Study on Phytochemical Screening, HPLC Analysis of Phenols and In vivo Assay on Mice by Using Traditional Herbal Medicinal Plant in Oman

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Abstract- The focus of this research was to find the effect of Rhamnus (***Rhamnus cathartica)*** and Henna (***Lawsonia inermis)*** an important medicinal plant extracts, phytochemical constituents and role on hair growth and wound healing. The extracts of the selected medicinal plants are prepared through two different methods namely soxhalation and maceration to determine the effective method of extraction.Phytochemical screening of both the extracts were carried out to check the preliminary phytoconstituents followed by HPLC analysis is performed to determine the quantity of phenol and flavanoids. Antimicrobial study was studied on selective microorganisms and in vivo study of extracts was carried out on mice with parameters like hair growth and wound healing. The Results of phytochemical screening clearly shows that presence of phenols(++) and flavanoids(+++) in all the tested seed and leaf extracts of *Rhamnus* while flavanoids are absent in henna extracts. The amount of phenol that recognized in HPLC varies according to the plant materials used and type of extraction methods, this is an indicator of extraction method also plays a role in presence or absence of particular constituent. In addition the *invivo* study on mice wound healing and hair growth study also showed a clear result of faster recovery from wound and hair growth.

Index Terms- Medicinal plants extraction methods, phytochemical screening, HPLC screening, *Invivo* assay

I. INTRODUCTION

According to World Health Organization (WHO) more than 80% of the world's population relies on traditional medicine for their primary healthcare needs. Use of herbal medicines in Asia represents a long history of human interactions with the environment. Plants used for traditional medicine contain a wide range of substances that can be used to treat chronic as well as infectious diseases. A vast knowledge of how to use the plants against different illnesses may be expected to have accumulated in areas where the use of plants is still of great importance (D. Diallo et al 1999). The medicinal value of plants lies in some chemical substances that produce a definite physiological action on the human body. The most important of these bioactive compounds of plants are alkaloids, flavanoids, tannins and phenolic compounds (H.O. Edeoga et al 2005). The use of medicinal plants as traditional medicines is well known in rural areas of many developing countries. Traditional healers claim that their medicine is cheaper and more effective than modern medicine.

1.1.***Rhamnus* (*Rhamnus cathartica)*** is a genus of about 150 species (fewer, if some species are placed in *Frangula*), which are shrubs or small trees, commonly known as buckthorns. It is part of the family Rhamnaceae. Its species range from 1 to 10 meters tall (rarely to 15 m) and are native mainly in East Asia and North America. Rigorous scientific studies have shown several pharmacological properties for some metabolites isolated from *Rhamnus.* In fact, the more interesting pharmacological properties of the species belonging to *Rhamnus* genus are due, in particular, to the presence, among several metabolites, of flavonoids, tannins, coumarins and anthraquinones (Izhaki, I., *et al*., 2002).

1.2. **Henna** (***Lawsonia inermis)***, also known as hina, the henna tree, the mignonette tree, and the Egyptian privet) is a flowering plant and the sole species of the Lawsonia genus. The name *henna* also refers to the dye prepared from the plant and the art of temporary tattooing based on those dyes. Henna has been used since antiquity to dye skin, hair, and fingernails, as well as fabrics including silk, wool, and leather. The name is used in other skin and hair dyes, such as black henna and neutral henna, neither of which is derived from the henna plant. Traditional healers have long used plants to prevent or cure infectious diseases. Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, and have demonstrated *in-vitro* antimicrobial (M. Cowan, 1999) and antiparasitic activities (El-Bashir & M Fouad, 2002). More recent findings demonstrated the usefulness of henna to treat headaches and skin diseases amongst many other usages in cosmetic preparations (M. Miller, M. Morris, 1988). Due to the above said medicinal importance, current project is aimed to investigate the following:To investigate various phytochemical constituent, HPLC analysis of phenolics and flavanoids, to screen Antimicrobial property and to determine the effect of the extract on hair growth and recovery the injury in *invivo.*

II. MATERIALS AND METHOD

2.1. Materials:

Rhamnus plant (leaves & seeds), Henna plant (leaves & seeds), petroleum ether, chloroform, blender, electric balance, filter paper, overnight cultures of *Bacillus, E.coli and Streptococcus*, Soxhlet apparatus, UHPLC system, ultra-pure water, phenol, white mice.

2.2. Method

The leaves and seeds of Rhamnus and henna were collected from different regions of Sultanate of Oman, A'Seeb -NorthAl Mabelle in January, 2014. The raw materials were washed with distilled water and driedfor 2 weeks under the shade dried, powdered in electric blender. The dried powdered material of both the plants were packed properly in sealed polyethene bags and further used for extraction.

2.2.1. Soxhalation Method:

20g of each individual dried powder was weighed and subjected for soxhalation in 200ml petroleum ether at 70^{o} C. The extractsare collected in round bottom flask and evaporated using a rotary evaporator at constant temperature (60) with reduced pressure for 2 hrs.

2.2.2. Maceration Method

20g of powdered dry leaf and seed materials were placed in 200ml of Hexane respectively for 48 hr at room temperature shaken periodically by in orbital rotary shaker at 100 RPM.The extracts were filtered twice with whatman no: 1 filter paper and filtrate was evaporated under reduced pressure at constant temperature 68^{0} C in rotary evaporator for 2 hrs.

2.3 Phytochemical Analysis:

The different extracts obtained after Rota evaporation were weighed and 1 g of each individual extract was dissolved in DMSO and used further for phytochemical screening, and *invivo* assy on mice. Phytochemical screening of individual extracts were qualitatively tested for the presence of various phytochemical constituents namely alkaloids, carboxylic acids, coumarins, flavonoids, phenols, quinones, resins, steroids, fixed oils, saponins, tannins, glycosides) according to Brain and Turner, 1975; Sofawora, 1982; Trease and Evans, 1983.

2.4. HPLC analysis

Quantitative analysis of the sample was performed according to the method of Singh et al. (2002). The peak area was calculated with a Winchrom integrator. Reverse-phase chromatographic analysis was carried out in isocratic conditions using a C-18 reverse phase column (250 x 4.6 mm , particle size 2 µm, Luna 5µ C-18 at 25°C. Running conditions included: injection volume, 20µl; mobile phase, methanol: 0.4% acetic acid (800: 200 v/v); flow rate, 1 ml/min; and detection at 290 mm. 2.5mg of crude extracts of Rhamnus plant (leaves & seeds) and Henna plant (leaves & seeds) samples were dissolved with 5 ml methanol solution. Then each samples were injected to UHPLC system with the same condition. Samples were filtered through an ultra-membrane prior to injection in the sample loop. Phenol solution was used as standard. Phenolic solution present in each sample were identified by comparing chromatographic peaks with the retention time (Rt) of individual standards and further confirmed by co-injection with isolated standards.

2.5. *Invivo* studies on the effect of plant extract on Hair Growth and Recovery from Injury:

Different aged female mice were used and housed in cages (20cm×32cm×14cm) under automatically controlled conditions of temperature (25◦C), humidity (about 60%), and lighting (light from 08:00am to 20:00pm). They were given free access to water and diet. The care and treatment of experimental animals conformed to the guidelines for the ethical treatment of laboratory.The 30 mice were randomly divided into 6 groups, 2 cages as control groups (one for hair growth treatment and the other cage for skin wound healing and the remaining 4 cages were used as treatment groups). 2 cages of mices were used to study the effect of Rhamnus on wound healing and hair growth while 2 other groups were subjected for henna treatment with same experiments. The treatment was given with the dosage of 50mg/Kg body weight of mice for a week duration while the control was given with distilled water and the feeding was remain maintained in the same condition.

III. RESULTS AND DISCUSSION

3.1 Phytochemical Screening

The percentage of phytoconstituents of different plant extracts were shown in the figure 3.1 and table 3.1. The alkaloid concentration in the leaf maceration extract found to be highest (33.3%) among the tested extract. Only the samples of leaves extract by maceration methods showed high content of flavonoids and Resin which was (100%). Content of phenol was found to be around (33.30%) in both seeds and leaves and in both methods soxhlation and maceration which show high percentage than tannins. Both seeds and leaves of Rhamnus and in both methods sexolation and maceration showed (0%) content of Tannins, Saponins, Quinines and Coumarins. In addition, the content of Resin was (50%) in leaves of Rhamnus by soxhalation method and it was observed that (33.30%) of resin in the seeds of Rhamnus by both soxhalation and maceration methods.

Table 3.1 preliminary phytochemical screening of leaves and seeds extract of Rhamnus.

[1] phytochemicals	[2] Leave Soxhalation	[3] Leave Maceration	[4] Seeds Soxhalation	[5] Seeds Maceration
[6] Alkaloids	[7] -	[8] +	[9] -	[10] -
[11] Carboxylic acid	[12] +	[13] +	[14] +	[15] +
[16] Coumarins	[17] -	[18] -	[19] -	[20] -
[21] Flavonoids	[22] +	[23] +++	[24] +	[25] ++
[26] Phenol	[27] +	[28] +	[29] +	[30] +
[31] Quinines	[32] -	[33] -	[34] -	[35] -
[36] Resin	[37] ++	[38] +++	[39] +	[40] +
[41] Steroids	[42] +	[43] +	[44] -	[45] -
[46] Fixed Oil	[47] +	[48] +	[49] +	[50] -
[51] Saponins	[52] -	[53] -	[54] -	[55] -
[56] Tannins	[57] -	[58] _	[59] -	[60] -

Note: the results represented in the above table are mean value of 3 replicates of each individual extracts.

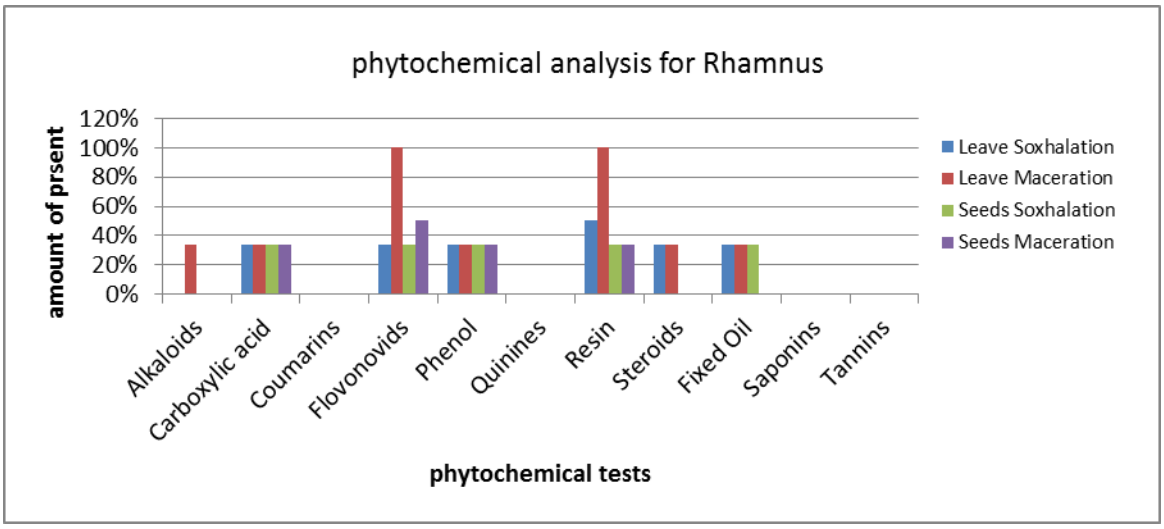

Figure 3.1.Phytochemical analysis of leaves and seeds extract of Rhamnus.

The plant extracts were screened for the presence of biologically active compounds like glycosides, phenolics, alkaloids, tannins, flavonoids, saponins and steroids.Phytochemical analysis of tested extracts demonstrated the presence of highest percentage of phytoconstituents like phenols in leaves of Rhamnus and Henna in leaves by maceration methods was (100%) ,by soxhalation methods was (50%) and for seeds by maceration methods was (33.30%)

Table 3.2 preliminary and phytochemical screening of leaves and seeds extract of Henna.

[61] phytochemicals	[62] Leave Soxhalation	[63] Leave Maceration	[64] Seeds Maceration
[65] Alkaloids	[66] +	[67] +	[68] +
[69] Carboxylic acid	[70] -	[71] ++	[72] +
[73] Coumarins	[74] +	[75] +	[76] -
[77] Flovonovids	[78] -	[79] -	[80] -
[81] Phenol	[82] ++	[83] +++	[84] +
[85] Quinines	[86] +	[87] +	[88] -
[89] Resin	[90] +	[91] +	[92] -

[93] Steroids	[94] ++	[95] +	[96] -
[97] Fixed Oil	[98] +	[99] -	[100]+
[101]Saponins	[102]-	[103]-	[104]-
[105]Tannins	[106]-	[107]-	[108]-

- Note: the results represented in the above table are mean value of 3 replicate.

However, condensed salt tannins was the lowest amount in henna extracts which was (0%) in both seeds and leaves in both methods maceration and soxhalation, in both resin and quinine were (33.3%)in both leaves maceration and soxhalation. Moreover, there were (0%) saponins and flavonoids in both seeds and leaves in both methods maceration and soxhalation. In all seeds and leaves in both methods maceration and soxhalation there were (33.3%) of alkaloids.

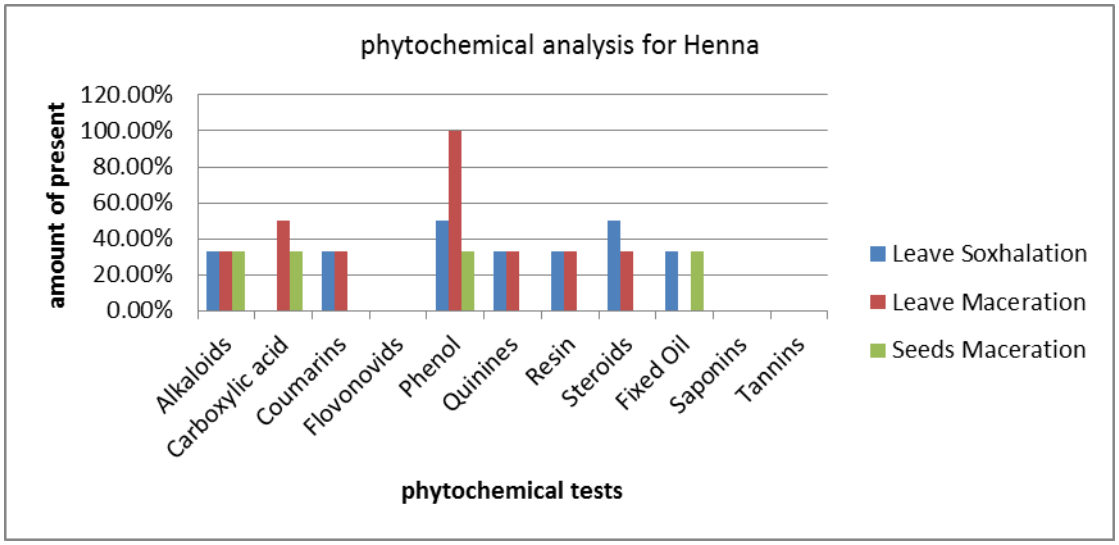

Figure 3.2 phytochemical analysis of different concentrations of phytoconstituents of leaves and seeds of Henna extract.

3.2. HPLC analysis for quantity of phenolic compounds present in Rhamnus and Henna extract

According to Bauer and Tittel (1997) and Springfied et al. (2005) HPLC fingerprinting is the best way for chemical characterization, and therefore this study also established HPLC fingerprint for the active phenlic acids that can act as antioxidant, antifungal, antibacterial and anti-inflammatory.

Phenols, a major group of antioxidant phytochemicals, have profound importance due to their biological and free radical scavenging activities, Tables 3.3 shows the total phenol contents of *Rhamnus* seed and leaves through soxhalation and macerationextracts. Much attention has been focused on the protective biochemical function of naturally occurring antioxidants in biological systems. The results of this study show that Rhamnus seed extract by using soxhalation and maceration extract both methods showed varied number of peaks. Rhamnus seed soxhalation extract showed 8 peaks with different height. Using HPLC led to isolation of eight compounds with a duration 17.96 min evidences the presence of varied compounds ranging from low to very high.

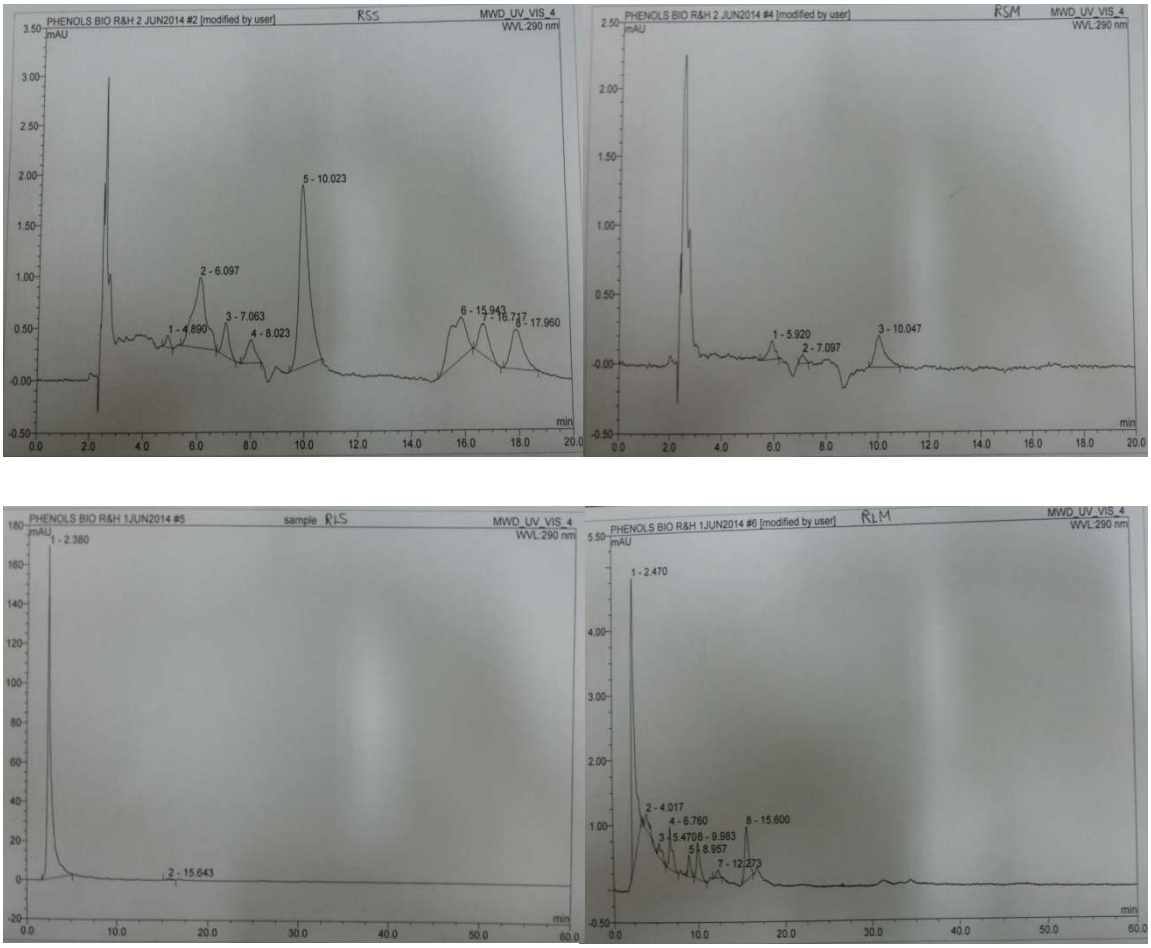

Figure 3. 3. HPLC analysis to recognize phenolic compounds in; A: Rhamnus Seed Soxhalation, B: Rhamnus Seed Maceration C: Rhamnus Leaves Soxhalation D: Rhamnus Leaf Maceration

The type of phenol compound that appeared in the peak number 3 of Rhamnus seed soxhalation which investigated at 7.06 min is exactly the same to the phenolic (Standard) compounds in the peak number 2 at 7.10 but it varied different in the highest is seed soxhalation 0.319 ʎ and seed maceration 0.066 ʎ. This peaks can indicate the present different type of phenol compounds that present such as; Kaempferol 3-O-robinobioside and Kaempferol 3-O-rutinoside according to earliest research (Hazane., f. et al 2005).However, from figure 3.3(C&D), were recognized 7 compounds of phenol in Rhamnus leaves maceration and two peaks in the Rhamnus leaves soxhalation but the first peak that recognized in all the figures is for the methanol that present in the mobile phase of all samples. Both Rhamnus leaves soxhalation and maceration have peak at 15.64 min and 15.60, respectively. But the peak in Rhamnus leaves maceration is 0.816 ʎ high so, this compound is one of the majority compound belongs phenol that present in high concentration.

HPLC analysis revealed presence of a variety of phenolic compounds in both extracts of Henna which might have been responsible for their effective therapeutic potential. However, Phenolic compounds can be defined as a large series of chemical constituents possessing at least one aromatic ring bearing hydroxyl and other sub constituents, including their functional derivatives (Strack, 1997). The peak number 3 at 3.91min and high 0.924 ʎ of Henna leaves Soxhalation is considered as major compound of phenol, As comparing to the peak that recognize in Henna leaves Maceration at 3.94min and its also major compound as comparing with the standard peaks. This peaks indicate phenolic compounds present in Henna leaves Soxhalation and Maceration such as Flavonol, Kaempferol, Chlorogenic acid , caffeoylquinic acid, p-hydroxybenzoic acid, Gallic acid and p-hydroxybenzoic acid(Sakakibara.H at all,.2003).

3.3. *In vivo* test for *Rhamnus* and *Henna* extracts:

The plant commonly known as Henna or Mehndi is abundantly available in tropical and subtropical areas. Ancient history of India describes its diverse uses and also plays appreciable role in Ayurvedic or natural herbal medicines (Lavhate M et al., 2007). Its use became popular in India because of its cooling effect in the hot Indian summers. Henna leaves, flowers, seeds, stem bark and roots are used in traditional medicine to treat a variety of ailments such as rheumatoid arthritis, headache, ulcers, diarrhea, leprosy, fever, leucorrhoea, diabetes, cardiac disease, hepatoprotective and as a coloring agent (Reddy K et al., 1988) (Chetty K et al., 2008). For the *in vivo* experiment, the extract was applied on the backs of shaved

mice and in small tails wounds. Since the mice were of the same age, they were known to be in the same phase of hair growth and cell of skin growth. The mice were then wipe by 1 ml of *Rhamnus* seeds *soxhalation* and henna leaves *soxhalation* extracts every day.

Hair growth and wound healing were seen after 3 weeks of wiping of *Rhamnus* seeds soxhalation and *Henna* leaves soxhalation but in control cages there were no observation of Hair growth and wound healing. So, it was noted that the *Henna* leaves soxhalation extract stimulated the normal hair cycle inducing hair follicles at the next anagen and telogen stages more than the *Henna* leaves soxhalation extract. In addition, the mice which wiped in the wound area by henna leaves soxhalation extract more became healing and forms new cells than the one which was wiped by *Rhamnus* seed soxhalation. However, the control cage of wounded mice were shown the negative results of no faster recovery from the wound healing as well hair growth

IV. Conclusion

The findings of the present work revealed the approaching use of *Rhamnus* and *Henna* in phytochemical screening show that the major component in Henna extract was phenol(+++) and the major components in *Rhamnus* were flavonoids(+++) and Resin. However, the results of *invivo* assay on mice on hair growth and recovery from injury show that the Henna leaves extract in Soxhalation was shown the effective role in wound healing as well hair growth within 7 days compared to control taken 12 days . Finally, the HPLC analysis of phenolics was shown that the Rhamnus seeds extract through maceration had the highest peak of phenol indicating that more amount of phenolics and are responsible for the biological activity.
Recommended that, the henna leaves are more useful for preparing cosmetics like soap and shampoo for hair treatment since it improves the hair growth effectively according to the experiment.

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