Original Research Article

Anticancer potential of bioactive 16-methylheptadecanoic acid methyl ester derived from marine Trichoderma

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\textbf{Abstract}

The present work aimed at purifying the intracellular fungal metabolites, such as 16-methylheptadecanoic acid methyl ester (HDA) and 9,12-octadecadienoic acid (ODA) from marine Trichoderma, Hypocrea lixii TSK8, Hypocrea rufa SKS2 respectively, and investigating their anticancer and antioxidant effects. The two fungal metabolites were tested against two human cancer cell lines, namely oral cancer (KB) and skin carcinoma (A431) by using MTT assay. The inhibitory concentrations (IC\textsubscript{50}) against KB oral cancer cells were found to be 18.75 \pm 0.12 \mu g/mL for HDA and 75.50 \pm 0.42 \mu g/mL for ODA. Whereas IC\textsubscript{50} values of HDA and ODA against A431 were found 37.5 \pm 0.42 \mu g/mL and 72.89 \pm 0.15 \mu g/mL, respectively. In addition, the down-regulation of heat shock protein 90 kDa (HSP90) was confirmed by using SDS-PAGE and Western blot analysis. The effect of HDA induced apoptosis via ROS-dependent internucleosomal DNA fragmentation was confirmed by AGE analysis. We further evaluated the \textit{in vivo} anti-skin cancer activity of HDA in Swiss albino mice induced with skin cancer by 7,12-dimethylbenz(a)anthracene (DMBA) and croton oil (CO). The \textit{in vivo} hematological, biochemical and histopathological results revealed that the fungal metabolite HDA was a highly potent anticancer compound against the skin cancer.

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Introduction

Globally, human diseases are increasing due to the development of pathogenic microbes such as virus and bacteria. Thus, the discovery of the potentially effective therapeutic bioactive metabolites to control the diseases is a challenge to pharmaceutical science (Miguel et al., 2012). To overcome this problem, highly potent bioactive agents have been searched from plant, animals and microorganisms. Marine flora is vital source for the isolation of novel compounds with high medicinal value (Sithranga Boopathy and Kathiresan, 2010) since 1960 almost 10,000 new compounds have been isolated from marine organisms. Some toxins produced by marine organisms are very potent to have anticancer activity (Folmer et al., 2009). Many types of the novel compounds and enzymes of marine origin have been reported with pharmaceutical applications (Martins et al., 2011).

Unlike terrestrial fungi, marine fungi are least utilized to produce valuable products, even though they are a rich source of structurally new natural products with a wide range of biological activities (Druzhinina et al., 2011). The fungi are capable of producing highly potent substances, Aspergillus niger and A. flavus var. Columnaris are now underway in the development of the antimicrobial compounds. The fungal genus Trichoderma/Hypocrea of terrestrial origin has received significant attention due to its biological and bio-control activities (Shibu et al., 2012). Our previous study has reported the marine Trichoderma as a potent source of phosphate solubilization and growth improvement of the mangroves species Avicennia marina (Saravanakumar et al., 2013). Trichoderma species are also producing the secondary metabolites with enormous pharmaceutical values such as antibacterial (Cheng et al., 2011), antiviral (Lu et al., 2002), antiprotozoal (Ciscotto et al., 2009), antifungal (Ande et al., 2008) and anticancer activities (Liu et al., 2009). However, the studies are limited on marine Trichoderma species in pharmaceutical applications. Marine T. virens isolated from ascidians and green algae is reportedly a producer of Trichodermanamides-A and B with high pharmaceutical value (Garo et al., 2003). Trichoderma strains isolated from marine sponges and seaweeds are also known to exhibit significant antimicrobial activity against human pathogens (Masuma et al., 2001). New polyketide-derivatives, Trichodermatides A–D (1–4) from the marine fungus T. reesei exhibits strong cytotoxicity against A375-S2 human melanoma cell line (Sun et al., 2008). Trichodenone A, B and C, extracted from marine T. harzianum OUPS115 shows significant cytotoxicity against leukemia P388 cell line (Thakur et al., 2003). T. longibrachiatum isolated from mussels produces the fatty acids (Ruiu et al., 2007). Recent genomic analysis of Trichoderma has emphasized the wide spread property within the genus (Druzhinina et al., 2011).

Human skin cancer is rapidly increasing throughout the world due to the UV and solar radiations; its incidence is about 30% of all types of human cancers (Armstrong and Kricker, 2001). Globally South Africa and Australia are affected by the skin cancer due to the high UV radiation (Staples et al., 2006). The DMBA is the site and an organ specific carcinogen, commonly employed to induce skin cancer in Swiss albino mice in experimental trials was used in the present study. DMBA could either be used as an initiator or promoter for inducing skin carcinogenesis. Repeated topical applications of DMBA (2 times per week for 12 weeks) induce skin carcinogenesis in the mice. According to the Norrheim et al. (1990), β-oxidation-resistant 3-thia analog of 3-thia heptadecanoic acid (THDA) can noticeably inhibit cancer cell growth in vitro. Recently Kim and coworkers (2010) have reported that palmitic acid, margaric acid (similar compound of HDA), and stearic acid from pacific oyster Crassostrea gigas increase apoptosis in human prostate cancer cells but do not show any apoptotic effects on normal liver cells. Trichoderma species are known to produce fatty acids. They also produce novel compounds like Trichodenone A, B, and C which exhibit significant cytotoxicity against the P388 leukemia cells.

The first report from our study has identified novel HDA and ODA from marine Trichoderma species and also reported that HDA has significant in silico docking effect against the skin cancer protein 4,5-diarylisoxazole HSP90 chaperone with a potent docking score of ~11.4592 kcal/mol than the ODA score of ~9.286 kcal/mol. This value is better than the known skin cancer drug “Dyclonine” with a docking score of ~8.935 (Saravanakumar et al., 2012). Heat shock protein 90 kDa (HSP90) is mainly responsible for chaperoning proteins involved in cell signaling, proliferation and survival of cancer. It seems to offer a novel unique target for anticancer drug development (Trepel et al., 2010; Franco et al., 2013). However, the present investigation made successfully the intracellular metabolites of HDA and ODA purified from Hypocrea lixii TSK8 and Hypocrea rufa SKS2 respectively to study their anticancer effects on selected human cancer cells to suppress HSP90 protein expression. In addition, an attempt was also made to evaluate the in vivo anti-skin cancer activity of the purified HDA metabolites. Hence, we presently report the role of purified bioactive compounds in several aspects of the anticancer effects including antioxidant, in vitro and in vivo, inhibition of HSP90 and ROS-dependent induction of apoptosis.

Materials and methods

Chemicals

Minimal Essential Media (MEM), penicillin, and streptomycin dodecyl sulfate polyacrylamide (SDS) and croton oil were procured from Hi Media Laboratories, Mumbai. Fetal bovine serum (FBS), trypsin, methylthiazolyl diphenyl-tetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO), 7,12-dimethylbenz anthracene (DMBA) and 1,2-diphenyl-1-picryl-hydrazyl (DPPH) and all other chemicals and reagents were procured from Sigma Aldrich Mumbai, India.

Microorganisms and culture conditions

H. lixii TSK8 (NCBI Accession No: JQ809340) and H. rufa strain SKS2 (NCBI Accession No: JQ619675) were isolated from the mangrove biotope and stored at 4°C. For the production of biomass, these Hypocrea strains were inoculated in a production medium containing (g L⁻¹ of 50% seawater) peptone (10), glucose (5), MgSO₄, NH₄NO₃ (2.4), yeasts extracts (5), ZnSO₄ (0.2), FeSO₄ (0.2) at pH 7.2 and incubated at 28°C for 12 days and then
the biomass was harvested for the extraction of intracellular metabolites.

**Extraction and purification of fungal metabolites**

The fungal biomass was homogenized with 80% methanol, filtered through Whatman No.1 filter paper and the filtrate was concentrated by using a rotary evaporator (VC 100A Lark Rotavapor) at 30°C. The extract was further purified according to the method proposed by Kim et al. (2010). In brief, this extract was further purified by using silica gel 80 vacuum column chromatography (2.5 cm × 50.0 cm and pore size of 15 μm), using a MeOH as eluent (CC). A total 80 fractions of crude methanol extract from *Trichoderma* was collected at a different retention time by CC. Followed by this, thin layer chromatography (TLC) methods, aluminum sheet pre-coated with silica gel 60 F254 nm (Merck) were used for TLC; the pots were visualized using both ultraviolet light (254 and 366 nm) and 50% H2SO4 as spray reagent. The pooled fractions were purified by HPLC using MeOH–H2O (1:1), and 40 fractions from *Trichoderma* sp., and tested for antioxidant potential. Further, the two potential fractions of each strain were purified by HPLC (Fig. 1). Two potential fractions were obtained on basis of antioxidant activity, and the compounds of each selected two fractions were confirmed by HPLC–MS as 16-methylheptadecanoic acid methyl ester (HDA) and 9,12-octadecadienoic acid (ODA) derived from *Trichoderma* sp., *H. lixii* and *H. rufa*, in addition to the purification of the extract verified by HPLC with the single intensity peak for each *Trichoderma* extracts. Further, the highly potential fraction of HDA was confirmed as 16-methylheptadecanoic acid methyl ester (HDA) by the 1H NMR, 13C NMR (600 MHz Nuclear Magnetic Resonance Spectrometer, Avance III 600 MHz). Fig. 1 illustrates the extraction and purification process of HDA and ODA from marine *Trichoderma*.

**In vitro antioxidant assays**

The antioxidant properties of purified HDA and ODA were determined by using six different assays: total phenol content using the Folin–Ciocalteu reagent (Singleton and Slinkard, 1997); radical scavenging activity using the DPPH (1,2-diphenyl-1-picrylhydrazyl) free radical (Duan et al., 2006); total antioxidant capacity (Prieto et al., 1999); reducing power (Oyaizu, 1986); nitric oxide radical scavenging property (Govindarajan et al., 2003); and, hydrogen peroxide radical inhibition activity (Gulcin et al., 2004).

**Cell culture and maintenance**

Human cell lines, such as oral carcinoma (KB cell line), skin carcinoma (A431 cell line) and normal cell line (VERO cell line,
African Green Monkey kidney cell line) were selected for the experiment. Cell lines were obtained from National Centre for Cell Sciences (NCCS), Pune, India. The cells were maintained in Minimal Essential Media supplemented with 10% fetal bovine serum (FBS), penicillin (100 U mL$^{-1}$), and streptomycin (100 μg/mL) in a humidified atmosphere of 5% CO₂ at 37 °C. The monolayer culture of the cell line of 48 h old at a concentration of 1 × 10⁶ cells/mL were seeded in 24 well plates. The plates were microscopically examined for confluent monolayer, turbidity and toxicity. The growth medium (MEM) was removed using micropipette. Care was taken so that the tip of the pipette did not touch the cell sheet. The monolayer of cells was washed twice with MEM without FCS to remove the dead cells and excess FCS. To the washed cell sheet, 1 mL of medium (without FCS) was added containing a defined concentration of the HDA and ODA in respective wells. Each dilution of the HDA and ODA ranged from 1:1 to 1:64 and they were added to the respective wells of the titer plate. To the control well, 1 mL of MEM without FCS was added. The plates were incubated for 24 h. The plates were microscopically examined for confluent monolayer, turbidity and toxicity. The growth medium (MEM) was removed using micropipette. Care was taken so that the tip of the pipette did not touch the cell sheet.

**Stock and working solutions for preparation of HDA and ODA**

The 0.05 mL of purified HDA and ODA was dissolved in 4.95 mL of DMSO giving a working concentration of 1 mg/mL. The working concentration was prepared freshly and filtered through a 0.45 μm filter before each assay. 5 mL of HDA and ODA was prepared in the concentration of 1 mg/mL. 500 μL of MEM without FCS was taken in 8 Eppendorf tubes for each sample. The samples were syringe filtered using 0.45 μM filter to remove contaminants. Then 500 μL of the working concentration of sample was added to the first Eppendorf tube and mixed well, then 500 μL of this volume was transferred from first to last tube by serial dilution to obtain the desired concentration of HDA and ODA. As a result the volume remained constant but there was a change in concentration.

**MTT assay**

MTT is called as (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide and the standard MTT-assay (Vivek et al., 2014). MTT is cleaved by mitochondrial dehydrogenase in viable cells, yielding a measurable purple product formazan. This formazan production is proportionate to the viable cell number and inversely proportional to the degree of cytotoxicity. In brief, after incubation, the medium from the wells was removed carefully for MTT assay. Each well was washed with MEM without FCS for 2–3 times and 200 μL of MTT (5 mg/mL) was added. The plates were incubated for 6–7 h in 5% CO₂ incubator for cytotoxicity. After incubation, 1 mL of DMSO was added to each well and mixed by pipette and left for 45 s. If any viable cells were present, they showed formazan crystals after adding solubilizing reagent (DMSO) and purple color formation. The suspension was transferred to the cuvette of spectrophotometer and the OD values were read at 620 nm by using DMSO as a blank. Standard Graph was plotted by taking concentration of the drug in X-axis and relative cell viability in Y-axis as follows:

Cell viability (%) = Mean OD/Control OD × 100

The concentration dependent (3.625–1000 μg/mL) cytotoxicity of HDA and ODA were tested on selected cell lines. After 24 h of incubation the cell viability of normal cell line and cell cytotoxicity of cancer cell lines was determined by the MTT assay. The cells were also observed under a microscope and taken for microphotographs to observe the changes in the cell morphology. Furthermore, to determine the IC₅₀ concentration the concentration of HDA and ODA at which 50% cancer cell inhibition concentration was observed, while CC₅₀ concentration of HDA and ODA at which 50% normal cell survival was also observed. Doxorubicin was used as a positive control. All experiments were done in triplicate and significant values as denoted as *p < 0.05. The therapeutic index value was calculated using the CC₅₀ value of normal cell line and IC₅₀ values of cancer cell lines as follows. Therapeutic index was calculated by CC₅₀/IC₅₀ (Mason, 1999). The induction of ROS activity was measured according to the earlier report (Thangam et al., 2014).

**Protein expression of Western blot analysis**

After the HDA treatments, HSP90 protein expression was detected by Western blot analysis. In brief, total protein was isolated, subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a polyvinylidene fluoride membrane. After being blocked, the membrane was incubated with primary polyclonal antibodies either anti-HSP90 or anti-β-actin overnight at 4 °C, and subsequently incubated with horseradish peroxidase-conjugated immunoglobulin G antibody as the secondary antibody for 1 h at room temperature. The protein bands were detected by an enhanced electrochemiluminescence detection system. After normalization by corresponding β-actin expression, protein expression levels were determined.

**DNA fragmentation analysis**

The cells were incubated with inhibitory concentration (IC₅₀) of HDA for the treatment of 24 h; DNA was extracted using a DNA isolation kit. The DNA was evaluated on a 1% agarose gel. The cells were incubated with inhibitory concentration (IC₅₀) of HDA for the treatment of 24 h; DNA was extracted using a DNA isolation kit. The DNA was evaluated on a 1% agarose gel. The cells were incubated with inhibitory concentration (IC₅₀) of HDA for the treatment of 24 h; DNA was extracted using a DNA isolation kit. The DNA was evaluated on a 1% agarose gel. The cells were incubated with inhibitory concentration (IC₅₀) of HDA for the treatment of 24 h; DNA was extracted using a DNA isolation kit. The DNA was evaluated on a 1% agarose gel.

**Animal model**

The purified HDA was used as a drug and the effect of HDA against skin cancer was tested in Swiss albino male mice. Swiss albino mice (weighing 25–30 g) was housed in well ventilated rooms (temperatures 23 ± 2 °C, humidity 65–70% and 10 h light/dark cycle) and fed with standard pellet diet and water ad libitum. The study was carried out in accordance with the Indian National law of animal care and use, and committee for the purpose of control and supervision of animals at Annamalai University (Reg. No.160/1999.CPCSEA).

**Experimental design**

Male mice with a weight between 25 and 30 g were kept under standard laboratory conditions for five days before the
Experiments. The hair was removed 2 cm² of the dorsal side of the experimental animals. The mice were randomly divided into four groups of six mice each. For the induction of the tumors, a two stage protocol consisting of initiation with a single topical application of carcinogen (DMBA dissolved in acetone) and two weeks later a promoter, croton oil was applied, three times a week for 12 months, were employed (Cibin et al., 2011). The HDA (100 µL/mL) was administered orally to the mice prior to promoter treatment, three times a week for 12 months. Animals were segregated into the following groups to investigate anti-tumor potential of the HDA.

**Group 1**: Control mice topically treated with 50 µL of acetone per animal.

**Group 2**: Mice were topically treated with DMBA (100 µL/100 µL of acetone) and croton oil (1% of 100 mL acetone).

**Group 3**: Mice were treated with DMBA (100 µg/100 µL of acetone) followed by croton oil (1% of 100 mL acetone) three times per week till the end of the experiment, applied topically over the shaven area of the skin of the mice, along with oral administration of the test sample 100 µg/mL of the HDA.

**Group 4**: Mice treated with 100 µg/mL of the HDA.

**Survival analysis**

Survival and body weight analysis of mice grouped under different treatment conditions were performed every week to check the possible toxic effects of administering compounds (Steele and Lubet, 2010). The animals were provided with food and water immediately after the drug administration. Mortality of the animals was observed up to 12 weeks. Animals were sacrificed by cervical dislocation after the termination of the experiment.

**Hematological observations**

The blood samples were analyzed using an automatic hematological assay analyzer (Swelab-24D) for hematological characteristics: red blood corpuscles (RBC), white blood corpuscles (WBC), mean cell volume (MCV), men corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet (PLT) counts, hemoglobin (HB) hematocrite (HCT), lymphocytes (LY) and total protein (Sithranga Boopathya et al., 2011).

**Biochemical observations**

The levels of acid phosphatase, alkaline phosphatase serum glutathione oxidase (SGOT), serum glutathione peroxides (SGPT) and lactate dehydrogenase (LDH) was estimated by the standard methods of King (1965).

**Statistical analysis**

The data were analyzed for analysis of variance (ANOVA) followed by DMRT and the results shown as mean ± standard error at the significance level $2\alpha = 0.05$.

**Results and discussion**

**Morphological and molecular characterization of marine Trichoderma**

Pictorial representation of the morphological characteristics of Trichoderma, H. lixii and H. rufa is presented in Fig. 2. *Trichoderma harzianum* is called as Anamorph but *H. lixii* is called as teleomorph (Chaverri and Samuels, 2003). The *Trichoderma virde* is called as Anamorph but teleomorph is called as *H. rufa* (Jaklitsch and Samuels, 2006). Colony was radius on potato dextrose agar (PDA) after 72 h at 30 °C. After 96 h at 30 °C in

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**Fig. 2** – Morphological observations of *Trichoderma* species; colonization in agar plates, light microscopic and scanning electron microscopic observations.
darkness on PDA conidia filled most of the Petri dishes. Conidia were formed densely over the center and in undulating concentric rings toward the edge and no pustules were formed. In many colonies, conidia were first at yellow, became yellow-green and often with yellow pigment diffusing in medium. After 96 h at 30 °C in darkness, Petri dish was filled with conidia that were dark green, uniformly dispersed except for the center of the colony, which remained sterile. The spore surface morphology was observed under light, and SEM images revealed that spores were spherical in shape and smooth surface. The colony nature of H. rufa was radius on PDA in darkness after 72 h at 25 °C and did not grow at above 35 °C; after 144 h in darkness, conidia were formed abundantly in conspicuous concentric rings. There was no diffusing pigment noted, occasionally with a sweet, ‘coconut’ odor and no distinctive odor were noted. Furthermore, Trichoderma species were confirmed by molecular characterization of ITS gene sequence of NCBI blast phylogenetic analysis (Fig. 3, Underlined). The isolated H. lixii strain TSK8 (JQ809340) was found to be closer by 90% of similarity with H. lixii strain C37. Further, H. rufa strain SKS2 (JQ619675) was 99% of similarity with H. rufa DAOM JBT1003. Hence, based on the morphological and phylogenetic analysis, the strains isolated from mangrove sediments were found belonging to the fungal genus Tricho-derma/Hypocrea.

**Extraction and purification of HDA and ODA from marine Trichoderma**

In our previous work, we have reported the unpurified compounds of HDA and ODA from Trichoderma by GC-MS (Saravanakumar et al., 2012). The identified ODA has the two double bond is located in acyl chain at C9 and C12 position and E or Z geometry of the olefins were determined as (Z,Z)-9,12-octadecadienoic acid. In this study the purified fraction of HDA was confirmed as the linear structure of 16-methylheptadecanoic acid methyl ester (HDA) by 1H NMR and 13C NMR. 1H NMR: 0.91 (d, 6H, J = 7.8 Hz), 1.25 (m, 6H), 1.26 (m, 14H) 1.29 (m, 8H), 1.62 (m, 3H), 2.32 (t, 2H, J = 7.8 Hz), 3.65 (s, 3H, OCH3) 13C NMR: 23.2, 25.0, 26.8, 28.1, 29.0, 29.6, 29.8, 29.9, 33.6, 39.6, 51.9, 173.1. The 1H NMR and 13C NMR spectra were recorded with an Avance III 600 MHz instrument operating in 600 (1H) and 150 (13C) MHz, using residual and deuterated solvent peaks as reference standards (Supporting information, Fig. 1a and b).

**In vitro antioxidant activity of HDA and ODA**

In our first in silico report (Saravanakumar et al., 2012) has recommended for further in vitro and in vivo studies toward the development of HDA as an anticancer drug in future. To the best of our knowledge, no other attempts, or reports from marine Trichoderma compound HDA as an anticancer potential for investigating in vitro and in vivo molecular level anticancer evaluation have not been successfully achieved. Therefore, we developed a way to investigate the HDA anticancer effect in molecular level is challenging and very attractive approach for both fundamental as well as developing molecular level understanding in cancer research. The present study investigated the antioxidant activity of HDA and ODA purified from potent strains of H. lixii and H. rufa, respectively. The purified compounds HDA and ODA showed significant antioxidant activity with increasing concentration from 50 μg/mL to 500 μg/mL. This observation was in accordance with the previous antioxidant studies in fungus, plants and bacterial extracts (Kim et al., 2002). The purified compound of HDA exhibited the highest total phenol content of HDA (90.01 ± 0.13%) than the ODA (80.61 ± 1.23%). The total antioxidant activity of HDA (85.61 ± 1.76%) was higher than ODA (80.91 ± 1.97%). The total DPPH radical scavenging activity of HDA was found to be more (90.21 ± 0.91%) than that of ODA (74.61 ± 1.55%). The nitric oxide radical scavenging activity determined the HDA was higher (83.3 ± 1.63%) than ODA (75.42 ± 0.41%). Further, the H2O2 radical scavenging activity was higher in HDA (89.03 ± 0.07%) and lower in ODA (79.18 ± 1.32%). The total reducing power was rich in HDA (89.76 ± 0.38%) and poor in ODA (85.61 ± 1.54%). Thus the overall...
antioxidant assays revealed that the HDA metabolite of *H. lixii* strain TSK8 was more effective than the ODA of *H. rufa* strain SKS2 (Fig. 4), due to the higher phenolic derivatives present in the HDA that is responsible for a potential antioxidant activity in removal of toxic free radicals (Branislav et al., 2011).

**In vitro anticancer effect of HDA and ODA**

To investigate the anticancer effect, two purified compounds of HDA and ODA were tested in vitro MTT-assay against the oral cancer cell line (KB) and skin cancer cell line (A431) for 24 h. The concentration-dependent effect of HDA and ODA on cell viability decreased with increasing concentrations of HDA and ODA. The cell viability of HDA and ODA inhibitory concentration of KB cells were found to be $125 \pm 0.32 \mu g/mL$ (IC$_{25}$), 18.75 ± 0.12 (IC$_{50}$), 3.625 ± 0.01 (IC$_{75}$ – 60% of cells viability), and 437 ± 0.36 μg/mL (IC$_{25}$), 75.50 ± 0.42 (IC$_{50}$) and 6.34 ± 0.02 (IC$_{75}$) for 24 h, respectively (Fig. 5). The concentration dependent effect of HDA and ODA on A431 cells was found to be 250 ± 0.36 μg/mL (IC$_{25}$), 37.50 ± 0.42 (IC$_{50}$), 4.21 ± 0.02 (IC$_{75}$), and 750 ± 0.16 μg/mL (IC$_{25}$), 72.89 ± 0.15 (IC$_{50}$), and 3.62 ± 0.01 (IC$_{75}$), respectively (Fig. 6). Results clearly indicated that KB and A431 cancer cells exhibited strong growth inhibitory effects when compared to the normal Vero cells (Supporting information, Fig. 2). Morphological changes of selected cancer cells after the incubation with HDA and ODA were observed under the light microscope. Selected cancer cells were incubated with HDA and ODA with inhibitory concentrations. The morphological changes were observed in HDA and ODA treated KB and A431 cell lines when compared with the untreated cells (Supporting

Fig. 4 – The antioxidant activity of purified compounds HDA and ODA were determined by using different assays. The total phenol content using the Folin-Ciocalteu reagent; radical scavenging activity using the DPPH, free radical, total antioxidant capacity, reducing power, nitric oxide radical scavenging property and hydrogen peroxide radical inhibition activity were compared with these two compounds. Results shown are mean ± SEM (n = 3), different superscript letters indicate statistically significant findings.
Fig. 5 – In vitro anticancer effects of purified compounds HDA and ODA were determined by using MTT-assay on the oral cancer KB cells. Results shown are mean (n = 3).

Fig. 6 – In vitro anticancer effects; the skin cancer A431 cells treated with HDA and ODA after 24 h of incubation. The cell viability of HDA and ODA were compared with anticancer drug DOX. Results shown are mean (n = 3).

information, Fig. 2). The most recognizable morphological changes of HDA and ODA treated cells observed in this study was the cytoplasmic condensation, cell shrinkage, production of numerous cell surface protuberances at the plasma membrane and aggregation of the of cells into dense masses beneath the membrane (Fig. 7). In the present study, a standard anticancer drug DOX was used as a positive control against the two selected cancer cell lines to compare with our purified compounds, and the inhibitory concentration of KB and A431 cells were found to be 5.81 ± 1.32 (IC50) and 18.75 ± 0.17 (IC50), respectively. From our results, we suggested that HDA and ODA could effectively inhibit in vitro human skin cancer as well as oral cancer cells. The comparison of HDA and ODA with known anticancer drug DOX revealed that the DOX showed better activity than the tested purified compounds. But, DOX induced higher cytotoxic effect on normal cells at higher concentration, this data supported with previous reports that DOX-induced apoptosis in normal bovine aortic endothelial cells and adult rat normal cardiomyocytes (Wang et al., 2004). The cytotoxic effect of HDA and ODA on normal cell lines revealed the less toxicity at a higher concentration of 750 μg/mL (Supporting information, Table 1 and Fig. 3). In this regard, therapeutic index (or selectivity index) is an important parameter as it includes both anticancer activity and eventual toxicity to the normal cells. In the present study, therapeutic index value was found to be 40 against oral cancer cell line and 20 against skin cancer cell line. A drug is considered to be worthy of further testing if it has a therapeutic index value of 16 or greater (Kryston et al., 2011). The early reports are comparable to our present study that the endophytic fungi A. Niger, Trichoderma sp., and Fusarium isolated from the mangrove plant Avicennia marina have been proved to have anticancer activity against human epidermal carcinoma of the oral cavity, KB cell line, human Burkitt’s lymphoma, Raji cell line and Hep2 cell line (Cheng et al., 2011). Reactive oxidative stress (ROS) such as hydroxyl radical (OH−), superoxide anions (O2−), hydrogen peroxide (H2O2) and peroxyl radicals (ROO−) are common products of aerobic metabolism that can be useful or harmful to biological systems. Low concentration of ROS may facilitate signal transduction, enzyme activation and other cellular functions, but high concentration of ROS generates DNA damage (apoptosis), protein and lipid which can lead to cell transformation (Holmstrom and Finkel, 2014). As the mitochondrial respiratory chain is the main source of intracellular ROS production in aerobic cells, mitochondrial dysfunction (∆ψm) may lead to oxidative stress. On the other hand, mitochondrial membrane is among the major susceptible targets of the deleterious effects associated with intracellular ROS (Sawarkar et al., 2012). Our results also showed that HDA and ODA could induce ROS generation in treated cancer cells. Concentration based ROS-dependent programmed cell death were also clearly demonstrated in skin cancer A431 cells (Fig. 8). With support of this data, further the purified compound HDA was tested against the HSP90 protein expression by Western blot analysis in skin cancer cells. Our earlier report has suggested that the ability of HDA suppresses the HSP90 protein with highest docking score value from in silico method (Saravanakumar et al., 2012). Hence, the present study further confirmed HSP90 protein expression in skin cancer cells by HDA treatment. The molecular mechanism of HDA targeted HSP90 protein expression was down-regulated, which was confirmed by the Western blot analysis (Fig. 9). The Western blot results clearly supported the inhibition of HSP90 protein expression after the treatment of the HDA. The molecular chaperone HSP90 promotes the maturation of several kinds of important proteins and plays a remarkable role in the development of cancer progression (Quinlan and Ellis, 2013). According to the published reports on HSP90, the specific “client” protein plays key role in the regulation of cancer. It also upregulated 10-fold in tumor cells, suggesting that HSP90 helps to maintain tumor cell growth and survival. Another role for HSP90 maintenance of tumor cells is its ability to inhibit apoptosis (Trepel et al., 2010). Apoptosis is a highly conserved phenomenon that plays a vital role in the regulation of the cellular activities and is characterized by chromatin condensation. Furthermore, the effect of HDA induced apoptosis via the DNA fragmentation in the selected cancer cells were confirmed by AGE analysis. Because of the formation of DNA ladder due to the fragmentation of DNA is widely regarded as a biochemical hallmark of late apoptosis. The formation of DNA fragmentation in the present study was verified by extracting the DNA from apoptotic cells treated
with HDA followed by the detection on agarose gel. It was (Fig. 10) clearly demonstrated that the DNA laddering pattern occurred in cancer cells treated with HDA and there was no laddering in untreated A431 cells. The apoptosis of DNA fragmentation induced by HDA might be the HSP90 inhibition and activation of apoptosis inducing factors such as caspase activation mainly responsible for the cell death as well as DNA fragmentation. According to our earlier reports, we have clearly proved the activation of caspase enzyme activity induced apoptosis by DNA damage in cancer cells by anticancer drugs (Vivek et al., 2014). Hence, these results clearly authenticate that, HDA is a potent inhibitor of HSP90 and induces apoptosis through the DNA fragmentation of selected anticancer cell lines.

**In vivo anticancer effect of HDA**

The effect of the purified HDA on experimental animals induced with skin cancer by using DMBA and Croton oil was tested in vivo skin cancer activity (Supporting information, Fig. 4). Here, the DMBA acts as a carcinogen to induce cancer and Croton oil acts as a promoter of cancer (Sithranga Boopathya et al., 2011). After the cancer induction by these DMBA and Croton oil the physical observations of the animal survival and weight loss were noted (Fig. 11). The low survival of 59.80 ± 1.5% observed in group 2 skin cancer induced animals and the significant survival of 79.80 ± 1.97% was recorded in group 3 skin cancer induced animals treated with HDA. These results suggested that survival was higher by 33.44% by the treatment of the HDA. The dose-dependent concentration of HDA was optimized on the animal survival by treating with different concentrations of HDA (50 μg/mL, 100 μg/mL and 150 μg/mL) to the healthy animals and 100 μg/mL was found to be appropriate concentration for the animal experiment. The 100% percentage of survival was observed in group 4 animals treated with HDA (100 μg/mL) and this revealed that the present compound was non-toxic in normal healthy animals. The significant weight loss of 50 g was observed in the group 2
animals induced with skin cancer with DMBA and Croton oil as compared with group 3 animals induced with skin cancer treated with HDA which exhibited weight loss of 15 g. Interestingly, HDA increased 8.8 g of animal body weight as compared to the group 1 control animals. HDA is a fungal metabolite of fatty acid enhanced the growth and survival of human and animal. The effect of the fungal metabolite of HDA on inflammatory and anemia factors in the treated animals is presented in Table 1. Compared to the cancerous animals (group 2), the group 3 and 4 were significantly noticed with the changes in WBC, RBC, monocyte, HGB, HCT, MCV, MCH, MCHC and PLT. These inflammatory and anemia, changes revealed that the cancerous animals recovered from the HDA treatment. A similar change has been reported with white blood cells, lymphocytes and neutrophils in oral tumor bearing mice treated with mangrove tea (Sithranga Boopathya et al., 2011). The white blood cell count is a single measurement, shown to predict risk for specific diseases, including cancer and cardiovascular diseases (Henk et al., 2003). The increased expressions of WBC, lymphocyte, neutrophils are often observed in various cancer patients (Henk et al., 2003). It is hypothesized that activated platelets in the tumor vasculature release TPO, and thereby stimulating the bone marrow generation of platelets. As a consequence, increased numbers of platelets, lymphocytes and neutrophils reach the circulation as they are often seen in cancer patients (Henk et al., 2003).
Mean corpuscular hemoglobin (MCH) is the average amount of hemoglobin per red blood cell in a blood sample. MCH is used to help diagnose the cause and severity of anemia. When MCH is low, it indicates that a person has iron-deficiency anemia. The biochemical parameters such as alkaline phosphate (U L⁻¹), SGPT (U L⁻¹) and LDH (g dl⁻¹) were significant between the experimental groups (Table 2). The lower level of the liver toxic enzymes in the HDA treated animals (group 4) revealed that the HDA did not have toxic effect on experimental animals. The effects of HDA on histopathology were observed in the experimental animals. DMBA and croton oil topically treated animals showed irregular multilayered epithelial cells indicating the skin cancer epithelial cell proliferation as evidenced from Fig. 12. The proliferation of skin cancer cells were significantly reduced by HDA. There was no noticeable abnormality in skin epithelial cell of experimental animals control untreated. The present hematological, biochemical and histopathological results revealed that the fungal metabolite of HDA of H. lixii TSK8 was a highly potent to prevent the skin cancer and dermatological changes in the skin. The molecular mechanism of HDA induced ROS-dependent programmed cell death (PCD) in cancer cells was well proved from this present investigation, HDA is a potential chemotherapeutic agent for treating cancers (Fig. 13). In general, the prediction of the biological compounds responses and

### Table 1 – Effect of the HDA on inflammatory and anemia factors in experimental animals.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Control</th>
<th>DMBA + Croton oil</th>
<th>DMBA + Croton oil + HDA</th>
<th>HDA</th>
<th>p Value significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (10⁶ μL⁻¹)</td>
<td>10.2 ± 1.2</td>
<td>15.6 ± 0.5</td>
<td>12.0 ± 0.5</td>
<td>10.5 ± 0.5</td>
<td>0.008*</td>
</tr>
<tr>
<td>Lymphocyte (%)</td>
<td>64.9 ± 2.3</td>
<td>83.2 ± 0.6</td>
<td>70.2 ± 0.6</td>
<td>58.4 ± 0.2</td>
<td>0.000*</td>
</tr>
<tr>
<td>Monocyte (%)</td>
<td>1.7 ± 0.2</td>
<td>1.5 ± 0.2</td>
<td>1.8 ± 0.2</td>
<td>1.6 ± 0.3</td>
<td>0.995</td>
</tr>
<tr>
<td>Granulocyte (%)</td>
<td>32.8 ± 0.5</td>
<td>24.8 ± 0.3</td>
<td>27.5 ± 0.3</td>
<td>19.5 ± 0.3</td>
<td>0.000*</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>7.3 ± 0.3</td>
<td>4.9 ± 0.3</td>
<td>6.8 ± 0.4</td>
<td>7.3 ± 0.2</td>
<td>0.023*</td>
</tr>
<tr>
<td>HGB (g dL⁻¹)</td>
<td>12.2 ± 0.1</td>
<td>10.0 ± 0.1</td>
<td>11.0 ± 0.5</td>
<td>12.7 ± 0.1</td>
<td>0.201</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>47.3 ± 0.3</td>
<td>34.9 ± 0.5</td>
<td>43.5 ± 0.6</td>
<td>47.9 ± 0.8</td>
<td>0.000*</td>
</tr>
<tr>
<td>MCHC (g dL⁻¹)</td>
<td>10.6 ± 0.6</td>
<td>76.0 ± 0.2</td>
<td>57.3 ± 0.6</td>
<td>60.7 ± 0.6</td>
<td>0.025</td>
</tr>
<tr>
<td>PMN (%)</td>
<td>3.2 ± 0.3</td>
<td>31.0 ± 0.3</td>
<td>32.3 ± 0.2</td>
<td>32.9 ± 0.2</td>
<td>0.457</td>
</tr>
<tr>
<td>PLT (10⁴ μL⁻¹)</td>
<td>692.8 ± 0.3</td>
<td>707.8 ± 0.3</td>
<td>662.8 ± 0.3</td>
<td>630.8 ± 0.2</td>
<td>0.000*</td>
</tr>
</tbody>
</table>

Results are mean ± SD (n = 6 mice in each group).
* Statistically significant.

### Table 2 – Effect of the HDA on blood biochemical parameters in experimental animals.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Control</th>
<th>DMBA + croton oil</th>
<th>DMBA + croton oil + HDA</th>
<th>HDA</th>
<th>p Value significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline phosphate (U L⁻¹)</td>
<td>54.8 ± 1.2</td>
<td>59.8 ± 1.3</td>
<td>56.8 ± 0.6</td>
<td>49.8 ± 0.6</td>
<td>0.000*</td>
</tr>
<tr>
<td>SGOT (U L⁻¹)</td>
<td>4.8 ± 1.1</td>
<td>6.8 ± 0.6</td>
<td>5.8 ± 0.4</td>
<td>4.8 ± 0.6</td>
<td>0.368</td>
</tr>
<tr>
<td>SGPT (U L⁻¹)</td>
<td>7.8 ± 0.9</td>
<td>11.8 ± 0.63</td>
<td>9.8 ± 0.6</td>
<td>8.8 ± 0.6</td>
<td>0.042*</td>
</tr>
<tr>
<td>Total protein (g dl⁻¹)</td>
<td>2.3 ± 0.3</td>
<td>3 ± 0.3</td>
<td>2.1 ± 0.5</td>
<td>2.5 ± 0.5</td>
<td>0.977</td>
</tr>
<tr>
<td>LDH (g dl⁻¹)</td>
<td>69.8 ± 2.6</td>
<td>75.8 ± 0.5</td>
<td>73.8 ± 0.6</td>
<td>68.8 ± 0.3</td>
<td>0.001*</td>
</tr>
<tr>
<td>Acid phosphatase (U L⁻¹)</td>
<td>2.8 ± 0.2</td>
<td>4.8 ± 0.6</td>
<td>3.8 ± 0.5</td>
<td>2.8 ± 0.3</td>
<td>0.368</td>
</tr>
</tbody>
</table>

Results are mean ± SD (n = 6 mice in each group).
* Statistically significant.

Fig. 12 – Histopathological changes in the skin samples of experimental animals (a) control, (b) DMBA + croton oil, (c) DMBA + croton oil + HDA and (d) HDA treatments.
underlying mechanism on cancer treatment is important for the successful drug discovery (Unger et al., 2015). Therefore this work revealed the basic to molecular level of drug responses and possible mechanism of anti skin cancer activity of HDA. Since the applied biomedicine has been improve the general economy on the drug discovery (Berger, 2011). Our results support the emerging needs of HDA from marine biological resources, in particular Trichoderma.

**Conflict of interest**

The authors declare that there are no conflicts of interest.

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**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jab.2015.04.001.

**Fig. 13 –** The proposed molecular mechanism of HDA induced ROS-dependent apoptosis in cancer cells is now well proved from this present investigation, HDA is a potential chemotherapeutic agent for treating cancers.

**REFERENCES**


