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Pharmacological investigation of hair growth promotional potential of *Phyllanthus niruri* Linn. extract against doxorubicin induced alopecia in experimental rats

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Abstract

The present study was aimed to assess the hair growth promotion activity of *Phyllanthus niruri* in doxorubicin induced alopecia in experimental rats. The experimental protocol was designed for 20 days as per reported model of doxorubicin induced alopecia. At day 0 all hair follicles were in resting stage (telogen). Hair growth was induced by depilating the hairs with a hair remover cream. At 9th day after depilation; all follicles were in anagen VI stage. As soon as induced anagen follicles, i.e. on 9 post depilation, had reached early anagen VI, At the 9th day freshly prepared doxorubicin solution 2mg/kg was administered through intra-peritoneal route from 9th to 15th day in groups I, II and III. Hence, hair follicle dystrophy was induced after doxorubicin administration. Animals of groups- II, III were orally administered with 250mg/kg body weight of extract solution of petroleum ether of *Phyllanthus niruri* from 10th day upto 19th day. At 20th day of experiment all the groups were sacrificed and the histopathology of skin was conducted. Histopathology and gross morphologic observations for hair regrowth at shaved sites revealed active follicular proliferation. It was observed that the petroleum ether extract of *Phyllanthus niruri Linn* showed the ability to prevent damage to hair follicles by doxorubicin. Animal of groups II, III treated with extracts of plant showed hair regrowth. The study was concluded that extracts of *Phyllanthus niruri Linn* shown to be capable of promoting follicular proliferation or preventing hair loss in doxorubicin induced hair fall.

Keywords: Doxorubicin, *Phyllanthus niruri*, hair loss, chemotherapy, alopecia.

Introduction

The three major and frequent toxicities of cytotoxic cancer chemotherapy are bone marrow suppression, gastrointestinal disturbances and alopecia. Alopecia negatively affects a patients perception of physical appearance, body image, sexuality and self-esteem, and deprives patients of the privacy of having cancer (Carelle *et al*.2002, Dorr *et al*.1998, Lindley *et al*.1999, Pickard-Holley *et al*.1995).Botchkarev *et al*. (2001) have shown that p53 is necessary for the development of chemotherapy-induced alopecia. This apoptosis largely depends on p53, a key mediator of cellular mechanism of stress response (Lakin*et al.*, 1999).

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Histologically, Doxorubicin induces two types of follicle dystrophy. More severely damaged anagen follicles are transformed into dystrophic catagen, with rapid shedding of the hair shaft and abrupt cessation of follicular melanogenesis. Less severe damage induces dystrophic anagen, with variable hair shaft shedding and continued, but abnormal follicular melanogenesis. Both dystrophic anagen and dystrophic catagen follicles run through a significantly shortened telogen phase and display very different recovery patterns (Paus *et al*., 1994).

Phyllanthus niruri (Euphorbiaceae) is an annual erect glabrous herb that is found in tropical and subtropical regions of the world (Unander*et al.* 1990;http://www.alergyresearchgroup.com; Adedapo*et al*. 2004). The plant is a rich source of phytochemicals such as alkaloids, astragalin, brevifolin, carboxylic acids, corilagin, geraniin, hypophyllanthin, lignans, methyl salicylate, terpenes, tanins, saponins and flavonoids, such as quercetin, quercetol, quercetrin and rutin(Singh *et al*; 1989;

Khanna *et al*., 2002, Mellinger *et al*., 2005).It has been used in herbal medicine worldwide for centuries where it grows (Kirtikar *et al*., 1935) for the treatment of various ailments including diabetes, malaria, colic, fever, jaundice, kidney and gall bladder stones, tuberculosis, bacterial infections such as cystitis, prostatitis, gonorrhea, urinary tract infections and viral infections (J Qian-Cutrone.,1996). But not much work has been carried out on hair growth promoting activity. The plant is highly valued in "Ayurvedic system of medication" for hair loss treatment and this plant isused in folk and traditional medicine for hair growth promotion.

The side effect of chemotherapy i.e. alopecia is used as model in the present study. Doxorubicin chemotherapy induces alopecia. In the present study the extract of *Phyllanthus niruri was* administered orally against Doxorubicin induced alopecia in albino rats, separately. The study was based on oral administration of the extracts in doxorubicin induced alopecia animals. The hair growth promotion activity was study on Doxorubicin treated animals.

Material and Methods

Materials and Plant Procurement

All the chemicals, solvents and reagents were purchased from commercial suppliers and were used as such without any further purification. Doxorubicin was obtained as generous gift from M/s Dabur India Ltd.,Tweenwas procured from Qualikens Fine Chemicals Pvt. Ltd, India. Ethanol was procured from Changshu Yangyuan, propylene glycol from Saiper Chemicals and petroleum ether from Fisher Scientific. The whole plant of *Phyllanthus niruri* was collected in the month of Sep-Oct 2011 from forests surrounding university campus, Dr. H. S. Gour University Sagar, India and were authenticated by Dr. P.K. Tiwari, Department of Botany, Dr. H.S. Gour Central University (Herbarium no. Bot/Her/1329).

Plant Extraction

The obtained plant material was dried in sunlight and reduced to a coarse powder. Collected whole plant was shade dried and subjected to size reduction to get coarse powder (sieve size 44) and stored in air light container at room temperature (35°C). Aerial parts of *Phyllanthus niruri* were powdered and used for extraction. The yields of petroleum ether extracts of *Phyllanthus niruri* was 2.3%.

Preparation of Doxorubicin Solution, Test Solution and its Dose

Doxorubicin solution

Doxorubicin solution (2mg/kg) was freshly prepared in water for injection. Orange color solution was formed. 0.1 ml of doxorubicin solution was given for each animal of all groups regularly for 7 days. Test solution of Pet. ether extract of *Phyllanthus niruri* was prepared in Tween (2%) and final volume was made with distilled water. 0.5ml of extract solution was given to each group by orally administration. Test solution was administered as per the pre designed protocol.

Animals

4 Swiss albino rats of either sex (5-6 month age, 140-150g) were used for experimental model and these were divided into 2 groups. Two animals were used for each group in all the experiments. The animals were maintained under standard housing condition with food and water provided *ad libitum*. The photoperiod was kept at 12 h of light and 12 h darkness. All experimentation was carried out after approval of the protocol by the Institutional Ethical Committee of Dr H.S. Gour University. The guideline of CPCSEA, India was strictly followed. The protocol approval no. is Eths. Comm./12/439.

Experimental Design And grouping of Animals

In this experimental model, it was proposed to evaluate the hair growth promoting effect of *Phyllanthus niruri* extract administered orally in doxorubicin induced alopecia.

The animals were randomly divided in 2 groups of 4 male Swiss albino rats and were treated as follows:

Group I: Doxorubicin solution (2 mg/Kg) (i.p.).

Group II: Doxorubicin solution (2 mg/Kg) (i.p.) + Petroleum ether extract solution (250 mg/Kg) of *Phyllanthus niruri* (orally).

The experimental protocol was designed for 20 days as per reported model of doxorubicin induced alopecia. At day 0 all hair follicles were in resting stage (telogen). Hair growth was induced by depilating the hairs with a hair remover cream. At 9th day after depilation; all follicles were in anagen VI stage. As soon as induced anagen follicles, i.e. on 9 post depilation, had reached early anagen VI, At the 9th day freshly prepared doxorubicin solution 2mg/kg was administered through intra-peritoneal route from 9th to 15th day in groups I and II. Hence, hair follicle dystrophy was induced after doxorubicin administration. Animals of groups- II was orally administered with 250mg/kg body weight of extract solution of petroleum ether of *Phyllanthus niruri* from 10th day upto 19th day. At 20th day of experiment all the groups were sacrificed and the histopathology of skin was conducted.

Statistical analysis

Results were expressed as mean±S.D. The treated groups were compared with control by analysis of variance following Dunnet's test. All the statistical

analysis was carried out using INSTAT version 2.1 software (Graphpad Software Inc., La Jolla, CA, USA).

Anagen induction

Swiss albino rats had gone through several postnatal hair cycles were induce to enter anagen by depilation of all hair shaft (having any cycle). It was done by applying hair remover to back skin and by peeling off this mixture after some time (Paus R). By this technique, all depilated hair follicles immediately start in on to transform into anagen follicles (Stage-I to Stage -VI) with their associated properties. After 9–10 days of depilation, they enter in Anagen -VI stage.

Induction of Alopecia

As soon as early anagen-VI stage reached by induced anagen follicles, i.e. on day 9th post depilation, a single intra peritoneal injection of doxorubicin was given (2 mg/kg body weight, freshly dissolved in distilled water).

Treatment of animals for study

The method reported by Paus was followed with slight modification. Swiss albino rats in-group I and group II were administered doxorubicin intraperitoneally (2 mg/kg body weight) once i.e. on 9th day of depilation. Animals of groups II was administered orally of petroleum ether extract of *Phyllanthus niruri* from the day next to doxorubicin administration upto 19 day of study. Animals of group I were treated with doxorubicin only. After 19th days of study, Swiss albino rats from each group was selected randomly and sacrificed.

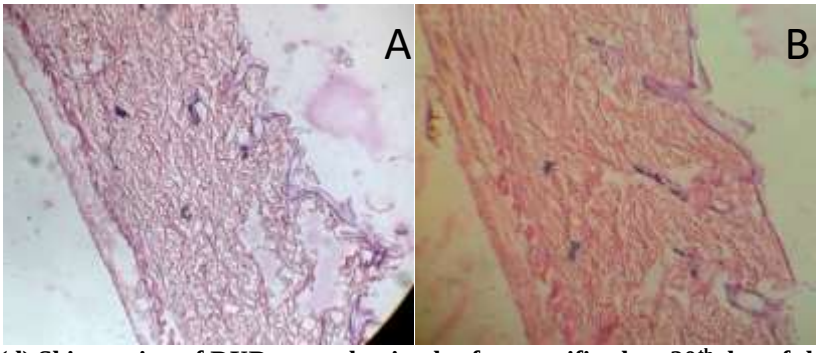
Results and Discussion

Number of follicles: The number of follicles/mm in section of skin of different groups of animals were counted and the observation were recorded n=12 after 19 day treatment duration (Table -1).

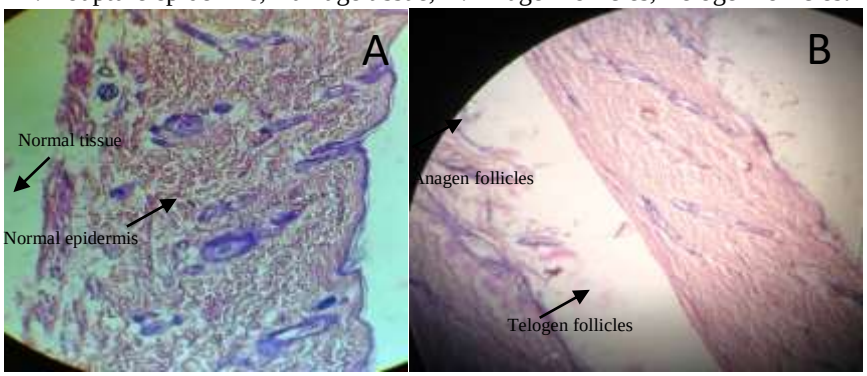
On examination of skin biopsy of different group's total no. of follicle, anagen and telogen follicles were found to be 20, 6 and 14 in group-I respectively. In group- II, total no. of follicles, anagen and telogen follicle were found to be 29, 16 and 12. (Photograph - 1 d, e).

Table 1: Number of follicles, anagen and telogen follicles per mm area in different groups of animals

No. of microscopic view	Group I			Group II		
	follicles	Anagen follicles	Telogen follicles	follicles	Anagen follicles	Telogen follicles
1	1	0	1	2	1	1
2	2	1	1	3	2	1
3	1	0	2	4	2	0
4	2	2	0	3	1	2
5	1	0	1	3	2	1
6	2	0	2	2	0	2
7	2	1	1	2	2	0
8	1	0	1	2	0	2
9	2	1	1	3	2	1
10	2	0	2	2	1	1
11	2	0	2	2	2	0
12	2	1	1	2	1	1
Total	20	6	14	29	16	12



Photograph-1(d) Skin section of DXR treated animals after sacrificed on 20th day of the experiment.
A: Roupture epidermis, Damage tissue; B: Anagen follicles, Telogen follicles.



Photograph-1(e) Skin section of Doxorubicin + Pet. ether extract of *Phyllanthus niruri* treated animals after sacrificed on 20th day of the experiment.
A: Normal epidermis, Normal tissue; B: Anagen follicles, Telogen follicles.

Hair follicular density: Hair follicular density was calculated based on number of follicle /mm observed. In addition, the anagen to telogen ratio was calculated. The follicular density and the A/T ratio of each group is shown in (Table-2)

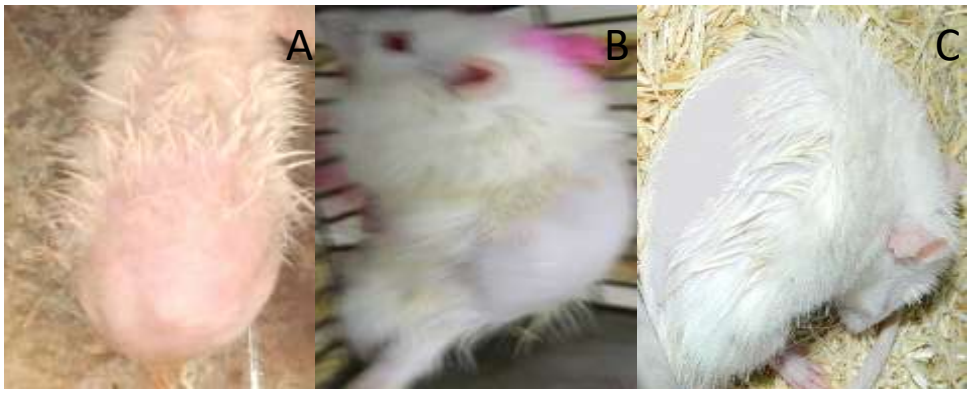
Table 2: Hair follicular density and A/T ratio in sections of skin of different groups of animals

Group	Treatment	Hair follicular density (no./mm)	Anagen to Telogen (A/T) ratio
I	DXR Solution (i.p.)	1.75 ± 0.4523	0.4 : 1
II	DXR Solution (i.p.) + Pet ether Extract solution of *Phyllanthus niruri*(orally)	2.5± 0.4924*	1.45 : 1

Values are mean ± SD, n=12, *p<0.05 compared to the value in doxorubicin treated animals/group- I. Each group was consisted of 3 animals.

Group-I

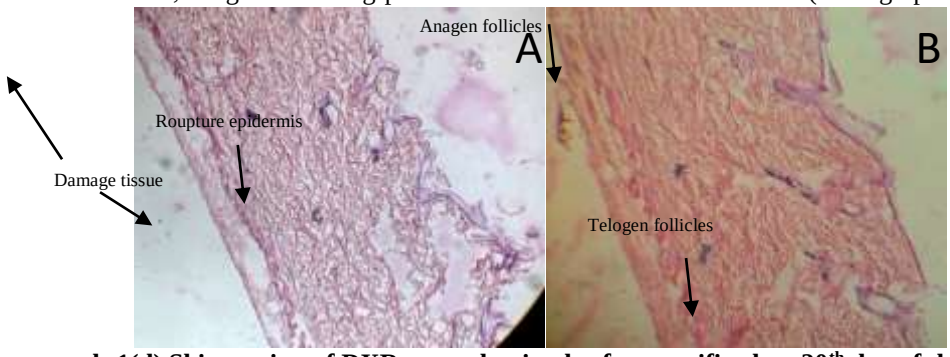
Visual observation: Normal feeding pattern was observed for 1st -11th day. After Doxorubicin administration on 9th day regularly upto 15th day, a slight decrease in feeding pattern was observed and a diminished feeding pattern was noted on 18th and 19th day of study. Animals became weak and fatigue during the study. Hair growth at depilated site was visibly inhibited in most of the animals by 14th day. On 16th day moderate alopecia was observed in head and neck region of some animals. After 19th day of study severe alopecia was observed at depilated site in all the animals (Photograph-1 a).



Photograph-1 (a) DXR treated animals

A: After depilation; B: After 9th day of study (DXR administered); C: After 19th day of study.

Histopathological Observation: The skin biopsy section was obtained from rats after treatment with DXR (2mg/kg per day IP) regularly for 7 days and sacrificing on 20th day of the experiment. The skin section showed rupture of some tissue, disruption of epidermis, less number of melanin pigment, less number of hair follicles, irregular banding pattern of hair shaft, irregular diameters of hair bulbs, distortion of hair follicles, stoppage of hair follicle growth and degeneration of hair follicles. Besides these observations, increase in telogen follicles as compared to anagen follicles was observed. Telogen follicle distinguished by smaller length and decreased diameter of follicles (Photograph-1 d).

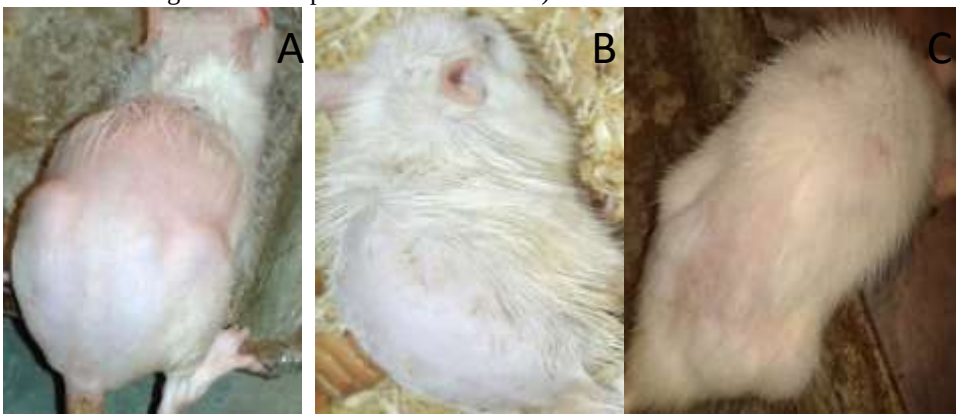


Photograph-1(d) Skin section of DXR treated animals after sacrificed on 20th day of the experiment.

A: Roupture epidermis, Damage tissue; B: Anagen follicles, Telogen follicl

Group-II

Visual observation: Normal feeding pattern during the study was observed and animals were found to be healthy and active. Hair re-growth at depilated site was slow upto 14th day and no significant changes were observed in head and neck hair of rats and on 19th day full re-growth was observed (Photograph-1 b).

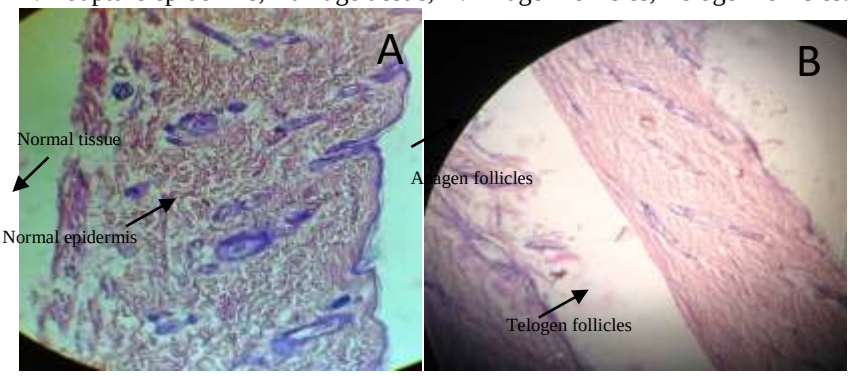


Photograph-1(b) DXR+Pet. ether extract of *Phyllanthus niruri* treated animals

A: After depilation; B: After 9th day of study (DXR administered); C: After 19th day of study.

Histopathological observation: Skin biopsy section was obtained from rats treated with DXR (2mg/kg per day IP) and Petroleum ether extracts of *Phyllanthus niruri* according to protocol. The observation of skin section indicated less number of ruptured tissue, normal epidermis, more number of melanin pigment, more number of hair follicles, regular banding pattern of hair shaft and regular diameter of hair bulbs were observed. Both anagen and telogen follicles were observed in skin section, but anagen follicles were more in number than telogen follicles. Anagen follicles were also more in number as compared to group I. Anagen follicles were longer in length and deeper than the control group (Photograph-1 e).

A: Roupture epidermis, Damage tissue; B: Anagen follicles, Telogen follicles.



Photograph-1(e) Skin section of DXR+ Pet. ether extract of *Phyllanthus niruri* treated animals after sacrificed on 20th day of the experiment.

A: Normal epidermis, Normal tissue; B: Anagen follicles, Telogen follicles.

The herbs in the present study were selected on the basis of their use in traditional medicines for hair care. Aerial parts of *Phyllanthus niruri* was evaluated during present investigation. Petroleum ether extract of *Phyllanthus niruri* evaluated for Doxorubicin induced alopecia models.

The experimental animals of group I in the first 9 days of study the animal of group-I Doxorubicin administration on 9th day regularly upto 15th day, a slight decrease in feeding rate was observed and a diminished feeding rate was noted on 18th and 19th day of study. Animals became weak and fatigue during the study. While in remaining groups, there were no significant variations in the feeding pattern and animals became active during the study.

In group-I, Hair growth at depilated site was visibly inhibited in most of the animals by 14th day. On 16th day moderate alopecia was observed in head and neck region of some animals. After 19th day of study severe alopecia was observed at depilated site in all the animals (Photograph-1 a). In group-II, Hair re-growth at depilated site was very slow upto 14th day and on 18th day are visible observed. No significant change was observed in head and neck hair of rats and on 19th day full re-growth was observed (Photograph-1 b). After 19th day of study, rat from each group were selected randomly and sacrificed. Skin biopsy was also undertaken from the balding site of each group of rats. The cyclic phase of hair follicles (anagen, telogen), number of hair follicles and the anagen/telogen ratio were determined from the sections.

Histologically, follicles of group-I showed characteristics of telogen follicles i.e. smaller in length, decreased the diameter of follicles, while follicles of group-II, group-III showed characteristics of anagen follicle i.e. follicle hair longer in length follicles dense, and (number increases as compared to group I), deeper in the epidermis. There was rupture of some tissue, distorted epidermis, less number of hair follicles. Less number of rupture tissue, normal epidermis, more number of hair follicle in group-II (Photograph-1 d, e).On examination of skin biopsy of different groups total no. of follicle, anagen and telogen follicles were found to be 20, 6 and 14 in group-I respectively. In group- II, total no. of follicles, anagen and telogen follicle were found to be 29, 16 and 12 respectively. (Table-1).The histological study showed that hair density was maximum i.e. 2.5 ± 0.4924* in case of petroleum ether extract of *Phyllanthus niruri* treated, 2.16 ±0.7177 in control (Doxorubicin) treated animals(Table-2, Figure-2).A/T ratio was maximum i.e. 1.45:1 in case of petroleum ether extract of *Phyllanthus niruri* treated, 0.73 :1 in Doxorubicin treated animals (Table-2,Figure-3).

Cancer treatment with chemotherapeutic agent is associated with severe side effects due to the occurrence of apoptosis in several sensitive tissues (such as hematopoietic system or epithelia of digestive tract) because of drug cytotoxicity (Hannun, 1997). This apoptosis largely depends on p53, a key mediator of cellular mechanism of stress response (Lakin*et al.,* 1999).

Doxorubicin administration will result in enhanced degeneration of the matrix, leading to premature catagen formation and thus increased telogen shedding (Braun-Falco, 1961). When Doxorubicin administered was found to inhibit mitosis in the hair bulb, leading to narrowing of the hair shaft, which subsequently breaks at this point. Apoptosis of hematopoietic cells and cells of the digestive tract associated with cancer treatment is known to be p53 dependent. Radiation or chemotherapy-induced DNA damage leads to the rapid accumulation of p53 protein in the susceptible cells (Giaccia*et al.,* 1998; Kastan*et al.,* 1991), followed by up-regulation of Fas, IGF-BP3, and Bax, encoded by the corresponding p53-responsive genes. Moreover, it was demonstrated that Fas and Baxupregulated in the HF during doxorubicin treatment (Vladimir *et al.,* 2000).

Hair follicles are strongly affected by many chemotherapeutic agent because of the rapid proliferative rate of hair matrix keratinocytes during anagen. In the experimental model of chemotherapy-induced hair loss, the active hair growth phase was first induced by depilation, and Doxorubicin administration during new anagen phase causes complete alopecia imitating changes seen in human chemotherapy induced hair loss. The drug treatment induces dystrophic changes in growing HF and, in more severely damaged follicles, premature regression as a result of massive apoptosis in the entire proximal hair bulb, with subsequent hair shedding (Paus*et al*., 1994; 1996).The decrease in feed/water consumption, week, feel fatigue and some cardiac disorder especially in animals that received only doxorubicin is attributed to the adverse systemic effect of doxorubicin (Pharmacology, 2000). Thus, petroleum ether extract of *Phyllanthus niruri* can be used to inhibit the hair loss due to chemoptherapy. Doxorubicin is one of the most often used anticancer drugs. Although cross-linking/scission of DNA strand/topoisomerase inhibite, is considered responsible for its cytotoxicity, the mechanism of initiation and execution of cell death is apoptosis .Anticancer drug mainly acting on rapid dividing cell (mainly tumour cell). The hair follicles are also rapid dividing cell, so these are also affected by anticancer drugs. Because of this region causes hair loss in chemotherapy, this is main drawback for treating the cancer by chemotheraphy. Doxorubicin also effective chemotheraphy drug but its side effect likes alopecia. So this drug used in protocol. Doxorubicin induce alopecia model, Doxorubicin promoting hair loss by affecting hair follicles causing apoptosis of hair follicles the result of this hair loss increases.

In our investigation, *P. niruri* extracts prevents hair loss by preventing the damage of hair follicles or by inhibiting the effect of Doxorubicin on hair follicles. In experimental model, extract solution were administered orally.

Thus, petroleum ether extract *Phyllanthus niruri* can be used to inhibit or stop the hair loss due to chemotherapy in experimental models They may be act by preventing accumulation or by downregulation of p53.

Conclusion

In Doxorubicin induced alopecia models, it was seen that petroleum ether extract of *Phyllanthus niruri* use to prevent hair loss or to treat the alopecia during chemotherapy.

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