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APPENDICES

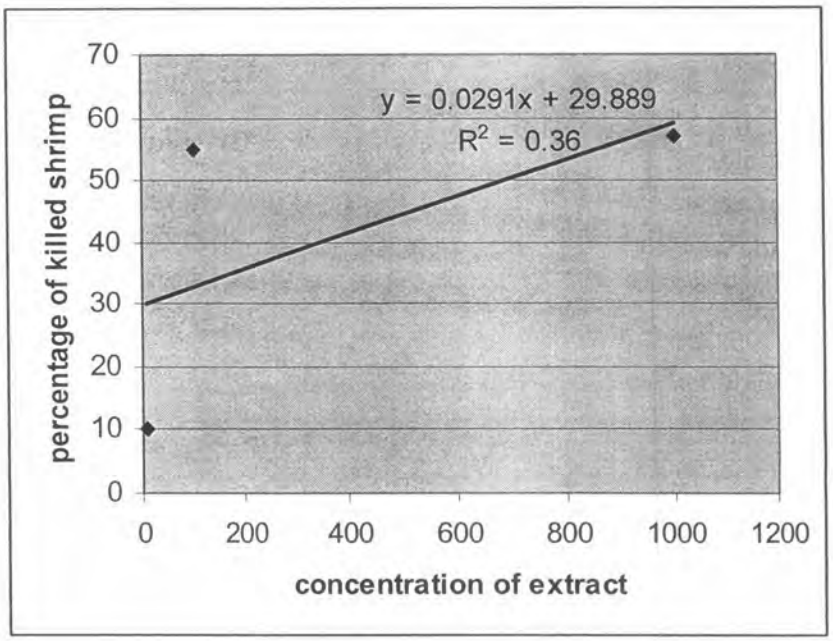
APPENDIX A
BRINE SHRIMP ASSAY

Component of artificial sea water

Component	**mmole/kg**
Na^+	444.64
K^+	9.52
Mg^{++}	50.09
Ca^{++}	8.16
Sr^{++}	0.08
Cl^-	508.50
SO_4^{2-}	27.19
Br^-	0.74
F-	0.07
I-	0.01
HCO_3-(CO_2+CO_3^{2-})	2.0-2.5
$B(OH)_3$+$B(OH)_4$-	0.43
$Si(OH)_4$+$SiO(OH)_3$-	0.01-0.1
H_2PO_4-+HPO_4^{2-} +PO_4^{2-}	nil
NO_3-	nil

The LC_{50} values of the extracts were obtained by a plot of percentage of the shrimp nauplii killed against the concentrations of the extracts and the best-fit line was obtained from the data by means of regression analysis (Krishnaraju *et al.*, 2005).

Example LC_{50} of Methanol extract from ixora



X= (50-29.889)/0.0291

X =686 µg/ml

APPENDIX B

PREPARATION OF REAGENTS FOR ANTIOXIDANT ASSAY

DPPH Reagent:

Chemicals

1. 150 μM DPPH$^{\bullet}$ (2,2'-diphenyl-1-picrylhydrazl) in 80% Methanol
2. 1.28 mM Trolox in 80% Methanol

   Standard Trolox was run in triplicate using several concentrations. (1.28, 0.64, 0.32, 0.16, 0.08 mM)

FRAP Reagent:

Chemicals

1. 300 mM Acetate buffer (pH 3.6)

   (3.1 g of sodium acetate trihydrate ($C_2H_3NaO_2.3H_2O$) plus 16 ml glacial acetic acid and made up to 1 L with distilled water.)
2. 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM HCl
3. 20 mM $FeCl_3.6H_2O$

   Mixing the reagent from 1-3 before use and heated to 37 °C

   300 mM Acetate buffer: 10 mM TPTZ solution: 20 mM $FeCl_3.6H_2O$ (ratio 10:1:1)
4. 1000 μM $FeSO_4.7H_2O$

   Standard $FeSO_4.7H_2O$ was run in triplicate using several concentrations. (1000, 500, 250, 125 and 62.5 μM)

Phenolics Reagent:

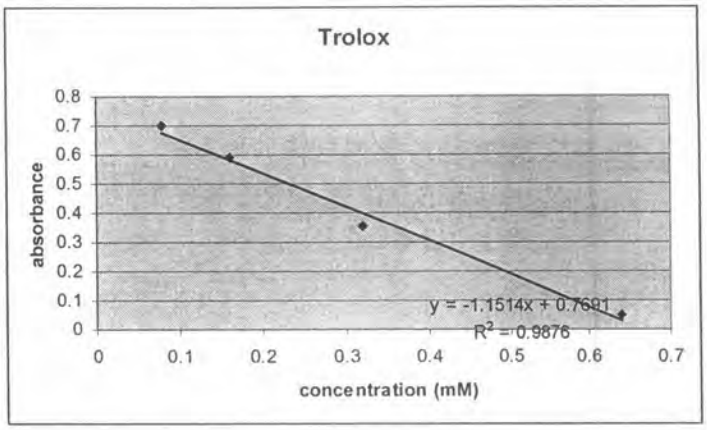
Chemicals

1. Folin-Ciocalteu reagent
2. Saturated sodium carbonate solution
3. 800 mg/l Gallic acid

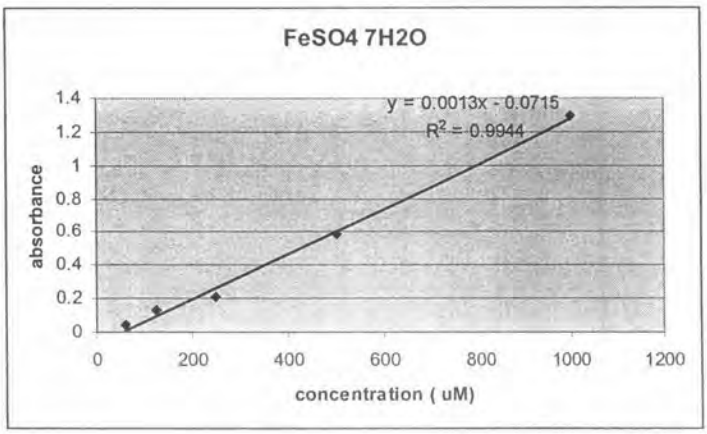
   Standard Gallic acid was run in triplicate using several concentrations. (800, 400, 200, 100, 50, and 25 mg/l)

Standard curve of antioxidant assay and total phenolic content

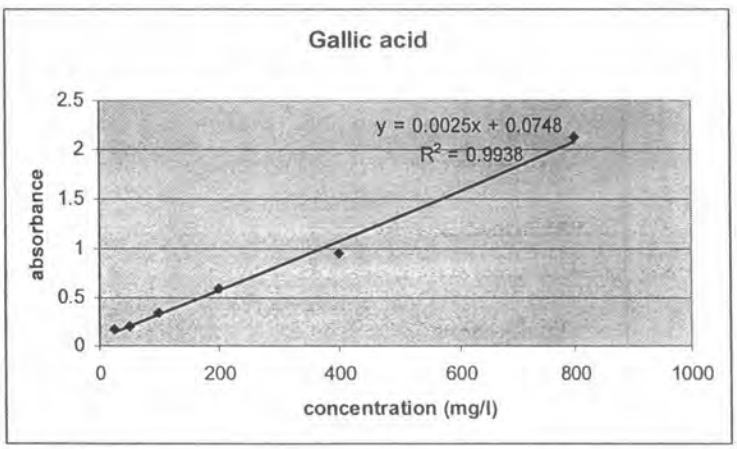
1. DPPH assay



2. FRAP assay



3. Total phenolic content



APPENDIX C

MANIPULATION OF THE TESTER STRAINS

1. Preparation of Stock Solution and Media (Maron and Ames, 1983)

1.1 Vogel-Bonner medium E stock salt solution (VB salt)

Use: Minimal agar

Ingredient	per liter
Distilled water	670 ml
Magnesium sulfate ($MgSO_4.7H_2O$)	10 g
Citric acid monohydrate	100 g
Potassium phosphate, dibasic (anhydrous) (K_2HPO_4)	500 g
Sodium ammonium phosphate ($NaNH_4HPO_4.4H_2O$)	175 g

Add salts in the order indicated above to warm water in a 2-liter beaker or flask placed on a magnetic stirring hot plate. Allow each salt to dissolve completely before adding the next. Adjust the volume to 1 liter. Filter the solutions and autoclave at 121°C for 20 min.

1.2 Minimal glucose agar plate

Use: Mutagenicity assay

Ingredient	350 ml
Bacto agar	5.25 g
Distilled water	330 ml
VB salts	7 ml
40% glucose	17.5 ml

Add Bacto agar to distilled water in a glass bottle. Autoclave at 121°C for 20 min. When the solution has cooled slightly, add sterile VB salts and sterile 40% glucose. After all the ingredients have been added, the solution should be stirred thoroughly. Pour 30 ml into each sterile petri dish. Keep in an incubator at 37°C for 48°C before using.

1.3 Oxoid nutrient broth No.2

Use: Growing culture

Ingredient	100 ml
Nutrient broth no. 2	2.5 g

Distilled water	100 ml

Dissolve nutrient broth no. 2 in distilled water. Mix it until dissolve completely. Transfer 12 ml of Nutrient broth for each 50ml Erlenmeyer flask (covered with sterile gauze). Autoclave at 121°C for 20 min.

1.4 Top agar

Use: Mutagenicity assay

Ingredient	100 ml
Bacto agar	0.6 g
Sodium chloride (NaCl)	0.5 g
Distilled water	100 ml

Dissolve ingredients in distilled water. Store the solution in a glass bottle. Autoclave at 121°C for 20 min and add 10 ml of a sterile solution of 0.5mM L-histidine/ biotin and mixed thoroughly by swirling.

1.5 0.1 M L-histidine HCl stock

Use: Preparation of 1mM L-histidine HCl stock

Ingredient	100 ml
L-histidine HCl	2.096 g
Distilled water	100 ml

Dissolve L-histidine HCl (MW 209.63) in distilled water. Autoclave at 121°C for 20 min. Store the solution in a glass bottle at 4°C until use.

1.6 1 mM L-histidine HCl stock

Use: Preparation of 0.5mM L-histidine/ biotin solution

Ingredient	100 ml
0.1 M L-histidine HCl	1 ml
Distilled water	99 ml

Dilute 1 ml of 0.1M L-histidine HCl in 99 ml of distilled water and autoclave at 121°C for 20 min.

1.7 1 mM biotin stock

Use: Preparation of 0.5mM L-histidine/ biotin solution

Ingredient	100 ml
Biotin	24.43 mg
Distilled water	100 ml

Dissolve biotin (MW 244.3) in distilled water. Warm it until dissolve completely. Autoclave at 121°C for 20 min.

1.8 0.5 mM L-histidine HCl-0.5 mM biotin

Use: Mutagenicity assay (add 10 ml to 100 ml of Top agar)

Ingredient	200 ml
1 mM L-histidine HCl	100 ml
1 mM biotin	100 ml

Mix ingredients and autoclave at 121°C for 20 min.

1.9 1 M potassium chloride (KCl)

Use: Preparation of 152mM Na_3PO_4-KCl buffer

Ingredient	100 ml
KCl	7.456 g
Distilled water	100 ml

Mix ingredients and autoclave at 121°C for 20 min.

1.10 0.5 M sodium dihydrogen phosphate (NaH_2PO_4)

Use: Preparation of 0.5M sodium phosphate pH 7.4

Ingredient	500 ml
NaH_2PO_4 (MW120)	30 g
Distilled water to	500 ml

Dissolve NaH_2PO_4 in distilled water. Stir it until dissolve completely. Adjust the final volume to be 500 ml.

1.11 0.5 M sodium phosphate (Na_3PO_4) pH 7.4

Use: Preparation of Na_3PO_4-KCl buffer

Ingredient	500 ml
Disodium hydrogen phosphate dehydrate ($Na_2HPO_4 \cdot 2H_2O$)	44.5 g
Distilled water to	500 ml

Dissolve $Na_2HPO_4 \cdot 2H_2O$ in 300 ml of distilled water. Add 0.5 M NaH_2PO_4 until to pH 7.4, and then adjust the final volume to be 500 ml. Autoclave at 121°C for 20 min.

1.12 152 mM Na_3PO_4-KCl buffer

Use: Mutagenicity assay

Ingredient	330 ml
0.5M Na_3PO_4 pH 7.4	100 ml
1M KCl	16.5 ml
Distilled water	213.5 ml

Mix ingredients and autoclave at 121°C for 15 min.

1.13 8 mg/ml ampicillin solution

Use: Tests of ampicillin resistance (to confirm R-factor strains)

Ingredient	10 ml
Ampicillin (sodium)	80 mg
Distilled water	10 ml

It necessary to sterilize ampicillin solutions but they can be filtered through a 0.22 μm membrane filter. Store it in a glass bottle with a screw cap at 4°C.

1.14 0.1% crystal violet

Use: Tests for crystal violet sensitivity (to confirm *rfa* mutation)

Ingredient	10 ml
Distilled water	10 ml
Crystal violet	10 mg

Store at 4°C in a glass bottle with a screw cap. Wrap the bottle with metal foil to protect against light.

2. Recipes for Some Reagents

2.1 2 M sodium nitrite

Use: Nitrosation

Ingredient	10 ml
Sodium nitrite	1.38 g
Distilled water to	10 ml

Store in a glass bottle with a screw cap (wrap the bottle with metal foil to protect against light). Autoclave at 121°C for 20 min.

2.2 2 M ammonium sulfamate

Use: Reaction mixture

Ingredient	10 ml
Ammonium sulfamate	2.28 g
Distilled water to	10 ml

Dissolve ammonium sulfamate in distilled water and adjust the final volume to be 10 ml. Autoclave for 20 min at 121°C.

2.3 0.2 N hydrochloric acid

Use: Reaction mixture

Ingredient	100 ml

Conc. Hydrochloric acid	1.66 ml
Sterile distilled water	98.34 ml

Dissolve conc. hydrochloric acid in sterile distilled water. Store in sterile glass bottles with screw caps.

Note: Preparation of 0.2 N hydrochloric acid must be used sterile technique because hydrochloric acid can not be autoclaved.

2.4 0.0375 mg/ml 1-aminopyrine

Use: Standard solution for mutagenicity assay

Ingredient	2 ml
0.3 mg/ml 1-aminopyrine	250 µl
Acetonitrite	1,750 µl

Dissolve 3 mg of 1-aminopyrine in 300 µl of acetonitrite and mix; and subsequently dilute 300 µl of this solution (3 mg/ml 1-aminopyrine) in 2,700 µl of acetonitrite, the solution obtained will be 0.3 mg/ml 1-aminopyrine. Then, dilute 250 µl of 0.3 mg/ml 1-aminopyrine in 1,750 µl of acetonitrite and mix. Store all solutions in sterile glass vials with screw caps in a freezer. The preparation must be used sterile technique.

3. Procedure for Reisolation and Growing Culture (Maron and Ames, 1983)

The tester strains (TA98 and TA100) are grown in oxoid nutrient broth no.2 and incubate overnight at 37°C in a shaking water bath. The rate of rotation should be decreased to about 120 rpm to avoid foaming and the growth period should not exceed 16 h. These cultures are reisolated by streaking the bacteria on minimal glucose agar plates enriched with 0.1 ml of 8 mg/ml ampicillin, 0.3 ml of 0.1M histidine HCl and 0.1 ml of 1mM biotin. Incubate at 37°C for 48 h. With a sterile wire loop, pick a well-isolated colony for overnight growth in oxoid nutrient broth no.2 at 37°C in a shaking water bath. Each culture is confirmed genotypes of the strains and keep the cultures as the source of bacteria for mutagenicity testing. For each 1 ml of culture, add 0.09 ml of spectrophotometric grade DMSO. Combine the culture and DMSO in a sterile tube. Swirl gently until the DMSO is dissolved and distribute 200 µl of the culture aseptically into sterile cryotube. Store the tubes in a freezer at -80°C.

4. Confirm Genotype of Tester Strains

The broth culturesnof strains TA 98 and TA 100 are used to confirm genotypes in the following ways.

4.1 Histidine requirement

The his$^-$ character of the strains is confirmed by demonstrating the histidine requirement for growth on selective agar plate.

Procedure:

plate a	no histidine and biotin
plate b	0.1 ml of 1 mM biotin
plate c	0.3 ml of 0.1 M his-HCl
plate d	0.3 ml of 0.1 M his-HCl + 0.1 ml of 1 mM biotin

Four minimal glucose agar plates are required for each tester strain. Apply 0.1 ml of 1 mM biotin, 0.3 ml of 0.1 M his-HCl and 0.3 ml of 0.1 M his-HCl plus 0.1 ml of 1 mM biotin on the surface of minimal glucose agsr of plate b, c and d, respectively; and no application for plate a. Make a single streak of each strain across these plates and incubate at 37 °C for 24 hours. Four strains could be tested on the same plate. The growing of bacteria on histidine plus biotin plate (plate d) is the result of histidine requirement.

4.2 R-Factor

The R-factor strains (TA 97, TA 98, TA 100 and TA 102) should be tested routinely for the presence of the ampicillin resistance factor because the plasmid is somewhat unstable and can be lost from the bacteria. For this test, nutrient agar plates are seeded with cultures of the strains to be test and a sterile filter paper disc (1/4 inch) containing ampicillin is placed on the surface of each seeded plate.

Procedure: For each tester strain (TA 98 and TA 100), add 0.3 ml of fresh overnight culture to a tube containing 0.1 ml of 0.1 M histidine-HCl. And then add 2 ml of molten top agar containing 0.5 mM histidine-HCl and 0.5 mM biotin. Vortex and pour on a minimal agar plate. The plate was rotated in order to distribute the mixtures and allowed several minutes for agar to become firm. R-factor and *rfa* mutation (see the next section) are performed in the same plate by dividing the plate into 2 parts, one for R-factor and the other for *rfa* mutation. For R-factor, filter paper disc containing 8 mg/ml solution of ampicillin is applied on the surface of the agar by using sterile forceps. Incubate at 37°C for 24 hours. The absence of the clear zone of inhibition around the indicates resistance to ampicillin.

4.3 *rfa* mutation

Strains having the deep rough (*rfa*) character should be tested for crystal violet sensitive. The method of this test is similar to the test of ampicillin resitance.

Procedure: Pipet 10 μl 0f 0.1 % solution of crystal violet to the center of sterile filter paper disc and transfer one disc to each of the seeded plates using sterile forceps. The following procedures are similar to the R-factor testing described above. The clear zone appeared around the disc indicate the presence of the *rfa* mutation that crystal violet is transferred into the cell and kill bacteria.

5. Spontaneous Reversion

Spontaneous reversion of the tester strains to histidine independence is measured routinely in mutagenicity experiments and is expressed as the number of spontaneous reveryants per plate. The revertant colonies are clearly visible in a uniform background lawn of auxotrophic bacteria. Each tester strain reverts spontaneously at a frequency that is characteristic of the strain. Nevertheless, there is variability in the number of spontaneous revertants from one experiment to another and from one plate to another, and it is advisable to include at least 2-3 spontaneous mutation control plates for each strain in a mutagenicity assay.

Procedure: Add 0.1 ml of DMSO to capped culture tube. Then add 0.5 ml of $NaPO_4$-KCl buffer pH 7.4 and 0.1 ml of fresh overnight culture of TA 98 or TA 100. Incubate the mixture in a shaking water bath at37°C for 20 min. After that 2.0 ml of top agar is added to the mixture. Mix and pour on the minimal glucose agar plate. Plates are rotated and left it to become harden. Incubate at °C for 48 h. The his^+ revertants colonies that grow on the minimal glucose agar plate are counted.

6. The Response to Standard Mutagen

Standard mutagens or positive mutagens are used routinely in mutagenicity experiments to confirm the reversion property and specificity of each strain. The standard mutagen, which used in this experiment, was nitrosated-aminopyrene. Tester strains that highly response to positive mutagens will be chosen.

Procedure: 0.02 and 0.04 ml of 0.0375 mg/ml aminopyrene in acetonitrile were pipetted to sterile capped tube. Then, 0.73 and 0.71 ml of 0.2 N HCl were added respectively, and followed by 0.25 ml of 2 M $NaNO_2$. The final concentrations of aminopyrene were 0.6 and 1.2 mg respectively, and the final concentration of nitrite

was 0.5 M. The solution was mixed and shaked in water bath at 37°C for 4 hours. The tube was placed in an ice bath and 0.25 ml of 2 M ammonium sulfamate ($NH_2SO_3NH_4$) was added and standed for 10 min in ice bath. 0.1 ml of each mixture was pipetted to cap culture tube for testing the stock culture TA 98 (equal to 0.06 mg of aminopyrene/plate) and TA 100 (equal to 0.12 mg of aminopyrene/plate). Then, the evaluation of their mutagenicity was tested as described in spontaneous reversion. The characteristic properties of the stock culture for TA 98 and TA 100 as the source of bacteria for mutagenic testing are:

1. Contain R-factor (pKM 101) and *rfa* mutation.
2. His^+ requirement.
3. Low spontaneous reversion.
4. Highly response to standard carcinogen.

The experiment was performed only when the characteristic properties of bacteria strain were done.

APPENDIX D
STATISTICAL CONSIDERATION

In experiments designed to assess the mutagenicity of a chemical, most often a treatment series were compared with a control series. One might like to decide whether the compound used in the treatment should be considered as mutagenic or non-mutagenic. The formulation of 2 alternative hypotheses allowed one to distinguish among the possibilities of a positive, inconclusive, or negative result of an experiment.

In the null hypothesis one assumes that there was no difference in the mutation frequency between control and treated series. Rejection of the null hypothesis indicated that the treatment resulted in a statistically increased mutation frequency. The alternative hypothesis postulated a priory that the treatment results in an increased mutation frequency compared to the spontaneous frequency. The alternative hypothesis was rejected if the mutation frequency was significantly lower than the postulated increased frequency. Rejection indicates that the treatment did not produce the increase requires to consider the treatment as mutagenic. If neither of the 2 hypotheses was rejected, the results were considered inconclusive, as one could not accept at the same time the 2 mutually exclusive hypotheses. In the practical application of the decision procedure, one defines a specific alternative hypothesis requiring the mutation frequency in the treated series be *m* times that in the control series and used together with the null hypothesis. It might happen in this case that both hypotheses had to be rejected. This should mean that the treatment was weakly mutagenic, but led to a mutation frequency which was significantly lower than *m* times the control frequency.

Testing against the null hypothesis (H_O) at the level α and against the alternative a hypothesis (H_A) at the level β led to the error probabilities for each of the possible diagnoses: positive, weakly but positive, negative, or inconclusive. The following four decisions were possible; 1) accept both hypotheses; these can not be true simultaneously, so no conclusions can be drawn--inconclusive result; 2) accept the first hypothesis and reject the second hypothesis--negative result; 3) reject the first hypothesis and accept the second hypothesis--positive result; 4) reject both hypotheses --weak effect (Frei and Würgler, 1988).

Calculation step by step

Estimation of spot frequencies and confidence limits of m_e

Particularly in the case that both hypotheses, H_O as well as H_A, had to be rejected, one might be interested in knowing the confidence interval of m_e, i.e., of the estimated multiple by which the mutation frequency in the experimental series was larger than the spontaneous frequency. The estimated value was

$$m_e = \frac{(n_t / n)\, N_c}{(n_c / n)\, N_t}$$

Where N_c and N_t represented the respective sample sizes in control and treatment series, n_c and n_t the respective numbers of mutations found, and n the total of mutations in both series together. Exact lower and upper confidence limits p_l and p_u for the proportion n_c/n on one hand, as well as q_l and q_u for the proportion n_t/n on the other hand, may be an easy method to calculate these values using an F-distribution table. To determined q_l and p_u one-sidedly at the level α, and q_u and p_l also one-sidedly at the level β. In this way and in agreement with the foregoing section, a confidence limit $m_l > 1$ led to rejection of H_o, while a confidence limit $m_u < m$ led to rejection of H_A.

In the first step, F-distribution were used to determine the value $F_{\nu l, \nu 2}$ at the level $\alpha = 0.05$, where the degrees of freedom (ν_1, ν_2) were given by the equations

$$\nu_1 = 2\,(n - nt + 1) \text{ and } \nu_2 = 2n_t$$

In the second step, the F-value so obtained was used to calculate the lower confidence limit (q_l) for the proportion of spots in the experimental series

$$q_l = n_t / [n_t + (n - n_t + 1)\, F_{\nu l, \nu 2}]$$

This gave a lower confidence limit for the frequency of spots per wing in the control, which was equal to

$$f_{t,l} = q_l n / N_c$$

This was the following complementarily, namely that the lower confidence limit for the number of spots in the experimental series ($q_l n$) plus the upper confidence limit for the number of spots in the experiment ($p_u n$) was equal to the total number of spots (n) found in experimental and control series together, i.e.,

$$P_u n = (1 - q_l)\, n$$

This gave an upper limit for the frequency of spots per wing for the control, which is

$$f_{c,u} = p_u n/N_c$$

The lower confidence limit m_l of the multiple m_e was determined as the ratio between the lower confidence limit for the frequency in the treated series and the upper confidence limit for the frequency in the control, i.e.,

$$m_l = \frac{f_{t,l}}{f_{c\,u}} = \frac{q_l\, n/N_t}{p_0 n/N_c}$$

Only in the case that m_l, the lower confidence limit of m_e, was larger than 1.0 would reject H_o. Since this was not the case, H_o remains accepted.

In the same way, the lower confidence limit of the spot frequency may be determined in the control $f_{c,l}$ which will give $f_{t,u}$, the upper confidence limit of the spot frequency in the experimental series. This is also done one-sidedly, at the level $\beta = 0.05$. The inverse ratio of these values will provide the upper 5% confidence limit m_u for the multiple m_e.

Again, the F-distribution was used and determined the value $F_{v1,v2}$ at the level $\beta = 0.05$, where the degrees of freedom $(_{v1,v2})$ were given by the equations

$$v_1 = 2(n\text{-}n_c + 1) \text{ and } v_2 = 2\, n_c$$

The F-value so obtained was used to calculate the lower confidence limit (p_l) for the proportion of spots in the control

$$P_l = n_c / [n_c + (n\text{-}n_c + 1)\, F_{v1,v2}]$$

This gave a lower confidence limit for the frequency of spots per wing in the control, which equal to

$$f_{c,l} = p_l n/N_c$$

Again, there was complementarily, in that the lower confidence limit for the number of spots in the control ($p_l n$) plus the upper confidence limit for the number of spots in the experiment ($q_u n$) was equal to the total number of spots (n), so that

$$q_u n = (1\text{-}p_l)n$$

This gave an upper limit for the frequency of spots per wing for this series, which is

$$f_{t,u} = q_u n/N_t$$

The upper confidence limit m_u of the multiple m_e can be determined as the ratio between the upper confidence limit for the frequency in the treated series and the lower confidence limit for the frequency in the control, i.e.,

$$m_u = \frac{f_{t,u}}{f_{c,l}} = \frac{q_u\, n/N_t}{p_l n/N_c}$$

H_A was rejected if m_u, the upper confidence limit of m_e, was less than m (m=2 for the total of all spots and for the small single spots, and m=5 for the large single spots as well as for the twin spots). Substitution of m_e by m_l or m_u in the above formulas provided the respective exact upper and lower confidence limits for the frequencies estimated.

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