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# *In vitro* cytotoxic and mutagenic evaluation of thirteen commercial herbal mixtures sold in KwaZulu-Natal, South Africa

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**Abstract**

Cytotoxic and mutagenic effects of thirteen commercial herbal mixtures sold in KwaZulu-Natal, South Africa were evaluated using the neutral red uptake (NRU) assay and the Ames test. The herbal mixtures tested included *Umzimba omubi*, *Umuthi wekukhwehlela ne zilonda*, *Mvusa ukunzi*, *Umpatisa inkosi*, *Imbiza ephuzwato*, *Vusa umzimba*, *Ingwe® muthi mixture*, *Ibhubezi™*, *Supreme one hundred™*, *Sejeso herbal mixture Ingwe®*, *Lion izifozonke Ingwe®*, *Stameta™ BODicare®* and *Ingwe® special muti*. The relative cytotoxicity of the herbal mixtures was established by determining their $NI_{50}$ values (50% inhibition of neutral red uptake). The test revealed that the most toxic herbal mixture was *Umpatisa inkosi* with an $NI_{50}$ value of 0.016 mg/mL and the least toxic mixture was Stameta™ BODicare® with an $NI_{50}$ value of 28.00 mg/mL. The herbal mixtures showed no mutagenic effects against *Salmonella typhimurium* tester strains TA98, TA100, TA102, TA1535 and TA1537 when the assay was done without S9 metabolic activation. However, four herbal mixtures, *Umpatisa inkosi*, *Imbiza ephuzwato*, *Vusa umzimba* and *Stameta™ BODicare®* showed mutagenic effects against TA98 but not the rest of the tester strains after using S9 metabolic activation. *Umpatisa inkosi* also exhibited weak mutagenic activity against TA1535 after metabolic activation. The remaining mixtures did not show mutagenic effects against all the tester strains after S9 metabolic activation. The cytotoxic and mutagenic results reported here offer a step toward determining the safety of commercial herbal mixtures in South Africa. Herbal mixtures showing higher cytotoxic and mutagenic effects need to be further investigated for their possible effects on humans.


*Keywords:* Ames test; Cytotoxicity; HepG2; Herbal mixtures; Mutagenicity; Neutral Red Uptake

## 1. Introduction

Herbal mixtures are concoctions of two or more plant species with the same or different medicinal uses (Cano and Volpato, 2004). These remedies are often well-known in local communities and are prepared at home for minor ailments such as insomnia, digestive disorders, coughs, diarrhoea and fever. In South Africa, commercialisation of such products has resulted in the production of complex mixtures for many conditions as well as energy boosters, detoxifiers, immune boosters and aphrodisiacs. The principle aim of making these herbal mixtures seems to be increasing the therapeuticity of a herbal product by mixing plant species of common or different uses (Cano and Volpato, 2004).

A growing number of the world's population are turning to plant derived remedies and medicines for many reasons, which include their low cost compared to western pharmaceuticals and seeking natural alternatives which are widely believed to have fewer side effects. The herbal medicine (*muthi* in isiZulu) industry is a multi million Rand industry in South Africa and is continually growing. Despite the existence of the Medicines and Related Substances Control Act 101 of 1965 and its amendments of 2002 (Department of Health, 1965, 2002) it is still difficult to regulate commercially labelled medicines, herbal formulations and nutritional supplements. Medicine regulation, is in the public interest, and comprises three integral aspects: quality, safety and

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efficacy. In terms of quality the hygiene and potential contamination of herbal products used in traditional medicines are a concern as they are sold on pavements and in markets where the materials are often exposed to sputum, urine and faeces, contrasting with the pharmaceutical manufacturing standards which are necessary for production and packaging of other medicines (Steenkamp et al., 2006). Regarding efficacy and safety, it is imperative to evaluate the relative risk from traditional African herbs and remedies.

Techniques involving *in vitro* tests are increasingly used as alternatives to whole animal toxicity tests due to their reduced use of experimental animals, their low cost, high specificity and rapidity (Asensio et al., 2007). Cell line methods such as the neutral red uptake (NRU) assay are used as screening tests for new therapeutic products to assess acute and chronic toxicity (Castell and Gómez-Lechón, 1996). The NRU cytotoxicity assay procedure is a cell survival/viability chemosensitivity assay based on the ability of viable cells to incorporate and bind neutral red (NR), a supravital dye. NR is a weak cationic dye that readily penetrates cell membranes by non-ionic diffusion and accumulates intracellularly in lysosomes. Alterations of the cell surface or the sensitive lysosomal membrane leads to lysosomal fragility and other changes that gradually become irreversible. Such changes brought about by the action of xenobiotics result in a decreased uptake and binding of NR. It is thus possible to distinguish between viable, damaged, or dead cells, which are the basis of this assay (Barile, 1994; Repetto et al., 2008).

Healthy mammalian cells, when maintained in culture, continuously divide and multiply over time. A toxic chemical, regardless of site or mechanism of action, will interfere with this process and result in a reduction of the growth rate as reflected by cell number. Cytotoxicity is expressed as a concentration dependent reduction of the uptake of the NR after chemical exposure thus providing a sensitive, integrated signal of both cell integrity and growth inhibition (Bohets et al., 1993; Repetto et al., 2008).

Damage of the genetic material by environmental mutagens can lead to mutations in many organisms, including humans. Mutations are associated with the development of most cancers and various degenerative disorders and genetic defects in offspring (Cariño-Cortés et al., 2007). To prevent mutagenic risk, it is important to identify the involved environmental mutagens and minimize human exposure to them. Short-term genetic bioassays like the Ames assay have been used as important tools in mutagenic studies because of their simplicity, sensitivity to genetic damage, speed, low cost of experimentation and small amount of sample required (Mathur et al., 2007).

Poisoning due to plant products (herbal poisoning) is not well documented because of the unwillingness of people to admit using traditional medicine derived from plant material and because of the fear that the cultural heritage of the people will be put under strong laws and regulations (Steenkamp et al., 2006). To date several studies have looked at the mutagenic effects of individual medicinal plants from South Africa (Elgorashi et al., 2003; Verschaeve et al., 2004; Reid et al., 2006; Verschaeve and Van Staden, 2008), but not at herbal preparations. This study was undertaken to evaluate cytotoxic and mutagenic effects of herbal mixtures that are commonly sold in traditional herbal shops in KwaZulu-Natal, South Africa.

## 2. Materials and methods

### 2.1. Sample procurement

Thirteen commercial herbal mixtures, *Umzimba omubi*, *Umuthi wekukhwehlela ne zilonda*, *Mvusa ukunzi*, *Umpatisa inkosi*, *Imbiza ephuzwato*, *Vusa umzimba*, *Ingwe® muthi mixture*, *Ibhubezi™*, *Supreme one hundred™*, *Sejeso herbal mixture Ingwe®*, *Lion izifozonke Ingwe®*, *Stameta™ BODi-care®* and *Ingwe® special muti* were bought at random from *muthi* shops around Pietermaritzburg, KwaZulu-Natal according to availability. Table 1 includes trade names, uses and packaging information for the thirteen herbal mixtures. We have evaluated nine of these herbal mixture for antibacterial, antifungal effects and the abilities to inhibit the cyclooxygenase (COX-1 and -2) enzymes (Ndhlala et al., 2009).

### 2.2. Sample preparation

The herbal mixtures (200 mL) were filtered through Whatman No. 1 filter paper and freeze dried. The dried material was weighed and resuspended in water and filtered through a sterile 0.22 μm filter unit (Millex® GV, Molsheim, France) to obtain a sterile 50 mg/mL concentration used with further dilutions in the concentration-range finding study. For $NI_{50}$ evaluation (see below) concentrations and dilutions were prepared based on the range finding data.

### 2.3. The neutral red uptake (NRU) assay

The NRU test was done according to Borenfreund and Puerner (1985). Cell suspension of human hepatocellular liver carcinoma cell line 2 (HepG2) in Dulbecco's modified Eagle's culture medium (DMEM) supplemented with 10% foetal calf serum was seeded into each well of a 96-well microtitre plate such that the cell density was forty thousand cells/well. Plates were incubated overnight at 37 °C, 5% $CO_2$ and humidity was maintained using a water bath containing milli-q water inside the incubator. After 24 h incubation, the cells were treated with dilutions of the herbal mixtures and a positive control sodium dodecyl sulfate (SDS). Cells were kept in the presence of the test mixture (0, 0.0005, 0.005, 0.05, 0.5, 5 and 50 mg/mL of each herbal mixture initially and thereafter suitable concentrations were used depending on the preliminary toxicity results) for another 24 h. At this point the medium was removed and cells were washed with phosphate-buffered saline (PBS) solution. Medium (200 μl) containing 0.05 mg/mL neutral red dye (NR) was added to each well using a multichannel pipette. The microtiter plates were incubated for 3 h in a humidified 5% $CO_2$ incubator at 37 °C. The medium was removed and the cells were rapidly washed with 0.2 mL of PBS solution. An acetic acid–ethanol mixture (0.2 mL) was used to extract the dye from the cells. The plates were agitated on a microtiter plate shaker for at least 1 h (or until a homogeneous stained medium was

Table 1
Selected commercial herbal mixtures sold in KwaZulu-Natal, South Africa.

| Product name / Trade name / Batch number | Medicinal uses | Directions of use | Expiry date / Shelf life | Packaging |
|---|---|---|---|---|
| Umzimba omubi | Used to treat wounds, skin rashes, fungal infections and boils | Not on label | Not on label | 500 ml |
| Umuthi wekukhwehlela ne zilonda | Used as a cough mixture, to treat chest infections and difficulty in breathing | Not on label | Not on label | 500 ml |
| Mvusa ukunzi | A 'man tonic' for increasing sexual prowess and can be used as an energiser | Not on label | Not on label | 500 ml |
| Umpatisa inkosi | An 'adult tonic' used for increasing sexual prowess, as an energiser also used to treat sexually transmitted diseases (STDs), to stop menstrual pains, increase appetite, treat high blood pressure and fight arthritis | Not on label | Not on label | 500 ml |
| Imbiza ephuzwato | A detoxifying and energising tonic used to increase sexual prowess, relieve constipation, reduce stress, reduce high blood pressure, clear skin conditions, boost energy, boost vitality, helps to prevent arthritis, kidney problems and relieve general body pains | ¼ cup in the morning after meals twice a week | Not on label | 1 Litre |
| Vusa umzimba<br>Ingwe®<br>Batch no. AMM 0016 | Used to treat wounds, rashes, fungal infections, boils chest infections, stop menstrual pains, increase stamina and fight against influenza virus | 4 tablespoons twice a day.<br>Not for children under 14 years of age and pregnant women | On label<br>6 months | 500 ml |
| Ingwe muthi mixture<br>Ingwe®<br>Batch no. AMM 0011 | A traditional African mixture for chest infections, STDs, arthritis, heartburn, relieving constipation and increasing sexual prowess | 3 tablespoons every morning | On label<br>6 months | 500 ml |
| Ibhubezi™ | Used for wounds, fungal infections, STDs, treatment of influenza, to reverse impotence, clean the body system and stimulate blood production | ¼ cup twice a week. Not for children under 14 years of age and pregnant women. Shake well before use | Not on label | 500 ml |
| Supreme one hundred<br>BODicare®<br>Batch no. 05020207 | Used for nervous disorders, skin conditions, stimulates blood production, boost sexual performance, treats back pains, fights influenza and strengthens the body | ¼ cup every night before sleeping after meals. Not for children under 14 years of age and pregnant women | On label<br>1 year | 500 ml |
| Sejeso herbal mixture<br>Ingwe ®<br>AMM 005 | Used to relieve heartburn, constipation, stomach ache, stomach cramps and indigestion | ¼ cup three times a day after meals.<br>Not to be taken by children and pregnant women | On label<br>6 months | 500 ml |
| Lion izifozonke<br>Ingwe®<br>AMM 003 | Used for chest infections, STDs, arthritis, heartburn, relieving constipation and increasing sexual prowess | ¼ cup three times a day after meals.<br>Not to be taken by children and pregnant women | On label<br>6 months | 500 ml |
| Stameta™<br>BODicare®<br>04020207 | Used for nervous disorders, skin conditions, boosts sexual performance, poor blood quality, high blood pressure. Chest, lung, kidney infections. Fever and flu. Heart problems, back pain, persistent tiredness. Menstrual pain, easy bruising, cleans out bile, bleeding gums, stomach gas, body sores. Strengthens bones and boost the immune system. | ½ or ¼ cup three or four times a week.<br>Not for children under 14 years of age and pregnant women. Drink water after using Stameta™. | On label<br>1 year | 500 ml |
| Ingwe special muti<br>Ingwe®<br>AMM 003 | Used for alleviating menstrual pain, general pain. | Take one 5 ml teaspoon in hot water or with tea every morning until the course is finished.<br>Not for children under 14 years of age and pregnant women. | On label<br>6 months | 500 ml |

obtained) and then absorbance against a blank reference was measured at 540 nm using a micro plate spectrophotometer.

For all wells optical density (OD) values were calculated as the measured value minus the control value (Vc). Results were expressed as percentage of the OD determined from the average of the blank control culture read at 540 nm and set at 100%. The $NI_{50}$, (50% inhibition of NRU) was determined from the dose response curve of the mean OD values of the seven concentrations as indicated.

For the positive control a separate plate was used where cells were treated with different concentrations of SDS and the $NI_{50}$ was determined as for the herbal extracts described above. The $NI_{50}$ was kept within limits that were determined from 10 independent experiments from which average $NI_{50}$ values and standard deviations were calculated (unpublished data). The calculated $NI_{50}$ for the positive control in an experiment should be within ±2.5 SD of the historical data for SDS. If this is not the case the results cannot be accepted and the test should be repeated.

### 2.4. Ames test

Mutagenicity was tested using the *Salmonella* microsome assay based on the plate-incorporation procedure with *Salmonella typhimurium* tester strains TA98, TA100, TA102, TA1535 and TA1537 with and without metabolic activation (Maron and Ames, 1983; Mortelmans and Zeiger, 2000). TA98 strain has a −1 frameshift mutation at *hisD3052* which affects the reading frame of a repetitive base pair –C–G–C–G–C–G–C–G- sequence, TA100 and TA1535 has a *hisG46* marker resulting from the substitution of a leucine (GAG/CTC) by a proline (GGG/CCC). The *hisG46* mutation can be reverted to a wild type by mutations that cause base pair substitution at the GC site. TA102 contains AT base pairs at the *hisG428* mutant site. The *hisG428* can be reverted by mutagens that cause oxidative damage. TA1537 carries a +1 frameshift mutation *hisC3076* located near the repetitive site –C–C–C- sequence and can be reverted by frameshift mutagens that are not readily detected by the *hisD3052* (TA98).

Overnight bacterial tester strains were grown in 10 mL Oxoid nutrient broth No. 2 for 16 h at 37 °C to obtain a density of $1–2\times10^9$ colony forming units (CFU/mL). The metabolic activation mixture (S9 mix) was prepared freshly before the assay and kept on ice throughout the assay procedure. The S9 mix consisted of 5% (v/v) S9 fraction (Sigma-Aldrich, Co., St Louis) pooled from Sprague-Dawley male rats in mixed enzymic cofactors containing NADP. At the beginning of the assay, top agar supplemented with 0.5 mM histidine and biotin was melted and kept in a 50 °C water bath. To sterile glass tubes, in triplicate, 100 µl of three dilutions (50, 500, 5000 µg/mL) per sample were added, followed by 500 µl phosphate buffer (0.1 mM, pH 7.4) or S9 mix. To the mixture, 100 µl of the overnight bacterial culture was added followed by 2 mL of the melted top agar. The contents of the tubes were then mixed and poured onto labelled minimal agar plates. As soon as the top agar had hardened (2–3 min), the plates were inverted and incubated at 37 °C for 48 h. The colonies were then counted with the aid of a binocular microscope. The assay was repeated twice for each bacterial strain and the results were expressed as the mean (±standard error) number of revertant colonies per plate. 4-Nitroquinoline-N-oxide (4NQO) (2 µg/plate) was used as a positive control for the assay without metabolic activation while 2-aminoanthracene (2-AA) (2 µg/plate) was used where the assay was carried out with S9 metabolic activation. Sterile distilled water was used as a negative control in both assays. The test herbal mixture/compound was classified as a 'mutagen' if the results satisfied two criteria (1) a dose dependent increase in the number of revertants is observed and (2) the number of revertants is equal to or greater than two times that of the negative control.

## 3. Results and discussion

### 3.1. The neutral red uptake (NRU) assay

The neutral red uptake inhibition in human liver (HepG2) cells was used to assess potential toxicity of thirteen commercial herbal mixtures sold in South Africa. The NRU assay is important for ranking of toxic components according to their potencies and structure–toxicity relationship studies (Bohets et al., 1993). The $NI_{50}$ values of the thirteen mixtures are summarised in Table 2. The results revealed that the most toxic herbal mixture was *Umpatisa inkosi*, with an $NI_{50}$ value of 0.016 mg/mL and a yield of $2.2\times10\ 10^{-4}$ mg/mL residue which implies that 72.72 mL of the herbal mixture will result in the stated $NI_{50}$ value (Table 2). The least toxic mixture was *Stameta*™ BODicare® with an $NI_{50}$ value of 28.00 mg/mL and a yield of $1.8\times10^{-4}$ mg/mL which implies that 115.56 L of the mixture is required to reach the $NI_{50}$ value of the mixture. The rest of the mixtures exhibited moderate toxicity with $NI_{50}$ values ranging within the two highlighted values.

HepG2 cells used were from a highly differentiated human hepatoma cell line that retains many of the cellular functions often lost by cells in culture. This cell line also has the enzymes involved in phase I (MFO) and phase II (glucuronic acid and sulphate conjugation) metabolism of xenobiotics, and it has been used as an *in vitro* system instead of human normal hepatocytes to study drug metabolism and toxicity (Nakama et al., 1995).

### 3.2. Ames test

The standard plate incorporation test methods for the Ames test using *Salmonella typhimurium* tester strains TA98, TA100, TA102, TA1535 and TA1537 exposed to three dilutions with and without S9 metabolic activation of the herbal mixtures was performed. Ames test without S9 metabolic activation can only detect direct mutagens while with S9 metabolic activation allows the detection of indirect mutagens, often caused by conjugation reactions of metabolic oxidation systems. Table 3 presents the spontaneous reversion response of the *Salmonella typhimurium* tester strain to the different dilutions of the herbal mixtures. The results revealed that all thirteen herbal mixtures were non-mutagenic towards the *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537 for the assay without metabolic activation. The average $His^+$ revertants observed for all the tester strains caused by the herbal mixtures at all the concentrations without metabolic activation did not

Table 2
$NI_{50}$ values (mg/mL) after 24 h treatment of HepG2 with herbal test mixtures.

| Sample | Yield[a] (mg/mL) | $NI_{50}$ (mg/mL) |
|---|---|---|
| *Umzimba omubi* | $2.4\times10^{-6}$ | 1.220 |
| *Umuthi wekukhwehlela ne zilonda* | $2.3\times10^{-6}$ | 1.010 |
| *Mvusa ukunzi* | $2.9\times10^{-6}$ | 1.680 |
| *Umpatisa inkosi* | $2.2\times10^{-4}$ | 0.016 |
| *Imbiza ephuzwato* | $2.1\times10^{-5}$ | 1.150 |
| *Vusa umzimba* Ingwe® | $5.5\times10^{-6}$ | 5.890 |
| *Ingwe muthi* mixture Ingwe® | $5.5\times10^{-6}$ | 3.610 |
| *Ibhubezi*™ | $1.1\times10^{-4}$ | 15.200 |
| Supreme one hundred BODicare® | $9.2\times10^{-5}$ | 17.900 |
| *Sejeso* herbal mixture Ingwe® | $0.5\times10^{-5}$ | 2.630 |
| Lion *izifozonke* Ingwe® | $5.3\times10^{-6}$ | 4.280 |
| *Stameta*™ BODicare® | $1.8\times10^{-4}$ | 28.000 |
| *Ingwe* special muti Ingwe® | $6.2\times10^{-6}$ | 2.080 |
| SDS (positive control) | | 0.082 |

[a] Yield was obtained by filtering and freeze drying 100 mL portions and the residue was weighed and expressed as yield (mg/mL).

Table 3
Number of His+ revertants in *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537 produced by herbal mixtures with and without S9 metabolic activation.

| Sample | μg/mL | Number of His+ revertants | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | TA98 | | TA100 | | TA102 | | TA1535 | | TA1537 | |
| | | $S9^-$ | $S9^+$ | $S9^-$ | $S9^+$ | $S9^-$ | $S9^+$ | $S9^-$ | $S9^+$ | $S9^-$ | $S9^+$ |
| *Umzimba omubi* | 5000 | 22.3±0.9 | 29.0±1.7 | 157.0±12.6 | 183.3±10.1 | 349.3±20.7 | 277.0±5.8 | 13.3±1.2 | 21.3±1.8 | 6.0±1.7 | 9.3±0.3 |
| | 500 | 21.7±1.7 | 24.3±3.8 | 195.0±2.9 | 234.3±15.7 | 374.0±26.4 | 269.0±9.1 | 14.3±4.5 | 18.0±0.5 | 6.7 ±0.6 | 8.0±0.5 |
| | 50 | 26.7±0.9 | 24.7±0.9 | 182.7±8.3 | 202.3±6.6 | 326.3±31.6 | 262.3±4.2 | 12.0±1.5 | 13.3±0.8 | 7.0±0.5 | 7.7±0.6 |
| *Umuthi wekukhwehlela ne zilonda* | 5000 | 26.0±0.6 | 21.3±0.7 | 168.7±4.4 | 209.7±5.9 | 331.7±12.8 | 284.7±6.8 | 17.0±1.2 | 33.0±0.5 | 9.0±0.5 | 7.7±1.4 |
| | 500 | 25.0±1.5 | 23.0±1.0 | 173.3±8.9 | 187.7±5.5 | 321.6±17.1 | 275.0±6.1 | 16.3±0.3 | 21.7±1.8 | 8.3±0.3 | 7.0±0.5 |
| | 50 | 23.3±0.9 | 19.3±0.9 | 183.0±6.5 | 188.3±6.1 | 301.0±2.1 | 269.3±3.7 | 14.3±2.4 | 18.3±0.6 | 7.7±0.8 | 6.7±1.2 |
| *Mvusa ukunzi* | 5000 | 22.7±0.7 | 19.3±0.3 | 163.0±4.6 | 191.0±6.6 | 306.3±10.1 | 287.0±1.2 | 12.0±0.6 | 21.0±4.5 | 7.0±0.0 | 10.0±0.5 |
| | 500 | 23.0±0.3 | 21.0±2.1 | 178.3±11.4 | 191.0±7.1 | 319.0±13.3 | 257.0±5.0 | 14.0±0.6 | 21.3±2.1 | 8.0±1.0 | 10.0±0.5 |
| | 50 | 25.0±2.0 | 26.0±4.4 | 200.3±6.0 | 198.0±0.6 | 320.0±2.0 | 257.0±2.1 | 17.7±2.6 | 21.3±0.8 | 7.7±1.3 | 8.7±0.3 |
| *Umpatisa inkosi* | 5000 | 23.3±2.6 | **219.6±16.3** | 180.7±8.0 | 207.0±2.6 | 281.3±9.3 | 274.0±1.5 | 17.0±4.0 | **96.3±1.7** | 6.3±0.8 | 8.3±0.6 |
| | 500 | 24.7±0.9 | **70.6±1.5** | 183.0±7.6 | 198.3±0.7 | 291.3±7.7 | 264.3±1.2 | 14.0±0.6 | **71.0±2.5** | 6.3±1.4 | 7.7±0.3 |
| | 50 | 25.7±1.2 | 32.3±4.1 | 195.3±5.7 | 200.0±2.1 | 289.3±6.9 | 272.3±7.9 | 15.7±0.9 | **47.3±2.0** | 5.7±0.8 | 7.3±0.3 |
| *Imbiza ephuzwato* | 5000 | 22.0±0.9 | **227.7±23.1** | 171.6±10.1 | 230.7±1.5 | 329.6±19.7 | 256.3±1.9 | 14.7±1.5 | **52.0±3.0** | 7.3±0.3 | 8.0±1.0 |
| | 500 | 24.0±1.6 | **71.5±5.5** | 173.7±2.9 | 194.7±1.8 | 320.7±50.3 | 236.3±3.9 | 15.3±1.5 | 34.0±1.5 | 5.0±0.5 | 9.3±0.3 |
| | 50 | 19.7±1.2 | 45.3±1.8 | 180.5±4.4 | 203.7±1.8 | 299.3±7.2 | 231.7±0.7 | 14.0±0.6 | 22.0±0.5 | 5.7±0.6 | 9.7±1.7 |
| *Vusa umzimba* Ingwe® | 5000 | 23.7±2.7 | **217.0±26.2** | 167.7±3.2 | 191.0±1.2 | 305.3±1.7 | 320.3±4.4 | 13.3±0.9 | 41.3±0.6 | 5.7±0.3 | 8.0±0.1 |
| | 500 | 19.3±0.7 | **106.0±18.0** | 173.6±9.1 | 181.3±14.8 | 314.3±26.5 | 223.7±1.2 | 17.0±0.6 | 32.0±0.5 | 7.0±0.5 | 6.7±0.3 |
| | 50 | 18.0±1.2 | **52.7±3.0** | 182.6±11.5 | 181.3±9.8 | 288.7±12.7 | 209.7±0.7 | 11.0±0.6 | 20.3±6.0 | 6.0±0.5 | 8.7±0.3 |
| *Ingwe muthi* mixture Ingwe® | 5000 | 25.0±1.0 | 32.7±5.2 | 197.0±9.7 | 209.0±3.2 | 350.3±14.1 | 288.0±0.6 | 10.3±2.4 | 21.3±1.2 | 7.3±0.8 | 7.3±0.3 |
| | 500 | 19.3±1.7 | 24.0±5.5 | 185.3±7.2 | 200.7±0.3 | 311.3±6.1 | 269.7±4.2 | 16.0±4.5 | 20.7±1.8 | 7.3±1.2 | 9.7±0.3 |
| | 50 | 20.0±1.0 | 33.7±8.0 | 179.7±6.3 | 207.3±3.8 | 288.3±5.7 | 215.7±0.9 | 12.3±0.9 | 15.7±2.0 | 6.0±0.5 | 8.3±0.6 |
| *Ibhubezi*™ | 5000 | 23.3±1.3 | 36.0±2.1 | 184.6±6.4 | 179.3±1.8 | 337.0±7.1 | 301.3±0.3 | 16.0±2.5 | 27.0±0.5 | 6.0±0.5 | 7.7±0.3 |
| | 500 | 23.0±1.0 | 39.7±2.7 | 177.0±5.2 | 180.3±14.8 | 319.3±3.9 | 266.0±6.1 | 13.7±4.8 | 19.7±1.7 | 6.0±0.5 | 8.0±0.5 |
| | 50 | 22.0±1.2 | 35.3±0.9 | 181.3±5.9 | 176.7±11.7 | 305.0±5.0 | 258.7±6.9 | 12.0±1.0 | 17.0±0.5 | 6.3±0.8 | 7.3±0.3 |
| Supreme one hundred BODicare® | 5000 | 28.0±1.5 | 31.6±6.7 | 178.0±9.9 | 223.3±1.5 | 388.7±12.7 | 244.0±5.8 | 12.3±0.3 | 26.0±0.5 | 8.0±1.5 | 7.7±0.8 |
| | 500 | 26.0±3.2 | 31.3±2.3 | 179.6±11.4 | 213.3±1.8 | 322.7±13.3 | 219.3±6.8 | 17.0±0.5 | 25.3±0.3 | 7.3±0.3 | 7.3±0.3 |
| | 50 | 26.0±1.5 | 31.0±1.7 | 176.7±4.1 | 204.0±2.1 | 305.0±2.5 | 206.3±1.8 | 13.0±2.0 | 24.3±0.6 | 6.0±1.5 | 9.3±1.2 |
| *Sejeso* herbal mixture Ingwe® | 5000 | 20.0±0.7 | 23.3±0.9 | 163.3±25.4 | 211.7±5.3 | 307.0±19.4 | 277.7±6.1 | 14.3±0.6 | 25.0±1.5 | 5.3±0.8 | 8.3±0.3 |
| | 500 | 22.0±0.6 | 25.0±3.5 | 155.7±1.8 | 203.7±0.3 | 302.3±12.3 | 255.3±5.8 | 14.0±1.5 | 18.3±0.8 | 5.0±0.5 | 8.7±1.3 |
| | 50 | 20.4±0.2 | 21.3±1.2 | 172.6±2.2 | 203.3±1.2 | 304.0±4.0 | 313.0±0.5 | 17.7±0.6 | 17.7±0.6 | 6.3±1.2 | 8.7±0.3 |
| Lion *izifozonke* Ingwe® | 5000 | 22.7±1.0 | 23.7±0.9 | 177.7±10.1 | 255.7±4.9 | 319.0±12.9 | 316.0±2.5 | 24.3±0.3 | 24.3±0.3 | 7.0±2.0 | 7.7±0.6 |
| | 500 | 23.1±1.7 | 25.3±3.5 | 177.7±13.3 | 235.7±2.9 | 298.0±13.2 | 256.0±6.4 | 22.0±1.5 | 22.0±1.5 | 6.3 ±0.8 | 7.7±0.8 |
| | 50 | 21.0±0.2 | 23.7±0.9 | 188.6±16.5 | 203.7±1.5 | 284.0±3.6 | 256.0±6.4 | 13.3±0.3 | 15.3±1.2 | 6.7±0.8 | 8.3±0.3 |
| *Stameta*™ BODicare® | 5000 | 22.0±2.0 | **131.3±6.9** | 172.6±6.9 | 187.0±15.0 | 395.3±9.3 | 257.0±5.5 | 13.0±0.5 | 28.0±1.5 | 6.3±0.3 | 8.7±0.3 |
| | 500 | 22.3±2.0 | 48.0±3.2 | 166.3±1.9 | 135.0±1.0 | 341.3±10.1 | 259.0±9.6 | 13.0±0.5 | 24.0±0.5 | 6.3±0.8 | 8.7±0.3 |
| | 50 | 19.0±2.0 | 39.3±8.0 | 197.7±4.9 | 125.3±3.3 | 325.7±9.5 | 255.3±5.8 | 13.3±0.8 | 17.7±0.6 | 7.3±1.2 | 8.7±0.3 |
| *Ingwe* special muti Ingwe® | 5000 | 21.3±0.3 | 23.6±2.4 | 175.0±5.7 | 173.3±4.2 | 320.3±6.9 | 262.0±3.1 | 11.3±0.3 | 19.0±0.5 | 6.7±0.8 | 10.3±1.2 |
| | 500 | 19.0±1.0 | 24.3±1.9 | 188.3±2.2 | 167.0±1.2 | 344.3±11.3 | 256.0±6.4 | 16.0±2.0 | 18.7±1.4 | 7.3±0.8 | 7.7±0.3 |
| | 50 | 21.0±0.9 | 28.0±1.5 | 204.3±4.5 | 163.0±3.5 | 318.7±18.2 | 247.7±8.8 | 12.7±1.8 | 18.3±0.8 | 7.7±0.6 | 7.0±0.5 |
| 4NQO | 2 | 64.0±0.6 | | 931.7±145.2 | | 2436.3±120.1 | | 1415.7±62.3 | | 65.0±6.9 | |
| 2–AA | 2 | | 218.0±13.4 | | 985.3±113.9 | | 1659.0±51.5 | | 162.3±4.0 | | 128.7±31.4 |
| Water (–ve control) | | 22.0±1.6 | 25.3±1.0 | 199.6±2.3 | 207.0±4.9 | 296.0±10.6 | 16.7±1.4 | 21.7±1.4 | 21.7±1.4 | 6.7±1.2 | 9.3±0.8 |

Number of His+ revertants/plate: mean values of three triplicates, the assay was repeated two times.
$S9^-$ refers to assay without metabolic activation; $S9^+$ refers to assay with metabolic activation.
4-NQO; 4-nitroquinoline-oxide, positive control for the $S9^-$ assays. 2-AA; 2-aminoathracene, positive control for $S9^+$ assays.
Values in bold represent mutagenic effects.

satisfy the criteria for mutagenicity. There were no notable dose dependent increase in the number of revertants and the numbers of revertants were all not equal to or greater than two times that of the negative control (Bulmer et al., 2007). There was also no decrease in the number of revertant colonies to levels far below the negative control (spontaneous reversion) which could also be classified as toxic.

The Ames test with metabolic activation was carried by addition of the S9 mix so as to detect indirect mutagenic effect cause by metabolites of the test herbal mixtures. The S9 fraction contains a mixture of xenobiotic metabolizing enzymes such as the cytochrome P450s and sulfotransferase. Four of the thirteen herbal mixtures (*Umpatisa inkosi*, *Imbiza ephuzwato*, *Vusa umzimba* and *Stameta*™ *BODicare*®) showed indirect mutagenic effect toward the tester strain TA98 after metabolic activation but not in the other tester strains (TA100, TA102, TA1535 and TA1537). *Umpatisa inkosi*, a man tonic induced 219.6, 70.6 and 32.3 TA98 revertant colonies from the highest to the lowest concentration, with revertant colonies increasing as the concentration of the mixture increases. However, the revertant colonies (32.3) induced at the lowest concentration (50 μg/mL) by *Umpatisa inkosi* did not satisfy the criteria for mutagenicity. The mixture also exhibited mutagenic effects towards TA1535 after metabolic activation. *Imbiza ephuzwato*, a multipurpose Zulu herbal tonic induced a high number of TA98 revertant colonies (227.7, 71.5 and 45.3) while *Vusa umzimba*, also a multipurpose herbal mixture induced 217.0, 106, 0 and 52.7 TA98 revertant colonies. The number of revertant colonies for *Imbiza ephuzwato* and *Vusa umzimba* satisfy the criteria of mutagenicity. *Stameta*™ *BODicare*® exhibited a weak mutagenicity potential against TA98. At the highest concentration (5000 μg/mL), *Stameta*™ *BODicare*®, induced 131.1 revertant colonies and 48.0 at 500 μg/mL. The rest of the mixtures did not show any mutagenic potential against all tester strains after metabolic activation.

TA98 has a −1 frameshift mutation *hisD3052* which affects the reading frame of a repetitive –C–G–C–G–C–G–C–G- sequence (Mortelmans and Zeiger, 2000). Therefore, the four herbal mixtures (*Umpatisa inkosi*, *Imbiza ephuzwato*, *Vusa umzimba* and *Stameta*™ *BODicare*®) cause a reversion of the *hisD3052* mutation back to the wild-type state. The herbal mixtures could contain various aromatic nitroso-derivatives of amine carcinogens that have been shown to cause such a reversal of *hisD3052* mutation back to the wild-type state.

Some carcinogenic compounds such as aromatic amines or polycyclic aromatic hydrocarbons like benzo-[a]-pyrene are biologically inactive unless they are metabolized to active forms. In humans, xenobiotic metabolizing enzymes present in the liver, lungs and kidneys, are constantly carrying out conjugation reactions and in some cases the compounds results in bioactive metabolites capable of damaging DNA (Mortelmans and Zeiger, 2000).

*Imbiza ephuzwato* contains plants that are known from previous investigations to possess mutagenic and toxic constituents (Verschaeve and Van Staden, 2008). *Imbiza ephuzwato*, consists of a mixture of 21 plant species consisting of bulbs, leaves and roots. Most of the 21 plant species that constitute *Imbiza ephuzwato* are used by traditional people to treat various conditions. For example, *Gunnera perpensa* or *Ugobo* in Zulu, one of the plant constituents of *Imbiza ephuzwato* contains bioactive compounds with uteroactive properties (Brookes and Dutton, 2007). Other plant species in the same mixture include toxic plants species such as *Scadoxus puniceus*, *Gomphocarpus fruticosa*, *Gnidia kraussiana* and *Drimia robusta* (Van Wyk et al., 2002). *Gnidia kraussiana* contains diterpenoids which causes fatalities in both humans and livestock in various parts of Africa including South Africa. The plant has been used as a fish poison. Diterpenoids have antitumour and antileukaemic activities (Van Wyk et al., 2002).

The evaluation of bacterial mutagenicity is important as an initial test for complex mixtures because of the possibility that one or more of their components can be a mutagen (Lee et al., 2005; Déciga-Campos et al., 2007). The fact that some of the tested herbal mixtures showed no mutagenic effects against all the tester strains could be due to antagonism on potential toxic compounds which could be a result of mixing a number of herbs (Leung, 2004).

Recently, there have been reports on increased global demands for herbal products that act as energy boosters, detoxifiers, immune boosters and aphrodisiacs (Cherdshewasart et al., 2008). The results of this study provide evidence to support the safe consumption of some herbal mixtures at low and medium doses and also have alerted us to mixtures that were found to be mutagenic.

Poisoning due to herbal mixtures and/or products most often occurs in the age group 1–5 years, however, according to descriptions on most of the labels of the herbal mixtures (Table 1), the mixtures are not recommended for children under the age of 14 years. Adult poisoning occur usually as a result of mislabeling of products, or products which are not stored in their original containers. Occasionally poisoning is through confusing or misidentifying a toxic plant with something that is thought to be edible (Van Wyk et al., 2002). In some instances, adults could be poisoned by taking incorrect doses or prolonged use, thus it is also important for the manufactures to clearly state the directions of use and possible side effects. Also interactions of these herbal products with western drugs could be lethal as often patients tend to mix western and traditional medicines.

## 4. Conclusion

The NRU assay revealed different levels of cytotoxicity while no mutagenic effects were observed in the thirteen herbal mixtures without S9 metabolic activation. The question of the nature of the toxic compounds observed in the NRU assay still remains unanswered. This calls for further work to identify the toxins involved. The observed cytotoxic and mutagenic effects against TA98 after S9 metabolic activation of *Umpatisa inkosi*, *Imbiza ephuzwato*, *Vusa umzimba* and *Stameta*™ *BODicare*® raises concerns as to the safety of these herbal products. At present the contents of most of the mixtures are not known. Further investigation and confirmation needs to be done with the objective of continuing research to determine and hopefully eliminate the source of the observed cytotoxic and mutagenic effects.

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