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UNIVERSITY OF CANTERBURY
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Bioactivity of Heartwood Compounds

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FOREST GROWERS RESEARCH

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EXECUTIVE SUMMARY

This report is based on work according to SWP Work Plan No. SWP-WP026. The results are part of Gayatri Mishra's PhD thesis, of which the relevant chapters are attached in the Appendix.

The primary product from plantations of naturally durable eucalypts is ground – durable timber, for example to be used as posts in the agricultural sector (Millen, 2009). The key wood property to ensure a quality product is the natural durability of the wood. It is well known that the natural durability of heartwood is a) highly variable within a species (Page et al., 1997) and b) lower at the centre of a tree (AS5604, 2005; Sherrard and Kurth, 1933). Therefore, NZDFI's strategy is to screen in the 1st instance for high heartwood extractive contents (Li and Altaner, 2016). Heartwood extractives are the key factor, which provide natural durability to wood (Hawley et al., 1924). However, heartwood extractives are numerous and their relative abundance is variable within a species (Haupt et al., 2003; Hillis, 1987; Rowe, 1989). Therefore, it is of interest to identify the most bioactive compounds in the naturally durable eucalypts (Davies et al., 2014; Van Lierde, 2013).
In previous work under the SWP programme (SWP-T037) we have developed a method to quantify individual heartwood compounds. This workplan describes the development of an assay to test the bioactivity of heartwood extractives (Appendix 1) which was subsequently used on a range of heartwood samples representing the genetic variation in the breeding population. By combing the bioactivity assay with the quantification of heartwood extractive compounds the bioactivity of those compounds was investigated (Appendix 2).

Key findings

- A method of testing the bioactivity of *E. bosistoana* heartwood extracts against *T. versicolor* and *C. cerebella* – two fungi commonly used to test wood decay – was developed. Extract DMSO solution was transferred to petri dishes filled with hardened agar. The dishes were inoculated with mycelium. The diameter of the fungus in the agar plates was measured daily. Controls were run with and without DMSO. The test had a CV of less than 11.13% within a batch. Variation in growth rate between different runs needed to be accounted for by normalising the fungal growth rate by that in the controls, i.e. calculating a relative growth rate.
- Ethanol extracts from *E. bosistoana* heartwood were less effective on the white rot *T. versicolor* with a relative growth rate of 83% than the brown rot *C. cerebella* (60%).
- No relationship was found between the growth rates of white rot and brown rot in extracts indicating that different compounds in the extracts inhibited the growth of the two fungi.
- No correlation was observed between the extractive content in the wood and the bioactivity of the extracts against the brown rot. Extractive content in the wood had a negative influence on the bioactivity towards the white rot for the Lawson, but not the Craven Road site. This suggested that the trees with elevated extractive content at the Lawson site deposited more compounds into the heartwood, which were not bioactive against white rot.
- Significant variability was found in the bioactivity of *E. bosistoana* heartwood extracts against white rot and brown rot between the trees. The difference in the relative growth rates of white rot between the sites was small and only significant for white rot. Therefore, the site influence on the bioactivity of the heartwood extracts was small.
- Thirty one compounds were quantified by GC in *E. bosistoana* ethanol extracts of which five were tentatively identified. Variation was present in composition of the extracts between trees and sites. Multivariate (PLSR) analysis identified compounds eluting at 10.2 and 11.5 min (Hexadecanoic acid, TMS ester) to be most related to the bioactivity of the *E. bosistoana* heartwood extracts against the tested white rot and brown rot.
- Significant variation in eight compounds (9.9, 11.2, 11.7, 22.3, 22.8, 23.3, 25.1, 33.3 min), out of 31 compounds was found between the sites. However, these did not have a large effect on the bioactivity of the heartwood extracts towards the two tested fungi.

Limitation

GC was chosen to quantify individual components in the ethanol extracts of *E. bosistoana* heartwood as the technique has a high resolution allowing the separation of numerous compounds - which was demonstrated in this work. However, the technique relies on the compounds entering the gas phase. It is likely that a large proportion of the extract (e.g. larger hydrophilic molecules like tannins) did not meet this criteria even after derivatisation and hence the analysis did not account for these compounds. Oligomeric tannins are known to a) interact with proteins and b) be present in eucalyptus tissue.

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APPENDICES

Appendix 1

Chapter 4
Development of a bioactivity assay for ethanol extracts from *Eucalyptus bosistoana* heartwood

Appendix 2

Chapter 5
Bioactivity of ethanol extracts from *Eucalyptus bosistoana* heartwood

Chapter 4

Development of a bioactivity assay for ethanol extracts from *Eucalyptus bosistoana* heartwood

Objective

The objective of this study was the development of an assay to test the bioactivity of heartwood extractives. The assay was subsequently used on a range of heartwood samples representing a range of the genetic variation in the *E. bosistoana* breeding population to assess the bioactivity of their extract.

4.1 Introduction

Durability of wood is attributed to secondary metabolites known as extractives (Hawley et al., 1924; Taylor et al., 2002). Their abundance is variable within a species (Haupt et al., 2003; Hillis, 1987; Li & Altaner, 2018; Rowe, 1989) and the extractive content has been linked to a timber's resistance to fungal decay (Mohareb et al., 2010; Taylor et al., 2002). However, extractives are composed of numerous bioactive compounds including flavonoids, phenols, phenolic glycosides, saponins and glucosinolates (Quiroga et al., 2001; Rowe, 1989), which in turn vary in their relative quantites within a species. Therefore, both, extractive content and the composition of the extractives contribute to the natural durability of a piece of wood.

In order to assess the bioactivity of an extract (here fungicidal activity) and further identify the most effective components among the numerous compounds, the influence of extractive content needs to be removed. In other words two pieces of wood can have the same extractive content but of different relative composition and therefore be of different natural durability.

The most active compounds in the naturally durable *E. bosistoana*, which inhibited fugal growth have been found to be in the ethanol extracts (Van Lierde, 2013). Therfore, this work focused on the ethanol extracts of *E. bosistoana*.

Numerous methods have been reported to test the bioactivity of plant extracts. Standard susceptibility methods are broth or agar dilution tests and disk diffusion assays (Balouiri et al., 2016). These assays have been used to analyse the antifungal and antimicrobial activity in countless medicinal plant extracts (Bafi-Yeboa et al., 2005; Chandrasekaran & Venkatesalu, 2004; Quiroga et al., 2001). The usage of different non-standardized approaches, inoculum preparation techniques, inoculum sizes, growth media, incubation conditions and endpoint determinations make the comparison between studies difficult (Balouiri et al., 2016). Dilution methods are the most commonly used techniques for the determination of minimal inhibitory or fungicidal concentration (MIC, MFC) values. Either broth or agar dilution methods may be used for quantitative in vitro measurements of the antifungal or

antimicrobial activity in extracts. Other methods that have been developed to determine antifungal or antimicrobial activity of extracts are the time-kill test (time-kill curve), an ATP bioluminescence assay, flow cytofluorometric and thin-layer chromatography (TLC)-bioautography, which includes several bioautographic techniques like agar diffusion, direct bioautography or the agar-overlay assay (Balouiri et al., 2016). These methods can also provide information on the nature of the inhibitory effect (bactericidal or bacteriostatic; time-dependent or concentration-dependent) or the cell damage inflicted to the test microorganism (Balouiri et al., 2016).

The agar dilution method involves the incorporation of different concentrations of extracts into agar medium followed by the application of a fungal or bacterial inoculum to the surface of the agar plate (Wiegand et al., 2008). This method can be used to determine the minimal inhibitory or fungicidal concentration (MIC, MFC) after a defined time of incubation. Most reports on the bioactivity of heartwood extracts against wood decaying fungi employed this method, which are summarised in Table 4.1.

Table 4.1: Summary of reports using the agar dilution method to investigate the bioactivity of wood extracts against wood decaying fungi

Tree species	**Extract type**	**Fungi**	**Reference**
Dipteryx odorata	Heartwood extracts	*Postia placenta*	(Wanschura et al., 2016)
Acacia mangium and *A. auriculiformis*	Heartwood extracts	*Phellinus noxius, P. badius*	(Mihara et al., 2005)
Pinus banksiana and *Pinus resinosa*	Pine bark cones (Stilbenes)	*Trametes versicolor, Phanerochaete chrysosporium, Neolentinus lepideus, Gloeophyllum trabeum and Postia placenta*	(Celimene et al., 1999)
Michelia formosana	Heartwood extracts	*Lenzites betulina, T. versicolor, Laetiporus sulphureus, G. trabeum, Fomitopsis pinicola*	(Wu et al., 2012)

Tree species	Extract type	Fungi	Reference
Calocedrus macrolepis	Heartwood extracts	*L. betulina, T. versicolor, Schizophyllum commune, L. sulphureus, G. trabeum, F. pinicola*	(Yen et al., 2008)
Juniperus virginiana	Heartwood extracts	*T. versicolor, G. trabeum*	(Mun & Prewitt, 2011)
Sequoia sempervirens	Heartwood extracts	*T. versicolor, G. trabeum*	(Davies et al., 2014)
Tectona grandis	Heartwood extracts	*T.versicolor, F. palustris, Rhizopus oryzae, Cladosporium cladosporioides, Chaetomium globosum*	(Lukmandaru, 2017)
Aquilaria crassna	Heartwood extracts	*Fusarium solani*	(Novriyanti et al., 2010).
Taiwania cryptomerioides	Heartwood extracts	*P. noxius*	(Chen et al., 2017)

The broth dilution procedure involves adding extracts at various concentrations to a liquid growth medium and inoculate with a microbial organism (Balouiri et al., 2016). After incubation the turbidity represents the growth of the test organism (Balouiri et al., 2016; Wiegand et al., 2008). Broth dilution assays require fungal spores for analysis. Therefore, these methods are difficult to apply to basidiomycetes, the main wood decaying fungi, as they do not form spores until developed into mycelia (Wanschura et al., 2016). However, they were successfully used to identify a naphthoquinone derivative from teak (*T. grandis*) heartwood extracts with fungicidal activity against *T. versicolor* (Niamké et al., 2012) and used to investigate the fungicidal activity of *Cinnamomum camphora* heartwood extracts against *G. trabeum* and *T. versicolor* (Li et al., 2014).

In disk diffusion assays agar plates are inoculated with a standardized inoculum of the test microorganism. Then, filter paper disks, which have been immersed in the extract solution, are placed on the agar surface. Extracts diffuse into the agar and inhibit the growth of the test microorganism. The gap of the inhibition zone around the filter paper disks is measured (Balouiri et al., 2016). This method is not appropriate to determine MIC or MIF, because it is not possible to quantify the amount of extract

that diffuses into the agar medium (Balouiri et al., 2016). A further difficulty to determine the bioactivity against fungi is that the hyphae can easily overgrow the disk (Wanschura et al., 2016). The method has beed applied to assess antimicrobial activities in crude extracts of *E. globulus* stump wood and bark (Luís et al., 2014).

A bioassay was developed by Kawamura et al. (2011) in which potato dextrose agar medium was dispensed with homogenised hyphae and poured into petri dishes onto which paper disks which had been immersed in extract solutions were placed. This method is a modified version of the disk diffusion assay. Antifungal activity in the heartwood extracts from the *C. porrectum, Mangifera indica* and *Endospermum malaccense* was assessed with this method (Kawamura et al., 2011).

The TLC method was used to screen wood extracts. First compounds in crude extracts were separated on a cellulose TLC plate, which was subsequently inoculated with the test fungi. This method allowed the isolation of bioactive compounds from root extracts of *Vernonanthura tweedieana* (Portillo et al., 2005) or stem extracts of *Rhodiola rosea* (Ming et al., 2005). Kawamura et al. (2004) used this method to isolate four constituents which showed antifungal activity against *T. versicolor* from the ethyl acetate soluble *Gmelina arborea* heartwood extracts.

Bioassays were also performed with the essential oils and leaf extracts from eucalypts. For example agar dilution and disk diffusion assays were used to determine the antibacterial and antifungal activities in the essential oils from *E. camendulensis* (Mouna & Segni, 2014), *E. bicostata, E. cinerea, E. maidenii, E. odorata and E. sideroxylon* (Elaissi et al., 2012), *E. tereticornis* (Maurya et al., 2016), *E. citriodora* (Javed et al., 2012) or the *Eucalyptus* hybrid *E. camaldulensis* × *E. tereticornis* (Varshney et al., 2012). A broth dilution assay was used to test antifungal activity of *E. maculata* leaves extracts (Takahashi et al., 2004).

Antifungal and antimicrobial activities of methanolic extracts from leaves, stems and flowers of *E. torquata and E. sideroxylon* were assessed by a modified disk diffusion assay (Ashour, 2008). Wells were drilled in the agar medium, which was previously inoculated with fungal or bacterial cultures. These wells were filled with extract and inhibition zones were recorded after incubation.

The approach taken in this work was to add a known, constant amount of bioactive substance (i.e. wood or ethanol extract) to agar in a petri dish and then measure the growth rate of wood decaying fungi. The resulting difference in growth rate between extracts should be due to the relative amounts of the individual components in the extract, not the amount of extract.

4.2 Materials

4.2.1 Wood

Heartwood and sapwood were sampled by drilling into the cross section of a disc from a 30 year-old *Eucalyptus bosistoana* tree. The collected drill dust was milled in a Wiley mill to pass a 20 mesh screen. The samples were oven dried at 60°C to stable the moisture content (MC) of ~2%.

4.2.2 Extracts

Heartwood powder was extracted using an Accelerated Solvent Extractor (Dinoex ASE 350, Thermo Scientific) equipped with 33 ml cells. In each run approximately 8 g milled heartwood was extracted with ethanol of HPLC grade as a solvent. The extraction conditions were two cycles at 70°C for 15 min (static time) followed by rinsing with 100% of the cell volume, resulting in approximately 70 ml of extract. The extract solutions were transferred to pre-dried labelled aluminium foil trays of known mass and placed in the fume hood overnight to evaporate the ethanol. The extracts were further dried using a vacuum oven at 60°C to remove moisture. The dried extract was stored in Eppendorf tubes.

4.2.3 Fungi and media

The white rot (*Trametes versicolor*) and brown rot (*Coniophora cerebella*) were obtained from the School of Biological Sciences, University of Canterbury. *T. versicolor* and *C. cerebella* (facultative synonym for *C. puteana*) are listed in European standards EN 113 (1996), EN 350–1 (1994) and by the Australian Wood Preservation Committee (2007) for accessing the durability of wood. The fungi were grown on potato dextrose agar media (Oxoid) containing 4.0 g/L potato extract, 20.0 g/L dextrose and 15.0 g/L agar.

4.3 Method development

4.3.1 Method 1: Heartwood powder mixed with agar

Although difficult to control the extractive content in the experiment, adding wood powder directly to agar has the advantage of not requiring preparing wood extracts, which is a) resource intensive and b) has the potential to alter the chemical nature of the extracts.

The media as well as *E. bosistoana* heartwood and sapwood powder were sterilized in an autoclave at 121°C for 12 min. Wood powders were added in concentrations of 0% (control), 1% $_{w/v}$, 10% $_{w/v}$ and 30% $_{w/v}$ to 50 ml hot media, respectively. Then 10 ml of the suspensions were poured into petri dishes (7 cm diameter). The dishes were inoculated with *T. versicolor* (0.5 mm diameter transplant) in sterile condition in a laminar flow cabinet and stored at 24°C in a temperature-controlled room. The radius of the fungus in each plate was measured every 24 h up to 6 days. The growth rates (cm/h) were calculated by fitting a linear regression for the diameter against time for each dish.

No consistent and pronounced effect on growth rate of wood type or wood concertation was observed (Table 4.2). This was likely caused by media surrounding the wood particles offering the fungi a healthy living environment and the extractive remaining confined to the wood particles. Furthermore, it was difficult to achieve a homogeneous suspension of the wood particles in the media and the mixing procedure was a source for frequently observed contamination of the plates (Figure 4.1a, b).

Table 4.2: Growth rate (cm/h) of *T. versicolor* on agar mixed with *E. bosistoana* wood powder (n = 1)

Heartwood			**Sapwood**			**Control (agar only)**
1%	***10%***	***30%***	***1%***	***10%***	***30%***	0.077
0.066	0.044	0.055	0.056	0.065	0.075	

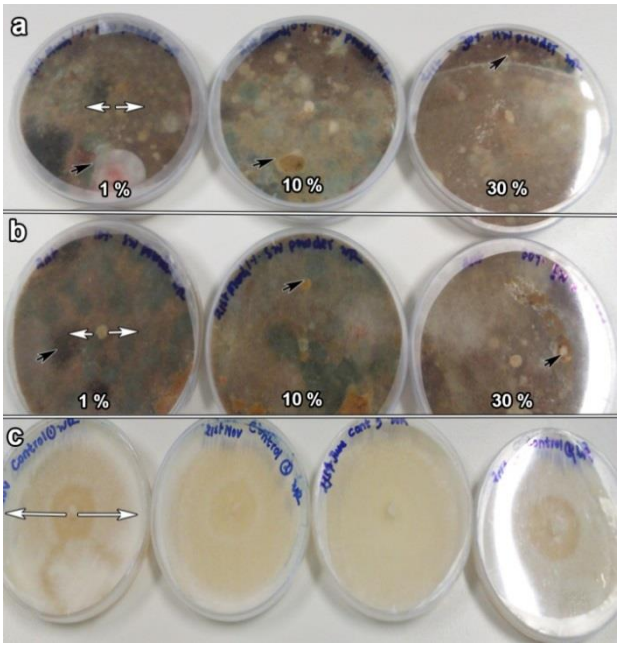

Figure 4.1: Growth of *T. versicolor* on agar mixed with different amounts of (a) heartwood and (b) sapwood powder as well as (c) the controls (agar only) after 168 h. Contamination (black arrows) were observed in the petri dishes with wood powder.

4.3.2 Method 2: Extractives added to the agar

4.3.2.1 *T. versicolor*

To improve mixing of the bioactive compounds with the agar, and therefore create a homogenous living environment for the fungi, a solution of extract was applied to solidified media in petri dishes.

In detail, 250 mg of dried *E. bosistoana* heartwood extract was added to 1 ml of dimethyl sulfoxide (DMSO). The mixture was placed in a shaker overnight. 10 ml media was poured into petri dishes (7 cm diameter) and left to cool and solidify. 10 µl, 25 µl, 50 µl and 100 µl of the extract solution (i.e. 2.5 mg, 6.25 mg, 12.5 mg, 25 mg of extract, respectively) were transferred into the petri dishes. Controls were prepared with 0 µl, 10 µl, 25 µl, 50 µl and 100 µl DMSO. The extracts and DMSO were spread on the surface of the media using disposable spreaders (Thermo Fischer). The plates were then inoculated with *T. vesicolor* in sterile condition in a laminar flow cabinet and stored at 24°C to monitor the growth of the fungi for 7 days at 24 h intervals. The radius of the fungi in each petri dish was measured using

a pair of digital callipers until the fungi covered the dish. The growth rates (cm/h) were calculated as described above. Observations (Table 4.3) suggested that:

a) DMSO of up to 100 µl added to 10 ml of agar did not interfere with the growth of *T. versicolor*.
b) The growth of fungi was not significantly inhibited by the used amount of extracts as the growth rate of the fungus exposed to *E. bosistoana* heartwood extracts did not conclusively differ from those without extracts. However, a slightly slower growth was observed with 100 µl extracts.
c) No contamination was observed, indicating that maintaining sterile conditions was easier when applying an extract solution compared to adding wood powder (Figure 4.2).

Table 4.3: Growth rate (cm/h) of *T. versicolor* on 10 ml agar dosed with *E. bosistoana* heartwood extract and DMSO (n = 1)

Extract				**DMSO + Agar**				**Control (agar only)**
10 µl (2.5 mg)	***25 µl (6.25 mg)***	***50 µl (12.5 mg)***	***100 µl (25 mg)***	***10 µl***	***25 µl***	***50 µl***	***100 µl***	
0.018	0.017	0.015	0.014	0.017	0.017	0.016	0.016	0.016

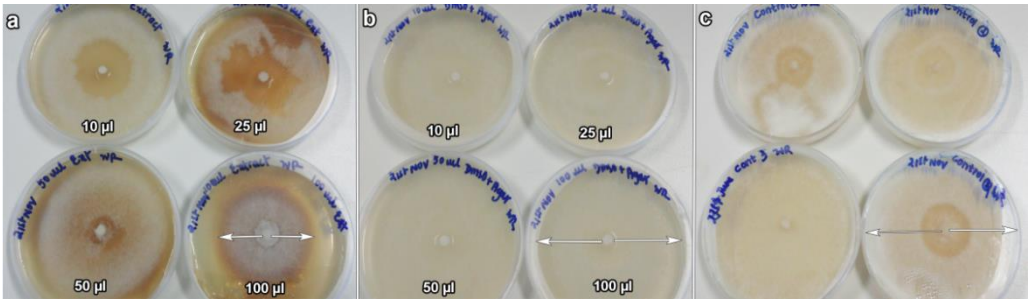

Figure 4.2: Growth of *T. versicolor* on agar (a) with *E. bosistoana* heartwood, (b) DMSO and (c) only agar after 168 h.

Consequently, higher amounts of *E. bosistoana* heartwood extracts were tested. This was achieved by adding larger amounts of the extract solution (100 µl, 500 µl, 1000 µl and 1500 µl) and DMSO to the agar. Observations (Table 4.4) suggested that:

a) 500 µl DMSO and above affected the growth of the white rot *T. versicolor*.
b) The growth of *T. versicolor* was completely inhibited with 1500 µl extract solution added to the petri dish.
c) Heartwood extractives slowed the growth of the fungus at each of the tested concentrations.

Table 4.4: Growth rate (cm/h) of *T. versicolor* on 10 ml agar dosed with *E. bosistoana* heartwood extract and DMSO (n = 1)

Extract				**DMSO + Agar**				**Control (only agar)**
100 µl (25 mg)	***500 µl (125 mg)***	***1000 µl (250 mg)***	***1500 µl (375 mg)***	***100 µl***	***500 µl***	***1000 µl***	***1500 µl***	
0.017	0.009	0.004	no growth	0.024	0.015	0.010	no growth	0.029

100 µl DMSO solution containing 25 mg of extract was chosen to assess the bioactivity of extracts against the white rot *T. versicolor*.

4.3.2.2 *C. cerebella*

To develop an assay for assessing the bioactivity of extracts against the brown rot *C. cerebella* the same procedures as described in 4.3.2.1 were followed.

C. cerebella was growing slower and more sensitive to the *E. bosistoana* heartwood extract (Table 4.5). The growth of the brown rot was completely inhibited at the used extract dosages. Like for the white rot DMSO inhibited the growth of *C. cerebella* above 100 µl and stopped growth when adding 1500 µl DMSO. Again no contamination was observed (Figure 4.3).

Table 4.5: Growth rate (cm/h) of *C. cerebella* on 10 ml agar dosed with *E. bosistoana* heartwood extract and DMSO (n = 1)

Extract				**DMSO + Agar**				**Control (only agar)**
100 µl (25 mg)	***500 µl (125 mg)***	***1000 µl (250 mg)***	***1500 µl (375 mg)***	***100 µl***	***500 µl***	***1000 µl***	***1500 µl***	
no growth	no growth	no growth	no growth	0.007	0.006	0.005	no growth	0.007

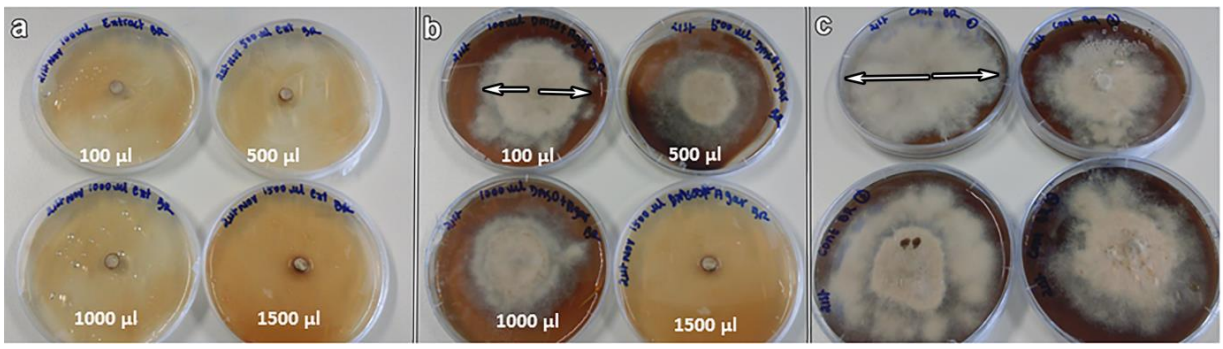

Figure 4.3: Growth of *C. cerebella* on agar (a) with *E. bosistoana* heartwood, (b) DMSO and (c) only agar after 432 h.

The dosage was optimised by diluting 300 µl of the above described *E. bosistoana* heartwood extract solution with 500 µl DMSO, resulting in a concentration of 93.75 mg/ml. 50 µl (i.e. 4.7 mg extract) and 100 µl (i.e. 9.4 mg extract) of this solution as well as corresponding amounts of DMSO were transferred to petri dishes and inoculated with *C. cerebella* as described above.

Consistent with the experiment above, the growth of *C. cerebella* was not affected by the amount of added DMSO (Table 4.6, Figure 4.4). The growth of the fungus was inhibited with 50 µl extract (~4.7 µg).

50 µl extract solution containing 4.7 µg *E. bosistoana* heartwood extract in DMSO were deemed suitable to assess the bioactivity against *C. cerebella*.

Table 4.6: Growth rate (cm/h) of *C. cerebella* on agar dosed with *E. bosistoana* heartwood extract and DMSO (n = 1)

Extract		**DMSO + Agar**		**Control (only agar)**
50 µl (4.7 mg)	***100 µl (9.4 mg)***	***50 µl***	***100 µl***	
0.0031	0.0033	0.0056	0.0058	0.0059

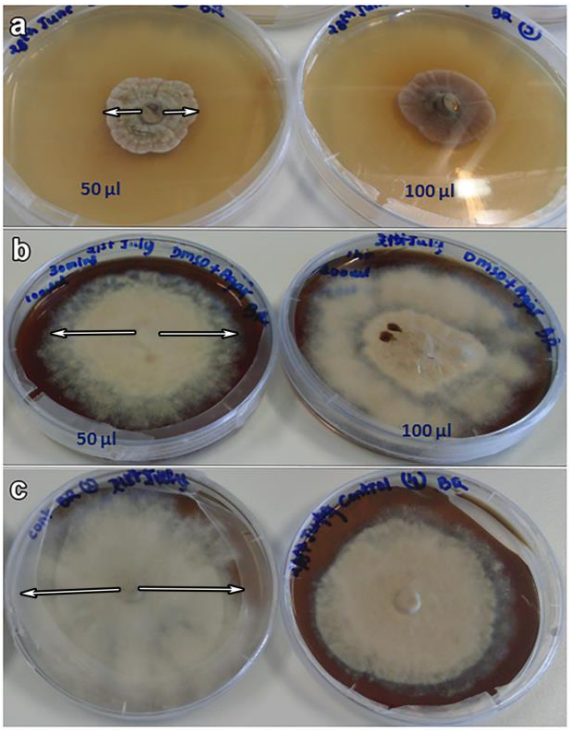

Figure 4.4: Growth of *C. cerebella* on agar (a) with *E. bosistoana* heartwood, (b) DMSO and (c) only agar after 432 h.

4.3.2.3 Diffusion of DMSO into agar

DMSO was found to affect the growth of the test fungi (Table 4.3, 4.4). After applying the DMSO extract solutions to the agar surface the DMSO starts to diffuse into agar, reducing the surface concertation. To avoid excessively high DMSO concentrations at the time of inoculation, different time intervals between extract application and inoculation were investigated. 100 µl and 500 µl DMSO was transferred to the petri dishes as described above and left for 15 min, 30 min, and 1 h to diffuse into the media. The plates were inoculated with *T. versicolor* and assessed as described above.

Again, while 100 µl DMSO did not affect the growth of the fungi, the higher concentration did (Table 4.7). Allowing at least 15 min for DMSO to diffuse into the agar was sufficient to obtain growth rates similar to that on pure agar. Again, no contamination was observed (Figure 4.5)

Table 4.7: Effect of diffusion time on growth rate (cm/hr) of *T. versicolor* exposed to 100 µl and 500 µl DMSO on 10 ml agar (n=1)

100 µl DMSO			**500 µl DMSO**			**Control (only agar)**
DMSO exposure time						
15 mins	***30 mins***	***1 hr***	***15 mins***	***30 mins***	***1 hr***	
0.013	0.013	0.013	0.009	0.010	0.009	0.015

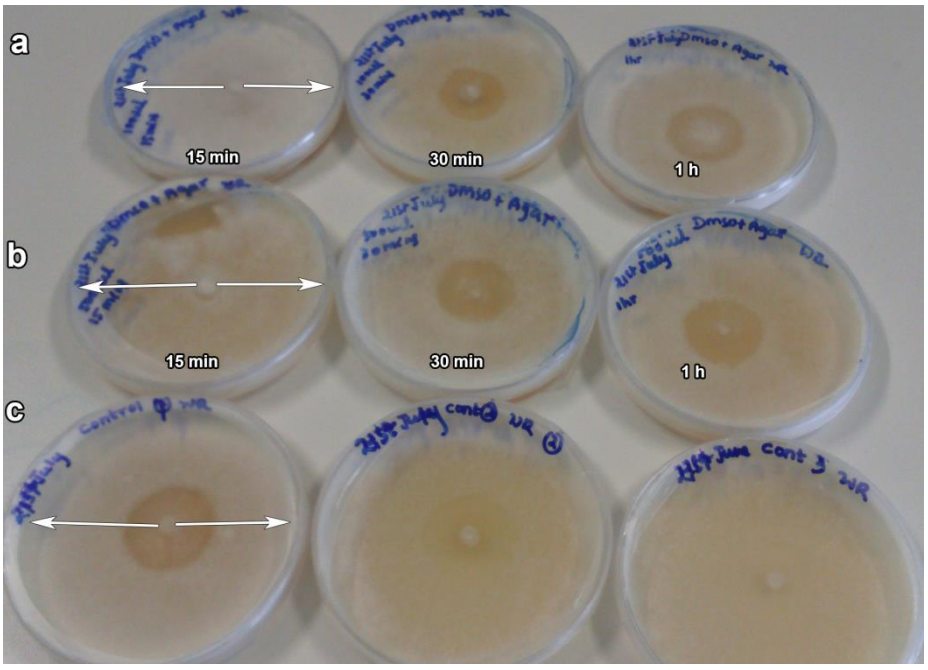

Figure 4.5: Growth r of T. versicolor exposed to (a) 100 µl and (b) 500 µl DMSO at different intervals and (c) only agar after 168 h.

4.3.3 Repeatability of the bioactivity test

To investigate the repeatability of the above described method, five solutions of the same *E. bosistoana* heartwood extract were prepared by adding 0.05 g dried extract to 0.2 ml DMSO. These were assessed for their bioactivity against white rot. 100 µl of the extract solutions were transferred to petri dishes. For brown rot tests, five solutions of 0.018 g dried extract added to 0.2 ml DMSO were prepared. 50 µl of these extract solutions were transferred to petri dishes. The subsequent procedure was as described above.

The diameter of the fungi increased linearly with time (Figure 4.6, 4.7). However, the fungi needed a certain lag time to establish, where no growth was observed. Restricting the calculation of the growth rate to the linear growth phase improved the correlation coefficients from 0.87-0.95 to > 0.95 for white rot (Figure 4.6) and 0.90-0.95 to > 0.95 for brown rot (Figure 4.7). Once the fungi came close the walls of the petri dishes the growth rate slowed again (data not shown).

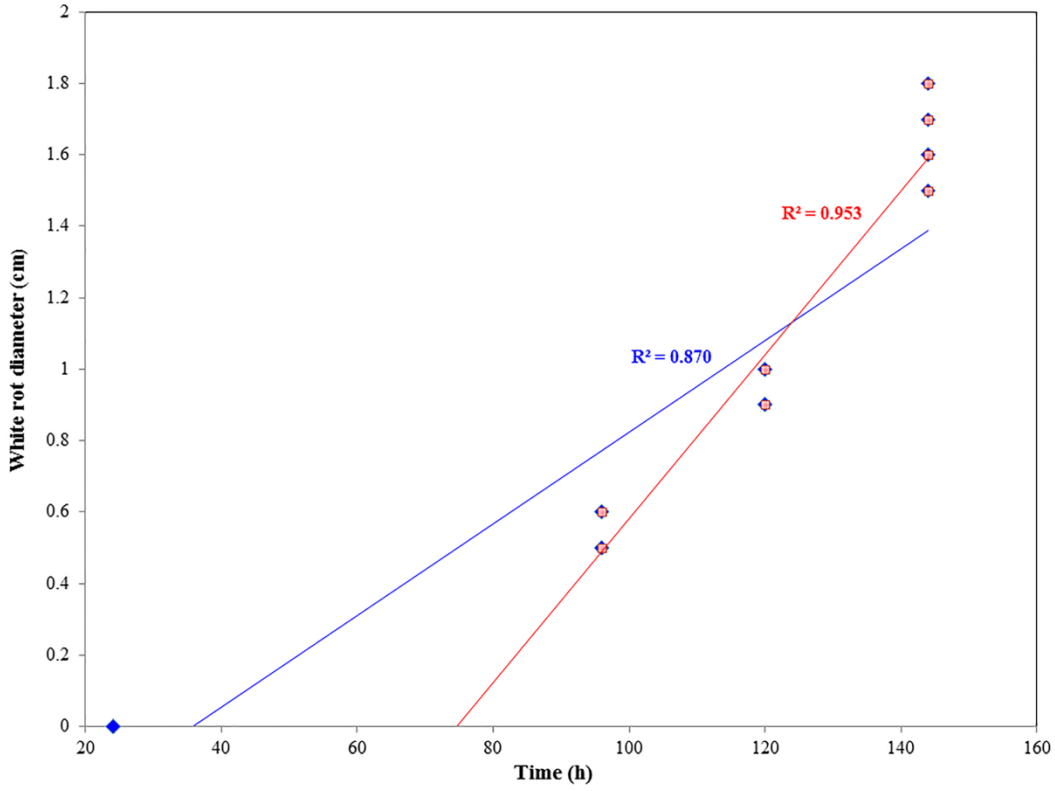

Figure 4.6: Fitting linear growth rates to white rot grown in petri dishes with heartwood extracts for 144 h; n = 5. Excluding a lag phase in which the fungi established (red) increased the linear fit compared to conserving all data (blue).

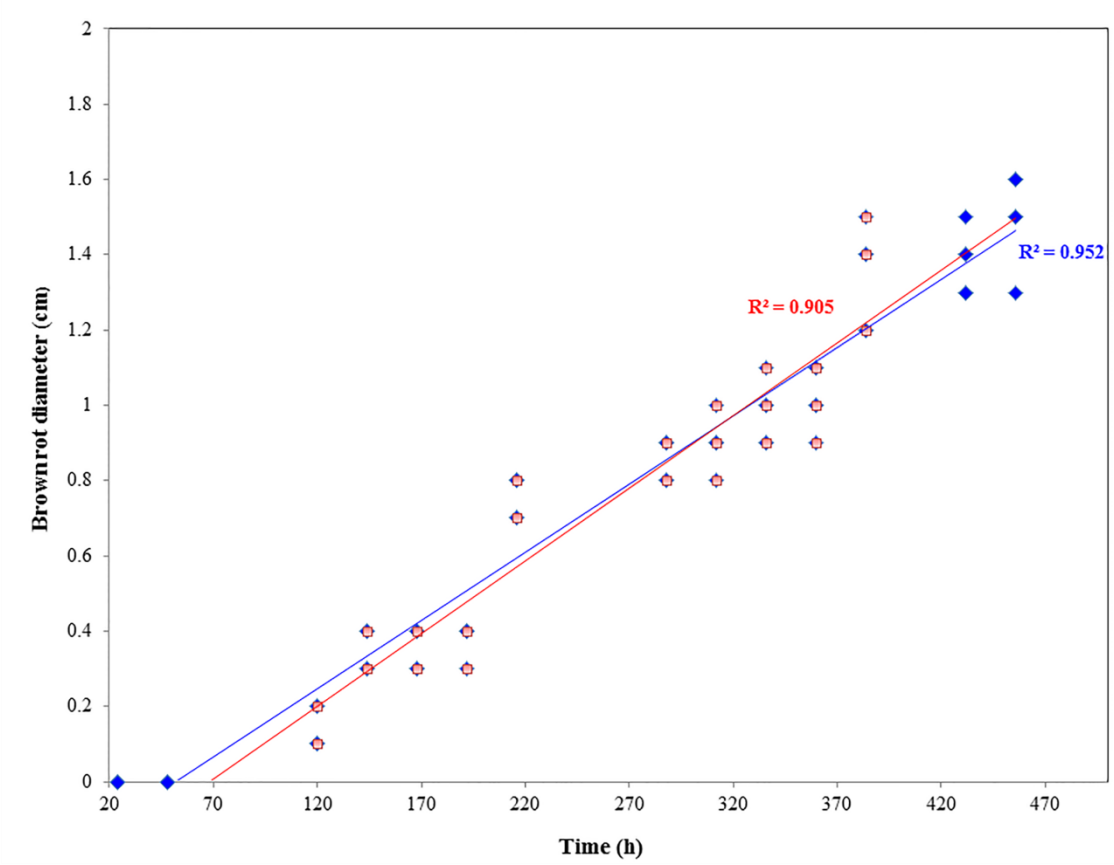

Figure 4.7: Fitting linear growth rates to brown rot grown in petri dishes with heartwood extracts for 456 h; n = 5. Excluding a lag phase in which the fungi established (red) increased the linear fit compared to conserving all data (blue).

The coefficient of variation (CV) was similar for the two fungi (Table 4.8, 4.9). The repeatability of the growth of the fungi on the controls was better (CV 3.2 to 5.4%) than for the plates with extracts (CV 11.1 and 9.6%). This suggested that while the fungi expressed consistent growth the preparation of the extract solutions or their application to the agar introduced some variability. The repeatability of the test was generally good but to get a more precise bioactivity measure the heartwood extracts should be measured as replicates. This would also account for potentially contamination, which had not occurred in these preliminary tests.

Table 4.8: Mean growth rate (cm/h), standard deviation (SDEV) and coefficient of variation (CV) of *T. versicolor* on 10 ml agar dosed with *E. bosistoana* heartwood extract and DMSO (n = 5)

Extract			**DMSO + Agar**			**Control (only agar)**		
Mean	***SDEV***	***CV (%)***	***Mean***	***SDEV***	***CV (%)***	***Mean***	***SDEV***	***CV (%)***
0.0229	0.0026	11.1	0.0363	0.0011	3.2	0.0346	0.0011	3.3

Table 4.9: Mean growth rate (cm/h), SDEV and CV of *C. cerebella* on 10 ml agar dosed with *E. bosistoana* heartwood extract and DMSO (n = 5)

Extract			**DMSO + Agar**			**Control (only agar)**		
Mean	***SDEV***	***CV (%)***	***Mean***	***SDEV***	***CV (%)***	***Mean***	***SDEV***	***CV (%)***
0.0039	0.0004	9.6	0.0072	0.0003	3.6	0.0068	0.0004	5.4

The differences in growth rate of the white rot fungus between the tests on the same medium (Table 4.3, 4.4, 4.8) indicted that the growth was also affected by variables not controlled in the test set up. This can include variations in the room temperature or the vitality of the fungus inoculum. These differences can be accounted for by normalising for the control growth rate, i.e. calculating the relative growth rate, which expresses by how much fungal growth was inhibited by the treatment. This a common parties for such tests (Balouiri et al., 2016)

Relative growth rate (%) = Ds / Dc × 100 ---------- Equation 1

Where, Ds is the diameter of growth in the plate containing the tested antifungal agent and Dc is the diameter of growth in the control plate.

4.4 Conclusion

A method of testing the bioactivity of *E. bosistoana* heartwood extracts against *T. versicolor* and *C. cerebella* was developed. 100 µl for white rot and 50 µl for brown rot of extract DMSO solution (250 mg/ml for white rot and 93.75 mg/ml for brown rot) were transferred to 7 cm petri dishes filled with 10 ml hardened agar. The extracts were spread on the surface of agar media using disposable spreaders. The dishes were inoculated with 0.5 mm mycelium after 15 minutes in sterile condition in laminar hood and stored at 24˚C. The diameter of the fungus in the agar plates was measured daily until it filled the petri dish. Controls were run with (100 µl for white rot and 50 µl for brown rot) and without (agar only) DMSO. Tests were done as replicates of five. The growth rates (cm/h) were calculated by fitting a linear regression for the diameter against time for each dish, considering only the linear growth of the fungi. Good sterility i.e. no contamination was observed and the test had a CV of less than 11.1% within a batch. Variation in growth rate between different runs needed to be accounted for by normalising the fungal growth rate by that in the controls, i.e. calculating a relative growth rate.

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Chapter 5

Bioactivity of ethanol extracts from *Eucalyptus bosistoana* heartwood

5.1 Introduction

In Eucalypts, heartwood extractives are predominately comprised of polyphenols such as tannins, the esters of gallic and ellagic acids or cinnamic acid derivatives (Conde et al., 1995; Hillis, 1971, 1991; Rudman, 1964). The quantity and composition of these extractives varies between species, between individual trees of a species and within a tree (Hillis, 1987; Taylor et al., 2002). The natural durability of timber does not necessarily correspond to extractive concentrations (Taylor et al., 2002). This suggests that also the composition of the extractive influences the durability of timber.

The variability in extractive content and composition, which exists between trees of a species is influenced by environmental and genetic factors. Variation in extractive content in *E. globulus* heartwood was observed between trees and sites (Morais & Pereira, 2012). Mosedale et al. (1996a) reported variation in the concentration of ellagitannins in the heartwood of oak species (*Quercus robur* and *Q. petraea*) between forests and found that the ellagitannins content is under strong genetic control (Mosedale et al., 1996b). Significant variation in the heartwood compounds (β-thujaplicin, ratio between γ- and β-thujaplicin and methyl thujate) was detected within and between western red cedar (*Thuja plicata*) within and between regions (Daniels & Russell, 2007).

Variation in the composition of heartwood flavonoids in regional populations of sweet cherry (*Prunus avium*) was reported under genetic and environmental control while age of the trees had no effect on the distribution of flavonoid aglycones (Vinciguerra et al., 2003). This lines with reports by Fries et al. (2000), who suggested, the phenotype of extractive compounds can be influenced by environmental conditions such as soil fertility. Site soil composition was reported to influence the amount of lipophilic extractives in *E. dunnii* and *E. grandis* (Kilulya et al., 2014). Also variation in decay resistance among individual trees of a single species has been reported to be extensive due to genetic and environmental factors (Yu et al., 2003).

Eucalyptus species were classified into four groups, based on the extractive composition (Hillis, 1991). Condensed tannins are characteristic in the heartwood of a group with pink to red brown heartwood (e.g. *E. camaldulensis*, *E. grandis*, *E. marginata*), while hydrolysable tannins are characteristic for a group with pale-light coloured outer heartwood (e.g. *E. delegatensis*). A third group with brown heartwood (e.g. *E. wandoo*) features stilbenes, ellagitannins and ellagic acids and a

fourth group with heartwood that has a greasy feel (e.g. *E. maculata*, *E. microcorys*) contain eucalenol and steroids in addition to ellagic acid and ellagitannins.

The quantification of individual extractive components is difficult because the numerous compounds have different physical and chemical properties. However, selective identification and quantification of some compounds are feasible by chromatographic methods such as Gas Chromatography (GC) and High Performance Liquid Chromatography (HPLC) combined with mass spectrometry (MS). Literature shows that the GC is now the most common technique for the characterisation of wood extractives (Fernandez et al., 2001; Gutiérrez et al., 1998; Sitholé et al., 1992). For GC analysis heartwood extractives often need to be derivatized by methylation, silylation or acetylation to allow analysis (Davies et al., 2014; Fernandez et al., 2001). Compounds identified in the heartwood extracts of *Eucalyptus* spp. by various chromatographic techniques are summarised in Table 5.1.

Table 5.1: Compounds identified in the heartwood extracts of Eucalyptus species by different chromatographic techniques

Species	**Compounds**	**Analytical technique**	**Reference**
E. marginata	Catechin, Proanthocyanidins, Ellagitannins, Stilbenes	Paper and liquid chromatography	(Hillis & Carle, 1962)
E. wandoo	Resveratrol (3,5, trihydroxystilbene), 3 β-D-glucoside	Paper chromatography	(Hathway, 1962)
E. sideroxylon	Resveratrol(3,5, trihydroxystilbene), Ellagic acid, Gallic acid, Catechin, Ellagitannins	Two dimensional paper chromatography	(Hillis & KoichiroIsoi, 1965)
	Resveratrol (resveratrol-β-glucoside), 3,3′-di- and 3,3′,4-tri-*o*-methylellagic acids and their glucosides	Paper chromatography with NMR spectroscopy	(Hillis et al., 1974)
E. sieberiana	Catechin	Paper chromatography with NMR spectroscopy	(Hillls & Carle, 1959)
E. delegatensis	Ellagitannins, Ellagic acid , 2,3- and 4,6-(hexahydroxydiphenoyl)-glucose,3- and 4,6-(hexahydroxydiphenoyl)-glucose, Pedunculagin, two complex tannins of incompletely determined structure	Paper chromatography with NMR spectroscopy	(Hillls & Carle, 1959; Seikel & Hillis, 1970)
E. polyanthemos	3,4,3'-Tri-O-methylellagic acid 4'-glucoside, Leucoanthocyanins	Thin layer chromatography	(Hillis & Yazaki, 1973)

Species	Compounds	Analytical technique	Reference
E. grandis *E. dunnii*	Octanoic acid, Decanoic acid, Octanedioic acid, 1-dodecanol, Dodecanoic acid, Nonanedioic acid, Tridecanoic acid, 12-methyltridecanoic acid, Tetradecanoic acid, 9-methyltetradecanoic acid, 5-octadecenoic acid, Pentadecanoic acid, 1-hexadecanol, 11-hexadecenoicacid, Heptadecenoic acid, 2-hydroxyhexadecanoic acid, 1-octadecanol,9,12-octadecadienoic acid, 9-octadecenoic acid, Octadecanoic acid, Nonadecanoic acid, Eicosanoic acid, Heneicosanoic acid, 1-tricosanol, Docosanoic acid, Tricosanoicacid, 2-hydroxydocosanoic acid; Tetracosanoic acid, Pentacosanoic acid; 2-methoxy-tricosanoic acid, Hexacosanoic acid, Docosanedioic acid, Stigmasta-3,5-diene, Sitosterol, Stigmastanol, Stigmasta-3,5-dien-7-one; Stigmast-4-en-3-one.	Gas chromatography-mass spectrometry (GC-MS)	(Kilulya et al., 2014)
E. camaldulensis	Polyphenol (Catechin), Resin acids, Fatty acids, Glycerides, Triterpenes, Steryl esters, Sterols and fatty alcohols, Stilbenes, Flavanols, Monosaccharides and cyclic polyols	GC-MS	(Benouadah et al., 2018)
E. wandoo	Reveratrol glucoside Aglucone	Paper chromatography	(Hathway & Seakins, 1959)
E. globulus *E. regnans*	Ellagitannins, Ellagic acid, Gallic acid, Catechin, 3-O-methylellagic acid-4'-rhamnoside, Chlorogenic acid	Thin layer chromatography	(Yazaki & Hillis, 1976)

Species	Compounds	Analytical technique	Reference
E. citriodora	Trans-calamenene, T-muurolol, α - cadinol, 2β-cadinol, 4-hydroxy-3,5-dimethoxybenzaldehyde, Dimethoxy benzoic acid, Linoleic acid, Squalene, Tocopherol, Erythrodiol, Morolic acid, Betulonic acid, Sesamin	Thin layer chromatography	(Lee & Chang, 2000)
E. astrigens	Stilbenes	Paper and liquid chromatography	Hillis and Carle (1962)

Van Lierde (2013) showed that ethanol extracts from *E. bosistoana* heartwood was found to have highest fungicidal activity against *Trametes versicolor* and *Gloeophyllum trabeum*. In order to assess the bioactivity of an ethanol extract and further quantify the most effective component among the numerous compounds by gas chromatography, the influence of extractive content needs to be removed. In other words two pieces of wood can have the same extractive content but of different relative composition (or vice versa) and therefore different natural durability.

In this study the bioactivity of 91 *E. bosistoana* extracts from two different sites was investigated. A constant amount of extract was added to agar in a petri dish and then the growth rate of wood decaying fungi on extracts was compared to the growth rate on pure agar. The resulting difference in growth rate between extracts was due to the relative amounts of the individual components in the extract, not the amount of extract. Variation in extractive components was quantified by gas chromatography for all 91 trees and subsequently correlated to the relative growth rates.

5.2 Materials and methods

5.2.1 Material

91 Heartwood disks were obtained from 7 year-old *E. bosistoana* breeding trials planted in 2009. These trees were grown at two different sites, Lawson and Craven Road (41°26´S, 173°56´E and 41°43´S, 173°02´E) in Marlborough, New Zealand. Two wood rot fungi, the white rot (*Trametes versicolor*) and the brown rot (*Coniophora cerebella*), obtained from the School of Biological Sciences, University of Canterbury were used for the fungal assays.

Heartwood powder was prepared and extracted with ethanol as described in chapter 4. The mass of each oven-dried extract was measured and the extractive content was calculated on a dry mass basis. Data for extractive content and relative growth rates of white rot (*T. versicolor*) and brown rot (*C. cerebella*) in heartwood extracts of 91 samples is shown in the Table 5.2. The largest variation was found for extractive content, with the samples from the Craven Road site having a lower extractive

content. Brown rot was more susceptible to the *E. bosistoana* heartwood extracts and the effect was also more variable than for white rot.

Table 5.2: Summary statistics of extractive content in *E. bosistoana* heartwood from 7 year-old trees and the effect of ethanol extracts on the relative growth rates of the white rot *Trametes versicolor* and the brown rot *Coniophora cerebella* for 2 sites. CV: Coefficient of variation, n: number of samples

	Lawson (n = 33)			**Craven Road (n = 58)**		
	Relative growth rate (%) (white rot)	**Relative growth rate (%) (brown rot)**	**Extractive content (%)**	**Relative growth rate (%) (white rot)**	**Relative growth rate (%) (brown rot)**	**Extractive content (%)**
Min	70.467	36.312	4.34	69.03	33.73	1.32
Max	94.834	95.598	14.789	94.66	80.36	7.85
CV (%)	7.5	26.6	24.1	7.2	16.6	37.4

5.2.2 Fungal assays

The method described in chapter 4 was used to test the bioactivity of the *E. bosistoana* heartwood ethanol extracts. In brief, 10 ml of autoclaved (121˚C, 12 min) potato dextrose agar (Oxoid - containing 4.0 g/L potato extract, 20.0 g/L dextrose and 15.0 g/L agar) was poured into 7 cm petri dishes. 100 µl (250 mg/ml) or 50 µl (93.75 mg/ml) of dimethyl sulfoxide (DMSO) extract solution were spread onto the surface of the solidified agar with a disposable spreader for white rot and for brown rot tests, respectively. Controls were run with pure agar and 100 µl or 50 µl DMSO, respectively. Five replicates for each fungi and extract were conducted resulting in 1160 dishes. The assays were conducted in 25 batches over a period of 20 weeks due to lack of space and time taken for growth rate measurements of the large number of dishes. The relative growth rate was calculated as defined in equation 1 (chapter 4) using the agar controls only.

5.2.3 Gas chromatography (GC)

For each sample, 10 mg of dried heartwood extract were mixed with 90 µl pyridine in Eppendorf (1.5 ml) tubes to which 10 µl internal standard solution (5 mg Betulin (Sigma Aldrich) dissolved in 1 ml of pyridine) was added. A 15 µl aliquot of this solution was trimethylsilylated at room temperature using 50 µl of N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA, Supelco Analytical) in a septum-sealed vial for 20 min according to the supplier's recommendations. The trimethylsilyl derivatives were analysed with a gas chromatograph (Agilent 7820A), fitted with a fused-silica capillary column (Agilent DB-5 - 30 m x 0.320 mm x 0.25 µm) using helium as the carrier gas and FID detection at 300°C. The initial oven temperature was set to 116°C, ramped up to 280°C at 7°C/min and held for 20 min. Each sample was analysed twice after a blank and a pyridine run.

Individual compounds were identified by comparing the *E. bosistoana* heartwood extracts with an *E. globoidea* heartwood extract. Some compounds were previously identified in *E. globoidea* heartwood extracts by GS-MS (Schroettke, 2018). These *E. globoidea* extracts were previously run on the same GC system but in the meantime shortening of the column resulted in a shift of the retention times of up to 0.7 min (i.e. the internal standard Betulin at ~27.9 min).

5.2.4 Data extraction from chromatograms and data analysis

Peaks of the chromatograms were integrated using integration tool in the Chem Station software (Agilent, Rev.c.01.07) and refined manually for the largest 32 peaks including the internal standard. Peak areas and retention times were extracted as .csv files and the retention times for 91 samples were aligned manually in Excel. Peak area of 31 retention times were normalised by the peak area of the internal standard before duplicate runs were averaged. Data analysis was performed using R programming language (Team, 2013).

Multivariate analysis (Partial Least Squares Regression) between the quantified heartwood compounds and the relative growth rates of white rot (*T. versicolor*) and brown rot (*C. cerebella*) was performed using the plsdepot (version-0.1.17) package in R (Sanchez, 2012). This analysis was performed to identify the most important compound showing bioactivity of the *E. bosistoana* heartwood extracts on the growth of white rot (*T. versicolor*) and brown rot (*C. cerebella*).

5.3 Results and discussion

5.3.1 Bioactivity of extracts

An F-test (ANOVA) of the growth rates for the controls between the batches showed significant differences for both fungi (Table 5.3). This confirmed the need to normalise the growth rate of fungi exposed to extracts by the growth rate of the controls (only agar) without extract in order to make the data comparable between the batches. The subsequent analysis was based on the relative growth rate, which expressed how much slower (or faster) the fungus grew when exposed to the extract compared to the control conditions.

Table 5.3: F-test (ANOVA) of the growth rate of the controls in different batches (white rot (*T. versicolor*): 15 batches with 5 replicates for each control type; brown rot (*C. cerebella*): 10 batches with 5 replicates for each control type)

Fungi	**F value**		**P value**	
	only agar	***DMSO + agar***	***only agar***	***DMSO + agar***
White rot	108.71	212.89	<2.2 e-16	<2.2 e-16
Brown rot	53.041	116.13	<2.2 e-16	<2.2 e-16

There was no evidence of different growth rates in the two type of controls for both, white rot ($t = 0.235$, $p = 0.814$) and brown rot ($t = 0.154$, $p = 0.878$) (Table 5.4). This confirmed that the used quantities of DMSO had no effect on the test, and the changes in growth rate were caused by the heartwood extracts.

Table 5.4: t-Test showing no difference in the growth rates of the white rot (*T. versicolor*) and brown rot (*C. cerebella*) between the controls, i.e. with and without DMSO (white rot: 15 batches with 5 replicates for each control type; brown rot: 10 batches with 5 replicates for each control type)

Fungi	**t test**	**p value**
White rot	0.235	0.814
Brown rot	0.154	0.878

The relative growth rate of white rot ($t = 42.65$, $p < 2.2$ e-16) and brown rot ($t = 27.131$, $p < 2.2$ e-16) was significantly lower when exposed to ethanol extracts of *E. bosistoana* heartwood compared to the controls (Table 5.5). The brown rot was more sensitive to the *E. bosistoana* extracts than the white rot as the growth was more retarded by smaller amounts of extract (Figure 5.1).

Table 5.5: t-Test showing lower relative growth rates of white rot (*T. versicolor*) and brown rot (*C. cerebella*) when exposed to ethanol extracts of *E. bosistoana* heartwood compared to the controls

Fungi	**t test**	**p value**	**Mean in group (sample estimate)**	
			Both controls	***Extracts***
White rot	42.65	p < 2.2 e-16	100.14 %	82.96%
Brown rot	27.131	p < 2.2 e-16	100.38%	60.47%

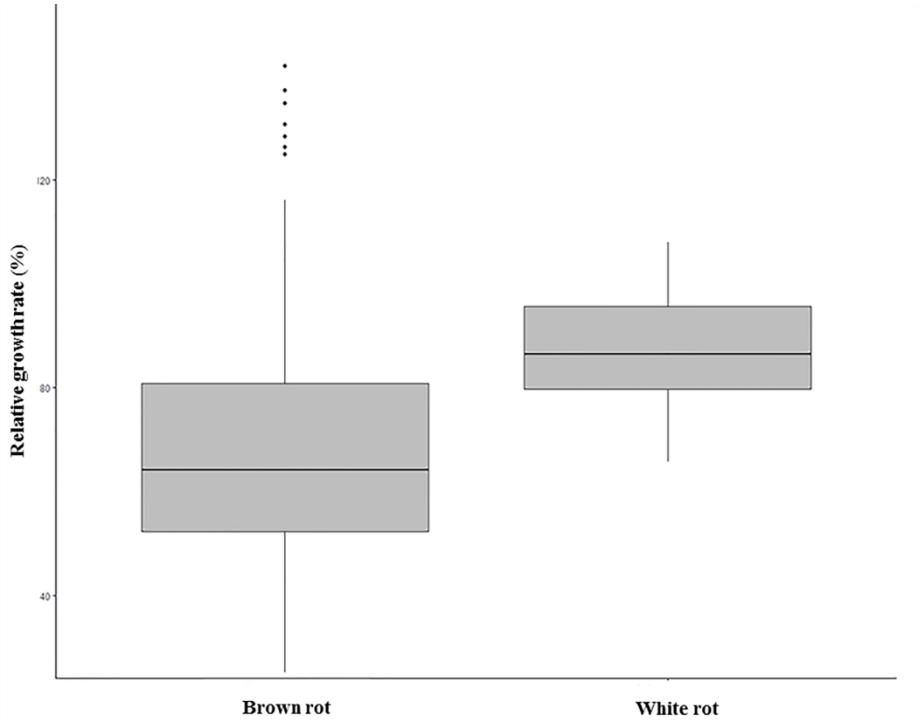

Figure 5.1: Box-and-whisker plot of relative growth rates of brown rot (*C. cerebella*) and white rot (*T. versicolor*) when exposed to ethanol extracts of *E. bosistoana* heartwood. The white rot was less affected.

No relationship between the growth rates of white rot and brown rot in extracts was observed for either of the two sites. A t-test showed that the slope was not different than 0, ($t = 0.086$, $p = 0.9316$) (Table 5.6). This was consistent with the small coefficient of determination ($R^2 = 0.009$) (Figure 5.2). Similar observations were reported by Davies et al. (2014) for *Sequoia sempervirens* (redwood) and Ohtani et al. (2009) for *Cryptomeria japonica* (Sugi) heartwood extracts. The observation suggested that the growth rates of the fungi were inhibited by different compounds of the extracts. Kirker et al. (2013) reported that individual components in extractives confer durability rather than bulk presence of extractive. For example in vitro tests suggested that thujaplicin, a natural fungicide in the heartwood extracts of *Thuja plicata* (western red cedar), was found to be toxic to brown rot but not so effective against white rot (Roff & Atkinson, 1954). Similarly naphthoquinone, a compound in the heartwood of *Tectona grandis* (teak) was found to have a stronger negative effect on the brown rots *Polyporus palustris* and *Gloeophyllum trabeum* than the white rots *T. hirsuta*, *T. versicolor* and *Pycnoporous sanguineus* (Thulasidas & Bhat, 2007). Therefore, as stated by Taylor et al. (2006) or Morris and Stirling (2012), it is not possible to focus on a single heartwood compound to understand the resistance of the wood against multiple biodegrading organisms.

In eucalypts, extractives are predominately comprised of tannins (Conde et al., 1995; Hillis, 1972, 1991; Rudman, 1964). Ellagitannins in the heartwood extractives from *Quercus alba* (white oak) were

reported to be less inhibitory to *T. versicolor* (white rot) than *Poria manticola* (brown rot) (Hart & Hillis, 1972). It was suggested that this variation was caused by the difference in the protein-tannin binding capacity of different tannin protein mixtures, which is influenced by molecular size and chemical structure (Rudman, 1963).

Table 5.6: t-Test and coefficient of determination between the growth rates of white rot (*T. versicolor*) and brown rot (*C. cerebella*) when exposed to ethanol extracts of *E. bosistoana* heartwood.

t test	p value	Coefficient of determination (R^2)
0.09	0.93	0.01

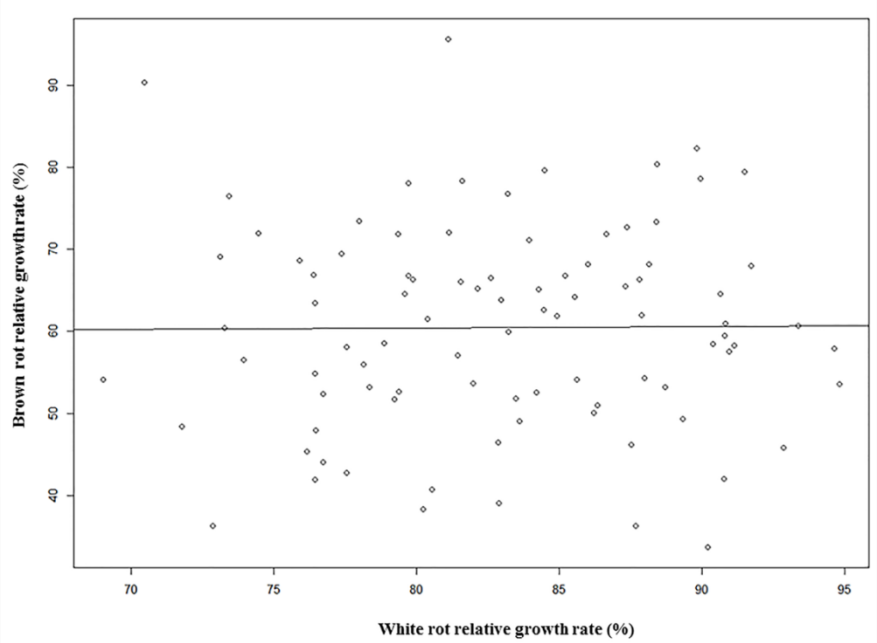

Figure 5.2: Relationship between the growth rates of white rot (*T. versicolor*) and brown rot (*C. cerebella*) when exposed to ethanol extracts of *E. bosistoana* heartwood. $R^2 = 0.009$.

No correlation was observed between the extractive content in the wood, which was variable, and the relative growth rates of the fungi exposed to a fixed quantity of extract (Table 5.7, Figure 5.3). However, when taking sites factors into account the extractive content had a negative influence on the bioactivity towards the white rot for the Lawson, but not the Craven Road site (Table 5.8, Figure 5.4a). This suggested that the trees with elevated extractive contents at the Lawson site deposited more compounds into the heartwood, which were not bioactive against *T. versicolor*. For the brown rot *C. cerebella* no evidence of a relationship between growth rate and extractive content in the wood was found in either of the two sites (Table 5.8, Figure 5.4b). This suggested that extractive content

was not associated with a change in extractive composition increasing or decreasing the amount of fungicidal compounds for this fungi.

Table 5.7: Coefficient of determination between the extractive content in heartwood of 7 year-old *E. bosistoana* and relative growth rates of white rot (*T. versicolor*) and brown rot (*C. cerebella*)

white rot	Brown rot
0.010	0.004

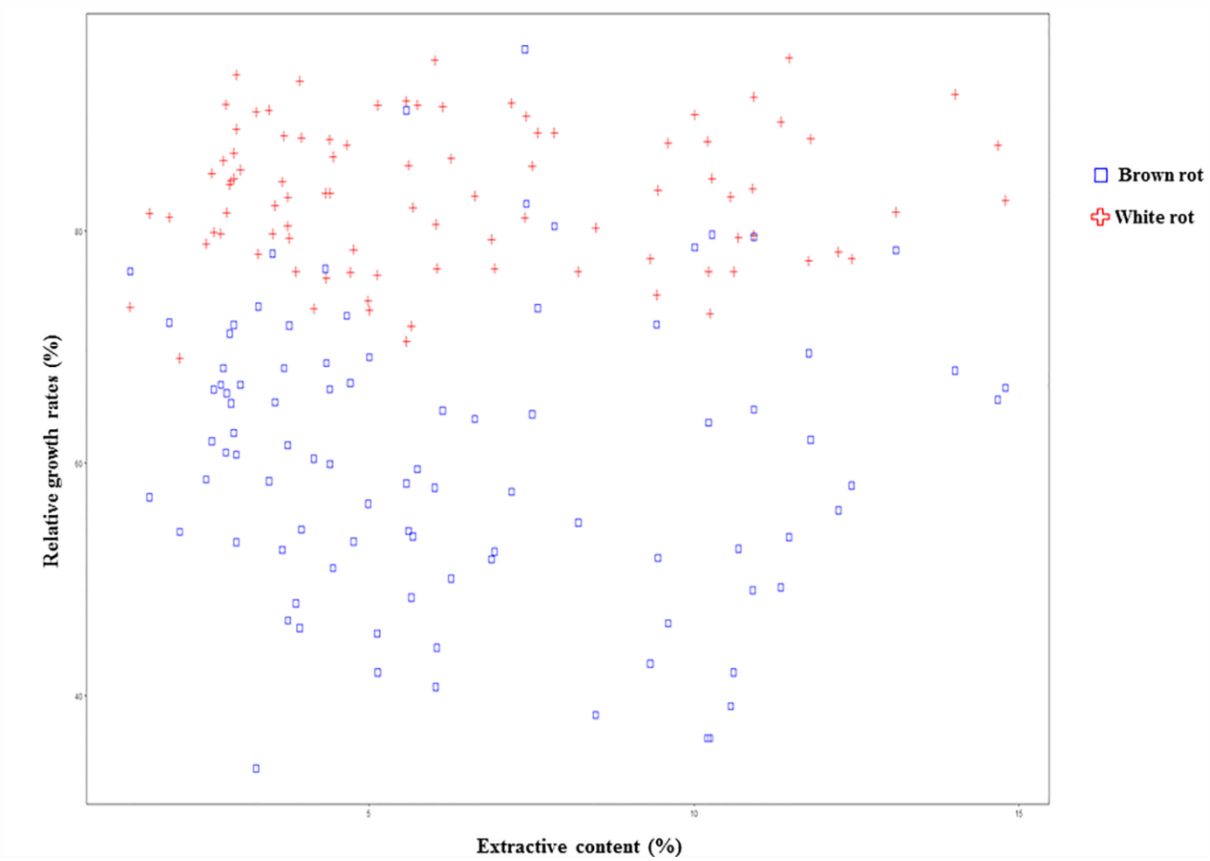

Figure 5.3: Relationships between the relative growth rates of white rot (*T. versicolor*) (R^2 = 0.010) and brown rot (*C. cerebella*) (R^2 = 0.004) with extractive content in *E. bosistoana*.

Table 5.8: Relationship between growth rates of white rot (*T. versicolor*) brown rot (*C. cerebella*) with extractive content in Lawson and Craven Road.

Sites	White rot			Brown rot		
	Slope	**t test**	**P value**	**Slope**	**t test**	**P value**
Lawson	1.0064	2.451	0.0201	-0.4193	-0.357	0.723
Craven Road	0.765	1.546	0.128	-0.8722	-1.04	0.303

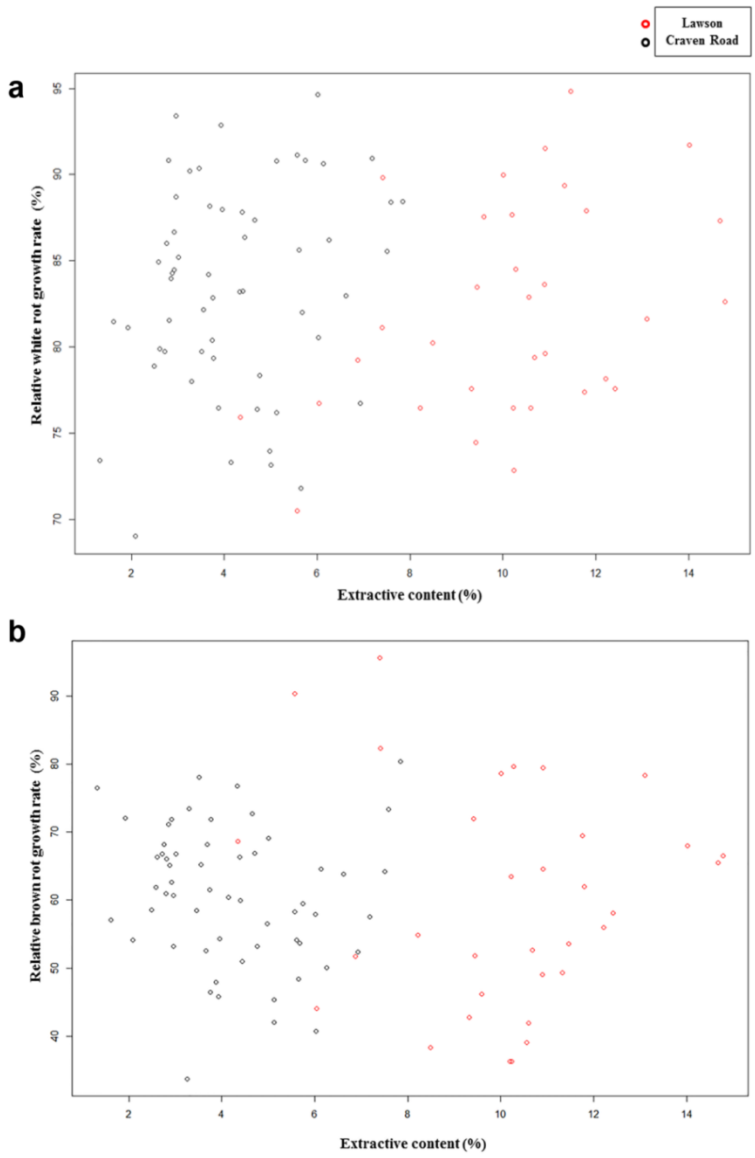

Figure 5.4: Relationship between the heartwood extractive content of 7 year-old *E. bosistoana* and the relative growth rate of a) white rot (*T. versicolor*) and b) brown rot (*C. cerebella*). Red: Lawson; black Craven Road.

5.3.2 Variation in bioactivity between trees

Variation in the relative growth rates of white rot (Figure 5.5a) and brown rot (Figure 5.5b) were observed between trees (see also Appendix 1). Tukey tests were conducted to compare the relative growth rates of fungi exposed to the sample extracts. The results showed that there were significant differences between the trees (Appendix 2) and consequently suggested an influence of genetic factors on the composition of heartwood extracts of *E. bosistoana*. Erdtman (1950) reported a regional influence on the amount of pinosylvin in *Pinus sylvestris* in Sweden. Later high heritability of the heartwood extractives pinosylvin, resin acids, fatty acids and sterols were found for this species (Fries et al., 2000). Gansel & Squillace (1976) reported strong genetic control of the terpene composition in *P. ellotti* with negligible plantation effects. Genetic variability in the quantity of 10 out of 42 terpenes was reported for *P. nigra* and based on the terpene composition *P. nigra* populations were divided into two geographical groups (Bojovic et al., 2005). Genetic analysis of β-pinene concentration in individual trees demonstrated monogenic heredity (Zavarin et al., 1990a, 1990b). Tree-to-tree phenotypic variation and high heritabilities were also reported for the bioactive heartwood compounds plicatic acid, thujaplicatin methyl ether, β-thujaplicin, γ-thujaplicin, β-thujaplicinol, thujic acid, and methyl thujate, in the ethanol extract of *Thuja plicata* (Daniels & Russell, 2007).

Puech et al. (1999) summarised natural variation in the concentration of heartwood tannins in oaks. High variation within and between trees and populations was observed. However, the relative importance of different factors such as species, forest origin (or provenance) that influenced the variation in the concentration of ellagitannins remained unclear. A study suggested that the variation in the concentration of heartwood ellagitannins was a heritable property and not influenced by the growth rate of the trees. This agreed with the observation that ellagitannins and volatile compounds were highly variable among individuals in heartwood of *Q. pyrenaica* (Fernández et al., 2006). Guilley et al. (2004) reported positive correlations between the amount of the ellagitannins roburine, grandinin, vescalagin and castalagin and decay resistance in *Q. petraea*.

Extractive content is known to increase radially from pith to the sapwood-heartwood boundary, including *Eucalyptus* species (Wilkes, 1984). This radial pattern of within tree extractive content cannot be considered for variation in decay resistance between *E. bosistoana* trees in this experiment as all trees were of the same age and sampled at the same location (i.e. stem base).

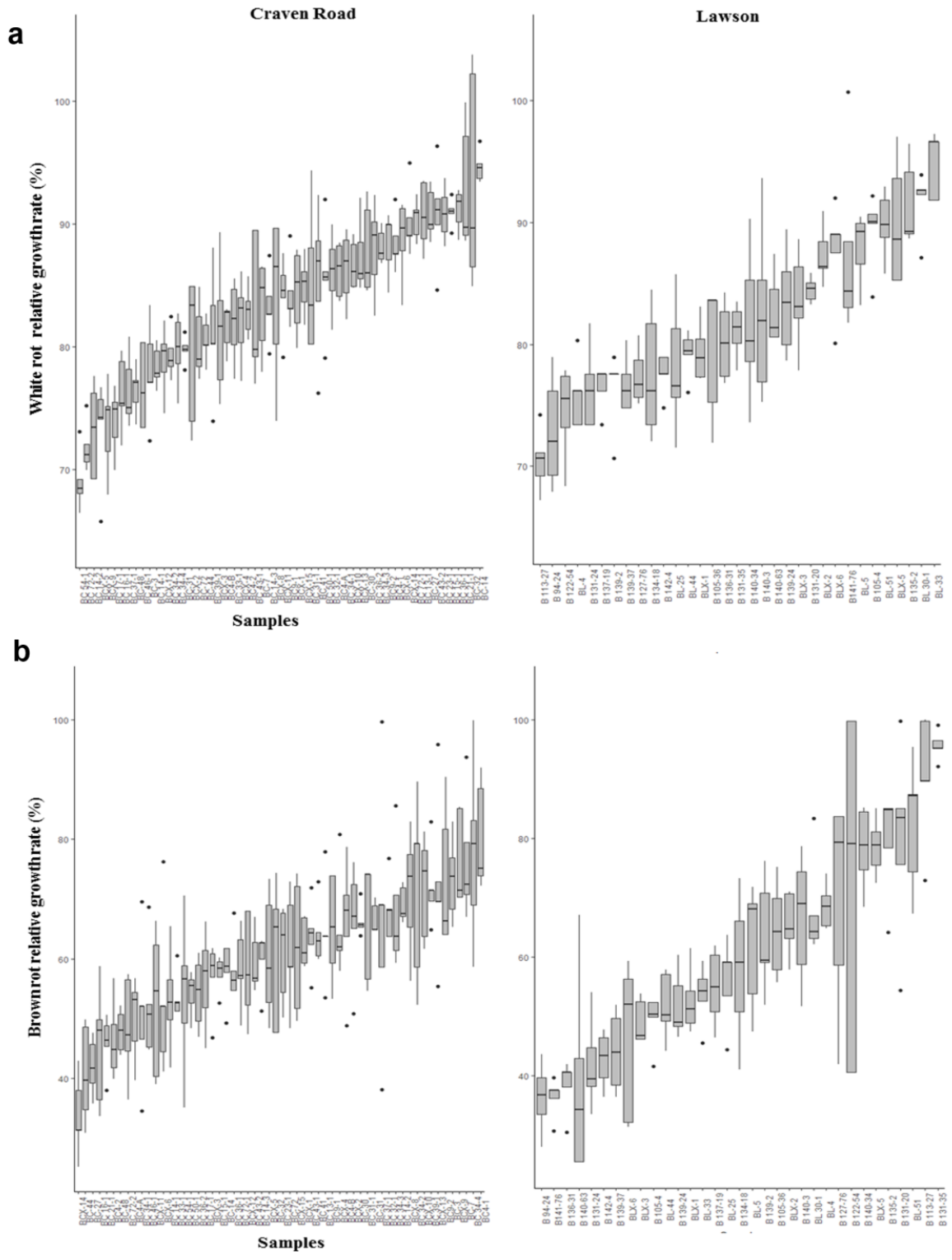

Figure 5.5: Box-and-whisker plots representing variation in relative growth rates of a) white rot (*T. versicolor*) and b) brown rot (*C. cerebella*) between the trees and sites.

No statistical significant difference in relative growth rates was observed between the two sites for the brown rot (Table 5.9). Although statistically significant (p = 0.028), the difference in the relative growth rates between both sites for the white rot was small, with 82.01% and 83.50% for Lawson and Craven Road, respectively (Table 5.9). Reports on differences in durability between sites were typically associated with a higher extractive content in wood, for which was normalised in this experiment. For example Harju et al. (2003) found that *P. sylvestris* from a site with more durable wood contained also higher amounts of heartwood extractives (total acetone-soluble extractives, resin acids, pinosylvin and the total phenolics quantified as tannin acid equivalents). This lines with observations on *T. grandis*, for which variability in natural durability, total extractive content as well as the amounts of individual compounds was reported between trees, plantations and geographical zones (Kokutse et al., 2006; Thulasidas & Bhat, 2007; Windeisen et al., 2003). Morais & Pereira (2012) reported an influence of site on the extractive content in *E. globulus*.

Table 5.9: Site effect on the relative growth rates of white rot (*T. versicolor*) and brown rot (*C. cerebella*) exposed to *E. bosistoana* heartwood extract

Fungi	**t test**	**p value**	**Mean in group (sample estimate)**	
			Lawson	Craven Road
White rot	2.2	0.028	82.01%	83.50%
Brown rot	0.267	0.79	60.19%	60.62%

5.3.3 Identification of compounds in ethanol extracts of *E. bosistoana* heartwood

The chemical composition in the ethanol extracts was analysed by GC. A typical chromatogram of silylated *E. bosistoana* heartwood extracts is shown in Figures 5.6 - 5.16. Numerous compounds were well separated. In Figures 5.6 - 5.16, the chromatogram was overlaid with that of a silylated *E. globoidea* heartwood ethanol extract. The heartwood extracts of the two species were similar and differed mainly in the relative amounts of the individual compounds. The *E. globoidea* heartwood extract was previously analysed by GC-MS, what allowed the identification of some compounds (Schroettke, 2018). By comparing the *E. bosistoana* and *E. globoidea* extracts five compounds were identified (Table 5.10). The unspecified ‘polyphenol‘ peak at 25.1 min might be catechin, which was observed in heartwood of *E. camaldulensis* (Benouadah et al., 2018). The authors also reported the presence of monosaccharides including fructose, glucose and hexose in the hydrophilic heartwood extract.

Similarities in the chemical composition of heartwood extracts of *Eucalyptus* species have been described (Hillis, 1991). While compounds such as stilbenes have been suggested as taxonomic marker for closely related eucalyptus species, the heartwood compound, 3',4-tri-o-methylellagic acid-4'-glucoside was considered a marker for the close relationship between the eucalyptus groups iron barks (e.g. *E. paniculata* and *E. sideroxylon*) and boxes (e.g. *E. bosistoana*) (Brooker, 2000; Hillis et al., 1974). Limited information is available about the identification of *Eucalyptus* species based on the chemical composition in heartwood. Chemotaxonomy studies were mainly based on leaf extracts (Hillis, 1966, 1967), however, it was possible to draw conclusions on the polyphenols in Eucalyptus wood from the leaf extracts.

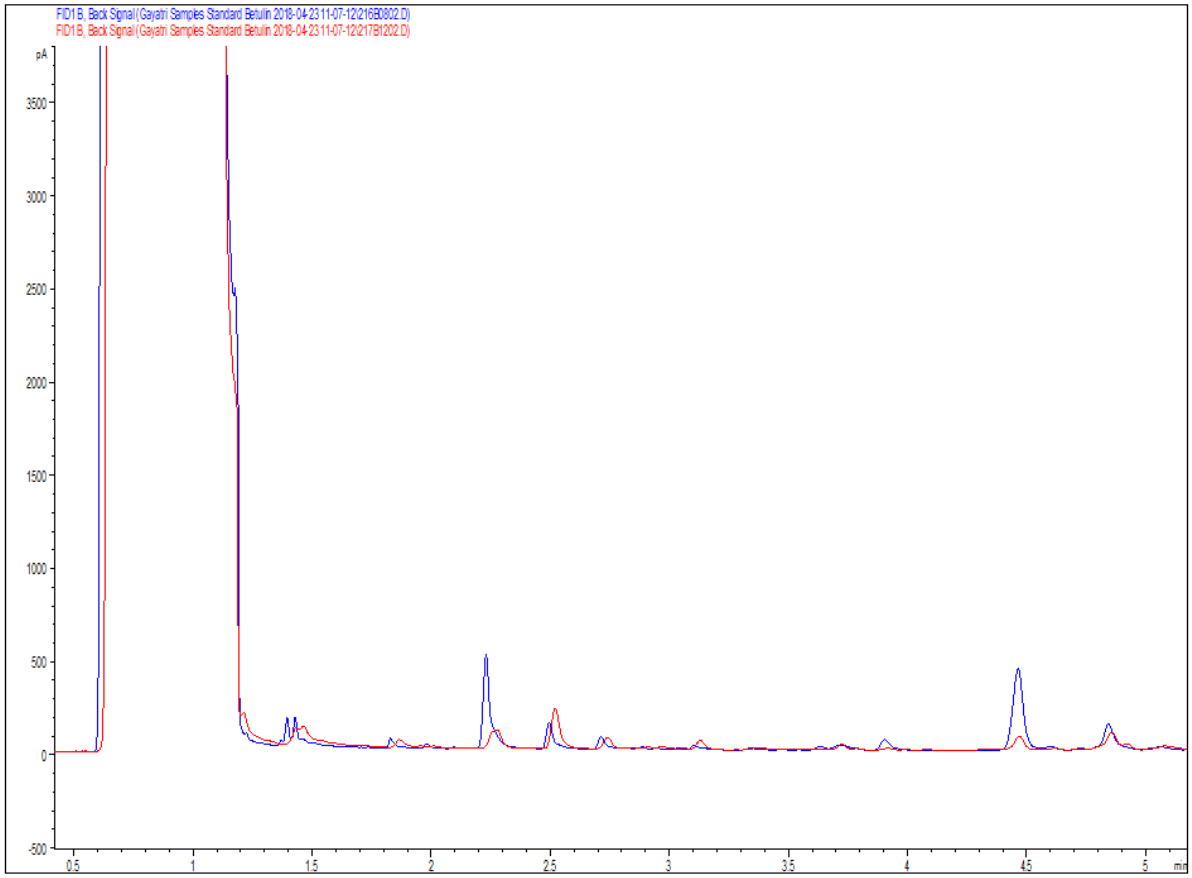

Figure 5.6: Gas chromatograms of silylated ethanol extracts from heartwood of *E. bosistoana* (blue) and *E. globoidea* (red) between 0.5 and 4.5 min retention time.

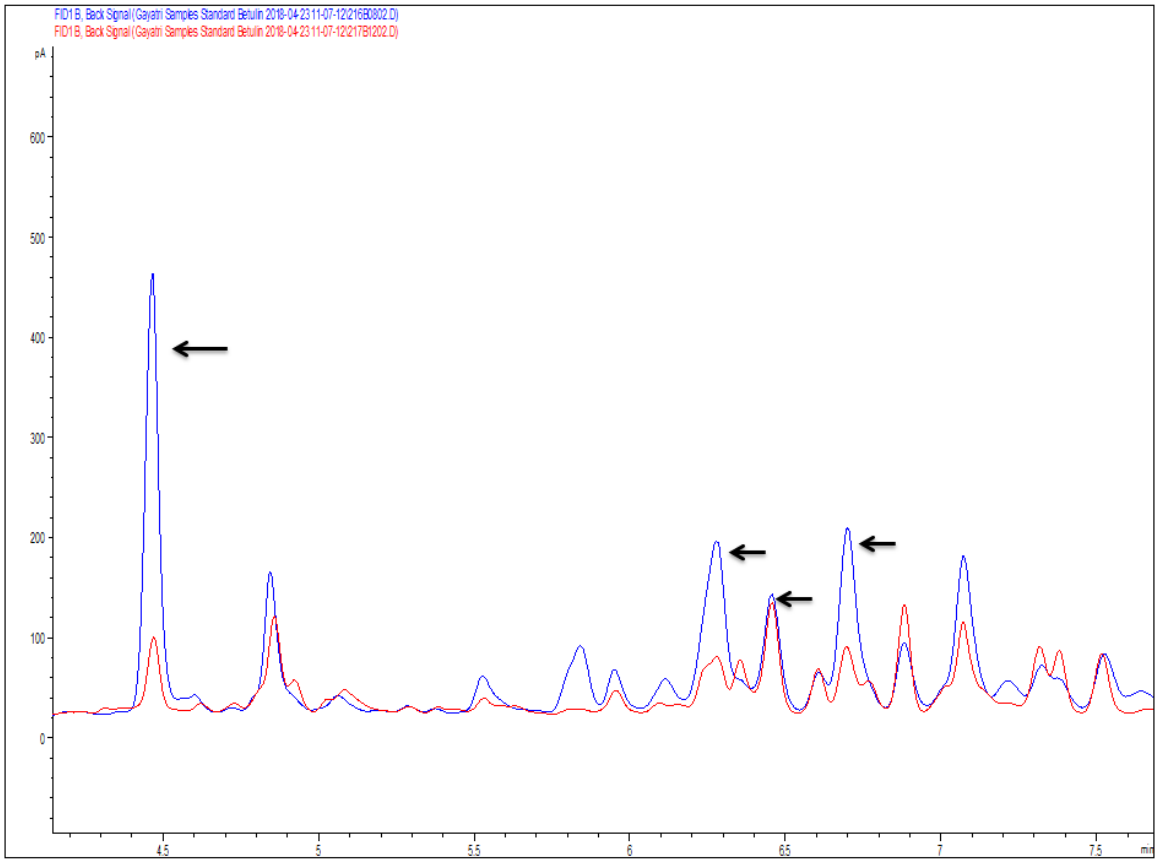

Figure 5.7: Gas chromatograms of silylated ethanol extracts from heartwood of *E. bosistoana* (blue) and *E. globoidea* (red) between 4.5 and 7.5 min retention time. Black arrows indicating peaks at retention times 4.5, 6.2, 6.4 and 6.7 were selected for further analysis.

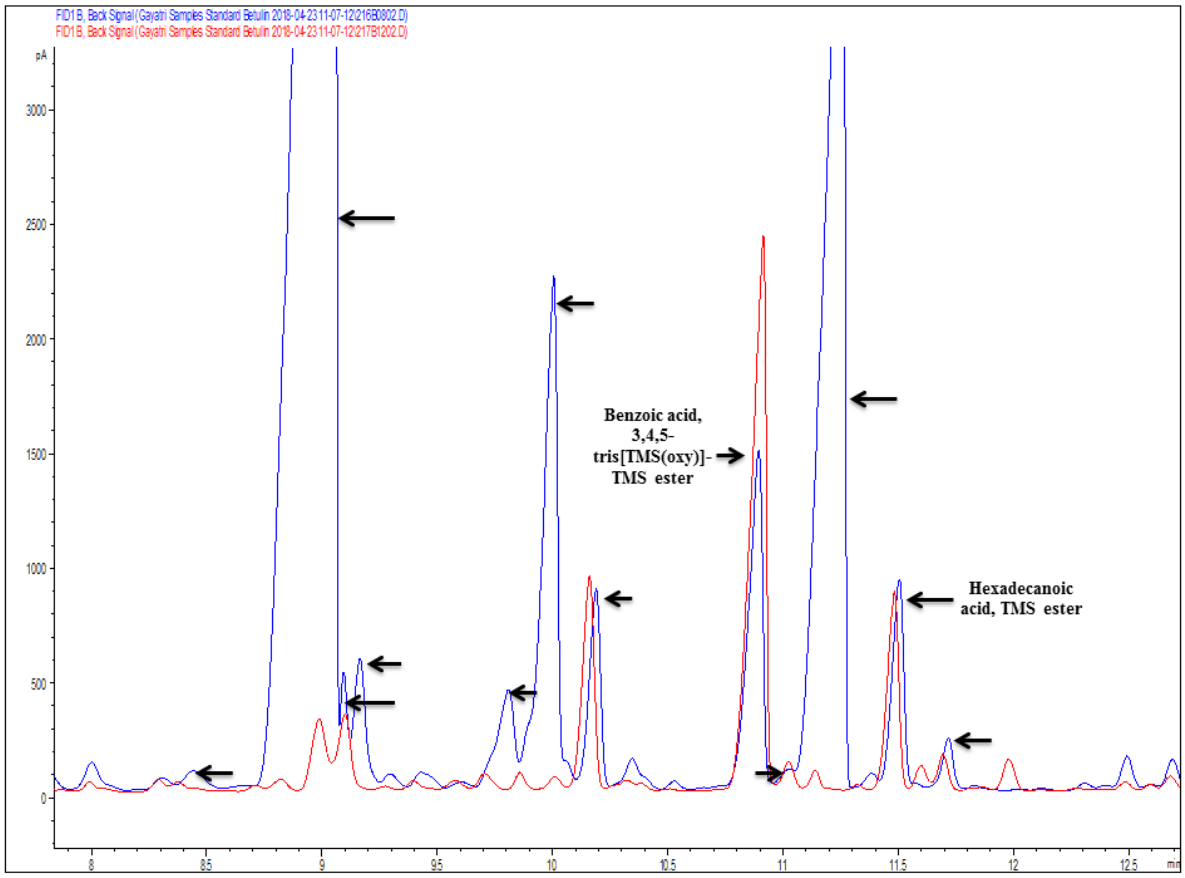

Figure 5.8: Gas chromatograms of silylated ethanol extracts from heartwood of *E. bosistoana* (blue) and *E. globoidea* (red) between 8 and 12.5 min retention time. Indicated peaks (arrows) at the retention times 8.4, 9.0, 9.1, 9.2, 9.7, 9.9, 10.2, 10.9, 11.1, 11.2, 11.5 and 11.7 were selected for further analysis. Compound identified at the retention times 10.9 and 11.5 min by comparing the chromatograms of *E. bosistoana* heartwood extracts with *E. globoidea* (Schroettke, 2018).

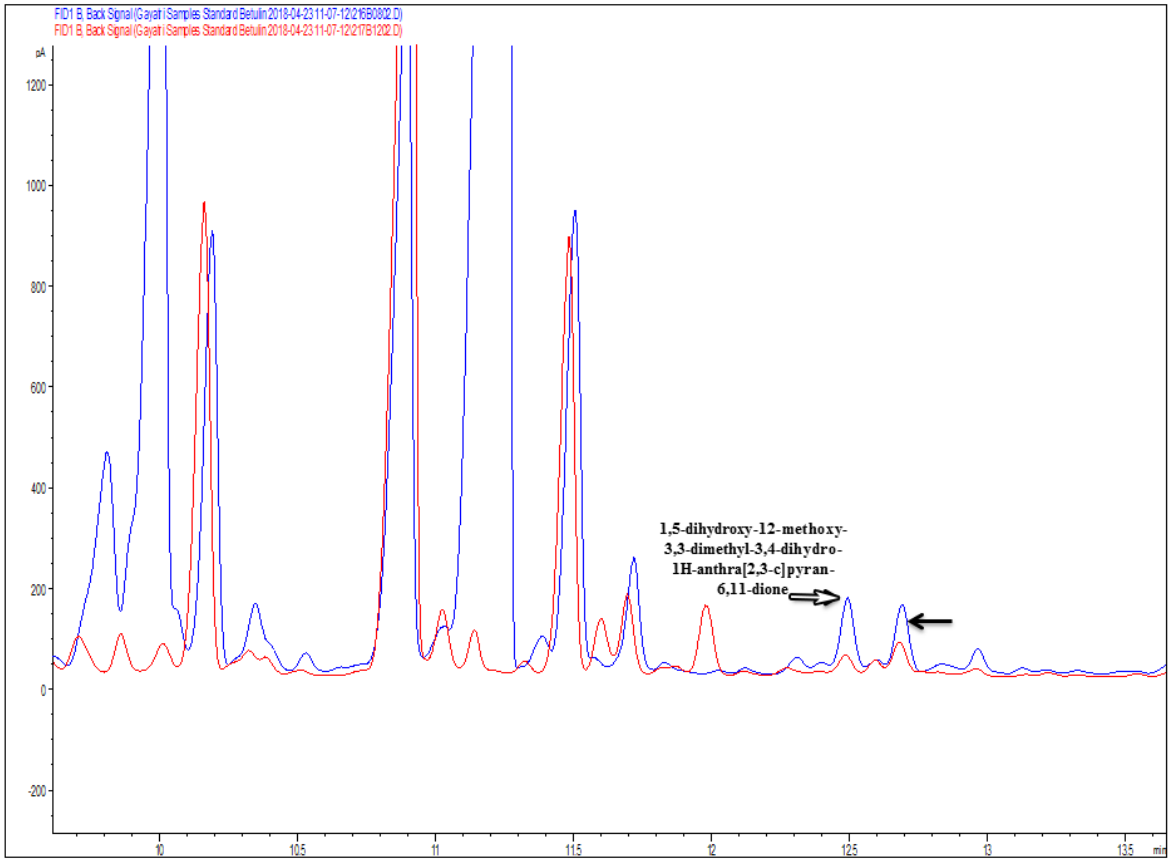

Figure 5.9: Gas chromatograms of silylated ethanol extracts from heartwood of *E. bosistoana* (blue) and *E. globoidea* (red) between 10 and 13.5 min retention time. Indicated peak (arrow) at the retention times 12.7 min was selected for further analysis. Compound identified at the retention time 12.5 min (arrow) by comparing chromatograms of *E. bosistoana* heartwood extracts with *E. globoidea* (Schroettke, 2018).

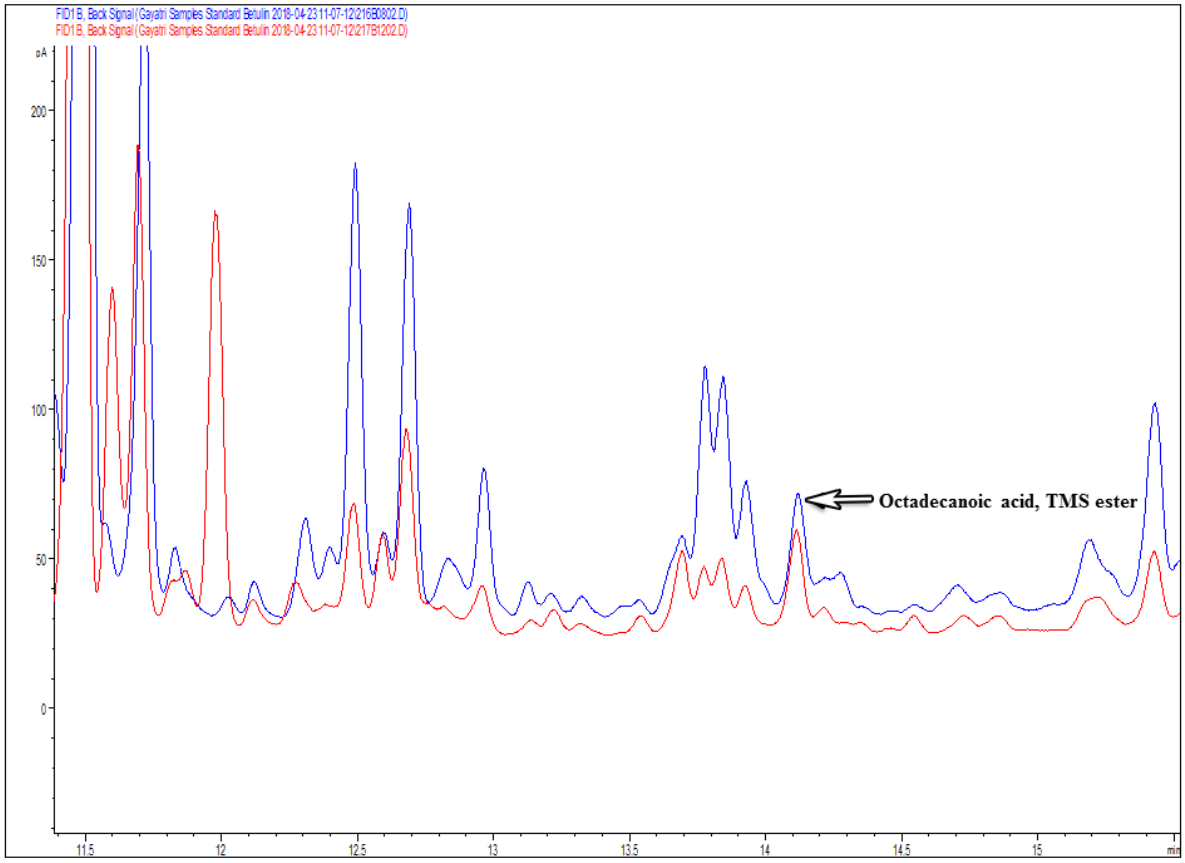

Figure 5.10: Gas chromatograms of silylated ethanol extracts from heartwood of *E. bosistoana* (blue) and *E. globoidea* (red) between 11.5 and 16 min retention time. Compound identified at the retention time 14.13 min (arrow) by comparing chromatograms of *E. bosistoana* heartwood extracts with *E. globoidea* (Schroettke, 2018).

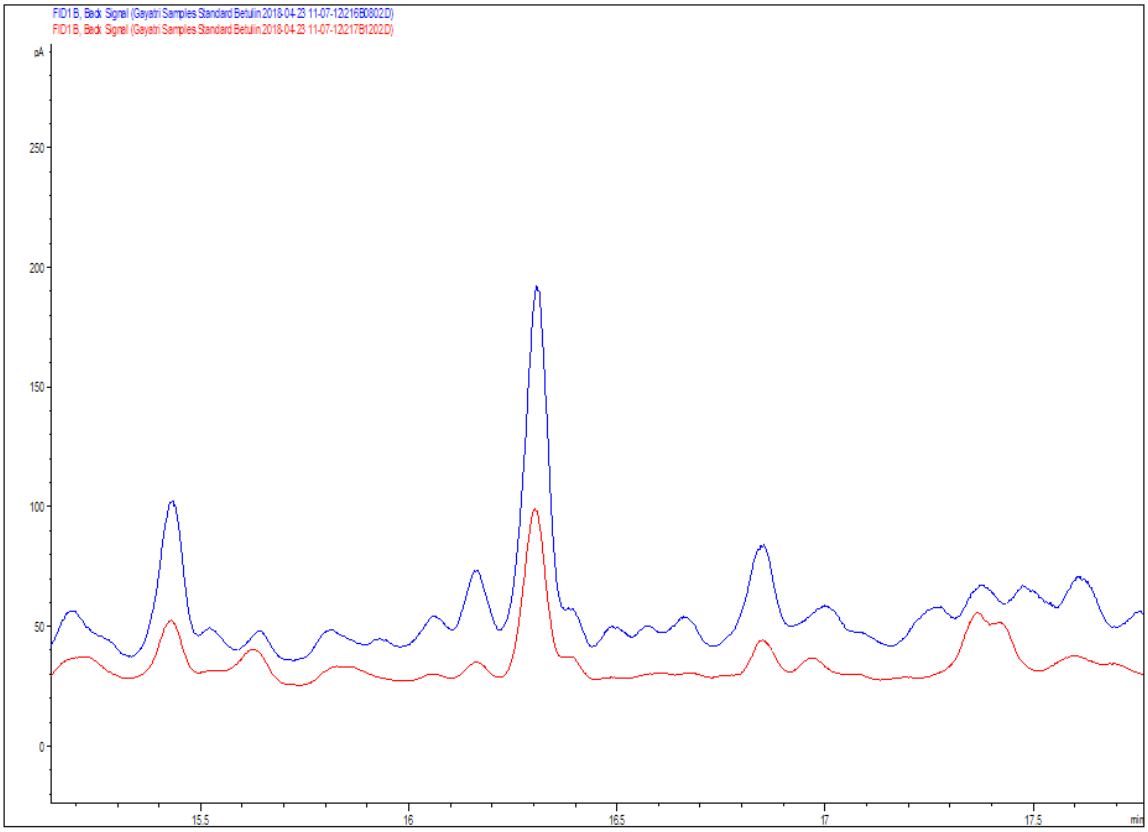

Figure 5.11: Gas chromatograms of silylated ethanol extracts from heartwood of *E. bosistoana* (blue) and *E. globoidea* (red) between 15.5 and 17.5 min retention time.

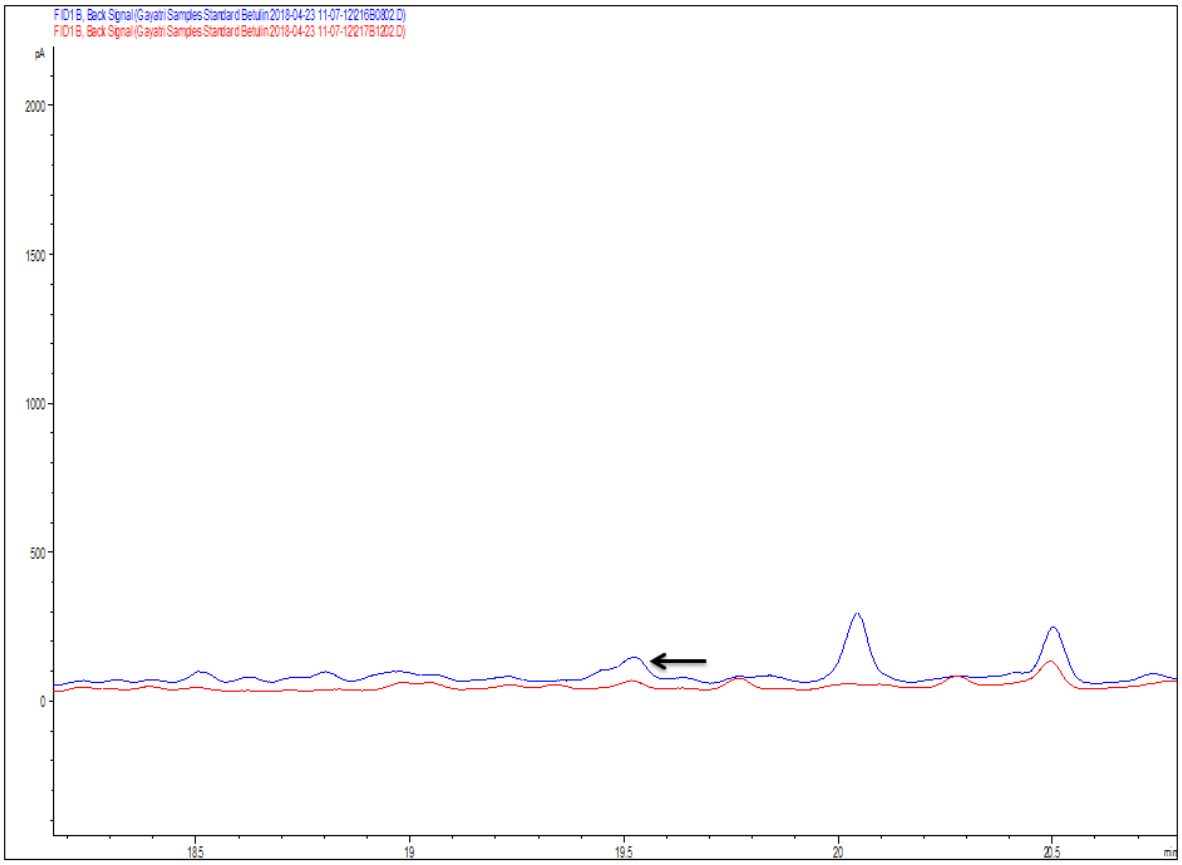

Figure 5.12: Gas chromatograms of silylated ethanol extracts from heartwood of *E. bosistoana* (blue) and *E. globoidea* (red) between 18.5 and 20.5 min retention time. Indicated peak (arrow) at the retention time 19.5 min was selected for further analysis.

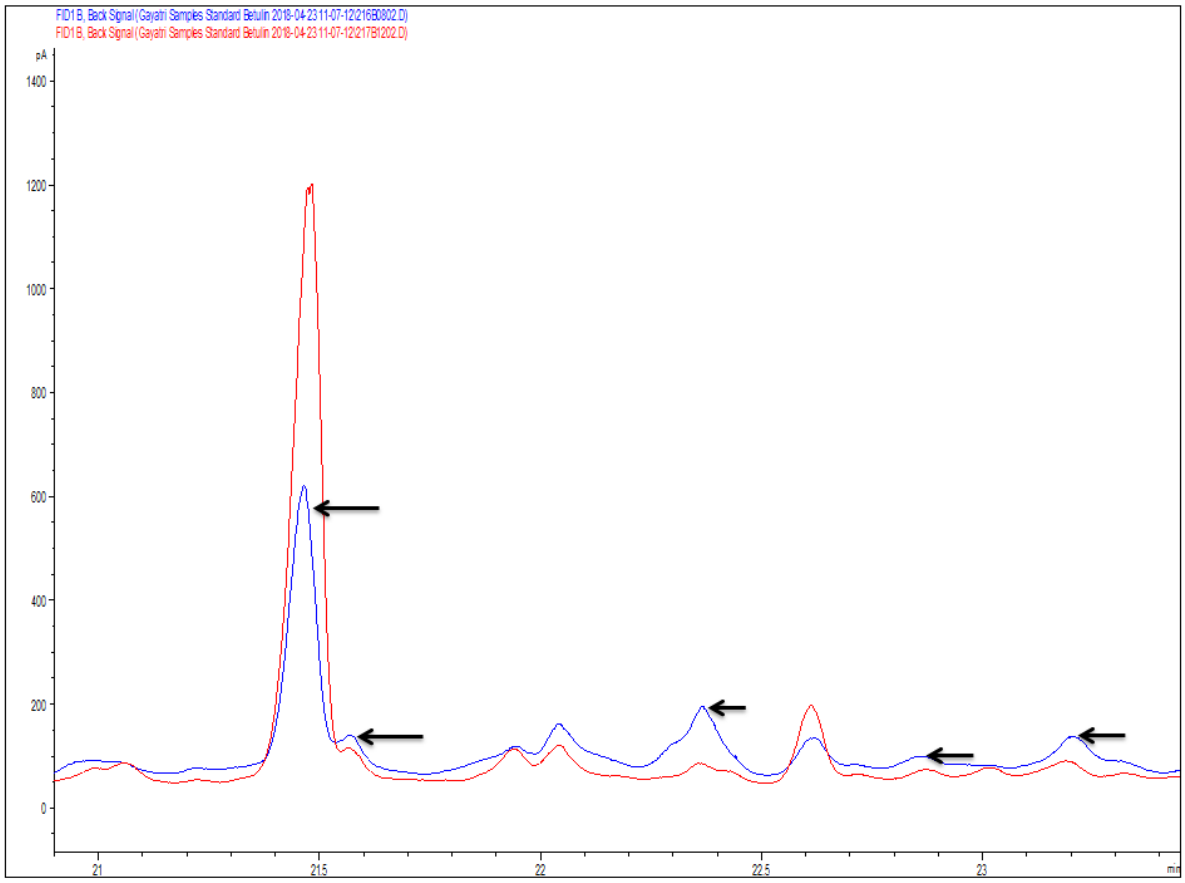

Figure 5.13: Gas chromatograms of silylated ethanol extracts from heartwood of *E. bosistoana* (blue) and *E. globoidea* (red) between 21 and 23.5 min retention time. Indicated peaks (arrow) at the retention times 21.4, 21.5, 22.3, 22.8 and 23.3 min were selected for further analysis.

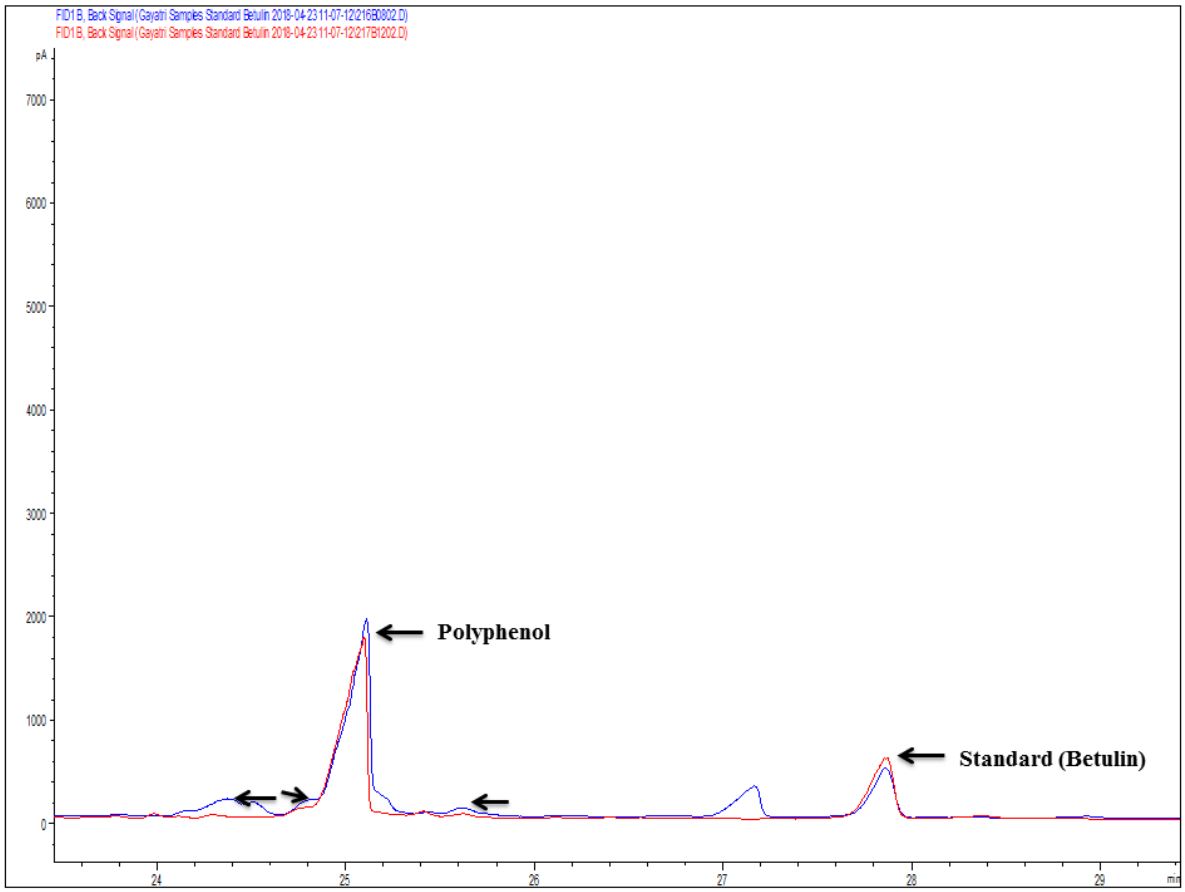

Figure 5.14: Gas chromatograms of silylated ethanol extracts from heartwood of *E. bosistoana* (blue) and *E. globoidea* (red) between 24 and 29 min retention time. Indicated peaks (arrow) at the retention times 24.8, 24.9, 25.1 and 25.4 min were selected for further analysis. Compound identified at the retention time 25.1 min (arrow) by comparing chromatograms of *E. bosistoana* heartwood extracts with *E. globoidea* (Schroettke, 2018). Standard (Betulin) at 27.9 min (arrow).

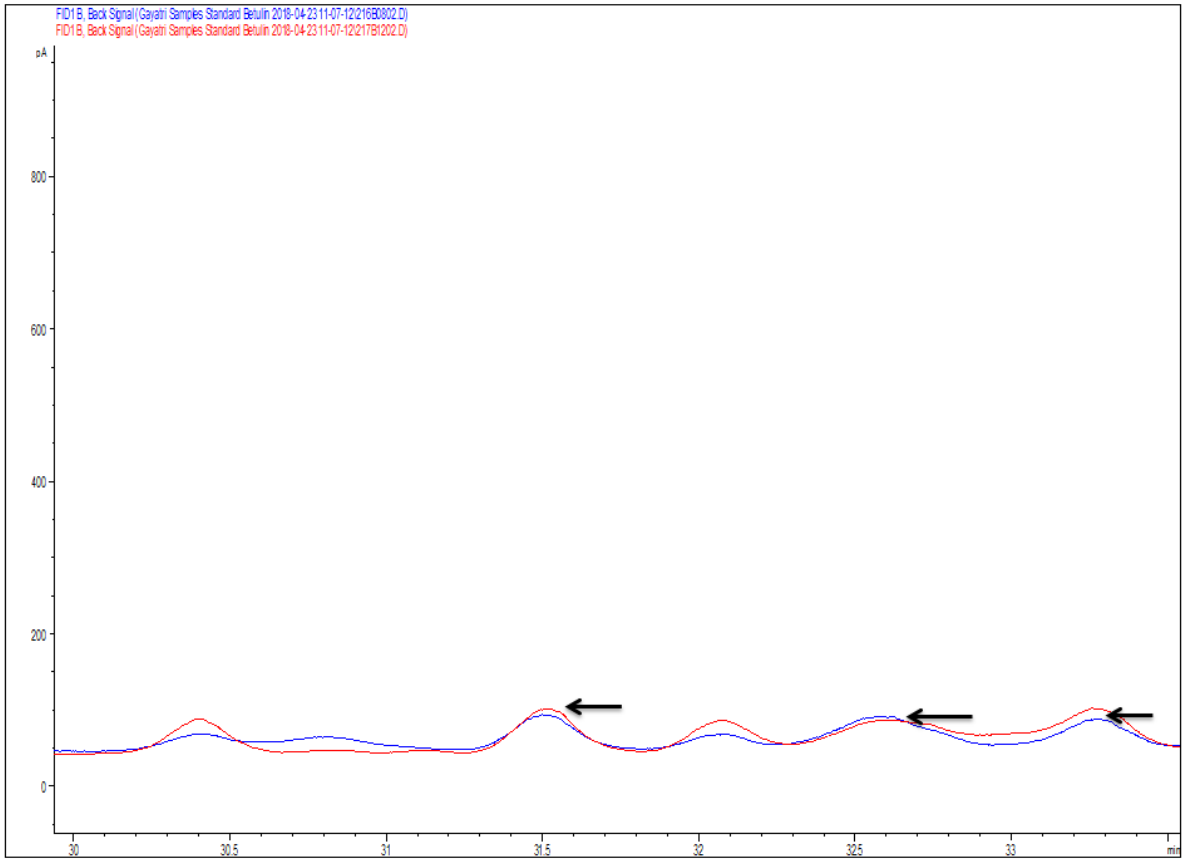

Figure 5.15: Gas chromatograms of silylated ethanol extracts from heartwood of *E. bosistoana* (blue) and *E. globoidea* (red) between 30 and 33 min retention time. Indicated peaks (arrow) at the retention times 31.5, 32.7 and 33.3 min were selected for further analysis.

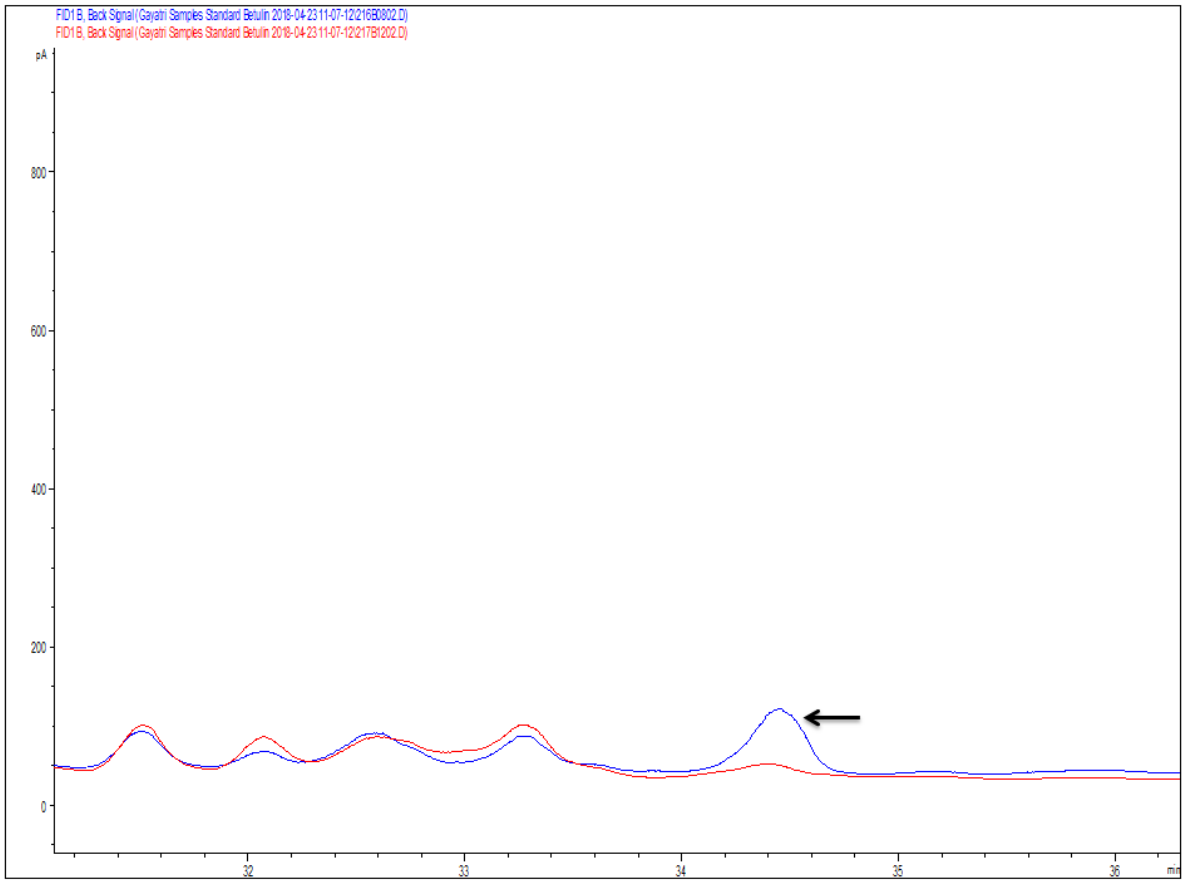

Figure 5.16: Gas chromatograms of silylated ethanol extracts from heartwood of *E. bosistoana* (blue) and *E. globoidea* (red) between 32 and 36 min retention time. Indicated peak (arrow) at the retention times 34.7 min was selected for further analysis.

Table 5.10: Compounds identified by comparing the chromatograms of *E. bosistoana* heartwood extracts with *E. globoidea* (Schroettke, 2018)

Retention times (min)	**Chemical compounds**
10.9	Benzoic acid, 3,4,5-tris[TMS(oxy)]-,TMS ester
11.5	Hexadecanoic acid, TMS ester
12.5	1,5-dihdroxy-12-methoxy-3,3-dimethyl-3,4-dihydro-1H-anthra[2,3-c]pyran-6,11-dione
14.1	Octadecanoic acid, TMS ester
25.1	Polyphenol

5.3.4 Chemical composition of extracts

5.3.4.1 Identification of bioactive compounds

Analysis was performed to correlate the normalised peak areas of the retention times with the relative growth rates of brown rot and white rot. None of the considered compounds showed a significant correlation to the bioactivity tests (Table 5.11).

Table 5.11: Coefficients of determination (R^2) between the relative amounts of *E. bosistoana* heartwood compounds and the relative growth rates of brown rot (*C. cerebella*) and white rot (*T. versicolor*).

Retention Time (min)	R^2 Brown rot	R^2 White rot
4.5	0.00596	0.01271
6.2	0.01322	0.00009
6.4	0.00089	0.00720
6.7	0.00628	0.00102
8.4	0.01429	0.00114
9.0	0.00319	0.00950
9.1	0.00015	0.00001
9.2	0.00003	0.00260
9.7	0.00040	0.00111
9.9	0.00367	0.00652
10.2	0.01164	0.06256
10.9 **(Benzoic acid, 3,4,5-tris[TMS(oxy)]- ,TMS ester)**	0.01608	0.00494
11.1	0.00014	0.01768
11.2	0.00030	0.00436
11.5 **(Hexadecanoic acid, TMS ester)**	0.01102	0.02149
11.7	0.00123	0.01270
12.7	0.02485	0.00269
19.5	0.00427	0.00014
21.4	0.00053	0.00134
21.5	0.00672	0.00101
22.3	0.00680	0.00095
22.8	0.00028	0.01359
23.3	0.00166	0.01546
24.8	0.00280	0.00711
24.9	0.00308	0.00605
25.1 **(Polyphenol)**	0.01252	0.04101
25.4	0.00006	0.00386
31.5	0.00098	0.00044
32.7	0.00270	0.02680
33.3	0.00744	0.00375
34.7	0.00069	0.01449

Multivariate analysis identified two groups of compounds (Figure 5.17). Compounds 10.2 and 11.5 were most important for predicting the bioactivity of the *E. bosistoana* heartwood extracts towards

white rot (*T. versicolor*) (Figure 5.17a) and brown rot (*C. cerebella*) (Figure 5.17b). The same compounds were affecting the growth of the brown rot (*C. cerebella*). The other 29 compounds were grouped together and differed in the second principle component for white rot and in the first principal component for brown rot.

However, for both fungi, only a small part of the variation in bioactivity was explained by two principal components (Appendix 3, 4). The total variance in relative growth rate explained by the first two principle components was 13.2% (9.2% and 3.9%, respectively) for white rot (*T. versicolor*) and 15.8% (8.9% and 6.9%, respectively) for brown rot (*C. cerebella*). This suggested that other compounds, not captured in the GC analysis contributed to the bioactivity against the two tested fungi. GC relies on compounds entering the gas phase, which is difficult for larger and more hydrophilic molecules. Derivatisation, like acetylation or silylation, can aid the transition of hydrophilic compounds into the gas phase; however, the size of a molecule makes this more difficult. Therefore, the GC analysis of the hydrophilic ethanol extracts form *E. bosistoana* heartwood was likely not to capture larger compounds. HPLC was suggested as an alternative method to quantify larger molecular mass compounds in wood extracts (Smeds et al., 2018).

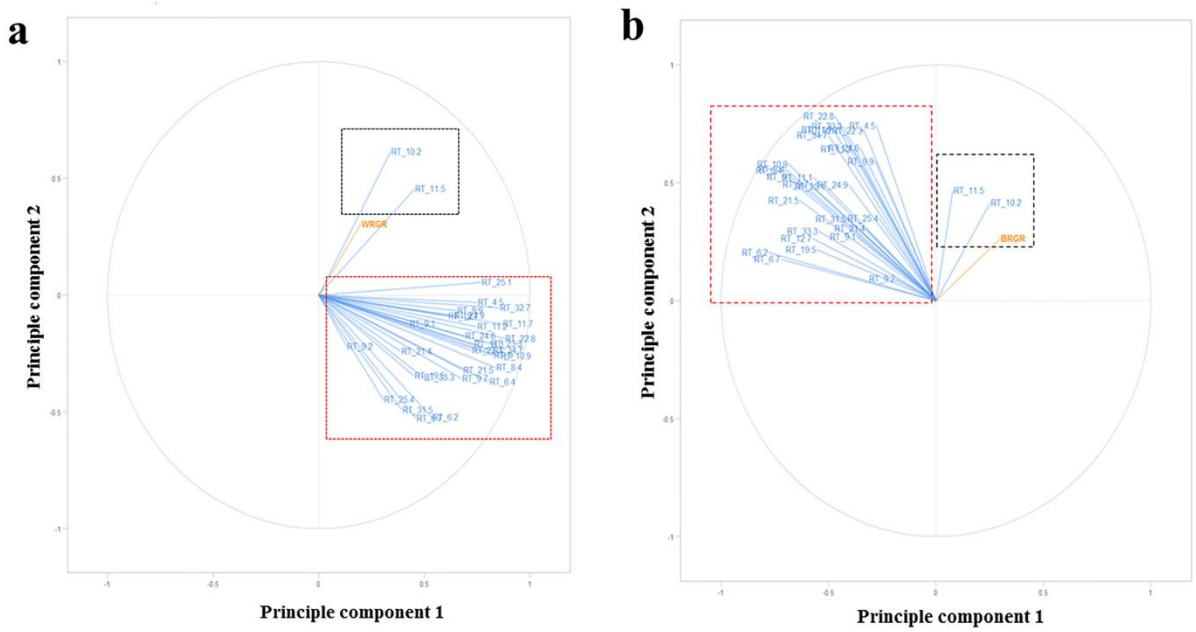

Figure 5.17: Influence of heartwood compounds in *E. bosistoana* heartwood on the growth of the a) white rot (*T. versicolor*) and b) brown rot (*C. cerebella*) analysed by partial least squares regression analysis (PLSR). Compounds that are near each other are highly correlated in the first two principal components. Compounds at 10.2 and 11.5 min in the black box aligned with the relative growth rate (orange line) and were considered different to the other 29 compounds grouped together in red box.

5.3.4.2 Site influence on the chemical composition

Figure 5.18 shows the variation in relative proportions of 31 *E. bosistoana* compounds between the two sites. Differences in the chemical composition between the sites were analysed with a t-test for the peak areas after normalisation by internal standard, for each of the quantified compounds. P values suggested significant variation for eight compounds between the sites (Table 5.12). One of the compounds could be identified as "polyphenol" and is potentially catechin.

The identified site influences are in agreement with the statement by Hillis (1987) that extractive composition is dependent on species, and varies within a species with the morphological part of the tree (roots, knots, bark, stem), age, season and location. Variation in heartwood extractives between species and locations (sites) are relevant to this study.

Investigation of the influence of geographical origin and species on extractives in oaks (*Q. alba, Q. robur* and *Q. petraea*) suggested variations in the content of whiskey lactone and ellagitannins differentiated species, whereas the content of eugenol, 2-phenylethanol, vanillin, and syringaldehyde were the most important features for distinguishing the geographical origin within species (Prida & Puech, 2006). Similar observations were reported by Emilia Guchu et al. (2006) who demonstrated variation in the content of volatile compounds (cis- and trans- methyl-γ-octalactones, furfural, 5-methylfurfural, guaiacol, eugenol and vanillin) between *Q. robur* and *Q. petraea*. The content of volatile compounds in *Q. petraea* was higher than in *Q. robur* and variation between geographical provenances was less than between species. Kilulya et al. (2014), evaluated the effect of site, species and tree sizes on the amount of lipophilic heartwood extractives from *E. dunnii* and *E. grandis*. GC–MS analysis showed that saturated fatty acids dominated in lipophilic extracts of *E. dunnii* from one site, whereas unsaturated fatty acids dominated in samples from another two sites. Correlation of the amount of lipophilic extractives with tree species/clones and soil type was revealed by principal component analysis (PCA), with trees grown on sites with high clay and organic matter content containing higher amounts of lipophilic extractives. However, in all the sampled sites *E. dunnii* was found to contain a higher amount of lipophilic extractives than *E. grandis*.

Table 5.12: *E. bosistoana* heartwood compounds with a significant difference between the Lawson and Craven Road sites.

Retention Time	p-values (Bonferroni corrected)
9.9	5.70E-13
11.2	4.20E-07
11.7	4.28E-05
22.3	0.000449849
22.8	0.000357398
23.3	0.001130283
25.1 (Polyphenol)	6.15E-07
33.3	0.000198083

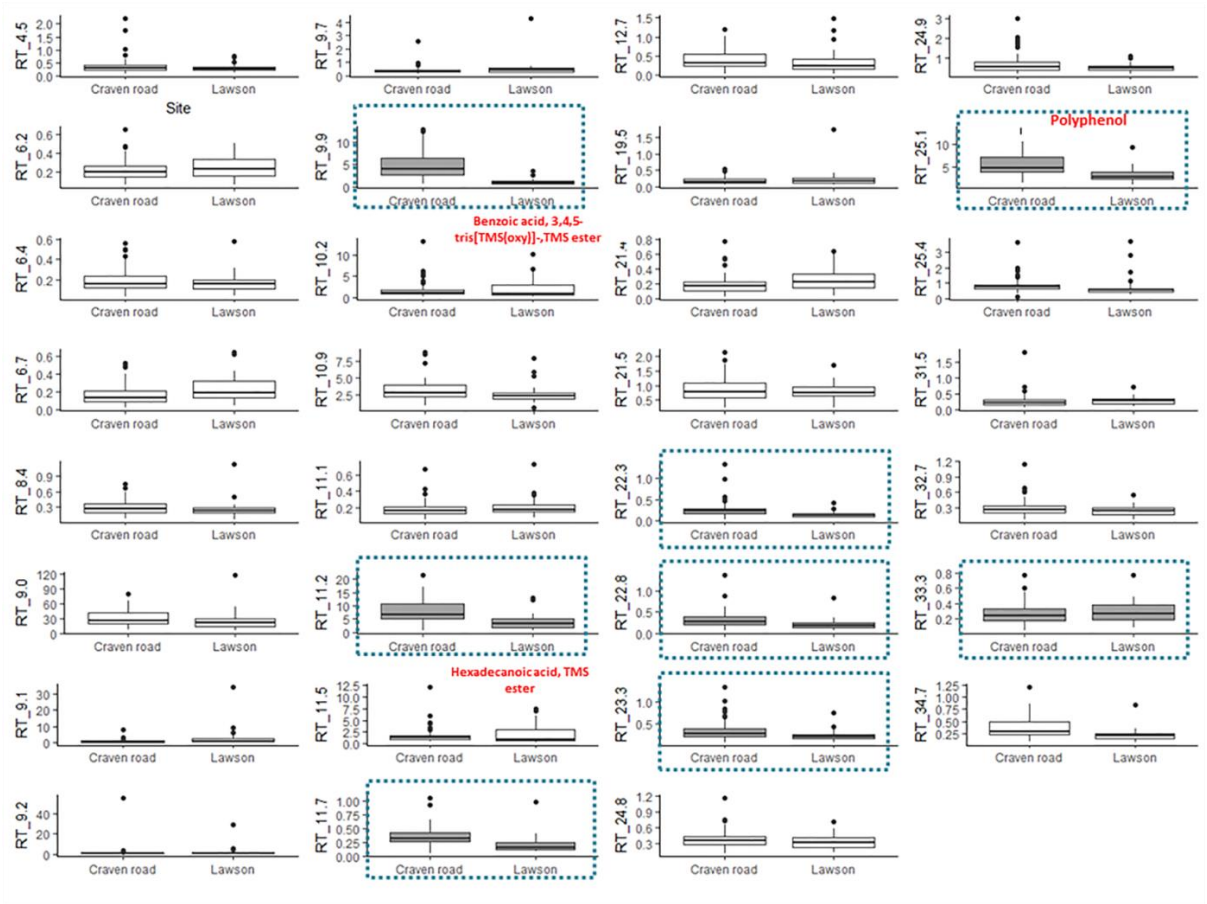

Figure 5.18: Box-and-whisker plots representing amount of heartwood compounds in *E. bosistoana* between Lawson and Craven Road. Eight compounds showing significant variation between the sites are highlighted by a box.

5.4 Conclusion

Ethanol extracts from *E. bosistoana* heartwood were less effective on the white rot *T. versicolor* with a relative growth rate of 82.96% than the brown rot *C. cerebella* (60.47%). No relationship was found between the growth rates of white rot and brown rot in extracts ($t = 0.086$, $p = 0.9316$) indicating that different compounds in the extracts inhibited the growth of the two fungi.

No correlation was observed between the extractive content in the wood and the bioactivity of the extracts against the brown rot ($R^2 = 0.004$). Extractive content in the wood had a negative influence on the bioactivity towards the white rot for the Lawson, but not the Craven Road site. This suggested that the trees with elevated extractive content at the Lawson site deposited more compounds into the heartwood, which were not bioactive against white rot.

Significant variability was found in the bioactivity of *E. bosistoana* heartwood extracts against white rot (stdev = 6.1 %, min = 69.0%, max = 94.8%) and brown rot (stdev = 12.5%, min =33.7%, max = 95.6%) between the trees. The difference in the relative growth rates of white rot between the sites

was small and only significant for white rot. Therefore, the site influence on the bioactivity of the heartwood extracts was small.

Thirty one compounds were quantified by GC in *E. bosistoana* ethanol extracts of which five were identified. Variation was present in composition of the extracts between trees and sites. Multivariate (PLSR) analysis identified compounds eluting at 10.2 and 11.5 min (Hexadecanoic acid, TMS ester) to be most related to the bioactivity of the *E. bosistoana* heartwood extracts against the tested white rot and brown rot. Significant variation in eight compounds (9.9, 11.2, 11.7, 22.3, 22.8, 23.3, 25.1, 33.3), out of 31 compounds was found between the sites. However, these did not have a large effect on the bioactivity of the heartwood extracts towards the two tested fungi.

5.5 References

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Appendix

Appendix 1: Extractive content (ethanol) of the *E. bosistoana* wood samples and the mean relative growth rates for white rot (*T. versicolor*) and brown rot (*C. cerebella*) when exposed to the extract as well as their standard deviation (SDEV) (n=5).

Samples	Sites	Extractive content (%)	Mean relative white rot growth rate (%)	SDEV relative white rot growth rate (%)	Mean relative brown rot growth rate (%)	SDEV relative brown rot growth rate (%)
B 105-36	Lawson	10.918	79.599	5.621	64.591	8.096
B 105-4	Lawson	11.334	89.346	3.165	49.292	4.427
B 113-27	Lawson	5.574	70.467	2.596	90.371	11.018
B 122-54	Lawson	9.424	74.466	3.883	71.939	29.843
B 127-76	Lawson	11.762	77.375	2.308	69.464	18.535
B 131-20	Lawson	10.274	84.496	1.026	79.642	16.610
B 131-24	Lawson	10.607	76.454	3.449	41.973	7.833
B 131-35	Lawson	7.396	81.113	2.226	95.598	2.496
B 134-18	Lawson	12.424	77.562	5.364	58.053	12.632
B 135-2	Lawson	10.918	91.506	3.532	79.454	9.025
B 136-31	Lawson	8.485	80.244	3.225	38.331	4.603
B 137-19	Lawson	8.216	76.454	1.806	54.881	6.494
B 139-2	Lawson	10.22	76.454	3.307	63.482	9.775
B 139-24	Lawson	9.444	83.485	4.384	51.852	5.734
B 139-37	Lawson	6.043	76.731	2.318	44.101	6.758
B 140-3	Lawson	14.789	82.609	7.346	66.474	11.156
B 140-34	Lawson	13.102	81.605	6.411	78.314	7.017
B 140-63	Lawson	10.562	82.898	2.971	39.064	17.225
B 142-4	Lawson	9.325	77.562	1.696	42.747	4.699
B 94-24	Lawson	10.244	72.853	4.656	36.312	5.940
B141-76	Lawson	10.204	87.681	7.711	36.316	3.378
BC 13-1	Craven road	6.131	90.651	2.799	64.519	8.733
BC 14	Craven road	6.009	94.660	1.317	57.873	5.133
BC 14-1	Craven road	4.762	78.358	1.689	53.240	8.690
BC 14-2	Craven road	5.002	73.130	3.843	69.117	3.059
BC 14-3	Craven road	4.399	83.234	2.897	59.919	5.019
BC 15-1	Craven road	17.190	90.956	1.128	57.550	3.010
BC 16-1	Craven road	5.125	76.177	3.115	45.324	10.279
BC 17-1	Craven road	4.984	73.938	2.718	56.495	8.288
BC 21-1	Craven road	3.932	92.873	5.225	45.813	4.828
BC 23-1	Craven road	2.797	90.830	2.206	60.924	8.198
BC 27	Craven road	5.133	90.792	2.087	41.992	4.781
BC 3	Craven road	3.293	77.987	4.100	73.468	6.949
BC 30	Craven road	4.392	87.823	3.774	66.350	2.652
BC 31	Craven road	2.719	79.735	1.107	66.741	2.598
BC 31-1	Craven road	3.018	85.220	6.028	66.741	10.211
BC 32	Craven road	2.956	93.396	8.936	60.712	12.290
BC 32-1	Craven road	2.758	86.011	3.279	68.161	5.338
BC 33-1	Craven road	5.675	81.990	3.332	53.699	3.916
BC 34-1	Craven road	4.444	86.341	3.037	50.955	12.643
BC 34-2	Craven road	3.773	79.354	1.965	71.848	15.690
BC 34-3	Craven road	3.69	88.153	1.410	68.161	10.692
BC 34-4	Craven road	3.514	79.715	2.935	78.038	15.531

Samples	Sites	Extractive content (%)	Relative white rot growth rate (%)	SDEV	Relative brown rot growth rate (%)	SDEV
BC 36-1	Craven road	5.570	91.153	1.706	58.261	5.405
BC 36-2	Craven road	3.959	87.993	3.848	54.268	5.971
BC 37-1	Craven road	4.71	76.395	2.989	66.896	22.281
BC 39-1	Craven road	1.926	81.132	5.167	72.073	6.635
BC 41	Craven road	7.506	85.554	6.091	64.187	5.232
BC 43-1	Craven road	6.625	82.976	6.018	63.787	6.001
BC 43-2	Craven road	5.743	90.818	4.220	59.491	5.190
BC 44	Craven road	6.026	80.536	1.779	40.741	8.356
BC 46-1	Craven road	6.93	76.731	3.476	52.366	9.733
BC 48	Craven road	3.873	76.464	2.003	47.941	3.603
BC 50-1	Craven road	5.606	85.625	4.575	54.146	3.733
BC 54-1	Craven road	2.084	69.030	2.459	54.102	13.069
BC 7	Craven road	4.327	83.214	3.839	76.725	10.547
BC 72	Craven road	3.746	80.393	3.127	61.531	9.676
BC 72-1	Craven road	3.457	90.384	1.619	58.450	6.756
BC 72-2	Craven road	5.647	71.785	2.066	48.420	8.777
BC4-1	Craven road	7.85	88.427	2.250	80.363	9.151
BC4-2	Craven road	3.75	82.865	1.999	46.452	6.695
BC4-A	Craven road	6.259	86.212	2.247	50.047	6.992
BC4-B	Craven road	2.809	81.548	1.916	66.013	11.056
BC9-1	Craven road	2.877	84.273	2.854	65.150	9.046
BC9-2	Craven road	7.593	88.407	2.648	73.333	12.059
BCX-1	Craven road	2.917	84.477	3.486	62.602	4.073
BCX-10	Craven road	2.917	86.658	1.995	71.895	8.761
BCX-11	Craven road	3.662	84.203	3.168	52.521	12.458
BCX-12	Craven road	2.492	78.873	2.846	58.588	9.270
BCX-13	Craven road	4.656	87.374	2.998	72.671	14.651
BCX-14	Craven road	3.261	90.208	2.854	33.733	6.854
BCX-15	Craven road	2.58	84.940	2.355	61.894	11.297
BCX-2	Craven road	2.61	79.874	6.230	66.322	9.713
BCX-3	Craven road	1.622	81.460	5.529	57.072	6.010
BCX-4	Craven road	3.553	82.151	3.464	65.235	8.992
BCX-5	Craven road	4.149	73.284	4.357	60.385	10.599
BCX-6	Craven road	2.957	88.723	3.347	53.177	13.737
BCX-8	Craven road	2.854	83.962	6.817	71.151	10.504
BCX-9	Craven road	1.323	73.422	3.812	76.510	8.002
BL 25	Lawson	12.219	78.158	5.481	55.929	7.425
BL 30-1	Lawson	14.014	91.724	2.657	67.968	8.799
BL 33	Lawson	11.46	94.834	2.740	53.618	5.169
BL 4	Lawson	4.34	75.900	2.838	68.601	3.833
BL 44	Lawson	6.881	79.242	1.940	51.703	5.768
BL 5	Lawson	11.794	87.885	3.006	62.001	10.704
BL 51	Lawson	7.418	89.826	2.780	82.320	11.278
BLX-1	Lawson	10.681	79.384	2.466	52.638	5.557
BLX-2	Lawson	14.673	87.327	2.419	65.460	5.470
BLX-3	Lawson	10.901	83.616	4.124	49.065	3.771
BLX-5	Lawson	10.010	89.967	5.208	78.571	4.871
BLX-6	Lawson	9.599	87.537	4.451	46.197	13.475

Appendix 2: Tukey test comparing the relative growth rates of both the fungi between all samples

Tukey multiple comparisons of means

95% family-wise confidence level

Fit: aov(formula = RGR ~ Sample + Site, data = NCBR)

Sample	diff	lwr	upr	p adj
B 113-27-B 105-4	41.07981582	12.23186227	69.92776937	0.0001338
B 122-54-B 105-4	22.64790385	-6.2000497	51.4958574	0.3657575
B 127-76-B 105-4	20.17197538	-8.67597817	49.01992893	0.60412
B 131-20-B 105-4	30.35079243	1.50283889	59.19874598	0.0271708
B 131-35-B 105-4	46.30677593	17.45882238	75.15472948	0.0000066
B 136-31-B 105-4	-10.96053265	-39.8084862	17.8874209	0.9994176
B 139-24-B 105-4	2.5602784	-26.28767515	31.40823195	1
B141-76-B 105-4	-12.9753915	-41.82334505	15.87256205	0.9931551
BC-31-B 105-4	17.44966443	-11.39828912	46.29761798	0.8434763
BC-44-B 105-4	-8.55083271	-37.39878626	20.29712084	0.9999925
BC-72-B 105-4	12.23983416	-16.60811939	41.08778771	0.9969278
BC 34-2-B 105-4	22.5562028	-6.29175075	51.40415635	0.3738544
BC 34-4-B 105-4	28.74602398	-0.10192957	57.59397753	0.0520307
BC 37-1-B 105-4	17.60434585	-11.2436077	46.4522994	0.8324878
BC4-1-B 105-4	31.07130163	2.22334809	59.91925518	0.0199918
BC9-1-B 105-4	15.85846144	-12.98949211	44.70641499	0.9318081
BCX-12-B 105-4	9.29654487	-19.55140868	38.14449842	0.9999646
BCX-14-B 105-4	-15.55814707	-44.40610062	13.28980648	0.9434432
BCX-15-B 105-4	12.60253542	-16.24541813	41.45048897	0.9953805
BCX-3-B 105-4	7.78026348	-21.06769006	36.62821703	0.9999988
BCX-5-B 105-4	11.09357098	-17.75438257	39.94152453	0.9992953
BCX-9-B 105-4	27.21849366	-1.62945989	56.06644721	0.0921092
BL-25-B 105-4	6.63746483	-22.21048872	35.48541838	1
BL-44-B 105-4	2.41113597	-26.43681758	31.25908952	1
BLX-6-B 105-4	-3.0947835	-31.94273705	25.75317005	1
B 122-54-B 113-27	-18.43191197	-47.27986552	10.41604158	0.7672013
B 127-76-B 113-27	-20.90784044	-49.75579399	7.940113109	0.5307805
B 131-20-B 113-27	-10.72902338	-39.57697693	18.11893017	0.9995865
B 131-35-B 113-27	5.22696011	-23.62099344	34.07491366	1
B 136-31-B 113-27	-52.04034846	-80.88830201	-23.19239491	0.0000002
B 139-24-B 113-27	-38.51953742	-67.36749097	-9.67158387	0.000538
B141-76-B 113-27	-54.05520732	-82.90316087	-25.20725377	0.0000001
BC-31-B 113-27	-23.63015139	-52.47810494	5.217802161	0.2849173
BC-44-B 113-27	-49.63064853	-78.47860208	-20.78269498	0.0000009

BC-72-B 113-27	-28.83998166	-57.68793521	0.00797189	0.0501563
BC 34-2-B 113-27	-18.52361302	-47.37156657	10.32434053	0.759354
BC 34-4-B 113-27	-12.33379184	-41.18174539	16.51416171	0.9965769
BC 37-1-B 113-27	-23.47546997	-52.32342352	5.372483582	0.2968872
BC4-1-B 113-27	-10.00851418	-38.85646773	18.83943937	0.9998702
BC9-1-B 113-27	-25.22135438	-54.06930793	3.62659917	0.1796908
BCX-12-B 113-27	-31.78327095	-60.6312245	-2.935317402	0.0146371
BCX-14-B 113-27	-56.63796289	-85.48591644	-27.79000934	0
BCX-15-B 113-27	-28.4772804	-57.32523395	0.370673153	0.0577317
BCX-3-B 113-27	-33.29955233	-62.14750588	-4.451598784	0.0073377
BCX-5-B 113-27	-29.98624484	-58.83419839	-1.138291292	0.0316239
BCX-9-B 113-27	-13.86132216	-42.70927571	14.98663139	0.984194
BL-25-B 113-27	-34.44235099	-63.29030454	-5.594397439	0.0042649
BL-44-B 113-27	-38.66867985	-67.5166334	-9.820726301	0.0004969
BLX-6-B 113-27	-44.17459932	-73.02255287	-15.32664577	0.000023
B 127-76-B 122-54	-2.47592847	-31.32388202	26.37202508	1
B 131-20-B 122-54	7.70288858	-21.14506497	36.55084213	0.999999
B 131-35-B 122-54	23.65887208	-5.18908147	52.50682563	0.2827277
B 136-31-B 122-54	-33.6084365	-62.45639005	-4.760482948	0.0063482
B 139-24-B 122-54	-20.08762545	-48.935579	8.760328097	0.612486
B141-76-B 122-54	-35.62329535	-64.4712489	-6.775341801	0.00239
BC-31-B 122-54	-5.19823942	-34.04619297	23.64971413	1
BC-44-B 122-54	-31.19873656	-60.04669011	-2.350783014	0.0189184
BC-72-B 122-54	-10.40806969	-39.25602324	18.43988386	0.9997487
BC 34-2-B 122-54	-0.09170105	-28.9396546	28.7562525	1
BC 34-4-B 122-54	6.09812013	-22.74983342	34.94607368	1
BC 37-1-B 122-54	-5.043558	-33.89151155	23.80439555	1
BC4-1-B 122-54	8.42339778	-20.42455577	37.27135133	0.9999943
BC9-1-B 122-54	-6.78944241	-35.63739596	22.05851114	0.9999999
BCX-12-B 122-54	-13.35135898	-42.19931253	15.49659457	0.9900782
BCX-14-B 122-54	-38.20605093	-67.05400448	-9.358097376	0.000635
BCX-15-B 122-54	-10.04536843	-38.89332198	18.80258512	0.9998618
BCX-3-B 122-54	-14.86764037	-43.71559392	13.98031318	0.9647155
BCX-5-B 122-54	-11.55433287	-40.40228642	17.29362068	0.9986804
BCX-9-B 122-54	4.57058981	-24.27736374	33.41854336	1
BL-25-B 122-54	-16.01043902	-44.85839257	12.83751453	0.9253313
BL-44-B 122-54	-20.23676788	-49.08472143	8.611185666	0.5976809
BLX-6-B 122-54	-25.74268735	-54.5906409	3.105266197	0.1523193
B 131-20-B 127-76	10.17881706	-18.66913649	39.02677061	0.999827
B 131-35-B 127-76	26.13480055	-2.713153	54.9827541	0.1339279
B 136-31-B 127-76	-31.13250802	-59.98046157	-2.284554474	0.0194695
B 139-24-B 127-76	-17.61169698	-46.45965053	11.23625657	0.8319555
B141-76-B 127-76	-33.14736688	-61.99532043	-4.299413328	0.0078766
BC-31-B 127-76	-2.72231095	-31.5702645	26.1256426	1
BC-44-B 127-76	-28.72280809	-57.57076164	0.125145459	0.0525031

BC-72-B 127-76	-7.93214122	-36.78009477	20.91581233	0.9999982
BC 34-2-B 127-76	2.38422742	-26.46372613	31.23218097	1
BC 34-4-B 127-76	8.5740486	-20.27390495	37.42200215	0.9999921
BC 37-1-B 127-76	-2.56762953	-31.41558308	26.28032402	1
BC4-1-B 127-76	10.89932626	-17.94862729	39.74727981	0.9994673
BC9-1-B 127-76	-4.31351394	-33.16146749	24.53443961	1
BCX-12-B 127-76	-10.87543051	-39.72338406	17.97252304	0.9994857
BCX-14-B 127-76	-35.73012245	-64.578076	-6.882168903	0.002266
BCX-15-B 127-76	-7.56943996	-36.41739351	21.27851359	0.9999993
BCX-3-B 127-76	-12.39171189	-41.23966544	16.45624166	0.9963441
BCX-5-B 127-76	-9.0784044	-37.92635795	19.76954915	0.999977
BCX-9-B 127-76	7.04651828	-21.80143527	35.89447183	0.9999998
BL-25-B 127-76	-13.53451055	-42.3824641	15.313443	0.9882148
BL-44-B 127-76	-17.76083941	-46.60879296	11.08711414	0.8209623
BLX-6-B 127-76	-23.26675888	-52.11471243	5.58119467	0.3135045
B 131-35-B 131-20	15.95598349	-12.89197006	44.80393704	0.9276983
B 136-31-B 131-20	-41.31132508	-70.15927863	-12.46337153	0.0001176
B 139-24-B 131-20	-27.79051404	-56.63846758	1.057439514	0.0748008
B141-76-B 131-20	-43.32618393	-72.17413748	-14.47823038	0.0000376
BC-31-B 131-20	-12.90112801	-41.74908155	15.94682554	0.9936578
BC-44-B 131-20	-38.90162515	-67.7495787	-10.0536716	0.0004389
BC-72-B 131-20	-18.11095828	-46.95891183	10.73699527	0.7937535
BC 34-2-B 131-20	-7.79458964	-36.64254319	21.05336391	0.9999988
BC 34-4-B 131-20	-1.60476845	-30.452722	27.2431851	1
BC 37-1-B 131-20	-12.74644658	-41.59440013	16.10150697	0.9946069
BC4-1-B 131-20	0.7205092	-28.12744435	29.56846275	1
BC9-1-B 131-20	-14.492331	-43.34028454	14.35562255	0.9734014
BCX-12-B 131-20	-21.05424757	-49.90220112	7.793705982	0.5162466
BCX-14-B 131-20	-45.90893951	-74.75689306	-17.06098596	0.0000083
BCX-15-B 131-20	-17.74825701	-46.59621056	11.09969654	0.8219038
BCX-3-B 131-20	-22.57052895	-51.4184825	6.2774246	0.3725838
BCX-5-B 131-20	-19.25722146	-48.10517501	9.590732091	0.6930189
BCX-9-B 131-20	-3.13229877	-31.98025232	25.71565478	1
BL-25-B 131-20	-23.71332761	-52.56128115	5.134625944	0.2786047
BL-44-B 131-20	-27.93965647	-56.78761002	0.908297083	0.0707682
BLX-6-B 131-20	-33.44557594	-62.29352949	-4.597622387	0.0068532
B 136-31-B 131-35	-57.26730857	-86.11526212	-28.41935503	0
B 139-24-B 131-35	-43.74649753	-72.59445108	-14.89854398	0.0000295
B141-76-B 131-35	-59.28216743	-88.13012098	-30.43421388	0
BC-31-B 131-35	-28.8571115	-57.70506505	-0.009157949	0.049821
BC-44-B 131-35	-54.85760864	-83.70556219	-26.00965509	0
BC-72-B 131-35	-34.06694177	-62.91489532	-5.21898822	0.0051072
BC 34-2-B 131-35	-23.75057313	-52.59852668	5.097380418	0.2758063
BC 34-4-B 131-35	-17.56075195	-46.4087055	11.2872016	0.8356258
BC 37-1-B 131-35	-28.70243008	-57.55038363	0.145523472	0.0529208

BC4-1-B 131-35	-15.23547429	-44.08342784	13.61247926	0.9542938
BC9-1-B 131-35	-30.44831449	-59.29626804	-1.60036094	0.0260792
BCX-12-B 131-35	-37.01023106	-65.85818461	-8.162277512	0.0011842
BCX-14-B 131-35	-61.864923	-90.71287655	-33.01696945	0
BCX-15-B 131-35	-33.70424051	-62.55219406	-4.856286958	0.0060676
BCX-3-B 131-35	-38.52651244	-67.37446599	-9.678558894	0.000536
BCX-5-B 131-35	-35.21320495	-64.0611585	-6.365251402	0.0029283
BCX-9-B 131-35	-19.08828227	-47.93623582	9.759671283	0.7087949
BL-25-B 131-35	-39.6693111	-68.51726465	-10.82135755	0.0002902
BL-44-B 131-35	-43.89563996	-72.74359351	-15.04768641	0.0000271
BLX-6-B 131-35	-49.40155943	-78.24951298	-20.55360588	0.000001
B 139-24-B 136-31	13.52081104	-15.3271425	42.36876459	0.9883634
B141-76-B 136-31	-2.01485885	-30.8628124	26.8330947	1
BC-31-B 136-31	28.41019708	-0.43775647	57.25815063	0.0592363
BC-44-B 136-31	2.40969993	-26.43825362	31.25765348	1
BC-72-B 136-31	23.2003668	-5.64758675	52.04832035	0.3189005
BC 34-2-B 136-31	33.51673544	4.66878189	62.36468899	0.0066281
BC 34-4-B 136-31	39.70655663	10.85860308	68.55451018	0.0002844
BC 37-1-B 136-31	28.5648785	-0.28307505	57.41283205	0.0558168
BC4-1-B 136-31	42.03183428	13.18388073	70.87978783	0.0000785
BC9-1-B 136-31	26.81899408	-2.02895946	55.66694763	0.1060685
BCX-12-B 136-31	20.25707751	-8.59087604	49.10503106	0.5956606
BCX-14-B 136-31	-4.59761443	-33.44556798	24.25033912	1
BCX-15-B 136-31	23.56306807	-5.28488548	52.41102162	0.2900719
BCX-3-B 136-31	18.74079613	-10.10715742	47.58874968	0.7403419
BCX-5-B 136-31	22.05410362	-6.79384993	50.90205717	0.4196161
BCX-9-B 136-31	38.17902631	9.33107276	67.02697986	0.0006442
BL-25-B 136-31	17.59799748	-11.24995607	46.44595103	0.8329468
BL-44-B 136-31	13.37166861	-15.47628494	42.21962216	0.9898843
BLX-6-B 136-31	7.86574914	-20.98220441	36.71370269	0.9999985
B141-76-B 139-24	-15.5356699	-44.38362345	13.31228365	0.9442534
BC-31-B 139-24	14.88938603	-13.95856752	43.73733958	0.9641539
BC-44-B 139-24	-11.11111111	-39.95906466	17.73684244	0.9992776
BC-72-B 139-24	9.67955576	-19.16839779	38.52750931	0.9999273
BC 34-2-B 139-24	19.9959244	-8.85202915	48.84387795	0.6215556
BC 34-4-B 139-24	26.18574558	-2.66220797	55.03369913	0.1316718
BC 37-1-B 139-24	15.04406745	-13.8038861	43.892021	0.9599651
BC4-1-B 139-24	28.51102324	-0.33693031	57.35897679	0.0569875
BC9-1-B 139-24	13.29818304	-15.54977051	42.14613659	0.9905718
BCX-12-B 139-24	6.73626647	-22.11168708	35.58422002	0.9999999
BCX-14-B 139-24	-18.11842547	-46.96637902	10.72952808	0.7931526
BCX-15-B 139-24	10.04225702	-18.80569653	38.89021057	0.9998625
BCX-3-B 139-24	5.21998509	-23.62796846	34.06793864	1
BCX-5-B 139-24	8.53329258	-20.31466097	37.38124613	0.9999927
BCX-9-B 139-24	24.65821526	-4.18973829	53.50616881	0.213157

BL-25-B 139-24	4.07718643	-24.77076712	32.92513998	1
BL-44-B 139-24	-0.14914243	-28.99709598	28.69881112	1
BLX-6-B 139-24	-5.6550619	-34.50301545	23.19289165	1
BC-31-B141-76	30.42505593	1.57710238	59.27300948	0.0263359
BC-44-B141-76	4.42455879	-24.42339476	33.27251234	1
BC-72-B141-76	25.21522566	-3.63272789	54.06317921	0.1800329
BC 34-2-B141-76	35.5315943	6.68364075	64.37954785	0.0025015
BC 34-4-B141-76	41.72141548	12.87346193	70.56936903	0.0000935
BC 37-1-B141-76	30.57973735	1.7317838	59.4276909	0.0246708
BC4-1-B141-76	44.04669313	15.19873958	72.89464668	0.0000248
BC9-1-B141-76	28.83385294	-0.01410061	57.68180649	0.0502767
BCX-12-B141-76	22.27193637	-6.57601718	51.11988992	0.3994803
BCX-14-B141-76	-2.58275558	-31.43070913	26.26519797	1
BCX-15-B141-76	25.57792692	-3.27002663	54.42588047	0.1606028
BCX-3-B141-76	20.75565498	-8.09229857	49.60360853	0.545936
BCX-5-B141-76	24.06896248	-4.77899107	52.91691603	0.2526095
BCX-9-B141-76	40.19388516	11.34593161	69.04183871	0.0002181
BL-25-B141-76	19.61285633	-9.23509722	48.46080988	0.6590406
BL-44-B141-76	15.38652747	-13.46142608	44.23448102	0.949421
BLX-6-B141-76	9.880608	-18.96734555	38.72856155	0.999896
BC-44-BC-31	-26.00049714	-54.84845069	2.847456408	0.1400208
BC-72-BC-31	-5.20983027	-34.05778382	23.63812328	1
BC 34-2-BC-31	5.10653837	-23.74141518	33.95449192	1
BC 34-4-BC-31	11.29635955	-17.551594	40.1443131	0.9990654
BC 37-1-BC-31	0.15468142	-28.69327213	29.00263497	1
BC4-1-BC-31	13.62163721	-15.22631634	42.46959076	0.9872342
BC9-1-BC-31	-1.59120299	-30.43915654	27.25675056	1
BCX-12-BC-31	-8.15311956	-37.00107311	20.69483399	0.999997
BCX-14-BC-31	-33.0078115	-61.85576505	-4.159857954	0.0084029
BCX-15-BC-31	-4.84712901	-33.69508256	24.00082454	1
BCX-3-BC-31	-9.66940094	-38.51735449	19.17855261	0.9999286
BCX-5-BC-31	-6.35609345	-35.204047	22.4918601	1
BCX-9-BC-31	9.76882923	-19.07912432	38.61678278	0.9999146
BL-25-BC-31	-10.8121996	-39.66015315	18.03575395	0.9995316
BL-44-BC-31	-15.03852846	-43.88648201	13.80942509	0.9601211
BLX-6-BC-31	-20.54444793	-49.39240148	8.303505618	0.5670101
BC-72-BC-44	20.79066687	-8.05728668	49.63862042	0.5424461
BC 34-2-BC-44	31.10703551	2.25908196	59.95498906	0.0196853
BC 34-4-BC-44	37.29685669	8.44890314	66.14481024	0.0010214
BC 37-1-BC-44	26.15517856	-2.69277499	55.00313211	0.1330218
BC4-1-BC-44	39.62213435	10.7741808	68.4700879	0.0002978
BC9-1-BC-44	24.40929415	-4.4386594	53.2572477	0.2292685
BCX-12-BC-44	17.84737758	-11.00057597	46.69533113	0.8144168
BCX-14-BC-44	-7.00731436	-35.85526791	21.84063919	0.9999999
BCX-15-BC-44	21.15336813	-7.69458542	50.00132168	0.5064423

BCX-3-BC-44	16.3310962	-12.51685735	45.17904975	0.9103283
BCX-5-BC-44	19.64440369	-9.20354986	48.49235724	0.6559835
BCX-9-BC-44	35.76932637	6.92137282	64.61727992	0.0022221
BL-25-BC-44	15.18829754	-13.65965601	44.03625109	0.9557431
BL-44-BC-44	10.96196868	-17.88598487	39.80992223	0.9994164
BLX-6-BC-44	5.45604921	-23.39190434	34.30400276	1
BC 34-2-BC-72	10.31636864	-18.53158491	39.16432219	0.9997832
BC 34-4-BC-72	16.50618982	-12.34176373	45.35414337	0.9013565
BC 37-1-BC-72	5.36451169	-23.48344186	34.21246524	1
BC4-1-BC-72	18.83146748	-10.01648607	47.67942103	0.7322372
BC9-1-BC-72	3.61862728	-25.22932627	32.46658083	1
BCX-12-BC-72	-2.94328929	-31.79124284	25.90466426	1
BCX-14-BC-72	-27.79798123	-56.64593478	1.049972317	0.0745944
BCX-15-BC-72	0.36270126	-28.48525229	29.21065481	1
BCX-3-BC-72	-4.45957067	-33.30752422	24.38838288	1
BCX-5-BC-72	-1.14626318	-29.99421673	27.70169037	1
BCX-9-BC-72	14.9786595	-13.86929405	43.82661305	0.9617784
BL-25-BC-72	-5.60236933	-34.45032288	23.24558422	1
BL-44-BC-72	-9.82869819	-38.67665174	19.01925536	0.999905
BLX-6-BC-72	-15.33461766	-44.18257121	13.51333589	0.9511359
BC 34-4-BC 34-2	6.18982118	-22.65813237	35.03777473	1
BC 37-1-BC 34-2	-4.95185695	-33.7998105	23.8960966	1
BC4-1-BC 34-2	8.51509884	-20.33285471	37.36305239	0.999993
BC9-1-BC 34-2	-6.69774136	-35.54569491	22.15021219	0.9999999
BCX-12-BC 34-2	-13.25965793	-42.10761148	15.58829562	0.9909167
BCX-14-BC 34-2	-38.11434987	-66.96230342	-9.266396322	0.0006665
BCX-15-BC 34-2	-9.95366738	-38.80162092	18.89428617	0.9998819
BCX-3-BC 34-2	-14.77593931	-43.62389286	14.07201424	0.9670112
BCX-5-BC 34-2	-11.46263182	-40.31058537	17.38532173	0.9988306
BCX-9-BC 34-2	4.66229086	-24.18566268	33.51024441	1
BL-25-BC 34-2	-15.91873797	-44.76669152	12.92921558	0.9292874
BL-44-BC 34-2	-20.14506683	-48.99302038	8.702886721	0.606791
BLX-6-BC 34-2	-25.6509863	-54.49893985	3.196967251	0.1568884
BC 37-1-BC 34-4	-11.14167813	-39.98963168	17.70627542	0.9992458
BC4-1-BC 34-4	2.32527765	-26.52267589	31.1732312	1
BC9-1-BC 34-4	-12.88756254	-41.73551609	15.96039101	0.9937463
BCX-12-BC 34-4	-19.44947911	-48.29743266	9.398474437	0.6747672
BCX-14-BC 34-4	-44.30417105	-73.1521246	-15.45621751	0.0000214
BCX-15-BC 34-4	-16.14348856	-44.99144211	12.70446499	0.9193285
BCX-3-BC 34-4	-20.96576049	-49.81371404	7.882193055	0.5250242
BCX-5-BC 34-4	-17.652453	-46.50040655	11.19550055	0.828988
BCX-9-BC 34-4	-1.52753032	-30.37548387	27.32042323	1
BL-25-BC 34-4	-22.10855915	-50.9565127	6.739394399	0.4145444
BL-44-BC 34-4	-26.33488801	-55.18284156	2.513065538	0.1252393
BLX-6-BC 34-4	-31.84080748	-60.68876103	-2.992853932	0.0142678

BC4-1-BC 37-1	13.46695578	-15.38099777	42.31490933	0.9889326
BC9-1-BC 37-1	-1.74588441	-30.59383796	27.10206914	1
BCX-12-BC 37-1	-8.30780098	-37.15575453	20.54015257	0.9999957
BCX-14-BC 37-1	-33.16249292	-62.01044647	-4.314539375	0.0078214
BCX-15-BC 37-1	-5.00181043	-33.84976398	23.84614312	1
BCX-3-BC 37-1	-9.82408237	-38.67203591	19.02387118	0.9999058
BCX-5-BC 37-1	-6.51077487	-35.35872842	22.33717868	1
BCX-9-BC 37-1	9.61414781	-19.23380574	38.46210136	0.9999355
BL-25-BC 37-1	-10.96688102	-39.81483457	17.88107253	0.9994122
BL-44-BC 37-1	-15.19320988	-44.04116343	13.65474367	0.9555937
BLX-6-BC 37-1	-20.69912935	-49.5470829	8.148824198	0.5515734
BC9-1-BC4-1	-15.2128402	-44.06079374	13.63511335	0.9549933
BCX-12-BC4-1	-21.77475677	-50.62271032	7.073196782	0.4459973
BCX-14-BC4-1	-46.62944871	-75.47740226	-17.78149516	0.0000054
BCX-15-BC4-1	-18.46876621	-47.31671976	10.37918734	0.7640608
BCX-3-BC4-1	-23.29103815	-52.1389917	5.5569154	0.3115443
BCX-5-BC4-1	-19.97773066	-48.82568421	8.870222891	0.6233514
BCX-9-BC4-1	-3.85280797	-32.70076152	24.99514558	1
BL-25-BC4-1	-24.43383681	-53.28179035	4.414116744	0.2276439
BL-44-BC4-1	-28.66016567	-57.50811922	0.187787883	0.0537965
BLX-6-BC4-1	-34.16608514	-63.01403869	-5.318131587	0.0048707
BCX-12-BC9-1	-6.56191657	-35.40987012	22.28603698	1
BCX-14-BC9-1	-31.41660851	-60.26456206	-2.568654964	0.0172042
BCX-15-BC9-1	-3.25592602	-32.10387957	25.59202753	1
BCX-3-BC9-1	-8.07819795	-36.9261515	20.7697556	0.9999975
BCX-5-BC9-1	-4.76489046	-33.61284401	24.08306309	1
BCX-9-BC9-1	11.36003222	-17.48792133	40.20798577	0.9989809
BL-25-BC9-1	-9.22099661	-38.06895016	19.62695694	0.9999694
BL-44-BC9-1	-13.44732547	-42.29527902	15.40062808	0.9891343
BLX-6-BC9-1	-18.95324494	-47.80119849	9.894708609	0.7212075
BCX-14-BCX-12	-24.85469194	-53.70264549	3.993261608	0.2010128
BCX-15-BCX-12	3.30599055	-25.541963	32.1539441	1
BCX-3-BCX-12	-1.51628138	-30.36423493	27.33167217	1
BCX-5-BCX-12	1.79702611	-27.05092744	30.64497966	1
BCX-9-BCX-12	17.92194879	-10.92600476	46.76990234	0.8086804
BL-25-BCX-12	-2.65908004	-31.50703359	26.18887351	1
BL-44-BCX-12	-6.8854089	-35.73336245	21.96254465	0.9999999
BLX-6-BCX-12	-12.39132837	-41.23928192	16.45662518	0.9963457
BCX-15-BCX-14	28.1606825	-0.68727105	57.00863605	0.0651326
BCX-3-BCX-14	23.33841056	-5.50954299	52.18636411	0.3077401
BCX-5-BCX-14	26.65171805	-2.1962355	55.4996716	0.1124043
BCX-9-BCX-14	42.77664074	13.92868719	71.62459429	0.0000515
BL-25-BCX-14	22.1956119	-6.65234165	51.04356545	0.4064888
BL-44-BCX-14	17.96928304	-10.87867051	46.81723659	0.8049938
BLX-6-BCX-14	12.46336357	-16.38458998	41.31131712	0.9960377

BCX-3-BCX-15	-4.82227194	-33.67022549	24.02568161	1
BCX-5-BCX-15	-1.50896444	-30.35691799	27.33898911	1
BCX-9-BCX-15	14.61595824	-14.23199531	43.46391179	0.9707442
BL-25-BCX-15	-5.96507059	-34.81302414	22.88288296	1
BL-44-BCX-15	-10.19139945	-39.039353	18.6565541	0.9998234
BLX-6-BCX-15	-15.69731892	-44.54527247	13.15063463	0.9382404
BCX-5-BCX-3	3.31330749	-25.53464606	32.16126104	1
BCX-9-BCX-3	19.43823018	-9.40972337	48.28618373	0.6758431
BL-25-BCX-3	-1.14279866	-29.99075221	27.70515489	1
BL-44-BCX-3	-5.36912752	-34.21708107	23.47882603	1
BLX-6-BCX-3	-10.87504699	-39.72300054	17.97290656	0.999486
BCX-9-BCX-5	16.12492268	-12.72303086	44.97287623	0.920185
BL-25-BCX-5	-4.45610615	-33.3040597	24.3918474	1
BL-44-BCX-5	-8.68243501	-37.53038856	20.16551854	0.9999899
BLX-6-BCX-5	-14.18835448	-43.03630803	14.65959907	0.9791472
BL-25-BCX-9	-20.58102883	-49.42898238	8.266924717	0.5633589
BL-44-BCX-9	-24.80735769	-53.65531124	4.040595856	0.2038925
BLX-6-BCX-9	-30.31327716	-59.16123071	-1.465323613	0.0276015
BL-44-BL-25	-4.22632886	-33.07428241	24.62162469	1
BLX-6-BL-25	-9.73224833	-38.58020188	19.11570522	0.99992
BLX-6-BL-44	-5.50591947	-34.35387302	23.34203408	1

Appendix 3: Loadings of the 1st and 2nd principal components for partial least squares regression (PLS R) model of the growth of the white rot *T. versicolor* by the 31 quantified compounds in *E. bosistoana* heartwood ethanol extracts.

Retention times	Pc 1	Pc 2
4.5	0.268	-0.020
6.2	0.192	-0.335
6.4	0.289	-0.238
6.7	0.165	-0.338
8.4	0.299	-0.198
9.0	0.295	-0.165
9.1	0.153	-0.079
9.2	0.047	-0.141
9.7	0.242	-0.229
9.9	0.233	-0.042
10.2	0.122	0.392
10.9	0.307	-0.167
11.1	0.262	-0.135
11.2	0.268	-0.086
11.5	0.162	0.290
11.7	0.311	-0.079
12.7	0.218	-0.057
19.5	0.161	-0.221
21.4	0.139	-0.154
21.5	0.244	-0.205
22.3	0.259	-0.151
22.8	0.315	-0.119
23.3	0.292	-0.133
24.8	0.247	-0.113
24.9	0.229	-0.056
25.1	0.275	0.035
25.4	0.110	-0.286
31.5	0.141	-0.315
32.7	0.306	-0.034
33.3	0.178	-0.226
34.7	0.293	-0.152

plsmod2$R2	
t1	0.03912522
t2	0.09245184
Sum (plsmod2$R2)	0.1315771

The results of Appendix 3 shows that the two PLS components have a r-square of 0.133 by adding val ues of t1 and t2 in predicting white rot (*T. versicolor*) relative growth rate (WRGR). Results can be fu rther simplified by the total variance in relative growth rate explained by the first two principle compo nents was 13.2% (9.2% and 3.9%, respectively) for white rot (*T. versicolor*).

Appendix 4: Loadings of the 1st and 2nd principal components for partial least squares regression (PL SR) model of the growth of the brown rot (*C. cerebella*) by the 31 quantified compounds in *E. bosisto ana* heartwood ethanol extracts

Retention times	Pc 1	Pc 2
4.5	-0.205	0.269
6.2	-0.574	0.074
6.4	-0.524	0.200
6.7	-0.531	0.064
8.4	-0.516	0.202
9.0	-0.511	0.191
9.1	-0.272	0.098
9.2	-0.136	0.033
9.7	-0.433	0.179
9.9	-0.209	0.215
10.2	0.185	0.151
10.9	-0.505	0.210
11.1	-0.419	0.190
11.2	-0.287	0.234
11.5	0.058	0.170
11.7	-0.352	0.264
12.7	-0.423	0.096
19.5	-0.407	0.078
21.4	-0.240	0.111
21.5	-0.466	0.154
22.3	-0.248	0.262
22.8	-0.346	0.284
23.3	-0.318	0.269
24.8	-0.261	0.236
24.9	-0.299	0.178
25.1	-0.375	0.176
25.4	-0.194	0.126
31.5	-0.304	0.126
32.7	-0.330	0.262
33.3	-0.402	0.106
34.7	-0.369	0.255

plsmod3$R3	
t1	0.08909579
t2	0.06913363
Sum (plsmod3$R3)	0.1582294

The results of Appendix 4 shows that the two PLS components have a r-square of 0.158 by adding values of t1 and t2 in predicting brown rot (*C. cerebella*) relative growth rate (BRGR). Results can be further simplified by the total variance in relative growth rate explained by the first two principle components was 15.8% (8.9% and 6.9%, respectively) for brown Rot (*C. cerebella*)