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Original Research Article

Effect of Moracin on DMBA-TPA induced skin cancer in the mice, *Mus musculus (L).*

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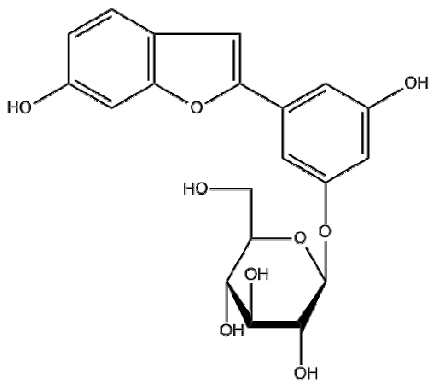
Abstract: The phytochemicals serves to orchestrate the healthy metabolism in the animal cells. The efforts in the study were conducted to assess the protective influence of Moracin, the major constituent of leaves of mulberry, *Morus alba* (L.) on tumor promotion in 7, 12 – dimethylbenz (alpha) anthracene (DMBA) – initiated and 12-O-tetradecanoylphorbol 13-acetate (TPA) – promoted mouse skin tumerigenesis model. The acetone solution of Moracin was topically applied to DMBA – initiated female mouse (*Mus musculus*) skin at the dosage of 2.5 and 5 mg twice per week for sixteen weeks, thirty minutes prior to each promotion treatment with TPA in the first experimental schedule. The significant reduction in tumor incidence and tumor multiplicity effects were evident in the treated group. The expression of tumor necrosis factor (TNF) – alpha protein and the level of 4-hydroxynoneal (4HNE) in the normal epidermis were significantly reduced in both moracin treated groups. Moracin at the dosage of 5 mg was topically applied to the dorsal surface of mouse skin 30 minutes before application of a TPA in the second effort in the study. And the same dosages of TPA and Moracin were applied twice at the interval of 24 hours. Moracin treatment was found inhibiting the double TPA treatment – induced morphological changes reflecting inflammatory response, including leucocyte infiltration, hyperplasia and cell proliferation. Moracin treatment furthermore significantly suppressed the elevation in 4-HNE level and elevated expression of *c-fos*, *c-myc* and cycloxygenase-2 (COX-2) in normal epidermis induced by double application of TPA. The moracin was found protective influence in tumor promotion. Utilization of Moracin may open a new avenue in the treatment of tumerigenesis.

Keywords: c-fos; c-myc; COX-2; Moracin Moracin; Cytokines; TPA and DMBA.

Introduction

The plants are the richest source of healthy and energy rich compounds for the animal life. The biochemical constituents of mulberry, *Morus alba* (L.) serve a lot to orchestrate the progression of life cycle of lepidepteran insects like silkworm, Bombyx mori (L). Mulberry leaves are also cut for food for livestock (Cattle, goat, etc.) in the areas where dry seasons restrict the availability of ground vegetation. In the traditional Chinese medicine, the mulberry fruits are used to treat the prematurely grey hair, to tonify the blood and to treat constipation and diabetes. Moracin-M; Steppogenin-4′-O-beta-D–glucoside and mulberroside-the novel compounds of mulberry, Morus alba (L) were found to produce hypoglycemic effects (Zhang, *et al.,* 2009). Ethanolic extract of leaves of mulberry, *Morus alba* (L) had antihyperglycemic, antioxidant and antiglycation effects in chronic diabetic rats (Naowaboot, *et al.,* 2009).

Moracin-M-3′-O-β-D-glucopyranoside



Structural Information:
Systematic Name: 3-hydroxy-5-(6-hydroxy-2-benzofuranyl) phenyl β-D-Glucopyranoside Common Name: Moracin-M-3′-O-β-D-glucopyranoside (Source Reference: wakndb.u.toyama.ac.jp/wiki/compound:moracin-M-3′-o-beta-D-Glucopyranoside).

Involvement of oxidative stress in cancer induction and its subsequent development, and associated molecular mechanism is becoming increasingly clear (Lahiri *et al.,* 1999 and Ames *et al.,* 1995).

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The skin is directly and frequently exposed to sun light. And the skin is always in contact with oxygen, which resulting in the production of reactive oxygen species (R O S) implying that, the skin is always in a state of being attacked by ROS (Garmyn and Degreef, 1997). There is a possibility of suppression of skin cancer promotion through the antioxidant activity of Moracin. For the purpose to screen the possibilities of cancer suppression through the phytocompounds there is a well known method on TPA-induced tumor promotion in DMBA initiated in mouse skin. Application of TPA triggers excessive R O S production by leucocytes in mouse skin ultimately leading tumor promotion (Dragsted, 1998 and Nakamura, *et al.,* 1998). This method had been undertaken to address whether the given compound suppress TPA-induced oxidative stress in mouse skin. TNF-alpha, one of the inflammatory cytokines, act as endogenous tumor promoter, and induces similar biochemical and biological responses as known tumor promoters (Suganuma, *et al.,* 1999 and Fuji, *et al.,* 1997). On this much background, the efforts has been planned to analyze the expression of epidermal TNF-alpha in DMBA-initiated TPA-promoted skin of mice, *Mus musculus* (L).

Material and Methods

Chemicals and Experimental Animals:

The chemicals like DMBA (7, 12-dimethylbenz alpha anthracene); TPA (12-0-tetradecanoylphorbol 13 acetate) and Moracin were procured from Sigma Chemicals. Six week old female CD-1 (ICR): Crj mice (*Mice musculus*) were obtained from Department of Zoology, University of Pune. The mice were housed in four cages and maintained at 28 degree celcious and subjected to a 09:15hours light-dark cycle (Lights on 8.00a.m. to 5.00p.m.). The mice in cages were acclimatized for one week before the experimental use. They were feed a commercial stock diet and deionized water. The mice were maintained in laboratory through the standard methods. The body weight was measured every week. The dorsal surface of the skin was shaved using electric clippers. The mice with hair cycle in the resting phase were used in the studies.

Experimental Effort-I:

The long term study entitled, Experimental Effort-I was carried through two steps, which include: A. Two stage carcinogenesis in the skin of mice; B. Histochemical observation and Immuno histochemical Staining.

A. Two Stage Carcinogenesis in the Skin of Mice:

The experimental animals were randomly divided into three groups with twenty individuals each. The groups include Control, 2.5 mg Moracin treated and 5 mg Moracin treated. The mice were initiated with single application of 190 nanomol of DMBA in 0.2 ml acetone painted to the dorsal surface of each individual mouse. After one week the mice in control groups were treated topically with 0.2 ml of acetone, and the individuals in the treated groups were treated with either 2.5 or 5.00 mg dosages of Moracin in 0.20 ml acetone. Half an hour after the treatments, a 3.20 nanomol dosage of TPA per individual was applied topically to the animals in all the three groups. The TPA alone or two dosages of Moracin plus TPA treatments were repeated two times per week up to the termination of experiment at sixteen weeks from the starting of TPA treatment. At the intervals of seven days, one of the mouse in each group was selected randomly; anaesthetized with chloroform soaked cotton pad and dissected for skin tissue. The skin tumor formation was recorded weekly, and tumors of greater than one milimetere in diameter were included in the cumulative total if they persisted for two weeks or more. The Latent periods for the onset of tumor in various groups were computed, and the tumors were diagnosed histologically at the termination of the experiment. At this point, the average volume of tumor per mouse was also recorded.

B. Histochemical Observation and Immunohistochemical Staining:

The skin tissue from each group was removed from the individual mouse and fixed in ten percent neutral-buffered formalin at four degree Celcious and embedded in paraffin. Biopsied tumors were sectioned to four micrimetere thickness, stained with haematoxylin and Eosin (H and E) and diagnosed histologically. For the Immunohistochemical analysis of apoptosis labeling was examined by TUNEL method. This method is based on TdT-mediated dUTP-biotin nick end labeling of fragmented DNA (Komatsu *et al.,* 2001). After deparaffinization, the specimens were stained by Apoptosis in situ Detection Kit (Wako Pure Chemicals, Osaka, Japan). The quantification was made by counting the apoptosis-positive cells as well as the total cells at ten arbitrarility selected fields at x40 magnification within the normal epithelial regions in a blinded manner. The percentage of the apoptosis –positive cells for different treatment samples was determined as: number of the positive cells x 100/total number of cells (labeling index cells). The TNF-alpha-and 4-HNE staining method was carried through the method described below:

After deparaffinization, affinity-purified goat polyclonal TNF-alpha (L-19) antibody (Santa Cruz Biochemistry, Santa Cruz, CA, USA) and monoclonal anti-4-HNE antibody (Nippon Yoshi, Tokyo, Japan) were put on the specimens. Then, the TNF-alpha (L-19) staining specimens were stained by a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) and the 4-HNE staining specimens were stained by Vectastain Universal Quick Kit (Vector Laboratories, Burlingame, CA, USA).

Images from immunostaining were obtained using Olympus light microscope and Olympus Smart Media 8MB film. These scaned and formatted as tiff images in Abode Photoshop 5.0 and Microsoft Powerpoint in order to make the composite figures.

Experimental Effort-II:

The short term study, entitled Experimental Effort-II was carried through: Double TPA treatment of mouse skin; Epidermal hyperplasia and leucocyte infiltration; Immunohistochemical Staining and 4-HNE, c-fos, c-mycand COX-2 staining method.

A. Double TPA Treatment of mouse Skin:

The double treatment protocol was based on the method of Nakamura, et al (1998). The dorsal surface of each mouse was shaved using electric clippers two days before each of the experiment. Each experimental group consisted of five mice. 5.0 mg of Moracin in 0.2ml acetone was topically applied to the shaved dorsal surface of the skin of individual mouse half an hour before the application of a TPA solution (8.1nanomol in 0.1 ml acetone).

B. Double Treatment Protocol:

In the double treatment protocol, the same dosages of TPA and the test compound or acetone were applied twice at the interval of twenty four hours. The mice were divided into three groups: AT (acetone-acetone / acetone-acetone); TPA (acetone-TPA / acetone-TPA) and Moracin + TPA (Sericin-TPA / Sericin-TPA).

C. Epidermal Hyperplasia and Leucocyte Infiltration:

The mice treated by double-treatment protocol were sacrificed one hour after the last TPA treatment. For the epidermal hyperplasia study, skin samples from different treatment groups were fixed in ten percent buffered formalin and embedded in paraffin.Vertical sections of four micrometer thick were cut, and stained with H and E. Epidermal hyperplasia was determined as mean vertical epidermal thickness and mean number of vertical epidermal cell layers by microscopic examination of different treated skin tissue sections. For each section of skin, the thickness of the epidermis from the basal layer to the stratum corneumwas measured at ten equal distance interfolicular sites using light microscope equipped with eye piece micrometer. A total of fifty sites (Ten sites per skin section per sample, a total of five skin samples were determined) were examined per group. The number nucleated cell layers was counted in the same areas. The number of infiltrating leucocytes was counted at five different areas of each section using a digital image analysis with micro analyzer (Poladigital Company Limited, Tokyo, Japan).

D. Immunohistochemical Staining:

For the purpose to document TPA induced increases in epidermal proliferation, the Immunhistochemical Staining method was used. The method may be named as Immunochemical Detection Combined with Antobody Directed against Proliferating Cell Nuclear Antigen (PCNA). After deparaffinization, the skin sections were treated with 1.2 percent Hydrogen peroxide in absolute methanol for twenty minutes. The Dako Epos anti-PCNA/HRP antibody (PC 10; Dako A/S, Demark) was put on the specimens. The quantitation of proliferating cells was made by counting the PCNA-positive cells as well as the tota cells at ten arbitrarily selected fields at X40 magnification within the normal epitjhelial regions in a blinded manner. The percentage of PCNA-positive cells per 10 x 40 fields for different treatment samples was determined as: number of the PCNA-positive cells x 100 / total number of cells (labeling index of PCNA). The 4-HNE, c-*fos*, c- *myc* and COX-2 staining methods were carried out as described here. After deparaffinization, monocle;onal anti-4-HNE antibody (Nippon Yushi, Tokyo, Japan), rabbit polyclonal anti-c - *fos* antibody (Oncogene Research Product, Cambridge, MA, USA) , rabbit polyclonal anti-c-*myc* antibody (Santa Cruz Biochemistry, Santa Cruz , CA, USA) and affinity-purified goat polyclonal COX-2 antibody (Santa Cruz Biochemistry, Santa Cruz, CA, USA) were put on the specimens. Then, the 4-HNE and COX-2 staining specimens were stained by a Vectstasin Universal Quick Kit and the c-*fos* and c-myc staining specimens were stained by counting the various positive cells as well as the total cells at ten arbitrarily selected fields at X40 magnification within the normal epithelial regions in a blinded manner. The percentage of various positive cells for different treatment samples was determined as: number of the positive cells x 100 / total number of cells (labeling index).

The experimentations were repeated for three times for consistency in the results. The data were subjected for statistical analysis. The values were presented as means +_ SE. The tumor incidence and tumor multiplicity were analyzed by x2 test and Wilcoxon rank sum test, respectively. Other data were analyzed by Student's t-test. Differences with $p < 0.05$ were considered significant. Some data were analyzed by regression analysis.

Results and Discussion

The efforts on the topical application of Moracin prior to each TPA application resulted in highly significant preventive effect against TPA-induced tumor promotion in DMBA-initiated ICR mouse skin. With reference to anti-tumor activity of Moracin, the percentage of tumor incidence was significant in the treated groups. Topical application of Moracin prior to that of TPA in DMBA-initiated ICR mouse skin resulted in significant protection. The time of appearance of the first tumor was delayed by one week in 2.5mg Moracin treated groups of mice. However, application of 5 mg Moracin did not produce any tumors on the skin by week 15, and only one small tumor in one mouse was seen at 16 week of the tumor promotion. Sixty one percent ($p<0.01$) of the mice in 2.5mg Moracin treated group was found calculated during the assessment at the week 10 of tumor promotion in comparison of hundred percent mice with skin tumors in non-Moracin-treated group. At the termination of the experiment, at 16 weeks, compared to hundred percent individuals with the skin tumors in the non-moracin-treated control group, only eighty eight percent of the individuals in the 2.5 mg and 5 mg moracin-treated groups respectively, exhibited skin tumors accounting for thirteen and ninety four percent ($p<0.01$) inhibition in tumor incidence, respectively. In the evalution of the data for tumor multiplicity (the number of tumors per individual), beginning with the first tumor appearance up to the termination of experiment, all the two dosages of Moracin were found responsible for highly significant protection against the TPA-induced complete tumor promotion in the mouse skin. At the end of experimentation, at sixteenth week, compared to 17 (+ / - 1.2) tumors per individual in non-moracin-treated group, only 5.5 (+/- 0.7) and 0.1 (+/- 0.01) tumors per individual were observed in the 2.5 and 5 mg Moracin-treated groups, respectively. This was found to account for 69 and 99 percent inhibition ($p<0.05$), respectively. With reference volume, the tumor volume per mouse and tumor volume per tumor were found to be significantly lower ($p<0.001$) in different dosages of Moracin-treated groups (Table.1). There was no difference in the average body weight between two dosages of Moracin-treated and non - Moracin-treated groups of mice in entire experimentation.

The tumors in each group of mice were histologically identified as papillomas. The labeling index of apoptosis cells was found unaffected. The strong cytoplasmic and membrane TNF-alpha exprerssion was observed in the epidermal cells from the non-moracin-treated-control group. Where as only rare staining for TNF-alpha was observed among the epidermal cells in the samples from both moracin-treatment groups. There was a weak 4-HNE-immunoreactivity observation in the cytoplasm of epidermal cells from both moracin –treatment groups. While, it was markedly increased in the non-moracin-treated-control group.

There was significant induction of epidermal hyperplasia, namely vertical epidermal thickness and vertical epidermal cell layers as compared to double application of acetone as vehicle, application of double dose of acetone and TPA to the shaved mouse skin. However, pre-application of Moracin at 5 mg dose prior to each TPA application protocol resulted in marked inhibition ($p<0.01$) of TPA-induced epidermal hyperplasia. Compared to acetone control, pre-application of Moracin to that of each TPA treatment did not result in an increase3 in either mean epidermal thickness or mean vertical epidermal cell layers. Greater number of leucocytes were found to have infiltrated the dermis by double-TPA application as compared with the acetone treated control, where as double pre- treatment with Moracin significantly inhibited (eighty two percent reduction, $p<0.01$) the leukocyte infiltration in comparision with TPA-treated group without receiving Moracin. There was no significant difference between acetone control and Moracin-treatment group ($p>0.05$). The PCNA-labeling index is the marker for cell proliferation. This index, in the epidermis of double application of TPA mice was found increased by 3.2 fold ($p<0.01$) over that of control group (Table.2). However, pre-treatment with Moracin at 5 mg dose prior to each TPA application significantly reduced (Eighty nine percent reduced, $p<0.01$) the PCNA-labeling index (Table-2) compared with the TPA-treated without receiving the Moracin treatment. Compared to the acetone treated control, pre-application of Moracin to that of each TPA treatment did not result in the increase in the PCNA-labeling.

Inhibitory effect of Moracin on TPA-induced elevations of 4-HNE, and of the expressions of expressions of *c-fos, c-myc* and *COX*-2 proteins in ICR mice skin are shown in the fig. As compared with acetone-treated, the double application of TPA resulted in the significant induction of epidermal 4-HNE labeling index and expression of *c-fos, c-myc* and *COX*-2. However, double pre - treatment with Moracin at a dose of 5 mg prior to that of each TPA application significantly reduced the labe4lling index of 4-HNE and expressions of *c-fos,c-myc* and *COX-2* (percent reduction: 83, 91 and 76 respectively), accounting for $p<0.01$, compared to the TPA-treated group without receiving Moracin. In addition, the labeling index of 4-HNE observed in all epidermis was significantly correlated with those of c-fos, c-myc and COX-2 proteins in all epidermis ($r = 0.782$. 0.691 and 0.513 respectively, $p<0.01$). The Moracin treatment was found most significant in the preventation of tumor growth.

Moracin, the beta-D-Glucopyranside exhibited strong anti-tumor promoting effect in the mouse skin two-stage-tumorigenesis model, and suggesting that, the Moracin could be useful as skin - cancer preventing agent. Double application of TPA to the mouse skin lead to excessive ROS production (Nakamura *et al.,* 1998). The available data suggest that each application induces two distinguishable biochemical events, namely priming and activation. The first event, priming is characterized by infiltration of inflammatory leukocytes. The second event, the activation is characterized by ROS production from accumulated leukocytes (Murakami, *et al.,* 2000). Induction of inflammatory response, as seen by dermal recruitment of inflammatory cells, is thus, integral part of response of mouse skin to TPA (Skarin, *et al.,* 1999). It has been also revealed that, the second TPA application significantly increases leukocyte infiltration in mouse skin (Nakamura, *et al.,* 1998). The present attempt demonstrated the significant induction of leukocyte infiltration in response to application of double dose of acetone and TPA on the mouse skin. The study, further demonstrated significantly reduced TPA-induced leukocyte infiltration in the cutis in response to double application of Moracin. This clearly implying that, there is suppressing inflammatory responses due to Moracin treatment.

The epidermal hyperplasia and proliferating cell nuclear antigen-positive cells in the epidermis mediated by inflammatory response, both, the hyperproliferative responses, seems to be the most common events after topical application of TPA on the mouse skin (Nakamura, *et al.,* 1998 and Lahiri-Chatterjee, *et al.,* 1999). Kim and Shine (1997) and Shin, *et al.,* (1993) have developed the method of measurement of proliferating activity to determine the grade of precancerous lesions during tumerigenesis, as well as in predicting the prognosis of malignant tumors. Treatment of mice with Moracin prior to TPA, in the present study, resulted in a highly significant inhibition of TPA-induced morphological changes, which suggest the reduction in epidermal hyper proliferation may be responsible for the protective effect of Moracin against the skin tumorigenesis. There is a close association between the ROS production through double or multiple treatment and activation of proximate carcinogen and increased levels of lipid peroxidation and oxidized DNA bases (Nakamura, *et al.,* 1998 and Zhao, et *al.,* 1999). The present attempt on Moracin treatment well illustrating the topical application suppressing one major TPA-induced epidermal oxidative stress marker, 4-HNE. It is one of the major products of membrane peroxidation and reacts with proteins to form stable adducts (Esterbauer, *et al.,* 1991). It has been demonstrated that, the level of 4-HNE was elevated in DMBA / TPA-induced mouse skin (Zhao, *et al.,* 2001). As per the expection, the elevation of 4-HNE in DMBA / TPA-induced mouse skin, as well in double-TPA treated skin was significantly suppressed by topical application of Moracin. The present attempt, further illustrates the suppression in the proliferation-related genes, *c–fos* and *c-myc,* by topical application of Moracin. The *c-fos* and *c-myc* have been associated with a variety of carcinogenesis (Yuen *et al.,* 2001). The attempts on in vitro have shown that, ROS stimulate the generation of proto-oncogenes *c-fos, c-myc* and others in various cell system (Nose, *et al.,* 1991 and Shibanuma, *et al.,* 1988), and that TPA could stimulate the generation of such active oxygen species *in vivo* (Frenkel, 1986 and Witz, 1991). Expression of *c-fos* was found elevated in DMBA / TPA-induced mouse skin (Zhao, *et al.,* 2001 and Kim, *et al.,* 2000), which revealed that, topical application of Moracin significantly reduced the expression of epidermal *c-fos* and *c-myc* and that labeling index of 4-HNE was correlated with those of *c-fos* and *c-myc*. These results imply the suppression of epidermal hyper cell proliferation through the reduction in oxidative stress in response to Moracin treatment.

The present attempt on the topical application indicated that, the Moracin involve in the suppression of epidermal COX-2 protein induced by TPA. The COX-2, as enzyme responsible for catalyzing the committed step in prostanoid biosynthesis, is the product of immediate early gene capable of being upregulated by diverse stimuli. The oxidative stress is associated with the upregulation of COX-2 (Nanji, *et al.,* 1997). 4-HNE, the end product of lipid peroxidation is a specific inducer of COX-2 gene expression (Kumagai, *et al.,* 2000). Further, the COX-2 is constitutively over expressed in epidermal tumors obtained from the initiation-promotion protocol in mouse skin (Muller, *et al.,* 1998 and Marks, *et al.,* 1998). Accordingly, as per expectation, the topical application of Moracin significantly inhibited the expression of epidermal COX-2 in the present attempt. There was significant correlation between the labeling index of 4-HNE and expression of COX-2 in all epidermal cells. The reduction of epidermal COX-2 expressions by Moracin, in the present attempt might be mediated by suppressing oxidative stress. In addition, COX-2 seems to be important pro-inflammatory mediator, such as release of pro-inflammatory cytokine, and plays important role in skin inflammation, cell proliferation and skin tumor promotion (Zhao, *et al.,* 1999). The inhibition of inflammatory responses through Moracin may be partly attributable to the suppression of COX-2 expression.

The present attempt, further demonstrated the Moracin inhibited expression of epidermal TNF-alpha protein in the mouse skin. The TNF-alpha, one of the pro-inflammatory cytokines that is produced by number of different cell types including keratinocytes under a variety of inflammatory conditions and it is known to prime inflammatory cells to produce enhanced levels of reactive oxygen (Robertson, *et al.,* 1996 and Marino, et *al.,* 1997). Significantly, the topical application of Moracin inhibited TPA-caused induction of inflammatory leucocytes, implying that, Moracin suppressed the expression of epidermal TNF-alpha protein possibly by inhibiting the inflammatory responses and further reduced epidermal oxidative stress. TNF-alpha acts as endogenous tumor promoter and central mediator of tumor promotion via a PKC alpha (one of majior receptor for TPA-induced signaling in basal keratinocytes) and AP-1-dependent pathway (Suganuma, *et al.,* 1999; Komori, *et al.,* 1993 and Arnott, *et al.,* 2002). The tumor promotion by TPA is critically dependent on TNF-alpha (Moore, *et al.,* 1999). The present attempt demonstrated that, the topical application of Moracin inhibited the expression of epidermal TNF-alpha in the mouse skin two-stage tumor formation model. According to Siqin Zhaorigetu *et al.,* (2003), the anti-tumor compound exerts chemopreventive effects against TPA-induced tumor promotion by inhibiting endogenous tumor promoter TNF-alpha. In a conclusion, the Moracin has a suppressing activity against TPA-induced tumor promotion in mouse skin and the underlying mechanism may involve inhibition of promoter-induced leukocyte infiltration, epidermal hyper-proliferation, oxidative stress and endogenous tumor promoter TNF-alpha. Moracin has suppressive actionagainst the chemical-induced skin tumorigenesis. Utilization of Moracin, the compound belongs to *Morus alba* (L.) may open a new avenue in the promotion of human health through the prevention of cancer.

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