1 **Genotoxic effects of *Dukhan:* A smoke bath from the wood of *Acacia***
2 ***seyal* used traditionally by Sudanese women**
3
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Abstract:

Ethnobotanical relevance: Smoke from the wood of *Acacia seyal* Delile has been used by Sudanese women for making a smoke bath locally called *Dukhan*. The ritual is performed to relieve rheumatic pain, smooth skin, heal wounds and achieve general body relaxation.

Aim of the study: The present study was designed to investigate the *in vitro* anti-inflammatory effect of the smoke condensate using cyclooxygenase -1 (COX-1) and -2 (COX-2) as well as its potential genotoxic effects using the bacterial-based Ames test and the mammalian cells-based micronucleus/ cytome and comet assays.

Material and methods: The smoke was prepared in a similar way to that commonly used traditionally by Sudanese women then condensed using a funnel. Cyclooxygenase assay was used to evaluate its *in vitro* anti-inflammatory activity. The neutral red uptake assay was conducted to determine the range of concentrations in the mammalian cells-based assays. The Ames, cytome and comet assays were used to assess its potential adverse (long-term) effects.

Results: The smoke condensate did not inhibit the cyclooxygenases at the highest concentration tested. All smoke condensate concentrations tested in the *Salmonella*/microsome assay induced mutation in both TA98 and TA100 in a dose dependent manner. A significant increase in the frequency of micronucleated cells, nucleoplasmic bridges and nuclear buds was observed in the cytome assay as well as in the % DNA damage in the comet assay.

Conclusions: The findings indicated a dose dependent genotoxic potential of the smoke condensate in the bacterial and human C3A cells and may pose a

health risk to women since the smoke bath is frequently practised. The study highlighted the need for further rigorous assessment of the risks associated with the smoke bath practice.

Keywords: smoke condensate, mutagenicity, DNA damage, micronucleus, nuclear bud, nucleoplasmic bridge

1. Introduction

Medicinal plants have been used for the treatment of diseases and illnesses by humankind for millennia. In some cultures their use is not limited to medicine but is part of religious celebrations and ceremonies (Berlowitz et al., 2020; Braithwaite et al., 2008; Mohagheghzadeh et al., 2006). There are different methods for preparation and routes of administration of medicinal plant extracts. Smoke is one of these forms and could be administered through inhalation or by directing it through to a specific organ or body part (Braithwaite et al., 2008; Ezekwesili-Ofili and Okaka, 2019; Mohagheghzadeh et al., 2006).

The use of smoke in the African traditional medicine dates back to 2800 BC during the Ancient Egyptian civilization. For instance, Ancient Egyptians used the smoke of *Commiphora myrrrha* (Nees) Engl. for the treatment of skin sores, inflammation, urinary tract diseases, for mummification, and as incense and perfumes (Grbić et al., 2018).

A. seyal Delile, Leguminosae, (known locally as *talh*) and occurs widely in Africa especially north of the equator is used traditionally in Sudan for various purposes including curing colds, jaundice, headache, burns, arthritis,

78 rheumatism and rheumatic fever (El Ghazali et al., 1997). Bark and wood of *A.*
79 *seyal* (Fig.1) and other *Combretum* species are used by Sudanese women for
80 making a smoke bath locally called *dukhan*. The practice is used traditionally
81 to ease rheumatic pain, smoothen skin, treat wounds and achieve general
82 body relaxation (Eldeen and van Staden, 2008). It is also used as a cosmetic
83 and a means of beautification (Ogbazghi and Bein, 2006). Recently the
84 smoke has been commercialized into a cosmetic product called cream
85 "*Dukhan*". It is used topically as a skin-softening and emollient agent (Eldeen
86 et al., 2016).

87

88 Figure 1: Wood *of A. seyal* used traditionally in making the smoke bath.

89 The use of *A. seyal* smoke bath by most women in Sudan makes it necessary
90 to assess its efficacy in the treatment of joint pain and inflammation.
91 Prostaglandins are formed from arachidonic acid by the cyclooxgenase
92 enzymes activity, play major roles in a number of biological processes
93 including the protection of the stomach mucosa. They are also involved in
94 major pathological functions including pain sensation, fever and inflammation

95 (Botting, 2006; Elgorashi and McGaw, 2019; Herschman, 2003). In this
96 context, assessing the smoke condensate against these enzymes will give an
97 insight on their efficacy in the treatment of joint pain and inflammation. On the
98 other hand, there is little toxicological information on *dukhan*. Given the
99 frequent and long term use of the smoke and the key role of genotoxicity in
100 risk assessment a thorough genotoxicological approach is warranted to
101 assess its long term harmful effects. The well-known Ames, cytocalassin-B-
102 blocked micronucleus test (cytome) and comet assays are widely used to
103 assess DNA damage, cytosis and cytotoxicity of natural products. The present
104 study aimed at evaluating the *in vitro* anti-inflammatory properties of the
105 smoke condensate using the cyclooxegenase assay as a possible mechanism
106 to explain the traditional use of smoke baths. It is also important to assess the
107 potential genotoxic risks associated with its long-term practice using three
108 different assays measuring different genotoxic endpoints.
109

110 ## 2. Materials and methods

111 ### *2.1 Chemicals and reagents*

112 Acetic acid, agarose, biotin, cyclooxygenase-1 and -2, cytochalasin B, L-
113 epinephrine, ethyl bromide, formaldehyde, Geimsa stain, glutathione, hematin,
114 histidine, May-Grünwald, neutral red dye, 4-nitroquiniline-1-oxide, Oxoid
115 nutrient broth and sodium dodecyl sulphate (SDS), were procured from
116 Sigma-Aldrich (St. Louis, Missouri, USA). [1-^{14}C] arachidonic acid was
117 purchased from AEC-Amersham (Kyalami, Gauteng, South Africa). Difco agar
118 was purchased from Becton Dickinson (Johannesburg, South Africa). Ethnaol
119 and methanol were procured from Merck (Germiston, South Africa),

120 *Salmonella typhimurium* strain TA98 and TA100 were purchased from Moltox
121 (Boone, USA). C3A cells were purchased from ATCC (Manassas, USA).
122

123 *2.2 Collection of wood material and smoke preparations*:
124 Dry wood of *Acacia seyal* Delile (locally known as *talh*) was collected from
125 *Alrwashda* forest in eastern Sudan (Latitude of 14.2° N and the Longitude of
126 35.6° E). The plant was identified by Dr Eldeen, Herbarium curator in the
127 Department of Silviculture, Faculty of Forestry, University of Khartoum. A
128 voucher specimen (A.var.*Seyal* 23) was deposited in the Departmental
129 Herbarium. The wood (10 kg) was chopped into small pieces and lodged to
130 burn in a hole 30 cm deep in the ground (diameter about 25 cm). The hole
131 was covered by a cylindrical shaped clay bucket (height about 50 cm) with an
132 opening (diameter 5 cm) on top. The opening at the top of the bucket was
133 covered by a stainless steel conical flask and sealed with clay to ensure
134 maximum concentration of smoke in the container. The smoke was
135 condensed in the container forming a dark brown layer. After two hours of
136 continuous burning, the container was removed and washed with ethanol
137 resulting in a thick dark brownish smoke solution. The solution was
138 distributed in petri dishes and left to dry under a fan at room temperature.
139

140 *2.3 Cyclooxygenase assay*
141 Cyclooxygenase (COX) inhibitory activity was determined using the COX-1
142 and COX-2 assays described by Jäger et al. (1996) and Noreen et al. (1998).
143 Briefly, 10 µL of COX-1 and COX-2 enzymes containing 3.0 enzyme units
144 were activated with 50 µL co-factor solution containing 0.9 mM L-epinephrine,

145 0.49 mM glutathione, and 1 µM hematin in 0.1 M Tris buffer, pH 8.0 on ice for
146 5 minutes. Enzyme solution (60 µl) and a 20 µl sample solution (0.0- 250.0
147 µg/ml) were incubated at room temperature for 5 minutes. Thereafter, 20 µL
148 [1-^{14}C] arachidonic acid (30 µM, 17CiMol^{-1}) were added. The mixtures were
149 incubated for 10 minutes at 37ºC, and the reaction was terminated by adding
150 10 µL of 2 M HCL. The prostaglandin products were separated by column
151 chromatography. Percentage inhibition of the enzyme by the smoke
152 condensate was calculated by comparing the amount of radioactivity present
153 in the test solution to that in the solvent control. Indomethacin was used as a
154 positive control.
155
156 *2.4 Salmonella/microsome assay*
157 Mutagenicity of the smoke condensate was investigated in the *Salmonella/*
158 microsome assay using the plate incorporation method described by Maron
159 and Ames (1983). Two *Salmonella typhimurium* tester strains, TA98 and
160 TA100, capable of detecting different mutation mechanisms were used
161 without metabolic activation. Three different concentrations of 500, 250 and
162 125 µg /plate were prepared from the smoke condensate. Bacterial stock (100
163 µL) was inoculated in Oxoid Nutrient broth (20 ml) and incubated for 16h at 37
164 °C. One hundred microliters of the test solution (smoke sample, solvent
165 control or the positive control 4 NQO) were mixed with 0.5 ml of phosphate
166 buffer before 100 µL of the overnight bacterial culture were added. Thereafter,
167 2 ml of top agar (containing biotin and histidine) were added to the mixture.
168 The mixture was poured onto the surface of a minimal agar plate and
169 incubated for 48 h at 37 °C. After incubation, the number of his+ revertant

170 colonies was counted. Test solutions were tested in triplicate whereas five
171 replicates were prepared for the solvent control. The experiment was repeated
172 twice.

173 *2.5 Cytotoxicity assay*

174 The cytotoxicity assay was conducted to determine the range of doses in the
175 comet and micronucleus/cytome assays. The cytotoxic effects of the smoke
176 condensate were determined against C3A cell line using the neutral red
177 uptake assay (Borenfreund and Puerner, 1985). The assay was chosen as it
178 measures cell viability based on the ability of living cells to absorb the dye into
179 their lysosomes. Toxic chemicals alter cell membrane properties and therefore
180 inhibit their ability to uptake neutral red (Repetto et al., 2008). The method is
181 described in detail elsewhere (Makhafola, 2014). Briefly, cells were cultured in
182 Dulbecco's modified Eagle's culture medium (DMEM) supplemented with
183 foetal calf serum (10%). Cell suspensions were plated into each well of a 96-
184 well microtitre plate and incubated at 37°C. Once subconfluent, the cells in the
185 microtitre plate were treated with three different concentrations of the smoke
186 condensate (100, 500 and 2500 µg/ml), the positive control sodium dodecyl
187 sulphate (SDS) and a solvent control for a further 24 hours. Thereafter, 200 µl
188 of cell culture medium containing neutral red dye (0.05 mg/ml) were added,
189 following the removal of the test sample, and the plate was incubated for
190 further 3 hours. The neutral red was aspirated and the cells were washed with
191 200 µl of PBS. The dye was extracted from the cells using a 200 µl acetic
192 acid-ethanol mixture. The plates were agitated for at least 90 minutes to
193 obtain a homogenously stained medium. Absorbance values were measured
194 against a blank reference without cells at 540 nm using a micro plate

195 spectrophotometer. The optical density (OD) values were calculated by
196 subtracting the measured value of the condensate from the blank control
197 value. Results are expressed as percentage cell viability calculated from the
198 OD obtained from the average of the blank control culture read at 540 nm and
199 set at 100%.
200
201 *2.6 Cytokinesis-block micronucleus/cytome assay*
202 The micronucleus test was performed on C3A cells following a protocol
203 described by Fenech and Morley (1985). The method is described detail
204 elsewhere (Makhafola, 2014). In brief, cells were cultured at 37 °C in DMEM
205 growth medium supplemented with foetal bovine serum (15%) for 24 hours.
206 Thereafter, the cells were treated with different concentrations of the smoke
207 condensate, the positive 4-nitroquinoline 1-oxide (4-NQO) and the negative
208 (solvent) controls and incubated for another 24 hours. All cells were treated
209 with 100 µl of cytochalasin B (0.6 µg/ml of culture medium) for 24 hours.
210 Thereafter, the cells were trypsinized and recovered by centrifugation at 1000
211 rpm for 10 minutes. The pellet was resuspended in cold acetic acid/methanol
212 fixation solution with one drop of 37% formaldehyde. The centrifugation and
213 fixation step was repeated twice. Finally, the cell pellets were resuspended in
214 4 ml of fixation solution and stored at -20 °C for three days. Thereafter, cells
215 were resuspended in fresh fixation solution and mounted on microscope
216 slides. The slides were stained with May-Grünwald followed by Giemsa stain
217 for 2 and 5 minutes respectively. The slides were viewed under a microscope
218 and the micronuclei scored per 2000 binucleated cells. Furthermore, the

219 Nuclear Division Index (NDI) was scored in five hundred cells and established
220 according to the method of Eastmond and Tucker (1989).

221 *2.7 Comet assay*

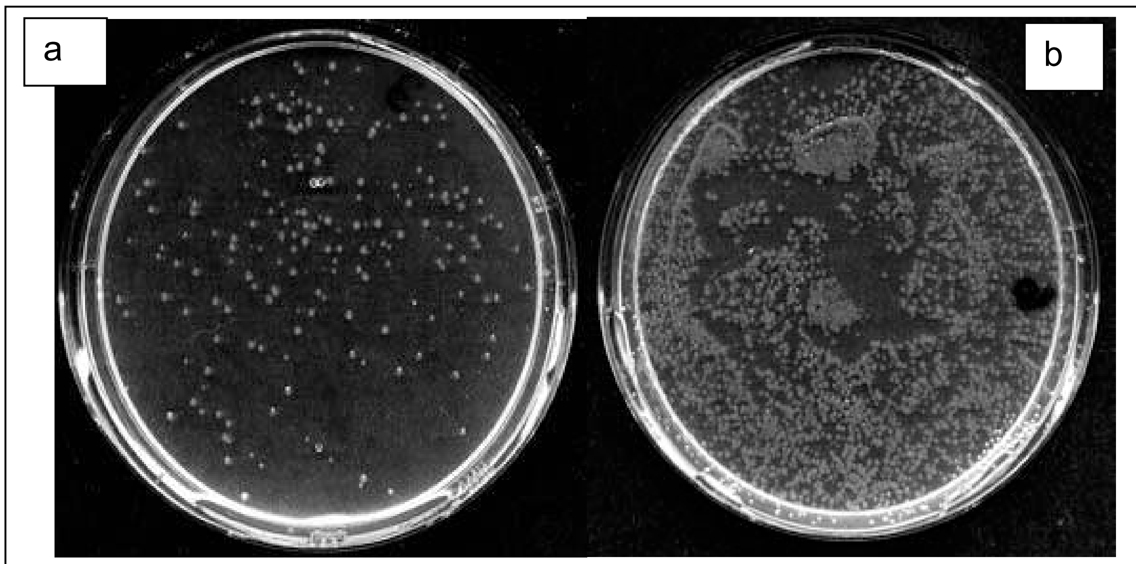
222 The protocol of Singh *et al*. (1988) was followed to determine the DNA
223 damaging effects of the smoke condensate. The method is described in more
224 detail elsewhere (Makhafola, 2014). Briefly, C3A cell suspension treated with
225 different concentrations of the smoke condensate were incubated for 24
226 hours. The cells were suspended in low melting point agar and pipetted onto a
227 glass slide precoated with normal melting point agarose which were then
228 covered with coverslips. The slides were allowed to harden at 4ºC. The
229 coverslips were removed and then the slides immersed in cold lysis buffer
230 overnight. Thereafter, the slides were covered with electrophoresis buffer at
231 17°C for 40 minutes to allow for denaturation and electrophoresed under 25V
232 and 300 mA for 20 minutes. Thereafter, the slides were placed in
233 neutralisation Tris buffer (pH 7.5), fixed in ice cold ethanol for 10 minutes and
234 dried at room temperature. The slides were stained with 100 µl of ethyl
235 bromide (20 µg/ml.) and analyzed using a fluorescent microscope supplied
236 with a camera and coupled to an image analysis system. The tail length, tail
237 DNA content (percentage of DNA in the comet tail) and the tail moment were
238 used to measure the DNA damage.

239 *2.8 Statistical analysis*

240 Data collected from smoke condensate treated and untreated cells were
241 analysed using Statistical Analysis System (SAS) package. Analysis of
242 variance (ANOVA) was used for detection of significant differences between
243 different experimental treatments in Ames test. Dunnet's test was applied to

244 determine significant differences between the means. A *t*-test was performed
245 to separate mean tail length, tail moment and % DNA in tail in treated and
246 untreated cells in comet assay and the $\varkappa^2$ test was used to determine
247 significant differences between mean frequencies of micronuclei (Mni),
248 nuclear buds and nucleoplasmic bridges (NPBs) in treated and untreated cells
249 in the micronucleus test. The level of statistical significance was set at
250 $P<0.05$.
251
252 3 Results
253 *3.5 Cyclooxygenase assay*
254 The smoke condensate had no inhibitory effect on both COX-1 and COX-2
255 activities at the highest concentration tested (250 µg/ml). The positive control
256 indomethacin had IC_{50} values of 3.3 ± 0.008 µM and 122.0 ± 5.7 µM for COX-
257 1 and COX-2 respectively.
258 *3.6 Ames test*
259 Representative photographs of revertant colonies obtained in the Ames assay
260 are shown in Fig 2. Results of the potential mutagenic effects of the smoke
261 condensate obtained from the *Salmonella*/microsome mutagenicity assay are
262 presented in Table 1. Test samples inducing revertant colonies numbering at
263 least twice the spontaneously induced revertant colonies are considered
264 mutagenic in this assay. Accordingly, all smoke concentrations tested induced
265 mutation in both TA98 and TA100 in a dose dependent manner. The
266 mutagenic effect of the smoke condensate observed was higher against TA98
267 (mutagenicity index of 8.5, 6.5 and 4.5 respectively) compared to that against
268 TA100 (mutagenicity index of 6.7, 4.6 and 2.8 respectively).

269



270 Figure 2: Revertant colonies induced in TA100 using (a) spontaneous control
271 (b) positive control (4NQO).

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283 Table 1: Mean number of his+ revertant colonies/plate in *Salmonella*
284 *typhimurium* TA98 and TA100 treated with different concentrations of the
285 smoke concentrate of *A. seyal.*
286

Sample	Concentration of smoke condensate (μg/ml)	Bacterial strain	
		TA98	TA100
Smoke	0.00	26.9±3.9	128.6±29.1
	1250	117.4±12.3*	364.7±35.7*
	2500	175.3±11.9*	597.6±66.1*
	5000	224.8±21.4*	859.6±103.3*
4NQO	2 μg/ml	234.1±15.2*	977.8±79.1*

287 *Significantly different from the solvent control (0.0 μg/ml) at $p<0.05$.

288 3.7 *Cytotoxicity assay*

289 In this study a C3A cell line was used to assess the cytotoxicity of the smoke
290 condensate. Cell viability decreased as the concentration of the smoke
291 condensate increased (Fig. 3). The concentration that resulted in 50% of cell
292 viability (IC_{50}) was 2560 ± 170 μg/ml. The positive control SDS, had an IC_{50} of
293 0.265 ±0.022 mM which is in agreement with the results previously reported in
294 our laboratories (Makhuvele et al., 2018b, Verschaeve et al., 2017). According
295 to the OECD guidelines for testing of chemicals in genotoxicity assays, a limit
296 of about 55% ± 5% toxicity is considered as an appropriate maximum dose
297 (OECD, 2016). Therefore, smoke concentrations that had ≥ 60% viability in
298 the NRU assay were chosen for the CBMN and comet assays.

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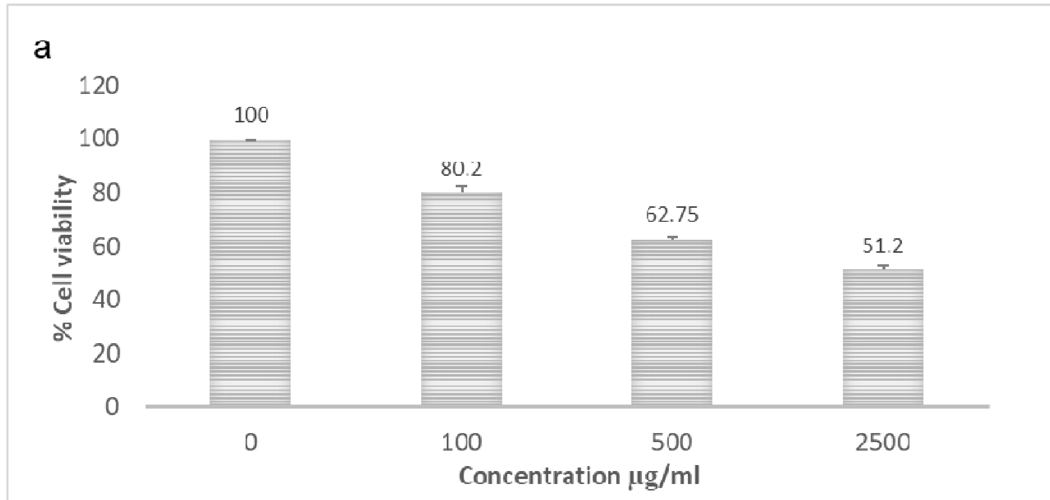

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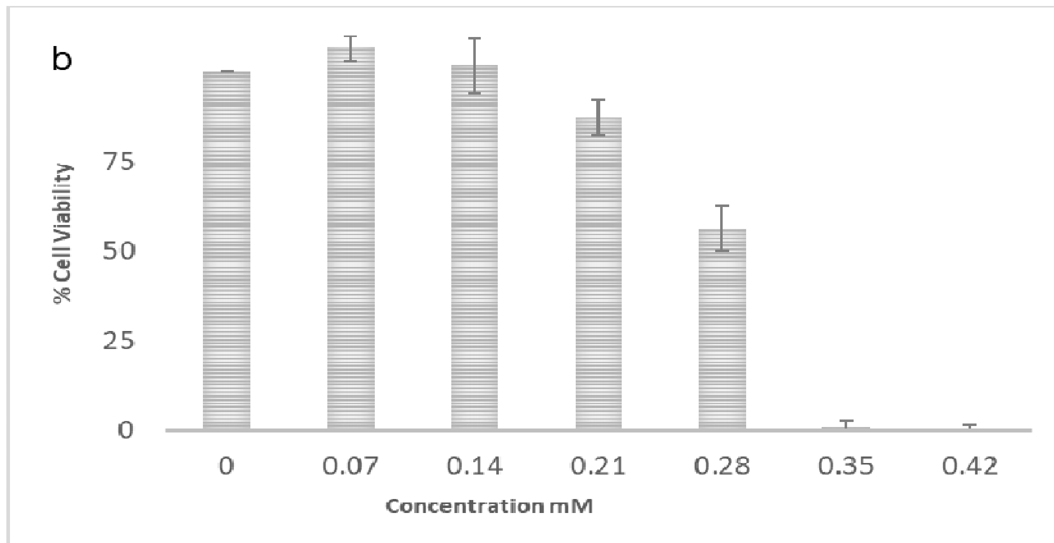


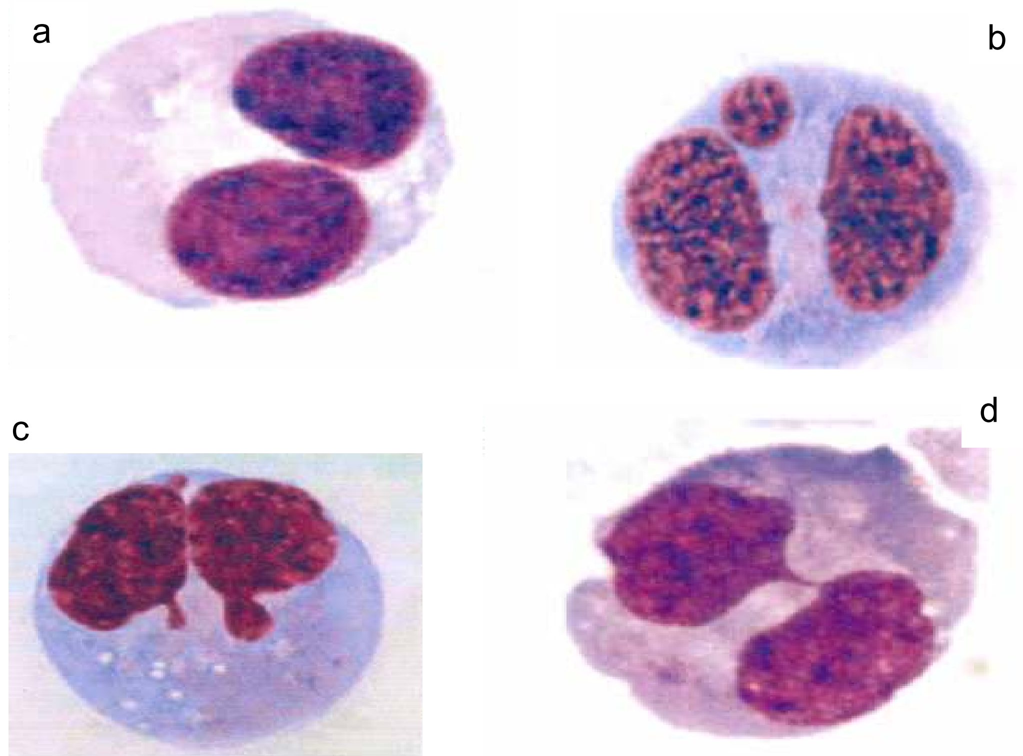
302 Figure 3: Percentage cell viability of C3A cells treated with (a) different
303 concentrations of the smoke condensate of *A. seyal* and (b) SDS.

304

305 *3.8 Micronucleus test*

306 Fig 4 (a-d) presents examples of end points scored in the cytome assay
307 following treatment with different concentrations of the smoke condensate and
308 the positive control. The frequencies of micronuclei, nucleoplasmic bridges
309 and nuclear buds in binucleated C3A cells following the treatment with the
310 smoke condensate are presented in Table (2). Smoke condensate increased

311 the frequency of the micronuclei in a dose dependent manner. However, the
312 increase was only significant at the highest concentrations tested (500 and
313 250 μg/ml) with a slight increase in micronucleated cells at a concentration of
314 125 μg/ml. The smoke also significantly increased the frequencies of
315 nucleoplasmic bridges for all concentrations tested ($p<0.05$) and caused an
316 increase of nuclear buds formation in a dose dependent pattern with statistical
317 significance within the range of 125-500 μg/ml ($p<0.05$). NDI was determined
318 for each treatment for evaluation of the cytotoxic effect of the smoke
319 condensate. No significant differences were observed between the treatments
320 and the negative control.



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322 Figure 4: Photomicrographs of (a) binucleated cell; (b) micronucleated
323 binucleated cell; (c) binucleated cell containing nuclear bud; and (d)
324 binucleated cell containing nucleoplasmic bridge scored in the cytome assay
325 in cells undergoing nuclear division after treatment with the smoke of *A. seyal*.

326

327 Table 2: Micronuclei, nucleoplasmic bridges and nuclear buds frequencies in
328 2000 binucleated C3A cells treated with different concentrations of the smoke
329 condensate.

330

Concentration of smoke condensate (ug/ml)	micronuclei	nucleoplasmic bridges	Nuclear buds	NDI
0	6±1.4	9±2.8	6±2.8	1.68±0.03
62.5	6±2.8	18±8.5*	10±2.8	1.706±0.02
125	15±7.1	20±5.6*	18±2.8*	1.631±0.01
250	18±2.8*	24±2.8*	18±4.2*	1.369±0.13
500	21±8.5*	23±7.1*	33±18.3*	1.414±0.25
4 NQO (1 μg)	13±4.2	28±14.1*	18±16.9*	1.764±0.06

331 *Significantly different from cells treated with negative control ($p<0.05$).

332

333 *3.9 Comet assay*

334 A representative example of undamaged and damaged cells in the comet
335 assay are presented in Fig (5). Results from the comet assay are presented in
336 Table 3. In this study, the DNA damaging effect of *dukhan* condensate was

expressed as %DNA in tail, tail length and tail moment (which is % DNA in the tail multiplied by the distance between the means of heads and tails distribution). The smoke increased DNA damage in all parameters measured in a dose dependent manner. However, significant DNA damage was observed within the range of 125-500 μg/ml when both % DNA and tail length were measured. Whereas DNA damage expressed as tail moment was significant only at the highest concentration tested. The positive control, ethyl methane sulfonate (1 mM), showed significant DNA damage in all of the parameters measured.

Figure 5: Photomicrographs of C3A cells treated with the smoke condensate of *Acacia seyal* after the comet assay was conducted (a) undamaged cell (b) damaged cell showing DNA migration.

378 Table 3. Mean tail length, percentage DNA in tail and tail moment in100 C3A
379 cells treated with different concentrations of the smoke condensate of *A.*
380 *seyal*.

Sample	Concentration of smoke condensate µg/ml	Tail length µm	Tail moment	% DNA in tail
SMOKE	500	26.80±4.6*	16.2±4.2*	63.60±6.4*
	250	18.56±3.4*	7.9±1.5	48.22±4.6*
	125	16.25±4.3*	7.8±1.8	43.83±2.2*
	62.5	7.22±3.03	2.8±1.8	5.40±2.1
Solvent Blank	0 mM	3.80±4.1	2.1±0.9	3.69±4.1
EMS	1 mM	56.21±10.3*	25.3±4.8*	43.40±3.4*

381 *Significantly different from cells treated with the solvent blank ($p<0.05$).
382

383 ## 4 Discussion

384 The use of smoke baths of wood of *A. seyal* in alleviating joint pain and
385 wounds healing motivated the investigation of its anti-inflammatory activity in
386 the cyclooxygenase model. The smoke condensate had no inhibitory effect on
387 the conversion of arachidonic acid to prostaglandins by both COX-1 and COX-
388 2. Previous studies revealed that crude extracts from leaves, wood bark and
389 roots of *A. seyal* had strong inhibitory effects against the activity of both COX-
390 1 and -2 (Eldeen and van Staden, 2008). This is expected as *Acacia* species
391 are rich sources of tannins in comparison to smoke which is rich in volatile

392 compounds. Furthermore the tannins may have been inactivated in the
393 burning process or may have not been volatilized to the smoke in the process.
394 Tannins are known for their ability to bind strongly with a target protein for the
395 cylooxygenase enzymes leading to inhibition of prostaglandin synthesis by
396 blocking the cyclooxygenase enzymes resulting in a false positive anti-
397 inflammatory activity (Eldeen et al., 2005). However, a negative result does
398 not reflect absence of bioactive constituents. The activity experienced by the
399 traditional users could be due to other *in vitro* pro-inflammatory mediators
400 such as tumour necrosis factor α, interleukins and activating nuclear factor κB
401 pathways.
402 Results obtained in this study clearly indicated that smoke condensate from
403 the wood of *A. seyal* collected from eastern parts of Sudan induced mutagenic
404 effects in the bacterial based Ames test, tester strain TA98 and TA100. The
405 tester strains used were selected based on their sensitivity, wide use in risk
406 assessment for detection of mutagens and carcinogens and are most
407 commonly used in the pharmaceutical industry (Makhafola et al., 2016;
408 Makhuvele et al. 2018). The number of revertant colonies obtained for the two
409 strains were in line with those reported in our laboratory and in accordance
410 with other published reports (Makhafola et al., 2016; Makhuvele et al., 2018;
411 Maron and Ames, 1983). The smoke condensate was tested in the Ames test
412 without metabolic activation. The use of metabolic activation with liver S9 was
413 not required given the fact that Sudanese women use the smoke bath
414 externally (skin and inhalation) or topically.
415 In this study, the human hepatocellular carcinoma-derived cell line C3A was
416 used for the detection of genotoxicity in both the cytome and comet assays.

417 This cell line together with HepG2 cell line have been widely used in
418 genotoxicity testing over the last two decades due to their ability to detect
419 direct and indirect mutagen and their ability to express metabolic enzymes
420 needed for the activation and detoxification of genotoxic compounds (Štampar
421 et al., 2021). The constituents of smoke condensate induced genotoxic effects
422 in the mammalian based CBMN assay in the C3A liver cell line. This through
423 inducing a significant increase in the frequency of NPBs (at all concentrations
424 tested), nuclear buds (between 125-500 µg/ml) and Mni (at the highest two
425 concentrations). NPBs provide a measure of chromosomes rearrangement
426 and originates from DNA mis-repair. On the other hand, nuclear buds are
427 associated with the process of gene amplification. While Mni are associated
428 with chromosome breakage and whole chromosome loss in nucleated cells
429 during the anaphase stage of cell division (Fench, 2007, Fench, 2006).
430
431 The comet assay was used to investigate the potential oxidative DNA damage
432 produced by *dukhan*. In the alkaline comet assay the % DNA damage and tail
433 length, which are considered the most suitable parameters to measure DNA
434 damage, were used to evaluate DNA damage of smoke condensate. Results
435 from the alkaline comet assay showed low levels of DNA damage in the
436 untreated cells and a significant DNA damage in cells treated with the positive
437 control EMS, as expected. *Dukhan* also induced a significant dose dependent
438 increase in the level of DNA strand breaks in C3A cells at concentrations
439 higher than 62.5 µg/ml. The toxicity was high with % DNA in tail almost 20
440 times more than the untreated cells at the highest concentration tested and
441 more than the positive control EMS. However, the tail length of the sample

442 was less than half of that of EMS. The difference in tail length may be due to
443 the fact that tail length depends on whether DNA breaks results from single
444 strand (SS) or double strand breaks (DS). SS breaks results in longer tails
445 whereas DS results in shorter tails. DS breaks occur much less frequently
446 than SS breaks but are major precursors in induction of chromosomal
447 aberrations and instability (Morgan et al., 1998). The smoke condensate may
448 be responsible for the induction of Ds breaks and may be considered a
449 potential mutagen (more % DNA in tail and short tail length). The results
450 demonstrate the strong genotoxic effects of *dukhan* to C3A cells.
451
452 In genetic toxicology, it is important to establish whether there is a relationship
453 between DNA damage caused by a test sample and the biological impact of
454 the damage i.e. if the DNA damage is converted into relevant genetic
455 instabilities and gene mutations (Merk and Speit, 1999). The results from
456 Ames, cytome and comet assays which are summarised in Fig. 6 clearly
457 indicated that smoke condensate had genotoxic effects at the concentrations
458 tested. It is also clear that DNA strand breaks in the comet assay could be
459 structurally incorporated into mutations other than gene mutations as
460 supported by a significant increase in the frequency of structural aberrations
461 measured in the micronucleus/cytome assay (Makhafola 2014). There is
462 considerable evidence that links gene and chromosomal mutations to
463 carcinogenesis (Fenech, 2002). Even though only certain mutations lead to
464 cancer, most of the known human carcinogens are detected in conventional
465 short-term genotoxicity tests (Waters et al., 2010). Given that the smoke bath
466 is applied externally, the skin is the organ most exposed to the smoke

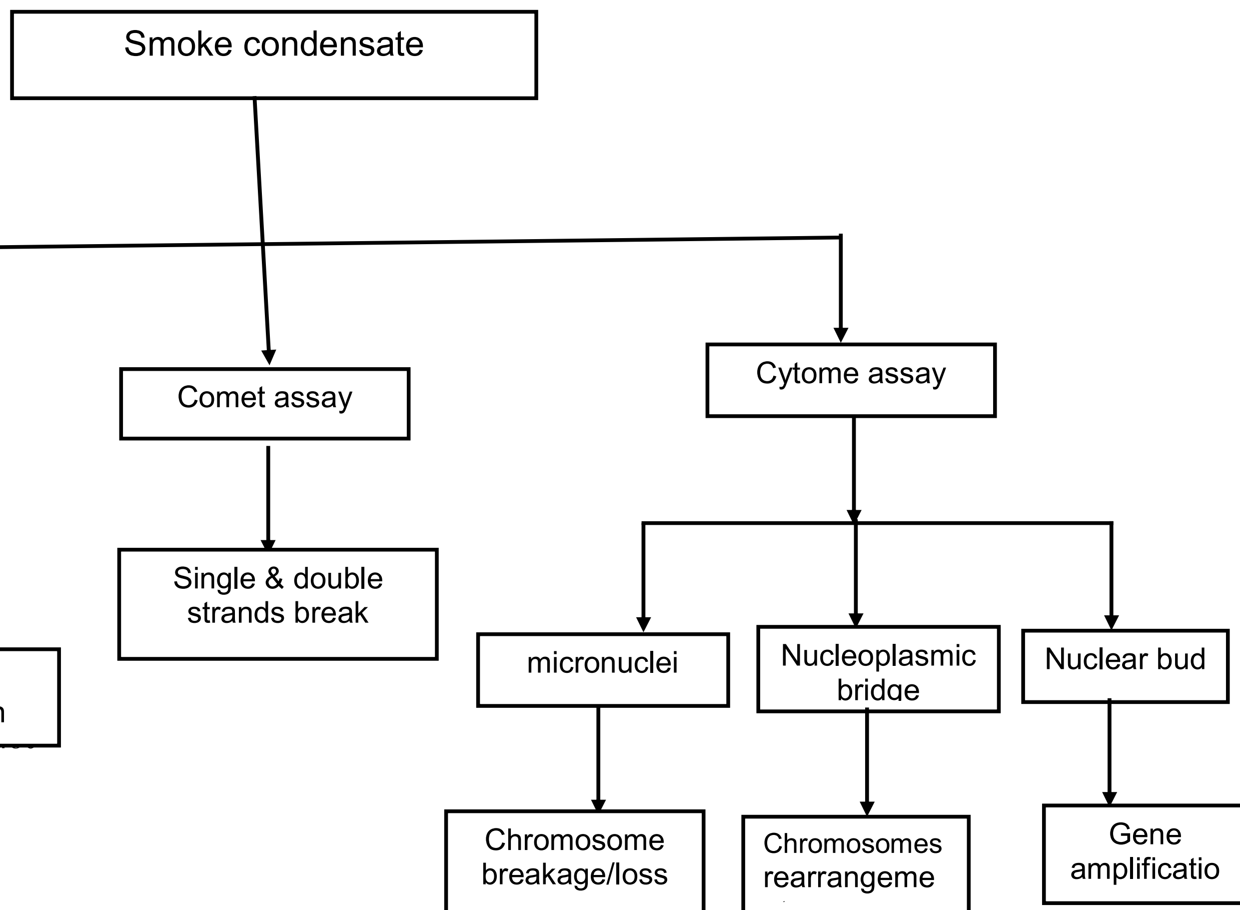

Figure 6: Schematic summary of the probable genotoxic potential of the smoke condensate of *A. seyal* in the *in vitro* model used.

484 followed by the respiratory system through inhalation. These body parts will
485 be most at risk to DNA damage caused by the potential genotoxins in the
486 smoke. The genotoxins in the smoke are potentially carcinogenic and can
487 also lead to other genetic disorders specific to these organs. Even if the
488 concentration of the smoke compounds in the bath are lower than the lowest
489 levels we tested, the results are still concerning.
490
491 Comprehensive epidemiological studies on cancer in Sudan as well as
492 national cancer and death registries are lacking. All published reports on
493 cancer incidence in Sudan are hospital-based surveys (Elamin et al,. 2015).
494 Available literature, however, indicate that breast, cervical and ovarian
495 carcinoma are the most frequent female tumours followed by leukaemia,
496 oesophageal carcinoma and lymphoma (Elamin et al., 2015; Saeed et al.,
497 2016) while the incidence of skin and lung cancers are generally low,
498 particularly among women (Hamad, 2006). It is clear that, from the above-
499 cited reports, there was no correlation between the use of *dukhan* and the
500 incidence of cancer in the body parts mostly exposed to it, i.e. the skin and
501 lungs.
502
503 The lack of correlation between the genotoxic effects of *dukhan* and results of
504 epidemiological studies causes a dilemma in assessing the risk of the
505 practice. However, it is well known that severe DNA damage is part of
506 apoptosis. Therefore, mammalian cells in which DNA damage is induced at
507 relatively high dose levels may not survive during cell division and are hence
508 unlikely to form mutant cell population. This means that the genetic changes

509 will not be passed to the next generation of cells. This applies to the results
510 obtained at high concentrations of 250 and 500 µg/ml used in this
511 study. Ideally mutations which take place at a non-cytotoxic concentrations
512 result in reciprocal translocations, inversions or small deletions in the cell
513 which will be passed from one cell to another during cell division (Ishidate Jr.
514 et al.,1998).
515 There is no literature information on the phytochemical composition of the
516 wood of *A. seyal*. However, linoleic acid and the flavonoids rutin and vicenin
517 have been reported in the seeds, leaf and flowers of *A. seyal* respectively
518 (Subhan et al., 2018). Investigation of other members of the genus *Acacia*
519 revealed that they are rich sources of phenols, alkaloids, saponins,
520 terpenoids, sterol, polysaccharide and fatty acids (Subhan et al., 2018;
521 Magnini et al., 2020). The mutagenic and genotoxic potentials of phenols and
522 terpenoids are well known and the possibility that the mutagenic and
523 genotoxic activities of the smoke of *A. seyal* is due to these compounds is
524 highly likely.
525
526 5 Conclusions
527 The results of this study suggest that *Dukhan* induced genotoxic effects in
528 both bacterial and mammalian cells genotoxicity assays. This was evident by
529 the increase of reverse mutations detected using the *Salmonella*/microsome
530 assay and the increase in the frequency of micronuceleated cells,
531 nucleoplasmic bridges and nuclear buds observed in the cytome assay as
532 well as the % DNA in the comet assay. The findings support the need for
533 further rigorous pharmacological and toxicological evaluation of *Dukhan* for

534 better understanding of the benefits as well as the risks associated with the
535 practise to Sudanese women. Further, characterization of the genotoxic
536 compounds present in the smoke is warranted given that these compounds
537 may help identify other plants of the same chemical prints which may pose
538 risks when used in traditional medicine.

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542

543 Declaration of Competing Interest

544 Authors declare no conflict of interest.

545

546 Authors' contribution

547 EEE performed Ames and cyclooxygenases assays, drafted the manuscript.
548 ISE conceptualised research, processed plant material, TJM performed the
549 mammalian cells based assays, JNE & LV reviewed and finalised the
550 manuscript.

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