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Staining of platyhelminthes with aqueous and ethanolic extract of onion (*Allium cepa*) tunic: Eco-friendly herbal stains

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Abstract
To develop eco-friendly herbal stains, Onion (*Allium cepa*) tunic were used and prepared aqueous and ethanolic Onion tunic extract stains by following standard protocol. Aqueous and ethanolic onion tunic extract stains showed good staining quality with appreciable contrast images of platyhelminthes parasites. Staining quality of aqueous and ethanolic onion tunic extract stains showed significant difference ((p<0.01) when compared with carmine stain and was found non-significant (p>0.05) when compared with different types of onion extract stains with each other for staining of platyhelminthes. Onion tunic extract herbal stain is eco-friendly, locally available and non-hazardous herbal extract stain could be a good substitute for commercially available hazardous and expensive conventional stains in the nearest future.

Keywords: Staining, platyhelminthes, aqueous and ethanolic extract, onion tunic and eco-friendly herbal stains

Introduction
Conventional method by microscopic examination is still considered as gold standard method for the diagnosis of various parasites and its ova, since it is simple and easy. Helminthic parasites are diagnosed conventionally by examination of faeces microscopically or gross examination of faeces in which adult or immature stages are detected. Generally staining of helminthic parasites is done by using commercial dyes which are expensive and hazardous chemical components. There appears that 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 newer and newer 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) [11]. Although preparation of herbals 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) [19], and the use of natural dyes has once again gained interest (Eom *et al*., 2001; Padly and Rathi,1990; Garg *et al*., 1991) [8, 17, 9]. Natural dyes can stand as much-needed alternatives to the complex world of chemical dyes (Prabhu and Bhute, 2012) [18]. Vibrant colours can be produced from natural dyes by mixing them with each other in different proportion. Furthermore, these natural dyes could consequently provide a new economy source to the country. The field veterinarians, who are residing in remote rural areas use to face problem of availability of commercial dyes whereas natural dyes are easily available which might be ready resources for helminth parasites.
The dry outer skins of red onion (*Allium cepa*) can be used for coloring natural textile materials. The process of achieving color from onion skins is one of the easiest sources of natural colour. Onion skins are simple for a few reasons as they are easy to procure and they do not require the aid of a mordant to achieve colorfast fabric. Onion skins do not need a mordant because they are naturally high in tannin, which binds to the color-molecules to the fabric, creating lasting colorfast fabrics. Dry papery skins of onion were known to be used to make plant-based dye for staining eggs or cloth (Hoffmann and Bauknecht, 1999) [10].

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) [12, 16]. Different kinds of mordants give a different hue to the staining dye in the cell or tissue.

Considering the very few number of stains for staining different types of parasites are available in the market, onion tunic stain was under taken for the microscopic study of platyhelminthes parasites.

Materials and Methods

Collection and preservation of plant materials

Onions (*Allium cepa*) of good quality having coloured outer tunic were purchased from the local market. Then dried outer scales (tunic) were separated from the whole blub by hand picking. Onions were critically examined so that it does not have any rotten area which might alter the dye composition.

Aqueous extraction

Twenty gram of grinded onion tunic 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 subjected to the purification process (Daryani *et al*., 2011) [7].

Ethanolic extraction

Twenty grams of grinded onion tunic 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) [14].

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 onion tunic extract with alum (as mordant), filtrate extracts of these herb were added to 2 % alum (potassium aluminum sulfate) solution and after proper mixing, dye solution so prepared was filtered using No. 1 Whatman grade filter papers..

Collection of platyhelminthic parasites

The whole or segments of platyhelminthes (*Fasciola* and *Moniezia* spp.) were collected from slaughter house as well as during post-mortem examination and also from departmental museum stored in 10 % formalin for staining. Collected specimens were kept in normal saline and brought to the laboratory. Specimens were washed with normal saline for 5 times to remove the stocking remains. Then, following protocol was followed for staining of platyhelminthic parasites (Kumar *et al*., 2015) [14]:

- Whole or segments of platyhelminthes were put between two slides and pressed on it hardly and gently to avoid any tearing. Two slides were bound by a cotton thread and preserved in a glass jar containing 10% formalin for 3-7 days.
- After fixation and flattening the specimens, the slides were opened and the specimens were transferred in 70% alcohol for 15 minutes.
- After that, specimens were immersed into aqueous or ethanolic onion tunic, henna leaves and China rose flowers extract with or without alum (as mordant) and incubated the same for 1-2 days at room temperature.
- Then specimens were incubated into acid alcohol (2 ml of concentrated HCl in 100 ml of 70% ethanol) for 1-5 minutes in order to remove excessive stain without loss of pigmentation.
- The destained specimens were again transferred into 70% ethanol for 1 hour for dehydration.
- Further dehydration was done by placing the specimens into 80%, 90% and 100% ethanol for 1 hour in each solution.
- After dehydration, the specimens were cleared by putting into clearing agent (Clove oil).
- After clearing, the specimens were mounted on to the slides using diastrenedibutyl phthalate xylene (DPX)

Stains composition used

Different stains like Aqueous onion tunic extract, Ethanolic onion tunic extract with alum (as mordant), Ethanolic onion tunic extract and Ethanolic onion tunic extract with alum (as mordant) of herbal dyes along with/without mordants were prepared

Scoring of staining quality

The scoring of staining quality was done according to clarity of staining of platyhelminthes 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 gotten from different groups was done with non-parametric Kruskal-Wallis Test and Mann Whitney U test.

Results and Discussion

For the evaluation of staining performance of aqueous and ethanolic extract of onion tunic (with/without alum), *Fasciola*, *Moniezia* and *Taenia* spp. specimens were used and the results so obtained have been presented in table no. 1 in respect of distribution pattern of staining quality and statiscal comparison of staining potential of stains prepared from different types of onion tunic extract. The microscopical images of different parts of stained flukes and tapeworms have been presented in Figs. nos 1-6 and the images clearly denotes that different parts of stained flukes and tapeworms have acquired varying degree of pigmentation with the distinction of their internal structures using onion extracts which is comparable to conventional carmine stain. It is noteworthy that the colour has been absorbed by the tissues of flukes and tapeworms but the colour intensity differs slightly between each extraction methods. The significant relationship (p<0.01) was observed between aqueous/ethanolic onion tunic extract with / without alum and carmine stain whereas non-significant relationship has found between aqueous and ethanolic onion tunic extract with or without alum. There is scanty information available regarding staining of parasites with onion extract. But Itodo *et al*., (2014) [13] have observed that *Allium cepa* (red onion) skin extract is a promising

histological stain that can serve as an useful stain for histological diagnosis and he also reported that presence of pigments, saponin, tannin, contributed to the staining ability of red onion skin extract. Dry onion skins may be used to make plant based dye for staining eggs or cloth (Hoffmann and Bauknecht, 1999) [10].

Here, alum has been used as mordant but in onion extract staining alum did not take significant role for enhancing the staining property. This might be due to the fact that onion tunic extract itself is a mordant and due to this reason, the effect of alum has not been reflected in the data. Our present finding bears conformity with the finding of Avwioro *et al*., (2005) [2] and Itodo *et al*., (2014) [13] where they found that alum had not significant role for enhancing staining quality. Itodo *et al*., (2014) [13] also reported that *Allium cepa* (red onion) skin extract is a promising stain that can serve as a useful stain for histopathological diagnosis.

The interaction or bonding or attractive forces between dye molecule and molecules within the cell or tissue had resulted the dyeing/staining of cells or tissues (Avwioro, 2002; Kusculuo and Benli, 2017) [3, 15] but the staining colour of cell is affected by cell medium. If the cell colour later staining is pink or blue, dye molecule or cell medium can be basic or acidic (Kusculuo and Benli, 2017) [5]. According to staining theory, acidic structures are stained by basic dyes while basic structures are stained by acidic dyes (Avwioro, 2002) [3]. The stains treated with acid and bases were reported to improve staining potentials for moulds (Chilton et *al.*, 2006) [6] and this can be applicable to other microbes. In addition, inadequate pH buffering during the staining process could have also affected the dyes' staining action as the ability to stain specific tissue structures is determined by the pH values of stain (Briggs *et al*., 2006) [4].

During staining, dye molecules in stain appear as a certain colour and attached to a specific site or cellular structure. Combination of stains may be necessary to affect certain tissue demonstrated. This goes to show that a dye must ionize in solution to produce coloured cations and anions which are capable of uniting with tissue components to form coloured compounds (Carleton *et al*., 1976) [5].

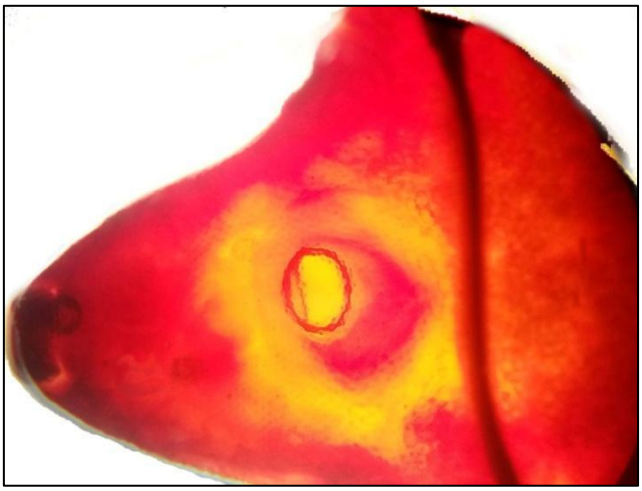

Fig 1: Anterior part of *Fasciola* sp. stained with aqueous onion tunic extract without alum

Fig 2: Posterior part of *Fasciola* sp. stained with aqueous onion tunic extract without alum

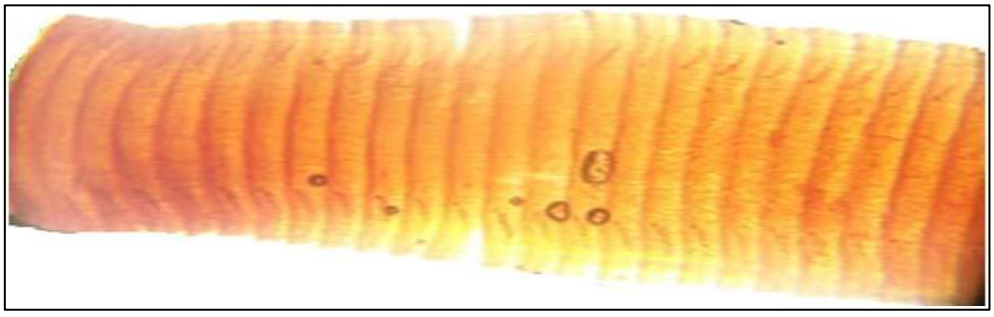

Fig 3: Proglottids of *Moniezia* sp. stained with aqueous onion tunic extract without alum

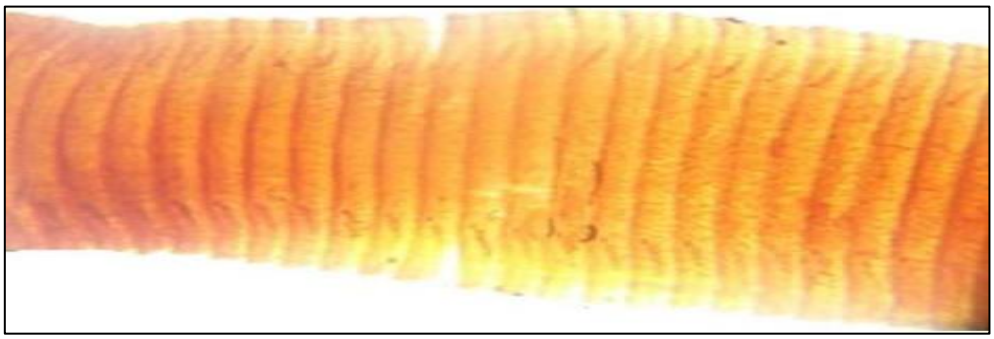

Fig 4: Proglottids of *Moniezia* sp. stained with aqueous onion tunic extract with alum

Fig 5: Proglottids of *Moniezia* sp. stained with ethanolic onion tunic extract without alum

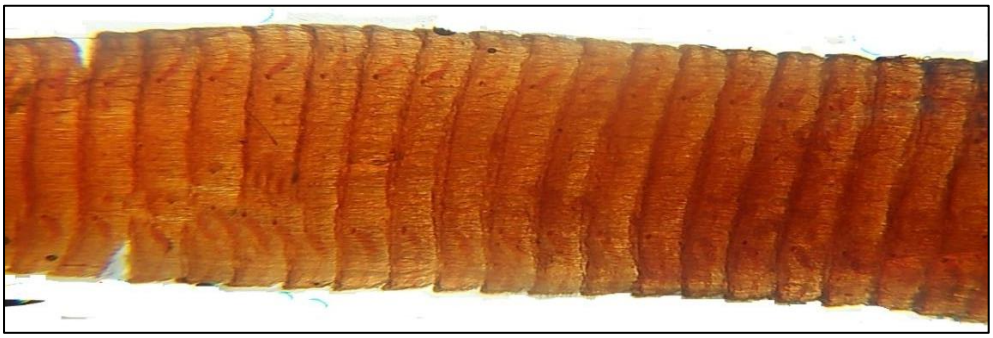

Fig 6: Proglottids of *Moniezia* sp. stained with ethanolic onion tunic extract without alum

Table 1: Comparison of aqueous and ethanolic onion (*Allium cepa*) tunic extract with / without alum (as mordant) and carmine stain for staining of flukes and tapeworms

Groups	No. of observations	Mean rank	Standard deviation	X^2-value	p - value
Aqueous onion tunic extract without alum (as mordant)	20	42.00	0.470	19.315**	0.001
Aqueous onion tunic extract with alum (as mordant)	20	44.50	0.489		
Ethanolic onion tunic extract without alum (as mordant)	20	44.50	0.489		
Ethanolic onion tunic extract with alum (as mordant)	20	49.50	0.510		
Carmine stain (control)	20	72.00	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.

Conclusion

Onion tunic extract stains give better staining quality of Platyhelminthes. Hence, the use of eco-friendly, locally available and non-hazardous onion herbal extract stains will reduce the problems associated with over-dependence on hazardous, expensive and scarcely available exotic stains and could mark the end of an era of dependence on foreign biological stains.

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