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1 # Anti-allergic and anti-inflammatory activities of black
2 cumin extracts in *in vitro* and *in vivo* model systems

3

4 **Nazma Shaheen [1,*], Afiatul Azam [1], Amlan Ganguly [2], Saeed Anwar [3], Md. Sorwer Alam**
5 **Parvez [3], Ujwal Punyamurtula [4], Md. Kamrul Hasan [5]**

6

7 [1] Institute of Nutrition and Food Science, University of Dhaka, Dhaka 1000, Bangladesh; nazmasha-
8 heen@du.ac.bd; nzmshaheen58@gmail.com (N.S.); afiatulazam@gmail.com (A.A.);
9 [2] Department of Clinical Pharmacy and Pharmacology, Faculty of Pharmacy, University of Dhaka,
10 Dhaka 1000, Bangladesh; gangulyamlan@du.ac.bd (A.G.);
11 [3] Department of Genetic Engineering and Biotechnology, School of Life Sciences, Shahjalal Uni-
12 versity of Science and Technology, Sylhet 3114, Bangladesh; saeedanwar.geb@gmail.com (S.A.);
13 sorwersust@yahoo.com (M.S.A.P.);
14 [4] COBRE Center for Cancer Research Development, Proteomics Core Facility, Rhode Island Hos-
15 pital, Providence, RI 02903, USA; ujwal_punyamurtula@brown.edu (U.P.);
16 [5] Department of Biochemistry and Molecular Biology, Tejgaon College, National University, Gazi-
17 pur 1704, Bangladesh; kamrulhasanhridoy205@gmail.com (M.K.H.).
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19
20 * Correspondence:
21 Dr. Nazma Shaheen
22 Professor,
23 Institute of Nutrition and Food Science,
24 University of Dhaka,
25 Dhaka-1000
26 Contact- +8801747799562
27 Email - nzmshaheen58@gmail.com, nazmashaheen@du.ac.bd
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42 # Abstract

43 Black cumin (*Nigella sativa*) is a widely used ingredient of traditional medicine for its broad-
44 spectrum pharmacological actions, including anti-allergic, bronchial asthma, and anti-in-
45 flammatory properties. We sought to evaluate BC extracts' efficacy for their anti-allergic and
46 anti-inflammatory properties using a comprehensive *in vitro*, *in vivo*, and *silico* experimental
47 setup. To investigate whether BC extract has anti-inflammatory, anti-allergic, and analgesic
48 therapeutic potentials *in vitro* and *in vivo*. The activity of BC was assessed through anti-
49 allergic activity on rat basophilic leukemia-2H3 cell line, anti-inflammatory activity on
50 J774.1A cell line, anti-inflammatory activity by carrageenan-induced rat paw edema, anal-
51 gesic activity by acetic acid-induced writhing test, and ingenuity analysis of the BC extracts
52 in inflammation control. BC exerted potent anti-allergic activity by inhibiting antigen-in-
53 duced degranulation. An anti-inflammatory effect is shown by inhibiting TNF-α production.
54 The acetic acid-induced writhing test shown a dose-dependent reduction of writhing number
55 following BC administration. Rat paw edema test shown the dose-dependent reduction of
56 paw edema volume following BC administration. Ingenuity Pathway Analysis (IPA) sug-
57 gested BC extracts containing ferulic acid, p-coumaric acid, kaempferol, and quercetin can
58 inhibit inflammation. This study suggests that bioactive compounds in BC extract act as an
59 anti-allergic and anti-inflammatory agent by regulating several downstream and upstream
60 inflammation pathways.

61 **Keywords:** Black cumin; *Nigella sativa*; anti-allergy; anti-inflammation; edema

62 **Abbreviations**

63 BC, Black cumin; RBL, Rat basophilic leukemia; IPA, Ingenuity Pathway Analysis; AWA,
64 Acetone-water-acetic acid; DMSO, Dimethyl sulfoxide.

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66 # Introduction

67 Inflammation is a fundamental part of the body's physiological defense mechanisms
68 against pathogenic infections and toxic substances [1]. It is involved in the body's response
69 to both the initial cause and the consequences of an injury. Often, however, inflammations
70 can be triggered inappropriately, leading to tissue destruction, which in turn can result in a
71 range of inflammatory disorders, including rheumatoid and gouty arthritis, psoriasis, and
72 Crohn's disease [2]. Many studies suggest that a persistent inflammatory condition can be
73 pervasive and develop into more clinically severe afflictions, including cardiovascular dis-
74 ease and cancer, often with fatal outcomes [3] [2] [4].
75
76 Current management strategies for inflammatory diseases include medications, relaxa-
77 tion, exercise, and surgery to correct joint damage. The medications used to treat inflamma-
78 tory diseases, such as non-steroidal anti-inflammatory drugs, corticosteroids, cyclophospha-
79 mide, hydroxy-chloroquine, and biologic drugs, possibly minimize disease progression by
80 lowering joint pain, swelling, and the inflammation itself [5] [6] [7] [8]. The type of manage-
81 ment strategy is contingent on many factors, including the patient's age, medical background
82 and comorbidity, immunity status, and the severity of the symptoms of inflammatory disease
83 [5] [8]. However, the efficacy of these management strategies can be questionable, and even
84 if the efficacy is satisfactory, many of these strategies are often not suitable for all patients
85 because of associated side effects [9]. Both steroidal and non-steroidal inflammatory drugs
86 are associated with a high range of adverse effects in the long-term [10] [11]. There is thus a
87 constant push toward the development of an effective and curative therapeutic strategy to
88 treat inflammatory diseases.
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90 Medicinal plants are an essential source of bioactive compounds with potential thera-
91 peutic efficacy [12]. Pharmacological investigations of medicinal plants can yield primers
92 for the effective treatment of inflammatory diseases [12]. Black Cumin (BC, *Nigella sativa*
93 L.) is a well-known medicinal plant used extensively in Unani, Ayurvedic, and Siddhi med-
94 icine for centuries [13]. This herb, endemic to South Asian and Mediterranean countries, is
95 rich in bioactive compounds, including tocopherols, alkaloids, saponins, and vitamins A and
96 C, all of which contribute to its biological functionality [14] [15] [16]. Overwhelming evi-
97 dence indicates the presence of bioactive ingredients in BC that can counteract the underlying
98 pathophysiology of many diseases, including cancers, inflammatory conditions, cardiovas-
99 cular defects, and autoimmune disorders [14] [17] [18]. Previous studies have highlighted
100 the probable anti-inflammatory and anti-analgesic activities of BC [19] [20]. However, only
101 a limited number of studies have explored BC's anti-inflammatory effects on subacute and
102 chronic models of inflammation [20].

103 As a result, we sought to investigate the dose-response effects of the anti-inflammatory
104 activity of BC in rats and mice with *in vitro* and *in vivo* anti-inflammatory, anti-allergic and
105 anti-analgesic activities. Using these findings, the present study has taken an attempt to de-
106 velop a probable mechanism of action for *N. sativa* through Ingenuity pathway analysis.
107

108 Materials and Methods

109 Collection and preparation of black cumin samples

110 Trained food sample collectors collected the samples from New market Kancha Bazar,
111 Dhaka, Bangladesh and then critical checking of BC seed samples by the expert faculty mem-
112 ber of the Department of Botanist, University of Dhaka. Preparation and processing of the

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113 collected samples were done using standard operating procedures. Processing involved dry-
114 ing of the fresh samples at 25 - 30°C, grinding by an electric blender, and preserving in an
115 airtight container until analysis.

116 **Extraction procedure**

117 Multiple rounds of sequential extraction (initially by hexane/dichloromethane (1:1 v/v)
118 (Merck, Germany; hexane- 296090, dichloromethane- 270997) and then by AWA (ace-
119 tone/water/acetic acid 70:29.5:0.5) (Merck, Germany; acetone- 650501, acetic acid- A16283)
120 was performed in an accelerated solvent extraction equipment known as ASE 200 (DIONEX,
121 USA, catalogue number: 055422). A detailed description of the extraction procedure has
122 been reported elsewhere [21] [22]. While ground samples were directly mixed with Dimethyl
123 sulfoxide (DMSO) (Sigma Aldrich, #D- 2650 Poland) to get the sample extracts for
124 assessment of anti-allergic and anti-inflammatory activities in *in vitro* cell line models, *in*
125 *vivo* assessments conducted in this study mixed dried AWA extracts with DMSO to prepare
126 the sample extracts.

127 **Experimental animal**

128 Swiss Albino mice (5 - 6 weeks of age; 20 - 30 grams) and Long-Evans rats (7 - 8 weeks of
129 age; 100 - 130 grams) were collected from the Animal Research Branch, International Centre
130 for Diarrheal Disease Research, Bangladesh (ICDDR, B). The animal were housed in poly-
131 vinyl cages and maintained under standard laboratory conditions (temperature 25 ± 2°C) and
132 a 12 h light - 12 h dark cycles for seven days. The animals were fed on standard laboratory
133 animal diet formulated by ICDDR, B and water *ad libitum*. To keep the hydration rate con-
134 stant, food and water were stopped 12 h before the experiments. The Ethical and Animal Care
135 Committee of the Institute of Nutrition and Food Sciences, University of Dhaka, Bangladesh,
136 critically reviewed and approved this study involving in vivo models. The procedures de-

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137 scribed in this study were conducted in accordance with the Bangladesh Biosafety and Bi-
138 osecurity guidelines 2019 and institutional oversight performed by qualified veterinarians
139 [23].

140 **Anti-allergic activity on rat basophilic leukemia-2H3 cell line**

141 Rat basophil leukemia (RBL)-2H3 cells [NIHS (JCRB), Tokyo, Japan: Cell No.: JCRB
142 0023] were used to study the anti-allergic activities of BC extracts. RBL-2H3 cells were
143 grown in minimal essential medium (Eagle) containing 15% fetal calf serum. We then inoc-
144 ulated 05 $\times$ 10^5 cells in each well of a 24-well plate and incubated at 37˚C in an environment
145 of 5% carbon dioxide for affluent growth. After overnight incubation (4 $\times$ 10^6 cells in each
146 well of a 24-well plate), mouse monoclonal anti-DNP IgE (Sigma Aldrich #A- 6661, UK)
147 solution was added to each of the 24 wells before incubation (for two hours at 37˚C).

148 The cells were then incubated with 10 µl of DNP labeled human serum albumin for 30
149 minutes. Supernatants were separated after lysis of cells with 500 µl of Triton X - 100. The
150 cell lysate (50 µl) along with the collected supernatant was transferred to the 96-well plate
151 and mixed with 100 µl of 0.1 M citrate buffer containing 3.3 mM para-nitrophenyl-2-acet-
152 amide-β-D-glucopyranoside (Wako, Japan). The supernatant-cell lysate mix was then kept
153 under incubation for 25 minutes at 37°C; it was followed by an addition (100 µl) of 2 M
154 glycine buffer of pH 10.0 which stopped the reaction in a microplate reader and measurement
155 of the absorbance. A stop solution was added to the lower four lanes which were used as
156 control, and then at the same time, the substrate was added to the experimental well.

157 Blank without antigen and another negative control were used as a positive control with
158 antigen and 5 µM of wortmannin (Wako, Japan) solution. Blank, negative control, and posi-
159 tive control were prepared by adding 490 µl of modified Tyrode's buffer and 200 folds diluted
160 wortmannin solution.

161 **Anti-inflammatory activity on J774.1A cell**

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162 Anti-inflammatory potential using J774.1A cell assay was assessed using the method as
163 reported by Herath et al. [24]. Shortly, ground BC seed samples were mixed with DMSO to
164 prepare 4mg/ml concentration and final strength of 40 μg/ml was employed in the assay me-
165 dia. For the assay, a clear supernatant was obtained by centrifuging that mixture at 3000 rpm
166 for 5 minutes. Mouse macrophages J774A.1 cell line [NIHS (JCRB) 9108] were cultured in
167 Dulbecco's Modified Eagle Media (DMEM) (Sigma Aldrich #D-5030**,** USA) with 10% fetal
168 calf serum and penicillin and streptomycin (Sigma Aldrich #F-7524, UK) at a concentration
169 of 100 U/mL. The cells were incubated at 37˚C in the incubator with an atmosphere of 5%
170 CO2, 95% air.

171

172 The cell suspension with the concentration of 5.0 x10^5 cell /ml (200 μl/well) was placed
173 in 96 well culture plates and incubated overnight (37˚C, 5% CO2-95% air). Overnight cul-
174 tured cells washed thrice with Hank's solution (Sigma Aldrich #6648, UK) (37˚C) after re-
175 moval of culture media prior to the addition of 180 μl DMEM and followed 20 μl of 10 times
176 diluted sample extracts (final concentration, 40 μg/ml) to the each well without lipopolysac-
177 charides (LPS). On the other hand, 160 μl DMEM with 20 μl of LPS (final concentration,
178 1.0 μg/ml) after the addition of the test samples and incubated further for more than four
179 hours. Cell supernatant was then collected for the assay of the TNF-α production, assayed by
180 using Mouse TNF-α ELISA kit (Ready-Set-Go, eBioscience, USA). In brief, 50 μl of TNF-
181 α capture antibody (250 times diluted) was added into 96 well plates and kept in the refrig-
182 erator (4˚C) overnight. The plate was washed with 300 μl of PBS-T (0.05%Tween20 in PBS)
183 3 times in a sera washer (Model MW-96F., Biotech, Japan) and 100 μl of assay diluents
184 solution was added as blocking solution. After incubation for 1hour at room temperature, the
185 plate was washed and 50 μl of cell supernatant /standard. TNF-α was added into the wells
186 according to plate design. Sample extract-treated cell supernatant was diluted 10 times with
187 assay diluents, whereas, the standard was added to maintain the final concentration from 0 to

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188 1000 pg/ml and further incubated for 2hour at RT. After at least 2 hrs incubation, plates were
189 washed again and TNF-α detection antibody (250 times diluted) was added (50 µl) into each
190 well and further incubated for 1hour. Avidin- HRP antibody solution (250 times diluted) was
191 added in the same volume as earlier after washing and kept 30 minutes at RT. Finally, 50 µl
192 of TMB-HRP substrate solution was added and incubated for 15 min., a stop solution (25 µl)
193 was added into each well to stop the reaction and the absorbance was read in the microplate
194 reader (model 550; Bio Rad, CA, USA) at 450 nm. From the absorbance data, the inhibition
195 effect of food extracts on TNF-α production was calculated from the standard graph. Dose-
196 response was done for the samples which had an inhibitory effect on TNF-α production at
197 the concentrations of 40, 10, 3, and 1 µg/ml.

198 **Assessment of anti-inflammatory activity by carrageenan-induced rat paw edema**

199 Evaluation of anti-inflammatory activity was conducted *in vivo* in the Long Evans rat
200 model. At first 200 mg of samples (i.e., dried AWA extract) were mixed with 5.0 ml of
201 DMSO in a shaking-incubator at 130 rpm overnight. Then the mixture was centrifuged at
202 3000 rpm for 5.0 minutes. Then the supernatant was collected and pipetted 500 µL of aliquots
203 to store at 25°C.

204 To determine anti-inflammatory activity, edema was induced by the phlogistic agent
205 carrageenan [25]. The control group (n=3) received normal saline per os (p.o) (10 ml/kg),
206 while group 2 received a standard drug, Diclofenac sodium (50 mg/kg, p.o). The rest of the
207 groups were given 800 (n=3), 400 (n=3), and 200 mg/kg body weight (n=3) p.o. of extract
208 preparations. Thirty minutes after administering sample extracts, each rat received 0.1 ml of
209 1% (w/v) carrageenan (injected into the sub-plantar region of the right hind paw subcutane-
210 ously) (Sigma Aldrich, # C-1013, Germany). Paw volumes of the right hind paw of each rat
211 were measured by plethysmometer before and 1, 2, 3, and 4 hours after carrageenan injection
212 to determine the edema volume. The hind paw volume was evaluated for anti-inflammatory

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213 activity and expressed as % inhibition of the hind paw volume, which was calculated by the
214 following equation:

215 % inhibition = [(Vc - Vt)/Vc] ×100

216 Here, Vc = average paw volume of the control group, and Vt = Average paw volume of
217 the treated group.

218 **Assessment of analgesic activity by acetic acid-induced writhing test**

219 Peripheral analgesic activity of the extracts was determined by the acetic-acid-induced
220 writhing inhibition method in mice [26]. The control group (n=3) (group 1) received normal
221 saline (10 ml/kg, p.o.). Group 2 received a reference drug Diclofenac sodium (50 mg/kg,
222 p.o). The rest of the groups received 800 (n=3), 400 (n=3), and 200 mg/kg body weight (n=3)
223 p.o. of dried AWA sample extracts. After 30 min of treatment, each mouse was administered
224 intraperitoneally with 0.6% acetic (10 ml/kg). Later, the writhing numbers of each mouse
225 were observed for 10 minutes. To evaluate the level of analgesia, writhing numbers of the
226 sample treated mice were compared with the writhing numbers of the control groups. Per-
227 cent-inhibition of writhing was calculated using the following equation:

228 % inhibition of writhing = [(Wc - Wt)/Wc] ×100

229 Here, Wc = Average number of the writhing of the control group, and Wt = Average
230 number of the writhing of the treated group.

231 **Ingenuity analysis of the BC extracts in inflammation control**

232 We evaluated the effects of the compounds extracted from BC using the knowledge-
233 based path explorer feature of the Ingenuity Pathway Analysis (IPA) tool [27] [28]. We ex-
234 plored new ingenuity-based pathways that showed the relationship between the genes in-
235 volved in the biosynthetic pathways of inflammation-related compounds (e.g., arachidonic
236 acids, prostaglandins, and leukotrienes) and compounds in the BC extracts, which includes
237 ferulic acid, quercetin, p-coumaric acid, and kaempferol. The shortest possible pathways

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238 were generated individually for each compound where the compounds in the BC extracts
239 were set as the initial compound, and inflammation-related compounds were selected as the
240 target.

241 ## Results

242 ### *In vitro* screening for anti-allergic effect

243 As compared to the negative control, BC extracts produced 46.9% (SD ±7.1) inhibition
244 of the antigen-induced degranulation of RBL-2H3 cells. AWA extracts produced increased
245 inhibition compared to the DMSO extracts for 5, 10, 20, and 40 μg/ml concentration. How-
246 ever, at higher concentrations, e.g., 40 μg/ml, both extracts produced comparable inhibition
247 (Figure 1).

248 ### *In vitro* screening for anti-inflammatory effect

249 In the preliminary evaluation of anti-inflammatory effects on J774A.1 cell, 40 μg/mL of
250 DMSO extracts of BC resulted in significant (70%) inhibition of TNF-α production. Dose-
251 response analysis showed that 1, 3, 10, and 40 μg/mL of DMSO extracts could produce up
252 to 70% inhibition in the TNF-α production.

253 ### *In vivo* anti-inflammatory activity

254 Four hours after carrageenan injection, the maximum volume of edema in control Long
255 Evans adult rat models was 1.18 ± 0.03 ml. Another group of rats pretreated with Diclofenac
256 sodium (reference drug; 50 mg/kg, p.o), showed significantly reduced ($p < 0.05$) edema in
257 the paw. Dose-response analysis of DMSO extract of BC produced a significant inhibition
258 of paw edema in Long Evans adult rats in a dose-dependent manner at 200, 400, and 800
259 mg/kg, p.o administration of carrageenan ($p<0.05$) (Table 1).

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260 The maximum inhibition of rat paw edema by BC was noted 4 hours after administration
261 of carrageenan at a dose of 800 mg/kg, p.o. compared to the control but less than the reference
262 drug. Figure 2 shows the changes of percent inhibition of carrageenan-induced paw edema
263 volume at 1, 2, 3, and 4 h at the doses of 800 mg/kg, p.o by the DMSO extract of BC when
264 compared to the control.

265 ***In vivo* analgesic activity**

266 The effects of DMSO extract of BC on 0.6% acetic acid-induced writhing in Swiss albino
267 mice are summarized in Table 2. A dose-dependent and significant ($p<0.05$) reduction in the
268 number of abdominal constrictions induced by intraperitoneal administration of 0.6% acetic
269 acid was observed with oral administration of BC, at the doses of 200, 400, and 800 mg/kg,
270 p.o when compared to the control. Among the different doses, the DMSO extract of BC at
271 the dose of 800 mg/kg exhibited the maximum inhibition of the number of writhing compared
272 to that of control.

273 The maximum inhibition of rat paw edema by BC was noted 4 hours after admin-
274 istration of carrageenan at a dose of 800 mg/kg, p.o. compared to the control but less than
275 the reference drug. Figure 2 shows the changes of percent decrease in carrageenan-induced
276 paw edema volume at first, second, third, and fourth hours post-administration of 800
277 mg/kg, p.o of DMSO extract of BC when compared to the control.

278 ***In silico* analysis provides insights into the mechanism of how BC extracts reduce in-**
279 **flammation**

280 The *in-silico* analysis of the bioactive compounds of BC illustrates a facet reactome de-
281 picting potential models of how BC extracts reduce inflammation in humans (Figure 3, S1
282 Table). Both prostaglandin and leukotriene synthesize from the arachidonic acid. Quercetin,
283 p-coumaric acid, and kaempferol reduce the expression of myc, MAPK, EGFR, and TNF, all

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284 of which are involved in the upregulation of the expression of arachidonic acid. Conse-
285 quently, quercetin, p-coumaric acid, and kaempferol downregulate the expression of arachi-
286 donic acids.

287 Ferulic acid, another component in the BC extract, negatively induces the synthesis of
288 phospholipids. Phospholipid biosynthesis is critically important for the synthesis of arachi-
289 donic acids. Also, ferulic acids upregulate the expression of HMOX and nrf2, resulting in the
290 reduction of prostaglandin E2 (PGE2). Moreover, all the compounds of the *Nigella* extract
291 directly reduce the expression of cox1 and cox2, two essential factors for the synthesis of
292 PGE2. Collectively, ferulic acid, quercetin, p-coumaric acid, and kaempferol help reduce
293 inflammation by interfering with the extended arachidonic acid pathway (Figure 3; S1 Ta-
294 ble).

295 **Discussion**

296 In this study, we demonstrate the *in vitro* dose-response of the DMSO extract of BC to
297 evaluate the anti-inflammatory and analgesic activities in the experimental models. The in-
298 hibition of the antigen-induced degranulation at the same concentration of 41 food samples
299 was assayed in the rat basophilic leukemia RBL-2H3 cell line (data not shown). A further
300 dose-response (40, 20, 10 and 5μg/mL) assay was carried out to confirm their anti-allergic
301 activity of the bioactive compound as present in food samples and only DMSO, as well as
302 AWA extract of BC, exhibited dose-response which support the concept that anti-allergic
303 activity belongs to the phenolic compounds. Recent research findings showed that polyphe-
304 nols, widely distributed among fruits, vegetables, and herbs, are widespread for their antiox-
305 idant capacity [29]. They also reported that some of them could exert anti-allergic activities
306 [30] [31] [29].

307 Carrageenan-induced paw edema in rat models is increasingly being employed to deter-
308 mine anti-inflammatory effects in animal models. In response to carrageenan injection, the

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309 formation of edema occurs in a biphasic process. Mast cells around the damaged tissue re-
310 leased prostaglandin with histamine and serotonin mediate the first phase (1 - 2 h) of the
311 carrageenan model. Bradykinin, leukotrienes, polymorphonuclear cells, and continuous se-
312 cretion of prostaglandins from macrophages tissue mediate the second phage (3 - 5h) of the
313 carrageenan model [32] [25]. Our study indicates that the DMSO extracts of BC exhibited
314 significant ($p < 0.05$) inhibition of paw edema in rats at the doses of 200, 400, and 800 mg/kg,
315 p.o in the second phases of inflammatory response. DMSO extracts of BC at the dose of 800
316 mg/kg produced the maximum inhibition of carrageenan-induced paw edema volume. The
317 result of the present study showed the maximum inhibition of carrageenan-induced paw
318 edema volume in rats by BC in the second phase of post carrageenan injection at 3-5 h, maybe
319 due to the modulatory principles acting with the prostaglandin alley. Moreover, the present
320 study supports the previous findings that BC seed polyphenol plays a crucial role as protec-
321 tive factors against

322 "Acetic acid-induced writhing experiment" is a well-known protocol for assessing the
323 analgesic potency of medicinal products [33]. Intraperitoneal injection of acetic acid that
324 triggers pain sensation is due to the prostaglandins and lipoxygenase products from arachi-
325 donic acid liberated from phospholipids by cyclooxygenase [34]. Thus, the significant
326 ($p<0.05$) reduction of writhing in this study by DMSO extracts of BC at the doses of 200,
327 400, and 800 mg/kg, p.o suggest analgesic activity peripherally mediated through inhibition
328 of prostaglandins and other endogenous pain mediators. The highest concentration (800
329 mg/kg) of the test samples exhibited a peak of analgesic effect significantly ($p<0.05$) in the
330 acetic acid-induced writhing test. These results indicate the peripheral analgesic potential of
331 the DMSO extracts of the test samples which could be exhibited due to the suppression of
332 peritoneal surface receptors through inhibited cyclooxygenase activity. The current study
333 findings show concordance with previous findings that BC seed polyphenol inhibits acetic
334 acid-induced writhing in the mouse model [35].

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335 Through an examination of the literature, we also note how to present findings confirm
336 previous work in the field. Specifically, the *in vitro* and *in vivo* experiments performed in this
337 study are in good agreement with those of prior experiments on BC's anti-inflammatory ac-
338 tivity, lending credence to the idea that BC is a potent medicinal plant for therapeutic uses.
339 In addition to the wet-lab analysis, ingenuity pathway analysis provides evidence for the
340 role of the *Nigella* extracts in the reduction of inflammation. Earlier studies revealed that
341 some molecules including myc, MAPK, EGFR, TNF, are involved in the cellular responses
342 for inducing the inflammation [36] [37] [38] [39]. We have found that compounds in the
343 *Nigella* extracts, including ferulic acid, quercetin, p-coumaric acid, and kaempferol, down-
344 regulates the inflammation-inducing signaling molecules in humans (Figure 3). The potential
345 role of the activation of pKA, HMOX, and nrf2 pathways led to anti-inflammatory effects
346 has been reported in many research groups [40] [41] [42] [43]. Our analysis uncovered that
347 compounds in the *Nigella* extracts upregulate the pKA, HMOX, and nrf2 (Figure 3).
348 Furthermore, we have shown the potential aspects of the anti-inflammatory mecha-
349 nism of BC that were previously only speculated upon. For example, Nasuti *et al.* found BC
350 to reduce only the acute inflammation suggested that BC's mechanism of action is tied to
351 interleukins, TNF, and prostaglandins [44]. In the ingenuity pathway analysis, we have
352 shown various components of BC act through IL32, IL8, IL1, TNF, and PGE2 to reduce
353 inflammation. In another study, researchers discovered that BC could decrease either IL-6
354 levels of IL-1B levels, depending on its storage conditions [45]. These findings establish a
355 connection between BC and interleukins in BC's anti-inflammatory activity, which we have
356 at least partially outlined in Figure 3. Similarly, other researchers speculated that BC WSE
357 inhibits COX2 and that BC inhibits prostaglandin synthesis through interaction with COX-1
358 and COX-2 [46]. Both hypotheses are supported by the results of our Ingenuity pathway
359 analysis, as depicted in Figure 3. Furthermore, Babar et al. suggested that phenols/polyphe-
360 nols in BC might be responsible for its anti-inflammatory activity [46]. Through our pathway

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361 analysis, we have found that these phenols and polyphenols are ferulic acid, p-coumaric acid,
362 kaempferol, and quercetin. It is speculated that the inhibitory effect on the secretion of leu-
363 kotrienes and prostaglandins by thymoquinone may be responsible for BC's anti-inflamma-
364 tory activity [47]. We have shown that this is the case, although, instead of thymoquinone,
365 quercetin and ferulic acid were found to be the components of BC responsible for this aspect
366 of its anti-inflammatory mechanism. In another study, *N. sativa* showed superior activity in
367 a screening of *Nigella* species for *in vitro* inhibition of PGE2 production catalyzed by COX1
368 and COX2 [48]. This lends credence to the mechanistic schematic we have depicted in Figure
369 3; as is shown in that diagram, several components of BC act to inhibit COX1/COX2 activity,
370 which is indeed necessary to produce PGE2. In yet another study, researchers discovered that
371 levels of erk are decreased upon *N. sativa* treatment of damaged rat lungs [49]. The erk re-
372 pression discussed here is supported by our mechanistic analysis, which has found that
373 kaempferol, a component of *N. sativa*, inhibits ERK1/2 activity. As evidenced by these ex-
374 amples from the literature, the results we have found build on previous research to provide
375 crucial insight into the medicinal properties of a common natural product.

376 Together with the *in vitro* and *in vivo* studies, the *in silico* analysis in this study provides
377 strong evidence that the bioactive compounds in BC extract act as an anti-inflammatory agent
378 by regulating several downstream and upstream pathways of inflammation.

379 **Conclusion**

380 In summary, the findings of the present study support that a BC extract has anti-inflamma-
381 tory, anti-allergic, and analgesic activity. This study, involving *in vitro* and *in vivo* systems,
382 indicates the possibility that BC extract might help treat pathologies that involve chronic
383 inflammation and pain. Also, *in silico* analyses suggested that the phenolic compounds in BC
384 extracts may play a key role in inhibiting inflammatory reactions in human? Besides, through

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385 the Ingenuity pathway analysis, the present study has supported and elaborated on sugges-
386 tions found in the literature regarding BC's biochemical mechanism of action. Thus, in un-
387 derstanding how to present findings relate to prior work in the field, we have gained insight
388 into the vitality of this research for future efforts to study the anti-inflammatory effects of *N.*
389 *sativa*. Future studies should focus on elucidating the mechanistic pathways through which
390 compounds in BC extracts exert such pharmacologic effects to prevent inflammation.

391 **Supplementary Materials:** Table S1: Components of the reactome *Nigella sativa* works on
392 to impact on human inflammations.

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396 A.A., A.G., S.A., M.K.H. and M.S.A.P.; software, S.A., and M.S.A.P; validation and scruti-
397 nization, N.S., M.K.H., and U.P; investigation, N.S., A.A., A.G., S.A. and M.S.A.P.; formal
398 analysis, N.S., A.A., A.G., S.A., and M.S.A.P.; writing—original draft preparation, A.A.,
399 A.G., and S.A.; writing—review and editing, N.S., M.K.H., and U.P.; supervision, N.S.; pro-
400 ject administration, N.S., and M.K.H.; funding acquisition, N.S.

401 **Competing Interest:** The authors do not have any conflicting interest.

402 **Approval for animal experiments:** Animals were housed at the Animal Housing Facility
403 of Institute of Nutrition and Food Sciences, University of Dhaka, Bangladesh, following
404 guidelines by their Ethics Committee for the Treatment of Laboratory Small Animals. All
405 procedures were reviewed and approved by the Ethical and Animal Care Committee of the
406 Biological Science faculty, University of Dhaka, Bangladesh. The committee's reference
407 number is not available.

408 **Approval for human experiments:** Not applicable.

409 **Approval for experimental procedures:** All experimental procedures were approved by the
410 internal committee of Biological Science faculty headed by Dean of Bioscience faculty, Uni-
411 versity of Dhaka, Bangladesh.
412

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414 in the study.

415 **Data availability:** Authors always welcome to share the data required for reviewers and
416 other researchers.

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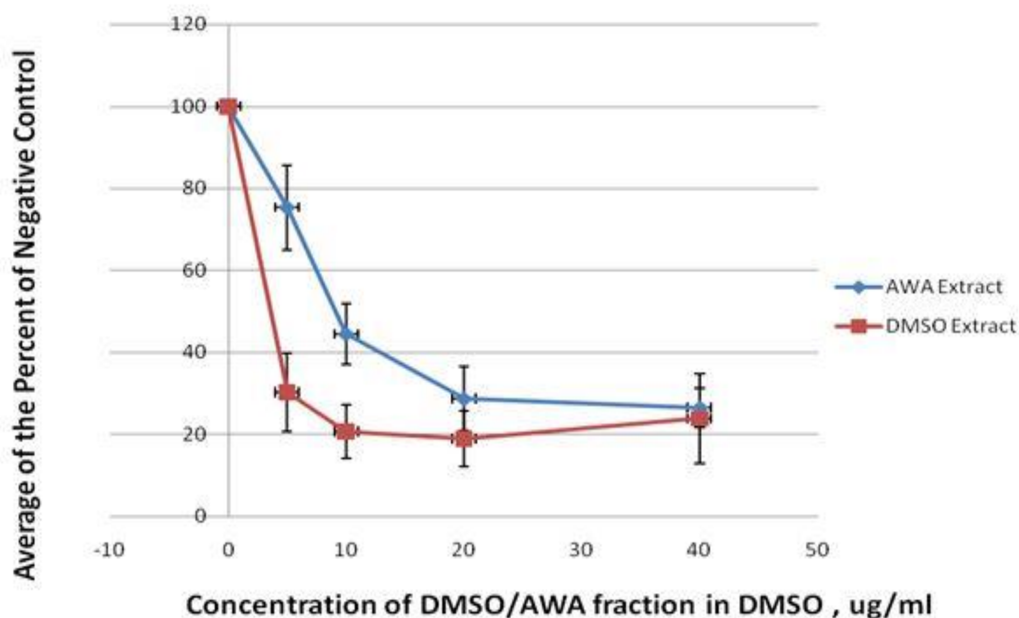
619 **Figure (s)**



620

621 **Fig.1.** Comparison of dose-response of the antigen induced degranulation of BC
622 extracts by DMSO (red color) and AWA (blue color) as percentages of negative
623 control at the level of 5, 10, 20 and 40 µg/ml concentration.

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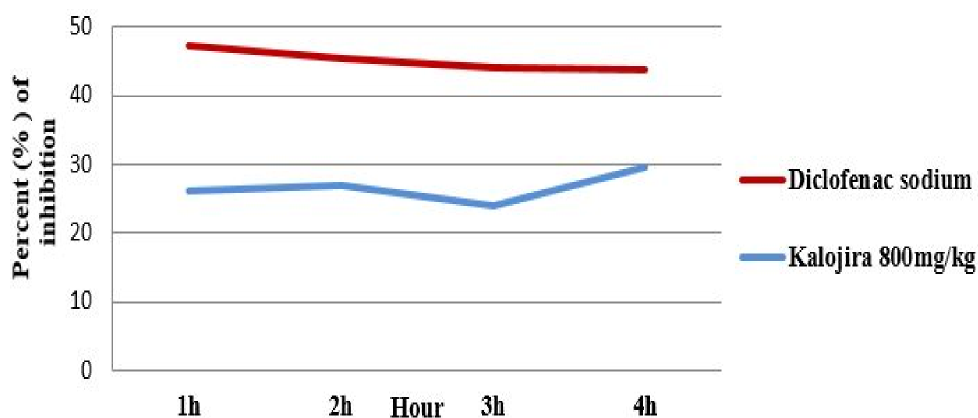

626

627 **Fig.2.** Time course in the changes of percent inhibition on carrageenan-induced paw
628 edema volume of BC at 1, 2, 3, and 4 h at the dose of 800 mg/kg, p.o.

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632

633 **Fig. 3.** Possible mechanism of action of BC extracts to reduce inflammation in hu-
634 mans. Phenols and polyphenols, e.g., ferulic acid, p-coumaric acid, kaempferol, and
635 quercetin in the BC extracts, inhibit the signaling molecules, stimulation of which
636 may directly or indirectly result in inflammation. Proteins linked to inflammation
637 participate in several canonical pathways underlying a range of biological activities.
638 Ingenuity pathway analysis (IPA) identified that the BC extracts' components could
639 target many of these proteins (Table S1) and control inflammation. Arrows (↓) in-
640 dicate direct stimulatory modification, whereas light up signs (┬) indicate an inhib-
641 itory modification. The Names of the proteins followed the Hugo nomenclature,
642 NCBI Entrez, UniProtKB/Swiss-Prot system.

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659 660 **Table 1**: Effects Black Cumin extracts by DMSO extracts of *BC* on carrageenan-induced paw in rats

Group	**Dose (mg/kg, p.o)**	**Paw edema volume [a] (ml) (% of inhibition)**			
		1 hr	**2 hr**	**3 hr**	**4 hr**
Control	--	0.83 ± 0.02	0.96 ± 0.02	1.07 ± 0.02	1.18 ± 0.03
Diclofenac sodium	50	0.44 ± 0.0* 47.18	0.53 ± 0.02* 45.32	0.60 ± 0.01* 44.10	0.66 ± 0.01* 43.79
Nigella sativa	200	0.71 ± 0.01* 14.15	0.78 ± 0.01* 19.03	0.89 ± 0.02* 16.26	0.94 ± 0.03* 20.62
	400	0.63 ± 0.001* 23.39	0.74 ± 0.01* 23.52	0.83 ± 0.001* 21.88	0.85 ± 0.001* 27.69
	800	0.61 ± 0.01* 26.21	0.70 ± 0.01* 26.99	0.81 ± 0.01* 24.06	0.83 ± 0.01* 29.66

[a] presented as mean ± SEM (n = 3)
*$p<0.05$ compared with the control group (Dunnett's test)

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663 664 **Table 2**: Effect of Black Cumin extracts by DMSO on acetic acid-induced (0.6%) writhing in mice

Group	**Dose (mg/kg,(p.o.)**	**No. of writhing [a]**	**% inhibition**
Control	--	23.67 ± 1.2	--
Diclofenac sodium	50	14.70 ± .89**	37.89
Nigella sativa	200	18.01 ± 1.0*	23.91
	400	17.35 ± 0.67*	26.65
	800	16.62 ± 0.33*	29.78

[a] presented as mean ± SEM (n = 3)
*$p<0.05$ compared with the control group (Dunnett's test)
**$p<0.001$ compared with the control group (Dunnett's test)

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