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ORIGINAL RESEARCH

Enzymatic Synthesis of Ricinoleyl Hydroxamic Acid Based on Commercial Castor Oil, Cytotoxicity Properties and Application as a New Anticancer Agent

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Video abstract

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Background: New anticancer agents that rely on natural/healthy, not synthetic/toxic, components are very much needed.

Methods: Ricinoleyl hydroxamic acid (RHA) was synthesized from castor oil and hydroxylamine using Lipozyme TL IM as a catalyst. To optimize the conversion, the effects of the following parameters were investigated: type of organic solvent, period of reaction, amount of enzyme, the molar ratio of reactants and temperature. The highest conversion was obtained when the reaction was carried out under the following conditions: hexane as a solvent; reaction period of 48 hours; 120 mg of Lipozyme TL IM/3 mmol oil; HA-oil ratio of 19 mmol HA/3 mmol oil; and temperature of 40°C. The cytotoxicity of the synthesized RHA was assessed using human dermal fibroblasts (HDF), and its application towards fighting cancer was assessed using melanoma and glioblastoma cancer cells over a duration of 24 and 48 hours.

Results: RHA was successfully synthesized and it demonstrated strong anticancer activity against glioblastoma and melanoma cells at as low as a 1 μg/mL concentration while it did not demonstrate any toxicity against HDF cells.

Conclusion: This is the first report on the synthesis of RHA with great potential to be used as a new anticancer agent.

Keywords: ricinoleyl hydroxamic acid, enzymatic reaction, anticancer agent, biomedicine, lipozyme and castor oil

Introduction

Castor oil is extracted from castor seeds that are native plants of India. From a historical point of view, castor oil has been used for lubricating leather goods, as fuel for lamps and as a Galenic medicine for some diseases. Castor oil is a triglyceride of fatty acids and almost 90% of them consist of ricinoleic acid. Ricinoleic acid is a poly-unsaturated fatty acid and its high concentration in castor seeds might be responsible for castor oil's remarkable properties.[1–3] It has been used in several applications, such as the production of biodiesels, biomass, antioxidants and drugs.[4,5] In the current work, castor oil was used as a raw material for the synthesis of nano ricinoleyl hydroxamic acid (RHA).

Hydroxamic acids and their derivatives are weak organic acids with low toxicity, which have been widely studied for over half of a century. Several biological

and chemical applications for hydroxamic acids and their derivatives have been reported, such as metal extraction chelators, cell-division factors, food additives, growth factors, antimicrobial agents, antimalarial drugs, tumor inhibitor drugs and enzyme inhibitors.[6–15] Hydroxamic acids can be synthesized using different methods and most of them consist of multistep reactions that are time-consuming and costly due to the use of expensive raw materials.[16–19] Enzymes have also been used as catalysts for the synthesis of hydroxamic acids.[20,21] Servat et al (1990) reported the biological synthesis of amphiphilic molecule-fatty hydroxamic acids using the *Mucor miehei* lipase by reacting hydroxylamine with fatty acids in their free or methyl ester form. They described a general method for the synthesis of fatty hydroxamic acids and investigated the effect of different synthesis parameters (including temperature, molar ratio of reagents, amount of biocatalyst and period of reaction) on its properties.[22]

The above-mentioned enzymatic method was modified by our group in 2011, in which the fatty hydroxamic acids were directly synthesized from canola oil by hydroxylaminolysis using lipozyme as a catalyst in nano dimensions.[23] For the separation of the products from the reaction mixture, it was converted to a copper fatty hydroxamate and precipitated in acetone. The precipitate was then treated with a nitric acid solution to release fatty hydroxamic acids and then extracted by chloroform. Finally, fatty hydroxamic acids were obtained through solvent evaporation. In another body of work, we have synthesized phenyl fatty hydroxamic acids from canola and palm oils using Lipozyme TL IM and RM IM as catalysts in a one-step reaction.[24] Similarly, methyl fatty hydroxamic acids, isopropyl fatty hydroxamic acids and benzyl fatty hydroxamic acids were successfully synthesized using Lipozyme TL IM and lipozyme RM IM catalysts in our lab.[25,26] In all of our above-mentioned works, we successfully used different plant oils as raw materials and substrates to synthesize a wide range of fatty hydroxamic acids. In this work, we assess the anticancer activity of the synthesized fatty hydroxamic acid for the first time.

Hydroxamic acids (HA) are potent anticancer moieties due to the histone deacetylase (HDAC) enzyme activity in cells.[27] Especially, HDAC inhibitors have shown the ability to impact the level of acetylation and deacetylation of histones. Histones are core proteins of nucleosomes in chromatin with a great impact on gene expression and transcription.[28] Therefore, HDAC inhibitors can induce tumor growth inhibition, cell differentiation, and consequently programmed cell death.[29] Due to the strong anticancer activity of HA, different types of HA have been synthesized from various fatty acids and tested in vitro and in vivo.[30–33] Here, we used an environment friendly plant extract (castor oil) to synthesize nano ricinoleyl hydroxamic acid (RHA) in a one-step reaction. Using plant extracts in medicine has attracted a lot of attention recently since it is a healthy, as opposed to a synthetic chemotherapeutic toxic technique[34]. This is further supported by their observed high biocompatibility and the stability of plant extracts compared to chemical synthetic agents. Moreover, there is a long history of castor oil in traditional medicine due to its benefits for anti-fungus and tissue regeneration applications.[35]

Altogether, RHA is a new product with the potential to be used as a newbioactive agent. In this study, RHA was separated from the mixture product containing some other fatty hydroxamic acids, such as oleoyl and linoleoyl hydroxamic acids. This new green medicine method was catalyzed by immobilized lipase catalysts, due to great advantages of enzymatic reactions including taking place in mild conditions, mediating energy saving, being environment friendly, and its high selectivity.

Materials and Methods

Materials

Hexane, heptane, toluene, xylene, chloroform, cyclohexane, diethyl ether, sodium hydroxide and hydroxylamine hydrochloride were purchased from Sigma Aldrich (Missouri, USA). Lipozyme TL IM was obtained from Novo Nordisk (Bagsvaerd, Denmark). American castor oil was purchased from Aria Starr Beauty Co., Sunny California, USA. Dulbecco's Modified Eagle Medium (DMEM) was purchased from Gibco. Eagle's Minimum Essential Medium (EMEM), Human Dermal Fibroblasts (HDF, CCL-110), Human Malignant Melanoma (CRL-1619) and Human Glioblastoma Multiforme (CRL-1690) were ordered from ATCC. MTS cell proliferation assays (CellTiter 96 AQueous One Solution Cell Proliferation Assay) were purchased from Promega. A LIVE/DEAD™ Viability/Cytotoxicity Kit for mammalian cells was purchased from Invitrogen. A DCFDA Cellular ROS Detection Assay Kit was ordered from Abcam.

Synthesis of RHA

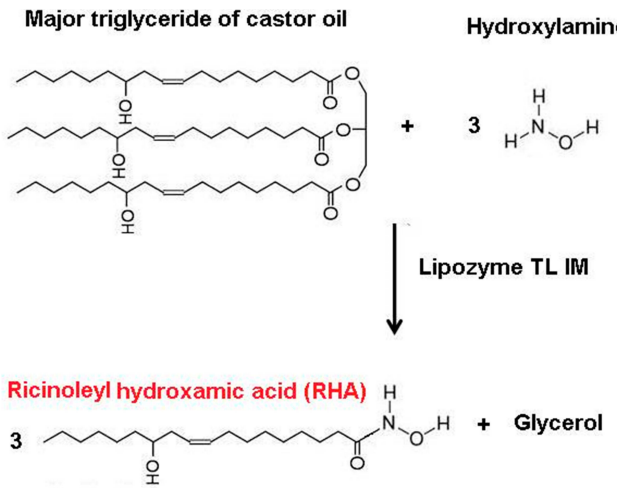
Hydroxylaminolysis was carried out by dissolving 1.31g hydroxylamine hydrochloride in 20 mL of distilled water. Next, the solution was neutralized to pH 7.0 by 1 M NaOH. Castor oil (2.79 g) was dissolved in 30 mL of the desired

solvent (eg, hexane, petroleum ether, cyclohexane, heptane, chloroform, xylene, and toluene). Then, 120 mg of the immobilized Lipozyme TL IM was placed in a 250 mL flask and sealed using Teflon film. The mixture was shaken at 120 rpm at 40°C in a water bath shaker for 48 hours. The product was separated from the reaction mixture as follows: first the enzyme was filtered and then the filtrate was transferred into a separation funnel for the separation of the aqueous phase from the organic phase. The organic phase in the funnel was shaken with 30 mL of distilled water for the removal of glycerol residue and cooled to 10°C and hexane and the oil residue were then separated from the solid product by filtration. The precipitate was dissolved in hot hexane (boiling), then cooled to 25°C and filtered to remove other formed fatty hydroxamic acids (FHAs) from the product. Finally, the product was washed two times using 30 mL hexane at 25°C to purify ricinoleyl hydroxamic acid (RHA). Scheme 1 shows the equation of the hydroxylaminolysis of castor oil.

For reaction optimization, the effect of different reaction parameters (such as type of organic solvent, reaction period, amount of enzyme, molar ratio of reactants, and temperature) was investigated. The conversion percentage of every experiment was calculated using the following equation (1):

$$\text{Conversion}, \% = \frac{\text{Amount of experimental RHA} \times 100}{\text{Amount of theoretical FHAs}} \quad (1)$$

where RHA = ricinoleyl hydroxamic acid and FHAs = fatty hydroxamic acids; we assumed all of the fatty acids of the triglycerides in the oil (ricinoleic acid (94%), linolenic acid (4.5%), oleic acid (0.5%) and stearic acid (1%)) were converted to FHAs that includes RHA, linolenyl hydroxamic acid, oleyl hydroxamic acid and stearyl hydroxamic acid. In order to obtain the fatty acid composition of castor oil, first the oil was hydrolyzed using a KOH solution (dissolved in aqueous ethanol 90% v/v) then the fatty acids were separated from glycerol after neutralization by 1N HCl using hexane as a solvent. In the next step, the fatty acids were converted to methyl esters using methanol and HCl. Finally, the composition was determined using a GC capillary column with a flame ionization detector.



Scheme 1 Reaction equation for the hydroxylaminolysis of castor oil.

Chemical Characterization

The amount of RHA was estimated based on the nitrogen content, determined by an elemental analyzer (model 932 LECO, USA). A Perkin-Elmer 1650 Infrared Fourier Transform Spectrometer was used for recording FTIR spectra. ^{1}H nuclear magnetic resonance (^{1}H-NMR) spectra were recorded using a NMR Spectrophotometer (a Bruker AV-III-600 FT-NMR 600 MHz spectrometer with a CryoProbe and also using chloroform–D as a solvent).

Cell Viability Assays

MTS cell proliferation assays were performed to determine the cytotoxic effects of RHA toward human dermal fibroblasts (HDF) as well as melanoma and glioblastoma cells. 5×10^3 cells ($\approx 1.5 \times 10^4$ cells cm^{-2}) were seeded per well with 200 µL of appropriate culture media with 10% FBS and 1% penicillin–streptomycin inside a 96-well plate that was tissue culture-treated. The plate was left to incubate for 24 h under static conditions at 37°C and in an environment enriched with 5% CO_2. After the incubation period, the cells adhered to the base surface of each well. The overlying media were carefully aspirated and replaced with appropriate media containing various concentrations of RHA (0, 10, 50, 100, 500, and 1000 µg/mL). At this step, due to low solubility of the synthesised RHA, 1% ethanol was mixed in the media to increase the dispersity of RHA inside the desired media. The plate was left inside the incubator at 37°C and 5% CO_2 for 1, 3 and 5 days. Following the incubation period, the contents of each active well were carefully aspirated and replaced by 100 µL of a mixture containing 20% MTS reagent in media. The cells were allowed to incubate with the MTS mixture for 4 h at 37°C and 5% CO_2, in the dark. Post incubation, the absorbance of each well was measured using a spectrophotometer (SpectraMax M3, Molecular Devices, Sunnyvale, CA) with λ_{ab}, the wavelength of absorbance, set to 490 nm. The procured OD data were analyzed graphically

and statistically. EMEM was used for HDF cells and DMEM was used for melanoma and glioblastoma cells. The half-maximal inhibitory concentration (IC_{50}) was calculated by GraphPad Prism 6.0 software using the variable slope (four parameters) equation from a concentration–response curve obtained with RHA concentrations ranging between 0 and 1000 µg/mL.

Live/Dead Assay

Live and dead cell staining was carried out using a calcein AM/ethidium homodimer-1 live/dead kit according to instructions from the manufacturer. 5×10^3 HDF, glioblastoma and melanoma cells ($\approx 1.5 \times 10^4$ cells cm^{-2}) were separately seeded per well with 200 µL of appropriate culture media with 10% FBS and 1% penicillin–streptomycin inside a 96-well plate that was tissue culture-treated. The plate was left to incubate for 1, 3, and 5 days under static conditions at 37°C and in an environment enriched with 5% CO_2. Following the incubation period, the contents of each active well were carefully aspirated and replaced by 100 µL of a mixture containing 2 µL/mL ethidium homodimer-1 and 0.5 µL/mL calcein AM reagents in PBS according to manufacturer's instructions. After 20 minutes of incubation at 37°C and a 5% CO_2 environment, the contents of each active well were carefully aspirated and cells were washed 3 times with PBS. Next, the wells were filled with 100 µL of PBS and fluorescent images were taken immediately. Fluorescent images were taken using a Zeiss Axio Observer Z1 inverted microscope and were analyzed using ImageJ software.

ROS Detection Assay

To assess intracellular reactive oxygen species (ROS) in melanoma cells, we used the ROS detection assay kit with 2′,7′-dichlorodihydrofluorescein diacetate (ab113851, DCFDA Cellular ROS Detection Assay Kit, Abcam). DCFDA is a fluorescent probe that can detect reactive oxygen species such as hydrogen peroxide, peroxyl radical and peroxynitrite. Briefly, melanoma cells were seeded into 96-well plates at a cell density of 5×10^4 cells per well in 100 µL of working media (DMEM media with 10% FBS and 1% P/S (penicillin/streptomycin)). After 4 h of incubation at 37 °C in a 5% CO_2-humidified atmosphere, melanoma cells were treated with different concentrations of RHA (0, 10, 50, 100, 500, and 1000 µg/mL). 4 h post-treatment, a TBHP (Tert-Butyl Hydrogen Peroxide) solution was added to 3 wells to the final concentration of 500 µM as a positive control. 4 h post-treatment, 100 µL of 50 µM DCFDA in working media was added to each well to make the final concentration of 25 µM DCFDA. End-point fluorescence from triplicate wells for each experimental condition was measured in a fluorescence microplate reader at 485 nm excitation and 535 nm emission wavelengths at 5 h post-treatment.

Statistical Analysis

At least 3 replicates were included in the experimental setup for each sample. Data analysis was carried out using a 1- or 2-way ANOVA test with GraphPad Prism 6.0 software. Error bars represent the mean ± standard deviation (SD) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$).

Results and Discussion

Effect of Organic Solvent

The effect of different organic solvents on the conversion reaction of hydroxylaminolysis is shown in Table 1. Based on the results, polar and non-polar organic solvents were shown to have a similar effect on castor oil hydroxylaminolysis which can be due to the semi-polar property of castor oil (as a substrate). On the other hand, in the mentioned reaction, non-polar aliphatic organic solvents acted better than the aromatic organic solvents. As shown in Table 1, hexane showed the highest conversion for hydroxylaminolysis of castor oil. Therefore, hexane was used as an organic solvent for the next experiments.

Effect of Reaction Time

Figure 1 shows the effect of the reaction period on the conversion percentage of castor oil hydroxylaminolysis. Based on the results, in the first 24 hours, the reaction was rapid and continued in a descending rate until reaching the equilibrium at 48 hours. This behavior can be explained due

Table 1 Effect of Organic Solvents on the Hydroxylaminolysis of Castor Oil

Organic Solvent	Conversion %
Hexane	55.6
Petroleum ether	53.2
Cyclohexane	51.5
Heptane	50.7
Chloroform	48.3
Xylene	34.7
Toluene	33.4

Notes: Reaction conditions: organic solvents = 30 ML, H_2O = 20 ML, NaOH (6M) = 2.4 mL, initial pH = 7.0, reaction time = 30 hours, Lipozyme TL IM = 90 mg, $NH_2OH{\cdot}HCl$ = 1.0 g, castor oil= 2.79 g (3 mmol), temperature = 37°C and shaking rate = 120 rpm.

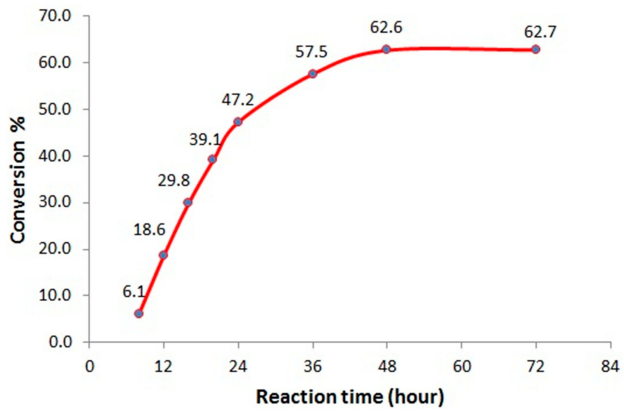


Figure 1 Effect of reaction time on the hydroxylaminolysis of castor oil. Reaction conditions: hexane = 30 mL, H_2O = 20 mL, NaOH (6M) = 2.4 mL, initial pH = 7.0, Lipozyme TL IM = 90 mg, $NH_2OH{\cdot}HCl$ = 1.0 g, castor oil = 2.79 g (3 mmol), temperature = 37°C and shaking rate = 120 rpm.

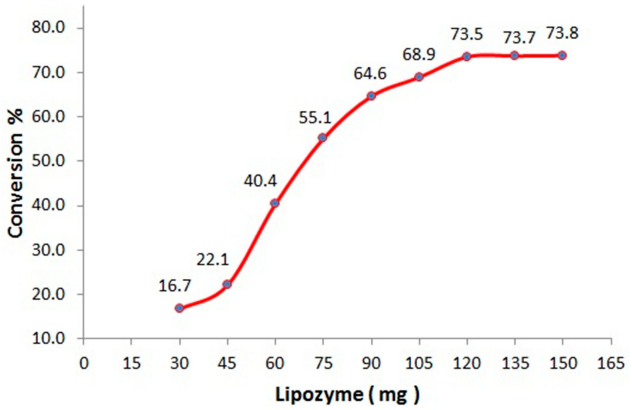


Figure 2 Effect of the amount of the enzyme on the hydroxylaminolysis of castor oil. Reaction conditions: hexane = 30 mL, H_2O = 20 mL, NaOH (6M) = 2.4 mL, initial pH = 7.0, reaction time = 48 hours, $NH_2OH{\cdot}HCl$ = 1.0 g, castor oil = 2.79 g (3 mmol), temperature = 37°C and shaking rate = 120 rpm.

to the nature of the enzymatic reactions in which the reaction rate is high at the beginning and it drops due to the decrease of mass transfer into the active sites of the enzyme. Our observation is similar to Servat et al (1990), whom reported the same behavior for oleyl hydroxamic acid synthesis.[22] We have also observed similar behavior for canola oil hydroxylaminolysis and methylhydroxylaminolysis of *Jatropha curcas* seed oil.[23–26] According to the results, a reaction time of 48 hours was selected for the following experiments.

Effect of the Amount of the Enzyme

In the current investigation, Lipozyme TL IM was used as a desirable enzyme due to our experience and results that previously have been reported.[6,7,23-25] In any enzymatic reaction, the active sites of the enzyme are the regions at which the reaction is carried out. Due to the accretion of active sites in Lipozyme, with increases in the amount of enzyme, the reaction conversion increased (Figure 2). At 120 mg of Lipozyme TL IM for 3 mmol oil, the highest conversion rate was observed. Further increases in the amount of Lipozyme did not show any significant effects on the conversion. This can be explained by the fact that when the highest amount of the enzyme is present in a reaction, most of the active sites of the enzyme cannot be accessed by the substrates and remain in the bulk of the enzyme.

Effect of the Molar Ratio of HA to Castor Oil

To obtain the maximum conversion percentage, the effect of the reactant's molar ratios was evaluated. The optimal molar ratios of the reactants depend on the nature of the reaction and its equilibrium constant. The fixed amount of oil and different amounts of HA were applied to the reaction mixtures (with the appropriate amount of 6M NaOH to obtain a pHof 7). Figure 3 shows that with an increase in molar ratio of HA to oil, the conversion of the reaction increased to its maximum point at 19 mmol of HA for 3 mmol of oil. This amount is slightly higher than the amount of hydroxylamine needed for hydroxylaminolysis of canola oil reported in our previous work.[23] Further increases in the molar ratio of HA to the oil decreased the conversion. Based on the results, 19 mmol of HA for 3 mmol of oil was used for the following experiments.

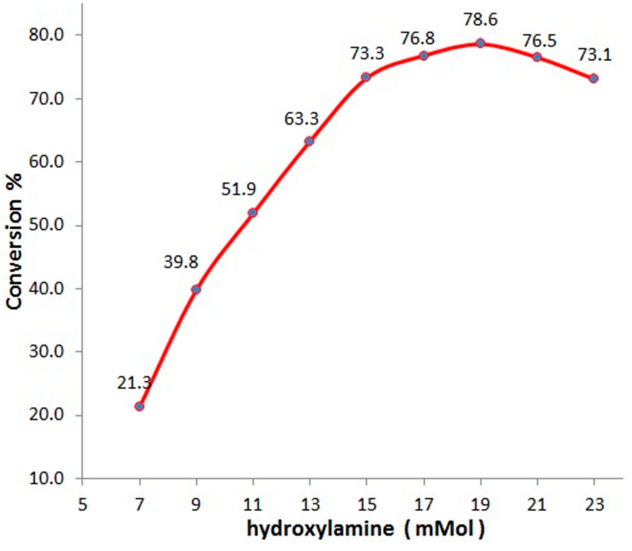


Figure 3 Effect of the molar ratio of HA to oil on the hydroxylaminolysis of castor oil. Reaction conditions: hexane = 30 mL, H_2O = 20 mL, initial pH = 7.0, reaction time = 48 hours, Lipozyme TL IM = 120 mg, castor oil = 2.79 g (3 mmol), temperature = 37°C and shaking rate = 120 rpm.

Effect of Temperature

Generally, the optimal temperature for enzymatic reactions depends on the thermostability of the enzyme. Enzymes are biomolecules that have the highest stability at room temperature. High temperatures (>50°C) affect lipase activity, resulting in reduction or even loss of activity.[36,37] In this investigation, a wide temperature range (31–45°C) was applied to the reaction. As shown in Figure 4, as the temperature rose, the reaction conversion increased until it reached its maximum at 40°C. Temperatures higher than 40°C resulted in lower conversions. Based on these results, 40°C was used for the reaction in the following experiments.

Characterization of RHA

Elemental Analysis

Previous studies have shown the nanostructured of RHA.[23] Based on the elemental analysis, the nitrogen content in the RHA was 4.41%, meaning that in one gram of the product, 3.17 mmol of ricinoleyl hydroxamic acid groups were present.

Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectra of castor oil and RHA are shown in Figure 5. In the castor oil spectra, the wide peak at 3438 (3200 to 3600) cm^{-1} corresponds to the O—H stretching and the peaks at 2923 and 2857 cm^{-1} correspond to the —C—H stretching for the long alkyl chain, while the peaks at 1739, 1454 and 1161 cm^{-1} correspond to C=O stretching, C=C stretching and C—O stretching, respectively. These results were generally confirmed for alkyl triglyceride oil.[38] In the RHA spectra, the wide peaks at 3401 (3200 to 3600) cm^{-1} correspond to O—H stretching (O—H bonded to nitrogen atom) which overlapped with the peak of O—H that bonded to the carbon atom on the alkyl branch of RHA, the sharp peak at 3273 cm^{-1} corresponds to N—H stretching that was confirmed by the peaks at 1559 and 719 cm^{-1} corresponds to N—H bending. The peaks at 2920 and 2853 cm^{-1} correspond to —C—H stretching for the long alkyl chain, the peak at 1656 cm^{-1} corresponds to C=O stretching that was shifted to upper frequencies due to the bonding of the nitrogen atom in the hydroxamic acid functional groups. These results are similar to the FTIR spectra of fatty hydroxamic acids obtained from canola oil that showed a C=O peak at 1642 cm^{-1}.[23] In addition, the peak at 1427 cm^{-1} corresponds to C=C stretching and finally the peak at 1064 cm^{-1} corresponds to the C—N stretching that confirmed the formation of hydroxamic acid functional groups and RHA.

1H Nuclear Magnetic Resonance (^{1}H-NMR)

The formulation of RHA based on castor oil is presented in Figure 6. ^{1}H-NMR spectra of the product showed peaks at a

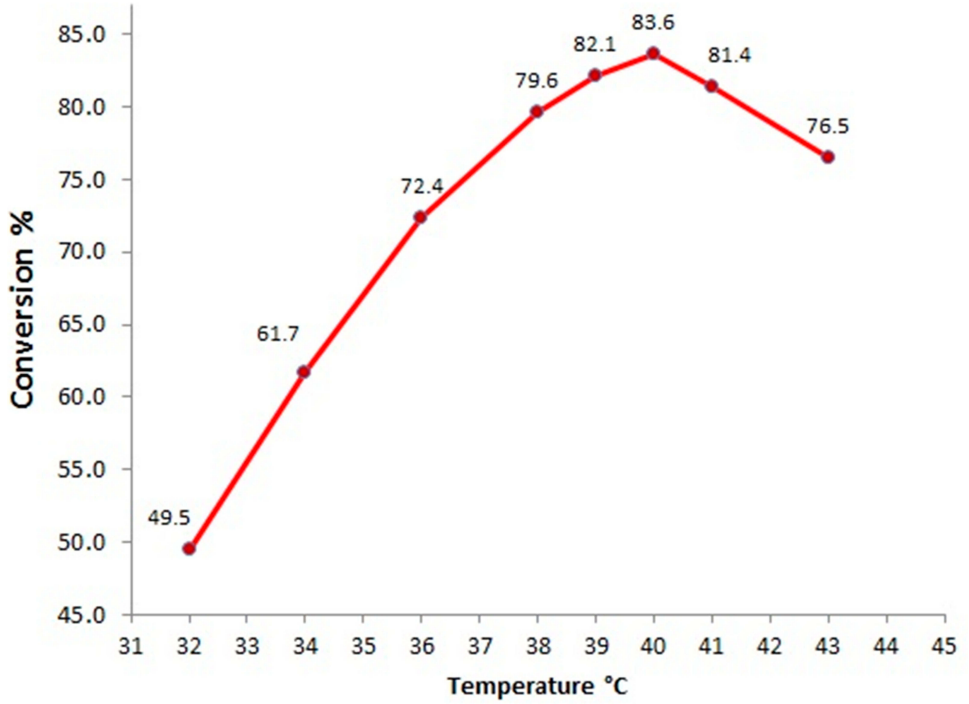


Figure 4 Effects of temperature on the hydroxylaminolysis of castor oil. Reaction conditions: hexane = 30 mL, H_2O = 20 mL, NaOH (6M) = 3.15 mL, initial pH = 7.0, reaction time = 48 hours, Lipozyme TL IM = 120 mg, $NH_2OH{\cdot}HCl$ = 19 mmol, castor oil = 2.79 g (3 mmol) and shaking rate = 120 rpm.

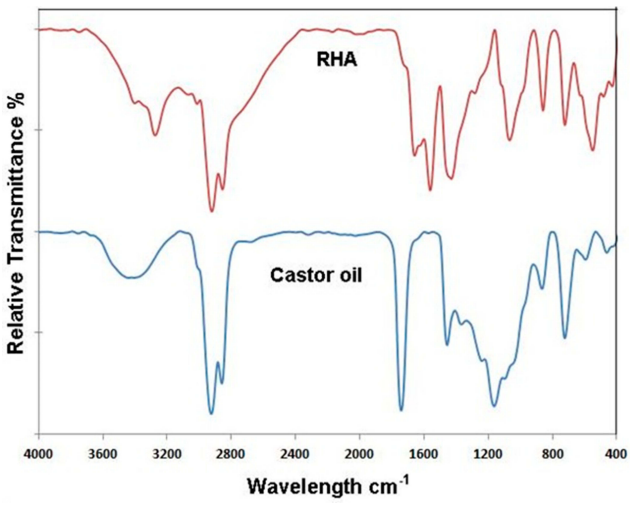


Figure 5 FTIR spectra of castor oil and RHA.

(0.88 ppm), b(1.29 ppm), c(1.47 ppm), d(1.63 ppm), e(2.04 ppm), f(2.18 ppm), g(2.21 ppm) h(3.63 ppm), i(5.38 ppm) and j(5.58 ppm) corresponding to the alkyl branch hydrogens of RHA that bonded to a carbon atom. The weak and wide peak at k(5.85 ppm) corresponds to the hydrogens of OH present on the alkyl branch of the RHA (Figure 6). These peaks confirmed the alkyl branch hydrogens of ricinoleic acids. In addition, the wide and weak peaks appeared at l (8.5 to 8.7 ppm) correspond to hydrogen which bonded to the nitrogen atom and the wide and weak signals at m(9.9 to 10.1 ppm) correspond to the hydrogens of -OH which bonded to the nitrogen atom, respectively. These results were confirmed by similar observations reported by other researchers[26,39] for the hydrogens of the hydroxamic acid functional groups.

Cell Viability Assays

To assess the cytotoxicity of RHA, we performed MTS assays after treatment of HDF as well as melanoma and glioblastoma cells with various concentrations of RHA for 1, 3, and 5 days (Figure 7). Representative live/dead stained images of HDF, glioblastoma and melanoma cells, incubated with different concentrations of RHA, are also presented in Figure 7A. Also, the average number of viable cells per well is reported in Figure 7B–D. The average number of cells inside wells containing 0 and 10 µg/mL RHA after 1 day of treatment were in the same range (4023 and 4579, respectively) for HDF cells with no statistical difference. However, 50 µg/mL RHA inhibited the growth of the HDF cells with significantly lower cell numbers as compared to negative control treated wells containing 1427 cell per well (Figure 7B). Higher concentrations

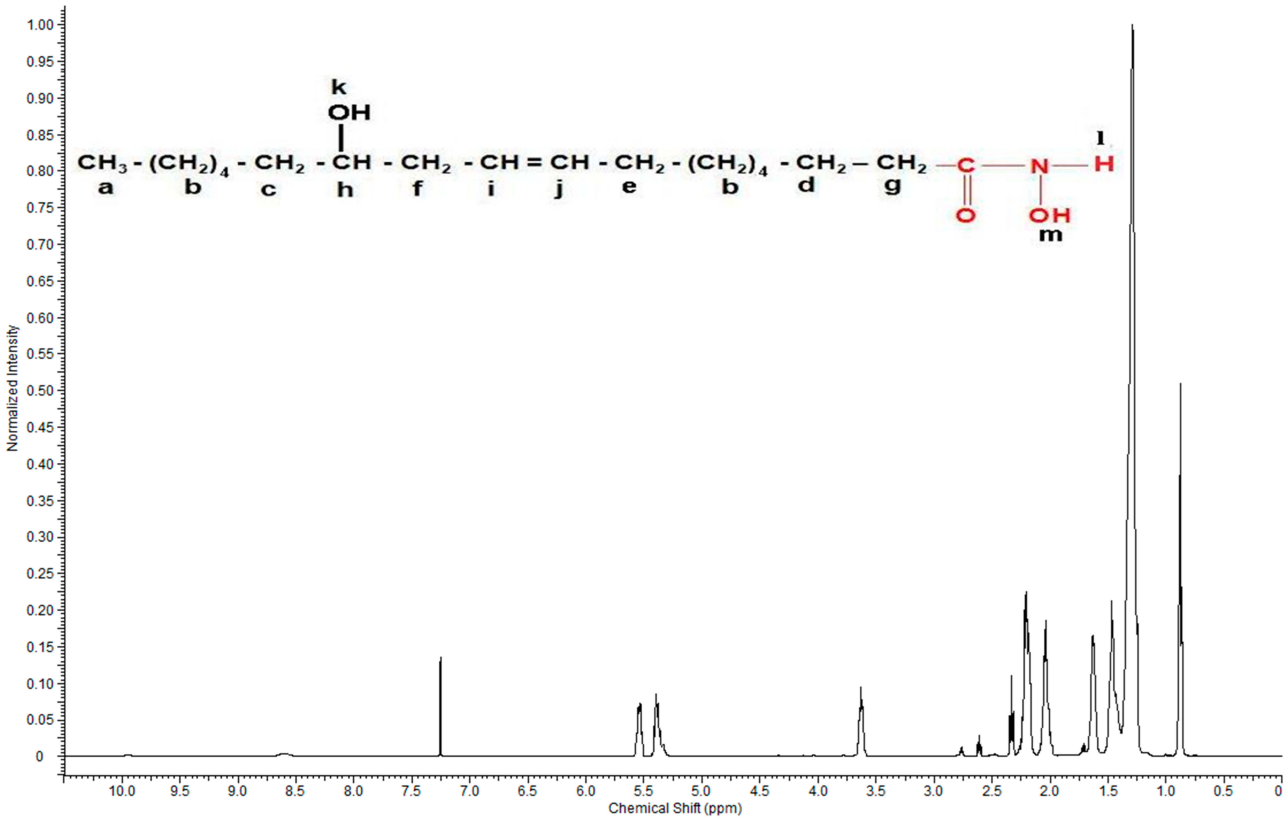


Figure 6 ^{1}H-NMR spectra and formula of RHA (hydrogen atoms are labelled by a, b, c, m).

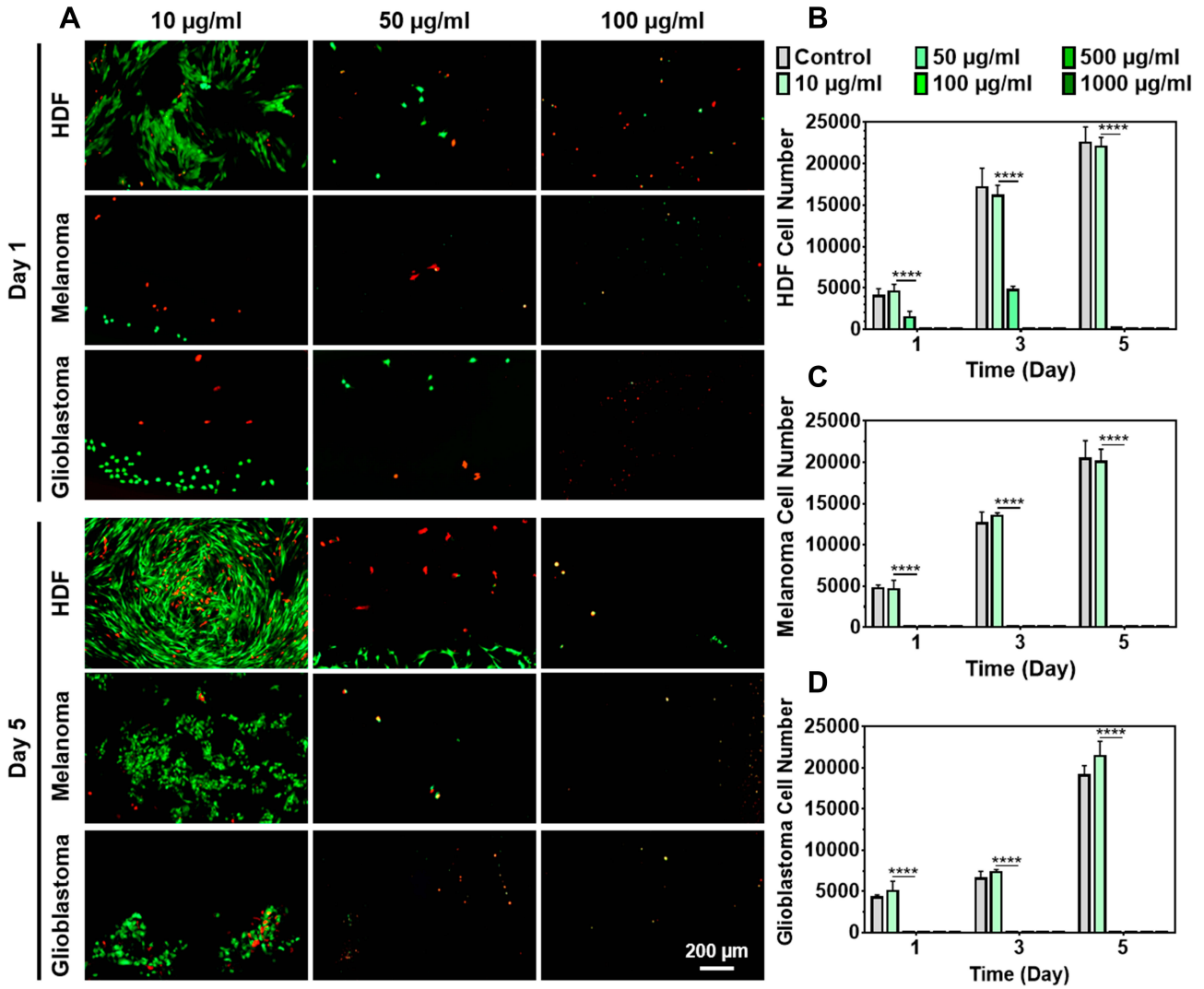


Figure 7 Cytotoxicity of RHA. (**A**) Representative live/dead images of HDF, melanoma, and glioblastoma cells at 1 and 5 days post-incubation. The green color represents live cells and red color represents dead cells. (**B**) HDF cell numbers in the presence of different concentrations of RHA at 1, 3, and 5 days post-treatment. (**C**) Melanoma cell numbers in the presence of different concentrations of RHA at 1, 3, and 5 days post-treatment. (**D**) Glioblastoma cell numbers in the presence of different concentrations of RHA at 1, 3, and 5 days post-treatment. Data are presented as the mean ± SD (n=6 and ****$p < 0.0001$).

of RHA (ie, 100, 500, and 1000 µg/mL RHA) demonstrated high toxicity and inhibited the growth of HDF cells completely. In addition, the same concentrations of RHA were incubated with HDF cells for 3 and 5 days. Wells treated with negative controls contained an average of 17,158 cells per well at day 3 which is more than 1427 at day 1 of treatment. Further, the results showed the continual growth of the cells until day 3. However, the HDF cell number did not alter significantly after day 3 of the treatment with 16,907 cells per well at day 5. This suggests that the area of the wells containing the negative control treatment was mostly covered with HDF cells after day 3 post-treatment (Figure 7B). The IC_{50} values obtained for the synthesized RHA were 48.87, 48.53, and 40.18 µg/mL at day 1, 3, and 5 post-treatment of HDF cells, respectively.

To assess the anticancer properties of the synthesized RHA, the cytotoxicity of various concentrations of RHA was tested against melanoma and glioblastoma cancer cell lines (Figure 7). Results showed that using as low as 50 µg/mL RHA inhibited the growth of melanoma and glioblastoma cells completely. The average number of melanoma cells inside of wells containing 0 and 10 µg/mL RHA were in the same range at 1, 3, and 5 days post-treatment (Figure 7C). Consequently, the IC_{50} values obtained for the synthesized RHA were 19.33, 34.38, and 13.22 µg/mL at 1, 3, and 5 days post-treatment of melanoma cells, respectively.

Nonetheless, IC_{50} values for melanoma cells were significantly lower than IC_{50} values for HDF cells which suggests higher toxicity of RHA against cancer cells as compared to healthy cells. Similarly, 50 µg/mL of RHA and higher concentrations (ie, 100, 500, and 1000 µg/mL) were able to inhibit the growth of glioblastoma cancer cells completely. The average number of glioblastoma cells inside wells treated with 0 and 10 µg/mL were at the same range at 1, 3, and 5 days post-incubation. The IC_{50} values obtained for RHA were in the same range (ie, 33.65, 33.58, and 32.97 µg/mL at day 1, 3, and 5 post-incubation, respectively) and lower than the IC_{50} values related to the HDF cells.

ROS Detection Assay

To assess the mechanism behind the anticancer properties of the synthesized RHA, ROS generation was measured for various concentrations of RHA (Figure 8). RHA treatment of melanoma cells increased the production of ROS in a dose-dependent manner after a concentration of 50 µg/mL. Previously, ROS accumulation was reported for other HDAC inhibitor (HDACi) treatments as well.[40] Although the reason behind the initially elevated ROS generation in response to HDACi remains unclear, elevated ROS generation activates different normal cell death pathways.[41] Nonetheless, it has been shown before that cancer cells seem to have increased endogenous ROS generation in vitro and in vivo, compared to normal cells.[42,43] Therefore, cancer cells are more vulnerable to further oxidative stress induced by exogenous ROS generating agents, such as RHA.[44] This is most likely the reason why RHA treatment showed lower toxicity against HDF cells as compared to melanoma cells after 1, 3, and 5 days post-treatment (Figure 7B–D).

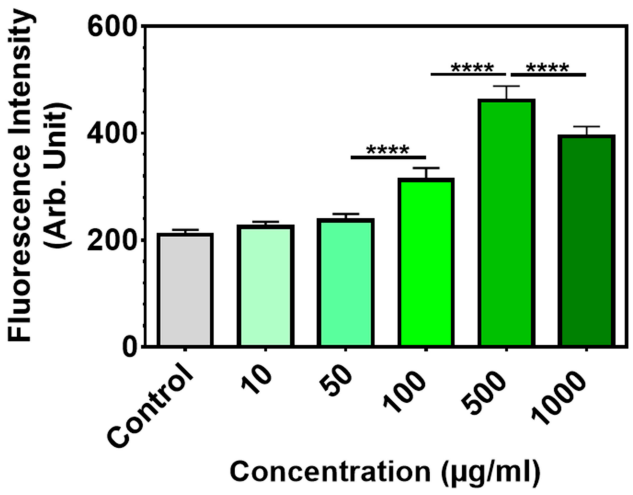


Figure 8 ROS generation of melanoma cells in the presence of various concentrations of RHA at 5 h post-treatment. Data are presented as mean ± SD (n=3 and ****$p < 0.0001$).

Conclusion

This paper is the first report describing the synthesis of RHA from HA and castor oil using a lipozyme as a catalyst. This is a new compound with potential to be used as a strong metal collector, an antioxidant, antimicrobial and anticancer agent. Some advantages of our synthesis method are as follows: use of easily available oils and utilization of enzymatic reactions for the purpose of saving energy and avoiding the use of toxic compounds in order to achieve green chemistries. Besides, the separation technique used in this study for the isolation of RHA from the reaction mixtures is a new method.

Elemental analysis, ^{1}H-NMR and FTIR spectra showed that RHA was produced from the reaction of HA and the oil through the optimized conditions of: hexane = 30 mL; H_2O =20 mL; reaction time of 48 hours; Lipozyme TL IM = 120 mg; HA 19 mmol; oil = 2.79 g (3 mmol); temperature =40°C and shaking rate of 120 rpm. The application of these optimized conditions to commercial castor oils resulted in RHA conversions of 83.9%. Evaluation of the cytotoxicity of the synthesized RHA using MTS cell proliferation assays revealed the anticancer activity of RHA against melanoma and glioblastoma cancer cells. The synthesized RHA showed toxicity against HDF cells also, however, HDF cells treated with 50 µg/mL RHA were able to grow up to day 3 of incubation. This suggests that this concentration can be used to inhibit the growth of melanoma and glioblastoma cancer cells while at the same time HDF cells were able to continue their growth after treatment.

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Disclosure

The authors report no conflicts of interest in this work.

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