Nigella sativa seed extract and its bioactive compound thymoquinone: the new melanogens causing hyperpigmentation in the wall lizard melanophores

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Abstract

Objective The effects of the lyophilized seed extract of *Nigella sativa* and its active ingredient, thymoquinone, were studied on the isolated melanophores of the wall lizard to find the mechanism of skin darkening at the cellular level.

Methods The integumental melanophores of the wall lizard, *Hemidactylus flaviviridis*, were assayed using the mean melanophore size index and their responses were recorded in the presence of various concentrations of the plant extract, thymoquinone, specific antagonists and potentiator.

Key findings Significant skin darkening activity of the extract of *N. sativa* and thymoquinone was observed on the isolated melanophores of the wall lizard. The pigment cells responded by distinct dispersion leading to skin darkening. The effect was physiologically significant as re-immersion in physiological saline made the melanophores return to their normal intermediate state. These melanin dispersal effects were antagonized by atropine as well as hyoscine and were also found to be highly potentiated by neostigmine, an anticholinesterase agent.

Conclusions These findings suggest that the extract of *N. sativa,* as well as its active principle, mimic the action of acetylcholine in melanin dispersion leading to skin darkening via stimulation of cholinergic receptors of muscarinic nature within the melanophores of wall lizard. This study opens new vistas for the use of *N. sativa* active ingredient, thymoquinone, as a novel melanogen for its clinical application in skin disorders such as hypopigmentation or vitiligo.

Keywords cholinergic receptors; extract; *Nigella sativa*; thymoquinone; wall lizard melanophores-dispersion

Introduction

Vertebrate melanophores are melanin-containing dark pigment cells of neural crest origin and interestingly have been designated as a disguised type of smooth muscles, which due to their intracellular movement of melanin granules, regulate skin colour leading to either darkening or lightening of the skin.[1] It is well known that the pigment cells are controlled by either nerves alone or by hormones or by a combination of both.[2,3] Involvement of cellular receptors of different types, such as adrenergic, cholinergic and histaminergic, leading to darkening or lightening of vertebrate skin has been suggested by several workers.[3–5] It has been well documented that some cellular receptors, such as adrenergic or cholinergic, present on the melanophore-membrane, via elevation of intracellular cyclic adenosine monophosphate (cAMP), cause melanophore dispersion leading to skin darkening. The lowering of adenyl cyclase via stimulation of α-adrenergic or histaminergic receptors causes the opposite responses, thereby making the skin appear pale.[1,2] It has been shown that melanophores of lower vertebrates, particularly those of amphibians and reptiles, are affected by cholinergic agents such as acetylcholine leading to skin darkening.[6,7] Since thymoquinone is a known cholinergic stimulant in some muscle systems, such as the respiratory tract of guinea-pigs,[8] it would be interesting to explore its role as a neuromodulator in reptilian pigment cells. Further, there are very few studies on the effects of pharmaceutical agents from natural plant extracts that have melanogenic action on vertebrate pigment cells, the melanophores, which offer excellent opportunities to study the cellular reactions in response to externally applied stimuli.

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The seeds of *N. sativa* have been subjected to a range of pharmacological investigations as they have been reported to contain thymoquinone, monoterpenes, nigellidine, nigellimine and saponins.[9–12] Several related studies have shown its seeds to possess a wide spectrum of activity, such as antioxidant, anti-tumour, anti-inflammatory, CNS depressant, analgesic, smooth muscle relaxant, cytotoxic, immunostimulant, and antidiabetic activity.[13–19] However there have been no reports dealing with the effects of the seed extract of *N. sativa* or any of its active ingredients on pigment cells, despite the fact that many plant extracts are known to be melanogenic in nature.[20–22] Recently, Lee *et al*.[23] reported that 5-chloroacetyl-2-piperidino-1,3-selenazole, a novel selenium-containing compound, caused depigmentation in the brown guinea-pig skin by inhibiting tyrosinase activity. In this study an attempt has been made to explore the possibility of initiation of the pigment cell machinery by the seed extract of *N. sativa* and its active ingredient thymoquinone to induce melanin displacement causing skin darkening. It may be mentioned here that this is the first report of its kind where *N. sativa* and its active ingredient has been found to cause skin darkening via melanin displacement within the melanophores. These plant extracts containing the active compound thymoquinone can be used as novel candidates for the treatment of vitiligous skin conditions.

Material and Methods

Nigella sativa L. seeds were collected from the local market (Bhopal), and were authenticated by the Minor Forest Processing and Research Centre, Bhopal. The macerated extract was prepared as follows: 50 g of the crushed seeds of the plant were extracted with 250 ml double-distilled water (on a shaker) for 48 h. The solvent of the macerated extract was removed by lyophilization using the modified method of Zhao *et al*.[24] The extract was collected and centrifuged (2000*g*, at 25°C) for 10 min using REMI R.24 (REMI Instrumental Ltd. Mumbai) to remove any water-insoluble materials. The supernatant was lyophilized in a freeze drier. The lyophilized extract was subjected to the analysis of its chemical constituent by using a high-performance liquid chromatography (HPLC) Perkin Elmer instrument. The dried powder was re-dissolved in distilled water with 0.2% dimethyl sulfoxide (DMSO) for in-vitro studies using different concentrations. Thymoquinone (Cat. No. 274666-1G) was used as standard in this study and was obtained from Sigma-Aldrich (St. Louis, MO, USA).

The ethical committee for Animal Experimentation and Research, Saifia College of Science, Bhopal, India certified the use of animals. The research work of the institute is done in strict compliance with the guidelines of Indian Council of Medical Research (ICMR), Guidelines for Laboratory animal in Medical College (2001) as per Breeding and Experiments of Animal Amendment Rules (2001) and The Prevention of Cruelty to Animal Act (1966).

H. flaviviridis Ruppell, commonly called wall lizard, was selected as an experimental animal because its pigmentary responses are uniform and the melanophores present in the skin are of almost equal size and are distributed uniformly.

The lizards were hand caught from houses and kept in a terrarium with constant lighting of 14 h light and 10 h dark. A temperature gradient of 20–35°C was maintained across the terrarium during light hours, while the minimal temperature attained at night was 18°C. The lizards were fed with flies, and the terrarium was sprayed twice daily with water, which the animal drank by licking the droplets off the vegetation. *H. flaviviridis* of either sex with a length of 12–15 cm were sacrificed by decapitating. The dorsal skin was peeled away and floated in reptilian physiological saline (8.0 g NaCl, 0.5 g KCl, 0.25 g CaCl$_2$, 0.1 g MgCl$_2$, 0.2 g NaHCO$_3$, 10.0 g dextrose dissolved in 1 l of double distilled water pH 7.2–7.4). A series of 5-mm diameter skin pieces were cut out with a sharpened steel scissor and the pieces were placed individually in small Petri dishes with fresh physiological saline, following the method of Goldman and Hadley,[4] modified accordingly by Ali *et al*.[5] The skin pieces were left to equilibrate in the physiological saline for about 30–45 min. Lyophilized extract of seeds of *N. sativa* and the pure compound thymoquinone in different Log dose concentrations (1×10^{-6}, 2×10^{-6}, 4×10^{-6}, 8×10^{-6}, 1.6×10^{-5}, 3.2×10^{-5} and 6.4×10^{-5} g/ml) were allowed to mix with the skin pieces through the saline for 15–20 min. The control and the plant extract/thymoquinone-treated skin pieces were first placed on a chambered glass slide with dermal skin up in the reptilian physiological saline. The slide was mounted, placed on an inverted microscope and observed accordingly in low power magnification. The reading was taken with the help of an ocular micrometer fitted in the microscope calibrated earlier, using the mean melanophore size index (MMSI) method of Bhattacharya *et al*.[25] based on Hogben and Slome.[26] In this method, an individual melanophore was measured by noting the maximum vertical and horizontal diameters. Ten such randomly selected melanophores were measured and the melanophore size index was calculated. When the melanophores disperse (i.e. the melanin granules within the melanophores move to the periphery) the diameter of the cell increases, which can be measured as melanophore size index and expressed as MMSI.

MMSI was prepared and the data were analysed by using analysis of variance followed by Dunnett's test. In all tests, the criterion for statistical significance was $P \leq 0.05$. Statistical data analyses are presented as mean + SEM (standard error of the mean). The number of experiments was $n = 7$. The following drugs were used: atropine sulfate (C.H. Boehringer, Sohn, Germany), hyoscine butylbromide (Cadila healthcare, Goa), L-adrenaline bitartrate (C.H. Boehringer, Sohn, Germany) and neostigmine methylsulfate (Neon Lab. Bombay, India).

Results

Chemical composition analysis of the lyophilized seed extract of *N. sativa*

HPLC analysis of the lyophilized extracts of *N. sativa* showed the presence of thymoquinone, the active ingredient. Its quantity was found to be 0.0356 % and the HPLC chromatograph is given in Figure 1.
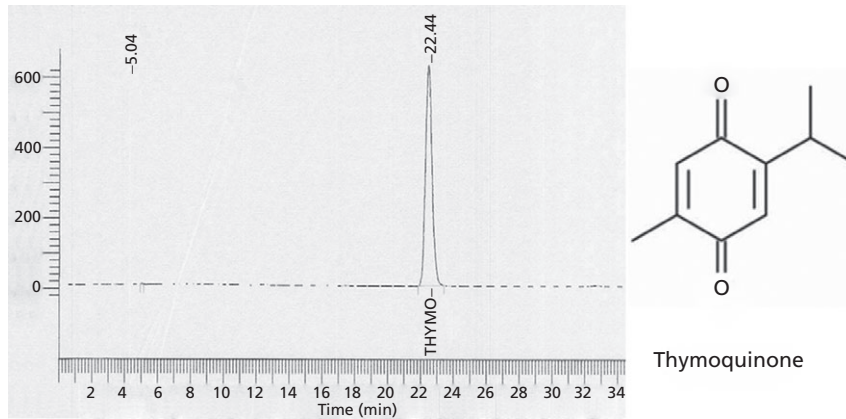
Figure 1 HPLC chromatograph and structure of thymoquinone.

Effect of the lyophilized seed extract of *N. sativa per se* on the isolated skin melanophores of *H. flaviviridis* along with specific antagonists and potentiator

It was found that lyophilized seed extract of *N. sativa per se* induced a statistically significant ($P < 0.0001$) skin darkening response in the isolated skin melanophores of *H. flaviviridis*. To ensure that the dispersion of melanophores was caused by the agents used in the study and not by saline itself, the skin melanophores of the wall lizard were initially incubated for 7–10 min in 2×10^{-8} g/ml of adrenaline, making them slightly aggregated so as to keep them in the intermediate state of neither dispersion nor aggregation. The different concentrations of lyophilized seed extracts of *N. sativa* (1×10^{-6} to 6.4×10^{-5} g/ml) highly dispersed the previously adrenalised skin melanophores, where the MMSI increased from a control (reptilian physiological saline + adrenaline 2×10^{-8} g/ml incubated melanophores) value of 3.43 ± 0.099 to 7.82 ± 0.32 in response to the maximal concentration of 6.4×10^{-5} g/ml of the seed extract. After repeated washings and re-immersion of the *N. sativa*-treated melanophores in reptilian physiological saline, the melanin dispersal effects returned to the control as the MMSI had become 4.77 ± 0.22. Atropine as well as hyoscine in a dose of 4×10^{-7} g/ml blocked the powerful per-se melanophore dispersal effects of the lyophilized seed extract of *N. sativa* (Figure 2).

The skin melanophores of the wall lizard were initially incubated for 7–10 min in 2×10^{-8} g/ml of adrenaline. Following this the skin pieces were treated with 2×10^{-7} g/ml neostigmine, a specific anticholinesterase agent. These skin pieces were further kept in a Petri plate containing increasing concentrations of lyophilized seed extract of *N. sativa* ranging from 1×10^{-6} to 6.4×10^{-5} g/ml. It was found that the per-se melanin dispersal effects of lyophilized extract of *N. sativa* became highly potentiated by 2×10^{-7} g/ml of neostigmine. The MMSI had become 18.47 ± 0.60 ($P < 0.0001$) in comparison to per-se (7.82 ± 0.32) treatment of the skin melanophores with lyophilized extracts of *N. sativa*. It was also found that the potentiated melanophore dispersal effects of lyophilized extract of *N. sativa* by neostigmine were completely blocked by 4×10^{-7} g/ml of atropine as well as hyoscine (Figure 3).
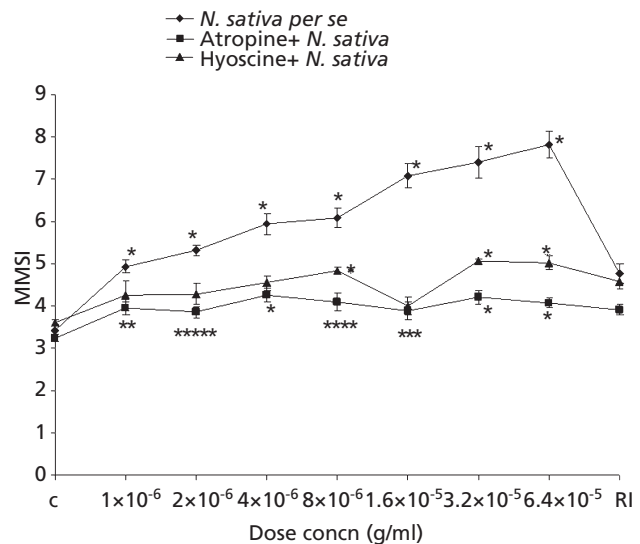

Figure 2 Dose–response curve for the melanophore dispersal effect of lyophilized *N. sativa* seed extract *per se* on the adrenalised melanophores of *H. flaviviridis*. The complete blocking effects of specific antagonists atropine (4×10^{-7} g/ml) and hyoscine (4×10^{-7} g/ml) against *N. sativa* seed extract dispersed melanophores are also shown. C, control; RI, MMSI after the re immersion of skin in reptilian physiological saline after repeated washings. Vertical bars represent the standard error of mean. *$P < 0.0001$, **$P = 0.001$, ***$P = 0.01$, ****$P = 0.0016$, *****$P = 0.0009$ vs control.

Effect of thymoquinone *per se*, its antagonists and potentiator on the isolated skin melanophores of *H. flaviviridis*

It was observed that pure thymoquinone induced physiologically and statistically significant ($P < 0.0001$) melanophore dispersion in all concentrations used. The highest degree of dispersion from the control value of 3.37 ± 0.13 to 11.63 ± 0.42 was induced by 6.4×10^{-5} g/ml of standard thymoquinone. The powerful melanin dispersal effects of thymoquinone were also found to be completely blocked by atropine and hyoscine in a pre-selected concentration of 4×10^{-7} g/ml (Figure 4). Isolated skin melanophores of the wall lizard
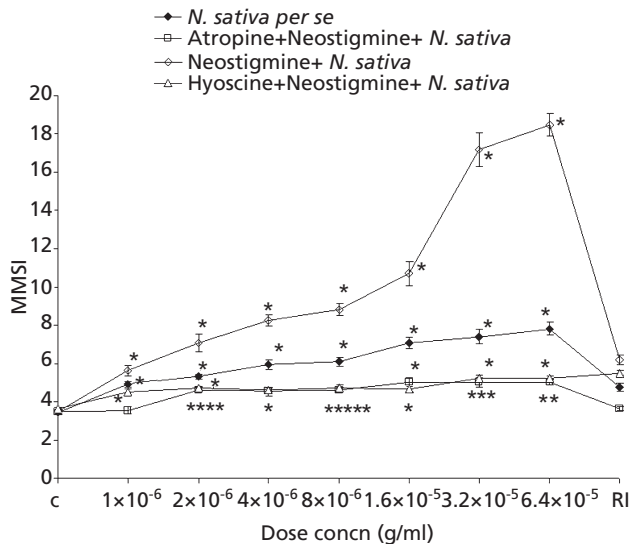
Figure 3 Dose–response curve for the melanophore dispersal effect of lyophilized extract of seed of *N. sativa per se*. Also shown is the effect of neostigmine (2×10^{-7} g/ml) on the dose–response curve of lyophilized seed extract of *N. sativa*. Note the potentiation of the dispersal response of *N. sativa* by neostigmine and the same blocked by (4×10^{-7} g/ml) of atropine, as well hyoscine. *$P < 0.0001$, **$P = 0.0005$, ***$P = 0.0006$, ****$P = 0.0007$, *****$P = 0.0024$.

Figure 5 Dose–response curve for the melanophore dispersal effect of thymoquinone *per se*. Neostigmine (2×10^{-7} g/ml) effect on the dose-response curve of thymoquinone is also shown. Note the potentiation of the dispersal response of thymoquinone by Neostigmine and the same blocked by (4×10^{-7} g/ml) of atropine, as well as hyoscine. *$P < 0.0001$, **$P = 0.0014$, ***$P = 0.0002$, ****$P = 0.0012$, *****$P = 0.0004$.

neostigmine potentiated the melanin dispersal effects of pure thymoquinone, which were also antagonized by atropine as well as hyoscine (Figure 5).

Discussion

We here demonstrate that the lyophilized seed extract of *N. sativa*, containing significant amounts of thymoquinone as shown by HPLC analysis, induced a distinct melanophore dispersion effect along with the pure compound thymoquinone, leading to darkening of the wall lizard skin. It appears that the seed extract of *N. sativa*, which principally contains thymoquinone, probably acts like acetylcholine, the cholinomimetic agonist, in activating the dominantly present cholinergic receptors of the neuro-melanophore junction of this species, to induce distinct and marked melanin dispersion. The physiologically significant dose-related melanin dispersion effects of lyophilized *N. sativa* and its active ingredient thymoquinone *per se* were found to be completely abolished by atropine and hyoscine, the specific cholino-muscarinic receptor blockers. There are few reports on the effects of any pharmaceutical agent like that of acetylcholine or related cholinergic agents on the integumental melanophores of lower vertebrates; moreover they are contradictory in nature. The earlier work of Parker *et al*.[27] showed that pale and dark skins of cat fish had different concentrations of acetylcholine and, according to these authors, it had an important role in melanophore dispersion. However, in another study acetylcholine was ineffective in inducing any responses in the American lizard (*Anolis*) melanophores, except in very high concentrations, where it had caused dispersion.[28] Similarly Ovais & Ali[7] also reported that acetylcholine had a varied effect on the melanophores of the wall lizard, *H. flaviviridis*. It had caused slight aggregation in lower concentra-
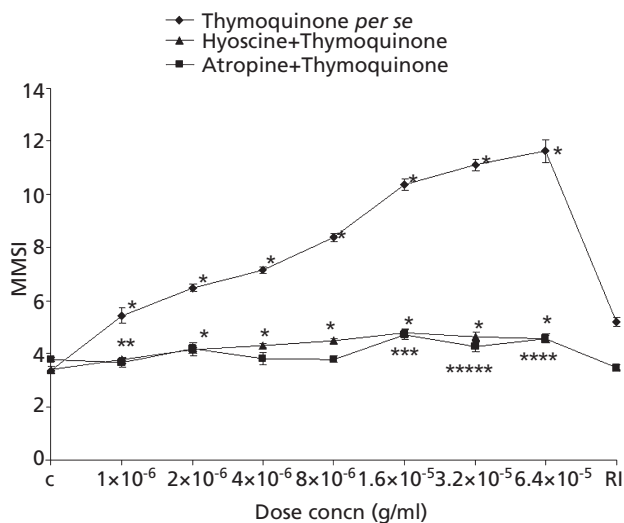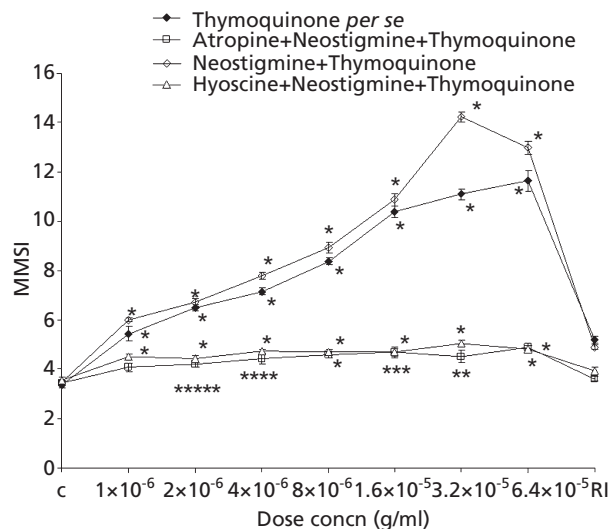

Figure 4 Dose–response curve for the melanophore dispersal effect of thymoquinone *per se* on the adrenalised melanophores of *H. flaviviridis*. The complete blocking effects of specific antagonists atropine (4×10^{-7} g/ml) and hyoscine (4×10^{-7} g/ml) against thymoquinone dispersed melanophores are also shown. *P*-values: *$P < 0.0001$, **$P = 0.0078$, ***$P = 0.0007$, ****$P = 0.0013$, *****$P = 0.0414$.

pre-incubated with neostigmine (2×10^{-7} g/ml) were further treated with increasing concentrations of active ingredient thymoquinone from 1×10^{-6} to 6.4×10^{-5} g/ml. The MMSI was increased to a very high value of 14.22 ± 0.22 ($P < 0.0001$) by treatment of the skin melanophores with 3.2×10^{-5} g/ml of thymoquinone. Thus it was found that

tions whereas higher concentrations of acetylcholine were found to cause melanophore dispersion. This variation in the response of the melanophores to cholinergic agents may be due to the species difference, which is a common phenomenon in lower vertebrates.

In this study, the melanin dispersal effects of *N. sativa* and its active ingredient thymoquinone seem to involve the cholinergic receptors of muscarinic nature as the data using specific blockers and the potentiator indicate. It has been reported by Tahir *et al*.[8] that *N. sativa* volatile oil induced respiratory effects in guinea-pigs, which were mediated via activation of muscarinic cholinergic mechanisms. In-vitro inhibitory activity exerted by the main constituents of essential oil obtained from the aromatic plant *Thymus vulgaris* L. containing thymoquinone on acetylcholinesterase has also been reported by some workers.[29] As it has been shown that melanophores of amphibians and reptiles are known to be affected by cholinergic agents, such as acetylcholine, leading to skin darkening,[6,7] the present data assume significance in corroborating this fact. The role of thymoquinone as a neuromodulator in reptilian pigment cells becomes established by the present findings. Similarly the data of the seed extract of *N. sativa* causing melanophore dispersal can also be compared with the muscle relaxant activity of volatile oil from this plant on different smooth muscles including rabbit aorta, rabbit jejunum and isolated tracheal muscles of guinea-pigs.[30–32] The present findings are also corroborated by the reports that acetylcholine has been found to be one of the principle neurotransmitter substances of the vertebrate epidermis and is responsible for melanin distribution and melanosome movement via cholinergic signal transduction.[33,34]

Our findings are quite interesting as for the first time it is being documented that extracts of the *N. sativa per se* can cause melanin stimulatory effects leading to skin darkening. In addition, in this study, neostigmine (an anticholinesterase agent) was found to potentiate the melanin dispersal effects of both *N. sativa* extract and its active ingredient thymoquinone. These data are also in corroboration with earlier findings that the muscarinic stimulating agent pilocarpine and the anticholinesterase agent eserine had potentiated the melanin dispersal effects of acetylcholine in some fishes[35,36] whereas in a strange deviation of sorts it has been demonstrated that there is an augmentation of the melanin aggregation response in the fish *Parasilurus asotus* by eserine.[37]

Interestingly in some amphibians, such as *Rana tigerina* and *Bufo melanostictus*, it has been shown that cholinergic receptors of muscarinic nature control melanin dispersion leading to skin darkening.[6] These findings are well supported by the recent observations of Gonzalez *et al*.,[38] who have reported that there is involvement of M1-M5 odd receptors of muscarinic nature in melanophore dispersion of the blue gill (*Lepomis macrochirus*) retinal pigment epithelium. Thus there is considerable complexity of the receptor mechanisms in response to pharmacological agents and the involvement of various receptors in the different species of lower vertebrates studied so far. This study opens new vistas for the clinical application of *N. sativa* active ingredient, thymoquinone, as a melanogenic compound for skin disorders such as hypopigmentation or vitiligo.

Conclusion

These data are quite interesting as for the first time it is being documented that seed extract of the *N. sativa per se* and its active ingredient thymoquinone cause melanin stimulatory effects leading to skin darkening thereby making them novel melanogenic candidates. These findings also suggest that the extract of *N. sativa*, as well as its active principle, mimic the action of acetylcholine in melanin dispersion leading to skin darkening via stimulation of cholinergic receptors of muscarinic nature within the melanophores of wall lizard. This study opens new vistas for the use of *N. sativa* active ingredient, thymoquinone as a novel melanogen for its clinical application in skin disorders such as hypopigmentation or vitiligo.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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