CODEN (USA)-IJPRUR, e-ISSN: 2348-6465

Original article

In-vitro Mutagenicity Study of Kumkumadi Taila and Kanakataila using the Ames Test

Venkataramana Sridhar[1], Jayagopalan Veena[1*], Donna Webster[2], Nagesh N C[1], Atanu Halder[1]

[1]HNL Business Services India Private Limited Prestige Shantiniketan, Mahadevapura Post, Bengaluru, India
[2]Herbalife Nutrition, 950 West 190th Street, Torrance CA 90502, United States

ARTICLE INFO:
Received: 05 Apr 2025
Accepted: 28 Apr 2025
Published: 30 Apr 2025

Corresponding author *
Dr. Jayagopalan Veena
Senior Manager, Scientific Affairs
HNL Business Services India Private Limited
Email: veenaj@herbalife.com

ABSTRACT:

Objective: Mutagenic characteristics of two Ayurvedic oils, Kumkumadi taila and Kanaka taila were evaluated as per OECD - 471 methodology of Ames test

Experimental approach:The oils were primarily authenticated using High-Performance Liquid Chromatography (HPLC) coupled with a photodiode array (PDA) detector by confirming the presence of glabridin in Kanaka Taila and both glabridin and safranal in Kumkumadi Tailas. Mutagenicity was assessed by exposing the oils to bacterial tester strains—TA1537, TA100, TA1535, and TA98 of Salmonella typhimurium, and WP2uvrA (pKM101) strain of Escherichia coli—at a maximum concentration of 5000 µg/plate, both with and without metabolic activation using the S9 fraction (prepared from Aroclor 1254-induced rat liver). Negative control (Dimethyl sulfoxide, DMSO) and appropriate positive controls (2-aminoanthracene, 2-nitrofluorene, sodium azide, 9-aminoacridine, and 4-nitroquinoline-1-oxide) were tested in parallel.Findings and discussion: The chromatographic peak observed in Kanaka Taila matched the glabridin reference standard. In Kumkumadi Taila, peaks corresponding to both glabridin and safranal were identified and confirmed against reference standards, thereby validating the authenticity of the test samples. At concentrations up to the maximum of 5000 µg/plate, neither test substance induced a two-fold increase in the mean number of revertant colonies nor a three-fold increase in strains, in either the presence or absence of the metabolic activation system. These findings were consistent across both the initial and confirmatory tests when compared with the respective negative controls.

Conclusion: On the basis of study conditions and assay results, it could be concluded that the test materials did not exhibit any mutagenic activity.

Keywords: Kumkumadi Thailam, Kanaka Thaila, OECD, mutagenicity, Glabridin, Safranal.

1. INTRODUCTION

The growing interest in global application of plant-based or herbal-based cosmetics is attributed to the rise in consumer awareness of traditional use and history along with advantage sassociated with use of natural ingredients, including belief of less side effects[1,2].Natural ingredients have been historically used in the science of cosmetics, which was mostly constituted from botanical and mineral sources. The dermatological and cosmetic rituals practices in the ancient world have paved way for the current revolution including natural ingredients in cosmetic products[3]. Natural ingredients have been used traditionally for millennia and the concept of using herbs for beautification is also well defined in Ayurveda, which is regarded as one of the oldest medical sciences known to man[4].Herbal oils are amongst the ingredients used in the cosmetics industry for skin health/benefits, some of which also find a special mention in ancient texts of Ayurveda as *Taila* (~oils) and *Ghrita* (~clarified butter or ghee) used for facial beautification.They are generally composed of selective extracts, single molecules purified from extracts, or a mixture of multiple extracts[5-7].

*Kanaka taila*is a classical Ayurveda oil formulation for skin described in *Chakradatta*, one of the treatise composed by Shri Chakrapanidatta[8]. The topical application utilities of this oil have been indicated briefly in the chapter named '*Kshudraroga'* for *Nilika* (~blackish patches) and *Vyanga* (facial eruptions)[9-11].

Another oil that is commonly described in ancient Ayurvedic texts is *Kumkumadi taila*. This*taila*is mentioned in ancient Ayurvedic texts such as *Ashtanga Hridaya*, *Bhaishjya Ratnavali*, *Yogaratnakara*, and also described in *Chakradatta*[8,12-14]. According to these texts, continuous use of *Kumkumadi taila* is believed to enhance complexion, giving it a golden glow and improving facial appearance. *Kumkuma* is the Sanskrit name for saffron (*Crocus sativus* L.).According to most Ayurveda books, this formulation typically contains saffron, along with the rich blend of other renowned Ayurvedic herbs, processed with sesame oil and milk. *Kumkumadi taila*is anAyurvedic recipe that has been

used for more than 5000 years in India as an integral part of a traditional beauty regime indicated for imparting young, healthy and radiant skin[8,12-14]

One challenge in utilizing traditional Ayurvedic oils such as *Kumkumadi taila* and *Kanakataila* in commercially available cosmetic products is the lack of available documented safety data. However, the products formulated with these oils have shown to offer substantial benefits for all skin types, effectively tackling various skin concerns. Clinical studies indicate significant improvements in patch color, as well as reductions in both the intensity and number of patches, with no reported adverse effects. Additionally, there are no studies looking into the mutagenicity, which would be next in line for the toxicology evaluation. Therefore, these oils were subjected to Amestest to evaluate any possible mutagenicity potentialin order to strengthen the data supporting safeuse of these oils in cosmeceutical preparations or herbal cosmetics.

2. MATERIAL AND METHODS

A. Test substances/Ingredients:

The detailed composition for the both*Kanaka taila* and *Kumkumadi taila* is presented in Table 1A and 1B, respectively**.**

B. Chromatographic conditions for determining markers for each of the oils

a. Glabridin in *Kanaka taila*

The mobile phase consisted of two solvents, A and B. Solvent A was prepared by dissolving 0.136 g of anhydrous potassium dihydrogen orthophosphate (KH_2PO_4) in 900 mL of HPLC grade water and 0.5mL of orthophosphoric acid. This was made upto 1000 mL with water, filtered through 0.45 µ membrane and degassed in a sonicator for 3 minutes. Acetonitrile (Solvent B) was prepared at different gradients. The diluent used in the experiment was 1:1:1 of n-Hexane, Isopropyl alcohol and Methanol, respectively. The instrument included the column (Phenomenex- Luna 2.5µ C-18(2)HST Size: 100x3 mm,2.5-micron) with photo diode array detector. Glabridin standard was used at the concentration 0.002mg/ml of glabridin in diluent. The test sample was prepared by diluting 8-10mg/ml of *Kanaka taila*, sonicating it for 10 minutes and filtered through 0.45µm PES filters. Twenty µL of the standard preparation (injection volume: 5µl and flow rate: 0.4 ml/min) was injected and recorded. This was performedin triplicates and, the mean area and the Relative Standard Deviation (RSD) were calculated. The RSD should not be more than 2.0%. 5 µL of sample preparation was injected and chromatogram peaks were recorded.

b. Glabridin and safranal in the *Kumkumadi taila*

In the experiment, the mobile phase consisted of two solvents, A and B. Solvent A was prepared by dissolving 0.136 g of anhydrous potassium dihydrogen orthophosphate (KH_2PO_4) in 900 mL of HPLC grade water and 0.5mL of orthophosphoric acid. This was made up to 1000 mL with water, filtered through 0.45µm membrane and degassed in a sonicator for 3 minutes. Acetonitrile (Solvent B) was prepared at different gradients. The diluent used in the experiment was n-Hexane, Isopropyl alcohol, and Methanol in 1:1:1 ratio. The instrument included the column (Phenomenex- Luna 2.5µ C-18(2)HST) with a photo diode array detector or for glabridin identification and Phenomenex- Luna 5µ C-18(2) column with photo diode array detector for safranal. Glabridin and safranal standards was used at concentrations 0.002mg/ml and 0.01 mg/ml, respectively in diluent.

The test samples were prepared by diluting 10-12 mg/ml of *Kumkumadi taila* in diluent, sonicating for 10 minutes and filtering through 0.45µm PES filters. Reference standards were injected (with injection volume: 5µl, flow rate: 0.4 ml/min for glabridin identification and with injection volume: 20µl, flow rate: 1 ml/min for safranal identification) and peaks were recorded. Each standard sample was injected in triplicate, and the mean area and the RSD was calculated. The RSD should not be more than 2.0%. 20 µL of test sample preparation was injected, and recorded for glabridin and safranal chromatogram peaks.

C. *In-vitro* assay

Study design and sample size

This study was conducted as per OECD Guideline No. 471 for testing of chemicals, “Bacterial Reverse Mutation Test”, adopted on 21 July 1997, corrected on June 26, 2020 (OECD, 2020). This is an *In-vitro* study and hence no sample size calculation is required for this study. The studies were carried out by a third-partyContract Research Organization (CRO).

D. Stock Solution Preparation

i. *Kumkumadi taila*and *Kanaka taila*

Separate stock solutions of 50000 µg/mL of *Kumkumadi taila*and*Kanaka taila*(both oils were sourced locally, India) were prepared by mixing 500 mg test ingredient in Dimethyl sulfoxide (DMSO) with a resulting volume of 10 mL. The above stock was further diluted in DMSO to prepare 5 concentrations 50, 158, 500, 1581, and 5000 µg/plate of the test ingredient, and 100µL of these concentrations were added to cultures.

ii. Negative and positive controls

DMSOwas used as a negative control for both the test ingredients. Specific positive controls were usedin order to confirm the specificity of the reversion properties of each tester strain and the efficacy ofthe metabolic activation system.Positive controlchemicals were specifically designed as highlymutagenic to each strain. For each strain, 2-Aminoanthracene (CAS# 613-13-8) was the positive control used with S-9 mix, whereas 2-Nitrofluorene, Sodium azide (CAS# 26628-22-8), 9-Aminoacridine (CAS#90-45-9), and

4-Nitroquinoline-1-oxide (CAS# 56-57-5) were positive controls used without S-9 Mix (Sigma Aldrich, St. Louis, USA).

Test system and genotypic characterization of test system

Histidine and Tryptophan auxotrophic strains were chosen based on the 1977 OECD guideline for the assessment of point gene mutation. Histidine auxotrophic strains of *Salmonella typhimurium*were TA98, TA100, TA1535, and TA1537[Health Protection Agency National Collection of Type Cultures (NCTC)]. Tryptophan auxotrophic strain of *Escherichia coli*wereWP2uvrA (pKM101)[The National Collection of Industrial and Marine Bacteria Ltd. (NCIMB)]. Stock cultures of tester strains were stored in Oxoid nutrient broth No.2 in the test facility as frozen permanents in liquid nitrogen. Laboratory stocks were maintained on respective minimal glucose agar plates as master plates of each strain for a maximum period of 2 months and refrigerated at 2-8ºC.

Each *S. typhimurium* tester strain contained a mutation in the histidine operon, along with additional mutations that enhanced sensitivity to some mutagens, including mutations in the *rfa* and *uvrB* genes. A mutation in the*rfa*gene results in a cell wall deficiency that increases the permeability of the cell to certain classes of chemicals, such as those containing large ring systems that would otherwise be excluded. A deletion in the *uvrB* gene results in a deficient DNA excision repair system. Tester strains TA98 and TA100 also contain the pKM101 plasmid (carrying the R factor). It has been suggested that the plasmid increases sensitivity to mutagens by modifying an existing bacterial DNA repair polymerase complex involved with the mismatch repair process. Each strain can be reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) through different mechanisms. The strainsTA98 and TA1537 can be reverted through frameshift mutagens,TA1535 can be reverted by a base substitution mutagen, andTA100 can be reverted by both frameshift and base substitution mutagens.

The *E. coli* tester strain had an AT base pair at the critical mutation site within the *trpE* gene[15]. Tester strain WP2*uvrA* (pKM101) had a deletion in the *uvrA* gene resulting in a deficient DNA excision repair system. Tryptophan revertants can arise due to a base change at the originally mutated site or by a base change elsewhere in the chromosome causing the original mutation to be suppressed. Thus, the specificity of the reversion mechanism is sensitive to base-pair substitution mutations[16,17].

Culture medium and chemicals

The media used in the study includes VB agar, soft agar, soft agar containing 0.5 mM histidine and biotin, soft agar containing 5 µg/mL tryptophan, nutrient agar, phosphate buffer solution (PBS) (pH 7.4) (Hi Media Laboratories Private Limited, Mumbai, India) and Oxoid Nutrient broth No.2 (Oxoid Ltd., Basingstoke, England).

Metabolic activation system (S9 Mix)

Aroclor1254-induced rat liver S9 homogenate[17] was used as the metabolic activation system. The S9 homogenate was prepared from male Wistar rats induced with a single intraperitoneal injection of Aroclor 1254 (0.7 mL/rat ready-to-use solution), 5 days prior to euthanasia. The S9 homogenate was prepared in batches and stored in a deep freezer maintained at -68 to -86ºC. S9 homogenate was thawed immediately before use and mixed with the cofactor solution containing 4 mM NADP, 5 mM glucose-6-phosphate, 8 mM $MgCl_2$ and 33 mM KCl in PBS to achieve a final concentration of 10% S9 (v/v) in the activation mixture.

Solubility test selection of vehicle

The test ingredients i.e., both *Kanakataila* and *Kumkumaditaila* were soluble in DMSO at 50 mg/mL.

Preliminary toxicity test for selection of doses for the mutation assay

The preliminary toxicity test was conducted to check the toxicity of the test ingredients to the *Salmonella typhimurium* tester strain TA100 at test concentrations of 50, 100, 200, 400, 800, 1600, 3200, and 5000 µg/plate along with the DMSO control. The stock solution of 50 mg/ml was diluted with DMSO to provide 100 µL each of the above respective dilutions. A loopful of the tester strain TA 100 was inoculated into a tube containing Oxoid Nutrient Broth No. 2 and the tube was incubated at 37 ± 1ºC for 17 hours. *Plating procedure:* One hundred microliters (100 µL) each of the respective dilutions (stock equivalent to doses of 50, 100, 200, 400, 800, 1600, 3200, and 5000 µg) were mixed with 2 mL of soft agar containing histidine and biotin, 500 µL of S9 mix (for the test in the presence of metabolic activation) or 500 µL of PBS (for the test in the absence of metabolic activation), 100 µL of overnight TA100 culture and overlaid onto pre-labeled VB agar plates in duplicate. A DMSO control, similarly treated, was maintained. After the agar had been set, these plates were incubated at 37 ± 1 °C for 67 hours. The number of revertant colonies on the VB agar plates was counted and the bacterial background lawn was evaluated for the evidence of test ingredient toxicity. The plates were also checked for any precipitation formation and results were reported.

Procedures

The principle of the Bacterial Reverse Mutation Test is that it detects mutations that revert the mutations already present in the test strains, thereby restoring the functional capability of the bacteria to synthesize an essential amino acid. Such revertant bacteria are detected by their ability to grow in the absence of the amino acid (histidine or tryptophan) in the culture medium as required by the parent strain.

In the initial mutation assay, which is a plate incorporation mode of exposure, the bacterial suspensions[Required Cell count: 1-2x10^9 Colony Forming Units (CFU)/mL] are exposed to the test ingredient, vehicle and the positive

controls in the presence and absence of an exogenous metabolic activation system. These bacterial suspensions are then mixed with overlay agar and plated immediately onto minimal medium viz., *his*$^-$ for *S. typhimurium* and *trp*$^-$ for *E. coli*, respectively.

In the confirmatory assay, which is a pre-incubation mode of exposure, the test ingredient, vehicle (negative control), and the positive controls in the presence and absence of an exogenous metabolic activation system are mixed with the bacteria inside a tube, incubated in an incubator shaker, mixed with overlay agar, and plated immediately onto minimal medium *his*$^-$ for *S. typhimurium* and *trp*$^-$ for *E. coli*, respectively.

After 48 to 72 hours of incubation at 37±1 ºC, the revertant colonies are counted and expressed as the number of Colony Forming Units (CFU) per mL of the bacterial suspension and compared with the number of spontaneous revertants in the negative control plates.

Colony counting

The number of colonies was counted manually. Each plate was counted three times and the average of the three counts was adopted as the number of revertant colonies on the plate. The average number of revertant colonies for each dose was calculated as the average plate count for a set of duplicates. Decimals of the average figures were rounded off.

Interpretation of the results

The test substance is judged to be negative when the number of revertant colonies is less than twice that of the negative control.The test is judged positive if the increase in mean revertant colonies at the peak of the dose response is equal to or greater than 2 times the mean negative control value for the strains TA98, TA100, and WP2*uvrA* (pKM101) or equal to or greater than 3 times the mean negative control value for the strains TA1535 and TA1537. An equivocal response is a biologically relevant increase in a revertant count that partially meets the criteria for evaluation as positive. This could be a dose-responsive increase that does not achieve the respective threshold cited above or a non-dose-responsive increase that is equal to or greater than the respective threshold cited. A response is evaluated as negative if it is neither positive nor equivocal.

3. RESULTS

A. HPLC analysis

a. *Kanakataila*

HPLC-PDA was utilized to develop an analytical method for the detection of glabridin in *Kanaka taila*. The consistent chromatogram peaks observed in multiple runs confirmed a retention time of 17.57 minfor the glabridin standard [Figure 1A]. The chromatogram peak of test sample *Kanaka taila* was identified at a retention time of 17.57 min [Figure 1B] matching with the glabridin reference standard. Therefore, glabridinwas determined as a suitable marker for *Kanaka taila.*

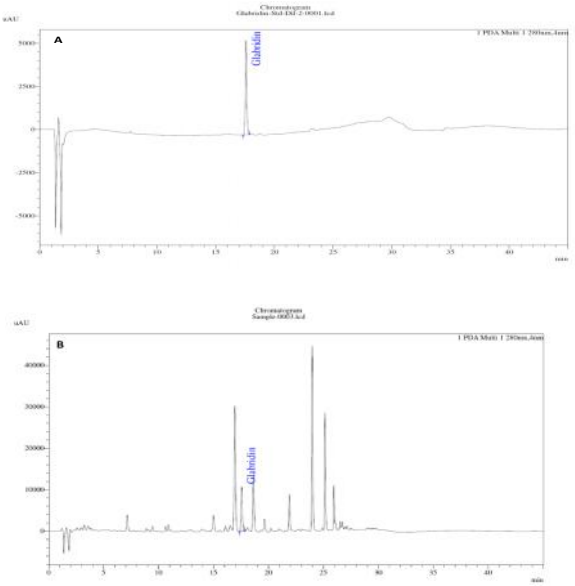

Fig 1: Representative HPLC chromatograms of (A) reference standard solution of glabridin (0.002mg/ml, RT:17.574 min) and (B) *Kanaka taila* (10-12 mg/ml) with glabridin peak identified at a retention time of 17.574 min that matches with the standard peak

b. *Kumkumadi taila*

The HPLC-PDA method was utilized to develop an analytical approach for the detection of glabridin and safranal markers in *Kumkumadi taila*. The consistent chromatogram peaks observed in multiple runs confirmed for both the standards at a retention time of 18.5 min [Figure 2A] and 20.3 min [Figure 2B] for glabridin and safranal, respectively.

In the *Kumkumadi taila*, the chromatogram peak at a retention time of 18.5 min matches with the Glabridin reference standard [Figure 3A]. Similarly, the safranal marker was identified at a retention time of 20.3 min [Figure 3B], aligning with the safranal standard. Consequently, both glabridin and safranal were successfully identified as markers in *Kumkumadi taila.*

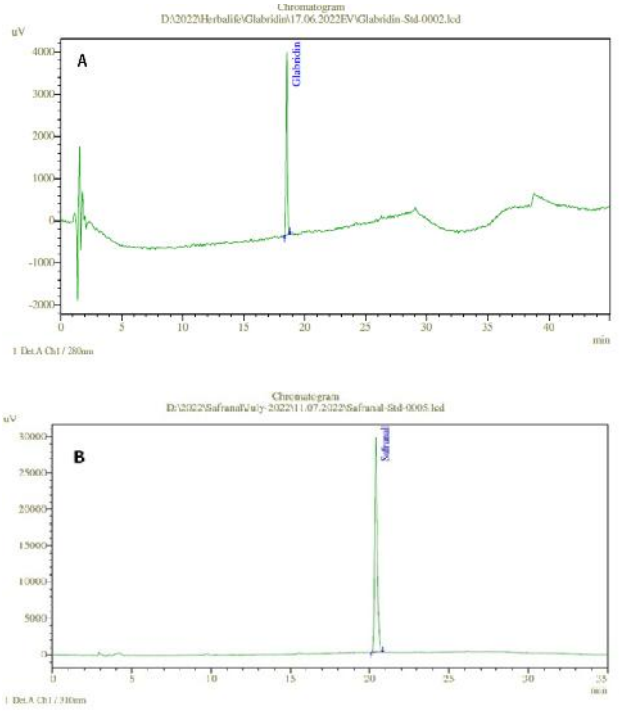

Fig 2: Representative HPLC chromatograms of (A) reference standard solution of glabridin (0.002mg/ml, RT:18.5 min) andRepresentative HPLC chromatograms of (B) reference standard solution of safranal (0.01mg/ml, RT:20.3 min)

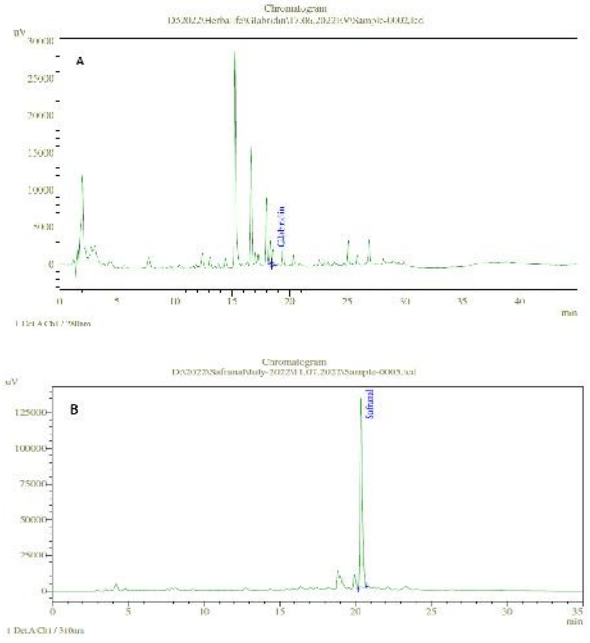

Fig 3: Representative HPLC chromatograms of (A) *Kumkumadi taila* (10-12 mg/ml) with glabridin peak identified at a retention time of 18.5 min and (B) *Kumkumadi taila* (10-12 mg/ml) with safranal peak identified at a retention time of 20.3 min that matches with the standard peak

B. *In-vitro* analysis

a. Preliminary toxicity test:

The results of the preliminary toxicity test are presented in Table 2 and 3. The test ingredients did not precipitate on the basal agar plates at any of the tested doses.

The test ingredients were not toxic to the test strain (TA100) at any of the tested doses as the intensity of the bacterial background lawn and the mean numbers of revertant colonies were comparable to the DMSO control. Based on the observations of the preliminary toxicity test, the following five test doses (with approximately half-log dose interval) were selected for testing in the mutation assays: a) 50, b) 158, c) 500, d) 1581 and e) 5000 µg/plate.

b. Mutation assay

Viable counts of all the test strains were within the required range of 1 to$2x10^9$ CFU/mL in both the assaysat the initial as well as the confirmatory mutation assay [Table 4 and 5].

The results of the initial mutation assay are summarized in Tables 6 to 9. The test ingredients did not precipitate on the basal agar plates at any of the tested doses. The test ingredients were not toxic to the test strains at any of the tested doses as the intensity of the bacterial background lawn was comparable to the respective DMSO control plates. The tested doses showed no positive mutagenic increase in the mean number of revertant colonies for all test strains when compared to the respective negative control plates, either in the presence or absence of metabolic activation. Agar plates tested simultaneously with positive control produced more than a three-fold increase in the mean numbers of revertant colonies for all the strains when compared to the respective negative control plates. No toxicity was observed in the positive controls as the intensity of the bacterial background lawn of all the test strains was comparable to that of the respective negative control plates.

Results of the Confirmatory Mutation Assay are presented in Tables10 to 13.Both the test ingredients did not precipitate on the basal agar plates at any of the tested doses. The test ingredients were not toxic to the tester strains at any of the tested doses as the intensity of the bacterial background lawn was comparable to the respective DMSO control plates. The tested doses showed no positive mutagenic increase in the mean number of revertant colonies for all tester strains when compared to the respective negative control plates, either in the presence or absence of metabolic activation. Agar plates tested simultaneously with positive control produced more than a three-fold increase in the mean numbers of revertant colonies for all the strains when compared to the respective negative control plates. No toxicity was observed in the positive controls as the intensity of the bacterial background lawn of all the tester strains was comparable to that of the respective negative control plates.

4. DISCUSSION

Although commonly described in ancient Ayurvedic texts and used in practice based on traditional knowledge, both *Kumkumadi taila* and *Kanaka taila*lack documented toxicological studies assessing their safety. In the current study,the Ames Test has been used to document that these oils are not mutagenic.

Bacterial reverse mutation studies in both *Kanakataila* and *Kumkumaditaila*, *S.typhimurium* and *E.coli* tester strains were found to be reliable and responsive to the different genotypic characterization tests like the amino acid requirement, *rfa* mutation, *uvr* mutation and the R-factor plasmids. Similarly, the spontaneous revertant counts of the negative control groups of these tester strains were in the range of the test facility's historical control data.

The positive controls produced more than 3-fold increase in the mean numbers of revertant colonies when compared to the respective negative controls, demonstrating the sensitivity of the assay procedure for the tested ingredients. Both the test ingredients, tested at doses up to the maximum level of 5000 µg/plate did not cause a two-fold increase in the mean numbers of revertant colonies in the strains TA98, TA100, and WP2*uvr*A (pKM101) or a three-fold increase in the mean numbers of revertant colonies in the strains TA1535 and TA1537 either in the presence or absence of the metabolic activation system when compared to the respective negative control plates both in initial and confirmatory tests.The assay results indicated that both oils tested negative, leading to the conclusion that they are non-mutagenic.

Table 1A: Composition of *Kumkumadi taila*

Ingredients	Part used	Quantity
Decoction of:		
*Neelotpala(Nymphaea nouchali*Burm.f.)	Rhizomes	1.6g
Manjishtha(*Rubia cordifolia* L.)	Root	1.6g
Kumkuma (*Crocus sativus* L.)	Stigma	1.6g
Usheera(*Chrysopogonzizaniodes* [L,] Roberty)	Root	1.6g
Daruharidra(*Cosciniumfenestratum* [Gaertn.] Colebr.)	Bark	1.6g
Laksha (*Lacciferlacca*)	Resinous secretion	1.6g
Yashtimadhu (*Glycyrrhiza glabra* L.)	Root	1.6g
Chandana (*Santalum album* L.)	Heart wood	1.6g
Nyagrodha (*Ficus bengalensis* L.)	Root	1.6g
Padmaka (*Prunus campanulate* Maxim)	Heart wood	1.6g
Kamala (*Nelumbo nucifera*Gaertn.)	Stem	1.6g
Paste of:		
Laksha (*Lacciferlacca*)	Resinous secretion	0.5g
Patranga (*Biancaeasappan* [L.] Tod)	Heart wood	0.5g
Rakta chandana (*Pterocarpus santalinus*L.f.)	Heart wood	0.5g
Yashtimadhu (*Glycyrrhiza glabra* L.)	Root	0.5g
Kumkuma (*Crocus sativus* L.)	Stigma	0.5g
Base and others:		
Tila taila (Sesame oil)	-	10ml
Goat milk	-	20ml

Table 1B: Composition of *Kanaka taila*

Ingredients	Part Used	Quantity (g)
Til taila (Sesame oil)	Seed	1000
Yashtimadhu (*Glycyrrhiza glabra* L.)	Dried stolon, roots and Rhizomes	400
Priyangu (*Callicarpa macrophylla* Vahl)	Dried Seed	33.33
Manjishtha (*Rubia cordifolia* L.)	Dried Stem	33.33
Utpala (*Nymphaea nouchali* var. nouchali)	Dried flower	33.33
Nagkesara (*Mesua ferrea* L.)	Dried stamen	33.33

Table2: Preliminary toxicity testof *Kumkumadi taila*

Treatment («g/plate)	TA 100 revertant colonies/plate*					
	Presence of Metabolic			Absence of Metabolic		
	Mean Revertant Colony	Background Lawn Intensity**	Precipitation	Mean Revertant Colony	Background Lawn Intensity**	Precipitation
DMS	101	4+	NPO	104	4+	NPO
5	104	4+	NPO	105	4+	NPO
100	102	4+	NPO	100	4+	NPO
200	99	4+	NPO	99	4+	NPO
400	100	4+	NPO	101	4+	NPO
800	99	4+	NPO	105	4+	NPO
1600	103	4+	NPO	104	4+	NPO
3200	100	4+	NPO	102	4+	NPO
5000	96	4+	NPO	97	4+	NPO

*: Mean of two replicates, **: Normal: Distinguished by a healthy background lawn comparable to Negative control plates, DMSO: Dimethyl sulfoxide; NPO: No precipitation observed

Table 3: Preliminary toxicity test of *Kanaka taila*

Treatment («g/plate)	TA 100 revertant colonies/plate*					
	Presence of Metabolic Activation			Absence of Metabolic Activation		
	Mean Revertant Colony Count	Background Lawn Intensity**	Precipitation	Mean Revertant Colony Count	Background Lawn Intensity**	Precipitation
DMSO	100	4+	NPO	105	4+	NPO
50	98	4+	NPO	99	4+	NPO
100	97	4+	NPO	98	4+	NPO
200	95	4+	NPO	98	4+	NPO
400	97	4+	NPO	95	4+	NPO
800	98	4+	NPO	96	4+	NPO
1600	90	4+	NPO	89	4+	NPO
3200	90	4+	NPO	93	4+	NPO

5000	93	4+	NPO	94	4+	NPO

*: Mean of two replicates, **: Normal: Distinguished by a healthy background lawn comparable to Negative control plates, DMSO: Dimethyl sulfoxide; NPO: No precipitation observed

Table 4: Viable counts of tester strains in the overnight culture (*Kumkumadi taila*)

Tester Strains	Viable Counts (x 10^9 CFU/mL*)	
	Initial Mutation Assay	Confirmatory Mutation Assay
TA98	1.53	1.53
TA100	1.54	1.52
TA1535	1.55	1.55
TA1537	1.59	1.58
WP2*uvr*A (pKM101)	1.67	1.65

* Required Cell count: 1-2x10^9 Colony Forming Units (CFU)/mL

Table 5: Viable counts of tester strains in the overnight culture (*Kanakataila*)

Tester Strains	Viable Counts (x 10^9 CFU/mL*)	
	Initial Mutation Assay	Confirmatory Mutation Assay
TA98	1.53	1.53
TA100	1.53	1.52
TA1535	1.56	1.54
TA1537	1.52	1.56
WP2*uvr*A (pKM101)	1.65	1.67

* Required Cell count: 1-2x10^9 Colony Forming Units (CFU)/mL

Table 6: Results of initial bacterial reverse mutation assay using plate incorporation mode of exposure in the presence of metabolic activation for *Kumkumadi taila*

Treatment [µg/plate]	No. of revertants/plate[a]														
	TA98			TA100			TA1535			TA1537			WP2*uvr*A		
	Mean	SD	Ratio[b]	Mean	SD	Ratio[b]	Mean	SD	Ratio[b]	Mean	SD	Ratio[b]	Mean	SD	Ratio[b]
DMSO	24	3	NA	104	4	NA	15	1	NA	10	2	NA	142	2	NA
50	24	2	1.00	99	4	0.96	14	1	0.93	11	1	1.03	140	3	0.98
158	23	3	0.97	95	3	0.91	12	2	0.82	12	1	1.19	139	4	0.98
500	22	2	0.92	93	4	0.90	14	2	0.96	10	2	1.00	139	2	0.98
1581	22	1	0.93	95	5	0.91	13	2	0.84	12	3	1.13	134	4	0.94
5000	22	2	0.92	94	3	0.90	12	2	0.82	11	3	1.10	137	4	0.96
Positive control	365[c]	9[c]	15.44[c]	766[c]	15[c]	7.37[c]	169[c]	4[c]	11.24[c]	164[c]	12[c]	15.90[c]	585[d]	11[d]	4.11[d]

[a] Values are means of three replicates calculated from individual values and are rounded off to the nearest whole number.
[b] Ratio of treated/Negative control (mean revertants per plate). The presentation was made using the mean values with decimals before rounding off to the nearest whole number. Hence, some of the values may not match if calculated using the rounded-off mean values of this summary table.[c] TA98, TA100, TA1535, TA1537: 2-Aminoanthracene (4 µg/plate), [d] WP2*uvrA* (pKM101): 2-Aminoanthracene (30 µg/plate),DMSO: Dimethyl sulfoxide,SD: Standard deviation, NA: Not applicable

Table 7: Results of initial bacterial reverse mutation assay using plate incorporation mode of exposure in the absence of metabolic activation for *Kumkumadi taila*

Treatment [µg/plate]	No. of revertants/plate[a]														
	TA98			TA100			TA1535			TA1537			WP2*uvr*A		
	Mean	SD	Ratio[b]	Mean	SD	Ratio[b]	Mean	SD	Ratio[b]	Mean	SD	Ratio[b]	Mean	SD	Ratio[b]
DMSO	23	2	NA	106	3	NA	13	2	NA	12	1	NA	142	4	NA
50	24	3	1.01	103	3	0.97	13	1	1.03	11	1	0.89	140	8	0.99
158	23	2	0.97	101	5	0.96	12	1	0.92	11	2	0.92	143	3	1.00
500	23	3	0.97	102	2	0.96	13	3	1.03	10	1	0.86	136	3	0.96
1581	22	2	0.96	97	3	0.91	13	2	1.00	10	2	0.86	141	4	0.99
5000	21	2	0.91	97	4	0.92	12	1	0.92	11	1	0.89	136	4	0.96
Positive control	274[c]	9[c]	11.76[c]	563[d]	8[d]	5.31[d]	166[d]	12	12.77[d]	156[e]	13[e]	13.03[e]	583[f]	4[f]	4.10[f]

[a] Values are means of three replicates calculated from individual values and are rounded off to the nearest whole number.[b] Ratio of treated/Negative control (mean revertants per plate). The presentation was made using the mean values with decimals before rounding off to the nearest whole number. Hence, some of the values may not match if calculated using the rounded-off mean values of this summary table.[c] TA98: 2-Nitrofluorene (2 µg/plate), [d] TA100, TA1535: Sodium azide (1 µg/plate), [e] TA1537: 9-Aminoacridine (50 µg/plate), [f] WP2*uvrA* (pKM101): 4-Nitroquinoline-1-oxide (4 µg/plate), DMSO: Dimethyl sulfoxide,SD: Standard deviation NA: Not applicable

Table 8: Results of initial bacterial reverse mutation assay using plate incorporation mode of exposure in the presence of metabolic activation for *Kanakataila*

Treatment [µg/plate]	No. of revertants/plate[a]														
	TA98			TA100			TA1535			TA1537			WP2*uvr*A		
	Mean	SD	Ratio[b]	Mean	SD	Ratio[b]	Mean	SD	Ratio[b]	Mean	SD	Ratio[b]	Mean	SD	Ratio[b]
DMSO	24	3	NA	103	3	NA	15	2	NA	13	4	NA	142	5	NA
50	23	3	0.96	103	6	1.00	13	3	0.85	11	3	0.85	137	3	0.96
158	24	2	0.97	104	2	1.01	14	4	0.91	10	2	0.78	139	7	0.98
500	26	2	1.08	100	3	0.97	15	2	0.96	12	2	0.88	138	4	0.97
1581	23	3	0.96	102	4	0.99	13	2	0.87	12	3	0.88	138	2	0.97
5000	22	2	0.89	100	6	0.98	13	2	0.83	10	1	0.75	138	4	0.97
Positive control	362[c]	15[c]	14.89[c]	723[c]	37[c]	7.04[c]	164[c]	14[c]	10.72[c]	164[c]	10[c]	12.33[c]	586[d]	5[d]	4.11[d]

[a]Values are means of three replicates calculated from individual values are rounded off to the nearest whole number; [b] Ratio of treated/Negative control (mean revertants per plate). The presentation was made using the mean values with decimals before rounding off to the nearest whole number. Hence, some of the values may not match if calculated using the rounded-off mean values of this summary table. [C] TA98, TA100, TA1535, TA1537: 2-Aminoanthracene (4 µg/plate), [d] WP2*uvrA* (pKM101): 2-Aminoanthracene (30 µg/plate), DMSO: Dimethyl sulfoxide SD: Standard deviation, NA: Not applicable

Table 9: Results of initial bacterial reverse mutation assay using plate incorporation mode of exposure in the absence of metabolic activation for *Kanakataila*

Treatment [µg/plate]	No. of revertants/plate[a]														
	TA98			TA100			TA1535			TA1537			WP2*uvr*A		
	Mean	SD	Ratio[b]	Mean	SD	Ratio[b]	Mean	SD	Ratio[b]	Mean	SD	Ratio[b]	Mean	SD	Ratio[b]
DMSO	24	3	NA	105	4	NA	15	3	NA	12	3	NA	146	4	NA
50	22	2	0.93	102	6	0.97	14	2	0.91	11	2	0.94	138	2	0.95
158	25	3	1.03	102	3	0.97	14	2	0.96	12	2	0.97	139	6	0.95
500	23	3	0.96	102	6	0.97	13	2	0.87	11	1	0.94	141	3	0.97
1581	24	1	1.00	101	6	0.96	12	3	0.82	12	3	1.00	142	6	0.97
5000	20	1	0.85	101	8	0.97	14	3	0.91	11	1	0.89	134	6	0.92
Positive control	272[c]	9[c]	11.35[c]	569[d]	15[d]	5.42[d]	172[d]	9[d]	11.49[d]	172[e]	12[e]	14.33[e]	585[f]	7[f]	4.01[f]

[a]Values are means of three replicates calculated from individual values are rounded off to the nearest whole number.[b] Ratio of treated/Negative control (mean revertants per plate). The presentation was made using the mean values with decimals before rounding off to the nearest whole number. Hence, some of the values may not match if calculated using the rounded-off mean values of this summary table.[c] TA98: 2-Nitrofluorene (2 µg/plate), [d] TA100, TA1535: Sodium azide (1 µg/plate), [e] TA1537: 9-Aminoacridine (50 µg/plate) ,[f] WP2*uvrA* (pKM101): 4-Nitroquinoline-1-oxide (4 µg/plate), DMSO: Dimethyl sulfoxide SD: Standard deviation NA: Not applicable

Table 10: Results of confirmatory bacterial reverse mutation assay using pre-incubation mode of exposure in the presence of metabolic activation for *Kumkumadi taila*

Treatment [µg/plate]	No. of revertants/plate[a]														
	TA98			TA100			TA1535			TA1537			WP2*uvr*A		
	Mean	SD	Ratio[b]	Mean	SD	Ratio[b]	Mean	SD	Ratio[b]	Mean	SD	Ratio[b]	Mean	SD	Ratio[b]
DMSO	24	4	NA	101	9	NA	15	2	NA	12	2	NA	143	5	NA
50	24	2	1.00	102	3	1.01	14	2	0.89	9	2	0.78	144	3	1.01
158	24	3	1.01	103	3	1.02	12	2	0.80	10	1	0.86	140	2	0.98
500	23	3	0.94	100	4	0.99	12	3	0.80	9	1	0.78	145	3	1.02
1581	23	2	0.97	103	2	1.02	12	1	0.76	10	2	0.86	137	3	0.96
5000	21	2	0.88	97	4	0.96	12	2	0.78	11	1	0.89	136	4	0.95
Positive control	357[c]	13[c]	14.89[c]	774[c]	17[c]	7.66[c]	166[c]	12[c]	10.83[c]	173[c]	7[c]	14.44[c]	586[d]	5[d]	4.11[d]

[a] Values are means of three replicates calculated from individual values rounded off to the nearest whole number, [b] Ratio of treated/Negative control (mean revertants per plate). The presentation was made using the mean values with decimals before rounding off to the nearest whole number. Hence, some of the values may not match if calculated using the rounded-off mean values of this summary table.[c] TA98, TA100, TA1535, TA1537: 2-Aminoanthracene (4 µg/plate), [d] WP2*uvrA* (pKM101): 2-Aminoanthracene (30 µg/plate),

Table 11: Results of confirmatory bacterial reverse mutation assay using pre-incubation mode of exposure in the absence of metabolic activation for *Kumkumadi taila*

Treatment [µg/plate]	No. of revertants/plate[a]														
	TA98			TA100			TA1535			TA1537			WP2*uvr*A		
	Mean	SD	Ratio[b]	Mean	SD	Ratio[b]	Mean	SD	Ratio[b]	Mean	SD	Ratio[b]	Mean	SD	Ratio[b]
DMSO	25	2	NA	104	4	NA	13	2	NA	12	1	NA	146	4	NA
50	23	3	0.92	100	3	0.96	14	2	1.03	10	2	0.86	138	5	0.95
158	23	2	0.89	103	9	0.99	14	2	1.03	10	2	0.89	143	3	0.98
500	23	3	0.91	101	3	0.97	12	1	0.88	11	1	0.91	144	3	0.99
1581	23	2	0.92	98	7	0.94	14	2	1.05	10	1	0.83	140	2	0.96
5000	24	2	0.93	99	7	0.96	10	1	0.78	11	1	0.97	136	3	0.93
Positive control	266[c]	10[c]	10.51[c]	573[d]	10[d]	5.51[d]	156[d]	12	11.73[d]	168[e]	9[e]	14.37[e]	591[f]	3[f]	4.06[f]

[a]Values are means of three replicates calculated from individual values are rounded off to the nearest whole number, [b] Ratio of treated/Negative control (mean revertants per plate). The presentation was made using the mean values with decimals before rounding off to the nearest whole number. Hence, some of the values may not match if calculated using the rounded-off mean values of this summary table.[c] TA98: 2-Nitrofluorene (2 µg/plate), [d] TA100, TA1535: Sodium azide (1 µg/plate),[e] TA1537: 9-Aminoacridine (50 µg/plate), [f] WP2*uvrA* (pKM101): 4-Nitroquinoline-1-oxide (4 µg/plate), DMSO: Dimethyl sulfoxide, SD: Standard deviation, NA: Not applicable

Table 12. Results of confirmatory bacterial reverse mutation assay using pre-incubation mode of exposure in the presence of metabolic activation for *Kanakataila*

Treatment [µg/plate]	No. of revertants/plate[a]														
	TA98			TA100			TA1535			TA1537			WP2*uvr*A		
	Mean	SD	Ratio[b]	Mean	SD	Ratio[b]	Mean	SD	Ratio[b]	Mean	SD	Ratio[b]	Mean	SD	Ratio[b]
DMSO	24	3	NA	107	3	NA	15	2	NA	12	2	NA	143	4	NA
50	24	2	0.99	100	4	0.94	14	3	0.91	12	3	1.00	141	3	0.98
158	26	2	1.10	101	2	0.95	14	2	0.91	10	2	0.81	142	8	0.99
500	23	2	0.96	98	3	0.92	13	2	0.89	12	3	0.97	139	3	0.97
1581	24	2	1.00	100	7	0.94	12	2	0.80	12	1	0.97	141	3	0.98
5000	22	3	0.92	105	3	0.98	12	2	0.82	12	3	0.97	140	5	0.97
Positive control	364[c]	11[c]	15.17[c]	767[c]	14[c]	7.19[c]	161[c]	12[c]	10.71[c]	164[c]	11[c]	13.64[c]	586[d]	6[d]	4.09[d]

[a]Values are means of three replicates calculated from individual values are rounded off to the nearest whole number. [b] Ratio of treated/Negative control (mean revertants per plate). The presentation was made using the mean values with decimals before rounding off to the nearest whole number. Hence, some of the values may not match if calculated using the rounded-off mean values of this summary table.[c] TA98, TA100, TA1535, TA1537: 2-Aminoanthracene (4 µg/plate), [d] WP2*uvrA* (pKM101): 2-Aminoanthracene (30 µg/plate),

Table 13. Results of confirmatory bacterial reverse mutation assay using pre-incubation mode of exposure in the absence of metabolic activation for *Kanakataila*

Treatment [µg/plate]	No. of revertants/plate[a]														
	TA98			TA100			TA1535			TA1537			WP2*uvr*A		
	Mean	SD	Ratio[b]	Mean	SD	Ratio[b]	Mean	SD	Ratio[b]	Mean	SD	Ratio[b]	Mean	SD	Ratio[b]
DMSO	23	2	NA	106	4	NA	14	2	NA	11	2	NA	145	3	NA
50	24	3	1.01	104	3	0.98	14	2	0.95	11	2	0.94	142	4	0.97
158	24	2	1.04	99	5	0.93	12	1	0.84	12	2	1.06	139	3	0.96
500	23	2	0.97	104	4	0.98	14	3	0.95	11	2	0.94	141	3	0.97
1581	22	4	0.96	99	4	0.93	14	2	0.95	10	1	0.91	144	3	0.99
5000	22	2	0.94	102	4	0.96	12	3	0.84	10	2	0.91	138	3	0.95
Positive control	269[c]	20[c]	11.51[c]	566[d]	11[d]	5.32[d]	165[d]	14	11.51[d]	163[e]	11[e]	14.38[e]	585[f]	7[f]	4.03[f]

[a] Values are means of three replicates calculated from individual values are rounded off to the nearest whole number, [b] Ratio of treated/Negative control (mean revertants per plate). The presentation was made using the mean values with decimals before rounding off to the nearest whole number. Hence, some of the values may not match if calculated using the rounded-off mean values of this summary table.[c] TA98: 2-Nitrofluorene (2 µg/plate), [d] TA100, TA1535: Sodium azide (1 µg/plate) [e] TA1537: 9-Aminoacridine (50 µg/plate), [f] WP2*uvrA* (pKM101): 4-Nitroquinoline-1-oxide (4 µg/plate), DMSO: Dimethyl sulfoxide,SD: Standard deviation, NA: Not applicable

5. CONCLUSION

Both the oils Kumkumaditaila and Kanakataila were not mutagenic in the bacterial reverse mutation test when tested up to the highest OECD 471-recommended dose of 5000 µg/plate under the conditions of use.

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ACKNOWLEDGEMENT: We would like to thank Dr. Pallavi Kiradi (MD, Ayurveda) from Herbalife Nutrition, India for assessing the composition details of oils for their correctness as per Ayurveda principles. The experiment was funded by HNL Business Services Private Limited and the experiments were performed at third party Contract Research Organization (CRO) in Bengaluru, India

CONFLICT OF INTEREST: The authors declare no conflict of interest, financial or otherwise.

SOURCE OF FUNDING: This experiment was funded by HNL Business Services private limited and performed at third party Contract Research Organization (CRO).

AVAILABILITY OF DATA AND MATERIALS: Not applicable.

CONSENT FOR PUBLICATION: Not applicable.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE: Not applicable