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First development of an eco-friendly herbal stain using aqueous and ethanolic extract of henna (*Lawsonia inermis*) leaves for staining of haemoprotozoa

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Abstract
Henna (*Lawsonia inermis*) has been used since antiquity to dye skin, hair and fingernails, as well as fabrics and it's local name is 'mehendi'. In this present study, henna leaves were used for preparation of aqueous and ethanolic henna leaves extract stains following standard protocol. Basing on clarity of staining, different gradations (+4, +3, +2, +1 & 0) were designed for extremely clear, clear, moderately clear, less clear and unclear, respectively for assessing four categories (aqueous extract with alum, ethanolic extract with, aqueous extract without alum, ethanolic extract .The data derived from the score values statistically (p>0.05) depicted equal clarity of the four categories of stains developed in the present study In this present experiment, herbal extract stain took less time (only 30 minutes) to give better staining quality of haemoprotozoa with good resolution whereas commercially available Giemsa stain generally takes 45 minutes. In the present study, henna leaves extract has got substantial worth to be used as eco-friendly, non-hazardous herbal stain in the field.

Keywords: Haemoprotozoa, aqueous and ethanolic extract, henna leaves and eco-friendly stains

Introduction
Despite the intensive efforts made towards immuno-parasitological and molecular diagnosis by different workers, conventional method by microscopic examination is still considered as gold standard method since it is simple, easy and does not involve the purchase and maintenance of expensive equipments. In conventional method, haemoprotozoan are generally diagnosed by staining with Giemsa, Leishman's or other Romanowsky's stain. Generally staining of blood parasites is done by using commercial dyes which are expensive and hazardous chemical components. Synthetic dyes are gradually decreasing in the market on account of an increased environmental awareness of the people about their bio-incompatibility absence/low biodegradability wrapped with several harmful propositions and as an obvious consequence; many workers are exponentially motivated to delve into search of natural colourants which are safe for use, unsophisticated and harmonized with nature. Although commercial dyes are simpler and staining with them is easier, the quality of herbal dyes is better and more appropriate than synthetic dyes (Ifeatu *et al*., 2017) [5]. Although preparation of herbal dyes are more complex but commercial dyes are not stable against light, washing and friction, whereas natural dyes are stable (Afshar, 2001) [1]. Recently, dyes derived from natural sources have emerged as important alternatives to synthetic dyes, the latter one having been reported to have carcinogenic effects (Sewekow, 1988) [15], and the use of natural dyes has once again gained interest (Eom *et al*., 2001; Padly and Rathi, 1990; Garg *et al*., 1991) [3, 13, 4]. Natural dyes can stand as much-needed alternatives to the complex world of chemical dyes (Prabhu and Bhute, 2012) [14]. The field veterinarians, who are residing in remote rural areas face problem of unavailability of commercial dyes; whereas natural dyes are easily available which might be ready resources for blood parasites and helminth parasites. A view paid on the existing staining agents reveals that only few commercially available stains requires more attention to formulate newer and newer staining agent particularly to explore the staining ability of various locally available natural dyes.
Previous studies using some plant extracts have shown that they can be explored in biology and medicine to reveal or identify cellular components or parasites infecting blood cells and tissues (Odinihirin, 1982; Okoli, 2008) [11, 12]. But it is worth-noticing that the use of natural colourants for staining of parasites/ parasitic stages, bacteria, fungi or other organisms is very less.

Herbal dyes (alizarin and henna) are cheap and safe and could be potential alternatives for staining *Fasciola hepatica* and other trematode (Daryani *et al.*, 2011) [2]. The active ingredient of henna is lawsone. The paste of powdered dry leaves of henna plant is used as body art called mehandi from ancient India. The lawsone will gradually migrate from the heena paste into the outer layer of the skin and bind to the proteins in it, creating a fast stain.

Mordants are regularly included in the dyeing protocols when natural dyes are used in order to fix or intensify the satins in cell or tissue preparations. They are used to set dyes on tissue by forming a coordination complex with the dye which then attaches to the tissues (IUPAC, 1997; Llewellyn, 2005) [6, 8]. Different kinds of mordants give a different hue to the staining dye in the cell or tissue.

It is very surprising that no research on stain development was carried out during last ten years which prompted us to take this research program to develop a stain using heena leaves. We have been able to develop an eco-friendly herbal stain using heena leaves which is the first work in the field of staining of haemoprotozoan and seek to communicate the work immediately amongst the mass audience for further development of the stain using different mordants or other substances.

Materials and Methods

Collection and preservation of plant materials

Fresh healthy leaves of henna (*Lawsonia inermis*) were gently collected from the plants. The leaves were washed gently by placing in distilled water and checked properly for presence of any dust and unwanted contaminants. All collected materials were dried in shadow and then grinding of dried plant parts were done with the help of electric grinder machine.

Aqueous extraction

Twenty gram of grinded plant materials were mixed with 100 ml of distilled water and heated to 70 °C for three hours in a water bath. After completion of the heating process, different extracts were allowed to cool. The extracts were then subject to the purification process (Daryani *et al*., 2011) [2].

Ethanolic extraction

Twenty grams of grinded henna leaves were mixed with 100 ml of 80% ethanol and the same was incubated at 4 °C for 24-48 hours for complete extraction of stains. The extracts were proceeded to purification procedure (Kumar *et al*., 2015) [7].

Purification of extract

At the end of each extraction procedure, the extracts were purified by a two steps filtration process. Primary filtration was done with the help of wire mesh followed by Whatman filter paper. Lastly, filtrates were centrifuged at 5000 r.p.m. for 15 minutes. The supernatant part was collected into a reagent bottle and stored at 4 °C until next usage.

Addition of alum

To evaluate the staining ability of henna leaves extract with alum (as mordant), filtrate extracts of these herbs were added to 2% alum (potassium aluminum sulfate) solution and after proper mixing, dye solution so prepared were filtered using No. 1 Whatman grade filter papers.

Blood smear preparation and staining

Blood smears of haemoprotozoa suspected cows, buffaloes and dogs were prepared from the ailing animals. Blood smears were prepared with proper labeling. Fixation and staining were done as per the protocol (Mc Cosker, 1975) [9]. Staining was done for 30 minutes in case of herbal extract. Stained smears were critically examined under 100X and visual cues were utilized for determination of staining quality pertaining to different 'scores' as mentioned later.

Stains composition used

Different stains like aqueous henna leaves extract, ethanolic henna leaves extract with alum (as mordant), ethanolic henna leaves extract and ethanolic henna leaves extract with alum (as mordant) of herbal dyes along with/without mordants were prepared Twenty (20) blood smears infected either with *Theileria* and *Babesia* spp. were used in each type of henna leaves extract stains.

Scoring of staining quality

The scoring of staining quality was done according to clarity of staining of haemoprotozoan parasites and graded as +4, +3, +2, +1 & 0 for Extremely clear, Clear, Moderately clear, Less clear and Unclear, respectively

Statistical Analysis

Statistical analysis of results was done using Statistical Package for Social Sciences (SPSS) version 20. Comparison of the various parameters obtained from different groups was done with non-parametric Kruskal-Wallis Test and Mann Whitney U test.

Results and Discussion

For this experimental trials, *Babesia* and *Theileria* infected blood smears were stained with or without alum mordant added aqueous and ethanolic henna leaves extract and the results thus obtained have been depicted in Table no. 1 and Figs. nos 1 to 5. Out of 20 stained blood smears for each types of herbal extract, the staining quality of aqueous and ethanolic henna leaves extract without alum stain pertains to the similar scoring grade like "extremely clear' in 17 (85%) and 'clear' in 03 (15%). The numerical values were slightly different in case of heena leaves extract with mordant i.e. 'extremely clear' in 18 (90%) & 'clear' in 2 (10%). Results of data were found statistically non-significant while compared amongst all four groups i.e. aqueous henna leaves extract without alum (Mean rank-48.50), aqueous henna leaves extract with alum (Mean rank-51.00), ethanolic henna leaves extract without alum(Mean rank-48.50), and ethanolic henna leaves extract with alum (Mean rank-51.00). Critical analysis of photographs revealed that haemoprotozoan parasites i.e. *Babesia* and *Theileria* were clearly visible with good contrast with all the four type of henna leaves extract stains.

There have no literature on use of henna leaves extract as stain for staining of haemoprotozoa so far. Nor Afandy *et al*., (2001) [10] used henna leaves extract for staining of intestinal protozoa by west staining method where good colour absorption was recorded. Daryani *et al.*, (2011) [2] reported that henna can be considered as alternatives to industrial dyes such as carmine for the staining of *Fasciola hepatica*. Henna (*Lawsonia inermis*) has been used since antiquity to dye skin, hair and fingernails, as well as fabrics. Historically henna has also been used for medicinal purposes, to dye cloth and leather as well as hair, to color the manes of horses and

the fur of other animals. In our present study, an encouraging result has been achieved while henna stain was used for some haemoprotozoan. Moreover, we found the same details of the parasite without mordant. This is a first work which bears much possibility for future research for development of more potent stain using henna leaves. It could be stated unequivocally that the present work has substantial worth.

Table 1: Comparison of aqueous and ethanolic henna (*Lawsonia inermis*) leaves extract with / without alum (as mordant) stain for staining of haemoprotozoan parasites.

Groups	No. of observations	Mean rank	Standard deviation	X^2-value	p - value
Aqueous henna leaves extract without alum (as mordant)	20	48.50	0.366	1.416	0.841
Aqueous henna leaves extract with alum (as mordant)	20	51.00	0.308		
Ethanolic henna leaves extract without alum (as mordant)	20	48.50	0.366		
Ethanolic henna leaves extract with alum (as mordant)	20	51.00	0.308		

*p<0.05- significant at 5%; **p<0.01- significant at 1%; p = 0.00- highly significant at 1%; p>0.05- non-significant.

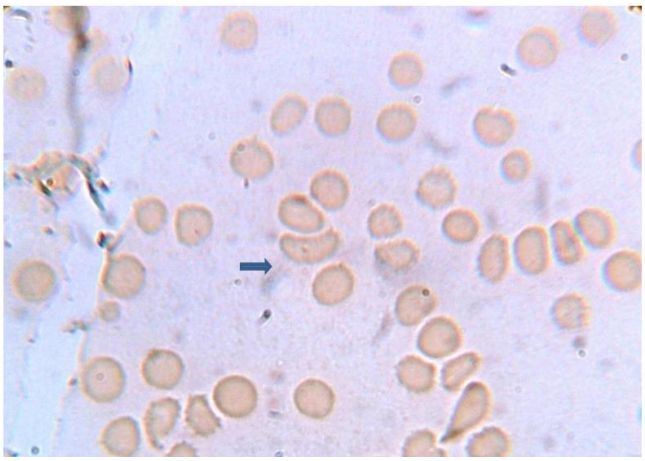

Fig 1: Blood smear stained with aqueous henna extract with alum showing *Babesia* organisms in RBCs

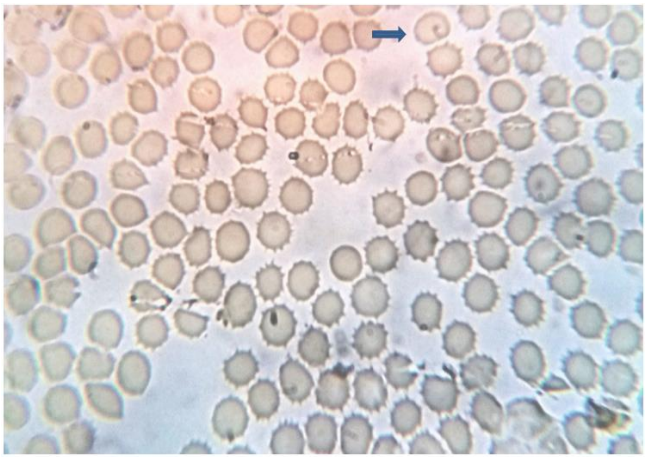

Fig 2: Blood smear stained with ethanolic henna leaves extract with alum showing *Babesia* organisms in RBCs

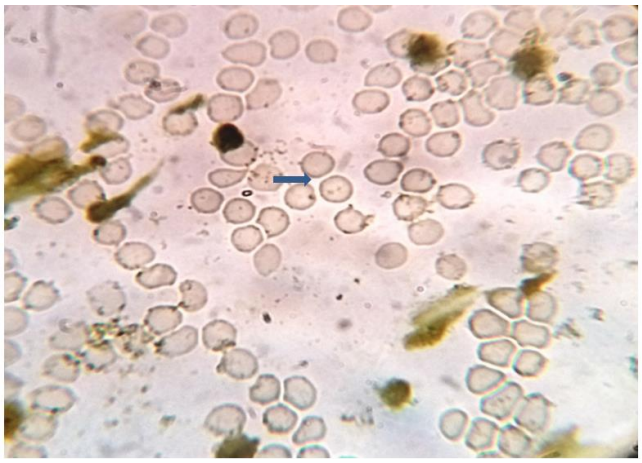

Fig 3: Blood smear stained with ethanolic henna leaves extract without alum showing *Babesia* organisms in RBCs

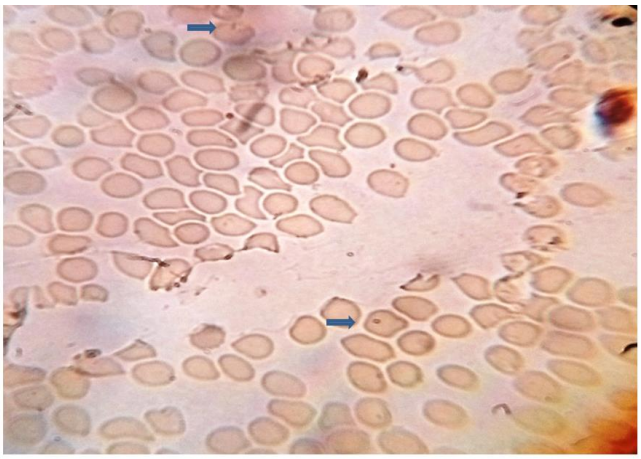

Fig 4: Blood smear stained with ethanolic henna leaves extract with alum showing *Babesia* organisms in RBCs

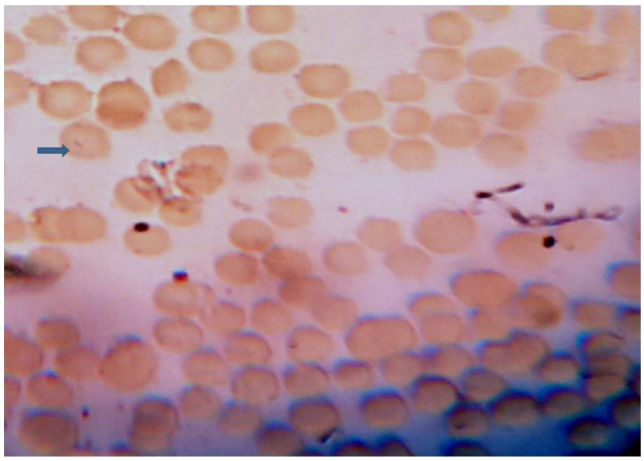

Fig 5: Blood smear stained with aqueous henna leaves extract with alum showing *Theileria* organism in RBC

Conclusion

Aqueous and ethanolic henna leaves extract stains produced remarkable staining quality for staining of haemoprotozoan. Henna leaves extract stain is eco-friendly, locally available and non-hazardous herbal extract stain which could be a good substitute for commercially available hazardous and expensive conventional stains in the nearest future.

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References

1. Afshar VJ. The process and methods of dyeing fibers with natural dyes. Art University Press, Tehran, Iran, 2001.
2. Daryani A, Sharif M, Meigouni, M. Staining of *Fasciola hepatica* by natural herbal dyes. Comparative Clinical Pathology. 2011;20:305-308.
3. Eom S, Shin D, Yoon K. Improving the dye ability of natural colourants on cotton by cationization. Indian Journal of Fiber &Textile Research. 2001;26(4):425-431.
4. Garg A, Shenda S, Gupta KC. Effect of mordants on colour of natural dye extracted from tissue flowers (*Butea mongsperma*).Colourage. 1991;38(2):50-53.
5. Ifeatu OC, Promise MDU, Nkoli OS, Mgbakogu R. Evaluating the staining potential of dye extracted from *Baphia nitida* (camwood) on malarial parasite. European Journal of Biomedical and Pharmaceutical Sciences. 2017;12(4):152-158.
6. International Union of Pure and applied Chemistry (IUPAC). Compendium of Chemical Terminology, 2nd ed. Complied by Mc Naught, A.D. and Wilkinson, A. Blackwell Scientific Publications, Oxford. 1997; 1622p.
7. Kumar N, Mehul J, Das B, Solanki JB. Staining of platyhelminthes by herbal dyes: An eco-friendly technique for the taxonomist. Veterinary World. 2015;8(11):1321-1325.
8. Llewellyn BD. Satins File: mordants. Available from: http;// stainsfile.info/stainsFile/theory/mordant.htm. 2005; Accessed date: Jan 18, 2012.
9. Mc Cosker PJ. Control of piroplasmosis and anaplasmosis in cattle. A practical manual, Rome, FAO. FAO Animal Health Programme, Bolivia, 1975, 1-64.
10. Nor Afandy H, Noor Hayati MI, Chang BTE, Ismail MG. Staining of intestinal protozoa (*Entamoeba* spp.) Using extracts from henna (*Lawsonia inermis*), pandan (*Pandanus odorus*) and a commercial dye. In Proceedings of the Seminar on Medicinal and Aromatic Herbals, 24-25 July 2001.
11. Odinihirin RA. Studies on extraction and use of red mangrove (*Rhizophora racemosa*) for staining animal and plant tissues. Journal of Science Association of Nigeria. 1982;10:4-6.
12. Okolie NJC. Staining of intestinal parasites with extract of *Hibiscus sabdariffa* and *Azadirachta indica*. International Science Research Journal. 2008;1(2):116-119.
13. Padly RN, Rathi D. Effect of mordants on dyeing of cotton with vegetable dyes. Text Dyer Print. 1990;23(25): 27-28.
14. Prabhu KP, Bhute, AU. Plant based natural dyes and mordants: a review. Journal of Natural Product and Plant Resource. 2012;2(6):649-64.
15. Sewekow U. Natural dyes- an alternative to synthetic dyes. Melliand Textilber. 1988;9(4):145-148.