RESEARCH ARTICLE

Toluidine Blue Stain as a Prognostic Indicator in Canine Cutaneous MCTs

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Abstract

Canine cutaneous mast cell tumours (MCTs) are common and exhibit variable biological behaviour, creating prognostic uncertainty. This study included 20 biopsy samples from 20 dogs with cutaneous MCT. Two prognostic groups (dead vs. alive at 24 months post-diagnosis) were compared. Tumours were characterized using cytomorphology, toluidine blue staining, and cKIT immunostaining. No significant differences between prognostic groups were observed for tumour location, size, sex, histological grade, cKIT immunostaining pattern, or cKIT immunoscore. A higher mitotic index was associated with poor prognosis. Toluidine blue staining score for cytoplasmic granulation differed significantly between groups, with poor granulation indicating worse prognosis, suggesting its utility as a better prognostic indicator.

Keyrords: Canine, Cutaneous, Mast cell tumour, Prognostic, Toluidine blue.

Ind J Vet Sci and Biotech (2026): 10.48165/ijvsbt.22.5.07

Introduction

Mast cell tumours (MCTs) represent 16-21% of all cutaneous neoplasms in dogs, manifesting as either solitary masses or metastatic lesions (Shoop *et al.,* 2015). Predilection sites include the trunk, perineal region, extremities, head and neck (Bae *et al.,* 2020). These tumours are categorised as cutaneous (dermal) or subcutaneous forms (De Nardi *et al.,* 2022). The biological behaviour of MCT is frequently unpredictable in clinical settings, largely due to mast cell degranulation, which may cause either localized effects (including ulceration, edema, or erythema) or systemic manifestations involving visceral organs (Mullins *et al.,* 2006). Previous studies have identified multiple prognostic markers for canine MCTs, including patient signalment, clinical presentation, tumour phenotype, histological grade, clinical stage, c-KIT (tyrosine-protein kinase) expression patterns, KIT gene mutations, Ki-67 proliferation index, and nodal metastasis status (Kiupel and Camus, 2019; Thamm *et al.,* 2019; Tamlin *et al.,* 2022).

MCT is histologically graded using a 3-tier (Patnaik *et al.,* 1984) and 2-tier grading system (Kiupel *et al.,* 2011). Grading of MCTs can also be done on the basis of cytological features (Camus *et al.,* 2016). The immunoexpression of c-KIT (CD117) in canine MCTs has been extensively characterized. Immunohistochemical analysis reveals distinct c-KIT staining patterns that correlate with mutations in the c-kit proto-oncogene, serving as valuable indicators of tumour biological behaviour (Webster *et al.,* 2006; da Costa *et al.,* 2015). Toluidine blue staining remains a widely used and reliable histochemical method for detecting granulated mast cells in canine MCTs. While this stain demonstrates consistent metachromatic staining in well- and moderately-differentiated tumours, its efficacy in poorly differentiated MCTs remains debated. Some studies (Cruz *et al.,* 2020) report diminished metachromasia in poorly differentiated tumours,

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How to cite this article: Srivastav, A., Prabhu, S. N., Singh, A. P., Gangwar, N. K., Purohit, S., Chaudhary, J. K., & Shukla, S. (2026). Toluidine Blue Stain as A Prognostic Indicator in Canine Cutaneous MCTs. Ind J Vet Sci and Biotech, 22(5), 32-38.

Source of support: Nil

Conflict of interest: None

Submitted 09/06/2026 **Accepted** 28/06/2026 **Published** 10/09/2026

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whereas more recent investigations (Rasche *et al.,* 2022; Logvinova *et al.,* 2024) demonstrate that optimized toluidine blue protocols can effectively identify mast cells across all differentiation grades, including poorly differentiated variants.

Despite extensive research, canine mast cell tumours (MCTs) often behave aggressively with limited therapeutic success, highlighting the need for rapid, accessible, and cost-effective prognostic tools. This study evaluated the prognostic significance of toluidine blue staining patterns in canine cutaneous MCTs using a standardized scoring system alongside established prognostic parameters, to determine whether staining patterns could serve as practical diagnostic and prognostic indicators in routine veterinary practice.

Materials and Methods

All the experimental protocols were undertaken following approval of the Institutional Animal Ethics Committee of U.P. Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go-Anusandhan Sansthan (DUVASU), Mathura, India (IAEC/21/24 No. 133/IAEC/21 dated 18.03.2022 and IAEC/22/19 No. 136/IAEC/22 dated 31.01.2021). Informed owner consent was obtained for the present study.

Selection of Cases & Histopathology

Dogs presented to the Veterinary Clinical Complex, College of Veterinary Science and Animal Husbandry, DUVASU, Mathura, India with a cutaneous mass or swelling were evaluated. Between January 2021 and December 2022, 20 biopsy samples were collected from 20 dogs diagnosed with cutaneous mast cell tumours (MCTs). Biopsies were obtained surgically via marginal excision. Inclusion criteria required tumours to be of cutaneous origin both grossly and on histopathology; subcutaneous tumours were excluded. Patient data, including breed, age, gender, lesion location, size, consistency, and ulceration, were recorded. A cytological examination was performed using a fine needle biopsy, and the smears were stained with Giemsa stain.

The positive slides for MCT were cytologically graded as high grade or low grade (Camus *et al.,* 2016). The tissue samples were fixed in 10% neutral buffered formalin and routinely processed for histological analysis. Sections of 5-µm thickness were cut in a microtome and stained with hematoxylin and eosin. A two-tier grading system was used in histopathological sections, as proposed by Kiupel *et al.* (2011). The mitotic index was calculated as number of mitotic figures per 10 high power fields (400x).

Toluidine Blue Stain

Cytological smears and histopathological tissue sections were stained with the toluidine blue special stain to differentiate mast cells (Sridharan and Shankar, 2012). Slides were deparaffinised in xylene and hydrated in descending grades of alcohol and distilled water for 3 min each, then subjected to toluidine blue working solution (pH 2.0-2.5) for 2-3 min. Sections were washed in distilled water, dehydrated in alcohol, cleared in xylene, and mounted with cover slips. The granularity in mast cells was graded based on the criteria as per earlier studies (Sabattini and Bettini, 2010). The density of granules was assessed manually from five different high power field (400x) areas with visibly stained cells as Score 1 (low density of granules in most neoplastic cells); score 2 (moderate density in most cells or high density in less than half the cells) or score 3 (high density in most cells).

Immunocytochemistry/ Immunohistochemistry

For immunocytochemistry (ICC), fine needle aspiration of suspected samples was carried on the 3-aminopropyltriethoxysilane (APES) coated slides that were then fixed in chilled acetone for 5 min. For immunohistochemistry (IHC), the tissue sections of 5-µm thickness were taken on 3-APES (Himedia) coated slides. The slides were then processed as described by Sabattini and Bettini (2010). The KIT pattern was assessed in immunostained sections as diffuse, focal or stippled and membraneous. The percentage of immunoreactive cells were calculated and assessed manually in five different high power fields (400x) randomly selected. The immunoscoring (KIT IS) was also done as the product percentage of positive cells (1, ≤10%; 2, 11 to 30%; 3, 31 to 60% or 4, >60%) and intensity of labelling (1, low; 2, moderate and 3, high) (Sabattini and Bettini, 2010).

Statistical Analyses

Various clinicopathological parameters were tested for significance using chi-square, Fisher exact test and logistic regression. For the toluidine blue stain score, ICC/IHC cKIT pattern and cKIT IS, the Mann Whitney U test was employed. The mitotic index and clinical outcome was tested for significance using independent samples test. All tests had significance set at $p < 0.05$, and SPSS 27.0 software was used for statistical analysis.

Results and Discussion

Signalment and Gross Appearance

MCT occurred in both sexes, with affected breeds including Labrador Retriever, Mongrel, German Shepherd, Beagle, Pug, Pomeranian, Pit Bull, and Great Dane. Mean age was 8 years (range: 4-13 years). Tumours were most common on the hind limbs (9/20), followed by trunk, neck, inguinal, and scrotal areas; two cases had multiple cutaneous sites. Tumour size was measured using a digital Vernier caliper (Ingco) as the largest diameter and a perpendicular measurement, ranging from 23 mm to 100 mm (for multiple-site cases, the area with the largest tumour dimensions was selected for grading). Most tumours (93.33%) were >30 mm, and 73.33% were non-ulcerated. Table 1 summarizes the signalment and clinical progression of the 20 dogs.

Previous studies have reported equal prevalence of MCT in both sexes, with an increased incidence in Labrador

Table 1: Signalment and clinical outcomes of 20 dogs diagnosed with cutaneous MCT

No.	Breed	Age (yrs)	Sex	Tumour location	Tumour size	Histological grade	Clinical course
1	Labrador retriever	11	M	hind limb (solitary)	57mm X 50mm	HG	4 month duration before excision; died 2 months after excision
2	Pug	4	M	hind limb (solitary)	23 mm	HG	3 months duration before excision; alive 21 months after excision
3	Beagle	6	M	Back (solitary)	40mm	HG	5 month duration before excision; alive 16 months after diagnosis
4	Labrador retriever	8	M	hind limb (solitary)	35mm	HG	2 months duration before excision; alive 16 months post diagnosis
5	Labrador retriever	9	F	hind limb (solitary)	39 mm	HG	8 month duration before excision; alive 16 months post diagnosis
6	Labrador retriever	10	F	Chest (solitary)	50mm	HG	5 month duration before excision; died 3 months after excision
7	Mongrel	13	F	Neck (solitary)	45mm	HG	3 month duration before excision; alive 16 months post diagnosis
8	Mongrel	9	F	hind limb (solitary)	36mm X 30 mm	HG	6 months duration before excision; alive 16 months post diagnosis
9	Labrador retriever	8	M	hind limb (solitary)	45mm X 40mm	HG	Operated 3 months back; First recurrence excised; alive 15 months post diagnosis
10	Labrador retriever	6	M	hind limb (solitary)	40mm X 50mm	HG	3 month duration before excision; died 2 months after excision
11	Mongrel	8	F	disseminated	23mm to 67 mm	HG	1 year duration before excision; Metastasis suspected; died 5 months after excision
12	Labrador retriever	9	M	disseminated	24mm to 45 mm	HG	4 month duration before excision; metastasis suspected; died 2 months after excision
13	Labrador retriever	7	F	Chest (solitary)	30 mm	HG	2 month duration before excision; died 1 month after excision
14	Spitz	9	F	ventral abdomen (solitary)	45 mm	HG	4 month duration before excision; died 2 months after excision
15	Mongrel	11	F	Chest (solitary)	47mm X 38 mm	HG	9 month duration before excision; alive 3 months after excision
16	Pit Bull	6	M	Chest (solitary)	60mm	HG	4 month duration before excision; died 1 month after excision
17	Labrador retriever	7	M	Abdominal (solitary)	70mm	LG	6 months duration before excision; alive 14 months post diagnosis
18	Mongrel	9	F	Perineum (solitary)	100mm	LG	8 month duration before excision; alive 10 months post diagnosis
19	Mongrel	5	F	hind limb (solitary)	30mm X 40 mm	LG	2 months duration before excision; alive 6 months post diagnosis
20	Mongrel	8	M	hind limb (solitary)	25mmX 30mm	LG	2 months duration before excision; alive 6 months post diagnosis

M: Male, F: Female; HG: High grade, LG: Low grade

retrievers (Pattnaik *et al.,* 1984; Camus *et al.,* 2016). MCTs are most commonly seen in the animals of the age group 6-11 years (Villamil *et al.,* 2011). The most common sites of MCT were hind limbs and trunk regions, with other locations including inguinal regions, head, neck, and abdomen as reported by Bae *et al.,* (2020). Previous studies have associated poorer prognosis with ulceration, erythema, pruritus, and larger tumour size >3cm diameter (Hahn *et al.,* 2004; Mullins *et al.,* 2006). However this study showed no association of tumour ulceration or size with prognosis.

Cytology and Histopathology

The cytoplasmic granulation seen was either dense (Fig. 1a), moderate (Fig. 1b), or poor (Fig. 1c). The cytological/histopathological grading showed that 16/20 were high-grade tumours with poor, moderate or dense granulation and nuclear details like mitotic figures, nuclear pleomorphism, binucleation, and more than 50% anisokaryosis. The low-grade tumours (4/20) showed dense granulation without these features. Most of cases under high Kiupel grade also had high MI. The mitotic figure count varied in high grade tumours in a range of 0 to 20 and in low grade tumours no mitotic figures were seen. Toluidine blue staining revealed that 6/20 cases were poorly granulated with a score 1 (Fig. 1d); 9/20 had a score 2 (Fig. 1e) and 5/20 had a score 3 (Fig. 1f). Surgical margin assessment was not included in our study, as the primary focus was on histological grading and prognostic

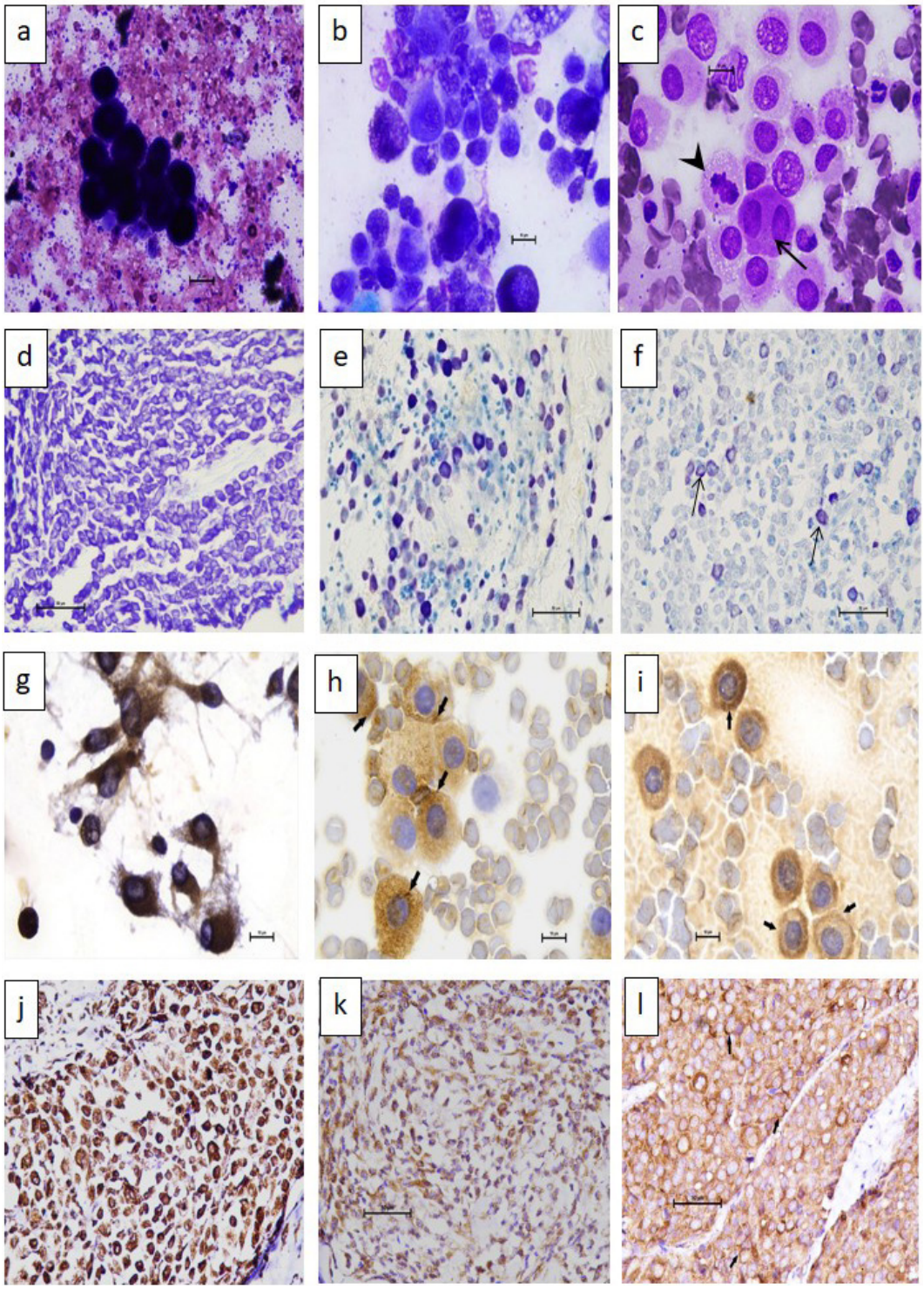


Fig. 1: a-c Fine Needle Aspiration cytology Giemsa stain 100x. a. The tumour cells are well differentiated well granulated and uniformly round discrete cells. b. Moderately granulated pleomorphic round cells. c. Poorly granulated discrete round cells. Binucleated cells (arrow) and mitotic figures (arrow head) are also seen. **d to f Toluidine blue stain 40x.** d. Well differentiated densely granulated neoplastic mast cells (score=3). e. Tumour with admixture of poorly granulated and densely granulated pleomorphic mast cells (score=2); f. Atypical poorly granulated pleomorphic tumour cells (score=1). **Cytological smear** showing positive reactivity for KIT protein. g. showing diffuse and intense cytoplasmic staining pattern of tumour cells for KIT protein; h. showing focal/ stippled cytoplasmic staining pattern (arrows) for KIT protein and i. shows the immunoreactivity for KIT is mainly membrane associated (arrows) ICC DAB chromogen & Mayer's haematoxylin counter stain, 1000X. Immunohistochemical staining shows that most of the cells show diffuse and intense staining in j. k. shows that majority of the cells have a stippled cytoplasmic staining and l. shows that most of the cells showing a membraneous pattern IHC DAB chromogen & Mayer's haematoxylin counter stain, 400X.

factors. However, margin status is recognized to play a crucial role in local recurrence risk and long-term outcomes, despite variability in defining clear versus compromised margins for canine cutaneous MCTs (de Nardi *et al.,* 2022). Notably, low-grade MCTs rarely relapse even with compromised margins (Scarpa *et al.,* 2012), whereas high-grade MCTs may recur despite free margins (Sledge *et al.,* 2016). Sentinel lymph node analysis was attempted when possible, but often limited by inadequate samples or non-palpable nodes.

Toluidine blue is the most sensitive and widely used stain for mast cell granules (Epstein and Sciubba, 2010; Ribbatti, 2018). Staining efficacy depends on pH, with an acidic pH (2.5-3) effectively demonstrating metachromatic granules in connective tissue mast cells and cutaneous mast cell tumors (Grigorev and Korzhevskii, 2021; Rasche *et al*., 2022). The cytoplasmic granulation based on toluidine blue stain score showed a significant difference between the two prognostic groups. A lower toluidine blue stain score, indicating poor granulation or less differentiation, was associated with an unfavorable prognosis, consistent with previous findings in feline mast cell tumors (Sabattini and Bettini, 2010).

Grading of canine cutaneous mast cell tumours (MCTs) was performed using both cytology and histopathology, as cytological evaluation allows better visualization of poorly granulated anaplastic cells due to superior resolution of cytoplasmic granules (Kiupel and Camus, 2019). Although histopathological grading, particularly the Kiupel 2-tier system, is an established prognostic tool for canine cutaneous MCTs (Murphy *et al.,* 2004), the present study found no significant difference in tumour grade between the two prognostic groups (p = 0.068). This finding aligns with previous reports indicating that some high-grade solitary MCTs, based on Kiupel criteria, may have a favourable prognosis or exhibit unpredictable biological behaviour irrespective of grade (Moore *et al.,* 2020). Histological grading has limited prognostic value, particularly for tumours of intermediate differentiation, which behave erratically (Weisse *et al.,* 2002), partly due to inter-pathologist variability in subjective parameters such as cellular/nuclear morphology and invasiveness (Northrup *et al.,* 2005). In our study, most high Kiupel grade cases also had a high mitotic index (MI), and consistent with prior reports that a higher MI was associated with a guarded prognosis (Berlato *et al.,* 2018).

Immunocytochemistry/ Immunohistochemistry

The samples were positive for c KIT immunostaining showing three patterns in ICC and IHC namely diffuse cytoplasmic (Fig. 1g, j) in most of the cases (12/20); focal/stippled (7/20) (Fig.1h, k) and membraneous (1/20) (Fig. 1i, l) patterns.

The cKIT immunostaining revealed three staining patterns - membrane-associated, focal cytoplasmic, and diffuse cytoplasmic - consistent with previous observations (Webster *et al.,* 2006). While cKIT immunostaining is generally considered a useful prognostic factor in canine mast cell tumors (Vascellari *et al.,* 2013), neither the cKIT staining pattern nor the KIT immunostaining score (IS) showed any association with clinical outcome in our study. This finding aligns with earlier reports indicating that KIT staining patterns and aberrant KIT expression lack prognostic relevance (Giantin *et al.,* 2012; Costa Casagrande *et al.,* 2015).

Clinical Course

The tumours were excised marginally in all the twenty cases. Out of the eight dogs that died, two dogs had disseminated tumours; hence only the largest mass was excised. Metastasis was suspected clinically in these two cases. One dog had its tumour excised 3 months prior, and the first recurrence was subsequently removed. The mean survival time in the eight dogs that died was 2.25 months (range, 1 to 5).

Table 2 compares alive and dead groups. Mean age was 8 years in both, with no significant relationship to prognosis (p=0.42). Tumour location (p=0.337), size, multiplicity, and ulceration also lacked prognostic relevance. Logistic regression showed higher odds for males (OR=1.7) and trunk location (OR=1.3), though requiring larger studies. Toluidine blue stain score differed significantly between groups (p=0.035), with poorly granulated (poorly differentiated) cells linked to worse prognosis. Mitotic index (MI) also showed significant association (p=0.012), where higher MI indicated poor prognosis. No significant differences were observed for cytologic/histologic grade, cKIT pattern, or KIT IS.

Table 2: A comparison of various clinicopathological parameters with the outcome in twenty dogs diagnosed with cutaneous mast cell tumors by 24 months of surgery *p<0.05

Parameters	Criteria	Alive (n=12)	Dead (n=8)	P value
Gender	Male: Female	6:6	4:4	1.0
Age years	Mean (range)	8 (6-11)	8 (4-13)	0.42
Location	Limbs	7	2	0.337
	Trunk	4	5	
	Others	1	1	
Size (cm)	<3	2	1	0.394
	>3-5	8	4	
	>5-7	1	3	
	>7	1	0	
Multiplicity	Solitary	11	7	0.76
	Multiple locations	1	1	
Ulceration	Non-ulcerated	10	6	0.648
	Ulcerated	2	2	
Toluidine blue stain score	Well granulated	4	1	0.035*
	Moderate	7	2	
	Poor	1	5	
Histology/cytology Grade	HG	8	8	0.068
	LG	4	0	
cKIT pattern	Stippled	4	3	0.418
	Diffuse	8	4	
	Membraneous	0	1	
cKIT IS	Mean (range)	7.33 (3-12)	8.75 (6-12)	0.077
MI	Mean (range)	0.33 (0-2)	7.13 (2-20)	0.012*

In the present study, survival data and post-treatment information was collected through telephonic interviews with the owners due to the practical limitations of extended follow-up in veterinary studies. Future studies may benefit from in-person clinical re-evaluations to enhance data consistency and reliability.

Conclusion

The canine cutaneous mast cell tumours remain therapeutically challenging due to unpredictable behaviour, with no single reliable prognostic marker to date. The present study identifies mitotic index (MI) and toluidine blue stain scoring as useful, cost-effective prognostic indicators suitable as point-of-care tests. Further research incorporating Ki-67 and KIT mutations across larger cohorts is warranted to validate these findings.

Acknowledgements

The authors are highly thankful for the indispensable assistance and support provided by the Vice Chancellor, DUVASU, Mathura.

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