STEP-BY-STEP PREPARATION PROTOCOL

– Preparation of Unmordanted Acidified Hibiscus Cytoplasmic Counterstain 800

mL

The working volume was calculated based on 142 tissue sections. Assuming 5

mL of stain per section = 710 mL, with an additional 10–15% to compensate for

:losses during extraction, filtration, and handling. Final preparation volume 800

.mL

Phase A: Aqueous Extraction

Weighing: Accurately weigh 80.0 g of dried, finely ground Hibiscus sabdariffa

.calyces using an analytical balance

Extraction: Transfer the ground material into a heat-resistant 1 L flask. Add 800.0

mL of distilled water. Ratio 1:10 w/v

Heating: Place the flask on a hot plate with a magnetic stirrer. Heat the mixture

at 80–90°C with continuous stirring for 15–20 minutes until a deep ruby-red

extract is obtained.

Cooling & Filtration: Allow the extract to cool to room temperature (20–25°C).

Filter the mixture through Whatman No. 1 filter paper into a clean volumetric

flask to obtain a clear, particulate-free dye solution.

Volume Adjustment: Adjust the final volume to 800 mL with distilled water if

necessary.

Phase B: Compounding the Cytoplasmic Counterstain Solution

Measure Extract: Measure 800.0 mL of the filtered hibiscus extract.

Acidification: Add 16.0 mL of Glacial Acetic Acid gradually with stirring. This

lowers the solution pH to approximately 2.5 – 3.0. Acidification increases the

positive charge on cytoplasmic proteins, ensuring selective binding of the dye.

Stabilization: Add 40.0 mL of Glycerol. Mix thoroughly. Glycerol enhances color

retention and solution stability.

Storage & Labeling: Pour the finalized solution into an amber glass bottle. Label

clearly as Unmordanted Acidified Hibiscus Cytoplasmic Counterstain with the

preparation date.

Storage Conditions: Store at 4°C. Use within one week of preparation. Measure

and record the pH of the final staining solution before use.

Note: If preliminary staining results indicate insufficient staining intensity, the concentration may be adjusted to 1:5 w/v.

HISTOPATHOLOGICAL STAINING PROCEDURES

 Routine Hematoxylin & Eosin (H&E) Staining – Control Group

For formalin-fixed paraffin-embedded (FFPE) tissue sections cut at 3–5 µm:

Deparaffinization: Clear slides in 2 changes of Xylene, 2 minutes each.

Rehydration: Hydrate in Absolute Alcohol for 10 dips. Then in 2 changes of 95%

Alcohol, 10 dips each.

Rinsing: Rinse in running tap water until the water runs off evenly.

Nuclear Staining: Stain nuclei in Mayer’s Hematoxylin for 15 minutes.

Rinsing: Rinse in 2 changes of tap water, 10 dips each.

Blueing: Blue in 0.25% Ammonia Water or 0.5% Lithium Carbonate until nuclei

turn blue.

Rinsing: Rinse again in 2 changes of tap water, 10 dips each.

Cytoplasmic Counterstaining: Counterstain in 1% Alcoholic Eosin for 10–20 dips.

Differentiation: Differentiate in 70% Alcohol for 10–15 dips.

Dehydration: Dehydrate in 2 changes of 95% Alcohol, followed by 2 changes of

Absolute Alcohol, 10 dips each.

Clearing: Clear in 2 changes of Xylene, 2 minutes each.

Mounting: Mount with DPX mounting medium and apply a glass coverslip.

 Hematoxylin & Hibiscus (H&H) Staining – Test Group

The same procedure as H&E, except that the Hibiscus sabdariffa aqueous

extract is used instead of Eosin as the cytoplasmic counterstain.

Deparaffinization: 2 changes of Xylene, 2 minutes each.

Rehydration: Absolute Alcohol 10 dips. Then 2 changes of 95% Alcohol, 10 dips

each.

Rinsing: Running tap water until water runs off evenly.

Nuclear Staining: Mayer’s Hematoxylin for 15 minutes, or Acidified Harris

Hematoxylin for 1–3 minutes.

Rinsing: 2 changes of tap water, 10 dips each.

Blueing: 0.25% Ammonia Water until nuclei turn blue.

Rinsing: 2 changes of tap water, 10 dips each.

Cytoplasmic Counterstaining: Immerse slides in the prepared Hibiscus

Counterstain for 2 to 5 minutes. Start with 3 minutes and adjust according to

desired intensity.

Quick Rinsing: Quick rinse in distilled water, 2–3 dips only. Do not over-rinse.

Differentiation & Fixation: Differentiate and fix the stain in 70% Alcohol, 10–15

dips.

Dehydration: Dehydrate rapidly in 2 changes of 95% Alcohol, then 2 changes of

Clearing: 2 changes of Xylene, 2 minutes each.

Mounting: Mount with DPX and apply a coverslip.