

Genotoxicity and Antigenotoxicity of selected South African indigenous plants

Rhulani Makhuvele^{a, b}, Ramadumetja G. Matshoga^a, Roel Antonissen^d, Luc Pieters^c, Luc Verschaeve^{d, e},
Esameldin E. Elgorashi^{a, b*}

^aToxicology and Ethnoveterinary Medicine, Public Health and Zoonosis Programme, Agricultural Research Council-Onderstepoort Veterinary Research, Private Bag X05, Onderstepoort, 0110, South Africa;

^bDepartment of Paraclinical Sciences, University of Pretoria, Private Bag X04, Onderstepoort, 0110, South Africa;

^cDepartment of Pharmaceutical Sciences, University of Antwerp, B-2610 Antwerp, Belgium;

^dLaboratory of Toxicology, O.D. Public Health and Surveillance, Scientific Institute of Public Health, Juliette Wytmanstreet 14, B-1050 Brussels, Belgium;

^eDepartment of Biomedical Sciences, University of Antwerp, B-2610 Antwerp, Belgium

Contact information for Corresponding Author

Dr Esameldin Elgorashi, Toxicology and Ethnoveterinary Medicine, Public Health and Zoonoses,
Agricultural Research Council-Onderstepoort Veterinary Research, Private Bag X05, Onderstepoort, 0110,
South Africa

Tel: 0027 12 5299260, Fax: 0027 12 5299258

e-mail: elgorashie@arc.agric.za

e-mail addresses:

R Makhuvele: makhuveler@arc.agric.za

RG Matshoga: MatshogaG@arc.agric.za

R Antonissen: Roel.Anthonissen@wiv-isp.be

L Pieters: luc.pieters@uantwerpen.be

L Verschaeve: Luc.Verschaeve@wiv-isp.be

ABSTRACT:

Twenty-two plant species extracted with dichloromethane and 90% methanol were investigated for their genotoxicity as well as antigenotoxicity against aflatoxin B₁ induced-mutagenicity using the Ames (*Salmonella typhimurium* strains TA98 and TA100) and Vitotox assays in the presence of S9 rat liver fraction. The results obtained from Ames assay for some plant extracts correlated well with the results obtained from the Vitotox assay. Dichloromethane and methanolic extracts of *Helichrysum petiolare*, *Protea hybrid*, *Protea roupelliae*, *Artabotrys brachypetalus (leaves)*, *Friesodielsia obovata*, *Hexalobus monopetalus*, *Monanthonotaxis caffra*, *Monodora junodis*, *Uvaria caffra*, *Xylophia parviflora*, *Podocarpus henkellii*, *Rhoicissus sekhukhuniensis*, *Podocarpus elongatus* and *Agapanthus praecox* had moderate to strong antimutagenic activities in both Ames and Vitotox assays. The methanolic extract of *Annona senegalensis* and dichloromethane extract of *Podocarpus falcatus* also showed antigenotoxic potentials against aflatoxin B₁ induced mutagenicity. Methanolic extracts of *Xylophia* sp., showed a co-mutagenic effect with aflatoxin B₁ in the Ames assay (strain TA 100). All extracts were not genotoxic in the Vitotox assay in the absence of S9. Plant extracts with promising antimutagenic effects could be used in the form of feed and food supplements as a preventative strategy against aflatoxin B₁ induced mutagenicity and carcinogenicity.

Keywords: Genotoxicity, Antigenotoxicity, Aflatoxin B₁, Vitotox assay, Ames assay, Plant extracts

1. Introduction

Deoxyribonucleic acid (DNA) damage in living organisms occurs spontaneously or could be induced by genotoxins and can lead to gene mutations, chromosomal aberrations and rearrangement of the chromosomes through translocation, deletion and inversion (Wang et al. 2003; Sloczynska et al. 2014). Mutagenicity plays a crucial role in carcinogenesis and it may lead to different types of cancers and genetic diseases, which are increasing at an alarming rate in human beings and animals (Nagarathna et al. 2013). Globally, cancer is one of the leading diseases and is expected to become the leading cause of morbidity and mortality in the next decades (Canceratlas.cancer.org 2014).

Aflatoxins, a class of mycotoxins, contaminate various foodstuffs including animal feeds and foods such as nuts, corn, cereals, oilseeds, and dehydrated foods during production, harvest, storage and food processing (Bennett and Klich 2003; Madrigal-Santillan et al. 2010). They are the most common known mutagens and linked with the incidences of genetic diseases, especially hepatocellular cancer and other liver diseases such as aflatoxicosis. Aflatoxins consist of four major groups namely, B₁, B₂, G₁ and G₂ (Zain 2011). However, aflatoxin B₁ is the most potent genotoxin, highly mutagenic and carcinogenic metabolite known so far. They are recognized as human carcinogens (class 1) by the international agency for research on cancer (IARC). Aflatoxin B₁ is metabolized in the liver cells by cytochrome P450 enzyme into a highly reactive aflatoxin B₁-8, 9-epoxide, which binds to the guanine residues forming G to T transversion mutation. This biotransformation of aflatoxin B₁ induces DNA adducts which leads to mutation, genetic and oxidative damage, thus resulting in cancer (Bhat et al. 2010; Ferrante et al. 2012; Tiemersma et al. 2001). Various strategies have been employed in the control and prevention of contamination with aflatoxins, but most of them have major drawbacks that limit their use, starting from limited efficacy due to limitless reservoir to loss of essential nutrients and high costs. Therefore, potential strategies that will detoxify aflatoxins without altering the nutritional value of food and feed are needed. Scientists today are exploring

the plant kingdom to search for antimutagens or anticarcinogens that are capable of decreasing or inhibiting the mutagenic effects of aflatoxins (Alabi et al. 2011; Sloczynska et al. 2014). Plants contain many bioactive compounds with promising activity against many diseases including genetic diseases such as cancer that could be explored for drug discovery and development (Palombo 2011; Street and Prinsloo 2013).

This study focused on the screening of South African indigenous plants for their antimutagenic or antigenotoxic potentials against aflatoxin B₁ induced mutagenicity. These plant extracts were also evaluated for their mutagenicity to confirm that they were not mutagenic. The plants were selected based on results from preliminary screening in our laboratory (unpublished results). The antigenotoxicity of the plant extracts was tested using the *Salmonella* microsome and Vitotox assays. These two assays are genotoxicity bioassays commonly used in the screening of genotoxic substances (Sloczynska et al. 2014; Verschaeve et al. 1999).

2. Materials and Methods

2.1. Sample collection and processing

Twenty-two plant species collected from South African National botanical gardens (Lowveld, Walter Sisulu and Pretoria) and in the university of Pretoria botanical garden (Manie van der Schijff Botanical Garden) are listed in Table 1. The table also shows the common names, plant part used as well as the accession number for the plants. The plant material (leaves, seeds or fruits) was dried in an oven set at 45°C. Thereafter, the plant material was ground to a fine powder and stored in airtight containers in the dark at room temperature until use. Voucher specimens for the collected plant species were deposited in the H.G.W.J. Schweickerdt herbarium of the University of Pretoria.

2.2. Sample extraction and preparation

Ten grams of ground powder of each plant material was sequentially extracted with 100 mL of dichloromethane (Merck) followed by 90% methanol (Merck) by vigorous shaking for 2 h in a rotary shaker. Thereafter, the crude extracts were filtered under vacuum using Whatman No.1 filter paper (Merck). Organic solvents were concentrated using a rotary evaporator (Buchi) and then dried under a stream of cold air. Stock solutions of 100 mg/mL extracts were prepared and dissolved in dimethyl sulfoxide (DMSO; Merck) or methanol.

2.3. Genotoxicity and antigenotoxicity assay

2.3.1. Ames assay

The Ames assay was performed using the pre-incubation test. Two *S. typhimurium* tester strains were used in the Ames test, including the frame shift mutation detecting strain TA98 and the base-pair substitution detecting strain TA100 (Moltox) as described by Maron and Ames (1983). Hundred microliters of stock bacterium (kept at -80 °C) were added to 20 mL of Oxoid nutrient broth No.2 and incubated on a rotary shaker at 37 °C for 16 h. An aliquot of 0.1 mL was added to 0.1 mL test solution or the solvent (negative control), 0.5 mL of 4% (v/v) S9 mixture from Sprague Dawley rat liver (Moltox) and 2 mL of top agar containing biotin (Sigma Aldrich) and histidine (Sigma Aldrich). For mutagenicity screening, the test solution contained 50 µL test sample and 50 µL solvent control. For antimutagenicity screening, the test solution contained 50 µL test sample and 50 µL Aflatoxin B₁ (2 µg/mL, Sigma Aldrich). The top agar mixture was poured over the surface of the minimal glucose agar plates and incubated at 37 °C for 48 h. The number of revertant colonies (mutants) in each plate were counted following incubation. All cultures were done in triplicate for all concentrations of plant extract (5, 0.5 and 0.05 mg/mL) with the exception of controls where five replicates were used. The positive control was 1 µg/mL aflatoxin B₁ and 10% (v/v) DMSO/methanol (Merck) was used as negative control. Antimutagenicity of the test sample was expressed as percentage inhibition of mutagenicity and calculated as follows:

$$\% \text{ inhibition} = [(1-T/M) \times 100]$$

where T is the number of revertants per plate in the presence of mutagen and the test solution and M is the number of revertants per plate in the positive control (Ong et al.1986). Absence of toxicity was confirmed by the presence of a background layer of bacterial growth in the plate.

2.3.2. Vitotox test

The Vitotox test was performed as described by Verschaeve et al. (1999) using the Genox (TA 104 *rec* N2-4) and Cytox (TA 104 *pr* 1) tester strains of *S. typhimurium* TA 104. Hundred microliters of each of the two bacterial strains were seeded into rich growth medium supplemented with tetracycline (Sigma Aldrich) and ampicillin (Sigma Aldrich) and incubated for 16 h on a rotary shaker at 300 rpm and 36 ± 1 °C. Various concentrations (0.02, 0.1 and 0.5 mg/mL) of the 22 plant species methanolic and dichloromethane extracts were added to 10-fold dilutions of 16 h cultures of the genox and cytox strains in the presence and absence of rat liver S9. Benzo[a]pyrene (B[a]P) and 4-nitroquinoline 1-oxide (4NQO) were used as controls in the presence and absence of rat liver S9, respectively. DMSO (Sigma Aldrich) was used as a vehicle control. Light production was measured every 5 min in each well for 4 h at 30 °C using a luminometer (Modulus Microplate Multimode Reader, Turner Biosystems). Antimutagenicity of the plant extracts against aflatoxin B₁ was measured by adding 1 µg/mL of the aflatoxin B₁ to each well. The signal to noise ratio (S/N) which is the light production of exposed cells divided by the light production of non-exposed (control) cells, was automatically calculated for each measurement. Genotoxicity of each sample was evaluated with the Genox/Cytox ratio. A ratio exceeding 1.5 shows genotoxicity in non-cytotoxic extracts provided that the signal is not generated in the first 20 min of measurement. However, the extract is considered toxic if S/N (for *rec* N2-4 and/or *pr* 1) rapidly decreases below 0.8. Antimutagenicity of the test sample expressed as percentage inhibition of mutagenicity was calculated as in Ames assay.

2.4. Statistical methods

Antigenotoxicity data obtained from the Ames assay was analysed using the Statistical Analysis System software package. Analyses of variance were performed using one-way ANOVA procedures and Dunnet's

test to determine the significant differences between the mean ($P < 0.05$). No statistical analysis was necessary for the Vitotox assay.

3. Results and Discussion

Dichloromethane and 90% methanolic extracts of the selected 22 plant species were investigated first for their potential mutagenic effects in the bacterial based Ames and Vitotox assays. This was done to rule out extracts that exhibited both genotoxic and antigenotoxic effects as they would not be good candidates in further studies. The number of revertant colonies obtained from TA98 and TA100 are in agreement with results generated in our laboratory and in accordance with those reported in literature (Maron and Ames 1983). The two strains are widely used in mutagenicity testing because they are sensitive in detecting most mutagens and carcinogens (Dhawan and Bajpayee 2013; Makhafola et al. 2016; Verschaeve and van Staden 2008). The assays were performed in the presence of S9 since aflatoxin B₁ is an indirect mutagen and need to be converted metabolically to its 8,9-epoxide active derivative (Hamid et al. 2013). The enzyme contains a mixture of xenobiotic enzymes such as cytochrome P450s and sulfotransferase which mimic mammalian metabolism in bacteria (Ndhlala et al. 2010; Verschaeve and Van Staden 2008).

Results on the mutagenic effects of methanolic and dichloromethane plant extracts tested in the Ames assay using *S. typhimurium* strain TA 100 and TA 98 are presented in Tables 2 and 3. In the Ames test for the used TA98 and TA100, an extract is considered mutagenic when the mean number of revertant colonies produced in each plate was double or greater than two times that of the negative control (Bierkens et al. 2004; Ndhlala et al. 2010). Accordingly, most of the plant extracts tested did not have any mutagenic properties. Only methanolic extracts of *M. junodis* were mutagenic on TA 98 strain in a dose dependent manner, while *P. hybrid* produced double the number of revertant colonies as the negative control at the highest concentration tested. Few more plant extracts produced double or more than double the number of revertant colonies as the negative control on strain TA 100 without showing a dose response. These

include the methanolic extracts of *X. parviflora*, *Xylopi*a sp. and *R. laetans*. While the dichloromethane extracts of *M. junodis* produced more than double the colonies compared to the negative control at the lowest concentration used when tested against TA 100 tester strain. The same was observed in the Vitotox test for dichloromethane extract of *U. caffra* (Figure 1B). In this instance, dichloromethane extracts of *U. caffra* induced signal to noise ratio of strain rec *N2-4* over the maximum signal to noise ratio of *pr1* signal to above 1.5, it was also not cytotoxic as the signal to noise ratio in *pr1* was not below 0.8 in a dose dependent manner. Moreover, all 44 plant extracts (methanolic and dichloromethane extracts) tested on Vitotox assay showed no evidence of genotoxicity at all tested concentrations in the absence of S9 metabolizing enzyme as none of the extracts had signal to noise ratio of more than 1.5 (Figure 1A, 2A). Methanolic plant extracts of *H. monopetalus*, *Xylopi*a sp., *L. rofulata* and *P. henkellii*, were genotoxic in the presence of S9 in a dose dependent manner (Figure 2B) while dichloromethane extract, *P. roupelliae* was genotoxic in the presence of S9 metabolizing enzymes (Figure 1B). However, there was an increase in light production in the cytox strain, therefore these plants extracts, which showed genotoxicity are considered not genotoxic because there was an interaction between the lux gene and plants extracts. There is usually a very good correlation, about 95%, between the Ames assay and Vitotox test (Westerinck et al. 2009). However, there may be also variations that may be observed between the two assays ascribed to the fact that different endpoints are tested (true gene mutations against SOS induction). This was also seen for a few plants investigated here. Actually, the Vitotox test was used as a first rapid screening test and Ames test was used as a confirmatory and complementary test to confirm Vitotox test results and identify mutagens that the Vitotox test could not clearly detect most likely due to high toxicity. It is indeed true that compounds, especially mixtures, can be toxic at much lower concentrations in the Vitotox test compared to the Ames assay (Schoonen et al. 2009; Westerink et al. 2009).

The Vitotox assay also allows detection of cytotoxic compounds. It uses the Cytox strain (*pr1*) which contains the plasmid with *lux* operon under transcriptional control of a constitutive promoter, thus

constitutively expresses the *lux* operon (Chichioco-Hernandez et al. 2011; Verschaeve and others 1999). In the presence of cytotoxic compounds, there is a decrease in light production. However, the Cyttox strains can also be used as the reference for non-specific enhancement of light emission (Verschaeve et al. 1999). Therefore, the lack of a dose response in the mutagenicity test using Vitotox is due to toxicity of the highest dose tested. The S/N curve for *pr1* strain, which is a useful tool in testing for toxicity alone, was below 0.8 and therefore clearly indicative of the toxicity of the highest dose used for these extracts. These plant extracts with mutagenic effects should be used with care in any form of prescription and further rigorous toxicological investigations are required before they are recommended in pharmaceuticals and drug discovery industries (Verschaeve and Van Staden 2008).

The results on cytotoxicity in the Vitotox assay showed that almost all of the methanolic and dichloromethane plant extracts were toxic at the highest concentration (0.5 mg/mL) when tested without metabolic activation. An exception was the methanolic extracts of *P. falcutus*, *A. brachypetalus* (fruit) and *R. laetans* and the dichloromethane extracts of *R. rhomboidea* and *L. roviolata*. However, in the presence of S9 metabolizing enzymes 95% of the methanolic extracts were not toxic at all tested concentrations. An exception was leaf extract of *A. brachypetalus*, which was toxic at 0.5 mg/mL. Whereas 73% of the dichloromethane extracts namely *P. hybrid*, *P. roupelliae*, *A. brachypetalus* (leaves), *A. senegalensis*, *F. obovata*, *H. monopetalus*, *M. caffra*, *M. junodis*, *X. parviflora*, *Xylopi* sp., *A. brachypetalus* (fruit), *R. sekhukhuniensis*, *P. falcutus*, *R. rhomboidea*, *L. roviolata* and *R. laetans* were not toxic at all concentrations tested (Figure 3, 4).

A test solution is considered antimutagenic when the frequency of genetic damage caused by the combined treatments (extracts and aflatoxin B₁) is substantially lower compared to the damage induced by the mycotoxin alone. Usually, an extract is considered to have no or only weak antimutagenic properties when the percentage inhibition of mutagenicity is less than 25. When the percentage inhibition is between

25 and 40%, the extract is considered to have moderate antimutagenic properties. Finally, the extract is said to possess a strong antimutagenic activity if the percentage inhibition is greater than 40% (Abdillahi et al. 2012; Ong et al. 1986; Verschaeve and Van Staden 2008). The statistical results from Dunnett's test showed that almost all the mean revertant colonies produced by all tested extracts were significantly different from the mean revertant colonies produced by aflatoxin B₁ alone, but not different to each other in most cases for strain TA 98 and TA 100. However, the mean number of revertant colonies for few extracts at 0.05mg/mL were not different from those produced by the aflatoxin B₁. For all plant extracts tested, no signs of toxicity to the bacteria were observed at all tested concentrations as evident from the background bacterial lawn observed after comparing with the negative control. The results on antimutagenicity in *S. typhimurium* TA100 (Figure 1) showed that the methanolic extracts of *H. Petiolare*, *P. hybrid*, *P. roupelliae*, *A. brachypetalus* (leaves), *F. obovata*, *H. monopetalus*, *M. caffra* and *M. junodis*, *U. caffra*, *P. henkelii*, *R. sekhukhuniensis*, *P. elongatus*, *P. falcutus* and *R. laetans* possessed strong antimutagenicity against aflatoxin B₁-induced mutagenicity in a dose dependent manner. Whereas 23% of the extracts including *P. cynaroides*, *A. senegalensis*, *X. parviflora*, *A. praecox* and *L. roviolata* showed moderate antimutagenicity in a dose response manner. *R. rhomboidea*, *A. brachypetalus* (fruit) and *Xylopi* sp., had low to co-mutagenic effect with the aflatoxin B₁ by enhancing the mutagenic effect of the mutagen.

The methanolic plant extracts tested against *S. typhimurium* strain TA 98 (Figure 5B) showed strong antimutagenic properties compared to the extracts tested with strain TA 100 (Figure 5A). The results showed that 86% of methanolic extracts namely, *H. petiolare*, *P. hybrid*, *P. roupelliae*, *A. brachypetalus* (leaves), *A. senegalensis*, *F. obovata*, *H. monopetalus*, *M. caffra*, *U. caffra*, *M. junodis*, *X. parviflora*, *A. brachypetalus* (fruit), *P. henkelii*, *R. sekhukhuniensis*, *P. elongatus*, *P. falcutus*, *R. rhomboidea*, *L. roviolata* and *R. laetans* had strong antimutagenic activities mostly at 5 mg/mL whereas extracts of *Xylopi* sp., and *A. praecox* possessed moderate antimutagenic effect in a dose dependent manner. Extracts of *P. cynaroides* had weak antimutagenicity.

The dichloromethane plant extracts tested on *S. typhimurium* strain TA 100 revealed that 45% of plant extracts (*H. petiolare*, *P. hybrid*, *P. roupelliae*, *A. brachypetalus* (leaves), *H. monopetalus*, *M. caffra* and *P. henkellii*) tested against aflatoxin B₁ induced mutagenicity had strong antimutagenicity (Figure 6A). Fifty five percent of plant extracts, namely, *H. petiolare*, *P. cynaroides*, *P. hybrid*, *P. roupelliae*, *A. brachypetalus* (leaves), *F. obovata*, *H. monopetalus*, *M. junodis*, *P. henkellii*, *R. sekhukhuniensis*, *P. elongatus*, and *P. falcutus*) demonstrated strong antimutagenic effect against aflatoxin B₁-induced mutagenicity on TA 98 (figure 6B).

In the Vitotox assay, the antigenotoxicity study of plant extracts against aflatoxin B₁ induced mutagenicity revealed that 41% of all methanolic extracts tested for antigenotoxicity, namely, *P. hybrid*, *A. brachypetalus* (leaves), *F. obovata*, *H. homopetalus*, *U. caffra*, *X. parviflora*, *R. rhomboidea*, *A. senegalensis* and *R. laetans* had moderate to strong antimutagenicity against aflatoxin B₁ mutagenicity in a dose dependent manner. About 45% of the plant extracts, namely *H. petiolare*, *P. cynaroides*, *P. roupelliae*, *M. caffra*, *M. junodis*, *Xylopi* sp., *P. henkellii*, *R. sekhukhuniensis*, *P. elongatus* and *A. praecox* had antimutagenicity of above 40% inhibition at the highest concentration tested whereas *A. brachypetalus* (fruit) had a co-mutagenic effect with aflatoxin B₁ mutagenicity though not in a dose dependent manner. About 50% of the plant extract had weak to moderate co-mutagenic effects, by enhancing the genotoxic effect of aflatoxin B₁, at the lowest concentration tested (Figure 7A). However, the antigenotoxicity of *M. junodis*, *Xylopi* sp., *H. petiolare*, *P. hybrid*, *A. brachypetalus* (leaves), *F. obovata*, *U. caffra*, *X. parviflora* and *R. rhomboidea* was due to the toxicity of the plant extracts observed at highest tested concentration in the Cyttox strain. For the dichloromethane plant extracts, 86% of the extracts had a percentage inhibition above 40% against aflatoxin B₁ mutagenicity in a dose dependent manner. However, only plant extract of *A. brachypetalus* (leaves), *A. senegalensis*, *M. junodis* and *P. falcutus* showed strong antimutagenicity without sign of toxicity whereas the antigenotoxicity of some plant extracts was influenced by the cytotoxicity of the extracts at higher concentration. Lower concentration of extract showed weak antigenotoxicity against aflatoxin B₁. Of

the 86% antigenotoxic extracts, 59% of the plant extracts had antigenotoxic activities of above 40% at 0.5mg/mL whereas *A. brachypetalus* (fruit) and *L. rofulata* showed moderate to weak antigenotoxicity and co-mutagenic effect against aflatoxin B₁ mutagenicity (Figure 7B).

Almost 73% of the dichloromethane plant extracts tested had antimutagenic effect in both *S. typhimurium* strain TA98 and TA 100 compared to 82% methanolic extracts. It is interesting to note that results obtained using TA98 correlates much better with those obtained using the Vitotox test than with those obtained with TA100 as 71% of the extracts tested had antimutagenic effects in both strain TA98 and Vitotox. There was, however, 40% concordance in the antimutagenicity results obtained using Vitotox with both Ames strains (TA98 and TA100). This concordance is more evident with the plant species of Annonaceae family. For instance, methanolic and dichloromethane extracts of *H. petiolare*, *P. hybrid*, *P. roupelliae*, *A. brachypetalus* (leaves), *F. obovata*, *H. monopetalus*, *M. caffra*, *M. junodis*, *U. caffra*, *X. parviflora*, *P. henkellii*, *R. sekhukhuniensis*, *P. elongatus* and *A. praecox* had antigenotoxic activity against AFB₁ mutagenicity in TA100, TA98 and Vitotox assays. Furthermore, methanolic extract of *A. senegalensis* and *R. laetans* as well as dichloromethane extracts of *P. falcutus* also showed interesting antigenotoxic activities in the Ames (TA100 and TA98) and Vitotox assays.

Plant extracts of *Xylopi*a sp. were not mutagenic when tested alone. However, they showed a co-mutagenic effect with aflatoxin B₁ by enhancing the mutagenic effect of the mycotoxin. Literature data on the interaction of the plant extracts investigated in this study with DNA are limited. However, the comutagenic effect of *P. henkellii* with 4-nitroquinoline-1-oxide (4NQO) mutagenicity has been recently reported (Makhafola et al., 2016). Extracts of *P. henkellii* were not comutagenic in this study which is an indication that the extracts exert their effect on direct mutagens such as 4 NQO rather than indirect mutagens. A number of previous studies suggest that other natural products including coumarins and flavonoids exerted synergistic effects on aflatoxin B₁-induced mutagenicity and other direct and indirect mutagens (Goeger et al. 1999; Snijman et al. 2007). However, the comutagenic effect with AFB₁ was attributed largely to an

increase in the bioactivation of aflatoxin B₁ to its AFB₁-8,9-epoxide (Goeger et al. 1999; Snijman et al. 2007).

This study investigated plant extracts from members of different families including Annonaceae, Asparagaceae, Asteraceae, Podocarpaceae, Proteaceae and Vitaceae. The mechanism by which some of these extracts reduced the mutagenicity of aflatoxin B₁ is so far unknown. However, members of these families have been reported to contain sterols, terpenes, alkaloids, acetogenins, glycosides, amino acids and proteins as well as phenolic compounds (Mulholland et al. 2000; Parmena et al. 2012). It is well established that AFB₁ requires activation by cytochrome B-450 microsomal mixed function oxidase system into AFB₁-8,9-epoxide. The epoxide form adducts with DNA or undergo a detoxification process through conjugation with glutathione to form AFB₁-glutathione conjugate, which are thereafter excreted. Various natural products, including those reported in species under investigation, exert their antimutagenic effect by either reducing metabolic activation of the promutagen or through interaction with its metabolic activation derivatives (Waters et al. 1990; Jeng et al. 2000). However, most compounds antimutagenic to AFB₁ are intracellular blocking agents i.e. bioantimutagens and act through prevention of AFB₁ from reacting with target sites, affecting DNA repair, scavenging of radicals or prevention of neoplastic expression of initiated cells (Water et al. 1990).

Conclusion

Most plant extracts investigated in this study had antigenotoxic activities against aflatoxin B₁ induced mutagenicity in either the Ames or Vitotox test or both. Although the mechanism of action of these extracts is unknown, however, it is well-known that AFB₁ exerts its mutagenic effect through oxidative stress. Few plant extracts such as *A. brachypetalus*, *H. petiole*, *M. caffra*, *P. hybrid* and *P. roupelliae* had strong to moderate antigenotoxic activity in both tests. The activity of the latter plant extracts is of particular interest and could be confirmed in other *in vitro* assays such as the mammalian cells-based comet and

micronucleus assays. Extracts with low toxicity could further be investigated in *in vivo* assays in rodents. The bioactive plant extracts contain a complex mixture of different classes of natural products that may act in a synergistic or antagonistic manner. Further studies to characterize the active antimutagenic compounds may therefore lead to the discovery of interesting molecules that may play an important role in liver cancer prevention.

Acknowledgments

The authors thank the National Research Foundation of South Africa (CPRR 87746 and NRF/FWO 87964), the Fund for Scientific Research (FWO – Flanders-Belgium), the Flemish Interuniversity Council (VLIR) and Agricultural Research Council (ARC-OVI) for financial support. We wish to acknowledge the University of Pretoria Botanical Garden, Lowveld National Botanical Garden, Pretoria National Botanical Garden, and Walter Sisulu National Botanical Garden for permission to collect plant material for the study. We also wish to thank Mrs Elsa Van Wyk for assisting with voucher specimen identification. The authors declare that there is no conflict of interest.

Figure Captions

Figure 1: Genotoxic effect of the dichloromethane plant extracts in Vitotox assay in the absence of S9 (A) and presence of S9 (B).

Figure 2: Genotoxic effect of the methanolic plant extracts in Vitotox assay in the absence of rat liver S9 (A) and presence of S9 (B).

Figure 3: Cytotoxic effect of methanolic plant extracts in Vitotox assay in the absence of S9 (A) and presence of S9 (B).

Figure 4: Cytotoxic effect of dichloromethane plant extracts in Vitotox assay in the absence of S9 (A) and presence of S9 (B).

Figure 5: Percentage inhibition of mutagenic effects of aflatoxin B1 by methanolic plant extracts using *S. typhimurium* strain TA 100 (A) and TA 98 (B). (*) present significant differences between the mean revertant colonies.

Figure 6: Percentage inhibition of mutagenic effects of aflatoxin B1 by dichloromethane plant extracts using *S. typhimurium* strain TA 100 (A) and TA98 (B). (*) present significant differences between the mean revertant colonies.

Figure 7: Percentage antigenotoxicity of methanolic (A) and dichloromethane (B) plant extracts against aflatoxin B1 induced mutagenicity in Vitotox assay in the presence of S9.

References

- Abdillahi, H.S., Verschaeve, L., Finnie, J.F., Van Staden, J., 2012. Mutagenicity, antimutagenicity and cytotoxicity evaluation of South African *Pordocarpus* species. *Journal of Ethnopharmacology* 139,728-738.
- Alabi, O.A., Anokwuru, C.P., Ezekiel, C.N., Ajibaye, O., 2011. Antimutagenic and Antigenotoxic effect of ethanolic extract of neem on dietary-aflatoxin induced genotoxicity in mice. *Journal of Biological Sciences* 11, 307-313.
- Bennett, J.W., Klich, M., 2003. Mycotoxins. *Clinical Microbiology Reviews* 16,497-516.
- Bhat, R., Rai, R.V., Karim, A.A., 2010. Mycotoxins in food and feed: Present status and future concerns. *Comprehensive Reviews in Food Science and Food Safety* 9, 58-81.
- Bierkens, J., Brits, E., Verschaeve, L., 2004. Environmental monitoring for genotoxic compounds. *Thompson/ Environmental Toxicology Testing*. p 229-256.
- Canceratlas.cancer.org., 2014. Taking action Research: Medicines and fundamental biology dominate cancer research in high-income countries. Fact sheet. The American Cancer society, Inc. p 32.
- Chichioco-Hernandez, C., Wudarski, J., Gevaert, L., Verschaeve, L., 2011. Evaluation of cytotoxicity and genotoxicity of some Philippine medicinal plants. *Pharmacognosy Magazine* 7,171-175.
- Dhawan, A., Bajpayee, M., 2013. Genotoxicity Assessment: methods and protocols. *Methods in Molecular Biology*. Volume 1044. Human Press, Totowa, NJ. p 3-26. DOI 10.1007/978-1-62703-529-3_1.

Ferrante, M., Sciacca, S., Conti, G.O., 2012. Carcinogen role of food by mycotoxins and knowledge gap, Carcinogen. (Pesheva, M., Ed). In Tech. Available from: <http://www.intechopen.com/books/carcinogen-role-of-food-by-mycotoxins-and-knowledge-gap>. Accessed 2014 January 15.

Goeger, D.E., Hsie, A.W., Anderson, K.E., 1999. Co-mutagenicity of coumarin (1,2-benzopyrone) with aflatoxin B1 and human liver S9 in mammalian cells. *Food and Chemical Toxicology* 37, 581-589.

Hamid, A.S., Tesfamariam, I.G., Zhang, Y., Zhang, Z.G., 2013. Aflatoxin-B1 induced hepatocellular carcinoma in developing countries: Geographical distribution, mechanism of action and prevention (Review). *Oncology Letters* 5, 1087-1092.

Jeng, S.N., Shih, M.K., Kao, C.M., Liu, T.Z., Chen, S.C., 2000. Antimutagenicity of ethanol extracts of bee glue against environmental mutagens. *Food and Chemical Toxicology* 38, 893-897.

Madrigal-Santillan, E., Morales-Gonzalez, J.A., Vargas-Mendoza, N., Reyes-Ramirez, P., Cruz-Jaime, S., Sumaya-Martinez, T., Perez-Pasten, R., Madrigal- Bujaidar, E. 2010. Antigenotoxic studies of different substances to reduce the DNA damage induced by aflatoxin B1 and Ochratoxin A. *Toxins* 2, 738-757.

Makhafola, T.J., Elgorashi, E.E., McGaw, L.J., Verschaeve, L., Eloff, J.N., 2016. The correlation between antimutagenic activity and total phenolic content of extracts of 31 plant species with high antioxidant activity. *BMC Complementary and Alternative Medicine* 16,1-13.

Maron, D.M., Ames, B.N., 1983. Revised methods for the Salmonella mutagenicity test. *Mutation Research* 113, 173–215.

Mulholland, D., Naidoo, N., Hutchings, A., Lavaud, C., Massiot, G., 2000. Crotepoxide, a cyclohexane diepoxide from *Monanthotaxis caffra*. *Biochemical Systematic and Ecology* 28, 595-597.

Ndhala, A.R., Anthonissen, R., Stafford, G.I., Finnie, J.F., Verschaeve, L., Van Staden, J., 2010. In vitro cytotoxic and mutagenic evaluation of thirteen commercial herbal mixtures sold in KwaZulu-Natal, South Africa. *South African Journal of Botany* 76,132-138.

- Nagarathna, P.K., Wesley, J.M., Reddy, P.S., Reena, K., 2013. Review on genotoxicity, its molecular mechanisms and prevention. *International Journal of Pharmaceutical Sciences Review and Research* 22, 236-243.
- Omar, H.E.M., 2013. Mycotoxins-induced oxidative stress and disease. Egypt. Available from: <http://dx.doi.org/10.5772/51806>. Accessed 2015 October 10.
- Ong, T.M., Whong, W.Z., Stewart, J., Brockman, H.E., 1986. Chlorophyllin: a potent antimutagen against environmental and dietary complex mixtures. *Mutation Research* 173, 111-115.
- Palombo, E.A., 2011. Traditional medicinal plant extracts and natural products with activity against oral bacteria: Potential application in the prevention and treatment of oral diseases. *Journal of Evidence-Based Complementary and Alternative Medicine* 1-15.
- Parmena, D.S., Mgina, C.A., Joseph, C.C., 2012. Composition of non-volatile oils and antimicrobial activities of extracts from *Mononothotaxis discolor* and an undescribed *Uvariondendron* species. *Tanzania Journal of Science* 38, 221-231.
- Schoonen, W.G., Westerink, W.M., Horbach, G.J., 2009. High-throughput screening for analysis of in vitro toxicity. *Molecular, Clinical and Environmental Toxicology* 99, 401-452.
- Snijman, P.W., Swanevelder, S., Joubert, E., Green, I.R., Gelderblom, W.C.A., 2007. The antimutagenic activity of the major flavonoids of roiboos (*Aspalathus linearis*): Some dose-response effects on mutagen activation -flavonoid interactions. *Mutation Research* 631, 111-123.
- Sloczynska, K., Powroznik, B., Pekala, E., Waszkielewicz, A.M., 2014. Antimutagenic compounds and their possible mechanisms of action. *Journal of Applied Genetics* 55, 273-285.
- Street, R.A., Prinsloo, G., 2013. Commercially important medicinal plants of South Africa. A review. *Journal of Chemistry* 2013, 1-3.
- Tiemersma, E.W., Omer, R.E., Bunschoten, A., van't Veer, P., Kok, F.J., Idris, M.O., Kadaru ,A.M.Y., Fedail, S.S., Kampman, E., 2001. Role of genetic polymorphism of glutathione-S-transferase T1 and

microsomal epoxide hydrolase in aflatoxin-associated hepatocellular carcinoma. *Cancer Epidemiology, Biomarkers & Prevention* 10, 785–791.

Van der Lelie, D., Regniers, L., Borremans, B., Provoost, A., Verschaeve, L., 1997. The Vitotox test, a SOS-bioluminescence *Salmonella typhimurium* test to measure genotoxicity kinetics. *Mutation Research* 389, 279-290.

Verschaeve, L., Van Gompel, J., Regniers, L., Van Parijs, P., Van der Lelie, D., 1999. Vitotox genotoxicity and toxicity test for the rapid screening of chemicals. *Environmental and Molecular mutagenesis* 33, 240-248.

Verschaeve, L., Van Staden, J., 2008. Mutagenic and antimutagenic properties of extracts from South African traditional medicinal plants. *Journal of Ethnopharmacology* 119, 575-587.

Waters, M.D., Brady, A.L., Stack, H.F., Brockman, H.E., 1990. Antimutagenicity profiles for some model compounds. *Mutation Research* 238, 57-85.

Westerink, W.M., Stevenson, J.C., Lauwers, A., Griffioen, G., Horbach, G.J., Schoonen, W.E., 2009. Evaluation of the Vitotox and RadarScreen assays for the rapid assessment of genotoxicity in the early research phase of drug development. *Mutation Research* 676, 113-130.

Zain, M.E., 2011. Impact of mycotoxins on humans and animals. *Journal of Saudi Chemical Society* 15, 129-144.

Table 1: Plant species investigated for antimutagenicity and mutagenicity.

Sample No.	Species name	Common name	Family name	Plant part	Accession No.
1	<i>Helichrysum petiolare</i> Hilliard & B.L. Burt	Silverbush everlasting	Asteraceae	Leaves	122773
2	<i>Protea cynaroides</i> (L.) L.	King protea	Proteaceae	Leaves	122756
3	<i>Protea hybrid</i>		Proteaceae	Leaves	122758
4	<i>Protea roupelliae</i> Meisn.subsp. hamiltonii Beard ex Rourke	Silver protea	Proteaceae	Leaves	122757
5	<i>Artabotrys brachypetalus</i> Benth.	Hook berry	Annonaceae	Leaves	122766
6	<i>Annona senegalensis</i> Pers.ssp. senegalensis	Wild custard apple	Annonaceae	Leaves	122755
7	<i>Friesodielsia obovata</i> (Benth.) Verdc	Dwaba berry	Annonaceae	Leaves	122759
8	<i>Hexalobus monopetalus</i> (A.Rich.) Engl. & Diels	Baboons breakfast	Annonaceae	Leaves	122760
9	<i>Monanthes affra</i> (Sond.) Verdc	Dwaba berry	Annonaceae	Leaves	122761
10	<i>Monodora junodii</i> Engl. & Diels		Annonaceae	Leaves	122768
11	<i>Uvaria affra</i> E. Mey. Ex Sond	Small cluster pear	Annonaceae	Leaves	122764
12	<i>Xylopiopsis parviflora</i> (A. Rich.) Benth	Bushveld bitterwood	Annonaceae	Leaves	122765
13	<i>Xylopiopsis</i> sp.		Annonaceae	Leaves	122763
14	<i>Artabotrys brachypetalus</i> Benth	Hook berry	Annonaceae	Fruits	122762
15	<i>Podocarpus henkelii</i> Stapf ex Dallim. & A.B. Jacks.	Henkel's yellow wood	Podocarpaceae	Seeds	122771
16	<i>Rhoicissus</i> <i>sekhukhuniensis</i> Retief, Siebert & A.E. van wyk	Sekhukhune grape	Vitaceae	Leaves	122774
17	<i>Podocarpus elongatus</i> (Aiton) L'Her.ex Pers	Breede river yellow wood	Podocarpaceae	Seeds	122772
18	<i>Agapanthus praecox</i> Willd.	Blue lily	Agapanthaceae	Leaves	122767
19	<i>Podocarpus falcatus</i>	Outeniqua	Podocarpaceae	Seeds	122770

	(Thunb) R.Br.ex Mirb.	yellow wood			
20	<i>Rhoicissus rhomboidea</i> (E.Mey ex Harv.) Planch	Glossy forest grape	Vitaceae	Leaves	^a NV
21	<i>Ledebouria revoluta</i> (L.f.) Jessop 1970	Bushveld grape	Asparagaceae	Leaves	^a NV
22	<i>Rhoicissus laetans</i> Retief		Vitaceae	Leaves	122769

^aNV – not voucher specimen due to lack of plant material

Table 2: number of His+ revertant colonies in *S. typhimurium* strains TA98 and TA 100 produced by 44 methanolic and dichloromethane plant extracts in the presence of S9 metabolizing enzyme.

<i>S. typhimurium</i>	TA 100						TA 98					
Plant extracts	Methanolic extracts			Dichloromethane extracts			Methanolic extracts			Dichloromethane extracts		
Concentration (mg/mL)	5	0.5	0.05	5	0.5	0.05	5	0.5	0.05	5	0.5	0.05
<i>H. petiolare limelight</i>	173±1	206±1	256±12	197±1	225±1	253±8	35±11	36±6	39±2	16±4	25±6	33±3
<i>P. cynaroides</i>	215±16	269±16	287±2	202±2	229±2	264±8	51±7	40±9	42±2	33±11	40±0	34±4
<i>P. hybrid</i>	228±3	241±3	259±9	203±6	266±6	337±6	69±9	40±6	51±4	44±11	44±4	44±2
<i>P. roupelliae</i>	231±5	270±5	280±8	194±1	220±1	232±5	65±4	43±11	36±4	40±2	36±1	40±3
<i>A. brachypetalus</i>	257±8	281±8	339±7	200±3	221±3	265±5	47±7	38±2	35±0	44±3	43±4	44±5
<i>A. senegalensis</i>	235±3	206±3	196±0	205±3	221±3	238±3	56±8	25±3	31±7	40±7	47±3	45±2
<i>F. obovata</i>	268±1	240±1	213±2	208±6	259±6	285±2	59±2	48±8	35±4	20±0.	46±5	33±1
<i>H. monopetalus</i>	275±1	231±1	198±1	181±6	243±6	313±2	28±4	38±3	34±3	43±1	55±5	49±1
<i>M. caffra</i>	208±1	201±1	191±1	192±2	216±2	251±2	36±5	33±8	23±7	23±1	28±2	24±1
<i>M. junodis</i>	259±1	224±1	187±1	206±5	362±5	516±11	162±1	101±5	22±3	27±3	35±1	43±1
<i>U. caffra</i>	334±7	263±7	229±7	203±4	214±4	237±4	45±8	38±6	31±5	23±3	27±1	26±4
<i>X. parviflora</i>	880±16	339±16	212±4	178±3	218±3	270±5	37±4	47±4	34±4	40±3	31±4	33±2
<i>Xylopi</i> sp.	831±9	261±9	208±14	220±4	240±4	277±4	66±8	30±4	31±8	35±2	65±6	34±4
<i>A. brachypetalus</i>	222±16	239±16	248±12	186±6	200±6	213±1	25±3	26±3	28±5	19±1	26±4	30±1
<i>P. henkelii</i>	213±11	221±11	246±1	201±2	211±2	215±4	29±5	35±6	29±2	16±4	29±2	21±3
<i>R. sekhukhuniensis</i>	211±0.	226±0	233±4	193±2	218±2	254±9	23±1	35±1	43±1	23±0	26±1	25±3
<i>P. elongatus</i>	213±2	234±2	241±11	201±1	218±1	259±11	30±2	44±2	33±4	33±6	32±10	28±5
<i>A. praecox</i>	212±7	235±7	248±7	200±2	216±2	275±4	43±3	28±4	37±1	23±4	34±1	33±5

<i>P. falcutus</i>	189±0	205±0	216±3	197±2	207±2	224±3	22±3	33±5	28±3	27±4	33±6	30±12
<i>R. rhomboidea</i>	203±17	244±17	232±14	196±3	213±3	275±3	33±2	29±1	32±2	^b ND	^b ND	^b ND
<i>L. rovilata</i>	344±10	377±10	424±6	191±2	205±2	228±3	34±4	29±1	37±4	^b ND	^b ND	^b ND
<i>R. laetans</i>	332±5	437±5	499±7	210±1	254±1	289±3	22±1	25±5	26±5	45±9.87	37±1	31±2
Solvent control	206±6			191±6			34±5			30±5.09		

^bND- the antimutagenicity of the plant extracts was not determined due to lack of plant material

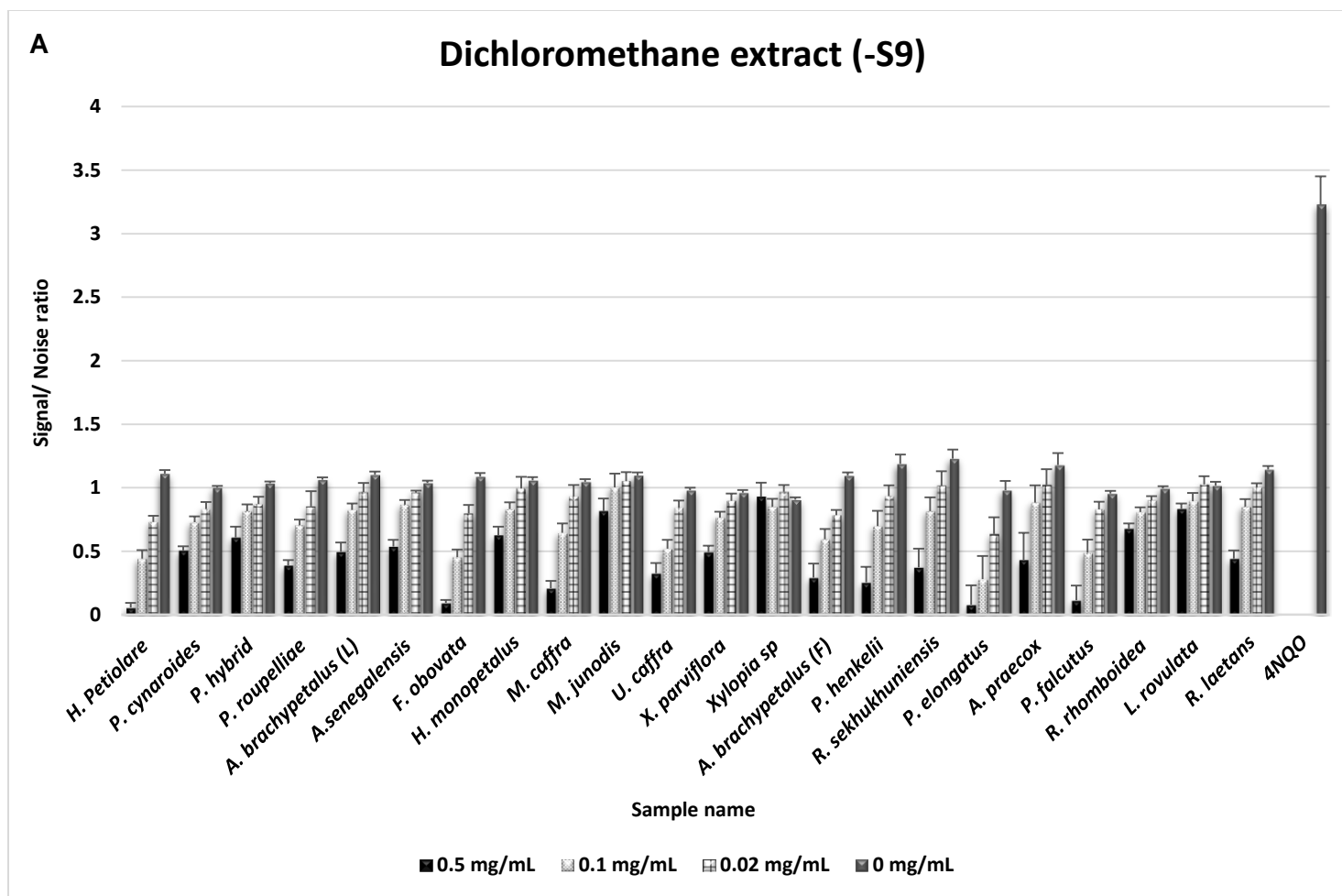


Figure 1(A)

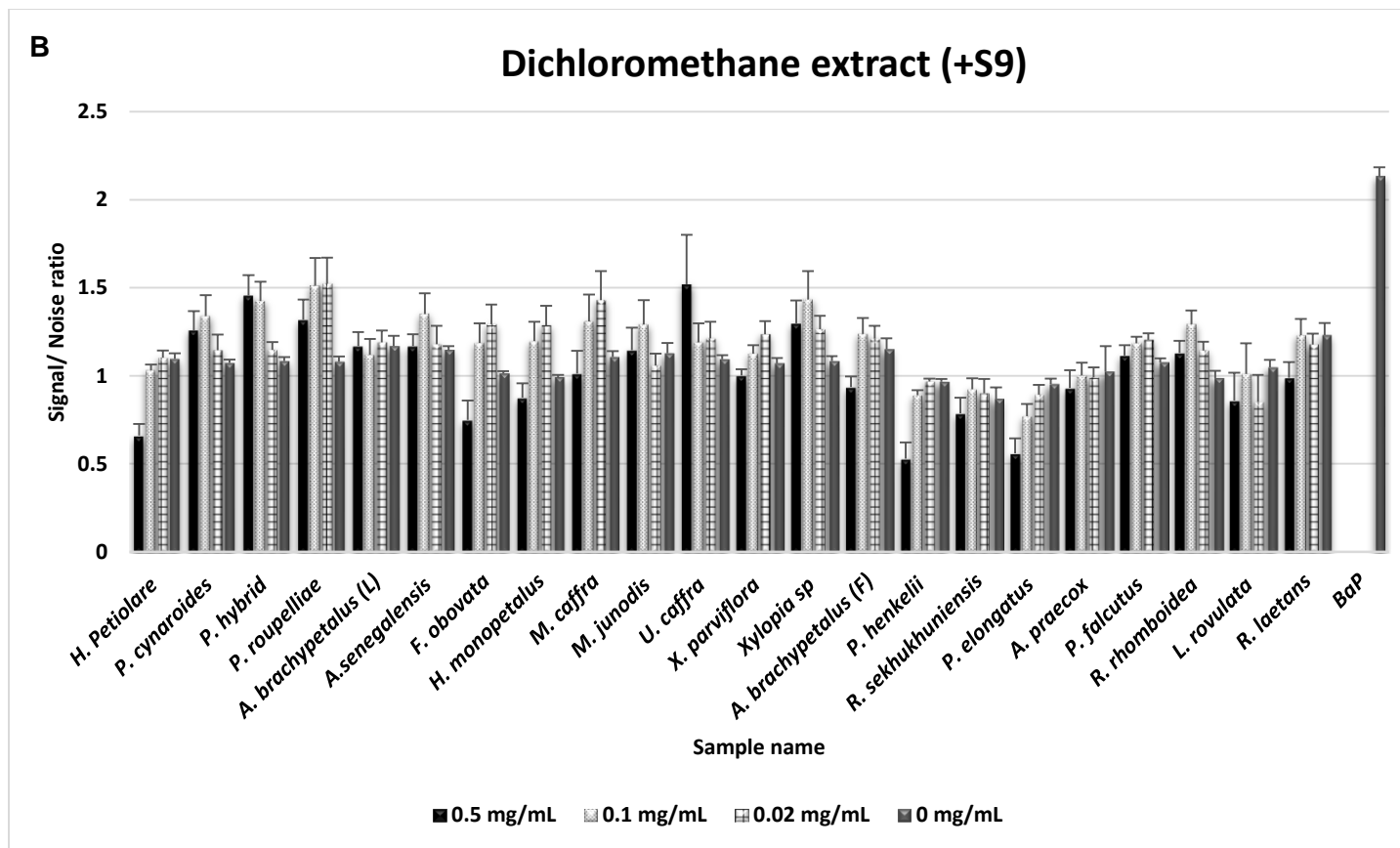


Figure 1(B)

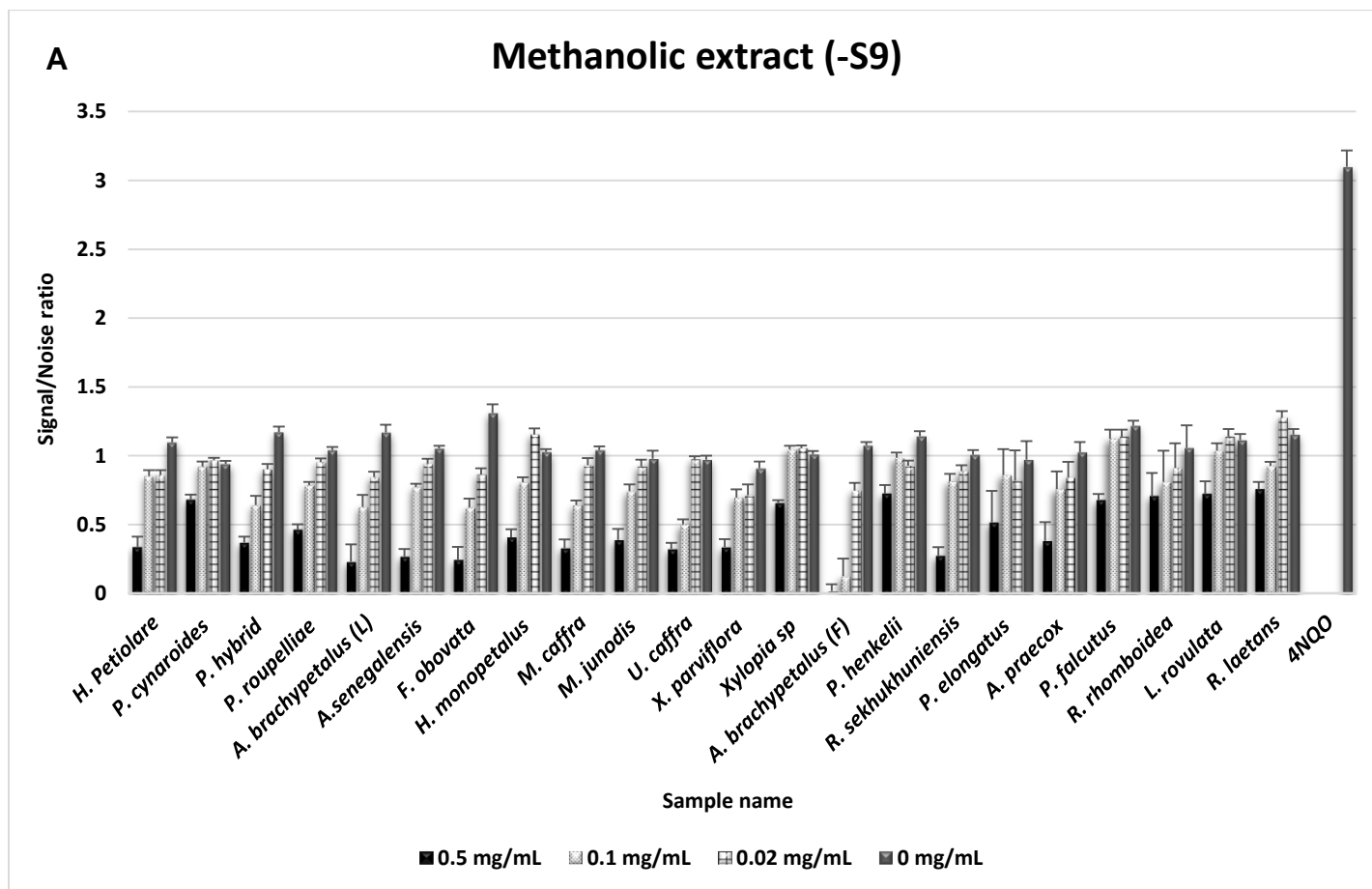


Figure 2(A)

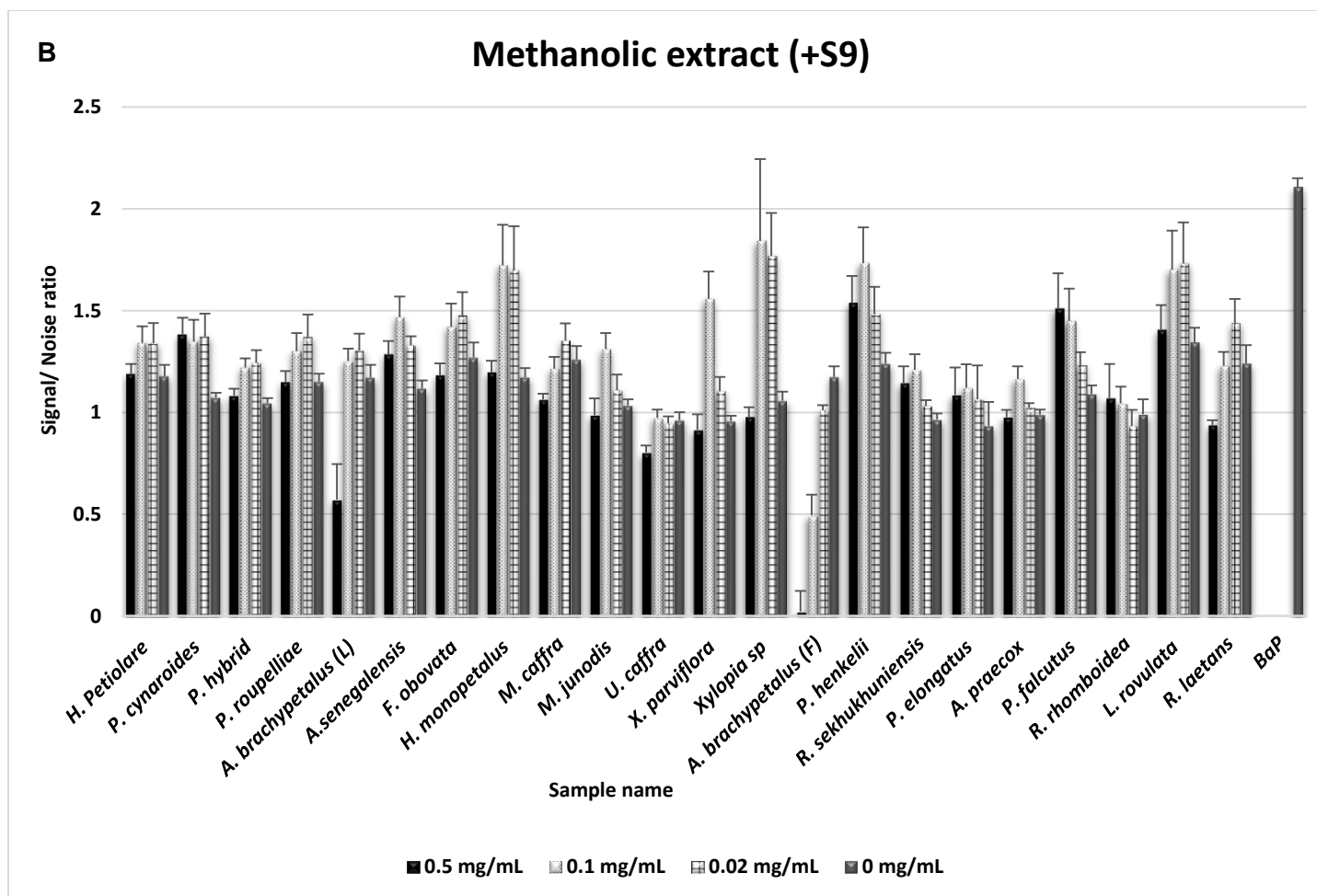


Figure 2(B)

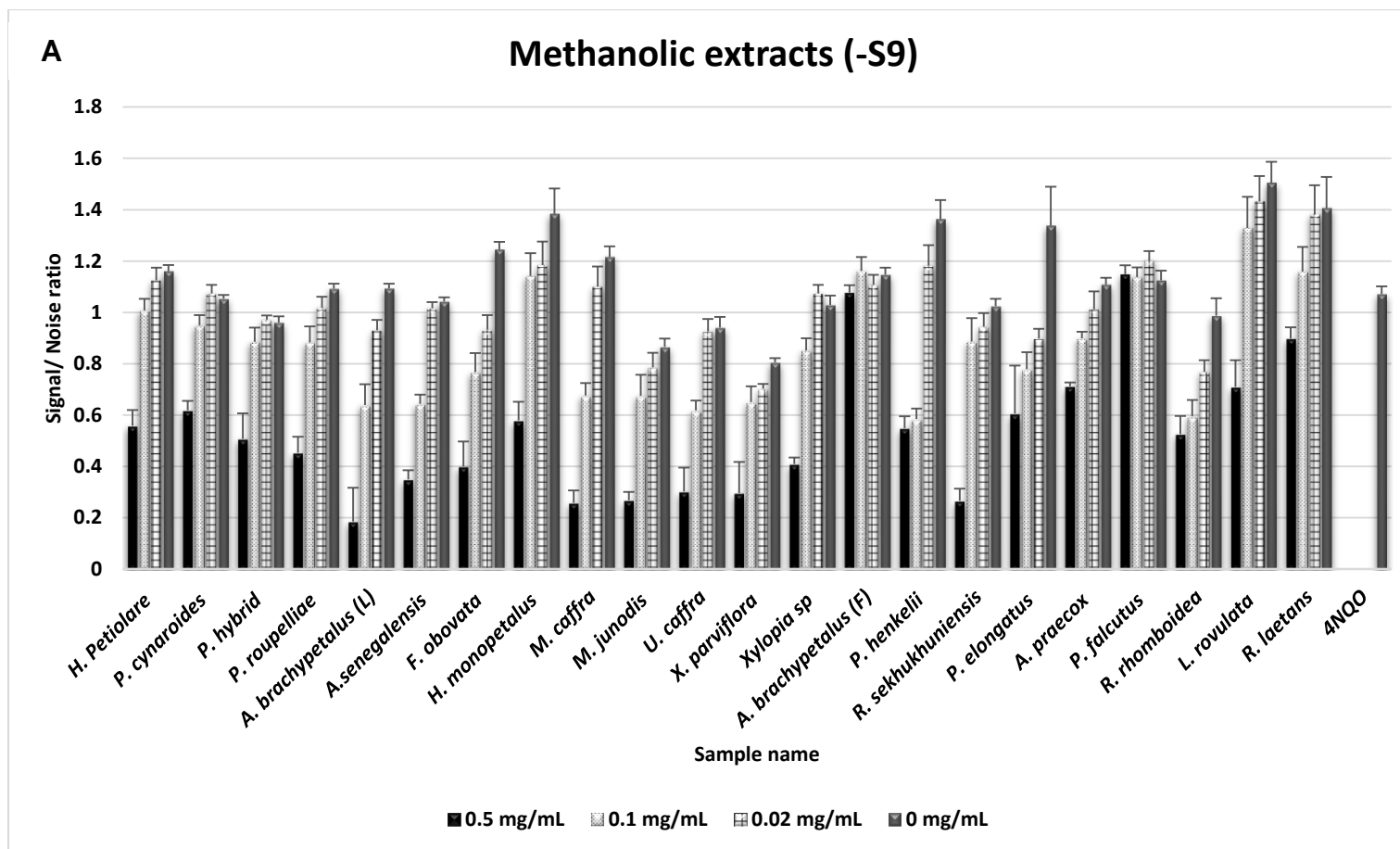


Figure 3(A)

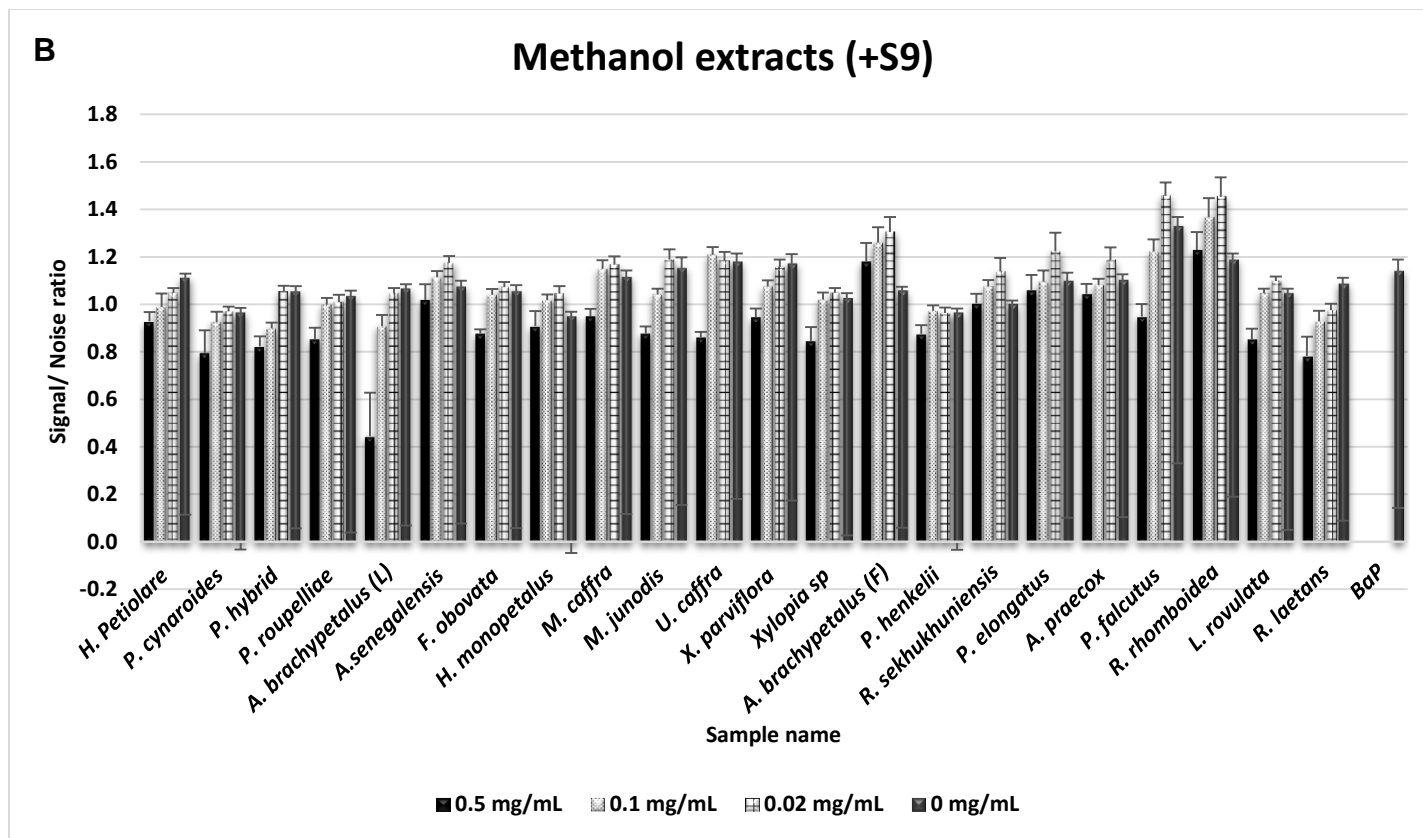


Figure 3(B)

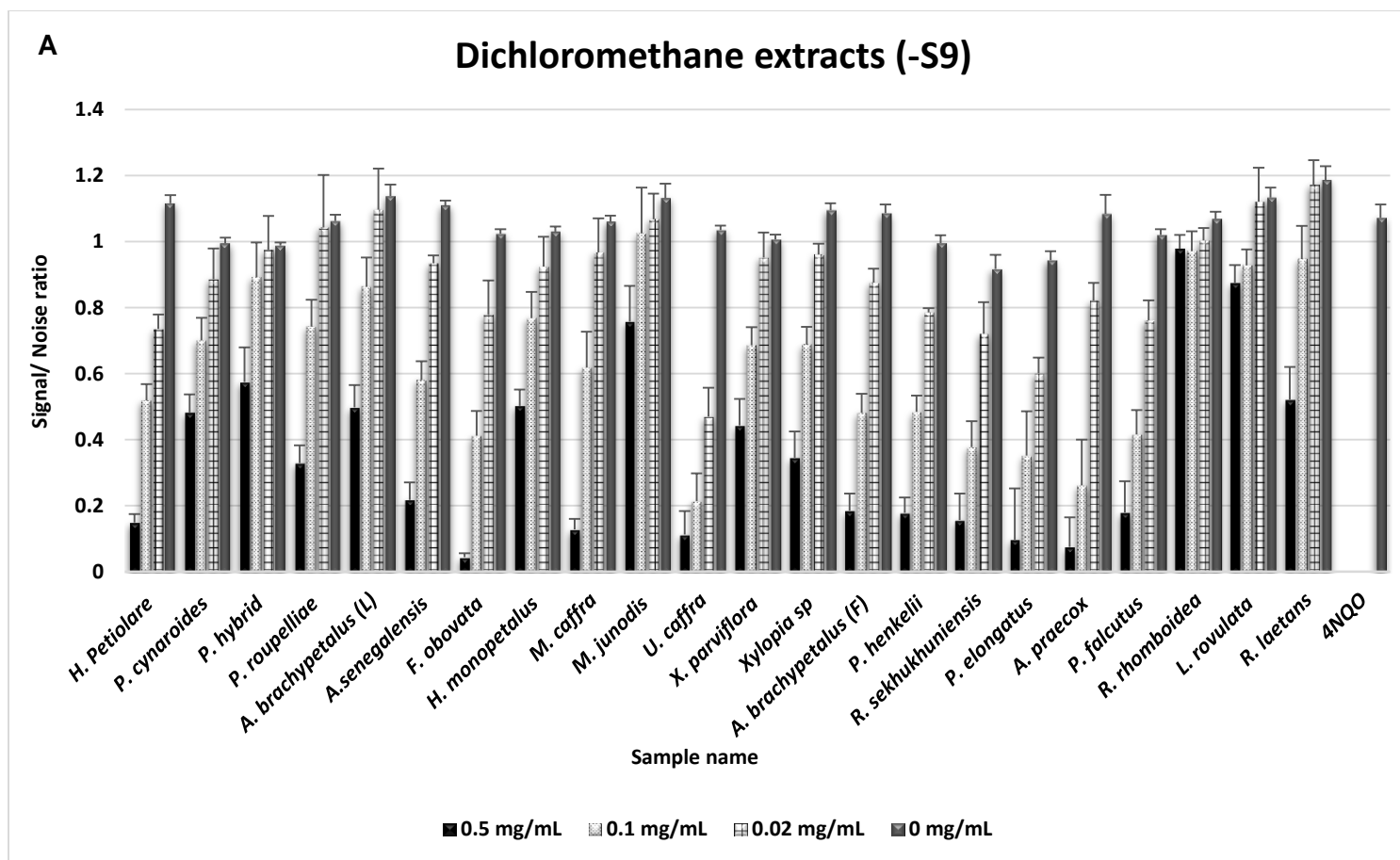


Figure 4(A)

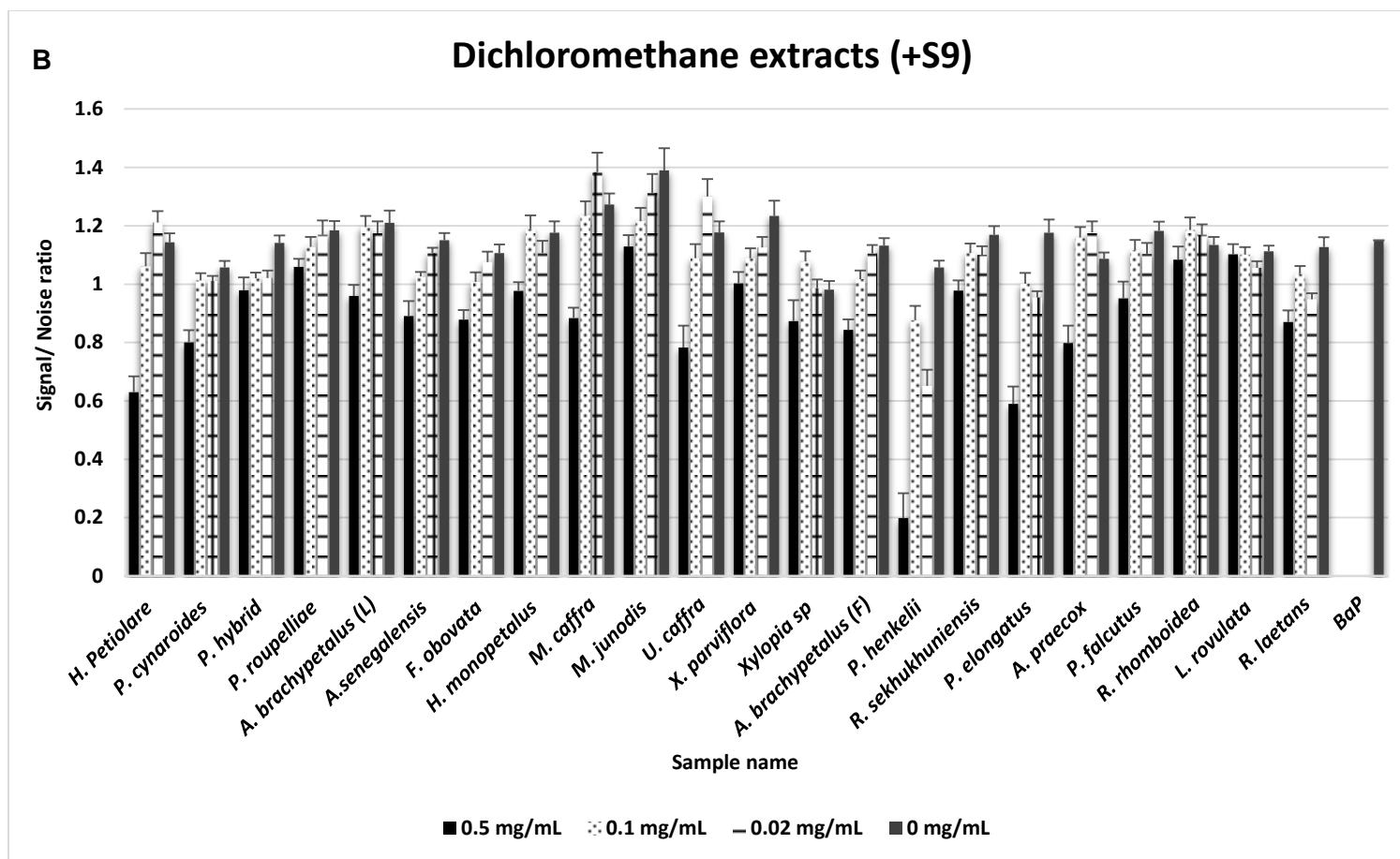


Figure 4(B)

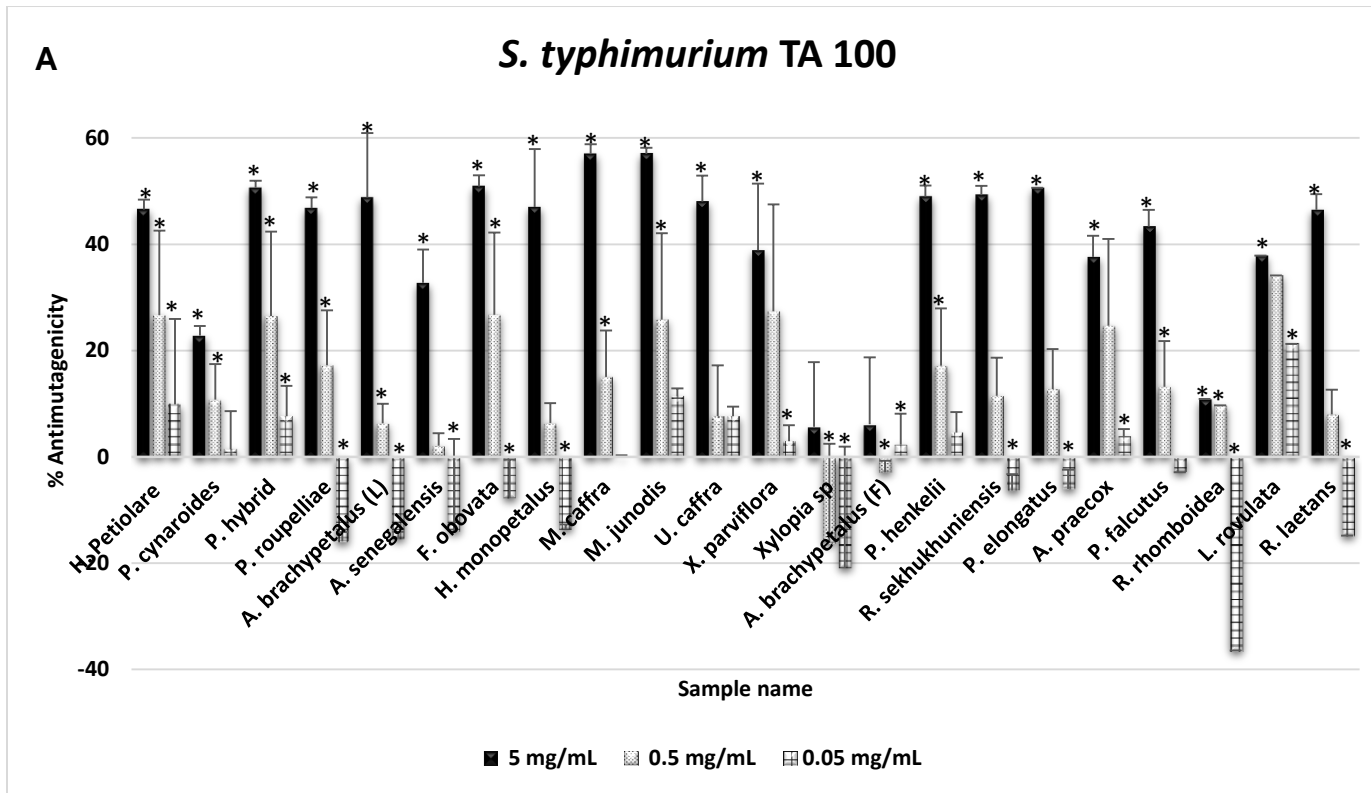


Figure 5 (A)

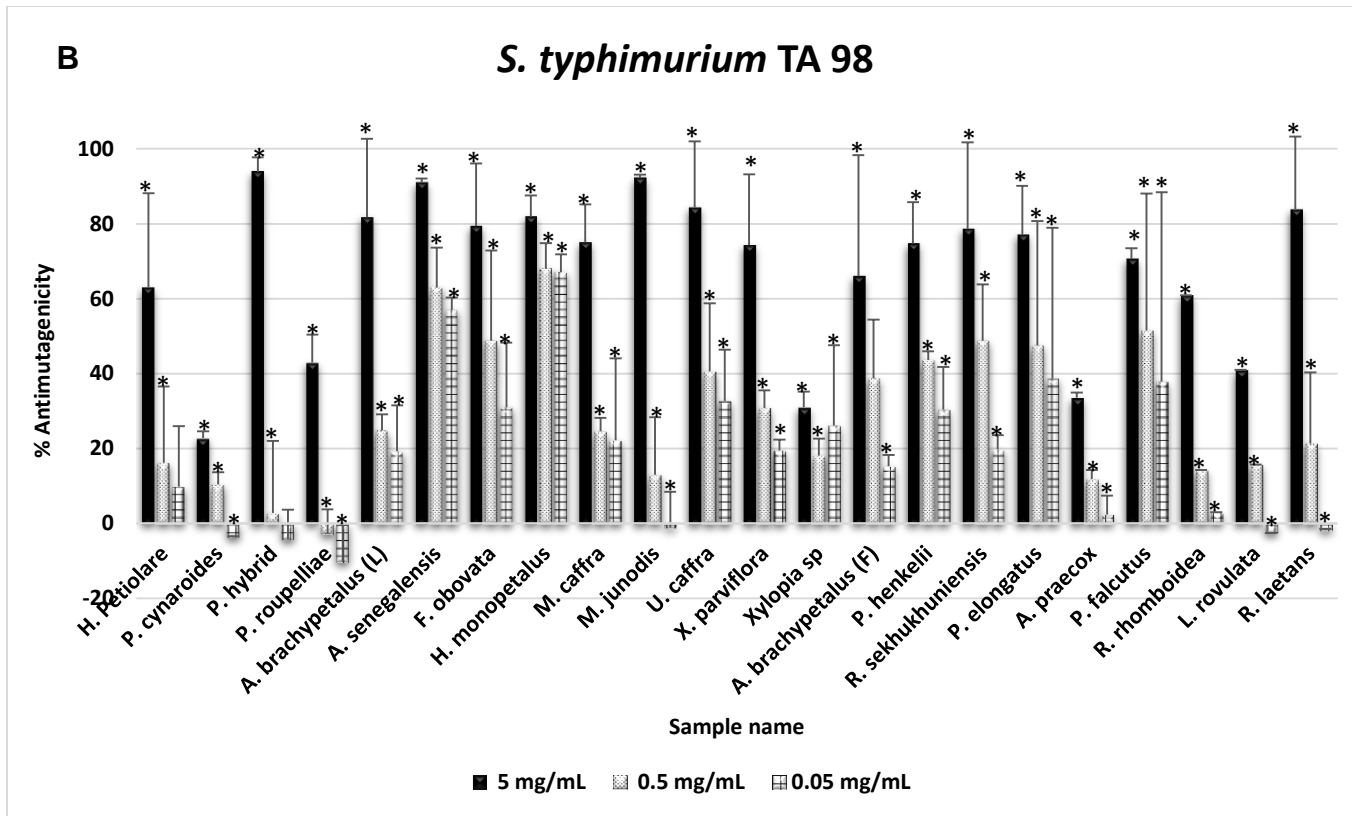


Figure 5(B)

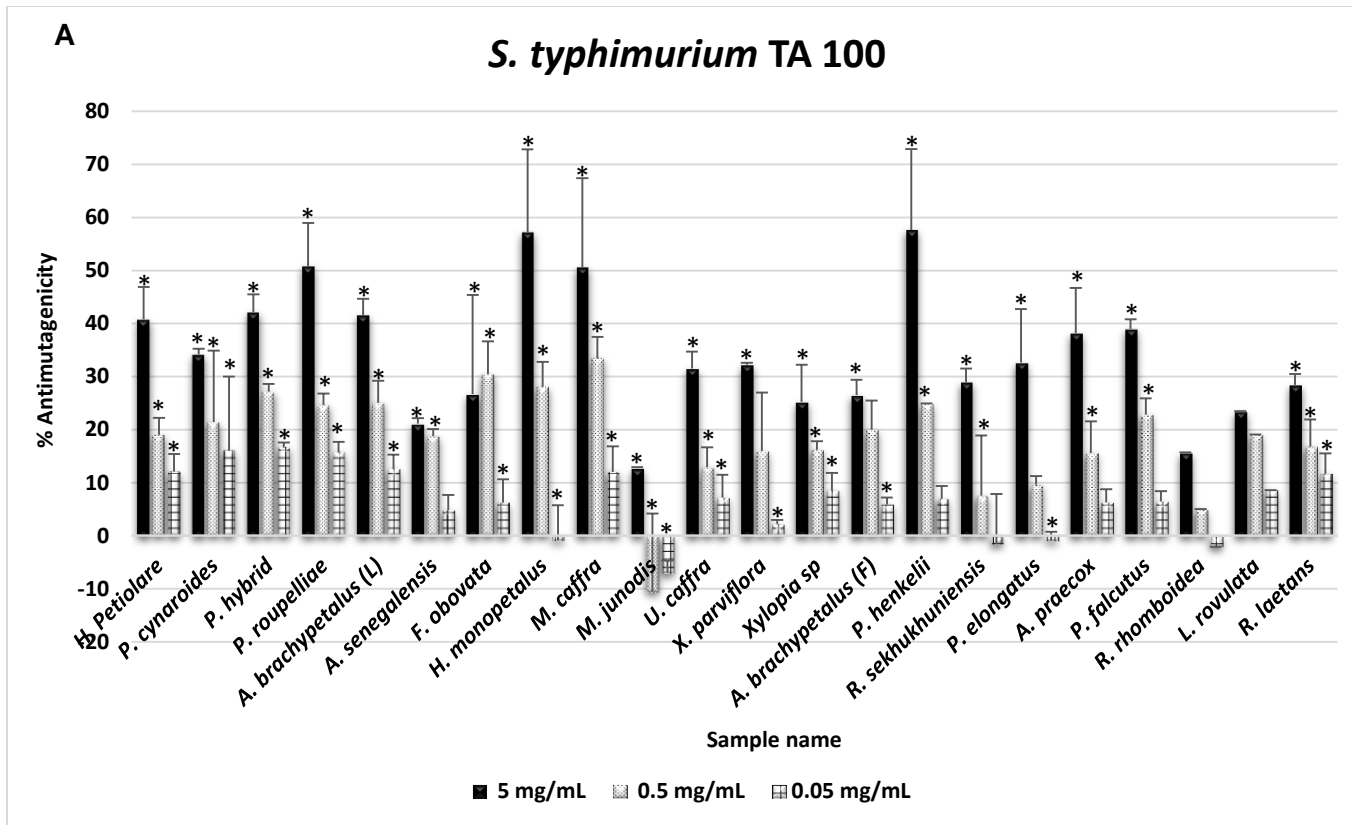


Figure 6(A)

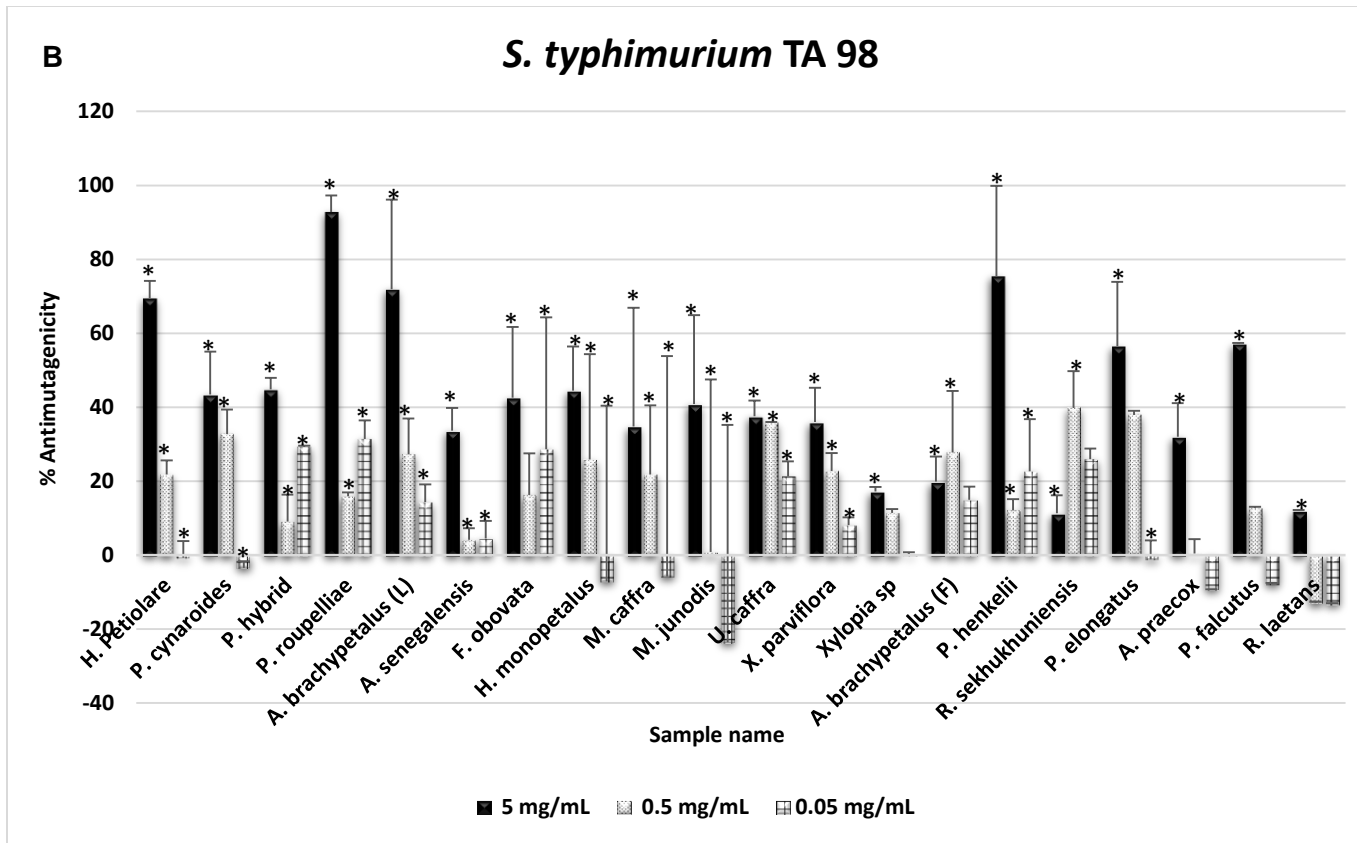


Figure 6(B)

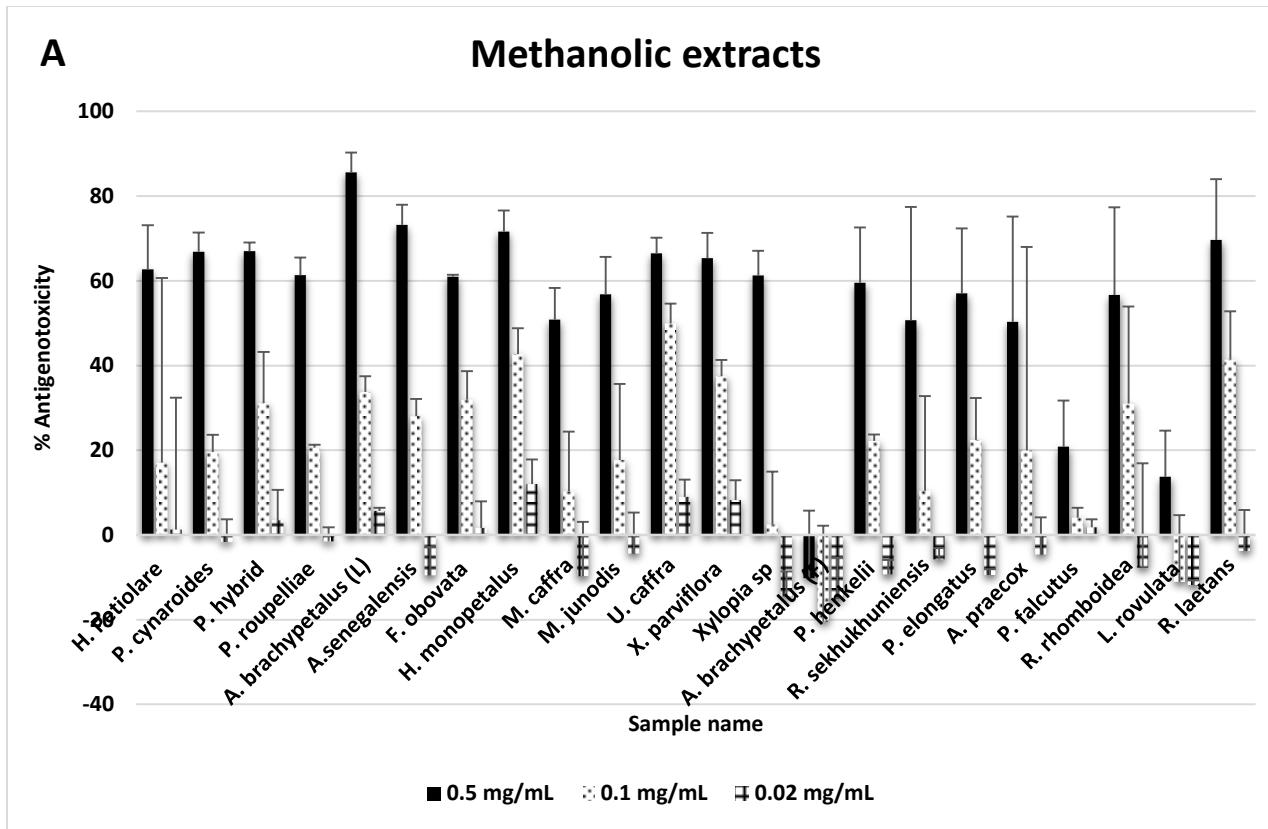


Figure 7 (A)

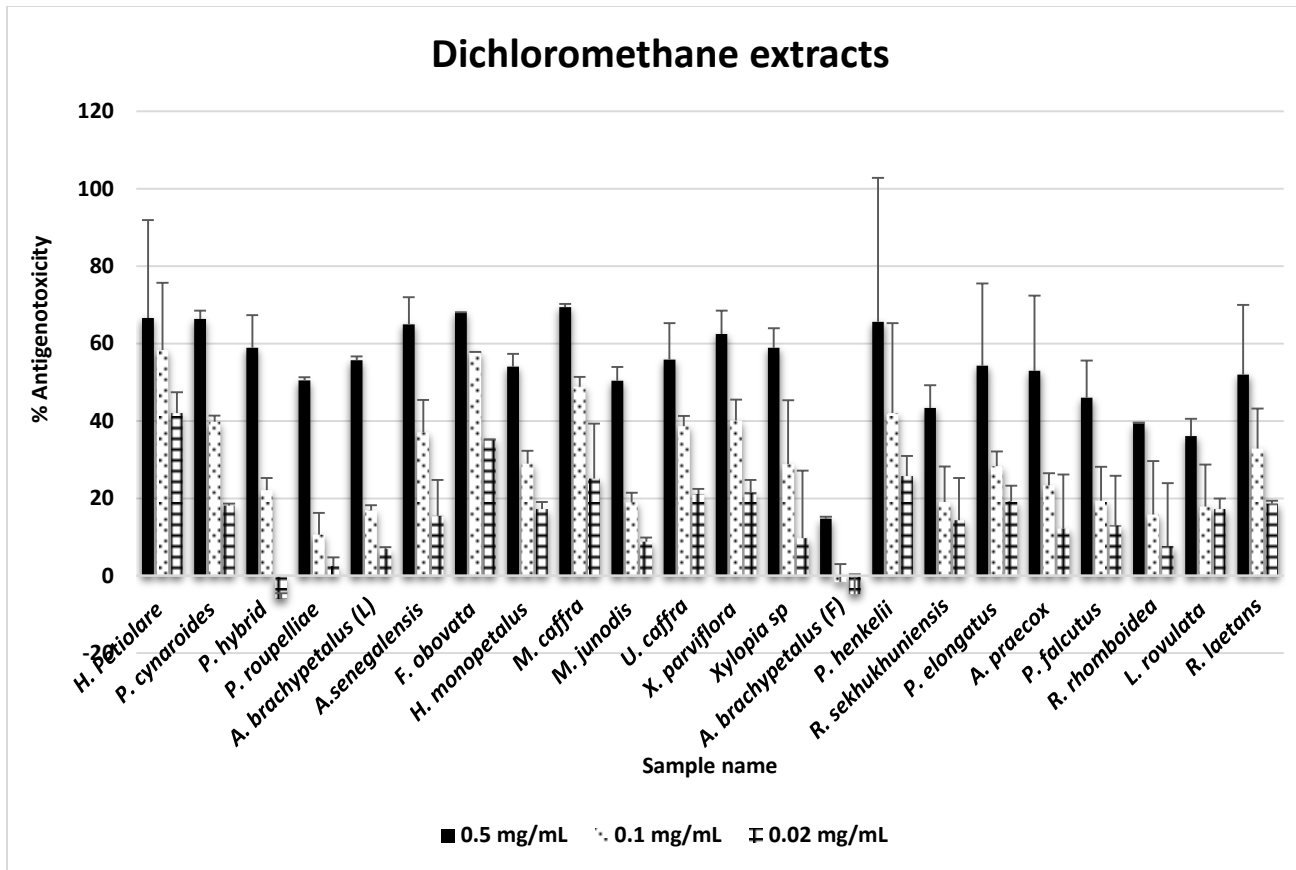


Figure 7(B)