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ANTIOXIDANT, PROXIMATE AND CYTO-GENOTOXIC EVALUATION OF ANTI-TYPHOID FEVER HERBAL RECIPE IN *ALLIUM CEPA* CELLS

Akinboro Akeem[1*], Adedosu Olaniyi Temitope[2], Famurewa Feyisetan Omobolanle[1], Olowe Temidayo[1], Oyewole Oyepeju Opeyemi[1]

[1]Department of Pure and Applied Biology, Ladoke Akintola University of Technology, Ogbomoso, Oyo State, Nigeria.
[2]Department of Biochemistry, Ladoke Akintola University of Technology, Ogbomoso, Oyo State, Nigeria.
*Corresponding author email: aakinboro@lautech.edu.ng

ABSTRACT

It is the common practice that herbal medicines are prepared with combination of more than one plant part to make recipes. This study evaluated anti-typhoid fever herbal recipe (ATFHR) containing 10 different plants parts for antioxidant, proximate and cytotoxicity and genotoxicity in *Allium cepa* cells. Extract of ATFHR was tested for ability to scavenge DPPH free radicals. Proximate analysis was performed to determine the kinds of nutrients and phytochemicals present. The effects of the ATFHR on mitosis and chromosomes morphology in *A. cepa* were assayed after 24 hour treatment. In the same manner, the possibility of affected cells recover from toxic effects of ATFHR after 24 hours post-treatment in distilled water was determined. ATFHR and ascorbic acid scavenged 50% free radicals at 531.1µg/ml and 147.7µg/ml, respectively. The proximate analysis revealed the percentage of carbohydrate, crude fibre, proteins and fats present in the ATFHR as 58.63%, 14.13%, 10.67% and 1.50%, respectively. The ATFHR was cytotoxic to the *A. cepa* cells, inhibiting cell division in a non-concentration dependent manner. However, there was recovery of mitotic inhibited cells in distilled water at 44.4%, 64.2% and 41.5% obtained at 6.25%, 12.5% and 25.0% concentrations, respectively, suggesting mitostatic effect. Induced chromosomal aberrations (CA) were not concentration dependent, indicating weak mutagenic potency of ATFHR. Nevertheless, the mutagenic effect was not reversible at the tested concentrations except 25% where the induced CA at both treatment and post treatment levels was 0.03. Therefore, ATFHR should be consumed with cautions based on the obtained results in this study.

KEY WORDS: Herbal recipe; Typhoid fever; chromosome aberration; *Allium cepa*; mitostatic; antioxidants.

INTRODUCTION

The use of herbal medicines to treat diseases is an age long practice of people in the developing and developed countries of the world. Various plants in different localities are known to be potent in alleviating disease conditions of their users. In Africa, a number of plants are used for the treatment of diseases such as fever, dysentery, cholera, diarrhoea, and others which are major diseases of any tropical African countries (Fadimu *et al*., 2014). Typhoid fever is an infectious disease caused by *Salmonella typhi* and *Salmonella paratyphi* A, B or C. The number of reported death cases as a result of typhoid fever vary from region to region, with highest reported from Indonesia, Nigeria and India (Miller *et al*., 1994). More than 80% of the of world's population relies on traditional medicine for their primary healthcare. Plants are significant sources of medicines that are used in the treatment of various diseases of both humans and animals.

Apart from the fact that plants are readily available in the bush and as well cheaper to buy than orthodox medicines, they also contain active phytochemicals or principles that are potent in treating chronic as well as infectious diseases, being the reasons for their acceptance as alternative medicines (Gupta *et al*., 2005). Herbal remedies are either prepared from dry or freshly collected plants in different solvents such as water, pure honey, lime and aqueous extract from fermented maize (Shosan *et al*., 2014). They may contain either single plant material or two or more plant materials that are combined to act synergistically in bringing about healing of affected persons or animals. More than 50% of the orthodox medicines are developed from plants containing phytochemicals that are active against a number of diseases. The secondary metabolites from higher plants are the source of compounds with possible biological activity (Santos *et al*., 2006). However, the evaluation of metabolites from higher plants used extensively in traditional medicines is necessary considering their role in modulating the activity of environmental genotoxicants (Sreeranjini and Siril, 2011). Although medicinal importance of some plants has been proved with scientific research, however, caution should be observed while taking the herbal recipes for the healing purpose.

Herbal recipe containing mixture of plant extracts is logically expected to contain more phytochemicals acting synergistically either as active principle (s) against a disease or as toxicants to inflict adverse effects on the consumers. In Southwestern Nigeria where this investigation was carried out most herbal recipes or formulations are prepared with more than one plant material. This perhaps is due to an ethnomedicinal belief that each of the plants in the recipe will have a role to play

in bringing about the expected healing effects. In this case, synergism plays a key role in the effectiveness or toxicity of the herbal recipe. Considering this common practice among the people of Southwestern Nigeria, it is worth investigating the cyto-genotoxicity of herbal recipe commonly employed to treat typhoid fever.

Thus far, this investigation sought to evaluate antioxidant, proximate and cyto-genotoxicity of anti-typhoid fever herbal recipe containing 10 different plants using the *Allium cepa* assay.

MATERIALS AND METHODS

Preparation of Herbal recipe

Anti-typhoid fever herbal recipe (ATFHR) containing ten plants for the treatment of typhoid fever was purchased at Jagun's market in Ogbomoso, Oyo State, Nigeria. The plants in the ATFHR were identified in the department of Pure and Applied Biology, Ladoke Akintola University of Technology (LAUTECH), Ogbomoso to be *Momordica charantia* (bark), *Ocimum gratissimum* (leaves), *Morinda lucida* (bark), *Cymbopogon citratus* (leaves), *Zingiber officinale* (bulb), *Allium sativum* (bulb), *Alstonia scholaris* (leaves), *Nuclea latifolia* (leaves), *Mangifera indica* (bark) and *Cochlospermum planchonii* (leaves). All the plant materials in ATFHR weighed 500 g, and thereafter, it was added with 2.5L distilled water before boiling for 20 minutes in order to prepare decoction of ATFHR. It was allowed to cool down, filtered through a muslin cloth and then stored in a refrigerator at 4°C for further evaluations.

DPPH Antioxidant test

Solution of DPPH at 0.33mM and stock solutions of ascorbic acid (positive control) and ATFHR were prepared at 1 mg/ml. The stock solutions were diluted serially to obtain 7 concentrations; 1, 0.5, 0.25, 0.125, 0.063, 0.031, 0.016 mg/ml for the experiment. To 1 ml each of ATFHR and ascorbic acid, 1 ml of DPPH solution was added and the reaction was incubated in the dark for 30 minutes (Akinboro *et al*., 2014). The blanks were prepared with the samples without DPPH solution. The absorbance of the colour change reaction was measured after 30 minutes using a Microfield SM 23A Spectrophotometer set at 517 nm wavelength. The free radical scavenging power of ATFHR was calculated in percentage using the equation below:

$$\text{Scanvenging power (\%)} = \frac{\text{Abs Control} - \text{Abs Sample}}{\text{Abs Control}} \times 100 \quad \text{.....1}$$

Abs = absorbance

Quantitative phytochemical screening

For this analysis, 1-2g of ATFHR was weighed for determining the presence and concentration of crude fibre, proteins, ash, fat, moisture contents, phenols, alkaloids, terpenoids, flavonoids, tannins, saponins and carotenoids following the methods of Marcano *et al*. (1991), Offor *et al.* (2014) and Ekwueme et al. (2015), but with some modifications.

Test for Carbohydrate

To test for carbohydrate in ATFHR, 1g of the recipe was dissolved in 50ml of distilled water and filtered. To 1ml of the filtrate, saturated aqueous solution of picric acid was added and the absorbance of reaction was read at 580 nm.

Ash content determination

The ash content in ATFHR was determined by weighing 2g of the sample into a crucible in a muffle furnace and heated at 550°C for six hours until it became gray ash. The burnt recipe was removed from the muffle furnace and placed in a desiccator to cool. It was re-weighed after cooling and the weight of ash was obtained by the difference.

Moisture content determination

2g of ATFHR was put in a preweighed Petri dish, and then placed in an oven to dry at 105°C for two hours. The dry sample of ATFHR was transferred to a desiccator to cool at room temperature before it was weighed again. The experiments were repeated until constant weight was obtained.

Fat content determination

This was determined by weighing and wrapped 2g of ATFHR in a filter paper. This was exhaustively extracted in 200ml of petroleum ether boiled at 60°C for six hours. The extract was concentrated in rotary evaporator. It was tranferred into a desiccator to cool before weighing.

Fibre content determination Crude Fibre

2g of ATFHR was weighed and dissolved in 200 ml of 1.25% H_2S0_4, the mixture was boiled under reflux for 30 minutes. The solution was filtered and rinsed thoroughly with hot water in order to remove the acid content. The residue was added with 200ml of 1.25% NaOH and boiled for 30minutes in a digestion apparatus. The mixture was filtered and rinsed with distilled water until the filtrate is of neutral P^H. The residue was transferred into a crucible and placed in an electric oven at 100°C for eight hours to dry. It was cooled inside a desiccator and weighed. Thereafter, this was incinerated, cooled in a desiccator and reweighed.

Protein determination

1 g of ATFHR was weighed into a Kjeldahl digestion flask in the presence of selenium. ATFHR was digested by heating in 20 ml of H_2SO_4, 10 g of $NaSO_4$, 1g of $CuSO_4$ inside a fume cupboard. Thereafter, the solution was dissolved in 100 ml distilled water. This was added with 60ml of 40% of NaOH and two pieces of zinc metal in the Kjeldahl distillation apparatus. To the mixture, boric acid was added into 50 ml distilate and titrated with 0.1N H_2SO_4 using methyl red indicator. The titration continued until the endpoint value was obtained, and this value was multiplied by 6.25 factor for the calculation total nitrogen atoms in the ATFHR.

Test for saponins

2 g of ATFHR was weighed and added with 100 ml Isobutyl alcohol in a beaker. The mixture was shaken for 5hours afterwhich the mixture was filtered and 20ml of 40% saturated solution of Magnesium carbonate was added. 1ml of the solution was pipetted into 50ml volumetric flask and 2 ml of 5% $FeCl_3$ solution was added. The reaction mixture was allowed to stand for 30 minutes. The absorbance of the reaction was measured at 380nm.

Test for Alkaloids

1g of ATFHR was mixed with 25 ml of distilled water in a beaker, after which 20 ml each of 10% acetic acid and ethanol was added, the mixture was covered and allowed to stand for 4 hours at 28^0C. The mixtrue was filtered and concentrated on a water-bath to one quarter of the original volume. Concentrated ammonium hydroxide (NH_4OH) was added drop wise to the mixture until there was a complete formation of precipitate. The precipitate was collected and washed with dilute ammonium hydroxide and then filtered. The residual alkaloid was dried, weighed and expressed as a percentage of sample dry weight.

Test for Tannin

1g of ATFHR was weighed and mixed with 25ml of solvent mixture of 80% Acetone, 10% glacial acetic acid for 5 hours. The reaction mixture was filtered and its absorbance was measured at 500nm.

Test for Flavonoids

To 1g of ATFHR, 4ml of distilled water was added in a test tube. 0.3ml of 5% sodium nitrite was added and after 5 minutes 0.3ml of 10% aluminium chloride was also added followed by 2ml of 1M sodium hydroxide. The reaction mixture was diluted with 2.4 ml of distilled water and mixed well after which the absorbance was read at 510nm.

Test for Terpenoids

1g ATFHR was dissolved in distilled water and filtered to 2.5 ml filtrate, 5% aqueous phosphomolybdic acid solution was added and 2.5 ml of concentrated H2SO4 was gradually added and mixed. The mixture was left to stand for 30 min and then made up to 12.5 ml with methanol. The absorbance was taken at 700 nm.

Test for Carotenoids

1g of ATFHR was added with 20ml Acetone and left for 1 hour. The mixture was added with 10ml of distilled water before filtration. In a separating funnel, 5ml of petroleum ether was added and allowed to react with the ATFHR sloution for some 10 minutes. The lower layer was discarded while the layer on top was collected and its absorbance was measured at 440nm.

Test for Phenols

2g of ATFHR was mixed with 0.5ml of Folin-Ciocalteau reagent and 1.5 ml of 20% Sodium Carbonate, the mixture was incubated at 40°C for 30 minutes to develop colour. The absorbance of chemical reaction was measured at wavelength of 765nm.

Cytotoxicity and mutagenicity tests

Onion bulbs (*Allium cepa*, 2n = 16) were bought at Wazo market in Ogbomoso, and sun dried for two weeks prior to the commencement of the experiment. The refrigerated ATFHR stock was diluted with distilled water to obtain 6.25%, 12.5%, and 25% concentrations for the *in vivo Allium cepa* cytotoxicity and mutagenicity tests. Thirty six onions were divided into five groups including negative and positive controls. They were suspended (pretreatment) in distilled water for 24 hours, roots from 4 onions were harvested and fixed in ethanol acetic acid (3:1) fixative, The remaining 32 onions (8 onions per concentration) divided into 4 groups, were transferred into 0.1% vincristine sulphate (positive control) and 6.25%, 12.5% and 25% concentrations of ATFHR for another 24 hours root growth (Treatment). Roots from 4 onions in each group were harvested and fixed in ethanol acetic acid fixative. The remaining four onions per concentration were transferred into distilled water for another 24 hours root growth (Post treatment) after which their roots were harvested and kept in the fixative for cytological studies. The harvested roots at the pretreatment, treatment and post-treatment levels were hydrolyzed in 1N HCL and treated for slide preparation as previously described (Akinboro *et al*., 2012). The hydrolyzed roots were homogenized and stained in aceto-orcein for 10 minutes (Akinboro and Bakare, 2007, Akinboro *et al*., 2011 a & b, Akinboro *et al*., 2014). A total of 6,000 cells per concentration in each of the treatment levels were scored under the immersion oil objective lens of a binocular compound microscope to check for the mitotic dividing stages and aberrant cells. Percentage mitotic index (MI) and chromosomal aberration (CA) were calculated using the formulae:

$$\% \text{ MI} = \frac{\text{Number of dividing cells}}{\text{Total number of counted cells}} \times 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 2$$

$$\% \text{ CA} = \frac{\text{Number of aberrant cells}}{\text{Total number of counted cells}} \times 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 3$$

The percentage mitotic-inhibition recovery (MIR) of *A. cepa* cells was calculated as follows:

$$\% \text{ MIR} = \frac{\text{MI(PT)} - \text{MI(T)}}{\text{MI(T)}} \times 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 4$$

Where: MI (PT) = mitotic index at post-treatment level.
MI (T) = mitotic index at treatment level.

RESULTS

The mitotic index (MI) caused by distilled water (negative control) after 24 hours pre-treatment in *A. cepa* was 4.6%. The treatment of *A. cepa* with ATFHR for 24 hours produced 3.6%, 5.3%, and 4.1%, MI values, while for the post-treatment exposure; 5.2%, 8.7% and 5.8% MI values were recorded at 6.25%, 12.5% and 25% concentrations, respectively. These translated to 44.4%, 64.2% and 41.5% recovery from mitotic inhibitory effects of ATFHR on *A. cepa* cells. Vincristine sulphate at 0.1% induced 3.6% MI value after 24 hours treatment, and this was reduced to 3.3% after another 24 hours post-treatment in distilled water, respectively. Cell division in *A. cepa* grown in vincristine (positive control) was further inhibited resulting to −8% mitotic inhibition (Table 1).

The percentage chromosomal aberration (CA) caused by distilled water was 0.19%. The treatment of *A. cepa* in ATFRH at 6.25% and 12.5% concentrations produced

0.43% and 0.18% CA, respectively after 24 hours. However, further 24 hours post-treatment in distilled water increased the percentage CA to 0.68% and 0.20%, respectively. The percentage CA induced at 25% concentration was 0.05% each after 24 hours treatment in ATFHR and post-treatment in distilled water. Vincristine sulphate (positive control) caused 0.30% and 0.25% CAs for treatment and post-treatment exposures, respectively (Table 2).

Phytochemical analysis of ATFHR revealed the presence of carbohydrate, protein, fat, fiber and ash contents at 58. 63%, 10.67%, 1.50%, 14.13% and 7.23%, respectively. Alkaloids, saponins, tannins, phenolics, trepenoids, flavonoids and carotenoids were also detected in varying amounts (Table 3).

Inhibitory concentration of ATFHR capable of scavenging 50% free radicals (IC_{50}) from DPPH salt was recorded at 531.1 µg/ml, and it was 147.7 µg/ml for ascorbic acid (positive control) (Figure 1).

TABLE 1: Effects of ATFHR on mitosis in *Allium cepa* cells

Treatment group	24-h. Pre-treatment in distilled water		24-h. Treatment in herbal recipe or positive control		24 h. Post-treatment in distilled water		
	No of dividing cells	Mitotic index (%)	No of dividing cells	Mitotic index (%)	No of dividing cells	Mitotic index (%)	% recovery from inhibition of mitosis
Distilled water	276	4.6	276	4.6	276	4.6	0.0
0.1% Vincristine	276	4.6	216	3.6	198	3.3	-8.3
Herbal recipe							
6.25%	276	4.6	216	3.6	312	5.2	44.4
12.5%	276	4.6	318	5.3	522	8.7	64.2
25%	276	4.6	246	4.1	348	5.8	41.5

Total counted cells = 6000

TABLE 2: Effects of ATFHR on the structure of chromosomes in *Allium cepa* cells

Treatment group	24-h. Pre-treatment in distilled water		24-h. Treatment in typhoid fever herbal recipe or positive control		24 h. Post-treatment in distilled water	
	Number of aberrant cells	Percentage of chromosome aberration (%)	Number of aberrant cells	Percentage of chromosome aberration (%)	Number of aberrant cells	Percentage of chromosome aberration (%)
Distilled water	-	-	-	-	-	-
0.1% Vincristine	7	0.12	12	0.20	10	0.17
Herbal recipe						
6.25%	4	0.07	17	0.28	27	0.45
12.5%	9	0.15	7	0.12	8	0.13
25%	9	0.15	2	0.03	2	0.03

TABLE 3: Amount of phytochemicals detected in ATFHR

Phytochemicals	Cocentration (X ± SD)
Moisture content (%)	7.83 ± 0.11
Protein (%)	10.67 ± 0.25
Fat (%)	1.50 ± 0.11
Ash (%)	7.23 ± 0.15
Crude fibre (%)	14.13 ± 0.15
Carbohydrate (%)	58.63 ± 0.06
Alkaloids (mg/100g)	2325 ± 15
Saponins (mg/100g)	478.33 ± 7.64
Tannins (mg/1000g)	1646.67 ± 15.28
Phenolics (GAE (mg/100g)	24.67 ± 0.58
Triterpenoids (mg/100g)	2171.67 ± 10.41
Flavonoids (mg/100g)	926.67 ± 5.78
Carotenoids (µg/100g)	1958.33 ± 2.89

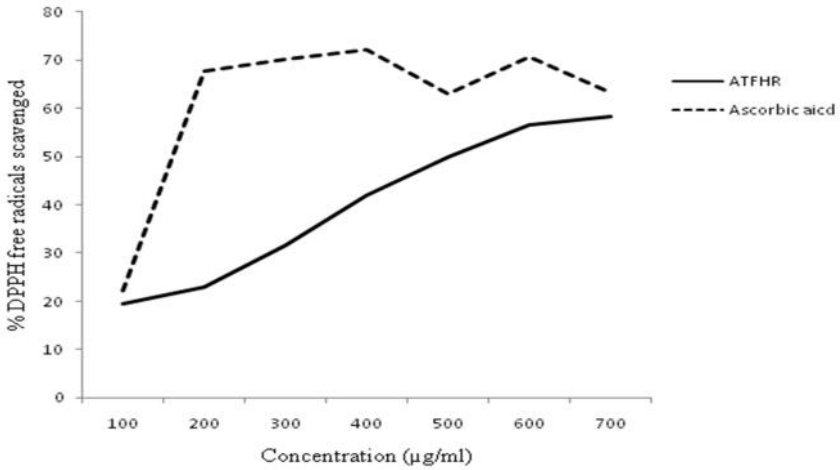

FIGURE 1: Percentage free radicals scavenged by ATFHR and ascorbic acid

DISCUSSION

The chemotherapeutic relief from medicinal plants is mainly due to a large number of active phytochemicals in them. Herbal recipes containing plants of different kinds or unpurified plant extracts containing several phytochemicals often work synergistically to either alleviate or aggravate the disease condition. Thus, toxicity evaluation of such herbal recipes is essential for safety of consumers. The non-concentration dependent reduction in mitotic index (MI) values obtained at the tested concentrations in *A. cepa* treated with ATFHR for 24 hours, compared to the mitotic index values obtained for the pretreatment group suggests mitostatic effect of ATFHR on *A. cepa* cells. This means that the mitotic inhibitory effect of ATFHR has been shown in this study to be reversible. This is evident in the increase in MI values obtained after post treatment in distilled water. The mitostatic effect of ATFHR in this study is contrary to the mitodepressive effect of aqueous extracts of herbal recipes containing *Luffa cylindrica* (L), *Nymphaea lotus* (L) and *Spondias mombin* (Atoyebi *et al*., 2015). The mitotic inhibition of ATFHR confirms its potency against *Salmonella typhi* which is the pathogenic organism that causes typhoid fever. Although the causative organism of typhoid fever is a prokaryote, while *A. cepa* used as the test organism in this study is a eukaryote. Nevertheless, the two types of cell have a number of similarities in their structural organization and compositional organelles; hence their response to toxic agent is logically comparable. The cytotoxic effect of ATFHR on *A. cepa* cells may be due to high amounts of alkaloids and triterpenoids in the recipe (Table 3). This is in accordance with the results of cytotoxic activity of five medicinal plants reported for *O. gratissium*, *Azadirachta indica*, *Mangifera indica*, *Morinda lucida* and *Cymbopogon citratus* (Akinboro and Bakare, 2007).

Decrease in MI observed herein was probably due to either chromatin dysfunction or disturbance in the cell cycle induced by the interaction of the phytochemicals with the DNA. Furthermore, most of the plants used to prepare the investigated ATFHR contain phenolic compounds (Polyphenols and flavonoids) which are one of the largest and most ubiquitous groups of plant metabolites possessing biological properties such as antiapoptosis, anticarcinogenesis, inhibition of angiogenesis and cell proliferation activities (Han *et al*., 2007; Singh *et al*., 2007). In some cases stress factors such as oxidative stress which could have been caused by a number of antioxidants which are able to behave as oxidants thereby provoking cell cycle retardation at G1/S and/or G2/M phases and contribute to reduction in mitotic index (Den Boer and Murray, 2000). In this study, it also possible there was an alteration in cell division causing the blockage of transition at the G2/M checkpoint since such arrest of cell division during mitosis is possibly caused by the complex phytochemicals mixture in the ATFHR. This is in line with the observation of Hussain *et al*. (2007) demonstrating that there was increase in root length of sorghum seedlings cultured in water extract of *Cassia angustifolia* Vahl (senna) suggesting that phytochemicals could act as plant growth stimulators (Dorota *et al*., 2011). This is suggesting that the consumption of this complex mixture of pytochemicals in the ATFHR prepared with 10 different plants parts should be at intervals when suffering from typhoid fever. The positive response of *A. cepa* cells in the mitotic recovery test may be expected in human cells exposed to this herbal recipe because of the eukaryotic nature of *A. cepa* and human cells. However, this has to be substantiated with animal model investigation on this herbal recipe.

The induction of chromosomal aberrations by ATFHR was not concentration dependent (Table 2). The percentage chromosomal aberrations induced at 25% concentration of ATFHR were the least (0.03%) and were not different after post-treatment in distilled water. This suggests mild mutagenic activity of ATFHR in the *A. cepa* cells. This is in contrary to the report of genotoxic effects of Zidovudine or Nevirapine at higher concentrations (Onwuamah *et al*., 2014). Carotenoids and phenolic compounds in ATFHR acting as antioxidants may be responsible for the observed low chromosomal damaging effect of the ATFHR at 12.5% and 25% concentrations. However, our observation on the mutagenic behaviour ATFHR in the *A. cepa* does not guarante its consumption without any cautions as no mutagenic effect of a chemical substance should be considered insignificant.

CONCLUSION

This study has demonstrated that complex mixture of pytochemicals in ATFHR possessed mitostatic and mild mutagenic effects on *A. cepa* cells. It may be surprising that *A. cepa* cells exposed to ATFHR containing combination of 10 different plant parts could still resume mitosis after their transfer into distilled water. However, these results suggest intermittent consumption of this herbal recipe only if it becomes necessary to use for typhoid fever treatment as an alternative therapy.

Conflict of interest

Authors declared no conflict of interest in connection with the finance of this investigation, preparation and submission of the manuscript to this journal.

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