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ABSTRACT:

Twenty-two plant species extracted with dichloromethane and 90% methanol were investigated for their genotoxicity as well as antigenotoxicity against aflatoxin B_1 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, Monanthotaxis caffra, Monodora junodis, Uvaria caffra, Xylopia 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 falcutus* also showed antigenotoxic potentials against aflatoxin B1 induced mutagenicity. Methanolic extracts of *Xylopia* sp.*,* showed a co-mutagenic effect with aflatoxin B_1 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 B_1 induced mutagenicity and carcinogenicity.

Keywords: Genotoxicity, Antigenotoxicity, Aflatoxin B_1, 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, B_1, B_2, G_1 and G_2 (Zain 2011). However, aflatoxin B_1 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 B_1 is metabolized in the liver cells by cytochrome P450 enzyme into a highly reactive aflatoxin B_1-8, 9-epoxide, which binds to the guanine residues forming G to T transversion mutation. This biotransformation of aflatoxin B_1 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

77 the plant kingdom to search for antimutagens or anticarcinogens that are capable of decreasing or
78 inhibiting the mutagenic effects of aflatoxins (Alabi et al. 2011; Sloczynska et al. 2014). Plants contain
79 many bioactive compounds with promising activity against many diseases including genetic diseases such
80 as cancer that could be explored for drug discovery and development (Palombo 2011; Street and Prinsloo
81 2013).
82 This study focused on the screening of South African indigenous plants for their antimutagenic or
83 antigenotoxic potentials against aflatoxin B_1 induced mutagenicity. These plant extracts were also
84 evaluated for their mutagenicity to confirm that they were not mutagenic. The plants were selected based
85 on results from preliminary screening in our laboratory (unpublished results). The antigenotoxicity of the
86 plant extracts was tested using the *Salmonella* microsome and Vitotox assays. These two assays are
87 genotoxicity bioassays commonly used in the screening of genotoxic substances (Sloczynska et al. 2014;
88 Verschaeve et al. 1999).
89
90 ## 2. Materials and Methods
91 ### 2.1. Sample collection and processing
92 Twenty-two plant species collected from South African National botanical gardens (Lowveld, Walter Sisulu
93 and Pretoria) and in the university of Pretoria botanical garden (Manie van der Schijff Botanical Garden) are
94 listed in Table 1. The table also shows the common names, plant part used as well as the accession
95 number for the plants. The plant material (leaves, seeds or fruits) was dried in an oven set at 45^{o}C.
96 Thereafter, the plant material was ground to a fine powder and stored in airtight containers in the dark at
97 room temperature until use. Voucher specimens for the collected plant species were deposited in the
98 H.G.W.J. Schweickerdt herbarium of the University of Pretoria.
99 ### 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 ^{o}C) were added to 20 mL of Oxoid nutrient broth No.2 and incubated on a rotary shaker at 37 ºC 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 B_1 (2 µg/mL, Sigma Aldrich). The top agar mixture was poured over the surface of the minimal glucose agar plates and incubated at 37 ^{o}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 B_1 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:

% inhibition = [(1-T/M) X 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 ºC. 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 ^{o}C using a luminometer (Modulus Microplate Multimode Reader, Turner Biosystems). Antimutagenicity of the plant extracts against aflatoxin B_1 was measured by adding 1 µg/mL of the aflatoxin B_1 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

148 test to determine the significant differences between the mean ($P<0.05$). No statistical analysis was
149 necessary for the Vitotox assay.
150
151 **3. Results and Discussion**
152 Dichloromethane and 90% methanolic extracts of the selected 22 plant species were investigated first for
153 their potential mutagenic effects in the bacterial based Ames and Vitotox assays. This was done to rule out
154 extracts that exhibited both genotoxic and antigenotoxic effects as they would not be good candidates in
155 further studies. The number of revertant colonies obtained from TA98 and TA100 are in agreement with
156 results generated in our laboratory and in accordance with those reported in literature (Maron and Ames
157 1983). The two strains are widely used in mutagenicity testing because they are sensitive in detecting most
158 mutagens and carcinogens (Dhawan and Bajpayee 2013; Makhafola et al. 2016; Verschaeve and van
159 Staden 2008). The assays were performed in the presence of S9 since aflatoxin B_1 is an indirect mutagen
160 and need to be converted metabolically to its 8,9-epoxide active derivative (Hamid et al. 2013). The
161 enzyme contains a mixture of xenobiotic enzymes such as cytochrome P450s and sulfotransferase which
162 mimic mammalian metabolism in bacteria (Ndhlala et al. 2010; Verschaeve and Van Staden 2008).
163 Results on the mutagenic effects of methanolic and dichloromethane plant extracts tested in the Ames
164 assay using *S. typhimurium* strain TA 100 and TA 98 are presented in Tables 2 and 3. In the Ames test for
165 the used TA98 and TA100, an extract is considered mutagenic when the mean number of revertant
166 colonies produced in each plate was double or greater than two times that of the negative control (Bierkens
167 et al. 2004; Ndhlala et al. 2010). Accordingly, most of the plant extracts tested did not have any mutagenic
168 properties. Only methanolic extracts of *M. junodis* were mutagenic on TA 98 strain in a dose dependent
169 manner, while *P. hybrid* produced double the number of revertant colonies as the negative control at the
170 highest concentration tested. Few more plant extracts produced double or more than double the number of
171 revertant colonies as the negative control on strain TA 100 without showing a dose response. These

include the methanolic extracts of *X. parviflora*, *Xylopia* 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 *pr*1 signal to above 1.5, it was also not cytotoxic as the signal to noise ratio in *pr*1 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*, *Xylopia* sp., *L. rovulata* 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

196 constitutively expresses the *lux* operon (Chichioco-Hernandez et al. 2011; Verschaeve and others 1999). In
197 the presence of cytotoxic compounds, there is a decrease in light production. However, the Cytox strains
198 can also be used as the reference for non-specific enhancement of light emission (Verschaeve et al. 1999).
199 Therefore, the lack of a dose response in the mutagenicity test using Vitotox is due to toxicity of the highest
200 dose tested. The S/N curve for *pr1* strain, which is a useful tool in testing for toxicity alone, was below 0.8
201 and therefore clearly indicative of the toxicity of the highest dose used for these extracts. These plant
202 extracts with mutagenic effects should be used with care in any form of prescription and further rigorous
203 toxicological investigations are required before they are recommended in pharmaceuticals and drug
204 discovery industries (Verschaeve and Van Staden 2008).
205 The results on cytotoxicity in the Vitotox assay showed that almost all of the methanolic and
206 dichloromethane plant extracts were toxic at the highest concentration (0.5 mg/mL) when tested without
207 metabolic activation. An exception was the methanolic extracts of *P. falcutus, A.brachypetalus* (fruit) and *R.*
208 *laetans* and the dichloromethane extracts of R. *rhomboidea* and *L. rovulata* . However, in the presence of
209 S9 metabolizing enzymes 95% of the methanolic extracts were not toxic at all tested concentrations. An
210 exception was leaf extract of *A. brachypetalus*, which was toxic at 0.5 mg/mL. Whereas 73% of the
211 dichloromethane extracts namely *P. hybrid, P. roupelliae, A. brachypetalus (leaves), A. senegalensis, F.*
212 *obovata, H. monopetalus, M. caffra, M. junodis, X. parviflora, Xylopia sp., A. brachypetalus* (fruit)*, R.*
213 *sekhukhuniensis, P. falcutus, R. rhomboidea, L. rovulata* and *R. laetans* were not toxic at all concentrations
214 tested (Figure 3, 4).
215
216 A test solution is considered antimutagenic when the frequency of genetic damage caused by the
217 combined treatments (extracts and aflatoxin B_1) is substantially lower compared to the damage induced by
218 the mycotoxin alone. Usually, an extract is considered to have no or only weak antimutagenic properties
219 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 B_1 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 B_1. 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. rovulata* showed moderate antimutagenicity in a dose response manner. *R. rhomboidea, A*. *brachypetalus* (fruit) and *Xylopia* 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. rovulata and R. laetans* had strong antimutagenic activities mostly at 5 mg/mL whereas extracts of *Xylopia 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. henkelii*) tested against aflatoxin B_1induced 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 B_1-induced mutagenicity on TA 98 (figure 6B).

In the Vitotox assay, the antigenotoxicity study of plant extracts against aflatoxin B_1 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 B_1 mutagenicity in a dose dependent manner. About 45% of the plant extracts, namely *H. petiolare, P. cynoroides, P. roupelliae, M. caffra, M. junodis, Xylopia 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 B_1 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 B_1, at the lowest concentration tested (Figure 7A). However, the antigenotoxicity of *M. junodis, Xylopia 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 Cytox strain. For the dichloromethane plant extracts, 86% of the extracts had a percentage inhibition above 40% against aflatoxin B_1 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 B_1. 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. rovulata* showed moderate to weak antigenotoxicity and co-mutagenic effect against aflatoxin B_1 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 AFB_1 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 *Xylopia* sp. were not mutagenic when tested alone. However, they showed a co-mutagenic effect with aflatoxin B_1 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. henkelii* with 4-nitroquinoline-1- oxide (4NQO) mutagenicity has been recently reported (Makhafola et al., 2016). Extracts of *P. henkelii* 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 B_1-induced mutagenicity and other direct and indirect mutagens (Goeger et al. 1999; Snijman et al. 2007). However, the comutagenic effect with AFB_1 was attributed largely to an

292 increase in the bioactivation of aflatoxin B_1 to its AFB_1-8,9-expoxide (Goeger et al. 1999; Snijman et al.
293 2007).
294 This study investigated plant extracts from members of different families including Anonnaceae,
295 Asparagaceae, Asteraceae, Podocarpaceae, Proteaceae and Vitaceae. The mechanism by which some of
296 these extracts reduced the mutagenicity of aflatoxin B_1 is so far unknown. However, members of these
297 families have been reported to contain sterols, terpenes, alkaloids, acetogenins, glycosides, amino acids
298 and proteins as well as phenolic compounds (Mulholland et al. 2000; Parmena et al. 2012). It is well
299 established that AFB_1 requires activation by cytochrome B-450 microsomal mixed function oxidase system
300 into AFB_1-8,9-epoxide. The epoxide form adducts with DNA or undergo a detoxification process through
301 conjugation with glutathione to form AFB_1-glutathione conjugate, which are thereafter excreted. Various
302 natural products, including those reported in species under investigation, exert their antimutagenic effect by
303 either reducing metabolic activation of the promutagen or through interaction with its metabolic activation
304 derivatives (Waters et al. 1990; Jeng et al. 2000). However, most compounds antimutagenic to AFB_1 are
305 intracellular blocking agents i.e. bioantimutagens and act through prevention of AFB_1 from reacting with
306 target sites, affecting DNA repair, scavenging of radicals or prevention of neoplasmic expression of initiated
307 cells (Water et al. 1990).
308
309 **Conclusion**
310 Most plant extracts investigated in this study had antigenotoxic activities against aflatoxin B_1 induced
311 mutagenicity in either the Ames or Vitotox test or both. Although the mechanism of action of these extracts
312 is unknown, however, it is well-known that AFB_1 exerts its mutagenic effect through oxidative stress. Few
313 plant extracts such as *A. brachypetalus*, *H. petiole*, *M. caffra*, *P. hybrid* and *P. roupeliae* had strong to
314 moderate antigenotoxic activity in both tests. The activity of the latter plant extracts is of particular interest
315 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 antimuatgenic compounds may therefore lead to the discovery of interesting molecules that may play an important role in liver cancer prevention.

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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.

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462 Table 1: Plant species investigated for antimutagenicity and mutagenicity.

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

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

463 [a]NV – not voucher specimen due to lack of plant material

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

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

P. falcutus	189±0	205±0	216±3	197±2	207±2	224±3	22±3	33±5	28±3	27±4	33±6	30±12
R. rhomboidea	203±17	244±17	232±14	196±3	213±3	275±3	33±2	29±1	32±2	[b]ND	[b]ND	[b]ND
L. rovulata	344±10	377±10	424±6.	191±2	205±2	228±3	34±4	29±1	37±4	[b]ND	[b]ND	[b]ND
R. laetans	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		

[b]ND- the antimutagenicity of the plant extracts was not determined due to lack of plant material

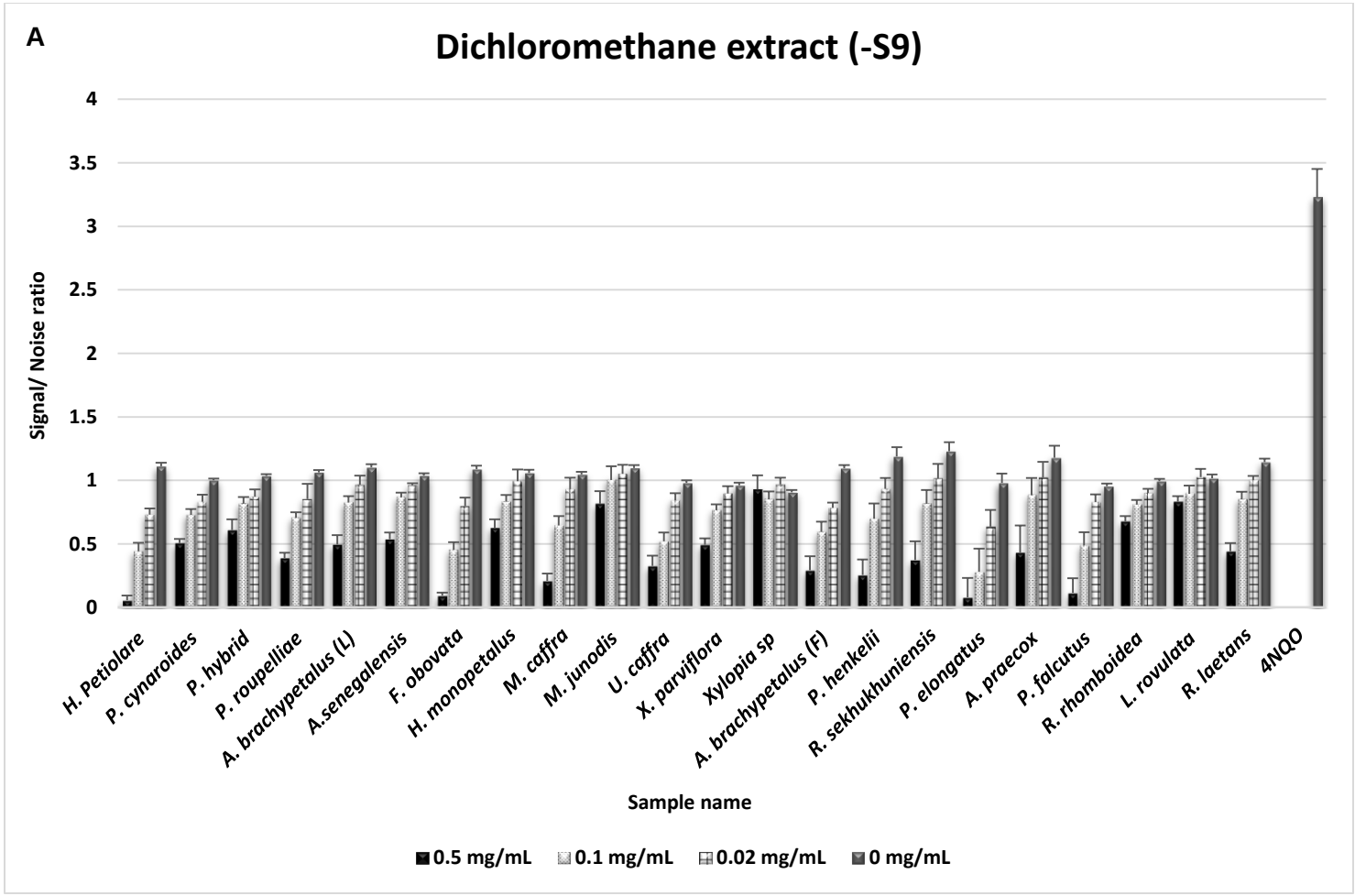

A
Dichloromethane extract (-S9)
Signal/ Noise ratio
4
3.5
3
2.5
2
1.5
1
0.5
0
H. Petiolare
P. cynaroides
P. hybrid
P. roupelliae
A. brachypetalus (L)
A.senegalensis
F. obovata
H. monopetalus
M. caffra
M. junodis
U. caffra
X. parviflora
Xylopia sp
A. brachypetalus (F)
P. henkelii
R. sekhukhuniensis
P. elongatus
A. praecox
P. falcutus
R. rhomboidea
L. rovulata
R. laetans
4NQO
Sample name
0.5 mg/mL
0.1 mg/mL
0.02 mg/mL
0 mg/mL

Figure 1(A)

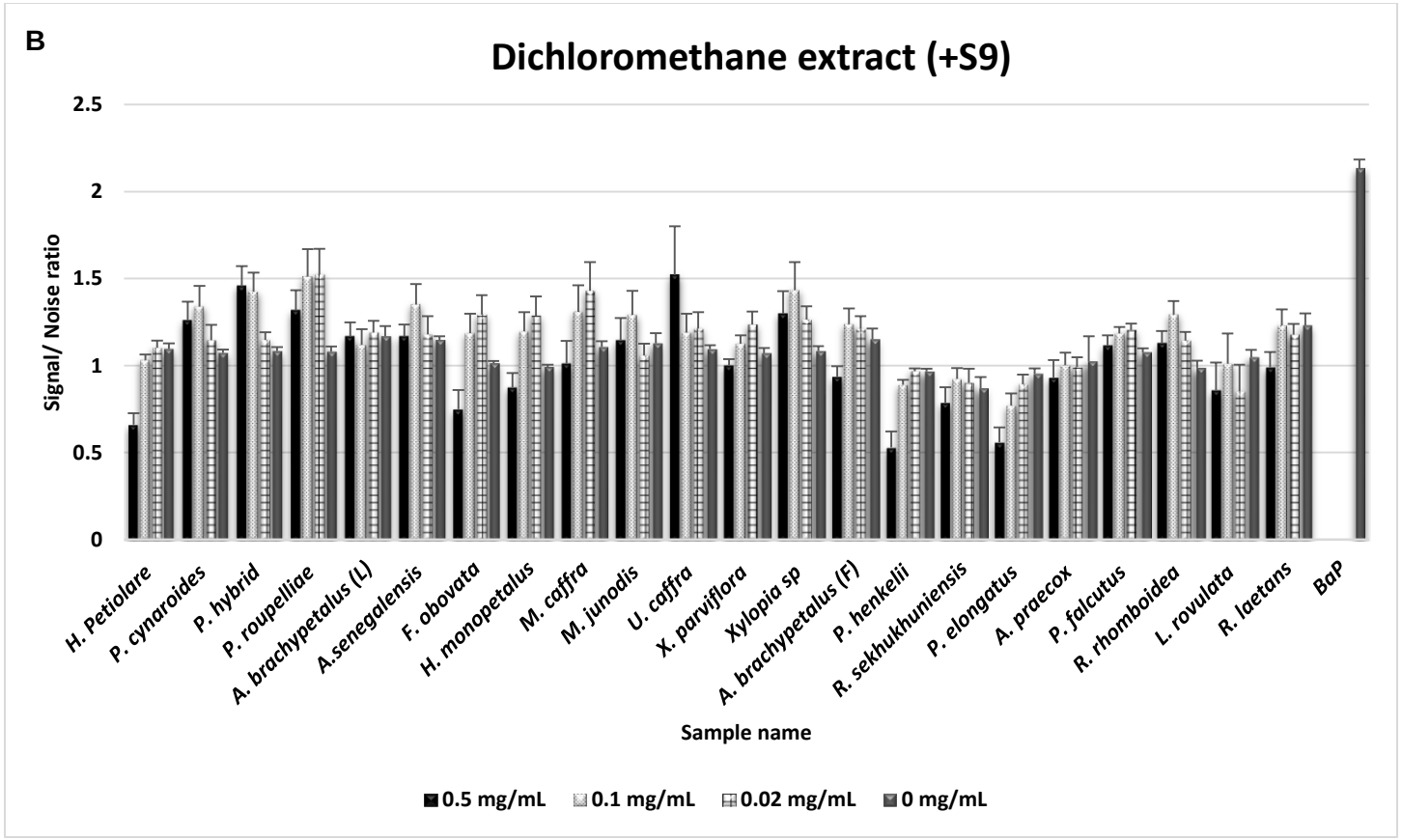

B
Dichloromethane extract (+S9)
Signal/ Noise ratio
2.5
2
1.5
1
0.5
0
H. Petiolare
P. cynaroides
P. hybrid
P. roupelliae
A. brachypetalus (L)
A.senegalensis
F. obovata
H. monopetalus
M. caffra
M. junodis
U. caffra
X. parviflora
Xylopia sp
A. brachypetalus (F)
P. henkelii
R. sekhukhuniensis
P. elongatus
A. praecox
P. falcutus
R. rhomboidea
L. rovulata
R. laetans
BaP
Sample name
0.5 mg/mL
0.1 mg/mL
0.02 mg/mL
0 mg/mL

Figure 1(B)

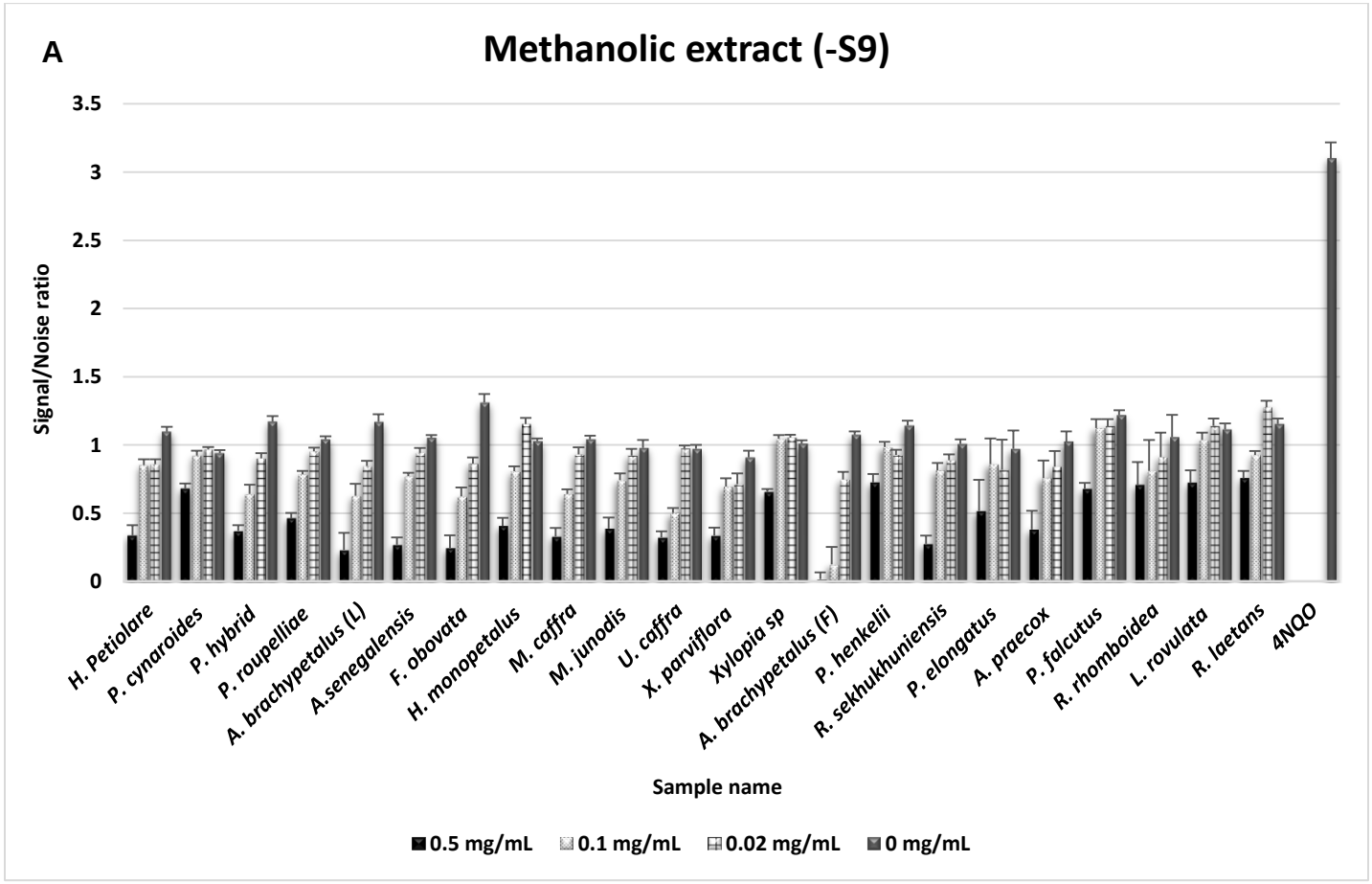

A
Methanolic extract (-S9)
Signal/Noise ratio
3.5
3
2.5
2
1.5
1
0.5
0
H. Petiolare
P. cynaroides
P. hybrid
P. roupelliae
A. brachypetalus (L)
A.senegalensis
F. obovata
H. monopetalus
M. caffra
M. junodis
U. caffra
X. parviflora
Xylopia sp
A. brachypetalus (F)
P. henkelii
R. sekhukhuniensis
P. elongatus
A. praecox
P. falcutus
R. rhomboidea
L. rovulata
R. laetans
4NQO
Sample name
0.5 mg/mL
0.1 mg/mL
0.02 mg/mL
0 mg/mL

Figure 2(A)

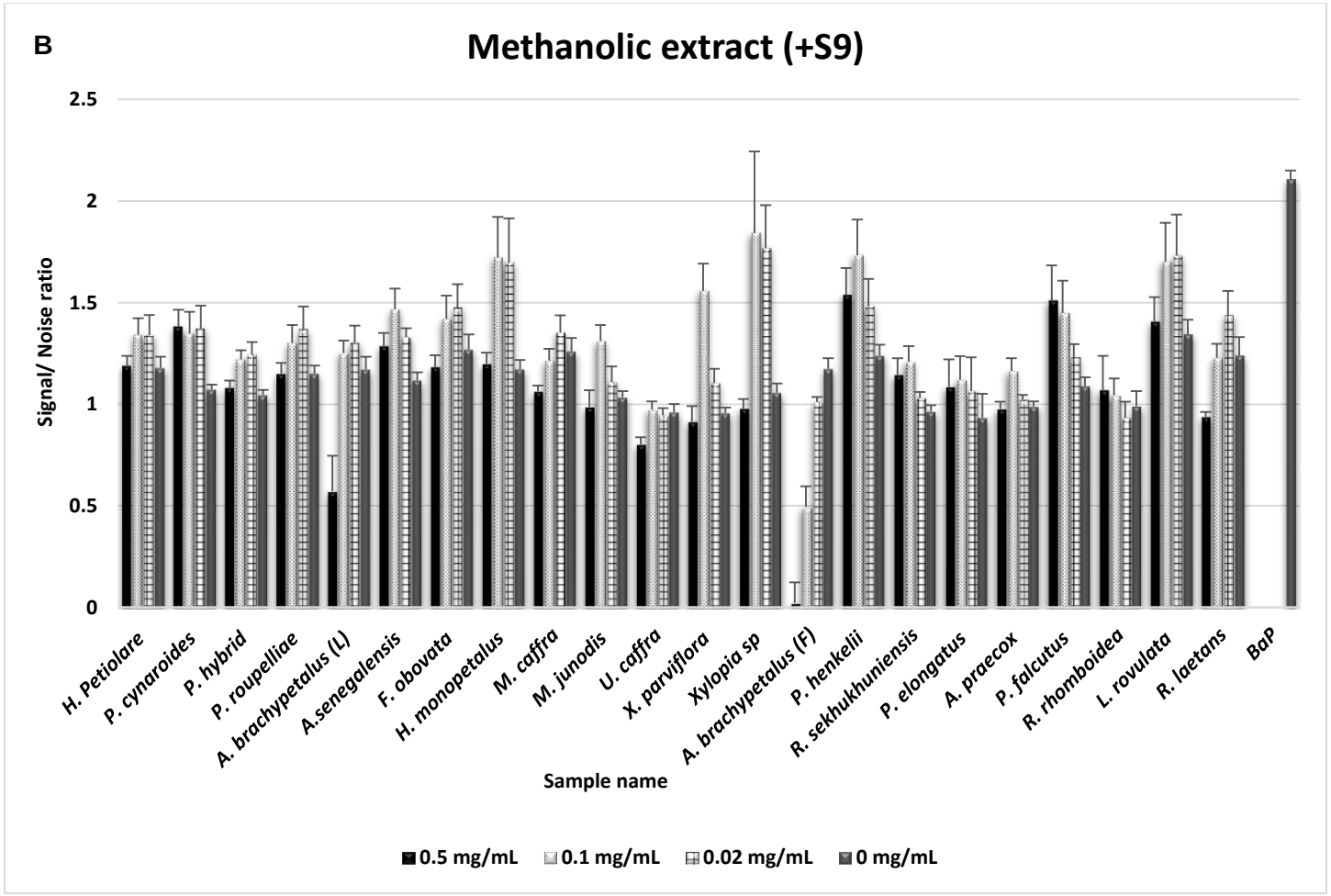

B
Methanolic extract (+S9)
Signal/ Noise ratio
2.5
2
1.5
1
0.5
0
H. Petiolare
P. cynaroides
P. hybrid
P. roupelliae
A. brachypetalus (L)
A.senegalensis
F. obovata
H. monopetalus
M. caffra
M. junodis
U. caffra
X. parviflora
Xylopia sp
A. brachypetalus (F)
P. henkelii
R. sekhukhuniensis
P. elongatus
A. praecox
P. falcutus
R. rhomboidea
L. rovulata
R. laetans
BaP
Sample name
0.5 mg/mL
0.1 mg/mL
0.02 mg/mL
0 mg/mL

Figure 2(B)

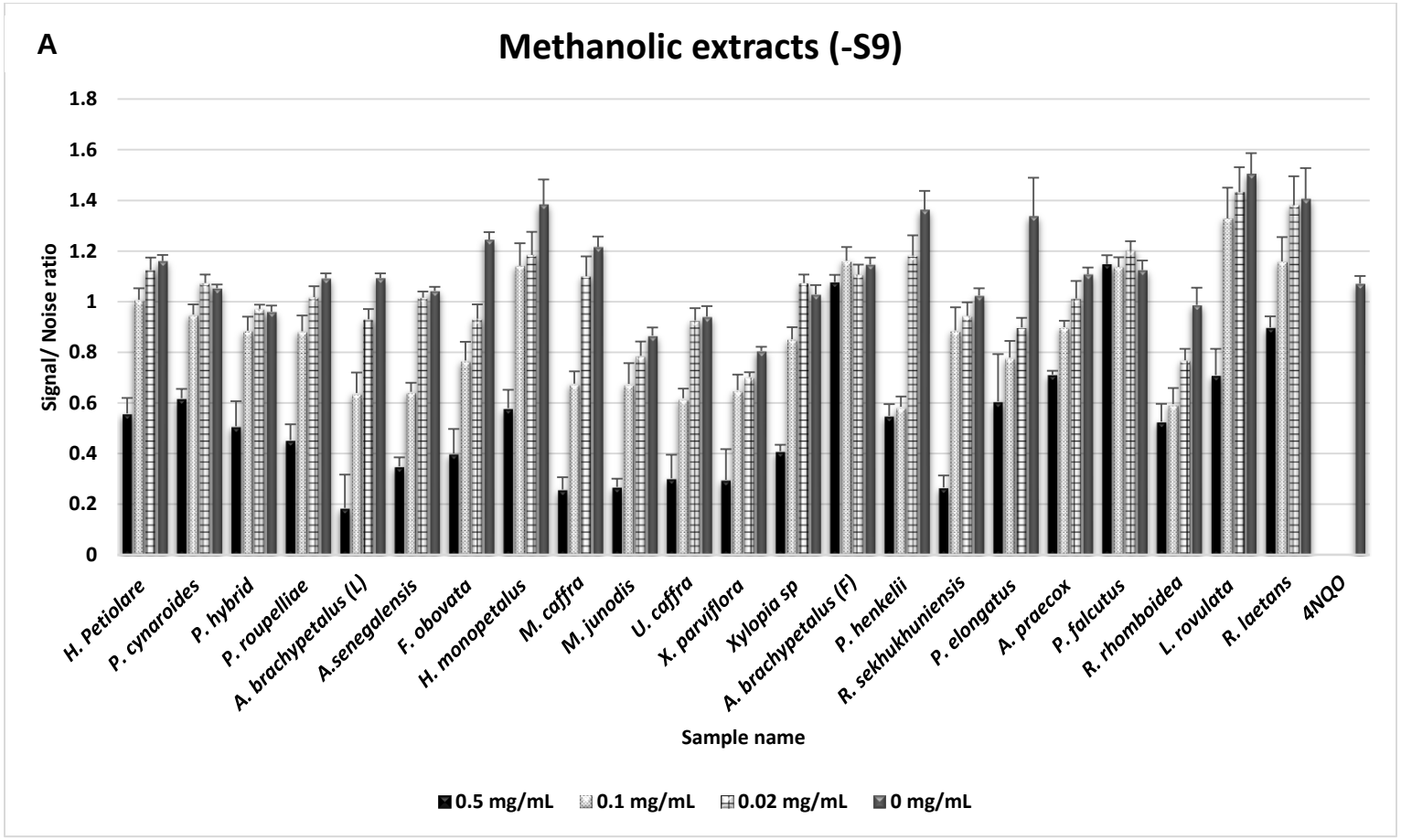
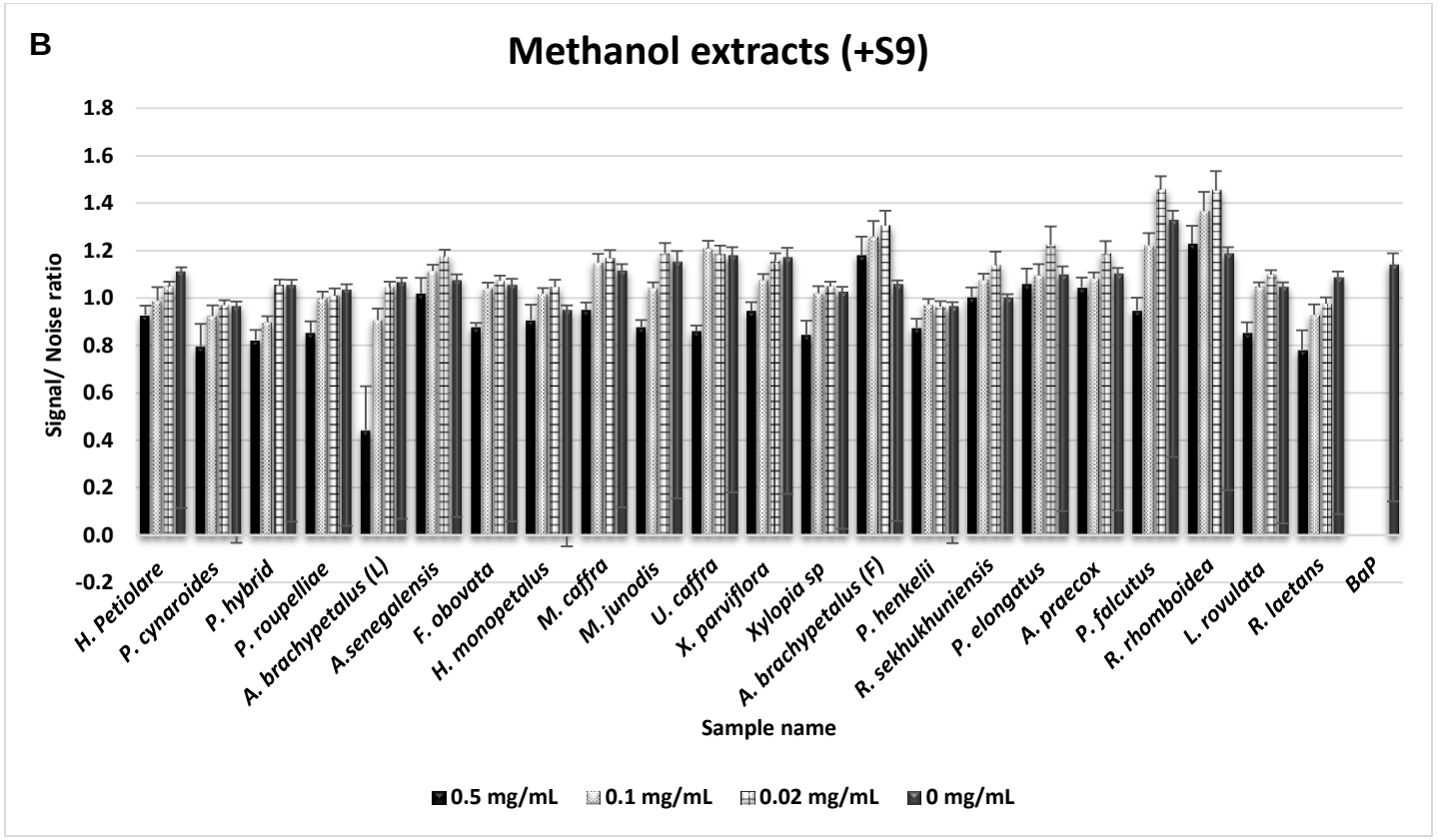

A
Methanolic extracts (-S9)
Signal/ Noise ratio
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
H. Petiolare
P. cynaroides
P. hybrid
P. roupelliae
A. brachypetalus (L)
A.senegalensis
F. obovata
H. monopetalus
M. caffra
M. junodis
U. caffra
X. parviflora
Xylopia sp
A. brachypetalus (F)
P. henkelii
R. sekhukhuniensis
P. elongatus
A. praecox
P. falcutus
R. rhomboidea
L. rovulata
R. laetans
4NQO
Sample name
0.5 mg/mL
0.1 mg/mL
0.02 mg/mL
0 mg/mL

Figure 3(A)



488

489 **Figure 3(B)**

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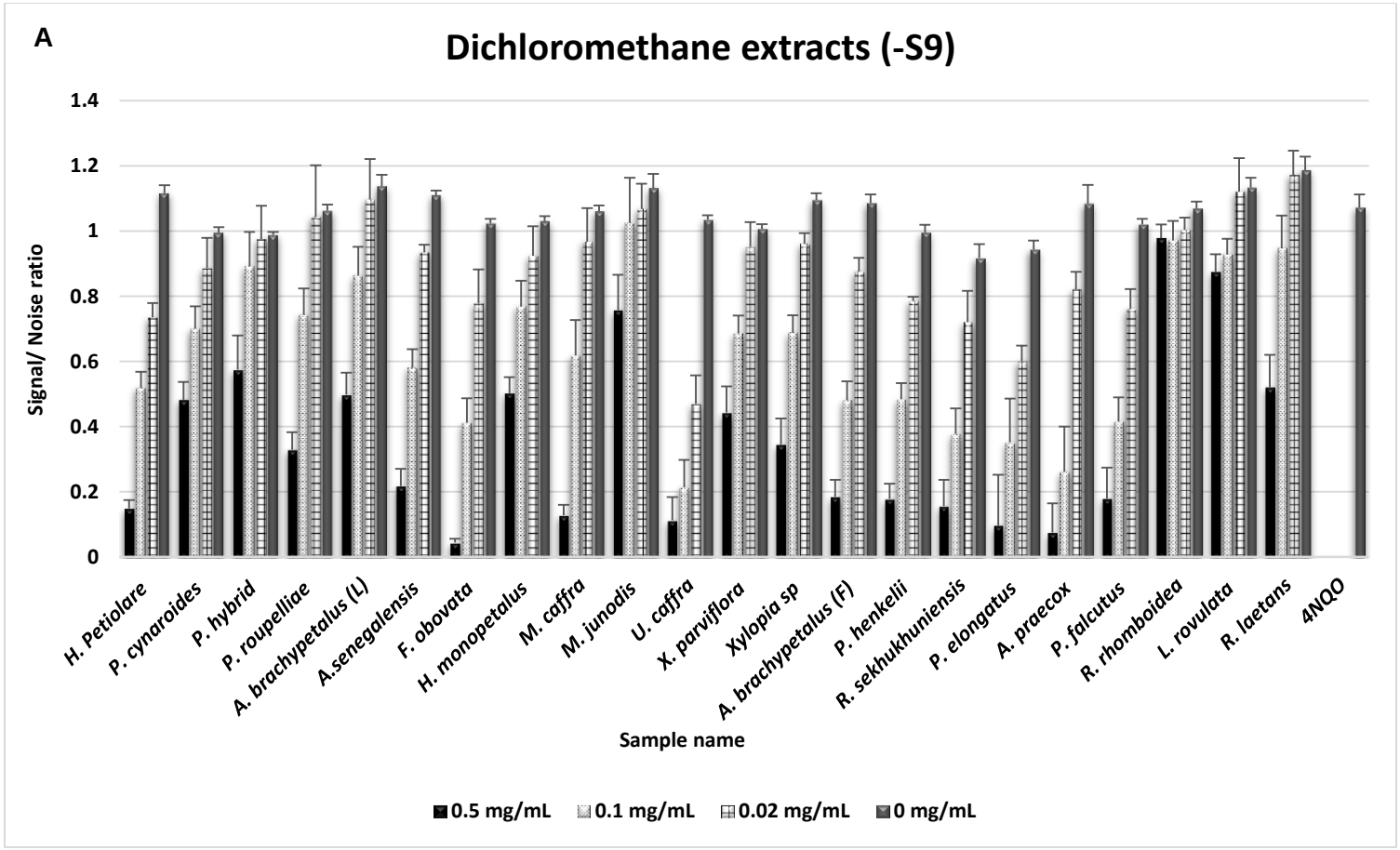


491

492 **Figure 4(A)**

493

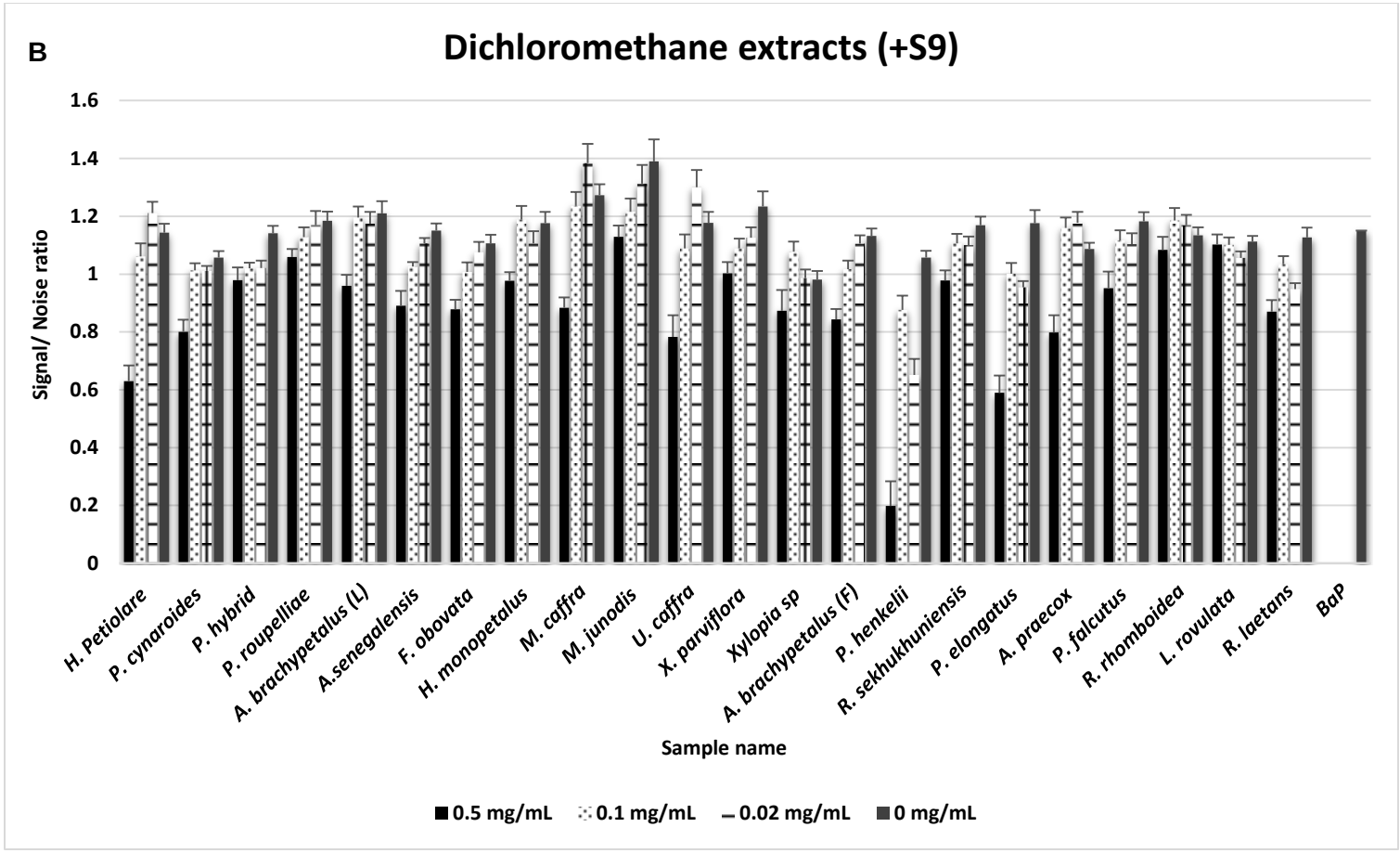

B
Dichloromethane extracts (+S9)
Signal/ Noise ratio
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
H. Petiolare
P. cynaroides
P. hybrid
P. roupelliae
A. brachypetalus (L)
A.senegalensis
F. obovata
H. monopetalus
M. caffra
M. junodis
U. caffra
X. parviflora
Xylopia sp
A. brachypetalus (F)
P. henkelii
R. sekhukhuniensis
P. elongatus
A. praecox
P. falcutus
R. rhomboidea
L. rovulata
R. laetans
BaP
Sample name
0.5 mg/mL
0.1 mg/mL
0.02 mg/mL
0 mg/mL

Figure 4(B)

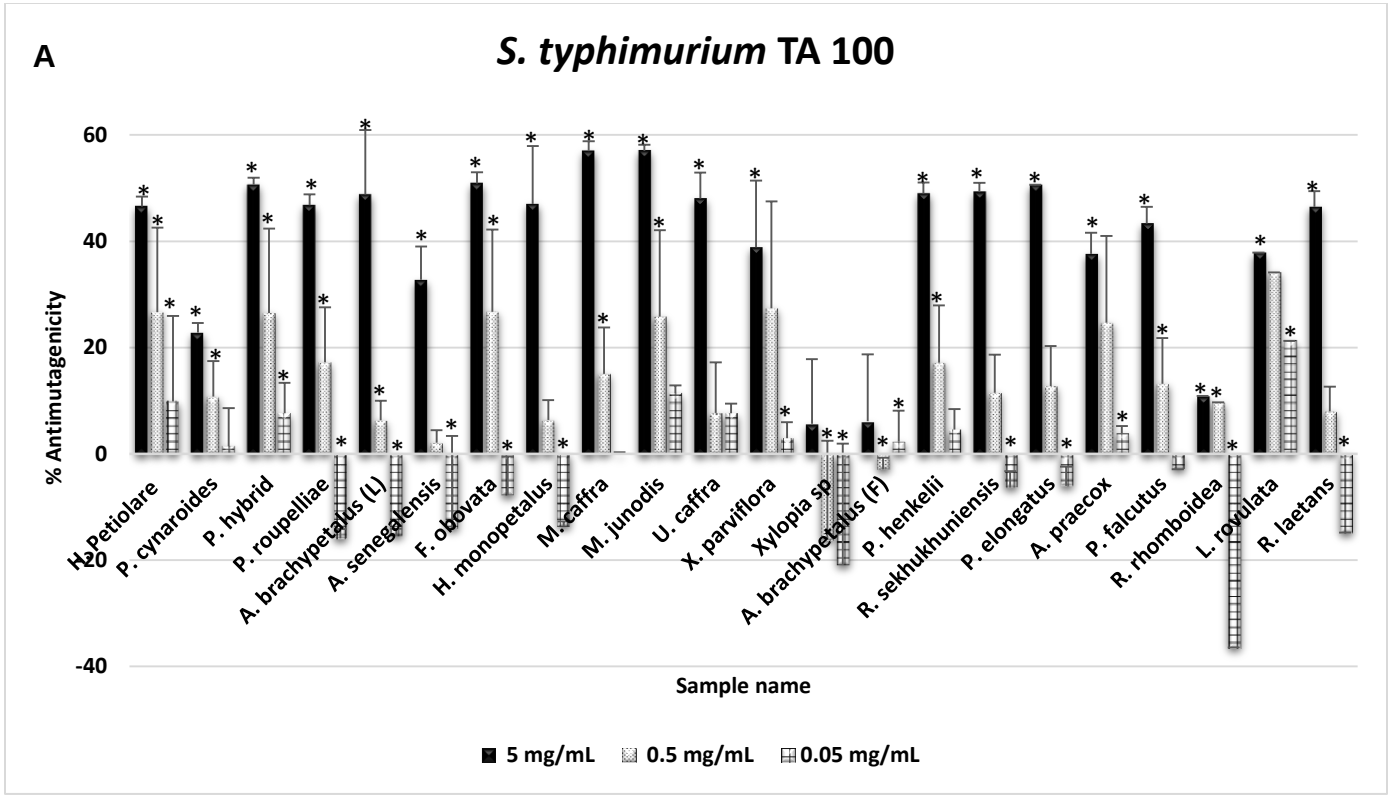

A
S. typhimurium TA 100
% Antimutagenicity
60
40
20
0
-20
-40
H. Petiolare
P. cynaroides
P. hybrid
P. roupelliae
A. brachypetalus (L)
A. senegalensis
F. obovata
H. monopetalus
M. caffra
M. junodis
U. caffra
X. parviflora
Xylopia sp
A. brachypetalus (F)
P. henkelii
R. sekhukhuniensis
P. elongatus
A. praecox
P. falcutus
R. rhomboidea
L. royulata
R. laetans
Sample name
5 mg/mL
0.5 mg/mL
0.05 mg/mL

Figure 5 (A)

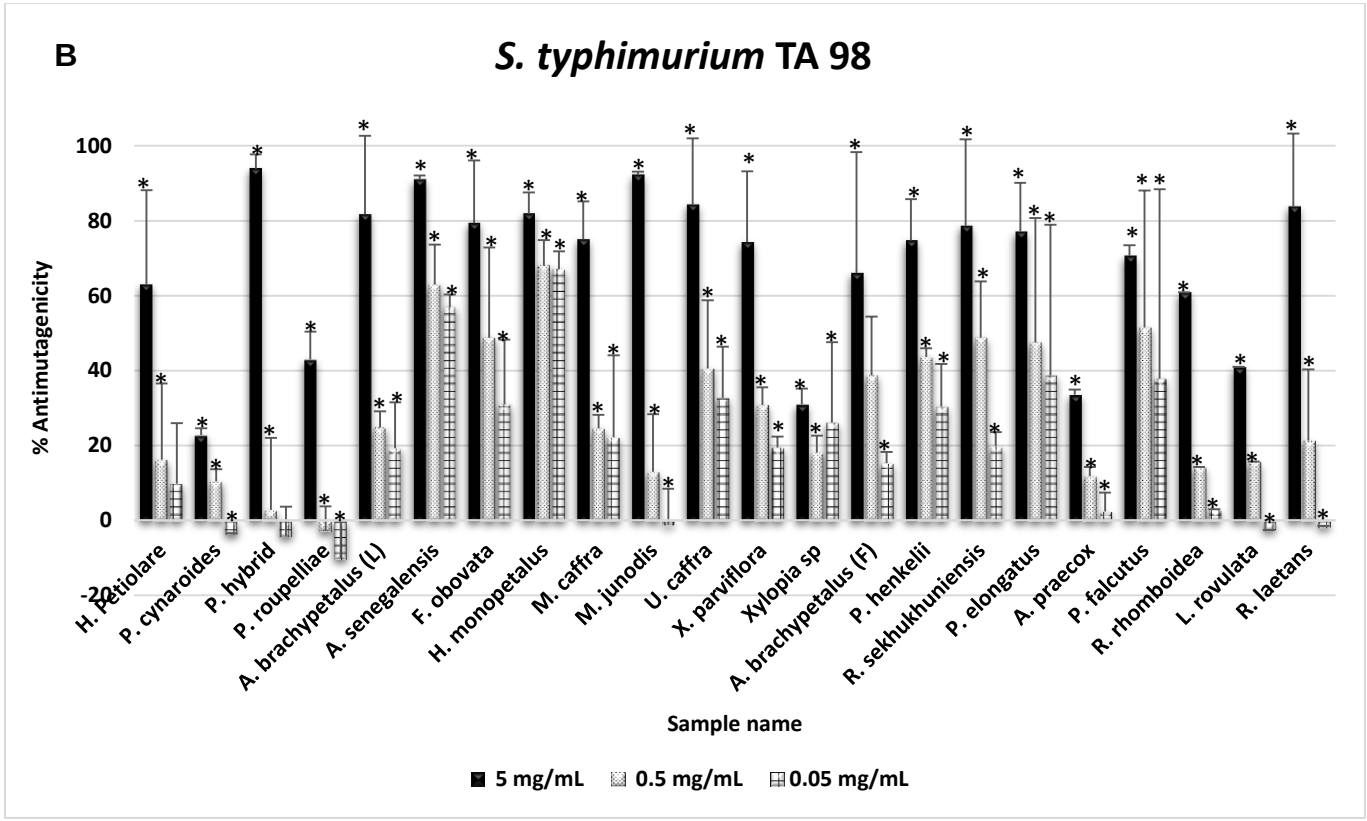

B
S. typhimurium TA 98
% Antimutagenicity
100
80
60
40
20
0
-20
H. Petiolare
P. cynaroides
P. hybrid
P. roupelliae
A. brachypetalus (L)
A. senegalensis
F. obovata
H. monopetalus
M. caffra
M. junodis
U. caffra
X. parviflora
Xylopia sp
A. brachypetalus (F)
P. henkelii
R. sekhukhuniensis
P. elongatus
A. praecox
P. falcutus
R. rhomboidea
L. rovulata
R. laetans
Sample name
5 mg/mL
0.5 mg/mL
0.05 mg/mL

Figure 5(B)

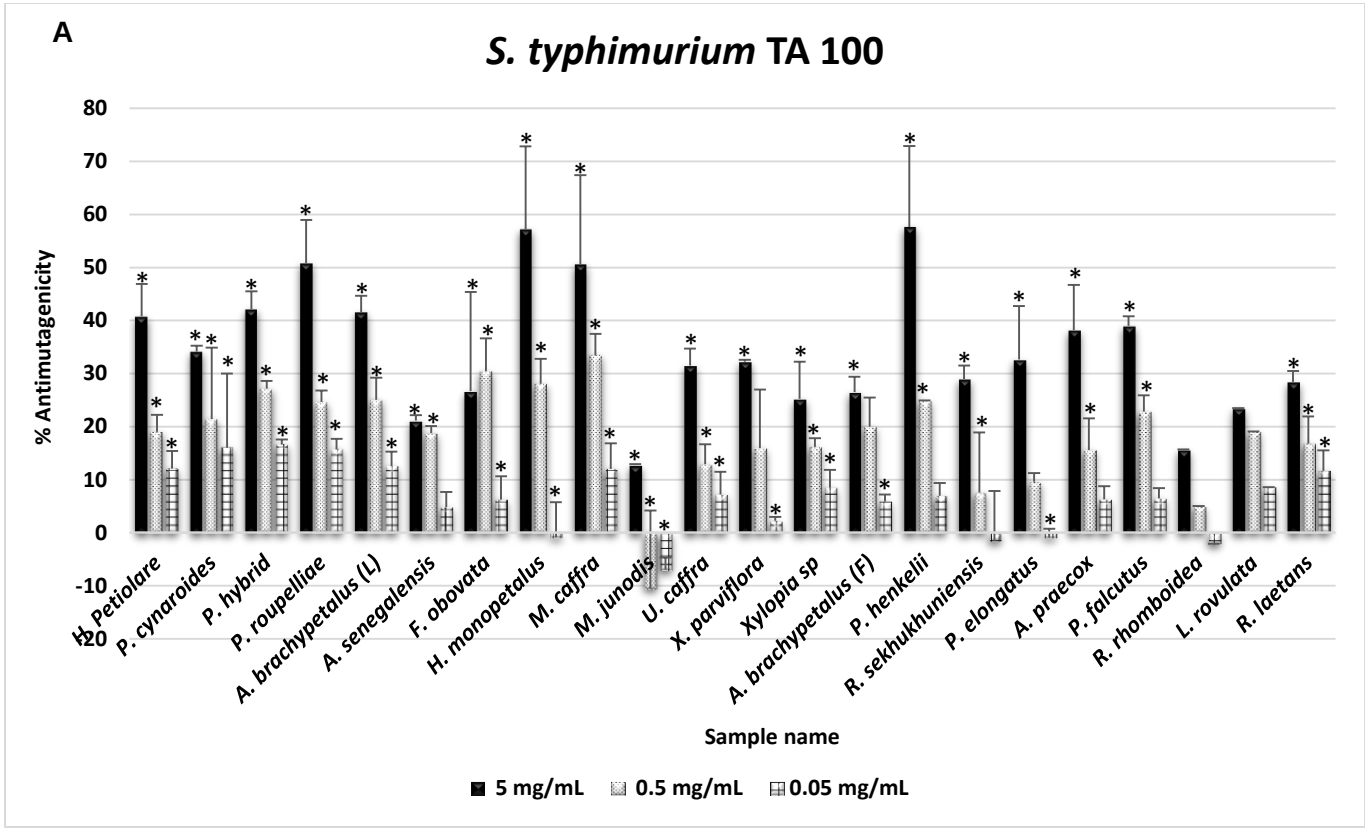

A
S. typhimurium TA 100
% Antimutagenicity
80
70
60
50
40
30
20
10
0
-10
-20
P. Petiolare
P. cynaroides
P. hybrid
P. roupelliae
A. brachypetalus (L)
A. senegalensis
F. obovata
H. monopetalus
M. caffra
M. junodis
U. caffra
X. parviflora
Xylopia sp
A. brachypetalus (F)
P. henkelii
R. sekhukhuniensis
P. elongatus
A. praecox
P. falcutus
R. rhomboidea
L. rovulata
R. laetans
Sample name
5 mg/mL
0.5 mg/mL
0.05 mg/mL

Figure 6(A)

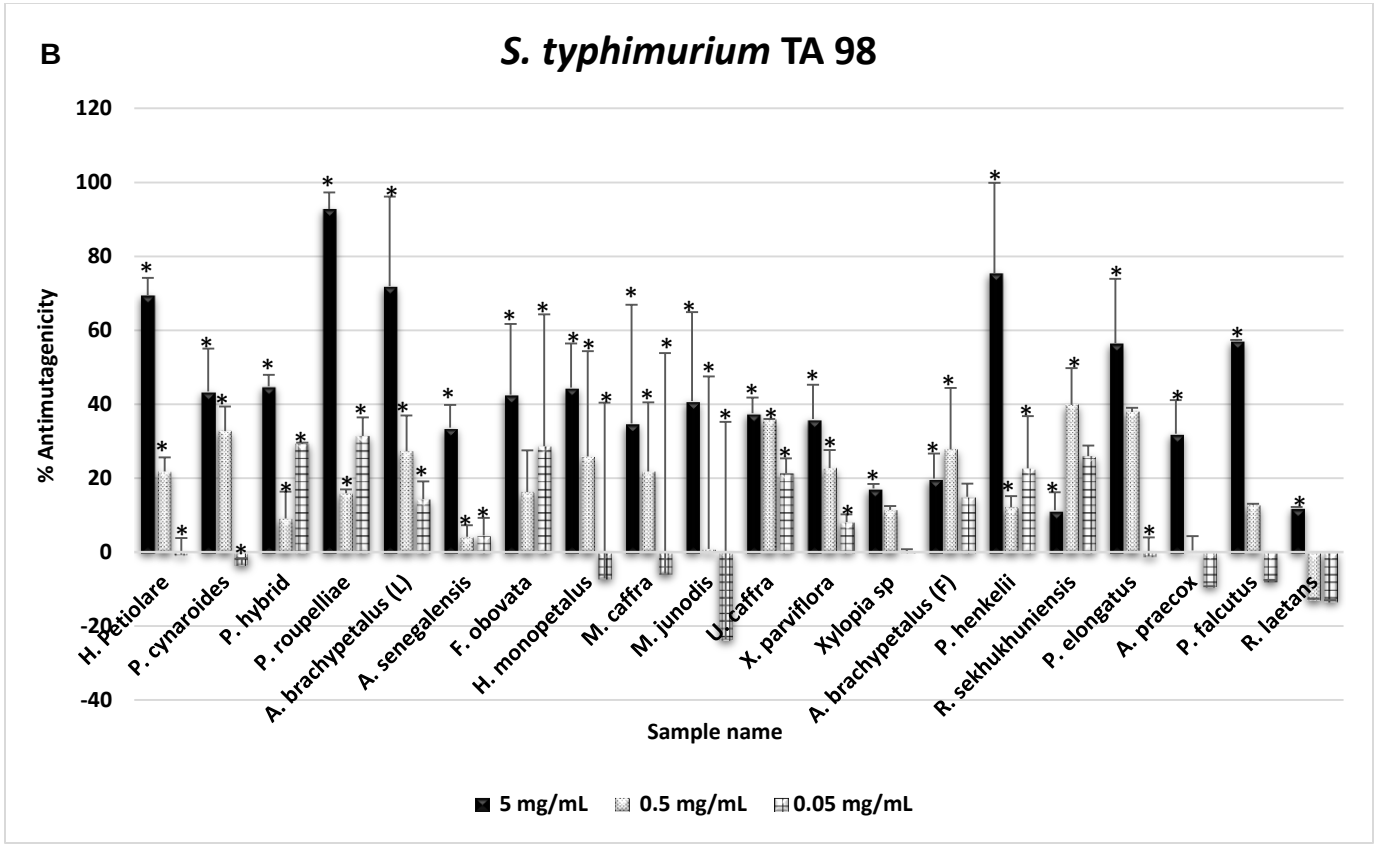

B
S. typhimurium TA 98
% Antimutagenicity
120
100
80
60
40
20
0
-20
-40
H. Petiolare
P. cynaroides
P. hybrid
P. roupelliae
A. brachypetalus (L)
A. senegalensis
F. obovata
H. monopetalus
M. caffra
M. junodis
U. caffra
X. parviflora
Xylopia sp
A. brachypetalus (F)
P. henkelii
R. sekhukhuniensis
P. elongatus
A. praecox
P. falcutus
R. laetans
Sample name
5 mg/mL
0.5 mg/mL
0.05 mg/mL

Figure 6(B)

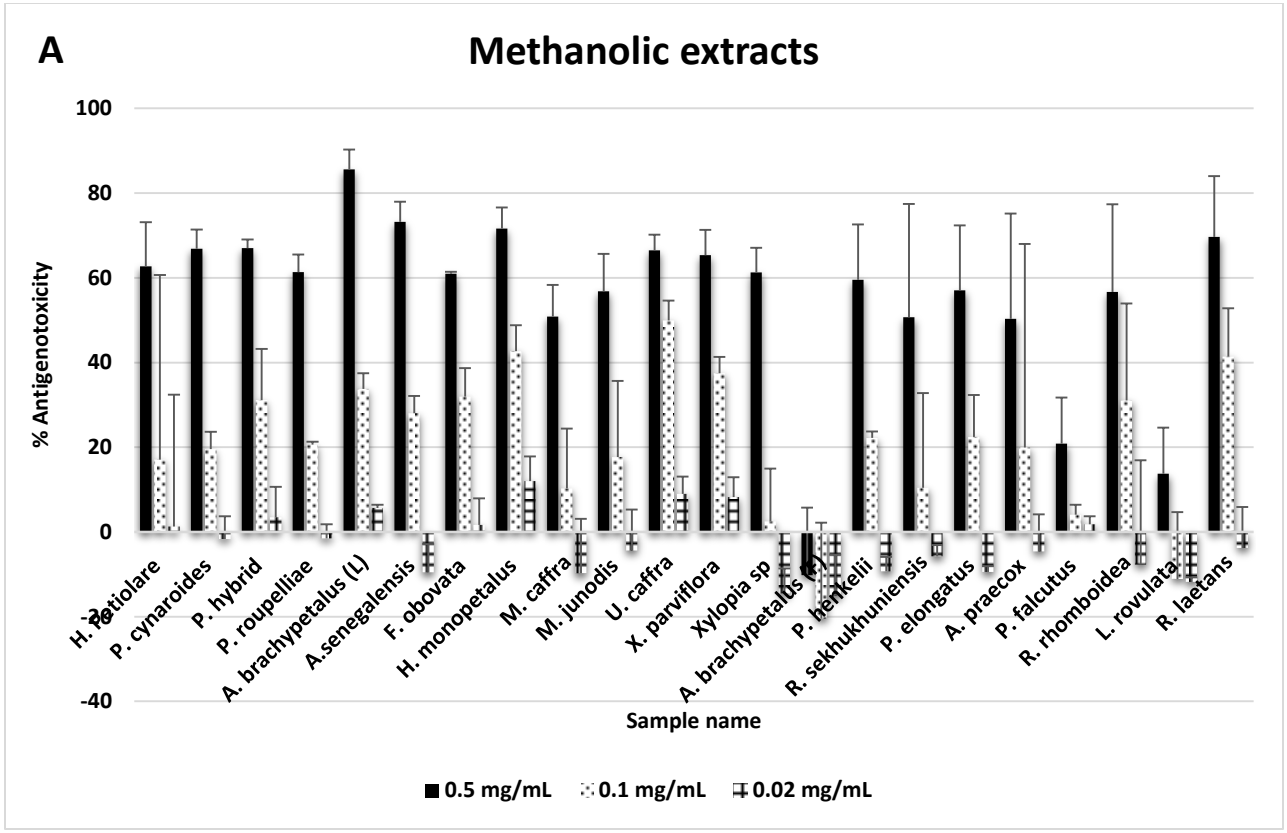
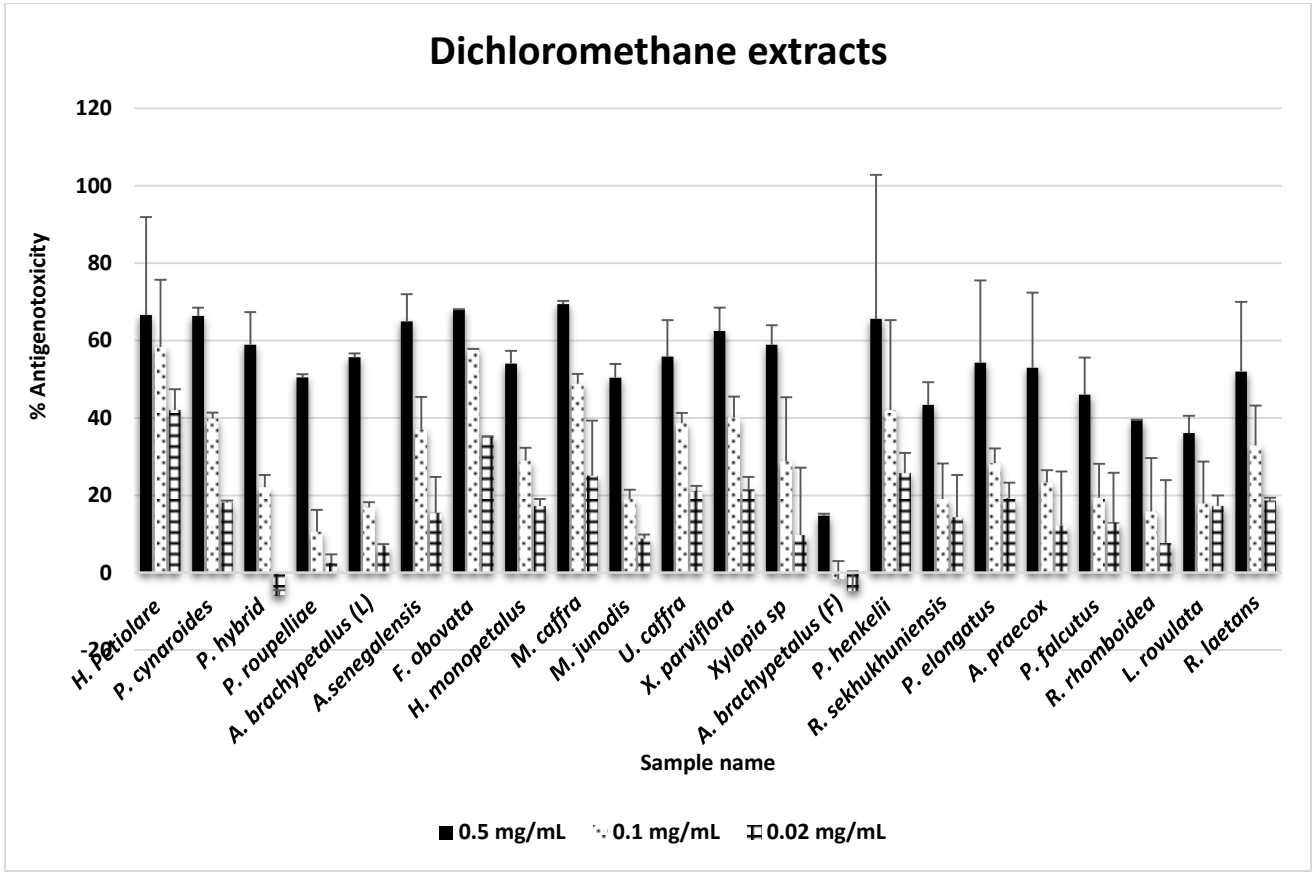

A
Methanolic extracts
% Antigenotoxicity
100
80
60
40
20
0
-40
H. petiolare
P. cynaroides
P. hybrid
P. roupelliae
A. brachypetalus (L)
A.senegalensis
F. obovata
H. monopetalus
M. caffra
M. junodis
U. caffra
X. parviflora
Xylopia sp
A. brachypetalus (K)
P. henkelii
R. sekhukhuniensis
P. elongatus
A. praecox
P. falcutus
R. rhomboidea
L. rovulata
R. laetans
Sample name
0.5 mg/mL
0.1 mg/mL
0.02 mg/mL

Figure 7 (A)



515

516 **Figure 7(B)**