai name	Family	Voucher number	Plant Part	Biological activity
11.	*Moringa oleifera* Lam.	Marum	Moringaceae	PRD-08009	Bark	Anti-cancer (Guevara *et al.*, 1999)
12.	*Phyllanthus amarus* Schumach. & Thonn.	Luk tai bai	Euphorbiaceae	PRD-08010	Whole plant	Anti-cancer (Rajeshkumar *et al.*, 2002)
13.	*Phyllanthus emblica* L.	Ma kham pom	Euphorbiaceae	PRD-08011	Fruit	Anti-cancer (Subchareon, 1998; Pinmai *et al.*, 2008)
14.	*Phyllanthus urinaria* L.	Ya tai bai	Euphorbiaceae	PRD-08012	Whole plant	Anti-cancer (Huang *et al.*, 2006)
15.	*Rauvolfia serpentina* (L.) Benth.	Rayom	Apocynaceae	PRD-08013	Root	Anti-cancer (Dassonneville *et al.*, 1999)
16.	*Rhinacanthus nasutus* (L.) Kurz	Thong phan chang	Acanthaceae	PRD-08014	Leaf	Anti-cancer (Saetung *et al.*, 2005)
17.	*Rhinacanthus nasutus* (L.) Kurz	Thong phan chang	Acanthaceae	PRD-08015	Root	Anti-cancer (Saetung *et al.*, 2005)
18.	*Sapindus rarak* DC.	Ma kham di khwai	Sapindaceae	PRD-08016	Fruit	Antimicrobial (Wuthi-udomlert, Luanratana, and Suriyawong, 2002)
19.	*Schleichera oleosa* (Lour.) Oken	Ta khro	Sapindaceae	BCH-08002	Root	Anti-cancer (Pettit *et al.*, 2000)
20.	*Stephania erecta* Craib	Bua bok	Menispermaceae	TTP-08001	Caudex	Anti-cancer, Antiplasmodial (Angerhofer *et al*, 1999)
21.	*Stephania pierrei* Diels	Sabu lueat	Menispermaceae	TTP-08002	Caudex	Anti-cancer, Antiplasmodial (Angerhofer *et al*, 1999)

Table 3.1 (continued)

No.	Plant Species	Thai name	Family	Voucher number	Plant Part	Biological activity
22.	*Stephania suberosa* Forman	Bora phet phung chang	Menispermaceae	TTP-08003	Caudex	Anti-cancer (Makarasen, 2004)
23.	*Terminalia bellirica* (Gaertn.) Roxb.	Samo phi pek	Combretaceae	PRD-08018	Fruit	Anti-cancer (Subchareon, 1998; Pinmai *et al.*, 2008)
24.	*Terminalia chebula* Retz. var. chebula	Samo thai	Combretaceae	PRD-08017	Fruit	Anti-cancer (Subchareon, 1998; Saleem *et al.*, 2002)
25.	*Tinospora baenzigeri* Forman	Chingcha chali	Menispermaceae	PRD-08019	Stem	Antipyretic (Salguero, 2003)
26.	*Usnea siamensis* Vain.	Foi lom	Usneaceae	NSR-08002	Whole plant	Hepatotoxic agent (Pramyothin *et al*, 2004)
27.	*Ziziphus attopoensis* Pierre	Kamlang suea khrong	Rhamnaceae	PRD-08020	Branch	Anti-proliferation (Numchaisermsuk and Cherdshewa sart, 2008)

3.1.2 Extraction methods

Plant materials were cut into small pieces and dried in a hot air oven at 55°C. Dried plant material was ground to a coarse powder and stored at an ambient temperature prior to extraction. The powdered plant material (50 g) was macerated with 95% ethanol (250 ml) for 3 days and filtered. The filtrate was evaporated under reduced pressure until dryness.

3.1.3 Yeast strain and growth

3.1.3.1 Construction of yeast

The *Saccharomyces cerevisiae* strain RS190 (ATCC 208354, *MATa*, *top1*Δ), genotype a *top1-8 [top1::LEU2] ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100*, was purchased from American Type Culture Collection. Genes of *Arabidopsis thaliana topoisomerase I.* was cloned into yeast. The gene of *A. thaliana topoisomerase I* was cloned by the Gateway Cloning Technology (Invitrogen), and the integrity of the constructs was verified by DNA sequencing. The Gateway™ expression vector pYES-DEST52 was used for expression in *S. cerevisiae*. This plasmid also contains *URA3*, selectable markers for the selection and maintenance of plasmid bone sequences in auxotrophic yeast strain. Furthermore this plasmid has strong inducible promoter (*pGAL1*) enable the study of topoisomerase I enzyme function.

3.1.3.2 Media for yeast cell culture

Growth media (YPD media) (Difco™)

YPD Agar and YPD Broth are used for maintaining and propagating yeasts in molecular microbiology procedures. The compositions of this culture media were described in Appendix A.

Synthetic complete media lacking uracil (S.C. ura$^-$ media)

S.C. ura$^-$ media was used for selecting and maintaining transformant yeast. The compositions of this culture media were described in Appendix A.

3.1.3.3 Storage of yeast cultures

Yeast cultures can be stored at -70°C in growth media containing 15% (v/v) glycerol. Yeast cell can be recovered from storage by transferring a small frozen sample to a YPD agar plate.

For storage of the transformant yeast strain, they were grown overnight at 30°C in YPD broth media. The 0.5 ml of cultures was mixed with 0.5 ml of sterile 30% (v/v) glycerol in 2ml vial and transferred to -70°C.

3.1.3.4 Yeast plate count

Yeast is quantitated by the fractional pour plate technique (modified from Fankhauser, 2005). Media can be rendered selective for transformant yeast by using synthetic complete media lacking amino acid uracil.

The transformant yeasts were grown 18 hr at 30°C in a shaking incubator set at 200 rpm in liquid S.C. ura$^-$ media containing glucose. The cultures were adjusted to an OD_{600} of 0.3 and serially diluted ten-fold. A hundred microliter of each dilutions was mixed with 15 ml of the S.C. ura$^-$ agar media (triplicate) before pouring plates. Culture plates were incubated at 30°C for 48 hrs. The yeast survival was counted in plates with no more than 300 colony forming units (CFU) and preferably not less than 30 CFUs. The counts of the triplicate plates were averaged. The number of yeast suspension at OD_{600} of 0.3 was calculated by average number of colony forming unit multiply with dilution factor.

3.1.4 Yeast cell-based assay for topoisomerase I inhibitory activity

Topoisomerase I inhibitory activities of the crude extracts were screened for their inhibitory growing culture by spotted transformant yeast. Crude extracts were prepared by solubilized in dimethylsulfoxide (DMSO), sonicated, and filtered sterilized using 0.25 µm millipored filter. For primary screening, the plant extractions were further diluted in S.C. ura$^-$ media to produce 3 concentrations of 125, 250, and 500 µg/ml of each extract and secondary screening the plant extract concentrations were 25, 50, 100, and 200 µg/ml. The medium were prepared under two different conditions for carbon source; glucose or

galactose. Positive controls were 2.5, 5, and 10 µg/ml camptothecin, and vehicle controls were 125, 250, and 500 µg/ml DMSO alone.

The transformant yeasts were grown 18 hr at 30°C in a shaking incubator set at 200 rpm in liquid S.C. ura$^-$ media containing glucose. The cultures were adjusted to and OD_{600} of 0.3 and serially diluted 10-fold and 5 µl aliquots spotted onto selective plates supplemented with 2% glucose or galactose and individual test extract. Incubated the culture plates at 30°C for 48 hr, and cell viability of yeast in the presence of plant extracts was observed. The yeast survival was determined by comparison of the viability of colonies in the vehicle control culture (DMSO plate) with those in the positive control culture (CPT plate) on glucose or galactose agar medium.

3.2 Results

3.2.1 Counting of the yeast cell

Yeast cell suspension was counted on S.C. ura$^-$ plates. The number of yeast colony forming unit per plate showed in Table A1 (Appendix A). The OD_{600} of 0.3 of yeast suspension were 5.86×10^6 CFU/ml.

3.2.2 Topoisomerase I inhibitory activity of Thai medicinal plants

The ethanolic extract of 27 Thai medicinal plants were assayed for topoisomerase I-targeted drugs by recombinant yeast expressing *Arabidopsis thaliana topoisomerase I* gene. The growth of transformant yeast on the agar plate contain individual compound can be used for demonstrating topoisomerase I inhibitory activity of the compound. In the repressed condition (glucose-containing medium), CPT, anti-cancer agent did not affect the growth of strain RS190 because topoisomerase I, the target of CPT, is absent in this strain. In contrast, overexpression of topoisomerase I by induction with galactose in the presence of CPT caused cell death because CPT block the correct reaction of topoisomerase I by stabilizing a covalent complex of nicked DNA and topoisomerase I leading to a DNA lesion (Reid *et al.*, 1998). On the other hand, selective medium contain vehicle control (DMSO), yeast cells can growth on induced condition just like repressed condition. The growing of recombinant yeasts was shown in Figure 3.1.

As a result, the growth of transformant yeasts on the agar plates contain individual crude extract can demonstrated topoisomerase I inhibitor activities as shown in Figure 3.2-3.28.

By this assay, 6 out of 27 extracts from Thai medicinal plants have topoisomerase I inhibitor activities. The transformant yeast model results we obtained indicated that ethanolic extract of rhizome of *Curcuma longa*, rhizome of *Curcuma zedoaria*, whole plant of *Grangea maderaspatana*, root and leaves of *Rhinacanthus nasutus*, and caudex of *Stephania suberosa* extracts demonstrated topoisomerase I inhibitor activity overall.

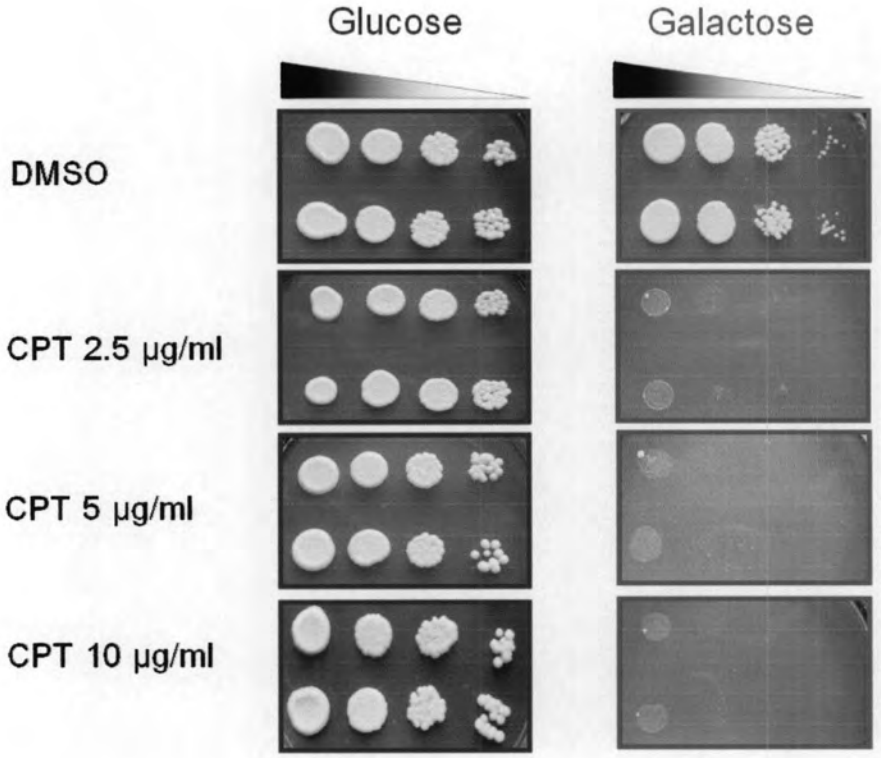


Figure 3.1 Spot test assay of control experiments with exponentially growing of transformant yeast expressing topoisomerase I. Five microliters of ten-fold serial dilutions were spotted on agar plates containing DMSO (vehicle control) and various concentrations of CPT (positive control) and incubated at 30°C for 48 hr. The expression of exogenous topoisomerase I was repressed by glucose (left panel) and induced by galactose (right panel).

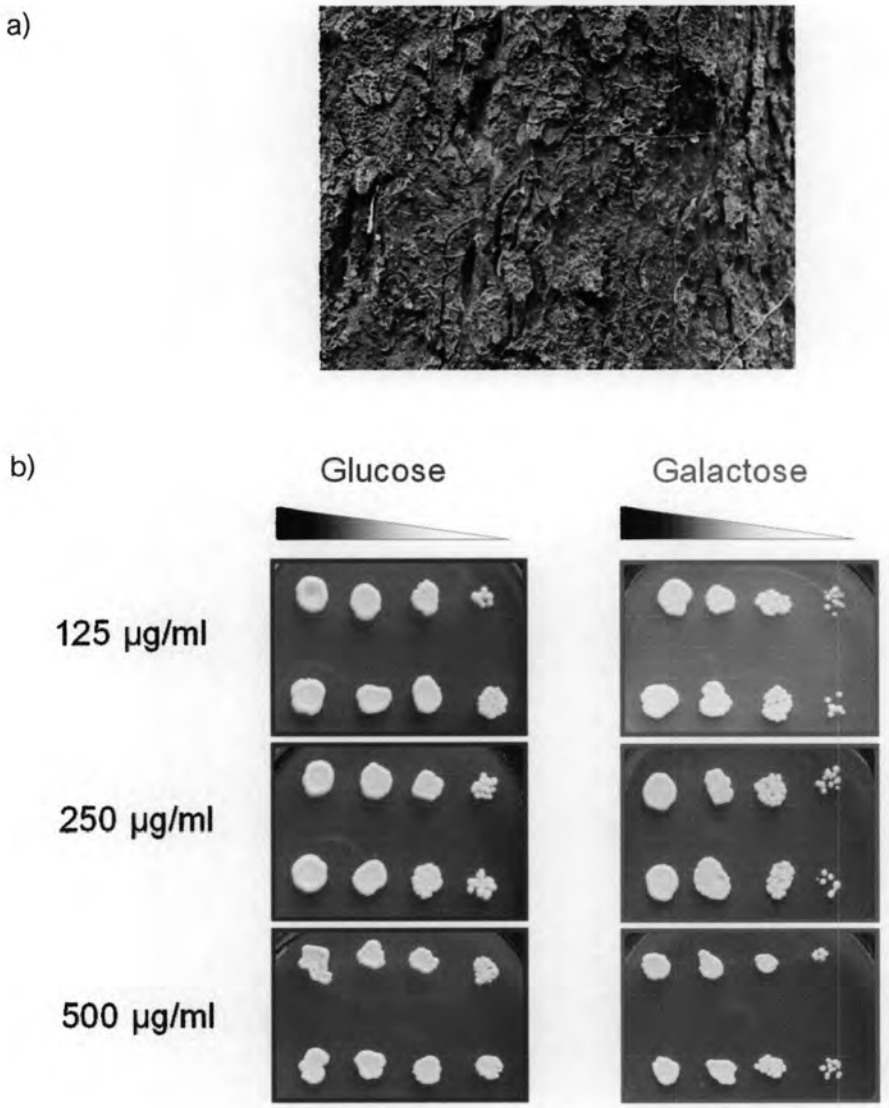


Figure 3.2 Yeast cell-based assay of *Artocarpus gomezianus* barks. a) Barks of *A. gomezianus* (http://www.biotik.org/india/species/a/artogoze/artogoze 02 en.html) b) Spot test assay of extracts from *A. gomezianus* bark with exponentially growing of transformant yeast expressing topoisomerase I. Five microliters of ten-fold serial dilutions were spotted on plates containing various concentrations of extract from bark of *A. gomezianus* and incubated at 30°C for 48 hr. The expression of exogenous topoisomerase I was repressed by glucose (left panels) and induced by galactose (right panels).

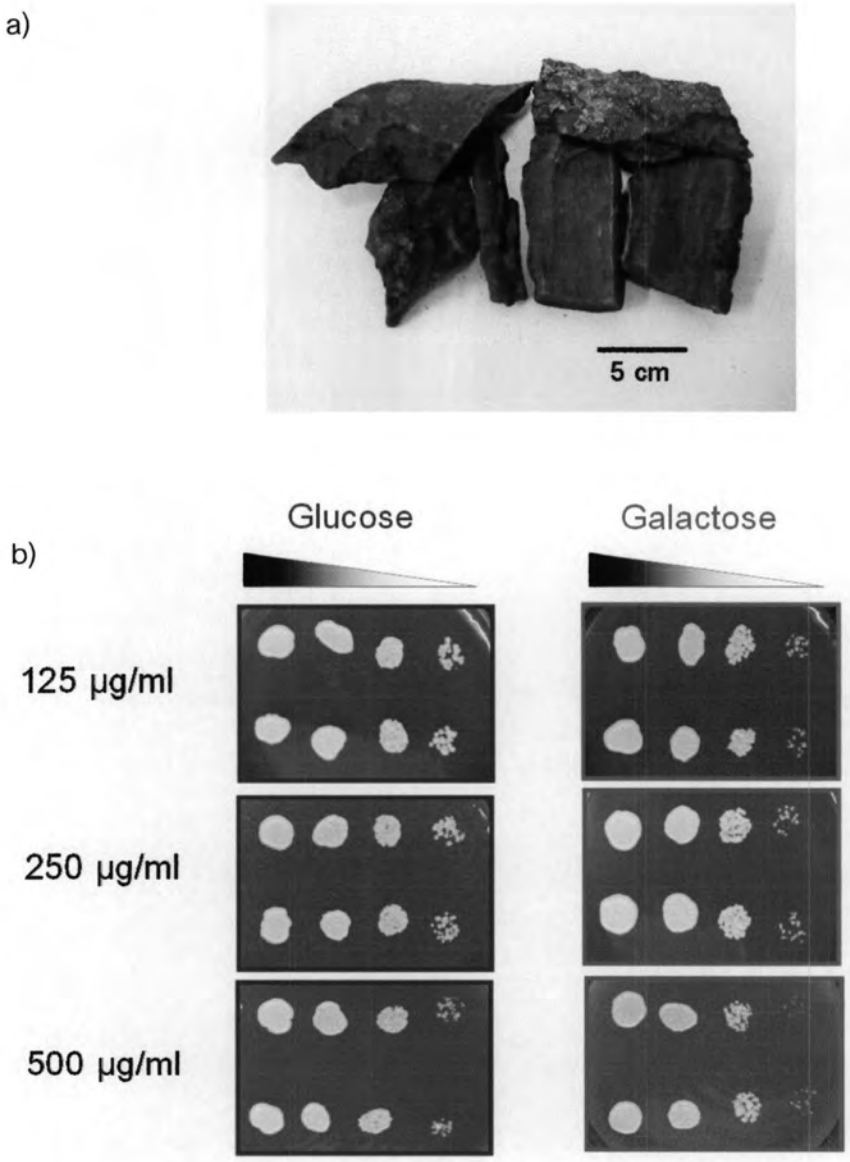


Figure 3.3 Yeast cell-based assay of *Betula alnoides* bark. a) Barks of *B. alnoides* b) Spot test assay of extracts from *B. alnoides* bark with exponentially growing of transformant yeast expressing topoisomerase I. Five microliters of ten-fold serial dilutions were spotted on plates containing various concentrations of extract from bark of *B. alnoides* and incubated at 30°C for 48 hr. The expression of exogenous topoisomerase I was repressed by glucose (left panels) and induced by galactose (right panels).

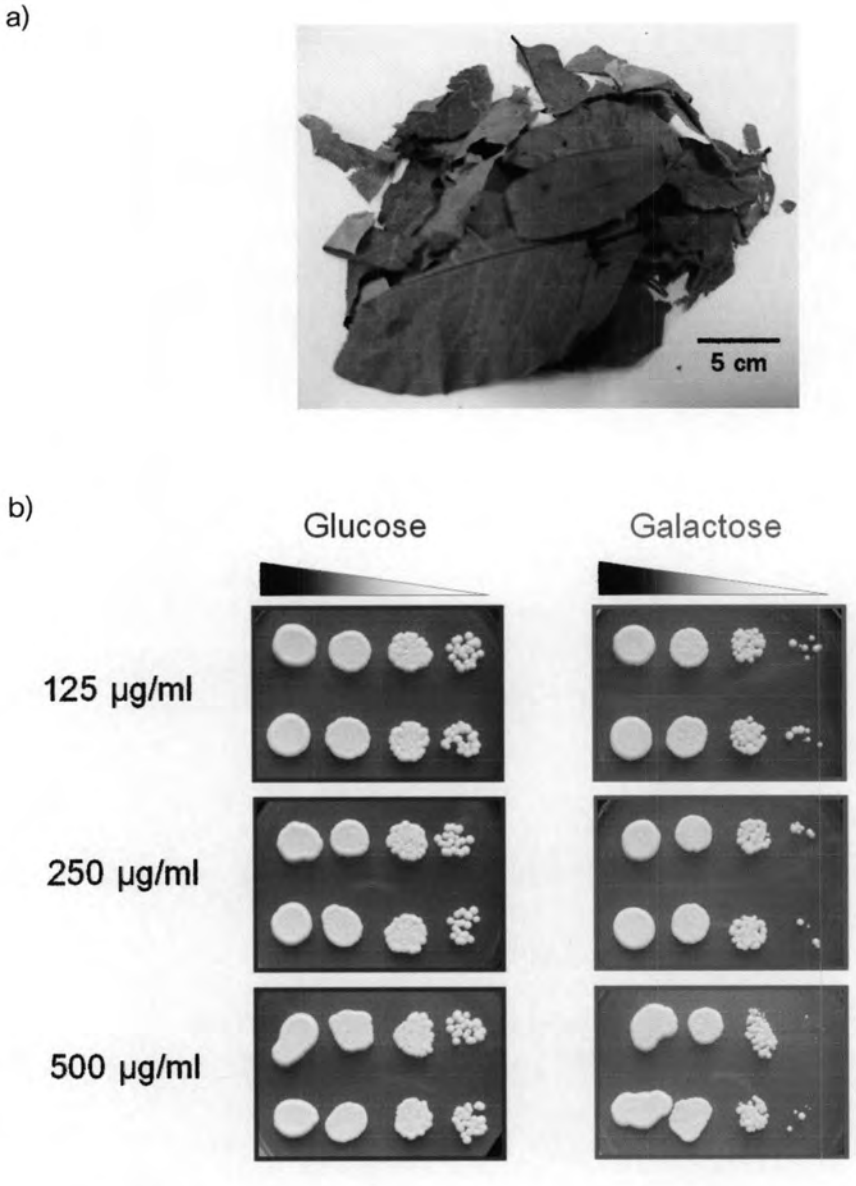


Figure 3.4 Yeast cell-based assay of *Bridelia ovata* leaves. a) Leaves of *B. ovata* b) Spot test assay of extracts from *B. ovata* leaves with exponentially growing of transformant yeast expressing topoisomerase I. Five microliters of ten-fold serial dilutions were spotted on plates containing various concentrations of extract from leaves of *B. ovata* and incubated at 30°C for 48 hr. The expression of exogenous topoisomerase I was repressed by glucose (left panels) and induced by galactose (right panels).

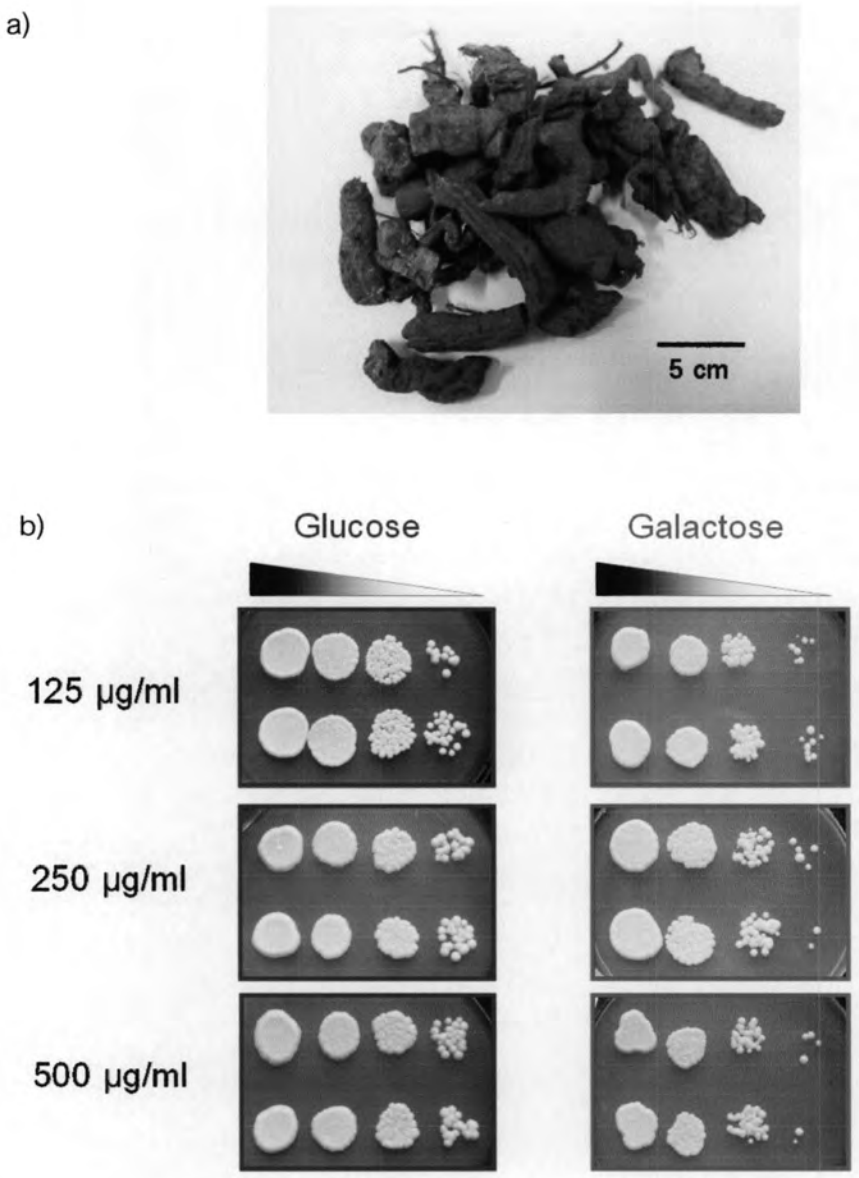
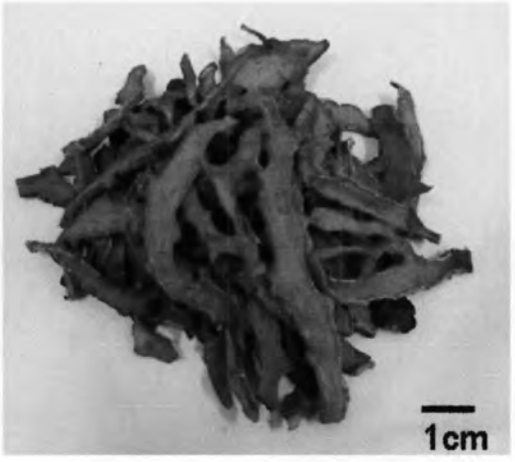


Figure 3.5 Yeast cell-based assay of *Cissampelos pareira* roots. a) Roots of *C. pareira* b) Spot test assay of extracts from *C. pareire* roots with exponentially growing of transformant yeast expressing topoisomerase I. Five microliters of ten-fold serial dilutions were spotted on plates containing various concentrations of extract from roots of *C. pareire* and incubated at 30°C for 48 hr. The expression of exogenous topoisomerase I was repressed by glucose (left panels) and induced by galactose (right panels).

a)



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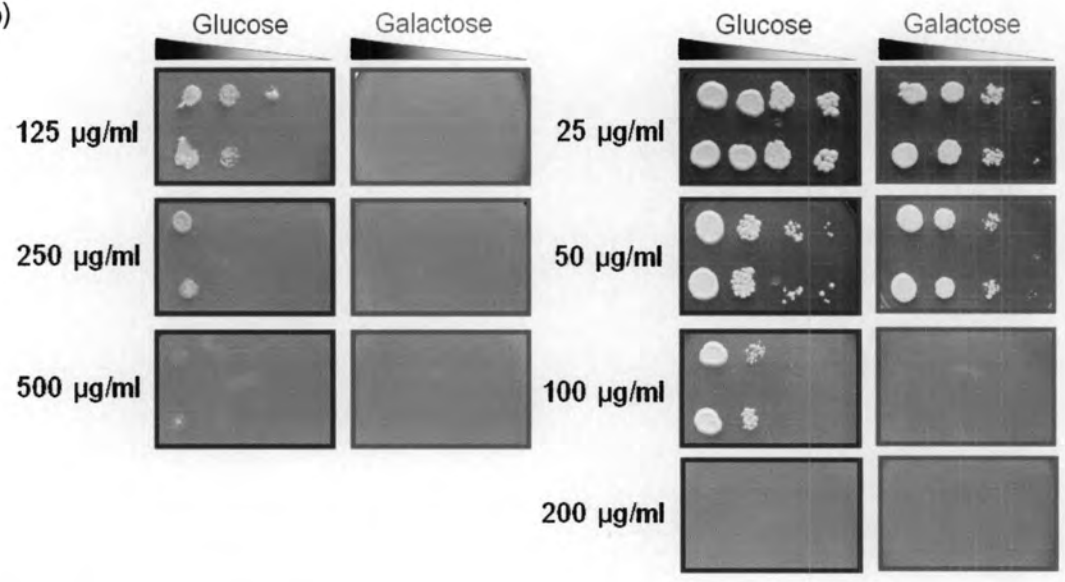

Figure 3.6 Yeast cell-based assay of *Curcuma longa* rhizomes. a) Rhizomes of *C. longa* b) Spot test assay of extracts from *C. longa* rhizomes with exponentially growing of transformant yeast expressing topoisomerase I. Five microliters of ten-fold serial dilutions were spotted on plates containing various concentrations of extract from rhizomes of *C. longa* and incubated at 30°C for 48 hr. The expression of exogenous topoisomerase I was repressed by glucose (left panels) and induced by galactose (right panels).

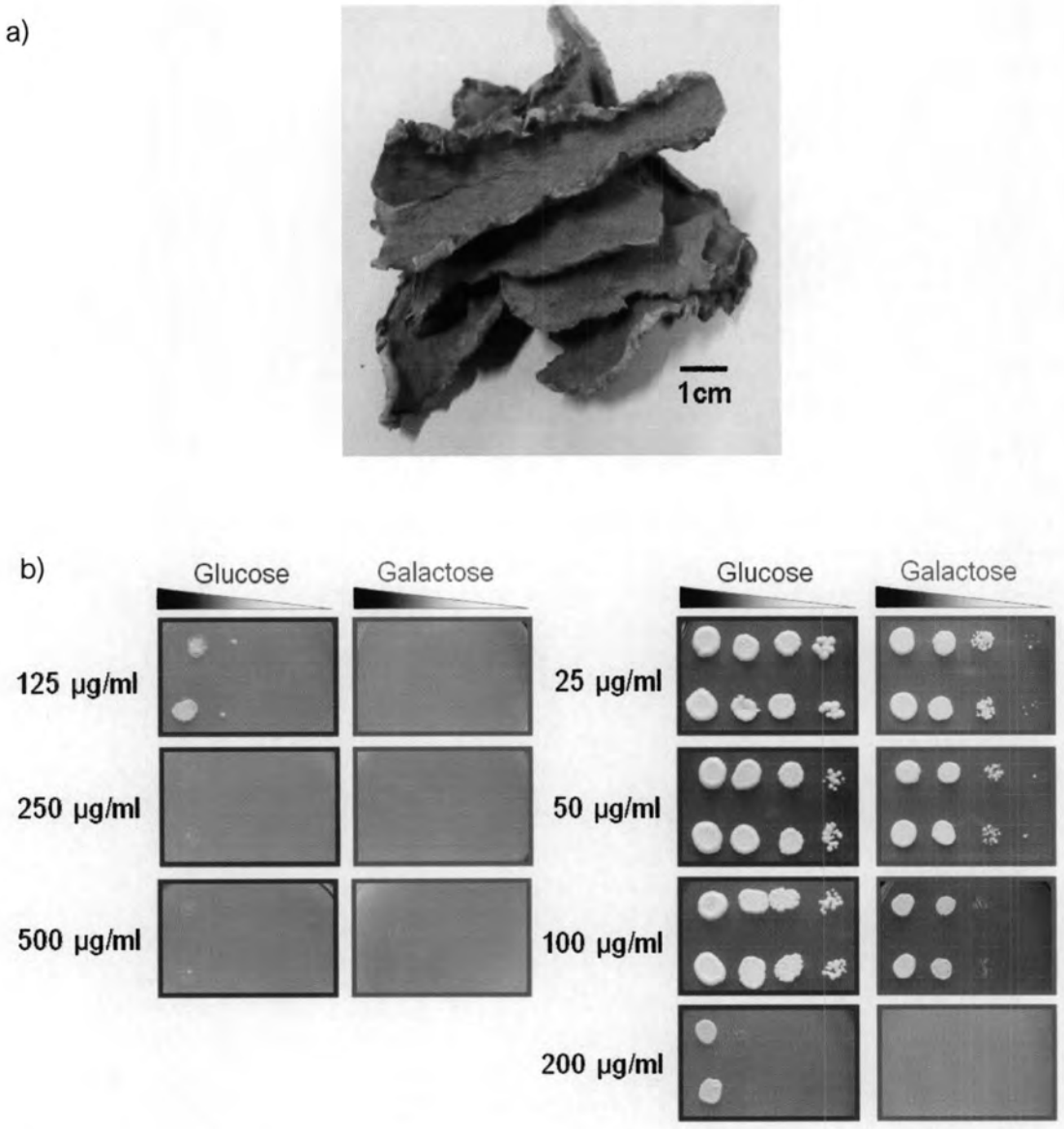

Figure 3.7 Yeast cell-based assay of *Curcuma zedoaria* rhizomes. a) Rhizomes of *C. zedoaria* b) Spot test assay of extracts from *C. zedoaria* rhizomes with exponentially growing of transformant yeast expressing topoisomerase I. Five microliters of ten-fold serial dilutions were spotted on plates containing various concentrations of extract from rhizomes of *C. zedoaria* and incubated at 30°C for 48 hr. The expression of exogenous topoisomerase I was repressed by glucose (left panels) and induced by galactose (right panels).

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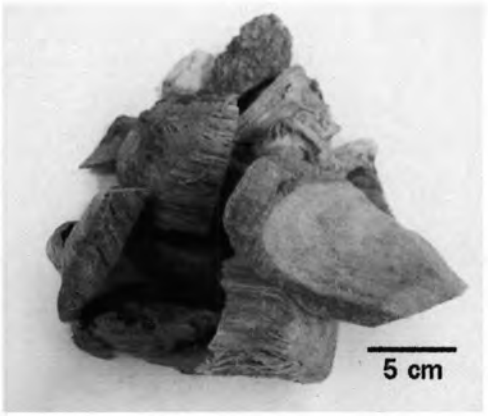

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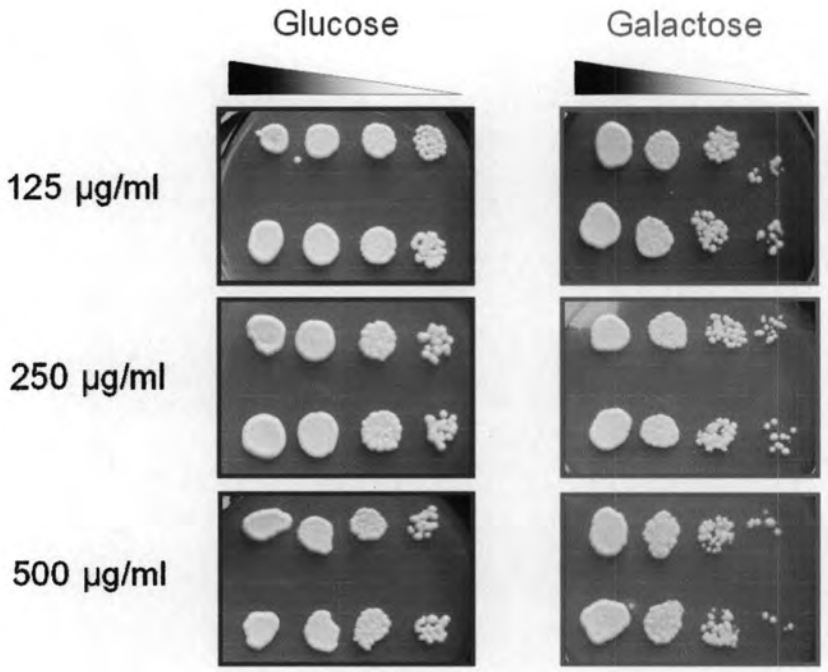

Figure 3.8 Yeast cell-based assay of *Derris scandens* stems. a) Stems of *D. scandens* b) Spot test assay of extracts from *D. scandens* stems with exponentially growing of transformant yeast expressing topoisomerase I. Five microliters of ten-fold serial dilutions were spotted on plates containing various concentrations of extract from stems of *D. scandens* and incubated at 30°C for 48 hr. The expression of exogenous topoisomerase I was repressed by glucose (left panels) and induced by galactose (right panels).

a)

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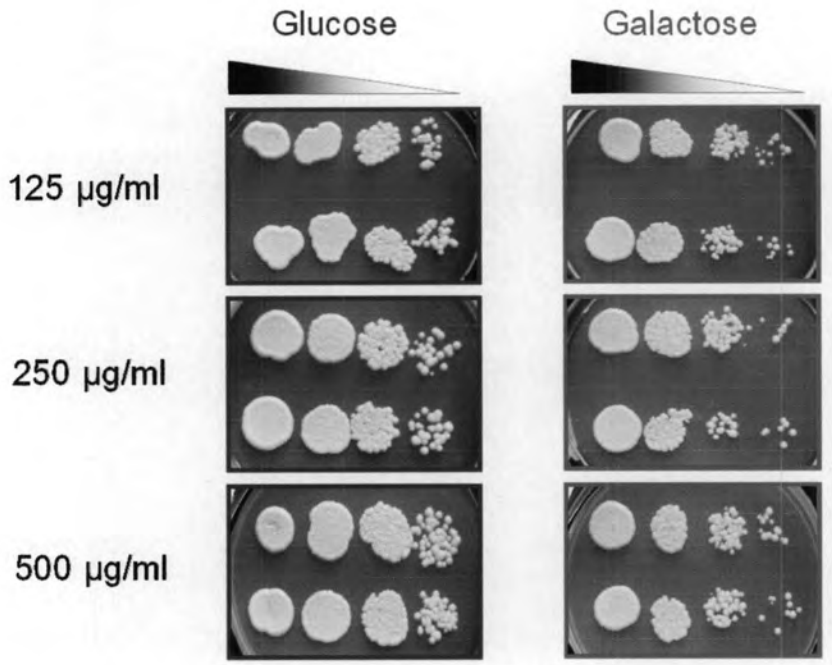

Figure 3.9 Yeast cell-based assay of *Drynaria quercifolia* stems. a) Stems of *D. quercifolia* b) Spot test assay of extracts from *D. quercifolia* stems with exponentially growing of transformant yeast expressing topoisomerase I. Five microliters of ten-fold serial dilutions were spotted on plates containing various concentrations of extract from stems of *D. quercifolia* and incubated at 30°C for 48 hr. The expression of exogenous topoisomerase I was repressed by glucose (left panels) and induced by galactose (right panels).

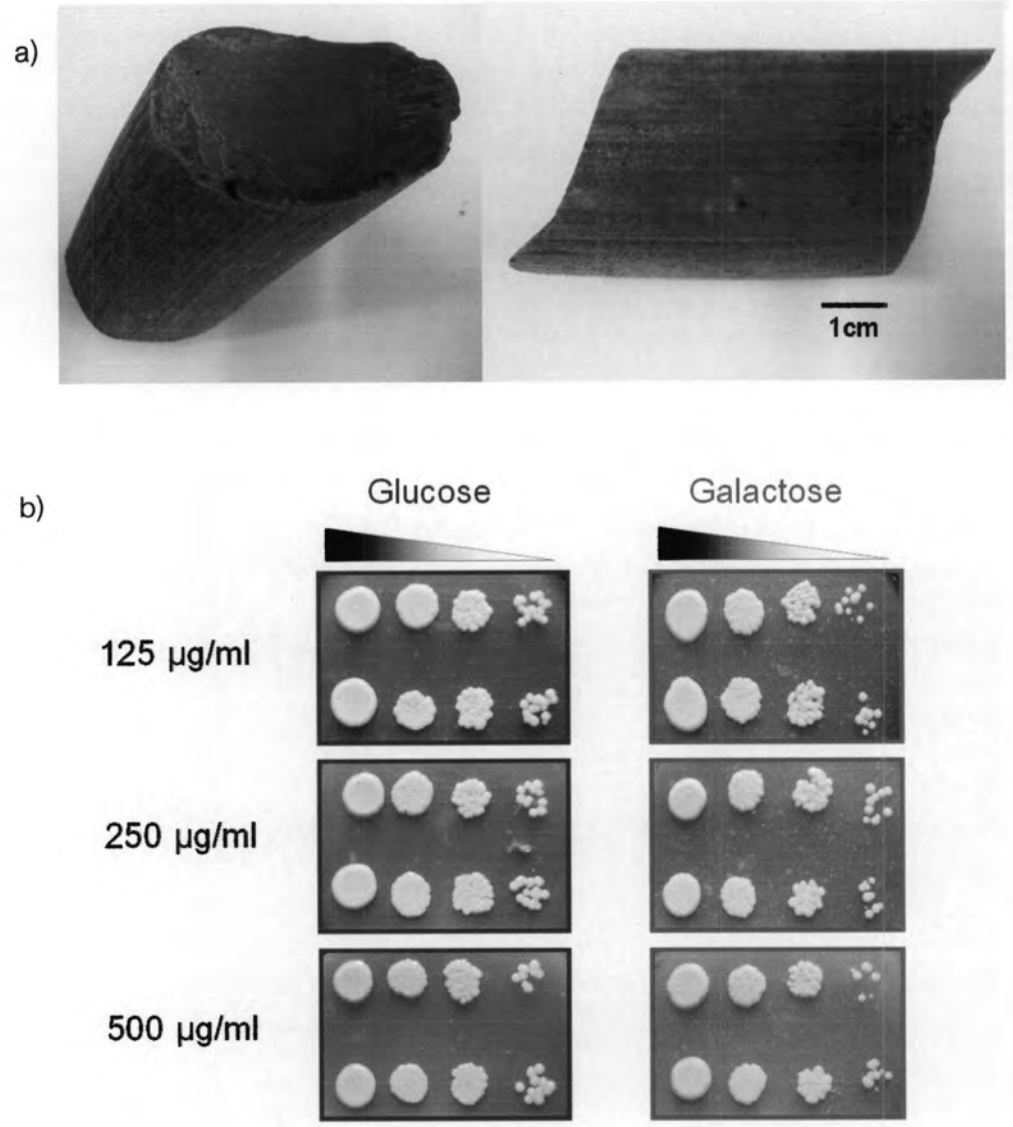

Figure 3.10 Yeast cell-based assay of *Garcinia hanburyi* secretion. a) Secretions of *G. hanburyi* b) Spot test assay of extracts from *G. acuminata* secretions with exponentially growing of transformant yeast expressing topoisomerase I. Five microliters of ten-fold serial dilutions were spotted on plates containing various concentrations of extract from secretions of *G. acuminata* and incubated at 30°C for 48 hr. The expression of exogenous topoisomerase I was repressed by glucose (left panels) and induced by galactose (right panels).

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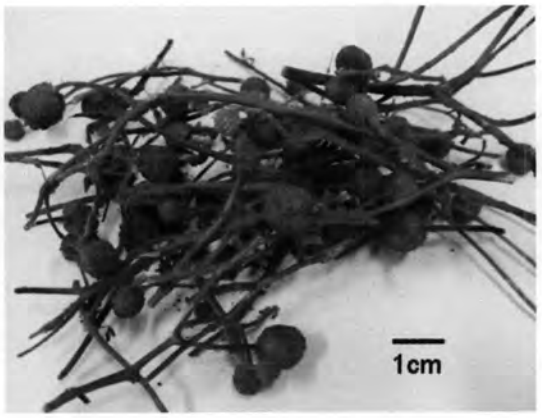

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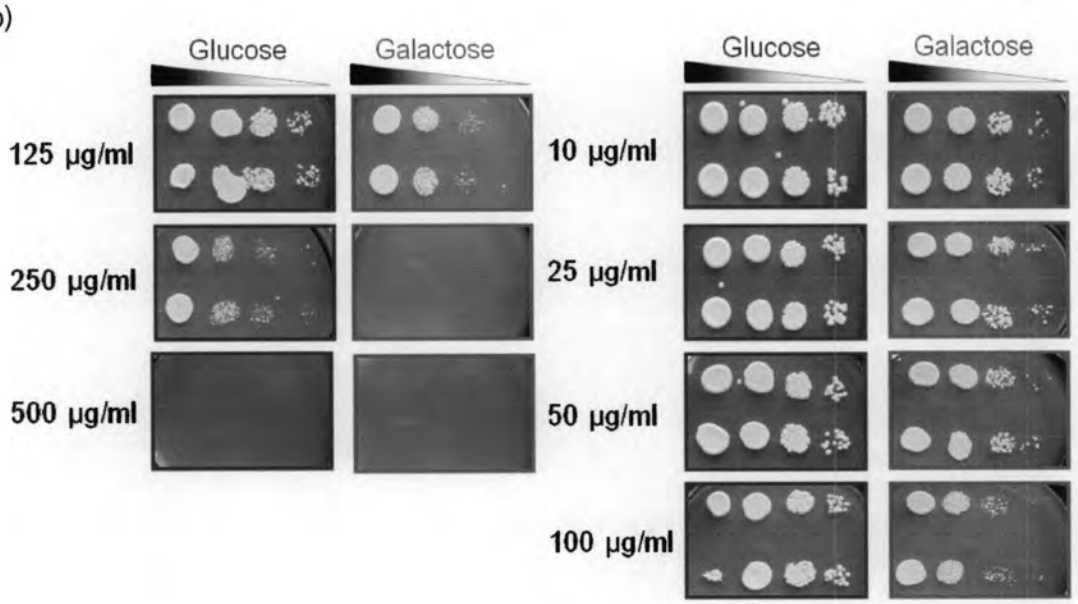

Figure 3.11 Yeast cell-based assay of *Grangea maderaspatana* whole plants. a) Whole plants of *G. maderaspatana* b) Spot test assay of extracts from *G. maderaspatana* plants with exponentially growing of transformant yeast expressing topoisomerase I. Five microliters of ten-fold serial dilutions were spotted on plates containing various concentrations of extract from whole plants of *G. maderaspatana* and incubated at 30°C for 48 hr. The expression of exogenous topoisomerase I was repressed by glucose (left panels) and induced by galactose (right panels).

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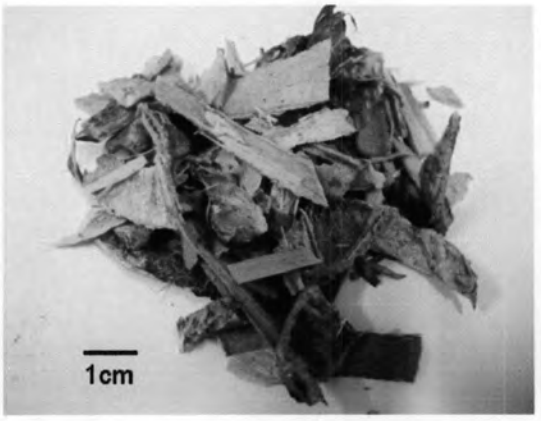

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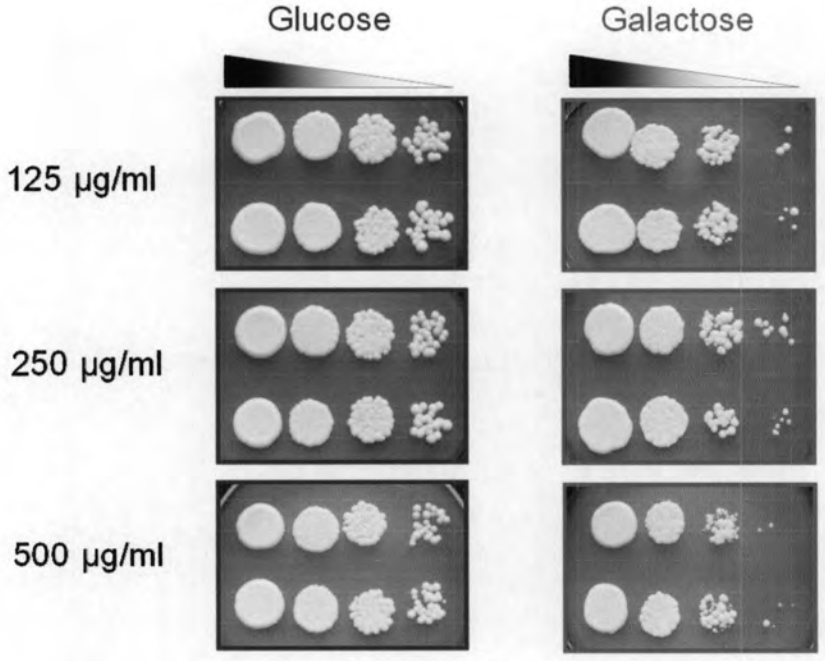

Figure 3.12 Yeast cell-based assay of *Moringa oleifera* barks. a) Barks of *M. oleifera* b) Spot test assay of extracts from *M. oleifera* barks with exponentially growing of transformant yeast expressing topoisomerase I. Five microliters of ten-fold serial dilutions were spotted on plates containing various concentrations of extract from barks of *M. oleifera* and incubated at 30°C for 48 hr. The expression of exogenous topoisomerase I was repressed by glucose (left panels) and induced by galactose (right panels).

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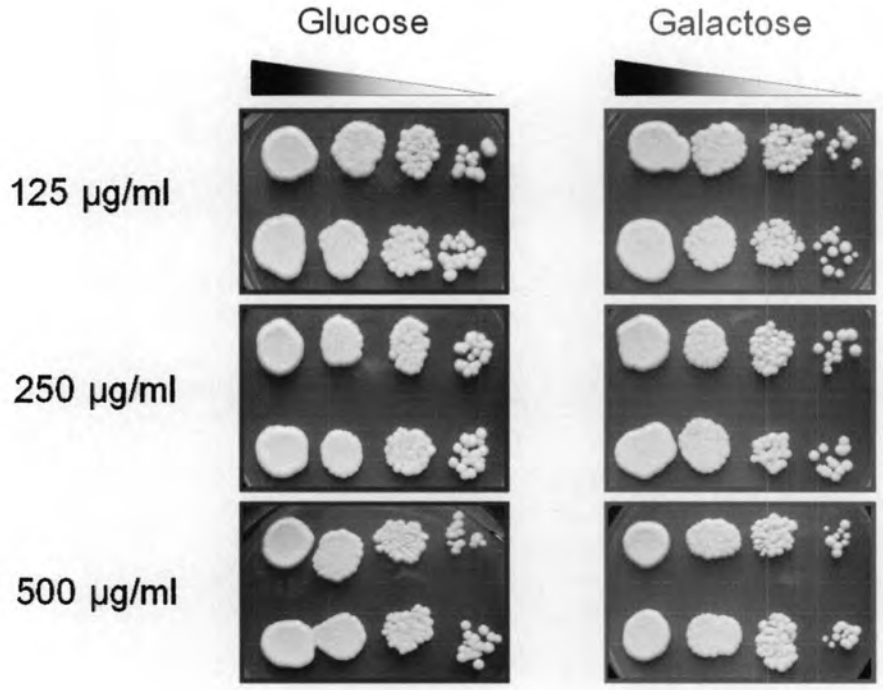

Figure 3.13 Yeast cell-based assay of *Phyllanthus amarus* whole plants. a) Whole plants of *P. amarus* b) Spot test assay of extracts from *P. amarus* plants with exponentially growing of transformant yeast expressing topoisomerase I. Five microliters of ten-fold serial dilutions were spotted on plates containing various concentrations of extract from whole plants of *P. amarus* and incubated at 30°C for 48 hr. The expression of exogenous topoisomerase I was repressed by glucose (left panels) and induced by galactose (right panels).

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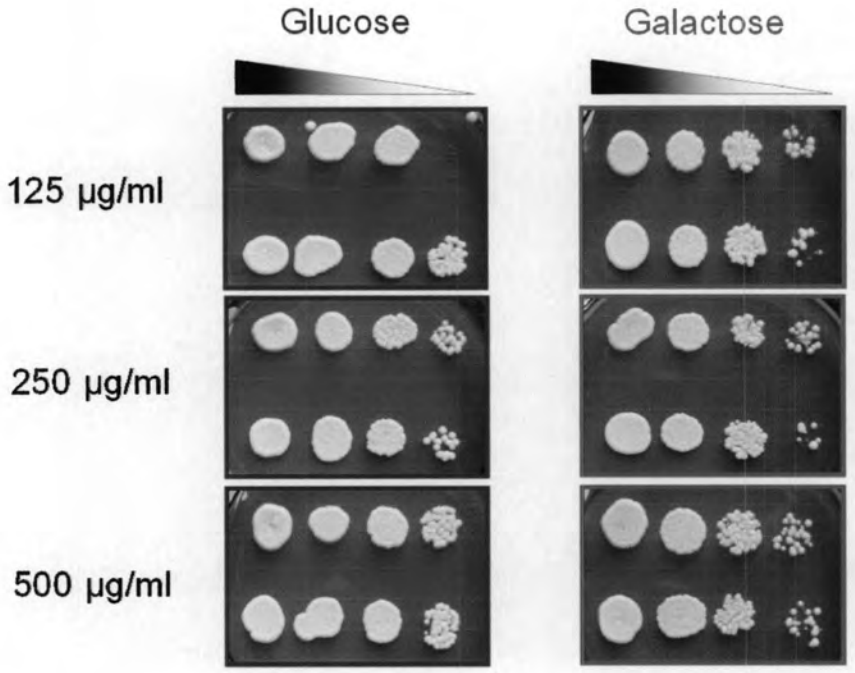

Figure 3.14 Yeast cell-based assay of *Phyllanthus emblica* fruits. a) Fruits of *P. emblica* b) Spot test assay of extracts from *P. emblica* fruits with exponentially growing of transformant yeast expressing topoisomerase I. Five microliters of ten-fold serial dilutions were spotted on plates containing various concentrations of extract from fruits of *P. emblica* and incubated at 30°C for 48 hr. The expression of exogenous topoisomerase I was repressed by glucose (left panels) and induced by galactose (right panels).

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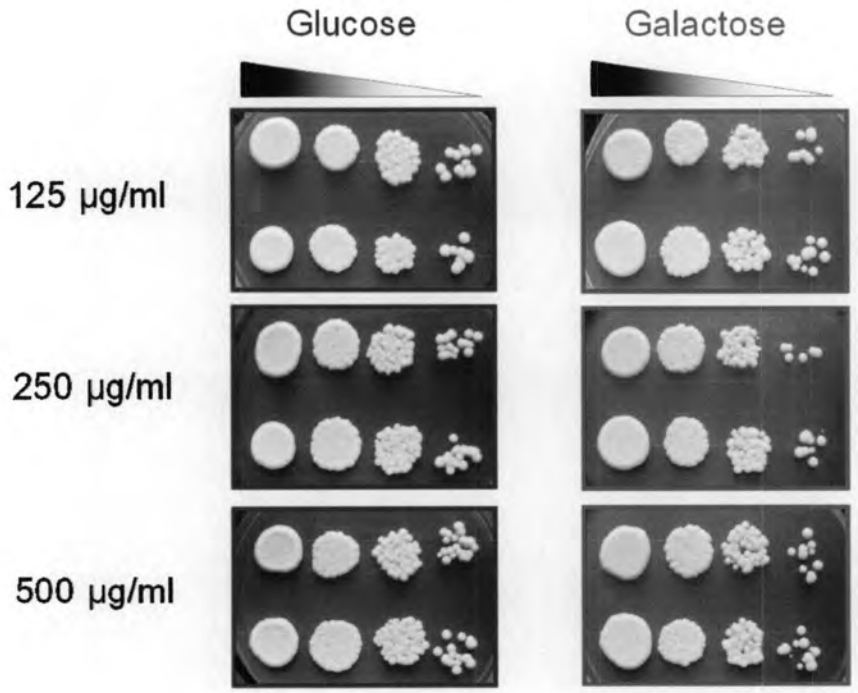

Figure 3.15 Yeast cell-based assay of *Phyllanthus urinaria* whole plants. a) Whole plants of *P. urinaria* b) Spot test assay of extracts from *P. urinaria* plants with exponentially growing of transformant yeast expressing topoisomerase I. Five microliters of ten-fold serial dilutions were spotted on plates containing various concentrations of extract from whole plants of *P. urinaria* and incubated at 30°C for 48 hr. The expression of exogenous topoisomerase I was repressed by glucose (left panels) and induced by galactose (right panels).

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b)

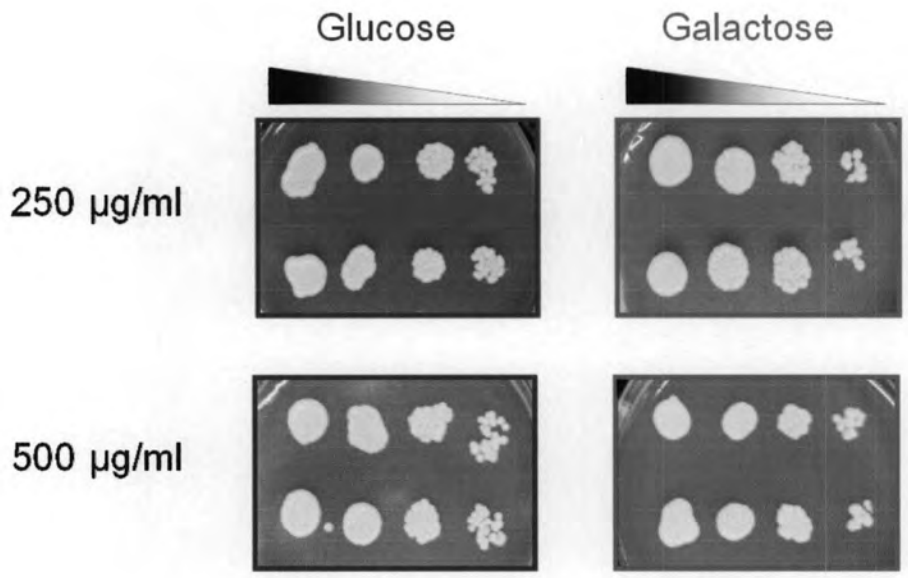


Figure 3.16 Yeast cell-based assay of *Rauvolfia serpentina* roots. a) Roots of *R. serpentine* b) Spot test assay of extracts from *R. serpentina* roots with exponentially growing of transformant yeast expressing topoisomerase I. Five microliters of ten-fold serial dilutions were spotted on plates containing various concentrations of extract from roots of *R. serpentina* and incubated at 30°C for 48 hr. The expression of exogenous topoisomerase I was repressed by glucose (left panels) and induced by galactose (right panels).

a)

b)

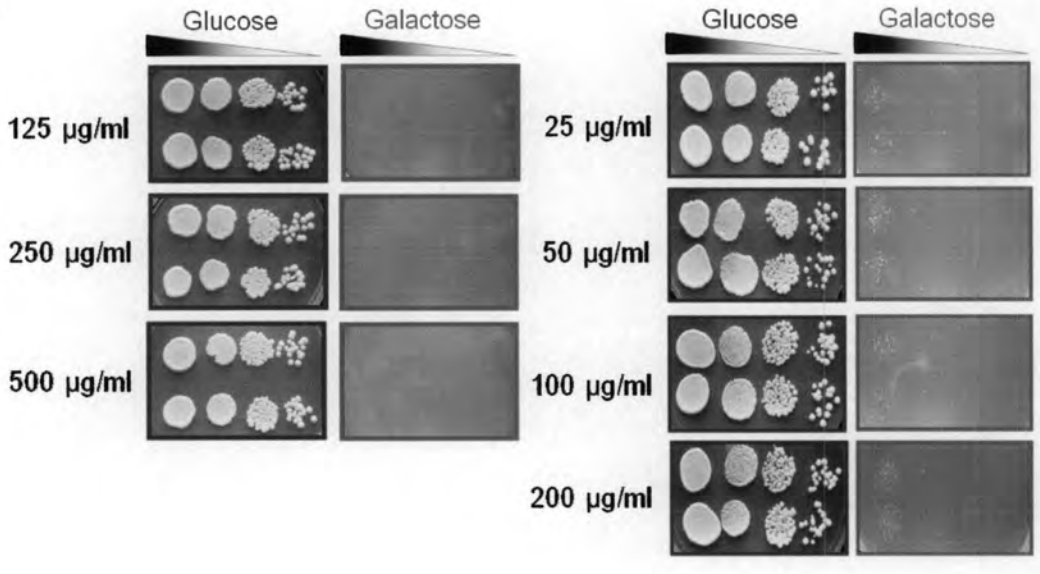


Figure 3.17 Yeast cell-based assay of *Rhinacanthus nasutus* leaves. a) Leaves of *R. nasutus* b) Spot test assay of extracts from *R. nasutus* leaves with exponentially growing of transformant yeast expressing topoisomerase I. Five microliters of ten-fold serial dilutions were spotted on plates containing various concentrations of extract from leaves of *R. nasutus* and incubated at 30°C for 48 hr. The expression of exogenous topoisomerase I was repressed by glucose (left panels) and induced by galactose (right panels).

a)

b)

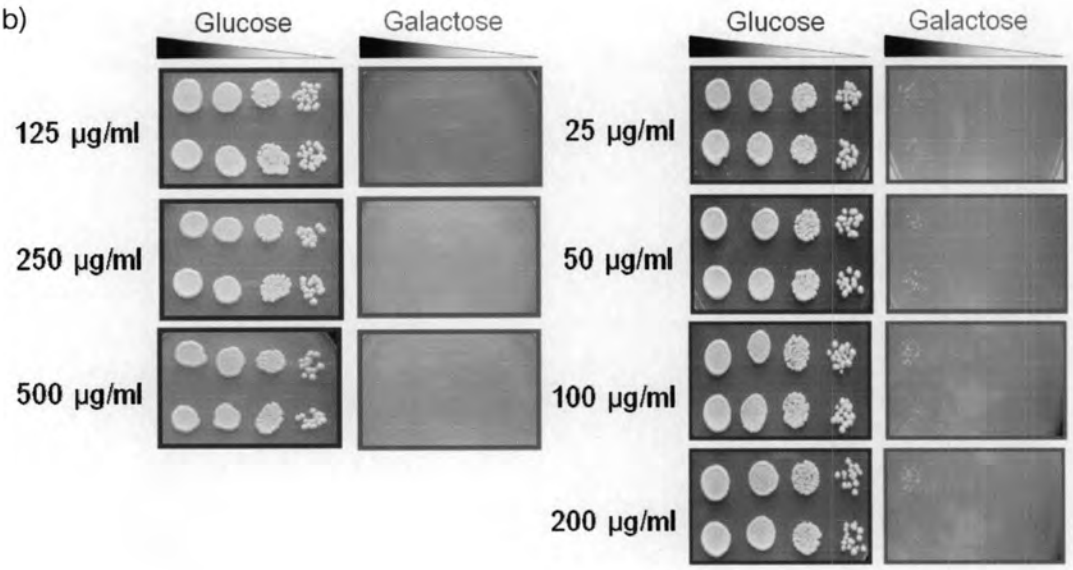


Figure 3.18 Yeast cell-based assay of *Rhinacanthus nasutus* roots. a) Roots of *R. nasutus* b) Spot test assay of extracts from *R. nasutus* roots with exponentially growing of transformant yeast expressing topoisomerase I. Five microliters of ten-fold serial dilutions were spotted on plates containing various concentrations of extract from roots of *R. nasutus* and incubated at 30°C for 48 hr. The expression of exogenous topoisomerase I was repressed by glucose (left panels) and induced by galactose (right panels).

a)

b)

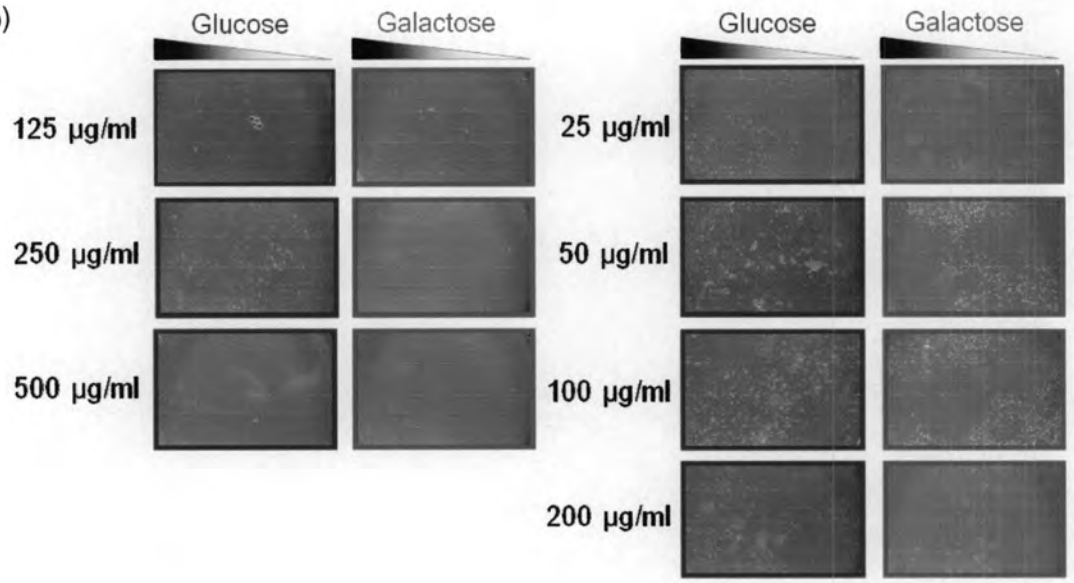


Figure 3.19 Yeast cell-based assay of *Sapindus rarak* fruits. a) Fruits of *S. rarak* b) Spot test assay of extracts from *S. rarak* fruits with exponentially growing of transformant yeast expressing topoisomerase I. Five microliters of ten-fold serial dilutions were spotted on plates containing various concentrations of extract from fruits of *S. rarak* and incubated at 30°C for 48 hr. The expression of exogenous topoisomerase I was repressed by glucose (left panels) and induced by galactose (right panels).

a)

b)

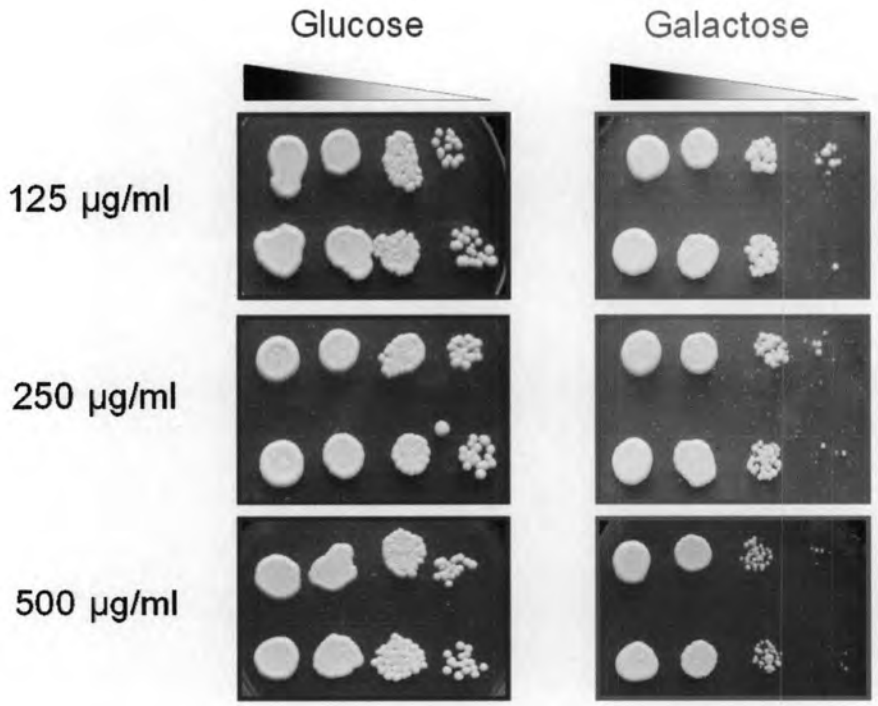
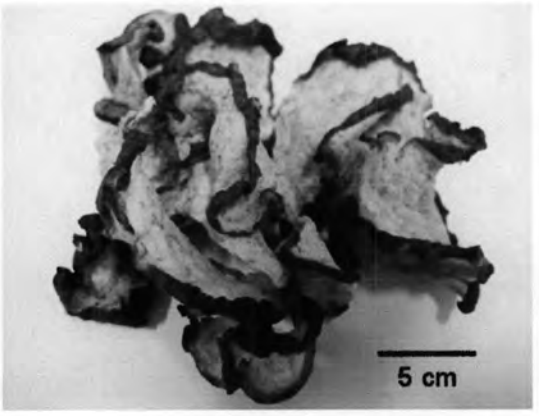


Figure 3.20 Yeast cell-based assay of *Schleichera oleosa* roots. a) Roots of *S. oleosa* (http://www.discoverlife.org/mp/20p?see=I_PAO126&res=640) b) Spot test assay of extracts from *S. oleosa* roots with exponentially growing of transformant yeast expressing topoisomerase I. Five microliters of ten-fold serial dilutions were spotted on plates containing various concentrations of extract from roots of *S. oleosa* and incubated at 30°C for 48 hr. The expression of exogenous topoisomerase I was repressed by glucose (left panels) and induced by galactose (right panels).

a)



b)

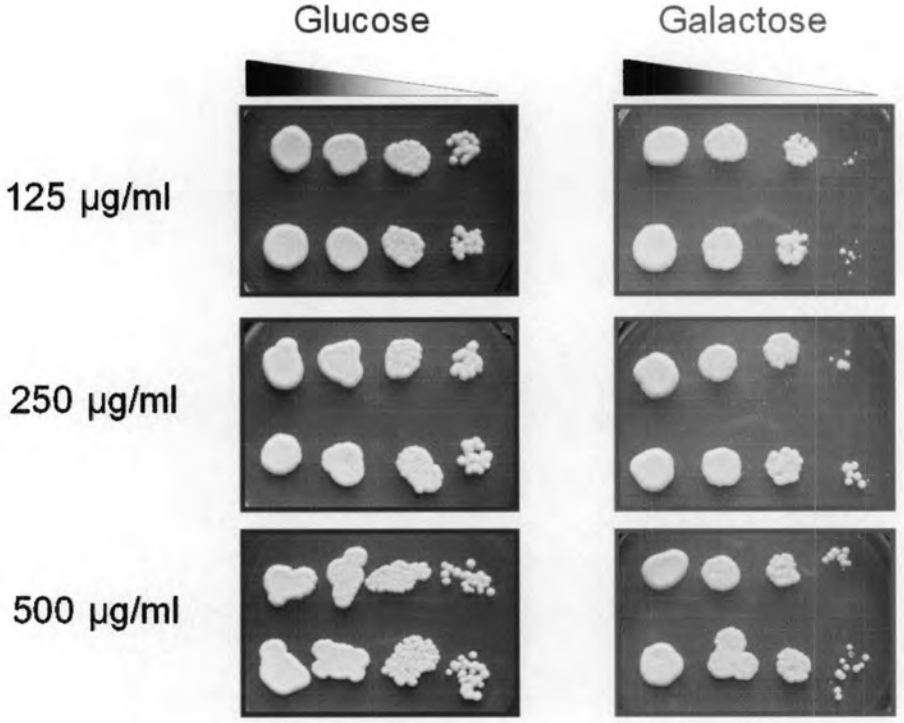


Figure 3.21 Yeast cell-based assay of *Stephania erecta* caudex. a) Caudex of *S. erecta* b) Spot test assay of extracts from *S. erecta* caudex with exponentially growing of transformant yeast expressing topoisomerase I. Five microliters of ten-fold serial dilutions were spotted on plates containing various concentrations of extract from caudex of *S. erecta* and incubated at 30°C for 48 hr. The expression of exogenous topoisomerase I was repressed by glucose (left panels) and induced by galactose (right panels).

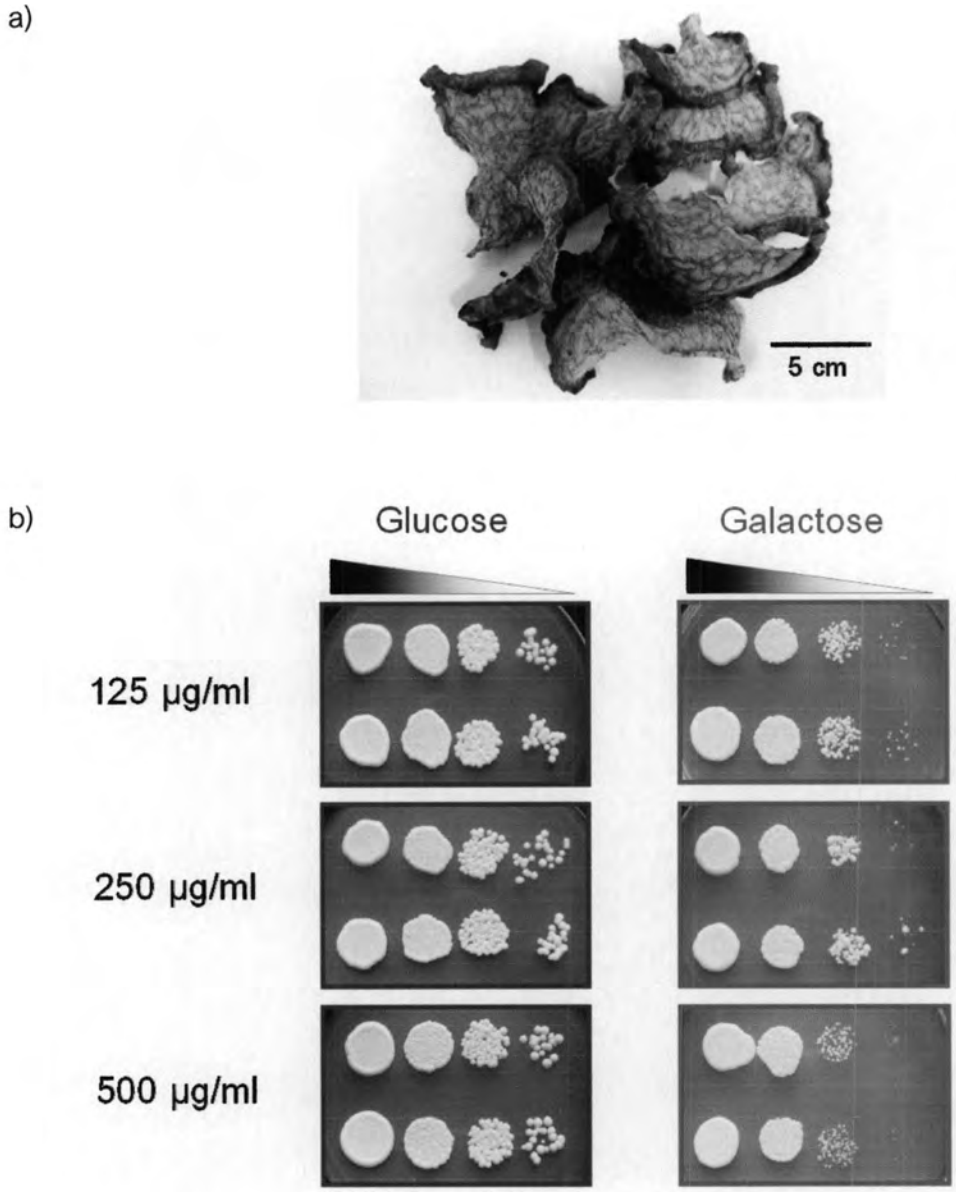


Figure 3.22 Yeast cell-based assay of *Stephania pierrei* caudex. a) Caudex of *S. pierrei* b) Spot test assay of extracts from *S. pierrei* caudex with exponentially growing of transformant yeast expressing topoisomerase I. Five microliters of ten-fold serial dilutions were spotted on plates containing various concentrations of extract from caudex of *S. pierrei* and incubated at 30°C for 48 hr. The expression of exogenous topoisomerase I was repressed by glucose (left panels) and induced by galactose (right panels).

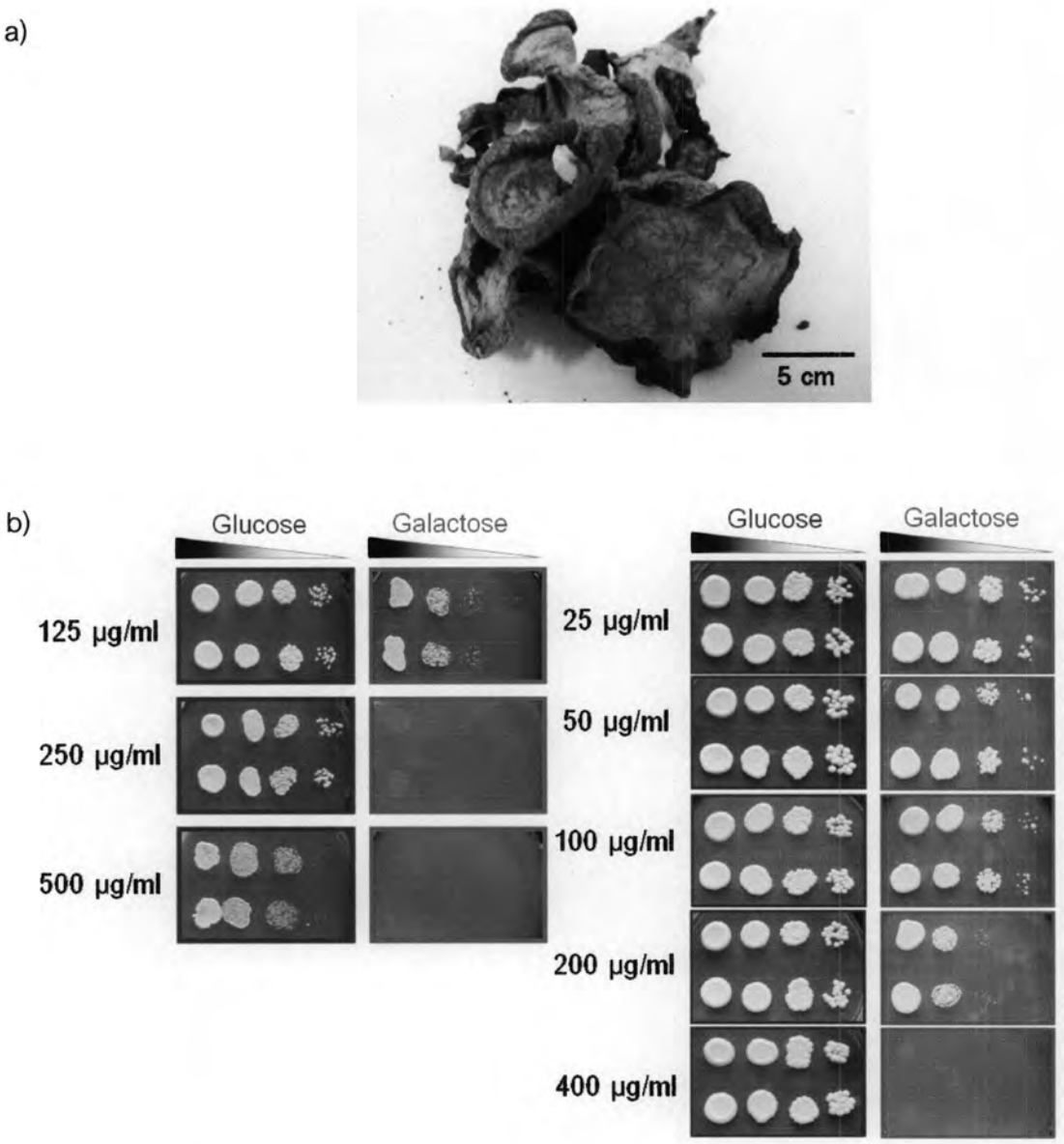


Figure 3.23 Yeast cell-based assay of *Stephania suberosa* caudex. a) Caudex of *S. suberosa* b) Spot test assay of extracts from *S. suberosa* caudex with exponentially growing of transformant yeast expressing topoisomerase I. Five microliters of ten-fold serial dilutions were spotted on plates containing various concentrations of extract from caudex of *S. suberosa* and incubated at 30°C for 48 hr. The expression of exogenous topoisomerase I was repressed by glucose (left panels) and induced by galactose (right panels).

a)

b)

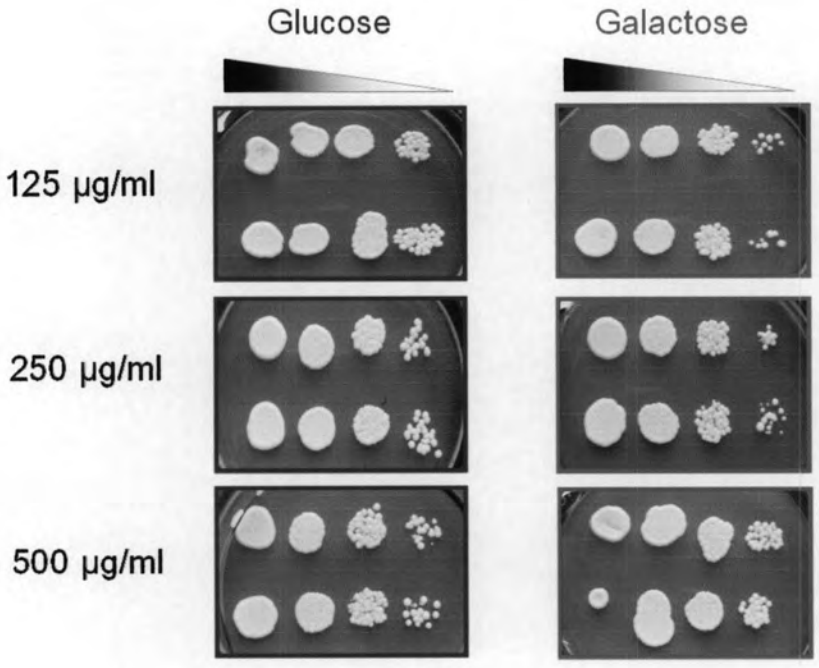


Figure 3.24 Yeast cell-based assay of *Terminalia bellirica* fruits. a) Fruits of *T. bellirica* b) Spot test assay of extracts from *T. bellirica* fruits with exponentially growing of transformant yeast expressing topoisomerase I. Five microliters of ten-fold serial dilutions were spotted on plates containing various concentrations of extract from fruits of *T. bellirica* and incubated at 30°C for 48 hr. The expression of exogenous topoisomerase I was repressed by glucose (left panels) and induced by galactose (right panels).

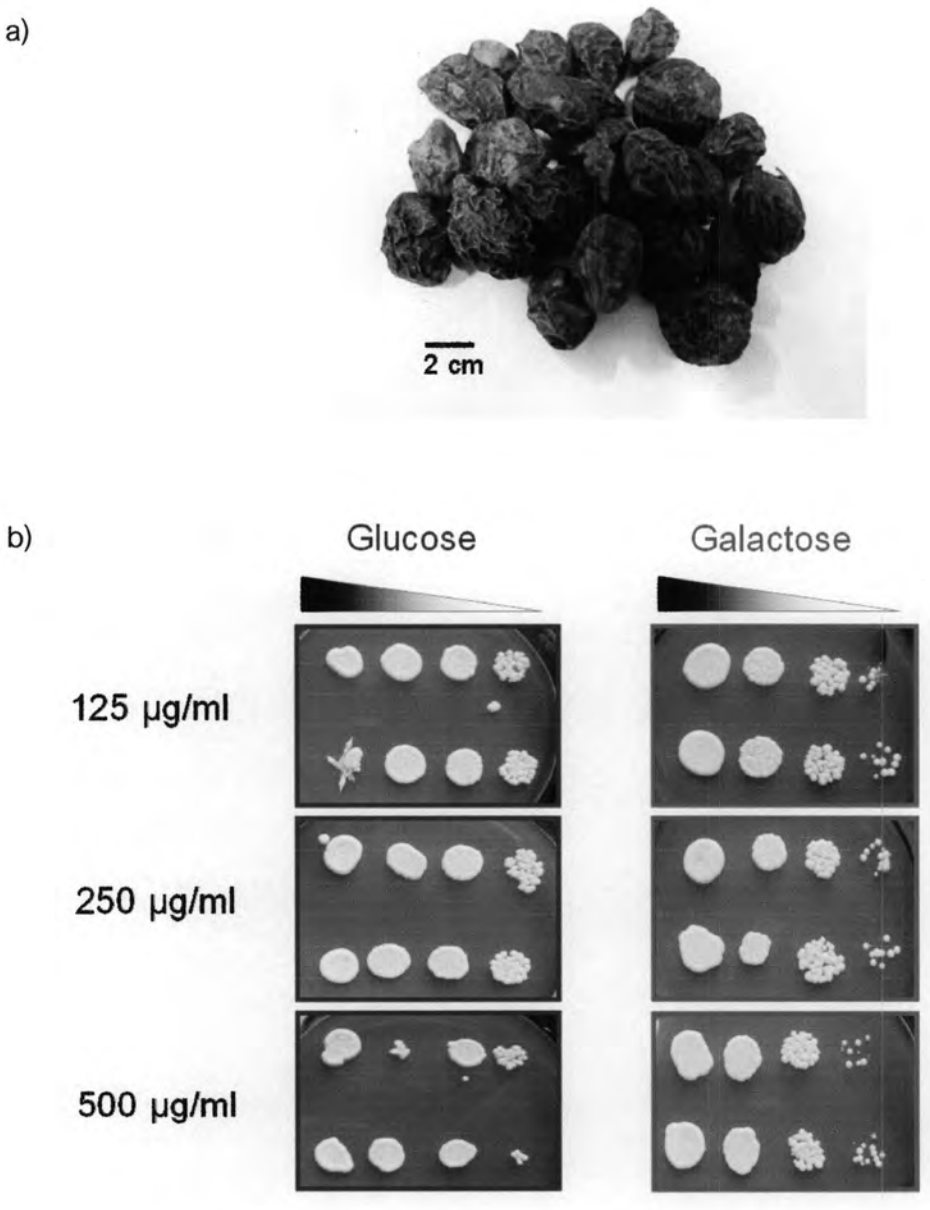
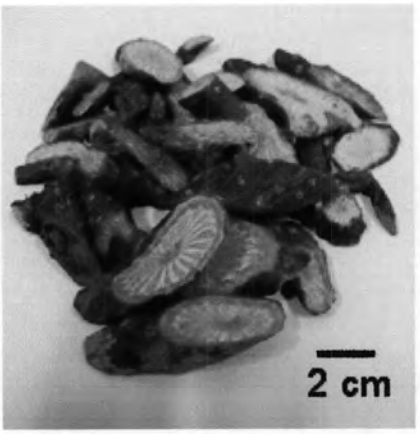


Figure 3.25 Yeast cell-based assay of *Terminalia chebula* fruits. a) Fruits of *T. chebula* b) Spot test assay of extracts from *T. chebula* fruits with exponentially growing of transformant yeast expressing topoisomerase I. Five microliters of ten-fold serial dilutions were spotted on plates containing various concentrations of extract from fruits of *T. chebula* and incubated at 30°C for 48 hr. The expression of exogenous topoisomerase I was repressed by glucose (left panels) and induced by galactose (right panels)

a)



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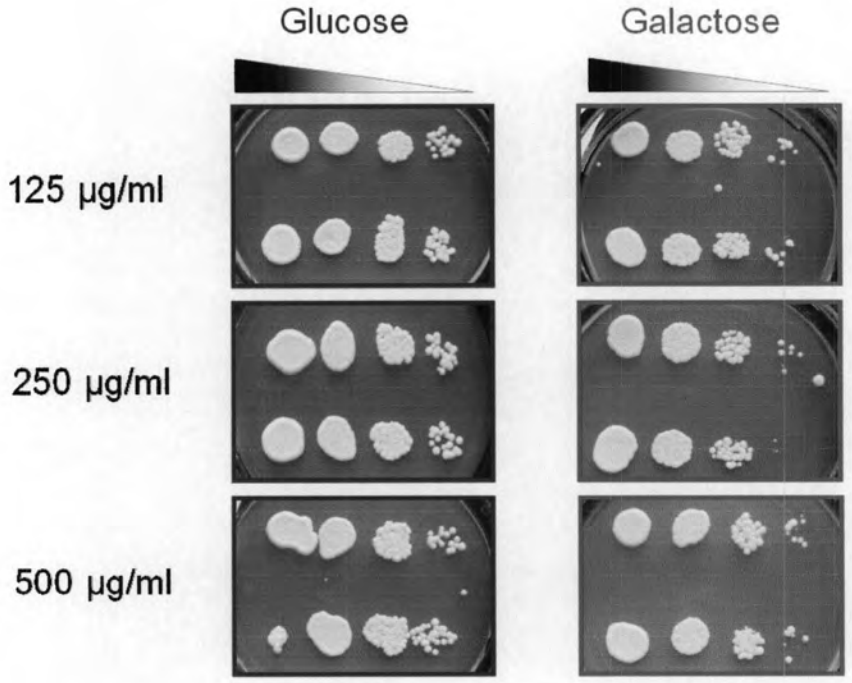


Figure 3.26 Yeast cell-based assay of *Tinospora baenzigeri* stems. a) Stems of *T. baenzigeri* b) Spot test assay of extracts from *T. baenzigeri* stems with exponentially growing of transformant yeast expressing topoisomerase I. Five microliters of ten-fold serial dilutions were spotted on plates containing various concentrations of extract from stems of *T. baenzigeri* and incubated at 30°C for 48 hr. The expression of exogenous topoisomerase I was repressed by glucose (left panels) and induced by galactose (right panels).

a)

b)

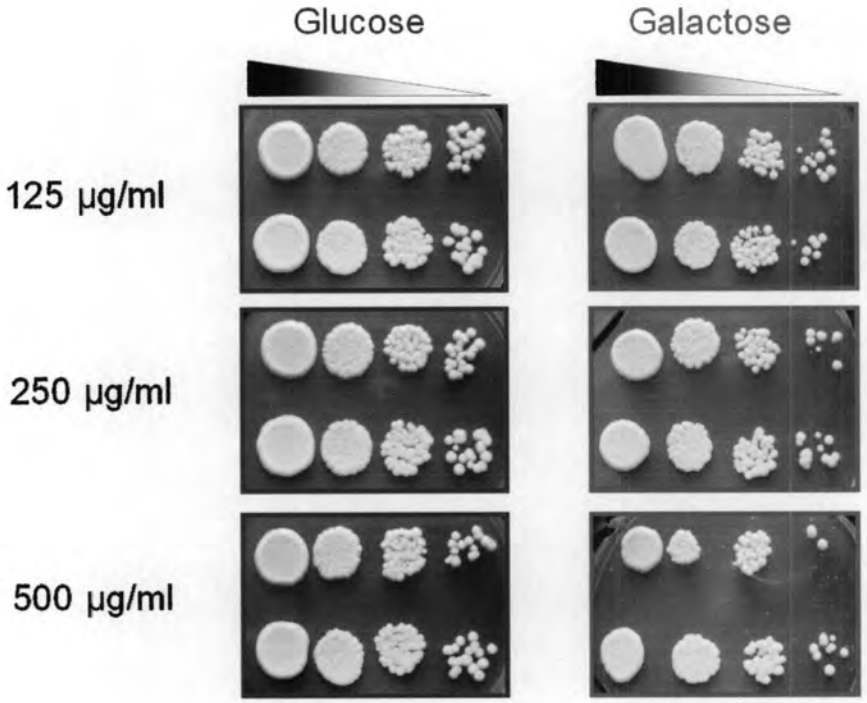
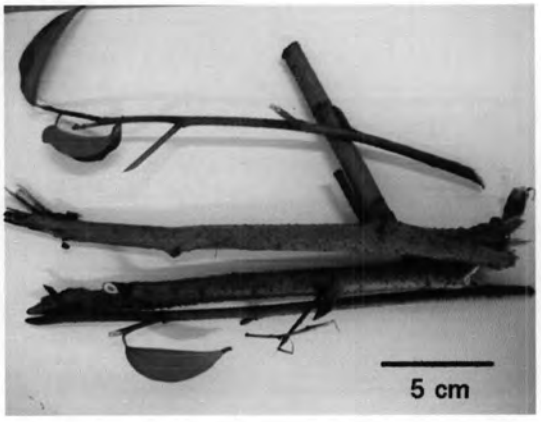


Figure 3.27 Yeast cell-based assay of *Usnea siamensis* whole plants. a) Whole plants of *U. siamensis* b) Spot test assay of extracts from *U. siamensis* plants with exponentially growing of transformant yeast expressing topoisomerase I. Five microliters of ten-fold serial dilutions were spotted on plates containing various concentrations of extract from whole plants of *U. siamensis* and incubated at 30°C for 48 hr. The expression of exogenous topoisomerase I was repressed by glucose (left panels) and induced by galactose (right panels).

a)



b)

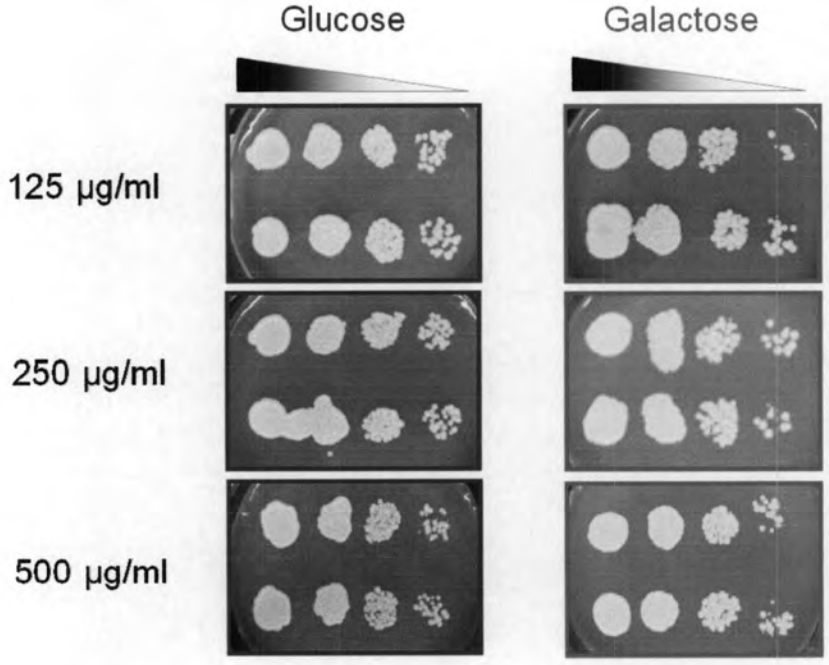


Figure 3.28 Yeast cell-based assay of *Ziziphus attopoensis* branches. a) Branches of *Z. attopoensis* b) Spot test assay of extracts from *Z. attopoensis* branches with exponentially growing of transformant yeast expressing topoisomerase I. Five microliters of ten-fold serial dilutions were spotted on plates containing various concentrations of extract from branches of *Z. attopoensis* and incubated at 30°C for 48 hr. The expression of exogenous topoisomerase I was repressed by glucose (left panels) and induced by galactose (right panels).

3.3 Discussions

The ethanolic extract of 27 Thai medicinal plants were assayed for topoisomerase I-targeted drugs by recombinant yeast expressing *Arabidopsis thaliana topoisomerase I* gene. The yeast cell-based assay has been used to study the interaction of enzyme topoisomerase I with topoisomerase I-targeted agents (Nitiss and Wang, 1988). We controlled *A. thaliana Top1* gene expression with the effortless induction of the *GAL1* promoter by the vector pYES-DEST52 contains the *GAL1* promoter and enhancer sequences for regulated expression. Transcription from the *GAL1* promoter is repressed in the presences of glucose and induced by remove glucose and adding galactose as a carbon source. In the repressed condition (glucose-containing medium), CPT, anti-cancer agent did not affect the growth of strain RS190 because topoisomerase I, the target of CPT, is absent in this strain. In contrast, overexpression of topoisomerase I by induction with galactose in the presence of CPT caused cell death because CPT block the correct reaction of topoisomerase I by stabilizing a covalent complex of nicked DNA and topoisomerase I leading to a DNA lesion (Reid *et al.*, 1998). On the other hand, selective medium contain vehicle control, DMSO yeast cells can growth on induced condition just like repressed condition.

As a result, the growth of transformant yeasts on the agar plates contain individual crude extract can demonstrated topoisomerase I inhibitor activities of Thai medicinal plants. If yeast cells can grow on repressed condition but death on induced condition that mean the extract was active for topoisomerase I inhibitory activities. If yeast cells grow on induced condition just like repressed condition that mean the extract was inactive for topoisomerase I inhibitory activities. In contrast, If yeast cells death on both induced and repressed conditions that not mean the extract was active because the death of yeasts from cytotoxicity not topoisomerase I inhibitory activitiy.

In this method, cell viability is scored by the formation of colonies either in the presence of topoisiomerase I-targeted agents. Spotting aliquots of serially diluted culture on agar plates provides a rapid and semi-quantitative measure of drug induced cytotoxicity. With this approach, one-four log decreases in cell viability can easily be scored as a

consequence of topoisiomerase I-targeted agents (Osheroff and Bjornsti, 2001). Therefore, it was a valuable model in establishing eukaryotic DNA topoisomerase I as the cellular target of specific antineoplastic agent.

The plants selection for the screening was base on two approaches. The first was ethnopharmacology approach which led to the selection of the plants use in traditional medicines to treat cancer. The second approach was the Thai medicinal plants reported cytotoxicity against cancer cell lines. In the primary screening, we chose three final concentrations of the ethanolic plant exctracts (125, 250, and 500 µg/ml of medium). The maximum concentration was 500 µg/ml because this concentration, all of plant exctracts can solubilized in DMSO. If the transformant yeasts on the agar plates contain 125 µg/ml of individual crude extracts can not grow both induced and repressed conditions, we will dilute concentration of the extracts to 25, 50, 100, 200 µg/ml of medium for the secondary screening.

By this assay, 6 of 27 extraction plants from Thai medicinal plants have topoisomerase I inhibitor activities. The transformant yeast model results we obtained indicated that ethanolic extract of rhizome of *Curcuma longa*, rhizome of *Curcuma zedoaria,* whole plant of *Grangea maderaspatana*, root and leaves of *Rhinacanthus nasutus*, and caudex of *Stephania suberosa* extracts demonstrated topoisomerase I inhibitor activity overall. Resulting in, on the plates included those extracts the growth of transformant yeasts on repressed condition was allowing but not on induced condition (Fig. 3.6, 3.7, 3.11, 3.17, 3.18, and 3.23 respectively). On the other hand, the ethanolic extraction of fruit of *Sapindus rarak* was the cytotoxic agent because the yeast cells can not grow on both repressed and induced conditions, when we diluted concentration of this extract to 25 µg/ml (Fig. 3.19). In contrast, another ethanolic plant extracts in this study allowed the growth of yeasts in all dilutions both repressed and induced conditions that mean those extracts were inactive for topoisomerase I inhibitor activities.

The topoisomerase I inhibitory activities of those species may be correlated with their use in the traditional medicine for treatment of cancer. The ethanolic extract of root and leaves of *R. nasutus* showed most inhibitory topoisomerase I activity against transformant

yeast models (Fig. 3.17-3.18 respectively). Many research demonstrated extraction of *R. nasutus* show potent cytotoxicity activity against cancer cell line (Wu *et al.*, 1988; Punturee *et al.*, 2004; Siripong *et al.*, 2006a). Naphthoquinones compounds isolate from *R. nasutus* had antiproliferative activity against human cancer cell lines by targeting DNA topoisomerase II (Siripong *et al.*, 2006b). Also, crude extract of rhizome of *C. longa* in this study showed topoisomerase I inhibitory activity. A previous study by Roth, Chandra, and Nair (1998) has shown fractionation of ethyl acetate extract from *C. longa* rhizomes displayed topoisomerase I and II enzyme inhibition activity. Our results confirmed that the transformant yeast model have accuracy for screening topoisomerase I-targeted drugs.

Previous studies showed potent cytotoxicity activities of sesquiterpene lactones compounds from *G. maderaspatana* (Ruangrungsi *et al.*, 1989), aporphine alkaloid compounds from *S. suberosa* (Makarasen, 2004), and sesquiterpene and curcumin compounds from *C. zedoaria* (Matsuda *et al.*, 1998). Interesting, by the ethanolic extract of *G. maderaspatana* whole plant, *S. suberosa* caudex, and *C. zedoaria* rhizome have never been reported for their topoisomerase I inhibitory activities before. It also the first time that topoisomerase I inhibitory activities of these plants are reported. Further chemical study of *G. maderaspatana* might lead to discovery of topoisomerase I-targeted drugs.