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Animals living in polluted environments are a potential source of anti-tumour

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molecule(s)

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10 **Short title: Animal-based antitumor compound(s)**

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17 **Summary**

18 Despite advances in in therapeutic interventions and supportive care, the morbidity
19 and mortality associated with cancer has remained significant. Thus there is a need for newer
20 and more powerful anti-tumour agents. The search for new anti-tumour compounds
21 originating from natural resources is a promising research area. Animals living in polluted
22 environments are a potent source of anti-tumour agents. Under polluted milieus, species such
23 as crocodiles, feed on rotten meat, are exposed to heavy metals, endure high levels of
24 radiation, are among the very few species to survive the catastrophic Cretaceous-Tertiary
25 extinction event with a prolonged lifespan. Thus it is reasonable to speculate that animals
26 such as crocodiles have developed mechanisms to defend themselves against cancer. The
27 discovery of antitumor activity in animals such as crocodiles, whales, sharks, etc will
28 stimulate research in finding therapeutic molecules from unusual sources, and has potential
29 for the development of novel antitumor compound(s) that may also overcome current drug
30 resistance. Nevertheless, intensive research in the next few years will be required to realize
31 these expectations.

32

33 **Key words:** Anticancer agents; Animals-based compounds; cancer resistance; antitumour
34 molecule(s).

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36 **Introduction**

37 The morbidity and mortality associated with cancer has remained significant, despite
38 advances in therapeutic interventions and supportive care. For example, the International
39 Agency for Research on Cancer reports that in 2012, there were approximately 14.1 million
40 new cancer cases, 32.6 million pre-existing cancer patients and 8.2 million deaths due to
41 cancer worldwide [1]. By 2030, the global cancer burden is expected to almost double,
42 growing to 21.7 million cases and 13 million deaths, , in part due to aging population [1-3].
43 Additionally, cancer cell dormancy and emergence of drug resistance contributes to poor
44 prognosis, resulting in a high number of pre-existing cancer cases. Thus there is a continuous
45 need to search for anti-cancer therapies.

46 Natural products have been used widely for medicinal purposes. In particular, natural
47 products derived from plants have led to the identification of anti-cancer agents such as Vinca
48 alkaloids (e.g., Vincristine, Vinblastine) [4], Podophyllotoxin (PPT) derivatives (e.g.,
49 etoposide) [5] and Taxol derivatives (e.g., paclitaxel) [6] but drug resistance remains a major
50 challenge and highlights the importance of new compounds. . Recently, we hypothesized that
51 crocodiles possess anti-tumour compound(s) and/or mechanisms to counter cancer
52 development [7]. The fact that animals such as crocodiles live in unhygienic conditions, feed
53 on rotten meat, are exposed to heavy metals such as arsenic, cadmium, cobalt, chromium,
54 mercury, nickel, lead, selenium, endure high levels of radiation, are among the very few
55 species to survive the catastrophic Cretaceous-Tertiary extinction event [8-14], with a
56 prolonged lifespan and rarely develop cancer suggest that they possess mechanisms to
57 counter cancer development. Having visited several crocodile sanctuaries in South-East Asia
58 and working together with expert veterinarians handling crocodiles/alligators over the past
59 few decades, it was intriguing to note the absence of cancer development in these animals
60 (personal communications with expert veterinarians, S. Vellayan, who dissected over 2000

61 crocodiles, post-mortem, and none showed cancer characteristics). This is corroborated with
62 the absence of scientific evidence on cancer incidence rate in these species. Similarly, other
63 animals such as whales, sharks, turtles, tortoises, elephants, snakes etc have long lifespan.
64 Although the incidence rate of cancer is not available for several vertebrates, cancer has been
65 reported in snakes [15], tortoise [16], crocodile [17], monitor lizards [18], whales [19], and
66 sharks [20]. Given the rarity of cancer development or associated complications together with
67 their prolonged life span of up to a 100 years [14,21-23], it is reasonable to speculate that
68 these animals may have developed protective mechanisms or possess bioactive molecules
69 with anti-tumour properties which may prevent them from developing cancer. Here, we
70 review the literature on the occurrence of anti-tumour compounds in animals living in
71 polluted environments and the potential for future investigation in these species.

72 ***Animal-based anti-cancer agents***

73 The discovery of anti-cancer agents from animals living in polluted environments is a
74 worthy area of research that offers an untapped biological source for the isolation of novel
75 anti-tumour molecules. Among mammals, animals such as elephants and whales are
76 perceived to be highly resistant to cancer [21-23]. Being one of the oldest mammal which has
77 existed since pre-historic times, whales have demonstrated their ability to survive evolution
78 by adaptation [21-23]. For example, Bowhead whales (*Balaena mysticetus*) are able to live
79 up to over 211 years [21-23]. Animals with large body size and longer lifespans were
80 presumed to have an increased risk of developing cancer, if organisms possess similar
81 malignant transformation risks and cancer suppression mechanisms when exposed to cancer
82 causing agents. Therefore, since larger sized animals contain more cells, they were presumed
83 to have a higher chance of developing cancer compared to smaller sized organisms [21-23].
84 Additionally, animals with longer lifespans were thought to have more time to mount up
85 mutations caused by cancer causing agents compared to animals with shorter lifespan [21-

86 23]. This concept was however proven wrong by Peto's paradox. Cancer was shown to have
87 no correlation with the body size and lifespan of an organism. The concept of Peto's paradox
88 explains the presence of lower oncogene (anti-apoptotic genes) expression and increase
89 tumour suppressor genes in large, long lived animals [21]. As a result, active apoptosis
90 activity within cells, prevents the proliferation or cell division of abnormal cells, leading to a
91 lower chance of developing cancer. Besides animals such as elephants have lower metabolic
92 rates, leading to reduced free radical accumulation. Additionally, elephants were found to
93 produce 'cheater' tumour cells which parasitizes the growth of other tumour cells [21]. As a
94 result, tumour cells are unable to grow leading to a reduced risk of developing cancer.
95 Additional studies revealed the possible mechanisms which are involved in lower cancer
96 development in bowhead whales and elephants compared to other mammals [21-23]. These
97 animals possess altered gene expression levels which makes them less likely to develop
98 cancer in comparison to other species. The *p53* tumour suppression gene activity was found
99 to be highly expressed in elephants. A higher expression of tumour suppressor gene,
100 increases cell sensitivity towards cancer causing agents, leading to the initiation of 'apoptosis
101 of tumour cells. Elephants which are the evolved version of mammals from the
102 Proboscideans family are also found to have a very low chance of developing cancer [21,23].
103 Studies have shown that the tumour suppressor gene, *p53* in elephants was retro-duplicated.
104 This retro-duplicated *p53* was highly expressed in elephants leading to enhanced sensitivity
105 of elephant cells towards genotoxic stress, resulting in induction of apoptosis [21]. Although
106 the discovery of multiple *p53* genes in elephants partially explains lower cancer risk in
107 elephants and other large sized animals, the link between the *p53* gene expression and cancer
108 suppression as well as the presence of potential anti-tumour molecule(s) is yet to be
109 determined.

110 Reptiles such as crocodiles are shown to contain many bioactive peptide which
111 exhibits anti-inflammatory, anti-oxidative and anti-microbial characteristics [24-26]. Song et
112 al., [27] showed that bile acids from crocodiles and snakes were found to contain anti-cancer
113 properties. Furthermore, ESC-3 was shown to be the active component in crocodile bile that
114 induced apoptosis in Mz-ChA-1 cells through the mitochondria-dependent pathway and it
115 was proposed as a potential chemotherapeutic drug against cholangiocarcinoma [27]. This is
116 consistent with Chinese Traditional Medicine where animal bile acids have been used in the
117 treatment of various diseases including cancer [28]. In particular, Siamese crocodiles
118 (*Crocodylus siamensis*) are one the most studied crocodile species in terms for cancer
119 research and their bile acids and white blood cell extracts were shown to exhibit anti-cancer
120 properties [25-29]. In particular, bile acid extract inhibited proliferation of human biliary
121 adenocarcinoma cells (Sk-ChA-1) and several other cholangiocarcinoma cells such as MZ-
122 ChA-1 [27] and QBC939 [29] and human hepatocellular carcinoma cells (SMMC7721) [30]
123 in a dose-dependent manner. Molecular studies revealed that the proliferation of cancer cells
124 were inhibited *via* the cell cycle arrest mechanism at the G0/G1 phase [228,29]. Later studies
125 revealed that Siamese crocodile bile extracts induce apoptosis *via* production of reactive
126 oxygen species, loss of mitochondrial membrane potential, resulting in the release of
127 cytochrome *c* into the cytosol, up-regulation of pro-apoptotic proteins such as p53 and Bax,
128 and down-regulation of anti-apoptotic proteins such as Bcl-2, Survivin and *c-Myc* [25-27]. In
129 addition to cytotoxic effects of crocodile bile acids on human cells, bile acid extract was
130 found to enhance the sensitivity of drug uptake by human cholangiocarcinoma multidrug
131 resistance cell line (QBC939/5-FU) suggesting that molecular constituents of bile acid
132 extracts of Siamese crocodiles can augment anti-cancer chemotherapeutic properties [31].
133 Notably, phase III trial of Ursodeoxycholic acid (UDCA) treatment, a component from
134 normally present in bile fluid showed a 39% reduction in malignant tumours [29,32]. This is

135 in contrast to bile from humans where secondary bile acids were shown to play a role in
136 intestinal tumour development [33] suggesting differences in composition of molecular
137 constituents of bile in difference species. More recently, white blood cell extracts from
138 Siamese crocodiles are shown to exhibit anti-angiogenic properties in cancer cells by
139 inhibiting the expression of matrix metalloproteinase such as MMP2 and MMP9, suggesting
140 the disruption of vascular endothelial growth factor (VEGF) and integrin-mediated signal
141 transduction [24]. The disruption of MMP2, MMP9 and VEGF activity directly inhibits
142 metastasis among cancer cells [24]. Patathananone et al., [24] demonstrated the anti-motility
143 effects of Siamese crocodile white blood cell extracts against HeLa cells, mediated by
144 disruption of Ras and p38 signalling pathway, however *in vivo* studies are needed to
145 determine the translational value of these findings.

146 Recently, our studies showed that the organ lysates of *Crocodylus palustris* exhibit
147 antitumor activity against prostate cancer cells (PC3). Among various body organs of
148 crocodile tested including the heart, brain, spleen, Gall bladder, lungs, liver, stomach,
149 intestines, blood, cerebrospinal fluid, testis and copulatory organs, the results revealed that
150 100 µg of sera, bile, gall bladder and heart lysates killed more than 60% PC-3 cells, however
151 lung, intestine, and brain lysates showed partial cytotoxic effects (unpublished findings).
152 When inoculated in fresh medium, PC3 cells treated with bile, gall bladder, sera, and heart
153 lysates did not revive, while PC3 cells treated with lung, intestine and brain lysates exhibited
154 partial growth. These findings suggest that crocodile organ crude extracts contain active
155 component(s) that affect the viability of PC3 cells. The broad-spectrum antitumor activity of
156 various organ lysates of the crocodile against cancer and primary cells and the chemical
157 identities of the active compound(s) are the subject of future studies. It is hoped that these
158 molecules can eventually be developed into treatments against cancer that are becoming
159 increasingly resistant to current available drugs. Crocodiles are one of the most ancient and

160 hardest species that have survived millions of years. The ability of crocodiles to survive
161 polluted environments together with the fact that crocodiles are an untapped source of
162 pharmaceutical drug-leads, suggests such species may possess antitumor compound(s),
163 endogenously and/or mechanisms to counter carcinogenic substance(s), however further work
164 is needed to realize the potential of these findings.

165 Snake venom has been tested for therapeutic interventions. Snake venom is made up
166 of a mixture of biologically active components such as neurotoxins, myotoxins, enzymes, and
167 pain inducing agents [34], some of which are shown to be of therapeutic value including
168 captopril (derived from *Bothrops jararaca*) for renal dysfunction and exenatide (derived from
169 Gila Monster lizard) for *diabetes mellitus* [34]. For anti-cancer properties, Phospholipase A₂
170 (PLA₂) from snake venom was shown to induce apoptosis and inhibition of cell metastasis
171 [35]. This was shown using BnSP-6, an isoform of PLA₂ derived from the venom of
172 *Bothrops pauloensis* that exhibited selective toxicity against MDA-MB-231 breast cancer
173 cells in a dose-dependent manner with lower toxicity against normal breast epithelial cells
174 (MCF10A) [35]. An acidic Asp49PLA₂ known as MVL-PLA₂, from the venom of
175 *Macrovipera lebetina* also showed antitumor properties by inhibiting the adhesion and
176 migration of melanoma cells (IGR39) [36]. The molecular mechanism of action of PLA₂
177 indicated that the hydrolytic activity of the PLA₂ targeting the phospholipid membrane
178 bilayer. The release of lysophospholipids (LysoPL) and fatty acids (FAs) from the membrane
179 results in membrane damage, disruption of membrane surface proteins and cellular cascade
180 functional disruption [34]. Ebrahim et al., [37] demonstrated the cytotoxic effects of
181 cytotoxin, CTX-1 and CTX-11, derived from the *Caspian Cobra (Naja oxiana)*, against
182 tumour cells (liver adenocarcinoma, HepG2, and breast adenocarcinoma, MCF7 cells) and
183 compared with the normal kidney cells (MDCK). It was shown that the cytotoxic effects are
184 mediated via the lysosomal pathway and by entry of cathepsin into the cytosol [37]. Overall,

185 snake venom components such as CTX-1, CTX-11, BnSP-6 are shown to induce apoptosis
186 [37] and inhibit cell adhesion and migration in cancer cell lines [35]. Cardiotoxin III from the
187 venom of *Naja naja atra* demonstrated anti-metastatic properties against human breast cancer
188 cells by suppressing the expression of hepatocyte growth factor (HGF)-induced *c*-Met
189 phosphorylation [38]. Besides venom, studies are needed to test snake organ lysates for
190 potential anti-tumour properties and the associated molecular mechanisms. For example,
191 organ extracts of *Cryptopodion scabrum*, a geckonid lizard and *Gekko swinhonis* Guenther
192 (GSPP) exhibited anti-proliferative activity and anti angiogenic effects against cancer cells
193 selectively and in a dose- and time-dependent manner *in vitro* and *in vivo* [38,39]. It was
194 demonstrated that cancer cells were unable to undergo metastasis due to disruption in bFGF
195 function, a growth factor responsible for angiogenesis [38].

196 Among small mammals, several mechanisms have been proposed that may inhibit
197 cancer development. For example, it was shown that Naked Mole Rats (NMRs;
198 *Heterocephalus glaber*) exhibit changes in p53 gene [40], and their non-coding RNAs
199 (lncRNAs) interact more with 4 types of high-molecular-mass hyaluronan (HMM-HA) from
200 the fibroblasts compared to other rodents, which may enables them to inhibit cancer
201 development [42-44]. Signals from HMM-HA triggers the activation of tumor suppressor
202 INK4 (Inhibitors of cyclin-dependent kinase 4) locus expression [42-44]. This results in the
203 activation of an alternate reading frame (ARF) and a novel product, pALT^{INK4a/b}, which is a
204 hybrid of the two tumor suppressor proteins, p15^{INK4b} and p16^{INK4a}. Interestingly, pALT^{INK4a/b}
205 were found to be present in NMRs but absent in humans, which suggests its role in the cancer
206 resistance of NMRs [45]. On the other hand, an equilibrium between cell proliferation and
207 cell death is essential. Extreme expression of cell proliferation proteins may result in
208 tumorigenesis whereas extreme expression of tumor suppressor proteins will contribute in
209 accelerated ageing. The tumor suppressor proteins, p15^{INK4b} and p16^{INK4a}, was also found to

210 be highly expressed in NMRs upon low levels of stress in addition to pALT^{INK4a/b} which
211 explains the reason NMRs do not develop cancer [42-46]. However, extreme tumor
212 suppression activities accelerate ageing which is not the case among NMRs. Although many
213 studies have been performed to discover the mechanism involved in NMR cancer resistance,
214 [42-46] reported the presence of 2 NMRs with tumor. This finding showed that NMRs do
215 develop cancer[47], albeit at a lower rate compared with humans. However, further studies
216 are needed for NMRs with cancer , to investigate the reliability of the anti-cancer mechanism
217 which is believed to protect NMRs against tumorigenesis.

218 Among Amphibians, the Bufonidae family which generally consists of toad species
219 were found to possess bioactive compounds with anti-cancer activities as well as other
220 therapeutic activities such as anti-microbial and anti-allergy activities [48-49]. Bufonidae
221 family are able to produce secretions from the parotids glands and skin which is rich in
222 bioactive secondary metabolites with anti-cancer properties [50]. The secretion from the
223 granular glands of the frog (*Physalaemus nattereri*) are poisonous and is normally used as a
224 defense mechanism against predators. Studies have revealed the anti-cancer ability of the
225 crude skin secretion containing this poisonous substance from *Physalaemus nattereri* against
226 B16F10 murine melanoma cells [51,52]. The crude skin extracts were cytotoxic against
227 murine melanoma cells in a concentration dependent manner via apoptosis and by cell cycle
228 arrest at S phase [51,5242]. This was consistent with the findings by [52]. Cinobufacini
229 compound from the skin of *Physalaemus nattereri* significantly inhibited the growth of
230 HepG2 via apoptosis, inducing cell cycle arrest at S phase and by downregulating the protein
231 expression of TOPO 1 and TOPO II [51,52]. Later studies demonstrated the anti-cancer
232 properties of skin extracts from the organisms belonging to the Bufonidae family [53]. The
233 skin extract of the *Bufo bufo gargarizans* toad exhibited anti-cancer effects against human
234 breast carcinoma cells by inducing apoptosis, cell cycle arrest and inhibiting metastasis via

235 the inhibition of cell migration and cell invasion of cancer cells [50-53]. The skin of the frog
236 has been used since ancient times in Chinese Traditional Medicines such as Cinobufacini [51-
237 53]. This water soluble extract is a cancer treatment compound which used widely in China
238 and approved by Chinese State Food and Drug Administration (SFDA) (ISO9002) [52]. The
239 active compounds from Cinobufacini such as bufalin and resibufogenin were found to inhibit
240 the proliferation of a wide range of cancer cell lines such as human hepatocellular carcinoma
241 cells (HEPG2) and prostate adenocarcinoma cells (PC3) [50,52]. Studies also demonstrated
242 the ability of this compound in inducing apoptosis among human hepatocellular carcinoma
243 cells (SMMC-7721) and gastric carcinoma cells by decreasing the expression of certain anti-
244 apoptotic proteins such as Bcl-2 [52]. Notably, the majority of aforementioned studies have
245 been conducted on human cells exposed to variety of cellular extracts from different
246 organisms. Future studies are needed to test the effects of selected compounds *in vitro* using
247 primary human cells of relevance as well as *in vivo* using relevant animal models.

248 Compounds derived from animals are preferred for anti-tumour therapy as they are
249 natural and can be readily synthesized. Being naturally-derived molecules, they are more
250 tolerated and potentially non-toxic to normal human cells, albeit there are exceptions. If
251 animals-derived drugs can demonstrate selectivity in research, are non-toxic to primary cells
252 and show cytotoxicity to cancer cell lines, these drugs can be lead into clinical trials for
253 further therapeutic development. Their potential mode of action is methytransferase
254 inhibitors, DNA damage preventive drugs or antioxidants, histone deacetylases (HDAC)
255 inhibitors and mitotic disruptors.

256 In summary. this review is timely and topical and further investigation is warranted to
257 explore various animals living in polluted environments as a large untapped source of
258 pharmaceutical drug-leads that may lead to the identification of novel antitumor compound(s)

259 and/or mechanisms of cancer resistance for the rational development of therapeutic
260 interventions.

261

262 **Compliance with Ethical Standards:**

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270

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