5.1 Introduction

Human infectious diseases have markedly increased during the past ten years, especially in immunocompromised patients. Among the animal and human pathogens, the dermatomycosis is the main cause of dermatomycosis (infections of the hair, skin, and nails), superficial infections that are not life threatening but are chronic also cause considerable morbidity (Bell-Syer et al., 1998). Commercial antifungal agents can have adverse effects such as gastrointestinal disturbances, hepatotoxicity and leucopenia and these primarily occur with systemic administration. Therefore, the development of more effective and less toxic antifungal agents is required for the treatment of dermatomycosis (Silva et al., 2005). Recent research showed that higher plants may serve as promising sources of novel antimycotics with no side effects on human and animals. Effective compounds play a great role in these investigations. Various plant materials are believed to have antifungal activity and many effective compounds have been reported to have antifungal activities with no side effects on humans and animals (Sokmen et al., 1999).

Mechanism of In-vivo antidermatophytic activity/wound healing

The response to injury, either surgically or traumatically induced, is immediate and the damaged tissue or wound then passes through three phases in order to affect a final repair:

- The inflammatory phase
- The proliferative phase
- The remodeling phase

The inflammatory phase prepares the area for healing and immobilizes the wound by causing it to swell and become painful, so that movement becomes restricted. The fibro plastic phase rebuilds the structure, and then the remodeling phase provides the final form.
The Inflammatory phase

The inflammatory phase starts immediately after the injury that usually last between 24 and 48 hrs and may persist for up to 2 weeks in some cases. The inflammatory phase launches the haemostatic mechanisms to immediately stop blood loss from the wound site. Clinically recognizable cardinal sign of inflammation, rubor, calor, tumor, dolor and function-laesa appear as the consequence. This phase is characterized by vasoconstriction and platelet aggregation to induce blood clotting and subsequently vasodilatation and phagocytosis to produce inflammation at the wound site (Li et al., 2007).

Proliferation phase

The proliferative phase essentially involves the generation of the repair materials and majority of the skeletal muscle injuries (Guo and LA 2010).

Remodeling phase

The remodeling phase is an essential component of tissue repair and is often overlooked. The final outcome of these combine events is that the damaged tissue will be repaired with the scar (Guo and LA 2010).

Previous in vitro and in vivo investigations of the antifungal activity of the effective compounds suggested that they could be used as effective antifungal agents (Adam et al., 1998). The selection of plants for evaluation was based on traditional usage for treatment of infection diseases (Janssen et al., 1986, Panizzi et al., 1993, Crespo et al., 1990). However, there are only limited data in the literature on the antifungal activity of effective compounds toward human fungal pathogens in vivo.

The purpose of this in vivo studies is to examine the antifungal potential of effective compounds and their components against dermatomycetes.
5.2 Review of literature

Medicinal plants having wound healing/In-vivo antidermatophytic activity

Leaves of Adhatoda vasica Linn. (Vinothapooshan et al., 2010), Aloe vera (Jettanacheawchankit et al., 2009), Hibiscus rosasinensis (Rajesh Mandade et al., 2011), Tephrosia purpurea L. (Chaudhari 2010), Tribulus terrestris Linn (Wesley 2009), Gymnema sylvestre R.Br. (Malik et al., 2009), Lawsonia inermis Linn. (Gagandeep chaudhary et al., 2010), Euphorbia hirta L. (Ayyanar et al., 2009), Moringa oleifera L. (Rathi et al., 2006), Tectona grandis (Majumdar et al., 2007), Acalypha indica L. (Ayyanar et al., 2009), Adhatoda zeylanica (Bhardwaj, Gakhar 2005), Calotropis procera Br, Cassia alata L., Cassia auriculata L., Datura stramonium L., Nerium indicum Mill, Pongamia pinnata Vent., Sida acuta Burm.F., Tridax procumbens L. (Chopda and RT 2009), Dodonae aviscosa Linn., Cleome viscosa L., Ficus bengalensis L., Mentha viridis L., Murraya paniculata Linn. (Sudersanam et al., 1995), Ocimum sanctum Linn. (Udupa et al., 2006), various plants were reported. Flower part of Catharanthus roseus (Magnotta, et al., 2006, Nayak et al., 2006), Ixora coccinia L. (Sudersanam et al., 1995), Bark of Acacia catechu Willd., Cassia auriculata L. Ficus religiosa L. (Chopda and Mahajan 2009), Jatropha curcas L. (PC Abhilash et al., 2011), also reported. Few of latex of Achyranthes aspera L. Argemone mexicana L. reported by Chopda and Mahajan (2009). Rajeswari et al., 2010, Dhulmal et al., (2007) were used seed of Sesamum indicum Linn. Stem of Calotropis gigantea L. (Sudersanam et al., 1995), Adhatoda vasica Linn. were recorded by Vinothapooshan et al., (2010). Whereas rhizome of Curcuma longa L. used by Chopda and Mahajan (2009) and fruit of Terminalia bellirica Roxb. (Choudhary et al., 2008), Anacardium occidentale L. Brassica juncea L. (Sudersanam et al., 1995), Areca catechu L. reported by Chopda and Mahajan (2009).

Morinda citrifoliais also known as Indian mulberry, belongs to family: Rubiaceae. It mainly contains saponins, tannins, triterpenes, alkaloids, flavonoids. It is mainly used for the bowel disorders, including arthritis, atherosclerosis, bladder infections, boils, burns, cancer, chronic fatigue syndrome, circulatory weakness, cold, congestion, constipation, diabetes, eye inflammations, fever, fractures, gastric ulcers, gingivitis, headaches, heart diseases, hypertension, immune weakness, indigestion, intestinal parasites, kidney disease,
malaria, menstrual cramps, mouth sores, respiratory disorders, ringworms, sinusitis, sprains, stroke, skin inflammation and wounds (Nayak et al., 2007)

*Terminalia bellirica* Roxb. belonging to the family Combretaceae, commonly known as belliric myrobalan. Fruit is astringent, antiseptic, rejuvenative, brain tonic, expectorant and laxative. It is used in coughs, sore throat, dysentery, diarrhoea and liver disorders. It is also useful in leprosy, fever and hair care. In folk medicine it has been used for the treatment of skin diseases as antiseptic and on all types of fresh wound. An ethanol extract of *Terminalia bellirica* Fruit has properties that render it capable of promoting accelerated wound healing activity compared with placebo control (Choudhary 2008).

*Moringa oleifera* Linn. (Moringaceae) has been an ingredient of Indian diet since centuries. The leaves of this plant have also been reported for its anti-tumor, hypotensive, antioxidant, radio-protective, anti-inflammatory and diuretic properties. The aqueous extract was studied and it was found that there was significant increase in wound closure rate, skin-breaking strength, granuloma breaking strength, hydroxyproline content, granuloma dry weight and decrease in scar area was observed.

*Sesamum indicum* is a member of family Pedaliaceae. Sesame oil obtained from the seeds of the plant is highly nutritive as it is rich source of natural oxidant such as sesamin and sesamol (Rajeswari et al., 2010). The methanolic extract of root *sesamum indicum* was obtained and was incorporated in gel and ointment bases. These preparations were evaluated for in vivo wound healing on rat using excision wound model (Dhulmal and Kulkarni 2007).

*Catharanthus roseus* is a key source of mono terpenoidindol alkaloid, vincristine and vinblastine which found useful in treatment of cancer (Magnotta et al., 2006). In a study of ethanolic extract of flower of this plant in a dose of 100 mg/ kg/day demonstrated to possess wound healing property (Nayak et al., 2006). *Aloe vera* is one of the oldest healing plants known to mankind Acemannan (1,4)-acetylated polymannose - the major polysaccharide of *A. vera*- stimulates expression of VEGF and other wound healing-related factors (e.g., keratinocyte growth factor-1 and type I collagen) in gingival fibroblasts. This can be especially beneficial in the case of oral wound healing. Thus, crude *Aloe vera* extract or isolated proangiogenic components may have potential pharmaceutical applications for the management of wounds (Jettanacheawchankit et al., 2009).
Aqueous extract of roots of *Radix paeonia* was screened for wound healing by excision, incision and dead space wound models on wistar rats. Parameters studied were tissue breaking strength, epithelialization, wound contraction and granulation tissue dry weight. The test group demonstrated significant wound healing activity as compared to nitrofurazone ointment treated control group (Malviya and Jain 2009).

*Lycopodium serratum* is commonly known as club moss. Wound activity of aqueous and ethanolic leaf extract of Lycoponium was studied by excision, incision and dead space wound model on rats as compared to the aqueous extract and controls the ethanolic extract showed significant decrease in the period of epithelialization and an increase in wound contraction rate, tissue breaking strength and hydroxyl proline content at the wound site (Manjunatha et al., 2007).

*Hippophaerhamnoides* L. (family Elaeagnaceae) is commonly known as seabuckthorn (SBT). Leaves, ripe fruits and seeds from seabuckthorn have been found to be a rich source of a large number of bioactive substances including flavonoids (isorhamnetin, quercetin, myricetin, kaempferol and their glycoside derivatives), carotenoids (carotene, lycopene), vitamins (A, C, E and K), tannins, triterpenes, glycerides of palmitic, stearic and oleic acids and some essential amino acids (Zu et al., 2006). The high content of bioactive substances has been reflected in its extensive exploitation by traditional medicine. Seabuckthorn has antioxidant (Upadhyay et al., 2010) and anti-inflammatory activity (Ganju et al., 2005) and has been reported to be useful in treating skin wounds (Upadhyay et al., 2009).

*Quercus infectoriai* (Fagaceae) is mainly used for the treatment of anti-inflammatory disorders and also used as dental powder, toothache treatment, gingivitis. Pharmacologically it acts as astringent, antidiabetic, antiviral, antitremorine, local anaesthetic, antibacterial, antifungal, anti-inflammatory and larvicidal activities. It mainly contains tannin (50-70%) and small amounts of gallicacid ellagic acid (SP Umachigi et al., 2008, Sanjay PrahaladUmachigi et al., 2009, Dayang Fredalina Basri et al., 2012).
5.3 Materials and Methods

Antifungal activity in vivo

Locally bred, 2-month-old male Wistar rats weighting about 250 g were used. The rats were maintained in propylene cages, separately, at room conditions (temperature of 22 ± 2 °C; relative humidity ~60%) in a 12-h light–dark cycle. They were given pelleted diet (Veterinary Institute, Subotica, Serbia) and tap water ad libidum. Protocols for animal use followed the Public Health Service Policy on Human Care and Use of Laboratory Animals and were approved by the institutional animal care and use committee.

Analysis of non harmful effects

To determine the non harmful concentrations of the effective compounds and thymol, we used 25 locally bred male wistar rats (170–240 g). The rats were maintained under the same condition as described previously. Rats used for tests were randomly divided into five groups according to concentration of applied compounds investigated. A 0.5 ml of prepared stock solution of the essential oils and components were diluted in ethanol (0.0 1–1%, vol/vol) and injected intraperitoneally. The ointment is considered as non harmful if all the four animals in a group survive 48 h after application (M. Soković, J et al., 2009). Concentrations that are no harmful (0.1%) to the feet of animals were used for further investigation.

In vivo fungi toxicity assay

The in vivo investigation of the antifungal activity of effective compounds and components was made according to Adam et al., (1998). T. rubrum, T. tonsurans and C. albicans were collected from MR medical college. Locally bred, 2-month-old male Wistar rats were divided into four groups for the five animals; untreated animals served as a control, treated animals with compound (every effective compound separately), treated animals with components (every components separately), Ketoconazole and Clotrimazole. On the back of each animal, 4 cm2 areas were cleaned and depilated. The fungal inoculum was prepared from 7-day-old cultures of T. rubrum, T. tonsurans and C. albicans, suspended in sterilized physiological saline containing 0.1% Tween 80. Following filtration through four layers of sterile gauze to remove hyphae fragments and agar ficks, the final conidial suspension was...
adjusted to 10^7 conidia/ml for use as the inoculum. The conidia were counted using a hemocytometer under a microscope. The inoculum was applied on the back of the animals immediately after depilation and left for 3 days. The establishment of active infection was confirmed on day 4 by isolation of the pathogens from skin scales cultured from infected loci on SDA plates containing 100 units/ml penicillin and streptomycin. Infections were also confirmed by visual examination of the animals on days 8–10. In the animals in which active infections were confirmed, treatment was initiated on day 20 post inoculation and continued until complete recovery from infection was achieved. The ointments contained 0.1% (vol/vol) of compounds, separately, mixed in petroleum jelly. The commercial fungicides Ketoconazole and Clotrimazole were used as standards. The treatments were applied once daily, and the infected areas were scored visually for inflammation and scaling. Clinical assessment of inoculates skin area was performed using a modified lesion score from 0 to 4 as indicated: score 0, no visible lesion; score 1, few slightly erythematous lesions on the skin; score 2, well-defined vesicles; score 3, large areas of marked redness incrustation, scaling, blade patches, ulcerated in places; score 4, mycotic foci well developed with ulceration in addition to a score 3 lesion (G. Petranky, J et al., 1987). The presence of the pathogens was confirmed by cultivation of skin scales from infected loci on SDA plates containing 100 units/ml ketoconazole and streptomycin each day.

5.4 Results

All the seven effective compounds have shown therapeutic activity against three dermatophytes. The effective in-vivo activity was observed early in *T. tonsurans* followed by *T. rubrum*, *C. albicans*. AR-1, AS-1, CO-1 and FR-1 compounds were showed 100% antifungal activity against *T. tonsurans* at 20th day followed by ET-1, P-1 and VN-1 showed 100% activity at 30th day (Tables 5.1, 5.2, 5.3). The animals treated with the commercial drug, ketoconazole, were cured after 10th days of treatment (Figure 5.2).

The standard Ketoconazole showed 100% antifungal activity against *T. rubrum* after 20th days of treatment. While isolated compounds AR-1, AS-1, CO-1 and FR-1 have shown therapeutic activity at 30th day of treatment followed by ET-1, P-1 at 40th day. Whereas the isolated compound VN-1 was shown slow therapeutic activity at 45 day (Figure 5.1). C.
albicans infection was 100% cured at 30th day, all the six compounds (AR-1, AS-1, Co-1, FR-1, ET-1 and VN-1) except P-1, showed at 40th day (Figure 5.3). After this period, cultures taken from the infected region were negative. For untreated rats (control group) symptoms were observed at the same time as in treated animals and were present at the end of the experiment (Plate 5.1).

Plate 5.1: In-vivo antidermatophytic activity of effective compounds using Wister rats

1A: Normal Wister rat, 1A: Treated Wister rat, 2. T. rubrum treated Wister rat, 3. T. tonsurans treated Wister rats, 4. C. albicans treated Wister rats

A: Before treatment, B: Middle of the treatment, C: After treatment.
Table 5.1: In vivo studies time-dependent changes in skin lesion scores in Wister rats infected with *Trichophyton rubrum* and treatment with 07 effective compounds, standards.

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C=control (Untreated infected Wister rats), K=Ketoconazole, CL=Clotrimazole, AR=Annona reticulata, AS=Annona squamosa, CO=Corchorus oleterius, ET=Euphorbia tirucalli, FR=Ficus racemosa, P=Pongamia pinnata, VN=Vitex negundo.

![In-vivo studies against Tr](image)

Fig. 5.1. Time-dependent changes in skin lesion scores in Wistar rats infected with *Trichophyton rubrum* and treatment with 07 effective compounds, and Ketoconazole.
Table 5.2: In vivo studies time-dependent changes in skin lesion scores in Wister rats infected with *Trichophyton tonsurans* and treatment with 07 effective compounds, standards.

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C=control (Untreated infected Wister rats), K=Ketoconazole, CL=Clotrimazole, AR=Annona reticulata, AS=Annona squamosa, CO=Corchorus oleterius, ET=Euphorbia tirucalli, FR=Ficus racemosa, P=Pongamia pinnata, VN=Vitex negundo.

**In-vivo studies against Tt**

![In-vivo studies against Tt](image)

**Fig.5.2.** Time-dependent changes in skin lesion scores in Wistar rats infected with *Trichophyton tonsurans* and treatment with 07 effective compounds, and Ketoconazole.
Table 5.3: In vivo studies time-dependent changes in skin lesion scores in Wister rats infected with *Candida albicans* and treatment with 07 effective compounds, standards.

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C=control (Untreated infected Wister rats), K=Ketoconazole, CL=Clotrimazole, AR=Annona reticulata, AS=Annona squamosa, CO=Corchorus olcterius, ET=Euphorbia tirucalli, FR=Ficus racemosa, P=Pongamia pinnata, VN=Vitex negundo.

Fig.5.3. Time-dependent changes in skin lesion scores in Wistar rats infected with *Candida albicans* and treatment with 07 effective compounds, and Ketoconazole.

From the previously reported results and results presented here, it can be concluded that the effective compounds used shown very good therapeutic and antifungal effect *in vivo*. These compounds could represent possible alternatives for the treatment of patients infected by dermatomycosis. Even more, because of the side effects of commercial fungicides and possible resistance of pathogens to the synthetic mycotics, the preparation with natural products have an advantage in treatment of fungal diseases. After all facts, and the obtained results, it can be said that the advantage of products based on medicinal plants that were
studied showed no harmful effects on humans and animals and also proved to be very good antifungal and therapeutic agents.

5.5 Discussion

Therapeutic and antifungal activity of isolated 07 compounds *in vivo* could be presented as follows: *Annona reticulata, Annona squamosa, Corchorus oleretius, Ficus racemosa*, showed the best antifungal activity *in vivo*, while *Euphorbia tirucalli, Pongamia pinnata, Vitex negundo* compounds had the moderate antifungal potential in this experiment.


These results fully confirm our results obtained *in vitro* research, which is in agreement with the literature (Adam et al., 1998, Janssen et al., 1986, Müller-Riebau et al., 1995, Reddy et al., 1998). In general, studies have shown that oxygenated terpenoids play a bigger role in antifungal activity of extract than monoterpenic hydrocarbons (Griffin et al., 2000).

After reviewing of the results of the antifungal activity of extracts and individual components *in vivo* experiment, knowing that the composition of extracts and the proportion of the tested individual components may be, to some extent, examine and explain the differences between the activities of the tested extracts.

In the present in-vivo activity the effective AS-1, AS-1, CO-1and FR-1 compounds cured within 25th days infection of *T. rubrum* and *C. albicans*, 15th day of *T. tonsurans*. This is supported by previous reports of crude extracts of *Annona reticulata* seed extract in vivo

In the present report maximum plant leaves and other parts used in treating skin diseases. It was supported by previous reports of Adhatoda vasica Linn. (Vinothapooshan et al., 2010), Aloe vera (Jettanacheawchankit et al., 2009), Hibiscus rosasinesis (Rajesh Mandade et al., 2011), Tephrosia purpurea L. (Chaudhari 2010), Tribulus terrestris Linn (Weskey 2009), Gymnema sylvestre R.Br. (Malik et al., 2009), Lawsonia inermis L. (Gagandeep chaudhary et al., 2010), Euphorbia hirta L. (Ayyanar et al., 2009), Moringa oleifera Linn. (Rathi et al., 2006), Tectona grandis (Majumdar et al., 2007), Acalypha indica L. (Ayyanar et al., 2009), Adhatoda zeylanica, (Bhardwaj , Gakhar 2005), Calotropis procera Br, Cassia alata L., Cassia auriculata L., Datura stramonium L., Nerium indicum Mill, Pongamia pinnata Vent., Sida acuta Burm.F., Tridax procumbens L. (Chopda 2009), Dodonaea viscosone Linn., Cleome viscosa L., Ficus bengalensis L., Mentha viridis L., Murraya paniculata Linn. (Sudersanam et al., 1995), Ocimum sanctum Linn. (Udpa et al., 2006).

Whereas fruit parts of Anacardium occidentale L. was used in the present study it support with previous studies like Terminalia bellirica Roxb. (Choudhary et al., 2008), Anacardium occidentale L. Brassica juncea L. (Sudersanam et al., 1995), Areca catechu L. (Chopda and Mahajan 2009), Ixora coccinia L. flower used in the present study it was also earlier reported by Sudersanam et al., 1995, catharanthus roseus (Magnotta et al., 2006, Nayak et al., 2006) used in dermatological treating.

In the present report the root of Cassia auriculata L. and leaf of Jatropha curcas L. were used, whereas in past studies bark of Acacia catechu Willd., Cassia auriculata L. Ficus religiosa L. Jatropha curcas L. used by Chopda and Mahajan (2009), Abhilash et al., (2011).

Achyranthes aspera L. and Argemone mexicana L. leaves and Curcuma longa L. rhizome were used in this study the latex and rhizome of same plants was used by Chopda and Mahajan (2009).