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Unveiling the potential toxicity and mutagenicity of traditional remedies used for gynaecological and obstetric ailments in Maputaland, South Africa

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ABSTRACT

Medicinal plants play an important role m the primary healthcare of lay people around the world, including the rural community ofMaputaland, South Africa. According to an ethnobotanical survey conducted in 2014, the lay people in northern Maputaland use plant species mdependently and m combination to treat gynaecology and obstetric ailments. These plant species were generally regarded as safe by the lay people except for one plant species, *Tnchilia dregeana* Harv. & Sand. The aim of this study was to investigate the safety of 16 plant combinations usmg the brme shnmp lethality assay (BSLA) for toxicity and Ames test using 5. *typhimurium* TA98 and TAlOO strains for mutagenicity. Toxicity studies on 33 medicinal plant species independently needed to be analysed first in order to make a comparison of combined with single use. The aqueous and organic (1:1 methanol-d1chloromethane) extracts were prepared rrom 51 plant samples (including leaf samples collected **as a** substllutton for the roots). There were three plant species *lAcalypha villicaulis* Horchst. Ex A.Rich. root, *Grewia occidentalis* L root and *Gymnosporia senegalensis* Loes. leaves) indicated neither toxicity nor mutagenicity when tested at 1 and 5 mg/ml using the BSLA and Ames test, respectively, *Hermannia* boraginiflora Hook., 5apium inregerrimum (Hochst. ex Krauss). 5cadoxus puniceus (L) Friis Nordal and Taber naemontana *elegans* Stapf demonstrated toxicity even after d1lutton to the lowest concentration of 0.031 mg/ml. The three plant combinations which were found to be non toxic in the BSLA (both aqueous and organic extracts) were Euphobia rirucalli L (root) with Ozoroa *engleri* R.Fern & A.Fern. (bark}, 5. puniceus (bulb) and 5enecro *serrarulo1des* DC. (whole plant); *Bridelia cathartica* G.Bertol. (root) with Opuntia *stricta* Haw. (stem) with *5earsia nebulosa* (Schoenland) Moffett (bark); and *B. cathartrca* (root) with *Erythnna humeana* Spreng (root). In the Ames test, the plant samples which appeared to be non-mutagenic against both 5. typhimurium TA98 and TA100 strains were *A.* villicaulis root, Cyperus *natalensis* Hochst ex Krauss root, *Euc/ea natalensis* A DC. leaves, *G. occidentalls* root, *Ochna* natallt1a Walp. leaves, *S. mtegerrimum* leaves and *S. puniceus* bulb. This study indicated that medicmal plant species (independently and in combination) may have toxic and/ or mutagenic effects, even without any obvious signs after consumption. More importantly, it was determined that tox1C1ty can be reduced by carefully managing the dose.

1. Introduction

Women can experience gynaecological ailments at any point in their lifetime, especially in their reproductive ages between 15 - 49 years (Ngene et al 2021). According to Abam (2015), gynaecological conditions can result in infertility, chronic diseases and may also be fatal. The prevalence of gynaecological and obstetric ailments can be further increased by sexual activity, or when women experience vaginal tract infections such as human papillomavirus (HPV) and candidiasis (Abam, 2015; Gao et al., 2021). According to Nergard et al. (2015), the use of medicinal plants during pregnancy is common, especially in Africa. Women rely on medicinal plant species to induce or facilitate labour, prevent preterm birth or miscarriage, or as a health tonic during pregnancy (Steenkamp, 2003; Malan and Neuba. 2011; Nergard et al., 2015). Women also rely on medicinal plants for conditions associated with menstruation i.e., dysmenorrhoea, amenorrhoea, menorrhagia and oligomenorrhoea (De Wet and Ngubane, 2014).

De Wet and Ngubane (2014) documented 35 traditionally used medicinal plant species for the treatment of gynaecological and obstetric ailments by the lay women in northern Maputaland. The

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most frequently reported plant species for various women ailments was *Bridelia cachartica* G.Bertol. (mentioned by 36 % of the participants), followed by *Ranunculus multifidus* Forssk. (mentioned by 26 % of the participants). Some of the plant species were used in combination by the women to treat gynaecological and obstetric ailments. In the study conducted by De Wet and Ngubane (2014), 16 plant species combinations were documented. These plant combinations incorporated two to a total of seven plant species in a combination. Although the study focused on medicinal plant species that are used by mostly women in their reproductive age (between menarche to menopause). These plant species were also reportedly used for other health conditions such as diarrhoea (De Wet et al., 2010), respiratory infections (York et al 2012), sexually transmitted infections (De Wet et at 2012), skin disorders (De Wet et al.. 2013) and hypertension (De Wet et al., 2016). These studies signify the importance of these medicinal plant species in the healthcare of the lay people of northern Maputa land.

It is plausible to assume that the long-term traditional use of medicinal plants and herbal remedies do not always guarantee safety (Madmgou et al., 2016). The most notable factors that influence human poisoning from using medicinal plants include the misidentification and unintentional use of toxic plant species, indirect consumption of plant toxins through eating animals. and/or over dosage of home preparations (Ndhlala et al., 2013; Wangpan et al., 2023). In South Africa, human intoxication by plants is common. According to hospital data, traditional herbal medicine has caused about 2.4 % cases of acute toxicity in South Africa (Malangu and Ogunbanjo, 2009; Malangu, 2011). Children have been reported to constitute most of these cases in South Africa (Fennell et al.. 2004). It should be acknowledged that there are many similar unreported incidents that are mostly treated at home and therefore cannot be found on health records.

Mutagenicity is the type of toxicity that results in the change of DNA sequence which can either be chromosomal or through gene mutation as a result of exposure to chemical substances (Richardson et al.. 2007). According to Ames et al. (1975). the majority (85 %) of carcinogens that have been tested reacted as mutagens, which implies that their sources may be similar. The mechanism of mutagenicity varies. Sometimes the mutagen may work directly by chemically promoting changes in the DNA sequence, or by damaging the building blocks of DNA (Reha-Krantz, 2013). The damage in the DNA may cause cell malfunction or induce tumour formations and other cancerous activities (Otlu et al.. 2023). Some medicinal plants in South Africa that have been reported to have mutagenic effects are: *£kebergia capensis* Sparrm, *Helichrysum herbacea* (Andrews) Sweet, *Helichrysum regulosum* Less and *Helichrysum simillimum* DC. (Reid et al., 2006; Verschaeve and Van Staden. 2008; Mulaudzi et al., 2013). It is thus important to make practitioners of medicinal plants aware of plant species that cause side effects, especially as these mutagenic effects are most likely to progress into cancer (Razak et al., 2007).

Ensuring safety is an important factor in continued traditional plant use and thus the plants identified by De Wet and Ngubane (2014) as traditionally used for gynaecological and obstetric ailments were assessed further for toxicology analysis. These plants are ingested orally, hence toxicity studies are recommended. Some plant species have toxicology data. however. 15 plant species have no toxicology data (Table 1), in spite of them frequently used for gynaecological conditions. Plant combinations are believed to be more effective in reducing toxicity (Van Vuuren and Viljoen, 201 I; Mundy et al.. 2016). According to Naidoo et al. (2013), in some cases. plant species combinations may exhibit a greater potential for toxicity. The latter results emphasise the importance of assessing safety of plant species combinations. None of the plant combinations mentioned in our previous study (De Wet and Ngubane, 2014}. have been assessed for toxicity, providing a rationale for the current study. Thus, the aim of this study was to Investigate the safety of 35 medicinal plant species and 16 plant combinations using the brine shrimp lethality assay (BSlA) for toxicity and Aines test using *Salmonella typhimurium* TA98 and TA100strains for mutagenicity.

2. M.1teri.1ls and methods

2.1. Preparation of plant extracts

Plant materials were collected from four areas (Mseleni. Kwajozana, Tshongwe and Mabibi) in northern Maputaland (KwaZulu-Natal), with ethical clearance (UZREC 171,110-030 PGM 2017/447) and permission from the land leaders and home owners. The leaves of all plant species that had the roots reported for medicinal purposes were also collected for comparison purposes. According to Manohar (2012), different plant parts can share the same properties. Therefore, it was important to assess further toxicity as a first step to find out whether the leaves can be used instead of the roots. for conservation purposes. Collected plant materials were chopped into small pieces and left to dry at room temperature. The dried plant material was then ground into fine powder using a Scientec RSA hammer mill. The powdered plant material was prepared into aqueous and methanol (MeOH}-dichloromethane (**DCM**) extracts.

2.2. Preparation of extracts

To prepare aqueous extracts. 10.00 g of dried plant material was boiled in 200 ml of distilled water for 30 min. The extract was then filtered and then lyophilised (CHRISTAlpha 1-2 LD plus). Methanol dichloromethane extracts were prepared by immersing 10 g of ground plant material into 200 ml of 1:1 methanol-dichloromethane (MeOH-DCM) for 24 hrs. This was kept in a platform shaker at 37 •C for 24 hrs. The extracts were then filtered through a 90 mm grade 3 hw filter paper (Whatman). The obtained liquid extract was kept in a fume hood to evaporate the solvent, after which a dry extract was collected.

2.3. The Amestestfor mutagenicity

The mutagenicity of plant extracts was evaluated using the standard Ames test method (Ames, 1973) against *S. typhimurium* TA98 and TA100 bacterial strains.These mutant bacteria can identify frameshift (TA98) and base-pair substitution mutations (Cross and DeMar• inl, 2023). The Ames test can be performed with or without a metabolic enzyme called S9 microsomal fraction which can activate the test samples. In the absence of the metabolic activation enzyme. the Aines test is used to identify direct acting mutagens (Sui tt al.. 2009; Cross and DeMarini, 2023). Plant extracts used in this study were dissolved to a concentration of 5 mg/ml using 10 % DMSO for MeOH-DCM extracts and distilled water for the aqueous extracts.

The assay was performed by adding 100 µI of the test sample, 500 µl of phosphate buffer (pH 7.4). and 100 µI of the overnight culture together in triplicate. Then 2 ml of the top agar was added into the sample-culture mixture, and then poured over the surface of the minimum glucose plates. Plates were incubated at 37 •C for 48 hrs. After the incubation period, bacterial colony forming units (CRJ) were counted, and the reversion rate was compared with the control plates. Positive controls for the assay were 4-Nitroquinoline oxide (4NQO) for TA98 strain at 2.5 mg/ml and sodium azide (Sigma-Aldrich) for TA100 at 2 mg/ml. The negative controls were distilled water for aqueous samples and 10 % DMSO for the MeOH-DCM samples The test sample was considered mutagenic if the number of colony-forming units (CFU) in a plate doubled compared to the negative control plates (distilled water and 10 % DMSO).

Table 1
Med1c111al plant species used ror gynaecolog al and obstetric conditions (De Wet and Ngub,me, 2014).

Plant names	Plant part used	Number or times mentioned for traditional use	Previous toxicological studies
Acolypha villicaulis Hochst ex A.Rich.	Roots	3	None Found
Acanthospennum gJabratum (DC) Wild	Whole plant	1	Contains toxic compounds (Saleh *et* al., 1980)
Bridelia cathartica G.Bertol	Roots	18	None Found
Cassytha filifonnis L	Whole plant		Cytotox1t m HepG2. IC,. of 143"3 g/ml, ethanol exrract (Prayong et al., 2008)
Commiphora neglecta I.Verd.	Roots		None Found
Crotalana monreiroiTaub.ex Baker f.	Roots		Pneumo1oxic ro horses (Botha et al . 2012)
Cyperus nataJensis Hochst ex.Krauss	Roots		*None* Found
Diospyros vilrosa (L)De Winter	Roots	1	None Found
Erythrina humeana Spreng	Roots	4	None round
Euclea naralensis A.DC.	Roots		Crude extract had IC,,, or 64.8 µg/ml in Vero cells (I.all et al.. 2005), toxic against selected cancer *cell* lines(Kishore et al., 2014).
Euphorbia Ilrucalli L	Stem		Latex was toxic to catfish (Heteropneusres/ossilis) with LC,,0 oFJ.45 111/1 after 24 hrs and 1 31111/1aFter 96 hrs (Kumar et al., 2010). Latex powder was toxic to the *CoJisafasdatus* with LC$_{14}$orB.14 mg/l (24 h)and 9.01 mg/l For *Channa punclatus* (Kumar et al., 20lO). sap had toxic effects on Tillap1a zflli with LC,. or 1.20 mg/l after 96 h period (Kumar et al..20l0 I.atex aqueous extract was non-toxic lo the embryo development orrat(S1va e1 •I.. 2007).
Gardnia *livingstonei* T.Anderson	Roots	2	Bark reported non-toxic in BSLA (Khumalo et al.. 202t)
Grtw1a ocodenta/1s L	Roots	1	Ethanol and aqueous extracts were non-mutagenic to *S. lyphimurium* TA98 strain (Mulaudzi et al., 2013)
Gymnospona senegalensis (I.am.) Loes.	Roots	3	Dichloromethane and methanol extracts *were* mutagenic in UmuC test without the activation S9 in *S. yphimurium* sir ain. The extracts were also genotoxic by inducingmicronucle1 in m1cronucleus test without S9 in human white blood cells (Vercheve and Van Staden. 2008)
Hennonnio *borag,niflora* Hook	Roots	1	None found
Hyphaene conacea Gaertn.	Stem	7	None round
Hypoxis *hemerocatlidea* Fisch.	Corm	6	Non-toxic to DU 145 prostate cancer cells. MDA-MB-23 CA.Mey. & Ave-I.all. breast cancer cells. MCF-7 bread cancer cells and McF 12A non-malignant breast cells (Steenkamp and Gouws, 2006). Aqueous and methanol-dichloromethane extracts were non-toxic in MTT assay usmg the human epilhelial cell line(Naidooet al., 2013). Methanol and dichloromethane extracts or the corm and leaves were non-mutagenic in the Ames test (S,*1yphimurium* **TA98** and TAI 00) with and without S9 activation (Elgorash1 et al., 2003; Reid *et* al., 2006).
Kigelia afn'cana (Lam.)Benth.	Bark	3	Non•mutagenic (Fru1t)(Elgorash1et al.. 2003; Verschaeve and Van Staden, 2008)
Ochna naroJitia Walp.	Roots	8	Concentration dependent when studied in the MTT assay (Suleiman er al., 20 lO).
Opunlla *slncta* Haw	Stem	3	Juice extract protects against induced hep.1to-nephrotoxicity (Zhu and Athmouni, 2002).
Ozaroa englen RFem & A.Fem	Bark	3	None Found
Pelrophorum africanum Sond.	Roots	3	Acetone extract were non-toxic on the BSLA and Vero monkey cell line (Mad1k1zela et al.. 2017)
Ranunculus mullifidus Fomk.	Whole plant	13	None Found
Rhoidssus digitata {Lf.J	Roots	3	None Found Gilg & M.Brandt
Sopium inregerrimum (Hochst.,	Roots	I	None Found ex Krauss)J.U:onard
Scadoxus puniceus (L) Friis Norda	Bulb		Aqueous extract was non-mutagenic in Ames test (S. *typhimurium* TA98) with and without S9 metabolic activation (Ndhlala et al., 2013). Contains toxic alkaloids (Nair and Van Staden, 2013)
Sderocarya birrea (A. Rich.) Hochsr.	Bark		Aqueous and methanol-dichloromethane extracts were non-toxic in MIT assay using human embryonic kidney epithelial cell line (Graham HEK-293) (Naidoo et al.. 2013). Methanol extract had LC,o <5000 mg/kg in BSLA (I.all and Kishore, 2014)
Searsia nebuJoso (Schoenland) Moffe!I	Bark	3	None found
Senecio serratuloides DC.	Whole plant	9	Aqueous and methanol-dichloromethane extract were non-toxic in MTT assay using hum.in kidney epithelial *cell* line (Na dooet al., 2013). Have toxic effects inmicronucleus test on human lymphocyte culture without metabolic activation S9 (Tamokou and Kuete, 2014). Methanol and dichloromethane lear extracts were non-mutagenic in Ames test (S. *typhimurium* TA98 andTAlOO strains) with and withour metabolic actiVa•lion S9 (Elgorashi et al. 2003)
Senegalia burkei (Benth.) Kyal. & Boatwr. (• *Acada burkei* Benth.)	Bark		None found
Tabemaemonrana eJegans Stapf	Roots		Ethanol extracts *were* cyroroxic towards human monocytic THP-1 cells with IC,.< 4 g/ml (Lou etal, 2011)
Trichilia dregeana Haiv. & Sond.	Roots	8	None found
	I.eaves	I	Aqueous extract were non•toxic in MTT assay on human embryonic kidney epithelial {Graham HEK-293) cell line (Naidoo et al 201l)
	Bark		Non-toxic in BSLA (Khumalo er al 2021)
Vangueria infausta Burch. subsp.	*I.eaves*		Toxic in MTT assay with *CC50* values higher than *infausta* positive control Berberine (Mthethwa et al 2014)

2.4. *The brine shrimp lethality assay for toxicity of plant extracts*

The brine shrimp eggs (0.5 g) of Artemiafranciscana(Animal Kingdom, Constantia Kloof, Johannesburg) were incubated for 18-24 hrs for hatching at 25 •C with a rotary pump (for aeration) in growth medium prepared by dissolving 16 g of Tropic Marine® Sea Salt in 500 ml of distilled water. An additional light source (220-240 V) was provided. To assess for toxicity, 400 µI of saltwater (containing 40-60 live brine shrimp) was added along with 400 µI of the plant extract into 48-well microtiter plates. According to Bussmann et al. (2011).

when a concentration above 1 mg/ml is required to exhibit toxic effect against Artemia spp., the extract is considered non-toxic. Studies were undertaken in duplicate and repeated in three cycles for each concentration. A negative control (toxin-free) consisting of artificial sea water was included. Potassium chloride (1.6 g/1}was used as a positive control. Dead shrimp were counted after 24 and 48 hrs using a stereo microscope. A percentage mortality of 50 and above indicated biological toxicity of the extract. This assay has been recommended as a suitable alternative model to mammals for pre-screening pharmaceutical substances for toxicity (Nunes et al. 2006; Okumu et al.. 2021).

The BSLA was also performed using 16 different plant combina tions encompassing a total of 25 different plant species. Plant combinations were selected based on combination remedies used for gynaecology and obstetric ailments in Mapuraland. Both aqueous (AC) and MeOH-DCM (organic) combinations (OCJwere tested. The plant extracts incorporated in a combination, were mixed in equal ratios depending on the number of plant species to be incorporated in the combination. Plant species were further assessed to determine whether the combination therapy is more or less toxic (antagonism or synergism, respectively) when compared to the toxicity of the extracts tested independently. This was determined using Eq. (1) to calculate the sum of fractional concentration (rFIC).

$$F/0^{1\cdot} \quad \frac{\text{Toxicity (a) in combination with (b)}}{\text{Toxicity (a) independently}}$$

$$Ff((ffl^{=} \quad \frac{\text{Toxicity (a) in combination with (b)}}{\text{Toxicity (a) independently}}$$

$$rf/C \quad fl((iJ+ \quad fl(liil \qquad (1l$$

The letters (a) and (b) represent different plant extracts. In some instances, more than two plants were combined and then the equation was expanded to include each of these components, and (iii), (iv) etc. were calculated. For the purpose of this study, the I:FIC value 0.50 was regarded as synergistic (reducing toxicity >0,50 - 1.00 as additive (slightly reducing toxicity); >1.00 - 4.00 as indifferent (no combined effect on toxicity) and >4.00 as antagonistic (increasing toxicity) (Hubsch et al., 2014). When 0.00 % toxicity was noted, the 1:FJC was not determined (ND) because it could not be calculated with zero as a denominator. For these instances a tentative value was documented based on comparative assessment with the results when reported independently.

3. Results and discussion

3.1. The Ames test

In the Ames test, 88% (Table 2) of the extracts demonstrated some mutagenicity against both *S. ryphtmurium* TA98 and TA100 strains. This includes the most frequently reported plant species for conditions related to gynaecological conditions such as *B. cathartica, H. hemerocallidea,* 0. *natalitia, R. multijidus, S.* serraru/oides and *T.* dre*geana.* There were six extracts (A. *villicaulis* root, C. *natalensis* root. *E. natalensis* leaves, G. *ocddentalis* root, 0. *natalitia* leaves, *S. integeni*mum leaves and *S. puniceus* bulb) that were non-mutagenic against both *Salmonella* strains.

The majority (92%) of the aqueous extracts showed no mutagenic effects towards the TA98 strain **(T.ible 2).** Only four aqueous extracts demonstrated a mutagenicity potential against the TA98 strain **i.e.,** f. *tirucalli* stem (by 1205 CFU), H. *hemerocal/idea* corm (by 1266 CFU O. *stricta* stem (by 2413 CFU) and 0. *engleri* bark (by 494 CFU), According to Paiva et al.(2011), the latex that exudes from the stem of *E. tiruca/li* has the potential to cause cancer. Waczuk et al. (2015) also reported that the aqueous extract off. *tiruca/lii* induced genetic damage in the Comet assay. The extracts which resulted in the highest number of revertant colonies against *S. typhimurium* TA98 were 0. *stricta* stem (2413 CFU) and *H. hemerocal/idea* corm (1266 CFU). There were approximately 28i aqueous extracts (Table 2) which demonstrated mutagenicity towards the TAtoO strain. Only two aqueous extracts (C. filiformis and *S. serratuloides)* have been previously assessed against the TAtoO strain. A previous study (Wu et al., 2017) was found to have similar results for C. *fi/iformis,* where it indicated that this plant species is mutagenic. However, *S. serraru/oides* had different results (non-mutagenic) in the Ames test according to Ramulond1 et al. (2019). The aqueous extracts which resulted in the highest number of revertant colonies were C. *natalensis* shoot (1814 CFU) and *E. humeana* roots (1350 CFU),

A total of 14 (27%) MeOH-DCMextracts demonstrated mutagenicity potential towards the TA98 strain (Table 2). *Euphorbia tirucalli* (Paiva et al, 2011), H. *coriacea* (Ramulondi et al.. 2019) and H. *hemer• ocallidea* (Elgorashi et al., 2003: Reid et al., 2006; Ramulondi et al., 2019) were reported in previous studies as non-mutagenic against *S. typhimurium* TA98. However, these plant extracts were mutagenic in the current study. The solvents used for the previous study were different from the current study, which could be the reason why the results are not the same. However,£ *tiruca/h* has other reports where the latex has genotoxic effects (Waczuk et al.. 201S and that could explain the mutagenicity found in the current study. In northern Maputaland, £ *tirucalli* was not among the popularly known plants to treat women aliments as it was only mentioned on one occasion to treat genital warts (De Wet and Ngubane, 2014). In this community the more frequently cited plant to treat genital warts was *R. multifidus. Ranunculus multijidus* demonstrated non-mutagenicity for both aqueous and MeOH-DCM extracts against both *S. ryphimurium* test strains. *Hypoxis hemerocallidea* and *S. serratu/oides* demonstrated different results to the previous study (Ramulondi et al., 2019), even though the same solvent (MeOH-DCM) was used. The extracts which resulted in the highest number of revertant colonies against 5. *typhimurium* TA98 were 0. *stricta* stem (2848 CFU) and H. boraginiflora leaves (2721 CFU). In terms of correlation to the use by the women in Maputaland, 0. *stricta* was not a popularly used plant (only three citations for the use against cervical pain and blood cleansing) **(Dt Wee** and Ngubane, 2014). The leaves of H. *boraginijlora* were only tested to compare with the roots for potential substitution. The results showed that the aqueous root extracts were non-mutagenic against both *S.* ryphimurium strains.

When the extracts were assessed against *S.* ryphimurium TAtoO. the majority (69%) of the MeOH-DCM extracts were mutagenic. Although there were no previous studies on the majority (65%) of the extracts, there were some extracts that have been previously assessed for mutagenicity. The MeOH-DCM extracts that were non-mutagenic against *S. typhimurium* TA100 correlated with previous studies with G. *occidentalis* (Mulaudzi et al., 2013), H. *coriacea* stem (Ramulondi et al., 2019), 0. *nata/itia* leaves (Makhafola et al., 2014) and *S.* puniceus bulb (Nair and Van Staden. 2013). Mutagenic extracts that correlated to previous studies were C jiliformis whole plant (Wu et al., 2017), *E. tiruca/li* stem (Waczuk et al.. 201S), *G. senegalensis* root (Verschaeve and Van Staden. 2008), 0. *engleri* bark (Ramulondi et al., 2019) and *S. serracu/oldes* whole plant (Tamokou and Kuete, 2014).

3.2. The brine shrimp lethality assay

The results (Table 3) indicated that 21% aqueous and 14% MeOH-DCM extracts indicated toxicity after 24 hrs. After 48 hrs, the toxicity increased to 47% and 53% in the aqueous and MeOH-DCM extracts, respectively. Toxicity of the aqueous extracts varied from O to 100% after 24 hrs and tto 100% after 48 hrs. The toxicity of MeOH-DCM extracts varied from Oto 78% after 24 hrs and Oto 100 % after 48 hrs of exposure to the brine shrimp nauplii. Among the 17 leafplant sample collected for potential substitution for roots (LPSR), the BSLA

ra111 2
Average revertant colonies (t1 • 3) using the Ames test on SolmonelJa *cyphimurium* TA98 **and** *S. l)'pltimurium* TA100.

Plant species	Plant palT	*S. typhimurium* TA98		*S.* typhimuriumTAlOO	
MeOH-DCM		Aqueous	MeOH-DCM	Aqueous	MeOH-OCM
Avillicaulis	Roots	78	116	98	54
Leaves	Leaves	29	115	91	**721**
Aglobrotum	Whole plant	29	185	73	**737**
B.*cocharnca*	Roots	10	21	**944**	**876**
Leaves	Leaves	20	7	126	**&J4**
C.filifonnis	Whole plant	255	**1617**	**652**	**1829**
C. negJecta	Roots	111	111	74	**693**
	Leaves	26	68	344	**733**
C. monreiroi	Roots	91	**1402**	81	**17DJ**
	Leaves	26	64	**900**	**1112**
C. natalensis	Roots	110	69	83	150
	Shoot	33	**1866**	**1814**	**584**
D.villasa	Roots	79	156	**431**	403
	Leaves	32	110	76	515
E. humeona	Roots	34	**1331**	**1350**	118
E. notole11sis	Roots	81	178	**509**	242
	Leaves	19	120	56	123
f. *cirucalli*	Stems	**1205**	**2069**	77	66]
G. livingsconei	Roots	62	117	177	**1151**
	Leaves	19	162	**715**	159
G. occidentolis	Roots	101	140	259	344
	Leaves	21	178	**979**	195
G. senegalensis	Roots	185	108	**1087**	**774**
	Leaves	28	76	63	**1019**
H. boraginijloro	Roots	94	**356**	74	328
	Leaves	29	**2721**	75	**2456**
H.coriocea	Stems	198	**2021**	122	76
H. hemerocaJlidea	Corms	**1266**	**1241**	**1307**	**1887**
K. *africana*	Bark	59	140	72	**948**
O. na1atiria	Roots	28	94	58	**1808**
	Leaves	28	196	319	283
O.sericta	Stems	**2413**	**2848**	**930**	**770**
O.*engleri*	**Bark**	**494**	145	**1000**	**1032**
P.africanum	Roots	58	47	26	**1142**
	Leaves	21	**2462**	73	84
R. muldfidus	Whole plant	103	235	166	**1875**
R. digirora	Roots	78	132	86	**1535**
	Leaves	23	**1006**	68	414
S. integerrimum	Roots	61	121	29	**2455**
	Leaves	34	91	61	74
S. puniceus	Bulb	133	90	99	71
S. birrtll	B.lrk	25	245	**659**	**1994**
S. nebulosa	B.lrk	78	**1836**	69	**1362**
S. serraculoides	Whole plant	77	170	**514**	**868**
S. burkei	B.lrk	21	62	75	**790**
T. elegans	Roots	177	**1583**	**1074**	**1088**
	Leaves	131	116	**984**	**1546**
T. dregeana	Roots	142	141	215	**814**
	Leaves	164	154	**895**	**2103**
	Bark	25	79	127	**717**
V. infausta	Leaves	33	81	234	**718**
Water (negalive control)		131	-	174	
DMSO (negative control)		-	142	-	274
4NQO (positive control)		]41	]41		
Sodium azide (positive control)		-	-	624	**624**

Bold-represents mutageniciiy.

indicated that most of the aqueous (76%) and MeOH-DCM (53%) leaf extracts as toxic. However, both aqueous and MeOH-DCM leaf extracts of C monteiroi, G. *senega/ensis* and *T. dregeana* were non-toxic. Hypothetically, this indicated the potential for root substitution, ifthe leaves were active.

In general, most of the aqueous extracts were non-toxic except for the seven aqueous extracts from C *monteiroi* root, ***E.*** *humeana* root, H. *boraginijlora* root, *T. e/egans* root, *T. dregeana* root and bark. and *V. infausra* leaves which were toxic. While evidence is lacking in the literature of toxicity for most of these species, C *monteiroi* was reported by Botha et al. (2012), to have somewhat toxic metabolites such as the pyrlizidine alkaloids. The pyrrolizidine alkaloids were reported to have a pneumotoxic effect when tested in a horse's respiratory system (Botha et al., 20 l 2) and were also associated with hepatotoxicity (Neuman et al., 2015). In another study, (Lou et al., 2011) reported that the ethanol root extracts of *T. e/egans* were cytotoxic towards the human monocytic THP - 1 cells with the LCso <4.00 µg/ml demonstrating some congruency with our findings. A previous BSI.J\ study by Ramulondi et al. (2019), reported that the aqueous leaf extract of *V. infausta* to be non-toxic with a mortality percentage of 5%. Our study demonstrated 73% mortality. The discrepancy between results could be associated with the variation of the individual plant chemotype. This variation usually causes the individuals of the species or sub-species to have differences in the quality and quantity of

Tallle l
Aver•ge percentage mortality or brine shrimp larvae (11• 9) against aqueous .i.nd MeOH-DCM plant extr.1.c1s in the BSlA

Plant names	Plant part	Aqueous extract		MeOH-DCM extract	
		24hrs	48 hrs	24hrs	48hrs
A. villicaulis	Roots	0	I	17	27
	leaves	90(0.43)	93(0.34)	4	24
Aglabrarum	Whole plant	10	17	29	34
& *calharrico*	Roots	J	9	2	**19(026)**
	I.eaves	3	87(0.57)	28	**93(0.22)**
C.filiformis	Whole plant	6	29	20	i!l(0.72)
C. ntglecra	Roots	18	32	0	**40**
	Leaves	ll	92(1174)	0	97(0.65)
C. ioonteiroi	Roots	**55(0.80)**	**74(0.48)**	0	&8(0.23)
	leaves	16	30	13	14
C narolensis	Roots	21	38	45	72(0.80)
	Shoot	47	**74(0.78)**	45	96(0.60)
D. villasa	Roots	6	14	35	**61** (0.35)
	Leaves	59(0.92)	80(0.50)	14	28
E. humeana	Roots	22	92(0.50)	5	49
f. norofensis	Roocs	2	24	I	H(0.12)
	leaves	15	**11** (0.75)	0	87(0.65)
f. rirucalfi	Stems	**100(0.15)**	100(050)	**71**(025)	**90(0.58)**
G. livingstonei	Roots	I	JO	8	45
	Leaves	27	i7(0.89)	0	22
C. occidentalis	Rools	0	2	4	**44**
	Leaves	**45**	**94(058)**	26	44
C. senegalensis	ROO(s	4	13	0	1
	Leaves	10	14	1	1
H. boraiinijlora	Roots	28	86(0.34}	13	**91 (Taxlc:)**
	Leaves	76(0.41)	**98(0.25)**	**59** (0.80)	89(0.10)
H. cotiacea	Stems	4	14	25	]8
H. htmerocallidea	Corms	4	40	28	66(0.80)
K.Africana	Bark	13	40	30	35
0. norolirio	Roots	1	15	52(0.70)	55(0,75)
	leaves	6	67 [0.64)	59(0.97)	68(0,83)
0. *$Crit'Ca*	Stems	79(0.33)	ll (0.25)	19	38
o. ren	Bark	**50(0.94)**	74(0.45)	23	54(0.45)
P.africonum	Roots	47	49	23	28
	Leaves	**45**	47	37	53(0.94)
R. mulfijidus	Whole plant	11	15	17	34
R.digilOlO	Roots	22	43	38	I0(0.71)
	Leaves	28	**51** (0.98)	27	56(0.85)
S. inregenimum	Roots	3	8	**65** (0.61)	**100(ToKk)**
	I.eaves	87(0.50)	**93** (0.33)	**61** (0.61)	**11** (0.81)
S. puniceus	Bulb	50(0.91)	93(0.30)	22	**94(Toxlc:)**
S. birrea	Bark	3	17	23	90(0.53)
S. nebulasa	Bark	1	I	1	2
S. serratuloides	Whole plant	4	14	0	0
S. burkei	Bark	17	48	3	4
T. *eleians*	Roots	19	85(0.83)	27	**!lll(Toxlc)**
	I.eaves	18	89(0.50)	0	0
T. *dregeana*	Roots	75(0.55)	92(0.44)	0	0
	leaves	I	27	I	I
	Barie	20	100(0.89)	25	79(0.70)
V. in/•usra	*I.eaves*	67(0.57)	73(0.48)	'6(0.71)	11S(0.34)
Potassium dichromate (positive control)		**100**	**100**	**100**	**1«1**
Disrilled water (negative control)		1	7	I	8
2:ii; DMSO (**negative** control)		0	1	I	**4**

Bold•lOX1C; values in brackets present the concentration (mg/ml) at which the sample demonstrates non-toxicity.

chemical compounds as a result of differences in genetic expression (Polatoglu, 2013). Overall. there was more toxicity observed with MeOH-DCM extracts (54i) when compared to aqueous extracts (20%). The most frequently used plant species *(B. cuthartica, H. hemerocallidea.* 0. *nata/itia* and *T. dregeana* according to De Wet and Ngubane (2014i was toxic when the MeOH-DCM extracts were tested in the BSLA. Two exceptions were *S. serratuloides* and *R. mu/tifidus* which were non toxic. These two plant species were also reported in previous studies to be non-toxic (Naidoo et al.. 2013; Ramulondi et ak 2019).

All plant extracts where toxicity was found at 1 mg/ml were subjected to further testing at lower concentrations (0.50, 0.25, 0.125, 0.063, and 0.031 mg/ml). Reducing the dose of toxic extracts is of importance to determine a safe concentration.The non-toxic concentrations of these extracts are presented in brackets (**Table** 3). These plant extracts indicated varying non-toxic concentrations which ranged between 0.98-0.25 mg/ml in the aqueous extracts, and 0.94-0.01 mg/ml in the MeOH-DCM extracts, with some samples still retaining toxicity at the lowest concentration tested. Although there are limited studies on a toxicity dose response of the plant species in this study, toxicity at lower concentrations correlated with previous studies (Ah et al., 2011; Aapu et al.. 2013; Abesede et al.. 2015; Kolbeck and Tintjer, 2016; Ahmed et al., 2018; Ramulondi et al.. 2019). Another correlation observed was the lower dosages used traditionally with plants having higher toxicity levels. For example, *R. multi.Ii• dus, S. burkei* root and *T. dregeana* leaves that were traditionally

Table4
Average percentage mortality or brine shrimp when exposed to aqueous and MeOH-DCM combinations or plant extracts.

Plant combinalion code'	Plants in combination	Times menlionedb	AC [ERC index, interaction[24 hrs	AC [ERC index, interaction[48 hrs	OC [I:FIC index, interaction] 24 hrs	OC [I:FIC index, interaction] 48 hrs
ACl/OCl	*8. catharli(f]* (roots)+ *C. neglecia* (roots)+ *C. monteiroi* (root)+ *G.* li•ings/onei (root)+ *G. ocddemalis* (root)+ o. noralirio (root)+ *R. digitala* (root)	1	0.67 IND, syn]	I0.33 [7.80,antl	45.67 [ND.ant'!	**75.33** [9.50,antl
AC2/0C2	*E.* tirucalli (root)+ 0. *engleri* (bark)+ *S.*puniceus (bulb)+ *S. mratuloides* (whole plant)	1	0.09 IO.OJ.syn]	0.23 [0.03.synl	32.00 IND,ant'I	49.00 [ND,ant'I
AO/OO	*B. carharlica* (root)+ f. humeono (root)+ 0. *nataliria* (rool) + T. *elegans* (rool) + *S. nebulosa* (bark)	I	0.67 [2.40.indl	23.33 [27.60, ant]	30.00 [52.70,anl]	**61.67** [34.70, anti
AC4/0C4	A. •illicoulis (root)+ *B. carhartica* (root)+ *S. nebuJosa* (bark)	I	1.67 IND.ind]	3.33 [6.30,antf	**68.33** I I0652, ant]	**75.33** [41.31, anti
AC5/0C5	*B. calhartica* (root)+ *P. africanum* {root)+ *R.* digiroto (root)	2	5.33 [2.00, ind I	22.00 [3.40,ind]	**55.67** 135.90, anti	**66.33** [3.90,indl
AC6/0C6	*B. cothartica* (root)+ *H. coriacea* (seem)+ 0. *engleri* (bark)	6	20.00 [12.10,ant]	38,33 17.50, anti	45.67 125.70, anti	**69.67** [3.90,indl
AC7/0C7	***R.*** mulrijidus (whole plant)+ *S. serraruloides* (whole plant)	5	11.67 I4.09.antl	**69.J]** [9.53 ant]	**86.I]** IND.ant']	**92,00** IND.ant]
ACB/OCB	***B.*** *cathartica* (root)+ 0. *s/ricta* (stem)+ S. *nebulosa* (bark)	3	0.02 I0.03,syn[	34.00 I38.16,antl	21.00 I32.61,ant[	40.00 121.so,aml
AC9/0C9	*R.*mullijidus (whole plane) + *H. hemerocallidea* {corm)	2	4.33 11.36, ind[	**66.67** I6.14,ant]	**74.67** I3.71,ind]	**88.67** [2.30, ind]
AClO/ OClO	*K. africana.* (bark)+ *C.filiformis* (whole plant)	1	0.00 (0.00,syn[	0.67 10.10, syn[	**78.00** 16.50, ant]	**78.67** 13.40, ind]
AC11/ OC11	*E. humeana* (root)+ 0. na/olitio (root)	1	9.67 [I0.11.anII	40.67 (3.15, ind]	26.33 (5.78, ant]	**60.67** 12,34, ind]
ACl2/ OCl2	***B.*** *catharrica* (root)+ f. *humeana* (root)	1	O.QI [0.00, syn[	36.00 14.39, anti	24.00 (16.80, ant]	48.00 (1.46, ind]
AC13/0C13	*G. senegolensis* (root)+ *H. hemerocallideo* (corm)	3	0.00 [0.00,syn[	**52.33** 5.30, anti	**5].67** IND.ant']	**82.33** I83.20,am]
AC14/0C14	*K.ofricana* (bark)+ *S.* nebulosa (bark)	1	0.00 10.00, syn]	0.67 II.10,ind]	44.00 145.50, ant]	**57.00** 130.10. ant]
AC15/0C15	*B. carharlica* (root)• *O. natalicia* (root)	1	0.00 I0,00, syn!	5.67 [1.I0,indJ	**56.I]** [29.08, ant]	**57.67** (1.70, ind]
AC16/0C16	*S. birrea* (bark)+ *T. dregeana* (bark)	1	16.67 I6.50.antl	49.33 IJ.40.indJ	36.00 [3.00, ind[	**57.67** p.40, ind[
Potassium dichromate (positive control)			**100**	**100**	**100**	**100**
Salt *water* (negative control for aqueous extracts)			0.00	2.00		
2 % OMSO (negative control for MeOH-DCM extracts)			-	-	0.00	0.00

• AC• Aqueous combination; OC • Organic combination.
b Number or times mentioned traditionally rrom total number or 50 (Oe Wet and Ngubane, 2014); **V.alul!S In bold** - Toxic. syn - synergistic. ind - indifferent, ant - antagonistic; ND - not determined where' is the tentative interactive value.

reported at higher dosages (Table 1) such as five or two handfuls per litre were non-toxic in the BS!J\. The lowest-reported dosage (quarter handful per litre) was with *V. infausta* leaves which was toxic and mutagenic in this study. There were three MeOH-DCM extracts in this study {H. *boraginijlora* root, *S. integerrimum* root, *S.* puniceus bulb and *T.* elegans root) that remained toxic even at the lowest concentration of0.031 mg/ml, and these extracts were considered highly toxic. However, these four highly toxic plants were the least popular for medicinal use{reported by only one to three respondents).

3.3. An overview *of toxicity and mutagen/city using* single *plant extracts*

In general, it was observed that some extracts were non-mutagenic while others were mutagenic against one strain or both. Similarly, with the BISA, some extracts were non-toxic while others were toxic only after 48 hrs or in both 24 and 48 hrs. An overview of the plant samples studied in both the BISA and Ames test indicate that there were 17 aqueous extracts and five MeOH-DCM extracts which demonstrated non-toxicity and non-mutagenicity. The popularity and selection of plant species by the women in Maputaland mostly did not show any correlation in terms of selection to prevent toxicity and mutagenicity. However, it was noted that some of the popularly known plants such as *H. coriacea, R. multifidus* and *S. serrntuloides* were non-toxic, but with mutagenic effects. There were two plant extracts (A *villicau/is* and G. *occidentalis* roots) that demonstrated non-toxicity and non-mutagenicity in both the aqueous and organic extracts.

3.4. Toxicity of plant combinations in the brine shrimp lethality assay

An overview of the 16 plant species combinations (Table 4) indicated that none of the aqueous plant combinations were toxic after 24 hrs. After 48 hrs. three (19 %) aqueous combinations (AC?, AC9, and AC13) were toxic and antagonistic. It was noted that in combinations where plant species such as *R. multijidus* and/or *H. hemeroca/lidea,* G. *senegalensis* and *S. serrntuloides* were included, the outcome was toxic. When these plant species were tested individually (aqueous extract). they were all non-toxic. According to Naidoo et al.

T• s

The highest conceniratton of aqueous and MeOH-DCM combinations which were found to **be** non-<oxic at 48 hrs in the BLSA -

Combinalion of plant species concentrations(mg/ml)	Code	Non-toxic aueous concentration (mg/ml)	Non-toxic MeOH-DCM concentration (mg/ml)
8. cothartico (roots)+ C. neglecta (roots) +C. monttiroi (root) • G. *Nvi11&5tonei* (root) +G. occidentalis (roor) + O. naroliria (root)+ ***R.*** digifata (root).	AC\ /OC1	-	0.42
8. corharrica (root)+£. humeona (root)+ 0.nofallrlo (root)+ *T. elegans* (root) +*S. nebulosa* (bark).	AO/OC.3	-	0.67
A. villicaulis (root)+ *8. cothorrica* (root)+ *S.* nebulosa (bark).	AC4/0C4	-	0.75
8. carharriro (root)+ *P.* africonum (root) +*R. digitato* (root)	AC5/0CS	-	0.68
8. catharriro (root]+ *H. coriacea* (stem)• 0. *engleri* **(bark)**	AC6/0C6	-	0,70
R. *multifidus* (whole plant) •*S.* semitu/oides (whole plant)	AC7/0C7	0.62	0.70
R. *mulrifidus* (whole plant) •H.hemerocollideo(corm)	AC9/0C9	0.59	0.66
K. *africana* (b.uk) +*C.filiformis* (whole plant)	AC10/0C10	-	0.71
£ *humtona* **(root)**• 0.notoliria **([*00()**	AC11/0C1\	-	0.74
C. senegalensis (rool) +H. hemerocal/idea (corm).	ACII / 0C\3	0.95	**0.60**
K. africana (bark)• *S. nebulosa* (bark)	AC\4/0(14	-	0.88
8. rothorriro (root)+ 0. *naraliria* (root)	ACIS / 0C15	-	0.47
*S.*birreo (bark)+ *T.* dregeona (bark).	AC\6 / 0C16	-	0.94

(2013), the interactions within the combination can potentially bring about toxic activity, regardless of the non-toxic nature of the individual extracts. When plant combinations were tested, it was determined that these plant combinations were toxic except for AC2/0C2 and AC8/OC8. Both combinations are used for blood cleansing by the women in Maputaland during pregnancy. The use of both these combinations implies safety of both the pregnant woman and the baby. The highest percentage mortality (most toxic) observed with the aqueous extract combinations was 69.33% from combination *AO,* which was reported five times for genital warts, and the lowest mortality (least toxic) was *023%* with combination AC2 where the combination was reported only once. The higher reported use for the toxic combination warrants concern.

When the MeOH-DCM extract combinations were tested in the BSIJ\ (24 hrs), there were seven **(44 %)** toxic extracts that include OC4, OCS, *00,* OC9, OC.10, OC13 and OC15. The highest percentage mortality (as seen with aqueous combination) was obtained with combination OC7 at 86.33%, and the lowest was 21.00 % with OC8. After 48 hrs, 81% **MeOH-DCM** extracts indicated toxicity, which was higher (as expected) compared to the aqueous extracts. The highest percentage mortality for MeOH-DCM combinations was 92.00% from the combination OC7; and the lowest mortality was 40.00% with the combination OC8. Toxicity results for these combinations have not previously been reported, hence no comparison with previous studies could be done.

When all plant combinations were further analysed for plant-plant interactions using the EFIC calculation (Eq. (1)). some combinations indicated a change in the interaction between 24 and 48 hrs. e. g_, a synergistic demonstration after 24 hrs changing to antagonistic/ indifferent interaction after 48 hrs. A longer period may have allowed these combinations to undergo some processes such as excretion. rnetabolization, transportation. uptake and binding at the target sites (Cock and Ruebhart, 2009; Cedergreen, 2014) and thus the difference in interaction was observed. For this reason. the discussion of the results in this section focuses more on the results obtained after the maximum period of 48 hrs.

Approximately 56% (including tentative interpretations) of aqueous combinations indicated an antagonistic interaction after 48 hrs. This indicated that the toxicity of these combinations was enhanced when compared to the toxicity of their respective independent extracts. The aqueous combination which demonstrated the highest EFIC value was OC8 with the EFIC value of 38.16 after 48 hrs. Synergistic interactions were detected in two aqueous combinations after 48 hr, namely the AC2 and AC10 with the EFIC value of 0.02 and 0.10. respectively. The toxicity of these combinations was considerably reduced compared to the respective individual extracts in these combinations. The EFIC of MeOH-DCM extract combinations **(Table** 4) indicated 50% of the extracts to be antagonistic and the other 50% being indifferent after 48 hrs. There were no synergistic or additive interactions observed with MeOH-DCM combinations. The lowest acquired EFIC value was 1.40 (indifferent) which was obtained with the combination OC16. The highest EFIC value obtained was 106.52 (antagonistic) with combination OC4.

As with the extracts evaluated independently. it was important to evaluate toxic combinations at reduced concentrations to determine a dose response to toxicity. Thus, the aqueous combinations (AG, AC9 and AC13) and the organic combinations that demonstrated toxicity after 48 hrs were tested further at varying concentrations for a dose response to toxicity (Table 5). It was noted that the aqueous extract combinations AC7 and AC9 were non-toxic at highest concentrations of 0.62 mg/ml and 0.59 mg/ml, respectively. Combination AC13 was non-toxic only at a concentration of0.95 mg/ml. When the toxic MeOH-DCM combinations **were** diluted, all combinations demonstrated reduced toxicity (0.42 to 0.94 mg/ml). Combinations AC7 and AC9 were among the most popularly used combinations for genital warts (De Wet and Ngubane. 2014) and reduced concentrations demonstrated a less toxic outcome. Although AC13 was only mentioned once, dosage at reduced toxicity is critical because it is recom• mended for use during pregnancy.

4. Conclusion

This study highlights that not all natural products are necessarily safe. Many of the organic extracts demonstrated toxic and mutagenic effects when tested in the Ames and BSIJ\ assays. Although the aqueous extracts (preferred traditional method of preparation) showed less toxicity. some instances show that the frequently used plants (e. g., *B. cathartica* root) and plant combinations (e.g., AC7) are toxic. This indicated the potential for these plants to cause adverse effects, especially because these plants are used regularly by the lay people of Maputaland. Toxic plants may cause liver, kidney and other organ damages. especially those that are used frequently for menstrual cycle conditions. Genetic disorders that may be caused by mutagenic plants can be transmitted to future generations. However. careful consideration of the dose when preparing herbal medicine can lower the toxicity. For example. toxic plant species (e.g.. 0. *engleri* bark, *T. e/egans* root etc.) and combinations (e.g., AG, AC9 and AC13) in this study demonstrated non-toxicity after reducing concentration by dilution. Another aspect that needs to be considered is that not all mutagens result in irreparable damage and the administration of plant needs to be carefully considered when reporting toxicity. It is recommended that toxicity at varied concentrations should always be considered when studying the efficacy of medicinal plants.

Declaration of competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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