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Inhibition of croton oil-induced inflammatory and tumor promoting activity in mice skin by henna (*Lawsonia inermis*)

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Abstract

Henna (*Lawsonia inermis*) inhibited inflammatory responses triggered by croton oil in mice ears and skin. Topical application of 0.5 or 1 mg henna extract (HE) to mice ears prior to application of 0.8 mg croton oil inhibited edema significantly ($P < 0.02$). The average weight of inflammated ear punches decreased by 64 and 87 %. Application of HE (5 or 10 mg) reduced, significantly ($P < 0.05$), Hyperplasia and leukocytes infiltration induced in the skin by application of croton oil (8 mg). Since tumor promotion is closely related to inflammation, the antitumor promoting activity of HE was investigated in two-stage model of skin carcinogenesis in mice. Twice weekly application of 5 and 10 mg HE prior to application of 8 mg croton oil on the shaved back skin prevented 40 and 50 % papilloma formation, respectively. HE, also, reduced, significantly ($P < 0.001$), the average number of papillomas per mouse and delayed their appearance. The anti-tumor promoting activity of henna could be ascribed to its anti-inflammatory potential.

Keywords: Henna (*Lawsonia inermis*), anti-inflammation, anti-tumor promotion, edema, papilloma.

INTRODUCTION

Henna (*Lawsonia inermis*) is a tropical and subtropical shrub native to Asia and North Africa. For centuries, henna leaves were renowned as the most extensively used natural hair dying and tattooing agent in many cultures. The plant was used as a cosmetic as early as the ancient Egyptian civilization where hieroglyphs depict regal women with painted nails and dyed skin and hairs. Besides its use in cosmetics, henna is used in folk medicine as a prophylactic against skin diseases and many other ailments (1, 2). Modern pharmacological research has confirmed a variety of biological activities for henna such as antimicrobial (3-5), antibacterial (6), antioxidant (7-10), fungtoxic (11), immunostimulant (10), antipyretic and analgesic activity (12). Henna has also been proved to be a hepatoprotective agent against carbon tetrachloride induced hepatotoxicity and oxidative stress (13, 14). In addition, henna and its major constituent,

lawsone, were found to possess anticarcinogenic effect against several cancer cell lines (8, 15).

Recently, considerable efforts have been made to identify chemopreventive agents of dietary and medicinal origin that could inhibit, retard or reverse the process of carcinogenesis at any of its stages, i.e. initiation, promotion and progression (16-18). Chemoprevention has become an emerging area of cancer research that, in addition to providing a practical approach to identifying potentially useful inhibitors of malignant transformation, affords opportunities to study the mechanisms of anticarcinogenesis (19). Chemopreventive agents are subdivided into blocking and suppressing agents according to application during initiation and promotion stages, respectively. Blocking agents prevent carcinogens from reaching the target sites, from undergoing metabolic activation or from subsequently interacting with crucial cellular macromolecules (for example, DNA, RNA and proteins). Suppressing agents, on the other hand,

inhibit the malignant transformation of initiated cells, in either the promotion or the progression stage (20).

When applied during the initiation stage of chemically induced carcinogenesis, henna proved a significant tumor inhibiting activity in the skin of mice (1). However, the promotion stage of carcinogenesis seems to be the best target for chemoprevention since promotion, unlike initiation or progression, is a reversible process that requires repeated and prolonged exposure to promoting agents (21-23). Therefore, the present study was undertaken to examine whether henna exerts chemopreventive effects when used during the promotion stage of skin carcinogenesis in mice. Since the process of tumor promotion is closely linked to inflammation (23), the anti-inflammatory effect of henna was also investigated.

MATERIALS AND METHODS

Preparation of henna extract (HE)

The air-dried powdered leaves of henna (*Lawsonia inermis*) were purchased from the local herbal stores in Alexandria, Egypt. Two hundred grams of the powder was extracted with ethanol for 3 days at room temperature. The extract (HE) was then filtered and evaporated at 40°C under reduced pressure and subsequently air dried. Finally, the residue (7 g) was lyophilized and stored at 4°C.

Treatment of animals

Eight weeks old female Swiss albino mice were obtained from Medical Research Institute (Alexandria, Egypt). They were maintained in a ventilated room at 25°C under a light-dark cycle. Mice were kept on shavings in polypropylene cages and received commercial laboratory chow and water *ad libitum*. For the morphological examination and skin carcinogenesis experiments, the dorsal portion of the mice skin were shaven with an electric clipper, two days before treatment. Only mice showing no signs of hair growth were used in the experiments.

Ear edema formation

Croton oil (Sigma Chemical, St Louis, MO) and HE were dissolved in acetone and applied in a volume of 20 µl to the left ear surface of mice (eight mice per group). HE (0.5 or 1 mg) and acetone as a vehicle were applied 30 min prior to application of croton oil (0.8 mg). The left ear of mice of other two groups was treated with acetone alone or HE (1 mg) in acetone. Seven hours after croton oil application, mice were sacrificed by cervical dislocation and 4 mm diameter (12.6 mm²) left ear punch biopsies were collected from all mice and weighed. The increase in the weight of the ear punch of each mouse was directly proportional to the degree of inflammation.

Morphological examination

The test substances were dissolved in acetone and topically applied in a volume of 200 µl to the back shaved skin of mice (eight mice per group). HE (5 or 10 mg) and acetone as a vehicle were applied 30 min prior to application of croton oil (8 mg). The back skin of mice of other two groups received topical applications of acetone alone or HE (10 mg) in acetone. Mice were sacrificed by cervical dislocation 27 h after croton oil application. The dorsal skin was excised and fixed in Bouin's solution. The paraffin-embedded skin samples were sectioned to a thickness of 5 µm and stained with haematoxylin and eosin. The leukocyte infiltration and epidermal thickness were used as indicators of morphological changes. The thickness of the epidermis was measured at five equidistant sites in each section of the skin utilizing a photomicroscope (100-fold magnification) and an ocular micrometer.

Skin carcinogenesis

Three groups of 20 mice each were initiated for skin carcinogenesis with a single topical application of DMBA (Sigma Chemical, St. Louis, MO) in acetone (0.05 mg/200 µl/ animal) applied on the shaved back mice skin. One week later, all mice were promoted with topical applications of croton oil in acetone (0.5 mg/200 µl/ animal) twice weekly till the termination of the experiment on the 20th week. Mice of one group were given no further treatment and served as positive control group. However, in anti-promotion groups, HE at a dose of 5 or 10 mg in 200 µl acetone was applied topically 30 min prior to each application of croton oil. Mice in all groups were weighed at the beginning of the experiment and were observed for any signs of toxicity during the entire period of the study. Skin tumors with a diameter larger than 1 mm were

recorded every week and were included in data analysis only if they persist for two weeks or more.

Statistical analysis

The student's *t*-test was employed to assess the significance of data. The data are presented as mean ± SE. The level of significance was taken at *p* < 0.05.

RESULTS

Inhibitory effects of HE on croton oil-induced mouse ear edema, hyperplasia and leukocyte infiltration.

The inflammatory response triggered by croton oil in mouse ears and skin was characterized in a previous study (24). In the present work, the anti-inflammatory activity of HE was evaluated by determining its effect on croton oil-induced edema of mouse ears, hyperplasia and infiltration of leukocytes in the skin. Topical application of 0.8 mg croton oil to the ear of a mouse increased the average weight of an ear punch (4 mm diameter) from 3.7 to 9.5 mg, i.e. edema formation, at 7 h after the dose. The application of 0.5 or 1 mg HE prior to croton oil inhibited edema significantly ($P < 0.02$). The average weight of ear punches decreased by 64 and 87 %.

Figure 1. Inhibitory effects of HE on croton oil-induced hyperplasia and leukocytes infiltration in mice skin. Mice back shaved skin received topical applications as following: (A) acetone; (B) 8 mg croton oil; (C) 5 mg HE prior to 8 mg croton oil; (D) 10 mg HE prior to 8 mg croton oil. All compounds were applied in 200 µl acetone. Mice were sacrificed 27 h after croton oil application and the skin tissue was taken for histological examination. All sections were stained with hematoxylin eosin. X 200.
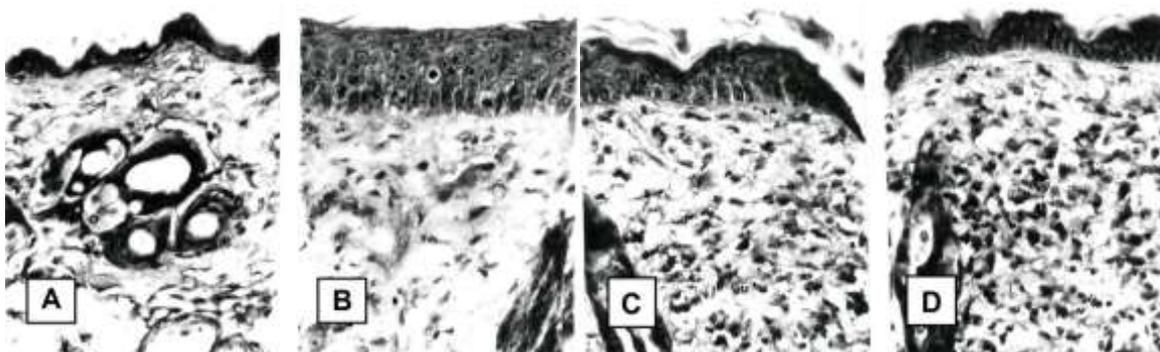

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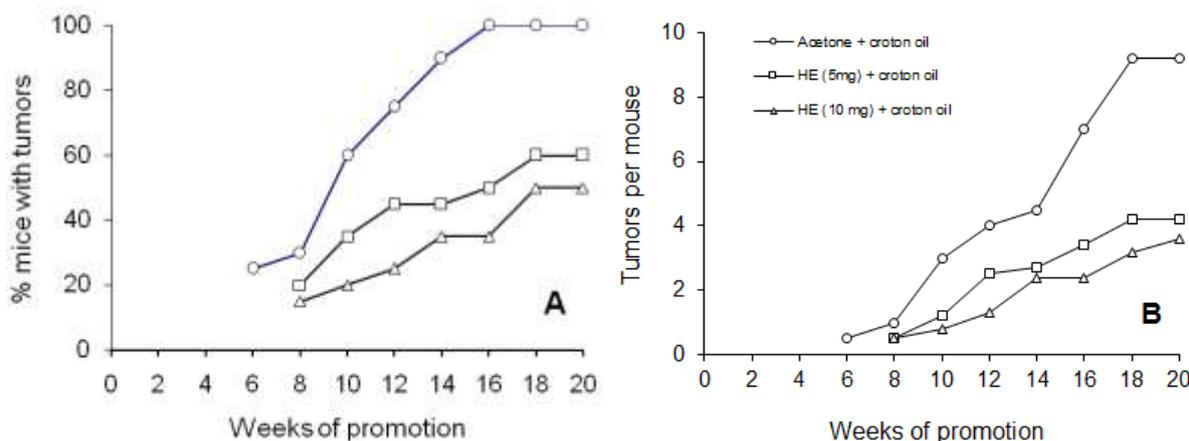
The effect of topical application of HE on croton oil-induced hyperplasia and leukocyte infiltration was, also, assessed (Fig. 1). A single topical application of croton oil (8 mg) to the shaved back skin and followed 27 h later killing of animals resulted in a 2.7-fold increase in epidermal thickness, i.e. hyperplasia, as compared to the acetone group. The topical application of HE (5 or 10 mg) before croton oil application resulted in a significant inhibition of hyperplasia ($P < 0.05$). The application of croton oil increased, also, the infiltration of leukocytes in the dermis which was reduced by the pre-application of HE to that of croton oil, as observed under microscope. The application of HE alone did not induce edema, hyperplasia or leukocyte infiltration in mice skin.

Inhibitory effects of HE on croton oil-induced skin tumors promotion.

Since tumor promotion is closely related to inflammation, the antitumor promoting effect of HE which has been proved to inhibit inflammation is expected . In support of this possibility, the inhibitory effect of HE on croton oil-induced skin tumor promotion was assessed (Fig. 2). All animals were observed for 20 weeks. The first tumor appeared at the 6th week in the positive control group which received treatments of DMBA and croton oil alone. In the anti-promotion groups, where the animals were given topical applications of HE (5 or 10 mg) twice weekly prior to the application of croton oil, the appearance of first tumor was prolonged to the 8th week. The positive control group showed 100 % incidence of papillomas within 16 weeks, while the animals in the anti-promotion groups showed 60 % and 50 % papilloma formation at the end of the experiment (Fig. 2A). The tumor inhibitory effect of HE was also seen as a reduction in the total number of papillomas. In the positive control group, the average number of papillomas per mouse was 9.2 which was significantly reduced ($P < 0.001$) to 4.2 and 3.6 in the groups which pretreated with 5 and 10 mg HE, respectively (Fig.2B). Throughout the promotion stage, the survival rate of mice in all groups was 100 % and all mice looked healthy. There was no significant difference in the body weight gain between mice of the positive control group and anti-promotion groups.

DISCUSSION

Two-stage skin carcinogenesis has been used as a convential model to study the mechanism of chemical carcinogenesis and modulation of sequential steps involved in this process. It is accomplished by a single topicl application of an initiating agent such as DMBA, followed by repeated applications of a promoting agent such as croton oil. Extensive research has identified numerous dietary and botanical natural compounds that modulate this process (25). Previously, we proved the effectiveness of some plants in inhibiting skin carcinogenesis in mice and mammary gland carcinogenesis in rat (26-31). Although henna has been proved to modulate skin carcinogenesis by inhibiting its initiation stage (1), data obtained in the present study clearly demonstrated that it inhibited also the promotion stage of this process. At its two examined doses, HE reduced significantly the papilloma incidence and the average number of papillomas per mouse when applied on the skin prior to each application of croton oil. It also delayed the tumors appearance.

An accumulating evidence has long documented an association between inflammatory tissue damage and the process of cancer development (32). Infammatory responses have been considered to occur during the process of tumor promotion (33-35). These responses include the development of edema, hyperplasia, induction of pro-inflammatory cytokine interleukin-1α, enhanced release of reactive oxygen species, induction of epidermal ornithine decarboxylase (ODC) and over-expression of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) proteins (36,37). Mice skin inflammation caused by croton oil has been shown to be a dose- and time-dependent process (24). At the appropriate dose and time, HE proved protection against croton oil-induced inflammation in the present study. It inhibited significantly cutaneous edema, epidermal hyperplasia and infiltration of leukocytes in the skin. Some compounds isolated from stem bark and root of henna showed anti-inflammatory activity against Carregenan induced paw edema in rats (12,38). Leaves, also, showed significant anti-inflammatory effect with some active principles (39,40).

Components of intracellular signal transduction pathways have been often considered as potential targets for studying the molecular mechanisms of carcinogenesis as well as chemoprevention. Exposure of cells and tissues to oxidative stimuli or electrophilic carcinogens forces the cell to turn on its antioxidant-detoxification arsenal as the first line of defense. Transcriptional regulation of antioxidant or phase 2 detoxifying genes is predominantly mediated by a redox-sensitive transcription factor NF-E2 related factor-2 (Nrf2). Many chemopreventive phytochemicals have been found to enhance cellular antioxidant and detoxifying capacity through activation of this particular transcription factor, thereby blocking initiation of carcinogenesis (41,42). This could explain the anticarcinogenic activity of henna extract which applied during initiation stage of skin carcinogenesis in the study of Dasgupta et al (1). On the other hand, aberrant activation of other transcription factors such as nuclear factor-kappa-B (NF-κB) and activator protein-1 (AP-1), which results in activation of genes involved in inflammation, cellular proliferation and growth, has been implicated in pathophysiology of various malignancies (43,44). Turning off improper activation of NF-κB and AP-1 by antioxidant and anti-inflammatory phytochemicals would prevent initiated cells from undergoing further proliferation at the stage of tumor promotion (45). It is most likely that chemopreventive phytochemicals with strong anti-inflammatory activities act as antitumor promoters as well (46). This comes in accordance with the correlation between anti-inflammatory and antitumor promoting activities of henna extract observed in the present study.

Several phytochemicals have been identified in henna extract including naphthoquinone derivatives, flavonoids, coumarins and gallic acid (47). Nearly all compounds isolated from henna exhibited free radical scavenging, immunostimulant, and antioxidant activities (10). In addition, naphthoquinones and flavonoids have important effects on inhibiting various stages of carcinogenesis (48, 49). Flavonoids of henna such as luteolin and apigenin have been proven to have cancer chemopreventive and chemotherapeutic potentials (50-53). Thus, henna is a unique source of various phytochemicals which could be responsible for the various activities of the plant. The results obtained in the present study together with those of Dasgupta et al (1) and Venkanna Lunavath and Estari Mamidala (54) indicate that henna is a double-acting chemopreventive agent since it inhibited several stages of skin carcinogenesis i.e. initiation and promotion. While the chemopreventive effect of henna during initiation stage could be ascribed to its antioxidant and detoxifying activity, our results indicate that its effect during promotion stage is related to its anti-inflammatory activity. Further investigations are warranted to elucidate the molecular mechanisms by which henna, or any of its components, exerts its tumor-inhibiting activity. As the current data suggest that henna is a strong chemopreventive agent, potentials of henna against other types of carcinogenesis should also be examined.

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