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Review

Carcinogenic potential of sanguinarine, a phytochemical used in 'therapeutic' black salve and mouthwash

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ABSTRACT

Black salves are escharotic skin cancer therapies in clinical use since the mid 19th century. *Sanguinaria canadensis,* a major ingredient of black salve formulations, contains a number of bioactive phytochemicals including the alkaloid sanguinarine. Despite its prolonged history of clinical use, conflicting experimental results have prevented the carcinogenic potential of sanguinarine from being definitively determined.

Sanguinarine has a molecular structure similar to known polyaromatic hydrocarbon carcinogens and is a DNA intercalator. Sanguinarine also generates oxidative and endoplasmic reticulum stress resulting in the unfolded protein response and the formation of 8-hydroxyguanine genetic lesions. Sanguinarine has been the subject of contradictory *in vitro* and *in vivo* genotoxicity and murine carcinogenesis test results that have delayed its carcinogenic classification. Despite this, epidemiological studies have linked mouthwash that contains sanguinarine with the development of oral leukoplakia. Sanguinarine is also proposed as an aetiological agent in gallbladder carcinoma.

This literature review investigates the carcinogenic potential of sanguinarine. Reasons for contradictory genotoxicity and carcinogenesis results are explored, knowledge gaps identified and a strategy for determining the carcinogenic potential of sanguinarine especialy relating to black salve are discussed. As patients continue to apply black salve, especially to skin regions suffering from field cancerization and skin malignancies, an understanding of the genotoxic and carcinogenic potential of sanguinarine is of urgent clinical relevance.

1. Introduction

Patients often associate natural therapies as being safe with a reduced risk of toxicity [1]. This can be a dangerous misconception with natural product exposures responsible for a range of toxicities and fatalities [2]. While natural products may possess anti-mutagenic properties, they also include some of the most potent carcinogens known to humankind [3]. Unlike pharmaceuticals, rigorous pre-clinical testing of natural therapies is not a regulatory requirement for their sale to consumers, placing patients at risk of adverse outcomes [4].

Topical skin cancer therapies are often applied to tissue that has been chronically exposed to carcinogenic ultraviolet radiation (UVR) with both UVA and UVB inducing genetic damage to skin cells [5]. Over time UVR results in the formation of abnormal clonal cellular areas, patches of damaged skin with altered p53 tumor gene expression levels, in a process referred to as field cancerization [6]. It is therefore especially important that topical therapeutics, which may be applied to UV-induced precancerous skin regions and skin malignancies, are assessed for their mutagenic and carcinogenic potential.

Black salve is an alternative skin cancer therapy available for purchase online. It contains a number of constituents that vary in composition and concentration between vendors, including bloodroot (*S. canadensis*), chaparral (*Larrea tridentata*), graviola (*Annona muricata*), oleander (*Nerium oleander*) and zinc chloride among others. A number of these botanical extracts and their constituent phytochemicals have not been assessed for mutagenic or carcinogenic potential.

Black salve has never been studied in a systematic clinical trial, with only a limited number of patient outcomes being reported in case

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studies [7]. Patients with melanoma [8], squamous cell carcinoma [9] and basal cell carcinoma [10] have experienced black salve treatment failures. Whether black salve altered the natural history or metastatic potential of these malignancies is currently unknown, as is the rate of new skin cancer formation in black salve treated areas.

S. canadensis is a key ingredient of black salve. It contains quaternary benzophenanthridine alkaloids, chelerythine, chelilutine, chelirubine, sanguilutine with sanguinarine being the main alkaloid in bloodroot and black salve formulations [11]. There are concerns about the carcinogenic potential of sanguinarine arising from reports of an association with mouthwash-induced leukoplakia and gallbladder carcinoma [12], [13]. The usual battery of *in-vitro* genotoxicity tests and murine studies on sanguinarine have given mixed and conflicting results [14–17]. This literature review explores the evidence relating to the carcinogenic potential of sanguinarine, the main alkaloid present in black salve.

2. Mechanisms of sanguinarine carcinogenesis

2.1. DNA intercalation

Sanguinarine can exist as a charged iminium (pH 2–6) or uncharged alkanolamine (pH 6.5–9.0) form, at physiological pH (7.4) both forms are present [18,19] (Fig. 1). Spectroscopic studies involving calf thymus DNA suggests the iminium form of sanguinarine binds to DNA, exhibiting GC base pair specificity [19]. Thermodynamic analysis has revealed DNA binding is exothermic and enthalpy driven, which is indicative of intercalative binding [20].

DNA intercalators are able to interfere with the action of DNA polymerase thus impairing DNA replication, especially in rapidly dividing cancer cells. As a result, the mechanism of DNA intercalation has been harnessed by the anthracycline chemotherapy agents doxorubicin and the topoisomerase II inhibitor mitoxantrone [21]. Sanguinarine cytotoxicity correlates with its DNA intercalating ability, with DNA single-strand breaks occurring before the initiation of apoptosis induced double strand breaks, suggesting genotoxic activity [22,23].

DNA intercalators are usually associated with frameshift mutations as they increase the distance between adjacent DNA base pairs [24]. Paradoxically some agents used to treat cancer may result in the development of secondary malignancies. For example, anthracycline intercalating agents can cause leukemias and myelodysplasia [25] by inducing chromosomal disruption with the subsequent development of tumor forming translocations [26]. Sanguinarine is a strong DNA intercalator [27], with a binding constant to calf thymus DNA of $1.00 \times 10^6\,M^{-1}$ [28] being similar to the binding constants of anthracycline chemotherapy agents daunorubicin ($1.27 \times 10^6\,M^{-1}$) and doxorubicin ($2.04–3.3 \times 10^6\,M^{-1}$) [29,30]. The strong intercalative binding action of sanguinarine, as with anthracycline agents, may damage DNA with carcinogenic consequences.

2.2. Reactive species generation

Free radicals are chemical moieties that contain orbiting unpaired electrons. They are unstable, reactive and able to interact with and damage cellular proteins, lipids and nucleic acids [31]. Reactive Oxygen Species (ROS) contain biologically active reactive oxygen functional groups such as hydrogen peroxide (H_2O_2) and singlet oxygen [32]. Oxidative stress is an indirect mechanism through which carcinogens can exert genotoxic effects. ROS have been shown to act in cancer initiation, promotion and progression [33–36].

Sanguinarine induces ROS in a range of cell lines [37,38], and also in a murine *in vivo* model [39], by mechanisms that are not as well established as for UVA radiation. While UVA activates nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) oxidase, facilitating electron transfer to molecular oxygen creating superoxide anions, sanguinarine appears to be reduced spontaneously by NADPH-producing superoxide anions without requiring a catalytic enzyme [40]. Subsequent intracellular sanguinarine re-oxidation can occur, suggesting redox cycling as a mechanism by which sanguinarine rapidly generates large amounts of ROS, in particular H_2O_2 [41]. Redox cycling is more

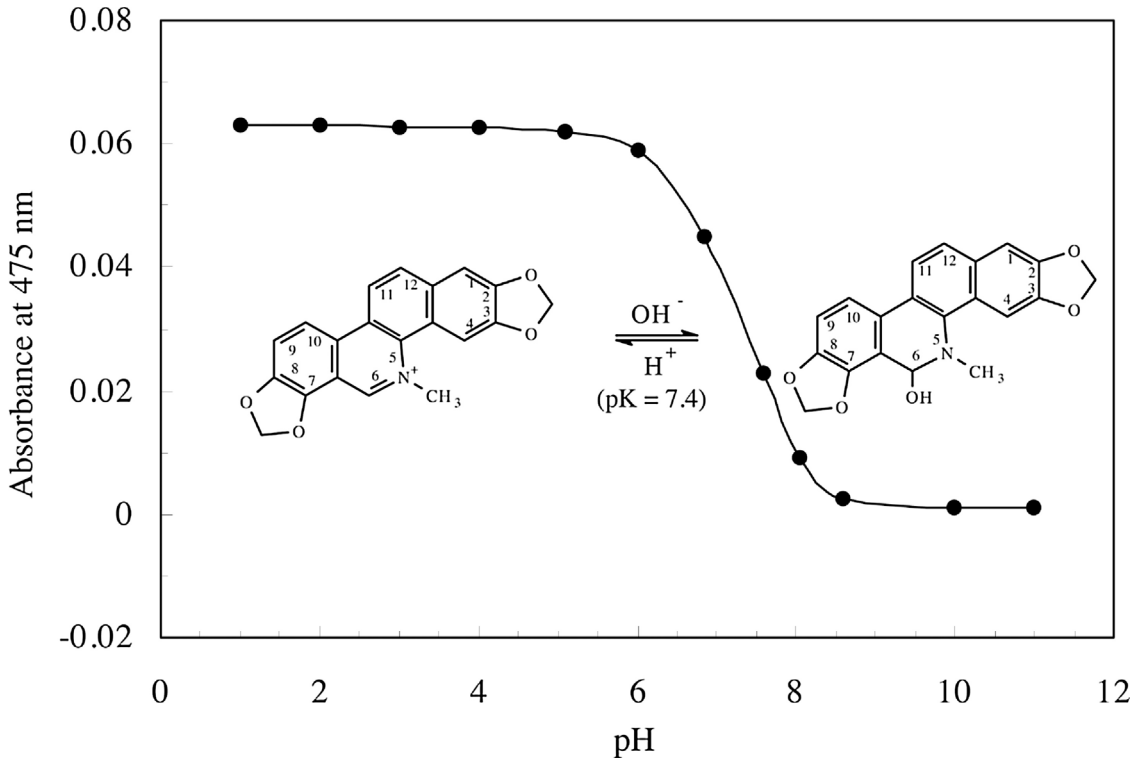

Fig. 1. pH induced transformation of sanguinarine between iminium and alkanolamine forms.
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likely to occur in proliferating cells with high NADPH concentrations that then reduce nucleotides for DNA synthesis, and less likely to occur in cells, such as the MCF breast cancer cell line, that contain higher levels of catalase [42].

When assessing the ROS effects of sanguinarine in SPCA1 human lung adenocarcinoma cells, endoplasmic reticulum (ER) stress was found to be induced [43]. It is well established that ROS can cause the misfolding or unfolding of proteins which accumulate in the ER lumen [44], resulting in the unfolded protein response (UPR) molecular cascade [45]. While the UPR is an anti-cancer target [46], continuous ER stress creates a positive ROS feedback loop [47]. From the current evidence, sanguinarine therefore appears to activate the ROS-ER stress cycle, and amplifies the oxidative damage sustained by cells [43].

Sanguinarine has also been shown to generate reactive nitrogen species (RNS), in LNCaP prostate cancer epithelial cells exposed to 3 μg/ml sanguinarine experience having increased nitric oxide (NO) production [48]. The mechanism for sanguinarine induced NO generation is currently unknown, although neoplastic prostate cancer cell lines express higher levels of inducible Nitric Oxide Synthase (iNOS) than their non-neoplastic counterparts [49]. In the epidermis, human keratinocytes express all three isoforms of NOS, including iNOS [50,51]. Human skin fibroblasts stimulated with cytokines and lipopolysaccharides have been shown to express both constitutive and inducible NOS [52]. Although not yet studied in human keratinocytes, if sanguinarine increases RNS production then the generation of highly reactive peroxinitrite ($ONOO^-$) is likely to increase its genotoxic potential in skin.

A number of factors affect antioxidant levels in human skin. The epidermis, being the body's main barrier to environmental exposures has significantly higher levels of enzymatic (superoxide dismutase (SOD), catalase) and non-enzymatic (Vitamin C, Vitamin E, uric acid and glutathione) antioxidants than the dermis [53]. Aged skin has normal levels of enzymatic anti-oxidants but non-enzymatic antioxidant levels are 60–70% of those found in younger skin [54]. Sanguinarine exposure can result in a rapid and severe depletion of cellular glutathione (GSH) in PC3 and L-929 cell lines. Similarly, in human hepatocytes sanguinarine has been shown to result in reduced GSH levels and cell death without an increase in malondialdehyde (MDA) production, a marker of lipid peroxidation [55]. Sanguinarine GSH depletion appears to occur via addition of GSH to iminium bond of sanguinarine in a 1:1 ratio and not due to the generation of ROS [56]. An analogous interaction is suggested with SH-enzymes [57]. Whether sanguinarine exerts a similar antioxidant depleting effect in human skin is currently not known.

UVA is a known carcinogen that exerts a significant proportion of its carcinogenic effect through the generation of reactive species [35,34]. Sanguinarine has been found in a number of normal and malignant cell lines to generate a reactive species profile similar to that of UVA [58]. Additionally, the process of redox cycling, ROS-ER stress cycle activation and antioxidant depletion may amplify the cellular oxidative stress and carcinogenic potential that may occur from sanguinarine exposure. Cells exposed to high levels of ROS undergo apoptosis or necrosis [59], although sublethal ROS doses can result in genotoxicity that a cell is unable to repair completely [60,61], increasing the likelihood of gene mutations that ultimately may lead to cancer [62,63].

Despite a number of studies investigating the action of sanguinarine as an oxidant and its ability to cause genetic damage, surprisingly only one study has searched for the presence of the ROS induced 8-hydroxyguanine. This effect in sanguinarine exposed HCT116 human colon cancer cells indicates that sanguinarine can induce guanine oxidative genetic damage [58].

2.3. DNA adduct formation and sanguinarine metabolism

In a ^{32}P-postlabelling assay, sanguinarine when incubated with β-naphthoflavone (β-NF) activated rat hepatic microsomes, resulted in DNA adduct formation [64]. This was concentration dependent, a sanguinarine concentration of 100 μM causing 36.3 DNA adducts per 10^8 nucleotides, 10 μM causing 3.2 DNA adducts per 10^8 nucleotides and 1 μM not resulting in the formation of detectable DNA adducts by using a nuclease P1 enriched ^{32}P- postlabeling assay [64]. Whether the DNA adducts detected in this study were genuine adducts or artefacts generated during oxidative DNA damage [65] has been questioned [42]. While DNA adducts may be formed by reactive oxygen species and lipid peroxidation [66], malondialdehyde generation was not detected, suggesting these processes were not responsible for the observed DNA adduct formation [55]. While adducts may have formed in a rat liver microsome system, they either do not form after an equivalent sanguinarine exposure in human hepatocytes, or any adducts that form are repaired and removed [67]. In a 90 day feeding trial of pigs, toxicity studies with 64 ppm sanguinarine ingestion failed to show liver DNA adduct formation utilizing ^{32}P-postlabeling [68]. Plasma sanguinarine levels of 0.11 μg/ml and liver levels of 0.13 μg/g in the highest exposure group were reported, although these are below the concentration at which DNA adducts were induced in the positive rat liver microsome study [64].

Once formed, DNA adduct stability and the risk of its ability to induce a persistent somatic mutation is determined by the cells replication rate and DNA repair capacity [69,70]. When DNA adducts are used in quantitative risk assessment, induced DNA adduct levels should be compared to the tissues background adduct formation level in each tissue [71]. Care should be exercised when extrapolating animal adduct results for assessing human risk, as humans have greater sensitivity to tumor induction than mice when exposed to some carcinogens [72].

With sanguinarine not resulting in definite adduct formation, research has subsequently focused on whether the metabolism of sanguinarine generates a DNA adduct forming metabolite. Observations that sanguinarine toxicity in mice is reduced by 3-methylcholanthrene, a CYP450 inducer, suggests sanguinarine is metabolized by CYP450 [73]. CYP1A, a member of the CYP450 family, has been found to metabolize polycyclic aromatic hydrocarbons (PAH) to generate reactive epoxides that have carcinogenic potential [74]. Sanguinarine shares a similar structure to PAHs, raising the possibility of sanguinarine also being metabolized by CYP450 to an epoxide metabolite [75].

Sanguinarine has been shown to undergo dihydro derivative formation by *in vitro* UV–vis spectrometry and fluorimetry [76], this being confirmed by an *in vivo* rodent study [77]. Kosina et al. in 2011 [78], further elucidated the multi-step metabolism of sanguinarine in human hepatocytes using electrospray quadrupole ion-trap mass spectrometry with reversed phase chromatographic analysis. They found that dihydrosanguinarine (DHSG) is oxidized by cytochrome P450 enzymes resulting in O-demethyl and hydroxyl metabolite formation. These metabolites subsequently appeared to undergo Phase II biotransformation, being conjugated by glucuronidases and sulfotransferases, as determined by the respective MS mass measurements and fragmentation patterns of the resultant compounds. Sanguinarine epoxide metabolites suspected of having carcinogenic potential were not detected in this analysis [78].

Dihydrosanguinarine is the main phase I metabolite of sanguinarine [78]. In a 90 day rat feeding study [79] DHSG reached a maximal plasma concentration of 28 ng/ml with a maximal liver concentration of 130 ng/ml. No liver adduct formation was detected following exposure to these low DHSG concentrations.

2.4. DNA repair mechanisms

Alkaloids may potentially disrupt DNA repair mechanisms. The catalytic subunit of human telomerase (hTERT) upregulates DNA repair genes and increases the nucleoside triphosphate (NTP) pool available for correcting DNA lesions [80]. A number of alkaloids inhibit telomerase activity by suppressing hTERT mRNA expression, including the isoquinoline alkaloids, chelidonine [81] and papaverine [82].

Sanguinarine has been shown to disrupt telomerase activity through G-quadruplex binding with an 8 μM concentration resulting in a 76% reduction in telomerase activity [83] but its effect on hTERT is not currently known. While telomerase is a valid anticancer target being overexpressed in 85% of human malignancies [84], the impact of telomerase targeting compounds on DNA repair processes is unclear. By impairing DNA repair, such agents may pose a mutagenic risk for normal and malignant cells.

Inflammation has also been shown to inhibit DNA repair by up to 70% in cholangiocarcinoma cells via NO mediated DNA repair enzyme nitrosylation [85]. While sanguinarine has been shown to induce NO production in prostate carcinoma cells [48], a number of studies have found sanguinarine to have an anti-inflammatory effect [86–88]. Black salve however has been associated with histological evidence of significant inflammation in humans [89], either due to the effect of sanguinarine or other salve constituents. This inflammation may impair DNA repair, aggravating any genotoxic effects.

Current genotoxicity testing protocols do not directly assess the impact of a compound on DNA repair processes. Due to human inter-individual variation in DNA repair capabilities, the developing field of personalized medicine may in the future incorporate an assessment of an individual's genetic repair capacity when determining toxicity risk. The effect of sanguinarine on DNA repair mechanisms is currently unknown.

2.5. Tumor immune surveillance

Organ transplant patients can have a 100-fold increased risk of non-melanoma skin cancer compared to the general population [90] arising from the use of anti-rejection medications [91]. Nearly 100% of patients diagnosed with non-melanoma skin cancers develop immune suppressive effects from UV exposure compared to 40% in the general population [92,93]. These findings highlight the importance that immune surveillance plays in limiting skin carcinogenesis.

UV-induced immunosuppressant cellular and cytokine effects are triggered either by DNA damage to skin immune cells or by oxidative stress [94,95]. Both mechanisms may result from sanguinarine exposure. Currently the effect of sanguinarine on skin immune cell numbers and function is unknown, as is its potential impact on skin immune surveillance and carcinogenesis.

3. Assessment of sanguinarine genotoxicity

In order to register a pharmaceutical compound there is a regulatory requirement to carry out and report genotoxicity testing to assess carcinogenicity, and in some instances to assess the risk of heritable mutation [96,97]. A battery of genotoxicity tests based on micro-organism and mammalian systems are routinely employed to detect compound-induced DNA damage (Table 1).

3.1. Sanguinarine in vitro genotoxicity results

Following S9 activation sanguinarine and sanguinaria extract yield positive Ames test results [98,99]. Sanguinarine has been shown to elicit a positive mutagenic response [98] in TA98, TA100, TA1537 and TA1538 strains, suggesting it may induce frame-shift mutations [99]. Interestingly in one study where sanguinaria extract, derived from *S. canadensis* rhizomes, contained the full complement of bloodroot phytochemicals, a positive Ames test result was observed with metabolic activation only in the one TA1538 tester strain [99]. Despite these positive Ames test results, sanguinarine has returned negative genotoxicity test results in the SOS chromotest [100] and *Saccharomyces cerevisiae* mutation test [98]. While the Ames test has a greater capacity for identifying carcinogens, the SOS chromotest has a lower false positive rate [101]. Unfortunately, the majority of these were industry studies where experimental methodology was not available for peer-reviewed scrutiny [99].

Sanguinarine has also produced conflicting *in vitro* mammalian results with Comet assays being positive while Micronucleus assays have yielded negative genotoxicity results. Positive Comet assays following sanguinarine exposure were reported from several studies in murine and human cell lines [102,40,103,104] at low concentrations from 1 μg/ml [102]. Human gingival fibroblasts develop 2 to 3-fold higher single DNA strand break levels in the Comet assay in response to sanguinarine compared to prostate cancer cell lines LNCaP and DU-145 [105]. This was observed in the absence of a significant increase in gingival fibroblast apoptosis, suggesting gingival fibroblasts may be more susceptible to sanguinarine genotoxic injury than prostate cancer cells [105].

There is evidence that sanguinarine can induce rapid *in vitro* genetic damage. In one study, after a one hour exposure a concentration of 4 μg/ml caused 10% of human CEM leukemia T cells to have comet assay detectable DNA damage, and this increased to 60% of cells by 3 h. These cells showed a bimodal mechanism of cytotoxicity with some displaying poly (ADP-ribose) polymerase-1 (PARP-1) fragmentation indicative of apoptosis and others being propidium iodide positive indicative of necrosis [14]. Another study investigated the timing of comet assay DNA single and double strand break formation in HCT116 colon cancer cells treated with 3 μM sanguinarine. Single strand breaks developed after 45 min, while double strand breaks developed after 90 min, around the time apoptosis markers such as PARP cleavage appeared, suggesting sanguinarine possesses reactive species genotoxicity apart from its cytotoxic apoptotic effects. The single strand breaks showed guanine oxidation to 8-Oxo-2′-deoxyguanosine characteristic of oxidative stress with antioxidant treatment preventing DNA single strand breaks and reducing DNA double strand breaks to control levels [58].

Due to the significant cytotoxicity displayed by sanguarine against a number of normal and malignant cell types [7], the comet assay may be prone to potentially false positive genotoxicity test results. [106]. Human keratinocytes and hepatocytes have shown greater resistance to sanguinarine cytotoxicity than other cell types [107,108] and may

Table 1
In vitro sanguinarine genotoxicity results.

Test	Description	Result	Ref
Ames: *Salmonella typhimurium*	Genetic mutation enables histidine production and bacterial colony growth	Positive	[98]
SOS Chromotest: *Escherichia coli* PQ37	Assesses lacZ gene damage by measuring its product β-galactosidase	Negative	[100]
Yeast: *Saccharomyces cerevisiae*	Mutations identifiable by colony colour and growth on certain media	Negative	[200]
Comet Assay	DNA strand breaks appear as a comet tail on agarose gel	Positive	[14]
Micronucleus Assay	Micronuclei chromosome fragments develop during mitosis following genotoxin exposure	Negative	[15]
Gamma-H2AX	Marker of DNA damage localizes to DNA strand break sites	Positive	[58]
GADD45a	Gene activated in response to DNA damage	Untested	
UDS Assay	Measures DNA nucleotide excision repair	Untested	
CHO-HPRT Mutation Assay	Exposure to mutagens establishes HPRT negative CHO cell mutants	Equivocal	[99]

represent better models for the *in vitro* Comet assay testing of sanguinarine.

Contradictory negative *in vitro* genotoxicity results for sanguinarine have however been reported using the micronucleus assay. In the presence of rat liver S9, human lymphocytes and the metabolically competent Hep-G2 human hepatoma cell line showed no micronucleus formation when exposed to sanguinarine concentrations up to 5 μM [15]. This study tested a total of fifteen natural products including five established rodent carcinogens arecoline, plant extract aristolochic acid, B-asarone, psoralen and safrole, promoters of carcinogenesis coumarin, isatidine dehydrate, monocrotaline, retrorsine, tannin and thiourea, in addition to compounds suspected of carcinogenic activity benzyl acetate, emodine, reserpine and sanguinarine chloride. All of the established carcinogens in this study with the exception of safrole showed genotoxicity, the suspected carcinogens monocrotaline and retrorsine also had negative genotoxicity results. While other groups also reported negative safrole results with rat hepatocytes [109], they did show safrole as clearly positive in the Hep-G2 system [110]. This suggested that the Hep-G2 subclone used by the authors may differ in its metabolic activation capacity and raises questions regarding the accuracy of sanguinarines negative micronucleus assay result.

The lack of clarity surrounding inferences from *in vitro* testing of sanguinarine include the equivocal results obtained when tested in the Chinese hamster ovary (CHO-HPRT) mutagenesis assay [99]. In one industry study relayed by Munro et al. [99] a significant increase in mutant CHO cell numbers occurred in the presence of S9 at a sanguinarine concentration of 10 μg/ml. This was in the absence of mutant frequency increases at lower dose levels. An industry study also reported by Munro et al. [99] produced similar results with a concentration of 10 μg/ml increasing mutant frequency with no mutants forming below this concentration. The CHO-HPRT mutagenesis assay results were considered inconclusive. The selective use of such data by Munro et al. [99] to refute concerns about the safety of sanguinarine, without the company that conducted the studies making the research available for public scrutiny, is a disappointing display where commercial interests seem to have taken precedence over patient safety.

The inconclusive assessment from sanguinarines *in vitro* genotoxicity testing has led to a need for clarification with *in vivo* toxicity studies. *In vitro* metabolism systems contain restricted Phase I and II enzyme subsets that preferentially enable oxidative cytochrome P450-mediated reactions [111]. Alternate metabolic processes are utilized by a number of mutagens to form genotoxic metabolites that are often not assessed by current *in vitro* assays [112]. *In vivo* toxicity studies however operate in a physiological system that utilizes reduction, hydrolysis and conjugation in addition to oxidation with a full complement of Phase I and II enzymes [113]. Current *in vitro* genotoxicity assays have high false positive rates, this being another reason for use of *in vivo* assessment of compounds. One analysis found that of 700 compounds that were known rodent non-carcinogens, 75–95% generated a positive result in at least one *in vitro* genotoxicity test [114]. This outcome is supported by a study assessing over 1000 pharmaceutical compounds [115,116]. Chemicals that are genotoxic *in vitro* therefore require further evaluation *in vivo* in order to establish their potential risk to humans.

3.2. Sanguinarine in vivo genotoxicity assessment

Several *in vivo* assays have been used in an attempt to determine the genotoxicity and carcinogenic potential of sanguinarine (Table 2). These have unfortunately not clarified the compound's toxicity status, with methodology problems, unpublished non-peer reviewed industry studies and contradictory results all contributing to uncertainty and a lack of consensus.

Regulatory agencies have increasingly accepted the *in vivo* Comet assay as a reliable genotoxicity test [117,118] following the development of a standardized protocol [119]. It has been validated showing 73.7% to 78.9% sensitivity with 19 known genotoxins in liver and stomach tissues respectively [120]. As with the *in vitro* comet assay, the *in vivo* assay may detect cytotoxic rather than genotoxic compounds. To reduce false positive results, histopathological correlation and cell analysis for cytotoxicity are often performed.

Male Swiss albino mice given a single intra-peritoneal injection of sanguinarine starting at a dose of 2.7 mg alkaloid/kg body weight, developed bone marrow and blood cell signs of genotoxicity starting with an increase in comet tail length. At 5.4 mg/kg Olive Tail Moment (OTM) and tail length increased by 14–32% while 10.8 mg/kg caused a significant increase of 33–51% OTM, tail length and tail DNA [121]. This suggests that a single sanguinarine exposure can result in cellular genetic damage in a dose dependent manner. The sanguinarine in this study however was of low purity (88%), being a precipitation product obtained from argemone oil. Argemone oil is an alternative botanical source of sanguinarine, that comes from the seeds of *Argemone mexicana* Linn, and has independently been shown to possess genotoxic potential [122,123]. In a murine model the intraperitoneal injection of argemone oil resulted in positive micronucleus and chromosomal aberration tests at a 1 ml/kg body weight dose and positive comet assays at a 0.25 ml/kg body weight dose in lymphocytes/hepatocytes and bone marrow cells [122]. Whether this is due to the sanguinarine contained within the argemone oil or other factors is yet to be determined. Therefore, any conclusions drawn from positive sanguinarine genotoxicity test results using low purity sanguinarine, with possible argemone oil contaminants, should be considered with caution. *Macleaya cordata* extract is primarily composed of quaternary benzophenanthridine alkaloids including sanguinarine 528.95 g/kg and chelerythrine 82.05 g/kg, with a minor quantity of protopine and allocryptopine [124]. A subsequent study using such an extract, orally administered to rats showed no hepatocyte adduct formation and no evidence of lymphocyte or hepatocyte genotoxicity in Comet assay [125]. Since less than 5% of ingested sanguinarine is absorbed [77], oral sanguinarine administration without the determination of plasma sanguinarine levels may translate to blood concentrations too low for comet assay positivity yielding false negative results.

In vivo micronucleus (MN) assays have also been developed as a genotoxicity screening tool [126]. *Sanguinaria* extract at doses up to 14.2 mg/kg bw administered intraperitoneally on two occasions 24 h apart were investigated by *in vivo* MN assay in CD-1 mice. While bone marrow polychromatic erythrocytes showed no increases in MN formation [99], this information derives from an industry study conducted by Vipont Pharmaceuticals Inc (manufacturer of Viadent) that is unpublished and not available for public scrutiny. This prevents an assessment of sample size and research methodology, undermining its value. Compared to the normal practice of sampling bone marrow 24 h after the second compound exposure [127], bone marrow was collected 6 h after the second dosing. This may not have allowed sufficient time for MN formation and detection. There have been no peer-reviewed reports to date of *in vivo* MN assessment of sanguinarine.

Sanguinarine has been assessed for its effect on *in vivo* chromosome aberration and sister chromatid exchange. Mice administered sanguinarine chloride intraperitoneally at 5, 10 and 15 mg/kg body weight concentrations had bone marrow cell changes suggestive of genotoxicity, with a minimum effective concentration of 10 mg/kg [128]. The sanguinarine in this experiment was a defined product from Sigma, which reduces concerns of purity and contamination. The chromosomal aberrations observed were largely chromatid breaks with occasional chromosome breaks. Positive control mitomycin C gave a 28-fold higher chromosomal aberration rate compared to the negative control, with a five fold higher rate than sanguinarines minimum effective concentration. The SCE induction and predominance of chromatid-type breaks were consistent with S phase-dependent clastogen induced DNA damage [129,130]. The 10 mg/kg dose induced 5.32 sister chromatid exchanges (SCE) per cell, while the 15 mg/kg dose induced 6.02 SCEs/cell.

Table 2
In vivo sanguinarine genotoxicity testing.

Test	Result	Dose	Comment	References
Micronucleus Assay			Not Tested	
Comet Assay	Positive	2.7 mg/kg IP	SG isol argemone oil 88% purity Murine lymphocyte/Bone marrow cells both positive	[121]
	Negative	367 ppm PO	SG isol *M. cordata* 98.1% purity 90 day ingestion rat lymph/liver neg Plasma SG conc not determined	[125]
CA & SC Exchange Assay	Positive	10 mg/kg IP	SG Sigma	[128]

SG = Sanguinarine; CA & SC Exchange Assay = Chromosomal aberration & Sister chromatid exchange assay.

When investigating the DNA damage caused by genotoxic carcinogens, different agents cause unique changes in the genomic sequence of cancer related genes [131]. These characteristic mutations act as carcinogen specific 'signatures', where their assessment is known as DNA-lesion footprinting or DNA-damage mapping [132]. For example ultraviolet radiation (UVR) induces dipyrimidine site C to T or CC to TT transitions within the RAS oncogene and TP53 tumor suppressor gene while polycyclic aromatic hydrocarbons from tobacco smoke tend to induce G to T transversions [133]. To date, DNA damage mapping has not been performed to determine whether sanguinarine or black salve result in characteristic genomic sequence mutations.

3.3. Contradictory murine sanguinarine carcinogenesis results

Two experiments have assessed sanguinarines murine carcinogenic potential. One study in female Swiss albino mice, sought to determine whether sanguinarine could initiate or promote cancer. Mice exposed to 1,3-dimethylbutylamine (DMBA) and sanguinarine (4.5 μM concentration) as a combination initiator did not develop increased tumor rates compared to DMBA initiation alone. Mice however, initiated with DMBA and subsequently exposed to topical twice weekly 1.5 μM sanguinarine for 25 weeks did develop earlier tumor onset with mean tumor numbers increasing from 5 to 7.07 [16], suggesting sanguinarine may act as a tumor promoter.

Another study sought to determine whether sanguinarine could protect mice from the effects of UVB. Female SKH-1 hairless mice had 5 μM topical sanguinarine applied as a pretreatment 30 min pre-UVB exposure or 5 min post-UVB exposure, both groups showed significantly reduced skin edema, leukocyte infiltration and hyperplasia [17]. There was also a significant reduction in H_2O_2 and ornithine decarboxylase (ODC) levels, suggesting sanguinarine's anti-inflammatory action may reduce oxidative stress with low concentration topical application. Sanguinarine's mixed cancer promotion and potentially protective effects in murine models requires clarification by futher research, especially as sanguinarine containing topical therapies are in current use by patients.

In addition to these studies that have assessed isolated sanguinarine, two dental industry-funded studies have explored the carcinogenic potential of *Sanguinaria* extract in rats [99]. The first study in 50 female and 50 male CD rats at doses up to 60 mg/kg body weight/day administered by gavage was terminated before the scheduled 104-week study period was reached due to early high dose female and male control group mortality. An explanation for rat mortality was not provided, no increased incidence of pre-neoplastic or neoplastic lesions was reported [99].

A follow-up 2-year dietary feeding study administered *Sanguinaria* extract up to 200 mg/kg body weight/day to 75 male and female Charles River CD rats. The incidence of fibrosarcomas and subcutaneous fibromas was increased in high dose males (6/75 animals) with high dose females developing an increase in uterine polyps (5/75 animals). These lesions did not occur in control animals. Despite the difference between the treatment and control groups, the authors cited the 5% historical incidence of such tumors in control animals [134] and concluded the fibrosarcomas/fibromas and uterine polyps seen in the high dose group were not a consequence of *Sanguinaria* exposure [99]. These studies highlight the lack of transparency and independent peer-review that may occur when commercially used botanical products undergo industry funded toxicity testing [135].

4. Human epidemiological evidence suggesting sanguinarine carcinogenic potential

4.1. Maxillary vestibule leukoplakia

There is epidemiological evidence linking sanguinarine exposure to the development of leukoplakia. Viadent, an antibacterial mouth rinse and toothpaste containing 0.3 μM sanguinarine chloride and 0.7 μM sanguinarine chloride respectively [136–138], has been associated with increased maxillary vestibule leukoplakia [12]. This area normally is rarely effected by leukoplakia [139]. The anterior portion of the maxillary vestibule has the highest concentration of intra-oral rinse residue due to a low salivary turnover this mucosal area has prolonged mucosal product contact [140]. 84% of patients that developed maxillary vestibule leucoplakia had used Viadent with the average period of use being 4.4 years. The epidemiological correlation between sanguinarine dental product use and the development of leukoplakia suggests sanguinarine may induce pre-malignant change in humans.

Histologically, Eversole et al. [141] showed sanguinaria-associated leucoplakias had borderline dysplasia in 55% of cases, mild dysplasia in 42.5% of cases and moderate dysplasia in 2.5% of cases. No lesions showed severe dysplasia with no instances of carcinoma arising within a sanguinaria-associated leukoplakia. A single case of oral squamous cell carcinoma that was confluent with a sanguinaria-associated leucoplakia has been reported. Using sanguinarine-containing products increases the risk for leukoplakia development 10 fold, which is significantly higher than the 2.5 times risk of leukoplakia reported from tobacco smoking [142,143]. The majority of sanguinaria related leukoplakias appear not to resolve even after Viadent is discontinued suggesting the possibility of a permanent alteration in the genome of epithelial cell lineages.

Based on evidence from molecular profiling, sanguinaria-related leukoplakias appear to lie between benign and dysplastic keratoses. With p53 expression, p16, proliferating cell nuclear antigen (PCNA) and cyclin D1 levels being intermediate between the two [144]. While dysplastic biopsy samples analyzed in this study were over 20 years old and this has been associated with reduced immunohistochemistry reactivity [145], another study by Eversole et al. utilizing archived pathology specimens showed similar results. In a further study PCNA levels were elevated in sanguinaria-associated leukoplakia compared to benign keratoses, while lower than those in dysplastic lesions [141]. In this analysis, whilst sanguinaria-associated leukoplakias did not display an elevation of total DNA content, 1.5% of their cell population were aneuploid, compared to 3.5% for dysplasias, and zero for benign keratoses. The authors recommended that sanguinaria-associated leukoplakia be classified as mildly dysplastic lesions of uncertain malignant potential.

Several studies have sought to determine the mechanism by which sanguinarine may have a carcinogenic effect on oral mucosa.

Sanguinarine's chemical structure shows considerable homology to polycyclic aromatic hydrocarbons (PAHs) [144]. Many PAHs undergo metabolic activation by cytochrome P450 to form mutagenic and carcinogenic compounds [146], or activate the aryl hydrocarbon receptor (AhR) causing an upregulation of AhR responsive genes that include the protocarcinogen activating enzymes CYP1A1 and CYP1B1 [147]. There has therefore been interest in determining the interactions of sanguinarine, cytochrome P450 enzymes and the AhR.

A study published in 2005 by Karp et al. suggested that sanguinarine may exert a carcinogenic effect by activating the aryl hydrocarbon receptor (AhR) in oral human keratinocytes [148]. These results were however challenged due to the inconsistency of findings and experimental control failures [149]. A subsequent study found that sanguinarine at 1 μM did not alter CYP1A1 mRNA or protein expression (key features of AhR activation) in human hepatoma cells, contrary to the proposed sanguinarine AhR carcinogenesis mechanism [67]. Sanguinarine also failed to induce AhR in H4IIE.luc rat hepatoma cells at a concentration of 1 μM when incubated for 48 h [150].

While sanguinarine does not appear to activate AhR, it may be metabolized to a carcinogenic compound by cytochrome P450 enzymes. CYP1A induction reduces sanguinarine *in vitro* rat hepatocyte and human hepG2 cytotoxicity, suggesting CYP1A converts sanguinarine into a less cytotoxic metabolite [151]. This reduced cytotoxic metabolite, may however have greater genotoxicity as evidenced by rat DNA adduct levels increasing with higher microsomal cytochrome P450 sanguinarine activation [64]. Sanguinarine incubated with human liver microsomes and NADPH for 150 min failed to generate HPLC detectable metabolites [151]. Serum free human hepatocyte cell cultures exposed to sanguinarine and analyzed with electrospray ionization quadrupole time-of-flight mass spectrometry, did detect and unambiguously identify new metabolites. However, their carcinogenic potential is unknown [78]. Currently the molecular mechanism by which *Sanguinaria* containing dental products cause oral leukoplakia has not been determined.

4.2. Sanguinarine associated gallbladder carcinoma

The northern Indian provinces of Uttar Pradesh and Bihar have a high incidence of gallbladder carcinoma, the disease being the most common biliary tract malignancy in these regions [152]. Indeed the highest gallbladder cancer incidence rates in the world of 21.5 per 100,000 are to be found in Indian women [153].

A number of etiological factors have been investigated in an effort to determine the cause for these increased rates. Up to 95% of gallbladder cancers have been associated with gallstones [154], with patients having gallstones > 3 cm carrying a 10-fold higher risk of gallbladder cancer [155,156]. However, gallbladder carcinoma prevalence does not always correlate with cholelithiasis. In the developed world, 10% of the population has gallstones yet gallbladder carcinoma only accounts for 0.5% of all malignancies [157]. Chronic inflammation is another possibility with chronic carriers of *Salmonella typhi* infection having an 8-fold increased risk of gallbladder cancer [158].

Environmental carcinogen exposure has also been explored. Northern Indian regions are transected by the river Ganges, which provides the main source of irrigation and drinking water. Carcinogen exposure from the water supply, as Gangetic waters receive industrial effluents and untreated domestic sewage has been suggested as a possible cause for elevated gallbladder carcinoma rates [159]. Dietary carcinogen exposure has also been investigated [159]. Repeatedly boiled sunflower oil, compared to single boiled and fresh sunflower oil has a significantly increased polycyclic aromatic hydrocarbon (PAH) content [160]. PAHs are known mutagens and can be carcinogenic. Heating vegetable oil at high temperatures also results in the formation of toxic compounds [161–163].

Argemone mexicana Linn, a herb originally of West Indies origin, grows extensively in sub-tropical and tropical countries being abundant on roadsides and in wastelands, with its seeds and oil closely resembling that of mustard seeds allowing their substitution [164]. Argemone oil contains sanguinarine, with a content varying between 0.044 to 0.5% [165]. Sanguinarine accounts for 5% of argemone seed oil alkaloids, dihydrosanguinarine 87%, the remainder composed of chelerythrine, protopine and berberine [166]. Mustard oil (obtained from *Brassica* species including *B. rapa* (syn. *campestris*) and *B. nigra*) is used as a frying and cooking medium in North India [167], [168]. Unscrupulous traders, for economic gain, often adulterate mustard oil with cheap argemone oil obtained from *Argemone mexicana* [169,170].

In those with gallbladder carcinoma the gallbladder sanguinarine concentration was 195.18 ng/mg while in those who had cholelithiasis it was 24.05 ng/mg. Blood sanguinarine levels were also reported as elevated in those with gallbladder carcinoma compared to cholelithiasis being 230.96 ng/ml and 14.01 ng/ml respectively [13]. While suggesting a causative link between sanguinarine tissue exposure and gallbladder carcinoma, the capillary gas chromatography method used by the authors is unlikely to assess sanguinarine concentration accurately. Unfortunately, no analytical data were supplied to assist with the scrutiny of this result. However, with sanguinarines ability to induce DNA damage and reactive oxygen species it may potentially be a contributing factor in gallbladder carcinogenesis [171].

5. Current sanguinarine carcinogen status and future directions

The carcinogenic risk of sanguinarine has not been definitively determined due to its conflicting test results as represented in Fig. 2. Sanguinarine has not been included in carcinogen databases, such as

in vitro Genotoxicity Test	Ref
Ames test	98
Comet assay	102
Gamma-H2AX	58
Micronucleus assay	15
Saccharomyces cerevisiae mutation test	98
SOS chromotest	100

in vivo Genotoxicity Test	Ref
Comet assay	121
Chromosome aberration & sister chromatid exchange	128
Micronucleus assay	99

Murine Models	Ref
Carcinogenesis promoter	16
UV photoprotection	17

Human Epidemiology	Ref
Oral leukoplakia	12
Gallbladder cancer	13

Positive

Negative

Fig. 2. Summary of current sanguinarine carcinogenic risk assessment.

the IARC monographs (http://monographs.iarc.fr/ENG/Classification/index.php), the International Workshop on Genotoxicity Testing UDS List [172], the 1547 chemicals of the Carcinogenic Potency Project Database (CPPD) (http://toxnet.nlm.nih.gov/cpdb/), and the 2300 chemicals of the US National Toxicology Program (http://www.predictive-toxicology.org/data/ntp/original_ntp_data.txt). Further toxicology work is required to clarify its status.

A mode of action approach is used to determine a chemical's intrinsic genotoxic properties [173] by taking all of the available genotoxicity information into account, combining it with pharmacokinetic, structure activity, ADME (absorption, distribution, metabolism and excretion) data and other biological responses to determine the risk a chemical may pose to humans. The mode of action approach assesses whether positive genotoxicity results pose a risk for human health. Currently this has not been performed for sanguinarine [174].

While the majority of genotoxicity testing has assessed compound-induced genetic damage in blood and liver cells, there is growing interest in using skin for genotoxicity testing [175–177]. This alternative is especially relevant for sanguinarine, since skin is the main target organ for black salve. The tissues used for risk assessment should have the highest toxicant exposure levels or be a site of biological action. Several skin genotoxicity assessment methods have been developed including the rodent skin *in vivo* MN assay [175], ex vivo human skin [178] and human reconstructed epidermal models [179].

Skin is the largest body organ and acts as a toxin barrier. Epidermal cells from discarded neonatal foreskin have been found to express 13 of 15 CYP1-4 gene mRNA at detectable levels [180]. CYP skin metabolism can inactivate reactive compounds having a protective role [181,182] but can also activate procarcinogens potentially leading to toxicity and skin cancer [183,146]. It is currently unknown whether sanguinarine interacts with cytochrome p450 metabolism in human skin.

The PAH benzo[a]pyrene (BP), which has a chemical structure similar to sanguinarine, is not toxic itself but is metabolized into mutagenic and carcinogenic metabolites. BP provides a useful test compound to assess the metabolic capability of various skin genotoxicity models. *Ex-vivo* human skin, cultured normal human keratinocytes and 3D human skin constructs generate all the major BP derived metabolites in sufficient quantity to induce significant DNA damage present in the alkaline comet assay [184]. Constructs are however not metabolically equivalent to normal skin, as they are insufficient for cyclophosphamide metabolic activation [185] with some constructs found to have lower basal CYP expression levels compared to human skin biopsies [186].

In March 2009 the European Union imposed a ban on the *in vivo* genotoxicity testing of cosmetics ingredients [187] which stimulated the development of animal free methods for assessing toxicity. A multilayered human skin construct (EpiDerm) has been developed based on differentiated foreskin-derived epidermal keratinocytes, that allows both basal and apical compound exposure to mimic topical therapy application [188]. This construct has metabolic gene expression similar to that in human skin, with Phase II enzymes more pronounced than Phase I enzymes [186]. Micronucleus [188] and comet assay [189] protocols have been developed and are undergoing validity testing. The construct has a low background frequency of MN and detects lower genotoxin induced MN levels than the Japanese rodent skin MN assay suggesting it may be the preferred method for testing sanguinarine [190]. False positive results often generated by significantly cytotoxic compounds and those that induce oxidative stress in other assays are less likely to occur [191].

Fresh *ex-vivo* human skin obtained as excess tissue from cosmetic surgery has provided another model for genotoxicity testing. Against a range of true negative, misleading positive and true positive genotoxins it was found to have a sensitivity, specificity and accuracy of 89%, 90% and 89% respectively [192]. Human skin has some advantage over reconstructed epidermal models as all skin cell types are present and the stratum corneum has a normal function [193] with comparable physiological permeability [194], [195]. To date sanguinarine and black salve have not been assessed using skin genotoxicity models.

At present, ADME factors cannot be determined for black salve, largely due to a lack of compositional data. Despite toxicity concerns, there has only been a single report in the literature that analyzed black salve constituents without determining compound concentrations [196]. According to ICH guidelines exposure to genotoxins or their degradation products must be limited to 1.5 μg/day in order to minimize the risk of carcinogenicity.

Current genotoxicity testing strategies were developed primarily for assessing single chemicals, and so applying these strategies to herbal therapies that contain multiple bioactive compounds has been challenging. According to current EU herbal product guidelines (EMEA 2007), the Ames test is the primary endpoint for genotoxicity testing. Compounds that test negative are accepted as probably non-genotoxic [197]. As discussed, the Ames test fails to detect a number of genotoxins with a sensitivity of 60%, while its specificity of 77% indicates a number of compounds will yield false positive results [198]. The lack of regulatory rigor in natural product genotoxicity testing is of ongoing concern, as some of the most potent carcinogens known are natural products [199]. Since black salve containing sanguinarine is currently in clinical use, accurately determining its carcinogenic potential should be a matter of some urgency.

6. Conclusion

Currently we do not know whether sanguinarine or products that contain it are carcinogenic. This is surprising, as sanguinarine has been the subject of a significant body of scientific investigation. Sanguinarine shares molecular mechanisms of action with known carcinogens such as UVA and intercalating DNA agents, structurally resembles PAHs – a chemical class containing carcinogens, has positive *in vitro* and *in vivo* genotoxicity results, has cancer promoter action in a murine model, has a causative role in the development of mouthwash induced human leukoplakia and a suspected role in gallbladder carcinogenesis. Despite these findings, other contradictory *in vitro* and *in vivo* results have prevented the carcinogenic classification of sanguinarine.

As patients are presently using *S. canadensis* containing topical therapies in areas of UV induced field cancerization, urgent research is needed to determine the carcinogen status of sanguinarine and assess the level of risk. If carcinogenic, patients using black salve may develop subsequent malignancies. If mutagenic, black salve may increase the malignancy of existing skin cancers, making them more invasive and treatment resistant. In this situation, as skin is the target organ of interest, assessing sanguinarine genotoxicity in a bio-equivalent human skin *ex vivo* model may provide the most relevant and accurate assessment of its carcinogenic risk.

Declaration of interest

The authors report no conflicts of interest.

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