Supplementary Data Set

Unlike morphine, long-term exposure to analgesic mitragynine, 7-hydroxymitragynine, paynantheine, and speciociliatine alkaloids does not contribute to antinociceptive tolerance of μ-opioid receptors

Fatemeh Elahian [1], Sorour Zahedian [1], Mohsen Safaei [1], Elham Pahlevani-Gazi [1], and Seyed Abbas Mirzaei [1,2*]

[1] Department of Medical Biotechnology, School of Advanced Technologies, Shahrekord University of Medical Sciences, Shahrekord, Iran.

[2] Cellular and Molecular Research Center, Basic Health Sciences Institute, Shahrekord University of Medical Sciences, Shahrekord, Iran.

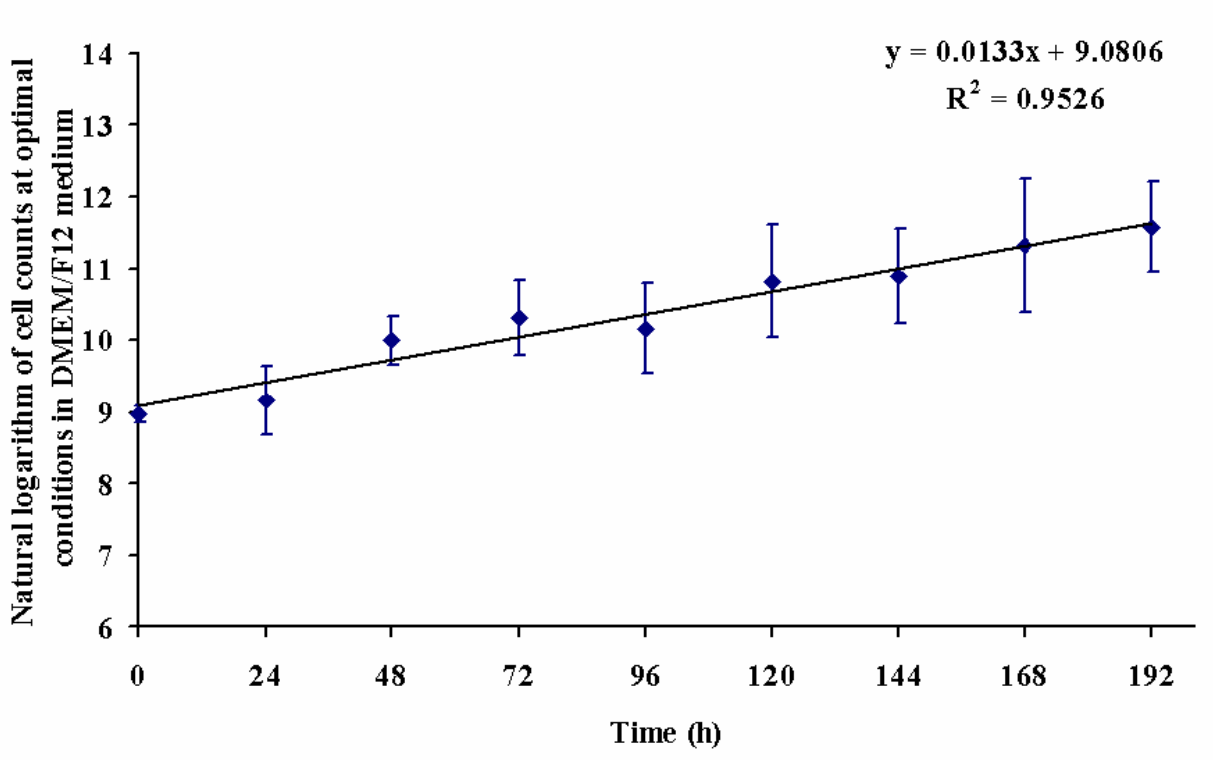
*Author for correspondence:

S. A. Mirzaei, Pharm.D, Ph.D.

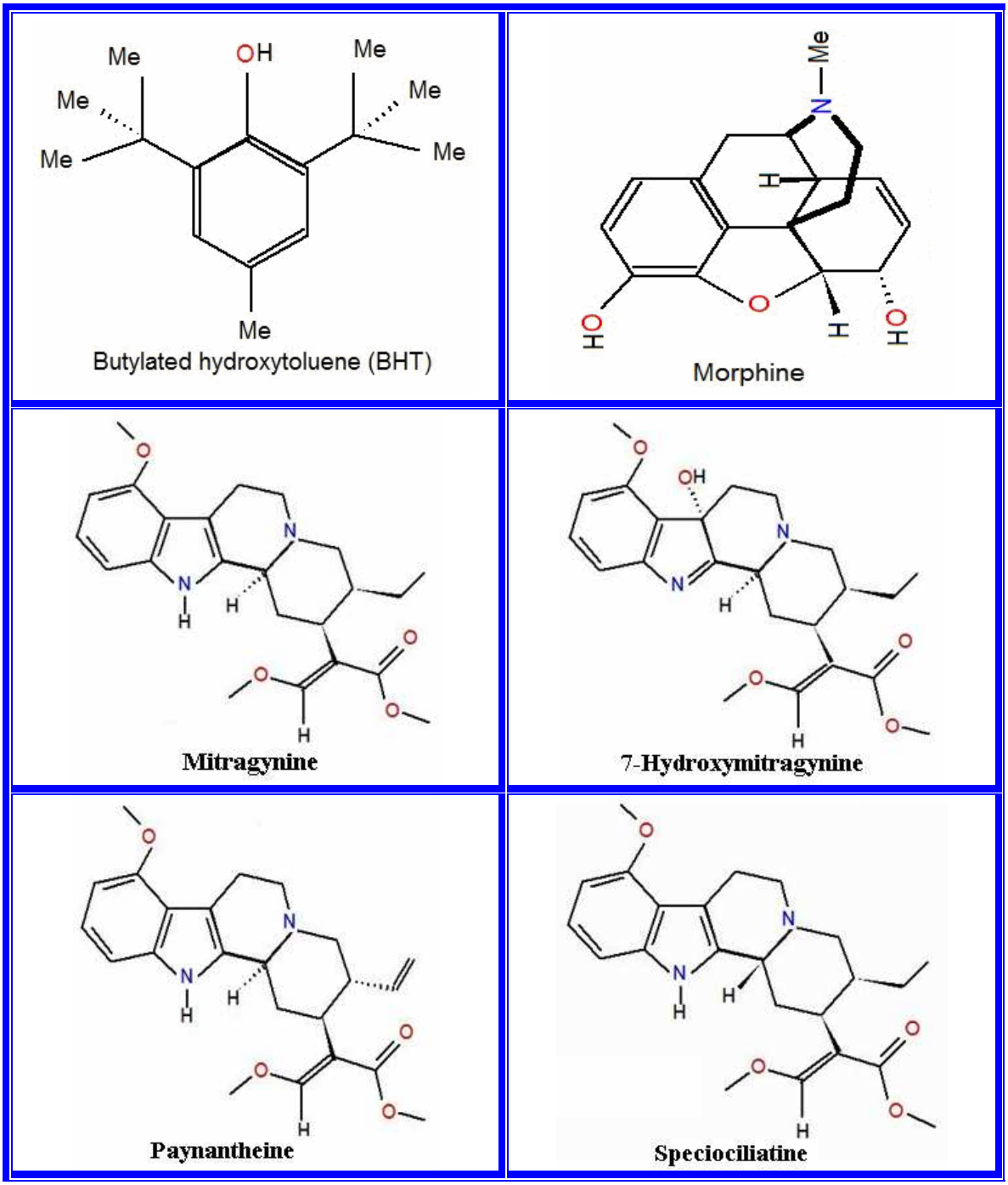
Cellular and Molecular Research Center, Basic Health Sciences Institute, Shahrekord University of Medical Sciences, Shahrekord, Iran.

Tel: (+98) 38 33331471, Fax: (+98) 38 33330709

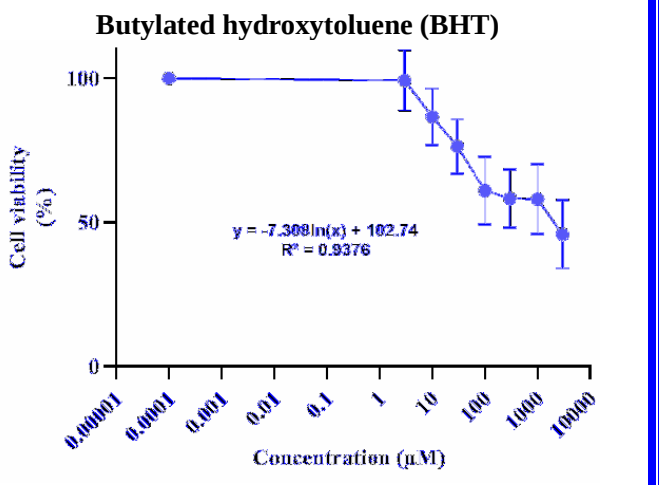
E-mail: mirzaei.a@skums.ac.ir, dr_amirzaei@yahoo.com

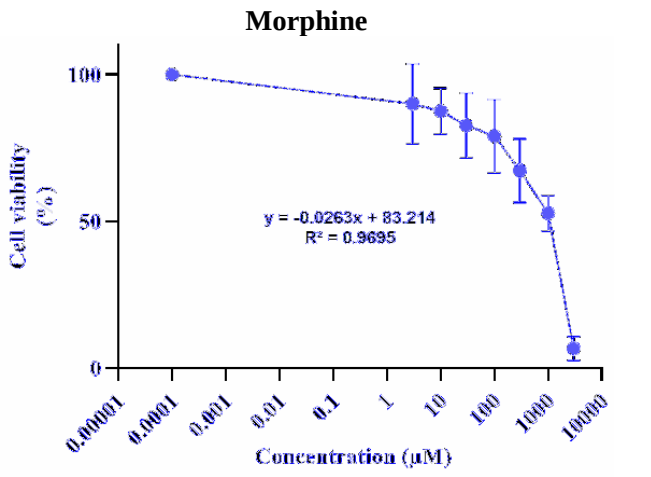


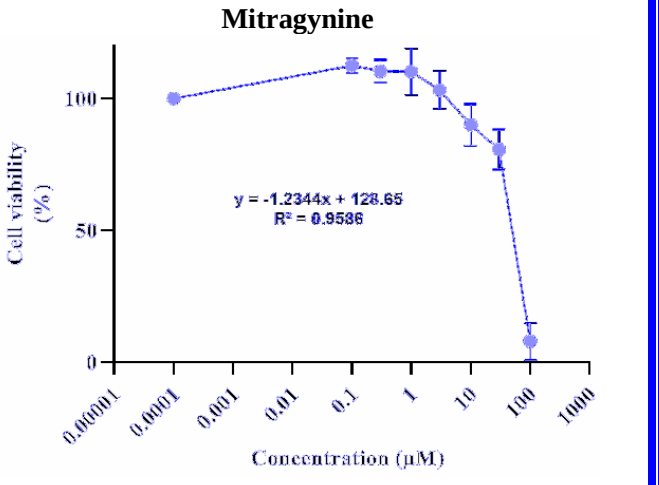
Supplementary Figure S1. Growth kinetics curve. SH-SY5Y cells were seeded at a density of 8000 cells /well in a 96-well plate and the growth curve was depicted during the 8 days in the DMEM/F12 medium at optimal condition.

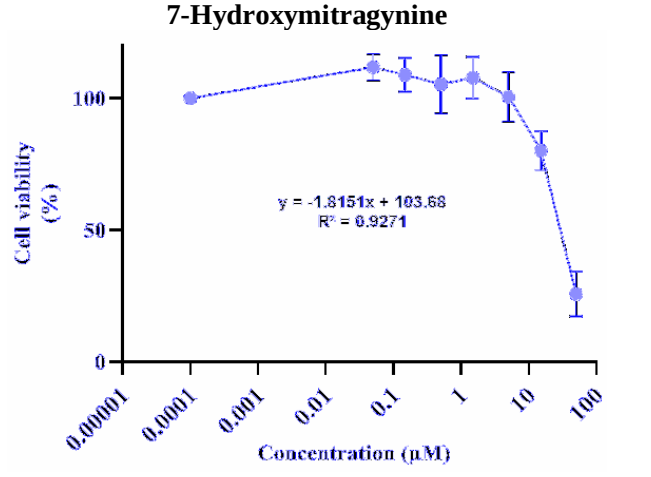


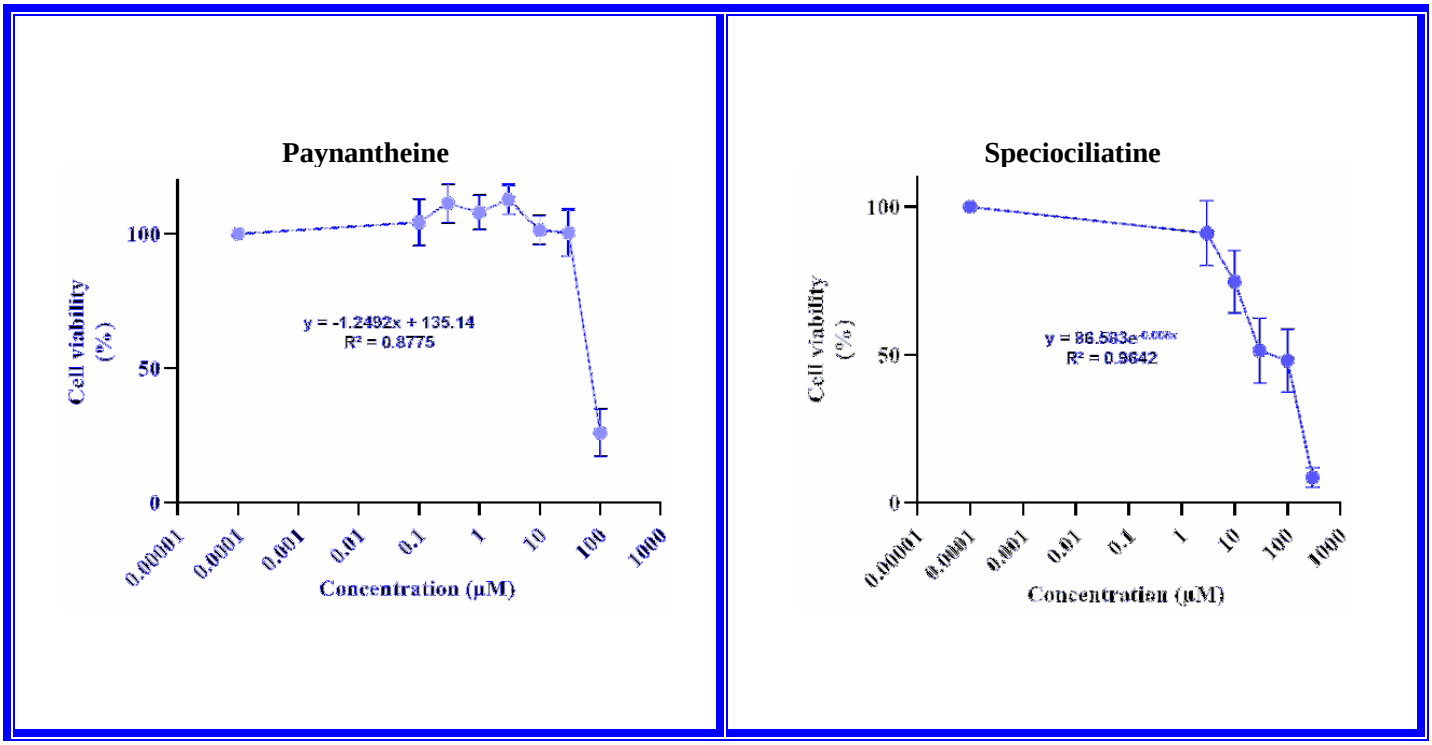
Supplementary Figure S2. Chemical structure of butylated hydroxytoluene, morphine, mitragynine, 7-hydroxymitragynine, paynantheine, and speciociliatine.


Butylated hydroxytoluene (BHT)
Cell viability (%)
y = -7.308ln(x) + 102.74
R² = 0.9376
Concentration (µM)


Morphine
Cell viability (%)
y = -0.0263x + 83.214
R² = 0.9695
Concentration (µM)

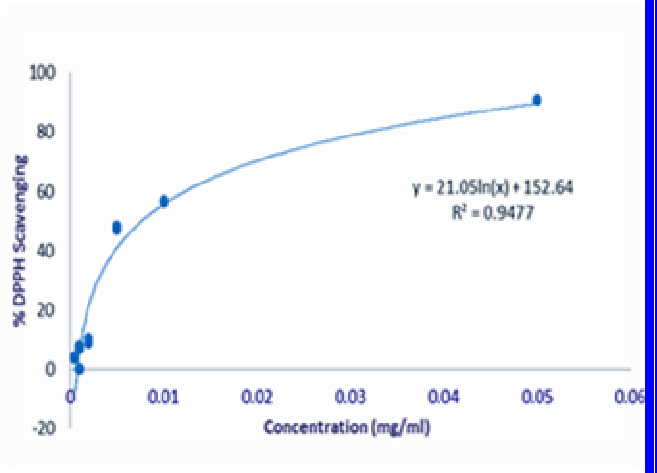

Mitragynine
Cell viability (%)
y = -1.2344x + 128.65
R² = 0.9586
Concentration (µM)


7-Hydroxymitragynine
Cell viability (%)
y = -1.8151x + 103.68
R² = 0.9271
Concentration (µM)

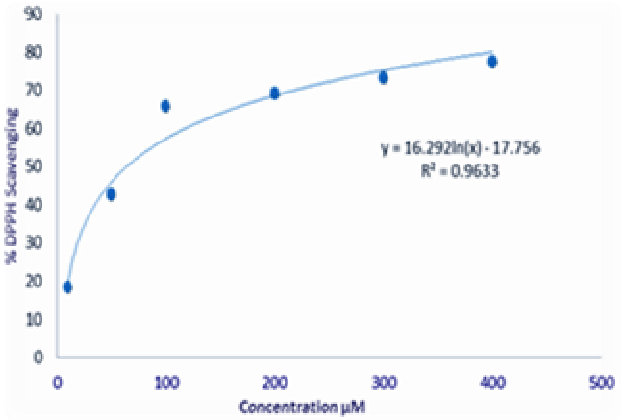


Supplementary Figure S3. Effects of Butylated hydroxytoluene (BHT), morphine, mitragynine, **7**-hydroxymitragynine, paynantheine, and Speciociliatine on the survival of SH-SY5Y cell. The cells were cultured for 5 days with increasing doses (0, 0.1, 0.3, 1, 3, 10, 30, 100 and 300 µM) of each these compounds. Cell survival was measured by MTT assay. The values represent the means of three independent experiments performed in triplicate (Mean ± SE).

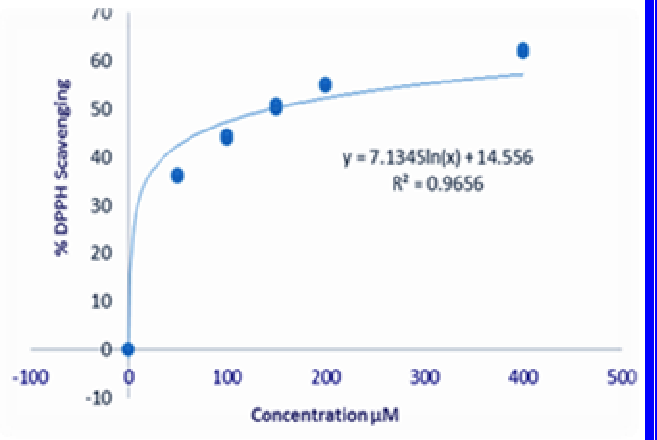
Butylated hydroxytoluene (BHT)



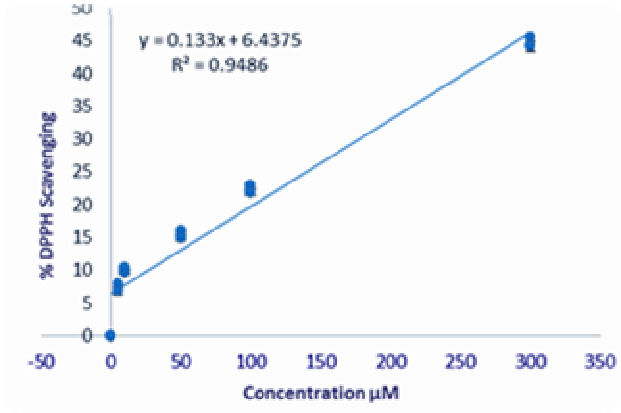
Morphine

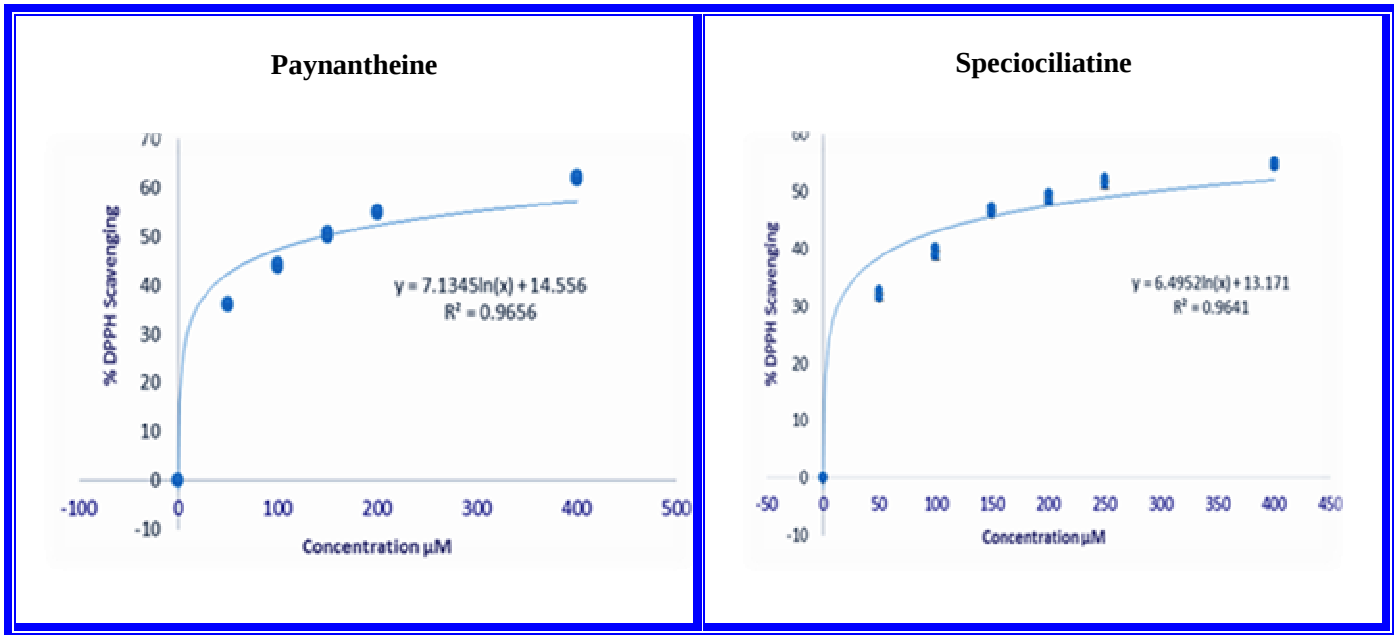


Mitragynine



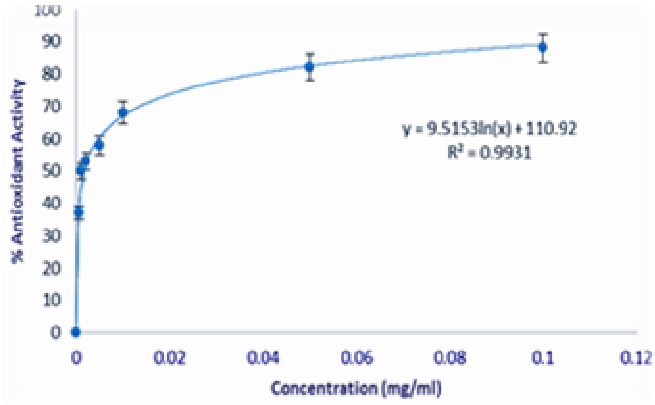
7-Hydroxymitragynine



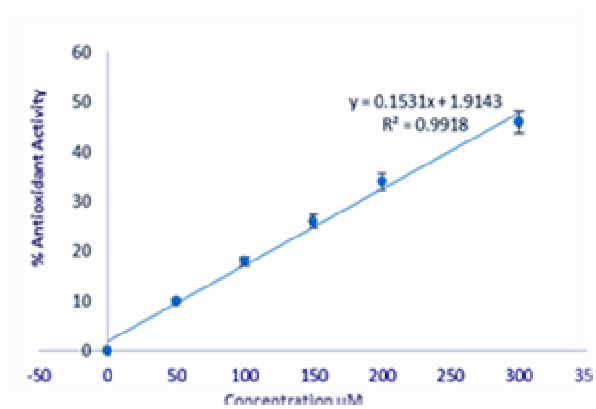


Supplementary Figure S4. Safety analysis of the alkaloids. Antioxidant effects of alkaloid compounds base on DPPH assay. Two milliliter of a fresh DPPH stock was added to various concentrations of each alkaloid (0-400 µM) and placed in the dark for 30 min. Scavenging capacity percentage was determined by [(control absorbance – sample absorbance) × 100 / control absorbance] equation. Where controls containing all reagents except the antioxidant factors. The scavenging capacity-50 (SC_{50}) is an alkaloid concentration required for scavenge 50 % of DPPH radicals. Scavenging capacity-50 was calculated from the calibration curve determined by linear or non-linear regression from the scavenging capacity percentage versus alkaloid concentrations.

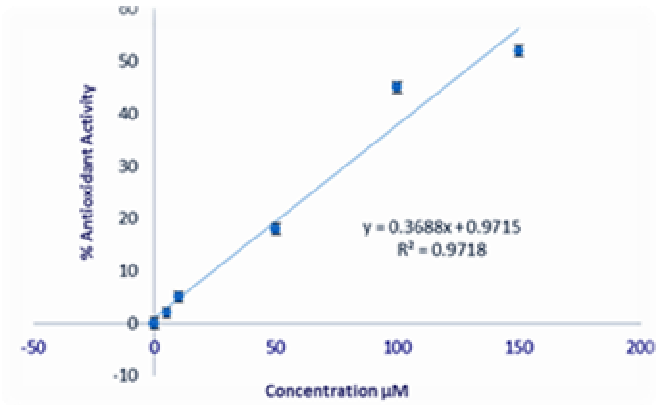
Butylated hydroxytoluene (BHT)



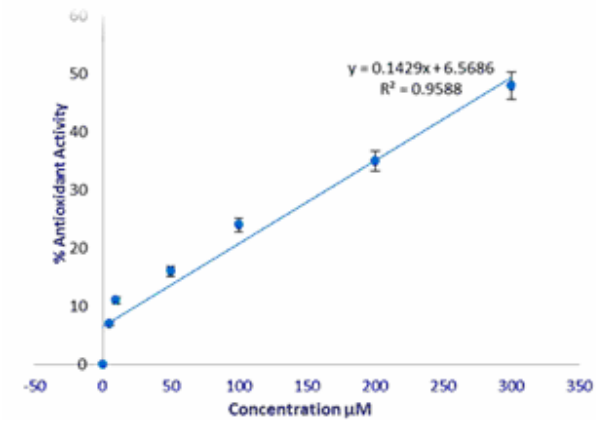
Morphine

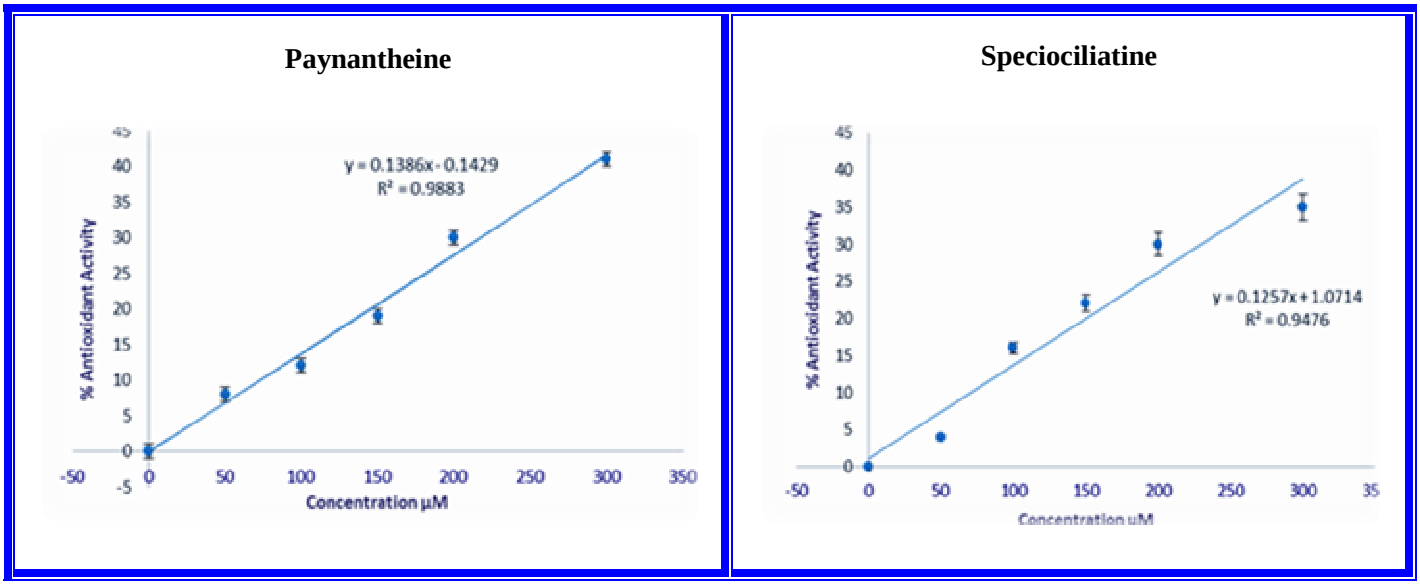


Mitragynine



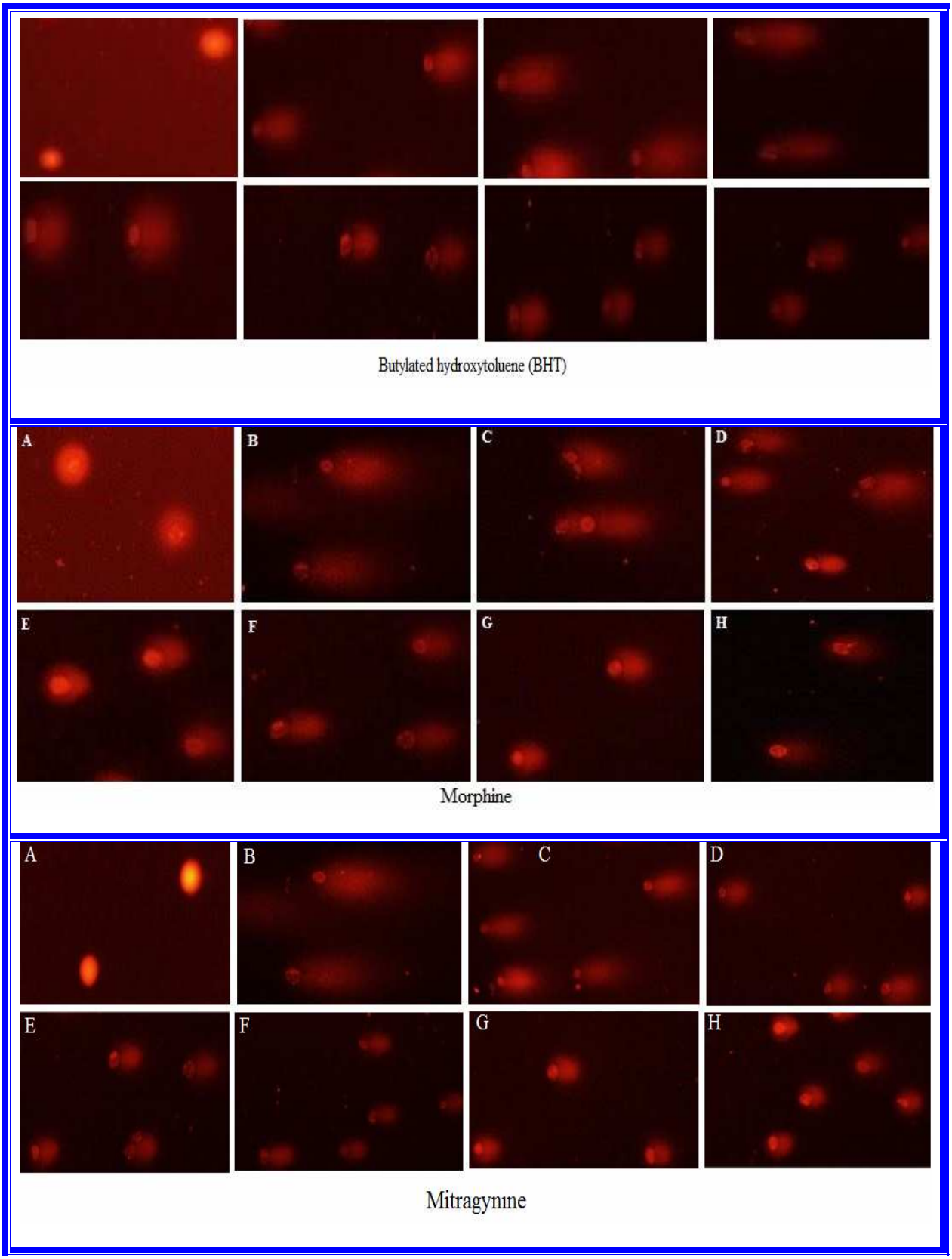
7-Hydroxymitragynine

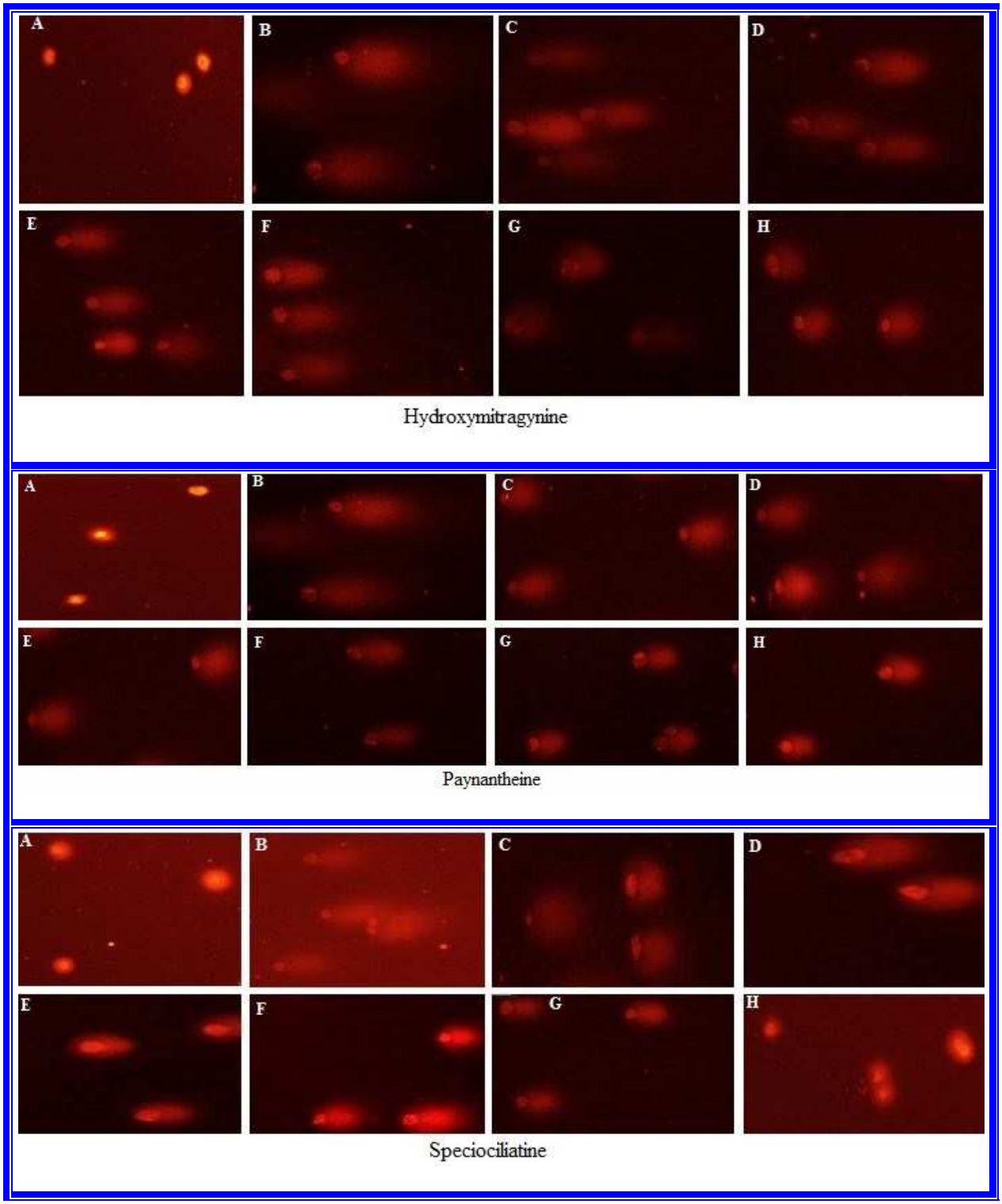




Supplementary Figure S5. Safety analysis of the alkaloids. Antioxidant effects of alkaloid compounds base on β-carotene bleaching assay. Bleaching assay was conducted using a β-carotene/linoleic acid emulsion method. Briefly, 1 mg β-carotene, 40 mg of linoleic acid, and 200 mg of Tween-20 were homogenized in 2 ml of chloroform. Then chloroform was rotary evaporated at 40 °C for 30 min and then 100 ml of oxygenated deionized water was added with vigorous shaking to form a stable emulsion. Then, 2.5 ml of the emulsion was added to 350 µl of various alkaloid concentrations (0-400 µM) and left at 50 °C in the light for 2 h and optical density was monitored at 470 nm. Bleaching inhibition capacity percentage was calculated from [(sample absorbance at time 0 – sample absorbance after 2 h) × 100 / (control absorbance at time 0 – control absorbance after 2 h)] equation. Where controls containing all reagents except the antioxidant factors. Bleaching inhibitory capacity-50 (BIC_{50}) is an alkaloid concentration required for protection of half percentage of β-carotene molecules from bleaching and is calculated from the calibration curve determined by linear or non-linear regression from the bleaching inhibition percentages versus alkaloid concentrations.

Supplementary Data Set



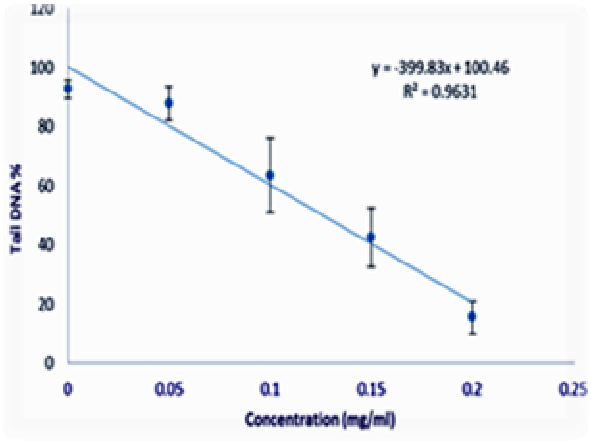


Supplementary Figure S6. Safety analysis of the alkaloids. The induced DNA damage by the alkaloids was evaluated on SH-SY5Y cells by the COMET assay conducted under alkaline

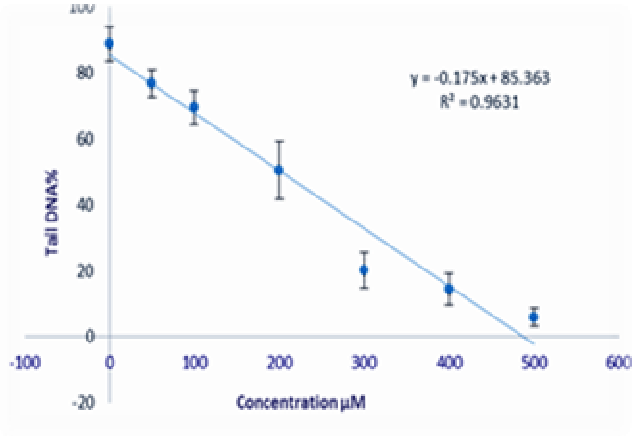
conditions. A serial dilution of each alkaloid (0 to 600 μM) was supplemented with 120 μM H_2O_2 and stored for 5 min at room temperature. Then, 10000 cells were transferred to each dilution and the suspension was stored at 4 °C for 30 min. Cells were harvested and sandwiched between two layers low melting agarose on a slide. Cells were lysed and electrophoresed. Finally, DNA were stained with ethidium bromide and pictured using a fluorescent microscope (BX51; Tokyo, Japan). Representative COMET-images from (A) untreated SH-SY5Y (negative COMET), (B) H_2O_2-treated cells (positive COMET) and (C–H) simultaneous treatment with butylated hydroxytoluene (BHT), morphine, mitragynine, 7-hydroxymitragynine, paynantheine, and speciociliatine.

Supplementary Data Set

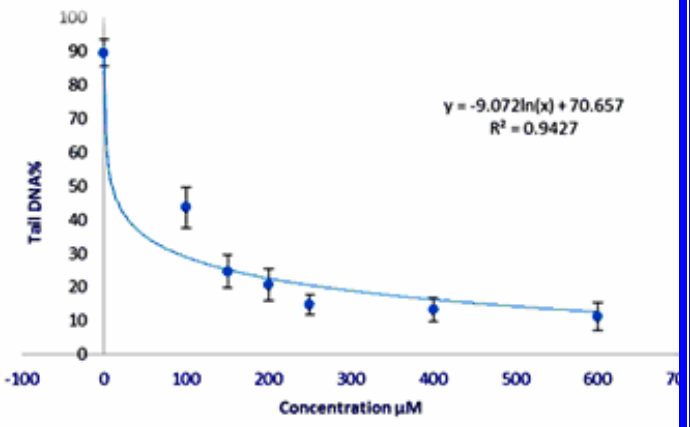
Butylated hydroxytoluene (BHT)



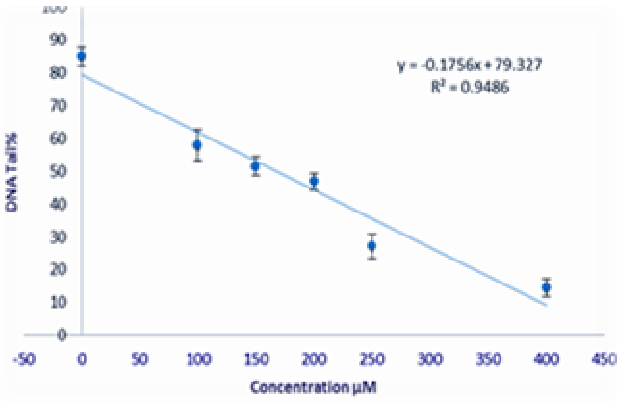
Morphine

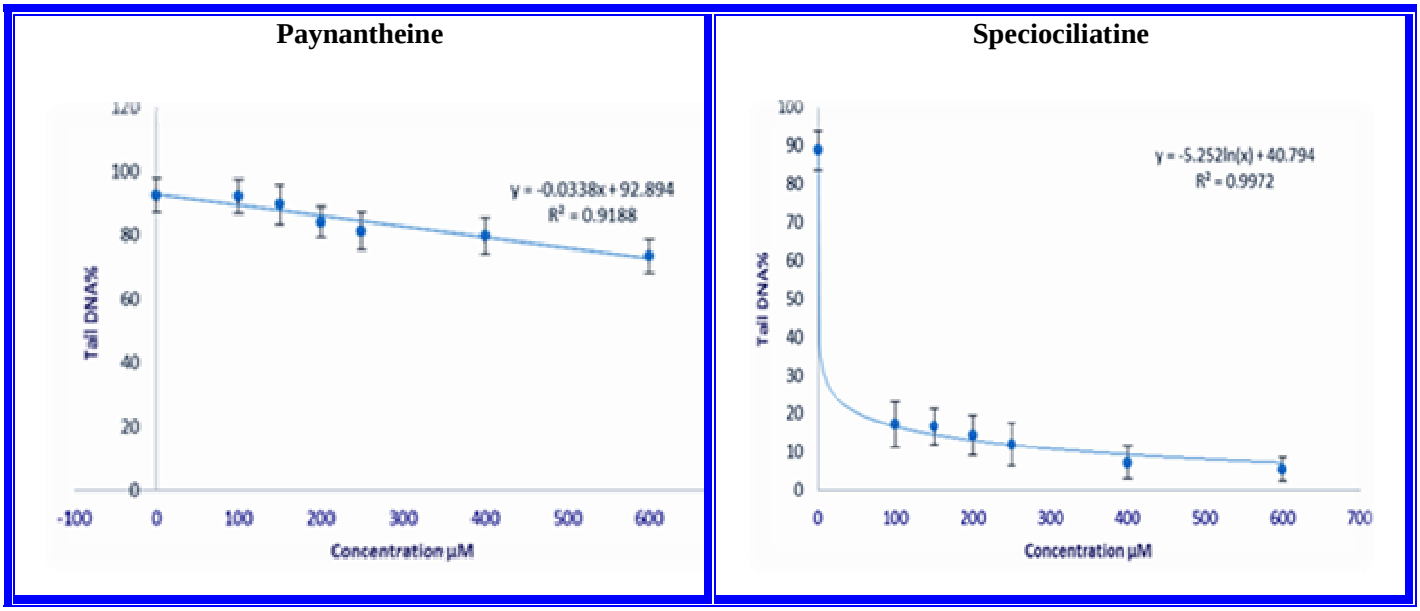


Mitragynine



7-Hydroxymitragynine





Supplementary Figure S7. Safety analysis of the alkaloids. The induced DNA damage by the alkaloids was evaluated on SH-SY5Y cells by the COMET assay conducted under alkaline conditions. The DNA damage was analyzed via Open-Comet software and Cells were determined undamaged to maximally damaged, according to tail DNA intensity [Tail DNA×100 ÷ (Head DNA + Tail DNA)], for each alkaloid concentrations. The COMET-inhibitory capacity-50 (CIC_{50}) defined as concentration of the alkaloid that diminish the tail DNA percent to 50 % against damage induced by H_2O_2, and calculated from the calibration curve determined by linear or non-linear regression from tail DNA percentage versus alkaloid concentrations.

Supplementary Data Set

Supplementary Table 1. According to the homogeneity of variances using an ANOVA analysis, Reagents and alkaloids in the same column are not statistical different (P> 0.05).

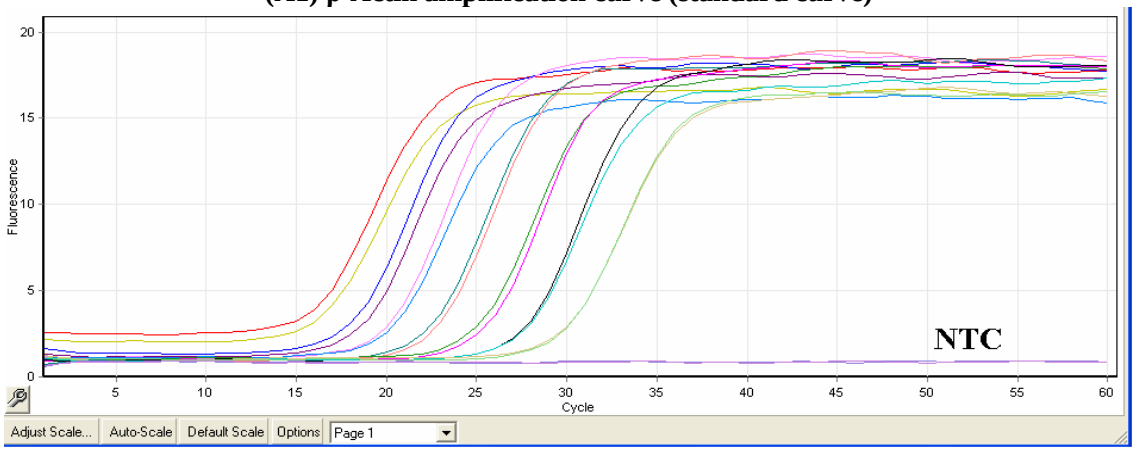
Homogenous Subsets for alpha= 0.05

	Group 1	Group 2	Group 3	Group 4	Group 5
IC_{10}	7-Hydroxy mitragynine	Paynantheine, Mitragynine, Speciociliatine,	Butylated hydroxytoluene, Morphine	--	--
IC_{50}	7-Hydroxy mitragynine	Paynantheine, Mitragynine, Speciociliatine,	Butylated hydroxytoluene, Morphine	--	--
SC_{50}	Butylated hydroxytoluene	Morphine	Mitragynine, Paynantheine	Speciociliatine	7-Hydroxy mitragynine
BIC_{50}	Butylated hydroxytoluene	Mitragynine	Morphine, 7-Hydroxy mitragynine	Paynantheine	Speciociliatine
CIC_{50}	Speciociliatine, Mitragynine	7-Hydroxy mitragynine	Morphine	Butylated hydroxytoluene	Paynantheine

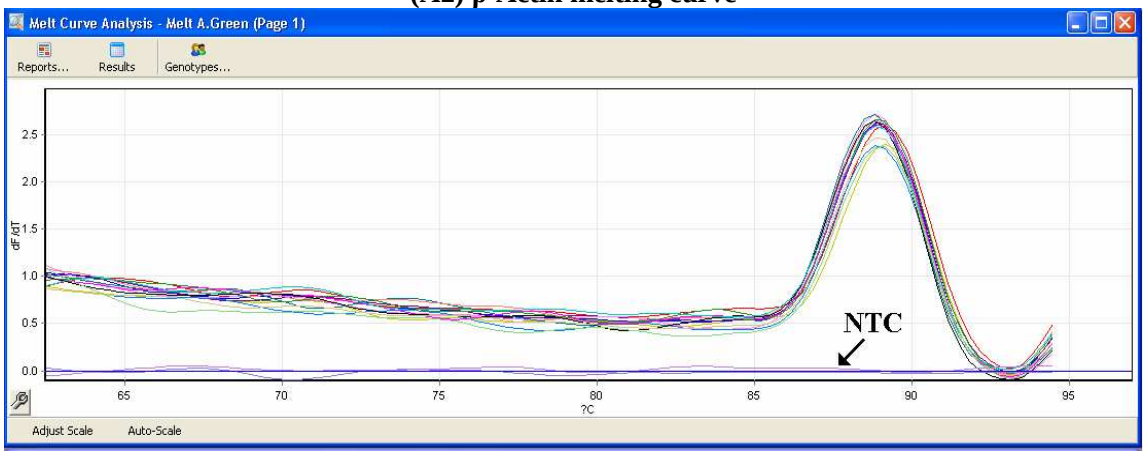
Supplementary Table 2. Primer design. The primer sets were successfully designed using Gene Runner software version 3.05 and then controlled on Primer BLAST database on NCBI

Gene Name	NCBI Gene Number	Primer Sequences	Annealing Temperture	Amplicone Size (bp)
β-Actin (ACTB)	NM_001101.5	Forward: 5′ TCA TGA AGT GTG ACG TGG ACA TC 3′ Reverse: 5′ CAG GAG GAG CAA TGA TCT TGA TCT 3′	60 °C	156
β-Arrestin-2 (ARRB2)	NM_001257330.2	Forward: 5' CGG GAC CAG GGT CTT CAA GAA G 3' Reverse: 5' CCT GGT AGG TGG CGA TGA ACA G 3'	60 °C	245
Mitogen-activated protein kinase 1 (MAPK1)	NM_002745.5	Forward: 5' GTT CTG CAC CGT GAC CTC AAG C 3' Reverse : 5' GCC AGA ATG CAG CCT ACA GAC C 3'	60 °C	224
Mitogen-activated protein kinase 3 (MAPK3)	NM_001040056.3	Forward: 5' CCT ATG ACC ACG TGC GCA AGA C 3' Reverse : 5' ATG GTC ATT GCT CAG CTG CTG G 3'	60 °C	251

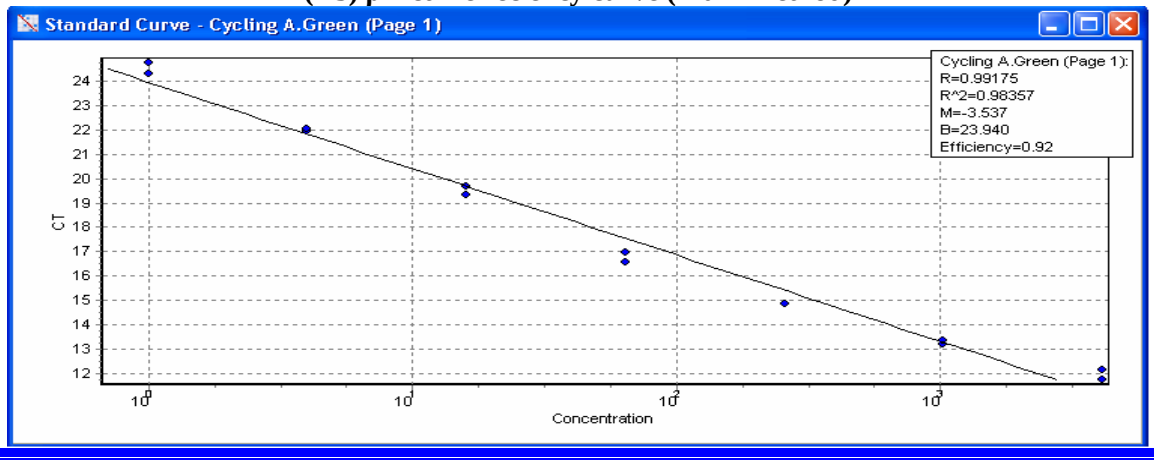
(A1) β-Actin amplification curve (standard curve)



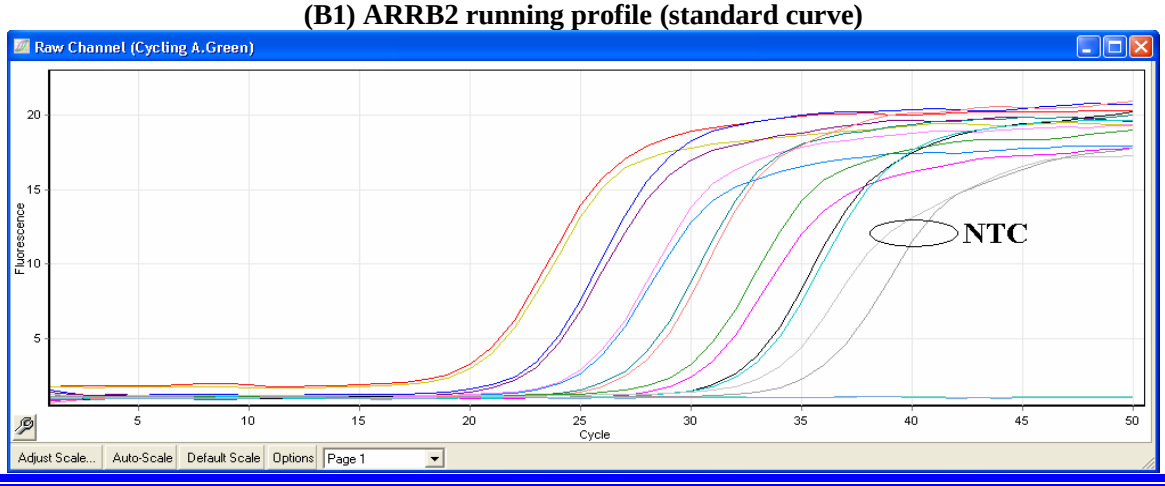
(A2) β-Actin melting curve



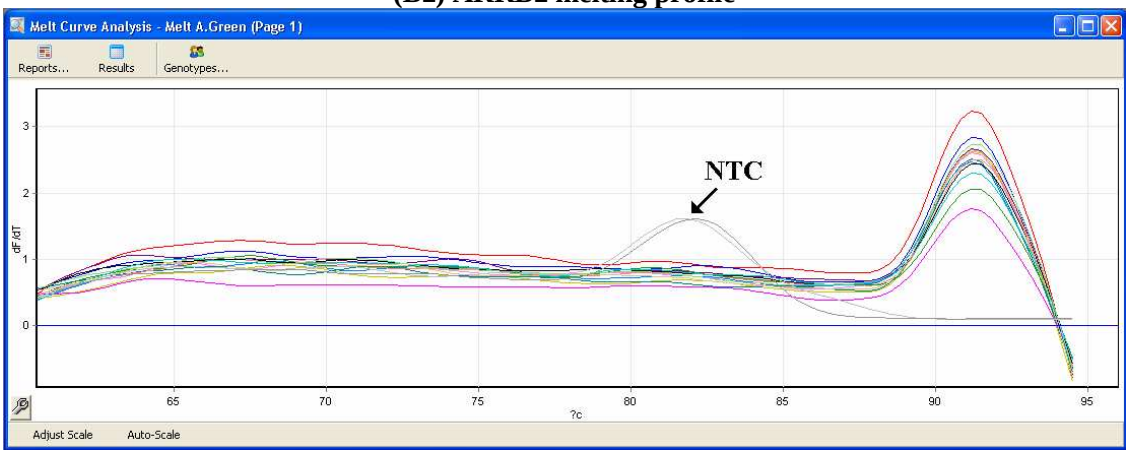
(A3) β-Actin effeciency curve (Pfaffl method)



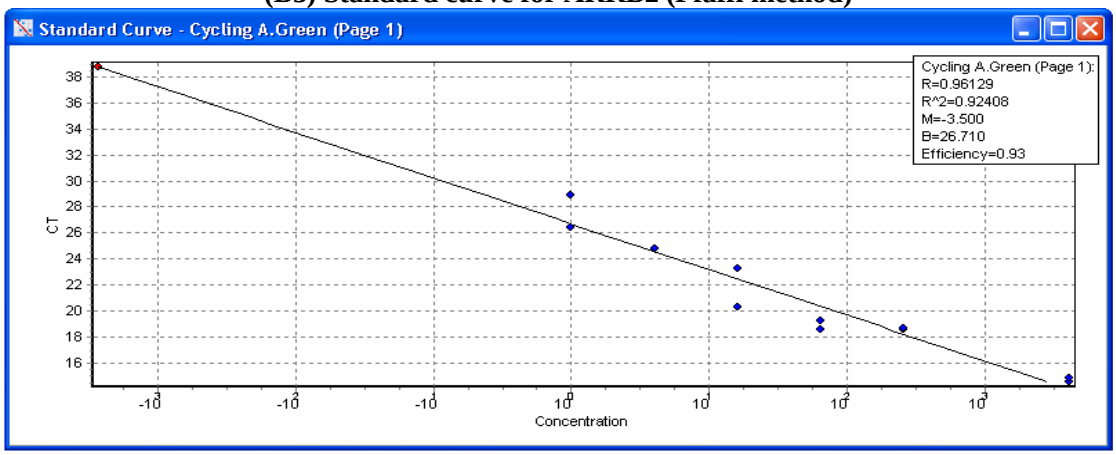
(B1) ARRB2 running profile (standard curve)



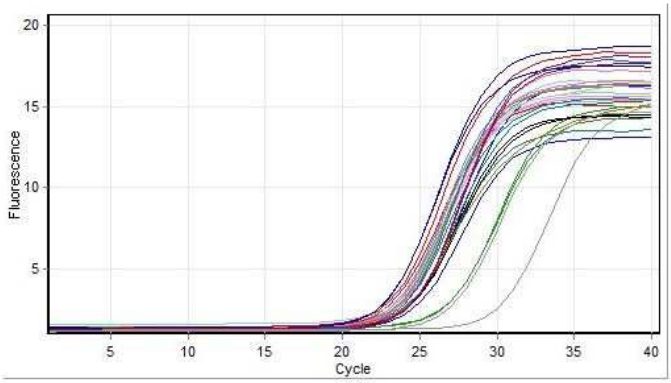
(B2) ARRB2 melting profile



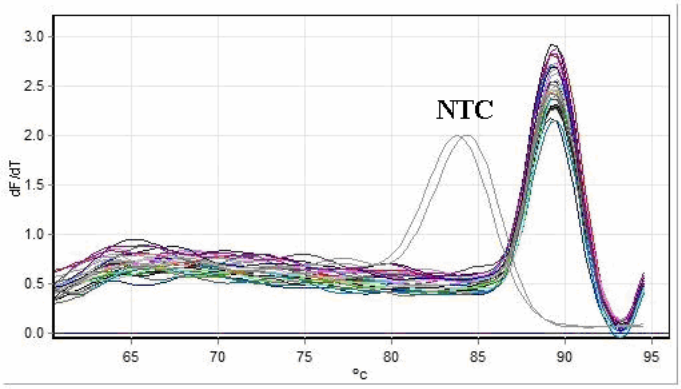
(B3) Standard curve for ARRB2 (Pfaffl method)



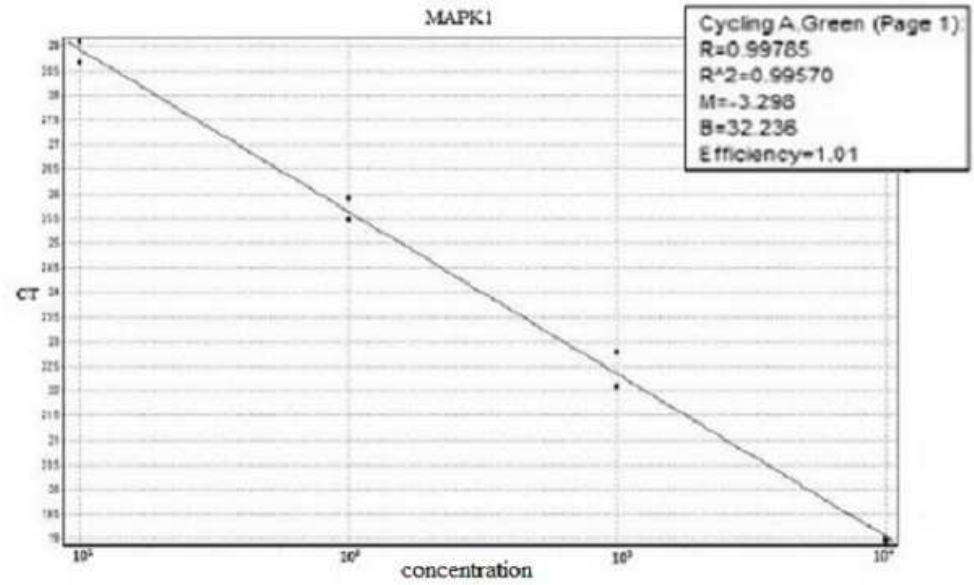
(C1) A sample of MAPK1 running profile

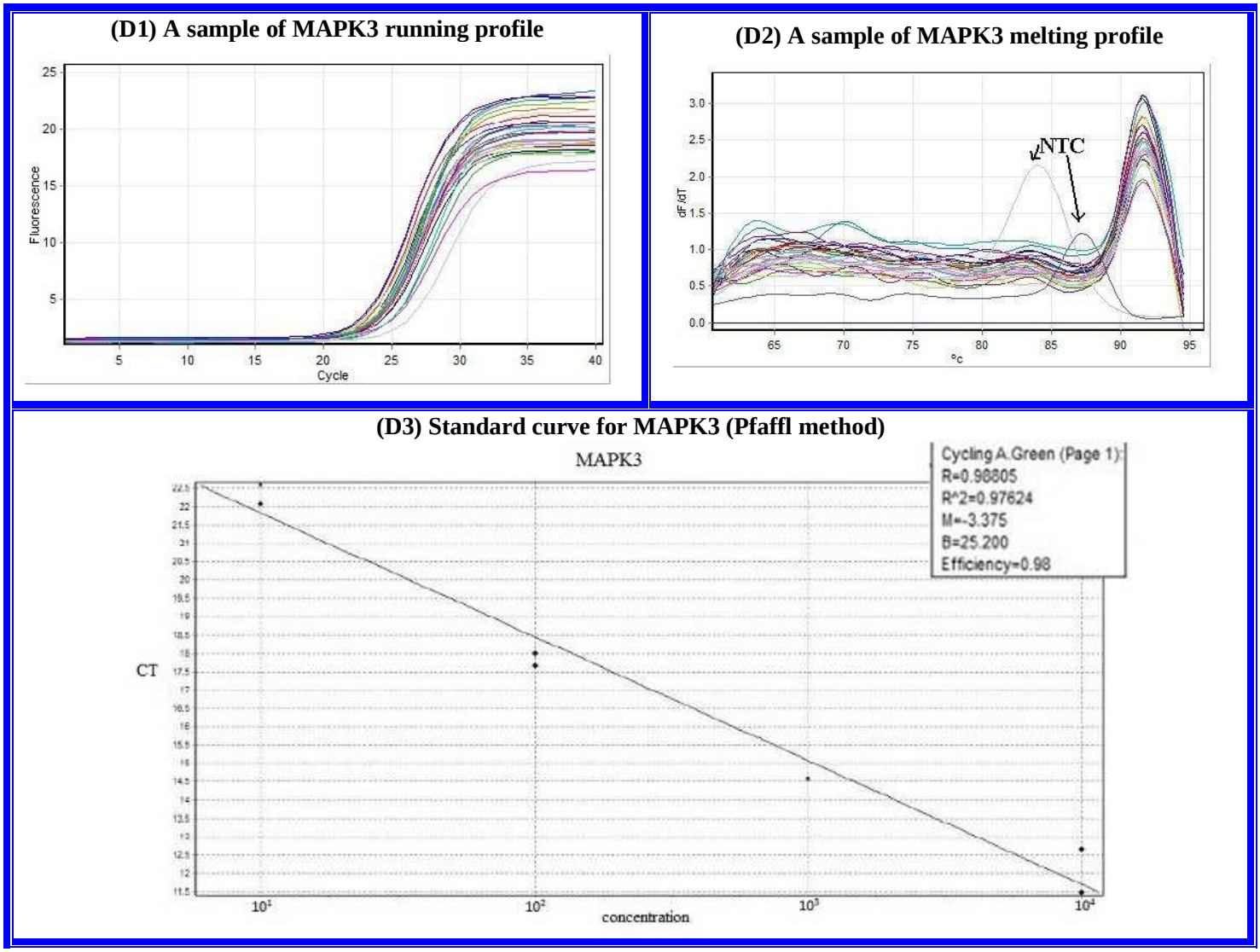


(C2) A sample of MAPK1 melting profile



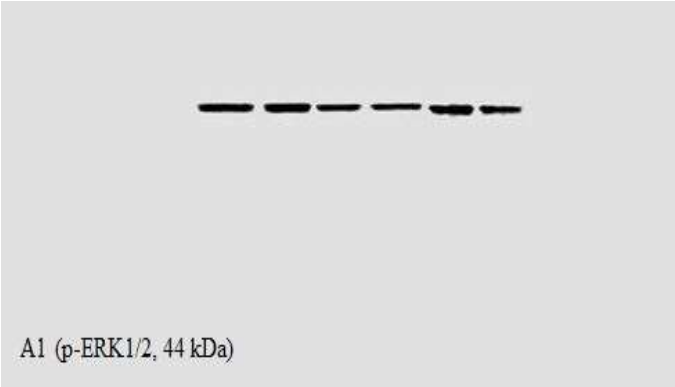
(C3) Standard curve for MAPK1 (Pfaffl method)



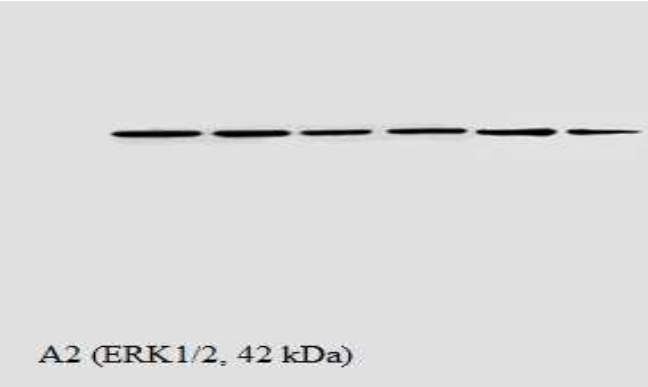


Supplementary Figure S8. Real time efficiency and sample analysis for (A) β-Actin, (B) ARRB2, (C) MAPK1, and (D) MAPK3. Standard curves were run to calculate real time efficiency as Pfaffl method. A serially four-fold dilution series of cDNA was used as the template within a range of 1:1, 1:4, 1:16, 1:64, 1:256, 1:1024, 1:4096. All experiments were repeated three independent times. A1, B1, C1, and D1 are amplification curves. A2, B2, C2, and D2 are respective melting curves. A3, B3, C3, and D3 are standard curves for the respective genes according to Pfaffl method.

Supplementary Data Set

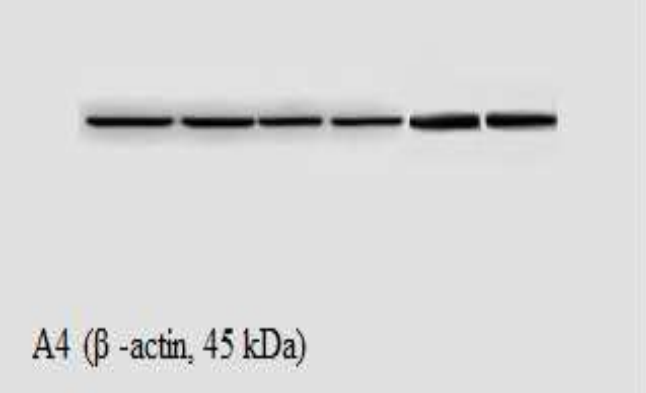


A1 (p-ERK1/2, 44 kDa)

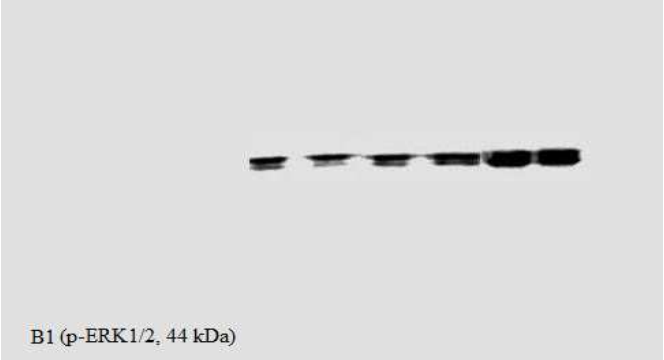


A2 (ERK1/2, 42 kDa)

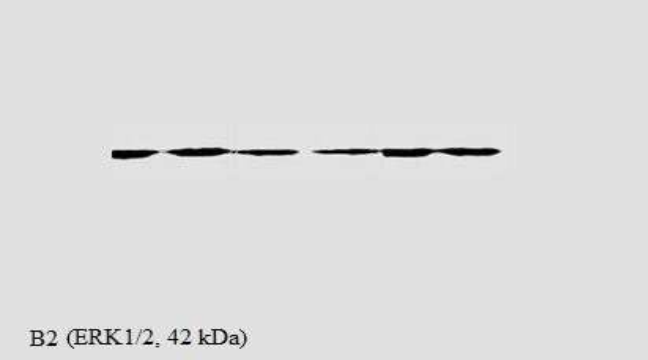
A3 (β–arrestin2, 50 kDa)



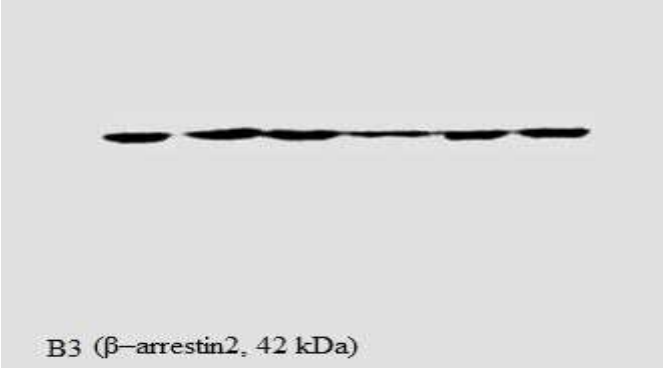
A4 (β -actin, 45 kDa)



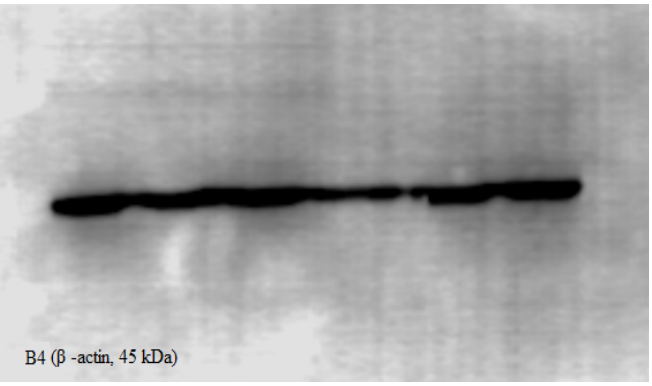
B1 (p-ERK1/2, 44 kDa)



B2 (ERK1/2, 42 kDa)

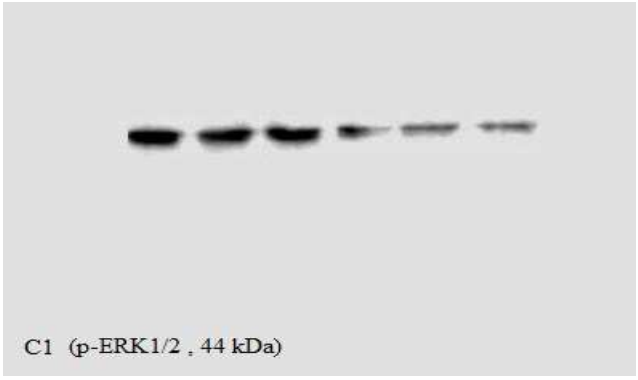


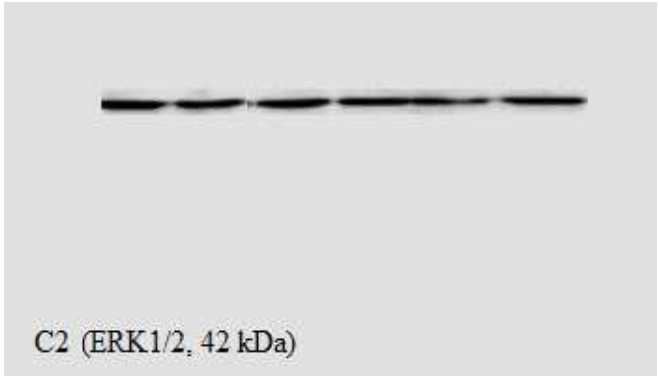
B3 (β–arrestin2, 42 kDa)

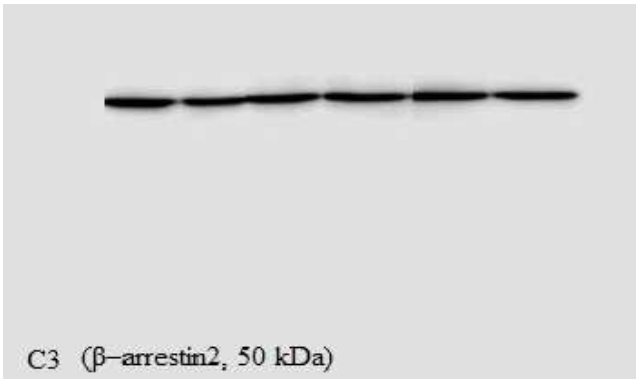


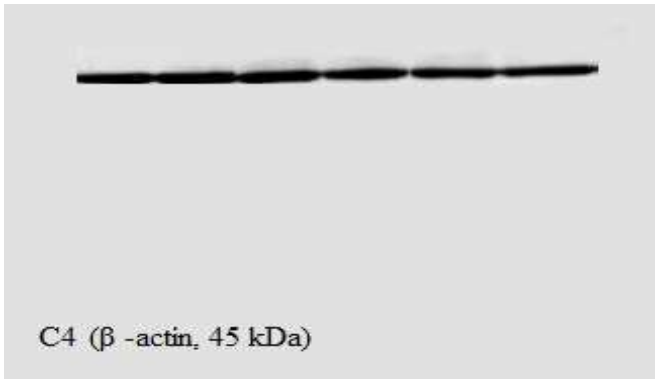
B4 (β -actin, 45 kDa)

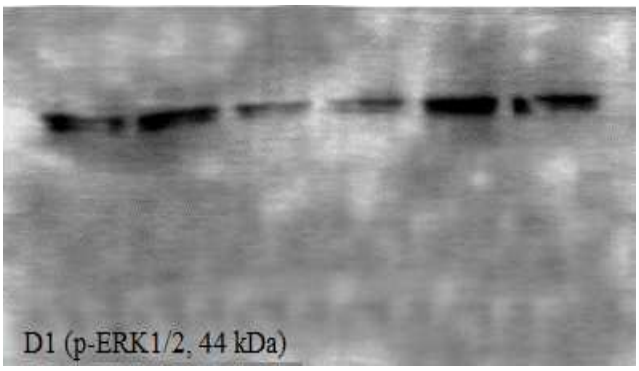
Supplementary Data Set

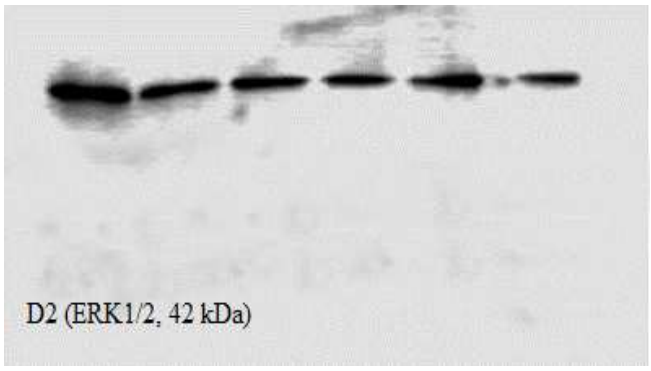


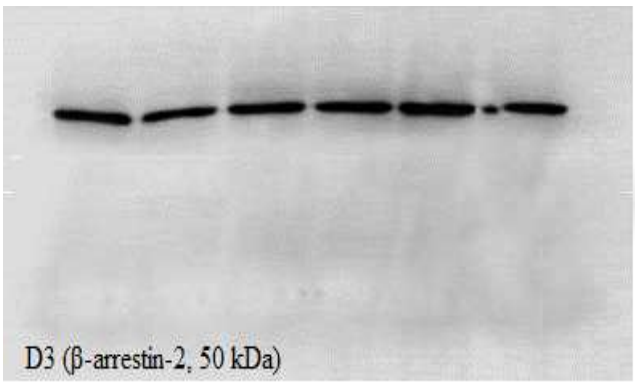


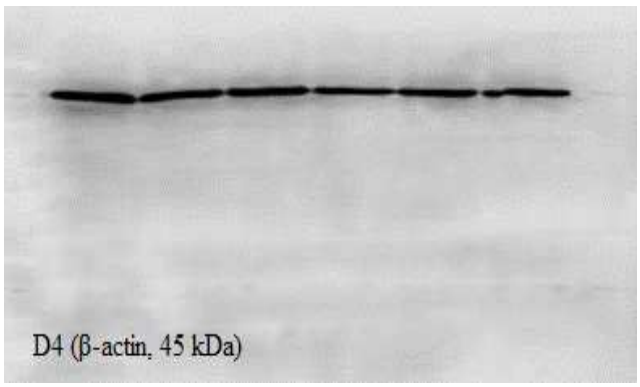






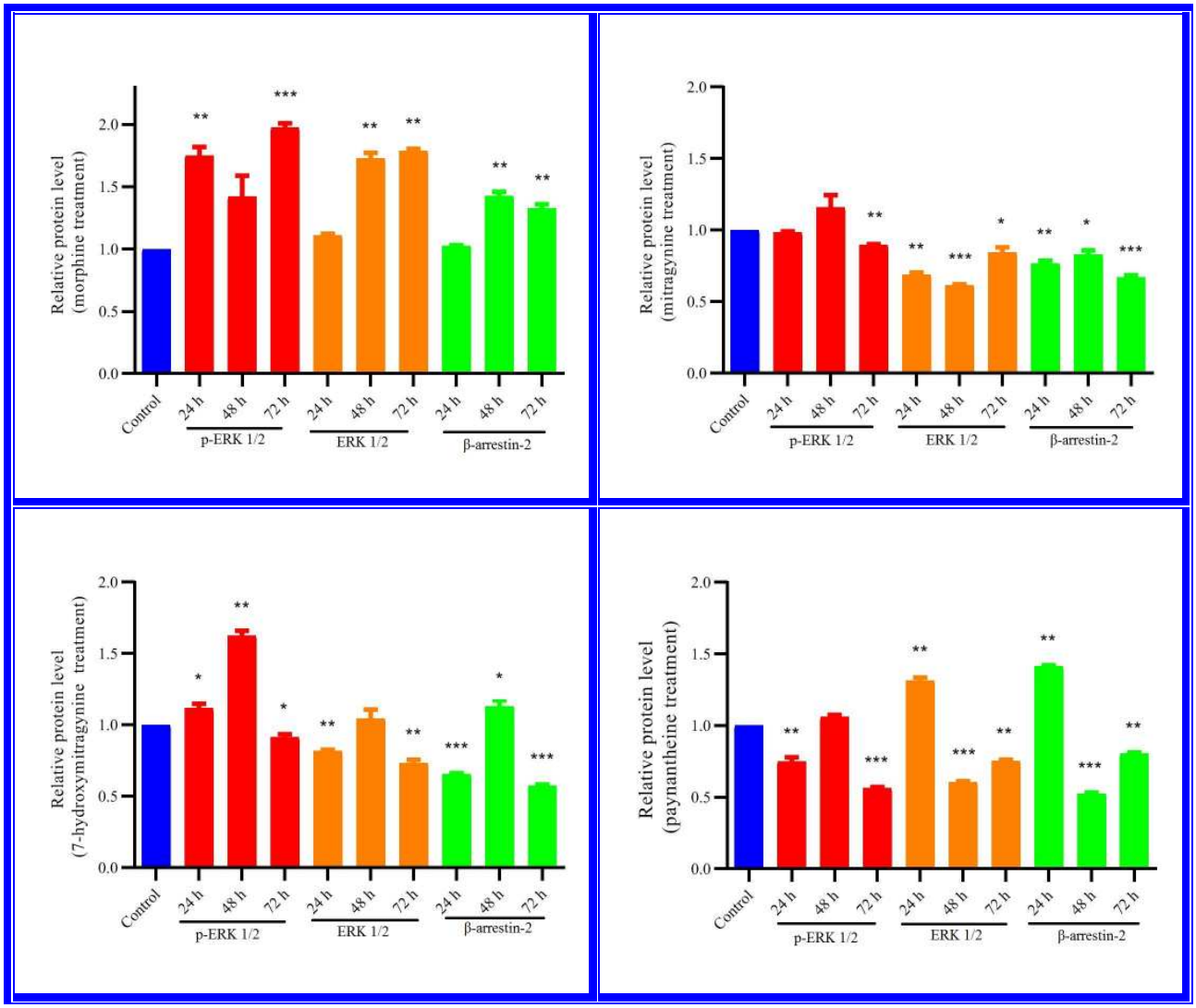






Supplementary figure S9. Authenticate pictures for western blots.

Western protein analyses of β-actin, β-arrestin-2, ERK1/2, and p-ERK1/2. SH-SY5Y cells were treated with morphine (A1 to A4), mitragynine (B1 to B4), 7-hydroxymitragynine (C1 to C4), and paynantheine (D1 to D4) for 24, 48, and 72 h. Briefly, whole protein extracts were prepared at each individual time point and subjected to western blotting. 50 μg protein was electrophoresed on SDS-PAGE. Proteins were transferred to nitrocellulose membranes. Then blots were incubated overnight at 4 ^{o}C with primary antibodies of β-arrestin-2 (Santa Cruz Biotechnology, sc-13140), ERK1/2 (Santa Cruz Biotechnology, sc-135900), or p-ERK1/2 (Santa Cruz Biotechnology, sc-7383) and then incubated with mouse IgG kappa binding protein conjugated to horseradish peroxidase secondary antibody (m-IgGκ BP-HRP, Santa Cruz Biotechnology, sc-516102) for 2 h at room temperature. β-Actin (Santa Cruz Biotechnology, sc-47778) was used as an internal standard protein.



Supplementary figure S10. Visual band quantification of western blots. A free version of Image J (version 1.8.0-112) from National Institutes for Health (NIH) was used as the software for band densitometry. Band intensities were normalized using internal control (β-actin) and then compared to normalized untreated samples. Stars (∗), (∗∗), and (∗∗∗) represent the mean differences between normalized treated and normalized untreated cells' protein level as $P<0.05$, $P<0.01$, and $P<0.001$using one-way ANOVA, respectively.