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REVERSE AGING IN *SACCHAROMYCES CEREVISIAE* THROUGH REDUCED *TOR1* EXPRESSION AND REPLICATIVE LIFE SPAN MODELLING WITH CELL VIABILITY ASSESSMENT USING PHYTOCHEMICAL EXTRACTS *ALPINIA GALANGAL, COMMIPHORA MUKUL, ACORUS CALAMUS* AND *MORSHELLA ESCULENTA*

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Keywords:

Reverse aging, Target of rapamycin, Replicative life span, *S. cerevisiae*, Cell viability

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ABSTRACT: To study reverse aging in *Saccharomyces cerevisiae* (*S. cerevisiae)* by gene expression analysis of target of rapamycin (*TOR1)* induced autophagy and replicative life span modelling (RLS) using *Alpinia galangal (A. galangal), Commiphora mukul (C. mukul), Acorus calamus (A. calamus) and Morshella esculenta (M. esculenta).* Methanolic extraction of plants was carried out using maceration. Activation of yeast was performed using the sugar fermentation method. Pharmacological anti-aging screens in yeast performed by RLS. Primers for Actin (*ACT1*) and *TOR1* were designed using Primer-BLAST. Gene expression analysis (RNA isolation and quantification, reverse transcription, and real-time polymerase chain reaction) of *TOR1* in *S. cerevisiae* was conducted using methanolic extracts. Phenolic and Flavonoid compounds were quantified using reversed-phase high-performance liquid chromatography (RP-HPLC). Cell counting and viability assessment of *S. cerevisiae* with trypan blueusing methanolic extracts was performed. Gene expression analysis results revealed under expression of *TOR1* in *S. cerevisiae* with mean normalized expression ratios < 1 using all four methanolic extracts. Results from RLS showed increased absorbances due to decreased *TOR1* activity. HPLC analysis showed the presence of antioxidants such as vanillic acid, caffeic acid, rutin, and quercetin in crude extracts. Cell viability assessment revealed nontoxicity effects of *M. esculenta* and *A. galangal* whereas toxicity effects of *A. calamus* and *C. mukul* on *S. cerevisiae* cells. Reverse aging in *S. cerevisiae* was studied by gene expression analysis of *TOR1* and RLS. *TOR1* gene role in the activation of autophagy was explored using real-time polymerase chain reaction.

INTRODUCTION: Autophagy, an evolutionary conserved catabolic process, involves the degradation of aberrant biomolecules, organelles, *etc*., including the elimination of aging cells by replacement with new cells and proteins required for maintaining cellular homeostasis [1].

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Aging has been characterized by the gradual failure over time in physiological functions, leading to an assemblage of molecular and cellular damage (autophagy decreases with age) with loss of physiological function [2].

The yeast, *S. cerevisiae was* chosen as a model organism to study reverse aging because it has been reported that there are several conserved hallmarks of aging between yeast and humans, remarkedly autophagy by a pharmacological screening method known as RLS, which provided a measure of several mitotic events an individual mother cell

could undertake before senescence in many eukaryotes [3, 4]. TOR proteins are highly evolutionary conserved proteins throughout eukaryotes activated in response to nutrient sensors; orthologous to human mTOR (mechanistic target of rapamycin kinase). *TOR1*, a phosphatidylinositol kinase homologue; part of TORC1 complex in *S. cerevisiae* isinvolved in several metabolic processes, remarkedly in inhibition of autophagy [5, 6]. Rapamycin (Sirolimus) increased lifespan by activating autophagy through mTOR inhibition in various model organisms. Studies have reported that reduced signalling (inhibition /decreased expression) of the TOR pathway, achieved by either genetic or pharmacological means, resulted in increased lifespan by up-regulation of autophagy in many organisms including yeast [6, 7, 8, 9, 10]. Several plant antioxidants, such as resveratrol have been extensively studied earlier for lifespan extension via the induction of autophagy [11, 12].

Amongst the various medicinal plants, we have chosen plants, viz., *A. galangal, C. mukul, A. calamus* and *M. esculenta. A. galangal* commonly known as kulanjan and greater galangal is found in Western Ghats, Malabar coasts and Gujrat in India. The rhizome of *A. galangal* has antioxidant and antiaging activities [13, 14, 15]. *C. mukul* commonly known as guggul and Indian bdellium, is found in arid regions of India, Assam, Karnataka, *etc*. Oleo-gum resin secreted by *C. mukul* has antioxidant and anti-aging activities [16, 17]. *A. calamus,* commonly known as vacha and sweet flag is found in areas of Jammu and Kashmir, Himachal Pradesh *etc*., in India. The rhizome of *A. calamus* exhibited antioxidant activity [18]. *M. esculenta* is one of the costliest edible morels commonly known as gnocchi and morel found in the North-West Himalayas mainly, Kashmir renowned for their gastronomic quality and nutritional health benefits due to their anti-oxidative properties [19]. Earlier, fungus such as *Aspergillus niger* has been studied using our selected plant *A. galangal* [20]. It was reported that *S. cerevisiae* autophagy was induced by starvation [21]. The present study evaluates gene expression of *TOR1* in the absence of starvation and RLS with cell viability assessment in *S. cerevisiae* using methanolic extracts *A. galangal, C. mukul, A. calamus, and M. esculenta.* Pharmacological activation of autophagy through medicinal plants by under expression of *TOR1* would cause depletion of damaged cells which will pave way for stem cell development and rejuvenation of new cells which might be a realistic and promising treatment for reverse aging.

MATERIALS AND METHODS:

Sample Collection: *A. calamus* rhizome was collected from Himachal Pradesh (India), *M. esculenta* mycelia were collected from Uttarakhand (India), *A. galangal* rhizome and *C. mukul* gum were collected from Karnataka (India).

Extract Preparation: Samples were thoroughly cleansed with distilled water and air dried to remove moisture and ground into a coarse powder. For methanolic extraction, 10g powder of all four plant samples was mixed with 40ml solvent to attain a minimum concentration of 250mg/ml, followed by maceration with occasional shaking for 5 days at room temperature with 100% methanol. The extracts were filtered and evaporated to obtain the crude extracts. All the extracts were preserved at 4°C for a further [22].

Growth of Yeast by Sugar Fermentation: Beaker was filled with distilled water (100ml) and microwaved until warmed. Sucrose (1g) was added to the prewarmed water, and the sugar solution was microwaved until boiled. The mixture was cooled at room temperature, followed by adding baker's yeast (0.5g) and mixed properly for yeast activation.

RLS Modelling in *S. Cerevisiae*: Activated yeast was streaked on sterilized Potato Dextrose Agar media (PDA; 0.2g potato extract, 2g dextrose, and 0.4g agar in 50ml of distilled water) followed by an incubation of 48 -72 hours; pH 5.6 ± 0.2). Single colonies were inoculated into sterilized Potato Dextrose Broth media (PDB; 0.4g potato extract and 4g dextrose in 100ml of distilled water; pH 5.6 ± 0.2) followed by incubation at 36°C for 48 hours along with constant agitation using an orbital shaker incubator [23].

Forty-eight hours old yeast cultures (5ml) were distributed in five test tubes first test tube contained only yeast culture marked as a control sample. In contrast, the other four test tubes consisted of four methanolic extracts (1ml) and were incubated at 36°C with unvarying agitation for 20 days.

Absorbance was recorded at 600 nm by spectrophotometer in triplicates [24].

RNA Isolation and Quantification: RNA was isolated from yeast previously incubated with four methanolic plant extracts and with a control sample (only yeast) using TriXtract™ RNA isolation method manufacturer's instructions. Qualitative analysis of isolated RNA was performed using 1.5% (w/v) Tris-borate-EDTA (TBE) - agarose gel electrophoresis at 100°C.

The gel was visualized with UV transilluminator at 302nm to obtain the image. RNA concentration and purity were assessed by determining the absorbance of RNA in RNase-free water at 260nm and 280nm using spectrophotometer. RNA yield was evaluated based on absorbance at 260 nm (A_{260}). The ratio A_{260}/A_{280} was assessed to calculate RNA purity.

Reverse Transcription: RNA (288-336µg/ml) from each sample was retrotranscribed to complementary DNA (cDNA). Briefly, the assay was performed with total RNA (5µl), Oligo $(dT)_{16}$ (2µl), and sterile RNase- free water to make a final volume of 14µl. The mixture was heated to 65°C for 5 minutes and immediately chilled on ice. It was followed by the addition of Moloney murine leukemia virus (MMLV) buffer (5X) (4µl), dNTPs (2µl), RNAsin (0.5µl), M-MLV (1µl) followed by incubation at 42°C for 60 minutes and successively heated up to 95°C for 15 minutes. Finally, the samples were chilled on ice for downstream experiments or stored at -20°C.

Primer Designing: *ACT1* and *TOR1* sequences were retrieved from NCBI data bank. Primers were designed and checked for specificity using Primer-BLAST software.

Polymerase Chain Reaction (PCR): To check the specificity of the designed primer pairs, conventional PCR amplification was carried out from cDNA template in a final volume (50µl) consisting of master mix (25µl), cDNA (5µl), each forward and reverse primer (1µl) (10mM) and nuclease-free water (18µl) in a thermal cycler.

The PCR thermal profile was 95°C for 3 minutes, 95°C for 20 seconds, 50°C (*Act1*) / 56°C (*TOR1*) for 20 seconds, 72°C for 40 seconds for 40x, 72°C for 3 minutes 4°C for 10 minutes.

Real-Time PCR (qPCR) Analysis: qPCR was conducted using a thermal cycler ABI PRISM 7000 sequence detection system. Amplifications were carried out in a final volume of 50µl, reaction solutions containing Kappa SYBR Fast Master Mix (25µl) (2X) with ROX reference dye, Primer F (2.0µl) (10mM), Primer R (2.0µl) (10mM), cDNA (5.0µl), nuclease-free water (16µl). The experiment was performed in triplicates for each test sample and control sample in a 96-well plate. qPCR conditions were used with an activation step of 2 minutes at 95°C followed by an initial denaturation step of 5 minutes at 95°C followed by 40 cycles of 95°C for 10 seconds, 52°C for 20 seconds, and 72°C for 10 seconds. Optimization of annealing temperatures and primer concentrations was done, and it confirmed that primer sets performed well as single plex. Standard curve assays were conducted to determine assay sensitivity, efficiency, and reproducibility of the assay at various concentrations of cDNA using initial concentration (N_0) 1ng and five 1: 2 serial dilutions (0.5ng, 0.250ng, 0.0625ng, 0.015625ng, and 0.0009765625ng). Normal yeast cells were selected as a biological control in standard curve assay. Primers with amplification efficiency below 90% and above 110% were not considered. Gene expression variations were evaluated regarding fold induction concerning the normal yeast cells by $2^{-\Delta\Delta Ct}$ comparative Ct method [25]. The real-time data analysis was conducted with ABI Prism 7000 SDS software, Data analysis gene expression (DAGE), LinReg, and Excel.

HPLC Preparation of Standards: Commercial standard compounds consisting of four phenolics and two flavonoid compounds namely: gallic acid, vanillic acid, caffeic acid, resveratrol, quercetin, and rutin were purchased. Working standard solutions (30µg/ml) were prepared in HPLC-grade methanol from the standard stock solution (100µg/ml) and stored in HPLC vials until use.

HPLC Sample Preparation: Samples (1.5ml) were briefly vortexed and centrifuged at 10000g for 10 minutes to collect the supernatant. Subsequently, the supernatant was sonicated for 30 minutes at 30°C and successively filtered through a filter (pore size 0.45µm), pre-washed with 70% ethanol, and stored in HPLC vials before injection in the sample loop.

Chromatographic Conditions: HPLC analysis of methanolic crude extracts was performed on a reverse phase Zorbax Eclipse XDB-C18 column (4.6 × 150mm, 5µm particle). The injection volume of samples was 20µl. Methanol: phosphate buffer (pH 6.8) was used as mobile phase adjusted with orthophosphoric acid solution 0.5%, v/v) in MilliQ water (63:37%, v/v) and filtered through a 0.22µm nylon membrane and ultrasonically degassed before use at a flow rate of 1ml/min in an isocratic mode and the column temperature was thermostatically stable at 30°C. The total runtime was 4 minutes. Detection wavelengths of different standard compounds were as follows: 254nm for vanillic acid, 320nm for caffeic acid, 307nm for resveratrol, 350nm for quercetin, 278nm for gallic acid and 256nm for rutin, respectively [24, 25, 26]. Identification of phenolic and flavonoid compounds was based on their respective retention times (RT) compared with commercial standards. Values for a limit of detection (LOD) and limit of quantification (LOQ) were calculated to identify and quantify phenolic, and flavonoid compounds in the extracts. The experiment was performed in independent triplicates. Data analysis was performed using MS Excel. The concentration of antioxidants was calculated from the concentration obtained by HPLC (µg/ml) divided by the weight of the plant sample (g), initially mixed with the volume of solvent (ml) for extraction.

Concentration (ug/g) = Concentration calculated (ug/ml)) / (weight of plant (g) / volume of solvent (ml)

Cell Counting and Viability Assessment of *S. Cerevisiae*: Aqueous plant extracts (0.2ml) were prepared and mixed with freshly activated yeast culture (0.5ml) for 20 days incubation. The resulting mixed aqueous extracts and freshly prepared control sample *(S. cerevisiae)* (0.1ml) were separately mixed with commercially available trypan blue dye (0.01ml) on a glass slide and immediately observed under a microscope. An appropriate amount of mixture (2µl) was then applied at the center of the hemocytometer using a micropipette. The mixture was drawn into the chamber by capillary action and left to sit for 2-3 minutes before counting. The cells in all 9 squares were counted in the order of "S." according to the conventional counting principle wherein cells on the top and the left boundaries are counted, whereas, cells on the bottom and the right boundaries are not counted [27]. Cells were manually counted using a 40× objective, and snapshot images were taken. With the supposition that dead cells retain stain while viable cells unretain stain, viable transparent (unstained), and dead blue (stained) cells were counted. Concentration was calculated, and cell viability, viable and dead cells were calculated using the following equations [28]:

Cell Viability = (Number of viable cells) / (Number of dead cells)

Viable cells (%) = (Number of live cells) / (Total number of cells) ×100

Dead cells (%) = (Number of dead cells) / (Total number of cells) × 100

Statistical Analysis: Methods such as replicative life span modelling and RNA quantification were analyzed by one-way ANOVA: Single factor. For HPLC, a t-test: paired two samples for mean was used. Significance was set at $P< 0.05$ unless otherwise stated.

RESULTS:

Growth of *S. Cerevisiae*: *S. cerevisiae* activated using sugar fermentation **Fig. 1A**. Single colonies appeared on PDA media **Fig. 1B,** and yeast culture obtained using PDB media **Fig. 1C.**

FIG. 1: *S. CEREVISIAE* GROWTH. (A) ACTIVATION OF YEAST IS CHARACTERIZED BY THE RELEASE OF ETHANOL AND FOAMING CAUSED BY CO_2 BUBBLES. (B) SINGLE COLONY ISOLATION (C) YEAST CULTURE

RLS of *S. Cerevisiae*: Methanolic extracts with yeast cultures for studying RLS in yeast ae shown in **Fig. 2 and Table 1**.

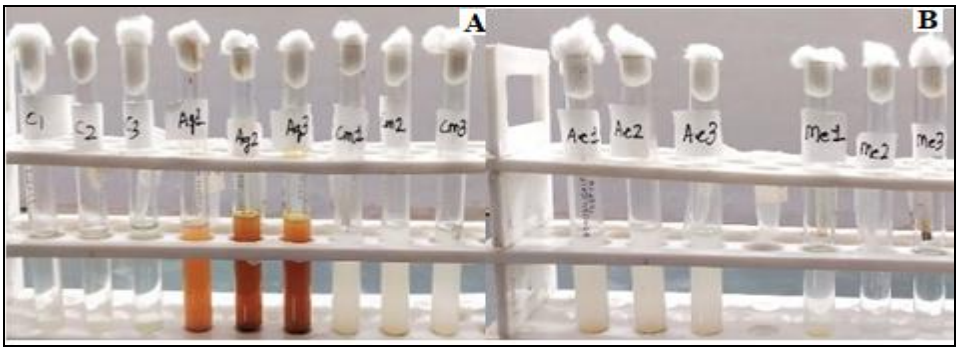

FIG. 2: SAMPLES IN TRIPLICATES. (A) L-R: TEST TUBES (1-3): CONTROL (YEAST), (4-6): *A. GALANGAL* + YEAST, (7-9): *C. MUKUL* + YEAST (B) L-R: TEST TUBES (1-3): *A. CALAMUS* + YEAST, (4-6): *M. ESCULENTA* + YEAST

TABLE 1: REPLICATIVE LIFE SPAN

Samples	Absorbance at 600nm
Control	0.012 ± 0.002
A. galangal	1.054 ± 0.001
C. mukul	0.335 ± 0.003
A. calamus	1.053 ± 0.002
M.esculenta	0.105 ± 0.024

Values are represented as Mean ± S.D. (n=5) and analyzed by one-way ANOVA: Single factor statistical method (*P* 0.00028).

Qualitative and Quantitative Assessment of RNA: Fig. 3 shows an agarose image of isolated RNA from *S. cerevisiae* and a quantitative analysis of RNA **Table 2**.

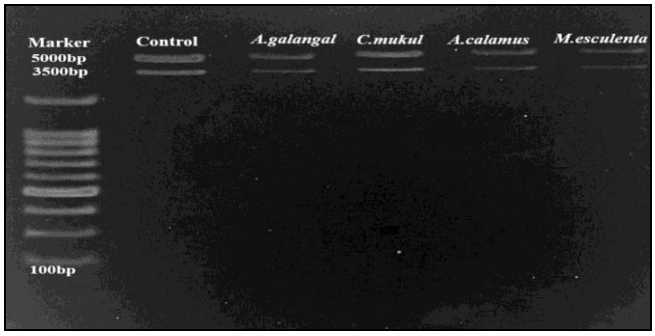

FIG. 3: RNA QUALITATIVE ANALYSIS. DENATURING AGAROSE GEL ELECTROPHORESIS OF TOTAL EXTRACTED RNA (7ML) STAINED WITH ETHIDIUM BROMIDE. FOR THE POSITIVE CONTROL, TOTAL RNA WAS EXTRACTED FROM *S. CEREVISIAE*

TABLE 2: ANALYSIS OF RNA CONCENTRATION, YIELD AND PURITY

Samples	Concentration of RNA sample (µg/ml ± S.D)	Total yield (µg ± S.D)	Purity (%)
Control	288 ± 13.063	20.16 ± 0.914	1.942 ± 0.212
A. galangal	336 ± 14.236	23.52 ± 0.996	2.120 ± 0.531
C.mukul	290.666 ± 11.469	20.346 ± 0.802	1.934 ± 0.187
A. calamus	292 ± 8.640	20.44 ± 0.604	2.050 ± 0.112
M.esculenta	332 ± 6.531	23.24 ± 0.457	2.166 ± 0.068

Values are represented as Mean ± S.D. (n=5) and analyzed by one-way ANOVA: Single factor statistical method (*P* 0.0021).

Primer Designing: Primers for *TOR 1* and *ACT1* were designed as shown in **Table 3**. *TOR1* showed significant homology only to *S. cerevisiae*, whereas *ACT1* was not only specific to *S. cerevisiae* but also to *Torulaspora delbrueckii* and *Scheffersomyces stipites.*

TABLE 3: PRIMER PARAMETERS FOR *ACT1* AND *TOR1*

Name /Gene ID	Gene Description	Accession number	Sequence (5'->3')	Tm (°C)	GC (%)	Amplicon length (bp)
TOR1	Phosphatidylinositol kinase-related protein kinase TOR1 [*Saccharomyces cerevisiae S288C]*	NM_001181724.1	F: TTACCACAC TGCCGTGATCC R:CTTTGTAGG TTCCCGCTGGT	60.04 59.96	F:55.00 R:55.00	492
ACT1	Actin[Saccharomyces cerevisiae *S288C]*	NM_001179927.1	F:TCGTTCCAATTTACGCTGGTT R: CGGCCAAATCG ATTCTCAA	58.50 56.01	F:42.86 R:47.37	60

TOR1: target of rapamycin, *ACT1:* actin, Tm: melting temperature, GC: guanine-cytosine, F: forward, R: reverse, bp: base pairs

Gene Expression Studies: Amplification plot and standard curves were generated from cDNA dilutions **Fig. 4**. Relative quantification of *TOR1* among test samples shown in **Table 4 and Fig. 5.**

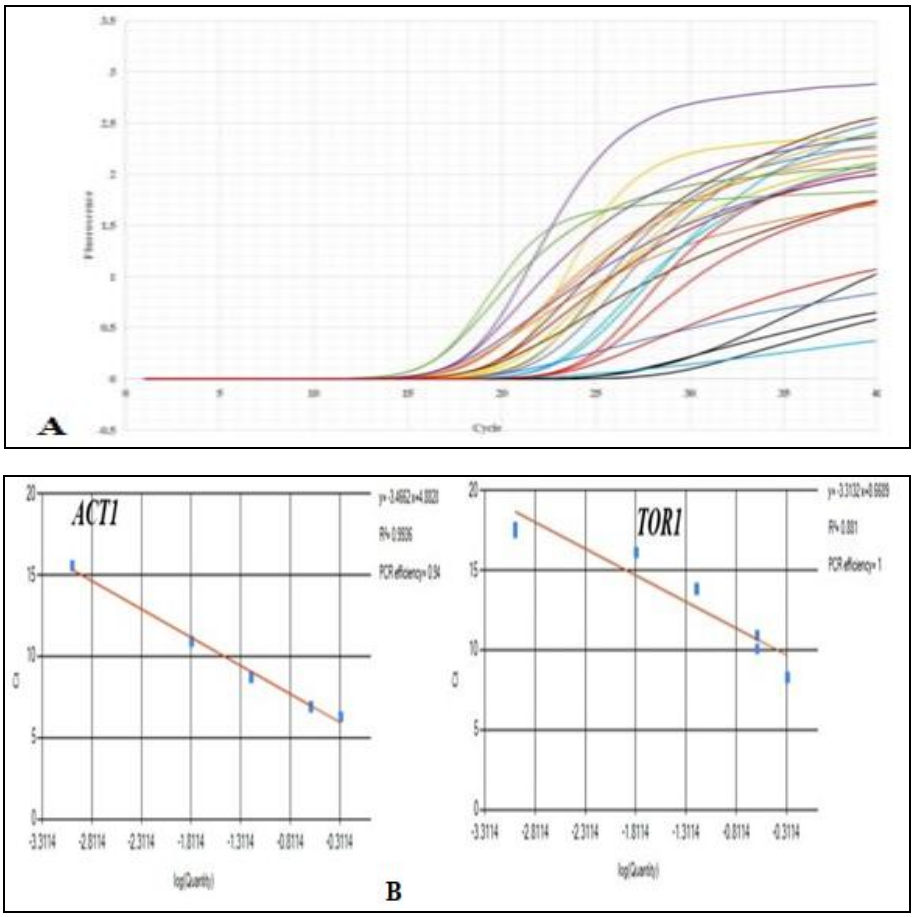

FIG. 4: REGRESSION CURVES (A) LOG AMPLIFICATION CURVE PLOTTED WITH FLUORESCENCE AGAINST CYCLE NUMBERS (B) STANDARD CURVES WITH C_T PLOTTED AGAINST LOG OF STARTING QUANTITY OF CDNA. EQUATION FOR THE REGRESSION LINE AND COEFFICIENT OF DETERMINATION (R^2) ARE SHOWN. PCR EFFICIENCY FOR *ACT1* AND *TOR1* 94.31% AND 100.36%

TABLE 4: RELATIVE QUANTIFICATION OF *TOR1* IN *S. CEREVISIAE*

Parameters	Control	*A. galangal*	*C. mukul*	*A. calamus*	*M. esculenta*
C_T ACT1	23	14.1	12.2	13.7	18.9
	23.2	14.3	12.3	13.6	18.87
	23.2	14.1	12.2	13.4	18.89
C_T TOR1	17	16.3	15.2	15.2	20.21
	17.3	16.6	15.28	15.21	20.26
	17.2	16.2	15.25	15.26	20.24
Δ C_T	-6	2.2	3	1.5	1.31
	-5.9	2.3	2.98	1.61	1.39
	-6	2.1	3.05	1.86	1.35
ΔΔ C_T	0	8.2	9	7.5	7.31
		8.2	8.88	7.51	7.29
		8.1	9.05	7.86	7.35
Negative ΔΔ Ct	0	-8.2	-9	-7.5	-7.31
		-8.2	-8.88	-7.51	-7.29
		-8.1	-9.05	-7.86	-7.35
Normalized	1	0.003	0.001	0.005	0.006
expression ratio	1	0.003	0.002	0.005	0.006
(2-ΔΔCt)	1	0.003	0.001	0.004	0.006
Mean (2-ΔΔCt)	1	0.003 ± 0.001	0.001 ± 0.001	0.005 ± 0.006	0.006 ± 0.001

TOR1: target of rapamycin, *ACT1*: actin, C_T: cycle threshold. Values expressed as Mean± S.D.

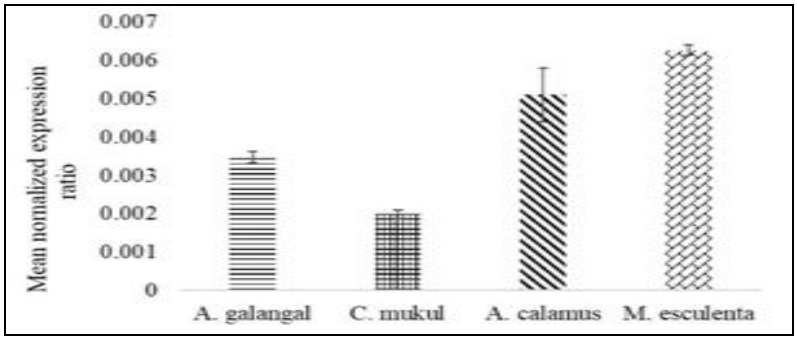

FIG. 5: RELATIVE COMPARISON OF *TOR1* EXPRESSION AMONG SAMPLES. NORMALIZED EXPRESSION RATIO / FOLD DECREASE VALUES OF *TOR1* IN *S. CEREVISIAE* IN THE PRESENCE OF TEST SAMPLES (METHANOLIC EXTRACTS)

HPLC of Standards and Crude Extracts: Chromatograms of standard antioxidants and samples are shown in **Fig. 6** and **Fig. 7**. Standard parameters for phenolics and flavonoids are shown in **Table 5**. Antioxidants in plant extracts were identified, and their concentrations were calculated in **Table 6**.

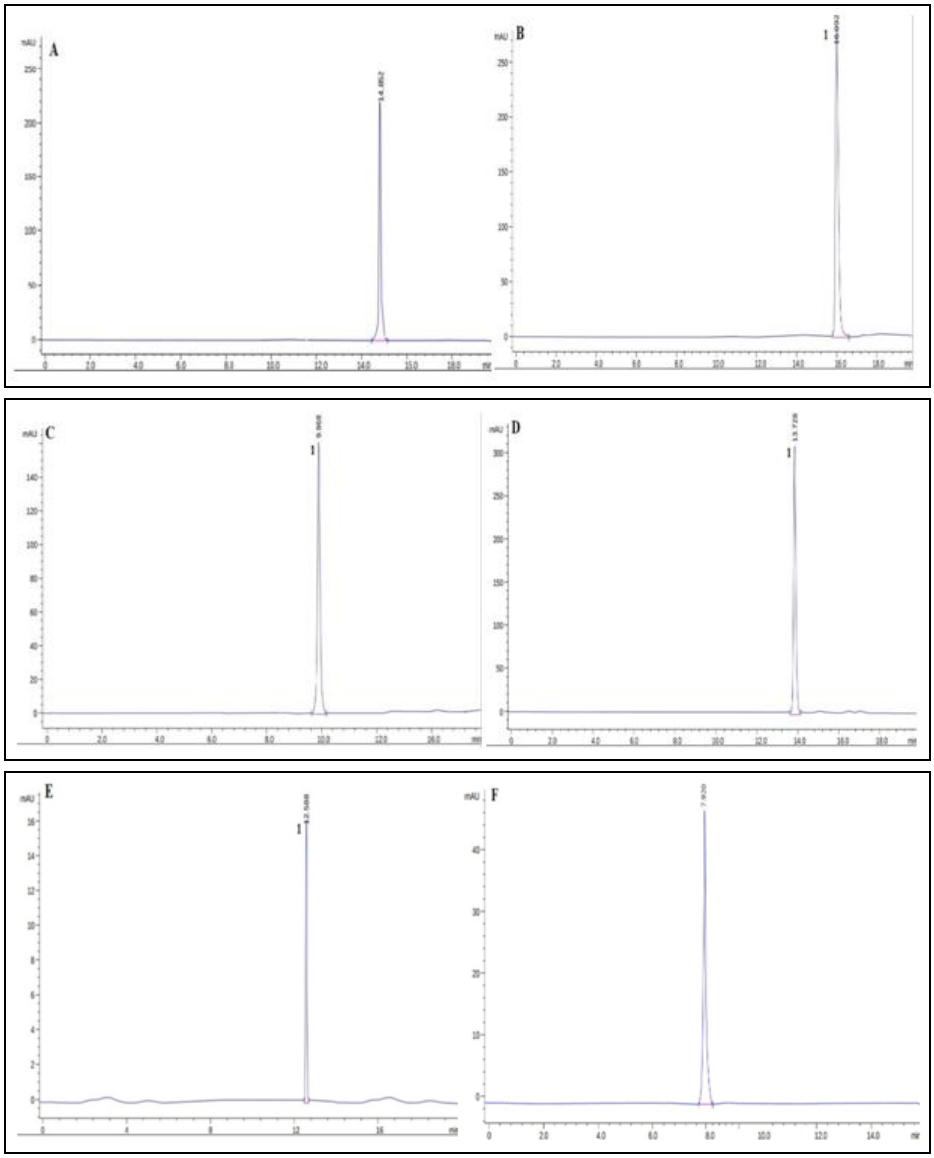

FIG. 6: HPLC CHROMATOGRAMS OF STANDARD PHENOLICS AND FLAVONOIDS (A) CAFFEIC ACID (RT: 14.852 MIN) (B) GALLIC ACID (RT: 16.092 MIN) (C) VANILLIN (RT: 9.968 MIN) (D) RUTIN (RT: 13.728 MIN) (E) RESVERATROL (RT: 12.588 MIN) (F) QUERCETIN (RT: 7.92 MIN)

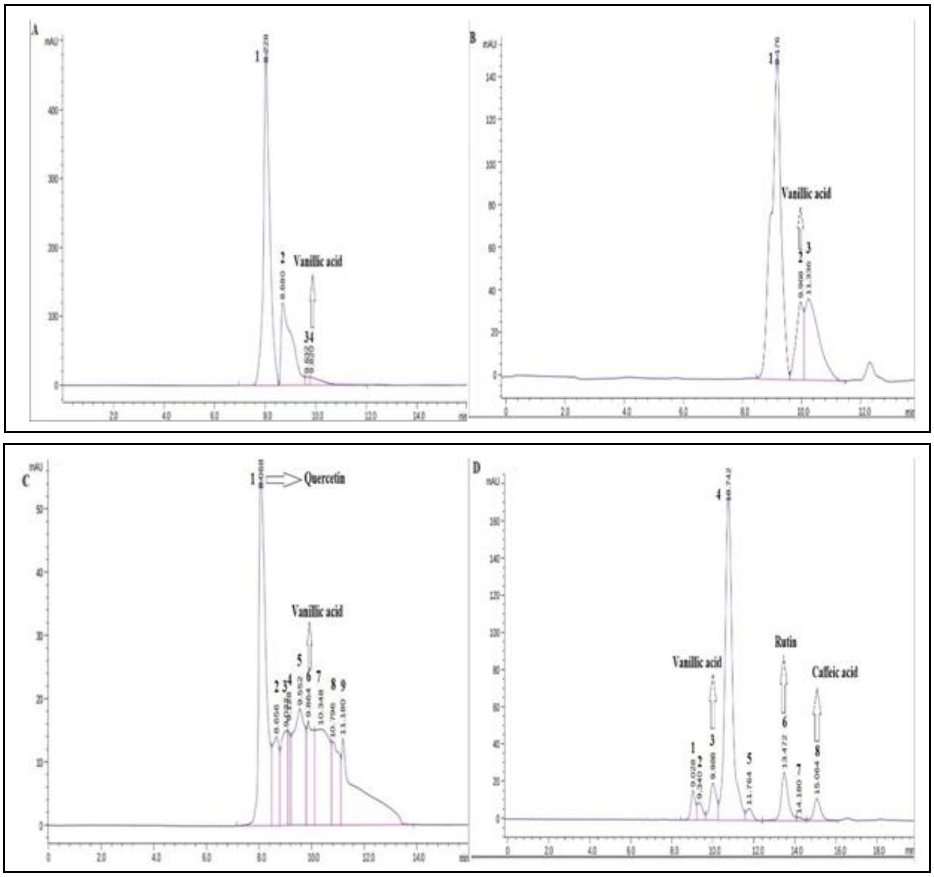

FIG. 7: HPLC CHROMATOGRAMS OF SAMPLES (A*) A. GALANGAL* EXTRACT (VANILLIC ACID AT 9.820 MIN) (B) *C. MUKUL* (VANILLIC ACID AT 9.968 MIN) (C) *A. CALAMUS* (QUERCETIN AND VANILLIC ACID AT 8.068 AND 9.864 MINS) (D) *M. ESCULENTA* (VANILLIC ACID, RUTIN, AND CAFFEIC ACID AT 9.988, 13.472 AND 15.064 MINS)

TABLE 5: ANALYTICAL PARAMETERS FOR HPLC ANALYSIS

Standards	Detection wavelength (nm)	RT (min)	m	c	R^2	LOD (µg/ml)	LOQ (µg/ml)
Gallic acid	278	16.001 ± 0.09	24.701	911.5	1	0.256	0.855
Caffeic acid	300	14.751±0.174	1.779	1058.8	1	0.298	0.996
Vanillic acid	254	9.966 ± 0.001	2.475	584.49	1	0.296	0.988
Resveratrol	305	12.587±0.005	2.508	3.884	1	0.128	0.427
Quercetin	254	7.92 ± 0.02	3.558	97.468	1	0.154	0.514
Rutin	300	13.727±0.005	24.937	1170	1	0.256	0.855

RT: retention time, m: slope, c: intercept, R^2: regression coefficient, LOD: limit of detection, LOQ: limit of quantification. Values expressed as Mean ± S.D.

TABLE 6: HPLC QUANTIFICATION OF STANDARD COMPOUNDS IDENTIFIED IN PLANTS

Antioxidant standards	*A. galangal*	*C.mukul*	*A. calamus*	*M. esculenta*
Gallic acid	nd	nd	nd	nd
Caffeic acid	nd	nd	nd	10.990 ± 0.001
Vanillic acid	17.652 ± 0.017	27.439 ± 0.0005	13.206 ± 0.0005	20.393 ± 0.001
Resveratrol	nd	nd	nd	nd
Quercetin	nd	nd	150.017 ± 0.003	nd
Rutin	nd	nd	nd	9.720 ± 0.002

n.d.: not detected. Values expressed as Mean ± S.D. in (µg/g)and analysed by t-test: paired two sample for means; significant at (P<0.05).

Cell Viability of *S. Cerevisiae*: *S. cerevisiae* live and dead cells were observed from trypan blue staining **Fig. 8**. Concentration and cell viability values were obtained for control and test samples **Table 7**.

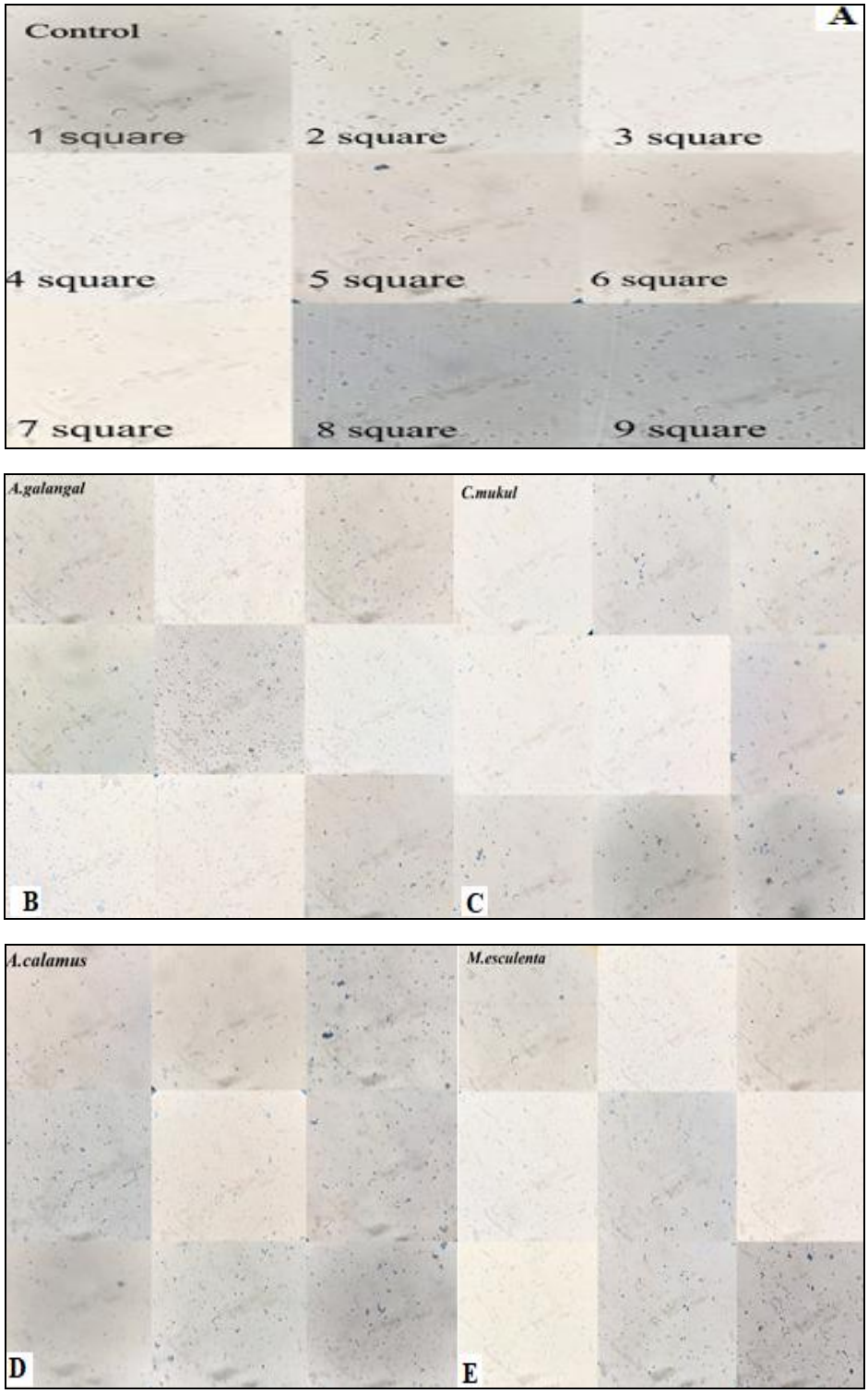

FIG. 8: IMAGES OF YEAST CELLS TAKEN FROM 9 SQUARES OF HEMOCYTOMETER IN WHICH DEAD CELLS ARE INDICATED BY BLUE COLOR AND LIVE CELLS ARE UNSTAINED. (A) YEAST AS CONTROL (B) YEAST+ *A. GALANGAL* (C) YEAST +*C. MUKUL* (C) YEAST +*A. CALAMUS* (D) YEAST +*M. ESCULENTA*

TABLE 7: CELL VIABILITY OF S. CEREVISIAE

Samples	Concentration (cells/ml)	Viable cells (%)	Dead cells (%)	Cell viability
Control	$4.52 *10^5$	87.057	12.942	6.726
A. galangal	$1.809*10^6$	86.677	13.322	6.506
C. mukul	$5.665*10^5$	46.513	53.486	0.869
A. calamus	3.98*105	28.607	71.392	0.400
M. esculenta	$1.114*10^6$	87.730	12.269	7.15

DISCUSSION: *S. cerevisiae* has been proven to be an appropriate reverse aging model because we have provided evidence about reduced *TOR1* activated autophagyon lifespan extension of yeast without starvation by all four methanolic plant extracts *A. galangal, C. mukul, A. calamus and M. esculenta*. Reverse aging was studied in two ways which are *TOR1* gene expression analysis and RLS. Pharmacological anti-aging screens in yeast performed by RLS in *S. cerevisiae* in the presence of extracts showed an increase in absorbances (0.105 ± 0.024 - 1.054 ± 0.001) compared to the control yeast sample (0.012 ± 0.002) due to decrement of *TOR1* activity. Gene expression studies revealed *M. esculenta* with the highest reverse aging effect with a mean normalized expression value of 0.006 and the lowest *C. mukul* with a value of 0.001.

Results from HPLC indicated the presence of vanillic acid as the major phenolic compound present in all four methanolic extracts. Only *M. esculenta* showed the presence of a maximum number of antioxidants consisting of vanillic acid (20.393 ± 0.001µg/g), rutin (9.720 ± 0.002µg/g), and caffeic acid (10.990 ± 0.001µg/g). Our result contradicts another study that reported the absence of vanillic acid, rutin, and caffeic acid in *M. esculenta* methanolic extract [29]. Cell viability assay performed to check the effect of aqueous extracts on the longevity of *S. cerevisiae* cells showed the highest percentage of viable cells in the presence of *M. esculenta* extract with a value of 87.73% followed by *A. galangal* extract (86.67%) as compared to control yeast sample (87.05%).

These results suggested the strongest survival effect of *S. cerevisiae* cells conferred by *M. esculenta* whereas *A. galangal* was at the borderline. By contrast, the lowest percentage of viable cells was observed by *A. calamus* (28.60%) followed by *C. mukul* (46.51%). Cell viability studies have been reported earlier in *A. calamus* using methylene blue for anticancer study [30]. A major controversy that the results of our study might raise is since it is proven from the results of gene expression studies that methanolic extracts of A. *calamus* and *C. mukul* elicit reverse aging effect in *S. cerevisiae* but results from cell viability assay are contradictory to the data that *A. calamus* and *C. mukul* are toxic to *S. cerevisiae* cells. Two main facts might resolve this controversy: the first is that plants make self - poisonous compounds and the second is heavy metal toxicity in plants due to their growth in contaminated areas. These two facts might be the reasons of death of *S. cerevisiae* cells by *A. calamus* and *C. mukul* extracts. Our study showed that *M. esculenta* might be a promising reverse aging herb based on gene expression studies, HPLC, and cell viability assay results. Results from HPLC showed the presence of specific phenols and flavonoids in plant extracts; however, other antioxidants might also contribute to the reverse aging property of these plants, which requires further research. Future directions for our study might be the development of natural drugs focused on improving the health of the aging population and the formulation of herbaceutical products for reverse aging using our proven extracts.

Furthermore, cultivating plants in a healthy environment free from heavy metal contamination might make the plants nontoxic to yeast cells. Alternatively, plants could be grown along the river bed so that heavy metals from plants get absorbed by water which can be treated by wastewater treatment. Moreover, the exact mechanisms by which plant antioxidants activate autophagy by reduced *TOR1* remains obscure and requires further exploration. In conclusion, our bodies will be biologically younger for longer if the rate of aging could be modulated at a genetic level since aging is amenable to therapeutic manipulations, regardless of our chronological age.

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