UNIVERSITY OF KWAZULU-NATAL

SYNTHESIS, CHARACTERISATION AND BIOLOGICAL ACTIVITIES OF HOMOISOFлавONOIDS

2012

KAALIN GOPAUL
SYNTHESIS, CHARACTERISATION AND BIOLOGICAL ACTIVITIES OF HOMOISOFлавONOIDS

KAALIN GOPAUL
2012

A thesis submitted to the School of Chemistry and Physics, University of KwaZulu-Natal, Westville, for the degree of Masters of Science.

As the candidate’s supervisor, I have approved this thesis for submission.

Supervisor:

Signed: ---------------------------Name: -------------------------- Date: --------------
To my family...
ज्ञान शक्ति है।

“Knowledge is power”
DECLARATIONS

DECLARATION 1- PLAGIARISM

I, **KAALIN GOPAUL**, declare that the experimental work described in this dissertation was carried out at the School of Chemistry and Physics, University of KwaZulu-Natal, Westville campus under the supervision of Dr. N. A. Koorbanally, and that:

1. The research reported in this thesis is my original research, except where otherwise indicated.

2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other persons’ data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

4. This thesis does not contain other persons’ writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:
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5. This thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References sections.

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v
DECLARATION 2- PUBLICATIONS

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis.

Publication 1
Kaalin Gopaul, Mahidansha Shaikh, Deresh Ramjugernath, Neil A. Koorbanally and Bernard Omondia, 3-(3-Methoxybenzylidene)chroman-4-one, Acta Crystallographica Section E, Accepted for publication: 4 March 2012

Publication 2
Kaalin Gopaul, Mahidansha M. Shaikh, Neil A. Koorbanally, Deresh Ramjugernath and Bernard Omondia, (E)-3-(4-Cyclohexyl-3-fluorobenzylidene)chroman-4-one, Acta Crystallographica Section E, Accepted for publication: 28 May 2012

Publication 3
Kaalin Gopaul, Neil Anthony Koorbanally, Mahidansha M. Shaikh, Hong Su and Deresh Ramjugernath, 3-(3,4-Dichlorobenzylidene)chroman-4-one, Acta Crystallographica Section E, Accepted for publication: 25 September 2012

From all the above publications, my role included carrying out the experimental work and contributing to the writing of the publications along with my supervisor. The other co-authors contribution was that of an editorial nature and checking on the scientific content and my correct interpretation. Based on their expertise, they have added minor parts to the manuscripts.

Signed: ...........................................
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<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>$^1$H NMR</td>
<td>proton nuclear magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>$^{13}$C NMR</td>
<td>carbon-13 nuclear magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>$^{19}$F NMR</td>
<td>fluorine-19 nuclear magnetic resonance spectroscopy</td>
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<tr>
<td>DEPT</td>
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<tr>
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<tr>
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</tr>
<tr>
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<td>trichloroacetic acid</td>
</tr>
<tr>
<td>EI-MS</td>
<td>electron impact mass spectrometry</td>
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<td>min</td>
<td>minutes</td>
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<td>t</td>
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</tr>
<tr>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>AMP</td>
<td>ampicillin</td>
</tr>
<tr>
<td>TSA</td>
<td>trypticase soy agar</td>
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<tr>
<td>MH</td>
<td>Mueller-Hinton</td>
</tr>
<tr>
<td>AI</td>
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</tr>
<tr>
<td>DPPH</td>
<td>2,2-diphenyl-1-picrylhydrazyl</td>
</tr>
<tr>
<td>FRAP</td>
<td>ferric reducing antioxidant power</td>
</tr>
<tr>
<td>ABTS</td>
<td>2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)</td>
</tr>
<tr>
<td>ORAC</td>
<td>oxygen radical absorbance capacity</td>
</tr>
<tr>
<td>INT</td>
<td>p-iodonitrotetrazolium</td>
</tr>
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<td>ORTEP</td>
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**ABSTRACT**

Fifteen homoisoflavonoids (3-17) were synthesised using the base-catalysed aldol condensation, thirteen of which were of the 3-benzylidene-4-chromanone type and the remaining two of the 3-benzyl-4-chromanone type. The substitution patterns of the homoisoflavonoids were varied by keeping the A-ring unsubstituted whilst changing the substituent’s on the 3' and 4' positions of the B-ring. Methoxy, hydroxy, chloro, fluoro and nitro groups were inserted on the B-ring of the homoisoflavonoids. All homoisoflavonoids were characterised by NMR (1D and 2D), IR, UV spectroscopy and GC-MS. The crystal structures were obtained for seven of the homoisoflavonoids. The homoisoflavonoids (3-17) were tested for their antibacterial activity against ten gram-positive and six gram-negative bacterial strains using the method of disc diffusion. Five compounds showed moderate antibacterial activity whilst compound 14 showed good antibacterial activity against the gram positive bacteria. The hydroxylated compounds were tested for their antioxidant activity using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging method as well as the FRAP (ferric reducing antioxidant power) method. Compound 15 showed good antioxidant activity, comparable to that of ascorbic acid, due to the presence of a catechol system within the molecule.
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Homoisoflavonoids are a group of naturally occurring oxygen heterocyclic compounds which consist of either a chromane or chromanone system with a benzyl or benzylidene group at the 3-position (Figure 1) (Adinolfi et al., 1986). They are referred to as homoisoflavonoids, homoisoflavanones or 3-benzyl-4-chromanones. The basic structure and nomenclature for the homoisoflavonoids are shown below (Figure 1).

![Figure 1: The basic structure of a 3-benzylidene homoisoflavonoid](image)

Homoisoflavonoids belong more broadly to the flavonoids, and differ from another subclass, the isoflavonoids in that they have a C-16 rather than a C-15 skeleton (du Toit et al., 2010). Whereas the isoflavonoids result from a phenyl shift from C-2 to C-3, the homoisoflavonoids have a varied biosynthetic pathway (Dewick, 1975), resulting in an extra carbon atom between the chromanone ring and the phenyl group, which distinguishes this subclass of isoflavonoids from the others (Dewick, 1975).

The first homoisoflavonoids, eucomin and eucomol, were isolated from *Eucomis bicolor* (Hyacinthaceae) in 1967 (Figure 2) (Böhler and Tamm, 1967). Since then, many homoisoflavonoids have been isolated from various plant families, but have remained to be a key chemotaxonomic marker amongst the Hyacinthaceae (Koorbanally et al., 2006). Apart
from the Hyacinthaceae, they have also been isolated from the Fabaceae, Liliaceae, Dracaenaceae, Leguminosae and Convallariaceae plant families (Abegaz et al., 2007). In recent studies, three new homoisoflavonoids which showed potent anti-inflammatory activity were isolated from *Ophiopogon japonicus* (Liliaceae) (Hung et al., 2010).

![Figure 2](image_url) **Figure 2**: The chemical structures of eucomin (a) and eucomol (b)

### 1.1 Classification, structure and biosynthesis of homoisoflavonoids

Homoisoflavonoids contain a chromanone ring, which is a benzene ring fused with a tetrahydropyran ring, and a phenyl ring joined together by a carbon atom at C-3, which makes them different from both the flavonoids and the isoflavonoids (Figure 3). In nature, different substitution patterns occur on both the chromanone and phenyl moieties, leading to various permutations of hydroxylated, methoxylated and acetylated compounds as well as others such as the prenylated compounds.

![Figure 3](image_url) **Figure 3**: The structural skeleton of an isoflavonoid (a) and a homoisoflavonoid (b)
Homoisoflavonoids are biosynthesised from chalcone precursors. The mechanism and biosynthetic pathways by which homoisoflavonoids are formed were determined by labeling studies with phenylalanine, sodium acetate and methionine in *Eucomis comosum*, where labeled precursors were incorporated into the chalcone intermediates and further into the homoisoflavonoid (Dewick, 1975).

Phenolic compounds can be biosynthesised by two pathways: the shikimate pathway or the polyketide pathway. Homoisoflavonoids are of mixed biosynthetic origin, the A-ring is polyketide derived and the B-ring is shikimate derived (Figure 4) (Mann *et al.*, 1994).

![Figure 4](image)

**Figure 4:** The formation of the A and B ring of the chalcone which is further cyclised into the homoisoflavonoid

The first step to the biosynthesis of homoisoflavonoids is the biosynthesis of the chalcone precursor (Figure 4). Chalcone biosynthesis (Scheme 1) starts with the deamination of L-phenylalanine to cinnamic acid and oxidized at the *para*-position to 4-coumaric acid, which is then converted to 4-coumaryl CoA (Bhandari *et al.*, 1992). This process is mediated by three enzymes, L-phenylalanine ammonia lyase, cinnamate-4-hydroxylase and coumarate-CoA-ligase. The 4-coumaryl CoA intermediate then combines with three molecules of malonyl CoA yielding the polyketide ester, which cyclises *via* a Claisen type condensation, producing the chalcone precursor, 2',4',6',4'-tetrahydroxychalcone. This process is catalyzed by
chalcone synthase. Methionine is the source of the extra carbon atom, methylating the 2'-hydroxy group (Scheme 1-E), a key step in the biosynthesis of the homoiso-flavonoids, since pyranone ring cyclisation involves this methoxyl group (Scheme 1).

![Scheme 1: The biosynthetic formation of the chalcone precursor (Hahlbrock and Grisebach, 1975)](image)

The biosynthesis of homoiso-flavonoids from chalcones was proposed by Dewick (1975). The 2'-methoxy group is pivotal in this biosynthesis, which is oxidized by the loss of a proton. Subsequent cyclisation resulting from a flow of electrons from the lone pair on the 4'-hydroxy oxygen atom to the oxidized methoxy oxygen at the 2-position leads to the
formation of the three basic types of homoisoflavonoids, the methoxy carbon ending up as C-2 on the homoisoflavonoid skeleton. Addition of a hydride ion or loss of a proton results in either the 3-benzyl-4-chromanone or the 3-benzylidene-4-chromanones. Water added across the double bond of the 3-benzylidene-4-chromanones leads to the third type, the 3-benzyl-3-hydroxy-4-chromanones. The 3-benzyl-3-hydroxy-4-chromanone can also be formed by oxidation of 3-benzyl-4-chromanone at the 3-position. The mechanism for the formation of the homoisoflavonoids is illustrated in Scheme 2.

Scheme 2: The biosynthetic pathway from 2,4,4'-trihydroxy-2'-methoxychalcone to its corresponding homoisoflavonoids (Dewick, 1975)
The fourth type of homoisoflavonoid, the scillascillin type, is formed by the cyclisation of the 3-benzyl-3-hydroxy-4-chromanone, forming a strained but stable four-membered ring (Scheme 3) (Dewick, 1975).

Scheme 3: The proposed biosynthesis of scillascillin (Dewick, 1975)

Due to the presence of the double bond at the 3-position, 3-benzylidene-4-chromanones may undergo chemical conversion and exist as either the trans (E) or cis (Z) isomer (Kirkiacharian et al., 1984) (Figure 5). In natural products, the E isomer is prevalent but can be converted to the Z isomer by light (Siddaiah et al., 2006).

Figure 5: The E and Z-isomers of 3-benzylidene-4-chromanone
1.2 A review of the methods used to synthesise the 3-benzylidene-4-chromanones

Homoisoflavonoids have been synthesised since the mid twentieth century (Farkas et al., 1968). The first synthesis of homoisoflavonoids was completed just a year after these compounds were first isolated (Farkas et al., 1968). A retrosynthetic analysis of these compounds results in aromatic aldehydes, phenols and carboxylic acid synthons. The carboxylic acid synthon has a halide functionality at the other end (Scheme 4).

\[
\begin{align*}
\text{Scheme 4: A retrosynthetic approach to 3-benzylidene-4-chromanone}
\end{align*}
\]

The synthesis of 3-benzylidene-4-chromanones involves formation of the 4-chromanone (2), which is then condensed with aromatic aldehydes in the presence of an acid or base catalyst via the mechanisms in Scheme 6 and Scheme 7. However, various chromanones are commercially available and need not be synthesised as a first step. A survey on the Aldrich website indicates the availability of nine derivatives (Table 1) (www.sigmaaldrich.com/4-chromanone).
Table 1: Comparative prices of commercially available 4-chromanones

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
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<th>R₅</th>
<th>R₆</th>
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<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>545.61/10 g</td>
</tr>
<tr>
<td>6-bromo-4-chromanone</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>Br</td>
<td>H</td>
<td>H</td>
<td>1377.61/0.25 g</td>
</tr>
<tr>
<td>6-chloro-4-chromanone</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>Cl</td>
<td>H</td>
<td>H</td>
<td>324.80/1 g</td>
</tr>
<tr>
<td>6-methyl-4-chromanone</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>CH₃</td>
<td>H</td>
<td>H</td>
<td>1177.61/5 g</td>
</tr>
<tr>
<td>6-fluoro-4-chromanone</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>F</td>
<td>H</td>
<td>H</td>
<td>476.80/1 g</td>
</tr>
<tr>
<td>7-fluoro-4-chromanone</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>F</td>
<td>H</td>
<td>1264.01/1 g</td>
</tr>
<tr>
<td>6,7-dimethoxy-2,2-dimethyl-4-chromanone</td>
<td>diCH₃</td>
<td>H</td>
<td>H</td>
<td>OCH₃</td>
<td>OCH₃</td>
<td>H</td>
<td>1072.01/1 g</td>
</tr>
<tr>
<td>2,2-dimethyl-5,7,8-trimethoxy-4-chromanone</td>
<td>diCH₃</td>
<td>H</td>
<td>OCH₃</td>
<td>H</td>
<td>OCH₃</td>
<td>OCH₃</td>
<td>708.80/0.05 g</td>
</tr>
<tr>
<td>2,2-dimethyl-7-ethoxy-6-methoxy-4-chromanone</td>
<td>diCH₃</td>
<td>H</td>
<td>H</td>
<td>OCH₃</td>
<td>OCH₃CH₂</td>
<td>H</td>
<td>708.80/0.1 g</td>
</tr>
<tr>
<td>7-acetoxy-3-acetyl-2-methyl-4-chromanone</td>
<td>CH₃</td>
<td>CH₃CO</td>
<td>H</td>
<td>H</td>
<td>CH₂CO₂</td>
<td>H</td>
<td>708.80/0.05 g</td>
</tr>
</tbody>
</table>
Several of the chromanone derivatives that are commercially available are relatively expensive, compared to the unsubstituted chromanone therefore derivatisation of the chromanone may be a better alternative to purchasing the derivatives.

1.2.1 Synthesis of the 4-chromanone (2) intermediate

Even though the 4-chromanone intermediates are available, researchers in the field still synthesise the intermediate en route to the homoisoflavonoids. Different methods were employed for their synthesis (Scheme 5) (Siddaiah et al., 2006; Foroumadi et al., 2007; Siddaiah et al., 2007; Shaikh et al., 2011a).

They can be formed from the reaction of:

(a) 3-bromo- or 3-chloropropanoic acids and phenols under basic conditions, producing a phenoxypropanoic acid which can be cyclised with polyphosphoric acid (Siddaiah et al., 2006; Shaikh et al., 2011a);

(b) 3-bromo- or 3-chloropropanoic acids and phenols under acidic conditions, producing a benzophenone alkyl chloride which can be cyclised with sodium hydroxide (Foroumadi et al., 2007);

(c) acrylonitrile and phenols under basic conditions forming a phenoxy nitrile which is followed by the cyclisation with sulfuric acid (Siddaiah et al., 2007).

In all cases, an activated carbon is produced in the intermediate. In the case of the acid and nitrile intermediates, an acid is used as a catalyst for the cyclisation by activitating the carboxyl or nitrile groups toward nucleophilic substitution and for the alkyl chloride, a base is needed for the abstraction of the proton of the hydroxyl group, which is then followed by nucleophilic substitution.
Scheme 5: (a) The synthesis of 3-phenoxypropanoic acid (1) and 4-chromanone (2) (Siddaiah et al., 2006); (b) The synthesis of 7-hydroxy-4-chromanone (Foroumadi et al., 2007); (c) The synthesis of 7,8-dihydroxy-4-chromanone (Siddaiah et al., 2007)

The method employed for the synthesis of chromanone depends on the functionalities on the A-ring of the molecule. Strong bases may not be used with more than one hydroxyl group on the A-ring, due to the unwanted complete deprotonation of the hydroxyl groups.

Methods a, b and c above has reasonable yields of 46%, 54% and 61% respectively (Siddaiah et al., 2006; Foroumadi et al., 2007; Siddaiah et al., 2007).

1.2.2 Synthesis of the 3-benzylidene-4-chromanones from the 4-chromanone intermediate

Benzaldehydes are condensed onto the 3-position of the 4-chromanone intermediate using either acids or bases as a catalyst. Acid-catalysed condensation requires protic acids, such as phosphoric (Desideri et al., 2011; Shaikh et al., 2011a) and hydrochloric acid (Evans and
Lockhart, 1966; Foroumadi et al., 2007; Cheng et al., 2011; Desideri et al., 2011), which protonate the carbonyl group of the 4-chromanone intermediate, promoting enol formation and also protonate the carbonyl group of the aldehyde, activating the carbonyl group, making it more electrophilic and more susceptible to nucleophilic attack (Scheme 6).

Scheme 6: The proposed reaction mechanism for the acid-catalysed preparation of 3-benzylidene-4-chromanone

Base-catalysed condensation involves the abstraction of the alpha proton by weak bases such as piperidine (Léval and Schág, 1979; Perjési et al., 2008; Shaikh et al., 2011a; Jacquot et al., 2012), which result in the enolate anion, a better nucleophile than the enol, thereby promoting the addition of the nucleophile to the aldehyde.
In the base-catalysed mechanism, the driving force for the elimination of water is the highly conjugated product that forms as a result of the elimination (Scheme 7). Other bases such as pyrrolidinidne have also been used (Shankar et al., 2012).

Scheme 7: The proposed reaction mechanism for the base-catalysed preparation of 3-benzylidene-4-chromanone

1.2.3 Other methods used for the synthesis of 3-benzylidene-4-chromanones

Rather than synthesising the chromanone and then condensing it with an aldehyde to prepare the homoisoflavonoid, Basavaiah and Bakthadoss (1998) applied the Baylis-Hillman reaction and started with the construction of the benzylidene moiety and then the chromanone ring system (Scheme 8). This method is better at synthesising homoisoflavonoids with different substituents on the chromanone A-ring as different phenols can be used in the first step.
Scheme 8: The reaction scheme for the preparation of 3-benzylidene-4-chromanone by the Baylis-Hillman reaction (Basavaiah and Bakthadoss, 1998)

The synthesis of several types of homoisoflavonoids has been reported in the literature (Farkas et al., 1968; Lévai and Schág, 1979; Basavaiah and Bakthadoss, 1998; Siddaiah et al., 2006; Foroumadi et al., 2007; Siddaiah et al., 2007; Perjési et al., 2008; Rao et al., 2008; Zhang et al., 2008; Das et al., 2009; Cheng et al., 2011; Desideri et al., 2011; Shaikh et al., 2011a; Jacquot et al., 2012; Shankar et al., 2012).

5,7-diacetoxy-3(4-methoxybenzal)-4-chromanone was synthesised by refluxing 5,7-dihydroxy-4-chromanone in acetic anhydride. Deacylation of 5,7-diacetoxy-3(4-methoxybenzal)-4-chromanone yielded eucomin (Farkas et al., 1968). Other natural homoisoflavonoids, such as punctatin, were also synthesised by refluxing chromanone and an aldehyde in hot acetic anhydride (Farkas et al., 1971), however this method was inefficient due to the long reaction times (Lévai, 2004).
1.3 Bioactivity of homoisoflavonoids

Homoisoflavonoids have been reported to have a wide range of biological activities (du Toit et al., 2010). They have been found to exhibit antibacterial (Das et al., 2009; Shankar et al., 2012), antioxidant (Farkas et al., 1968; Siddaiah et al., 2006; Siddaiah et al., 2007; Lin et al., 2010), anti-inflammatory (Hung et al., 2010; Shaikh et al., 2011b), antifungal (Rao et al., 2008), antiviral (Tait et al., 2006), antimutagenic (Miadoková et al., 2002), anticancer (Yan et al., 2012) and antirhinovirus (Conti and Desideri, 2009) activity. Naturally occurring homoisoflavonoids serve as potent protein tyrosine kinase inhibitors (Lin et al., 2008).

Since the homoisoflavonoids synthesised in this work were tested for their antioxidant and antibacterial activity, these activities are reviewed below.

1.3.1 Antioxidant activities of substituted 3-benzylidene-4-chromanones

Polyphenolic homoisoflavonoids such as 7-hydroxy-3-(3,4-dihydroxybenzylidene)chroman-4-one (sappanone A) and 7-hydroxy-3-(3,4,5-trihydroxybenzylidene)chroman-4-one (Figure 6) showed potent antioxidant activity, stronger than that of ascorbic acid, a commonly consumed antioxidant (Siddaiah et al., 2006). This was attributed to the catechol like system within these molecules. This finding was also confirmed by Foroumadi et al. (2007) who studied a range of C1-C4 (methoxy through to n-butoxy). The 7-substituted alkyloxy benzylidene-4-chromanones showed the best antioxidant activity (Foroumadi et al., 2007).
1.3.2 Antibacterial activities of substituted 3-benzylidene-4-chromanones

Flavonoids have been shown to be active against many species of bacteria, both gram positive and gram negative strains (du Toit et al., 2010). Das et al. (2009) tested a range of naturally occurring homoisoflavonoids and their derivatives against three gram positive (Staphylococcus aureus, Bacillus subtilis, Bacillus sphaericus) and three gram negative (Klebsiella aerogenes, Chromobacterium violaceum, Pseudomonas aeruginosa) bacterial strains. Of the compounds tested, the benzylidene-4-chromanone with a hydroxy group at C-7 and a 3’4’-methylenedioxy group showed good antibacterial activity against Staphylococcus aureus (gram positive), Klebsiella aerogenes and Chromobacterium violaceum (gram negative) (Figure 7) (Das et al., 2009).

Figure 7: 3-(Benzo[1,3]dioxol-5-ylmethylene)-7-hydroxychroman-4-one, a 3-benzylidene-4-chromanone with good antibacterial activity
A series of thirteen naturally occurring homoisoflavonoids, of all structural types, was screened against *Staphylococcus aureus* (ATCC 12600) (du Toit *et al.*, 2007), where two homoisoflavonoids of the 3-benzylidene-4-chromanone type (Figure 8) oxygenated at the 5,7,4' and 5,7,8,4'-positions showed good antibacterial activity with minimum inhibitory concentrations (MIC) of 0.52 and 0.24 mM respectively.

![Figure 8: The substituted 3-benzylidene-4-chromanones with good antibacterial activity against *Staphylococcus aureus* (ATCC 12600)](image)

**1.4 Methodology used for the bioassays**

Antioxidant assays are carried out using several different types of assays, a few of them being ABTS, DPPH, FRAP, and ORAC assays. The antibacterial assays include the Kirby-Bauer disk-diffusion method and the bioautographic method. Minimum inhibitory concentration may be determined using a microdilution method on a 96 well microtitre plate.

**1.4.1 Methodology for the antioxidant assays**

Several methods have been established to determine the antioxidant potential of compounds, each providing unique information on the way the compounds exhibit this antioxidant activity. The main methods included the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical
scavenging, ferric reducing antioxidant power (FRAP) and the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ABTS method (Thaipong et al., 2006).

1.4.1.1 DPPH radical scavenging assay

The DPPH radical scavenging method is the simplest of all those mentioned above and the most commonly used. DPPH is a stable free radical containing compound, which absorbs light at 517 nm. In the presence of a compound with antioxidant potential, the DPPH radical is reduced, by accepting an electron or a hydrogen radical, to form a stable diamagnetic molecule, resulting in a decrease in absorbance. Upon reduction, a colour change from purple to yellow is observed. The amount by which the absorbance decreases is a measure of the strength of the antioxidant. The structure of DPPH and the reaction that occurs between it and the antioxidant compound is illustrated below (Scheme 9) (Shyam et al., 2012).

![Scheme 9: The reaction of DPPH with a hydrogen radical](image)

1.4.1.2 Ferric reducing antioxidant power assay

The ferric reducing antioxidant power (FRAP) measures the ability of a compound to reduce Fe$^{3+}$ to Fe$^{2+}$. Ferric chloride (Fe$^{3+}$Cl$_3^-$) (dark green in colour) is reduced in the presence of an antioxidant at a low pH, resulting in ferrous chloride (Fe$^{2+}$Cl$_2^-$), which is deep blue in
solution. The absorbance of the resulting solution, measured at 700 nm, is an indication of the strength of the antioxidant \textit{i.e.} the more \( \text{Fe}^{3+} \) is reduced, the more blue the solution is and the higher the absorbance, indicating a higher activity of the antioxidant (Figure 9).

![Figure 9: The reduction of Fe\(^{3+}\) to Fe\(^{2+}\) in the presence of an antioxidant](image)

**1.4.1.3 ABTS assay**

The 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay is a colorimetric assay which involves the conversion of the coloured ABTS radical to the colourless ABTS. Colourless neutral ABTS is oxidised creating the blue-green ABTS radical cation. In the presence of a compound with antioxidant potential, the ABTS radical cation reacts with the antioxidant and is neutralized. The measure of antioxidant capacity is measured spectrophotometrically at a wavelength of 734 nm. The decrease in the absorbance is an indication of how much the ABTS radical cation has been neutralised and hence the compounds’ antioxidant potential (Scheme 10) (Erel, 2004; Osman \textit{et al.}, 2006).

![Scheme 10: The reaction between ABTS and the antioxidant (Osman \textit{et al.}, 2006)](image)

(Note the radical formation on the nitrogen in the top half of the molecule)
1.4.2 Methodology for the antibacterial assays

Antimicrobial activities may be determined in one of three general ways i.e. by dilution, diffusion or bioautographic methods. Diffusion and bioautographic methods are qualitative methods whereas dilution is a quantitative method. Compounds are screened initially using either the diffusion or bioautographic method to determine if the compound has antibacterial activity. Once this has been established, the minimum inhibitory concentration may be determined using the method of dilution.

1.4.2.1 Kirby-Bauer disk-diffusion method

The Kirby-Bauer disk-diffusion method, also known as the agar disc diffusion method, involves the inhibition of the growth of bacteria on a Müller-Hinton agar surface in the presence of an antibacterial agent. Petri dishes are filled with Müller-Hinton agar, onto which a bacterial strain is swobbed. Test compounds are impregnated onto filter discs and placed onto the agar surface. The plates are then incubated for bacterial growth. The test compound diffuses from the filter paper onto the agar. Depending on the activity of the compound, the bacterial growth surrounding the disc is inhibited. After incubation is complete, the zones of inhibition are measured, i.e. the area around the filter disc on which the bacterial growth was inhibited. The zones of inhibition are a clear indication whether a specific test compound shows antibacterial activity against bacterial strains.

1.4.2.2 Bioautographic methods

This method involves the inhibition of growth of bacterial strains on a silica based surface in the presence of an antibacterial agent. Test compounds are spotted onto thin layer chromatography (TLC) plates at various concentrations. The plates are then coated with a bacterial strain and incubated for bacterial growth. Test compounds diffuse inhibiting
bacterial growth. After incubation the plates are sprayed with an indicator solution of p-iodonitrotetrazolium (INT) violet. This indicator solution colours the plate where the bacteria is present, thereby clearly showing areas in which the bacterial growth was inhibited. Zones of inhibition are measured as an indication of the compounds antibacterial activity (Valgas et al., 2007).

1.4.2.3 Method of dilution

The method of dilution is employed to determine the minimum inhibitory concentration of test compounds. A range of concentrations of the test compound are prepared. A 96-microwell plate is prepared by adding a standard amount of test compound, Müller-Hinton broth and bacterial standard into each well. The plate is then incubated for bacterial growth. A small volume of INT violet is then added to each well. In the wells which have a negative result, i.e. where the bacterial growth was not inhibited, the INT changes from yellow to purple. The results may be read spectrophotometrically or visually. The lowest concentration at which the INT remained yellow is the minimum inhibitory concentration (Valgas et al., 2007).

1.5 Hypothesis, aims and objectives

Since naturally occurring homoisoflavonoids of the 3-benzylidene type have shown antibacterial and antioxidant activities (Siddaiah et al., 2007; Das et al., 2009), it was hypothesised that various derivatives with chemical modifications to the phenyl ring could produce enhanced activity in antibacterial and antioxidant assays. Investigations into various constituents on the phenyl ring of the homoisoflavonoids were explored to see which of the groups are essential for good biological activity.
The aim of the study was to synthesise and characterise a series of homoisoflavonoids with modified phenyl rings and to test them for their antibacterial and antioxidant activity. To this end, substitution on the B-ring of the homoisoflavonoids were varied with fluoro, chloro, nitro, hydroxy and methoxy groups in order to determine which substituents as well as their position on the phenyl ring will be the most biologically active. Mono- and di-substituted derivatives were prepared to see whether or not substitution at more than one position could also lead to enhanced activity.

The objective of the study is to find new target molecules for the development of more potent antibacterial and antioxidant drugs.
CHAPTER 2 RESULTS AND DISCUSSION

This chapter includes a discussion of the synthesis and characterisation of 3-benzylidene-4-chromanones as well as the antioxidant and antibacterial activities of the synthesised compounds. The methods used to synthesise the compounds are discussed together with mechanisms for the reactions. The characterisations of the compounds include a discussion of the NMR data along with other data such as mass spectrometry, IR and UV to validate the structures assigned to the synthesised products. The data for the antioxidant and antibacterial assays as well as the interpretation of it are also included in this chapter.

2.1 Synthesis and Characterisation

Thirteen 3-benzylidene-4-chromanones and two 3-benzyl-4-chromanones with different substitution patterns on the phenyl ring (B-ring) (Scheme 11) were synthesised in good yields of between 50 and 90% according to the modified procedure by Shaikh et al. (2011a). The target molecules were chosen to examine the effect that the fluorine, chlorine, methoxy, hydroxy and nitro groups have on different positions of the phenyl ring with regard to reactivity and biological activity. This would enable us to study the structure-activity relationship of the substituted benzylidene-4-chromanones with regard to antioxidant and antibacterial activity.

The synthesis of the homoiso flavonoids from phenol is a three step reaction process; the synthesis of 3-phenoxypropanoic acid (1) from phenol, the cyclisation of 3-phenoxypropanoic acid to 4-chromanone (2), and the condensation of 4-chromanone with the aromatic aldehyde to the homoiso flavonoid (3-17) (Scheme 11).
**Scheme 11:** The synthetic scheme for the synthesis of 3-phenoxy propanoic acid (1), 4-chromanone (2) and homoiso flavonoids (3-17)

**2.1.1 Synthesis and characterisation of the 4-chromanone (2) intermediate**

The 4-chromanone intermediate was synthesised in two steps (Scheme 11). The first step involves the reaction of phenol with bromopropanoic acid and a strong base (NaOH) to abstract the proton of the hydroxyl group on phenol. The resultant phenolate anion is stabilised by resonance structures. It then attacks the electrophilic carbon alpha to the bromine in probably a $S_N2$ type mechanism forming 3-phenoxypropanoic acid (1), which
cyclises upon addition of polyphosphoric acid (PPA). PPA forms a phosphate ester with the 3-phenoxypropanoic acid, which is followed by electrophilic substitution of the ester on the aromatic ring forming a pyran ring. Polyphosphoric acid is an oligomer of phosphoric acid and commonly used to activate the carboxyl group, making nucleophilic substitution possible (Clayden et al., 2001). A proposed mechanism is given below (Scheme 12).

Scheme 12: The proposed reaction mechanism for the synthesis of 3-phenoxypropanoic acid (1) and 4-chromanone (2)

The reaction was carried out at ambient conditions for 30 min and then heated under reflux for 2 hrs. A yield of 52% was obtained from the reaction of 3-phenoxypropanoic acid with polyphosphoric acid to 4-chromanone, which compares well to the yields reported in the literature (Siddaiah et al., 2006). On heating for longer durations, no improvement in the yield was observed. Purification of the target compound was necessary after the reaction occurred. This was carried out on silica gel using column chromatography.

The infrared spectrum of the first intermediate, 3-phenoxypropanoic acid (1), showed a sharp band at 1688 cm\(^{-1}\) and a broad band at 2931 cm\(^{-1}\), indicating the presence of a carbonyl and
hydroxy group respectively. This serves as an indication for the formation of the acid. After the cyclisation of 3-phenoxypropanoic acid to 4-chromanone (2), the carbonyl band shifted from 1688 cm$^{-1}$ to 1682 cm$^{-1}$. Other characteristic absorption bands observed in 2 were that of the aromatic ring, C=C stretching vibrations (1599, 1476, 1453 cm$^{-1}$) and the C-O stretching vibration at 1258 cm$^{-1}$.

The mass spectrum confirmed the presence of 4-chromanone (2) by displaying the molecular ion peak [M$^+$] of 148 mass units. The base peak was observed at 120 mass units as a result of a retro Diels-Alder cleavage. Other intense peaks were seen at 92 and 64 mass units. The proposed fragmentation scheme of 4-chromanone is illustrated below (Scheme 13).

The $^1$H NMR spectrum of 3-phenoxypropanoic acid (1) is characterised by two methylene triplets (t) at $\delta_H$ 2.83 and $\delta_H$ 4.23 with coupling constants of $J = 6.24$ Hz, with the methylene proton resonance adjacent to the oxygen being more deshielded than that next to the acid group. The aromatic protons of H-6/10 appear at $\delta_H$ 6.89 and that of H-7/9 appear at $\delta_H$ 7.26. The H-6/10 resonance appears more upfield due to the electron donation by resonance from the oxygenated group. The H-8 proton resonance appears as a triplet, due to the coalescing of doublets with similar coupling constants, at $\delta_H$ 6.94 with $J = 7.42$ Hz. The $^{13}$C NMR spectrum shows the two methylene carbon resonances at $\delta_C$ 34.49 and 63.05 and the aromatic carbon resonances between $\delta_C$ 114.65 to $\delta_C$ 129.50 with the aromatic C-O resonance at $\delta_C$
158.39 and an additional carbonyl resonance at $\delta_C 176.93$ for the carboxylic acid carbonyl group.

The $^1$H NMR spectrum of the 4-chromanone (2) intermediate differs from the phenoxypropanoic acid intermediate in that the two H-2 and two H-3 resonances are now not equivalent. Due to chemical shift overlap of H-2a and H-2b and also H-3a and H-3b, the coupling pattern is no longer first order, but is now second order (Figure 10). H-2 and H-3 splitting patterns are therefore difficult to interpret and are reported as multiplets.

![Figure 10: The chromanone ring (2) showing protons H-2a, H-2b, H-3a and H-3b](image)

The aromatic resonances for H-5 and H-7 are seen to occur distinctly from the H-6/8 resonances. The H-6/8 resonances overlap, but can be distinguished with H-8 occurring as a doublet at $\delta_H 6.93$ ($J = 8.44$ Hz) and H-6 as a triplet of doublets showing both ortho and meta coupling at $\delta_H 6.97$ ($J = 7.84, 0.76$ Hz). The H-7 and H-5 protons are both meta to the oxygenated group at C-8a and are therefore more deshielded due to the electronic effects discussed below (Scheme 15). The H-7 resonance appears as a double double doublet (ddd) at $\delta_H 7.42$ with $J = 8.64, 7.16$ and 1.64 Hz, showing ortho coupling with H-8 and H-6 and meta coupling with H-5. The H-5 resonance is the most deshielded due to the magnetic
anisotropic effect. The H-5 proton is in the deshielded part of the cone formed by the π-electrons of the carbonyl group and appears as a double doublet (dd) at $\delta_H 7.85$ ($J = 7.84, 1.72$ Hz) (Figure 11). The $^{13}$C NMR resonance of the carbonyl group appears at $\delta_C 191.80$, indicating the conversion from the acid to the cyclised chromanone in that electron donation from the hydroxy group no longer occurs in the chromanone resulting in the carbonyl resonance being more deshielded and closer to that of a pure ketone resonance. The oxygenated aromatic C-O resonance occurs at $\delta_C 161.86$ and the other aromatic carbon resonances occur between $\delta_C 117.88$ and $\delta_C 135.96$ (C-4a, C-5-8). The methylene group closest to the oxygen, C-2 occurs at $\delta_C 67.00$ and that close to the carbonyl group appears at $\delta_C 37.78$.

### 2.1.2 Synthesis of the 3-benzylidene-4-chromanones

The condensation of 4-chromanone with the aromatic aldehyde is achieved with piperidine as a base, which abstracts the most acidic proton at the alpha carbon (C-3) resulting in the formation of an anion, followed by an aldol addition to the aldehyde forming a $\beta$-hydroxy carbonyl compound. The extensive conjugation in the molecule drives the elimination of water from this intermediate without the addition of acid to form a highly conjugated molecule, the 3-benzylidene-4-chromanone (Scheme 7).

The reaction of 4-chromanone with various substituted benzaldehydes to give the homoisoflavonoids were carried out under reflux at 80-90 °C. Reaction temperatures were monitored and kept below 90 °C, due to exocyclic to endocyclic bond migration which may occur at ~ 150 °C (Jacquot et al., 2012). Exocyclic to endocyclic bond migration, however
did occur at 80-90 °C with the electron withdrawing nitro groups resulting in the 3-benzyl homoisoflavonoid rather than the 3-benzylidene homoisoflavonoid (Valkonen et al., 2012).

All homoisoflavonoids synthesised were obtained in good yields of between 50-80%. For the deactivating groups, chloro and fluoro substituents, higher yields were obtained for the meta substituted than the para substituted compounds. For the activating groups, the hydroxy and methoxy group, a higher yield was obtained for the para substituted product. In the case of all disubstituted benzaldehydes, the product yields were lower than that of the mono-substituted benzaldehydes. For the hydroxy substituents, separation of these compounds from the reaction mixture was problematic. In the extraction process with ethyl acetate, a clear distinction could not be made between the phases and although careful care was taken to recover the amount of ethyl acetate used, some of the product could have been sacrificed in this procedure. This may account for the lower yields with the hydroxy groups as opposed to the other substituents.

2.1.3 Structural elucidation of homoisoflavonoids (3-17)

Infrared spectroscopy was used to confirm the presence of functional groups within the homoisoflavonoids synthesised. The infrared spectrum of 3 showed a sharp peak at 1665 cm\(^{-1}\) which is attributed to the carbonyl group (C=O, C-4). The low frequency of the absorption is indicative of the conjugation in the molecules resulting in greater single bond character and lower wavenumbers. The peaks at 1466 and 1450 cm\(^{-1}\) are as a result symmetrical stretching of the aromatic alkene (C=C) groups. The asymmetrical stretching peaks for the aromatic C=C bonds are observed at 1308 and 1267 cm\(^{-1}\). The ether group (C-O-C) stretching frequency is observed at 1209 cm\(^{-1}\).
The infrared spectrum of compounds 13, 14 and 15 showed broad bands at 3093, 3255, 3117 cm\(^{-1}\) respectively, confirming the presence of a hydroxyl group. The compounds containing a nitro group, 16 and 17, showed two strong bands for the stretching of the N-O bond at 1462 cm\(^{-1}\) (asymmetrical) and 1341 cm\(^{-1}\) (symmetrical) for 17, and 1464 cm\(^{-1}\) (asymmetrical) and 1339 cm\(^{-1}\) (symmetrical) for 16. For the mono-fluorinated compounds, a single band is observed at 1145 cm\(^{-1}\) for 10, and at 1144 cm\(^{-1}\) for 11 which is characteristic of the C-F bond. The di-fluorinated compounds shows two strong bands at 1116 and 1145 cm\(^{-1}\) as a result of symmetric and asymmetric stretching.

Flavonoids are commonly identified by the existence of two characteristic bands in the UV spectrum. These two bands occur in the region of 300 to 550 nm, attributed to B-ring and 240 to 285 nm attributed to the A-ring (Heller and Tamm, 1981). For the homoisoflavonoids (3-17) synthesised, the same trend was observed with two bands occurring in the region of 280-300 nm and 340-360 nm indicating that this skeletal structure was also present in the synthesised compounds.

The mass spectra for the homoisoflavonoids are all similar in that they display the same type of fragmentation pattern. The differences in the mass spectra are associated with that of the phenyl ring, due to there being different substituents on the ring and hence fragment with different masses. The fragmentation pattern of the chromanone moiety of the homoisoflavonoid is illustrated in Scheme 13. The mass spectra of the chloro containing homoisoflavonoids (7, 8 and 9) show peaks for both the chlorine isotopes (\(^{35}\text{Cl}\) and \(^{37}\text{Cl}\)) with a peak height ratio of 3:1.
For 4, the para methoxy derivative, the molecular ion peak [M+] is observed at 266 mass units which confirm the formation of the homoisoflavonoid. The proposed fragmentation pattern of the B-ring of the homoisoflavonoid is illustrated below (Scheme 14). Thereafter the chromanone moiety is fragmented as described above (Scheme 13).

Scheme 14: The fragmentation pattern of (E)-3-(4’-methoxybenzylidene)chroman-4-one (4)

The proton NMR spectrum of compound 3 showed the characteristic resonances for the benzylidene proton (H-9) as a singlet (s) at δH 7.86 and the H-2 proton resonance, a two proton resonance occurring as a doublet (d) at δH 5.33 with a small coupling constant of J = 1.32 Hz due to the germinal coupling between the two H-2 protons. The oxygenated moiety and the carbonyl moiety attached to C-8a and C-4a respectively play a significant role in the chemical shift of the proton resonances on the chromanone ring. For example, in 3, the H-6 and H-8 resonances are more upfield at δH 7.05 and δH 6.95 due to the electronic effects of the oxygenated moiety, similar to that occurring in a phenol substituted structure (Scheme 15).
Scheme 15: Resonance structures of phenol showing electronic effects of the hydroxyl group and the build-up of electron density at the ortho and para positions, resulting in protons occurring more upfield.

In the same manner, the H-5 and H-7 proton resonances are more downfield similar to that which occurs in an acetophenone substituted structure (Scheme 16).

Scheme 16: Resonance structures of acetophenone showing electronic effects of the acyl group and the withdrawal of electron density from the ortho and para positions resulting in protons occurring more downfield.

The H-5 resonance however is noticeably more downfield than H-7 due to an anisotropic effect (Figure 11), and occurs at $\delta_{H}$ 8.00 away from the other aromatic resonances. The H-7 resonance occurs at $\delta_{H}$ 7.47. The phenyl proton resonances in the absence of substituents on this ring occur typically in the aromatic region between $\delta_{H}$ 7.29 to 7.42.
The splitting pattern of the protons on the chromanone ring in the absence of substituents shows a doublet of doublets for H-5 with $J = 7.86$ Hz, typical of ortho coupling and 1.10 Hz attributed to meta coupling with H-7. Para coupling was not observed in the $^1$H NMR spectra. In the case of the H-8 proton resonance, only ortho coupling with $J = 8.32$ Hz was present and meta and para coupling could not be detected, but in the COSY spectrum H-8 was seen coupled to H-6. The H-6 proton resonance appeared as a triplet due to the coalescing of the double doublets that occurs because of similar coupling constants between H-6 and H-5 and H-6 and H-7 with $J = 7.50$ Hz. Although this should also be observed for H-7, this cannot be distinguished because of overlapping with the aromatic protons of the phenyl ring. In the case of the phenyl ring protons, the H-3'/5' resonance and the H-2'/6' resonances can both be distinguished as doublets with similar $J$ values of 7.36 and 6.96 Hz respectively. There is a small difference between the coupling constants of coupled proton resonances, for example, the H-5 proton resonance has $J = 7.86$ Hz, but the triplet of H-6 has a $J$ value of 7.50 Hz. We attribute this to the coalescing of resonances, where peaks overlap and also to the broadened resonances for some of the proton peaks. However, we confirmed the coupling of all the resonances with the aid of the COSY spectrum.

The $^{13}$C NMR spectrum of compound 3 showed the presence of fourteen carbon resonances with two of the resonances being equivalent and therefore amounting to sixteen carbon
resonances, which confirms the presence of a homoisoflavonoid skeleton. The oxygenated aliphatic carbon resonance of C-2 occurs at $\delta_C$ 67.61 typical for C-2, with that of C-4, the carbonyl resonance occurring at $\delta_C$ 182.27 and the oxygenated aromatic carbon of C-8a occurring at $\delta_C$ 161.14 typical for these resonances in benzylidene homoisoflavonoids (Jacquot et al., 2012). This was confirmed by the presence of HMBC correlations between C-4 and H-5, C-2 and H-9 and C-8a with H-2, H-5 and H-8.

The C-6, C-8 and C-4a are the most shielded of all the aromatic resonances due to the electronic effects explained above (Scheme 15 and Scheme 16). The remaining two aromatic carbon resonances on the chromanone ring, C-5 and C-7 occur at $\delta_C$ 127.96 and 135.89 respectively. The resonances of C-5 to C-8 were determined by their corresponding proton resonances in the HSQC spectrum. The equivalent phenyl carbon resonances of C-2/6' occurs slightly more downfield at $\delta_C$ 129.99 compared to the C-3/5' resonance of $\delta_C$ 128.74. This was confirmed by a HMBC correlation between C-2/6' and H-9. The C-4' carbon resonance lies in between these two resonances at $\delta_C$ 129.48. The C-1' carbon resonance was assigned at $\delta_C$ 134.39 due to a HMBC correlation with H-3/5'. The remaining olefinic carbon resonance of C-3 was assigned to $\delta_C$ 130.92 because of a HMBC correlation to H-2. The $^1$H and $^{13}$C NMR resonances compare well with that in the literature (Jacquot et al., 2012).

2.1.4 Structural elucidation of the para substituted derivatives (except 4'-fluoro)

For the para substituted B-ring benzylidene homoisoflavonoids (4, 7 and 13), excluding the para fluoro substituted compound (10), the $^1$H NMR spectrum showed the ortho coupled proton resonances of H-2/6' and H-3/5' as doublets with coupling constants between 8.4 and
8.8 Hz for the three compounds. These are located at $\delta_H$ 7.26 and 6.96 for the methoxy derivative (4), $\delta_H$ 7.32 and 6.89 for the hydroxy derivative (13) and $\delta_H$ 7.22 and 7.40 for the chloro derivative (7) for the H-2'/6' and H-3'/5' resonances respectively. For compounds 4 and 13, with an activating electron donating methoxy or hydroxy group at the para position, the H-3'/5' resonance is more shielded due to electron donation and build-up of electron density at these carbon atoms. In contrast, the para chloro derivative had the H-3'/5' proton resonances more deshielded. Even though the chloro group has lone pairs and is capable of electron donation toward the ring, the inductive effects of the deactivating chloro group is responsible for this effect. In the unsubstituted benzylidene homoiso flavonoid (3), the H-7 and H-3'/5' resonances overlapped at $\delta_H$ 7.47 but when a substituent was placed at the para position of this ring, causing the H-3'/5' resonance to be shifted, the H-7 resonance can now be clearly seen as a triplet of doublets (td) in the chloro (7) and hydroxy derivatives (13) with $J_{7,8}$ and $J_{6,7}$ being the same at approximately 7.7 Hz. In 4, a double double doublet (ddd) was seen with $J_{7,8}$ being slightly larger than $J_{6,7}$. Both coupling constants were in the region of 8.0 Hz. The $J_{5,7}$ coupling constant was seen to be approximately 1.70 Hz. A singlet resonance for the methoxy group was seen at $\delta_H$ 3.83 in 4.

For 4 and 13, the C-4' resonance shifted more downfield in the region of C-8a at approximately $\delta_C$ 160 because of the oxygenated substituent at this position. In 7, the para chloro derivative, C-4' occurred more in the region of C-7 and C-9 at $\delta_C$ 135.59 due to the chloro group being less electronegative than the oxygenated groups. The $^1$H and $^{13}$C NMR data of 4, 7 and 13 compare well with that in the literature (Jacquot et al., 2012; Valkonen et al., 2012).
2.1.5 Structural elucidation of the meta substituted derivatives (except 3'-fluoro)

For the meta substituted compounds (5, 8 and 14, excluding the fluorinated compound 11), all the NMR spectra were similar with some subtle changes brought about by the different substituted groups. For 5, the 3-(3'-methoxybenzylidene)chroman-4-one, the proton resonances for the aromatic ring and the chromanone ring are well resolved. The H-5 resonance appears as a double doublet at $\delta_H$ 8.00 ($J = 7.82, 1.46$ Hz), deshielded due to hydrogen bonding as described above (Figure 11), the H-9 resonance appears as a singlet at $\delta_H$ 7.82 and the H-7 and H-6 resonances occurred at $\delta_H$ 7.47 (td, $J = 7.72, 1.60$ Hz) and $\delta_H$ 7.05 (t, $J = 7.52$ Hz) respectively. The H-8 and H-4' resonances overlap at $\delta_H$ 6.94, however the doublet resonance of H-8 could be distinguished and its coupling constant was determined to be $J = 8.12$ Hz. The multiplicity of the H-4' resonance could not be determined due to overlap with H-8. Due to the meta substitution on the phenyl ring, the H-2' resonance should show meta coupling with either H-6' or H-4', but this was not observed. Rather, a broad singlet for this resonance was observed at $\delta_H$ 6.82, however coupling in the COSY spectrum was observed between this resonance and that of H-4' and the H-6' resonance which appears as a doublet at $\delta_H$ 6.87 ($J = 7.60$ Hz). The H-5' resonance appears as a triplet at $\delta_H$ 7.34 ($J = 7.92$ Hz). HMBC correlations between H-5 and the carbonyl resonance, C-4 at $\delta_C$ 182.23 and H-8 and C-4 confirm these assignments. The H-2 resonance appears at $\delta_H$ 5.33, which was confirmed by a HMBC correlation to C-4. The methoxy resonance was seen at $\delta_H$ 3.82 as an intense singlet resonance in the $^1$H NMR spectrum.

All the protonated carbon resonances were identified from their corresponding proton resonances using the HSQC spectrum. There were five carbon resonances beside the carbonyl resonance, which were non-protonated. These occurred at $\delta_C$ 122.02, 131.15, 135.69, 159.69, and 161.18 and were identified using HMBC correlations. The C-8a
resonance was identified at $\delta_C$ 161.18 by a HMBC correlation to H-2 and the other oxygenated carbon resonance at $\delta_C$ 159.60 was then attributed to C-3'. This was corroborated by HMBC correlations between C-3' and both H-4' and H-5'. The resonance at $\delta_C$ 131.15 shows a correlation to H-2 and was therefore attributed to C-3, and the resonance at $\delta_C$ 135.69 shows a correlation to H-5' and was attributed to C-1'. The remaining resonance at $\delta_C$ 122.02 showed a correlation to H-6 and was therefore assigned to C-4a.

The $^1$H NMR spectrum of 14, the 3'-hydroxy derivative was very similar to 5, but had the notable difference in that the H-4' resonance which overlapped with H-8 in 5, was now seen overlapping with the H-6' resonance and could be seen as a two proton doublet resonance for H-4'/6' at $\delta_H$ 6.86 with $J = 8.08$ Hz. This allowed the H-8 resonance to be clearly seen as a doublet at $\delta_H$ 7.06 with $J = 8.28$ Hz. The rest of the resonances have similar splitting patterns and chemical shifts to 5. There was an added hydroxyl proton resonance at $\delta_H$ 9.71 for 14. The carbon resonances in both 5 and 14 were similar. In 14, the resonances for C-4' and C-6' could not be unequivocally assigned from their HMBC correlations and we have based their assignments on those made for 5.

In the meta chloro derivative 8, all the proton resonances for the chromanone ring, H-2 and H-5 to H-9 were all similar to 5. Changes were observed for the H-2', and H-4' to H-6' resonances due to a now weakly deactivating group. The H-2' resonance shifted more downfield from $\delta_H$ 6.82 in 5 to $\delta_H$ 7.27 in 8, probably by the electron withdrawal inductive effects of the chloro group. The same can be seen for the H-4' and H-6' resonances which now shifted more downfield to $\delta_H$ 7.37 and occur as overlapping resonances. It is observed that the H-5' resonance for the chloro compound is shifted slightly more upfield from 7.34 in
to 7.17 in 8, probably due to a greater resonance effect by the chloro group, donating more
electron density to the meta position.

In the $^{13}$C NMR spectrum of 8, there was a noticeable shift of the C-2', C-3', C-4' and C-6' resonances. The C-2', C-4' and C-6' resonances were all shifted more downfield to $\delta_C$ 130.00, 129.42 and 129.62 in 8, from 115.42, 115.06 and 122.28 in 5. The C-3' resonance shifted more upfield to $\delta_C$ 134.76 in 8, from 159.69 in 5, due to the weaker electronegativity of the chloro group as opposed to oxygen, resulting in less electron withdrawal by induction from the chloro group.

2.1.6 Structural elucidation of the 3',4'-disubstituted derivatives (except 3',4'-difluoro)

The proton NMR resonances for the chromanone ring (including that of H-9) of compounds 6, 9 and 15 were similar to that described for 5, 8 and 14 above with regard to chemical shift and splitting patterns. The carbon resonances of C-2 to C-9 including C-4a and C-8a of the chromanone moiety were also similar to 5, 8 and 14.

With regard to the phenyl group, the 3',4'-substitution in 15 resulted in H-2', H-5' and H-6' having the expected splitting patterns of doublets for H-2' and H-5' and a double doublet for H-6' at $\delta_H$ 6.81 ($J = 2.04$ Hz), $\delta_H$ 6.79 ($J = 9.16$ Hz) and 6.73 ($J = 9.14$, 1.88 Hz) respectively. Their corresponding carbon resonances occurred at $\delta_C$ 117.76 (C-2'), 115.85 (C-5') and 123.47 (C-6'). These assignments were confirmed by HMBC correlations between H-9 and C-2' and C-6'. The two resonances at $\delta_C$ 145.38 and 147.87 were attributed to C-3' and C-4' respectively. They were assigned as such because of a HMBC correlation between C-4' and all the protons on the phenyl ring, H-2', H-5' and H-6', whereas C-3' showed a HMBC correlation to H-2' and H-5' only. The extra HMBC correlation between C-4' and H-6'
allowed the unambiguous assignment of these two carbon resonances (C-3' and C-4'). The C-1' carbon resonance was identified at $\delta_{C} 125.18$ due to a HMBC correlation between this carbon resonance and H-2', H-6' and H-5'.

For the dimethoxy compound 6, the resonances in the $^1$H NMR spectrum are slightly different to that of 15 due to solvent effects, 15 being acquired in deuterated DMSO and 6 being acquired in deuterated CDCl$_3$. This resulted in H-5' and H-6' overlapping at $\delta_{H} 6.88$-$6.90$ and H-2' being a doublet at $\delta_{H} 6.83$ ($J = 1.72$ Hz). The C-5' and C-6' resonances were difficult to identify using the HSQC spectrum because of the overlap of H-5' and H-6', but in comparison to 15 they were made at $\delta_{C} 123.64$ (C-6') and $\delta_{C} 111.06$ (C-5'). This was confirmed by a HMBC correlation between H-9 and C-6'. The H-2' resonance occurred as a doublet at $\delta_{H} 6.83$ ($J = 1.72$ Hz) with a corresponding carbon resonance at $\delta_{C} 113.31$. Two methoxy proton resonances can be seen in the $^1$H NMR spectrum at $\delta_{H} 3.89$ and 3.91 as two intense singlets, both corresponding to the overlapping carbon resonance at $\delta_{C} 55.99$. The aromatic C-O resonances occurred at $\delta_{C} 148.99$ and 150.41 as for 15 above.

In the disubstituted chloro compound 9, the proton resonances for H-2', H-5' and H-6' all kept their splitting patterns as that for above, but all these resonances shifted more downfield in comparison to 6 and 15. The H-5' resonance now occurred as a doublet at $\delta_{H} 7.50$ ($J = 8.40$ Hz), H-2' as a doublet at $\delta_{H} 7.38$ ($J = 1.92$ Hz) and H-6' as a double doublet at $\delta_{H} 7.12$ ($J = 8.28, 1.96$ Hz). This shift downfield was probably due to the greater inductive effect of chlorine versus its electron donating effect. In 6 and 15, the oxygenated groups probably had a greater electron donating effect than an inductive effect. This is consistent with the chloro group being deactivating and the hydroxy and methoxy groups being activating. Due to the chloro substitution at C-3' and C-4', these resonances shifted more upfield to $\delta_{C} 133.70$ and
Similar to the proton resonances, the corresponding carbon resonances, C-2', C-5' and C-6' shifted more downfield to $\delta_C$ 131.41, 130.79 and 128.94 respectively.

2.1.7 Structural elucidation of the fluorine containing compounds (10, 11 and 12)

As above, the proton and carbon resonances for the chromanone ring did not change and therefore a discussion for this part of the molecule will not be repeated. The only changes that occurred were in that of the phenyl ring. In compound 10, the para-fluorinated compound, the H-2'/6' and H-3'/5' proton resonances, which appeared as a pair of doublets in 7 (the para-chlorinated compound) at $\delta_H$ 7.22 and 7.40 now appeared as a doublet of doublets with $J = 8.60$ and 5.40 Hz at $\delta_H$ 7.28 (H-2'/6'), and a triplet with $J = 8.62$ Hz at $\delta_H$ 7.13 (H-3'/5'). The H-3'/5' resonance was most affected by the substitution with fluorine in terms of chemical shift, resulting in the resonance being shifted from $\delta_H$ 7.40 in 7 to $\delta_H$ 7.13 in 10. The H-2'/6' resonance did not shift notably from $\delta_H$ 7.22 to $\delta_H$ 7.28. However, the splitting patterns of the two resonances in the fluoro compound were markedly different to that of the chloro compound. The H-2'/6' resonance was split into a double doublet by the fluorine with $J_{HF} = 5.40$ Hz and the proton ortho to it with $J = 8.60$ Hz. The H-3'/5' resonance being closer to the fluorine substituent experienced the same coupling constant for both $J_{HF}$ and the ortho coupled protons $J_{H-2'/6', H-3'/5'}$ with $J = 8.62$ Hz. These resonances were distinguished by the H-2'/6' resonance showing a HMBC correlation with C-9 at $\delta_C$ 136.27 and the C-3'/5' resonance a HMBC correlation to C-1' at $\delta_C$ 130.52.

In the $^{13}$C NMR spectrum, all the carbon resonances of the phenyl ring were seen coupled to fluorine and appeared as doublets. The ipso carbon (C-4') directly bonded to the fluorine atom appeared at $\delta_C$ 163.21 and had a coupling constant of $J = 250.87$ Hz. Care must be
taken when making these assignments so as not to confuse this resonance for two separate carbon resonances. This resonance was confirmed by correlations to both the H-2'/6' and H-3'/5'. Also present in the $^{13}$C NMR spectrum were resonances at $\delta_{C} 115.98$ as a doublet with $J = 21.59$ Hz, typical of ortho coupled carbon resonances and $\delta_{C} 131.97$ ($J = 8.55$ Hz) typical for meta coupling and were assigned to C-3'/5' and C-2'/6' respectively. The C-2'/6' carbon resonance was coupled to H-9 in the HMBC spectrum as expected. The remaining doublet at $\delta_{C} 130.52$ ($J = 2.97$ Hz) was assigned to C-1' and the fact that this was a doublet was used to distinguish between the C-1' and C-3 carbon resonances which were located in the same region of the $^{13}$C NMR spectrum.

In compound 11, the 3'-fluoro derivative, the fluorine couples to the H-2', H-4' and H-5' protons. Both the H-4' and H-5' proton resonances appear as triplets of doublets, however they can be distinguished by their coupling constants. The H-4' resonance at $\delta_{H} 7.09$ experiences $J_{HF}$ and $J_{HH}$ coupling with similar coupling constants, hence the triplet with $J = 8.44$ Hz and the meta coupling ($J = 2.40$ Hz) results in the triplet being further split into a triplet of doublets. The H-5' resonance at $\delta_{H} 7.39$ shows a larger second coupling ($J = 5.96$ Hz) since this is due to the H-F coupling, the triplet ($J = 8.00$ Hz) being due to the coupling between the two protons ortho to it, H-4' and H-6'. The H-2' proton resonance, ortho to the fluorine atom, appears as a doublet with $J = 9.52$ Hz at $\delta_{H} 6.98$. Similar to 10, all the carbon resonances of the phenyl ring were doublets displaying ipso ($\delta_{C} 162.70$, $J = 245.90$ Hz, C-3'), ortho ($\delta_{C} 116.35$, $J = 15.56$ Hz, C-4'; $\delta_{C} 116.57$, $J = 16.28$ Hz, C-2'), meta ($\delta_{C} 130.31$, $J = 8.24$ Hz, C-5', $\delta_{C} 136.40$, $J = 7.80$, C-1'), and para ($\delta_{C} 125.66$, $J = 2.92$ Hz, C-6') coupling. Since the C-9 carbon atom is now only four bonds away from fluorine, this carbon resonance is also coupled by fluorine with a coupling constant of $J = 2.27$ Hz. HMBC correlations
between C-9 and H-2' and H-6' as well as between C-1' and H-5' are also present confirming the assignments of these carbon resonances.

In the $^1$H NMR spectrum of 12, the 3',4'-difluoro derivative, the H-5' ortho coupled proton appears as a triplet of doublets at $\delta_H$ 7.22 ($J$ = 9.88 and 8.28 Hz), the triplet being due to the same coupling constant between H-5' and the ortho fluorine and H-5' and H-6', which was split further into a triplet of doublets by the other meta fluorine atom. The H-2' resonance was split into a ddd because of coupling to both the ortho ($J$ = 10.08 Hz) and meta ($J$ = 7.52 Hz) fluorine atoms as well as the meta proton ($J$ = 2.04 Hz, H-6'). The H-6' proton resonance overlaps with the multiplet of resonances at $\delta_H$ 7.04. HMBC correlations between H-2', H-6' and C-9 as well as C-1' and H-5' confirm the assignments of these resonances.

The carbon resonances of the phenyl moiety are once again all split by the fluorine atoms. However in this instance, double doublets are experienced by all but the ortho coupled C-2' and C-5' resonances which appear as doublets at $\delta_C$ 118.74 ($J$ = 17.66 Hz) and $\delta_C$ 117.85 ($J$ = 17.69 Hz). This is due to there being two fluorine atoms present on the phenyl ring. The other four resonances are present at $\delta_C$ 150.25 ($J$ = 248.55 and 12.80 Hz, C-3'), $\delta_C$ 150.78 ($J$ = 261.53 and 12.86 Hz, C-4'), $\delta_C$ 131.40 ($J$ = 5.98 and 3.80 Hz, C-1') and $\delta_C$ 126.48 ($J$ = 6.25 and 3.47 Hz, C-6'). HMBC correlations are also seen for H-9 with C-2' and C-6' confirming the assignment of these carbon resonances.

2.1.8 Structural elucidation of the nitro containing compounds (16 and 17)

In comparison to the other compounds previously described, there were changes to the chromanone ring proton resonances in the nitro derivatives 16 and 17 indicating that some kind of conversion must have taken place. Of particular importance was the HMBC
correlation between the proton resonance at $\delta_H 7.45$ ($J = 8.48$ Hz), attributed to the H-2'/6' resonance and the methylene carbon resonance at $\delta_C 31.89$ in 16. This was not encountered for the other molecules. This now meant that the methylene group, which occurred at C-2 in the other molecules, was now present at C-9 in 16. This change was accompanied by a change in chemical shift of the methylene carbon resonance from approximately $\delta_C 67$ to $\delta_C 31.89$ in 16, which also indicated that the methylene carbon was no longer situated next to the oxygen atom. There was however no change in the olefinic methine resonance at $\delta_H 7.79$ when compared to the other compounds, but the C-2 resonance was now more deshielded at $\delta_C 153.14$ in comparison to the C-9 resonance in the other compounds. This was due to being situated close to the oxygen atom on the chromanone ring. This meant that an exo-endo bond migration had occurred in 16 and 17 due to the highly electron withdrawing nitro group. This was reported to occur previously (Valkonen et al., 2012).

For the H-5 to H-8 proton resonances in 16, all the resonances were shifted downfield in comparison to the 4-chloro derivative 7, between 0.15 to 0.50 Hz. However, these resonances retained their splitting patterns, being present as a doublet of doublets at $\delta_H 8.18$ ($J = 7.98$, 0.66 Hz, H-5), $\delta_H 7.65$, a triplet of doublets ($J = 8.32$, 1.10 Hz, H-7), $\delta_H 7.41$, a doublet ($J = 8.60$ Hz, H-8) and $\delta_H 7.38$ as a triplet ($J = 7.54$ Hz, H-6). For the carbon resonances in 16 there is not much difference in chemical shift of the carbon resonances C-4a through to C-8a with the largest shift being experienced by C-8a, which is only a 5 ppm shift upfield from $\delta_C 161$ in 7 to $\delta_C 156$ in 16. A 5 ppm shift was also experienced by the carbonyl carbon resonance from $\delta_C 182$ in 7 to $\delta_C 177$ in 16.

In the phenyl ring of 16, due to the electron withdrawing nitro group, a downfield shift was experienced by both the H-3'/5' and H-2'/6' resonances by 0.72 Hz and 0.23 Hz respectively,
H-3'/5' now occurring at $\delta_H$ 8.12 ($J = 8.56$ Hz) and H-2'/6' at $\delta_H$ 7.45 ($J = 8.48$ Hz) in comparison to the para-chloro compound 7. In comparison to 7, the carbon resonances of the phenyl ring C-1' through to C-6' were all shifted upfield by approximately 2 to 11 ppm in 16 at $\delta_C$ 123.06 (C-1'), 129.65 (C-2'/6'), 123.79 (C-3'/5') and 146.76 (C-4'). In 7, there was conjugation between the phenyl ring with the C-3 (9) double bond, delocalising the electrons amongst more carbon atoms, hence the more downfield chemical shift. In 16, due to the double bond migrating to the $\Delta^2$ position, this delocalisation does not occur and the electrons are localised to the phenyl group, hence more upfield chemical shifts are experienced.

In 17, there are now four separate resonances for the phenyl group protons as opposed to only two in 16. This was due to the nitro group now being at the 3' position. The H-2' resonance occurred as a broad singlet at $\delta_H$ 8.13, the H-4' and H-6' proton resonances occurred as doublets at $\delta_H$ 8.06 ($J = 8.16$ Hz) and $\delta_H$ 7.69 ($J = 7.68$ Hz) and the H-5' resonance occurred at $\delta_H$ 7.45 as a triplet with $J$ being 8.16 Hz. The other proton resonances of 17, the chromanone proton resonances as well as H-2 and H-9 were the same as that for 16 as were the carbon resonances of C-2 to C-9. There were however changes in chemical shifts for the carbon resonances on the phenyl ring. The C-3' carbon to which the nitro group was attached had a resonance of $\delta_C$ 148.41 and the carbon resonances meta to the nitro group, C-1' and C-5' resonated at $\delta_C$ 141.02 and $\delta_C$ 129.42 respectively, while the ortho positioned carbon atoms (C-2' and C-4') resonated at $\delta_C$ 123.52 and $\delta_C$ 121.71. This is consistent with the ortho and para carbon resonances being more shielded and the meta resonances being more deshielded due to electron withdrawal by resonance by the nitro group.
2.2 Bioactivity of the synthesised homoisoflavonoids

The synthesised homoisoflavonoids were subjected to antibacterial tests using the disc diffusion assay, and antioxidant testing by the DPPH radical scavenging and the ferric reducing antioxidant power assay. The results are reported and discussed in relation to the chemical structures of the homoisoflavonoids.

2.2.1 Antioxidant activity of the synthesised homoisoflavonoids

The three hydroxylated homoisoflavonoids (13-15) were tested for their antioxidant activity by the DPPH radical scavenging assay and the FRAP assay as hydroxylated flavonoids are commonly known to be antioxidants. Ascorbic acid, a common antioxidant, served as the positive control for these experiments. A sample, containing all required reagents except for the antioxidant was used as a blank.

2.2.1.1 DPPH radical scavenging assay

The principles for this assay are discussed in chapter 1 (1.4.1.1), and the methodology described in chapter 3 (3.2.1.1).

Polyhydroxylated compounds are known to have good antioxidant activity. Compounds 13, 14 and 15 are hydroxylated compounds and were therefore tested for their antioxidant activity. As expected, compound 15, which has two hydroxy groups at the 3' and 4' positions, showed strong antioxidant activity, comparable to that of ascorbic acid, and compounds 13 (the 4’-hydroxy) and 14 (the 3'-hydroxy) which have one hydroxy group showed weak antioxidant activity.
Graph 1: The scavenging ability of compounds 13, 14 and 15 in comparison to ascorbic acid at various concentrations

Graph 1 shows the percentage scavenging activity (donation of hydrogen to the DPPH radical) of the tested compounds at different concentrations in comparison to ascorbic acid. At 30-50 µg/mL, the scavenging ability of compound 15 is much higher than that of ascorbic acid, indicating its ability to act as a scavenger at low concentrations. At 500 µg/mL, compound 15 shows 99% scavenging activity, higher than that of ascorbic acid which showed 96% scavenging activity at the same concentration.

Compound 13 shows a higher scavenging ability than compound 14 indicating that it is preferable for antioxidants to have a hydroxy group on the 4'-position than the 3'-position. This may be as a result of the stability of the quinone resonance structures that form for the para substituted product (13) when a hydrogen atom is transferred to a radical, making it
more stable than the radical that results when the meta product (14) transfers its hydrogen atom.

When comparing the chemical structure of ascorbic acid to that of compound 15, it can be seen that ascorbic acid is polyhydroxylated and that a catechol moiety is present in 15. The similarity in the two structures is having two hydroxy groups adjacent to each other as in catechol (Figure 12).

![Figure 12: The chemical structures of ascorbic acid and compound 15](image)

Ascorbic acid is a known antioxidant which is a polyhydroxylated compound. The weak O–H bond present in phenolic compounds is responsible for their radical scavenging activity as this bond can easily be cleaved homolytically and the hydrogen atom transferred to a radical species. Other hydrogen atoms, such as those bonded to the aromatic ring and a methyl group are bonded to a carbon atom and are not easily scavengable since the C-H bond is much stronger and hence compounds containing these groups only are not good antioxidants. Compound 15, which has two hydroxy groups, shows results comparable to that of ascorbic acid. By further derivatising compound 15, for example substituting hydroxy groups on the A-ring in a catechol like manner may enhance its antioxidant activity. A proposed mechanism by which compound 15 is thought to scavenge radicals is illustrated below (Scheme 17). From this mechanism, it can be seen that the many resonance structures stabilise the resultant radical homoisoflavonoid that results when a hydrogen atom is
transferred to a radical species and that the diketone structure is also a stable structure resulting in the excellent antioxidant activity shown by this compound.

Scheme 17: The proposed mechanism for the antioxidant activity of (E)-3-(3',4'-dihydroxybenzylidene)chroman-4-one

2.2.1.2 Ferric reducing antioxidant power assay

The results of the FRAP assay confirm the conclusions made from the DPPH radical scavenging assay in that compound 15 compares well to ascorbic acid and that compound 13 and 14 are weak antioxidants. The methodology and experimental data are reported in chapter 3 (3.2.1.2).
Graph 2: The reducing power of compound 15 and ascorbic acid

Graph 2 shows the concentration against absorbance curves for compound 15 in comparison to ascorbic acid in the FRAP assay. The results of this assay differs from that of the DPPH assay in that it shows that compound 15 is a slightly weaker antioxidant than ascorbic acid at all concentrations. The trend observed is that as the concentration increases, the reducing power (transformation of Fe$^{3+}$ to Fe$^{2+}$) increases.

Graph 3: The reducing power of compound 13 and compound 14
Graph 3 shows, by the low absorbance values as compared to those in graph 2 for ascorbic acid and 15 that compounds 13 and 14 are weak antioxidants at low concentrations. At concentrations higher than 200 µg/mL, a sharp increase in its antioxidant activity is observed but its activity is still nowhere near that of 15 and ascorbic acid. The para hydroxy derivative (13) shows slightly better antioxidant activity than the meta hydroxy derivative (14), which is consistent with the results from the DPPH assay. The reducing power of the tested compounds in decreasing order was found to be: ascorbic acid > compound 15 > compound 13 > compound 14.

2.2.2 Antibacterial activity of the synthesised homoisoﬂavonoids

The antibacterial activities of the homoisoﬂavonoids synthesised were tested against ten gram positive and six gram negative bacterial strains using the Kirby-Bauer disk-diffusion method.

In general, the homoisoﬂavonoids tested were more active against the gram positive than the gram negative bacteria. No antibacterial activity was shown against the following gram negative strains of bacteria: *Salmonella arizonae* (ATCC 13314), *Escherichia coli* (ATCC 35219), *Pseudomonas aeruginosa* (ATCC 27853 and ATCC 35037), and *Klebsiella pneumoniae* (ATCC 70063). This is because gram negative bacteria have an extra membrane, known as the outer membrane which is made up of lipopolysaccharides and protein, which is difficult to diffuse or penetrate through. Therefore gram negative bacteria are more difficult to destroy. Since the homoisoﬂavonoids show good antibacterial activity against gram positive bacteria, they may be tested for antituberculosis activity and against nosocomial pathogens.
These homoisoflavonoids show the best inhibitory activity against the bacterial strain, *Staphylococcus aureus*, therefore two strains of *Staphylococcus aureus* were used in the antibacterial tests, *Staphylococcus aureus* (ATCC 29212) and *Staphylococcus aureus* (ATCC 43300).

The *para* substituted derivatives showed weaker activity than the *meta* substituted derivatives except for the *para* fluoro derivative against *Staphylococcus saprophyticus* (ATCC 35552). The *para* methoxy (4), chloro (7) and nitro (16) derivatives showed no activity against the bacterial strains tested.

Compound 14, the *meta* substituted hydroxylated homoisoflavonoid, showed the broadest range of antibacterial activity. It was the only homoisoflavonoid to show mild activity against the gram negative strain, *Escherichia coli* (ATCC 29522). Compound 14 was strongly active against *Staphylococcus aureus* (ATCC 29212 and ATCC 43300) and was moderately active against *Staphylococcus saprophyticus* (ATCC 35552), *Staphylococcus scuiri* (ATCC 29062), *Staphylococcus xylosus* (ATCC 35033) and *Streptococcus pyogenes* (ATCC 19615).
Graph 4: The zone of inhibition of compounds 3 (unsubstituted), 11 (3’-F), 12 (3’,4’-diF), 13 (4’-OH), 14 (3’-OH) and 15 (3’,4’-diOH) against *Staphylococcus aureus* (ATCC 43300)

With respect to *Staphylococcus aureus* (ATCC 43300), compounds 3, 11 and 14, the unsubstituted, the 3’-fluoro and the 3’-hydroxy homoisoflavonoids respectively showed better activity than the common antibiotic ampicillin, with activity indexes of 1.31, 1.23 and 1.46 respectively (Graph 4). Compound 3, the unsubstituted homoisoflavonoid, has an activity index of 1.31, which is decreased when substituents are introduced onto the B-ring of the homoisoflavonoid, except for when a hydroxyl group is introduced at the 3’-position of the B-ring. The meta hydroxy substituent enhances its antibacterial activity, increasing the activity index from 1.31 to 1.46 for ampicillin and 0.52 to 0.58 for tetracycline. The activity of the 3’-fluoro derivative 11, is only slightly decreased by the introduction of fluorine at the 3’-position and therefore the positive effects of having a fluorine atom in the molecule for the development of an antibacterial agent such as increased lipophilicity makes it an interesting subject for further research.
By comparing the activity indices of the fluorinated compounds 10, 11 and 12 against *Staphylococcus aureus* (ATCC 43300), it can be seen that the most favourable substitution pattern for antibacterial activity is the *meta* substitution with activity indices of 1.23 (AMP) and 0.48 (TET). The *para* substituted fluoro derivative showed no activity. The disubstituted fluorinated derivative, 12, showed a decrease in activity when compared to the *meta* fluoro derivative. By introducing an additional fluorine atom on the 4'-position of the *meta* substituted derivative, the activity is decreased from 1.23 to 0.85. This may be due to the mechanism by which the antibacterial agent works. Substitution at the 4'-position may inhibit binding to the active site of enzymes required for normal functional of the bacterial species. This explains why the activity decreases when the homoisoflavonoid is disubstituted. In contrast, substitution at the 3'-position with a molecule or group capable of hydrogen bonding, such as fluorine (11) or the hydroxy group (14) may be perfect for binding to enzymes responsible for the functioning of bacterial cells. Once bound to these enzymes, they may alter their function and hence lead either to cell death of the bacterial species or inhibit replication of the bacterial species.

*Staphylococcus aureus* (ATCC 43300) is resistant to both methicillin and oxacillin ([http://www.straininfo.net/strains/54914/](http://www.straininfo.net/strains/54914/)). Such strains of bacteria are referred to as methicillin resistant *Staphylococcus aureus* (MRSA). Compound 14 may therefore serve as a useful antibacterial agent against *Staphylococcus aureus* (ATCC 43300). MRSA is one of the most dangerous bacterial infections that occur in the commercial health care sector (hospitals), as these bacterial infections cannot be easily destroyed. As compound 14, shows good antibacterial activity against *Staphylococcus aureus* (ATCC 43300), it may be applied to combat such infections. Derivatisation of compound 14, by varying the substitution on the A-ring can be conducted in order to develop better antibacterial agents.
CHAPTER 3 EXPERIMENTAL

This chapter includes the experimental techniques employed to synthesise and characterise the thirteen benzylidene and two benzyl homoisoflavonoids. Characteristic data, such as the \(^1\text{H}\) and \(^{13}\text{C}\) NMR, UV, IR and MS data are reported in the chapter. New crystal structures for seven of the homoisoflavonoids were obtained. The methodology used to determine the antioxidant and antibacterial activities of the test compounds are also included here.

3.1 Chemistry

The experimental procedure for the synthesis of the intermediates 3-phenoxypropanoic acid (1) and 4-chromanone (2) as well as the homoisoflavonoids (3-17) are included here. The instrument details and parameters used for the characterisation techniques employed are also reported.

3.1.1 General experimental procedures

The reagents and chemicals used in this study were purchased from Sigma Aldrich via Capital Lab, South Africa and were reagent grade. All organic solvents were redistilled and dried according to standard procedures. The melting points were recorded on an Ernst Leitz Wetziar micro-hot stage melting point apparatus. IR spectra were recorded on a Perkin Elmer Spectrum 100 FTIR spectrometer with universal ATR sampling accessory. UV spectra were obtained on a Varian Cary UV-VIS Spectrophotometer in dichloromethane (1-12, 16-17) and methanol (13-15). For GC-MS analyses, the samples were analysed on an Agilent GC–MSD apparatus equipped with DB-5SIL MS (30 m x 0.25 mm i.d., 0.25 µm film thickness) fused-silica capillary column. Helium (at 2 mL/min) was used as a carrier gas. The MS was operated in the EI mode at 70 eV. The NMR spectra were recorded using a
Bruker Avance III (400 MHz) spectrometer at room temperature (25 °C). The chemical shifts (δ) were referenced against an internal standard, tetramethylsilane (TMS) for ¹H and ¹³C and trifluorotoluene for ¹⁹F NMR. Solution NMR was performed in deuterated solvents, CDCl₃ (1-12, 16-17) and DMSO (13-15).

3.1.2 The synthesis of 3-phenoxypropanoic acid (1)
To a 100 mL round bottom flask, a solution of phenol (5.27 g, 56 mmol) in EtOH (30.00 mL) and a solution of NaOH (3.401 g, 85 mmol) in deionized water was added under cool conditions (10-15 °C) and stirred for 45 min for the deprotonation of phenol. A solution of 3-bromopropionic acid (10.57 g, 69 mmol) in EtOH (30.00 mL) was then added using a dropping funnel at 0 °C and the reaction mixture stirred for 12 hrs at 50 °C under reflux. The reaction mixture was cooled and acidified with 10% HCl and extracted with ethyl acetate (3 × 50 mL). The ethyl acetate layers were combined, washed with brine (1 × 20 mL), water (2 × 10 mL) and dried over anhydrous magnesium sulfate. The residue obtained after evaporation of the solvent was purified by column chromatography using silica gel with 10% ethyl acetate in hexane as the mobile phase. Compound 1 has an Rf of 0.36 in an ethyl acetate: hexane (20:80) solvent system (Scheme 18) (3.35g, 36%).

![Scheme 18: The preparation of 3-phenoxypropanoic acid (1)](image)
3.1.3 The synthesis of 4-chromanone (2)

In a 250 mL round bottom flask, a mixture of 3-phenoxypropanoic acid (4.51 g, 27 mmol) and polyphosphoric acid (13.52 g, 40 mmol) was stirred at 85–90 °C under reflux for 2 hrs (solvent free reaction). The viscous reaction mixture was poured onto crushed ice and extracted with diethyl ether (3 × 30 mL). The extract was washed with NaOH (30 mL), water (50 mL) and dried over magnesium sulfate. The residue obtained after evaporation of the solvent was purified by column chromatography using silica gel with 5% ethyl acetate in hexane as the mobile phase. Compound 2 has an $R_f$ of 0.53 in an ethyl acetate: hexane (20:80) solvent system (Scheme 19) (2.08 g, 52%).

![Scheme 19: The preparation of 4-chromanone (2)](image)

3.1.4 The base catalysed preparation of homoisoflavonoids (3-17)

In a 50 mL round bottom flask, a mixture of 4-chromanone (68 mmol), the desired substituted benzaldehyde (81 mmol) and 10–15 drops of piperidine was stirred at 80–90 °C under reflux for 12–36 hrs (Scheme 20). Typically masses between 1.01 g and 1.21 g of 4-chromanone were used. The reaction mixture was monitored for completion by thin layer chromatography. Upon completion, the reaction mixture was cooled, diluted with water and neutralized using 10% HCl. For compounds 16 and 17, 10 mL of ethyl acetate was added to the viscous solution and the homoisoflavonoids precipitated out upon addition of hexane. The powdered product was filtered, washed with hexane and dried under vacuum.
For the other compounds, the reaction mixture was extracted with ethyl acetate (3 × 30 mL). The ethyl acetate layers were combined, washed with brine (20 mL), water (2 × 10 mL), dried over anhydrous magnesium sulfate and the solvent evaporated. On slow evaporation, crystals of compound 6 were obtained. In this case, the supernatant liquid was decanted. The crystals were then filtered and dried under vacuum. The remaining compounds were subjected to column chromatography on silica gel using various ethyl acetate: hexane mixtures as the mobile phase depending on the polarity of the compound. A 2 cm diameter column was used and 50 mL fractions were collected until the compound was eluted from the column. Upon slow evaporation of the solvent, crystals of the homoisoflavonoids were obtained. The crystals were then filtered and dried under vacuum.

\[
\text{Scheme 20: The preparation of homoisoflavonoids (3-17)}
\]

3.1.5 The physical and spectroscopic data of synthesised compounds (1-17)

The chemical formula, molecular mass, physical description, melting points and yields of the synthesised compounds are listed below. Spectroscopic data including the UV, IR, MS and NMR data are listed. The \(^1\)H and \(^{13}\)C NMR data of 3-17 are contained in Table 2, 3, 4 and 5.
3-phenoxypropanoic acid (1)

\[ \text{C}_9\text{H}_8\text{O}_3 \text{ (166.17 g mol}^{-1}\text{)}; \]

Cream powder,

m.p.: 98-100 °C, yield: 36%

Rf: 0.36 (hexane: ethyl acetate, 80:20)

UV \( \lambda_{\text{max}} \) (nm) (log \( \varepsilon \)): 270 (2.68), 277 (2.60)

IR \( \nu_{\text{max}} \) (cm\(^{-1}\)): 2931 (O=H), 1688 (C=O), 1596, 1493 (C=C), 1231 (C-O)

EI MS \( m/z \) (%): 166 (38) [M\(^+\)], 94 (100), 77 (15), 66 (15), 65 (15), 55 (8), 51 (9)

\(^1\)H NMR (400 MHz, CDCl\(_3\)) \( \delta \): 2.83 (2H, t, \( J = 6.24 \text{ Hz} \), H\(_{32}\)), 4.23 (2H, t, \( J = 6.24 \text{ Hz} \), H-3), 6.89 (2H, d, \( J = 8.04 \text{ Hz} \), H-6/10), 6.94 (1H, t, \( J = 7.42 \text{ Hz} \), H-8), 7.26 (2H, dd, \( J = 8.44, 7.48 \text{ Hz} \), H-7/9)

\(^13\)C NMR (100 MHz, CDCl\(_3\)) \( \delta \): 34.49 (C-2), 63.05 (C-3), 114.65 (C-6/10), 121.17 (C-8), 129.50 (C-7/9), 158.39 (C-5), 176.93 (C-1)

4-chromanone (2)

\[ \text{C}_9\text{H}_8\text{O}_2 \text{ (148.16 g mol}^{-1}\text{)}; \]

White solid,

m.p.: 39-40 °C, yield: 52%

Rf: 0.53 (hexane: ethyl acetate, 80:20)

UV \( \lambda_{\text{max}} \) (nm) (log \( \varepsilon \)): 249 (3.91), 318 (3.53)

IR \( \nu_{\text{max}} \) (cm\(^{-1}\)): 1682 (C=O), 1599, 1476, 1453 (C=C), 1258 (C-O)

EI MS \( m/z \) (%): 148 (89) [M\(^+\)], 120 (100), 92 (77), 64 (18), 63 (17)

\(^1\)H NMR (400 MHz, CDCl\(_3\)) \( \delta \): 2.76 (1H, m, H-3a), 2.78 (1H, m, H-3b), 4.49 (1H, m, H-2b), 4.50 (1H, m, H-2a), 6.93 (1H, d, \( J = 8.44 \text{ Hz} \), H-8), 6.97 (1H, td, \( J = 7.84, 0.76 \text{ Hz} \), H-6), 7.42 (1H, ddd, \( J = 8.64, 7.16, 1.64 \text{ Hz} \), H-7), 7.85 (1H, dd, \( J = 7.84, 1.72 \text{ Hz} \), H-5).
\(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\): 37.78 (C-3), 67.00 (C-2), 117.89 (C-6/4a), 121.36 (C-8), 127.12 (C-5), 135.96 (C-7), 161.86 (C-8a), 191.79 (C-4)

\((E)\)-3-benzylidene-chroman-4-one (3)

\[\text{C}_{16}\text{H}_{12}\text{O}_{2} (236.27 \text{ g mol}^{-1})\];

Colourless crystalline solid,

m.p.: 108-110 °C, yield: 77%

R\(_f\): 0.69 (hexane: ethyl acetate, 80:20)

UV \(\lambda_{\text{max}}\) (nm) (log \(\varepsilon\)): 299 (4.13), 344 (3.91)

IR \(\nu_{\text{max}}\) (cm\(^{-1}\)): 1665 (C=O), 1601, 1466, 1450 (C=C), 1209 (C-O)

EI MS m/z (%): 236 (100) [M\(^+\)], 235 (99), 207 (20), 178 (8), 131 (13), 121 (65), 115 (88), 92 (22), 79 (8), 63 (16)

\((E)\)-3-(4'-methoxybenzylidene)chroman-4-one (4)

\[\text{C}_{17}\text{H}_{14}\text{O}_{3} (266.29 \text{ g mol}^{-1})\];

Pale yellow crystalline solid,

m.p.: 135-137 °C, yield: 75%

R\(_f\): 0.56 (hexane: ethyl acetate, 80:20)

UV \(\lambda_{\text{max}}\) (nm) (log \(\varepsilon\)): 247 (4.34), 351 (4.33)

IR \(\nu_{\text{max}}\) (cm\(^{-1}\)): 1663 (C=O), 1595, 1509, 1463 (C=C), 1210 (C-O)

EI MS m/z (%): 266 (100) [M\(^+\)], 265 (51), 251 (20), 237 (14), 235 (14), 223 (8), 207 (14), 165 (5), 146 (32), 145 (19), 131 (26), 121 (64), 103 (29), 92 (12), 77 (18), 63 (9)
Crystal structure:

**Figure 13:** ORTEP diagram of compound 4 drawn at the 50% probability level

\((E)-3-(3'\text{-methoxybenzylidene})\text{chroman-4-one (5)}\)

\[
\begin{align*}
\text{C}_{17}\text{H}_{14}\text{O}_3 (266.29 \text{ g mol}^{-1}); \\
\text{Pale yellow crystalline solid,} \\
\text{m.p.: 85-86 °C, yield: 72%}
\end{align*}
\]

\(R_f: 0.63 \text{ (hexane: ethyl acetate, 80:20)}\)

\(\text{UV } \lambda_{\text{max}} \text{ (nm) (log } \varepsilon) : 267 (4.06), 298 (4.04), 342 (3.99)\)

\(\text{IR } \nu_{\text{max}} \text{ (cm}^{-1}) : 1667 (\text{C=O}), 1598, 1460 (\text{C=C}), 1264 (\text{C-O})\)

\(\text{EI MS } m/\text{z } \% : 266 (95) [\text{M}^+], 265 (42), 251 (11), 235 (30), 146 (17), 145 (13), 131 (14), 121 (100), 115 (17), 103 (23), 92 (12), 77 (17), 63 (10)\)

Crystal structure:

**Figure 14:** ORTEP diagram of compound 5 drawn at the 50% probability level
**(E)**-3-(3',4'-dimethoxybenzylidene)chroman-4-one (6)

\[
\text{C}_{18}\text{H}_{16}\text{O}_4 \quad (296.32 \text{ g mol}^{-1})
\]

Pale yellow crystalline solid,
m.p.: 128-129 °C, yield: 61%

Rf: 0.34 (hexane: ethyl acetate, 80:20)

UV λ\text{max} (nm) (log ε): 266 (4.22), 366 (4.28)

IR ν\text{max} (cm\textsuperscript{-1}): 1661 (C=O), 1585, 1510, 1480 (C=C), 1241 (C=O)

EI MS m/z (%): 296 (100) [M\textsuperscript{+}], 295 (24), 281 (23), 265 (29), 221 (10), 176 (22), 161 (20), 131 (10), 121 (99), 92 (14), 77 (12), 63 (11)

Crystal structure:

![Image of ORTEP diagram of compound 6 drawn at the 50% probability level](image)

**Figure 15:** ORTEP diagram of compound 6 drawn at the 50% probability level

**(E)**-3-(4'-chlorobenzylidene)chroman-4-one (7)

\[
\text{C}_{16}\text{H}_{11}\text{ClO}_2 \quad (270.71 \text{ g mol}^{-1})
\]

White needle-like crystals,
m.p.: 173 °C, yield: 67%

Rf: 0.69 (hexane: ethyl acetate, 80:20)

UV λ\text{max} (nm) (log ε): 302 (4.20), 345 (3.88)

IR ν\text{max} (cm\textsuperscript{-1}): 1670 (C=O), 1603, 1475 (C=C), 1217 (C=O), 748 (C-Cl)
EI MS m/z (%): 270 (100) [M⁺], 269 (55), 241 (14), 235 (29), 207 (13), 179 (11), 178 (15),
150 (19), 149 (21), 134 (14), 131 (14), 121 (71), 120 (17), 117 (20), 115 (77), 92 (29), 89
(14), 76 (11), 63 (19)

(E)-3-(3'-chlorobenzylidene)chroman-4-one(8)

\[
\begin{align*}
\text{C}_{16}\text{H}_{11}\text{ClO}_2 & (270.71 \text{ g mol}^{-1}) ; \\
\text{White needle-like crystals,} \\
\text{m.p.: 123-124 °C, yield: 71%} 
\end{align*}
\]

Rf: 0.56 (hexane: ethyl acetate, 80:20)

UV λ\text{max} (nm) (log ε): 291 (4.17), 345 (3.71)

IR ν\text{max} (cm\textsuperscript{-1}): 1671 (C=O), 1604, 1464 (C=C), 1218 (C-O), 750 (C-Cl)

EI MS m/z (%): 270 (100) [M⁺], 269 (54), 241 (11), 235 (41), 207 (10), 178 (15), 149 (20),
131 (17), 121 (81), 120 (25), 117 (28), 115 (59), 92 (36), 76 (11), 63 (19)

(E)-3-(3',4'-dichlorobenzylidene)chroman-4-one (9)

\[
\begin{align*}
\text{C}_{16}\text{H}_{10}\text{Cl}_2\text{O}_2 & (305.16 \text{ g mol}^{-1}) ; \\
\text{White needle-like crystals,} \\
\text{m.p.: 165-167 °C, yield: 59%} 
\end{align*}
\]

Rf: 0.66 (hexane: ethyl acetate, 80:20)

UV λ\text{max} (nm) (log ε): 297 (3.96), 346 (3.53)

IR ν\text{max} (cm\textsuperscript{-1}): 1668 (C=O), 1604, 1464 (C=C), 1218 (C-O), 832, 746 (C-Cl)
EI MS m/z (%): 306 (57), 304 (90) [M+], 275 (12), 269 (23), 234 (11), 205 (8), 183 (15), 178 (13), 151 (23), 149 (65), 134 (41), 121 (100), 120 (38), 114 (15), 113 (15), 93 (11), 92 (51), 76 (14), 64 (20), 63 (24)

Crystal structure:

Figure 16: ORTEP diagram of compound 9 drawn at the 50% probability level

(E)-3-(4'-fluorobenzyldiene)chroman-4-one (10)

\[ C_{16}H_{11}FO_2 \text{ (254.26 g mol}^{-1}) ; \]

Pale yellow needle-like crystals,

m.p.: 152-153 °C, yield: 59%

Rf: 0.63 (hexane: ethyl acetate, 80:20)

UV \( \lambda_{\text{max}} \text{ (nm) (log } \varepsilon) \): 301 (3.97), 344 (3.63)

IR \( v_{\text{max}} \text{ (cm}^{-1}) \): 1671 (C=O), 1597, 1477 (C=C), 1217 (C-O), 1145 (C-F)

EI MS m/z (%): 254 (100) [M+], 253 (53), 237 (5), 225 (22), 207 (6), 196 (5), 134 (41), 133 (87), 131 (14), 121 (55), 120 (17), 107 (8), 92 (25), 63 (11)

\(^{19}\text{F NMR (376.5 MHz, CDCl}_3\text{) } \delta: \text{-110.16} \]

(E)-3-(3'-fluorobenzyldiene)chroman-4-one (11)

\[ C_{16}H_{11}FO_2 \text{ (254.26 g mol}^{-1}) ; \]

Pale yellow needle-like crystals,

m.p.: 95-96 °C, yield: 61%
Rf: 0.66 (hexane: ethyl acetate, 80:20)

UV $\lambda_{\text{max}}$ (nm) (log $\varepsilon$): 291 (3.99), 346 (3.55)

IR $\nu_{\text{max}}$ (cm$^{-1}$): 1666 (C=O), 1598, 1477 (C=C), 1213 (C-O), 1144 (C-F)

EI MS $m/z$ (%): 254 (100) [M$^+$], 253 (55), 237 (5), 225 (17), 134 (23), 133 (62), 121 (55), 120 (20), 92 (26), 63 (14)

$^{19}$F NMR (376.5 MHz, CDCl$_3$) $\delta$: 112.15

\begin{center}
(E)-3-(3',4'-difluorobenzylidene)chroman-4-one (12)
\end{center}

\begin{center}
\begin{tabular}{c}
\includegraphics[width=0.3\textwidth]{image1.png}
\end{tabular}
\end{center}

C$_{16}$H$_{10}$F$_2$O$_2$ (272.25 g mol$^{-1}$);

Pale yellow needle-like crystals,

m.p.: 138-139 °C, yield: 50%

Rf: 0.69 (hexane: ethyl acetate, 80:20)

UV $\lambda_{\text{max}}$ (nm) (log $\varepsilon$): 267 (3.96), 387 (4.05)

IR $\nu_{\text{max}}$ (cm$^{-1}$): 1672 (C=O), 1603, 1477 (C=C), 1217 (C-O), 1116, 1145 (C-F)

EI MS $m/z$ (%): 272 (100) [M$^+$], 271 (29), 243 (19), 152 (19), 151 (62), 134 (10), 131 (16), 121 (46), 120 (27), 92 (34), 63 (11)

$^{19}$F NMR (376.5 MHz, CDCl$_3$) $\delta$: -136.2 (d, $J = 21.65$ Hz), -134.83 (d, $J = 21.46$ Hz)

\begin{center}
(E)-3-(4'-hydroxybenzylidene)chroman-4-one (13)
\end{center}

\begin{center}
\begin{tabular}{c}
\includegraphics[width=0.3\textwidth]{image2.png}
\end{tabular}
\end{center}

C$_{16}$H$_{12}$O$_3$ (252.26 g mol$^{-1}$);

Yellow powder,

m.p.: 222-224 °C, yield: 54%

Rf: 0.22 (hexane: ethyl acetate, 80:20)

UV $\lambda_{\text{max}}$ (nm) (log $\varepsilon$): 331 (3.98), 359 (3.97)
IR $\nu_{\text{max}}$ (cm$^{-1}$): 3093 (O-H), 1651 (C=O), 1608, 1557, 1509 (C=C), 1291, 1209 (C-O)

EI MS $m/z$ (%): 252 (100) [M$^+$], 251 (28), 235 (13), 223 (9), 207 (13), 132 (11), 131 (23), 121 (82), 77 (15), 63 (6)

(E)-3-(3'-hydroxybenzylidene)chroman-4-one (14)

C$_{16}$H$_{12}$O$_3$ (252.26 g mol$^{-1}$);

Pale yellow powder,

m.p.: 199-200 °C, yield: 51%

$R_f$: 0.31 (hexane: ethyl acetate, 80:20)

UV $\lambda_{\text{max}}$ (nm) (log $\varepsilon$): 267 (4.20), 340 (4.04)

IR $\nu_{\text{max}}$ (cm$^{-1}$): 3255 (O-H), 1668 (C=O), 1593, 1461 (C=C), 1220 (C-O)

EI MS $m/z$ (%): 252 (100) [M$^+$], 251 (23), 235 (14), 234 (17), 223 (8), 206 (22), 205 (12), 131 (18), 121 (83), 92 (8), 77 (16)

Crystal structure:

Figure 17: ORTEP diagram of compound 14 drawn at the 50% probability level
\((E)-3-(3',4'-dihydroxybenzylidene)chroman-4-one\) (15)

\[
\begin{align*}
\text{C}_ {16} \text{H}_{12} \text{O}_4 \ (268.26 \ \text{g mol}^{-1});
\end{align*}
\]

Yellow powder,

m.p.: 230-231 °C, yield: 45%

\(R_f\): 0.16 (hexane: ethyl acetate, 80:20)

UV \(\lambda_{\text{max}}\) (nm) (log \(\varepsilon\)): 268 (3.62), 374 (3.68)

IR \(\nu_{\text{max}}\) (cm\(^{-1}\)): 3453, 3117 (O-H), 1649 (C=O), 1601, 1558, 1531 (C=C), 1286, 1187 (C-O)

\(3-(4\text{-nitrobenzyl})-4H\text{-chromen-4-one}\) (16)

\[
\begin{align*}
\text{C}_ {16} \text{H}_{11} \text{NO}_4 \ (281.26 \ \text{g mol}^{-1});
\end{align*}
\]

White powder,

m.p.: 179-180 °C, yield: 80%

\(R_f\): 0.34 (hexane: ethyl acetate, 80:20)

UV \(\lambda_{\text{max}}\) (nm) (log \(\varepsilon\)): 265 (4.07), 295 (4.15)

IR \(\nu_{\text{max}}\) (cm\(^{-1}\)): 1624 (C=O), 1603, 1464 (C=C), 1145 (C-O), 1505, 1339 (N-O)

EI MS \(m/z\) (%): 281 (100) [M\(^+\)], 264 (24), 235 (14), 234 (35), 205 (14), 178 (26), 121 (65), 120 (19), 117 (19), 115 (31), 92 (22), 77 (18), 63 (18)

Crystal structure:

\[\text{Figure 18: ORTEP diagram of compound 16 drawn at the 50% probability level}\]
3-(3-nitrobenzyl)-4H-chromen-4-one (17)

C_{16}H_{11}NO_4 (281.26 g mol^{-1});

Cream powder,

m.p.: 129-130 °C, yield: 72%

R_f: 0.38 (hexane: ethyl acetate, 80:20)

UV \lambda_{\text{max}} (nm) (log \varepsilon): 296 (3.96)

IR \nu_{\text{max}} (cm^{-1}): 1623 (C=O), 1605, 1464 (C=C), 1142 (C-O), 1523, 1341 (N-O)

EI MS m/z (%): 281 (100) [M^{+}], 264 (83), 234 (89), 205 (26), 178 (18), 117 (17), 115 (18), 92 (11), 89 (9), 77 (8), 76 (10), 63 (10)

Crystal structure:

Figure 19: ORTEP diagram of compound 17 drawn at the 50% probability level
Table 2: $^1$H NMR data of compounds 3-10

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Table 3: $^1$H NMR data of compounds 11-17

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Table 4: $^{13}$C NMR data of compounds 3-10

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Table 5: $^{13}$C NMR data of compounds 11-17

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<td>147.87</td>
<td>123.79</td>
<td>148.41</td>
</tr>
<tr>
<td>4'</td>
<td>116.35</td>
<td>150.78 (dd, $J = 261.53, 12.86$ Hz)</td>
<td>159.30</td>
<td>116.87</td>
<td>145.38</td>
<td>146.76</td>
<td>121.71</td>
</tr>
<tr>
<td>5'</td>
<td>130.31</td>
<td>117.85 (d, $J = 17.69$ Hz)</td>
<td>115.78</td>
<td>129.84</td>
<td>115.85</td>
<td>123.79</td>
<td>129.42</td>
</tr>
<tr>
<td>6'</td>
<td>125.66</td>
<td>126.48 (dd, $J = 6.25, 3.47$ Hz)</td>
<td>132.74</td>
<td>121.08</td>
<td>123.47</td>
<td>129.65</td>
<td>135.29</td>
</tr>
</tbody>
</table>
3.2 Biochemistry

The experimental techniques employed to determine the antioxidant and antibacterial activities of the homoisoflavonoids are stated below as well as the experimental data.

3.2.1 Antioxidant activity of the homoisoflavonoids synthesised

The antioxidant activities of the homoisoflavonoids were determined using two common simple methods, i.e. the DPPH radical scavenging assay and the ferric reducing antioxidant power assay.

3.2.1.1 DPPH radical scavenging assay

The DPPH scavenging activity of homoisoflavonoids was determined according to the modified method by (Murthy et al., 2012). The free radical scavenging activity was determined spectrophotometrically using a stable free radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH). Stock solutions of each compound were prepared by dissolving 10 mg of the compound in 10 mL of methanol (1000 µg/mL). The stock solutions were used to prepare a series of eight concentrations (500, 200, 100, 50, 40, 30, 20, 10 µg/mL). A solution of DPPH was prepared by dissolving 1.97 mg of DPPH in 50 mL of methanol (0.1 mM) and protected from light by covering the volumetric flask with aluminum foil. An aliquot of each dilution of the compound (150 µl) was mixed with methanolic solution of DPPH (2850 µl) in glass test tubes. The mixtures were shaken vigorously and set in a dark cupboard at ambient temperature for 30 min. The absorbance was measured at 517 nm against methanol as a blank. All measurements were done in triplicate and the average absorbance was used. The percent scavenging activity of the compounds were calculated using the following formula:
The calculated scavenging activities are displayed below in Table 6.

Table 6: The DPPH free radical scavenging activity of the homoisoflavonoids (13, 14, 15) and ascorbic acid

<table>
<thead>
<tr>
<th>Scavenging activity (%)</th>
<th>Compound 13</th>
<th>Compound 14</th>
<th>Compound 15</th>
<th>Ascorbic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 µg/mL</td>
<td>10.57</td>
<td>3.38</td>
<td>1.01</td>
<td>5.88</td>
</tr>
<tr>
<td>20 µg/mL</td>
<td>10.37</td>
<td>5.90</td>
<td>7.60</td>
<td>7.22</td>
</tr>
<tr>
<td>30 µg/mL</td>
<td>9.93</td>
<td>5.32</td>
<td>13.25</td>
<td>7.36</td>
</tr>
<tr>
<td>40 µg/mL</td>
<td>9.83</td>
<td>5.22</td>
<td>15.89</td>
<td>8.13</td>
</tr>
<tr>
<td>45 µg/mL</td>
<td>11.03</td>
<td>6.14</td>
<td>20.13</td>
<td>10.56</td>
</tr>
<tr>
<td>50 µg/mL</td>
<td>9.70</td>
<td>5.85</td>
<td>19.60</td>
<td>12.76</td>
</tr>
<tr>
<td>100 µg/mL</td>
<td>8.83</td>
<td>3.28</td>
<td>55.52</td>
<td>51.77</td>
</tr>
<tr>
<td>200 µg/mL</td>
<td>11.43</td>
<td>4.68</td>
<td>91.29</td>
<td>96.12</td>
</tr>
<tr>
<td>500 µg/mL</td>
<td>10.40</td>
<td>6.42</td>
<td>99.00</td>
<td>96.46</td>
</tr>
</tbody>
</table>

3.2.1.2 Ferric reducing antioxidant power assay

A series of methanolic standard solutions of varying concentrations (500, 200, 150, 100, 50, 40, 30, 20, 10 µg/mL) were prepared from a 1000 µg/mL stock solution. A 2.5 mL volume of the different concentrations were mixed with 2.5 mL phosphate buffer solution (0.1 M, pH = 6.6) and 2.5 mL of aqueous potassium hexacyanoferrate [K₃Fe(CN)₆] solution (1%) in test tubes. After 20 min of incubation at 50 ºC in a water bath, a volume of 2.5 mL of 10% trichloroacetic acid (TCA) was added to the mixture and mixed thoroughly. A volume of 2.5
mL from the mixture was added to 2.5 mL of distilled water and 0.5 mL of FeCl₃ (0.1% solution). The resulting mixture was mixed thoroughly and allowed to stand for 10 min after which the absorbance was taken at 700 nm using a UV-Vis spectrophotometer. Ascorbic acid was used as a positive control. All measurements were taken in triplicate and the average absorbance is displayed below (Table 7).

<table>
<thead>
<tr>
<th>Absorbance</th>
<th>Compound 13</th>
<th>Compound 14</th>
<th>Compound 15</th>
<th>Ascorbic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 µg/mL</td>
<td>0.015</td>
<td>0.044</td>
<td>0.045</td>
<td>0.062</td>
</tr>
<tr>
<td>20 µg/mL</td>
<td>0.033</td>
<td>0.023</td>
<td>0.085</td>
<td>0.323</td>
</tr>
<tr>
<td>30 µg/mL</td>
<td>0.034</td>
<td>0.024</td>
<td>0.193</td>
<td>0.867</td>
</tr>
<tr>
<td>35 µg/mL</td>
<td>0.029</td>
<td>0.024</td>
<td>0.157</td>
<td>0.957</td>
</tr>
<tr>
<td>40 µg/mL</td>
<td>0.014</td>
<td>0.024</td>
<td>0.544</td>
<td>1.071</td>
</tr>
<tr>
<td>45 µg/mL</td>
<td>0.016</td>
<td>0.020</td>
<td>0.617</td>
<td>1.566</td>
</tr>
<tr>
<td>50 µg/mL</td>
<td>0.017</td>
<td>0.019</td>
<td>0.735</td>
<td>1.932</td>
</tr>
<tr>
<td>100 µg/mL</td>
<td>0.021</td>
<td>0.025</td>
<td>1.741</td>
<td>2.799</td>
</tr>
<tr>
<td>200 µg/mL</td>
<td>0.033</td>
<td>0.042</td>
<td>3.000</td>
<td>3.000</td>
</tr>
<tr>
<td>500 µg/mL</td>
<td>0.163</td>
<td>0.146</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**3.2.2 Antibacterial activity of the homoisoflavonoids synthesised**

The fifteen synthesised homoisoflavonoids were screened for their antibacterial activity using the disc diffusion method. The principles of the technique are explained in Chapter 1 (1.4.2.1).
3.2.2.1 Disc diffusion antimicrobial susceptibility testing

The antibacterial activities of the synthesised homoisoflavonoids were determined using the Kirby-Bauer disk-diffusion method. The homoisoflavonoids synthesised were tested against ten gram positive and six gram negative bacteria (Table 8). Stock solutions (5 mg/mL) of each homoisoflavonoid were prepared by dissolving compounds in 1 mL of DMSO. Blank discs (5 mm; MAST, UK) were impregnated with 50, 100 and 200 µg/mL of each homoisoflavonoid and allowed to dry. Bacterial isolates were grown overnight on TSA agar plates and the turbidity of cell suspensions were adjusted equivalent to that of a 0.5 McFarland standard. These were used to inoculate Mueller-Hinton (MH) agar plates by streaking swabs over the entire agar surface followed by the application of the respective homoisoflavonoid discs. Plates were then incubated for 21 hrs at 30 °C. Testing was done in duplicate and tetracycline (TET) and ampicillin (AMP) discs were used as standard antimicrobial agent controls. The negative control was 5 µl of DMSO (100%). Zone diameters were measured physically and averaged. Activity indices of each compound were calculated by comparing zones of inhibition obtained with each of the compounds with those obtained with the standard antimicrobial agents, tetracycline and ampicillin. The following equation was used:

\[
\text{Activity index (AI)} = \frac{\text{Inhibition diameter (mm) with test compound}}{\text{Inhibition diameter (mm) with standard antimicrobial agent}}
\]
Table 8: The strains of bacterial cultures tested against in the disc diffusion assay

<table>
<thead>
<tr>
<th>Gram positive bacteria</th>
<th>Gram negative bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus subtilis (ATCC 6633)</td>
<td>Escherichia coli (ATCC 29522)</td>
</tr>
<tr>
<td>Enterobacter aerogenes (ATCC 13048)</td>
<td>Escherichia coli (ATCC 35219)</td>
</tr>
<tr>
<td>Enterococcus faecalis (ATCC 5129)</td>
<td>Klebsiella pneumoniae (ATCC 70063)</td>
</tr>
<tr>
<td>Staphylococcus aureus (ATCC 29212)</td>
<td>Pseudomonas aeruginosa (ATCC 27853)</td>
</tr>
<tr>
<td>Staphylococcus aureus (ATCC 43300)</td>
<td>Pseudomonas aeruginosa (ATCC 35037)</td>
</tr>
<tr>
<td>Staphylococcus saprophyticus (ATCC 35552)</td>
<td>Salmonella arizonae (ATCC 13314)</td>
</tr>
<tr>
<td>Staphylococcus scuiri (ATCC 29062)</td>
<td></td>
</tr>
<tr>
<td>Staphylococcus xylosus (ATCC 35033)</td>
<td></td>
</tr>
<tr>
<td>Streptococcus agalactiae (ATCC 13813)</td>
<td></td>
</tr>
<tr>
<td>Streptococcus pyogenes (ATCC 19615)</td>
<td></td>
</tr>
</tbody>
</table>

The zone diameters obtained for the three concentrations of each homoisoflavonoid are displayed below (Table 9 and Table 10). The activity indexes were calculated for each compound at the highest concentration (200 µg/mL) and are displayed in Table 9 and Table 10. Compounds 4, 7, 16 and 17 showed no antibacterial activity and are therefore omitted from the tables. The homoisoflavonoids were tested but showed no activity against the following bacterial strains, which are also omitted from the data tables: Salmonella arizonae (ATCC 13314), Escherichia coli (ATCC 35219), Pseudomonas aeruginosa (ATCC 27853 and ATCC 35037), and Klebsiella pneumoniae (ATCC 70063).
Table 9: The zone diameters and activity indices of compounds 3-6 and 8-15 against bacterial strains: *Staphylococcus aureus*, *Staphylococcus saprophyticus* and *Staphylococcus scuiri*

<table>
<thead>
<tr>
<th>Bacteria Cultures</th>
<th>50 µg/mL</th>
<th>100 µg/mL</th>
<th>200 µg/mL</th>
<th>50 µg/mL</th>
<th>100 µg/mL</th>
<th>200 µg/mL</th>
<th>50 µg/mL</th>
<th>100 µg/mL</th>
<th>200 µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus</em> (ATCC 29212)</td>
<td>Activity index</td>
<td>Activity index</td>
<td>Activity index</td>
<td>TET</td>
<td>AMP</td>
<td>TET</td>
<td>AMP</td>
<td>TET</td>
<td>AMP</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
<td>12</td>
<td>21</td>
<td>0.67</td>
<td>0.84</td>
<td>11</td>
<td>15</td>
<td>17</td>
<td>0.52</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>9</td>
<td>10</td>
<td>12</td>
<td>0.39</td>
<td>0.48</td>
<td>7</td>
<td>10</td>
<td>11</td>
<td>0.33</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>8</td>
<td>10</td>
<td>0.32</td>
<td>0.40</td>
<td>-</td>
<td>-</td>
<td>9</td>
<td>0.27</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>-</td>
<td>8</td>
<td>0.26</td>
<td>-</td>
<td>-</td>
<td>9</td>
<td>10</td>
<td>0.30</td>
</tr>
<tr>
<td>9</td>
<td>9</td>
<td>10</td>
<td>12</td>
<td>0.39</td>
<td>0.32</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>10</td>
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</tr>
<tr>
<td>11</td>
<td>9</td>
<td>12</td>
<td>15</td>
<td>0.48</td>
<td>0.60</td>
<td>10</td>
<td>14</td>
<td>16</td>
<td>0.48</td>
</tr>
<tr>
<td>12</td>
<td>-</td>
<td>7</td>
<td>7.5</td>
<td>0.23</td>
<td>0.30</td>
<td>-</td>
<td>7</td>
<td>11</td>
<td>0.33</td>
</tr>
<tr>
<td>13</td>
<td>9</td>
<td>10</td>
<td>12</td>
<td>0.39</td>
<td>0.48</td>
<td>-</td>
<td>8</td>
<td>10</td>
<td>0.30</td>
</tr>
<tr>
<td>14</td>
<td>17</td>
<td>19</td>
<td>22</td>
<td>0.71</td>
<td>0.88</td>
<td>14</td>
<td>16</td>
<td>19</td>
<td>0.58</td>
</tr>
<tr>
<td>15</td>
<td>10</td>
<td>12</td>
<td>13</td>
<td>0.42</td>
<td>0.52</td>
<td>7</td>
<td>8</td>
<td>11</td>
<td>0.33</td>
</tr>
</tbody>
</table>

Tetracycline (TET) | 31 | 33 | 30 | 25 |
Ampicillin (AMP) | 25 | 13 | 37 | 35 |
DMSO | - | - | - | - |
Table 10: The zone diameters and activity indices of compounds 3-6 and 8-15 against bacterial strains: *Staphylococcus xylosus*, *Streptococcus agalactiae*, *Streptococcus pyogenes* and *Escherichia coli*

<table>
<thead>
<tr>
<th>Bacteria Cultures</th>
<th>Staphylococcus xylosus (ATCC 35033)</th>
<th>Streptococcus agalactiae (ATCC 13813)</th>
<th>Streptococcus pyogenes (ATCC 19615)</th>
<th>Escherichia coli (ATCC 29522)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration/ µg/mL and Activity index at 200/ µg/mL</td>
<td>TET</td>
<td>AMP</td>
<td>TET</td>
<td>AMP</td>
</tr>
<tr>
<td>50</td>
<td>100</td>
<td>200</td>
<td>Activity index</td>
<td>50</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>8</td>
<td>10</td>
<td>0.36</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>7</td>
<td>9</td>
<td>0.32</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>-</td>
<td>8</td>
<td>0.29</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>-</td>
<td>7</td>
<td>0.25</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
<td>-</td>
<td>8</td>
<td>0.29</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>8</td>
<td>9</td>
<td>10</td>
<td>0.36</td>
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<tr>
<td>12</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>13</td>
<td>-</td>
<td>-</td>
<td>8</td>
<td>0.29</td>
</tr>
<tr>
<td>14</td>
<td>13</td>
<td>15</td>
<td>16</td>
<td>0.57</td>
</tr>
<tr>
<td>15</td>
<td>-</td>
<td>-</td>
<td>8</td>
<td>0.29</td>
</tr>
</tbody>
</table>

Tetracycline (TET): 28 24 33 24
Ampicillin (AMP): 32 - 37 21
DMSO: - - - -
CHAPTER 4 CONCLUSION

A series of fifteen homoisoflavonoids, three of the 3-benzylidene and two of the 3-benzyl type, were synthesised in a three step reaction. The solvent free base-catalysed aldol condensation of 4-chromanone and substituted benzaldehydes in the presence of piperidine resulted in the formation of the homoisoflavonoids in good yields of between 50 and 90%. Substitution of the phenyl ring was varied at the 3′-position, 4′-position and 3′,4′-positions with methoxy, hydroxy, chloro, fluoro and nitro groups. Compounds containing the electron withdrawing nitro groups resulted in the formation of the 3-benzyl-4-chromanone rather than the desired 3-benzylidene-4-chromanone. The synthesised compounds were fully characterised by $^1$H, $^{13}$C and $^{19}$F NMR, IR and UV spectroscopy and EI-MS. Crystal structures of seven homoisoflavonoids were also obtained and reported for the first time in this work.

The antioxidant testing of the homoisoflavonoids, using the DPPH radical scavenging assay and the ferric reducing antioxidant power assay, showed that the polyhydroxylated compounds have good antioxidant activity due to the fact that the hydrogen on an O-H group is scavengable. Compound 15, a disubstituted hydroxyl compound, showed good antioxidant activity comparable to that of ascorbic acid. This was attributed to the fact compound 15 has a catechol moiety. Mono-substituted hydroxy containing homoisoflavonoids (13 and 14) showed weak antioxidant activity compared to compound 15. Further derivatisation of compound 15, by substituting a catechol moiety on the A-ring of the homoisoflavonoid, may result in increased antioxidant activity. Compound 15 is therefore an interesting target molecule to derivatise in the pursuit of good antioxidants.
The synthesised homoisoflavonoids were also subjected to antibacterial testing which showed that homoisoflavonoids are more active against gram positive than gram negative bacteria. The synthesised homoisoflavonoids showed good antibacterial activity against a methicillin resistant strain of bacteria, *Staphylococcus aureus* (ATCC 43300). Compounds 3, 11 and 14 showed better antibacterial activity than the common antibiotic ampicillin. Compound 14, the meta hydroxy homoisoflavonoid, showed the highest activity index of 1.46. Derivatisation of compound 14 with respect to the A-ring can be conducted and a structure-activity relationship determined.
REFERENCES


$^1$H NMR spectrum of compound 1
$^1$H NMR spectrum of compound 1 (expanded)
$^{13}$C NMR spectrum of compound 1
HSQC spectrum of compound 1
UV-Vis spectrum of compound 1
Infrared spectrum of compound 1
Mass spectrum of compound 1
\(^1\)H NMR spectrum of compound 2
¹H NMR spectrum of compound 2 (expanded)
$^{13}$C NMR spectrum of compound 2
UV-Vis spectrum of compound 2
Infrared spectrum of compound 2
Scan 467 (7.496 min): CHROMANONE.Dvdata.ms

Mass spectrum of compound 2
$^1$H NMR spectrum of compound 3
$^1$H NMR spectrum of compound 3 (expanded)
$^{13}$C NMR spectrum of compound 3
HMBC spectrum of compound 3
UV-Vis spectrum of compound 3
Infrared spectrum of compound 3
Mass spectrum of compound 3
$^1$H NMR spectrum of compound 4
$^1$H NMR spectrum of compound 4 (expanded)
DEPT 135 spectrum of compound 4
HSQC spectrum of compound 4
Overlay Spectrum Graph Report

UV-Vis spectrum of compound 4

<table>
<thead>
<tr>
<th>Wavelength/ nm</th>
<th>Absorbance</th>
<th>Log ε</th>
</tr>
</thead>
<tbody>
<tr>
<td>247</td>
<td>0.329</td>
<td>4.34</td>
</tr>
<tr>
<td>351</td>
<td>0.323</td>
<td>4.33</td>
</tr>
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</table>
Scan 1362 (14.044 min): 4-METHOXY.D 
data.ms

Mass spectrum of compound 4
$^1$H NMR spectrum of compound 5
$^1$H NMR spectrum of compound 5 (expanded)
DEPT spectrum of compound 5
HSQC spectrum of compound 5
NOESY spectrum of compound 5
UV-Vis spectrum of compound 5
Infrared spectrum of compound 5
Mass spectrum of compound 5
$^1$H NMR spectrum of compound 6
$^1$H NMR spectrum of compound 6 (expanded)
$^{13}$C NMR spectrum of compound 6
COSY spectrum of compound 6
NOESY spectrum of compound 6
UV-Vis spectrum of compound 6
Infrared spectrum of compound 6
Mass spectrum of compound 6
$^1$H NMR spectrum of compound 7
$^1$H NMR spectrum of compound 7 (expanded)
$^{13}$C NMR spectrum of compound 7
COSY spectrum of compound 7
HSQC spectrum of compound 7
HMBC spectrum of compound 7
UV-Vis spectrum of compound 7
Infrared spectrum of compound 7
Mass spectrum of compound 7
$^1$H NMR spectrum of compound 8
$^1$H NMR spectrum of compound 8 (expanded)
$^{13}$C NMR spectrum of compound 8
HSQC spectrum of compound 8
HMBC spectrum of compound 8
NOESY spectrum of compound 8
UV-Vis spectrum of compound 8
Infrared spectrum of compound 8
Mass spectrum of compound 8
$^1$H NMR spectrum of compound 9
$^1$H NMR spectrum of compound 9 (expanded)
HSQC spectrum of compound 9
UV-Vis spectrum of compound 9
Infrared spectrum of compound 9
Mass spectrum of compound 9
4-fluoro in cdc13

$^{1}H$ NMR spectrum of compound 10
4-fluoro in CDCl3

$^1$H NMR spectrum of compound 10 (expanded)
4-fluoro in d cereal 

- 182.1012
- 161.0762
- 157.8972
- 156.3081
- 151.8792
- 147.8972
- 146.3081
- 141.8792
- 139.3081
- 134.8792
- 132.3081
- 128.8792
- 126.3081
- 122.8792
- 120.3081
- 116.8792
- 114.3081
- 110.8792
- 108.3081
- 104.8792
- 102.3081
- 98.8792
- 96.3081
- 92.8792
- 90.3081
- 86.8792
- 84.3081
- 80.8792
- 78.3081
- 75.8792
- 72.3081
- 68.8792
- 66.3081
- 62.8792
- 60.3081

* carbons split by 19F 

13C NMR spectrum of compound 10
4-fluoro in CDCl₃
$^{19}$F NMR spectrum of compound 10
COSY spectrum of compound 10
HMBC spectrum of compound 10
NOESY spectrum of compound 10
UV-Vis spectrum of compound 10
Infrared spectrum of compound 10
Mass spectrum of compound 10
3-fluoro in CDCl3

1H NMR spectrum of compound 11
$^1$H NMR spectrum of compound 11 (expanded)
$^{13}$C NMR spectrum of compound 11
$^{13}$C NMR spectrum of compound 11 (expanded)
$^{19}$F NMR spectrum of compound 11
3-fluoro in cdcl3

COSY spectrum of compound 11
3-fluoro in cdcl3

HMBC spectrum of compound 11
NOESY spectrum of compound 11
UV-Vis spectrum of compound 11
Infrared spectrum of compound 11
Mass spectrum of compound 11
3,4-difluoro(4)

1H NMR spectrum of compound 12
$^1$H NMR spectrum of compound 12 (expanded)
3,4-difluoro(4)

$^{13}$C NMR spectrum of compound 12
3,4-difluoro(4)

![Chemical structure diagram]

- Interchangeable
- $3^1, 4^1$, J = 61.5 Hz, 12.26 Hz
- J = 248.55 Hz, J = 12.80 Hz
- J = 5.98 Hz
- J = 3.30 Hz
- J = 6.35 Hz
- J = 2.47 Hz

$^{13}$C NMR spectrum of compound 12 (expanded)
$^{19}$F NMR spectrum of compound 12
HSQC spectrum of compound 12 (expanded)
HMBC spectrum of compound 12
NOESY spectrum of compound 12
UV-Vis spectrum of compound 12

<table>
<thead>
<tr>
<th>Wavelength/ nm</th>
<th>Absorbance</th>
<th>Log ε</th>
</tr>
</thead>
<tbody>
<tr>
<td>267</td>
<td>0.455</td>
<td>3.96</td>
</tr>
<tr>
<td>387</td>
<td>0.557</td>
<td>4.05</td>
</tr>
</tbody>
</table>
Infrared spectrum of compound 12
Mass spectrum of compound 12
4-hydroxy in dmso

\(^1\)H NMR spectrum of compound 13
$^1$H NMR spectrum of compound 13 (expanded)
4-hydroxy in dmso

\[\text{13C NMR spectrum of compound 13}\]
COSY spectrum of compound 13
HSQC spectrum of compound 13
HMBC spectrum of compound 13
4-hydroxy in dmso

NOESY spectrum of compound 13
UV-Vis spectrum of compound 13

<table>
<thead>
<tr>
<th>Wavelength/nm</th>
<th>Absorbance</th>
<th>Log ε</th>
</tr>
</thead>
<tbody>
<tr>
<td>331</td>
<td>0.956</td>
<td>3.98</td>
</tr>
<tr>
<td>359</td>
<td>0.941</td>
<td>3.97</td>
</tr>
</tbody>
</table>
Infrared spectrum of compound 13
Mass spectrum of compound 13
$^1$H NMR spectrum of compound 14
3-hydroxy in DMSO

^1H NMR spectrum of compound 14 (expanded)
3-hydroxy in DMSO

$^{13}$C NMR spectrum of compound 14
3-hydroxy in DMSO

$^{13}$C NMR spectrum of compound 14 (expanded)
COSY spectrum of compound 14
HSQC spectrum of compound 14
HMBC spectrum of compound 14
3-hydroxy in DMSO

NOESY spectrum of compound 14
UV-Vis spectrum of compound 14
Infrared spectrum of compound 14
Mass spectrum of compound 14
Scan 1318 (13.722 min): 3-HYDROYX.Dicata.ms

[ M+ ]

loss of OH
18
$^{1}H$ NMR spectrum of compound 15
3,4 dihydroxy(2)

$^{1}$H NMR spectrum of compound 15 (expanded)
$^{13}$C NMR spectrum of compound 15
3,4 dihydroxy(2)

$^{13}$C NMR spectrum of compound 15 (expanded)
COSY spectrum of compound 15
HSQC spectrum of compound 15
HMBC spectrum of compound 15
UV-Vis spectrum of compound 15
Infrared spectrum of compound 15
Mass spectrum of compound 15
\(^1\)H NMR spectrum of compound 16
4-nitro

\[ \text{\textsuperscript{1}H NMR spectrum of compound 16 (expanded)} \]
4-nitro

\[ \text{13C NMR spectrum of compound 16} \]
$^{13}$C NMR spectrum of compound 16 (expanded)
COSY spectrum of compound 16
HSQC spectrum of compound 16
HMBC spectrum of compound 16
HMBC spectrum of compound 16 (expanded)
NOESY spectrum of compound 16
UV-Vis spectrum of compound 16
$^1$H NMR spectrum of compound 17
$^1$H NMR spectrum of compound 17 (expanded)
$^{13}$C NMR spectrum of compound 17
$^{13}$C NMR spectrum of compound 17 (expanded)
COSY spectrum of compound 17
HSQC spectrum of compound 17 (expanded)
HMBC spectrum of compound 17 (expanded)
3-nitro

NOESY spectrum of compound 17
UV-Vis spectrum of compound 17
Infrared spectrum of compound 17