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High potential biological stains from the traditional dyes of Manipur, India

Dhananjoy Singh Chingangbam[a,*], Thangjam Anand Singh[b] & Oinam Brojendra Singh[c]

[a]District Epidemiologist, O/o Chief Medical Officer, Ukhrul, Manipur 795 142, India

[b]Directorate of Research, Central Agricultural University Iroisemba, Imphal Manipur 795 004, India

[c]M/S Eastern Dyeing & Printing Industries, Heirangoithong, Imphal Manipur 795 003, India

[*]E-mail: dhananjoych@gmail.com

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Cytogenetics depends to a large extent on the biological stains and advancement of the microscopic devices. Most important and oldest biological stain is carmine, an animal extract. The application of natural dyes for staining of various biological tissues from an alternative source will decrease the expense for purchasing the synthetic dye and reduce their effects on human and environment. The plant derivative dyes were screened for biological staining in the present study. Two of the most popular traditional vegetative dyes in Manipur are extracted from *Bixa orellana* (local name *Ureirom,* UR) and *Strobilanthes cusia* (local name KUM, KU). The water extracts of two the plants were taken to study for the stainability of nuclear on root tip cells of *Allium ascalonicum* L. to test the feasibility of the dyes as the biological stains. The different stages of mitosis cell division in *A. ascalonicum* were stained with the dyes of KU and UR and compared with the standard stain acetocarmine. The UR stain is nonspecific as it stains whole cytoplasm as well as the nuclear parts. The KU stained the nuclear parts more precisely than UR and was as good as acetocarmine. The nuclear stainability of KU or UR is significant in the sense that these are natural products with no allergic response as that of carmine and it is time tested (particularly in Manipur). Hence, KU and UR are promising candidates for cytological/biological application in future that will be cost effective and environmental friendly. In future these two could be used as food colourant for human consumption.

Keywords: *Kum*, Manipur, Mitosis, Nuclear stain, *Ureirom*

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Biological stains are the backbone of both cytogenetics and histology. Two types of dyes were being used for staining and dyeing *viz.,* synthetic and natural dyes. The synthetic dyes are efficient but are hazardous to human and animal health and environment[1]. Thus, alternative natural dyes for biological staining that are eco-friendly and biodegradable are preferred. Some trees, herbs and fruits yield many colours that could be used for dyeing varieties of articles[1]. The vegetative dyes or plant dyes were used all over the world in the past for enhancing food quality, medicines, clothing and so on. Rome[2], China[3], and India[4,5] were few examples. Dye extracted from certain plants imparts honey fragrance to rice[6]. The cucurmin dye which is one of the most important food colorants of the ancient world has antitumour, anti-inflammatory, antioxidant properties and is useful in treatment of rheumatism and prevent the formation of gallstones[7] and is used in daily life of the valley inhabitants of Manipur.

The two most important traditional vegetative dyes of Manipur are *Kum* (KU) dyes obtained from leaves and stems of *Strobilanthes cusia* (Nees) Kuntze (Fig. 1 A) and *Ureirom* (UR, (Fig. 1 B) from *Bixa orellana* L. The technology of extraction and dyeing of KU dates back to 11th century in Manipur[8]. It was considered to be a valued item and some subordinate ethnic tribes of Manipur used *S. cusia* to pay tribute to the Meitei kings[8,9]. The formal dress of elderly ladies in Manipur on occasions related with happiness and wellbeing known as *phanekmayeknaiba* is exclusively dyed with KU[5]. The initial colour of the extract is indigo but it gradually darkens after dyeing. The indigo dyed clothes were used to hide from prey during hunting in China[3]. In addition to this, *S. cusia* has many other medicinal values like bioactivity against influenza and viral activity[10] which is related with the production of secondary metabolite[11], to clear heat, relieve sore throat, antimicrobial activity kill pathogenic microorganisms and improve immunity[7]. The active compound from *S. cusia,* Isatin or indole -2- 3 – dione was used in processing of oilfield water to remove or

*Corresponding author

Fig. 1 — The materials used in the present studies. The leaves of *Strobilanthes cusia* (A), flower pods of *Bixia orallana*, seeds and paste of seeds (B), coloured of the two dyes – indigo from *S. cusia* and deep redfrom *B. orallana*

reduce bacteria before the water is re- injected into formations via wells[12].

The extract of UR seeds are deep red in colour and used to dye cloths to give saffron colouration. In Meitei community, the dye is used starting from day to day towel known as faddhi/washcloth to shirts, loin loom woman dresses, particular trousers, hats for man etc. This dyeing gives attraction and increase longevity besides having some beneficial effect on skin[13]. Particular attire known as *poungouphanek* exclusively dyed with *B. orellana* is used in all cultural functions that signify sadness or humility or mourning by womenfolk of Manipur. Hinduism also identifies with this colour and all the garments of monks/priest of ISKCON (International Society for Krishna Consciousness) wear UR dyed clothes. So these dyes also have cultural significance other than its colouring properties. The extract of this plant has been used for dyeing various articles and drinks in South America during the Mayan civilization[14].

Though these dyes have been used since medieval times, their application as biological stains is yet to be explored. The present work reports the stainability of the two selected vegetable dyes for nuclear materials of meristematic cells from plant as compared to the acetocarmine for application in cytogenetics and other biological works in both plant and animal materials.

Materials and Methods

Method of dye preparation from KU and UR

Four leaves of *S. cusia* (Fig. 1 A) were crushed with 25 mL of water in mortar and pestle and left for two days to ferment in our laboratory. The material was filtered with muslin/nylon cloth; the filtrate was boiled for 15 min with $NaHCO_3$ and pinch of Allum, left for 15 min to cool down. After filtration, the filtrate having pale yellow colour was discarded. The black paste residue collected on the filter paper (Whatman No. 1) was kept for further use.

The young seed pods of UR (Fig. 1 B) were collected from different parts of Imphal valley. The pinkish young seeds were crushed to small pieces with mortar and pestle with water. To 100 mL of this dye 1.5 g of Allum was added and boiled for 5 min. Then it was filtered and dried at room temperature to obtain the dye powder. The difference in colouration of the two dyes were shown in Figure 1C.

Acetocarmine stain (1%) was prepared by dissolving 1 g of carmine powder (Merck, India- C. I. No. 75470, S.No. 1381) in 100 mL of 45% glacial acetic acid, boiled in a beaker for 1 h and filtered, used as standard stain to compare the present dyes in the study.

Plant material used to study the mitosis

The local shallot, *A. ascalonicum* L. were grown in our laboratory and after 24 h, root tips of average 0.5 cm were fixed in fixative (Carnoy's Fluid) for 24 h and preserved in 70% ethanol. Preserved root tips were processed for slide preparation.

Slide preparation

In a test tube 1 mL of dye with 20 µL each of 45% glacial acetic acid and 1N HCl was taken along with seven root tips of *A. ascalonicum* and heated for 10 min over sprit lamp. Meristematic cells from the softened root tips were used for micro slides preparation by squashing method to obtain the different stages of mitosis cells. Fifty cell plates were used for each stage and photographs of best 10 were selected for each stages starting from Interphase, Prophase, Metaphase, Anaphase and Telophase.

Gamete staining

The slides prepared from the testes of male rat by flame drying method was stained with 5% KU water solution for 30 min in a coupling jar at room temperature and results were compared the against 5% UR stain of the same aged slides.

Results and Discussion

Two of the most common traditional dyes used in dyeing cloths: KU and UR were explored for their application as biological stains. The dyes extracted by improvised traditional method could efficiently stained both plant and animal materials. The two extracts were soluble in water as well as in glacial acetic acid (99.5%) and sparingly soluble in chloroform. The water soluble extracts could stain the meristematic cells, however the cells were shrunken and the cytoplasms were distorted.

The meristematic cells of local shallot stained with three dyes with the reference as non-stained cells are displayed in Figure 2 (A-H) for more specific cell stages. The KU stains both cell membrane and chromosomes more precisely. The clarity of chromatins in prophase, metaphase, anaphase and telophase stages was much better in cells stained with KU than acetocarmine or UR. In the telophase stage, the chromatin materials were stained specifically by KU which was more effective than both UR as well as aceotocarmine stained slides (Fig. 3). Cell plates that were stained with KU stain were more distinct and exhibited higher contrast than either acetocarmine or UR stained cells. The different phases of mitosis from meristematic cells of the onion root tip cells could be identified with the three dyes (Fig. 3 A - O).

The UR stain worked specifically to the cytoplasmic as well as the nucleolus and less to nuclear portions that allowed specific and distinctive staining. The stages of mitosis from the root tips of local shallot (Fig. 4 A) could be seen but it lacked contrast between the cytoplasm and nuclear parts. In the elongated cells, the nuclear parts were properly stained by UR. It stained the entire cells and no differentiation of cytoplasm and protoplasm could be made, as was the case with the KU or aceotocarmine. However, it stained the nucleolus clearly which was not the case with either KU or acetocarmine (Fig. 4 B, C and D). Therefore UR may not be suitable for cytological studies but could be considered for staining cytoplasmic materials and as a food colourant. The reddish orange colour of the annatto mainly comes from the resinous outer covering of the seeds of the plant and is composed of the carotenoid pigments *bixin, norbixin* and their esters[15]. The UR has been utilised as food colourant since Mayan Civilization[14].

Both KU and UR were also examined for staining animal cells. The two dyes were found to be effective in staining the sperm cells/male gametes of *Rattus rattus* species (Fig. 4 E and F) but were unable to stain the dividing cells of the germinal cells. Thus their applications may also be extended to morphological as well as histological purposes.

A biological stain is a dye for making microscopic objects more clearly visible than when they are unstained. Cytologists and histologists emphasized the importance of the biological stains for their low cost, minimum or no hazards as compared to the synthetic dyes and easy availability[16-18]. The most important natural dyes for biologists are haematoxylin, indigo, cochineal (and its derivatives), orcein and litmus[19]. This is exemplified by use of *S. cusia* for textile in China[3] and in Manipur[5]. *B. orellana* has been

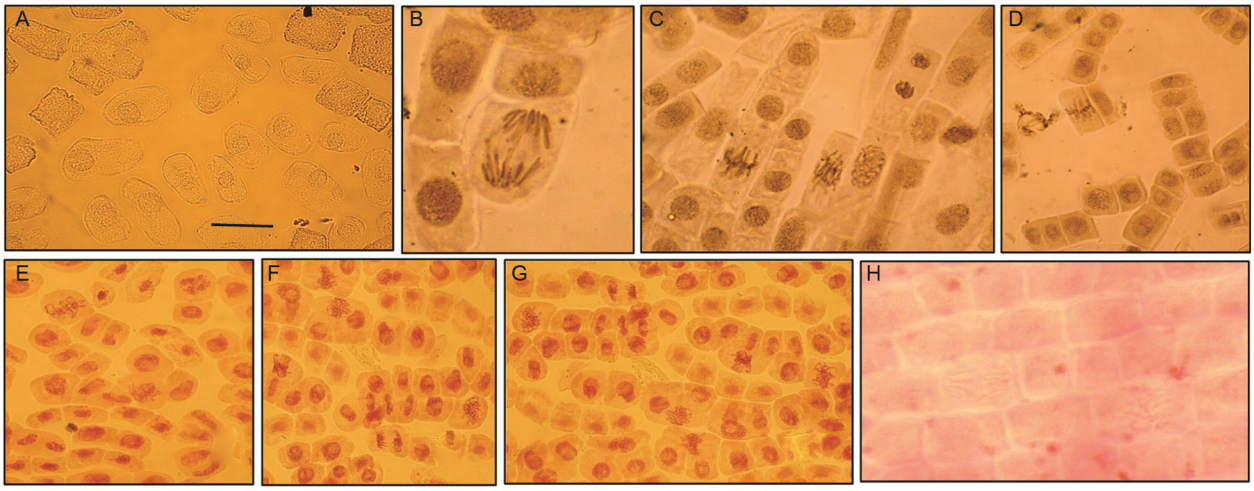

Fig. 2 — Cell plates stained with the three stains. The cell plate without any stain (A), the anaphase and interphase nuclei stained with Kum (B), Kum stained metaphase, prophase and interphase cell plate (C), Kum stained metaphase and interphase cell plate (D), Acetocarmine stained cell plates (E, F and G) and the cell plate stained with UR (H). Bar represents 10 µ and all photographs were taken in 40X.

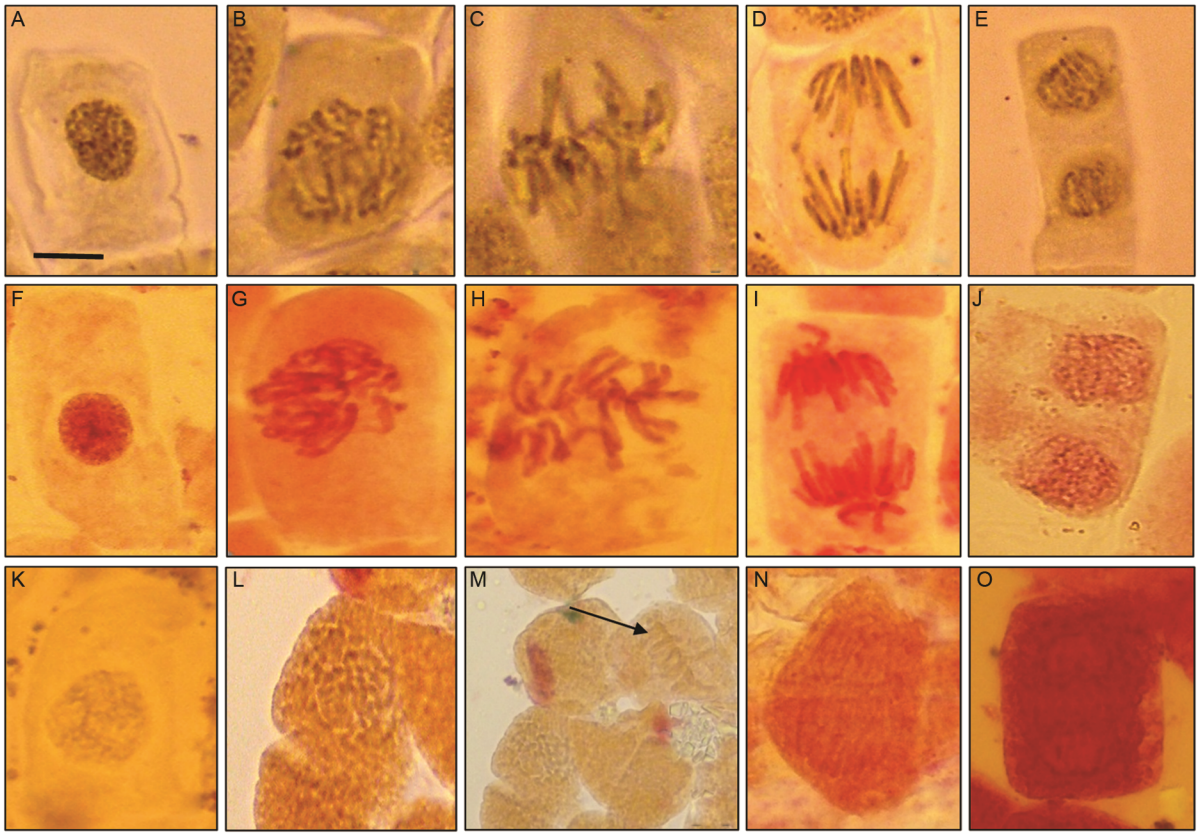

Fig. 3 — The different mitosis stages from meristematic cells of local shallot: Interphase, prophase, metaphase, anaphase and telophase; stained with three stains. A, B, C, D and E were stained with Kum; F, G, H, I and J were acetocarmine stained, K, L, M, N and O were stained with UR. Bar represent 10 µ and all photographs were taken in 40 X.

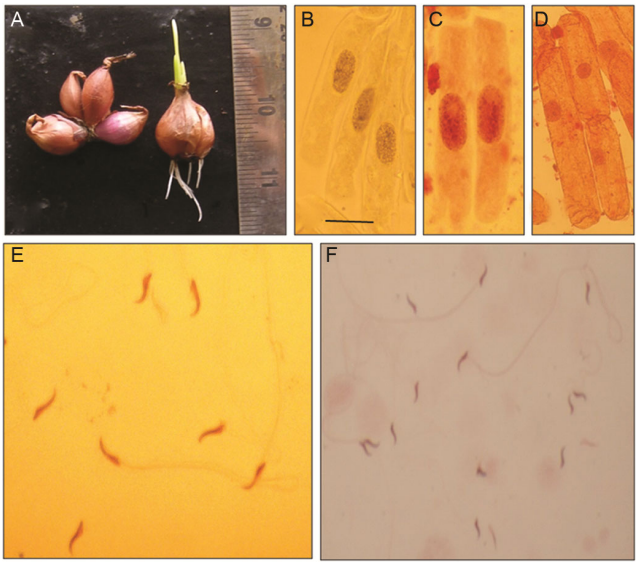

Fig. 4 — The photograph of shallot with roots coming out (A), the elongated cells stained with three stains: Kum (B), Acetocarmine (C), and UR (D). The male gametes of *Rattus rattus* species stained with UR (E) and Kum (F). Bar represent 10 µ and all photographs were taken in 40 X.

favoured in food, pharmaceutical and cosmetic industries as it has been considered safer for human consumption than synthetic dyes[14], as well as economically advantageous for these industries. But the effectiveness of such stains depends on certain factors like pH, solvent etc. The applicability of a dye to stain specific tissue structures is usually determined by factors such as the electrostatic attractions. Acidic tissues (e.g. nucleus) are likely to be stained by basic dyes like haematoxylin while basic structures (e.g. cytoplasm) would be stained with acidic dyes (e.g. eosin)[19]. Natural traditional dyes particularly from Manipur have high potential for application in biological staining studies.

45% glacial acetic acid has been used in the cytological studies in the present work. It is in accordance with the report by Chaisomparn[20] that used 45% acetic acid to extract dye from black glutinous rice, Malabar night shade fruit and butterfly pea sepal for the chromosome staining of multiplier onion root (*Allium cepa* var. *aggregatum*). The results support the three division of the natural dyes[21].

The UR stain most of the membrane boundaries both nuclear and cell membrane and both are distinct as the rest two *viz.,* acetocarmine and KU. So UR could be used to study the cell structure but not the nuclear structures as this stain is unable to distinguish between nuclear part and protoplasm. Interestingly, the two dyes in the present study showed different affinities: KU penetrated deep and stained the nucleus to a greater extent to the cytoplasm to a lower extent and UR stained nucleus to lower extent and cytoplasm to a greater degree. Moreover UR stains the nucleolus more than nucleus. So it is assumed that KU is acidophilic and UR is basophilic so they could be used as a substitute of Haematoxylene and Eosin respectively. There was a hint of applicability in staining cells of animal origin as observed in staining cells of male gametes of rat. *Strobilanthes cusia* had the highest QI (mention index) value of 1.0 and AI (availability index) value of 1.8 for indigo production and has been used against respiratory diseases[22]. The most important precursor of indigo is the indican (indoxyl-3-O-β-D-glucoside) which are stored in vacuoles of the plants[23] after steeping in water indican hydrolysed and produce indoxyl and thereby producing indigo pigment which are in common blue[24]. Future studies will focus on extraction of dye using different solvent to enhance its staining intensity and mixing the two dyes for staining both nuclei and cytoplasm of cells simultaneously.

Conclusion

The present study screened the traditional natural dyes of Manipur for biological staining two natural dye *Kum* and *Ureirom* were explored for their potential to stain biological materials such as cytoplasm and protoplasm of plant cell besides animal cells. Both *Kum* and *Ureirom* could be used as biological stains. The KU may be recommended for nuclear studies while UR may be favoured for cell structure staining, particularly the cytoplasmic parts. However, further studies with some modifications in temperature, duration of staining and pH are highly recommended to obtain better results. Both the stains could also be used in animal materials also. The two dyes are cost effective and have the potential to replace the toxic synthetic dyes for cytogenetic studies.

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Conflict of Interests

There is no conflict of interests among the authors.

Author's Contributions

O B initiated the idea, T A S design the experimental setups and C D S performed the experiments and drafted manuscript.

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